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Mechanical stretch inhibits myoblast-to-adipocyte differentiation through Wnt signaling

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Abstract

Myoblasts are able to differentiate into other mesenchymal lineages including adipocytes and osteoblasts. However, it is not known how this differentiation is normally regulated in intact animals and humans. Here, we subjected cultured C2C12 myoblasts to cyclic mechanical stretch (20% elongation) during differentiation into adipocytes. Mechanical stretch inhibited the myoblast-to-adipocyte differentiation significantly, concurrent with an enhanced expression of Wnt10b mRNA. Inhibition of the Wnt signaling by incubation of the myoblasts with a soluble Wnt ligand, sFRP-2, abolished the inhibitory function of mechanical stretch on adipogenesis. These findings provide evidence that mechanical stretch inhibits myoblast-to-adipocyte differentiation possibly through Wnt signaling.

Keywords: Mechanical stress; Mechanical stretch; Trans-differentiation; C2C12; Wnt10; Myoblasts; Adipocytes

Mechanical stimuli are thought to be important for regulation of tissue development, as somatic cells are normally exposed to a variety of mechanical stimuli in living animals [1]. Mechanical stretch has a variety of effects on the structure and function of cells, and is responsible for post-natal adaptations through pre- and post-transcriptional regulation [2], which has been investigated extensively in smooth and cardiac muscle cells [3–8]. However, little is known about the effect of mechanical stress on cell differentiation [1,9].

Myogenic stem cells in adult skeletal muscle (satellite cells) maintain multi-differentiation potential and are able to differentiate into non-muscle cells including osteoclasts and adipocytes when exposed to certain inductive factors. C2C12 cells, a skeletal muscle myogenic progenitor cell line, possess pluripotent differentiation potential to mesenchymal cell lineages in vitro and in vivo [10,11]. This transition between one differentiated lineage to another is also called trans-differentiation. The signaling mechanisms that are responsible for trans-differentiation are poorly understood [12]. The normal development of skeletal muscle is controlled by myogenic regulatory factors (MRFs), such as myf-5, myogenin, and MyoD [13]. On the other hand, differentiation of adipocytes appears to be controlled by peroxisome proliferator activating receptor (PPAR)-γ and the C/EBP families of transcription factors. The balance between these myogenic and adipogenic transcriptional factors is related to commitment of cell

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differentiation since forced expression of MyoD causes myogenic differentiation of pre-adipocytes, while forced expression of PPAR-γ leads to differentiation of myoblasts to adipocytes [10].

We have previously shown that mechanical stretch regulates expression of transcriptional factors influencing cell differentiation [14]. Application of cyclic mechanical stretch down-regulates expression of MyoD and MNF-α in C2C12 myoblasts. Based on these findings, we hypothesized that mechanical stretch affects the balance between myogenic and adipogenic transcriptional regulatory factors, thereby promoting the commitment of pluripotent precursor cells, such as muscle satellite cells, into the myogenic lineage and inhibiting their differentiation into the adipogenic lineage.

In this study, we investigated the effect of mechanical stretch on C2C12 muscle satellite cells during induced adipogenesis. We show that mechanical stretch inhibits C2C12 cell adipogenic differentiation through activated Wnt signaling. These findings suggest a mechanism by which the commitment of muscle satellite cells to the myogenic lineage is maintained by mechanical stimuli in vivo.

Materials and methods

Cell culture. Murine C2C12 cells were generously provided by Riken Cell Bank (Tsukuba, Japan). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and antibiotics in 5% CO_2 atmosphere. To inhibit Wnt signals, C2C12 cells were cultured in the presence of 100 nM secreted Frizzled-related protein (sFRP)-2 (R&D Systems, Minneapolis, MN).

Induction of adipogenesis. For adipogenic differentiation, C2C12 cells were grown to confluency in DMEM containing 10% FBS before switching to an adipogenic induction medium (4.5 g/L glucose, 10% FBS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 μg/ml insulin in DMEM). After 72 h, the medium was changed to adipogenic maintenance medium (4.5 g/ml glucose, 10 μg/ml insulin, and 10% FBS in DMEM) and was changed every other day. After 7 days, the cells were used for further analyses.

Oil red-O staining. The cells were rinsed twice with PBS, fixed with 4% para-formaldehyde for 5 min, and washed with distilled water. Cytoplasmic lipid droplets in cell cultures were stained with the Oil red-O solution for 30 min. The cultures were then washed thoroughly with distilled water.

Mechanical stretch of C2C12. Cyclic mechanical stretch was applied to cultured C2C12 cells in vitro using a BioFlex strain unit (Flexcell International, Hillsborough, NC) as previously described [15]. Briefly, C2C12 myoblasts were cultured in a 6-well plate with flexible collagen-coated silicone rubber membranes at the bottom of each well. A vacuum (17 kPa) was applied at a frequency of 6 cycles/min (3-s on-time, 7-s off-time) from the base of the plate. The maximal percent elongation of the culture surface was 20%. Cells cultured on the same type of plates without stretch served as control.

RNA isolation and semiquantitative reverse transcriptional polymerase chain reaction. Semiquantitative reverse transcriptional polymerase chain reaction (RT-PCR) analysis was performed as described previously [16] with modification. Briefly, total RNA was isolated from the cultured C2C12 by using ISOGEN (Nippongene, Tokyo, Japan). Four micrograms of total RNA was used in each RT-reaction (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Complementary DNA (2 μl) was then used as a template for the PCR in a 20-μl reaction volume including 100 ng of each primer, 2 mM $MgCl_2$, PCR buffer, and 1 U *Taq* polymerase (Toyobo, Osaka, Japan). Ten microliters of each PCR was loaded on a 1% agarose gel as described. PCR primer pairs used for this study include glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-GTG GCA AAG TGG AGA TTG TTG CC-3′ and 5′-GAT GAT GAC CCG TTT GGC TCC-3′; PPAR-γ: F, 5′-ATC AGC TCT GTG GAC CTC TC-3′ and R, 5′-ACC TGA TGG CAT TGT GAG AC-3′; and Wnt10b: F, 5′-CGG CTG CCG CAC CAC AGC GC-3′ and R, 5′-CAG CTT GGC TCT AAG CCG GT-3′.

The RT-PCR exponential phase was determined on 15–35 cycles to allow semiquantitative comparisons among cDNAs developed from identical reactions. Each PCR regime involved a 94 °C, 2 min initial denaturation step followed by 25 cycle at 94 °C for 1 min, 55–57 °C for 1 min, 72 °C for 1 min on a GeneAmp PCR system 9700 (Perkin-Elmer, Wellesley, MA). The PCR products were separated by electrophoresis on 1% agarose gel. We included primers for GAPDH in the PCR as an internal control. The linearity of the PCR was confirmed for each gene (data not shown).

Statistical analysis. Data were presented as means ± SEM from separated experiments. To assess the effect of mechanical stretch on temporal data, one-way analysis of variance (ANOVA) was used, and Bonferroni corrections were made for multiple comparisons. P values < 0.05 were considered significant.

Results

C2C12 cells can differentiate into adipocytes

To induce adipogenic differentiation, C2C12 cells were cultured in adipogenic induction medium for 7 days. The cells were stained with Oil-red O to visualize the degree of lipid accumulation (Fig. 1A). To further confirm adipogenic differentiation, PPAR-γ mRNA was measured by RT-PCR in these cells. There was a

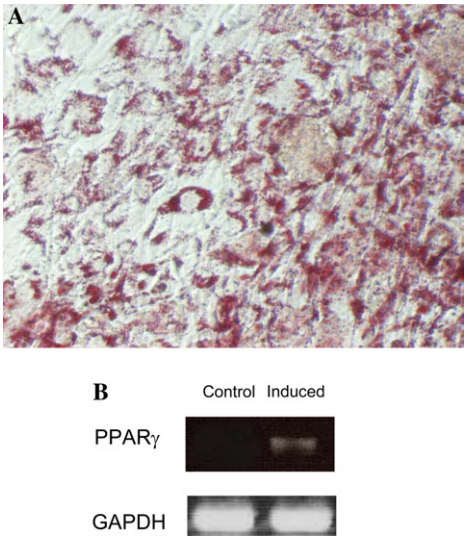

Fig. 1. C2C12 cells differentiate into adipocytes. Cells cultured in adipogenic induction medium (10% FBS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 μg/ml insulin) for 7 days stain positive with Oil red-O (A) and express PPAR-γ mRNA (B) as markers of adipogenic differentiation.

4.2-fold increase of PPAR-γ in cells was induced adipogenesis (Fig. 1B).

Mechanical stretch inhibits adipogenic differentiation in C2C12 cells

To determine the effect of mechanical stretch on trans-differentiation of C2C12 cells into adipocytes, we subjected C2C12 cells to cyclic mechanical stretch during induced-adipogenic differentiation. Mechanical stretch maintained C2C12 cells in spindle shape while cells without stretch treatment acquired a round shape during adipogenic differentiation (Figs. 2A and B). Furthermore, the expression of PPAR-γ mRNA was decreased by stretch in adipogenic induction medium (Figs. 2C and D).

Mechanical stretch inhibits adipogenesis via Wnt signaling

To determine the mechanism of stretch-induced inhibition of adipogenesis in C2C12, we compared the expression of Wnt10b, which is known to stabilize cytosolic β-catenin in Wnt signaling, between stretched and unstretched cells under adipogenic conditions. Wnt10b mRNA was increased 2.5-fold in mechanical stretched cells compared with sister cultures without stretch (Fig. 3). To confirm that the profound effects of mechan-

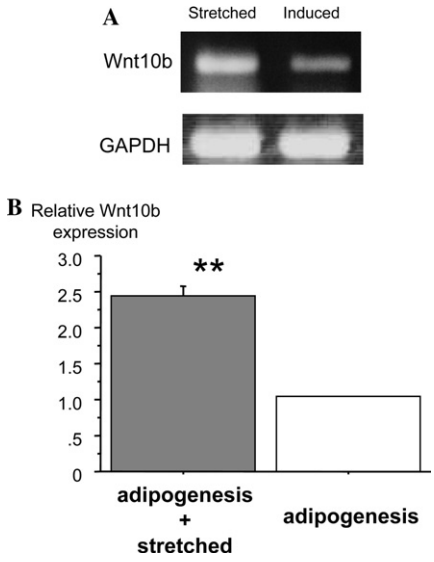

Fig. 3. Mechanical stretch increases expression of Wnt10b. Expression of Wnt10b mRNA was enhanced by mechanical stretch (A,B). $^{**}p < 0.01$ versus control by ANOVA.

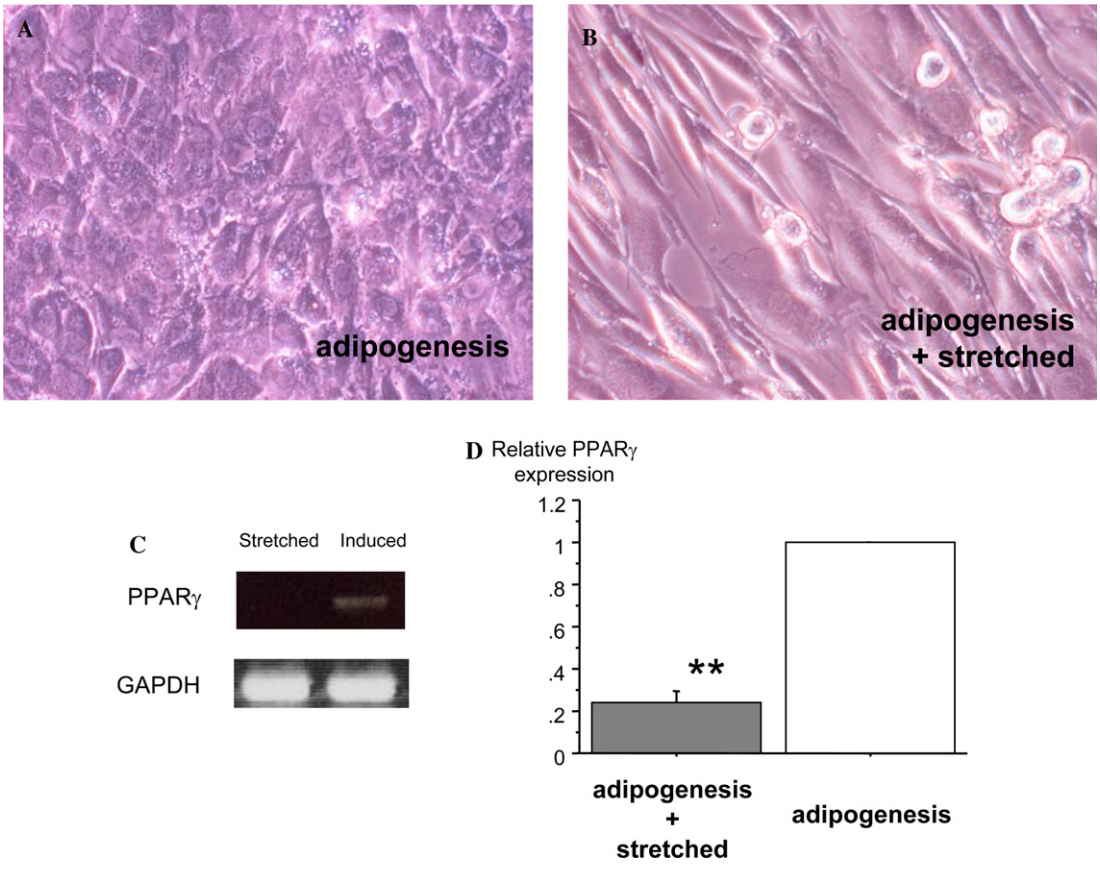

Fig. 2. Mechanical stretch inhibits adipogenic differentiation of C2C12 cells. Unstretched cells in adipogenic medium assume the rounded shape of adipocytes (A) while stretched cells remain spindle-shaped (B). Stretch reduced expression of PPAR-γ mRNA (C,D). $^{**}p < 0.01$ versus control by ANOVA.

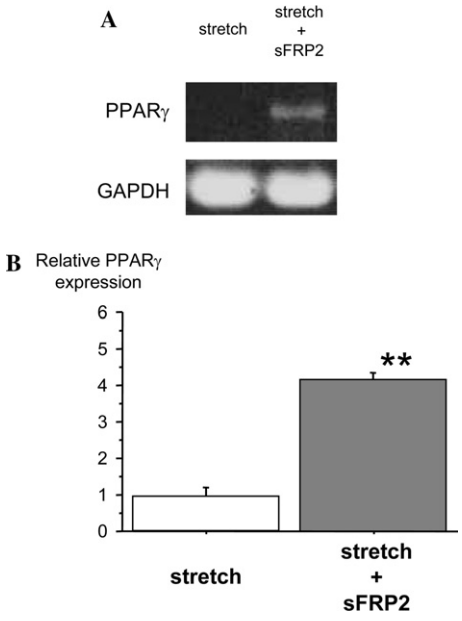

Fig. 4. Mechanical stretch-induced inhibition of adipogenesis requires Wnt signaling. C2C12 cells cultured in adipogenic induction medium with sFRP2 to inhibit Wnt signaling expressed PPAR-γ mRNA in the manner of unstretched cells.

ical stretch were indeed attributable to Wnt signaling, we added sFRP-2, a soluble receptor of Wnt10b, into the culture. sFRP-2 can block the binding of Wnt10b to its receptor on the cell membrane [17]. Addition of sFRP-2 completely blocked stretch-induced expression of PPAR-γ mRNA (Fig. 4).

Discussion

This study demonstrates that differentiation from myoblasts to adipocytes is inhibited by cyclic mechanical stretch. Blocking Wnt signaling by a soluble receptor of Wnt, secreted Frizzled-related protein-2, abolished the mechanical stretch-induced inhibition of adipogenesis. These results suggest that mechanical stretch inhibits the myoblast-to-adipocyte differentiation via Wnt signaling.

To our knowledge, this is the first report to show that mechanical stress affects myoblast-to-adipocyte differentiation. The term trans-differentiation refers to the phenotypic change of one differentiated cell type to another [11,12]. Recent studies have shown that trans-differentiation occurred in adult mammals. Adult stem cells were shown to differentiate into cell types that are not normally present in their residing tissues/organs [11,12]. It has been postulated that some tissue/organ injury might be repaired by trans-differentiation of reservoir cells, such as satellite cells.

Our results that myoblast-to-adipocyte trans-differentiation is inhibited by mechanical stretch suggest that mechanical cues play important roles in the development, maintenance, and regeneration of mammalian tissues. Experimentally, the in vitro myoblast-to-adipocyte differentiation system used here should allow us to identify extracellular and intracellular signaling molecules that control cross-lineage commitment of myogenic stem cells. Identification of such signaling molecules will not only shed light on the fundamental mechanisms regulating trans-differentiation, but also provide novel approaches for cell-based therapeutics. In addition, our findings may also be relevant to understanding the pathogenesis of obesity and skeletal muscle diseases. For example, aged and obese patients have a high fat to muscle ratio compared with young and healthy individuals [18], an outcome possibly due to the conversion of muscle to fat cells. Understanding the signaling pathways that mediate trans-differentiation between myoblasts and adipocytes should be of relevance to the development of therapeutics for the treatment of obesity and impaired metabolism in sedentary population.

Our results might also provide new insights into the relationships between exercise and body composition. It is generally known that exercise decreases fat mass [19] due to increased energy expenditure during muscle contraction. However, our current data suggest that exercise-associated mechanical stimuli may block pathogenic trans-differentiation of myogenic satellite cells into pre-adipocytes that may migrate to sites of fat deposition. It is also possible that signaling molecules elaborated within contracting muscles circulate as humoral factors to exert similar effects on adipogenic progenitor cells to reduce fat body mass.

Adipogenic differentiation can be regulated by both inhibitory and stimulatory factors [20]. One of the endogenous factors proposed to repress adipogenesis is Wnt10b, which belongs to a large family of secreted, cysteine-rich proteins that regulate diverse cellular and developmental processes [21,22]. When the Wnt signaling in pre-adipocytes is blocked by overexpression of axin or dominant-negative TCF4, these cells differentiate into adipocytes spontaneously. Although Wnt regulates cell fate through several signaling pathways, activation of the canonical Wnt/β-catenin pathway is sufficient to inhibit differentiation and apoptosis of pre-adipocytes [17]. Mechanical stretch-induced inhibition of adipogenesis was prevented by addition of a Wnt inhibitor, sFRP2. Activity of Wnt proteins is regulated by secreted inhibitors, such as Wnt inhibitory factor-1, and the family of secreted Frizzled-related proteins appears to bind and sequester Wnts from their receptors. While activation of Wnt signaling inhibits adipogenic differentiation through C/EBPα and PPAR-γ, disruption of endogenous Wnt signaling results in adipogenesis [17]. Consistent with this model, trans-differentiation in the presence of Wnt is partially rescued by addition of sFRP-2, highlighting the importance of this pathway not only in adipocyte differentiation but also in mesodermal cell fate determination.

Mechanical stress provides signals to several types of cells during development, growth, and remodeling of tissues [1]. This is certainly the case in skeletal muscle in which changes in phenotype result from differential gene expression in response to mechanical signals [23]. The process by which mechanical stimuli initiate intracellular signaling is termed mechano-transduction. The mechanosensory mechanisms that are responsible for changes in gene transcription and expression are incompletely understood, but externally applied mechanical stimuli influence nuclear events either directly or indirectly via stimulation of signaling molecules [7,24]. The mechanism(s) of increased expression of Wnt10b by mechanical stretch remains to be explored.

In summary, this study demonstrates that trans-differentiation from myoblasts to adipocytes is inhibited by application of mechanical stretch, through mechanisms that include the activation of Wnt10b signaling. We provide the first evidence for the inhibition of adipogenesis in C2C12 myogenic cells in vitro during application of mechanical stretch. This finding suggests that satellite cells of intact animals may resist entry into the adipogenic program by mechanical stretch-induced secretion of Wnt10b.

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References

[1] J.J. Tomasek, G. Gabbiani, B. Hinz, C. Chaponnier, R.A. Brown, Myofibroblast and mechano-regulation of connective tissue remodelling, Nat. Rev. Mol. Cell. Biol. 3 (2002) 349–363.
[2] C.A. Powell, B.L. Smiley, J. Mills, H.H. Vandenburgh, Mechanical stimulation improves tissue-engineered human skeletal muscle, Am. J. Physiol. Cell Physiol. 283 (2002) C1557–C1565.
[3] R. Aikawa, T. Nagai, M. Tanaka, Y. Zou, T. Ishihara, H. Takano, H. Hasegawa, H. Akazawa, M. Mizukami, R. Nagai, I. Komuro, Reactive oxygen species in mechanical stress-induced cardiac hypertrophy, Biochem. Biophys. Res. Commun. 289 (2001) 901–907.
[4] J. Atance, M.J. Yost, W. Carver, Influence of the extracellular matrix on the regulation of cardiac fibroblast behavior by mechanical stretch, J. Cell Physiol. 200 (2004) 377–386.
[5] J. Lammerding, R.D. Kamm, R.T. Lee, Mechanotransduction in cardiac myocytes, Ann. NY Acad. Sci. 1015 (2004) 53–70.
[6] J.A. Mitchell, O. Shynlova, B.L. Langille, S.J. Lye, Mechanical stretch and progesterone differentially regulate activator protein-1 transcription factors in primary rat myometrial smooth muscle cells, Am. J. Physiol. Endocrinol. Metab. 287 (2004) E439–E445.
[7] H.H. Vandenburgh, Mechanical forces and their second messengers in stimulating cell growth in vitro, Am. J. Physiol. Regul. Integr. Comp. Physiol. 262 (1992) R350–R355.
[8] Y. Zou, H. Akazawa, Y. Qin, M. Sano, H. Takano, T. Minamino, N. Makita, K. Iwanaga, W. Zhu, S. Kudoh, H. Toko, K. Tamura, M. Kihara, T. Nagai, A. Fukamizu, S. Umemura, T. Iiri, T. Fujita, I. Komuro, Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II, Nat. Cell Biol. 6 (2004) 499–506.
[9] L. Song, R.S. Tuan, Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow, FASEB J. 18 (2004) 980–982.
[10] E. Hu, P. Tontonoz, B.M. Spiegelman, Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha, Proc. Natl. Acad. Sci. USA 92 (1995) 9856–9860.
[11] D. Tosh, J.M. Slack, How cells change their phenotype, Nat. Rev. Mol. Cell Biol. 3 (2002) 187–194.
[12] P.A. Tsonis, Regenerative biology: The emerging field of tissue repair and restoration, Differentiation 70 (2002) 397–409.
[13] K. Yeow, B. Phillips, C. Dani, C. Cabane, E.Z. Amri, B. Derijard, Inhibition of myogenesis enables adipogenic transdifferentiation in the C2C12 myogenic cell line, FEBS Lett. 506 (2001) 157–162.
[14] T. Akimoto, T. Ushida, S. Miyaki, T. Tateishi, T. Fukubayash, Mechanical stretch is a down-regulatory signal for differentiation of C2C12 myogenic cells, Mater. Sci. Eng. C 17 (2001) 75–78.
[15] J.A. Gilbert, P.S. Weinhold, A.J. Banes, G.W. Link, G.L. Jones, Strain profiles for circular cell culture plates containing flexible surfaces employed to mechanically deform cells in vitro, J. Biomech. 27 (1994) 1169–1177.
[16] Z. Yan, S. Choi, X. Liu, M. Zhang, J.J. Schageman, S.Y. Lee, R. Hart, L. Lin, F.A. Thurmond, R.S. Williams, Highly coordinated gene regulation in mouse skeletal muscle regeneration, J. Biol. Chem. 278 (2003) 8826–8836.
[17] S.E. Ross, N. Hemati, K.A. Longo, C.N. Bennett, P.C. Lucas, R.L. Erickson, O.A. MacDougald, Inhibition of adipogenesis by Wnt signaling, Science 289 (2000) 950–953.
[18] J.M. Taylor-Jones, R.E. McGehee, T.A. Rando, B. Lecka-Czernik, D.A. Lipschitz, C.A. Peterson, Activation of an adipogenic program in adult myoblasts with age, Mech. Ageing Dev. 123 (2002) 649–661.
[19] D.A. Brodie, Techniques of measurement of body composition. Part I, Sports Med. 5 (1988) 11–40.
[20] C.N. Bennett, C.L. Hodge, O.A. MacDougald, J. Schwart, Role of Wnt10b and C/EBPalpha in spontaneous adipogenesis of243 cells, Biochem. Biophys. Res. Commun. 302 (2003) 12–16.
[21] K.A. Longo, W.S. Wright, S. Kang, I. Gerin, S.H. Chiang, P.C. Lucas, M.R. Opp, O.A. MacDougald, Wnt10b inhibits development of white and brown adipose tissues, J. Biol. Chem. 279 (2004) 35503–35509.
[22] Z. Wu, P. Puigserver, B.M. Spiegelman, Transcriptional activation of adipogenesis, Curr. Opin. Cell Biol. 11 (1999) 689–694.
[23] G. Goldspink, A. Scutt, P.T. Loughna, D.J. Wells, T. Jaenicke, G.F. Gerlach, Gene expression in skeletal muscle in response to stretch and force generation, Am. J. Physiol. 262 (1992) R356–R363.
[24] B. Han, X.H. Bai, M. Lodyga, J. Xu, B.B. Yang, S. Keshavjee, M. Post, M. Liu, Conversion of mechanical force into biochemical signalling, J. Biol. Chem. 279 (2004) 54793–54801.