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The Expression of Insulin-like Growth Factor-I, II and Their Cognate Receptor 1 and 2 during Mouse Tongue Embryonic and Neonatal Development

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ABSTRACT—While the signal of insulin-like growth factors (IGF) in skeletal muscle cells is known to be mediated by IGF receptor (IGFR) 1, not IGFR 2, there is no report even on the expression of IGFRs during tongue myogenesis. Here we examined changes in the mRNA level of IGFR 1 and 2, in addition to IGF-I and II, during mouse tongue myogenesis between embryonic day (E) 11 and newborn by competitive reverse-transcriptase polymerase chain reaction. Immunolocalization of IGF-I, II, IGFR 1 and 2 was examined using confocal laser scanning microscope at E13, E15 and newborn stages. Immunolocalization of fast myosin heavy chain was also examined to detect differentiating myoblasts, myotubes and myofibers.

IGF-I, II and IGFR 1 mRNAs were highly expressed between E13 and E15 during differentiation of myoblasts and formation of myotubes. IGF-I and II proteins were co-localized to differentiating myoblasts, myotubes and myofibers with IGFR 1 protein. High level expression of IGFR 2 mRNA was also observed between E13 and E15. However, the expression of IGFR 2 protein was sparsely observed throughout the whole tongue tissues and not restricted to the striated muscle tissue. These data suggest that IGFR 1 is related to the IGF signal transduction and the differentiation of mouse tongue striated muscle, whereas IGFR 2 is not directly involved in them.

INTRODUCTION

The roles of insulin-like growth factor (IGF)-I and II in myogenesis of cultured myoblasts have been well evaluated (reviewed by Florini *et al.*, 1994; 1996). The autocrine secretions of IGF-I and II stimulate proliferation and subsequently differentiation of cultured myoblasts (Engert *et al.*, 1996; Ewton *et al.*, 1994; Florini *et al.*, 1991; Rosenthal and Cheng, 1995; Yoshiko *et al.*, 1996). The mitogenic action of IGFs utilizes mitogen-activated protein (MAP) kinase signaling pathway, while phosphatidylinositol 3-kinase/$p70^{s6k}$ signaling pathway is essential for the IGF-stimulated differentiation (Coolican *et al.*, 1997). Recently it has been reported that IGF-I is involved in the regulation of skeletal muscle hypertrophy and a shift in myofiber phenotypes through Ca^{2+}-calcineurin signaling pathway (Semsarian *et al.*, 1999).

It is known that both the IGF-I and II can bind to IGF receptor (IGFR) 1, 2, and insulin receptor (reviewed by Florini *et al.*, 1996). However, the IGF signalings during skeletal myogenesis are shown to be mediated only by IGFR 1 (Liu *et al.*, 1993; Navarro *et al.*, 1997). It appears that IGFR 2 serves IGF-II turnover in skeletal muscle tissue (Ewton *et al.*, 1987; Kiess *et al.*, 1987; Lau *et al.*, 1994; Ludwig *et al.*, 1996; Wang *et al.*, 1994).

The actions of the IGFs appear to be modulated by a family six high-affinity IGF binding proteins (IGFBP), designated to IGFBP-1 to 6 (reviewed by Jones and Clemmons, 1995). The IGFBPs function not only as modulators of the IGF actions, but also as carrier proteins for the IGFs in the circulation. It has been reported that five IGFBPs except for IGFBP-3 are expressed and modulate the IGF actions in skeletal muscles (Ferguson *et al.*, 1992; Florini *et al.*, 1996).

It has been already reported that several unique developmental characteristics differentiate tongue striated muscle from other skeletal muscles such as limb and trunk muscles. The maturation of myofibers in tongue striated muscle occurs earlier than in other skeletal muscles (Prigozy *et al.*, 1997; Yamane *et al.*, 2000). Transforming growth factor α promotes the early myogenesis in mouse tongue (Yamane *et al.*, 1997; 1998a; b), while it inhibits myogenesis in mouse hind limb muscle (Luetteke *et al.*, 1993). Hepatocyte growth factor is not involved in the migration of tongue precursor cells

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(Mackenzie *et al.*, 1998), but is involved in the migration of hind limb muscle precursor cells (Bladt *et al.*, 1995).

In vivo expressions of IGFs, IGFRs and IGFBPs during the development of skeletal muscles have not been examined intensively (Ferguson *et al.*, 1992; Ishii, 1989; Kleffens *et al.*, 1999). Particularly, despite the unique developmental characteristics, there are only a few reports on the expression of IGFs and IGFBPs (Ferguson *et al.*, 1992; Kleffens *et al.*, 1999) and no report on the expression of IGFRs during the development of tongue striated muscle. We thus examined changes in the mRNA level and the immunolocalization of IGFR 1 and 2, in addition to IGF-I and II, during the development of tongue striated muscle. The immunolocalization of fast myosin heavy chain was also examined to characterize differentiating myoblasts, myotubes and myofibers (Dalrymple *et al.*, 1999).

MATERIALS AND METHODS

Tissue

Pregnant ICR mice were purchased (Nippon Clea, Tokyo, Japan). Tongues were dissected from embryos at E11, 13, 15, and 17, and from newborn mice. The tissues for PCR analysis were immediately frozen and stored at –80°C until use. Five or six samples were collected at each developmental stage. Four preparations at E11 and two preparations at E13 were pooled due to their small amounts of RNA. The tissues obtained from E13, E15 and newborn mice for immunohistochemistry were immediately fixed in Bouin's solution.

RNA extraction and competitive RT-PCR amplification

Total RNA extraction and competitive RT-PCR amplification were performed as previously described (Yamane *et al.*, 2000; Yamane *et al.*, 1998b). Briefly, total RNA extraction was performed according to the manufacturer's specifications of Rapid total RNA isolation kit (5 Prime->3 Prime Inc., Boulder, CO, USA). The RNA was treated with 2 units of ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD, USA), and then was transcribed with 200 units of reverse transcriptase (SuperScript II, Life Technologies, Gaithersburg, MD, USA).

In conventional PCR technique, due to the exponential nature of PCR, any variations affecting amplification efficiency could result in dramatic changes in product yield and the plateau effect after many cycles could lead to inaccurate estimate of product yield. To overcome these problems, the competitors (internal standard), DNA fragment with the same sequences as each gene-specific primer at 3' and 5' ends, was used for the competitive RT-PCR amplification (Gilliland *et al.*, 1990; Siebert and Larrick, 1992; Yamane *et al.*, 1998b; 2000). The competitors constructed according to the manufacturer's instructions of PCR MIMIC Construction Kit (Clontech Laboratory Inc., Palo Alto, CA, USA) were amplified with 50 ng of the total cDNA in the presence of primer pair specific to target gene in a thermal cycler (TP3000, TaKaRa Biochemicals, Shiga, Japan). The sequences and product size for target gene-specific primers except for glyceraldehyde-phosphate dehydrogenase (GAPDH) are shown in Table 1 and those for GAPDH were previously reported (Zhao *et al.*, 1995). To determine the specificities of primers for IGFR 1 and 2, the sequences of the resultant PCR products were analyzed by an automated sequencer. The specificities of primers for IGF-I and II were previously confirmed by Hannon *et al.* (1992). Amplification products were separated by electrophoresis on an agarose gel containing ethidium bromide. Fluorescent intensities of the bands of the target genes and their respective competitors were measured by an image analyzer (Argus-100, Hamamatsu Photonics K.K., Hamamatsu, Japan). We then calculated the ratios of fluorescent intensities of the target gene bands to those of their respective competitor bands.

Table 1. Sequences of PCR primers and product sizes for the target genes

IGF-I	
Forward	5' –GCT CTT CAG TTC GTG TGT GG-3'
Reverse	5' –TTG GGC ATG TCA GTG TGG– 3'*
Product size	221 bp
IGF-II	
Forward	5'–CGT GGA AGA GTG CTG CTT CC–3'
Reverse	5'–GAC ATC TCC GAA GAG GCT CC–3' *
Product size	329 bp
IGFR 1	
Forward	5'–TCT TGG ATG CGG TGT CCA ATA AC–3'
Reverse	5'–GCA GCA CTC ATT GTT CTC GTT GC–3' **
Product size	215 bp
IGFR 2	
Forward	5'–TGC ACA CTC TTC TTC TCC TGG CA–3'
Reverse	5'–GCA GAT GTT GAT ATA GAA GTC AGG–3'#
Product size	186 bp

* Hannon *et al.*, 1992
** Wada *et al.*, 1993
# Ludwig *et al.*, 1994
bp, base pairs

To calculate the cDNA quantity of each target gene from the calculated ratio of fluorescent intensity, we generated a standard curve for each target gene by following ways. Each target gene was amplified by PCR in the presence of primers specific to the target genes without competitor. The amplified PCR product, identified as a single band on the electrophoretic gel, was purified by passing it through a spin column (CHROMA SPIN+TE 100, Clontech Laboratory Inc., Palo Alto, CA, USA). The quantity of cDNA in the purified product was measured by a spectrophotometer at a wavelength of 260 nm and 1:2 serial dilutions of the cDNA were made. The diluted cDNA (cDNA standard) and the respective competitor, the concentration of which was identical to that used for the experimental samples, were co-amplified by PCR and analyzed in the same manner as the experimental samples. The logarithmic values of the ratios of fluorescent intensities in the bands of known quantities of diluted cDNA to those in the bands of the respective competitors were plotted against the logarithmic values of the known quantities of the diluted cDNA. From this relation (i.e. standard curve), the quantity of cDNA for target genes was estimated in the experimental samples and divided by the quantity of GAPDH to normalize the variations in the yield of mRNA and efficiency of reverse transcription. The cDNA quantity seems to be equivalent to the mRNA quantity, because the cDNA was obtained by reverse transcription of the mRNA. The resulting value was expressed as a percent relative to the mean value of each target gene at the newborn stage.

Immunohistochemistry

Specimens for immunohistochemistry were fixed in Bouin's fixative for two hours at 4°C, immersed in a graduated series of sucrose solutions (5–40% w/v) in phosphate buffered saline (PBS) at 4°C, embedded in Tissue-Tek Oct Compound (Miles Laboratory, Elkhart, IN, USA) and frozen. Sagittal sections of tongues were prepared at a 10 μm thickness in a cryostat and air-dried for 1 hr at room temperature. The frozen sections were post-fixed in acetone at –20°C, rehydrated in PBS, and incubated with 5% of normal goat serum for 30 min to block non-specific immunostaining. For double immunostaining, the sections were incubated with a mixture of the rabbit polyclonal antibodies against IGF-I, II (AUSTRAL Biologicals, San Ramon, CA, USA), IGFR 1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)

or IGFR 2 (a kind gift from Dr. S.D. Scott, Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, NSW, Australia; Scott and Baxter, 1987), and the mouse monoclonal antibody against fast skeletal muscle myosin heavy chain (Sigma-Aldrich Inc.). After washing 3 times in PBS, the sections were incubated with a mixture of the FITC-conjugated goat antibody against rabbit IgG and the rhodamine-conjugated goat antibody against mouse IgG (Sigma-Aldrich Inc.). After washing in PBS and subsequent incubating with Equilibration buffer of SlowFade-Light Antifade Kit (Molecular Probes Inc., Eugene, OR, USA), the stained sections were mounted in SlowFade-Light Antifade reagent and visualized with a confocal laser scanning microscope (PCM2000, Nikon, Tokyo, Japan). For control staining, the primary antibodies were replaced with normal rabbit, mouse IgG or PBS; all controls showed no staining.

Statistical Analyses

Scheffe's method was used to compare the mean values between two groups.

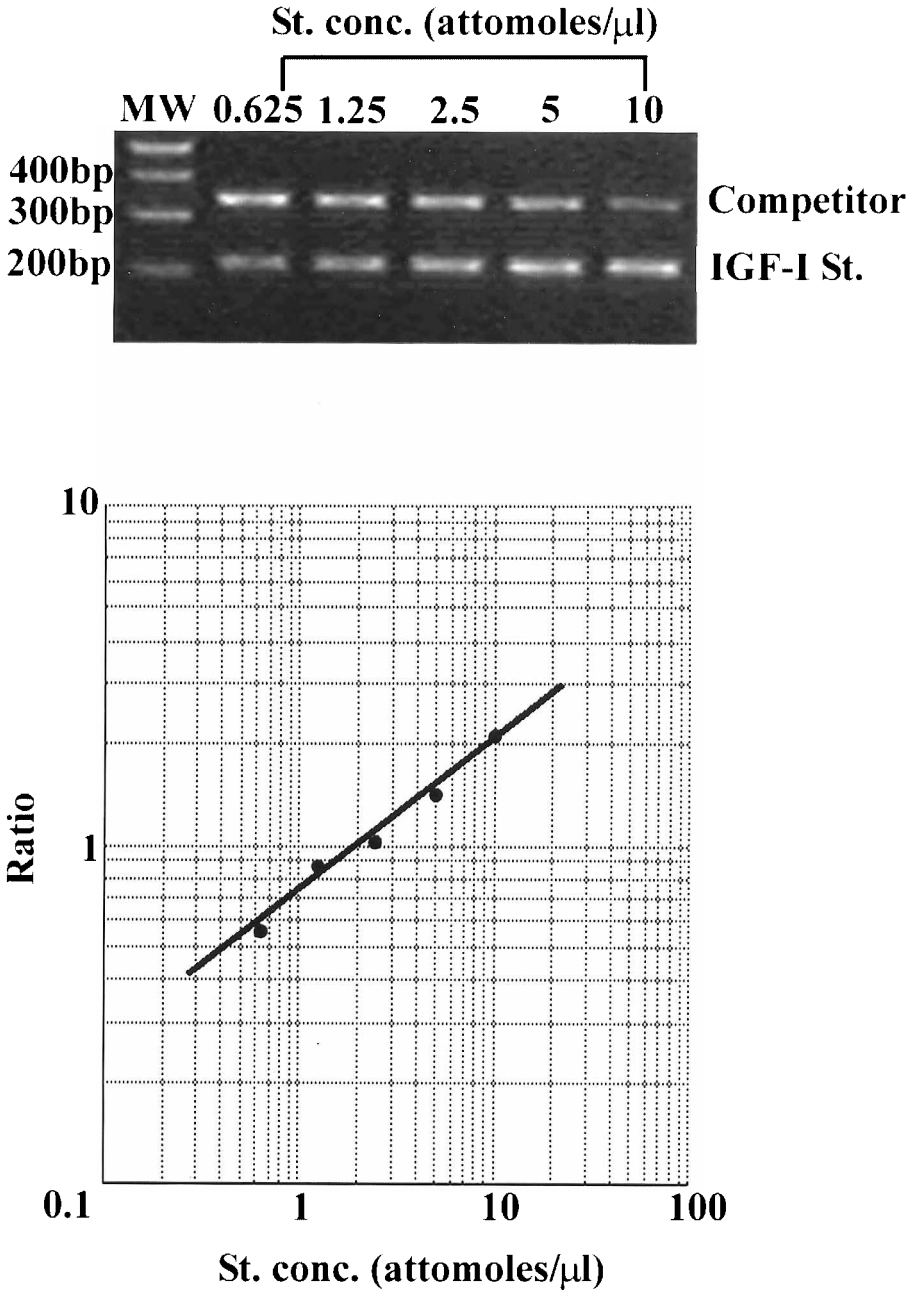

Fig. 1. Electrophoretic gel pattern of IGF-I cDNA standard and its competitor (upper panel) after competitive PCR and the standard curve (lower panel). The standard curve in the lower panel was generated from the result of image analysis of electrophoretic bands in the upper panel. The formula of the regression line is represented by y= 0.46X – 0.15, where y is the logarithmic value of the ratio of the fluorescent intensity in the IGF-I cDNA standard band to that in its competitor band and x is the logarithmic value of the concentration of the cDNA standard. St. conc.; Standard concentrations. MW; molecular weight markers. bp; base pairs.

RESULTS

Standard curves

Fig. 1 shows the electrophoretic gel pattern (upper panel) of IGF-I cDNA standard and its competitor after competitive PCR to generate a standard curve (lower panel). The sizes of PCR products for IGF-I cDNA standard and its competitor were 221 and 338 bp, respectively. The fluorescent intensities of IGF-I cDNA standard and competitor bands on the gel appeared to be inversely related. The formula of the regression line is represented by $y=0.46X-0.15$.

The formulae for the regression lines and correlation coefficients for IGF-I, II, IGFR 1 and 2 are included in Table 2. The correlation coefficients were greater than 0.99 for all the genes and were statistically significant from zero ($p<0.001$). This result indicated that the quantities of the target gene cDNAs could be reliably determined from these standard curves.

Table 2. Formulae for the regression lines and correlation coefficients for IGF-I, II, IGFR 1 and 2.

Target gene	Formula	r	significance
IGF-I	y = 0.46x – 0.15	0.99	p<0.001
IGF-II	y = 0.38x – 0.39	0.99	p<0.001
IGFR 1	y = 0.52x – 0.64	0.99	p<0.001
IGFR 2	y = 1.45x – 1.57	0.99	p<0.001

y, the logarithmic value of the ratio of the fluorescent intensity in the target gene band to that in its respective competitor band.
x, the logarithmic value of the concentration of the cDNA standard.
r, correlation coefficient.

Changes in the mRNA level of IGF-I, II, IGFR 1 and 2 during the development of mouse tongue

The mRNAs of IGF-I (Fig. 2A), II (2B), IGFR 1 (2C) and IGFR 2 (2D) were actively expressed between E13 and E15 stages in the mouse tongues. This time period corresponds with differentiation of myoblasts and formation of myotubes in tongue striated muscle. IGF-I mRNA was highly expressed between E11 and E15, then decreased in the level of expression until birth. Its quantity at the newborn stage was less than 40% ($p<0.0001$) of the E15 value. The quantity of IGF-II mRNA increased by 43% ($p<0.05$) between E11 and E15 and showed a peak value at E15. After E15, the quantity decreased and became 53% ($p<0.001$) of the E15 value at the newborn stage. IGFR 1 mRNA was expressed at E11 and the expression decreased throughout all subsequent developmental stages. However, the quantities at E13 and E15, during which

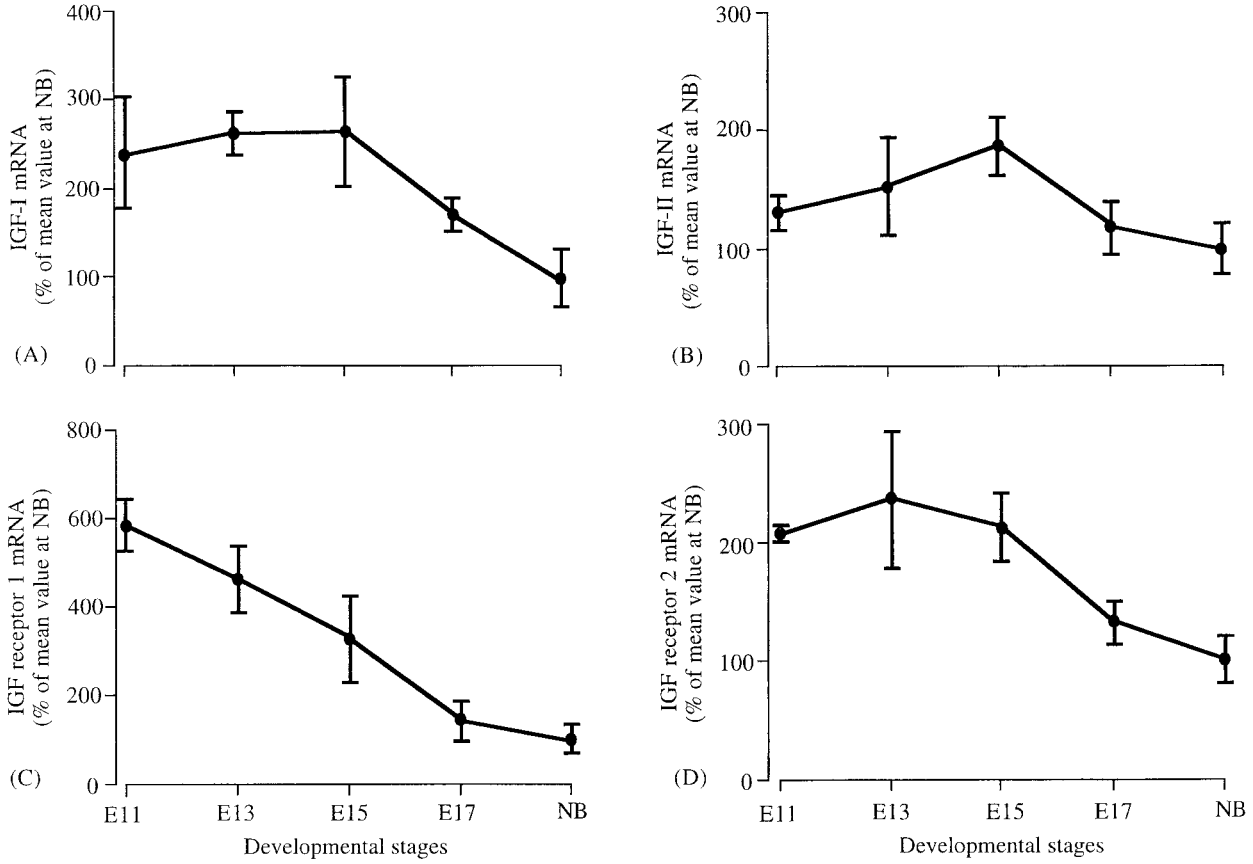

Fig. 2. Relative changes in the mRNA level for IGF-I (A), II (B), IGFR 1 (C) and IGFR 2 (D) in mouse tongues at stages E11, 13, 15 and 17, and at birth assessed by using competitive RT-PCR. Each point with its vertical bar represents the mean±1 SD of five or six samples. The vertical axis is expressed as a percentage of the mean value at the newborn stage. All the four mRNAs studied were highly expressed between E13 and E15 during which differentiation of myoblasts and formation of myotubes actively occurred. NB, newborn.

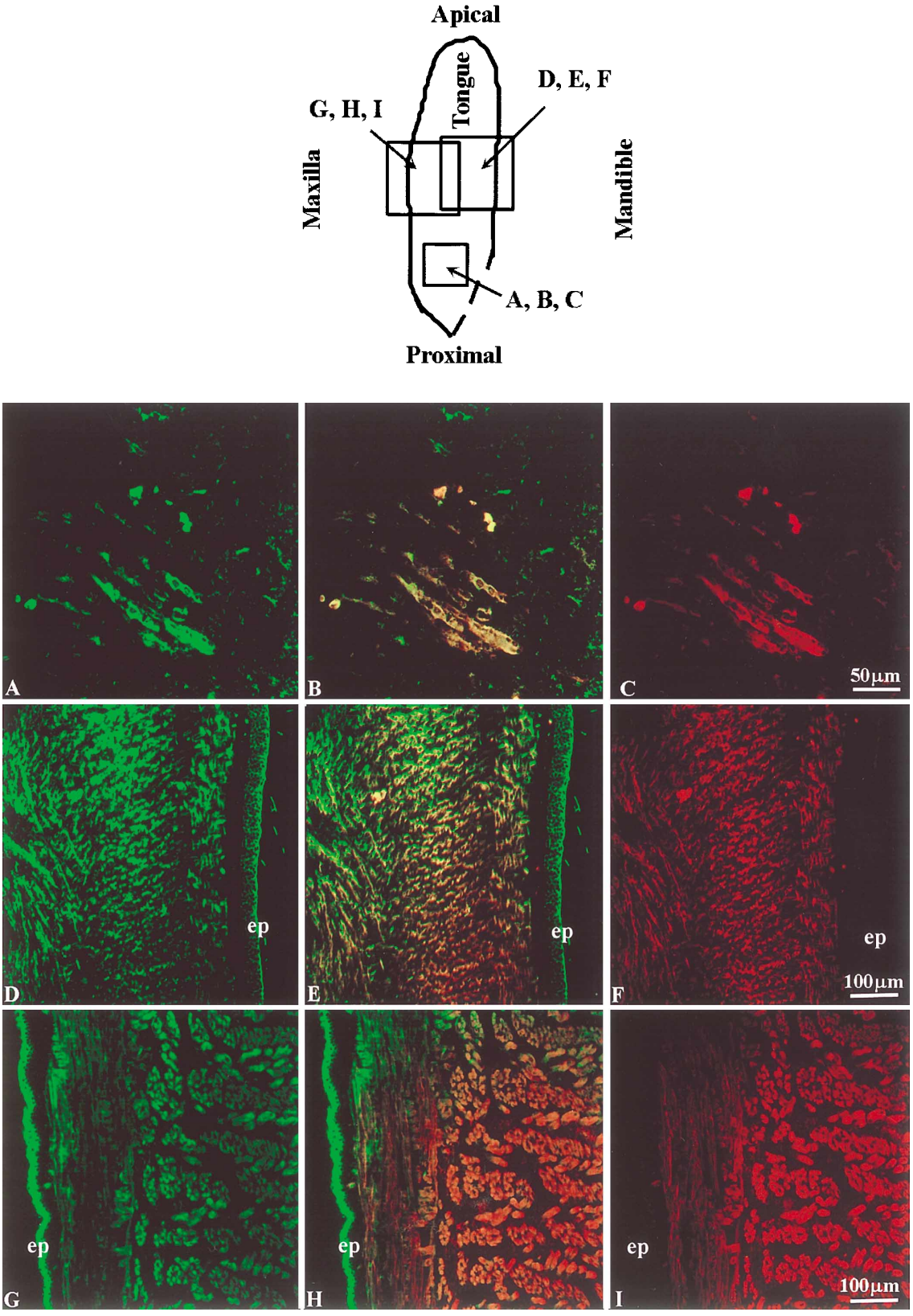

Fig. 3. Confocal microscopic images of sagittal sections of tongues obtained from E13 (A, B, C) and E15 (D, E, F) mouse embryos, and newborn mice (G, H, I). A, D and G show immunostaining for IGF-I; C, F and I show immunostaining for fast myosin heavy chain; B, E and H show double-staining. Immunostaining for IGF-I was observed in differentiating myoblasts, myotubes and myofibers. The epithelial tissue (ep) displayed strong immunostaining for IGF-I at E15 (D) and newborn (G) stages. The top diagrammatic representation shows a sagittal section of tongue viewed from the buccal side. The squares indicate the regions shown in A~ I.

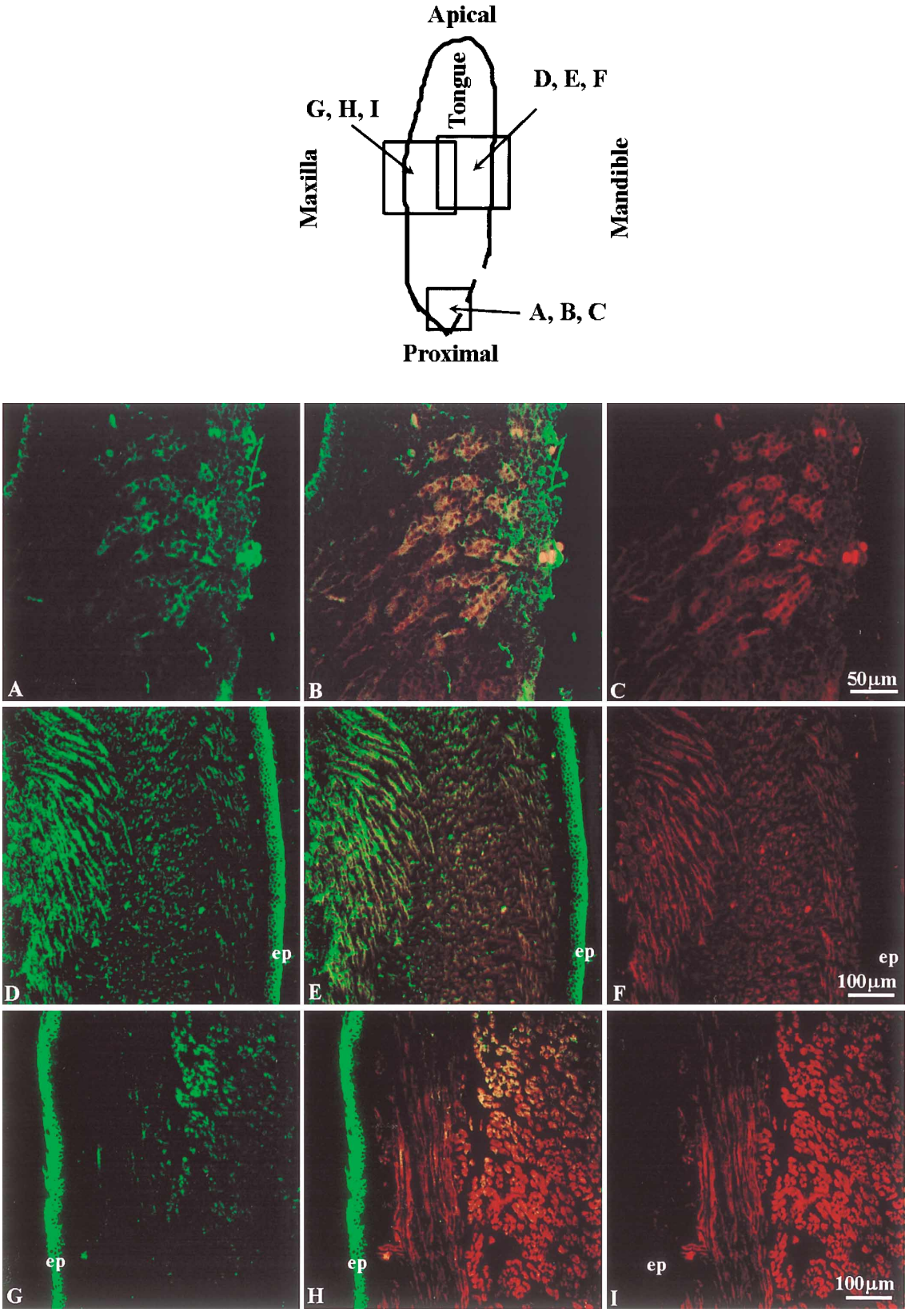

Fig. 4. Confocal microscopic images of sagittal sections of tongues obtained from E13 (A, B, C) and E15 (D, E, F) mouse embryos, and newborn mice (G, H, I). A, D and G show immunostaining for IGF-II; C, F and I show immunostaining for fast myosin heavy chain; B, E and H show double-staining. Differentiating myoblasts, myotubes and myofibers were immunostained for IGF-II. The epithelial tissue (ep) displayed strong immunostaining for IGF-II at E15 (D) and newborn (G) stages. The top diagrammatic representation shows a sagittal section of tongue viewed from the buccal side. The squares indicate the regions shown in A ~ I.

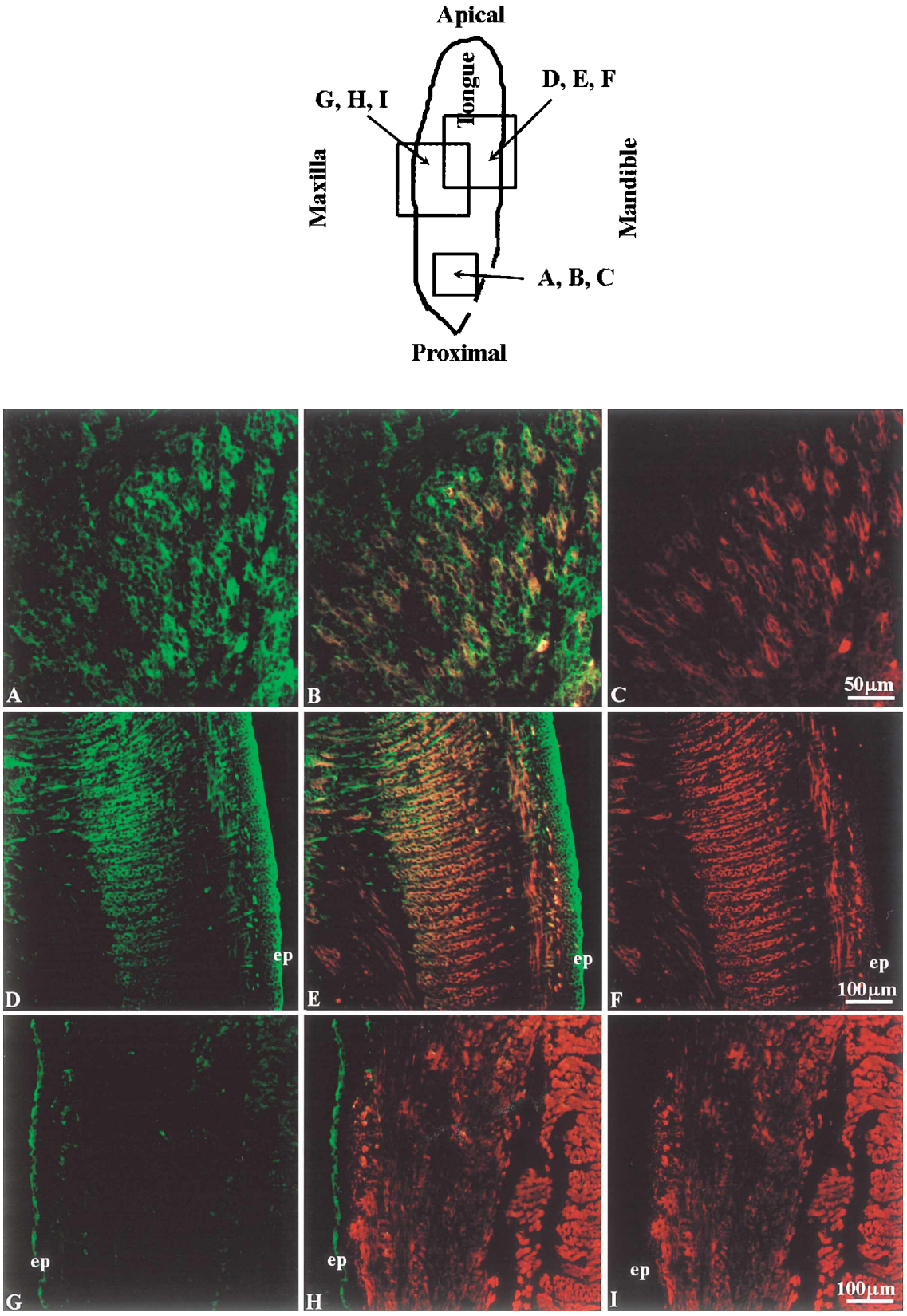

Fig. 5. Confocal microscopic images of sagittal sections of tongues obtained from E13 (A, B, C) and E15 (D, E, F) mouse embryos, and newborn mice (G, H, I). A, D and G show immunostaining for IGFR 1; C, F and I show immunostaining for fast myosin heavy chain; B, E and H show double-staining. Immunostaining for IGFR 1 was also observed in differentiating myoblasts, myotubes and myofibers. ep, epithelial tissue. The top diagrammatic representation shows a sagittal section of tongue viewed from the buccal side. The squares indicate the regions shown in A~ I.

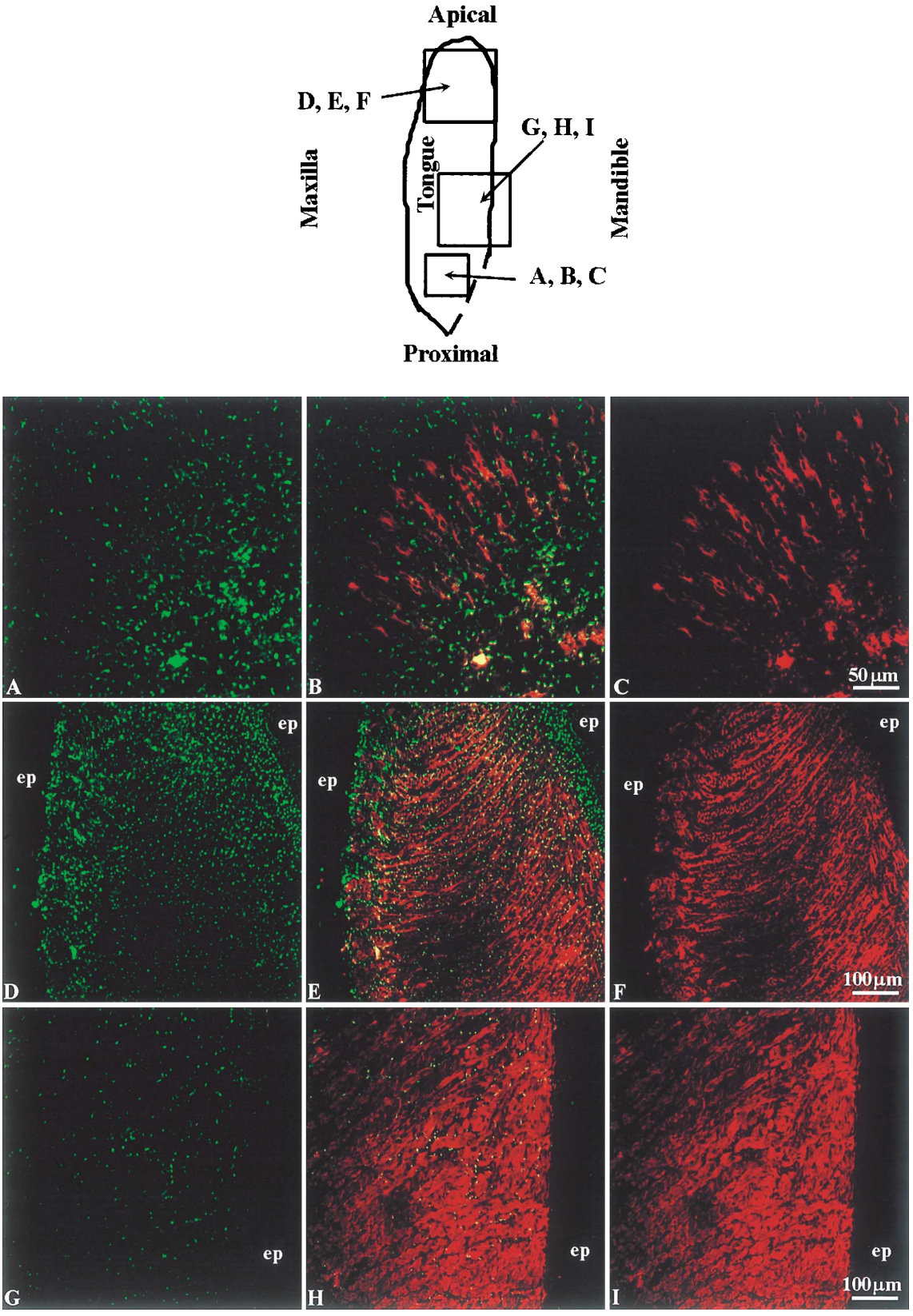

Fig. 6. Confocal microscopic images of sagittal sections of tongues obtained from E13 (A, B, C) and E15 (D, E, F) mouse embryos, and newborn mice (G, H, I). A, D and G show immunostaining for IGFR 2; C, F and I show immunostaining for fast myosin heavy chain; B, E and H show double-staining. Immunostaining for IGFR 2 was sparsely distributed in the whole tongue and not restricted to differentiating striated muscle. ep, epithelial tissue. The top diagrammatic representation shows a sagittal section of tongue viewed from the buccal side. The squares indicate the regions shown in A~ I.

the tongue muscle differentiation actively occurred, were over 2-fold greater than those at E17 and newborn stages ($p<.01 \sim 0.0001$). High level expression of IGFR 2 mRNA was observed between E11 and E15. After E15, the expression decreased and ultimately became less than 50% ($p<0.0001$) of the E15 value at the newborn stage.

Localization of IGF-I, II, IGFR 1 and 2 during the development of mouse tongue

IGF-I (Fig. 3) and II (Fig. 4) displayed similar immunostaining patterns and the proteins were present in differentiating myoblasts, myotube and myofibers in the developing mouse tongue. At E13, several cells with strong immunostaining for IGF-I and II were found in the proximal region of the developing tongue (Figs. 3A and 4A). These cells were spindle-shaped and displayed strong immunostaining for fast myosin heavy chain, indicating that these cells were differentiating myoblasts (Figs. 3B, 3C, 4B and 4C). At E15, strong immunostaining for IGF-I and II was observed in the myotubes and myofibers which displayed strong immunostaining for fast myosin heavy chain (Figs. 3D, 3E, 3F, 4D, 4E and 4F). At the newborn stage, immunostaining for both IGF-I and II was observed in well-developed muscle fibers (Figs. 3G and 4G), but was weak in comparison with that in the myotubes at E15 (Figs. 3D and 4D). The epithelial tissue of the developing tongue had a strong immunopositive reaction for both IGF-I and II at E15 and newborn stages (Figs. 3D, 3G, 4D and 4G).

The immunostaining pattern for IGFR 1 was very similar to those for IGF-I and II in the developing mouse tongue (Fig. 5). At E13, immunopositive cells for both IGFR 1 and fast myosin heavy chain were observed in the proximal region (Fig. 5A, 5B and 5C). At both the E15 and newborn stages, the immunostaining for IGFR 1 was observed in myotubes and myofibers, which also contained fast myosin heavy chain (Fig. 5D ~ 5I). The immunoreaction for IGFR 1 at the newborn stage was very weak (Fig. 5G) in comparison with that at the E13 and E15 stages (Fig. 5A and 5D). The epithelial tissue in the developing tongue was immunopositive for IGFR 1 at both the E15 and newborn stages (Fig. 5D and 5G).

The immunostaining pattern for IGFR 2 in the developing mouse tongue differed from the other three proteins studied (Fig. 6). Dot-shaped immunostaining for IGFR 2 was distributed sparsely throughout the developing tongue except for the epithelial tissue at all stages studied (Fig. 6A, 6D and 6G). The immunostainig was not restricted to differentiating myoblasts, myotube and myofibers (Fig. 6B, 6E and 6H). Immunostaining at the newborn stage (Fig. 6G) was less than at the E13 (Fig. 6A) and E15 (Fig. 6D) stages.

DISCUSSION

We observed that mRNA and protein of IGF-I and II were highly expressed in differentiating myoblasts and myotubes of mouse tongue (Figs. 2, 3 and 4). Previously, few studies examined *in vivo* expression of IGF-I and II in skeletal muscle tissues (Ferguson *et al*., 1992; Ishii, 1989). Ferguson *et al*. (1992) detected IGF-I and II gene expression in developing mouse tongue between E12 and E15, which is consistent with our present observation. Ishii (1989) reported that IGF-II mRNA increased with accumulation of polyneuronal innervation and decreased with elimination of superfluous synapses in rat hind limb muscle. Polyneuronal innervation accumulated between E11 and E15, and elimination of superfluous synapses occurred after E15 in mouse tongue striated muscle (Yamane *et al*., unpublished data). The present results seem to accord with the results in rat hind limb muscle (Ishii, 1989).

It has been already reported that autocrine secretion of IGF-I and II stimulates differentiation of cultured myoblasts such as C2C12 and L6 (Florini *et al*., 1991; Yoshiko *et al*., 1996). Thus the expression of IGF-I and II in differentiating myoblasts and myotubes (Figs. 3 and 4) suggests that the autocrine signals of IGF-I and II regulate differentiation of mouse tongue myoblast and formation of myotube, too. IGF-I and II gene expression persisted in the mouse tongue myofibers at the newborn stage (Figs. 3 and 4). Recently, it has been reported that stable expression of IGF-I in C2C12 myogenic cells results in a switch to glycolytic metabolism, suggesting that IGF-I may be related to a change in myofiber phenotypes (Semsarian *et al*., 1999). Since mouse tongue myofibers mature to be fast-twitch glycolytic between E15 and newborn stages (Prigozy *et al*., 1997; Yamane *et al*., 2000), IGF-I and II expression in tongue striated muscle at the newborn stage may be involved in maturation into fast-twitch glycolytic fibers.

Strong immunostaining for IGF-I and II was observed in the mouse tongue epithelial tissue adjacent to the striated muscle tissue at E15 and newborn stages (Figs. 3 and 4). The development of craniofacial organs such as tooth and Meckel's cartilage is thought to depend on inductive interactions between epithelium and mesenchyme (reviewed by Hall 1992 and Slavkin, 1988). The present data suggest the potential that paracrine secretion of IGF-I and II from the tongue epithelial tissue may play a role in differentiation and maturation of mouse tongue striated muscle. Since there has been no report on the involvement of interactions between epithelial and muscle tissues in the development of mouse tongue striated muscle, further studies seem to be needed.

IGFR 1 was co-localized to differentiating myoblasts, myotubes and myofibers with IGF-I and II (Figs. 3, 4 and 5). Since many studies have shown that IGF-I and II control differentiation of myoblasts *in vitro* (reviewed by Florini *et al*., 1994; 1996), the co-localization suggests that IGFR1 is closely involved in the autocrine signal transduction of IGFs, differentiation of myoblasts and formation of myotubes in the developing mouse tongue. It has been reported that null mutants for the IGFR 1 gene exhibit a severe growth deficiency with poor skeletal muscle formation (45% of normal size) (Liu *et al*., 1993); overexpression of IGFR 1 affects proliferation and differentiation (Quinn and Roh, 1993; Quinn *et al*., 1993; 1994), and abolishes the exogenous IGF requirement for differentiation of cultured myoblasts (Navarro *et al*., 1997). These results support our conclusion.

IGFR2 displayed a different localization pattern from IGFs and IGFR 1 in the developing mouse tongue (Fig. 6). IGFR 2 protein was expressed sparsely throughout the developing tongue. This expression pattern suggests that IGFR 2 is not directly related to the signal transduction of IGFs, differentiation of myoblasts and formation of myotubes in the developing mouse tongue. There are several reports that provide supportive evidence for this conclusion. Blocking antiserum against IGFR 2 does not inhibit IGF-induced myogenesis in L6 cells (Kiess *et al.*, 1987) and IGF-I analogs with a low affinity for IGFR 2 exhibit the identical activity to native IGF-I for myogenesis in L6 cells (Ewton *et al.*, 1987).

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