Specific loss of chondromodulin-I gene expression in chondrosarcoma and the suppression of tumor angiogenesis and growth by its recombinant protein in vivo

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Abstract Chondromodulin-I (ChM-I) was previously identified as an angiogenesis inhibitor in cartilage. Here, we demonstrated that the level of ChM-I transcripts was substantially reduced to 100 or even less in the lower-grade chondrosarcomas, in articular cartilage or other benign cartilage tumors. We implanted human chondrosarcoma OUMS-27 cells into nude mice that reproducibly produced tumors with cartilaginous matrix. Tumor-induced angiogenesis was evident when the tumors were excised 30 days after implantation. However, the local administration of recombinant human ChM-I almost completely blocked vascular invasion and tumor growth in vivo. Moreover, ChM-I also inhibited the growth of HT-29 colon adenocarcinoma in vivo, implying its therapeutic potential for solid tumors.
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Key words: Chondromodulin-I; Angiogenesis inhibitor; Tumor angiogenesis; Tumor suppression

1. Introduction

Vascularity of tissues is regulated by the balance of the cumulative levels of inducer and inhibitor signals that maintain the endothelial cells in the alternative 'angiostatic' and 'angiogenic' states[1,2]. Thus, the angiogenic switching could be activated either by increasing angiogenic inducers or by reducing inhibitors. Cartilage is avascular and markedly resistant to vascular invasion. Despite the apparent avascularity, several angiogenic molecules have been described in cartilage, including fibroblast growth factor (FGF) [3]. Due to an intrinsic inhibitor [4], cartilage remains avascular except during endochondral bone development. When chondrocytes become hypertrophic and calcified, capillaries invade from the surrounding periosteum and trigger the replacement of cartilage by bone. The angiogenic switching of cartilage from 'angiostatic' to 'angiogenic' thus takes place at a precise stage during development. However, no tissue-specific macromolecule explicable for the angiogenic resistance of cartilage has been identified.

We previously isolated a novel endothelial cell growth inhibitor, chondromodulin-I (ChM-I), from fetal bovine cartilage [5]. It is a 25 kDa glycosylated protein that inhibits angiogenesis in the chick chorioallantoic membrane in vivo [6]. In situ hybridization defined the specific expression of ChM-I

transcripts within the avascular zone of cartilage in developing bone [5,7]. However, the expression of the gene was markedly reduced in the late hypertrophic and calcified zones of cartilage, allowing for vascular invasion. The localization of ChM-I protein completely overlapped the area of its gene expression [5]. The secreted mature ChM-I was accumulated in the interterritorial space of cartilage matrix so that an anti-angiogenic barrier of ChM-I could wrap up the angiogenic FGF-containing pericellular space [5,8].

A considerable body of evidence has suggested that the growth of solid tumor and metastasis require persistent new blood vessel growth [1]. A malignant cartilage-forming tumor is not an exception. As we previously reported [7,9], normal cartilage contains abundant ChM-I transcripts in comparison to those for other growth factors. However, during the molecular cloning of human ChM-I cDNA [6], we noticed that expression of the ChM-I gene was extremely low in chondrosarcoma (CS). Transcripts for ChM-I were hardly detectable in CS even in the tissues from the differentiated type lesion (grade I). Thus, we hypothesized that the marked reduction of ChM-I expression is involved in loss of the 'anti-angiogenic' property of cartilage during malignant transformation. In this study, we first compared the level of ChM-I mRNA expressed in CS with those in normal articular and other benign tumors. We then examined whether the anti-angiogenic properties could be restored in tumors by local administrations of recombinant human ChM-I (rhChM-I) protein.

2. Materials and methods

2.1. Materials and clinical samples

rhChM-I was expressed in Chinese hamster ovary cells and purified from the culture supernatant to homogeneity by sequential chromatography, as described [6]. Clinical samples were obtained from the patients with informed consent. Six cases with CS (grade I periosteal CS: two cases, grade I CS: three cases, grade II CS: one case), six cases with enchondroma (Ench), six cases with exostosis (Exo) and five cases with articular cartilage (AC) were examined. Each specimen was processed separately for pathological examination and for mRNA extraction. Sections were evaluated by a hospital staff pathologist.

2.2. Cell culture

Human CS OUMS-27 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 in air [10]. HCS-TG cells were maintained in α-modified Eagle's medium (αMEM) containing 20% FBS at 37°C in 5% CO_2 in air. AC was obtained from the femoral head of a 51 year old female who was suffering from osteoarthritis and underwent a total hip arthroplasty. Chondrocytes were then isolated by sequential digestions

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with 0.1% EDTA for 20 min, 0.2% trypsin for 1 h and 0.15% collagenase for 3 h [5]. The cells were grown in αMEM containing 10% FBS at 37°C in 5% CO_2 in air. The medium was replaced every other day.

2.3. Northern blot analysis

Total RNA was prepared from various human tissues and cultured cells by the single-step method of Chomczynski and Sacchi [11]. Total RNA (20 μg) was denatured with 6% formaldehyde, separated by 1% agarose electrophoresis and transferred onto Nytran membranes (Schleicher and Schuell, Germany). Hybridization was performed overnight at 42°C with an appropriate probe (10^6 cpm/ml) as previously described [7]. Hybridization probes were prepared by the random primer method with a BcaBEST Labelling kit (Takara, Japan) using the appropriate cDNA fragments: a 1.4 kb EcoRI fragment of pKT1180 [7] for α1(II) collagen mRNA and a 0.9 kb EcoRI fragment of pCRII-hChM-I [6] for ChM-I mRNA. The filters were washed twice for 15 min at 55°C in $0.1 \times$ SSPE and 0.1% SDS and exposed to Kodak X-OMAT film at −80°C.

2.4. Reverse transcription (RT)-PCR and competitive PCR

To correct for any variation in RNA content and cDNA synthesis between the different preparations, samples were equalized on the basis of their β-actin content which was determined by using the human β-actin Competitive PCR set (Takara, Tokyo, Japan) in accordance with the manufacturer's instructions. First strand cDNA was synthesized using SuperScript II RNAse H⁻ Reverse Transcriptase (Gibco, Grand Island, NY, USA) with the corrected amount of total RNA isolated from human tissues or cultured cells according to the manufacturer's instructions. Portions of total RNA were reverse-transcribed. For RT-PCR, aliquots of 1/20 of the cDNA were used to amplify ChM-I, α1(II) collagen, aggrecan and β-actin genes. The following primer sets were synthesized: 5′-CATCGGGGGCCTTC-TACTTCT-3′ and 5′-GGCATGATCTTGCCTTCCAG-3′ for ChM-I mRNA [6], 5′-AACTGGCAAGCAAGGAGACA-3′ and 5′-AG-TTTCAGGTCTCTGCAGGT-3′ for α1(II) collagen [12], 5′-ATGCC-CAAGACTACCAGTGG-3′ and 5′-TCCTGGAAGCTCTTCTCA-GT-3′ for aggrecan [12], 5′-AGCCATGTACGTTGCTA-3′ and 5′-AGTCCGCCTAGAAGCA-3′ for β-actin [13]. Expected sizes of the PCR products were 312, 621, 501 and 800 bp for ChM-I, α1(II) collagen, aggrecan and β-actin, respectively. For the competitive PCR analysis of ChM-I mRNA, the 479 bp competitive template for human ChM-I was generated using the composite primers (5′-CATCGGGGGCCTTCTACTTCTCAAGTTTCGTGAGCTGATTG-3′ and 5′-GGCATGATCTTGCCTTCCAGTTGAGTCCATGGGGA-GCTTT-3′) with a PCR MIMIC™ Construction kit (Clontech, CA, USA). One-fiftieth of the product was re-amplified by using the gene-specific primer described above. Following the second PCR amplification, the competitive template was purified by Chroma SPIN+TE-100 and diluted to 100 attomol/μl for competitive experiments. A fixed amount of cDNA was co-amplified with serial loga-

rithmic dilutions of the competitive template (from 1 to 10^{-6} attomol/μl). The competitive template yields a larger PCR product of 479 bp. The amplification reaction was carried out with initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 90 s and a final extension at 72°C for 4 min. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and photographed. The fluorescence intensity of each PCR product was estimated on NIH image 1.60, as previously described [14].
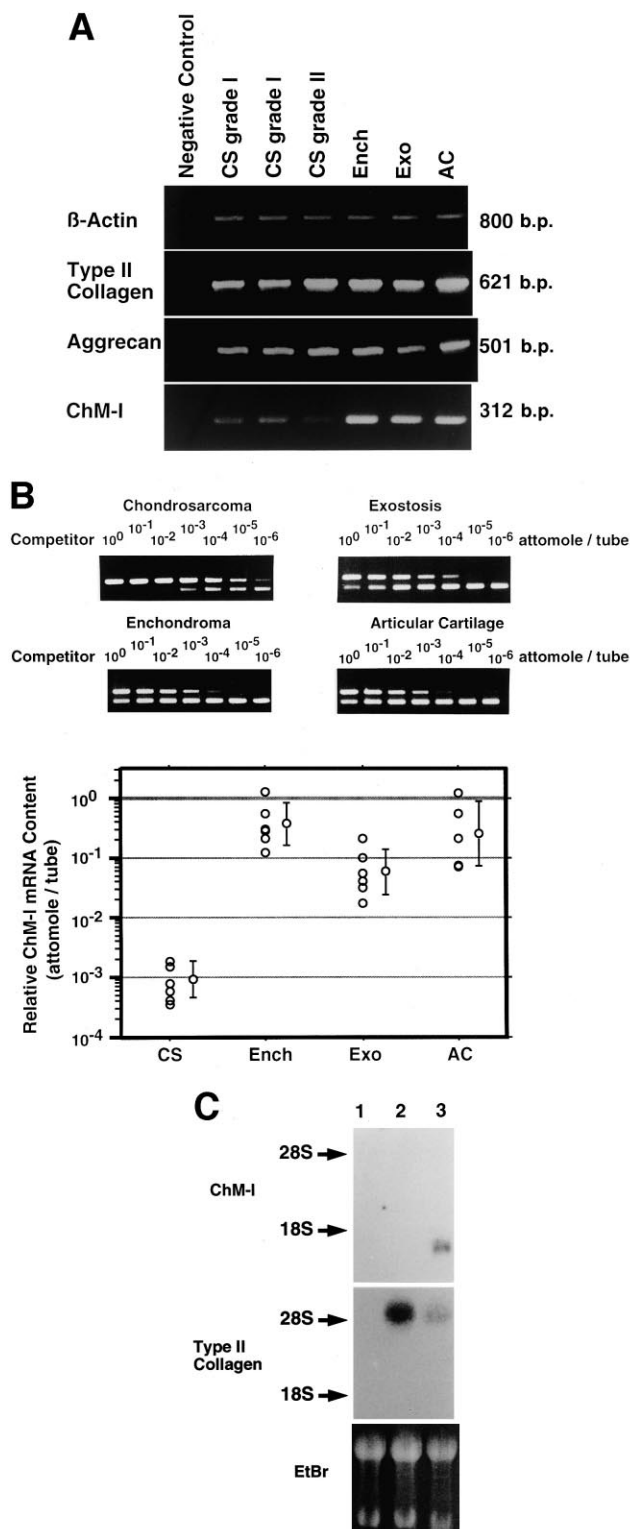

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Fig. 1. Expression of ChM-I mRNA in human chondrogenic tumors and AC. Total RNA was isolated from human CS, Ench, Exo and AC. A: Reverse-transcribed cDNA was amplified by PCR using primer pairs specific for human β-actin, α1(II) collagen, aggrecan or ChM-I. B: The relative level of ChM-I mRNA was estimated by competitive PCR using cDNA from CS (grade I) and other cartilaginous tissues. Each reverse-transcribed cDNA was co-amplified by gene-specific primers in the presence of a 10-fold dilution series of competitive template (upper panels). Estimated ChM-I mRNA levels were plotted in a logarithmic scale with means ± S.D.s ($n = 6$ for grade I CS, $n = 6$ each for Ench and Exo and $n = 5$ for AC) in the lower panel. C: Total RNA (20 μg/lane) isolated from human CS cells HCS-TG (lane 1), OUMS-27 (lane 2) or human articular chondrocytes (lane 3) was separated by a 1% agarose gel for Northern blot analysis. The transferred filter was hybridized with human ChM-I cDNA (upper panel) or rat α1(II) collagen cDNA (lower panel). Equivalent loading of RNA was verified by ethidium bromide staining (bottom panel). The positions of 28S and 18S ribosomal RNAs are indicated. The nucleotide sequence of human ChM-I cDNA is available from the GenBank database under accession numbers AB005999 and AB006000.

2.5. Xenograft tumor models

Cultured OUM-27 cells (5×10^6 cells) in 0.1 ml phosphate-buffered saline (PBS) were inoculated subcutaneously in the back of 4 week old Balb/c nu/nu mice. When the tumors developed at the injection sites to become about 45 mm^3 in volume (6 days after inoculation), mice were randomized into two groups. One group received 5 μg rhChM-I in 50 μl PBS, injected subcutaneously around the tumor daily for the initial 5 days. The other group received PBS alone. Tumor volumes were determined as width$^2 \times$length$\times 0.52$ [15]. At the last time point, tumors were excised and fixed for histological examinations. Similarly, nude mice were inoculated by subcutaneous injection of HT-29 cells (3.5×10^6 cells). When the tumor volume reached about 75 mm^3, 11 days after inoculation, each mouse was injected with 20 μl PBS alone or PBS containing 20 μg rhChM-I, daily for the initial 5 days.

3. Results and discussion

CSs have been traditionally divided into three histological grades [16]. Low-grade lesions infrequently metastasize. However, when they do, it is usually to the lung and other areas of the body through the invaded capillaries [16]. Although high-grade (grade III) lesions are characterized by the most atypical matrix, low-grade CSs are essentially indistinguishable from growing Enchs. CSs of grades I and II excised from patients expressed the cartilage-specific matrix components such as type II collagen and aggrecan at a level similar to those in benign tumors and normal AC (Fig. 1A). Consequently, the biochemical techniques have failed to help in the most difficult discrimination of the low-grade malignant entities [16].

Interestingly, the expression of ChM-I transcripts was only barely detectable even in low-grade CSs, while they were readily detected in Ench or Exo as well as normal AC (Fig. 1A). The competitive PCR revealed a striking difference in the ChM-I mRNA level between CS and other specimens. Grade I CSs contained ChM-I mRNA only at a level from 1/100 to 1/3500 of AC, while Ench contained a similar level of ChM-I mRNA to normal cartilage (Fig. 1B). Exo expressed the transcripts a little less than AC. On the other hand, there was no detectable difference in the content of type II collagen and aggrecan transcripts (data not shown), indicating that ChM-I is one of the most sensitively affected genes by the malignant transformation of cartilage. Further study on the basis of a larger number of clinical samples, which is now underway, may enable us to distinguish low-grade CSs from Enchs in terms of the ChM-I gene expression.

Among a few human CS cell lines available, we examined the expression of ChM-I mRNA in HCS-TG and OUMS-27 derived from grade III CS by Northern blotting and it was compared to articular chondrocytes in primary culture (Fig. 1C). Although HCS-TG was devoid of type II collagen expression, OUMS-27 maintained the differentiated phenotype of chondrocytes which included an active synthesis of matrix metachromatically stained with toluidine blue and the expression of type I, type II and type III collagens [10]. The cells grew, taking on the elongated polygonal shape in monolayer culture, and formed cartilage-like nodules after reaching confluence. However, the expression of ChM-I mRNA was also absent in these cell lines (Fig. 1C).

The balance hypothesis on angiogenic switching [1] may predict that cartilaginous tissue becomes angiogenic to allow for vascular invasion by the loss or withdrawal of anti-angiogenic molecules even without any change in the level of angiogenic molecules such as FGF-2. In fact, intralesional administration of anti-FGF-2 monoclonal antibody inhibited the
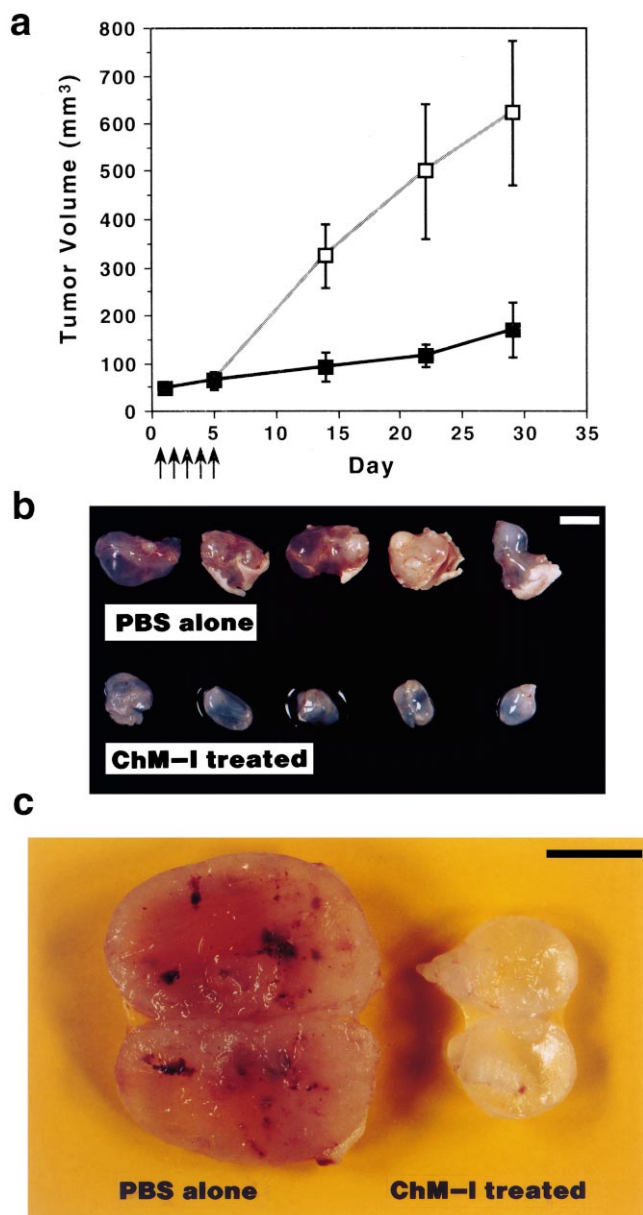
Fig. 2. Effect of rhChM-I on OUMS-27 human CS xenografts in nude mice. OUMS-27 cells (5×10^6 cells) were inoculated subcutaneously in the back of 4 week old nude mice. A: When the tumor volume reached about 45 mm^3 after inoculation, each mouse was injected with 50 μl PBS alone (□) or 50 μl PBS containing 5 μg rhChM-I (■) around the tumor for the initial 5 days indicated by arrows. Values represent means ± S.D. ($n = 5$). B: Gross appearance of tumors excised on day 29. Tumors were treated with PBS alone (upper panel) or with rhChM-I (lower panel) for the initial 5 days. C: A cross-section of the representative tumor treated with PBS alone (upper panel) or with rhChM-I (lower panel). Bars represent 5 mm in B and C.

growth and vascularization of rat CS in vivo [17]. Then, it must be similarly possible to restore the angiogenic resistance in tumors by the supplementation of ChM-I protein. As we demonstrated previously [7], exogenously added ChM-I protein inhibited the vascular invasion into cartilage, ectopically induced by demineralized bone matrix in vivo, leading to the suppression of bone formation [7]. Taking advantage of the OUMS-27 xenograft tumor model [10], we tested our hypoth-
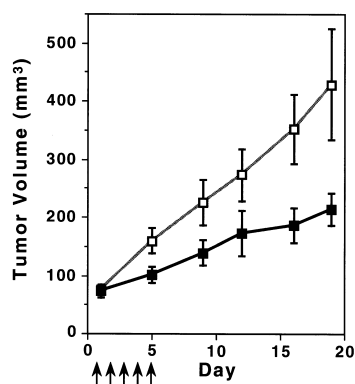
Fig. 3. Effect of rhChM-I on HT-29 human colon adenocarcinoma xenografts in nude mice. HT-29 cells (3.5×10^6 cells) were inoculated subcutaneously in the back of 8 week old nude mice. When the tumor volume reached about 75 mm^3, each mouse was injected with 20 μl PBS alone (□) or 20 μl PBS containing 20 μg rhChM-I (■) around the tumor for the initial 5 days indicated by arrows. Values represent means ± S.E.M. ($n = 7$).

esis by the administration of rhChM-I. When OUMS-27 cells formed a tumor about 45 mm^3 in size, mice received 5 μg rhChM-I dissolved in PBS at the site of the tumor for the initial 5 days. In contrast to the control mice receiving PBS alone, the tumor size was significantly suppressed (Fig. 2A). Fig. 2B shows all of the tumor tissues excised from mice (five in each experimental group) used on day 29. The wet weight (156 ± 48 mg on average) was about 30% of that in the control mice (492 ± 100 mg on average). As shown in the cross-sections (Fig. 2C), tumors in the control mice allowed for a considerable amount of capillary invasion, whereas those in the treated mice did not.

Vascular invasion into cartilage may require a complex series of events including an abnormal setting of angiogenic switching as well as an inappropriate matrix organization. Low-grade CSs have only a subtle anomaly in their extracellular matrices and are free of any appreciable invasion by capillaries. Higher-grade CSs are accompanied by the loss of major matrix components such as type II collagen as exemplified by HCS-TG cells (Fig. 1C). Loss of ChM-I expression seems an important prerequisite for acquisition of an angiogenic property in CS, prior to the atypical matrix formation. Supplementation of exogenous ChM-I protein resulted in restoration of the anti-angiogenic property (Fig. 2B,C).

O'Reilly and coworkers identified anti-angiogenic peptides termed angiostatin and endostatin in tumor-bearing mice [15,18]. These peptides were released from plasminogen and type XVIII collagen by a mechanism as yet unidentified. Administration of these peptides resulted in tumor suppression in mice, suggesting that cartilage-derived angiogenesis inhibitor ChM-I can generally be applicable for the growth suppression of solid tumors. We thus administered rhChM-I at the tumor site of mice implanted with human colon adenocarcinoma HT-29 cells (Fig. 3). When tumors grew to about 75 mm^3 in size, 20 μg rhChM-I was injected for 5 days. The tumor growth was evidently suppressed. As we suggested [5], ChM-I is localized in cartilage matrix and bound to anchoring molecules as a complex. The presence of anchoring molecules in cartilaginous matrix may account for the effective suppression of CS, when compared to that of HT-29 colon carcinoma.

Identification of anchoring molecules and the cell-surface receptor may prove the usefulness of ChM-I as a therapeutic agent.

At this moment, it is not known how the expression of ChM-I mRNA was lost or suppressed in CSs. However, Yamaguchi and coworkers reported that local recurrence of CSs was significantly associated with loss of heterozygosity on 13q and/or 17p, indicating the presence of the retinoblastoma (*Rb*) and/or *p53* gene mutations [19]. The human ChM-I gene was mapped on chromosome 13 q14-21 by fluorescence in situ hybridization (submitted elsewhere). The NCBI database revealed that the ChM-I gene (the human genomic map marker: SHGC-31236) was mapped closely to the *Rb* gene (SHGC-12474). The mouse ChM-I gene was also mapped at D3 on chromosome 14, the region that contained the *Rb* gene locus (C. Shukunami, unpublished data). The available evidence suggests a cooperative role for the Rb and p53 tumor suppressors in growth signaling and that the need for angiogenic factors is balanced with the elimination, via mutation, of p53 [20]. Human CS OUMS-27 xenograft tumor was apparently angiogenic (Fig. 2), while expression of the *ChM-I* gene could not be detected in OUMS-27 cells by either Northern blot (Fig. 1C) or RT-PCR. Kunisada and coworkers recently described that OUMS-27 cells bear the mutant type *p53* gene [10], suggesting that p53 tumor suppressor may participate in the *ChM-I* gene expression in cartilage. As reported [20], mutations in the *p53* gene were never selected in teratocarcinoma. In fact, the teratocarcinoma-derived chondrogenic cell line ATDC5 expressed ChM-I mRNA in a manner dependent on cartilage differentiation [7]. Experiments are currently underway to elucidate the role of the Rb and p53 tumor suppressors in *ChM-I* gene expression.

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