

# A novel chordin-like BMP inhibitor, CHL2, expressed preferentially in chondrocytes of developing cartilage and osteoarthritic joint cartilage

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Accepted 2 October 2003

Development 131, 229–240

Published by The Company of Biologists 2004

doi:10.1242/dev.00901

## Summary

We have identified a novel chordin-like protein, CHL2, which is structurally most homologous to CHL/neuralin/ventroptin. When injected into *Xenopus* embryos, CHL2 RNA induced a secondary axis. Recombinant CHL2 protein interacted directly with BMPs in a competitive manner to prevent binding to the type I BMP receptor ectodomain, and inhibited BMP-dependent induction of alkaline phosphatase in C2C12 cells. Thus, CHL2 behaves as a secreted BMP-binding inhibitor. In situ hybridization revealed that *CHL2* expression is restricted to chondrocytes of various developing joint cartilage surfaces and connective tissues in reproductive organs. Adult mesenchymal progenitor cells expressed *CHL2*, and its levels decreased during chondrogenic differentiation. Addition of CHL2 protein to a chondrogenic culture system

reduced cartilage matrix deposition. Consistently, *CHL2* transcripts were weakly detected in normal adult joint cartilage. However, *CHL2* expression was upregulated in middle zone chondrocytes in osteoarthritic joint cartilage (where hypertrophic markers are induced). CHL2 depressed chondrocyte mineralization when added during the hypertrophic differentiation of cultured hyaline cartilage particles. Thus, CHL2 may play negative roles in the (re)generation and maturation of articular chondrocytes in the hyaline cartilage of both developing and degenerated joints.

Key words: Secreted protein, Chordin, BMP, Inhibitor, Chondrocyte, Cartilage, Superficial zone, Joint, Osteoarthritis

## Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)  $\beta$  super family. These molecules play important roles during many organogenic processes, even though they were originally identified as factors promoting the ectopic formation of cartilage and bone. The concentration of active BMP is controlled in part by inhibitors from three BMP binding protein families: short gastrulation/chordin, noggin and cerberus (reviewed by Balemans and Van Hul, 2002). Among these, chordin and noggin were the first proteins found to inhibit the activity of bound BMPs by preventing interactions with their BMP receptors (Piccolo et al., 1996; Zimmerman et al., 1996). Although noggin is encoded by a single gene in mammals, chordin belongs to a family of proteins that share a cysteine-rich pro-collagen repeat [or chordin-like cysteine-rich repeat (CR)], which is also found in various extracellular matrix proteins (reviewed by Garcia Abreu et al., 2002). Without exception, the

homology between chordin family members lies within their CRs.

The chordin polypeptide contains four CRs, of which the first and the third (CR1 and CR3) are responsible for BMP binding (Larrain et al., 2000). Binding of chordin to BMP4 is specific and tight (Piccolo et al., 1996). Proteolysis by Tolloid (or BMP1), which liberates CR1 and CR4 from chordin, is required to release bound BMP4 (Piccolo et al., 1997; Scott et al., 1999). The importance of CR for BMP interactions has been strengthened by the recent finding that connective tissue growth factor functions as a BMP-binding inhibitor, and that its single CR domain is essential for this activity (Abreu et al., 2002).

We previously described a small chordin-like secreted protein, CHL1 (for chordin-like 1, re-designated from CHL), a novel BMP-binding inhibitor with three CRs (Nakayama et al., 2001). CHL1 was isolated originally from mouse bone marrow stromal cells. Interestingly, *CHL1* expression

correlates with the stem/progenitor-support activities of over 19 stromal cell lines established from the aorta-gonads-mesonephros region, the site at which definitive hematopoietic stem cells first arise during embryogenesis (Oostendorp et al., 2002). However, *CHL1* mRNA is also detected in various mesenchymal derivatives associated with (1) the dermatome, limb bud and chondrocyte precursors of the skeleton during embryogenesis, and (2) digestive tract connective tissues, kidney tubules and marrow stromal cells in adults. In addition, *CHL1* is expressed in olfactory bulb and cerebellum, suggesting a wider array of physiological functions. Two other groups have independently isolated *CHL1*, naming it neuralin-1 and ventroptin (Coffinier et al., 2001; Sakuta et al., 2001) and demonstrating its ability to correctly specify retinotectal projections along the dorsoventral retinal axis during development.

We provide evidence that *CHL2*, a novel chordin family member with structural homology to *CHL1*, is a BMP-binding inhibitor whose expression is uniquely restricted to the superficial layers of developing joint cartilage, in contrast to that of other family members. Potential downregulation of cartilage matrix accumulation and/or cartilage mineralization by *CHL2* is suggested by in vitro observations using cartilage particles derived from embryonic stem (ES), cell-derived mesodermal cells (Nakayama et al., 2003) and with marrow-derived mesenchymal stem/progenitor cells (MSCs). *CHL2* is also induced in osteoarthritic joint cartilage, implying a potential role during cartilage regeneration in the adult.

## Materials and methods

### Cells and reagents

Enzymes for the polymerase chain reaction (PCR) and cDNA library constructions; recombinant human proteins (and corresponding antibodies for western blot detection) for BMP4, BMP5, BMP6, activin A, TGF $\beta$ 2, and BMP receptor 1B-Fc fusion protein (BMPR1B-Fc); human platelet-derived growth factor (PDGF)-BB, human TGF $\beta$ 3, and mouse noggin-Fc fusion protein (noggin-Fc); monoclonal antibodies against collagens type II (COL2, clone 2B1.5) and type X (COL10, clone X53); other staining reagents, and all culture vessels were obtained as described previously (Nakayama et al., 2003; Nakayama et al., 2001). Mouse chordin (mCHD-His); human BMP2, BMP7 and TGF $\beta$ 1 (and mouse monoclonal antibodies against them); mouse monoclonal anti-human TGF $\beta$ 3; mouse growth and differentiation factor 5 (GDF5); and affinity-purified goat polyclonal anti-mouse GDF5 were from R&D Systems (Minneapolis, MN). Goat polyclonal anti-human IgG1-Fc fragment (IgG-Fc) was obtained from Sigma. Human kidney epithelial cell lines 293 and 293T were maintained as described previously (Nakayama et al., 2001). The human MSCs (hMSCs) were obtained from BioWhittaker (Walkersville, MD). The E14 mouse ES cells were grown, and embryoid body (EB) cells were harvested, treated and sorted as described previously (Nakayama et al., 2003).

### Isolation of mouse, rat and human *CHL2* cDNAs

Mouse placentas were isolated at E18. A signal-trap cDNA library and a regular full-length cDNA library were constructed as described previously (Nakayama et al., 2001). From 400 trap-positive clones sequenced, a cDNA fragment encoding the NH<sub>2</sub>-terminal sequence of a putative secreted protein with significant homology to *Xenopus* chordin was identified (designated mouse *CHL2* or mCHL2) by a BLAST search (Accelrys, San Diego, CA). Using this partial cDNA as a probe, the corresponding full-length cDNA (approximately 1.8 kb) was isolated, and designated as pSPORTmCHL2.

A human placenta library was constructed with size-selected (>1.5 kb) oligo(dT)-primed cDNAs in the pSPORT1 vector (Gibco). A full-length human cDNA clone (hCHL2, 1.5 kb in length) was isolated using the mouse cDNA probe, and designated pSPORThCHL2. Rat *CHL2* (rCHL2) cDNA was cloned by PCR from a rat fetal liver cDNA library (Stratagene) using the following primers: sense, 5'-TCCTCTCATCCTCACCTTAG-3' (based on mCHL2-5'UTR), and antisense, 5'-GAGGGTAATGCGACTTCTTT-3' (based on mCHL2-3'UTR). A 1.2 kb fragment was amplified using Advantage-HF2 enzyme (Clontech), cloned into pTOPO2.1 (Invitrogen), and designated pTOPOrCHL2.

### Production, purification, and detection of recombinant *CHL2* protein

To prepare mCHL2, the mCHL2 open reading frame (ORF) was mutated by PCR to replace the stop codon with a *SalI* site, inserted into a pFLAG-CMV5a expression vector (Sigma) to attach the FLAG sequence in-frame to mCHL2 at its COOH terminus (mCHL2-FLAG), and designated pFLAGmCHL2. Expression was checked by transient transfection of 293T cells, followed by direct western blot analysis of conditioned media, using the anti-FLAG monoclonal antibody M2 as described previously (Nakayama et al., 2001).

A large-scale, transient transfection-based expression was performed as described with 293T cells bearing pFLAGmCHL2 (Nakayama et al., 2001), yielding approximately 10  $\mu$ g/ml of mCHL2-FLAG. The protein was purified by affinity chromatography using anti-FLAG M2 affinity gel (Sigma) under high-salt conditions, as described by Piccolo et al. (Piccolo et al., 1997), after which positive fractions were subjected to hydroxyapatite column chromatography (equilibrated with 10 mM phosphate, with gradient elution from 10 mM to 400 mM phosphate) at pH 6.9. Purity was confirmed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. Approximately 5 mg of >95% pure mCHL2-FLAG protein were obtained from 2.5 l of conditioned medium.

A rabbit polyclonal antibody for mCHL2 ( $\alpha$ CHL2-COOH) was raised to the peptide NH<sub>2</sub>-CPEDEAEDDHSEVISTR-COOH, and affinity purified against the corresponding peptide (Harlow and Lane, 1988).

### Co-immunoprecipitation analysis

Immunoprecipitations to demonstrate direct interactions between BMPs, TGF $\beta$ s and activin A were performed as described previously (Nakayama et al., 2001) except that only one condition was used: 200 ng mCHL2-FLAG were mixed with 100 ng of BMP/GDF/activin/TGF $\beta$  in 1 ml binding buffer, followed by 12  $\mu$ g/ml of  $\alpha$ CHL2-COOH. The BMP/GDF/activin/TGF $\beta$  immunocomplex was precipitated with 20  $\mu$ l protein A agarose beads (Santa Cruz), fractionated on an SDS-polyacrylamide gel (NuPAGE, Invitrogen), blotted, and visualized with the corresponding antibody as described previously (Nakayama et al., 2001), or with 1  $\mu$ g/ml of anti-BMP2, anti-BMP7, anti-TGF $\beta$ 1 or anti-TGF $\beta$ 3 or 0.3  $\mu$ g/ml of anti-GDF5. Each blot then was treated with 4.4  $\mu$ g/ml M2 to confirm the precipitation of mCHL2-FLAG. The inhibitory effect of mCHL2-FLAG (0.1–1  $\mu$ g, in 1 ml binding buffer) on BMP4 binding (at 100 ng/ml) to the BMPR1B ectodomain (BMPR1B-Fc at 1  $\mu$ g/ml) was performed as described by Nakayama et al. (Nakayama et al., 2001), except that BMP4 visualization on blots was followed by *CHL2* and BMPR1B-Fc detection using 4.4  $\mu$ g/ml M2 and 2.2  $\mu$ g/ml anti-IgG-Fc, respectively.

### Ectopic axis formation in the *Xenopus* embryo

Inhibition of BMP4 by mCHL2 was assessed in *Xenopus* embryos. The *EcoRI*-*NotI* fragment of pSPORTmCHL2 was cloned into the *EcoRI*-*NotI* sites of pCS2+ (Rupp et al., 1994), and the resulting plasmid was linearized with *NotI*. Capped mRNAs were synthesized with SP6 polymerase, quantified, diluted and injected into two ventral blastomeres as described previously (Nakayama et al., 2001).

**Table 1. Oligonucleotide primers for human genes**

Gene		Sequence	Product (bp)
Aggrecan	Sense	5'-ACAGCCACCTCCCCAACAG-3'	414
	Antisense	5'-ATTCCACTCGCCCTTCTCGT-3'	
COMP	Sense	5'-CAGAAGAACGACGACCAAAAG-3'	1000
	Antisense	5'-GCAGGAACCAACGATAGGAC-3'	
COL1	Sense	5'-AGGGCTCCAACGAGATCGAGATCCG-3'	222
	Antisense	5'-TACAGGAAGCAGACAGGGCCAACGTCG-3'	
COL2	Sense	5'-CGTCTACCCCAATCCAGCAAAC-3'	412
	Antisense	5'-GGAGGCGTGAGGTCTTCTGTG-3'	
COL10	Sense	5'-CCAGCAGGAGCAAAGGGAATG-3'	831
	Antisense	5'-GTGGGGTAGAGTTAGAGAATG-3'	
CHL1	Sense	5'-GGGTTGGTTTACTGCGTGAA-3'	647
	Antisense	5'-GACTCGCCATGAGAATAGGTCTT-3'	
CHL2	Sense	5'-TGTGAGTTGTATCCGCCTCCAC-3'	839
	Antisense	5'-GGCTTGGGGATACCGATGTGT-3'	
SOX9	Sense	5'-AGGTAAAAGGCAAGCAAAGGAG-3'	993
	Antisense	5'-CTGGGAGGGAAACAAGTGAAAC-3'	
GAPD	Sense	5'-ACCACAGTCCATGCCATCAC-3'	450
	Antisense	5'-TCCACCACCTGTTGCTGTA-3'	

### Alkaline phosphatase induction in C2C12 cells by BMPs

Promyoblast C2C12 cells were maintained and differentiated according to the method of Kirsch et al. (Kirsch et al., 2000a). Briefly, cells were plated at  $3 \times 10^4$  cells/well in a 96-well plate, and after 1 day, stimulated to differentiate for 72 hours in 120  $\mu$ l of Dulbecco's Modified Eagle's medium with 2% calf serum (Gibco) in the presence or absence of BMP and/or CHL2. Cells were washed and lysed. Alkaline phosphatase (AP) activity was measured with *p*-nitrophenyl phosphate (Sigma), with specific activity calculated as the amount of *p*-nitrophenol produced in 30 minutes at 37°C, normalized to total protein content, as determined with BCA reagent (Pierce).

### In situ hybridization and northern blot analysis

Northern blotting was performed against the entire open reading frame (ORF) of *CHL2* on human and mouse multiple tissue RNA blots (Clontech) using the  $^{32}$ P-labeled *Xba*I-*Sal*I fragment from pSPORT<sub>h</sub>CHL2 as a probe (Sambrook et al., 1989).

In situ hybridization (ISH) was performed on 5  $\mu$ m paraffin sections of zinc formalin-fixed, formic acid-decalcified tissue according to the method of Wilcox (Wilcox, 1993), including a high stringency wash with  $0.1 \times$  SSC at 55°C. Slides hybridized with  $^{33}$ P-labeled RNA probes were exposed to NTB2 emulsion (Kodak) for 3 weeks, developed and counterstained with Hematoxylin and Eosin. Expression was subjectively evaluated under dark-field microscopy.

For *mCHL2*, the 1234-bp *Sal*I-*Not*I fragment and the 653 bp *Hind*III fragment were deleted from pSPORT<sub>m</sub>CHL2 to obtain pSP<sub>m</sub>CHL2-COOH and pSP<sub>m</sub>CHL2-NH<sub>2</sub>, respectively. Both plasmids were linearized with *Eco*RI to generate non-overlapping probes. An *hCHL2*-bearing plasmid, pSP<sub>h</sub>CHL2NH<sub>2</sub>, was made by deleting the 811 bp *Bam*HI fragment from pSPORT<sub>h</sub>CHL2, leaving the 746 bp *hCHL2* NH<sub>2</sub>-terminal ORF. The plasmid was linearized with *Sal*I. RNA probes for *hCHL2* and *mCHL2* were synthesized with SP6 RNA polymerase. For *rCHL2*, an approximately 1.2 kb fragment of *rCHL2* cDNA derived from pTOP<sub>Or</sub>CHL2 was cloned into the pBluescript vector (pBS<sub>r</sub>CHL2). The plasmid was linearized with *Xba*I and a labeled RNA probe synthesized with T7 RNA polymerase.

*CHL2* expression was assessed by ISH in mouse embryonic/adult tissues, and in structurally normal and arthritic joints of adult humans (knee cartilage) and rats (hind paws). Human diseased cartilage samples from rheumatoid arthritis (RA) or osteoarthritis (OA)/degenerative joint disease (DJD) patients were provided by Cooperative Human Tissue Network, and normal control samples were from Zoion Diagnostics (New York, NY), with pre-approval of the Institutional Review Board (758WIRB and CP1098/01, respectively). Collagen-induced arthritis (CIA) was induced, as described (Trentham et al., 1977), in anesthetized female Lewis rats

(7-8 weeks old, 150-170 g) by intradermal injection of porcine COL2 (Chronochem, Seattle, WA) emulsified 1:1 in incomplete Freund's adjuvant (Difco, Detroit, MI) at 10 different sites over the back (50  $\mu$ g COL2/100  $\mu$ l/injection). Disease developed between 10 and 12 days after injection, as determined by caliper measurements (Cole Parmer, Vernon Hills, IL) of ankle width and ambulatory difficulties. Paws were harvested for ISH 7 days after CIA onset. These experiments were conducted in accordance with federal animal care guidelines and were pre-approved by the Amgen Institutional Animal Care and Use Committee.

### Chondrogenic differentiation of MSCs and gene expression analysis

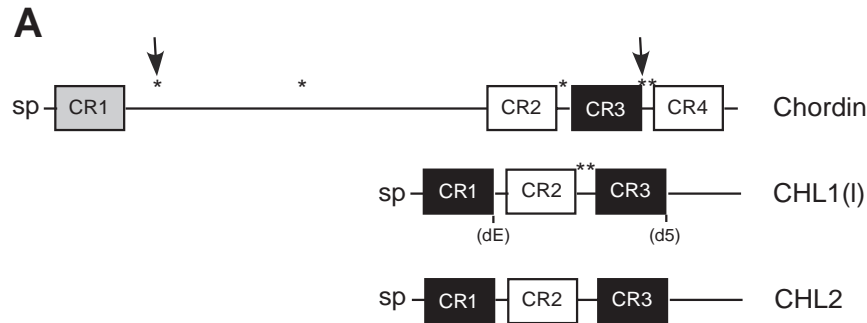
Human MSCs were cultured and differentiated as described (Mackay et al., 1998). Briefly, the pellet culture was performed in serum-free chondrogenesis medium supplemented with 10 ng/ml TGF $\beta$ 3, with or without 2  $\mu$ g/ml mCHL2-FLAG, 1  $\mu$ g/ml noggin-Fc, or 1  $\mu$ g/ml IgG-Fc. On days 21-28, cartilage-like particles were formalin-fixed, paraffin-embedded, sectioned centrally and stained with Toluidine Blue to detect sulfated glycosaminoglycans (Nakayama et al., 2003; Sheehan and Hrapchak, 1987). Three sections from different regions were examined to confirm staining reproducibility.

To analyze gene expression, two to five particles were harvested at designated times and disrupted immediately in guanidine isothiocyanate solution (RNeasy kit, Qiagen). Total RNA was purified using the manufacturer's protocol, including DNase I treatment. Reverse transcription (RT) and PCR were performed as described previously (Nakayama et al., 1998), except that the PCR used 30 cycles, an annealing temperature of 62°C and one primer set per gene. Primers for aggrecan, cartilage oligomeric matrix protein (COMP), COL1, COL2, COL10, SOX9, CHL1, CHL2 and glyceraldehyde-3-phosphate dehydrogenase (GAPD) are shown in Table 1.

### Cartilage mineralization in vitro

Mineralizing cartilage particles were produced as described by Nakayama et al. (Nakayama et al., 2003). Briefly,  $3.4 \times 10^5$  FACS-purified EB cells were pellet-cultured in serum-free chondrogenesis medium with 10 ng/ml TGF $\beta$ 3 and 50 ng/ml PDGF-BB. On day 10, TGF $\beta$  and PDGF were replaced by 50 ng/ml BMP4 to generate a hyaline cartilage particle. On day 15, cultures were adjusted to the hypertrophic differentiation medium without T3 (Sigma), containing 3  $\mu$ g/ml mCHL2-FLAG, 2  $\mu$ g/ml noggin-Fc or 50 ng/ml BMP6 for 3 days; 10 nM T3 was added to induce hypertrophic differentiation on day 18. On days 24-26, each particle was stained with Toluidine Blue as described above (Sheehan and Hrapchak, 1987). Additional serial sections were immunostained with 2B1.5 for COL2 and X53 for





**B**

1	50	51	100
mCHL2	MVPGVRIIPS LLGLVMFWLP LDSQALSRSRG KVCFLFGEKIY TPGQSWHPYLY	EPQGTIYCVLR CTCSENGHVN CYRLRCPPPLH CSQPVMEPQQ CCPRCVDPHV	
rCHL2	MVPRVRIIPS LLGLVMFWLP LDSQARARPG KVCFLFGEKIY TPGQSWHPYLY	EPQGTIYCVLR CTCSENGHVN CYRLRCPPPLH CSQPVMEPQQ CCPRCVDPRV	
hCHL2	MVPEVRVLSS LLGLALLWFP LDSHARARPD MFLFLFHGKRY SPGESWHPYLY	EPQGLMYCLRL CTCSEGAHVS CYRLHCPVPH CPQPVTEPQQ CCPKCVEPHT	
101	150	151	200
mCHL2	PSGLRVPLKS CQLNETTYQH GEIFSAQELF PARLSNQCVL CSCIEGHTYC	GLMTCPEPSC PTTPLPLPDS CQTCQDRTTE SSTEENLTQL QHGERHSQDP	
rCHL2	PSGLRVPLKS CQLNETTYQH GEIFSAQELF PARLSNQCVL CSCIEGHTYC	GLMTCPEPSC PSTLPLPDS CQTCQDRTME SSTEENLTQL QHGERHSQDP	
hCHL2	PSGLRAPPKS CQHNGTMYQH GEIFSAHELF PSRLPNQCVL CSCIEGHTYC	GLTTCPEPGC PAPLPLPDS CQACQKDEASE QSDEEDSVQS LHGVRHPQDP	
201	250	251	300
mCHL2	C.SE...RRG PSTPAPTSLS SPLGFIPRHF QSVGMGGTTI KIILKEKHKK	ACTHNGKTYH HGEVWHPTVL SFGPMPCILC TCIDGYQDCH RVTCTPTQYPC	
rCHL2	C.SDDGKRRG PSTPAPTSLS SPLGFIPRHF QSVGMGGTTI KIILKEKHKK	ACTHNGKTYH HGEVWHPTVL SFGPMPCILC TCMDGYQDCH RVTCTPTQYPC	
hCHL2	CSSDAGRKRK PGTPAPTGLS APLSFIPRHF RPKGAGSTTV KIVLKEKHKK	ACVHGKTYH HGEVWHPTVL AFGPLPCILC TCEDGRQDCQ RVTCTPTQYPC	
301	350	351	400
mCHL2	SQPKKVAGKC CKICPEDEAE DDHSEVISTR CPKVPQGQFV YTLASPSQDS	LHRRFVLEHEA SDQVEMYIWK LVKGIYHLVQ IKRVRKQDFQ KEAQNFRLLT	
rCHL2	SQPKKVAGKC CKICPEDEAD DDHSEVISTR CPKVPQGQFV YTLVSPSPDS	LHRRFVLEHEA SDQVEMYIWR LVKGIYHLVQ IKRVRKQDFQ KEAQNFRLLT	
hCHL2	RHPEKVAGKC CKICPEDEAD DDHSEVISTR CPKVPQGQFV YTLVSPSPDN	LRRFALEHEA SDLVEIYLWK LVKGIYHLVQ IKKVRKQDFQ KEAQHFRLLA	
401	430		
mCHL2	GTHEGYWTVF LAQTPELVKT ASPDKVTKTL		
rCHL2	GTHEGYWTVF LAQTPELVKT ASPDKVTKTL		
hCHL2	GPHEGHWNVF LAQTPELVKT ASPDKVTKT.		

**C**

CVF.QDKKYRV.GEKWHPYLYGLVYVNCICS.ENGVLCSRVRGPSLHCLSPVHI.PHLCCPRC	mCHL1 CR1
CLF.GEKIYTP.GQSWHPYLYEPQGTIYVRCICS.ENGHVNCRRLCPPLHCSQPVME.PQQCCPRC	mCHL2 CR1
CYFDGDRSWRAAGTRWHPVPPFGLIKCAVCTCKGATGEVHCEKVOCPRLAQAQVRANPTDCCKQC	mChordin CR3
CVSNG.KTY.SHGESWHPNLRFAFGIVEVLTCTNVTKQE..CKKIHCNRYPCYPQKIDGK.CCKVC	mCHL1 CR3
CTHNG.KTY.SHGEVWHPTVLSFGPMPCILCTCIDGYQD..CHRVCTPTQYPCSQPKKVAGK.CCKIC	mCHL2 CR3
CYFDGDRSWRAAGTRWHPVPPFGLIKCAVCTCKGATGEVHCEKVOCP.RLAQAQVRANPTDCCKQC	mChordin CR3

COL10, and stained to reveal mineral deposition (von Kossa). Immunostained sections were counterstained with Gill 2 Hematoxylin, and von Kossa sections with Nuclear Fast Red (Sheehan and Hrapchak, 1987).

## Results

### Cloning of CHL2, a novel chordin-like gene

Random nucleotide sequencing of a mouse placenta library

**Fig. 1.** Primary structure of CHL2.

(A) Schematic representation of chordin, CHL1(I), and CHL2. SP stands for signal peptide. The CR1 and CR3 regions in CHL1 and CHL2 (black boxes) are most homologous to CR3 of chordin (also in black). The chordin CR1 (in gray) and CR3 possess the BMP-binding capability (Larrain et al., 2000). Putative BMP1/Tolloid cleavage sites are indicated with an asterisk, while actual Tolloid cleavage sites (Scott et al., 1999) are shown by vertical arrows. The CHL1 ORF had two sites with amino acid sequence variations (dE and d5) (Nakayama et al., 2001). (B) Amino acid sequences of mouse, rat and human CHL2 protein precursors. The three CRs are indicated by boxes. The vertical arrow indicates the NH<sub>2</sub>-terminal amino acid of mature mCHL2-FLAG (Leu<sup>26</sup>), as determined by amino acid sequencing of purified recombinant protein. (C) Amino acid sequence alignment showing sequence similarities between CR1 or CR3 of mouse CHL1 and CHL2, and CR3 of mouse chordin. Ten conserved cysteines (highlighted in black) are found in the spacing typical of vertebrate chordin. Other conserved amino acids are highlighted in gray.

enriched for genes of secreted and membrane-bound proteins revealed a cDNA encoding a protein precursor consisting of a potential signal peptide followed by three chordin-like cysteine-rich repeat (CRs) homologous to those of chordin and CHL (Fig. 1A,C). A GAP search (Accerlys) revealed amino acid sequence identities with mCHL(13) and chordin of 41% and 33%, respectively. The predicted precursor size of 426 amino acids resembled mCHL(13) (448 amino acids) more than mouse chordin (999 amino acids). Because this novel cDNA was clearly homologous to CHL, we designated it CHL2 and renamed CHL as CHL1. Human and rat CHL2 cDNAs were cloned also. The human gene encoded a 429 amino acid precursor with 73% amino acid homology to mCHL2, while the rat variant was 95% identical to mCHL2 (Fig. 1B).

A genomic DNA fragment containing a region spanning at least the 5'-half of *mCHL2* was cloned from a 129SV genomic BAC library (Genome Systems).

Using this clone, *mCHL2* was localized to chromosome 7, mapping to a position 66% of the distance from the heterochromatic-euchromatic boundary to the telomere. A search of public genome databases confirmed that *mCHL2* is on 7E1 and *hCHL2* is on 11q13. Therefore, unlike *CHL1*, *CHL2* is an autosomal gene. Interestingly, the exon-intron junctions of *CHL2* were nearly identical to those of *CHL1* (not shown), suggesting that both genes may originate from a common ancestor.

**Table 2. Secondary axis formation by CHL2**

Type of CHL injected	Embryos with axis duplication/total injected embryos (%)
Uninjected	0/31 (0)
mCHL1(s2)* (10 pg RNA/blastomere)	16/20 (80)
mCHL2 (1 pg RNA/blastomere)	29/39 (74)
mCHL2 (5 pg RNA/blastomere)	27/28 (96)

\*pcDNAmCHL(s2) (Nakayama et al., 2001).

### MCHL2 induced a secondary axis in the *Xenopus* embryo

Chordin is known to dorsalize the gastrulating *Xenopus* embryo by inhibiting BMP4 activity, so the impact of CHL2 on *Xenopus* embryo development was examined (Table 2). Injection of 1 pg mCHL2 RNA per blastomere induced trunk duplication in 74% of embryos, compared with 0% for uninjected controls and embryos given EF1 $\alpha$  mRNA. As a positive control, injection of 10 pg mCHL1(s2) RNA yielded an axis duplication rate of 80% (Nakayama et al., 2001). These results indicated that mCHL2 actively antagonized an endogenous ventralizing factor (presumably BMP4). The improved efficacy afforded by a 10-fold lower CHL2 dose suggested that it might be a more stable and/or potent BMP inhibitor than CHL1.

### Direct interaction of mCHL2 with BMPs prevents their binding to BMP receptor

mCHL2-FLAG protein was purified to near homogeneity (Fig. 2A). Unlike mCHL1(s2)-FLAG, the overall yield was higher, and degradation products were not detected.

Purified mCHL2-FLAG co-immunoprecipitated BMP2, BMP4, BMP5, BMP6, BMP7 and GDF5 (Fig. 2B). Like chordin and CHL1, mCHL2-FLAG did not bind activin A, TGF $\beta$ 1 or TGF $\beta$ 3. However, unlike CHL1, no interaction between mCHL2-FLAG and TGF $\beta$ 2 was found. The control protein noggin-Fc exhibited comparable qualitative binding specificity (not shown), suggesting that CHL2 may be a pan-BMP-binding protein like noggin and chordin.

Chordin and CHL1 inhibit BMP activity by blocking their interactions with their receptors. Therefore, we determined whether CHL2 had a similar function by mixing an Fc-fusion

protein, incorporating the extracellular domain of BMP receptor 1B (BMPR1B-Fc), with BMP4 and mCHL2-FLAG, followed by precipitation of the BMPR1B-Fc complex with protein A beads. As shown in Fig. 2C, BMP4 co-precipitated specifically with BMPR1B-Fc, but not IgG-Fc, in the absence of mCHL2-FLAG. However, the signal for co-precipitated BMP4 weakened appreciably as increasing amounts of mCHL2-FLAG were added (particularly at 0.3  $\mu$ g/ml or higher). Co-precipitation of mCHL2-FLAG was not detected. These results suggest that CHL2 acts like chordin and CHL1 to prevent BMP4 interacting with its receptor.

### CHL2 inhibits BMP in vitro

Next, we demonstrated that the recombinant mCHL2 inhibited BMP activity by quantifying BMP-dependent AP induction in C2C12 cells. A serially diluted BMP inhibitor [chordin (mCHD-His), mCHL2-FLAG, or noggin-Fc] was mixed with BMP2, BMP4, BMP6 or BMP7 at a concentration corresponding to the EC50 for AP induction in C2C12 cells and then cultured for 3 days. Cell-bound AP activity was then measured (Fig. 3, Table 3). CHL2 inhibited AP induction by all four BMPs. Noggin-Fc and mCHL2-FLAG reproducibly elicited similar, dose-dependent inhibitions of BMP4, with complete suppression occurring at concentrations of 1–3  $\mu$ g/ml (20–60 nM). The mCHD-His activity was weakest for BMP4; it was approximately fivefold less potent than mCHL2-FLAG. Partially purified mCHL1(s2)-FLAG and mCHD-FLAG (Nakayama et al., 2001) inhibited BMP4 with a potency indistinguishable from that of mCHD-His (not shown). In contrast, both noggin-Fc and mCHD-His displayed weaker inhibitory activities than mCHL2-FLAG toward BMP6 and BMP7. In particular, noggin-Fc was approximately sevenfold less potent on BMP6 than mCHL2-FLAG. Thus, CHL2 inhibits BMP2, BMP4, BMP6 and BMP7 as well as or better than noggin and chordin.

### CHL2 mRNA expression in normal cartilage

In mouse embryos, ISH for CHL2 mRNA revealed expression restricted to the surface chondrocytes of developing joint cartilage (Fig. 4) and to the connective tissue of reproductive organs (Fig. 5). In the adult mouse, CHL2 was weakly expressed in cartilage of the femoral head and patella (Fig. 4C,D) and articular facets of vertebrae (Fig. 4E,F); the latter

**Table 3. Inhibition of BMP action by CHL2, noggin and chordin**

BMP	EC50 $\pm$ s.d. BMP $\ddagger$ (nM)	IC50* $\pm$ s.d. mCHL2 $\S$ (nM)	IC50 $\dagger$ $\pm$ s.d.		
			mCHL2 (nM)	noggin $\P$ (nM)	chordin** (nM)
BMP2	14.2 $\pm$ 4.6 (n=14)	4.1 $\pm$ 1.7 (n=6)	ND $\dagger\dagger$	ND	ND
BMP4	10.0 $\pm$ 3.5 (n=16)	5.0 $\pm$ 1.5 (n=6)	3.0 $\pm$ 0.9 (n=12)	3.6 $\pm$ 0.6 (n=5)	15.4 $\pm$ 2.2 (n=7)
BMP6	15.0 $\pm$ 4.3 (n=12)	5.8 $\pm$ 3.0 (n=7)	5.2 $\pm$ 2.2 (n=3)	34.6 $\pm$ 16.6 (n=2)	10.3 $\pm$ 4.9 (n=2)
BMP7	33.4 $\pm$ 5.4 (n=8)	12.5 $\pm$ 2.6 (n=5)	3.2 $\pm$ 1.1 (n=3)	9.4 $\pm$ 3.0 (n=2)	7.4 $\pm$ 1.8 (n=2)

C2C12 cells were cultured for 3 days with various BMP concentrations or with a fixed BMP level and various quantities of CHL2, chordin or noggin. Alkaline phosphatase activities were measured, normalized to total protein level, and plotted to determine EC50s and IC50s. Mean values ( $\pm$ s.d.) of 2–16 experiments (n=2–16), are shown.

\*BMP2: 14 nM, BMP4: 10 nM, BMP6: 15 nM, BMP7: 25 nM.

$\dagger$ BMP4: 4–7.7 nM, BMP6: 13 nM, BMP7: 19 nM (Fig. 3).

$\ddagger$ BMP2: 26 kD, BMP4: 26 kD, BMP6: 30 kD, BMP7: 31.4 kD, as dimer (R&D).

$\S$ mCHL2-FLAG: mature peptide, 46.5 kD (415 amino acids) as monomer.

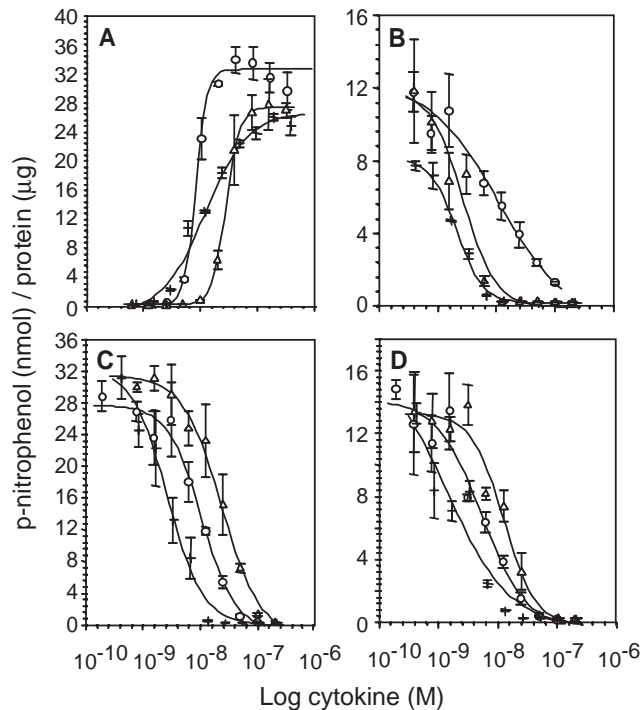
$\P$ noggin-Fc: 50 kD as monomer (R&D).

\*\*mCHD-His: 101.5 kD as monomer (R&D).

$\dagger\dagger$ Not determined.







**Fig. 3.** Inhibitory effects of mCHL2 on BMP-dependent differentiation of C2C12 cells in vitro. Dose-dependent inhibition by CHL2, chordin or noggin of BMP-dependent alkaline phosphatase (AP) induction in C2C12 cells. (A) Cells were cultured in differentiation medium for 2 days with various concentrations of BMP4 (plus sign), BMP6 (circle), or BMP7 (triangle). (B–D) Cells were also differentiated with a constant concentration of BMP4 (10 nM; B), BMP6 (15 nM; C) or BMP7 (19 nM; D) and various concentrations of mCHL2-FLAG (plus sign), noggin-Fc (triangle), or mCHD-His (circle) for 2 days. Mean specific activity of AP (triplicate assays) is shown; vertical bars denote s.d. Data are representative of three independent experiments.

### Normal, non-skeletal expression of the CHL2 gene

Northern analyses revealed little mCHL2 mRNA on the mouse embryo blot, in contrast to the abundant expression of mouse chordin and mouse CHL1 (Nakayama et al., 2001). On the mouse adult tissue blot, a faint 2 kb transcript was detected in liver and kidney extracts, and a trace amount of a >2.4 kb transcript was detected in skeletal muscle and testis, although ISH failed to localize a signal in these tissues (not shown). The adult mouse blot did not include uterus. On the human adult tissue blot, a 2 kb CHL2 transcript was detected readily in uterus and weakly in colon, heart, prostate, stomach and skeletal muscle (Fig. 5A); only the uterus and colon had expression patterns consistent with the ISH signals in mouse tissues (Fig. 5B). Traces of hCHL2 mRNA were found in placenta, testis, small intestine, trachea and bone marrow. Expression was not detected in either human liver or kidney. Thus, low levels of *CHL2* expression in soft tissues may differ between humans and mice.

In mouse reproductive organs, ISH detected CHL2-positive connective tissues such as ligaments of the ovary and oviduct in females, and of testis, epididymis and certain male accessory sex glands in males. Expression was high in uterine myometrium (Fig. 5Ba), as was that of CHL1 (not shown).

CHL2 was also present in maternally derived placental tissues (not shown). A trace CHL2 signal was found on colonic serosa (Fig. 5Bb); in contrast, CHL1-positive cells lie between the colonic submucosa and muscularis (Nakayama et al., 2001). Interestingly, *CHL1* but not *CHL2* was expressed in stomach and small intestine (Nakayama et al., 2001). In rat tissues, CHL2 occurred at low to moderate levels in cervical muscles and discrete regions of the placenta (not shown).

### CHL2 mRNA expression in diseased cartilage

Degenerating cartilage from human arthritis patients and rats with CIA were assessed by ISH (Fig. 6). In two relatively normal specimens from knees of adult humans, CHL2 mRNA was expressed in a few chondrocytes in the superficial zone as well as in the middle zone (Fig. 6A). In 19 OA cases, expression was limited to chondrocytes in the middle zone, where numerous well-labeled cells were observed (Fig. 6B,C); positive cells were not found in the superficial zone in any OA sample. Interestingly, 50–90% of such *CHL2*-expressing chondrocytes existed in clusters of 2–3 cells. Unlike OA, two RA specimens exhibited weak expression in both the superficial and middle zones (Fig. 6D). As with humans, scattered chondrocytes in normal articular cartilage of rats expressed *CHL2* (Fig. 6E), while CIA paw joints had similar (Fig. 6F) or fewer labeled chondrocytes relative to controls.

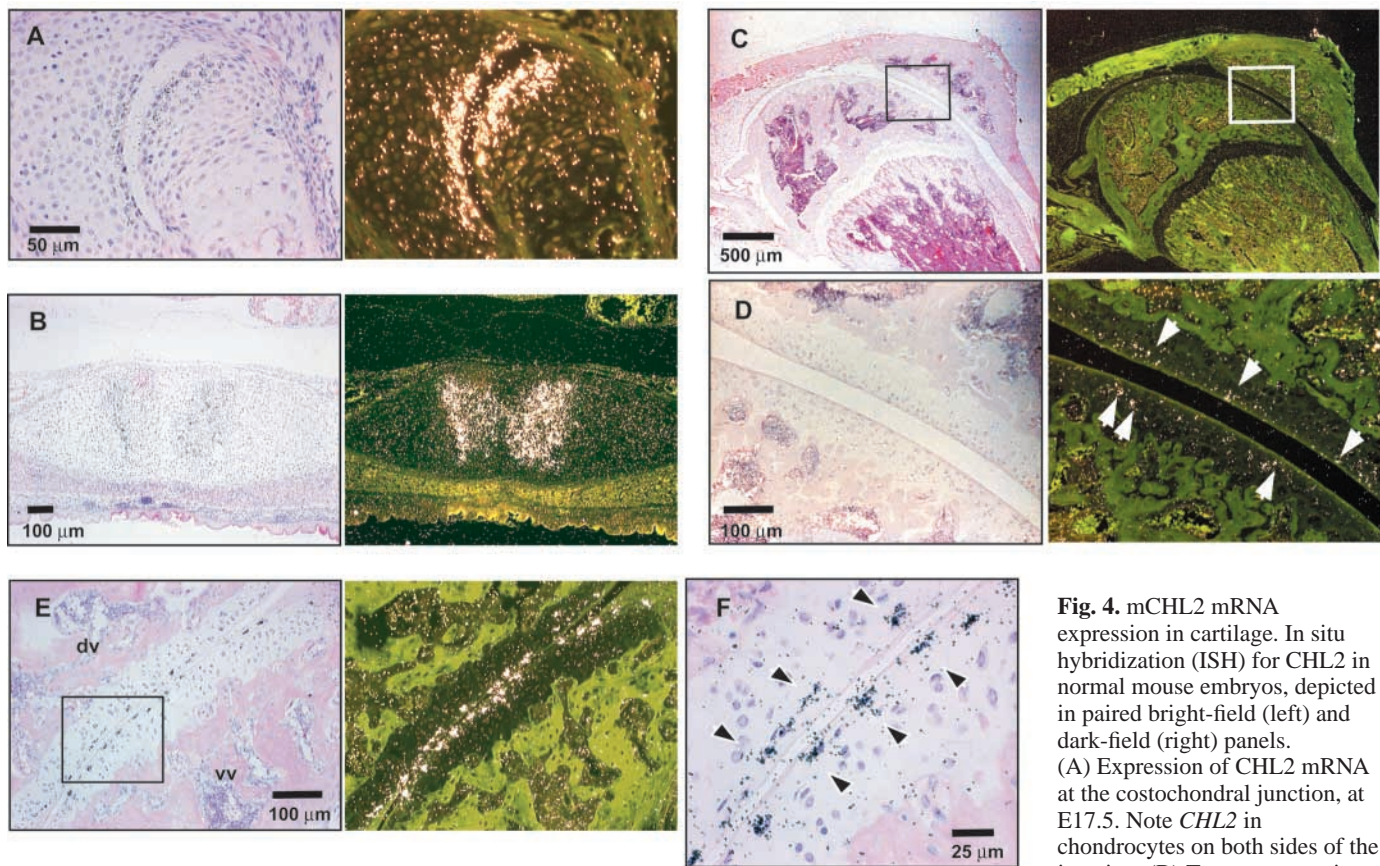
In summary, *CHL2* was expressed in normal and diseased cartilage in humans and rats. It was expressed most strongly in human OA patients, although the signal had shifted to the middle zone. Interestingly, *CHL2* expression levels and patterns were not significantly altered, relative to normal cartilage, in the rat CIA model and human patients with RA.

### Effects of CHL2 on MSC differentiation into chondrocytes

We further addressed the relevance of CHL2 to cartilage formation using MSC, which can differentiate into chondrocytes in vitro (Mackay et al., 1998) in association with upregulated BMP transcription (Roh et al., 2001). As shown by RT-PCR in Fig. 7A, hCHL2 mRNA, but not COL2 mRNA, was expressed by undifferentiated MSCs. Transcripts for SOX9, COL1 and COL10 (not shown) as well as CHL1, aggrecan and COMP were also detected. The CHL2 signal fell as chondrogenesis progressed; those for SOX9 and COL10 (not shown) as well as CHL1, aggrecan and COMP (Fig. 7A) maintained a similar level throughout the culture period. In contrast, COL2 mRNA was tightly regulated, with production induced by day 7 in culture conditions favoring chondrogenesis but absent under osteogenic conditions (not shown) (Jaiswal et al., 1997).

Next, mCHL2-FLAG protein was added at various concentrations to the chondrogenic pellet culture of MSC. mCHL2-FLAG (2 μg/ml) significantly inhibited cartilage nodule formation (Fig. 7B, Table 4), yielding definitive cartilage nodules in less than 10% of particles. Inhibition was absent at 0.1–0.2 μg/ml, while complete inhibition occurred at 3–10 μg/ml. Noggin-Fc at 0.1 and 1 μg/ml provided similar, dose-dependent inhibition.

These results implicate CHL2 as a negative regulator of cartilage formation/regeneration from immature mesenchymal cells, by preventing or reducing the rate of matrix accumulation.



**Fig. 4.** mCHL2 mRNA expression in cartilage. In situ hybridization (ISH) for CHL2 in normal mouse embryos, depicted in paired bright-field (left) and dark-field (right) panels. (A) Expression of CHL2 mRNA at the costochondral junction, at E17.5. Note *CHL2* in chondrocytes on both sides of the junction. (B) Transverse section

through sternum, at E18.5. Signal is present in areas where ribs converge. (C,D) ISH for *CHL2* in the adult knee. Weak *CHL2* expression is present over the articular cartilage surface of the femoral head and patella, but not in growth plate chondrocytes. Boxed areas in C shown at a higher magnification in D in which the signal can be seen in chondrocytes on both sides of the joint. (E,F) Expression of *CHL2* in adult vertebral articulation. Signal occurs in superficial articular chondrocytes on both sides of the zygapophyseal or facet joint. The boxed area in E is shown at a higher magnification in F, revealing signal localization over the superficial zone chondrocytes. dv, dorsal (superior) vertebra; vv, ventral (inferior) vertebra. Arrowheads indicate *CHL2*-positive chondrocytes.

**Table 4. Inhibition of cartilage-matrix deposition by CHL2**

Factors*	Cartilaginous particles† (% total)	Weak-positive particles‡ (% total)	Negative particles§ (% total)
TGFβ3	20 (87.0)	3 (13.0)	0 (0)
TGFβ3+noggin-Fc	2 (18.2)	2 (18.2)	7 (63.6)
TGFβ3+mCHL2-FLAG	1 (6.7)	3 (20.0)	11 (73.3)

Human mesenchymal progenitor cells, cultured as a pellet for 21–28 days, were fixed, sectioned and stained with Toluidine Blue. Particles were classified on the basis of their degree of metachromatic staining.

\*10 ng/ml TGFβ3, 1 μg/ml (20 nM) noggin-Fc, 2 μg/ml (43 nM) mCHL2-FLAG.

†Particles containing definitive cartilage nodules, consisting of well-separated chondrocytes embedded in proteoglycan-rich extracellular matrix (with metachromatic Toluidine Blue staining and positive Alcian Blue staining, pH 1.0) (Fig. 7Ba,b,d,e). These particles also contained regions with fusiform cells that stained lightly with Toluidine Blue (Fig. 7Bb,d).

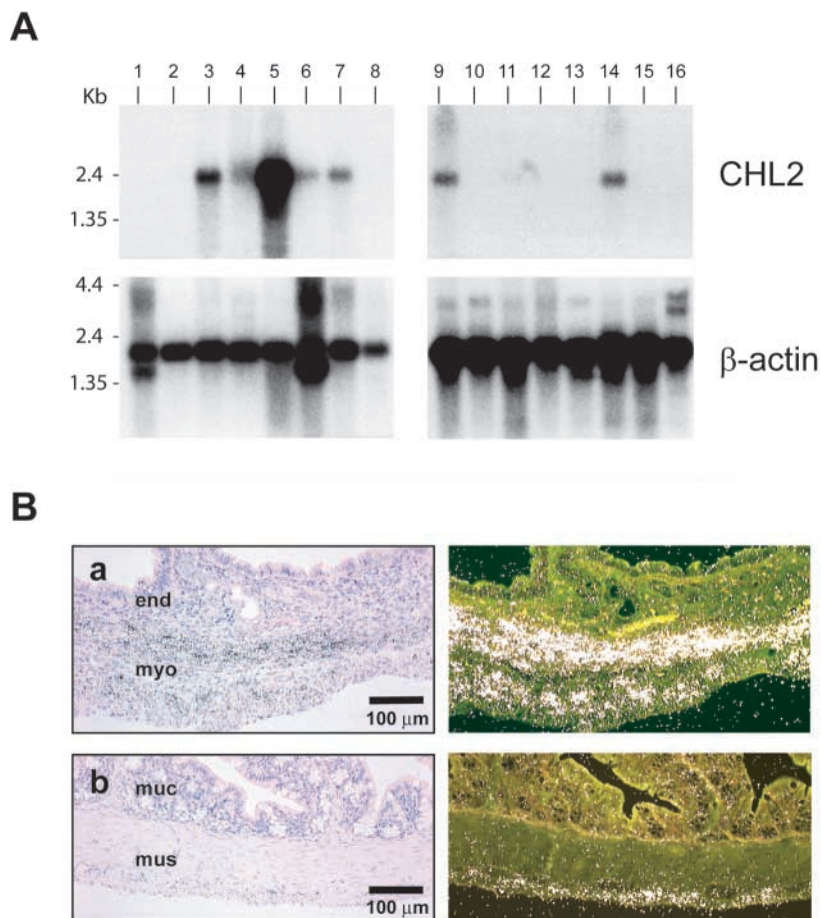
‡Particles consisting of Toluidine Blue-negative cells as well as relatively larger areas of fusiform cells that stained lightly with Toluidine Blue. The latter areas occasionally contained a small cartilaginous nodule, consisting of a few chondrocytes, as shown in Fig. 7Bf.

§Particles containing no cartilage nodules, but instead with a surface layer of spindle-shaped cells that stained lightly with Toluidine Blue, as shown in Fig. 7Bc.

**Effects of CHL2 on chondrocyte maturation**

Chondrocytes from OA joints express markers of hypertrophy, such as COL10 and AP (Kirsch et al., 2000b; Von der Mark et al., 1992), so we addressed whether CHL2 induction in OA cartilage would affect differentiation and mineralization of hypertrophic chondrocytes. We demonstrated previously that mesodermal progenitor cells, purified from differentiating ES cells, can form hyaline cartilage particles in vitro that will undergo further mineralization (Nakayama et al., 2003). We isolated FLK1<sup>+</sup> PDGFRα<sup>+</sup> mesodermal cells, subjected them to pellet micromass culture, and induced cartilage matrix mineralization (verified by von Kossa staining) in the presence or absence of mCHL2-FLAG, noggin-Fc or BMP6 (Fig. 7C, Table 5). Addition of 3 μg/ml mCHL2-FLAG significantly reduced the von Kossa-positive matrix area in 75% of particles examined, of which half showed near-complete inhibition. In contrast, COL10 expression was reduced slightly, while no significant change was detected in COL2 (Fig. 7C). Noggin-Fc at 2–3 μg/ml provided similar, but somewhat weaker, inhibition. A positive in vitro role for BMP6 has been suggested in chondrocyte hypertrophic differentiation (Grimsrud et al., 1999). However, BMP6 at 50 ng/ml, which





**Fig. 5.** Expression of CHL2 mRNA in soft tissues.

(A) Northern blot analyses with human multiple tissue blots IV (lanes 1-8) and I (lanes 9-16) are shown. Blots were first probed with a DNA containing the *hCHL2* ORF (CHL2), and then re-probed with human  $\beta$ -actin (Clontech). Essentially identical results were achieved with three different blot batches. Lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, uterus (without endometrium); lane 6, small intestine; lane 7, colon; lane 8, peripheral blood leukocytes; lane 9, heart; lane 10, whole brain; lane 11, placenta; lane 12, lung; lane 13, liver; lane 14, skeletal muscle; lane 15, kidney; lane 16, pancreas. (B) In situ hybridization for *CHL2* in adult mouse tissues, shown in paired bright-field (left) and dark-field (right) panels. (a) Uterus (transverse section) showing *CHL2* expression primarily in the myometrium. (b) Colon, showing weak *CHL2* expression in serosal cells – a different pattern to that of *CHL1* (Nakayama et al., 2001). muc, submucosa; mus, muscularis; end, endometrium; myo, myometrium.

damaged joint cartilage may reduce the extent or speed of hypertrophic differentiation in articular chondrocytes.

## Discussion

We have demonstrated here that CHL2, the second chordin-like gene, encodes a protein that directly interacts with different BMPs, inhibiting their actions in vitro as well as in vivo in a manner similar to chordin, noggin and CHL1. Expression analysis suggests a possible role for CHL2 during formation and maintenance of articular cartilage and reproductive organs. We have also provided evidence that CHL2 might negatively regulate cartilage formation/regeneration in diseased joints.

### Structure and function of CHL2

Searches of human and mouse genome databases indicated that CHL2 is most homologous to CHL1. Injection of CHL2 RNA induced trunk duplication in early *Xenopus* embryos similar to those produced by chordin and CHL1(s2) RNAs (Table 2) (Nakayama et al., 2001). Recombinant mCHL2 protein interacted directly with five BMPs and one GDF (Fig. 2 and Nakayama et al., 2001) thereby inhibiting, in vitro, several BMP/GDF-dependent processes including, osteogenic differentiation of C2C12 mesenchymal progenitor cells by several BMPs (Fig. 3, Table 3), ATDC5 embryonal carcinoma cells by GDF5 (not shown) and BMP4-dependent lymphohematopoietic (CD34<sup>+</sup>CD31<sup>hi</sup> and CD34<sup>+</sup>CD31<sup>lo</sup>) progenitor cell development from ES cells (not shown) (Nakayama et al., 2000). Under our conditions, CHL2 provided 50% inhibition (IC<sub>50</sub>) by blocking a half to a third of available BMP dimers, suggesting that tight CHL2 binding to one BMP subunit might be sufficient for full inhibition. Furthermore, as with related factors (chordin, noggin, CHL1), CHL2 prevented BMP interactions with the BMP receptor (Fig. 2C), although CHL2 activity was two- to sevenfold more potent than chordin (Table 3) and CHL1 (not shown). Thus, CHL2 is structurally and functionally similar to chordin and CHL1.

was sufficient to enlarge the particle size, did not affect the degree of mineralization.

Taken together, these results suggest that CHL2 induction in

**Table 5. Reduced levels of cartilage matrix mineralization in the presence of CHL2**

Factors*	Mineralizing cartilage <sup>†</sup> (% total)	Weak-positive <sup>‡</sup> (% total)	Negative <sup>§</sup> (% total)
none	5 (71.5)	2 (28.5)	0 (0)
noggin-Fc	1 (25)	2 (50)	1 (25)
mCHL2-FLAG	2 (25)	3 (37.5)	3 (37.5)
BMP6	3 (75)	1 (25)	0 (0)

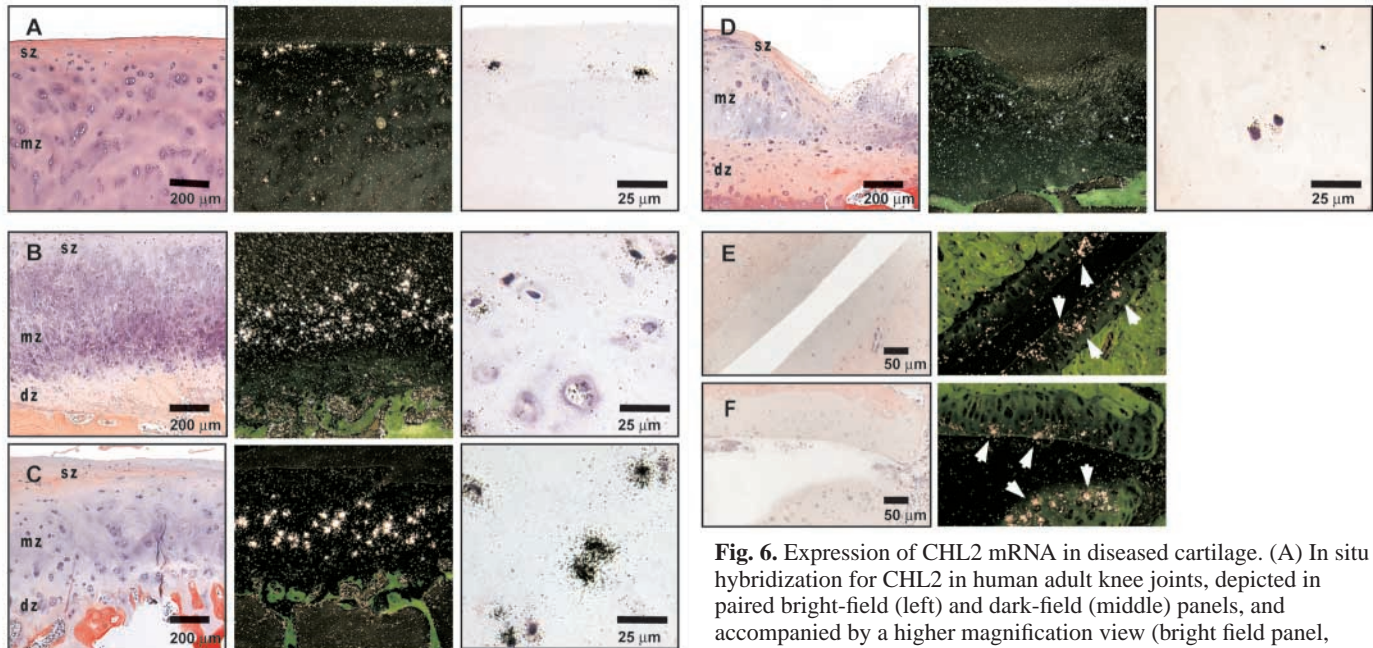
ES cell-derived mesodermal progenitor cells were cultured as a pellet for 18 days and then induced to mineralize for 6-8 days. Cartilage particles were fixed, sectioned, stained with von Kossa, and classified on the basis of the degrees of mineral deposition in matrix. Occasional strong von Kossa staining on the non-cartilaginous surface layer of the particles was not considered to be cartilage mineralization.

\*2  $\mu$ g/ml (40 nM) noggin-Fc, 3  $\mu$ g/ml (64.5 nM) mCHL2-FLAG, 50 ng/ml (1.7 nM) BMP6 were added on day 15.

<sup>†</sup>Particles containing areas of cartilaginous extracellular matrix that stained with von Kossa (Fig. 7Ca). The von Kossa-positive areas were located adjacent to the surface cell layer, which was COL2/COL10-negative and thereby non-cartilaginous (Fig. 7Cb).

<sup>‡</sup>Particles containing smaller von Kossa-positive nodules.

<sup>§</sup>Particles containing no von Kossa-positive areas or a very small von Kossa-positive spot inside the particle (Fig. 7Cd,g).



**Fig. 6.** Expression of *CHL2* mRNA in diseased cartilage. (A) In situ hybridization for *CHL2* in human adult knee joints, depicted in paired bright-field (left) and dark-field (middle) panels, and accompanied by a higher magnification view (bright field panel, right). Expression of *CHL2* mRNA in normal knee cartilage (femur, 55-year old female). Note that *CHL2* is localized on articular chondrocytes (right). However, *CHL2*<sup>+</sup> chondrocytes are scattered throughout the cartilage. Weak signals are also visible in superficial zone chondrocytes. (B,C) Expression of *CHL2* in osteoarthritic (OA) knee cartilage (B: 73-year-old male with OA, degenerative joint disease (DJD), and chronic proliferative synovitis; C: 48-year-old female with DJD). Signal is strongly induced in middle zone chondrocytes, but absent in superficial zone chondrocytes. (D) Expression of *CHL2* in rheumatoid arthritic knee cartilage (80-year-old female with RA). Weakly positive chondrocytes are scattered throughout the cartilage. (E,F) Expression of *CHL2* mRNA in hind paws of rats with collagen-induced arthritis, depicted in paired bright-field (left) and dark-field (middle) panels. *CHL2* signal is weakly expressed in normal chondrocytes (E), and is not substantially elevated in animals with severe immune-mediated disease (F). Arrowheads indicate *CHL2*-positive chondrocytes. sz, superficial zone; mz, middle zone; dz, deep zone.

55-year old female). Note that *CHL2* is localized on articular chondrocytes (right). However, *CHL2*<sup>+</sup> chondrocytes are scattered throughout the cartilage. Weak signals are also visible in superficial zone chondrocytes. (B,C) Expression of *CHL2* in osteoarthritic (OA) knee cartilage (B: 73-year-old male with OA, degenerative joint disease (DJD), and chronic proliferative synovitis; C: 48-year-old female with DJD). Signal is strongly induced in middle zone chondrocytes, but absent in superficial zone chondrocytes. (D) Expression of *CHL2* in rheumatoid arthritic knee cartilage (80-year-old female with RA). Weakly positive chondrocytes are scattered throughout the cartilage. (E,F) Expression of *CHL2* mRNA in hind paws of rats with collagen-induced arthritis, depicted in paired bright-field (left) and dark-field (middle) panels. *CHL2* signal is weakly expressed in normal chondrocytes (E), and is not substantially elevated in animals with severe immune-mediated disease (F). Arrowheads indicate *CHL2*-positive chondrocytes. sz, superficial zone; mz, middle zone; dz, deep zone.

### Potential roles of *CHL2* in joint formation

Cartilages within hip and knee joints and at the costochondral junction were the major *CHL2* expression sites during embryogenesis (Fig. 4). *CHL2* mRNA was also expressed strongly in connective tissues anchoring reproductive organs (Fig. 5). *CHL2* in developing joints was restricted to superficial zone chondrocytes; expression was substantially diminished in adult joint cartilage (Fig. 4). The *CHL2*-expressing areas did not overlap domains expressing chordin (non-chondrogenic mesenchyme of limb buds), *CHL1* (condensing mesoderm, hypertrophic chondrocytes) and gremlin (non-chondrogenic regions of limb buds, including interdigital mesenchyme) during limb formation (Nakayama et al., 2001; Scott et al., 1999; Scott et al., 2000), suggesting that these four factors have divergent biological roles. However, as with *CHL1* (Nakayama et al., 2001), *CHL2* expression in developing cartilage overlapped with *noggin* expression (Brunet et al., 1998; Capdevila and Johnson, 1998; Merino et al., 1998; Nifuji and Noda, 1999; Pathi et al., 1999).

As *CHL2* is a BMP-binding inhibitor, and BMPs regulate multiple steps during chondrogenesis, expression of *CHL2* in superficial chondrocytes in developing joints suggests a role in joint specification. The ability of exogenous m*CHL2* to inhibit chondrogenesis by hMSCs supports this hypothesis (Fig. 7A,B, Table 4). The surface of developing cartilage consists of proliferating mesenchymal cell layers that are differentiating into chondrocytes. By its location, *CHL2* might act as an important boundary in joint formation. A possible role could be to prevent articular cartilage from becoming too massive,

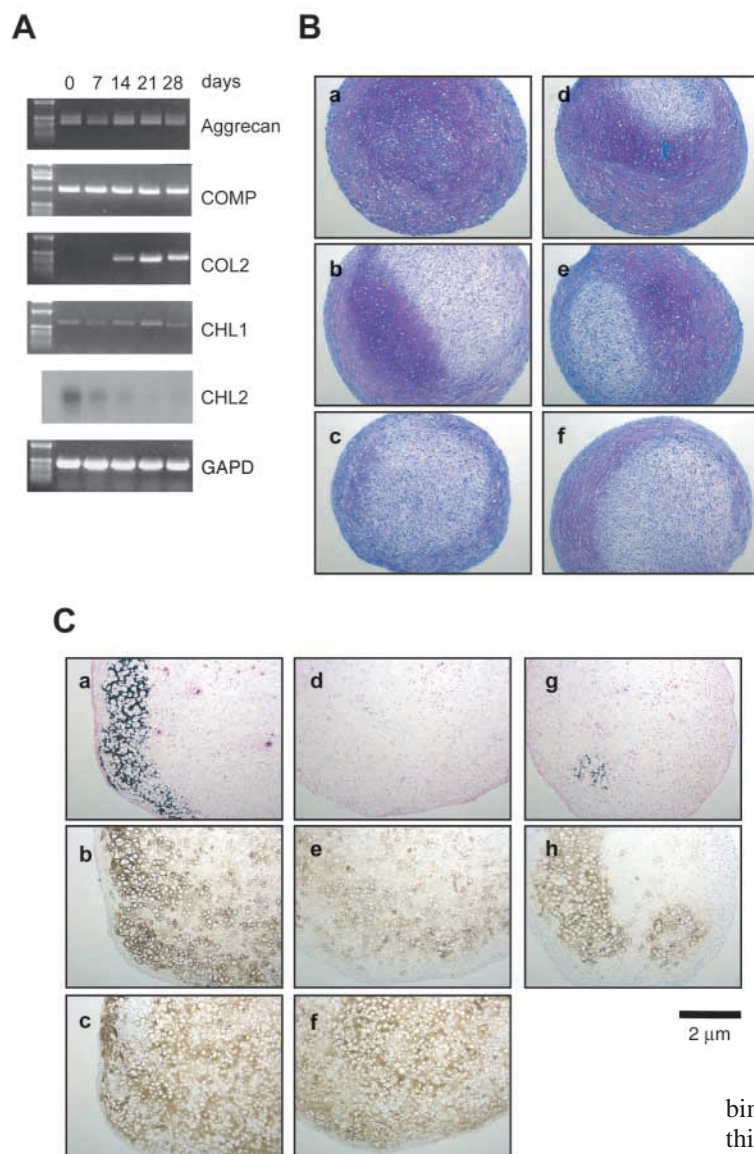
by keeping mesenchymal cells in the joint space from being recruited to the chondrocyte developmental pathway.

Alternatively, *CHL2* could play more subtle roles. The superficial zone of articular cartilage is composed of flattened chondrocytes separated by tangential arrays of thin collagen fibrils, but no proteoglycan matrix. In contrast, the middle zone consists of rounded chondrocytes surrounded by a proteoglycan-rich matrix containing radial bundles of thick collagen fibrils. Osteogenic BMPs accumulate in the pericellular matrix of articular cartilage, with highest levels in the middle to deep zone (Anderson et al., 2000). Conversely, the osteogenic antagonist BMP3 (Daluiski et al., 2001) is more highly expressed in the superficial zone. We failed to detect an interaction between *CHL2* and BMP3 (not shown), suggesting that preferential expressions of *CHL2* and BMP3 in the surface chondrocytes act to regulate a BMP gradient in normal articular cartilage.

### Potential involvement of *CHL2* in osteoarthritis

*CHL2* mRNA was never detected in the growth plate, where proliferation and hypertrophic differentiation of pre-hypertrophic chondrocytes normally occur, implying that *CHL2* is not relevant to normal pathways of chondrocyte proliferation and maturation. However, the up-regulation of *CHL2* transcripts specifically in middle zone cartilage of adult joints with OA (Fig. 6) prompted our speculation that *CHL2* has a role in cartilage repair. We examined the associations between *CHL2* and three principal phenotypes of OA cartilage: (1) reduced proteoglycan levels (which precede overt





**Fig. 7.** Effect of CHL2 on in vitro chondrogenesis. (A) CHL mRNA expression during chondrogenic culture. hMSCs were grown in pellet culture in the presence of TGF $\beta$ 3. On the indicated day, RNA was extracted from particles, treated with DNase I, and subjected to RT-PCR using primers for aggrecan, COMP, COL2, CHL1, CHL2 and GAPD. The CHL2 signal was confirmed with a  $^{32}$ P-labeled *Xba*I-*Sal*I fragment from pSPORTCHL2. (B) hMSCs were grown in pellet culture in the presence of TGF $\beta$ 3 alone (a,d), or TGF $\beta$ 3 with either 0.2  $\mu$ g/ml mCHL2-FLAG (b), 2  $\mu$ g/ml mCHL2-FLAG (c), 0.1  $\mu$ g/ml noggin-Fc (e), or 1  $\mu$ g/ml noggin-Fc (f). On day 21, particles were harvested and stained with Toluidine Blue. Data are representative of five independent experiments. Addition of IgG-Fc did not affect growth and maturation of cartilage-containing particles (not shown). Note that cartilage nodules, in which well-separated cells were embedded, stained more intensely with Toluidine Blue. (C) Effect of CHL2 on in vitro mineralization of EB cell-derived cartilage. FACS-purified FLK1 $^{+}$ PDGFR $\alpha^{+}$  EB cells were grown in pellet micromass culture to produce hyaline cartilage particles. On day 18, the medium was changed to a hypertrophic differentiation medium in the absence (a-c) or presence of 3  $\mu$ g/ml mCHL2-FLAG (d-f) or 2  $\mu$ g/ml noggin-Fc (g,h). On day 24, particles were harvested, stained with von Kossa (a,d,g), and immunostained with X53 for COL10 (b,e,h) or 2B1.5 for COL2 (c,f). Data are representative of four independent experiments. The Scale bar for B and C is shown in C.

histological changes), (2) aberrant chondrocyte proliferation (resulting in clonal chondrocyte expansion), and (3) upregulation of molecules (e.g., COL1, COL3, COL10, and AP) found in hypertrophied or de-differentiated chondrocytes but not normal articular chondrocytes (Aigner et al., 1993; Kirsch et al., 2000b; Von der Mark et al., 1992). First, the proteoglycan content in CHL2-expressing regions of OA cartilage was not reduced, as detected in Toluidine Blue-stained sections by the retained metachromasia (not shown). Second, CHL2-expressing chondrocytes in OA cartilage were typically found as aggregates; however, middle to deep zone chondrocytes are normally arranged in a cylindrical fashion, so this association might reflect normal middle zone anatomy. In contrast, the weak but significant inhibition of cartilage mineralization by CHL2 (Fig. 7C, Table 5) suggested that in OA cartilages this molecule might delay and/or reduce the degree of chondrocyte hypertrophy, thereby ameliorating cartilage degeneration. Further support for this premise is that medium turbidity, which indicates mineral deposition and excess  $\text{Ca}^{2+}$  excretion, was delayed during hypertrophic

differentiation culture of cartilage particles by CHL2 or noggin (not shown). However, we have not addressed whether CHL2 is involved in the de-differentiation of mature articular chondrocytes. Co-localization analyses between cells expressing the CHL2 mRNA and those expressing transcripts for COL1, COL10 or proliferating cell-nuclear antigen are underway to answer this question.

In conclusion, abundant evidence suggests that BMP functions are regulated by numerous extracellular BMP-binding proteins in developing joints. Our current data support this paradigm and add a new BMP inhibitor, CHL2, to this pathway. Our findings also provide the first evidence that a chordin-like BMP-binding inhibitor might be intimately involved in the pathogenesis of degenerative joint disease.

First, we wish to acknowledge the many scientists and technicians in the Amgen Genomics and Bioinformatics groups who constructed and analyzed the nucleotide sequence databases. We greatly appreciate the superb technical support provided by R. Haldankar, H. Yamane, and M. Haniu for mid-scale mCHL2 preparation and  $\text{NH}_2$ -terminal amino acid sequence determination, and by R. Manoukian for cell sorting. We are grateful to D. Chang, L. Daugherty, C. Baikalov and H. Yamane for preparing and purifying anti-mCHL2 peptide antibodies. Finally, we warmly thank W. Boyle and S. Simonet for support and encouragement. R.N. and T.Y. are supported by Amgen Japan, K.K. Cooperative Human Tissue Network is funded by the National Cancer Institute.

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