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Original Article

Somatic *GNAQ* Mutation is Enriched in Brain Endothelial Cells in Sturge–Weber Syndrome

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ABSTRACT

BACKGROUND: Sturge–Weber syndrome (SWS) is a rare congenital neurocutaneous disorder characterized by facial and extracraniofacial capillary malformations and capillary-venule malformations in the leptomeninges. A somatic mosaic mutation in *GNAQ* (c.548G>A; p.R183Q) was found in SWS brain and skin capillary malformations. Our laboratory showed endothelial cells in skin capillary malformations are enriched for the *GNAQ* mutation. The purpose of this study is to determine whether the *GNAQ* mutation is also enriched in endothelial cells in affected SWS brain. **METHODS:** Two human SWS brain specimens were fractionated by fluorescence-activated cell sorting into hematopoietic (CD45), endothelial (CD31, VE-Cadherin, and vascular endothelial growth factor receptor 2), and perivascular (platelet-derived growth factor receptor beta) cells and cells negative for all markers. The sorted cell populations were analyzed for *GNAQ* p.R183Q mutation by droplet digital polymerase chain reaction. SWS patient-derived brain endothelial cells were selected by anti-CD31-coated magnetic beads and cultured in endothelial growth medium *in vitro*. **RESULTS:** The *GNAQ* p.R183Q mutation was present in brain endothelial cells in two SWS specimens, with mutant allelic frequencies of 34.7% and 24.0%. Cells negative for all markers also harbored the *GNAQ* mutation. The mutant allelic frequencies in these unidentified cells were 9.2% and 8.4%. SWS patient-derived brain endothelial cells with mutant allelic frequencies of 14.7% and 21% survived and proliferated *in vitro*. **CONCLUSIONS:** Our study provides evidence that *GNAQ* p.R183Q mutation is enriched in endothelial cells in SWS brain lesions and thereby reveals endothelial cells as a source of aberrant Gαq signaling. This will help to understand the pathophysiology of SWS, to discover biomarkers for predicting cerebral involvement, and to develop therapeutic targets to prevent neurological impairments in SWS.

Keywords: Sturge–Weber syndrome, capillary malformation, *GNAQ*, endothelial cell, ddPCR

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Introduction

Sturge–Weber syndrome (SWS) is a sporadic, congenital neurocutaneous disorder that affects 1 in 20,000 to 1 in 50,000 newborns.[1,2] It is characterized by cutaneous capillary malformation with ocular and/or brain abnormalities. The clinical features are largely variable and they can be progressive. Glaucoma is the most common ophthalmology complication, and typically the neurological problems often

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include medically resistant epilepsy, hemiparesis, and neurocognitive impairment.

Facial capillary malformations (also known as port-wine stain) with specific distribution patterns are associated with an increased risk of SWS.[3,4] If the birthmark involves the forehead and upper eyelids, the risk of having SWS is approximately 26%.[5] The strong association of facial capillary malformation and SWS suggests that they are caused by the same somatic mutation and that the timing of when the mutation occurs during the development likely impacts the clinical sequela and disease severity.

Recent genetic studies discovered a somatic mosaic mutation in *GNAQ* (c.548G>A; p.R183Q) in SWS brain and skin lesions and nonsyndromic capillary malformation lesions.[6-8] *GNAQ* encodes Gαq, an alpha subunit of heterotrimeric G proteins. G proteins are a family of membrane-bound guanosine triphosphatases (GTPase) that transmit signaling from transmembrane G-protein–coupled receptors. The p.R183Q mutation impairs GTPase activity and maintains Gαq in its GTP-bound, active state.[9] Our laboratory recently showed that the *GNAQ* p.R183Q mutation is enriched in the endothelial cell population in SWS skin lesions and sporadic capillary malformations.[10] Another group reported *GNAQ* p.R183Q mutant cells located around blood vessels in port-wine stains.[11] In this study we set out to determine whether the *GNAQ* p.R183Q mutation is also enriched in endothelial cell population in SWS brain lesions.

Materials and Methods

SWS brain specimens and cell isolation

Two individuals with SWS who underwent hemispherectomy as part of their clinical care were enrolled in the study. The Committee on Clinical Investigation at Boston Children's Hospital approved this study. SWS brain lesions were collected during the neurosurgical procedure. The brain tissue of Patient 1 (male, 11 months old) was from his left lateral temporal lobe and of that of Patient 2 (male, 12 years old) was from the occipital lobe. Both were about 0.5 cm^3, including leptomeninges, cortex, and superficial white matter. Single cell suspensions were prepared by digestion with LiberaseTM (Roche, Indianapolis, IN). Cells were fractionated into distinct populations by fluorescence-activated cell sorting (FACS) using the following antibodies: anti-human CD45 and anti-human Glycophorin A, both conjugated to allophycocyanin (eBioscience; San Diego, CA); anti-human platelet-derived growth factor receptor beta (PDGFRβ) conjugated to fluorescein isothiocyanate (FITC) (R&D Systems, Minneapolis, MN); and a mixture of anti-human CD31 (BD Biosciences Pharmingen; Bedford, MA), anti-human VE-Cadherin (R&D Systems), and anti-human vascular endothelial growth factor receptor 2 (VEGFR2, R&D Systems), each conjugated to phycoerythrin (PE). Genomic DNA was extracted from the sorted cells using the QIAamp DNA Mini kit (Qiagen, Germantown, MD).

Nonsorted brain cells from the single cell suspension were seeded on fibronectin-coated (1 μg/cm^2) tissue culture plates in EGM-2 complete medium (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum. After 10 days of culture, endothelial cells were selected using anti-human CD31 DynaBeads (Thermo Fisher Scientific, Cambridge, MA). Genomic DNA was extracted from CD31-positive and CD31-negative cell populations using the QIAamp DNA Mini kit.

Droplet digital polymerase chain reaction–based analysis for GNAQ p.R183Q

Primers and probes were designed to target *GNAQ* p.R183Q as described previously.[10] Sequences used were: forward primer (5′-CCTGCCTACGCAACAAGAT-3′); reverse primer (5′-GTAAGTCAAAGG GGTATTCGAT-3′); reference probe (5′-TGCTTAGAGTTCGAGTCCCCACC-3′); mutant probe (5′-TGCTTAGAGTTCAAGTCCCCACC-3′). Briefly, the 20 μL droplet digital polymerase chain reaction (ddPCR) reaction mixture was composed of 1X SuperMix for Probes (Bio-Rad, Hercules, CA), mutant and reference probes (0.25 μM each), forward and reverse primer (0.9 μM each), and template DNA (30 ng). The PCR reaction was partitioned into approximately 20,000 droplets using the QX100 Droplet Generator (Bio-Rad) and subjected to the following PCR cycle profile: 95°C for 10 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 60 seconds, and 72°C for 30 seconds; and final 98°C for 10 minutes. Droplets were measured in the QX100 Droplet Reader (Bio-Rad) and results were analyzed with the QuantaSoft software (Bio-Rad).

Immunostaining and confocal microscopy

Formalin-fixed, paraffin-embedded SWS brain sections were deparaffinized and immersed in antigen retrieval solution (Citrate– ethylenediaminetetraacetic acid [EDTA] buffer: 10 mM citric acid, 2 mM EDTA, 0.05% Tween 20, pH 6.2, or 1 mM EDTA pH 8.0) for 20 minutes. Sections were blocked for 30 minutes and stained with anti–human VE-Cadherin antibody (Sigma, HPA030562), anti-human CD31 antibody (Santa Cruz, C-20), and anti-human VEGFR2 antibody (55B11, Cell Signaling Technology, Danvers, MA) overnight. Purified class- and species-matched IgGs (Vector Lab) were the controls. Afterward, sections were incubated with appropriate biotinylated secondary antibodies followed by DyLight 488 Streptavidin (Vector Lab). All slides were mounted using 4',6-diamidino-2-phenylindole (Molecular Probe, Eugene, OR) to visualize nuclei. Images were acquired using a Leica TCS sp2 Acousto-Optical Beam Splitter confocal system equipped with DMIRE2 inverted microscope camera (Leica Microsystems, Wetzlar, Germany).

Flow cytometry

Cultured cells (1×10^5) were incubated at 4°C for 30 minutes in 100 μl of phosphate-buffered saline plus 1% bovine serum albumin with PE-conjugated anti-human CD31 antibody, washed three times, and analyzed by flow cytometry (Becton Dickinson, San Diego, CA). PE-conjugated mouse IgG1,k (R&D Systems) was used as isotype control. Data were analyzed using Flowjo_10v software (Tree Star, Inc Ashland, OR).

Results

Two patients with SWS underwent hemispherectomy due to extensive brain parenchyma abnormalities causing drug-resistant epilepsy. Magnetic resonance imaging (MRI) in Fig 1A,B demonstrated marked abnormalities in the left hemisphere including the extensive leptomeningeal enhancement, cerebral atrophy, choroid plexus hypertrophy, and tortuous and enlarged veins. Hematoxylin and eosin staining highlighted the malformed vessels in the leptomeninges (Fig 1C,D). Anti-human VE-Cadherin (Fig 1E,F), anti-human CD31 (Fig 1G,H), and anti-human VEGFR2 (Fig 1I,J) staining shows strong staining along vessel lumens in the leptomeninges and cortex, consistent with endothelial cells, which validated the use of these antibodies for brain endothelial cell sorting. Sparse anti-VEGFR2 staining of nonvascular cells was also observed (Fig 1I,J). Red blood cell autofluorescence was evident in sections stained with anti-VEGFR2, which may be due to the antigen retrieval method that was different from anti-VE-Cadherin and anti-CD31 or to the weak but specific anti-VEGFR2 signal wherein red blood cell autofluorescence was similar in intensity.

Small pieces of brain tissue from Patient 1 and Patient 2 were digested to prepare single cell suspensions. The brain

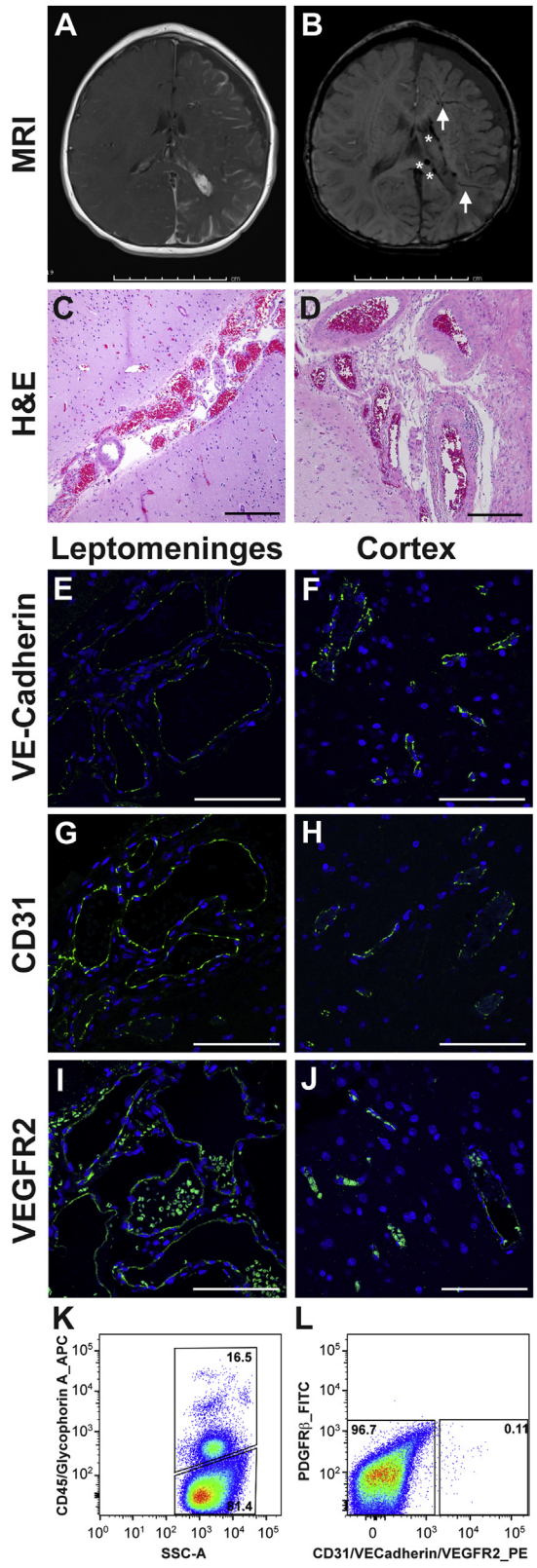

FIGURE 1.
Brain specimens from two patients with SWS. (A) Postcontrast axial T1-weighted magnetic resonance imaging (MRI) in Patient 1 shows extensive left hemisphere leptomeningeal enhancement, left choroid plexus hypertrophy, and marked left cerebral atrophy. (B) Axial susceptibility-weighted MRI in Patient 1 shows enlarged medullary veins (arrows) and dilated deep veins (*). (C) Hematoxylin and eosin staining of Patient 1 brain tissue section shows abnormal clusters of leptomeningeal vascular channels of variable calibers. The underlying cortex demonstrates focal loss of neurons in layer 2 and subpial gliosis. Scale bar, 100 μm. (D) Hematoxylin and eosin (H&E) staining, viewed at higher magnification, shows some of these vascular walls are thick and rigid. Scale bar, 200 μm. The histologic findings are consistent with leptomeningeal angiomatosis. (E-J) Anti-human VE-cadherin, anti-human CD31, and anti-human vascular endothelial growth factor receptor 2 (VEGFR2) staining highlights enlarged vessels in leptomeningeal angiomas (E, G, and I, green) and shows positive staining predominately in the endothelium in the cortex (F, H, and J, green) in Patient 1. Nuclei counterstained with 4',6-diamidino-2-phenylindole (blue). Scale bar, 100 μm. (K-L) Fluorescence-activated cell sorting of freshly isolated brain cells from Patient 2. Cells were sorted into hematopoietic and nonhematopoietic cells based on CD45/Glycophorin A expression (G). Nonhematopoietic cells were further fractioned into endothelial cells (using a set of antiendothelial antibodies against CD31, VE-Cadherin, and VEGFR2), platelet-derived growth factor receptor beta (PDGFRβ)-positive cells and cells negative for all markers (H). Percentages of cells in each population are shown. APC, allophycocyanin; FITC, fluorescein isothiocyanate; SSC-A, side scatter-A. (The color version of this figure is available in the online edition.)

cell preparations from each patient with SWS were fractioned into different cellular populations by fluorescence-activated cell sorting. Hematopoietic cells were detected and gated using anti-human CD45 and anti-human Glycophorin A antibodies (Fig 1G). Endothelial cells were labeled with a panel of PE-conjugated antibodies against endothelial markers CD31, VE-Cadherin, and VEGFR2 to ensure all endothelial cells were captured. Fluorescein isothiocyanate–conjugated anti-human PDGFRβ antibody was used to detect pericytes.[12,13] However, very few PDGFRβ-positive cells were detected in these two SWS brain specimens (Fig 1H). The hematopoietic cells, endothelial cells, and cells negative for all markers from SWS brain lesions were sorted for ddPCR to determine the *GNAQ* p.R183Q allelic frequency.

The two SWS brain lesions were first confirmed to have the *GNAQ* p.R183Q mutation by performing ddPCR on unfractionated brain cells. Mutant allelic frequencies were 8.7% and 6.2% for Patient 1 and Patient 2, respectively (Table 1). Remarkably, the endothelial cell fraction from Patient 1 showed a mutant allelic frequency of 34.7% and endothelial cells from Patient 2 showed a mutant allelic frequency of 24.0%. This corresponds to 70% and 48% mutant cells, respectively, for each of the sorted endothelial populations. These results indicate that *GNAQ* p.R183Q mutation is enriched in endothelial cells in SWS brain lesions, similar to what we reported for skin capillary malformation lesions.[10] Mutant cells were also detected in the cell population negative for all markers, which may include uncaptured endothelial cells, neuronal cells, pericytes, or other stromal cells. Additional specimens and studies are needed to determine the identity of the mutant cells in this population. Very few mutant cells were found in the hematopoietic cell population.

An aliquot of unfractionated brain cells from each patient was plated on a fibronectin-coated dish and cultured in endothelial growth medium. A viable and robust primary culture was observed after 10 days of *in vitro* culture (Fig 2A). Endothelial cells and nonendothelial cells were isolated from the primary culture using anti-CD31-coated magnetic beads. CD31-positive and CD31-negative cells were analyzed by flow cytometry to verify purity (Fig 2B). The *GNAQ* mutation was detected in CD31-positive endothelial cells but

TABLE 1.
GNAQ p.R183Q Mutational Analysis in SWS Brain Cell Populations

Cell Population	Mutant Allelic Frequency (%)	
	Patient 1	Patient 2
Unfractionated cells from brain lesion	8.7	6.2
CD45/Glycophorin A–positive cells	0.2	0.5
Endothelial cells	34.7	24.0
PDGFRβ-positive cells	na	na
All negative cells	9.2	8.4

Abbreviations:
na = Not available
PDGFRβ = Platelet-derived growth factor receptor beta
SWS = Sturge–Weber syndrome

TABLE 2.
GNAQ p.R183Q Mutational Analysis in Cultured Cells Isolated From Sturge–Weber Syndrome Brain Lesion

Sample	Mutant Allelic Frequency (%)	
	Patient 1	Patient 2
CD31–positive, p4	14.7	21.0
CD31–negative, p4	0.1	2.6

not in CD31-negative cells (Table 2). These results demonstrate that we were able to isolate and culture SWS patient-derived *GNAQ* mutant brain endothelial cells for further characterization. However, the mutant allelic frequencies of 14.7% and 21% demonstrate that the cultures are a mix of mutant and nonmutant endothelial cells. For the Patient-1-derived EC culture at passage 4, it is approximately 30% mutant: 70% nonmutant; the Patient 2-derived endothelial cell culture at passage 4 is 42% mutant: 58% nonmutant.

Discussion

In this study, we demonstrated that the *GNAQ* p.R183Q mutation resides within endothelial cells in SWS brain lesions. This is consistent with our previous findings for the *GNAQ* p.R183Q mutation in skin capillary malformations.[10] Here we extend our studies to show the SWS brain endothelial cells can be isolated and expanded in culture and further confirm that the *GNAQ* p.R183Q mutation is present in brain endothelial cells.

The *GNAQ* mutant allelic frequency in the CD45-negative/endothelial marker–negative/PDGFRβ-negative cell population from the SWS brain specimens was 9.2% and 8.4%, higher than what was found in the same population in capillary malformation.[10] These mutant cells may include endothelial cells that were not sorted due to insufficient antibody capture; addition of anti-glucose transporter 1 (GLUT1) may provide a more complete capture of all brain endothelial cells as GLUT1 is specifically expressed in brain endothelial cells.[14,15] We also observed GLUT1 expression in SWS brain endothelium by immunostaining (data not shown). Alternatively, some cells could be neuronal cells; subsorting of CD45-negative/endothelial marker–negative/PDGFRβ-negative cell population with antibodies against neuronal markers would be needed to address this. These refinements will be incorporated into future studies as additional SWS brain specimens become available.

We used anti-VE-Cadherin, anti-CD31, and anti-VEGFR2 antibodies to sort endothelial cells from the SWS brain specimens based on previous studies.[10,16] Anti-VEGFR2 was included to capture cells that may be undergoing angiogenesis and thereby have increased VEGFR2 on the cell surface.[17,18] A limitation of this strategy is that VEFGR2 expression is not strictly confined to endothelial cells and therefore the sorted endothelial population may contain nonendothelial cells. We can rule out that the mutant cells are composed entirely of nonendothelial VEGFR2-positive cells because the CD31-selected cells from a primary culture of total brain cells showed high mutant allelic frequency for *GNAQ* p. R183Q (Fig 2, Table 2). Together, our data demonstrate that the predominate mutant cells in SWS brain are endothelial cells.

One feature of SWS is tortuous, malformed blood vessels in thickened leptomeninges (shown in Fig 1C,D). The aberrant pattern of arterial and venous cortical circulation underneath meningeal angiomas is also documented.[19,20] In particular, venous congestion and stasis is recognized in SWS brain, which causes cerebral hypoperfusion and intraparenchymal dystrophic calcification, and potentially leads to frequent seizures, long epilepsy duration, severe cerebral atrophy, and brain functional deterioration. Given that the *GNAQ* mutation appears to reside primarily in endothelial cells in SWS brain, we speculate that *GNAQ* mutation causes some type of endothelial dysfunction, which in turn leads to

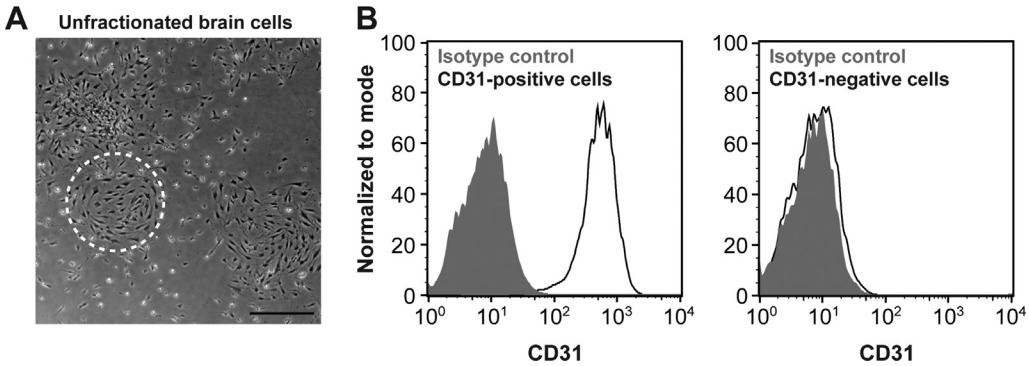

FIGURE 2.
Cells isolated from SWS brain lesion. (A) Primary culture from Patient 1 brain lesion. Dashed circle highlights cells with endothelial morphology. Scale bar, 50 μm. (B) Flow cytometric analysis of cells in the primary culture after selection using anti-CD31-coated magnetic beads: CD31-positive cells and CD31-negative cells. Black lines depict cells incubated with anti-CD31 antibody; shaded gray areas are cells incubated with isotype-matched control antibody.

the abnormal vascular structure and perhaps abnormal interaction with adjacent cells. The abnormal vessels in the leptomeninges and cortex may lead to faulty neurovascular interactions, which could in turn contribute to the cortical malformation, epileptogenesis, and ultimately neurological deficits in SWS. Therapies directed at the impaired endothelial cell function and/or vascular-neuronal crosstalk at the early stage might prevent or alleviate some of the most devastating consequences of SWS.

The extent of leptomeningeal angiomas has been correlated with the severity of seizures, brain atrophy, and cognitive loss.[21] In our previous study, we noticed an association between capillary malformation severity and endothelial *GNAQ* mutant allelic frequency. That is, patients with more severe capillary malformations had higher endothelial *GNAQ* mutant allelic frequency.[10] Therefore, it would be of interest to know whether endothelial *GNAQ* mutant allelic frequency in skin capillary malformation is correlated with mutant allelic frequency in the brain, and associated with the extent of leptomeningeal angiomas, and can further predict the severity of neurological impairment. If so, a biopsy of capillary malformation along with neuroimaging could potentially be performed in early childhood to predict the neurological problems. For example, if the endothelial mutant allelic frequency is high in skin capillary malformation, the patient may be expected to have a larger leptomeningeal angioma, and a higher risk of neurological impairments. Future studies can test this hypothesis.

Recently, somatic mutations in *GNAQ* and *GNA11* had been identified in congenital hemangioma,[22] indicating *GNAQ* plays essential roles in vascular growth and vascular development. In addition, somatic mutations in *GNA14*, another Gαq family member, were recently reported in tufted angiomas, kaposiform hemangioendotheliomas, and pyogenic granulomas.[23] In these studies, the cellular residence of the mutations in the vascular tumors was not determined, although Lim and colleagues expressed the *GNA14* p.Q205L mutation in human umbilical vein endothelial cells. Further studies aimed to understand the mechanism by which altered Gαq signaling in endothelial cells contributes to pathophysiology of SWS and capillary malformation, as well as congenital hemangiomas and vascular tumors, may help to determine early biomarkers for predicting brain involvement and provide therapeutic targets to prevent skin and brain lesion progression.

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