Introduction

The naturally occurring cell death of developing motor neurons is considered to be guided by the influence of their peripheries (Hamburger, 1934, 1958). Skeletal muscle cells have been implicated as a key source of peripheral-derived survival factors, as the absence of them causes a near total loss of developing motor neurons (Grieshammer et al., 1998). Many neuronal cell types are controlled by a small number of survival factors, with the genetic deletion of a single factor leading to a profound loss of neurons (Lindsay, 1996). Motor neurons, in contrast, appear to be controlled by numerous survival factors. The null mutation of a single, or even multiple, factors only leads to partial loss of motor neurons (Liu and Jaenisch, 2000; Oppenheim et al., 2000; Oppenheim et al., 2001). This may indicate that there are distinct subpopulations of motor neurons (Oppenheim et al., 2000). Equally, it implies that muscle fibers produce more than one regulator of motor neurons, as the consequence of the genetic deletion of them type is more profound than the deletion of any growth factor.

BMPs were originally identified by their ability to induce bone differentiation (Wozney et al., 1988). Because this bone-inducing activity was shown to be sensitive to protease digestion, it was named bone morphogenetic protein. To date, more than 20 BMPs have been identified, comprising the largest subgroup within the transforming growth factor (TGF)-β superfamily. The TGF-β superfamily signals through a complex involving a type I and a type II receptor. In general, the type II receptors control the ligand binding specificity, while the type I receptors determine which downstream signaling pathway is activated. There are five type II receptors and seven type I receptors (Shi and Massague, 2003). The type II receptors associate with the subfamilies of the superfamily and are referred to as the TGF-β (TβRII), BMP (BMPRII), MIS (MISRII) and activin (ActRIIA, ActRIIB) type II receptors. The type I receptors are shared by the various subfamilies, and are termed the activin receptor-like kinases (ALKs) 1 through 7.

Several members of the TGF-β superfamily, including Müllerian Inhibiting Substance (MIS), TGF-β2 and BMP6 are involved in the regulation of motor neuron survival (McLennan and Koishi, 2002; Wang et al., 2005; Wang et al., 2007). The expression of TGF-β related ligands and receptors in the NMJ or nerve terminals has also been demonstrated in mammals (McLennan and Koishi, 1994; Jiang et al., 2000). Despite studies suggesting that the *Drosophila* BMP ortholog Gbb may regulate signal transduction and maturation of the neuromuscular junction (NMJ) in flies (Marques et al., 2002; McCabe et al., 2003; Eaton and Davis, 2005), the function of BMPs in the mammalian neuromuscular system remains unclear. In this report, we identify BMP4 as a muscle-derived factor that mediates neuron-muscle interactions.

Materials and methods

1. Neuron and muscle co-culture

C2C12 cells were grown as myoblasts in medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 10% fetal bovine serum, 100U/ml penicillin G, and 100μg/ml streptomycin. NG108-15 cells were maintained undifferentiated in medium containing DMEM supplemented with 10% FBS, 100μM hypoxanthine, 1μM aminopterin, and 16μM thymidine. For neuron and muscle co-cultures, C2C12 myoblasts were induced to differentiate and fuse into myotubes by using the culture medium composed of DMEM supplemented with 2% horse serum. Differentiation of NG108-15 cells was induced by adding 1mM of dibutyryl cAMP (Sigma) into the culture medium. In the co-cultures, the 4-day-old differentiated NG108-15 cells were plated onto the 6-day-old C2C12 for 3 days in DMEM medium with 2% horse serum. All cultures were grown in a humidified incubator at 37°C, 95% air and 5% CO_2.

2. RNA preparation, cDNA synthesis, and real-time PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen). The RNA was treated with DNase and converted to cDNA using oligo-d$(T)_{15}$ (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) as described before (Wang et al., 2005). Real-time PCR reactions were performed using a 7300 Fast Real-Time PCR System (Applied Biosystems), SYBR Green Master Mix (Applied Biosystems) and gene specific primers (Table 1). A two-step PCR reaction was carried out with denaturation at 95°C for 15 seconds, annealing and extension combined at 60°C for one minute in a total of 40 cycles. The uniqueness of amplicons was analyzed using dissociation.

Table 1. Oligonucleotide primer sequences

Primer	Sequence	Accession no.	Product size (bp)
BMP4-F	5'-TCGCCATTCACTATACGTGGACTT-3'	BC052846	201
BMP4-R	5'-CACAACAGGCCTTAGGGATACTAGA-3'		
BMP6-F	5'-GCCATCTCGGTTCTTTACTTCGAT-3'	NM_007556	168
BMP6-R	5'-GTGGTTTAAGGCAGATGTTGTTGTT-3'		
BMPRII-F	5'-AGGATCAGGTGAAAAGATCAAGAGA-3'	NM_007561	165
BMPRII-R	5'-GCAAGGTACACAGCAGTGCTAGATT-3'		
GAPDH-F	5'-CTTCATTGACCTCAACTA-3'	NM_008084	300
GAPDH-R	5'-TTCACACCCATCACAAAC-3'		

3. Immunocytochemistry

Cells were fixed in different concentrations of paraformaldehyde (PFA) at 4°C or room temperature (RT) for 30 minutes. PFA were prepared in 0.1M phosphate buffer (PB, pH 7.4). The cells were sequentially incubated in 0.01M PB containing 0.1M glycine (5 minutes for 2 times), 0.01M PB (5 minutes for 2 times), 5% donkey serum (30 minutes, Jackson ImmunoResearch) and various primary antibodies (24 hours at 4°C). Antibodies were diluted in a buffer containing 0.01M PB, 1% NaCl, 0.2% Tween 20 and 1% bovine serum albumin (BSA, Jackson ImmunoResearch). Unbound primary antibodies were removed by washing with 0.01M PB containing 2% NaCl and 0.1% Tween 20 (6 times, 2 minutes each), and biotinylated secondary antibodies were added and incubated for 1 hour. Cells were washed with 0.01M PB containing 2% NaCl and 0.5% Tween 20 (3 times, 5 minutes each), treated with 0.3 % H_2O_2 in methanol for 10 minutes to inhibit endogenous peroxidase, and washed with 0.01M PB (3 times, 1 minute each) followed by streptavidin–horseradish peroxidase (HRP) complex (1:200 diluted in secondary antibody diluting buffer, Amersham) for 1 hour. After washing in 0.01M PB (3 times, 2 minutes each), cells were incubated in 0.1M acetate buffer (pH=5.2) for 3 minutes. Immunoreactivity was developed in 0.1M acetate buffer containing 3-amino-9-ethylcarbamide (AEC, Sigma) and 0.03% H_2O_2 for 12 minutes. In other experiments, immunoreactivity was visualized using a fluorescent BODIPY-fl conjugated secondary antibody (1:200, Molecular Probes). Non-specific binding will be controlled by replacing the primary antibody with non-immune IgG (Sigma). The neuromuscular junctions (acetylcholine receptor clusters) were labeled with rhodamine-conjugated-α-bungarotoxin (1:200, Molecular Probes).

4. Motor neuron survival assay

The spinal cords of 13 to 15-day-old (E13-15) fetuses were dissected and incubated for 15 minutes at 37 ºC in Dulbecco's PBS (DPBS, pH = 7.2, Sigma) containing 10 M beta-mercaptoethanol, 0.05 % trypsin (Sigma) and 0.04 % EDTA. The trypsin digestion was stopped by adding 0.033 % of trypsin inhibitor (Sigma) and the spinal cords were mechanically dissociated by multiple drawing up-and-down through a 21 and a 23 gauge needle. The resulting cell suspension was passed through a 100 m mesh (Sigma), overlaid onto 10.4 % Optiprep (Sigma) in DPBS and centrifuged for 20 minutes at 400 g. The isolated motor neurons (2000 cells/cm^2) were cultured under serum-free conditions in Neurobasal medium (Invitrogen) with B27 supplement and 500 M glutamine at 37 ºC with 5 % CO_2. Growth factors including rhBMP4 (R&D System) and rhBMP6 (R&D System) were added immediately after seeding. Half the volume of medium was changed every 2 days. Four days after plating, the cultures were stained with an antibody to the motor neuron marker, anti-islet-1 (Ericson et al., 1992). The number of surviving motor neurons

was determined by counting islet-1^{+ve} neurons with neurites in three randomly selected fields in each well. Three wells were used for each concentration of the growth factor and the experiment was replicated four times. Virtually all cells were stained by islet-1 indicating that the cultures were pure.

Results and discussions

1. Neuron and muscle co-culture

We began this study with neuron-muscle co-culture to mimic the neuromuscular system *in vivo*. Figure 1A shows differentiated NG108-15 neurons with long branched neurites, whereas figure 1B shows differentiated polynucleates C2C12 myotubes. When C2C12 myotubes were cultured along, only a few acetylcholine receptor clusters could be observed (Figure 1C, arrows). Upon co-culture of C2C12 myotubes with NG108-15 neurons, the acetylcholine receptor clusters were significantly increased (Figure 1D, arrows).

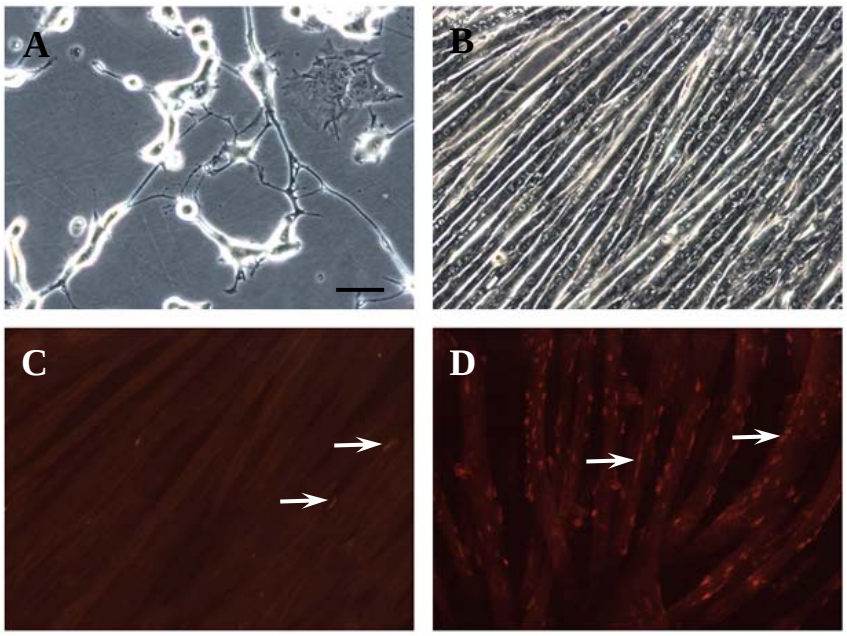

Figure 1. Acetylcholine receptor clusters can be detected in co-cultures of differentiated NG108-15 neurons and C2C12 myotubes. NG108-15 (A) and C2C12 cells (B, C) were induced to differentiate into neurons and myotubes. The acetylcholine receptor clusters (arrows) can be observed when C2C12 myotubes were co-clutured with NG108-15 neurons (D). The acetylcholine receptor clusters were labeled with rhodamine-conjugated -α-bungarotoxin. The scale bars = 20 μm.

2. BMP4 may mediate neuron-muscle interactions

It has been shown that motor neuron-derived agrin can induce acetylcholine receptor clusters at synaptic sites in the muscles (Jones et al., 1997; Meier et al., 1997), and muscle-derived signals are important for motor neuron survival and axon guidance (Nguyen et al., 1998; Oppenheim et al., 2000). The cross talk between motor neurons and muscle cells is therefore very important for the maintenance of neuromuscular junctions. We investigate whether BMPs mediate communications between motor neurons and muscle cells. We found that BMP4 mRNA is highly expressed in C2C12 myotubes but not in NG108-15

neurons (Figure 2A). BMP4 mRNA expression is also up-regulated in C2C12 myotubes when agrin is added in culture medium (Figure 2B). Furthermore, addition of BMP4 to the medium caused an increase in motor neuron survival (Figure 2C). Together, these data indicates that BMP4 may act as a survival factor in response to nerve innervations.

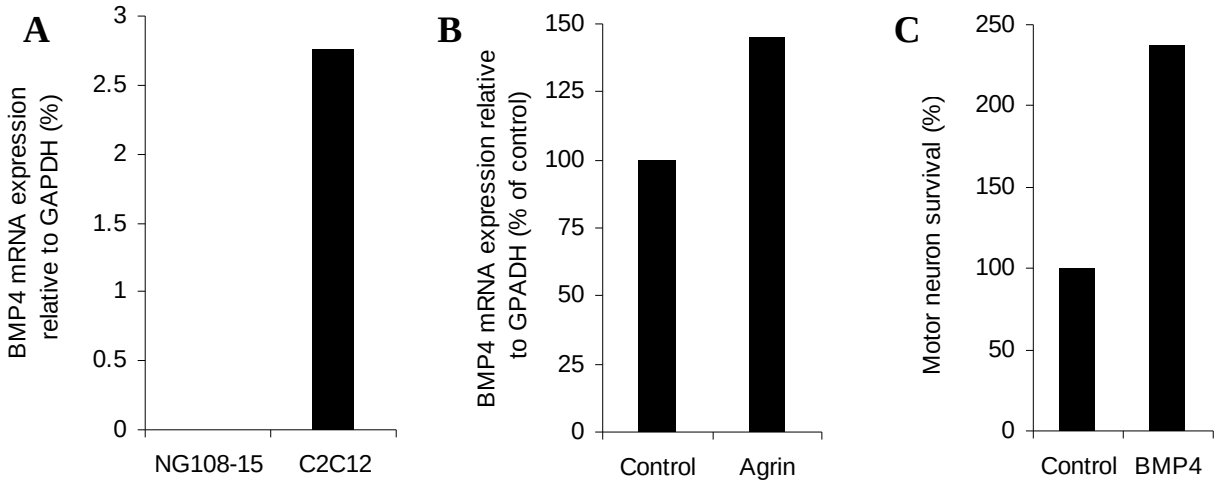

Figure 2. BMP4 is a muscle-derived factor that mediates neuron-muscle interactions. (A, B) BMP4 mRNA expression was measured in differentiated NG108-15 neurons and C2C12 myotubes treated with or without 2 ng /ml agrin using real-time PCR. (C) BMP4 (50ng/ml) promotes motor neuron survival *in vitro.*

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