Characterization of Regenerative Phenotype of Unrestricted Somatic Stem Cells (USSC) from Human Umbilical Cord Blood (hUCB) by Functional Secretome Analysis*[S]

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Stem cell transplantation is a promising therapeutic strategy to enhance axonal regeneration after spinal cord injury. Unrestricted somatic stem cells (USSC) isolated from human umbilical cord blood is an attractive stem cell population available at GMP grade without any ethical concerns. It has been shown that USSC transplantation into acute injured rat spinal cords leads to axonal regrowth and significant locomotor recovery, yet lacking cell replacement. Instead, USSC secrete trophic factors enhancing neurite growth of primary cortical neurons *in vitro*. Here, we applied a functional secretome approach characterizing proteins secreted by USSC for the first time and validated candidate neurite growth promoting factors using primary cortical neurons *in vitro*. By mass spectrometric analysis and exhaustive bioinformatic interrogation we identified 1156 proteins representing the secretome of USSC. Using Gene Ontology we revealed that USSC secretome contains proteins involved in a number of relevant biological processes of nerve regeneration such as cell adhesion, cell motion, blood vessel formation, cytoskeleton organization and extracellular matrix organization. We found for instance that 31 well-known neurite growth promoting factors like, *e.g.* neuronal growth regulator 1, NDNF, SPARC, and PEDF span the whole abundance range of USSC secretome. By the means of primary cortical neurons *in vitro* assays we verified SPARC and PEDF as significantly involved in USSC mediated neurite growth and therewith underline their role in improved locomotor recovery after transplantation. From our data we are convinced that USSC are a valuable tool in regenerative medicine as USSC's secretome contains a comprehensive network of trophic factors supporting nerve regeneration not only by a single process but also maintained its regenerative phenotype by a multitude of relevant biological processes. ***Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.049312, 2630–2643, 2015.***

Injury to the spinal cord leads to a multiple damaging process including axonal contusion and transection with subsequent degeneration, massive apoptosis of oligodendrocytes and break-down of the blood-spinal cord barrier accompanied by invasion of immune cells resulting in sustained motoric and sensory impairments. Glial-fibrotic scarring and the lack of growth promoting factors impair axonal regrowth, which is currently the main target for therapeutic interventions to treat spinal cord injury. In addition, modulation of neuronal survival, remyelination of axons, and the immune reaction could promote functional regeneration (1–3). The inhibition of axonal regeneration might be overcome by exogenous application of growth factors or by transplantation of stem cells directly into the lesion site, which locally release trophic factors and thus support axonal regrowth. For clinical applications, stem cells should be ideally available on a clinical scale without ethical concerns or invasive interventions. Human umbilical cord blood (hUCB)[1] is

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[1] The abbreviations used are: hUCB, human umbilical cord blood; BDNF, brain derived neurotrophic factor; CSPG, chondroitin sulfate; proteoglycan; CST3, Cystatin C; CSF-1, colony-stimulating factor 1; CV, coefficient of variation; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; FDR, false discoverer rate; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; GO, gene ontology; GDNF, glial derived neurotrophic factor; GMP, good manufacturing

an alternative stem cell source including cells similar to mesenchymal stem cells from bone marrow (BM-MSC) and can be easily expanded as adherent cells *in vitro* without any risk for the donor. Besides MSC, hUCB contains unrestricted somatic stem cells (USSC) (4), which can be clearly distinguished from BM-MSC as well as from hUCB derived MSC (CB-MSC) by their immunological behavior (5–6), their transcriptome (7), the inability to differentiate into adipocytes (8), and by a specific Hox-gene expression pattern (9). Additionally, USSC exhibit a significant lower senescence rate and possess longer telomeres compared with CB-MSC and BM-MSC. As USSC can be easily isolated at GMP (good manufacturing practice) grade (10) and expanded on a clinical scale, USSC are a promising tool for transplantation studies. In a recent study, we have demonstrated that after transplantation into the acute injured rat spinal cord, USSC induce significant axon regrowth into the lesion side and effectively improve long-term functional locomotor recovery (11). Moreover, USSC transplantation promotes tissue sparing, which might contribute to the locomotor improvement. Although USSC were shown to differentiate into neuronal-like cells under *in vitro* conditions (4, 12) replacement of endogenous cells in the injured spinal cord was not detected supporting the hypothesis that transplanted stem cells despite their lack of differentiation enhance regeneration by paracrine regulation or direct interactions with endogenous cells. *In vitro* studies confirmed the potential of USSC to promote neurite growth. Incubation of primary rat dorsal root ganglion neurons or cortical neurons with USSC conditioned medium (USSC-CM) significantly enhanced neurite growth, hence considering USSC-CM is an ideal tool to investigate USSC-derived neurite growth promoting factors. Kögler *et al.* (13) provided first evidence using cytokine specific antibody arrays that USSC secrete axon growth promoting and neuroprotective factors *in vitro* such as stromal derived factor-1 (SDF-1) (14–15), leukemia inhibitory factor (LIF) (16), and vascular endothelial growth factor (VEGF) (17–18). In comparison, BM-MSC also secrete trophic factors, which enhance neurite growth *in vitro*, but the main trophic factors responsible for beneficial effects on neurite growth remain to be elucidated (19). Neurite growth promoting effects of BM-MSC seem to be at least partially mediated by brain derived neurotrophic factor (BDNF) and glial derived neurotrophic factor (GDNF) as demonstrated by neutralization of these neurotrophins using antibodies (20–21). Classical neurotrophic factors, *e.g.* nerve growth factor (NGF), BDNF, and neurotrophin-3 (NT-3), have not been identified in the USSC secretome yet, and other factors released by USSC, which promote neurite outgrowth, have not been described in detail. Antibody array analysis of the secretome of other somatic stem cell populations of hUCB revealed that the cells release a large panel of cytokines and growth factors (22). Additionally, these cells express genes related to neurogenesis and blood vessel development as determined by gene expression profiling. For the unbiased identification of secreted proteins proteomic approaches including mass spectrometry is the method of choice. For example, CB-MSC secrete typical cartilage-related proteins during chondrogenic differentiation shown by nanoLC MALDI-TOF/TOF (23). Currently, the secretome of several stem cell types has been investigated by mass spectrometry (reviewed by (24–25)). However, a secretome study of USSC derived from hUCB by LC-MS/MS focusing on nerve regeneration has not been performed, yet.

Here, we present the first study aiming at the characterization of the regeneration-supportive phenotype of USSC by detailed secretome analysis using protein mass spectrometry and exhaustive bioinformatic interrogation as well as subsequent functional validation of candidate neurite growth promoting proteins.

EXPERIMENTAL PROCEDURES

Cultivation of USSC—SA 5/73 USSC were isolated from human umbilical cord blood as described by Kögler *et al.* (4), and have been provided by the Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University Medical Center, Düsseldorf, Germany. In brief, cells were separated from fresh umbilical cord blood by Ficoll followed by red blood cell lysis with ammonium chloride. Mononuclear cells were plated in Dulbecco's modified Eagle's medium supplemented with 30% fetal bovine serum (FBS), dexamethasone (10^{-7} M, Sigma Aldrich, Steinheim, Germany), 2 mM glutamine and penicillin/streptomycin (100 U/ml). Subsequently, USSC clones were picked, expanded in dexamethasone-free medium, and characterized by Hox gene (Hox-negative) (9) and DLK-1 (DLK-positive) (8) expression. For expansion, USSC were cultured in expansion medium containing DMEM (Lonza, Cologne, Germany) supplemented with 30% heat-inactivated FBS (Biochrom, Berlin, Germany), 2 mM glutamine (Invitrogen, Darmstadt, Germany) and penicillin/streptomycin (100 U/ml, Invitrogen) at 37 °C, 5% CO_2 and 98% humidity. USSC have been used in passage 6–7 for all experiments.

Preparation of Serum-free Conditioned Medium—To identify secreted proteins USSC were cultured in serum-free medium. USSC were grown to a subconfluency of 80–90% in expansion medium, washed with PBS for three times to remove FBS and then incubated with serum-free N2 medium modified according to Bottenstein and Sato (26) for 48 h. Medium was changed and cells have been incubated for another 48 h. For secretome studies, the second batch of conditioned medium was used to exclude FBS effects. N2 medium is a 1:4 mixture of Dulbecco's modified Eagle's medium with Ham's F12, 2 mM glutamine (all Invitrogen), 5 μg/ml insulin, 30 nM sodium selenite, 100 μM putrescine, 20 nM progesterone and 5 μg/ml transferrin (all Sigma Aldrich). The conditioned medium has been collected, centrifuged at 1500 $\times$ *g* for 5 min at 4 °C to remove cell fragments and

practice; GDF-15, growth/differentiation factor 15; HDGF, hepatoma-derived growth factor; CB-MSC, derived MSC; LIF, leukemia inhibitory factor; MIF, macrophage migration inhibitory factor; MMP, matrix metallopeptidases; MANF, mesencephalic astrocyte-derived neurotrophic factor; BM-MSC, mesenchymal stem cells from bone marrow; MIEN-1, migration and invasion enhancer 1; NGF, nerve growth factor; NGPF, neurite growth promoting factor, NDNF, neuron-derived neurotrophic factor; NT-3, neurotrophin-3; PEDF, pigment epithelium-derived factor; PDGFA, platelet-derived growth factor A; p0, postnatal day 0; rec, recombinant; SPARC, secreted protein acidic and rich in cysteine; SCI, spinal cord injury; SDF-1, stromal derived factor-1; TSP-1, thrombospondin-1; TGF-β, transforming growth factor-beta; USSC, unrestricted somatic stem cells; USSC-CM, USSC conditioned medium; VEGF, vascular endothelial growth factor.

then stored at −80 °C until application to cortical neurons or secretome analysis.

Protein Concentration—Different strategies have been used to concentrate proteins from USSC conditioned medium. Proteins have been (A) precipitated using a modified method published by Chevallet *et al.* (27), or (B) concentrated by lyophilization. Briefly, for protein precipitation (method A) 20 ml of conditioned medium was mixed with 10% sarkosyl NL (Sigma Aldrich). After adding trichloroacetic acid (Sigma Aldrich) to a final concentration of 7.5%, samples were incubated on ice for 2h with subsequent centrifugation at 10,000 × *g* and 4 °C for 10 min. The pellet was resuspended in 2 ml ice cooled tetrahydrofuran (THF, Fluka, Buchs, Switzerland). Afterward, samples were centrifuged again at 10,000 × *g* and 4 °C for 10 min. The pellet was washed carefully with 2 ml THF and dissolved in 100 μl SDS sample buffer consisting of 600 mM DTT, 30% Glycerin, 12% SDS, 150 mM Tris/HCl, pH 7.0 (all Sigma Aldrich). After SDS-gel-electrophoresis (2 cm, 10 min) and silver staining according to Nesterenko *et al.* (28), the resulting lane was cut out in one slice. The gel slice was decolorized with a 1:1 mix of 30 mM sodium thiosulfate and 100 mM potassium hexacyanoferrate (III) (both Sigma Aldrich), washed, reduced (10 mM DTT) and alkylated (55 mM iodacetamide). Proteins were digested with 2 μg trypsin (Serva, Heidelberg, Germany) overnight at 37 °C. Resulting peptides were extracted with 50% acetonitrile (Biosolve, Valkenswaard, The Netherlands) and 0.05% TFA. For protein concentration by lyophilisation (method B) 20 ml of conditioned medium were lyophilized, reconstituted in 250 μl SDS sample buffer and 10 μl (2 μg/μl) was run into short SDS-gel, silver stained and subsequently digested and extracted as described above. Furthermore, a SDS-PAGE was performed and 12 lanes have been cut out for further analysis (method C). In supplemental Table S1 the methods are summarized.

LC-MS/MS—Peptides were separated using a UltiMate 3000 RSCLnano System (Dionex LC Packings (now Thermo Scientific)) with a C18-Pepman column (2 cm, 75 μm I.D., 3 μm particle size, 100 AA) as precolumn and a C18-Pepmap column (25 cm or 50 cm, 75 μm I.D., 2 μm particle size, 100 AA) as main column. A gradient of 0.1% FA (Fluka) to 0.1% FA/60% acetonitrile over 120–150 min and a constant flow rate of 300 nl/min was used to elute peptides directly via electrospray (voltage 1.2–3.0 keV, capillary temperature 310 °C) into the LTQ Orbitrap Velos or LTQ Orbitrap Elite mass spectrometer (both Thermo Fisher Scientific, Bremen, Germany). All MS spectra were recorded in positive ion mode with a mass range of 300–2000 *m/z* and a resolution of 35.000 (Orbitrap Velos) or 350–1700 *m/z* and a resolution of 60.000 (Orbitrap Elite). +2, +3 and higher charged (only Orbitrap Velos) monoisotopic precursors were isolated with a width of 2.0 Da and fragmented using a normalized collision energy of 35% for CID within the linear ion trap. A TOP20-data-dependent acquisition with polysiloxan as a lock mass was applied and dynamic exclusion activated (repeat count 1, duration 30 s on Orbitrap Velos and 45 s on Orbitrap Elite). Seven independent USSC secretomes were analyzed.

MS Data Analysis—Proteins were identified using Proteome Discoverer (Version 1.4, Thermo Fisher Scientific http://www.thermoscientific.com/en/product/proteome-discoverer-software.html) including Mascot search engine (Version 2.4.1, Matrix Science) (29). The UniProtKB/Swiss-Prot database (version from 2014/09, total entries: 546.439) was searched with a mass tolerance of 10 ppm (MS-mode) and 0.4 Da (MS/MS mode), enzyme specificity was set to trypsin and two missed cleavage sites were considered during the search against human subdatabase. Mass range setting was 350–5000 Da. Carbamidomethylation of cysteine was set as fixed modification. Oxidation of methionine was accepted as variable modification. Proteins were assembled by Proteome Discoverer with a Mascot threshold for protein identification set ≥ 50.0 and one unique peptide. For positive identification we considered a FDR of < 1% on peptide level (high peptide confidence, default $p < 0.01$). For FDR calculation we applied a decoy approach based on reversed protein sequences using Proteome Discoverer. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002241 (30). Proteins were grouped by Gene ontology (GO) slim term cellular component and biological process.

Bioinformatic Analyses—The prediction of secreted proteins was performed with SecretomeP (31) as well as with SignalP (32). A NN-score > 0.6 (FDR < 0.05) was interpreted as a high probability of secretion via a non-classical pathway using Secretome P. We further applied functional annotation clustering to further characterize the USSC secretome using DAVID database (DAVID Bioinformatics Resources 6.7, (33), http://david.abcc.ncifcrf.gov/home.jsp) including Gene Ontology (GO) terms containing the sub-ontology *biological process* (defined by the Gene Ontology Consortium) with a false discoverer rate (FDR) < 0.05. Secretion of all UniProt listed proteins were predicted by SecretomeP and supplemented by proteins that are assigned to the UniProt term *secreted* or *extracellular* as well as classically secreted proteins with signal peptide predicted by SignalP. The 11.238 proteins served as background list for GO term annotation in DAVID database.

An absolute quantification of protein concentration was performed using Hi-N in Progenesis QI for proteomics 2.0 (Nonlinear Dynamics, Newcastle upon Tyne, see http://www.nonlinear.com/progenesis/qi-for-proteomics/v2.0/faq/how-does-hi-n-work.aspx for further details) based on a publication by Silva *et al.* (34) using proteins concentrated by method A (TCA precipitation) and B (lyophilisation). Briefly, the Hi-N method considers all peptides for quantification. In the case if more than three peptides are available Hi-N includes the three most abundant unique (non-conflicting) peptides to calculate the absolute concentration. In contrast to top-3 method (34) the Hi-N algorithm applies the average and not the sum of the peptide intensities. Transferrin with a known concentration of 5 μg/ml (64.8 pmol/ml) was applied as single point calibrator and the protein signals were adjusted to transferrin for absolute quantification.

Preparation and Cultivation of Primary Rat Cortical Neurons—To test potential neurite growth promoting factors found in USSC secretome, primary cortical neurons were prepared from E15 Wistar rats as described before (11, 35) and subsequently incubated with serum-free conditioned medium derived from USSC. N2 medium as well as conditioned medium derived from primary cortical astrocytes prepared from p0-p1 Wistar rats (11, 36) served as negative and positive controls, respectively. Embryonic rat cortical neurons were seeded on 96 well plates (Costar, Corning, Wiesbaden, Germany) pre-coated with 1 mg/ml poly-D-lysine and 13 μg/ml laminin (both Sigma Aldrich). A cell density of 40,000/cm^2 was proven to be optimal for automated neurite growth analyses. Cells were incubated at 37 °C, 10% CO_2 and 98% humidity.

Neutralization and Stimulation Experiments—To influence activity of potential neurite growth promoting factors in USSC-CM, neutralizing antibodies have been incubated for 2 h at 37 °C with USSC-CM prior to incubation of primary cortical neurons. Following antibodies have been used: anti-SPARC (secreted protein acidic and rich in cysteine, also known as Osteonectin, human, monoclonal produced in mouse, sc-33645, Santa Cruz Biotechnology, Heidelberg, Germany), 4 μg/ml; anti-PEDF (pigment epithelium-derived factor, also known as serpinF1, human, polyclonal produced in rabbit, sc-25594, Santa Cruz Biotechnology), 4 μg/ml; anti-CST3 (Cystatin C, human, polyclonal produced in goat, P01034, R&D System, Wiesbaden-Nordenstadt, Germany), 3 μg/ml. Because antibodies have been solved in PBS, USSC-CM with appropriate amounts of PBS has been used as control.

On the other hand, selected recombinant proteins were applied in nonconditioned N2 control medium to stimulate neurite growth. Recombinant proteins have been diluted as follows: SPARC (C388, Novoprotein Scientific, Summit, NJ), 3 μg/ml; PEDF (P3662, Abnova, Taipei, Taiwan), 30 ng/ml.

Immunocytochemical Staining of Cortical Neurons—After 48 h of cultivation, cortical neurons were fixed with 3.7% formaldehyde (Merck, Schwalbach, Germany) for 15 min and carefully washed with PBS. For immunocytochemical analysis, fixed cells were incubated with blocking solution containing 10% normal goat serum (Sigma Aldrich), and 0.03% Triton x-100 (Sigma Aldrich) for 1 h. Staining was performed using a neuron specific anti-β-III-tubulin (Tub) antibody (rabbit, polyclonal, Sigma Aldrich, 1:500 dilution in blocking buffer) over night at 4 °C. After washing with PBS, secondary antibody (goat anti-rabbit, Alexa 488, Invitrogen, 1:500 dilution) was incubated for 4 h at room temperature. DAPI (4′,6-diamidino-2-phenylindole) staining was performed to label all cell nuclei.

Analyses of Neurite Growth—High content screening of cortical neurons was performed *in vitro* with a Thermo Scientific Cellomics® ArrayScan® VTI HCS Reader and the Neuronal Profiling v3.5 BioApplication software (http://www.thermoscientific.com/en/products/cellular-imaging-analysis.html). Plates were imaged with a 10× objective (25 images per well) and simultaneously analyzed. For quantification, only Tub-positive structures with a DAPI-positive nucleus were included in cell counts. Following parameters were used for quantification: nucleus identification (iso data > -0.119; segment intensity > 80), nucleus validation (size 7–110), cell body identification (iso data > 0; minimum 1 cell body; seed segmentation; cell body segmentation $= 2$), cell body validation (1 nucleus; cell body size 30–1050; total cell intensity 20,450–521,307), neurite identification (identification modifier $= -0.938$; length 255; point resolution 2; gap tolerance $= 5$), neurite validation (neurite length 10–600; neurite intensity 0–4095). Absolute numbers of neurons per well and total neurite length per neuron were used for analyses of different culture conditions. Significance of neurite growth was assessed by paired Student's *t* test comparing median neurite growth from six wells per condition and experiment with appropriate control condition. Neurite growth was considered to be significantly different at $p < 0.05$. All data are presented as a mean relative neurite growth (%) of at least three experiments ± S.E.

RESULTS

Characterization of USSC Secretome by Mass Spectrometry—The first evidence that the regenerative potential of USSC is likely to rely on paracrine mechanisms was obtained because we showed that USSC secrete factors that stimulate neurite growth of cultured dorsal root ganglion neurons and cortical neurons (11). As a first approach to test which molecular class mediates the trophic functions of USSC the supernatant was heated to 80 °C for 5 min. The abolished neurite growth potential (data not shown) suggests that temperature-sensitive proteins are likely to enhance neurite growth. Therefore, we set-up a workflow analyzing serum-free supernatants of USSC using nano LC-ESI-MS/MS to identify proteins secreted by USSC. For protein preparation, we applied different methods to obtain a comprehensive protein catalogue of the USSC secretome (supplemental Table S1). Altogether, we identified 1520 proteins in USSC secretome (supplemental Table S2). Because of possible contaminations of conditioned medium by proteins originating from dead cells, not all identified proteins are likely to be secreted or extracellularly localized. Based on UniProt annotation, 993 proteins were classified as being potentially localized extracellularly and 118 proteins to be present at the cell surface (Fig. 1*A*, multiple protein entries possible). The USSC secretome further includes 884 membrane associated proteins, which could result from shedding. For further identification of secreted proteins, SignalP 4.0 algorithm was applied. We categorized 385 proteins to be classically secreted and—using SecretomeP 2.0 algorithm—276 proteins to be potentially secreted via non-classical pathways, remaining 487 proteins to be localized extracellular predicted by UniProt annotation (supplemental Table S2). Eight proteins, which have been found to be localized at the cell surface as identified by UniProt annotation, were not identified by SignalP and SecretomeP databases (Fig. 1*B*). Overall, 1156 proteins were determined to be candidate secretory proteins of USSC secretome, whereas 364 proteins were presumably contaminants from dead cells.

Neurite Growth Promoting Factors Secreted by USSC—Several USSC secreted proteins were found to be associated with neurite growth or axonal regeneration. Comparison of all secreted proteins or proteins predicted to be secreted according to the literature revealed that USSC secrete at least 31 proteins that are known to be involved in neurite growth or axonal regrowth (Table I) including the neurotrophic factors mesencephalic astrocyte-derived neurotrophic factor (MANF) (37), neudesin (38), and neuron-derived neurotrophic factor (NDNF) (39) as well as the growth factors angiopoietin-2 (40), growth/differentiation factor 15 (GDF-15) (41), hepatoma-derived growth factor (HDGF) (42), PDGF (43), and transforming growth factor-beta1/-2 (TGF-β1/TGF-β2) (44–45). Moreover, we identified several matrix- and membrane-associated proteins, *e.g.* decorin (46), fibulin-1 (47), netrin-4 (48), neuronal growth regulator 1 (49), neuroplastin (50–51), noelin-2 (52), SPARC (53), tenascin (54), and the cytokines colony-stimulating factor 1 (CSF-1) (55), macrophage migration inhibitory factor (MIF) (56) as well as the chemokine stromal cell-derived factor 1 (SDF-1) (15). In addition, the serine protease inhibitors glia-derived nexin (serpinE2) (57), neuroserpin (58), and plasminogen activator inhibitor 1 (serpinE1) (59) as well as the extracellular chaperone clusterin (60–61) were secreted by USSC. In summary, these USSC secreted proteins represent a network of trophic factors that are likely to promote neurite growth *in vitro* as well as axonal regrowth after transplantation into the injured spinal cord in a synergistic way.

Clustering of USSC Secreted Proteins into Biological Processes—In order to gain an understanding of the complex composition of the USSC secretome, we assigned all proteins (1156 proteins) to biological processes by UniProt annotation resulting in biological processes associated with regeneration after spinal cord injury. USSC secreted proteins were assigned to *cell communication* (455 proteins), *development* (426 proteins), *cell differentiation* (307 proteins), *cell prolifer-*

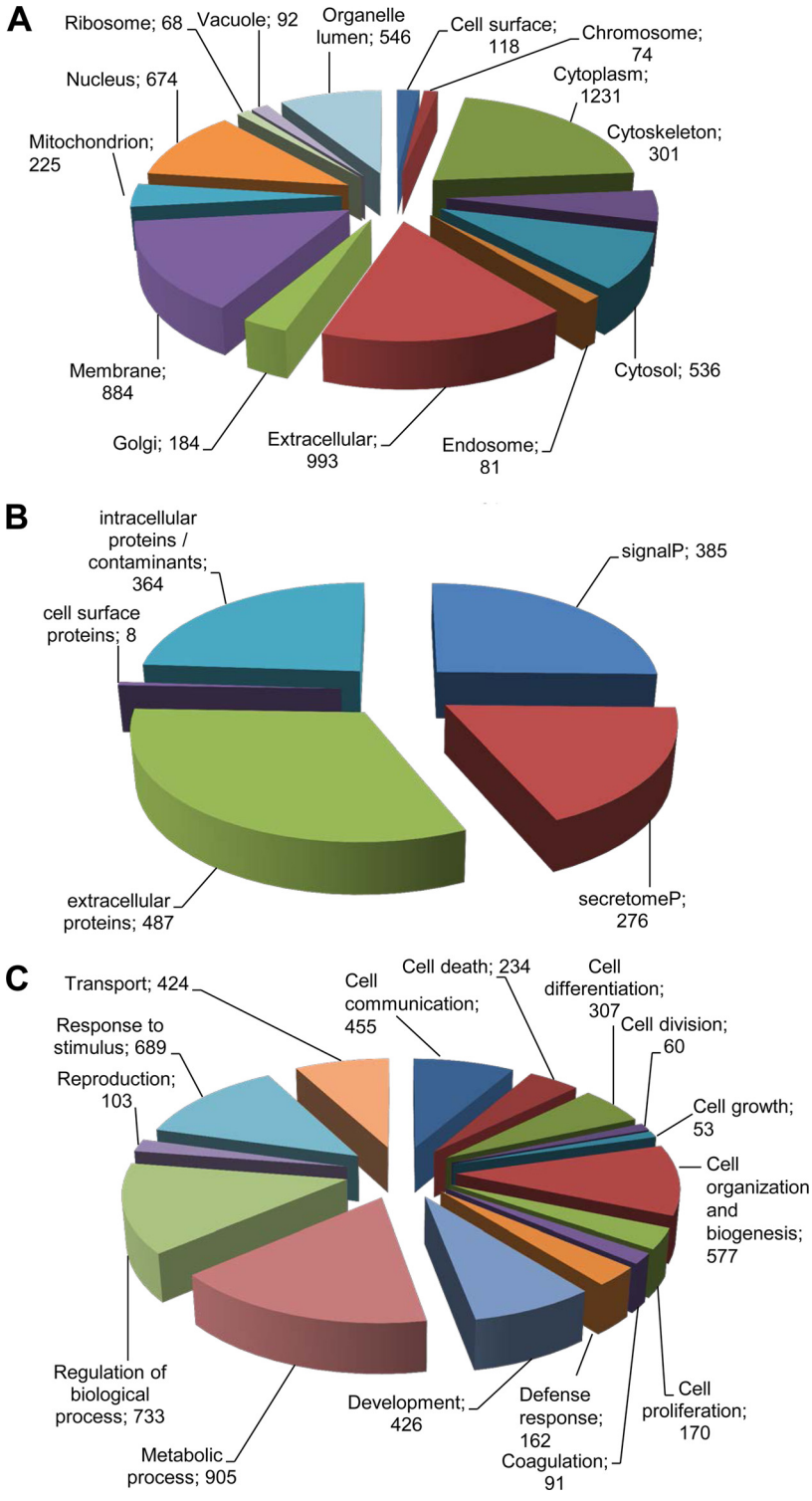

FIG. 1. **Categorization of proteins identified in USSC secretome by LC-MS/MS.** *A*, Based on UniProt annotation, 993 proteins were found to be localized extracellularly. The USSC secretome further included 884 membrane associated proteins that might be found in the secretome because of shedding and 118 cell surface proteins (multiple protein entries possible). In total, 1520 proteins were determined. *B*, SignalP and SecretomeP algorithms revealed that 385 proteins are classically secreted and 276 proteins are nonclassically secreted. GO slim term *extracellular* included 487 proteins that were not predicted as secreted by SignalP and SecretomeP. Additionally, eight proteins were predicted to be localized at the cell surface. Overall, we identified 1156 proteins predicted to be secreted and 364 contaminants. *C*, These 1156 were assigned biological processes that in part play important roles during nerve regeneration (multiple protein entries possible) such as *cell differentiation*, *development*, *cell communication* and *cell division*.

TABLE I

Neurite growth promoting factors identified in the USSC secretome. Overall, 31 proteins were identified in the USSC secretome (contaminants excluded) which are known to be directly involved in neurite growth

ID	Neurite growth promoting factor	Short description	Secretion via/ localization	Reference
O00468	Agrin	Heparan sulfate basal lamina glycoprotein	signal peptide	72
O15123	Angiopoietin-2	Growth factor	signal peptide	40
P10909	Clusterin (Clu)	Extracellular chaperone	signal peptide	60–61
P09603	Colony-stimulating factor 1 (CSF-1)	Cytokine	signal peptide	55
P07585	Decorin	Matrix proteoglycan	signal peptide	46
P23142	Fibulin-1	ECM-associated	signal peptide	47
P06396	Gelsolin	Actin-binding protein	signal peptide	80
P07093	Glia-derived nexin (serpinE2)	Secreted serine protease inhibitor	signal peptide	57
P28799	Granulins	Secreted glycosylated peptide, cytokine-like activity	signal peptide	68–69
Q99988	Growth/differentiation factor 15 (GDF-15)	Growth factor	signal peptide	41
P51858	Hepatoma-derived growth factor (HDGF)	Growth factor	extracellular	42
P14174	Macrophage migration inhibitory factor (MIF)	Pro-inflammatory cytokine	non-classical pathway	56
P55145	Mesencephalic astrocyte-derived neurotrophic factor (MANF)	Neurotrophic factor	signal peptide	37
Q9HB63	Netrin-4	ECM-associated	signal peptide	48
Q9UMX5	Neudesin	Secreted neurotrophic factor	signal peptide	38
Q8TB73	Neuron-derived neurotrophic factor (NDNF)	Neurotrophic factor	signal peptide	39
Q7Z3B1	Neuronal growth regulator 1	Membrane-associated	signal peptide	49
Q9Y639	Neuroplastin	Type I transmembrane protein	signal peptide	50–51
Q99574	Neuroserpin	Secreted serine protease inhibitor	signal peptide	58
P14543	Nidogen-1	Sulfated glycoprotein in basal lamina	signal peptide	71
O95897	Noelin-2	secreted glycoprotein	signal peptide	52
Q15063	Periostin	ECM-associated	signal peptide	67
P36955	Pigment epithelium-derived factor (PEDF)	Secreted neurotrophic factor	signal peptide	84–86
P05121	Plasminogen activator inhibitor 1 (serpinE1)	Secreted serine protease inhibitor	signal peptide	59
P04085	Platelet-derived growth factor (PDGFA)	Growth factor	signal peptide	43
P09486	Secreted protein, acidic, cysteine-rich (SPARC)	Matrix-asscociated	signal peptide	53
P48061	Stromal cell-derived factor 1 (SDF-1)	Chemokine	signal peptide	14–15
P24821	Tenascin	ECM-associated	signal peptide	54
P07996	Thrombospondin-1 (TSP-1)	Adhesive glycoprotein	signal peptide	63
P01137	Transforming growth factor, beta-1 (TGF-b1)	Growth factor	signal peptide	45
P61812	Transforming growth factor, beta-2 (TGF-b2)	Growth factor	signal peptide	44

ation (170 proteins), and *cell growth* (53 proteins) suggesting that USSC transplantation affects these biological processes relevant for nerve regeneration (Fig. 1C, multiple protein entries possible). Next, we performed enrichment analysis using DAVID bioinformatics database (DAVID Bioinformatics Resources 6.7, (33)) to reveal biological processes overrepresented in USSC secretome. Several biological processes were identified to be significantly enriched that are associated with neurite growth, neuronal differentiation and survival as well as nerve regeneration, *e.g. cell adhesion (*111 proteins*), cell motion* (58 proteins*), cytoskeleton organization* (42 proteins), and *extracellular matrix organization* (45 proteins) (Fig. 2 and supplemental Table S3). Numerous proteins involved in *cell adhesion* expressed by USSC including cadherins, neuronal growth regulator 1, neuropilin 1, neuroplastin, roundabout axon guidance receptor and tenascin C are known to have a strong impact on nerve regeneration. Moreover, the USSC secretome included proteins associated with the functional category *blood vessel development* (44 proteins). The latter is known to be important for regeneration after neuronal trauma, containing angiopoietin 2, SDF-1, matrix metallopeptidases MMP-2, MMP-14, MMP-19, platelet-derived growth factor A (PDGFA), and TGF-β2. In addition, the biological process *collagen fibril organization* (18 proteins) included proteins associated with extracellular matrix, *e.g.* lumican as well as aggrecan and TGF-β2.

Quantification of USSC Secreted Proteins—Further we were interested to determine the concentration of identified proteins involved in relevant biological processes of neuronal regeneration. Therefore, we determined the protein concentrations based on a publication by Silva *et al.* (34) which enabled us to quantify 1020 proteins. For the USSC secretome we found that the protein concentrations varied in the range of 2–3 orders of magnitude with fibronectin as the highest abundant (88 pmol/ml) and quinone oxidoreductase (2 fmol/ml) as the lowest abundant protein detected by MS

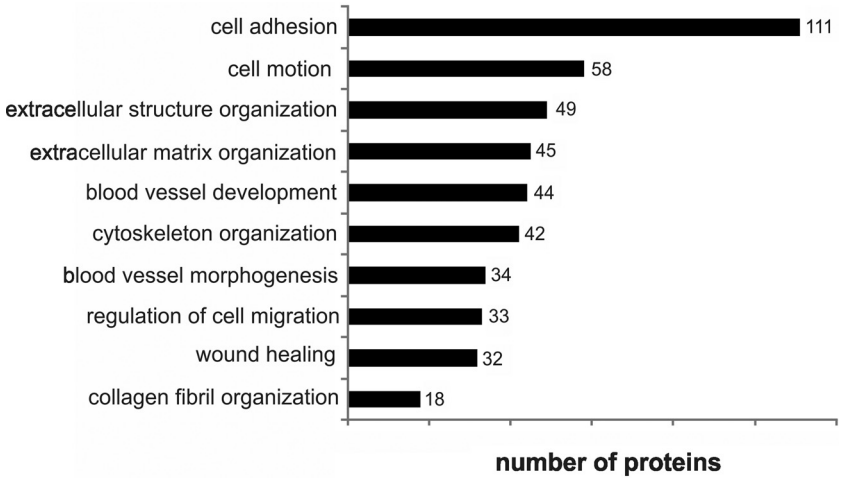

FIG. 2. **Subset of biological processes associated with neurite growth, neuron differentiation, survival and regeneration enriched in comparison to all secreted proteins.** All proteins identified in the USSC secretome by LC-MS/MS excluding contaminants were included (multiple protein entries possible). GO terms were evaluated with DAVID database with a false discoverer rate (FDR) $p < 0.05$.

(supplemental Table S4). Quantified proteins were ranked into three classes based on their abundance. Enrichment analysis of the three abundance classes by DAVID bioinformatics database thereby using all secreted proteins as background revealed that the high abundant proteins (class I) are linked to *extracellular matrix organization* and *ectoderm development* (Fig. 3). Class II included proteins likewise associated with *extracellular matrix organization* but also with *cytoskeleton organization* and *actin filament-based processes* whereas class III proteins are linked to *biological adhesion* and *cell adhesion*. Analysis further revealed that 26 of the 31 identified neurite growth promoting factors were quantifiable and evenly distributed in class II (71–2457 fmol/ml) and class III (2–71 fmol/ml). Class II included growth factors and extracellular matrix (ECM) associated proteins whereas class III mainly included growth factors and small cytokine-like proteins. In class I, four neurite growth promoting proteins (2–88 pmol/ml) have been found that are all associated with ECM.

Validation of Potential Neurite Growth Promoting Factors in a Neurite Outgrowth Assay—

Neutralization of Candidate Neurite Growth Promoting Factors—With intend to validate neurite growth promoting function of potential candidates we investigated selected proteins assigned to first two abundance classes in a neurite outgrowth assay *in vitro*. Therefore, we inhibited functionality of candidate proteins by neutralizing antibodies applied to USSC-CM 2 h before application to primary rat cortical neurons. Cortical neurons were incubated for 48 h, fixed and stained with a neuron-specific antibody (β-III-tubulin) to visualize cell bodies and neurites and analyzed by automated scanning with a Cellomics® ArrayScan® VTI HCS Reader. Astrocyte conditioned medium known to enhance neuronal survival and neurite growth *in vitro* (11, 36, 62) served as positive control for culture preparations in each experiment (data not shown). Our data indicate that neutralization of SPARC as well as PEDF (also known as serpinF1) in USSC-CM was effective with 4 μg/ml neutralizing antibody each resulting in significantly decreased neurite growth compared with control cultures treated with USSC-CM (Fig. 4). Neurites were significantly shorter (67.6% $\pm$ 2.3 as well as 74.8% $\pm$ 4.1 of control) because of neutralization of SPARC and PEDF, respectively. Neutralizing antibodies against cystatin C (CST3) (Fig. 4) had no effect on neurite growth, which also indicates that application of immunoglobulin does not in general suppress neurite growth. Cell survival was unchanged upon neutralization of proteins (data not shown).

Stimulation of Neurite Growth by Application of Candidate Neurite Growth Promoting Factors—To further validate candidate proteins, we applied recombinant proteins in N2 (negative) control medium on primary cortical neurons and analyzed neurite growth after 48 h of incubation. Recombinant SPARC led to significantly enhanced neurite growth after application on cortical neurons up to 364.6% $\pm$ 74.4 (Fig. 5). PEDF application alone had a small effect on neurite growth (139.7% $\pm$ 18.6, $p = 0.07$), which did not meet our significance criteria. Again, cell survival was not influenced by application of recombinant proteins (data not shown). In summary, neutralization of SPARC and PEDF in USSC-CM resulted in significantly decreased neurite growth. However, the decline in neurite growth did not reach the low basal levels of N2 control medium. We demonstrated further that application of recombinant SPARC to N2 medium enhanced neurite growth, but less strong than USSC conditioned medium.

DISCUSSION

USSC are a promising tool to support regeneration after spinal cord injury (SCI), because this stem cell type was previously shown to improve axonal regeneration and tissue sparing after transplantation into an acute rat SCI model (11). Beside axonal regeneration, support of cell survival and modulation of the ECM as well as angiogenesis are main targets for therapeutic interventions after SCI. As USSC secrete factors enhancing neurite growth and neuronal survival *in vitro*, we were interested to identify and characterize these factors in the USSC secretome to gain insight into potential paracrine mechanisms leading to en-

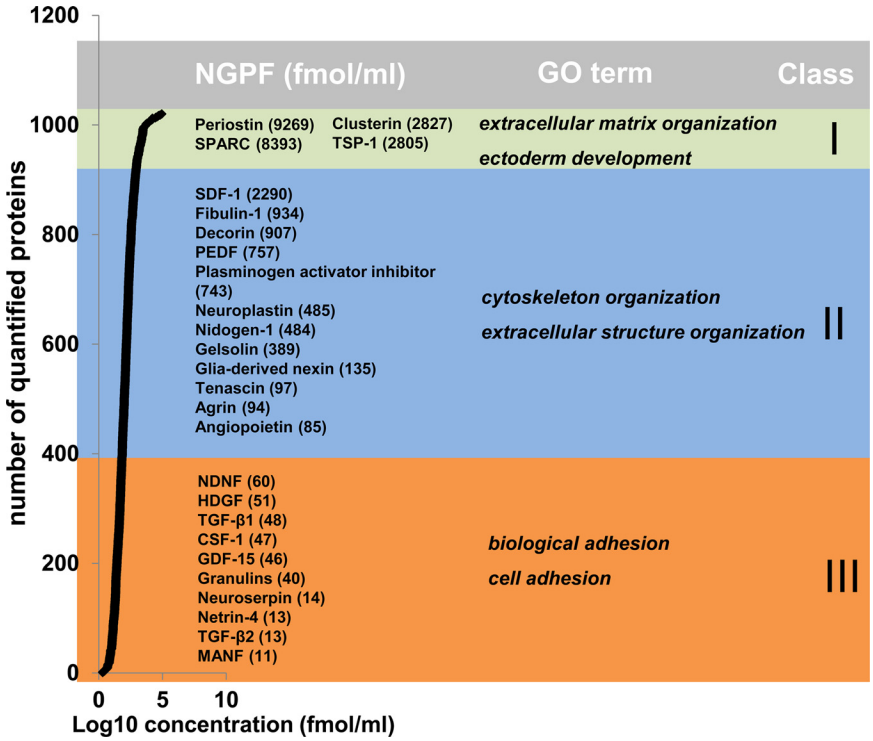

FIG. 3. **Concentration range of neurite growth promoting factors within USSC secretome.** Protein concentrations (Log10 fmol/ml) of USSC secretome were ranked into three classes. For each class neurite growth promoting factors (NGPF) with corresponding concentration (fmol/ml) were assigned. Enrichment analysis was applied for each abundance class. GO terms important for regeneration are indicated.

hanced regeneration after USSC transplantation. In the present study we established a USSC secretome comprising 1156 proteins and revealed that USSC release at least 31 well-established neurite growth promoting factors. In addition, USSC release proteins that are related to several biological processes involved in nerve regeneration. We were able to functionally validate two of the candidate neurite growth promoting factors by neurite outgrowth assays *in vitro* validating our proteomic approach and confirming a paracrine mechanism of regeneration support for transplanted USSC in SCI.

Secretion of Proteins Involved in Neurite Growth or Axonal Regeneration—By means of secretome approach applying LC-MS/MS we determined 1156 proteins, whereof at least 31 proteins in the USSC secretome are already known to be associated with neurite growth or axonal regeneration. The group of 31 proteins include SPARC and thrombospondin-1 (TSP-1), which together with tenascin C modulate interactions with ECM molecules (63). We further identified netrin-4 known to attract or repel growing axons depending on neuron type (48). PDGF and TGF-β1, both secreted by USSC, are known to interact with SPARC (64–66), indicating a solid network between the secreted factors. Moreover, USSC secrete MIF, which directs the initial neurite outgrowth from the statoacoustic ganglion (SAG) to the developing inner ear (56), and NDNF, which could together with MANF, HDGF, and neudesin stimulate neurite growth as well as migration, cell growth, and survival (37–39, 42). Recently, periostin, which is normally expressed in bone and muscles, has been shown to play an essential role in neurite growth and axonal regeneration after SCI to overcome the inhibitory effect of scar-associated molecules by signaling through focal adhesion kinase and Akt (67). Progranulin has similar characteristics like neurotrophins as it is cleaved into mature granulins by extracellular proteases and has been shown to stimulate neurite growth (68–69). TGF-β2, which is an immunomodulatory factor, normally facilitating ECM synthesis resulting in enhanced scar formation has also been shown to stimulate neurite growth in cultured dorsal root ganglion neurons (44). Using Hi-N method we showed that the identified proteins are distributed over a concentration range from fmol/ml - pmol/ml. As expected, growth factors were present at low concentration (fmol/ml range), whereas ECM associated proteins were highly abundant (pmol/ml range) in the USSC secretome. However, it has been shown that this kind of quantification method is associated with large quantification errors of small proteins (70), which likely depend on limited number of peptides as well as protein-to-protein variation. As our experiments were not designed for protein quantification in the first instance we took prior advantage of Hi-N method allowing quantification of hundreds of proteins in parallel to get a first impression about the distribution of proteins' concentration in USSC secretome. Although, this global approach gave acceptable results

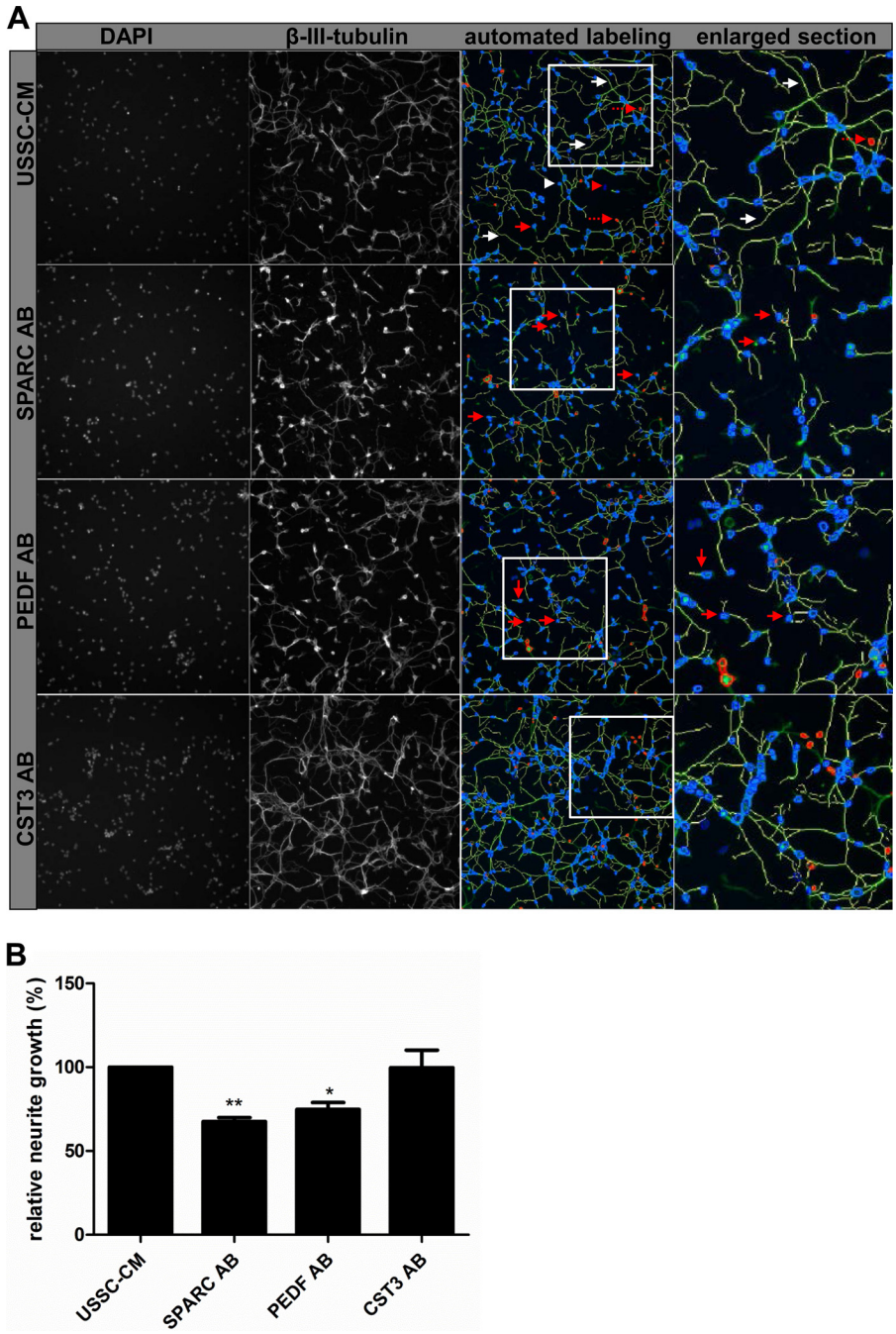

FIG. 4. **Reduced neurite growth after immune neutralization of neurite growth promoting factors.** *A*, Neutralization of SPARC and PEDF leads to reduced neurite growth of cortical neurons compared with USSC-CM. Neutralization of CST3 had no significant effect on neurite growth. Red arrow: neuron with short neurite; white arrow: long neurite; red arrowhead: nuclei but no neuron, excluded from quantification; white arrowhead: counted cell body of a neuron; interrupted red arrow: artifact excluded from quantification. Neurites labeled by the Neuronal Profiling v3.5 BioApplication software (yellow labeled neurites) were included into quantification. See also enlarged sections for further details. *B*, Quantification of relative neurite length (%) compared with USSC-CM control. Results derived from at least three independent experiments are presented as mean values ± SEM. * $p < 0.05$, ** $p < 0.01$ (Student's *t* test).

with mean standard deviation from 49–115% concentrations of specific proteins need confirmation by complementary techniques.

Altogether, these different neurite growth promoting factors, which were expressed by USSC, either alone or in combination, are likely to participate in neurite growth stimulation of cultured primary cortical neurons as well as to support axonal regrowth observed after USSC transplantation into the injured spinal cord. The combined secretion of neurite growth promoting factors as well as inhibitory proteins led to en-

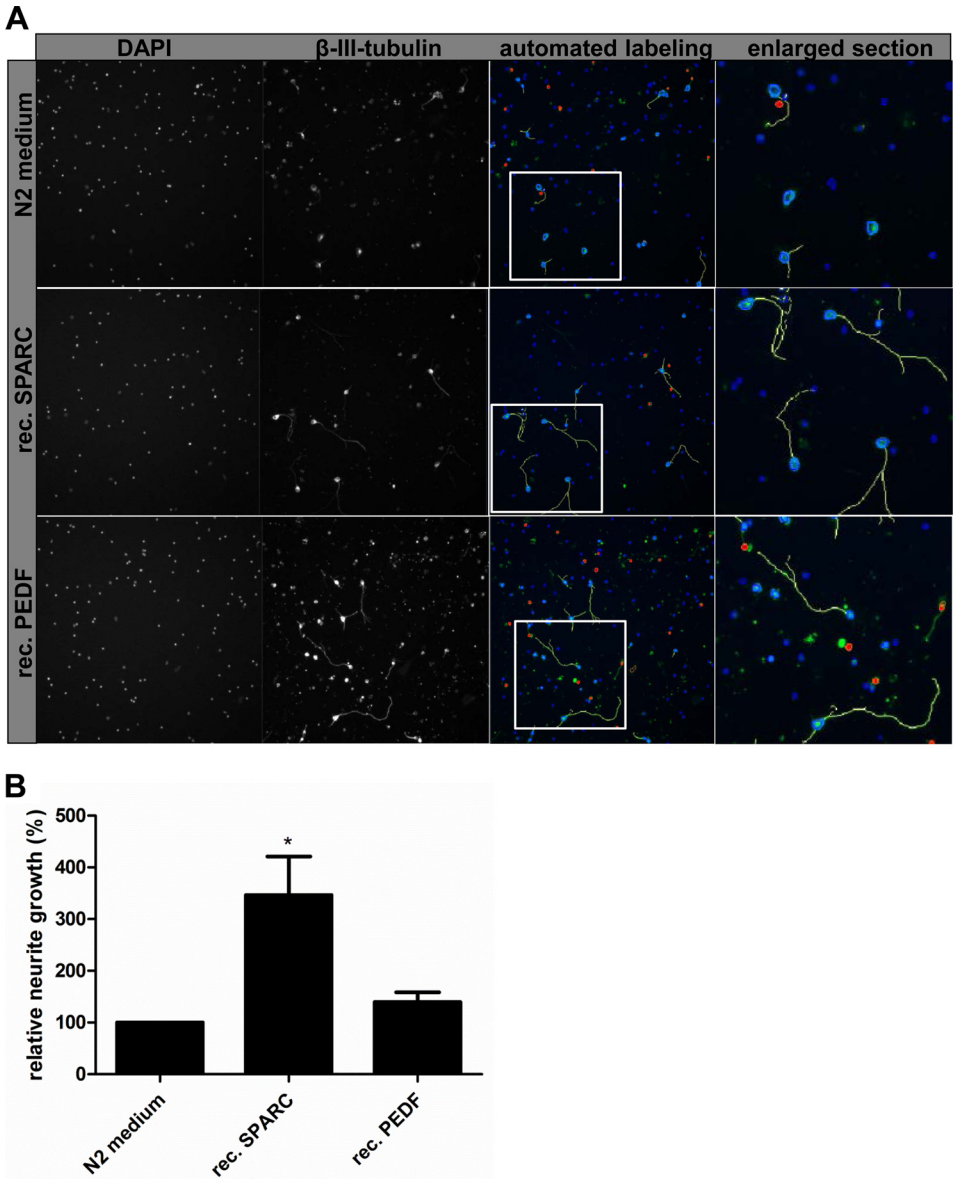

FIG. 5. **Enhanced neurite growth by application of recombinant proteins.** *A*, Application of recombinant SPARC leads to enhanced neurite growth of cortical neurons compared with N2 control medium. Application of recombinant PEDF has only minor effects on neurite growth. Neurites labeled by the Neuronal Profiling v3.5 BioApplication software (yellow labeled neurites) were included into quantification. See also enlarged sections for further details. *B*, Quantification of relative neurite length (%) compared with N2 control medium. Results derived from at least three independent experiments are presented as mean values ± SEM. * $p < 0.05$ (Student's *t* test).

hanced neurite growth as well as to axonal regeneration indicating an over-all supportive balance of USSC secreted proteins for regeneration associated processes.

Native USSC cultured in serum-containing medium were shown to release several cytokines and growth factors (13). In the present study, expression of CSF and SDF-1α, which are known to be directly or indirectly involved in neurite growth or axonal regeneration (14–15, 55), was confirmed under serum-free conditions. However, expression of the other proteins identified in the former work of Kögler *et al.* (13) was not detected in our experiments, suggesting changes in the USSC expression profile under different culture conditions as well as the different detection modalities of the applied analytical platforms.

USSC secrete proteins involved in multiple regeneration associated processes—To establish the USSC secretome we performed rigorous bioinformatics data analysis. We considered accessible knowledge about proven extracellular localization and further we took advantage of the two bioinformatics tools SignalP and SecretomeP allowing prediction of classical and non-classical protein secretion, respectively. From the 1520 proteins identified by LC-MS/MS 1156 are known or were predicted to be secreted including 385 classically secreted proteins and 276 proteins secreted via non-

classical pathways. Three hundred and sixty-four proteins did not match our criteria and were removed from our candidate list. We are confident that our approach of combining experimental data with bioinformatics prediction tools is straightforward to establish USSC secretome. This holds true especially for classical protein secretion by signal peptide that is well studied and prediction algorithms like *e.g.* SignalP were successfully proven. In contrast, SecretomeP, which, based on simplified assumption that extracellular proteins share the same characteristics, have to be taken with caution and should only be applied in combination with experimental data.

Secretion of Proteins Involved in Extracellular Matrix Organization—Further data interrogation by annotation clustering revealed that USSC secrete proteins are involved in differentiation and developmental processes. For more detailed characterization of the USSC secretome, proteins were assigned GO terms by DAVID bioinformatics database revealing that USSC secrete proteins associated with biological processes involved in regeneration after SCI.

Several proteins of the USSC secretome were assigned to *cell adhesion*, *extracellular structure organization,* and *extracellular matrix organization*, *e.g.* the chondroitin sulfate proteoglycan (CSPG) aggrecan is known to inhibit axonal regrowth but also the growth promoting decorin, which antagonizes scar formation (46). USSC further secrete laminin subunits, which might promote neural differentiation and migration of neural precursor cells after CNS lesion and together with nidogen-1 could guide growth cone turning (71) as well as the heparan sulfate proteoglycan agrin, which is involved in synaptogenesis during regeneration (72). Thus, our results suggest that USSC strongly interact with the local environment after transplantation into the injured spinal cord via modifications of ECM structures as well as cell-cell-contact. Previous studies described that even in the presence of inhibitory molecules successful axon growth after injury were achieved when a prevalence of growth permissive signals is present (73–74). Thus, USSC release ECM proteins that seem to shift the balance toward an axon growth permissive microenvironment thereby enhancing axonal regrowth *in vivo*.

Secretion of Proteins Involved in Angiogenesis—Beside axonal regrowth, regulation of cell adhesion and ECM modulation, angiogenesis is essential for proper regeneration as neurons have high metabolic requirements. Despite massive blood vessel sprouting, new blood vessels exhibit abnormal permeability after SCI suggesting limited functionality (75). USSC are likely to promote angiogenesis because these cells secrete several proteins assigned to *blood vessel development* or *blood vessel morphogenesis*, *e.g.* angiopoietin-2, angiopoietin-like 4, MMP-2, −14, and −19, PDGF, and TGF-β, which together with guidance molecules such as netrin-4, semaphorin-7 and slit-3, could enhance blood vessel formation during regeneration.

USSC further secrete proteins grouped into GO terms *cell motion* and *regulation of cell migration* indicating that USSC might influence migration of endogenous neural precursor cells and endothelial as well as immune cells. Enhanced migration of precursor cells derived from the ependymal zone of the central canal could led to better recovery because these cells have been shown to differentiate into oligodendroglial cells (76). Because the SDF-1/CXCR4 axis is suggested to play a key role in migration and differentiation of adult endogenous stem cells and oligodendrocyte precursor cells after SCI (77–79), USSC expressed SDF-1 could influence precursor cell properties. In addition, secreted CSF-1, laminin subtypes, neuropilin-1, PDGF, migration and invasion enhancer 1 (MIEN-1) and TGF-β could regulate migration of precursor cells, astrocytes, fibroblasts, and immune cells, which contribute to proper regeneration.

Secretion of Proteins Involved in Cytoskeletal Organization—Most of the above mentioned proteins are known to be secreted via the classical pathway. In addition, the USSC secretome includes several proteins predicted to be either secreted by nonclassical pathways or annotated as extracellular by UniProt database. These proteins were found to be enriched in biological processes *cytoskeleton organization*, *e.g.* capping proteins, actin-related protein complexes, and gelsolin, which is known to play a role in neurite growth (80). Together with syntaxin, synaptotagmin, and kinesins, which were also found in the USSC secretome these proteins potentially result from exocytosis processes as these proteins are known to be involved in exocytosis. Furthermore, cytoskeleton proteins could originate from shedding because of their localization at the cell membrane, in particular ezrin and moesin both identified in the USSC secretome that link cell surface receptors and the actin cytoskeleton. It is unclear whether these proteins were secreted under certain conditions (25), but we cannot exclude that these proteins partially originate from dead cells. However, the biological processes related to the cytoskeleton included classically secreted proteins normally directly or indirectly regulating the dynamic remodeling of actin cytoskeleton (PDGF, SDF-1) indicating that USSC could also influence cytoskeleton dynamics of regenerating axons. By protein quantification we revealed that biological processes like, *e.g. extracellular matrix organization* and *ectoderm development* are enriched among highly abundant proteins, whereas proteins involved in *cell adhesion* are enriched within the group of low abundant proteins. We only can speculate if the abundance of a protein or a protein family is a measure for relevance of the associated biological process because both low abundant proteins like neurotrophins and high abundant ECM proteins are known to be critical for regeneration.

Functional Analysis of Candidate Proteins—SPARC is one of the most abundant proteins identified in the USSC secretome. It is a multifunctional glycoprotein that binds to collagens and vitronectins and modulates the activity of PDGF, FGF-2 and VEGF. It was further demonstrated that SPARC stimulates Schwann cells to facilitate axonal regeneration via

laminin-1 and TGF-β1 signaling (53). Schwann cells also express SPARC, which have been shown to promote both neurite growth and survival of purified retinal ganglion cells (81). SPARC is discussed to have synergistic effects on classical neurotrophins enhancing neurite outgrowth of retinal ganglion cells (82–83). USSC-CM has been shown to stimulate neurite growth of primary cortical neurons (11). In the present study, we demonstrated that neurite growth is significantly decreased when a SPARC neutralizing antibody is applied to USSC-CM. On cortical neurons, recombinant SPARC directly enhances neurite growth. Therefore, we conclude that cortical neurons respond to SPARC signaling and that SPARC is one of the major neurite growth promoting factors secreted by USSC.

Recombinant PEDF (serpinF1) has been applied at low concentration representing the rather low amounts identified in USSC-CM, resulting only in a slightly increased neurite growth. On the other hand, neutralization resulted in a significant decrease in neurite growth, similar in range as SPARC antibody, indicating that PEDF may act in combination with other USSC secreted factors to stimulate cortical neurite growth. PEDF has been shown to enhance neuronal survival as well as neurite growth of retinoblastoma cells (84), cerebellar granule cells (85) and spinal motor neurons (86). Under our culture conditions, SPARC and PEDF did not affect neuronal survival of cortical neurons, indicating that other USSC secreted proteins promote neuron survival of cultured cortical neurons. *In vivo*, both proteins are likely to stimulate axonal regrowth directly but could also have indirect effects on regeneration via stimulation of glial cells or cell survival.

CONCLUSIONS

The lack of regeneration after SCI might be overcome by transplantation of stem cells directly into the lesion site, which locally release trophic factors and thus support axonal regrowth. The present study demonstrates that the regenerative phenotype of USSC secretome bases on a complex network of proteins associated with several biological processes that lead to enhanced neurite growth *in vitro* and therefore suggest a relevant role on axonal regeneration *in vivo*. The complex network of trophic factors secreted by USSC seems to act synergistically on several levels of nerve regeneration. With this observation it appears that transplantation of somatic stem cells instead or in combination with defined factors has great potential in regenerative medicine.

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S This article contains supplemental Tables S1 to S4.

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