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The effect of Runx2 immobilization on poly (ϵ -caprolactone) on osteoblast differentiation of bone marrow stromal cells *in vitro*

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Abstract

In vivo regenerative gene therapy is a promising approach for bone regeneration and can help to address cell-source limitations through surgical implantation of osteoinductive materials and subsequent recruitment of host-derived cells. Localized viral delivery may reduce risk of virus dispersion, enhance transduction efficiency, and reduce administration/injection dosing, which subsequently increases patient safety. In this manuscript, we present a custom-tailored strategy to immobilize adenovirus expressing runt-related transcription factor 2 (AdRunx2) by using reactive polymer coatings to enhance *in vitro* osteoblast differentiation of bone marrow stromal cells (BMSCs). A thin polymer film of poly[*p*-xylylene carboxylic acid pentafluorophenol ester-*co-p*-xylylene] equipped with amine-reactive active ester groups was deposited on the surface of poly (ϵ -caprolactone) (PCL) using the CVD polymerization technique and then anti-adenovirus antibody was conjugated on the material with an amide chemical bond. Following antibody conjugation, AdRunx2 was conjugated to the PCL surface through antibody-antigen interaction. Osteoblast differentiation of BMSCs was induced by incubation in osteogenic medium. Alkaline phosphatase (ALP) activity, calcium deposition, and matrix mineralization were confirmed as markers of osteoblast formation. Incubation of the BMSCs in the presence of AdRunx2 modified PCL resulted

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in a 6.5-fold increase in ALP activity and significant increases in matrix mineralization when compared to controls.

1. Introduction

While traditional gene therapy transports genetic materials into cells to treat disease, regenerative gene therapy uses vectors to infect implanted or host-derived cells to produce continuous regenerative signals at desired sites [1–3]. Gene vectors can be delivered to cells outside of the patient before implantation (*ex vivo*) or directly to the patient for local modification of host cells (*in vivo*) [4,5]. Although fewer viral particles are delivered with the *ex vivo* approach, the method is limited by its requirement for *in vitro* transduction and reloading of infected cells into implantation scaffolds. This requires large cell numbers and critical sterilization conditions. *In vivo* gene delivery pursues direct injection of viral vectors, which increases risks to the patient [6].

Localized gene delivery directly from biomaterials may be a solution that can avoid the risk of virus dispersion and infection of surrounding tissue, increase transduction efficiency and reduce the administration dose. Many approaches have been explored to localize viral vectors to biomaterials [7–10]. For example, the net negative surface charge of retrovirus has been exploited to immobilize virus on polylysine-coated collagen scaffolds via electrostatic interactions [11]. Viral capsid proteins have also been modified to facilitate conjugation to material surfaces. Amine groups on chitosan surfaces were used for bioconjugation to bind virus via avidin-biotin [12] and antibody-antigen interactions [13]. Viral surfaces were covalently modified by biotin or digoxigenin while the infectivity was preserved. Compared to randomly conjugating avidin to biomaterials, immobilization of avidin onto previously biotinylated materials increases avidin orientation and therefore may result in enhanced binding efficiency [12]. The potential shortcoming of these methods, however, is that they may be limited to materials having inherent functional groups on the surface.

Chemical vapor deposition (CVD) polymerization is a surface modification technique that uses reactive coatings for two- and three-dimensional surface engineering of a broad range of biomaterials, while maintaining biocompatibility [14,15]. Theoretically, this method could provide customized coatings for implants with variable surface chemistry [16] and pore sizes [17]. The CVD method has been used to create a number of surface coatings with functional groups, including amine, carboxylic acid, ketones and aldehyde [18]. These specialized polymer coatings can be used to immobilize biomolecules with controllable patterns [19,20] and gradients of signals [21]. In a previous study, we used CVD coating technology to generate a thin polymer film with amine groups on the surface of PCL. Biotin was then conjugated on CVD-modified PCL materials and biotinylated AdLacZ particles were bound on the material through an avidin linker [22]. While effective, this procedure is complicated by the need for both the virus and the biomaterial to be modified with biotin prior to binding via an avidin interaction. The aim of this study was to develop methods to overcome this limitation by using antibody immobilization. Antibody immobilization is frequently used to tether virus to materials and has been successfully used to deliver adenovirus to cells without diffusing away from the scaffold [23–25]. Here, we combined antibody-antigen interactions with reactive coatings and present a new method that can provide robust immobilization of viral vectors to custom-tailored scaffolds.

Runx2 is a master regulator that plays an essential role in osteoblast differentiation [26]. Many studies have provided insight into Runx2 overexpression in different systems, including bone marrow stromal cells (BMSCs) [27,28], adipose-derived stem cells [29], and myoblasts [30]. A gap remains in understanding how local expression of Runx2 affects osteoblast

differentiation. Here, we immobilized AdRunx2 on inert PCL surfaces using the CVD technique to drive osteogenic differentiation of BMSCs *in vitro*.

2. Materials and methods

2.1. CVD-coated poly (ϵ -caprolactone) films

A PCL film was prepared as previously reported [22]. Briefly, PCL (Aldrich, MW 65000, St Louis, MO) was dissolved in glacial acetic acid at 60 °C for 2 days to generate a 1% w/v solution. One milliliter per well of PCL solution was added to 12-well cell culture plates and the plates were transferred into a 50 °C oven overnight. The PCL film was then treated with 0.2N NaOH solution and washed with phosphate buffered saline (PBS, Hyclone, Logan, Utah).

Cell culture plates with thin PCL films were fixed in a self-designed CVD chamber at 45 °C. Thirty milligrams of [2,2] paracyclophane –4-carboxylic acid pentafluorophenol ester was added into the sublimation zone and then sublimated following by pyrolysis and polymerization [31]. The final polymer poly[*p*-xylylene carboxylic acid pentafluorophenol ester-*co-p*-xylylene] [32] was deposited on the PCL films at room temperature.

2.2. Adenovirus immobilization on CVD-coated PCL films

Thirty micrograms of goat anti-adenovirus antibody (AbD Serotec, Raleigh, NC) in PBS (pH 7.4) was added into 12-well plates and incubated at room temperature. After 2 hours, the antibody solution was aspirated and the wells were washed three times with PBS. Tris-HCl solution (0.1M, pH8.0) was added and incubated at room temperature for 1 hour. Later, 4% gelatin (w/v) in a PBS solution was applied for 1 hour at 37 °C. Finally, adenovirus encoding Runx2 (generated as described before [33]) or LacZ (purchased from the University of Michigan Vector Core, Ann Arbor, MI) in a Tris buffered saline (TBS) containing 0.5% gelatin (w/v) was added and reacted at 4 °C for two hours. Free virus in solution was removed by extensive washing with PBS.

2.3. Bone marrow cell preparation and culture

Bone marrow was harvested from C57BL6 mice as reported previously [34]. The femora, tibia and humeri of mice were removed under aseptic conditions, and surrounding connective tissue and muscle was dissected. The epiphyses of the bone were excised to expose the marrow cavity. The marrow was flushed out using Hank's buffered salt solution in 5-mL syringe with a 23-gauge needle. Single cell suspension was obtained by aspirating several times through the syringe and then filtered through a 70 μ m-mesh nylon cell strainer (BD Biosciences, San Diego, CA) to remove debris. The cell suspension was collected and transferred into a T-75 tissue culture flask and incubated in alpha modified Eagle's medium (α -MEM, Gibco) with 10% fetal calf serum (Gibco) and 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco) until confluence (10–14 days).

2.4. Adenovirus transduction

Bone marrow stromal cells, up to 3 passages, were used for the experiments. Before cell seeding, the CVD-coated plate was conjugated with the antibody and was sterilized with 70% ethanol three times and rinsed with sterile PBS five times before binding the adenovirus. After the virus was bound on the plate and free virus was removed by rinsing with sterile PBS, BMSCs (10,000 cells/cm² unless otherwise noted) were seeded into the 12-well cell culture plate with AdRunx2 or AdLacZ (0 to 1000 m.o.i.). After 6 hours of infection, the growth culture medium was replaced with osteogenic medium, which is growth medium supplemented with 50 μ g/ml of ascorbic acid (Sigma, St Louis, MO) and 10mM of β -glycerolphosphate (Sigma, St Louis, MO). Cell culture medium was replaced every other day thereafter.

2.5. Western blot assay

BMSCs were seeded at a density of 2.5×10^4 cells/cm² in 12-well plates with AdRunx2 conjugated to the CVD coating. After 48 hrs of infection, cells were washed with ice-cold PBS, lysed in radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated on ice for 30 min. Protein concentration was determined by the DC protein assay kit (Bio-Rad Laboratories, Richmond, CA). Equal amounts of protein (20 µg) were boiled in hot water and fractionated by 10% polyacrylamide gels. Protein samples were transferred onto a PVDF membrane (Bio-Rad Laboratories, Richmond, CA) and blocked with 5% (w/v) dry milk in TBS-Tween for 2 hours, followed by the addition of 1 µg/ml of mouse anti-Runx2 (MBL, Beverly, Japan) overnight at 4 °C. The membrane was rinsed with Tris buffered saline, containing 0.1% tween-20 (v/v), three times and then incubated with goat anti-mouse IgG-HRP (1:1500, Santa Cruz Biotechnology, Santa Cruz, CA). Finally, the membrane was developed with a SuperSignal West Pico Luminol enhancer solution and stable peroxide solution (Thermo Scientific) then exposed 30 sec onto the film. After exposure, the same membrane was incubated in stripping buffer (2% SDS, 62mM Tris-HCl pH6.8, 0.7% 2-mercaptoethanol) at 60 °C for 15 min, rinsed with Tris buffered saline 0.2% tween-20 (TBST) three times, and re-blocked and blotted for GAPDH expression.

2.6. Alkaline phosphatase activity and DNA content evaluation

After cells were cultured in osteogenic medium for defined periods, cells were washed with ice-cold PBS and treated with 200 µl of lysis buffer (10mM Tris-HCl, pH7.4, 0.2v/v% NP-40 (Sigma, St Louis, MO) and 2mM phenylmethanesulphonyl fluoride (Sigma, St Louis, MO)) and placed on ice for 20 min. The cell extracts were sonicated for 10 seconds and 20 µl of suspension was taken for DNA content measurement using Quant-iT PicoGreen dsDNA Reagent and Kits (Invitrogen, Eugene, OR). Fluorescence intensity was measured on a Spectramax Gemini XPS microplate reader (Molecular Devices, Sunnyvale, CA). The leftover lysate was centrifuged at 10,000xg for 5 min at 4 °C and 20 µl of supernatant was removed to quantify ALP activity using a p-nitrophenol phosphate as standard and pNPP substrate solution (Sigma, St Louis, MO). The solutions were read at 405 nm using a microplate reader (Biotek-µQuant, Bio-Tek Instruments Inc, Winooski, VT). The amount of p-nitrophenol phosphate in the lysis solution was normalized to DNA content. The cell pellet was used to determine calcium content.

2.7. Quantification of calcium deposition

The cell pellet obtained in the above procedure was washed twice with Dulbecco's PBS and then resuspended in 0.5M HCl for 16–18 hours at 37 °C. The suspension was centrifuged at 13,000xg for 15 min and the supernatant was used to measure calcium content by QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA). The solution was read at 575 nm and calcium content was normalized to the DNA concentration.

2.8. Alizarin red staining

After 14 or 21 days, cells grown on tissue culture plates with osteogenic medium were rinsed with ice-cold PBS and fixed using Z-FIX, an aqueous buffered zinc formalin solution (Anatech Ltd, Battle Creek, MI). After 15 min, formalin was aspirated and deionized (DI) water was added to remove formalin. Subsequently, cells were stained with 40mM alizarin red (pH4.2, Sigma, St Louis, MO) for 15 min at room temperature. Excess alizarin red dye was removed with DI water and PBS.

2.9. Statistical analysis

All experiments were repeated at least three times and experimental data was reported as mean value $\pm$ standard deviation. The Student's *t* test was used for statistical analysis and *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Antibody conjugation and virus binding efficiency

Biodegradable materials including polylactic acid (PLA), its copolymer with polyglycolic acid (PLGA), and polycaprolactone (PCL), have been widely used in tissue engineering. These materials are considered inert due to the absence of reactive groups on their side chains. Although these properties are useful from a biological standpoint, they may limit certain applications. Many strategies have been developed to functionalize biomaterials by changing their basic chemical structure [35,36] or physical nature through radiation methods [37,38]. Chemical vapor deposition polymerization is a newer technology that can be used to functionalize the surface of any material with a custom chemical film in a tailored pattern. Here, we utilized the CVD technique to facilitate gene delivery to cells cultured on PCL surfaces (Figure 1). A thin polymer film with pentafluorophenol ester groups was coated onto PCL films. Subsequently, goat anti-adenovirus antibody was conjugated onto the surface with an amide bond, followed by AdRunx2 or AdLacZ immobilization via antibody-antigen interaction.

Before adding adenovirus, we optimized antibody binding to the surface of CVD film-coated 24-well tissue culture plates. Saturation of the CVD functional groups was observed at antibody levels above 5 $\mu\text{g}/\text{well}$ (Figure 2). After determining optimal antibody amounts, we further assessed virus binding efficiency with three different antibody concentrations. We determined that 5 μg of antibody per well would saturate the functional groups on the surface, while virus binding efficiency increased from 85% to 100% by increasing antibody amount from 5 $\mu\text{g}/\text{well}$ to 15 $\mu\text{g}/\text{well}$ (Figure 3a). Further, results shown in Figure 3b indicate that binding efficiency was nearly 100% within a wide range (from 1×10^8 to 1×10^{11} viral particles) with 15 $\mu\text{g}/\text{well}$ of antibody. These results demonstrate establishment of an efficient method to tether adenovirus (or other antibody recognized particles) on the surface of inert biomaterials *in vitro*.

3.2. Runx2 protein expression

After developing a strategy to immobilize AdRunx2, we examined its ability to enhance osteoblast differentiation of BMSCs. Before seeding any cells on CVD-coated PCL materials, we evaluated cell viability on the CVD coating. There was no detectable cytotoxicity within 7 days after incubating with cells (data not shown). Next, primary BMSCs were seeded in 12-well plates with AdRunx2 conjugated to the CVD coating at multiplicity of infection (MOI) ranging from 400 to 1000 pfu/cell. After 48 hrs of infection, Runx2 protein levels were assessed by Western blot analysis. Strong expression of Runx2 at all test conditions was observed (Figure 4) and indicated that the viruses do not lose bioactivity after immobilization. There was no detectable signal in the control group without virus.

3.3. Runx2 upregulation of osteoblast differentiation

Alkaline phosphatase (ALP) is a hydrolase enzyme that functions to remove phosphate groups from proteins in the bone matrix. Though it is expressed throughout the body, the tissue non-specific isoform is concentrated in organs such as the bone. ALP activity is an important parameter to indicate early osteoblast differentiation *in vitro* [39]. We investigated ALP activity in the AdRunx2 treatment groups and the AdLacZ control groups using the *p*-nitrophenol assay

during *in vitro* osteogenic differentiation. Overexpression of Runx2 promoted a 6.5-fold increase in ALP activity as early as seven days post-differentiation when compared to controls (Figure 5a). ALP activity increased most rapidly with 700 MOI AdRunx2 transduction (Figure 5b). However, both AdRunx2 groups showed peak ALP activity at day 10 post-transduction with a similar maximum value (Figure 5a). These results indicate that overexpression of Runx2 enhances early osteoblast differentiation and demonstrate that increased Runx2 immobilization leads to faster stimulation of ALP activity.

As a second marker of *in vitro* osteoblast differentiation and function, we quantified calcium deposition in the extracellular matrix at time points up to 21 days. All data were normalized to DNA content in the same well (Figure 6a). Calcium content increased significantly at day 10 post-transduction and increased with extended incubation time until the end of the experiment. The amount of calcium in Runx2 groups was significantly higher than in the LacZ control group after 10 days.

Alizarin Red is a dye that can bind selectively with calcium and is widely used for visualization of matrix mineralization and calcium mineral histochemistry. In our case, mineralization was observed at day 14 in both Runx2 groups, while, no mineralization was observed in the LacZ group (Figure 6b). Following an incubation time of 21 days, matrix mineralization could be visualized in all groups (Figure 6b). Cells formed multilayers after 14 days incubation and some cell aggregates detached from the plate yielding a somewhat uneven pattern of staining. This finding provides additional evidence to demonstrate that immobilized AdRunx2 increases the rate of osteoblast differentiation of bone marrow stromal cells *in vitro*.

4. Discussion

With an aim to improve patient safety and reduce the need to transplant cells, *in vivo* regenerative gene therapy strategies are focused on spatial and temporal control of genetic factor release. Viral vectors released from polymeric microparticles may reduce the immunogenicity *in vivo* and prolong release time with diffusion and polymer degradation [40,41]. However, physical absorption on the outside of particles and limited loading efficiency may limit the effectiveness of this method. Solid-phase delivery is another approach to deliver genes from materials and typically relies on embedding virus or plasmid DNA within, or immobilized on a material surface [42]. Using this method, an injectable hydrogel has been used to treat head and neck tumors [43] and AdBMP2 lyophilized in gelatin sponges have been used to regenerate critical-size calvarial defects in preclinical studies [7].

Virus immobilization on biomaterials via antibody-antigen [44,45] and biotin-avidin binding [12,22,46] systems are currently used to localize and sustain release of virus. While, the lack of surface functional groups is one challenge that limits conventional inert biomaterials such as PLA, PLGA, and PCL for matrix-mediated virus delivery, this shortcoming may be overcome by generating reactive coatings with CVD. We previously demonstrated the feasibility of using a CVD technique to conjugate AdLacZ with a biotin-avidin-biotin system [22]. A potential limitation of this approach is that it requires modification of both adenovirus and material with biotin, which involves harsh chemical conditions. The objective of this study was to develop an easy and widely applicable strategy to tether adenovirus, specifically AdRunx2, using antibody-antigen interactions to accelerate osteoblast differentiation of BMSCs *in vitro*. This method can be widely applied in various systems by a simple change of antibody and antigen.

To reduce need of expensive antibodies, we optimized the conditions for antibody conjugation and determined the minimum antibody concentration necessary to saturate the functional groups on the CVD surface (Figure 2). Subsequently, we determined the virus binding

efficiency at saturation conditions to reduce unbound virus. Our methods demonstrate high efficiency of virus conjugation within a wide range of virus concentration (Figure 3a and b).

After optimization, we conjugated adenovirus encoding Runx2 on the surface and infected BMSCs. Transduction of BMSCs with immobilized AdRunx2 significantly increased Runx2 protein expression at all MOIs tested (Figure 4). Previous studies demonstrated that overexpression of Runx2 will upregulate osteoblast differentiation of BMSCs *in vitro* [27]. Three phenotype assays, including ALP activity, deposited calcium content and extracellular matrix mineralization, were characterized at different time points. ALP activity peaked at day 10 post-transduction which is a little later than results previously reported when using a free viral delivery system [27]. This may result from antibody-antigen interaction that delays the release of AdRunx2. All parameters show that AdRunx2 significantly stimulates osteoblast differentiation of BMSCs. Moreover, when more AdRunx2 is immobilized on the surface, faster and stronger of osteogenesis is observed.

Overexpression of transcription factors such as Runx2, unlike secreted proteins, will only affect cells that are directly transduced on the biomaterial scaffold. Thus, if a designed surface immobilization pattern is established, spatial control of infection may be accomplished to direct tissue regeneration. Pioneering studies have demonstrated the utility of selective surface modification and gradient signals with the CVD technique [18–20]. Combining this with our strategy will allow conjugation of multiple bioactive factors on the same matrix in specific patterns that may approach the gradients of signals that function *in vivo*.

5. Conclusions

In this study, we developed a simple and widely applicable strategy to immobilize cell-signaling adenovirus on inert biomaterials using a functionalized CVD coating method. A thin polymer film with functional groups was coated on PCL surfaces using a CVD coating technique. Subsequently, goat anti-adenovirus antibody was conjugated onto the surface followed by adenovirus immobilization via antibody-antigen interaction. Our application of this strategy demonstrated successful immobilization of AdRunx2 and enhancement of osteoblast differentiation of BMSCs *in vitro*. Our strategy could be widely applied in other systems using different antibody-antigen interactions. Combined with custom-tailored properties of the CVD technique, our method may be used to localize multiple bioactive factors with specific patterns for tissue regeneration.

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Figure 1.

Adenovirus immobilization on modified PCL surfaces. Chemical Vapor Deposition (CVD) was used to generate a thin polymer film with PFP functional groups on 2-D PCL films. Anti-adenovirus antibodies were conjugated to customized functional groups on the material surface. Finally, adenovirus was incubated and bound on the material via antibody-antigen interaction.

Figure 2. Saturation curve of antibody conjugation on CVD-coated PCL films. To test the capacity of antibody conjugation, goat IgG was used as model antibody and was conjugated on PCL films. An ELISA assay was used to determine the OD value (405nm) and the results were normalized to a standard amount of goat IgG. Values are reported as means $\pm$ SD, n=3.

Figure 3.

Virus binding efficiency evaluated in different conditions. (a) Three different amounts of antibodies near saturation levels (■ 5 µg/well, ▒ 10 µg/well, □ 15 µg/well) were conjugated on the CVD-coated PCL surfaces and then Ad-LacZ were added in a range of viral particles; (b) 15 µg/well of antibody was added to assess binding efficiency with wider range of viral particles.

Figure 4.

Runx2 protein expression in BMSCs transduced with different levels of immobilized AdRunx2 was assessed with a Western blot assay. Different amounts of AdRunx2 were bound on CVD-coated PCL films and then BMSCs (2.5×10^4 cells/cm² in 12-well cell culture plates) were seeded on the modified surfaces. After 48hrs infection, whole cell protein extracts were detected with antibodies against Runx2 and GAPDH.

Figure 5. Effects of AdRunx2 immobilization on alkaline phosphatase activity in BMSCs. (a) Time-course of ALP activity in three experimental groups: BMSCs transduced with AdRunx2 (MOI=400 Δ, 700 ○) or AdLacZ (MOI=700 ■) as control group; (b) ALP activity on day 10 post-transduction with different immobilized AdRunx2 and same cell seeding density (MOI=100, 400, 700, 1000). * p value < 0.05, n=5.

Figure 6.

Calcium deposition in extracellular matrix. a) AdRunx2 or AdLacZ with same MOI (700) were immobilized on CVD-coated PCL films and then incubated with BMSCs. (■ AdRunx2, □ AdLacZ). ** p value < 0.01, n=4. b) Effects of AdRunx2 immobilization on matrix mineralization. BMSCs were transduced with AdRunx2 (MOI=400, 700) or LacZ (MOI=700) bound to CVD-coated PCL films and then stained with Alizarin Red dye solution after 14 or 21 days.