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TGFβ3 is expressed in differentiating muscle of the embryonic mouse tongue

AKIRA YAMANE*,[1], TADAYOSHI FUKUI[2], TAKEO SUGA[3], RYOHEI IIDA[3] and MITSUHIKO MORITO[3]

Departments of [1]Biophysics, [2]Orthodontics and [3]Geriatric Dentistry, Tsurumi University School of Dental Medicine, Yokohama, Japan

ABSTRACT The purpose of the present study was to elucidate the involvement of transforming growth factor betas (TGFβs) in the differentiation of tongue striated muscles by analyzing the expression of TGFβs, their receptors and factors of TGFβ signal transduction in the mouse tongue between embryonic days 11 (E11) and E15. The expression levels of *TGFβ3* mRNA and protein were much higher than those of *TGFβ1* and *TGFβ2* , and the immunolocalization of TGFβ3 was more consistent with the differentiating muscle cells in comparison with those of TGFβ1 and 2 between E12 and E15. TGFβRI and II were localized to the differentiating muscle cells between E11 and E15. Phosphorylated-smad2/3 was localized to the nucleus of muscle cells which just began to differentiate. These results suggest that the signal of TGFβ3, but not that of β1 or β2, may be involved in the early stages (particularly the beginning) of differentiation of mouse tongue muscle cells through TGFβRI, TGFβRII, and smad2/3.

KEY WORDS: *TGFβ, TGFβR, smad2/3, tongue, muscle differentiation*

Introduction

Tongue striated muscles are involved in several important physiological tasks such as mastication, swallowing, respiration, and human speech, and their development has been studied extensively. Tongue muscle progenitor cells undergo commitment in the occipital somites (somites 2 ~5) between embryonic days (E) 8 and E11 (Mayo *et al.*, 1992). They migrate to the first branchial arch between E9 and E11, and become myoblasts (Mayo *et al.*, 1992) before fusing to form myotubes between E13 and E15 (Yamane, 2005; Yamane *et al.*, 2000a). These myotubes then mature to fast-twitch myofibers between E15 and birth (Yamane, 2005; Yamane *et al.*, 2000a).

We previously investigated the roles of growth factors such as the transforming growth factors α (TGFα), insulin-like growth factor (IGF), and hepatocyte growth factor (HGF) by using an organ culture system of mouse tongue or mandible (Yamane *et al.*, 2003). The roles of IGF-I, HGF, and TGFα in tongue myogenesis are almost identical to those in the myogenesis of limb and cultured myogenic cell lines, such as C2 and L6, suggesting that the program that governs tongue myogenesis is similar to the programs for limb and cultured myogenic cell lines. However, there are several reports suggesting that the program actually differs from the limb and cultured cell lines; they indicate that the myogenesis and synaptogenesis of mouse tongue are completed at much earlier stages than those of limb muscles (Yamane, 2005) and that the expression profiles of miRNAs in the developing tongue striated muscle, which were recently reported to play important roles in myogenesis, are different from those in limb and cultured myogenic cell lines (Yamane and Fukui, 2007).

The mammalian transforming growth factor-β (TGFβ) family consists of three different proteins, TGFβ1, TGFβ2, and TGFβ3, which play essential roles in the development of many kinds of tissues and cells (Dunker and Krieglstein, 2000; Nilsen-Hamilton, 1990). The three individual TGFβ null-mutants show distinct and only partially overlapping phenotypes. In mice, targeted disruption of the TGFβ1 gene results in diffuse and lethal inflammation about 3 weeks after birth, suggesting a prominent role of TGFβ1 in the regulation of immune cell proliferation and extravasation into tissues (Kulkarni *et al.*, 1993; Shull *et al.*, 1992). TGFβ2 null mice exhibit a broad range of developmental defects, including cardiac, lung, craniofacial, limb, eye, ear and urogenital defects (Sanford *et al.*, 1997). TGFβ3 gene null-mutation results exclusively in defective palatogenesis and delayed pulmonary development (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995). However, in these TGFβ null mice, the development of tongue striated

Abbreviations used in this paper: TGF, transforming growth factor; TGFβR, TGFβ receptor.

***Address correspondence to:** Dr. Akira Yamane. Departments of Biophysics, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama, 230-8501 Japan. e-mail: yamane-a@tsurumi-u.ac.jp

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muscle have not been extensively analyzed using immunohistochemistry, PCR and Western blotting.

In the development of skeletal muscles, the function of TGFβs are mainly studied using cultured myogenic cells, such as C2C12 (De Angelis *et al.*, 1998; Florini *et al.*, 1986; Olson *et al.*, 1986), L6 (Matsushita *et al.*, 2004), and cultured organ (Stern *et al.*, 1997), and there are few *in vivo* studies in the literature (Cusella-De Angelis *et al.*, 1994; Kollias and McDermott, 2008). The *in vivo* studies indicate that TGFβ1 inhibits the differentiation of skeletal muscle cells, suggesting that TGFβs play a role in the development of tongue striated muscles. However, to date, there are no reports focused on the expression and roles of TGFβs in the development of tongue striated muscles. The purpose of the present study was to elucidate the involvement of TGFβs in the *in vivo* development of tongue striated muscles by analyzing the expression of TGFβs, their receptors, and factors of TGFβ signal transduction.

Results

The expression levels of TGFβs, their receptors and a member of the signal transduction pathway in the mouse embryonic tongue

We analyzed the expression levels of TGFβs, TGFβRs, smad2/3, and p-smad2/3 in developing tongue of E11, E13, and E15 mice (Fig. 1). Fig. 1A shows a representative pattern of the Western blot analysis by antibodies against TGFβ3, TGFβR1, and TGFβRII. The expression level of TGFβRI protein in the E15 tongues was 37% ($p<0.01$) and 32% ($p<0.05$) less than those in the E11 and E13 tongues, respectively, whereas no significant difference in the expression levels of TGFβ3 and TGFβRII proteins was found among the E11, 13, and 15 tongues. Since TGFβ1 and 2 proteins were not detectable by Western blot analysis, we analyzed the mRNA expression of TGFβs by RT-PCR analysis (Fig. 3C). The expression levels of TGFβ1 and 2 mRNAs were much less than that of TGFβ3 (Fig. 1C). The expression level of TGFβ3 mRNA in the E15 tongues was 60% and 63% less than those in the E11 and E13 tongues, repectively, ($p<0.05$)(Fig. 1C). The expression level of p-smad2/3 in the E15 tongues was 64% less than that in the E11 tongues ($p<0.05$), whereas the expression of smad2/3 was nearly constant between E11 and E15 (Fig. 1D).

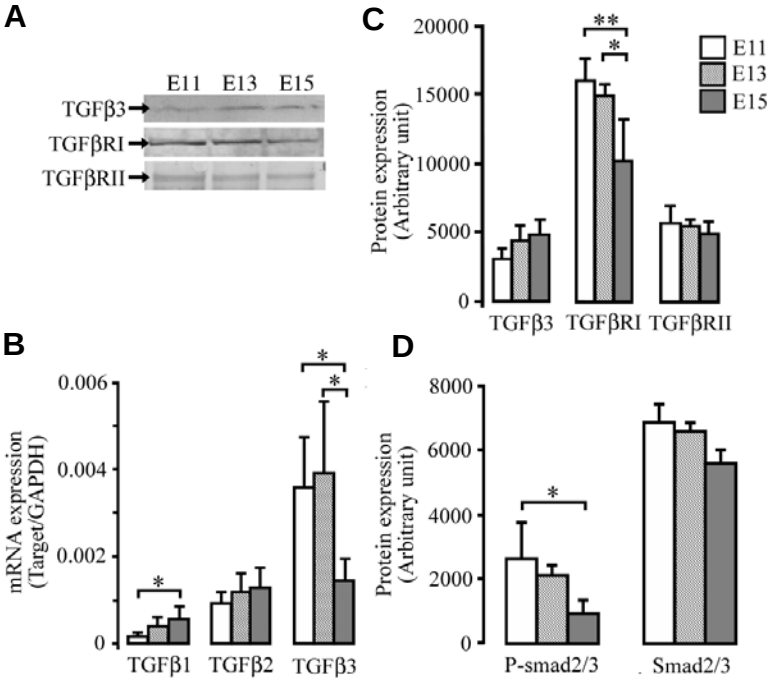


Fig. 1. TGFβs and their receptors in E11, E13, and E15 tongues. (A) *Representative Western blotting patterns of TGFβ3, TGFβRI and TGFβRII.* **(B)** *Quantification of corresponding protein expression levels of TGFβ3, TGFβRI, and TGFβRII in E11, E13, and E15 tongues.* **(C)** *The mRNA expression levels of TGFβ1, TGFβ2, and TGFβ3 in E11, E13, and E15 tongues as analyzed by competitive RT-PCR.* **(D)** *The protein expression levels of p-smad2/3 and smad2/3 in E11, E13, and E15 tongues as analyzed by Western blotting. Significant differences between the two groups, *p < 0.05, **P<0.01. Each column and vertical bar represent the mean + 1 SD of five samples.*

Immunolocalization of TGFβs in the differentiating muscle tissues of embryonic mouse tongue

To determine whether TGFβs play a role in the differentiation process of tongue muscle cells, we analyzed the relationship between cells that express TGFβs and the differentiating muscle cells identified by immunolocalization of fast myosin heavy chain (fMyHC) between E13 and E15, which was the most active period for the differentiation of tongue muscle cells (Nagata and Yamane, 2004; Yamane, 2005; Yamane *et al.*, 2000a). TGFβ1 and TGFβ2 exhibited a similar immunostaining profile in developing tongues of E11~E15 mice; faint immunostainings for both TGFβ1 and 2 began to appear in the E12~E13 tongues and were observed in the differentiating skeletal muscle with intense staining for fMyHC and in the connective tissues with non-staining for fMyHC until E15 (data not shown).

TGFβ3 exhibited a distinct immunostaining pattern from those of TGFβ1 and 2. Intense immunostaining for TGFβ3 began to be observed at E12 in skeletal muscle cells which just began to express fMyHC, a marker of tongue muscle differentiation (Fig. 2A~F). As the differentiation continued at E13 (Fig. 2G~L) and E15 (Fig. 2M~K), immunostaining for TGFβ3 was observed in not only muscle tissues with intense immunostaining for fMyHC, but also in the tissues with no or weak immunostaining for fMyHC (probably undifferentiated muscle tissue or connective tissues).

Immunolocalization of TGFβRs in the differentiating muscle tissues of embryonic mouse tongue

TGFβ ligands initiate signaling by first directly binding TGFβRII, which forms a heteromeric receptor complex with TGFβRI (Derynck and Zhang, 2003; Kollias and McDermott, 2008; Massague and Chen, 2000). To better understand the involvement of TGFβs in the differentiation of tongue muscle cells, we analyzed the immunolocalization of TGFβRI (data not shown) and TGFβRII (Fig. 3) in the tongues of E12, E13, and E15 mouse embryos. TGFβRI and TGFβRII exhibited a similar immunostaining profile in developing tongues of E11~E15 mice; immunostainings for both TGFβRI and II were observed in the differentiating muscle tissues identified with intense

staining for fMyHC and in other tissues with no or weak stainings for fMyHC during all of the developmental periods studied.

Immunolocalization of p-smad2/3 and smad in the differentiating muscle tissues of embryonic mouse tongue

The signaling of TGFβs through TGFβRs phosphorylates smad2/3 which, in turn, enters into the nucleus and regulates the translation of the target genes (Derynck and Zhang, 2003; Kollias and McDermott, 2008; Massague and Chen, 2000). To determine whether TGFβ signaling plays a role in the differentiation of tongue myoblasts, we analyzed the immunolocalization of p-smad2/3 and smad2/3 in the tongues of E13 and E15 mouse embryos. In E12 tongue, immunostaining for p-smad2/3 was found in the nucleus (DAPI-positive and fMyHC-negative) (arrows in Fig. 4D~I) of the differentiating muscle cells (fMyHC-positive and DAPI-negative), whereas immunostaining for smad2/3 was found in the whole tongue tissues, with intense staining localized mostly in the cytoplasm of muscle cells that were fMyHC-positive and DAPI-negative (arrowheads in Fig. 4M~R). These results indicate that p-smad2/3 was located to the nucleus of the muscle cells which just began to differentiate and in which the TGFβ signal entered the nucleus, suggesting that TGFβ signal is involved in the beginning of the differentiation of tongue muscle cells.

Discussion

In the present study, the expression levels of TGFβ3 mRNA and protein were much higher than those of TGFβ1 and 2, and the immunolocalization of TGFβ3 was more consistent with the differentiating muscle cells of mouse tongue in comparison with TGFβ1 and 2 between E12 and E15. This higher expression and better consistency of localization of TGFβ3 in the differentiating tongue muscle cells suggest that TGFβ3, not TGFβ1 and 2, may be involved in the early stages of differentiation of tongue muscle cells. There is supportive evidence that the treatment of TGFβ3, not TGFβ1 and 2, inhibits the differentiation of L6 myogenic cells (Matsushita *et al*., 2004).

There are several reports that TGFβ inhibits the differentiation of skeletal muscle cells by repressing the myoD family, including myf5, myoD, myogenin, and MRF4, which are key regulators of the development of skeletal muscle (Kollias and McDermott, 2008). TGFβ inhibits the transcriptional activity of myogenin without affecting its DNA binding affinity (Brennan *et al*., 1991); TGFβ targets the basic helix-loop-helix (bHLH) region of all myoD family members, decreasing their DNA transcriptional activity without affecting their binding properties (Martin *et al*., 1992); and, in addition to inhibiting the transcriptional activity of the MyoD protein, TGFβ also inhibits the transcription of the MyoD gene, thus reducing both its levels and activity (Vaidya *et al*., 1989). Since the expression levels of myoD family members, except for

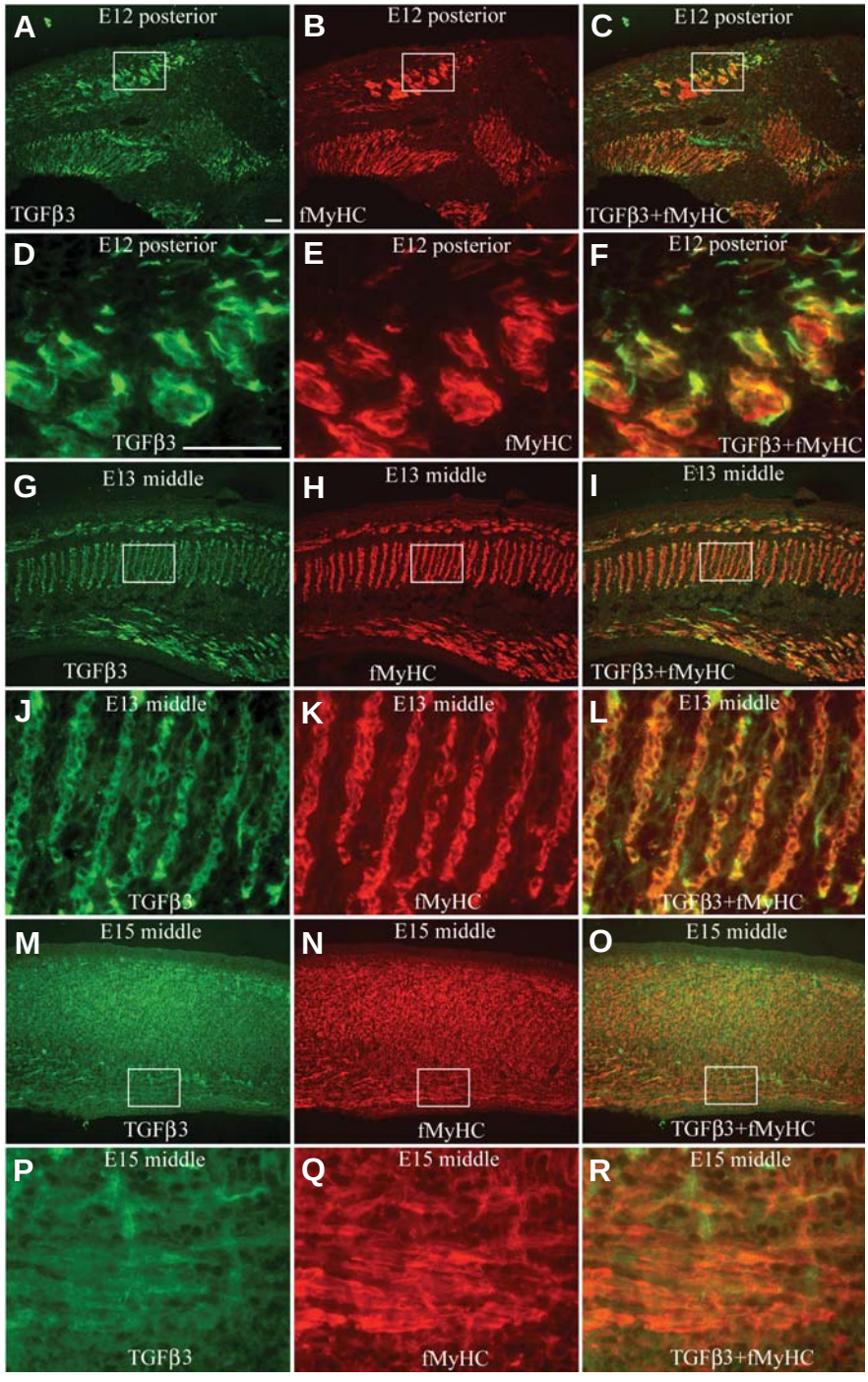
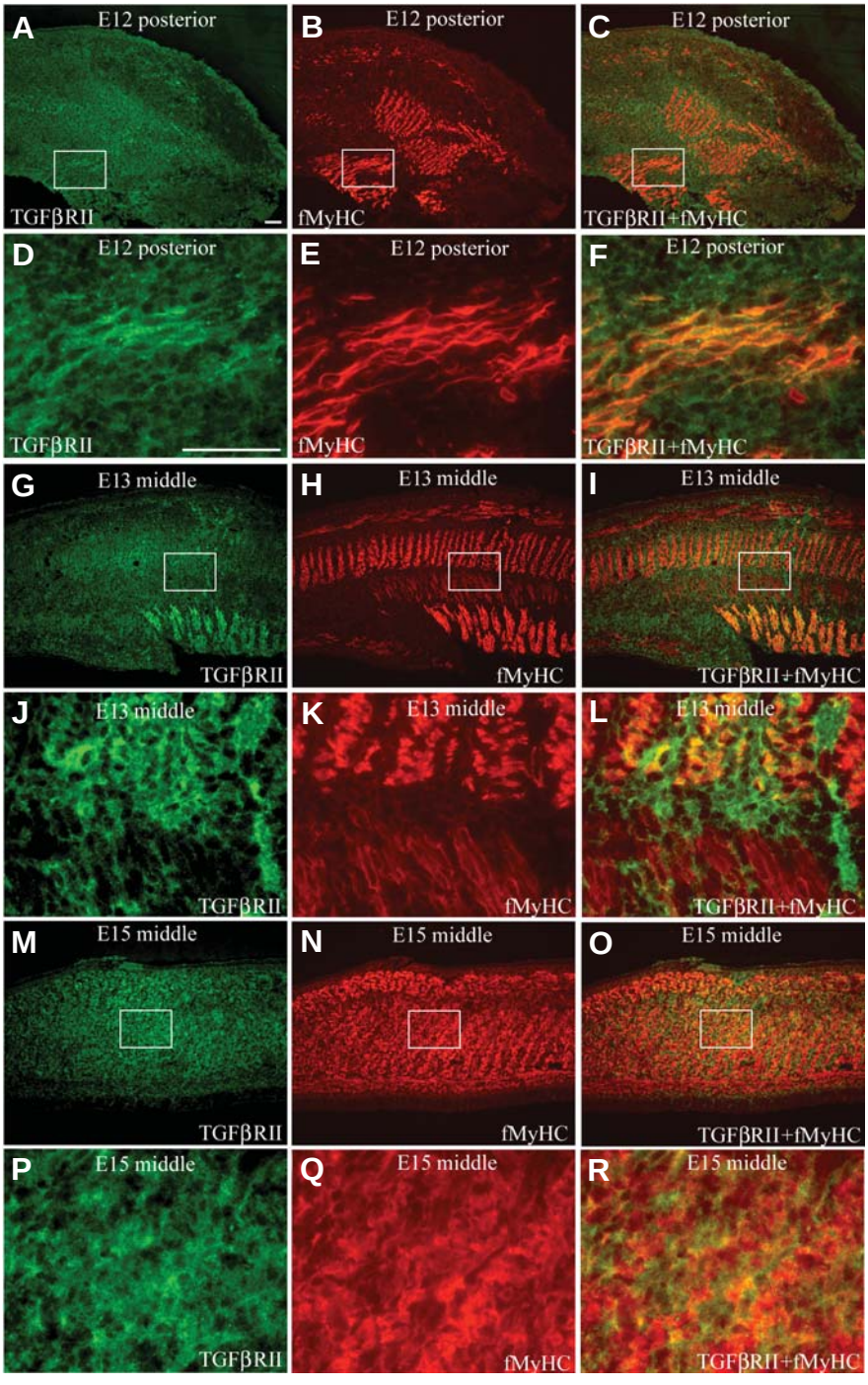


Fig. 2. Immunostaining for TGFβ3 (A,D,G,J,M,P) and fMyHC (B,E,H,K,N,Q), and merged images (C,F,I,L,O,R) *in the posterior portion of tongue at E12* **(A-F)** *the middle portion of tongue at E13* **(G-L)**, *and the middle portion of tongue at E15* **(M-R)**. *The areas of the rectangles in (A-C), (G-I), and (M-O) are enlarged to (D-F, (J-L), and (P-R), respectively. Scale bars in (A,D) are 30 μm. The same scale as (A) is used in (B,C,G-I, M-O). The same scale as (D) is used in (E, F, J-L, and P-R.*

MRF4, are highly maintained between E11 and E15, and since tongue muscle cells actively differentiate between E12 and E15 (Yamane, 2005), the TGFβ signal may be involved in controlling the progress of differentiation in tongue muscle cells within the physiological range and in preventing an excess differentiation by repressing the transcriptional activity of high expression levels of myf5, myoD, and myogenin.

The phosphorylating TGFβRI in the heteromeric receptor complex activates smad2/3 by carboxy-terminal phosphorylation of smad2/3. The p-smad2/3 is released from the receptor complex, translocate into the nucleus, and regulate the translation of target genes (Derynck and Zhang, 2003). In the present study, p-smad2/3 was localized to the nucleus of differentiating muscle cells in the mouse tongue at E12, whereas smad2/3 was localized to the cytoplasm of differentiating muscle cells. Since the differentiation of tongue muscle cells began at around E12 (Yamane, 2005; Yamane *et al.*, 2000a), the localization of p-smad2/3 to the nucleus may be involved in the beginning of differentiation of tongue muscle cells.

In the present study, in the differentiating tongue muscle cells, only TGFβ3 protein detectable by the Western blotting analysis and the analysis of RT-PCR demonstrated the higher expression levels of TGFβ3 mRNA in comparison with those of TGFβ1 and 2. These results of Western blotting and RT-PCR were consistent and imply that TGFβ3, not TGFβ1 and 2, is involved in the differentiation of tongue muscle cells. The present result of Western blotting analysis showed that the expression levels of TGFβRI and p-smad2/3 gradually decreased between E11 and E15. The present immunohistochemical analysis demonstrated that the differentiation of tongue muscle cells actively progresses from E11 and E15 and almost completed at around E15, which is consistent with our previous reports (Yamane, 2005; Yamane *et al.*, 2000b). These results imply that TGFβ3 signal is involved in the early stage (particularly beginning) of differentiation of tongue muscle cells at around E12.



Materials and Methods

Experimental animals

Pregnant ICR mice were purchased from Nippon Clea Co., Ltd. (Tokyo, Japan) and killed by cervical dislocation at embryonic days (E) 11, E12, E13, and E15. Embryos were isolated from uterine deciduas and were removed from their membranes under a dissection microscope. Tongues were removed for PCR and Western analyses, immediately frozen, and then stored at -80°C until use. Tongues for immunohistochemical analysis were immediately fixed in Bouin's solution or 4% paraformaldehyde. All experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine.

Western blot analysis

The tissues of tongue muscles were homogenized in 2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 10% glycerol. The homogenate was centrifuged at 8,000 rpm for 15

Fig. 3. Immunostaining images for TGFβRII (A,D,G,J,M,P) and fMyHC (B,E,H,K, N,Q), and merged images (C,F,I,L,O,R) *in the posterior portion of tongue at E12* **(A~F)**, *the middle portion of tongue at E13* **(G~L)**, *and the middle portion of tongue at E15* **(M~R).** *The areas of the rectangles in (A~C), (G~I) and (M~O) are enlarged to (D~F), (J~L), and (P~R), respectively. Scale bars in (A,D) are 30 μm. The same scale as (A) is used in (B,C,G~I, M~O). The same scale as (D) is used in (E,F,J~L,P~R).*

minutes and the supernatant was stored at -20∞C until use. The protein concentration of each supernatant was measured using the BCM protein assay (Pierce, Rockford, IL, USA). After β-mercaptoethanol was added to the supernatant (final concentration, 5%), the supernatant was heated at 100∞C for 5 minutes. The samples containing 10 μg total protein were subjected to a 5-20% gradient SDS-PAGE. After electrophoresis, the proteins were transferred onto a PVDF membrane (Hybond-P PVDF Membrane, Amersham Biosciences Corp., Piscataway, NJ, USA). The membranes were then treated with Casein solution (Vector Laboratories, Inc., Burlingame, CA, USA) for 3 hours at 25∞C, and incubated overnight at 4∞C with the rabbit polyclonal antibody against TGFβ1, TGFβ2, TGFβ3, p-smad2/3, or smad2/3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunoreactions were made visible using the Vectastain Elite ABC Kit and 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories, Inc., Burlingame, CA, USA) and images were acquired through a scanner (Epson, GT9700F, Tokyo, Japan) and loaded into a personal computer. The intensity in the bands was measured using a image analyzing software, Densitograph (ATTO Corp., Tokyo, Japan).

RNA extraction, reverse transcription, and competitive PCR amplification

Total RNA extraction, reverse transcription, and competitive PCR amplification were performed according to the manufacturer's specifications (Trizol, Life Technologies, Gaithersburg, MD, USA). The RNA was treated with 2 units of ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD, USA), and was then reverse transcribed with 200 units of reverse transcriptase (SuperScript II, Life Technologies, Gaithersburg, MD, USA).

The competitors were constructed according to the manufacturer's instructions (Competitive DNA Construction Kit, TaKaRa Biochemicals, Shiga, Japan) and amplified with 50 ng of the total cDNA, in the presence of a primer pair specific to the target genes, in a thermal cycler (TP3000, TaKaRa Biochemicals, Shiga, Japan). The amplification products were separated by electrophoresis on an agarose gel containing ethidium bromide. The fluorescent intensities of the bands of the target cDNAs and their respective competitors were measured by an image analyzer (Molecular Imager FX, Bio-Rad, Hercules, CA, USA). We then calculated the ratios of the fluorescent intensities of the target cDNA bands to those of their respective competitors. The logarithmic value of the fluorescent intensity ratio was used to calculate the amount of endogenous target mRNA based on the line formula derived from a standard curve for each target gene. The standard curve was generated as described previously (Ohnuki *et al.*, 2000; Yamane *et al.*, 2000a). The quantities of TGFβ mRNAs were normalized by the quantity of GAPDH mRNA. The sequences of primers for TGFβs and GAPDH are described elsewhere (Yoshida *et al.*, 2005).

Immunohistochemistry

Sagittal sections of tongues at E11, E12, E13, and E15 were prepared

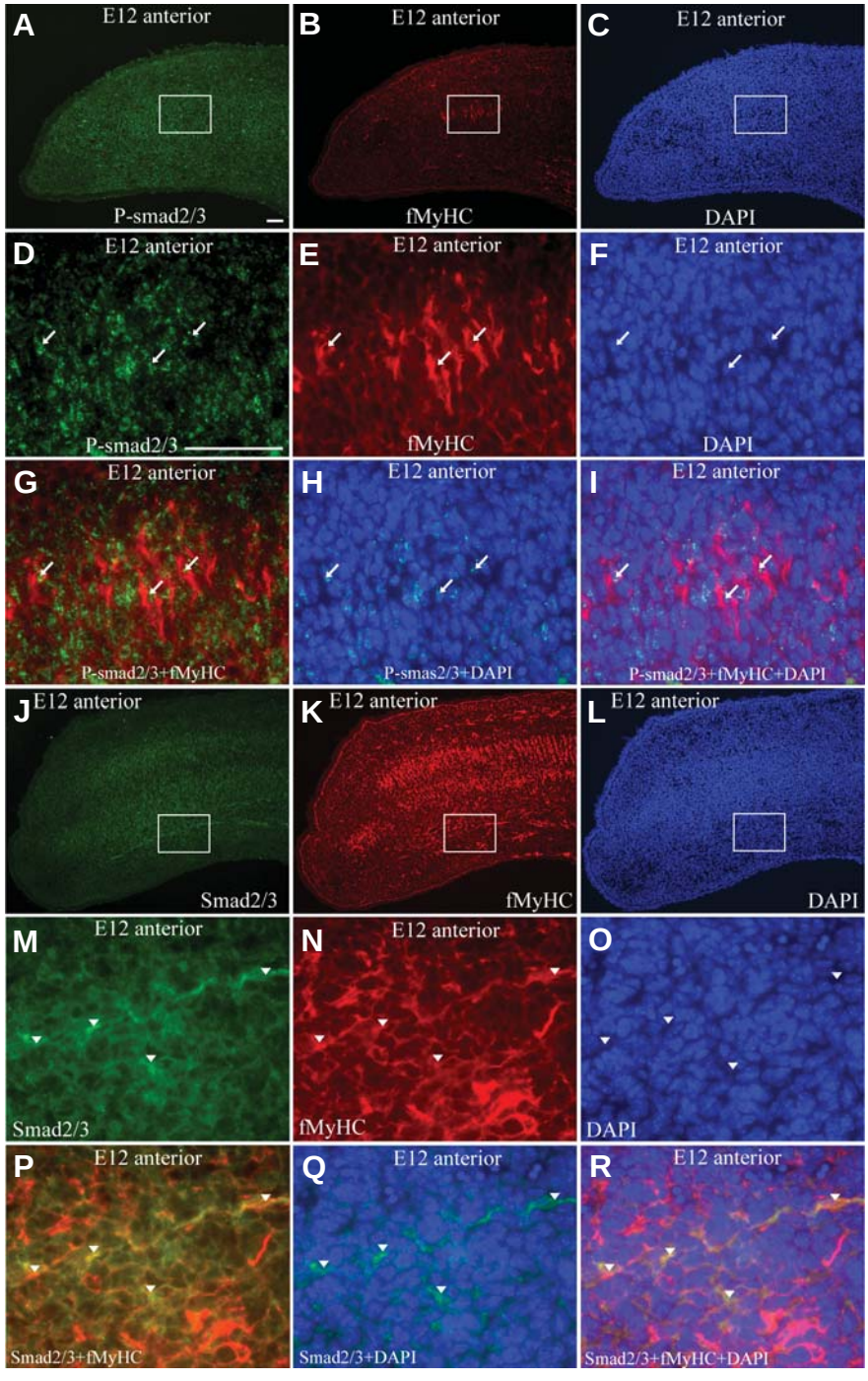


Fig. 4. Immunostaining images for p-smad2/3 (A,D), smad2/3 (J,M), and fMyHC (B,E,K,N); *DAPI staining image of DNA* **(C,F,L,O);** *and merged images for p-smad2/3 + fMyHC* **(G),** *p-smad2/3 + DAPI* **(H),** *p-smad2/3 + fMyHC + DAPI* **(I),** *smad2/3 + fMyHC* **(P),** *smad2/3 + DAPI* **(Q),** *and smad2/3 + fMyHC + DAPI* **(R)** *in the anterior portion of tongue at E12* **(A~R).** *The area of the rectangle in (A) is enlarged to (D,G), that of (B) to (E,H), that of (C) to (F,I), that of (J) to (M,P), that of (K) to (N,Q), and that of (L) to (O,R). Scale bars in (A,D) are 30 μm. The same scale as (A) is used in (B,C, J~L). The same scale as (D) is used in (E,F,G~I, M~R). Arrows in (D~I) indicate the immunostaining for p-smad2/3 which was found in the nucleus (DAPI-positive) of the differentiating muscle cells (fMyHC-positive). Arrowheads in (M~R) indicate the immunostaining for smad2/3 which was observed in the cytoplasm of muscle cells with fMyHC-positive and DAPI-negative stainings.*

at a 10 μm thickness with a cryostat and were double-stained with a rabbit polyclonal antibody against TGFβs, TGFβRs, P-smad2/3, and smad2/3 in combination with a mouse monoclonal antibody against fMyHC. The immunolocalization of fMyHC was determined to identify differentiating muscle tissues in the mouse tongue. The anterior, middle and posterior portions of tongue on the sagittal sections, which were defined in our previous study (Nagata and Yamane, 2004), were observed and marked difference in the intensity and localization of all genes studied among the three portions was not observed. The monoclonal antibody against fMyHC was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). FITC-conjugated donkey antibody against rabbit IgG and the rhodamine-conjugated donkey antibody against mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (Baltimore, PA, USA) and used as the secondary antibodies. The stained sections were mounted in Vectashield Hardset mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and visualized with an immunofluorescence microscope (PCM2000, Nikon, Tokyo, Japan). The visualized image was taken by a digital camera (AxioCam, Carl Zeiss Japan Co., Tokyo, Japan) and imported into a personal computer. For control staining, the primary antibody was replaced with normal goat IgG, normal mouse IgG, or PBS. None of the controls showed staining in the differentiating muscle tissues (data not shown).

Statistical analyses

Tukey's method was used to compare the median values between two groups of multiple groups of E11, E13, and E15.

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