ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

High-Throughput Screening of a Small Molecule Library for Promoters and Inhibitors of Mesenchymal Stem Cell Osteogenic Differentiation

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ABSTRACT: The use of high-throughput screening (HTS) techniques has long been employed by the pharmaceutical industry to increase discovery rates for new drugs that could be useful for disease treatment, yet this technology has only been minimally applied in other applications such as in tissue regeneration. In this work, an assay for the osteogenic differentiation of human mesenchymal stem cells (hMSCs) was developed and used to screen a library of small molecules for their potential as either promoters or inhibitors of osteogenesis, based on levels of alkaline phosphatase activity and cellular viability. From a library of 1,040 molecules, 36 promoters, and 20 inhibitors were identified as hits based on statistical criteria. Osteopromoters from this library were further investigated using standard culture techniques and a wider range of outcomes to verify that these compounds drive cellular differentiation. Several hits led to some improvement in the expression of alkaline phosphatase, osteogenic gene expression, and matrix mineralization by hMSCs when compared to the standard dexamethasone supplemented media and one molecule was investigated in combination with a recently identified biodegradable and osteoconductive polymer. This work illustrates the ability of HTS to more rapidly identify potential molecules to control stem cell differentiation.

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KEYWORDS: tissue engineering; stem cells; high-throughput screening; small molecules; differentiation

Introduction

As research has expanded toward the application of stem cells as a cell source for tissue regeneration and cell replacement therapies, the importance of driving differentiation and cell behavior in a controlled fashion is evident. Human mesenchymal stem cells (hMSCs) are readily available adult stem cells that have been investigated extensively in recent years as an autologous cell source and have been shown to differentiate along chondrogenic (Chung et al., 2009; Mackay et al., 1998; Pittenger et al., 1999), adipogenic (Benoit et al., 2008; Treiser et al., 2010), osteogenic (Benoit et al., 2008; Brey et al., 2010; Engler et al., 2006; Jaiswal et al., 1997; Nuttelman et al., 2004; Treiser et al., 2010), and neuronal (Tao et al., 2005) lineages when provided the appropriate chemical or physical cues. Physical cues may include mechanical stiffness of the surrounding matrix (Engler et al., 2006), the presentation of functional groups in the encapsulating cell environment (Benoit et al., 2008), or even the shape of the cell (Ruiz and Chen, 2008). These are important signals that may be incorporated into scaffold design for regenerative medicine to obtain specific differentiation paths of delivered or interacting cells.

The assessment of the proper chemical cues has also been extensively investigated, and can be divided between the use of either growth factors or the use of small molecules. Growth factors can naturally drive cell differentiation, such as during development and wound repair, through interactions with cell receptors and via cell signaling cascades. For example, transforming growth factor beta (TGF-β) is crucial for the chondrogenic differentiation of hMSCs (Chung and Burdick, 2009; Huang et al., 2008), while combinations of basic fibroblast growth factor, epidermal growth factor, and

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platelet-derived growth factor may lead to neuronal differentiation (Tao et al., 2005). Likewise, bone morphogenetic proteins (BMPs) have been important drivers of hMSC osteogenic differentiation (Friedman et al., 2006; Sasano et al., 1993). Of the many variations in the BMP family, the use of BMP-2, -4, and -7 have all been shown to stimulate osteoblast markers in cells that already have a predisposition to osteoblast differentiation and have lost their multipotency, termed osteoprogenitor cells (Diefenderfer et al., 2003; Kanczler et al., 2010; Li et al., 2005; Viereck et al., 2002). However, this effect appears mitigated when explored in hMSCs that still retain the ability to differentiate into other cell lines (Diefenderfer et al., 2003; Friedman et al., 2006; Gruber et al., 2003; Osyczka et al., 2004; Shui et al., 2003). Similarly, while BMPs have shown great promise in stimulating bone growth in rat and rabbit models (Brey et al., 2010; Kakudo et al., 2006a,b; Okubo et al., 1999, 2000; Peng et al., 2005; Sasano et al., 1993; Yasko et al., 1992), there has only been limited success in human clinical studies (Giannoudis and Einhorn, 2009; Schmidmaier et al., 2009), which often require large quantities of the expensive molecules for even modest improvements. BMP-6 has also been shown to drive the osteogenic differentiation of hMSCs (Friedman et al., 2006; Gruber et al., 2003; Vukicevic and Grgurevic, 2009) in vitro as well as in animal models, though these treatments have not progressed to clinical trials.

These limitations have directed many researchers towards the identification of small molecules as osteogenic promoters (Jaiswal et al., 1997; Peters et al., 2009; Wu et al., 2002). In particular, dexamethasone (Dex) is commonly used for in vitro cell studies. Dex is a synthetic glucocorticoid that is often used as an anti-inflammatory drug and in cancer treatments, but has also acted as a potent osteopromoter in 2D cultures. Dex has been shown to induce the osteogenic differentiation of MSCs, pericytes, bovine vascular smooth muscle cells, and mouse NIH3T3 fibroblasts (Kirton et al., 2006). Several studies have shown optimal Dex concentrations to be between 10 and 100 nM (Jaiswal et al., 1997; Kuznetsov et al., 1997; Meinel et al., 2004; Otto and Rao 2004; Pittenger et al., 1999) in the presence of 5–10 mM beta-glycerol phosphate (βGP) and ascorbic acid (or ascorbic acid-2-phosphate, AA2P). Our lab has previously used a formulation of 10 nM dexamethasone, 10 mM βGP, and 25 μg/mL AA2P to drive robust hMSC osteogenic differentiation (Brey et al., 2010).

Unfortunately, these osteogenic effects studied in vitro have not translated into clinical practice. First, while Dex, like many glucocorticoids, are potent anti-inflammatory agents, prolonged treatment can actually lead to osteoporosis (Adachi 1997; Augat et al., 2005; Sawin et al., 2001; Walsh et al., 2001). This is suspected to be due to the suppression of the proliferation of osteoblastic precursors, which allows osteoclasts to break down bone without subsequent replacement with new bone (Walsh et al., 2001). Next, intramuscular injection of Dex at the site of a spinal fusion actually inhibited bone graft incorporation (Sawin et al., 2001). Finally, while there has been some success with implanted Dex releasing hMSC-seeded scaffolds (Kim et al., 2003, 2005), this technique of delivery adds a level of clinical complexity, cost, and time that could be better avoided if native progenitor cells could be recruited and differentiated in situ.

While control over tissue formation is of interest for many applications, there are some diseases where the prevention of differentiation and matrix production may be desired. In the case of bone, progenitor cells have been found to form heterotopic bone in models for fibrodysplasia ossificans progressiva (FOP) (Lounev et al., 2009). This disease is caused by a mutation to a BMP receptor, ACVR1, that causes connective tissues to spontaneously form bone (Shore et al., 2006). Interestingly, Dex has also been implicated in stimulating osteogenic differentiation of pericytes that can lead to the formation of vascular calcification present in many cardiovascular diseases (Kirton et al., 2006). Additionally, heterotopic bone formation can be fairly common in elbow and acetabular fractures, especially when accompanied by an injury to the central nervous system (Pape et al., 2004; Shehab et al., 2002). Ballistic and blast injuries can also lead to heterotopic bone formation (Forsberg et al., 2009; Volgas et al., 2005). Therefore, new therapies to inhibit progenitor cells from differentiating into osteoblasts could be beneficial for a variety of clinical conditions.

These limitations of Dex and the high cost and limited effectiveness of BMP therapy motivates the need for the identification of alternative factors that stimulate osteogenic differentiation. Likewise, conditions of excess bone formation suggest a need for new molecules that inhibit osteogenesis. Little effort has been made to identify new soluble factors for their osteogenic potential in stem cells (Wu et al., 2002); however, high-throughput screening (HTS) tools and techniques are being developed to explore large libraries of molecules and materials for a variety of applications (Desbordes et al., 2008; Ding et al., 2003; Huang et al., 2008; Peters et al., 2009; Underhill and Bhatia, 2007; Zhao and Ding, 2007). The advantages of HTS methods allows for more combinations to be assessed faster, and with fewer reagents, so that new compounds can be discovered and more complex delivery schemes can be tested.

In this study, we developed techniques for the assessment of a library of soluble factors for promoters and inhibitors of the osteogenic differentiation of hMSCs using HTS tools. Hits were identified using statistical relationships to controls, as well as meeting criteria related to viability. Towards our interests in tissue engineering, promoters were then screened further using dose–response studies and traditional stem cell differentiation outcomes to confirm these effects and one molecule was used for the culture of hMSCs in combination with a biodegradable and osteoconductive polymer.

Materials and Methods

HTS Assay Development

Human MSCs (Lonza) were cultured in growth media (αMEM, 17% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine) and plated onto 384-well,

flat bottom plates (Corning, NY) at passage 3 or 4 in 40 μL of media per well using a microplate dispenser (Matrix Wellmate, Thermo Scientific, Hudson, NH). Plates were sealed with Breath-Easy gas permeable membranes (Research Products International Corp., Mt. Prospect, IL) to minimize evaporative losses. The following day, media was changed to incomplete osteogenic media (OG−, growth media plus 10 mM βGP and 25 μg/mL ascorbic acid-2-phosphate) or complete osteogenic media (OG+, OG− plus 10 nM dexamethasone), depending on the goal of identifying either promoters or inhibitors, respectively.

After 7 days of culture, 4 μL of Alamar Blue was added to each well and the fluorescence was measured (535 nm excitation/595 nm emission, EnVision, Perkin Elmer, Waltham, MA) after incubation for 15 and 30 min to assess relative viability. Cells were then washed with PBS, and lysed in the wells with 5 μL of CelLytic M (Sigma, St. Louis, MO) at 37°C for 15 min. Plates were cooled for 5 min on ice before the addition of 45 μL of the fluorescent ALP detection reagents (40 μL fluorescent buffer, 5 μL dilution buffer, 0.25 μL 4-methylumbelliferyl phosphate disodium salt, APF, Sigma). Plates were then read every 10 min for 1 h at 355 nm excitation/450 nm emission to determine ALP activity.

Various aspects of this protocol were optimized prior to the library screening. These included the timing of media change (no change for the entire 7 days vs. changing once after 4 days), the cell seeding density (24, 18, 12, and 6 thousand cells/cm^2), and the concentration of DMSO (1.0%, 0.75%, 0.50%, 0.25%, 0.10%, and 0% DMSO) used for addition of the small molecules. Ultimate ALP activity, separation between ALP activity in cells grown in OG− media (negative controls) and OG+ media (positive controls) using *Z*-factor analysis (Zhang et al., 1999) (see the Statistical Analysis Section), and viability were used to identify the optimal conditions.

HTS NINDS Library Screen

Once the HTS assay optimization was complete, the approach was used with the National Institute of Neurological Disorders and Stroke (NINDS) chemical library of 1,040 small molecules (provided and listed by Microsource Discovery Systems, Inc., Gaylordsville, CT, www.msdiscovery.com/ninds.html). The specific procedure used is shown in Figure 1. Cells were plated in each well at 12,000 cells/cm^2 (~720 cells per well) in 40 μL of growth media. The next day, the media was switched to OG− (for promoter studies) or OG+ (for inhibitor studies). Soluble factors were diluted in DMSO and added using a robotic liquid handling system (Evolution P3 Pipetting Platform, PerkinElmer) for a final concentration of 10 μM and 0.1% DMSO. In each plate the first and last two columns were reserved for positive (OG+ with 0.1% DMSO) and negative (OG− with 0.1% DMSO) controls. At day 5 (4 days since molecules were added), the media and soluble factors were refreshed. Finally, 7 days after soluble factors were added, cell viability and ALP activity were measured as described above.

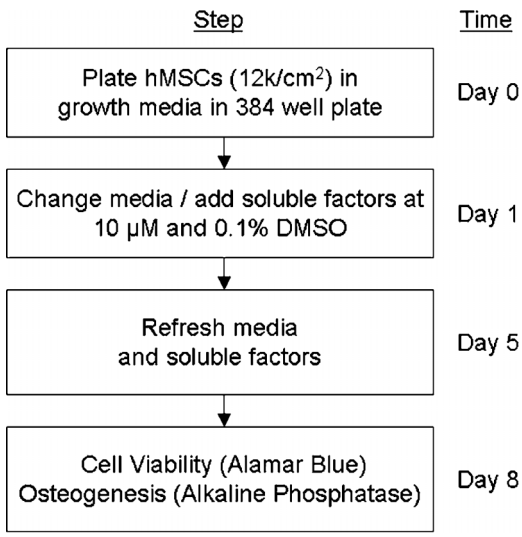

Figure 1. Flow chart for HTS experiments. First, cells were plated in 384-well plates overnight to allow for attachment. On the next day, the media was changed to OG− for promoter and OG+ for inhibitor experiments, and soluble factors were added at a concentration of 10 μM. Four days later, the media was refreshed and factors were added again. After 7 days, the cell viability was assessed using Alamar Blue and the cells were lysed to determine their ALP activity.

Hits were determined only in plates with a *Z*-factor >0.50 (see the Statistical Analysis Section). For promoters, hits were wells that had an ALP activity level greater than three times the coefficient of variation (CV) above the negative control and cell viability >75% of the negative controls. For the inhibitors, hits were wells that had an ALP activity level less than three times the CV below the positive control and cell viability >75% of the positive controls. Promoters were then studied in a dose–response experiment (3–1 × 10^5 nM) to confirm the osteogenic effects and determine the most potent hits.

Traditional Cell Culture

The most potent hits were studied further using traditional cell culture techniques. From the initial library, three factors were selected for further study: triamcinolone diacetate (TD), fludrocortisone acetate (FC), and medrysone (Med). For these studies, hMSCs were plated on 6-, 12-, or 24-well plates at 6,000 cells/cm^2 and cultured for up to 3 weeks using protocols previously developed in the lab (Brey et al., 2010). Cells were cultured in OG− (negative control), OG+ (positive controls), and 1, 10, and 100 nM concentrations of TD, FC, and Med, with the media changed every 2–3 days. Cells were lysed in 150 μL of CelLytic M at 37°C for 20 min at 3, 7, 11, 14, and 21 days, spun down at 12,000 G for 15 min and the supernatant collected for analysis.

Cell proliferation was measured using the Quant-iT™ PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA). Samples were prepared by diluting 2 μL of cell lysate in 23 μL of TE buffer and reacting with 25 μL of working solution (40 μL of PicoGreen reagent with 8 mL TE buffer) and fluorescence was measured at 480 nm excitation and 520 nm emission. The ALP activity was measured using the procedure as described previously with 5 μL of lysate. On some plates, cells were fixed in formalin after 3 weeks and calcium deposits were visualized with Alizarin Red staining (Wang et al., 2006). Alizarin Red staining was quantified by eluting the dye in 10% (w/v) cetylpyridinium chloride, and measuring absorbance at 562 nm. Additionally, RNA was extracted at 14, 18, and 21 days using TRIzol® (Invitrogen) and gene levels were measured using quantitative PCR for the osteogenic markers osteocalcin and CBFA1/Runx2 and normalized to the housekeeping gene GAPDH (Table I) followed by normalization to the cell pellet gene expression levels at day 0.

Culture With an Osteoconductive Polymer

One of the most promising promoter molecules was then used in conjunction with a previously identified osteoconductive poly(β-amino ester), termed A6 (Brey et al., 2010), to test for the osteogenesis of hMSCs. A6 is a photocrosslinkable and biodegradable polymer that can be processed into a range of scaffolds using radical polymerization techniques. Thin films of the polymer were photocrosslinked on the bottom of 12- and 24-well plates as in (Brey et al., 2010), and cells were plated and cultured as described above. Cell proliferation, ALP expression, and mineralization were investigated as described above, for hMSCs grown in OG−, OG+, and in the presence of one osteopromoter molecule.

Statistical Analysis

The quality of assay was determined using *Z*-factor analysis (Zhang et al., 1999) comparing the positive controls to the negative controls.

$$Z = 1 - 3 \times \frac{\sigma_p + \sigma_n}{\mu_p - \mu_n} \quad (1)$$

where σ_p and σ_n are the standard deviation of the positive and negative control values, respectively, and μ_p and μ_n are their means. A score >0.5 indicates an excellent assay, which delineates possible hits from statistical noise on a given plate. The percent activity of a given well was determined as:

$$\%\text{Activity} = \frac{x - \mu_n}{\mu_p - \mu_n} \times 100 \quad (2)$$

where x was the fluorescence reading of a given well. A "hit" was determined as a factor whose percent activity was more than three times the CV above the negative control values for promoters and three times the CV below the positive control for inhibitors. Again, "hits" were only explored further if determined to be non-toxic, by rationale of viability that is at least 75% of control values.

Statistical analysis for non-HTS studies were performed using ANOVA with Tukey's post hoc among the groups with significance defined as a P-value <0.05. All values are reported as the mean and the standard deviation of the mean.

Results and Discussion

HTS Assay Development

The advantage of HTS is the ability to rapidly test many conditions while using fewer reagents on smaller platforms. In this work, our goal was to optimize an HTS assay to assess the osteogenic differentiation of hMSCs. We chose to use standard osteogenic media in these studies and ALP activity as the primary marker of osteogenesis, given its common use in stem cell differentiation studies and its compatibility with the HTS process. The development of any HTS assay requires the optimization of a variety of culture parameters (e.g., media changes, seeding density, media components).

For this study, the optimization of MSC culture conditions was completed to best demonstrate osteogenic differentiation (i.e., ALP activity), as shown in Figure 2. In normal culture, cell media is changed once every 2–4 days to remove waste and provide new nutrients, but in HTS, because of the scale, these changes are not always necessary. In this study, hMSCs grown for 7 days in OG+ media with a media change at 4 days showed nearly a threefold increase in ALP expression over hMSCs grown in OG+ media without refreshing the media (Fig. 2A). Thus, a media change was determined to be necessary after 4 days. More frequent changes were not explored due to this regime meeting acceptable criteria and that additional changes would increase complexity of experiments.

Beyond the culture conditions, the cell seeding density has been shown to be important in determining stem cell

Table I. Quantitative PCR primers and probes.

Gene	Forward primer	Reverse primer	Probe
GAPDH	AGGGCTGCTTTTAACTCTGGTAAA	GAATTTGCCATGGGTGGAAT	CCTCAACTACATGGTTTAC
Osteocalcin	CTGGCCGCACTTTGCAT	CTGCACCTTTGCTGGACTCT	CACCTGCCTGGCCAGC
CBFA1/Runx2	TGGACCTCGGGAACCCA	GCGGTCAGAGAACAAACTAGGTT	AAGGCACAGACAGAAGC

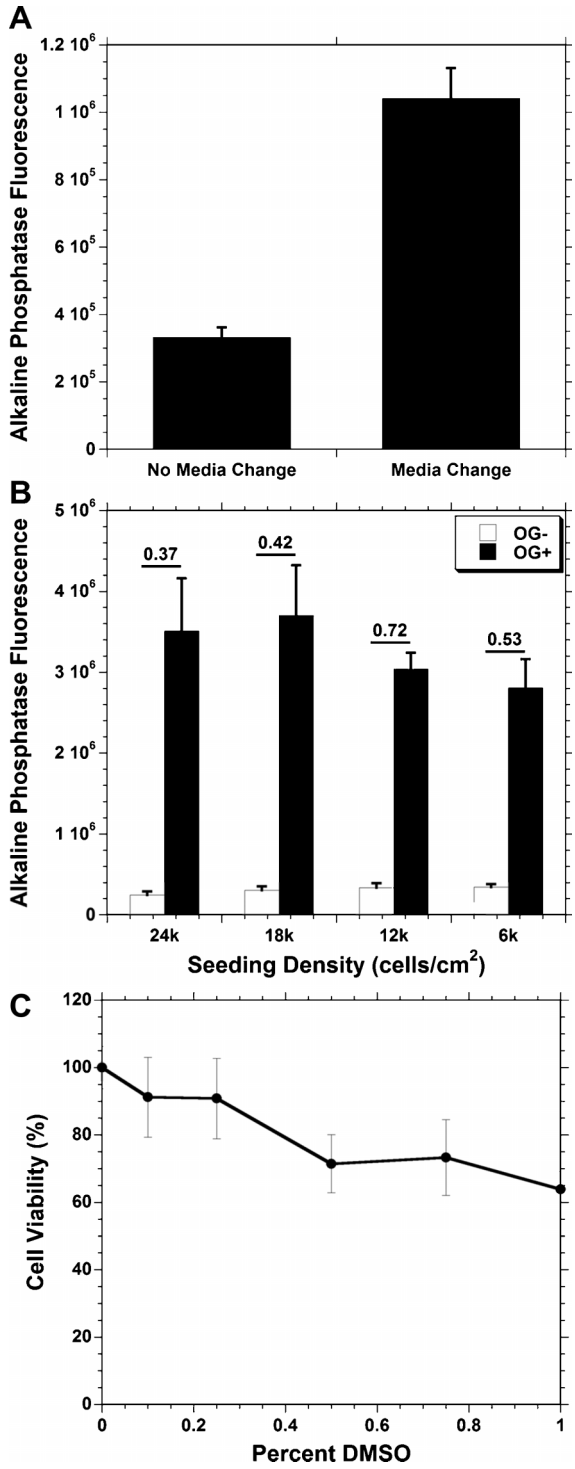

Figure 2. Assessment of conditions for optimal osteogenesis and viability of hMSCs in 384-well plates. **A**: ALP levels of hMSCs cultured in OG+ media for 7 days with a media change at 4 days and without. **B**: Differences in ALP activity of the positive and negative controls for osteogenesis for various cell seeding densities, the *Z*-factor for each pairing is reported above each bar. **C**: MSC sensitivity (reported as viability assessed with Alamar Blue) to DMSO since the small molecules are added in a DMSO solution.

differentiation (Ruiz and Chen, 2008; Treiser et al., 2010). Thus, cells were plated at 24, 18, 12, and 6 thousand cells/cm^2 in OG− or OG+ medium to determine the density for optimal distinction of osteogenic differentiation between these positive and negative controls. While there was several fold differences in ALP expression in all cases (Fig. 2B), the lower variability of the cells seeded at 12,000 cells/cm^2 led to a *Z*-factor of 0.72, while samples at a higher density failed to achieve a *Z*-factor score >0.5. Thus, a seeding density of 12,000 cells/cm^2 was used in all subsequent studies (except for long-term studies). Finally, the soluble factors are dissolved in DMSO for addition to the media, so the DMSO toxicity to hMSCs was tested using a cell viability dose–response study (Fig. 2C). The cells were still ~90% viable at 0.1% and 0.25%, but dropped below 75% as DMSO quantities increased. To minimize cell death from DMSO, the final concentration of DMSO in all studies was set at 0.10%. These studies illustrate the sensitivity of the cells to culture conditions and parameters, which can diminish statistical relevance. With this in mind, it is important to characterize and optimize these procedures for each cell type and outcome of interest.

Identification of Osteogenic Promoters and Inhibitors

After optimization of the cell culture conditions for HTS and hMSC osteogenesis, the NINDS library was screened to identify potential promoters and inhibitors of hMSC osteogenic differentiation, using ALP activity and viability as outcome parameters. Although there are many outcomes that could have been chosen as markers of osteogenesis, it was important to choose a marker that is easily measured in high throughput and with the available equipment; thus, ALP was used.

Representative plots for the HTS assay are shown in Figure 3. From the screen of 1,040 compounds, 36 potential promoters (high ALP activity compared to controls, high cell viability), and 20 potential inhibitors (low ALP activity compared to controls, high cell viability) were identified, as shown in Table II, which met the requisite requirements as a "hit." Many of the inhibitors that met the criteria for low ALP did so only by killing the cells, illustrating the importance of assessing cell viability. Also, many of the compounds stimulated cell viability >100%, indicating potential proliferative changes due to the small molecules. There is wide diversity in these outcomes and the molecules identified, illustrating the potential of HTS to identify molecules that could not have led to predictable outcomes.

The inhibitors display a wide range of molecules that include antifungals (griseofulvin), anticancer drugs (mechlorethamine, isotretinoin), and sex hormones. Interestingly, there are several progestins (hydroxyprogesterone, norgestrel, norethynodrel, norethindrone, cyproterone acetate) which act similarly as estrogens and are often elevated during pregnancy. There is some indication that FOP disease is inhibited during pregnancy (Fox et al., 1987; Thornton et al., 1987), and although only speculative, these hits may be an indicator of how that occurs. Estrogen has been implicated as

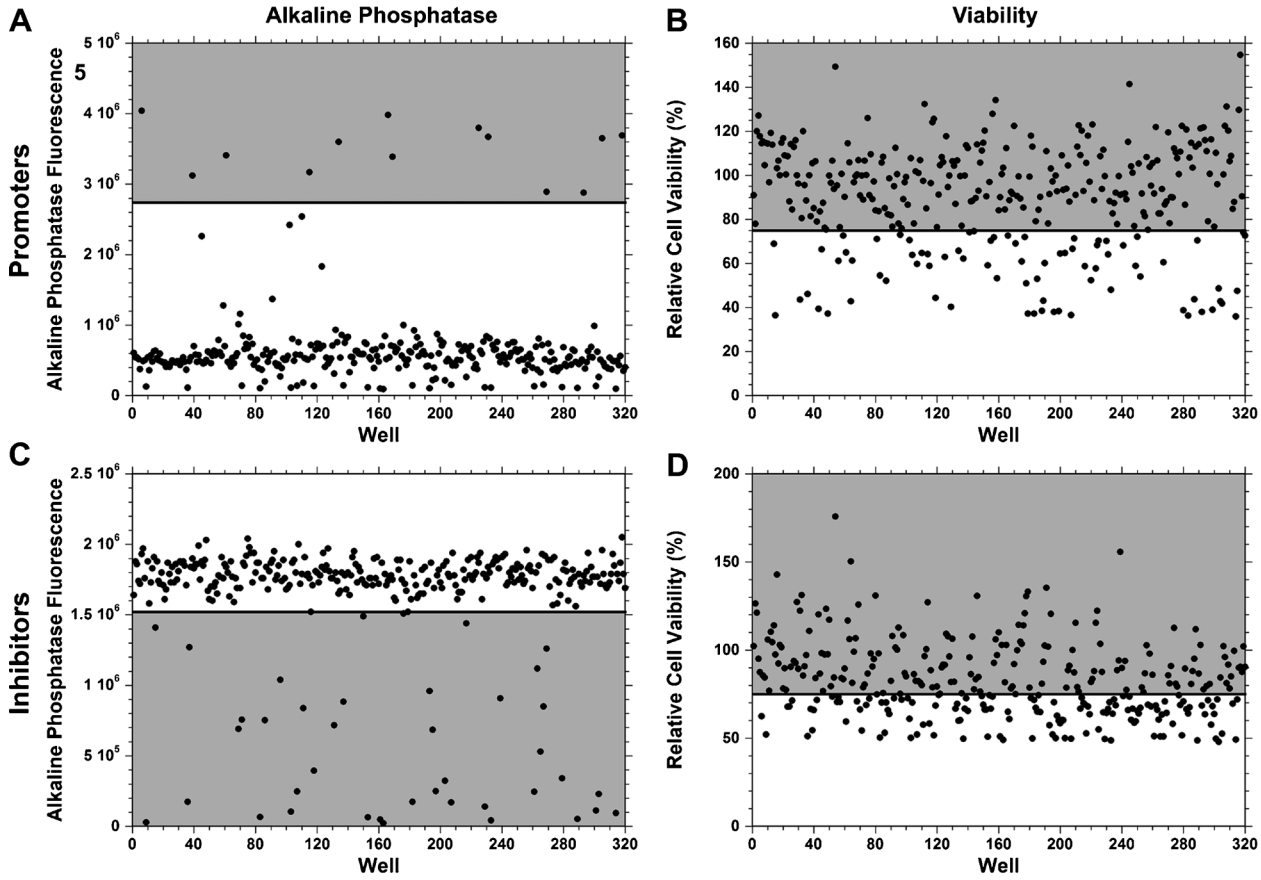

Figure 3. Sample plots of plates for promoter (**A** and **B**) and inhibitor (**C** and **D**) experiments with outcomes of ALP activity and viability. "Hits" for promoters (gray) were defined as having (A) ALP levels greater than three times the CV above cells in OG− and (B) cell viability at least 75% that of cells in OG−. "Hits" for inhibitors (gray) were defined as having (A) ALP levels less than three times the CV below cells in OG+ and a (B) cell viability at least 75% that of cells in OG+ (gray).

being beneficial to coronary heart disease (CHD) (Klinge et al., 2005; Solomon and Dluhy, 2003) and inhibition of osteogenesis of pericytes could prevent vascular calcification (Kirton et al., 2006), although recent concerns with CHD associated with hormone replacement therapy has further complicated this picture. At the same time, testosterone is also a potential inhibitor, even though testosterone and estrogen have both been shown to help prevent osteoporosis (Riggs et al., 1998, 2002; Snyder et al., 1999). Although these inhibitors may have great potential in these and other disease applications, they were not investigated further in this study.

In addition to inhibitors and promoters, it is possible to use the inhibitor setup to investigate ALP activity that is statistically above the level of the positive controls. This could help identify potential supplements to media that may not have an osteogenic effect on hMSCs alone, but may be synergistic with other compounds. While not expanded upon here, edoxudine did enhance ALP activity in cells grown in OG+ during the study for inhibitors.

The promoters of hMSC osteogenic differentiation were also a diverse collection of factors. These included several glucocorticoids (hydrocortisone acetate, metamethasone, fluorometholone, halcinonide, medrysone) that could act along the same pathways as Dex. While many of the hits for osteopromoters were statistically improved from negative controls, many did not necessarily show improvements that were greater than the response to OG+ media. The top hits were then screened again using the HTS assay for a dose–response (Fig. 4). The results of the dose–response found that many of the identified hits were not as potent as observed in the initial HTS screen (dose of 10 μM). This highlights the importance of performing additional assays after the initial screening to clarify the activity of specific molecules. Only a handful of molecules stimulated ALP activity at concentrations at the nM range that is often used in Dex experiments. These compounds were considered the most potent, while some of the other hits were only osteogenic at very high concentrations or were false hits and were not explored further.

Traditional Cell Culture

Several previous studies have used HTS techniques to identify small molecules that governed stem cell fate, but

Table II. Osteogenic promoters and inhibitors of hMSCs identified by the initial screen of the NINDS library.

Promoters
Acetanilide
γ-Aminocutyric acid
Aminohippuric acid
Antipyrine
Aspirin
Avobenzone
Cephalexin
Chloroguanide hydrochloride
Cholecalciferol
Clopamide
Dienestrol
Doxycycline hydrochloride
Ebselen
Fenthion
Fludrocortisone acetate
Fuorometholone
Furegrelate sodium
Gentamicin sulfate
Halcinonide
Inositol
Isopropamide iodide
Medroxyprogesterone acetate
Medrysone
Minocycline hydrochloride
Nalbuphine hydrochloride
Nateglinide
Olanzapine
Protoveratrine A
Ropinirole
Spiperone
Tolazamide
Triamcinolone diacetate
Trimebutine maleate
Tuaminoheptane sulfate
Valacyclovir hydrochloride
Zolpidem
Inhibitors
7,4'-Dihydroxyflavone
Anethole
Clorsulon
Cyproterone acetate
Desoxycorticosterone acetate
Ethacrynic acid
Griseofulvin
Hydroxyprogesterone
Hydroxyprogesterone caproate
Isotrentinon
Mechlorethamine
Menadione
Norethindrone
Norethynodrel
Norgestrel
Phentolamine hydrochloride
Securinine
Tannic acid
Testosterone
Testosterone propionate

these often involved mouse cell lines (Ding et al., 2003; Wu et al., 2002, 2004) or did not confirm the identified molecule activity using traditional cell culture techniques (Huang et al., 2008). Therefore, some promoters from the dose–response study were investigated further in larger cultures to confirm the osteogenic effects beyond ALP activity. The most potent hits from the dose–response study were mainly glucocorticoids, similar to Dex, though with different levels of potency (Grossmann et al., 2004) for the glucocorticoid receptor (GCR). Therefore, three compounds were chosen that had low GCR potency (Med), medium GCR potency (TD), and a potent mineralocorticoid (FC) to further investigate with traditional cell culture techniques.

These compounds were tested for up to 21 days at 1, 10, and 100 nM concentrations and compared to the OG+ media with the highly GCR potent 10 nM Dex. The cell proliferation was very similar in all cases (Fig. 5A), but in samples with <100 nM of drug, the cells eventually detached from the tissue culture polystyrene (TCPS) in a sheet. This is common in MSCs grown in growth media if they are allowed to grow beyond confluency. Interestingly, this does not occur in cells in OG+ media, likely due to the mineralized matrix being produced that alters how the cells are interacting with the substrate. Similarly, cells treated with 100 nM of the FC, TD, and Med all remained attached throughout the 3-week study. The ALP activity (Fig. 5B) showed a slight increase in all of the 100 nM samples at 14 days and ~25% increase at 21 days as compared to cells grown in OG+ media.

RNA expression of osteogenic genes of interest provided additional information (Fig. 6) at later time points once cells were dense enough to collect DNA. CBFA1 is a transcription factor that has been associated with osteoblast differentiation, and while these levels are slightly elevated from the starting levels in hMSCs, there does not appear to be much variation between the different inducers. While CBFA1 levels may be marginally increased, this assay does not investigate the activity post-translation, which is even more important in human cells (Rajgopal et al., 2007). Conversely, osteocalcin is a bone-specific matrix protein that is usually active late in fracture healing just as mineralization is beginning, and the expression levels improve with time. At 21 days, the osteocalcin expression in the 100 nM TD samples was ~2.5-fold better than OG+ levels, which could indicate increased matrix production.

Alizarin Red staining for calcium deposits, a component of bone matrix, show that only the hMSCs grown in FC media deposit comparable matrix to OG+ positive controls (Fig. 7). Staining was significantly less than OG+ controls on the Med and TD samples, even though osteogenic genes were upregulated. Once again, this difference could be due to a post-translational change in the cells that could further activate CBFA1. While levels of CBFA1 may be similar between groups, their activity could be hampered or enhanced accordingly.

These results indicate that these factors newly identified through HTS methods show improvement in some of the general indicators of osteogenic differentiation and mineralized matrix production when compared to Dex. ALP expression is elevated 2 and 3 weeks for all three factors studied, with comparable cell survival and proliferation. Osteocalcin expression was increased for TD samples at all

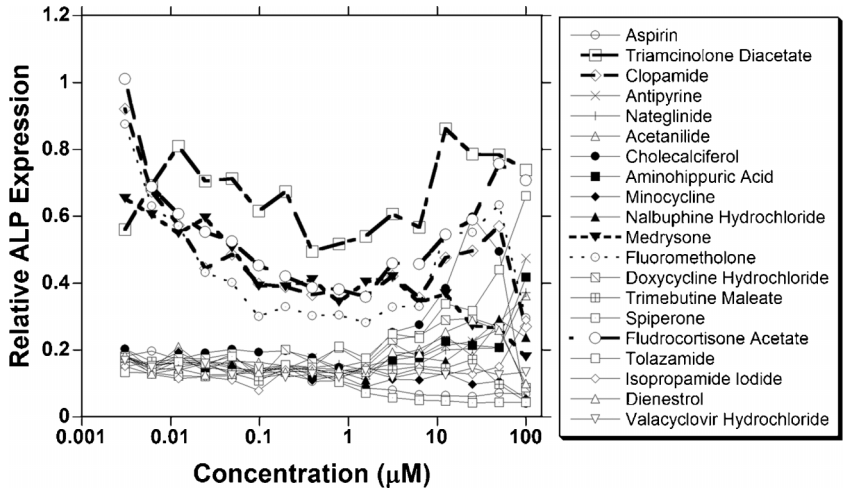

Figure 4. Alkaline phosphatase activity dose–response to the top 20 osteopromoters relative to OG+ controls. Note that many of the molecules did not perform as well in the dose–response study as in the initial screening.

time points when compared to all other groups. Unfortunately, this did not translate to improvements in mineralized matrix production, as shown in the Alizarin Red staining.

Interestingly, the FC samples that showed the most staining for calcium deposits only had modest increases in CBFA1 and OC expression. The staining showed the presence of many nodules on the FC-treated cells, which was

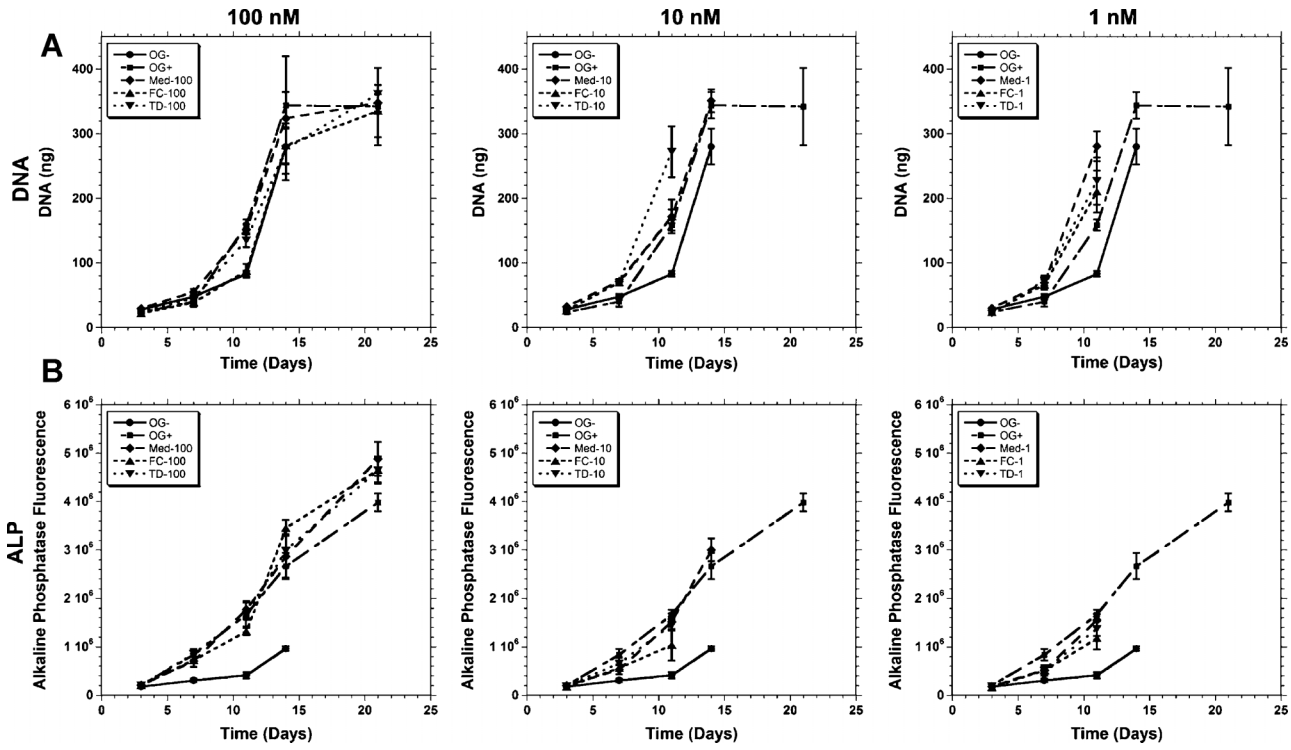

Figure 5. Temporal response of hMSCs with exposure to three of the top osteopromoters at various concentrations. The amount of (**A**) DNA and (**B**) ALP expression with exposure to 100, 10, and 1 nM of medrysone (Med), fludrocortisone acetate (FC), and triamcinolone diacetate (TD) is shown over 21 days. Note: Lines that do not continue through entire time course indicate cell layer detaching from monolayer culture.

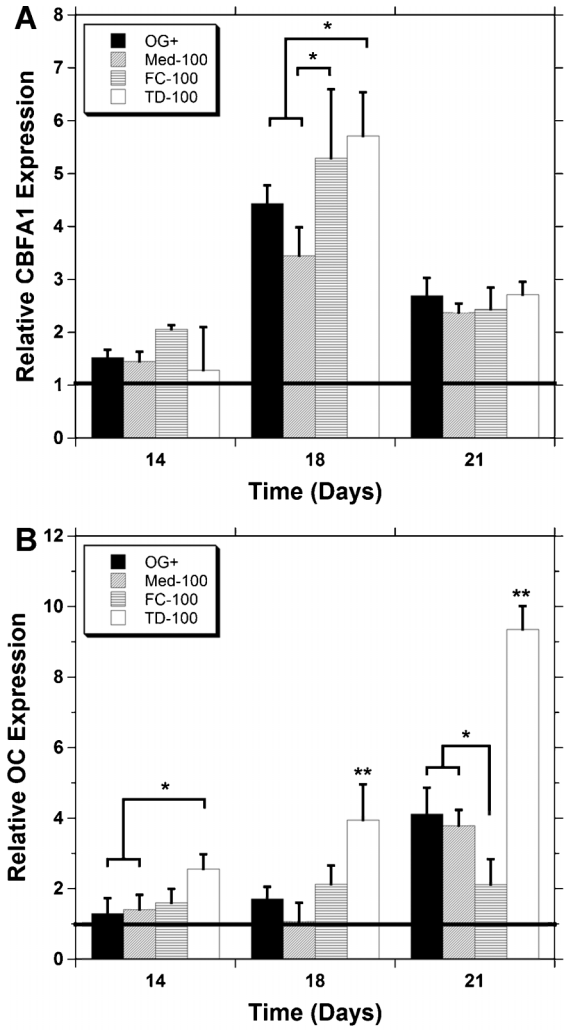

Figure 6. Expression of osteogenic markers in hMSCs compared to cell pellet at day 0. Expression of (**A**) CBFA1 and (**B**) osteocalcin after culture in OG+ media or 100 nM of medrysone (Med), fludrocortisone acetate (FC), and triamcinolone diacetate (TD) for 14, 18, and 21 days. *Significance between indicated groups, **Significance from all groups at that time point.

comparable to the staining observed on the Dex-treated hMSCs. Unlike Dex, Med, and TD, which are glucocorticoids, FC is a potent mineralocorticoid, which may demonstrate another pathway to stimulate osteogenic differentiation of stem cells.

These results also indicate some of the limitations of HTS techniques in identifying molecules that dictate stem cell fate. First, many of the "hits" were not as effective in the dose–response study as they appeared in the initial screen. Then once some of the best hits were screened using traditional cell culture techniques, the increase in ALP activity (the marker for a "hit") did not always lead to improvement in outcomes (e.g., matrix mineralization).

While these results show that there may be other steroids that may stimulate osteogenic differentiation in vitro, this effect has not been replicated in vivo. Dex has been used for years in culture, but there are no reports of using Dex alone to recruit progenitor cells and stimulate differentiation in animal models. This may be a limitation of the Dex that these newly identified compounds may help overcome. There may also be limitations to using these steroids in general since they could have counteracting effects on neighboring cells as well.

Although this is just the first step towards assessment of these identified molecules and no in vivo work has been performed, this study emphasizes the utility of techniques such as HTS to identify new molecules to control stem cell behavior. For instance, these molecules could be readily incorporated into disease therapies or tissue engineering scaffolds to modulate cellular behavior. It is the hope that this technology can be useful to identify molecules that would have been difficult to isolate using traditional culture techniques. In addition, this study only looked at a relatively small library to test the concept, and several potential candidates were identified. Larger libraries could be probed using these techniques to identify other compounds to control osteogenesis in hMSCs.

Culture With an Osteoconductive Polymer

Mineralized tissue regeneration approaches typically use a combination of osteoinductive factors, osteoconductive matrices, and cells (e.g., stem cells) for tissue production and repair. Towards our interests in using principles of tissue engineering, the best promoter identified in this study, FC, was used to investigate the osteogenesis of hMSCs grown on an osteoconductive and biodegradable polymer (A6, macromer structure shown in Fig. 8A). This polymer has been shown previously to support the osteogenic differentiation of hMSCs in vitro using Dex and new bone formation occurred in vivo with three-dimensional scaffolds of A6 in combination with BMP-2 (Brey et al., 2010).

HMSCs were cultured for up to 3 weeks on thin films of A6 in OG−, OG+, and 100 μM FC media formations. Cells proliferated most rapidly in OG− media, but after 14 days, the cells detached from the surface as a sheet (Fig. 8B). The cells proliferated more slowly on the OG+ and FC-treated wells, but they remained attached throughout the duration of the experiments. The ALP activity was elevated in the OG+ and FC-treated wells compared to cells grown in OG− (Fig. 8C). Notably, hMSCs proliferated better in FC media than OG+, and ALP activity was improved as well.

HMSCs grown for 21 days in FC and OG+ media stained positive (dark red nodules) for mineralization using Alizarin Red (Fig. 8D). However, the polymer has some level of background staining with this dye and quantification could not be completed. These studies highlight that a molecule identified using HTS can be used to enhance mineralization

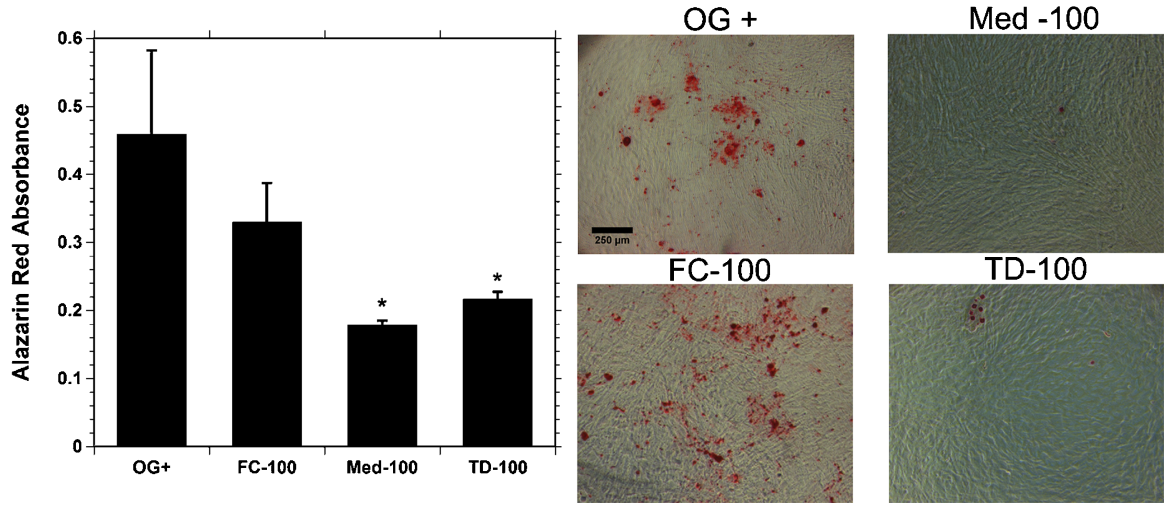

Figure 7. Alizarin Red staining of hMSCs on TCPS. hMSCs were grown for 21 days in OG+ and OG− media supplemented with 100 nM Med, FC, or TD and stained for calcium deposition, indicating mineralized matrix production. No staining was observed in OG− cultures. *Significance between indicated group and OG+. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

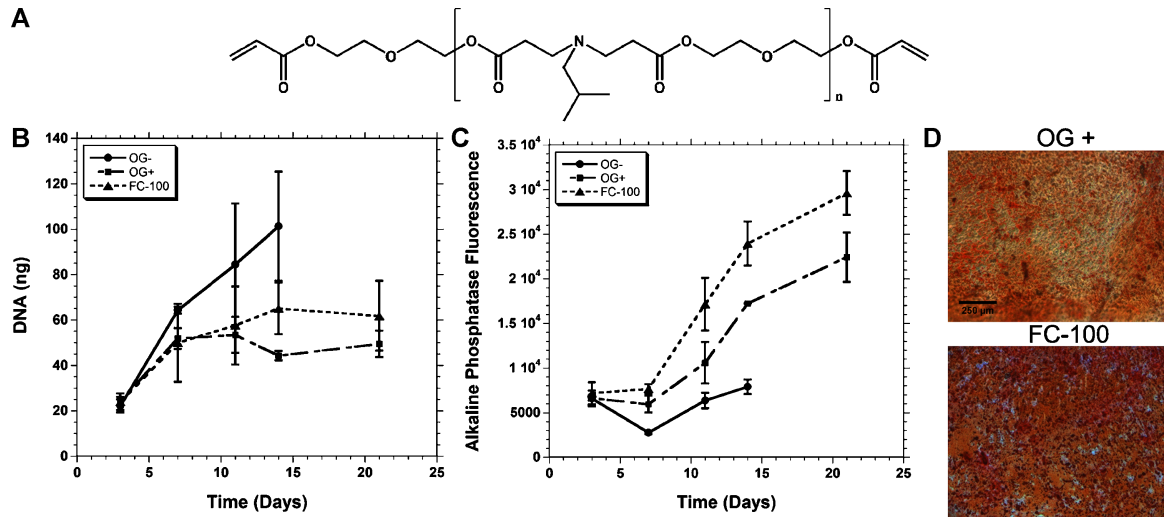

Figure 8. HMSCs were cultured on thin films of an osteoconductive polymer, A6 (**A**), in OG−, OG+, and 100 μM FC media. The cell proliferation (**B**) and ALP activity (**C**) were assessed over 21 days; however, the OG− samples detached after 14 days in culture. Alizarin red staining for cell mineralization of hMSCs after 21 days in OG+ or FC supplemented media (**D**), the dark red spots are indicative of mineralized nodules, whereas the lighter orange background is due to dye absorption into the A6 films. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

of hMSCs in combination with a biodegradable polymer, offering a new avenue for exploration of novel molecule and material formulations for mineralized tissue repair.

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