Role of the P2X7 Receptor in the Osteogenesis of Heterotopic Ossification of the Achilles Tendon

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Research Article

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Abstract

Background

Heterotopic ossification (HO) refers to a painful and complex disease. HO occurs in the setting of persistent systemic inflammation and appears in flare-ups during inflammation, following injury. In the recent research, the P2X7 receptor (P2X7R) is tightly involved in the osteogenesis of periodontal ligament stem cells under the inflammatory conditions. The ionotropic P2X7 receptor (P2X7R) is an ATP-gated ion channel expressed in the majority of stem cells. However, the function of P2X7R in the pathological formation of HO is unclear. Here, this paper hypothesizes that in the model of Achilles tendon ectopic ossification, P2X7R is overexpressed in tendon-derived stem cells and promote osteogenesis of tendon-derived stem cells under inflammatory conditions.

Methods

The tenotomy puncture and burn injury-induced HO model was constructed. The qPCR and immunofluorescence were used to detect the expression of P2X7R at the site of injured Achilles tendon where HO occurs. Achilles tendon stem cells (SCs) from control group and experimental group sources were cultivated separately under inflammatory conditions. The cells from the two groups were cultured for osteogenic analysis. In addition, a specific antagonist of P2X7R, BBG was used to detect whether reversed the above process. At last, BBG was used to intervene in animal models of heterotopic ossification.

Results

Under inflammatory conditions, P2X7R expression of the Achilles tendon and osteogenic capability of SCs is higher in heterotopic ossification group (HOG) than in other two groups. The P2X7R expression was positive correlated with the capacity of osteogenesis of SCs. BBG can inhibit osteogenic differentiation and subsequent bone formation in the P2X7R overexpress of SCs. BBG impeded the heterotopic bone formation in animal model.

Conclusions

P2X7R is one of the crucial mediators in the formation of the HO, blocking which may represent a potential therapeutic target for HO.

Background

Heterotopic ossification (HO) refers to a complex, reactive, musculoskeletal condition characterized by ectopic bone formation in muscles, tendons, or other soft tissues [1]. HO tends to be accompanied by
trauma, burns, and orthopedic surgeries etc. There are many theories explaining why it develops. However, the mechanism remains largely unknown. Therefore, there are no completely effective treatment methods that can be taken to prevent this clinically devastating pathological condition.

Tissues vulnerable to HO act out a dysregulated inflammatory response to injury. Dwelling in a prolonged or abnormally heightened inflammatory microenvironment [2, 3], stem cells including mesenchymal stem cells (MSCs) that attempt to repair tissue differentiate into osteogenic cells instead of its original fate. Moreover, such osteogenic cells have witnessed the intensifying formation of bone which has been recognized as a fundamental process for HO. Adenosine triphosphate (ATP) is a key modulator of inflammation [4]. Prior research has illustrated that extracellular ATP is of importance to inflammation-induced HO, as it interacts with bone morphogenetic protein–mediated canonical SMAD signaling [5]. Moreover, with the application of apyrase, an enzyme that hydrolyzes ATP to adenosine monophosphate (AMP), sees a rise in intracellular adenylate cyclase activity and cyclic AMP (cAMP). The reduction of bone formation was achieved at the site of HO [5].

The ionotropic purine type 2X7 receptor (P2X7R) is a nonspecific cation channel expressed in most stem cells. Activated by extracellular ATP, P2X7R plays a wide range of physiological and pathological roles in inflammation and inflammation-related condition[6]. It is noteworthy that P2X7R expression on bone marrow MSCs has been found to regulate the formation of osteoblasts at different stages to orchestrate bone metabolism [7]. Also, recent researches have confirmed the crucial role for P2X7R in periodontal ligament stem cells (PDLSC) osteogenesis under inflammatory condition [8]. Nonetheless, the expression pattern of P2X7R on Achilles tendon stem cells (SCs) from the HO site and whether it is essential to the mediation of ATP signaling-related bone formation remain unknown.

Brilliant Blue G, BBG, is a blue dye. It is also known as acid blue 90 and Coomassie brilliant blue G. It was found to be a highly selective P2X7R antagonist[9]. BBG has been used in biological fields, as it binds nonspecifically to most proteins. However, the pharmacological function of the dye remains unconfirmed[10]. It have been proved to be useful in treating glaucoma[11]. BBG has been investigated in a number of neurological and inflammatory diseases[12]. BBG has been shown to attenuate multiple disease characteristics of the neurology department, such as depressive like effect, Parkinson's disease, Graft versus Host Disease, and spinal cord injury[12–15]. BBG as a neuroprotective agent in retinal degeneration linked to excessive extracellular ATP[16]. However, the effects of BBG on the formation of HO has not been reported in the literature as far as we know.

Here, this paper applied an Achilles tendon puncture and burn injury-induced HO model. The increasing expression of P2X7R emerged at the site of injured Achilles tendon where HO occurs. Subsequently, Achilles tendon stem cells (SCs) from HO group have a stronger osteogenic capability under the inflammatory conditions compared to the sham group. An enhancement of osteogenesis was also observed along with an overexpression of P2X7R of SCs under inflammatory conditions. In addition, BBG, an P2X7R specific antagonist successfully reversed the above process, which may suggest that it could be a potential therapeutic target for HO.
Method

Animal model

The animal experiment was grouped into three cohorts, sham group (Sham), Achilles tendon puncture group (Tendon puncture group, TPG), 30% total body surface area (TBSA) partial thickness burn injury combined Achilles tendon puncture group (HOG), respectively. Partial thickness scald injury was performed as Peterson JR et al. described\cite{17}. In brief, 8 weeks, male, C57BL/6 mice in sham group, the Achilles tendons were exposed alone. In TPG, after Achilles tendon exposed, a 27G needle was punctured into the Achilles tendon body perpendicularly at different parts from proximal to distal and this process was repeated three times. In HOG, apart from the Achilles tendon punctured, mice also received about 30% TBSA burn injury with exposure to 60°C for 18 s (Fig. 1a) \cite{18}.

Cell harvest and in vitro culture assays

Achilles tendon stem cells (SCs) were harvested from the different groups. Stem cells (SCs) from Achilles tendon were isolated based on the research Xu et al. reported \cite{8}, and modified slightly. In short, freshly harvested tendon was washed with sterile phosphate-buffered saline (PBS; Gibco, New York, USA), and then tendon tissue was placed into 30mm dish. The tissue was cut into fragments and digested in 3 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, USA) for 45 minutes. Then, the digested fragments were cultivated in 35 mm dish (Corning, New York, USA) in α-minimum essential medium (α-MEM, Gibco) containing 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), and 10% fetal bovine serum (FBS; Gibco, Australian). In in vitro experiment, the complete culture medium was changed every 3 days. Cell passage was performed until the cell fusion rate reached about 80~90%. SCs at passage 2-5 (P2-P5) were used for further experiment. For osteogenic differentiation, complete osteo-inductive medium was replaced every 2 days when the percentage of cell fusion dipped to 60–70%. Osteo-inductive medium contained α-MEM and 10% FBS supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM β-glycerophosphate, 50 µg/mL L-ascorbic-2-phosphate and 10 nM dexamethasone (all from Cyagen). TNF-α (10 ng/ml) and IL-1β (5 ng/ml) was added to the medium of SCs to simulate inflammation environment in vitro\cite{19}.

Micro CT

Micro CT imaging of HO of Achilles tendon was obtained from all animals by means of the high-resolution micro-CT equipment (Siemens, Munich, Germany). CT scan settings: 80 kV, 500µA, and 1100-ms exposure. The bone mineral in the soft tissues was quantified for HO bone volume using standard protocols as Peterson et al. described\cite{20}.

Immunofluorescent Staining

MSCs were seeded into a 96-well culture plate designed for confocal microscopy and cultivated in α-MEM containing 10% FBS. When the confluence of cells reached 80~90% (day 0), different condition was added respectively for intervention. After 7d culture or 14d osteogenesis inducing, adherent cells were
fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 15 min and blocked with 10% goat serum for 40 min at room temperature. The cells were then incubated at 4°C with an antibody against P2X7R (1:200, Abcam, ab48871) overnight. The samples were washed with PBST, and then incubated in the dark place at 25°C with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:500; Abcam, ab150077) for 2 h. Finally, the cells were stained in a dark room at 25°C with DAPI for 30 min. Images were captured by a confocal laser scanning microscopy (A1R S1; Nikon, Tokyo, Japan).

**ALP staining**

ALP activity in SCs was analyzed with ALP staining. In brief, cells were seeded at 1×10⁴ cells/well in 96-well plates. ALP staining was performed after 14 days of osteo-inductive culture using an ALP staining kit (Beyotime Biotechnology, China) according to the instructions. The quantitative analysis of ALP staining was performed with the help of an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, China).

**ARS staining**

According to the manual instructions, Alizarin red staining (ARS, Cyagen, Suzhou, China) was completed which was used to evaluate the osteogenic differentiation of cells. To quantify the staining for statistical analysis, the mineralized nodes were dissolved in 2% cetylpyridinium chloride for 20 min, and then OD values were measured at 560 nm wavelength[21].

**qRT-PCR assays**

Total RNA was extracted using RNAeasy Kit (Vazyme, RC112-01) and 1 µg of total RNA was used to generate cDNA using HiScript II Q RT SuperMix (Vazyme, R223-01) for qPCR. Quantitative Real time PCR (qRT-PCR) was performed on cDNA samples diluted twentyfold in double-distilled water using SYBR Green Master Mix (Vazyme, Q711). The CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used. To amplify them under the following conditions: denaturation at 95°C for 5 min; 40 cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s, with melt curve analysis from 65 to 95°C in increments of 0.5°C. The gene-specific qRT-PCR primers were specific for mouse. Relative mRNA gene expression levels were measured by qRT-PCR. Relative quantification for qRT-PCR was calculated using the 2−ΔΔCT method. The sequences of primers used in the present study are shown in supplementary Table. 1.

**Statistical Analysis**

All results are calculated by the mean and standard deviation (mean ± S.D.). There are more than three samples. Experiments for each cell were performed independently for three times. GraphPad Prism 9 software was utilized for statistical tests. The Student's t-test was used for analysis of two unpaired groups, and one-way ANOVA followed by Tukey's posttest for analysis of multiple groups. Statistical significance was established at p < 0.05.

**Ethics statement**
The ethics committee of the general hospital of People Liberate Army of China and Harbin Medical University approved this experiment. All methods were performed in accordance with the relevant guidelines and regulations from the same.

Results

**P2X7R expression of the Achilles tendon is higher in HOG than in other two groups**

In this study, TPG and HOG all significantly had given rise to the HO. The largest size of heterotopic ossification formation was in HOG, and the no HO was observed in the sham group (Fig. 1b). Then, this paper compared the bone volume (BV) of ectopic ossification (EO) in different groups (Fig. 1c). These disparities were of statistical significance. BV in HOG and TPG outstripped that of Sham, \( p \) values were less than 0.0001 and 0.0001, respectively. There was a significantly higher BV in the HOG than in the TPG \( (p<0.01) \) (Fig. 1b). Burn injury enhanced bone formation as previous reports [22, 23].

After the establishment of the model, at 7 days, the Achilles tendon was resected for further study. Then, the Achilles tendon tissues of different groups received pathological analysis. Among three groups (HOG, TPG and Sham), the fluorescence intensity of P2X7R in Achilles tendon was considerably different from each other \( (p<0.05) \) (Fig. 1d). In HOG and TPG, the immunofluorescence (IF) intensity of P2X7R was significantly higher compared with sham group \( (p<0.001) \) (Fig. 1e).

As can be seen from the above results, positive relationship between the expression of P2X7R and the capacity of osteogenesis of Achilles tendon tissue under inflammatory condition. The result indicates that the higher expression of P2X7R, the higher BV of EO of Achilles tendon tissue. Therefore, we speculated that the higher expression of P2X7R in the inflammation condition plays an important role in the formation of HO.

**The P2X7R overexpression of SCs promoted osteogenesis under inflammation condition**

To further investigate the role of increased expression of P2X7R on osteogenesis under inflammatory condition, SCs of Achilles tendon extracted from Sham and HOG separately were used. Evidenced by IF, P2X7 receptor expression is lower in SCs from Sham compared to HOG (Fig. 2a). Then, osteogenic differentiation was evaluated by alkaline phosphatase (ALP) staining. After 7 days of the osteogenic induction of SCs under inflammatory condition, osteogenesis differentiation was assessed by ALP (Fig. 2b). The results of the ALP positive cells suggested the difference between the two groups. SCs from HOG had better osteogenesis compared with another groups. The software of Image J was employed to quantify ALP staining. The ability of osteogenesis of SCs from HOG was higher than that of the TPG and sham on account of a higher cell ALP staining positive area rate (Fig. 2b) \( (p<0.0001, 0.001, \text{ respectively}) \).

To test the osteogenic differentiation variances of SCs between the Sham and HOG, Alizarin Red staining(ARS) were performed, and then ARS solution absorbance was measured. Under inflammatory
conditions, SCs overexpressing P2X7R brought about a remarkable surge of mineralized nodes formation. The results of ARS staining was also similar to ALP \((p<0.05)\) (Fig. 2c). ALP activity and ARS staining revealed higher osteogenic activity in overexpressing P2X7R of SCs. According to the qRT-PCR analysis, mRNA expression levels of osteogenic-associated genes such as OCN, RUNX2, OPN were significantly higher in SCs overexpressing P2X7R (Fig. 2d). Based on the above data, under inflammation condition, the ability of osteogenesis of SCs overexpressing P2X7R were enhanced.

**Under inflammatory conditions, BBG inhibit SCs overexpressing P2X7R osteogenic differentiation**

To prove the effect of overexpression of P2X7R on osteogenic differentiation under inflammatory conditions, the P2X7R antagonist BBG was employed. SCs overexpressing P2X7R was used. BBG (10 \(\mu\)M) was added into osteo-inductive medium under inflammation condition as intervention group. Control group is no added BBG. Osteogenic differentiation of SCs was evaluated by ALP and ARS staining. There is a significant difference of osteogenic differentiation of SCs between intervention and control groups. The intervention groups presented weak ALP staining. The ARS staining results were consistent with the ALP staining results (Fig. 3a). The results of ALP activity revealed that ALP activity in control group was higher than intervention group (Fig. 3b) \((p<0.05)\). The results of ARS staining also had the similar result as ALP (Fig. 3b) \((p<0.01)\). Additionally, the expression levels of osteogenesis-related genes were assessed by qRT-PCR. According to the qRT-PCR, the mRNA expression of osteogenic-related genes in the intervention group, such as OCN, RUNX2, OPN, deceased significantly \((p<0.0001, p<0.0001, p<0.01, \text{respectively})\) (Fig. 3c). Taken together, under inflammation condition, the intervention of BBG could control the enhanced osteogenesis of SCs overexpressing P2X7R. Cytological results of BBG intervention may provide a novel treatment for HO.

**Intervention of BBG reduced heterotopic bone formation in animal model**

To further confirm whether the therapeutic effects of intervention of BBG in animal model, animal experiments were carried out. HO animal model of the 30% TBSA partial thickness burn injury combined Achilles tendon puncture was used. 6 hours after the modeling, the intervention of BBG (50 mg/kg) by intraperitoneal administration was employed. The BBG administered once daily for 14 consecutive days, and the mice were watched for 12 weeks. Saline solution was injected as the control. Micro-CT analysis was conducted to evaluate heterotopic bone formation at 1st, 6th and 12week (Fig. 4a). One day after BBG injection, the skin of mice showed blue pigmentation (Fig. 4b). At the 1st week, there was no heterotopic bone formation between two groups. At the 6th week, there was heterotopic bone formation in two groups. The difference of bone volume (BV) of heterotopic bone between two groups was statistically significant \((p<0.05)\). At the 12th week, the difference of BV was more significant between the two groups \((p<0.0001)\) (Fig. 4c). Based on the result of animal experiment, treatment of BBG reduced heterotopic bone formation in HO model.

**Discussion**
Inflammation has been confirmed to be a key driver of HO [24]. A commonality across many of the conditions that predispose to HO formation such as autoimmune diseases, trauma, burn etc. is that they are more or less related to inflammation [22]. Clinical application of NSAIDs for HO prophylaxis rests on the theory that reducing postoperative inflammation will likewise reduce HO formation albeit that no robust evidence has been provided [1]. Prior review by Benjamin Levi et al. summarized the crucial role of inflammation in development of HO[25]. Among which, processes involved in innate immunity, adaptive immunity, secretion of inflammatory cytokines had all been demonstrated to be of importance underlying the mechanism of HO [25].

In our study, P2X7R in the Achilles tendon tissue of HO animal model is overexpressed. The latest research results indicated that P2X7R overexpression promoted MSCs to osteogenic differentiation under inflammatory conditions[8]. But Effect of P2X7R in HO were not reported. In HO model, prior research has shown that extracellular ATP acted as a signal transducer, which interacts with bone morphogenetic protein mediated canonical SMAD signaling to promote HO development [5]. Moreover, with application of apyrase, a rise occurs in intracellular adenylate cyclase activity and cyclic AMP (cAMP) and a reduced bone formation was achieved at the site of HO [5]. The reduced volume of heterotopic ossification with the use of ATP hydrolase may be due to a reduction in the number of ATP. Additional, in the inflammatory conditions, due to the presence of large amounts of ATP, the P2X7 receptor may be overactivated, leading to the entry of osteogenic-related substance into cells. Similar results have been reported[26].

Almost all living organisms, from protozoa to higher mammals, have evolved a sophisticated receptor set for extracellular nucleotides. In higher mammals, the nucleotide receptor family (P2 receptors, P2Rs) is comprised of two subfamilies: G protein- coupled metabotropic P2Y (P2YR) and ligand (ATP)-gated ionotropic P2X receptors (P2XR)[27]. Among these family, P2X7R is the most studied and outstanding as the single member of the P2XR family with a firmly established role in multiple inflammatory and immune responses [6]. Prior studies have shown that P2X7R plays a crucial role of bone homeostasis with controversial conclusions [27, 28]. The P2X7R has previously been shown that it plays a crucial role in the bone anabolic under the mechanical loading [29]. P2X7R involves bone formation through Wnt/β-catenin signaling[30]. The P2X7R plays animportant role in osteogenesis procession, but its role is controversial. Earlier studies have suggested that P2X7R activation leads to apoptosis of human osteoblasts[31]. However, recent studies suggest that activation of P2X7R can promote MSC vesicle formation and bone formation in mice[32–34]. P2X7R-deficient mice showed reduced bone mass[35]. In this study, in vivo model showed an intensified expression of P2X7R in the injured Achilles tendons together with a formation of exotic bone. Using in vitro model mimicking an inflammatory microenvironment, osteogenic differentiation of cells from Achilles tendons from two different models was used to validated. As exposed to an osteogenesis condition medium, an enhancement in osteogenesis was achieved in SCs expressed higher P2X7R. Moreover, this enhancement was attenuated when BBG was added into the medium. These results suggested that P2X7R plays a crucial role in the mechanism underlying inflammation-induced HO.
Previous studies have confirmed that P2X7 receptor is involved in the process of osteogenic differentiation. It has been reported that the osteogenic differentiation ability of MSC is weakened under inflammatory conditions, but the osteogenic differentiation ability is enhanced after the expression of P2X7 receptor is increased. Xu et al. found in their study that under inflammatory conditions, activation of P2X7R promotes osteogenesis through PI3K-Akt-mTOR signaling pathway [8]. Our findings are consistent with the results of other studies.

BBG is a P2X7R antagonist of low toxicity and high selectivity. In vitro studies have shown that BBG can inhibit osteogenic differentiation of SCs with overexpressed P2X7R under inflammatory condition. Therefore, BBG was selected to treat the HO model to test whether BBG can inhibit ectopic bone formation. Previous studies have reported that BBG treatment reduces tissue injury and promotes motor function recovery after spinal cord injury (SCI)[36, 37]. The inhibiting ectopic bone formation effect of BBG was also proved in our in vitro study.

There are some limitations in this research. Although the treatment of BBG reduces progression of heterotopic ossification in animal model, the role of BBG in the initiation occurrence of heterotopic ossification is uncertain. In clinical research, progression of heterotopic ossification in tendon leads to restricted joint mobility and pain [38]. Nevertheless, there are differences in current HO prophylaxis. Taken together, the treatment of reducing the rate of surgery is a key for HO prevention and management. Because BBG inhibits osteogenesis of SCs under inflammation condition through block P2X7R, it may provide a new therapeutic target for HO in the future. Thus, further studies are required in order to gain deeper insight into these mechanisms.

Conclusion

The expression of P2X7R was found increasing in tendon puncture and burn injury- induced HO. Under inflammatory conditions, overexpression of P2X7R promotes ectopic osteogenesis in mice model. An antagonist of P2X7R attenuated ectopic osteogenesis, which may suggest that P2X7R is a crucial mediator underlying the mechanism of HO. This study may bring forward a novel therapy for the HO.

List Of Abbreviations
| Abbreviation | Full Form |
|-------------|-----------|
| AMP         | Adenosine Monophosphate |
| ARS         | Alizarin Red Staining |
| ATP         | Adenosine Triphosphate |
| BBG         | Brilliant Blue G |
| BV          | Bone Volume |
| cAMP        | Cyclic AMP |
| HO          | Heterotopic Ossification |
| HOG         | Heterotopic Ossification Group |
| IF          | Immunofluorescence |
| MSCs        | Mesenchymal Stem Cells |
| P2 receptors| P2Rs |
| P2X7R       | P2X7 Receptor |
| P2XR        | P2X Receptors |
| PDLSC       | Periodontal Ligament Stem Cells |
| SCI         | Spinal Cord Injury |
| SCs         | Stem Cells |
| TBSA        | Total Body Surface Area |
| TPG         | Tendon Puncture Group |

**Declarations**

**Ethics approval**

The ethics committee of the general hospital of People Liberate Army of China and Harbin Medical University approved this experiment. Our manuscript reporting adheres to the ARRIVE guidelines. All methods were performed in accordance with the relevant guidelines and regulations from the same.

**Consent to participate**

Not applicable

**Consent for publication**

Not applicable
Availability of data and material

Availability of data and materials Data supporting the conclusions of this article are included within the article and/or the supplementary materials.

Competing interests

The authors declare that they have no competing interests.

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Code availability

The code included in this study may be available on request, with a clear statement of purpose.

Authors' contributions

The study was designed by Shi Cheng, Siqi Zhang, Jinglong Yan, Songcen Lv. Acquisition of the data was performed by Shi Cheng and Siqi Zhang. Analysis and interpretation of the data were performed by Shi Cheng and Siqi Zhang. Drafting of the manuscript, including critical revision, was performed by all authors. All authors accept responsibility for the integrity of the data analysis.

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Figures
**P2X7R expression of the Achilles tendon is higher in HOG than in other two groups**

a) Schematic of the model in three groups. b) Micro CT images of the Achilles tendon. The red represents the tissue of HO, and the white represents the normal bone tissue. c) Quantitative analysis of the bone volume (BV) of HO on the right graph. d) The protein expression levels of P2X7R (green) in Achilles tendon in three groups were quantified based on immunofluorescence staining. DAPI (blue) stained the nuclei. e) Scale bar = 100 μm. f) Quantitative analysis of the fluorescence intensity in three groups. HOG and TPG domains show higher fluorescence intensity with respect to the sham group ($p < 0.001$, $p < 0.001$, respectively). Data are presented as the mean ± S.D.; NS indicates no significant difference between the indicated columns; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ indicate significant differences between the indicated columns.
The P2X7R overexpression of SCs promoted osteogenesis under inflammation condition a) Comparison of the P2X7R (green) IF staining (confocal micrograph) of SCs from HOG and Sham separately. DAPI (blue) stained the nuclei. (Scale bar =25 μm). b) Under inflammatory condition, SCs from HOG and Sham separately were stained by ALP after 14 days of culture and quantitative results of ALP staining ($p<0.05$). c) Under inflammatory condition, SCs from HOG and Sham separately were stained by ARS after 21 days.
of culture and quantitative results of ARS staining ($p<0.01$). d) qRT-qPCR results of osteogenesis marker genes (OCN, RUNX2, OPN) of SCs from HOG and Sham separately with osteogenic differentiation medium culture. There is significant difference between two groups ($p < 0.0001$, $p < 0.0001$, $p < 0.01$, separately). Data are presented as the mean ± S.D.; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ indicate significant differences between the indicated columns.

**Figure 3**

**Under inflammatory conditions, BBG inhibit SCs overexpressing P2X7R osteogenic differentiation** a) ALP and ARS staining of SCs from HOG and Sham separately under 14 days or 21 days osteogenic differentiation culture with or without BBG. b) ALP activity and ARS absorbance is higher in no BBG group than another group. c) The mRNA expression of OCN, RUNX2 and OPN (determined by qRT-PCR) in SCs from HOG and Sham separately with osteogenic differentiation medium following a 7-day culture. With the BBG intervention, gene expression of OCN, RUNX2 and OPN is lower than the other group ($p<0.001$,
Figure 4

Intervention of BBG reduced heterotopic bone formation in animal model. a) Flow chart for animal experiment procedures. The result of Micro CT in 1st week was used as a baseline. The HO formation changes were evaluated in two groups at baseline, 6 and 12 weeks of with or without BBG treatment based on the Micro CT. b) The injected BBG turned mouse skin visibly blue, whereas no color change
occurred in another group (not shown). Blue skins are within the red circles. c) The red represents the tissue of HO, and the white represents the normal bone tissue. Quantitative analysis of BV of HO on the right graph. Data are presented as the mean ± S.D.; *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 indicate significant differences between the indicated columns

**Supplementary Files**

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- SupplementaryTable1.docx