

# イヌ乳腺腫瘍の筋上皮細胞におけるコンドロモジュリン-I(ChM-I)及び骨形成タンパク-6 (BMP-6)の共発現

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## Co-localization of Chondromodulin-I (ChM-I) and Bone Morphogenetic Protein-6 (BMP-6) in Myoepithelial Cells of Canine Mammary Tumors

Atsushi KAWABATA<sup>1,2</sup>, Kumiko OKANO<sup>2</sup>, Kazuyuki UCHIDA<sup>2</sup>, Ryoji YAMAGUCHI<sup>2</sup>, Toshiharu HAYASHI<sup>3</sup> and Susumu TATEYAMA<sup>2</sup>\*

<sup>1</sup>Department of Pathogenetic and Preventive Veterinary Science, The United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi 753-8515, <sup>2</sup>Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2155 and <sup>3</sup>Department of Veterinary Pathology, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan

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**ABSTRACT.** To compare the roles of chondromodulin-I (ChM-I) and bone morphogenetic protein-6 (BMP-6) in ectopic mesenchymal tissue formation in canine mammary gland tumors, 33 tumors and 2 normal mammary glands were examined. Immunohistochemical analysis revealed co-expression of ChM-I and BMP-6 in canine mammary tumors. In mixed tumors, newly formed woven bone with ossified cartilage matrix was observed in 4/9 cases. The osteoblasts lining the woven bone showed moderate immunoreactivity to ChM-I and BMP-6. Almost all chondrocytes and proliferative myoepithelial cells within the basement membrane showed intense immunoreactivity to both, and the myoepithelial cells adjacent to the mature cartilage showed the most intense immunoreactivity. The immunoreactivity to ChM-I and BMP-6 of the interstitial myoepithelial cells in the myxomatous stroma varied in each focus of mixed tumors. Similar findings were found in complex adenomas. In simple adenomas, hyperplastic myoepithelial cells within the basement membrane showed moderate immunoreactivity to both markers. Western blot analysis detected a 25 kDa band of ChM-I in fresh tissue samples from three mixed tumors. Our results support the hypothesis that proliferating myoepithelial cells with ChM-I and BMP-6 expression play important roles in mesenchymal metaplasia in canine mammary tumors.

**KEY WORDS:** BMP-6, canine, chondromodulin-I, mammary tumor, myoepithelial cell.

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Mammary gland tumors are the most common neoplasms in female dogs [15]. Their most unique morphological features are prominent proliferation of myoepithelial cells and formation of ectopic mesenchymal tissue, including cartilage and bone, especially in complex adenomas and benign mixed tumors. Although proliferation of myoepithelial cells is rare in human breast tumors, salivary pleomorphic adenoma is characterized by mixed proliferation of both glandular epithelial and myoepithelial cells with cartilage or bone formation. Several reports suggest that neoplastic myoepithelial cells contribute to the formation of these ectopic mesenchymal elements [1, 20].

Chondromodulin-I (ChM-I), a 25 kDa glycosylated protein composed of 335 amino acids, was first extracted and cloned from fetal bovine cartilage [3, 11]. The major biological functions of ChM-I are to stimulate the proliferation of chondrocytes and inhibit angiogenesis [8-10]. Several reports have indicated that, in human salivary pleomorphic adenomas, ChM-I might be expressed in the lacunar cells of the chondroid matrix and in the myoepithelial cells [13, 14]. These observations suggest that myoepithelial cells with ChM-I expression play major roles in the formation of ectopic cartilage and bone in these human tumors. However, there is little information on the roles played by ChM-I in canine mammary gland tumors.

Bone morphogenetic proteins (BMPs), belonging to a subgroup of the transforming growth factor- $\beta$  (TGF- $\beta$ )

superfamily, and their receptors, have been detected in myoepithelial cells in canine mammary tumors [1, 20]. BMPs were originally identified as important factors for endochondral ossification [17, 21, 22]. BMP-6 is also involved in the development of the embryonic urinary system and in the differentiation of keratinocytes [4, 5]. Myoepithelial cells in human salivary pleomorphic adenomas are intensely positive for BMPs, suggesting that BMPs might be involved also in ectopic cartilage or bone formation in the tumor [7, 23].

Our aim was to examine the association of ChM-I with ectopic mesenchymal tissue formation and compare the distribution patterns of ChM-I and BMP-6 among several types of canine mammary gland tumors.

### MATERIALS AND METHODS

**Tissue samples:** Surgical specimens from 33 mammary tumors and two normal mammary gland tissues were used. The tissues were fixed in methanol-Carnoy's solution. Paraffin sections 4  $\mu$ m thick were made and stained with hematoxylin and eosin (HE) for routine histopathological examination. The diagnosis of each tumor was based on the World Health Organization (WHO) classification [15]. The diagnoses are summarized in Table 1.

**Antibodies:** Immunohistochemistry was performed using an avidin-biotin-peroxidase complex (ABC) kit (PK4000, Vectastain, Burlingame, CA, U.S.A.). Goat antisera against human ChM-I (1:20, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and BMP-6 (1:20, Santa Cruz Biotechnology) were used as primary antibodies. The secondary

\* CORRESPONDENCE TO: TATEYAMA, S., Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2155, Japan.

Table 1. Diagnosis of canine mammary tumors examined

Histological diagnosis	Number of cases
Normal	2
Simple adenoma	
Tubular	2
Papillary	2
Complex adenoma	8
Benign mixed tumor	9
Adenocarcinoma	
Tubular	1
Papillary	3
Solid	4
Complex adenocarcinoma	2
Carcinoma in mixed tumor	2
Total	35

antibody was a biotinylated rabbit serum against goat immunoglobulin (1:20, Dako-Japan, Kyoto, Japan).

**Immunohistochemistry:** Sections were incubated with 3% hydrogen peroxide in methanol at room temperature for 10 min to block endogenous peroxidase activity. Sections were incubated at 37°C for 40 min with phosphate-buffered saline (PBS) (pH 7.4) containing 3% bovine serum albumin (BSA) to avoid nonspecific binding. The sections were then incubated with primary antibodies at 37°C for 45 min, followed by incubation with secondary antibody and ABC reagent at 37°C for 45 min, respectively. The sections were exposed to 3, 3'-diaminobenzidine-4HCl (DAB, Sigma, St. Louis, MO, U.S.A.) and then counterstained with Mayer's hematoxylin. In accordance with the findings of previous reports [1, 5, 16, 17], mammary cells were classified into 4 types: (1) glandular epithelial cells, (2) resting and proliferating myoepithelial cells within the basement membrane, (3) proliferating myoepithelial cells at the interstitial myxomatous areas, and (4) chondrocytes in the ectopic cartilage. The intensity and distribution of immunoreactivity to the antibodies were quantified by assessing the labeled cells in 10 high power fields ( $\times 400$ ) as follows: ( $\pm$ )=0%, (+)=0%–5%, (++)=5%–10%, (+++)=10%–50%, and (++++)=>50%.

**Western blot analysis:** Fresh tissue samples from three benign mixed tumors were used for Western blot analysis. Western blot analysis was performed according to a previous report [16]. Briefly, samples were homogenized in extraction buffer and sonicated on ice for 2.5 min; this was repeated 5 times. Then the extracts were centrifuged at  $10,000 \times g$  for 10 min. The supernatants were dialyzed through a cellophane tube (Wako, Osaka, Japan) and freeze-dried. The samples obtained were separated by SDS-12% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Atto, Tokyo, Japan). After incubation with antibody against human ChM-1 (1:20, Santa Cruz Biotechnology) at 37°C for 1 hr, the membrane was incubated with biotinylated rabbit anti-goat IgG (1:20, Dako-Japan) at 37°C for 1 hr. The membrane was then reacted with ABC reagents at 37°C for 30

min. The attached antibodies were visualized using DAB (Sigma).

## RESULTS

The distribution patterns of ChM-I- and BMP-6-positive myoepithelial cells were almost the same. The results of immunohistochemical analysis for ChM-I and BMP-6 are summarized in Tables 2 and 3.

**Simple adenomas (n=4):** The tumors were composed of well-differentiated glandular or tubular epithelial cells with resting myoepithelial cells. The neoplastic epithelial cells showed two proliferating patterns, tubular (two cases) or papillary (two cases). In three cases, weak to moderate immunoreactivity to both ChM-I and BMP-6 was observed in the resting myoepithelial cells (Fig. 1). A few neoplastic glandular epithelial cells were weakly positive for both. Myoepithelial cells in tubular adenomas tended to show more intense immunoreactivity to BMP-6 than those in papillary adenomas.

**Complex adenomas (n=8):** The tumors were composed of glandular or tubular epithelial cells and myoepithelial cells with various amounts of myxomatous stroma. Moderate to mild immunoreactivity to ChM-I was observed in the proliferative intraductal myoepithelial cells in all cases. Although immunoreactivity to ChM-I was weak in the interstitial myxomatous myoepithelial cells, the intraductal myoepithelial cells in most cases were intensely positive for ChM-I (Table 2 and Fig. 2). Most neoplastic glandular epithelial cells were negative for ChM-I. The distribution pattern of BMP-6 in 8 complex adenomas was consistent with that of ChM-I (Fig. 2). Immunoreactivity to BMP-6 in the proliferating myoepithelial cells in both intraductal and interstitial regions was mild compared with that to ChM-I (Table 2).

**Benign mixed tumors (n=9):** In this type of tumor, ectopic cartilage formation was observed in addition to the proliferation of both myoepithelial and glandular epithelial cells. The most intense reaction to both ChM-I and BMP-6 was detected in the lacunar cells of the chondroid matrix in 6 of 9 cases (Fig. 3). Proliferating myoepithelial cells adjacent to the ectopic cartilage also showed intense immunoreactivity to both (Fig. 4). Woven bone with calcified cartilage matrix surrounded by osteoblasts was observed in 4 of 9 cases (Table 2). The osteoblasts lining the newly formed woven bone showed moderate immunoreactivity to both markers (Fig. 5). Immunoreactivity to ChM-I in the proliferating intraductal myoepithelial cells varied among cases. Intense reaction to BMP-6 was observed in the intraductal and interstitial myxomatous myoepithelial cells of mixed tumors (Table 2). Proliferating myoepithelial cells in the interstitial myxomatous regions showed mild immunoreactivity to ChM-I and intense immunoreactivity to BMP-6 (Fig. 6). The immunoreactivity of the interstitial myoepithelial cells tended to mild compared with that of the intraductal myoepithelial cells. The neoplastic glandular epithelial cells were negative for both markers.

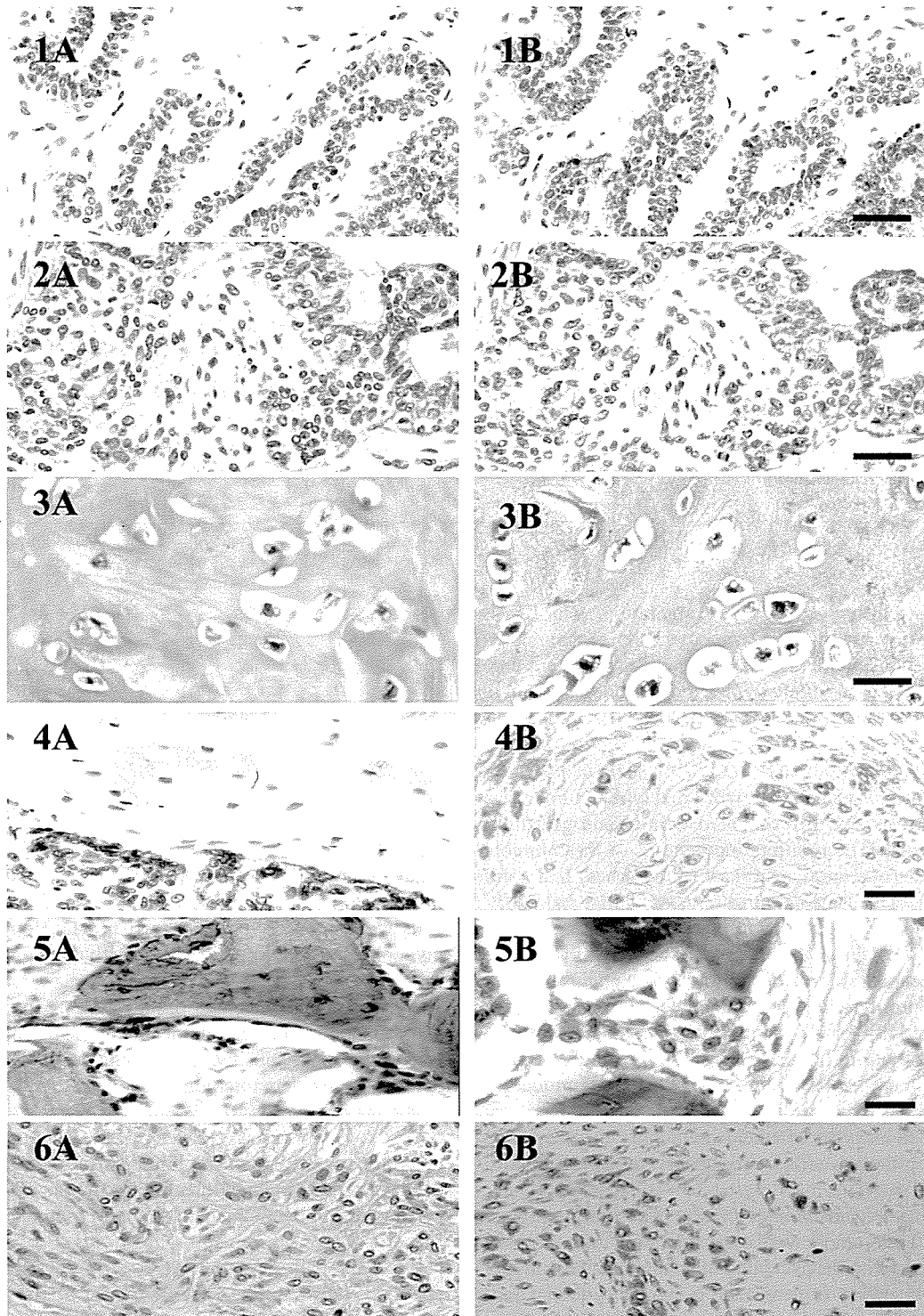


Fig. 1. Mammary gland; simple adenoma (case No. 3). Immunoreactivity of resting myoepithelial cells. ChM-I (A) and BMP-6 (B). Bar=60  $\mu$ m.  
 Fig. 2. Mammary gland; complex adenoma (case No. 14). Intense immunoreactivity of proliferating myoepithelial cells within the basement membrane and weak reactivity of interstitial myoepithelial cells. ChM-I (A) and BMP-6 (B). Bar=60  $\mu$ m .  
 Fig. 3. Mammary gland; benign mixed tumor (case No. 22). Intense immunoreactivity of mature cartilage. ChM-I (A) and BMP-6 (B). Bar=70  $\mu$ m.  
 Fig. 4. Mammary gland; benign mixed tumor (case No. 21). Intense immunoreactivity of the proliferating myoepithelial cells adjacent to the ectopic cartilage. ChM-I (A) and BMP-6 (B). Bar=50  $\mu$ m.

Table 2. Immunoreactivity to ChM-I and BMP-6 in canine benign mammary tumors

Diagnosis	Case No.	*Glandular ECs		Intraductual MCs		Interstitial MCs		Chondrocytes		Osteoblasts		
		ChM-I	BMP-6	ChM-I	BMP-6	ChM-I	BMP-6	ChM-I	BMP-6	ChM-I	BMP-6	
Normal (n=2)	1	-	-	±	+							
	2	-	-	-	-							
Simple adenoma (n=4)	Tubular	3	±	±	2+	+						
		4	-	±	±	2+						
Papillary		5	±	-	2+	2+						
		6	-	-	+	-						
Complex adenoma (n=8)		7	±	-	2+	2+	2+	±				
		8	-	-	2+	+	±	2+				
		9	±	±	2+	2+	±	2+				
		10	-	±	2+	2+	+	±				
		11	-	-	+	±	±	±				
		12	±	-	2+	+	±	±				
		13	±	-	+	±	±	-				
		14	±	±	2+	2+	±	±				
Benign mixed tumor (n=9)		15	-	-	+	2+	+	+	2+	2+	+	+
		16	±	±	2+	2+	±	±	3+	+		
		17	±	±	2+	2+	±	+	2+	3+	+	+
		18	±	±	2+	2+	+	2+	2+	2+	+	+
		19	-	-	+	±	±	+	+	+		
		20	±	±	2+	2+	2+	2+	+	2+	+	+
		21	±	±	3+	2+	2+	2+	2+	2+		
		22	±	±	2+	2+	2+	2+	2+	3+		
		23	±	±	±	2+	+	±	2+	+		

\* EC=epithelial cells; MC=myoepithelial cells.

(-)=0%, (±)=0-5%, (+)=5-10%, (2+)=10-50% and (3+)=>50% positive cells.

**Simple adenocarcinomas (n=8):** This type of tumor was composed of poorly differentiated tubular and glandular epithelial cells. The proliferating patterns of these tumors were tubular (one case), papillary (three cases) and solid (four cases). The distribution patterns of ChM-I and BMP-6 in the tumors were consistent with those of simple adenomas (Table 3). Proliferating myoepithelial cells were rare in solid adenocarcinomas. In tubular adenocarcinomas, resting and proliferating myoepithelial cells showed intense immunoreactivity for BMP-6 and ChM-I compared with the level of reactivity in tubular adenomas. The myoepithelial cells in papillary adenocarcinomas showed mild immunoreactivity to both markers compared with those in papillary adenomas. In all types of adenocarcinomas, the neoplastic glandular epithelial cells were weakly positive for both markers.

**Complex adenocarcinomas (n=2):** The morphological features of these tumors were consistent with those of complex adenoma, with the exception of apparent cellular atypia and invasive activity of neoplastic glandular epithelial and/or myoepithelial cells. The results of ChM-I- and BMP-6-immunostaining were consistent with those in complex ade-

nomas (Table 3).

**Adenocarcinomas in benign mixed tumors (n=2):** The morphology of these tumors was similar to that of benign mixed tumors, except for the malignant features of neoplastic epithelial cells. Intraductal and interstitial myoepithelial cells were intensely positive for ChM-I. However, these myoepithelial cells were weakly positive for BMP-6 (Table 3).

**Normal mammary gland tissues (n=2):** In intact mammary glands around the tumor mass, resting myoepithelial cells were weakly positive for both markers. The glandular epithelial cells were totally negative (Table 2).

**Western blot analysis:** Western blot analysis was performed to examine the presence of ChM-I protein in benign mixed tumors. Bands immunopositive for ChM-I were detected at 25 kDa in all three mixed tumors examined; this corresponds to the molecular weight of human ChM-I (Fig. 7).

## DISCUSSION

ChM-I was distributed in neoplastic myoepithelial cells

Fig. 5. Mammary gland; benign mixed tumor (case No.18). Mild or moderate immunoreactivity of the osteoblastic cells. ChM-I (A) and BMP-6 (B). Bar=70  $\mu$ m.

Fig. 6. Mammary gland; benign mixed tumor (case No. 21). Moderate immunoreactivity of interstitial myxomatous myoepithelial cells. ChM-I (A) and BMP-6 (B). Bar=50  $\mu$ m.

Table 3. Immunoreactivity to ChM-I and BMP-6 in canine malignant mammary tumors

Case No.	* Glandular ECs		Intraductal MCs		Interstitial MCs		Chondrocytes		
	ChM-I	BMP-6	ChM-I	BMP-6	ChM-I	BMP-6	ChM-I	BMP-6	
Adenocarcinoma (n=8)									
Tubular									
24	±	-	2+	2+					
Papillary									
25	-	-	+	+					
26	-	-	-	-					
27	±	-	+	±					
Solid									
28	±	±							
29	-	±							
30	-	-							
31	±	-							
Complex adenocarcinoma (n=2)									
32	-	±	2+	2+	+	+			
33	±	±	2+	+	2+	±			
Carcinoma in mixed tumor (n=2)									
34	±	-	2+	+	±	±	2+	2+	
35	±	±	2+	2+	2+	+	3+	2+	

\* EC=epithelial cells; MC=myoepithelial cells.  
 (-)=0%, (±)=0-5%, (+)=5-10%, (2+)=10-50% and (3+)=>50% positive cells.

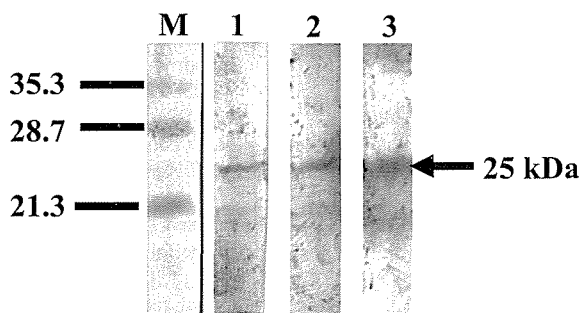


Fig. 7. Western blot analysis for a 25 kDa protein of ChM-I from fresh tissue samples of 3 benign mixed tumors. M. Low molecular weight marker. Lanes 1-3. Fresh tissues samples from benign mixed tumors.

in canine mammary tumors. Arai *et al.* [2] indicated that class II  $\beta$ -tubulin was associated with cartilaginous metaplasia of proliferative myoepithelial cells in canine benign mixed tumors. Gama *et al.* [6] also suggested p63 as an additional marker of myoepithelial histogenesis. Although previous studies have suggested that metaplasia of proliferative myoepithelial cells might be involved in the formation of cartilage or bone in canine benign mixed tumors, this hypothesis has not been completely supported. Our present results indicate that expression of ChM-I in myoepithelial neoplastic cells might be involved in the formation of cartilage in canine benign mixed tumors. Expression of ChM-I was observed in myoepithelial cells within several types of canine mammary tumors, especially in complex and mixed tumors. The basic distribution pattern of ChM-I-immunopositive cells was similar to that of BMP-6 positive cells, as previously reported [1, 20]. This suggests that ChM-I and

BMPs play important roles in the metaplastic process.

Immunoreactivity to BMP-6 was observed in intraductal and interstitial myxomatous myoepithelial cells in complex adenomas and benign mixed tumors. Although intense immunoreactivity to ChM-I was observed in chondrocytes, interstitial myxomatous myoepithelial cells showed weak immunoreactivity to ChM-I. The most intense immunoreactivity to ChM-I was observed in the proliferative myoepithelial cells adjacent to ectopic cartilage and mature chondrocytes. The intense expression of ChM-I in these cells may be explained by the biological nature of this protein as an inducer of cartilage and bone formation [3, 9-11, 19]. These observations suggest that both ChM-I and BMP-6 play roles in the metaplastic change of myoepithelial cells, and especially that ChM-I might contribute to metaplastic change of the cells into mature chondrocytes.

In addition, the osteoblasts lining the woven bone showed moderate immunoreactivity for ChM-I and BMP-6. Although ChM-I was first isolated as a growth-promoting factor from chondrocytes [12], Suzuki [18] suggested that ChM-I and -II might be associated with endochondral ossification. Nakamichi *et al.* [16] also reported that ChM-I knockout mice showed a significant increase in bone mineral density and suggested that ChM-I might play a role in endochondral bone development. In agreement with these observations, our results suggested that ChM-I might be associated with not only chondral metaplasia but also osseous metaplasia of mixed tumors. Interestingly, intraductal myoepithelial cells showed distinct immunoreactivity to ChM-I and BMP-6, even in simple adenomas and in histologically intact mammary glands. This indicated that they might have certain roles in regulating the proliferation and/or transformation of myoepithelial cells. However, immunoreactivity to both markers was slightly decreased in the

malignant counter parts of these tissues, suggesting that the intense expression of ChM-I and BMP-6 in myoepithelial cells might decrease with proliferation outside the basement membrane.

We observed the expression of ChM-I and BMP-6 in canine mammary gland tumors. Co-localization of ChM-I and BMP-6 in myoepithelial cells might be associated with mesenchymal metaplasia of canine mammary gland tumors. BMP-6 might be involved in all stages of metaplasia of myoepithelial cells to cartilages or bone, and ChM-I might especially participate in the latter process of cartilage formation, and also in endochondral ossification. To confirm some other additional roles of ChM-I, such as inhibition of the proliferation of neoplastic cells, further studies *in vivo* and *in vitro* will be required.

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