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Involvement of the T-box transcription factor Brachyury in early-stage embryonic mouse salivary gland

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ABSTRACT

The mouse submandibular gland (SMG) is important organ for embryonic development, and branching morphogenesis is regulated by many molecules containing transcription factors. Real-time reverse transcriptase polymerase chain reaction revealed that the expression of *Brachyury* increased in the SMG and peaked between E12.5–E13.5, concomitant with the early stage of branching morphogenesis. The expression of Brachyury in SMG rudiments between E12.5–E13.5 was confirmed by western blotting. In addition, fibronectin and Btbd7 (regulated by fibronectin), which are both essential for cleft formation, were expressed strongly during the same period. The *Sox2* and *Wnt3a*, which regulate cell growth, were also expressed strongly during E12.5–E13.5. On the other hand, cleft formation and branching morphogenesis was suppressed by knockdown of *Brachyury* gene, suggesting that Brachyury plays a central role in regulating cell growth and cleft formation in early-stage embryonic mouse salivary gland development.

1. Introduction

Salivary gland disorders, which can result from Sjögren's syndrome, radiation therapy for head and neck cancer, and aging [1], lead to xerostomia [2,3]. Regenerative medicine has become increasingly important in recent years, and the regeneration of various organs, including skin and cartilage, has been attempted; however, the use of regenerative medicine in salivary glands has not been studied sufficiently. Therefore, novel therapies that can restore salivary gland function are needed.

Branching morphogenesis, which is defined as the growth and branching of epithelial buds [4], is a fundamental embryologic process in many developing organs including SG, lungs, kidneys, pancreas, and mammary glands [4–9]. Many studies have demonstrated that organogenesis requires epithelial-mesenchymal tissue interactions, which are mediated by growth factors [10], cytokines, and the extracellular matrix [11–20]. Murine submandibular gland (SMG) organogenesis begins at embryonic day 11.5–12 (E11.5–E12) as an outgrowth of the oral floor epithelium, which extends into the underlying mandibular mesenchyme [21].

Earlier, it was reported that the T-box transcription factor Brachyury is required for mesoderm formation during early development [21]. During vertebrate development, Brachyury regulates downstream signaling pathways such as transforming growth factor (TGF) and Wnt. In mature tissues, Brachyury also regulates the epithelial-mesenchymal transition. In addition, it may play a role in cancer stem cells, which are important during the invasion and metastasis of cancer cells [22].

In this study, we examined the role of Brachyury during mouse SG development. Our results suggest that Brachyury plays a central role in regulating cell growth and cleft formation in early-stage embryonic mouse SG development.

Abbreviations: ES cell, embryonic stem cell; iPS cell, induced pluripotent cell; SG, salivary glands; SMG, submandibular gland; TGF, transforming growth factor.

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2. Materials and Methods

2.1. Reagents

The following rabbit polyclonal antibodies were used: mouse Brachyury (Santa Cruz Biotechnology, Dallas, TX, USA), mouse Btbd7 (Novus, Oakville, ON, Canada), mouse E-cadherin and fibronectin (Millipore, Billerica, MA, USA), mouse Sox2 (Cell Signaling Technology, Danvers, MA, USA), mouse Wnt3a (Bioss, Woburn, MA, USA), and mouse β-actin (Sigma Aldrich, St Louis, MO, USA).

2.2. Tissue preparation and organ culture

Tissue preparation and organ culture were performed as described previously [10]. Pregnant time-mated ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The plug day was considered to be the day of gestation initiation. Pregnant mice were anesthetized using isoflurane and sacrificed by cervical dislocation on E12.5–E16.5. The embryos were washed to remove blood and dissected in cold phosphate buffered saline (PBS). The submandibular rudiments and peripheral tissues were isolated using fine needles under a stereomicroscope. Submandibular rudiments obtained from E12.5–E15.5 embryos were used for organ cultures. They were placed on cell culture inserts (0.4 μm pore size: Becton Dickinson, Franklin Lakes, NJ, USA), which were then placed in the individual wells of a 24-well tissue culture dish (Becton Dickinson) that contained 200 μL of culture medium. Culture consisted of BGjb (Gibco-BRL, MD, USA) that was supplemented with 100 μg/mL of ascorbic acid (Wako Pure Chemical, Osaka, Japan), 100 U/mL of penicillin G potassium (Banyu Pharmaceutical Co., Ltd. Tokyo), and 100 μg/mL of streptomycin (Meiji Seika, Tokyo). For organ cultures, isolated submandibular rudiments were incubated on cell culture inserts at 37 °C in a humidified atmosphere of 5% CO_2, and the culture media were changed at 2-day intervals. The Animal Care and Use Committee of Kyushu University approved all experiments.

2.3. Total RNA preparation and real-time RT-PCR

Total RNA was extracted from E12.5–E16.5 submandibular rudiments using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 3 μg of total RNA using Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) and random hexanucleotide primers. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification using oligonucleotide primers that were specific for the constitutively expressed mouse gene *β-actin*, and normalized. All PCRs were performed using the Light Cycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). The following sets of primers were used: Brachyury forward 5′-ATA ACG CCA GCC CAC CTA-3′, reverse 5′-GGG AGC CTC GAA AGA ACT-3′; and β-actin forward 5′-TGG GAC GAC ATG GAG AAA-3′; reverse 5′-AGC ACA GCC TGG ATA GCA-3′. Amplification was performed using the following conditions: denaturation at 95 °C for 10 min, followed by 46 cycles of annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. Dissociation curve analyses confirmed that the signals corresponded to unique amplicons. Expression levels were normalized to β-actin for each sample obtained from parallel assays and analyzed using the LightCycler 2.0 System software package (Roche Applied Science, Indianapolis, IN, USA).

2.4. Western blotting

Isolated submandibular rudiments or organ culture rudiments were analyzed by western blotting. Samples were rinsed with PBS, and then lysed by sonication in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 6% mercaptoethanol) containing protease inhibitor cocktail (Sigma-Aldrich). The protein content of the lysates and fractionated samples was quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein from

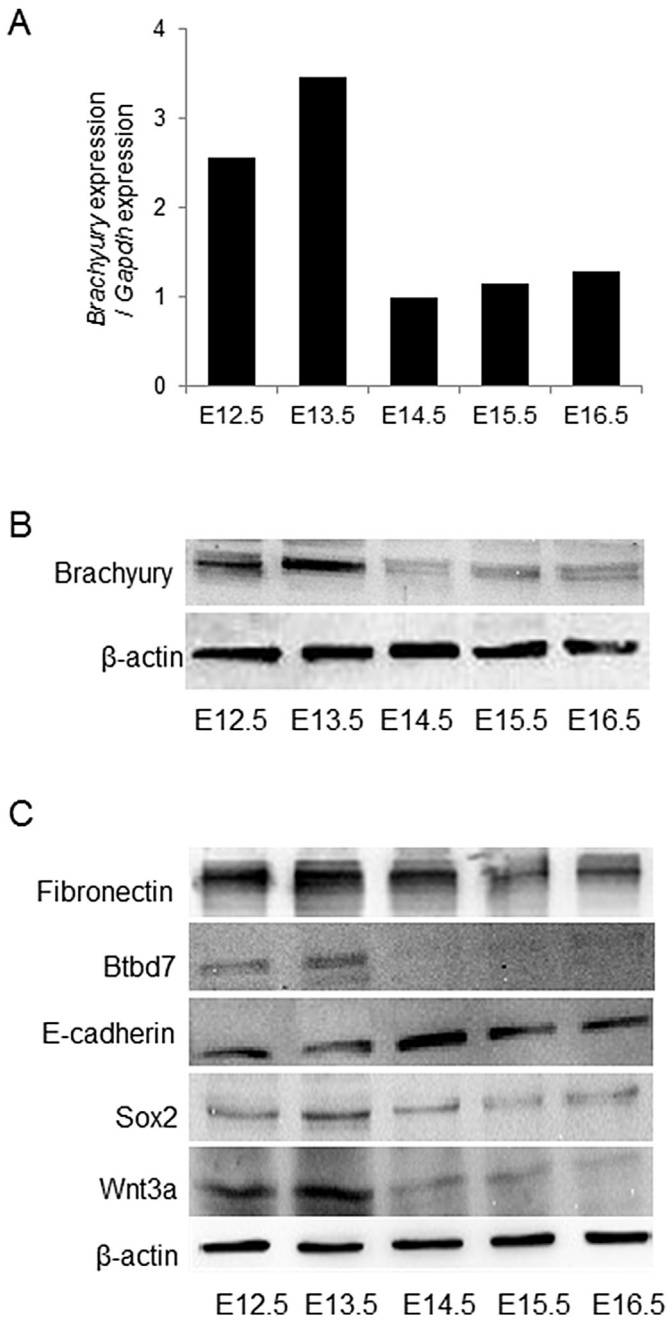

Fig. 1. Relationship between the Brachyury and fibronectin, E-cadherin, Btbd7, Sox2, and Wnt3a in early-stage embryonic mouse salivary gland A: Total mRNA was isolated from the submandibular gland rudiments at the indicated embryonic days. Relative mRNA levels of Brachyury are shown. **B**: Equal amounts of protein from the total lysates of submandibular gland rudiments at the indicated embryonic days were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting using anti-Brachyury antibodies. **C**: Equal amounts of protein from the total lysates of submandibular gland rudiments isolated at the indicated embryonic days were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-Btbd7, Sox2, Wnt3a, fibronectin, E-cadherin, and β-actin antibodies.