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Investigation of the Biochemical Mechanism for Cell-Substrate Mechanical Sensing

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Vincent Anthony Ricotta

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Abstract of the Thesis

Investigation of the Biochemical Mechanism for Cell-Substrate Mechanical Sensing

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Advancements in stem cell biology and materials science have enabled the development of new treatments for tissue repair. Dental pulp stem cells (DPSCs), which are highly proliferative and can be induced to differentiate along several mesenchymal cell lineages, offer the possibility for pulpal regeneration and treatment of injured dentition. Polybutadiene (PB) may be used as a substrate for these cells. This elastomer can be spun casted into films of different thicknesses with different moduli. DPSCs grown on PB films, which are relatively hard (less than 1500 Å thick), biomineralize depositing crystalline calcium phosphate without a requirement for the typical induction factor, dexamethasone (Dex). The moduli of cells track with the moduli of the surface suggesting that mechanics controls mineralization. The purpose of this study was to determine whether the major effect of Dex on biomineralization is the result of its ability to alter cell mechanics or its ability to induce osteogenesis/odontogenesis. DPSCs sense substrate mechanics through the focal adhesions, whose function is in part regulated by the Ras homolog gene (Rho) and its downstream effectors Rho associated kinases (ROCKs). ROCKs control actin filament polymerization and interactions with myosin light chain. Because cells sense substrate mechanics through focal adhesion proteins whose function is regulated by ROCKs, the impact of a ROCK inhibitor, Y-27632, was monitored. Blocking this pathway with Y-27632 suppressed the ability of DPSCs to sense the PB substrate. The cell modulus, plasma membrane stiffness, and cytosol stiffness were all lowered and biomineralization was suppressed in all cultures independent of substrate modulus or the presence of Dex. In other words, the inability of DPSCs to sense mechanical cues suppressed their ability to promote mineralization. On the other hand the expression of osteogenic/odontogenic markers (alkaline phosphatase and osteocalcin) was enhanced, perhaps due to Y-27632 induced changes in Wnt signaling as seen in other mesenchymal stem cells. How mechanical sensing regulates matrix proteins to promote their mineralization remains an open question.

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Acknowledgments

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Introduction

1.1 Tooth Development and Regeneration

Tooth development arises from a series of interactions between oral epithelial cells and mesenchymal cells [1-3]. This process begins with induction, where the dental epithelial cells signal the cranial neural crest derived mesenchymal cells to initiate development. The next few stages involve the differentiation and localized proliferation of the dental epithelial cells, which lead to the bell stage. Here, the epithelial cells form the enamel producing ameloblasts and the mesenchymal cells produce the dental pulp stem cells, which differentiate into the dentine-forming odontoblasts [2-3]. The last stage occurs postnatally and involves tooth eruption from bone resorption and root development [2].

The interactions between the dental epithelial cells and mesenchymal cells involved in the tooth development process are regulated by the bone morphogenetic proteins BMP-2 and BMP-4 and by transforming growth factor beta (TGF-β) [3-7]. On the other hand, fibroblast growth factors (FGFs) are associated with cell proliferation [3, 8-10]. Out of the molecules above, BMP-4 and FGF-8 account for vital preliminary signals from the dental epithelial cells that have a major part in the initiation of mesenchyme specific homeobox genes [3]. The development and morphology of teeth appear to be regulated by these transcription factors [3, 11].

There is a natural regenerative potential of odontoblasts in the formation of tertiary dentine [12]. After tooth eruption, odontoblasts are capable of forming a reparative dentine matrix in response to mild injury. Unfortunately, advanced caries or dental procedures may deteriorate pre-existing odontoblasts [12-14]. In response to great dental trauma, preodontoblasts from the

dentine-pulp complex migrate to the site of injury, differentiate, and promote deposition of reparative dentin, a poorly structured mineralized matrix that acts as a protective barrier to the pulp [1, 12]. Our understanding of tooth development, repair, and regeneration via advancements in stem cell biology and materials science will allow us to produce innovative treatments using engineered biological compounds [3].

1.2 Human Dental Pulp Stem Cells (DPSCs)

There has been an increasing interest for the potential application of stem cells in the field of dentistry with dental tissue regeneration. Stem cells may be characterized as a unique class of clonogenic cells with the potential for self-renewal and multilineage differentiation [15]. These include embryonic stem cells (ESCs), adult or postnatal stem cells (ASCs), or induced pluripotent stem cells (iPSCs) [15-19]. The ASCs are undifferentiated cells identified from tissues following the embryonic development [20]. Among the ASCs, mesenchymal stem cells (MSCs) are multipotent progenitor cells that have been identified in most postnatal tissues with the potential to differentiate into bone, cartilage, muscle, and fat [15, 21]. The gene-expression profiles of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) may control the functioning and behavior of MSCs [22]. The activation of several adhesion and extracellular matrix (ECM) proteins in MSCs are regulated through proteolysis by MMPs [23]. Several studies have indicated that MMPs and TIMPs influence crucial processes of cell differentiation, such as, proliferation, morphogenesis, and apoptosis [22, 24, 25]. These MMPs are a family of calcium and zinc dependent endopeptidases that have the capability of cleaving a majority of the ECM units [25]. There are several classes of MSCs derived from different dental tissue [15], including dental pulp stem cells (DPSC) [1], stem cells from human exfoliated deciduous teeth (SHED) [26], stem cells from apical papilla (SCAP) [27-28],

periodontal ligament stem cells (PDLSCs) [29], dental follicle progenitor cells (DFPCs) [30], and gingiva-derived MSCs (GMSCs) [31]. Therefore, teeth are a practical source of MSCs for a vast range of clinical applications [2].

Gronthos and co-workers first reported the isolation and characterization of stem cell populations in dental pulp in 2000. These DPSCs displayed high proliferation rates with a tendency to form colonies that produced sparse and dense calcified nodules [1]. Additional characterization of DPSCs revealed their multipotent differentiation capabilities in vitro, including differentiating into odontoblasts, adipocytes, chondrocytes, and osteoblasts [2, 32-35]. The capability of DPSCs to differentiate into a distinct cell lineage is regulated by microenvironment factors, such as, signaling molecules, growth factors, transcription factors, and extracellular matrix proteins [15]. Also, the DPSC donor tooth conditions have a strong effect on the distribution and expression of ECM proteins [20].

Previous studies have shown the potential clinical application of using TGF-βs to cap exposed pulp [36, 37]. Human dentin formation is regulated by TGF-β1, which can direct ECM formation, cell proliferation, and plays a major role in the differentiation of DPSCs into odontoblasts [38-40]. In general, TGF-β1 regulates gene expression through use of the SMAD pathway [41]. The stimulation of collagen is a vital step in the process of pulpal repair and regeneration. It was demonstrated that the TGF-β1 and TGF-β2 released in the pulp in response to trauma might regulate pulpal repair by maximizing the deposition of collagen [38]. When treated with dentin matrix protein-1 (DMP-1), TGF-β with or without FGF-2, BMPs, insulin-like growth factors (IGFs), FGFs, or platelet-derived growth factor (PDGF), DPSCs induces odontoblast formation [15, 38, 42-44]. Odontoblasts and osteoblasts exhibit similar mineralized matrix protein gene expression, including osteocalcin (OCN), alkaline phosphate (ALP), DMP-1,

collagen type I, and bone sialoprotein [39, 45, 46]. However, there are odontoblast-specific gene products, such as, dentin sialoprotein and dentin phosphoprotein, which are encoded by dentin sialophosphoprotein (DSPP) [47, 48]. The expression of both of these gene products arises once a collagenous predentin matrix has formed and is connected to the process of dentinogenesis [49]. The runt-related transcription factor-2 (RUNX2) protein is identified in the nuclei of preodontoblasts, immature odontoblasts, and DPSCs. Overexpression of this protein inhibits the terminal differentiation of odontoblasts. In order to reach complete odontoblast differentiation for dentinogensis, RUNX2 expression must be down-regulated [50]. These DPSCs may prove to be a prominent aspect in tooth repair and regeneration.

1.3 Effects of Dexamethasone on DPSCs

Previous studies have shown that treatment with dexamethasone (Dex), with or without other FGFs, can induce the differentiation of DPSCs into hard-tissue-forming cells [51, 52]. Dex is a synthetic glucocorticoid that is taken up by cells and binds to the glucocorticoid receptor (GR). The receptor-ligand complex migrates into the nucleus and binds to glucocorticoid responsive elements (GREs). Differentiation is initiated after the binding of Dex-GR complex to these GREs, which exist on genes of many bone markers, including OCN and ALP [41]. The treatment of Dex displays a stimulatory effect on these markers. In culture, Dex inhibits cell proliferation and stimulates ALP activity [52]. Thus, suggesting that Dex treatment promotes differentiation of DPSCs into odontoblast-like cells.

Fibronectin has displayed a vital role in the development and repair process of dentin [41, 53]. Fibronectin is an adhesive protein that regulates cell adhesion to ECM and the formation of microfilaments involved in cell spreading and migration [54]. An increase in the production of

this protein may assist in the migration and proliferation of DPSCs to the area of trauma. The treatment of Dex, with or without TGF-β1, stimulates fibronectin synthesis while inhibiting the secretion of the nerve growth factor (NGF) in DPSCs. An increased level of NGF displayed a connection with pulpal pain and dentin hypersensitivity [41]. Promoting the production of fibronectin and suppressing the secretion of NGFs indicates the relevance of Dex treatment in clinical applications.

1.4 Regulation and Function of Rho-Associated Kinase (ROCK)

The Ras homolog gene (Rho) is a small GTPase protein that serves as a molecular switch of several cellular activities. This GTPase regulates signals to various molecules by alternating among the inactive, guanosine diphosphate (GDP)-bound form and the active, guanosine triphosphate (GTP)-bound form [55-58]. The active form of Rho binds to specific downstream effectors of several biological actions, and mediates cell-to-substrate adhesion and motility, cytokinesis, advancement of the G_1-S phase of the cell cycle, and gene expression [56-60]. Included in these effectors is the Rho-associated kinase (ROCK), which is involved in several cellular processes downstream of Rho [55].

ROCK is a serine/threonine protein kinase that was recognized as an effector by binding to the GTP-bound form of Rho [61]. The ROCKs have displayed a connection in the Rho-induced production of actin stress fibers and focal adhesions [58, 62-64], and in inactivation of myosin phosphatase [58, 65]. Out of the Rho GTPase family, RhoA has been shown to be the principal mediator of actomyosin and focal adhesion dynamics [59]. Focal adhesions contain transmembrane receptors (integrins) that connect the cell to the ECM and at the same time to the actin cytoskeleton via a network of various actin-binding proteins [66-68]. The integrins relay

signals produced by the ECM and transmit mechanical forces to mediate cell proliferation and movement [69-71]. The primary regulator of this integrin-ECM signaling complex is focal adhesion kinase (FAK) [66, 72]. This ubiquitously expressed kinase is needed for several standard cellular activities [73]. During microfilament formation and production of focal adhesions, the proteins FAK and paxillin are phosphorylated [74]. It was shown that the phosphorylation of FAK is followed by the phosphorylation of paxillin [75, 76]. In response to integrin interaction with the ECM, FAK is activated and delivered to focal adhesions [66, 72, 73]. ROCK is capable of mediating the phosphorylation of myosin light chain (MLC) via direct phosphorylation of MLC plus the down-regulation of myosin-binding subunit (MBS) [77]. This regulation of MLC is believed to induce stress fiber production in nonmuscle cells [62-64]. Actin is viewed to be constantly polymerizing and depolymerizing based on several extracellular signals throughout cell movement, adhesion, and cytokinesis [78]. The activity of the actin-binding and actin-depolymerizing protein, Cofilin, is disturbed by phosphorylation at Serine-3 by LIM-kinases 1 and 2 downstream of Rho [55, 79]. The glucocorticoid induction of actin polymerization did not promote changes in actin gene transcription [80]. However, the Dex-induced formation of actin is linked with an increase in tyrosine phosphorylation levels of FAK and paxillin [81]. The LIM-kinases 1 and 2 have been classified as substrates of ROCK [82]. The phosphorylation of these substrates by ROCK stimulates the ability of the LIM-kinases to phosphorylate cofilin [82]. Therefore, ROCK steadies filamentous actin via phosphorylation of LIM-kinases, which results in the phosphorylation and inactivation of cofilin [55].

Rho has also been proposed to be a mechanotransducer of matrix stiffness. The differentiation of MSCs into various cell lineages has shown to be regulated by ECM stiffness and growth factors. Soft substrates cannot tolerate high levels of force applied by cells [83].

Once this limit of resistance to cell traction forces is surpassed, the ECM can yield and the cells may adjust by lowering their traction forces, integrin binding, and cell stiffness [83-85]. These factors regulate cell proliferation and differentiation [86, 87]. It was demonstrated that soft substrates could only permit weak cell adhesion, which leads to a decrease in stress fiber production and actin assembly [83]. This matrix stiffness is also directly related to the expression of lamin-A intermediate filament proteins. It was displayed that tissue stiffness and stress were directly proportional to lamin-A levels [88].

The morphology of cells is mediated through the modification of the actin cytoskeleton by ROCK [55]. There are two isoforms of ROCK, which are ROCK-I and ROCK-II. These isoforms have distinctive functions, in which the knockdown of ROCK-I decreases adhesion in keratinocytes on fibronectin and stimulates keratinocyte terminal differentiation, whereas the knockdown of ROCK-II suppresses this differentiation [89]. The isoform ROCK-I has also been connected to the promotion of stress fiber production and facilitation of focal adhesions in fibroblasts [90]. The synthetic compound Y-27632 [(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] has been reported to be a specific inhibitor of ROCK [58]**.** Treatment with this inhibitor has displayed a suppressive effect on stress fiber formation and an associated decline in tyrosine phosphorylation of FAK and paxillin [91]. Therefore, this ROCK inhibitor decreases the cell-ECM contact [92]. Even though Y-27632 inhibits both isoforms of ROCK, it has a greater knockdown potential of ROCK-I than ROCK-II. This compound inhibits the activity of the ROCK isoforms by competing with ATP for binding to the kinases and possibly other specific protein substrates. Also, this ROCK inhibitor was found to be cell-permeable, in which the intracellular concentration was equal to the extracellular concentration of the compound. This proposes that micromolar concentrations of Y-27632 can

be applied to employ specific inhibition of cellular actions, such as, stress fiber initiation and neurite retraction. In regards to the role of ROCK in the role of cytokinesis and G_1-S progression, Y-27632 only delayed these processes instead of blocking them [58].

There are two factors that influence the mechanics of a tissue, such as, cell and ECM density [92]. Adjusting either the cell or ECM density affects cellular differentiation, proliferation, and apoptosis [93-95]. Increasing the cell density exhibited a decrease in cell adhesion and spreading against the substrate, whereas there was an increase in cell-cell contact and paracrine signaling. It was shown that the cell shape, altered from the plating density, mediates an adipogenic-osteogenic lineage commitment for MSCs. This commitment was regulated through the RhoA-ROCK signaling pathway, which exhibits that cell shape directly influences the activity of RhoA and ROCK [87]. It was displayed that the kinase activities of ROCK, FAK, and extracellular-signal-regulated kinases 1 and 2 (ERK1/2) are stimulated on stiffer matrices. The inhibition of FAK and ROCK exhibited a lowered expression of osteogenic markers during osteogenic induction [96]. It was demonstrated that FAK influences osteogenic differentiation through ERK1/2, while ROCK mediates both FAK and ERK1/2 [96, 97]. Treatment with Y-27632 to decrease focal adhesions by the inhibition of ROCK-I has displayed differing effects on cells by altering the microenvironment [92].

1.5 Purpose

Our knowledge of oral tissue repair with advancements in stem cell biology and materials science will enable us to create innovative treatments through the use of engineered biological substrates. DPSCs, which are highly proliferative and can be induced to differentiate along several mesenchymal cell lineages, offer the possibility for pulpal regeneration and treatment of

injured dentition. Following the dental procedure for root canal, gutta percha, or polyisoprene, is used to obdurate and fill the empty space of the tooth. Polybutadiene (PB) is a viscoelastic polymer similar in structure to polyisoprene **(Figure 1)**. At room temperature, PB is rubber-like due to its elastomeric characteristics. In general, PB has a "soft" texture and low modulus from the unsaturated carbons and diene groups. The elastomeric characteristics, or rubber-like behavior is a result of the polymer chain structure and crosslinking. PB may be used as a substrate for DPSCs in the process of dental tissue regeneration and repair. In this study, *in vitro* tests of DPSCs cultured on different thicknesses of spun casted PB films on silica wafer were performed. Altering the thickness of the PB thin films would maintain the chemistry of the substrates while varying the mechanical cues signaled to the cells. A thinner PB film (200 Å) has a higher Young's modulus than thicker polybutadiene films (above 1500 Å). The higher modulus of the thinner PB films makes the substrate harder than the thicker films, which enables it to tolerate higher traction forces generated by the DPSCs. In the literature, it was demonstrated that the differentiation of DPSCs into certain cell lineages is regulated by biological factors and mechanical cues from the cell-ECM interactions. Previous studies have shown that treatment with Dex can stimulate the differentiation of DPSCs into odontoblasts. These *in vitro* studies were performed on tissue culture plastic, which is comprised of polystyrene **(Figure 1)**. The purpose of this study was to determine whether the major effect of Dex on biomineralization is the result of its ability to alter cell mechanics or its ability to induce osteogenesis/odontogenesis. DPSCs sense substrate mechanics through the focal adhesions whose function is in part regulated by Rho and its downstream effector ROCK. The polymerization of actin filaments and interactions with myosin light chain is controlled by ROCK. Because cells sense substrate mechanics through focal adhesion proteins whose function

is regulated by ROCK, the impact of a ROCK inhibitor, Y-27632, was monitored. The differentiation and mineralization of the DPSCs should be regulated by these factors.

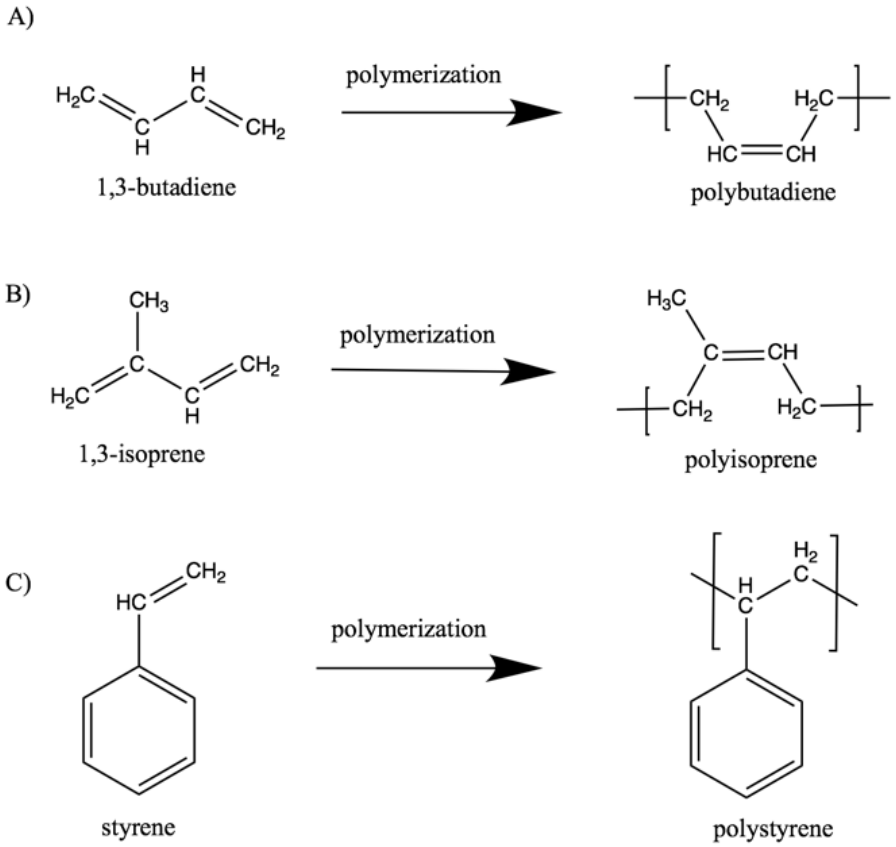

Figure 1. General Reaction Scheme of Polymerization to Illustrate Molecular Structural Differences Among Polymers Used. These include A) polybutadiene, B) polyisoprene, and C) polystyrene. This figure was produced via ChemDraw and does not take stereochemistry into consideration.

Materials and Methods

2.1 Polymer Substrates

The silica wafers (Wafer World) were cut into 1 cm^2 and 2 cm^2 squares. The wafers were soaked in methanol and sonicated for 5 minutes to remove any dust or debris from cutting them. To remove any organic compounds, the wafers were treated in a 3:1:1 solution of deionized water, ammonium hydroxide, and hydrogen peroxide, respectively, and heated to boil for 10 minutes. After washing with deionized water, the wafers were treated in a 1:1:3 solution of sulfuric acid, hydrogen peroxide, and deionized water, respectively, and washed after heated to boil for 5 minutes. This step made the surfaces hydrophilic. In order to induce hydrophobic behavior, the wafers were rinsed in a diluted solution of hydrofluoric acid in deionized water for 15 seconds. The wafers were placed on the spin caster and spun at 2500 rpm for 30 seconds after adding enough of the polybutadiene solution drop wise to cover the entire surface. The substrates were placed in an ultra high vacuum oven at 10^{-8} Torr and 120°C for 12 hours to sterilize surfaces. The polybutadiene (Scientific Polymer) had a M_W of 205,800 g/mol and a M_w/M_n of 1.49. The polybutadiene was dissolved in toluene to give a thickness ranging from 200 Å (thin polybutadiene, or Tn PB) to 1500 Å and 3000 Å (think polybutadiene, or Tk PB), which were measured by ellipsometry after spin casting and annealing the substrates. The uniformity of the polymer thin films was measured via AFM.

2.2 ROCK Inhibitor

Stock solutions (10 mM) of Y-27632 dihydrochloride, (Enzo Life Sciences) were prepared by dissolving the ROCK inhibitor in 10% DMSO. For cell culture, the stock solution was diluted 1:1000 in medium.

2.3 Cell Culture

Dental pulp stem cells (strain Ax3) used for this study were previously obtained under IRB approval (2007-6778). Cells were plated either on tissue culture plastic or on spun cast elastomers (polybutadiene or polyisoprene) and grown in MEM alpha media containing 2 mM L-glutamine (Life Technologies), supplemented with 10% fetal bovine serum (FBS; HyClone, Logan UT), 1% Penicillin-Streptomysin, 200 μM L-ascorbic acid-2-phosphate, and 10 mM β-gylcerophosphate and incubated at 37°C in 5% CO_2. Culture medium was changed one day post plating and every other day thereafter. For experiments, cells were incubated in media above with or without 10^{-8} M Dex (Sigma) and 10 μM Y-27632 in combination or separately. The media containing the ROCK inhibitor Y-27632 was prepared fresh each time before adding to the cell culture.

2.4 Shear Modulation Force Microscopy (SMFM)

Cell mechanics was measured using SMFM. DPSCs were plated on 1 cm^2 spun casted polybutadiene substrates in 24-well plates (Nunc) at an initial cell density of 5,000 cells/well. Cells were then cultured for 4 and 7 days in growth medium with and without Dex and with and without Y-27632 prior to measuring the cell modulus via SMFM [98].

2.5 Optical Tweezers

To further explore the mechanical properties of the cell plasma membrane and cytosol, analyses with optical tweezers were employed using 1 μm polystyrene beads and 2.88 μm polystyrene beads coated with anti-integrin alpha 5. For these experiments, cells were plated on polyisoprene coated 12 mm diameter microscope cover glass slides (Electron Microscopy Sciences); polybutadiene dewets on glass and hence could not be used. Glass slides were used instead of silica wafers because of the need for a transparent sample in order to be analyzed by optical tweezers. To prepare the surface slides were first soaked in methanol and sonicated for 5 minutes to remove any dust. The slides were then placed on the spin caster and spun at 2500 rpm for 30 seconds after adding enough of the polyisoprene solution drop wise to cover the entire surface. The substrates were placed in a vacuum oven at 10^{-3} Torr and 150°C for 3 hours to anneal and sterilize surfaces. The polyisoprene (Scientific Polymer; M_W of 80,300 g/mol and a M_w/M_n of 1.36) was dissolved in toluene to give a thickness ranging from 200 Å (thin polyisoprene: Tn PI) to 3000 Å (think polyisoprene, or Tk PI). PI thickness was measured by ellipsometry after spin casting and annealing the substrates. The uniformity of the polymer thin films was measured via AFM. The DPSCs were plated on the spun cast polyisoprene substrates in 24-well plates (Nunc) at an initial cell density of 5,000 cells/well. The cells were cultured for 4 and 7 days under the conditions listed above (± Dex, ± Y-27632). The polystyrene particles were added in the medium the day prior to analysis by optical tweezers. Steven Ming-Tzo Wei at Lehigh University then measured the plasma membrane and cytosolic stiffness via optical tweezers.

2.6 Histochemistry

The DPSCs were plated on 1 cm^2 spun casted polybutadiene substrates in 24-well plates (Nunc) at an initial cell density of 5,000 cells/well and cultured for 4, 7, and 15 days under the conditions listed above. For histochemical analyses, each culture was then washed in PBS, fixed with 10% formalin for 15 minutes and permeabilization by incubation with 0.1% Triton-X (Sigma) in PBS for 15 minutes. The cells were then incubated with Alexa Fluor 488 Phalloidin for 20 minutes and then propidium iodide for 5 minutes to stain F-actin and DNA, respectively. Following each treatment, the cell cultures were washed with 0.01% Triton-X/PBS and stored in PBS until analyzed. After staining, the samples were analyzed via confocal microscopy.

2.7 Biomineralization

The DPSCs were plated on 1 cm^2 spun casted polybutadiene substrates in 24-well plates (Nunc) at an initial cell density of 5,000 cells/well. The cells were cultured for 28 days under the conditions listed above. The samples were left out of the incubator for 1 day in the media to let the cells die. After removing the media, the samples were washed gently with deionized H_2O and then air-dried. To identify mineral deposits, the samples were analyzed using SEM/EDAX.

2.8 Total mRNA Extraction

The DPSCs were plated on 2 cm^2 spun casted polybutadiene substrates in 6-well plates (BD Falcon) at an initial cell density of 20,000 cells/well or on tissue culture plastic as a control. The cells were cultured for 7, 14, 21, and 24 days under the conditions listed above. After culturing, the media was removed and the samples were washed with PBS. The cells were trypsinized in 0.1% trypsin/0.01% EDTA for 5 minutes, neutralized with the control cell culture media, and centrifuged at 1500 rpm for 6 minutes. The supernatant was aspirated and the cell

pellet resuspended in 350 μL of lysis buffer (Qiagen, Lysis Buffer RLT) with 1% β-Mercaptoethanol (Sigma). RNA was isolated from each sample using the RNeasy Mini Kit from Qiagen. The purity of the RNA was determined by reading the optical density ratios at 260 and 280 nm using a Beckman DU 530 Life Sciences UV/Vis Spectrophotometer [99]; the quantity was calculated from the absorbance at 260 nm.

2.9 qRT-PCR Technique

A Perkin Elmer Cetus DNA Thermal Cycler was used to synthesize cDNA from the extracted mRNA. The cDNA synthesis was performed by using a first-strand cDNA synthesis Superscript II RT kit (Invitrogen) with 200 ng random primers (Invitrogen), 10 mM dNTP Mix (Invitrogen), and 40 units of RNaseOut (Invitrogen). The cDNA was diluted in a 20 μL qRT-PCR reaction using a QuantiTect SYBR Green PCR kit (Qiagen) with 4 pmol of each human-specific primer sets **(Table 1)**. The reactions were incubated in the MJ Research DNA Engine Opticon machine, Opticon 2, from Stony Brook University's DNA Sequencing Facility, at 95°C for 15 minutes and then 94°C/(30 s), 55°C/(30 s), 72°C/(30 s) for 40 cycles. Known concentrations of human 18S tRNA was used with the human 18S primer set for a standard curve. The housekeeping gene used to normalize the target gene expression values was human 18S.

Human Gene Sequence Name	Direction	Primer Sequence 5' to 3'	Product Size (bp)
18S	F	GTAACCCGTTGAACCCCATT	151
	R	CCATCCAATCGGTAGTAGCG	
OCN	F	ATGAGAGCCCTCACACTCCTCG	255
	R	GTCAGCCAACTCGTCACAGTCC	
ALP	F	GTACTGGCGAGACCAAGCGCAA	282
	R	ACCCCACACAGGTAGGCGGT	
COL10	F	ATCTCCAGGAACTCCCAGCA	303
	R	GGACTTCCGTAGCCTGGTTT	
DSPP	F	GCATTTGGGCAGTAGCATGG	108
	R	CTGACACATTTGATCTTGCTAGGAG	
LMNA	F	AATGATCGCTTGGCGGTCTAC	255
	R	CTTCTTGGTATTGCGCGCTTT	
ACAN	F	TGGCCTGCCTGACTTTAGTG	197
	R	GGAGTCCACTAGCTCTCCCA	
RUNX2	F	CTCCCTGAACTCTGCACCAA	130
	R	CGTCATCTGGCTCAGGTAGG	

Table 1. List of Human Gene Primer Sets Used for qRT-PCR.

Results

3.1 Modulus of DPSCs on Polymer Films

The relative modulus of DPSCs grown on PB in media with or without Dex and with or without Y-27632 was measured 4-days and 7-days post-plating. As can be seen in **Figure 2**, at passages 6 and 7, the relative modulus of the DPSCs grown on Tn PB was greater than on Tk PB for all medium conditions. When incubated with Dex, there is a 2 and 3-fold increase in the relative modulus for the DPSCs cultured at passage 6 on Tn PB and Tk PB, respectively. Incubation with Y-27632 reduces the relative modulus of cells grown on Tn PB and Tk PB in the absence of Dex and limits the increase in modulus in response to Dex. In cultures grown on Tn PB for 4-days with Dex and Y-27632, there is no increase in relative modulus and although there is an increase in cultures grown on Tk PB, it is one-fourth that observed in cultures grown with Dex alone. This trend in relative modulus for the induced and noninduced DPSCs on the PB films is also seen at passage 7. The lowered relative modulus from the Y-27632 is more apparent at day 7 than day 4 for DPSC passage 7.

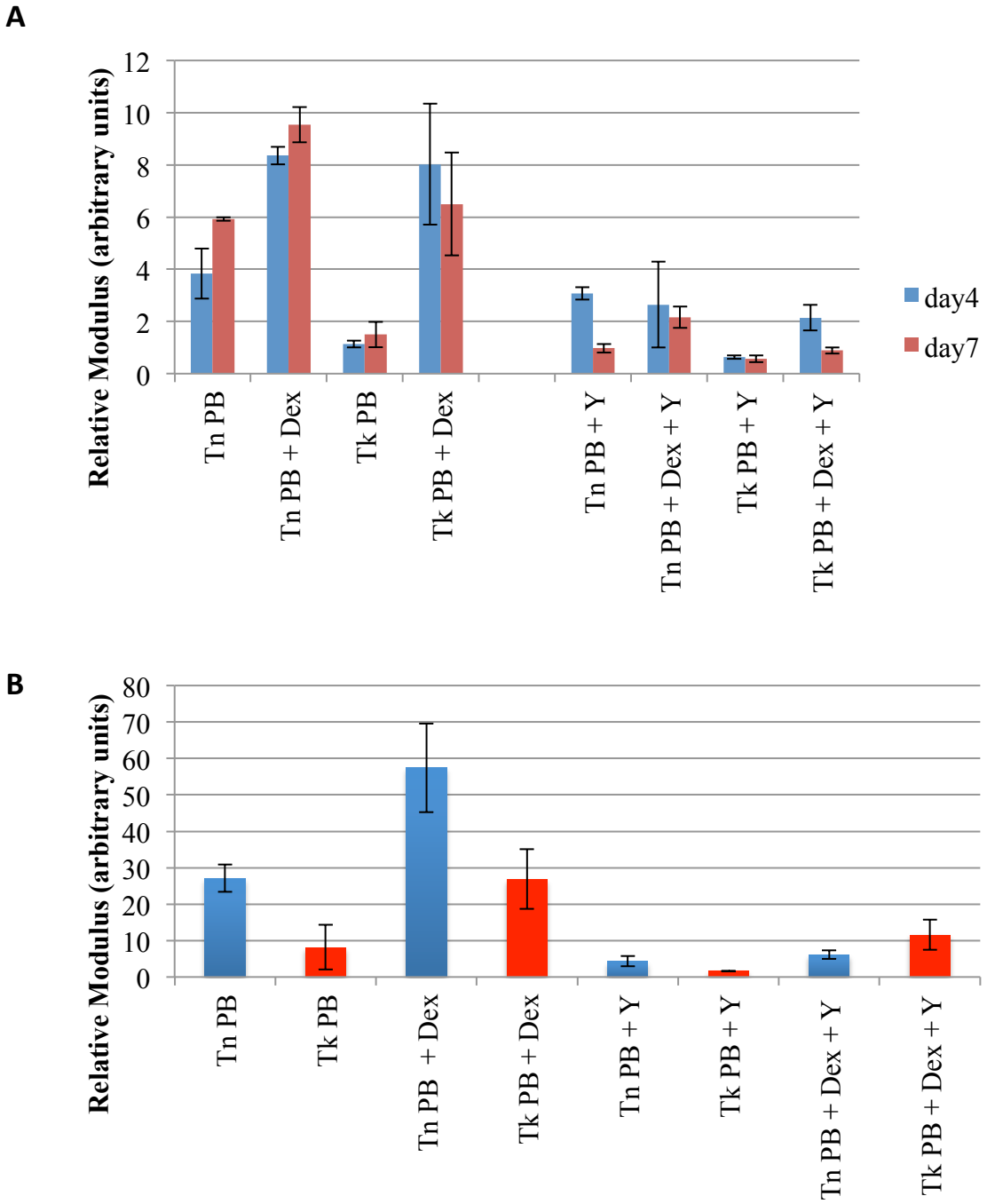

Figure 2. Shear Modulation Force Microscopy of DPSCs on Polybutadiene Films. The thicknesses of the polybutadiene films were 200 Å (Tn PB) and 1500 Å (Tk PB). (A) Relative modulus of DPSCs cultured on spun casted polybutadiene substrates for 4 and 7 days at passage 7. (B) Relative modulus of DPSCs cultured on spun casted polybutadiene substrates for 7 days at passage 6.

The stiffness of the plasma membrane and the cytosol of DPSCs cultured on the PI films were measured after day 4 and 7 of incubation using optical tweezers **(Figure 3, 4)**. As can be seen in **Figure 3**, by day 7 Dex enhancement of plasma membrane stiffness (G') was observed in cultures on Tn PI, Tk PI, and tissue culture plastic; at day 4 Dex-induced increases in stiffness were significant only in cultures on tissue culture plastic. Incubation with Y-27632 inhibited all Dex-induced increases in G' consistent with the requirement for ROCK and FAK in mediating cell stiffness [90-92].

As can be seen in **Figure 4**, in contrast to the effects of Dex on plasma membrane stiffness, Dex enhanced cytosolic stiffness only in the day 4 cultures grown on Tn PB. However, in the absence of Y-27632, cytosolic stiffness was significantly greater in all day 7 samples compared to day 4 samples; incubation with Y-27632 inhibited all increases in cytosolic stiffness. Hence, ROCK inhibition affects both Dex-mediated and non-mediated events altering cellular mechanics.

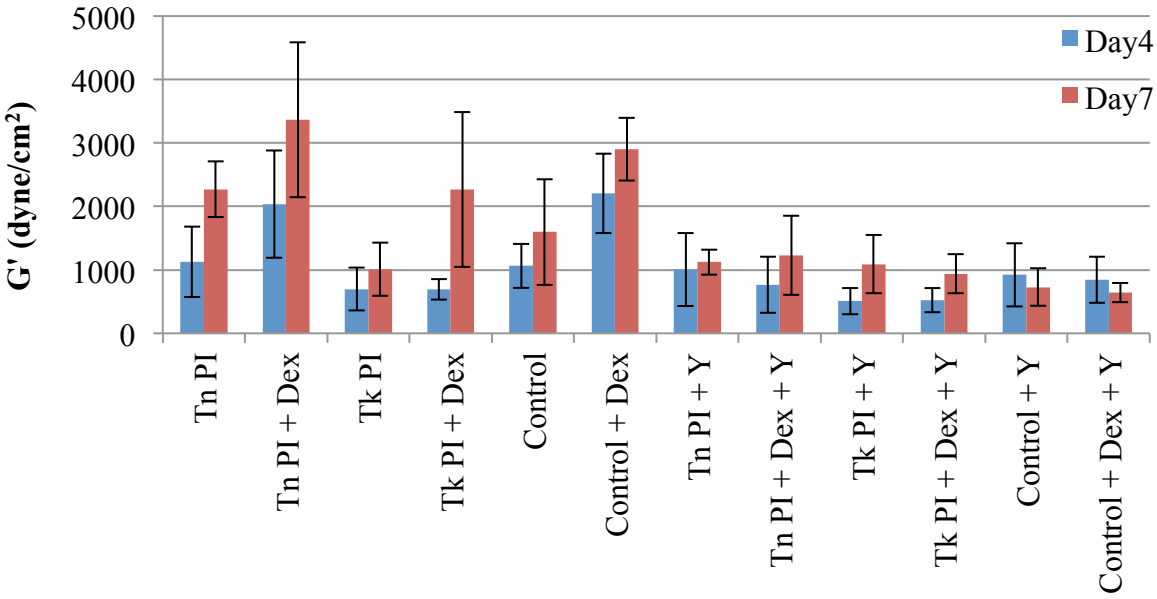

Figure 3. Plasma Membrane Stiffness of DPSCs on Polyisoprene Films. Modulus of DPSCs cultured on spun casted thin and thick polyisoprene films and coverglass (control). Steven Ming-Tzo Wei took the measurements for Day 4 and 7 via optical tweezers at Lehigh University.

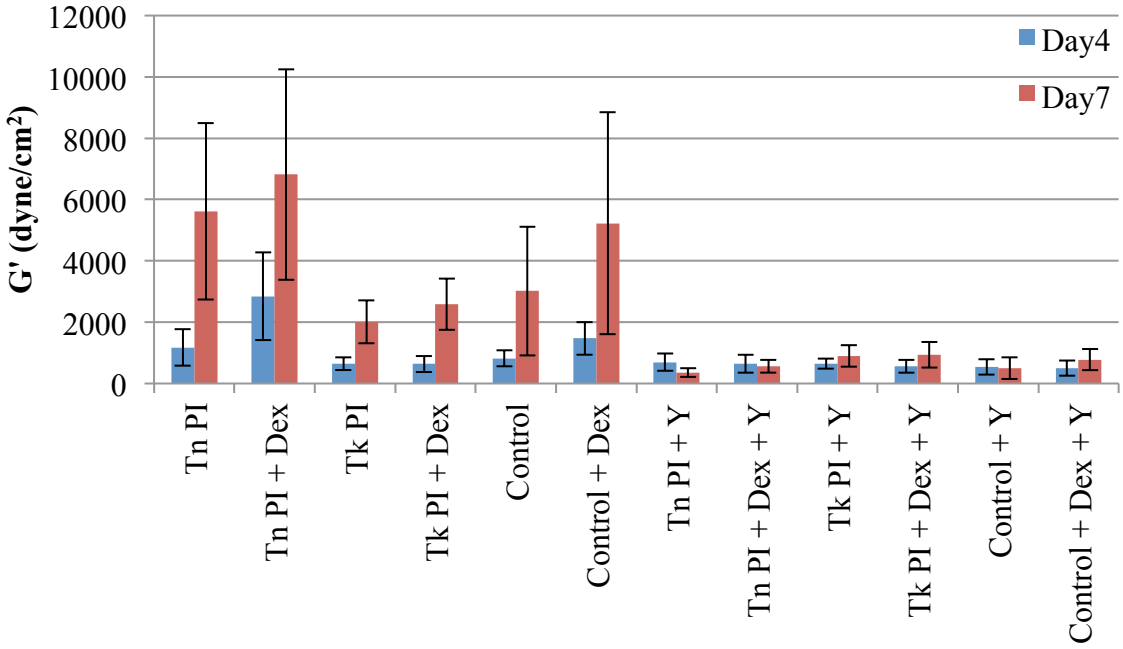

Figure 4. Cytosol Membrane Stiffness of DPSCs on Polyisoprene Films. Modulus of DPSCs cultured on spun casted thin and thick polyisoprene films and coverglass (control). Steven Ming-Tzo Wei took the measurements for Day 4 and 7 via optical tweezers at Lehigh University.

3.2 Cell Morphology of DPSCs on Polybutadiene Films

After day 4, 7, and 15 of incubation, cell morphology of the DPSCs cultured at passage 7 on the PB films was analyzed using confocal microscopy **(Figure 5)**. There is no distinctive difference among the morphology of the DPSC colonies for each condition on Tn PB and Tk PB. The DPSCs appear to be fibroblast-like and are confluent in culture by day 15 of incubation.

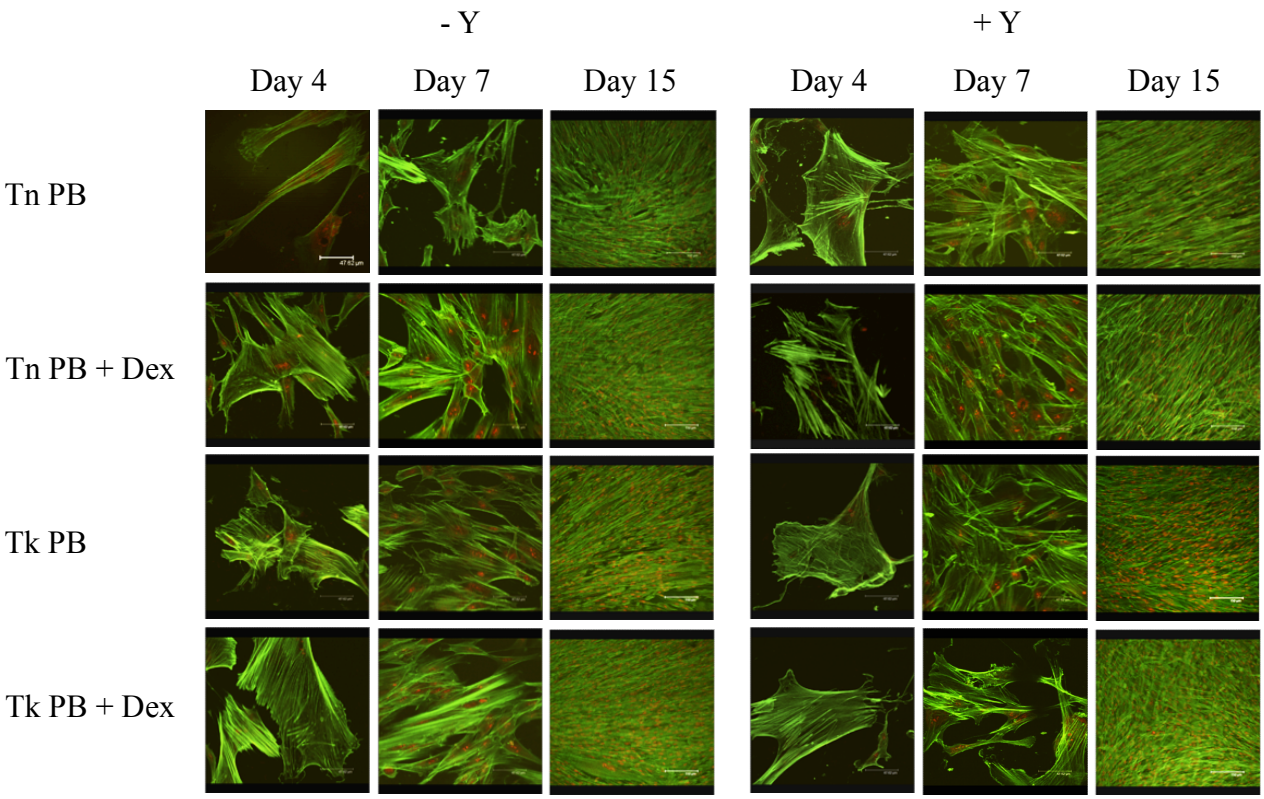

Figure 5. Cell Morphology of DPSCs on PB Films. The DPSCs were cultured at passage 7 on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. Confocal microscopy was used to analyze the DPSCs at day 4, 7, and 15 after treating with alexa fluor 488 phalloidin and propidium iodide.

3.3 Biomineralization of DPSCs on Polybutadiene Films

After day 28 of incubation, biomineralization by the DPSCs cultured on the PB films was analyzed by SEM/EDAX. The DPSCs appeared to sense the mechanics of the PB films. As can be seen in **Figure 6a**, Panels A and B, biomineralization on Tn (200 Å) PB was apparent in cultures grown without or with Dex. In contrast cultures grown on Tk (1500 Å) PB showed little mineralization with only sporadic deposits detected; a modest increase in deposits were observed in cultures incubated with Dex. When grown with Y-27632, which causes a reduction in both plasma membrane and cytosolic stiffness, almost all mineralization was suppressed (**Figure 6a**, Panels C and D). Taken together the data suggest that cell stiffness regulates the cell's ability to promote mineralization.

At passage 8, the DPSCs did not appear to sense the mechanics of Tn PB (**Figure 6b**). The DPSCs at passage 8 only biomineralized when induced with Dex and cultured on Tn PB. There was no biomineralization from the induced DPSCs at passage 8 cultured on Tk (3000 Å) PB. The biomineralization of these samples was determined based on the production of any calcium phosphate crystals. The crystals formed by the samples that did not biomineralize were determined to be calcium carbonates from EDAX.

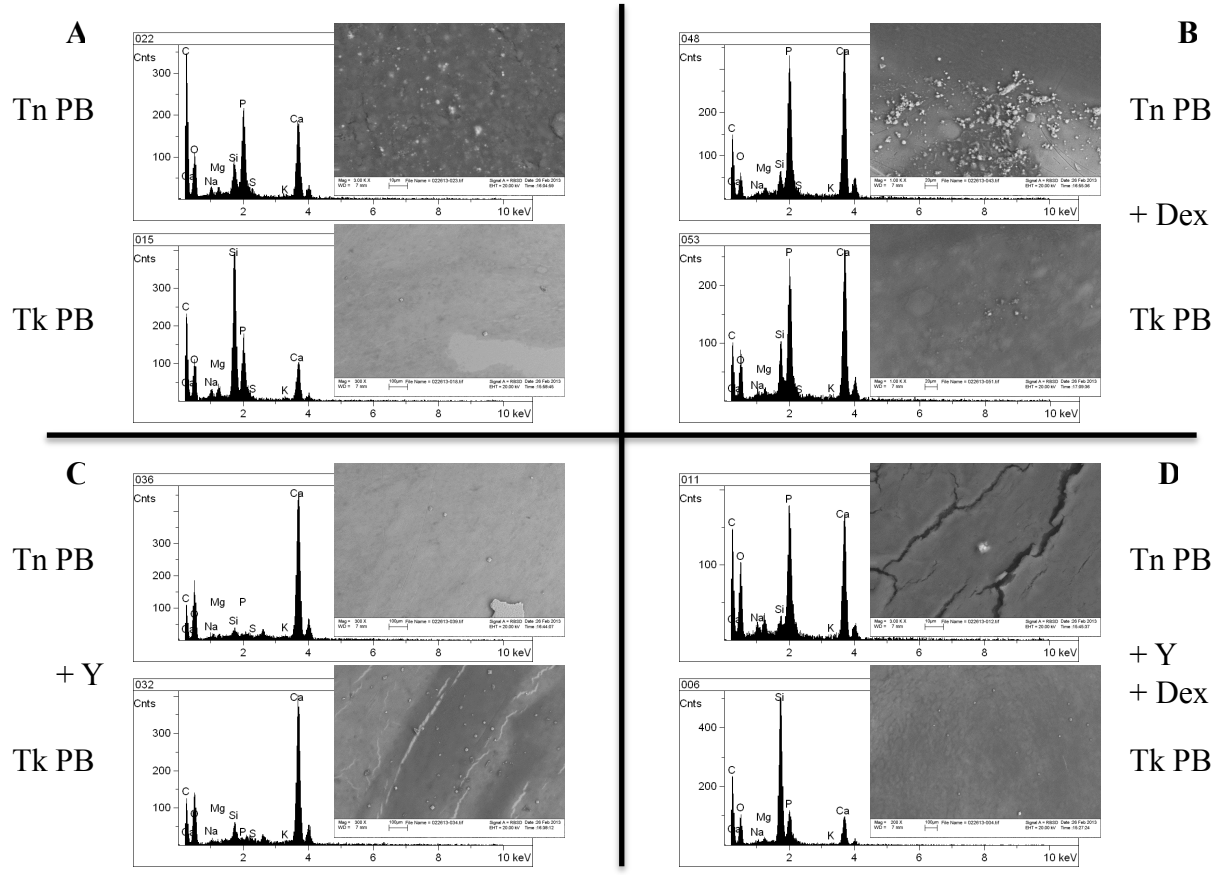

Figure 6a. SEM/EDAX of DPSCs on Polybutadiene Films After 28 Days of Culture. The DPSCs were cultured at passage 7 on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions (A) no Dex or Y, (B) only Dex, (C) only Y, and (D) both Dex and Y.

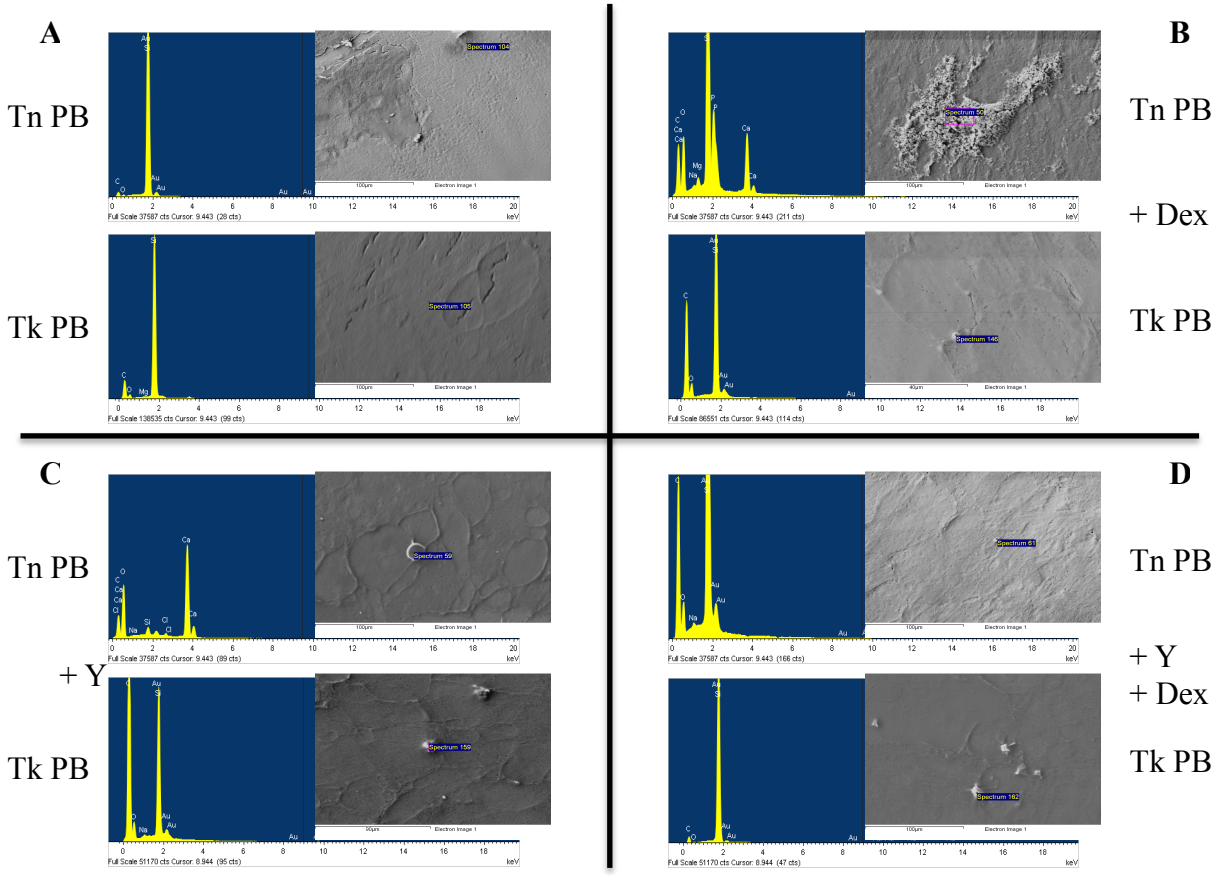

Figure 6b. SEM/EDAX of DPSCs on Polybutadiene Films After 28 Days of Culture. The DPSCs were cultured at passage 8 on thin and thick polybutadiene films with thicknesses of 200 Å and 3000 Å, respectively. The cells were maintained with the medium conditions (A) no Dex or Y, (B) only Dex, (C) only Y, and (D) both Dex and Y.

3.4 Gene Expression of DPSCs on Polybutadiene Films

The DPSCs were cultured at passage 7 on tissue culture plastic and on Tn PB and Tk PB, with thicknesses of 200 Å and 1500 Å, respectively. At day 0, 14, 21, and 24, cells were harvested, and RNA was isolated. To monitor gene expression, cDNA was prepared and used for qRT-PCR using the primer sets in **Table 1**. For the day 24 samples, cDNA was synthesized from all conditions except for the tissue control plastic samples with Dex and Y factor due to insufficient available RNA isolated. The mRNA expression levels of the genes COL10, DSPP, LMNA, and ACAN were below detection.

The OCN expression levels of the DPSCs cultured on tissue culture plastic, Tn PB, and Tk PB throughout incubation are displayed in **Figure 7**. Treatment with Y-27632 up-regulates OCN independent of substrate and Dex. OCN is promoted after day 14 of incubation when treated with Y-27632 **(Figure 8)**. After day 21, this stimulation is not significant among each condition for Tn PB and Tk PB **(Figure 9)**. At day 24, the up-regulation of OCN from the Y factor is significant only for Tn PB, while there is distinction among each condition for Tk PB **(Figure 10)**. This stimulation of OCN is not seen for the tissue culture plastic until day 21 **(Figure 9)**. There appears to be no effect of Dex for the OCN expression from the DPSCs cultured on either the tissue culture plastic, Tn PB, or Tk PB.

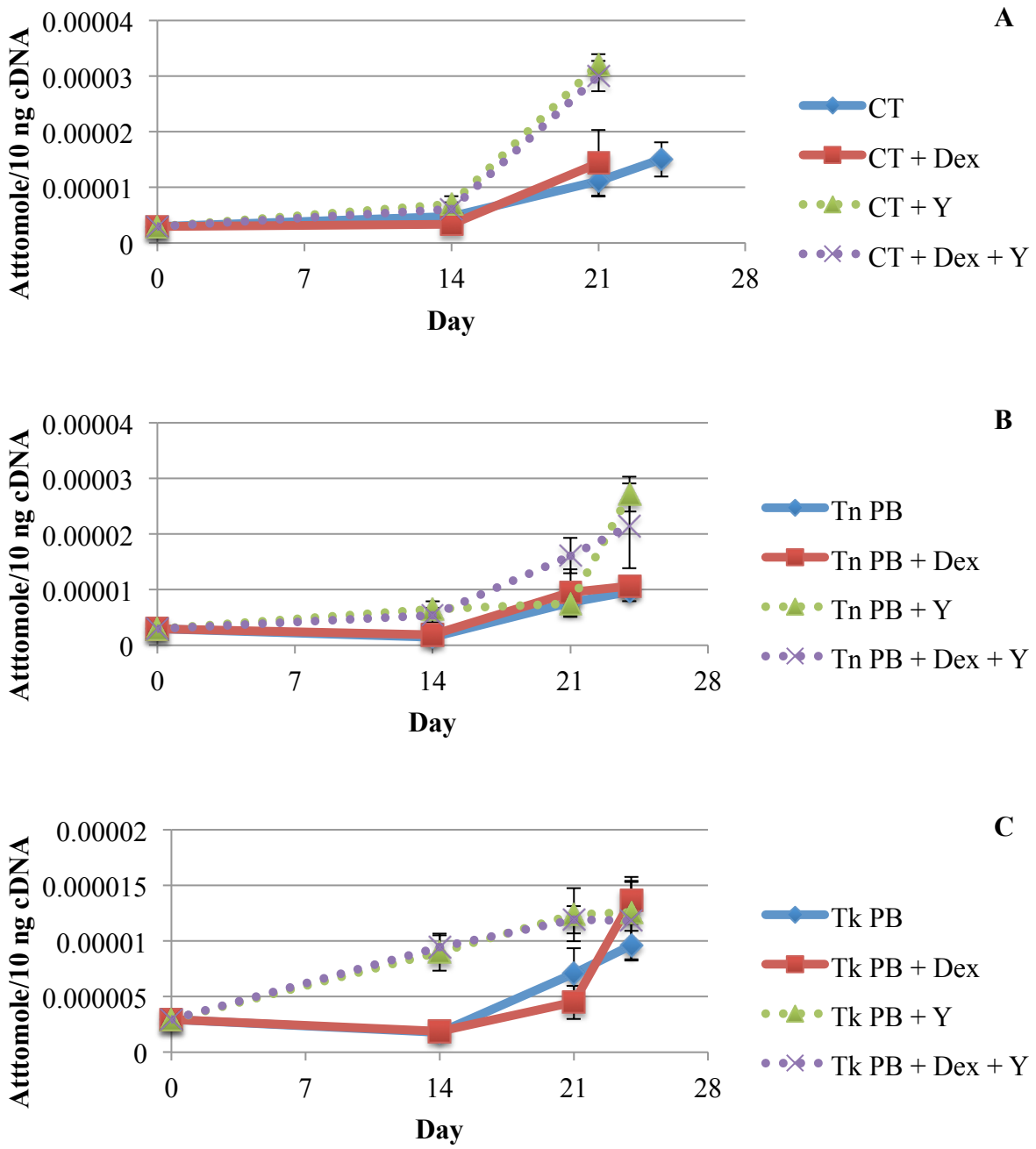

Figure 7. OCN Expression For Samples Over Time. The DPSCs were cultured at passage 7 on (A) tissue culture plastic (control) and on (B) thin and (B) thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

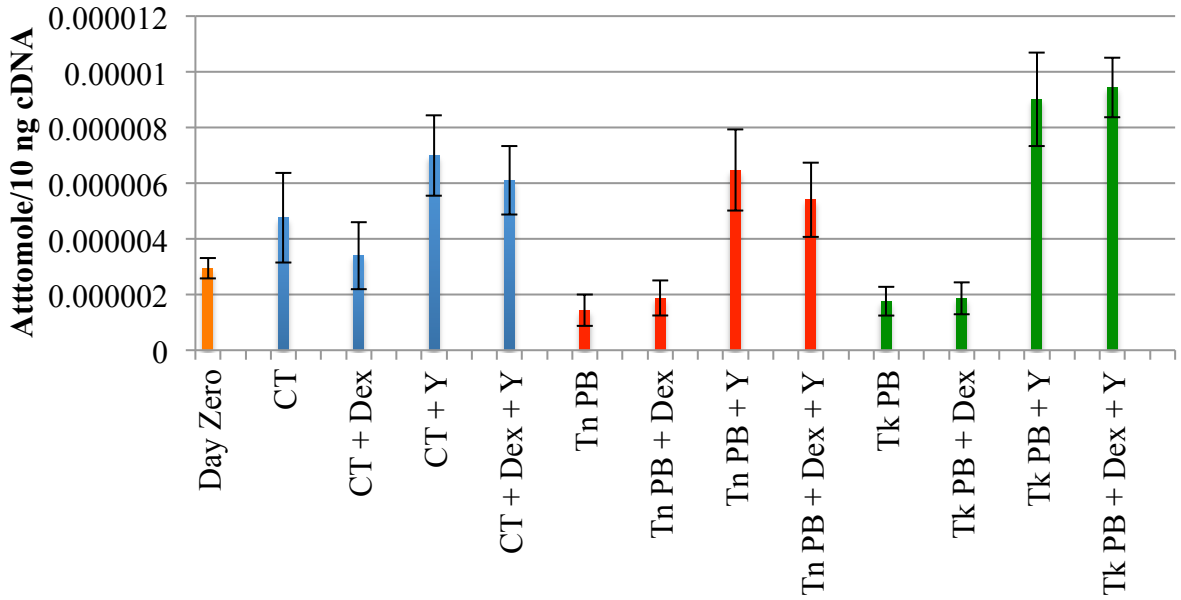

Figure 8. OCN Expression for Day 14 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

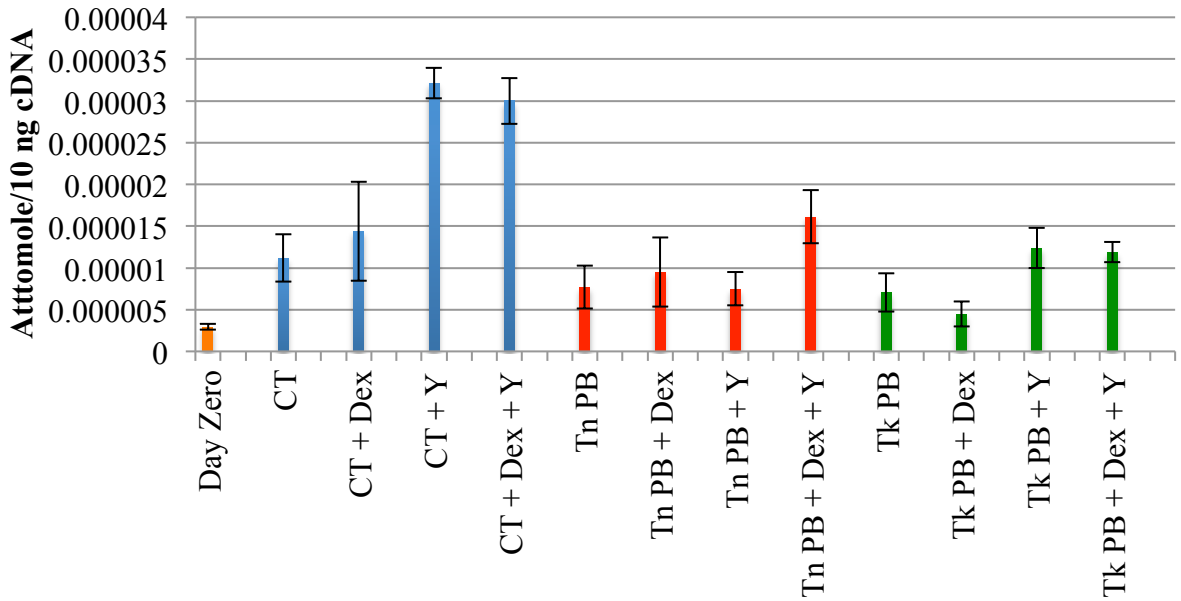

Figure 9. OCN Expression For Day 21 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

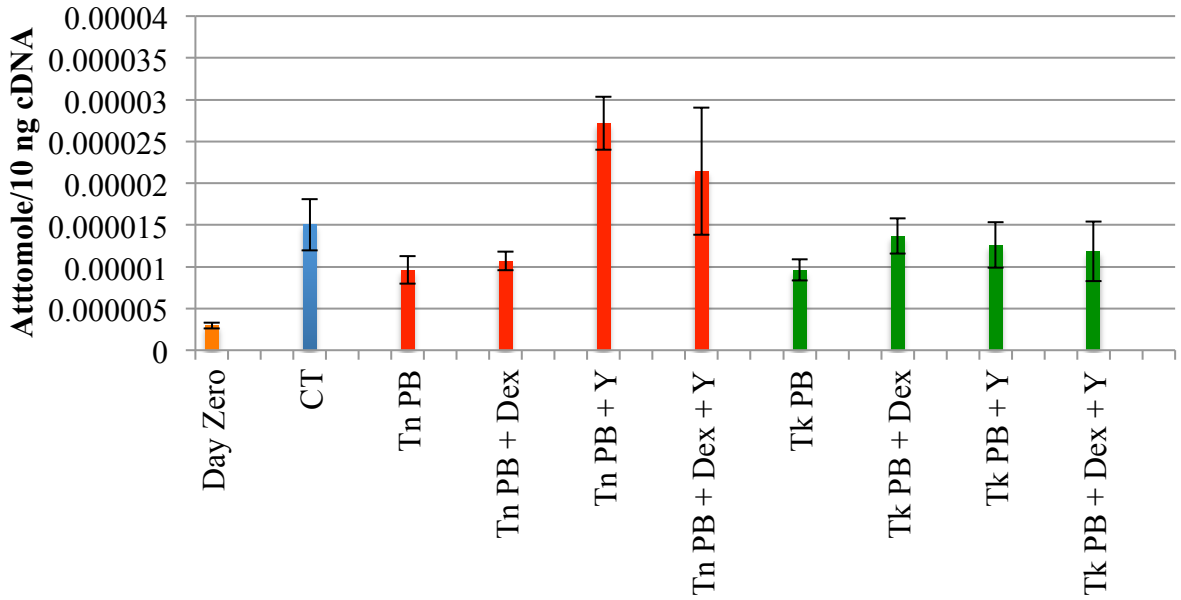

Figure 10. OCN Expression For Day 24 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

The ALP expression levels of the DPSCs cultured on tissue culture plastic, Tn PB, and Tk PB throughout incubation are displayed in **Figure 11**. The ALP expression levels of the DPSCs displays that treatment with Dex stimulates ALP after day 14, 21, and 24 of incubation independent of substrate **(Figure 11-14)**. Treatment with Y-27632 does not exhibit any effect on the expression level of ALP for the Tn PB samples without Dex. Also, Y-27632 does not show any suppressive effect of the Dex-induced stimulation of ALP on the Tn PB and Tk PB samples. However, treatment with Y-27632 stimulates ALP for the DPSCs on tissue culture plastic at day 21 and for Tk PB at day 14 and 21. When induced DPSCs were treated with Y-27632, ALP expression was up-regulated significantly more than other medium conditions, independent of substrate.

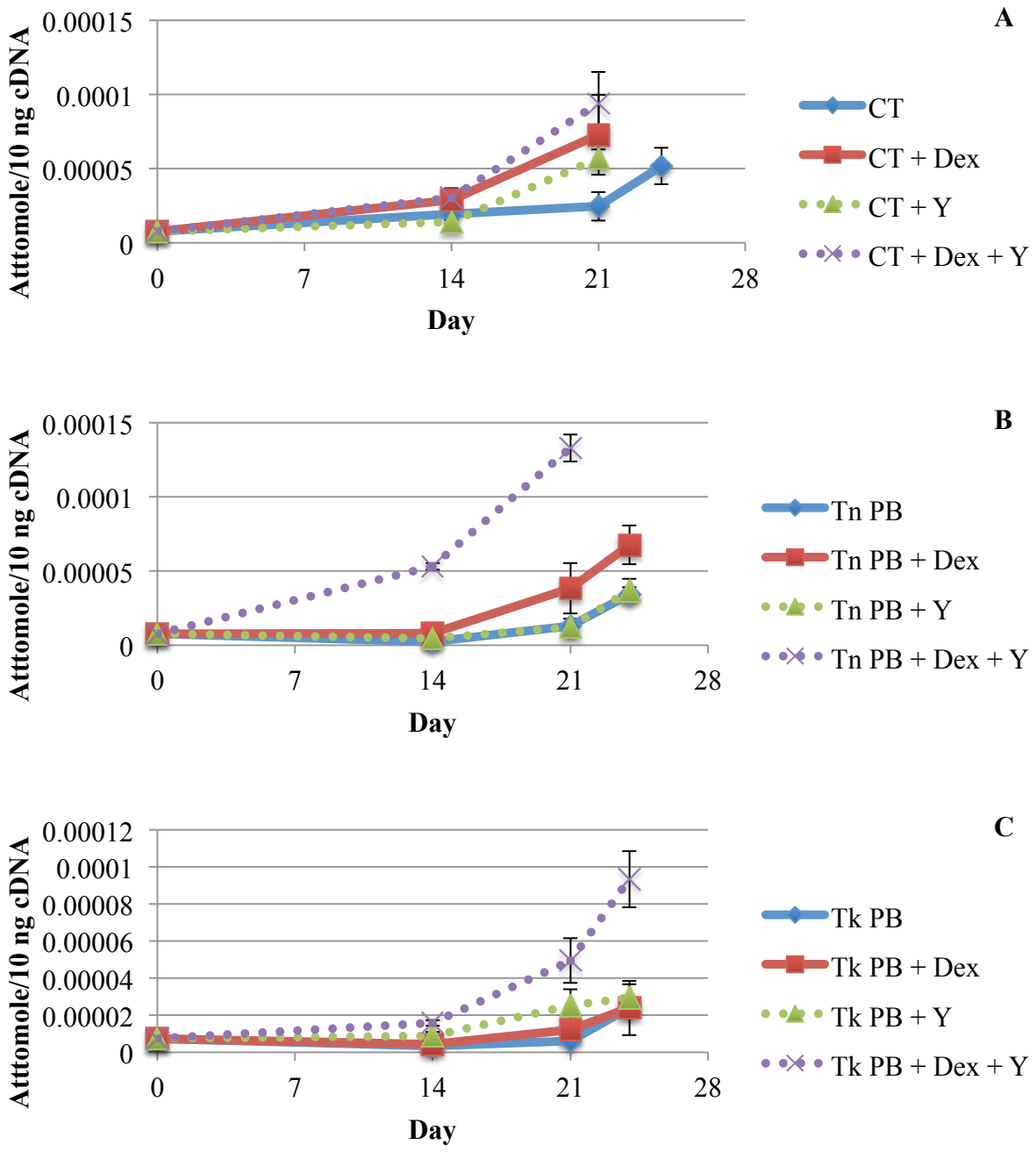

Figure 11. ALP Expression For Samples Over Time. The DPSCs were cultured at passage 7 on (A) tissue culture plastic (control) and on (B) thin and (B) thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

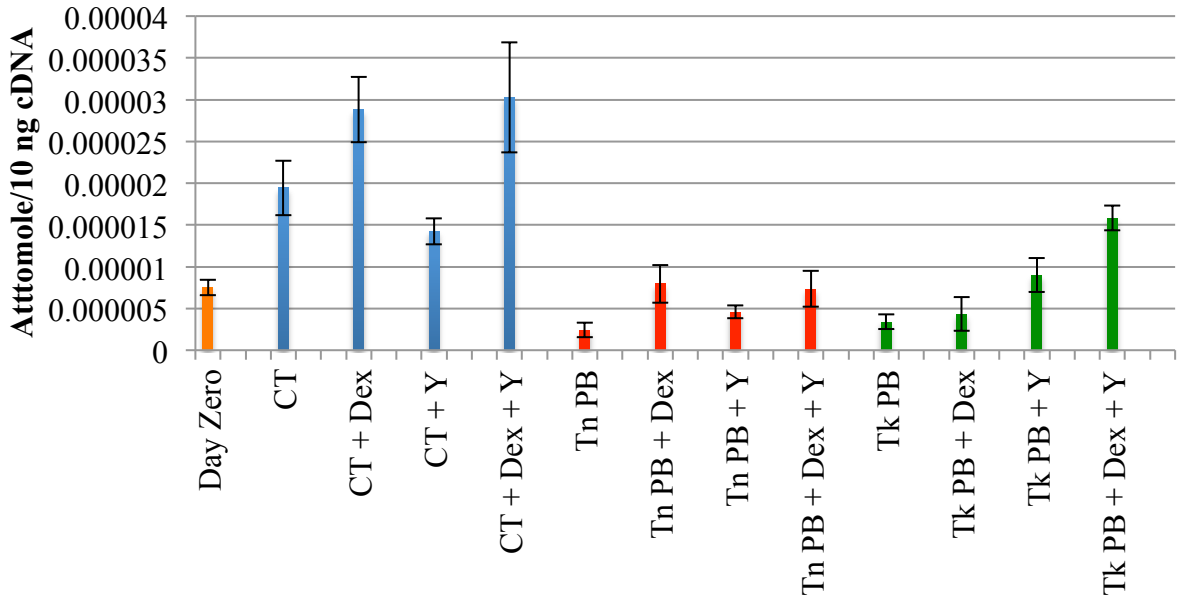

Figure 12. ALP Expression For Day 14 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

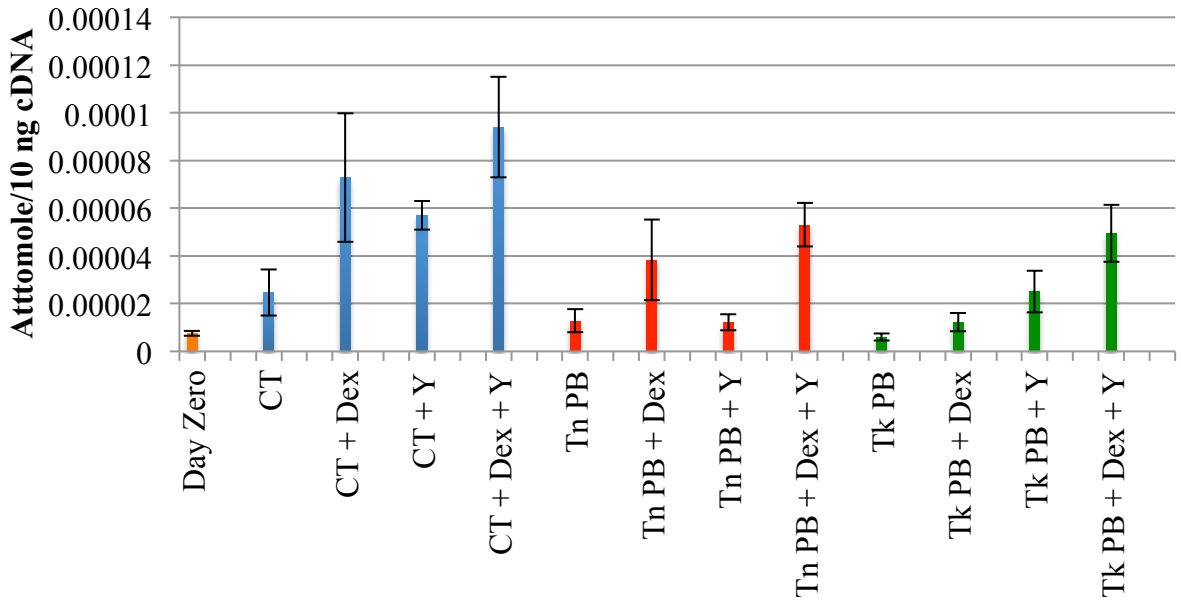

Figure 13. ALP Expression For Day 21 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

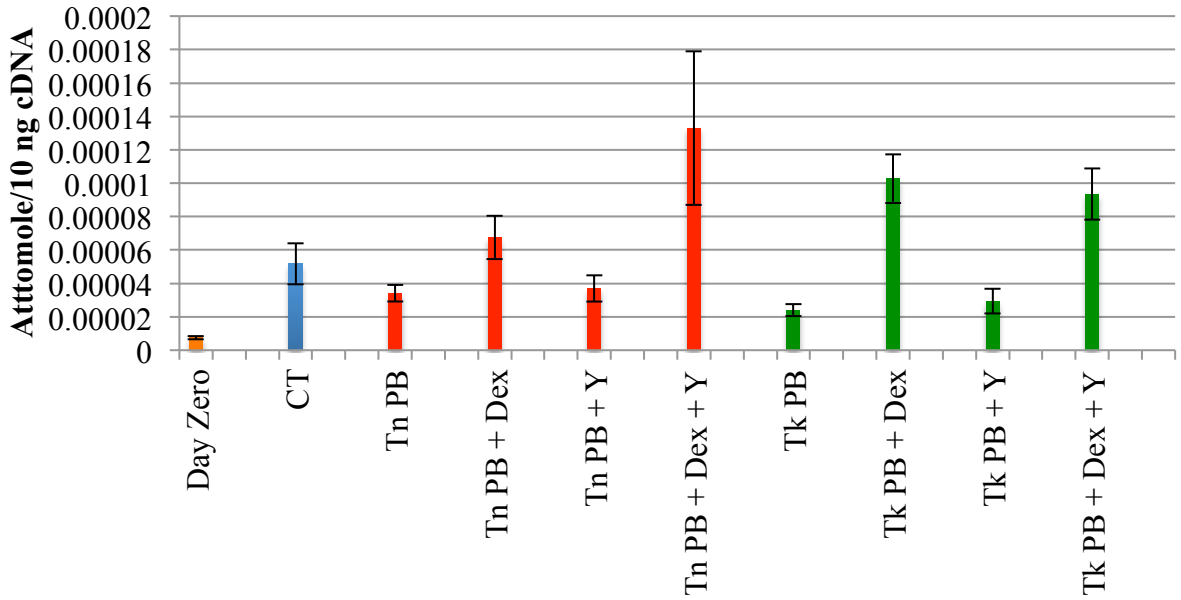

Figure 14. ALP Expression For Day 24 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

The RUNX2 expression levels of the DPSCs cultured on tissue culture plastic, Tn PB, and Tk PB throughout incubation are displayed in **Figure 15**. The RUNX2 expression levels of the DPSCs suggest that treatment with Y-27632 may up-regulate RUNX2. After day 14 of incubation, RUNX2 expression for the DPSCs plated on tissue culture plastic, Tn PB, and Tk PB remained lower than the expression level for the day zero sample **(Figure 16)**. At day 21 of incubation, the only samples that expressed higher RUNX2 levels than day zero were the samples treated with Y-27632, except for noninduced DPSCs on Tn PB **(Figure 17)**. There is a distinctive increase of RUNX2 expression for Tk PB treated with Y-27632 at day 21.

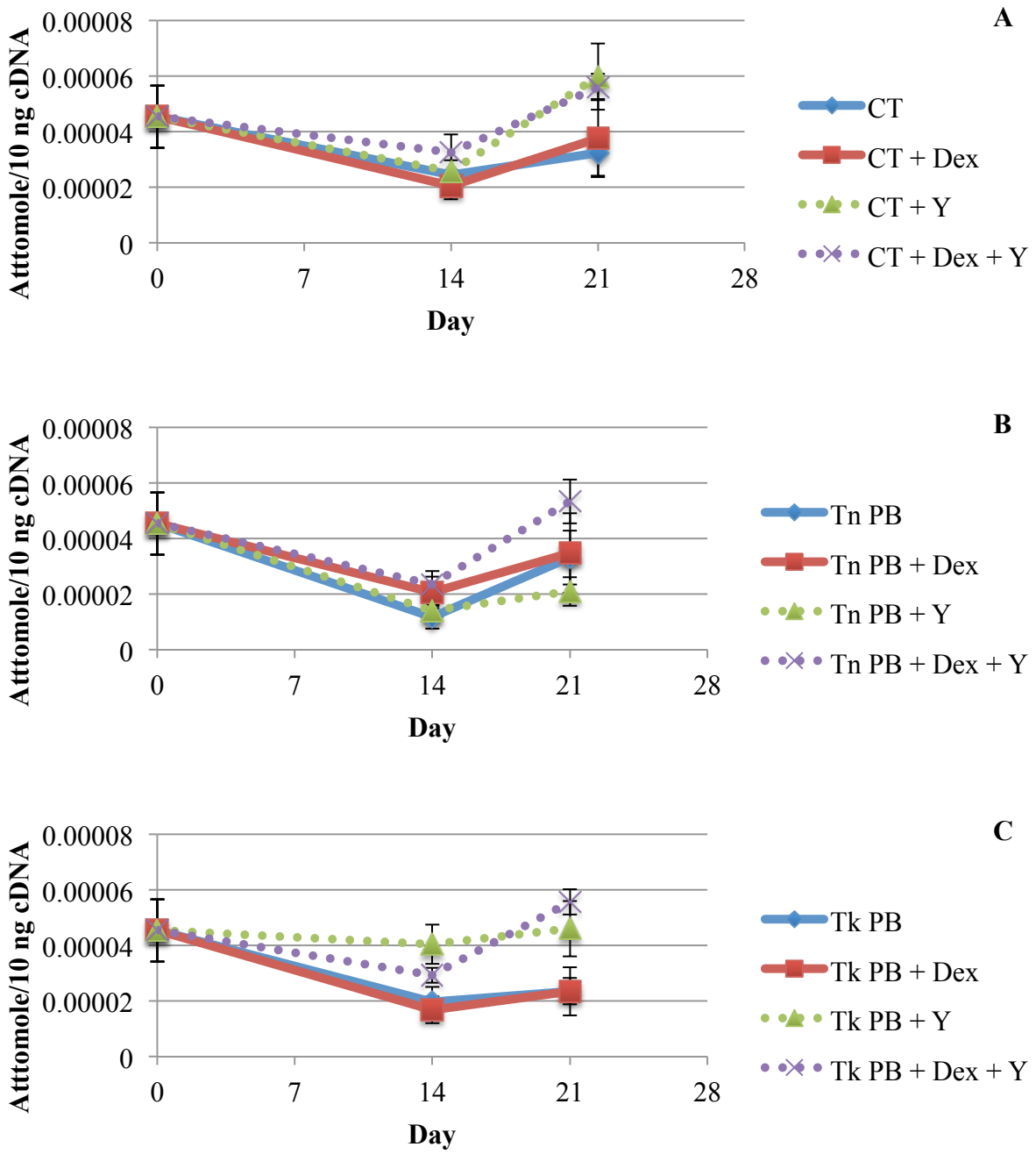

Figure 15. RUNX2 Expression For Samples Over Time. The DPSCs were cultured at passage 7 on (A) tissue culture plastic (control) and on (B) thin and (B) thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

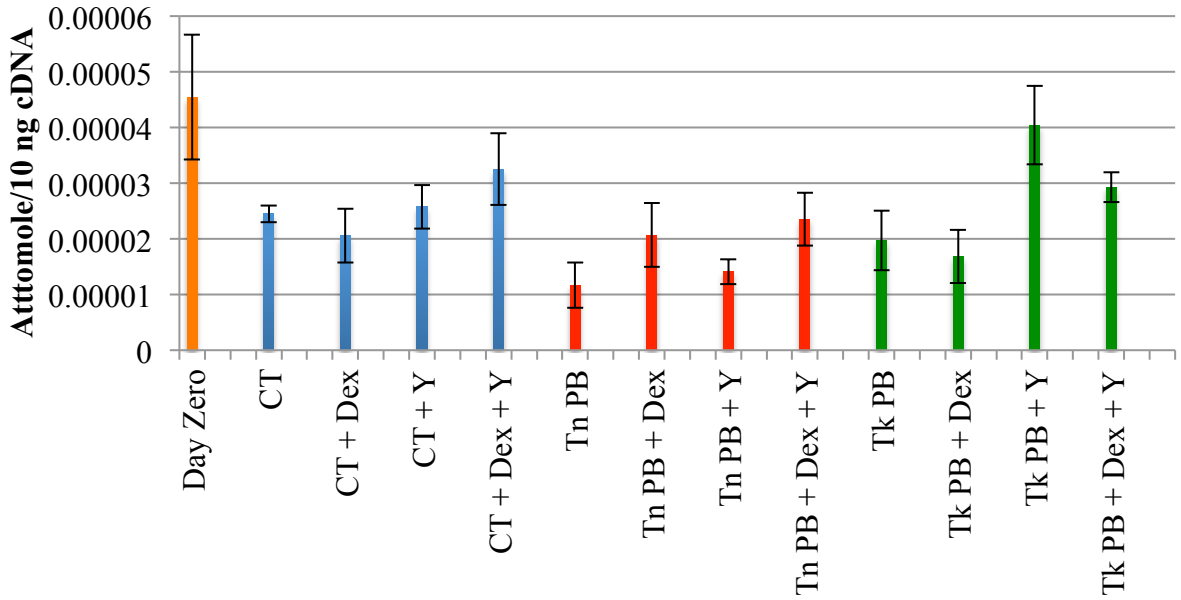

Figure 16. RUNX2 Expression For Day 14 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

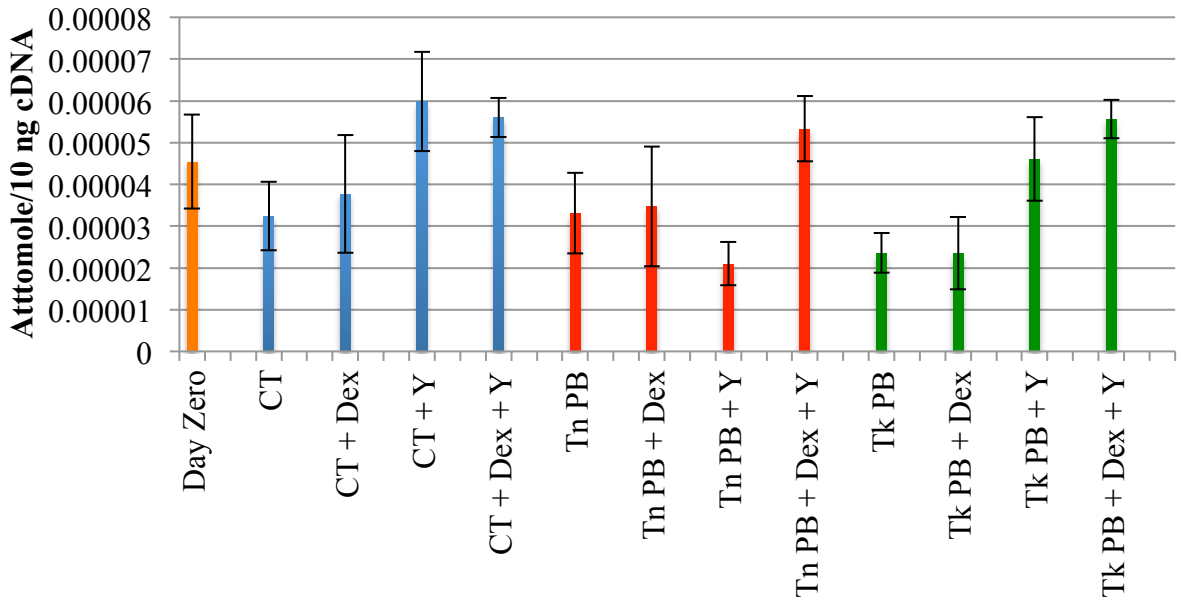

Figure 17. RUNX2 Expression For Day 21 Samples. The DPSCs were cultured at passage 7 on tissue culture plastic (control) and on thin and thick polybutadiene films with thicknesses of 200 Å and 1500 Å, respectively. The cells were maintained with the medium conditions listed in the materials and methods section. The mRNA expression levels were analyzed by qRT-PCR.

Discussion

Advancements in stem cell biology and materials science will enable us to form new treatments in tissue repair using engineered biological compounds [3]. The idea of using stem cells in the field of dentistry with oral tissue repair has been on the rise. Of the many MSCs derived from dental tissue, DPSCs have displayed high proliferation rates and multipotent differentiation capabilities, which are regulated by the microenvironment [1, 15]. These cells in combination with therapies designed to promote angiogenesis and soft tissue repair may be used to regenerate the dental pulp in developing teeth following injury. This alternative to the more typical root canal therapy is of particular value for teeth with incomplete root elongation.

The purpose of this study was to determine whether the major effect of Dex on biomineralization is the result of its ability to directly or indirectly alter cell mechanics, rather than enhance the expression of genes promoting osteogenesis/odontogenesis. Because cells sense substrate mechanics through focal adhesion proteins whose function is regulated by ROCK, the impact of a ROCK inhibitor, Y27632, was monitored. These transmembrane integrins, which are part of the focal adhesions, connect cells to the ECM while adapter proteins and FAK on the cytoplasmic surface regulate binding to the actin cytoskeleton [66-68, 72]. ROCK regulates the Rho-induced formation of actin stress fibers and focal adhesions [58, 62-64]. In this manner, Rho serves as a mechanotransducer of matrix stiffness.

Blocking the Rho-ROCK pathway with Y-27632 exhibited a suppressive effect on the ability of the DPSCs to sense the PB substrate mechanical cues. The mechanical cues from the PB films did not have an effect on the morphology of the noninduced or induced DPSCs. The

Tn PB films have a higher Young's modulus than the Tk PB films. Therefore, Tn PB could tolerate higher levels of traction forces applied by the DPSCs. This was displayed via SMFM, where the relative modulus of the noninduced DPSCs cultured on Tn PB was harder than that on Tk PB. The addition of Dex significantly increased the relative modulus of the DPSCs independent of substrate. Treatment with Y-27632 significantly lowered the relative modulus of the noninduced DPSCs on Tn PB, while suppressing the effect of Dex by lowering the relative modulus of the induced DPSCs on Tn PB and Tk PB. The Y factor exhibited no significant effect on the noninduced DPSCs cultured on Tk PB because Tk PB films are already soft and cannot tolerate high traction forces generated by the DPSCs. In studies carried out with DPSCs on Tn PI and Tk PI, it was found that Dex increased the plasma membrane stiffness. Cytosolic stiffness was increased only at the day 4 time point and only in cells grown on Tn PI. By day 7, Dex was without effect, but cytosolic stiffness was 3-times greater in cells grown on Tn PI compared to Tk PI. Independent of the substrate, the cell modulus, plasma membrane stiffness, and cytosol stiffness of cells treated with Y-27632 were all reduced to levels comparable to or below that of DPSCs grown on Tk PB. The reductions noted were independent of growth with Dex. Thus while previous studies by us and others have shown that treatment with Dex can induce the differentiation of DPSCs into hard-tissue-forming cells [51, 52], the cell's ability to sense its substrate is necessary.

Concomitant with the reduction in cell mechanics and stiffness was the reduction in biomineralization supporting the notion that mechanosensing is necessary for mineralization. Compared to the literature, SEM/EDAX showed that the DPSCs formed dense calcified growths [1] when cultured on Tn PB. When induced with Dex, the calcification increased significantly for the DPSCs on Tn PB. Biomineralization only became slightly visible on Tk PB when

induced with Dex. After treating with the Y-27632, this biomineralization diminished for both the noninduced and induced DPSCs on Tn PB and Tk PB. This exhibits the importance of ROCK in the cell-substrate mechanical sensing. Treatment with Y-27632, plus or minus Dex, inhibits the ability of the DPSCs to biomineralize on the PB films. At a later cell passage, the DPSCs were unable to sense the mechanical cues from the PB films. There was no calcification formed by the noninduced DPSCs on Tn PB or Tk PB. The DPSCs at the later cell passage were only able to biomineralize on Tn PB when induced with Dex. By increasing the thickness of Tk PB from 1500 Å to 3000 Å, the induced DPSCs were unable to biomineralize. This displays the role of cell culture passage and substrate stiffness in the biochemical mechanism for DPSC-PB mechanical sensing.

Evaluation of OCN, ALP, and RUNX2 revealed another aspect of regulation. On all substrates, cells treated with Y-27632 up-regulated OCN with or without Dex; Dex by itself did not alter OCN gene expression independent of substrate. The regulation of ALP was more complex. On tissue culture plastic, ALP expression was enhanced more by Dex than by Y-27632. On Tn PB, ALP expression was enhanced by Dex alone, but not by Y-27632. However, treatment with Dex and Y-27632 was synergistic. On Tk PB, treatment with Dex and Y-27632 was also synergistic, but Dex or Y-27632 alone were without effect. At day 14, the expression of RUNX2 was lower than day 0 with modest increases in expression seen only in cells grown on tissue culture plastic or Tk PB in medium containing Y-27632 with or without Dex, and in cells grown on Tn PB in medium containing both Y-27632 and Dex. This expression pattern is reminiscent of that found in mouse embryonic pre-osteoblasts (MC3T3-E1) grown on polished titanium surfaces. In this system, treatment with Y-27632 resulted in a 2-3 fold increase in ALP and a 4-5 fold increase in OCN [100]. In these experiments, β-catenin expression was also up-

regulated consistent with ALP and OCN being Wnt target genes [101] and with the ability of Y-27632 to suppress BMP-4 stimulated OCN synthesis through p38 MAP kinase [102]. Taken together the data further suggests that expression of osteogenic/odontogenic markers cannot support biomineralization in the absence of proper mechanosensing.

Conclusion

The Rho-ROCK pathway is crucial for the DPSC mechanical sensing. The noninduced DPSCs were able to sense the mechanical cues from Tn PB, which lead to the production of hard-tissue-forming cells and dense calcification. Treatment with Y-27632 had an inhibitory effect on the ability of the DPSC to sense these mechanical cues and limited the ability of these cells to promote mineralization, in spite of their promotion of ALP, OCN, and RUNX2 expression. The impact of mechanosensing on the proteins, protein folding, or protein modifications, required for proper biomineralization remains to be determined.

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