

# Fos- and Jun-related transcription factors are involved in the signal transduction pathway of mechanical loading in condylar chondrocytes

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**SUMMARY** The chondrocytes of the articular condylar cartilage proliferate, hypertrophy and ultimately undergo apoptosis (programmed cell death), being replaced by osteoblasts. Converging results consolidate activator protein-1 (AP-1) transcription factor as the pivotal downstream effector in the early response of stress-sensitive cells to mechanical loading, and the Fra-1, Fra-2, JunB and JunD members of the AP-1 transcription factor family, as mediators in bone remodelling and apoptotic phenomena. The aim of the present study was to examine the involvement of the Fra-1, Fra-2, JunB and JunD proteins in the biochemical response of functionally loaded mandibular condylar cartilage, and the subsequent initiation of cartilage maturation and apoptotic phenomena.

Thirty, female, 14-day-old Wistar rats were assigned to two groups: one group was fed a soft diet and the other a hard diet. At day 21 after weaning, experimental animals from both groups were killed at 6, 12 and 48 hours and their condyles harvested. The condylar cartilage of both groups was immunostained using specific antibodies against Fra-1, Fra-2, JunB and JunD. Statistical analysis of the data revealed over-expression of Fra-1, Fra-2, JunB and JunD proteins in all stages of differentiation of chondrocytes derived from the mandibular condylar cartilage of animals fed on a hard diet. Moreover, the involvement of these proteins significantly increased with time in both groups.

Since the aforementioned proteins play key roles in remodelling phenomena of bone and cartilage tissue, influencing pivotal cellular functions such as maturation, differentiation and apoptosis, the results of the present study suggest that mandibular condylar chondrocytes sense functional loading changes and respond by induction of proteins associated with biological phenomena that ultimately influence the growth of the condylar cartilage.

## Introduction

In dentofacial orthopaedics an effort is made to influence mandibular growth. The main target in this effort is the condylar cartilage of the mandible. There has been a great deal of controversy over the years concerning the true effects of this treatment rationale. Animal experiments and condylar cartilage tissue examination revealed that the condylar cartilage responded favourably in functional mandibular advancement and extra growth of the cartilage tissue was evident (McNamara and Carlson, 1979).

Despite the clear anatomical evidence of extra condylar cartilage growth, the biological explanation of the phenomenon remains obscure. Research data have demonstrated that mechanical loading influences the growth of the condylar cartilage and the mechanical environment of the temporomandibular joint (TMJ) regulates the biological and histological features of the cartilage tissue, through the differentiation of proliferative cells into mature chondroblasts (Meikle, 1973; Koski, 1974). While

intermittent mechanical loading can serve as maintenance of cartilage growth within the joint, unloading or static loading seem to restrain growth in organ cultures (Kantomaa and Hall, 1988; Pirttiniemi and Kantomaa, 1996).

Tissue culture experiments on condylar chondrocytes under hydrostatic pressure application demonstrate changes in the phosphorylation state of proteins associated with the differentiated phenotype (Basdra *et al.*, 1994). These changes would potentially allow entry into more mature phases of chondrocytes, i.e. the hypertrophic state, accelerating the differentiation/maturation process and subsequently influencing overall growth of the tissue. Thus, unravelling the biological phenomena occurring after force application to mandibular condylar chondrocytes will help elucidate the biological mechanisms of growth and development of the mandibular condyle.

The Jun family proteins are components of the activator protein-1 (AP-1) transcription factor. They are activated by a wide variety of extracellular stimuli and play critical roles

in two distinct and important processes: cell growth and cell apoptosis. The *fos* gene family encodes nuclear proteins (c-Fos, Fra-1, Fra-2) that dimerize with the Jun family proteins, such as c-Jun, JunB, and JunD, to form the AP-1 transcription factor complex (Curran and Franza, 1988; Angel and Karin, 1991; Papavassiliou *et al.*, 1995). Thomas *et al.* (2000), by examining the role of c-Fos in chondrocyte differentiation, showed that induction of c-Fos resulted in a concomitant increase in the expression of Fra-1 and c-Jun, further highlighting the importance of AP-1 transcription factors in chondrocyte differentiation. Moreover, these data demonstrated that c-Fos over-expression directly inhibits chondrocyte differentiation *in vitro*.

After static compressive application to bovine cartilage explants for periods between 1 and 24 hours, the response of 28 genes using reverse transcriptase polymerase chain reaction (RT-PCR) analyses was measured (Fitzgerald *et al.*, 2004). Immediate early genes, *c-fos* and *c-jun*, were up-regulated 6- and 30-fold, respectively, during the first 8 hours of 50 per cent compression and remained elevated after 24 hours. Recent data has identified the Fos-related AP-1 transcription factors Fra-1 and Fra-2 as key elements in cartilage development (Eferl *et al.*, 2004; Karreth *et al.*, 2004).

The aim of this study was to examine the involvement of components of the AP-1 transcription factor family such as Fra-1, Fra-2, JunB and JunD in the signalling pathway of mechanical loading of the condylar cartilage, and subsequently the association of mechanical loading to cell differentiation and apoptosis through the involvement of these proteins.

## Materials and methods

Thirty, female, 14-day-old Wistar rats were assigned to two groups: a group fed on whole pellets (Hankkija, Finland) and a group fed on a soft, powdery diet. Water was available *ad libitum* (Pirttiniemi *et al.*, 2004). At day 21 immediately after weaning, animals from both groups were killed after 6, 12 and 48 hours. At each time point the condyles of the animals were removed and dissected. Specimens from five different animals were used at each time point. All condylar specimens were fixed in 10 per cent neutral formalin for 24 hours.

The sections were then demineralized for five days in 5 per cent formic acid and embedded in paraffin. Sections from paraffin-embedded tissue were used for immunohistochemical staining.

The protocols were approved by the animal experimentation committee of the University of Oulu (Pirttiniemi *et al.*, 2004).

### Immunohistochemistry

The classic biotin-streptavidin-peroxidase method was applied on 4- $\mu$ m thick sections, obtained from the paraffin-

embedded material. Following deparaffinization, the sections were placed in 0.01 M sodium citrate buffer (pH 6.0) and heated in a 1000-W microwave oven twice for 5 minutes. The sections were allowed to cool for 20 minutes at room temperature, rinsed for 5 minutes with distilled water and washed twice with 0.05 M Tris buffered saline (TBS), pH 7.6, for 10 minutes. Endogenous peroxidase activity was quenched by incubation for 20 minutes with 0.6 per cent hydrogen peroxide in methanol. To prevent non-specific binding, following an additional wash in TBS, the sections were incubated for 20 minutes with 0.1 per cent bovine serum albumin (BSA). Incubation with the following commercially available primary antibodies followed overnight at room temperature (Santa Cruz Biotechnology, Santa Cruz, California, USA): anti-Fra-1 (monoclonal, sc-7345/dilution 1:50), anti-Fra-2 (monoclonal, sc-6254/dilution 1:50), anti-JunB (monoclonal, sc-822/dilution 1:70), and anti-JunD (polyclonal, sc-253/dilution 1:100).

Specific binding was detected by utilizing the streptavidin-biotinylated peroxidase-based system (Super Sensitive Ready-to-Use Detection Kit, BioGenex Laboratories Inc., San Ramon, California, USA). To develop a colour reaction, a 0.05 M Tris solution containing 0.05 per cent 3',3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, Missouri, USA) and 0.01 per cent hydrogen peroxide was added to the slides for 3 minutes at room temperature. Finally, the slides were counterstained in Harris haematoxylin, the tissue was dehydrated in graded ethanol and the slides were mounted using Permount (Fisher Scientific, Loughborough, Leicestershire, UK).

Stain intensity and the proportion of immunopositive cells were assessed by light microscopy and evaluated by two investigators (DJP and EKB). A minimum of 1000 cells was detected in each specimen. Immunohistochemical staining was graded on a scale of 0–4+, according to the following assessment (McCarty *et al.*, 1985; Kloen *et al.*, 2003): 0 = no detectable staining, 1+ = weak staining, 2+ = moderate staining, 3+ = strong staining, 4+ = very strong staining. Since the examined proteins were equally distributed throughout the articular chondrocytes, the percentage of immunopositive cells was not evaluated.

To assess the statistical effect of both food types (hard, soft) and time (6, 12 and 48 hours) on the intensity of protein immunostaining, two-way analysis of variance (ANOVA) was used.

## Results

### *Mechanical load induces the expression of Jun-related proteins in articular cartilage chondrocytes*

JunB was detected in both the nucleus and the cytoplasm of articular cartilage chondrocytes. At 6 and 12 hours, JunB was located almost exclusively in proliferating-prehypertrophic

chondrocytes, whereas at 48 hours it was observed in all growth plate zones (Figure 1). Two-way ANOVA showed statistical significance for JunB values, both for food type [ $F = 16.89$ , degrees of freedom (df) = (1.39),  $P < 0.001$ ] and time [ $F = 9.36$ , df = (4.39),  $P < 0.001$ ]. Animals fed with a hard diet showed statistically significant JunB values compared with soft diet animals. The statistical significance of JunB values increased with time in both animal groups, but the influence of both food type and time in the interaction was not statistically significant [ $F = 0.98$ , df = (4.39)].

The other member of Jun family, JunD, displayed both nuclear and cytoplasmic localization in all animals. At 6, 12 and 48 hours JunD was observed mainly in the proliferating cartilage zone (Figure 2). Two-way ANOVA showed statistical significance for JunD values, both for food type [ $F = 20.64$ , df = (1.40),  $P < 0.001$ ] and time [ $F = 15.89$ , df = (4.40),  $P < 0.001$ ], while animals fed with a hard diet showed statistically significant JunD values compared with animals fed with a soft diet. The statistical significance of JunD values increased with time in both groups but the influence of both factors (diet and time) was not statistically significant [ $F = 1.54$ , df = (4.40)].

#### *Mechanical load induces the expression of Fos-related proteins in articular cartilage chondrocytes*

Fra-1 demonstrated both nuclear and cytoplasmic immunolocalization in the majority of the assessed chondrocytes. Hypertrophic cartilage cells exhibited immunopositivity for Fra-1 principally at 48 hours, while prehypertrophic proliferating chondrocytes were Fra-1-reactive in almost all animals, at all time points (Figure 3). Two-way ANOVA showed statistical significance for Fra-1 values both for food type [ $F = 24.85$ , df = (1.41),  $P < 0.001$ ] and time [ $F = 13.53$ , df = (4.41),  $P < 0.001$ ]. Data analysis showed that animals fed with a hard diet had statistically significant higher Fra-1 values compared with animals fed with a soft diet. The statistical significance of Fra-1 values increased with time in both groups. The influence of both parameters (food type and duration) was not statistically significant [ $F = 1.30$ , df = (4.41)].

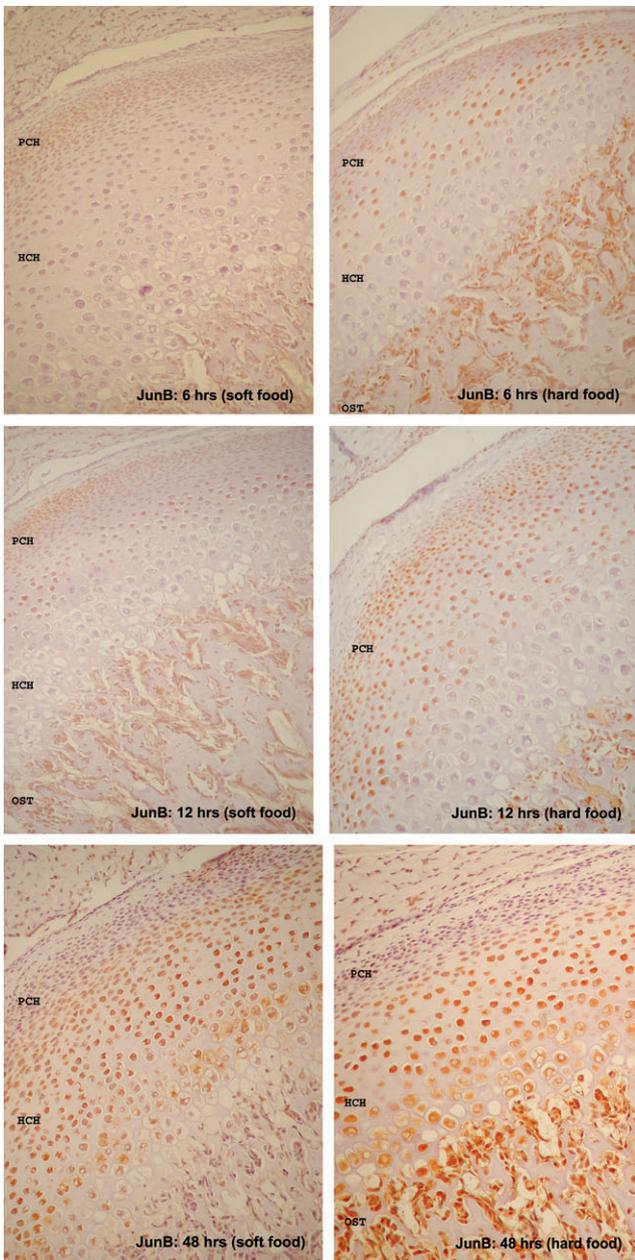
Fra-2 exhibited nuclear and cytoplasmic immunolocalization, in all histological sections evaluated. At 6 and 12 hours Fra-2 was detected in the prehypertrophic zone, and at 48 hours in both the prehypertrophic and hypertrophic zones of the condylar growth plate (Figure 4). Two-way ANOVA showed statistical significance for Fra-2 values both for food type [ $F = 6.95$ , df = (1.41),  $P < 0.05$ ] and time [ $F = 13.52$ , df = (4.41),  $P < 0.001$ ]. Hard diet animals showed statistically significant higher Fra-2 values compared with soft diet animals. The statistical significance of Fra-2 values increased with time in both groups. The influence of type of food and time in interaction was not statistically significant [ $F = 0.35$ , df = (4.41)]. The results are summarized in Table 1.

## **Discussion**

Mechanobiological influences associated with normal function play an important role in the regulation of joint morphogenesis. The development and well being of normal joints depend greatly on mechanical function (Wong and Carter, 2003). Experimental studies have shown that the normal surface topography of diarthrodial joints fails to form in paralysed embryos (Persson, 1983). Heegaard *et al.* (1999) implemented a mathematical model for joint morphogenesis exploring the hypothesis that the stress distribution created in a functional joint may modulate the growth of the cartilage anlagen and lead to the development of congruent articular surfaces. Cyclic hydrostatic stress caused by joint motion may modulate the baseline biological growth, with compression slowing it and tension accelerating it.

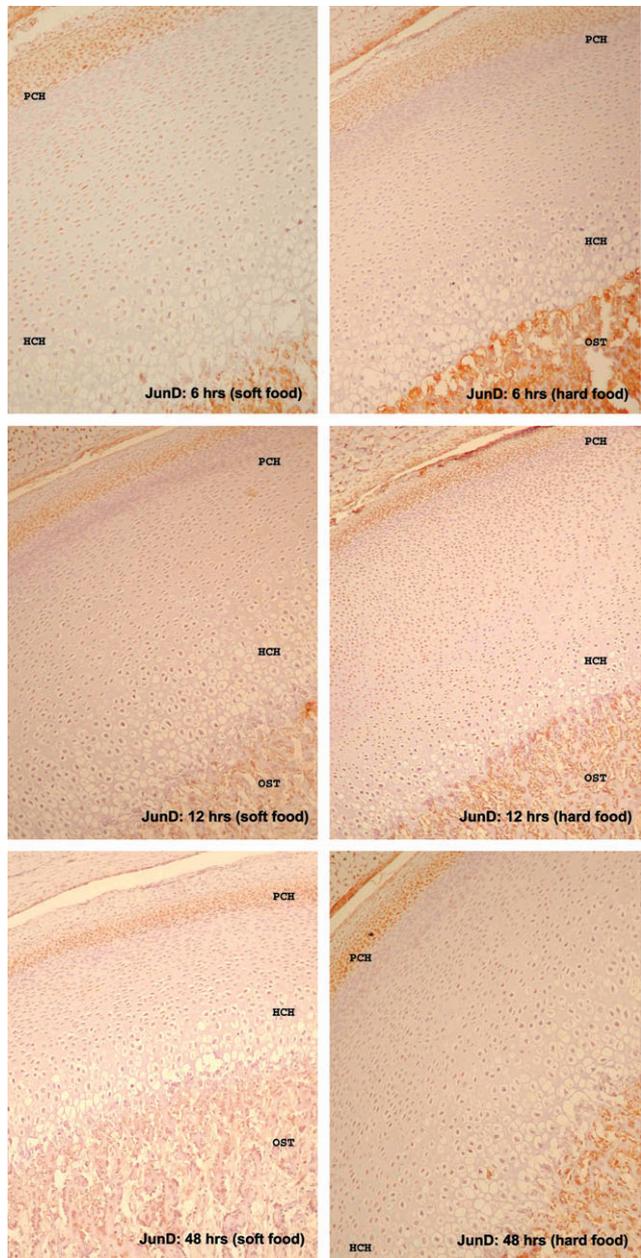
More specifically, for condylar cartilage, Kantomaa and Pirttiniemi (1996) implied that the most rewarding aspect of the regulation of condylar growth seems to be the possibility of regulating the maturation rate of cartilage cells. Furthermore, Kantomaa *et al.* (1994) posed that the rate of differentiation and maturation of mesenchymal cells into chondrocytes seems to be controlled by mechanical forces. Indeed, when condylar cartilage cells were loaded *in vitro* with hydrostatic pressure, the phosphorylation status of proteins associated with the differentiated phenotype was markedly affected, suggesting that mechanical loading signals influence the differentiation and maturation of condylar cartilage chondrocytes (Basdra *et al.*, 1994).

In an effort to explore the *in vivo* role of mechanical loading in the maturation/differentiation process of the whole condylar cartilage, condylar heads of animals fed with different types of diet (soft and hard), hence different types of functional loading, were examined. Immunohistochemical staining was performed at different time points of loading (6, 12 and 48 hours) for both groups (soft and hard), against the JunB, JunD, Fra-1 and Fra-2 proteins. JunB, JunD, Fra-1 and Fra-2 are DNA-binding proteins that operate as AP-1 dimers. The AP-1 transcription factor is involved in cellular proliferation, transformation and apoptosis (Whitmarsh and Davis, 1996; Wisdom *et al.*, 1999). Apoptosis, or programmed cell death, plays a key role in embryogenesis, immunological competence and tissue homeostasis for cell removal and can be distinguished biochemically and morphologically from cell necrosis, which is a passive, energy-independent form of cell death. Chondrocyte degradation and death occurs in endochondral ossification as well as in age-associated arthropathies such as osteoarthritis (Roach *et al.*, 1995). There is evidence that AP-1 proteins, mostly those that belong to the Jun group, control cell life and death through their ability to regulate the expression and function of cell-cycle regulators such as cyclins (Schreiber *et al.*, 1999; Wisdom *et al.*, 1999; Shaulian and Karin, 2001). Moreover, it has been shown that the Fos



**Figure 1** Immunostaining of JunB at 6, 12 and 48 hours in the condyles of animals fed with soft or hard food [ $\times 200$ ]. JunB was detected in both the nucleus and the cytoplasm of articular cartilage chondrocytes. At 6 and 12 hours JunB was located almost exclusively in prehypertrophic chondrocytes, whereas at 48 hours it was observed in all growth plate zones of the temporomandibular joint (PCH, proliferating chondrocytes, HCH, hypertrophic chondrocytes, OST, osteoblasts).

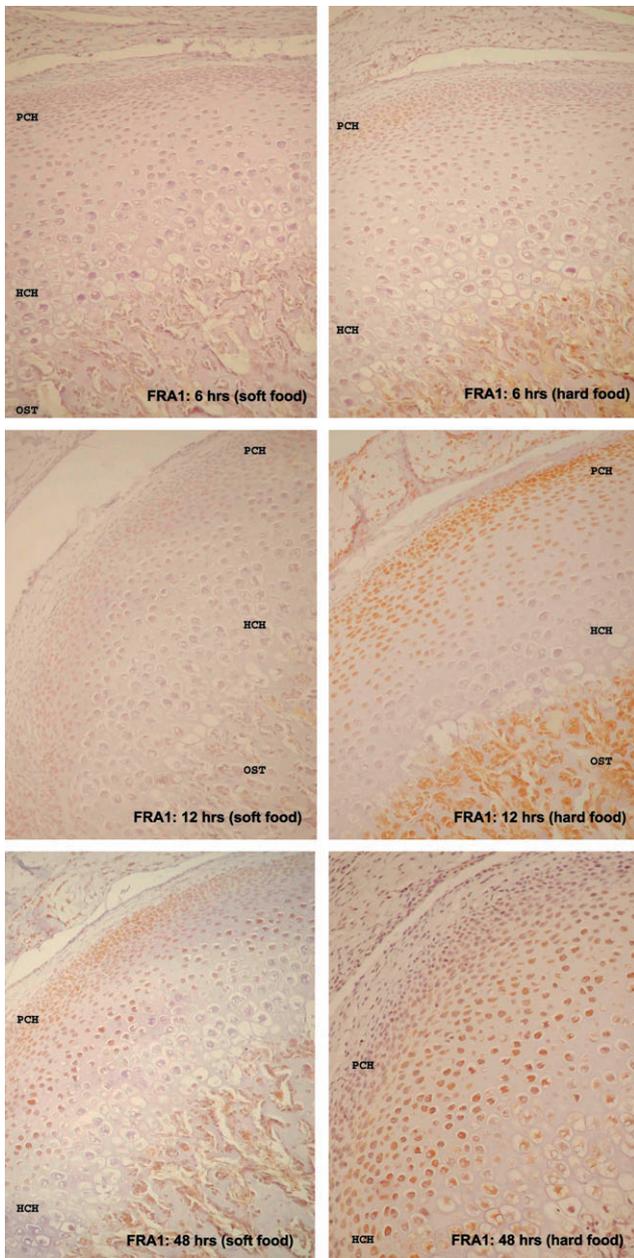
protein group is involved in bone development and growth (Wang *et al.*, 1992). Ectopic expression of the transcription factor Fra-1 in transgenic mice leads to osteosclerosis, a bone disorder characterized by increased bone mass. Recent data demonstrated a novel function of Fra-1 in regulating bone mass through bone matrix production by osteoblasts and chondrocytes (Eferl *et al.*, 2004). Moreover, the AP-1



**Figure 2** Immunostaining of JunD at 6, 12 and 48 hours in condyles of animals fed with soft or hard food [ $\times 200$ ]. JunD displayed both nuclear and cytoplasmic localization in temporomandibular joint chondrocytes in all rats and was observed mainly in the proliferating cartilage zone (PCH, proliferating chondrocytes, HCH, hypertrophic chondrocytes, OST, osteoblasts).

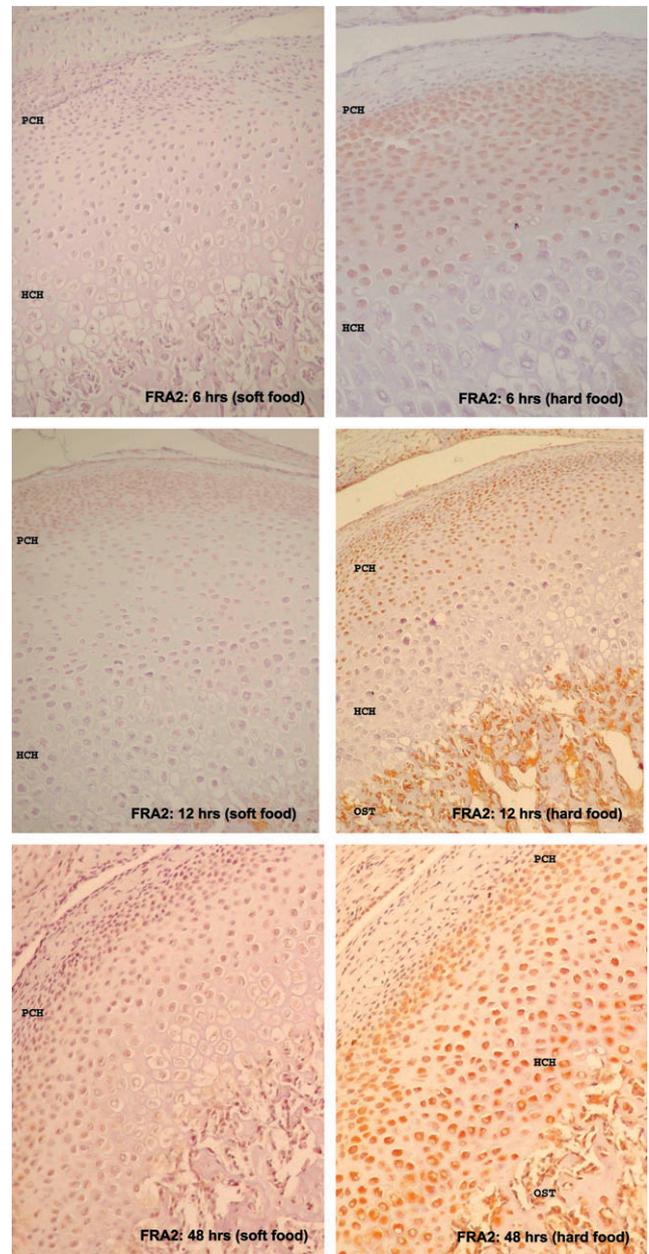
transcription factor Fra-2 is required for efficient cartilage development (Karreth *et al.*, 2004).

Converging results consolidate AP-1 as the pivotal downstream effector in the early response of stress-sensitive cells to continuous mechanical stretching, via the coordinate stimulation of *de novo* synthesis and post-translational regulation of AP-1 proteins (Kletsas *et al.*,



**Figure 3** Immunostaining of Fra-1 at 6, 12 and 48 hours in condyles of animals fed with soft or hard food [ $\times 200$ ]. Fra-1 demonstrated both nuclear and cytoplasmic immunolocalization in the majority of the assessed chondrocytes. Hypertrophic cartilage cells exhibited immunopositivity for Fra-1 principally at 48 hours (PCH, proliferating chondrocytes, HCH, hypertrophic chondrocytes, OST, osteoblasts).

2002; Peverali *et al.*, 2001). This ‘integrating’ function of AP-1 is mediated through a mechanotransduction circuit that incorporates elements of well-defined upstream signalling protein kinase systems (Basdra, 1997; Kletsas *et al.*, 2002). Thus JunB, JunD, Fra-1 and Fra-2 proteins/transcription factors are excellent candidates for studying mechanical load signalling in stress-sensitive condylar cartilage cells. Indeed, the results of the present study



**Figure 4** Immunostaining of Fra-2 at 6, 12 and 48 hours in condyles of animals fed with soft and hard food [ $\times 200$ ]. Fra-2 exhibited nuclear and cytoplasmic immunolocalization, in all histological sections evaluated. At 6 and 12 hours Fra-2 was detected in the prehypertrophic and at 48 hours in both the prehypertrophic and hypertrophic zones of the condylar growth plate (PCH, proliferating chondrocytes, HCH, hypertrophic chondrocytes, OST, osteoblasts).

showed involvement of JunB, JunD, Fra-1 and Fra-2, members of the AP-1 transcription family, in the mechanotransduction cascade in condylar chondrocytes. Notably, the involvement of JunB, JunD, Fra-1 and Fra-2 significantly increased with time in the animal groups fed with a soft or hard diet.

It is well documented that condylar cartilage cells proliferate, hypertrophy and undergo apoptosis, being

**Table 1** Grading of immunohistochemical staining, according to the type of food (soft, hard) and the duration of mastication/functional loading.

	Duration of mastication/functional loading		
	6 hours (n = 5)	12 hours (n = 5)	48 hours (n = 5)
<i>Soft food</i>			
JunB	0(0), 5(+1), 0(+2), 0(+3), 0(+4)	1(0), 3(+1), 1(+2), 0(+3), 0(+4)	0(0), 3(+1), 1(+2), 1(+3), 0(+4)
JunD	1(0), 4(+1), 0(+2), 0(+3), 0(+4)	0(0), 3(+1), 2(+2), 0(+3), 0(+4)	0(0), 1(+1), 4(+2), 0(+3), 0(+4)
Fra-1	0(0), 4(+1), 1(+2), 0(+3), 0(+4)	0(0), 4(+1), 1(+2), 0(+3), 0(+4)	0(0), 0(+1), 5(+2), 0(+3), 0(+4)
Fra-2	0(0), 3(+1), 2(+2), 0(+3), 0(+4)	0(0), 2(+1), 3(+2), 0(+3), 0(+4)	0(0), 0(+1), 3(+2), 2(+3), 0(+4)
<i>Hard food</i>			
JunB	0(0), 3(+1), 2(+2), 0(+3), 0(+4)	0(0), 3(+1), 2(+2), 0(+3), 0(+4)	0(0), 0(+1), 2(+2), 2(+3), 1(+4)
JunD	0(0), 4(+1), 1(+2), 0(+3), 0(+4)	0(0), 3(+1), 2(+2), 0(+3), 0(+4)	0(0), 0(+1), 2(+2), 2(+3), 1(+4)
Fra-1	0(0), 3(+1), 2(+2), 0(+3), 0(+4)	0(0), 0(+1), 3(+2), 2(+3), 0(+4)	0(0), 0(+1), 2(+2), 2(+3), 2(+4)
Fra-2	0(0), 1(+1), 4(+2), 0(+3), 0(+4)	0(0), 0(+1), 4(+2), 1(+3), 0(+4)	0(0), 0(+1), 2(+2), 2(+3), 2(+4)

The number in parenthesis (+1, +2, +3 or +4) indicates the intensity of staining, i.e. +1 = one grade of intensity, +2 = two grades of intensity, etc., while the number in front of the parenthesis reflects the number of animals (N).

replaced by osteoblastic cells (Provot and Schipani, 2005). The present *in vivo* data revealed over-expression of AP-1 proteins JunB, JunD, Fra-1 and Fra-2, at all stages of condylar cartilage differentiation in animals fed on a hard diet. Immunohistochemistry of the condylar heads of the experimental animals fed with a soft and hard diet showed statistical differences with time in the intensity of staining, indicating that the condylar cartilage cells sense increased functional loading and respond by induction of proteins associated with apoptotic phenomena. Early onset of apoptosis due to a change/increase in functional demands will eventually lead to early replacement by osteoblasts, thus early maturation of the tissue and, finally, reduced overall cartilage growth. Indeed, once chondrocytes become hypertrophic they are destined for rapid cell death and replacement by bone (de Crombrughe *et al.*, 2001).

The gradual intensifying of immunostaining in relation to time suggests that the effect of mechanical loading greatly depends on the duration of force application. It can therefore be speculated that a longer duration of loading will create more stable effects. It remains to be proven, however, whether the time variant is of significance or whether other factors are also involved (for example muscular forces).

The results of the present study correlate well with clinical findings after functional load alteration of condyles. Animal experiments have demonstrated increased proliferative activity after unloading of the cartilage, while loading had the reverse effect (McNamara and Carlson, 1979). In dentofacial orthopaedics many treatment modalities are based on the notion that condylar cartilage growth can be influenced by changes in mechanical loading of the condylar head. The present data provide evidence on the mode of the cellular response. Further studies will elucidate the signalling pathway of mechanical loading to condylar cartilage cells, providing answers for future

innovative treatment approaches in young and maybe adult patients (Ruf and Pancherz, 2004).

## Conclusions

The results of the present study clearly demonstrate over-expression of the JunB, JunD, Fra-1, and Fra-2 members of the AP-1 transcription family in the response of condylar cartilage chondrocytes to functional loading alterations, suggesting that mechanical loading in chondrocytes triggers biochemical responses associated with AP-1 cellular functions such as maturation, differentiation and apoptosis. Downstream, these biological phenomena influence the overall growth of the condylar cartilage.

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