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Abstract: Introduction: The balance between periapical tissue inflammation and regeneration is pivotal in determining the success of endodontic treatment. This study was designed to investigate the effect of silicate-based root canal sealer BioRoot™ RCS (BRCS) on modulating the inflammatory response and early steps of regeneration initiated by human periodontal ligament (PDL) fibroblasts.

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Conclusions: These results report that the endodontic sealers inflammatory and regeneration modulation can be predicted in vitro. It demonstrates that BRCS, unlike PCS, has an anti-inflammatory effect and promotes regeneration.

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BioRoot™ RCS modulates the initial steps of inflammation and regeneration

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Clinical relevance

Root canal sealers inflammatory and regeneration potentials can be predicted using *in vitro* models. Within the limits of this work performed in vitro, BioRoot™ RCS has anti-inflammatory and regenerative potentials.

- The initial steps of inflammation and regeneration can be investigated in vitro.
- BioRoot™ RCS decreased pro-inflammatory cytokine secretion and inflammatory cell recruitment.
- BioRoot™ RCS induced growth factor secretion by PDL fibroblasts and their proliferation.
- BioRoot™ RCS exerts an anti-inflammatory effect and has the potential to promote periodontal tissue regeneration.

ABSTRACT

Introduction: The balance between periapical tissue inflammation and regeneration is pivotal in determining the success of endodontic treatment. This study was designed to investigate the effect of silicate-based root canal sealer BioRoot™ RCS (BRCS) on modulating the inflammatory response and early steps of regeneration initiated by human periodontal ligament (PDL) fibroblasts.

Methods: Samples of BRCS and Pulp Canal Sealer (PCS) were incubated in culture medium to obtain material extracts. To simulate bacterial infection and endodontic sealer use, PDL fibroblasts were stimulated with lipopolysaccharides (LPS) and cultured with material extracts. Secretion of pro-inflammatory cytokine (IL-6) and growth factor (TGF- β 1) were quantified by ELISA. Adhesion of inflammatory (THP-1) to endothelial cells (HUVEC) was studied using fluorescent THP-1, their migration using Boyden chambers and their activation using a cell adhesion assay. Proliferation of PDL fibroblasts was quantified by MTT assay. PDL stem cell migration was investigated using Boyden Chambers after immunofluorescence and RT-PCR characterization.

Results: IL-6 secretion decreased with BRCS while it increased with PCS. TGF- β 1 secretion significantly increased only with BRCS. The material extracts did not affect THP-1 adhesion to HUVECs but only BRCS inhibited their migration. Moreover, activation of THP-1 decreased with BRCS and to a lesser extent with PCS. Finally, BRCS increased PDL fibroblast proliferation without affecting PDL stem cell migration. By contrast, PCS decreased PDL cell proliferation and migration.

Conclusions: These results report that the endodontic sealers inflammatory and regeneration modulation can be predicted *in vitro*. It demonstrates that BRCS, unlike PCS, has an anti-inflammatory effect and promotes regeneration.

Keywords: Endodontic sealer, periodontal ligament, inflammation, regeneration, tricalcium silicates.

INTRODUCTION

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2
3 Endodontic treatments are required to heal or prevent apical periodontitis which may
4 appear as a result of bacterial infection of the root canal space. Adequate root shaping,
5 cleaning and filling are prerequisites of endodontic treatment. The filling procedure combines
6 the use of gutta percha with a root canal sealer to seal the root canal space and avoid bacterial
7 contamination. Even though root canal sealers are used in a cleaned root canal, these materials
8 may exceed the apical foramina and be in contact with inflamed osseous or periodontal
9 tissues. Root canal sealers may play a role in the consecutive inflammatory response, its
10 resolution and the initiation of the lost tissue regeneration.

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13 The initial steps of inflammation include pro-inflammatory cytokine secretion and
14 subsequent immune cell recruitment. The resolution of this inflammation is required to initiate
15 the regenerative events initiated by growth factors secretion, cell proliferation and stem cell
16 migration to regenerate the lost tissues (1). In addition, it is well known that these events can
17 be modulated by dental materials (2,3).

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20 Root canal sealers have been developed since decades with the first being based on
21 zinc eugenates followed by resin-based sealers, calcium hydroxide or glass ionomers. Pulp
22 Canal Sealer™ (PCS) (SybronEndo, Orange, CA, USA) is one of the widely used Zinc Oxide
23 Eugenol (ZOE) sealers which contains eugenol and has a potential cytotoxicity to
24 periodontal/gingival cells (4,5). Silicate-based materials are successfully used in canal
25 perforation treatment, apexification and pulp therapies such as direct pulp capping, thanks to
26 their biological and sealing properties (6). For these reasons, silicate-based formulations with
27 appropriate handling and viscosity have been also developed for root canal filling procedures.
28 BioRoot™ RCS (Septodont, Saint-Maur-des-Fossés, France) (BRCS), has been developed as
29 a calcium silicate-based root canal sealer. It is composed of a powder which contains tricalcium
30 silicates, zirconium oxide and povidone while the liquid contains polycarboxylate and
31 calcium chloride. Recently conducted studies on BRCS have demonstrated calcium release,
32 alkalizing activity, apatite-forming ability (7) and anti-microbial activity (8). Moreover its
33 biocompatibility has been demonstrated on human periodontal ligament (PDL) cells (9), PDL
34 stem cells (10) and pulp-derived mouse stem cells (11). Indeed, after direct contact with pulp
35 cells, BRCS induced a high level of mineralization indicating the high potential of this
36 material to induce regeneration (12). Similarly, investigating the interaction of BRCS with
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1 PDL cells demonstrated an increased secretion of angiogenic growth factors such as VEGF
2 and FGF-2 and the osteogenic BMP-2 (13).
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4 On the other hand, PDL cell secreted Interleukine (IL) 6, 8 and TNF- α after
5 stimulation with Lipopolysaccharides (LPS) indicating that these cells may play a role in
6 initiating the inflammatory reaction (14–16). Additionally, under similar conditions, they also
7 expressed and secreted Transforming Growth Factor beta 1 (TGF- β 1) which have been
8 demonstrated to play a significant role in mesenchymal stem cell recruitment (17) and
9 regulation of PDL osteoblastic differentiation (18). However, investigations to evaluate the
10 effect of the material on modulating PDL cells regeneration and inflammatory potentials are
11 still lacking.
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20 This study was designed to evaluate, the effects of BRCS on periapical tissue
21 inflammatory response and on the early steps of regeneration. To simulate bacterial infection
22 of the root canal space and the filling procedure, PDL fibroblasts were stimulated with
23 lipopolysaccharides (LPS) and cultured with sealers' extracts. PDL fibroblast secretion of IL-
24 6 was quantified and the involvement of these cells in the inflammatory cell recruitment
25 sequence was evaluated by analyzing cell adhesion to endothelial cells, migration and
26 activation at the injury site. Furthermore, the early regenerative events were studied by
27 investigating TGF- β 1 secretion, fibroblast proliferation and PDL stem cell migration.
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MATERIALS AND METHODS

Reagents

Media, reagents and cell culture supplies were from Dutscher (Brumath, France).

Human periodontal ligament cell culture and characterization

Primary PDL cell cultures

PDL cells were obtained from immature third molars, freshly extracted (males and females under 18 years old) in compliance with French legislation (informed patients consent and institutional review board approval of the protocol used), by the explant outgrowth method (19). Cells were cultured in minimum essential medium (MEM) supplemented (10% fetal bovine serum, glutamine 2mM, penicillin 100UI/mL, streptomycin 100µm/mL, and amphotericin B 0.25µg/mL) at 37°C, 5%CO₂ atmosphere.

Magnetic stem cell sorting

PDL STRO-1 positive cells were directly sorted from primary PDL cell cultures at passages 1 to 5 with mouse anti-human STRO-1 IgM with immune magnetic beads according to the manufacturer's protocol (Dyna, Oslo, Norway) as described (20). STRO-1 negative cells were characterized as fibroblasts as described (2).

Immunofluorescence PDL STRO-1 positive cell characterization

PDL STRO-1 positive cells were cultured in 8-well glass culture chambers up to 70% confluence. Cells were washed with Phosphate Buffered Saline with calcium and magnesium (PBS) and fixed with 4% paraformaldehyde (15min, 4°C). Then non-specific binding sites were blocked with 1% Bovine Serum Albumine (BSA) (1h). Cells were incubated with primary antibodies (1h) against STRO-1 (5µg/mL), CD90 (2.5µg/mL), CD105 (2.5µg/mL), CD45 (2.5µg/mL) or their respective isotypes. After washing, cells were incubated (45min) with their respective secondary antibodies Alexa Fluor-488 (2µg/mL) and with DAPI (1µg/mL) for fluorescence microscopy.

RT-PCR characterization

Total RNAs were isolated from PDL STRO-1 positive cells using a PureLink RNA mini kit. RNA samples (2µg) were reverse-transcribed using a reverse transcription AMV

1 system. PCR were performed to detect RNA expression of four stem cell transcription factors:
2 KLF4, NANOG, OCT3/4 and SOX2. The RT-PCR primer list is presented on Table 1.
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4 **Material extract preparation**

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7 Samples of BRCS and PCS were prepared according to the manufacturers'
8 instructions and incubated in serum-free MEM (24h, 37°C) to obtain unset materials extracts.
9 The contact between the unset material and the culture media volume was 20 mg/mL. The
10 resulting material extracts were filtered on 0.22 µm filters and diluted in serum-free MEM to
11 obtain the working concentration (0.2 mg/mL).
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16 **Cytokine secretion by PDL fibroblasts**

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18 Cytokine expression

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20 PDL fibroblasts were cultured in 8-well glass culture chambers up to 70% confluence. Cells
21 fixed and permeabilized with 70% Ethanol (20min, 4°C). Then non-specific binding sites
22 were blocked with 5% BSA (1h). Cells were incubated with primary antibodies (1h) against
23 IL-6 (1µg/mL), TGF-β1 (1µg/mL) or their isotypes. After washing, cells were incubated
24 (45min) with their respective secondary antibodies Alexa Fluor-488 (1µg/mL) or Alexa
25 Fluor-594 (1µg/mL) and with DAPI (1µg/mL) for fluorescence microscopy.
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34 Cytokine quantification

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36 PDL fibroblasts were cultured at confluence in 12-well plates. Cells were stimulated with LPS
37 (1µg/mL, 4h) and incubated with material extract or serum-free MEM control media. After
38 24h the supernatants were used for IL-6 and TGF-β1 quantification by the enzyme-linked
39 immunosorbent assay (ELISA) using DuoSet kits (R&D Systems, Minneapolis, MN, USA)
40 according to the manufacturer's instructions.
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47 **Inflammatory cell recruitment sequence**

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49 Human umbilical vein endothelial cell (HUVECs) and inflammatory (THP-1) cell culture

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51 HUVECs (PromoCell, Heidelberg, Germany) were cultured in Endothelial Cell
52 Growth Medium 2 (ECGM 2) (PromoCell). THP-1 cells, a human monocytic cell line (Sigma,
53 St Quentin Fallavier, France) were cultured in RPMI medium (supplemented with 10% FBS,
54 100UI/mL Penicillin, 100µg/mL streptomycin, 0,25µg/mL amphotericin B, 2mM L-
55 glutamine, 1mM sodium pyruvate), as described (2).
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Conditioned media preparation

PDL fibroblasts were cultured until they became confluent in 12-well plates and then stimulated with LPS (1µg/mL, 4h). Next, they were incubated with material extract or serum-free MEM control media for 24h. The supernatants were then harvested and will be called *conditioned media*.

THP-1 cell adhesion on HUVECs

Confluent HUVECs (80000cells/cm²) were cultured in 12-well plates with the *conditioned media* (4h, 37°C). THP-1 cells were first incubated with 0.1µM of BCECF acid (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein) for 1h. These BCECF-labeled THP-1 cells (25000 cells/mL) were added to the HUVECs monolayers (30 min, 4 °C) under gentle orbital rotation as described (21). HUVEC monolayers were washed four times with PBS and labelled-THP-1 adherent cells were counted in 5 random fields using a fluorescent microscope. Results are expressed as percentage of control.

THP-1 cell migration

Migration was studied using Boyden chambers (8µm pore size) in 12-well plates. Confluent PDL fibroblasts were cultured in the lower chambers. They were stimulated with LPS (4h, 1µg/mL) and then incubated with 1 mL of material extract or serum-free MEM (control) (24h, 37°C). THP-1 cells were activated with Phorbol Myristate Acetate (400ng/mL, 24h) and seeded (15000cells /100µL) in the upper chambers. After 24h of migration, THP-1 cells on the top side of the upper chamber were eliminated using a cotton bud, and cells on the lower surface of the upper chamber were fixed (cold ethanol 70%, 15min) and stained (eosin, 20min). The number of migrating cells was counted in 5 random fields using light microscopy. Results are expressed as percentage of control (cells migrating in response to LPS-stimulated PDL fibroblasts incubated in MEM).

THP-1 cell activation

THP-1 suspension (25000cells/mL) was cultured with PDL *conditioned medium* in 12-well plates (24h, 37°C). Then, the wells were washed four times with PBS to remove non-activated and dead cells. Adherent cells were fixed (cold ethanol 70%, 15min) and nuclei stained with DAPI (4',6-diamidino-2-phenylindole) (1µg/mL). The number of activated

1
2 (adherent) cells was counted in 5 random fields using a fluorescent microscope. Results are
3 expressed as percentage of control.

4 **PDL fibroblast proliferation**

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7 PDL fibroblasts were cultured at a low density (1000 cells/cm²) in 12-well plates for
8 24h and then stimulated with LPS (1µg/mL, 4h). Next, media were replaced by material
9 extracts or serum-free MEM control media. After 3, 6 and 9 days the supernatants were
10 removed and a MTT assay was performed as described (17) to measure cell proliferation (22).
11 Results are expressed as percentage of control at day 3 considered as the baseline.
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16 **PDL stem cell migration**

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18 Migration assays were performed using Boyden chambers (12-well plates fitted with
19 8µm pore Boyden inserts). PDL fibroblasts were cultured in the lower chamber at confluence.
20 Fibroblast stimulation (LPS, 1µg/mL, 4h), they were incubated either in serum-free MEM
21 (control) or in material extracts (1mL). The upper chambers (inserts) were seeded with PDL
22 stem cells (10⁴cells/100µL). After 24 hours, migrating cells on the lower side of the
23 membrane were fixed (cold ethanol 70%, 15min) and then stained (hematoxylin, 20min).
24 Migrating cells were counted in 5 random fields using light microscopy. Results are expressed
25 as a percentage of control (cells migrating in response to LPS-stimulated PDL fibroblasts
26 incubated in MEM).
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37 **Statistical analysis**

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39 All experiments were done in triplicate and repeated three times. Data are expressed as
40 means ± standard error of mean. Student t-test was used to determine statistical significance
41 (p-value < 0.05).
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RESULTS

BRCS induced TGF- β 1 and inhibited IL-6 secretion by PDL fibroblasts

After LPS stimulation of PDL fibroblasts and incubation with MEM (control) or with BRCS or PCS extracts, immunofluorescence showed that PDL fibroblasts expressed TGF- β 1 (Fig. 1Aa-d) and IL-6 (Fig. 1Ae-h). An intense TGF- β 1 labeling was observed with BRCS extract (Fig. 1Ab) while that of IL-6 was weakly stained (Fig. 1Af) as compared to control (Fig. 1Aa and e) and PCS (Fig. 1Ac and g). No expression was observed with isotype controls (Fig. 1Ad and h). ELISA showed that BRCS significantly induced TGF- β 1 secretion and significantly reduced IL-6 secretion as compared to the control and to PCS. On the opposite, PCS induced IL-6 secretion as compared to the control (Fig. 1B).

BRCS modulated inflammatory cell recruitment sequence

Use of *conditioned media* from both materials did not affect THP1-cell adhesion on HUVEC monolayer as compared to the control (Fig. 2A). THP-1 migration towards LPS-stimulated PDL fibroblasts was significantly reduced with BRCS as compared to the control and PCS (Fig. 2B). Finally, as illustrated with representative pictures (Fig. 2Ca-c), BRCS and PCS *conditioned media* significantly reduced THP-1 activation as compared to the control. However, this decrease was significantly more important with BRCS as compared to PCS (Fig. 2Cd).

BRCS induced PDL fibroblast proliferation

Pictures of LPS-stimulated PDL fibroblast MTT assay show an increased staining after incubation with BRCS (Fig. 3Aa-c) and a decrease with PCS (Fig. 3Ad-f). Quantitative analysis of the results shows a significant increase of PDL fibroblast proliferation after 6 and 9 days with BRCS extracts as compared to the control. By contrast, this proliferation significantly decreased after 6 and 9 days with PCS as compared to the control (Fig. 3B).

PCS decreased PDL stem cell migration

The STRO-1 sorted PDL cells proliferated forming Colony Forming Units (CFU), a characteristic of stem cells (Fig. 4Aa-c). Cell characterization was performed by immunofluorescence with 3 characteristic stem cell markers (STRO-1, CD90 and CD105) and 1 hematopoietic cell marker (CD45). Results showed that sorted cells expressed STRO-1 (Fig. 4Ad), CD90 (Fig. 4Ae) and CD105 (Fig.4Af). On the opposite, they did not express CD45

1 (Fig. 4Ag). Isotype controls were negative (Fig. 4Ah). RT-PCR confirmed the stem cell
2 nature of the sorted PDL cells as characteristic transcription factors genes KLF4, NANOG,
3 OCT3/4 and SOX2 were expressed (Fig. 4Ai).
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5 Investigation of the characterized PDL stem cells migration showed that this migration
6 was not affected by BRCS but was significantly reduced by PCS as compared to the control
7 (Fig. 4B).
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DISCUSSION

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This study demonstrates that applying endodontic sealers' extracts on LPS-stimulated PDL fibroblasts modulates the initial steps of inflammation and regeneration. The modulatory effect of inflammation was studied by investigating pro-inflammatory IL-6 cytokine secretion and the consequences of adding the materials' extracts on the initial steps of inflammation which include adhesion of inflammatory cells onto the vascular endothelium, their migration to the stimulation site and their activation. The effect on modulating the initial steps of regeneration was investigated through TGF- β 1 secretion and the consequences of adding the sealers extracts on PDL fibroblast proliferation and migration of PDL stem cells to the stimulation site. These steps play a key role as they determine the overall outcome of healing/regeneration after endodontic treatment.

In this experimental design, LPS-stimulated PDL fibroblasts were used as the inflammatory response of the periodontal tissue results from root canal bacterial infection. Gram-negative bacteria involvement in root canal infection has been well demonstrated (23). In addition, the presence of LPS, a gram-negative bacterial component (24) was correlated to endodontics symptoms in necrotic teeth (25), and its stimulation of periapical tissue inflammation and destruction has been demonstrated (26,27). The rationale in choosing interactions between materials extracts and LPS-stimulated PDL cells is that, after root canal filling, a direct contact usually occurs between the filling material which may exceed the apex and the periapical tissues including the periodontal ligament and/or the alveolar bone. The success of endodontic treatment depends not only on the sealer's mechanical sealing function in preventing recurrent infection of the periapical space (28,29) but also on the sealer's capacity to arrest the inflammation and induce apical hard tissue formation. PDL is known to have a population of mesenchymal stem cells (30) and these cells are responsible for hard tissue formation during the regeneration process (31). This explains why we isolated and characterized PDL stem cells and investigated their migration towards LPS-stimulated fibroblasts to check the effect of the presence of sealers on their migration and subsequent hard tissue formation.

This work shows that PCS significantly increased pro-inflammatory IL-6 secretion and inhibited PDL stem cell migration. IL-6 secretion may be due to the previously reported PCS toxicity *in vitro* (13,32,33).

1 This increased pro-inflammatory activity may appear surprising as PCS is a ZOE and
2 these sealers are widely used for their reported anti-inflammatory effects due to eugenol. Yet,
3 eugenol effects on dental tissues are controversial (34). Indeed, a localized inflammation with
4 ZOE sealers has been observed, both in soft and bone tissue (35). Additionally, *in vivo* studies
5 with ZOE intentional overfilling in monkeys over a 6-month period (36), demonstrated a
6 severe irritation over the full 6-month duration.
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11 By contrast, BRCS decreased IL-6 pro-inflammatory cytokine secretion and
12 inflammatory cell recruitment while it increased TGF- β 1 secretion, and induced PDL
13 fibroblast proliferation. IL-6 is considered as a potent cytokine playing a major role in the
14 acute inflammation process (37). Its decrease, together with the subsequent decreased
15 inflammatory cell migration/activation, suggests a BRCS anti-inflammatory potential. This
16 result is similar to previously reported data with another tricalcium silicate-based material.
17 Indeed, Biodentine™ has been shown to induce TGF- β 1 secretion from pulp cells. After
18 encapsulation of this factor in Polylactic/Polyglycolic acid microspheres, its sustained release
19 has been shown to guide pulp stem cell recruitment (17,38) and induced the initial steps of
20 regeneration (38). Moreover, the recent investigation of pro-inflammatory cytokine secretion
21 by pulp cells and the initial steps of inflammation, demonstrated that Biodentine™ has an
22 anti-inflammatory potential (2). The observed increase in TGF- β 1 secretion is also in
23 agreement with a previously reported work showing that BRCS induced a significant increase
24 in VEGF, FGF-2 and BMP-2 from PDL cells (13). This growth factor has been shown to play
25 a significant role in mesenchymal stem cell recruitment (17) and osteoblastic differentiation
26 (18).
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42 Overall, this work reports that the root canal sealers can modulate the periapical tissue
43 inflammatory and regeneration potentials and that the initial steps of these processes can be
44 predicted *in vitro*. This demonstrates BRCS anti-inflammatory potential and highlights the
45 fact that the sealers' bioactive properties should be considered to improve the clinical
46 outcome of endodontic treatment.
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FIGURE LEGENDS

Figure 1: TGF- β 1 and IL-6 secretion by PDL fibroblasts after LPS stimulation and incubation with materials' extracts.

(A) Representative immunostaining pictures of TGF- β 1 (green, a-d) and IL-6 (red, e-h) expression under control (a,e) BRCS (b,f), PCS (c,g) conditions and isotype controls (d,h). A strong expression of TGF- β 1 (b) and a weak labelling of IL-6 (f) were observed with BRCS. Scale bars: 50 μ m. (B) ELISA quantification of the secretion in pg/mL after incubation for 24h with the materials' extracts. BRCS significantly increased TGF- β 1 secretion and inhibited IL-6 secretion as compared to the control and PCS. PCS had no effect TGF- β 1 secretion and significantly increased the IL-6 secretion as compared to the control and BRCS. (*) Indicates a statistical difference with the control condition. (**) Indicates a statistical difference between the two materials.

Figure 2: Inflammatory cell recruitment sequence modulation by the materials' extracts.

(A) Inflammatory cell (THP-1) adhesion on endothelial cell (HUVEC) monolayer using *conditioned media* from both materials' extracts or MEM media (control). No significant differences were observed between all conditions. (B) THP-1 cell migration in Boyden chambers towards LPS-stimulated PDL fibroblasts incubated with materials' extracts. BRCS significantly decreased THP-1 cell migration as compared to the control and PCS conditions. (C) THP-1 cell activation using *conditioned media* from both materials' extracts. Representative pictures used for cell count from control (a); BRCS (b) and PCS(c). Scale bars: 50 μ m. BRCS and PCS significantly decreased inflammatory cell activation as compared to the control. The reduction with BRCS was also significant as compared to PCS (d). Results are expressed as percentage of control. (*) Indicates a statistical difference with the control condition. (**) Indicates a statistical difference between the two materials.

Figure 3: Materials' effects on PDL fibroblast proliferation.

(A) Representative pictures of fibroblasts with MTT assay after 3, 6 and 9 days using BRCS (a-c) or PCS (d-f) extracts. Scale bars: 50 μ m. (B) Quantitative analysis showed a significant increase of proliferative cells with BRCS after 6 and 9 days while a significant decrease was observed with PCS. Results are expressed as percentage of the control (3 days baseline). (*) Indicates a statistical difference with the control condition.

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Figure 4: PDL stem cell characterization and effect of sealers on their migration.

(A) Pictures showing PDL STRO-1 positive Colony Forming Unit (a-c). Immunofluorescence expression of STRO-1(d), CD90 (e), CD105 (f), CD45 (g) and isotype control (h). Scale bars: 50 μ m for a, 500 μ m for b and c and 100 μ m for d,e,f,g and h. RT-PCR of KLF4, NANOG, OCT3/4 and SOX2 on STRO-1 isolated PDL stem cells (i). (B) Effect of sealers on PDL stem cell migration in Boyden chambers towards LPS-stimulated PDL fibroblasts. PCS extracts significantly decreased stem cell migration. No effect on this migration was observed with BRCS. Results are expressed as percentage of the control. (*) Indicates a statistical difference with the control condition.

TABLE LEGEND

Table 1: RT-PCR primer sequence.

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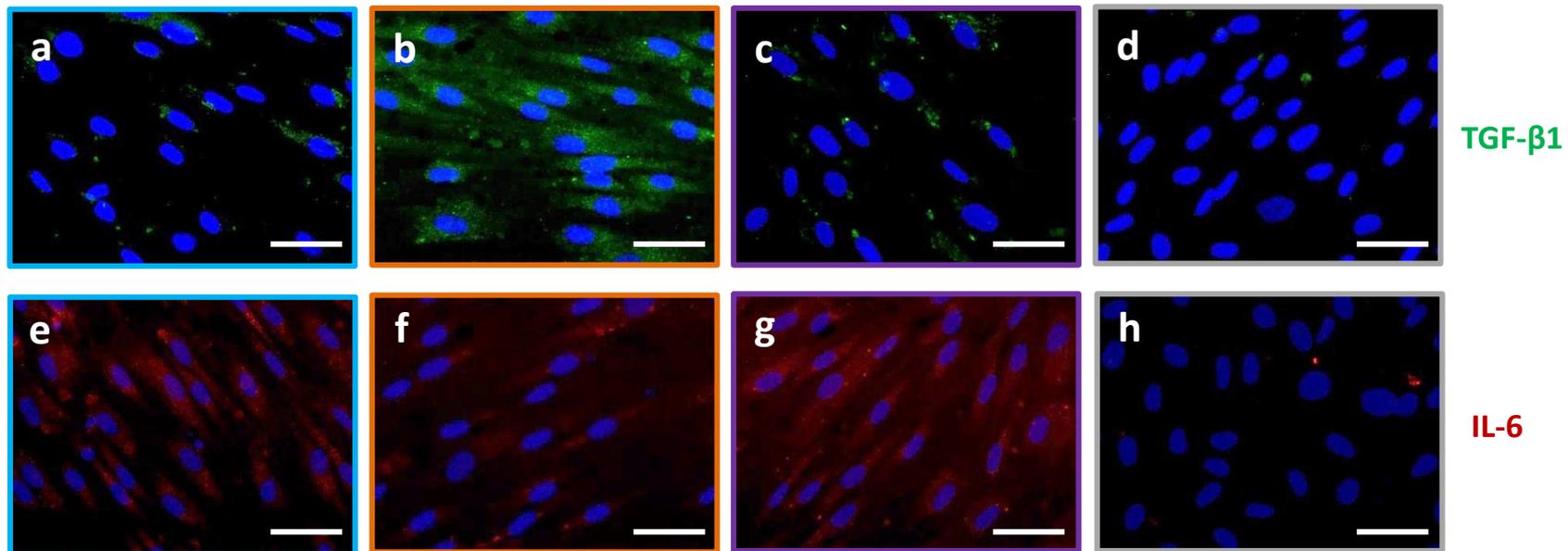
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Table1

	Primer	Sequence
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3	OCT3/4-1	5' -CAGTGCCCGAAACCCACAC-3'
4	OCT3/4-2	5' -GGAGACCCAGCAGCCTCAA-3'
5		
6	KLF4-1	5' -GGGAGAAGACACTGCGTCAA-3'
7	KLF4-2	5' -TCCAGGTCCAGGAGATCGTT-3'
8		
9	SOX2-1	5' -GTTGCCTGGCTTCTCTTTTG-3'
10	SOX2-2	5' -GCTGATTGGTCGCTAGAAAC-3'
11		
12	NANOG-1	5' -AAGGTCCCGGTCAAGAAACAG-3'
13	NANOG-2	5' -CTTCTGCGTCACACCATTGC-3'
14		
15	GAPDH-1	5' -GAAGGTGAAGTTCGGAGTC-3'
16	GAPDH-2	5' -GAAGATGGTGATGGGATTTTC-3'
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Figure 1

A



B

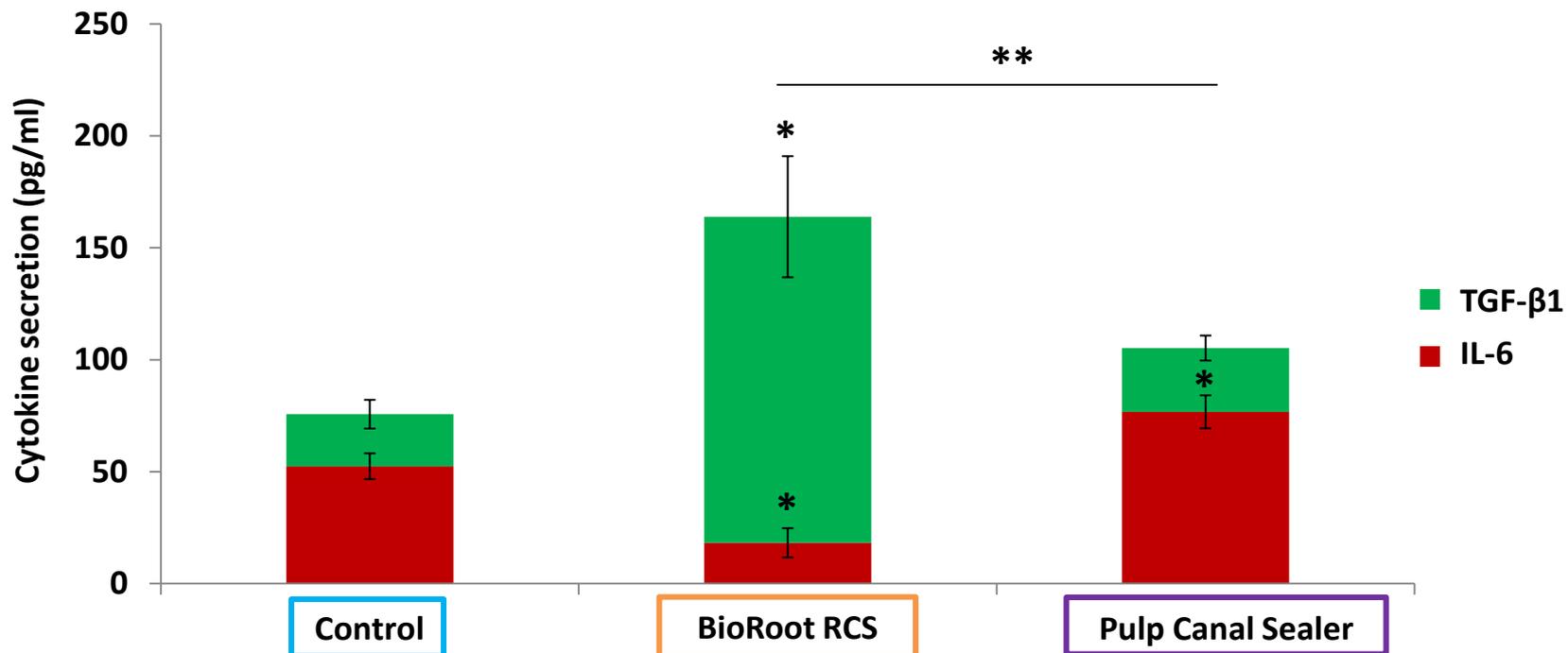


Figure 2

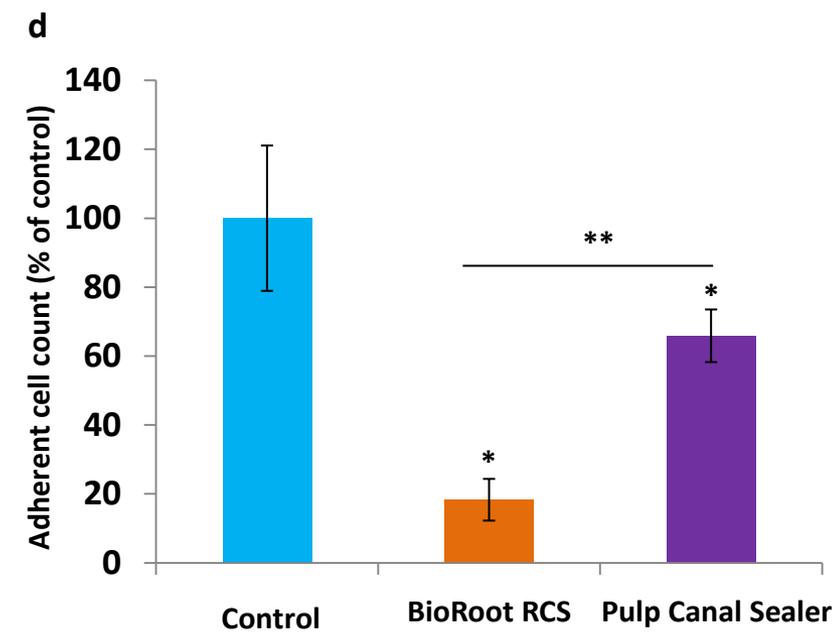
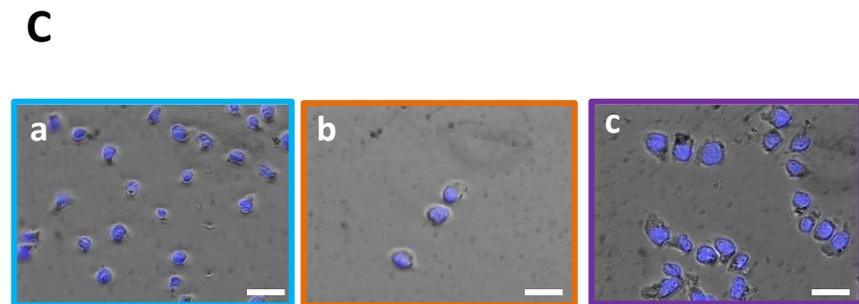
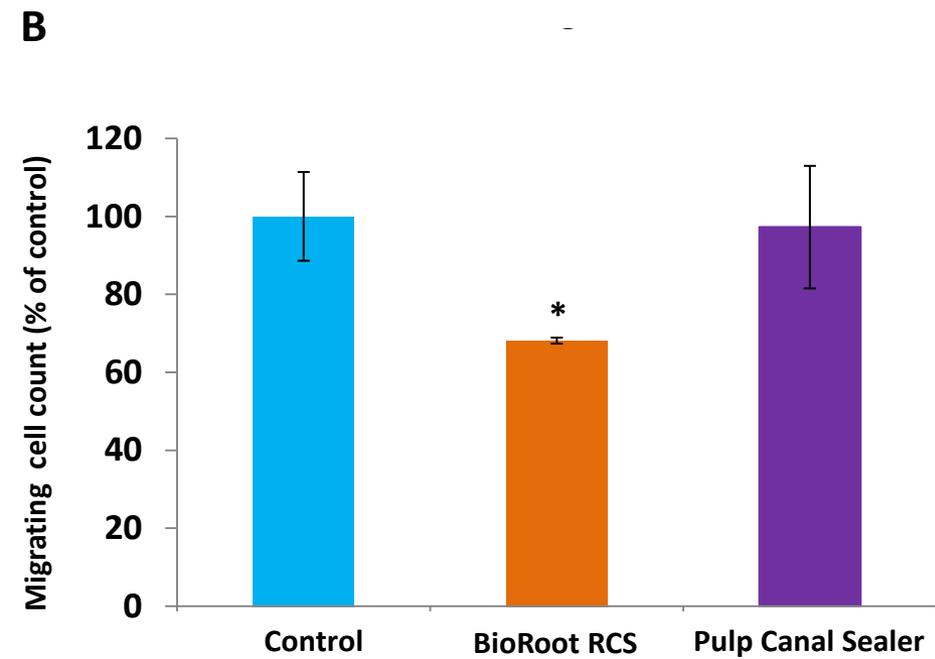
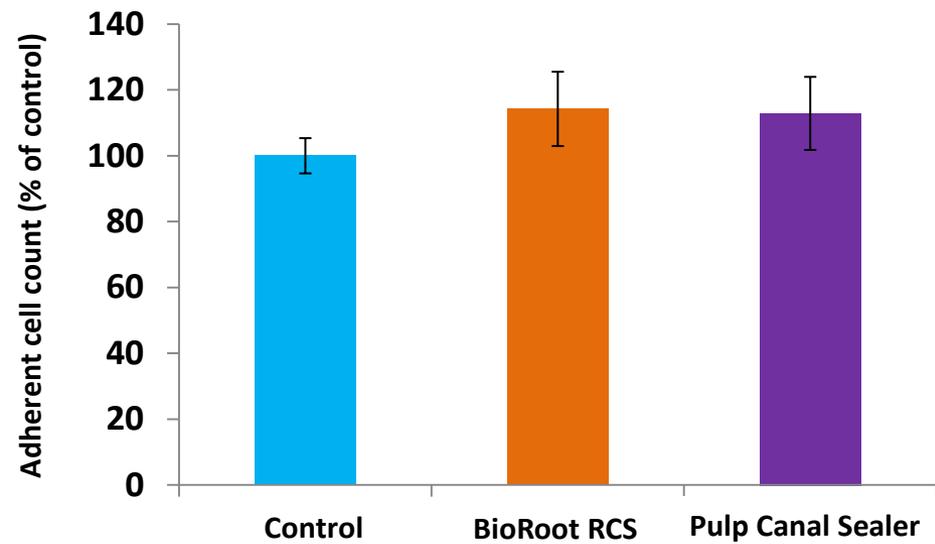
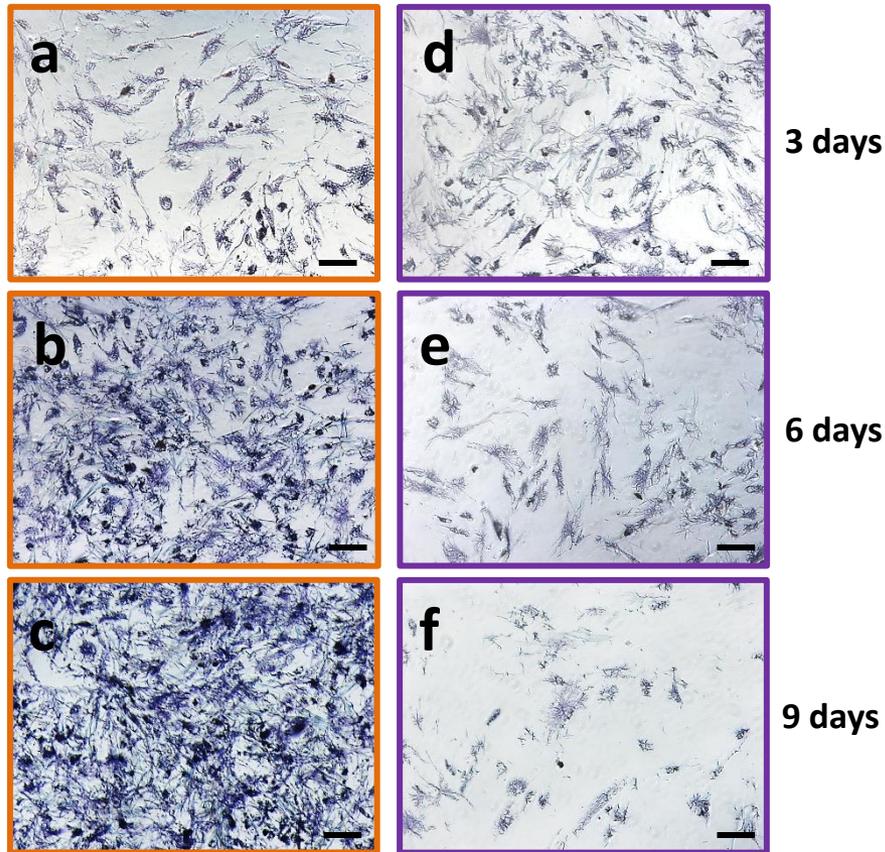


Figure3

A



B

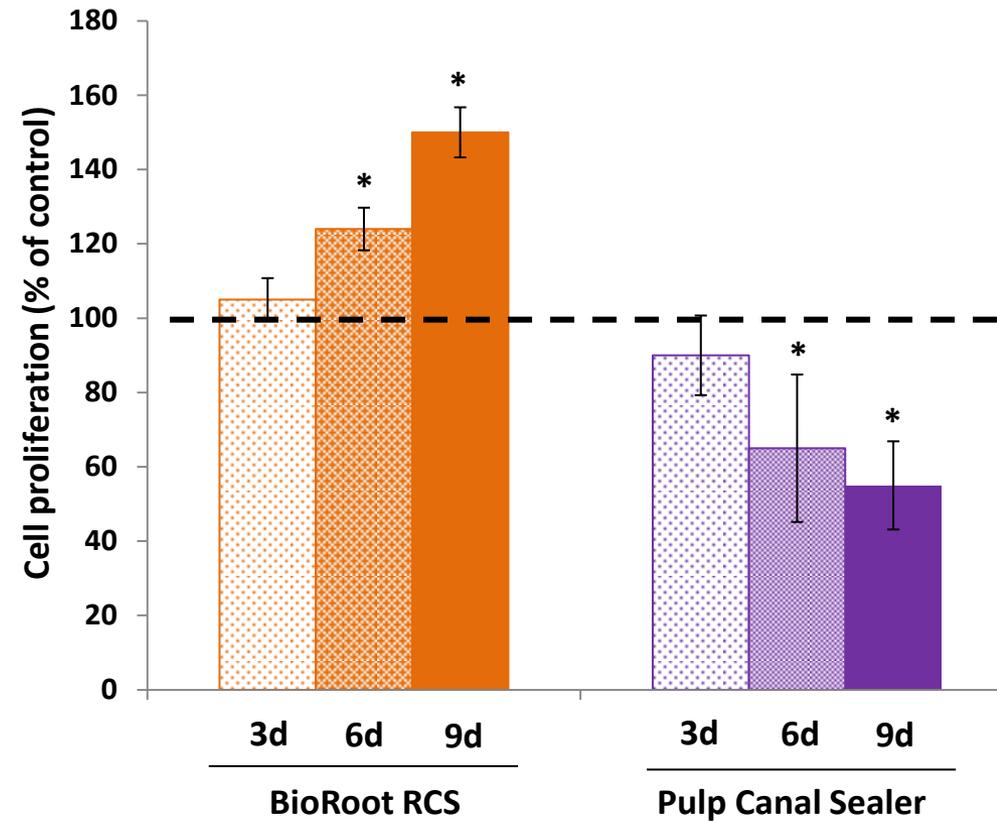
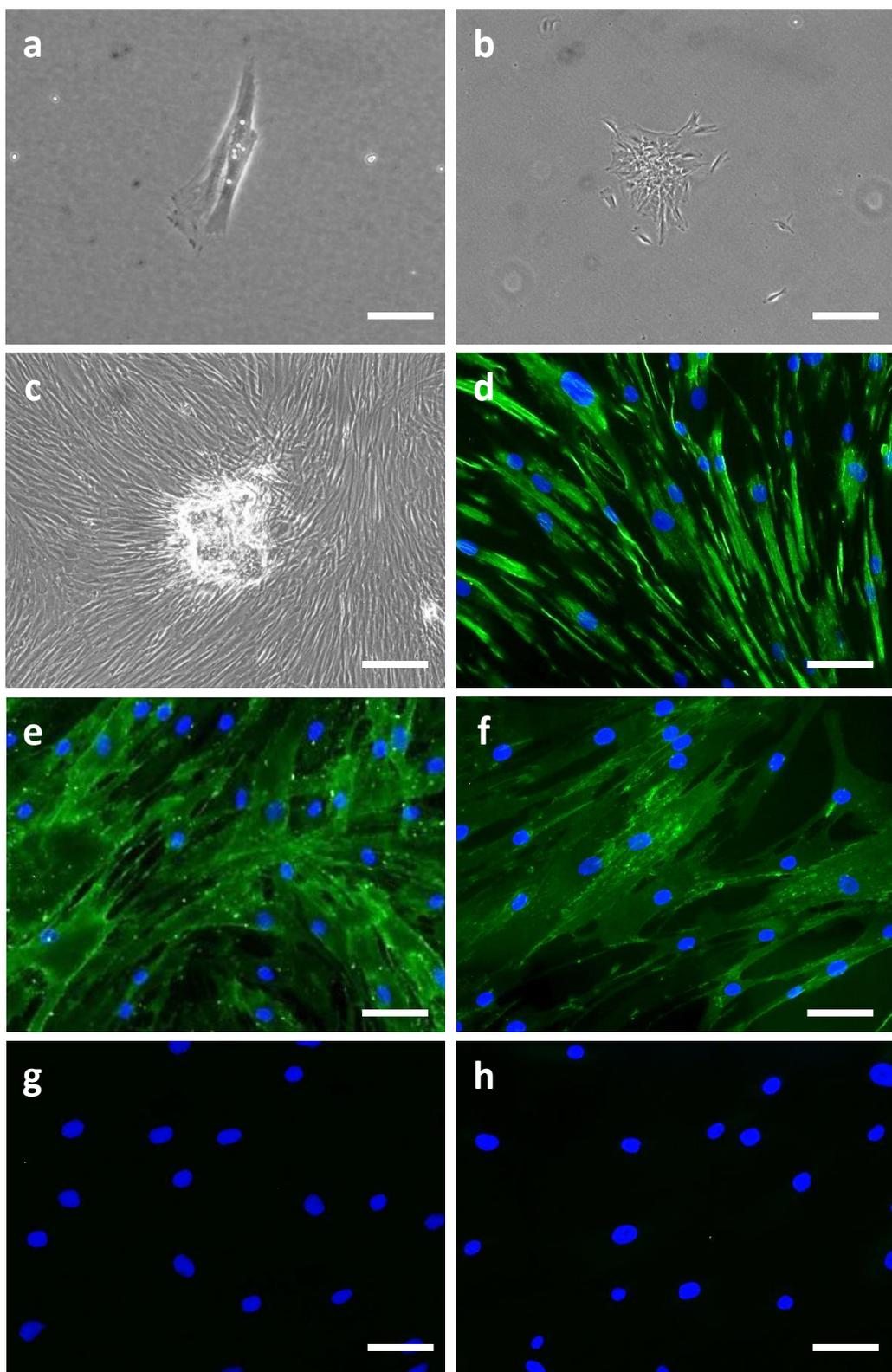


Figure4A
A



GAPDH KLF4 NANOG OCT3/4 SOX2

Figure4B
B

