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# Distribution and characterization of proliferative cells in the rat mandibular condyle during growth

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SUMMARY Distribution of proliferative cells and localization of types I and II collagen were examined in the rat mandibular condylar cartilage of 36 long-Evans/Turku strain rats during normal postnatal growth using an immunohistochemical method combined with histomorphometry. There were considerable differences in the thickness of the proliferative cell layer in the condylar head, with most mitoses occurring in the postero-superior area. It was found that the extracellular matrix of the proliferative cells does not stain for type II collagen in 20-day-old and older rats, and that besides the subchondral bone, the strongest intensity for type I collagen stain was always localized in the articular surface of the condylar head. Statistically significant overlapping of the proliferative cell layer and the one secreting type II collagen occurred during the earlier stages of development, particularly in the postero-superior area of the condylar head. As type II collagen is considered to be a marker for identification of typical cartilage cells, the findings indicate that, in addition to undifferentiated cells, a portion of the proliferative cells can be characterized as chondroblasts during the early postnatal period in rats, but not in the later stages of development. The developmental phase of the condylar cartilage should therefore be taken into consideration when the effect of various biomechanical and humoral/hormonal factors on growth of the condylar cartilage is examined.

## Introduction

Studies on humans and experimental animals have shown that the morphology and micro-anatomy of the mandibular condyle undergoes changes during normal growth (Furstman, 1966; Wright and Moffett, 1974; Thilander *et al*., 1976; Carlson *et al*., 1978; Vinkka, 1982; Luder, 1983, 1996; Copray and Liem, 1989; Kantomaa *et al*., 1992). Furstman (1966) described condylar cartilage of newborn rats as embryonic cartilage and reported that articulation is not fully formed earlier than the age of 7 days. The typical oblong shape of the condylar process of rats only develops postnatally, more precisely during the first 3 weeks after birth (Copray and Liem, 1989).

The change in the morphology and micro-anatomy has been attributed to the maturation of the masticatory apparatus, i.e. changes in the mode of feeding and development of the dentition. It has also been reported that the condylar cartilage of rats shows transient degenerative features at the time of weaning at the age of 21 days (Kantomaa *et al*., 1992), which is considered to be the 'major starting point of postnatal development' of the rat condylar process (Luder *et al*., 1988). Less attention has been paid to concomitant changes in the distribution of proliferative cells. The general view that proliferative cells form a narrow zone under the articular zone is based mainly on findings in mature animals. Most often reference is made to Blackwood's study (1966), where 40-day-old rats were used. A comprehensive study of age-related changes in the rat condylar cartilage has recently been presented by Luder (1996), but animals younger than 3 weeks of age were not included.

Proliferative cells in the mandibular condylar cartilage are considered to be undifferentiated cells (Blackwood, 1966; Folke and Stallard, 1967; Öberg *et al*., 1967; Joondeph, 1972; Petrovic *et al*., 1974; Heeley *et al*., 1983; Luder *et al*., 1988), which is at variance with the situation in long bone epiphyseal cartilages where dividing cells are mainly chondroblasts (Kember, 1978; Mizoguchi *et al*., 1990). This difference in the dividing cell population is regarded as being the essential differentiating characteristic between primary and secondary cartilages, and provides a clue to the different behaviour of the cartilages (Koski, 1981). However, there are no studies to characterize the proliferative cells during early postnatal changes in morphology. The immunohistochemical method for analysing extracellular matrix collagen, especially type I and II collagen, offers a useful means to characterize proliferative cells (Mayne and von der Mark, 1983).

The aims of the present investigation were to examine the distribution of proliferative cells in the rat mandibular condylar cartilage during normal postnatal growth and to characterize the proliferative cells. Particular emphasis was paid to the early developmental stages, which have so far not been adequately studied.

## Materials and methods

The material consisted of 36 Long-Evans/Turku strain rats, three in each of 12 age groups, ranging from 1 to 70 days. In order to detect proliferative cells, the animals were injected intraperitoneally with bromodeoxyuridine at a dose of 40 mg/kg body weight in a solution of 10 mg/ml saline. Two hours after the injection, the rats were killed with an overdose of carbon dioxide and thereafter decapitated. The heads were fixed in 10 per cent neutral formalin for 1 day, decalcified in 5 per cent formic acid for 1 week, and cut sagittally into two equal parts. After paraffin embedding, the specimens were sectioned sagittally at 6 μm. Three mid-sagittal representative sections of the condylar area of each animal were selected for examination. One section was used for labelling mitotic cells and the others for cell characterization.

### *Cell proliferation*

To detect proliferative cells, anti-bromodeoxyuridine (anti-BrdU, DAKO, Copenhagen, Denmark) was applied according to the manufacturer's instructions using an automatic immunostainer (DAKO TechMate™ 500). Briefly, sections were dewaxed with xylene, treated with 0.1 per cent trypsin solution diluted 1:1500 in distilled water and denaturated with 1 M HCL. The BrdU antibody (DAKO, monoclonal mouse anti-BrdU) was then applied at a dilution of 1:10 for 1 hour. The sites of antibody binding were also visualized using the immunostainer with the streptavidin-biotin method and DAB substrate. Finally, the specimens were lightly counterstained with haematoxylin. Negative controls, i.e. sections from animals that had not been injected with BrdU, were similarly treated.

### *Cell characterization*

To characterize the extracellular matrix of the proliferative cells, antibodies for type I and II collagen were used. For type II collagen the sections were digested with 0.4 per cent pepsin (Sigma, St Louis, MO, USA) for 1 hour at 37°C and stained with a monoclonal antibody (CIID3). The antibody was used at ×200 dilution and allowed to react overnight in a moist chamber at 4°C. For type I collagen, the sections were treated with normal serum and stained with a monoclonal antibody (Sigma, monoclonal mouse anti-collagen type I) at ×1000 dilution, which was also allowed to react overnight in a moist chamber at 4°C. In both cases the reaction product was visualized with a Vectastain® ABC Elite kit (Vector Laboratories, Burlinghame CA, USA) and DAB substrate. Negative controls without the primary antibody were also prepared. The sections were counterstained with haematoxylin.

### *Histomorphometry*

In order to measure the depth of the proliferative cell layer and the start of the type II collagen secreting cell layer, the condylar head was divided into four regions: (1) the anterior, (2) superior, (3) postero-superior, and (4) posterior (Figure 1).

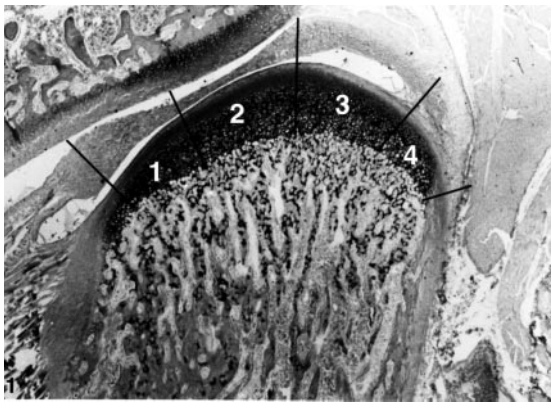


**Figure 1** The depth of the proliferative cell layer and the start of the type II collagen secreting cell layer were measured perpendicular to the articular surface in four areas of the condylar head: (1) the anterior; (2) superior; (3) postero-superior; (4) posterior.

The mean of five equally distributed measurements of each area (three animals in each age group) was used to indicate the depth of the mitotic layer and the first appearance of the type II collagen secreting cell layer. The measurements were made perpendicular to the articular surface using a computerized image analysis system (MicroScale TC 2,1, Digithrust Ltd, Liverpool UK), the images being acquired by means of a microscope connected to a CCD camera.

Overlapping or no-overlapping of the proliferative cell layer, and the one secreting type II collagen was calculated for each animal and region of the condylar head, and its statistical significance was tested using the *t*-test. In addition, regression analysis was used to evaluate the association of the overlapping with age. In both analyses, the measurements of animals younger than 21 days were pooled and evaluated separately from the older age groups. This was because of the known alterations occurring in the condylar cartilage structure due to a change in function of the temporomandibular joint (Copray and Liem, 1989; Kantomaa *et al*., 1992).

To test the repeatability of the measurements, one section labelled for BrdU and one for type II collagen in each age group were measured twice and the measurement error calculated using Dahlberg's method (1940) from the formula $\sqrt{\Sigma d^2/2n}$, in which $d$ is the difference between the two duplicate measurements in each case and $n$ is the number of duplicates.

Reliable measurement of type I collagen distribution was not possible due to the thin layers. Therefore, the localization of type I collagen stain was registered visually.

## Results

### *Cell proliferation*

The measurements indicate that there was a systematic and considerable difference in the depth of the proliferative cell layer among the four areas measured, irrespective of the age of the animals. Most mitoses occurred in the postero-superior area, while there were few, if any, labelled cells in the very posterior area. There was an age-related decrease in the depth of the proliferative cell zone, the most notable changes occurring during the first three weeks. From 21 days, the depth of the labelled zone, as measured perpendicular to the articular surface, remained fairly constant, except in the postero-superior area, where 'adult depth' was attained between 42 and 56 days (Figure 2).

### *Cell characterization*

As in the proliferative cell layer, regional differences were noted in the depth of the start of the type II collagen secreting cell layer along the condylar head. The greatest depth unstained for type II collagen was noted in the postero-superior area of the condyle in every age group. The most marked changes were noted again during the first three weeks: the undifferentiated, mitotic cell layer (matrix unstained for type II collagen) reaching deeper into the condylar cartilage in the young than in the older animals (Figure 2).

The reaction of type I collagen stain was localized in the fibrous articular layer of the condylar head in all age groups and particularly in the area of newly-formed subchondral bone. The most intense staining was always found in the postero-superior area and the intensity seemed to increase with the age of the animals (Figure 2).

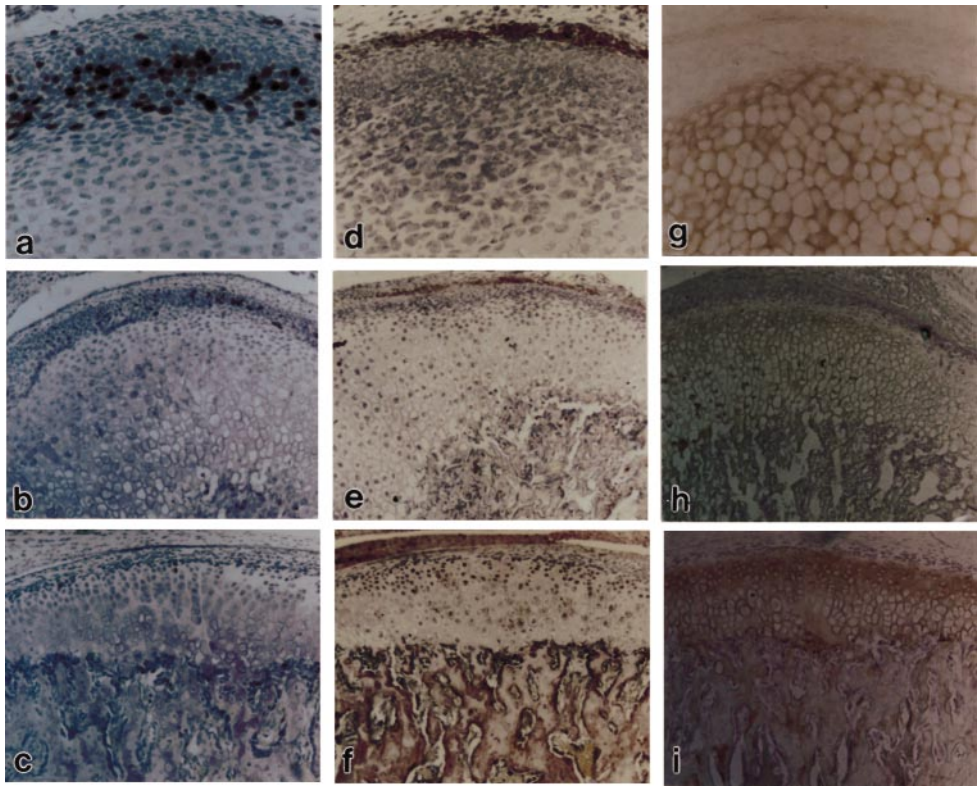


**Figure 2** Sagittal section through the postero-superior area of the rat condylar cartilage stained for bromodeoxyuridine (a–c), type I collagen (d–f), and type II collagen (g–i). In 3-day-old rats (a, d, and g), there is considerable overlapping of the proliferative and the type II collagen secreting layers, whereas type I collagen is localized in the fibrous articulating layer. At 28 days (b, e, and h), there are proliferative cells only under the fibrous articular layer which is not stained for type II collagen. In addition to the articulating layer, type I collagen is found in the subchondral bone. At 70 days (c, f, and i) only a narrow layer of proliferative cells has collagen type II positive matrix, whereas staining for type I collagen is similar to that of the younger rats. For staining, see text. 3-day group, ×160; 28- and 70-day groups, ×54.

*Cell proliferation and characterization (Figures 2–4)*

Comparison of the measurements showed a statistically significant overlapping of the proliferative and the type II collagen secreting cell layers in the postero-superior area of young animals (mean 27.19 μm, SD 32.51 μm, $P = 0.001$). No deposition of type II collagen around the proliferative cells was seen after approximately 17 days of age. In the anterior and posterior area statistically significant differences were also noted (mean –4.53 μm, SD 6.66 μm, $P = 0.005$; mean –3.5 μm, SD 7.19 μm, $P = 0.037$, anterior and posterior area, respectively). However, in these areas and in the superior areas (mean 0.87 μm, SD 15.69 μm, $P = 0.802$) the analysis indicated no overlapping of the two stains.

Regression analysis revealed a statistically significant age-related change in the overlapping or no-overlapping in every area of the condylar head ($P < 0.05$). During the first three postnatal weeks of rats, there was a reduction in the overlapping of the proliferative and the type II collagen secreting cell layers in the postero-superior area. In the superior area overlapping

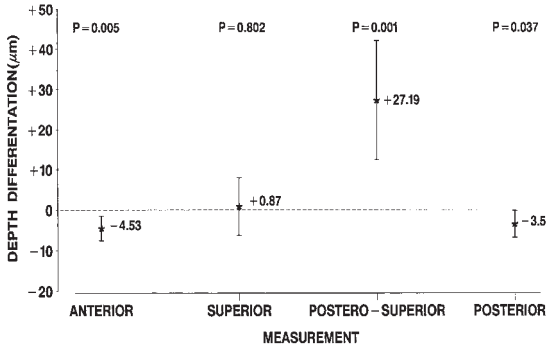


**Figure 3** Mean values and 95 per cent confidence intervals for measurements of overlapping or no-overlapping (depth difference in μm) of the proliferative cell layer, and the type II collagen secreting cell layer in the four areas of 1–17-day-old rat condylar heads. *P*-values are from one sample *t*-test ($n = 21$).

was seen during the first week only, which seemed to be the case in the anterior and posterior area during the entire postnatal period.

In the two youngest age groups, there was type I collagen secretion (intra- and extra-cellular) around the proliferative cells in the postero-superior area, whereas no overlapping of type I and II collagen secreting layers was noted.

Negative controls did not show any immunoreactivity.

### *Repeatability of measurements*

Measurement errors ranged from 2.93 to 7.77 μm and were regarded as insignificant as far as the reliability of the measurements was concerned.

## Discussion

The present findings provide new information on the growth dynamics of the mandibular condylar cartilage, particularly during the first three postnatal weeks. These results are in agreement with the generally accepted opinion that proliferative cells in the condylar cartilage are undifferentiated cells in 20-day-old and older rats, as inferred from the finding that no immunoreactivity for type II collagen was noted around the proliferative cells. However, there was considerable overlapping

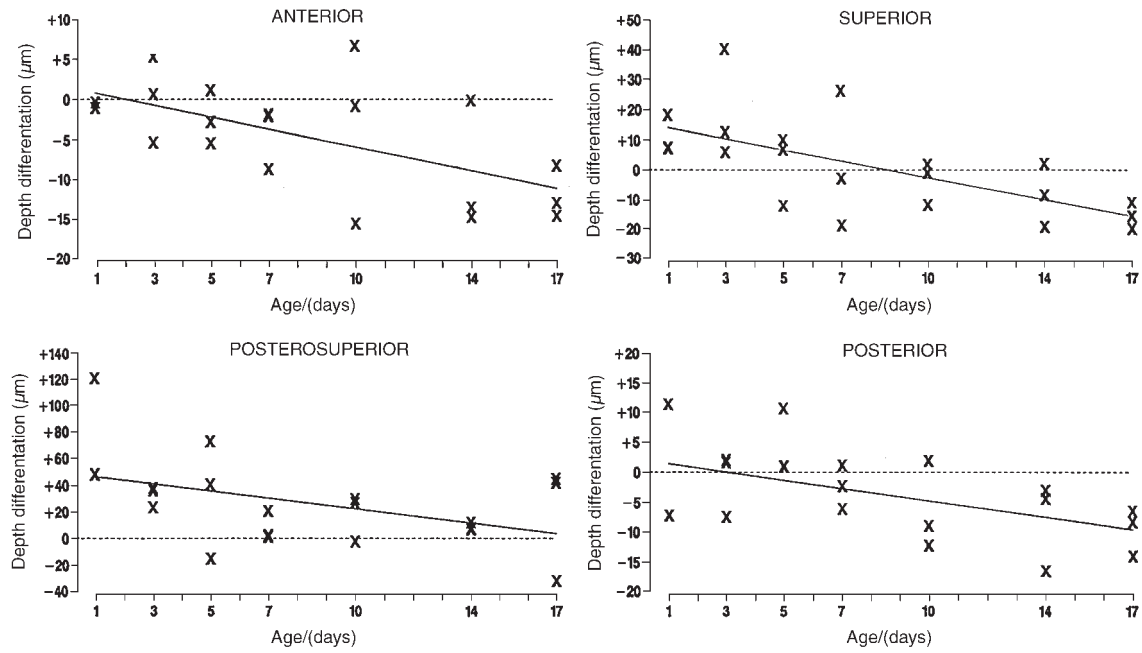


**Figure 4** Scatterplot of age and the measurements of depth difference (DD) in the four areas of 1–17-day-old rat condylar heads. Continous line is the regression line ($n = 21$).

of the proliferative cell layer and secreting type II collagen was found during the earlier stages of development, particularly in the postero-superior area, which represents the main growth direction of the rat condylar head. Interestingly, Luder *et al.* (1988) also reported some staining for type II collagen in the lower part of the proliferative cell layer in 20-day-old rats. As type II collagen is considered to be a marker for identification of typical cartilage cells (Mayne and von der Mark, 1983; Luder *et al.*, 1988), the finding can be interpreted to mean that, in addition to undifferentiated cells, there are, up to a certain age, proliferative cells in the condylar cartilage, which can be characterized as chondroblasts. Furthermore, on the basis of transplantation experiments, it has been speculated that cell types other than undifferentiated cells may proliferate in the condylar cartilage (Garcia and Gray, 1995).

The present results show that, in parallel with the changing morphology, the depth of the proliferative cell layer and the appearance of the type II collagen secreting cell layer undergo distinct changes during normal growth. This finding supports the view that, during the early stages of development, condylar cartilage functions more like a growth cartilage, even comparable to epiphyseal cartilage of a long bone, and following maturation of the masticatory complex, similar to articular cartilage (Vinkka, 1982; Copray *et al.*, 1988). The present finding of increasing intensity of type I collagen in the fibrous articular layer with age is in line with earlier reports, which suggest a relationship between joint loading and deposition of type I collagen (Pirttiniemi *et al.*, 1996). The shift of the condyle from having mainly a growth function to an essentially articular function evidently occurs during a short time period in rats, since proliferative cells can already be viewed as undifferentiated cells by about 20 days of age also in the postero-superior area of the condylar head. The variable findings from transplantation experiments of different age condylar cartilage are probably related to the observed rapid changes, as particularly extensive growth and maintenance of the cartilage structure have been reported when very young transplants have been used (Rönning, 1966; Copray and Duterloo; 1986; Garcia and Gray, 1995).

The present findings also indicate that the developmental phase of the condylar cartilage should be taken into consideration when the effect of various biomechanical and humoral/hormonal factors on the growth of the condylar cartilage is examined. It is known, for example, that early postnatal growth is not affected by growth hormone, and administration of growth hormone at different times will lead to differences in response, depending on the developmental stage of the responding tissue (Isaksson *et al.*, 1987; Buchanan and Preece 1992; Vogl *et al.*, 1993).

The noted regional differences in the depth of the proliferative cell layer and in the appearance of the type II collagen-secreting cell layer along the condylar head are in agreement with previous findings (Carlson *et al.*, 1978; Luder, 1983; Salo and Kantomaa, 1993; Mizoguchi *et al.*, 1996). Differences in the region of the condyle are interpreted as indicating that environmental factors, such as posture and functioning of the lower jaw, and attempts to change these, do not have an equal effect on different areas of the condyle (Kantomaa, 1986; Hinton and Carlson, 1986; Kantomaa and Hall, 1988). Probably as a consequence of the various regional response of the condylar cartilage, different kinds of growth rotations of the lower jaw can be clinically seen and achieved by orthodontics/orthopaedics. It seems also that in the anterior and posterior area of the rat condylar head, where no overlapping of the proliferative and type II collagen secreting cell layers were noted, the adult stage of the condylar cartilage is attained much earlier than in the superior and postero-superior areas.

## Conclusions

Using immunohistochemistry and histomorphometry, it has been demonstrated that there are proliferative cells in the condylar cartilage of rats that secrete type II collagen during the first two postnatal weeks. This indicates that a portion of mitotic cells can be characterized as chondroblasts contrary to the later stages of development. As the nature of cartilage and particularly its ability to respond to various

(intrinsic and extrinsic) factors is determined by the proliferative cells, i.e. chondroblasts, condylar cartilage seems to have properties similar to growth cartilage, such as the epiphyseal growth plate, during early development.

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