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Progressive mRNA decay establishes an *mkp3* expression gradient in the chick limb bud

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Abstract

The apical ectodermal ridge (AER) controls limb outgrowth and patterning, such that its removal causes changes in mesodermal gene expression, cell death and limb truncation. Fibroblast growth factor (FGF) family members are expressed in the AER and can rescue limb bud outgrowth after AER removal. Cells localized underneath the AER are maintained in an undifferentiated state by the FGFs produced by the AER. *MAPK phosphatase 3* (*mkp3*) is a downstream effector of FGF8 signalling during limb bud development and is expressed in the distal limb mesenchyme. The present work evidences a gradient of *mkp3* transcripts along the chick limb bud, in a distal to proximal direction. *mkp3* transcription occurs only in the most distal limb bud cells and its mRNA gradient throughout the limb results from progressive mRNA decay. We show that FGF8-soaked beads induce ectopic *mkp3* expression, indicating that AER-derived FGF8 protein may activate *mkp3* in the distal mesenchyme.

Keywords: FGF8; *mkp3*; mRNA decay; mRNA gradient; Limb bud distal mesenchyme; Chick embryo development

Initiation of limb bud development occurs at defined positions in the flank of the embryo through differential cell proliferation of mesenchymal cells of two embryonic origins, lateral plate and somitic mesoderm. Fibroblast growth factors (FGFs) are essential during embryonic development namely, for the regulation of cellular proliferation and differentiation [1].

Intracellular responses to FGFs are mediated by several signal transduction cascades, including the MAPK and the phosphatidylinositol-3-OH kinase (PI(3)K)/Akt pathways [2]. The *MAPK phosphatase 3* (*mkp3*) encodes a dual-specificity phosphatase, known to be a negative regulator of the MAPK/ERK pathway [3–6]. Kawakami and colleagues (2003) found that *mkp3* is a downstream effector of the FGF8 signalling pathway during limb development, and that its induction is mediated through the PI(3)K pathway [7]. Thus, *mkp3* mediates the cellular response to FGF8 signalling in the vertebrate limb [7].

The growing limb mesenchyme is surrounded by ectoderm, whose distal tip forms a specialized epithelial structure, the apical ectodermal ridge (AER) [8]. AER removal leads to limb truncation, evidencing that the AER is responsible for proximal-distal (p–d) limb outgrowth and its activity keeps the subjacent mesoderm in an undifferentiated state [8,9]. Several members of the FGF family, *fgf2*, *fgf4*, and *fgf8* are expressed in the chick AER and rescue limb outgrowth and patterning following ridge removal [10–12].

FGFs are diffusible proteins whose signals are mediated by a group of four transmembrane proteins with intrinsic tyrosine kinase activity, known as FGF receptors (FGFRs) [13,14]. FGFRs are expressed in a variety of tissues and organs throughout vertebrate development with a distinct expression pattern for each receptor. FGFR1, 2, and 3 exist in two alternative splice variants: IIIb and IIIc. FGF4 and FGF8 are produced by the limb epithelium AER

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and preferentially bind to the IIIc isoforms [15], which are expressed in the mesenchyme [16–18].

In this work, we show that FGF8 protein produced in the AER induces the expression of its downstream effector *mkp3* in the distal limb mesenchyme. *mkp3* presents an expression gradient in the limb mesenchyme and we show that this gradient results from the decay of mRNA transcribed by the distal limb bud progenitors.

Materials and methods

Eggs and embryos. Fertilised chick (*Gallus gallus*) eggs obtained from commercial sources were incubated at 37.2 °C in a 49% humidified atmosphere and staged according to the Hamburger and Hamilton (HH) classification [19].

RNA probes. Antisense digoxigenin-labelled RNA probes *mkp3* [7] was prepared as previously described.

An intronic *mkp3 in situ* hybridisation probe was generated. Reverse transcription and polymerase chain reactions (RT-PCR) were used to isolate the fourth chick *mkp3* intron, using the sense oligo 5′-AGCA GAACCCCCATTCCCTTCT-3′ and the antisense oligo 5′-CCAG CATGGAAATCCTGCA-3′. The DNA fragment generated was cloned into the pCR®II-TOPO® vector (Invitrogen) and plasmid DNA was isolated. Antisense *mkp3* intronic RNA probe was produced using standard procedures.

RNA extraction and reverse transcription. Limb buds at stage HH24 were dissected into three portions of equivalent size along the p–d axis. Total mRNA was extracted from 5 distinct batches of each portion of the limb using the RNeasy Mini Kit Protect (Qiagen, Germany). Total mRNA quantification was done by spectrophotometry (NanoDrop Technologies, Inc., USA). Total RNA was digested with DNAse RNAse-Free (Promega, USA) according to manufacturer's instructions.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR was performed as previously described [20]. Primer design was based on available sequences in GenBank (NCBI-NLM-PubMed- Gene). The following intron-spanning primers were used, sense and antisense, respectively: MKP3 5′-ATCTCTTTCATAGATGAAGCGCGG-3′ and 5′-TGAGCTTCTGCATGAGGTAGGC-3′; FGF8 5′-CTGATCGGCAA GAGTAACGGC-3′ and 5′-CCATGTACCAGCCCTCGTACTTG-3′; RALDH2 5′-CAAGGAGGAGATTTTTGGGCCTGTTC-3′ and 5′-GGCCTTGTTTATGTCATTTGTAAAGACAGC-3′; the primers used for the reference gene 18S Ribosomal RNA were: 5′-TGCTCTTAACT GAGTGTCCCGC-3′ and 5′-CCCGTTTCCGAAAACCAACAA-3′. Primer sets standard amplification curves were made for MKP3, FGF8, RALDH2 and 18S Ribosomal RNA with cDNA samples from chick limb buds at stage HH24, setting $r = 0.99$. In all the samples, gene expression was normalized for 18S Ribosomal RNA and for limb region 1 values.

Statistical analysis. All quantitative data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA on ranks and the Student–Newman–Keuls test was used for post-test analysis. Statistical significance was set at $p < 0.05$.

Whole-mount in situ hybridization. Embryos and limb buds were collected from stage HH24 and fixed overnight at 4 °C in fresh 4% formaldehyde 2 mM EGTA in phosphate-buffered saline (PBS), rinsed in PBT (PBS, 0.1% Tween 20), dehydrated through a methanol series and stored in 100% methanol at –20 °C. Whole-mount *in situ* hybridization was performed according to the previously described procedure [21].

Immunohistochemistry (IHC). Forelimb buds were collected from stage HH25 chick embryos and immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) with 0.12 mM $CaCl_2$ and 4% sucrose overnight at 4 °C, washed in buffer only, and then in PB with 15% sucrose, both overnight at 4 °C. Embryos were then incubated in PB with 15% sucrose and 7.5% gelatin for 1 h at 30 °C, immediately frozen in liquid nitrogen chilled isopentane, and stored at −80 °C until sectioned. Approximately 10-μm-thick serial sections were collected on Super Frost slides, and blocked for 30 min with 10% normal goat serum in PBS containing 1% bovine serum albumin. The primary anti-FGF8 antibody (KM1334 from BioWa Inc.; [22,23]) was diluted at 1:500 in the blocking solution and incubated overnight at 4 °C, followed by washing in PBS, and detected with Alexa Fluor 488-conjugated anti-rabbit IgG, all F(ab′)2 fragments (Molecular Probes) diluted 1:1000 in blocking solution, followed by PBS washes. Slides were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma), mounted in Vectashield (Vector Laboratories) and sealed after cover-slipping.

Bead implants. A window was cut in fertilised eggs and the vitelline membrane was removed. Heparin acrylic beads (Sigma) soaked in FGF8 protein at 1 mg/ml (R&D Systems) or PBS were microsurgically implanted in the proximal limit of the zone of *mkp3* expression in the right limb buds of stage HH24 embryos. The embryos ($n = 12$) were incubated for 4 h and hybridized with *mkp3* probe. The same experiment was performed using AG1-X2 ion exchange beads (Bio-Rad) incubated in 4 mg/ml SU5402 (a drug known to specifically block the kinase activity of FGF receptors) (Calbiochem) or in DMSO ($n = 6$).

Imaging. Embryos processed for *in situ* hybridization were photographed in PBT/0.1% azide, using a Sony DXC-390P-3CCD colour video camera coupled to a Leica MZFLIII microscope. Embryos processed for IHC were photographed using an Olympus DP70 camera coupled to an Olympus BX61 microscope with epifluorescence. Images were treated in Adobe Photoshop CS.

Results and discussion

We performed *mkp3 in situ* hybridization studies in chick limb buds and observed that *mkp3* shows a graded distribution of its transcripts throughout the limb mesenchyme (Fig. 1). *mkp3* expression is stronger in the most distal part of the limb (underneath the AER), fading out in the direction of the more proximal part (Fig. 1A). Real-time polymerase chain reaction (real-time RT-PCR) was performed to characterise the observed *mkp3* mRNA gradient in the limb bud. Limb buds at stage HH24 were dissected into three portions of equivalent size along the p–d axis (Fig. 1A). Total mRNA was extracted from multiple batches of each portion of the limb ($n = 5$) and *mkp3* mRNA content variation in the three regions was analysed by real-time RT-PCR. This high-resolution analysis clearly demonstrates a gradient of *mkp3* transcripts in the limb bud, in a distal to proximal direction (Fig. 1B).

Real-time RT-PCR experiments showed that *fgf8* is strongly expressed in the distal tip of the limb bud, and no transcripts can be significantly measured in the more proximal limb regions (Fig. 1B). We could also observe an opposing gradient of *raldh2* expression, which presents higher transcript levels in the proximal limb region (Fig. 1C). An antagonism between FGF and RA signalling pathways is thought to operate during the development of several structures (see e.g. [24,25]). Namely, during somitogenesis, FGF8 and RA are mutually inhibitory [26]. In fact, it has been shown that FGFs promote limb distalization by counteracting the RA pathway. This happens through inhibition of the proximalizing RA signal required to maintain the Meis activity, providing a molecular basis for the role of RA in specifying proximal limb fates in different vertebrates [27].

To further characterise limb bud *mkp3* expression gradient, we constructed an intronic *in situ* hybridisation probe

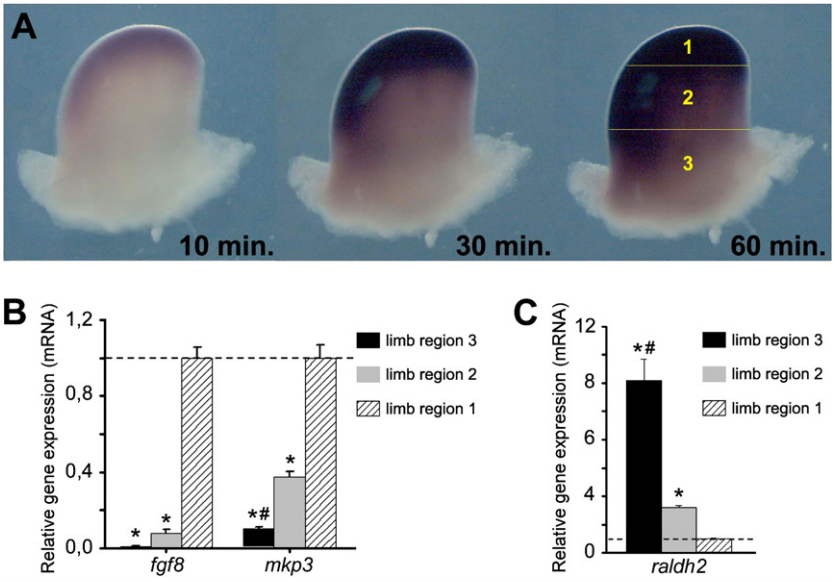

Fig. 1. Characterization of the *mkp3* mRNA gradient in the chick limb bud. (A) Visualization of the *mkp3* mRNA gradient in stage HH24 chick wing bud mesenchyme. The wing bud was photographed after increasing staining reaction times: 10, 30, and 60 min. The three regions of wing bud collected for real-time RT-PCR are represented in the wing bud with 60 min of staining. (B) *mkp3* and *fgf8* mRNA levels in the different limb regions analyzed obtained by real-time RT-PCR, expressed in arbitrary units normalized for 18S Ribosomal RNA and for limb region 1 values ($n = 5$ for each region); $p < 0.001$: ∗ *vs.* limb region 1, # *vs.* limb region 2. (C) *raldh2* mRNA levels in the different limb regions analyzed obtained by real-time RT-PCR, expressed in arbitrary units normalized for 18S Ribosomal RNA and for limb region 1 values ($n = 5$ for each region); $p < 0.05$: ∗ *vs.* limb region 1, # *vs.* limb region 2.

for *mkp3* (see Materials and methods) that allows the detection of *mkp3* nascent transcripts. Whereas *mkp3* expression detected by the exonic probe spans through a wide area of the limb mesenchyme (Fig. 2A), cells recognized by the intronic probe were restricted to the most distal part of the limb, even after a long exposure time to developing solution (Fig. 2B). These observations suggest that the *mkp3* mRNA gradient seen in the limb mesenchyme may not result from a gradient of active transcription, but from the progressive decay of the mRNA molecules produced by the distal limb progenitors.

Kawakami et al. (2003) proposed that *mkp3* is able to mediate cellular response to FGF8 signalling in the vertebrate limb by observing that FGF8-soaked beads can rescue the loss of *mkp3* expression resulting from AER removal [7]. As has been previously described, *fgf8* mRNA is expressed in the AER [28]. Moreover, we detected strong immunoreactivity for the FGF8 protein in the AER (Fig. 3A and B). Thus, FGF8 produced at the AER is most likely capable of inducing *mkp3* expression in the closely located distal limb mesenchymal cells. To further address this issue, we placed FGF8-soaked beads in stage HH24 chick embryo right wings at the proximal end of the *mkp3* expression domain. After 4 h of incubation, we observed ectopic *mkp3* expression extending proximally (Fig. 4Aii). In order to validate our hypothesis, this experiment was repeated using SU5402-soaked beads, a drug

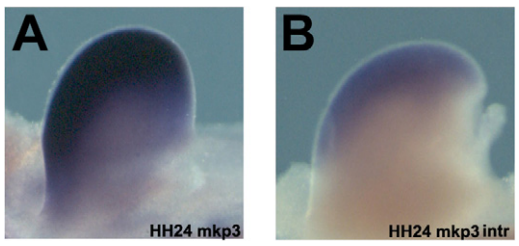

Fig. 2. Expression patterns obtained using *mkp3* exonic *versus* *mkp3* intronic probes. (A) *mkp3* expression is detected throughout the limb mesenchyme by the exonic probe at stage HH24. (B) In the same stage, *mkp3* expression recognized by the intronic probe was restricted to the distal part of the limb. *In situ* hybridization was performed for both probes at the same time, ensuring the same conditions, including the same time of labelling.

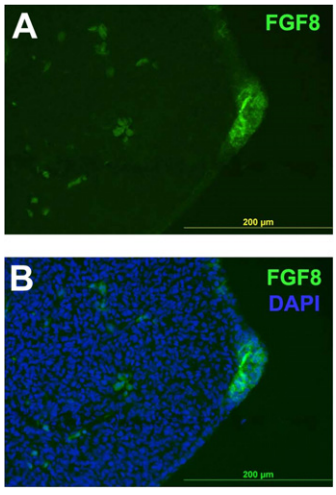

Fig. 3. FGF8 protein is expressed in the AER. (A) Longitudinal section of stage HH25 forelimb bud confirms the production of FGF8 protein (green) by the AER. (B) Same section as (A) counterstained with DAPI.

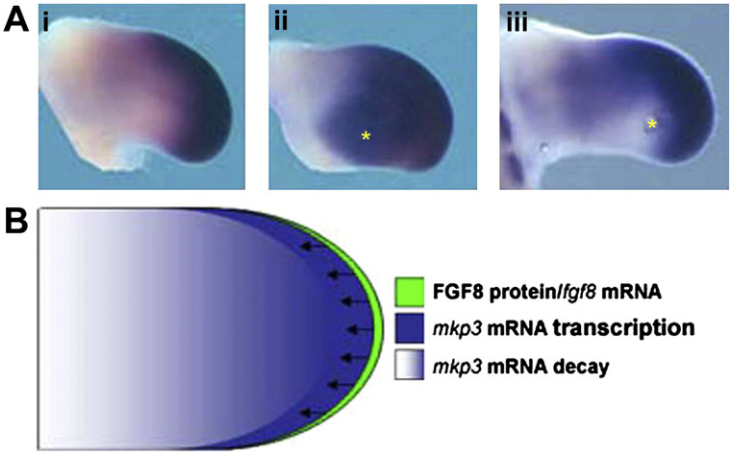

Fig. 4. *mkp3* is unregulated by FGF8. (A) *mkp3* expression pattern in the distal limb mesenchyme of stage HH24 embryo. (i) control (ii) 4 h after incubation with an FGF8-soaked bead located at the proximal limit of *mkp3* expression; (iii) 4 h after incubation with SU5402-soaked bead. (B) Model proposed for *mkp3* expression pattern in the chick limb bud (see text for details).

known to specifically block the kinase activity of FGF receptors. As expected, SU5402 caused downregulation of *mkp3* expression (Fig. 4Aiii).

The results obtained throughout this work lead us to propose the following model for *mkp3* expression pattern in the chick limb bud (schematized in Fig. 4B): FGF8 protein produced in the AER induces *mkp3* gene transcription in the cells located immediately underneath, in the distal limb mesenchyme. As cell division and limb outgrowth proceeds, these cells are progressively displaced away from the FGF8 source. At a certain distance from the AER, *mkp3* transcription is no longer induced, but *mkp3* mRNA molecules are still present in these cells and decay over time. This results in a spatial gradient of mRNA expression in the chick limb bud.

Our description of the *mkp3* expression gradient in the chick limb bud is reminiscent to the *fgf8* mRNA gradient present in the chick presomitic mesoderm. In fact, Dubrulle and Pourquié have shown a mechanism based on progressive RNA decay involved in the autonomous establishment of the caudal *fgf8* mRNA gradient [29]. Our work suggests for the first time that a similar process can be operating during limb bud development.

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