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Etv1 and Ewsr1 cooperatively regulate limb mesenchymal *Fgf*10 expression in response to apical ectodermal ridge-derived fibroblast growth factor signal

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ABSTRACT

Fibroblast growth factors (FGFs) expressed in the apical ectodermal ridge (AER) and FGF10 expressed in the underlying mesoderm are essential for limb bud outgrowth. Their expression is maintained through a positive feedback loop. We identified the *cis*-regulatory element and *trans*-acting factors involved in the AER-FGF-dependent transactivation of *Fgf*10. Etv1 and Ewsr1 stimulated transcription from the *Fgf*10 promoter in the sub-AER mesenchyme of mouse and chick limb buds in a conserved AGAAAR cluster-dependent manner. We found that both *Etv*1 and *Ewsr*1 were necessary for *Fgf*10 expression and elongation of the limb bud. In addition, Etv1 and AER-FGF synergistically stimulated *Fgf*10 promoter activity in an Ewsr1-dependent manner. We also found that Etv1 and Ewsr1 bound to the segment of DNA containing the AGAAAR cluster *in vivo* and *in vitro*. Moreover, Etv1 directly bound to the AGAAAR sequence *in vitro*. Our results suggest that Etv1 and Ewsr1 transactivate *Fgf*10 directly and cooperatively in response to AER-FGFs.

Introduction

During organogenesis, biased tissue growth directed by local epithelial-mesenchymal interactions is important for morphogenesis (Goldin, 1980). Systems consisting of diffusible signaling molecules and their receptors play major roles in these epithelial-mesenchymal interactions. FGFs and their receptors (FGFRs) are known to be involved in epithelial-mesenchymal interactions in various organ primordia (Pownall and Isaacs, 2010). In appendage primordia and in some primordia of the digestive organ, urinary organ and masticatory organ, different FGFs are expressed in the epithelium and mesenchyme, and bidirectional signaling by these different FGFs is important for tissue growth and the elongation of organ primordia (Ohuchi et al., 1997; Xu et al., 1998; Al Alam et al., 2012; Trueb et al., 2013; Thesleff and Mikkora, 2002). In the limb bud, an appendage primordium, FGF8, 4, 9, and 17 are expressed in the AER (Sun et al., 2000), and FGF10 is expressed in the underlying mesenchyme (Ohuchi et al., 1997). FGF10 is necessary for *Fgf*8 expression in the AER (Ohuchi et al., 1997), and AER-FGFs are required for the maintenance of mesenchymal *Fgf*10 expression (Ohuchi et al., 1997; Boulet et al., 2004; Sun et al., 2002). This regulatory loop formed by AER-FGFs and FGF10 is essential for limb bud elongation (Sekine et al., 1999; Mariani et al., 2008). In this regulatory loop, FGF10 is transmitted by FGFR2b in the AER and activates *Wnt*3 expression (Danopoulos et al., 2013). Wnt3 signaling, in turn, activates the AER-specific transcription factor Sp8, which directly activates *Fgf*8 transcription (Kawakami et al., 2004: Sahara et al., 2007). On the other hand, AER-FGFs are transmitted by FGFR2c in the limb mesenchyme and activate the Mek–Erk and PI3K pathways (Kawakami et al., 2003; Eblaghie et al., 2003; Smith et al., 2006; Mariani et al., 2008; Sheeba et al., 2012). However, how AER-FGF signaling leads to *Fgf*10 activation in the limb mesenchyme remains an open question.

In the present study, we sought to determine the AER-FGF signaling-dependent molecular mechanism that activates sub-AER *Fgf*10 transcription. For this purpose, we first identified a *cis*-regulatory element necessary for sub-AER *Fgf*10 promoter activity and the *trans*-acting factors that stimulate the *Fgf*10 promoter. One of these *trans*-acting factors is Etv1, an Ets transcription factor and a known target of the Mek–Erk pathway (Charlot et al., 2010; Janknecht, 1996; Abe et al., 2012). Another is the transcriptional co-activator Ewsr1 (Park et al., 2013; Lee et al., 2005; Araya et al., 2003). These *trans*-acting factors were found to activate the *Fgf*10 promoter *in vitro* cooperatively with AER-FGF and to be necessary

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for *Fgf*10 expression in the limb bud. Furthermore, we found that these *trans*-acting factors bound to the *Fgf*10 promoter region and directly regulated *Fgf*10 transcription.

Material and methods

DNA constructs and cDNA cloning

A murine genomic DNA fragment containing 5.9 kb upstream of the *Fgf*10 transcription start site and 0.7 kb of 5′ UTR (*Mm*6.6kb) was a kind gift from Dr. Shigeaki Kato. Using this fragment as a PCR template, a 768 bp promoter region fragment (*Mm*768bp) and partial deletion fragments (*Mm*768bpΔGGAA, *Mm*768bpΔAGAAAR, and *Mm*768bpΔAGAAAR/ΔGGAA) were generated (Fig. 1A). A chicken genomic DNA fragment containing 5.1 kb upstream of the *Fgf*10 transcription start site and 0.8 kb of 5′ UTR (*Gg*5.9kb) was amplified by PCR. Two 5′ truncated fragments (*Gg*1.3kb and *Gg*1.0kb) and a fragment deleting the AGAAAR cluster (*Gg*5.9kbΔAGAAAR) were isolated by restriction enzyme digestion (Fig. 1J). These fragments were inserted into the SDK-*LacZ* plasmid (a kind gift from Dr. Hiroshi Sasaki) or the pGL2-basic plasmid for *LacZ* and *Luciferase* reporter gene expression, respectively. Mouse and chicken *Ewsr*1 cDNAs were amplified by RT-PCR using the primers shown in Supplementary Table 1 and RNA prepared from an E11.5 mouse embryo and St. 21–23 chick embryos, respectively. Chick embryos were staged according to Hamburger and Hamilton (1951). pCMV-SPORT6-*Etv*1 and pCMV-SPORT6-*Etv*5 were purchased from Open Biosystems (EMM1002-3796055, CloneID3257346; EMM1002-5800626, CloneID5324125; and EMM1002-6822456, CloneID6510019). pCAGGS-*Etv*1 and pCAGGS-*Ewsr*1 were constructed using full-length coding DNA fragments of murine *Etv*1 and *Ewsr*1, respectively. pCS2-ca*Mek* was a kind gift from Dr. Hiroshi Hanafusa. pMiw-*Sp*1 has been described previously (Suzuki et al., 2003).

Transgenic (Tg) mice

Fertilized eggs were obtained by the intercross of the F1 progeny of C57BL/6 and C3H/He or the outcross of these F1 mice with C57BL/6. After DNA injection, transgenic F0 embryos were implanted into pseudo-pregnant ICR recipients and harvested at E10.75. Transgene insertion into the genome was confirmed by PCR.

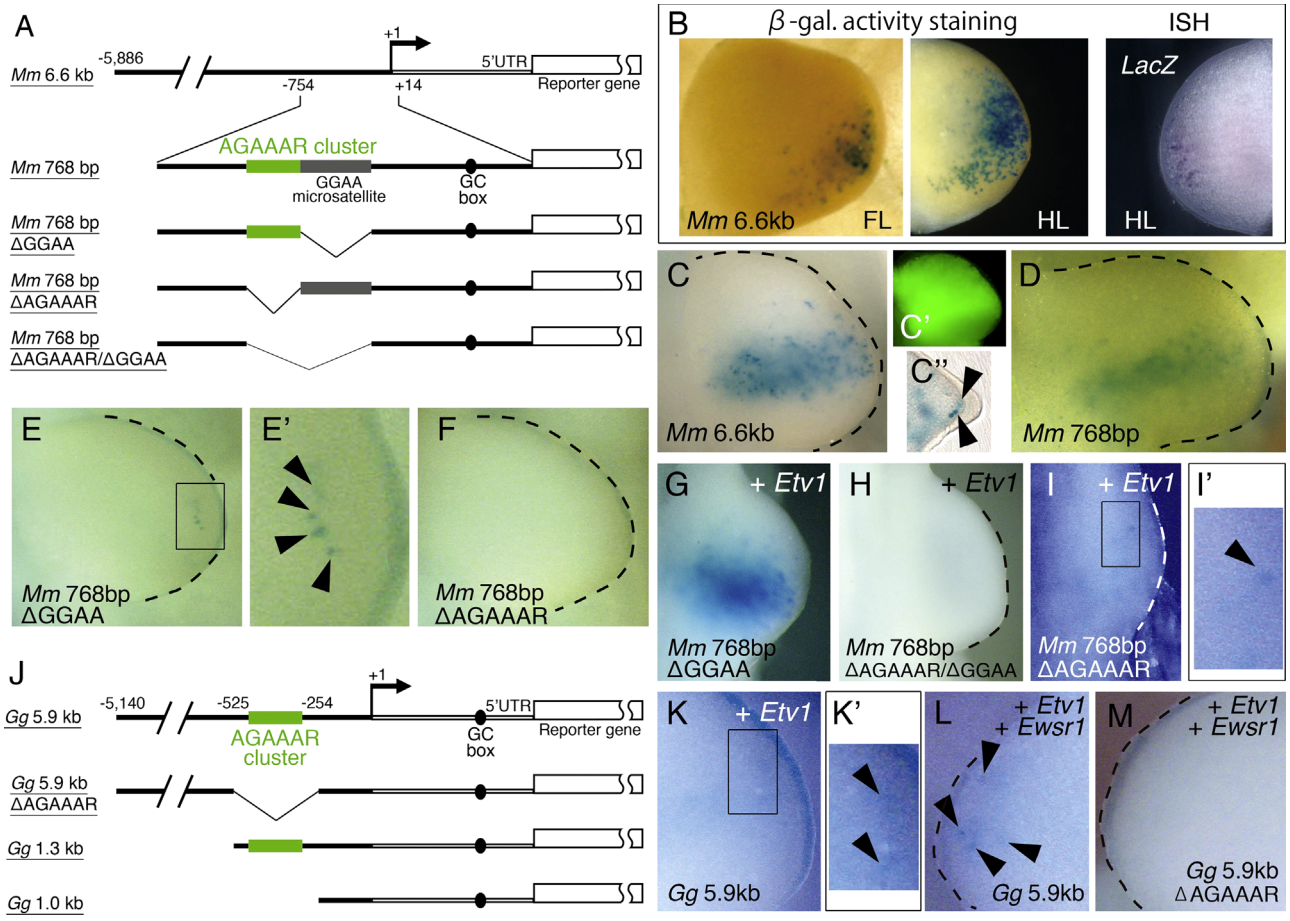

Fig. 1. In the sub-AER mesenchyme, *Etv*1 and *Ewsr*1 stimulate *Fgf*10 promoter activity in an AGAAAR cluster-dependent manner. (A) Schematics of the mouse *Fgf*10 reporter constructs. AGAAAR cluster, green box; GGAA microsatellite, gray box; GC box, black oval. 5′ UTR: 5' untranslated region. (B) Distribution of beta-galactosidase activity and *LacZ* mRNA in forelimb (FL) and hindlimb buds (HL) of *Mm*6.6kb-*LacZ* Tg mice at E10.75. (C–E) Beta-galactosidase activity in chicken limb buds transfected with *Mm*6.6kb-, *Mm*768bp- or *Mm*768bpΔGGAA-*LacZ* reporter. (C′) EGFP fluorescence in the limb bud in C for visualization of introduction area. (C″) Sagittal section of the limb bud in C. Arrowheads indicate the signal in the sub-AER mesenchyme. (E′) High magnification version of the image in E. Arrowheads indicate the signal. (F) *Mm*768bpΔAGAAAR-*LacZ* reporter showed no beta-galactosidase activity ($n=0/21$). Result of binomical test showed a statistically sufficient difference between *Mm*768bp-*LacZ* ($n=2/11$) and *Mm*768bpΔAGAAAR-*LacZ* reporters ($p<0.05$). (G–I) Beta-galactosidase activity in chicken forelimb buds into which pCAGGS-*Etv*1 was co-transfected with *Mm*768bpΔGGAA-, *Mm*768bpΔAGAAAR/ΔGGAA-, or *Mm*768bpΔAGAAAR-*LacZ* reporter. (I′) High magnification version of the image in I. Arrowhead indicates the signal. (J) Schematics of the chicken *Fgf*10 reporter constructs. (K) Beta-galactosidase activity in the chicken forelimb bud co-transfected with pCAGGS-*Etv*1 and *Gg*5.9kb-*LacZ* reporter. (K′) High magnification version of K. Arrowheads indicate the signal. (L, M) Beta-galactosidase activity in chicken forelimb buds into which pCAGGS-*Etv*1 and pCAGGS-*Ewsr*1 were co-transfected with *Gg*5.9kb- or *Gg*5.9kbΔAGAAAR-*LacZ* reporter. Arrowheads indicate the signal. Anterior is at the top in B, C, C′, D–I′ and K-M. Dorsal is at the top in C″.

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