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Dopaminergic enhancement of cellular adhesion in bone marrow derived mesenchymal stem cells (MSCs)

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Abstract

Dopamine (DA) is a well-known neurotransmitter and critical element in the mussel adhesive protein that has gained increasing attention for its role in cellular growth enhancement in biomaterials, including cellular adhesion improvement. As the mechanism underlying this remains unclear, the objective of this study was to explore the effects of DA on the adhesion properties of bone marrow derived rat mesenchymal stem cells (rMSCs) using an hydroxyapatite gelatin nanocomposite biomaterial and to test whether the effects are mediated through various endogenously expressed DA receptors. Primary rMSCs were pretreated with D1-like antagonist, D2-like antagonist, or a combination of these antagonists followed by treatment with 50 μM DA and cellular adhesion quantification at 0.5, 1, 2 and 4 hours post DA addition. DA was found to increase rMSC adhesion and spreading at the 0.5 hour time-point and the dopaminergic effect on cell adhesion was partially blocked by DA antagonists. In addition, the D1-like and D2-like antagonists appeared to have a similar effect on rMSCs. Immunofluorescent staining indicated that the rMSC spreading area was significantly increased in the DA treated group versus the control group. Treatment of the D1-like DA antagonists with DA revealed that the actin filaments of rMSCs could not connect the membrane with the nucleus. In summary, DA was found to enhance early rMSC adhesion partially via DA receptor activation.

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Conflict of Interests

The authors declare no conflict of interests.

Keywords

Dopamine; Biomimetic biomaterial; Mesenchymal stem cells; Cellular spreading; Cellular adhesion

Introduction

Cellular attachment is a complex event, affected by cellular characteristics, material surface chemistry, and environmental factors. When cells contact the material surface, they adhere to the surface by adhesion molecules including selectins, syndecans, cadherins and especially integrins [1]. Integrins are transmembrane heterodimers, which possess cytoplasmic domains to transduce signals from the binding surface to the cell and thereby play a key role in cellular adhesion [2]. Poor cellular adhesion onto biomaterials can lead to the incomplete or poor bone formation after implantation *in vivo*. Thus, various surface modification methods such as physical adsorption, chemical immobilization of bioactive molecules, and plasma treatment have been introduced to enhance cellular adhesion onto artificial surfaces [3-5]. However, these methods are largely limited in application due to lack of stability, the requirement for sophisticated procedures, and high equipment cost.

Recently, coating with polydopamine (PDA) has been introduced to improve surface wettability for cellular adhesion within tissue engineering research. Studies have shown that PDA coating can increase the attachment of various cell types including osteoblasts, endothelial cells, and fibroblasts [6-9]. Inspired by those PDA characteristics, Ko et al. developed a 3D composite structure using an amalgamation of PDA to make hydroxyapatite-gelatin calcium silicate with poly-dopamine crosslinking (HGCS-PDA). HGCS-PDA can be fabricated through 3D printing techniques with porosity control, which can provide more surface area for cellular attachment. This novel material also has nearly equivalent mechanical properties as compared to cortical bone, with the incorporation of DA shown to increase the mechanical strength by 30% [13]. The HGCS-PDA material likely interacts with cells by providing PDA for cell adhesion and as DA released from the material induces cellular responses via receptor binding. The affinity of mesenchymal stem cells (MSCs) toward the biomaterial is an early event considered crucial for bone regeneration.

One possible mechanism driving this enhanced cellular attachment and proliferation on the PDA coating is likely the latent reactivity of PDA to various nucleophiles with amine or thiol groups [10], which extracellular matrix (ECM)-derived adhesion proteins and growth factors can be efficiently attached to [11, 12]. In addition, significantly improved MC3T3-E1 cell adhesion through integrin on the surface of PDA-modified materials has been described [13-15] and there is evidence that a PDA coating can promote osteogenic differentiation via the focal adhesion kinase (FAK) cascade [16]. Inhibition of FAK has been shown to prevent the expression of ERK-dependent alkaline phosphatase activity in osteoblast-like cell lines, consequently preventing further osteogenic differentiation. Furthermore, reduction of FAK activity in the osteoblasts of osteoporotic or osteoarthritic patients has been indicated [17]. ECM responsiveness during osteogenesis is caused by the aggregation of the $\alpha 2\beta 1$ and $\alpha V\beta 3$ integrin transmembrane receptors, which convey information from the ECM to the

intracellular compartment and subsequently activate intracellular signaling cascades such as FAK [18].

Another possible mechanism behind enhanced cellular attachment and differentiation may be the DA receptor mediated effect. Recent studies suggest that mesenchymal stem cells (MSCs) and osteoblasts expressed DA receptors and responded to DA agonists. For example, Shome S et al showed that DA acted through the D2 receptors present in the MSCs to inhibit their mobilization to the wound beds [19]. In addition, MC3T3-E1 osteoblasts are known to express their functional DA receptors to enhance proliferation and mineralization in vitro [20]. These findings implicate that MSCs may respond in a receptor mediated manner to free DA monomers leaching out of PDA to stimulate their early adhesion via adhesion molecules. Still, whether the PDA coating itself can directly promote cell adhesion or if leached DA monomers are the driving factor behind cellular adhesion via the DA receptors remains unclear.

We have hypothesized that free DA, released from a biomaterial containing PDA crosslinking, promotes rat MSC (rMSC) adhesion by increasing integrin expression and activity. This type material likely interacts with cells by providing PDA for cell adhesion and as DA released from the material induces cellular responses via receptor binding, with the affinity of rMSCs toward the biomaterial an early event considered crucial for bone regeneration. To test this hypothesis, we examined cell adhesion, cell spreading, and FAK activation to determine whether free DA can promote this integrin-mediated cell activity. The adhesion of rMSCs responding to both free and polymerized DA was investigated by checking the expression of five types of DA receptors and using a bone composite of hydroxyapatite-gelatin calcium silicate with PDA (HGCS-PDA, the only bone biomaterial containing dopamine). The mechanism of rMSC adhesion was also examined by whether they were regulated by free DA and HGCS-PDA. The adhesion and spreading of rMSCs was illustrated through actin cytoskeleton formation and formation of cell–matrix adhesions at different time points. The expression of integrin genes was also identified. We found that free DA can enhance early cell adhesion and rMSC spreading by activating FAK-transduced ECM/integrin signal pathways, suggesting that PDA-laced biomaterials could serve as a cytophilic surface during the cell seeding process in tissue engineered bone regeneration.

Materials and Methods

Preparation of HGCS and HGCS-PDA coated dishes

HGCS and HGCS-PDA materials were coated onto the surface of a 35 mm culture dish using the P-6000 spin coater (Specialty Coating Systems, Inc., Indianapolis, IN). The mixture of HGCS and HGCS-PDA slurry was prepared as described previously [21]. Briefly, for HGCS coating, calcium hydroxide powder (40.32 mg) was added to 1 mL of hydroxyapatite-gelatin (HAp-Gel slurry) and cross-linked with enTMOS [bis [3-(trimethoxysilyl)-propylethylenediamine, (Gelest, Inc., Morrisville, PA)]. After vigorous mixing, 50 μL of the mixture was sprayed in the center of a spinning culture dish on the spin coater to form a thin, homogenous layer of coating. Each dish was spun for 20 seconds at 6,000 rpm. For the HGCS-PDA coating, DA (Acros Organics, New Jersey, NY) was added to the mixture of calcium hydroxide powder (40.32 mg) and 1 mL of HAp-Gel slurry. After

crosslinking with enTMOS, DA was polymerized by interaction with 7.5% ammonium sulfate (Sigma-Aldrich, St. Louis, MO) to generate a HGCS-PDA mixture for culture dish coating, using the same method as the HGCS coating described above. The coated dishes were air-dried for 24 hours, sterilized under UV light for 72 hours, and washed with PBS before use.

Contact angle measurement on the HGCS and HGCS-PDA coating

A contact angle was measured with a contact angle analyzer (CAM 200, KSV Instruments, Finland) to determine the hydrophilicity/hydrophobicity of the HGCS and HGCS-PDA coated surface. Approximately 1 μL of water was dropped onto the surface of the coated dishes and the contact angle was measured after a static time of 30 seconds. A total of three coated dishes from each material and five data points from each dish were used to calculate the mean and standard deviation (SD).

DA release and measurement by ELISA

To measure the DA release, ultra-pure water (2 mL) was added in HGCS and HGCS-PDA coated dishes. A test sample (500 μL) was collected from each dish after vigorous mixing every 24 hours for 7 days. After the sample was collected, it was immediately added with 1 mM EDTA and 100 mM HCl to stabilize dopamine and the pH was adjusted (6.0 ± 0.5). Collected samples were then stored at 4°C until use. DA concentrations were measured using an ELISA kit (DLD Diagnostika GmbH, Hamburg Germany), following the manufacturer's instructions and the optical density was measured at 450 nm in a microplate reader within 15 minutes.

Isolation of rat bone marrow mesenchymal stem cells (rMSCs) and characterization

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill (Approved protocol number 15-273). Sprague-Dawley rats (Charles River, Wilmington, MA, about 250g, 7 weeks old) were euthanized primarily using carbon dioxide gas and cardiac puncture as secondary method. The femurs were isolated aseptically and retrieved bone marrow by flushing with Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS, Equitech-bio, Kerrville, TX, USA), 250 μL of GlutaMax (Invitrogen, Grand Island, NY, USA) and 2% of penicillin/streptomycin (Invitrogen, Grand Island, NY, USA) after removal of the epiphysis at both ends. The suspension of all nucleated cells from bone marrow was seeded into 100 mm culture dishes (Corning, NY, USA) and cultured at 37°C and 5% CO_2. After three days, non-adherent cells were removed and adherent cells were cultured for 21 days in Media was refreshed every three days. When rMSCs reached approximately 80% confluency, the cells were passaged for further expansion. At passage 8, the rMSCs were seeded at a density of 4.5×10^5 cells per 35 mm dish for adipogenic and osteogenic differentiation. To make rMSC aggregates for chondrogenesis, 2×10^6 cells per 15 mL conical tube were centrifuged at 1,200 rpm for 3 minutes. Osteogenic differentiation was induced by replacing osteogenic media (growth media with 10 mM β-glycerophosphate, 0.2 mM L-ascorbic acid, and 10^4 mM dexamethasone) every 3 days for 21 days. Then, cells were fixed with 10% formalin and stained with Alizarin Red S for mineral nodule detection. Adipogenic differentiation was

induced through use of adipogenic media (Stem Cell Technology, USA), replenished every 3 days for 14 days. Cells were then stained with Oil Red-O (Company info, USA). Chondrogenic differentiation was induced using 3D pellet cultures and replenishing chondrogenic media (Stem Cell Technology, USA) every 3 days for a total of 21 days. The aggregates were fixed in 10% formalin, embedded in Optical Cutting Temperature (OCT), sliced in 5 μm sections using a cryostat. Safranin O staining was completed followed by fast green counter staining. For the single cell colony forming assay, 50 rMSCs were seeded on a 35 mm dish. After 14 days, the culture dishes were fixed with 10% neutral formalin for 30 minutes and stained with 0.5 % crystal violet solution for 10 minutes. All images were acquired using a light microscope (Nikon Eclipse T*i*-U, Chiyoda-ku, Tokyo, Japan).

RT-PCR and Western Blot Analysis of DA Receptor Expression

Total RNA extraction and RT-PCR (nested PCR) for DA receptor gene expression were performed as described previously [20], with primer sequences described in Table 1. For western blots, a total of 20 μg of protein was used and separated using SDS-PAGE with GAPDH serving as the loading control. The detailed procedures for western blotting are described in the previous study [20].

Cell Adhesion and Spreading Assay

After overnight starvation, rMSCs were incubated with/without the DA receptor antagonists SCH23390 (Tocris Bioscience, Bristol, UK), Eticlopride (Tocris Bioscience, Bristol, UK), GR103691 (Tocris Bioscience, Bristol, UK), and L741742 (Tocris Bioscience, Bristol, UK) with final concentrations of 1 mM, 1 mM, 0.5 mM and 1 mM, respectively. After 30 minutes, DA (50 μM) was added to the cells before seeding at a density of 2×10^4 per well in a 96-well plate that was pre-coated with type I collagen solution (10 μg/mL) and incubated at 4°C overnight. At 0.5, 1, 2 and 4 hours, MTS [(3-(4, 5-dimethylthiazol-2-5-(3 carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-tetrazolium), (Promega, Madison, WI, USA)] assay was performed to quantify attached cells.

For spreading assay, rMSCs were plated at 5×10^4 cells on each coverslip coated with type I collagen, HGCS, and HGCS-PDA. Collagen solution (10 μg/mL) was added on the coverslip placed in 35 mm dish and stored at 4°C overnight. rMSCs in the DA+D1-like receptor blocker (RB) group were first treated with the D1/5 receptor antagonist SCH23390 (Tocris Bioscience, Bristol, UK) at a final concentration of 1 mM. After incubation at 37°C for 30 minutes, both DA only and DA+D1-like RB group were treated with 50 μM of DA. The rMSCs were directly seeded onto the coated dishes for 0.5, 1, 2 and 4 hours, respectively and then fixed with 4% paraformaldehyde (PFA). This was followed by permeabilization in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) for 5 minutes followed by blocking with 1% of bovine serum albumin (BSA: Gibco, Gran Island, NY, USA) for 30 minutes. Anti-Vinculin antibody (1:100) was incubated at 4°C overnight. After vigorous washing, Alexa Fluor® 488 (Invitrogen Gran Island, NY USA) and TRITC-conjugated phalloidin (Millipore, Billerica, MA USA) antibody (1:500) were incubated together at room temperature for 1 hour. Nuclei were counterstained with DAPI for 5 minutes. Antibodies were diluted using 1% BSA in PBS. The vinculin, F-actin phalloidin, and DAPI staining were observed with a Nikon fluorescence microscope with TRITC, FITC

and DAPI filters and images were acquired with a digital camera (Nikon Instruments, Melville, NY USA). Cell numbers and spreading areas were measured with Image J software (National Institutes of Health, Bethesda, MD USA). Ten random images from each slide (n=5) were acquired for analysis for each condition.

qPCR of cell adhesion related genes

For qPCR analysis, rMSCs treated with 50 μM DA were seeded on 35 mm culture dishes coated with type I collagen, HGCS, or HGCS-PDA. Non-treated rMSCs served as a control. Integrin β1, β3, α2, αV and FAK gene expression were measured using qPCR. Briefly, RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) at 10, 15, 30, 45, 60 minutes after DA treatment. Then, cDNA was synthesized using iScript™ (BioRad Laboratories, Hercules, CA). The qPCR gene analysis was performed using the Applied Biosystems 7500 (Applied Bio-systems, Foster City, CA USA). PCR was performed for 1 min at 95°C. A total of 40 amplification cycles were run, which consisted of 30 seconds denaturation at 95°C, 30 seconds of annealing at 60°C, and 30 seconds of extension at 72°C. Samples were assayed in triplicate and the target gene expression levels were normalized in relation to the housekeeping gene GAPDH (n=3).

Statistical analysis

All experimental samples were carried out in triplicate. Quantitative data was represented as the mean ± standard deviation (SD). Statistical analysis was performed with a one-way analysis of variance (ANOVA); p-values less than 0.05 were considered statistically significant.

Results

HGCS-PDA surface characteristics and release of DA

Water contact angles in the HGCS-PDA group (25.5 ± 5.6°) were lower than that in HGCS group (82.2 ± 5.0°), indicating that the HGCS-PDA surface has greater hydrophilicity than the HGCS surface (Figs 1A and 1B). Free DA release from the coating was measured using ELISA and the peak value of leached free DA was detected and converted to concentration based on the standard curve, which was 2398.04 ± 847.50 nmol/L. This was significantly higher than control levels of 8.79 ± 2.02 nmol/L. DA concentration was found to decrease 75-fold from day 1 to Day 7. The DA concentration at day 7 was 31.84 ± 1.27 nmol/L, which was similar to the HGCS group at day 7 (42.74 ± 30.49 nmol per L) (Fig 1C).

Expression of DA receptors in rMSCs

rMSCs were isolated from the bone marrow of the Sprague Dawley rat (6 weeks old, male) according to the procedure in the previous study [22]. rMSCs were expanded to passage 5 and investigated for DA receptor presence through use of PCR and Western blotting. Results were compared to control lysates containing DA receptors expressed in the brain. PCR showed expression of all five DA receptor genes in rMSCs and their size were identical compared to the DA receptors genes of mouse brain (Fig 2A). Western blot analysis of rMSCs showed the presence of all five DA receptors in these cells. The bands for these receptors showed similar molecular weights as those in mouse brain (Fig 2B). The bands of

five DA receptors, D1, D2, D3, D4, and D5 were detected around 70, 50, 50, 50 and 70 KDa respectively in both mouse brain and rMSCs.

rMSC adhesion and spreading

To assess the effect of adding free DA on cell adhesion and spreading, we counted the number of cells attached to each substrate as well as their cell areas. rMSCs in the DA treated group indicated significantly higher cell adhesion (51 ± 13 cells) at 30 minutes compared with the untreated control (45 ± 12 cells). The increased binding was blocked by DA+D1-like receptor blocker (RB) (28 ± 4 cells). Although there is no statistical difference in cell adhesion between the DA treated group and DA+D1-like receptor blocker group at 1, 2 and 4 hours, blocking rMSCs with D1-like RB showed a significant decrease in the number of attached cells on collagen coating after 30 minutes (Fig 3A).

We also found DA has an effect on cell spreading, with rMSCs treated with DA indicating higher spreading area (2108.7 ± 567.1 μm^2) with more filopodia processes compared to cells in the control group (1272.7 ± 419.5 μm^2) and DA+D1/5 RB treated group (1528 ± 330.4 μm^2), which presented smaller circular area with fewer or no cell processes evident at 30 minutes. No significant difference of rMSC spreading was found at 1, 2 and 4 hrs in DA treated group compared with 30 minutes (Figs 3B and 3C), likely as the spreading at 30 minutes already reached the maximum.

Consistent with results from free DA experiments, rMSCs directly cultured on HGCS coated dishes have round morphology. This indicated that the cells were poorly spread out on HGCS-coated surfaces. In strong contrast, the rMSCs projected area increased progressively over time grown on HGCS-PDA coating (Fig 4C), similar to that on a collagen coated control group (Fig 3C). Counting the number of cells showed that there were more cells attached to HGCS-PDA coated dishes at 30 minutes and 1 hour than to HGCS and that this trend reversed at the 2 and 3 hour time points (Fig 4A). This is likely because DA has a short-term effect on cellular attachment. Measurement of cell areas confirmed no significant difference was found until 4 hours after seeding between HGCS (1149.4 ± 289.5 μm^2) and HGCS-PDA (1473.2 ± 312.3 μm^2) groups (Fig 4B).

In this study, confocal images showed that both rMSCs cultured on HGCS-PDA and treated with free DA had a positive effect on cell spreading. Enhanced cell spreading by DA treatment showed larger area with greater formation of filopodia, lamellipodia and cellular extensions compared to the HGCS only or the DA antagonist blocked group (Figs 3C and 4C). In particular, cells seeded on HGCS-PDA coating exhibited enhanced spreading versus those treated with free DA, due to the combined effect from free DA and the PDA in the material.

Adhesion related gene expressions

To evaluate whether cell adhesion receptor expression is affected by DA treatment, gene expression of integrin β1, β3, α2, αV and FAK were measured by the qPCR. For the collagen coated group, the peak level of integrin β1 activity in cells treated with 50μM DA was observed at 30 minutes, which is earlier than the 45 minutes observed in the “no DA” control group. In contrast, no change in the expression of integrin β3 was observed for both

treatment groups. The expression of integrin α2 in 50 μM DA treated group was significantly higher than in the control group without DA at 30 and 45 minutes. Significantly higher expression of integrin αV was observed at 30 minutes, whereas significant higher expression of FAK was observed at 30, 45 and 60 minutes in 50μM DA treated group than in the “no DA” control group (Fig 5).

Cell adhesion receptor gene expression was also evaluated in cells coated on HGCS-PDA and HGCS coated groups. Significantly higher expressions of integrin β1, α2, αV and FAK were detected in the HGCS-PDA coated group at 30 minutes after seeding than in the HGCS coated group. While the peak of integrin β3 expression occurred earlier at 10 minutes, no difference was found for other time points. Activity of integrin β1 and FAK remained higher at 45 minutes in the HGCS-PDA coated group than the HGCS coated group (Fig 6). The differences in gene expression level between the HGCS-PDA or HGCS coating groups were more distinctive versus when cells were treated with free DA as shown in Figure 5. The peak level of integrin β3 gene expression was evident at 10 minutes for the HGCS-PDA group, while the rest of the genes reached peak expression at 30 minutes.

Discussion

PDA coating as a surface modification has been reported to effectively improve cell attachment to materials without causing adverse effects on biological cell behaviors [23] and DA functional groups such as catechol and amine [18] may play key roles on the cell adhesion. Enhanced cell attachment to materials has been reported when a PDA coating has been utilized [23]. rMSC morphology was correlated with their physiological behavior and the shape was suggested to be a key regulator of rMSC commitment [24]. On rough surfaces, the cells were polygonal in shape with many thin filopodia for surface attachment [25]. Cell spreading increased osteoblast differentiation in pre-osteoblastic progenitors [26].

In this study, DA was incorporated into the HGCS-PDA biomaterial instead of coating on the surface to improve mechanical strength [27]. The HGCS-PDA can be fabricated through 3D printing techniques with excellent porosity control, which can provide greater surface area for cellular attachment. Among the numerous approaches to improve cell seeding on the biomaterial surface, the most common techniques involve the use of a biomolecular coating such as collagen, chondroitin sulfate, sulfated hyaluronan, diamines, heparin, and RGD motif [28]. However, these coating methods experience limitations in regard to practical applications due to lack of stability and the requirement for complicated or expensive procedures [29-31]. Thus, the HGCS-PDA material with DA incorporated can be a good alternative candidate for these conventional methods. Furthermore, water contact angle measurements revealed the addition of PDA greatly improved the hydrophilicity of HGCS for favorable cell attachment conditions.

We further asked whether contact between the DA substrate and cells or free DA released from the material and interacting with cells via DA receptors was responsible for the observed cellular effects. Free DA effect on rMSCs was analyzed through comparison among DA-treated, no DA treatment, and DA receptor antagonist treated groups on type I collagen coating. Additionally, the combined material effect was analyzed between the

HGCS-PDA and HGCS coated groups. According to the previous study, 50 μM DA was verified to be a non-toxic and effective concentration in regard to MC3T3-E1 cell proliferation and osteogenic differentiation *in vitro* [20]. The same concentration was therefore used to treat rMSCs. Cell counting assays confirmed that significantly higher cell adhesion was achieved in the DA treated group (Fig 3A). Blocking with D1-like DA receptor blockers resulted in a significant decrease in cell adhesion at 30 minutes, while blocking with D2-like receptors had little or no effect on inhibiting cell adhesion. The suppressive effect on the rMSCs' adhesion resulting from D1-like RB returned to a level that was statistically higher than the control group at 4 hours, though the adhesion was lower than that of the unblocked DA group. This phenomenon can be explained by the time-related antagonistic effect. The DA treated group maintained significantly higher cell adhesion results within the four groups at all-time points. Overall, it was found that DA led to increased cell spreading and that adhesion can be blocked by DA receptor blockers, indicating that binding is receptor specific.

Formation of focal adhesions (FA) initiates the interaction between cells and substrate. Studies have suggested that rMSC gene expression can be induced by nanotopographical signals through FA and actomyosin cytoskeleton contractility [32]. FAK is one of the two major influencing agents in the FA processes activated by integrin-fibronectin interactions [33]. Matrix responsiveness during osteogenesis is caused by the α2β1 and αVβ3 integrin binding that subsequently activate intracellular signaling cascades [34]. Therefore, the expression levels of integrin β1, β3, α2, αV and FAK genes were measured after free DA treatment and direct material interaction. The HGCS-PDA group demonstrated enhanced biological performance compared with HGCS, while the free DA treatment increased rMSC adhesion and cell spreading, though further studies are needed to verify the effect on later proliferation and osteogenic differentiation as well as for elucidation of the underlying mechanism. It remains unclear whether a DA receptor mediated pathway or other transmembrane receptor mediated pathways are involved. As the present study focused on the early stage of rMSC behavior, results are limited. Future studies should focus on the downstream targets of the integrin-FAK signaling complex (i.e. phosphorylation of FAK, expression of ERK), which is related to osteoblast differentiation.

Conclusions

This study examined the effects of DA leaching from the HGCS-PDA and free DA on rMSCs for their adhesion and spreading characteristics. Our results indicate that DA can increase early adhesion and spreading of rMSCs, partially via activation of DA receptors. Furthermore, we found that cell adhesion related gene expression of rMSCs (Integrin and FAK) is upregulated by free or immobilized DA by interaction with HGCS-PDA. Further investigation is needed to elucidate the dopaminergic signaling pathway responsible for modulating rMSC cell adhesion and whether a PDA coating directly promotes cell adhesion. HGCS-PDA could be a good candidate for bone scaffolding materials because it improves MSC adhesion on the porous surface, which enhances the bone formation *in vivo*.

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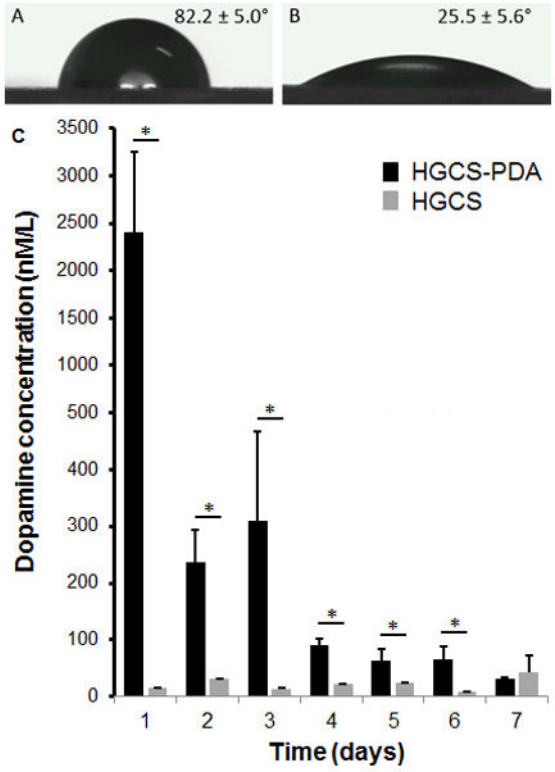

Figure 1.

Measurements of Water contact angle of dish surfaces coated with HGCS and HGCS-PDA (A), and amount of dopamine released from HGCS-PDA dish coating at different time points was measured by ELISA method (B).

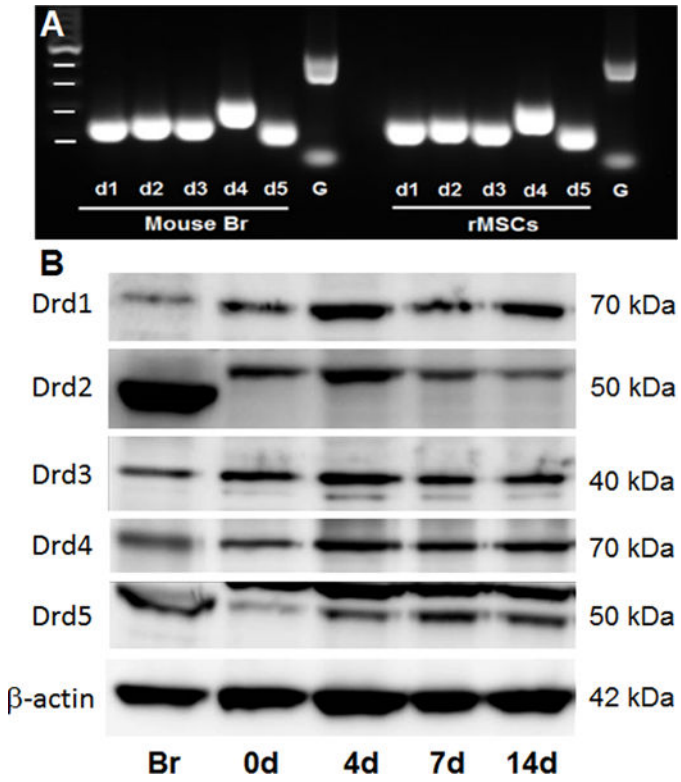

Figure 2.

DA receptor expression detected by Nested PCR or western blot. DA receptor gene expression in cultured rMSCs was detected by Nested PCR. Mouse brain tissue were used as positive controls (A). Western blot analysis of DA receptors on the rMSCs were evaluated during osteogenic differentiation at day 0, 4, 7 and 14. Brain tissue protein was used as a control to identify the correct size of each DA receptor protein and binding specificity against each DA receptor antibody. The relative molecular mass of significant bands identified on immunoblots was labelled on the right side. Br: brain, bp: base pair, G: GAPDH.

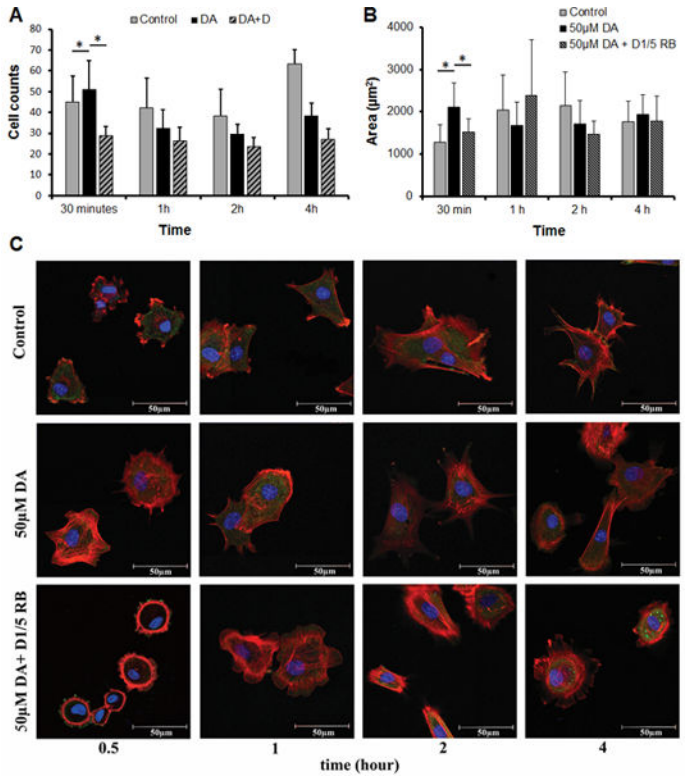

Figure 3.

Number of rMSCs on type I collagen coated plate treated without DA (controls), with DA, with DA+D1-like RB or DA+D2-like RB counted as cell number after 0.5, 1, 2 and 4 hours, respectively (A) and the cellular area was measured as the area/cell using Image J (B). All images were taken by selecting ten random areas and data were represented as Average ± Standard Deviation (n=3, *p< 0.05). Immuno-fluorescent confocal images of cytoskeletal actin (red), vinculin (green) and nucleus (blue) for rMSCs were acquired using confocal microscope (C).

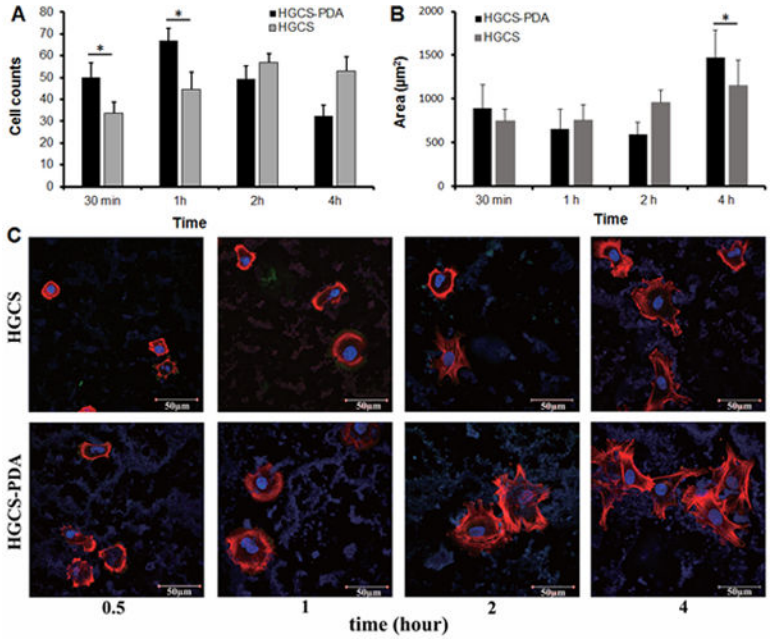

Figure 4.

Number of rMSCs on HGCS-PDA and HGCS coated coverslips was counted as cell number after 0.5, 1, 2 and 4 hours, respectively (A) and the cellular area was measured as the area/cell using Image J (B). Images were taken by selecting ten random areas and data were represented as Average ± Deviation (n=3, *p< 0.05). Immuno-fluorescent images of cytoskeletal actin (red), vinculin (green) and nucleus (blue) for rMSCs were acquired using confocal microscope (C).

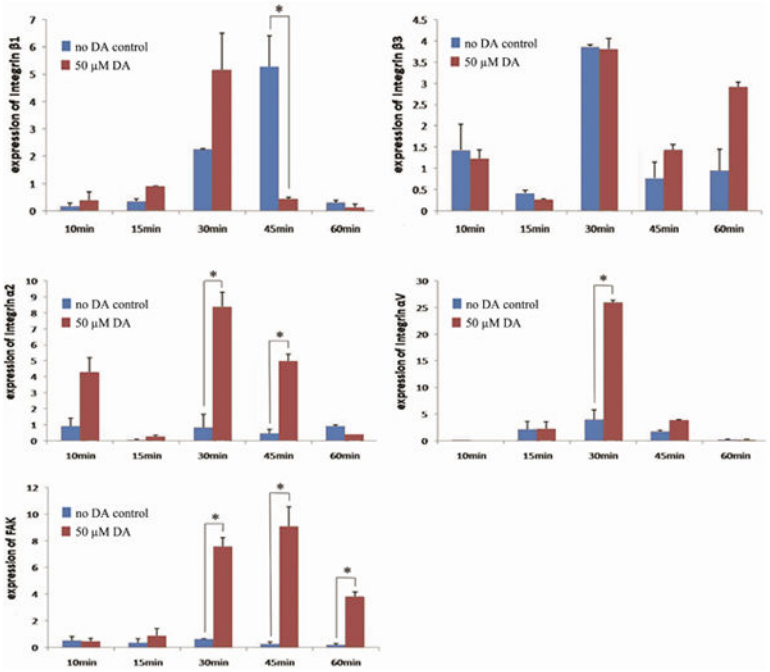

Figure 5.

Gene expression of Integrin β1, β3, α2, αV and FAK in rMSCs treated with or without 50μM dopamine (DA) at 10, 15, 30, 45 and 60 minutes after seeding on type I collagen coated dishes (*$p < 0.05$).

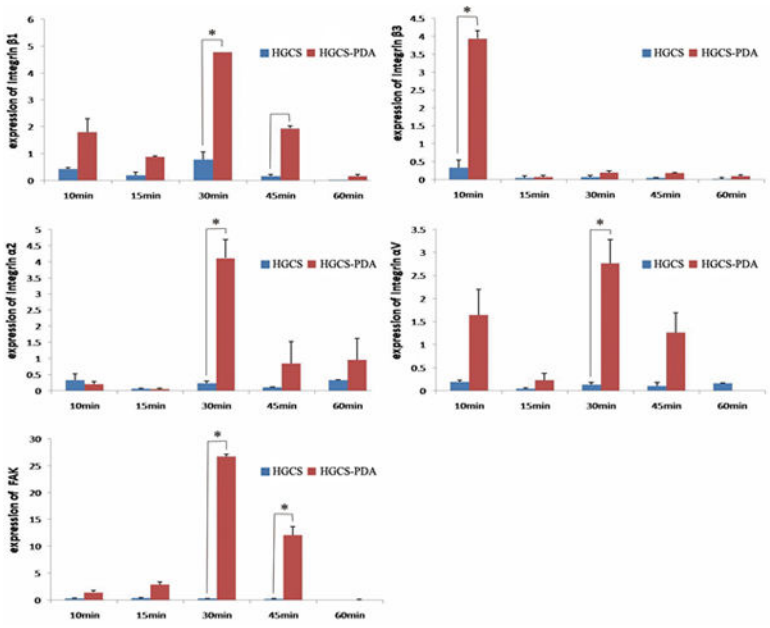

Figure 6.

Gene expression of Integrin β1, β3, α2, αV and FAK in rMSCs at 10, 15, 30, 45 and 60 minutes after seeding on HGCS-PDA or HGCS coated dishes (*p< 0.05).

Table 1

Primer sequences used for qPCR in this study. FAK: focal adhesion kinase and DAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Gene	Forward primer sequence (5′ – 3′)	Reverse primer sequence (5′ – 3′)	Size (bp)
Integrin β1	CAAGTGCCATGAGGGAAATGG	ATTGGGATGATGTCGGGACC	107
Integrin β3	TCCTATGGAGACACCTGCGA	AGGTACAGTTCACCGCGTTT	132
Integrin αV	GGCTCCAGGGAGGAGTTCTA	CTTGGCCCAGACTCGGAAAT	140
Integrin α2	AGGGTACCATTCGCACCAAG	AGTTGAACCACTTGCCCCAA	154
FAK	CTTAATCTGGCCAGGACGGT	ACTAAGCTTCCCCTGTGCTC	150
GAPDH	ACCCACGGCAAGTTCAACGG	GCATGTCAGATCCACAACGG	477