

Regulation of Matrix Metalloproteinase-1 Expression by the Homeodomain Transcription Factor Caudal in *Drosophila* Intestine

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The matrix metalloproteinase (MMP) family plays essential roles in physiological processes such as embryonic development, angiogenesis, wound healing, and tissue homeostasis as a consequence of MMP capacity for breaking down many types of extracellular matrix proteins. Imbalanced regulation of MMP expression can also lead to pathological conditions such as tumor progression. We recently reported that the *Drosophila Mmp1* gene is highly expressed in the digestive tract and is required for the maintenance of intestinal homeostasis such as by restriction of uncontrolled intestinal stem cell proliferation. However, the regulatory mechanisms of MMP gene expression in the intestine remain unclear. In this study, we determined that the expression of *Mmp1* is regulated by the homeodomain transcription factor Caudal. Experiments using the targeted expression of Caudal under the regulation of Gal4-UAS system indicated that endogenous Caudal is required for the *Mmp1* gene expression in the adult *Drosophila* intestine and that exogenous Caudal induces *Mmp1* expression. Transient transfection experiments indicated that Caudal can activate the promoter activity of *Mmp1* and that several putative Caudal binding sites in the 5'-flanking region of the *Mmp1* gene may be critical to the up-regulation by Caudal. Our data suggest that *Mmp1* is one of the target genes of Caudal in physiological normal condition and in tumorigenesis.

Key words : Matrix metalloprotease-1, Caudal, *Drosophila* intestine

Introduction

Extracellular proteases, such as matrix metalloproteinase (MMP), are crucial regulators for cell functions. The essential roles of MMP within physiological processes such as embryonic development, angiogenesis, wound healing, and tissue homeostasis result from their capacity to break down all kinds of extracellular matrix (ECM) proteins [22]. As a result of their potent proteolytic activity, imbalanced regulation of MMP can lead to excessive ECM degradation and patho-

logical conditions such as rheumatoid arthritis and tumor progression [5,22]. As the importance of MMP, the activity of MMP is tightly regulated at various steps: transcription, post-translational activation through the cleavage of inactive prodomain, and interaction with tissue inhibitors of metalloproteinases [23,31]. However, almost investigations for the regulatory mechanisms of MMP expression are limited to the stressed and pathological conditions. The regulatory mechanisms of MMP in physiological normal condition are not fully understood.

In *Drosophila* genome, only two MMPs have been identified as having conserved domain structures, which differ from the 26 MMPs found in mammals [17,18]. Both fly MMPs have the canonical MMP domain structure, with a signal sequence, prodomain, catalytic domain, hinge and hemopexin domain [7]. Although there are not clear orthologous relationships between the *Drosophila* and vertebrate MMPs [7], fly *Mmp1* represents the typical secreted form of MMP and fly *Mmp2* represents the membrane anchored form of MMP on the basis of their structure [17,18]. Recently, we reported that the *Drosophila Mmp1* gene is highly expressed in the digestive tract in both third instar larvae and adult flies and is required for the maintenance of intestinal

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homeostasis such as the restriction of uncontrolled intestinal stem cell proliferation, and the maintenance of intestinal architecture [15]. It was reported that *Mmp1* expression is regulated by JNK and Fos to induce invasive tumor [28]. However, the underlying mechanisms of *Drosophila Mmp1* gene expression in the intestine remain unknown.

Caudal-related homeobox genes play crucial roles in axial patterning of embryonic development and intestinal development and differentiation [20,21,26]. The *Drosophila caudal* gene contributes to defining the anteroposterior axis during development, patterning of the posterior segments, and gut development [16,30]. In addition, *caudal* gene was recently reported to be involved in the innate immune system in the gut [24,25]. Moreover, CDX1 and CDX2, mammalian homologues of *Drosophila caudal*, are highly expressed in intestinal epithelium, and are required for the maintenance of intestinal integrity in the adult intestine [11]. Abnormal *Cdx* expression have been well known to be associated with intestinal tumorigenesis [8].

In this study, we investigated whether the expression of *Mmp1* is regulated by Caudal transcription factor in *Drosophila*. We found that endogenous Caudal is required for the *Mmp1* gene expression in the adult *Drosophila* intestine and exogenous Caudal expression can activate *Mmp1* promoter *in vitro* and *in vivo*.

Materials and Methods

Immunohistochemistry

Immunostaining was performed as previously described [3]. Briefly, the fly guts and larval epidermis were fixed in 4% formaldehyde (Sigma-Aldrich, MO, USA) for 1 hr at 25°C, washed in PBS/0.1% Triton X-100 (PBST) and incubated with primary antibodies in 1% bovine serum albumin (BSA, Sigma-Aldrich) overnight at 4°C. The samples were then washed in PBST, incubated with secondary antibodies for 45 min at 25°C, washed and mounted with Vectashield (Vector Labs, CA, USA). The images were analyzed using a Zeiss AxioSkop 2Plus microscope (Carl Zeiss Inc., Germany). Primary antibodies were diluted as follows: mouse anti-Mmp1 1:50 (Developmental Studies Hybridoma Bank, IA, USA); rabbit anti-GFP 1:500 (Molecular Probes, OR, USA). Secondary antibodies used included Cy3-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit (Jackson ImmunoResearch, PA, USA); all were diluted 1:300.

Western blot

Proteins were extracted from whole third instar larvae using PRO-PREP™ protein extract solution (iNtRON Biotechnology, Korea) and separated by SDS-PAGE. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, UK), and incubated with primary antibodies for 16 hr at 4°C. After washing with Tris-based buffered saline (TBS)/0.1% Tween 20 (TBST), membranes were incubated with secondary antibodies for 45 min at 25°C. Bound antibodies were then detected using the WEST-One™ Western Blot detection system (iNtRON Biotechnology). Mouse anti-Mmp1, 1:20; mouse anti- α -tubulin, 1:10,000 (BioGenex, CA, USA) were used in combination with HRP-conjugated anti-mouse IgG secondary antibodies (1:5,000, Santa Cruz Biotechnology Inc., CA, USA).

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from whole third instar larvae with TRIzol reagent (Molecular Research Center Inc., OH, USA), according to manufacturer's instructions, and 1 μ g total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, WI, USA). Samples underwent 30 PCR cycles, were separated on 2% agarose gels, and stained with ethidium bromide (Sigma-Aldrich). All oligonucleotides were chemically synthesized. Oligonucleotide sequences for the analysis of *Mmp1* [15], *cad* [12], and *rp49* [1] were previously reported.

Construction of reporter plasmids

To construct reporter plasmids of *Mmp1*, the 5'-flanking region of *Mmp1* contained 2,345 bp fragments spanning from 1,980 bp upstream and 365 bp downstream with respect to the transcription initiation site was PCR-amplified from Kc genomic DNA and cloned into pGL2-basic (Promega), and the sequences were confirmed by sequencing. The primers for the promoter region of *Mmp1* containing linker sequences recognized by 5' and 3' *KpnI* were as follow: Forward, 5'-CCG GGT ACC AGA TGG CTT GCA CAA GCA-3'; Reverse, 5'-CCG GGT ACC TTC CAG CTC TCT CTG CCA TT-3'. The deletion constructs were generated by digestion of p*Mmp1-luc* with *SmaI*, *SmaI-KpnI* and *HindIII*. The resulting deletion constructs were designated -1627p*Mmp1-luc*, -708p*Mmp1-luc* and -570p*Mmp1-luc*, respectively. Additional two deletion constructs were cloned (-1119p*Mmp1-luc* and -170p*Mmp1-luc*). All oligonucleotides were chemically synthesized.

DNA transfection and luciferase assay

Drosophila S2 cells were grown at 25°C in Schneider medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS, GIBCO-BRL, MD, USA). All plasmids for transfection were prepared by using Qiagen Plasmid Midi Kit (Qiagen, CA, USA). Dimethyldioctadecyl ammonium bromide (DDAB) mediated transfection of *Drosophila* cultured cells were performed using 6-, 12-, or 24-well tissue culture plates as described previously [9]. Each transfection mixture contained 100 ng of the appropriate reporter plasmids and 300 ng of pAc-lacZ internal control plasmids. For co-transfection experiments, 300-900 ng of effector plasmids and pAc-HisA for the control of effector plasmids were used. Forty-eight hr after transfection, cell extracts were prepared by adding 50-200 µl of Cell Culture Lysis Reagent (Promega) to each well after removing the media by aspiration. The well plate was shaken gently on a rotary shaker for 2-5 min and cell extracts were transferred to a microcentrifuge tube. After centrifugation for 5 min, these supernatant were subjected to β-galactosidase and luciferase assays. Luciferase assay was performed with a luminometer (TD-20/20, Turner Designs, CA, USA). Normalized luciferase activity was calculated by determining the luciferase/β-galactosidase activity ratio and by averaging the values from 3-8 experiments and the average values, and standard errors were calculated.

Drosophila stocks

Drosophila stocks were consistently maintained at 25°C on standard cornmeal-sugar-yeast medium under a 12 hr/12 hr light/dark cycle. The food consisted of 79.2% water, 1% agar, 7% cornmeal, 2% yeast, 10% sucrose, 0.3% bokinin, and 0.5% propionic acid. To avoid larval overpopulation in culture vials, 25-30 adult flies were cultured in a vial and transferred to new vials containing fresh food every 2-3 days. *tub-Gal80^{ts}* and *hs-Gal4* fly strains were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, IN, USA). Temperature-inducible differentiated enterocyte-specific *Myo1A-Gal80^{ts}* flies were obtained from B. A. Edgar [13], and *UAS-cad^{RNAi}* flies from W. J. Lee [10]. The fly stocks of *UAS-cad* were described previously [12].

Results

Requirement of Caudal for *Mmp1* expression in the adult *Drosophila* intestine

To understand the regulatory mechanism of *Mmp1* ex-

pression, we assessed whether the homeodomain transcription factor Caudal is a potential regulator of *Mmp1* gene in the adult gut. To investigate whether the endogenous Caudal can regulate *Mmp1* expression in the adult intestine, we used GAL4/UAS system [6]. The GAL4/UAS ectopic expression system is valuable machinery for the overexpression or knockdown of a transgene under specific conditions, such as cell-, tissue-, temperature-, and stage-specific conditions in *Drosophila* [6]. When the GAL4 transcription factor is expressed under the control of various promoters, GAL4 can drive the expression of a transgene under an upstream activating sequence (UAS) [6]. We crossed the flies *UAS-cad^{RNAi}*, which expressing the interference RNAi (RNAi) against to the Caudal (*cad^{RNAi}*), with the heat-shock inducible enterocytes-specific *Myo1A-Gal80^{ts}* flies. Since these flies contained *UAS-GFP*, the cells expressing *cad^{RNAi}* construct were detected via the expression of GFP protein (Fig. 1A). When *cad^{RNAi}* was expressed in the enterocyte of the fly guts, the cell expressing *cad^{RNAi}* decreased the *Mmp1* level detected by anti-*Mmp1* antibody (inset in Fig. 1B). These

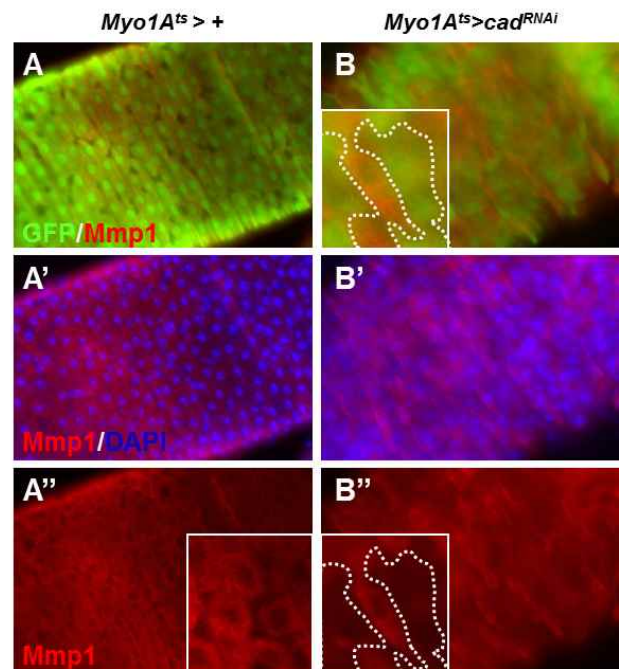


Fig. 1. *Mmp1* expression is decreased by *cad^{RNAi}* expression in the enterocytes of adult intestine. The flies expressing *cad^{RNAi}* under enterocyte-specific *Myo1A-Gal80^{ts}* driver (*Myo1A^{ts}>cad^{RNAi}*, B-B'') and driver alone (*Myo1A^{ts}>+*, A-A'') were cultured at 29°C for 4 days and the guts were stained with anti-GFP (green), anti-*Mmp1* (red), and DAPI (blue). Dashed line indicates *cad^{RNAi}* expression region.

results indicate that Caudal is required for the maintenance of Mmp1 levels in the adult midgut.

Caudal overexpression induces ectopic *Mmp1* expression in larval gut

To know whether the ectopic expression of Caudal can increase *Mmp1* expression, we crossed the flies *UAS-cad* which expressing the wild type Caudal protein, with the heat-shock inducible *hs-Gal4* driver. We previously reported that ectopic overexpression of Caudal under *hs-Gal4* driver induce melanotic tumors in the epidermis and guts of the third instar larvae [12]. As reported previously, the third instar larvae expressing Caudal under *hs-Gal4* showed melanotic tumor in their epidermis and guts. We found that the expression of *Mmp1* was drastically increased in the epidermis and guts having melanotic tumors (Fig. 2A-D).

Furthermore, the regulation of *Mmp1* by Caudal was confirmed at protein level using Western blot (Fig. 2E). These results indicate that ectopic Caudal expression increased *Mmp1* expression.

Transcriptional regulation of *Mmp1* by Caudal transcription factor

To understand whether Caudal transcriptionally regulate *Mmp1* expression, we analyzed the mRNA level of *Mmp1* in *cad* expressing flies. In the third instar larvae expressing Caudal protein under *hs-Gal4*, the mRNA level of *Mmp1* was increased compared to control (Fig. 3A).

Next, to determine the effect of Caudal protein on *Mmp1* promoter activity, we investigated whether the Caudal protein can regulate the activity of *Mmp1* promoter, using a *pMmp1-luc* reporter plasmid containing the promoter region

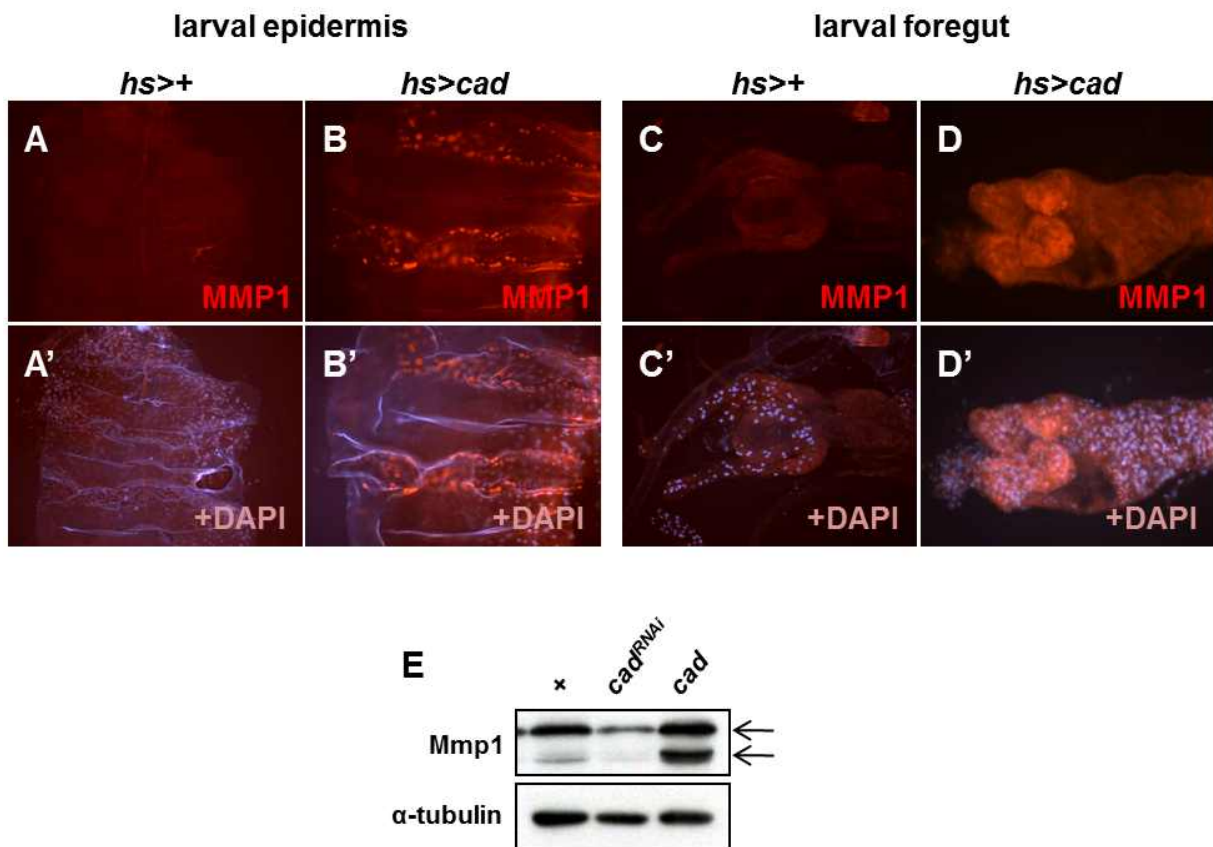


Fig. 2. *Mmp1* expression is increased by *cad* expression in the larval gut and epidermis. A-D. The third instar larvae expressing *cad* under heat shock-inducible *hs-Gal4* driver (*hs>cad*) and driver alone (*hs>+*) were incubated at 25°C for 24 hr after heat shock and the epidermis (A and B) and guts (C and D) were stained with anti-Mmp1 (red), and DAPI (blue). E. Mmp1 expression level was dependent with *cad* expression in larvae. The third instar larvae expressing *cad^{RNAi}* or *cad* under heat shock-inducible *hs-Gal4* driver (*hs>cad^{RNAi}* or *hs>cad*) and driver alone (*hs>+*) were incubated at 25°C for 24 hr after heat shock and the whole larvae extracts were blotted with anti-Mmp1 antibody. Upper arrow indicates full length, inactive form of Mmp1. Lower arrow indicates cleaved, activated form of Mmp1. Alpha-tubulin was used as internal control.

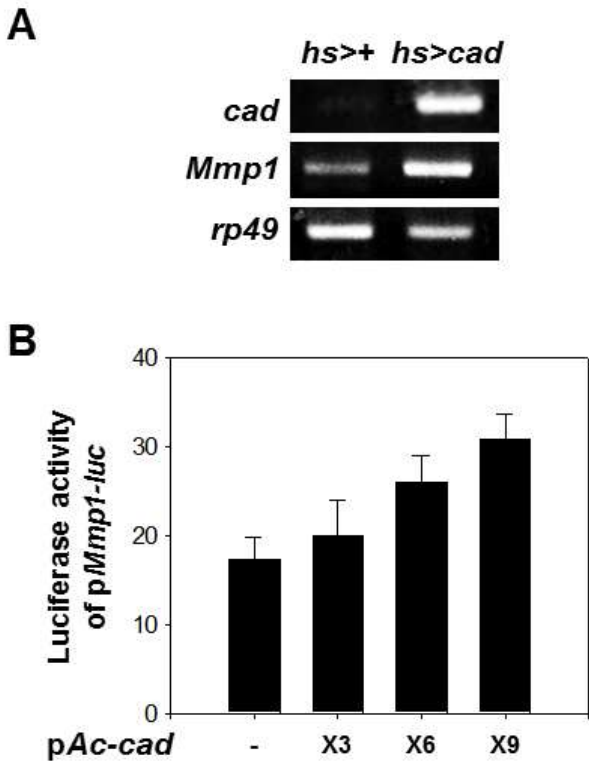


Fig. 3. *Mmp1* expression was regulated by *cad* expression. A. Increase in *Mmp1* mRNA level by *cad* expression in the third instar larvae. The third instar larvae expressing *cad* under heat shock-inducible *hs-Gal4* driver (*hs>cad*) and driver alone (*hs>+*) were incubated at 25°C for 24 hr after heat shock and the mRNA extract from whole larvae was analyzed by RT-PCR. *Ribosomal protein 49* (*rp49*) was used as internal control. B. Activation of *Mmp1* promoter by *cad* expression in S2 cells. The luciferase activity of p*Mmp1-luc* was increased by expression of *cad*. The reporter plasmid p*Mmp1-luc* (100 ng) was co-transfected with the indicated amount of p*Ac-cad* into *Drosophila* S2 cells and the luciferase activities were normalized with co-transfected β -galactosidase activity. Average values obtained from 3 independent experiments with \pm SE values are given.

(-1,980 to +365) of the *Mmp1* gene fused with the luciferase reporter gene which was constructed as described in the Materials and Methods. Effect of Caudal on *Mmp1* gene promoter activity was examined in transient transfection experiments using p*Mmp1-luc* reporter plasmids and a p*Ac-cad* expression plasmid in the *Drosophila* S2 cell line. *Mmp1* promoter was transactivated depending on the amount of p*Ac-cad*, up to nearly 2-fold (Fig. 3B). These results indicate that *Mmp1* expression is directly regulated at transcriptional level by the Caudal protein.

Putative Caudal recognition sites in the 5'-flanking region of the *Drosophila Mmp1* gene

Within the promoter region of *Mmp1* gene, we found eight putative Caudal binding sites similar to the Caudal binding consensus sequences, A/CTTTATA/G, named cad0, 1, 2, 3, 4, 5, 6 and 7 in the 5'-flanking region of the *Mmp1* gene (Fig. 4A). To investigate the potential of Caudal binding sites in *Mmp1* gene, deletion constructs of p*Mmp1-luc* were constructed as described in the Materials and Methods and named from their sites deleted (Fig. 4A).

The promoter activity of -1627p*Mmp1-luc* reporter was not significantly decreased compared to control -1850p*Mmp1-luc* reporter, in which three most distal Caudal binding sites (cad5-7) were deleted (Fig. 4A). The promoter activity of -1119p*Mmp1-luc* reporter dramatically decreased compared to control, in which further three distal Caudal binding sites (cad2-4) were deleted and possessed only two putative Caudal binding sites (cad0, cad1) (Fig. 4A). The reporter lines -708, -570 and -170p*Mmp1-luc* contained only one Caudal binding sites (cad0) in the upstream of transcriptional initiation site did not have significant promoter activities (Fig. 4A). Since the marked reduction of *Mmp1* promoter activity between the -1627 to -1119 regions, the positive regulator might be a function in the promoter region of *Mmp1* gene between the -1627 to -1119 regions.

To assess whether this promoter region is responsible for Caudal, co-transfection experiments were carried out with the wild type or deleted reporter plasmids and Caudal expressing p*Ac-cad* plasmid. The wild-type promoter reporter activity was activated by Caudal expression; while the deletion reporter -1119p*Mmp1-luc* did not respond to Caudal expression (Fig. 4B). In addition, the other deleted reporter lines also were not activated by Caudal (Fig. 4B). Since the promoter activity of -1627p*Mmp1-luc* was not significantly altered compared with -1850p*Mmp1-luc*, the Caudal binding sites cad2-4 may be critical in regulating *Mmp1* expression.

These results indicate that Caudal increases the promoter activity of the *Mmp1* gene and the Caudal binding sites cad2-4 may be critical in the upregulation of *Mmp1* expression by Caudal.

Discussion

In the present study, we documented that the expression of *Mmp1* gene is regulated by the homeodomain tran-

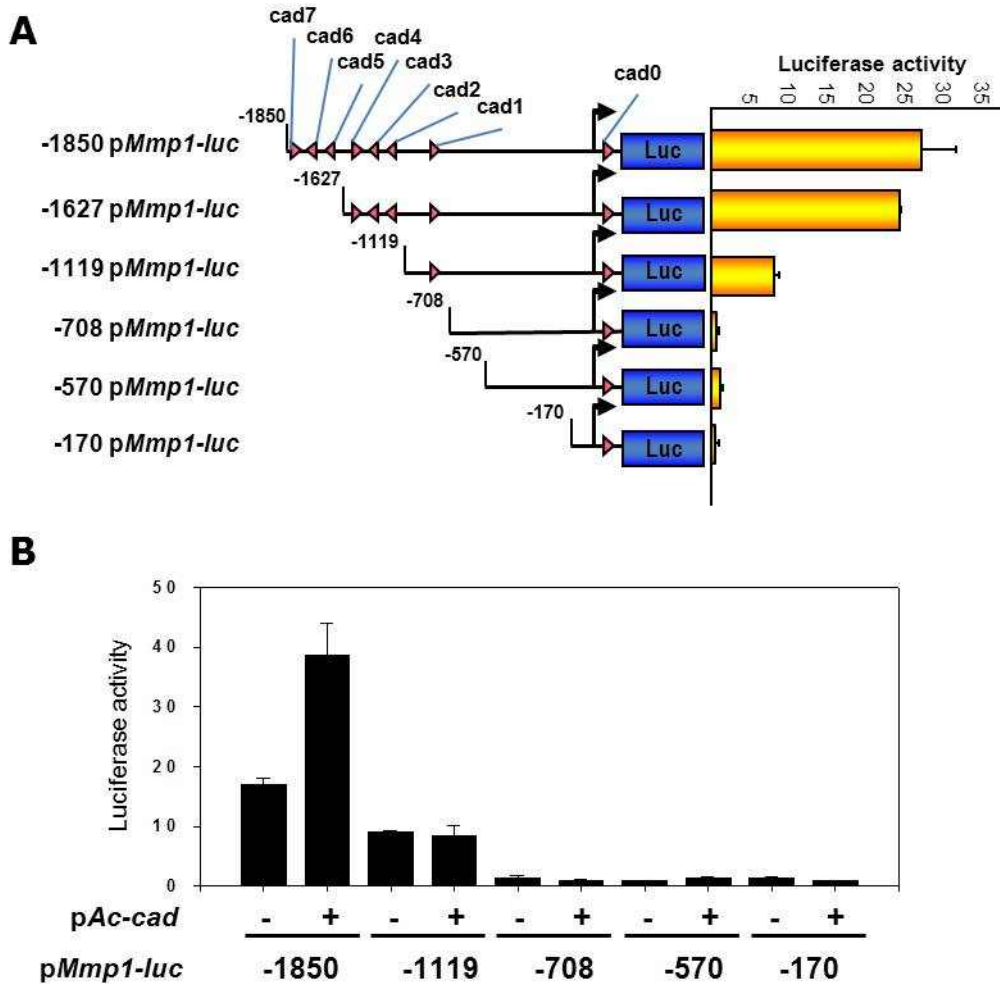


Fig. 4. Caudal responsible region in the *Mmp1* promoter. A. Promoter activity of the deletion constructs of p*Mmp1-luc*. Red triangles indicate putative Caudal binding sites in the *Mmp1* promoter region. Putative 8 Caudal binding sites were existed in the *Mmp1* promoter region. Wild type p*Mmp1-luc* or deletion reporter plasmids (100 ng) were transiently transfected into S2 cells and the luciferase activities were normalized with co-transfected β -galactosidase activity. Average values obtained from 3 independent experiments with \pm SE values are given. B. Effect of Caudal on the activity of *Mmp1* deletion reporter plasmids. Wild type p*Mmp1-luc* or deletion reporter plasmids (100 ng) were co-transfected with the p*Ac-cad* (600 ng) into S2 cells and the luciferase activities were normalized with co-transfected β -galactosidase activity. Average values obtained from three independent experiments with \pm SE values are shown.

scription factor Caudal in *Drosophila*.

Recently, we reported that the *Drosophila Mmp1* gene is highly expressed in the digestive tract in both third instar larvae and adult flies and is required for the maintenance of intestinal homeostasis such as the restriction of uncontrolled intestinal stem cell proliferation, and the maintenance of intestinal architecture [15]. The *Drosophila cad* gene encoding the homeodomain transcription factor Caudal and its mammalian counterpart *Cdx* genes are well known to be a master regulator of intestinal development [21,30]. Interestingly, mammalian *Cdx1* has been known to be ex-

pressed in the intestinal crypt, where stem cells and progenitor cells are resided, and *Cdx2* is expressed in the differentiated enterocytes [8] as similar with the expression of *Drosophila Mmp1* in intestinal enterocytes [15]. It was reported the extinction of *CDX1* in human colorectal tumors, suggesting a tumor suppression function of *Cdx1* [19,29]. In addition, *Cdx2* has been well known to function as tumor suppressor gene in the intestine [2,14]. From these facts, our results suggest that the regulation of MMP expression by endogenous CDX in physiological normal condition may be associated with the role of CDX as tumor suppressor.

Furthermore, previous our report showed that the expression of *Mmp1* is increased with age and exposure to oxidative stress [15]. Caudal was also reported to be accumulated in the midguts with age and oxidative stress, which is mediated with NF κ B [4]. Thus, the increase of MMPs expression with age and exposure to oxidative stress may also be regulated by the Caudal transcription factor, although further investigations are required. The overexpression of *Mmp1* also induces intestinal stem cell proliferation [15] as consistent with a great deal of reports to show the tumorigenic function of MMP in mammal. The ectopic expression of *Drosophila* Caudal has been reported to induce melanotic tumor in third instar larvae [12] and an increase of *cad* expression to induce hyperproliferation of the adult midgut [4]. Ectopic expression of human CDX1 has been known to be associated with intestinal metaplasia in gastric and esophageal tumors [27], suggesting that Cdx1 has an oncogenic potential. From these studies, it will be interesting to further investigate the specific functions of Caudal/CDX and MMPs in regulating ISC proliferation and tumorigenesis.

Although further investigations including rescue experiment to assess the involvement of *Mmp1* in the Caudal-regulated intestinal homeostasis was needed, our data suggests that *Mmp1* is one of the target genes of Caudal in physiological normal condition and tumorigenesis

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초록 : 초파리 장조직에서 Caudal 전사조절인자에 의한 matrix metalloproteinase-1 발현 조절

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Matrix metalloproteinase (MMP)는 세포외골격의 주요 조절효소로, 배아발생, 혈관생성, 상처치료 및 조직 재생과정에 중요한 인자로 알려져 있다. MMP의 조절 이상은 비정상적 세포외골격 분해로 인해 암 전이와 같은 질병을 일으킨다. 따라서, MMP의 발현과 활성은 엄격하게 조절되고 있다. 최근, 초파리 Mmp1이 소화기관에서 강하게 발현되며, 장줄기세포의 비정상적인 활성을 억제하여 장의 항상성 유지에 중요함을 밝혔다. 하지만, 장조직에서 Mmp1의 발현 조절 기전은 아직 밝혀지지 않았다. 본 연구에서는, 장조직에서 Mmp1의 발현이 장 발생과 항상성 유지에 중요한 Caudal homeobox 유전자에 의해 조절되는지를 연구하였다. GAL4/UAS 조절계를 이용하여 장조직 특이적으로 Caudal의 발현을 감소시켰을 때, Mmp1의 발현이 감소함을 확인하였으며, Caudal을 과발현 시켰을 때, Mmp1의 발현이 증가함을 *in vitro*와 *in vivo* 실험 모두에서 확인하였다. 또한, Mmp1 promoter에 Caudal 전사인자 결합 부위가 존재하며, 이 부위가 Mmp1 발현에 중요한 역할을 함을 확인하였다. 이상의 본 연구는, 정상적 혹은 암화 과정에서 Mmp1이 Caudal의 표적 유전자일 수 있음을 의미한다.