Growth hormone (GH) participates in the postnatal regulation of skeletal muscle growth, although the mechanism of action is unclear. Here we show that the mass of skeletal muscles lacking GH receptors is reduced because of a decrease in myofiber size with normal myofiber number. GH signaling controls the size of the differentiated myotubes in a cell-autonomous manner while having no effect on size, proliferation, and differentiation of the myoblast precursor cells. The GH hypertrophic action leads to an increased myonuclear number, indicating that GH facilitates fusion of myoblasts with nascent myotubes. NFATc2, a transcription factor regulating this phase of fusion, is required for GH action because GH is unable to induce hypertrophy of NFATc2−/− myotubes. Finally, we provide three lines of evidence suggesting that GH facilitates cell fusion independent of insulin-like growth factor 1 (IGF-1) up-regulation. First, GH does not regulate IGF-1 expression in myotubes; second, GH action is not mediated by a secreted factor in conditioned medium; third, GH and IGF-1 hypertrophic effects are additive and rely on different signaling pathways. Taken together, these data unravel a specific function of GH in the control of cell fusion, an essential process for muscle growth.

Growth hormone (GH) coordinates the postnatal growth of multiple target tissues, including skeletal muscle (1). This anabolic action has been exploited to increase lean body mass and protein synthesis in GH-deficient patients and muscle wasting diseases (2, 3). However, the mechanisms of GH anabolic actions on skeletal muscle are not fully elucidated. With respect to the somatomedin hypothesis, the growth-promoting actions of GH are mediated by circulating or locally produced insulin-like growth factor 1 (IGF-1) (4), which is a critical myogenic agent involved in muscle growth (1, 5). Circulating IGF-1 is mostly derived from the liver and may act in an endocrine manner. In addition, GH-induced growth could also be mediated by local production of IGF-1 in target tissues, where IGF-1 may act in an autocrine/paracrine fashion. The expression of GHR has been reported in skeletal muscles, and several studies have shown that GH treatment increases IGF-1 mRNA in skeletal muscle tissues as well as in the myoblast cell line C2C12 (1, 6, 7). That intact IGF-1 receptor signaling is required for the GH effects on skeletal muscle growth and function has been recently inferred by overexpressing a dominant-negative IGF-1 receptor in muscle (8). However, the comparative analysis of mice lacking GHR, IGF-1, or both genes suggests that there are anabolic effects of GH that are not mediated by IGF-1 (9). Because the growth retardation of double GHR/IGF-1 mutants is more severe than that observed with single mutants, it is likely that GHR signaling also exerts specific and direct effects on skeletal muscles, which do not depend on IGF-1 expression.

Skeletal muscle growth and regeneration rely on satellite cells that are located between the basal lamina and sarcolemma of myofibers. After the initial steps of satellite cell activation, proliferation, and differentiation, the fusion of myoblasts with the growing multinucleated muscle cells is essential to increase cell size (10). Two stages of fusion can be distinguished: the initial myoblast–myoblast fusion to form nascent myotubes and the subsequent myoblast–myotube fusion. In mammals, the regulatory mechanisms that control myoblast fusion are only beginning to be understood at the molecular level (11). Recent studies have identified NFATc2, a member of the nuclear factor of activated T cell (NFAT) family of transcription factors, as essential for the second phase of fusion. NFAT proteins are regulated through dephosphorylation by calcineurin, a calcium-dependent phosphatase, resulting in nuclear translocation, DNA binding, and regulation of transcription (12). Interestingly, NFATc2 directs fusion by stimulating the expression of IL4, suggesting that extracellular cytokines are involved in this regulation (13).

In addition to the modulation of myonuclear number, the control of cytoplasmic volume also determines the final size of muscle fibers by altering the balance between protein synthesis and protein degradation (14). Central to this regulation are the kinases Akt and mTOR. The shutdown of this pathway results in the up-regulation of two E3 ubiquitin ligases, MuRF1 and MAFb, thus accelerating proteolysis via the ubiquitin–proteasome pathway and leading to muscle atrophy. Conversely, the activation of Akt and mTOR produces large muscle fibers, an effect that requires the mTOR substrate S6 kinase 1 (15).

To understand how skeletal muscle responds to GH, we used mutant mice lacking GHR (GHR−/−) (16). GHR−/− mice present a postnatal growth retardation with elevated GH and low IGF-1 circulating levels, indicating a complete GH resistance. By using muscle cell cultures, we show that GH signaling affects muscle mass by controlling myofiber size in a cell-autonomous manner. Next we identify a mechanism by which GH exerts its hypertrophic action: GH facilitates the second phase of myoblast fusion when nascent myotubes are present and develop to mature myotubes. Finally, we provide strong evidence suggesting that GH action on fusion is not mediated by local IGF-1.

Results

Myofiber Cross Section Area (CSA) Is Reduced in GHR−/− Muscles. As the body weight of GHR−/− mice was reduced, the reduction of muscle mass was evaluated by weighing two distinct hind limb muscles of female and male mice and normalizing to total body weight (Table 1, which is published as supporting information on www.pnas.org).
the PNAS web site). The absolute muscle weight and the muscle/body weight of soleus and tibialis anterior were reduced in GHR<sup>-/-</sup> mice of both genders as compared with wild type. Thus, the disproportionate reduction in muscle mass is not only a consequence of the global dwarfism of the GHR<sup>-/-</sup> mice and suggests a specific action of GH as a muscle growth factor.

To study muscle fiber composition and morphometric characteristics, we performed immunohistological analysis of transversal cross sections from female soleus muscles with anti-myosin heavy chains (MyHCs) antibodies. Consistent with the reduction of muscle mass, the CSA of the entire GHR<sup>-/-</sup> soleus was reduced as compared with wild type (Fig. 6A, which is published as supporting information on the PNAS web site). Because muscle mass is determined by the number and size of individual fibers, we first counted the total number of soleus myofibers, which is set during embryonic development, and we observed no difference between the numbers of wild-type and mutant myofibers (Fig. 1B). These data, combined with the onset of growth retardation at the second week after birth (16), indicate that GH is not involved in the control of muscle growth at the embryonic and perinatal stages.

Muscles are composed of different fiber types that vary in their contractile and metabolic status. Slow myofibers express type I MyHC and are oxidative, whereas fast myofibers express type II MyHC and are glycolytic. In addition, hybrid fibers expressing MyHC type I and II are also found. In GHR<sup>-/-</sup> soleus, we measured a 26% decrease of type I fiber number and a 16% increase of type II fibers (Fig. 1C), indicating that the lack of GH signaling causes a switch from type I and hybrid fibers to type II fibers. Consistent with these data, overexpression of human GH (hGH) in transgenic mice led to an increased percentage of type I fibers, confirming that GH has a positive role in type I fiber specification (17). Next, we measured the CSA of both fiber types in soleus. GHR deletion decreased type I and type II fiber size by 36% and 40%, respectively (Fig. 1A, B, and D). Similar reductions in CSA were measured in male GHR<sup>-/-</sup> soleus and in GHR<sup>-/-</sup> tibialis anterior from both genders (Fig. 1B). Taken together, these data show that the reduced mass of GHR<sup>-/-</sup> muscles is due to smaller individual myofiber size rather than to a change in their number. Thus, GHR deletion specifically affects postnatal myofiber growth and specification, whereas myofiber formation during embryogenesis is unchanged.

**GH Induces Myoblast Hypertrophy.** The dwarf phenotype of GHR<sup>-/-</sup> mice is associated with low circulating IGF-1 levels, a defect that may lead to growth retardation (16). To distinguish between humoral or cell-autonomous control of muscle size in GHR<sup>-/-</sup> mice, we established primary muscle cell cultures from wild-type and GHR<sup>-/-</sup> mice. Myoblast cultures of both genotypes expanded similarly in mitogen-rich medium as assessed by cell counting and by FACS analysis (data not shown and Fig. 2A). Next, we assessed GH-stimulated cell-cycle progression by FACS analysis of cell-cycle phase distribution and by BrdU incorporation. In starvation conditions the percentage of cells in S-phase was largely reduced as compared with complete medium conditions (Fig. 2A). Of note, GH concentrations ranging from 10 to 1,000 ng/ml failed to induce S-phase entry of wild-type cells as compared with starved conditions (Fig. 2A, Fig. 7, which is published as supporting information on the PNAS web site, and data not shown). In contrast, serum treatment stimulated both wild-type and GHR<sup>-/-</sup> cell-cycle progression. To control whether the lack of GH-induced proliferation was not due to the absence of GH expression and signaling, we checked GH expression and phosphorylation of Janus kinase 2 (Jak2), the tyrosine kinase associated to GHR. GH expression and GH-induced Jak2 phosphorylation were detected in wild-type cells, indicating that the first steps of GH signaling were intact (Fig. 2B). These data show that wild-type myoblasts express functional GHR receptors, although their stimulation by GH does not favor cell-cycle progression.

To measure cell size, the parameter of forward scatter height was determined by flow cytometry. Because cell size also depends on DNA content, forward scatter height was measured in G<sub>1</sub>-phase gated cells, and similar values were obtained for myoblasts of both genotypes (Fig. 7B). Thus, the growth defect of GHR<sup>-/-</sup> muscles...
is not accompanied by impaired myoblast cell growth and proliferative capacity.

To determine whether the size of differentiated cells was affected by GHR deletion, we switched myoblasts to mitogen-poor differentiation medium (DM) and followed their differentiation. Forty-eight hours after mitogen removal, both wild-type and GHR−/− cells differentiated to multinucleated myotubes (Fig. 3A). The kinetics of muscle differentiation were followed by the expression of appropriate molecular markers. The expression of myogenin and the myofibrillar proteins α-skeletal actin, MyHC, and troponin T showed similar onset in GHR−/− and wild-type cells (Fig. 8A, which is published as supporting information on the PNAS web site). In addition, we did not observe an effect of GH treatment on wild-type cell differentiation (data not shown). Altogether, these observations suggest that GH signaling does not affect muscle cell differentiation.

Strikingly, 48 h after differentiation GHR−/− cells formed myotubes that were 25% smaller than wild type (Fig. 3A). To further assess the implication of GH in myotube size control, cells were induced to differentiate in the presence of GH, and myotube diameter was measured after 48 h. GH treatment increased the myotube size by 20% in wild-type cells and had no effect in GHR−/− cells. Myotube diameter was measured and is expressed as percentage change over +/+ cells transduced by β-galAdlox. Histograms are means ± SEM of four experiments on two independent cultures. *P < 0.005, vs. +/+ control.

GH Controls Cell Fusion. The reduction of myofiber size could be due to an impairment of myoblast cell fusion and/or a reduction of the cytoplasmic domain regulated by one myonucleus. Fusion consists of two distinct phases: myoblast/myoblast fusion to form nascent myotubes and subsequent myoblast/myotube fusion, resulting in a rapid accretion of size. The efficiency of the first phase can be evaluated by measuring the fusion index, which represents the proportion of the total cell population that has fused. After 48 h in DM, the fusion index did not differ as a function of the genotype or stimulation by GH (Fig. 4A), suggesting that GH signaling does not control nascent myotube formation.

To determine whether GH increased muscle cell size by recruiting new nuclei to existing myotubes, the number of nuclei per myotube was determined in wild-type and GHR−/− cultures. The nuclear number and the percentage of myotubes having five or more nuclei were diminished in GHR−/− myotubes as compared with wild type (Fig. 4A and Fig. 9A, which is published as supporting information on the PNAS web site). In addition, GH significantly increased the nuclear number in wild-type and not in GHR−/− cells. Of note, 48 h after differentiation the fusion process was completed, because the myotube nuclear number from both geno-
types did not vary at later time points (Fig. 8B). To rule out the possibility that the increased myonuclear number was due to an effect of GH on cell proliferation/survival in DM, the number of nuclei per square millimeter was assessed after 48 h in DM (Fig. 9B). No difference in the DNA content was observed between GHR−/− and wild-type cells treated or not with GH. These data, together with the lack of GH-induced proliferation, suggest a specific role of GH in enhancing cell fusion rather than controlling the number of nuclei available for fusion. To further examine the kinetics of GH-stimulated cell fusion, cells were treated with GH at different stages of differentiation. Cells were treated once at the onset of differentiation (time 0) or at 24 h, when cells begin to fuse and nascent myotubes appear (time 24). In both cases, myotube size was analyzed at 48 h in DM. GH did not have a hypertrophic effect when added at the onset of differentiation (Fig. 4C). However, when administered at 24 h, GH increased the size of myotubes by 25%. Administration of GH at 24 h alone was sufficient to stimulate growth as much as the daily administration at time 0 and 24 h (0 + 24). Thus, GH acts when nascent myotubes are already formed to allow an increase in muscle cell size by enhancing muscle cell fusion.

The calcineurin/NFATc2 pathway has been implicated in myotube cell size control by regulating the second phase of fusion in part through the up-regulation of IL4 gene expression (13). Because the timing of GH action on cell fusion parallels NFATc2 activation (18, 19), we investigated the implication of NFATc2 and IL4 in the GH hypertrophic action. NFATc2−/− and IL4−/− cells were differentiated in the presence of GH, and myotube size was measured after 48 h. As already reported, at the basal state NFATc2−/− and IL4−/− myotubes were smaller than wild type (Fig. 4D) (13). Strikingly, GH treatment had no effect on NFATc2−/− myotube size. The complete resistance of NFATc2−/− cells to the hypertrophic action of GH was not due to the lack of expression of GHR mRNAs in those cells as assessed by RT-PCR analysis (Fig. 4D).

Interestingly, the size of IL4−/− myotubes increased upon GH addition, indicating that this downstream target of NFATc2 is not involved in GH-induced cell fusion. These data suggest that NFATc2 activity is required for the growth-promoting action of GH. NFATc2 could be a direct target of the GHR signal transduction pathway or may belong to a parallel pathway that is permissive for cell fusion.

To investigate whether the reduced nuclear number of GHR−/− myotubes was also observed in mouse muscles, we counted the number of nuclei inside the sarcolemma of soleus and tibialis anterior transversal sections. A 21% decrease in myonuclear number was observed in both GHR−/− muscles as compared with wild type (Fig. 9C). These data suggest that the defect of muscle cell fusion contributes to the impaired muscle growth of GHR−/− mice.

**GH Action on Muscle Cell Fusion Is Independent of IGF-1.** The action of GH on muscle cell size could be mediated by IGF-1, a potent muscle growth factor whose expression is induced by GH in different experimental models. To discriminate between direct or IGF-1-mediated hypertrophic effects of GH, we first estimated the levels of IGF-1 mRNA in wild-type cells by semiquantitative RT-PCR during differentiation and GH stimulation. In the IGF-1 gene two distinct promoters drive IGF-1 transcripts. Class 1 transcripts contain exon 1 and are mainly expressed in skeletal muscle, whereas class 2 transcripts contain exon 2 and are expressed in both liver and muscle (5). Because both classes of transcripts may be regulated by GH (20), we analyzed the expression of IGF-1 mRNA isoforms containing exon 1, exon 2, or the common exons 3 and 4. As reported, the expression of all IGF-1 transcripts reached a peak 24 h after the onset of differentiation (Fig. 5A) (21). Importantly, GH stimulation did not further increase IGF-1 transcript levels at both differentiation time 24 and 48 h, indicating that the GH action on myotube hypertrophy does not correlate with IGF-1 levels. Shorter incubation times (1, 2, or 4 h) of wild-type cells with doses of GH ranging from 30 to 600 ng/ml also failed to affect IGF-1 expression (data not shown). This finding differs from what was reported in the C2C12 cell line, where GH increases the expression of IGF-1 (6, 7). The transcription factor Stat5 has been shown to
participate in the control of IGF-1 expression by GH (20, 22). In wild-type myotubes we failed to detect GH-induced phosphorylation of Stat5, and adrenoviral overexpression of a Stat5 dominant-negative protein (20) had no effect on the basal and GH-stimulated myotube size, indicating a marginal role of Stat5 in mediating GH action in our muscle cultures (data not shown). Moreover, we did not note any significant difference in IGF-1 levels between wild-type and GHR−/− cells (Fig. 5B). Finally, induction of GH expression in GHR−/− cells did not affect IGF-1 expression (Fig. 5B) but enhanced myotube size (Fig. 5B). Taken together, these data suggest that in our cellular system there is no regulation of IGF-1 expression by GH and no correlation between GH-regulated myotube size and IGF-1 levels.

If the reduced GHR−/− myotube growth was due to the absence of a secreted factor, such as IGF-1 or others, we would expect that the conditioned medium (CM) from wild-type cells would rescue GHR−/− myotube size. To test this possibility, CM were collected from wild-type or GHR−/− cells and added to GHR−/− cells during differentiation. After 48 h of differentiation the myotube size was analyzed and compared with GHR−/− cells in DM. The CM from untreated or GH-stimulated wild-type cells did not affect GHR−/− cell size (Fig. 5C). This finding suggests that GHR−/− cells are not defective for the production of a secreted factor controlling myotube growth. Of note, wild-type CM did not affect wild-type myotube size, whereas CM from cells treated with exogenous IGF-1 rescued GHR−/− myotube size and exerted a hypertrophic action on wild-type cells. These data indicate that this assay is scoring the lack or presence of secreted factors rather than small variations in their amount. To evaluate the activity of CM, we tested whether it was able to reverse the small size of NFATc2−/− cells, which results from their defect in producing IL4 (13). When NFATc2−/− cells were cultured in wild-type CM they formed large myotubes similar to wild-type cells. Importantly, CM from GHR−/− cells also rescued NFATc2−/− cell size, indicating that GHR−/− cells do not produce inhibitory factors precluding growth. In conclusion, CM rescues fusion of the NFATc2−/− cells but not the GHR−/− cells. These data do not support the defective production of a secreted factor in the control of GHR−/− cell size.

To study the relationship between the growth-promoting actions of GH and IGF-1, we analyzed whether their effects were additive or mediated by a common mechanism. IGF-1 stimulated an 20% increase in myotube diameter of wild-type cells, indicating that cells are responsive to an increase in the extracellular concentration of IGF-1 (Fig. 5D). The effect of IGF-1 in GHR−/− cells was comparable to the one in wild-type cells, demonstrating that mutant cells are not refractory to this growth stimulus. Strikingly, when wild-type cells were stimulated with the combination of GH and IGF-1, myotube size increased in an additive manner as compared with cells treated with GH or IGF-1 alone. Finally, to determine whether NFATc2 activity was required for IGF-1 hypertrophic action, NFATc2−/− cells were treated with IGF-1. Interestingly, IGF-1 induced a 20% increase in NFATc2−/− myotube size and myonuclear number comparable with that observed for wild-type cells, whereas GH had no effect (Fig. 5D and Fig. 10, which is published as supporting information on the PNAS web site). This finding suggests that GH and IGF-1 use different signaling pathways to induce fusion and growth. In conclusion, our data suggest that GH promotes a cell-autonomous increase in myotube cell size that occurs through a facilitation of cell fusion and is independent of local IGF-1 up-regulation.

Discussion

Here we show that GH acts as a muscle growth factor in a cell-autonomous manner. The myofiber atrophy observed in the GHR−/− mice is recapitulated in cultures, where the GHR deletion causes a sharp decrease in myotube diameter. This difference in size at the basal state suggests that GHR signaling is active during muscle differentiation, possibly because of the presence of GH in the serum-containing differentiation medium or to the autocrine production of GH (23). Treatment of wild-type cells with exogenous GH enhances myotube growth, whereas transient expression of GHR is sufficient to partially rescue the growth defect of GHR−/− cells. This rapid and efficient control of muscle growth by GH signaling is observed at a specific developmental stage, during the differentiation to multinucleated myotubes.

In contrast, GHR deletion has no effect on myoblast cell size. Moreover, the modulation of GHR signaling does not affect myoblast proliferation and differentiation. This lack of effect is not because of limiting expression of GHR in muscle cell cultures, as exogenous GH activates intracellular transduction elements and promotes myotube growth. In the literature it has not been conclusively established whether GH has a direct effect on proliferation and differentiation of satellite cells (24). Recently Kim et al. (8) measured BrdU-positive nuclei after GH treatment of mice and showed that GH induced cell proliferation in muscle. However, the nature of the proliferating cells was not precisely determined. Moreover, the mitogenic action of GH required IGF-1 receptor activity in differentiating muscle cells, because overexpression of a dominant-negative form of IGF-1 receptor under the muscle creatine kinase promoter abrogated this effect. Because the muscle creatine kinase promoter should not be active in proliferating satellite cells (25), one interpretation of the results is that the in vivo proliferative effect of GH is mediated by environmental factors rather than being intrinsic to satellite cells. Our study is consistent with this possibility, because in a pure population of myoblast cells under controlled extracellular milieu GH signaling does not promote proliferation.

GH increases the size of myotubes by acting on a specific feature of differentiated cells: cell fusion. At the onset of differentiation, a subset of mononucleated cells initially fuses to form nascent myotubes. Subsequently, additional cells fuse with the nascent myotube, and muscle growth occurs. In this article we demonstrate that GH acts at the later stage of muscle cell fusion when nascent myotubes are present. Several lines of evidence support this finding: (i) GH does not affect the fusion index, suggesting that GH does not act on the initial fusion of muscle cells; (ii) GH increases the number of nuclei per myotube; and (iii) GH exerts its hypertrophic effect when added at 24 h of differentiation, when nascent myotubes are present. Our data show that GH enhances myonuclear accretion in nascent myotubes, leading to muscle growth, and indicate that a defect in fusion contributes to the atrophy of GHR−/− muscles. The fusion process requires multiple steps involving cell motility, alignment, recognition, adhesion, and membrane fusion, all of which could potentially be regulated by GH (26). Thus, we have identified GH as one of the few ligands, in addition to prostaglandin F2α, and IL4, that have been shown to regulate myotube growth by enhancing the fusion of myoblasts to nascent myotubes (13, 19).

Pavlath and colleagues (13) demonstrated that the calcineurin/NFATc2 pathway regulates the second stage of cell fusion during myotube growth. NFATc2 belongs to a family of transcription factors that are activated by dephosphorylation upon the activation of a calcium-dependent phosphatase, calcineurin. Interestingly, we find that GH does not increase the size of NFATc2−/− cells, suggesting that NFATc2 acts downstream of or parallel to the GH signaling pathway. Consistent with the first possibility, many studies reported an increase of intracellular Ca2+ by GH in different cell types (27). In addition, the involvement of calcineurin in GH effects has been suggested in cardiomyocytes, where protection against apoptosis by GH is inhibited by a treatment with cyclosporin, an inhibitor of calcineurin (28). However, when cardiac hypertrophy was induced in vivo by GH and IGF-1 injection, NFAT activity was not altered in the heart (29). To gain more insights on a direct activation of NFATc2 pathway by GH, we transduced wild-type cells with a NFAT−luciferase reporter construct (29) and measured luciferase activity after GH stimulation. However, the effect of GH
on NFAT activity was low and scarcely reproducible (A.S., unpublished data), and further investigations are needed. Interestingly, IL4, a myoblast recruitment factor acting downstream of NFATc2, is not required for GHR action on fusion. We have shown that GHR action is not mediated by a secreted factor, because the CM of wild-type cells has no effect on GHR−/− myotube size. Furthermore, GHR stimulates IL4−/− myotube hypertrophy. Thus, GHR action on cell fusion depends on NFATc2 expression but is exerted through a mechanism different from secretion of IL4, suggesting that additional targets of NFATc2 may render muscle cells competent for fusion.

The use of mice lacking GHR and/or IGF-1 expression demonstrated that there must be independent and additive effects of both hormones on tissue growth (9). Here we suggest that the control of skeletal muscle cell fusion and size by GHR is not mediated by local IGF-1 up-regulation. This conclusion is supported by several data. We have shown that the change in size of myotubes in response to GHR activation is not correlated with changes in IGF-1 gene expression. That IGF-1 does not contribute to the GHR action on fusion is also indicated by functional data. First, the secreted factors in the CM of wild-type cells do not rescue the atrophy of GHR−/− myotubes, whereas CM from IGF-1-treated wild-type cells does. Second, the additive effect of GHR and IGF-1 on cell size argues for independent actions of both hormones. Furthermore, NFATc2 activity is required for GHR but not IGF-1 hypertrophic action, suggesting that the GHR and IGF-1 rely on different signaling pathways to facilitate fusion. Our study unraveled a role of GHR on myotube size control, i.e., the specific control of muscle cell fusion that participates in muscle growth. This action may be exploited in therapies against muscle disorders, such as Duchenne muscular dystrophy. The success of stem cell transfer therapies will rely on the efficient recruitment of stem cells to striated muscles, an event mediated by NFAT factors (30). Activation of GHR signaling by promoting the NFAT-dependent fusion of myoblasts with growing myotubes may improve these protocols.

Materials and Methods

Animals and Histology. Wild-type and GHR−/− littermates were bred from heterozygous mice of the genetic background Sv129Ola, and offspring were genotyped as described (16). Mice were handled in accordance with institutional animal care policies. Histological immunostaining were performed as described in Supporting Text, which is published as supporting information on the PNAS web site.

Cell Cultures, Proliferation, and Differentiation Assays. Primary cultures were derived from gastrocnemius and tibialis anterior muscles of 4-week-old mice as described (ref. 15 and Supporting Text). BrdU incorporation and FACS analysis were performed as described in Supporting Text.

To differentiate muscle cells, 20,000 myoblasts per square centimeter were plated on Matrigel-coated dishes in DMEM/Ham F12/2% horse serum (DM). To measure the hypertrophic response, cells were incubated with 600 ng/ml hGH and/or 250 ng/ml IGF-1-R3 (Sigma) in DM. Hormones were added daily. After 48 h, the diameter of at least 400 myotubes was measured. The nuclear number was determined after staining with Hoechst 33258. The fusion index was determined by dividing the number of nuclei within multinucleated myotubes by the total number of nuclei analyzed.

To evaluate the effect of CM, culture medium was collected from muscle cells after 24 and 48 h in DM with or without 250 ng/ml IGF-1-R3 or 600 ng/ml hGH. Myoblasts were seeded in CM, which was replaced after 24 h. Myotube diameter was analyzed after 48 h.

Viral Vectors and Infection. Adenovirus vectors containing either β-galactosidase (β-galAdlox) or the murine GHR cDNA (GHRA-dlox) were used (31). For the infection, myoblasts were infected with 30, 100, or 300 multiplicities of infection in DM for 1.5 h. After washing, cells were incubated in DM and analyzed 48 h later.

Immunoprecipitation and Immunoblotting. Cell lysates were prepared as described (15). For immunoprecipitation studies, cells were starved overnight and stimulated with 300 ng/ml hGH for 5 min. After cell lysis, 500 μg of total protein extract was incubated overnight with protein A Sepharose and anti-Jak2 antibodies (Upstate Biotechnology, Lake Placid, NY). Protein extracts were analyzed by immunoblotting by using the indicated primary antibodies (Supporting Text).

RNA Extraction and RT-PCR. RNA was isolated by using Trizol Reagent (Life Technologies). Reverse transcription was performed from 2 μg of total RNA with Moloney murine leukemia virus reverse transcriptase (Life Technologies) and poly dT primers (Amersham Pharmacia). Amplifications were done specific primers as described in Supporting Text.

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