Developmentally regulated expression of mouse HtrA3 and its role as an inhibitor of TGF-ß signaling

Doctor Dissertation

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Contents

SUMMARY

HtrA is a highly conserved family of serine proteases that contain a catalytic domain with at lease one C-terminal PDZ domain. Bacterial HtrA functions as a chaperon at low temperature and a serine protease that degraded misfolded proteins at high temperature. Mammals have four HtrA genes: HtrA1, -2, -3 and -4. HtrA2 contains a mitochondrial localization signal and a transmembrane motif in the N-terminal region and localized in the intermembrane space of mitochondria. HtrA1, 3 and 4 are secretory proteins that share similar domains that containing the insulin-like growth factor binding protein domain and the Kazal-type serine protease inhibitor domain at N-terminus. The expression of mouse HtrA1 is developmentally regulated and restricted in embryo tissues which depend largely on TGF-β signaling for their differentiation. I examined whether mouse HtrA3, another HtrA family member very close to HtrA1, shows similar expression patterns. HtrA3 and -1 were expressed mostly in the same embryonic organs but exhibited complementary patterns in various tissues; lens epithelial cells in day 12.5 embryo expressed HtrA3 whereas the ciliary body and pigment retina expressed HtrA1. In the vertebrae of day 14.5 embryo, HtrA3 was expressed in the tail region, but HtrA1 was predominantly expressed in the thoracic and lumbar regions. In adult tissues, both HtrA3 and -1 are strongly expressed in the placenta. It seems likely that HtrA3 was more highly expressed in the placenta. This result suggested that HtrA3 may function in placental development.

Similar to HtrA1, HtrA3 bound to various TGF-ß proteins and inhibited the signaling of BMP-4, -2 and TGF-ß1. HtrA3 did not inhibit signaling originated from a constitutively active BMP receptor, indicating that the inhibition occurred upstream of the cell surface receptor. HtrA3 also showed proteolytic activities indistinguishable from those of HtrA1 toward ß-casein and some extracellular matrix (ECM)

proteoglycans. The protease activity was absolutely required for the TGF-ß signal inhibition activity.

All these data suggest that HtrA3 and -1 have the overlapping biological activities but can function in complementary fashions in certain types of tissues.

1. INTRODUCTION

1.1 General Introduction

High temperature requirement A (HtrA, also called DegP or Protease Do) was initial identified in *E.coli* by two phenotypes of corresponding null mutants and named accordingly. Mutant did not grow at either elevate temperature (HtrA) (Lipinska et al, 1988) or failed to digest misfold protein in the periplasm (Deg P)(Strauch and Beckwith, 1988). HtrA shows an ATP-independent proteolytic activity and plays an important role in the degradation of misfolded proteins accumulated by heat shock of other stresses (Lipinska et al, 1989). Therefore, its activity seems to be essential for bacterial thermotolerance and for cell survival at high temperatures. HtrA is also involved in pathogenesis of Gram-negative and Gram-positive bacteria by degrading damage proteins that are produced by reactive oxygen species released from the host defense system (Jones et al, 2001). In addition to proteolytic activity, HtrA is known to have a molecular chaperon activity (Spiess et al, 1999; Misra et al, 2000). The chaperone function is dominant at low temperature, whereas the proteolytic activity is turned on at elevated temperatures (Spiess et al, 1999). HtrA is a highly conserved protein found in species ranging from bacteria to humans (Fig. 1). Recently mammalian homologues of bacterial HtrA have been identified. Those are HtrA1, HtrA2, HtrA 3 and HtrA4. Similar to bacterial HtrA, the mammalian HtrA family shares a modular architecture composed of a conserved trypsin-like protease domain, and PDZ domain at C-terminal. PDZ domain is a protein modules that mediate specific protein-protein interactions and bind preferentially to the C-terminal three to four residues of membrane receptor and ion channels. The crystal structure of HtrA has shown that bacterial HtrA forms a hexameric complex composed of two trimer (Krojer et al, 2002) and human HtrA2 forms a homotrimer (Li et al, 2002).

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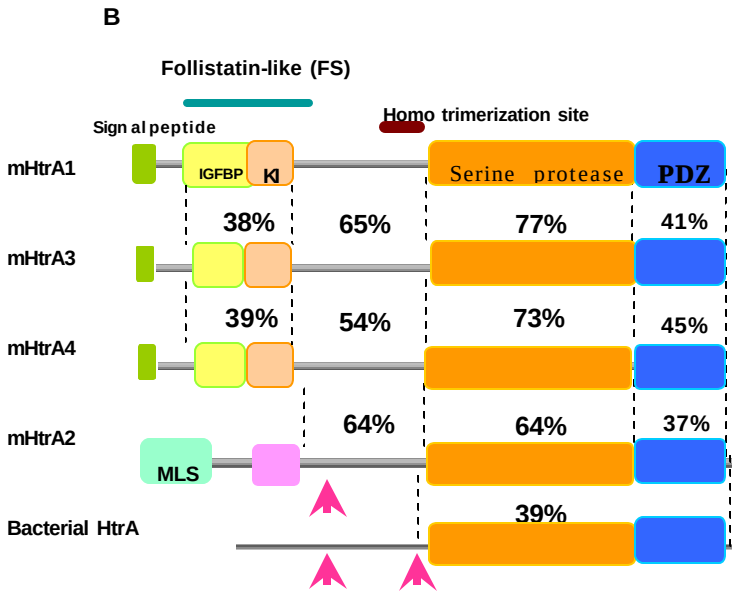

Figure 1 Domain organization of HtrA family members (A) The domain organization of selected HtrA family from prokaryotes to eukaryotes. The position of signal peptides (SS) and transmembrane segments (TM) are taken from database entries. The domain or family identifiers used are IPR001254 trypsin family, IPR001940 S2C(HtrA)serine protease family, IPR001478 PDZ domain, IPR000867 insulin growth factor binding domain (IGFBP), and IPR002350 Kazal proteaseinhibitor (KI) domain. The trypsin and PDZ total numbers include those detected by sequence similarity. (B) The domain organization of mouse HtrA family members compared with bacterial HtrA. IGFBP: IGF binding protein domain; KI: Kazal type inhibitor domain; MLS: mitochondria localization; TM: transmembrane, ▲ cleavage site

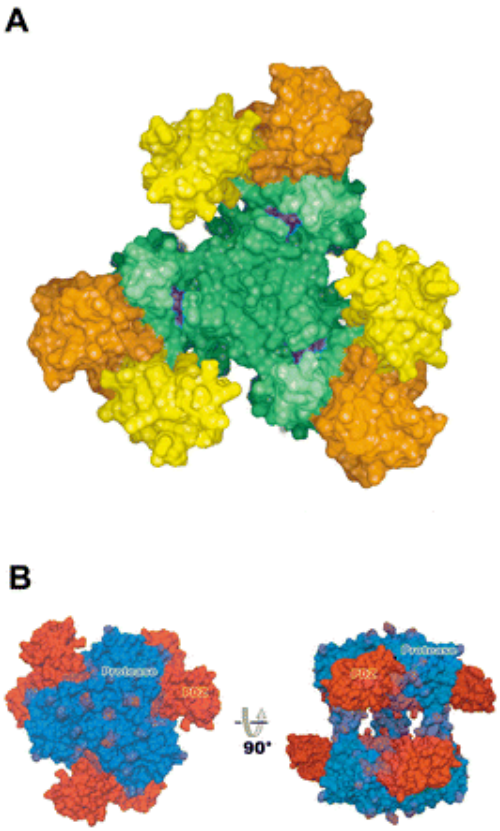

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Figure 2 Overall Architecture of HtrA

(A) Molecular surface of one trimeric funnel that represents the functional unit of HtrA proteins. The location of different DegP domains (protease in green, PDZ1 in yellow, and PDZ2 in orange) and the proteolytic sites (blue) are indicated.

(B) Molecular surface of the DegP hexamer colored by the thermal motion factors (blue, rigid portions; red, flexible portions). The protease domains build up a solid molecular cage with the PDZ domains acting as tentacular arms controlling the lateral access to the inner cavity

Each subunit is composed of one protease domain at the amino terminus and one or two PDZ domains at the carboxyl terminus. In the crystal structures of *E.coli* HtrA and human HtrA2, PDZ domains are proposed to mediate the initial binding of substrates (Krojer et al, 2002) or to be involved in modulation of protease activity (Li et al, 2002) (Fig. 2). This trimer structure is essential for the proteolytic activity. Structure of N-terminal regions of mammalian HtrA1, 3 and 4 are distinct from that of HtrA2 (Clausen et al, 2002). They commonly contain secretory signals at the N-terminus followed by two domains, one similar to the insulin-like growth factor (IGF) binding protein and the other to the Kazal-type serine protease inhibitor. HtrA2 contains a mitochondrial localization signal and a transmembrane region in the N-terminal region and localized in the intermembrane space of mitochondria. HtrA2 was originally described as an apoptosis inducer that binds to inhibitor of apoptosis proteins (IAPs), thereby suppressing the caspase-inhibitory activity of IAPs (Martin et al, 2002; Suzuki et al, 2001). Recently, it was reported that mutation of mouse HtrA2 caused a neurodegenerative disease due to progressive mitochondrial damage (Vaux and Silke 2003). The primary function of HtrA2, therefore, seems to handle misfolded proteins in mitochondria. In contrast with HtrA2, the precise function of HtrA1, -3 and -4 are less understood. HtrA1 was initially identified as a gene that was downregulated in SV40-transformed human fibroblasts (Zumbrunn and Trueb 1996). The expression of HtrA1 was also decreased in progression and invasion of ovarian cancers and melanomas (Shridhar et al, 2002; Baldi et al, 2002; Chien et al, 2004). Overexpression of HtrA1 in highly invasive melanomas was shown to suppress proliferation and migration of tumor cell (Baldi et al, 2002). Moreover, HtrA1 can induce cell death in ovarian cancer via protease domain suggesting a potential role of these proteases in caspases-independent cell death. Thus, HtrA1 may function as a tumor suppressor

gene that involved in promoting serine-protease-mediated cell death (Chien et al, 2004). Another interesting aspect of human HtrA1 may be its possible involvement in the pathogenesis of osteoarthritis. Hu et al. reported that the content of HtrA1 protein was increased in the joint cartilage of human osteoarthritis patients (Hu et al, 1998). They notice a high degree of homology between the N-terminal region of HtrA1 and mac25, a presumed member of IGFBPs. Although mac25 was initially identified as an IGFBP, the affinities of mac25 to IGFs are orders of magnitude lower than those of authentic IGF binding proteins, such as IGFBP-3 (Oh et al, 1996). Indeed, Kato et al. noted that mac25 was more closely related to follistatin, an activin-binding protein and showed that mac25 actually bound to activin (Kato et al, 2000). It was therefore proposed that HtrA1 regulates biological process by modulating growth factor systems other than IGF, such as the system mediated by the transforming growth factor-ß (TGF-ß) family to which activin belongs. Oka et al. have investigated expression pattern of mouse HtrA1 during embryogenesis in detail and found that HtrA1 was characteristically expressed in a distinct set of embryo tissues where the development was largely regulated by TGF-ß family proteins (Oka et al, 2004). For instance, HtrA1 is expressed in skeletal tissues, such as rudimentary tendons and ligaments, cells surrounding mesenchymal condensations that later forms bones, and cells in future joint areas. Development of these tissues is regulated by BMPs, GDFs and TGF-ßs (Brunet et al, 1998; Capdevila and Belmonte 2001; Francis-West et al, 1999; Schweitzer et al, 2001). HtrA1 was also expressed in the developing endocardial cushion where epithelial-to-mesenchymal transformation (EMT) was mainly regulated by TGF-ß2 (Nakajima et al, 2000; Sandford et al, 1997). TGF-ß-regulated EMT is also a fundamental process involved in the malignant progression and metastasis of cancers (Moustakas et al, 2002). Maintenance of normal chondrocytes in the joint cartilage requires intricate regulation

by TGF-ßs, and transgenic mice with disturbed TGF-ß signaling show phenotypes very similar to human osteoarthritis (Serra et al, 1997; Yang et al, 2001). All these findings suggest close association of HtrA1 and TGF-ß signaling. Our previous investigation has proved that HtrA1 binds to a variety of TGF-ß family proteins, and that HtrA1 inhibits signaling of at least BMP2, -4 and TGF-ß1 not only in vitro but also in vivo. Surprisingly, the signal inhibition activity depends totally on the proteolytic activity of HtrA1 (Oka et al, 2004). The actual mechanism how the secretory protease inhibits TGF-ß signaling remains elusive. HtrA1 seems not to degrade TGF-ß molecules or their receptors. The most plausible mechanism may be degradation by HtrA1 of some components of ECM that support signaling of various TGF-ß family proteins (Kresse and Schönherr 2001).

1.2 TGF-b signaling

The TGF-β family comprises over 30 different cytokines including TGFβs, BMPs, GDFs and activin. These factors control biological processes such as embryogenesis, organogenesis, morphogenesis of tissues like bone and cartilage, vasculogenesis, wound repair and angiogenesis, hematopoiesis and immune regulation (Massague and Chen, 2000).

TGF-βs family exert their biological effects through binding to a heteromeric complex of type I and type II transmembrane serine/threonine kinase receptor. The type II receptors then phosphorylates the type I receptors, which in turn phosphorylate activating R-Smads proteins (Attisano and Wrana, 2000; Massague and Wotton, 2000; Miyazono et al, 2001). In vertebrates, R-Smads include Smad2 and Smad3 (which are activated in response to TGFβ and activins) and Smad1, Smad5 and Smad8 (which are specifically regulated by BMP and GDF factors). Once activated, receptor-regulated

Smad (R-Smad) bind Smad4 and the complexes translocate to the nucleus and in conjunction with other nuclear cofactors, regulate the transcription of target genes. A third group of Smad proteins, I-smads or inhibitory Smads, including Smad6 and Smad7, negatively regulate TGF-β signaling by competing with R-Smads for receptor or Co-Smad and by targeting the receptors for degradation. (Christian and Nakayama 1999) (Fig. 3).

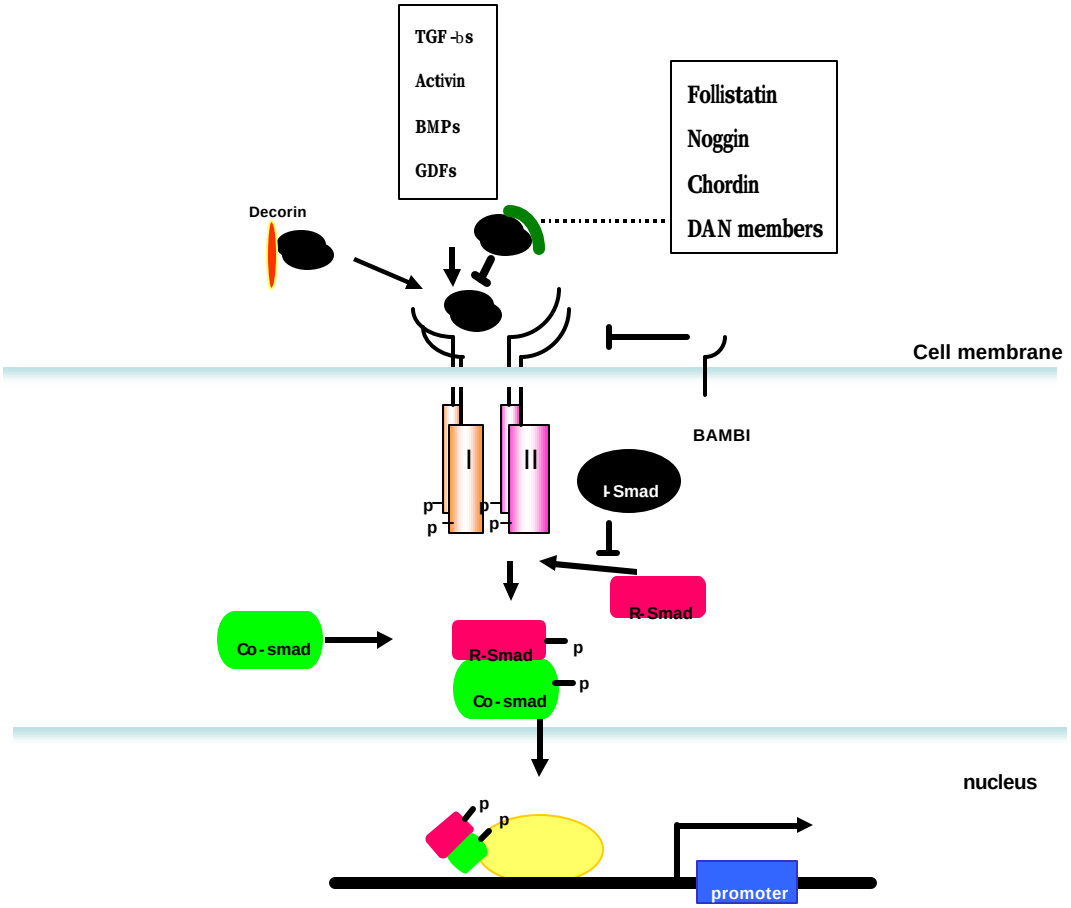

Figure 3 Transforming growth factor-β (TGF-β) signaling pathway. Ligand binding-induced heteromerization of type II and type I receptors leads to type I receptor phosphorylation by type II receptor followed by R-smad phophorylation by type I receptors. A complex is formed of phosphorylated R-Smad with the Co-Smad, and this complex translocates to the nucleus where it regulates transcription.

1.3 Modulation of TGF-b signaling

The signals between cells are necessary to coordinate all aspects of development, from patterning of the embryonic body axis to homeostasis of adult tissues. For this reason, TGF-β signaling is subjected to be regulated at multiple levels: intracellularly, at the membrane site, and extracellularly. The first level of regulation resides within the cell cytoplasm, where inhibitory Smads and Smurfs act as modulators. The second level of regulation at the membrane site, the transmembrane protein BAMBI (BMP and Activin membrane-bound inhibitor), playing a role in attenuating BMP signaling, was identified in Xenopus. BAMBI encodes a TGF-β pseudoreceptor, which stably associates with TGF-β family receptors and interferes with BMP, Activin, and TGF-β signaling (Onichtchouck et al., 1999; Grotewold et al, 2001). Finally, in the extracellular space, several proteins such as Follistatin, Noggin, Chordin and the related factors have been identified that bind TGF-β family members and prevent their contacts with signaling receptors (Fig. 3).

1.3.1 Intracellular Modulation

Inhibitory Smads (I-Smads) consist of Smad6 and Smad7, antagonize the TGF-β signaling pathway either by interacting with phosphorylated type I receptors and thereby preventing the activation of R-Smads, or through competition with Co-Smads for the formation of the R-Smad/Co-Smad complexes (Itoh et al, 2001). Multiple stimuli, including epidermal growth factor (EGF) and other TGF-β signaling members such as TGF-β1, Activin, and BMP-7 induced the expression of I-Smads. The expression of I-Smads appears to be regulated by a negative feedback loop.

1.3.2 Membrane Receptor Modulation

The accessory receptors betaglycan and endoglin

The TGF-β receptor complex contains two auxiliary co-receptors named endoglin and betaglycan (Cheifetz et al, 1992; Lopez-Casillas et al, 1991; Wang et al, 1991). These are transmembrane proteins with large extracellular domains and serine/threonine-rich cytoplasmic regions without consensus signaling motifs. Endoglin is a homodimeric integral membrane glycoprotein composed of disulfide-linked subunits of 90-95 kDa. Endoglin has been shown to modulate cellular responses to TGF-β1 (Letamendia et al, 1998; Barbara et al, 1999; Kastres et al, 1996). However, the molecular mechanism of this modulation is not known. Endoglin was shown to bind to TGF-β1, TGF-β3, BMP-2 and BMP-7 in the presence of the signaling receptor type I and II and modulates TGF-β1-dependent cellular responses (Letamendia et al, 1998; Lastres et al, 1996; Li et al, 2000). In human, it is expressed at high levels on vascular endothelial cells and several evidence support an important role for endoglin in cardiovascular development and vascular remodeling (Qu et al, 1998; Vincent et al, 1998). Endoglin has also been implicated in the regulation of trophoblasts differentiation, a process stimulated by activin and inhibited by TGF-β1 and TGF-β3 (Gougos et al, 1992). Endoglin also interact with activin type II receptors, it might be functioning in activin as well as TGF-β receptor complexes during placental development.

Betaglycan (type III TGF-β receptor) is a membrane-anchored proteoglycan whose core protein binds with high-affinity to TGF-β1, TGF-β2 and TGF-β3. The heparin sulfate and chondroitin sulfate chains of betaglycan do not have a role in TGF-β binding or interaction with signaling receptors. Betaglycan binds all three TGF-β forms with high affinity and facilitates TGF-β binding to the type II receptor. The role of

betaglycan as a facilitator of TGF-β binding to the signaling receptors is most evident with TGF-β2. Transfection of betaglycan promotes TGF-β2 binding and activity in hematopoietic progenitor cells (Massague et al, 1998).

Pseudoreceptor BAMBI

BMP and activin membrane bound inhibitor (BAMBI) has been identified as an inhibitor of BMP signaling during Xenopus embryonic development (Onichtchouck et al, 1999). BAMBI/Nma is structurally related to type I serine/threonine kinase receptors in the extracellular domain, but it lacks the intracellular serine/threonine kinase domain (Onichtchouck et al, 1999;Grotewold et al, 2001). The pseudoreceptor antagonizes the effects of TGF-β, Activin, and BMPs by stably associating with serine/threonine kinase receptors, thus preventing the formation of active receptor complexes (Onichtchouck et al, 1999)

1.3.3 Extracellular Modulation

Various families of diffusible ligand-binding proteins modulate the activity of TGF-β factors. These proteins prevent ligand access to the signaling receptors (Wharton et al, 1993; Dale and Wardle, 1999; Dale and Jones, 1999; De Robertis et al, 2000). These proteins may contribute to the formation of morphogen gradients during embryogenesis, to the relay of signals by extracellular signal transduction pathways, and to homeostasis of signaling in tissues (Barth et al, 1999; Liem et al, 2000; Hama and Weinstein, 2001). In vertebrate, the BMP antagonists comprises more than seven proteins, including noggin, chordin, chordin-like, follistatin, FSRP, the DAN/Cerberus protein family and sclerostin.

Noggin

The Spemann organizer, a signaling center at the dorsal lip of the Xenopus gastrula blastopore, secreted BMP antagonists, Noggin and Chordin. Both Noggin and Chordin bind to BMP4 but not to TGF-β or activin. In mouse, a noggin homologue is expressed in specific regions of the nervous system (McMahon et al, 1998). However, knockout experiments in mice did not show any effect of noggin in the formation of neural tissue, notochord, or somites during the early gastrulation stages (McMahon, 1998). The result of noggin/chordin double knockout mice showed that loss of noggin function could be rescued by chordin (Bachiller et al, 2000). Both proteins showed an overlapping expression at the midgastrula stage. Absence of both noggin and chordin expression led to inappropriate anteroposterior, dorsoventral, and left-right patterning. In later stage of mouse embryonic development, noggin expression was seen in the notochord, the roof of the neural tube, the dorsal aspect of the somites, and the limbs. Noggin is known to bind and antagonize BMP-2 and -4 (Zimmerman et al, 1996). Several studies demonstrated that noggin inhibits BMP-4 activity in a competitive manner by binding to BMP-4 and consequently interfering with the ability of BMP-4 to bind to cell-surface receptors (Zimmermann et al, 1996). Additionally, noggin has been shown to interact directly with GDF-5 in vitro and influences its effect in vivo. Merino et al. suggested a possible antagonistic role for noggin in regulating the signaling of GDF-5 during digit skeletogenesis in the embryonic chick digit bud (Merino et al, 1999).

Chordin

Like Noggin, chordin plays major roles in dorsoventral axis formation and in the induction, maintenance, and/or differentiation of neural tissues during gastrulation (Streit et al, 1998; Sasai et al, 1994; Miller-Bertoglio et al, 1997). When chordin is

secreted from the organizer, it acts by interfering in BMP signaling, allowing dorsally derived tissues, such as neuroectoderm and somatic muscle, to develop (Miller-Bertoglio et al, 1997). Chordin expression in the mouse is detected in most major organs, like the brain, lung, liver and kidney, suggesting multiple functions in organogenesis. Chordin expression is relatively high in condensing and differentiating cartilage elements, where it is coexpressed with BMP-2, -4 and -7, indicating an important role in embryonic skeletogenesis (Scott et al, 2000). Chordin binds to BMP-2-,4 and -7 in the same way as noggin, with a higher affinity for BMP-2 and -4 and antagonizes BMP signaling by blocking the binding to the BMP receptors (Piccolo et al, 1996).

Follistatin

Follistatin was identified as an activin-binding protein that prevents activin from binding to its receptor (Nakamura et al, 1990; Hemmati-Brivanlou et al, 1994). It has been shown that follistatin binds BMP-2, -4 and -7 with higher affinities for BMP-7 (Yamashita et al., 1995; Iemura et al, 1998). Iemura et al. demonstrated that follistatin can inhibit the effect of BMP-2, -4 and -7 differently from noggin and chordin, by binding to BMP receptors through BMPs, forming a trimeric complex (Iemura et al, 1998). Activin signaling has been implicated in early limb development (Stern et al, 1995) and in skeletal muscle differentiation (Link and Nishi, 1997), and probably also in limb skeletogenesis. Merino et al. suggested the role of activin in the control of digit formation (Merino et al, 1999).

Glycoprotein antagonists of TGF-b superfamily ligands

Small leucine-rich proteoglycans (SLRP)

The biological implications of the interaction between small proteoglycans and the isoforms of TGF-β have been most intensely investigated. At least four SLRP members (decorin, biglycan, lumican and fibromodulin) interact with TGF-β (Hildebrand et al, 1994). Many studies have confirmed a regulatory role of decorin for the activity of all isoforms of TGF-β (Yamaguchi et al, 1990; Ruoslahti and Yamaguchi, 1991). Biglycan and fibromodulin, two other members of the family of leucine-rich proteoglycans have also been reported to bind to TGF-β with distinct affinities (Hildebrand et al, 1994), but their effect on the bioactivity of TGF-β is unknown. The transfer of decorin cDNA into rat skeletal muscle increased the amount of decorin protein in skeletal muscle as well as in the kidney, where decorin had a marked therapeutic effect on fibrosis of glomerulonephritic rat. Interestingly, the levels of glomerular TGF-β1 mRNA and TGF-β1 protein were significantly reduced in decorin-transduced glomerulonephritic rat (Isaka et al, 1996). This result suggested that decorin not only affects TGF-β bioactivity by complex formation but, by some other direct or indirect mechanism, affects TGF-β synthesis or stability. The ectopic expression of decorin in glioma cells greatly inhibited TGF-β synthesis and release into the cell culture medium in these cells (Stander et al, 1998). The strong antifibrotic properties of decorin in vivo have recently been confirmed in a hamster model of lung fibrosis induced by bleomycin (Giri et al, 1997). Addition of recombinant decorin blocks TGF-β -dependent growth stimulation or inhibition of cells, indicating that the mechanism of decorin activity is the neutralization of TGF-β activity. Both blocking anti-TGF-β antibodies and decorin work equally well in preventing glomerulosclerosis (Border et al, 1992; Isaka et al, 1996; Zhao et al, 1999; Kolb et al, 2001), a pathological process that can be prevented by gene therapy utilizing decorin cDNA transfected into the skeletal muscle of affected animals (Isaka et al, 1996). Similarly, the growth

response of arterial smooth muscle cells towards TGF-β was reduced upon retrovirally over-expression of decorin (Fischer et al, 2001) as was the chemotactic response of microglial cells in experimental rat glioma (Engel et al, 1999). Recent studies indicated that collagen-bound decorin might sequester the cytokine in the extracellular matrix (Markmann et al, 2000). Interestingly, no beneficial effects were seen in pulmonary fibrosis when biglycan instead of decorin was adenovirally induced (Kolb et al, 2001). On the other hand, it was shown that in several different experimental systems TGF-β remained unchanged in spite of an up to 10000-fold molar excess of decorin (Hausser et al, 1994). As biglycan is located predominantly pericellularly, this proteoglycan would be much less suited to trap TGF-β in the extracellular matrix and hence explain the unsuccessful treatments of fibrotic disorders with this proteoglycan. Other experiments indicate that biglycan-deficient bone marrow stroma cells (BMSCs) are less responsive to exogenous TGF-ß and show an increased rate of apoptosis. Studies in human diabetic nephropathy led to the conclusion that small proteoglycans might be able to remove the cytokine via the circulation or the urinary tract, again postulating that it is not the inactivation of the cytokine but the different localization of the TGF-β/decorin complexes application (Schaefer et al, 2001). In other studies, TGF-β/decorin complexes were not inhibiting but even of greater biological activity than the growth factor alone (Takeuchi et al, 1994; Riquelme et al, 2001). This may indicate that in given cells or in given biological constellations the proteoglycan/ TGF-β complex may be more efficiently presented to the TGF-β signaling receptors than the free cytokine. Controversy exists about the biological activity of the proteoglycan/ TGF-β complex. The potential role of small proteoglycans in TGF-β activation has not yet been investigated.

DAN/Cerberus Protein Family

DAN

The DAN family of vertebrate BMP antagonists includes mammalian DAN, Dante, Drm/Gremlin, Cerl and protein related to DAN and Cerberus (PRDC) (Hsu et al, 1998; Stanley et al, 1998; Simpson et al, 1999). Similar to Noggin and Chordin, DAN antagonized BMP signaling by interfering their interaction with the signaling receptors (Hsu et al, 1998). Xenopus injection studies, which implies a role for DAN in early gastrulation stages (Hsu et al, 1998). During mouse embryogenesis, DAN was expressed in cranial mesenchyme and somites, later in limb and facial mesenchyme. DAN was also expressed in the developing myotome (Stanley et al, 1998; Eimon and Harland, 2001). DAN expression was seen in all adult tissues, including lung, kidney, brain, intestine, stomach, and spleen. The expression profiles suggest that DAN is a modulator of inductive processes and cell growth during embryonic as well as adult life. DAN has been shown to bind BMP-2 in vitro (Hsu et al, 1998); however, interaction between DAN and any specific TGF-β family member under physiological conditions remains to be demonstrated.

Cerberus

Cerberus is a secreted growth factor expressed in the Spemann organizer and anterior endomesoderm of gastrula-stage embryos. Microinjection of Cerberus mRNA into Xenopus embryos has the extraordinary ability to induce ectopic heads, neutralize the ectoderm, duplicate heart and liver (Bouwmeester et al, 1996). Cerberus is a high-affinity BMP4-binding protein, and some of its effects are mediated by its ability to block BMP (Piccolo et al, 1999).

Gremlin

Gremlin induce a secondary axis in the Xenopus embryo (Hsu et al, 1998). Gremlin may have a role in neural crest induction and patterning, as its expression starts at the tailbud stage and is correlated with neural crest lineages (Pearce et al, 1999). Gremlin plays a role as mediator during vertebrate limb outgrowth, in both mouse (Zuniga et al, 1999) and avian (Merino et al, 1999), where it is coexpressed with BMP-2, 4 and -7, secreted factors known to play important roles in limb development. Outgrowth and patterning of the vertebrate limb are controlled by reciprocal interactions between the posterior mesenchyme (polarizing region) and a specialized ectodermal structure, the apical ectodermal ridge (AER). Sonic hedgehog (Shh) and fibroblast growth factors (FGFs) are key signaling molecules produced in the polarizing region and AER, respectively. Shh signaling by the polarizing region modulates FGF signaling by the AER, which in turn mediates the polarizing region. The BMP antagonist Gremlin regulates the signal from the polarizing region to the AER, inducing FGF4 and establishing the SHH/FGF4 feedback loop (Zuniga et al, 1999).

1.3 Rationales for Work and Inference

From previous data, HtrA1 function as inhibitor of TGF-ß family members that can bind to a broad range of TGF-ß proteins and inhibits signaling of at least BMP 2, -4 and TGF-ß1 not only in vitro but also in vivo. The expression patterns of HtrA1 are characteristically expressed in distinct embryonic tissues where TGF-ß family proteins play major roles in regulation of differentiation. HtrA3 that shares high degree of sequence and domain homologies with HtrA1, but its precise function have not yet to be

determined. I hypothesize that HtrA3 may share the functional similarity to TGF-ß antagonist with HtrA1 in developing mice, although the precise mechanism of inhibition is still unknown.

In this study, I analyzed HtrA3 expression in embryonic stages using in situ hybridization or immunohistochemical analysis to identify cell types differentially expressing these two genes. HtrA3 and-1 were mostly expressed in the same embryonic organs but exhibited complementary patterns in various tissues. However, in adult tissues, HtrA3 and -1 were highly expressed. They showed overlapping expression in ovary and placenta. A striking correlation between the expression patterns of HtrA3 and HtrA1 with the functional sites of various TGF-β proteins together with the presence of a domain similar to follistatin in HtrA1 protein suggest that HtrA3 may play a role in the TGF-β-mediated signaling. To confirm the involvement of HtrA3 in TGF-β signaling, I examined the binding activity of HtrA3 to TGF-β family proteins. Similar to HtrA1, HtrA3 can bind to all of TGF-β members. Next, I examined whether HtrA3 was able to inhibit signaling mediated by TGF-β proteins. I examined the effect of HtrA3 on BMP-2, -4 and TGF-β1 signaling by transcriptional assay in mouse C2C12 myoblast cell.

Hu et al. showed that HtrA1 was upregulated in articular chondrocytes of osteoarthritis patients (Hu et al, 1998). To identify physiological substrates for HtrA1 and -3, I tested if HtrA proteases could degrade components of cartilage, because both HtrA1 and -3 were up-regulated in the arthritic cartilage. I examined fibrillar collagens, proteoglycans, and other glycoproteins whose expression was known to be up- or downregulated in osteoarthritis (Tsuchiya unpublished data). Decorin, biglycan and a glycoprotein, fibronectin were examined, as candidates for HtrA3 substrates. HtrA3 and HtrA1 digested decorin and biglycan but not fibronectin. HtrA3 and -1,

therefore, exhibit similar substrate specificity. From these data, HtrA3 and -1 may work bifunctional molecules; as inhibitors of TGF-ß signaling and as degrading enzymes of ECM.

2. MATERIALS AND METHODS

2.1 Preparation of Tissue samples

Mouse tissues were fixed in 4% (w/v) paraformaldehyde in PBS at 4°C overnight. The tissue specimens were then incubated in 30% sucrose in PBS at 4°C overnight, embedded in OCT compound and frozen in liquid nitrogen. Frozen tissues were kept in a -80° C freezer until sectioning.

Mouse embryos at 12.5 dpc were fixed in 4% paraformaldehyde in PBS at 4°C overnight. The fixed embryos were dehydrated in methanol and stored at -20°C.

Bone samples were fixed in 4% (w/v) paraformaldehyde in PBS at 4°C, and decalcified in 10% EDTA (pH 8.0) for 2 weeks at 4°C. The buffer was changed every 2 days. The bone specimens were then dehydrated in grading concentrations of methanol and chloroform and finally embedded in paraffin.

2.2 Preparation of HtrA1 and HtrA3 probe

The HtrA1 probe (554 bp, from nucleotide 1197 to 1750) was described previously (Oka et al., 2004). Two fragments of HtrA3 cDNA cloned in pBSKS (Stratagene) were used to prepare hybridization probes; one fragment (500 bp, from nucleotide 219 to 718, refer to the sequence registered as AY037300 for nucleotide number) was derived from the 5' coding region and the other fragment (790 bp, from 1730 to 2519) was derived from the 3' noncoding region. The 5' coding probe was used in this report for Northern blot analysis after ^{32}P-labeling and for in situ hybridization after digoxigenin-labeling. The 3' noncoding probe was used to confirm the specificity of in situ hybridization signals.

Sense and antisense digoxigenin-labeled RNA probes were synthesized with a kit

(Roche Diagnostics) using T3 and T7 RNA polymerases (Boehringer Mannheim). Specificity of the HtrA1 and HtrA3 probes was confirmed by genomic Southern blotting; each probe detected only the corresponding gene fragments under standard conditions.

2.3 In situ hybridization

2.3.1 Section in situ hybridization

Air-dried sections were fixed in 4% paraformaldehyde in PBS for 30 min. The sections were incubated with proteinase K (10 mg/ml) in 10mM Tris-HCl (pH 7.6) and 1 mM EDTA for 10 min at 37°C and post fixed in 4% paraformaldehyde, in PBS for 10 min. The sections were briefly washed in PBS and acetylated two times for 10 minutes with 0.4% (v/v) acetic anhydride in 0.1M triethanolamine. The sections were briefly washed in PBS, incubated with 0.2M HCl for 10 min, and then washed in PBS for 5 min before hybridization. Hybridization was carried out in a buffer containing 50% formamide, 5xSSC, 0.1 mg/ml tRNA, and 1 μg/ml antisense probe overnight at 55°C. Hybridized sections were washes with 2xSSC (15 min at 55° C), twice with 0.2xSSC (15 min at 55° C) and then incubated with the blocking solution containing 0.5% blocking reagent (Boehringer Mannheim) and 20% sheep serum for 1 hr at room temperature. After blocking, the samples were incubated with alkaline phosphatase-labeled an anti-digoxigenin antibody at 4°C overnight. The bound antibodies were detected using BCIP and NBT. Control was incubated with an equivalent amount of sense probe in the same way as described above.

2.3.2 Whole mount in situ hybridization

The embryos were rehydrated in PBS and treated with 50μg/ml proteinase K for 1 hr.

The embryos were further fixed in 0.2% Glutaldehyde and 4% paraformaldehyde in PBST for 20 min. After two washes with PBST for 5 min each, the embryos were incubated in a prehybridization solution (50% formamide, 5xSSC and 1%SDS) at 70°C for 1 hr. Hybridization was carried out at 70°C overnight in the prehybridization solution supplemented with 1µg/ml digoxigenin-labelled probe, 50µg/ml tRNA and 50µg/ml heparin. After hybridization, excess probes were removed by washing in 5xSSC. The embryos were further washed with 2xSSC at 70°C and 65°C for 1 hr each. The embryos were then treated with 1.5% blocking reagent (Boehringer Mannheim), containing 20% sheep serum for 2 hr and incubated overnight at 4°C with alkaline phosphatase-conjugated antidigoxigenin antibody diluted 1:1000 in the blocking reagent The next day the embryos were washed and incubated with NBT/BCIP.

2.4 Immunohistochemical staining

Immunohistochemical staining was performed according to the ABC procedure (vector). Paraffin embedded sections were washed in xylene, followed by rehydration in a series of ethanol solutions. After washing with PBS, sections were incubated with antiHtrA1 serum (1:3000) or antiHtrA3 serum (1:2000) followed by incubation with biotin–labeled secondary antibody (1:1000). This was followed by tyramide signal amplification-avidin-biotin-complex method as previous described (Today et al, 1999) The sections were developed in diaminobenzidin-H_2O_2 and counterstained with haematoxylin.

2.5 RNA isolation and Northern blot analysis

RNA was isolated from different stages of embryos or from different adult mouse tissues using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) following

manufacturer's instructions. Total RNA (20μg) was size-fractionated by electrophoresis on a 1% agarose-formaldehyde denaturing gel at 40V for 3 hr and visualized with ethidium bromide. The RNA was transferred overnight to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Little Chalfont, UK) and crosslinked by UV light. Membranes were prehybridized for 2 hrs at 42°C in 50% (v/v) formamide, 6xSSPE, 5xDenhardt's solution, 1%SDS, 10mM EDTA and 10μg/ml Salmon sperm DNA. Probes were ^{32}P-labelled by random priming with the use of a-^{32}P-dCTP (Amersham-Pharmacia Biotech, Inc). Hybridization with ^{32}P-labeled cDNA probes was carried out at 42°C in the same solution overnight, followed by washing three times with 2xSSC/0.1%SDS at 65°C and washes twice with 1xSSC/0.1%SDS at 65°C. The membrane was exposed to X-ray film (Fuji film, Japan). After stripping, blots were reprobed with a β-actin probe for an mRNA loading control. The β-actin probe used was a 410 base-pair *Bgl*II-*Xba*I fragment of mouse β-actin cDNA (nucleotide 173-582).

2.6 Expression of HtrA3 in mammalian cells

A DNA fragment encoding HtrA3 tagged with tandem-aligned three myc epitopes at the C-terminus (HtrA3-myc) was cloned into pFasBac1(Gibco), yielding a bacmid transfer vector. The transfer vector was used for transformation of DH10Bac cell (Gibco). Identification and isolation of the recombinant bacmid were performed according to the instruction of the Bac-to-Bac baculovirus expression kit (Gibco).

Sf21 cells (*Spodoptera frugiperda*) were propagated as monolayer at 27°C in Grace's medium (Gibco) containing 8% fetal bovine serum (FBS) (Wako), 3.3g/L of lactalbumin hydrolysate and 3.3g/L of yeastolate, 50 mg/L penicillin (Wako), and 80 mg/L streptomycin (Wako). Transfection of sf21 cells with recombinant bacmid DNA

was performed by using Cellfectin Reagent (Gibco) according to the instructions of the manufacturer. Positive viral clones were identified by their ability to direct the expression of secreted HtrA3 protein as revealed by immunoblot analysis of culture supernatants using anti myc antibody. Optimal infection conditions were determined by varying the multiplicity of infection and time course.

For expression of HtrA3, Sf21 cells ($2x10^6$ cells/ml) were infected with the recombinant baculovirus. After 48 h, the culture medium was harvested, clarified by centrifugation at 1000 x g for 10 min and stored at −80 °C.

293T cell was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 mg/L penicillin, 80 mg/L streptomycin. The DNA fragment coding the full length HtrA1 tagged with myc epitopes at the c-terminus (HtrA1-myc) was cloned into pCDNA3 (Invitrogen). 293T cells were transiently transfected with the HtrA1 expression vector using calcium phosphate precipitation method (Jordan et al, 1996). The culture medium containing secreted HtrA1 was collected 48 hr after transfection and used for binding assay.

2.7 GST pull-down assay

Full length TGF-β1, TGF-β2, BMP4 and GDF5 were cloned into pGEX-4T-1 and transformed into DH5a. Expressions of GST-fusion proteins were induced at 37°C for 5 hr with 0.2M isopropyl-1-thiol-ß-D-galactopyranoside (IPTG). The cells were then sonicated in 20mM Tris-HCl (pH8.0), 500mM NaCl, 0.05% NP-40 and 10% glycerol. Insoluble GST-fusion proteins were solubilized as described (Groppe et al, 1998). The cell extract was centrifuged at 15000 rpm at 4°C. The pellet containing insoluble GST-fusion proteins were solubilized in solubilization buffer containing 8M urea, 50 mM Tris-HCl (pH8.0), 2mM EDTA, 1mM DTT and the solution was kept on ice for 2

hr. The solubilized GST-fusion proteins were mixed with 50 volumes of folding buffer containing 50mM Tris-HCl (pH8.0), 2mM EDTA, 1.8%CHAPS, 1.25M NaCl, 2mM reduced glutathione, 1mM oxidized glutathione and incubated at 4°C for 72 hr. Then the solution was dialyzed against the pull-down buffer that contained 20mM Tris-HCl (pH8.0), 500mM NaCl, 0.05%NP-40, 10% glycerol.

GST fusion proteins or control GST protein was incubated at 4°C overnight with HtrA3-myc produced by sf21 cells or HtrA1-myc produced by 293T in the pull-down buffer. After incubation, total extract was clarified by centrifugation at 15000 rpm at 4°C. Supernatant was incubated with glutathione 4B beads (Pharmacia) at 4°C for 1 hr. Beads were then gently centrifuged, washed five times with the pull-down buffer and boiled in 2xSDS sample buffer (100mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% Bromophenol Blue, 20% glycerol). The eluted proteins were separated by SDS gel electrophoresis and detected by Coomassie brilliant blue (CBB) staining or by Western blotting using anti myc antibody.

2.8 Proteolysis Cleavage Assay

A HtrA3 mutant with truncation in N-terminal 137 amino acids, ΔN HtrA1, a HtrA1 mutant with truncation in N-terminal 150 amino acids, ΔN HtrA1 and their derivatives with mutation of active serine replaced with alanine, ΔN S311A HtrA3 and ΔN S328A HtrA1 were produced as histidine-tagged proteins in BL21 using the pET 32a vector. Production of histidine-fusion proteins were induced at 20°C for 24 hr with 0.2 M IPTG. The cell was then sonicated in 20mM Tris-Cl (pH7.6), 500mM NaCl. The cell extract was centrifuged at 15000 rpm at 4°C and the supernatant containing soluble proteins were purified using Ni-NTA agarose (Qiagen).

The proteolysis activity of these proteins was detected by incubating with various

substrates in a 20µl reaction mixture containing (5-10 µg of β-casein, BSA, decorin and biglycan, or fibronectin) in 50 mM Tris-HCl (pH 7.6) for 12 hr at 37°C. When decorin or biglycan was used, the reaction was terminated with 1 µl of 100 mM phenylmethylsulphonyl fluoride (PMSF) and then glucosaminoglycan (GAG) chains were removed by treatment with 0.02 u of Chondrotinase ABC (Seikagaku, Japan) at 37°C for 1 hr. The reaction products were boiled for 5 min with SDS sample buffer and separated by SDS gel electrophoresis and stained with CBB

2.9 Luciferase assay in C2C12 cells

C2C12 myoblasts were maintained in DMEM (Gibco) containing 15% FBS (FBS, Gibco). The C2C12 cells were seeded in 24-well plates at a density of 1.5 x 10^4 cells/well. After an overnight incubation, the medium was replaced with DMEM containing 2% FBS. Four hours later, cells were transfected by calcium phosphate method (Jordan, 1996). To assay signaling of BMP-4, cells were transfected with 250 ng/well of Id985wt-luc reporter plasmid, 125 ng/well of pME-ß-gal, 50 ng/well each of pcDEF3-smad1 and pcDEF3-smad4 , 50 ng/well of pcDNA-BMP4 or 3 ng/well of CaBMPR-1B and the expression vector for HtrA1-myc or HtrA3-myc (0-200 ng/well). After 16 hrs of transfection, medium was replaced with fresh medium containing 2%FBS. Twenty four hour after medium change, culture medium was collected for protein assay and cell lysate was prepared for luciferase and β-galactosidase assay. Luciferase activity was measured using a kit (PicaGene, Toyo Ink) and normalized with β-galactosidase activity. For BMP-2 signaling assay, pcDNA3-BMP-4 was omitted from the transfection and recombinant human BMP-2 (15 ng/well) was added to the medium at the end of transfection. For TGF-ß1 assay, the SBE-luc reporter was used and recombinant human TGF-ß1 (5 ng/well) was added at the end of transfection. To

assay the effect of decorin on TGF-ß1 signaling, cells were transfected similarly with TGF-ß1 assay and pcDNA3-Decorin was added.

2.10 Antibody against HtrA3

An N-terminal peptide of mouse HtrA3 (aa 25 to 128) was produced in *E. coli* as a histidine-fusion protein using pET-28 vector (Novagen). The bacteria were sonicated in PBS(-) and clearified by centrifugation at 15000 rpm for 10 min. The supernatant containing soluble proteins were purified using Ni-NTA agarose (Qiagen). The purified protein was used to immunize two rabbits (NZW). About 1 mg/ml of fusion protein was mixed with an equal volume of Freund's complete adjuvant (Wako) and injected intradermal. The animals received a booster injection with 0.5 mg/ml of the same antigen in Freund's incomplete adjuvant five times every 3 weeks. Two weeks after infection, blood samples were collected. The sera were prepared and tested for sensitivity and specificity for HtrA3 protein. The antiserum produced did not cross react with HtrA1 (Fig. 11C).

2.11 Purification of anti HtrA3

The antibody against HtrA3 was purified by immunoaffinity. The GST fusion N-terminal HtrA3 was produced in *E.coli* and was purified by glutathione 4B beads (Pharmacia). The purified proteins were coupled with Hitrap NHS-activated HP column (Pharmacia) and washing with buffer A: 0.5M ethanolamine, 0.5M NaCl, pH8.3 and buffer B: 0.1M acetate, 0.5M NaCl, pH4.0 for deactivating column for 30 min. For purification antibody, the column was equilibrated with a binding buffer containing 75mM Tris-Cl, pH 8.0 and then the antibody was applied to column. The column was washed 10 volumes of binding buffer and eluted with an elution buffer containing

100mM glycine-HCl, 0.5M NaCl, pH 2.7. The eluent was immediately neutralized with 1M Tris, pH8.0. In each step, the solution passed through column was collected and measured at OD280 nm (Fig. 16). The purified antibody was checked for its specificity and sensitivity by western blotting.

2.12 Immunoabsorption and western blotting

For immunoabsorption, N-terminal of HtrA3 protein was mixed with Ni-NTA agarose (Qiagen). The mixture was incubated with rotation at 20°C for 1 hr then the anti HtrA3 was added and incubated with rotation at 20°C overnight and subsequently centrifuged at 4000 g for 1 min. The supernatant was collected and checked for western blotting.

2.13 Induction of mouse arthritis

Arthritis was induced in Balb/c mice by injecting a mixture of anti-collagen type II monoclonal antibodies followed by lipopolysaccharide injection using a kit (Arthritogenic mAb Cocktail, Immuno-Biological Laboratorie, Japan). Swelling of joints appeared 3 days after lipopolysaccharide injection and reached maximum after 6-9 days as reported (Wallace et al, 1999). The joints were collected 6, 9 and 13 days after lipopolysaccharide injection and fixed in 4% paraformaldehyde for immunohistochemical analysis. For western blotting, the condylar cartilage and articular cartilage from the tibial plateau was separated at the cartilage-bone interface with the aid of a dissecting microscope, extraneous tissue removed, and the cartilage finely diced. Tissues were extracted at 4°C in 4M GuHCl in 50 mM potassium acetate buffer (pH 5.9),10 mM EDTA, 5mM benzamidine-HCl, 100 mM 6-amino-n-caproic acid, 0.35 mg/ml Pefabloc-SC (4-(2-aminoethyl)-benzenesulphonyl-fluoride,

hydrochloride) (Roughley et al, 1994).

2.14 Preparation of protein extract from mouse placentae

Mouse placentae from 10.5 dpc were collected and homogenized at 4°C in 75 mM Tris-Cl, pH 7.4, 0.1% SDS. The mixture was centrifuge at 15000 rpm at 4°C for 10 min and supernatant was collected and checked by western blotting.

3. RESULTS

3.1 Expression patterns of mouse HtrA3

HtrA3, a new member of the mammalian HtrA serine protease family, shares the similar domain structure and high homology with HtrA1 (Fig. 4). Base on their structural resemblance, HtrA3 and -1 have been speculated to have similar activities. However, the precise function of HtrA3 is still largely unknown. To elucidate physiological roles of HtrA3 protein, I examined its expression pattern by Northern blotting, in situ hybridization and immunohistochemical staining by using antibody against mouse HtrA3. Result from Northern analysis indicated that transcription of HtrA3 was developmentally regulated during mouse embryogenesis similar to HtrA1. In E9.5, a very low level of HtrA3 transcripts could be detected, while HtrA1 was first detected at a slightly later stage (E10.5) stage (Oka et al, 2004). The HtrA3 transcript was gradually increased and became most abundant at E16.5 and its level continued until after birth (Fig. 5A).

Expression patterns of HtrA3 and HtrA1 in developing eye

HtrA3 and HtrA1 in embryonic eye showed different expression patterns. In whole mount in situ hybridization, the HtrA3 probe showed a round-shaped expression pattern, while the HtrA1 probe stained a ring-shaped area just outside the HtrA3 expression area (Fig. 6 a-b). The sections of embryo eye indicated that HtrA3 was expressed in the anterior surface of the lens but was not expressed in the lens fibers (Fig. 7, a-d). The expression still continued during embryonic stages but could no longer detected in the adult eye. The onset of the expression of HtrA3 was found in E10.5 embryo when the lens placode has began to invaginate and separates from the surrounding ectoderm to

form a lens vesicle, and eventually the cells of the lens vesicle differentiated into fiber cells characteristic of the adult lens. At the same time, the optic cup has started to form and gives rise to the neural retina, the retina pigment epithelium and the epithelia of the iris and the ciliary body where HtrA1 was expressed (Fig. 7, f-i). The high expression in the ciliary body, iris and cornea was retained in adult mouse (Oka et al., 2004). In adult eye, HtrA3 and HtrA1 show the similar expression pattern in retina at the ganglion cell layer and the inner nuclear layer of the retina. However, HtrA3 showed very weak expression compared with HtrA1. These data agree well with the Northern blot analysis, which showed that HtrA1 was expressed at a much higher level than HtrA3 in the adult eye (Fig. 5B). These data suggested that these two very similar genes could have complementary functions in the developmental regulation of the eye. Several studies have reported that BMP signaling is required for eye development. BMP family genes are expressed during eye development (Luo et al., 1995; Dudley et al., 1995; Dudley and Robertson, 1997; Furuta and Hogan, 1998; Hung et al, 2002). The BMP4 transcript is present in both the presumptive lens and presumptive retina but is expressed predominantly in the dorsal optic cup where primary fiber cell differentiation begins at E11.5 (Furuta and Hogan, 1998). Knockout studies have shown that BMP-4 and-7 are essential for early morphogenesis of eye (Furuta and Hogan, 1998; Luo et al., 1995; Dudley et al., 1995; Jena et al, 1997; Wawersik et al, 1999). Inhibition of BMP signaling by Noggin can suppress lens size in explant culture (Faber et al, 2002) and block the formation of the ciliary processes (Zhao et al., 2001)

Expression of HtrA3 and HtrA1 in developing heart and blood vessels

In E16.5 embryo heart, a weak expression of HtrA1 was detected by Northern blot but

HtrA3 was barely expressed (Fig. 5B). To localize the HtrA3 expression regions in the heart, the situ hybridization and immunohistochemical analysis were carried out. In the E14.5 heart, HtrA1 was detected in the endocardial cushion. (Fig. 8, c and d) and it showed strongly expressed in the outflow tracts including valves (Oka et al., 2004). Development of artrioventricular endocardial cushion and outflow tracts depends on signaling by TGF-β proteins. TGF-β induces epithelial-mesenchymal transition (EMT) of endothelial cells in these regions, endowing them with mesenchymal properties (Nakajima et al, 2000). Indeed, TGF-β2 knockout mice show defects in the cardiac septa and valves due to disturbed EMT (Sanford et al, 1997).

In the trachea of E16.5 embryo, HtrA3 was expressed in the outer layers, whereas HtrA1 was localized predominantly in lamina propria (Fig. 8, e, f, h and j). At the same stage in aorta, HtrA3 was expressed in the adventitia and HtrA1 in the intima, the innermost layer, made up of endothelial cells (Fig. 8, e, g, h and i). The outer most layer of large vessels, the adventitia, consists of loose connective tissue containing smaller blood vessels and nerves. The blood vessels in the embryo are formed by vasculogenesis, where vessels form de novo in embryonic mesenchyme. Endothelial precursor cells that are dispersed throughout embryonic mesenchyme form an aggregate and produce a small single layered endothelial tube with a lumen. Vasculogenesis sets up the initial major blood vessels in the developing embryo in a primitive vascular labyrinth (Carmeliet, 2000; Risau and Flamme, 1995; Risau, 1997). TGF-β superfamily regulates vascular smooth muscle recruitment, development and maintenance. Several TGF-β superfamily proteins, receptors and intracellular signal transduction proteins are expressed in the vasculature (Pelton et al, 1990; Schmid et al, 1991; Roberts and Sporn, 1992; Pepper 1997). During the development of the chicken arterial system, type I, II, and III receptors for TGF-β are expressed in both the

neural-crest-derived smooth muscle cells in the pharyngeal arch arteries and the mesoderm-derived smooth muscles cells in more distal parts of the arteries (Topouzis and Majesky 1996). In addition, the type II receptor is also expressed in the endothelial cells of the developing vessels, suggesting a possible important role for TGF-β in endothelial cell cytodifferentiation (Brown et al, 1996). The expression patterns of TGF-β3 and its receptors during vascular development suggest that TGF-β3 acts in an autocrine fashion to regulate smooth muscle cell differentiation during the formation of the tunica media, and that it may play a role in the epithelial differentiation of the vascular endothelium via a paracrine mode of action. In vivo studies focusing on loss of function of various TGF-β genes in the mouse indicate roles of the TGF-β signaling pathway in vascular development.

Expression of HtrA3 and HtrA1 in ovary and placenta

The placenta expressed the highest levels of both HtrA3 and HtrA1. (Fig. 5B). In situ hybridization revealed that HtrA3 are expressed diffusely in the labyrinthine layer with patchy areas showing strong expression (Fig. 9, a and b). On the other hand, HtrA1 showed a patchy expression pattern (Fig. 9, c and d). As judged from the distribution, shape and size of the stained cells, it is most likely that HtrA1 expressing cells are labyrinthine trophoblasts (Maekawa et al, 1999). The labyrinthine area which consists of syncytiotrophoblast, chorionic trophoblast, blood vessels and stoma, functions to provide a pivotal nutrient-waste exchange compartment between the fetal and maternal blood supply in the mammalian placenta. The trophoblast branching morphogenesis is essential for the vascularization of the labyrinth, providing a framework into which vessels can grow.

In ovary, HtrA1 showed a high level of expression (Fig. 10, c and d) while HtrA3

expression seemed to be relatively lower than that of HtrA1 (Fig. 10, a and b). In consistent with the result of Northern blot analysis, the in situ hybridization signals were much lower for HtrA3 than for HtrA1 in ovary. The expression of both HtrA1 and HtrA3 was detected in granulosa cell. Granulosa cell is a major source of activin and inhibin. The function of Activin is to promote granulosa cell proliferation and to potentiate FSH action by increasing FSH receptor expression on granulosa cells. Activin can also modulate steroidogenesis in granulosa and thecal cells (Findlay, 1993; Knight and Glister, 2001; Ethier and Findlay, 2001). Moreover, the BMP receptor mRNAs are present in the ovary, with the strongest expression in the granulosa cells and the oocytes, which is consistent with the BMP actions observed on the granulosa cells (Shimasaki et al, 1999; Wilson et al, 2001). BMPRIB deficient mice showed that the absence of BMPRIB signaling leads to infertility (Yi et al, 2001).

Expression of HtrA3 in skeletal tissues

HtrA1 is characteristically expressed in the skeletal tissues of the embryo and adult mouse (Oka et al, 2004) and because HtrA1 expression was upregulated in the cartilage of human osteoarthritis patients (Hu et al., 1998). Therefore, we examined the later stage fetal, infant and adult mice in detail for the expression pattern of HtrA3 in the bone and tissues associated with the bone (Fig. 11, a-e). In E12.5 embryo, HtrA3 was expressed in the vertebral rudiments in the tail region (Fig. 6). This was in contrast with the expression of HtrA1, which showed predominant expression in the thoracic and lumbar parts of the vertebrae (Fig. 6). HtrA3 was strongly expressed in the radial part while HtrA1 was expressed in the future elbow joint of forelimb (Fig. 6). In hindlimb, the expression of HtrA1 was strongly detected at the future knee and the tarsal region of hind limb but expression of HtrA3 was weak and localized mostly in the tibial part. (Fig.

6).

In the forelimb of E14.5 embryos, HtrA1 expression was detected widely in the rudimentary tendons and ligaments and mesenchymal cells surrounding cartilaginous condensations, which later developed to bones (Fig. 11A, f). Especially, the cells in the future carpal and elbow joints showed strong expression of HtrA1. On the other hand, HtrA3 was expressed in a limited number of the tendons and ligaments (Fig. 11A, a). Mesenchymal cells in the carpal and elbow joint regions barely expressed HtrA3. Both HtrA3 and -1 were not expressed in the core of the cartilaginous condensations. Interestingly, however, when the blood vessels invaded into the condensations and ossification started in the peri- and postnatal periods, the expression of HtrA3 and -1 were tremendously upregulated (Fig. 11A, b, c, g and h). Chondrocytes probably undergoing degeneration in the ossification center produced HtrA1 and -3.

In the adult bone, HtrA3 was largely localized in the bone matrix (Fig. 11A, d and i) as in the case of HtrA1 (Oka et al, 2004). HtrA3 exhibited significant expression in the articular chondrocytes (Fig. 11A, d). On the contrary, HtrA1 was hardly expressed by mature articular chondrocytes (Oka et al, 2004). Both HtrA3 (Fig. 11A, e) and HtrA1 (data not shown) were not expressed in the hypertrophic and proliferating chondrocytes in the epiphyseal cartilage of normal mouse.

Damages in chondrocytes in osteoarthritis induced expression of HtrA1 several folds in the joint cartilage (Hu *et al.* 1998; Tsuchiya unpublished data). The expression of HtrA3 was similarly induced in mouse experimental arthritis but to a lesser extent (1.5-2.5 folds) (Fig. 3D, western). The size of the HtrA3 detected in the cartilage was smaller than the size expected from the cDNA structure or the size of HtrA3 protein produced by sf21 cells. When Western blot analysis was carried out with anti-HtrA3 serum absorbed with bacterially produced HtrA3 protein this smaller band was not

detected (data not shown). Western blotting of placenta proteins revealed the presence of both the full-length form and a smaller form of HtrA3 (data not shown). These data strongly supported that the detected band was a truncated HtrA3 that was probably produced by proteolytic cleavage. Since the polyclonal antibody against HtrA3 recognizes the N-terminal half of the molecule, it is likely that this smaller molecule is a form of HtrA3 that has intact N-terminal and protease domains but lacks the PDZ domain (predicted *Mr*=40,000). Similar degradation products have been reported for human HtrA1 in the joint cartilage (Hu et al., 1998).

Mostly chondrocytes in the superficial layer of the joint cartilage produced HtrA3 after arthritis induction (Fig. 3B, a). Chondrocytes in the epiphyseal cartilage displayed strong elevation in HtrA3 protein production (Fig. 3B, b). These findings were in contrast with the induction of HtrA1 expression, which was most prominent in the chondrocytes of the deep layer in the joint cartilage and minimum in the epiphyseal cartilage (Tsuchiya unpublished data). Again, this result suggested a complementary function of HtrA3 and HtrA1 not only in normal developmental processes but also in pathological conditions.

3.2 Antibody against HtrA3

The polyclonal antibody against HtrA3 was checked for specificity and sensitivity by western blotting using HtrA3 produced by Sf21 cells and HtrA1 produced by 293T cells. The antibody specifically recognized HtrA3, did not cross react with HtrA1, and did not show the non-specific binding with other proteins. The antibody can detect 1 ng of HtrA3 at 3000 fold dilution.

The antibody was purified by HiTrap NHS-activated HP column (Amersham Pharmacia Biotech) coupled with GST-fusion HtrA3 N-terminal fragment that was used to

immunize rabbits. The elution profile of this column chromatography was shown in figure 12A. The purified antibody lost its activity. (Fig. 12B, a-d). From this result, I used non-purified serum in all experiments of this study.

3.3 Interaction of HtrA3 with TGF-b family proteins

The above-mentioned results on expression patterns indicated that HtrA3 and -1 were expressed in common tissues and, in some regions, they were expressed in complementary manners. Based on the characteristic expression pattern of HtrA1 and its molecular similarity to follistatin, we have proposed that HtrA1 functions as an inhibitor of TGF-β signaling. Actually, we have shown that HtrA1 binds to various TGF-β family proteins and inhibits their signaling (Oka et al, 2004). To examine if HtrA3 has the similar activities, we first tested the binding of HtrA3 to various TGF-β proteins. The myc-tagged HtrA3 (HtrA3-myc) produced by sf21 cells were used for GST pull-down assay with GST-TGF-β1, -TGF-β2, -BMP4, and -GDF5. HtrA3-myc bound to all of these TGF-β proteins (Fig. 13) but not to control GST protein. As judged from the recovery of HtrA3 protein through the pull-down assay, HtrA3-myc showed preferable binding to GDF5 and BMP-4 rather than to TGF-β1 and -ß2. This is consistent with the result with HtrA1-myc, which bound to all these TGF-β proteins with the highest affinity for BMP4 (Fig. 13, Oka et al, 2004). Because HtrA1 was recovered less than 3% of input and HtrA3 more than 3% in the pull-down assay with BMP4, it was likely that HtrA3 had the same or slightly higher binding activity to BMP4 than HtrA1.

3.4 Inhibition of BMP2, BMP4 and TGF-b signaling

Next, I investigated the effects of HtrA3 on BMP2, -4 and TGF-β1 signaling in

C2C12 myoblastic cells. The transfection of C2C12 cells with the BMP4 expression vector resulted in approximately 19-fold induction of a luciferase reporter activity under the control of a BMP-responsive promoter element (Id985wt) (Fig. 14A). Similar to HtrA1, HtrA3 suppressed the BMP4 activation of this reporter in a dose-dependent manner. The effect of HtrA3 on TGF-β1 signaling in C2C12 cells was next examined using a luciferase reporter driven by a TGF-β responsive promoter element (SBE). Both HtrA1 and HtrA3 inhibited moderately the signaling induced by the recombinant TGF-β1 protein (Fig. 14C). HtrA1 displayed moderate inhibitory activity, as compare with noggin, on the activation of the Id promoter induced by recombinant BMP2 protein in C2C12 cells (Oka et al, 2004). HtrA3 also showed a weaker but significant inhibition on the recombinant BMP2 (Fig. 14D).

HtrA3 did not inhibit the signaling generated by a constitutively active BMP type-1 receptor, caBMPR-1B, which is able to originate signal transduction without ligand binding (Akiyama et al, 1997; Katagiri et al, 2002) (Fig. 14E). This clearly showed that the inhibition by HtrA3 occurred in the extracellular space and not on the signal transduction pathways in the cytoplasm.

Inhibitory activities of HtrA3 appeared weaker than those of HtrA1. However, if the levels of protein expression in the C2C12 culture media were compared by Western blotting (see inlets of Fig. 14A and E), the amounts of HtrA3 expressed were always lower that those of HtrA1. This indicates that HtrA3 may be as potent, if not more potent, an inhibitor of TGF-β proteins as HtrA1.

HtrA1 absolutely required its protease activity to inhibit TGF-β signaling (Oka et al, 2004). Consistent with this finding, a mutation of serine in the protease active center eliminated the inhibitory activity of HtrA3 on the BMP4 signaling (Fig. 14B) simultaneously with the proteolytic activity (Fig. 15A).

3.5 Proteolytic assay

β-casein could be used as a generic substrate to monitor the protease activity of HtrA3. We produced truncated forms of HtrA3 in bacteria as histidine-fusion proteins; ΔN HtrA3 with deletion in the N-terminal IGF binding protein and KI domains and ΔN HtrA3 S311A which contains the same N-terminal deletion and a mutation at the serine residue in the active center. The proteolytic activities of these proteins were compared with similarly mutated forms of HtrA1. Fig. 15A shows that ΔN HtrA3 rapidly degraded β-casein. The protease activity of ΔN HtrA3 was comparable to that of ΔN HtrA1. ΔN HtrA3 S311A and ΔN HtrA1 S328A were very inert as proteases. Recent our study on HtrA1 (Yano, unpublished data) revealed that several proteoglycans in ECM served as substrates for HtrA1. We examined decorin, biglycan and a glycoprotein, fibronectin, as candidates for HtrA3 substrates (Fig. 15B). HtrA3 digested decorin and biglycan fairly rapidly, when HtrA3 did not significantly cleave none-denatured BSA, which was a poor substrate for HtrA1 (Yano, unpublished data). Fibronectin was hardly digested by ΔN HtrA3 or by ΔN HtrA1. HtrA3 and -1, therefore, exhibit similar substrate specificity.

The mechanism for the inhibitory activity of HtrA1 on the TGF-β signaling remains elusive. One intriguing possibility is that HtrA1 digests some components of ECM that are essential for efficient TGF-β signaling. The proteolytic activity of HtrA3 on decorin and biglycan may support this notion, but physiological substrates of HtrA3 and -1 should be identified to explore the unique inhibitory activity of these serine proteases on the TGF-β signaling.

3.6 Effect of decorin in TGF-b signaling in C2C12 cells

It has been reported that decorin can inhibit physiological signaling of TGF-βs by sequestering TGF-β molecules from the vicinity of the receptor, or in some cases, decorin enhances the TGF-β signaling probably by effectively concentrating TGF-β molecules near the receptor (Yamaguchi et al. 1990; Markmann et al. 2000). In the previous Figure (Fig. 15), I showed that HtrA3 was able to digest decorin. Yano et al. also showed HtrA1 digested decorin (Yano et al, unpublished data). Therefore, I examined the effect of decorin on the TGF-β1 signaling in C2C12 cells. The active recombinant TGF-β1 protein stimulated 4 folds the induction of luciferase reporter activity under the control of the TGF-β responsive element. Transfection of pcDNA3-decorin protein at the concentration of 1 to 200 ng/well showed weak inhibition on TGF-β1 signaling (Fig. 16A). In addition, the recombinant decorin at the concentration of 1-50 ng/well also weakly inhibited TGF-β1 signaling (Fig. 16B). Next, I checked the effect of decorin on HtrA1 inhibition of TGF-β1 signaling. Figure 16C-D showed that decorin did not suppress the inhibitory activity of HtrA1 on the TGF-β1 signaling (Fig. 16C and D). These data suggest that decorin alone is not able to efficiently regulate the TGF-β signaling. Decorin was used to prevent glomerlosclerosis that was indirectly enhanced TGF-β signaling. Exogeneously administered decorin formed a complex with TGF-β signaling, the decorin/TGF-β complex should interact with other extracellular matrix molecules. Probably, HtrA1 may inhibit TGF-β signaling by digesting not only decorin but also other ECM molecules that enhance TGF-β binding to its receptor. Inhibition TGF-β1 signaling by HtrA1 may mediated indirectly by digesting decorin and the other ECM molecules, triggered upon binding of TGF-β1 to its receptor.

5. DISCUSSION

Complementary expression of HtrA3 and -1

This study characterized a new member of the mammalian HtrA serine protease family, HtrA3 that shares the similar domain structure and high homology with HtrA1. Base on their structural resemblance, HtrA3 and -1 have been predicted to have similar activities. However, since precise expression patterns of the HtrA3 gene had not been examined and compared with those of HtrA1, there remained possibility that the two genes could have non-redundant, complementary functions in different regions of organs.

Nie et al. analyzed expression of human HtrA1, -2 and -3 using multiple tissue expression arrays and showed that HtrA1 and -3 displayed a very different pattern of expression among human adult tissues (Nie et al, 2003a). The previous study revealed that the expression of HtrA1 was under intricate developmental regulations (Oka et al, 2004). It is, therefore, of particular interest to analyze HtrA3 expression in embryonic stages using in situ hybridization or immunohistochemical analysis to identify cell types differentially expressing these two genes. HtrA1 is characteristically expressed in distinct embryonic tissues where TGF-β family proteins play major roles in regulation of differentiation. For instance, HtrA1 is expressed in the developing skeletal tissues, such as rudimentary tendons, ligaments, mesenchymal cells surrounding the future bones and cells in future joint regions. HtrA3 was also expressed in these tissues, but the expression was less robust and restricted to a limited number of them. In vertebral rudiments, HtrA1 and -3 are expressed in non-overlapping regions; HtrA3 at the tail region and HtrA1 at the thoracic and lumbar regions. The embryo eye shows the most

striking differential expression patterns for HtrA1 and -3. HtrA1 is expressed in the pigment retina and ciliary body, whereas HtrA3 is expressed in the lens epithelium. Non-overlapping expression patterns were also seen in the blood vessels; the outer adventitial layer expressed HtrA3 and the inner intimal layer HtrA1. No significant expression of HtrA3 was observed in the endocardial cushion (Fig. 8, c and d), the choroids plexus of the brain and the gonad (data not shown), where HtrA1 was expressed at high levels and supposed to play important roles in regulation of TGF-β signaling (Oka et al, 2004). It is likely that HtrA1 and -3 have non-redundant functions at least in some developing tissues.

In adult mouse tissues, both HtrA1 and -3 are expressed at the highest levels in the placenta and ovary. In situ hybridization of the ovary showed that HtrA1 and -3 were produced by the granulosa cells, which are the major source of activin (Harlow et al, 2002). More robust hybridization signals were observed for HtrA1 than HtrA3 in the ovary. In the placenta, HtrA3 displayed more diffuse expression pattern than HtrA1 whose expression seemed to be restricted to the trophoblasts in the labyrinthine region. Northern blot analysis indicated that expression of HtrA3 in the placenta was much higher than in the ovary (Fig. 5B). These data suggest that HtrA3 plays important roles in the placental development and functions, and probably contributes more to them than HtrA1. This is contradictory to the result of human expression patterns (Nie et al, 2003a). In human, placental HtrA1 was expressed relatively higher than HtrA3. The usage of HtrA genes may be different in mouse and human. Based on the unique expression pattern of HtrA3 in the peri- and post-implantation uterus, Nie et al. proposed that HtrA3 plays an important role in the formation/function of the placenta (Nie et al, 2003a; Nie et al, 2003b); one possible mechanism predicted HtrA3 being an IGF binding and degrading protease. The IGF-IGF binding protein system has been

repeatedly reported to participate in the growth and development of placenta. The preliminary data indicated that HtrA1 bound to but did not cleave IGF-I in vitro. Future study will establish the intrinsic function of HtrA1 and -3 in the placenta during pregnancy.

From this result, both HtrA3 and -1 share similar domain structure and may share roles in regulating tissue function during development. Recently, the cDNA sequence of fourth member of the family, named HtrA4, has been reported (GenBank accession no. AK075205.1). Comparison of the sequence of this newly identified HtrA4 with those of HtrA1 and -3 revealed that HtrA4 share similar conserved domains with HtrA1 and -3 with high homology (Fig. 4). A comparison of the sequences of HtrA4 with all entires in the GenBank and Swissport database identified a number of expressed sequence tags (ESTs) that were highly homologous. It is of interest that these ESTs were predominantly from placenta, and from other tissues such as ovary, uterus, whole joint, bone, total fetus, and that in all these tissues, high levels of both HtrA3 and-1 were detected.

HtrA1 is expressed in joint interzones and may regulate joint development, a process closely regulated by BMP/GDF signaling (Brunet et al, 1998; Francis-West et al, 1999). It has also been proposed that signaling mediated by TGF-β subfamily participates in the maintenance of adult joints. TGF-β is required to keep articular chondrocytes in their normal, undifferentiated state. Transgenic mice carrying a dominant negative, kinase-defective TGF-β type II receptor gene (Serra et al, 1997) or Smad3 knockout mice (Yang et al, 2001) showed phenotypes very similar to human osteoarthritis. In these mice, suppression of TGF-β signaling probably promoted terminal differentiation of articular chondrocytes, leading to osteoarthritis-like phenotypes. Previous study showed that expression of human HtrA1 increased substantially in articular cartilage

cells of patients with osteoarthritis (Hu et al, 1998). Data from this present study also show that HtrA3 is increased in arthritic mice. It is possible that both HtrA3 and -1 antagonized TGF-β and aggravated osteoarthritis.

The size of the HtrA3 detected in the cartilage was smaller than the size expected from cDNA structure. After absorption with bacterial produced HtrA3 proteins, antiserum cannot recognize anymore this smaller band. Western blotting of placenta proteins revealed the presence of both the full-length form and a smaller form of HtrA3 (Fig. 11E). These data strongly supported that the detected band was a truncated HtrA3 that was probably produced by proteolytic cleavage. Since the polyclonal antibody recognizes the N-terminal half of the molecule, it is likely that this smaller molecule is a form of HtrA3 that has intact N-terminal and protease domains but lacks the PDZ domain (predicted Mr =40,000). It has been reported that autoproteolysis was occurred in the family of HtrA proteins. Gray et al. demonstrated that the autoproteolysis of human HtrA2 was occurred during import of protein to mitochondria that leads to release of pro-apoptotic HtrA2 into mitochondrial intermembrane space (Gray et al, 2000). The autoproteolysis also found in mouse HtrA1 in physiological condition (Yano, unpublished data). The recent study in bacterial HtrA has demonstrated that the autocleavage is triggered specifically under reducing conditions, and is a physiological process occurring in cells (Skorko-Glonek et al, 2003). However, the physiological meaning of the autocleavage still remain unclear.

TGF-ß signal inhibition activity of HtrA3

We have reported that mouse HtrA1 binds to various TGF-β proteins and inhibits their signaling in C2C12 myoblast cells (Oka et al, 2004). Likewise, HtrA 3 also bound to a broad range of TGF-β family proteins in the GST pull-down assay and

actually inhibited BMP4, BMP2 and TGF-β1 in co-transfection assay in C2C12 cells. Although HtrA1 is able to inhibit in vivo BMP signaling in the chick eye development, it is still un-tested if HtrA3 has the same in vivo activity. The TGF-β signal inhibition activities of HtrA3 and -1 were both absolutely dependent on their protease activities. In case of HtrA1, a deletion of a small linker region between the Kazal protease inhibitor domain and the protease domain eliminated both the protease and TGF-β signal inhibition activities (Oka et al, 2004). This small region is conserved among all mammalian HtrA proteins and essential for homotrimer formation of HtrA2 (Li et al, 2002) and HtrA1 (M. Yano, unpublished data). Since this region is also highly conserved in HtrA3, it is likely that HtrA3 forms trimer to become active as a protease and as an inhibitor of TGF-βs.

Proteolytic activity of HtrA3

The way in which HtrA3 and HtrA1 protease activity inhibits signaling by TGF-β proteins has yet to be clarified. One possibility that have to be considered is that binding to TGF-β proteins is irrelevant to the apparent inhibition of their signaling. So far, we do not have obtained evidence indicating that the binding is essential for the inhibition of TGF-β signaling. It is possible that HtrA1 digested extracellular matrix (ECM), indirectly inhibiting TGF-β signaling. Because both HtrA1 and -3 were up regulated in the arthritic cartilage, the extracellular matrix components of cartilage were tested for substrates for HtrA1 and -3. We examined fibrillar collagens, proteoglycan, and other glycoprotein whose expression was known to be up-or down-regulated in osteoarthritis (Tsuchiya unpublished data). Bacterially produced ΔN HtrA3 displayed proteolytic activity towards ß-casein. The protein of HtrA3 was as potent as ΔN HtrA1. Deletion of the N-terminal end enhanced the protease activity of HtrA1

3-times as compared with the intact, full length HtrA1 (M. Yano, unpublished result). Similarly, ΔN HtrA3 exhibited elevated protease activity (data not shown), suggesting that N-terminal regions exert regulatory function over the catalytic domains of both HtrA1 and -3.

We found that ΔN HtrA3 cleaved decorin and biglycan. TGF-β signaling and small, leucine-rich proteoglycans, such as decorin, biglycan and fibromodulin exhibit a mutual, functional interactions (Hildebrand et al, 1994). Decorin and biglycan are known to bind to several specific TGF-β proteins. The TGF-β signaling induces synthesis of these proteoglycans and they in turn modulate signal transduction of TGF-βs. Due to its TGF-β binding ability, decorin has been successfully used as an antifibrotic agent to overcome the overproduction of TGF-β in experimental glomerulonephritis (Border et al, 1992; Isaka et al, 1996). Decorin can inhibit physiological signaling of TGF-βs by sequestering TGF-β molecules from the vicinity of the receptor, or in some cases, decorin enhances the TGF-β signaling probably by effectively concentrating TGF-β molecules near the receptor (Yamaguchi et al. 1990; Markmann et al. 2000). Based on in vitro studies, a complex picture is beginning to emerge indicating the proteoglycan control cell differentiation and proliferation in a cell-specific fashion (Schonherr et al, 2001; Kresse and Schonherr, 2001) with each cell type responding with unique sets of signaling factors (Santra et al, 1997; Iozzo et al, 1999a,b; Xaus et al, 2001). It may be plausible that the primary targets of the extracellular HtrA proteases may be ECM proteoglycans, including decorin and biglycan.

Therefore, I have examined the effect of decorin on TGF-β1 signaling in C2C12 myoblast cells. In this experiment, decorin did not show any effect on either TGF-β1 signaling or did not suppress the inhibition of TGF-β1 signaling by HtrA1. Similarly, it was shown that in several different experimental systems, decorin did not affect the

TGF-β1 signaling. For example, the signal transduction of TGF-β1 in monocytes was still fully active in spite of an up to 10,000-fold molar excess of decorin (Hausser et al, 1994). It still needs to be further studied at this point. Decorin also contains binding domains for other proteins such as collagen, fibronectin, thrombospondin and TGF-β (Kresse et al, 1993; Iozzo and Murdoch, 1996; Hocking et al, 1998). Decorin binds several types of collagen in vivo, among them being types I, II and VI, and promotes fibril stability (Vogel et al, 1984; Danielson et al, 1997). By interacting with ECM molecules like fibronectin and thrombospondin, it influences cell adhesion. Additionally, its core protein interacts with TGF-β, which interact independent binding sites of collagen. Schonherr et al. has shown that collagen-bound decorin was able to interact with TGF-β and regulated the biological function of TGF-β (Schonherr et al, 1998). Decorin can modulate cellular function by interacting with other extracellular matrix molecules. Moreover, biglycan, another ECM component, has also been reported to bind to TGF-β but their effect on the bioactivities of TGF-β is unknown. Decorin and biglycan are ubiquitous components of ECM of various tissues, such as the blood vessel, dermis, tendon, ligament, sclera and articular cartilage (Rosenberg et al, 1986). The breakdown of ECM is essential in various developmental processes such as blastocyst implantation, embryonic development, tissue morphogenesis and remodeling as well as in pathological processes such as arthritis or tumor growth and invasion. The breakdown required precisely coordinated and timely controlled expression and activation of growth factors and a host of enzymes that degrade ECM proteins. HtrA3 and -1 may contribute a great deal in these processes as bifunctional molecules; as inhibitors of TGF-β signaling and as degrading enzymes of ECM.

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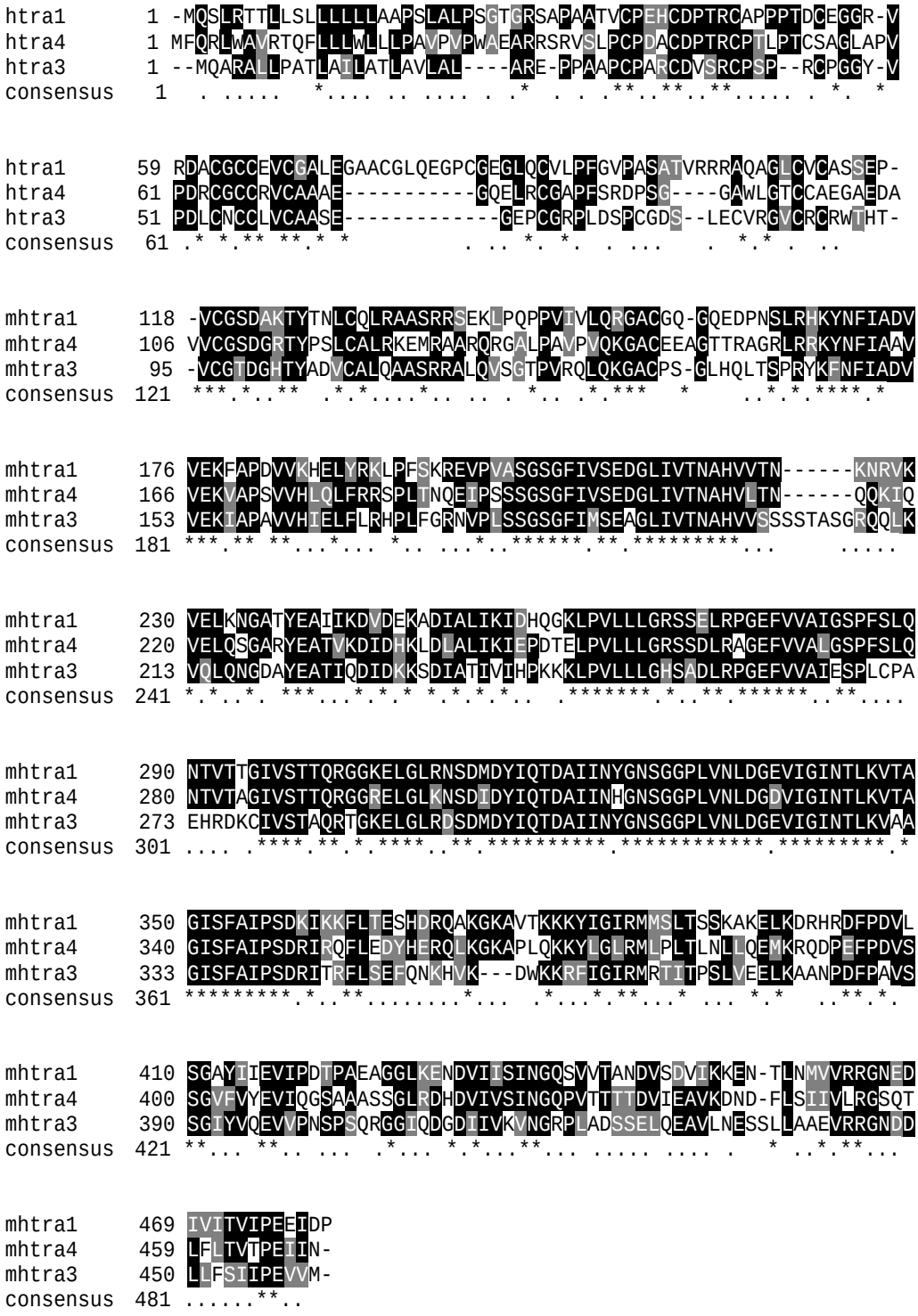


Figure 4 Comparison of the amino acid sequences of the mouse HtrA1, 4 and 3 proteins. Amino acid residues that are conserve in alignment are placed in shaded boxes.

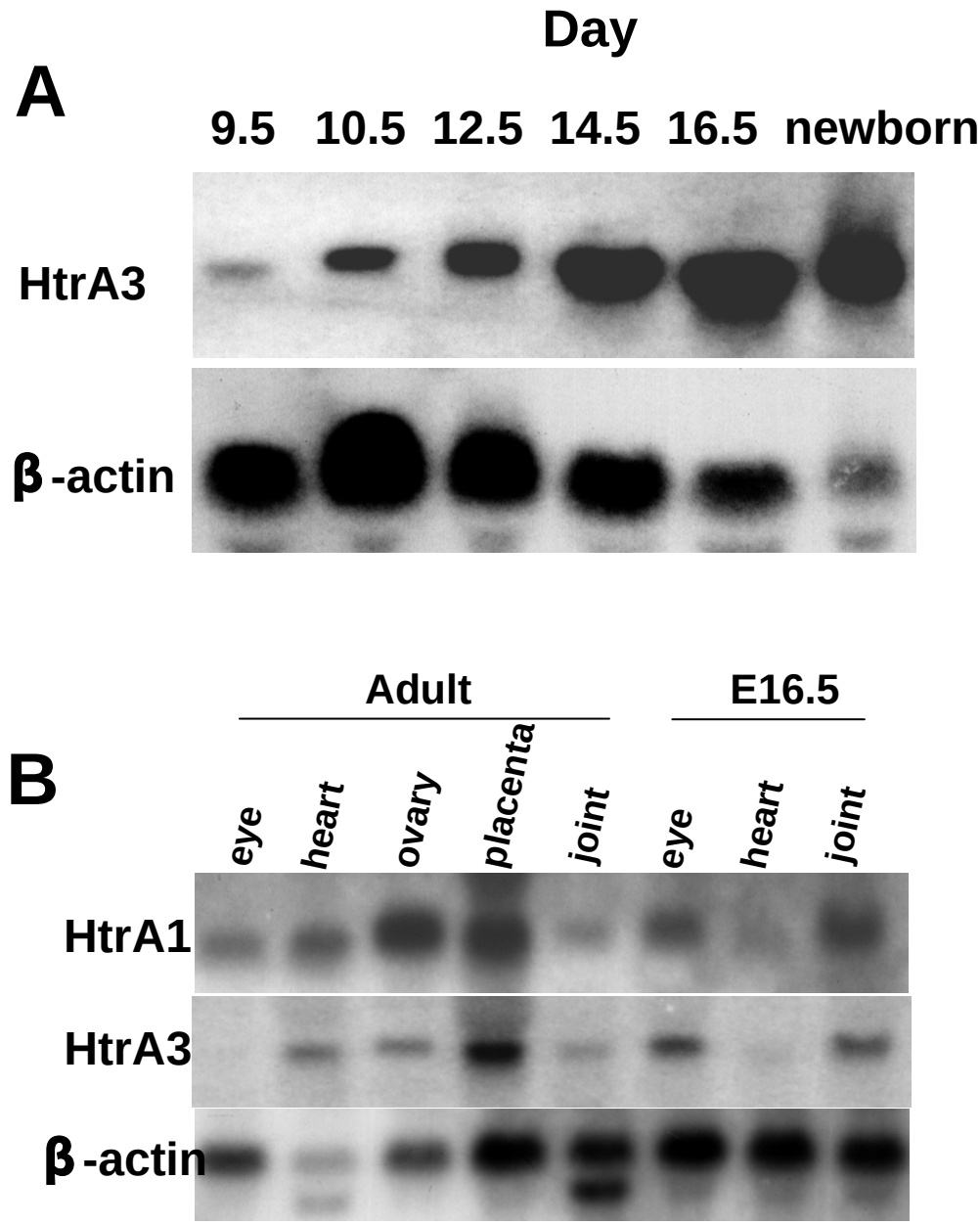


Figure 5. Northern analysis of HtrA3 expression. (A) Total RNA was isolated from mouse embryos of different embryonic days as indicated. A ^{32}P-labeled HtrA3 fragment was used as a probe. A 2.8 Kb major band was detected (top panel). After exposure to x-ray film, the filter was stripped and rehybridized with a β-actin probe for an RNA loading control (bottom panel). (B) Total RNA was isolated from tissues of E16.5 embryos and adult mice. Hybridization was carried out as above.

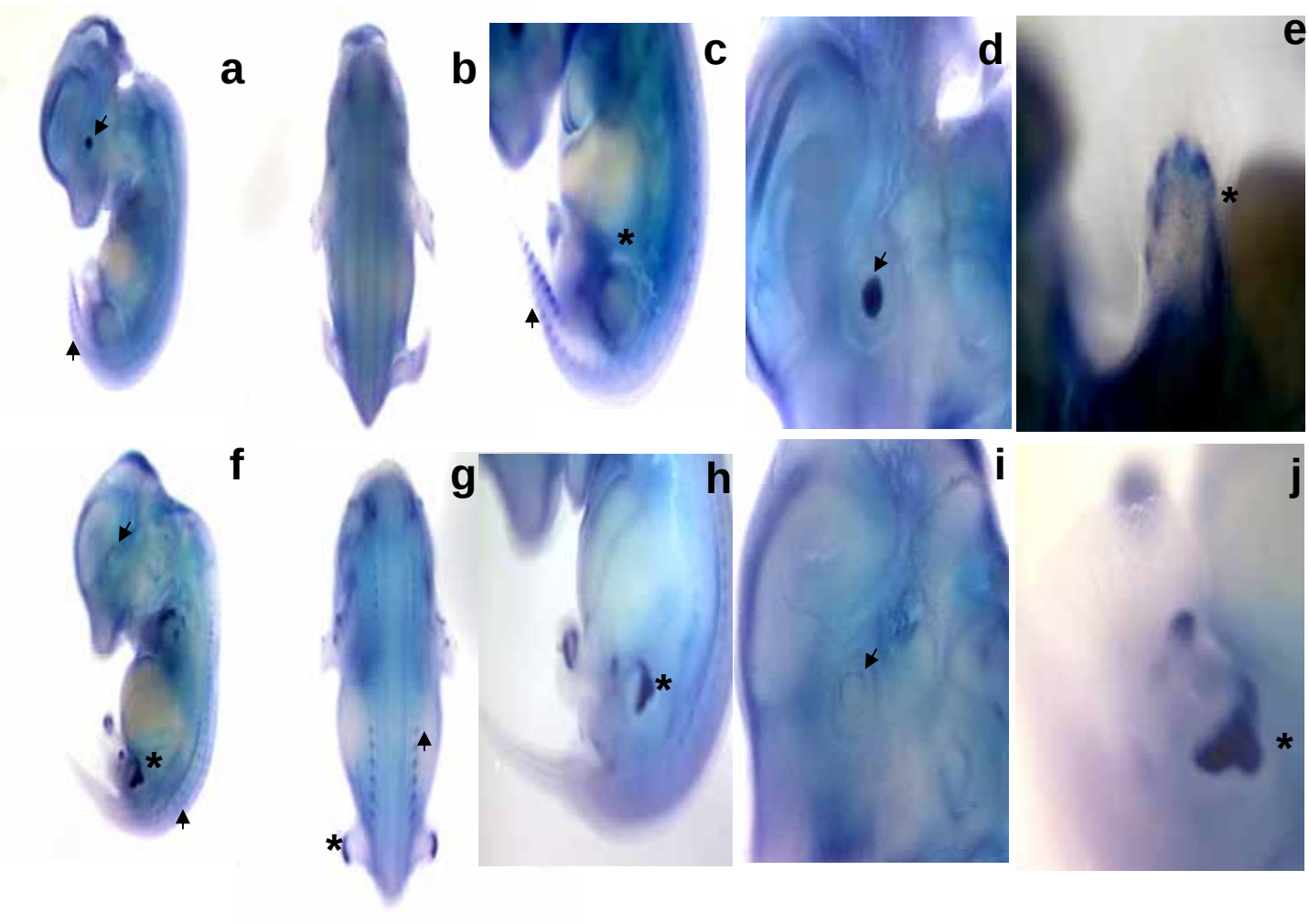


Figure 6 Whole mount in situ hybridization of E12.5 embryos showing expression of Htra3 (a-e) and HtrA1
Shaped expression of HtrA3 and a ring shaped expression of HtrA1 in the eye rudiments. Arrowheads indic
Region and HtrA1 expression in the trunk region of the vertebrae. Asterisks show strong expression of HtrA
Joint of the hind limb and weak expression of HtrA3 in the tibial region.

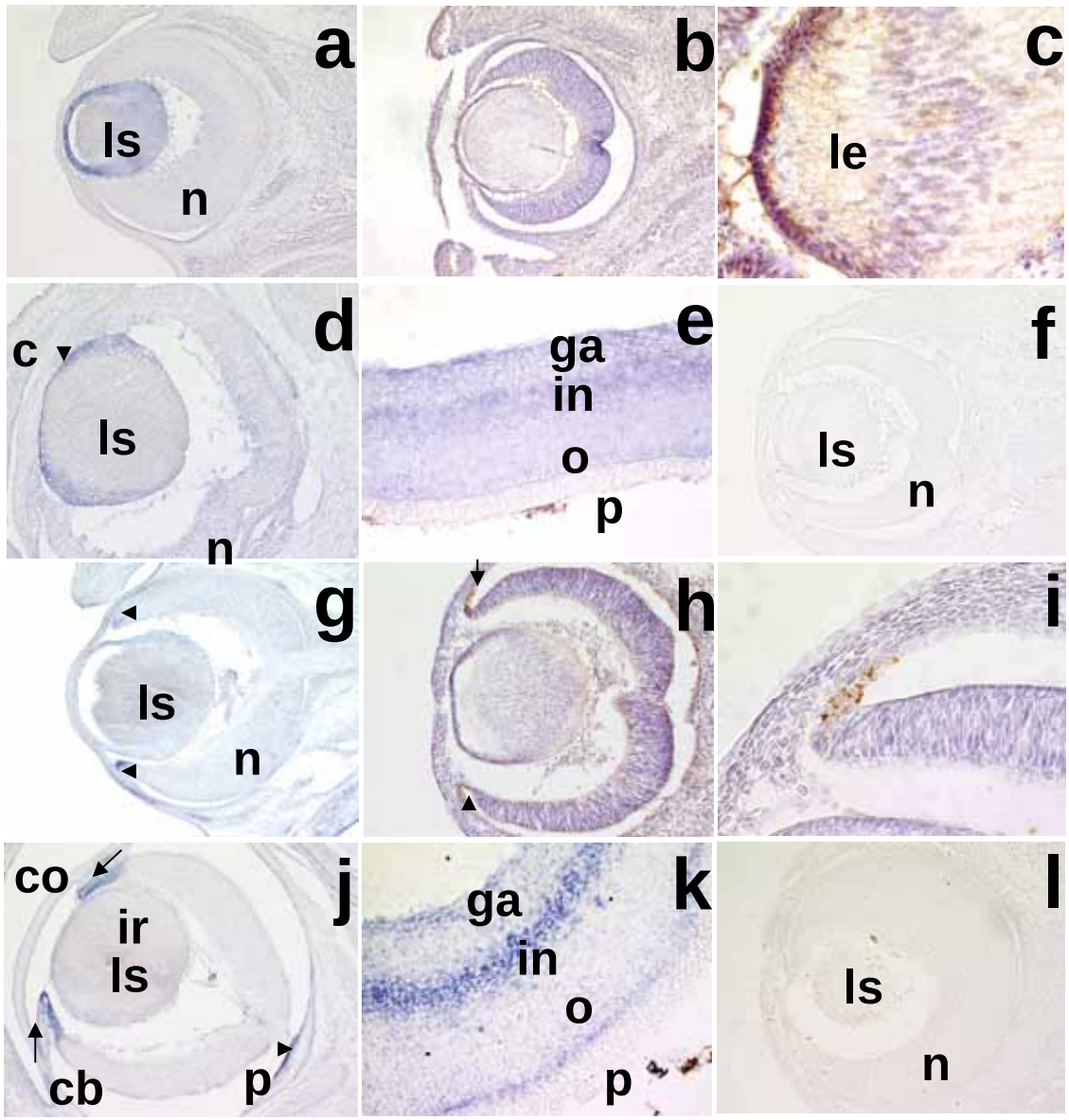


Figure 7 Sections of embryonic eyes and adult retina showing expression of HtrA3 (a-e) and HtrA1 (f-j). Sections of eyes from embryos of E12.5 (a-c and f-h), E16.5 (i), E18.5 (d) or from adult (e and j) were analyzed by in situ hybridaion (a,d,e,f,I, and j) or immunostaining (b,c,g, and h). L, lens; n, neural retina; g, ganglion cell layer; in, inner cell layer; ir, iris; o, outer cell layer; p, pigment retian; cb, ciliary body; co, cornea.

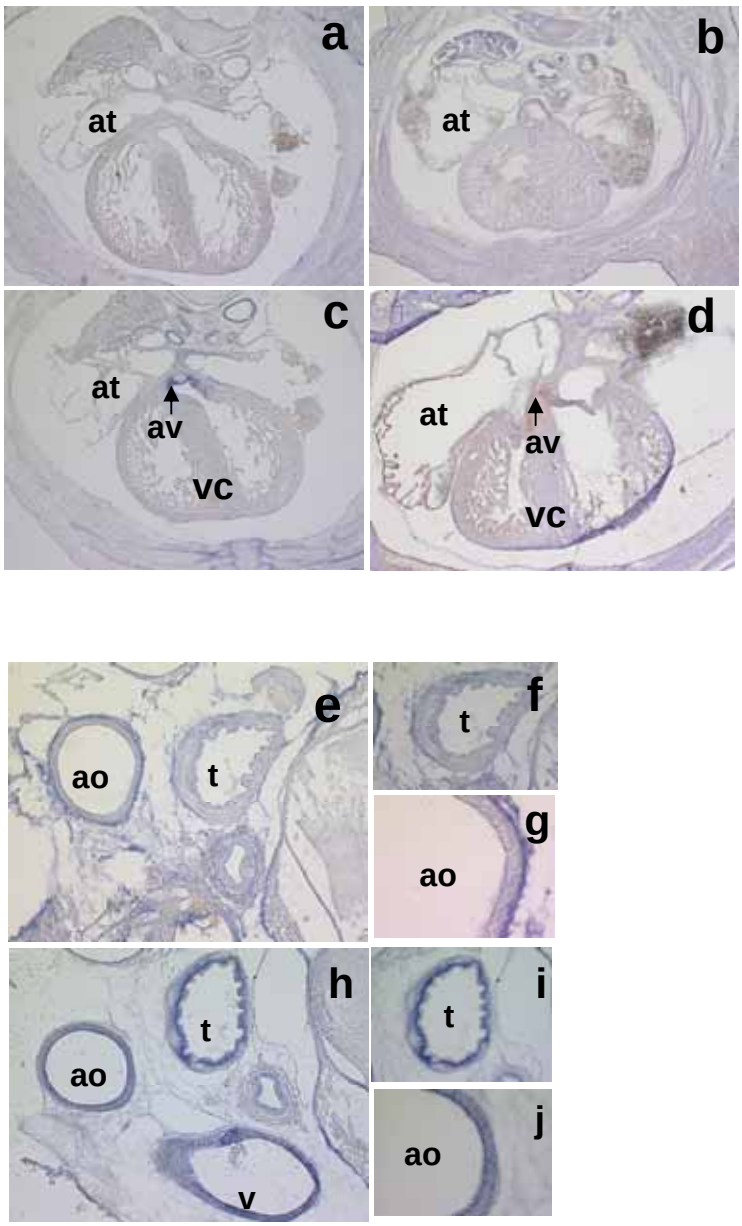


Figure 8 Section of embryo at the thoracic level showing expression of HtrA3 (a, b, e,f,g) and HtrA1 (c,d,h,i,j) Section of E14.5 embryo were analyzed by insitu hybridization (a,c) or by immunostaining (b,d) and E16.5 Embryo were analyzed by in situ hybridization for HtrA3(e,f,g) and for HtrA1 (h,i,j) heart, blood vessels and trachea.at, atrium; ao, aorta; av, atrio-ventricular cushion; v, ventricular septum;vc, vena cava; e, esophagus ; t, trachea.

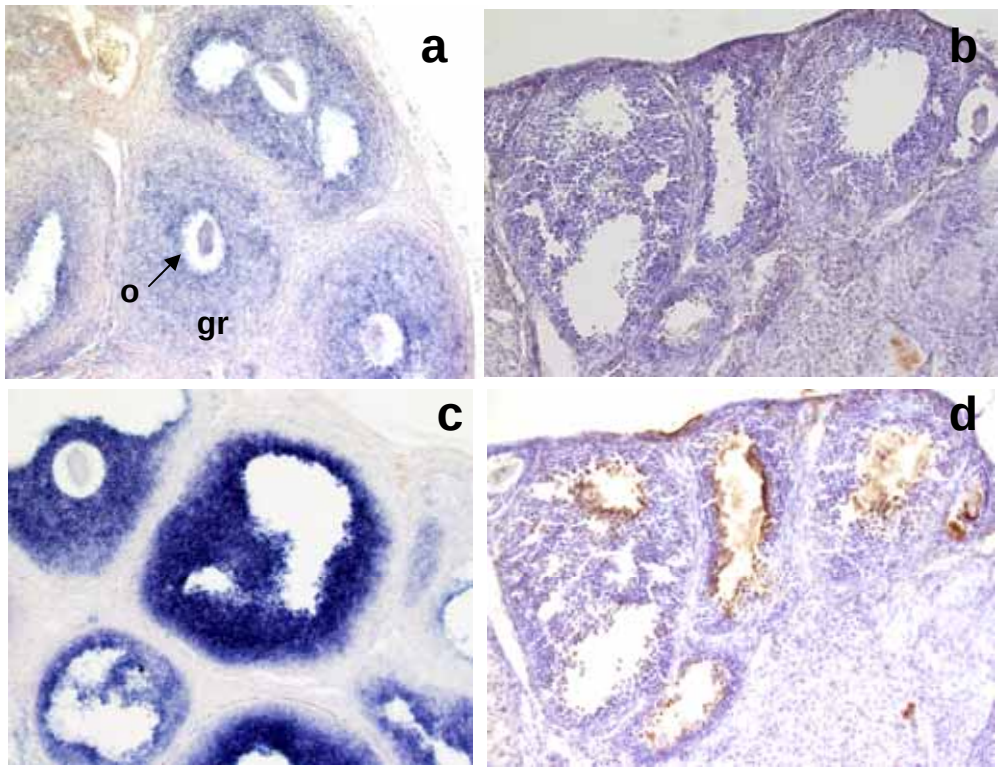


Figure 10 In situ hybridization and Immunohistochemical analysis showing expression of HtrA3 (a and b) and HtrA1 (c and d) in ovary. gr, granulosa cell; o, oocyte.

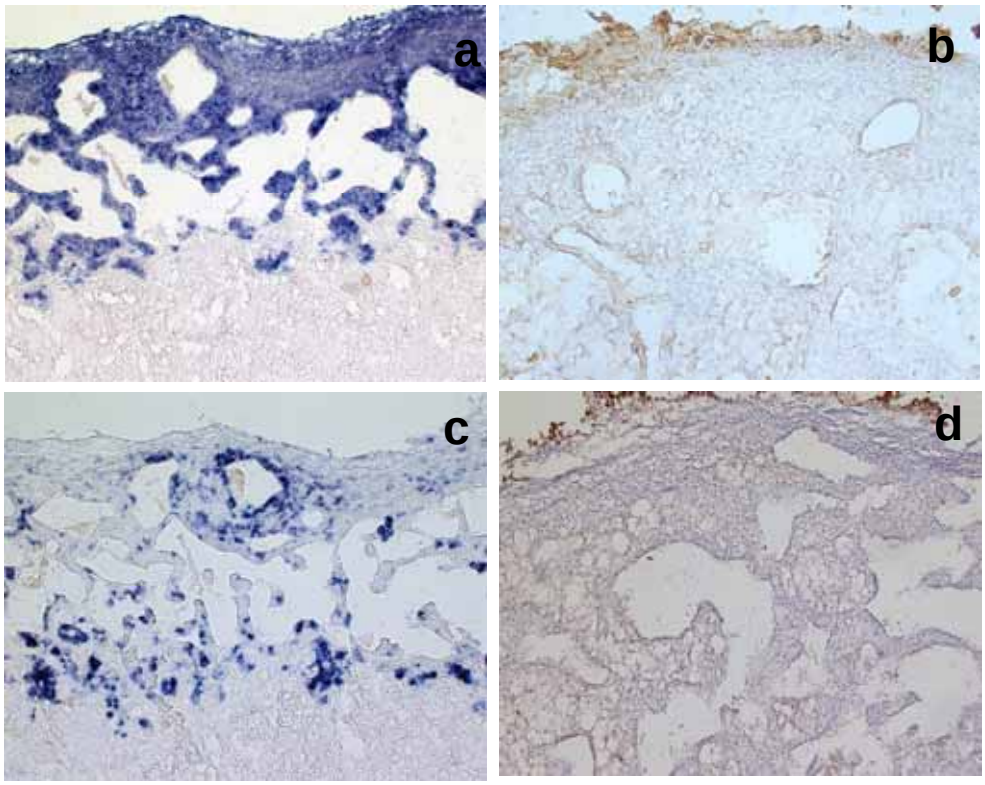


Figure 9 Sections of placenta showing expression of HtrA3 (a and b) and HtrA1(c and d). Sections of placenta of day 10.5 of gestation were analyzed by in situ hybridization (a and c) and immunostaining (b and d).

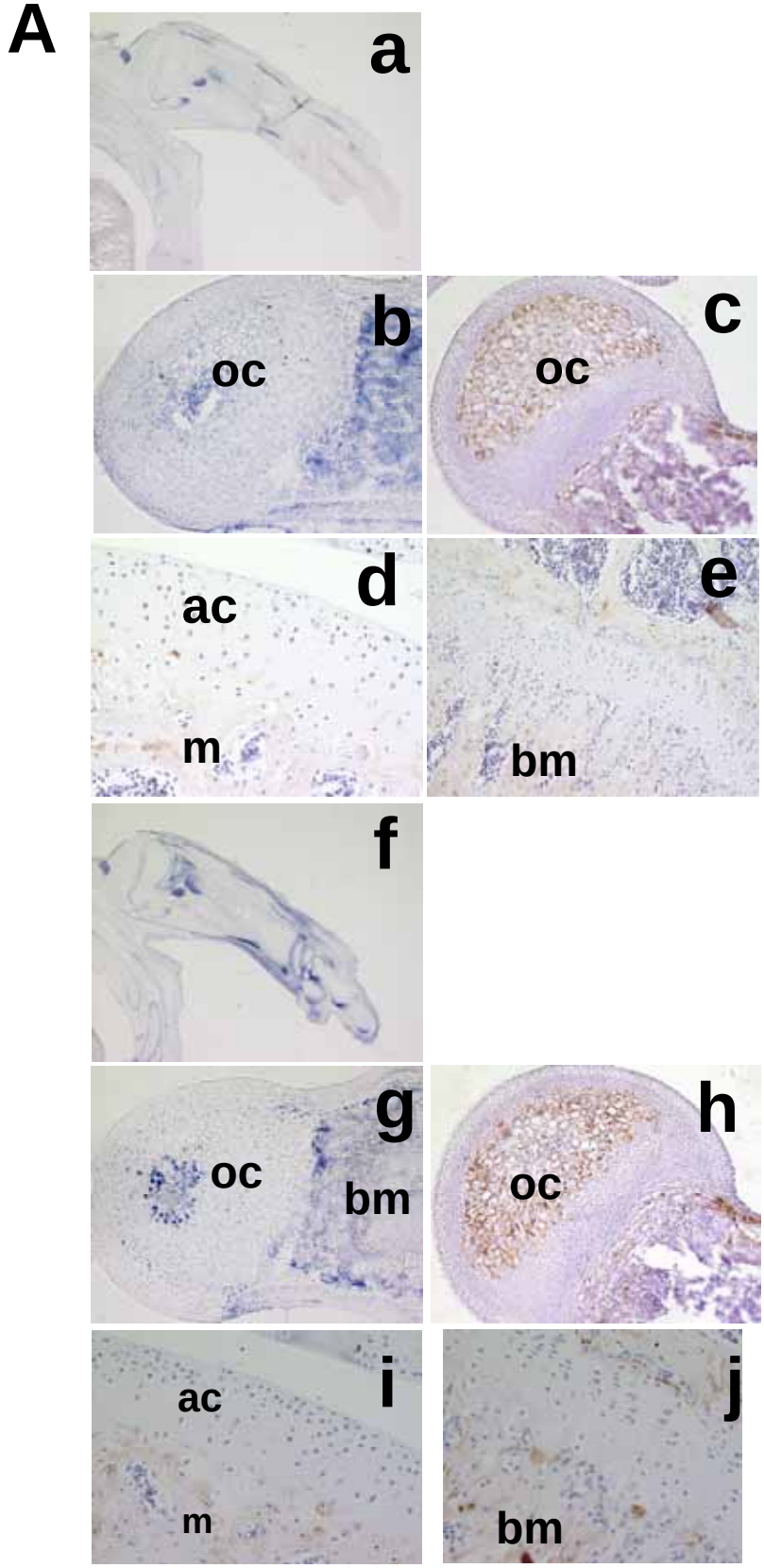


Figure 11A Expression of HtrA3 and HtrA1 in skeletal tissues. (A) Expression pattern of HtrA3 (a-e) and HtrA1 (f-j) in various stages of bone development. The forelimb of E14.5 embryo was analyze by in situ hybridization (a,f). The femur of postnatal day 7 infant were analyzed by in situ hybridization (b and g) and immunostaining (c and h). The adult joints were analyzed by inmunostaining (d, e, i and j)

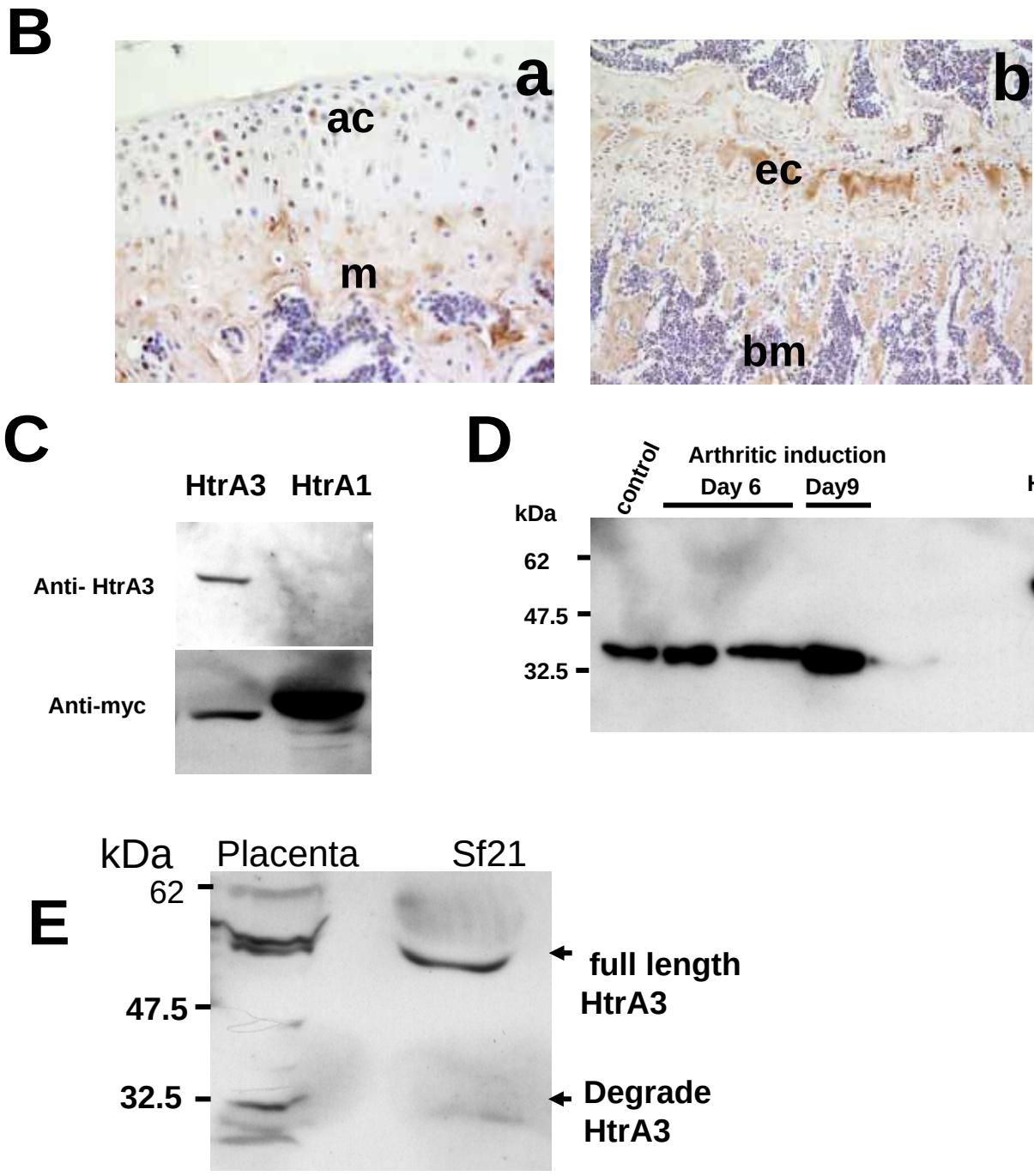


Figure 11B-D (B) Increased expression of HtrA3 in joint cartilage affected by arthritis. The joint samples were prepared from mouse 6 days after lipopolysaccharide injection. The joints sectioned were stained with HtrA3 antibody (a and b). The Superficial Chondrocytes in the articular cartilage (a) and chondrocytes in the epiphyseal cartilage (b) displayed increased staining as compared with (i and j)were prepared 6 or 9 days after lipopolysaccharide injection. (C) Western blotting showing specificity of anti-HtrA3 antibody. HtrA3-myc and HtrA1- myc proteins were produced in sf21 cell using the baculovirus system. Culture supernatants were electrophoresed and blotted on membranes. The membrane was stained with anti-HtrA3 antibody (upper panel) or with anti-myc antibody (lower panel) to show the amounts of loaded HtrA3-myc and HtrA1-myc proteins. (D) Increase in HtrA3 contents in arthritic joints. Knee joints from each mouse were prepared 6 or 9 days after lipopolysaccharide injection. The joint cartilage was scraped off and extracted with a guanidine buffer. The same amounts of the extracted proteins (8.0 mg each lane) were separated by SDS electrophoresis, blotted on a membrane, and probed with anti-HtrA3 antibody. The joints from two mice prepared at day 6 both showed 1.5 fold increase, and the joints of a mouse prepared at day 9 showed 2.5 fold increase in HtrA3 protein. ac, articular cartilage; bm, bone marrow; m; bone matrix; oc, ossification center; ec, epiphyseal cartilage. (E) Western blotting showing the degradation of HtrA3 protein in placenta.

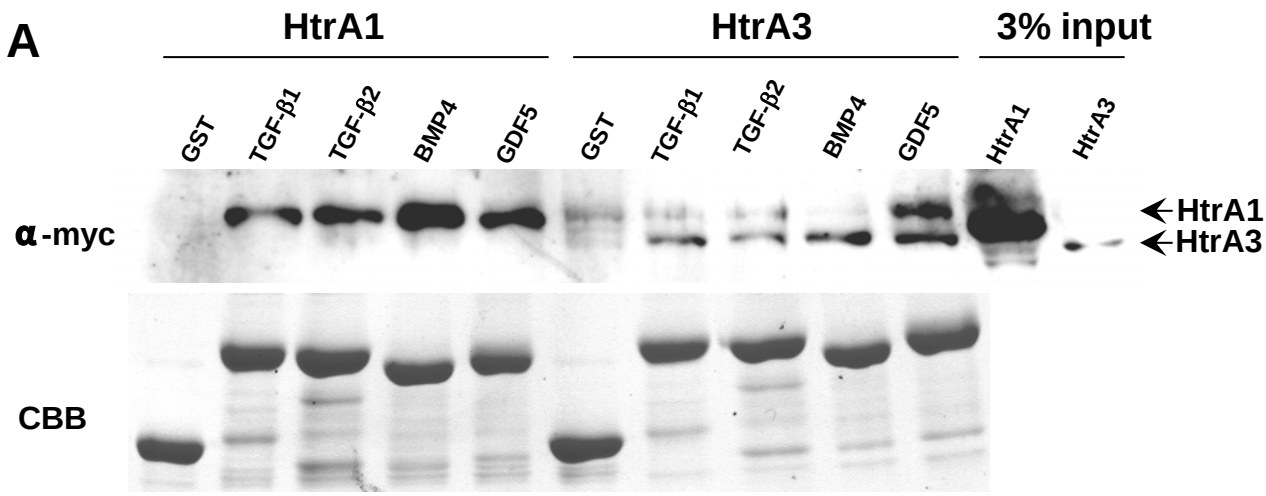


Figure 13. GST pull-down assay of HtrA3 and HtrA1 with various TGF-b proteins. GST protein (control) or GST-fused various TGF-b proteins were incubated with conditioned medium containing HtrA1-myc or HtrA3-myc protein and pulled down with Ni beads. Pulled-down samples were separated by SDS gel electrophoresis and detected by anti myc antibody (top panel) to assay the bound HtrA proteins or by CBB staining (bottom panel) to confirm the amount of GST or GST-fusion proteins recovered. The right two lanes contain HtrA1-myc and HtrA3-myc proteins corresponding to 3% of the amounts used for the GST-pull down assay. An unknown protein band appeared above the HtrA3 protein.

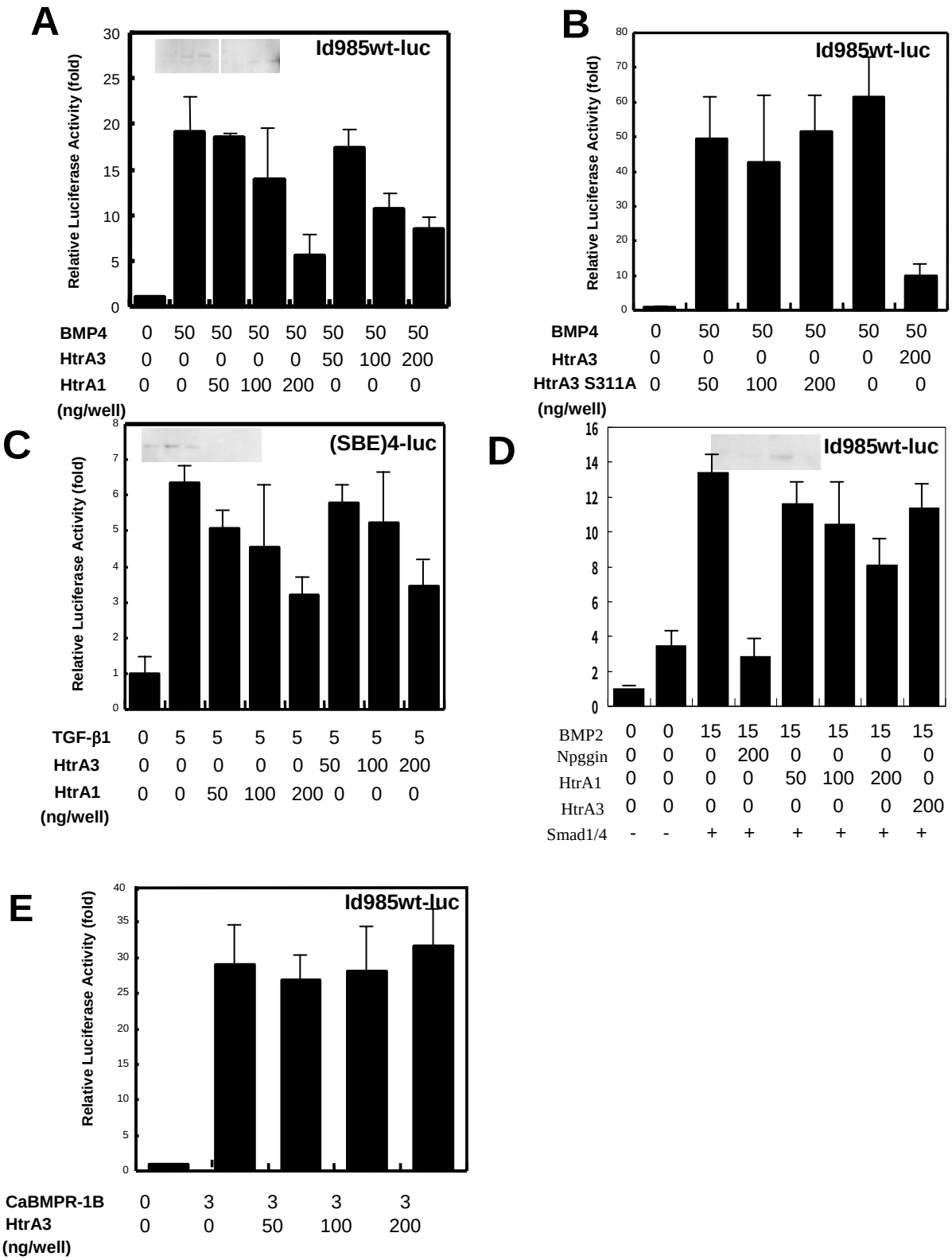


Figure 14. Inhibition of TGF-β signaling by HtrA3. (A) Inhibition of BMP4 signaling by HtrA3. C2C12 cells were co-transfected with the BMP4 expression vector (50 ng DNA/well containing 0.5 ml of medium) and various amounts of expression vectors (50-200 ng DNA/well) for HtrA3 and HtrA1 as indicated. (B) An HtrA3 mutant lacking protease activity was inactive as a signal inhibitor. Co-transfectoin was carried out as described in (A) except that the expression vector for the protease mutant, HtrA3 S311A, was used. Inset depicts the result of Western blot analysis of culture medium (10 ml each) showing the expression levels of HtrA proteins; from left, HtrA1 produced at 50, 100 and 200 ng DNA/well, HtrA2 at 50, 100 and 200 ng DNA/well. (C) Inhibition of TGF-β1 signaling by HtrA3. Co-transfectoin was carried out as described in (A) except that the SBE reporter plasmid was used and recombinant human TGF-β1 (5 ng protein/well that is 10 ng/ml) was added in place of the BMP4 expression vector. Inset shows the expression levels of HtrA proteins as in B.(D) Inhibition of BMP 2signaling by HtrA3. Co-transfectoin was carried out as described in (A) and recombinant human BMP2 (15 ng protein/well that is 30 ng/ml) was added in place of the BMP4 expression vector. (E) HtrA3 did not inhibit signaling from the constitutively active BMP type 1 receptor. Co-transfection was carried out using the Id985wt reporter, the expression vector for caBMPR-1B and various amounts of HtrA3 expression vector as indicated. The amount of caBMPR-1B plasmid added (3 ng DNA/well) was chosen so that the

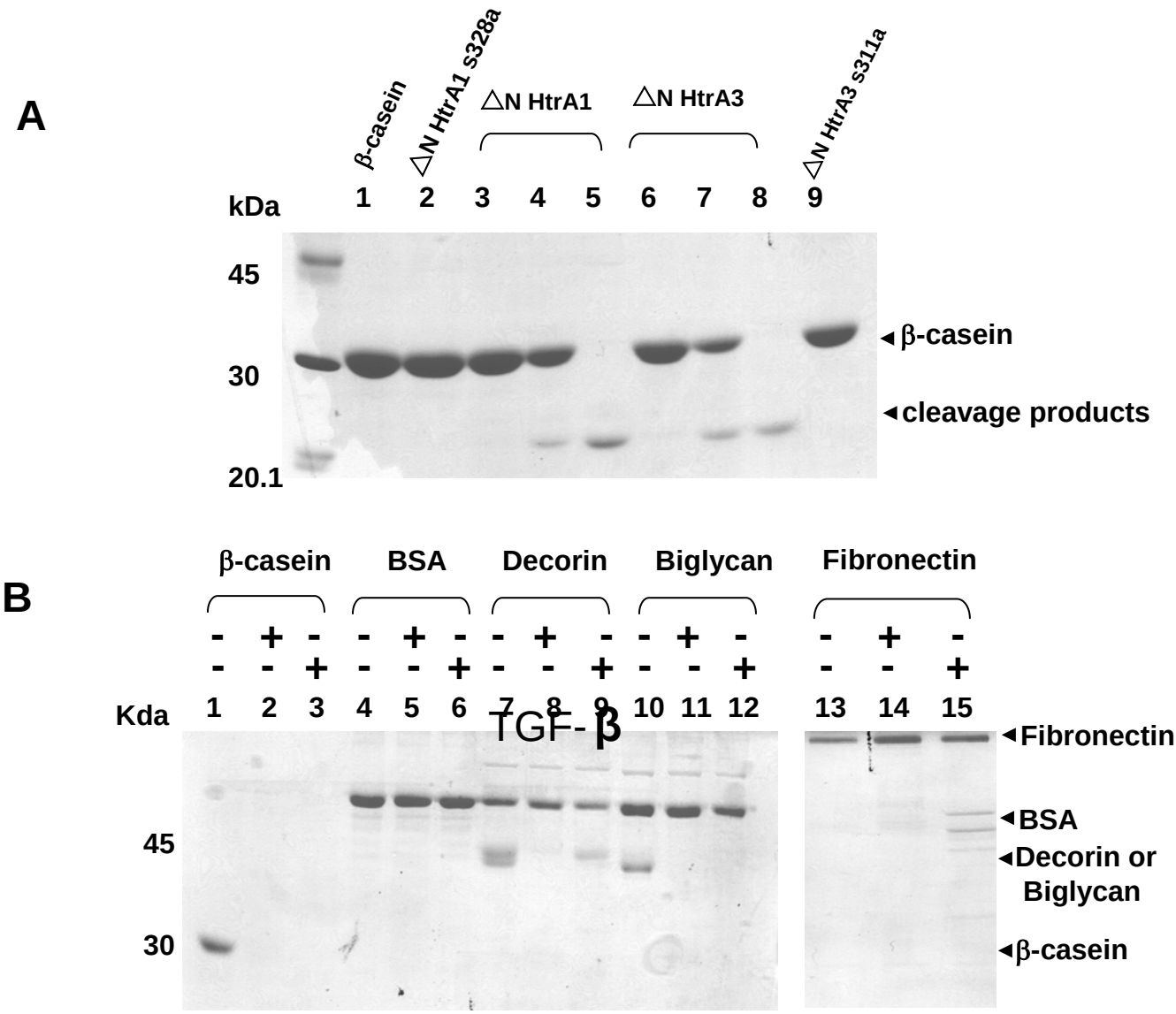
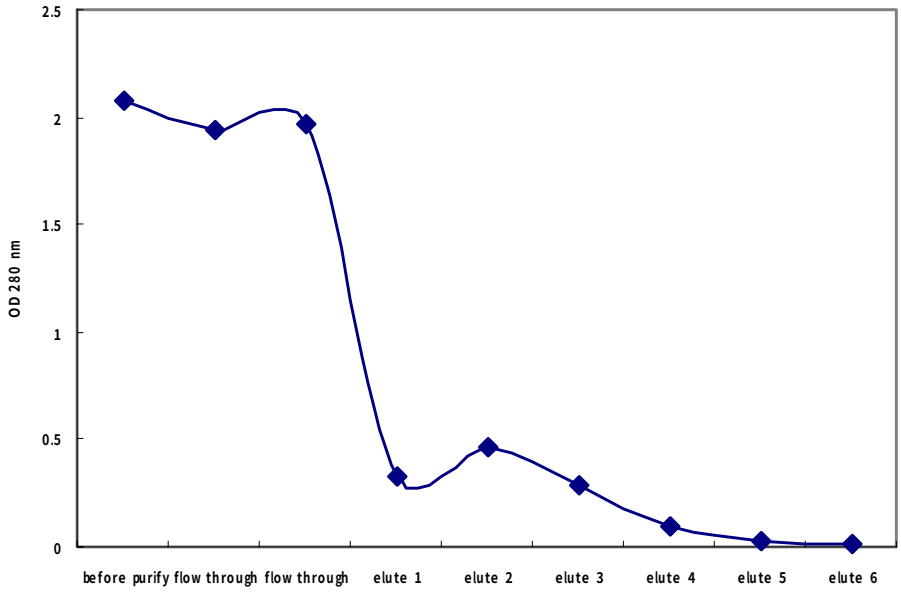


Figure 15 In vitro assay of the proteolytic activity of His-Δ(1-150) HtrA3. **(A)** 10 μg β-caseine was incubated with alone (lane 1) or with 0.1 μg of His-Δ(1-150) S328A HtrA1 (lane 2) or with 0.1 μg of His-Δ(1-150) HtrA1 (lane 3-5) or 0.1 μg of His-Δ(1-150) HtrA3 (lane 6-8) or with 0.1 μg of His-Δ(1-150) S311A HtrA3 (Lane 9) in a 20 μl reaction volume for 30 min at 37°C. The reactions were resolved on SDS-PAGE and the gel stained with Coomassie Blue. **(B)** Digestion of BSA, biglycan, decorin and fibronectin by HtrA1 and HtrA3.10μg β-caseine, 0.5μg BSA, 10μg biglycan, 5μg decorin were incubated with buffer alone (lane 1,4,7,10, respectively) or His-Δ(1-150) HtrA1 0.1μg (lane2,5,8,11,respectively) or His-Δ(1-150) HtrA3 (lane3,6,9,12, respectively) at 37°C for 12 hr. For biglycan and decorin, after termination of the reaction with PMSF, the samples were treated again with Chondrotinase ABC at 37°C for 1 hr and subjected to SDS/PAGE (10% acrylamide) under reducing conditions.

A



B

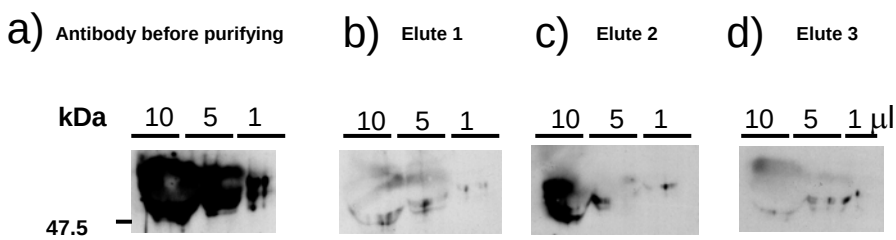


Figure 12 (A) The profile of purification of polyclonal antibody against HtrA3. (B) Western blotting of purified antibody to HtrA3 producing by Sf21 10, 5 and 1 μl in 1000 fold dilution. a) antibody before purifying b) elute 1 c) elute 2 d) elute 3

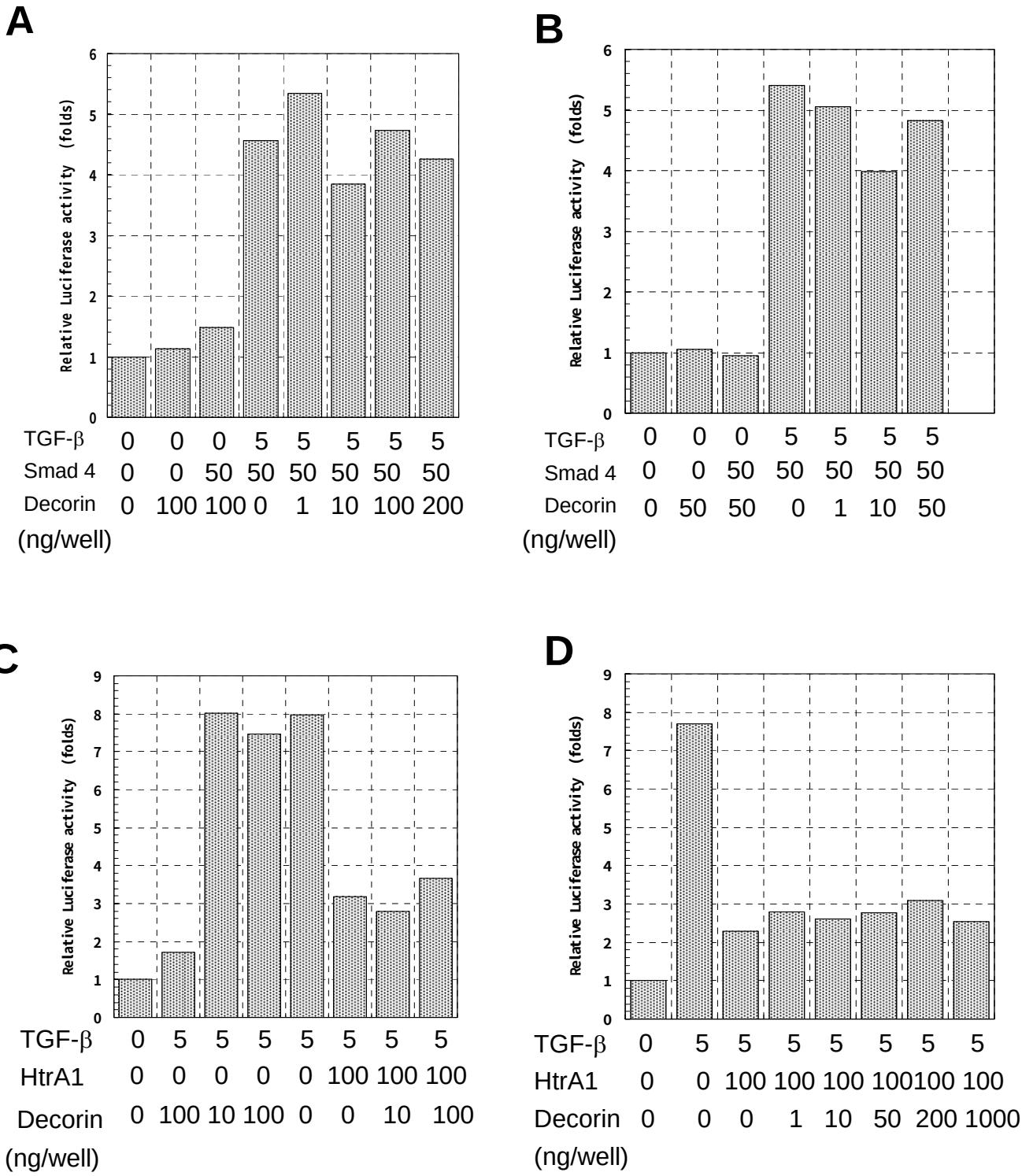


Figure 16 Effect of decorin on TGF-β signaling. (A) Effect of decorin an inhibtion of TGF-β signaling by HtrA1. C2C12 cells werer co-transfected with SBE reporter plasmid and various concentration of HtrA1. Then 24 hr after transfection the recombinant human TGF-β1 and decorin were added. (B) Co-transfection was carried out as described in (A) except that the expression vector for decorin was used instead of recombinant decorin (C) Effect of decorin on inhibitory of TGF-β signaling by HtrA1. Co-transfection was as describe in (A) except HtrA1 was cotransfected at concentration 100 ng/well. (D) Effect of decorin on inhibitory of TGF-β signaling by HtrA1. Co-transfection was as describe in (B) except HtrA1 was cotransfected at concentration 100 ng/well.