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Dichloroacetate (DCA) Promotes a De-Differentiated Phenotype in Dental Pulp-Derived Stem Cells *in vitro*

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Abstract
Mesenchymal dental pulp-derived stem cells (DPSC) may be capable of regenerating many tissue types, including bone, adipocyte, vascular, and neural tissues. Methods currently under development direct pluripotent stem cells to differentiate, however, spontaneous *in vitro* differentiation may also occur – although the mechanisms that direct this process have not been fully elucidated. The goal of this study was to evaluate the potential of dichloroacetate (DCA), a cell proliferation inhibitor, to mediate growth and promote de-differentiation of DPSC exhibiting markers of different lineages. Thirty DPSC isolates were isolated and characterized using standard *in vitro* assays. Two cell lines exhibited evidence of spontaneous, non-directed in vitro differentiation. mRNA screening revealed these isolates may have spontaneously differentiated into neuronal progenitors and odontoblast progenitors. The remaining cell lines remained uncommitted. A dose-dependent inhibition of proliferation was observed following administration of DCA (10 – 1000 nmol), with the growth inhibitory maximum observed at 200 nmol. This inhibition was associated with a statistically significant decrease in cellular adhesion among the neuronal progenitors to Fibronectin, as well as Collagen. This provides evidence that growth of one lineage of spontaneously *in vitro* differentiated mesenchymal stem cells derived from dental pulp (neuronal progenitors) may be modulated, in part, through administration of exogenous metabolic regulators including DCA. Moreover, this inhibition may be associated with alterations in adhesion to extracellular matrix proteins, as well as down-regulation of key differentiation markers – suggesting a potential new mechanism for tissue engineering involving DPSC.

1. Introduction

Mesenchymal stem cells (MSC) are resident in, and may be obtained from, a variety of adult tissues (1,2).Many studies have demonstrated that waste tissue from medical procedures such as liposuction and childbirth can yield MSC, although harvesting may be costly from these sources and may involve controversial ethical and moral considerations when obtained from *in vitro* fertilization (IVF) or other embryonic sources (3,4). However, recent clinical studies have also demonstrated that dental pulp may also provide a rich supply of multi-potent, highly proliferative MSCs that can be obtained less invasively, more cost effectively, and with fewer ethical considerations. In fact, dental pulp-derived stem cells (DPSC) have now been shown to be capable of differentiating into many different tissue types, including dental structures, bone forming

cells, such as osteoblasts, chondroblasts, adipocytes, as well as vascular and neural tissues (5-9).

Although some technical issues remain, several recent experiments have now elucidated *in vitro* processes to direct uncommitted (and some partially committed) DPSC isolates to differentiate using methods that include cell-matrix adhesion molecules, growth factors, biomechanical scaffolding, tension and pressure (10,11). For example, DPSC migration and differentiation can be directed using extracellular matrix (ECM)-coated culture materials, including Fibronectin, laminin, collagen, and fluorapatite (12-14). Other research has demonstrated the potential to use bio scaffolding, tension and pressure to induce DPSCs toward specific differentiated phenotypes (15-18). In addition, biochemical stimulation using growth factors has been demonstrated to induce DPSCs into differentiated phenotypes, including Insulin-transferrin-sodium selenite supplement (ITS), bone morphogenic protein 2 (BMP2), growth differentiation factor 11 (Gdf11), platelet-derived growth factor (PDGF-AB), transforming growth factor (TGF-β1), dexamethasone, and basic fibroblast growth factor (bFGF) (19-25).

Although these studies describe how to influence and modulate and differentiate uncommitted DPSCs, or to influence further differentiation of partially committed DPSCs, few (if any) studies have focused on the possibility of de-differentiating partially or fully committed DPSC lines, knowledge that will be needed if DPSCs are to be used to treat the growing list of age-related illnesses in the adult population. One compound that may prove useful in this area is dichloracetate (DCA), which has been demonstrated to inhibit development in vivo, as well as altering and reversing differentiation *in vitro* (26,27). DCA is a metabolic modulator that is non-toxic to normal cells, but has been used as an anti-proliferative and anti-tumor agent in many types of cancers, including glioblastomas, endometrial, and breast cancers (26-30). The administration of DCA appears to shift cellular metabolism from aerobic glycolysis to glucose oxidation, which induces apoptosis in cancer cells, increases p53 expression and decreases Survivin-transcript levels – suggesting both mitochondrial-dependent and –independent processes that leave normal cells unaffected (28,29). Recent studies suggest that the metabolic profiles of MSC tend to shift towards glycolysis and away from oxidative phosphorylation while undergoing differentiation *in vitro*, suggesting that DCA may provide a non-toxic method to induce and select for de-differentiated phenotypes among DPSC isolates (31,32).In addition, DCA appears to also select for clonal expansion of anchorage-independent cells in culture, although the mechanism has not been fully elucidated (26). Based upon this evidence, the primary objective of this project was to evaluate the effects of DCA on the phenotype and differentiation status of DPSC isolates *in vitro*.

2. Materials and Methods

2.1. Human Subjects

The protocol for this study titled "Isolation of Non-Embryonic Stem Cells from Dental Pulp" at the University of Nevada, Las Vegas - School of Dental Medicine (UNLV-SDM) dental clinic was filed, amended, and approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#0907-3148) on February 5, 2010.In brief, subjects were randomly recruited by members of the UNLV-SDM clinic during their dental visits between February and June, 2010.Informed Consent was required and was conducted onsite. Inclusion criteria: subjects had to be between eighteen (18) and sixty five (65) years old and must agree to participate. In addition, all potential subjects must have sound, unrestored, vital teeth (teeth that have healthy pulp tissue), and need to have one or more extractions that are necessary for oral health, as determined by the clinical faculty member in charge. Exclusion criteria: Any subject under eighteen (18) or over sixty five (65) years of age, any subjects having dental extractions involving compromised pulp, and any subject that refuses to donate their extracted teeth.

2.2. DPSC Isolation and Culture

In brief, dental pulp was extracted from the vital teeth of healthy adults who agreed to participate. The majority of the teeth included in this study were extracted due to severe periodontal disease, necessity for fabrication of complete dentures, or impaction and/or crowding (e.g., third molars). Following extraction, teeth were placed into sterile solution and transported to the laboratory for sectioning. The teeth were sectioned at the cemento-enamel junction (CEJ) using a diamond rotary disc and the dental pulp was removed with an endodontic broach. The dental pulp was then immediately placed into sterile micro centrifuge tubes containing 1X phosphate buffered saline (PBS) and transferred to the laboratory for culture. Tubes were pre-assigned a unique, randomly-generated number to prevent research bias. Demographic information regarding the sample was concurrently collected, which consisted of age, gender, and ethnicity only.

Dental pulp samples brought to the laboratory for culture were processed either using enzymatic digestion (DPSC-ED) or direct outgrowth (DPSC-OG).In brief, DPSC-ED were digested in a solution of 0.25% Trypsin-EDTA (0.02%) containing Collagenase Types I, II and IV (200 units/mL) obtained from Invitrogen (Carlsbad, CA) for 30 – 60 minutes at 37C.Dental pulp processed using DPSC-ED and direct outgrowth (DPSC-OG) were then processed similarly; Extracted dental pulp was vortexed for 10 – 30 seconds to dislodge cells and centrifuged for five (5) minutes at 2,100 relative centrifugal force (RCF) or g. Supernatant (PBS) was aspirated from the tube and dental pulp-derived cells were resuspended in 1.0 mL of RPMI-1640 medium from

Hyclone(Logan, UT) with 2mM L-Glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Media was supplemented with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 ug/mL) solution and 10% fetal bovine serum (FBS), obtained from Hy Clone (Logan, UT).Cells were cultured in 75 cm2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO2 in humidified chambers. Media was changed every 48 hours until adherent cells reached 70% confluence. Cells were subsequently passaged at a 1:4 ratio.

2.3. Materials

Methyl dichloroacetate (DCA) MW= 42.97, d=1.381 g/cm3 (10.8M) was obtained from Acros Organics – Thermo Fisher Scientific (Fair Lawn, NJ).Media were supplemented with DCA in cell culture media for final concentrations ranging between 10 and 1000 nM DCA.

2.4. Cell Proliferation and Doubling Time

Cell proliferation assays were performed in the appropriate complete media, as described above, with and without the addition of DCA. In brief, cells at 70% confluence were trypsinized and plated 1:4 into new 75 cm2 BD Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO2 in humidified chambers and their confluence was measured with a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany).Doubling time (DT) from passages one (P1) though ten (P10) were recorded. Three separate, independent replications of each experiment were performed to determine doubling time for each cell culture. Averages from the first five passages (P1-5), last five passages (P6-10) and overall average DT (P1-10) were calculated. Data were analyzed and graphed using Microsoft Excel (Redmond, WA).

2.5. Cell Adhesion

In brief, cell adhesion assays were performed in the appropriate complete media, as described above using Costar 96-well cell culture cluster plates, coated with either poly-L-lysine or Fibronectin solution at a protein concentration of 20 µg/mL for 1 hour (60 min.) at room temperature, 25°C. Wells were then washed twice with phosphate-buffered saline with 0.2% Tween-20 (PBST) prior to assay. Cells were seeded at a concentration of 1.2×10^5 in each of 96-transwell chamber filters (100 µL of 1.2×10^6cells/mL solution) with and without ECM-coating (described above) and allowed to attach for 30 minutes at 37°C. Following adhesion, non-adherent cells were removed by suspending plates upside down in a rotating tank of PBS for 10 minutes at room temperature, 25°C. Adherent cells were fixed using 50 µL of 10% buffered formalin, and were stained with crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ). The relative absorbance was measured at 630 nm using a Bio-Tek ELx808 microplate reader (Winooski, VT). Data were analyzed and graphed using Microsoft Excel (Redmond, WA) and SPSS (Chicago, IL). Three separate, independent replications of each experimental condition were performed.

2.6. Statistical Analysis

The differences between treatments were measured using a t distribution, $\alpha = 0.05$. All samples were analyzed using two-tailed t-tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed t-test (33). As long as the sample size is at least moderate (>20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses. The analyses involving multiple two sample t-tests have a higher probability of Type I error, leading to false rejection of the null hypothesis, H_0.To confirm the effects observed from these experiments and minimize the possibility of Type I error, further analysis of the data was facilitated using ANOVA with SPSS (Chicago, IL) to more accurately assess relationships and statistical significance among and between groups.

2.7. RNA Isolation, Concentration, and Yield

To determine if any cells derived from dental pulp were dental pulp stem cells (DPSC), RNA was isolated from 1.5 x 10^7 cells of each of the experimental cell lines, using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) in accordance with the procedure recommended by the manufacturer. RNA concentration and purity were calculated using UV spectroscopy. The absorbance of diluted RNA samples (10 uL of RNA sample in 490 uL nuclease-free water, pH 7.0) was measured at 260 and 280 nm. RNA purity was determined by calculating the ratio of A260:A280, which should be > 1.80. Concentration for RNA samples was determined by the A260 reading of 1 = 40 ug/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water. Concentration was calculated as 40 x A260 absorbance measure x dilution factor (50).Total yield was determined by concentration x sample volume in mL.

Example: RNA standard
A260 = 0.75
Concentration = 40 x 0.75 x 50 = 1,500 ug/mL
Yield = 1,500 ug/mL x 1.0 mL = 1,500 ug or 1.5 mg RNA

2.8. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

To quantify the expression of DPSC-specific mRNA, RT-PCR was performed on total RNA using the ABgene Reverse-iT One-Step RT-PCR Kit (Ready Mix Version) and a Mastercycler gradient thermocycler (Eppendorf: Hamburg, Germany) using the following mesenchymal stem cell (MSC) primers synthesized by Seq Wright (Houston, TX):
CD44 FORWARD: GAAAGGCATCTTATGGATGTGC;
CD44 REVERSE: CTGTAGTGAAACACAACACC;
NANOG FORWARD: GCTGAGATGCCTCACACGGAG;
NANOG REVERSE: TCTGTTTCTTGACTGGGACCTTGTC;
Oct4 FORWARD:

TGGAGAAGGAGAAGCTGGAGCAAAA;

Oct4 REVERSE: GGCAGATGGTCGTTTGGCTGAATA;

One ug of template (total) RNA was used for each reaction. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C.Thirty-five amplification cycles were run, consisting of 20 second denaturation at 94°C, 30 seconds of annealing at 58°C, and 6.5 minutes of extension at 72°C.Final extension was run for 5 minutes at 72°C.Reaction products were separated by gel electrophoresis using Reliant 4% Nu Sieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME).Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY).Quantitation of RT-PCR band densitometry and relative mRNA expression levels were performed using Adobe Photoshop (San Jose, CA) imaging software, Image Analysis tools.

3. Results

Thirty one (31) individual dental pulp samples were collected from twenty four different (24) UNLV-SDM patient clinic between February and June, 2010 and processed for cell culture using direct outgrowth (DPSC-OG) as described in Methods. This resulted in thirty DPSC isolates, with at least one from each patient, yielding an overall success rate greater than 95% percent (n = 30/31 or 96.8%).

3.1. Cell Proliferation and Doubling Time

The average doubling time (DT) was established and calculated for all potential DPSC isolates, revealing characteristic average DTs ranging from approximately two to ten days (Figure 1A). More specifically, DT averages remained fairly stable for the initial five passages P1 – 5, which varied from 2.5 to 10.25 days. Twenty five DPSC isolates were observed to have fairly rapid DTs (rDT that ranged between 2.5 and 4 days (n=25/30 or 83.3%).Three DPSC isolates exhibited a much slower DT (sDT), ranging between 8 to 10.25 days. Two DPSC isolates, however, exhibited intermediate DTs (iDT) of 5.4 and 6.4 days, respectively. Although the iDT isolates proliferated significantly faster than sDT cells (p = 0.04), the change in proliferation rates was not sufficient to be significantly different than the average of rDT isolates (p = 0.11).

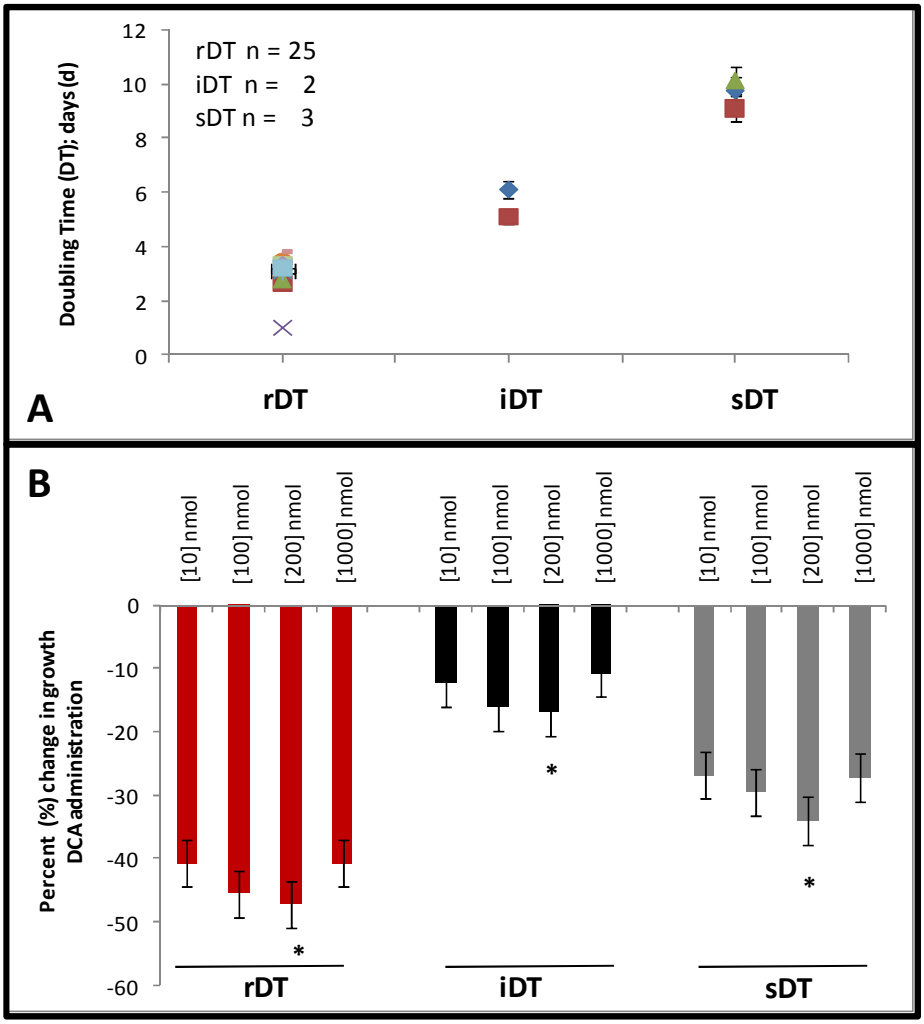

Figure 1. *Doubling time (DT) established for the isolates yielded three statistical groups rapid doubling (rDT), intermediate doubling (iDT), and slow doubling (sDT). rDT (n=25) is defined by 2.5-4 days for doubling, iDT (n=2) is 5.4 to 6.4 days, and sDT (n = 3) is 8 to 10.25 days.*

3.2. Effects of DCA Administration

DPSC isolates were plated in 96-well assay plates and their proliferation measured to determine if the administration of DCA was sufficient to alter cellular proliferation (Figure 1B).A dose-dependent relationship was observed in among rDT isolates, with increasing concentrations of DCA resulting in a more robust inhibition of cell growth. The lowest concentration of DCA evaluated (10 nmol) was sufficient to inhibit the growth of rDT isolated by 40.8%, with increasing concentrations exhibiting greater proliferation inhibition effects up to the growth inhibitory maximum (GI_{MAX}) observed at 200 nmol (-47.2%); higher concentrations elicited less robust proliferation-inhibiting effects extending to 1000 nmol (-39%).

Administration of DCA was also sufficient to inhibit iDT proliferation, although equivalent concentrations were less effective at inhibiting iDT cell growth than rDT cells. More specifically, the lowest concentrations of DCA (10 nmol) were sufficient to inhibit the growth of iDT cells by 12.3%, up to a GI_{MAX} of -16.9% (200 nmol), with decreasing effects observed up to 1000 nmol (-10.8%).The DCA-induced proliferation inhibiting effects were also observed among sDT cells, inhibiting sDT cell growth by 26.9% at 10 nmol up to the GI_{MAX} of -34.1% (200 nmol), with decreasing effects observed up to 1000 nmol (-27.2%).

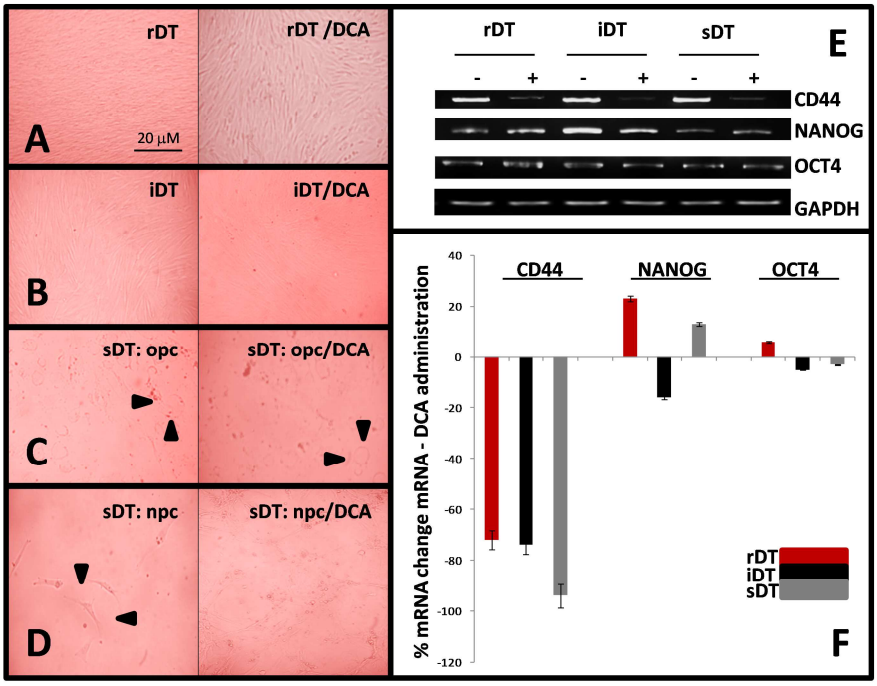

Figure 2. *Boxes A through D display light microscopy of sample DPSC isolates from each of the groups as defined by doubling time.*

The columns represent the control (left) and cultures exposed to DCA (right).rDT and iDT cultures show no significant variation between control and DCA isolates. However, sDTodonotgenic progenitor (opc) and sDT neuronal progenitor (npc) both showed morphologic variation between control and DCA exposed isolates. E. RT-PCR results demonstrated all three cell groups expressed mesenchymal stem cell markers CD44, NANOG and Oct4 with changes observed between the control (-) and experimental exposure to DCA (+).F, Densitometry from RT-PCR results shows significant statistical reduction in expression of CD44 mRNA in all isolates between control and DCA exposed groups. Similar reduction was not seen in expression of NANOG and OCT4.

To more accurately assess the qualitative effects DCA at GI_{MAX} concentrations, sufficient to inhibit proliferation of rDT, iDT and sDT DPSC isolates, microscopy and RT-PCR were performed (Figure 2).Although administration of the GI_{MAX} concentration of DCA (200 nmol) significantly inhibited both rDT (A) and iDT (B) cell growth, no significant alterations to cellular morphology or cell spreading were observed under experimental conditions. In addition, sDT cells established distinctive morphologies, such as large, ovoid-shaped cells (C) that were suggestive of odontoblast progenitor cells (sDT:opc), which did not exhibit any phenotypic changes in morphology. However, two sDT isolates formed long, narrow cells with axon- or dendrite-like projections (D), suggestive of neuronal progenitor cells (sDT:npc) that appeared to under stark changes in cellular morphology under DCA administration – in stark contrast to the effects of DCA on other cells.

To provide more qualitative assessments of the changes induced by DCA administration on these potential DPSC isolates, RNA was successfully isolated from all DPSC cultures and relative endpoint (RE) RT-PCR performed on equal concentrations of total RNA from each cell line (Figure

2E).Expression of mRNA for the mesenchymal stem cell marker CD44, NANOG and Oct4 was observed in all rDT, iDT and sDT isolates (representative samples shown).However, the addition of DCA at the GI_{MAX} concentration of 200 nmol significantly reduced expression of the cell surface MSC marker CD44, but not NANOG or Oct4. Densitometry measurements of band intensity using RE-RT-PCR for mRNA expression following DCA administration were compared to baseline expression from the untreated cells (Figure 2F), revealing a stark reduction in CD44 expression in all three types of isolates, rDT, iDT and sDT (-72%, -74%, -94%, respectively).

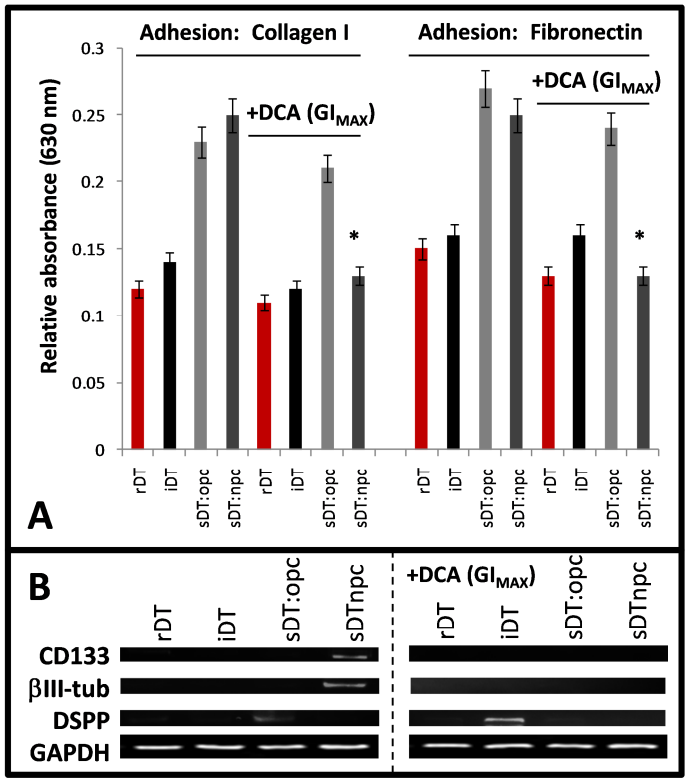

Figure 3. *A. Adhesion assays with Collagen 1 (CG) and Fibronectin (FN) were performed to assess effect of DCA.*

sDT showed more adherence to both CG and FN as compared to rDT and iDT controls. Isolates grown in DCA showed no change in adherence except for a significant decrease in sDT:npc. BRT-PCR evaluating odontoblast progenitor differentiation marker dentin sialophosphoprotein (DSPP) and neural progenitor differentiation markers CD133 and βIII-tubulin expression. DCA isolates showed decreases in βIII-tubulin and CD133 but not DSPP in all 3 isolate groups.

Finally, to further evaluate the effects of DCA administration on the phenotype of DPSCs, isolates were plated on Collagen I (CG) and Fibronectin (FN) to assess cellular adhesion and anchorage to the ECM (Figure 3). These results demonstrated that sDT:opc and sDT:npc adhered strongly to both CG and FN compared with rDT and iDT (Figure 3A). In addition, the administration of DCA did not alter adhesion of rDT, iDT or sDT:opc, but significantly inhibited sDT:npc cell adhesion ($p < 0.001$).The effects of DCA administration on the odontoblast progenitor differentiation marker dentin sialophosphoprotein (DSPP) and neural progenitor differentiation markers CD133 and βIII-tubulin were also evaluated (Figure 3B). These results suggest that DCA administration significantly down-regulated mRNA expression of both CD133 and βIII-tubulin, the neural progenitor (sDT:npc) markers, but not DSPP, the odontoblast progenitor (sDT:opc) marker.

4. Discussion

The primary objective of this project was to evaluate the potential for DCA administration to alter the phenotype or differentiation status of DPSC *in vitro*. The main results of this study strongly suggest that DCA administration is sufficient to inhibit proliferation and growth of DPSCs at levels that are demonstrated to be non-toxic to normal, non-cancerous cells. However, DCA administration may also influence cellular adhesion and expression of differentiation-specific markers in DPSC isolates that are partially committed to become neural progenitors; although no such effects were observed among the DPSC odontoblast progenitors.

These results appear to confirm previous observations, which demonstrated that DCA inhibits growth and proliferation, while selecting for anchorage-independent cells and promoting de-differentiation (26-30, 34). Although these effects in this initial pilot study appear to have specificity for neural progenitors, they do not seem to extend to odontoblast progenitors – results that may potentially limit the application

and use of DCA for these purposes. However, much work remains to be done to elucidate the possible mechanisms that might explain these effects and their specificity for particular types of DPSC isolates that are partially committed towards a specific cell lineage.

5. Conclusions

Although few studies have evaluated the possibility of de-differentiating DPSC that have spontaneously committed to a particular cell lineage, some evidence suggests the administration of DCA may be one potential mechanism for achieving this goal. This study provides the first evidence that DCA administration not only inhibits the growth and proliferation of uncommitted DPSC isolates, but more specifically reduces the expression of neuronal-specific biomarkers associated with specific DPSC isolates that appear to be neuronal progenitors. Future research in this area, however, will be needed for dental researchers and clinicians to more fully explore the feasibility and potential for isolating, culturing, and re-directing differentiation of DPSC extracted from adult human teeth in this new, rapidly developing field of regenerative medicine.

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