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# A pilot trial on the molecular pathophysiology of traumatic temporomandibular joint bony ankylosis in a sheep model. Part II: The differential gene expression among fibrous ankylosis, bony ankylosis and condylar fracture

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ABSTRACT

*Objective:* The purpose of the study was to preliminarily explore the differential expressions of a series of genes regulating bone formation in temporomandibular joint (TMJ) fibrous ankylosis, bony ankylosis and condylar fracture healing.
*Methods:* The cDNA from either the bony ankylosed callus or fracture callus of the 6 sheep, as described in the part I, were both used in the study. The differences of gene expressions between bony ankylosis and condylar fracture at 1, 3, and 6 months postoperatively were measured by real-time PCR, with 2 samples at each time point. In addition, another 2 sheep were added to have fibrous ankylosis induced on the right TMJ, and 1 sheep was sacrificed at 3 and 6 months after surgery, respectively. The differences of gene expressions between fibrous and bony ankylosis at 3 and 6 months postoperatively were measured by real-time PCR.
*Results:* Bony ankylosis showed higher mRNA expression trends in Wnt2b, Wnt5a, β-Catenin, Lef1, CyclinD1, Runx2, Osterix, Sox9, Col10a1, Alp, Ocn, Bmp2, and Bmp7 compared to fibrous ankylosis, although no statistical analysis was performed due to the very small sample size. Whereas bony ankylosis showed a significant lower expression of Wnt5a, β-Catenin, Lef1, Runx2, Osterix, Sox9, Col10a1, Alp, Ocn and Bmp4 compared to condylar fracture at several time points ($P < 0.05$).
*Conclusion:* Our data provided a preliminary molecular evidence for the hypothesis that the development of traumatic TMJ bony ankylosis was the course of delayed bone healing or hypertrophic nonunion, and deserved to be further studied.


## 1. Introduction

Traumatic temporomandibular joint (TMJ) bony ankylosis, characterized by excessive bone apposition around the joint and the radiolucent zone in the bony fusion area (Yan et al., 2011, 2012a), has generated great interest in the craniomaxillofacial surgeons, yet remains an enigma. So far, the understanding of the pathogenesis of the condition stagnates in the stage of hypotheses, lacking evidences from experimental and clinical studies. The classical hypothesis is that the organization and ossification of the hematoma leads to bony ankylosis after condylar fractures (Norman, 1978; Sawhney, 1986; Ferretti et al., 2005). Meng et al. (2009) consider that distraction osteogenesis of the lateral pterygoid muscle may play an important role in the formation of TMJ ankylosis. Hall (1994) thinks that it is not trauma but biological behavior of specific gene populations that relate to the traumatic TMJ ankylosis.

As a supporter to the classical hypothesis, we previously made a hypothesis that traumatic TMJ bony ankylosis may be a course similar to the hypertrophic nonunion (Yan et al., 2012a). After the similarity between the bony ankylosis and fracture healing was confirmed by histological analysis in a sheep model (Yan et al., 2012b), we designed an experiment based on the model with the aim to further verifying the hypothesis in the molecular level. The experimental results were

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divided into two parts due to the complexity of the data. In the part I, we found that the Wnt signaling, which played important roles in the fracture healing and bone regeneration, was also activated in the bony ankylosis (Yan et al., 2013). This result confirmed the similarity between the bony ankylosis and fracture healing from the perspective of signal pathway.

Advanced understanding of bone healing has demonstrated that the deficiency of key growth factors and the reduced bone-forming activity may contribute to fracture healing progressing toward nonunion by comparing the standard and delayed bone healing (Kwong et al., 2009; Lienau et al., 2010). Therefore, in this report (part II), the primary aim was to investigate whether or not bone-forming activity in the joint space of bony ankylosis is reduced by exploring the differential expression of a series of genes regulating bone formation between the bony ankylosis and condylar fracture healing in a sheep model.

Fibrous ankylosis, an important category of TMJ ankylosis, is traditionally considered as a variation of the same pathology as bony ankylosis (Miller et al., 1975). However, our study on the large animal model showed that bony ankylosis can only progress gradually from fibro-osseous ankylosis, not fibrous ankylosis (Yan et al., 2012b); and that the degree of damage to the glenoid fossa made the difference between fibrous and bony ankylosis (Yan et al., 2012b). In view of the differences of histological process and outcome between the fibrous and bony ankylosis in our sheep model, exploring the molecular basis underlying the two conditions will contribute to revealing the pathogenesis of bony ankylosis. Therefore, another aim of the part II was to preliminarily explore the differences of target gene expression between the fibrous and bony ankylosis in the model.

## 2. Materials and methods

### 2.1. Experimental design

The cDNA from either the bony ankylosed callus or the fracture callus of the 6 sheep, as described in the part I (Yan et al., 2013), were both used in the study. The differences of gene expressions between bony ankylosis and condylar fracture at 1, 3, and 6 months postoperatively were measured respectively by real-time PCR, with 2 samples at each time point.

To explore the differences of target gene expression between the fibrous and bony ankylosis, 2 male sheep with the weight of 18–21 kg were added to the study group and had fibrous ankylosis induced in the right TMJ and condylar fracture in the contralateral side according to the same methods previously described (Yan et al., 2012b). One animal was sacrificed at 3 and 6 months after surgery, respectively. The harvesting of the specimens of fibrous ankylosed callus, the RNA extraction and the cDNA synthesis were performed as described previously (Yan et al., 2013). The differences of gene expressions between fibrous and bony ankylosis at 3 and 6 months postoperatively were measured respectively by real-time PCR.

### 2.2. Real-time PCR

The sequences of primers for GAPDH, Wnt2b, Wnt5a, β-Catenin, Sfrp1, Lef1, CyclinD1, Col10a1, Alp, Ocn, Runx2, Osterix, and Sox9 were described previously in the part I (Yan et al., 2013). The primers used for another 3 genes, BMP2/4/7, are presented in Table 1. Real-time PCR analysis using SYBR green was performed for the assessment of changes in gene expression as previously described (Yan et al., 2013).

### 2.3. Statistical analyses

Because the fibrous ankylosis group has only 1 sample at the 2 time points, no statistical analysis was performed for the comparisons between the fibrous and bony ankylosis groups, however the trends of differential gene expressions were shown descriptively. For the bony ankylosis and condylar fracture, means were calculated and statistical comparisons between the groups were performed using Independent-Samples *T* Test (SPSS 17.0) per time point. The statistical significance level was set at $P < 0.05$. Due to

**Table 1**
Sequences of primers for real-time PCR.

| Gene | GenBank accession no. | Sequence of forward and reverse primers | Product size |
|---|---|---|---|
| BMP2 | AF508028.1 | 5′ ATGGTTTCGTGGTGGAGGTAG 3′<br>5′ ACTTGAGGCGTTTCCGCTGTT 3′ | 210 bp |
| BMP4 | NM_001110277.1 | 5′ CGGAAGAAGAATAAGAACTGTCGG 3′<br>5′ CAATGGCGTGGTTGGTTGAGT 3′ | 166 bp |
| BMP7 | DQ192015.1 | 5′ AGTCTGACCTGTTCCTGCTCG 3′<br>5′ GTGGTTGCTGGTGGCTGTG 3′ | 89 bp |

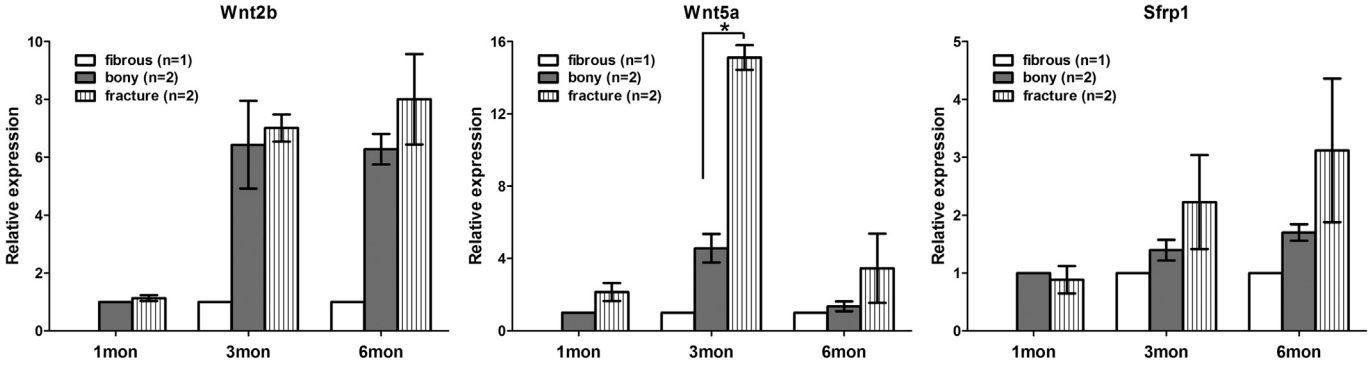


**Fig. 1.** Temporal gene expression of Wnt2b, Wnt5a and Sfrp1 in the fibrous ankylosis, bony ankylosis, and condylar fracture. Results were normalized to GAPDH levels and compared to the target signals in the bony ankylosis at 1 month, in the fibrous ankylosis at 3, and 6 month using the comparative $C_t$ method (*$P < 0.05$).

the small sample size and the exploratory nature of the study, the *P*-values have to be interpreted descriptively.

## 3. Results

### 3.1. Wnt2b, Wnt5a, and Sfrp1

The expression of Wnt2b and Wnt5a in the bony ankylosis tended to be higher than that in the fibrous ankylosis at 3, and 6 months. There was a maximum 6.4-fold change at 6 months in Wnt2b expression, a maximum 4.6-fold change at 3 months in Wnt5a expression between fibrous and bony ankylosis. The gene expressions of Sfrp1 seemed to be similar between fibrous and bony ankylosis. Compared with condylar fracture, the expression of Wnt5a was inclined to be lower in the bony ankylosis at 3 time points, and at 3 months, bony ankylosis showed a significantly lower Wnt5a expression ($P = 0.005$) with the maximum change of 3.3 folds. For Wnt2b and Sfrp1, no significant differences of gene expression were found between condylar fracture and bony ankylosis at 3 time points. (See Fig. 1).

### 3.2. β-Catenin, Lef1, and CyclinD1

The expression of β-Catenin, Lef1, and CyclinD1 in the bony ankylosis tended to be higher than that in the fibrous ankylosis at both 3 and 6 months. The maximum changes of expression of β-Catenin, Lef1, and CyclinD1 between the fibrous and bony ankylosis were 3.2-fold, 5.1-fold and 2.7-fold at 6 months, respectively. Bony ankylosis showed a significantly lower β-Catenin expression at 1 month ($P = 0.047$) and at 3 months ($P = 0.002$) in comparison to condylar fracture, with a maximum 3.9-fold change at 1 month. The expression of Lef1 in the bony ankylosis tended to be lower than that in the condylar fracture at 1, and 3 months, and be similar to that in the condylar fracture at 6 months. Statistically significant differences of Lef1 expression were found at 1 month ($P = 0.026$) with the maximum change of 5.9 folds. The CyclinD1 expression in the bony ankylosis was inclined to be lower than that in the condylar fracture at 1 month, with a maximum 1.9-fold changes, however no statistically significant different CyclinD1 expressions were found at 3 time points. (See Fig. 2).

### 3.3. Alp, Ocn, and Col10a1

Bony ankylosis showed an apparent higher expression of Alp, Ocn, and Col10a1 in comparison to fibrous ankylosis both at 3 and 6 months. The maximum change was 737.5-fold for Alp, 3634.5-fold for Col10a1 at 3 months, and 1061.5-fold for Ocn at 6 months. Compared with condylar fracture, the expression of Alp and Ocn in bony ankylosis at 3 time points was inclined to be lower, and the statistically significant differences were found at 3 months both for Alp expression ($P = 0.028$) and Ocn expression ($P = 0.044$). The Col10a1 expression in the bony ankylosis tended to be lower than that in condylar fracture at 1 and 3 months, and be similar to that in the condylar fracture at 6 months. Significant difference of Col10a1 expression was found at 3 month ($P = 0.031$) with the maximum change of 3.87 folds. (See Fig. 3).

### 3.4. Runx2, Sox9, and Osterix

The expression of Runx2, Sox9, and Osterix in bony ankylosis was inclined to be higher than that in the fibrous ankylosis at 3 and 6 months. And the maximum change of Runx2, Sox9, and Osterix was 11.2-fold at 6 months, 5.1-fold at 3 months and 33.4-fold at 3 months between fibrous and bony ankylosis. Compared with

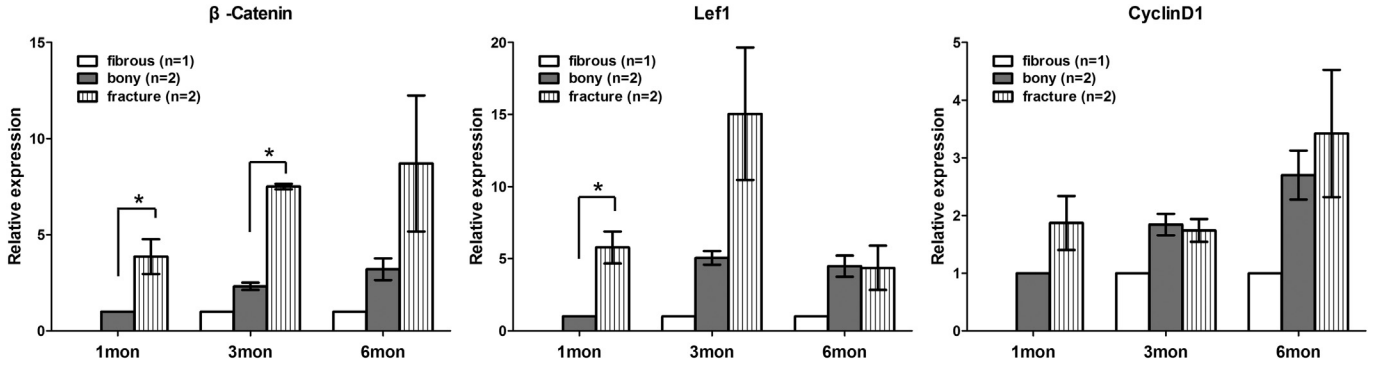


**Fig. 2.** Temporal gene expression of β-Catenin, Lef1 and CyclinD1 in the fibrous ankylosis, bony ankylosis, and condylar fracture. Results were normalized to GAPDH levels and compared to the target signals in the bony ankylosis at 1 month, in the fibrous ankylosis at 3, 6 month using the comparative $C_t$ method (*$P < 0.05$).

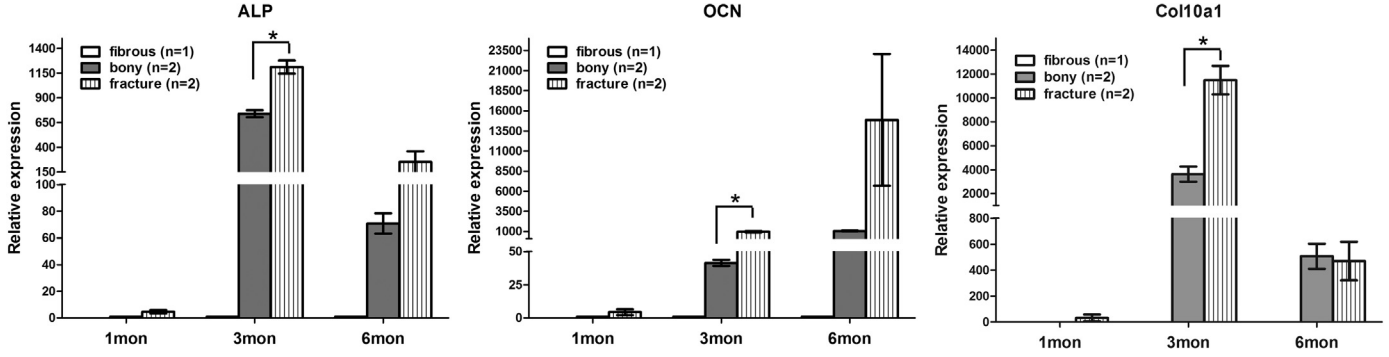


**Fig. 3.** Temporal gene expression of ALP, OCN and Col10a1 in the fibrous ankylosis, bony ankylosis, and condylar fracture. Results were normalized to GAPDH levels and compared to the target signals in the bony ankylosis at 1 month, in the fibrous ankylosis at 3, 6 month using the comparative $C_t$ method (*$P < 0.05$).

condylar fracture, the expression of Runx2 and Osterix in bony ankylosis tended to be lower at 3 time points. Statistically significant differences were found for Runx2 expression at 1 month ($P = 0.048$) with the maximum change of 2.7-fold, and for Osterix expression both at 3 months ($P = 0.005$) and at 6 months ($P = 0.043$) with the maximum change of 4.8-fold at 6 months. As for Sox9, bony ankylosis showed a lower expression at 1 months, a similar expression at 3 months, and a higher expression at 6 months in comparison to condylar fracture, but statistically significant difference for Sox9 expression was found only at 6 months ($P = 0.022$). (See Fig. 4).

### 3.5. *Bmp2, Bmp4, and Bmp7*

The expression of Bmp2 and Bmp7 in bony ankylosis was inclined to be higher than that in the fibrous ankylosis, with a maximum 2.5-fold change for Bmp2 and a maximum 4.9-fold change for Bmp7 at 6 months. A similar expression of Bmp4 was found between fibrous and bony ankylosis both at 3 and 6 months. Compared with condylar fracture, the expression of Bmp2, Bmp4, and Bmp7 in the bony ankylosis tended to be lower at 3 time points. Although the maximum change for Bmp2 was 2.6-fold at 6 months and for Bmp7 was 5.6-fold at 3 months, the statistically significant difference was only found for Bmp4 at 6 months ($P = 0.037$) with the maximum change of 3.68-fold. (See Fig. 5).

## 4. Discussion

For the weight-bearing bones, the limited range of motion (ROM) of the animal due to prolonged immobilization can impact the normal healing process, and disuse osteoporosis and bone loss may occur on account of a lack of muscle contractions and decreased weight bearing (Doyle, 2004). In our animal model, the sheep were allowed free, unrestricted weight bearing in animal house after recovery from anesthesia. One week after operation, they were transferred back to the farm and their ROM is the same as the preoperation (Yan et al., 2012b). Therefore, we believe that the ROM of sheep has no influence on the healing of TMJ trauma in the model.

The nutritional status of diet of animals may be another notable factor affecting the normal fracture healing. Studies have shown that malnutrition can lead to intermediate bone healing and nonunion for experimental long fractures (Guarniero et al., 1992; Day and DeHeer, 2001). Rodrigues et al. (2011) found the impaired callus formation during condylar fracture healing in rats with protein undernutrition. In our animal model, although the mouth opening decreased for the sheep with bony ankylosis, they could eat a normal diet, albeit with a prolonged feeding time. In addition, their body weights increased significantly in comparison to the preoperative values (Yan et al., 2012b). Therefore, we did not consider that the animals were in the condition of malnutrition due to the limited mouth opening, and the healing of TMJ trauma in the model did not seemed to be impacted by the status of diet.

Lack of signaling molecules and growth factors is considered to be one of reasons for delayed bone healing and nonunion (Harwood et al., 2010). Kwong et al. (2009) found altered relative expression of BMPs and BMP inhibitors in human fractures progressing toward nonunion. Fajardo et al. (2009) found that the expression of BMP7 decreased, and the expression of several BMP antagonists increased in the fracture nonunion in comparison to healing bone. Lienau et al. (2010) found that a series genes expression, such as BMP2,

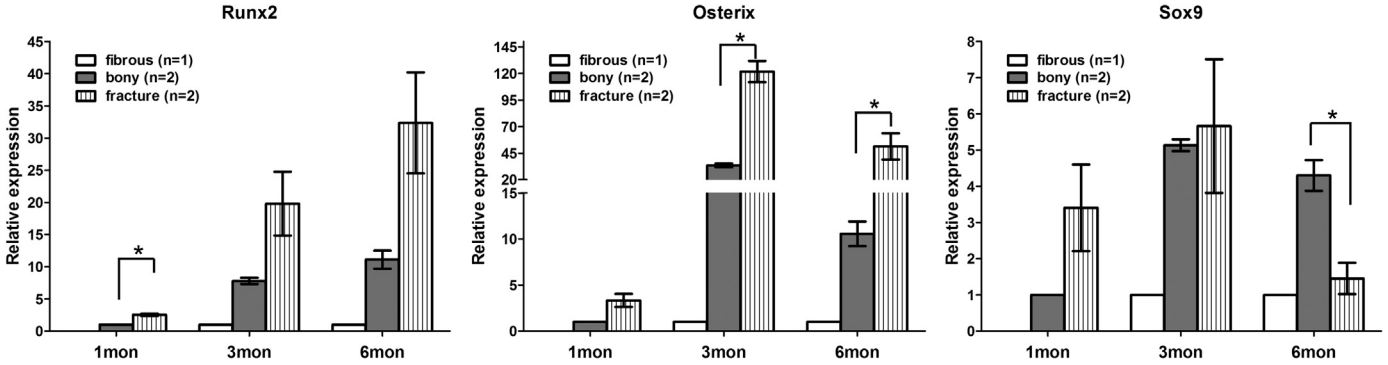


**Fig. 4.** Temporal gene expression of Runx2, Osterix and Sox9 in the fibrous ankylosis, bony ankylosis, and condylar fracture. Results were normalized to GAPDH levels and compared to the target signals in the bony ankylosis at 1 month, in the fibrous ankylosis at 3, 6 month using the comparative $C_t$ method ($^*P < 0.05$).

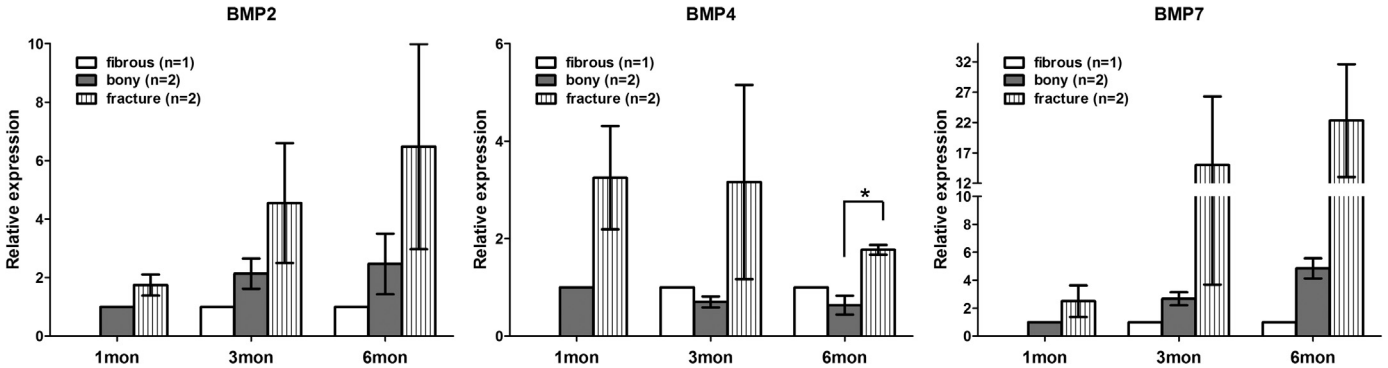


**Fig. 5.** Temporal gene expression of BMP2, BMP4 and BMP7 in the fibrous ankylosis, bony ankylosis, and condylar fracture. Results were normalized to GAPDH levels and compared to the target signals in the bony ankylosis at 1 month, in the fibrous ankylosis at 3, 6 month using the comparative $C_t$ method ($^*P < 0.05$).

BMP4, BMP7, noggin, and Col1a1, was lower in delay healing at several time points compared with the standard healing. In the study, we found bony ankylosis showed a lower expression of several members of Wnt signaling and BMP signaling in comparison to condylar fracture at 1, 3, and 6 months. The result indicated that the development of traumatic TMJ bony ankylosis was a course of delayed bone healing or hypertrophic nonunion with low activity of osteogenesis from the molecular level.

We also found a series of genes expression, such as β-Catenin, Lef1, Runx2, Alp, Ocn, and Bmp2 in bony ankylosis was inclined to be higher than that in fibrous ankylosis both at 3 and 6 months. Accordingly, histological analysis in the previous study showed that no cartilage and new bone formation in the joint space during the course of fibrous ankylosis (Yan et al., 2012b). We believed that the higher activity of BMP and Wnt signalings in the bony ankylosis in comparison to fibrous ankylosis was the molecular base leading to the continuous new bone formation in joint space during the development of traumatic TMJ bony ankylosis.

In the hypothesis of hypertrophic nonunion proposed by Yan et al. (2012a), the Perren's strain theory can well explain, for patients with bony ankylosis, why the radiolucent zone exists in the bony fusion area and how the traumatic articular surfaces fuse gradually under the interference of open movement. In this study, the deficiency of key growth factors in the formation of bony ankylosis preliminarily verified one of the characteristics of the condition, namely the long-standing radiolucent zone, from the molecular level. However, like the hypertrophic nonunion, another characteristic of bony ankylosis manifesting excessive bone apposition around the joint, which seems to be paradoxical to the radiolucent zone, has not been addressed in the study. We did not consider that the low activity of osteogenesis in the joint space can explain it.

In order to clarification of the paradox between the radiolucent zone indicating inhibition of bone formation in the joint space and the excessive bone apposition around the joint, the complex mechanical microenvironment after condylar fracture should be taken into account. A supplemental hypothesis which did not contradict the hypothesis of hypertrophic nonunion was proposed here. When sagittal or comminuted fracture with high risk to cause ankylosis occurs, disc displacement often leads to bone-to-bone contact or close approximation of the traumatic articular surfaces, which provides the most favorable condition for bony ankylosis (Laskin, 1978). However, opening movement is unavoidable because of eating, and the repeated condylar motion produces relative movement between the injured articular surfaces where bony fusion will occur in the future. Although the condylar movement is complex after condylar fracture, at least two types of forces in the traumatic joint can be postulated in the simplified theoretical model. One is the cyclic shear force from the condylar gliding; the other is the dynamic compressive loading from the repeated impact of the condyle against the glenoid fossa or zygomatic arch. Furthermore, the compressive loading on both of the condyle and glenoid fossa may increase because of the attenuation or lost of buffer effect due to disc displacement.

On one hand, the cyclic shear force in the joint gap favors the formation of cartilage (Le et al., 2001) and leads to disruption of the nascent microvasculature growing into hematoma, thus inhibiting calcification of hematoma and ultimately resulting in delayed bone healing just as happens in non-stabilized fractures (Augat et al., 2003; Schell et al., 2005). The molecular mechanism underlying the course was preliminarily investigated in the present study.

On the other hand, bone tissue has the capacity to adapt its mass and architecture to meet the prevailing mechanical loading environment. It is clear that dynamic, compressive loading induces periosteal and endocortical bone formation in long bone and that static, stationary loading does not (Robling et al., 2001; Turner et al., 2009). Therefore the increased dynamic compressive loading can continuously stimulate periosteal and endocortical bone formation in the condyle, articular fossa and zygomatic arch, thus presenting as excess bone formation around the joint, disappearance of bone marrow cavity and osteosclerosis (Yan et al., 2011). During this complex course, osteocytes are thought to be the major bone cell type responsible for sensing the mechanical strain (Bonewald and Johnson, 2008). The decrease of sclerostin secretion by osteocyte and the upregulation of Wnt/β-catenin signaling pathway in osteoblast may be the potential mechanism for the excess bone formation around the joint (Robling et al., 2006, 2008; Lin et al., 2009). This is our current research focus.

In summary, the specificity of TMJ traumatic bony ankylosis which is distinguished from other bone-forming diseases such as spondylarthritis, is that the osteogenesis around the joint may be stimulated, whereas the osteogenesis in the joint space may be inhibited. The biomechanical factors working on the cellular level as described by Frost and his Mechanostat theory (Frost, 2003, 2004) may play key roles in the development of TMJ bony ankylosis, and deserved to be studied further.

Based on the low incidence of ankylosis after condylar fracture and the infrequent patients with TMJ ankylosis even after arthroscopy, Hall (1994) considers that gene biological behavior of specific populations may relate to the traumatic TMJ ankylosis. No previous published data support a role of genetic factors in the susceptibility of the condition, although Gu et al. (2008) reports that with the deficiency of Shox2, the disc will fuse with the fibrous layers of the condyle and glenoid fossa in the development of mice TMJ. In our sheep model, we did not think that genetic predisposition played any role in the formation of traumatic TMJ bony ankylosis because all the sheep developed into bony ankylosis as long as the induced conditions were provided (Yan et al., 2012b). From this perspective, the differential gene expressions among fibrous, bony ankylosis and condylar fracture are not the "cause", but just the "symptom" of differentiation under initially different traumatic microenvironments. For the role of the genetic factors, a lack of evidence to support does not mean they do not exist. These genetic factors may be share with other bone-forming diseases such as heterotopic calcification, ankylosing spondylitis and fibrodysplasia ossificans progressiva, and deserved to be further studied.

There are apparent limitations to the study design that need to be considered. Firstly, the sample size for each time point is so small that our results only showed the trend of gene expression differences among the fibrous ankylosis, bony ankylosis and condylar fracture. These gene expression differences in the study should be verified by a larger sample size. Secondly, given the presence of these gene expression differences, it does not mean the expression of the gene products also have the same changes, so the methods for protein quantitative analysis, such as Western blot, should be applied to verify the expression differences of interesting proteins. Thirdly, the study did not measure the cell-specific measurement of gene expression, whereas both ankylosis and fracture callus are heterogenous mixture of various cells and tissues. Therefore, the spatial patterns of gene expression among various cell types were not addressed. We intend to resolve the problem using immunohistochemistry in the next step. Fourthly the signal molecules examined in the study were only a small part of genes related to the bone healing and regeneration. By use of gene chip technology in the future, we can acquire more beneficial information and may discovery new signal molecules which are specifically linked to the TMJ bony ankylosis. In addition, the healing of condylar fracture created in the model was not the standard cortical healing since the fracture fragments were not fixed. Even in the case of fracture fixation, the healing of the condylar head will be that of cancellous bone in the first place as the condylar head is made up mainly by spongious bone with very thin cortical layers. Therefore, for the

bony ankylosis, a better control may be the condylar neck fracture with fixation. Last but not least, we only studied the progressive events since the first time point selected for tissue harvesting is at 1 month after surgery, next challenge will be to identify the target cells and early events triggering cell differentiation to gain better insights into the pathogenesis of bony ankylosis.

## 5. Conclusion

Taking fibrous ankylosis and condylar fracture as controls, the study has demonstrated for the first time that the activity of bone formation in the bony ankylosis was inclined to be higher than that in the fibrous ankylosis, but lower than that in the condylar fracture in a sheep model. These results provide preliminary evidences for our hypothesis that the development of traumatic bony ankylosis is the course of delayed bone healing or hypertrophic nonunion, and deserved to be studied further.

## Conflicts of interest statement

The authors indicate no potential conflicts of interest.

## Acknowledgment

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