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Development process of traumatic heterotopic ossification of the temporomandibular joint in mice

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A R T I C L E I N F O

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Purpose: The exact development process underlying traumatic heterotopic ossification of the temporomandibular joint (THO-TMJ) is largely unclear. In this study, we try to explore the histological development process of THO-TMJ.

Materials and methods: Condylar cartilage of one-month-old male mice was partially removed from the left joint with small scissors to induce THO-TMJ. The phenotypes were observed using gross observation, microcomputed tomography (micro-CT) scans and histological examination from one month to six months after surgery.

Results: The micro-CT examination results showed that the injured condyle integrated with ectopic bone tissue to form an osteophyte and that the volume and density of the osteophyte grew exponentially with time. Hematoxylin and eosin (H&E), safranin O and fast green staining of the THO-TMJ specimens revealed that the ectopic bone tissue was mainly nonmineralized fibrous tissue 1 month after surgery. This tissue gradually transformed into cartilage 3 months after surgery. Finally, the tissues transformed into mature bone tissue 6 months after surgery. Immunofluorescence staining showed VEGF-α expression in the heterotopic tissue 1 month after surgery, and the expression of Sox9 in the heterotopic tissue was obvious 3 months after surgery. Furthermore, OCN expression was evident in most of the heterotopic tissue 6 months after surgery. The results also showed clear hypoxia-inducible factor 1-alpha (Hif-1α) expression in the injured chondrocytes of the condyle, especially in the articular proliferative zone and fibrocartilaginous zone.

Conclusions: The THO-TMJ imaging characteristics indicated an exponential change with time. Histologically, the development process of THO-TMJ is an endochondral ossification process and includes three stages, fibroproliferative, chondrogenic and osteogenic stage. In addition, Hif-1α, which was expressed in some of the injured chondrocytes, may play an essential role in the initial THO-TMJ.

1. Introduction

Heterotopic ossification (HO) means the formation of ectopic bone in muscles, tendons, ligaments, and other soft tissues (Amar et al., 2015). HO is divided into acquired nongenetic HO and inherited genetic HO (Xu et al., 2018). For the rare genetic HO, fibrodysplasia ossificans progressiva (FOP) involves endochondral ossification, while progressive osseous heteroplasia (POH) and Albright hereditary osteodystrophy (AHO) leads to HO through intramembranous ossification (Shore and Kaplan, 2010). Neurogenic trauma-induced HO is a common type of acquired nongenetic HO and occurs through both intramembranous and endochondral ossification (Huang et al., 2018). However, in trauma-induced myositis ossificans circumscripta, cartilage formation occasionally occurs through an intramembranous or endochondral process (Walczak et al., 2015). Trauma-induced HO is solely triggered by injury, while genetic HO has both injury-mediated and noninjury-mediated components.

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Trauma is the main cause of HO around the temporomandibular joint (TMJ) (Cetinkaya, 2012). When substantial ectopic bone formation occurs around the condyle, patients may suffer from the symptoms of TMJ ankylosis and may eventually experience limited mouth opening and facial deformity (Song and Yap, 2017). TMJ ankylosis is often divided into fibrous and bony; in addition, fibrous ankylosis can progress into bony ankylosis. Although a close relationship exists between traumatic condyle and TMJ ankylosis, the pathogenesis of the disease remains indefinite.

Currently, the main treatment for THO-TMJ is surgical resection (Zhu et al., 2015). However, there is currently no effective preventive method for THO-TMJ because the exact cellular and molecular mechanisms have not been completely elucidated (Mercuri and Saltzman, 2017). Fortunately, development of animal models which serve as powerful tools to study the unique traumatic injuries has greatly facilitated the mechanistic understanding of trauma-induced HO(Dey et al., 2017). We have successfully established a mouse model with traumatic HO of the TMJ (THO-TMJ) by partially removing condylar cartilage in our previous studies and showed that injured cartilage, but not injured bone and uninjured cartilage, plays a crucial role in the development of THO-TMJ (Dai et al., 2016). In this study, we mainly try to explore the histological development process of THO-TMJ, which will shed light on proper and effective treatments for THO-TMJ (Ohrbach and Dworkin, 2016).

2. Materials and methods

2.1. Mouse strains and study design

Male mice with a C57BL/6J genetic background were used in this study. All animal experiments complied with the ARRIVE guidelines. Animal experimental procedures were performed in compliance with the Institutional Animal Care and Use Committees of the Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. The procedures were reviewed and approved by the Ethics Committees of the Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, China (approval number 2016-45). In this study, condylar cartilage was partially removed from the left joint of one-month-old male mice with small scissors to induce THO-TMJ (Yan et al., 2014). The right condyle was used as the control. A total of 15 mice were killed at specific time points, 30, 90 and 180 days after surgery, and 20 mice were examined through microcomputed tomography (micro-CT) scans at 30, 60, 90, 120, 150, and 180 days after surgery.

2.2. Surgical procedures

The THO-TMJ mouse model was established as previously described (Ouyang et al., 2018). Briefly, one-month-old male mice were anesthetized with 0.8% pentobarbital sodium intraperitoneally. Surgery was performed on the left joint, whereas the right side was used as the control. The preauricular skin was sterilized with 75% alcohol, and a 1-cm linear preauricular skin incision was made. The preauricular fascia was cut open just above the superior border of the facial vessel to expose the zygomatic arch. Then, the joint capsule was cut open to expose the condyle, and some of the condylar cartilage was then removed using small scissors. Finally, the incision was closed using a 3-0 suture. The mice were given soft food for 2 weeks after surgery.

2.3. Micro-CT tomography scans

Total of twenty mice were collected and anesthetized with pentobarbital sodium intraperitoneally at 30, 60, 90, 120, 150 and 180 days after surgery (Bouxsein et al., 2010). Micro-CT scans were performed, and the density of ectopic bone was measured using an eXplore Locus Micro-CT scanner (GE Healthcare, Milwaukee, Wisconsin, USA). The slice thickness for micro-CT scans was 40 μm. Three-dimensional (3D) reconstruction of the skulls was conducted using GE MicroView software (GE Healthcare, Milwaukee, Wisconsin, USA). The ectopic bone volume was measured using Geomagic Studio software (Geomagic, North Carolina, USA) (Sousa et al., 2012).

2.4. Histological analysis

Whole heads of mice were freely dissected and collected 30, 90 and 180 days after surgery, and the samples were fixed in 4% paraformaldehyde (PFA) and demineralized in 0.5 M ethylenediaminetetraacetic acid (EDTA) for 2–3 weeks (Ballal et al., 2016). Subsequently, the samples were embedded in paraffin and cut to a thickness of 5 μm for regular hematoxylin and eosin (H&E) staining and to a thickness of 4 μm for safranin O (Sigma–Aldrich, S2255) and fast green (Sigma–Aldrich, F7252) staining. Briefly, slides were incubated in a safranin O solution for 5 min. Then, the slides were washed in differentiation solution and stained with a fast green solution for 10 min at 37 °C (Wang et al., 2018). Subsequently, the stained slides were washed with running tap water and cleared in xylene. Finally, the slides were mounted with resinous mounting medium for observation and image acquisition.

2.5. Immunofluorescence staining

Whole heads of mice were collected 30, 90 and 180 days after surgery and were embedded in paraffin. Five-micrometer thick sections were obtained, and the slides were then deparaffinized and hydrated (Donaldson, 2015). Next, antigen retrieval was performed using an antigen restoration liquid kit (Sunteam Biotech, China). The slides were subsequently blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h and incubated with anti-VEGF-α (Abcam, 1/200), anti-sox-9 (Abcam, 1/100), anti-OCN (Santa Cruz Biotechnology, 1/300), and anti-hypoxia-inducible factor 1-alpha (Hif-1α; Abcam, 1/100) primary antibodies overnight at 4 °C. Subsequently, secondary antibodies (AlexaFluor 568 donkey anti-mouse, AlexaFluor 488 donkey anti-rabbit, Jackson) were diluted in PBS (1/300) and incubated at room temperature for 1 h. Finally, the slides were mounted with Vectashield mounting medium for visualization under a fluorescence microscope (Dey et al., 2016).

2.6. Statistical analyses

The data are expressed as the mean ± standard deviation. Data analysis was performed using a Students T test using SPSS 18.0 software (International Business Machines, Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. General observation and Micro-CT examination

During the experiment, 1 mouse in the experimental group died 3 days after the operation, and the other mice survived until the end of the experiment. There were two mice with a restricted mouth opening due to soft tissue adhesions 30 days after surgery.

Micro-CT examination from different views showed that the deviation of the mandible and adhesion around the region with condylar trauma gradually changed over time. In addition, the shape and volume changed more obviously in the region with condylar trauma than at the side with the healthy condyle at

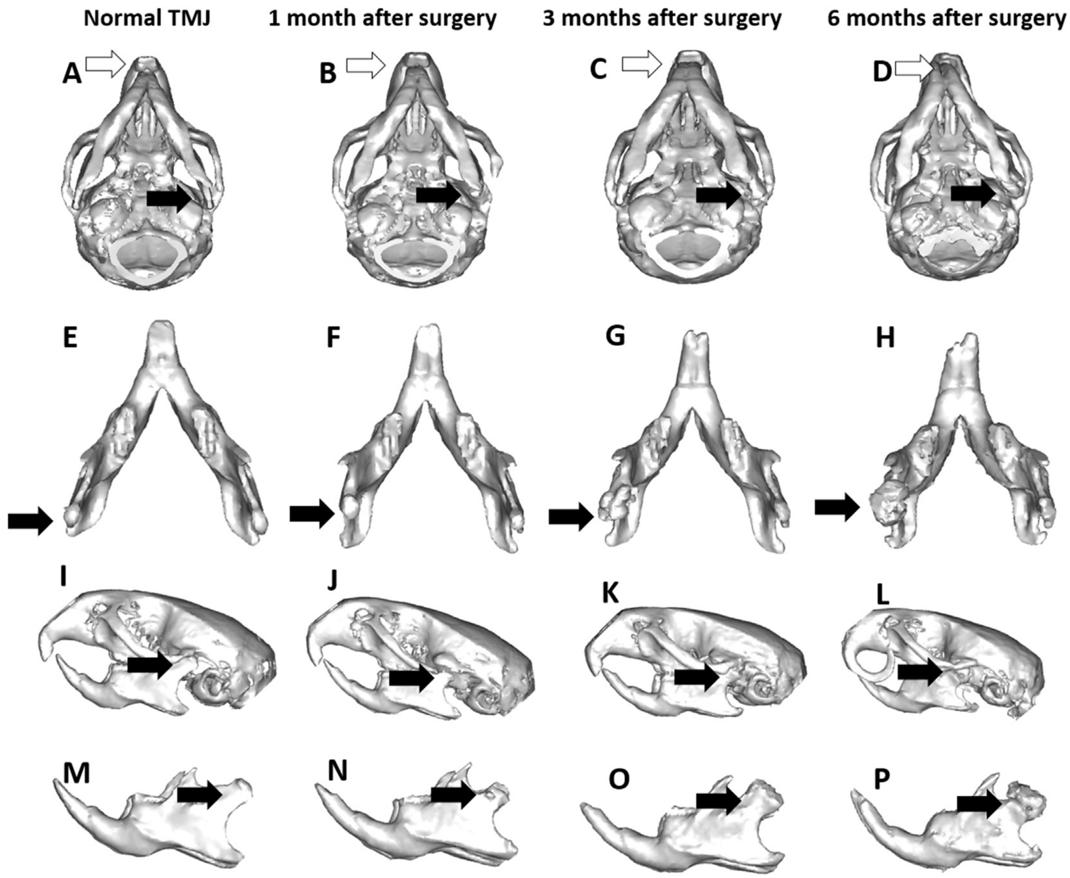

Fig. 1. Micro-CT examination of the THO-TMJ mouse model. (A–D) The bottom view shows the normal condyle structure in the control mice without surgery (A, black arrow head), the hyperplasia in the left condyle, and the gradual increase in the ectopic bone volume (B, C, D, black arrow head). The deviation of the mandible also gradually became obvious (B, C, D, white arrow head). (E–H) In the isolated mandible, the volume of bilateral condyles did not differ from each other for control mice without surgery (E, black arrow head), but there were apparent changes in the shape and volume of the left condyle compared to the control side (F, G, H, black arrow head). (I–L) The lateral view shows the normal relationship between the condyle and the surrounding bone tissues in the control mice without surgery (I), but the volume and shape of the left condyle gradually increased, resulting in disruptive adhesion to the surrounding bone tissue (J, K, L). (M–P) The lateral view of the isolated mandible shows the normal shape and volume of the condyle in the control mice without surgery (M), but the volume and shape of the condyle gradually changed and increased 1, 3 and 6 months after surgery (N, O, P).

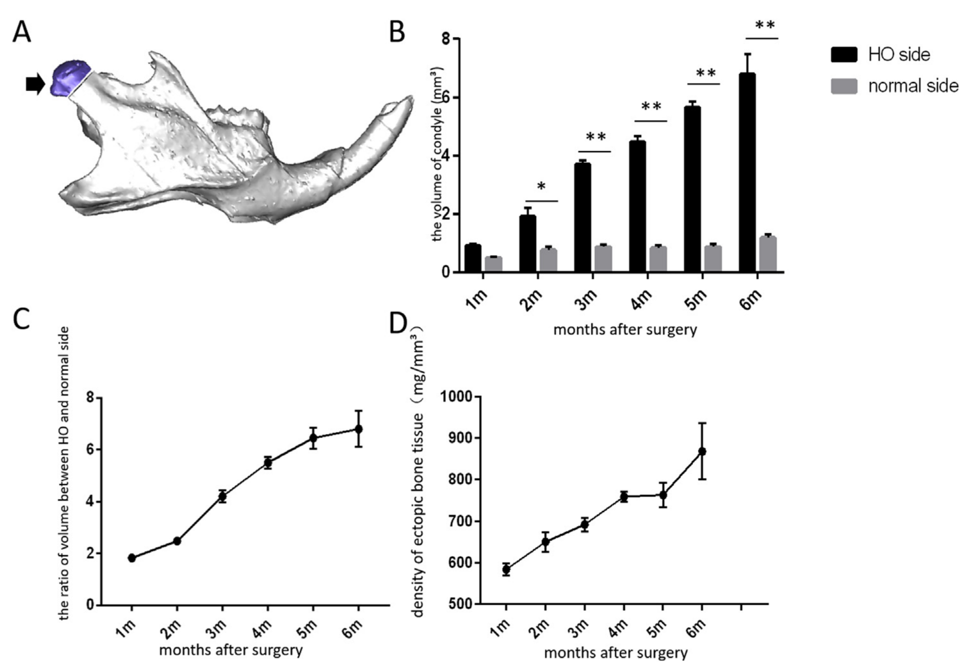

Fig. 2. Quantitative data for the volume and density changes in the condyles and ectopic bone tissue. (A) The blue area represents the region used to calculate the volume of the condyle (black arrow head). (B) The volume of injured condyle and surrounding ectopic bone tissues is larger than the volume of the control side at different time points ($^{*}p < 0.05$, $^{**}p < 0.01$). (C) The change trend of the volume ratio between the injured side and the control side corresponded to an exponential growth pattern. (D) The change trend for the density of ectopic bone tissue also corresponded to an exponential growth pattern except at 6 months after surgery.

different time points (Fig. 1A–H). The ectopic bone tissues and injured condyle fused to become an osteophyte, which led to a gradual increase in the volume of the condyle (Fig. 1I–P), and the trend for the volume change between this region and the healthy condyle corresponded to exponential growth (Fig. 2B, C). Furthermore, the density of the ectopic bone tissues also showed an exponential growth trend from 1 month to 5 months but exhibited a fast increase from 5 to 6 months after surgery (Fig. 2D).

3.2. Safranin O and fast green and H&E staining

Safranin O and fast green staining showed that there was no obvious ectopic cartilage and bone tissue around the region with condylar trauma 30 days after surgery. The early HO may resemble reactive fibroblastic lesions (Fig. 3A, B). However, there was obvious ectopic cartilage in the periarticular region and glenoid fossa 90 days after surgery (Fig. 3C, D). Then, the ectopic cartilage became

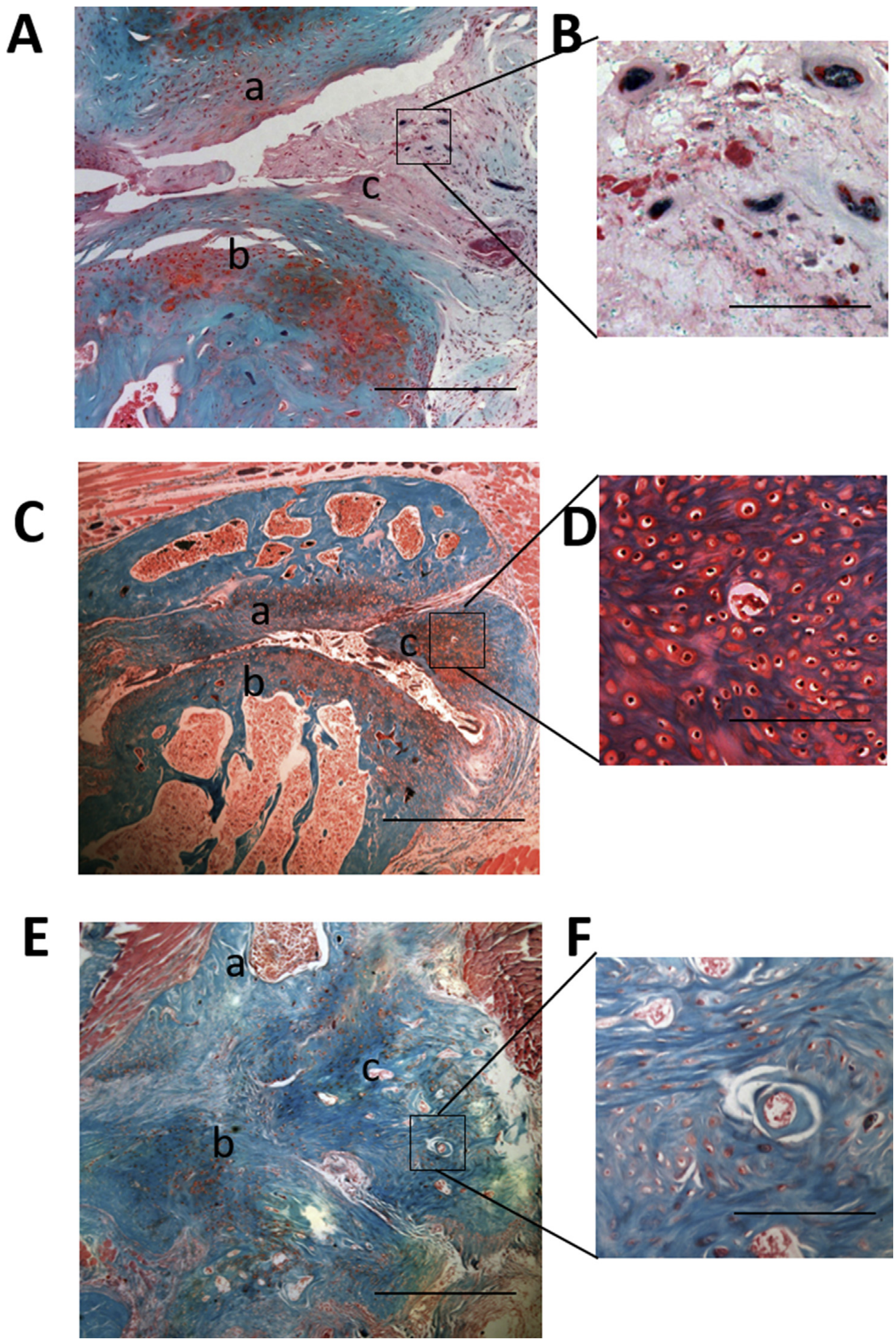

Fig. 3. Safranin O and fast green staining of specimens in THO-TMJ mouse models at different time points. (A, B) The ectopic tissues at 30 days after surgery mainly were fibrous and vascular tissues, and there was no obvious cartilage or bone tissue surrounding the region with condylar trauma. (C, D) The ectopic tissues at 90 days after surgery were cartilage tissues that exhibited reddish-orange coloring around the region with condylar trauma and even the glenoid fossa. (E, F) The ectopic tissues at 180 days after surgery were bone tissues and exhibited light green and cyan staining around the region with condylar trauma. a: glenoid fossa. b: condyle. c: ectopic tissue. Scale bars: A, C, E 200 μm. B, D, F 25 μm.

mature bone tissue in the periarticular region 180 days after surgery (Fig. 3E, F). H&E staining showed that the area of ectopic cartilage/bone tissues gradually increased from 30, 90 and to 180 days after surgery (Fig. 4A–F).

3.3. Immunofluorescence staining

We used immunofluorescence staining to measure the expression of factors related to angiopoiesis, chondrogenesis and osteogenesis, and the results showed that there was obvious expression of VEGF-α in the soft tissues around the region with condylar trauma 30 days after surgery (Fig. 4G, J). In contrast, there was stronger expression of Sox9 in the periarticular region 90 days after surgery, as well as stronger expression of OCN in the periarticular region 180 days after surgery; these features were consistent with the endochondral ossification development process of THO-TMJ that was revealed by safranin O and fast green staining (Fig. 4H, I, K, L). At 30 days after surgery, immunofluorescence staining also showed evident Hif-1α expression in the injured chondrocytes of the condyle, especially in the fibrocartilaginous zone and proliferative zone (Fig. 5A, B, C).

4. Discussion

The TMJ is a special and complex structure that is found only in mammals (Iwasaki et al., 2010). Due to the specific location and function of the TMJ, the incidence of trauma in the mandibular condyle is particularly high; this trauma will sometimes lead to THO-TMJ or TMJ ankylosis in some patients, followed by serious damage to the mouth function and facial appearance of these patients (Arakeri et al., 2012). For TMJ ankylosis, the new tissue formation is not physiological but pathological, because the continued ectopic tissue formation replaces the normal structure of TMJ and it seems that no remodeling takes place. The formation of HO is complex since it may involve many kinds of cells and specific osteogenesis process (Agarwal et al., 2016b).

Endochondral ossification and intramembranous ossification are two distinct processes for bone development (Hayashi et al., 2014). For intramembranous ossification, mesenchymal cells directly differentiate into osteoblasts (Vieira et al., 2015). However, endochondral ossification refers to the process of bone formation wherein a cartilage intermediate is formed and gradually replaced by osteoblasts. Firstly, mesenchymal cells differentiate into chondrocytes. Then, the chondrocytes undergo apoptosis, the cartilage stroma suffers degradation and finally the degraded cartilage is replaced by mature bone tissue (Gawlitta et al., 2010). The physiologic processes of osteogenesis and angiogenesis are highly coupled and interdependent. In this study, we found that the histological development process of THO-TMJ was an endochondral ossification process and included three stages, fibrosis with vascularization, chondrogenesis and osteogenesis. Especially, angiogenesis is an important stage in the formation of THO-TMJ. Thus, prevention of the occurrence and development of THO-TMJ through inhibiting angiogenesis may be a possible choice, and should be investigated in future.

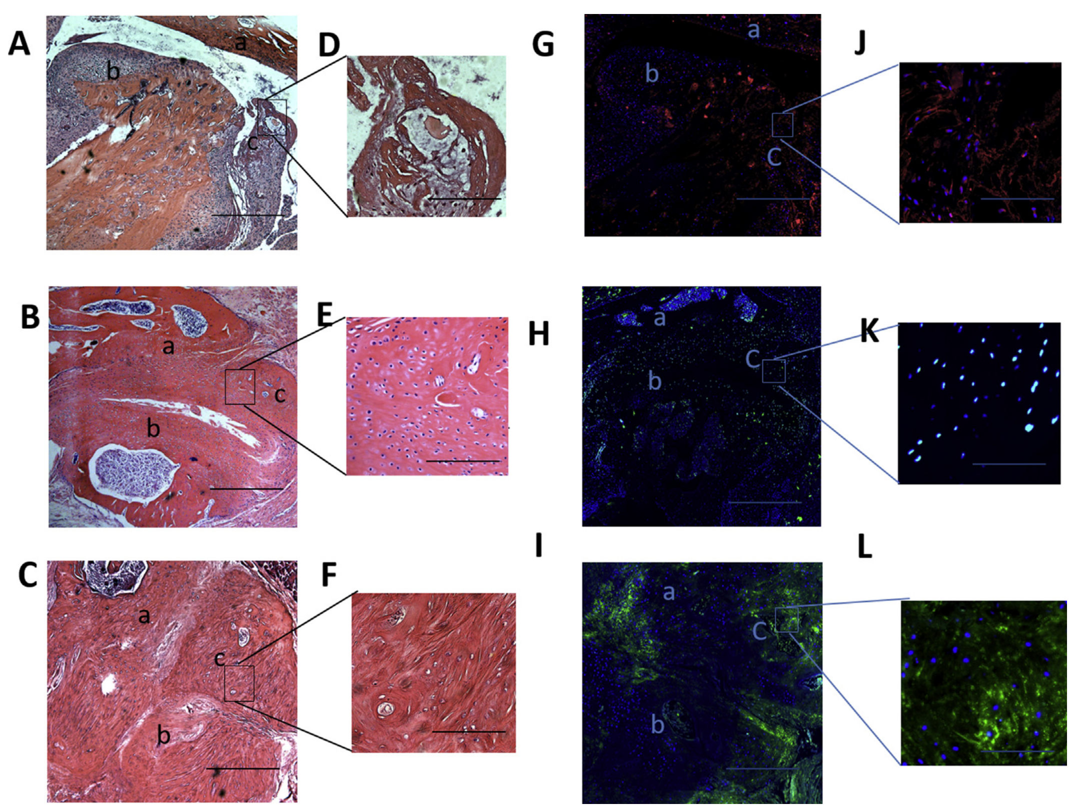

Fig. 4. H&E staining and immunofluorescence staining of specimens in THO-TMJ mouse models at different time points. (A, D) The H&E staining results showed that ectopic tissues around the region with condylar trauma had started to invade the joint gap 30 days after surgery. (B, E) The H&E staining results showed that the range of ectopic tissues around the region with condylar trauma became larger and that these tissues fused with the glenoid fossa of the TMJ at 90 days after surgery. (C, F) The H&E staining results showed that the gap between the injury and the glenoid fossa had been filled with ectopic tissues and that the residual condyle had been wrapped by the ectopic tissues at 180 days after surgery. (G, J) The immunofluorescence staining results showed the expression of VEGF-α in the ectopic tissues 30 days after surgery. (H, K) The immunofluorescence staining results showed the expression of Sox9 in most of the ectopic tissues 90 days after surgery. (I, L) The immunofluorescence staining results showed the expression of OCN in most of the ectopic tissues 180 days after surgery. a: glenoid fossa. b: condyle. c: ectopic tissue. Scale bars: A, B, C, G, H, I 200 μm. D, E, F 25 μm. J, K, L 15 μm.

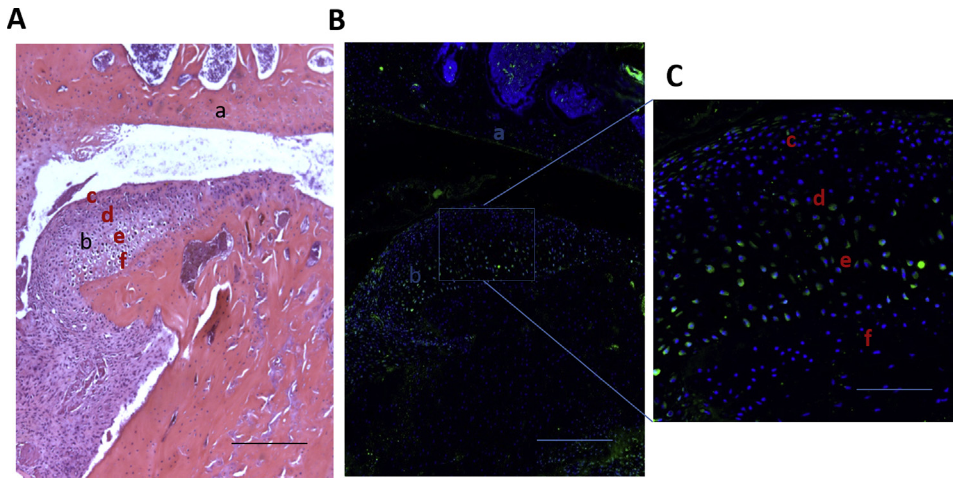

Fig. 5. H&E staining and immunofluorescence staining of specimens from THO-TMJ mouse models at 30 days after surgery. (A) The H&E staining results showed the four-layer structure of the injured condyle cartilage clearly. (B, C) The immunofluorescence staining results showed the expression of HIF-1α in the injured chondrocytes at the time of the initial THO-TMJ, especially in the articular proliferative zone and fibrocartilaginous zone. a: glenoid fossa. b: condyle. c: articular zone. d: proliferative zone. e: fibrocartilaginous zone. f: calcified cartilage zone. Scale bars: A, B 200 μm. C 50 μm.

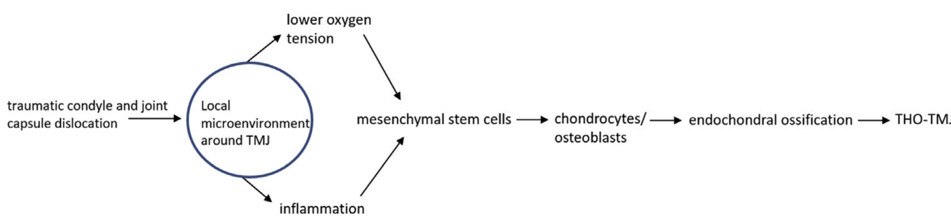

Fig. 6. A schematic diagram of the proposed development process of traumatic heterotopic ossification of the temporomandibular joint.

Trauma is the primary trigger of THO-TMJ, which will lead to changes in the local microenvironment around the injured TMJ. Previous study showed that the traumatic cartilage plays an important role in the development of HO (Hinton et al., 2017), which may be due to the injured chondrocytes secreting some essential bioactive factors that could initiate and contribute to the ectopic tissue formation (Coimbra et al., 2004). Thus, it is important to explore the exact role and molecular mechanism of traumatic condylar cartilage on the formation of THO-TMJ, which may help us to properly preserve the residual condylar cartilage in younger individuals to simultaneously preserve the function and growth ability, and avoid the promotion effect of injured cartilage on the formation of THO-TMJ.

Previous studies showed that some mesenchymal stem cells in the soft tissues may contribute to traumatic HO, including local mesenchymal stem cells or some other progenitors recruited to the lesions from bone marrow (Agarwal et al., 2017). Previous study has shown that vascular endothelial cells have emerged as the chief candidate for the cellular origin of HO in FOP, which was induced by a combination of genetic mutation and acute inflammatory responses. Especially, endothelial-mesenchymal transition (EndMT) of some cells may act as an important role in the process of ectopic bone formation (Medici et al., 2010). These cell lineage tracing studies provide new insight into the cellular pathophysiology of heterotopic ossification (Lounev et al., 2009). Thus, identifying the definite precursor cells which contribute to THO-TMJ through cell tracking and animal models is necessary in future.

Local factors, such as oxygen tension, pH, micronutrients, and mechanical stimuli, may play an essential role in the development of HO (Ranganathan et al., 2015). A large amount of various inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, could induce some mesenchymal stem cells to initiate ectopic endochondral ossification (Huang et al., 2015). Nonsteroidal anti-inflammatory drugs (NSAIDs) prevent HO by inhibiting the osteogenic differentiation of progenitor cells, but lower oxygen tension will facilitate this process (Joice et al., 2018). HIF-1α is a key transcriptional regulator for cellular response to ischemia through stimulation of vascular endothelial cell precursors, and also plays a crucial role in the development of HO (Agarwal et al., 2016a). Therefore, local inflammatory and lower oxygen tension microenvironment may mediate the differentiation of mesenchymal stem cells into chondrocytes and osteoblasts, and finally resulted in THO-TMJ through endochondral ossification (Medici and Olsen, 2012) (Fig. 6). These findings implied that anti-inflammation or application of small-molecule drugs for intervention of these factors may suitable methods for inhibition of HO formation around the traumatic TMJ, which also are research hotspots for the future.

However, the exact progenitors, as well as the exact molecule and mechanism that triggers this endochondral ossification in THO-TMJ, have not been determined clearly. Moreover, the role of mechanical force also cannot be overlooked in the formation of THO-TMJ due to the specific location and function of TMJ, and should be explored in future (Ruggiero et al., 2015).

5. Conclusion

In this study, we first revealed that the histological development process of THO-TMJ is an endochondral ossification process, which included three stages: fibrosis and vascular ingrowth, cartilage formation and bone formation. It provided useful information for advanced understanding of the molecular and cellular pathogenesis of THO-TMJ (Downey et al., 2015). Future therapeutic strategies may focus on targeted inhibition of local factors and signaling pathways to inhibit the endochondral ossification process in THO-TMJ (Juarez et al., 2018).

Disclosure statement

The authors have nothing to disclose.

Authors' roles

Study design: YZ, JD and GS. Study conduct: YZ, PL, QC, WZ. Data analysis: YZ, NO, YL, JD and GS. Drafting manuscript: GS. Revising manuscript content: JD and GS. Approving final version of manuscript: YZ, JD and GS. JD and GS take responsibility for the integrity of the data analysis.

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