Stem cells from human apical papilla decrease neuro-inflammation and stimulate oligodendrocyte progenitor differentiation via activin-A secretion

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Abstract:	Secondary damage following spinal cord injury (SCI) leads to non-reversible lesions and hampering of the reparative process. The local production of pro-inflammatory cytokines such as TNF-α can exacerbate these events. Oligodendrocyte death also occurs, followed by progressive demyelination leading to significant tissue degeneration. Dental stem cells from human apical papilla (SCAP) can be easily obtained at the removal of an adult immature tooth. This offers a minimally invasive approach to re-use this tissue as a source of stem cells, as compared to biopsying neural tissue from a patient with a spinal cord injury. We assessed the potential of SCAP to exert neuroprotective effects by investigating two possible modes of action: modulation of neuro-inflammation and oligodendrocyte progenitor cell (OPC)

	differentiation. SCAP were co-cultured with LPS-activated microglia, LPS-activated rat spinal cord organotypic sections (SCOS) and LPS-activated co-cultures of SCOS and spinal cord adult OPC. We showed for the first time that SCAP can induce a reduction of TNF-α expression and secretion in inflamed spinal cord tissues and can stimulate OPC differentiation via activin-A secretion. This work underlines the potential therapeutic benefits of SCAP for spinal cord injury repair.
Response to Reviewers:	see attachment

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Prof. Klaus Eichmann
Max-Planck-Institute of Immunobiology and Epigenetics
Stübeweg 51, D-79108 Freiburg, Germany
Editor-in-Chief

Brussels, Januray 8th 2018

Dear Prof. Eichmann,

My co-authors and I are pleased to submit a revised manuscript entitled "**Stem cells from human apical papilla decrease neuro-inflammation and stimulate oligodendrocyte progenitor differentiation via activin-A secretion**" that we would like to be considered for publication in *Cellular and Molecular Life Sciences*.

We saw that the reviewers were enthusiastic about the submission, though had several suggestions and requests for additional data prior to publication. Following the reviewer's advice, clarifications have been made in the manuscript. We also added a graph in figures 4 and 5 showing the impact of SCAP and SCOS on OPC differentiation on the number of GalC positive cells versus the total number of cells. We corrected the mistakes in the references.

All others comments have been fully addressed and the manuscript modified accordingly. We are grateful for the reviewer comments that, taken together, have substantially strengthened the manuscript

Thank you for your consideration of this manuscript for publication in *Cellular and Molecular Life Sciences*. We look forward to your reply.
Yours sincerely,

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Article Title: Stem cells from human apical papilla decrease neuro-inflammation and stimulate oligodendrocyte progenitor differentiation via activin-A secretion.

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COMMENTS FOR THE AUTHOR:

Reviewer #1:

This paper describes a study regarding possible molecular mechanisms how transplanted human apical papilla cells (SCAP) can improve reconstruction of spinal cord neuronal tissues after injury. By using several co-culture systems, the authors demonstrated that SCAP can reduce both expression and secretion of TNF-α, and stimulate OPC differentiation via activing-A secretion in inflamed spinal cord. It is interesting and worth publishing. The present manuscript is, however, lacking in several respects.

Major points

1- <Fig2> The authors claimed that SCAP induced a decrease in TNF-expression and secretion via interaction with LPS-activated BV-2 cells and spinal cord organotypic section (SCOS). To explain the phenomena, they also claimed a possible involvement of the mechanism that SCAP could induce an alternative activated microglia (arginase-positive M2 microglia) through secreting growth factors, cytokine, and etc… This reviewer thinks that data from Fig. 2b, and f should not be compatible with their theory, and that the authors should explain the discrepancy.

The reviewer is right and we rephrased this part more precisely as an increase of arginase expression has been observed only in SCAP co-cultures with microglia.

- p13:

"SCAP could induce an alternatively activated microglia phenotype by secretion of active molecules such as HGF, TGF-β, IDO, PGE2 and cytokines like IL-10 and IL-4 [38,39]."

Has been changed for:

"SCAP could have an anti-inflammatory action by secretion of active molecules such as HGF, TGF-β, IDO, PGE2 and cytokines like IL-10 and IL-4 [38,39]."

2- ELISA kit for TNF-α (Fig.2b, e) may detect both human and murine TNF-α, which should be described somewhere appropriate.

The supplier tested the kit against human TNFa and did not observe cross-reactivity. However, we agree that this is an important element and this information has been added in the M&M section.

- p8- TNF-α and activin-A quantification by ELISA.

"The TNF-α ELISA kit has been tested against human TNF-α by the manufacturer and didn't show cross-reactivity with human TNF-α."

3- ＜Fig.3＞ If SCAP has similar multi-potency as dental pulp stem cells, it can differentiate into MBP/GalC-positive oligodendrocytes. Although the authors described that no tissue with cell attachment was observed (p6, l55), SCAP should be labeled with fluorescent-dye and be monitored, especially for Fig.3-fig.5, to exclude the possibility that oligodendrocytes evaluated should not be involved SCAP-derived cells

We are sorry but it seems the reviewer misunderstood the meaning of those sentences.
In the mentioned sentence: "We checked that the cells attached properly by bright field microscopy but, as the inserts are designed for cell culture and were coated with collagen, no issue with cell attachment was observed (data not shown)." We really meant "issue" and not "tissue". Our purpose was to say that "the SCAP attached properly on the insert" not that "the tissue didn't include SCAP".

- p6: We changed the sentence to make it clearer.

We checked that SCAP attached properly on the inserts by bright field microscopy. SCAP attached and spread on the inserts with the same morphology as when grown in culture flasks (data not shown).

Regarding figure 3, it is possible that SCAP could differentiate into MBP positive cells. However, we checked the literature again and we didn't find any evidence of it. It is true that DPSC have been reported to differentiate in Schwann cells (Martens, W., et al. (2014). "Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro." Faseb J 28(4): 1634-1643) but it was after 2 weeks and a very specific culture protocol.
It was also described that it is possible to differentiate DSPC into OPC (Moayeri, A., et al. (2017). "Transdifferentiation of Human Dental Pulp Stem Cells Into Oligoprogenitor Cells." Basic Clin Neurosci 8(5): 387-394.) but here too, it required 12 days and specific culture conditions.
Since the SCAP were co-cultured with the SCOS for 48h only, we think it is unlikely that the SCAP could migrate in the sections and differentiate into mature oligodendrocytes.

In regard of this comment, we however, modified the title of the section as well as of the figure legend.

- p10:

"SCOS-SCAP co-culture promotes differentiation of OPC from SCOS" has been replaced by "SCOS-SCAP co-culture increased MBP staining in SCOS".

- p23:

"Fig.3 SCOS-SCAP co-culture increases the expression of MBP in SCOS and induces activin-A secretion."

Regarding Figure 5, the OPC we showed are the adult OPC from spinal cord that were seeded on coverslips under the inserts on which SCAP and SCOS were grown (see figure 1). As the porosity of the filter is 0.45 um, we are almost positive that no cells could have crossed the insert and that the GalC positive cells are only isolated rat adult OPC.

＜Fig.4, 5＞ The authors evaluated differentiation of the oligodendrocytes by the positive area. These evaluations would be difficult to distinguish between morphological changes or percentages of the differentiated cells. This reviewer prefers expression with the percentage of positive cells against total cells. In addition, were survivals of the oligodendrocytes not altered

in all the experiments? Any cells migration were not observed from the insert to bottom of the dishes?

As requested by the reviewer, we counted the number of positive cells vs the total number of cells and added this result to the manuscript (Fig 4c and 5d), although we believe that the positive area reflects better the extent of OPC differentiation. We thus propose to keep the graphs with the percentage of positive area in addition to the percentage of positive cells.

Regarding Figure 4, whether we quantified the positive area or the positive cells, the conclusions remained the same.

The text has been modified accordingly as followed:

- p11:

"The area of GalC$^+$ staining was significantly higher, as well as the percentage of positive cells, when OPC were co-cultured with both SCOS and SCAP relative to OPC alone, SCAP and OPC or SCOS and OPC (Fig. 4 b and c, respectively). Consistent with the hypothesis that activin A promotes OPC differentiation, a higher activin-A concentration was detected only in culture media of SCOS cultured with SCAP and OPC compared to the other conditions (Fig. 4d)."

Regarding Figure 5, the conclusions remain also similar whether we quantified the number of positive cells or the positive surface area, except for the fact that the difference between OPC-SCOS-exogenous activing A and OPC-SCOS-SCAP was not significant. So, we can conclude that the percentage of GalC+ surface was higher when OPC were co-cultured with SCOS and SCAP than with SCOS and exogenous activin A but, regarding the percentage of positive cells, no significant difference was observed. So, exogenous activin A and SCAP induced the same number of GalC+ cells but the extent of the differentiation is higher with SCAP.

The text has been modified accordingly as followed:

- p12:

When treated with exogenous activin A, the percentage of GalC+ cells and area tended to increase in OPC-SCOS cultures (Fig. 5c and d). The percentage of surface area significantly increased only when OPC were co-cultured with SCOS and SCAP (Fig. 5c), as the percentage of positive cells was not significantly different between activin A treated OPC-SCOS cultures and OPC-SCOS-SCAP tri-cultures (Fig. 5c). When treated with follistatin, the percentage of adult rat OPC that were GalC+ decreased significantly in the tri-cultures (Fig. 5b and d).

In the discussion, we modified p15:
"As the percentage of GalC+ area was higher in the tri-cultures than in cultures supplemented with exogenous activin-A, and as this percentage was reduced nonetheless at the same level by follistatin than in exogenous activin-A supplemented cultures, we concluded that activin-A was required but did not induce OPC differentiation alone."

Minor points

p6, l55: no issue with cell attachment… → no tissue with cell attachment ….
See answer to the comment above.

Primers sets used in this study should correspond to individual figure. Otherwise, readers might misread meaning of the figures.
We are not sure to exactly understand what the reviewer requires but we added a column in Table 1 with the figure number for which the primers were used and the accession numbers.

Gene name	accession number	Cell type	Forward primer (5'-3')	Reverse primer (3'-5')	Figure
Rat RPL13	NM_173340.2	SCOS	GGCTGAAGCCTACCAGAAAG	CTTTGCCTTTTCCTTCCGTT	Fig 2 d & f, 3 d
Rat Arginase	NM_017134.3	SCOS	GAAGGTCTCTACATCACAGAAGAAA	CAAGGTCAACGCCACTGC	Fig 2 f
Rat TNF-α	NM_012675.3	SCOS	AGTGACAAGCCCGTAGCC	TTGAAGAGAACCTGGGAGTAGA	Fig 2 d
Mouse RPL19	NM_009078.2	BV-2 cells	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT	Fig 2 a & c
Mouse Arginase	NM_007482.3	BV-2 cells	GGTTCTGGGAGGCCTATCTT	TGAAAGGAGCCCTGTCTTGT	Fig 2 c
Mouse TNF-α	NM_013693.3	BV-2 cells	AGCCCCCAGTCTGTATCCTT	GGTCACTGTCCCAGCATCTT	Fig 2 a
Human RPL13	NM_012423.3	SCAP	CATAGGAAGCTGGGAGCAAG	GCCCTCCAATCAGTCTTCTG	Fig 3 c
Human Activin	NM_002192.3	SCAP	TCCCTTGTGAGCCTTGAATC	CCTGGGTAATTGGGTAGGAAAG	Fig 3 c
UNIVERSAL Activin	NM_002192.3 (Human) NM_017128.2 (Rat) NM_008380.2 (Mouse)	SCOS and SCAP	TCATCACCTTTGCCGAGTCA	CTGGTTCTGTTAGCCTTGGG	Fig 3 d

Data from statistical analyses as p-values should be indicated for all the figures. Instead of symbols of the p-value, there are semantic alphabetical-ordered symbols above each bar in all the figures.

They are several ways of indicating significance. We allocated a different letter to conditions that are statistically different and the same letter to conditions that are not significantly different. It is, to our opinion, clearer than to have bars and stars crossing each other on the graphs, especially with multiple conditions.

We modified the sentence in the "Statistical analysis" part explaining this with the hope that it is now clearer. We also added this information in the figure legends.

- p9 and p23-24:

Conditions not related by the same letter are significantly different.

Reviewer #2:

I would like to suggest that two of the studies mentioned in Methods (Gerardo-Nava et al, 2014; Bianco et al, 2017) be included in References.
("Spinal cord organotypic slice cultures for the study of regenerating motor axon interactions with 3D scaffolds.
Gerardo-Nava J1, Hodde D2, Katona I2, Bozkurt A3, Grehl T4, Steinbusch HW5, Weis J6, Brook GA)

We are sorry for the issue with the references. It seems we had a problem with Endnote that resulted in these references missing. The problem has been fixed and we checked that all the references are in the text at their appropriate place and accurate.

Bianco et al, 2017 is ref 26 and Gerardo-Nava et al, 2014 is ref 27.

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Stem cells from human apical papilla decrease neuro-inflammation and stimulate oligodendrocyte progenitor differentiation via activin-A secretion.

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Original research

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Running Title: Neuroprotective potential of SCAP

Summary

Secondary damage following spinal cord injury (SCI) leads to non-reversible lesions and hampering of the reparative process. The local production of pro-inflammatory cytokines such as TNF-α can exacerbate these events. Oligodendrocyte death also occurs, followed by progressive demyelination leading to significant tissue degeneration. Dental stem cells from human apical papilla (SCAP) can be easily obtained at the removal of an adult immature tooth. This offers a minimally invasive approach to re-use this tissue as a source of stem cells, as compared to biopsying neural tissue from a patient with a spinal cord injury. We assessed the potential of SCAP to exert neuroprotective effects by investigating two possible modes of action: modulation of neuro-inflammation and oligodendrocyte progenitor cell (OPC) differentiation. SCAP were co-cultured with LPS-activated microglia, LPS-activated rat spinal cord organotypic sections (SCOS) and LPS-activated co-cultures of SCOS and spinal cord adult OPC. We showed for the first time that SCAP can induce a reduction of TNF-α expression and secretion in inflamed spinal cord tissues and can stimulate OPC differentiation via activin-A secretion. This work underlines the potential therapeutic benefits of SCAP for spinal cord injury repair.

Keywords: Spinal cord, dental stem cells, inflammation, oligodendrocyte progenitor cells, differentiation

Introduction

The cascade of secondary neuro-degenerative events following spinal cord injury (SCI) includes bleeding, necrotic or apoptotic processes and lesion area enlargement [1,2].

Among these, inflammation has been highlighted as a key regulator of degeneration and regeneration. Independent studies have shown that immunosuppression is permissive for central nervous system (CNS) tissue restoration [3,4], while a beneficial role of inflammation on neuro-regeneration has also been described [5]. Both detrimental and favorable effects of the inflammatory response depend on the abundance of cell types (microglia and leukocytes), signaling molecules (chemokines and cytokines) and the post-traumatic time-line (acute, subacute or chronic phase) [5]. Microglial activation is associated with both detrimental and beneficial neuro-regenerative outcomes depending on their activation state [5]. The presence of pro-inflammatory cytokines, such as TNF-α, may enhance cell death and intensify secondary damage in SCI [6,7]. Furthermore, an increase in arginase-1 positive macrophages has already been associated with axon preservation, decrease of scar formation, increase in myelin sparing and functional recovery after SCI [8].

Oligodendrocyte apoptosis, that can be induced by inflammation, in and around the lesion site leads to progressive demyelination, inducing axonal dysfunction and degeneration [9]. To restore the oligodendrocyte population, oligodendrocyte progenitor cell (OPC) proliferation and differentiation has to be activated. In the presence of pro-inflammatory cytokines and in the absence of growth promoting factors following SCI, remyelination is limited, among other factors, by impaired OPC differentiation [10]. Demyelination and inflammation are both crucial issues that must be addressed when developing therapeutic strategies aimed at the restoration of spinal cord function after injury.

Mesenchymal stem cell (MSC)-based therapies have proved to be promising strategies for spinal cord repair. Indeed, MSC therapy allows for multi-targeted and environmentally responsive benefits [11,12]. The positive effects of MSC treatment for CNS diseases/disorders result from their ability to differentiate into neural cell lineages, secrete neurotrophic factors, reduce cell apoptosis and modulate inflammation [13]. MSC improve outcomes after neural trauma by inhibiting the activation of pro-inflammatory microglia and by promoting their stimulation to an anti-inflammatory phenotype [14]. The pro-inflammatory phenotype of microglia has increased expression of pro-inflammatory cytokines such as TNF-α or IL-1β, whereas anti-inflammatory microglia releases neuroprotective factors including

anti-inflammatory cytokines (IL-10, IL-1 receptor antagonist) as well as neurotrophic factors (nerve growth factor and transforming growth factor β) [15].

Human dental stem cells have been increasingly studied as an alternative source of MSC to bone marrow due to their accessibility, their neural crest origin and their high proliferation rate [16]. Indeed, dental stem cells can be easily isolated with a limited invasiveness and display a higher proliferation rate and expression of specific neural stem cell transcripts and proteins than bone marrow-derived MSC [17,18]. Although SCAP are less studied and exploited than dental pulp stem cells (DPSC), they have a greater migratory and tissue regenerative capacity, as well as higher proliferative potential [19]. SCAP can regulate trigeminal nerve outgrowth *in vitro* and support an increased hind limp muscle strength in a rat spinal cord injury model [20]. We showed that implantation of SCAP embedded in their original niche (whole apical papilla tissue) into a rat hemi-section SCI model promoted functional recovery [21].

Activin-A is a member of the transforming growth factor β superfamily. It presents plethoric effects that has been extensively studied in various organs but it was only recently that Miron et al. showed that activin-A stimulates OPC differentiation. In addition, activin-A has an anti-inflammatory effect and is produced by alternatively-activated macrophages [22]. Jeong et al. showed that activin-A is constitutively expressed in healthy spinal cord and that levels increased up to 4 days post-injury to decrease again at day 7 [23]. Activin-A is synthesized by either neurons or inflammatory cells (possibly macrophages) or both and thus may participate in the protection of neuronal tissues after SCI. Additionally, activin-A levels increased in astrocytes and oligodendrocytes at the peripheries of SCI lesions [23]. Thus, activin A may protect glial cells, in addition to neurons, in either a paracrine or autocrine manner [23].

In this study, we examined the *in vitro* and *ex vivo* impact of SCAP on microglia activation and OPC differentiation. We demonstrated that SCAP can reduce the expression and secretion of TNF-α in an LPS-activated microglial cell line, and in LPS-activated spinal cord tissue. In addition, we showed that OPC differentiate when co-cultured with spinal cord tissue and SCAP, due, at least partially, to activin-A secretion. This study provides evidence that SCAP could support spinal cord repair by a neuroprotective action, driven by reduction of pro-inflammatory cytokines and the stimulation of OPC differentiation via activin-A secretion.

Methods

SCAP culture

Human SCAP were isolated from a healthy donor wisdom tooth and characterized [24]. SCAP were used between passages 5 and 8 [24,25]. SCAP were cultured in minimum essential medium eagle (MEM, Sigma-Aldrich, St Louis, USA) supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine (ThermoFisher, Merelbeke, Belgium), 1 % Penicillin/Streptomycin (PEST) (ThermoFisher) (SCAP medium). Cells were either grown in normoxia (N) in 21 % O_2 and 5 % CO_2 or in hypoxia (H) in 1 % O_2 and 5 % CO_2 (InVivo2 400 hypoxia Workstation, Ruskinn, Bridgend, UK). SCAP medium was equilibrated at 1 % O_2 before medium change for SCAP grown in hypoxia.

BV-2 mouse microglial cell culture

BV-2 cells (ATCC) were maintained in Dulbecco's modified eagle's medium (DMEM) with Glutamax™ (ThermoFisher), supplemented with 10 % FBS (Sigma-Aldrich) and 1 % PEST (BV-2 medium) at 37 °C in 21 % O_2 and 5 % CO_2. BV-2 cells were seeded in 24-well plates at a density of $5x10^5$ cells/well.

OPC isolation and culture

All animal-related experiments were approved by the local ethical committee for animal care (2016/UCL/MD/011). OPC were isolated and plated as previously described [26]. Briefly, adult Wistar rat spinal cords were isolated and dissociated into single cells through enzymatic digestion with trypsin-EDTA (ThermoFisher). The cell suspension was then strained, centrifuged and the resulting pellet re-suspended in DMEM/F12 containing 1 % PEST, 10 mM HEPES buffer (ThermoFisher), 10 ng/ml FGF_2 (PeproTech, Rocky Hill, USA), 10 ng/ml platelet-derived growth factor-AA ($PDGF_{AA}$) (PeproTech), and 10 ng/ml IGF-I (PeproTech) and plated in uncoated culture flasks. Fresh growth factors were added every other day, and media was changed completely after 7 days of culture. Free floating OPC spheres were collected and passaged following dissociation with Accutase® (ThermoFisher). OPC (4^{th} passage) were seeded as single cells onto poly-D-lysine (PDL) (Sigma-Aldrich) coated 13 mm glass coverslips at a density of 10.000 cells/cm^2 following dissociation of spheres with Accutase®. For seeding, cells were maintained in their growth media with the addition of 0.5 % FBS for 60 minutes to allow the cells to adhere. Seeded coverslips were then transferred to a 30 mm petri dish (3 coverslips per dish) and maintained in SCAP medium.

Spinal cord organotypic section (SCOS) preparation and culture

Spinal cords of P7 Wistar rats were extracted, dissected as previously described [27] and 350 µm sections were transversally cut with a tissue chopper (McIlWain Tissue Chopper, The Mickle Laboratory Engineering Co. Ltd., Surrey, UK). Four sections were placed on type I collagen-coated (10 µg/cm^2, Sigma-Aldrich) Millicell® Organotypic Cell Culture Inserts (Merck Millipore, Billerica, USA). One ml of medium consisting of 48.37 % MEM, 24.18 % horse serum, 24.18 % HBSS (ThermoFisher)) supplemented with 2 % D-glucose, 0.97 % Glutamax™, 0.97 % PEST, 1.21 % HEPES and 0.12 % amphotericin B (Thermofisher) (pH 7.4, SCOS medium) was added in the lower compartment of each insert. SCOS were maintained at 37 °C in a humidified incubator for 2 weeks before use. Medium was changed 24 hours after the dissection and then twice a week until SCOS were used for the experiments.

BV-2 cells-SCAP co-culture and activation by LPS

BV-2 cells (5x10^5 cells/well) were seeded in 24 well plates. Eight hours later, resting cells were stimulated by incubation with 100 ng/ml of lipopolysaccharides from Escherichia coli (LPS, O55:B5) (Sigma-Aldrich) for 16 hours. SCAP previously grown in normoxia or hypoxia were then added to the LPS-activated BV-2 cells at the same density (5x10^5 cells/well). The BV-2 cells-SCAP co-cultures were maintained in SCAP medium supplemented with 100 ng/ml of LPS at 37 °C in normoxia for 48 hours. LPS-activated BV-2 cells cultured without SCAP and non-activated BV-2 cells were used as controls (N=3, n=4).

SCOS-SCAP co-culture and activation by LPS

SCAP grown in normoxia and hypoxia (2x10^5 SCAP/insert in 200 µl of SCAP media) were co-cultured with SCOS by seeding them on the upper side of inserts (Fig.1a). Medium was changed from SCOS medium to SCAP medium and cells were allowed to attach on the inserts for 8 hours. We checked that SCAP attached properly on the inserts by bright field microscopy. SCAP attached and spread on the inserts with the same morphology as when grown in culture flasks (data not shown).

(data not shown). The medium was then changed for SCAP medium containing LPS (100 ng/ml). Co-cultures were maintained for 48 hours before analysis. LPS-activated SCOS cultured without SCAP and non-activated SCOS were used as controls (N=3; n=4-5).

SCOS-SCAP-OPC tri-culture and activation by LPS

SCAP cultured in either normoxia or hypoxia were seeded on the inserts as described above. Coverslips seeded with adult rat OPC were placed under seeded inserts (3 coverslips per insert). Medium was then changed for serum-free SCAP medium supplemented with LPS (100 ng/ml) (Fig.1b). Cultures were maintained for 7 days before analysis. OPC without SCOS or without SCAP were used as controls (N=3, n=3-4).

Impact of activin-A inhibition on OPC differentiation

SCOS-SCAP-OPC were tri-cultured as described above. Follistatin, a natural activin-A inhibitor [28], was added in the lower compartment of SCOS-SCAP-OPC cultures (50 ng/ml for 48 hours and then 10 ng/ml for 3 days (R&D system, Minneapolis, USA)). Cultures were maintained for 7 days before analysis. Cultures of SCAP alone, OPC alone, SCAP with OPC, and SCOS with OPC were used as controls. OPC alone, SCOS-OPC and SCOS-SCAP-OPC were supplemented with exogenous recombinant activin-A at the same concentration as detected using enzyme-linked immunosorbent assay (ELISA) in SCOS-SCAP supernatants (10 ng/ml) and were used as positive controls (N= 2, n=3).

RNA extraction and real-time qPCR

For mRNA analysis, media was removed and TriPure reagent (Roche, Basel, Switzerland) was added to each well of BV2 cells or co-/tri-cultures at the end of the incubation period. Regarding SCOS, each section was carefully picked up and placed in a 0.5 ml tube with TriPure. The plates were then stored at -80°C for later assessment. Total RNA was extracted using the TriPure reagent according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Promega corporation, Leiden, The Netherlands) from 1 µg of total RNA. qPCR was performed with a STEP one PLUS instrument and software (Applied Biosystems, Foster City, CA, USA) as previously described [29]. Data were normalized to the 60S ribosomal protein L19 (RPL19) mRNA expression for the BV-2

cells, and to the 60S ribosomal protein L13 (RPL13) mRNA expression for the SCAP and SCOS. For each experiment, the absence of treatment effect on reference gene expression was verified. Primer sequences and accession numbers are listed in table 1. Primers were designed to study the gene expression of activin-A. Activin-A mRNA sequence shows substantial homology between rodents and humans, however the mRNA sequence of human Activin-A is longer. Thus, primers were designed specifically for human activin-A (human specific primers) while it was not possible to design primers specific for rat activin-A. Therefore, the designed primers were able to recognize human, rat and mouse activin-A (universal primers).

TNF-α and activin-A quantification by ELISA

TNF-α and activin-A were quantified in undiluted cell supernatants using a Murine TNF-α Standard TMB ELISA Development Kit (PeproTech) and a DuoSet ELISA kit (R&D System, Abingdon, UK), respectively. The TNF-α ELISA kit has been tested against human TNF-α by the manufacturer and didn't show cross-reactivity with human TNF-α. ELISA assays were performed as recommended by the manufacturer. TNF-α was quantified 48 hours after treatment in BV-2 cells-SCAP and SCOS-SCAP co-cultures. Activin-A was quantified 48 hours after treatment in SCOS-SCAP co-cultures and in SCOS-SCAP-OPC tri-cultures (N=3, n=3-5). All measurements were performed in duplicate.

Immunofluorescence

SCOS were stained for Myelin Basic Protein (MBP), chemokine (C-C motif) ligand 1 (CCL1) and neural/glial antigen 2 (NG2) after co-culture with SCAP. SCOS were fixed in 4 % paraformaldehyde (PFA) for 1 hour and rinsed twice in PBS. Samples were blocked for 1 hour at room temperature in 2.5 % horse serum containing 0.03 % triton X100 and incubated with primary antibodies at room temperature for 1 hour (Table 2). Sections were then washed in PBS and incubated for 2 hours at room temperature with the secondary antibodies (Table 2). Native OPC were identified by NG2 immunoreactivity and mature oligodendrocytes by MBP immunoreactivity.

OPC derived from adult rat spinal cord were stained for GalC after tri-culture with SCOS and SCAP. OPC were fixed in 4 % PFA for 10 minutes, washed with PBS and incubated in blocking solution (2 % bovine serum albumin, 10 % normal goat serum, and 0.03 % Triton X100 in PBS) for 1 hour. Samples were incubated with primary antibodies diluted in blocking solution (Table 2) for 1 hour at room

temperature. After being washed in PBS, coverslips were incubated with secondary antibodies (diluted in PBS containing 0.5 % BSA, Table 2) for 1 hour at room temperature. Negative controls were performed by omitting primary antibodies. Vectashield hardset (containing 4', 6-diamidino-2-Phenylindole, dihydrochloride (DAPI)) (Vectorlabs, Burlingame, CA) was used as mounting medium. Quantification of positive cell number and area was performed on 3 random images per coverslips. MBP and GalC positive staining was quantified using Volocity (Perkin Elmer, Coventry, UK) and ImageJ (1.43u, National Institutes of Health, Bethesda, MD, USA), respectively.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) with an appropriate post-hoc test was performed. Paired T-test was performed for MBP quantification. Error bars represent the standard error of the mean in all figures. Statistical significance was accepted at the 5 % level (p-value < 0.05). Conditions not related by the same letter are significantly different. "N" and "n" indicate number of independent experiments and number of biological replicates, respectively. Between 3 and 5 sections per condition were analyzed depending of the total number of sections isolated from rat spinal cords.

Results

SCAP modulate the expression of inflammatory markers

The impact of SCAP on TNF-α and arginase-1 expression was investigated in LPS-activated BV-2 cells (microglia). Spinal cord lesions present a hypoxic environment that could impact MSC behavior and its secretome [30]. We previously demonstrated that hypoxia induced an up-regulation of SCAP gene expression of neurotrophic factors [25]. Thus, we assessed the impact of SCAP hypoxia-preconditioning treatment on their immunomodulatory properties.

LPS treatment induced an increased expression of TNF-α, while decreasing the expression of arginase-1 in comparison to non-activated BV-2 cells (supplementary data a and b). Co-culture of SCAP with LPS-treated BV-2 cells induced a significant decrease of TNF-α mRNA expression (Fig. 2a) and secretion (Fig. 2b) compared to BV-2 cells cultured alone. There was no influence of SCAP culture condition (i.e. hypoxia or normoxia). Arginase-1 mRNA expression was increased in BV-2 cells when co-cultured with SCAP relative to BV-2 mono-cultures. This effect was more pronounced when BV-2 cells were cultured with SCAP grown in normoxia than when cultured with SCAP grown in hypoxia (Fig. 2c).

The same experiment was performed to evaluate the effect of SCAP on TNF-α and arginase-1 mRNA expression by LPS-activated SCOS. This culture model enables the preservation of the basic cytoarchitecture and neuronal-glial interactions [31], while allowing us to assess responses of both endogenous and exogenous cells as well as their interactions [32]. SCAP, whether grown in normoxia or hypoxia, were able to adhere to inserts and displayed a normal cell morphology compared to cells grown in flasks (data not shown). LPS treatment of SCOS induced an increased expression of TNF-α mRNA compared with gene expression of non-activated SCOS (supplementary data c). Co-culture of SCAP (grown in normoxia or hypoxia) with LPS-treated SCOS significantly decreased TNF-α expression (Fig. 2d) compared to SCOS cultured alone. A significant decrease of TNF-α secretion was observed in the supernatants of SCOS co-cultured with SCAP in comparison to SCOS alone (Fig. 2e). However, SCAP co-culture with SCOS did not influence arginase-1 gene expression (Fig. 2f).

SCOS-SCAP co-culture increased MBP staining in SCOS

Remyelination is a key regenerative process, promoting functional recovery following SCI [33-35]. Therefore, proliferation and differentiation of OPC is crucial to restore and maintain myelin. Co-culture

of SCOS with SCAP showed an increase in staining for mature oligodendrocyte markers (MBP and CC1 positive cells) in SCOS compared to SCOS alone (Fig. 3a). Pixel quantification showed that significantly more cells were positive for MBP in SCOS-SCAP co-cultures than in SCOS alone (Fig. 3b).

SCOS-SCAP co-culture induces activin-A expression and secretion

Since SCAP co-culture with SCOS increased OPC to oligodendrocyte differentiation, we assessed the expression and secretion of activin-A in co-cultures in the presence of LPS. SCAP and SCOS cultured alone expressed activin-A (Fig. 3c and d). A significant increase of human activin-A gene expression was observed when SCAP were co-cultured with SCOS compared to SCAP alone (Fig. 3c). Using primers that recognized both human and rat activin-A, we observed that co-culture of SCAP with SCOS also showed a significant increase of activin-A gene expression (Fig. 3d). Importantly, SCAP co-culture with SCOS induced a higher concentration of activin-A in supernatants compared to SCAP or SCOS alone (Fig. 3e). No effect of hypoxia was observed on activin-A expression (Supplementary data d) so only SCAP grown in normoxia were used for the following experiments.

SCOS-SCAP promotes adult OPC differentiation

To confirm the impact of SCAP on adult spinal cord OPC differentiation, we developed an original tri-culture model where SCAP and SCOS were co-cultured with OPC isolated from adult rat spinal cord (Fig. 1b). The area of $GalC^+$ staining was significantly higher, as well as the percentage of positive cells, when OPC were co-cultured with both SCOS and SCAP relative to OPC alone, SCAP and OPC or SCOS and OPC (Fig. 4b and c, respectively). Consistent with the hypothesis that activin A promotes OPC differentiation, a higher activin-A concentration was detected only in culture media of SCOS cultured with SCAP and OPC compared to the other conditions (Fig. 4d).

Activin A produced by SCAP and SCOS co-culture induces OPC differentiation

We next investigated whether activin-A was responsible for OPC differentiation by treating the cultures with the activin-A-sequestering protein follistatin, which prevents its action and its detection by ELISA. Follistatin impact on activin-A concentration in culture media was first evaluated, and we observed that follistatin was able to reduce the amount of activin-A detected in the medium of the tri-cultures (Fig.

5a). We observed a similar effect of follistatin when exogenous activin-A was added to the cultures (Fig. 5a (grey bars)), confirming activin-A inhibition by follistatin.

When treated with exogenous activin A, the percentage of GalC$^+$ cells and area tended to increase in OPC-SCOS cultures (Fig. 5c and d). The percentage of surface area significantly increased only when OPC were co-cultured with SCOS and SCAP (Fig. 5c), as the percentage of positive cells was not significantly different between activin A treated OPC-SCOS cultures and OPC-SCOS-SCAP tri-cultures (Fig 5c). When treated with follistatin, the percentage of adult rat OPC that were GalC$^+$ decreased significantly in the tri-cultures (Fig. 5b and d).

Discussion

Stem cells can stimulate tissue repair primarily via two mechanisms: cell replacement and secretion of bioactive molecules. MSC have been described to have immunomodulatory properties [13] but little information is available on dental stem cells, and in particular SCAP, a promising source of MSC for CNS repair. In this study, we demonstrate that SCAP impact microglial function and stimulate OPC differentiation in the presence of a pro-inflammatory stimuli. We also showed for the first time that SCAP express activin-A and that this expression was increased in the presence of inflamed spinal cord tissue. We established a link between activin-A secretion by SCAP-SCOS and the differentiation of adult spinal cord OPC into mature oligodendrocytes.

LPS treatment stimulated TNF-α expression and secretion by both BV-2 cells and SCOS. The impact of LPS on BV-2 cells has been extensively described by others [36,37] but this is the first time that spinal cord organotypic cultures have been used to study the impact of stem cells on the activation of spinal cord tissue. We showed that when SCOS were treated with 100 ng/ml of LPS, gene expression for TNF-α significantly increased. Thus, SCOS reactivity to LPS makes this *ex-vivo* model a useful tool to study the effect of new therapies on neuro-inflammation.

We demonstrated that SCAP induced a decrease in TNF-α expression and secretion in LPS-activated BV-2 cells and SCOS. The ability of two types of dental stem cells (dental pulp stem cells and stem cells from human exfoliated deciduous teeth) to reduce secretion of pro-inflammatory cytokines like TNFα has already been described [38,39] but little is known about SCAP. SCAP could have an anti-inflammatory action by secretion of active molecules such as HGF, TGF-β, IDO, PGE2 and cytokines like IL-10 and IL-4 [40,41]. MSC could also act via extracellular vesicles containing miRNA (i.e. miRNA 146 and 155) or via an EV surface molecule like PD-L1 or galectin-1 [42]. Another possible explanation could be the production of TNF-stimulated gene 6 protein (TSG-6), a protein that inhibits the NF-kB and MAPK activation pathway, by SCAP activated by LPS-treated microglial cells. Expression of pro-inflammatory cytokines such as TNF-α increases during the acute phase (first hours) of SCI and exacerbates secondary tissue degeneration [43,44]. TNF-α induces neuron and oligodendrocyte apoptosis [43], lesion area enlargement and promotes Wallerian degeneration [9]. Down-regulation of TNF-α has also been linked with greater neuronal survival and reduced apoptosis [7]. Furthermore, MSC have been reported to reduce BV-2 cell proliferation and TNF-α expression

following LPS stimulation [37,45]. Similarly, our data demonstrates the impact of SCAP on pro-inflammatory cytokine expression. The transplantation of MSC after SCI has already been correlated with an increase of alternatively activated macrophages (i.e. arginase-1 positive cells) that were associated with functional recovery [8]. Stem cells from human exfoliated deciduous teeth have been reported to be associated with the induction of an anti-inflammatory macrophage phenotype when injected into the spinal cord after injury. This effect was attributed to the secreted proteins MCP-1 and ED-Siglec-9 [46]. Thus, SCAP could protect the spinal cord after injury by acting on macrophages and microglia, limiting damage that is associated with prolonged inflammation.

We observed that culturing SCAP in hypoxia did not offer any advantage but was not detrimental to their immunomodulation properties nor to their activin-A expression (supplementary data d). Although Jiang and colleagues reported that MSC can be grown in hypoxia to enhance their immunomodulatory properties, migration, proliferation, and survival [47], these effects depend on the origin of the MSC, the serum used [48] and O_2 tension under which the study was performed [49]. Since SCAP properties are not limited by hypoxia, we may expect that local hypoxic conditions in spinal cord lesion should not affect their immunomodulation properties while supporting neurotrophic factor production.

It has already been demonstrated that dental stem cells could support remyelination *in vitro* and *in vivo* [50,51]. In addition, other dental stem cells (dental pulp stem cells and stem cells from human exfoliated deciduous teeth) demonstrate neuro-regenerative properties, which they exert via different mechanisms, including the prevention of neural apoptosis, the blocking of axon growth inhibitors, and the replacement of dead oligodendrocytes [51]. These findings suggest that dental stem cells could promote remyelination after SCI. Our objective was thus to evaluate the impact of SCAP on adult OPC differentiation via paracrine actions. To that end, we developed an innovative tri-culture model composed of SCAP, SCOS and isolated adult OPC from rat spinal cord. This model can be used to investigate and optimize treatments for SCI before resorting to the more challenging *in vivo* SCI models. We showed that SCAP, when associated with spinal cord tissue, promoted the differentiation of adult OPC into mature oligodendrocytes. MSC have been described to support remyelination, reduce demyelination and cell loss by decreasing the astroglial response and apoptosis in a cuprizone model [52]. The mechanisms involved are not clearly elucidated yet but the positive action of MSC are attributed to the protection of damaged axons and immunomodulation.

This study reports, for the first time, that activin-A is expressed by SCAP and SCOS. Moreover, we showed that activin-A was expressed by both SCAP and SCOS separately, but that the interaction of SCAP and SCOS induced a higher degree of activin-A expression and secretion. Djouad and colleagues observed that activin-A secretion by MSC, as well as the activin-A:follistatin ratio, depends on their origin [53]. Activin-A expression is required for the maintenance of stemness and the regulation of MSC functions. Activin-A also plays a functional role in the suppression of inflammatory or immune processes [54]. A correlation between activin-A and MSC-mediated immunosuppression has been made and suggested that lower concentrations of activin-A produced by tonsil-derived-MSC (compared with other MSC types), corresponded to their significantly lower immunosuppressive potential [53]. Activin-A produced by human umbilical cord-derived MSC suppressed interferon gamma (INF-γ) production by natural killer cells [55]. An up-regulation of activin-A has been observed in the early stage of SCI [23]. This higher level of activin-A was correlated with neuroprotection and immunomodulation, potentially by stimulating proliferation of alternatively activated macrophages [23]. In the present study, a significant increase of activin-A concentration in the medium of SCAP-SCOS co-cultures correlated with increased MBP staining in SCOS. Recently, it has been shown that activin-A stimulates OPC differentiation to MBP positive oligodendrocytes [22]. In support of the earlier report, we demonstrated that OPC differentiation was correlated with increased activin-A production, which was enhanced by the presence of SCAP. Interestingly, we observed that the presence of spinal tissue was indispensable for the increased activin-A secretion and OPC differentiation. Our findings suggest the presence of a potential synergy between SCAP and spinal tissue that leads to an increase of activin-A production and OPC differentiation.

In order to determine if activin-A secreted by SCAP/SCOS was solely responsible for OPC differentiation, we treated the tri-cultures with follistatin. This protein inhibits activin-A actions by forming a complex consisting of one activin-A dimer and two follistatin molecules, thus preventing activin-A from binding to its receptor [28]. Activin-A was no longer detected by ELISA when culture supernatants were supplemented with follistatin, confirming that the majority of activin-A had been sequestered and inactivated. Also, blocking activin-A with follistatin in tri-cultures suppressed OPC differentiation, which was not rescued when exogenous activin-A was added in absence of SCAP. As the percentage of $GalC^+$ area was higher in the tri-cultures than in cultures supplemented with exogenous activin-A, and as this percentage was reduced nonetheless at the same level by follistatin

than in exogenous activin-A supplemented cultures, we concluded that activin-A was required but did not induce OPC differentiation alone. Supporting this hypothesis is the fact that as follistatin is mostly known to inhibit activin-A, it also inhibits factors from the TGF superfamily such as the bone morphogenic proteins [56]. It may be hypothesized that SCAP produce other essential bioactive molecules that stimulate OPC differentiation along with activin-A, the actions of which are also inhibited by follistatin. Several other related factors, such as growth factors (e.g. PDGF, IGF-1) or cytokines (e.g. TGF-β1, CXCL12), have been reported to induce OPC differentiation [57] and could, therefore, be involved in this phenomenon.

This study highlights the neuroprotective potential of SCAP via two mechanisms: modulation of neuro-inflammation and the promotion of OPC differentiation to mature oligodendrocytes. For the first time, we demonstrate that SCAP, in association with spinal tissue, produce activin-A and that this protein supports OPC differentiation. Our data demonstrate that SCAP may provide therapeutic benefits for treating acute SCI.

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References

1. Bianco J, De Berdt P, Deumens R, des Rieux A (2016) Taking a bite out of spinal cord injury: do dental stem cells have the teeth for it? Cell Mol Life Sci 73 (7):1413-1437. doi:10.1007/s00018-015-2126-5

2. Hall ED, Springer JE (2004) Neuroprotection and acute spinal cord injury: a reappraisal. NeuroRx 1 (1):80-100. doi:10.1602/neurorx.1.1.80

3. Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O (2003) Inflammation is detrimental for neurogenesis in adult brain. Proceedings of the National Academy of Sciences of the United States of America 100 (23):13632-13637. doi:10.1073/pnas.2234031100

4. Fitch MT, Silver J (2008) CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp Neurol 209 (2):294-301. doi:10.1016/j.expneurol.2007.05.014

5. Kyritsis N, Kizil C, Brand M (2014) Neuroinflammation and central nervous system regeneration in vertebrates. Trends Cell Biol 24 (2):128-135. doi:10.1016/j.tcb.2013.08.004

6. Held KS, Lane TE (2014) Spinal cord injury, immunodepression, and antigenic challenge. Semin Immunol 26 (5):415-420. doi:10.1016/j.smim.2014.03.003

7. Xue H, Zhang XY, Liu JM, Song Y, Liu TT, Chen D (2013) NDGA reduces secondary damage after spinal cord injury in rats via anti-inflammatory effects. Brain Res 1516:83-92. doi:10.1016/j.brainres.2013.04.016

8. Nakajima H, Uchida K, Guerrero AR, Watanabe S, Sugita D, Takeura N, Yoshida A, Long G, Wright KT, Johnson WE, Baba H (2012) Transplantation of mesenchymal stem cells promotes an alternative pathway of macrophage activation and functional recovery after spinal cord injury. Journal of neurotrauma 29 (8):1614-1625. doi:10.1089/neu.2011.2109

9. Beattie MS (2004) Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. Trends Mol Med 10 (12):580-583. doi:10.1016/j.molmed.2004.10.006

10. Alizadeh A, Dyck SM, Karimi-Abdolrezaee S (2015) Myelin damage and repair in pathologic CNS: challenges and prospects. Front Mol Neurosci 8:35. doi:10.3389/fnmol.2015.00035

11. Badner A, Siddiqui AM, Fehlings MG (2017) Spinal Cord Injuries: How Could Cell Therapy Help? Expert Opin Biol Ther. doi:10.1080/14712598.2017.1308481

12. Murphy MB, Moncivais K, Caplan AI (2013) Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. Exp Mol Med 45:e54. doi:10.1038/emm.2013.94

13. Martinez AM, Goulart CO, Ramalho Bdos S, Oliveira JT, Almeida FM (2014) Neurotrauma and mesenchymal stem cells treatment: From experimental studies to clinical trials. World J Stem Cells 6 (2):179-194. doi:10.4252/wjsc.v6.i2.179

14. Hsuan YC, Lin CH, Chang CP, Lin MT (2016) Mesenchymal stem cell-based treatments for stroke, neural trauma, and heat stroke. Brain Behav 6 (10):e00526. doi:10.1002/brb3.526

15. Chio CC, Lin MT, Chang CP (2015) Microglial activation as a compelling target for treating acute traumatic brain injury. Curr Med Chem 22 (6):759-770

16. Giuliani A, Manescu A, Langer M, Rustichelli F, Desiderio V, Paino F, De Rosa A, Laino L, d'Aquino R, Tirino V, Papaccio G (2013) Three years after transplants in human mandibles, histological and in-line holotomography revealed that stem cells regenerated a compact rather than a spongy bone: biological and clinical implications. Stem Cells Transl Med 2 (4):316-324. doi:10.5966/sctm.2012-0136

17. Huang GT, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88 (9):792-806. doi:10.1177/0022034509340867

18. Karaoz E, Demircan PC, Saglam O, Aksoy A, Kaymaz F, Duruksu G (2011) Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells. Histochem Cell Biol 136 (4):455-473. doi:10.1007/s00418-011-0858-3

19. Pomerat CM, Contino RM (1965) The Cultivation of Dental Tissues. Oral Surg Oral Med Oral Pathol 19:628-632

20. Yang C, Li X, Sun L, Guo W, Tian W (2017) Potential of human dental stem cells in repairing the complete transection of rat spinal cord. J Neural Eng 14 (2):026005. doi:10.1088/1741-2552/aa596b

21. De Berdt P, Vanacker J, Ucakar B, Elens L, Diogenes A, Leprince JG, Deumens R, des Rieux A (2015) Dental Apical Papilla as Therapy for Spinal Cord Injury. Journal of dental research 94 (11):1575-1581. doi:10.1177/0022034515604612
22. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, van Wijngaarden P, Wagers AJ, Williams A, Franklin RJ, ffrench-Constant C (2013) M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nature neuroscience 16 (9):1211-1218. doi:10.1038/nn.3469
23. Jeong J, Ahn M, Sim KB, Moon C, Shin T (2014) Immunohistochemical analysis of activin A expression in spinal cords of rats with clip compression injuries. Acta Histochem 116 (5):747-752. doi:10.1016/j.acthis.2014.01.002
24. Ruparel NB, de Almeida JF, Henry MA, Diogenes A (2013) Characterization of a stem cell of apical papilla cell line: effect of passage on cellular phenotype. J Endod 39 (3):357-363. doi:10.1016/j.joen.2012.10.027
25. Vanacker J, Viswanath A, De Berdt P, Everard A, Cani PD, Bouzin C, Feron O, Diogenes A, Leprince JG, des Rieux A (2014) Hypoxia modulates the differentiation potential of stem cells of the apical papilla. J Endod 40 (9):1410-1418. doi:10.1016/j.joen.2014.04.008
26. Bianco J, Carradori D, Deumens R, des Rieux A (2017) Rapid Serum-Free Isolation of Oligodendrocyte Progenitor Cells from Adult Rat Spinal Cord. Stem Cell Rev 13 (4):499-512. doi:10.1007/s12015-017-9742-4
27. Gerardo-Nava J, Hodde D, Katona I, Bozkurt A, Grehl T, Steinbusch HW, Weis J, Brook GA (2014) Spinal cord organotypic slice cultures for the study of regenerating motor axon interactions with 3D scaffolds. Biomaterials 35 (14):4288-4296. doi:10.1016/j.biomaterials.2014.02.007
28. Refaat B (2014) Role of activins in embryo implantation and diagnosis of ectopic pregnancy: a review. Reprod Biol Endocrinol 12:116. doi:10.1186/1477-7827-12-116
29. Alhouayek M, Masquelier J, Cani PD, Lambert DM, Muccioli GG (2013) Implication of the anti-inflammatory bioactive lipid prostaglandin D2-glycerol ester in the control of macrophage activation and inflammation by ABHD6. Proceedings of the National Academy of Sciences of the United States of America 110 (43):17558-17563. doi:10.1073/pnas.1314017110
30. Rouanet C, Reges D, Rocha E, Gagliardi V, Silva GS (2017) Traumatic spinal cord injury: current concepts and treatment update. Arq Neuropsiquiatr 75 (6):387-393. doi:10.1590/0004-282X20170048
31. Gerardo-Nava J, Mayorenko, II, Grehl T, Steinbusch HW, Weis J, Brook GA (2013) Differential pattern of neuroprotection in lumbar, cervical and thoracic spinal cord segments in an organotypic rat model of glutamate-induced excitotoxicity. J Chem Neuroanat 53:11-17. doi:10.1016/j.jchemneu.2013.09.007
32. Daviaud N, Garbayo E, Schiller PC, Perez-Pinzon M, Montero-Menei CN (2013) Organotypic cultures as tools for optimizing central nervous system cell therapies. Experimental neurology 248:429-440. doi:10.1016/j.expneurol.2013.07.012
33. Cao Q, Xu XM, Devries WH, Enzmann GU, Ping P, Tsoulfas P, Wood PM, Bunge MB, Whittemore SR (2005) Functional recovery in traumatic spinal cord injury after transplantation of multineurotrophin-expressing glial-restricted precursor cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 25 (30):6947-6957. doi:10.1523/JNEUROSCI.1065-05.2005
34. Hofstetter CP, Holmstrom NA, Lilja JA, Schweinhardt P, Hao J, Spenger C, Wiesenfeld-Hallin Z, Kurpad SN, Frisen J, Olson L (2005) Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nature neuroscience 8 (3):346-353. doi:10.1038/nn1405
35. Plemel JR, Keough MB, Duncan GJ, Sparling JS, Yong VW, Stys PK, Tetzlaff W (2014) Remyelination after spinal cord injury: is it a target for repair? Progress in neurobiology 117:54-72. doi:10.1016/j.pneurobio.2014.02.006

36. Jeong YH, Park JS, Kim DH, Kang JL, Kim HS (2017) Anti-inflammatory mechanism of lonchocarpine in LPS- or poly(I:C)-induced neuroinflammation. Pharmacol Res 119:431-442. doi:10.1016/j.phrs.2017.02.027
37. Jose S, Tan SW, Ooi YY, Ramasamy R, Vidyadaran S (2014) Mesenchymal stem cells exert anti-proliferative effect on lipopolysaccharide-stimulated BV2 microglia by reducing tumour necrosis factor-alpha levels. Journal of neuroinflammation 11:149. doi:10.1186/s12974-014-0149-8
38. Demircan PC, Sariboyaci AE, Unal ZS, Gacar G, Subasi C, Karaoz E (2011) Immunoregulatory effects of human dental pulp-derived stem cells on T cells: comparison of transwell co-culture and mixed lymphocyte reaction systems. Cytotherapy 13 (10):1205-1220. doi:10.3109/14653249.2011.605351
39. Yamagata M, Yamamoto A, Kako E, Kaneko N, Matsubara K, Sakai K, Sawamoto K, Ueda M (2013) Human dental pulp-derived stem cells protect against hypoxic-ischemic brain injury in neonatal mice. Stroke 44 (2):551-554. doi:10.1161/STROKEAHA.112.676759
40. Kyurkchiev D, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T, Belemezova K, Kyurkchiev S (2014) Secretion of immunoregulatory cytokines by mesenchymal stem cells. World journal of stem cells 6 (5):552-570. doi:10.4252/wjsc.v6.i5.552
41. Zachar L, Bacenkova D, Rosocha J (2016) Activation, homing, and role of the mesenchymal stem cells in the inflammatory environment. J Inflamm Res 9:231-240. doi:10.2147/JIR.S121994
42. Dostert G, Mesure B, Menu P, Velot E (2017) How Do Mesenchymal Stem Cells Influence or Are Influenced by Microenvironment through Extracellular Vesicles Communication? Front Cell Dev Biol 5:6. doi:10.3389/fcell.2017.00006
43. Lee YB, Yune TY, Baik SY, Shin YH, Du S, Rhim H, Lee EB, Kim YC, Shin ML, Markelonis GJ, Oh TH (2000) Role of tumor necrosis factor-alpha in neuronal and glial apoptosis after spinal cord injury. Experimental neurology 166 (1):190-195. doi:10.1006/exnr.2000.7494
44. Amini Pishva A, Akbari M, Farahabadi A, Arabkheradmand A, Beyer C, Dashti N, Moradi F, Hassanzadeh G (2016) Effect of Estrogen Therapy on TNF-alpha and iNOS Gene Expression in Spinal Cord Injury Model. Acta Med Iran 54 (5):296-301
45. Liu Y, Zhang R, Yan K, Chen F, Huang W, Lv B, Sun C, Xu L, Li F, Jiang X (2014) Mesenchymal stem cells inhibit lipopolysaccharide-induced inflammatory responses of BV2 microglial cells through TSG-6. Journal of neuroinflammation 11:135. doi:10.1186/1742-2094-11-135
46. Matsubara K, Matsushita Y, Sakai K, Kano F, Kondo M, Noda M, Hashimoto N, Imagama S, Ishiguro N, Suzumura A, Ueda M, Furukawa K, Yamamoto A (2015) Secreted ectodomain of sialic acid-binding Ig-like lectin-9 and monocyte chemoattractant protein-1 promote recovery after rat spinal cord injury by altering macrophage polarity. J Neurosci 35 (6):2452-2464. doi:10.1523/JNEUROSCI.4088-14.2015
47. Jiang CM, Liu J, Zhao JY, Xiao L, An S, Gou YC, Quan HX, Cheng Q, Zhang YL, He W, Wang YT, Yu WJ, Huang YF, Yi YT, Chen Y, Wang J (2015) Effects of hypoxia on the immunomodulatory properties of human gingiva-derived mesenchymal stem cells. Journal of dental research 94 (1):69-77. doi:10.1177/0022034514557671
48. Page P, DeJong J, Bandstra A, Boomsma RA (2014) Effect of serum and oxygen concentration on gene expression and secretion of paracrine factors by mesenchymal stem cells. Int J Cell Biol 2014:601063. doi:10.1155/2014/601063
49. Paquet J, Deschepper M, Moya A, Logeart-Avramoglou D, Boisson-Vidal C, Petite H (2015) Oxygen Tension Regulates Human Mesenchymal Stem Cell Paracrine Functions. Stem cells translational medicine 4 (7):809-821. doi:10.5966/sctm.2014-0180
50. Martens W, Sanen K, Georgiou M, Struys T, Bronckaers A, Ameloot M, Phillips J, Lambrichts I (2014) Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro. FASEB J 28 (4):1634-1643. doi:10.1096/fj.13-243980
51. Sakai K, Yamamoto A, Matsubara K, Nakamura S, Naruse M, Yamagata M, Sakamoto K, Tauchi R, Wakao N, Imagama S, Hibi H, Kadomatsu K, Ishiguro N, Ueda M (2012) Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple

neuro-regenerative mechanisms. The Journal of clinical investigation 122 (1):80-90. doi:10.1172/JCI59251
52. El-Akabawy G, Rashed LA (2015) Beneficial effects of bone marrow-derived mesenchymal stem cell transplantation in a non-immune model of demyelination. Ann Anat 198:11-20. doi:10.1016/j.aanat.2014.12.002
53. Djouad F, Jackson WM, Bobick BE, Janjanin S, Song Y, Huang GT, Tuan RS (2010) Activin A expression regulates multipotency of mesenchymal progenitor cells. Stem Cell Res Ther 1 (2):11. doi:10.1186/scrt11
54. Abe M, Shintani Y, Eto Y, Harada K, Kosaka M, Matsumoto T (2002) Potent induction of activin A secretion from monocytes and bone marrow stromal fibroblasts by cognate interaction with activated T cells. J Leukoc Biol 72 (2):347-352
55. Chatterjee D, Marquardt N, Tufa DM, Hatlapatka T, Hass R, Kasper C, von Kaisenberg C, Schmidt RE, Jacobs R (2014) Human Umbilical Cord-Derived Mesenchymal Stem Cells Utilize Activin-A to Suppress Interferon-gamma Production by Natural Killer Cells. Front Immunol 5:662. doi:10.3389/fimmu.2014.00662
56. Fodor J, Gomba-Toth A, Olah T, Almassy J, Zador E, Csernoch L (2017) Follistatin treatment suppresses SERCA1b levels independently of other players of calcium homeostasis in C2C12 myotubes. J Muscle Res Cell Motil. doi:10.1007/s10974-017-9474-8
57. Boulanger JJ, Messier C (2014) From precursors to myelinating oligodendrocytes: contribution of intrinsic and extrinsic factors to white matter plasticity in the adult brain. Neuroscience 269:343-366. doi:10.1016/j.neuroscience.2014.03.063

Tables

Table1. Primer sequences

Gene name	Accession number	Cell type	Forward primer (5'-3')	Reverse primer (3'-5')	Figure
Rat RPL13	NM_173340.2	SCOS	GGCTGAAGCCTACCAGAAAG	CTTTGCCTTTTCCTTCCGTT	Fig 2 d & f, 3 d
Rat Arginase	NM_017134.3	SCOS	GAAGGTCTCTACATCACAGAAGAAA	CAAGGTCAACGCCACTGC	Fig 2 f
Rat TNF-α	NM_012675.3	SCOS	AGTGACAAGCCCGTAGCC	TTGAAGAGAACCTGGGAGTAGA	Fig 2 d
Mouse RPL19	NM_009078.2	BV-2 cells	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT	Fig 2 a & c
Mouse Arginase	NM_007482.3	BV-2 cells	GGTTCTGGGAGGCCTATCTT	TGAAAGGAGCCCTGTCTTGT	Fig 2 c
Mouse TNF-α	NM_013693.3	BV-2 cells	AGCCCCCAGTCTGTATCCTT	GGTCACTGTCCCAGCATCTT	Fig 2 a
Human RPL13	NM_012423.3	SCAP	CATAGGAAGCTGGGAGCAAG	GCCCTCCAATCAGTCTTCTG	Fig 3 c
Human Activin	NM_002192.3	SCAP	TCCCTTGTGAGCCTTGAATC	CCTGGGTAATTGGGTAGGAAAG	Fig 3 c
UNIVERSAL Activin	NM_002192.3 (Human) NM_017128.2 (Rat) NM_008380.2 (Mouse)	SCOS and SCAP	TCATCACCTTTGCCGAGTCA	CTGGTTCTGTTAGCCTTGGG	Fig 3 d

When several variants exist, the primers were designed to recognize all variants.

Table2. List of antibodies used for immunofluorescence analysis.

Primary antibody	Dilution	Supplier	Secondary antibody	Dilution	Supplier
MBP	1:250	BioRad[a]	anti-rat IgG2a Alexa 555 (mouse)	1:1000	Life Technologies[d]
CC1	1:100	Abcam[b]	anti-mouse Alexa 647	1:1000	Life Technologies[d]
NG2	1:200	Merck Millipore[c]	anti-rabbit IgG Alexa 488 (goat)	1:1000	Life Technologies[d]
GalC	1:800	Merck Millipore[c]	anti-mouse IgG3 Alexa 594 (goat)	1:400	Life Technologies[d]

[a]BioRad, Hercules, USA

[b]Abcam, Cambridge, UK

[c]Merck Millipore, Temecula, CA

[d]Life Technologies, Eugene, OR, USA

Figure legend

Results obtained for LPS treated samples are presented in this manuscript while results for non-LPS treated samples are in supplementary data. In all figures, groups without SCAP are presented in white, groups with SCAP grown in normoxia (N) in light grey and groups with SCAP grown in hypoxia (H) in dark grey.

Fig.1 Graphical experimental plan

A: Co-culture model of SCOS with SCAP on insert. B: Tri-culture model of SCOS with SCAP on inserts and with OPC isolated from adult rat spinal cord seeded on coverslips.

Fig.2 SCAP modulate the inflammatory markers gene expression and secretion

A-C: SCAP impact on LPS-activated BV-2 cells, 48h after incubation. A: SCAP impact on TNF-α gene expression of BV-2 cells evaluated by RT-qPCR (N=2, n=4). B: Impact of SCAP co-cultured with BV-2 cells on BV-2 cells TNF-α production in culture media (ELISA) (N=2, n=4). C: SCAP impact on arginase-1 gene expression of BV-2 cells (N=2, n=4). D-F: SCAP impact on LPS-activated SCOS, 48h after incubation. D: Impact of SCAP on SCOS TNF-α gene expression (N=2, n=4). E: Impact of SCAP co-cultured with SCOS on TNF-α production in culture media (ELISA) (N=2, n=4). F: Impact of SCAP on SCOS arginase gene expression (N=2, n=4). Conditions not related by the same letter are significantly different.

Fig.3 SCOS-SCAP co-culture increased the expression of MBP in SCOS and induces activin-A secretion

A: OPC were identified in SCOS by immunofluorescence using NG2 (green) staining and oligodendrocytes were identified using MBP (red) and CC1 (white) staining. B: MBP positive pixels were quantified in SCOS cultured with and without SCAP (N=2, n=3). C: Activin-A gene in SCAP 48h after incubation with SCOS (N=2, n=3). B: Activin-A gene expression in SCOS 48h after incubation with SCAP (N=2, n=3). C: Activin-A quantification in culture media using ELISA (N=3, n=3). Conditions not related by the same letter are significantly different.

Fig.4 SCOS-SCAP promotes adult OPC differentiation and induces activin-A secretion

A: Oligodendrocytes were identified by immunofluorescence using GalC staining 7 days after incubation of adult rat spinal cord OPC with SCOS and SCAP. B: GalC positive pixels were quantified by ImageJ software (N=2, n=3 (3 pictures/coverslips)). C: Activin-A quantification in culture media after 48h of incubation (ELISA) (N=3, n=3). Conditions not related by the same letter are significantly different.

Fig.5 Activin-A produced by SCAP and SCOS induces OPC differentiation

A: Activin-A quantification in culture media (ELISA) 48h after incubation (N=2, n=3). B: Oligodendrocytes were identified by immunofluorescence using GalC staining 7 days after incubation. C: GalC positive pixels were quantified by ImageJ software (N=2, n=3 (3 pictures/coverslips)). Conditions not related by the same letter are significantly different.

Figure 1

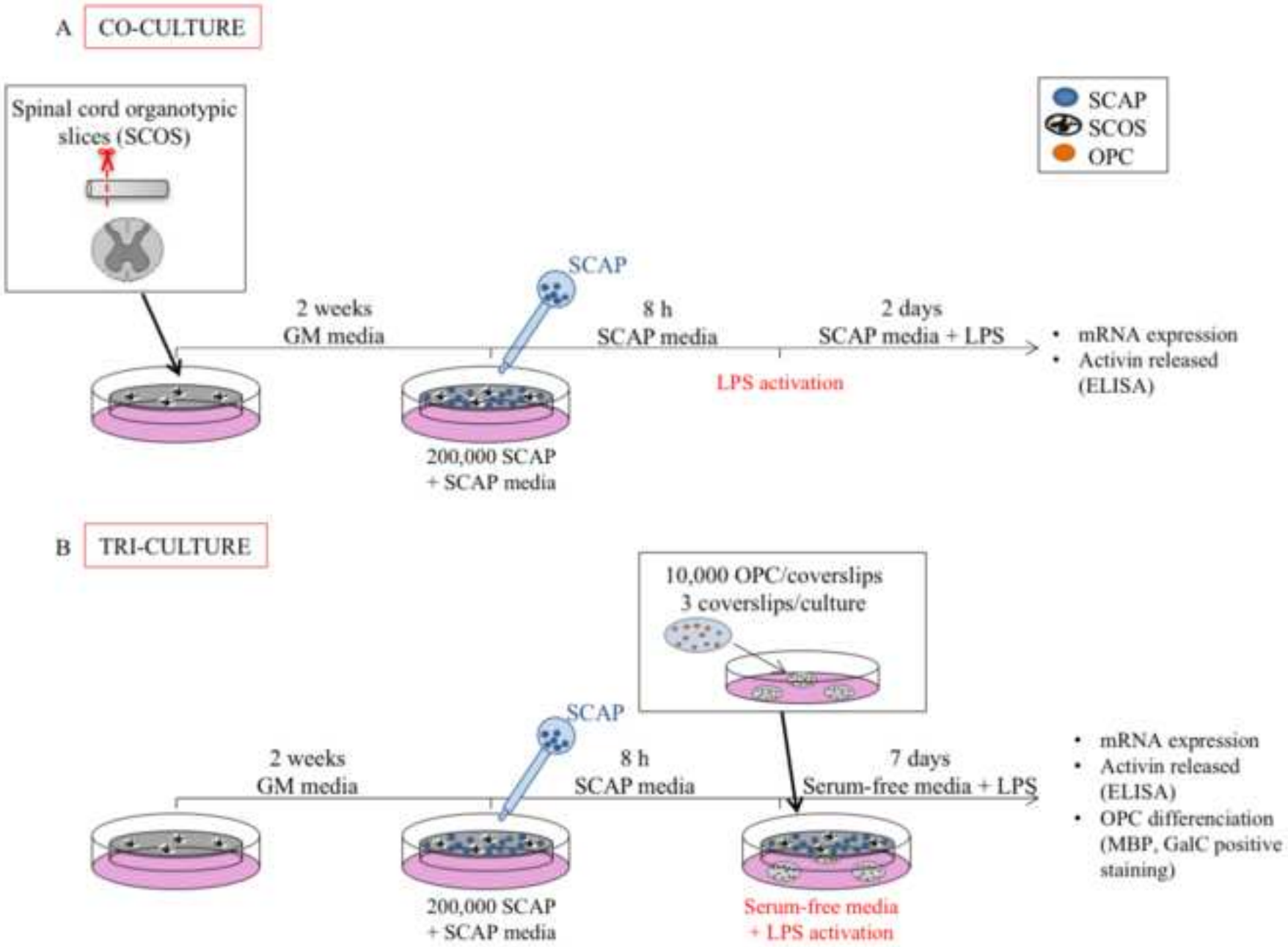


Figure 2

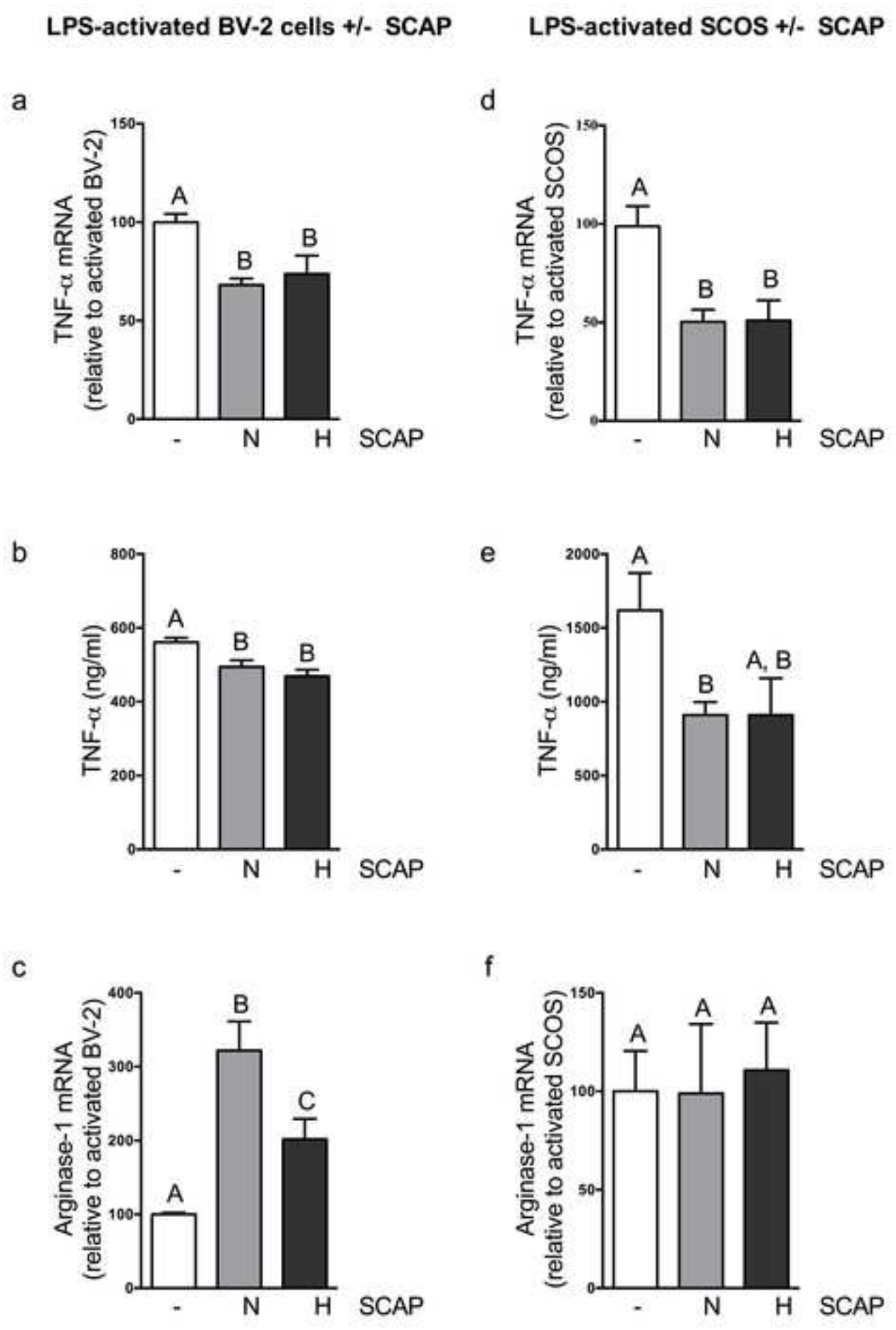
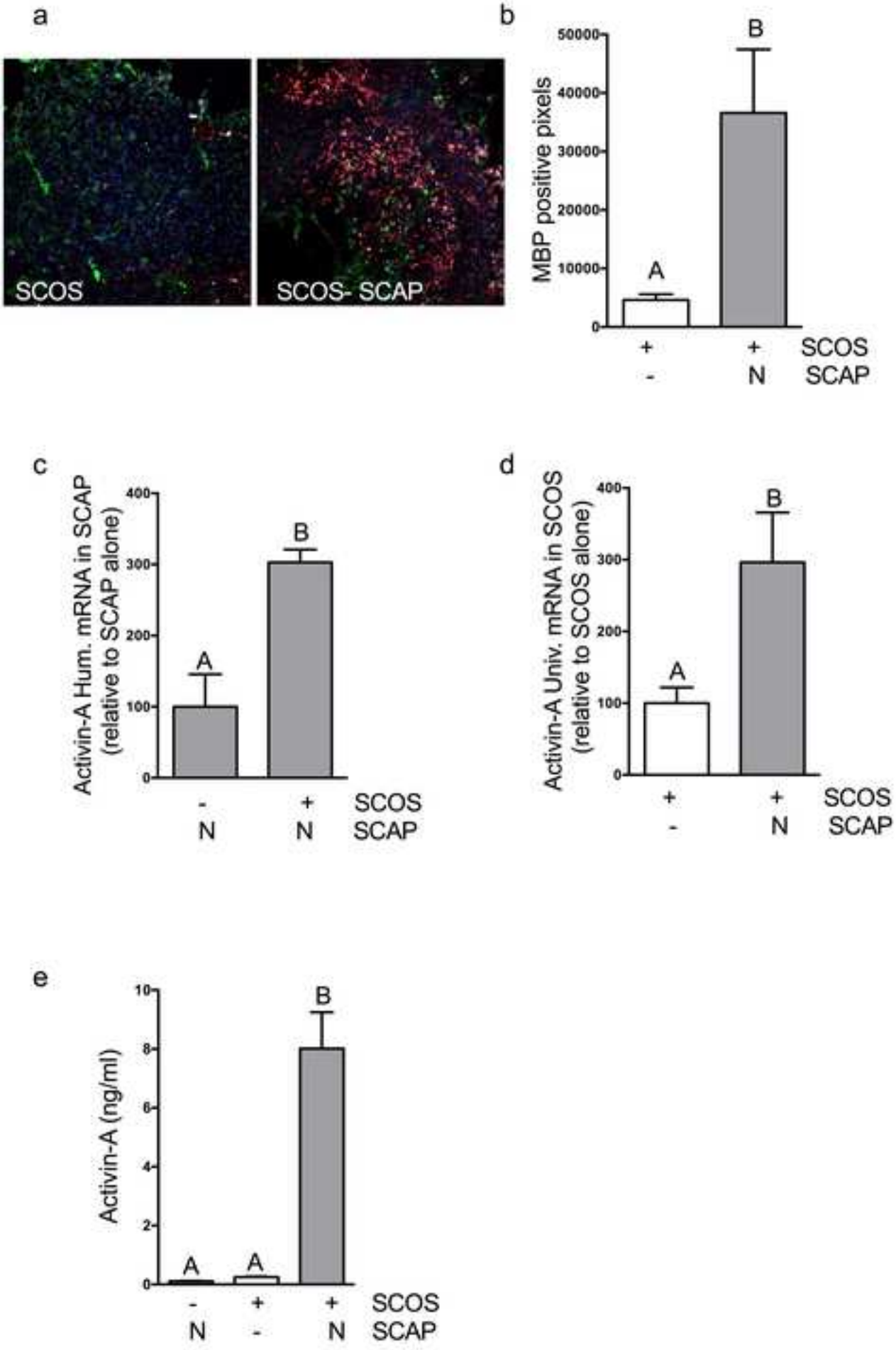


Figure 3



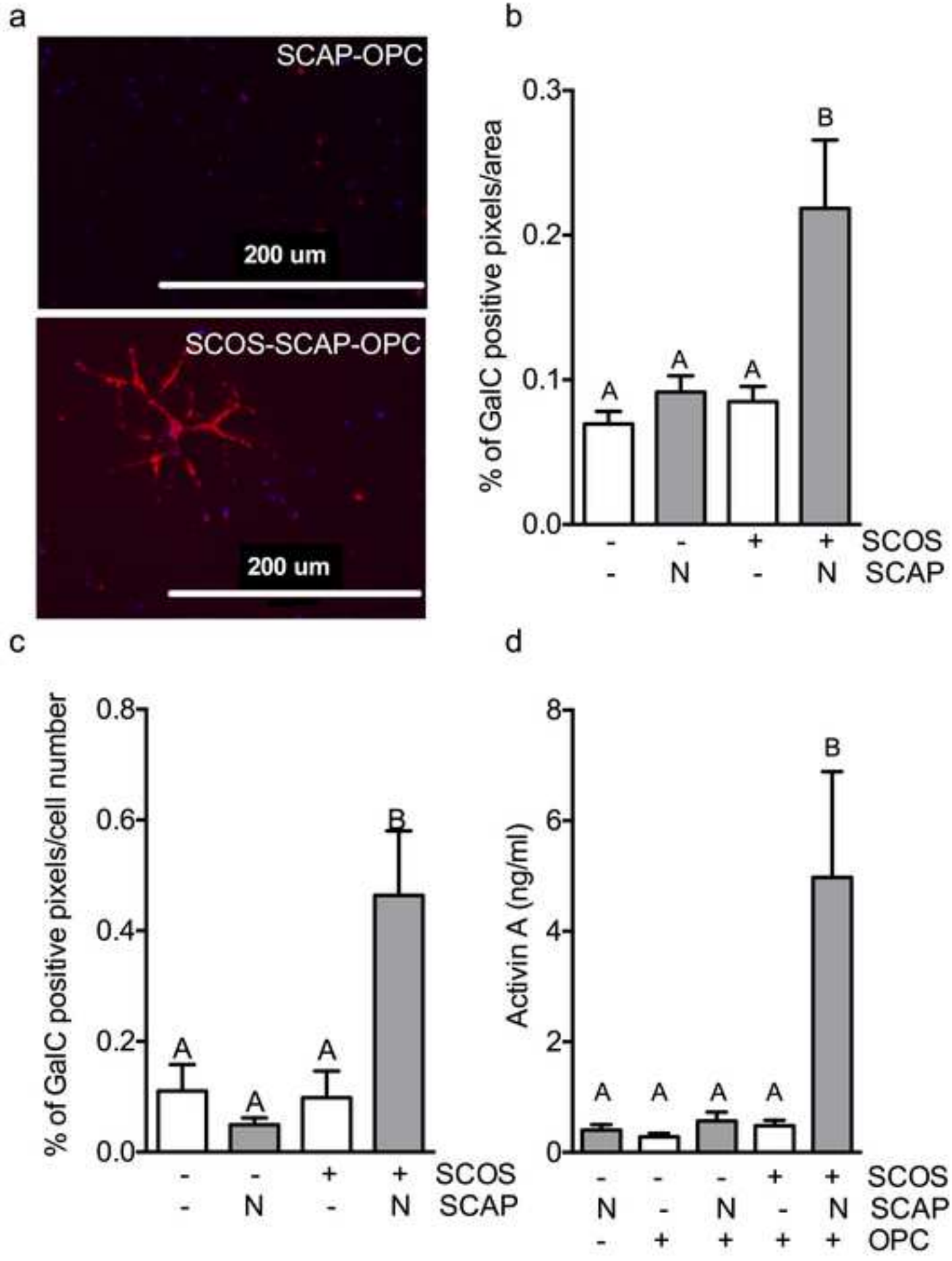


Figure 5 revised

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