Quantitative Proteomic Analysis of Rat Condylar Chondrocytes during Postnatal Development

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Objective: *To investigate differentially expressed proteins in rat mandibular condylar cartilage (MCC) chondrocytes caused by initial mastication for short postnatal periods.*
Methods: *Four groups of protein samples were extracted from primary cultured rat MCC chondrocytes, harvested from eigthy postnatal SD rats aged 1,7,14 and 28 days, with twenty in each group. Total proteins were labelled with isobaric tags for relative and absolute quantification (iTRAQ) reagents. Two-dimensional nano-high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization-time-of-flight/ time-of-flight (MALDI-TOF/TOF) mass spectrometry analysis with iTRAQ technique were performed. All data were analysed by MASCOT software with the SWISSPROT protein database. Furthermore, bioinformatics and statistical analysis were performed to classify their cellular components, biological processes, molecular functions and metabolic pathway by the PANTHER database.*
Results: *In total, 137 differentially expressed proteins were identified during MCC growth and were assigned to one or more cellular components. According to the PANTHER analysis, a significant proportion of proteins are involved in the metabolic process, cellular process, biological regulation, developmental process and response to stimulus. The most extensive molecular function was 43% in catalytic activity. In addition, it was found that proteins in MCC chondrocytes change markedly on the growth stage of eruption of the teeth.*
Conclusion: *This study provides an integrated perspective of molecular mechanisms regulating early normal postnatal growth and development of rat MCC at the protein level.*
Key words: *Bioinformatics analysis, Chondrocyte, iTRAQ, Mandibular condylar cartilage, Proteomic analysis*
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Temporomandibular joint disorders (TMD) are one of the most common chronic diseases adversely affecting speaking, masticating and swallowing. The causes of TMD are complicated and multifaceted, and cannot be predicted or prevented[1]. The pathophysiology of TMD is characterised by various hard and soft tissue changes of the joint[2], and inflammation mediated by several cytokines and growth factors[3,4]. Recently, tissue engineering of TMJ has shown potential for patients, who suffer afflictions from condyle dysfunctions or osteoarthritis, and offers the possibility of biologically and mechanically functional regeneration on both mandibular condylar cartilage and bone[5]. However, it is still a challenge to merge the three essential elements: cells, signals and scaffolds into a functional replacement similar to the native tissue. Therefore, a better and comprehensive understanding of TMJ biology on its normal growth and development is needed to prepare for all kinds of hurdles during the TMJ-related research.

It is widely known that mandibular condylar cartilage (MCC), characterised as secondary cartilage, is an important growth zone in the TMJ complex. It consists of chondrocytes and cartilaginous extracellular matrix (ECM). Previous studies showed that the MCCchondrocytes were responsible for cartilage and bone formation[6]. Further studies found[7] that MCC synthesis from the undifferentiated mesenchymal cells undergo sequential changes of cell proliferation, hypertrophy, apoptosis and bone formation, while these procedures are controlled by multiple networks of systemic factors, growth factors, signalling molecules, transcription factors and angiogenic mediators. Many studies have implicated the importance of signalling from indian hedgehog (Ihh)/-PTHrP[8,9], bone morphogenetic protein signalling[10], insulin-like growth factors[11], vascular endothelial growth factor (VEGF)[12], fibroblast growth factor-β (TGF-β)[13], transcription factors and other cytokines for the proliferation and differentiation of MCC[14]. In addition, much progress has recently been made in the identification of key growth factors and transcription factors in the condylar cartilage adaptations after altering the natural position of the mandible transversely or functional loading[15,16].

Although many investigations of MCC were based on human and animal genetic studies, the changes of protein properties in MCC caused by initial biting loading for short postnatal periods is still unclear. There has been very few studies on proteomic analysis providing direct information about cellular phenotype[17]. A comprehensive examination of proteome change would facilitate further understanding of the MCC development and illuminate the biological basis of TMJ tissue engineering.

Jiao et al[18] showed that the histomorphometric characteristics of rat condylar cartilage and subchondral bone changed with age and sex, but the data about young rats, aged from 1 to 28 days were not described. In the current study, drastic changes in rat MCC occurred during the short postnatal period. Furthermore, the purpose of this study was to identify and quantify the differential protein expressions of primary normal rat MCC chondrocytes. Such results will be important to understand the mechanisms of MCC growth or the response of young postnatal rat to mechanical stress from weaning to mastication. In this study, iTRAQ technique was standardised for quantitative analysis, and the authors combined 2D nano-HPLC with MALDI-TOF/TOF technology, and focused on biological information analysis to identify released proteins associated with development and other important biological functions during postnatal growth in rats.

Materials and methods

Cartilage isolation from rats

All animal experiments were conducted under an institutionally approved protocol by the Ethics Committee for animal care and the use of the research center for experimental medicine of Ruijin Hospital.

Eighty postnatal male SD rats aged 1,7,14 and 28 days (20 in each group) were divided into four groups according to the different time points. After euthanasia, the condyle tissues were removed from temporomandibular joint immediately. Cartilage tissues were aseptically microdessected, as described previously[19] for proteomic analysis, and some samples stored at 4ºC for histological analysis.

Hematoxylin and Eosin (H&E) staining and immunohistochemical (IHC) staining

For histological observations, the condyle tissues from 1,7,14 and 28 day-old rats were harvested and immersed in 4% paraformaldehyde (pH = 7.4) for 24 h to 48 h at 4ºC and then they were decalcified in 10% Na_2 Ethylene diamine tetraacetic acid (EDTA) solution until the pin could pierce. The specimens from four groups were prepared following a standard procedure for paraffin preparation. The 5 μm-thick frontal condylar sections were deparaffined and stained by H&E[20].

Immunohistochemical staining was carried out with a standard avidin-biotin complex (ABC) method according to the manufacturer‘s recommended protocol. Collagen I positive cells (ab34710, abcam, Cambridge, USA), Collagen II positive cells (ab34712, abcam) and Collagen III positive cells (ab7778, abcam) were identificated by their antibodies (at 1:100 dilution), respectively. Negative controls were incubated under similar preparations without primary antibody.

Cell isolation and culture

After all condylar cartilages were collected, the cartilage slices were combined into one pool respectively according to the time points. Chondrocytes were released from their surrounding matrix by standard enzyme digestion techniques[21]. Briefly, the cartilages were separately digested with 0.25% trypsin (pH 7.2 to 7.4) (Sigma, Saint Louis, USA) solution for 30 min, followed by 0.1% type II collagenase (Sigma, Saint Louis, USA) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, USA) for 2h. The cell suspensions were filtered through 120 μm mesh nylon sieves. After being centrifuged and

washed three times, the cell suspensions were cultured in 100 mm culture dishes for each group at a concentration of 1×10^5 cells/ml and incubated with DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, USA), 100 IU/mL penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 and 95% air at 37°C (Shellab, Cornelius, USA). The culture medium was changed every 2 days. Chondrocytes were used at 80% to 90% confluence in primary culture. Typan blue exclusion revealed that > 95% of the MCC chondrocytes were viable. The cultured MCC chondrocytes were stained with toluidine blue and identification of chondrocytes was carried out by positive immunohistochemistry staining to type II collagen (ab34712, abcam) and insulin-like growth factor-1 (IGF-1) (sc-1422, Santa Cruz, Texas, USA).

Protein isolation

The primary MCC chondrocytes (1×10^7 cells) from each group were collected, centrifuged for 5 min at 1000 rpm and cellular pellets were washed with ice-cold 1× PBS two times. Cells were then lysed by adding a mixture of ice-cold RIPA lysis buffer, containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS). Protease inhibitor (Roche, Mannheim, Germany) was added into cell lysis buffer to prevent protein degradation. Cellular pellets were vigorously vortexed on ice for 30 min at 10 min intervals until the pellets turned transparent. The lysed cellular suspension was centrifuged for 15 min at 12000 rpm at 4°C to remove cellular debris and protein supernatant was transferred to a new microtube. Protein concentrations were estimated by BCA protein assay kit (Beyotime, China), according to the manufacturer's instructions. Protein samples were frozen at -80°C until further use.

iTRAQ labelling

Briefly, 100 μg protein of different groups was labelled with 4-plex iTRAQ reagents, respectively, according to the standard manufacturer supplied protocol (iTRAQ Reagent Multi-plex Kit, Applied Biosystems, California, USA). Four iTRAQ reagents (iTRAQ reporter ions of 114.1, 115.1, 116.1, 117.1 mass/charge ratio) were assigned 114.1, 115.1, 116.1, 117.1 to the 1,7,14 and 28 day-old protein samples, respectively. The labelled protein samples were precipitated and resuspended in 20μl iTRAQ dissolution buffer (pH 8.5). 1μL denaturant (2% SDS) buffer was added to each sample tube and mixed. After that, 2μl reducing reagent (contains 50mM tris-(2-carboxyethyl) phosphine, TCEP) was added and mixed. The sample tubes were incubated at 60°C for 1 h, centrifuged for 1 min and then placed at room temperature for 10 min after adding 1μl cysteine blocking reagent. Then 40μl MilliQ water was used to dissolve 8 μg trypsin (Sigma, Saint Louis, USA), and then mixed and centrifuged for 1 min. Ten microlitres of trypsin solution was added to each tube and the digestion was performed overnight (12 h to 16 h) at 37°C. After that, iTRAQ reagents were taken out, placed at room temperature for 10 min, centrifuged for 1 min and then were diluted with 70μl of Ethanol, where it was mixed and centrifuged for 1 min and later added into the four protein samples, respectively. Each tube was mixed respectively and incubated for 1h at room temperature, diluted with 10 μl of ethanolamine (1M) to end the reaction. Prior to the final step, 10 μl of aliquot of the four samples was taken out, passing column by C18 Ziptip (Millipore, Billerica, MA) and eluted by 90% Acetonitrile (ACN), then concentrated by SpeedVac and evaluated with regard to their labelling efficiency using mass spectrometer.

2D nano-HPLC and MALDI-TOF/TOF MS experiments and bioinformatics analysis

After the labelled peptide mixture was combined and dried, it was fractionated by two-dimensional separation using 2D nano-high performance liquid chromatography (2D nano-HPLC) (SHIMADZU, Tokyo, JAPAN). The first dimension involved using a SCX column, Buffer A i.e. 10 mM ammonium formate/0.1% Formic acid; and Buffer B i.e. 500mM ammonium formate/0.1%Formic acid. The gradient elution started with 0% Buffer B and rose to 20%, 50% and 100% Buffer B over 24 min (6 min per gradient). The second dimension involved using a reverse-phase (RP) analytical capillary column (Thermo, Massachusetts, USA), where Buffer A was 5% Acetonitrile/0.1 Trifluoroacetic acid and Buffer B was 90% Acetonitrile/0.1 Trifluoroacetic acid. Peptides eluting from the capillary column were mixed at a continuous flow of CHCA matrix solution (a total column flow rate of 2μl/min were spotted onto target plates for MALDI-TOF-TOF-MS measurements with accuspot system (Shimadzu, Tokyo, Japan). For every separation run, 768 fractions in total were collected.

Tandem mass spectrometry (MS/MS) detection of peptides was carried out using a MALDI-TOF-TOF 4700 mass spectrometer (ABSCIEX, Massachusetts, USA). Five precursor ions per well were selected from four plates (192 wells per plate) for tandem mass spectrometry identification and there were approximately 3000 precursor ions being selected for the target plates.

Identification of peptide precursors and fragments were accomplished by database searching against the MASCOT software (version 2.1, Matrix Science, London, UK) and the rat SWISSPROT protein database (Release 2010_04). Filtering parameters were set where all reported data had a confidence level of more than 80%. The following mass search parameters were set: mass spectra over the m/z between 800 to 4000 Da; mass spectrometry (MS) tolerance ± 0.15M/Z and MS/MS tolerance ± 0.1M/Z; allowance of missed cleavage: 1; consideration for variable modifications such as methionine oxidation. Cut-off confidence values on accepting protein identification for Mascot was 80%. Finally, the ratios of proteins were normalised by the median average protein ration of the equal mix of other labelled samples. Peptides with no quantification value were removed. iTRAQ ratios were mathematically analysed after log transformation by GPS explorer v3.6 software. Only protein detected in every biological replicate were included and it had to contain at least two unique high-scoring peptides. The quantification results for all proteins found to be differentially expressed were examined manually according to iTRAQ ratio > 1.25 or < 0.75[22].

Identified proteins were classified according to their cellular component, biological process and molecular function. The PANTHER database (http://www.pantherdb.org/) and the web-based DAVID software (https://david.ncifcrf.gov/)[23] were used to analyse and annotate the functions of a large number of proteins. Hypergeometric distribution[23] was used for GO analysis, as the *P*-values were adjusted for multiple comparisons and a total of differentially expressed proteins were under the control of *P*-values < 0.05.

Western blot analysis

Western blot analysis of type I/II/III collagen was performed to verify the results of iTRAQ analysis. According to standard procedures, chondrocytes were lysed and homogenised by RIPA lysis buffer (50 mM Tris(pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Beyotime, China)). The lyastes were centrifuged at 12000 rpm for 20 min at 4ºC, separated by 10% SDS-PAGE gel and then transferred to a PVDF membrane (Millipore, Massachusetts, USA) with a semi-dry transfer apparatus. The blots were blocked for 2 h at room temperature with 5% w/v nonfat milk in Tris-Buffered Saline and Tween 20 (TBST) buffer. The membranes were incubated with primary antibodies as follows: anti-collagen I antibody (1:500, ab34710, abcam, Cambridge, USA), anti-collagen II antibody (1:250, ab34712, abcam), anti-collagen III antibody (1:1000, ab7778, abcam), anti-GAPDH antibody (1:1500, ab8245, abcam), and incubated overnight in TBST (10 mM Tris-HCL, pH 7.5, 150mM NaCl, 0.1% Tween-20), supplemented with 1% BSA at 4ºC. After three washes, the membrane was incubated with corresponding secondary antibodies for 2h: Anti-mouse IgG, HRP-linked Antibody (1:2000, 7076S, Cell Signaling, Massachusetts, USA), Anti-rabbit IgG, HRP-linked Antibody (1:2000, 7074S, Cell Signaling), and were visualised using ECL kits (#170-5060, Bio-Rad, California, USA). The results were digitised using GE Image Quant LAS 4000 mini analyser. This experiment was repeated thrice.

RNA extraction and Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from four time points with TRIZOL reagent (Invitrogen Life Technologies, California, USA). For Real-time PCR, total RNA according to its concentration was reverse transcribed with PrimeScript Reverse Transcriptase reagent kit (TaKaRa, Kusatsu, Japan). The primers of the following four genes involved in the calcium signalling pathway (which was screened from the proteomic results) included:

PLCB-2 (Gene accession No. O89040)-For,
5‘-GCTTCCCATCTAGTGTCCCTGTA-3‘,
PLCB-2-Rev, 5‘-GTTTCCTGACTCCTCCTCTTC-CT-3‘,
IP3KA (Gene accession No. P17105)-For,
5‘-ATGGACCTTGCGTGCTTGACTG-3‘,
IP3KA-Rev, 5‘-ATGCGGAAGCCGAGTGTAGT-GC-3‘,
PMCA3 (Gene accession No. Q64568)-For,
5‘-CCAGAGTTCGCTCGCTACACCA-3‘,
PMCA3-Rev, 5‘-GTCGTTGAAGACACGGCG-GATG-3‘,
CAC1C (Gene accession No. P22002)-For,
5‘-TTTTCGTTCTAAATCTGGTTCTCG-3‘,
CAC1C-Rev, 5‘-TCATTCTCAGGGTCTAT-GTCTTCC-3‘.

The cycle profile was as follows: stage 1: hold at 95ºC for 30s, stage 2: 40 cycles at 95ºC for 5s and 60ºC for 30s. The relative expression levels of target mRNAs were normalised against GAPDH mRNA and the fold changes were calculated by the formula $2^{-\Delta\Delta Ct}$. This experiment was repeated three times, and a comparison was made by ANOVA.

Statistical analysis

All statistical data were expressed as mean ± SD using SPSS version 13.0 (SPSS Inc., Chicago, USA). Comparisons were analysed by one-way ANOVA and statistical differences were considered significant at $P < 0.05$.

Results

Histology and immunohistochemical (IHC) staining

The focus was on early developmental stages of condylar cartilage during the transition from lactation to mastication to account for the altering mechanical load. The 1-day-old rats represented the beginning of the birth group; 7-day-old rats represented the lactation stage group[7]; 14-day-old rats were the group beginning from weaning to mastication stage; and the 28-day-old rats represented the early mastication stage. The histological staining showed that the cartilage layers were not clear and most chondrocytes of MCC were homogeneous at the age of day 1. From the age of 14 days, the layers of condylar cartilage became clearer and could be divided into four layers: the fibrous layer, the proliferative layer, the maturing layer and the hypertrophic layer. Also type I, II and III collagens expressed in MCC chondrocytes were detected among the four time points according to IHC staining (Fig 1).

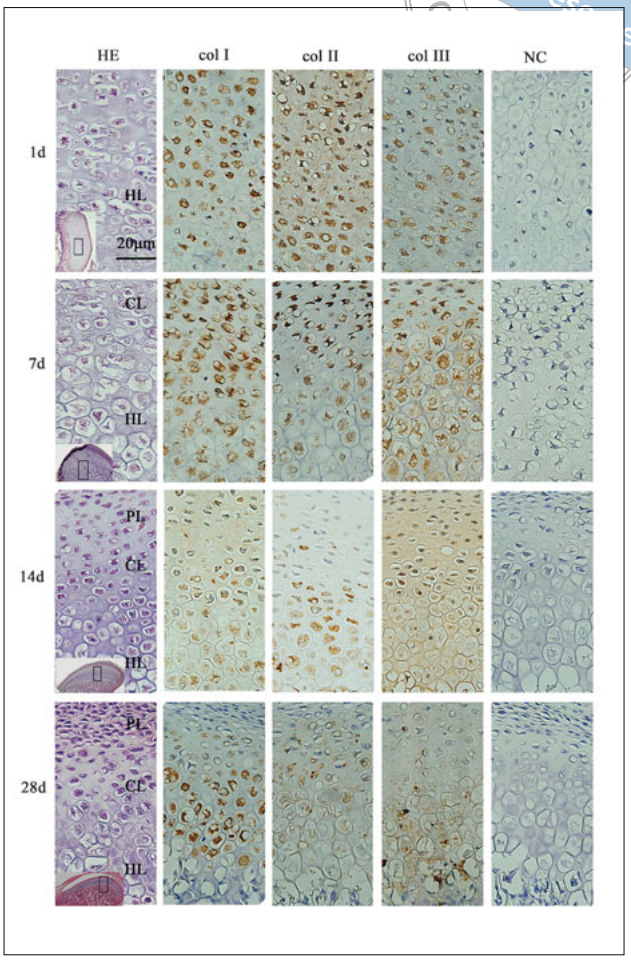

Fig 1 Histology and localisation of type I/II/III collagen in rat condylar cartilage. Type I/III collagen were observed from the fibrous layer to the chondrocyte layer and type II collagen were mainly observed in the chondrocyte layer and hypertrophic layer. FL: fibrous layer; PL: proliferative layer; CL: chondrocyte layer; HL: hypertrophic layer; NC: negative control of immunohistochemical (IHC) staining. Bar = 20μm. Each experiment was repeated thrice.

Isolation of condylar chondrocyte population from rats

When stained with toluiding blue, the cellular matrix of MCC chondrocytes in each group showed metachromasia, which stained partly blue and partly light purple (Fig 2). The cells stained positive because of sulfated gylcosaminoglycans. The detection of both type II collagen and IGF-1 further confirmed the presence of MCC chondrocytes; the latter was also proven to be expressed in the MCC[24]. In four groups, the immunoreactivity of type II collagen and IGF-1 antibody was intense in the cytoplasm of MCC-chondrocytes, especially in the cell gathering region, while the nucleus was not stained according to the preliminary experiment. This indicated that the cultured cells had the characteristics of condylar chondrocytes.

Relative quantitation of proteins from rat MCC-chondrocytes in each group by iTRAQ technology

Protein samples were cleaved into peptide fragments using trypsin, and their absolute masses were accurately measured with a mass spectrometer. In this study, less than 3000 precursor ions were found after background corrections were made for each peak individually. In a typical mass spectrum, the x-axis represents the mass-to-charge ratio (m/z), while the y-axis represents the absolute intensity of peaks. The peak height of peptides was relative to the signal-to-noise ratio and each peak corresponded to a peptide isotopic cluster. Five precursor ions in each well were selected for random mass spectrogram identification. All the peptide masses were compared to SWISS-PROT protein databases by MASCOT software. The iTRAQ-coupled 2D nano-HPLC and MALDI-TOF/TOF technology is a gel-free quantitative

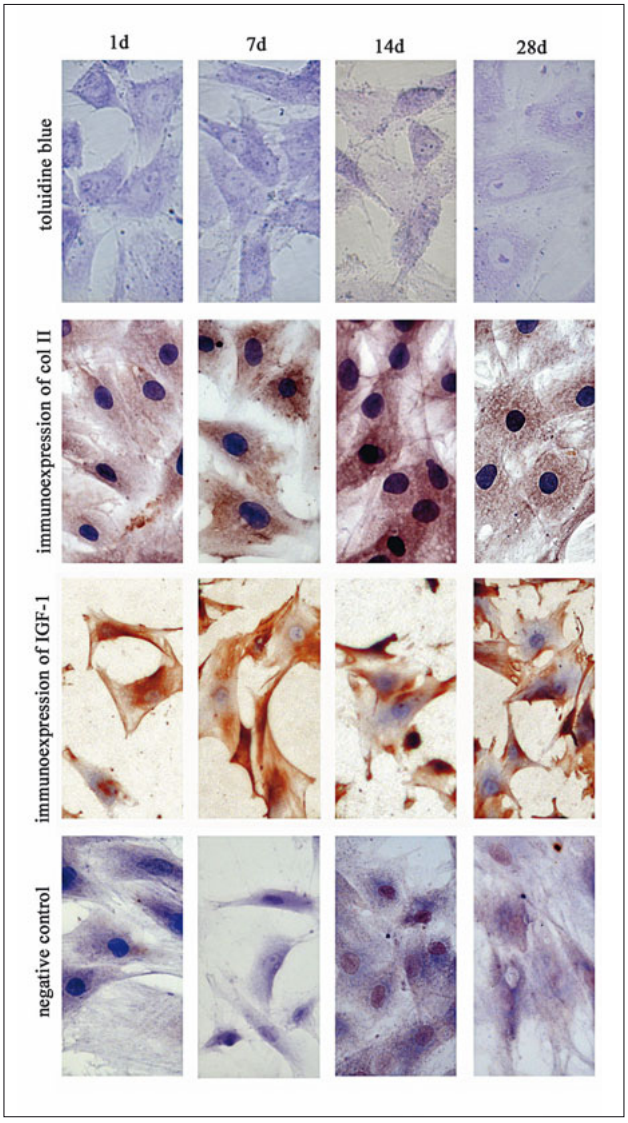

Fig 2 Photomicrographs of 1-,7-,14- and 28-day-old rat mandibular condylar chondrocytes as a function of time in toluidine blue staining, type II collagen, IGF-1 immunohistochemical staining and its negative control (× 400). Each experiment was repeated thrice.

proteomic technology that significantly increases the efficiency of detecting differentially expressed proteins. Relative quantification of proteins using iTRAQ labelling was based on the ratio of peak areas of reporter ions m/z 114.1 to 117.1 in this study. As iTRAQ has a potential advantage in terms of sensitivity to low-level proteins, 137 proteins were identified in the 115/114, 116/114 and 117/114 comparisons, and several interesting proteins were screened for further analysis.

Bioinformatics of differentially expressed proteins of rat MCC chondrocytes

To obtain a detailed view of the protein classification, the list of proteins were submitted as how their gene names were identified in the web-based PANTHER and DAVID software. The protein classification was then retieved according to their cellular components, biological processes and molecular functions. The list of submitted genes was then compared with the list of all the Rattus norvegicus genes.

According to PANTHER software, a significant proportion of proteins were involved in metabolic processes (49.1%), cellular processes (52.6%), biological regulation (28.9%), developmental processes (23.7%), cellular component organisation or biogenesis (19.3%), the immune system process (12.3%) and responses to stimuli (9.6%). Specifically, IL6RB, MYH9, MYH10, PGCB, JIP1, CO3A1, CO1A1, CO2A1, ACTC, TBG1, FAF1, SSH3, ACTC and ILK were detected in the MCC developmental process. PANTHER biological process analysis of the selected dataset was shown in Table 1 and mainly included the following classes: developmental process, apoptotic process, biological adhesion and response to stimulus.

In the molecular function, most of these proteins were found to play a role in catalytic activity (43.0%), binding (38.6%), structural molecule activity (15.8%), enzyme regulator activity (9.6%), receptor activity (6.1%) and protein binding transcription factor activity (0.9%). Furthermore, the identified proteins were categorised into enzyme modulators (14%), cytoskeletal proteins (11.4%), transporters (7.9%), calcium-binding protein (7%), signalling molecules (7%), receptors (7%) and cell adhesion molecules (4.4%), according to their cellular components by PANTHER analysis.

Validation of detected proteins by western blot and RT PCR

In this study, three representative proteins: type I/II/III collagen were chosen to validate the differentially expressed proteins by western blot (Fig 3) and IHC staining in condylar cartilage (Fig 1). Type III collagen expression levels were maintained, while type II collagen expression levels increased during the early dentition phase (at 14 days old), and decreased thereafter. The results were in close agreement with those of the iTRAQ analysis. The trend of Type I collagen expression levels was found to be up-regulated in 7- and 14-day-old rats, but as shown by the iTRAQ analysis, it was maintained (Fig 3).

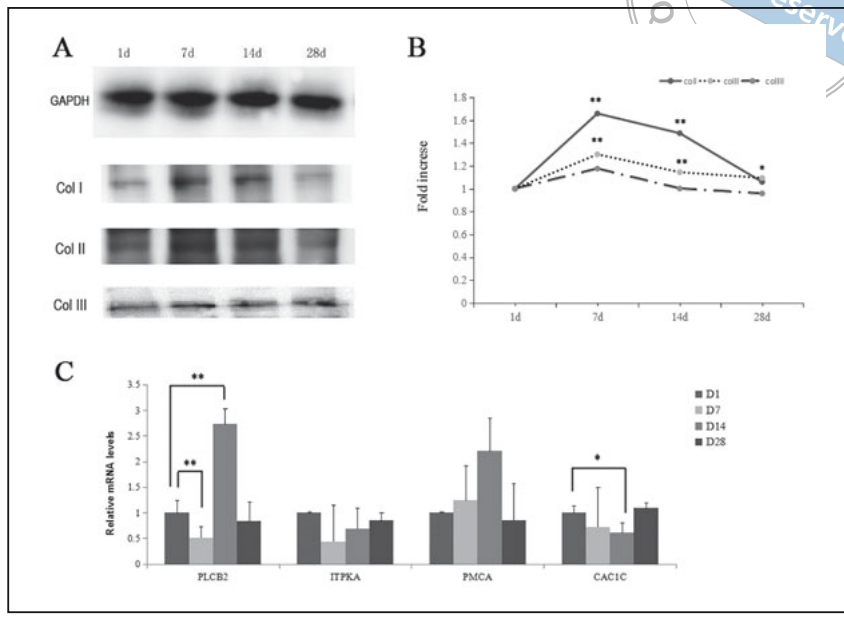

Fig 3 (A) and (B) shows the expression of three identified proteins (Col I/II/III) in each group by western blot. (C) shows the relative gene expression of PLCB2, ITPKA, PMCA and CAC1C in each group, as determined by RT-PCR. With regard to statistical analysis, these experiments were repeated thrice and values were expressed as Mean ± SD. Comparisons were indicated by bars. $^{*}P < 0.05, ^{**}P < 0.01$.

DAVID analysis suggested that the most prominent and relevant pathway is the calcium signalling pathway ($P = 0.021$), including 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-2, inositol-trisphosphate 3-kinase A, plasma membrane calcium-transporting ATPase 3 and voltage-dependent L-type calcium channel subunit alpha-1C encoded by PLCB2, IP3KA, PMCA3 and CAC1C genes, respectively. Four genes were chosen for validating the turnover of four differentially expressed proteins. Although the extent of mRNA alterations is not the same as the proteomic results, the trend of the change was consistent with the iTRAQ well (Fig 3). All the data showed that the quantitative proteomic analysis based on iTRAQ was convincing.

Discussion

It has been shown that MCC and its subchondral bone responds to the mechanical stress from its normal mechanical microenvironment or functional mechanical loading through a great deal of growth factors, transcription factors and cytokines, which leads to cell proliferation, differentiation and extracellular matrix synthesis. Different growth stages influence the expression of various proteins of MCC. However, characterisation of their protein profiles following MCC development has not been clearly performed. This study focused on the protein expression associated with MCC functional changes in young postnatal rats, during the growth stage of newborns (1 day old), lactation (7 days old), eruption of teeth (14 days old) and mastication (28 days old).

The authors have succeeded in quantifying the differentially expressed proteins of single MCC chondrocytes during the early postnatal growth stage for the first time. In the present study, MCC-chondrocytes were isolated from postnatal rat mandibular condylar cartilage by the sequential digestion with trypsin and collagenase. Monolayer cultures of chondrocytes served as useful techniques despite their limitations. The results of toluidine blue staining and type II collagen IHC staining indicated that the cultured cells had the characteristics of chondrocytes. As isolated chondrocytes are phenotypically unstable and chondrocytes dedifferentiate rapidly[25], the primary chondrocytes in the monolayer were used to minimise these phenotypic changes and to harvest sufficient chondrocytes under similar conditions, in order to retain a similar chondrocytic phenotype and protein expressions of each group. Previous studies also show that IGF-1 has a role in the chondrogenesis and growth of the condylar cartilage[11], thus the IGF-1 expression in the MCC-chondrocytes of four groups were further detected, respectively.

A comprehensive understanding of cartilage at the proteomic level is limited because of its complicated biochemical composition and its unique features. Nowadays, proteomic analysis of cartilage has targeted cartilage tissues in some pathological cases, cartilage explants and isolated chondrocytes. The first two-dimensional electrophoresis (2-DE) map of condylar chondrocytes obtained from a normal neonatal rat MCC response to mechanical stress was achieved[17] in 2010, and seven differentially expressed proteins were subsequently identified by MALDI-TOF

technology *in vitro*. The identified proteins contained MAPK pathway inhibitor and cytoskeleton proteins. Although traditional 2-DE is widely used as a standard method for proteomics, together with the subsequent identification of proteins by mass spectrometry (MS) in complex biological samples, it is not very efficient for some hydrophobic, highly-acidic/basic, low-abundance protein separations[26]. The iTRAQ labelling, which can simultaneously analyse 4 to 8 plex, coupled with LC-MS/MS, is multidimensional, accurate, sensitive and automated[27]. Application of current LC-MS/MS-based quantitative methods can identify various signalling molecules efficiently and their interacting proteins in cartilage and bone metabolism, creating new possibilities in the elucidation of changes in cell signal transduction and the regulatory mechanism of cell differentiation[28,29]. In this study, iTRAQ-based quantitative proteomic approaches provide in total, 137 differentially expressed proteins in the rat MCC chondrocytes during short postnatal periods, which were categorised according to different protein functions. However, the results of mass spectroscopy are not absolutely accurate due to the current limitations of proteomics[26], meaning false-positive or false-negative errors are unavoidable. In this study, the expression changes of type I collagen shown by the iTRAQ analysis did not completely agree with those shown by the western blot.

In this study, IL6RB, MYH9, MYH10, CO3A1, CO1A1, CO2A1, ACTC, TBG1, FAF1, SSH3, ACTC and ILK was detected in the MCC developmental process. The markers of mature MCC chondrocytes are type II collagen encoded by the CO2A1 gene[30]. In the cellular proteomic findings (shown in Table 1), type II collagen expression levels increased during the early dentition phase (at 14 days old), and decreased thereafter. This result agrees with results reported previously[7]. Type III collagen (encoded by the CO3A1 gene) expression levels were maintained from newborn to mastication. These iTRAQ quantification results were validated by western blot (Fig 3). It indicates that the collagen network in MCCs endure biomechanical or functional factors of the cartilage tissue and type II collagen, where the major collagen in MCC appears more sensitive to the changes of mechanical loading and play an important role in condylar development. In addition, the cytoskeleton is the other highly abundant chondrocytic protein in MCC chondrocytes. The microfilaments were detected to be made of actin (encoded by the ACTC gene), microtubules made of tubulin (encoded by the TBG1 gene) and myosin (encoded by the MYH9 and MYH10 genes). Unlike the expression levels of tubulin and myosin, actin expression levels tend to decrease markedly at 14 days.

Although a part of flat bone structures, the development of condylar cartilage resembles epiphyseal cartilage and endochondral ossification[31]. In this process, mesenchymal cells differentiate into proliferating chondrocytes, which further develop into Col II positive flattened chondrocytes and progress to mature into extracellular matrix synthesising hypertrophic chondrocytes[32]. After their terminal differentiation, these chondrocytes undergo programmed cell death. Moreover, in the current study, several interleukins, including interleukin-6 receptor subunit beta precursor (encoded by the IL6RB gene) and interleukin-4 precursor were detected. Although these protein expression levels changed very little in normal MCC development, whether they are phosphorylated or not needs to be confirmed, and it has been reported that the response of chondrocytes to mechanical stimulation may be mediated via integrins and interleukin-4[33], and inflammatory pathways will be activated in some pathological statuses[34]. Among heat shock protein binding proteins, FAS-associated factor 1 (encoded by the FAF1 gene) is another important protein during the developmental process of MCC chondrocytes. Its expression levels increased at 7 and 28 days then decreased at 14 days, which is contrary to one of the type II collagens. As Fas and its ligand are associated with chondrocyte apoptosis physiologically[35], FAS-associated factor 1 is involved in the regulation of programmed cell death and the NF-kappaB cascade according to DAVID analysis. It can be speculated from these results that although Fas-mediated MCC chondrocyte apoptosis increases postnatally, functional remodelling of MCC happens due to the alterations in mechanical loading with the eruption of teeth and thus chondrocyte apoptosis decreases, with type II collagen expression being upregulated. The data also indicated that the protein expression levels of MCC chondrocytes changed markedly on the growth stage of the eruption of teeth (at 14 days old).

In the biological process, biological adhesion may be modulated by mechanical loading. Mechanical stimulation in cartilage growth relies on the interactions between chondrocytes and their surrounding ECM by means of particular cell surface receptors, such as integrin. As reported by Liu et al[36], integrin, operating as mechanotransducers[37], and integrin-linked protein kinase may regulate the cellular response due to increased or decreased mechanical loading induced by the mandibular lateral shift on rats. Integrin may trigger several signalling pathways via collagen fibers and proteoglycans and may cross talk with other signalling

Table 1 List of differentially expressed proteins in rat condylar cartilage according to the PANTHER biological process analysis.

Protein name	Mapped IDs	iTRAQ quantification		
		7d/1d	14d/1d	28d/1d
Developmental process (GO:0032502)				
Interleukin-6 receptor subunit beta precursor	IL6RB	0.98	0.94	1.10
Myosin-9	MYH9	1.12	0.90	0.95
Myosin-10	MYH10	1.00	0.89	1.01
Nucleoside diphosphate kinase B	NDKB	1.03	0.77	0.98
Brevican core protein precursor	PGCB	1.07	0.93	1.06
JNK-interacting protein 1	JIP1	1.03	0.83	1.04
Serine/threonine-protein phosphatase with EF-hands 1	PPE1	0.98	0.99	0.98
Collagen alpha-1(III) chain precursor	CO3A1	1.03	0.93	1.06
Collagen alpha-1(I) chain precursor	CO1A1	0.94	0.98	0.86
Collagen alpha-1(II) chain precursor	CO2A1	0.73	1.35	0.73
Serine/threonine-protein kinase MRCK beta	MRCKB	1.29	0.59	1.18
Protein phosphatase Slingshot homolog 3	SSH3	0.76	0.91	0.83
Neurofascin precursor	NFASC	2.07	2.77	1.56
Integrin-linked protein kinase	ILK	1.08	0.75	0.87
FAS-associated factor 1	FAF1	1.18	0.64	1.18
TRAF3-interacting protein 1	MIPT3	0.88	1.05	0.91
Multiple epidermal growth factor-like domains 3	CELR2	1.05	0.85	1.00
Neuronal PAS domain-containing protein 4	NPAS4	1.02	0.88	1.02
Tubulin gamma-1 chain	TBG1	1.07	0.95	1.06
Cadherin-23 precursor	CAD23	1.07	0.66	1.12
Actin	ACTC	1.08	0.76	1.07
Filensin	BFSP1	1.51	3.32	0.43
apoptotic process(GO:0006915)				
Nucleoside diphosphate kinase B	NDKB	1.03	0.77	0.98
JNK-interacting protein 1	JIP1	1.03	0.83	1.04
IGF2 mRNA-binding protein 1	IF2B1	1.12	0.74	1.02
Integrin-linked protein kinase	ILK	1.08	0.75	0.87
FAS-associated factor 1	FAF1	1.18	0.64	1.18
Serine/threonine-protein phosphatase with EF-hands 1	PPE1	0.98	0.99	0.98
Biological adhesion (GO:0022610)				
Mucin-2 precursor	MUC2	1.23	0.70	1.06
Brevican core protein precursor	PGCB	1.07	0.93	1.06
Integrin-linked protein kinase	ILK	1.08	0.75	0.87
Multiple epidermal growth factor-like domains 3	CELR2	1.05	0.85	1.00
Response to stimulus (GO:0050896)				
Interleukin-6 receptor subunit beta precursor	IL6RB	0.98	0.94	1.10
Heat shock 70 kDa protein 9	GRP75	0.76	0.91	0.83
Collagen alpha-1(III) chain precursor	CO3A1	1.03	0.93	1.06
Collagen alpha-1(I) chain precursor	CO1A1	0.94	0.98	0.86
Collagen alpha-1(II) chain precursor	CO2A1	0.73	1.35	0.73
Integrin-linked protein kinase	ILK	1.08	0.75	0.87
Corticoliberin precursor	CRF	0.85	1.11	1.12
Cytochrome P450 5A1	THAS	0.93	0.92	1.06
Serine/threonine-protein kinase TAO1	TAOK1	0.89	0.96	0.91
Interleukin-33 precursor	IL33	1.04	1.09	1.11
Serine/threonine-protein phosphatase with EF-hands 1	PPE1	0.98	0.99	0.98

molecules[37]. However, in this study, the expression of integrin-linked protein kinase (encoded by ILK gene) decreases in the early dentition phase (14 days old). In other words, on the growth stage of the eruption of teeth, type II collagen, integrin-linked protein kinase and actin was found to be altered relatively, which indicates these proteins play an important role in MCC with the eruption of teeth, through several intracellular signalling cascades. Further investigation *in vivo* and *in vitro* should be carried out.

In conclusion, the combination of iTRAQ-based 2D nano-HPLC and MALDI-TOF/TOF technology provided extensive information on protein expression. This proteomic study on the MCC chondrocytes of postnatal rats has revealed that mechanical stimulation from mastication ignites mechanotransduction mainly through the calcium signalling pathway, focal adhesion and ECM-receptor interaction, during the early MCC growth stage. These signalling proteins together with ECM proteins are involved in chondrocyte differentiation, proliferation and cell fate. This data may be used as an effective tool to investigate various signalling pathways or biological functions and may facilitate comparative proteomic human or animal experiments of important biological proteins, in order to contribute to the understanding of MCC biology.

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Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Liting Jiang for preparing the manuscript; Dr Yinyin Xie for carrying out the proteomic experiment and analysis; Dr Li Wei for carrying out the western blot and PCR experiments; Dr Qi Zhou for carrying out the IHC experiments; Dr Xing Shen for completing the data analysis; Dr Yiming Gao and Dr Xinquan Jiang for the design of the study and revision of the manuscript.

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