Rskα-actin/hIGF-1 transgenic mice with increased IGF-I in skeletal muscle and blood: Impact on regeneration, denervation and muscular dystrophy

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Abstract

Human IGF-I was over-expressed in skeletal muscles of C57/BL6 × CBA mice under the control of the rat skeletal α-actin gene promoter. RT-PCR verified expression of the transgene in skeletal muscle but not in the liver of 1- and 21-day old heterozygote transgenic mice. The concentration of endogenous mouse IGF-I, measured by an immunoassay which does not detect human IGF-I, was not significantly different between transgenic mice and wild-type littermates (9.5 ± 0.8 and 13.3 ± 1.9 ng/g in muscle; 158.3 ± 18.6 and 132.9 ± 33.1 ng/ml in plasma, respectively). In contrast, quantitation with antibodies to human IGF-I showed an increase in IGF-I of about 100 ng/ml in plasma and 150 ng/g in muscle of transgenic mice at 6 months of age. Transgenic males, compared to their age matched wild-type littermates, had a significantly higher body weight (38.6 ± 0.53 g vs. 35.8 ± 0.64 g at 6 months of age; P < 0.001), dry fat-free carcass mass (5.51 ± 0.085 vs. 5.08 ± 0.092 g; P < 0.001) and myofibrillar protein mass (1.62 ± 0.045 vs. 1.49 ± 0.048 g; P < 0.05), although the fractional content of fat in the carcass was lower (167 ± 7.0 vs. 197 ± 7.7 g/kg wet weight) in transgenic animals. There was no evidence of muscle hypertrophy and no change in the proportion of slow type I myofibres in the limb muscles of Rskα-actin/hIGF-I transgenic mice at 3 or 6 months of age. Phenotypic changes in Rskα-actin/hIGF-I mice are likely to be due to systemic as well as autocrine/paracrine effects of overproduction of IGF-I due to expression of the human IGF-I transgene.

The effect of muscle specific over-expression of Rskα-actin/hIGF-I transgene was tested on: (i) muscle regeneration in auto-transplanted whole muscle grafts; (ii) myofibre atrophy following sciatic nerve transection; and (iii) sarolemmal damage and myofibre necrosis in dystrophic mdx muscle. No beneficial effect of muscle specific over-expression of Rskα-actin/hIGF-I transgene was seen in these three experimental models.

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1. Introduction

Insulin-like growth factor (IGF-I) is produced by a wide range of tissues. About 75% of circulating IGF-I is derived from liver and the remaining 25% is contributed by other tissues, with muscle and adipose being the likely candidates. Investigation into the somatic growth of mice with Cre/loxP recombination of the IGF-I gene in liver concluded that circulating IGF-I has very little or no effect on postnatal growth [1,2], whereas other studies suggest that the importance of an endocrine role for IGF-I in regulation of somatic growth could be underestimated [3,4]. Thus, the overall
effect of circulating IGF-I compared to the locally expressed IGF-I is yet to be fully defined.

It is well established that IGF-I gene transcripts can be alternatively spliced and give rise to different isoforms of precursor IGF-I peptide that might exert different biological effects. Classes 1 and 2 IGF-I mRNA transcripts are initiated at exons 1 and 2, respectively, and encode different IGF-I signal peptides [5]. In addition, in rodents, exons 4 and 6, exons 4, 5 and 6 and exons 4 and 5 splice variants exist, encoding IGF-I precursor proteins with different termination sequences (Ea, Eb and the third yet unnamed termination sequence, respectively) [5]. IGF-I precursors undergo post-translational modification to yield the 70 amino acid mature IGF-I peptide.

Two alternatively spliced IGF-I mRNA transcripts have been described in skeletal muscle, which encode IGF-I protein isoforms with Ea and Eb termination peptides. Both of these transcripts are upregulated with muscle damage and increased loading, indicating their involvement in muscle repair and growth [6,7]. Two transgenic mouse strains have been generated to over-express the IGF-I Ea isoform under skeletal muscle specific promoters: SK7733 IGF-I 3’SK mice over-express human IGF-I under the avian skeletal α-actin promoter [8], whereas MLC/mhIGF-I mice over-express the rat IGF-I driven by the rat MLC1/3 promoter [9]. Although these transgenic strains exhibit phenotypic discrepancies (reviewed in [5]), skeletal muscle specific over-expression of IGF-I results in elevation of total muscle IGF-I protein content and pronounced muscle hypertrophy in both strains. Such skeletal muscle hypertrophy is due to local function of IGF-I since the levels of circulating IGF-I are not increased [9,10]. Other studies show that tissue targeted over-expression of IGF-I can result in increased systemic IGF-I levels (in addition to the increase in local levels), which could lead to systemic effects of IGF-I derived from a transgene [11,12]. Increased body weight appears to be a common characteristic of the transgenic mice with elevated systemic IGF-I [13,11,12].

The transgenic mice described in this study were generated over 15 years ago, before the pattern of IGF-I mRNA transcription was fully characterized. The construct was designed to increase the muscle and serum concentration of IGF-I. In order to achieve this, we over-expressed a human IGF-I cDNA, encoding only the 70 amino acid mature IGF-I peptide under the control of a rat skeletal α-actin gene promoter in transgenic mice. A somatostatin signal peptide sequence was included in the transgene to ensure secretion of human IGF-I, body composition and skeletal muscle growth.

The transgenic mice heterozygous for Rskα-actin/hIGF-I have increased skeletal muscle and serum levels of IGF-I derived from the transgene, increased total body weight and lean tissue content, and decreased fat mass. No muscle hypertrophy is detected.

The Rskα-actin/hIGF-I transgenic mice were used to study the combination of local and systemic actions of elevated IGF-I on: muscle regeneration in auto-transplanted whole muscle grafts; muscle atrophy after sciatic nerve transection and muscle dystrophy in the mdx mouse model of Duchenne Muscular Dystrophy (DMD). In all of these experimental models systemic (endocrine) and local (autocrine and paracrine) actions of transgenic hIGF-I were expected. These three models have previously been used by various groups (see Section 4) to investigate the ability of elevated IGF-1 to improve muscle regeneration, reduce the rate of myofibre atrophy and ameliorate the adverse effects of muscular dystrophy.

2. Materials and methods

Experimental protocols were approved and conducted under the Animals (Scientific Procedures) Act 1986 in the United Kingdom, the Animal Experimentation Ethics Committee of CSIRO Health Sciences and Nutrition, Adelaide, Australia and the guidelines of the University of Western Australia Animal Ethics Committee and the National Health and Medical Research Council, Australia.

2.1. Generation of Rskα-actin/hIGF-I transgenic mice

The founder Rskα-actin/hIGF-I transgenic mice were generated in 1991 and have been maintained continuously, initially at the Roslin Institute, Edinburgh, Scotland for 4 years, subsequently at CSIRO Health Sciences and Nutrition in Adelaide, South Australia and now the Animal Resource Centre in Perth, Western Australia. The phenotype has remained essentially the same throughout this period. This is the first report describing the Rskα-actin/hIGF-I transgenic line.

The hybrid Rskα-actin/hIGF-I transgene was constructed from the muscle specific expression vector pJB5 (Fig. 1).

This expression vector containing a lacZ reporter gene was previously used to verify the tissue specificity of the rat skeletal α-actin promoter in transgenic mice [14]. Plasmid pJB5 was derived from pCV [15], which contains the 5’-flanking region and exons 1–6 of the rat skeletal α-actin gene fused to the 3’ end of human foetal ε-globin. A 3 kb EcoR1–BamH1 fragment containing the α-actin gene was cloned into pBluescript™.
A DraIII–BamHI fragment was replaced with a 17 bp oligonucleotide linker to remove the translation initiation and coding sequence for α-actin. The remaining 1.8 kb fragment containing the 5′-flanking region, exon 1, intron 1 and the 5′-end of exon 2 was sub-cloned into the EcoRI(BamHI) restriction site of pBluescript II sk+ to create pJB5 [14].

Plasmid pJB5 was modified to introduce sequences that should lead to the high level expression of human IGF-I in skeletal muscle. When the transgene was constructed in 1991, the sequences regulating the secretion of IGF-I were not known and we introduced a synthetic translation initiation signal (135 bp) derived from the rat somatostatin gene. A 1350-bp BamHI fragment containing a human IGF-I cDNA [51] was created as follows. A signal sequence based on rat somatostatin was synthesized by annealing 5′ and 3′ oligonucleotides with a 15 bp overlap and the complementary strands were filled in with Klenow polymerase. The resulting BamHI fragment was subcloned into pBS and sequenced (pJB6). A human IGF-I cDNA fragment was isolated from a cDNA clone kindly provided by Dr. Stuart Gilmour (Babraham Institute). A 777 bp PstI fragment was isolated, cut with BamHI, phosphatized with CIP and digested with AvaII. The 358-bp human IGF AvaII-BamHI-OH fragment was then isolated. Plasmid pJB6 was digested with BamHI and phosphatized with CIP and digested with AvaII to release 135bp HO-BamHI-AvaII fragment containing the signal peptide. This was ligated with the 358 bp human IGF AvaII-BamHI-OH fragment. Three fragments of 270, 500 and 716 bp were produced. The 500-bp BamHI-signal peptide-human IGF-I cDNA fragment was isolated and cloned into pBS to form pJB7.[14]

The next step was to introduce a polyadenylation signal derived from SV40 small T antigen to promote a high level of transcription. In order to achieve this, the plasmid pSV2-DHFR [16] was digested with BglII and ligated to BamHI digested pJB7. The ligated products were digested with BamHI and a 1350 bp somatostatin signal peptide-human IGF-I-polyA fragment was isolated and cloned into pBS (pJB8). The final construct, pJB9, was made by sub cloning the somatostatin signal peptide-human IGF-I-polyA BamHI fragment from pJB8 into the BamHI site in pJB5 to produce a plasmid pJB9 in which the 1.8 kb α-actin domain was upstream from the 1350 bp insert.

For microinjection, the whole 3150 bp fragment was excised intact. This was achieved by fragmenting the plasmid with PvuI, and then cutting out the fragment using BstXI and SalI, which flank the insert. The Rskα-actin/hIGF-I transgene was purified by gel electrophoresis.

Transgenic mice were produced using a previously described technique [14]. Transgenic offspring were identified by Southern Blot analysis of high molecular weight DNA prepared from tail biopsies. The presence of the transgene was identified by Southern blotting using the 1350 bp fragment containing a portion of the rat skeletal α-actin gene and extending to the first intron in the SV40 polyadenylation signal. Positive mice were subsequently mated and the lines in which the gene was transmitted were selected for breeding and maintained as a heterozygote colony.

After several generations the mice were routinely screened for the presence of the transgene by a PCR technique using the following oligonucleotide primers: 5′ AGC CTC ATC ATC ATC AGA TGG C 3′ and 5′ GAG CTG TGA TCT AAG GAG G 3′ (Geneworks, Adelaide, Australia).

2.2. Expression of transgene

Initial expression of the human IGF-I transgene was detected in RNA prepared by UltraSpec™ RNA (Biotec Laboratories, Houston, TX, USA) from whole 1-day-old embryos, using the same oligonucleotides used for identification of transgenic mice by PCR. These oligonucleotides were designed to take advantage of the fact that the transgene contains an intron sequence in the SV40 small T antigen domain, which is spliced out when expressed. The resulting amplified cDNA is 370 bp and can be easily separated from any contaminating genomic DNA (430 bp) amplified in the reaction.

In subsequent studies, expression of the human IGF-I transgene was assayed in liver and skeletal muscles of...
1- and 21-day-old transgenic mice and their wild-type littermates. Groups of mice were sacrificed by CO₂-induced asphyxiation. Liver and skeletal muscles were removed, immediately frozen in liquid nitrogen and stored at −80 °C until analysed. RNA was prepared from liver and muscle of transgenic and non-transgenic littermates using a commercially available kit (RNeasy, Qiagen Pty Ltd., Vic.). After treatment with RNase-free DNase (FPLC pure, Pharmacia Biotech Inc., NJ) the samples were incubated with reverse transcriptase (Superscript II, Gibco Life Technologies, MD) and the cDNA amplified by PCR as described above. Samples were also incubated without reverse transcriptase as an additional control to ensure that all the tissue DNA had been destroyed. The protocols used were essentially those provided by the suppliers of the kits and reagents.

2.3. Quantitation of plasma and muscle IGF-I

Six-month old male mice were sacrificed and trunk blood was collected into heparinized tubes. Plasma was obtained by centrifugation and stored at −20 °C until the concentration of IGF-I was determined. Samples of mixed hind limb muscle were extracted in 1 M acetic acid as previously described [8].

The concentration of IGF-I was measured in plasma and muscle extracts by radioimmunoassay after dissociation and separation of IGF-I from IGF binding proteins by size-exclusion chromatography at pH 2.5 [17]. The size exclusion column was characterized using [125I]-IGF-I and the recovery was 94% (n = 3 samples). Sample fractions were collected and neutralized by the addition of Tris (0.4 M; 0.6 v/v) and assayed in triplicate using two separate radioimmunoassays. Human IGF-I (resulting from the transgene) was measured as previously described [18], using a polyclonal antibody (GroPep Limited) at a final concentration of 1:80,000 with human IGF-I as standard and radioligand. Rat IGF-I exhibits a 25% cross-reactivity in this assay and since rat and mouse IGF-I differ by only a single amino acid (Ser69 to Ala69) [19], we have assumed that mouse IGF-I has a similar cross-reactivity. Mouse IGF-I was measured using a radioimmunoassay specific for rat IGF-I using a polyclonal antibody (GroPep Limited) at a final concentration of 1:60,000 with rat IGF-I as standard and radioligand. This assay shows no cross-reactivity to human IGF-I [20].

2.4. Carcass composition

Six-month old male transgenic mice and age matched wild-type littermates were fasted for 6 h, weighed and sacrificed by CO₂ induced asphyxiation. The pelt, head, tail and paws were removed, care being taken not to remove any subcutaneous fat with the pelt. The remaining carcass was opened down the midline and all of the body components, including the visceral organs, were then dried under vacuum at ambient temperature to determine body water and dry-matter content. The musculo-skeletal carcass and viscera were minced and a representative sample ground to a powder using a freezer mill (Spex Industries Inc., NJ). Carcass fat was determined gravimetrically after chloroform:methanol extraction of an aliquot of the powdered carcass. In order to gauge the contribution of muscle protein to the fat-free mass (FFM) the carcass myofibrillar protein was also determined gravimetrically after extraction from the powdered carcass essentially as described previously [21].

2.5. Histochemical staining and myofibre morphometric analysis

The tibialis anterior (TA), extensor digitorum longus (EDL) and gastrocnemius muscles were excised from 3-month old male transgenic mice and their non-transgenic wild-type littermates, dissected transversally in the mid belly region, mounted on tragacanth gum and frozen in liquid nitrogen quenched isopentane. In addition, gastrocnemius muscles were sampled from 6-month old transgenic mice and their non-transgenic littermates. Transverse sections (8 μm) of the mid belly region of the frozen muscles were cut on a cryostat (Leica CM3050), collected onto silane (3-aminopropyl-triethoxysilane) coated glass slides, air-dried and stored at −20 °C until used for histochemical or immunocytochemical staining. Histochemical staining of skeletal muscle of transgenic and wild-type mice was independently performed by two laboratories, using nicotinamide adenine dinucleotide – nitro-blue tetrazolium (NADH-TR) or succinic dehydrogenase (SDH). Staining with NADH-TR and SDH yield comparable results [22] and are used to differentiate fast, intermediate and slow type myofibrils [23,24]. Histochemistry with NADH-TR was performed on the limb muscle (EDL, TA and gastrocnemius) of 3-month old transgenic and wild type mice following described procedures [25]. Histochemistry with SDH was performed on gastrocnemius muscles of 6-month old transgenic and wild-type mice using standard techniques [26]. Non-overlapping images of the cross-section of TA, EDL and gastrocnemius muscles stained with NADH-TR were captured using a Leica PM RBE microscope connected to a video camera (Hitachi HV-C20M) and a personal computer equipped with ImagePro Plus 4.0 (Microsoft) software. Myofibre cross-sectional area (CSA) was measured by tracing each individual myofibre using ImagePro Plus 4 imaging software. The whole muscle cross-sectional area (CSA) was measured for gastrocnemius muscles of 6-month old transgenic and wild-type mice stained with SDH. For this purpose, images of the total muscle
cross-section were captured using digital imaging software (ImagePro Plus, Media Cybernetics, MD). The total CSA of the muscle and the area of dark-staining (slow type I) myofibres was estimated using SigmaScan Pro (Jandel Inc., CA).

2.6. Whole muscle grafts

Whole intact extensor digitorum longus (EDL) muscles were auto-grafted onto the surface of the tibialis anterior (TA) muscles following well described procedures [27,28] in female 6-week old Rskα-actin/hIGF-I transgenic and wild-type C57BL/6 mice. Numbers of animals and muscles sampled at each time point for each strain are summarized in Table 1. Between 3 and 21 days after grafting, mice were anesthetized and sacrificed by cervical dislocation. Day 3, 5, 7 and 21 grafts were paraffin-embedded or frozen as previously described [28]. Paraffin and frozen sections were used for Haematoxylin and Eosin (H&E) and immunocytochemical staining. H&E stained sections were used for the overall morphological assessment of samples as well as to confirm the orientation of grafts.

Polyclonal rabbit anti-desmin (DAKO, CA, USA) primary antibodies were used to identify muscle cells (myoblasts and myotubes) and monoclonal rat anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) (PharMingen, Sydney, Australia) primary antibodies were used to identify blood vessel endothelial cells in regenerating grafts. The primary antibodies were detected using biotinylated secondary antibodies: donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, PA, USA) and rabbit anti-rat IgG (Vector Laboratories, CA, USA), respectively, in conjunction with avidinD-peroxidase (Vector Laboratories, CA, USA) and streptavidin ALEXA488 (Molecular Probes, OR, USA). Procedures for immunocytochemical staining have been described previously [28].

For whole muscle graft analysis the digital images were tiled together to reconstruct the entire view of a transverse section of the graft. The extent of regeneration was assessed on paraffin-embedded and frozen transverse sections by counting total numbers of activated myoblasts in day 3 grafts and newly formed myotubes in day 5 and 7 grafts. The percentage vascularity of the grafts was assessed in day 5 grafts as previously described [28]. Statistical analysis was performed using Student’s t test.

2.7. Denervation experiments

Denervation surgery was performed on 8-week old Rskα-actin/hIGF-I transgenic mice and their wild-type control littermates by excising a ~10 mm sciatic nerve segment at the gluteal level [25]. The contra-lateral leg was left unoperated to allow normal activity of the animals. The mice were sacrificed by cervical dislocation at 28 days post-surgery. For every mouse, close examination of tissues in the thigh region under a surgical microscope confirmed that the severed sciatic nerve was still retracted and no re-innervation had occurred.

The tibialis anterior (TA) muscles were excised from denervated legs, dissected transversally in the mid belly region, mounted on tragacanth gum and frozen in liquid nitrogen quenched isopentane. The TA muscles from age-matched (12-week old) intact transgenic and wild-type mice were also sampled and used as controls for denervated muscles. Transverse sections (8 μm) of the mid belly region of the frozen muscles were stained with H&E for overall morphological assessment and morphometric analysis. Myofibre cross-sectional area (CSA) was measured on non-overlapping images of the entire cryosection of intact and denervated TA muscles of wild-type and Rskα-actin/hIGF-I mice stained with H&E by tracing each individual myofibre. For each muscle, CSA of 500–700 myofibres was measured from five non-overlapping fields. Variables obtained by combined effect of strain (wild-type control or transgenic) and treatment (intact or denervated) were compared using two-factor analysis of variance.

2.8. Experiments on dystrophic mdx/hIGF-I mice

2.8.1. Generation of mdx/hIGF-I mice

Transgenic Rskα-actin/hIGF-I male mice were bred with dystrophic mdx female mice (mdx/mdx) (C57Bl/10ScSn background) [29]. Male mice from the F1 generation (all carrying the mdx-affected maternal X-chromosome (mdx/Y)) that screened positive for the Rskα-actin/hIGF-I transgene were bred with female mdx mice. All male and female mice in the F2 generation were dystrophin-negative as they carried the

<table>
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<tr>
<th>Days after grafting</th>
<th>Wild-type</th>
<th>Rskα-actin/hIGF-I</th>
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<tbody>
<tr>
<td></td>
<td>Number of mice</td>
<td>Number of grafts sampled</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>4 P</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>4 P + 3 F</td>
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<tr>
<td>7</td>
<td>4</td>
<td>4 P + 1 F</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>3 P</td>
</tr>
</tbody>
</table>
mdx-affected X-chromosome(s) and these mdx mice were either positive or negative for the Rskα-actin/hIGF-I transgene. The transgene negative mdx littersates are hereafter referred to as mdx mice.

2.8.2. Evans blue dye (EBD) injection, sampling and analysis of dystrophic muscle

Experiments were conducted on young (21-, 22-, 26- and 40-day old) and adult (12-week old) mdx/hIGF-I mice and their non-transgenic control littersmates (mdx mice). Young 21 and 22 days old mdx/hIGF-I and mdx mice were injected intraperitoneally with 1% EBD (Sigma, St. Louis, MO, USA) 24 h before sacrifice [30]. Older (26- and 40-day old and 12-week old) mice were not injected with EBD. Mice were sacrificed by cervical dislocation and TA and diaphragm muscles were excised.

TA and diaphragm muscles from 21- to 26-day old mice were mounted onto tragacanth gum and frozen in liquid nitrogen quenched isopentane. The detailed procedures for sampling TA and diaphragm muscles were described previously [30]. From each frozen specimen 8 μm transverse sections were cut for H&E staining. Cryosections from day 21 to 22 muscles were also mounted for EBD analysis [30]. TA muscles from 40-day old mice and TA and diaphragm muscles from 12-week old mice were fixed in 4% paraformaldehyde (pH 7.6) for 30 min and processed in a Shandon automatic tissue processor for paraffin embedding. TA muscles were dissected in the mid-region and embedded with both cut surfaces at the top of the paraffin block. Diaphragm muscles were cut in two strips and embedded in the paraffin block for transverse sectioning. Myofibre “leakiness”, which is an indication of sarcolemmal damage [31,32] was measured on muscle cryosections from 21- to 22-day old animals injected with EBD. Areas of cumulative muscle damage, which represents the total area occupied by necrotic and/or regenerated myofibres [30] was measured on H&E stained muscle cryosections from 21, 22, 26 old mdx and mdx/hIGF-I mice. In TA muscles of 40-day old mdx and mdx/mIGF-I mice, muscle damage was assessed by calculating the proportion of centrally nucleated myofibres, which represent regenerated myofibres following necrosis. Morphometric analysis was performed on digital images of the tissue. Non-overlapping images of the entire cross-sectional area of the muscles were taken using ImagePro Plus 4.0 software and tiled to reconstitute the whole image of the muscle. The images for EBD analysis were captured using an excitation filter BP 515–560 and emission LP 590 (green wavelength filter set). Under this wavelength EBD fluoresces bright red. Areas of muscle damage were measured using ImagePro Plus 4.0 software and expressed as a proportion of the whole muscle cross-sectional area.

The myofibre CSA was measured on the cross-sectional images of the TA muscles of 12-week old mdx and mdx/mIGF-I mice by tracing individual myofibres. For each muscle, CSA of 500–700 myofibres was measured from five non-overlapping fields. The diaphragm width was also measured in 12-week old mdx/hIGF-I and mdx mice. Since the width of the diaphragm muscle can vary along the same cross-section [30], five measurements were taken from different areas within each diaphragm and the average value calculated.

2.9. Statistics

Data were analysed using statistical software (Systat 7.01, SPSS, Chicago, IL, USA). Body composition data were initially analysed by two-way analysis of variance (ANOVA) with genotype and generation number as independent variables. These data were further analysed by analysis of covariance using final body weight as the covariate. All other analyses were by one-way ANOVA. In some cases data were transformed to ensure homogeneity of variance. Student t-test was used for comparison of myofibre CSA between transgenic and wild-type mice.

3. Results

3.1. Production and characterization of transgenic mice

3.1.1. Production of Rskα-actin/hIGF-I transgenic mice

A 3150 bp fragment containing the transgene, prepared as described above, was microinjected into pronuclei of fertilized mouse eggs and cultured overnight to the two-cell stage. From one series of microinjections a total of 38 mice were born out of 101 two-cell stage embryos transferred to pseudo-pregnant recipient females. Of these, eight founder animals were identified by southern blotting analysis of DNA prepared from tail biopsies. Seven of the eight founder mice were able to transmit the transgene by hemizygous backcross to F1 mice (C57/BL6 × CBA). Expression of the transgene was initially tested in four of the seven founder lines and two were found to express the transgene in RNA samples prepared from whole 1-day old mice. One of these founder lines (71.5) was selected as representative and further studies described in detail below were carried out after the mice were moved to Australia.

3.1.2. Tissue expression of the transgene

RNA was isolated from liver and skeletal muscle tissues of newborn (1-day old) and 21-day old mice and subject to RT-PCR in order to determine expression of the transgene in these tissues. Expression of the Rskα-actin/hIGF-I transgene was detected in the skeletal muscle but not the liver of transgenic mice at both ages (Fig. 2). Expression of the transgene was not detected in either liver or skeletal muscle of the wild-type non-transgenic littersmates.
3.1.3. IGF-I concentration

The concentration of IGF-I was measured in plasma and skeletal muscle using two separate assay systems. The results of these analyses are shown in Table 2. When the concentration of IGF-I was measured using an assay specific for rat IGF-I that does not cross-react with human IGF-I, there was no difference in IGF-I concentration in plasma or muscle samples from transgenic mice compared to their wild-type littermates at 6 months of age. Since mouse and rat IGF-I differ by only one amino acid, we have made an assumption that this assay is measuring only endogenous mouse IGF-I.

In contrast, when the same samples were analysed using an assay for human IGF-I, the mice over-expressing the human IGF-I gene (in muscle) had significantly higher concentrations of IGF-I in plasma and muscle samples from transgenic mice compared to their wild-type littermates at 6 months of age. Since mouse and rat IGF-I differ by only one amino acid, we have made an assumption that this assay is measuring only endogenous mouse IGF-I.

Table 2

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Wild-type</th>
<th>Transgenic</th>
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<tbody>
<tr>
<td>IGF-I assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat Plasma (ng/ml)</td>
<td>132.9 ± 33.1</td>
<td>158.3 ± 18.6</td>
</tr>
<tr>
<td>Human</td>
<td>50.8 ± 8.9</td>
<td>159.9 ± 16.9</td>
</tr>
<tr>
<td>Rat Muscle (ng/g)</td>
<td>13.3 ± 1.9</td>
<td>9.5 ± 0.8</td>
</tr>
<tr>
<td>Human</td>
<td>56.9 ± 7.3</td>
<td>200.7 ± 39.9</td>
</tr>
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</table>

Data are mean ± SEM for 8–10 mice in each group.

Fig. 2. Expression of the Rskz-actin/hIGF-I transgene detected by reverse transcription (RT)-PCR in the skeletal muscles and liver of 1- and 21-day old transgenic mice. Rskz-actin/hIGF-I transgene was expressed in the skeletal muscles but not the liver of transgenic mice. Transgene expression was not detected in the skeletal muscle and liver samples of the transgenic mice incubated without reverse transcriptase (–RT). Transgene expression was not detected in either skeletal muscle or liver of wild-type littermates (not shown).
3.2. Body weight and carcass composition

Male transgenic mice were heavier compared to the age matched male wild-type mice at 3 and 6 months of age (Fig. 3, also see legend of Table 4).

Carcass measurements and body composition measurements were performed in 6-month old male transgenic mice and their wild-type littermates. The carcass (body less pelt, head, tail and paws) total wet weight, wet fat-free weight, water content and dry fat-free weight were 8–9% higher in the transgenic mice compared to the wild-type littermates (Fig. 3). Myofibrillar protein content was also 8–9% higher in the transgenic mice. The higher weights of these tissues and components in transgenic mice were all statistically significant (Fig. 3). On the other hand, the total dry weight of the carcass and the fat content were similar between transgenic mice and controls (Fig. 3). After accounting for the differences in body weight by covariance analysis, the transgenic mice had an 8% lower dry carcass weight, increased water content and increased wet fat-free mass (Table 3). However, differences in the dry fat-free mass and myofibrillar protein content were no longer evident. The data in Table 2 show that there was a substantial reciprocal shift in the relative proportions of fat and water in the carcass, with transgenic mice having a lower fat content at 6 months of age.

3.3. Muscle histology

Myofibre cross-sectional area (CSA) was measured on transverse sections of gastrocnemius (white part), TA and EDL muscles of 3-month old male wild-type and transgenic mice corrected for body weight by covariance analysis

<table>
<thead>
<tr>
<th>Carcass measure</th>
<th>Mouse genotype</th>
<th>Wild-type</th>
<th>Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet weight (g)</td>
<td>26.5 ± 0.14</td>
<td>26.3 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Dry weight (g)</td>
<td>10.64 ± 0.20</td>
<td>9.77 ± 0.17</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Water content (g)</td>
<td>15.8 ± 0.17</td>
<td>16.51 ± 0.15</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Fat content (g)</td>
<td>5.79 ± 0.21</td>
<td>4.65 ± 0.192</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Wet fat-free mass (g)</td>
<td>21.1 ± 0.20</td>
<td>21.8 ± 0.19</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Dry fat-free mass (g)</td>
<td>5.26 ± 0.059</td>
<td>5.34 ± 0.054</td>
<td>NS</td>
</tr>
<tr>
<td>Myofibrillar protein (g)</td>
<td>1.52 ± 0.049</td>
<td>1.59 ± 0.046</td>
<td>NS</td>
</tr>
<tr>
<td>Fat content (% WW)</td>
<td>19.7 ± 0.77</td>
<td>16.7 ± 0.70</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Fat content (% DW)</td>
<td>48.3 ± 1.28</td>
<td>44.6 ± 1.17</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Data are means ± SEM for 32 transgenic and 28 wild-type mice.

3.4. Experiments on transgenic mice

3.4.1. Regeneration of whole muscle grafts

The pattern of whole muscle autograft regeneration was compared from 3 to 21 days after transplantation between wild-type control C57/BL6 mice and transgenic RSKα-actin/hIGF-I mice. At day 3 after transplantation, inflammatory cells, degenerating myofibres and desmin positive cells (activated myoblasts) were present at the periphery of both wild-type and transgenic grafts (Fig. 5A and B). The average numbers of desmin positive cells (myoblasts) was not significantly different between wild-type (28 ± 25, n = 3) and transgenic (23 ± 9, n = 4) grafts at this time. One wild-type graft had more desmin positive cells than any other wild-type or transgenic graft examined, emphasizing the biological variability that can be encountered. Myofibre necrosis and regeneration were pronounced in day 5 (Fig. 5C and D) and day 7 wild-type and transgenic grafts. The average area of the persisting necrotic zone was similar in day 5 wild-type control (41 ± 36%, n = 3) and transgenic (44 ± 8%, n = 4) grafts. At day 7, the average area of the necrotic zone was smaller (1 ± 2%, n = 5) in wild-type control compared to transgenic grafts (16 ± 19%, n = 4), however, this difference was not statistically
significant. Desmin immunostaining was pronounced at days 5 and 7 in myotubes and myoblasts, which often appeared as “cuffing cells” lining the contour of the persisting basement membrane of the necrotic myofibres (Fig. 5C and D). Many desmin positive myotubes, which represent “plump” cells with well defined cytoplasm were observed in the regenerating zone of the wild-type and transgenic grafts. Quantitation of myotubes in the single transverse section of the day 5 and 7 grafts did not reveal any significant differences between wild-type control and transgenic grafts, and this was also the case when the data were standardized to take into account variations in the total area of the grafts (Fig. 6A and B). By day 21, all wild-type and (n = 3) and transgenic (n = 3) grafts were composed of densely packed small myofibres with centralized nuclei and had a similar appearance (Fig. 5E and F).

The vascular network was visualized using antibodies to the cell surface marker PECAM-1 and percentage vascularity was calculated at day 5 after transplantation. There was no difference between the percentage vascularity of the wild-type (9.4 ± 0.4%, n = 3) and transgenic (9 ± 0.4%, n = 2) grafts at this time. Due to the absence of any significant differences in the pattern of regeneration between wild-type and transgenic grafts, percentage vascularity was not quantitated at earlier (day 3) and later (day 7) times.

3.5. Muscle denervation

At 12 weeks of age Rskα-actin hIGF-I transgenic mice were significantly heavier compared to their wild-type littermates (Table 6). The average total body weight was reduced at 28 days following denervation for both wild-type and transgenic mice, however, this decrease was significant only for wild-type mice (Table 6). Myofibre CSA was measured on transverse cryosections of intact and denervated TA muscles stained with H&E (Table 7). At day 28 following sciatic nerve transection, a significant decrease of the myofibre mean CSA was detected in TA muscles of both wild-type and Rskα-actin/hIGF-I mice, but there was no significant difference in the extent of myofibre CSA reduction between wild-type and transgenic mice (Table 7).

3.6. Muscular dystrophy

3.6.1. Weights of mdx and mdx/hIGF-I littermate mice

There was no difference between the average weights of 21-day old mdx control and mdx/hIGF-I transgenic littermate mice (Table 8). The average weight of 40 days
old transgenic females was higher compared to the average weight of their mdx control female littermates (Table 8). At 12 weeks of age, only three transgenic mice (one male and two females) were available: the single 12-week old male mdx/hIGF-I mouse was heavier than any of the non-transgenic male littermates ($n = 4$), whereas weights of both 12-week old female mdx/hIGF-I mice were in the range of the four age-matched female mdx control littermates (Table 8).

3.7. Quantitation of muscle damage in young mdx and mdx/hIGF-I

3.7.1. 21–22 days old mice

Damage to the sarcolemma (myofibre leakiness) was assessed on transverse cryosections of the TA and diaphragm muscles of 21–22-day old mdx and mdx/hIGF-I littermate mice by the presence of EBD positive myofibres. Composite muscle damage, which represents a cumulative area of myofibre necrosis and regeneration was assessed histologically on H&E stained sections of the same muscles. An abrupt onset of muscle necrosis has been previously described in mdx TA muscles at 21 days of age [30]. In the TA muscles of mdx mice the myotubes are first seen at around 22 days of age [33,34,30], whereas in the mdx diaphragms myotubes are seen from 19 days of age, which is consistent with the earlier onset of myofibre necrosis (17 days of age) in this muscle [30]. Quantitative morphometric analysis of the average areas occupied by EBD positive myofibres and areas of cumulative muscle damage did not reveal any difference for the TA and diaphragm muscles between 21- and 22-day old non-transgenic mdx and transgenic mdx/hIGF-I littermate mice (Table 9).

It should be noted that the area of the average cumulative muscle damage in the TA muscles of 21–22-day old mdx/hIGF-I mice (4.6 ± 2.6%) and their non-transgenic mdx littermates (7.5 ± 2.8%) was lower compared to the earlier reported value of the average cumulative muscle damage for the TA muscles of the 21–22-day

Fig. 5. Typical transverse sections of wild-type (A, C and E) and Rskα-actin/hIGF-I (B, D and F) muscle grafts. Antibodies for desmin are detected using DAB (brown) staining. Activated satellite cells (arrows), shown at high magnification in (A) and (B) are detected at the periphery of wild-type (A) and transgenic (B) day 3 grafts. Most of the day 3 graft area is occupied by necrotic myofibres (necrotic core). By day 5, many small desmin positive myotubes (asterisks) are present in the grafts (C and D). The center of the day 5 grafts is still occupied by necrotic muscle and activated satellite cells (arrows) are seen “cuffing” necrotic myofibres. At day 21 (E and F), new myofibres occupy the entire cross-section of the wild-type and transgenic grafts. The appearance of regenerating grafts was similar in wild-type and Rskα-actin/hIGF-I mice at all time points examined.
old non-transgenic littermates of the mdx/mIGF-I mice (~18% of the whole muscle cross-sectional area) [30] and 21-day old conventional mdx mice (~17% of the whole muscle cross-sectional area) [34]. The extent of muscle necrosis was comparable between the diaphragm muscles in 21–22-day old mdx/hIGF-I mice and their non-transgenic mdx littermates and previously described mdx/mIGF-I mice and their non-transgenic mdx littermates [30].

3.7.2. Twenty-six- and forty-day old mice

Twenty-six- and forty-day old mdx and mdx/hIGF-I mice were not injected with EBD. The extent of the composite muscle damage was quantitated on H&E stained cryosections of the TA and diaphragm muscles of 26-day old transgenic mice and their non-transgenic mdx littermates. There was no significant difference in the extent of the composite muscle damage in any of the analysed muscles between 26-day old mdx and mdx/hIGF-I mice (Table 9).

Only TA muscles were analysed in 40-day old mdx and mdx/hIGF-I mice. The overall histological examination of the H&E stained paraffin embedded sections of the TA muscle showed the presence of very small areas of active muscle necrosis: areas occupied by necrotic myofibres and very small myotubes infiltrated with inflammatory cells. However, many myofibres were centrally nucleated, which indicated that they had previously undergone necrosis and regeneration. The proportion of centrally nucleated myofibres was similar in the TA muscle of mdx (39 ± 0.45%, n = 2) and mdx/hIGF-I (44 ± 2%, n = 4) mice indicating no difference in the pattern of pathology in this transgenic strain.

3.7.3. Muscle morphology in 12-week old mdx and mdx/hIGF-I mice

The overall morphological examination did not reveal any marked differences for the TA and diaphragm muscles between 12-week old mdx and mdx/hIGF-I mice. Since myofibre hypertrophy and increased diaphragm width has been reported previously in mdx/mIGF-I mice [35,30], the present study compared the average myofibre CSA in the TA muscles and the average width of the diaphragms between 12-week old mdx and mdx/hIGF-I mice (Table 10). Due to the limited availability of mdx/hIGF-I mice (one male and two females), it was not possible to perform statistical analysis of these morphometric data. The average CSA of the myofibres was similar in the TA muscles of male and female mdx and mdx/hIGF-I mice. The average diaphragm width was also similar in male non-transgenic and transgenic mice; although the average width of the female mdx/hIGF-I diaphragms was larger compared to the female mdx diaphragms, this difference may be due to the few mice analysed, since myofibre hypertrophy was not detected in other muscles for either sex.

4. Discussion

The initial objective of this work was to design a line of transgenic mice that expressed human IGF-I under control of the skeletal muscle specific rat skeletal α-actin promoter. At the same time we wished to ensure that the expressed IGF-I protein is secreted into the muscle to
act as an autocrine/paracrine factor and contribute to the overall concentration of circulating IGF-I.

Preliminary data from the Rskα-actin/hIGF-I transgenic mouse strain suggested that endogenous mouse IGF-I measured using assay specific for rodent IGF-1 was lower in the plasma and skeletal muscles of 6-month old transgenic mice compared to the wild-type litter-mates [5]. Further analysis of the plasma and muscle IGF-I concentrations showed that the levels of endogenous (mouse) IGF-I were similar in 6-month old transgenic and wild-type littermate mice. The human IGF-I assay detected an additional 100 ng/ml of IGF-I in the plasma and an additional 150 ng/g of IGF-I in skeletal muscles of Rskα-actin/hIGF-I transgenic mice. The fact that the concentration of (transgenic) human IGF-I in skeletal muscles is higher than could be accounted for by residual plasma in the samples confirms that the transgene is expressed at high levels in skeletal muscle.

While increased muscle IGF-1 protein is a common characteristic of transgenic mice with muscle specific IGF-I over-expression [8,9], the increase in plasma levels of IGF-I in Rskα-actin/hIGF-I mice is in marked con-trast with the situation in the other strains where there is no significant change of overall serum IGF-I levels [8,9].

The fact that a signal sequence from the somato-statin gene was included in the Rskα-actin/hIGF-I

<table>
<thead>
<tr>
<th>Table 7</th>
<th>Mean cross-sectional area (CSA) of myofibres (µm²) in intact and denervated tibialis anterior (TA) muscles of wild-type and Rskα-actin/hIGF-I littermate mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>Intact</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2884 ± 183 (n = 4)</td>
</tr>
<tr>
<td>Rskα-actin/hIGF-I</td>
<td>2943 ± 479 (n = 7)</td>
</tr>
</tbody>
</table>

All numbers are based on the analysis of 500–700 myofibres from randomly chosen fields of superficial and deep regions of TA muscles. Denervation significantly reduced the myofibre mean CSA in both wild-type and transgenic TA muscles (P < 0.005 calculated using Student’s t-test). There was no significant difference between the extent of myofibre CSA reduction following denervation (compared using two-factor analysis of variance) between wild-type and transgenic mice. Data represent mean ± SD. (n) represents number of muscles analysed.

<table>
<thead>
<tr>
<th>Table 8</th>
<th>Average weights (g) of young and adult mdx control and mdx/hIGF-I littermate mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/muscle</td>
<td>mdx</td>
</tr>
<tr>
<td>21 days (male and female)</td>
<td>7.4 ± 1.1 (n = 5)</td>
</tr>
<tr>
<td>40 days (female)</td>
<td>14 ± 0 (n = 2)</td>
</tr>
<tr>
<td>12 weeks</td>
<td>25 ± 2 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>24 ± 4 (n = 4)</td>
</tr>
</tbody>
</table>

Data represent mean ± SD. (n) represents number of animals.

<table>
<thead>
<tr>
<th>Table 9</th>
<th>Areas (%) of whole muscle sections occupied by Evans blue dye (EBD) positive myofibres and composite muscle damage (area of necrosis plus area of regeneration) in the TA and diaphragm muscles of young (21–26-day old) mdx and mdx/hIGF-I littermate mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/muscle</td>
<td>EBD</td>
</tr>
<tr>
<td>Day 21–22</td>
<td>TA</td>
</tr>
<tr>
<td></td>
<td>Diaphragm</td>
</tr>
<tr>
<td>Day 26</td>
<td>TA</td>
</tr>
<tr>
<td></td>
<td>Diaphragm</td>
</tr>
</tbody>
</table>

Data are mean ± SE. (n) represents numbers of animals.

<table>
<thead>
<tr>
<th>Table 10</th>
<th>Myofibre CSA (µm²) in the TA muscles and the diaphragm width (µm) in 12-week old mdx and mdx/hIGF-I mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myofibre CSA in TA muscles (µm²)</td>
<td>mdx</td>
</tr>
<tr>
<td>1911 ± 312 (n = 4) Male</td>
<td>1852 (n = 1) Male</td>
</tr>
<tr>
<td>1356 ± 309 (n = 4) Female</td>
<td>1379 ± 49 (n = 2) Female</td>
</tr>
<tr>
<td>Average diaphragm width (µm)</td>
<td>270 ± 47 (n = 4) Male</td>
</tr>
<tr>
<td>264 ± 15 (n = 4) Female</td>
<td>350 ± 60 (n = 2) Female</td>
</tr>
</tbody>
</table>

For each TA muscle myofibre, measurements are based on 500–700 myofibres from five non-overlapping fields.
construct to ensure secretion of the transgenic human IGF-I from the muscle cells is the most likely explanation for the increased circulating IGF-I levels in Rskα-actin/hIGF-I mice. Similar to the Rskα-actin/hIGF-I strain, another early line of transgenic mice which expressed human IGF-I in visceral tissues under control of the metallothionein-1 promoter, exhibited a significant (1.5 fold) increase in circulating levels of IGF-I [13]. The transgenic construct introduced into this transgenic mouse strain also contained a somatostatin signal sequence to direct the secretion of the human IGF-I [13].

Data from several studies suggest that skeletal muscle normally contributes to levels of circulating IGF-I. Mice with the IGF-I gene deleted in the liver maintain 25% of the total circulating IGF-I and the level of non-bound ‘free’ IGF-I in the circulation is unchanged [4]. The source of this portion of systemic IGF-I is yet to be established, but it has been speculated that skeletal muscle and adipose tissue might secret IGF-I into the bloodstream [36]. Exercising muscle also releases IGF-I into the circulation [37]. In our experience, intravenous infusion of 0.3 mg hIGF-I/kg/day to growing rats, equivalent to about 0.75 μg/g muscle/day is able to maintain plasma IGF-I levels of 250–300 ng/ml (P. Owens and S. Knowles, unpublished data). The design of the Rskα-actin/hIGF-I construct incorporating a somatostatin signal sequence to direct secretion of IGF-I from the transgene has led to increased IGF-I in the bloodstream in a way that mirrors the release of IGF-I from muscle in ‘normal’ physiology.

Rskα-actin/hIGF-I mice show broad phenotypic changes associated with enhanced somatic (lean tissue) growth. At 3 and 6 months of age transgenic mice were heavier compared to the age-matched wild-type mice. The carcass total wet weight, wet fat-free weight, dry fat-free weight, myofibrillar protein content and water content were increased proportionally to the total body weight in transgenic mice. The fat content was similar between transgenic and wild-type mice and, after accounting for the differences in body weight by covariance analysis, the transgenic mice appeared to be leaner and had a lower fat content. Increased carcass dry fat-free weight and myofibrillar protein content was an indication of the effect of IGF-I over-expression on muscle composition.

Changes in the body weight and fat composition observed in Rskα-actin/hIGF-I mice are in agreement with phenotypic changes reported in animals with the increased levels of systemic IGF-I. For example, systemic administration of recombinant human IGF-I to normal rats increases body weight and decreases fat content [38,39] and increased growth rate seen in transgenic mice with cardiac-specific [11] and smooth muscle specific [12] over-expression of IGF-I is also likely to be associated with elevated systemic IGF-I. It is interesting that in mice carrying an IGF-I transgene fused to a smooth muscle α-actin promoter, body weight gain was increased only in the lines with increased plasma IGF-I concentration [12].

On the other hand, it has been shown that the fractional weight of the carcass is generally reduced with exogenous IGF-I administration indicating a lack of muscle hypertrophy (relative to the body weight) [39], which accords with the view that the autocrine/paracrine mode of IGF-I action is the principal means for stimulating muscle growth. The results from the carcass analysis in Rskα-actin/hIGF-I mice show that carcass fat-free mass and myofibrillar protein mass increased at least in proportion to the higher body weight of transgenic mice, which is likely to be accounted for by the local muscle specific (rather than systemic) effects of the transgenic hIGF-I. Although we made no estimate of the linear growth of Rskα-actin/hIGF-I mice, other reports describing transgenic mice with IGF-I over-expression indicate little or no effect on tibial length [13,11,10,40]. This points to the increase in body weight being due, at least in part, to a relative increase in muscle weight. Although no statistically significant increase in myofibre CSA was observed in transgenic Rskα-actin/hIGF-I mice, the increase in myofibrillar protein content relative to the body weight might reflect a very mild not statistically significant myofibre hypertrophy or myofibre hyperplasia (increased proliferation and fusion of myoblasts), that could have occurred during the developmental phase. The factors that account for the relative increase of myofibre protein content in Rskα-actin/hIGF-I mice require further investigation.

The lack of increase in myofibre CSA in Rskα-actin/hIGF-I transgenic mice was unexpected, since muscle specific over-expression of transgenic IGF-I has been shown to cause muscle hypertrophy in other mice [8–10]. One of the explanations for the absence of myofibre hypertrophy in Rskα-actin/hIGF-I mice compared to two other transgenic mouse strains [8–10] might be the differences in the nature of the transgene over-expressed in the muscles of these different mouse strains. While previously described transgenic mice over-expressed Class 1 IGF-I Ea isoform [8–10,25], the Rskα-actin/hIGF-I construct was designed to over-express fully processed 70 amino acid IGF-I. It is now appreciated that not all IGF-I isoforms over-expressed in skeletal muscles result in myofibre hypertrophy. For example, transgenic mice with skeletal muscle specific over-expression of IGF-I Eb isoforms do not exhibit increased myofibre size (N. Winn, personal communication).

A significant increase in systemic IGF-I levels in Rskα-actin/hIGF-I mice appears to be another factor that accounts for the dramatic phenotypic differences between these mice and the other transgenic mouse strains characterized by elevated total IGF-I in only skeletal muscles, but not blood [8–10]. It should be
noted that the phenotype of the Rskα-actin/hIGF-I mice accords with the phenotype of recently described transgenic pigs which over-express human IGF-I (Class I Ea isoform) under the avian skeletal α-actin promoter and exhibit significantly elevated IGF-I in blood [41]: in particular there was a 19% and 11% increase of circulating IGF-I levels in transgenic gilts and boars respectively compared to non-transgenic littermates. These transgenic pigs had less fat and more lean tissue compared to the wild-type littermates and the difference became more pronounced as the pigs grew older [41]. These transgenic pigs had lower fat accretion rate, whereas there was no difference in the lean tissue accretion rate compared to the non-transgenic pigs. This indicates the absence of muscle hypertrophy in the transgenic pigs, which is also the case in the Rskα-actin/hIGF-I mice. It is noted that the same transgene over-expressed in mice did cause myofibre hypertrophy [8,10], thus indicating different responses between species (pigs and mice) and the role of epigenetic factors.

It is expected that increased systemic IGF-I in Rskα-actin/hIGF-I mice would cause perturbations in levels of other components of the IGF-I system and the overall growth hormone (GH)/IGF-I axis. It is known that circulating IGF-I negatively regulates the release of GH by the pituitary gland. While we have not measured GH levels in the Rskα-actin/hIGF-I mice, decreased circulating GH has been reported in transgenic mice with elevated circulating IGF-I [13]. It is now appreciated that IGF-I and GH have overlapping as well as independent effects on somatic growth [42]: it is estimated that the overlapping GH/IGF-I effect makes 34% contribution to the total weight, IGF-I alone contributes 35% and GH alone 14%. Therefore, it is possible that the absence of muscle hypertrophy in Rskα-actin/hIGF-I mice is a result of decreased systemic GH.

High concentrations of IGF-I can down-regulate IGF-I receptors (IGF-1R) and this might be another reason for the absence of muscle hypertrophy in Rskα-actin/hIGF-I mice. A decrease in IGF-1R number with increased IGF-I concentration, due to internalization of the complex, has been shown in cultured human lymphoid cells [43] and bovine chondrocytes [44].

Changes in levels of IGF binding proteins (BP) in an environment of increased systemic IGF-I could be another factor contributing to the phenotypic differences between Rskα-actin/hIGF-I mice and the other transgenic mice with increased IGF-I levels exclusively in skeletal muscles. Changes in IGFBP levels were described in mice with liver specific deletion of the IGF-I gene, where up to a 75% decrease of serum IGFBP-3 and IGFBP-1 was demonstrated [4]. Such a dramatic decrease in IGFBP levels is likely to account for the maintenance of free IGF-I in circulation of these mice. However, at present, there is no information about changes of IGFBP levels in the transgenic mice with muscle specific over-expression of IGF-I transgenes and this issue remains to be addressed.

4.1. Experiments on transgenic mice

The present study used Rskα-actin/hIGF-I transgenic mice to look at the effect of increased systemic and local levels of IGF-I on the remodelling of skeletal muscle using three established experimental models: (a) regenerating whole muscle autografts; (b) denervation induced muscle atrophy; and (c) mdx muscular dystrophy.

4.1.1. Regeneration

The pattern of the whole muscle graft regeneration was not affected by over-expression of the Rskα-actin/hIGF-I transgene. Results from this study do not support any aspect of our initial hypothesis that increased levels of hIGF-I in skeletal muscle in conjunction with increased systemic levels of IGF-I, would accelerate regeneration by enhancing myoblast proliferation, myotube formation and revascularisation of the grafts. We have previously shown that muscle specific over-expression of mIGF-I, with no systemic increase in IGF-I, does not affect early events of regeneration of whole muscle autografts in MLC/mIGF-I transgenic mice [28]. We suggested that the absence of any effect of mIGF-I on whole muscle autografts was due to the fact that necrosis occurs throughout the entire length of the individual myofibres in the grafts and the IGF-I protein (endogenous and transgenic) is no longer expressed by the necrotic myofibres [28]. Since proliferating undifferentiated myoblast do not express the MLC1/3 promoter, there is no possibility of any autocrine action of mIGF-I on undifferentiated myogenic precursor cells. Similarly, the skeletal α-actin promoter is not expressed by undifferentiated myoblasts and therefore hIGF-I would not be expressed by Rskα-actin/hIGF-I muscle precursor cells nor the necrotic myofibres. Moreover, it has been shown that at the early stages of regeneration muscle switches to the embryonic pattern of gene expression, which includes a switch from skeletal α-actin to cardiac α-actin expression [45]. Quantitation of skeletal α-actin mRNA expression in regenerating whole muscle grafts in rats shows that at 10 days after grafting less then 10% of skeletal α-actin mRNA remains, whereas expression of cardiac α-actin mRNA is considerably increased [45]. Thus, downregulation of the skeletal α-actin promoter in regenerating whole muscle grafts of Rskα-actin/hIGF-I mice will reduce expression of the transgenic hIGF-I. However, the elevated systemic IGF-I, resulting from over-expression of Rskα-actin/hIGF-I transgene was expected to have an endocrine effect on muscle precursor cells in regenerating Rskα-actin/hIGF-I grafts. Systemic IGF-I is delivered to the grafted muscle by infiltrating plasma and by blood vessels, which start growing into the graft from the host tissue.
by day 3 after transplantation [27,46]. However, despite the increased systemic levels of IGF-I, no effect was seen on the early events of whole muscle graft regeneration in Rskα-actin/hIGF-I transgenic mice with respect to proliferation of activated myogenic cells, formation of new myotubes or revascularisation. We suggest that the increased total plasma levels of IGF-I in Rskα-actin/hIGF-I mice might stimulate an increase of IGF-I binding proteins that could diminish the availability of free IGF-I (reviewed in [5]) or this could also result from modulation of other aspects of the overall IGF-I/GH system as discussed above.

4.1.2. Denervation
The second experimental model examined was muscle atrophy following sciatic nerve transection. Several recent studies report beneficial effects of IGF-I for the reduction of myofibre atrophy in denervated muscle. Intramuscular injection of human recombinant IGF-I reduced myofibre atrophy in short (7 days) [47] and relatively long-term (up to 6 weeks) [48] denervated muscles in rats. Muscle specific over-expression of rat mIGF-I also reduced myofibre atrophy in MLC/mIGF-I TA muscles by ~30% at 28 days following denervation [25]. However, the protective effect of mIGF-I on muscle atrophy was not sustained and no difference was seen between the atrophic wild-type and MLC/mIGF-I TA muscles at 2 months following denervation [25]. It is suggested that the main mechanism that provides for the protective effect of IGF-I on muscle atrophy is inhibition of the atrophy related genes via activation of PI3K/Akt signalling pathway [49,47,50]. Based on these data we hypothesized that increased muscle and systemic levels of IGF-I will reduce myofibre atrophy in denervated muscles of Rskα-actin/hIGF-I mice. About 60% of skeletal α-actin expression is maintained in atrophic EDL muscles of rats at 10 and 40 days following denervation [45] and therefore it was expected that at 28 days following sciatic nerve transection the denervated muscle of Rskα-actin/hIGF-I mouse will maintain expression of the transgenic hIGF-I, in addition to the elevated systemic IGF-I available through the circulating blood. However, no beneficial effect of Rskα-actin/hIGF-I over-expression was seen on denervated myofibres and the denervated TA muscles of the transgenic mice atrophied to the same extent over 28 days. The reduction of average myofibre CSA at 28 days following denervation observed in the TA muscle of the wild-type littermates of the transgenic Rskα-actin/hIGF-I mice (~58%) was similar to the previously observed rate of myofibre atrophy in 28 day denervated TA muscles of the FVB strain of mice (~59%), which were used as wild-type controls for the MLC/mIGF-I transgenic mice [25]. However, in contrast to the present study, where over-expression of Rskα-actin/hIGF-I did not have a significant effect on the myofibre atrophy at 28 days following sciatic nerve transection, over-expression of MLC/mIGF-I transgene did significantly slow down the myofibre atrophy in the TA muscles and the myofibre CSA was reduced by only ~37%. The reasons for the lack of effect of the elevated IGF-I on the denervated muscles of Rskα-actin/hIGF-I mice are not known but, as indicated above, it is possible that increased levels of IGBP5 in response to the considerable elevation of the systemic IGF-I reduces the availability of the free IGF-I.

4.1.3. Dystrophy
Tremendous interest was generated by the improved dystrophopathology demonstrated in MLC/mIGF-I mice [35] that is attributed to reduced myofibre necrosis [30] due to elevated mIGF-I within skeletal myofibres. We have previously shown that muscle specific over-expression of mIGF-I reduces sarcolemmal damage (assessed by EBD infiltration) and myofibre necrosis in the limb muscles (TA and quadriceps) of mdx/mIGF-I mice during the acute onset of dystrophic muscle degeneration around 3 weeks of age. This protective effect was most pronounced in the limb muscles of mdx/mIGF-I mice at 21–22 days of age, when severe degenerative changes were observed in non-transgenic mdx mice. Although adult mdx/mIGF-I mice exhibit myofibre hypertrophy [35,30], the increased myofibre size is not an absolute prerequisite for the protective effect of mIGF-I over-expression, since it is not seen in young mdx/mIGF-I mice [28]. A similar protective effect of IGF-I was expected in mdx mice that over-express the Rskα-actin/hIGF-I transgene. However, quantitation of the sarcolemmal damage and myofibre necrosis in the TA and diaphragm muscles of the young (21–40-day old) mdx/hIGF-I mice did not show any protective effect of hIGF-I over-expression on the dystrophopathology. This lack of effect of the transgenic hIGF-I was further substantiated by morphological examination of adult (12-week old) mdx/hIGF-I mice where muscle hypertrophy was not seen compared to the age matched non-transgenic mdx controls. In contrast, myofibre hypertrophy was pronounced in the limb and diaphragm muscles of 12-week and 1-year old mdx/mIGF-I mice [35,30].

It is important to note that the extent of necrosis in TA muscles of 21–22-day old mdx/hIGF-I mice and their non-transgenic mdx littermates was much lower (~4–8%) compared to the extent of necrosis in the TA muscles of the conventional mdx mice or transgene negative littermates (~20%) of mdx/mIGF-I mice at the same age. These differences might be accounted for by the differences in background strains used to generate the transgenic mice, since Rskα-actin/hIGF-I mice are on a C57BL/6J background and MLC/mIGF-I mice are on a FVB background.

Overall, in light of the published data, our results suggest that the combined increase in body weight and lean
tissue content in Rskα-actin/hIGF-I mice may result from both autocrine/paracrine and systemic (endocrine) actions of human IGF-I derived from the transgene, in addition to the endogenous mouse IGF-I. The absence of skeletal muscle hypertrophy in Rskα-actin/hIGF-I mice as well as the absence of any effect of the elevated muscle and circulating IGF-I in the experimental models of regeneration, atrophy and dystrophy might be due to the perturbations of the systemic IGF-I system and growth hormone/IGF-1 axis resulting from the dramatically increased systemic IGF-I. This study further demonstrates the complexity associated with transgenic manipulation to elevate IGF-1 in vivo.

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