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Decreased expression of neuropilin-1 as a novel key factor contributing to peripheral microvasculopathy and defective

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Decreased expression of neuropilin-1 as a novel key factor contributing to peripheral microvasculopathy and defective angiogenesis in systemic sclerosis

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3 **Decreased expression of neuropilin-1 as a novel key factor contributing to**
4 **peripheral microvasculopathy and defective angiogenesis in systemic sclerosis**
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ABSTRACT

Objectives: In systemic sclerosis (SSc), vascular involvement is characterised by VEGF-A/VEGFR system disturbances. Neuropilin-1 (NRP1), a receptor for both class-3 semaphorins (Sema3s) and VEGF-A, is required for optimal VEGF-A/VEGFR-2 signalling. Here, we investigated the possible involvement of Sema3A/NRP1 axis in SSc.

Methods: Circulating Sema3A and soluble NRP1 (sNRP1) were measured in SSc patients and controls. NRP1 and Sema3A expression in skin biopsies was evaluated by immunofluorescence and Western blotting. NRP1 expression was assessed in SSc and healthy dermal microvascular endothelial cells (SSc-MVECs and H-MVECs), and in SSc and control endothelial progenitor cell (EPC)-derived ECs. The possible impact of transcription factor Fli1 deficiency on endothelial NRP1 expression was investigated by gene silencing. The binding of Fli1 to NRP1 gene promoter was evaluated using chromatin immunoprecipitation. Capillary morphogenesis was performed on Matrigel.

Results: Decreased sNRP1 levels in SSc were associated with active and late nailfold videocapillaroscopy patterns and digital ulcers. No difference in Sema3A was found between patients and controls. NRP1 was significantly decreased in SSc-MVECs both *ex vivo* and *in vitro*. NRP1 and Fli1 significantly decreased in H-MVECs challenged with SSc sera, while they were not different in SSc and control EPC-derived ECs. Fli1 occupied the NRP1 gene promoter and Fli1 gene silencing reduced NRP1 expression in H-MVECs. NRP1 gene silencing in H-MVECs resulted in a significantly impaired angiogenic capacity comparable to that of cells treated with SSc sera.

Conclusions: In SSc, NRP1 deficiency may be an additional factor in the perturbed VEGF-A/VEGFR-2 system contributing to peripheral microvasculopathy and defective angiogenesis.

Keywords: systemic sclerosis, neuropilin-1, Fli1, dermal microvascular endothelial cells, angiogenesis, peripheral microvasculopathy

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is a life-threatening connective tissue disorder of unknown aetiology, characterised by widespread vascular injury and dysfunction, impaired angiogenesis, immune dysregulation and progressive fibrosis of the skin and internal organs [1,2]. The dysregulation of vascular tone control, clinically evident as Raynaud's phenomenon, and microcirculatory abnormalities paralleled by nailfold capillaroscopic changes are the earliest clinical manifestations of SSc and may precede skin and visceral involvement by months or years [1-3]. The whole process is characterised by an uncontrolled regeneration of the microvasculature and subsequent loss of microvessels, due to defects in both vascular repair and expected increase in new vessel growth (angiogenesis), leading to severe peripheral ischaemic manifestations, such as digital ulcers and gangrene [2,3].

Recent studies have highlighted the anatomic and structural similarities between blood vessels and nerves [4]. The two networks are often aligned, with nerve fibers and blood vessels following parallel routes. Furthermore, both systems require precise control over their guidance and growth. Several molecules with attractive and repulsive properties have been found to modulate the guidance both of nerves and blood vessels [4]. These include the neuropilin receptors and their semaphorin ligands, as well as netrins, slits and their receptors [4]. Among these, neuropilin-1 (NRP1) was initially described as an axonally expressed receptor for secreted class-3 semaphorins (Sema3s), a family of soluble molecules which modulate the development of the nervous and vascular systems [5,6]. NRP1 also serves as specific vascular endothelial growth factor-A (VEGF-A) co-receptor on endothelial cells (ECs) and regulates VEGF receptor (VEGFR) signalling, leading to enhanced migration [7] and survival of ECs *in vitro* [8,9]. Furthermore, NRP1 has been implicated in VEGFR-2-mediated endothelial permeability [10] and in VEGF-A-induced three-dimensional EC biology, such as vessel sprouting and branching [11]. The absence of functional NRP1 in mice results in embryonic death due to impaired heart and blood vessel development, thus suggesting that this receptor plays a central regulatory role during developmental angiogenesis [4]. The exact molecular mechanisms by which NRP1 modulates VEGF-A biology remain to be elucidated. It has, however, been shown that NRP1 potentiates the VEGF-A/VEGFR-2 signalling pathways implicated in the migratory response of ECs [12].

The evidence that NRP1 functions as a receptor for both VEGF-A and Sema3s suggests that these latter may also play a role in the modulation of angiogenesis. In particular, it has been reported that Sema3A acts as an antiangiogenic molecule impairing EC adhesion, migration and survival *in vitro* [13-15], and regulates tumour-induced angiogenesis *in vivo* [16]. Moreover, Sema3A null mice

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3 exhibit defects in blood vessel reshaping [15,17,18]. The molecular mechanisms underlying the
4 antiangiogenic effects of Sema3A are complex. Hence, it was initially suggested that Sema3A
5 competes with VEGF-A for NRP1 binding, thus inhibiting VEGF-A-induced angiogenesis.
6 However, recent reports have also shown that Sema3A increases vascular permeability, inhibits EC
7 proliferation, and induces apoptosis even in the absence of VEGF-A, suggesting that Sema3A may
8 activate its own signalling pathways [19,20].
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11 On these bases, we hypothesised that the Sema3A/NRP1 axis might play a role in the pathogenesis
12 of SSc-related microvascular abnormalities. Therefore, the aim of the present study was to
13 investigate whether the levels of Sema3A and NRP1 could be altered in the circulation, skin and
14 ECs of SSc patients, as well as the mechanism explaining the dysregulated expression of these
15 molecules and their possible contribution to the disturbed angiogenesis of SSc.
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25 **METHODS**

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27 An extended methods section is provided in the online supplementary material.
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29 **Patients, controls, serum samples and skin biopsies**

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31 Serum samples were obtained from 49 SSc patients [1] (45 women and 4 men; median age 64 years,
32 range 37 to 80 years, and median disease duration 10 years, range 2 to 31 years) classified as
33 limited cutaneous SSc (lcSSc; n=32) or diffuse cutaneous SSc (dcSSc; n=17) [21], and from 39
34 age-matched and sex-matched healthy individuals. All patients were clinically assessed as described
35 elsewhere [3,22,23]. Clinicodemographic characteristics of SSc patients used for collection of
36 serum samples are shown in online supplementary table S1. Full-thickness skin biopsies were
37 obtained from the clinically involved skin of one-third of the distal forearm of 18 patients with SSc
38 (15 women, 3 men; median age 48.5 years, range 29 to 73 years, and median disease duration
39 7.2 years, range 1 to 18 years). Skin samples from the same forearm region of 11 age-matched and
40 sex-matched healthy donors were used as controls. Each skin biopsy was divided into two
41 specimens and processed for immunohistochemistry and biomolecular analysis as described
42 elsewhere [23]. The study was approved by the local institutional review board at the Azienda
43 Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, and all subjects provided written
44 informed consent.
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Isolation, culture and stimulation of dermal microvascular endothelial cells (MVECs)

Dermal MVECs were isolated from biopsies of the involved forearm skin from 5 patients with dcSSc and from 5 healthy subjects, as described elsewhere [3,23]. MVECs from healthy subjects (H-MVECs) and SSc patients (SSc-MVECs) were used between the third and seventh passages in culture. For stimulation experiments, H-MVECs were grown to 70% confluence, and then were washed three times with serum-free medium and serum-starved overnight in MCDB medium supplemented with 2% FBS. Medium was removed and cells were incubated with 2% FBS-MCDB medium containing recombinant human VEGF-A165 (10 ng/ml; R&D Systems, Minneapolis, Minnesota, USA), or 10% serum from SSc patients (n = 5) and healthy subjects (n = 5) for 24 hours.

Late-outgrowth peripheral blood endothelial progenitor cell (EPC)-derived ECs

Late-outgrowth EPC-derived ECs were obtained from the peripheral blood of 15 SSc patients (13 women and 2 men; n=9 with lcSSc and n=6 with dcSSc; median age 60 years, range 42 to 78 years) and 8 healthy individuals (all women; median age 55 years, range 30 to 65 years), as described elsewhere [3,24,25].

Enzyme-linked immunosorbent assay (ELISA) for serum Sema3A and soluble NRP1 (sNRP1)

The levels of Sema3A and sNRP1 in serum samples were measured by commercial quantitative colorimetric sandwich ELISA (catalogue number ABIN481720 and ABIN415191, respectively; Antibodies-on line, Atlanta, Georgia, USA) according to the manufacturer's protocol. Each sample was measured in duplicate.

Immunofluorescence

Immunofluorescence on paraffin-embedded skin sections was performed as previously described [3]. For primary and secondary antibodies, refer to the online supplementary material.

Western blotting

Proteins were extracted from skin biopsies, dermal MVECs and late-outgrowth peripheral blood EPC-derived ECs as described elsewhere [3,25,26]. Western blotting was carried out according to previously published protocols [3]. For primary antibodies, refer to the online supplementary material.

Gene silencing of Friend leukaemia integration 1 (Fli1) and NRP1

MVECs were seeded shortly before transfection. The cells were transfected with 10 nM of Fli1 small interfering RNA (siRNA), 10 nM of NRP1 siRNA or non-silencing scrambled RNA (SCR) (Santa Cruz Biotechnology, Dallas, Texas, USA) using HiPerfect transfection reagent (Qiagen, Milan, Italy) for 72 hours.

RNA purification, cDNA synthesis and quantitative real-time PCR

Total RNA isolation from MVECs, first strand cDNA synthesis and mRNA quantification by SYBR Green real-time PCR was performed as reported elsewhere [27]. For predesigned oligonucleotide primer pairs obtained from Qiagen, refer to the online supplementary material.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as previously described [28] using a rabbit polyclonal anti-Fli1 antibody (catalogue number ab15289, Abcam, Cambridge, UK). Putative Fli1 transcription factor binding site was predicted by Tfsitescan. The primers were as follows: NRP1 Forward, 5'-CTAGGGGTGCAGAGCGAG-3'; NRP1 Reverse, 5'-GAAGGAAGGCGCTGGGAG-3'.

In vitro capillary morphogenesis assay

In vitro capillary morphogenesis assay on Matrigel was performed according to previously published protocols [27] as detailed in the online supplementary material.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 20.0 (SPSS, Chicago, Illinois, USA). Data are expressed as mean \pm SD or median and interquartile range (IQR). The Student's *t*-test and nonparametric Mann-Whitney *U*-test were used where appropriate for statistical evaluation of the differences between two independent groups. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Serum Sema3A and sNRP1 levels in SSc

No significant differences in serum levels of Sema3A were detected between SSc patients (median 2.22 ng/ml, IQR 1.84 to 3.17 ng/ml) and healthy controls (median 3.86 ng/ml, IQR 1.64 to 4.73

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3 ng/ml) (figure 1A). Circulating sNRP1 levels were significantly reduced in SSc patients (median
4 0.22 ng/ml, IQR 0.0 to 0.6 ng/ml) compared with healthy individuals (median 0.69 ng/ml, IQR 0.0
5 to 2.5 ng/ml; $p=0.001$) (figure 1B). Next, we evaluated the possible correlation of serum sNRP1
6 levels with the nailfold videocapillaroscopy (NVC) pattern as a measure of peripheral
7 microvascular involvement. sNRP1 levels were significantly decreased either in SSc patients with
8 active (median 0.14 ng/ml, IQR 0.0 to 0.4 ng/ml) or late (median 0.09 ng/ml, IQR 0.0 to 0.72
9 ng/ml) NVC patterns than in controls ($p=0.003$ and $p=0.01$, respectively) (figure 1C). Conversely,
10 no difference in serum sNRP1 was found between SSc patients with early NVC pattern (median
11 0.45 ng/ml, IQR 0.22 to 0.66 ng/ml) and healthy controls (figure 1C). Moreover, sNRP1 levels were
12 significantly decreased in SSc patients with digital ulcers (median 0.06 ng/ml, IQR 0.0 to 0.27
13 ng/ml) compared both with patients without digital ulcers (median 0.43 ng/ml, IQR 0.17 to 0.71
14 ng/ml; $p=0.009$) and controls ($p=0.001$) (figure 1D). No significant association was found with
15 other clinicodemographic and laboratory parameters or with clinical SSc subset.
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26 **Decreased expression of NRP1 in SSc dermal ECs *ex vivo* and *in vitro***

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28 The expression of Sema3A and NRP1 protein in forearm skin biopsies from SSc patients and
29 controls was investigated by immunofluorescence and Western blot. No significant differences in
30 Sema3A expression were detected between SSc and control skin (figure 2A-C). On the contrary,
31 NRP1 expression was decreased in clinically affected skin biopsies from SSc patients compared to
32 healthy skin, in particular in dermal ECs and perivascular stromal cells (figure 3A-F). The
33 localisation of NRP1 staining in vascular ECs was confirmed by NRP1/CD31 double
34 immunofluorescence staining (figure 3C,F). Moreover, Western blot analysis confirmed that NRP1
35 protein expression levels were significantly reduced in SSc skin in respect to control skin ($p<0.001$)
36 (figure 3G,H).
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39 Western blot analysis on cultured dermal MVECs revealed that NRP1 protein expression levels
40 were significantly reduced in SSc-MVECs compared to H-MVECs ($p<0.005$) (figure 4A).
41 Moreover, NRP1 expression in H-MVECs significantly increased after treatment with healthy sera
42 compared to basal condition, while it decreased after challenging with SSc sera (both $p<0.005$ vs
43 basal H-MVECs). As expected, stimulation with recombinant human VEGF-A165 strongly
44 upregulated NRP1 expression in H-MVECs ($p<0.005$ vs basal H-MVECs) (figure 4A). On the
45 contrary, no obvious differences in NRP1 protein levels could be found between late-outgrowth
46 EPC-derived ECs from SSc patients and healthy controls (figure 4B).
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Fli1 deficiency contributes to the downregulation of NRP1 gene in SSc-MVECs

We next examined the potential mechanism by which NRP1 expression is downregulated in dermal SSc microvessels. As the expression of the transcription factor Fli1 is markedly downregulated at least partially *via* an epigenetic mechanism in SSc dermal ECs, and experimental endothelial Fli1 deficiency reproduces the histopathological and functional abnormalities characteristic of SSc vasculopathy [28-30], we hypothesised that endothelial Fli1 deficiency could inhibit the expression of NRP1 in SSc-MVECs.

First, we analysed Fli1 protein expression in cultured dermal MVECs and late-outgrowth EPC-derived ECs from SSc patients and controls (figure 4C,D). As shown in figure 4C, Fli1 protein expression in H-MVECs closely paralleled that of NRP1 in the different experimental conditions assayed, and was strongly downregulated in SSc-MVECs. Similarly to what observed for NRP1 expression, Fli1 protein levels did not differ between SSc and control late-outgrowth EPC-derived ECs (figure 4D).

Moreover, we examined the effect of Fli1 gene silencing on mRNA levels of the NRP1 gene in H-MVECs. As displayed in figure 4E, gene silencing of Fli1 significantly suppressed the mRNA expression levels of the NRP1 gene in H-MVECs ($p < 0.01$). In addition, ChIP analysis revealed that Fli1 occupied the promoter region of the NRP1 gene in H-MVECs (figure 4F). These results indicate that Fli1 directly targets the NRP1 gene promoter and is required for homeostatic NRP1 expression in ECs.

NRP1 deficiency contributes to the impaired angiogenesis of SSc-MVECs

To verify whether endothelial NRP1 deficiency has a role in the modulation of angiogenesis, we carried out *in vitro* capillary morphogenesis on Matrigel matrix. Consistent with previous findings [26], capillary morphogenesis was significantly impaired in SSc-MVECs compared with H-MVECs ($p < 0.01$) (figure 5). H-MVECs stimulated with healthy sera produced an abundant network of branching cords (figure 5). On the contrary, as previously reported [31,32] angiogenesis was significantly reduced upon challenge with SSc sera ($p < 0.01$ vs basal H-MVECs) (figure 5). The addition of recombinant human VEGF-A165 or anti-VEGF-A165b blocking antibodies to SSc sera significantly increased H-MVEC angiogenesis compared with cells treated with SSc sera alone (both $p < 0.05$) (figure 5). NRP1 gene silencing in H-MVECs resulted in a significant impairment of angiogenic capacity comparable to that of cells treated with SSc sera ($p < 0.01$ vs basal H-MVECs) (figure 5). Stimulation of NRP1-silenced H-MVECs with recombinant human proangiogenic

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3 VEGF-A165 or antiangiogenic VEGF-A165b could only slightly increase or decrease angiogenesis,
4 respectively (figure 5).
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8 9 10 **DISCUSSION**

11 Here, we investigated for the first time the possible involvement of the *Sema3A/NRP1* axis in the
12 pathogenesis of SSc. Our present findings clearly demonstrate that serum levels and dermal
13 expression of NRP1 are significantly decreased in SSc patients and that lower circulating sNRP1
14 levels correlate with the severity of NVC abnormalities and the presence of digital ulcers. In
15 contrast to constitutive endothelial expression of NRP1 in healthy skin, NRP1 was found to be
16 strongly reduced *ex vivo* in SSc dermal microvessels, and NRP1 downregulation was maintained *in*
17 *vitro* in MVECs obtained from SSc dermis. On the contrary, we could not find any difference in
18 NRP1 protein levels between peripheral blood EPC-derived ECs from SSc patients and healthy
19 controls. This evidence suggests that the dysregulated expression of this receptor is restricted to
20 locally injured microvasculature in an overt disease without affecting bone marrow-derived
21 circulating endothelial progenitors. As far as *Sema3A* is concerned, no difference in its expression
22 was observed between SSc and controls either in the circulation or in the cutaneous tissue.
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31 The importance of NRP1 for vascular development is well established and shown by the generation
32 of knockout mice, which display an embryonic lethal phenotype characterised by severe vascular
33 defects due to impaired angiogenic sprouting and branching very much resembling the disturbed
34 vessel morphology seen in SSc patients [4,33,34]. Moreover, conditional NRP1 knockout in ECs is
35 associated with important cardiac and vascular defects, thus suggesting a crucial role for NRP1 in
36 EC functions [35]. Even though NRP1 was originally identified as an adhesion molecule in the
37 nervous system, it is more commonly studied as receptor for the neuronal guidance molecule
38 *Sema3A* and as co-receptor for the VEGF-A165/VEGFR-2 complex with key roles in neuronal and
39 vascular development [12,36]. Of note, several studies have implicated a dysfunctional VEGF-
40 A/VEGFR-2 system in the impaired angiogenic process characteristic of SSc [2,26,37-39].
41 Moreover, besides VEGF-A, it is well known that a dysregulated expression of a large array of
42 proangiogenic and antiangiogenic (angiostatic) factors present in the circulation of SSc patients may
43 be mostly responsible for such angiogenic deficit [2,37-40].
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53 In our study, not only we observed that the proangiogenic NRP1 receptor was constitutively
54 downregulated in dermal SSc-MVECs, but also that treatment with SSc sera could significantly
55 reduce NRP1 expression in H-MVECs, which is in line with the reported antiangiogenic properties
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3 of SSc sera [2,31,32,37,41]. Strikingly, we also found that NRP1 gene silencing in H-MVECs
4 resulted in a significantly impaired angiogenic process comparable to that of cells treated with SSc
5 sera, further supporting the implication of NRP1 deficiency in the disturbed angiogenesis of SSc.
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8 Consistent with previous studies, stimulation with recombinant proangiogenic VEGF-A165
9 strongly upregulated NRP1 expression in H-MVECs suggesting that this growth factor cannot only
10 activate ECs directly, but can also contribute to angiogenesis by a mechanism that involves
11 upregulation of its homologous receptor NRP1 [36]. The findings of NRP1 downregulation in H-
12 MVECs upon challenge with SSc sera are in agreement with the evidence that the majority of
13 VEGF-A detected in SSc circulation is not the proangiogenic VEGF-A165, but rather the
14 antiangiogenic VEGF-A165b isoform [26,42]. Further, it has been reported that VEGF-A165b is
15 unable to bind the co-receptor NRP1 because the basic carboxyterminal amino acids essential for
16 NRP1 binding are absent in this splice variant [43,44]. Interestingly, we observed that stimulation
17 of H-MVECs silenced for the NRP1 co-receptor with proangiogenic VEGF-A165 slightly increased
18 angiogenesis, while stimulation with VEGF-A165b isoform resulted only in a slight decrease in
19 their angiogenic capacity. These data are consistent with the antiangiogenic action of VEGF-A165b
20 being mainly dependent on its inability to recruit VEGFR-2/NRP1 co-receptor complex and activate
21 downstream signalling. In a recent study, it was demonstrated that VEGF-A165 and VEGF-A165b
22 may control the balance between VEGFR-2 recycling, degradation and signalling. In particular, due
23 to the lack of NRP1 co-receptor binding, VEGF-A165b may induce differential intracellular
24 vesicular trafficking of VEGFR-2 toward the degradative pathway [45]. Thus, both a switch from
25 the proangiogenic to the antiangiogenic VEGF-A isoform and the concomitant NRP1 co-receptor
26 downregulation may have a crucial role in the insufficient angiogenic response found in SSc (figure
27 6). Indeed, here we also demonstrated that both the addition of recombinant human VEGF-A165 or
28 anti-VEGF-A165b blocking antibodies could significantly dampen the antiangiogenic effects of SSc
29 sera on H-MVECs.
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32 The clinical correlation of serum sNRP1 levels with the severity of SSc-related peripheral
33 microvasculopathy also deserves discussion. Indeed, circulating levels of sNRP1 progressively
34 decreased reaching the lowest values in SSc patients with the active and late NVC patterns, which
35 are characterised by severe architectural changes of microvessels and progressive capillary loss with
36 formation of avascular areas [46]. In addition, patients with active/late NVC patterns and digital
37 ulcers showed serum sNRP1 levels significantly lower than healthy controls, whereas sNRP1 levels
38 did not differ significantly between controls and SSc patients with early NVC pattern and lack of
39 digital ulcers. However, since sNRP1 may be largely released by ECs, we should also consider that
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3 the reduction in circulating levels of sNRP1 might be either a cause or a consequence of the disease,
4 which is characterised by progressive loss of the peripheral microcirculation [2,3,46]. Circulating
5 levels of sNRP1 could even serve as a biomarker reflecting the severity and progression of SSc
6 microvasculopathy. Accordingly, further prospective studies on larger cohorts of SSc patients are
7 warranted.
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11 Finally, our mechanistic findings indicate that in SSc, endothelial NRP1 expression is suppressed at
12 least partially due to Fli1 transcription factor deficiency. In fact, here we provide the first evidence
13 that NRP1 is a member of the angiogenesis-related gene program regulated by Fli1 in dermal
14 MVECs. In this context, the impact of Fli1 deficiency in the loss of EC integrity and the
15 development of peripheral microvasculopathy during SSc has been well established [28-30]. Of
16 note, it has been demonstrated that in SSc Fli1 expression is markedly suppressed at least partially
17 through an epigenetic mechanism [29,30]. Thus, such an epigenetic modification might partly
18 explain the persistence of the multiple downstream effects of Fli1 deficiency in an *in vitro* culture
19 system, as supported by the downregulation of endothelial NRP1 observed *ex vivo* in SSc dermal
20 microvessels and maintained in cultured dermal SSc-MVECs. Interestingly, NRP1 was found to be
21 also a target of the antiangiogenic microRNA miR-320, and a dysregulated microRNA profile is
22 being increasingly reported in SSc [47,48].
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31 In conclusion, we shed light on NRP1 deficiency as a novel key factor contributing to peripheral
32 microvasculopathy and defective angiogenesis in SSc. Further studies are warranted to decipher
33 whether therapeutic modulation of VEGF-A/VEGFR-2/NRP1 co-receptor signalling might pave the
34 way to boost angiogenesis and block the progression of peripheral microvasculopathy in SSc.
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43 **Author contributions**

44
45 Study conception and design: ER, MM, LI-M, MM-C, SG. Acquisition of data: ER, IC, MM, CM,
46 IR, SB-R, JB, JA, YA. Interpretation of data: ER, IC, MM, RS, LI-M, MM-C, SG. Manuscript
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Competing interests

None declared.

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10 11 12 13 FIGURE LEGENDS

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16 **Figure 1.** Serum levels of semaphorin3A (Sema3A) and soluble neuropilin-1 (sNRP1) determined
17 by colorimetric sandwich ELISA. (A) Serum Sema3A levels in healthy controls and patients with
18 systemic sclerosis (SSc). (B) Serum sNRP1 levels in healthy controls and SSc patients. (C) Serum
19 sNRP1 levels in healthy controls and patients with SSc according to nailfold videocapillaroscopy
20 (NVC) pattern (early, active and late). (D) Serum sNRP1 levels in healthy controls and patients
21 with SSc according to the presence/absence of digital ulcers (DU). Data are shown as box plots.
22 Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines
23 outside the boxes represent the 10th and the 90th percentiles. Circles indicate outliers, and asterisks
24 indicate the extreme values. Mann-Whitney *U*-test was used for statistical analysis.
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28 **Figure 2.** Expression of semaphorin3A (Sema3A) in skin biopsies. (A and B) Representative
29 microphotographs of skin sections from (A) healthy controls (n = 11) and (B) systemic sclerosis
30 (SSc) patients (n = 18) immunostained for Sema3A (red) and counterstained with 4',6-diamidino-2-
31 phenylindole (DAPI; blue) for nuclei. Arrows indicate microvessels. Original magnification: x40.
32 Scale bar = 50 μ m. (C) Western blotting of total protein extracts from the skin of healthy subjects (n
33 = 10) and SSc patients (n = 15). A protein band with the expected molecular weight of 90 kDa was
34 detected with the anti-Sema3A antibody. Representative immunoblots are shown. The densitometric
35 analysis of the bands normalised to α -tubulin is reported in the histograms. Data are mean \pm SD of
36 optical density in arbitrary units (a.u.).
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39 **Figure 3.** Expression of neuropilin-1 (NRP1) in skin biopsies. (A, B, D, and E) Representative
40 microphotographs of skin sections from (A and B) healthy controls (n = 11) and (D and E) systemic
41 sclerosis (SSc) patients (n = 18) immunostained for NRP1 (red) and counterstained with 4',6-
42 diamidino-2-phenylindole (DAPI; blue) for nuclei. Arrows indicate microvessels. (C and F)
43 Representative microphotographs of skin sections from healthy controls (C) and SSc patients (F)
44 double immunostained for NRP1 (red) and the pan-endothelial cell marker CD31 (green) and
45 counterstained with DAPI (blue). Original magnification: x20 (A and D), x40 (B and E), x100 (C
46 and F). Scale bar = 100 μ m (A and D), 50 μ m (B and E), 10 μ m (C and F). (G and H) Western
47 blotting of total protein extracts from the skin of healthy subjects (n = 10) and SSc patients (n = 15).
48 A protein band with the expected molecular weight of 103 kDa was detected with the anti-NRP1
49 antibody. Representative immunoblots are shown. The densitometric analysis of the bands
50 normalised to α -tubulin is reported in the histograms. Data are mean \pm SD of optical density in
51 arbitrary units (a.u.).
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55 **Figure 4.** (A-D) Expression of neuropilin-1 (NRP1) and Friend leukaemia integration 1 (Flt1) in
56 dermal microvascular endothelial cells (MVECs) and late-outgrowth peripheral blood endothelial
57 progenitor cell (EPC)-derived endothelial cells. (A and C) Western blotting of total protein extracts
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3 from healthy MVECs (H-MVECs) at basal condition and treated with recombinant human vascular
4 endothelial growth factor-A165 (VEGF-A165), or 10% serum from SSc patients (n = 5) and healthy
5 subjects (n = 5) for 24 hours, and from basal systemic sclerosis MVECs (SSc-MVECs) assayed
6 with anti-NRP1 (A) and anti-Fli1 (C) antibodies. Representative immunoblots are shown. The
7 densitometric analysis of the bands normalised to α -tubulin is reported in the histograms. Data are
8 mean \pm SD of optical density in arbitrary units (a.u.). Student's *t*-test was used for statistical
9 analysis. **p*<0.005 vs. basal H-MVECs. Results are representative of three independent experiments
10 performed with each one of the 5 H-MVEC and 5 SSc-MVEC lines. (B and D) Western blotting of
11 protein lysates from control (n = 8) and SSc (n = 15) late-outgrowth EPC-derived endothelial cells
12 assayed with anti-NRP1 (B) and anti-Fli1 (D) antibodies. Representative immunoblots are shown.
13 The densitometric analysis of the bands normalised to α -tubulin is reported in the histograms. Data
14 are mean \pm SD of optical density in arbitrary units (a.u.). (E) mRNA levels of the Fli1 and NRP1
15 genes in H-MVECs transfected with Fli1 siRNA or non-silencing scrambled RNA (SCR) were
16 measured by quantitative real-time PCR and normalised to mRNA levels of the 18S ribosomal
17 RNA gene. The relative values compared with SCR are expressed as mean \pm SD of three
18 independent experiments. Statistical analysis was carried out with Student's *t*-test. **p*<0.01 vs. SCR.
19 (F) Chromatin was isolated from H-MVECs and immunoprecipitation was conducted with rabbit
20 anti-Fli1 antibody or rabbit IgG. To exclude exogenous DNA contamination, negative controls were
21 obtained by omitting the cell preparation in some specimens. PCR amplification was carried out
22 using NRP1 promoter-specific primers. One representative of three independent experiments is
23 shown.
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27 **Figure 5.** *In vitro* angiogenesis. (A) Representative images of capillary morphogenesis on Matrigel
28 after 24 hours. (B) Capillary morphogenesis of healthy and systemic sclerosis (SSc) dermal
29 microvascular endothelial cells (H-MVECs and SSc-MVECs, respectively) quantified as percent
30 field occupancy of capillary projections. Capillary morphogenesis of H-MVECs was evaluated at
31 basal condition and after stimulation with sera from healthy subjects (n = 5) and SSc patients (n =
32 5), these latter alone or in combination with recombinant human vascular endothelial growth factor-
33 A165 (VEGF-A165) or anti-VEGF-A165b blocking antibodies. Stimulation with VEGF-A165
34 added to basal medium was used as positive control of angiogenesis. H-MVECs transfected with
35 NRP1 siRNA, at basal condition or challenged with recombinant human VEGF-A165 or VEGF-
36 A165b, or non-silencing scrambled RNA (SCR), were also assayed. Capillary morphogenesis of H-
37 MVECs at basal condition was set to 100%; the other results are normalised to this value. Data are
38 the mean \pm SD of three independent experiments performed in triplicate with each one of the 5 H-
39 MVEC and 5 SSc-MVEC lines. Six to 9 photographic fields from 3 plates were scanned for each
40 experimental point. Student's *t*-test was used for statistical analysis. **p*<0.01 vs. basal H-MVECs;
41 #*p*<0.05 vs. H-MVECs challenged with SSc sera. (C) mRNA levels of the NRP1 gene in H-MVECs
42 transfected with NRP1 siRNA or non-silencing SCR were measured by quantitative real-time PCR
43 and normalised to mRNA levels of the 18S ribosomal RNA gene. The relative values compared
44 with SCR are expressed as mean \pm SD of three independent experiments. Statistical analysis was
45 carried out with Student's *t*-test. **p*<0.01 vs. SCR.
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49 **Figure 6.** Schematic illustration of the potential mechanisms steering dysregulated angiogenesis in
50 systemic sclerosis (SSc). SSc is characterised by progressive loss of peripheral microvasculature
51 and lack of compensatory angiogenesis. In most angiogenic states, vascular endothelial growth
52 factor-A165 (VEGF-A165) interacts with VEGF receptor-2 (VEGFR-2) *via* the VEGF homology
53 domain (VHD) and with the b1 and b2 domains of neuropilin-1 (NRP1) *via* exons 7 and 8. Co-
54 expression of NRP1 and VEGFR-2 enhances VEGF-A165 binding to VEGFR-2, VEGFR-2
55 phosphorylation and downstream signalling cascades. In nonangiogenic conditions, such as SSc,
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3 both a switch from proangiogenic VEGF-A165 to antiangiogenic VEGF-A165b isoform, which is
4 unable to bind the co-receptor NRP1, and concomitant NRP1 downregulation may result in an
5 insufficient tyrosine phosphorylation/activation of VEGFR-2 and incomplete or transient
6 downstream signalling along with a differential intracellular vesicular trafficking of VEGFR-2
7 towards the degradative pathway, ultimately leading to an impaired angiogenic response.
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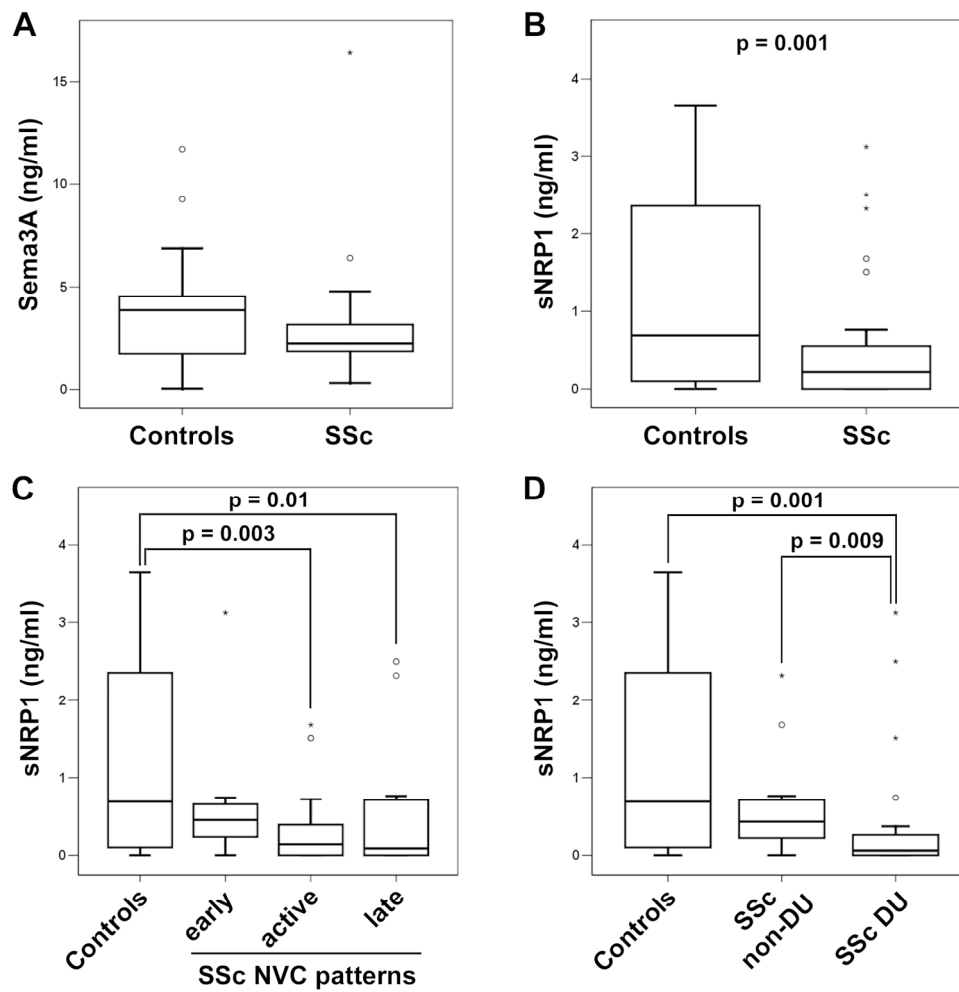


Figure 1
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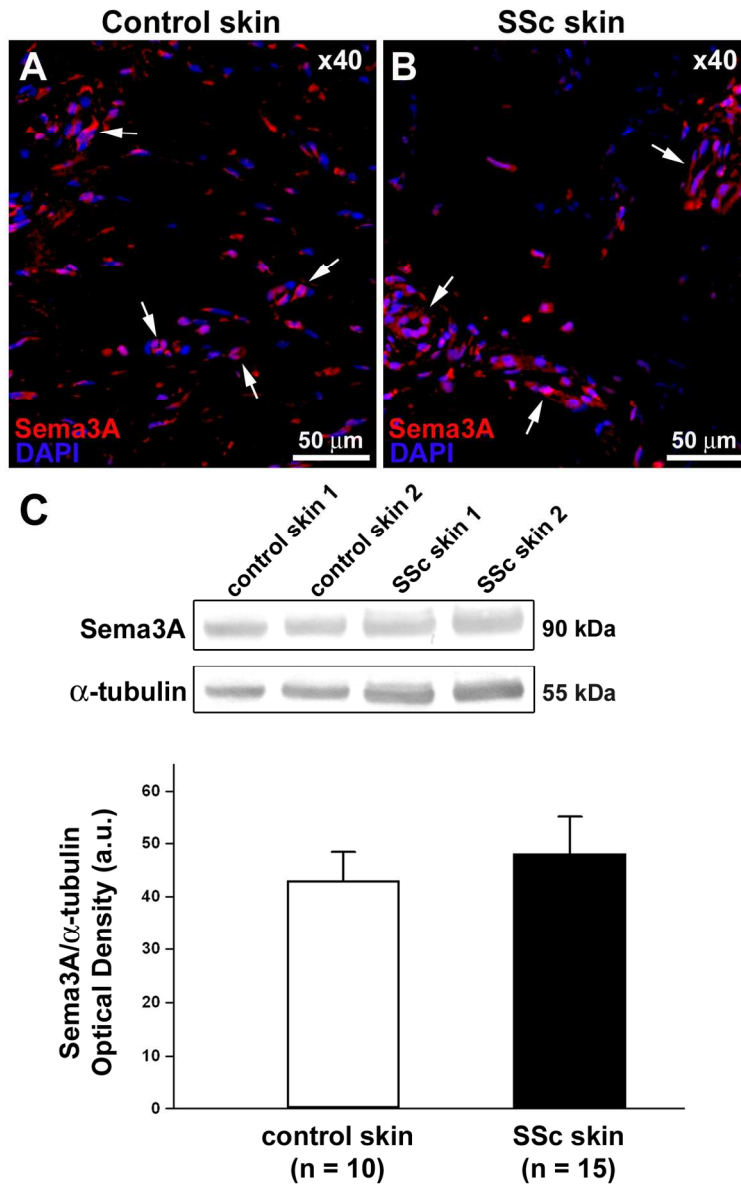


Figure 2
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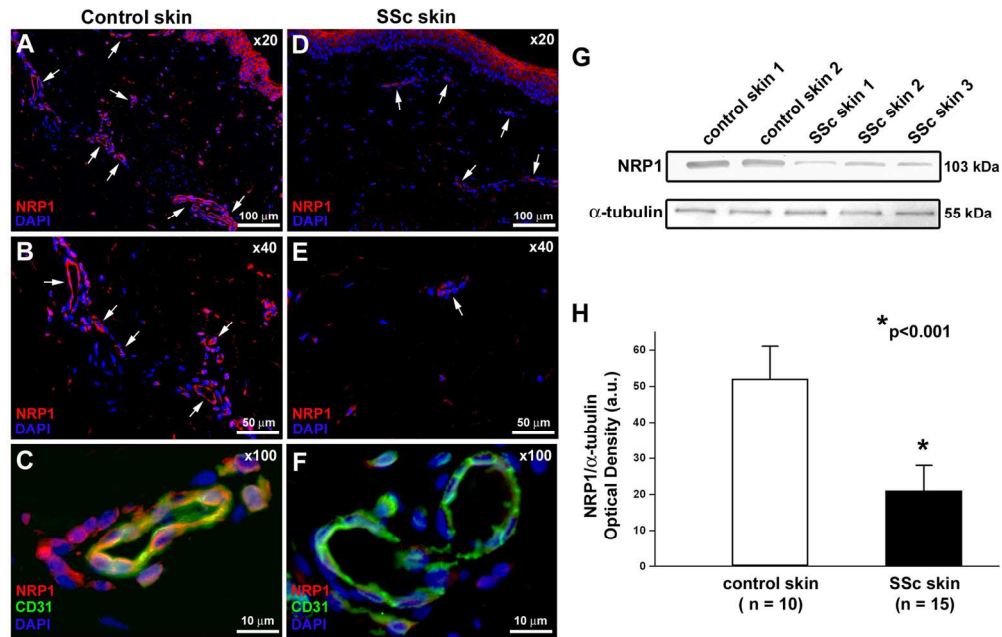


Figure 3
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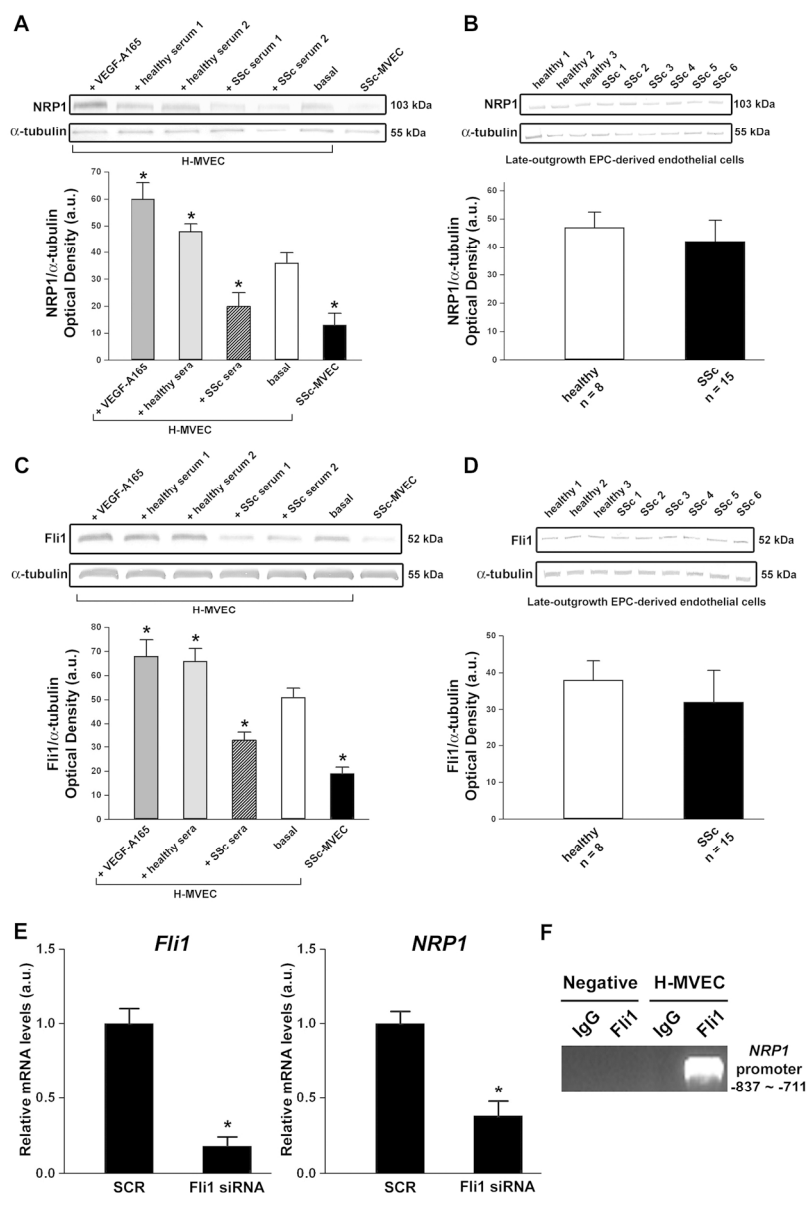


Figure 4
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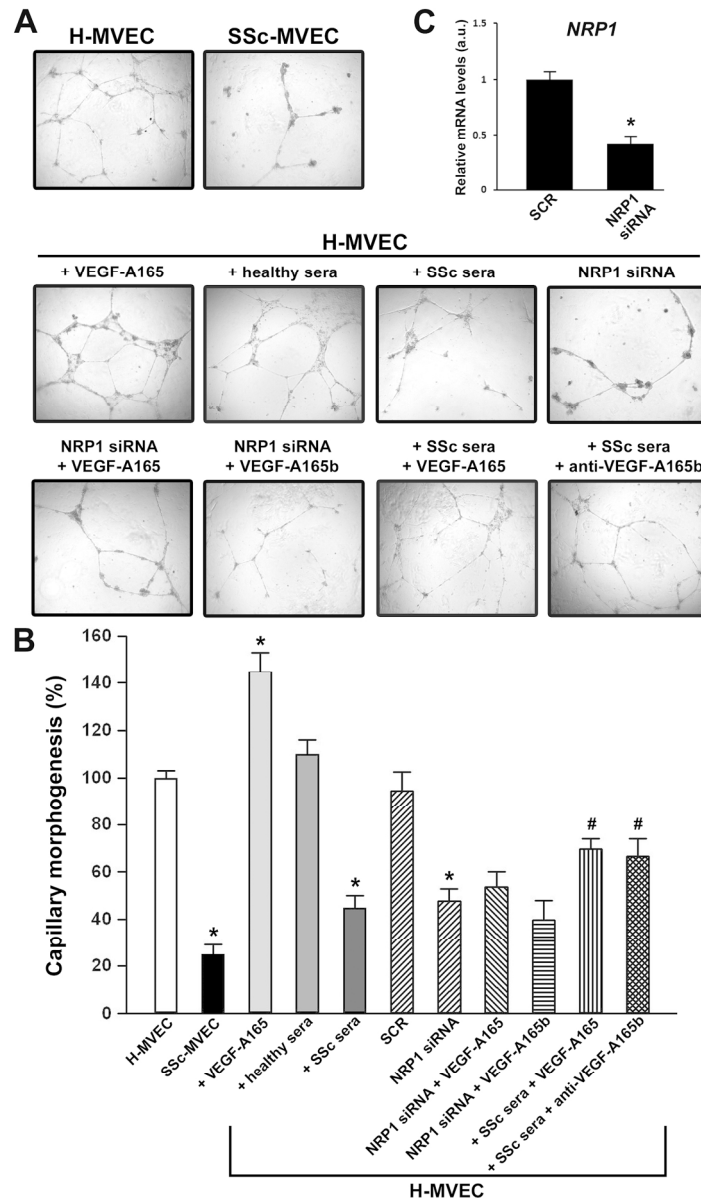


Figure 5
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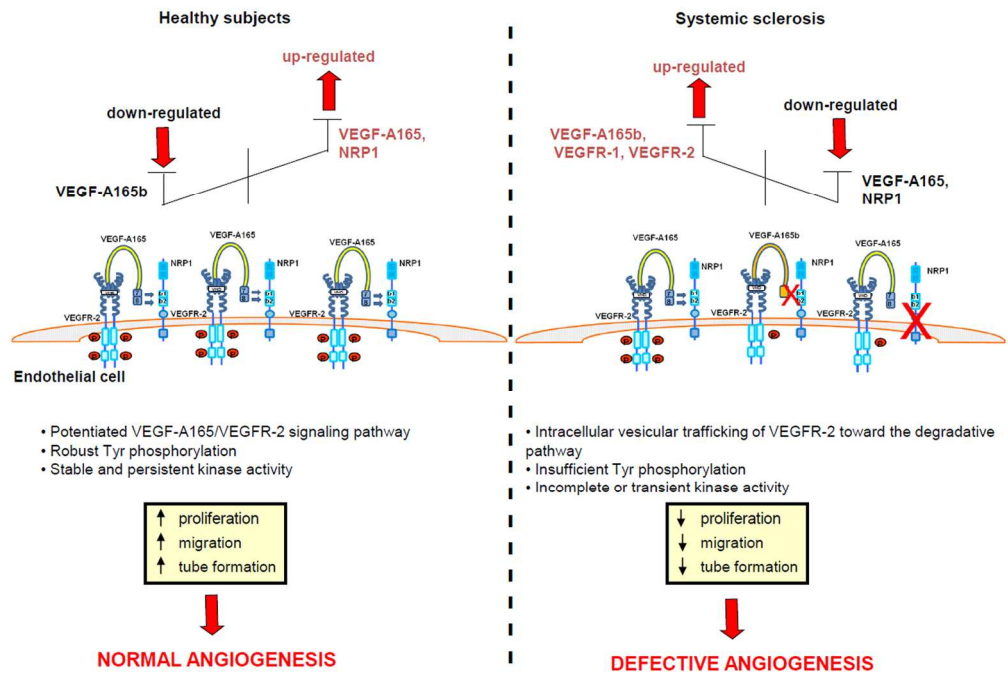


Figure 6
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Pre-Review Only

Online supplementary material

METHODS

Patients, controls, serum samples and skin biopsies

Serum samples were obtained from 49 systemic sclerosis (SSc) patients [1] (45 women and 4 men; median age 64 years, range 37 to 80 years, and median disease duration 10 years, range 2 to 31 years) classified as limited cutaneous SSc (lcSSc; n=32) or diffuse cutaneous SSc (dcSSc; n=17) [2], and from 39 age-matched and sex-matched healthy individuals. All patients reported the occurrence of Raynaud's phenomenon. At the time blood was drawn, the presence of digital ulcers was recorded. Nailfold videocapillaroscopy (NVC) was performed on all 10 fingers by a single rheumatologist and images were scored blindly to divide patients into three capillaroscopic patterns (i.e., early, active and late) [3,4]. Clinicodemographic characteristics of SSc patients used for collection of serum samples are shown in online supplementary table S1. Patients were not on immunosuppressive medications, corticosteroids or other disease-modifying drugs. Before blood sampling, they were washed out for 10 days from oral vasodilating drugs and for 2 months from intravenous prostanoids. Fresh venous blood samples were drawn, left to clot for 30 minutes before centrifugation at 1,500 g for 15 minutes, and serum was collected and stored in aliquots at -80°C until used. Full-thickness skin biopsies were obtained from the clinically involved skin of one-third of the distal forearm of 18 patients with SSc (15 women, 3 men; median age 48.5 years, range 29 to 73 years, and median disease duration 7.2 years, range 1 to 18 years). Skin samples from the same forearm region of 11 age-matched and sex-matched healthy donors were used as controls. Each skin biopsy was divided into two specimens and processed for immunohistochemistry and biomolecular analysis, respectively. For immunohistochemistry, the specimens were fixed in 10% buffered formalin, dehydrated in graded alcohol series and embedded in paraffin. For protein extraction, skin specimens were immediately immersed in liquid nitrogen and stored at -80°C until use. The study was approved by the local institutional review board at the Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, and all subjects provided written informed consent.

Isolation, culture and stimulation of dermal microvascular endothelial cells (MVECs)

Dermal MVECs were isolated from biopsies of the involved forearm skin from 5 patients with dcSSc and from 5 healthy subjects, as described elsewhere [4,5]. Patients were not taking immunosuppressive or disease-modifying drugs at the time of biopsy. Briefly, skin biopsies were

mechanically cleaned of epidermis and adipose tissue in order to obtain a pure specimen of vascularised dermis, and were treated as previously described [4]. Colonies of polygonal elements were detached and CD31-positive cells were subjected to immunomagnetic isolation. Isolated cells were further identified as MVECs by labelling with anti-factor VIII-related antigen and anti-CD105, followed by reprobing with anti-CD31 antibodies. MVECs were maintained in MCDB medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 30% fetal bovine serum (FBS), 20 µg/ml endothelial cell growth supplement (Calbiochem, Nottingham, UK), 10 µg/ml hydrocortisone, 15 IU/ml heparin, and antibiotics. MVECs from healthy subjects (H-MVECs) and SSc patients (SSc-MVECs) were used between the third and seventh passages in culture. For stimulation experiments, H-MVECs were grown to 70% confluence, and then were washed three times with serum-free medium and serum-starved overnight in MCDB medium supplemented with 2% FBS. Medium was removed and cells were incubated with 2% FBS-MCDB medium containing recombinant human vascular endothelial growth factor (VEGF)-A165 (10 ng/ml; R&D Systems, Minneapolis, Minnesota, USA), or 10% serum from SSc patients (n = 5) and healthy subjects (n = 5) for 24 hours.

Late-outgrowth peripheral blood endothelial progenitor cell (EPC)-derived endothelial cells (ECs)

Late-outgrowth EPC-derived ECs were obtained from the peripheral blood of 15 SSc patients (13 women and 2 men; n=9 with lcSSc and n=6 with dcSSc; median age 60 years, range 42 to 78 years) and 8 healthy individuals (all women; median age 55 years, range 30 to 65 years), as described elsewhere [5-7]. The study was approved by the local institutional review board at the Cochin Hospital, Paris, France, and all patients and control subjects provided written informed consent. Briefly, EPC isolation was performed on a 50-ml heparinised venous blood sample obtained from the forearm. Samples from hospitalised patients were obtained in the morning, at rest, during routine analysis. Patient and control samples were immediately transported to the laboratory for testing. The blood mononuclear cell fraction was collected by Ficoll density-gradient centrifugation and was resuspended in complete endothelial cell growth medium 2 (EGM-2; Lonza, Basel, Switzerland). Cells were then seeded onto separate wells of a 12-well tissue culture plate (2×10^7 cells/well) precoated with type I collagen (rat tail; BD Biosciences, Le Pont de Claix, France) and stored in an atmosphere of 5% CO₂ at 37°C. After 24 hours of culture, non-adherent cells and debris were aspirated, adherent cells were washed once with phosphate-buffered saline (PBS), and complete EGM-2 was added to each well. The medium was changed daily for 7 days

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3 and then every other day until the first passage. Colonies of ECs appeared between 8 and 26 days of
4 culture and were identified as well-circumscribed monolayers of cells with a cobblestone
5 appearance. After the third passage, phenotyping of EPC-derived cells was performed by flow
6 cytometry, as previously described [6]. After confirmation of the endothelial phenotype, cells were
7 suspended in FBS supplemented with 20% dimethyl sulfoxide, frozen in liquid nitrogen, and stored
8 until used.
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14 **Enzyme-linked immunosorbent assay (ELISA) for serum semaphorin3A (Sema3A) and** 15 **soluble neuropilin-1 (sNRP1)**

16 The levels of Sema3A and sNRP1 in serum samples were measured by commercial quantitative
17 colorimetric sandwich ELISA (catalogue number ABIN481720 and ABIN415191, respectively;
18 Antibodies-on line, Atlanta, Georgia, USA) according to the manufacturer's protocol. Each sample
19 was measured in duplicate.
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27 **Immunofluorescence**

28 For antigen retrieval, paraffin-embedded skin sections (5 µm thick) were deparaffinised and boiled
29 for 10 minutes in sodium citrate buffer (10 mM, pH 6.0). The sections were washed three times in
30 PBS, incubated in 2 mg/ml glycine for 10 minutes to quench autofluorescence caused by free
31 aldehydes, and then blocked for 1 hour at room temperature with 1% bovine serum albumin in PBS.
32 The slides were incubated overnight at 4°C with rabbit monoclonal antihuman NRP1 antibody (1:50
33 dilution; catalogue number ab81321, Abcam, Cambridge, UK) or rabbit polyclonal antihuman
34 Sema3A antibody (1:50 dilution; catalogue number ab23393, Abcam). After extensive washing in
35 PBS, the sections were incubated with Rhodamine Red-X-conjugated goat anti-rabbit IgG (1:200
36 dilution; Molecular Probes, Eugene, Oregon, USA) for 45 minutes at room temperature in the dark.
37 Irrelevant isotype-matched and concentration-matched rabbit IgG (Sigma-Aldrich) were used as
38 negative controls. For double immunofluorescence staining, we used a mouse monoclonal antibody
39 against CD31/pan-endothelial cell marker (1:25 dilution; catalogue number ab9498, Abcam)
40 followed by Alexa Fluor-488-conjugated goat anti-mouse IgG (1:200 dilution; Molecular Probes).
41 Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The immunolabelled
42 sections were then observed under a Leica DM4000 B microscope equipped with fully automated
43 fluorescence axes (Leica Microsystems, Mannheim, Germany). Fluorescence images were captured
44 using a Leica DFC310 FX 1.4-megapixel digital colour camera equipped with the Leica software
45 application suite LAS V3.8 (Leica Microsystems).
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Western blotting

Proteins were extracted from skin biopsies, dermal MVECs and late-outgrowth peripheral blood EPC-derived ECs as described elsewhere [7,8]. Twenty micrograms of total proteins were electrophoresed on NuPAGE 4 to 12% Bis-Tris Gel (Invitrogen, Carlsbad, California, USA) and blotted onto polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked with blocking solution included in the Western Breeze Chromogenic Western Blot Immunodetection Kit (Invitrogen) for 30 minutes at room temperature on a rotary shaker and incubated for 1 hour at room temperature with rabbit monoclonal antihuman NRP1 (1:1,000 dilution; catalogue number ab81321, Abcam), rabbit polyclonal antihuman Sema3A (1:1,000 dilution; catalogue number ab23393, Abcam), rabbit polyclonal antihuman Friend leukaemia integration 1 (Fli1) (1:1,000 dilution; catalogue number ab180902, Abcam) and rabbit polyclonal anti- α -tubulin (1:1,000 dilution; catalogue number #2144, Cell Signaling Technology, Danvers, Massachusetts, USA) antibodies, assuming α -tubulin as control invariant protein. Immunodetection was performed as described in the Western Breeze Chromogenic Immunodetection protocol (Invitrogen). Densitometric analysis of the bands was performed using the free-share ImageJ software (NIH, Bethesda, Maryland, USA; online at <http://rsbweb.nih.gov/ij>) and the values were normalised to α -tubulin.

Gene silencing of Fli1 and NRP1

MVECs were seeded shortly before transfection. The cells were transfected with 10 nM of Fli1 small interfering RNA (siRNA), 10 nM of NRP1 siRNA or non-silencing scrambled RNA (SCR) (Santa Cruz Biotechnology, Dallas, Texas, USA) using HiPerfect transfection reagent (Qiagen, Milan, Italy) for 72 hours.

RNA purification, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from MVECs using the RNeasy Micro Kit (Qiagen). First strand cDNA was synthesised using the QuantiTect Reverse Transcription kit (Qiagen). For mRNA quantification, SYBR Green real-time PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Milan, Italy) with melting curve analysis. Predesigned oligonucleotide primer pairs were obtained from Qiagen (QuantiTect Primer Assay). The assay IDs were QT00023009 Hs_NRP1, QT00078372 Hs_FLI, and QT00199367 Hs_RRN18S. The PCR mixture contained 1 μ l cDNA, 0.5 μ M sense and antisense primers, 10 μ l 2 \times QuantiTect SYBR Green PCR Master Mix containing SYBR Green I dye, ROX passive reference dye, HotStarTaq DNA Polymerase, dNTP mix and MgCl₂ (Qiagen). Amplification was performed according to a

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3 standard protocol recommended by the manufacturer. Non-specific signals caused by primer dimers
4 or genomic DNA were excluded by dissociation curve analysis, non-template controls and samples
5 without enzyme in the reverse transcription step. 18S ribosomal RNA was measured as an
6 endogenous control to normalise for the amounts of loaded cDNA. Differences were calculated with
7 the threshold cycle (Ct) and comparative Ct method for relative quantification. All measurements
8 were performed in triplicate.
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14 15 16 **Chromatin immunoprecipitation (ChIP) assay**

17 The ChIP assay was carried out using EpiQuik ChIP kit (Epigentek, Farmingdale, New York,
18 USA). Briefly, cells were treated with 1% formaldehyde for 10 minutes. The cross-linked chromatin
19 was then prepared and sonicated to an average size of 300-500 bp. To exclude exogenous DNA
20 contamination, negative controls were obtained by omitting the cell preparation in some specimens.
21 The DNA fragments were immunoprecipitated with rabbit polyclonal anti-Fli1 antibody (catalogue
22 number ab15289, Abcam) or normal rabbit IgG (catalogue number ab37415, Abcam) at 4°C. After
23 reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of
24 specific region of the NRP1 gene promoter. Putative Fli1 transcription factor binding site was
25 predicted by Tfsitescan. The primers were as follows: NRP1 Forward, 5'-
26 CTAGGGGTGCAGAGCGAG-3'; NRP1 Reverse, 5'-GAAGGAAGGCGCTGGGAG-3'. The
27 amplified DNA products were resolved by agarose gel electrophoresis.
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39 ***In vitro* capillary morphogenesis assay**

40 *In vitro* capillary morphogenesis assay was performed in 96-well plates covered with Matrigel (BD
41 Biosciences). Matrigel (50 µl; 10-12 mg/ml) was pipetted into culture wells and polymerised for 30
42 minutes to 1 hour at 37°C. H-MVECs (30x10³ cells/well) were incubated in basal MCDB medium
43 containing 10% healthy serum (n = 5) or 10% SSc serum (n = 5), this latter used alone or in
44 combination with recombinant human VEGF-A165 (10 ng/ml; R&D Systems), mouse monoclonal
45 anti-human VEGF-A165b antibody (10 µg/ml; catalogue number ab14994, Abcam) or irrelevant
46 mouse IgG. SSc-MVECs and H-MVECs transfected with NRP1 siRNA, at basal condition or
47 challenged with recombinant human VEGF-A165 or VEGF-A165b (both 10 ng/ml; R&D Systems),
48 or non-silencing SCR, were also assayed. Stimulation with recombinant human VEGF-A165 added
49 to basal MCDB medium was used as positive control of angiogenesis. Plates were photographed at
50 6 and 24 hours. Results were quantified at 24 hours by measuring the percent field occupancy of
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capillary projections, as determined by image analysis. Six to nine photographic fields from 3 plates were scanned for each experimental point.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 20.0 (SPSS, Chicago, Illinois, USA). Data are expressed as mean \pm SD or median and interquartile range (IQR). The Student's *t*-test and nonparametric Mann-Whitney *U*-test were used where appropriate for statistical evaluation of the differences between two independent groups. A *p*-value less than 0.05 was considered statistically significant.

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Supplementary Table S1. Demographic and clinical characteristics of the 49 patients with systemic sclerosis (SSc) included for collection of serum samples.

Characteristics	SSc patients
Age, years, median (range)	64 (37 to 80)
Sex	
Male	4 (8)
Female	45 (92)
Disease subset	
lcSSc	32 (65)
dcSSc	17 (35)
Disease duration, years, median (range) ^a	10 (2 to 31)
Autoantibody positivity	
ANA	49 (100)
ACA	32 (65)
Anti-topo I	11 (22)
Digital ulcers	27 (55)
Digital pitting scars	15 (31)
Nailfold videocapillaroscopy pattern	
Early	11 (22)
Active	21 (43)
Late	17 (35)
Skin score, median (range) ^b	6 (0 to 35)
Interstitial lung disease ^c	27 (55)
2013 ACR/EULAR score, median (range)	14 (10 to 28)

Except where indicated otherwise, values are the number (%) of subjects.

^aDisease duration was calculated since the first non-Raynaud's symptom of SSc; ^bmodified Rodnan skin thickness score; ^cDetermined by thoracic high-resolution computer tomography. ACA, anticentromere antibodies; ACR, American College of Rheumatology; ANA, antinuclear antibodies; Anti-topo I, anti-topoisomerase I antibodies; dcSSc, diffuse cutaneous SSc; EULAR, European League Against Rheumatism; lcSSc, limited cutaneous SSc.

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3 **Decreased expression of neuropilin-1 as a novel key factor contributing to**
4 **peripheral microvasculopathy and defective angiogenesis in systemic sclerosis**
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ABSTRACT

Objectives: In systemic sclerosis (SSc), vascular involvement is characterised by VEGF-A/VEGFR system disturbances. Neuropilin-1 (NRP1), a receptor for both class-3 semaphorins (Sema3s) and VEGF-A, is required for optimal VEGF-A/VEGFR-2 signalling. Here, we investigated the possible involvement of Sema3A/NRP1 axis in SSc.

Methods: Circulating Sema3A and soluble NRP1 (sNRP1) were measured in SSc patients and controls. NRP1 and Sema3A expression in skin biopsies was evaluated by immunofluorescence and Western blotting. NRP1 expression was assessed in SSc and healthy dermal microvascular endothelial cells (SSc-MVECs and H-MVECs), and in SSc and control endothelial progenitor cell (EPC)-derived ECs. The possible impact of transcription factor Fli1 deficiency on endothelial NRP1 expression was investigated by gene silencing. The binding of Fli1 to NRP1 gene promoter was evaluated using chromatin immunoprecipitation. Capillary morphogenesis was performed on Matrigel.

Results: Decreased sNRP1 levels in SSc were associated with active and late nailfold videocapillaroscopy patterns and digital ulcers. No difference in Sema3A was found between patients and controls. NRP1 was significantly decreased in SSc-MVECs both *ex vivo* and *in vitro*. NRP1 and Fli1 significantly decreased in H-MVECs challenged with SSc sera, while they were not different in SSc and control EPC-derived ECs. Fli1 occupied the NRP1 gene promoter and Fli1 gene silencing reduced NRP1 expression in H-MVECs. NRP1 gene silencing in H-MVECs resulted in a significantly impaired angiogenic capacity comparable to that of cells treated with SSc sera.

Conclusions: In SSc, NRP1 deficiency may be an additional factor in the perturbed VEGF-A/VEGFR-2 system contributing to peripheral microvasculopathy and defective angiogenesis.

Keywords: systemic sclerosis, neuropilin-1, Fli1, dermal microvascular endothelial cells, angiogenesis, peripheral microvasculopathy

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is a life-threatening connective tissue disorder of unknown aetiology, characterised by widespread vascular injury and dysfunction, impaired angiogenesis, immune dysregulation and progressive fibrosis of the skin and internal organs [1,2]. The dysregulation of vascular tone control, clinically evident as Raynaud's phenomenon, and microcirculatory abnormalities paralleled by nailfold capillaroscopic changes are the earliest clinical manifestations of SSc and may precede skin and visceral involvement by months or years [1-3]. The whole process is characterised by an uncontrolled regeneration of the microvasculature and subsequent loss of microvessels, due to defects in both vascular repair and expected increase in new vessel growth (angiogenesis), leading to severe peripheral ischaemic manifestations, such as digital ulcers and gangrene [2,3].

Recent studies have highlighted the anatomic and structural similarities between blood vessels and nerves [4]. The two networks are often aligned, with nerve fibers and blood vessels following parallel routes. Furthermore, both systems require precise control over their guidance and growth. Several molecules with attractive and repulsive properties have been found to modulate the guidance both of nerves and blood vessels [4]. These include the neuropilin receptors and their semaphorin ligands, as well as netrins, slits and their receptors [4]. Among these, neuropilin-1 (NRP1) was initially described as an axonally expressed receptor for secreted class-3 semaphorins (Sema3s), a family of soluble molecules which modulate the development of the nervous and vascular systems [5,6]. NRP1 also serves as specific vascular endothelial growth factor-A (VEGF-A) co-receptor on endothelial cells (ECs) and regulates VEGF receptor (VEGFR) signalling, leading to enhanced migration [7] and survival of ECs *in vitro* [8,9]. Furthermore, NRP1 has been implicated in VEGFR-2-mediated endothelial permeability [10] and in VEGF-A-induced three-dimensional EC biology, such as vessel sprouting and branching [11]. The absence of functional NRP1 in mice results in embryonic death due to impaired heart and blood vessel development, thus suggesting that this receptor plays a central regulatory role during developmental angiogenesis [4]. The exact molecular mechanisms by which NRP1 modulates VEGF-A biology remain to be elucidated. It has, however, been shown that NRP1 potentiates the VEGF-A/VEGFR-2 signalling pathways implicated in the migratory response of ECs [12].

The evidence that NRP1 functions as a receptor for both VEGF-A and Sema3s suggests that these latter may also play a role in the modulation of angiogenesis. In particular, it has been reported that Sema3A acts as an antiangiogenic molecule impairing EC adhesion, migration and survival *in vitro* [13-15], and regulates tumour-induced angiogenesis *in vivo* [16]. Moreover, Sema3A null mice

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3 exhibit defects in blood vessel reshaping [15,17,18]. The molecular mechanisms underlying the
4 antiangiogenic effects of Sema3A are complex. Hence, it was initially suggested that Sema3A
5 competes with VEGF-A for NRP1 binding, thus inhibiting VEGF-A-induced angiogenesis.
6 However, recent reports have also shown that Sema3A increases vascular permeability, inhibits EC
7 proliferation, and induces apoptosis even in the absence of VEGF-A, suggesting that Sema3A may
8 activate its own signalling pathways [19,20].
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11 On these bases, we hypothesised that the Sema3A/NRP1 axis might play a role in the pathogenesis
12 of SSc-related microvascular abnormalities. Therefore, the aim of the present study was to
13 investigate whether the levels of Sema3A and NRP1 could be altered in the circulation, skin and
14 ECs of SSc patients, as well as the mechanism explaining the dysregulated expression of these
15 molecules and their possible contribution to the disturbed angiogenesis of SSc.
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24 **METHODS**

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26 An extended methods section is provided in the online supplementary material.
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29 **Patients, controls, serum samples and skin biopsies**

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31 Serum samples were obtained from 49 SSc patients [1] (45 women and 4 men; median age 64 years,
32 range 37 to 80 years, and median disease duration 10 years, range 2 to 31 years) classified as
33 limited cutaneous SSc (lcSSc; n=32) or diffuse cutaneous SSc (dcSSc; n=17) [21], and from 39
34 age-matched and sex-matched healthy individuals. All patients were clinically assessed as described
35 elsewhere [3,22,23]. Clinicodemographic characteristics of SSc patients used for collection of
36 serum samples are shown in online supplementary table S1. Full-thickness skin biopsies were
37 obtained from the clinically involved skin of one-third of the distal forearm of 18 patients with SSc
38 (15 women, 3 men; median age 48.5 years, range 29 to 73 years, and median disease duration
39 7.2 years, range 1 to 18 years). Skin samples from the same forearm region of 11 age-matched and
40 sex-matched healthy donors were used as controls. Each skin biopsy was divided into two
41 specimens and processed for immunohistochemistry and biomolecular analysis as described
42 elsewhere [23]. The study was approved by the local institutional review board at the Azienda
43 Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, and all subjects provided written
44 informed consent.
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Isolation, culture and stimulation of dermal microvascular endothelial cells (MVECs)

Dermal MVECs were isolated from biopsies of the involved forearm skin from 5 patients with dcSSc and from 5 healthy subjects, as described elsewhere [3,23]. MVECs from healthy subjects (H-MVECs) and SSc patients (SSc-MVECs) were used between the third and seventh passages in culture. For stimulation experiments, H-MVECs were grown to 70% confluence, and then were washed three times with serum-free medium and serum-starved overnight in MCDB medium supplemented with 2% FBS. Medium was removed and cells were incubated with 2% FBS-MCDB medium containing recombinant human VEGF-A165 (10 ng/ml; R&D Systems, Minneapolis, Minnesota, USA), or 10% serum from SSc patients (n = 5) and healthy subjects (n = 5) for 24 hours.

Late-outgrowth peripheral blood endothelial progenitor cell (EPC)-derived ECs

Late-outgrowth EPC-derived ECs were obtained from the peripheral blood of 15 SSc patients (13 women and 2 men; n=9 with lcSSc and n=6 with dcSSc; median age 60 years, range 42 to 78 years) and 8 healthy individuals (all women; median age 55 years, range 30 to 65 years), as described elsewhere [3,24,25].

Enzyme-linked immunosorbent assay (ELISA) for serum Sema3A and soluble NRP1 (sNRP1)

The levels of Sema3A and sNRP1 in serum samples were measured by commercial quantitative colorimetric sandwich ELISA (catalogue number ABIN481720 and ABIN415191, respectively; Antibodies-on line, Atlanta, Georgia, USA) according to the manufacturer's protocol. Each sample was measured in duplicate.

Immunofluorescence

Immunofluorescence on paraffin-embedded skin sections was performed as previously described [3]. For primary and secondary antibodies, refer to the online supplementary material.

Western blotting

Proteins were extracted from skin biopsies, dermal MVECs and late-outgrowth peripheral blood EPC-derived ECs as described elsewhere [3,25,26]. Western blotting was carried out according to previously published protocols [3]. For primary antibodies, refer to the online supplementary material.

Gene silencing of Friend leukaemia integration 1 (Fli1) and NRP1

MVECs were seeded shortly before transfection. The cells were transfected with 10 nM of Fli1 small interfering RNA (siRNA), 10 nM of NRP1 siRNA or non-silencing scrambled RNA (SCR) (Santa Cruz Biotechnology, Dallas, Texas, USA) using HiPerfect transfection reagent (Qiagen, Milan, Italy) for 72 hours.

RNA purification, cDNA synthesis and quantitative real-time PCR

Total RNA isolation from MVECs, first strand cDNA synthesis and mRNA quantification by SYBR Green real-time PCR was performed as reported elsewhere [27]. For predesigned oligonucleotide primer pairs obtained from Qiagen, refer to the online supplementary material.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as previously described [28] using a rabbit polyclonal anti-Fli1 antibody (catalogue number ab15289, Abcam, Cambridge, UK). Putative Fli1 transcription factor binding site was predicted by Tfsitescan. The primers were as follows: NRP1 Forward, 5'-CTAGGGGTGCAGAGCGAG-3'; NRP1 Reverse, 5'-GAAGGAAGGCGCTGGGAG-3'.

In vitro capillary morphogenesis assay

In vitro capillary morphogenesis assay on Matrigel was performed according to previously published protocols [27] as detailed in the online supplementary material.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, version 20.0 (SPSS, Chicago, Illinois, USA). Data are expressed as mean \pm SD or median and interquartile range (IQR). The Student's *t*-test and nonparametric Mann-Whitney *U*-test were used where appropriate for statistical evaluation of the differences between two independent groups. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Serum Sema3A and sNRP1 levels in SSc

No significant differences in serum levels of Sema3A were detected between SSc patients (median 2.22 ng/ml, IQR 1.84 to 3.17 ng/ml) and healthy controls (median 3.86 ng/ml, IQR 1.64 to 4.73

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3 ng/ml) (figure 1A). Circulating sNRP1 levels were significantly reduced in SSc patients (median
4 0.22 ng/ml, IQR 0.0 to 0.6 ng/ml) compared with healthy individuals (median 0.69 ng/ml, IQR 0.0
5 to 2.5 ng/ml; $p=0.001$) (figure 1B). Next, we evaluated the possible correlation of serum sNRP1
6 levels with the nailfold videocapillaroscopy (NVC) pattern as a measure of peripheral
7 microvascular involvement. sNRP1 levels were significantly decreased either in SSc patients with
8 active (median 0.14 ng/ml, IQR 0.0 to 0.4 ng/ml) or late (median 0.09 ng/ml, IQR 0.0 to 0.72
9 ng/ml) NVC patterns than in controls ($p=0.003$ and $p=0.01$, respectively) (figure 1C). Conversely,
10 no difference in serum sNRP1 was found between SSc patients with early NVC pattern (median
11 0.45 ng/ml, IQR 0.22 to 0.66 ng/ml) and healthy controls (figure 1C). Moreover, sNRP1 levels were
12 significantly decreased in SSc patients with digital ulcers (median 0.06 ng/ml, IQR 0.0 to 0.27
13 ng/ml) compared both with patients without digital ulcers (median 0.43 ng/ml, IQR 0.17 to 0.71
14 ng/ml; $p=0.009$) and controls ($p=0.001$) (figure 1D). No significant association was found with
15 other clinicodemographic and laboratory parameters or with clinical SSc subset.
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26 **Decreased expression of NRP1 in SSc dermal ECs *ex vivo* and *in vitro***

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28 The expression of Sema3A and NRP1 protein in forearm skin biopsies from SSc patients and
29 controls was investigated by immunofluorescence and Western blot. No significant differences in
30 Sema3A expression were detected between SSc and control skin (figure 2A-C). On the contrary,
31 NRP1 expression was decreased in clinically affected skin biopsies from SSc patients compared to
32 healthy skin, in particular in dermal ECs and perivascular stromal cells (figure 3A-F). The
33 localisation of NRP1 staining in vascular ECs was confirmed by NRP1/CD31 double
34 immunofluorescence staining (figure 3C,F). Moreover, Western blot analysis confirmed that NRP1
35 protein expression levels were significantly reduced in SSc skin in respect to control skin ($p<0.001$)
36 (figure 3G,H).
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43 Western blot analysis on cultured dermal MVECs revealed that NRP1 protein expression levels
44 were significantly reduced in SSc-MVECs compared to H-MVECs ($p<0.005$) (figure 4A).
45 Moreover, NRP1 expression in H-MVECs significantly increased after treatment with healthy sera
46 compared to basal condition, while it decreased after challenging with SSc sera (both $p<0.005$ vs
47 basal H-MVECs). As expected, stimulation with recombinant human VEGF-A165 strongly
48 upregulated NRP1 expression in H-MVECs ($p<0.005$ vs basal H-MVECs) (figure 4A). On the
49 contrary, no obvious differences in NRP1 protein levels could be found between late-outgrowth
50 EPC-derived ECs from SSc patients and healthy controls (figure 4B).
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Fli1 deficiency contributes to the downregulation of NRP1 gene in SSc-MVECs

We next examined the potential mechanism by which NRP1 expression is downregulated in dermal SSc microvessels. As the expression of the transcription factor Fli1 is markedly downregulated at least partially *via* an epigenetic mechanism in SSc dermal ECs, and experimental endothelial Fli1 deficiency reproduces the histopathological and functional abnormalities characteristic of SSc vasculopathy [28-30], we hypothesised that endothelial Fli1 deficiency could inhibit the expression of NRP1 in SSc-MVECs.

First, we analysed Fli1 protein expression in cultured dermal MVECs and late-outgrowth EPC-derived ECs from SSc patients and controls (figure 4C,D). As shown in figure 4C, Fli1 protein expression in H-MVECs closely paralleled that of NRP1 in the different experimental conditions assayed, and was strongly downregulated in SSc-MVECs. Similarly to what observed for NRP1 expression, Fli1 protein levels did not differ between SSc and control late-outgrowth EPC-derived ECs (figure 4D).

Moreover, we examined the effect of Fli1 gene silencing on mRNA levels of the NRP1 gene in H-MVECs. As displayed in figure 4E, gene silencing of Fli1 significantly suppressed the mRNA expression levels of the NRP1 gene in H-MVECs ($p < 0.01$). In addition, ChIP analysis revealed that Fli1 occupied the promoter region of the NRP1 gene in H-MVECs (figure 4F). These results indicate that Fli1 directly targets the NRP1 gene promoter and is required for homeostatic NRP1 expression in ECs.

NRP1 deficiency contributes to the impaired angiogenesis of SSc-MVECs

To verify whether endothelial NRP1 deficiency has a role in the modulation of angiogenesis, we carried out *in vitro* capillary morphogenesis on Matrigel matrix. Consistent with previous findings [26], capillary morphogenesis was significantly impaired in SSc-MVECs compared with H-MVECs ($p < 0.01$) (figure 5). H-MVECs stimulated with healthy sera produced an abundant network of branching cords (figure 5). On the contrary, as previously reported [31,32] angiogenesis was significantly reduced upon challenge with SSc sera ($p < 0.01$ vs basal H-MVECs) (figure 5). The addition of recombinant human VEGF-A165 or anti-VEGF-A165b blocking antibodies to SSc sera significantly increased H-MVEC angiogenesis compared with cells treated with SSc sera alone (both $p < 0.05$) (figure 5). NRP1 gene silencing in H-MVECs resulted in a significant impairment of angiogenic capacity comparable to that of cells treated with SSc sera ($p < 0.01$ vs basal H-MVECs) (figure 5). Stimulation of NRP1-silenced H-MVECs with recombinant human proangiogenic

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3 VEGF-A165 or antiangiogenic VEGF-A165b could only slightly increase or decrease angiogenesis,
4 respectively (figure 5).
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8 9 10 **DISCUSSION**

11 Here, we investigated for the first time the possible involvement of the *Sema3A/NRP1* axis in the
12 pathogenesis of SSc. Our present findings clearly demonstrate that serum levels and dermal
13 expression of NRP1 are significantly decreased in SSc patients and that lower circulating sNRP1
14 levels correlate with the severity of NVC abnormalities and the presence of digital ulcers. In
15 contrast to constitutive endothelial expression of NRP1 in healthy skin, NRP1 was found to be
16 strongly reduced *ex vivo* in SSc dermal microvessels, and NRP1 downregulation was maintained *in*
17 *vitro* in MVECs obtained from SSc dermis. On the contrary, we could not find any difference in
18 NRP1 protein levels between peripheral blood EPC-derived ECs from SSc patients and healthy
19 controls. This evidence suggests that the dysregulated expression of this receptor is restricted to
20 locally injured microvasculature in an overt disease without affecting bone marrow-derived
21 circulating endothelial progenitors. As far as *Sema3A* is concerned, no difference in its expression
22 was observed between SSc and controls either in the circulation or in the cutaneous tissue.
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31 The importance of NRP1 for vascular development is well established and shown by the generation
32 of knockout mice, which display an embryonic lethal phenotype characterised by severe vascular
33 defects due to impaired angiogenic sprouting and branching very much resembling the disturbed
34 vessel morphology seen in SSc patients [4,33,34]. Moreover, conditional NRP1 knockout in ECs is
35 associated with important cardiac and vascular defects, thus suggesting a crucial role for NRP1 in
36 EC functions [35]. Even though NRP1 was originally identified as an adhesion molecule in the
37 nervous system, it is more commonly studied as receptor for the neuronal guidance molecule
38 *Sema3A* and as co-receptor for the VEGF-A165/VEGFR-2 complex with key roles in neuronal and
39 vascular development [12,36]. Of note, several studies have implicated a dysfunctional VEGF-
40 A/VEGFR-2 system in the impaired angiogenic process characteristic of SSc [2,26,37-39].
41 Moreover, besides VEGF-A, it is well known that a dysregulated expression of a large array of
42 proangiogenic and antiangiogenic (angiostatic) factors present in the circulation of SSc patients may
43 be mostly responsible for such angiogenic deficit [2,37-40].
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53 In our study, not only we observed that the proangiogenic NRP1 receptor was constitutively
54 downregulated in dermal SSc-MVECs, but also that treatment with SSc sera could significantly
55 reduce NRP1 expression in H-MVECs, which is in line with the reported antiangiogenic properties
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3 of SSc sera [2,31,32,37,41]. Strikingly, we also found that NRP1 gene silencing in H-MVECs
4 resulted in a significantly impaired angiogenic process comparable to that of cells treated with SSc
5 sera, further supporting the implication of NRP1 deficiency in the disturbed angiogenesis of SSc.
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8 Consistent with previous studies, stimulation with recombinant proangiogenic VEGF-A165
9 strongly upregulated NRP1 expression in H-MVECs suggesting that this growth factor cannot only
10 activate ECs directly, but can also contribute to angiogenesis by a mechanism that involves
11 upregulation of its homologous receptor NRP1 [36]. The findings of NRP1 downregulation in H-
12 MVECs upon challenge with SSc sera are in agreement with the evidence that the majority of
13 VEGF-A detected in SSc circulation is not the proangiogenic VEGF-A165, but rather the
14 antiangiogenic VEGF-A165b isoform [26,42]. Further, it has been reported that VEGF-A165b is
15 unable to bind the co-receptor NRP1 because the basic carboxyterminal amino acids essential for
16 NRP1 binding are absent in this splice variant [43,44]. Interestingly, we observed that stimulation
17 of H-MVECs silenced for the NRP1 co-receptor with proangiogenic VEGF-A165 slightly increased
18 angiogenesis, while stimulation with VEGF-A165b isoform resulted only in a slight decrease in
19 their angiogenic capacity. These data are consistent with the antiangiogenic action of VEGF-A165b
20 being mainly dependent on its inability to recruit VEGFR-2/NRP1 co-receptor complex and activate
21 downstream signalling. In a recent study, it was demonstrated that VEGF-A165 and VEGF-A165b
22 may control the balance between VEGFR-2 recycling, degradation and signalling. In particular, due
23 to the lack of NRP1 co-receptor binding, VEGF-A165b may induce differential intracellular
24 vesicular trafficking of VEGFR-2 toward the degradative pathway [45]. Thus, both a switch from
25 the proangiogenic to the antiangiogenic VEGF-A isoform and the concomitant NRP1 co-receptor
26 downregulation may have a crucial role in the insufficient angiogenic response found in SSc (figure
27 6). Indeed, here we also demonstrated that both the addition of recombinant human VEGF-A165 or
28 anti-VEGF-A165b blocking antibodies could significantly dampen the antiangiogenic effects of SSc
29 sera on H-MVECs.
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32 The clinical correlation of serum sNRP1 levels with the severity of SSc-related peripheral
33 microvasculopathy also deserves discussion. Indeed, circulating levels of sNRP1 progressively
34 decreased reaching the lowest values in SSc patients with the active and late NVC patterns, which
35 are characterised by severe architectural changes of microvessels and progressive capillary loss with
36 formation of avascular areas [46]. In addition, patients with active/late NVC patterns and digital
37 ulcers showed serum sNRP1 levels significantly lower than healthy controls, whereas sNRP1 levels
38 did not differ significantly between controls and SSc patients with early NVC pattern and lack of
39 digital ulcers. However, since sNRP1 may be largely released by ECs, we should also consider that
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3 the reduction in circulating levels of sNRP1 might be either a cause or a consequence of the disease,
4 which is characterised by progressive loss of the peripheral microcirculation [2,3,46]. Circulating
5 levels of sNRP1 could even serve as a biomarker reflecting the severity and progression of SSc
6 microvasculopathy. Accordingly, further prospective studies on larger cohorts of SSc patients are
7 warranted.
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11 Finally, our mechanistic findings indicate that in SSc, endothelial NRP1 expression is suppressed at
12 least partially due to Fli1 transcription factor deficiency. In fact, here we provide the first evidence
13 that NRP1 is a member of the angiogenesis-related gene program regulated by Fli1 in dermal
14 MVECs. In this context, the impact of Fli1 deficiency in the loss of EC integrity and the
15 development of peripheral microvasculopathy during SSc has been well established [28-30]. Of
16 note, it has been demonstrated that in SSc Fli1 expression is markedly suppressed at least partially
17 through an epigenetic mechanism [29,30]. Thus, such an epigenetic modification might partly
18 explain the persistence of the multiple downstream effects of Fli1 deficiency in an *in vitro* culture
19 system, as supported by the downregulation of endothelial NRP1 observed *ex vivo* in SSc dermal
20 microvessels and maintained in cultured dermal SSc-MVECs. Interestingly, NRP1 was found to be
21 also a target of the antiangiogenic microRNA miR-320, and a dysregulated microRNA profile is
22 being increasingly reported in SSc [47,48].
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31 In conclusion, we shed light on NRP1 deficiency as a novel key factor contributing to peripheral
32 microvasculopathy and defective angiogenesis in SSc. Further studies are warranted to decipher
33 whether therapeutic modulation of VEGF-A/VEGFR-2/NRP1 co-receptor signalling might pave the
34 way to boost angiogenesis and block the progression of peripheral microvasculopathy in SSc.
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43 **Author contributions**

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45 Study conception and design: ER, MM, LI-M, MM-C, SG. Acquisition of data: ER, IC, MM, CM,
46 IR, SB-R, JB, JA, YA. Interpretation of data: ER, IC, MM, RS, LI-M, MM-C, SG. Manuscript
47 preparation: ER, IC, MM, IR, LI-M, MM-C, SG.
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54 Medicine in Europe (FDIME).
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Competing interests

None declared.

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FIGURE LEGENDS

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16 **Figure 1.** Serum levels of semaphorin3A (Sema3A) and soluble neuropilin-1 (sNRP1) determined
17 by colorimetric sandwich ELISA. (A) Serum Sema3A levels in healthy controls and patients with
18 systemic sclerosis (SSc). (B) Serum sNRP1 levels in healthy controls and SSc patients. (C) Serum
19 sNRP1 levels in healthy controls and patients with SSc according to nailfold videocapillaroscopy
20 (NVC) pattern (early, active and late). (D) Serum sNRP1 levels in healthy controls and patients
21 with SSc according to the presence/absence of digital ulcers (DU). Data are shown as box plots.
22 Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines
23 outside the boxes represent the 10th and the 90th percentiles. Circles indicate outliers, and asterisks
24 indicate the extreme values. Mann-Whitney *U*-test was used for statistical analysis.
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28 **Figure 2.** Expression of semaphorin3A (Sema3A) in skin biopsies. (A and B) Representative
29 microphotographs of skin sections from (A) healthy controls (n = 11) and (B) systemic sclerosis
30 (SSc) patients (n = 18) immunostained for Sema3A (red) and counterstained with 4',6-diamidino-2-
31 phenylindole (DAPI; blue) for nuclei. Arrows indicate microvessels. Original magnification: x40.
32 Scale bar = 50 μ m. (C) Western blotting of total protein extracts from the skin of healthy subjects (n
33 = 10) and SSc patients (n = 15). A protein band with the expected molecular weight of 90 kDa was
34 detected with the anti-Sema3A antibody. Representative immunoblots are shown. The densitometric
35 analysis of the bands normalised to α -tubulin is reported in the histograms. Data are mean \pm SD of
36 optical density in arbitrary units (a.u.).
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39 **Figure 3.** Expression of neuropilin-1 (NRP1) in skin biopsies. (A, B, D, and E) Representative
40 microphotographs of skin sections from (A and B) healthy controls (n = 11) and (D and E) systemic
41 sclerosis (SSc) patients (n = 18) immunostained for NRP1 (red) and counterstained with 4',6-
42 diamidino-2-phenylindole (DAPI; blue) for nuclei. Arrows indicate microvessels. (C and F)
43 Representative microphotographs of skin sections from healthy controls (C) and SSc patients (F)
44 double immunostained for NRP1 (red) and the pan-endothelial cell marker CD31 (green) and
45 counterstained with DAPI (blue). Original magnification: x20 (A and D), x40 (B and E), x100 (C
46 and F). Scale bar = 100 μ m (A and D), 50 μ m (B and E), 10 μ m (C and F). (G and H) Western
47 blotting of total protein extracts from the skin of healthy subjects (n = 10) and SSc patients (n = 15).
48 A protein band with the expected molecular weight of 103 kDa was detected with the anti-NRP1
49 antibody. Representative immunoblots are shown. The densitometric analysis of the bands
50 normalised to α -tubulin is reported in the histograms. Data are mean \pm SD of optical density in
51 arbitrary units (a.u.).
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54 **Figure 4.** (A-D) Expression of neuropilin-1 (NRP1) and Friend leukaemia integration 1 (Flt1) in
55 dermal microvascular endothelial cells (MVECs) and late-outgrowth peripheral blood endothelial
56 progenitor cell (EPC)-derived endothelial cells. (A and C) Western blotting of total protein extracts
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3 from healthy MVECs (H-MVECs) at basal condition and treated with recombinant human vascular
4 endothelial growth factor-A165 (VEGF-A165), or 10% serum from SSc patients (n = 5) and healthy
5 subjects (n = 5) for 24 hours, and from basal systemic sclerosis MVECs (SSc-MVECs) assayed
6 with anti-NRP1 (A) and anti-Fli1 (C) antibodies. Representative immunoblots are shown. The
7 densitometric analysis of the bands normalised to α -tubulin is reported in the histograms. Data are
8 mean \pm SD of optical density in arbitrary units (a.u.). Student's *t*-test was used for statistical
9 analysis. **p*<0.005 vs. basal H-MVECs. Results are representative of three independent experiments
10 performed with each one of the 5 H-MVEC and 5 SSc-MVEC lines. (B and D) Western blotting of
11 protein lysates from control (n = 8) and SSc (n = 15) late-outgrowth EPC-derived endothelial cells
12 assayed with anti-NRP1 (B) and anti-Fli1 (D) antibodies. Representative immunoblots are shown.
13 The densitometric analysis of the bands normalised to α -tubulin is reported in the histograms. Data
14 are mean \pm SD of optical density in arbitrary units (a.u.). (E) mRNA levels of the Fli1 and NRP1
15 genes in H-MVECs transfected with Fli1 siRNA or non-silencing scrambled RNA (SCR) were
16 measured by quantitative real-time PCR and normalised to mRNA levels of the 18S ribosomal
17 RNA gene. The relative values compared with SCR are expressed as mean \pm SD of three
18 independent experiments. Statistical analysis was carried out with Student's *t*-test. **p*<0.01 vs. SCR.
19 (F) Chromatin was isolated from H-MVECs and immunoprecipitation was conducted with rabbit
20 anti-Fli1 antibody or rabbit IgG. To exclude exogenous DNA contamination, negative controls were
21 obtained by omitting the cell preparation in some specimens. PCR amplification was carried out
22 using NRP1 promoter-specific primers. One representative of three independent experiments is
23 shown.
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27 **Figure 5.** *In vitro* angiogenesis. (A) Representative images of capillary morphogenesis on Matrigel
28 after 24 hours. (B) Capillary morphogenesis of healthy and systemic sclerosis (SSc) dermal
29 microvascular endothelial cells (H-MVECs and SSc-MVECs, respectively) quantified as percent
30 field occupancy of capillary projections. Capillary morphogenesis of H-MVECs was evaluated at
31 basal condition and after stimulation with sera from healthy subjects (n = 5) and SSc patients (n =
32 5), these latter alone or in combination with recombinant human vascular endothelial growth factor-
33 A165 (VEGF-A165) or anti-VEGF-A165b blocking antibodies. Stimulation with VEGF-A165
34 added to basal medium was used as positive control of angiogenesis. H-MVECs transfected with
35 NRP1 siRNA, at basal condition or challenged with recombinant human VEGF-A165 or VEGF-
36 A165b, or non-silencing scrambled RNA (SCR), were also assayed. Capillary morphogenesis of H-
37 MVECs at basal condition was set to 100%; the other results are normalised to this value. Data are
38 the mean \pm SD of three independent experiments performed in triplicate with each one of the 5 H-
39 MVEC and 5 SSc-MVEC lines. Six to 9 photographic fields from 3 plates were scanned for each
40 experimental point. Student's *t*-test was used for statistical analysis. **p*<0.01 vs. basal H-MVECs;
41 #*p*<0.05 vs. H-MVECs challenged with SSc sera. (C) mRNA levels of the NRP1 gene in H-MVECs
42 transfected with NRP1 siRNA or non-silencing SCR were measured by quantitative real-time PCR
43 and normalised to mRNA levels of the 18S ribosomal RNA gene. The relative values compared
44 with SCR are expressed as mean \pm SD of three independent experiments. Statistical analysis was
45 carried out with Student's *t*-test. **p*<0.01 vs. SCR.
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49 **Figure 6.** Schematic illustration of the potential mechanisms steering dysregulated angiogenesis in
50 systemic sclerosis (SSc). SSc is characterised by progressive loss of peripheral microvasculature
51 and lack of compensatory angiogenesis. In most angiogenic states, vascular endothelial growth
52 factor-A165 (VEGF-A165) interacts with VEGF receptor-2 (VEGFR-2) via the VEGF homology
53 domain (VHD) and with the b1 and b2 domains of neuropilin-1 (NRP1) via exons 7 and 8. Co-
54 expression of NRP1 and VEGFR-2 enhances VEGF-A165 binding to VEGFR-2, VEGFR-2
55 phosphorylation and downstream signalling cascades. In nonangiogenic conditions, such as SSc,
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3 both a switch from proangiogenic VEGF-A165 to antiangiogenic VEGF-A165b isoform, which is
4 unable to bind the co-receptor NRP1, and concomitant NRP1 downregulation may result in an
5 insufficient tyrosine phosphorylation/activation of VEGFR-2 and incomplete or transient
6 downstream signalling along with a differential intracellular vesicular trafficking of VEGFR-2
7 towards the degradative pathway, ultimately leading to an impaired angiogenic response.
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