JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2009, 60, Suppl 3, 77-81
www.jpp.krakow.pl

T. GREDES[1], A. SPASSOV[1], R. MAI[1], H. MACK[2], B.W. LOSTER[3], M. MAZURKIEWICZ-JANIK[3], D. KUBEIN-MEESENBURG[4], J. FANGHANEL[1], T. GEDRANGE[1]

CHANGES IN INSULIN LIKE GROWTH FACTORS, MYOSTATIN AND VASCULAR ENDOTHELIAL GROWTH FACTOR IN RAT *MUSCULUS LATISSIMUS DORSI* BY POLY-3-HYDROXYBUTYRATE IMPLANTS

[1]Department of Orthodontics, Preventive and Pediatric Dentistry, Ernst-Moritz-Arndt University, Greifswald, Germany; [2]Department of Comprehensive Adult Dental Care in the School of Dentistry and Oral Health, Griffith University Queensland, Australia; [3]Institute of Dentistry, Jagiellonian University Medical College, Cracow, Poland; [4]Department of Orthodontics, Georg August University, Gottingen, Germany

The present study aimed at researching the synergistic effect between an ectopic bone substitute and surrounding muscle tissue. To describe this effect, changes of insulin like growth factors (IGF1, IGF2), myostatin (GDF8) and vascular endothelial growth factor (VEGF) mRNA content of 12 Wistar-King rats *musculus latissimus dorsi* with implanted poly-3-hydroxybutyrate (PHB) scaffold were examined after 6 and 12 weeks. At each time interval six rats were killed and implants and surrounding tissues prepared for genetic evaluation. Eight rats without any implants served as controls. RNA was extracted from homogenized muscle tissue and reverse transcribed. Changes in mRNA content were measured by Real-Time PCR using specific primers for IGF1, IGF2, GDF8 and VEGF. Comparing the level of VEGF mRNA in muscle after 6 and 12 weeks to the controls, we could assess a significant increase of VEGF gene expression ($p<0.05$) whereas the level of mRNA expression was higher after 6 than after 12 weeks of treatment. Expression of IGF1 gene was also significantly increased as compared to the controls over the observed period of time ($p<0.05$). In the case of the IGF2 gene, the expression was significantly elevated after 6 weeks ($p<0.05$), but not significantly increased after 12 weeks ($p>0.05$). We observed a significantly decreased GDF8 gene expression ($p<0.05$) both after retrieval of implants after 6 as well as after 12 weeks. Moreover, mRNA level of GDF8 after 6 and 12 weeks were comparable the same. Our results show that PHB implants in rat *musculus latissimus dorsi* interact with the surrounding muscle tissue. This interaction works itself on growth potential of the muscle.

Key words: *mRNA, muscle, rat, poly(3)hydroxybutyrate, real-time PCR*

INTRODUCTION

Many investigations have shown that it is practicable to create bone in a convenient anatomical area with good vascular supply, for instance in the muscle (1-5). The extracellular matrix (ECM) of muscle is a conglomerate of substances in which biochemical and biophysical properties allow for the construction of a flexible network that integrates information from loading and converts it into mechanical capacities. In developing skeletal muscle, an important interplay between muscle cells and the ECM is present, and some evidence from adult human muscle suggests common signalling pathways to stimulate contractile and ECM components. Unaccustomed overloading responses indicate an important role of ECM in the adaptation of myofibrillar structures in adult muscle (6).

Vascular endothelial growth factor (VEGF) is secreted by myocytes, diffuses through and interacts with ECM and basement membranes, and binds to VEGF receptors and on neuropilin-1 endothelial cell surfaces of blood vessels (7). It induces endothelial cell proliferation and migration to sprout neovessels, and provides a survival signal until mural cells, i.e. pericytes and smooth muscle cells, stabilize these immature vessels (8-10).

Particularly the adaptation of the muscle is dependent on extra- and intracellular signalling pathways. Growth and transcription factors play a significant role in muscle development and growth. Important growth factors for muscle regeneration and differentiation are insulin like growth factor 1 (IGF-1) for positive adjustment of cell proliferation and myostatin (GDF8) as a negative growth factor. Myostatin is a potent negative regulator of myogenesis (11). Besides, IGF-2 is also involved in muscle development.

Although IGF-2 plays a crucial role in muscle development (12), its role in the repair/regeneration process is secondary to other growth factors, for example IGF-1 (13). Age-associated decrements in muscle repair process have also been shown to be associated with levels of IGF gene expression. Marsh *et al*. (14) compared young, adult, and old rats exposed to bupivicaine induced muscle damage and showed that young rats exhibit

relatively higher levels of IGF-II expression and better recovery of muscle mass and protein concentration postinjury.

The aim of this study was to assess the effects of poly-3-hydroxybutyrate (PHB) implants on factors that regulate vascularisation or interaction with the extracellular matrix of the surrounding muscle tissue.

Because of PHB unique combination of biodegradability and biocompatibility it is of great interest for medical applications (15). PHB completely degrades to release a normal component of blood and tissue, D, L-β-hydroxybutyrate (HB). In recent studies PHB has been evaluated as a scaffold for ectopic bone formation *in vivo* (1).

Literature reports show that the tissue response to the PHB implants was characterized by mild inflammatory reaction of short time period, increasing macrophage infiltration and granulation response with formation of a highly vascularized thin fibrous capsule at the microspheres/tissue interface, with was resorbed after several weeks of implantation. Neither necrosis nor any other adverse morphological changes and tissue transformation in response to the implantation of PHB implants were recorded (16).

The synergistic effects between PHB scaffold and m. latissimus dorsi have not been sufficiently investigated. It has been demonstrated in a previous study that PHB implants can have an influence on the myosin isoform composition of the surrounding muscle tissue. The mRNA of myosin isoform type I was increased while the studied muscle tissue showed a decrease in isoform IIx compared to the controls (17). This result is in accordance with other studies, where changes in the expression of myosin isoforms I and IIx occurred, but no or very little changes in the expression of myosin isoform IIb were recorded (18).

MATERIALS AND METHODS

Poly-3-hydroxybutyrate (PHB)

Fully biodegradable biotechnologically produced polyester PHB was used in powder form as raw material. The powder with a molecular weight of 540000 g/mol was granulated using a twin screw extruder equipment. The PHB multifilaments were produced using a high-speed melt spinning and spin drawing process (1). Round embroidery patches with a thickness of 1.2 mm and a diameter of 12 mm were generated using an embroidery automat follwed by coating with calf skin collagen type I. The average macro mesh pore size of the embroidery was 200 µm and the total weight of the implant was approximately 12 mg. A total of 24 implants were prepared. All implants were ultrasonically cleaned in 70 % ethanol for 15 minutes and sterilised by ethylene oxide before the surgical procedure.

In order to determine the optimal sampling site, histological specimens of the whole region were produced from the test animals. The entire region was immediately fixed in 4% formalin and subsequently embedded in Technovit 9700 (19, 20). Specimen preparation was done using the hard tissue cutting-grinding technique to avoid affecting the substitute bone structure and the connected soft tissues (*Fig. 1*).

Experimental design and surgical procedure

Experiments were performed on twelve six-month-old adult male Wistar-King rats, with approximately 200 g body mass. All surgical and experimental procedures were approved by the Animal Welfare Committee of the State Government (no. 24-9168.11-1-2004-2).

Animals were anesthetized with intraperitoneal pentobarbital at an approximate dosage of 75 mg/kg. For the intra muscular implants a 3 cm sagittal incision was made in the skin in the midline of the back. In a intramuscular pocket a PHB embroidery was inserted. The muscle pocket around the implant was closed with one resorbable suture and for skin closing a continuous suture was used. The implants were located using a complete statistical randomisation. Eight rats without any intervention served as controls.

The biopsies were carried out 6 and 12 weeks after implantation under anesthesia without resuscitation of the rats. The implants with the surrounding tissues were retrieved and prepared for molecular investigation. Subsequently, the surrounding tissue after preparation was retrieved from the implant by blunt dissection and placed in liquid nitrogen.

RNA-isolation and reverse transcription

RNA-Isolation was conducted by means of the Qiagen Fibrous Tissue Mini Kit and the Fibrous Tissues Protocol (Qiagen, Valencia, CA, USA). In the beginning the frozen muscle tissue was homogenised under liquid nitrogen using a mortar and pistle and than the cells were lysed and the RNA released by centrifugation of the cell-homogenate through a biopolymer shredder (Qiashredder, Qiagen, Valencia, CA, USA). The quality and yield of the RNA was determined by spectrophotometry at 260 nm and the integrity examined by agarose gel electrophoresis with ethidium-bromide staining. The Quantification of the total RNA was performed using a NanoDrop®ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies), which minimized the loss of RNA material during the measurement procedure; only 2 µl RNA solution were used per sample.

Forthwith 1 µg of the isolated RNA was transcribed in cDNA according to the manufacturer´s instructions using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA).

Reversce transcriptase-polymerase chain reaction - Real-Time PCR

Changes in mRNA content were measured by Real-Time PCR using specific primers for IGF1, IGF2, VEGF, and GDF8 (Qiagen). The concentrations of the primers were adjusted as described in a manual for the Power Sybr-Green Master Mix (Applied Biosystems, Foster City, CA, USA). For every sample double-appointments were measured and No Template Controls were accomplished for each primer pair on each 96-well plate. From each sample, Real-Time PCR with primers for Igf1, Igf2, Gdf8, VEGF and β-actin was performed. In each well 12.5 µl of Master Mix was added and 300 nM of each primer, which was used at 50 nM. 2 µl of 1:50 diluted cDNA of a sample served as a template. The specifity of the reaction was examined by creating a dissociation curve for each sample and finally by checking the PCR products by agarose gel-electrophoresis.

Quantification of expression of target genes - 2(-ΔΔCt) method

To determine the quantity of the target-gene specific transcripts present in treated cells relative to untreated ones, their respective Ct values were first normalized by subtracting the Ct value obtained from the β-actin control (ΔCt = Ct, target – Ct, control). The concentration of gene-specific mRNA in treated cells relative to untreated cells was calculated by subtracting the normalized Ct values obtained for untreated cells from those obtained from treated samples (ΔΔCt = ΔCt, treated – ΔCt, untreated) and the relative concentration was determined (2–ΔΔCt) (21).

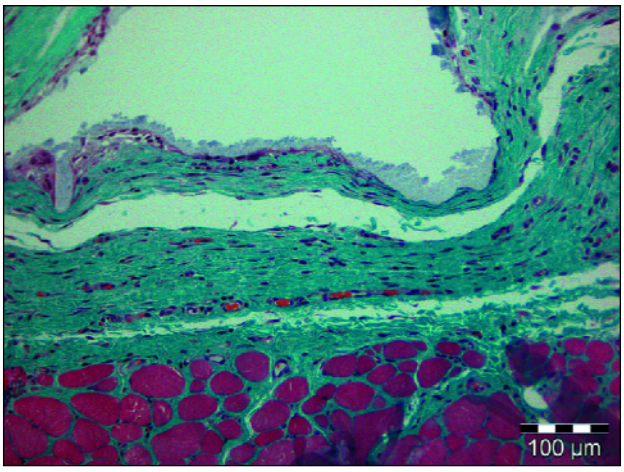

Fig. 1. Histologic specimen of the entire treatment area in Technovit 9700. The specimen was stained with Masson-Goldner trichrome stain. A connective tissue layer with bone substitute can be noticed on the musculature. The square shows the sampling site for molecular-biological analyses.

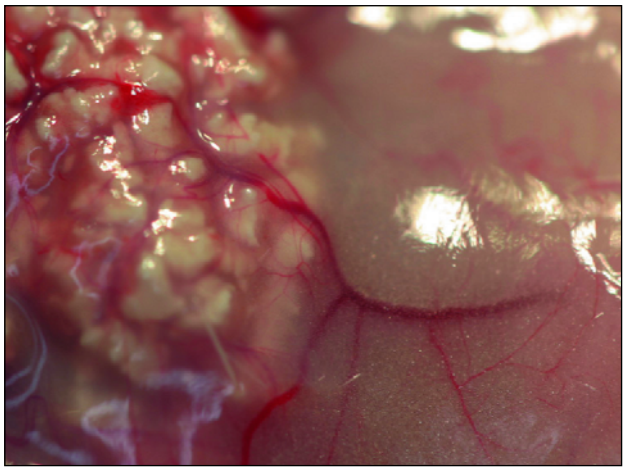

Fig. 2. Rat *musculus latissimus dorsi* with implanted poly-3-hydroxybutyrate (PHB) scaffold examined after 12 weeks. Morphologic image after skin dissection.

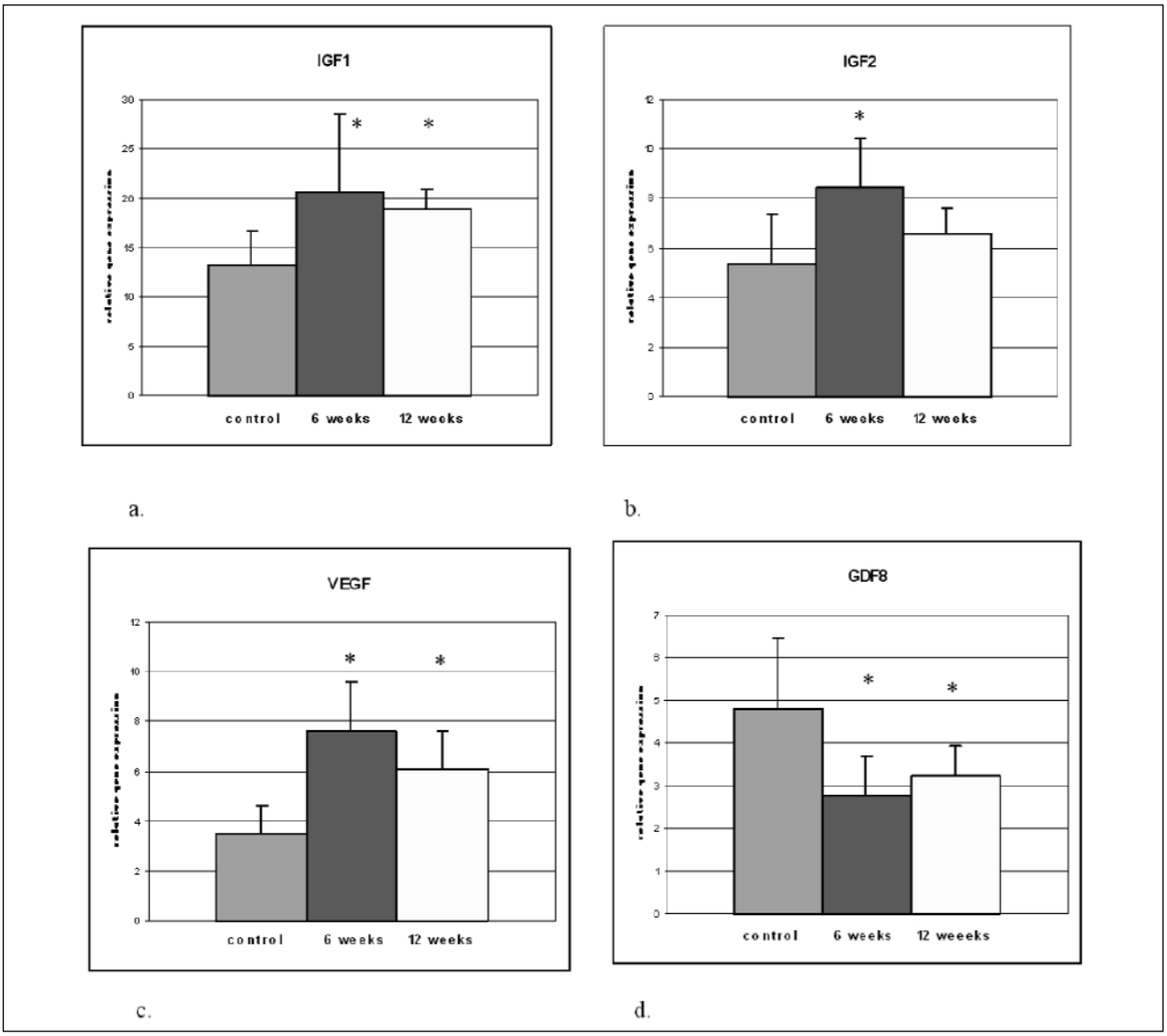

Fig. 3 a-d. : Relative expression of genes : a. IGF1, b. IGF2, c. VEGF and d. GDF8 in the *m. latissimus dorsi* of control rats and in the *m. latissimus dorsi* of rats carrying implants 6 weeks and 12 weeks after surgery. Given are the arithmetic means with standard deviations, significance at $p<0.05$; (*)-indicates a significant difference between investigated groups.

RESULTS

Morphologically, the ingrowth of applied bone substitute and a marked formation of capillaries are noticed (*Fig. 2*).

Figs 3 (*a-d*) show the values of 2(–ΔΔCt) reflecting the fold change in gene expression level in muscle surrounding PHB scaffold after 6 and 12 weeks, calculated relative to the level of β-actin expression. Comparing the level of VEGF mRNA in muscle after 6 and 12 weeks to the controls, we could assess a significant increase of VEGF gene expression ($p<0.05$) whereas the level of mRNA expression was higher after 6 than after 12 weeks of treatment. Expression of IGF1 gene was also significantly increased as compared to the controls over the observed period of time ($p<0.05$). In the case of the IGF2 gene, the expression was significantly elevated after 6 weeks ($p<0.05$), but not significantly increased after 12 weeks ($p>0.05$). We observed a significantly decreased GDF8 gene expression ($p<0.05$) both after retrieval of implants after 6 as well as after 12 weeks. Moreover, mRNA level of GDF8 after 6 and 12 weeks were comparable the same.

Macroscopic we could observe that one of the early responses to the PHB implants was capillary generation in the muscle tissue surrounding the implants which indicates a good compatibility of the PHB implants.

DISCUSSION

Mack *et al.,* (17) had shown that PHB implants can have an influence on the myosin isoform composition of the surrounding muscles. Muscles adapt to stress by changing of fiber types and respective mRNA content. Accordingly the basically fast rat *musculus latissimus dorsi* adopted a slower phenotype with a more efficient energy utilisation and greater potential for regeneration (17). Our investigation focused on factors that regulate vascularisation and interaction with the extracellular matrix.

Some investigation indicated that administration of vascular endothelial growth factor (VEGF) as gene, protein, or factor-overexpressing cell transplants can stimulate resident endothelial cells to form new capillary tubes, thereby improving regional blood flow in ill-perfused adult tissues (22, 23). In our study an increased VEGF mRNA expression was detected in muscles surrounding the PHB scaffold. However, this expression tended to decrease by and by. Recent studies suggest that newly formed vessels are eliminated by natural elimination (24). The VEGF-induced growth of vessels is not accompanied by increased metabolic demand, their durable presence is not essential for the function of the tissue.

Differentiation, maturation, maintenance, and repair of skeletal muscle require ongoing cooperation and coordination between an intrinsic regulatory program controlled by myogenic transcription factors and signalling pathways activated by hormones and growth factors (25, 26).

Insulin-like growth factors (IGFs) were the other decisive growth factors that we have investigated. IGF1 and IGF2 play key roles in normal muscle development in the embryo (27) and are important for coordinating muscle regeneration and re-innervation following injury (28, 29). However, their mechanisms of interaction with other muscle regulatory networks remain undefined (5).

We confirmed according to other studies that IGFs are expressed in muscles (30) and could show that muscle fibers express increased IGF mRNA while in contact with PHB scaffolds. It is well established that endogenously produced IFG1 and IFG2 can exert a strong positive effect on skeletal muscle differentiation (31). Combined with the observation that elevated levels of IGFs are sufficient to promote interstitial cell proliferation in otherwise untreated adult skeletal muscle (28, 29), other findings support the hypothesis that the early production of IGF1 by the inactive muscle fiber is involved in the initiation of the proliferation reaction of muscle. IGF1 was identified as a possible initiator of restorative reactions in injured muscles (29).

Recent results suggest that myostatin (GDF8) is a potent regulator of cell-cycle progression and functions by regulating both the proliferation and differentiation steps of myogenesis. The role of myostatin has been demonstrated in several studies not only during embryonic myogenesis, but also in postnatal muscle growth. It is not known whether myostatin influences only muscle formation or has also a function in the regulation of muscle metabolism (32). Lack of myostatin results in accelerated regeneration and reduced fibrosis (33). We observed after 6 as well as after 12 weeks a significantly decreased myostatin expression. The decrease after 6 and 12 weeks was about the same.

Several studies indicate that myostatin might function as an inhibitor of satellite cells proliferation (34), suggesting a role for myostatin in postnatal muscle growth and repair (35). Consistent with this hypothesis, recent results from McCroskery *et al*. (33) indicate that myostatin is indeed expressed in satellite cells. Myostatin is known to block hematogenesis and enhance chondrogenesis as well epithelial cell differentiation (36). Our results show that PHB implants in rat musculus latissimus dorsi interact with the surrounding muscle tissue. This interaction works itself on growth potential of the muscle.

Acknowledgments: The authors wish to thank the research group of the clinics and policlinics for inner medicine C: Prof. Dr. G. Doelken, Prof. Dr. C. A. Schmidt, Dr. F. Schueler, Kathrin Assmus for providing the real-time PCR machine and Dr. P. Grabarczyk for helpful comments on the RT-PCR analysis.

Conflict of interest statement: None declared.

REFERENCES

1. Mai R, Hagedorn MG, Gelinsky M *et al*. Ectopic bone formation in nude rats using human osteoblasts seeded poly(3)hydroxybutyrate embroidery and hydroxyapatite-collagen tapes constructs. *J Craniomaxillofac Surg* 2006; 34: 101-109.
2. Mai R, Lux R, Proff P *et al*. O-phospho-l-serine: a modulator of bone healing in calcium-phosphate cements. *Biomed Tech* 2008; 53: 229-233.
3. Warnke P, Springer I, Wiltfang J *et al*. Growth and transplantation of a custom vascularised bone graft in a man. *Lancet* 2004; 364: 766-770.
4. Weingartner J, Proff P, Fanghanel J *et al*. Different bone sensitivity to malformations induced by procarbazine in fetal rats. *J Physiol Pharmacol* 2008; 59 (Suppl. 5): 17-25.
5. Wilson EM, Hsieh MM, Rotwein P.. Autocrine growth factor signaling by insulin-like growth factor-II mediates MyoD-stimulated myocyte maturation. *J Biol Chem* 2003; 278: 41109-41113.
6. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 2004; 84: 649-698.
7. Ji JW, Mac Gabhann F, Popel AS. Skeletal muscle VEGF gradients in peripheral arterial disease: simulations of rest and exercise. *Am J Physiol Heart Circ Physiol* 2007; 293(6): H3740-H3749.
8. Ehrbar M, Zeisberger SM, Raeber GP, Hubbell JA, Schnell C, Zisch AH. The role of actively released fibrin-conjugated VEGF for VEGF receptor 2 gene activation and the enhancement of angiogenesis. *Biomaterials* 2008; 29: 1720-1729.

9. Gedrange T, Walter B, Tetzlaff I *et al*. Regional alterations in fiber type distribution, capillary density, and blood flow after lower jaw sagittal advancement in pig masticatory muscles. *J Dent Res* 2003; 82: 570-574.
10. Gedrange T, Lupp A, Kirsch C, Harze W, Klinger R. Influence of the sagittal advancement of mandibulae on myofibrillar ATPase activity and myosin heavy chain content in the masticatory muscles of pigs. *Folia Histochem Cytobiol* 2002; 40: 277-284
11. McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 1997; 387: 83-90.
12. DeChiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* 1990; 345: 78-80.
13. MacGregor J, Parkhouse WS. The potential role of insulin-like growth factors in skeletal muscle regeneration. *Can J Appl Physiol* 1996; 21: 236-250.
14. Marsh DR, Criswell DS, Hamilton MT, Booth FW. Association of insulin-like growth factor mRNA expressions with muscle regeneration in young, adult, and old rats. *Am J Physiol* 1997; 273: 353-358.
15. Vogel R, Tandler B, Haussler L, Jehnichen D, Brunig H. Melt spinning of poly(3-hydroxybutyrate) fibers for tissue engineering using α-cyclodextrin/polymer inclusion complexes as the nucleation agent. *Macromol Biosci* 2006: 6; 730-736.
16. Shishatskaya E, Voinova O, Goreva A, Mogilnaya O, Volova T. Biocompatibily of polyhydroxybutyrate microspheres: in vitro and in vivo evaluation. *J Mater Sci Mater Med* 2008; 19: 2493-2502.
17. Mack HB, Mai R, Lauer G *et al*. Adaptation of myosin heavy chain mRNA expression after implantation of poly(3)hydroxybutyrate scaffolds in rat *m. latissimus dorsi*. *J Physiol Pharmacol* 2008; 59(Suppl. 5): 95-103.
18. Talmadge RJ. Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. *Muscle Nerv* 2000; 23: 661-679.
19. Gedrange T, Mai R, Mack F *et al*. Evaluation of shape and size changes of bone and remodelled bone substitute after different fixation methods. *J Physiol Pharmacol* 2008; 59(Suppl. 5): 87-94.
20. Zietek M, Gedrange T, Mikulewicz M. Long term evaluation of biomaterial application in surgical treatment of periodontitis. *J Physiol Pharmacol* 2008; 59(Suppl. 5): 81-86.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001; 25: 402-408.
22. Lee LY, Patel SR, Hackett NR, Mack CA, Polce DR, El-Sawy T. Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for vascular endothelial growth factor 121, *Ann Thorac Surg* 2000; 69: 14-23.
23. Rutanen J, Rissanen TT, Kivela A, Vajanto I, Yla-Herttuala S. Clinical applications of vascular gene therapy. *Curr Cardiol Rep* 2001; 3: 29-36.
24. Peirce SM, Price RJ, Skalak TC. Spatial and temporal control of angiogenesis and arterialization using focal applications of VEGF164 and Ang-1. *Am J Physiol Heart Circ Physiol* 2004; 286: H918-H925.
25. Lassar A, Munsterberg A. Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. *Curr Opin Cell Biol* 1994; 6: 432-442.
26. Naya FS, Olson E. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Curr Opin Cell Biol* 1999; 11: 683-688.
27. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 1993; 75: 59-72.
28. Bauer R, Gedrange T, Bauer K, Walter B. Intrauterine growth restriction induces increased capillary density and accelerated type I fiber maturation in newborn pig skeletal muscles. *J Perinat Med* 2006; 34: 235-242.
29. Caroni P, Schneider C, Kiefer MC, Zapf J. Role of muscle insulin-like growth factors in nerve sprouting: suppression of terminal sprouting in paralyzed muscle by IGF-binding protein 4. *J Cell Biol* 1994; 124: 893-902.
30. Beck F, Samani NJ, Peuscow JD, Thorly B, Tregear GW, Coshlan JP. Histochemical localization of IGF-I and -II mRNA in the developing rat embryo. *Development* 1987; 101: 175-184.
31. Montarras D, Aurade F, Johnson T, Ilan J, Gros F, Pinset C. Autonomous differentiation in the mouse myogenic cell line, C2, involves a mutual positive control between insulin like growth factor II and MyoD, operating as early as the myoblast stage. *J Cell Sci* 1996; 109: 551-560.
32. Ji S, Losinski RL, Cornelius SG *et al*. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. *Am J Physiol* 1998; 275(4): H1265-H1273.
33. McCroskery S, Thomas M, Platt L *et al.* Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. *J Cell Sci* 2005; 1: 3531-3541.
34. McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R. Myostatin negatively regulates satellite cell activation and self-renewal. *J Cell Biol* 2003; 162: 1135-1147.
35. Wehling M, Cai B, Tidball JG. Modulation of myostatin expression during modified muscle use. *FASEB J* 2000; 14: 103-110.
36. Cieslak D, Blicharski T, Kapelanski W, Pierzchala M. Investigation of polymorphisms in the porcine myostatin (GDF8; MSTN) gene. *Czech J Anim Sci* 2003; 48: 69-75.

Received: January 12, 2009
Accepted: April 14, 2009

Author's address: Assoc. Prof. Bartlomiej W. Loster, Department of Orthodontics, Institute of Dentistry, Jagiellonian University Medical College, 4 Montelupich Street, 31-155 Krakow, Poland; Phone: +48-12-424-54-02; e-mail: bw@loster.pl