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Version of Record: https://www.sciencedirect.com/science/article/pii/S0197018621000073
Manuscript_5dbee3243ca1449a1193fd46a75bd486

Identification of secreted factors in dental pulp cell-conditioned medium optimized for neuronal growth

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Abbreviations[1]

Abstract

With their potent regenerative and protective capacities, stem cell-derived conditioned media emerged as an effective alternative to cell therapy, and have a prospect to be manufactured as pharmaceutical products for tissue regeneration applications. Our study investigates the neuroregenerative potential of human dental pulp cells (DPCs) conditioned medium (CM) and defines an optimization strategy of DPC-CM for enhanced neuronal outgrowth. Primary sensory neurons from mouse dorsal root ganglia were cultured with or without DPC-CM, and the lengths of βIII-tubulin positive neurites were measured. The impacts of several manufacturing features as the duration of cell conditioning, CM storage, and preconditioning of DPCs with some factors on CM functional activity were assessed on neurite length. We observed that DPC-CM significantly enhanced neurites outgrowth of sensory neurons in a concentration-dependent manner. The frozen storage of DPC-CM had no impact on experimental outcomes and 48 hours of DPC conditioning is optimal for an effective activity of CM. To further understand the regenerative feature of DPC-CM, we studied DPC secretome by human growth factor antibody array analysis and revealed the presence of several factors involved in either neurogenesis, neuroprotection, angiogenesis, and osteogenesis. The conditioning of DPCs with the B-27 supplement

DPCs: human dental pulp cells
CM: conditioned medium
DRG: dorsal root ganglia
PBS: phosphate-buffered saline
C^-: negative control
C^+: positive control
DPC-CM *pre* B-27: conditioning medium obtained from DPCs cultured with B-27
DPC-CM *post* B-27: B-27 added directly in the CM obtained from DPCs cultured without any supplement

enhanced significantly the neuroregenerative effect of their secretome by changing its composition in growth factors. Here, we show that DPC-CM significantly stimulate neurite outgrowth in primary sensory neurons. Moreover, we identified secreted protein candidates that can potentially promote this promising regenerative feature of DPC-CM.

Keywords:

Dental Pulp; Conditioned Medium; Neuron; Neurite; Outgrowth.

1. Introduction

The peripheral nervous system is fragile and easily damaged. After an injury, functional recovery depends on the regeneration of peripheral nerve axons. However, the mechanism is slow and the results are often unsatisfactory (Scheib and Höke, 2013). Unsuccessful regeneration leads to post-traumatic neuropathies, which are mostly resistant to current treatments (Martin et al., 2013).

The current standard of care for peripheral nerve injury is autologous nerve transplantation. Complications include loss of function at the donor site, limited availability of donor nerve tissue, and donor site morbidity (Grasman and Kaplan, 2017). The use of stem cells as a regenerative therapy process is an appealing strategy to overcome these limitations. Mesenchymal stem cells have been of particular importance in central and peripheral nervous system repair, due to their regenerative effects (Gögel et al., 2011; Teixeira et al., 2013). Their therapeutic potency is mainly associated with their ability to secrete multiple factors, namely the secretome, that induce survival and regeneration of host neurons (Crigler et al., 2006; Blurton-Jones et al., 2009). These secreted factors can be harvested from the supernatant of cell cultures, referred to as the conditioned medium (CM). Thus, the administration of CM into injury sites could be used as a better alternative to the grafting of stem cells. Indeed, the use of CM has several advantages compared to the use of stem cells, as it can be manufactured, freeze-dried, packaged, and transported more easily. Moreover, as it is devoid of cells, there is no need to match the donor and the recipient to avoid rejection problems (Pawitan, 2014).

The present study focuses on the secretome of dental pulp cells (DPCs). The human dental pulp is enriched in mesenchymal stem cells that were described for the first time by Gronthos et al. (Gronthos et al. 2000). One advantage of this source of mesenchymal stem cells is the absence of morbidity and the fact that it does not require additional surgical procedures (Alkhalil et al. 2015). Dental pulp stem cells originating from the neural crest (Huang et al. 2009), express neuron-related markers (Foudah et al. 2014) and can differentiate into neuron-like cells (Mead et al. 2014). The neurotrophic factors secreted by dental pulp stem cells are remarkably higher than bone marrow mesenchymal stem cells and adipose-derived stem cells (Mead et al., 2014). For all these reasons, denal pulp stem cells are considered as an excellent candidate for stem cell-related therapies in nerve diseases (Luo et al. 2018), and the leading role of dental pulp stem cell-conditioned medium in neuroprotection and neuritogenesis was described notably in many *in vitro* and *in vivo* studies (Kichenbrand et al., 2019).

Herein, we use dental pulp cell-conditioned medium (DPC-CM) to enhance the neurite growth of dorsal root ganglia (DRG) sensitive neurons. We study the DPC-CM potential for axonal growth and we define an optimization strategy of DPC-CM to aid axonal growth.

2. Materials and methods

2.1. Isolation and culture of human DPCs

DPCs were isolated from extracted wisdom teeth from young healthy patients (15 to 23 years of age). Informed consent was obtained from the patients after receiving approval by the local ethics committee (Comité de protection des Personnes, Centre Hospitalier de Montpellier). We used a previously described protocol to recover pulp cells (Collart-Dutilleul et al., 2014; Panayotov et al., 2014). Briefly, after disinfection, teeth were cut along the cementum–enamel junction using a diamond disc and were broken into two pieces. Pulps were then recovered and incubated for 1 hour in a collagenase-dispase solution (3 mg/ml type I collagenase and 4 mg/ml dispase). Digested pulps were filtered, centrifugated, and recovered cells were incubated in α-MEM (Gibco) with 1% Penicillin-Streptomycin, 10% fetal bovine serum, and 0.02% Recombinant Human FGF basic (R&D System). The medium was changed after 24 hours and then changed twice a week.

2.2. Preparation of DPC-CM

When DPCs (passage number (P) = 3 or 4) reached 80% confluence, two phosphate-buffered saline (PBS) washes were carried out and the medium was replaced with a serum-free neurobasal (Gibco), 1% Penicillin-Streptomycin. 48 hours later, the medium was collected by centrifugation for 5 min at 450 g and was centrifuged again for 3 min at 1800 g to remove cell debris. The CM was used fresh or stored at -20°C until use.

2.3. Optimization of DPC-CM

To optimize DPC-CM, 1% glutamine (200 mM, Sigma-Aldrich) and 2% B-27 (Supplement 50X, serum-free, Gibco) were added to the neurobasal medium, after the PBS washing step (DPC-CM *pre* B-27).

Following media conditioning of DPCs cultured with B-27, this supplement could still be partially present in the medium. Therefore, as comparison, we added B-27 directly in the CM obtained with DPCs cultured without any supplement (DPC-CM *post* B-27). We used neurobasal medium with B-27 only as negative control (C-). And neurogenic medium containing BDNF, NGF-ß, NT-3 (10 ng/ml each), and B-27 served as positive control (C+). Thus, all experiments were conducted with these four conditions: DPC-CM *pre* B-27, DPC-CM *post* B-27, C-, and C+.

2.4. Flow cytometry analysis

DPCs were collected and analyzed by flow cytometry before and after conditioned medium collection, named as follow: (1) DPCs Control for cells collected when they reached 80% confluence before starting medium conditioning, (2) DPCs after CM for cells collected after medium conditioning performed in the absence of B-27, and (3) DPCs after B27-CM for cells collected after medium conditioning carried out in the presence of B-27 (DPCs grown with B-27 and which gave the optimized DPC-CM). The antigen profiles of these cells were analyzed by detecting the expression of the cell surface markers CD90, Stro-1, CD117 using flow cytometry. CD90 is a widely accepted marker for mesenchymal stem cells, Stro-1 is the best-known mesenchymal stem cell marker, and CD117 is the receptor of stem cell factor. An analysis of 15,000 events was performed in a Gallios B01751 cytometer (Beckman-Coulter). Data were analyzed with the FCS Express software.

2.5. Primary sensory neurons isolation and culture

All animal protocols were approved by the national ethics committee and all procedures were performed following relevant institutional guidelines and regulations. Adult Swiss mice (6 to 10-week-old, CERJ, Le Genest St Isle, France) were sacrificed by CO_2 inhalation followed by cervical dislocation, and their DRG were then removed. Ganglia were successively treated by two incubations with collagenase A (1 mg/ml, Roche Diagnostic, France) for 45 min each (37°C) and then with trypsin-EDTA (0.25%, Sigma, St Quentin Fallavier, France) for 30 min. A mechanical dissociation was performed in a neurobasal medium supplemented with 10% fetal bovine serum and DNase (50 U/ml, Sigma). Isolated cells were collected then by centrifugation and suspended in neurobasal supplemented with 2% B-27, % glutamine, 1% Penicillin-Streptomycin. Dissociated neurons were plated on D,L-polyornithine (0.5 mg/ml)-laminin (5 mg/ml)-coated glass coverslips, and incubated in an incubator with a 5% CO_2 atmosphere. The culture medium was carefully replaced 4 hours later, according to the experiment, by DPC-CM or unconditioned medium for control.

2.6. Immunocytochemistry

After 24 hours of culture, neurons were fixed at room temperature in 4% paraformaldehyde in PBS for 20 minutes, washed two times with PBS, and blocked using PBS, 10% of donkey serum, and 0.1% of Triton x100. Then, cells were washed twice and incubated with primary antibodies against rat monoclonalβ-tubulin III (1:1000, ab6160, Abcam) overnight at 4°C. Cells were washed three times again, 10 min each, and incubated with Donkey anti-rat Alexa 488 secondary antibodies (1:500, ab150153, Abcam) for 1 h, in dark at room temperature. Primary and secondary antibodies were diluted in PBS, 1% of donkey serum (GeneTex, Irvine, CA, USA) and 0.01% of Triton x100. Next, cultures were washed three times, 10 min each, and were counterstained with 4,6-Diamidino-2-Phenylindole, Dilactate (DAPI, 1:1000, Sigma). Glass slides were mounted in prolong mounting media, and preparations were cured overnight at +4°C protected from light until microscopy analysis.

2.7. Neurites length measurements

A microscope slide scanner (ZEISS Axio Scan.Z1) was used to scan immunostained glass slides. A 20x objective scan image was obtained for each glass slide, containing tens of neurons. Then, neuron images were separately obtained using the Zen® acquisition software, neurites extensions of each cell were traced and the length of all the neurites per neuron was measured manually by NeuronJ plugin for ImageJ analyzing software. All cells were considered, except neurons presenting neurites connected to adjacent neurons (for technical reasons). Each experimental condition was replicated in four wells (four glass slides) per mouse. A total of 3898 neurons was measured throughout this study.

2.8. Bicinchoninic Acid Assay (BCA)

The total protein content of DPC-CM samples was determined using the Pierce® BCA Protein Assay Kit (Thermo Fisher) in 96-well plates. The assay mixture contained 200 µl of the reagent (solution A + B) and 20 µl of a sample containing either CM or bovine serum albumin standard. Absorbance was read at 540 nm using an Infinite 200 plate reader.

2.9. Growth factors array for DPC-CM

The profiles of DPC-CM were screened with Human Growth Factor Antibody Array (40 Targets) – Quantitative (ab197445 Abcam), following the manufacturer's protocol. The slide was then scanned using an InnoScan 300 Microarray Scanner (Innopsys, Carbonne, France). Data extraction and quantification of signal intensities were performed using Mapix software (Innopsys, France). Data analysis was done with GraphPad Prism (GraphPad Software, La Jolla California USA).

2.10. Statistical analyses

Statistical analyses were performed using SigmaPlot version 11.0 (Systat Software, Inc., San Jose California USA). Multiple group comparisons were performed by analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Comparisons between two groups were performed by Student's t-test. $P < 0.05$ was considered statistically significant. Data were presented as mean ± standard deviation (SD).

3. Results

3.1. Cell passage number and conditioning period impacts the quantity of secreted proteins

We first assessed the efficiency of our protocol to recover secreted proteins, by comparing total protein concentration in DPC-CM to that without cell conditioning. BCA test was used to determine protein concentration in the supernatant of various culture media. As expected, protein concentration in DPC-CM was significantly higher compared to the neurobasal medium (402 ± 14µg/ml vs. 52 ± 28µg/ml) (Figure 1a). The number of cell passage significantly affect the concentration of protein: 438 ± 30 µg/ml with DPCs at the 3rd passage vs. 330 ± 27 µg/ml with DPCs at the 5th passage (Figure 1b). The impact of secretion duration was also investigated; protein concentration increased significantly and markedly over the first two days, to reach 392 ± 16 µg/ml after 48 h. The kinetics of factors secretion (for up to three days) revealed higher levels of secreted factors over time but did not reveal any significant difference following 48 h of conditioning (Figure 1c).

3.2. DPC-CM concentration influences sensory neuron outgrowth

After 24 hours in culture, neuron growth was mostly in stellar morphology with many ramifications. Neurons were cultured in neurobasal medium complemented or not with either 50%, 75% DPC-CM or only in DPC-CM. After fixation and immunostaining, slides were scanned and neuron ramifications were measured using NeuronJ. Results showed a dose-dependent effect of DPC-CM on neurites outgrowth. Neurites length per neuron increased from 1018 ± 157 µm (135 neurons from 3 independent experiments) without DPC-CM to 4128 ± 179 µm (89 neurons from 3 independent experiments) with 100% DPC-CM (Figure 2). Therefore, DPC-CM was used directly without any prior dilution for all the next experiments.

3.3. The effect of DPC-CM on neurite outgrowth is reproducible between donors

Next, we sought to determine the reproducibility of our protocol between donors. We harvested DPC-CM from 3 different donors (15-23 years old) following the protocol previously described, i.e 4th passage at 80% confluency at the beginning of conditioning.

We first showed that secreted protein concentration did not statistically differ between DPC-CM obtained from the three different donors (Figure 3a).

Conditioned medium were also tested on primary sensory neurons isolated from 3 different mice: neurons isolated from the various mice were cultured in DPC-CM and neurobasal as a negative control. No statistically significant difference could be observed between mice (isolated neurons) when considering neurite length per neuron, while a significant impact of DPC-CM was always present compared to neurobasal (Figure 3b).

Taken together, these results showed no significant impact of DPC donors on the efficiency of their secretomes. However, to avoid variability in our study, we decided to continue the experiments with CM produced from a single donor.

3.4. 48 hours of DPC conditioning is optimal for effective activity of DPC-CM

The impact of the DPC conditioning period on CM efficiency was investigated. Neurons were cultured in DPC-CM harvested after 48 or 72 hours. Extending the conditioning time by one more day does not improve the effect of DPC-CM (3028.5 ± 358 µm, 86 neurons vs 3238.4 ± 328.3 µm, 100 neurons for 48h-CM and 72h-CM respectively) (Figure 3c).

3.5. Frozen storage does not influence the regenerative properties of DPC-CM

Next, we determined whether the storage conditions of DPC-CM might influence their regenerative capacities. Neurons were cultured with either freshly harvested DPC-CM or the same DPC-CM frozen at -20°C for a few hours. Frozen storage of DPC-CM did not affect its positive effect on total neurite length per neuron (Figure 3d). Therefore, for the next experiments, multiple volumes of DPC-CM were prepared at once, aliquoted, and frozen until use (after one month).

3.6. Impact of DPC culture with B-27 supplement on the neurogenic potential of DPC-CM

Further, we investigated whether the culture of DPCs with B-27 culture supplement could influence the neurogenic potential of DPC-CM. We, therefore, compared DPC-CM *pre* B-27 with DPC-CM *post* B-27, a neurogenic medium containing BDNF, NGF-ß, NT-3, and

B-27 (C+), and the neurobasal medium containing B-27 only (C-). We observed that CM was more effective when B-27 was added to DPCs before conditioning than when it was added after CM production: 2714 ± 97 µm (809 neurons) vs 1630 ± 95 µm (883 neurons) for DPC-CM *pre* B-27 and DPC-CM *post* B-27, respectively. Both CM were more effective than C- (1147 ± 11 µm, 727 neurons) but less effective than C+, which induced the longest neurites (3563 ± 115 µm, 681 neurons) (Figure 4).

3.7. DPCs produce a complex combination of neurotrophic and growth factors

We aimed at identifying the secreted factors that potentially promote the neurite outgrowth effect of DPC-CM in sensory neurons. An antibody arrays test, which targets 40 factors, was performed for the CM obtained from DPCs cultured in the presence or absence of B-27 supplement. A total of 34 factors was above the detection threshold.

The expression levels of NT-3, PDGF-AA, HGF, IGFBP (1-6), EGF R, OPG, and VEGF were significantly higher in CM obtained from DPCs cultured with B-27 supplement. GDF-15, SCF R, and Insulin were significantly detected only in this CM. However, some factors (BMP-7, FGF-7, and IGF-1) were significantly higher when DPCs were cultured without B-27, and FGF-4, GH, and VEGF-D were significantly detected only in that CM. This total of factors is involved in cellular proliferation and migration, neurogenesis, neuroprotection, angiogenesis, and osteogenesis (Table 1). The levels of other factors were not significantly modified by the presence of B-27 during the cell conditioning, or they were below the detection limit. The results are summarized in Figure 5.

3.8. Impact of DPC culture with B-27 supplement on cell profiles

We analyzed and compared dental pulp cell profiles before (DPCs control) and after medium conditioning in the presence (DPCs after B-27 CM) or not (DPCs after CM) of B-27 supplement. We obtained similar profiles for the 3 conditions, with 94 to 97% CD90+ cells, 2 to 4% Stro-1+ cells and 45 to 50 % CD117+ cells (Figure 6). This indicates that the addition of B-27 supplement to dental pulp cells did not significantly alter the type of cells present in the culture, although it influenced the release of growth factors from these cells.

4. Discussion

Previous studies have shown that dental pulp stem cells significantly enhance axon regeneration, with neuroprotective effects on DRG neurons (Kolar et al., 2017). Dental pulp stem cells release neurotrophic factors that enhance neurite guidance, promote neuronal growth both *in vivo* and *in vitro*, stimulate rescue survival of neurons, and induce neurogenesis at the site of injury (Sultan et al., 2019). The field of paracrine-mediated processes involving secreted trophic factors is increasingly studied, with a specific interest in optimizing neurotrophic factors production (Mead et al., 2017). Altering DPC culture conditions to prime and/or to predifferentiate the cells is a way to improve secreted factors production. Thus, it has been demonstrated that, following predifferentiation into Schwann-like glial cells, dental pulp stem cells secreted significantly more neurotrophins and were able to further stimulate neurite outgrowth in an *in vitro* model of spinal cord injury as compared to nondifferentiated cells (Martens et al., 2014). In another study, dental pulp stem cell stimulation with neuregulin1-β1, basic fibroblast growth factor, platelet-derived growth factor, and forskolin significantly increase protein levels of neurotrophic factors compared to unstimulated controls (Kolar et al., 2017). In this work, we defined the optimal preconditioning of DPCs to enhance neurites outgrowth of DRG sensory neurons. B-27 used to stimulate DPCs, is the most cited neuronal cell culture supplement and it is serum-free. While its composition has been published (Brewer et al., 1993), the exact concentrations of its components are not known (Sünwoldt et al., 2017). B-27 is commercially available as GMP-grade and has been already used in clinical-scale cell productions (Chimenti et al., 2014), which does not alter the GMP character of our CM.

The levels of DPC secreted factors, in our study, are similar to that of many other studies that show a neuroregenerative potential of mesenchymal stem cell-conditioned medium: NGF, BDNF, NT-3, with concentration levels varying between 0 and 70 pg/ml (Sugimura-Wakayama et al., 2015). However, differences in CM preparation procedures may explain why some factors present in CM of some studies are not present in ours and inversely. Some studies used fetal bovine serum or other supplements as human platelet lysate, while we used serum-free media (Pawitan, 2014). The washing step before adding a serum-free medium is important to remove any trace of the serum. Moreover, mesenchymal stem cells

might be cultured in different kind of basal medium, which affect their secretory potential (Sagaradze et al., 2019). Furthermore, in our study, we did not concentrate CM before use.

A great variety of extracellular signals are already known to induce axon growth. For instance, a family of peptide trophic factors called Neurotrophins, which in mammals include NGF, BDNF, NT-3, and NT-4/5, has been thoroughly studied (Goldberg, 2003). The effects of neurotrophins on neuronal outgrowth have been well described in different types of neuron populations in both the central (Tucker et al., 2001; Goldberg et al., 2002; O'Donovan, 2016; Martins et al., 2017) and the peripheral nervous system (Tuttle and Matthew, 1995; Kimpinski et al., 1997; Tucker et al., 2001). The DRG sensory neurons from adult mice in primary culture express the cognate receptors of the neurotrophins NGF, BDNF, NT4, and NT3, which are members of the tropomyosin-related kinase (TrkA, B, and C) receptor tyrosine kinase family and probably account for part of CM effects (Barbacid, 1994).

In addition to neurotrophins, some other factors like VEGF (Sondell et al., 2000), HGF (Maina et al., 1997), IGF-1 (Xiang et al., 2011) have been known for their neurotrophic action and shown to promote DRG neurites growth. The results of microarrays suggest that neurotrophins might not be the only effective growth factors on DRG sensory neurons, since they are almost present equally in CM with and without stimulation of DPCs, except for NT-3 which increased significantly with stimulated DPCs, but it acts only on 10% of DRG neurons.

Other than HGF and VEGF, various factors present in stimulated DPC-CM may be involved in its promoted neuro-potential. Further studies are needed to confirm whether this effect is attributed to the release of these factors, not yet studied for this effect, such as IGFBP (3-6), GDF-15, PDGF-AA…

Other studies investigating mesenchymal stem cells secretome effect on neurites growth predicted as well the existence of undetermined factors responsible for the neurite outgrowth, other than the well-known neurotrophic factors (Nakano et al., 2010; Miura-Yura et al., 2019). Park et al. asked whether this effect is attributed to the release of paracrine acting factors, such as IGFBP-4 and -6, secreted at high levels by stimulated human mesenchymal stem cells (Park et al., 2010). IGFBP-6 is already indicated as an important neuronal survival factor secreted from human mesenchymal stem cells (Jeon et

al., 2017), but its potential for neurites growth is not yet studied. Additional work must be done to determine the factors secreted by stimulated DPCs that are responsible for this regenerative effect.

5. Conclusion

In this study, we demonstrate that DPC-CM enhances axonal outgrowth of primary sensory DRG neurons *in vitro*. We identified several growth-promoting factors in the secretome of DPCs and we show that the B-27 supplement drastically changes the secretome's profile, further stimulating neurite outgrowth. Importantly, our work points towards promising avenues for the application of dental pulp stem cell conditioned media to aid neuronal regeneration.

6. Funding

This research was possible thanks to Lebanese University for the research award granted to Ph.D. student Batoul Chouaib. The work was mainly supported by the association *Fondations des "Gueules Cassées"*.

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8. Graphical abstract

Dental pulp cell-conditioned medium (DPC-CM) promotes neurites outgrowth of sensory neurons. Conditioning of DPCs with the B-27 supplement enhances significantly the neuroregenerative effect of their secretome, called "optimized DPC-CM". Analysis of the altered composition of the optimized secretome identifies candidate factors that can potentially promote this promising regenerative feature of DPC-CM.

9. Figure captions

Figure 1: Total protein concentration in DPC-CM: (a) DPC-CM compared to the basal medium. (b) CM obtained from DPCs at passage P3, P4, and P5. (c) CM obtained after 6, 24, 48, and 72 hours of conditioning with DPCs. Data are presented in µg/ml as mean ± SD. ***P < 0.001, **P < 0.01 and *P < 0.05 indicate significance between conditions as determined by two-tailed Student's t-tests for (a) and one-way ANOVA followed by Bonferroni post hoc test for (b) and (c).

Figure 2: Effect of DPC-CM on neurites growth: (a) After 24 h of incubation, DRG neurons were fixed and stained with DAPI (blue) or βIII-Tubulin (green), then neurites length of each neuron was measured with NeuronJ. (b) Neurites outgrowth of dorsal root ganglion (DRG) neurons when cultured with neurobasal, 50% DPC-CM + 50% neurobasal, 75% DPC-CM + 25% neurobasal and 100% DPC-CM. (c) Box plot diagram presenting the quantitative analyses for neurite outgrowth of DRG neurons. ***P < 0.001 indicates significance from other CM concentrations and **P<0.01 indicates significance between indicated concentrations, as determined by two-way ANOVA followed by Bonferroni post hoc test. The results represent the mean of triplicate cultures of three independent experiments; n= 3 mice.

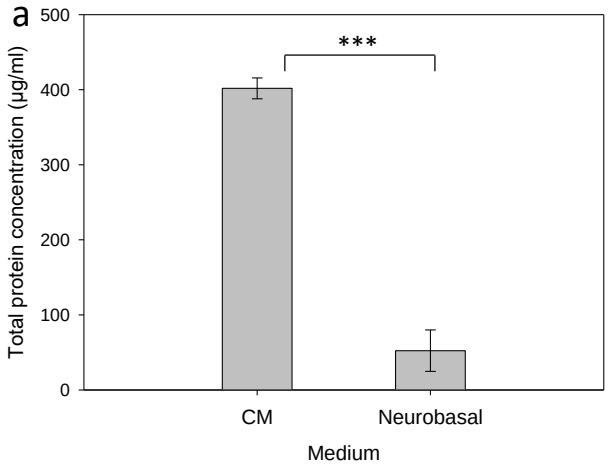
Figure 3:(a) Total protein concentration in DPC-CM obtained from 3 different donors. Results are the Means ± SD. (b) Impact of the recipient on the effect of CM. DRG neurons of three mice (M1, M2, and M3) were treated with unconditioned or DPC-conditioned medium. (c) Effect of time conditioning elongation (72 hours compared to 48 hours) on neurites length. (d) Effect of frozen DPC-CM on neurites length. The results in (c) and (d)

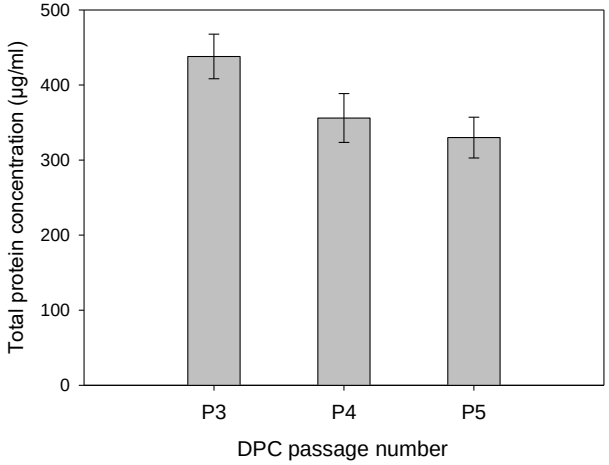
represent the mean of triplicate cultures of two independent experiments; n= 2 mice. Box plot diagrams in (b), (c), and (d) presenting the quantitative analyses for neurite outgrowth of DRG neurons. ***P< 0.001 in (b) indicate significance between CM and control for each mouse as determined by one-way ANOVA followed by Bonferroni post hoc test. One-way Anova and two-ways Anova tests were used in (a) and (c; d) respectively.

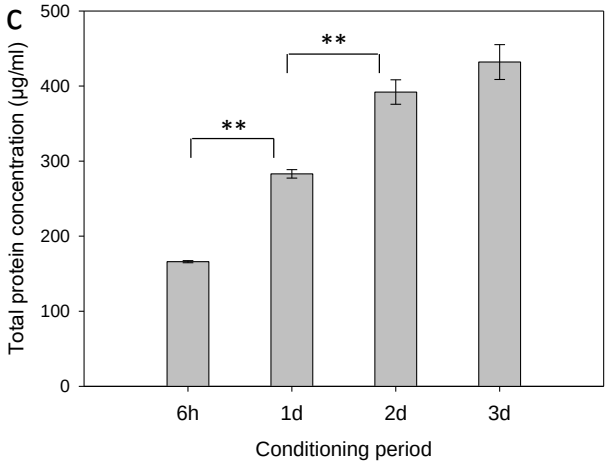
Figure 4: (a) Illustration of neurites outgrowth from DRG neurons treated with the unconditioned neurobasal medium but supplemented with B-27 (C-); CM obtained from DPCs cultured with media containing B-27 (DPC-CM Pre B-27), DPC-CM where B-27 was added only following conditioning (DPC-CM Post B-27); Neurobasal containing B-27 and neurotrophic factors served as a positive control (C+). (b) Box plot diagram presenting the quantitative analyses for neurites outgrowth of DRG neurons with these different mediums. ***P < 0.001 indicates significance from other treatments and **P<0.01 indicates significance between indicated treatments, as determined by two-way ANOVA followed by Bonferroni post hoc test. The results represent the mean of quadruplicate cultures of six independent experiments; n= 6 mice.

Figure 5: Quantitative Antibody microarray analysis of 40 human growth factors in CM obtained from DPCs cultured with (black bars) or without (gray bars) B-27 supplement. The array was scanned, and the intensities of signals were quantified. The expression levels of growth factors are presented in pg/mL as Means ± SD. ***P < 0.001, **P<0.01, *P<0.05 indicate significance between both CM, as determined by two-tailed Student's t-test.

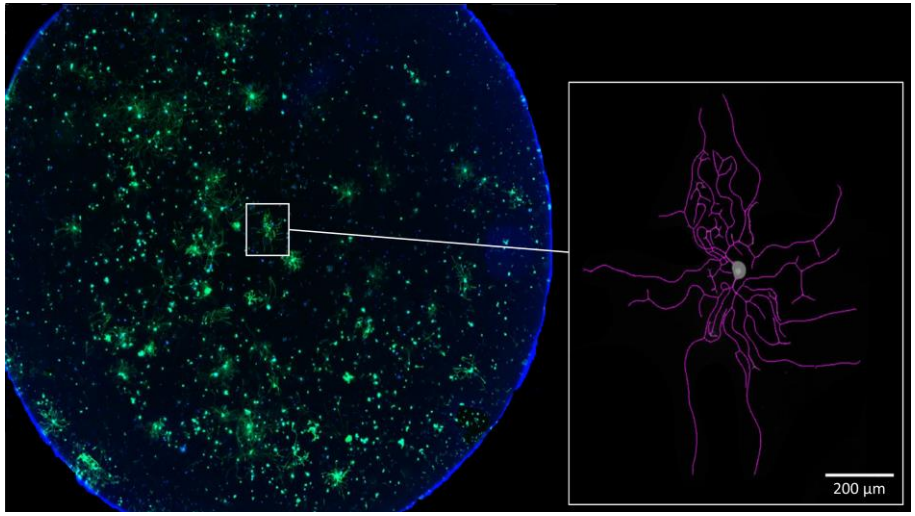
Figure 6: Flow cytometry analysis of dental pulp cells before (DPCs control) and after medium conditioning in the presence (DPCs after B-27 CM) or not (DPCs after CM) of B-27 supplement. The histograms showing the expression of markers CD 90, Stro-1, and CD 117.


a
Total protein concentration (µg/ml)
500
400
300
200
100
0
***
CM
Neurobasal
Medium

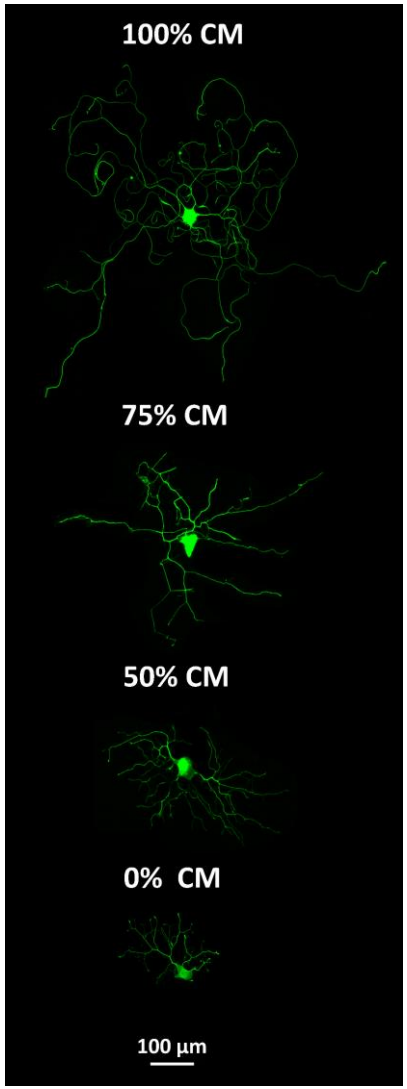

Total protein concentration (µg/ml)
500
400
300
200
100
0
P3
P4
P5
DPC passage number


c
Total protein concentration (µg/ml)
500
400
300
200
100
0
**
**
6h
1d
2d
3d
Conditioning period

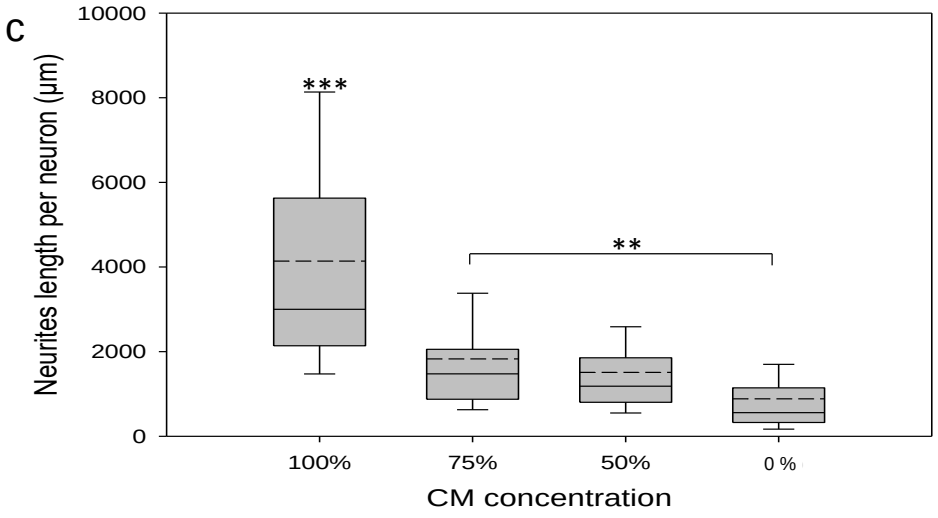
a

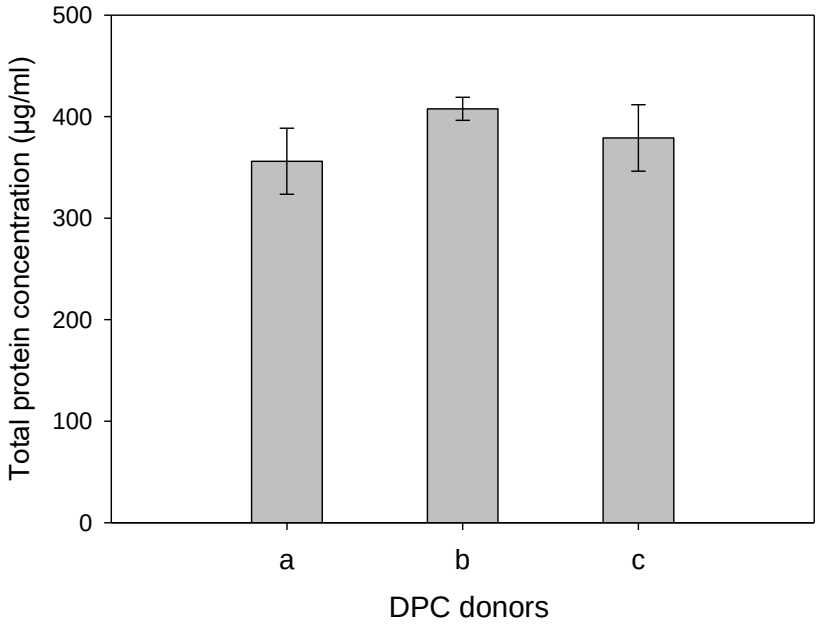

200 µm

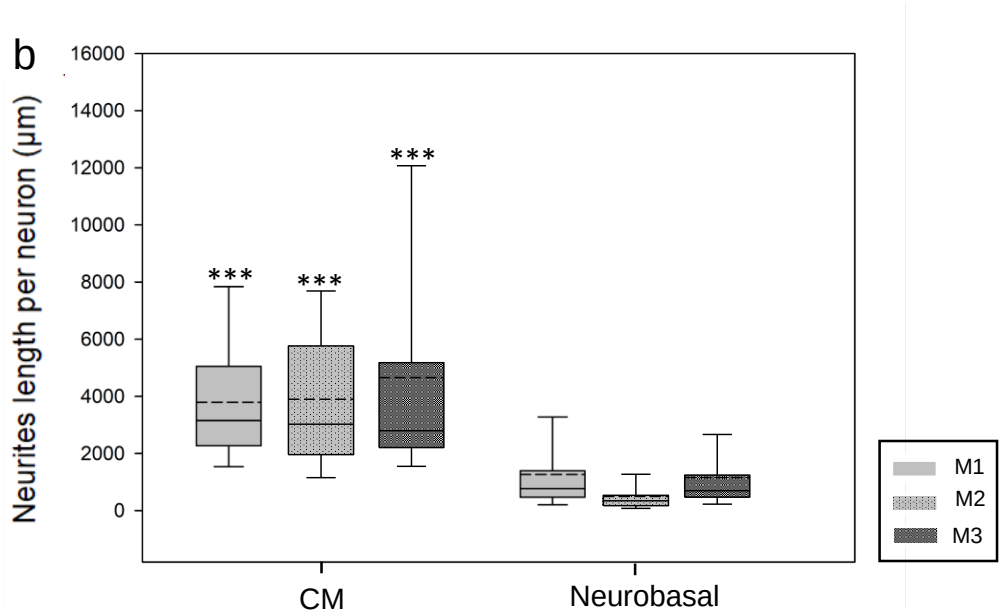
b

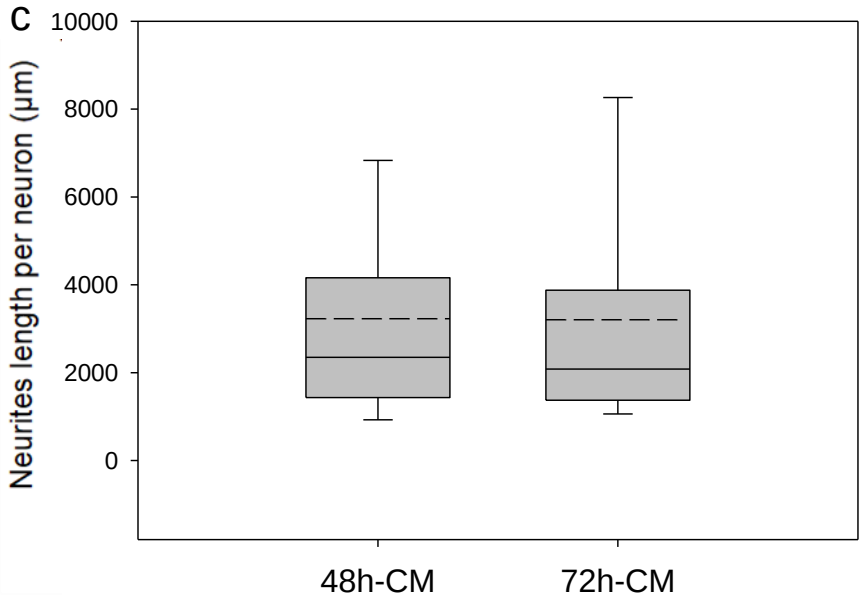

100% CM
75% CM
50% CM
0% CM
100 µm

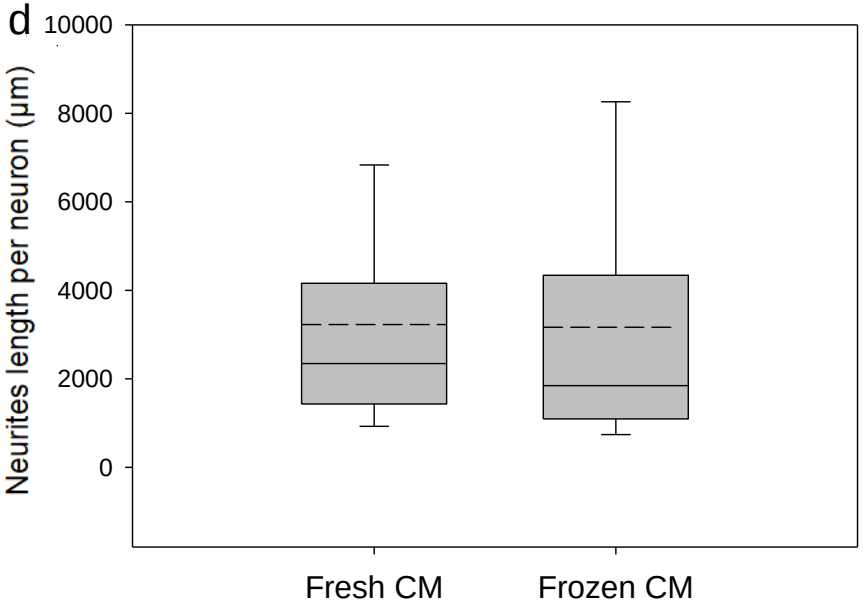
c


Neurites length per neuron (µm)
10000
8000
6000
4000
2000
0
***
**
100%
75%
50%
0 %
CM concentration

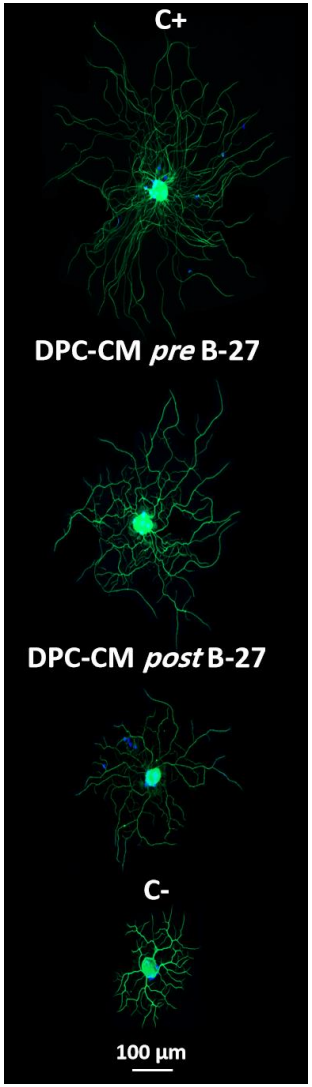

Total protein concentration (µg/ml)
500
400
300
200
100
0
a
b
c
DPC donors


b
Neurites length per neuron (µm)
16000
14000
12000
10000
8000
6000
4000
2000
0
***
***
***
CM
Neurobasal
M1
M2
M3

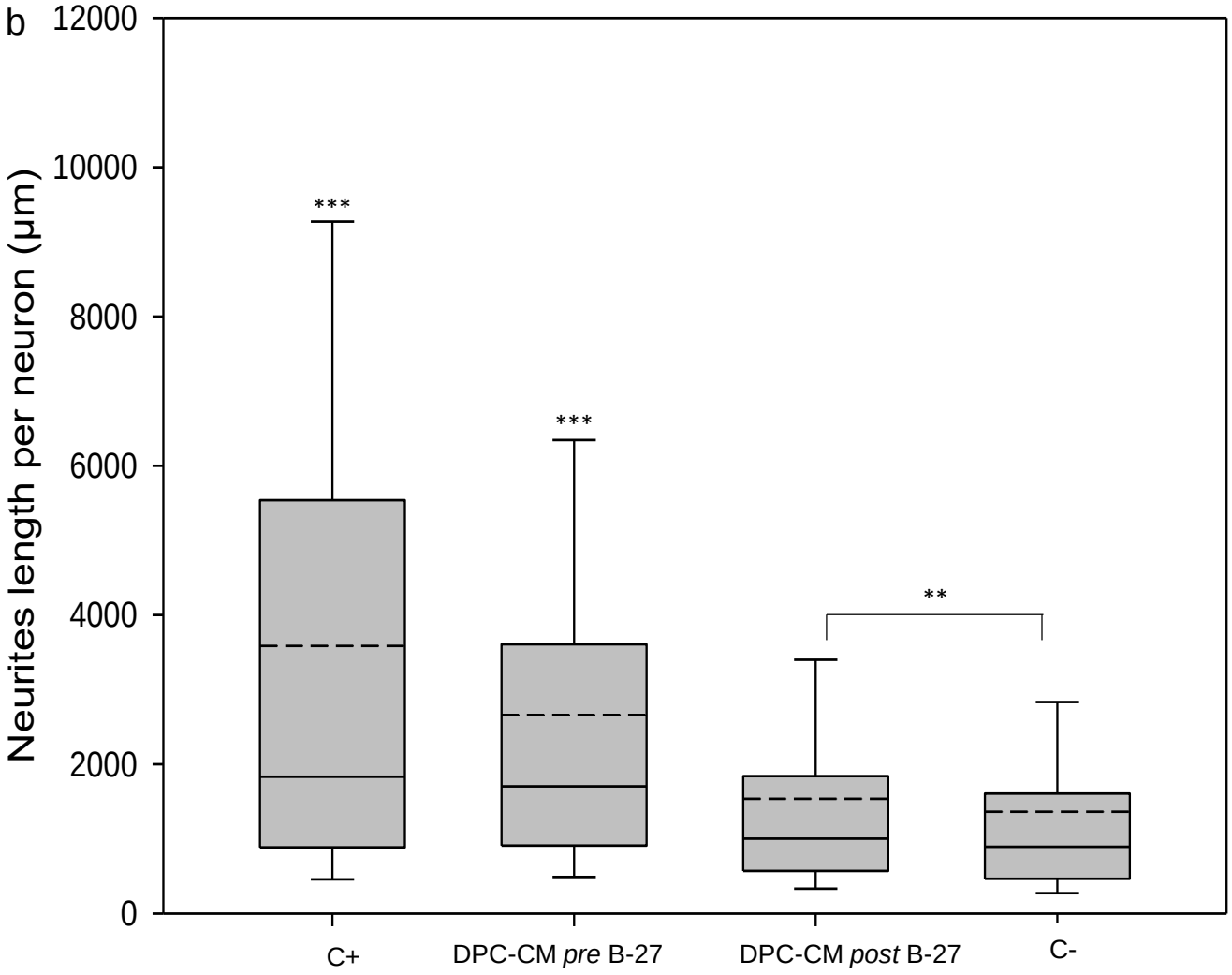

c
Neurites length per neuron (µm)
10000
8000
6000
4000
2000
0
48h-CM
72h-CM

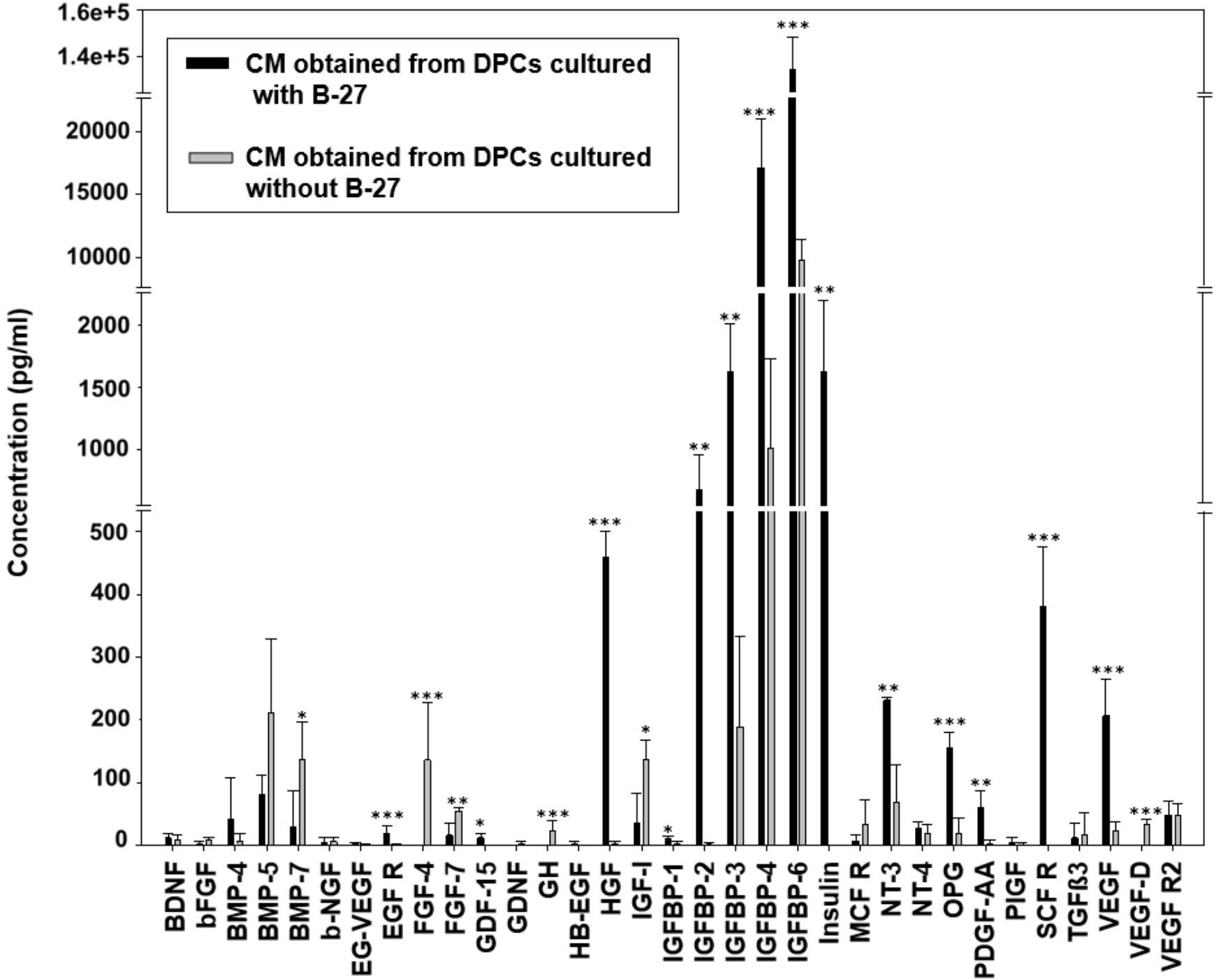

d
Neurites length per neuron (µm)
10000
8000
6000
4000
2000
0
Fresh CM
Frozen CM

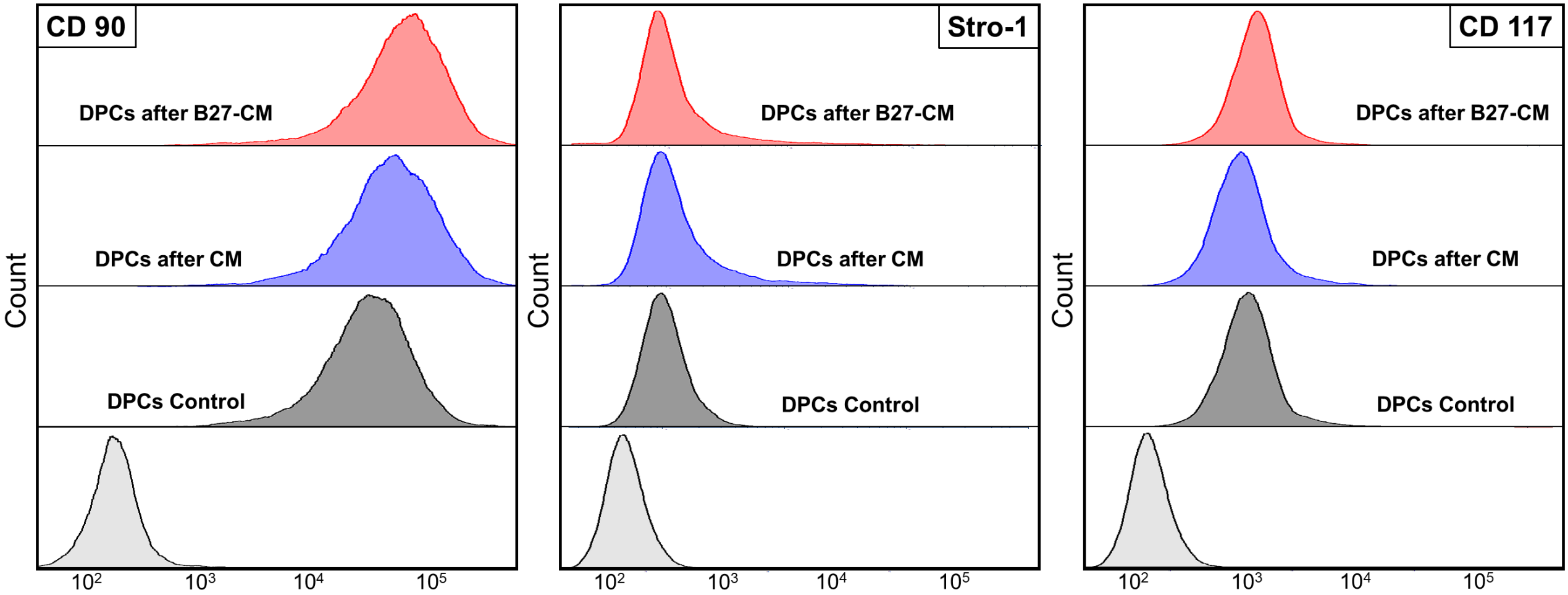
a


C+
DPC-CM *pre* B-27
DPC-CM *post* B-27
C-
100 µm

b

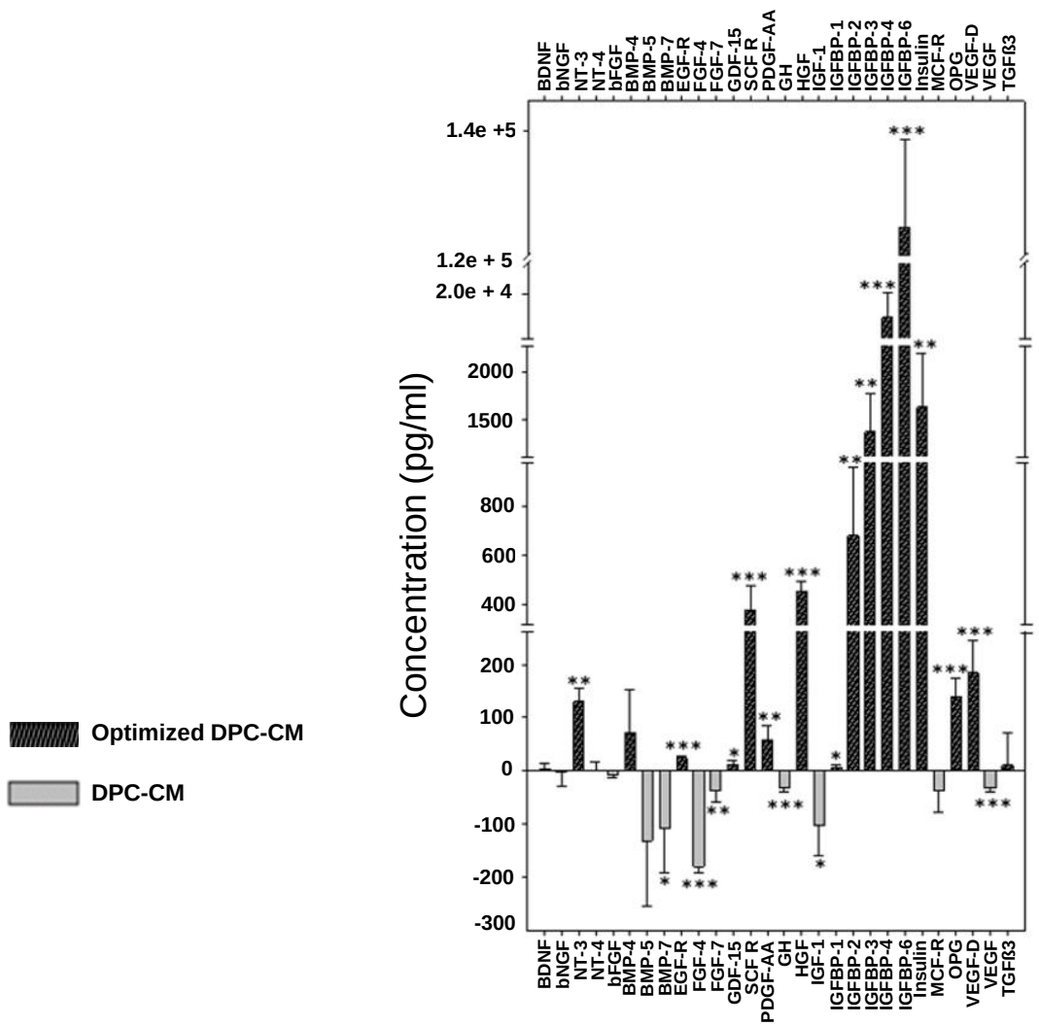

Neurites length per neuron (µm)
12000
10000
8000
6000
4000
2000
0
***
***
**
C+
DPC-CM *pre* B-27
DPC-CM *post* B-27
C-


CM obtained from DPCs cultured with B-27
CM obtained from DPCs cultured without B-27
Concentration (pg/ml)
1.6e+5
1.4e+5
20000
15000
10000
2000
1500
1000
500
400
300
200
100
0
BDNF
bFGF
BMP-4
BMP-5
BMP-7
b-NGF
EG-VEGF
EGF R
FGF-4
FGF-7
GDF-15
GDNF
GH
HB-EGF
HGF
IGF-I
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-6
Insulin
MCF R
NT-3
NT-4
OPG
PDGF-AA
PIGF
SCF R
TGFß3
VEGF
VEGF-D
VEGF R2


CD 90
DPCs after B27-CM
DPCs after CM
DPCs Control
Count
10^2
10^3
10^4
10^5
Stro-1
DPCs after B27-CM
DPCs after CM
DPCs Control
Count
10^2
10^3
10^4
10^5
CD 117
DPCs after B27-CM
DPCs after CM
DPCs Control
Count
10^2
10^3
10^4
10^5

Growth factors (pg/mL)	Functions
NT-3	Neurotrophins
BMP-7	BMPs induce the formation of both cartilage and bone (Chen et al., 2004)
EGF-R	EGF-R activation mediates inhibition of axon regeneration (Koprivica et al., 2005)
FGF-4	FGF4 induces cell proliferation (Tanaka et al., 1998) (Bosetti et al., 2007) and has angiogenic properties (Yun et al., 2010)
FGF-7	FGF7 induces cell growth (Finch et al., 1989; Tagashira et al., 1997; Thomson and Cunha, 1999), migration (Tsuboi et al., 1993; Tagashira et al., 1997), and differentiation (Werner et al., 1993)
GDF-15	GDF15 is a stress-induced cytokine released in response to tissue injury (Emmerson et al., 2018)
SCF R	SCF induces the outgrowth of c-kit-positive neurites from DRGs (Hirata et al., 1993). 20% of all DRG neurons expressed c-Kit (SCFR) (Milenkovic et al., 2007)
PDGF-AA	PDGF-AA may function to regulate bone formation (Sulzbacher et al., 2000). PDGF-AA myelinates nerve fibers throughout the CNS (Andrae et al., 2008). PDGF-AA is important for neuroprotection (Mead et al., 2014)
GH	GH promotes axon growth (Baudet et al., 2009; Bianchi et al., 2017)
HGF	HGF cooperates with NGF to enhance axonal outgrowth from cultured DRG neurons (Maina et al., 1997)
IGF-1	IGF-1 promotes neurite outgrowth of DRG neurons (Xiang et al., 2011; Zhu et al., 2018)
IGFBP-1	IGFBPL1 promotes axon growth (Guo et al., 2018)
IGFBP-2	IGFBP-2 participates in some aspect of axonal growth (Wood et al., 1990)
IGFBP-3	IGFBP-3 has a role in cell death and survival in response to a variety of stimuli (Baxter, 2013)
IGFBP-4	IGFBP-4 was shown to inhibit IGF1 action (Rao and Pearse, 2016)
IGFBP-6	IGFBP-6 is an important neuronal survival factor secreted from human MSCs (Jeon et al., 2017). The BP6 labeled cells represent approximately only 10%–20% of the total neuronal population in a DRG (Naeve et al., 2000)
Insulin	Insulin receptor signaling has a role in regulating neurite growth (Govind et al., 2001; Choi et al., 2005)
OPG	OPG inhibits osteoclastogenesis and bone resorption (McCarthy et al., 2009; Gutierrez et al., 2013). It prevents the neurite growth-inhibitory signal in sympathetic and sensory neurons (Gutierrez et al., 2013)
VEGF	VEGF is an angiogenic factors (Shibuya, 2011). It stimulates axon outgrowth from DGR (Sondell et al., 2000)
VEGF-D	VEGF-D can control the length and complexity of dendrites (Stacker and Achen, 2018)

Table 1: Physiological effects of the human growth factors in DPC-CM, significantly modified when DPCs were cultured with B-27 supplement.


Optimized DPC-CM
DPC-CM
Concentration (pg/ml)
1.4e +5
1.2e + 5
2.0e + 4
2000
1500
800
600
400
200
100
0
-100
-200
-300
BDNF
bNGF
NT-3
NT-4
bFGF
BMP-4
BMP-5
BMP-7
EGF-R
FGF-4
FGF-7
GDF-15
SCF R
PDGF-AA
GH
HGF
IGF-1
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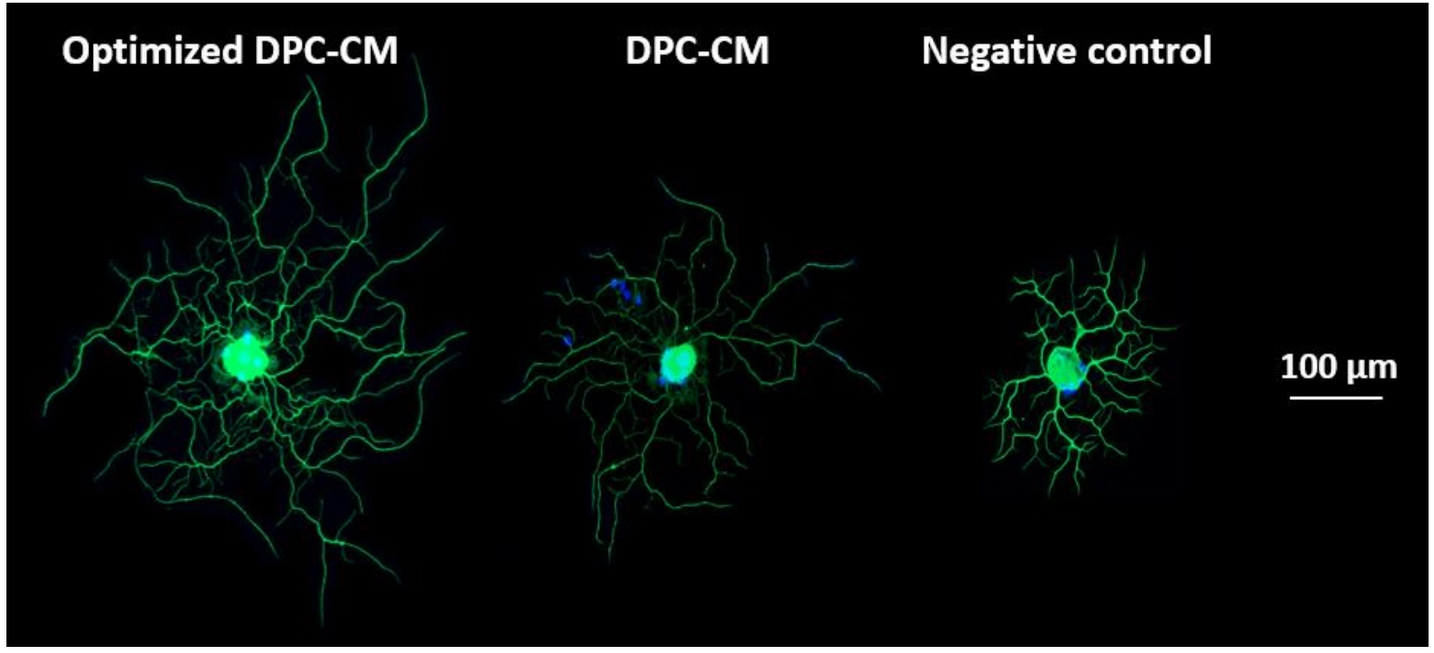
Insulin
MCF-R
OPG
VEGF-D
VEGF
TGFß3


Optimized DPC-CM
DPC-CM
Negative control
100 µm