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Shh regulates chick *Ebf1* gene expression in somite development

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ABSTRACT

The chick early B-cell factor 1 (*cEbf1*) is a member of EBF family of helix loop helix transcription factors. Recently, we have proved that *cEbf1* expression in feather is regulated by Shh. It is therefore possible that the somitic expression of *cEbf1* is controlled by *Shh* signals from the notochord. To assess this hypothesis, the expression profile of *cEbf1* was first detailed in somites of chick embryos (from HH8 to HH28). *cEbf1* expression was mainly localised in the medial sclerotome and later around the vertebral cartilage anlagen of body and pedicles. Tissue manipulations (notochord ablation) and Shh gain and loss of function experiments were then performed to analyse whether the notochord and/or Shh regulate *cEbf1* expression. Results from these experiments confirmed our hypothesis that the medial somitic expression of *cEbf1* is regulated by Shh from the notochord. In conclusion, *cEbf1* gene is considered as a medial sclerotome marker, downstream to and regulated by the notochord derived Shh, which may be functionally involved in somitogenesis.

1. Introduction

The chick *cEbf1* is a member of a novel highly conserved EBF family of atypical HLH transcription factors (Dubois and Vincent, 2001; Liberg et al., 2002). *Ebf1* was originally discovered in rodents as regulators of B-lymphocyte and olfactory neuron differentiation (Hagman et al., 1993; Wang and Reed, 1993). Since then, the expression pattern and role of *Ebf1* have been studied extensively in the immune (Fields et al., 2008; Lukin et al., 2008) and nervous tissues (Davis and Reed, 1996; Garel et al., 1997). These studies have demonstrated the importance of this molecule for specification, differentiation and cell movements during development of these tissues.

Somites are segmented, paired blocks of mesodermal cells originated from the cranial end of the presomitic mesoderm in an anterior to posterior direction. According to Takahashi (2005), this cranial end is called somite 0 (S0) and its separation border (between S0 and the remaining crPSM) is called the segmenter. Somite segmentation has a defined time course with each cycle producing a somite every 90 min in chick embryo (Goldbeter and Pourquie, 2008). The furthest posterior somite is the most newly formed somite, somite I (SI), the next cranial somite is SII, and so on (nomenclature according to, Pourquie and Tam, 2001). The immature somites (from SI to SIII) are spherical and composed of an outer columnar epithelial layer and a mesenchymal core in the centre, the somitocoele. In response to ventral signals (*Shh* and *Noggin*) from the notochord and floor plate of the neural tube, the ventro-medial portion of somite IV which is epithelial undergoes epithelial–mesenchymal transition (EMT) to form the sclerotome giving rise to the first mature somite. Once formed, the sclerotome is subdivided along the anterior posterior axis into anterior and posterior halves by von Ebner's (intra-somitic) fissure (Christ and Wilting, 1992). The anterior half is invaded by neural crest cells and their derivatives, dorsal root ganglia and nerve axons (Bronner-Fraser and Stern, 1991). The sclerotome is the primordium for the entire vertebral column and also gives rise to the proximal ribs (Dietrich et al., 1997). Functionally, the sclerotome is subdivided into: medial (precursor of vertebral bodies and intervertebral discs), central (precursor of pedicle part of the neural arches, proximal ribs and transverse processes), dorsal (precursor of dorsal portion of the neural arches and the spinous processes) and lateral (precursor of distal ribs) domains (Christ et al., 2004, 2007; Monsoro-Burq and Le Douarin, 2000).

Recently, *Ebf2* was shown to be important during the late stage of skeletogenesis in mice (Kieslinger et al., 2005, 2010), however, to date there is scarce available data on the expression of *Ebf* genes during the early stages of skeletogenesis, particularly during somite formation and differentiation. Some members of vertebrate *Ebfs* were expressed in different domains of somites. For example, chick *cEbf2,3* (El-Magd et al., 2013) and mouse *mEbf1-3* genes (Garel et al., 1997; Kieslinger et al., 2005) were expressed in the sclerotome. In *Xenopus*, *xEbf2* was

Abbreviations: Ci, cubitus interruptus; *Col*, collier; crPSM, cranial presomitic mesoderm; DRG, dorsal root ganglia; Ebf1, early b-cell factor 1; EMT, epithelial–mesenchymal transition; HBC, hydroxypropyl-β-cyclodextrin; HLH, helix loop helix; NT, neural tube; PSM, presomitic mesoderm; SI-X, somite 1–10; Shh, sonic hedgehog.

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expressed in cells located at the lateral portion of somites (Dubois et al., 1998).

The majority of the notochord function has been linked to the signalling molecule *Shh* which is expressed in the notochord at the time of sclerotome formation and controls the expression of sclerotomal markers such as, *Pax1*, *Pax9* and *Bapx1*/*Nkx3.2* (Brand-Saberi et al., 1993; Christ et al., 2004; Dockter and Ordahl, 2000). *Shh* can also promote axial chondrogenesis (Britto et al., 2000), since its inhibition results in absence of vertebral column (Chiang et al., 1996). Likewise, an ectopic grafting of the notochord in the PSM leads to a conversion of the entire somite into cartilage (Murtaugh et al., 1999). In addition, *Shh* is essential for proliferation and survival of the somitic cells and their precursors (Fan et al., 1995).

Studies in *Drosophila* have established that high levels of Hh (Shh ortholog) controls anterior–posterior patterning of the middle region of the wing through up-regulation of the *cEbf1* ortholog, collier (*Col*) (Vervoort et al., 1999; Mohler et al., 2000). Gain and loss of function experiments have determined the position of *Col* in the *Hh* signalling cascade downstream of cubitus interruptus (Ci, a key transducer for Hh signalling) (Vervoort et al., 1999; Mohler et al., 2000). Another study has confirmed that *Ci* can directly bind to, and activate, *Col* gene expression (Hersh and Carroll, 2005). This means that *Col* is a direct specific target for *Ci* during wing development. Interestingly, vertebrate *Ebfs* are also expressed in tissues patterned by Shh, such as somite, limb bud, and feather (El-Magd et al., 2013, 2014b; Kieslinger et al., 2005; Mella et al., 2004). Recently, we have proved that *cEbf1* expression in feather is regulated by Shh (El-Magd et al., 2014b). It is possible that the somitic expression of *Ebf1* in chick is controlled by *Shh* signals from the notochord. Therefore, this study was conducted to assess this hypothesis.

2. Materials and methods

2.1. Embryo preparation

Fertile hens' eggs (White Leghorn; supplied by Henry Stewart & Co Ltd) were incubated at 38 °C, 80% relative humidity to give embryos at stages HH8–HH28 (Hamburger and Hamilton, 1951). One hundred and twenty eight chick embryos were used in this study.

2.2. Embryo manipulations

For technical difficulty, notochord removal was performed on in vitro cultured embryo. The embryo culture was prepared as described by (Chapman et al., 2001). The embryo was first turned upside down (ventrally) and the endoderm above the area of operation was removed then a drop of 0.05% pancreatin (Gibco-BRL) in calcium free PBS was applied for 1 min. The notochord at the cranial presomitic mesoderm (crPSM) was excised, while the complete neural tube was left in situ, and the embryo was then turned dorsally. Unless stated otherwise, all operations were performed at HH11-12.

2.3. Application of cyclopamine

The embryos were treated in agar/albumin culture plate containing cyclopamine/2-hydroxypropyl-β-cyclodextrin (HBC) mixture. These plates were prepared by adding 100 μl 2.5 mM cyclopamine/HBC mixture to 38 ml agar/albumin mixture as described by Kolpak et al. (2005).

2.4. Application of beads

Affigel beads (100–150 μm, Bio-rad) were washed in PBS and then incubated in 2 μl of 0.5, 1 and 2 μg/μl SHH (Sigma) for 1 h at 37 °C. A small slit was made in the crPSM and then one to three beads were picked up with fine curved forceps and forced into the slit. For Shh rescue experiments, the notochord was removed, as described above, and the bead(s) were then inserted at the ablated site.

2.5. Cloning of cEbf1

Chick *Ebf1* was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using primers based on highly conserved regions in HLH and DBD of mouse *mEbf1* as described by Garcia-Dominguez et al. (2003). The forward primer was 5′AGAAGGTTATCCCCCGGCAC3′ and the reverse was 5′ CATGGGGGGAACAATCATGC 3′. Total RNA was isolated from 3 day old whole chick embryos using easy-RED™ following the manufacturer's protocol (iNtRON Biotechnology, #17063, Korea). The concentration and purity of the extracted RNA were determined using Nanodrop (UV–Vis spectrophotometer Q5000, Quawell, USA). The RT-PCR was carried out as previously described by us (El-Magd et al., 2014a). PCR was performed with the following cycling parameters: 95 °C for 5 min for initial denaturation, followed by 40 cycles with 94 °C for 30 s, annealing temperature 60 °C for 1 min, 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR products were analysed by 1% gel electrophoresis, and products of the correct size (696 bp) purified and ligated into pGEM-T Easy vector. Cloning procedure was as described by the manufacturer (Promega). Plasmid DNA was linearised using Nco1 restriction enzymes.

2.6. Whole mount in situ hybridisation

Harvested embryos were washed in phosphate buffered saline and fixed in 4% paraformaldehyde, overnight at 4 °C. Whole-mount in situ hybridization using DIG-labelled RNA probes was performed as described previously (Nieto et al., 1996). *cEbf1* 696 bp probe was prepared as previously described by us (El-Magd et al., 2013). Embryos were photographed using a Nikon E990 digital camera mounted on a Nikon dissecting microscope with both side and underneath illumination. For cryo-sectioning, embryos were frozen in tissue embedding medium (Jung) and sectioned at 30 μm for HH8-22 and at 15–20 μm for HH 28 embryos. After hydro-mounting and drying overnight, sections were photographed using a Leica DMRA2 microscope and DC300 camera system.

3. Results

3.1. Expression of cEbf1 in somitic mesoderm

At stage HH8, whole mount in situ hybridization revealed a strong *cEbf1* expression in the crPSM and the newly formed somites (SI), very weak expression in the second somites (SII), and weak expression in the most cranial two somites (SIII-IV) (Fig. 1A, n = 7). Transverse section at the level of SI showed this expression in the ventromedial epithelial somites (i.e. the sclerotome precursor area) and the adjacent somitocoele leaving the lateral and dorsal domains negative (Fig. 1E). Interestingly, *cEbf1* showed an asymmetrical expression in crPSM and SI, whereby the left crPSM/SI stained intensely (Fig. 1A, E; n = 7 embryos: 6 embryos showed asymmetrical expression and 1 embryo showed symmetrical expression). By HH11, *cEbf1* continued to be expressed in the crPSM, especially at the boundary of the prospective area of the somites that located at the separation border (also known as, segmenter) (Fig. 1B, n = 7). The differential expression of *cEbf1* was maintained during this stage, with stronger and broader expression in old mature (more cranial) somites and moderate medial expression in the two newly formed immature somites (SI and SII), and very weak throughout the remaining somites. However, no asymmetrical expression was found during this stage. At stage HH12, *cEbf1* expression remained in the crPSM with robust labelling at the segmenter and became obvious in all somites with stronger expression in the caudal halves and at the intersomitic boundaries (Fig. 1C, n = 7). The asymmetrical expression