Effects of the matricellular protein SPARC on human retinal pigment epithelial cell behavior

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Purpose: To determine the effects of the matricellular protein SPARC (Secreted Protein, Acidic and Rich in Cysteine) on human retinal pigment epithelial (HRPE) cell behavior in vitro.

Methods: Proliferation and migration assays were performed on HRPE cells exposed to various concentrations of SPARC. Additionally, HRPE cells were seeded on top of collagen matrices (a 2D model of the retinal scarring disorder known as proliferative vitreoretinopathy or PVR) and were exposed to SPARC over a 7 day period. Changes in matrix contraction were recorded.

Results: HRPE cell proliferation was significantly inhibited at 1 and 10 µg/ml SPARC ($p<0.01$). SPARC protein did not stimulate HRPE cell migration at any of the concentrations used. SPARC did not significantly affect fibronectin-induced HRPE cell migration at SPARC concentrations up to 10 µg/ml. HRPE cell-seeded collagen matrices demonstrated a significant inhibition of matrix contraction by 1 and 10 µg/ml SPARC (t-test; $p<0.02$ and 0.001, respectively) compared to controls.

Conclusions: SPARC protein has anti-proliferative effects on HRPE cells in vitro. In addition, SPARC appears to have an inhibitory effect on HRPE-mediated contraction of 2D collagen matrices. These results are consistent with an important role for SPARC in modulating cell behavior in vitro and may indicate a role for SPARC in modifying HRPE cell activities during the development of PVR and other proliferative retinal diseases.

Matricellular proteins are a non-homologous group of extracellular regulatory proteins that mediate cell-matrix interactions [1]. The most well-characterized matricellular proteins include the tenascins, thrombospondins-1 and -2 and SPARC (Secreted Protein, Acidic and Rich in Cysteine). These proteins are secreted by numerous cell types and have recently been grouped together as anti-adhesive glycoproteins [2-4]. They are believed to exert their anti-adhesive effects on many cell types, resulting in cell rounding and partial detachment from the cell substrata. Furthermore, they are all expressed by tissues undergoing morphogenesis and they modulate the attachment and spreading of various cell types on extracellular matrices [3,4]. Although matricellular proteins can be associated with structural elements including collagen fibrils or basement membranes, they generally do not appear to contribute to their structural integrity.

SPARC is a 43 kD matricellular protein which is also known as BM-40 or osteonectin [5,6]. The precise functions of SPARC are unclear, but it appears to counter cellular adhesion when it is in solution or incorporated into a substrate [2]. This anti-adhesive property may cause loss of focal adhesion in strongly adherent cells and, if exposure to SPARC is prolonged, cell rounding [2]. Changes in cell adhesion mediated by SPARC are thought to influence a number of cellular activities including proliferation, migration, shape, differentia-tion, and possibly cell-mediated matrix contraction [7-9]. These cellular activities are fundamental to major biological processes such as embryogenesis, tumorigenesis and wound repair.

Proliferative vitreoretinopathy (PVR) is an anomalous wound healing response to rhegmatogenous retinal detachment that is characterized by the formation of contractile, fibrocellular membranes on the surfaces of the neuroretina [10,11]. PVR membranes typically contain a predominance of retinal pigment epithelial (RPE) cells and it is thought that development of the membranes is critically dependent on the proliferative, migratory and matrix-remodeling behavior of these cells. It has been previously established that several matricellular proteins are secreted by fibroblastic RPE cells and may therefore have a role in the development and contraction of PVR membranes [12-15]. We have demonstrated that RPE cells in PVR membranes are co-distributed with SPARC [16,17] and, moreover, that human RPE (HRPE) cells synthesize and secrete SPARC in vitro [15]. Furthermore, we have shown that SPARC is counter-adhesive to HRPE cells in culture [17]. Therefore, we hypothesized that SPARC is likely to influence key adhesion-dependent PVR-related RPE cell activities. Hence we investigated the effects of exogenous SPARC on HRPE cell proliferation, migration, and HRPE cell-mediated matrix contraction.

METHODS

Cell culture: HRPE cells were obtained using previously established methods [18,19] from three different donors (a male age 29 years, a female aged 1 year, and a male aged 10 months). The cultures were maintained at 37 °C, 5% CO_2 in Hams F10

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medium (Life Technologies, Paisley, Scotland, UK), containing 20% fetal calf serum (FCS; Harlan Sera Laboratory Ltd., Crawley Down, UK), 1% each of glutamine, fungizone (Life Technologies), and 1% each glucose, penicillin-streptomycin (Sigma, Poole, UK). Cells were grown to confluence in 150 cm² flasks before passaging with 0.25% trypsin and 0.02% EDTA in sterile PBS, for 2 min. Detached cells were mixed with serum and centrifuged for 10 min at 800 rpm. Positive cytokeratin staining was used to confirm the purity of all HRPE cell cultures [20]. Cells were employed in the experiments at between sixth and eight passage.

Proliferation assay: At confluence, HRPE cells were trypsinized. The enzyme was neutralized with serum and the cells were precipitated by centrifugation for 10 min. Cells were counted and the volume adjusted to give a final density of 2000 cells per well of a 96 well plate (Nalge Nunc Int., Naperville, IL, USA). Plates were incubated overnight in complete media (F10 containing 20% FCS) at 37 °C in a 5% CO_2 atmosphere and the media was aspirated after 24 h. Wells were rinsed twice with serum-free media and 200 µl of 0.1, 1 and 10 µg/ml of human platelet SPARC (<3.5 E.U. endotoxin per milligram per lot; Haematologic Technologies, VT, USA) was diluted in F10/20% FCS and added to each well. Plates were then returned to the incubator for 4 days. Thereafter, a working solution of the MTS assay (Promega, WI, USA) was added to each well and the plate was returned to the incubator for 3 h. The MTS assay is a colorimetric method for the determination of viable cells in proliferation, which is composed of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron-coupling agent (phenazine methosulfate; PMS). The MTS reagent is converted by dehydrogenase enzymes in metabolically active cells into a formazan that is soluble in tissue culture medium. The absorbance of the formazan in each well was read at 525 nm using a microtiter plate reader, which was directly proportional to the number of living cells in culture. Procedural controls included cell-free wells, wells containing serum-free F10 media, F10/2% FCS and F10/20% FCS (maximum proliferation). Each dilution under test was performed in quadruplicate and the experiment was performed (with different SPARC lots) at least three times.

Migration assay: The migratory behavior of HRPE cells was assessed in a 48-well microchemoattraction (Boyden) chamber (Neuroprobe Ltd., Maryland, USA) as developed by Falk and coworkers [21]. The apparatus consists of upper and lower wells separated by a silicone gasket and a semi-permeable polycarbonate membrane composed of pores 10 µm in diameter (Nucleopore Inc., USA), and pre-coated with the wetting agent polyvinylpyrolidine (PVP) and denatured porcine gelatin (300 bloom, Sigma, Dorset, UK). Cultured HRPE cells were detached from flasks by trypsinization (as described above) and were centrifuged for 10 min. Cells were counted and diluted in serum-free media to give a final cell density in each upper well of 4x10⁴ cells. The test substance SPARC was diluted to 0.1, 1 and 10 µg/ml in serum-free F10 media and was pipetted into the lower wells of the chamber. Addi-

tionally, the same concentrations of SPARC in serum-free media were each combined with a standard 10 µg/ml of the HRPE chemoattractant human fibronectin [22] (FN; Sigma) and added to wells, in order to investigate the effects of SPARC on FN-induced HRPE cell migration. The chamber was placed in a humidified 37 °C incubator with an atmosphere of 5% CO_2 for 4 h. Thereafter, the membrane was fixed for 30 s in 100% ethanol, counterstained with haematoxylin (Shandon Scientific Ltd., Cheshire, UK) for 30 min and was mounted onto a glass slide with Aquaperm™ (Immunon, Pittsburgh, USA). Procedural positive controls were wells containing human FN alone [22] (10 µg/ml), and negative controls were included of FN-free media. Each dilution under test was performed in quadruplicate and the experiment was performed at least three times. Twenty random fields of cells that had migrated to the underside of the membrane were counted per well.

Preparation of collagen matrices: Collagen matrices were prepared following an established method [23]. Briefly, collagen matrices were prepared from a stock solution of rat tail type I collagen (Sigma) at 5 mg/ml in 0.1% acetic acid (Sigma). A collagen matrix with a final concentration of 1.5 mg/ml for each assay was prepared with 2.1 ml of concentrated culture medium (15 ml 10X MEM, 35 ml distilled water, 1.5 ml penicillin/streptomycin, 1.5 ml glutamine, 1.5 ml fungizone and 3 ml of 7.5% sodium bicarbonate) and 0.9 ml newborn calf serum (NCS) was added to 3.6 ml collagen solution at 4 °C. Matrix contraction studies were performed in 24-well plates (Corning, NY, USA), in which each well received 0.4 ml of the final mixture and was allowed to set at 37 °C for 30 min. Following polymerization, 1x10⁴ HRPE cells were seeded onto each matrix and were allowed to settle for 1 h at 37 °C. Test media was prepared in the meantime, with 0.1, 1 and 10 µg/
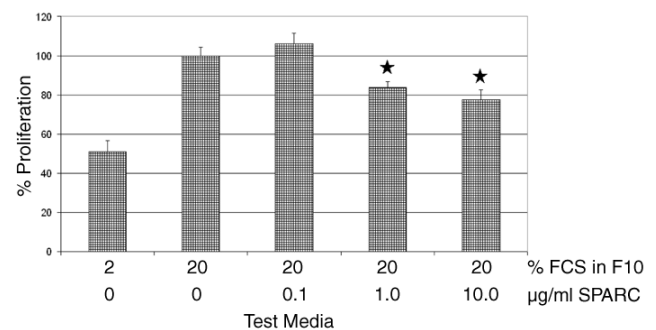

Figure 1. Effects of SPARC protein on HRPE cell proliferation. MTS assays were conducted on cultured HRPE cells. Four days after cells were seeded into the wells of 96 well plates with the test media, MTS reagent was added and the absorbance of the formazan produced in each well was read at 525 nm using a microtiter plate reader. Test media constituents were as noted in figure. The results are expressed as a percentage of the positive control, which was F10 medium with 20% fetal calf serum (FCS). A significant decrease in cellular dehydrogenase activity (absorbance) was observed with 1 and 10 µg/ml SPARC, represented as a decrease in percent proliferation (*$p < 0.01$). Error bars represent the standard error of the mean.

ml of SPARC (diluted in F10/20% FCS). Thereafter, matrices were overlaid with 1 ml of control media or test media and were detached from the well sides and base using a sterile pipette tip, before being returned to the incubator. Procedural controls included matrices overlaid either with F10/2% FCS (negative control, minimal contraction) or with F10/20% FCS (positive control). The percentage contraction of matrices was measured at 1, 4, and 7 days post-seeding, with a dissecting microscope (Olympus S240) as previously described [23]. Each dilution under test was performed in triplicate and the experiment was performed at least three times.

Statistical evaluations: All results are expressed as the mean with the standard error of the mean. The data were analysed using the statistics package Minitab, using a 2 sample t-test. p values of 0.05 or less were considered statistically significant.

RESULTS

SPARC and HRPE cell proliferation: HRPE cells grown in the presence of complete media (F10/20% FCS) showed maximal proliferation rates (100%) and served as a positive control. To show minimum proliferation rates, cells were exposed to F10/2% FCS, and these cells exhibited only half the proliferation level of the positive controls (51%). SPARC (in F10/20% FCS) at 0.1 μg/ml had no effect on HRPE cell proliferation compared to F10/20% FCS alone (Figure 1). As SPARC concentrations increased beyond 0.1 μg/ml, there was a concomitant decrease in HRPE cell proliferation. At 1 μg/ml SPARC (in F10/20% FCS), cell proliferation had decreased to 83.7% of controls, which was found to be significant (p<0.006). The proliferation rate of cells exposed to the highest concentration of SPARC (10 μg/ml in F10/20% FCS) was found to have decreased significantly, to 77.7% of the positive controls (p<0.002).

SPARC and HRPE cell migration: Positive controls (FN) showed maximal levels of HRPE cell migration (100%), and there was a significant difference in the migration rates observed between FN and FN-free negative controls (p<0.001, Figure 2A). HRPE cells did not migrate to any concentration of SPARC (0.1, 1, and 10 μg/ml) and this was found to be significant compared to the positive control, FN (p<0.001, Figure 2A).

No significant change in HRPE cell migration was observed for any concentration of SPARC in the presence of the standard concentration of FN (10 μg/ml, above), when compared to FN alone (Figure 2B). At concentrations of 0.1 μg/ml SPARC in FN-containing medium, HRPE cell migration was 68.6% of the positive control value. Migration was 81.2% for cells exposed to 1 μg/ml SPARC. At the highest concentration of SPARC used (10 μg/ml), migration was 55.2% of the cells compared to FN alone. However, the apparent trend for decreasing HRPE migration to FN in the presence of SPARC was not found to reach significance for any of the SPARC concentrations tested (e.g. p=0.076 for 10 μg/ml).

HRPE-seeded collagen matrices and SPARC: No contraction of 2D collagen matrices was observed at day 1 post-

seeding. However, by day 4 the positive controls (F10/20% FCS) showed a 15.2% contraction (Figure 3). Conversely, all of the SPARC-containing wells demonstrated reduced matrix contraction, concomitant with increasing SPARC concentration. HRPE cells exposed to 0.1, 1, and 10 μg/ml SPARC (in F10/20% FCS) underwent contractions of 14.9%, 8.8%, and 8.4%, respectively. Inhibition of contraction of matrices exposed to 1 and 10 μg/ml SPARC concentrations reached significance (p<0.02 and 0.001, respectively). By 7 days post-seeding (Figure 3), positive controls had undergone 46% contraction, with 0.1, 1, and 10 μg/ml SPARC-treated HRPE cells showing similar levels of matrix contraction as the F10/20% FCS controls (43.7%, 36.2% and 41%, respectively).
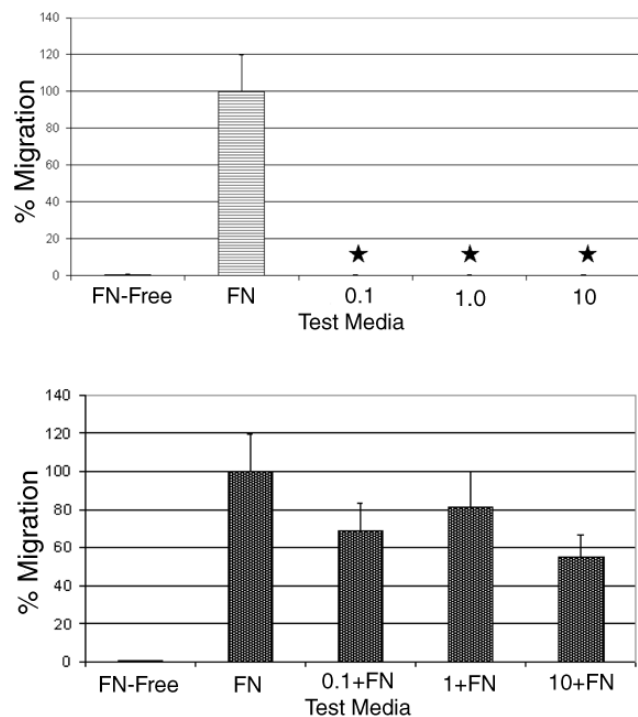

Figure 2. Effects of SPARC on HRPE and FN-induced HRPE cell migration. The migratory behavior of HRPE cells was assessed in standard 48-well microchemoattraction (Boyden) chamber experiments. The results are expressed as a percentage of the positive control (10 μg/ml fibronectin in F10 media), which is set at 100%. **A**: Test media: "FN-free" was F10 media only (negative control); "FN" was fibronectin at 10 μg/ml in F10 media (positive control); "0.1", "1.0", and "10" were the respective concentrations of SPARC (μg/ml) as indicated in the figure, diluted in F10 media only. No migration towards SPARC was observed for any of the concentrations used and this was found to be significantly different from the FN positive controls (*p<0.001). **B**: Test media: "FN-free" was F10 media only (negative control); "FN" was fibronectin at 10 μg/ml in F10 media (positive control); "0.1+FN", "1+FN", and "10+FN" were the respective concentrations of SPARC (μg/ml) as indicated in the figure, in the presence of 10 μg/ml FN in F10 media. Migration towards a source of FN was diminished with all concentrations of SPARC used, however, these results did not reach significance. Error bars represent the standard error of the mean. **A. B.**

DISCUSSION

Our results show that SPARC modulates several HRPE cell activities in vitro. We have demonstrated that concentrations of SPARC of 1 and 10 µg/ml significantly inhibited HRPE cell proliferation. Indeed, 10 µg/ml SPARC inhibited HRPE cell proliferation by almost 25% over a four day period. Previous work by Funk and Sage [24] has demonstrated an antiproliferative effect of high concentrations of SPARC on endothelial cells in culture, but low concentrations of SPARC appeared to support endothelial cell proliferation. Thus, during studies on a transformed human umbilical vein endothelial (HUVE) cell line, this group observed a slight increase (<10%) in thymidine incorporation after exposure to lower doses of SPARC (1-2.5 µg/ml). Yet after exposure to doses of >25 µg/ml SPARC, a 25-30% decrease in thymidine incorporation was noted [24]. Our results did not demonstrate an increase in HRPE cell proliferation at the lowest SPARC concentration used (0.1 µg/ml), although we did observe the inhibitory effect at higher SPARC levels. However, in comparison to HUVE cells, the inhibitory effect on HRPE cell proliferation was observed at lower concentrations of SPARC. The differences in results with respect to cell proliferation between the two studies may be explained by experimental differences. Alternatively, cultured HRPE cells might be more sensitive to changes in exogenous SPARC concentrations than endothelial cell-lines.

The migration bioassays demonstrated that HRPE cells do not migrate to a source of SPARC (i.e. SPARC did not appear to be a chemoattractant for HRPE cells). Nor did SPARC influence FN-induced HRPE cell migration at the levels of protein investigated. By contrast, SPARC has been shown to inhibit the migration of some cell types to bFGF. For example, it has been demonstrated that, in the absence of serum,

exogenous SPARC inhibits bFGF-induced migration of bovine aortic endothelial cells [8]. Differences between the previous study and the present investigation may, at least in part, reflect differences between the cell types and/or the chemotactic agents employed.

HRPE-populated collagen matrices were used in this study because previous work has established them as good in vitro models for evaluating the behavior of RPE cells in PVR and also because of the similarities in composition between the collagen matrices and PVR membranes [23,25-29]. RPE cells are known to reside both within the collagenous fibrous tissue, and on the surface of, PVR membranes [13,30,31]. These cells are known to synthesize collagen I in vitro [32,33] and are able to efficiently contract collagen I-containing matrices [23]. In the 2D PVR models (which mimic early PVR [29]), SPARC inhibited HRPE cell-mediated contraction of 2D gels in a concentration-dependent manner. The observation that all SPARC-treated matrices had contracted to levels similar to the positive controls by day 7 suggests that SPARC had been proteolytically degraded in the extracellular milieu by this time point. It is well known that SPARC is readily degraded, once it is secreted outside of the cell, by MMPs and other proteases [34,35]. Therefore, it seems likely that the SPARC protein had been digested by 7 days, which may explain the increased matrix contraction by the end of the experiment. When newly plated cultured cells are exposed to exogenous SPARC, there is subsequent modulation of cell:ECM interactions, possibly by a variety of pathways. One such pathway is thought to be via the abrogation of focal adhesions [36]. Diminution of focal adhesions would prevent interaction of the cells with the collagen fibres and bundling of the collagen. It therefore seems feasible that, in our model, SPARC reduced HRPE cell focal adhesion formation and attachment to the collagen, and thereby diminished the collagen remodelling upon which matrix contraction appears to depend.

Conversely, it is more difficult to reconcile our HRPE cell-populated collagen matrix results with the notion that SPARC and collagen type I together enhance matrix contraction. Iruela-Arispe and colleagues [37] observed less extracellular accumulation of SPARC in the tissues of collagen I-null mice embryos than in controls. Subsequent in vitro collagen matrix analyses demonstrated that these collagen I-null fibroblasts did not contract gels as efficiently as their heterozygous counterparts, but that addition of SPARC did enhance matrix contraction [37]. However, the latter study employed 3D collagen matrices. We have shown that 3D matrices behave differently to 2D matrices with regard to cell:matrix interactions [29]. Hence differences between the present study and that of Iruela-Arispe and coworkers may reflect experimental variation.

In recent years SPARC expression has been demonstrated in both developing and mature structures of vertebrate eyes. There is particular interest in the adult eye because high levels of SPARC are reported in various mature ocular structures, including the retina and lens [38-42]. Additionally, analyses of SPARC knock-out mice have demonstrated that the absence of SPARC within the eye results in severe early onset cataract
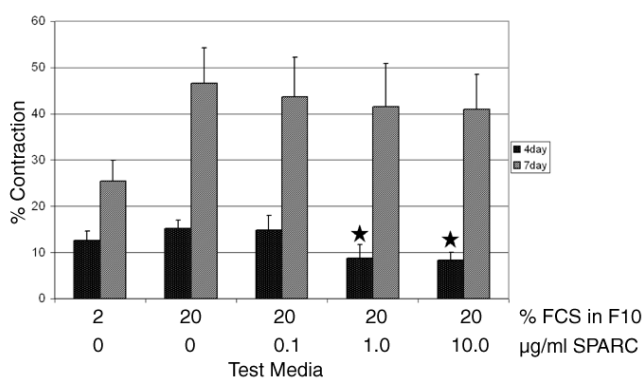

Figure 3. Effects of SPARC on HRPE cell-mediated contraction of collagen matrices. The histogram illustrates the effects of SPARC on HRPE cell-mediated contraction of collagen matrices over a 7 day period (a 2D model of PVR). Test media constituents were as noted in figure. At 4 days (black bars), post-seeding matrix contraction was significantly diminished by 1 and 10µg/ml SPARC (*p<0.01). However, by 7 days (gray bars), there was no significant difference in matrix contraction between SPARC-exposed cells and positive controls. Error bars represent the standard error of the mean.

formation [43,44]. Locally-derived SPARC therefore seems to represent a potentially crucial modulator of cell behavior in the eye. Furthermore, in the early stages of PVR, hematogenous SPARC may gain access to the retinal surfaces along with other matricellular proteins like thrombospondin 1 [17]. Hence during PVR development, RPE cells may have access to SPARC from a variety of sources. Once established, PVR membranes often contain a predominance of RPE cells [13,33]. In this respect it is interesting that we have shown that SPARC can be identified within RPE cells of PVR membranes [16,17] and that cultured HRPE cells synthesize and secrete the protein [15]. Thus the possibility arises that in PVR membranes, RPE cells are also influenced by SPARC in an autocrine-like way. SPARC can modulate matrix remodeling, migration, and proliferation by cells. Since migration and proliferation and matrix contraction by HRPE cells are essential processes during PVR membrane formation and development we examined how exogenous SPARC influenced these PVR-related activities by HRPE cells in vitro. Our findings suggest an important role for this protein in HRPE cell behavior. It seems possible that, at least in higher concentrations, SPARC disrupts HRPE focal adhesions, and promotes cell rounding and detachment. The reduced cell attachment may explain our observations with regard to the effects of the protein on HRPE cell proliferation and matrix contraction. On the other hand, there is evidence that SPARC could affect RPE cell behavior by other mechanisms such as by interacting with growth factors [9]. Thus, in addition to its ability to directly modulate cell:matrix interactions, SPARC is known to regulate the activity of a number of growth factors including platelet-derived growth factor (PDGF) [45], transforming-growth factor-β1 (TGF-β1) [46], fibroblast growth factor (FGF)-2 [47], and vascular endothelial growth factor (VEGF) [48]. High concentrations of exogenous SPARC protein may act to bind many of these growth factors present in serum, which would otherwise stimulate the proliferative, adhesive, migratory and contractile responses of HRPE cells. Additionally, SPARC appears to modulate (either directly or indirectly) collagen synthesis by HRPE cells. Therefore, taken together, the available data is supportive of the concept that SPARC modulates the behavior of RPE cells in PVR membranes by a variety of pathways and this concept may help explain the apparently complex role of matricellular proteins in proliferative retinal disease [17]. Nevertheless, since these proteins appear to influence so many of the cellular activities key to the development of conditions like PVR, they may represent an avenue for pharmacological intervention in the disease.

ACKNOWLEDGEMENTS

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