The protein kinase MLTK regulates chondrogenesis by inducing the transcription factor Sox6

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SUMMARY
Sox9 acts together with Sox5 or Sox6 as a master regulator for chondrogenesis; however, the inter-relationship among these transcription factors remains unclear. Here, we show that the protein kinase MLTK plays an essential role in the onset of chondrogenesis through triggering the induction of Sox6 expression by Sox9. We find that knockdown of MLTK in Xenopus embryos results in drastic loss of craniofacial cartilages without defects in neural crest development. We also find that Sox6 is specifically induced during the onset of chondrogenesis, and that the Sox6 induction is inhibited by MLTK knockdown. Remarkably, Sox6 knockdown phenocopies MLTK knockdown. Moreover, we find that ectopic expression of MLTK induces Sox6 expression in a Sox9-dependent manner. Our data suggest that p38 and JNK pathways function downstream of MLTK during chondrogenesis. These results identify MLTK as a novel key regulator of chondrogenesis, and reveal its action mechanism in chondrocyte differentiation during embryonic development.

KEY WORDS: MLTK, Sox6, Sox9, Chondrocyte differentiation, Xenopus

INTRODUCTION
Neural crest cells, which are generated at the lateral edge of the neural plate, migrate extensively and differentiate into diverse derivatives, including much of the facial skeleton and peripheral nervous system. Neural crest cells are multipotent and give rise to various types of cells, including craniofacial cartilage, pigment cells, autonomic and enteric ganglia, neurons and smooth muscle cells (Sauka-Spengler and Bronner-Fraser, 2008). The neural crest can be divided into two main domains; cranial and trunk neural crest domains. Only the cells of cranial neural crest have a potential to generate chondrocytes and osteoblasts (Le Douarin et al., 2004). However, the mechanism of the differentiation of cranial neural crest cells into chondrocytes has not been well understood.

Sox5, Sox6 and Sox9 constitute a transcription factor trio that is essential for cartilage formation in mice (Lefebvre and Smits, 2005). Sox9 is expressed in multipotent mesenchymal cells (Ng et al., 1997; Zhao et al., 1997) and is required for cell survival and chondrocyte differentiation in cartilage primordia (Bi et al., 1999; Akiyama et al., 2002). Sox5 and Sox6, which are related to each other and functionally redundant in chondrogenesis, are expressed with Sox9 in pre-cartilaginous condensations and are essential for cartilage formation (Smits et al., 2001). Sox9 has a transactivation domain, but Sox5 and Sox6 do not (Lefebvre et al., 1998). Sox5 and Sox6 bind to the enhancers of cartilage matrix genes and enhance the activity of Sox9 (Han and Lefebvre, 2008; Nagy et al., 2011). The three Sox genes together are able to confer on non-chondrogenic cells the ability to activate Col2a1, Agc1 and several other cartilage markers (Ikeda et al., 2004). Taken together, Sox5, Sox6 and Sox9 are not only required but also sufficient for chondrocyte differentiation. Although it has been reported that Sox9 induces Sox5 and Sox6 expression (Ikeda et al., 2004), neither the mechanism of this induction nor the inter-relationship among the three factors has been elucidated.

The mitogen-activated protein kinase (MAPK) pathways play an important role in transducing extracellular signals to the transcriptional program. The MAPK pathways consist of three sequentially activated protein kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK (Nishida and Gotoh, 1993; Robinson and Cobb, 1997; Chang and Karin, 2001). MLTKs (MLK-like mitogen-activated protein triple kinases) are a subfamily of the MLK family (Galio and Johnson, 2002). Two splicing variants are identified in MLTKs that have identical amino acid sequences in the N-terminal region (residues 1-311), which contains a kinase domain and a leucine zipper motif. MLTKα has a longer C-terminal region containing a sterile-alpha-motif (SAM), which is absent from MLTKβ (Liu et al., 2000; Bloem et al., 2001; Gotoh et al., 2001). MLTKα and MLTKβ form homo- and heterodimers, and can be activated by osmotic stress (Liu et al., 2000; Gotoh et al., 2001). Activated MLTKα and MLTKβ activate the p38 and JNK pathways through MKK3/6 and MKK4/7 phosphorylation (Gotoh et al., 2001; Gross et al., 2002). In cultured cells, knockdown of MLTK completely blocks anisomycin- and UV-induced p38 and JNK activation and apoptosis (Wang et al., 2005). Transgenic mice with cardiac-restricted overexpression of MLTKβ have increased p38 pathway activation and characteristics of a cardiac compensatory phenotype (Christe et al., 2004). However, a role of MLTK in embryonic development remains unknown in any organism.

To study the function of MLTK in embryonic development, we isolated and knocked down MLTK in the Xenopus embryo. MLTK-depleted embryos showed severe defects in chondrocyte differentiation, while displaying normal neural crest formation and behavior. We also found that Sox6, but not Sox5 or Sox9, is specifically induced during the onset of chondrogenesis, and the induction of Sox6 is required for chondrocyte differentiation. Importantly, our analysis demonstrated that MLTK plays a key role in inducing Sox6 expression. These results identify MLTK as a key player in the differentiation of neural crest cells into chondrocytes.
MATERIALS AND METHODS

**Molecular cloning**

A partial fragment (502 bp) of *Xenopus laevis MLTKβ* was obtained by PCR from stage 20 cDNA using a forward primer that was designed based on the *Xenopus tropicalis* EST sequence (GenBank Accession Number AL898835) homologous to mammalian *MLTKβ* (B23012/0H23Rik – Mouse Genome Informatics; *ZAK* – Human Gene Nomenclature Committee) and a reverse primer that was designed based on the sequence of mouse *MLTKβ* (Accession Number AB049732). A *Xenopus* gastrula stage cDNA library was then screened by plaque hybridization using this fragment as a probe, and the full-length clone of *xMLTKβ* was isolated. The entire coding region of *xMLTKα* was isolated by PCR from adult testis cDNA using a forward primer that was designed based on the N-terminal sequence of *xMLTKβ*, and a reverse primer that was designed based on the 3' UTR sequence of *Xenopus tropicalis MLTKα* (XM_002934606). Partial fragments of *Xenopus laevis* *Agc1* and *Crt1* were amplified by PCR from stage 46 cDNA using primers designed based on *Xenopus laevis* EST (EE137851), which contains a partial sequence of *xAgc1*, and *Xenopus tropicalis Crt1* (XM_002933930), respectively. The coding regions of *Crtl1* which contains a partial sequence of *Xenopus tropicalis MLTK* primer that was designed based on the 3' UTR sequence of *Xenopus tropicalis MLTKα* (XM_002934606). Partial fragments of *Xenopus laevis* *Agc1* and *Crt1* were amplified by PCR from stage 46 cDNA using primers designed based on *Xenopus laevis* EST (EE137851), which contains a partial sequence of *xAgc1*, and *Xenopus tropicalis Crt1* (XM_002933930), respectively. The coding regions of *Xenopus laevis* *Matn1* and *Sox6* were amplified by PCR from stage 18 and 48 cDNA using primers designed based on deposited sequences (*Matn1*, BC054272; *Sox5*, BC142559), respectively. As the obtained sequence of *xSox5* was different from the deposited sequence (BC142559), we submitted the obtained sequence and used this clone in our experiments. *Xenopus laevis* *Sox6*, including coding and untranslated regions, was amplified by PCR from stage 37/38 cDNA using primers designed based on *Xenopus tropicalis Sox6* (BC161247). Sequences of *xMLTKα*, *xMLTKβ*, *xCrt1*, *xSox5* and *xSox6* have been deposited in GenBank under Accession Numbers AB682774, AB682773, AB682775, AB682776, and AB682777, respectively.

**DNA constructs**

To generate MO-resistant constructs for rescue experiments, eleven silent mutations were introduced into the MO target sequence of *xMLTKα* and *xMLTKβ* by site-directed mutagenesis, which substitutes ATGTTG - to GAGGTG underlines indicate mutated nucleotides). The constitutively active form of *xMKK6* was constructed by replacing Ser208 and Thr212 of *xMKK6* with aspartic acid and glutamic acid, respectively. The constitutively active form of the line were measured. The relative cartilage size in Fig. 1F was calculated as the ratio of the length of the right (projected side) to that of the left (unprojected side) of the line.

**Immunoblotting**

*xMLTK*-MO and mRNAs were injected into the animal pole of the two-cell stage, and the embryos were harvested for lysis. Lysates were resolved by SDS-PAGE and subjected to immunoblotting according to a standard protocol. Mouse anti-Myc (9E10, Santa Cruz Biotechnology) or mouse anti-GFP (JL-8, Clontech) antibodies were used as a primary antibody, and sheep anti-mouse IgG HRP-conjugated (1:10,000; GE Healthcare) was used as a secondary antibody.

**Embryo manipulation**

*Xenopus* embryos were obtained by in vitro fertilization and cultured in 0.1× MBS at 22°C. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Antisense morpholino oligonucleotides (MOs), plasmid DNA and mRNA were injected into the animal pole of two or all blastomeres at the four-cell stage in 4% Ficoll in 0.1× MBS. In vitro synthesis of capped mRNA was performed using the T3700 reagent (Invitrogen) with random hexamer primers. Quantitative RT-PCR was performed on LightCycler (Roche Diagnostics) with QuantiTect SYBR Green PCR Kit (QIAGEN). The gene expression levels were normalized to those of *xODC* (ornithine decarboxylase). The sequences used for qRT-PCR are as follows: *xMLTKα* (AB682774) (F, TCCGGTGAAAGGATCGTT; R, CAGTGCTGATGTTGAGTT; *xMLTKβ* (AB682773) (F, GCCCTGGAAAGGATCGTT; R, AGAGGCTGCTGATGTTGAGTT; *xAgc1* (AY035397), 109-461; *xCrt1* (AB682775), 1-453; *xSox5* (AB682776), 1077-1263; *xSox6* (AB682777), 1172-1734.

**Alcian Blue staining**

Embryos were fixed in MEMFA for 1-2 hours, dehydrated and stored in methanol. Embryos were replaced with 100%, 75% and 50% ethanol, then stained overnight in the staining solution consisting of 0.01% Alcian Blue 8GX, 10% acetic acid and 60% ethanol.

**Quantification of cartilage size**

The length of cartilaginous structures was measured using the Photshop measure tool, the distance tips of the right and left cartilage structures of each embryo were connected with a line. Then, the distance between this line and the midline of the embryo was plotted to define the right and left parts of the line. Third, the lengths of the right and left parts of the line were measured. The relative cartilage size in Fig. 1F was calculated as the ratio of the length of the right (projected side) to that of the left (unprojected side) of the line.

**Quantitative RT-PCR analysis**

Total RNA was extracted from embryos using TRIzol reagent (Invitrogen), and cDNA was synthesized using reverse transcriptase M-MLV (Invitrogen) with random hexamer primers. Quantitative RT-PCR was performed on LightCycler (Roche Diagnostics) with QuantiTect SYBR Green PCR Kit (QIAGEN). The gene expression levels were normalized to those of *xODC* (ornithine decarboxylase). The sequences used for qRT-PCR are as follows: *xMLTKα* (AB682774) (F, TCCGGTGAAAGGATCGTT; R, CAGTGCTGATGTTGAGTT; *xMLTKβ* (AB682773) (F, GCCCTGGAAAGGATCGTT; R, AGAGGCTGCTGATGTTGAGTT; *xAgc1* (AY035397), 109-461; *xCrt1* (AB682775), 1-453; *xSox5* (AB682776), 1077-1263; *xSox6* (AB682777), 1172-1734.

**Microarray experiments**

The injection procedure and embryo collection are described in the figure legend. Total RNA was extracted using TRIzol reagent, treated with DNase (TURBO DNase, Ambion) and then purified using RNAeasy Mini Kit
The quality of total RNA was assessed using the Agilent 2100 BioAnalyzer. Reverse transcription to synthesize first-strand cDNA, second-strand cDNA synthesis, in vitro transcription to synthesize Biotin-modified aRNA, aRNA purification and fragmentation of the labeled aRNA were performed using the GeneChip 3' IVT Express Kit and hybridization to the Affymetrix GeneChip Xenopus laevis Genome 2.0 Array was performed according to the manufacturer's instructions. Craniofacial cartilage is outlined with a dashed yellow line.

**Fig. 1. xMLTK is required for craniofacial cartilage formation.**

(A) Whole-mount in situ hybridization for xMLTKα and xMLTKβ was performed on embryos at stages 17, 24, 37/38 and 41. OV, otic vesicle; G, glomus; PD, pronephric duct; No, notochord; F, left-rostral furrow in yolk mass; PM, paraxial mesoderm; FM, facial muscle; BA, branchial arch. (B) Magnified view of embryos at stage 41 shown in A. M, Meckel's cartilage; CH, ceratohyal cartilage; CB, ceratobranchial cartilage; OV, otic vesicle; G, glomus; F, left-rostral furrow in yolk mass; MQH, m. quadrato-hyoangulares; MOH, m. orbitohyoideus; MIH, m. interhyoideus; MLM, m. levator mandibulae. (C) xMLTK-MO or 5misMO was injected into the animal pole of all blastomeres (10 ng/cell) at the four-cell stage, and the embryos were fixed at stage 45. (D) Magnified view of the head of the embryo shown in C. Craniofacial cartilage is outlined with a dashed yellow line. (E) xMLTK-MO (n=29), xMLTK-MO plus xMLTKα-res-myc plasmid (n=25), or xMLTK-MO plus xMLTKβ-res-myc plasmid (n=25) was injected unilaterally into the animal pole of two blastomeres (xMLTK-MO, 10 ng/cell; plasmid, 50 pg/cell) at the four-cell stage. Unjected embryos (n=31) and injected embryos were fixed at stage 45, and cartilages were stained with Alcian Blue. Brackets indicate the injected side. M, Meckel's cartilage; CH, ceratohyal cartilage; CB, ceratobranchial cartilage; BHB, basihyoangulares. (F) The relative cartilage size (the rate of the length of the right side to that of the left side of ceratohyal cartilages) was quantified. Values are mean±s.d. **P<0.01.
Statistical analysis
Statistical differences were assessed with the unpaired Student’s t-test. Data are presented as mean±s.d., and P values less than 0.05 were considered to be statistically significant.

RESULTS
Isolation and expression of Xenopus MLTK\(\alpha\) and MLTK \(\beta\)
We isolated genes encoding *Xenopus laevis* MLTK\(\alpha\) (xMLTK\(\alpha\)) and MLTK\(\beta\) (xMLTK\(\beta\)). xMLTK\(\alpha\) and xMLTK\(\beta\) consist of 793 and 438 amino acids, respectively. Like human MLTKs, xMLTK\(\alpha\) and xMLTK\(\beta\) have the identical amino acid sequence in the N-terminal region (residues 1-330), which contains a kinase domain (16-277) and a leucine zipper motif (287-322). xMLTK\(\alpha\) has a longer C-terminal region containing a sterile-\(\alpha\)-motif (SAM) (339-406) (supplementary material Fig. S1A). At the amino acid level, xMLTK\(\alpha\) and xMLTK\(\beta\) shared 68% and 86% identity with human MLTK\(\alpha\) and MLTK\(\beta\) (NP_057737 and NP_598407), respectively. MLTKs are highly conserved in vertebrates (supplementary material Fig. S1B).

Quantitative RT-PCR (qRT-PCR) analysis at different stages of development showed that xMLTK\(\alpha\) and xMLTK\(\beta\) were expressed maternally, and their expression levels were decreased at the gastrula and neurula stages (stages 11-19). Increased expressions were detected at the early tailbud stage and persisted through the tadpole stage (supplementary material Fig. S2A). We next performed whole-mount in situ hybridization (Fig. 1A,B and supplementary material Fig. S2B). At stages 37/38 and 41, xMLTK\(\alpha\) expression was detected in various tissues including the head, gill, pronephron and others. xMLTK\(\beta\) was expressed in the neural tissues and paraxial mesoderm at stages 17, 24 and 37/38. Histological section showed that xMLTK\(\beta\) was also expressed in the presumptive epidermis (supplementary material Fig. S2C). At stage 41, xMLTK\(\beta\) expression was increased in the head, heart and left-rostral furrow in yolk mass (Fig. 1A,B).

xMLTK is required for craniofacial cartilage formation
To investigate the function of xMLTK during embryonic development, we performed knockdown of xMLTK by morpholino antisense oligonucleotides (MOs). An xMLTK-MO was designed to interfere with translation of both xMLTK\(\alpha\) and xMLTK\(\beta\) mRNAs. Immunoblotting analysis confirmed that xMLTK-MO blocked xMLTK protein production in a dose-dependent manner (supplementary material Fig. S3A). We next injected xMLTK-MO into the animal pole of all blastomers of four-cell stage embryos. Although the injected embryos appeared normal until stage 35/36, they exhibited several defects at stage 45, including edema formation, body axis bending, small eyes and a drastic reduction of craniofacial cartilage (Fig. 1C,D). Injection of a five-base mismatched MO (5misMO) at the same dose had little or no effect on embryonic development (Fig. 1C,D). Alcian Blue staining revealed that all cartilaginous elements, especially Meckel’s and quadrate cartilage, were drastically reduced on the injected side of the embryos unilaterally injected with xMLTK-MO (Fig. 1E, upper right panel).

To further assess the specificity of xMLTK-MO, we next asked whether the reduction of craniofacial cartilages in xMLTK-MO injected embryos could be rescued by expressing xMLTK\(\alpha\) or xMLTK\(\beta\). In rescue experiments, we used xMLTK\(\alpha\)-res-myc and xMLTK\(\beta\)-res-myc, which carried an 11 bp mutation within the target sequence of xMLTK-MO and had a myc tag at the C-terminus. Immunoblotting analysis confirmed that xMLTK-MO failed to block translation of xMLTK\(\alpha\)-res-myc and xMLTK\(\beta\)-res-myc mRNAs (supplementary material Fig. S3B). We unilaterally injected xMLTK-MO with or without xMLTK\(\alpha\)-res-myc or xMLTK\(\beta\)-res-myc plasmid into the animal pole of two blastomeres of four-cell stage embryos, and compared the length of ceratohyal cartilage between the injected and uninjected sides of each embryo. Co-injection of xMLTK\(\alpha\)-res-myc or xMLTK\(\beta\)-res-myc plasmid with xMLTK-MO significantly restored the size of cartilage when compared with siblings that received injection of xMLTK-MO alone (Fig. 1E,F). Collectively, these results show that xMLTK is required for craniofacial cartilage formation in *Xenopus*.

xMLTK is dispensable for neural crest formation and migration but required for chondrocyte differentiation
Craniofacial cartilage is mostly derived from neural crest cells, which pass through two fundamental steps: the formation and migration steps. We next examined whether xMLTK depletion disturbed neural crest formation and migration. Injection of xMLTK-MO did not substantially affect the expression levels and patterns of an early neural crest marker *xTwist* (Pohl and Knöchel, 2001; Sasai et al., 2001) (Fig. 2A), a migrating neural crest marker *xSox9* (Hopwood et al., 1989) (Fig. 2B) and a cranial neural crest marker *xSox9* (Spokony et al., 2002; Kenney et al., 2007) at stage 35/36, when neural crest cells reside in the branchial arches after migration (Fig. 2C). These results indicate that all steps of neural crest development are normal in xMLTK-depleted embryos.

We then examined whether xMLTK depletion affected chondrocyte differentiation. So far, few chondrocyte markers had been characterized in *Xenopus*. Therefore, we isolated the *Xenopus laevis* orthologs of matrilin 1 (*Matl1*), aggrecan (*Agc1*) and cartilage link protein 1 (*Crl1*), which are known as chondrocyte markers (Lefebvre and Smits, 2005). Whole-mount in situ hybridization analysis showed that their expressions were detected in the prospective ceratohyal cartilage at stage 39, and increased strongly in all cartilaginous elements at stage 41 (supplementary material Fig. S4A-C). However, *xSox9* was expressed at stage 35/36 in the branchial arch and also in cartilages at stage 41 (Kenney et al., 2007) (supplementary material Fig. S4D). These results demonstrated that *xMatl1*, *xAgc1* and *xCrl1* could be used as chondrocyte markers in *Xenopus*. qRT-PCR analysis also revealed that the expression levels of these three chondrocyte markers began to increase at around 60 hours after fertilization (corresponding to stage 39) (control lines in Fig. 2D). These results indicate that chondrocyte differentiation occurs around stage 39 in *Xenopus*.

We next analyzed their expression in xMLTK-depleted embryos by qRT-PCR. The heads of xMLTK-MO-injected embryos were collected in order to minimize contamination from other tissues than cartilages. The rise in the expression levels of *xMatl1*, *xAgc1* and *xCrl1* was strongly suppressed in xMLTK-depleted embryos (Fig. 2D). Whole-mount in situ hybridization for chondrocyte markers showed that their expression levels in the cartilaginous skeletal elements, including the Meckel’s, ceratohyal and ceratobranchial cartilages, were markedly reduced on the xMLTK-MO-injected side (Fig. 2E). Injection of a standard control MO (CoMO) or 5misMO did not reduce their expression levels (supplementary material Fig. S5A-C). These results taken together demonstrate that chondrocyte differentiation is inhibited by xMLTK knockdown, and thus indicate that xMLTK is required for chondrocyte differentiation, which starts around stage 39.
xMLTK is required for xSox6 expression

In mice, xSox5, xSox6 and xSox9 play a pivotal role in chondrogenesis. So, we analyzed the expression of their Xenopus orthologs before and after chondrocyte differentiation. qRT-PCR analysis showed that although the expression levels of xSox5 and xSox9 were not changed substantially during this period, xSox6 expression was increased markedly in the heads of embryos (Fig. 3A, blue bars). Interestingly, the increase in xSox6 expression was suppressed in xMLTK-depleted embryos (Fig. 3A). It should be also noted that the expression levels of xSox5 and xSox9 were not substantially decreased by xMLTK knockdown (Fig. 3A). Time-course measurements of xSox6 expression confirmed the marked upregulation of xSox6 during the onset of chondrocyte differentiation and its suppression by xMLTK knockdown (Fig. 3B). Whole-mount in situ hybridization also showed that the increased xSox6 expression in the head region at stage 41 (around 60 hours after fertilization) was dramatically reduced in the xMLTK-knockdown embryos (Fig. 3C). These results indicate that xMLTK is required for xSox6 expression.

We then inquired whether xMLTK is able to induce xSox6 expression. Overexpression of xMLTKα and xMLTKβ by injecting their mRNAs in Xenopus embryos, however, did not induce xSox6 expression (Fig. 3D). Therefore, we considered the possibility that xMLTK could collaborate with xSox9 to induce xSox6 expression, as xSox9 can induce xSox6 expression in mammalian cultured cells (Ikeda et al., 2004). As expected, although overexpression of xSox9 alone did not induce a substantial increase in xSox6 expression, co-expression of xMLTKα and xMLTKβ with xSox9 induced marked increases in xSox6 expression in a dose-dependent manner (Fig. 3E). Moreover, we examined whether the induction of xSox6 expression by xMLTK requires xSox9 and vice versa. For longer culture, we employed a sandwich-culture method of craniofacial cartilage induction in vitro (Furue et al., 2002) (Fig. 3F). Although injection of xMLTKα and xMLTKβ mRNAs increased xSox6 expression in 3-day sandwiched explants, co-injection of xSox9-MO markedly decreased xSox6 expression (Fig. 3G). Whereas injection of xSox9 mRNA increased xSox6 expression, co-injection of xMLTK-MO substantially decreased xSox6 expression (Fig. 3H). These results indicate that xMLTK functions to induce xSox6 expression during chondrocyte differentiation through xSox9.

xSox6 is essential for craniofacial cartilage formation

Is the increased xSox6 expression essential for craniofacial cartilage formation? In Xenopus, although xSox9 has been studied extensively (Spokony et al., 2002; Lee and Saint-Jeannet, 2011), the roles of xSox5 and xSox6 during embryonic development have not been studied. First, we analyzed the expression patterns of xSox5. Whole-mount in situ hybridization analysis confirmed the previously published expression patterns of xSox5 from stage 26 to 41 (Martin and Harland, 2001) and also showed that xSox5 was expressed at stage 15 in the anterior neural fold, paraxial mesoderm and neural crest (supplementary material Fig. S6A-D). We then isolated xSox6 (supplementary material Fig. S6A) and analyzed its expression. In contrast to xSox5, xSox6 expression at stages 15 to 23 was very low (supplementary material Fig. S6B) and undetectable by in situ hybridization (Fig. 4A). xSox6 expression was first detected in the otic vesicle, somite and branchial arch at stage 29/30, and in the brain and posterior notochord at stage 35/36. At stage 39, when mesenchymal cells begin to differentiate into chondrocytes, xSox6 expression in the branchial arches was dramatically increased, and each cartilaginous element was clearly distinguishable (Fig. 4A, B).

We then knocked down xSox5 and xSox6 by using xSox5-MO and xSox6-MO, respectively. Injection of xSox5-MO resulted in a modest reduction of craniofacial cartilage at stage 45 (supplementary material Fig. S7A, B). Conversely, injection of the...
same dose of xSox6-MO resulted in a drastic reduction of craniofacial cartilage (Fig. 4C and supplementary material Fig. S7B). Moreover, Alcian Blue staining showed that all cartilaginous elements in the head region were drastically reduced by xSox6-MO injection (Fig. 4D). These results suggest that xSox6 plays an essential role in craniofacial cartilage formation, and thus the increase in xSox6 expression at the onset of chondrocyte differentiation may be a key step in triggering chondrogenesis.
xSox6 is required for chondrocyte differentiation but dispensable for neural crest formation and migration

To further characterize the role of xSox6, we analyzed neural crest markers in xSox6-depleted embryos. Whole-mount in situ hybridization demonstrated that the expression patterns of xFoxD3 (Fig. 5A) and xTwist (Fig. 5B) were not substantially affected by xSox6 knockdown. These results indicate that xSox6 is dispensable for the induction and migration of neural crest cells. We then analyzed chondrocyte markers. qRT-PCR analysis showed that the strong induction of xMatn1 and xAgc1 during chondrogenesis was inhibited in xSox6-depleted embryos (Fig. 5C). Whole-mount in situ hybridization also showed that these two chondrocyte markers were drastically downregulated in xSox6-depleted embryos (Fig. 5D). Thus, xSox6 is essential for chondrocyte differentiation but not for neural crest formation and migration. This phenotype of...
xSox6-depleted embryos is essentially the same as that of xMLTK-depleted embryos. Therefore, these results together suggest that xMLTK regulates chondrogenesis through induction of xSox6.

**xSox6-depleted embryos closely resemble xMLTK-depleted embryos in the gene expression profile**

To further characterize the phenotypes of xMLTK-depleted and xSox6-depleted embryos, we performed genome-wide gene expression analysis. The heads of embryos injected with CoMO, xMLTK-MO or xSox6-MO were collected at stage 41 and used for microarray analysis. Scatter plot analysis showed a close similarity between xMLTK-MO and xSox6-MO-injected embryos in the gene expression profile (Fig. 6A). A Venn diagram demonstrated a remarkable overlap between xMLTK-dependent and xSox6-dependent genes (Fig. 6B). Gene set enrichment analysis (Subramanian et al., 2005) showed that genes whose mouse orthologs were induced during chondrogenesis in vivo (Cameron et al., 2009) were highly enriched in both the subsets of xMLTK-dependent and xSox6-dependent genes (Fig. 6C). Clustering analysis also revealed that the subsets of xSox6-dependent genes closely resemble those of xMLTK-dependent genes (Fig. 6D). Moreover, there was a remarkably high correlation between xMLTK-MO- and xSox6-MO-injected embryos in the expression levels of cartilage-expressed genes, such as *Matn1*, *Col11a1* (Li et al., 1995), *Lect1* (Shukunami et al., 1999), *Col9a2* (Muragaki et al., 1996; Annunen et al., 1999), *Papss2* (Stelzer et al., 2007), *Epyc*...
xMLTK regulates chondrogenesis through p38 and JNK pathways

Previous reports showed that MLTK activates both the p38 MAPK and JNK pathways (Gotoh et al., 2001; Gross et al., 2002). Therefore, we performed rescue experiments with constitutively active forms of xMKK6 (a direct activator of p38) and xMKK7 (a direct activator of JNK) (CA-xMKK6 and CA-xMKK7) in order to investigate whether p38 and JNK pathways are involved in chondrogenesis as downstream signaling effectors of xMLTK. Co-injection of HA-CA-xMKK6 or HA-CA-xMKK7 plasmid with xMLTK-MO significantly restored the size of cartilage when compared with siblings that received injection of xMLTK-MO alone (Fig. 7A,B). These results suggest that xMLTK chondrogenic activity is dependent upon p38 and JNK signaling pathways.

DISCUSSION

Cartilages are formed as the first skeleton of the embryo before endochondral bone formation, and also function in joints, airways and ears of adults as a supporting organ that has the flexibility. Therefore, abnormalities in chondrogenesis cause congenital disorders in human, such as campomelic dysplasia and dwarfism. In this study, we show that MLTK plays an essential role in chondrocyte differentiation during embryonic development through inducing Sox6, which is shown to be an essential transcription factor for chondrogenesis in Xenopus. Thus, xMLTK depletion resulted in complete loss of Meckel’s cartilage, which is derived from only neural crest cells (Sadaghiani and Thiébaut, 1987), and drastic reduction of ceratohyal and ceratobranchial cartilages, which are derived from neural crest and mesodermal cells. Importantly, the formation, migration and maintenance of neural crest cells were normal in xMLTK-depleted embryos, indicating that xMLTK is specifically required for a differentiation process of neural crest cells into chondrocytes. Although the mechanism of neural crest formation and migration has been studied extensively, how neural crest cells differentiate into chondrocytes remains poorly understood in organisms. Our findings introduce xMLTK as a key signaling factor in the differentiation of neural crest cells into chondrocytes.

Sox5, Sox6 and Sox9 play a central role in chondrocyte differentiation in mice (Lefebvre and Smits, 2005). Although Sox9 is required for both mesenchymal condensations and subsequent chondrocyte differentiation, Sox5 and Sox6 are specifically required for chondrocyte differentiation in mouse embryonic development (Smits et al., 2001; Akiyama et al., 2002). Interestingly, xMLTK depletion resulted in marked suppression of xSox6 expression at stage 41, but it did not affect the expression of xSox5 and xSox9. However, xSox6 function was unknown in Xenopus. Our analysis then shows that xSox6 expression markedly increases in the prospective cartilage at around stage 39, when precartilaginous condensations are occurring (Kerney et al., 2007; Lee and Saint-Jeannet, 2011), and that chondrocyte differentiation is severely inhibited in xSox6-depleted embryos, whereas prior neural crest development is normal. These results indicate that xSox6 is specifically required for chondrocyte differentiation, very similar to xMLTK. Collectively, our results show that xMLTK is required for xSox6 expression, which is essential for chondrocyte differentiation.

In mice, although Sox9 is required for Sox5 and Sox6 expression in chondrogenesis (Akiyama et al., 2002; Ikeda et al., 2004), the mechanism by which Sox9 induces Sox5 and Sox6 expression remains unknown. We have found that ectopic expression of xMLTK can induce Sox6 expression in a xSox9-dependent manner. Moreover, using the in vitro model system of cartilage formation, we showed that Sox6 induction by xMLTK requires Sox9. Based on these findings, we propose a hypothetical model in which xMLTK activates Sox9, thereby inducing Sox6 expression and promoting chondrocyte differentiation. It has been reported that p38 signaling can increase the transcriptional activity of Sox9, and transgenic mice in which p38 signaling is constitutively activated on these findings, we propose a hypothetical model in which xMLTK acts as a Sox9 activator. Importantly, overexpressing Sox9 in chondrocytes (Zhang et al., 2006). In addition, many reports have suggested that the p38 pathway is a positive regulator of the differentiation of mesenchymal cells into chondrocytes (Bobick and Kulyk, 2008). Our analysis suggests that p38 and JNK MAPK pathways play an important role as downstream signaling components of xMLTK during chondrogenesis, and that xMLTK can induce chondrogenesis through p38 and JNK pathways.
this is the first to describe the expression patterns of chondrocyte markers in *Xenopus laevis*. Our analysis revealed that the expression of chondrocyte markers is first detected in the prospective craniofacial cartilage at stage 39, when pre-cartilaginous condensations are occurring (Kerney et al., 2007; Lee and Saint-Jeannet, 2011). Sox6 expression begins to increase at around stage 35/36 and then markedly increases at stage 39 in the prospective cartilage, while Sox9 and Sox6 are continuously expressed in the neural crest from the neural stage to the late tailbud stage. Thus, studies examining spatiotemporal expression patterns of chondrocyte markers and key transcription factors complement functional analysis of the factors involved in chordogenesis, suggesting that *Xenopus laevis* is a useful model organism to study the mechanism of the differentiation of neural crest cells into chondrocytes.

In conclusion, this study identifies a novel player, xMLTK, in chordogenesis, and reveals that xMLTK functions to trigger the induction of Sox6 by Sox9 in chordogenesis during embryonic development.

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Competing interests statement
The authors declare no competing financial interests.

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