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ABSTRACT

We previously hypothesized that the development of traumatic temporomandibular joint (TMJ) ankylosis was similar to that of hypertrophic non-union. Besides similarities in etiology, hypertrophic bone stumps, and long-term development, the radiolucent zone, frequently located in the ankylosed bone, is another common feature. In this study, we demonstrated that the radiolucent zone also contained multilineage potential cells (RZs, radiolucent-zone-related cells) as the non-union tissues. RZs were characterized and compared with mandibular bone marrow stem cells (BMSCs) by analysis of MSC-related markers, colony-forming-unit assays, multipotential differentiation assays, alkaline phosphatase (ALP) activity assays, and cell transplantation *in vivo*. Both cell types were positive for CD105, CD166, and Stro-1 expression, negative for CD34 and CD45 expression, and exhibited osteogenic, adipogenic, and chondrogenic differentiation potentials. However, compared with mandibular BMSCs, RZs showed lower colony-forming-unit abilities and proliferation rates. The mineralization and bone-forming ability of RZs was weaker than that of mandibular BMSCs, with Runx2 and ALP mRNA expression and ALP activity significantly lower in RZs. All these results suggest that RZs possess the properties of MSCs but lower proliferation and osteogenic differentiation capacity similar to that of stromal cells in hypertrophic non-union tissues.

KEY WORDS: bone non-union, pathogenesis, condyle fracture, mesenchymal stem cells, bone formation, bone proliferation.

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INTRODUCTION

Traumatic temporomandibular joint (TMJ) ankylosis, a severe joint disability, is characterized by obliteration of joint space and abnormal bone formation in and around the joint (Sawhney, 1986). Previous studies have been limited to clinical or histological studies, or the development of animal models of TMJ ankylosis (Cheung *et al*., 2007 ; Yan *et al*., 2011; Yan *et al*., 2012b). However, the pathological mechanism of TMJ bony ankylosis remains to be elucidated.

Several hypotheses have been proposed in attempts to explain the pathological mechanism of TMJ ankylosis (Arakeri *et al*., 2012). Our group hypothesized that the development of traumatic TMJ ankylosis was the result of mouth-opening interfering with the bone healing of 2 injured articular surfaces (Yan *et al.*, 2012a). Considering the etiology, hypertrophic bone mass, and long-term development of TMJ ankylosis (Aggarwal *et al*., 1990), we further hypothesized that the development of TMJ ankylosis was similar to that of hypertrophic non-union, which has also been linked to inadequate immobilization (Yan *et al.*, 2012a). Strikingly, a radiolucent zone exists in both TMJ ankylosis and hypertrophic non-union (Yan *et al*., 2012a). Furthermore, histological analysis has revealed that the radiolucent zone in both diseases contains a large quantity of fibers, cartilage, and vessels, and that neoformative endochondral ossification occurs in or at the edge of the radiolucent zone (Reed *et al*., 2002; Cheung *et al*., 2007; Yan *et al.*, 2012b). As above, the radiolucent zones in both diseases have anatomical and histological similarities. However, the cytological similarity has yet to be elucidated.

Hypertrophic non-union is considered to be a biological process whereby lack of mechanical stability interferes with union. As a result, the osseous transformation of non-union tissues fails to occur adequately with the failure of the osseous bridge at the non-union tissues (Iwakura *et al*., 2009). In addition, the osteogenesis of stromal cells derived from the non-union tissues (radiolucent zone) also decreases (Hofmann *et al*., 2008; Bajada *et al*., 2009). However, once mechanical stability is attained, the mesenchymal progenitor cells in the radiolucent zone are still believed to contribute to bone formation for healing (Iwakura *et al.*, 2009). Thus, the radiolucent zone seems like a potential reservoir of mesenchymal progenitor cells for hypertrophic non-union healing.

However, in traumatic TMJ ankylosis, no previous reports are available on whether the radiolucent zone contains mesenchymal progenitor cells as do the hypertrophic non-union tissues, and, if so, whether the osteogenesis of these cells deserves to be elucidated. In this study, we hypothesized that

multipotential cells (herein called 'radiolucent-zone-related cells', RZs) existed in the radiolucent zone and further investigated their proliferative and osteogenic capacity.

MATERIALS & METHODS

Patients

In total, eight patients with type II or type III post-traumatic TMJ ankylosis (Sawhney classification) (Sawhney, 1986) were included in this study. Detailed patient information is listed in Appendix Table 1. The control group consisted of eight volunteers (four women and four men; mean age, 31.7 ± 6.4 yrs) who had undergone tooth extraction or orthopedic surgery. None of these individuals had infections or systemic metabolic bone disease. The study was approved by the Ethics Committee of the Peking University Health Science Center (IRB00001052-11002), and written informed consent was obtained from all participants.

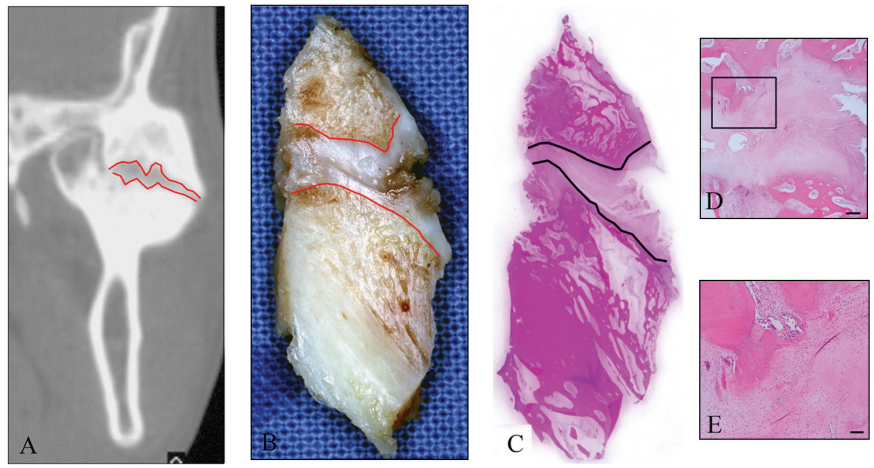

Figure 1. The specific site for cell isolation. The radiolucent zone containing fibro-cartilage tissues between the upper and lower lines was excised for cell isolation. **(A)** Computed tomography coronal view of the ankylosis TMJ. **(B)** Macroscopic view of the radiolucent zone. **(C,D,E)** Hematoxylin and eosin (H&E) staining of the radiolucent zone. (E) The black framed area of (D). Scale bar, 500 μm in (D) and 100 μm in (E).

Isolation and Culture of RZs

Isolation of RZs was conducted as previously described (Bajada *et al*., 2009). Briefly, fibro-cartilage tissues were excised from the radiolucent zone between the surgically resected upper and lower bone masses of the ankylosed TMJ (Fig. 1). The procedures are described in the Appendix.

Isolation of MSCs from Mandibular Bone Marrow

Trabecular bone was obtained from the mandibles of eight volunteers following resection of redundant bones during third molar extraction or orthopedic surgery. Bone was cut into small pieces, and then digested with collagenase type XI for 20 min at 37°C. MSCs were then isolated and cultured by the same method used for the isolation of RZs.

Colony-forming-unit Assays

Colony-forming-unit assays were performed according to a previously described method (Cheng *et al*., 2009) (see Appendix).

Cell Proliferation Assays

Third-passage cells were seeded at a density of 20,000 cells *per* well in 12-well plates in culture medium and counted by means of a hemacytometer on days 2, 4, 6, 8, 10, and 12 post-inoculation.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Alsalameh *et al*., 2004). Briefly, cells were grown to sub-confluence on coverslips in 6-well plates in culture medium. R-phycoerythrin-conjugated (PE) anti-human CD105 antibody (Ancell, Bayport, MN, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-human CD166 antibody (Ancell) were used to detect the cell surface cluster of differentiation (CD) markers. Fluorescently labeled cells were visualized and photographed by fluorescence microscopy (Olympus BX51, Olympus Optical Co., Ltd., Tokyo, Japan).

Flow Cytometry Analysis

Cells were harvested at the third passage. After 3 washes with PBS-1% FBS, the cells were incubated in the dark for 45 min at 4°C with anti-human CD34-FITC, CD45-PE (BD Biosciences, San Jose, CA, USA), CD105-PE, CD166-FITC (Ancell), and Stro-1 antibodies. The anti-mouse IgM-PE secondary antibody (R&D Systems Inc., Minneapolis, MN, USA) was used to stain Stro-1 antibody. The recommended non-specific mouse PE- and FITC-conjugated IgGs (BD Biosciences) and IgM (R&D Systems Inc.) were used as isotype controls. Flow cytometry was performed on a Coulter Epics XL (Beckman Coulter, Inc., Brea, CA, USA).

Multilineage Differentiation Potential

The third-passage cells were cultured in osteogenic, adipogenic, and chondrogenic conditions *in vitro* and also transplanted *in vivo* as described in the Appendix.

Reverse-transcription PCR and Real-time PCR Analysis

Total RNA was isolated from the third-passage cells with TRIzol® reagent (Invitrogen Life Technologies, Grand Island, NY, USA). Reverse-transcription PCR (RT-PCR) and real-time PCR were performed as previously described (Wu *et al*., 2010). The primer sets used are listed in Appendix Table 2. Glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA expression was used as internal control. The relative mRNA

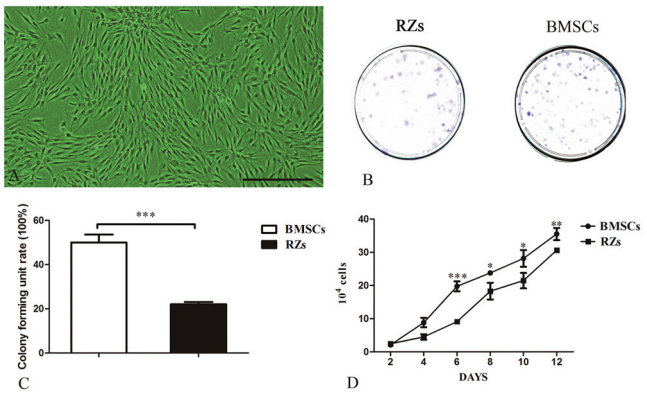

Figure 2. Proliferation of radiolucent-zone-related cells (RZs). **(A)** Fibroblast-like cells derived from radiolucent zone tissues (scale bar, 500 μm). **(B)** Colony-forming units of RZs and bone marrow stem cells (BMSCs). **(C)** Statistical analysis showed that colony-forming ability of RZs was significantly lower than that of BMSCs (n = 5, $***p < .001$). **(D)** Comparison of proliferative rates of BMSCs and RZs (third passage) over 12 days showed that the BMSCs sustained higher proliferative rates than RZs (n = 6, $*p < .05$,$**p < .01$,$***p < .001$).

expression of genes was calculated as described previously (Livak and Schmittgen, 2001).

Alkaline Phosphatase Activity

Alkaline phosphatase activity (ALP) activity was measured with the ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The protocol is in accordance with a previous study (Zhang *et al*., 2011) and the manufacturer's instructions. Concentrations of protein extracted from the cells were determined by the bicinchoninic acid protein assay (BCA reagent, Thermo Fisher Scientific Inc., Waltham, MA, USA). The relative ALP activity was normalized to protein concentration.

Statistical Analysis

Data are presented as the mean ± standard error (SEM) and were analyzed with SPSS software, version 18.0. (SPSS Inc., Chicago, IL, USA). We performed the Shapiro-Wilk test to test the normality of all values. The normally distributed values were tested by independent Student's *t* tests, and non-normally distributed values were tested by the Mann-Whitney U test. Here, $p < .05$ was considered to indicate statistical significance.

RESULTS

RZs Showed Lower Proliferative Ability than Mandibular BMSCs (bone marrow mesenchymal stem cells)

Cells derived from the radiolucent zones of all eight patients were mainly composed of fibroblast-like cells (Fig. 2A) and reached 80% to 90% confluence after 10 to 14 days. The colony-forming efficiency of RZs (22 ± 1.1%) was lower than that of mandibular BMSCs (50 ± 3.6%) (Figs. 2B, 2C). The proliferation rate of RZs was also lower at days 6, 8, 10, and 12 (Fig. 2D).

Immunophenotype of RZs

Both RZs and mandibular BMSCs were positive for the MSC-related markers CD105, CD166, and Stro-1 and negative for CD34 and CD45 (Appendix Fig.1A). Fluorescent analysis confirmed co-expression of CD166 and CD105 by RZs and mandibular BMSCs at the single-cell level (Appendix Fig. 1B).

RZs Exhibited Multilineage Differentiation Potential

After 21 days of osteogenic induction, both RZs and mandibular BMSCs formed mineralized matrix, as shown by alizarin red staining, with staining of mandibular BMSCs tending to be more extensive (Fig. 3A), which was further confirmed by *in vivo* bone formation. Corresponding induction of mRNA expression of Runx2, ALP, and osteocalcin (OCN) gave additional support (Fig. 3B).

To determine whether RZs undergo adipogenesis, we cultured RZs and mandibular BMSCs in adipogenic medium for 21 days and stained them with Oil Red O. Both cell types exhibited intracellular lipid droplet accumulation (Fig. 3C). Correspondingly, mRNA expression of peroxisome proliferator-activated receptor gamma-2 (PPARγ2) and lipoprotein lipase (LPL) increased in the cells under adipogenic conditions for 14 days (Fig. 3D).

Chondrogenic differentiation was induced in RZs and mandibular BMSCs from four patients and four donors. After 28 days, cell pellets from both RZs and mandibular BMSCs had a spherical and glistening appearance; cartilage development in the cell pellets was detected by type II collagen immunohistochemistry (Fig. 3E). Toluidine blue staining of both RZs and mandibular BMSCs also confirmed their chondrogenic capability (Fig. 3F).

Comparison of Osteogenesis between RZs and Mandibular BMSCs

We then examined whether the osteogenic potential of the RZs was different from that of BMSCs by evaluating the bone-related mRNA expression under osteogenic induction and *in vivo* bone formation. Expression of Runx2 mRNA by BMSCs was 3-fold (3.00 ± 0.70 *vs*. 1.04 ± 0.13) and 4.2-fold (4.20 ± 1.30 *vs*. 1.00 ± 0.25) higher than that of RZs at days 7 and 14, respectively, while the mRNA expression of ALP of BMSCs was 23-fold (23.76 ± 7.60 *vs.* 1.00 ± 0.8) and 19-fold (19.00 ± 6.30 *vs.* 1.00 ± 0.40) higher than that of RZs, respectively (Fig. 4A). Correspondingly, ALP activity was significantly higher in mandibular BMSCs than in RZs at days 3, 7, and 11 (Fig. 4B). However, no difference in the mRNA expression of the mature osteoblastic gene OCN was detected between the 2 cell types. Moreover, alizarin red staining of calcium deposition showed that the relative intensity of the staining in BMSCs was 1.9-fold (102.70 ± 9.80 *vs*. 53.60 ± 4.73) higher than that in RZs after osteogenic induction for 21 days (Fig. 4C). Although both RZs and BMSCs showed bone-regenerative ability *in vivo*, BMSCs had a greater capacity for bone formation than did RZs (Figs. 4D, 4E).

DISCUSSION

In the present study, we demonstrated for the first time that RZs derived from the radiolucent zone of clinically resected ankylosed masses of traumatic TMJ ankylosis possess MSC-like properties. Self-renewal and differentiation potential are 2 important properties of stem cells, in addition to being the key characteristics used to define MSCs (Pittenger *et al.*, 1999). RZs derived from the radiolucent zone of resected ankylosed masses of traumatic TMJ ankylosis were successfully expanded and subcultured and shown to form colonies efficiently. RZs had the potential to differentiate into osteoblasts, chondrocytes, and adipocytes under appropriate inductions. Moreover, their phenotypic characteristics were very similar to those of mandibular BMSCs, although RZs showed lower proliferative and osteogenic capacity. All these results indicated that RZs possess the 2 essential properties characteristic of stem cells. Therefore, we defined them as MSC-like stromal cells. These results also suggest that RZs in the radiolucent zone are capable of differentiation into chondrocytes and osteoblasts under certain pathological conditions, resulting in progressive endochondral ossification. The observation that the radiolucent zone contains MSC-like cells is very similar to that in hypertrophic non-union (Iwakura *et al*., 2009).

Compared with mandibular BMSCs, RZs showed not only lower proliferative and osteogenic capacity *in vitro* but also less bone formation *in vivo*. Correspondingly, their Runx2 and ALP expression and activity were lower as well. The above results are consistent with those for hypertrophic bone non-union (Hofmann *et al*., 2008). Furthermore, the osteogenesis of RZs could not be improved by a widely used osteogenic-inducing pressure (Appendix Fig. 2). This may explain why RZs retain a decreased rather than increased osteogenic potential under mechanical stimulation *in vivo*. Moreover, the large quantity of fibers and cartilage in the radiolucent zone can be inferred to provide indirect evidence of suppressed osteogenesis in RZs (Fig. 1). In addition, in animal models, we obtained the same result, that the bone-related gene expression in the radiolucent zone of TMJ ankylosis was suppressed compared with that of fractured bone (unpublished observations). Therefore, we reasonably deduce that osteogenesis was compromised due to mechanical stimulation in the radiolucent zone, similar to mechanically delayed bone healing (Schell *et al*., 2008).

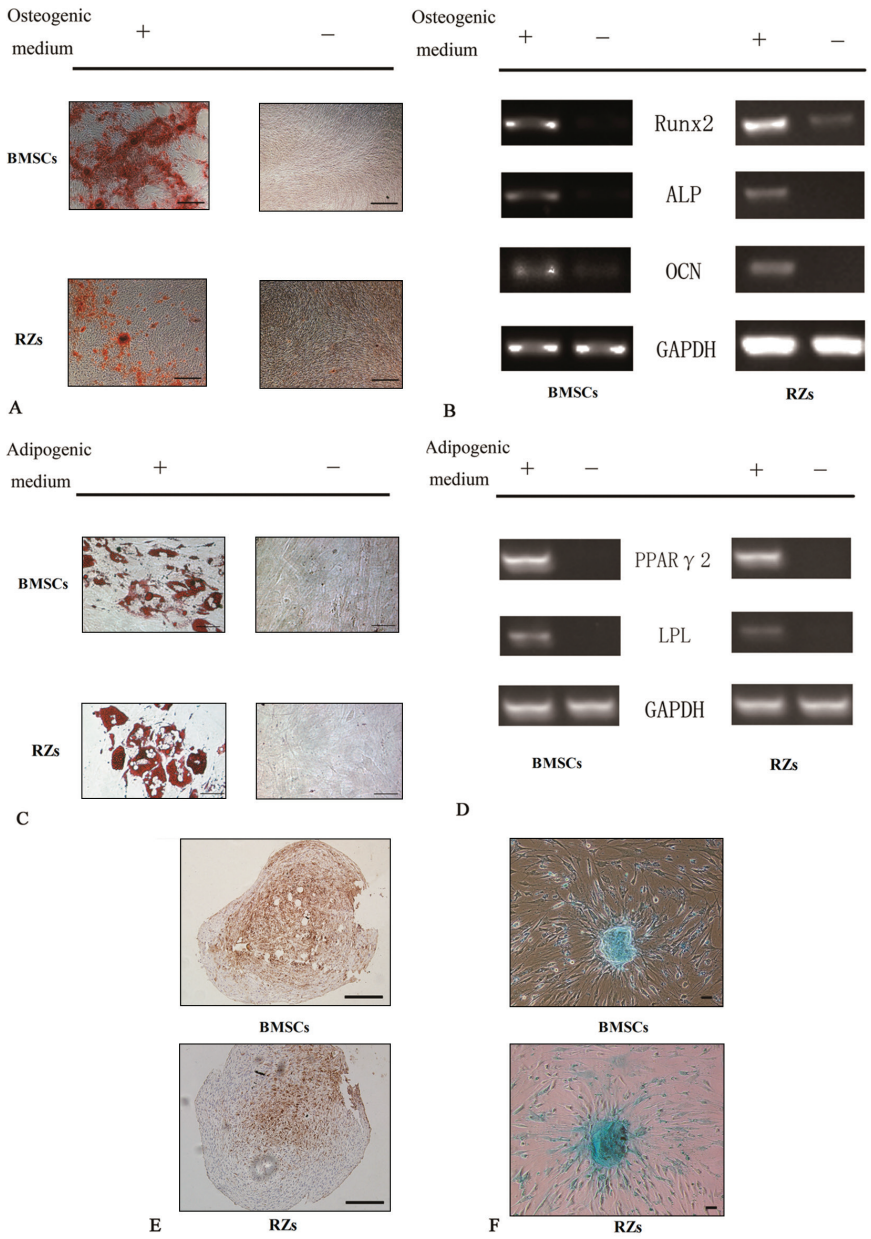

Figure 3. Multi-differentiation of RZs and mandibular BMSCs. **(A)** Alizarin red staining of bone marrow stem cells (BMSCs) and radiolucent-zone-related cells (RZs) after 3 wks of culture in osteogenic medium (scale bar, 500 μm). **(B)** Total RNA was analyzed by RT-PCR for bone-related gene mRNA expression at day 14. **(C)** Oil-red O staining of BMSCs and RZs after 3 wks of culture in adipogenic medium (scale bar, 50 μm). **(D)** Total RNA was analyzed by RT-PCR for adipose-related gene mRNA expression. **(E)** Chondrogenic differentiation was assessed by type II collagen immunohistochemistry (scale bar, 100 μm). **(F)** Toluidine blue staining showed blue-stained chondrocytes (scale bar, 50 μm).

The origin of these RZs is unclear. MSCs have previously been isolated from joint tissues such as synovium (Sakaguchi *et al*., 2005), condylar cartilage (Bibb *et al*., 1992), muscle, periosteum (Kisiel *et al.*, 2012), and mandibular bone marrow (Akintoye *et al*., 2006). MSCs in TMJ tissues, surrounding tissues, and peripheral blood can be recruited to the injured

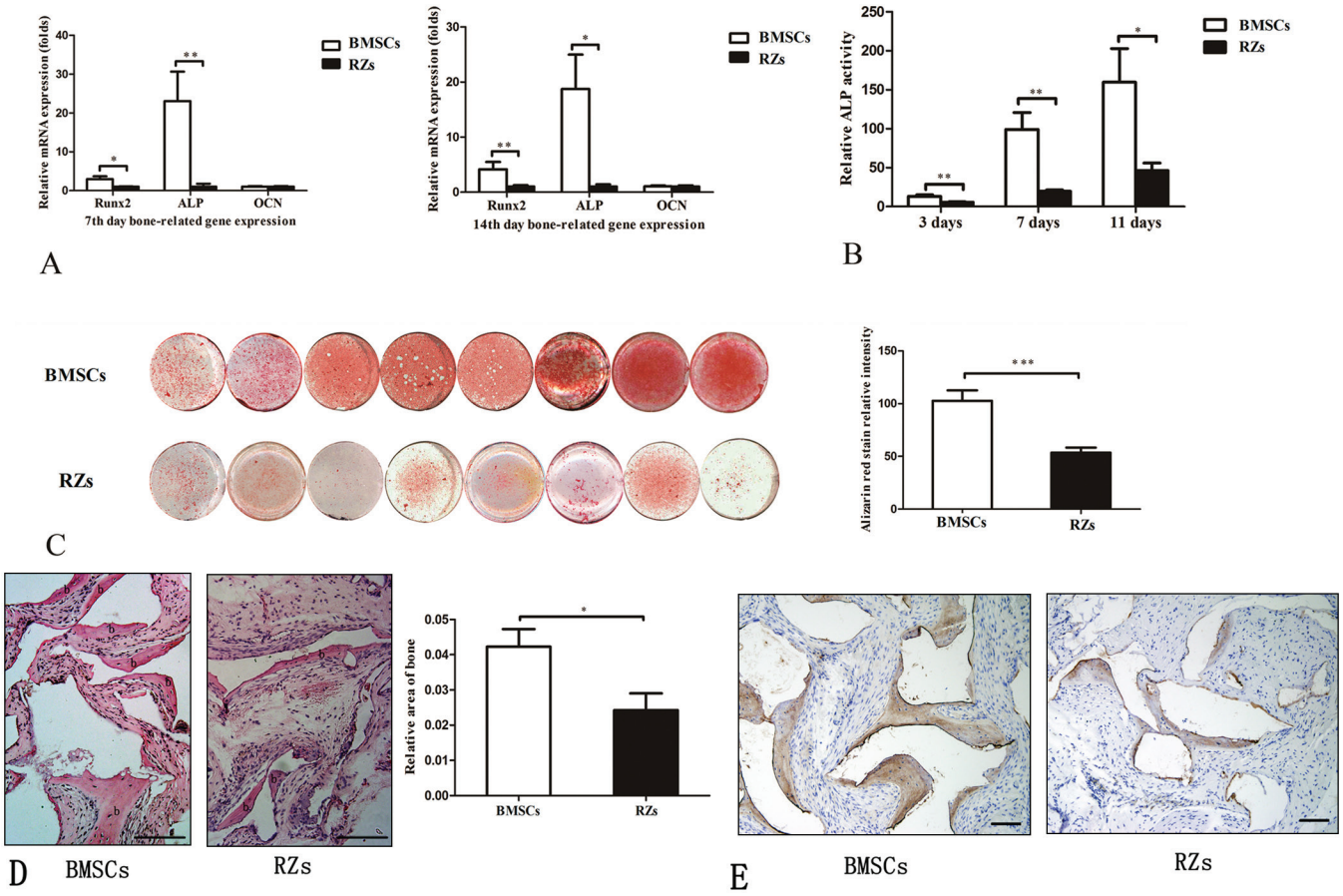

Figure 4. Comparison of osteogenic differentiation potential. **(A)** Real-time PCR assays of bone-related gene mRNA expression after osteogenic induction for 7 and 14 days (n = 6, $*p < .05$, $**p < .01$). **(B)** ALP activity assay at days 3, 7, and 11 during the course of osteogenic induction (n = 6, $*p < .05$, $**p < .01$). **(C)** Alizarin Red S staining of bone marrow stem cells (BMSCs) and radiolucent-zone-related cells (RZs) after culture in mineralization medium for 3 wks and quantification of alizarin red staining (n = 8, $***p < .001$). **(D)** H&E staining of BMSC and AC transplants. The quantification of bone tissue area is shown in the bar chart (n = 4; b = bone; scale bar, 100 μm; $*p < .05$). **(E)** Osteocalcin immunohistochemistry of BMSC and AC transplants. The brown area shows the positive staining of bone tissue (scale bar, 100 μm).

sites for repair (Lories and Luyten, 2011), and then reside in the joint for later tissue regeneration, which may be the major source of RZs. It is less likely that the RZs detected are actually MSCs from the adjacent tissues present, due to contamination during the isolation procedure, since the resected ankylosed mass was thoroughly rinsed with PBS, and only the middle portion of the radiolucent zone was used for cell isolation.

RZs may play a potential role in the development of TMJ ankylosis. As in bone non-union, the radiolucent zone, an indicator of impaired bone healing, contains large quantities of fibers and cartilage. Located between the upper and lower trabecular bones, like TMJ discs, they contribute to the residual mouth-opening of patients (Yan *et al.*, 2011). At the early stage of TMJ ankylosis, when jaw mobility is relatively greater, it is easy to understand that RZs with decreased osteogenesis and proliferation are not sufficiently active to form bone bridges, while limitation of jaw motion would hasten the progress of ankylosis (Miyamoto *et al.*, 2000). As ankylosis progresses, when the radiolucent zone is affected by endochondral bone formation, mouth-opening decreases with the width of radiolucent zone. At this time, with the joint obtaining more stability, RZs might rather contribute to bone formation, worsening ankylosis. Thus, at the later stage, patients commonly show narrower radiolucent zones with obvious bone bridges connecting the upper and lower bone stumps (Appendix Figs. 4, 6), in contrast to the early stage (Fig. 1). Therefore, we speculate that the radiolucent zone may be a potential reservoir of mesenchymal progenitors for bone formation.

According to our hypothesis (Yan *et al.*, 2012a), this study is the first to provide additional cytological evidence, besides their similar etiology, a hypertrophic bone stump, of long-term development history and a radiolucent zone. However, some differences remain. In anatomy, hypertrophic non-union implicates only one bone but rather the joint cartilage or disc, while TMJ ankylosis simultaneously implicates 2 bones and the joint cartilage and disc. Also, ankylosis is affected by biological and mechanical factors. Unfortunately, here we present only the cytological evidence that initially supported our hypothesis; whether RZs did take part in TMJ ankylosis, as we supposed, needs to be further clarified. In addition, whether mechanical factors play a role in the decreased osteogenesis of RZs is

another important question. A larger sample size would certainly be beneficial.

In conclusion, the present study demonstrated the existence of MSC-like RZs that exhibit the properties of lower proliferation and osteogenesis. These results provide innovative cytological evidence supporting the hypothesis that the development of traumatic TMJ ankylosis is similar to the course of hypertrophic bone non-union.

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REFERENCES

Aggarwal S, Mukhopadhyay S, Berry M, Bhargava S (1990). Bony ankylosis of the temporomandibular joint: a computed tomography study. *Oral Surg Oral Med Oral Pathol* 69:128-132.

Akintoye SO, Lam T, Shi S, Brahim J, Collins MT, Robey PG (2006). Skeletal site-specific characterization of orofacial and iliac crest human bone marrow stromal cells in same individuals. *Bone* 38:758-768.

Alsalameh S, Amin R, Gemba T, Lotz M (2004). Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. *Arthritis Rheum* 50:1522-1532.

Arakeri G, Kusanale A, Zaki GA, Brennan PA (2012). Pathogenesis of post-traumatic ankylosis of the temporomandibular joint: a critical review. *Br J Oral Maxillofac Surg* 50:8-12.

Bajada S, Marshall MJ, Wright KT, Richardson JB, Johnson WE (2009). Decreased osteogenesis, increased cell senescence and elevated Dickkopf-1 secretion in human fracture non union stromal cells. *Bone* 45:726-735.

Bibb CA, Pullinger AG, Baldioceda F (1992). The relationship of undifferentiated mesenchymal cells to TMJ articular tissue thickness. *J Dent Res* 71:1816-1821 (*erratum* in *J Dent Res* 72:88, 1993).

Cheng MT, Yang HW, Chen TH, Lee OK (2009). Isolation and characterization of multipotent stem cells from human cruciate ligaments. *Cell Prolif* 42:448-460.

Cheung LK, Shi XJ, Zheng LW (2007). Surgical induction of temporomandibular joint ankylosis: an animal model. *J Oral Maxillofac Surg* 65:993-1004.

Hofmann A, Ritz U, Hessmann MH, Schmid C, Tresch A, Rompe JD, *et al*. (2008). Cell viability, osteoblast differentiation, and gene expression are altered in human osteoblasts from hypertrophic fracture non-unions. *Bone* 42:894-906.

Iwakura T, Miwa M, Sakai Y, Niikura T, Lee SY, Oe K, *et al*. (2009). Human hypertrophic nonunion tissue contains mesenchymal progenitor cells with multilineage capacity in vitro. *J Orthop Res* 27:208-215.

Kisiel AH, McDuffee LA, Masaoud E, Bailey TR, Esparza Gonzalez BP, Nino-Fong R (2012). Isolation, characterization, and in vitro proliferation of canine mesenchymal stem cells derived from bone marrow, adipose tissue, muscle, and periosteum. *Am J Vet Res* 73:1305-1317.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402-408.

Lories RJ, Luyten FP (2011). The bone-cartilage unit in osteoarthritis. *Nat Rev Rheumatol* 7:43-49.

Miyamoto H, Kurita K, Ogi N, Ishimaru JI, Goss AN (2000). Effect of limited jaw motion on ankylosis of the temporomandibular joint in sheep. *Br J Oral Maxillofac Surg* 38:148-153 (*erratum* in *Br J Oral Maxillofac Surg* 38:575, 2000).

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al*. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143-147.

Reed AA, Joyner CJ, Brownlow HC, Simpson AH (2002). Human atrophic fracture non-unions are not avascular. *J Orthop Res* 20:593-599.

Sakaguchi Y, Sekiya I, Yagishita K, Muneta T (2005). Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* 52:2521-2529.

Sawhney CP (1986). Bony ankylosis of the temporomandibular joint: follow-up of 70 patients treated with arthroplasty and acrylic spacer interposition. *Plast Reconstr Surg* 77:29-40.

Schell H, Thompson MS, Bail HJ, Hoffmann JE, Schill A, Duda GN, *et al*. (2008). Mechanical induction of critically delayed bone healing in sheep: radiological and biomechanical results. *J Biomech* 41:3066-3072.

Wu YW, Bi YP, Kou XX, Xu W, Ma LQ, Wang KW, *et al*. (2010). 17-Beta-estradiol enhanced allodynia of inflammatory temporomandibular joint through upregulation of hippocampal TRPV1 in ovariectomized rats. *J Neurosci* 30:8710-8719.

Yan Y, Zhang Y, Sun Z, Li J, Xiao E, An J (2011). The relationship between mouth opening and computerized tomographic features of posttraumatic bony ankylosis of the temporomandibular joint. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 111:354-361.

Yan YB, Duan DH, Zhang Y, Gan YH (2012a). The development of traumatic temporomandibular joint bony ankylosis: a course similar to the hypertrophic nonunion? *Med Hypotheses* 78:273-276.

Yan YB, Zhang Y, Gan YH, An JG, Li JM, Xiao E (2012b). Surgical induction of TMJ bony ankylosis in growing sheep and the role of injury severity of the glenoid fossa on the development of bony ankylosis. *J Craniomaxillofac Surg* [Epub ahead of print 4/14/2012] (in press).

Zhang Y, Su J, Yu J, Bu X, Ren T, Liu X, *et al*. (2011). An essential role of discoidin domain receptor 2 (DDR2) in osteoblast differentiation and chondrocyte maturation via modulation of Runx2 activation. *J Bone Miner Res* 26:604-617.