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Connect Tissue Res. Author manuscript; available in PMC 2023 July 01.

Published in final edited form as:

Connect Tissue Res. 2022 July ; 63(4): 339–348. doi:10.1080/03008207.2021.1924696.

C5a Complement Receptor Modulates Odontogenic Dental Pulp Stem Cell Differentiation Under Hypoxia

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Abstract

Aim: Alterations in the microenvironment change the phenotypes of dental pulp stem cells (DPSCs). The role of complement component C5a in the differentiation of DPSCs is unknown, especially under oxygen-deprived conditions. The aim of this study was to determine the effect of C5a on the odontogenic differentiation of DPSCs under normoxia and hypoxia.

Material and Methods: Human DPSCs were subjected to odontogenic differentiation in osteogenic media and treated with the C5a receptor antagonist-W54011 under normal and hypoxic conditions (2% oxygen). Immunochemistry, western blot, and PCR analysis for the various odontogenic differentiation genes/proteins were performed.

Results: Our results demonstrated that C5a plays a positive role in the odontogenic differentiation of DPSCs. C5a receptor inhibition resulted in a significant decrease in odontogenic differentiation genes, such as DMP1, ON, RUNX2, DSPP compared with the control. This observation was further supported by the Western blot data for DSPP and DMP1 and immunohistochemical analysis. The hypoxic condition reversed this effect.

Conclusions: Our results demonstrate that C5a regulates the odontogenic DPSC differentiation under normoxia. Under hypoxia, C5a exerts a reversed function for DPSC differentiation. Taken together, we identified that C5a and oxygen levels are key initial signals during pulp inflammation to control the odontogenic differentiation of DPSCs, thereby, providing a mechanism for potential therapeutic interventions for dentin repair and vital tooth preservation.

Keywords

C5a; C5a receptor; DPSC; differentiation; odontoblast; hypoxia

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Disclosure statement

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article. I affirm that I/We have no financial affiliation, or involvement with any commercial organization with a direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed.

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Introduction

Stem cells have gained interest in the scientific community due to their astonishing ability in tissue regeneration and modulating potential inflammatory responses[1]. Similarly, regenerative dentistry is an emerging field of medicine involving dental and maxillofacial sciences[2]. Currently, among several strategies, root canal therapy is a typical treatment for permanent teeth with chronic inflammation or necrosis of pulp tissue. The regenerative ability of stem cells promises their translational potential for repairing damaged teeth and dentin[3, 4]. Human dental pulp stem cells (DPSCs) can differentiate into odontoblast-like cells that produce dentin and growth factors[5, 6]. Several factors, such as scaffolds, growth factors, and microenvironment play important roles in modulating stem cell differentiation, migration, and proliferation. Alterations in the microenvironment alters the phenotypes of stem cells. Specifically, oxygen has been well documented to influence stem cell differentiation[7-10]. Many laboratories use 20% oxygen for *in vitro* tissue culture. However, the natural microenvironment inside the body has a considerably lower oxygen concentration, ~3% inside the tissue[11]. Thus, careful evaluation of oxygen concentration effects on stem cell differentiation *in vitro* needs to be performed.

Certain pro-inflammatory mediators are actually helpful in tissue regeneration and bone healing in a choreographed and regulated manner by the body, while inflammatory mediators also contribute to the microenvironment[12]. However, the continued inflammatory response may also cause suboptimal tissue regeneration, therefore, it must be regulated and well managed. Several therapeutics can help in early diagnosis[13] and it can be treated, whereas natural compounds could be an option as an alternative therapy compare with synthetic NSAID's[14]. Complement system activation in pulp has been suggested to provide a connection between pulp inflammation and its regeneration ability. The complement active fragment C5a recruits pulp progenitor cells to the injured pulp[15]. We have demonstrated its critical roles in pulp fibroblast-mediated nerve regeneration under caries-like inflammation. Our previous studies[16, 17] demonstrated that the complement fragment C5a, released after complement activation by Lipoteichoic acid (LTA)-treated pulp fibroblasts, governs the production of brain-derived nerve growth factor (BDNF). This BDNF secretion promotes neurite outgrowth towards the carious injury site. Spagnuolo et al.,[1] have summarized that DPSCs present a higher tendency towards odontoblastic differentiation compared with MSCs, and the presence of un-treated pulp-periodontal infection does not preclude the possibility of finding resident DPSCs.

We now extend these observations in the DPSC odontogenic differentiation to further identify C5às role in this process, especially under oxygen-deprived conditions. Here, we evaluated the role of C5a in odontogenic differentiation of human DPSCs and further identify the effects of hypoxia on C5a-mediated DPSC differentiation.

Materials and Methods

Chemicals and reagents

Dental pulp stem cells (DPSCs) were collected from immature human healthy molars and the primary cells were cultured and expanded using the explant outgrowth method[18]. Human

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third molars were extracted for orthodontics purposes in the clinic (N=3, UIC protocol #20110129). The DPSCs were evaluated in culture for STRO-1, a stem cell marker (IHC and FACS analysis). Our analysis confirmed that over 99% of cells (398/402, N=6) used for our study were DPSCs. The PCR experiments were done using primary DPSCs. We used commercially available DPSCs (Celprogen, #36086) for the western blot, Alizarin red staining, and immunohistochemistry analysis. The reason that we used two types of cells in this study is that the initial preliminary PCR experiment was done 5 years ago, thus, we decided to use commercially available and verified DPSCs. The two types of DPSCs yielded similar DSPP and DMP-1 expression data and both DPSCs were frequently screened using STRO-1 marker analysis (Fig. 1B-D)

DPSCs were cultured in regular/osteogenic media (The regular growth medium consisted of alpha MEM medium (Life Technologies, Milan, Italy) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. The osteogenic medium consisted of alpha MEM medium supplemented with 10% FBS and 1% penicillin/streptomycin, 250 μmol/L ascorbic acid phosphate, 10 mmol/L beta glycerophosphate, and 10 nmol/L dexamethasone) at 37°C and treated with C5a antagonist, W54011 (10 nM), obtained from (Calbiochem, San Diego, CA, USA) for 72 h in regular growth media, which was replaced with with osteogenic media for 21d treated with the inhibitor W54011. The medium was changed every 2–3 days. DPSCs between the 3rd and 5th passages were used throughout the study. This methodology was used in the hypoxic (2% oxygen) and normoxic (20% oxygen) conditions. The differentiation procedure was conducted using different sets of DPSCs, 4 times. The experimental protocols used for this study were in accordance with the guidelines according to the Institutional Animal Care and Use Policy and approved by the IRB Protocol Committee at the University of Illinois at Chicago (UIC protocol #20110129).

Quantitative PCR

Primary DPSCs were cultured according to the differentiation protocol above in 6-well plates with 40,000–50,000 cells/well at the specified concentration of the C5a receptor inhibitor (W-54011). The experiments were done in triplicate. Total mRNA was extracted with 0.8 ml Trizol (Invitrogen, Waltham, MA, USA) and analyzed using the Fisher Scientific NanoDrop 2000 device. The cDNA samples were analyzed using the Applied Biosystems SYBR green reagent system according to the manufacture's protocol. Briefly, cDNA was synthesized from 1 μg of total RNA and qPCR was performed in reaction buffer (20μl) containing SYBR™ Green Master Mix (10μl), DEPC treated water (7μl), 0.5μM of forward and reverse primers (1μl), and cDNA (2μl). The mixture was incubated for 3 min at 95°C. Then, 40 cycles were performed for 3 sec at 95°C and 30 sec at 60°C. Primers (Integrated DNA Technologies, IDT) used are listed in the Table 2.

Immunohistochemistry

Differentiating and differentiated DPSCs were fixed and permeabilized as previously described[16,17]. Subsequently, the cells were incubated for 24 h with rabbit anti-C5a receptor (1:200, Proteintech, ST. Louis, MO, USA), mouse anti-DMP-1 (1:1000, R&D System/ Sigma, ST. Louis, MO, USA), mouse anti-STRO-1 (1:200, Fisher Scientific, Waltham, MA, USA) and/or mouse anti-DSPP (1:500, Santa Cruz, Dallas, Texas, USA) or their respective

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control/isotypes. The cells were then treated for 3 h with a mix of Alexa Fluor-594 anti-mouse IgG, Alexa Fluor-488 anti-rabbit IgG (1 μg/mL) and/or DAPI (2 μg/mL). The coverslips were mounted, and images were taken using a Zeiss Axiovert microscope. The fluorescence immune density was statistically analyzed using ImageJ 1.49v software.

Alizarin Red Staining

Fourth passage DPSCs control or with treatment were fixed with 10% paraformaldehyde for 15 min, then washed three times with distilled water. 1 mL Alizarin red dye was added for 20 min then washed four times with deionized water with gentle rocking to remove any additional Alizarin red. 1 mL 10% acetic acid was added to prevent the cells from drying. The cells were then subjected to visual inspection and imaging.

Western Blotting

Western blotting was performed on total protein samples from the differentiating/ differentiated cells in each group: control (normoxia), W-54011 (normoxia), and W-54011 (hypoxia). Briefly, 20 μl cell homogenates were separated on 10% SDS gels and transblotted onto nitrocellulose membranes. The membranes were incubated/blocked for 4 h, in 3% skim milk powder in TTBS (0.1% Tween20 in TBS). Subsequently, the membrane was treated with mouse monoclonal-DSPP (1:500) (Santa Cruz, Dallas, Texas, USA) and rabbit polyclonal tubulin (1:5000) primary antibodies (Sigma, ST. Louis, MO, USA) followed by incubation in goat anti-mouse IRDye 800 (Li-Cor Biosciences, Lincoln, NE, USA) secondary antibodies. The immunocomplexed bands were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Data Analysis

ImageJ was used to quantify the immunohistochemistry staining intensity,. Fixed areas of 1mm × 1mm or 2mm × 2mm were selected to analyze the number of differentiated cells. The significant differences were determined using the Student's t test ($P < 0.05$ was considered significant).

Results

Odontogenic lineage cell markers are expressed in the differentiating and differentiated cells from DPSCs.

To determine the specific *in vitro* role of the complement component C5a in the odontoblastic differentiation and proliferation of DPSCs in normal and oxygen-deprived experiments were performed using a C5a specific inhibitor W54011. DPSCs were cultured in regular growth or osteogenic medium for 24 d (repeated 4 times with different setss of DPSCs). The W54011 was added during differentiation from d1 to d4 (timeline presented in Fig. 1A). The homogeneity of the DPSC population was indicated by the co-localization of the stem cell marker STRO-1 (Fig. 1B-D). RT-PCR, western blot, and immunohistochemistry analysis demonstrated that the differentiated cells expressed DSPP (Fig. 2B, D, Fig. 3) and DMP-1 (Fig. 3, Fig. 4). Alizarin staining also showed mineralization of the matrix (Fig. 1E, F), which is a characteristic feature of functional ondontoblasts. Taken together, our gene and protein expression analysis indicated that this differentiation

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protocol successfully induced the odontogenic differentiation of isolated DPSCs under normoxic and hypoxic conditions.

DPSC-derived odontoblast-like cells express C5a receptor.

The previous studies[15, 16] has shown that STRO-1-positive pulp progenitor/stem cells sorted from the tooth pulp express the C5a receptor. We next examined the C5a receptor expression in the differentiated odontoblast-like cells. Immunohistochemistry staining with anti-C5a receptor and DSPP antibodies showed that the differentiated cells expressed both molecules. Double immunofluorescence staining demonstrated that C5a receptor -positive cells express DSPP (Fig. 2A-C).

C5a receptor inhibition resulted in a significant decrease in odontogenic differentiation gene expression, and hypoxia reversed this effect

C5a receptor inhibition using W-54011 demonstrated a significant decrease in odontogenic differentiation genes, such as DSPP (Fig. 2D, E optical density analysis gray: 0.66 ± 0.06, N=4 versus orange: 1.34 ± 0.09, N=4, $p<0.005$). Furthermore, the real-time PCR analysis of p38, NKFB, BMP1, FGF, ON, DSPP, RUNX2, OSX, IL6, IL6-R, and DMP-1 in the W-54011 treatment group compared with control (value '1' of the respective genes. The statistical results presented in Table 1.) demonstrate a significant downregulation of odontoblast lineage genes, such as DSPP, RUNX2, and DMP-1 in the W-54011 treatment group compared with control (Fig. 3A).

Under hypoxic conditions with C5a receptor inhibition, the odontogenic lineage genes, such as DMP-1 and DSPP, expression were significantly increased compared with the control (without C5a receptor inhibition) and normoxia groups (with C5a receptor inhibition) (Fig. 2D, E, Fig. 3B). The individual gene expression values are presented in Table 1. Taken together, our results demonstrate that C5a constitutes a positive regulator of the odontogenic DPSC differentiation in the normal oxygen condition, while C5a reverses its effect on DPSC differentiation in hypoxia.

DMP-1 expression in the differentiated odontoblast-like cells decreased with C5a receptor inhibition and this effect is altered in hypoxia.

To examine the complement gene expression data, we performed DMP-1 and DSPP immunocytochemistry with fully differentiated cells. In the control group, without W54011 treatment or hypoxia induction, DMP-1 immunoreaction was observed evenly throughout the cytoplasm of the differentiated cells (Fig. 4A, D). W54011 treatment resulted in a significant decrease in DMP-1 expression (Fig. 4B, E) demonstrating C5às positive role in odontogenic DPSC differentiation. In contrast, hypoxia reversed this effect and lead to a significant increase in DMP-1 expression (Fig. 4C, F, G). Several differentiated cells revealed a variety of cell morphology. Large and elongated cytoplasm (Fig. 4F) and small round shapes were frequently observed (Fig. 4G). The W54011 treatment group under hypoxia demonstrated a significant increase in DMP-1 expression in the differentiated cells compared with the normoxia group (gray: 0.73 ± 0.12, N=7 versus orange: 1.38 ± 0.07, N=6, $p<0.005$). Taken together, the gene and protein analysis confirm that C5a has a positive role in odontogenic DPSC differentiation and the hypoxia reverses this effect.

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C5a receptor inhibition and hypoxia induction do not significantly alter odontoblast-like cell proliferation.

To identify whether the increase in odontogenic gene expressions is due to an increased cell number during DPSC differentiation, we next examined the effect of C5a receptor inhibition and hypoxia on DPSCs-derived odontoblast-like cell proliferation. The number of DMP-1 immunoreactive cells was counted in a fixed 1×1 mm^2 area in the captured images. The cell number was similar among all four groups (Fig. 4H). The number of differentiated cells was not significantly different between the normoxia (1.123 ± 0.21, N=5) and hypoxia (0.96 ± 0.12, N=6) groups. These data show that C5a signaling and hypoxia do not have significant effects on DPSC cell number during odontogenic differentiation.

Discussion

The revascularization of traumatized pulp tissue due to luxation or avulsion trauma to young permanent teeth has been an ideal treatment of choice in regenerative endodontic treatment protocols. It has been widely accepted that a necrotic pulp can act as a matrix for new cells to grow on [19-21]. Regenerative endodontics, as seen today, began with a case report by Banchs and Trope[22]. At the time, it was a relatively new technique to re-vascularize immature permanent teeth and offered as an alternative to apexification. However, these studies could not determine whether vital pulp tissue was present, but hypothesized that due to continued root development, the entirety of the pulp could not be necrotic. This hypothesis sparked further research into DPSCs and their role in regenerative endodontics. Although the AAE guidelines for regeneration differ from the protocol in Banchs and Trope's[22] study, the article began a research trend toward a new treatment method, regeneration. Later on, Hargreaves et al.[23] suggested three essential parts of pulp regeneration research: 1. Cells capable of differentiating into odontoblasts, 2. A scaffold that promotes cell growth and differentiation, and 3. Molecules capable of signaling and stimulating cellular proliferation and directing differentiation. The present study examined the role of C5a as a key signaling factors in the odontogenic differentiation of DPSCs. The role of the complement system in stem cells has been addressed mainly regarding the complement active fragment C3a and C5a. Initially, C3a and C5a showed their ability as chemotactic factors for attracting mesenchymal stem cells (MSCs)[24].

Here, we differentiated primary DPSCs into odonto-lineage cells using a modified protocol in normal and oxygen-deprived conditions. The main findings are that C5a plays a significant role in the odontogenic differentiation of DPSCs because C5a receptor inhibition significantly decreased several key odontogenic differentiation genes' expression, such as RUNX2, DMP,1and DSPP. Interestingly, when cultured in hypoxic conditions, the effect of C5a inhibition was reversed. Both C5a and oxygen level have critical regulatory roles in odontogenic DPSC differentiation. This study affirms that alterations in the microenvironment, such as oxygen-deprivation, are crucial to the established DPSC phenotype[25].

To determine the role of C5a in DPSCs differentiation, we found co-localization of the stem cells marker STRO-1 in W54011 treated DPSCs. This observation is also supported by our previous reports[5, 6]. Consistent with this observation, Chmilewsky et al.[15] showed

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that C5a can recruit STRO-1-positive pulp progenitor cells to the injured pulp site. After 24 d, the differentiation status of cells was examined by several odontoblast lineage markers using PCR and immunohistochemistry. The differentiated cells at D24 demonstrated odontoblast-like characteristics as they expressed several key odontogenic cell molecules, including dentin matrix protein (DMP)-1 and dentin sialophosphoprotein (DSPP). These molecules are well-established odontoblast-specific lineage markers[26, 27]. DMP-1 is a key molecule in controlling mineralization and also has a significant role in the differentiation of DPSCs into functional odontoblasts[26]. A previous study has revealed that hypoxia promotes mineralization through increased expression of DMP-1 and DSPP[28], while another study reported that low oxygen causes stabilization of hypoxia inducible factor 1 alpha (HIF-1) and then the HIF-1 complex binds to hypoxia response elements (HRE) in the genome and initiates the transcription of target genes like VEGF and SOX, and pro-inflammatory/anti-inflammatory related factors (IL-6 and IL-8)[29]. C5a also has been implicated in the maintenance and proliferation/survival of different types of stem cells. Exogenous C5a treatment in embryonic stem cells (ESCs) and human-induced pluripotent stem cells (iPSCs) maintained their pluripotent status and promoted cell survival[30]. In disease conditions, such as diabetes, C5a induced MSC apoptosis[31]. This early mediator of the inflammatory environment has multiple roles affecting fundamental cell processes. We reported that the C5a receptor in pulpal fibroblast has a crucial role in nerve regeneration[16, 17]. C5a secreted by inflammatory fibroblasts, regulates BDNF secretion in an inflammatory context. This resultant BDNF secretion significantly promotes neurite outgrowth towards the injury site.

Low oxygen tension is beneficial to maintain undifferentiated stem cells and cell growth. ESCs remain undifferentiated for a much longer period of time in hypoxia[9]. There are also other reports regarding the role of hypoxia in stem cell differentiation. We [32] have shown that p38 is a negative regulator of the odontogenic DPSC differentiation in normoxia, while in hypoxia it exerts a positive function. Human ESCs produce more cartilage matrix proteins in oxygen-deprived conditions (2%)[8]. A similar observation was reported in rat MSCs-derived osteocytes. Low oxygen level, ~5%, significantly increased bone formation markers and differentiated osteocyte proliferation[7]. However, a subsequent study revealed controversial results where low oxygen was inhibited chondrogenesis and osteogenesis in adipose-derived mesenchymal cells[33]. The C5a receptor has been reported for its role in neural progenitor proliferation[34]. However, in the present study no significant increase in cell proliferation was observed at the terminal stage of differentiation in hypoxia. The number of the differentiated cells did not show a significant difference between the normoxia (1.123 ± 0.21, N=5) and hypoxia (0.96 ± 0.12, N=6) groups, indicating that C5a signaling and hypoxia do not have significant effects on DPSC proliferation. Several factors, including the differentiation stage, stem cell type, degree of hypoxia and culture conditions, play significant roles in determining specific hypoxia effects.

A significant risk in clinical stem cell treatment is the unpredictable behavior of the transplanted cells, such as in vivo differentiation into non-specific cell types[35, 36]. More lineage-specified and engineered stem cells are desirable for clinical application. In he present study, we show the complement fragment C5a as a crucial modulator in odontogenic DPSC differentiation. Furthermore, the microenvironment, as exemplified by low oxygen tension, can reverse this differentiation effect. Understanding the microenvironment control

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in stem cell differentiation is essential for successful dentin repair strategies in the injured pulp.

In the future, genetic animal models should be performed using genetic manipulation of complement signaling to understand C5a function in dentin regeneration. We are in the process of evaluating in vivo whether the complement system activation participates in the pulpal response to caries- induced or modeled dentinogenesis. Further, we plan to transplant our engineered DPSCs into our mouse dentinogenesis model. The current study provides an important foundation for exploration of potential therapeutic interventions and stem cell engineering.

Acknowledgments

The manuscript has been read and approved by all authors; and all authors agree to the submission of the manuscript to Connective Tissue Research. Dr. Chung and Dr. Alapati equally contributed to the manuscript as a senior author.

Funding

This study was supported from the NIH/NIDCR grants: R03 DE028637 (SC), R56 DE029816 (SC), and R01 DE011657 (AG).

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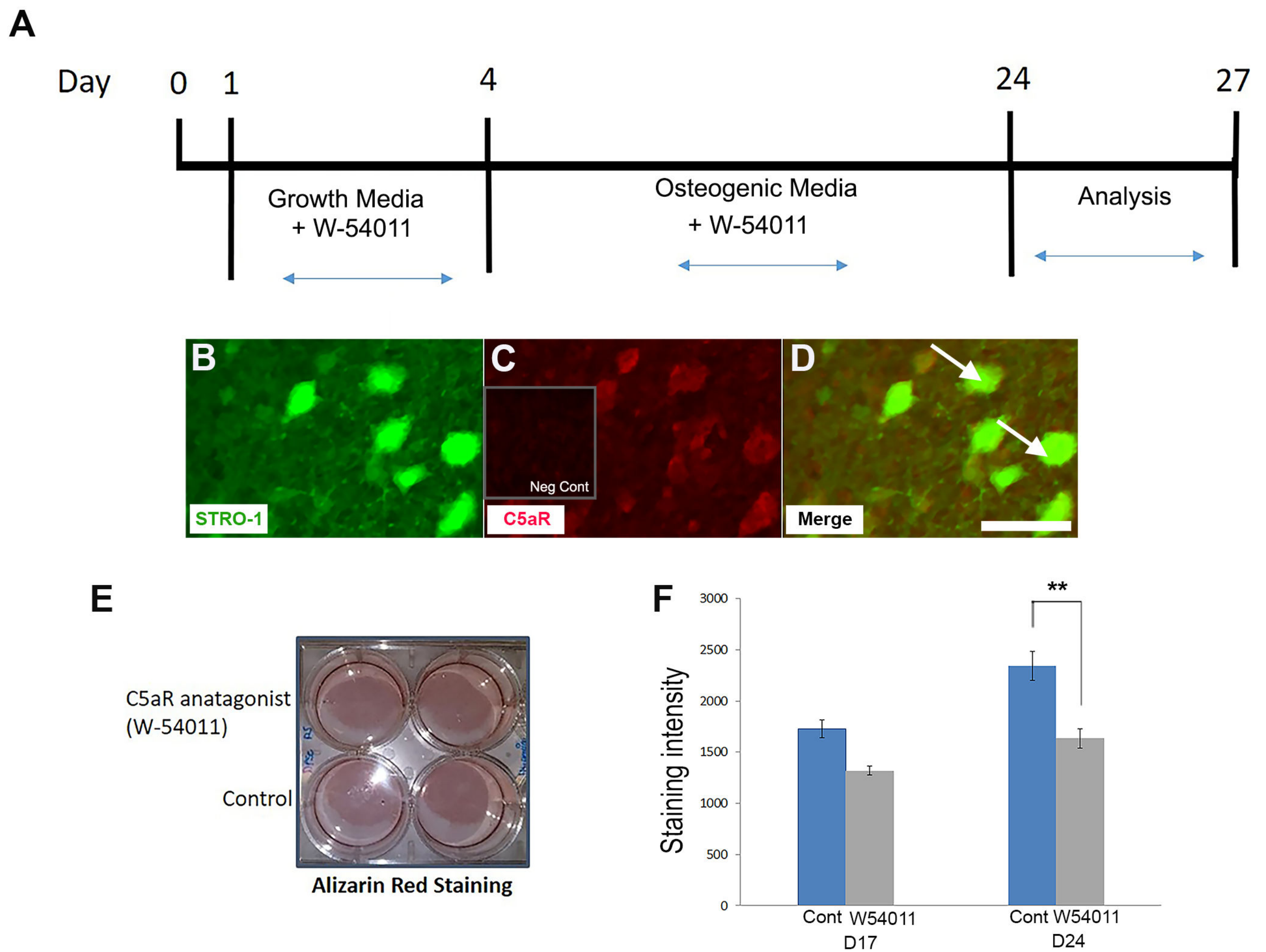

Figure 1. Schematic diagram of odontogenic DPSC differentiation.

B-D: Double immunofluorescence staining using anti-C5a receptor (red) and anti-STRO-1 (green) in DPSCs demonstrates that the majority of C5a receptor-positive cells express STRO-1. E: Alizarin red staining shows calcium compound deposition in the C5a receptor treatment and control groups. F: Statistical representation of the Alizarin staining intensity at D17 and D24 of DPSC differentiation in the W-54011 treatment group and control. ** = P<0.01. Scale bar: D = 50 μm (B-D).

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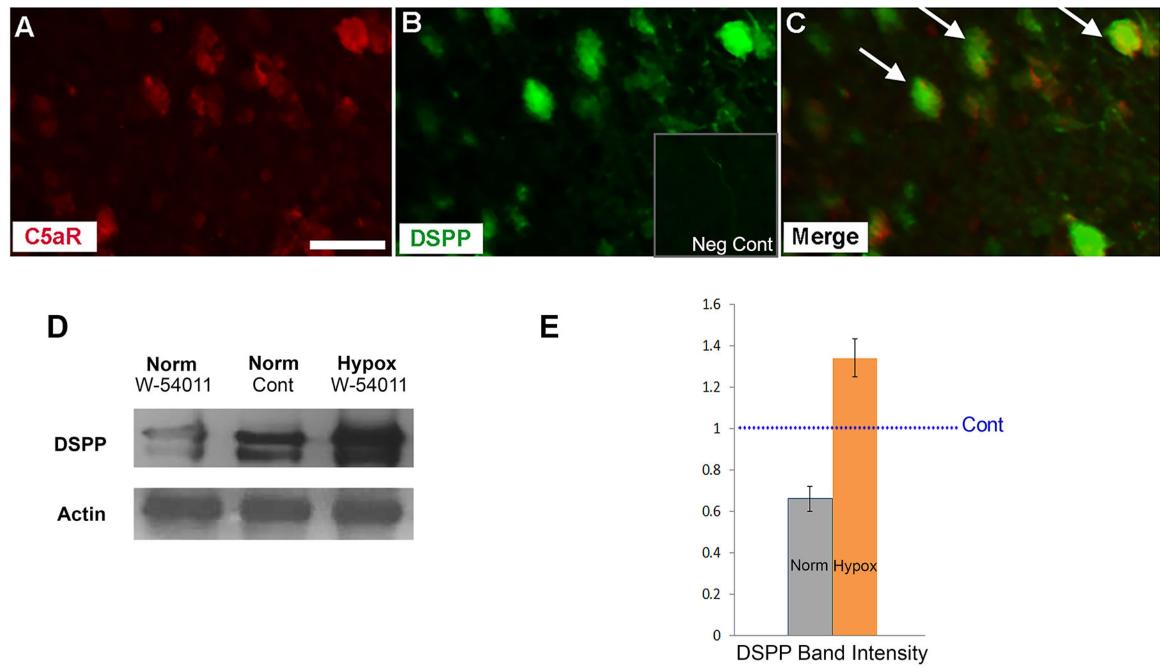

Figure 2. C5a receptor is expressed in DPSC-derived odontoblast-like cells and C5a receptor inhibition reduces DSPP expression.

A-C: Double immunofluorescence staining using anti-C5a receptor (red) and anti-DSPP (green) in DPSC-derived cells at D24. D, E: Western blot of DSPP expression with C5a receptor inhibition. E: Statistical representation of the DSPP bands at D24 show the staining intensity. Scale bar: A = 50 μm (A-C).

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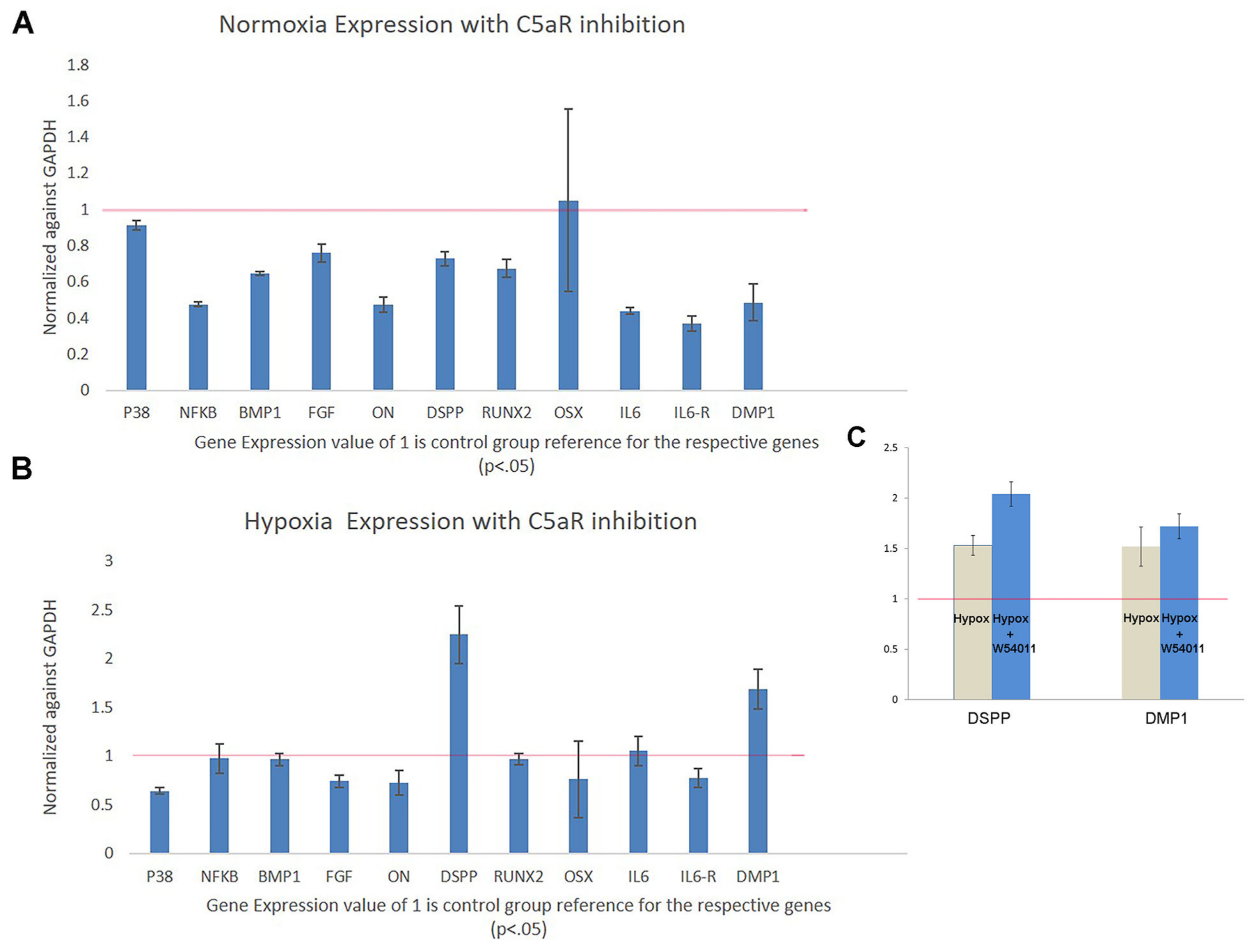

Figure 3. PCR analysis of DPSC-derived odontoblast-like cells in normoxia and hypoxia.

A: Real-time PCR analysis of p38, NKFB, BMP1, FGF, ON, DSPP, RUNX2, OSX, IL6, IL6-R, and DMP-1 in the W-54011 treatment group compared with control (as represented value '1' of respective genes). B: Real-time PCR analysis of p38, NKFB, BMP1, FGF, ON, DSPP, RUNX2, OSX, IL6, IL6-R, and DMP-1 in the W-54011 treatment group compared with control (as represented value '1' of respective genes) in hypoxia. The individual gene expression values are provided in Table 1. C: DSPP and DMP-1 expression data (control vs. hypoxia alone vs. hypoxia +C5aR inhibitor). Hypoxia alone resulted in a significant effect (increased the odontogenic gene expression) on DPSC odontogenic differentiation, and the C5aR inhibition enhanced this phenotype.

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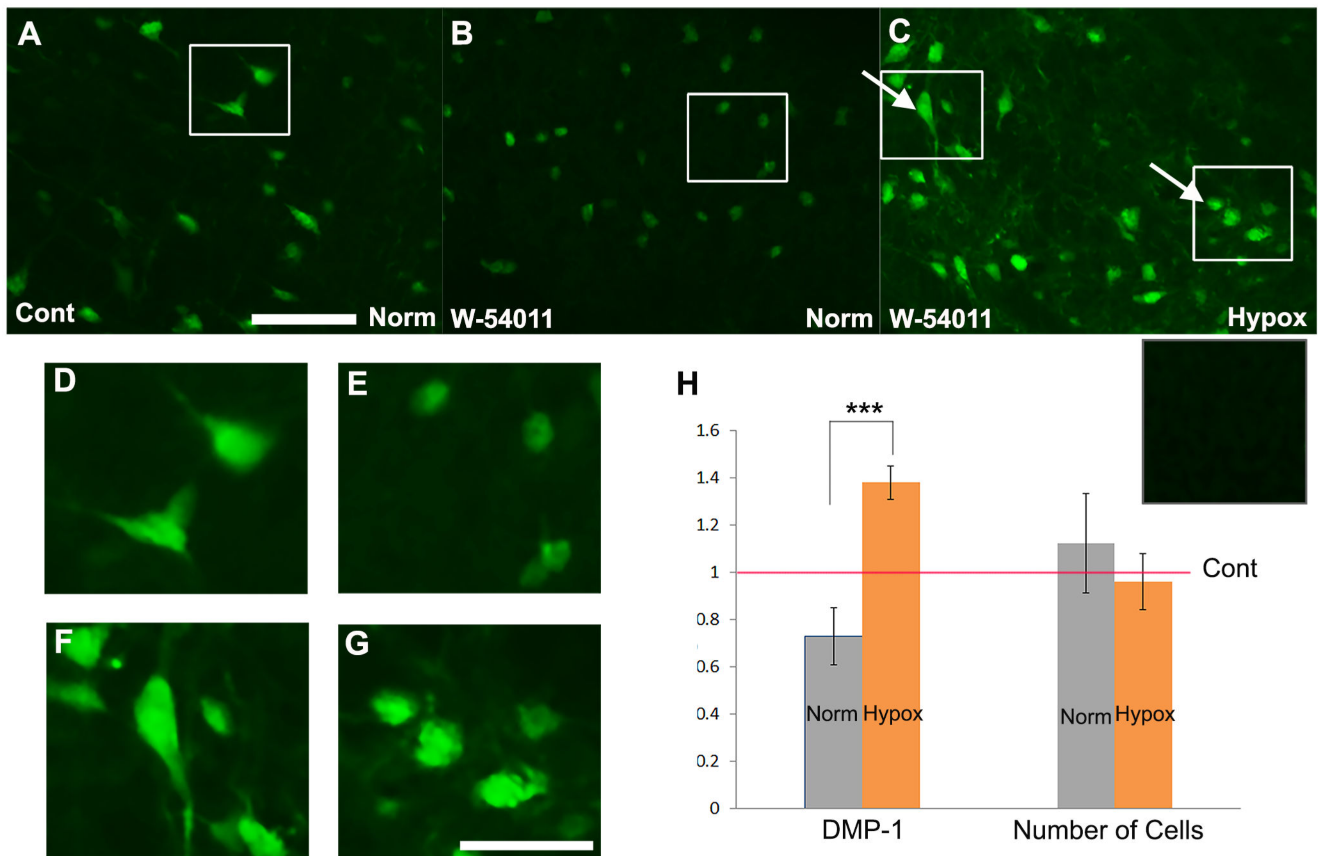

Figure 4. DMP-1 expression in the DPSC derived odontoblast-like cells in normoxia and hypoxia.
A-G: Immunofluorescence staining with anti-DMP-1 (green) after 24 d of differentiation showing the differentiated cells in control (A, D), W-54011 treatment group in normoxia (B, E) and W-54011 treatment group in hypoxia (C, F, G). H: DMP-1 immunostaining intensity was quantified using image J (Left) and the number of the cells in a fixed area was counted and compared (Right). Scale bar: A = 250 μm (A-C); Scale bar: G = 125 μm (D-G).

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Table 1.

PCR analysis of DPSC-derived odontoblast-like cells in normoxia and hypoxia.

Real-time PCR analysis of p38, NKFB, BMP1, FGF, ON, DSPP, RUNX2, OSX, IL6, IL6-R, DMP-1 in the W-54011 treatment group compared to control (as represented value '1' of respective genes) in normoxia and hypoxia.

Genes	Normoxia with C5aR blocking	p-value	stdev
P38	0.911	0.0043	0.0275
NFKB	0.475	0.0000	0.0141
BMP1	0.647	0.0000	0.0116
FGF	0.758	0.0017	0.0509
ON	0.473	0.0002	0.0409
DSPP	0.729	0.0006	0.0392
RUNX2	0.671	0.0008	0.0495
OSX	1.049	0.0753	0.5035
IL6	0.438	0.0000	0.0187
IL6-R	0.368	0.0001	0.0410
DMP1	0.485	0.0028	0.1020

Genes	Hypoxia with C5aR blocking	p-value	stdev
P38	0.642	0.0001	0.0306
NFKB	0.974	0.0024	0.1504
BMP1	0.964	0.0002	0.0660
FGF	0.741	0.0029	0.0632
ON	0.727	0.0180	0.1254
DSPP	2.247	0.0043	0.2946
RUNX2	0.972	0.0001	0.0588
OSX	0.763	0.0342	0.3927
IL6	1.052	0.0024	0.1484
IL6-R	0.773	0.0004	0.0935
DMP1	1.687	0.0019	0.2060

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Table 2.

List of primers and their sequences used.

FGFR3 forward	5’-CCC AAA TGG GAG CTG TCT CG-3’
FGFR3 Reverse	5’-CCC GGT CCT TGT CAA TGC C-3’
DMP1 forward	5’-CAC TCA AGA TTC AGG TGG CAG-3’
DMP1 reverse	5’-TCT GAG ATG CGA GAC TTC CTA AA-3’
P38 forward	5’-ACT CAG ATG CCG AAG ATG AAC-3’
P38 reverse	5’-GTG CTC AGG ACT CCA TCT CT-3’
NFKB forward	5’- AGA CAT CCT TCC GCA AAC TC- 3’
NFKB reverse	5’-GGC GAC CGT GAT ACC TTT AAT-3’
BMP1 forward	5′-CAG TCC TTT GAG ATT GAG CGC-3′
BMP1 reverse	5′-TGC TGC TCT CAC TGT GC CC-3′
FGF forward	5′-AAG GGC TTT TAT ACG GCT CG-3′
FGF reverse	5′-CCC ACA AAC CAG TTC TTC TCC-3′
DSPP forward	5’-CTG TTG GGA AGA GCC AAG ATA AG -3′
DSPP reverse	5’-CCA AGA TCA TTC CAT GTT GTC CT -3′
RUNX2 forward	5′-CCT GAA CTC TGC ACCAAG TG-3′
RUNX2 reverse	5′-GAG GTG GCA GTG TCA TCA TC-3′
OSX forward	5’-GGC ACA AAG AAG CCG TAC TC-3′
OSX reverse	5’-GCC TTG TAC CAG GAG CCA TA-3′
IL6 forward	5’-GTA GTG AGG AAC AAG CCA GAG-3’
IL6 reverse	5’-GGACTG CAG GAA CTC CTT AAA-3’
IL6-R forward	5’-ATA GCC TGC CTC TTC TCT ACT-3’
IL6- R reverse	5’-CCA CTC TGG AAT ACC CTG ATA AC-3’
GAPDH forward	5’-GGC ATC CAC TGT GGT CAT GAG -3′
GAPDH reverse	5’-TGC ACC ACC AAC TGC TTA GC -3′