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# Transfection of Human Gingival Fibroblasts with Cementum Protein 1 does not Alter the Ultrastructure of the Interchromatin Granule Clusters in the Cell Nucleus

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## Abstract

The cell nucleus contains domains related to gene expression. The nucleolus is involved in pre-rRNA metabolism while interchromatin granules are involved in pre-mRNA metabolism. It was recently shown that no ultrastructural changes are detected in the nucleolar organization, but no data of the effect of transfection in speckles are known. The aim of this work is to know if the interchromatin granule clusters display ultrastructural changes when introducing cement protein 1 (CEMP1) genes into human gingival fibroblasts (HGFs), a cell line of interest since they do not mineralize and upon transfection they perform mineralization, a potential to use in treatments of periodontal disease. Here we explored ultrastructural changes in interchromatin granule clusters after transfection of CEMP1. Non transfected and transfected cells display similar nuclear particles that form clumps in the interchromatin space and contain 10 - 15 nm in diameter individual granules intermingled with fibers, which are composed of ribonucleoproteins. We conclude that they correspond to interchromatin granule clusters. The absence of ultrastructural changes in the morphology of interchromatin granule clusters suggests that after transfection the general ultrastructure of particles related to pre-mRNA metabolism is not altered. These data may support the development of new dental materials.

***Keywords:*** *Cementum Protein 1; Human Gingival Fibroblasts; Interchromatin Granule Clusters; Cell Nucleus; Transfection*

## Introduction

The cell nucleus is a multifunctional and highly dynamic organelle [1-3]. The nucleus contains dynamic bodies known as nuclear bodies and also nuclear domains including those composed of RNA molecules and proteins. It also includes the genetic information of the cell, is the largest site of gene expression and is the site of synthesis and processing of cytoplasmic messenger RNA [1,4,5].

The genes of eukaryotic cells are segmented and their primary expression produce the pre-messenger RNA (pre-mRNA), which has to be processed to produce messenger RNA (mRNA). This maturation includes three major reactions: 1) a 7-methylguanosine group is added

to the 5' end forming a "cap"; 2) a reaction called polyadenylation adds a tail of poly A residues to the 3' end; 3) a reaction called splicing cuts the introns and ligate the resulting exons to generate the mature mRNA. The morphology of transcription and splicing analyzed by fluorescence microscopy corresponds to a nuclear pattern known as nuclear speckles [1]. The speckle pattern is formed by a bright pattern of speckles surrounded by a diffuse labeling. The ultrastructure of the bright elements or speckles corresponds to ribonucleoprotein particles known as interchromatin granule clusters (IGCs), while the diffuse surrounding staining corresponds to the perichromatin fibers [6-8]. The interchromatin granules clusters are composed of 20 - 25 nm in diameter granules and perchromatin fibers of 9 - 10 nm width, connected with the granules [1,6-9]. Interchromatin granules are a reservoir of splicing factors such as the hyperphosphorylatd SR proteins [10-11]. However, no transcription or splicing activities have been detected in the clumps of interchromatin granules clusters, as seen by experiments using pulses of tritiated uridine or bromouridine. Nonetheless, transcription activity takes place in the diffuse pattern or perichromatin fibres [1,7-9]. By using dynamic experiments with transfected genes of splicing factors, it has been concluded that the speckled pattern is a reflection of the activity of transcription and splicing [1,4]. In fact, the IGCs are highly dynamic structures and the speckled pattern varies depending on the expression of the genes. If a gene is over expressed, the morphology of speckles is irregular and if transcription is inhibited, the morphology of the speckles is compacted and rounded [2-4,8,9,12,13].

The human gingival fibroblasts are members of periodontal connective tissue and produce types I, III and V collagens. They are involved in the cellular remodeling of connective tissue. The human gingival fibroblasts play a role in wound healing [14-17]. Those cells are not mineralizing. However, when transfected with human cementum protein 1 (CEMP1) genes, they are induced to display a mineralizing phenotype since root cements proteins are expressed [18]. CEMP1 is highly expressed as mRNA in cementoblasts and also is expressed in subpopulations of the periodontal ligament [19,20]. It has been previously shown that when CEMP1 is over expressed it induces the expression of bone and cement matrix proteins in non-osteogenic cells and when transfecting CEMP1 into human gingival fibroblasts it is converted to a mineralizing type phenotype [19].

Recently, it was shown that after transfection with cementum protein 1 into human gingival fibroblasts, the general nucleolar ultrastructure is not altered, suggesting therefore that pre-rRNA metabolism is not altered either [20]. Here we show that the general ultrastructure of interchromatin granule clusters is neither altered, suggesting also that metabolism of cellular pre-mRNA is not affected.

## Materials and Methods

### Cell cultures

Human gingival fibroblasts (HGFs) were obtained as previously described [20], based on the initial isolation protocols [21]. Briefly, cells were grown and maintained in DMEM medium supplemented with 10% fetal bovine serum. When they reach confluence, the cells were harvested, using 0.025% trypsin-EDTA and transferred for expansion. The cells were kept into an incubator at 37°C in a humid atmosphere with 5% $CO_2$. Experiments were performed with cells from the fourth through fifth pass. Non-transfected (HGFs) and transfected cells with the CEMP1 (HGFs-CEMP1) were used.

Construction of a pcDNA40-CEMP1-expressing vector and transfection of HGFs. It was performed as originally described [19] and recently used [22]. Briefly, the CEMP1 coding region was used to produce the HGF-CEMP1 cell line.

### Transmission electron microscopy

The samples were processed by the standard transmission electron microscopy technique. Samples were fixed in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in saline phosphate buffer solution for 2 hours, postfixed in 1% osmium tetroxide for 1h, dehydrated using a graded series of ethanol and included in epoxy resin (epon 812, Electron Microscopy Science). The ultrathin sections (50 - 60 nm-thick) were mounted onto formvar coated copper grids and were stained with 5% uranyl acetate and 0.5% lead citrate. The samples were analyzed with a transmission electron microscope (Jeol 1010, Peabody, MA, USA) operating at 80 kV. Images were recorded with an MTI camera model CCD-300-RC (MTI, USA), and the Scion Image program (Scion Corporation, USA). A goniometer coupled to

the microscope was used to different inclinations (+10 and -10 degrees) to generate stereo pairs images. Stereo pair images were also processed through an anaglyph program called Anaghlyph Maker. Some samples were processed without adding osmium tetroxide to use the EDTA regressive technique for ribonucleoproteins [23]. Ultrathin sections were mounted on formvar coated copper grids for ultrastructural magnification.

### Interchromatin granule counting

The IGC counting was made by each speckle and a total of 50 speckles per cell line was considered. Speckles were chosen randomly among 100 images per cell line to avoid bias. The counting was also performed by an area of 430 $\mu m^2$ per cell line. Counting was carried out manually with the Image J program.

### Statistical analysis

Data were analyzed using the Mann-Whitney U test, a non-parametric test for two independent samples, using the Sigma plot 12 software.

## Results and Discussion

The nuclear ultrastructure of the HGFs and the HGF-CEMP1 human gingival fibroblast cell line was analyzed by standard transmission electron microscopy after contrast with uranyl acetate and lead citrate. We observed fibro-granular elements in the nucleoplasm of both cell types (Figure 1).

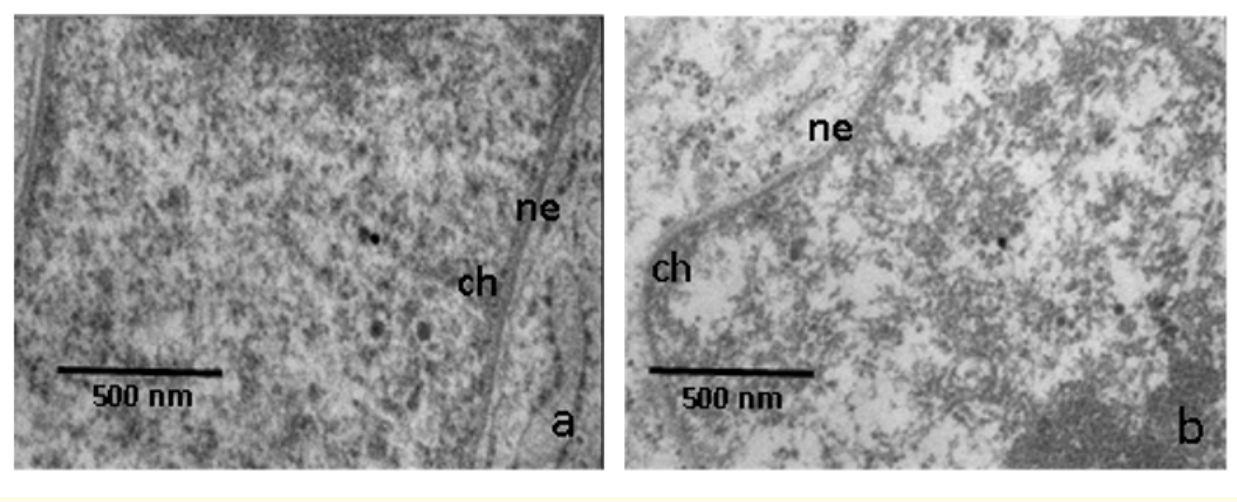


***Figure 1:*** *Electron micrographs of HGFs (a) and HGF-CEMP1 cells after standard fixation and contrast with uranyl acetate and lead citrate. Low magnification shows the general structure of nuclei. Ne: Nuclear Envelope; ch: Compact Chromatin.*

The granular part was present as clusters of interchromatin granules with connecting fibres among them (Figure 1a and 1b). The interchromatin granules are about the size of ribosomes found on the outside part of the nuclear envelope. These granules were positive for HGFs and for HGF-CEMP1 cells after staining with the regressive technique for ribonucleoproteins (Figure 2).

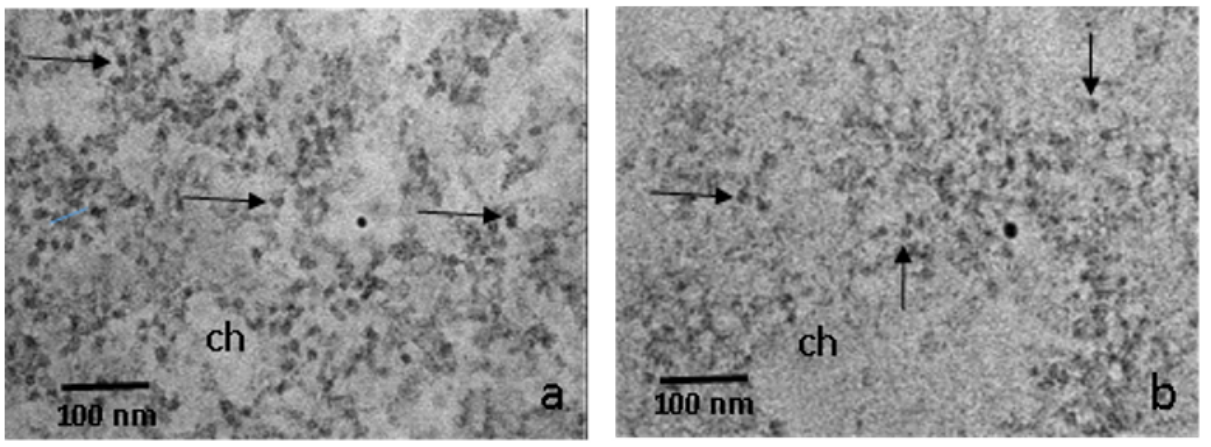


***Figure 2:*** *Transmission electron microscopy of HGFs (a) and HGF-CEMP1 (b) cells after the EDTA regressive staining for ribonucleoproteins. The arrows point to individual interchromatin granules. The compact chromatin (ch) is bleached by the technique, enhancing the contrast of the granules.*

In order to better know the association between granules and fibres in the clusters of interchromatin granules, we use a goniometer coupled to the transmission electron microscope to generate stereo-pairs of images. In both cell types, there are fibres among granules (Figure 3 and 4).

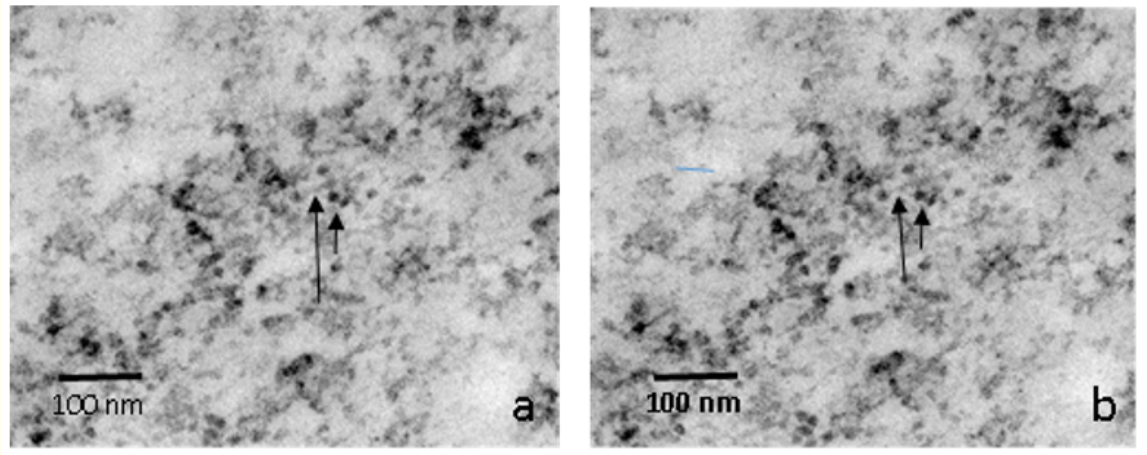


***Figure 3:*** *Stereo pair images from electron micrographs of HGFs after the EDTA regressive staining for ribonucleoproteins. Individual interchromatin granules (large arrows) are surrounding and connect granules by fibres (small arrows).*

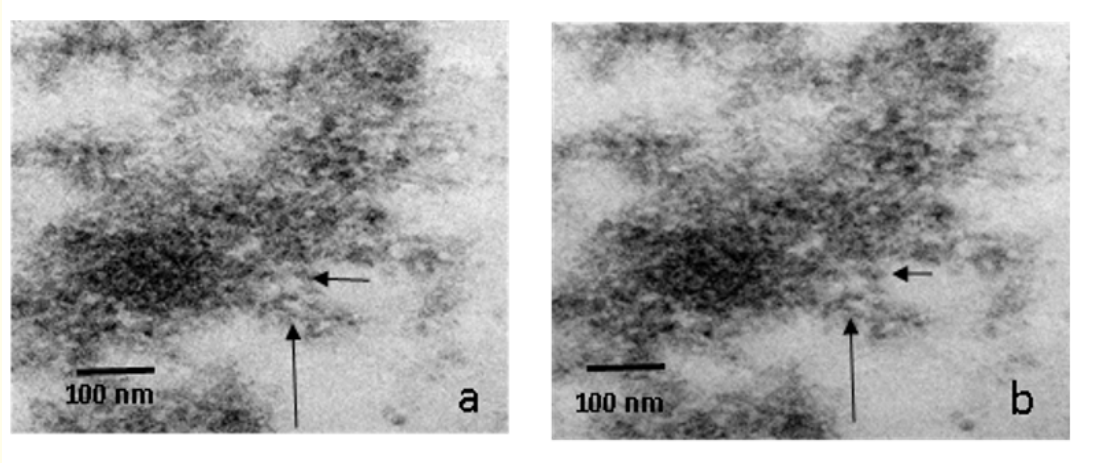


***Figure 4:*** *Stereo pair images from electron micrographs of HGF-CEMP1 transfected cells after the EDTA regressive staining for ribonucleoproteins. Individual interchromatin granules (large arrows) are surrounding and connect granules by fibres (small arrows).*

To analyze whether the ultrastructure of IGCs displays differences in the number of granules among HGFs and HGF-CEMP1 cells, we measured the density of granules for several clusters. The Mann-Whitney U statistical analysis showed differences. There are higher density of granules in HGFs than in HGF-CEMP1 cells ($P < 0.01$) (Figure 5). We counted granules in different clusters, but in a similar area of about 430 $\mu m^2$.

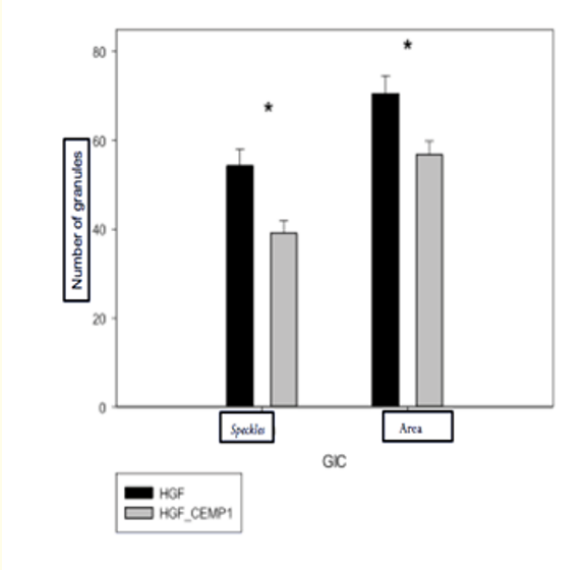


***Figure 5:*** *Density of interchromatin granules in the nuclei of HGFs and HGF-CEMP1 cells. The amount of granules (speckles) per area is shown. The HGFs display more granules than HGF-CEMP1.*

We have shown that nuclear interchromatin granule clusters (IGCs) in human gingival fibroblasts (HGFs) and CEMP1 transfected (HGF-CEMP1) cells display similar ultrastructural patterns. We use IGCs to evaluate putative alterations in the basic process of splicing, since it has been demonstrated that pre-mRNA splicing factors are localized in the IGC [1,8-10]. Although there are no differences among these cells in relation to the basic ultrastructure of IGCs, i.e. the fibro-granular nature of the IGCs clusters, we found differences in the density. This may indicate that every cell type is using different amount of splicing factors because of a difference in the overall gene expression. Alternatively, a higher number of granules in HGFs may also indicate a lower recycling of factors from clusters to sites of active transcription and splicing.

Previously, the cementum-specific protein known as CEMP1 has been purified and characterized [18-20,24-25]. The expression of CEMP1 induces a mineralizing phenotype [18,20]. HGFs were grown in mineralizing media and analyzed by Alizarin Red S staining. Calcium nodules characteristics of mineralization-like cells were detected in cells expressing CEMP1 but were not detected in control HFG cells transfected with the empty vector. Therefore HGF-CEMP1 cell line may have a potential use in periodontal therapies, in the formation of cement and bone [19].

Both cell types (HGF and HGF-CEMP1) were analyzed by transmission electron microscopy through a general staining of uranyl acetate and lead citrate, which allowed us to know in the major structural components of the nuclei. Then, we used the preferential and regressive contrast technique of EDTA for ribonucleoproteins [23] since it removes uranyl more rapidly from the chromatin than from the ribonucleoprotein particles. We focused particularly on interchromatin granules clusters, or otherwise known as speckles when analyzed by fluorescence microscopy. As mentioned, the clumps and the granules within are not altered in form and diameter. Therefore, the nature of the structures remain similar either in HGFs and HGF-CEMP1 cells. The observed changes are present only in the density of granules. This indicates that the HGFs present quantitative differences when compared to the HGF-CEMP1 cell line, suggesting differences in transcriptional and splicing activities, may be because of the overexpression of a particular gene.

Once we observed IGCs by standard and by the EDTA regressive technique for transmission electron microscopy, we analyzed the samples by means of the three-dimensional reconstruction using stereo pairs. We found granules immersed in a fibrous environment, characteristic of IGCs clusters [1,6,8-9].

Taken together, our results suggest that the splicing cellular morphology is a reflection of transcription and splicing activities, as proposed for cells and tissues [1,4,8-13].

Our results indicate that upon transfection of HGFs with CEMP1, no ultrastructural changes are detected in the ICGs, which suggests that the production of mRNA is normal. Future work will focus to study whether or not changes at molecular level are present. It is tempting to mention that after transfection, of CEMP1, the cells do not display ultrastructural changes and this may be taken into account to consider in the putative use for gene therapy. This notion fits with previous results indicating that no ultrastructural changes are detected for nucleolar morphology [22].

## Conclusion

This work shows that upon transfection of CEMP1 genes into human gingival fibroblasts, there are not changes in the ultrastructure arrangement of interchromatin granule clusters. The detected differences observed are present in the density of the granules.

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## Conflict of Interest

The authors declare no conflict of interest.

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