General Introduction

Tongue is a complex muscular organ constituted of several intrinsic and extrinsic muscles and involved in several important physiological tasks such as suckling, swallowing, mastication, respiration and vocality. Tongue striated muscles have several unique characteristics different from other skeletal muscles such as limb and trunk muscles as follows. The tongue muscles are capable of moving in three-dimension. The embryonic origin of connective tissue cells in tongue striated muscles is neural crest, whereas that in trunk and limb skeletal muscles is somite (Noden, 1983, 1986a, b; Jacob *et al.*, 1986). Fast types of myosin and troponin C are already expressed in undifferentiated tongue myoblasts, but it is expressed only in myotubes and myofibers of other skeletal muscles (Prigozy *et al.*, 1997; Dalrymple *et al.*, 1999).

Myogenesis, including the differentiation and maturation of myofibers, is known to be closely related to innervation and subsequent synaptogenesis (Hall and Sanes, 1993; Buonanno *et al.*, 1998). During synaptogenesis, the expression level, distribution, subunit composition, and properties of the nicotinic acetylcholine receptor (nAChR) change (Brehm and Henderson, 1988; Hall and Sanes, 1993). The embryonic-type of nAChR, composed of $\alpha_2\beta\gamma\delta$ subunits, is expressed throughout muscle cells. With development, the γ subunit is replaced by an ε subunit to become the adult-type nAChR ($\alpha_2\beta\varepsilon\delta$). The adult type of nAChR is eliminated outside the neuromuscular junction and preferentially expressed at the neuromuscular junction in adult myofibers.

MyoD family, which includes myoD, myf5, myogenin and MRF4, is known to play a key role in the regulation of the development of skeletal muscle (reviewed by Weintraub, 1993; Buckingham, 1994, 1996). It appears that myoD and myf5 control

myoblast determination (Braun *et al.*, 1992; Rudnicki *et al.*, 1992, 1993), myogenin is essential for myotube formation (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993) and MRF4 is involved in myofiber maturation and maintenance (Braun and Arnold, 1995; Patapoutian *et al.*, 1995; Block *et al.*, 1996; Zhu and Miller, 1997). In spite of unique developmental characteristics and important function of the tongue, little is known about embryonic tongue muscle development including myogenesis, synaptogenesis, and the expression and function of myoD family.

Many reports have shown that peptide growth factors regulate myogenesis; transforming growth factor (TGF) β and fibroblast growth factor type 2 inhibit differentiation of cultured myoblasts by repressing activities of myogenin (Brennan *et al.*, 1991; Li *et al.*, 1992; Martin *et al.*, 1992; Kong *et al.*, 1995). EGF and FGF can act synergistically to stimulate proliferation in BC3H1 myoblasts (Kelvin *et al.*, 1989), and maintenance of human skeletal muscle in culture (Askanas and Gallez-Hawkins, 1985). TGFα significantly inhibits DNA synthesis in primary cultures of fetal bovine skeletal muscle cells and rat L6 myoblasts (Błachowski *et al.*, 1993). Particularly, insulin-like growth factors (IGFs) play very important roles in both the proliferation and differentiation of myoblasts (Florini *et al.*, 1991a, 1994).

The roles of IGF-I and II in myogenesis of cultured myoblasts have been well evaluated (reviewed by Florini *et al.*, 1996). The autocrine secretions of IGF-I and II stimulate proliferation and subsequently differentiation of cultured myoblasts (Florini *et al.*, 1991b; Ewton *et al.*, 1994; Rosenthal and Cheng, 1995; Engert *et al.*, 1996; 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; Wang *et al.*, 1994; Ludwig *et al.*, 1996).

The actions of the IGFs appear to be regulated and coordinated by a family of six high-affinity IGF binding proteins (IGFBP), designated to IGFBP-1 to 6 (reviewed by Jones and Clemmons, 1995). The IGFBPs have been proposed to have four major functions that are essential to regulate and coordinate the biological activities of the IGFs. These are 1) to act as transport proteins in plasma and to control the efflux of IGFs from the vascular space; 2) to prevent IGFs from being degraded and prolong half-lives of IGFs; 3) to provide a means of tissue and cell type-specific localization and 4) to directly modulate interaction of the IGFs with their receptors and thereby indirectly control biological actions. In addition, recent evidence has emerged that the IGFBPs can have direct actions on cellular functions.

It has been reported that all six IGFBPs are expressed in skeletal muscles and IGFBP4, 5 and 6 play important roles in the regulation of skeletal myogenesis (Ferguson *et al.*, 1992, 1996). IGFBP4, 5 and 6 inhibit the proliferation and differentiation of cultured myogenic cell lines such as C2 and L6, although IGFBP5

has the additional capability of stimulating myogenesis of these cell lines under the proper conditions (James *et al.*, 1993; Ewton and Florini, 1995; Rotwein *et al.*, 1995; Silverman *et al.*, 1995; Ewton *et al.*, 1998).

In addition to several unique developmental characteristics of tongue striated muscle described above, the roles of growth factors such as transforming growth factor α (TGFα) and hepatocyte growth factor (HGF) in tongue myogenesis are reported to differentiate from those in other skeletal muscles. TGFα promotes the early myogenesis in mouse tongue (Yamane *et al.*, 1997, 1998a, 1998b), while it inhibits myogenesis in mouse hind limb muscle (Luetteke *et al.*, 1993). HGF is not involved in the migration of avian tongue precursor cells (Mackenzie *et al.*, 1998), but is involved in the migration of hind limb muscle precursor cells (Bladt *et al.*, 1995).

There are only a few reports on the expression of IGFs and IGFBPs during embryonic development of tongue striated muscles (Ferguson *et al.*, 1992; Kleffens *et al.*, 1999), but roles of IGFs and IGFBPs have not been examined. Thus, the main purpose of the present study is to elucidate roles of IGFs and IGFBPs in the differentiation of mouse tongue myoblasts. The present study was designed to consist of four parts: 1) Temporal expressions of myogenic marker mRNAs including desmin, muscle creatine kinase and troponin C, and myoD family mRNAs during the development of mouse tongue were examined by competitive polymerase chain reaction in combination with reverse-transcription (Competitive RT-PCR) to determine the stage for the myoblast differentiation and myofiber maturation. 2) Temporal mRNA expressions of nAChR α, δ, ε and γ subunits were examined to study the time course of nAChR subunit switch and elimination. 3) Temporal

expressions of IGF-I, II, IGFR1 and 2 mRNAs and immuonolocalization of their proteins during the development of mouse tongue were analyzed to elucidate in vivo roles of IGFs and IGFRs. 4) Mouse tongue organ culture system with serum-free and chemically-defined medium was established to study the differentiation of tongue myoblasts. The effects of exogenous IGF-I, exogenous IGFBP4, 5, 6 and des(1-3)IGF-I, an IGF analogue with the reduced affinity of IGFBPs, on the differentiation of mouse tongue myoblasts were examined by using this organ culture system to understand the roles of IGFs and IGFBPs in the differentiation of tongue myoblasts.