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Identification and analysis of a novel *bmp4* enhancer in Fugu genome

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ABSTRACT

Spatiotemporal expression of bone morphogenetic protein 4 (Bmp4) in epithelial and mesenchymal cells is critical for the development of many organs including teeth. Since *Bmp4* has a complex and widespread regulatory area in mammals, the tissue-specific enhancers that are responsible for mesenchymal expression of Bmp4 are difficult to identify in mammals. *TakiFugu rubripes* (Fugu, pufferfish) has a highly compact genome size and is widely used in comparative genomics studies of gene regulatory mechanisms. In this study, we used the Fugu genome to evaluate the 15 kb promoter region upstream of the Fugu *bmp4* gene. By DNA segmental cloning and luciferase assay with two dental odontoblast-like cell lines, a dental ameloblast-like cell line, and a kidney fibroblast cell line, we identified a 485 bp *cis*-regulatory enhancer between −4213 and −3728 bp of the Fugu *bmp4* gene. This enhancer showed strong transcriptional activity in all three dental cell lines and, to a lesser extent, also in kidney fibroblast cells. Though not located in an evolutionary conserved region, the enhancer activity for the DNA segment is intense. This is the first time a *bmp4* enhancer sequence with activity in both mesenchymal and epithelial cells has been identified, which will help to decode the mechanism of tooth development in vertebrates.

1. Introduction

Bone morphogenetic protein 4 (Bmp4) is a member of the Tgfβ family of signaling factors and displays a dynamic spatiotemporal expression pattern throughout embryogenesis. Its expression is required for the development and patterning of skeletal structures, kidneys, urogenital system, eyes, teeth, palate, lung, heart, and other organs.[1,2] During tooth development Bmp4 plays an essential role and is specifically expressed in either odontogenic epithelium or in mesenchyme depending on the developmental stage. Prior to epithelial thickening, antagonistic signaling of Bmp4 and Fgf8 in the mandiblular ectoderm determines the position and type of prospective tooth buds.[3,4] As the epithelial placode thickens, *Bmp4* expression shifts from epithelium to mesenchyme, concurrently with the tooth inducing potential.[5] Subsequently, the formation of enamel organs, induction of enamel knots as well as differentiation of ameloblasts and odontoblasts also depend on Bmp signaling.[6–9] At a later stage, Bmp4 is expressed during root formation, and functional studies have implicated its role in the regulation of this process.[6,10] The mechanism that

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directs this complex spatiotemporal expression of *Bmp4* has not been fully understood. Previous studies attempting to assay dental specific *cis*-regulatory elements of *Bmp4 in vivo* have not succeeded.[11] The major reason for this is that *Bmp4*, like other developmentally regulated genes, has a complex and widespread *cis*-regulatory landscape.[12] No other genes are found within a 1100 kb range of the *Bmp4* gene in human and mouse genomes. A recent study using transgenic mice with two overlapping 227 kb mouse bacterial artificial chromosome (BAC) reporter constructs confirmed that dental specific regulatory elements of *Bmp4* reside between −28 kb and −199 kb upstream to the *Bmp4* transcription start site.[13] These results are consistent with the previous finding that the −2.4 kb proximal *Bmp4* promoter failed to drive expression in dental epithelium or mesenchyme during early stages.[11] There are three evolutionary conserved regions (ECRs) in a stretch of 400 kb of the *Bmp4* gene. By genome comparison, the position of the ECRs can be precisely located in the *Bmp4* promoters of human, mouse, chicken, zebrafish and pufferfish. These comparisons also show that the pufferfish (Fugu) genome including the *Bmp4* gene is small and compact, around one tenth the size of human and mouse genomes.[13]

These findings inspired us to take a closer look at the *Bmp4* promoter area during tooth development in Fugu. As teeth are ancient vertebrate structures, it would not be surprising if the developmental and regulatory processes were also evolutionary conserved. Fugu has teeth in both the jaws and the pharyngeal cavity.[14] In its oral cavity, Fugu has unusual beak-like jaws that contains one large tooth plate in each quadrant. Although the Fugu tooth structure is quite unique histologically, these teeth have similar dentin and enamel layers compared to other vertebrates. Furthermore, the developmental process of Fugu pharyngeal odontogenesis is very close to mammalian tooth formation beginning with the interaction between epithelium and the adjacent mesenchyme.[14] *Bmp4*, as well as *pax9*, *pitx2* and *shh*, have conserved expression patterns in dental epithelium and/or mesenchyme during Fugu pharyngeal and oral tooth development.[15] All these facts should make it possible to use the much more compact Fugu genome to study dental specific elements in the *bmp4* promoter.

In this study, we evaluated the 15 kb promoter region upstream of the Fugu *bmp4* gene with a luciferase reporter system. By analyzing three dental cell lines and a kidney fibroblast cell line, we identified *cis*-regulatory elements in the Fugu *bmp4* promoter, which may involve in tooth development.

2. Materials and methods

2.1. Cell culture and transfection

Preodontoblast cells (kindly provided by Dr. A. Poliard, France[16]), odontoblast-like cells (MDPC-23[17]), ameloblast-like cells (LS-8, kindly provided by Dr. ML Snead, CA, USA[18]), and kidney fibroblast cells (COS7, ATCC® CRL-1651™) were maintained in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) with L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum (Invitrogen, CA, USA). Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA) was used for plasmid DNA transfections according to the manufacturer's protocols.

2.2. Plasmid construction

3–5 kb long DNA fragments from the 5′-proximal region of the *bmp4* gene were amplified by polymerase chain reaction (PCR) using Fugu genomic DNA (Source BioScience Plc, Nottingham, UK). The primers and restriction enzymes used for cloning are listed in Suppl. 1. We used the Finnzymes Phusion High-fidelity PCR kit (New England Biolabs, MA, USA) for amplification. The PCR cycling conditions were 98 °C 2 min initial denaturation followed by 30 cycles of 98 °C for 10 s, 64–66 °C for 30–60 s and 72 °C for 2 min 30 s, and 72 °C 10 min final extension. The pGL4.26 luciferase reporter vector (Promega, WI, USA) was selected for cloning since this vector contains a minimal promoter for enhancer analysis. The truncated *bmp4* promoter constructs were generated by either restriction enzyme digestion or PCR amplification from the established constructs (see Suppl. 1). All constructs were confirmed by multiple restriction enzyme digestions and both ends were sequenced to verify the correct insertion sequence.

2.3. Luciferase assay and β-galactosidase assay

Luciferase reporter constructs and the pCMV-SPORT β-Gal plasmid (as an internal control for transfection efficiency) (Invitrogen, CA, USA) were co-transfected into cells in a 12-well plate. For each well, 300 ng of promoter–reporter construct and 200 ng of pCMV-SPORT β-Gal were used. Transfection was performed in duplicate and repeated three times. Twenty-four hours after transfection, cell extracts were harvested with 1× reporter lysis buffer and assayed by the luciferase assay system (E4030 Promega, WI, USA) and a β-gal assay kit (E2000 Promega, WI, USA) according to manufactory's instruction respectively.

2.4. Transcription factor binding sites prediction

We use ConSite,[19] http://www.phylofoot.org/consite, to search for potential transcription factor binding sites for the enhancer sequence. The sequence was analyzed from vertebrates subgroup with a TF score cutoff of 80%.

2.5. Statistical analysis

The results were presented as the mean values and standard deviations.

3. Results

3.1. Establishing the promoter–reporter constructs for the Fugu bmp4 gene

To analyze the Fugu *bmp4* promoter, we designed four pairs of primers to amplify approximately 4–5 kb long DNA fragments of the 15 kb region upstream of the *bmp4* transcription start site (Fig. 1). The two evolutional conserved regions ECR1 and