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Role of RhoA/Rho kinase signaling pathway in microgroove induced stem cell myogenic differentiation

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In our previous report, the authors have demonstrated that direct laser machined microchannels would trigger upregulation of myogenic markers in human mesenchymal stem cells (hMSCs) through promotion of cell elongation. However, the molecular basis signaling pathways behind this observation remains unclear. In this work, three types of microchannels generated by femtosecond laser were utilized to investigate possible mechanisms behind the induction of hMSCs myogenesis by microchannels. The authors hypothesized that small G-proteins RhoA and Rac1 play a vital role on myogenesis of hMSCs through regulating cytoskeleton rearrangement, via cell tension signaling cascades. The RhoA and Rac1 activities were evaluated for cells cultured on the micropatterned substrates, using a flat unpatterned substrate as control. It was found that significant activation of RhoA GTPase was exhibited for cells cultured on narrow microchannels (20-20-20 and 30-30-20), while no obvious differences were obtained on wide ones (80-30-20). Meanwhile, no significant difference was found for Rac1 activities on all tested groups. To further deduce the role of RhoA signaling pathway in microchannel directed stem cell myogenesis, the effectors of Rho, Rho kinase (ROCK) was chosen to explore how cell shape regulate myogenesis of hMSCs cultured on laser micropatterned substrate. A pharmacological ROCK inhibitor, Y-27632, was used to treat the cells and the effect on RhoA activation was investigated. Our data on the role of RhoA/ROCK in regulating cell myogenic differentiation on lasered microchannels substrates may provide a mechanistic insight on hMSCs fate directed by substrate topography. © 2015 American Vacuum Society. [<http://dx.doi.org/10.1116/1.4916624>]

I. INTRODUCTION

It has been shown that the unspecialized precursor stem cells are attracting more and more attention due to their multipotent properties and potential application in tissue engineering.¹ Bone-marrow derived adult human mesenchymal stem cells (hMSCs), which is currently one of the most studied stem cell types, have been proven capable of self-renewal and differentiation into a wide variety of lineages in response to proper induction cues.²⁻⁴ The cues include soluble/immobilized factors, chemical and physical signals from the extracellular matrix.⁵ Increasing evidence has shown that the diverse physical environment factors can also control the stem cell activities,⁶ and meanwhile can avoid the side effects associated with biological or chemical additions. Our previous studies demonstrated that topography has great

potential in regulating cell behavior in the absence of any biological or chemical induction factors.⁷⁻¹⁰ Different techniques have been utilized to create different kinds of topographies wherein cells were cultured. Extensive studies have been carried out on using femtosecond laser induced highly uniform microchannels directly on the selected biodegradable polymer, poly(L-lactide-co-ε-caprolactone) (PLC) substrate, for cell growth.^{7,8,11} The channel dimensions were optimized to cause the cells to align, elongate and consequently induce myogenic differentiation in the absence of any induction medium. Other cellular behaviors such as proliferation and cellular organization were also investigated. However, the molecular mechanism of this microtopography induced stem cell myogenic differentiation has not been well documented yet.

Rho family GTPases are important regulators for many features of cellular activities,¹² such as cell adhesion, cell polarity, endocytosis, vesicle trafficking, cell differentiation,

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and gene transcription.¹³ RhoA and Rac1 are two widely studied proteins in Rho family GTPases. The RhoA mainly contributes to contractility formation of stress fibers and focal adhesions. Rac1 was first described to stimulate the formation of lamellipodia and membrane ruffles. It has been demonstrated that RhoA and Rac1 GTPases were two of the most vital molecular signals, which would make signals transductions from extracellular to nuclear, and then influence stem cell differentiation.¹⁴ Rac1 is a small G-protein in the Rho family that drives polymerization of actin and formation of lamellipodia.¹⁵ It is activated by guanine nucleotide exchange factors and repressed by GTPase-activating proteins. Activation of Rho is required for the formation of stress fibers, through one of its downstream effector, Rho kinase (ROCK), to phosphorylate myosin light chain kinase, then activate myosin driven cell contractility. ROCK was discovered as the first effectors of Rho, and it is one the major regulator of cytoskeleton to induce the formation of stress fibers and focal adhesions by phosphorylating myosin light chain.¹⁶ Established work were done to show myosin contractility resulted tension drives the formation of stress fibers.¹⁵ Recent studies have led to interesting conclusion that the Rho GTPases especially the RhoA is a key molecular regulator in hMSCs differentiation induced by both mechanical (cell spreading, intracellular tension) and soluble (insulin/IGF1) factors.^{14,17} For example, MSCs with an undifferentiated state need high RhoA activity to maintain, while down-regulation of RhoA lead to myogenesis.¹⁸ Some reports had already characterized the functionality of Rho family proteins in cell myogenesis.^{19–21} However, much is unknown, on the function of Rac1 in this process.¹⁹ One report from Chen demonstrated that activation of Rac1 inhibited chondrogenesis but was needed for smooth muscle cell differentiation.²² While other researchers have reported that Rac1 activation played an important role in myogenesis inhibition where activated Rac1 suppressed the activity of MyoD in promoting muscle differentiation.^{23,24} One interesting study had shown Rac1 played a dual role in stem cell

cardiac lineage differentiation.²⁵ At early stages, Rac GTPase inhibited cardiomyocytes differentiation, and then promoted cardiomyocytes proliferation and myofibrillogenesis. It would be very interesting to understand the crucial complex cross-talk between RhoA and Rac1 during stem cell differentiation.²⁶

In our previous work, we had already demonstrated that myogenesis is favorable for hMSCs cultured on laser machined narrower microchannels with groove and pitch widths of 30 μm .⁷ The narrow microchannel-induced cell elongation would generate higher intracellular tension than the wider one, which could play a critical role in stem cell myogenesis.^{27,28} In this study, three types of microchannels (20-20-20, 30-30-20, and 80-30-20) were fabricated and then utilized to modulate stem cell differentiation. This topography driven molecular mechanism was elucidated specifically on the chosen RhoA/Rac1 signaling pathway in this study.

II. EXPERIMENT

A. Preparation of film substrate

PLC film was fabricated by the solvent casting method. The block copolymer PLC (70:30), comprised of 70% L-lactide and 30% ϵ -caprolactone (Purac Biomaterials, Lincolnshire, IL), was used in this study. The copolymer PLC granules were dissolved in dichloromethane (Tedia, Fairfield, OH) in 1:5 mass/volume ratio to achieve an optimal viscosity that is suitable for solvent casting process. The films were cast using an industrial film applicator (Paul N. Gardner Company, Inc., Sheen automatic film applicator, FL, USA). The polymer films were dried in a 37 $^{\circ}\text{C}$ vacuum oven for 7 days. The thickness of the dry cast films were approximately 100 μm .

B. Femtosecond laser machining on film substrate

Scheme 1 shows the scaffold design on a 100- μm -thick polymer film. The scaffolds were fabricated using direct femtosecond laser machining as mentioned previously.^{7,8}

SCHEME 1. Design of laser machined scaffolds with three kinds of microchannels.

In brief, the microchannels were machined onto a 10 mm × 10 mm polymer film. Three feature sizes with a targeted channel width W_i of 80, 30, or 20 μm were produced. The length of each channel is 8 mm. The femtosecond laser scanning speed was 10 mm·s⁻¹. The channels with 20 and 30 μm widths were created by scanning two passes on the toolpath at the laser power of 50 mW and 100 mW, respectively. The wider channels with 80 μm width only need one pass at the laser power of 450 mW, to get the same depth as that of narrower channel.

C. Cell culture

hMSCs from an 18 years old male donor and cell culture medium were both obtained from Lonza (Cambrex, UK). hMSCs were expanded and cultured in mesenchymal stem cell basal medium according to the vendor's instruction. For the experiments, the cells were cultured with low-glucose Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (Sigma Aldrich, USA) supplemented with 10% FBS (PAA, Pasching, Austria) and 1% antibiotic/antimycotic solution (PAA, Pasching, Austria), at 37 °C in a humidified atmosphere of 5% CO₂. Culture medium was changed every 2–3 days. PBS and 0.05% trypsin-EDTA obtained from Invitrogen were used for washing and cell detachment purposes, respectively. Scaffolds were sterilized using 70% ethanol for 30 min. Then, scaffolds were washed with deionized water and PBS three times for 15 min each time.

D. Immunocytochemistry and microscopy

Briefly, cells were fixed with 4% paraformaldehyde for 15–20 min and then permeabilized with 0.1% Triton X-100 for 5 min and followed by blocking with 1%–2% bovine serum albumin or 5% goat serum. The primary antibodies used to target interested proteins were vinculin, MAP2, MHC, and fibronectin. Secondary antibodies were Alexa Fluor[®] 488 goat antimouse, Alexa Fluor[®] 568 goat anti-chicken, and Cy3 goat antirabbit IgG (1:400, Millipore). C2C12 myoblast and neural stem cell were used as positive controls for MHC and MAP2, respectively. Negative controls in the absence of primary antibodies were also performed. Filamentous actin (F-actin) was stained with tetramethyl rhodamine iso-thiocyanate conjugated-phalloidin (1:400, Chemicon), and cell nucleus were counter stained with 4'-6-diamidino-2-phenylindole (DAPI) respectively (1:500, Chemicon). Fluorescence images were visualized with a Nikon 80i eclipse (Nikon, Japan) upright microscope and captured with the Nikon DS-Fi1 (Nikon, Japan) using two 20× and 40× objective lens. The confocal laser scanning microscope system (Leica TCS SP5) was also used to capture 3D images using a 40× objective lens (oil).

E. G-LISA kit

The RhoA and Rac1 activity was checked by RhoA and Rac1 G-LISA specific Activation Assay (luminescence, Cat. BK121 and BK126, Cytoskeleton, Inc.) according to the

vendor's instructions as reported in the literatures.^{29,30} The signals were read by measuring absorbance at 490 nm using a microplate spectrophotometer (Infinite200[®] microplate reader, Tecan Inc., Maennedorf, Switzerland).

To evaluate if RhoA regulates the microchannel induced myogenesis, hMSCs were treated with 10 μM ROCK inhibitor, Y27632 (Millipore, USA). Y27632 was dissolved in dimethyl sulfoxide to make a 10 mM stock solution. 10 mM Y27632 was diluted in DMEM to a final concentration of 10 μM to make the block medium. After 7 days culture of cells on micropatterned scaffolds, the normal DMEM was replaced with block medium and changing of block medium every 24 h.

F. Quantitative real time polymerase chain reaction

Total RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA). The concentration and quality of the extracted total RNA was determined spectrophotometrically (Nano drop-N100, Thermo Scientific). Subsequently, cDNA was obtained by reverse transcription of total RNA with random hexamers and superscript III reverse transcriptase (Invitrogen). Quantitative real time polymerase chain reaction (qRT-PCR) was performed on a CFX96 real time PCR detection system (Bio-Rad Laboratories, Inc., USA) for 40 cycles. Primers specific to the targeted genes (listed in Table I) were obtained from primerbank.³¹ Relative quantification of gene expression was determined by comparing the target-amplified product against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin (both were used as internal standard). The results were analyzed with the relative expression software tool software.³¹

G. Statistical analysis

All data are presented as mean $\pm$ standard deviation. Statistical analysis was carried out using one way ANOVA, and the values are considered significantly different when $p < 0.05$.

III. RESULTS AND DISCUSSION

A. Direct lasered microchannels on PLC substrates

PLC is one of the commonly used biodegradable materials in tissue engineering and regenerative medicine. In our previous studies, it was chosen because of its mechanical property, which is elastic and less rigid as compared to most other aliphatic polyesters. Moreover, it has a relatively mild degradation rate, which avoids an abrupt drop in pH value during degradation, similar to PLA and PGA scaffolds.^{32,33}

Femtosecond laser is able to apply highly focused energy for the removal of materials. By changing the laser power, scan speed, and scan pass, the microchannels with three desirable dimensions were machined onto a 10 mm × 10 mm PLC film. The channels were fabricated at a predetermined width, depth, as well as interval distance. When the distance between two microchannels is much narrower than the cell width, there is possibility that cells may migrate from one

TABLE I. Compiled list of gene targets probed in this study.

Genebank accession number	Gene target	Sequence (5'-3')	Amplicon length (bp)
NM 001101	β -actin	CATGTACGTTGCTATCCAGGC CTCCTTAATGTCACGCACGAT	250
NM 002046	GAPDH	CATGAGAAGTATGACAACAGCCT AGTCCTTCCACGATACCAAAGT	113
NM 000364	Cardiac troponin T (cTnT)	TCTCCGAAACAGGATCAACGA GCCCGGTGACTTTAGCCTT	68
NM 002052	GATA-4	CCCAGACGTTCTCAGTCAGTG GCTGTTCCAAGAGTCCTGCT	146
NM 002478	MyoD1	CGGCGAACTGCTACGAAG GCGACTCAGAAGGCACGTC	172
NM 000257	β -myosin heavy chain (MHC)	CACTGATAACGCTTTTGATGTGC TAGGCAGACTTGTCAGCCTCT	165
NM 006086	Class III β -tubulin	GGCCAAGGGTCACTACACG GCAGTCGCAGTTTTCACATC	85
X65964	Nestin	CAACAGCGACGGAGGTCTC CCTCTACGCTCTCTTCTTTGAGT	163
NM 002500	NeuroD1	GCCTTGCTATTCTAAGACGCA GTGGGTTGGGATAAGCCCTT	156
NM 002374	Microtubule associated protein (MAP2)	CAGGAATTGACTCCCTCTACAGC TCTTCACCAGGCTTACTTTGC	80
NM 004348	RUNX2	TCCTATGACCAGTCTTACCCCT GGCTCTTCTTACTGAGAGTGGAA	190
BC 021289	ALPL	CTCTCCAAGACGTACAACACC AATGCCCACAGATTTCCACG	201
NM 003118	Osteonectin (ON)	AGCACCCCATGACGGGTA GGTCACAGGTCTCGAAAAAGC	138

track to another. However, the occurrence is also dependent on the depth (height) of microchannels. Endothelial cells adhered smoothly on surface with 10 μm depth grooves, while fibroblast cells encapsulated in grooves with 10 μm depth.³⁴ The depth of microchannels was selected as 20 μm as this is sufficiently deep to prevent cells from crossing to another track and thus more pronounced contact guidance could occur.³⁵ The microchannel 30-30-20 (channel/interval ratio = 1) was selected because those channels and intervals are similar to cell width, whereby cells can elongate efficiently. The microchannel 80-30-20 was selected because it has higher channel/interval ratio than 30-30-20. The microchannel 20-20-20 was selected to study if channels and intervals with narrower width have more significant effect on

stem cell behavior. As can be seen in Fig. 1, uniform microchannel topography with lined structure was achieved on the PLC film surface through femtosecond laser machining.

B. Micropatterned substrates induce stem cell myogenic differentiation

Our previous report had already presented that laser cut microchannel 30-30-20 on the polymeric PLC substrate would promote stem cell lineage differentiation toward myogenesis.⁷ The mRNA expression level of myogenic markers (cTnI, GATA4, MHC7, MyoD1, and Nkx2.5) were significantly upregulated for cells cultured on laser micropatterned 30-30-20 substrates for all 7, 11, and 14 days of cell culture.

FIG. 1. SEM images of laser induced microchannels in PLC substrate.

However, only transient expression of myogenic markers was detected for cells on laser micropatterned 80-30-20 substrates on the time-point of Day 11. Immunostaining for lineage-specific myocardial marker beta-MHC was performed to confirm that those special 30-30-20 microchannels steer plated hMSCs into myogenic lineage. In this study, direct laser machining of even narrower 20 μ m width microchannels on PLC substrates were applied to influence stem cell differentiation which could further prove that narrower microchannels (30-30-20 and 20-20-20) with smaller radii of curvature would direct stem cell myogenic differentiation. As shown in Fig. 2(a), MHC staining on the laser patterned scaffolds with microchannels was detected on narrower microchannels (30-30-20 and 20-20-20), while no positive

staining of MHC was observed on micropatterned 80-30-20 substrates.

C. RhoA and Rac1 GTPases activation on laser micropatterned substrates

A mechanistic study is performed here to elucidate the potential molecular basis of microchannels modulated stem cell differentiation through the elongation of cell shape. It has been demonstrated that Rho family of small GTPases (including three mainly studied proteins, RhoA, Rac1, and CDC 42) can regulate cell shape through cytoskeleton reorganizations.¹³ They act as transducers which are responsive for translating extracellular signals to cell movement and

FIG. 2. Overlaid fluorescent image of immunostained cellular components (merged) for the nonpatterned and micropatterned hMSCs after 11 days (a) untreated and (b) with ROCK inhibited (10 μ M Y27632). F-actin (red), DAPI (blue), and immunostained myogenic marker MHC (green). (c) The negative control and positive control of MHC. Scale bar, 50 μ m.

spatial dynamics of cytoskeleton.³⁶ One interesting report presented RhoA activity mediated cell-shape dependent modulation of hMSCs differentiation toward osteoblast or adipocytes, through the cell shape regulation by restricting adhesive areas.¹⁴ More recent evidence show that small Rho family GTPases monitor corneal epithelial cell alignment on substrate with anisotropic nanotopographies.³⁷ However, the role of Rho family GTPases in modulation of hMSCs contact guidance, shape elongation, and lineage commitment on laser micropatterned surface still remain to be explored.

G-LISA assay was applied to investigate the involvement of RhoA or Rac1 GTPases activation in our micropatterned induced hMSCs myogenic differentiation. The G-LISA assay results in Fig. 3 showed that there is a significant increase of RhoA GTPases in hMSCs on scaffolds with narrow microchannels (20-20-20 and 30-30-20) compared with that on scaffolds with wide microchannels (80-30-20) after 7, 11, and 14 days of cell culture. There was a gradual increase of RhoA GTPase activity in cells on 20-20-20 and 30-30-20 and with the culture time, from 28% at day 7 to 42% at day 11 and 51% at day 14, respectively. However, no significant differences in the activation of RhoA GTPase were exhibited for hMSCs on wide microchannels (80-30-20).

As for Rac1, there was no obvious difference in the activities presented in hMSCs on all three micropatterned groups, comparing with that of unpatterned control group. Based on the results showed previously,⁷ and G-LISA assay results here, activation of RhoA, but not Rac1, might be involved in the myogenic differentiation of hMSCs on laser machined substrate.

D. Role of RhoA/ROCK on topography induced stem cell differentiation

As mentioned previously, the small G-protein Rho leads to the reorganization of actin filaments as well as assembly of contractile stress fiber formations, which subsequently influence stem cell differentiation. Myogenic differentiation is associated with induction of a group of muscle-specific genes, many of which encrypt cytoskeletal proteins.³⁸ Contractility in cytoskeleton is essential for stem cell myogenic differentiation.³⁹ It has been reported that the existence

of cytoskeletal tension resulted in the differentiation of fibroblasts into myofibroblasts.⁴⁰ Through the manipulation of cell adhesion area, cytoskeleton rearrangement always stems from activation of RhoA/ROCK, which is important to detect biophysical stimuli and direct stem cell differentiation. One well-known report by McBeath revealed that the changes of RhoA/ROCK activity and cytoskeletal tension influence hMSCs lineage commitment through the control of cell shape.¹⁴ Another report showed that the micropatterns induced changes in cell shape alter RhoA-mediated cell contractility.⁴¹ Several critical reviews had already demonstrated surface topography would affect the reorganization of cytoskeleton and formation of cellular tension. Two more recent studies also showed RhoA/ROCK pathway is involved in topography induced actin cytoskeleton organization.^{42,43} Therefore, based on the literatures presented above demonstrating the important role of RhoA/ROCK in regulating cytoskeletal organization in general and subsequently myogenesis which coincides with our study, this specific pathway is also deemed to may be involved in our microchannel committed stem cell myogenic differentiation. To investigate that, hMSCs were treated with ROCK inhibitor (Y27632) after cells were cultured for 7 days, to check the role of RhoA/ROCK signaling in the microchannel directed myogenic differentiation of hMSCs.

To characterize the effect of Y27632 on cell morphology and microfilament formation, the cell morphology of each group was stained and observed under confocal fluorescent microscope [Fig. 2(b)]. Cells on the control group without exposure to ROCK inhibitor (10 μ M Y27632) were mostly well spread out with randomly distributed actin fibers [as shown in Fig. 2(a), control]. Cells on the laser micropatterned scaffolds treated with ROCK inhibitor lose their elongated cell shapes, became stellatelike cells, without clearly formed microfilaments, which is significantly different to the morphologies demonstrated in Fig. 2(a). Despite of that, it is noted that the inhibition of ROCK induced lamellipodia outgrowth accompanied with dendritic structures [white arrowhead in Fig. 2(b)]. From the images, more dendritic structures were found in cells cultured on scaffolds with wider microchannels (80-30-20) than those structures found

FIG. 3. RhoA and Rac1 GTPases activation in hMSCs on control PLC films and laser micropatterned PLC scaffolds measured by G-LISA. Each reaction was performed in triplicate. Student's t test, compared with unpatterned control * $p < 0.05$.

in cells on scaffolds with narrow microchannels (20–20–20 and 30–30–20). When investigated further, qPCR results (Fig. 4, see quantitative data in supplementary material Table S1)⁴⁴ revealed that neuromarkers of MAP2, Nestin and NeuroD1 were upregulated in cells on micropatterned scaffolds with the treatment of ROCK inhibitor, when cells were normalized to the unpatterned control group without ROCK inhibition. Most of osteo-markers remained unchanged as compared to control, while myogenesis markers were even downregulated after ROCK inhibition. Immunostaining of MHC in cells on all scaffolds after drug treatment was also found to be negative as shown in Fig. 2(b). Therefore, presence of ROCK inhibitor not only disrupted the formation of actin filament and elongated cell shape, interrupted myogenic differentiation of hMSCs, but also stimulated surface protrusions (lamellipodia). This was accompanied by upregulation of neurogenic markers especially for wider channels (80–30–20).

It is well known that RhoA and Rac1 GTPases have antagonistic functions during many processes, like dendritic branching in neurons,⁴⁵ cell–cell contact formation,⁴⁶ epithelial morphogenesis,⁴⁷ etc. Based on the Rac1 G-LISA assay results shown above (Fig. 3), a slight up-regulation of Rac1 activity was revealed on scaffolds with wide microchannels (80–30–20), and a down-regulation of Rac1 activity was

appeared on scaffolds with narrow microchannels (20–20–20 and 30–30–20). Although the decrease in Rac1 activity was not significant, these results showed that RhoA and Rac1 might have opposite effect on cell functions. Similar results were reported on the promotion of myogenic differentiation through RhoA positive and Rac1 negative regulation triggered by N-cadherin.⁴⁸ Hence, the possible mechanism was that RhoA activation in cells on scaffolds with narrow microchannels (20–20–20 and 30–30–20), initiated clustering of focal adhesion and polymerization of stress fibers, which caused increase in cellular tension, and promoted myogenic differentiation.^{6,30} On the other hand, activation of Rac1 in cells on scaffolds with wide microchannels (80–30–20) might encourage the lamellipodia formation and dendritic branching, which is in agreement with another report.⁴⁹ Therefore, these results suggested that activation of RhoA GTPase, but not Rac1, might be engaged in the microchannels induced actin cytoskeleton reorganization and subsequent myogenic lineage commitment.

IV. SUMMARY

The results presented here show that narrower microchannels are favorable for myogenic differentiation of hMSCs due to cell shape elongation. Rho GTPase molecular signaling was examined to elucidate the possible molecular mechanism behind topography of microchannels induced hMSCs myogenesis. RhoA/ROCK pathway is the key regulator for hMSCs elongation on laser micropatterned scaffolds. Inhibition of ROCK suppressed myogenesis of hMSCs, demonstrating that RhoA/ROCK signaling is involved in microchannel directed myogenesis of hMSCs.

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FIG. 4. Relative gene level expression analyses of neurogenic, osteogenic and myogenic markers after 14 days of cell culture untreated and with ROCK inhibition (10 μ M Y27632) are illustrated in the form of heat map. The unpatterned PLC substrate without addition of inhibitor was used as control.

¹N. S. Hwang, S. Varghese, and J. Elisseeff, *Adv. Drug Delivery Rev.* **60**, 199 (2008).

²M. F. Pittenger *et al.*, *Science* **284**, 143 (1999).

³H. Song, B.-W. Song, M.-J. Cha, I.-G. Choi, and K.-C. Hwang, *Expert Opin. Biol. Ther.* **10**, 309 (2010).

⁴A. W. Lund, B. Yener, J. P. Stegemann, and G. E. Plopper, *Tissue Eng., Part B* **15**, 371 (2009).

⁵A. M. Ross and A. B. Jason, *Adv. Mater.* **22**, 175 (2010).

⁶F. Guilak, D. M. Cohen, B. T. Estes, J. M. Gimble, W. Liedtke, and C. S. Chen, *Cell Stem Cell* **5**, 17 (2009).

⁷H. Li, F. Wen, Y. S. Wong, F. Y. C. Boey, V. S. Subbu, D. T. Leong, K. W. Ng, G. K. L. Ng, and L. P. Tan, *Acta Biomater.* **8**, 531 (2012).

⁸H. Li, Y. S. Wong, F. Wen, K. W. Ng, G. K. L. Ng, S. S. Venkatraman, F. Y. C. Boey, and L. P. Tan, *Macromol. Biosci.* **13**, 299 (2013).

⁹C. Y. Tay *et al.*, *Small* **7**, 1416 (2011).

¹⁰C. Y. Tay, H. Yu, M. Pal, W. S. Leong, N. S. Tan, K. W. Ng, D. T. Leong, and L. P. Tan, *Exp. Cell Res.* **316**, 1159 (2010).

¹¹W. Y. Yeong, H. Yu, K. P. Lim, K. L. G. Ng, Y. C. F. Boey, V. S. Subbu, and L. P. Tan, *Tissue Eng., Part C* **16**, 1011 (2010).

¹²A. Hall, *Science* **279**, 509 (1998).

¹³S. Etienne-Manneville and A. Hall, *Nature* **420**, 629 (2002).

¹⁴R. McBeath, D. M. Pirone, C. M. Nelson, K. Bhadriraju, and C. S. Chen, *Dev. Cell* **6**, 483 (2004).

¹⁵K. Burridge and K. Wennerberg, *Cell* **116**, 167 (2004).

- ¹⁶T. Leung, X. Q. Chen, E. Manser, and L. Lim, *Mol. Cell. Biol.* **16**, 5313 (1996), available at <http://mcb.asm.org/content/16/10/5313>.
- ¹⁷R. Sordella, W. Jiang, G.-C. Chen, M. Curto, and J. Settleman, *Cell* **113**, 147 (2003).
- ¹⁸S. Beqaj, S. Jakkaraju, R. R. Mattingly, D. Pan, and L. Schuger, *J. Cell Biol.* **156**, 893 (2002).
- ¹⁹B. A. Bryan, D. Li, X. Wu, and M. Liu, *Cell. Mol. Life Sci.* **62**, 1547 (2005).
- ²⁰R. L. Perry and M. A. Rudnick, *Front. Biosci.* **5**, D750 (2000).
- ²¹S. Travaglione, G. Messina, A. Fabbri, L. Falzano, A. M. Giammarioli, M. Grossi, S. Rufini, and C. Fiorentini, *Cell Death Differ.* **12**, 78 (2004).
- ²²G. Lin, M. Rowena, and S. C. Christopher, *Stem Cells* **28**, 564 (2010).
- ²³H. Heller, E. Gredinger, and E. Bengal, *J. Biol. Chem.* **276**, 37307 (2001).
- ²⁴M. Meriane, P. Roux, M. Primig, P. Fort, and C. Gauthier-Rouvière, *Mol. Biol. Cell* **11**, 2513 (2000).
- ²⁵M. Pucéat, P. Travo, M. T. Quinn, and P. Fort, *Mol. Biol. Cell* **14**, 2781 (2003).
- ²⁶B. J. Dickson, *Curr. Opin. Neurobiol.* **11**, 103 (2001).
- ²⁷S. Jakkaraju, X. Zhe, and L. Schuger, *Trends Cardiovasc. Med.* **13**, 330 (2003).
- ²⁸V. Dugina, L. Fontao, C. Chaponnier, J. Vasiliev, and G. Gabbiani, *J. Cell Sci.* **114**, 3285 (2001), available at <http://jcs.biologists.org/content/114/18/3285>.
- ²⁹W. S. Leong, S. C. Wu, M. Pal, C. Y. Tay, H. Yu, H. Li, and L. P. Tan, *J. Tissue Eng. Regen. Med.* **6**, S68 (2012).
- ³⁰H. Yu *et al.*, *Stem Cells Dev.* **22**, 136 (2013).
- ³¹M. Pfaffl, G. Horgan, and L. Dempfle, *Nucleic Acids Res.* **30**, E36 (2002).
- ³²Z. Yang, Y. Wu, C. Li, T. Zhang, Y. Zou, J. H. P. Hui, Z. Ge, and E. H. Lee, *Tissue Eng., Part A* **18**, 242 (2011).
- ³³L. Chao, Z. Jingjing, L. Yijiang, M. Shamus, K. Gilson, and G. Zigang, *Biomed. Mater.* **8**, 025005 (2013).
- ³⁴B. Chehroudi, T. R. L. Gould, and D. M. Brunette, *J. Biomed. Mater. Res.* **24**, 1203 (1990).
- ³⁵R. G. Flemming, C. J. Murphy, G. A. Abrams, S. L. Goodman, and P. F. Nealey, *Biomaterials* **20**, 573 (1999).
- ³⁶A. J. Ridley, M. A. Schwartz, K. Burridge, R. A. Firtel, M. H. Ginsberg, G. Borisy, J. T. Parsons, and A. R. Horwitz, *Science* **302**, 1704 (2003).
- ³⁷A. M. Rajniecek, L. E. Foubister, and C. D. McCaig, *Biomaterials* **29**, 2082 (2008).
- ³⁸J. Dhawan and D. M. Helfman, *J. Cell Sci.* **117**, 3735 (2004).
- ³⁹H. Yao, X. Xie, D. Wang, and X. Pei, *Chin. Sci. Bull.* **52**, 3025 (2007).
- ⁴⁰M. B. Vaughan, E. W. Howard, and J. J. Tomasek, *Exp. Cell Res.* **257**, 180 (2000).
- ⁴¹K. Bhadriraju, M. Yang, S. Alom Ruiz, D. Pirone, J. Tan, and C. S. Chen, *Exp. Cell Res.* **313**, 3616 (2007).
- ⁴²C. H. Seo, K. Furukawa, K. Montagne, H. Jeong, and T. Ushida, *Biomaterials* **32**, 9568 (2011).
- ⁴³A. Calzado-Martín, A. Méndez-Vilas, M. Multigner, L. Saldaña, J. L. González-Carrasco, M. L. González-Martín, and N. Vilaboa, *Acta Biomater.* **7**, 1890 (2011).
- ⁴⁴See supplementary material at <http://dx.doi.org/10.1116/1.4916624> for the following information: In order to complement the heat map, Table S1, showing the relative quantification of gene expression, has been added here in the supplementary material. Relative gene expression level was analyzed with CFX manager software (Bio-Rad Laboratories, Inc.) and Relative Expression Software Tool.
- ⁴⁵M. Negishi and H. Katoh, *Neuroscientist* **11**, 187 (2005).
- ⁴⁶F. Comunale, M. Causeret, C. Favard, J. Cau, N. Taulet, S. Charrasse, and C. Gauthier-Rouvière, *Biol. Cell* **99**, 503 (2007).
- ⁴⁷B. K. Chauhan, M. Lou, Y. Zheng, and R. A. Lang, *Proc. Natl. Acad. Sci.* **108**, 18289 (2011).
- ⁴⁸S. Charrasse, M. Meriane, F. Comunale, A. Blangy, and C. Gauthier-Rouvière, *J. Cell Biol.* **158**, 953 (2002).
- ⁴⁹D. L. Xie, H. Lill, G. Hauska, M. Maeda, M. Futai, and N. Nelson, *Biochim. Biophys. Acta* **1172**, 267 (1993).