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The *in vitro* viability and growth of fibroblasts cultured in the presence of different bone grafting materials (NanoBone® and Straumann Bone Ceramic®)

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Different clinical applications, including dentistry, are making increasing demands on bone grafting material. In the present study we have analysed the viability, proliferation and growth characteristics of fibroblasts cultured in vitro *together with two different bone grafting materials, NanoBone® and Straumann Bone Ceramic®, over a period of 24 and 28 days respectively. Viability was measured at least every 72 hours by using the alamarBlue assay, a test that measures quantitatively cell proliferation and viability but does not require cell fixation or extraction. After one week of culture fibroblast viability was as high as in controls for both grafting materials and remained high (> 90%) for the duration of the experiment. Cell growth was evaluated microscopically. Scanning electron microscopy revealed a dense fibroblast growth at the surface of both bone grafting materials after three weeks of* in vitro *culture. Generally, our* in vitro *analyses contribute to further insights into cell — scaffold interactions.*

Key words: bone defects, bone remodelling, scanning electron microscopy, SEM, alamarBlue assay

INTRODUCTION

Increasing demands are being made of bone grafting and replacement materials by many clinical applications, including surgery, orthopaedics and dentistry. So far the preferred choice for treating bone defects is autologous bone obtained from the patient and usually taken from the iliac crest [1]. The major advantage of autologous bone is that it does not activate immune reactions leading to inflammation and rejection. However, the bone harvesting process requires surgery in a second body part of the patient, which may cause additional pain, scarring and discomfort, as well as carrying the risk of infection, generally seen as a disadvantage in this procedure. It comes as no surprise, therefore, that more and more bone grafting and replacement materials have been developed and have entered the market. These materials should bear characteristics which support the wound healing process in general, but they should also promote natural bone reconstruction in the defect area by their osteoconductive and osteoinductive properties.

In vitro culture of cells and bone grafting materials is needed to approve scaffolds before they can be applied *in vivo*. The procedure of *in vitro* culture of stem cells and bone grafting material is, furthermore,

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regarded as a tool in obtaining autologous bone *ex vivo* and is known as "tissue engineering" and referred to as the "technology of the future" [4, 8]. Whereas the culture of bone is still in an early stage [10], great progress has been made in, for example, *in vitro* culture of skin transplants [5, 7, 9] and of cartilaginous tissue [2, 6].

In the current study we present data on the *in vitro* culture of fibroblasts and two different bone grafting materials (NanoBone®, Straumann Bone Ceramic®) which are currently used in clinical applications, especially for tooth implants.

MATERIAL AND METHODS

Bone grafting materials

NanoBone®. According to the description of the manufacturer (ARTOSS GmbH), NanoBone is a fully synthetic, granular, highly porous and nanostructured bone grafting material produced by a sol-gel process [3]. It is composed of nanocrystalline, non-sintered hydroxyapatite and silica gel (SiO_2) up to 76% and 24% respectively. During processing nanocrystals of this material become tidily connected but form interconnecting pores of different sizes. The smallest pores are of a nanometre in diameter. NanoBone® acts osteoinductively and is biocompatible and biodegradable.

Straumann Bone Ceramic®. According to the manufacturer's description (Straumann Biologics®), Straumann Bone Ceramic® is a fully synthetic bone graft material of medical grade purity in particulate form (400–700 μm) and commercially available. It is a biphasic calcium phosphate composed of 60% hydroxyapatite and 40% beta-tricalciumphosphate. It is up to 90% porous with interconnecting pores of 100–150 microns in diameter, biocompatible as well as resorbable and replaced by vital bone during bone remodelling.

Cell culture. Human fibroblasts (HFIB; oligene) and mice connective tissue fibroblasts (L-929; DSMZ GmbH Germany) were cultured in flasks (25 cm^2) with a culture medium (CM) consisting of RPMI-1640 medium (PAA) containing 5% foetal calf serum (FBS; GIBCO) and antibiotics (10.000 U/ml penicillin; 10.000 μg/ml streptomycin) for 2 days at 37°C and 5% CO_2. The confluent cell monolayer was harvested by adding trypsin (PAA) for 2 min at room temperature. The detached cells were washed twice (7 min; 1500 rpm) in PBS (PAA). Viability was checked by using trypan blue. Cell titer was determined by using a Neubauer chamber.

1×10^4 fibroblasts per well were added to culture plates containing bone grafting material and incubated for 24 or 28 days respectively at 37°C and 5% CO_2 in CM. Every third day viability/proliferation was analysed for treated and control cells by using the alamarBlue assay and fresh CM was added.

AlamarBlue assay. The alamarBlue™ assay measures quantitatively cell proliferation as well as relative cytotoxicity. It incorporates a water soluble colorimetric oxidation-reduction (Redox) indicator that changes colour in response to the chemical reduction of the culture medium resulting from cell growth (metabolic activity).

One hundred microlitres of indicator (0.4 ml alamarBlue stock solution diluted in 10 ml CM) were added to each test well and incubated for 4 hours at 37°C, 5% CO_2. Eighty μl of the culture supernatant were transferred to a fresh 96 well plate and read spectrophotometrically to monitor absorbance by 570 nm (reduction) and 630 nm (oxidation).

Relative cytotoxicity was calculated on the basis of the difference between the treated and the control cells by using the following formula:

$$\frac{(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2 \text{ of the test agent dilution}}{(\varepsilon_{ox})\lambda_2 A°\lambda_1 - (\varepsilon_{ox})\lambda_1 A°\lambda_2 \quad \text{of untreated positive growth control}} \times 100$$

ε_{ox} — molar extinction coefficient of alamarBlue in oxidised form
A — absorbance of test well
A — absorbance of positive growth control well
λ_1 — 570 nm
λ_2 — 630 nm

SEM

Fibroblasts incubated with or without bone grafting material as well as bone grafting materials were analysed by scanning electron microscopy after *in vitro* culture. Cells and bone grafting material were fixated by glutaraldehyde (2.5% in CM without FBS and antibiotics) for 2 h at 4°C, incubated in 1% tannin for 1 h at 4°C and then dehydrated by ethanol and finally processed in a critical point drying. Samples were examined using a digital scanning microscope (DSM 940A; ZEISS Oberkochen).

Statistics

Data presented result from at least three tests performed in duplicate for both bone-grafting materials.

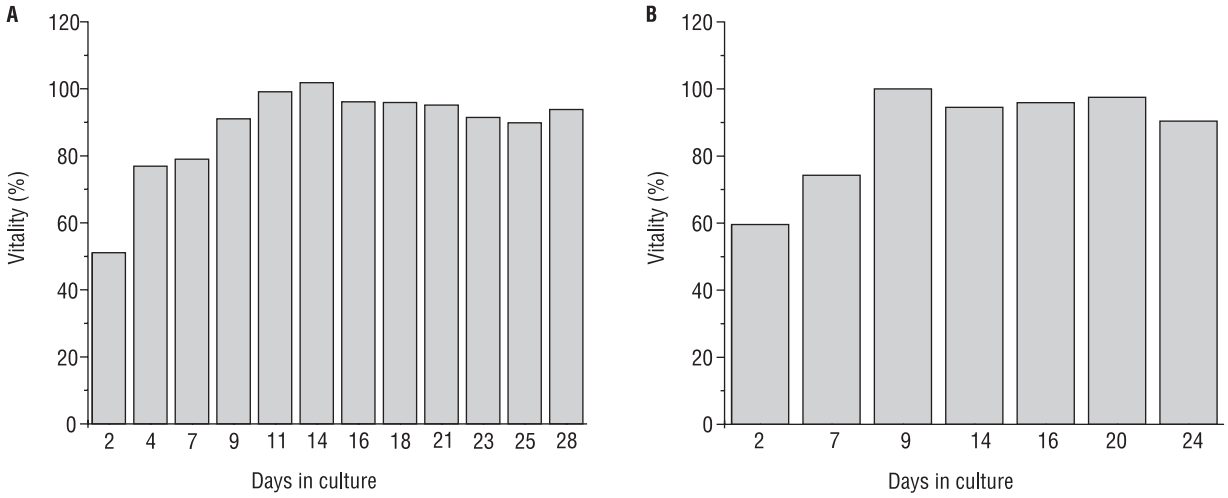

Figure 1. Viability of mice (L-929 cells) and human fibroblast cultured *in vitro* together with Straumann Bone Ceramic (**A**) and NanoBone (**B**) respectively for more than three weeks. Viability was measured as metabolic activity by using the alamarBlue assay. Viability is expressed as a percentage compared to control cells cultured without bone grafting material.

RESULTS

Viability

L-929 cells co-cultured with Straumann Bone Ceramic. Mice fibroblasts (L-929) were co-cultured with Straumann Bone Ceramic over a period of about 28 days. Fibroblast viability was checked by using the alamarBlue assay at the days displayed in Figure 1A and in comparison with L-929 cells cultured without bone grafting material. The metabolic activity of L-929 cells was decreased in the presence of grafting material at day 2 of cell culture. At day 4 of culture their metabolic activity had already reached 80% of that of control cells and about 100% at day 14. Cell viability remained high (> 90%) until the experiment was terminated at day 28 (Fig. 1A).

Human fibroblasts co-cultured with NanoBone. Human fibroblasts were co-cultured with NanoBone over a period of 24 days and their viability analysed by using the alamarBlue test. As for L-929 cells in the presence of bone ceramic, a decrease in viability was recorded for human fibroblasts in the presence of NanoBone at day 2 (60%) and day 7 (70%). At day 9 the viability was as high as in the controls. A cell viability of about 95% was measured until the end of the experiment at day 24 (Fig. 1B).

Scanning electron microscopy. L-929 cells and human fibroblasts differ in their morphology as shown in Figures 2C–F and 3C–F. However both fibroblast types grew and proliferated at the surface of the bone grafting material added to the culture, Straumann Bone Ceramic and NanoBone respectively.

The surface of Straumann Bone Ceramic is structured as seen in Figures 2A and B, but apparently not as much as NanoBone (Fig. 3A). Moreover NanoBone has micropores and nanopores (Fig. 3B), which may allow cells but also growth factors to get deep into the grafting material. After 24 days of culture NanoBone was covered by a nearly homogenous layer of human fibroblasts, where single cells were barely distinguishable.

L-929 cells grow in several layers. Their morphology is mainly fibroblast-like but in areas of high cell density they appear as round cells (Fig. 2C, D). Both types show several cell processes and some are interconnecting neighbour cells. This phenomenon can be observed at the surface of bone grafting material but also at the surface of a glass cover slip (Fig. 2E, F).

The cells at the surface of NanoBone are characterised by long thin processes, which are often connected and give the impression of a three-dimensional network (Fig. 3E, F).

DISCUSSION

Human fibroblasts as well as those from mice (L-929) can be cultured *in vitro* and remain viable for several weeks. From an overall view of the experiments, the bone grafting material does not affect cell viability. Nevertheless, the metabolic activity of cells cultured together with bone grafting material is reduced compared to that of control cells for a period of one to four days. This may be evidence of a lower proliferation rate of fibroblasts in the presence of bone grafting material and might be due to surface characteristics. Differences in cell spreading

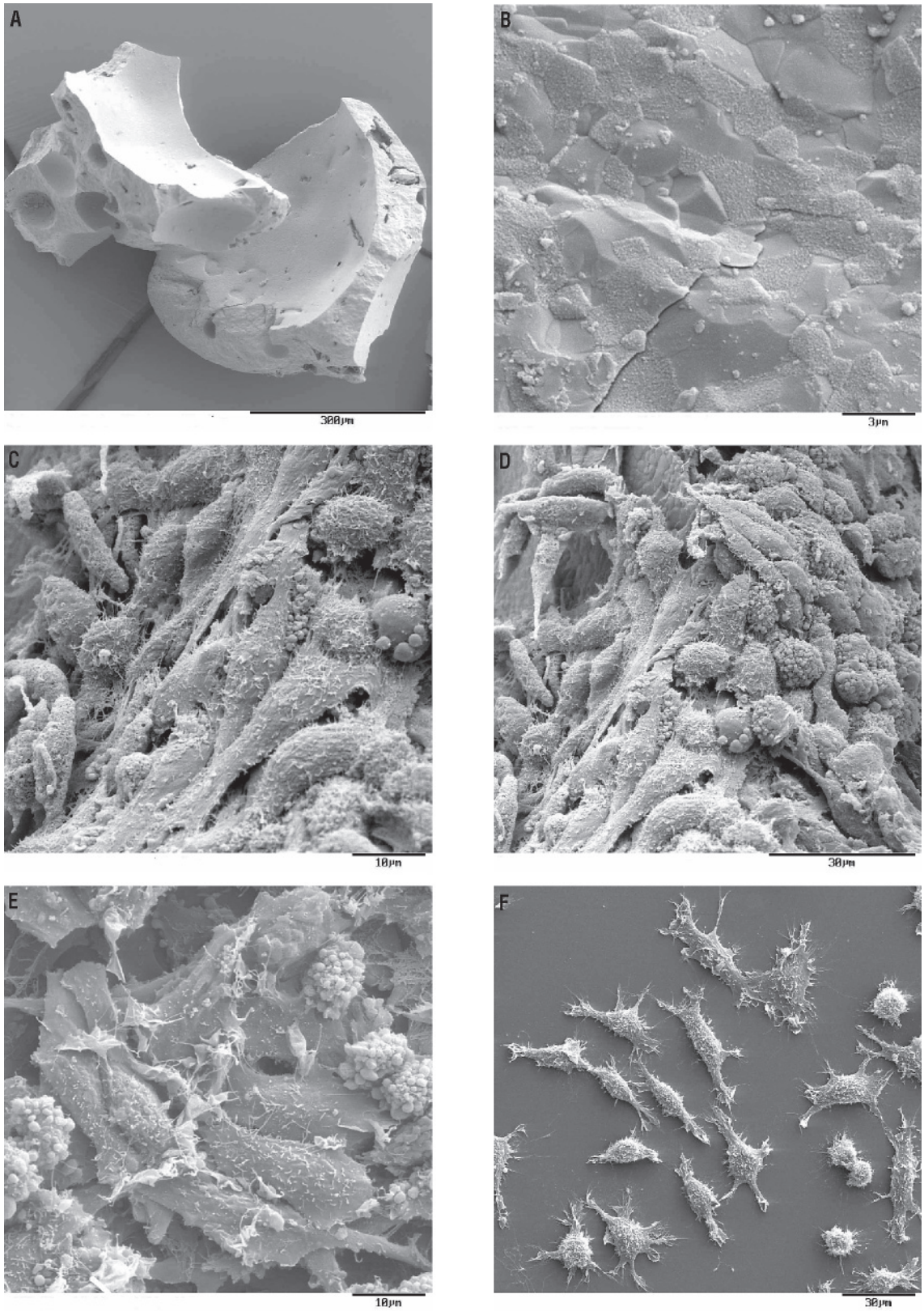

Figure 2. Scanning electron micrographs of Straumann Bone Ceramic cultured *in vitro* without (**A–B**) and together with L-929 cells (**C–D**) for 28 days. L-929 cells cultured *in vitro* on glass cover slips for 10 days and 48 hours are shown in **E** and **F** respectively. Scale bars are given in the lower right corner of the micrographs.

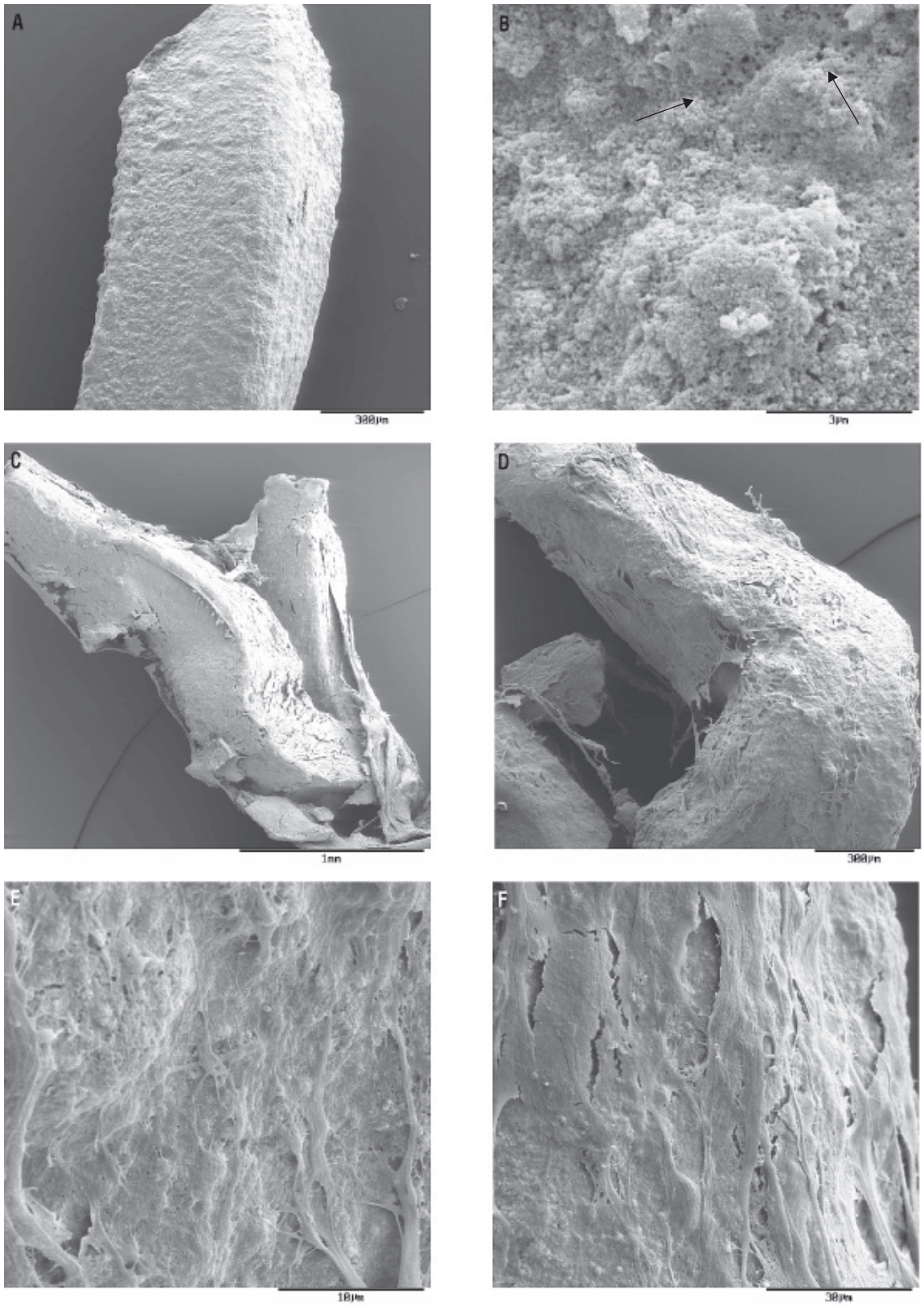

Figure 3. Scanning electron micrographs of NanoBone cultured *in vitro* without (**A–B**) and together with human fibroblasts for 24 days are shown at different magnifications (**C–F**). Arrows in Figure **3B** indicate nanopores in NanoBone. Scale bars are given in the lower right corner of the micrographs.

and proliferation are also known for plastic and glass-bottom wells used for cell culture.

It cannot be assumed that there is a toxic effect of the grafting materials upon fibroblasts, since earlier experiments using different amounts of NanoBone did not display a dose-dependent effect [11]. After one week of culture viability was as high as in controls for both grafting materials and remained high (> 90%) for the duration of the experiment.

Cells proliferated at the surface of the both bone grafting materials, since the surfaces were densely covered by fibroblasts after three weeks of culture, as visualised by SEM (Fig. 2C, D, 3C–F).

Fibroblast growing behaviour and proliferation cannot be compared directly for both bone grafting materials, since different cell lines were used in the experiments. However, interconnecting cellular processes were seen for both types of fibroblast. Human fibroblasts at NanoBone seem to produce longer fibrils, more intercellular substance and develop a network, where borders of single cells are hard to distinguish (Fig. 3B–D).

It would be preferable for human cells to be used in further studies and these may contribute to a better understanding of the interactions between cells and bone grafting materials *in vitro* and *in vivo*. These data could deliver further insights, especially valuable with respect to tissue engineering, which is gaining increasing attention and is currently progressing [5, 7, 8, 10].

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