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Expression of $P2Y_6$ receptors in the developing mouse skeletal muscle and after injury and repair

Donghui Chen,[1]* Wei Wang,[1]* Wei Guo,[2] Qiang Yu,[2] Geoffrey Burnstock,[3] Cheng He,[2] Zhenghua Xiang[2] and Hongliang Zheng[1]

[1]*Department of Otolaryngology & Head and Neck Surgery, Changhai Hospital, Shanghai, China*
[2]*Institute of Neuroscience and Key Laboratory of Molecular Neurobiology, Ministry of Education, Neuroscience Research Centre of Changzheng Hospital, Second Military Medical University, Shanghai, China*
[3]*Autonomic Neuroscience Centre, University College Medical School, Royal Free Campus, London, UK*

Abstract

In this study, single and double-labeling immunofluorescence histochemistry, Western blot and real-time polymerase chain reaction were used to study the expression of $P2Y_6$ receptors in developing mouse skeletal muscle and during injury and repair. The results show that $P2Y_6$ receptor immunoreactive (ir) cells were first detected in the dermamyotome at embryonic (E) day 9. The number and immunostaining intensity of the $P2Y_6$ receptor-ir cells increased from E9 to E13, but decreased from E15 to postnatal day 60 in the developing skeletal muscle system. The expression levels of $P2Y_6$ receptor protein and mRNA increased rapidly from 1 to 5 days after skeletal muscle injury and then decreased almost to the control level from 7 to 10 days, at the beginning of regeneration. $P2Y_6$ receptor-immunoreactivity was mainly localized to the ends of single myoblasts and myotube processes in the developing and injury-repair skeletal muscle tissues. These data suggest that the $P2Y_6$ receptor may be involved in the development and regeneration of skeletal muscle, especially in the migration and extension of the myoblast and myotube in developing and regenerating skeletal muscle.

Key words: development; $P2Y_6$ receptor; regeneration; skeletal muscle.

Introduction

Extracellular adenosine-5′-triphosphate (ATP) acts as a signaling molecule on both pre- and postjunctional membranes at neuroeffector junctions and synapses, as well as acting as a trophic factor during development and regeneration (Burnstock, 2007). Recently, ATP, acting via P2X and P2Y receptors, has been shown to be involved in the process of muscle regeneration in rats (Ryten et al. 2002, 2004; Banachewicz et al. 2005). $P2X_{1–7}$ receptors are intrinsic ligand-gated ion channels and activation of these receptors by ATP evokes a flow of cations across the plasma membrane, whereas P2Y receptors are G-protein-coupled receptors. There are two subgroups of G proteins which P2Y receptors are coupled with, Gq and Gi. $P2Y_{1,2,4\ and\ 6}$ receptors are linked to activation of phospholipase C (PLC), inositol lipid signaling and the mobilization of intracellular Ca^{2+}, $P2Y_{12,}$ $P2Y_{13}$ and $P2Y_{14}$ receptors are coupled with Gi protein, resulting in inhibition of cAMP formation, and $P2Y_{11}$ (only in humans) is coupled with Gs and Gq, resulting in increased cAMP (Burnstock, 2007). Within the family of P2Y receptors, $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors respond to adenosine diphosphate (ADP). $P2Y_2$ and $P2Y_4$ receptors respond to both ATP and uridine 5′-triphosphate (UTP), and $P2Y_6$ receptors to uridine 5′-diphosphate (UDP). The expression of specific P2X receptor subtypes during rat skeletal muscle development (Ryten et al. 2001, 2002, 2004) and in regenerating skeletal muscle (Ryten et al. 2004) has been demonstrated. Among these receptors, $P2X_5$ and $P2X_6$, and $P2X_2$ and $P2X_5$, have been found to be expressed in chick and rat skeletal muscle development, respectively (Meyer et al. 1999b; Bo et al. 2000, 2001; Ryten et al. 2001;). In contrast, there are few studies on the expression and function of P2Y nucleotide receptors in developing skeletal muscle. The expression of $P2Y_1$ receptors was detected during the first 10 days of chick embryonic development (Meyer et al.

Correspondence
Zhenghua Xiang, Institute of Neuroscience and Key Laboratory of Molecular Neurobiology, Ministry of Education, Neuroscience Research Centre of Changzheng Hospital, Second Military Medical University 200433 Shanghai, China. E: zhxiang@hotmail.com

Hongliang Zheng, Department of Otolaryngology & Head and Neck Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, China. E: zheng_hl2004@163.com

*These two authors contributed equally to this work.

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1999a). In rat embryonic skeletal muscle cells, Cheung et al. (2003) found early expression of the $P2Y_1$ receptor, whereas $P2Y_2$ receptor expression became gradually stronger in later development and $P2Y_4$ receptor expression was high at both early and late embryonic days. C2C12 cells (a murine myoblast cell line) were reported to express $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ receptor mRNAs in myoblasts, but were expressed less in myotubes. In contrast, $P2Y_2$ receptor mRNA was not detected in the myoblast, but high levels were detected in myotubes (Banachewicz et al. 2005). When we studied expression patterns of $P2Y_6$ receptors in the early mouse embryo, high levels of $P2Y_6$ receptor immunoreactivity were found in the dermamyotome. These findings suggest that $P2Y_6$ receptors might be involved in some activities during the development and regeneration of skeletal muscle cells. Thus, in the present paper we have studied $P2Y_6$ receptor expression in precursor cells of skeletal muscle from embryo day 9 to adult. The results showed that expression of $P2Y_6$ receptors is down-regulated during development. After myotrauma, the expression of $P2Y_6$ receptors was up-regulated rapidly in the early days and then down-regulated in the later days during regeneration. The expression pattern of $P2Y_6$ receptors in the precursor cells and myotube suggested that $P2Y_6$ receptors might be involved in the migration of the precursor cells and myotubes.

Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University. Kunming mice at the prenatal stages of development (E 9, E11, E13, E15, E18), postnatal stages [postnatal day (P) 3, P10, P25, P60] and adult mice were used for the developmental study. Seventy-two male Kunming mice (25–35 g) received an extensive crush injury to the tibialis anterior muscle of the right leg as described in detail by a previous study (McGeachie & Grounds, 1987). The mice were divided into six groups –control, day 1, day 3, day 5, day 7 and day 10 after muscle injury. Four mice in each group were used for immunohistochemistry, Western blot and real-time RT-PCR, respectively. For the immunohistochemistry, the mice were anesthetized with sodium pentobarbitone and perfused through the aorta with a 0.9% NaCl solution and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The specimens were removed and refixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight. The specimen blocks were then transferred to 20% sucrose in phosphate-buffered saline (PBS) and kept in the solution until they sank to the bottom. Thereafter, the specimen blocks were rapidly frozen. Sections of 10 μm thickness were cut in a cryostat and thaw-mounted on gelatin-coated slides.

Immunohistochemistry

The following protocol was used for immunostaining of $P2Y_6$ receptors. The preparations were washed 3×5 min in PBS and then preincubated in antiserum solution 1 (10% normal bovine serum, 0.2% Triton-X-100, 0.4% sodium azide in 0.01 M PBS, pH7.2) for 30 min, followed by incubation with the $P2Y_6$ antibody diluted 1 : 200 (goat polyclonal antibody; Santa Cruz) at room temperature. Subsequently, the preparations were incubated with Cy3-conjugated donkey anti-goat IgG diluted 1 : 400 for $P2Y_6$ receptors. All the incubations and reactions were separated by 3×10 min washes in PBS.

The following protocol was used for double immunostaining of $P2Y_6$ receptors with laminin-1 (a basement membrane marker) or α-sarcomeric actin (skeletal and cardiac muscle specific marker) or C-Met (a skeletal muscle satellite cell marker). The preparations were washed 3×5 min in PBS and then preincubated in antiserum solution1 for 30 min, followed by incubation with different combinations of $P2Y_6$ antibody diluted 1 : 200, laminin-1 diluted 1 : 400 (rabbit anti-rat; Abcam), α-sarcomeric actin (mouse-anti-rat; Abcam), C-Met diluted 1 : 100 (rabbit anti-rat; Boster) in antiserum solution 2 (1% normal bovine serum, 0.2% Triton-X-100, 0.4% sodium azide in 0.01 M PBS, pH 7.2), at room temperature. Subsequently, the preparations were incubated with fluorescein-labelled antibody (FITC)-conjugated donkey anti-goat diluted 1 : 400 for $P2Y_6$, Cy3-conjugated donkey anti-rabbit IgG diluted 1 : 200 for laminin-1 and C-Met antibodies, and Cy3-conjugated donkey anti-mouse IgG in antiserum solution 2 for 2 h at room temperature. All the incubations and reactions were separated by 3×10 min washes in PBS.

Control experiments were carried out with the $P2Y_6$ antibody preabsorbed with its peptide. No staining was observed in those preparations incubated with antiserum solutions preabsorbed with its peptide. A further negative control by omitting the primary antibody was also carried out. No staining was observed in those preparations.

Western blot

Mice were deeply anesthetized by sodium pentobarbital (60 mg kg^{-1}) and killed by decapitation. The injured muscle was rapidly removed and lysed with 20 mM Tris–HCl buffer, pH 8.0, containing 1% NP-40, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.1% mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma). Protein concentration was determined by the BCA protein assay method using bovine serum albumin as standard (BCA protein assay kit from Beyotime). Protein samples (100 μg) from the colon were loaded in each lane, separated by SDS-PAGE (10% polyacrylamide gels) and then electrotransferred onto nitrocellulose membranes. The membranes were blocked with 10% non-fat dry milk in Tris-buffered saline for 1 h and incubated overnight at 4 °C with the $P2Y_6$ antibody (Santa Cruz) diluted 1 : 200 or α-tubulin diluted 1 : 400 (loading control) in 2% bovine serum albumin (BSA) in PBS. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-goat IgG (Beyotime) diluted 1 : 1000 in 2% BSA in PBS for 1 h at room temperature. The color development was performed with 400 μg mL^{-1} nitro-blue tetrazolium, 200 μg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate and 100 mg mL^{-1} levamisole in TSM2 (0.1 M Tris–HCl_2 buffer, pH 9.5, 0.1 M NaCl and 0.05 M $MgCl_2$) in the dark. Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories).

Real-time quantitative PCR

Muscle tissues were homogenized in 1 mL of Trizol® reagent (Invitrogen Co., Carlsbad, CA, USA). Total RNA was extracted

with chloroform, precipitated with isopropanol, and resuspended in 30 μL of RNAse-free water. The concentration of isolated total RNA was determined by measuring the optical density at A260 nm with an ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reverse transcription of 1 μg of total RNA was performed to synthesize complementary DNA (cDNA) using the PrimerScriptTM RT reagent kit (TaKaRa Biotechnology Co. Ltd, Dalian, China) with the Geneamp PCR System 9700 (Applied Biosystems Inc., Foster City, CA, USA). Primers for the messenger RNA (mRNA) of interest were designed using the online software PRIMER 3PLUS (Wageningen University and Research Centre, Gelderland, Netherlands). Nucleotide sequences for mouse $P2Y_6$ receptors were obtained from GenBank, with β-actin as the calibrator housekeeping gene (internal control). The location and specific sequences of primers were chosen to exclude the detection of genomic DNA by placing one of the primers over a junction between two exons. The primer sequences for these target genes were as follows: (i) $P2Y_6$ (NM_183168): forward primer 5′-ATC AGC TTC CTG CCT TTC C-3′, reverse primer 5′-CTG TGA GCC TCT GTA AGA GAT CG-3′, yielding a fragment of 214 bp; (ii) β-actin (NM_007393): forward primer 5′-AGC CAT GTA CGT AGC CAT CC-3′, reverse primer 5′-CTC TCA GCT GTG GTG GTG AA-3′, yielding a fragment of 228 bp.

Real-time quantitative PCR was performed using the reagent and protocol contained in the SYBR® Premix Ex Taq TM II kit (TaKaRa Biotechnology Co. Ltd, Dalian, China). Primers were used at a concentration of 20 mM. Reactions were run in a volume of 20 μL, containing 0.5 μL cDNA. The program was set up as follows: (i) 95 °C, 2 min; (ii) 95 °C, 15 s $\rightarrow$ 62 °C, 20 s $\rightarrow$ 72 °C, 10 s, for 40 cycles; (iii) 72 °C, 10 min. Data were collected and analyzed on the Rotor-Gene Q real-time PCR cycler (QIAGEN Pty Ltd, Doncaster, Victoria, Australia). The specificity of the PCR product was examined based on the melt curves of the reactions. Relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta CT}$ quantitative method.

Photomicroscopy

Images were taken with a Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (ADOBE PHOTOSHOP).

Quantitative analysis

Quantitative analysis for the $P2Y_6$ receptor immunostaining in normal and injured muscle tissues of the adult mice and mice of different developmental ages was performed as follows: five random fields (each area 0.62 mm^2) for one section were chosen and the number of positive cells was counted and expressed as the positive cell number per mm^2. Five fields for each of the five sections from each of four mice were used. The mean number of positive cells per mm^2 from each mouse was calculated and data expressed as the mean ± SE of the mean (n = number of mice). The ratio of $P2Y_6$ to α-tubulin in Western blot and the relative transcript level in real-time PCR are also calculated and expressed as mean ± SE of the mean (n = number of mice). Statistical significance was tested by a one-way analysis of variance (ANOVA) followed by an unpaired t-test. A probability of $P < 0.05$ was considered significant for each test.

Results

Expression of $P2Y_6$ receptors in developing muscle system

In the coronal section of embryo at E9, $P2Y_6$ receptor immunoreactive (ir) cells were first detected in the dermamyotome, located at the ventrolateral side and under the epidermis. These $P2Y_6$ receptor-ir cells formed a cell band in the dorsal to the ventral direction (Fig. 2A). The number of the $P2Y_6$ receptor-ir cells and the intensity of the $P2Y_6$ receptor immunostaining increased from E9 to E11 (Fig. 1A,B). $P2Y_6$ receptor-ir cells were detected widely in the skeletal muscle system from E13 to E15. From E13 on, the strongly $P2Y_6$-immunostained cells were mainly detected in the borders of the skeletal muscle bundles, although low to median immunostained cells were also detected in the medial part of the skeletal muscle bundles (Figs 2C,D and 3D1). At high power magnification, the distribution of immunostaining signals in the $P2Y_6$ receptor-ir cells exhibited heteropolarity at these stages (E13–E18). As Figs 1C,D and 2D1 show, strong $P2Y_6$ receptor immunostaining signals were detected at the sides near vertebrae/bone or the proximal/distal ends of the limb muscle mass and weak signals were detected in the medial part at E13 and E15. These $P2Y_6$ receptor-ir asymmetrical cells were mainly detected in the border regions of the skeletal muscle bundle. Scattered $P2Y_6$ receptor-ir cells were also detected in the tissues from E9 to E15. These scattered single $P2Y_6$ receptor-ir cells also possessed the asymmetrical pattern of $P2Y_6$ receptor localization (Fig. 2C), the $P2Y_6$ receptor-ir signal being localized at both ends of some myoblasts (Fig. 2A,B). During the postnatal days, the number and immunostaining intensity of $P2Y_6$ receptor-ir cells decreased dramatically and the asymmetrical pattern of $P2Y_6$ receptor-ir disappeared at P3 (Fig. 1E). The $P2Y_6$ receptor-ir cells were weak around some of the skeletal muscle cells at P25. The number and immunostaining intensity of $P2Y_6$ receptor-ir cells were similar to those of adult mice (Fig. 1F). To confirm that the $P2Y_6$ receptor-ir positive cells were skeletal muscle, the specific antibody for the skeletal muscle specific marker (α-sarcomeric actin) was used. The developing muscle bundle-like structures and scattered single cells with P2Y6 receptor-ir were also immunoreactive for α-sarcomeric actin (Fig. 2C,D1,D2,D3).

Expression of $P2Y_6$ receptor mRNA and protein after skeletal muscle injury

The $P2Y_6$ receptor-ir cells were just detectable around limited regions of skeletal muscle cells in normal conditions. To confirm whether the $P2Y_6$ receptor-ir cells were satellite cells, the C-Met antibody was used. The results confirmed this, as $P2Y_6$ receptor-ir cells were also immunoreactive for C-Met (Fig. 2E1,E2,E3). One day after muscle injury, the

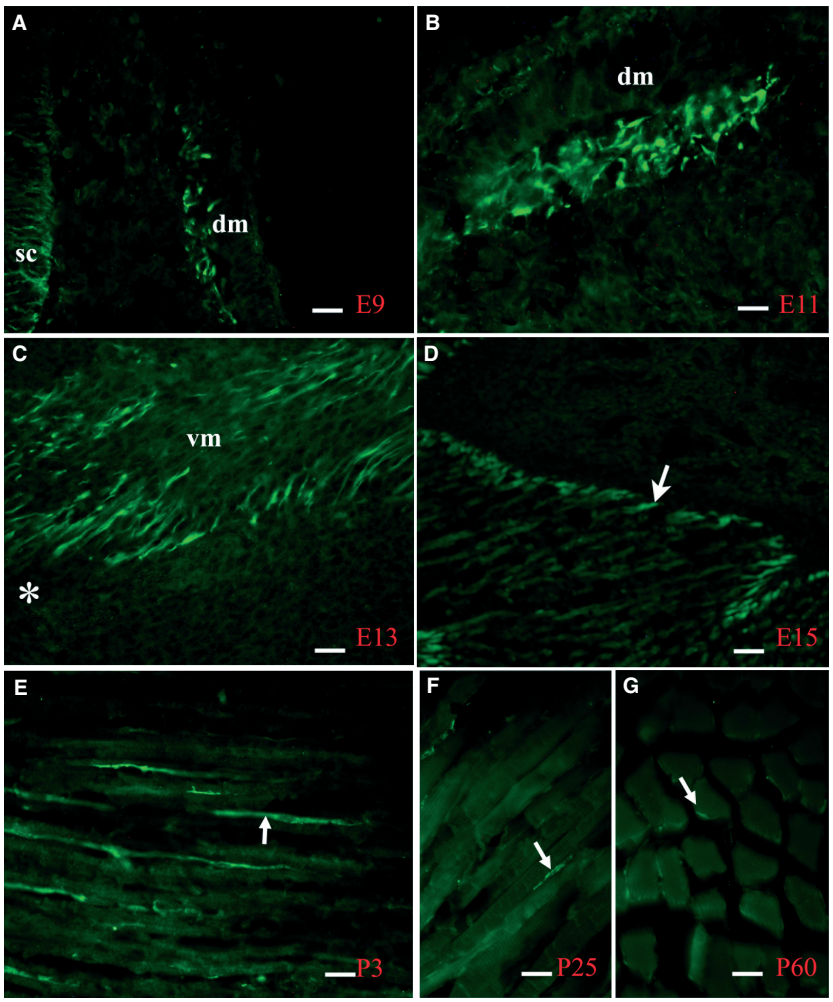

Fig. 1 Expression of $P2Y_6$ receptors in developing skeletal muscle at E9–25. (A) Expression of $P2Y_6$ receptors in the coronal section of embryo in E9. Dm, dermamyotome; sc, spinal cord. Note that strong $P2Y_6$ receptor-ir was detected in the dermamyotome. (B) $P2Y_6$ receptor-ir in the coronal section of the dermamyotome at E11. (C) $P2Y_6$ receptor-ir in the vertebral muscle (vm) mass at E13. A star shows the location of the developing cartilage. (D) Expression of $P2Y_6$ receptors in the coronal section of trunk muscle mass at E15. Note that strong $P2Y_6$ receptor-ir was detected in the border of the muscle mass. An arrow indicates the ventral part. (E) $P2Y_6$ receptor-ir in the longitudinal section of the limb muscle mass at P3. An arrow indicates a positive cell with a symmetrical pattern of $P2Y_6$ receptor-ir, and an arrow head indicates a negative muscle cell. (F) $P2Y_6$ receptor-ir in the longitudinal section of the limb muscle mass at P25. An arrow indicates a $P2Y_6$ receptor-ir cell. Scale bars: 50 μm.

number and immunostaining intensity of $P2Y_6$ receptor-ir cells significantly increased compared with the control group (Figs 1G and 3A). The majority of the cross-sections of the muscle cells in the injury area showed one to three $P2Y_6$ receptor-ir cells. At 3 and 5 days after injury, the number and immunostaining intensity of the $P2Y_6$ receptor-ir cells increased rapidly (Fig. 3B,C). The cell volume of the $P2Y_6$ receptor-ir cells was also larger than that 1 day after injury. From 3 days, myotube-like $P2Y_6$ receptor-ir cells appeared. In the longitudinal sections, the distribution pattern of the $P2Y_6$ receptor-ir signals in single myotube-like structure was also asymmetrical, similar to those cells observed at E13–E18 (Figs 2F1 and 3E,F). At the proximal or distal parts of the $P2Y_6$ receptor-ir myotube-like structure, the immunostaining signals were much more intense than in the median parts of the myotube-like structure. The ends of these positive myotube-like structures were conical in shape. In the longitudinal sections the majority of the cones at the proximal or distal part were orientated in one direction, towards the ends of the muscle mass (Fig. 3E,F). Most of the $P2Y_6$ receptor-ir cells in the injured muscle were also reactive for the skeletal muscle specific marker (α-sarcomeric actin) (Fig. 2F1,F2,F3). The majority of the $P2Y_6$ receptor-ir cells surrounded the injured muscle fibers and were circumvoluted by a basement membrane as shown by the laminin antibody (Fig. 2G), which also indicated that the majority of the $P2Y_6$ receptor-ir cells in the injured areas were from skeletal muscle cells. The number of $P2Y_6$ receptor-ir cells including the positive satellite cells, myoblasts and myotubes in the normal and different injured groups of adult mice are summarized in Fig. 4B. The number of $P2Y_6$ receptor-ir cells in the skeletal muscle mass of hind legs from E15 to P60 mice were also quantitatively studied and are summarized in Fig. 4A. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptor antibodies, have also been used to immunostain the muscle tissues under normal and injury conditions (see Supporting Information Figs S1 and S2). We found that only expressions of $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors were detected and that only the $P2Y_6$ receptor changed significantly after injury.

Western blotting, performed on tissue extracts derived from the mouse tibialis anterior muscle, assessed the specificity of the polyclona $P2Y_6$ receptor antibody. An immunoreactive band was detected at about 45 kDa. Preadsorption

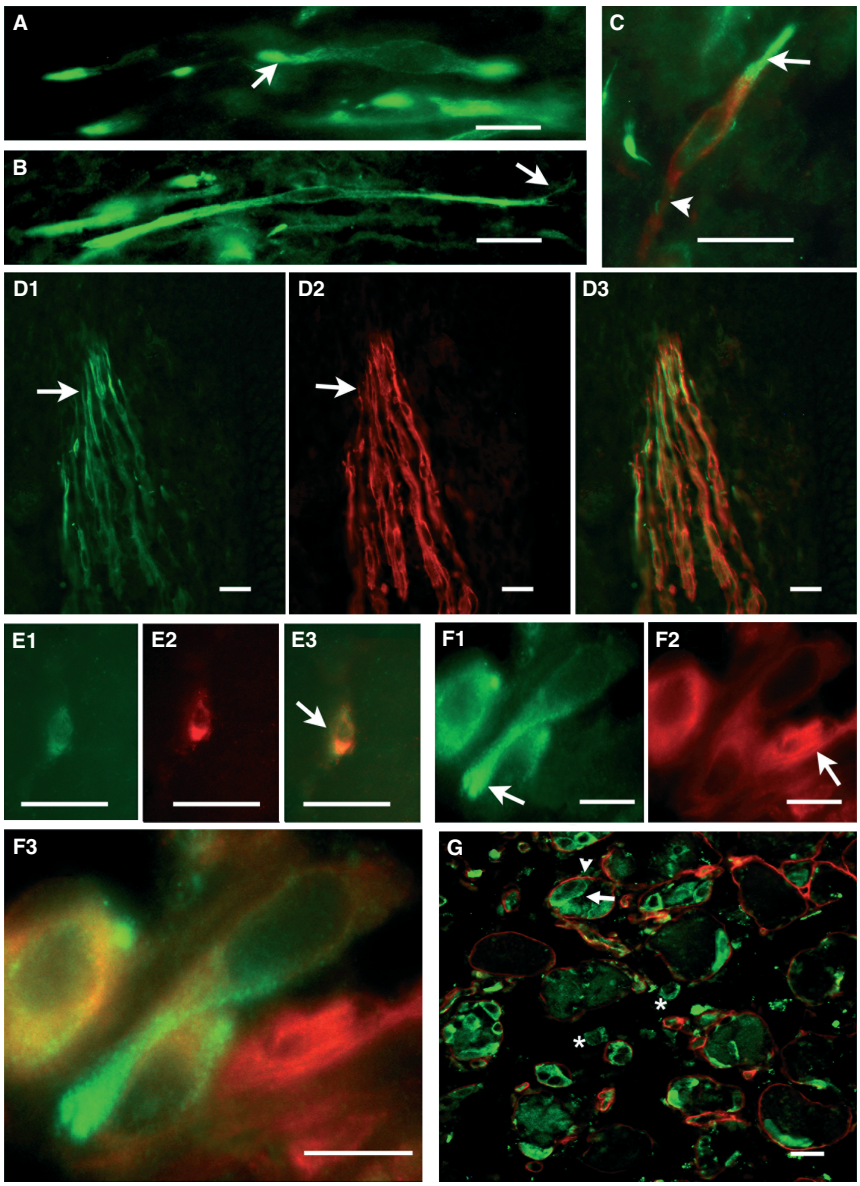

Fig. 2 Expression of $P2Y_6$ receptors in developing and injured skeletal muscle. (A) $P2Y_6$ receptor-ir in the longitudinal section of the dermamyotome at E11 at high magnification. Note that arrows indicate protrusions of the $P2Y_6$ receptor-ir cells. (B) $P2Y_6$ receptor-ir in the longitudinal section of limb muscle at E13 at high magnification. Note that the immunoreactive signals occurred in both ends of the cell. An arrow indicates a fine secondary protrusion. (C) Expression of $P2Y_6$ receptors (green) and α-sarcomeric actin (red) in the longitudinal section of an embryo at E10. Note that $P2Y_6$ receptor-ir was mainly detected in one protrusion of the cell, but α-sarcomeric actin-ir was detected in the whole cell. An arrow indicates the protrusion with $P2Y_6$ receptor-ir and an arrowhead indicates the protrusion only with α-sarcomeric actin-ir. (D1) Expression of $P2Y_6$ receptor-ir in the longitudinal section of limb muscle at E13. Note that stronger immunostaining was detected in the end of the developing skeletal muscle, in the direction of the distal part of the limb. An arrow indicates the end of the muscle. (D2) α-Sarcomeric actin immunostaining in the same section as D1. An arrow indicates the end of the muscle, which is closer to the distal part of the limb. (D3) The merged image of D1 and D2. Note that all the $P2Y_6$ receptor-ir cells also expressed α-sarcomeric actin. Expression of $P2Y_6$ receptor and C-Met in the adult skeletal muscle (E1, E2, E3). E1: expression of $P2Y_6$ receptors in the adult skeletal muscle. (E2) Expression of C-Met at the same field as E1. E3 is the merged image of E1 and E2, showing that $P2Y_6$ receptor-ir cells also express C-Met. An arrow indicates the coexistence of $P2Y_6$ receptors and C-Met. F1, F2, F3 show the coexpression of $P2Y_6$ receptors and α-sarcomeric actin in the longitudinal section of the muscle mass 5 days after injury. (F1) $P2Y_6$ receptor-ir cells. An arrow indicates the protrusion of $P2Y_6$ receptor-ir. (F2) α-Sarcomeric actin-ir cells. (F3) the merged image of F1 and F2. Note that all the $P2Y_6$ receptor-ir cells were also immunoreactive for α-sarcomeric actin (yellow), although some of the α-sarcomeric actin (red) cells were not immunoreactive for $P2Y_6$ receptors. An arrow indicates the cell with α-sarcomeric actin-ir, but not with $P2Y_6$ receptor-ir. (G) Co-localization of laminin-ir and $P2Y_6$ receptor-ir in the injured muscle 3 days after injury. Note that the majority of the $P2Y_6$ receptor-ir cells (green) were surrounded by laminin-ir basement membranes. An arrow indicates a myoblast (green) surrounded by lamina (red), which is indicated by an arrowhead. Stars show $P2Y_6$ receptor-ir cells not surrounded by lamina. Scale bars: 50 μm.

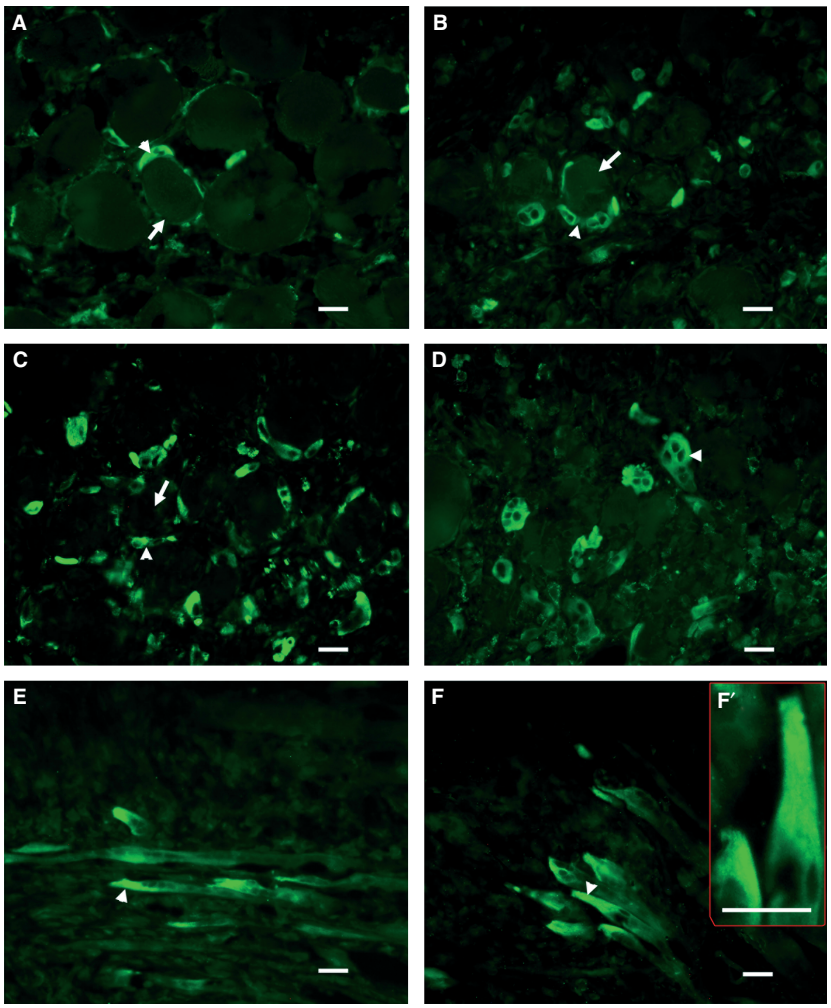

Fig. 3 Expression of $P2Y_6$ receptors in injured skeletal muscle. (A,B, C and D) Expression of $P2Y_6$ receptors in skeletal muscle 1, 3, 5 and 7 days after injury, respectively. Note that skeletal muscle fibers were surrounded by $P2Y_6$ receptor-ir cells and the number and the volume of the positive cells increased in A–C. Arrows indicate the degenerating muscle fibers and arrowheads the myoblasts in (A–C). Polynuclei myotubes were detected in (D). Arrow indicates a $P2Y_6$ receptor-ir polynuclei myotube. (E,F) $P2Y_6$ receptor-ir in the longitudinal sections of muscles 5 and 7 days after injury, respectively. Note that there were many myotubes with $P2Y_6$ receptor-ir detected and the immunostaining signals were mainly detected at the ends of the myotubes, which are directed towards the distal or proximal ends of the injured muscle. (F′) High magnification of an area indicated by an arrowhead in (F). Scale bars: 50 μm.

of the antiserum with the peptide antigen resulted in the absence of the band (Fig. 5A), indicating that the $P2Y_6$ receptor antibody detected the appropriate antigen sequence.

Western blot analysis was used to detect $P2Y_6$ receptor protein levels in the intact and injured tibialis anterior muscles of mice (Fig. 5B). Using the same antibody as for the immunohistochemistry, a single band of approximately 45 kDa was identified in the intact group (Fig. 5B, control lane). As shown in Fig. 5B, lanes 1d, 3d and 5d, the $P2Y_6$ receptor protein levels increased significantly as compared with that of the control. In the 7-day group, the $P2Y_6$ receptor protein level decreased compared with that of the 5-day group. By 10 days after injury, the $P2Y_6$ receptor protein level had almost decreased to that of the control. Real-time PCR analysis was also used to detect $P2Y_6$ receptor mRNA levels in the intact and injured tibialis anterior muscles of mice (Fig. 6). A similar pattern of $P2Y_6$ receptor mRNA change was seen during the injury-repair process. The peak duration of $P2Y_6$ receptor mRNA was 5 days after injury and by 10 days, the level of $P2Y_6$ receptor mRNA was similar to that of the control group (Fig. 6).

Discussion

In this study, using immunohistochemistry, Western blot and real-time PCR, we have shown that the $P2Y_6$ receptor is expressed in developing skeletal muscles of the pre- and postnatal mouse, and in normal and injured skeletal muscle cells of the mouse. Specificity of $P2Y_6$ antibodies on mouse skeletal muscle tissues was tested by Western blotting with normal and injured muscle tissue crude protein extracts. A single $P2Y_6$ receptor reactive band of approximately 45 kDa was detected. The preabsorption of $P2Y_6$ receptor antibodies with their relative peptides abolished nearly all immunoreactivity to these antibodies in skeletal muscle tissues. The molecular weight of the reactive band for the $P2Y_6$ receptor was higher than that expected (a theoretical molecular weight of 37 kDa). Post-translational modification of the $P2Y_6$ protein, by glycosylation for example, would result in the detection of proteins with different molecular weights.

The expression characterization of $P2Y_6$ receptors during development and in injury and repair of adult mouse skeletal muscle implies that $P2Y_6$ receptors might be involved in the development and regeneration of mouse skeletal

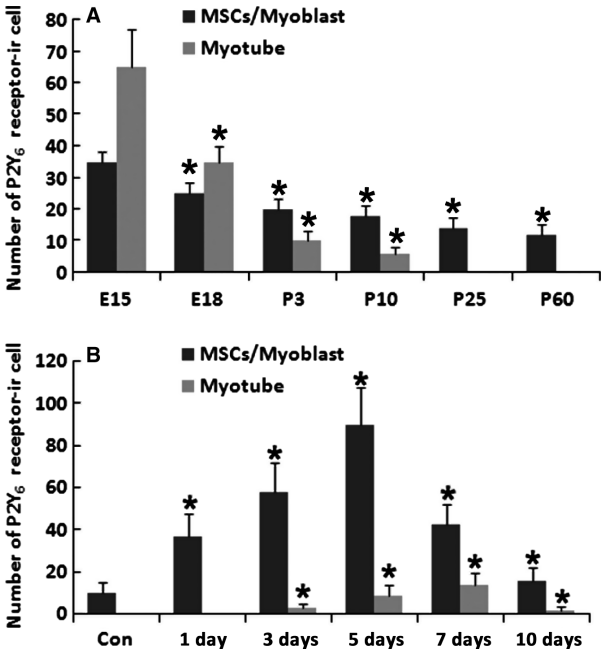

Fig. 4 Bar charts showing the number of cells expressing $P2Y_6$ receptor immunoreactivity at different developmental ages and the different injured groups of mice. (A) Con: control group; 1d, 3d, 5d, 7d and 10d are groups 1, 3, 5, 7 and 10 days after injury. All bars show mean ± SE of the mean. Statistical analyses were by one-way ANOVA followed by unpaired *t*-test. Significant differences in $P2Y_6$ receptor-ir cells between the control group and the injury groups (1d, 3d, 5d and 7d) were detected (*$P < 0.05$). (B) E15, E18, P3, P10, P25 and P60 are groups E15, E18, P3, P10, P25 and P60. All bars show mean ± SE of the mean. Statistical analyses were by one-way ANOVA followed by unpaired *t*-test. The differences in $P2Y_6$ receptor-ir cells between E15 and the other age groups (E18, P3, P10, P25 and P60) were significant (*$P < 0.05$).

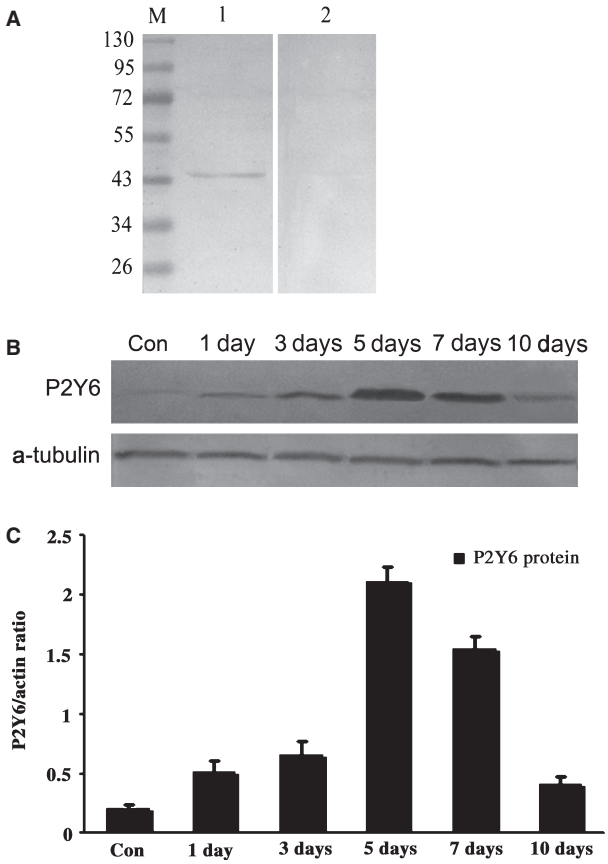

Fig. 5 Expression levels of $P2Y_6$ receptors in the tibialis anterior muscle of normal and injured mice (A–C). (A) M: molecular weight marker. Lane 1: $P2Y_6$ receptor immunoreactive band is located at about 45 kDa. Lane 2: preabsorption of the $P2Y_6$ receptor antisera with its peptide antigen, which resulted in the absence of the band. (B) Control lanes (con), 1d, 3d, 5d, 7d and 10d represent the expression levels of $P2Y_6$ receptors and α-tubulin in the control and 1-, 3-, 5-, 7- and 10-day groups, respectively. (C) Ratio of $P2Y_6$ receptors and α-tubulin immunostaining signals in control (con) and injury groups. The ratio of $P2Y_6$ to α-tubulin ratio in Western blot is expressed as mean ± SE of the mean ($n = 4$). Significant differences in $P2Y_6$ receptor expression levels between the control group and each of the injury groups (1-, 3-, 5-, 7- and 10-days) were detected (*$P < 0.05$).

muscle, such as the differentiation, proliferation, migration, fusion and extension of myoblasts and myotubes.

The distribution pattern of $P2Y_6$ receptor-ir in a single cell is polar or asymmetrical. $P2Y_6$ receptor-ir signals were mainly in one protrusion or both protrusions in these cells, where the immunostaining signals were very strong. It has been reported that after migration into the forming myotome, the round myoblasts start to elongate along the rostrocaudal axis, growing in the rostral and caudal directions at the same time, until they span the somite completely (Denetclaw et al. 1997; Gros et al. 2004). On the basis of $P2Y_6$ receptor distribution patterns during early embryo development and regeneration after skeletal muscle injury, we suggest that $P2Y_6$ receptors might be involved in the guidance of the myoblast and myotube migration and extension. Communication between myotubes and tendon cells is very important for myotube migration and, finally, attachment to the tendon cell (Schnorrer & Dickson, 2004). ATP and ADP have been reported previously to act as chemotatic factors. ATP binding to $P2X_4$ receptors and ADP binding to $P2Y_{12}$ receptors induces microglial chemotaxis via the PI3K pathway (Honda et al. 2001; Ohsawa et al. 2007). ATP or UTP binding to $P2Y_2$ receptors guides neutrophil chemotaxis via the PKC-ERK pathway (Chen et al. 2006; Meshki et al. 2006).This suggests that the tendon precursors might release UDP, which guides the myoblasts and myotubes together.

The endogenous ligand of mouse, rat and human $P2Y_6$ receptors is UDP (Chang et al. 1995; Communi et al. 1996; Nicholas et al. 1996; Lazarowski et al. 2001). Previous data have shown that UDP, via activation of $P2Y_6$ receptors, can mediate physiological functions through cAMP-protein kinase A (an indirect effect possibly mediated by prostaglandins) and Ca^{2+}-dependent protein kinase C pathways (Chang et al. 1995; Robaye et al. 1997; Kottgen et al. 2003).

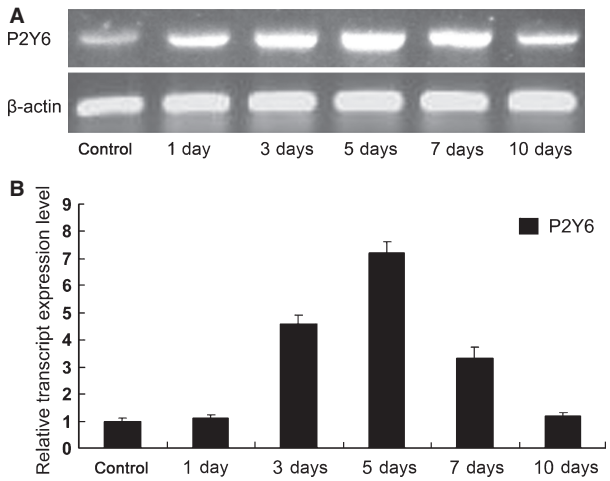

Fig. 6 Transcription level changes in $P2Y_6$ receptor mRNA in the tibialis anterior muscle after injury (A,B). (A) Control lane (con), 1d, 3d, 5d, 7d and 10d represent the transcription levels of $P2Y_6$ receptors and α-tubulin mRNA in the control and 1-, 3-, 5-, 7- and 10-day groups, respectively. (B) Relative transcription level of $P2Y_6$ receptors and α-tubulin mRNA in control and injury groups. The relative transcript level in real-time PCR is expressed as mean ± SE of the mean (n = 4). The differences in $P2Y_6$ receptor expression levels between the control group and the injury groups (3-, 5- and 7-day) were significant (*$P < 0.05$).

Cell locomotion involves changes of the cell cytoskeleton, such as microtubule and actin, which play crucial roles in growth cone motility, axon outgrowth, and guidance (Dent & Gertler, 2003) and in the shape, interaction, movement and extension of myoblasts (Swailes et al. 2004; Guerin & Kramer, 2009). As mentioned previously, the signal pathways of UDP-activated $P2Y_6$ receptors may be involved in cAMP-protein kinase A and Ca^{2+}-dependent protein kinase C pathways. Thus these two pathways might take part in the regulation of the cytoskeleton in the protrusion of the myoblast and myotube during the development and regeneration of mouse skeletal muscle.

In summary, we show for the first time that $P2Y_6$ receptors can be detected during the development, injury and regeneration processes of mouse skeletal muscle, using the methods of immunohistochemistry, Western blot and real-time PCR. Expression of the $P2Y_6$ receptor was first detected in the dermamyotome at E9. The number and immunostaining intensity of the $P2Y_6$ receptor-ir cells increased from E9 and decreased from E15 to adulthood. The expression levels of $P2Y_6$ receptor protein and mRNA increased rapidly from 1 to 5 days after skeletal muscle injury and decreased almost to the control level at 10 days. The distribution pattern of $P2Y_6$ receptors in developing skeletal muscle cells and myotubes was polar during the embryo days and regeneration after injury. These data suggest that $P2Y_6$ receptors may be involved in the development of skeletal muscle and regeneration processes after skeletal muscle injury.

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Authors' contributions

Z. Xiang, H. Zheng and G. Burnstock designed the research. D. Chen, W. Wang, W. Guo and Q. Yu performed the research. Z. Xiang, D. Chen and W. Wang analyzed data. Z. Xiang H. Zheng and G. Burnstock wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Immunostaining with $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor antibodies in normal and injured skeletal muscle tissues.

Fig. S2. Immunostaining with $P2Y_{12}$ and $P2Y_{13}$ receptor antibodies in normal and injured skeletal muscles.

Fig. S3. Double immunolabeling of $P2Y_6$ and ED1 (a marker for macrophages) in injured skeletal muscle.

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