Quality of placental RNA: effects of explant size and culture duration

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Abstract: We evaluated the impact of placental explant size (micro ≤50mg; macro ~200mg), oxygen concentration, culture method and duration on RNA quality. Our findings show that micro explants cultured at 8% oxygen have the best RNA quality and tissue structure after 6 days culture. Explants from early cultures produced poor quality RNA and should be avoided.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Highlights: Placental Viability and RNA Quality

- Placental explant size and duration of culture affect explant RNA quality.
- Placental micro explants (≤50mg) in long-term cultured at liquid gas interface in 8% oxygen produced RNA with quality akin to pre-culture explants
- Quality of RNA from explants in early cultures is compromised
- Macro explants (~200mg) after culture produced poor quality RNA and should be avoided.

Introduction

Previous studies have established very clearly that placental tissue, particularly placental explants [1-3] show loss of the syncytiotrophoblast (STB) layer during initial culture (2-4 days), followed by regeneration of the syncytium over longer culture periods (5 – 11 days) [4,5]. Studies from a number of groups confirm these data [6-12], and a review summarised the value of such explant models [13]. One key factor (included in the review by Miller et al) is the consideration of oxygen tension used during culture, which has considerable impact on explant functions [14,15], particularly as it is now clear that first trimester and term placental tissues are exposed to very different oxygen tensions in vivo, ~3% in the first trimester and ~8% thereafter [13].

In all tissues, the quantification of RNA can be used to determine how gene expression changes in a variety of *in vivo* or *in vitro* conditions. However, it is well established that RNAs are inherently unstable molecules [16], and therefore methods used for handling *ex vivo* substrates must sustain not merely the rather global measure of 'cell viability', but the more subtle integrity of mRNAs. There seem to have been no systematic studies on cultured human placenta, so we investigated the quality of RNA that could be obtained from cultured placental explants, and how this was affected by the size of the explants and the oxygen tensions used during in vitro culture. Optimising the conditions that allow the extraction of high quality mRNA is a necessary precursor for quantitative high throughput studies.

Methods

Collection of Placentae

Placentae (n = 12) were collected at term (38-39 weeks of gestation) from normal pregnancies at the Imperial College NHS Trust following informed consent from mothers. Ethics permission was granted by the Hammersmith and Queen Charlotte's & Chelsea Hospitals Research Ethics Committee. Samples were taken from non-smoking mothers without gestational diabetes, pre-eclampsia or other complications who were delivered by elective Caesarean section. Each placenta was visually inspected for signs of excessive tears, necrosis, and infarction, and samples taken from healthy looking areas about 5 cm away from the placental cord with sterile sharp scissors. Approximately 2 cm^3 of the placental tissue were cut with sterile scissors from three different sampling sites. Each sample was carefully excised to include an intact chorionic plate, intervillous space, basal plate and decidua. The samples were collected from the maternity operating theatre, washed in phosphate-buffered saline (PBS) solution (pH 7.4) containing 10% penicillin, streptomycin and L-glutamine (Sigma) to remove excess blood and blood clots and transported in 150 ml sterile pots containing warm PBS with 10% penicillin, streptomycin and L-glutamine immediately to the laboratory. Placental explants were then aseptically dissected from the intervillous space of each sample, fragmented with scissors and incubated within 30 minutes of delivery.

Explant Culture

Placental explants were cultured in RPMI 1640 culture medium (Invitrogen, UK) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin for up to 6 days (the culture media were changed at days 2 and 4), either in 5% CO_2, 95% air at 37°C (20% oxygen) or in 5% CO2, 8% oxygen (nitrogen used to decrease the oxygen tension). Tissue samples were either

macro explants (approximately 200 mg wet weight, 1 explant/ml medium; n = 24), or micro explants (<50 mg wet weight, 3 explants/ml medium; n = 24). 12-well culture plates were used throughout (Costar UK Ltd., High Wycombe, Bucks, UK), and the micro explants were cultured on 15mm diameter Netwell inserts with 74µm polyester mesh bottoms attached to polystyrene inserts (Corning, UK) and incubated at the liquid-gas interface [13].

Explants were cultured to time 24h, 48h, 72h, 96, 120h and 144h. At the end of each 24 hour period of culture, explants were either fixed in neutral phosphate-buffered 4% formaldehyde solution for 24 hours, or were stored in RNAlater (Ambion) at -80°C. Pre-culture, 0 hour control samples obtained from fresh placentae were stored similarly fixed or in RNAlater.

RNA Quality Assessment

RNA was extracted with TRIZOL (Invitrogen, UK) according to manufacturer's instruction. Briefly, 1 mL TRIZOL reagent per 50–100 mg of tissue sample was homogenised with Ultra-Turrax T8 homogeniser (IKA-Werke, Germany) in a type II flow hood (Gelaire, Flow Laboratory), and phase separated with 0.2 mL of chloroform per 1 mL of TRIZOL. RNA was precipitated with 0.5 mL of 100% isopropanol per 1 mL of TRIZOL reagent. RNA concentration was normalised to wet weight, and purity assessed by measuring UV 260/280 absorbance ratios (NanoDrop® ND-1000 UV-Vis Spectrophotometer). RNA quality was assessed by measuring RNA Integrity Number (RIN), rRNA ratio and microcapillary electrophoresis with Agilent Bioanalyzer 2100 with RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA, USA).

Immunohistochemistry

Placental explants fixed as described above were processed for immunohistochemistry and stained with haematoxylin and eosin as described previously for fetal membrane explants [17]. In addition, immunohistochemistry with antibody against β-HCG (5µg/ml), using the method recommended by the supplier (Abcam, UK) was performed. Positive staining (brown) was developed with diaminobenzidine chromogen solution, followed by counterstaining with hematoxylin. Nikon Eclipse ME600 (Nikon, Tokyo, Japan) microscope was used. Photomicrographs were taken with a Nikon D5100 digital SLR camera saved to a PC.

Results and Discussion

RNA Quality in Pre-culture, Micro and Macro Explants

The RIN for the RNA extracted from control tissues (0h culture) ranged between 7 and 10. After 6 days of culture, the mean RIN for RNA from 6 days micro explants was 8.28 ± 0.15, compared with 3.04 ± 1.1 for RNA from macro explants (means ± SEM, $p < 0.0001$, Bonferroni/Dunn; supplementary Table 1). Oxygen tension did not affect the RIN data obtained. Micro and macro explant samples obtained after 2-4 days of culture showed RIN values of 3.35 ± 0.64, demonstrating that the integrity of RNA in the macro samples was poor throughout the culture period used, whereas in the micro explants the RNA quality is poor transiently, and then improves by 6 days of culture. Typical electropherograms from which the RIN data are derived are shown in Figure 1; 18S and 28S rRNA peaks were clearly delineated in 0h control explants (Figure 1A) and micro samples cultured for 6 days in 8% (Figure 1D) or 20% (Figure 1E) oxygen. Little or no intact RNA could be seen in the macro explants after 2 days (Figure 1C) or 6 days (Figure 1F).

No correlation between RNA purity assessed with Spectrophotometer 260/280 OD ratio and RNA quality assessed with RIN was observed. However, the difference between the mean 260/280 ratio for macro explants (2.02 ± 0.008 SEM, n= 18) and micro explants (1.8 ± 0.028 SEM, n = 29) was significant ($p < 0.0001$), and implies a higher purity in the macro explants. No significant difference in RNA purity was observed between macro explants cultured to 48 hours or 120 hours (data not shown). No relationship was observed between the oxygen concentration and macro explant RNA purity. However, RNA samples from all 6 days micro explants (n = 23) passed quality control (QC) with the Agilent 2100 Bioanalyzer (RIN ≥7.0) whereas only 3 out of 18 macro explants RNA samples from same culture time met this standard (S1: Table) These suggest that it is the quality of extracted RNA that varies, and also that the 260/280 ratio is not an appropriate method to detect the difference between intact and degraded RNAs; electropherograms giving the RIN data are needed.

The mean concentration of RNA extracted from macro explants was slightly lower but not significantly different ($p> 0.05$) from micro explants (mean ± SEM = 703.78 ± 96.4 ng/μl vs 845.8 ± 58.4 ng/μl respectively), although it must be noted that as this depends on the intensity of absorbance at 260nm, the value for the macro explants reflects heavily degraded RNA, rather than intact RNA species (Figure 1).

Morphology of Micro Explants in Low and High Oxygen

Many previous studies [2-6,9] have shown the degeneration and regeneration of the STB layer of placental villi during culture, so we show only sufficient data (Figure 2) to confirm that the tissue morphology in this study was comparable with the earlier work. STB regeneration was thus defined according Siman *et al* [5] as the

progressive degeneration of the original STB layer and its replacement by a newly formed layer, evidenced by gradual thickening of the new STB as the original syncytium is sloughed off and lost. hCG-positive syncytium is clearly visible in the control tissue (Figure 2A), but less positive staining can be seen in micro explants after 1-3 days in culture (Figure 2C-F). More intense staining, consistent with STB regeneration, can be seen after 5 days of culture (Figure 2G-H).

A further point to note is that while syncytium may be seen during the shorter periods of culture, it is often detached from the underlying tissue (black arrows) (Figure 2 C-F). The time 0h controls, and micro explants after 5 days culture in 8% oxygen, show little or no such damage, suggesting that reformation of the syncytium is better at 8% oxygen tension than at 20% oxygen tension.

While our findings are consistent with previous studies showing STB degeneration after 24 hours and regeneration after 5 days or more in culture [2,5,10], they further suggest that high oxygen concentration (20%) decreases the quality of explant regeneration, so that 'standard' culture conditions of 5% CO2: 95% air are not optimal for placental explant culture.

Importantly, while the morphological evaluation in our study was limited to STB, the viability of other cell populations in the villi was not examined directly. Nonetheless, it is interesting to note that the RNA data showed a generic decline in quality in tandem with STB degeneration (Figure 1D), suggesting that the STB viability could be a proxy measure of explant quality if further work confirms that RNA integrity in many if not all cells in the villi is affected during the initial days of culture [18].

Overall, the findings suggest that micro explants cultured at 8% oxygen have the best mRNA quality and tissue structure, and support previous observations that micro explants in low oxygen improves explant viability in long-term culture [1,3-12,19]. Culture of micro explants at a liquid-air interface for 5 days would therefore seem to be required to generate a reliable system in which to study the functions of placental explants.

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Figure Legend

Figure 1: RNA electropherograms and electrophoresis for placental explants after culture

Figures show typical electropherogram images for placental explants: (A) Time 0h placentae (pre-culture) explants; (B) micro explant after 2 days culture in 8% oxygen; (C) macro explant after 2 days culture in 20% oxygen (D & E) placentae in optimal culture at 8% and 20% oxygen respectively for 6 days; ((F) macro explant after 6 days culture. Intact RNA is indicated by clear 18S and 28S rRNA peaks on the electropherograms. Micro explants samples after 6 days culture showed clear and distinct rRNA bands respectively for 18s (2000nt) and 28s (4000nt) rRNA, with negligible low molecular weight (MW) RNA molecules between the internal marker (peak at 25nt) and the 18s rRNA peak. Mean rRNA ratio for the micro explants samples after 6 days was 1.8 (n = 23) with an average RIN = 8.23 (RIN range = 7 – 9.5, n =23), indicating high quality intact total RNA. Typical 2 days macro or micro explant showed high levels of low molecular weight (MW) RNA molecules with negligible (macro) 18S and 28S rRNA peaks. The 6 days macro explant RNA showed no detectable rRNA (F). nt = nucleotide (product size). FU = fluorescence units.

Figure 2: Photomicrographs of placental micro explant demonstrating localisation of β-hCG to the 8 % and 20% Oxygen treated syncytiotrophoblast

Low power (x200) photomicrographs demonstrating localisation of β-hCG to the syncytiotrophoblast of (A) 0h control (Day 0, pre-culture explant); (B) 0h (Day 0, pre-culture explant) immunoglobulin G (IgG) antibody control; and after 1 (C&D), 3 (E&F) and 5 (G&H) days of culture in 20% and 8% oxygen respectively. All tissue samples in the figure were stained in the same staining run. Black arrows indicate syncytial detachment. All samples shown are micro explants. Scale bars: 100 µm. Photomicrographs were taken with a Nikon D5100 digital SLR camera.

Figure 1

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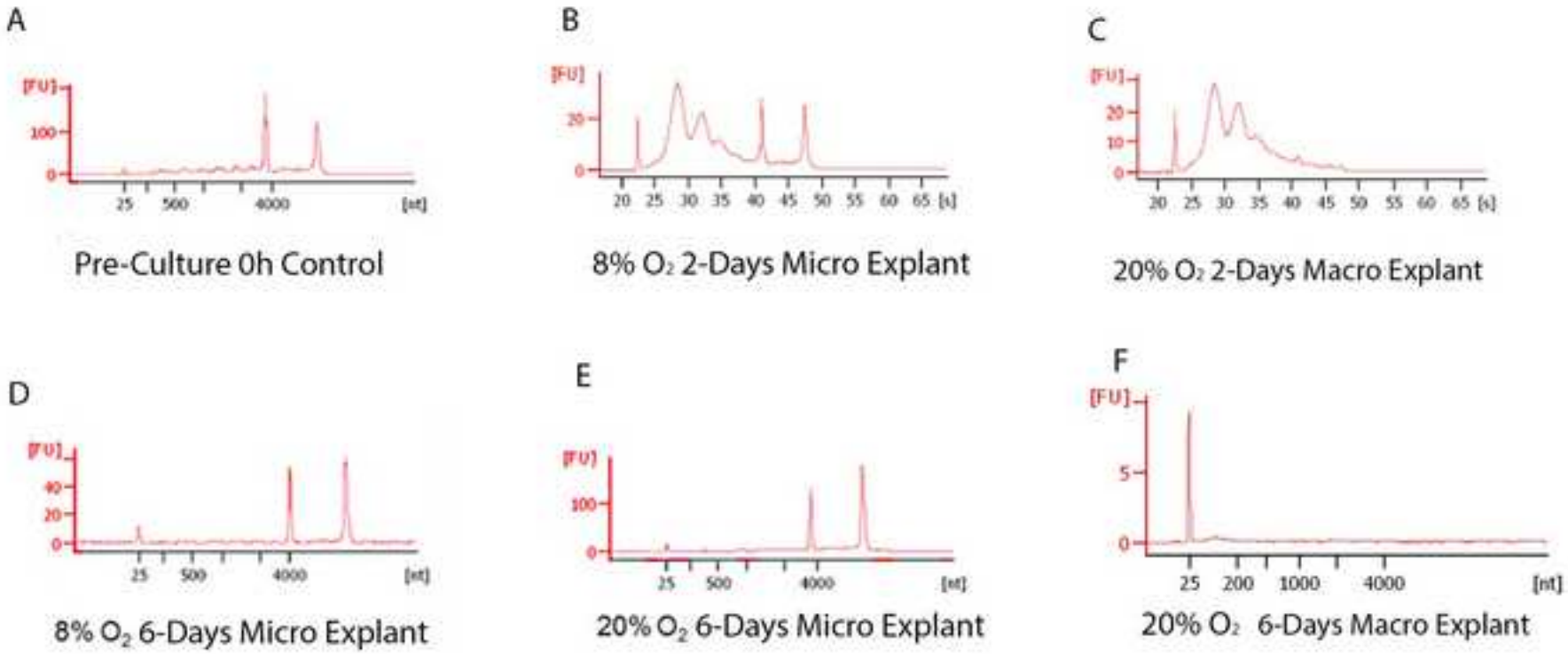

Figure 2

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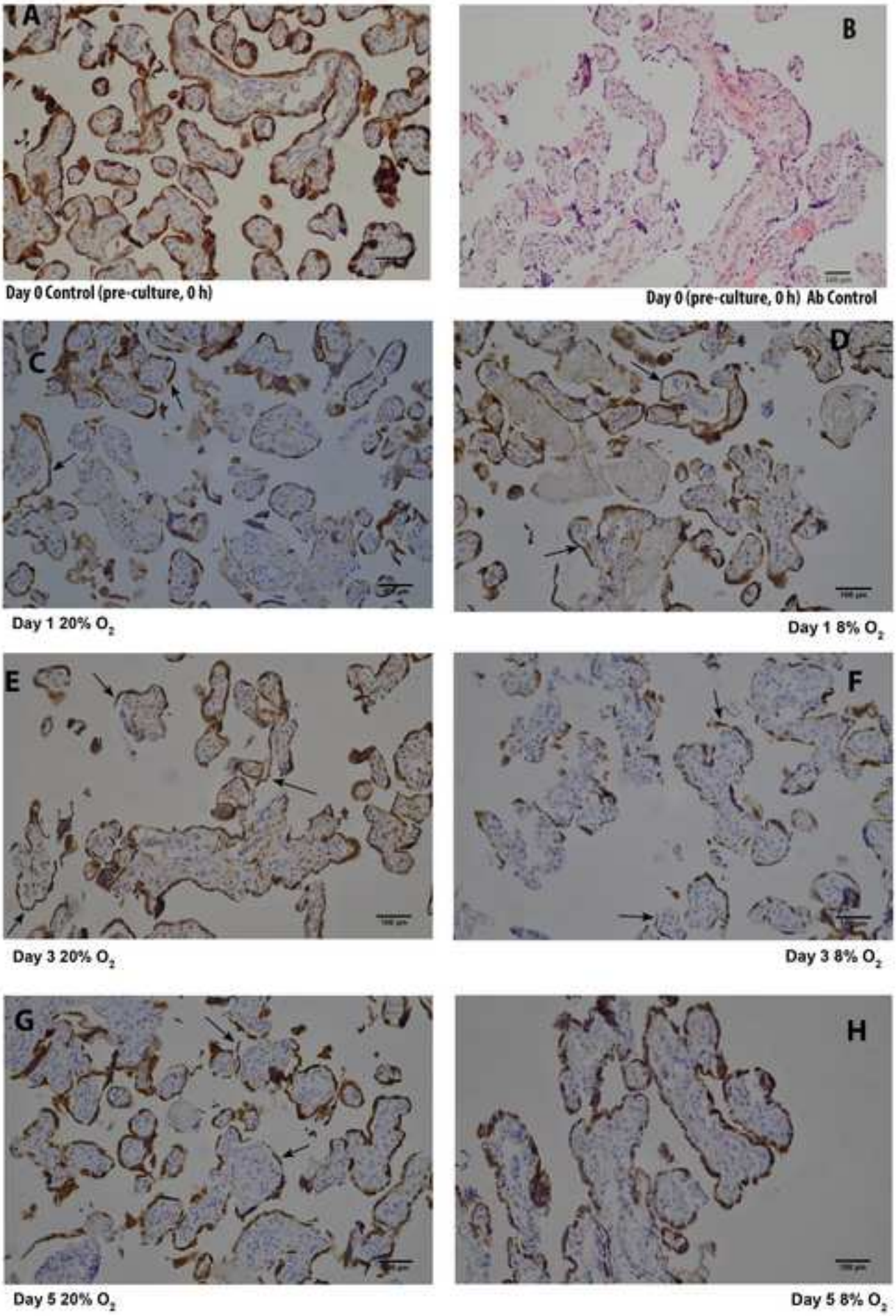

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