# Levels of circulating endothelial progenitor cells in systemic sclerosis

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## Abstract

### Objective

*Contradictory results have been reported regarding vasculogenesis in systemic sclerosis (SSc). Our aim was to investigate bone marrow-derived circulating endothelial precursors (EPCs) and activated circulating endothelial cells (CECs) in SSc patients.*

### Methods

*Peripheral blood from consecutive patients with SSc hospitalised for systemic follow-up was analysed and compared with blood from patients with active refractory rheumatoid arthritis (RA) and osteoarthritis (OA). EPCs were quantified by cell sorting and flow cytometry and were identified as circulating $CD34^+CD133^+$ cells. Activated CECs were defined as $CD105^+CD62^+$ or $CD105^+CD102^+$ or $CD105^+CD106^+$ cells.*

### Results

*Patients with SSc had higher putative EPC levels than OA patients, but lower levels than RA patients. In SSc patients, EPC levels increased with European disease activity score. Activated CEC levels were high in SSc patients and RA patients, but not correlated with EPC levels.*

### Conclusion

*These results together and previous data suggest that EPCs may be recruited during active vascular disease but that the sustained ischaemic conditions of SSc may eventually lead to EPCs depletion.*

**Key words**

Systemic sclerosis, endothelium, endothelial progenitor cells, vasculature.

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*Abbreviations:*

ACE inhibitors: angiotensin-converting enzyme inhibitors
CECs: circulating endothelial cells
EPCs: endothelial progenitor cells
FITC: fluorescein isothiocyanate
OA: osteoarthritis
PE: phycoerythrin
RA: rheumatoid arthritis
SSc: systemic sclerosis
VEGF: vascular endothelial growth factor

## Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterised by early generalised microangiopathy and culminating in systemic fibrosis. The key steps in the disease are endothelium injury, immune activation and collagen deposition by activated fibroblasts. Vasculature has a major effect on SSc prognosis, with outcome depending on the extent and severity of the vascular lesions (1).

Vascular changes are thought to occur at an early stage and changes may include endothelial cell apoptosis, endothelium activation with cell adhesion molecule expression, inflammatory cell recruitment, intimal proliferation and adventitial fibrosis, which may lead to vessel obliteration (2). Capillaroscopy can be used for microvascular investigation, showing disturbed angiogenesis with changes in permeability and architecture, giant capillaries, branched capillaries and avascular areas (3). Despite suggestions that SSc patients display deficiencies in angiogenesis, we and others have reported high circulating vascular endothelial growth factor (VEGF) concentrations in these patients (4) and chronic and uncontrolled VEGF upregulation has been implicated in the disturbed skin vessel morphology of SSc patients (5).

In human adults, new blood vessels may form in two ways: via endothelial sprouting from pre-existing endothelial cells/angioblasts (angiogenesis) or via the peripheral recruitment of endothelial progenitor cells (EPCs) (vasculogenesis). EPCs, first described by Asahara *et al*. (6), are a population of bone marrow-derived cells capable of differentiating into mature endothelial cells and participating in the formation of new blood vessels. The molecular phenotype of the putative endothelial progenitor cells and the processes leading to their mobilisation from the bone marrow and homing to sites of neovascularisation remain unclear (7). Populations of peripheral mononuclear cells expressing CD34 and/or CD133 have been shown to include putative EPCs, but it has long been thought that EPCs could be cultured from peripheral blood mononuclear cells (PBMCs) on fibronectin, and that these cells might then go on to express receptors for low density lipoprotein and lectin (7). However, most of the acetylated low density lipoprotein$^+$BS-lectin$^+$ cells derived from the monocyte/macrophage CD14$^+$ subpopulation do not proliferate and late outgrowth endothelial cells have been shown to develop exclusively from the CD14$^-$ fraction (8). Thus, many investigators base their estimates of the number of functional EPCs in the bloodstream on the expression of various cell surface markers, including CD34, CD34$^+$CD45-, CD34$^+$CD133$^+$, CD34$^+$VEGFR-2$^+$, VEGFR-2$^+$CD133$^+$ and CD34$^+$VEGFR-2$^+$CD133$^+$ (7, 9, 10).

EPCs and circulating endothelial cells (CECs) differ from the surface expression of CD133, a stem cell marker that is expressed in EPCs but not on mature endothelial cells (9). Other surface markers like CD34, CD146, CD45 and VEGFR2 could be present at the surface of either EPCs and CECs (11, 12).

Two previous studies have investigated EPCs levels in SSc: Del Papa *et al*. (13) reported significantly higher numbers of EPCs in SSc patients than in healthy subjects, mostly early in disease and concomitantly with increases in activated CECs levels. By contrast, Kuwana *et al*. (14) found that SSc patients had fewer EPCs than patients with rheumatoid arthritis or healthy controls, and that these cells displayed a lower ability to differentiate into endothelial mature cells *in vitro* despite the paradoxically high concentrations of angiogenic factors in SSc patients. We thus aimed to quantify EPCs and activated CECs in our SSc patients, as a means of evaluating endothelial injury.

## Patients and methods

*Patients*

We included all successive patients hospitalized for systematic follow-up during a four-month period with a diagnosis of SSc according to the criteria established by the American Rheumatism Association (15). Patients with symptoms overlapping those of other connective tissue diseases were excluded from the study. All patients gave

informed consent for all procedures, which were carried out with local ethics committee approval. Three months of stable current treatment was necessary for inclusion, and prednisone use, at a dose of less than 10 mg/day, was authorised. Vasodilators, including calcium channel blockers and angiotensin-converting enzyme (ACE) inhibitors, had to be withdrawn at least three days before inclusion (corresponding to more than five times the drug half life), and none of the controls was treated with vasodilator drugs. Smoking, diabetes or current treatment for dyslipidaemia were exclusion criteria.

The control groups were recruited over a period of four months. They were patients with rheumatoid arthritis (RA), defined according to American College of Rheumatology criteria (16), referred for active refractory disease and patients with osteoarthritis (OA) (17), referred for evaluation to assess treatment possibilities and treated with paracetamol only at the time of inclusion.

*Clinical assessment*

The following clinical data were collected: age, sex, cutaneous SSc subtype according to the definition of LeRoy *et al*. (18), disease duration (date of first non Raynaud symptom), duration of Raynaud phenomenon, digital ulceration and prostacyclin use. We diagnosed pulmonary fibrosis on the basis of computed tomography scan and abnormal results for respiratory function tests (forced vital capacity [FVC] and carbon monoxide diffusion capacity divided by alveolar volume [DLCO/AV]) (19). Patients with high risk of pulmonary arterial hypertension (PAH) were identified on the basis of pulmonary arterial pressure higher than 40 mmHg at rest on Doppler-echocardiography and the diagnosis was established following confirmation by right heart catheterisation. The following biological tests were carried out: usual blood tests, antinuclear and anti-centromere antibodies (immunofluorescence on Hep2 cells), anti-topoisomerase I and anti-ribonucleoprotein (counter immunoelectrophoresis). Von Wille-brand factor activity and antigen measurements were conducted to provide markers of endothelial injury (20). The von Willebrand antigen concentration was determined using an ELISA (VIDAS von Willebrand, BioMérieux, Marcy l'étoile, France). The von Willebrand ristocetin cofactor activity was measured, using the BC von Willebrand Reagent (Dade Behring, Marburg, Germany), on a PAP4 aggregometer (Biodata).

*Flow cytometry analysis*

Peripheral blood was taken at rest, in the morning, at forearm, together with routine analysis in hospitalised patients. Samples were immediately provided for the laboratory. Two hundred micro-liters of peripheral blood in sodium heparin was labelled with 10 µg of a panel of fluorescein isothiocyanate (FITC)-, R-phycoerythrin (PE)-, or Cychrome-conjugated antibodies: Mouse anti-human CD34-Cychrome (BD Bioscience, Le pont de Claix, France), anti-CD133-PE (Mitlteny biotech, Paris, France), anti-CD105-PE, anti-CD62-FITC, anti-CD102-FITC, anti-CD106-FITC (Serotec, Cergy Saint Christophe, France), for one hour at 4°C. After conjugation, red blood cells were lysed by incubating in FACS lysing solution (BD Bioscience) for 15 minutes at room temperature. White blood cell pellets were then washed three times in FACSFlow solution (BD Bioscience). Appropriate analysis gates were used to enumerate total and activated EPCs and CECs and to exclude debris. Putative EPCs were defined as positive for anti-CD34 and anti-CD133 (7, 9, 11, 21). Activated CECs were defined as positive for CD105 (endoglin), which expression is mainly restricted to vascular endothelial cells (18), and positive for CD62 (selectin) or CD102 (ICAM-2) or CD106 (VCAM-1). Five-parameter, 3-color flow cytometry was performed with a FACSCalibur flow cytometer with an argon laser (excitation at 488 mm) (BD Bioscience). The sensitivity of fluorescence detectors was set and monitored using CaliBRITE beads (Becton Dickinson) according to the manufacturer's recommendations. Cells stained with FITC-, PE- and Cychrome-isotypic controls were used as negative controls. At least 250, 000 cells per sample were acquired; analyses were considered informative when adequate numbers of events (> 100) were collected in the CECs enumeration gates. Data were analyzed with CellQuest software (Becton Dickinson). Results are expressed as % cells gated.

*Statistical analysis*

We compared levels of EPCs and CECs between SSc patients and controls groups, using the Mann-Whitney rank sum test. Correlations between EPC levels, CEC levels and various quantitative SSc disease parameters were assessed using Spearman's rank correlation test. Multiple logistic regression was used for multivariate analysis. P values less than 0.05 were considered significant.

## Results

*Demographic and clinical features of patients*

The characteristics of the 32 SSc patients included are summarised in Table I. All the SSc patients recruited (32/32) were regularly taking calcium channel blockers and 18 of the 32 (56%) were also treated with ACE inhibitors, which were withdrawn at least three days before evaluation. Control patients with RA (n = 15) had a mean age of 52 ± 12 years and a mean disease duration of 10 ± 9 years. They also presented the following characteristics (mean values): disease activity score (DAS28) of 4.7 ± 1.2, health assessment questionnaire (HAQ) score of 1.4 ± 0.8, C reactive protein concentration of 33 ± 27 mg/l, one-hour erythrocyte sedimentation rate of 39 ± 22 and creatininaemia of 74 ± 10 µmol/l. Ten of the 15 RA control patients had positive rheumatoid factor and 11 of the 15 had anti-cyclic citrullinated peptide antibodies. All were treated with methotrexate (12.5-20 mg/week) and low-dose prednisone. Patients with OA (n = 15) had a mean age of 56 ± 6 years and were treated with paracetamol only. All patients had white cells blood count within normal range (4 to 10 G/l).

*Quantitative studies of EPCs and association with disease phenotype*

SSc patients had significantly higher EPC levels than controls with osteo-ar-

**Table I.** Characteristics of systemic sclerosis patients.

| n patients (%), unless otherwise stated | SSc patients (n = 32) |
|---|---|
| Age (years); mean ± SD / sex (F/M) | 54 ± 15 / 30-2 |
| Diffuse / limited cutaneous form | 12 (37%) / 20 (63%) |
| Disease duration (years): mean±SD, n < 5 years | 8 ± 6 / 10 (31%) |
| Current digital ulcers | 8 (25%) |
| Pulmonary arterial hypertension | 2 (6%) |
| Pulmonary fibrosis on CT scan | 13 (41%) |
| Positive anti-nuclear antibodies | 30 (94%) |
| Positive anti-topoisomerase I antibodies | 9 (28%) |
| Positive for anti-centromere antibodies | 12 (37%) |
| Low FVC (<75% of predicted value) | 8 (25%) |
| Low DLCO/AV (<75% of predicted value) | 15 (47%) |
| Erythrocyte sedimentation rate; 1st hour | 18 ± 14 |
| C reactive protein; mean±SD | 8.9 ± 11.1 |
| Von Willebrand factor; mean±SD and n with high values | 165 ± 50; 11 (34%) |
| HAQ: mean ± SD and n > 1.5 | 1.02 ± 0.8 (29%) |
| Disease activity score: mean ± SD; n > 3 | 2.3 ± 1.7; 15 (47%) |
| Platelet anti-aggregating | 21 (66%) |
| Prednisone < 10 mg per day | 11 (34%) |

FVC: Forced vital capacity; DLCO/AV: Carbon monoxide diffusion capacity of the lung divided by alveolar volume.

thritis [median % of positive cells 0.92 (0.31-3.04) vs 0.57 (0.20-1.20)] but that patients with RA had even higher EPC levels than SSc patients [(median % of positive cells 1.37 (0.54-4.77) vs 0.92 (0.31-3.04)]. These results are detailed in Table II and illustrated in Figure 1.

Univariate analyses of associations with disease phenotype showed that SSc patients with active disease, according to European disease activity score (17) (score > 3; n = 15), had higher EPC levels than patients without active disease (score < 3) [(median % of positive cells 1.49 (0.38-3.04) vs 0.62 (0.31-1.8)]. We also found that SSc patients with high concentrations of endothelial injury markers (plasma concentration of von Willebrand factor; n = 11) had higher EPC levels than patients with normal concentrations of endothelial marker injury markers [(median % of positive cells 1.3 (0.76-3.04) vs 0.58 (0.31-1.8)]. In addition, patients in the early stages of the disease (first non Raynaud's symptom < 5 years ago; n = 10) had higher EPC levels than patients with a longer disease duration [median % of positive cells 1.51 (0.49-3.04) vs 0.715 (0.31-2.5)]. These results are shown in figure 2A to 2C. EPC levels did not depend on the subtype of the disease, current vascular complications or on the age of the patients (Table II).

Multivariate analysis revealed a significant association between EPC levels and disease activity score (95% CI: 1.02-13.14; p = 0.04) but associations between EPC levels and disease duration (95% CI 0.93-10.9; p = 0.06) and between EPC levels and plasma von Willebrand factor concentration (95% CI 0.9-11; p = 0.07) did not reach significance.

For the control group of RA patients, EPC levels correlated with acute phase reactants, as assessed by erythrocyte sedimentation rate (r = 0.58; p = 0.03) and C reactive protein concentration (r = 0.54; p = 0.04); no association with disease duration was observed.

*Quantitative studies of CECs and association with disease phenotype*

Detailed results are provided in Table II. SSc patients had higher values than OA controls for $CD105^+CD102^+$ cells and for $CD105^+CD106^+$ cells. Patients with RA had higher values than OA controls for $CD105^+CD62^+$, $CD105^+CD102^+$

**Table II.** Levels of EPCs and activated CECs in SSc patients and controls.

| Median (range) of % (absolute number) | OA patients (n=15) | SSc patients (n = 32) | Diffuse cutaneous SSc patients (n = 12) | Limited cutaneous SSc patients (n = 20) | RA patients (n =15) |
|---|---|---|---|---|---|
| EPCs: CD34+CD133+ | 0.57 (0.20-1.20) [1410(500-3000)] | 0.92 (0.31-3.04)* [2300(775-7600)] | 0.84 (0.31-2.04) [2100(775-6000)] | 1.03 (0.32-3.04) [2575(800-7600)] | 1.37 (0.54-4.77)* [3425(1350-11925)] |
| CECs: CD105+CD62+ | 2.69 (0.80-4.20) [6710(2000-10500)] | 2.23 (0.41-9.12) [5575(1025-22800)] | 2.23 (0.87-9.12) [5575(2175-22800)] | 2.25 (0.41-6.85) [5625(1025-17125)] | 3.78 (1.29-7.04)* [9440(3225-17600)] |
| CD105+CD102+ | 5.03 (1.01-8.03) [12555(2525-20075)] | 6.03 (2.13-16.85)* [15075(5325-42125)] | 6.04 (2.54-16.85) [15100(6350-42125)] | 5.99 (2.13-12.87) [14975(5325-32175)] | 6.92 (2.05-11.2)* [17300(5100-28000)] |
| CD105+CD106+ | 1.21 (0.48-1.82) [3005(1200-4550)] | 1.87 (0.58-8.64)* [4675(950-21600)] | 2.13 (1.04-8.64) [5325(2600-21600)] | 1.66 (0.38-4.75) [4150(950-11875)] | 2.39 (0.21-7.94)* [5950(525-19650)] |

*Patients with SSc and RA had higher EPC levels than patients with OA (p = 0.01 and 0.0001 respectively) and patients with RA had higher EPC levels than SSc patients (p = 0.03).

*SSc patients had higher CEC levels than OA controls for CD105+CD102+ (p = 0.047) and for CD105+CD106+ (p = 0.004) cells; patients with RA had higher values than OA controls for CD105+CD62+ (0.007), CD105+CD102+ (p = 0.01) and CD105+CD106+ (p = 0.007) cells; patients with RA had higher values than SSc patients for CD105+CD62+ cells (p = 0.03); there was no other difference between SSc and RA patients.

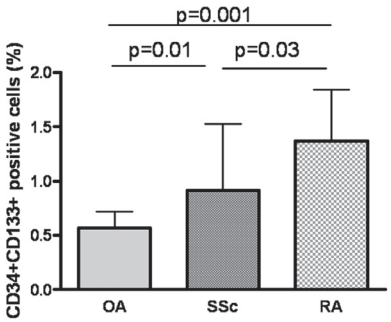


**Fig. 1.** Quantification of EPCs in patients with systemic sclerosis and controls with osteoarthritis and rheumatoid arthritis (median and interquartile range)

and $CD105^+CD106^+$ cells, and patients with RA had higher values than SSc patients for $CD105^+CD62^+$ cells. There was no correlation between EPC and activated CEC levels. Strong correlations were found between the levels of various CECs in SSc and RA patients (data not shown). In SSc patients, no disease phenotype association was observed in particular there was no relationship with current vascular complications.

## Discussion

The main findings of this study were that putative EPC levels are higher in SSc patients than in patients with osteoarthritis but lower than in patients with rheumatoid arthritis and that EPC levels increase with disease activity in SSc.

The development of vascular tissues, supplying blood, is essential for both normal organ development and the pathogenesis of several diseases. Considerable effort has therefore been devoted to unravelling the molecular pathways regulating angiogenesis and vasculogenesis and developing new strategies for treating refractory vascular diseases. There is increasing evidence that adult peripheral blood contains endothelial cell progenitors, which express some endothelial cell markers, migrate to sites of new vessel formation and vascular remodelling and are susceptible to therapeutic modulation (6-9, 22, 23).

Systemic sclerosis is associated with major disturbances in the vascular system and prognosis depends primarily on the severity of vascular lesions. Two studies have previously investigated putative EPCs in SSc, and gave apparently contradictory results (13, 14). Our objective was to clarify the situation regarding EPC levels. As Del Papa *et al*. (13), we detected EPCs on peripheral blood samples by flow cytometry, considering EPCs as $CD34^+CD133^+$ cells. Unlike Kuwana *et al*. (14), we did not perform an immunomagnetic isolation of $CD34^+$ cells before quantification and did not use VEGFR2 combined with CD34 and CD133 to quantify EPCs. Detection of adequate numbers of the rare event population of putative EPCs defined as $CD34^+CD133^+VEGFR2^+$ cells from peripheral blood without enrichment needs a large collection of events (more than $10^6$) (12). Direct analysis of 200 µl of peripheral blood did not permit us to analyse enough events to detect these triple marked cells. Thus, we identified putative EPCs as $CD34^+CD133^+$ (21) in order to improve the detection sensitivity. Moreover, we did not consider CD45 negativity to identify EPCs because some data suggest that EPCs may be positive for this surface marker (11).

We found that our group of SSc patients had more circulating $CD34^+CD133^+$ cells than patients with osteoarthritis. Although levels of EPCs should be investigated in healthy subjects, we have selected OA patients free of any vascular or systemic disease and only treated with paracetamol. We also found that EPC levels were associated with disease activity, endothelial injury and disease duration in univariate analysis. In multivariate analysis, the association with disease activity remained significant, but the associations with disease duration and endothelial injury did not reach significance, probably because of the small number of patients investigated. Disease phenotype associations were not demonstrated in the two previous studies because Kuwana's study (14) included only 11 patients and Del Papa (13) focused on analysis of the circulating endothelial cells. However, Del Papa *et al*. did report higher levels of EPCs in patients in the early stages of SSc. This may be of major importance, because the 11 patients included in Kuwana's study (14) had a median disease duration of 10 years (7-10, 13) and all had chronic ischaemic conditions (pit-

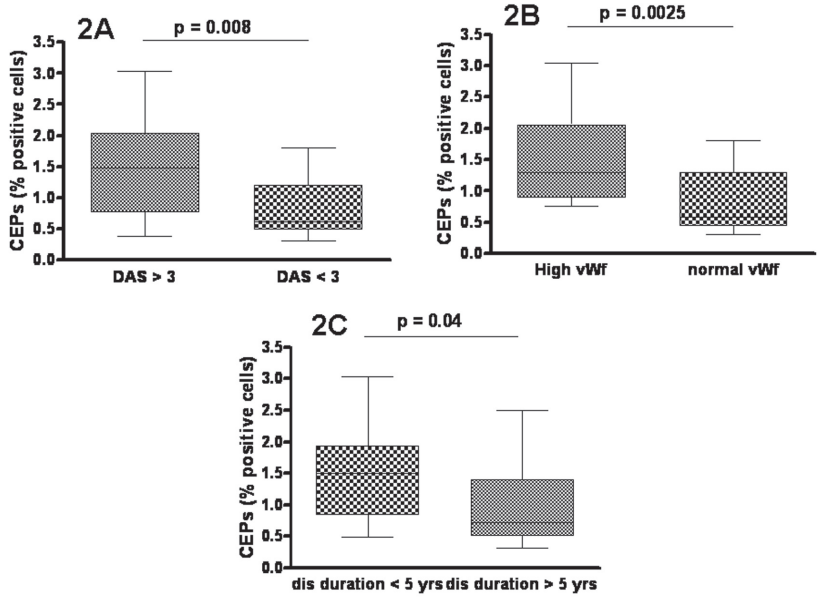


**Fig. 2.** Disease phenotype association with endothelial progenitor cell levels in patients with systemic sclerosis (n = 32; 25th to 75th percentile and median value).
2A: EPC levels as a function of disease activity score (19).
2B: EPC levels as a function of endothelial injury marker levels (plasma Von Willebrand Factor concentrations).
2C: EPC levels as a function of disease duration (less or more than 5 years after the first non Raynaud's symptom).

ting scars, ulcers and gangrene). Thus, our hypothesis is that EPCs are recruited during active disease but that chronic ischaemic conditions may eventually lead to EPC depletion. This speculation will require confirmation with further investigations. The first step in this process would be the isolation of EPCs and analysis of the ability of these cells to differentiate into mature endothelial cells taking into account the disease duration (14). Moreover, some more cell surface markers should be investigated for EPCs evaluation and maturation stage.

Even if we have previously shown that vasodilatators may decrease endothelial injury marker levels (4) and although our patients were studied after washout of these drugs, no conclusion can be driven from our results regarding the influence of this treatment on EPC recruitment. This will require specific investigations.

Our control group of patients with rheumatoid arthritis had very high EPC levels, even higher than those in SSc patients. The RA patients were homogeneous, all displaying high dis-ease activity, resistance to conventional therapy and high-grade inflammation, and we found that EPC levels were correlated with systemic inflammation. These results, combined with previous data suggesting presence of EPCs in the synovial tissue of RA patients and enhanced generation of endothelial cells derived from $CD34^+$ cells of the bone marrow in RA are consistent with the possible contribution of EPCs to synovial neovascularisation (24, 25). Some contradictory results regarding EPC quantification have also been reported in other studies (26, 27) suggesting a low number of EPCs in RA patients. This is consistent with previous findings showing a reduced number of EPCs in patients with diseases associated with an increased risk for cardiovascular events but further studies are needed to clarify these points taking into account the RA disease activity, the presence of the classical cardiovascular risk factors and also the potential effects of RA drugs.

Activated circulating endothelial cell levels were high in SSc patients, as previously reported (13), representing direct evidence of endothelial disease in SSc, as a probable result of shedding from affected walls of the blood vessels (13). This was also found in patients with RA and may reflect the vascular target of this inflammatory disorder. However, CECs were not correlated with EPC levels, suggesting that EPCs and CECs are involved in different biological processes or at different step of the disease.

In conclusion, our results and previous data suggest that EPCs are recruited during active disease in SSc patients but we hypothesize that chronic ischaemic conditions may eventually lead to EPCs depletion. Future studies should investigate the influence of disease duration on the ability of EPCs to differentiate and the predictive value of EPC levels in SSc (28). Such work may lead to the introduction of innovative therapies (29, 30), particularly for certain vascular complications, such as for example refractory ulcers (31).

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