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Therapeutic liver repopulation in a mouse model of hypercholesterolemia

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Liver repopulation constitutes an attractive approach for the treatment of liver disorders or of diseases requiring abundant secretion of an active protein. We have described previously a model of selective repopulation of a normal liver by Fas/CD95-resistant hepatocytes, in which we achieved up to 16% hepatocyte repopulation. In the present study, we investigated the therapeutic efficacy of this strategy. With this aim, apolipoprotein E (ApoE) knockout mice were transplanted with Fas/CD95-resistant hepatocytes which constitutively express ApoE. Transplanted mice were submitted to weekly injections of non-lethal doses of the Fas agonist antibody Jo2. After 8 weeks of treatment, we obtained up to 30% of the normal level of plasma ApoE. ApoE secretion was accompanied by a drastic and significant decrease in total plasma cholesterol, which even fell to normal levels. Moreover, this secretion was sufficient to markedly reduce the progression of atherosclerosis. These results demonstrate the efficacy of this repopulation approach for correcting a deficiency in a protein secreted by the liver.

INTRODUCTION

Hepatocyte transplantation could be an interesting alternative to whole-liver transplantation (1). Nevertheless, there are some drawbacks limiting the use of this approach, as clinical applications would demand significant hepatic replacement, which would, in principle, require either the transplantation of a large number of hepatocytes or proliferation of the transplanted ones.

Normal hepatocytes, which do not express the anti-apoptotic protein Bcl-2, are extremely sensitive to Fas/CD95-mediated apoptosis. We have reported previously that hepatocytes expressing human Bcl-2 are resistant to death induced by a Fas agonist antibody (2). Transplanted Bcl-2-expressing hepatocytes were able to repopulate normal mouse liver by up to 16% after 8–12 once-weekly injections of non-lethal doses of Fas agonist antibody Jo2 (3). These results indicated that Bcl-2 expression conferred a selective advantage on the transplanted hepatocytes compared with resident ones, allowing them to repopulate a normal liver.

To demonstrate the therapeutic efficacy of our repopulation strategy, we chose a liver-secreted protein deficiency model, apolipoprotein E (ApoE) knockout mice (4). ApoE is a key protein in the metabolism of plasma lipoproteins, as it mediates the uptake and degradation of very low density lipoproteins (VLDL) and chylomicron remnants (5). In humans and normal mice, the major site of ApoE synthesis is the liver (5). ApoE-deficient mice constitute a clinically relevant mouse model, since they are severely hypercholesterolemic and develop premature atherosclerosis on a chow diet (4).

We show, in the present study, that selective repopulation of $ApoE^{-/-}$ liver by Bcl-2 hepatocytes constitutively expressing ApoE results in the correction of cholesterol levels and the reduction of atherosclerotic lesions.

RESULTS

Repopulation of $ApoE^{-/-}$ mice livers by transplanted Bcl-2 hepatocytes

Seven-week-old ApoE knockout mice (n = 10) received 1 million Bcl-2 hepatocytes with normal ApoE gene expression by injection into the spleen. These animals were submitted to eight weekly injections of anti-Fas antibody Jo2 at sublethal doses and to daily immunosuppressive treatment with FK506, in order to avoid immunization against Jo2. A control group (n = 10) of $ApoE^{-/-}$ mice received non-transgenic normal hepatocytes in parallel.

Liver DNA was analyzed by PCR for the presence of the Bcl-2 transgene. In order to estimate the percentage of repopulation, we compared the signals obtained from our animals with those from serial dilutions of liver DNA of a Bcl-2 transgenic mouse (Fig. 1). Repopulation percentages ranged from 1

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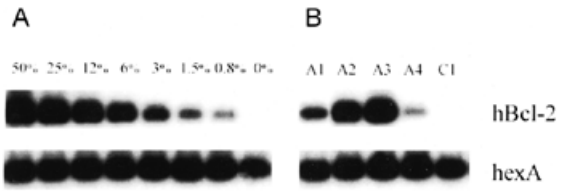


Figure 1. PCR quantitation of Bcl-2 DNA in transplanted mouse livers. Genomic liver DNA amplified by PCR for human Bcl-2 and murine hexosaminidase A (hexA) genes. (**A**) Serial dilutions of Bcl-2 transgenic mouse DNA in non-transgenic DNA. (**B**) Liver DNA of four representative animals of the experimental group (A1 to A4) transplanted with Bcl-2 transgenic hepatocytes and of the control group (Cl) transplanted with non-transgenic hepatocytes at termination, after eight weekly injections of Jo2.

to 19% of the total liver mass (animals A4 and A3, respectively). In fact, as only ~60% of total liver DNA originates from hepatocytes (6), the best repopulation percentage of 19% of the total liver mass, therefore means that 30% of the hepatocytes of the A3 mouse were derived from transplanted cells. This is almost 2-fold higher than the best percentage obtained in our previous study (3). The greater repopulation efficacy achieved in this study could be due to (i) an improvement in the transplantation protocol, as we have added a percoll centrifugation step in order to exclude non-parenchymal and dead cells from the preparation, and (ii) the fact that both donor and recipient animals had the same genetic background.

Immunohistochemical analysis of Bcl-2 expression in the liver of transplanted ApoE$^{-/-}$ mice confirmed the expected clonal proliferation of Bcl-2 hepatocytes (Fig. 2).

ApoE expression in ApoE$^{-/-}$ transplanted animals

We then evaluated the therapeutic efficacy of our protocol by analyzing ApoE expression in plasma and liver samples, as well as the cholesterol levels and lipoprotein profile. All animals in the experimental group showed ApoE expression both in their livers (data not shown) and in their plasma at termination (Fig. 3). The average ApoE plasma level was 17% of normal. Such a plasma level of ApoE has already been shown to be therapeutic, inducing a lowering of plasma cholesterol to control levels (7,8). Animal A3, which had the highest repopulation percentage, also had the highest ApoE level, reaching 29% of the normal level, as estimated by comparison with dilutions of a normal plasma. These results confirm that the repopulating hepatocytes conserved their physiological ability to express and secrete ApoE. In contrast, no ApoE expression was detected in plasma samples of animals of the control group (Fig. 3).

Effect of ApoE expression on cholesterol metabolism and on lipoprotein profile

To examine the effect of the ApoE expression on plasma cholesterol levels, we measured total plasma cholesterol at the end of treatment (Fig. 4). When compared with non-transplanted ApoE$^{-/-}$ mice or ApoE$^{-/-}$ mice transplanted with non-transgenic hepatocytes, the experimental group showed a marked reduction in cholesterol levels (489 ± 29 or 418 ± 30 versus 168 ± 19 mg/dl, respectively; $P < 0.005$). Furthermore, plasma cholesterol of animal A3 was 82 mg/dl, which is within the normal range for C57Bl/6 mice. In order to verify that the reduction of plasma cholesterol was due to a change in the distribution of plasma lipoprotein induced by ApoE, we performed a fast protein liquid chromatography (FPLC) analysis of plasma samples (Fig. 5). Animals in the experimental group showed a marked decrease in VLDL and low density lipoproteins (LDL) fractions, accompanied by an increase in high density lipoproteins (HDL) cholesterol. In contrast, the lipoprotein profile in the control group remained similar to that of non-treated ApoE-deficient mice.

Quantification of atherosclerotic lesions

We studied the effect of plasma cholesterol lowering on the long-term progression of atherosclerotic lesions. For this purpose, an experimental group, transplanted with Bcl-2 hepatocytes ($n = 7$), and a control group, transplanted with non-transgenic hepatocytes ($n = 5$), were submitted to the same treatment as described above, but were killed 2 months after the last Jo2 injection. Since FK506 has been shown to increase atherosclerosis (9), a control group of non-transplanted ApoE$^{-/-}$ mice

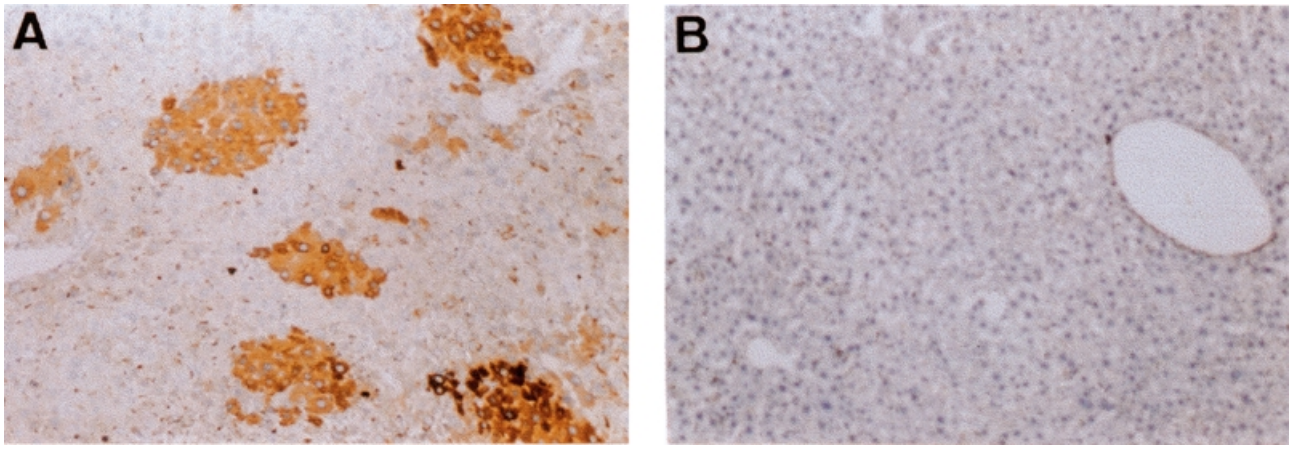


Figure 2. Immunohistochemistry for Bcl-2 expression. Bcl-2 immunostaining analysis of liver sections. (**A**) Liver of a mouse (A3) in the experimental group; (**B**) liver of a mouse in the control group (Cl). Note in (A) the staining of groups of hepatocytes, underlining the clonal expansion of transplanted hepatocytes. No staining is observed in (B). Original magnification: 100×.

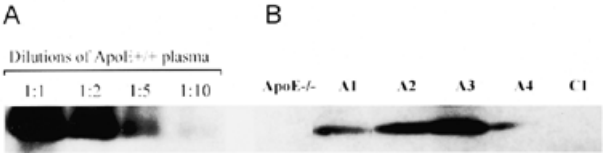


Figure 3. Western blot analysis of transplanted mice for expression of ApoE in their plasma. (**A**) Serial dilutions of normal mouse plasma. (**B**) Plasma of representative animals transplanted with Bcl-2 hepatocytes (mice A1–A4) or with non-transgenic hepatocytes (Cl) at termination.

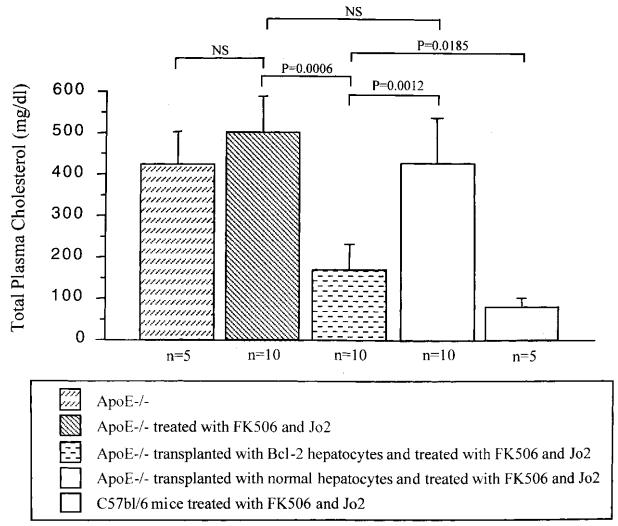


Figure 4. Effects of repopulation on plasma total cholesterol levels. Cholesterol values of animals at termination. Only ApoE$^{-/-}$ mice transplanted with Bcl-2 hepatocytes and treated with Jo2 show a significant decrease in cholesterol levels compared with other ApoE$^{-/-}$ groups. Error bars indicate SEM. The number of mice used in each group is shown below the graph.

receiving only FK506 ($n = 5$) was added. The mean lesion area per section was significantly lower in the experimental group ($68 \pm 18 \times 10^4$) compared with the two control groups ($158 \pm 20 \times 10^4$ and $201 \pm 16 \times 10^4$), as shown in Figure 6 ($P < 0.005$).

Discussion

The liver is of particular interest for gene and cell therapy as it is the site of many metabolic disorders and as hepatocytes can be targeted to secrete proteins in the general circulation. However, to date, the efficacy of *in vivo* viral-vector-based gene therapy has been limited by a transient therapeutic effect (10) and by a low level of hepatocyte transduction in mice (11–13). In addition, liver cell transplantation has usually been hampered by the high number of hepatocytes needed to be transplanted to achieve a therapeutic result.

Liver repopulation constitutes an attractive alternative strategy to these approaches as it could greatly enhance the efficiency of hepatic gene transfer by providing a selective growth advantage for genetically modified hepatocytes. Spectacular liver repopulation results have previously been described in the urokinase-type plasminogen activator (uPA) transgenic (14–16) and the fumarylacetoacetate hydrolase

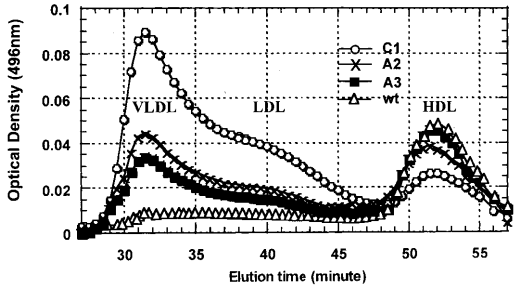


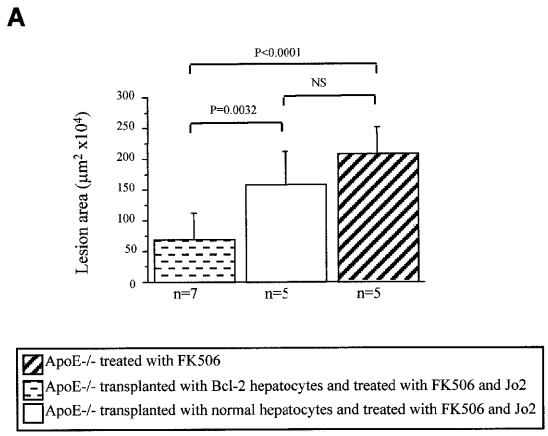
Figure 5. Effect of hepatocyte repopulation on the lipoprotein distribution of plasma cholesterol. Plasma samples were obtained at termination. Representative profiles obtained from two mice of the experimental group (A2 and A3), one mouse of the control group (Cl) and a wild-type C57Bl/6 mouse (wt) are shown. Plasma was fractionated by gel filtration chromatography with a Superose-6 column, and the cholesterol content of the eluted fraction was determined as described in Materials and Methods.

(FAH) null mice (17). However, they cannot be used as general study models, as they are based on diseased and continuously regenerating livers.

Recently, Laconi *et al.* (18–20) and our group developed two experimental models of normal liver repopulation. In the first model, repopulation of rat liver by transplanted cells is based on a selective proliferation disadvantage of resident hepatocytes. The regenerative property of resident hepatocytes is blocked by a drug, retrorsine, while the subsequently transplanted cells are stimulated to proliferate, either by partial hepatectomy or by successive triiodothyronine (T3) treatments. On the other hand, we have developed a liver repopulation approach based on the selective proliferation advantage of transplanted cells, which resist Fas/CD95-mediated apoptosis (3).

We now demonstrate that our strategy could actually be of therapeutic value. The transplantation of a limited number of Bcl-2 transgenic hepatocytes into ApoE$^{-/-}$ mice, followed by the induction of their selective expansion, is sufficient to repopulate the liver of these mice. More interestingly, this strategy can restore a therapeutic ApoE plasma level, and induce a very significant lowering in cholesterol levels of these hypercholesterolemic mice. In contrast to the experimental group, in which all animals had various levels of ApoE in their plasma, no ApoE secretion was observed in the control group transplanted with non-transgenic hepatocytes. Because ApoE is mainly synthesized by hepatocytes, the ability to reach a normal cholesterol level illustrates the power of this repopulation strategy for restoring a physiological function of the liver. Moreover, the reduction in plasma cholesterol was associated with a marked diminution in atherosclerotic lesions.

In addition to apolipoprotein deficiencies, there are several pathological circumstances in which a 15–30% restoration of the normal expression would be therapeutic, for instance, hypercholesterolemia with LDL receptor defects (21), Crigler Najjar disease (22), urea cycle deficiencies (23,24), hemophilias A and B (25,26) and afibrinogenemia (27). More generally, genetically modified hepatocytes engineered to secrete any type of therapeutic protein could be used to treat different types of genetic or acquired diseases using autologous hepatocyte transplantation.



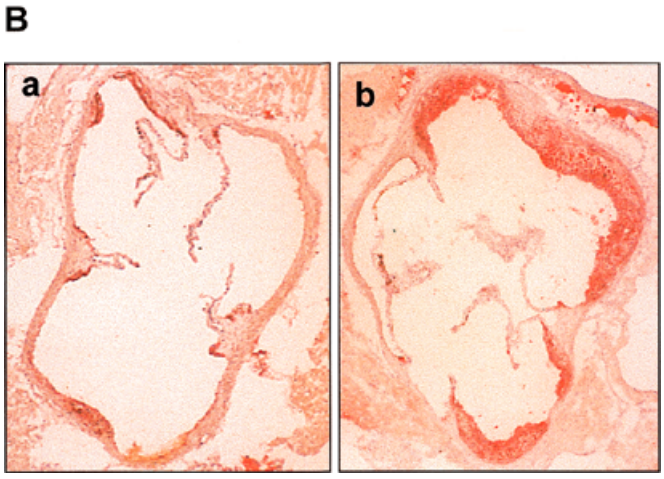


Figure 6. Quantitative analysis of atherosclerotic lesions. Atherosclerotic lesions of animals killed 2 months after the end of the treatments. (**A**) Mean aortic lesion area. Error bars indicate SEM. The number of mice used in each group is shown below the graph. (**B**) Section of an ApoE$^{-/-}$ mouse transplanted with Bcl-2 hepatocytes (a), and section of an ApoE$^{-/-}$ mouse transplanted with normal hepatocytes (b).

While we are aware that the exact strategy used in this work to repopulate the liver would probably not be applicable in humans, we are confident that more acceptable methods can be developed. They could also rely on apoptosis protection, as in this work. To this end, we have demonstrated that long term Bcl-2 expression in the livers of transgenic mice was not only not tumorigenic, but actually protected mice from hepatocellular carcinoma due to the expression of a c-*myc* transgene (28). If this type of hepatocyte protection is to be used, milder inducers of hepatocyte apoptosis than Fas agonist antibodies will be needed. Since pro-apoptotic molecules are actively being developed by different laboratories for the purpose of anticancer therapy, this is perfectly plausible. Furthermore, we are also engaged in testing other pairs of hepatotoxic xenobiotics and protector genes.

In this work, we have described the repopulation of the liver by cell transplantation. An alternative strategy would be to directly transfer, *in vivo*, integrative constructs encoding both the protector gene and the gene of therapeutic value. This method would avoid the immune problems related to allogenic cell transplantation, or if autologous hepatocytes were used, the need for prior hepatectomy. We have preliminary evidence that gene therapy vectors are indeed very effective for conferring a selective advantage on hepatocytes *in vivo* (J.E. Guidotti, unpublished data).

In conclusion, the possibility of selectively amplifying the number of hepatocytes previously engineered *ex vivo* or *in vivo* should greatly increase the prospects of liver-mediated gene therapy in the future.

MATERIALS AND METHODS

Animal procedures

Hepatocytes were isolated according to a standard protocol (29) from non-transgenic or L-PK-hBcl-2 mice (2) of C57Bl/6 background. Viable hepatocytes were separated from other cells using an isodensity Percoll centrifugation (30). One million hepatocytes (>95% viability) were then injected into the spleens of 7-week-old ApoE-deficient mice (4) fed a chow diet. In the experimental group (n = 10), ApoE$^{-/-}$ mice were transplanted with Bcl-2 hepatocytes, whereas in the control group (n = 10), ApoE$^{-/-}$ mice were transplanted with non-transgenic normal hepatocytes. Both groups received sublethal doses of Jo2, a hamster monoclonal anti-Fas antibody (Pharmingen, San Diego, CA), 0.1 mg/kg being administered intravenously once per week, beginning 48 h after hepatocyte transplantation. Mice were killed 1 week after the eighth Jo2 injection. All mice were immunosuppressed with daily intramuscular injection of 2.5 mg/kg of FK506 (kindly provided by Fujisawa GmbH, Germany) until termination. For atherosclerosis quantification, additional 7-week-old ApoE$^{-/-}$ mice were treated: (i) mice transplanted with Bcl-2 hepatocytes and submitted to eight weekly cures of Jo2 (n = 7); (ii) mice transplanted with non-transgenic normal hepatocytes and submitted to eight weekly cures of Jo2 (n = 5); (iii) mice treated only with FK506 (n = 5). These mice were killed 2 months after the end of Jo2 treatment. All procedures were in accordance with institutional guidelines.

Semi-quantitative PCR analysis

Liver genomic DNA was extracted according to standard protocols (31). PCR primers for the Bcl-2 transgene were 5′-CCAGGAGAAATCAAACAGAG-3′ and 5′-ACGGTGGTGGAGGAGCTCTT-3′, using the following conditions: 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, for 25 cycles. PCR products were hybridized with an internal probe (5′-GATGACTGAGTACCTGAAC-3′) labeled with [γ-^{32}P]ATP. The murine hexosaminidase A (*hexA*) gene was used as an amplification control (3). Bcl-2 amplification was quantitated in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), using *hexA* amplification for normalization.

Histology and immunohistochemistry

Liver samples fixed in 10% phosphate-buffered formalin were embedded in paraffin. Sections of 3 μm were stained with hematoxylin–eosin–safran for standard microscopy. For Bcl-2 immunohistochemistry, a monoclonal mouse antibody raised against human Bcl-2 (Clone 124; DAKO, Glostrup, Denmark) was used at a dilution of 1:50 at 37°C for 1 h. Endogenous

peroxidase activity was blocked with 3% H_2O_2 and methanol. Slides were developed with a peroxidase/DAB detection kit (ChemMate; DAKO).

Detection of plasma ApoE

Plasma samples (1 µl diluted 10-fold with PBS) underwent 12% (w/v) SDS–PAGE. The gel was then transferred onto a nitrocellulose membrane for 1 h. After blocking with 7% (w/v) dry milk, the membrane was incubated with polyclonal mouse ApoE antibody (Biodesign International, Kennebunkport, ME) overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit IgG was used as secondary antibody (DAKO), and signals were detected by the enhanced chemiluminescence method (Amersham-Pharmacia Biotech, Little Chalfont, UK). Dilutions of a normal mouse plasma in the plasma of an $ApoE^{-/-}$ were used to estimate the relative percentage of ApoE present in the plasma of transplanted animals. Signals were quantitated using a light densitometer (Shimadzu Corporation, Kyoto, Japan).

Plasma lipid measurement and fast protein liquid chromatography

Total cholesterol was measured in plasma samples using an enzymatic colorimetric test (Boehringer Mannheim, Mannheim, Germany). Plasma lipoprotein distribution (two measures per mouse plasma sample) was assayed by analytical gel filtration chromatography, with a Superose 6 HR 10/30 column (Pharmacia, Uppsala, Sweden). The elution flow rate was 0.4 ml/min in a running buffer consisting of 0.15 mol/l NaCl, 1 mmol/l EDTA and 0.02% NaN_3 pH 8.2. Fractions of 0.5 ml were collected and cholesterol levels determined.

Morphometric analysis

Analysis and quantification of atherosclerotic lesions were performed as described previously (32). Briefly, 10 µm cryo-sections of the aortic sinus with valves were stained with Oil Red O (Sigma-Aldrich, St Louis, MO), counterstained with hematoxylin and examined using light microscopy. Lesion area value for each mouse was obtained from the mean of 10 sections separated by 100 µm and calculated by use of computer planimetry after slide scanning on a Nikon scanner.

Statistical analysis

Plasma cholesterol data were analyzed using the Mann–Whitney test. Comparison of atherosclerotic lesion area values in the different groups was done by using a two-way ANOVA. Values are expressed as means ± SEM. $P < 0.05$ was considered significant.

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