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Rapid Self-Assembly of Tubular Arterial Media Layer from Smooth Muscle Cells in Transient Fibrin Gel

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Abstract

Background: Tissue engineered blood vessels could address the large clinical need for small caliber vascular grafts. Self-assembly approaches that employ transient scaffolds to form tissues from only cells and secreted matrix could form completely autologous vascular grafts that rapidly remodel and integrate with host tissue in vivo. The objective of this study was to develop a simple and rapid method to self-assemble vascular cells into vascular grafts.

Hypothesis: We hypothesized that entrapment in rapidly degrading fibrin gels could facilitate self-assembly of vascular smooth muscle cells into a tubular tissue comprised mainly of SMCs and secreted matrix.

Methods: Baboon SMCs were entrapped in fibrin around a silicone tube and cultured for 14 days without fibrinolysis inhibitor. Spontaneous delamination from the inner tube allowed for simple isolation of constructs with forceps.

Results: Engineered tissues are tubular, handleable, and highly cellular, with substantial collagen deposition. Fibrin is largely degraded within 14 days. Tensile elastic modulus of ring segments is 36.2 kPa and 1.60 MPa for the toe and heel regions of the stress-strain relation, respectively.

Conclusion: Fibrin entrapment without fibrinolysis inhibitor can facilitate rapid self-assembly of SMCs into tubular tissues. Future work will focus on mechanical conditioning and co-culture with vascular endothelial cells to improve mechanical strength and impart antithrombogenicity.

Keywords: Tissue Engineering; Self-assembly; Blood vessel; Artery; Smooth muscle; Fibrin gel

Abbreviations: SMC: Smooth Muscle Cell; EML: Engineered Medial Layer; PBS: Phosphate Buffered Saline; H&E: Hematoxalin and Eosin; DAPI: 4', 6-Diamidino-2-Phenylindole; OCT: Optimal Cutting Temperature Compound

Introduction

Cardiovascular disease is the leading cause of death in the United States, of which coronary artery disease claims more lives than any other [1]. When angioplasty and stenting is not possible, coronary artery disease is treated by bypass surgery, for which vessel autografts remain the standard of care [2]. However, 1 in 3 patients lack suitable autografts due to vascular disease or previous harvest [3], and autografting requires additional surgery and causes donor site morbidity [4,5]. Tissue engineered arteries produced from autologous cells can potentially replace autografts for bypass grafting. However, there are still major challenges including maintaining antithrombogenicity, matching arterial mechanical properties, and reducing production cost and time [4,5].

Engineering arteries entirely from autologous cells and secreted matrix, often referred to as "self-assembly", is an attractive approach because it avoids potential interference of scaffold materials with the mechanical and biological properties of the constructed tissue [6,7]. Recent advances in cell sheet engineering [8] and bioprinting [6,9] attest to the technical feasibility of this approach, but clinical feasibility could be greatly improved by reducing culture time and equipment costs. Self-assembly of vascular cells by seeding into rapidly degrading scaffolds [10] could improve translatability by employing inexpensive seeding techniques and using simple equipment.

Vascular smooth muscle cells (SMC) are the primary cellular component of the vascular tunica media, which is crucial for arterial mechanical properties and vascular tone [11-13]. Fibrin gel can be rapidly degraded *in vitro*, but typically fibrinolysis inhibitors are used to prevent fibrin degradation in fibrin-based tissue engineering [14-16]. We hypothesized that SMCs entrapped in fibrin gel without fibrinolysis inhibitor could self-assemble into tubular tissues comprised entirely of SMCs and the matrix they produce. These engineered media layers (EMLs) could serve as the basis for a completely autologous arterial graft that could rapidly remodel and integrate with host tissue *in vivo*. Here we report for the first time the rapid assembly of a tubular EML from fibrin entrapped SMCs without the use of a fibrinolysis inhibitor.

Methods

Cell isolation and culture

Baboon SMCs were obtained from fresh carotid arteries of 17–20 kg juvenile male baboons as described previously [17]. Briefly, arteries were denuded of connective tissue and adventitia, and endothelial cells were removed by filling the artery lumen with 600 U/mL collagenase for 5 min, then rinsing. Arteries were subsequently digested in 300 U/mL collagenase in SMC culture medium for 10 hours to release SMCs. SMCs were plated on tissue culture plastic with medium changes every

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48 h. SMC culture medium consisted of MCDB 131 with 10% fetal bovine serum (FBS), 1.0% L-glutamine, and 50 mcg/ml ascorbic acid, with 100 IU/ml penicillin, 100 mcg/ml streptomycin, and 0.25 mcg/ml amphotericin B as antibiotics and antimycotic.

EML fabrication and culture

EMLs were fabricated as diagrammed in Figure 1 and shown in Figure 2. Passage 4 SMCs were used in all EMLs except those shown in Figure 1 and 2D, which were Passage 22. Fabrication and culture consisted of 4 steps:

Fibrin gel entrapment: 1 x 10^6 SMCs were suspended in 4 mg/mL bovine fibrinogen and 10 U/mL thrombin in phosphate buffered saline (PBS) and injected into the tubular space between an outer silicone mold (3.175 mm inner diameter) and an inner silicone tube (1.96 mm outer diameter).

Isolation of silicone-supported tubular gel: After gelation, fibrin-entrapped SMCs and the inner silicone tube were extruded from the mold with a plunger.

Culture: Gels were suspended in well plates by wire and cultured for 14 days without fibrinolysis inhibitor.

EML isolation: EMLs were isolated from the inner silicone tube. Spontaneous delamination of EMLs from the silicone allows for simple isolation with forceps (Figure 1 - 4A, 4B). Delamination begins at 10-14 days culture. Data is presented from EMLs isolated on day 14. Longitudinal retraction occurs concurrent with delamination (Figure 1 - 4A, 4B). To determine if retraction can be prevented, an EML was secured to the inner silicone tube on day 13 by tying both ends and the center with 4-0 polypropylene suture (Ethicon, Somerville, NJ).

Histology and immunofluorescence

EMLs were embedded in optimum cutting temperature compound (OCT), frozen at -80°C, and sectioned to 5 µm. Axial cross-sections were stained using hematoxylin and eosin (H&E), Masson"s trichrome, or by immunofluorescence. Immunofluorescence samples were blocked in 5% normal goat serum (NGS), then incubated in with primary antibody at 37°C for 1 h. Slides were rinsed in 1% NGS and incubated in secondary antibodies at 37°C for 1 h. Primary and secondary antibodies are listed in Table 1. Slides were co-stained with 4',6-diamidino-2-phenylindole (DAPI) nuclear using a mounting medium containing 1.5 mcg/mL DAPI.

Mechanical properties

To determine tensile elastic modulus and ultimate tensile stress, EMLs were analyzed using a material testing system (Insight, MTS Systems, Eden Prairie, MN). Ring segments (2 cm length) were mounted around two steel pins (Figure 4 inset) and loaded uniaxially at a rate of 0.01 mm/s in a 37°C PBS bath (Bionix EnviroBath, MTS Systems). Segments were first precycled between 10 and 40% strain until reproducible stress-strain curves were obtained. Strain to failure was then performed. Circumferential ("ring") strain was calculated from camera images with Image J software (NIH) using the following equations [18].

Ring strain:

$$\varepsilon_{ring} \frac{C_{lumen} - C_{lumen}(0)}{C_{lumen}(0)}$$

Where C_{lumen} is the inner circumference, and $C_{lumen}(0)$ is the unloaded luminal circumference

Luminal circumference: At strains where ring segment is ellipse-shaped:

$$C_{lumen} = \pi(a+b)(1+\frac{3h}{10+\sqrt{4-3h}})$$

Where 'a' and 'b' are semi minor and semi major ellipse dimensions, respectively, and

$$h = \left(\frac{a-b}{a+b}\right)^2$$

At strains where ring segment is pill-shaped:

$$C_{lumen} = 2\left(b - D_{pin}\right) + \pi * D_{pin}$$

Where D_{pin} is the diameter of the mounting pins. Stress was calculated from:

$$\sigma = \frac{F}{A}$$

Where F is the axial load, and A is the loaded cross sectional area, given by:

$$A = 2(t_{min})\ (L)$$

Primary and secondary antibodies for immunofluorescence. Protein	Primary	Secondary
Collagen I	Rabbit polyclonal anti-human collagen I	Goat polyclonal anti – rabbit, Alexa Fluor 594 conjugated
Collagen III	Rabbit polyclonal anti-human collagen III	Goat polyclonal anti – rabbit, Alexa Fluor 594 conjugated
α-Smooth muscle actin (SMA)	Mouse monoclonal anti-human α-SMA, FITC conjugated	N/A
Calponin	Mouse monoclonal anti-human calponin	Goat polyclonal anti – mouse, Alexa Fluor 594 conjugated
Elastin	Rabbit polyclonal anti-human elastin	Goat polyclonal anti – rabbit, Alexa Fluor 594 conjugated
Myosin heavy chain	Rabbit polyclonal anti-human myosin heavy chain	Goat polyclonal anti – rabbit, Alexa Fluor 594 conjugated

Table 1:

	Ultimate Tensile Stress (kPa)	Toe Modulus (kPa)	Heel Modulus (kPa)
EML	667.3	36.22	1593
Rat Artery	2158	101.0	6342

Table 2: Ultimate Tensile Stress and Tensile Elastic Moduli Ultimate Tensile Stress (kPa).

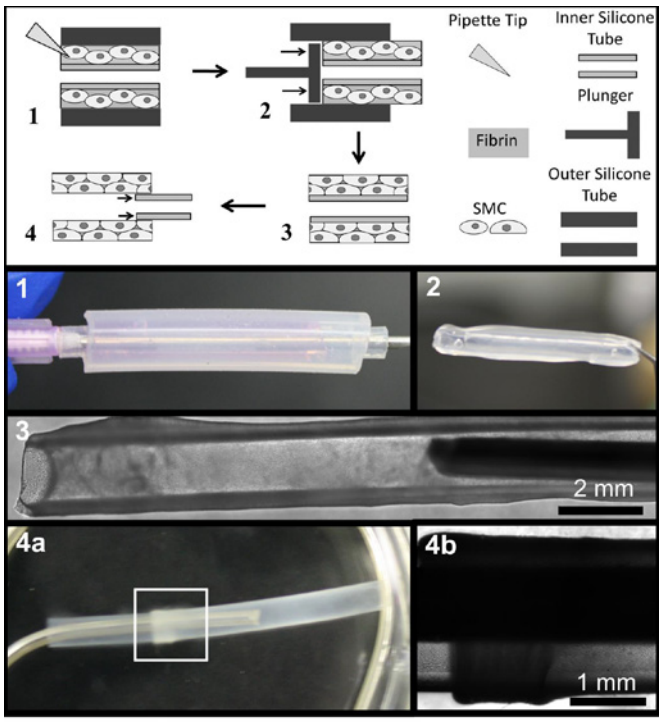

Figure 1: EML fabrication. Longitudinal view of EML fabrication steps. **1.** SMCs are entrapped in fibrin between an outer silicone mold and an inner silicone tube. **2.** Fibrin-entrapped SMCs and inner silicone tube were extruded from the mold with a plunger. **3.** SMCs are cultured in fibrin for 14 days to form EMLs. **4.** EMLs are isolated from the inner silicone tube using forceps. Spontaneous delamination from the inner silicone tube allows for simple isolation of EMLs with forceps **(4a, b)**. Longitudinal retraction occurs concurrent with delamination (**4a, b**).

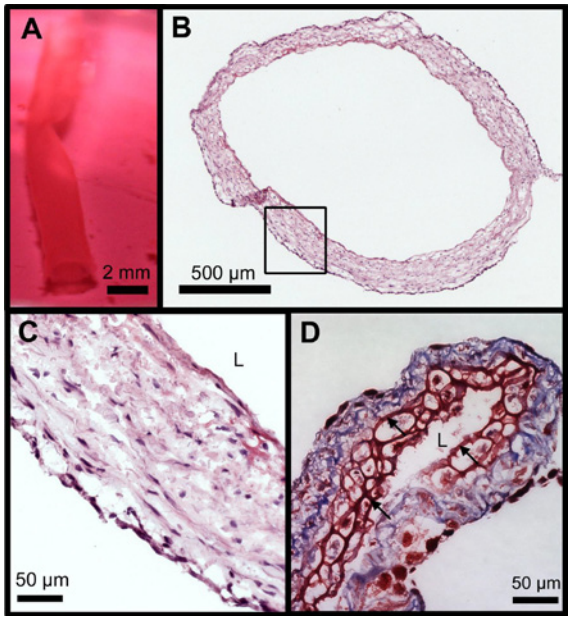

Figure 2: Construct morphology and histology. **A.** Macroscopic image of construct after 14 days culture. **B.** H&E staining of axial cross-section. Boxed region shown in **C. D.** Masson's Trichrome stain of axial cross-section. Collagen stains blue, residual fibrin stains red (arrows). "L" indicates lumen of the construct.

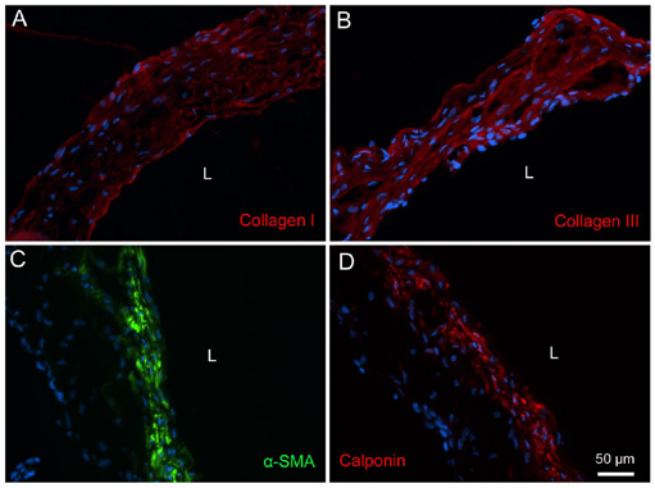

Figure 3: Immunofluorescent staining of matrix proteins and SMC markers. A. Collagen I (red) **B.** Collagen III (red). **C.** α-SMA (green). **D.** Calponin (red). Nuclei co-stained with DAPI (blue).

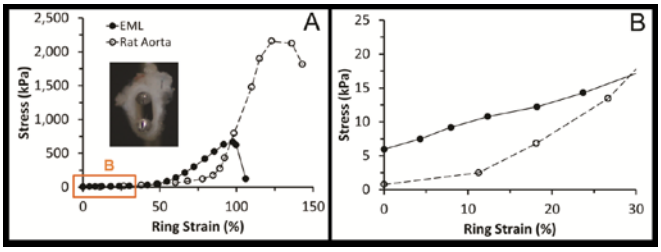

Figure 4: Mechanical properties. A. Stress-strain curves for EML and rat artery. Tissue rings were mounted on steel wires in a material testing system (inset) and strained axially to failure. **B.** Boxed region from **A** showing the toe (shallow) region of the stress-strain curve. Elastic moduli for the toe (shallow) and heel (steep) regions listed in **Table 2**.

Where t_{min} is the minimum wall thickness and L is the longitudinal length of the ring segment.

Ultimate tensile stress was taken to be the peak of the stress-strain relation. Tensile elastic modulus was calculated from:

$$E = \frac{\Delta\sigma}{\Delta\varepsilon_{Ring}}$$

Tensile moduli were calculated from linear segments of both the initial shallow region ("toe region") and the steep region ("heel region") of stress-strain curves.

Results

Morphology and histological characterization

EMLs are tubular and translucent, with 2 mm inner diameter and 1.5 cm length (Figure 2A). EMLs contain cells throughout the whole construct (Figure 2B,C). Fibrin is largely degraded within 14 days. The residual fibrin is visible as a red stain in Masson's trichrome and is present mostly near the EML lumen (Figure 2D). Since the lumen was attached to the silicone mandrel, this is possibly a consequence of reduced mass transfer to luminal SMCs. EMLs contain substantial amounts of evenly distributed collagen I and III (Figure 3A,B), but do not contain elastin. SMCs express α-SMA (pan-SMC marker) and

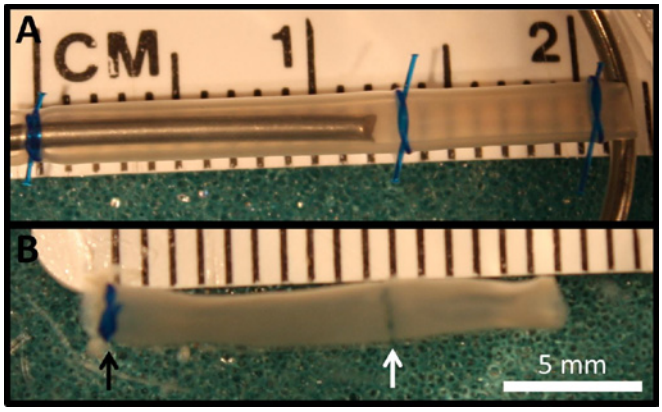

Figure 5: Prevention of EML Retraction. A. Polypropylene suture (blue) applied on day 13 prevents retraction. Image taken on day 21. **B.** Same EML isolated on day 21. The leftmost suture is still present (black arrow). The middle suture was removed, pulling off some tissue in the process (white arrow). The rightmost suture was removed with no damage to the EML.

calponin (mid-stage contractile differentiation marker; Figure 3C,D), but are not fully contractile (stain negative for myosin heavy chain). Interestingly, α-SMA and calponin are expressed only in SMCs near the lumen of EMLs, where mass transfer is likely limited by the inner silicone tube. This finding was unexpected, as vascular smooth muscle is normally in close proximity to the vessel lumen or vaso vasorum, where mass transfer is high.

Mechanical properties

The stress-strain relation for EMLs is shown in Figure 4. Stress vs. strain curves for constructs share a similar shape with rat arteries, but exhibit a shorter "toe" (shallow) region. Ultimate tensile stress and tensile elastic moduli for both the "toe" and "heel" (steep) regions are smaller than those of rat arteries, but are within 1 order of magnitude. (Table 2).

Prevention of EML retraction

EML culture is extended to 21 days by securing the EML to the inner silicone tube with suture on day 13 (Figure 5A). EML can be isolated after loosening or removing suture (Figure 5B).

Discussion

Self-assembled engineered tissues have many theoretical advantages over traditional scaffold based engineered tissues. Self-assembly should minimize the effects of scaffold topography [19-21] and mechanical properties [21,22] on the phenotypes of seeded cells. Importantly, self-assembled tissues avoid scaffold biocompatibility concerns including immunogenicity, foreign body response, disease transmission, and infection [6,7] because they are devoid of scaffold material and completely autologous at the time of implantation. Ideally, self-assembled tissues should also have the ability to completely remodel, self-renew, and respond to environmental signals immediately after implantation because they are comprised completely of autologous cells and matrix [7]. Self-assembled tissues could also achieve higher cell density *in vitro*, since there will ultimately be no scaffold material displacing cells [6].

Despite its theoretical advantages, the clinical translatability of self-assembly in vascular tissue engineering is limited for several reasons. Firstly, complex fabrication and/or long maturation times increase production costs. To self-assemble tissues into tubular structures, fabrication often requires expensive equipment (e.g. bioprinter [6,9]) or multiple manual steps (e.g. isolation of cell sheets and rolling around a mandrel [7,8,23]). Long maturation times are characteristic of self-assembly [7,8,23] because the final tissue relies only on cells and their secreted matrix for mechanical support. An additional disadvantage of long maturation time is that it prevents tissues from being available to patients with more urgent need for implantation [24,25]. Currently the only self-assembled vascular graft to be used clinically is Cytograft's Lifeline™ graft, which is effective as a hemodialysis shunt [23], but the high cost ($15,000 per graft) and 9 month lead time may limit its use in other applications [24,25]. Our approach avoids these disadvantages by offering simple fabrication and short culture time. Cell harvesting and *in vitro* expansion is another limitation for self-assembly as well as for many scaffold based tissue engineering approaches because of donor site morbidity and additional culture time. However, we believe that the potential of self-assembled tissues to immediately integrate, biologically function, and rapidly remodel supersedes the drawbacks of cell harvesting.

We chose SMCs to produce EMLs because SMCs and their secreted extracellular matrix comprise the tunica media of arteries, which is responsible for the majority of artery mechanical properties and for vascular tone [11-13]. SMCs produce a tightly organized matrix comprised largely of both collagen and elastin [13]. Our EMLs produced both collagen I and collagen III (Figure 3A, B), collagens found in arteries and other tissues [13]. Elastin in particular is crucial for arterial compliance [13,25], without which grafts fail by restenosis due to neointimal hyperplasia [26-28] or dilation due to fatigue of collagen fibers [25]. Recent work in our laboratory has demonstrated that SMCs can produce substantial amounts of cross-linked elastin in vitro when cultured on an elastomeric material and subjected to mechanical conditioning [29]. Previous work has also demonstrated that SMCs can produce elastin in fibrin disks *in vitro* [30], and that various bioactive molecules can be added to culture medium to improve elastin production [31-35]. These results suggest that, although our constructs did not yet produce elastin, altering culture conditions (e.g. culture medium or mechanical conditioning) could likely improve elastin production.

Fibrin gel has multiple qualities which make it attractive for self-assembly of SMCs. Firstly, fibrin is rapidly degraded by cells in vitro by plasminogen [30], allowing it to be quickly replaced with more cells and extracellular matrix proteins. Additionally, fibrin degradation products are bioactive and have been shown to promote SMC proliferation and collagen and elastin production *in vitro* [30]. Cells also can be distributed uniformly throughout the gel prior to gelation, enabling high cellularity and seeding efficiency. Lastly, fibrin can be produced from autologous plasma [15], thereby enabling the production of completely autologous vascular conduits.

This is the first report of self-assembly of SMCs in fibrin without the use of fibrinolysis inhibitor. Previous work by others has produced conduits from SMCs in fibrin *in vitro,* but constructs retain substantial fibrin because fibrinolysis inhibitors were used [36-38]. The purpose of fibrinolysis inhibitors in tissue engineering is to maintain the structural integrity of engineered constructs [36]. However, native vessels do not contain fibrin except in post-injury and pathologic conditions, suggesting its complete degradation *in vitro* could produce a more biomimetic graft. Recently, Syedain et al. [10] demonstrated that fibroblasts entrapped in fibrin without fibrinolysis inhibitor rapidly

degrade the majority of fibrin in vitro and form strong, compliant conduits [10]. Our results similarly demonstrate that SMCs in tubular fibrin gels without fibrinolysis inhibitor rapidly degrade the fibrin in vitro (Figure 2D) and can retain a tubular morphology with a little residual fibrin (Figure 2A). This fibrin will likely be degraded upon longer culture time, especially if mechanical conditioning is applied.

The spontaneous retraction of EMLs from their inner silicone tube at 10-14 days culture allows simple isolation with forceps (Figure 1-4a,b). Our preliminary studies indicated that SMCs attach poorly to silicone. Consequently, fibrin gel should be largely responsible for the anchoring of constructs to the inner silicone tube. This anchoring was likely weakened by progressive fibrin degradation. Additionally, compaction of fibrin gels due to cell tractional forces should place the EMLs under both circumferential and longitudinal tension [39-41]. Although circumferential tension stabilizes the construct onto the inner tubes, longitudinal tension should produce shearing forces which destabilize the construct's original attachments to the tube. We believe that the combined effect of fibrin degradation and longitudinal gel compaction resulted in the observed spontaneous retraction. While retraction is helpful for isolation, delaying retraction could extend the duration of EML culture on the silicone tube to allow additional maturation. We demonstrated that EML culture can be extended to 21 days by fixing EMLs to the silicone tube with suture (Figure 5). Retraction could also be delayed by doping fibrin with collagen fibers to stabilize the fibrin matrix [39].

EML mechanical properties must be improved for implantation to be possible. Additionally, SMCs did not express myosin heavy chain, a marker of the fully contractile phenotype found *in vivo*, although its expression is seldom reported in engineered vessels in vitro [12,42-44]. To improve mechanical properties and promote SMC contractile phenotypes, we plan to subject constructs to dynamic mechanical conditioning. We can apply biomimetic distension as early as day 0 by pressurizing the inner silicone tube with a peristaltic pump. Cyclic radial strain alone has been shown to improve EML strength through matrix remodeling and altered matrix production [45-47]. Once strong enough to be pressurized themselves, constructs will be cannulated to a pulsatile flow bioreactor, with pressure gradually being increased to physiologic ranges. This approach has also been shown previously to further improve mechanical strength [12,48-50] and vasoresponsiveness [12]. Additional future work will involve endothelialization of EMLs to impart vessel antithrombogenicity.

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