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## Extracellular matrix synthesis, proliferation and death in mechanically stimulated human gingival fibroblasts in vitro

Received: 13 January 2004 / Accepted: 6 July 2004 / Published online: 20 April 2005  
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**Abstract** Gingival tissues are constantly exposed to the effect of physical forces. Mechanical stimuli are regulators of connective tissue homeostasis and sustained mechanical stimulation may lead to modifications in cell activity and extracellular matrix (ECM) composition. This study examined in vitro ECM synthesis, proliferation, and death in mechanically stimulated human gingival fibroblastlike cells. Four primary human cell strains were established and subjected to intermittent stretching in FX-3000 Flexercell Strain Unit for 10 days, 45 min/day, at 1 Hz, 10% strain, and cell proliferation, cell death, and synthesis of collagens types I, III, and V, matrix metalloproteinase 1, elastin, and tenascin were assessed. In some of the cell strains mechanical stimulation led to changes in synthesis of the ECM molecules, proliferative activity, and death of stimulated cells as shown by statistically significant differences between the experimental and unstimulated control cultures. Although not seen in every culture investigated, these findings suggest that prolonged mechanical stimulation might lead to conspicuous modifications in the metabolic activity of gingival fibroblasts and cause changes in the ECM composition of the gingival connective tissue. The results indicate a pronounced interindividual variation in reactions of gingival fibroblasts to mechanical stimulation.

**Keywords** Gingiva · Fibroblasts · Mechanical stimulation · Proliferation · Cell death · Extracellular matrix

### Introduction

The cells of various tissues appear to modify their activity in accordance with the mechanical environment. In recent years there has been a rapid advance in knowledge of the effect of mechanical stimuli on tissue composition and function [6, 7], development of the methods of studying mechanical stimulation at the cellular level [4], and understanding of the intra- and intercellular mechanical signal transduction [8, 14]. With respect to the tissues of the oral cavity substantial knowledge has been acquired of the role of physical forces in regulating the form and function of the periodontal ligament [17]. However, very few investigations have addressed the role of mechanical forces in gingival connective tissue (GCT) functioning, and hardly any information is currently available regarding the effect of physical forces on gingiva which may be exposed to considerable mechanical loading from mastication, speech, orthodontic forces or ill-fitting dentures.

Changes in ECM metabolism in vitro [24, 28] and increased fibroblast progenitor cell proliferation in vivo [30] were recently reported as features of GCT modifications under the effect of mechanical stimulation. It has been proposed [25], for instance, that prolonged mechanical stimulation of gingival fibroblasts may lead to increased production of glycosaminoglycans, activation of collagen and elastin synthesis, to reduction in collagenase activity, and thus to significant changes in the ECM composition. These changes may render GCT more elastic and, analogous to compressed rubber, resistant to retention of the orthodontically corrected tooth position [25].

However, at present there is insufficient experimental evidence lending support to the above hypothesis, nor is there enough information about the potential role of mechanical stimulation in GCT homeostasis and pathological changes. The aim of this study was to obtain an insight into the remodeling events in GCT under the effect of mechanical environment. For this purpose we examined the effects of mechanical stimulation on proliferative activity, incidence of cell death, and synthesis of a number of typical ECM constituents by cultured human gingival fibroblastlike cells

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(HGF) subjected to cyclic mechanical forces of extended duration.

## Materials and methods

### Cell culture and mechanical stimulation

Ethical approval was obtained, and four primary HGF cell strains, designated as A–D, were grown from explant cultures [11] from four different healthy young adult donors and routinely maintained in  $\alpha$  minimum essential medium supplemented with 200 mM L-glutamine, 15% fetal calf serum, 1000 U/ml penicillin, 10  $\mu$ g/ml streptomycin, and 25  $\mu$ g/ml Fungizone at 37°C, 100% humidity and 5% CO<sub>2</sub>. To facilitate the secretion and maintain solubilization of matrix proteins we added to the medium 50  $\mu$ g/ml ascorbic acid, a cofactor in the hydroxylation of proline residues, and 50  $\mu$ g/ml  $\beta$ -aminopropionitrile, an inhibitor of cross-linking [13]. The explants were obtained from healthy attached gingiva during surgical third molar extractions. For the experiments cells of passages 4–8 were used, explant culture being the passage 1. Cell suspensions were seeded at standard initial densities of  $5 \times 10^4$  cells/ml into fibronectin-precoated, custom-designed, flexible-bottomed, multiwell tissue culture plates (BioFlex, Flexcell International, Hillsborough, N.C., USA). After cell attachment, spreading, and growth to subconfluency growth medium was replaced by cell culture medium as above but without fetal calf serum, and plates containing cultured cells were subjected to intermittent equibiaxial stretching in FX-3000 Flexercell Strain Unit for 10 days, 45 min/day, at 1 Hz, 10% strain. This regime of experimental mechanical stimulation was determined empirically in precursor trials by variation of frequency, magnitude, and duration of stimulation as the one without apparent adverse effects on cell maintenance. The experiments were carried out four times each time using six wells containing cells of the 4th, 5th, 7th, and the 8th passage of each cell strain, giving a total sample of 24 wells for each individual cell strain. Parallel, equally sized groups of multiwell plates containing cell cultures not subjected to mechanical stimulation and otherwise kept under identical conditions were employed as controls. The same serum-free cell culture medium was left in all wells during the entire experimental period, and after each experiment it was collected, frozen, and stored for further analysis as below.

### Assessment of cell proliferation, cell death, and synthesis of ECM constituents

Where available, calibrated commercially manufactured assay kits were employed (Table 1). Otherwise enzyme-linked immunosorbent assays (ELISAs) specific for individual ECM constituents were designed using commercially available antibodies (Table 2). At completion of the mechanical stimulation cell monolayers were used to determine cell proliferation using an appropriate assay kit (Table 1) according to the manufacturer's instructions. Occurrence of cell death

**Table 1** Calibrated assay kits used in the study

Assay	Assay kit	Manufacturer
Proliferation	Cell proliferation assay kit (based on mitochondrial dehydrogenase activity)	Chemicon International Inc., Temecula, Calif., USA
Cell death	Cell death detection (nuclear matrix protein 41/7) ELISA	Oncogene Research Products, Boston, Mass., USA
COL I	Prolagen-C IEMA	Metra Biosystems Inc., Mountain View, Calif., USA
MMP-1	MMP-1 ELISA	Oncogene Research Products

was assessed retrospectively in samples of cell culture medium from all HGF cultures simultaneously by means of an appropriate assay kit (Table 1) following the manufacturer's instructions.

After completion of all experiments samples of the cell culture medium from all stimulated and control HGF cultures were thawed and simultaneously subjected to measurement of concentrations of the ECM constituents collagens (COL) types I, III, V, elastin (soluble precursor tropoelastin), tenascin, and matrix metalloproteinase 1 (MMP-1). COL I and MMP-1 were measured using appropriate commercially available assay kits (Table 1). Individual ELISAs specific for other ECM constituents (Table 2) were designed, optimal conditions determined, and calibration curves for known standard concentrations obtained in a series of empirical tests according to the protocols described elsewhere [29].

Coating, blocking, and washing buffer solutions, peroxidase-labeled streptavidin and the reagents for the chromogenic signal (Protein Detector ELISA Kit) were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, Md., USA). All incubation steps were carried out at 4°C. In the final form of the ELISAs of COL III, COL V, and tenascin the following procedure was adopted. We incubated 100- $\mu$ l aliquots of the first antibody diluted in coating buffer for 6 h in microtiter plates. Nonspecific binding was blocked by incubating 300  $\mu$ l blocking solution for 60 min in each well. Samples of cell culture medium, 100  $\mu$ l in volume, containing the ECM constituents to be assessed were applied to each well and incubated for 6 h. After sample removal the plates were washed four times with 300  $\mu$ l washing buffer, and 100- $\mu$ l aliquots of the biotinylated second antibody were incubated for 6 h, followed by 100- $\mu$ l aliquots of peroxidase-labeled streptavidin solution (1:1000) for 30 min with washings as before. Chromogenic peroxidase substrate, 100  $\mu$ l/well, was applied and the reaction stopped after 30 min with 100  $\mu$ l stop solution. Samples of the resulting reaction product, 100  $\mu$ l each, were transferred from each well into new microtiter plates, and the optical density of the chromogenic reaction product read at 405 nm on an MR 5000 microplate reader (Dynatec Laboratories, Sussex, UK).

**Table 2** Antibodies and their working dilutions used in specially designed enzyme-linked immunosorbent assays

Extracellular matrix constituent (antigen)	Antibody	Working dilution	Manufacturer
COL III	Mouse monoclonal antibody to collagen type III	1:500	Silenus Labs Pty. Ltd., Boronia, Victoria, Australia
	Goat monoclonal antibody to human type III collagen (biotin conjugate)	1:500	Southern Biotechnology Associates Inc., Birmingham, Ala., USA
COL V	Mouse monoclonal antibody to collagen type V	1:500	Silenus Labs Pty. Ltd.
	Goat monoclonal antibody to human type V collagen (biotinylated)	1:500	Southern Biotechnology Associates Inc.
Elastin	Rabbit polyclonal antibody to tropoelastin	1:1000	DPC Biemann, Bad Nauheim, Germany
	Goat polyclonal antibody to rabbit IgG (biotinylated)	1:50000	DPC Biemann
Tenascin	Mouse monoclonal antibody to human tenascin	1:600	DPC Biemann
	Rabbit polyclonal antibody to human tenascin	1:600	DPC Biemann
	Goat polyclonal antibody to rabbit IgG (biotinylated)	1:20000	DPC Biemann

As a biotinylated anti-tenascin antibody for the ELISA of tenascin was unavailable, 100  $\mu$ l aliquots of an additional biotinylated antibody (Table 2) were applied before the incubation of the peroxidase-labeled streptavidin reagent. As only one suitable anti-elastin antibody was obtainable, instead of the sandwich design described above, a solid-phase indirect ELISA design was chosen for this ECM constituent, and the agent of interest was first immobilized from cell culture medium samples on microtiter plates followed by application of the specific first and conjugated second antibodies (Table 2). Both positive and negative controls were used in all assays according to the protocols described elsewhere [29].

### Statistical analysis

Differences between the measurements obtained from mechanically stimulated and unstimulated control groups of each cell strain were tested for statistical significance using the Mann-Whitney rank sum test with the level of statistical significance set at  $\alpha=0.01$ . For data presentation mean values and standard deviations were calculated from the optical density readings obtained from the above assays.

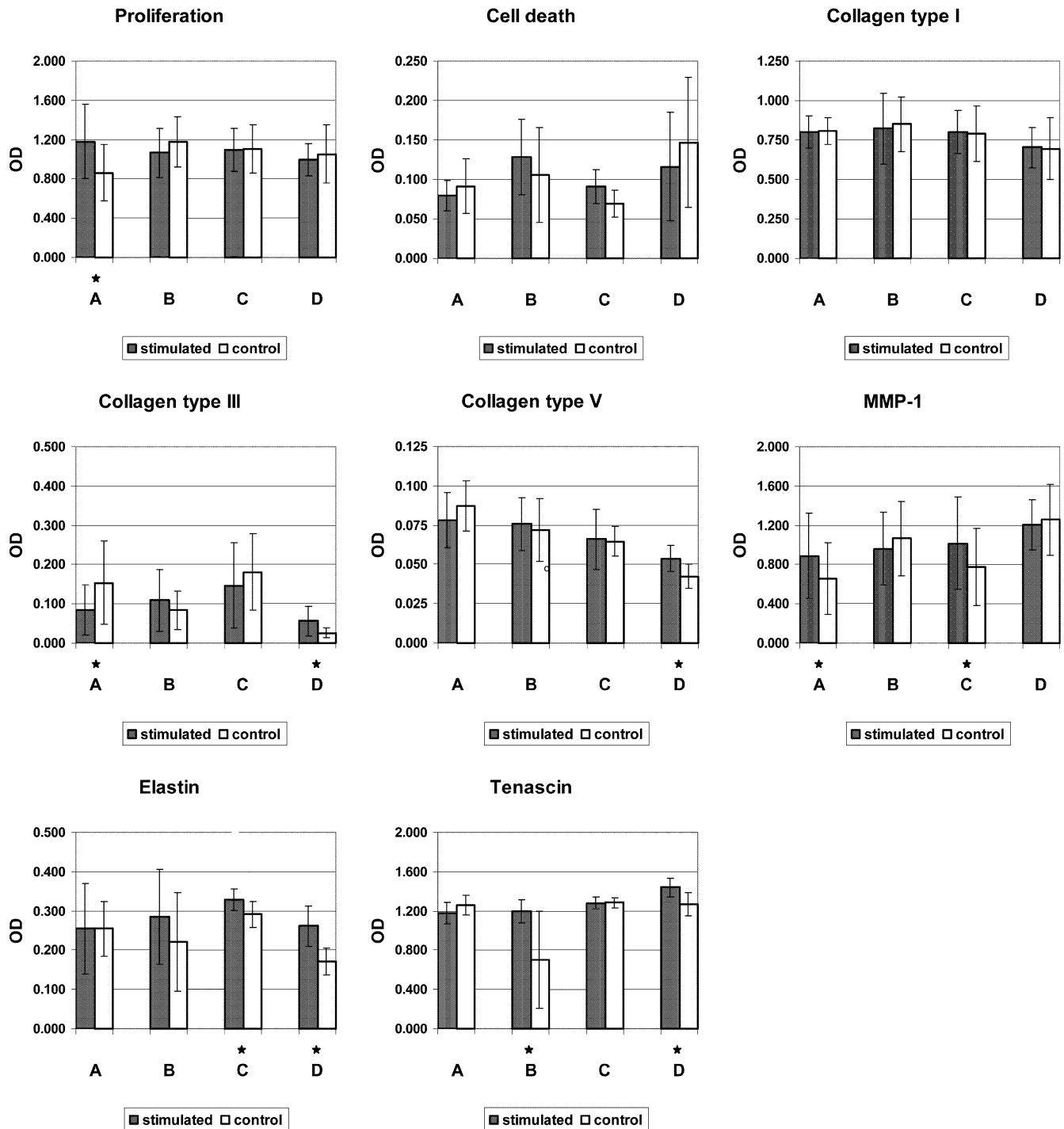
## Results

The effect of cyclic mechanical forces of extended duration on HGF cells and differences between the measurements obtained from experimental and control groups of each cell strain are shown in Fig. 1. Under the conditions of mechan-

ical stimulation there was a statistically significant increase in cell proliferation in cell strain A. In the remaining cell cultures there was no significant difference in cell proliferation or death between mechanically stimulated and control samples. No statistically significant differences in COL I production occurred in any of mechanically stimulated cell cultures. Under the conditions of mechanical stimulation COL III synthesis was significantly reduced in strain A and increased in strain D, remaining unchanged in the other cell cultures. The amounts of COL V were significantly higher in the medium of mechanically stimulated strain D and remained unchanged in the other HGF cultures. MMP-1 production was statistically significantly increased in stimulated strain A and C, showing no difference in the remaining cultures. Mechanical stimulation caused a significant increase in elastin synthesis in strains C and D. Higher concentrations of tenascin were detected in the medium of strains B and D, while production of this ECM constituent remained unchanged in both other cell cultures when measurements obtained from mechanically stimulated cells were compared with the corresponding controls.

## Discussion

Experimental models employing monolayer cell cultures as used in the present study for testing of the postulated effects of mechanical stimulation on HGF facilitate more accurate control of experimental conditions, replicate sampling, and quantification [11]. It has been shown for periodontal ligament fibroblasts, for instance, that they express similar phenotypes in vivo and in vitro, and that in vitro models



**Fig. 1** Assessment of cell proliferation, cell death, collagen type I, collagen type III, collagen type V, matrix metalloproteinase-1 (MMP-1), elastin, and tenascin in cell strains A–D. Results are expressed as

mean values and standard deviations of optical densities (OD) measured in 24 wells in quadruplicate assays. \* $P < 0.01$  mechanically stimulated vs. control groups (Mann-Whitney rank sum test)

used for studying cell differentiation are appropriate and independent of sampling method [16]. It is recognized, however, that lack of cell-cell and cell-matrix interactions which are typical for integrated cell populations in living tissues may constrain the relevance of these in vitro observations to the more complex events of cell behavior and ECM remodeling in vivo.

After a few passages of cells grown from a tissue explant, cultured cells assume a homogeneous, or at least uniform, constitution as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a more homogeneous culture of the most vigorous cell clones [11]. Presumably each of the four HGF strains established in this investigation comprised a mixture of a



few homogeneous cell clones with individual phenotypic characteristics representing some of the fibroblast subtypes resident *in vivo* in structurally and functionally heterogeneous GCT [12]. Continuing propagation of an individual cell strain may by means of the selective pressure of culture conditions support the survival of some cell clones and elimination of others [11] and thus modify the overall phenotypic setup of the cell strain and introduce a potential confounding factor, the present experiments were therefore carried out on cells derived from lower passages of each individual cell strain. It has also been demonstrated that passage levels of cultured periodontal ligament fibroblasts had no significant effect on the response to the applied mechanical strain when cells were used between the 3rd and 8th passages [13]. For this reason 4th–8th passages of HGF were used in the present investigation. In addition, several HGF strains from different donors were used as it was intended to test a potential interindividual variation and/or to exclude the confounding effect of such eventuality. Use of serum-free medium during mechanical stimulation assured that cells were relatively serum-starved and in synchronized growth phase [11]. The validity of this procedure was substantiated under similar experimental conditions [22].

Synthesis of ECM molecules was measured by means of commercially available and specifically designed ELISAs, which are considered to be convenient, highly sensitive, and reliable [29]. However, in some of the assays optical density values tended to be located in the lower part of the linear range, which should be duly considered as a potential limiting factor in the data interpretation.

In this investigation intermittent equibiaxial mechanical stretching was applied for extended periods by means of a computer-controlled vacuum pump to cells attached to a flexible substratum. It is unlikely that this type of experimental mechanical stimulation entirely equals the complexity of the mechanical environment to which GCT is subjected *in vivo* during mastication, speech, orthodontic forces, and irritation from ill-fitting dentures. Notwithstanding this consideration, this apparatus was employed for reasons of an accurate control of experimental mechanical parameters [1, 4], and on the basis of the information that intermittently acting forces are more effective in eliciting cellular responses in periodontal fibroblasts than continuous loads [17]. Taking all these considerations together, the test conditions employed in this study can be considered to represent a simplified but reasonably adequate experimental model of the mechanical environment of GCT.

The mechanical stimulation applied in this study affected the proliferative activity in one of the HGF cultures tested while it had no effects on the other cultures. These findings suggest (a) increased incidence of cell proliferation in HGF subpopulations subjected to prolonged mechanical stimulation and (b) the existence of a marked variation in these reactions between different individuals, on the one hand, and different cell subpopulations in the tissue of the same individual, on the other. Interindividual heterogeneity and variability in HGF proliferation rates are well known, and it has been demonstrated that proliferation rates of HGF are under strong genetic control, which emphasizes the im-

portance of carefully matching control and test HGF in *in vitro* assays [9].

Important GCT changes under conditions of stimulation, such as during inflammation, may be due to clonal imbalance and selection of certain fibroblast subtypes from the structurally and functionally remarkably heterogeneous gingival fibroblast population [12]. It has been suggested [15] that regulation of the balance of gingival fibroblast subpopulations with various metabolic capacities and responses to environmental signals has a fundamental effect on tissue form and function in both steady-state and pathological conditions of GCT. Although there are overt differences between pathological inflammatory conditions and mechanically induced tissue remodeling, it is reasonable to assume that basic cellular responses to stimuli, irrespective of the nature of the latter, has many features in common. For instance, analogously to inflammation-induced changes in homeostasis and proliferative behavior of gingival fibroblast progenitor cells [21], discrete progenitor subpopulations located in different GCT domains [23] react differently to mechanical stimulation *in vivo* with respect to the occurrence, magnitude, and duration of the proliferative response [30]. The results of the assessment of HGF proliferation in the present study are in agreement with the reported variability in the proliferative response of gingival fibroblast progenitor cell populations to mechanical stimulation [30].

Marked ECM changes are known to occur in GCT under inflammatory conditions and in drug-induced gingival overgrowth [2]. Characteristically these include altered ratios of synthesis of various different collagen types, changes in activity of matrix-degrading enzymes, and production of noncollagenous ECM molecules. Of primary importance for these compositional changes are the archetypal gingival ECM constituents COL I and COL III, which, compared with the steady-state conditions, are synthesized in altered ratios in inflamed gingiva [20] and in mechanically stimulated periodontal ligament [5] and collagenase 1 (MMP-1 or fibroblast type collagenase), which is an essential enzyme for periodontal connective tissue remodeling [2].

It appears from the results of the present investigation that mechanical stimulation by tensional stress alters the patterns of ECM protein secreted into the medium by HGF. While, in accordance with other reports [13], no effect on COL I was observed, in strain A the synthesis of COL III was reduced which was accompanied by a corresponding increase in MMP-1, an enzyme involved in GCT remodeling and whose substrate specificity is directed in particular at the hydrolysis of COL I and COL III [2]. This finding is in agreement with the induction of MMP-1 expression in mechanically stimulated HGF reported in other studies [3, 19, 26]. However, the enhancement of MMP-1 production in strains A and C contrasts with the decrease in collagenase mRNA levels in canine gingival fibroblasts subjected to experimental gravitational forces reported previously [24]. Most likely this disparity is based on functional heterogeneity [12] and pronounced variation in gingival fibroblast reactions to mechanical stimulation, on the one hand, and

the nature of the experimental mechanical stimuli, on the other. It has been suggested, for instance, that periodontal fibroblasts are capable of modifying their responses even to varying magnitudes of tensional stress [13].

In addition, in the present study mechanical stimulation led to changes in synthesis of COL III and COL V in some of the HGF cultures tested, which parallels altered ratios of collagen synthesis found in inflamed GCT [20]. These observations extend currently available information on collagen metabolism in mechanically stimulated GCT and call for further more detailed studies.

Other ECM constituents of potential relevance to compositional changes in GCT are elastin and tenascin. Elastin, which endows tissues with elastic properties and is known to occur in altered concentrations in diseased and aging tissues [10], is minimally expressed in human gingiva in the physiological steady state [2]. Parallel to the report of increased tropoelastin expression in mechanically stimulated human periodontal ligament fibroblasts [27], the present finding of the increased amounts of elastin in the medium of mechanically stimulated strains C and D suggests that prolonged mechanical stimulation, at least in distinct cell populations, leads to increased production of this ECM molecule. If applicable to in vivo events, this finding provides novel information regarding possible compositional changes in GCT during orthodontic tooth movement, which might bestow the tissue with modified mechanical properties such as increased elasticity.

Tenascin, whose expression in the physiological steady state is limited, has been shown to affect cell behavior during developmental and wound healing events [18]. This ECM constituent may be synthesized by fibroblasts under experimental gravitational loads [28], indicating involvement of this protein in modulation of cell-matrix interactions in dynamic environments [7, 28]. The results of the present study are in accordance with the previous report [28] and suggest that modification in the expression of this ECM molecule is one of the features of GCT remodeling under mechanical loading. Interestingly, there was a parallel increase in both the elastin and tenascin synthesis in the same HGF cultures, that is, in mechanically stimulated strain D. If extrapolated to in vivo events, this finding suggests the capability of mechanical stimulation as an environmental signal to incite distinct HGF phenotypes to activation of synthesis of ECM constituents which are untypical for GCT composition in the physiological steady state. These observations suggest that mechanical stimulation exhibits differential effects on the production of individual ECM constituents rather than simply having an overall effect on total protein synthesis, and support previous reports that instead of inducing a generalized hypertrophy of connective tissue, tensile loads show specific and differential effect on gene expression of distinct ECM proteins [8].

## Conclusions

In summary, the results of the present study show that cyclic stretching of extended duration may elicit changes in

HGF activity with regard to cell proliferation and ECM remodeling. They suggest that prolonged mechanical stimulation of GCT might, at least in some individuals and/or at distinct tissue locations, affect the clonal balance of the fibroblast subpopulations and, by means of selection of cell phenotypes with distinct synthetic capacities, result in significant modifications in the ECM composition and possibly its mechanical properties. The findings of this investigation also suggest a substantial functional heterogeneity of gingival fibroblasts and interindividual variations in their metabolic reactions to mechanical stimulation.

**Acknowledgements** This work was supported by a research grant from the European Orthodontic Society.

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