



# Nanog, Stat-3, and Sox-5 involvement in human fetal temporomandibular joint late development

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## ABSTRACT

**Background and aim:** The temporomandibular joint (TMJ) is a synovial joint that allows the complex movements essential for life. It connects the jawbone to the skull, working as a sliding hinge. Moreover, pluripotent stem cells are a source of precursors and tissue-specific cells in developing organisms, however, their biodistribution in developing fetal tissues is weakly studied. The aim of our study was analyse immunohistochemical expression of Nanog, Oct-4, Sox-2 and Stat-3 and Sox-5, in TMJ tissue samples from human fetuses aged between the 12th and 20th weeks of intrauterine life.

**Materials and methods:** We fixed and processed TMJ tissue samples from human fetuses, histological sections and immunohistochemical procedures were carried out.

**Results:** TMJ histological studies examination did not reveal any difference in the tissue organization between the samples in the studied periods. Immunohistochemical analysis demonstrated that Oct-4 and Sox-2 lack their expression in TMJ. In contrast, Nanog was expressed in nucleus of proliferative layer of mandibular condyle, Stat-3 was expressed in nuclear cells of articular disc, Stat-3 and Sox-5 showed positive nuclear and cytoplasmic immunostaining in chondrocyte layers and in ossification areas.

**Conclusions:** Nanog acts in maintenance of pluripotency, Stat-3 in articular disc acts as a transcriptional factor. Stat-3 and Sox-2 act in chondrocyte and osteoblast differentiation. Distribution of the cells, which express Nanog, Stat-3, and Sox-5 in TMJ tissue during fetal development, can help further understand its physiology, pathology, and repairing capacities.

## 1. Introduction

The temporomandibular joint (TMJ) is an interdependent bilateral synovial joint, allowing rotation and translation movements of the jaw around a fixed bone.<sup>1,2</sup> In adulthood, TMJ comprises bone structures, mandibular condyle (MC), articular tubercle, and mandibular fossa (MF) of the temporal bone, covered by articular fibrocartilage. The articular disc (AD) regulates the discrepancy between the anatomic joint surfaces, absorbs shock, and promotes smooth movements of the TMJ.<sup>3</sup> The fibrous capsule allows broad movements of the joint, and the synovial membrane produces a viscous lubricant liquid that nourishes fibrocartilage.<sup>4</sup> The development of the human TMJ is a unique process and Mérida-Velasco et al. (1999)<sup>5</sup> identified three phases in TMJ development. Our study focused on the twelfth to twentieth weeks of

intrauterine life, which is the third phase identified as maturation, with the differentiation of cells present in the proliferation layer.

The TMJ has several developmental stimuli, including intrauterine and postnatal ones. Intrauterine stimuli include sucking, tongue expulsion, and facial movements,<sup>6</sup> and postnatal ones also include dental occlusion, masticatory forces, facial morphology, and also pathological stimulus like vitamins, hormonal deficits, and infections or inflammatory diseases, all leading to TMJ cellular response and in some cases to temporomandibular disorders (TMD).<sup>7,8</sup>

The TMJ response in all developmental stages is associated with cell response. In early development, pluripotent stem cells transcriptional factors (TF) mainly rule this responsiveness. TMJ, like most craniofacial structures, is originated from the neural crest. Neural crest stem cells are known to migrate and subsequently participate in the morphogenesis of

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all craniofacial structures such as cartilage, bone, ligaments, cranial sutures, muscles, tendons, teeth, and periodontium.<sup>9</sup>

Pluripotent cells show a vast differentiation capacity in early development due to the expression of such TFs as Sox-2, Nanog, Stat-3, and Oct-4.<sup>10–12</sup> In addition, some TFs, like Sox-5, plays an important role in chondrogenesis.<sup>13,14</sup> However, the expression of these TFs in TMJ during prenatal human development is unknown.<sup>1</sup> Therefore, in this study, we investigate the expression of such TFs as Sox-2, Nanog, Stat-3, Oct-4 and Sox-5 in TMJ in human fetuses aged 12–20 weeks.

## 2. Methods

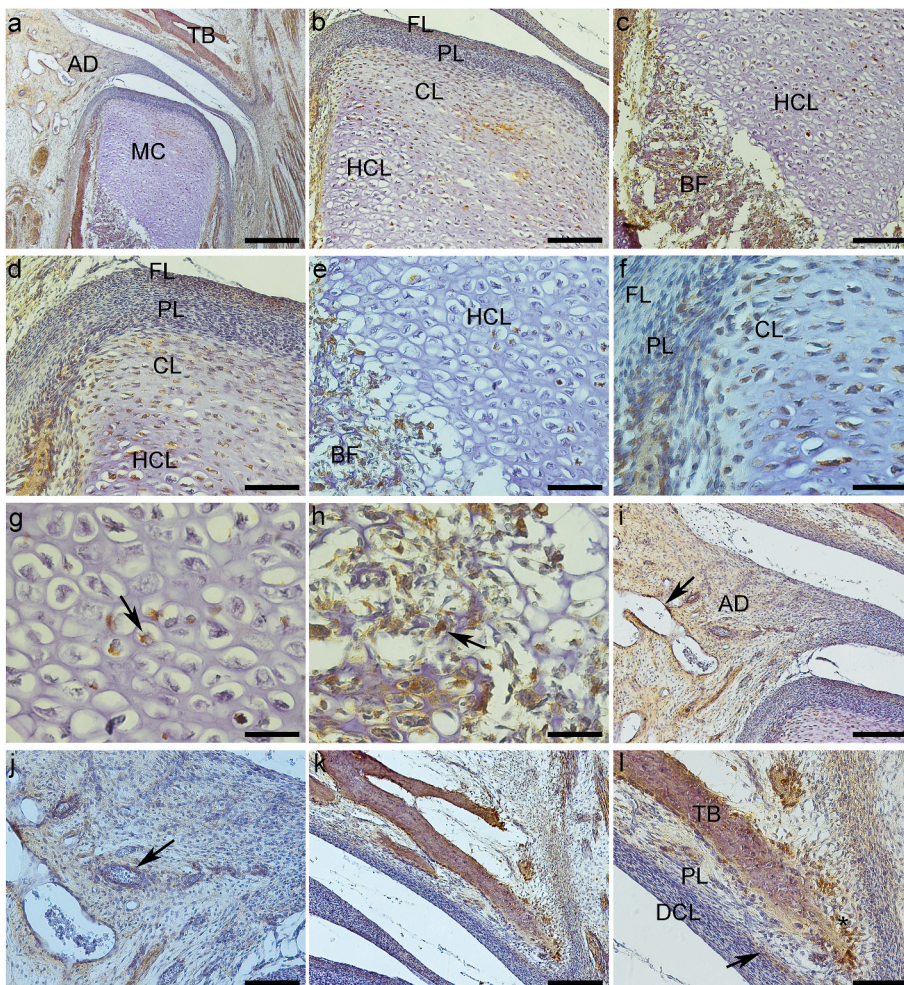
According to the Ethical Committee of São Paulo Federal University/São Paulo Hospital (#1906/09), fetuses were collected in the Pathology Department Archives of São Paulo Federal University. Samples of both genders, ranging from 12 to 20 weeks of development were used, and those presenting aberrations or genetic changes were excluded. Twenty fetal TMJ were dissected and fixed in neutral buffered formalin solution at 10%. The decalcification process was in formic acid at 10% for two days. After that, the tissue was processed and embedded in paraffin. Paraffin-embedded samples were cut by cross-section using the microtome Accu-Cut® SRMTM (Sakura Finetek, USA) into thin slices of 3  $\mu$ m. StarFrost® adhesive microscope slides silane coating (Knittel Glass, Germany) were used to keep the section attached to the slide. The histological sections were stained with hematoxylin and eosin (H/E) and analyzed under a microscope before immunohistochemical staining.

## 2.1. Immunohistochemistry

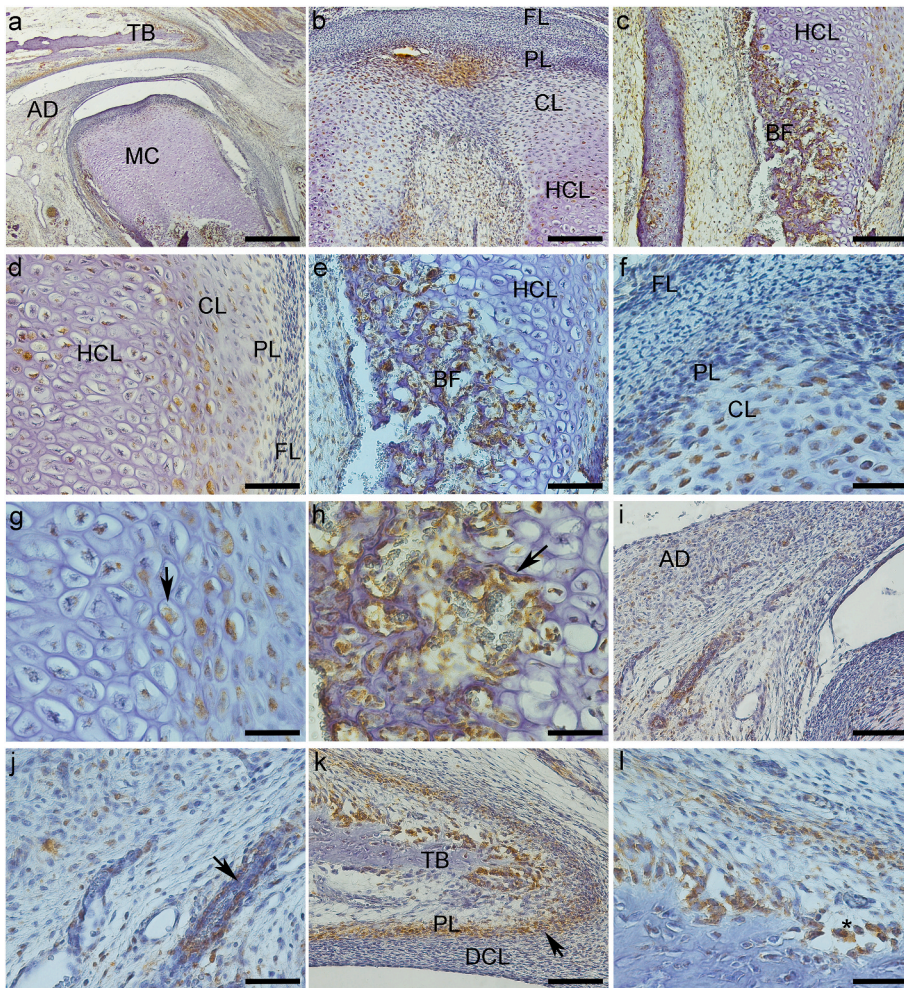
Sections (3  $\mu$ m thick) from specimens were deparaffinized and rehydrated. The slides were heated to 95 °C for 35 min in citrate buffer pH 6.0 for antigen retrieval and incubated in methanol and in 6% hydrogen peroxide solution (1:1) for 30 min to quench endogenous peroxidase activity. The sections were incubated with 1% BSA (Bovine Serum Albumin; Sigma Aldrich) for 45 min to block nonspecific reactions. The slides were incubated with primary antibodies: Oct-4 (C52G3, Cell Signaling Technology, Danvers, MA) 1:25, Sox-2 (D6D9 XP, Cell Signaling Technology, Danvers, MA) 1:50, Nanog (1E6C4, Cell Signaling Technology, Danvers, Massachusetts) 1:60, Stat-3 (79D7, Cell Signaling Technology, Danvers, Massachusetts) 1:50 and Sox-5 (AV33323, Sigma Aldrich, St Louis, Missouri) 1:50, overnight at 4 °C. The Advanced HRP system (Dako, Glostrup, Denmark) detected antibodies. The specimens were counterstained with Harris's hematoxylin (Sigma Aldrich, St Louis, Missouri) dehydrated and mounted for observation. Nonimmune serum was used as a negative control, bell stage of dental formation was used as a positive control for Oct-4, Sox-5 and Nanog, lung carcinoma for Sox-2 and colon adenocarcinoma for Stat-3. Immunohistochemistry was not evaluated by software, staining was analyzed by three researchers specialized in morphology. The assessment was carried out independently.

## 3. Results

Histological analysis confirmed that between 12 and 20 weeks of human fetus gestational age, the TMJ (Fig. 1a, 2a and 3a) comprises the



**Fig. 1.** Expression of Nanog protein (a–l). a – General TMJ image with mandibular condyle (MC), articular disc (AD) and temporal bone (TB). b and d – Mandibular condyle with fibrous (FL), proliferative (PL) and chondrocytes layers (CL). d and f – Chondrocytes layer (CL) of mandibular condyle with nuclear staining (arrow). e and g – Hypertrophic chondrocytes layer (HCL) of mandibular condyle with nuclear staining (arrow). c, e and h – Bone formation (BF) in the mandibular condyle (MC), with osteoblastic expression (arrow). i and j – Articular disc showing immunostaining surrounding vessels (arrow). k and l – Mandibular fossa of temporal bone (TB) with expression in osteoblasts (\*), fibroblasts of dense connective layer (DCL) and in undifferentiated cells of proliferative layer (PL) (arrow). a – Bar = 500  $\mu$ m b, c, i and k – Bar = 200  $\mu$ m d, e, j and l – Bar = 100  $\mu$ m. f, g and h – Bar = 50  $\mu$ m.



**Fig. 2.** Expression of Stat-3 protein (a–l). a – General TMJ image with mandibular condyle (MC), articular disc (AD) and temporal bone (TB). b and d – Mandibular condyle with fibrous (FL), proliferative (PL) and chondrocytes layers (CL). d and f – Chondrocytes layer (CL) of mandibular condyle. e and g – Hypertrophic chondrocytes layer (HCL) of mandibular condyle with nuclear staining (arrow). c, e and h – Bone formation (BF) in the mandibular condyle (MC), with osteoblastic expression (arrow). i and j – Articular disc showing immunostaining surrounding vessels (arrow). k and l – Mandibular fossa of temporal bone (TB) with expression in osteoblasts (\*), and in undifferentiated cells of proliferative layer (PL) (arrow). a-Bar = 500  $\mu$ m. b and c - Bar = 200  $\mu$ m d,e, i and k-Bar = 100  $\mu$ m. f, g, h, j and l- Bar = 50  $\mu$ m.

mandibular condyle (MC), mandibular fossa (MF) belonging to the squamous portion of the temporal bone (TB) and articular disc (AD). No changes were observed in TMJ structures between the fetuses of different gestation ages.<sup>5</sup>

The articular disc was between the mandibular condyle and the mandibular fossa of the temporal bone and divided the articular cavity into supra- and infra-discal cavities. The disc proved to be thinner in the center and thicker at the periphery. Its upper portion was flat to slightly convex, and its bottom was concave. In TMJ sagittal sections, the articular disc was connected to the capsule, and two divisions were seen; its upper portion was directed to the temporal bone and the lower portion to the mandible condyle. The lateral pterygoid muscle was attached in the anterior region of these two portions. The articular disc is composed of dense fibrous connective tissue and fibrocartilage, similar to the tissue surrounding the condylar process of the mandible and the mandibular fossa. Its structure consists of bundles of collagen fibers, fibroblasts, fibrochondrocytes, and undifferentiated cells (Fig. 1i and 2i). The mandibular condyle, in sagittal sections, is a convex structure, we can observe the fibrous layer (FL), consisting of dense connective tissue, proliferative layer (PL), which is composed of undifferentiated cells, chondrocytes layer (CL), hypertrophic chondrocytes layer (HCL) and formation of bone tissue (BT) (Fig. 1b–f; 2b–f; 3b–e, g). The mandibular fossa of the temporal bone (MF) presents its lower surface as slightly concave, a dense connective layer (DCL) which is very similar to the fibrous layer of the condyle, a fibrocartilage proliferative layer (PL) with undifferentiated cells, and temporal bone (TB) (Fig. 1l and 2k).

### 3.1. Immunohistochemistry (Table 1)

#### 3.1.1. Oct-4 and Sox-2

We did not observe immunostaining for Oct-4 and Sox-2 in TMJ of human fetuses (See Supplementary Material-Fig. S1 e S2).

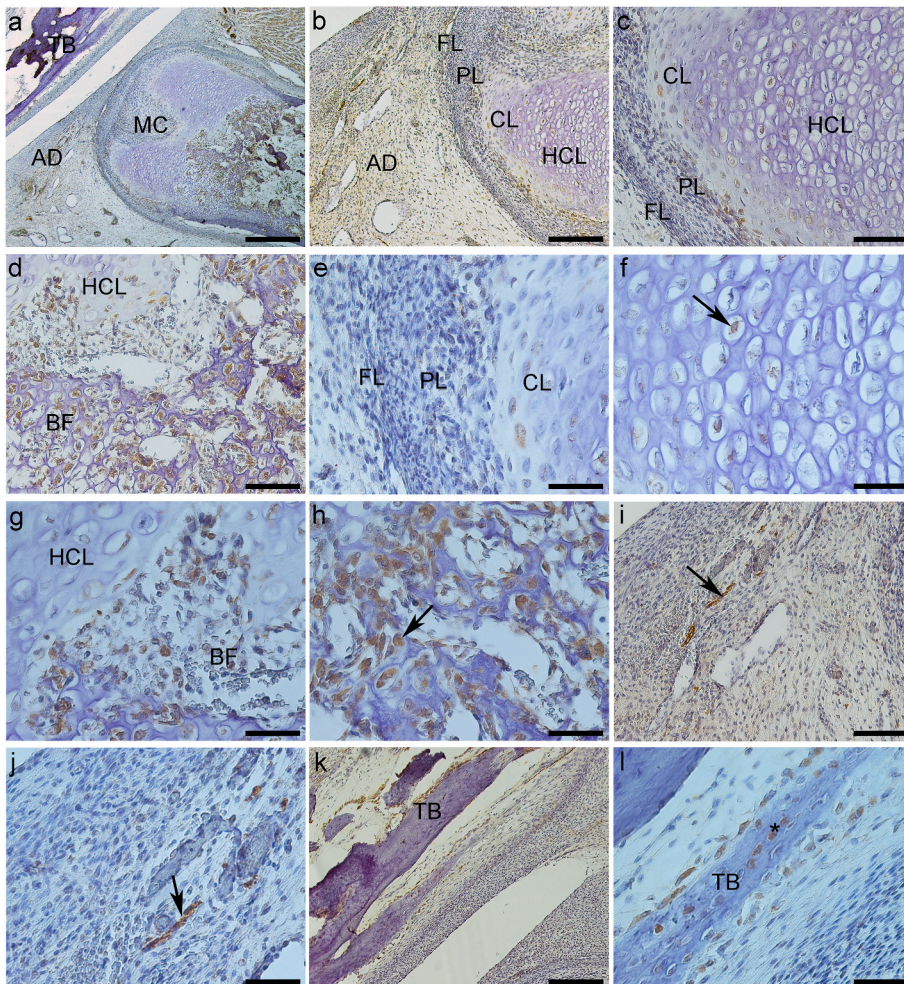
#### 3.1.2. Nanog

More precisely, in the mandibular condyle in the FL (fibroblasts and fibrochondrocytes) and PL, there was intense cytoplasmic and nuclear staining (Fig. 1b, d, and f). CL (Fig. 1b, d, and f) and HCL presented predominantly nuclear staining (Fig. 1b, c, e, and g). The mandibular condyle ossification area showed intense immunostaining mainly in the cytoplasm of osteoblasts and in the extracellular matrix (Fig. 1c, e, and h).

In the articular disc, fibroblasts, fibrochondrocytes, undifferentiated cells, and cells surrounding blood vessels showed mainly cytoplasmic staining (Fig. 1i and j). Analyzing the mandibular fossa (temporal bone), Nanog presented essentially cytoplasmic staining in DCL fibroblasts and in undifferentiated cells of PL (Fig. 1k and l). Osteoblasts surrounding recently formed temporal bone expressed Nanog in the cytoplasm (Fig. 1l) (See Table 1).

#### 3.1.3. Stat-3

Weak immunostaining was observed in FL and PL fibroblasts and fibrochondrocytes (Fig. 2b, d, and f). On the other hand, CL (Fig. 2b, d, and f) and HCL (Fig. 2b, c, d, e, and g) expressed Stat-3 in the nucleus while the ossification area (Fig. 2c, e, and h) was positive in cytoplasm of osteoblasts and the extracellular matrix.



**Fig. 3.** Expression of Sox-5 protein (a–l). a – General TMJ image with mandibular condyle (MC), articular disc (AD) and temporal bone (TB). b, c and e – Mandibular condyle with fibrous (FL), proliferative (PL) and chondrocytes layers (CL). c and e– Chondrocytes layer (CL) of mandibular condyle. f and g– Hypertrophic chondrocytes layer (HCL) of mandibular condyle with nuclear staining (arrow). d, g and h – Bone formation (BF) in the mandibular condyle (MC), with osteoblastic expression (arrow). i and j – Articular disc showing immunostaining surrounding vessels (arrow). k and l– Mandibular fossa of temporal bone (TB) with expression in osteocytes (\*). a-Bar = 500 µm. b and k - Bar = 200 µm. c, d and i-Bar = 100 µm. e, f, g, h, j and l- Bar = 50 µm.

**Table 1**  
Expression of Nanog, Stat 3 and Sox-5 in TMJ analyzed layers/areas.

| TMJ layers/areas               | Transcriptional Factors/Intracellular |          |            |
|--------------------------------|---------------------------------------|----------|------------|
|                                | Localization                          |          |            |
|                                | NANOG                                 | STAT-3   | SOX-5      |
| <b>Mandibular Condyle</b>      |                                       |          |            |
| Fibrous Layer                  | N+C (Strong)                          | C (Weak) | –          |
| Proliferative Layer            | N+C (Strong)                          | C (Weak) | C (Weak)   |
| Chondrocyte Layer              | N                                     | N        | N          |
| Hypertrophic Chondrocyte Layer | N                                     | N        | N          |
| Ossification Area (Osteoblast) | C                                     | C        | C          |
| <b>Articular Disc</b>          |                                       |          |            |
| Fibroblast/fibrochondrocytes   | C                                     | N        | C (Weak)   |
| Perivascular                   | C                                     | N        | C (Strong) |
| <b>Mandibular Fossa</b>        |                                       |          |            |
| Dense Connective Layer         | C                                     | –        | –          |
| Proliferative Layer            | C                                     | C        | –          |
| Ossification Area (Osteoblast) | C                                     | C        | C          |

(N-nuclear, C-cytoplasmic, N+C similar expression in nucleus and cytoplasm, - no immunostaining).

Cells of the articular disc presented Stat-3 essentially in the nucleus of fibroblasts, fibrochondrocytes, and cells surrounding blood vessels (Fig. 2i and j). Mandibular fossa showed Stat-3 immunorexpression mainly in undifferentiated cells cytoplasm of proliferative layer (Fig. 2k and l). Stat-3 was observed in osteoblasts' cytoplasm surrounding the recently formed bone matrix (Fig. 2k and l) (See Table 1).

### 3.1.4. Sox-5

The mandibular condyle showed weak immunoreactivity in the cytoplasm of proliferative layer cells (Fig. 3b, c, and e). CL and HCL showed weak nuclear staining (Fig. 3c, e, and f). The ossification area presented intense immunostaining in the cytoplasm of osteoblasts, surrounding the recently formed bone matrix (Fig. 3d and h). It was possible to see positive Sox-5 immunostaining in the nucleus of cells near HCL (Fig. 3d and g).

Fibroblasts, fibrochondrocyte, and poorly differentiated cells of the articular disc showed weak staining in the cytoplasm (Fig. 3i and j). Cells in the perivascular region showed intense cytoplasmic immunorexpression (Fig. 3i and j). Sox-5 did not show immunostaining in DCL fibroblasts and PL of the mandibular fossa (Fig. 3k); on the other hand, osteoblasts surrounding recently formed temporal bone and osteocytes were positive for Sox-5 (Fig. 3l) (See Table 1).

## 4. Discussion

TMJ is a synovial joint that allows the complex movements necessary for life. Therefore, we focused our study on the morphological features of TMJ development in human fetuses aged between the 12th and 20th weeks of intrauterine life, which has not been studied previously. TMJ starts to develop at the 7th week, thus adopting its postnatal form around the 12th week of intrauterine life.<sup>5</sup> We demonstrated that TMJ histological characteristics showed no differences in studied period of fetal development; for that reason, we analyzed the specimens as a single group. In addition to morphological analysis and taking into account the use of human fetuses, we also for the first time investigated the

expression of the pattern of selective markers of pluripotency and those related to normal chondrogenesis, bone growth, and ossification. The limitation of our study was that we were not able to study the immunohistochemical expression of these transcription factors in the stages prior to the maturation stage that is, the blastemastic stage (weeks 7–8 of development) and the cavitation stage (week 9–11 of development), according to Mérida-Velasco et al. (1999).<sup>5</sup> Another limitation refers to the fact that it was not possible to use other techniques, such as q-PCR, as the tissue studied was previously fixed in formalin and embedded in paraffin, so future studies using molecular techniques should be necessary.

Our immunohistochemical studies demonstrate that Sox-2 and Oct-4 were not expressed in this period of TMJ development. Sox-2 and Oct-4 express at high level in early embryo and in pluripotent stem cells and loss of expression of these proteins results in progressive loss of pluripotency and cell differentiation.<sup>15,16</sup> The lack of staining for Oct-4 in areas of cell proliferation may be associated with the presence of osteochondrogenically-committed cells or their precursors and may confirm that it is not required for self renewal and differentiation ability of somatic cells.<sup>17</sup> Sox-2 is expressed at the earliest nervous system developmental stages, and it functions as a marker protein for neural development and also regulates a variety of other tissue-specific stem/progenitor cells.<sup>18,19</sup> Thus, the lack of expression of Sox-2 suggests that in TMJ this protein is possibly not involved in regulation of stem cells self-renewal potential.

We also showed that Nanog had a robust nuclear and cytoplasmic localization in the PL and FL of the mandibular condyle. In addition, its cytoplasmic localization was observed in articular disc cells and DCL and PL cells of MF. Our data suggest that Nanog showed differential expression in TMJ tissues, thus indicating its ambiguous role in pluripotency regulation events that occur during TMJ development.<sup>20,21</sup> Active Nanog is typically localized in the cell nucleus of pluripotent stem cells.<sup>22</sup> Recently, cytoplasmic, more precisely centrosome localization of Nanog, during the cell cycle was detected in various tumor and non-tumor cell types.<sup>23</sup> Fan et al., 2021<sup>24</sup> recently described a fibrocartilage stem cell population in a fibrous superficial zone of the adult human mandibular condyle. The formation of these cells occurs during the late period of the embryonic stage,<sup>25</sup> which corroborate with our data.

Stat-3 is an important TF mediating intracellular signaling in osteoblasts and osteoclasts.<sup>26</sup> In humans, Stat-3 mutations reduce bone mass and increase bone resorption and trauma fractures, caused by rises in osteoclast number, thus suggesting its important role in osteoclast recruitment and activity. The literature has shown that Stat-3 is required for osteoblast and chondrocyte differentiation.<sup>26</sup> Stat-3 nuclear trafficking is pivotal to its function as a TF; to influence transcription Stat-3 should enter the nucleus.<sup>27</sup> According to Stat-3 biological function, we observed its expression in ossification areas of MF and MC. In articular disc cells, Stat-3 presented nuclear localization in fibrochondrocytes suggesting its transcriptionally active state. While in PL and FL of the mandibular condyle, Stat-3 showed weak cytoplasmic staining.

Our results demonstrate Sox-5 nuclear positive immunostaining in CL and HCL and in the cells near HCL. As reported, Sox-5 belongs to the Sox family. Its normal function avoids defects in chondrogenesis, bone growth, and ossification.<sup>13,14,28–30</sup>

We may conclude that Sox-2 and Oct-4 apparently are not involved in signaling pathway that regulates self-renewal potential and undifferentiated state of cells in fetus TMJ. Nanog, Stat-3 and Sox-5 are transcriptionally active in the chondrocyte hypertrophic and chondrocyte layers of the mandibular condyle. Additionally, Nanog was active in the fibrous and proliferative layers of the mandibular condyle. Stat-3 was expressed in fibroblast/fibrochondrocytes and perivascular region of the articular disc (Table 1). All these markers are implicated with cells proliferation, thus Sox-5 regulates cell proliferation, apoptosis and migration in Kaposi's sarcoma cells.<sup>31</sup> Nanog is a player in intrascriptonal networks in embryonic stem cell pluripotency,<sup>32</sup> and Stat-3 is a

signal transducer that regulates multiple cell processes, including proliferation.<sup>33</sup> Therefore, we may suppose that TMJ layers/areas positive for all three markers are areas of cell proliferation and growth. This conclusion follows the biological function of the condyle, which provides structural support to the overlying cartilage with a meager self-repair potential.

## Ethical approval

Ethical Committee of Sao Paulo Federal University/Sao Paulo Hospital (#1906/09)

## Declaration of competing interest

The authors declare they have no competing interests.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jobcr.2023.08.002>.

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## List of abbreviations

**AD:** Articular Disc  
**BSA:** Bovine Serum Albumine  
**BT:** Bone tissue  
**CL:** Condroytes Layer  
**DCL:** Dense connective Layer  
**FL:** Fibrous Layer  
**H/E:** Hematoxilin and Eosin  
**HCL:** Hypertrophic Condroytes Layer  
**HRP:** Horseradish Perioxidase  
**MC:** Mandibular Condyle  
**MF:** Mandibular Fossa  
**NCSC:** eural Crest Stem Cells  
**Oct:** 4 Octamer-binding - 4  
**PL:** Proliferative Layer  
**Sox-2:** SRY (sex determining region Y)-box 2  
**Sox-9:** SRY (sex determining region Y)-box 2  
**Stat-3:** signal transducers and activators of transcription)  
**TB:** Temporal BoneTF-Transcriptional Factors  
**TMD:** Temporomandibular Disorders  
**TMJ:** Temporomandibular Joint