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## ORIGINAL ARTICLE

Male Health

# Regeneration of rat corpora cavernosa tissue by transplantation of CD133+ cells derived from human bone marrow and placement of biodegradable gel sponge sheet

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The objective is to develop an easier technique for regenerating corpora cavernosa tissue through transplantation of human bone marrow-derived CD133+ cells into a rat corpora cavernosa defect model. We excised 2 mm × 2 mm squares of the right corpora cavernosa of twenty-three 8-week-old male nude rats. Alginate gel sponge sheets supplemented with $1 \times 10^4$ CD133+ cells were then placed over the excised area of nine rats. Functional and histological evaluations were carried out 8 weeks later. The mean intracavernous pressure/mean arterial pressure ratio for the nine rats (0.34258 ± 0.0831) was significantly higher than that for eight rats with only the excision (0.0580 ± 0.0831, $P = 0.0238$) and similar to that for five rats for which the penis was exposed, and there was no excision (0.37228 ± 0.1051, $P = 0.8266$). Immunohistochemical analysis revealed that the nine fully treated rats had venous sinus-like structures and quantitative reverse transcription polymerase chain reaction analysis of extracts from their alginate gel sponge sheets revealed that the amounts of mRNA encoding the nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) were significantly higher than those for rats treated with alginate gel sheets without cell supplementation (NGF: $P = 0.0309$; VEGF: $P < 0.0001$). These findings show that transplantation of CD133+ cells accelerates functional and histological recovery in the corpora cavernosa defect model.

## INTRODUCTION

Such conditions as congenital anomalies, penile cancer, traumatic penile injury, and some types of severe vasculogenic erectile dysfunction (ED) often require penile reconstruction.[1–3] Various reconstructive procedures have been attempted to achieve functional and cosmetic properties, but these procedures are often limited by a shortage of native penile tissue.[4–7] Even after multi-stage surgery, corporal tissue function is usually not restored.

The corpora cavernosa, a pair of cylindrical bodies that lie along the shaft of the penis, make up the body of the penis and are responsible for ED. These corporal bodies are composed of a sponge-like tissue containing sinusoid blood-filled spaces lined with endothelium and separated by connective tissue septa. Under normal conditions, erection is initiated by the release of nitric oxide from the endothelial cells, which triggers smooth muscle relaxation and inflow of blood into the corporal spaces. Due to the unique tissue structure and complex cellular function within the corpora cavernosa, reconstruction of functional erectile tissue has been especially challenging.[8]

The CD133 epitope is a marker of human hematopoietic and endothelial progenitors.[9] Although its function is not clear, this subpopulation of cells has great potential for the repopulation of bone marrow and differentiation into mature endothelial cells in animal models.[10] In this study, we focused on the bone marrow-derived CD133+ cells, which are very accessible human cells for clinical application. We hypothesized that CD133+ cells transplanted locally into corpora cavernosa defects in animal models induces corpora cavernosa regeneration through vasculogenesis and neurogenesis. We previously reported that transplantation of human CD133+ cells is potentially useful for cavernous nerve reconstruction after operations such as radical prostatectomy.[11]

The aim of this study was to develop an easier technique for regenerating corpora cavernosa including both the tunica albuginea and the endothelial tissue through transplantation of human bone marrow-derived CD133+ cells into a rat corpora cavernosa defect model in combination with application of an alginate gel sponge sheet.

## METHODS

### Animal usage

We used twenty-eight 8-week-old male athymic nude rats (F344/N Jcl rnu/rnu; CLEA Japan, Inc., Tokyo, Japan), each weighing 230–250 g.

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The rats were fed a standard maintenance diet and continuously provided with water. All the animal procedures were approved by the Animal Care and Animal Use Committees of Hiroshima University.

### Alginate gel sponge sheet preparation
Alginate gel sponge sheets (Koyo Sangyo, Tokyo) were washed with 2.5 mmol l$^{-1}$ calcium chloride and 143 mmol l$^{-1}$ sodium chloride and rinsed with distilled water. The sheets were then freeze-dried and sterilized with ethylene oxide gas.

### Cell preparation
CD133$^+$ cells derived from human bone marrow (Lonza, Walkersville, MD, USA) were thawed immediately before use in accordance with the supplier's instructions. They were then contained with 10 ml of DMEM (Dulbecco's Modified Eagle Medium) and centrifuged at 400 *g* for 5 min. Their subsequent resuspension in phosphate-buffered saline (PBS) demonstrated a concentration of $1 \times 10^4$ cells per 40 µl. The prepared cells were simply dropped onto a sterilized sheet and added directly to the penis without any bioreaction or incubation. That is, the graft was not seeded before transplantation.

### Animal treatment and grouping
The rats were anesthetized by administration of pentobarbital sodium (50 mg kg$^{-1}$, intraperitoneally). A lower abdominal midline incision was made from the symphysis pubis to mid-abdomen, and the penis was identified and exposed (Step 1). This step only was applied to five rats (*n* = 5), and they were designated the sham (SH) group. A portion of the corpora cavernosa, measuring about 2 mm × 2 mm, was excised with microscissors (Step 2). Steps 1 and 2 only were applied to eight rats, and they were designated the excision (EX) group. Then, a 2 mm × 2 mm alginate gel sponge sheet was placed, without suturing, over the excised area (Step 3). Steps 1, 2, and 3 only were applied to six rats, and they were designated the alginate (AL) group. Finally, CD133$^+$ cells ($1 \times 10^4$ per 40 µl PBS) were dropped onto a sheet immediately before placement (Step 4). All four steps were applied to nine rats, and they were designated the CD133$^+$ (CD) group (**Figure 1a** and **1b**).

### Erectile function evaluation
The erectile function of all rats was assessed at 16 weeks on the basis of intracavernous pressure (ICP) response elicited by elemental stimulation of the major pelvic ganglion (MPG). After the rats were anesthetized by pentobarbital injection, the MPG was exposed, and 25- and 24-gauge needles were inserted into the corpora cavernosum and the femoral artery, respectively, to monitor ICP and blood pressure. The MPG was electrically stimulated (0.5 mA, 1 ms, 20 Hz, 30 s) using a bipolar hook electrode connected to an electrostimulator, and the pressure signals were digitized and stored in a measuring instrument (Unique Acquisition, version 3, Unique Medical, Tokyo, Japan). The ICP and arterial pressure (AP) during electrostimulation were measured simultaneously, and the ratio of the maximum ICP divided by the mean AP (ICP/MAP) was calculated. The mean ICP/MAP ratio for the 28 rats was then compared among the four groups.

### Morphologic analysis
After erectile function evaluation, the penis of one rat in each group was harvested en block with the surrounding tissue and placed in 10% neutral-buffered formalin. The tissue was embedded in paraffin and cut into 4-µm-thick sections at 20-µm intervals. The sections were then stained with hematoxylin-eosin (H and E) and observed using light microscopy. In addition, the penises were dissected longitudinally and fixed overnight in 4% paraformaldehyde at 4°C.
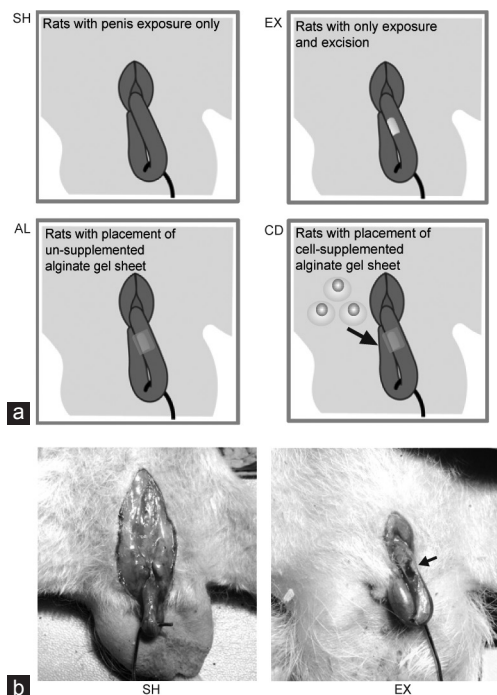


**Figure 1:** (**a**) Illustration of treatment for SH, EX, AL, and CD group. (**b**) Photograph of exposed penis of SH group rat and of treated penis of EX group rat. AL: alginate group; CD: CD133$^+$ group; EX: excision group; SH: sham group.

### Immunohistochemical analysis
A Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis. In brief, the sections were pretreated by microwave treatment in a citrate buffer for 30 min to retrieve antigenicity. Peroxidase activity was blocked with 3% H$_2$O$_2$ methanol for 10 min. The sections were then incubated with a primary antibody for 1 h at room temperature followed by incubation with a secondary antibody for 1 h at room temperature. Immunohistochemistry was completed by using 30-s incubation with a substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. Anti α-smooth muscle actin (α-SMA; 1A4, 1:200, Dako, Carpinteria, CA, USA) was used as a primary antibody to detect vascular smooth muscle. Anti-S-100 protein (polyclonal, 1:200, Dako, Carpinteria, CA, USA) was used as a primary antibody to detect neuron tissue, especially Schwann cells.

### Quantitative reverse transcription polymerase chain reaction analysis
We carried out quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of the rat nerve growth factor (NGF) and rat vascular endothelial growth factor (VEGF) in an alginate gel sheet to evaluate the upregulation of intrinsic growth factors. Four days after, the corpora cavernosa of the rats in the AL and CD groups were excised and an alginate gel sheet was placed over the excised area, the sheets on only the right side of the corpora cavernosa were retrieved from three rats in each group.

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 µg of total RNA was converted into cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences Corp., Piscataway, NJ, USA). qRT-PCR analysis was carried out with an SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was done with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA,

USA). Additional PCR conditions are available on request. The primer sequences for the qRT-PCR are listed in **Table 1**.

We used the $\Delta\Delta$Ct method to normalize the expression of the target genes, assuming approximately equal amplification efficiencies for both target and reference genes. Rat-beta actin (ACTB)-specific PCR products were amplified from the same RNA samples and used as an internal control for PCR normalization. The normalized results are expressed as the mean ratio of rat-ACTB mRNA. Three assays were conducted for each sample.

### Statistical analysis

All values are expressed as mean ± standard error. The Kruskal–Wallis test with a Steel–Dwass *post hoc* test was used to assess the ICP/MAP ratios. Analyses were conducted using the JMP version 10 statistical software package (SAS Institute Inc., Cary, NC, USA). Statistical significance was established at $P < 0.05$.

## RESULTS

### Restoration of erectile function

Representative changes in ICP and MAP and the mean ICP/MAP ratio for each group are shown in **Figure 2a** and **2b**. While no ICP increase elicited by electrical stimulation was observed for the EX group, there were obvious responses for the other groups. The mean ICP/MAP ratio for the CD group (0.34258 ± 0.0831) was significantly higher than that for the EX group (0.0580 ± 0.0831, $P = 0.0238$) and similar to that for the

**Table 1: Characteristics of primers used in this study**

| Gene | Primer pair sequence |
| --- | --- |
| rNGF | F: 5'-AGGGCAGACCCGCAACATC-3' |
| | R: 5'-GGTGGAGGCTGGGTGCTAAAC-3' |
| rVEGF | F: 5'TACCTCCACCATGCCAAGTG-3' |
| | R: 5'-TCTGCTCCCCTTCTGTCGTG-3' |
| rACTB | F: 5'-TCACCGAGCGCGGCT-3' |
| | R: 5'-TAATGTCACGCACGATTTCCC-3' |

rNGF: rat nerve growth factor; rVEGF: rat vascular endothelial growth factor; rACTB: rat beta actin
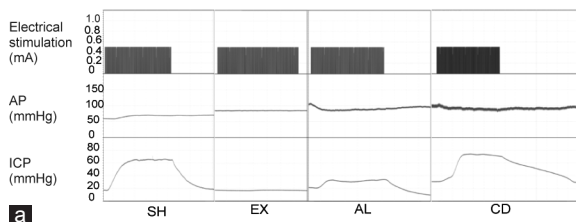


**Figure 2:** Intracavernous pressure (ICP) and arterial pressure (AP) response elicited by MPG electrical stimulation (0.5 mA and 20 Hz for 1 min) 8 weeks later. (**a**) Representative findings. (**b**) Mean ICP/mean arterial pressure (MAP) ratio. Bars show mean value for each group; error lines show standard error. AL: alginate group; CD: CD133⁺ group; EX: excision group; SH: sham group.

SH group (0.37228 ± 0.1051, $P = 0.8266$). For the AL group, although electrical stimulation yielded a certain response (0.25640 ± 0.0960), it did not reach significance ($P = 0.1318$). The mean ICP/MAP ratio was not significantly different between the CD and AL groups ($P = 0.5041$).

### Morphologic analysis

**Figure 3a** and **3b** (upper row) shows micrographs of the longitudinally dissected penises. Morphological analysis revealed that corpora cavernosa tissue had developed in the CD rat, evidenced by its appearance in H and E-stained tissue. The structure of the corpora cavernosa tissue had disappeared in the EX rat penis and had been replaced with fibrous tissue. The structure of the corpora cavernosa was not evident in the AL rat penis although regenerated corpora cavernosa in the horizontal direction was evident. The tissue of regenerated corpora cavernosa was evident in the CD rat penis; the structure resembled that of the SH rat penis as it appeared in H and E-stained tissue.

### Immunohistochemical analysis

**Figure 3a** and **3b** (middle and lower rows) show micrographs of the longitudinally dissected penises prepared for immunohistochemical analysis. The vascular smooth muscles and neuron tissues were stained using anti-$\alpha$-SMA (middle row) and anti-S-100 protein (lower row), respectively.

Vascular smooth muscles were detected in the central part of the corpora cavernosum of the SH rat penis, and neuron tissues were detected in the lateral sides of the venous sinus. Regenerated vascular smooth muscles and neuron tissues were barely detected in the EX rat penis. Vascular smooth muscles and neuron tissues were detected in the
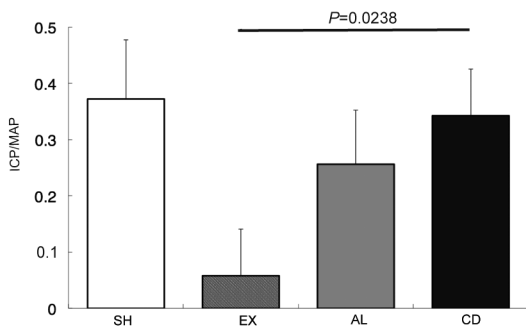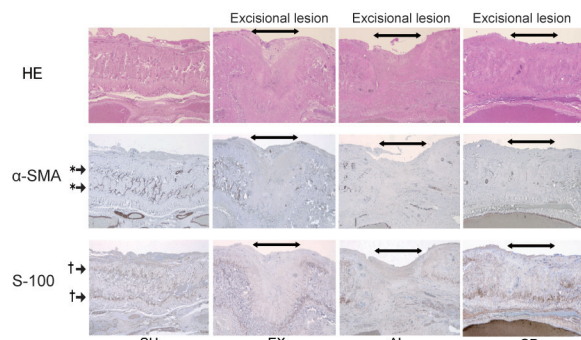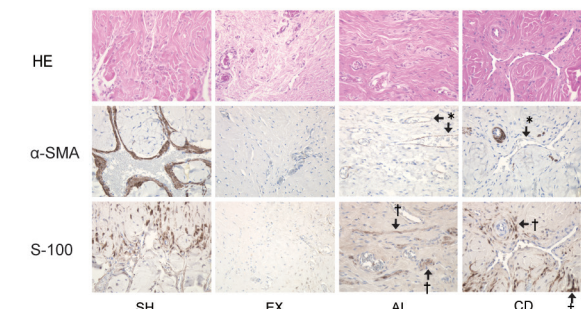




**Figure 3:** Representative H and E staining and immunohistochemical findings of corpora cavernosa tissues at 16 weeks for each group. (**a**) Low-power field (×40); (**b**) High-power field (×400). AL: alginate group; CD: CD133⁺ group; EX: excision group; SH: sham group.

horizontal direction in the AL rat penis, but their constructions differed from those of the SH rat penis. Similarly, vascular smooth muscles were detected in the CD rat penis. Moreover, venous sinus-like structures without vascular smooth muscles were detected and neuron tissues were evident in the lateral sides of the structures. These sinus-like structures were assumed to be immature venous sinuses.

### *VEGF and NGF mRNA expression in alginate gel sponge sheet*
**Figure 4** shows the relative levels of NGF and VEGF mRNA expression for the AL and CD groups. Expression of rat-ACTB was used as an internal control. The qRT-PCR analysis of the extracts from the alginate gel sponge sheets retrieved from the CD group rats revealed that the amounts of mRNA encoding the NGF, and VEGF were significantly higher than those for the AL group (NGF: $P = 0.0309$, VEGF: $P < 0.0001$). These results indicate that upregulation of intrinsic growth factors can be derived from rats.

### DISCUSSION
Erectile dysfunction is a serious medical condition that greatly affects the quality of life in 52% of men aged 40–70 and 22% of men under 40.[12,13] Therefore, current treatments are aimed at increasing smooth muscle relaxation of the corpora cavernosa, but this strategy is ineffective for men with smooth muscle loss due to apoptosis.[14] For example, 56%–59% of diabetic patients do not respond to smooth muscle relaxation agents, such as phosphodiesterase type 5 (PDE5) inhibitors, because of the loss of critical smooth muscle.[15,16]

Many of the recent advances in our understanding of erectile function have arisen from the use of animal models. While the complex nature of human sexual function cannot possibly be replicated fully, these animal models provide a reproducible paradigm enabling the investigation and evaluation of ED. Various animal models have been used over time for both ED and Peyronie's disease. Diabetes mellitus (DM) has long been recognized as a major risk factor in the development of ED with the underlying pathophysiologic mechanism thought to be related to endothelial dysfunction, neurological effects, and small and large blood vessel injury.[17]

The cavernosal tissue in men with ED secondary to DM exhibits impairment in both endothelium-dependent and endothelium-independent smooth muscle relaxant capacity, and this is strongly correlated with the duration of DM.[18] While the abnormalities in vasoreactivity contribute directly to initial vascular injury, persistent vascular smooth muscle-mediated vasoreactivity further exacerbates and promotes the cycle of injury within the diseased tissue.[19]

The majority of studies of diabetes-related ED have been conducted on animals with DM. Animals treated with streptozotocin provide an excellent model of diabetic ED, and this model is well-established as an experimental ED model.[17] However, for patients with severe ED and penile tissue injury, a new type of therapy for recovering the architectural changes in the penis is essential. Novel regenerative study requires the development of a rat corpora cavernosa defect model. The best human model of this situation is the one for Peyronie's disease and plaque surgery, which has been used to create a human model of severe ED.

We focused on CD133^{+} cells, which exist in bone marrow because they have a high potential for tissue regeneration.[11] The CD133^{+} cell fraction is a subpopulation of CD34^{+} cells, which are well characterized as a hematopoietic/endothelial progenitor fraction and are recognized as highly proliferating and early committed stem/progenitor cells, compared with CD34^{+} cells.[20]

We used alginate gel sponge sheets as a scaffold for transplanting CD133^{+} cells. Alginate is a well-established biodegradable polysaccharide that is extracted from brown seaweed. A novel alginate gel dressing cross-linked with covalent bonds has no inhibitory effect on cell proliferation *in vitro* and induces little foreign body reaction when implanted in tissue *in vivo*.[21,22] Alginate gel appears to provide neural formation more effectively than collagen sponge and fibrin glue.[23] Alginate gel provides a favorable environment for neural outgrowth as it prevents the ingrowth of fibrous scar tissue.

Consequently, the ICP during electrical stimulation of the MPG for the rats in the CD group was much higher than that for those in the EX group and it was more marked than that for those in the AL group although the difference did not reach significance. These findings suggest that transplanted human CD133^{+} cells accelerate functional penile development.

This study also showed that the regenerated corpora cavernosa tissue of the rats in the CD group resembled that of those in the SH group histologically. Immunohistochemical analysis revealed venous sinus-like structures without vascular smooth muscle in the rats in the CD group. We assumed that they were immature venous sinuses. Neuron tissues were also detected in the lateral sides of the venous sinus-like structures, similar to those in the rats in the SH group. A previous study demonstrated that cord blood-derived CD133^{+} cells can differentiate into neural cells *in vitro*.[24] CD133^{+} cells have been shown to play an important role in the interaction between neurons and vasculature.[25,26] Our findings thus suggest that venous sinus and neuron structures were detected in the rats in the CD group. While the mechanism underlying the CD133^{+} cells differentiating into the venous sinus and neural structures remain to be established, our finding that transplanted CD133^{+} cells contribute to vasculogenesis and nerve formation through upregulation of endogenous VEGF and NGF in a rat neuron deficient model[11] indicates that a similar mechanism might contribute to regeneration.

The CD133^{+} cells developed the corpora cavernosa through upregulation of endogenous VEGF and NGF, resulting in stimulation of angiogenesis in the host rat, as evidenced by the rat-derived VEGF and NGF expressed in the extracts from the alginate gel sponge sheet and by the finding that the amounts of these growth factors were significantly higher in the cell-treated rats than they were in the alginate sheet alone-treated rats.

Thus, our results strongly suggest that bone marrow-derived CD133^{+} cells have great potential as a therapeutic agent for injured
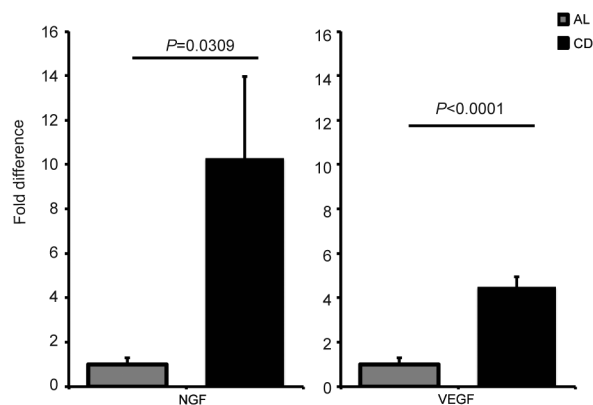


**Figure 4:** Quantitative reverse transcription polymerase chain reaction analysis of nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) in alginate gel sheet between AL and CD groups. Y-axis indicates relative ratio of retrieved alginate gel sheet mRNA to ACTB mRNA. mRNA expression of NGF and VEGF was higher for CD group than for AL group.

corpora cavernosa. There are, however, still several problems to overcome before the clinical application can be tried. One is that the number of CD133^{+} cells available is extremely limited.[27] This problem can be solved by using growth-factor-driven *ex vivo* expansion of CD133^{+} cells.[28] While the methods used in this study are promising, their safety and efficacy must be investigated. To achieve better penile regeneration, we must also further investigate the regeneration mechanism. A limitation of this study is that the results were from an in the xenogeneic animal model. Recently, clinical studies involving CD133^{+} cells implanted in patients undergoing coronary bypass surgery have been reported.[29,30] These studies may prove useful in our field.

## CONCLUSION

Human peripheral blood-derived CD133^{+} cells have the functional neurogenic potential for penile regeneration; however, the mechanism accounting for functional recovery is still unclear. Our results show that the transplantation of human-circulating CD133^{+} cells may help in establishing a regenerative environment like a "biological bridge" and that the proposed procedure is a promising treatment for penile regeneration in a clinical setting. We believe CD133^{+} cell transplantation is applicable to the treatment of penile regeneration and severe ED, which is not responsive to PDE5 inhibitors.

## AUTHOR CONTRIBUTIONS

SI designed the study, analyzed the data and wrote the paper. KM and KS participated in the evaluation of erectile dysfunction. SS and KS performed morphologic analysis, immunohistochemical analysis, and quantitative reverse transcription polymerase chain reaction. JT supervised the project and wrote the paper. AM and WY supervised the project.

## COMPETING INTERESTS

All authors declare no competing interests.

## ACKNOWLEDGMENTS

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