Insulin-like Growth Factor-I Extends in Vitro Replicative Life Span of Skeletal Muscle Satellite Cells by Enhancing G1/S Cell Cycle Progression via the Activation of Phosphatidylinositol 3'-Kinase/Akt Signaling Pathway*

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Interest is growing in methods to extend replicative life span of non-immortalized stem cells. Using the insulin-like growth factor I (IGF-I) transgenic mouse in which the IGF-I transgene is expressed during skeletal muscle development and maturation prior to isolation and during culture of satellite cells (the myogenic stem cells of mature skeletal muscle fibers) as a model system, we elucidated the underlying molecular mechanisms of IGF-I-mediated enhancement of proliferative potential of these cells. Satellite cells from IGF-I transgenic muscles achieved at least five additional population doublings above the maximum that was attained by wild type satellite cells. This IGF-I-induced increase in proliferative potential was mediated via activation of the phosphatidylinositol 3'-kinase/Akt pathway, independent of mitogen-activated protein kinase activity, facilitating G1/S cell cycle progression via a down-regulation of p27Kip1. Adenovirally mediated ectopic overexpression of p27Kip1 in exponentially growing IGF-I transgenic satellite cells reversed the increase in cyclin E-cdk2 kinase activity, pRb phosphorylation, and cyclin A protein abundance, thereby implicating an important role for p27Kip1 in promoting satellite cell senescence. These observations provide a more complete dissection of molecular events by which increased local expression of a growth factor in mature skeletal muscle fibers extends replicative life span of primary stem cells than previously known.

Repair or growth of skeletal muscle requires satellite cells for new myonuclei because myonuclei are postmitotic (1). Satellite cells are a population of mononuclear myogenic precursor cells wedged between the basal lamina and muscle fiber sarcolemma (2). Satellite cells differ from immortalized myogenic cell lines in that, like other normal diploid cells, they have a limited capacity to divide, and after a finite number of cell divisions enter a state of irreversible growth arrest, termed replicative senescence (3). Indeed, proliferative potential of these cells has been shown to decrease in muscular dystrophies that are characterized by multiple rounds of regeneration (4), ultimately resulting in their cellular senescence. Therefore, when satellite cells exhaust their finite proliferative reserves, the effectiveness of regeneration, hypertrophy, and myoblast-mediated gene therapy for the reconstruction of muscle is constrained.

Given the previously documented effects of insulin-like growth factor-I (IGF-I)1 to stimulate myoblast proliferation, myogenic differentiation, and myotube hypertrophy in cultured immortalized myogenic cell lines (5), and its ability to induce skeletal muscle hypertrophy in both young and old rodents (6–9), we asked if IGF-I might enhance the proliferative potential of the resident satellite cells within animal skeletal muscles. Such understanding, which is presently lacking in non-immortalized primary satellite cells, could be used to counteract many intractable muscle-wasting conditions.

A mouse lacking the igf-1 gene in a liver-specific manner demonstrated that IGF-I's action on growth and development is largely autocrine/paracrine (10). Therefore, a transgenic mouse expressing an IGF-I transgene driven by a skeletal α-actin promoter was selected (6) to allow the local overexpression of IGF-I in skeletal muscles in order to mimic its autocrine effect in the resident satellite cells. We hypothesized that satellite cells from the IGF-I transgenic (IGF-I Tg) skeletal muscles exposed to continuous high levels of IGF-I might possess a prolonged ability to proliferate in culture, reflecting an increased proliferative potential, relative to those cells isolated from their FVB wild type (WT) littermates.

We next asked how the IGF-I Tg satellite cells were able to have an enhanced replicative life span, when the corresponding WT satellite cells were growth-arrested? Based upon studies in other cell types (11, 12), it seemed likely that this growth arrest in WT satellite cells might be at the G1/S boundary of the cell cycle, a nodal point suggested as one of the biochemical hallmark.

1 The abbreviations used are: IGF-I, insulin-like growth factor I; PI 3-kinase, phosphatidylinositol 3-kinase; BrdUrd, bromodeoxyuridine; PD, population doubling; FACS, fluorescence-activated cell sorter; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; WT, wild type; MEK, mitogen-activated extracellular signal-regulate kinase.
mark of replicative senescence. While senescent cells are refractory to mitogens and are terminally non-dividing even under the most optimal growth conditions, quiescent cells can be initiated to induce DNA synthesis by mitogenic stimulation or subcultivation (13). Recent findings have demonstrated an accumulation of cell cycle inhibitors such as p16Ink4a, p21Cip1/Waf1, and p27Kip1 (14, 15) in various senescent cell types, further implicating a defect in G1/S transition in cellular senescence. Since IGFs act as progression factors stimulating progression from G1 to S phase of the cell cycle in other cell types (5), we reasoned that IGF-I might extend the in vitro replicative life span of satellite cells by modulating cell cycle regulatory molecules at the G1/S boundary. IGF-I exerts its pleiotropic effects by binding to the type I IGF receptor (IGF-IR) (16), which leads to the activation of both PI 3’-kinase and MAPK pathways (for review, see Ref. 17), in turn producing an increase in cellular proliferation in immortalized myogenic cell lines (18, 19) and other tumor cell lines (20). Therefore, we hypothesized that the IGF-I enhanced enhancement of cell cycle progression via modulation of G1/S regulatory molecules in the IGF-I Tg satellite cells might be signaled by either one, or both, of these pathways.

Here we report that IGF-I Tg satellite cells have an enhanced in vitro replicative life span, and continued to maintain cell cycle progression via activation of the PI 3’-kinase/Akt pathway, which in turn mediated the down-regulation of p27Kip1 even in late-passage (the point at which WT cells were senesced). Adenovirally mediated ectopic overexpression of p27Kip1 in growing late-passage IGF-I Tg satellite cells induced a G1 arrest, and mimicked the biochemical events seen in senescent late-passage WT satellite cells, indicating that p27Kip1 has a critical role in the regulation of satellite cell senescence.

**EXPERIMENTAL PROCEDURES**

**Materials**—The IGF-I analog, long R3-IGF-I (LR3) was purchased from GroPep Ltd., Adelaide, Australia. PI 3’-kinase inhibitor (LY294002), MEK inhibitor (PD98059), bromodeoxyuridine (Brdu), and goat polyclonal anti-IGF-I receptor antibody, were all obtained from Sigma. Fluorescein isothiocyanate-conjugated anti-Brdu monoclonal antibody was purchased from Caltag (Burlington, CA), and Hoechst 33342 and propidium iodide were from Molecular Probes (Eugene, OR). Cell culture media and reagents were purchased from Life Technologies, Inc. (Rockville, MD). The human p27Kip1 adenoviral construct was a generous gift from Dr. Perry Nisen, Abbott Laboratories (Chicago, IL). Myogenic cells secreting anti-desmin (clone D3) and anti-sarcomeric myosin antibody (clone MF20) were obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA. Animals—FVB WT mice (aged ~1 month) and their littermates harboring a single copy of a skeletal a-actin/hIGF-I hybrid transgene (IGF-I Tg), which express IGF-I exclusively in striated muscle have been previously described (6). Animals were housed in pathogen-free facilities and fed ad libitum. Protocols were approved by the Institutional Animal Care and Use Committees.

**Satellite Cell Isolation and Culture**—Immediately after the animals were euthanized by cervical dislocation, gastrocnemius muscles from both hindlimbs were removed, trimmed of excess connective tissue and fat, and weighed. Muscles from 5–6 animals from each of the two groups were pooled to provide sufficient tissue for satellite cell isolation; the methods for their isolation have been described in detail previously (9). These cells were cultivated on collagen-coated tissue culture plates using high serum conditions (Ham’s F-10 nutrient mixture containing 20% fetal bovine serum, 1% chicken embryo extract, with 1% penicillin/streptomycin antibiotic mixture, and 1% l-glutamine) to minimize any loss of proliferative cells due to either apoptosis or terminal differentiation. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2, and were passaged before they reached confluence. Both IGF-I Tg and WT satellite cell cultures at late-passage conditions were monitored for myogenic purity, apoptosis, and terminal differentiation using previously described methods by staining these cells with anti-desmin antibody (9, 21), Hoeschst 33342 (9, 22), and anti-sarcomeric myosin antibody (9), respectively.

**Determination of Cumulative Population Doublings**—Cumulative number of population doublings obtained over the course of the in vitro culture life span of WT and IGF-I Tg satellite cells was determined as described previously (9, 22). At the time of cell isolation from the animal, cell populations from both groups were considered to be at 1 population doubling (PD). The number of PDs at every passage thereafter was calculated as 1 + Log(N2/N1), where N is the number of cells harvested divided by the number of cells inoculated. Cell number was determined in triplicate using a Coulter counter. Cultures were deemed to have reached the end of their in vitro life span when they failed to reach subconfluence after 3 weeks of refeeding, at which point they were reassessed again with MF20 and Hoescht 33342 to ensure the loss of the ability to differentiate or proliferate. Lack of proliferation was also assessed by BrdUrd labeling (see below).

** Colony Size Distribution Assays**—Non-confluent late-passage WT and IGF-I Tg satellite cells were trypsinized and counted using a hemacytometer to calculate cell number. About 100 cells from each group were inoculated into each of several collagen-coated 100-mm tissue culture dishes, and left for ~7 weeks at 37°C, 5% CO2 atmosphere in serum-rich proliferation medium. Fresh growth medium was given to the cells weekly. Our previous protocol was then followed (9). Each of the groups was cloned in triplicate and the colony sizes attained were averaged. A total of three such separate experiments were done for each of the two groups. Parallel set of cultures at the end of the 7-week growth period were stained with anti-sarcomeric myosin and anti-desmin as described above to ensure that the cells constituting the colonies were undifferentiated and myogenic.

**Cell Synchronization and BrdUrd Labeling Studies**—Late-passage (day 76 in culture) satellite cells from the IGF-I Tg and WT groups were established overnight in proliferation media at 1 × 105 and 1 × 106 cells per 100-mm tissue culture dish, respectively. The cells were washed in Dulbecco’s modified Eagle’s medium and then maintained in serum-free media (Dulbecco’s modified Eagle’s medium with 1% penicillin-streptomycin) for 48 h to arrest any cycling cells at G0. A set of plates from each group was processed for subsequent analysis immediately at the end of the 48-h period (time 0) or after serum stimulation for an additional 6, 12, or 24 h. During the last 60 min of each incubation period, the cultures were pulsed with 10 µM BrdUrd. The cells were then analyzed by flow cytometry (see below) to determine the percentage of BrdUrd-positive cells (labeling index). To further document that the lack of proliferation seen in the late-passaged WT cells was due to an irreversible growth arrest, early passage (day 23 in culture) and late-passage (day 76 in culture) IGF-I Tg and WT cells were treated for 48 h with the long R3-IGF-I (LR3) in serum-free media. The response to LR3 was measured by determining the BrdUrd labeling index after cultures were pulsed with 10 µM BrdUrd during the last 24 h of the LR3 incubation. Percent of nuclei labeled with BrdUrd was quantitated as described below.

**Flow Cytometric Analysis of Cell Cycle Distribution**—Late-passage WT and IGF-I Tg satellite cells from either synchronized or asynchronous cultures were pulse-labeled with 10 µM BrdUrd for 60 min at 37°C, 5% CO2. Cells were then harvested by trypsinization, and pre pared for fluorescence-activated cell sorting (FACS) analysis (described previously (24, 25)). BrdUrd-labeled nuclei were detected using fluorescein isothiocyanate-conjugated anti-BrdUrd monoclonal antibody (1 µg/ml) and counterstained with propidium iodide (20 µg/ml). Stained nuclei were analyzed in a FACS Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). DNA histograms were modeled using the ModFit-LT software (Verity, Topsham, ME).

**Adenoviral Infection with p27Kip1**—Replication-defective adenovirus directing expression of human p27Kip1 (Adp27) under the transcriptional control of the cytomegalovirus promoter have been previously described (26, 27). Exponentially growing late-passage cultures of IGF-I Tg cells were cultured in proliferation media to ~50% confluence, and then incubated with the indicated amounts of adenovirus for 48 h (27). Cells were then washed with phosphate-buffered saline and processed for either FACS analysis for cell cycle distribution as detailed above, or harvested for the preparation of whole cell extracts. For mock infection, cells were infected with control adenovirus with empty vector.

**Immunoblot and Immunoprecipitation Analyses**—Whole cell extracts of late-passage IGF-I Tg and WT satellite cells were prepared by lysing cells in the lysis buffer, pH 7.5, 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 200 mM NaF, 20 mM sodium pyrophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 200 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) on ice for 30 min. Protein yield was quantified by Bio-Rad DC protein assay kit (Bio-Rad). Equal amounts of total protein were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with appropriate
antibodies. The antibodies against p27Kip1 (60-445), cdk2 (60-505), cdk4 (60-139), cyclin E (60-459), cyclin A (60-138), cyclin D1 (5D4), p85 subunit of PI 3-kinase (60-195), phosphotyrosine (4G10), and p21/Cip1/Waf1 (06-139) were purchased from Upstate Biotechnology, Lake Placid, NY. Antibody kits to detect phosphorylated Erk/Erk2 (9100) and Ser473-Akt (9270) were obtained from New England Biolabs, Beverly, MA. Antibodies against pRb (G5-245) and p53 (E-17) were acquired from Pharmingen (San Diego, CA) and Santa Cruz (Santa Cruz, CA), respectively. Bound antibodies were detected using horse-radish peroxidase-linked sheep anti-mouse or donkey anti-rabbit antibodies (Amer sham Pharmacia Biotech), and visualized by enhanced chemiluminescence (PerkinElmer Life Sciences) after exposure to x-ray film (Kodak X-Omat AR).

For immunoprecipitations, 500–1000 μg of total protein were incubated with antibodies to cyclin E, cyclin A, cyclin D1, cdk2, or p85 subunit of PI 3-kinase at 4 °C for 2 h, followed by 1 h incubation with protein A-agarose (Amer sham Pharmacia Biotech) at 4 °C. After washing in lysis buffer, the immunocomplexes were resolved by SDS-PAGE and transferred to nitrocellulose for subsequent immunoblot analysis.

Measurement of Cyclin-associated cdk Activity—Cyclin E- and cyclin-A associated kinase activities on the respective immunoprecipitates were performed using histone H1 as a substrate, as described previously (12, 24). Reaction mixtures were stopped with 2 times Laemmli sample buffer and the phosphorylated histone H1 resolved on 10% SDS-PAGE gels. The gels were subsequently dried and exposed to x-ray films. In another set of experiments, the kinase reaction was stopped by spotting 25 μl of the samples onto P81 phosphocellulose squares (Upstate Biotechnology) followed by extensive washing in 1% phosphoric acid. The filters were then dried in acetone, and counted in a β-counter with scintillation fluid.

Measurement of Phosphatidylinositol 3'-Kinase Activity—Using previously described procedures (19), PI 3-kinase activity in anti-phosphotyrosine and anti-p85 immunoprecipitates of late-passage IGF-I Tg and WT satellite cells was measured using phosphatidylinositol as a substrate. The products of the kinase reaction were spotted onto Silica Gel 60 plates coated with 1% potassium oxalate, and resolved by thin-layer chromatography in chloroform/methanol/water/ammonium hydroxide (60:47:11.3:2). The phosphorylated lipid products were then visualized by autoradiography.

Measurement of MAPK/ERK Activity—MAP kinase activity in whole satellite cell extracts was determined using myelin basic protein as a substrate provided as part of the MAP kinase assay kit (Upstate Biotechnology), following the manufacturer’s protocol. 25 μl of the reaction mixtures were spotted onto P81 phosphocellulose squares, and the radioactivity incorporated in the filters was determined by liquid scintillation counting.

RESULTS

IGF-I Overexpression Enhances the In Vitro Replicative Life Span of Satellite Cells Isolated from the Hypertrophied Gastrocnemius Muscles of IGF-I Tg Mice—The gastrocnemius muscles of 1-month-old IGF-I Tg mice were significantly larger (22% increase, p < 0.05) than their WT littermates. Resident satellite cells from the IGF-I Tg muscles secreted more hIGF-I peptide into the media (114.9 ± 4.5 ng/ml) compared with WT satellite cells (values were below detection limits of the assay). Overexpression of the IGF-I transgene did not affect endogenous mouse IGF-I peptide levels in the satellite cells from either of the two groups, consistent with our previous observations in whole muscle (28). IGF-I Tg satellite cells started to proliferate earlier (4 days) in culture and proliferated in greater numbers than those from WT muscles (lag period of ~10 days before they started to double) (Fig. 1A). Both populations underwent a period of rapid proliferation, after which the growth rate slowed, and finally ceased in WT cells after approximately 76 days in culture. Satellite cells from the IGF-I Tg group had a significantly greater proliferative capacity than the WT group, as reflected by the 119% higher (p < 0.0001) cumulative population doublings attained by the IGF-I Tg satellite cells (11.43 ± 2.1) compared with WT (5.21 ± 1.6) cells, after 76 days in culture. Thus, IGF-I Tg satellite cells achieved at least five additional rounds of divisions above the maximum that was attained by WT satellite cells. The population of satellite cells from both IGF-I Tg and WT groups at this 76-day point are henceforth referred to as the “late-passage” satellite cells, which were then used for all subsequent analyses described below. Analysis of these cultures with anti-desmin antibody routinely revealed >95% desmin-positive cells, hence ensuring that these cultures at late passages continued to be enriched in muscle-specific cells.

Late-passage satellite cells from the IGF-I Tg group showed a greater preponderance to form large-sized colonies after 7 weeks in proliferation media, compared with corresponding cells from the WT group (Fig. 1B, upper panel). Quantitation of these colony sizes (Fig. 1B, lower panel) demonstrated a dramatic shift to the right (i.e. colonies with >256 cells or >8 cell doublings) in IGF-I Tg satellite cells, thereby indicating a greater proliferative potential of individual cells, compared with the WT group. Profiles with ≥8 cell doublings accounted for 20–25% of the total 200–250 colonies in satellite cells from the IGF-I Tg group, in contrast to <2% for the WT group.

Long-R3 IGF-I (LR3), a synthetic analog of IGF-I is more biologically potent than unmodified IGF-I as it has low affinity for IGF-I-binding proteins (18). Addition of LR3 at 100 ng/ml resulted in 27 and 29% incorporation of the BrdUrd label in satellite cells from WT and IGF-I Tg muscles, respectively, in early passages (day 23 in culture) (Fig. 1C). The percent of labeled nuclei at all the other doses were also comparable in both WT and IGF-I Tg satellite cells in early-passage. However, by late-passage (day 76 in culture) (Fig. 1C), BrdUrd incorporation dramatically decreased to <5% in WT cells, whereas it was maintained at 26% in the IGF-I Tg cells when stimulated by 100 ng/ml LR3, thereby confirming that WT satellite cells in late-passage had exhausted their proliferative reserves. Even without any added LR3 (Fig. 1C), the percentage of BrdUrd-labeled nuclei in late-passage IGF-I Tg satellite cells remained at levels similar to that seen in early-passage WT and IGF-I Tg cells (~12.5%), validating that satellite cells overexpressing IGF-I possessed a higher proliferative capacity, compared with WT cells. The dramatic increase in proliferation potential seen in IGF-I Tg satellite cells was due to the growth-promoting effects of IGF-I since cell proliferation (measured with BrdUrd incorporation) was reduced by ~65% when these late-passage IGF-I Tg cells were cultivated in the presence of 1 μg/ml IGF-I-receptor antibody for 48 h, relative to control cultures grown without the blocking antibody (data not shown). Of note, <3% of late-passage IGF-I Tg and WT satellite cells demonstrated sarcomeric myosin and Hoescht 33342 positive cells when cultivated in high serum conditions, indicating that the growth arrest seen in WT cells was likely not due to terminal differentiation or apoptosis, respectively. Furthermore, IGF-I Tg satellite cells from 1-month-old mice were unlikely to be immortalized secondary to continuous IGF-I overexpression, because satellite cells cultured from 18-month-old IGF-I Tg muscles (i.e. these cells were exposed to high levels of IGF-I for 18 continuous months) had lesser proliferation capacity than their WT littermates, and failed to respond to exogenously added LR3-IGF-I.²

² M. V. Chakravarthy and F. W. Booth, manuscript in preparation.
IGF-I Enhances G1/S Progression in Satellite Cells

Analyzing asynchronous populations of late-passage WT satellite cells (Fig. 2B), revealed the characteristic profiles (29–31) expected of G1/S arrested cells (lack of phosphorylated pRb) and decrease in cyclin A protein levels, whereas the corresponding IGF-I Tg cells revealed a pattern more consistent with that of cycling cells (hyperphosphorylated pRb and increase in cyclin A protein). In contrast, there was an insignificant difference in protein levels of the early-mid G1 cyclins (cyclin D1 and E), and cdk2 (Fig. 2B), consistent with an interpretation (32) that WT cells were likely arrested in the G1 phase of the cell cycle. Furthermore, hypophosphorylation of pRb and the dramatic decrease in cyclin A protein levels in late-passage WT cells supports a potential arrest at the R-point in these cells since phosphorylation of pRb is required for passage through this point (12, 31), and consequently for synthesis of late-G1 genes (29).

To confirm that the G1/S arrest was due to cellular senescence, and not an extended state of quiescence, we analyzed the same cell cycle regulatory molecules after defined intervals following release from serum starvation in late-passage WT and IGF-I Tg cells, since it has been previously shown that senescent cells are refractory to further mitogenic stimulation (13). Mean generation time for the IGF-I Tg cultures were determined to be between 24 and 29 h, and hence the time intervals chosen approximated early-mid G1 (6 h), late G1/S (12 h), and G2/M (24 h) phases of the cell cycle (12). Approximately 80–85% of late-passage WT satellite cells remained in G0/G1 throughout 24 h of serum stimulation (Fig. 2C, upper panel), whereas the IGF-I Tg cells showed a time dependent decrease in the percentage of G0/G1 cells, and a corresponding increase in the percentage of S phase cells (<10% at time 0 to ~35% at the end of 24 h) with serum stimulation, indicating cell cycle progression. The failure of late-passage WT satellite cells to be recruited into S-phase confirms a G1 cell cycle arrest secondary to replicative senescence, and is supported by the following. (i) An unchanged predominance of the unphosphorylated form of pRb, before (lane 1, Fig. 2C, lower panel) and after 24 h of serum stimulation (lane 4 versus IGF-I Tg cells in lane 8, Fig. 2C, lower panel). (ii) Cyclin A levels were extremely low and indistinguishable from amounts seen in unstimulated IGF-I Tg cells (lane 1 versus lane 5, Fig. 2C, lower panel). Serum stimulation resulted in no change in cyclin A in late-passage WT satellite cells (lane 1 versus lane 5, Fig. 2C, lower panel). Serum stimulation caused a 15-fold increase in cyclin A by G2 phase (24 h) (lane 5 versus lane 8). (iii) In marked contrast, there were high constitutive abundance of both cyclin D1 and cyclin E proteins in unstimulated (lane 1) WT cells (15–20-fold increase relative to controls). B, colony size distribution of late-passage IGF-I Tg and WT satellite cells (i.e. cells from 76-day cultures from Fig. 1A). Satellite cells from each group were seeded at clonal density and grown in proliferation media for 7 weeks. A representative set of such plates after staining with 0.5% crystal violet is shown in the upper panel. Colony size was then estimated by counting the number of cells per colony for up to 256 colonies from each group. The percent of total colonies that were able to attain a specified size, denoted by the number of cell doublings attained in culture over the 7-week growth period is shown in the lower panel. A total of 200–250 colonies were scored for each distribution and the results are representative of three separate experiments. C, late-passage WT satellite cells were unable to be further stimulated with exogenous IGF-I-LR3. Early-passage (day 23 in culture) and late-passage (day 76 in culture) satellite cell cultures from IGF-I Tg and WT muscles were treated with the indicated concentrations of LR3 for 48 h, following which the labeling index was determined by scoring the percentage of BrdUrd-positive nuclei. Results are mean ± S.E. of three independent experiments in triplicate.
Serum stimulation resulted in only a modest further accumulation of these cyclins (15–20% for cyclin D1 and 10% for cyclin E in WT cells (lanes 2–4)), whereas serum stimulation of IGF-I Tg cells resulted in a dramatic accumulation (10-fold) of cyclin D1 by mid-G1 phase (6 h) and of cyclin E (5–7-fold increase) by mid- to late-G1 phase (12 h) (Fig. 2C, lower panel, lanes 6 and 7). (iv) The abundance of total cdk2 protein remained unchanged with serum stimulation and was similar in both WT and IGF-I Tg satellite cells. The above molecular events delineated in WT satellite cells are consistent with earlier findings reported in serum-stimulated senescent human fibroblasts (12, 33). Hence, the ability of IGF-I Tg satellite cells to continue doubling even in late-passages suggest that IGF-I may help overcome this G1/S arrest to subsequently enhance their in vitro replicative life span.

Fig. 2. Late-passage wild type satellite cells are arrested in the G1 phase of the cell cycle. A, flow cytometry analysis of cell cycle distribution. Asynchronous populations of late-passage (day 76 in culture) satellite cells from IGF-I Tg and WT groups were pulse-labeled with 10 μM BrdUrd for 1 h. BrdUrd incorporation was analyzed by fluorescein-conjugated anti-BrdUrd antibody and DNA content was measured by propidium iodide staining. The percentage of cells in S phase (upper boxes), G0/G1 phase (lower-left boxes), and G2/M phase (lower-right boxes) are as indicated. Results are a representative set from four separate experiments. B, Western blot analysis of G1/S cell cycle regulatory molecules in late-passage asynchronous WT and IGF-I Tg satellite cells. 35 μg of total protein was resolved by SDS-PAGE and immunoblotted with the indicated antibodies. The antibody used against retinoblastoma protein detects both the phosphorylated (slower-migrating band; ppRb) and unphosphorylated (faster-migrating band; pRb) forms. For normalization, the resolved proteins were subsequently probed with anti-actin antibody that recognizes all isoforms from skeletal, cardiac, and smooth muscle actin. Representative blots of three independent experiments are shown. C, failure of cell cycle progression upon release from serum starvation in late-passage WT satellite cells. After 48 h of serum starvation, late-passage WT and IGF-I Tg satellite cell cultures were stimulated with serum-rich media for the indicated times, at the end of which, the cells were prepared for FACS analysis for cell cycle distribution (upper panel) or were analyzed by Western blotting with the indicated antibodies (lower panel). The cell cycle profiles (percentages) are means of three separate experiments performed in duplicate. Each lane contains 35 μg of total protein from the whole cell lysates of the late-passage WT and IGF-I Tg satellite cells. Representative blots from three independent experiments are shown.
G1 Cyclin-associated Kinase Activities Are Decreased in Primary Cultures of Late-passage WT Satellite Cells—To understand in greater detail the regulatory events contributing to the G1/S arrest observed in late-passage WT satellite cells, we analyzed cdk2 as well as cyclin E- and cyclin A-associated kinase activities. These kinase activities were reduced 8–12-fold in late-passage asynchronous WT satellite cells, compared with IGF-I Tg cells (Fig. 3A). High levels of cyclin E protein in late-passage WT satellite cells (Fig. 2C, lower panel, lanes 1–4) were not coordinated with increased cyclin E-associated kinase activity up to 24 h (Fig. 3B, upper panel), or 72 h after serum stimulation (data not shown for 48–72 h). WT cells also lacked cyclin A-associated kinase activity (Fig. 3B, lower panel), consistent with the lack of cyclin A protein in these cells (Fig. 2C, lower panel, lanes 1–4). In contrast, cyclin E-associated kinase activity in IGF-I Tg satellite cells (Fig. 3B, upper panel) paralleled the maximum increase seen in cyclin E protein after 12 h of serum stimulation (Fig. 2C, lower panel, lane 7). Cyclin A-associated kinase activity on the other hand peaked at 24 h after serum stimulation (when cells have progressed past S phase), and correlated with the maximum accumulation of cyclin A protein as shown in Fig. 2C (lower panel, lane 8). Furthermore, the observed kinetics of the activation of cyclin E-associated kinase correlated well with the kinetics of pRb phosphorylation (compare Fig. 2C, lower panel, lane 7, with Fig. 3B, upper panel, at the 12-h time point). Taken together, these results suggest that IGF-I overexpression likely maintains cyclin E/cdk2 kinase activity to promote the phosphorylation of pRb, and consequently cell cycle progression in the late-passage IGF-I Tg satellite cells. Conversely, the lack of these kinase activities in late-passage WT satellite cells could impair the ability of these cells to pass the R-point, leading to the observed G1/S arrest. Thus, cyclin E/cdk2 kinase activity may be a key regulatory mechanism contributing to cell cycle transit beyond the R-point in satellite cells.

Up-regulation of p27^Kip1 and Increased Association of the Inhibitor with cdk2 in Late-passage WT Satellite Cells—Similar amounts of cyclin E-cdk2 complexes were observed in the late-passage WT and IGF-I Tg satellite cells (Fig. 4A, upper panel). However, the kinase activities of these same immunocomplexes were 8–12-fold lower in the WT cells, relative to that of the IGF-I Tg cells (Fig. 3A, middle panel). Hence, the low cyclin E-associated kinase activity observed in late-passage WT satellite cells is due to mechanisms other than a failure of cyclin E to bind to its kinase partner. In contrast, cyclin A immunoprecipitates exhibited a 5–10-fold decrease in the ability of cyclin A to complex with cdk2 in late-passage WT satellite cells (Fig. 4A, middle panel), relative to IGF-I Tg cells, and the same immunoprecipitates also exhibited a markedly decreased cyclin A-associated kinase activity (Fig. 3A, lower panel). Thus, the decrease cyclin A-cdk2 association, in part, may explain the decreased cyclin A-associated kinase activity seen in the late-passage WT cells. Association between cyclin D1 and cdk4 also was decreased to similar levels as cyclin A-cdk2 (5–10-fold) in late-passage WT satellite cells relative to the corresponding IGF-I Tg cells (Fig. 4A, lower panel).

Activities of the cdk5s can be regulated in part via cdk inhibitors (34). When the protein abundance of p27^Kip1, p21^Cip1/Waf1, and p57^Kip2, all of which are known to play crucial roles in G1/S transition (35), were analyzed in asynchronous populations of late-passage WT and IGF-I Tg satellite cells, only the level of p27^Kip1 protein (Fig. 4B, upper panel) was affected (3–4-fold decrease) by IGF-I overexpression, consistent with previous reports (15) seen with other growth factors. On the other hand, protein levels of p21^Cip1/Waf1 and p57^Kip2 (members of Waf/Kip family) remained unchanged in late-passage IGF-I Tg satellite cells (Fig. 4B, middle and lower panels), relative to WT cells. This was in marked contrast to the high levels of p21^Cip1/Waf1 that was reported previously in senescent human fibroblasts (14). Therefore, these results suggest that the cdk inhibitor

![Fig. 3](Image) Enhanced histone H1 kinase activity in late-passage IGF-I Tg satellite cells. A, histone H1 kinase activity of anti-cdk2, anti-cyclin E, and anti-cyclin A immunoprecipitates (IP) from aliquots of the same asynchronous, late-passage IGF-I Tg and WT satellite cell extracts used for Fig. 2B. 500 μg of total protein was immunoprecipitated with the indicated antibodies, and the kinase assay was performed using histone H1 as the substrate (lanes 3 and 4), or by omitting the substrate (S) (lane 2) to determine background phosphorylation, as well as with a species-specific immunoglobulin (lg) for control (lane 1). The products of the kinase reaction were resolved on 10% SDS-PAGE gels and autoradiographed to visualize the 32P-labeled histone H1. A representative set from one of the triplicate experiments is presented. B, histone H1 kinase activity of anti-cyclin E (upper panel) and anti-cyclin A (lower panel) immunoprecipitates from aliquots of the serum-stimulated, late-passage IGF-I Tg and WT satellite cells extracts used in Fig. 2C. Results are presented as the radioactivity (cpm) incorporated into the substrate after correcting for background, and is representative of three independent experiments.
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FIG. 4. Association of G1 cyclins and cdk-inhibitors with cdk2 in late-passage WT and IGF-I Tg satellite cells. A, cyclin-cdk complexes in WT and IGF-I Tg satellite cells. 350 μg of total protein from late-passage asynchronous cultures used in Fig. 2B were first immunoprecipitated with anti-cyclin E (upper panel), anti-cyclin A (middle panel), or anti-cyclin D1 (lower panel). The immunocomplexes were then resolved by SDS-PAGE gels and immunoblotted with antibodies against cdk2 or cdk4, as indicated. Representative blots from three independent experiments are presented. B, up-regulation of cdk-inhibitor p27Kip1 in total extracts of late-passage WT satellite cells. 35 μg of whole cell lysates were resolved by SDS-PAGE and probed with antibodies against p27Kip1 (upper panel), p21Cip1/Waf1 (middle panel), and p53 (lower panel). Representative blots from three separate experiments are shown. C, increased p27Kip1-ckd2 binding in late-passage WT satellite cells. 750 μg of total protein from WT and IGF-I Tg whole cell lysates was first immunoprecipitated (IP) with anti-cdk2 antibody, and then the immunoprecipitate was examined by Western blot analysis using the indicated antibodies. For each immunoprecipitation, an appropriate species-specific immunoglobulin (Ig) was used as background control. A set of representative blots from three independent experiments is shown.

The binding of cdk inhibitors to their respective cdkks leads to the inhibition of the activities of these kinases (34). The amount of p27Kip1 bound to cdk2 was ~7-fold higher in late-passage WT anti-cdk2 immunoprecipitates, compared with the corresponding IGF-I Tg immunoprecipitates (Fig. 4C, upper panel). These same immunoprecipitates from WT cells had a significantly decreased histone H1 phosphorylation, relative to IGF-I Tg IPs (similar to that shown in Fig. 3A, upper panel). Thus, the up-regulation of total p27Kip1 protein levels and increased p27Kip1-ckd2 binding (Fig. 4, B and C, upper panels) could contribute to the decreased cdk2 kinase activity (Fig. 3A, upper panel), and may lead to the G1/S arrest seen in late-passage WT satellite cells. The amount of total cdk2 immunoprecipitated from both WT and IGF-I Tg cells were similar as shown in Fig. 4C (lower panel), and hence it is unlikely that the decreased cdk2 kinase activity in late-passage WT satellite cells is due to insufficient amounts of total cdk2 precipitates. Also, overexpression of IGF-I in late-passage transgenic satellite cells did not change the amount of association of p21Cip1/Waf1 to cdk2 (Fig. 4C, middle panel), nor the association of p53 with cdk2 (data not shown; results are identical to that seen with p21Cip1/Waf1).

Ectopic Overexpression of p27Kip1 in Late-passage IGF-I Tg Satellite Cells Results in Decreased cdk2 Kinase Activity and G1 Arrest—To demonstrate that p27Kip1 is important in mediating the cell cycle arrest observed in late-passage WT satellite cells, we infected growing late-passage IGF-I Tg cells with an adenovirus encoding p27Kip1, with or without virus as a control (mock infection). Adp27-infection was documented by Western blot analysis (Fig. 5A, upper panel) and resulted in a dose-dependent inhibition of the BrdUrd labeling index, whereas cellular proliferation was not inhibited by mock infection (Fig. 5A, lower panel). As shown in Fig. 5B, the mean doubling time of Adp27-infected IGF-I Tg satellite cells, compared with mock-infected cells was prolonged ~2.5-fold, providing further evidence for an inhibition of cellular proliferation with ectopic p27Kip1 overexpression. Indeed, overexpression of p27Kip1 in IGF-I Tg satellite cells was sufficient to abolish the cdk2 kinase activity in cdk2 immunoprecipitates, failed to phosphorylate expression pattern in senescent cells is cell-type dependent.

Thus, overexpression of p27Kip1 in IGF-I Tg satellite cells induces G1 arrest, and inhibits cdk2 kinase activity and phosphorylation of pRb. A, upper panel, Western blot analysis using anti-p27Kip1 antibody to document p27Kip1 overexpression in IGF-I Tg satellite cells, infected at a multiplicity of infection of 5, 10, or 20 plaque forming units (pfu) per cell, or adenovirus with empty vector (mock), at a multiplicity of infection of 20 pfu/cell. Results are representative of three independent observations. During the last hour of the 48-h virus inoculation, parallel sets of plates were pulse-labeled with 10 μM BrdUrd, and subsequently processed to determine the BrdUrd labeling index (lower panel). The solid black bar represents mock-infected cells, and the hatched bars correspond to Adp27 concentrations in the upper panel. Values are mean ± S.E. from three separate experiments. B, growth curves of Adp27-infected, and mock-infected late-passage IGF-I Tg cells. The number of cells in equivalent subconfluent cultures of mock-infected (○) and Adp27-infected (□) IGF-I Tg satellite cells (both at a multiplicity of infection of 20 pfu/cell) were counted daily for 5 days. The average doubling time (from three separate experiments) of Adp27-infected cells was 71 ± 2.5 h versus 29 ± 1.8 h for mock-infected cells. C, 350 μg of total protein from mock- and Adp27-infected cells (at multiplicity of infection of 20 pfu/cell) that were cultured at low density (5.5 × 10^6 cells/60-mm dish). A representative set of histograms of the distribution of the cells with percentages in G1, G0, S, and G2/M phases from three separate experiments is presented.

pRb, and caused a 3–4-fold reduction in cyclin A protein abundance (Fig. 5C). In addition, FACS analysis of Adp27-infected cells demonstrated a significant accumulation in G1, with a concomitant decrease in the percentage of S phase cells, compared with mock-infected cells (Fig. 5D). Thus, overexpression of p27Kip1 alone is sufficient to reverse the IGF-I-mediated rescue from G1 arrest in late-passage IGF-I Tg cells, and con-
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**Fig. 6. Up-regulation of both the MAPK and PI 3′-K pathways in late-passage IGF-I Tg satellite cells.** A, phosphorylation status and kinase activity of Erk1/Erk2 MAPK in late-passage WT and IGF-I Tg satellite cells. 35 μg of total protein from each whole cell extract were resolved by SDS-PAGE and subjected to Western blot analysis to determine phosphorylated (upper panel) and total (middle panel) abundance of Erk1/Erk2 proteins in WT and IGF-I Tg cells. Results are representative of three separate experiments. Lower panel, 10 μg of each lysate was also subjected to MAP kinase assay using myelin basic protein as a substrate. Mean ± S.E. of the amount of 32P radiolabel incorporated into myelin basic protein substrate from four separate experiments in triplicate are presented. * indicates significantly (p < 0.0001) increased, compared with WT. B, PI 3′-kinase activity in the anti-phosphotyrosine (4G10 antibody) immunoprecipitated complexes was determined using phosphatidylinositol (PI) as a substrate, and the phosphorylated lipid product, phosphatidylinositol phosphate (PIP) was visualized by autoradiography (upper panel) and quantitated using a PhosphorImager. 350 μg of total protein was immunoprecipitated (IP) with anti-p85 antibody, followed by immunoblot (IB) with 4G10 (middle panel). The same membrane was then stripped and reprobed with a p85 polyclonal antibody to determine the abundance of total p85 protein (lower panel). Species-specific immunoglobulins (lg) were used as background control for each of the immunoprecipitations. Results are representative of four independent experiments. C, 35 μg of protein from late-passage IGF-I Tg and WT whole cell extracts were subjected to Western blot analysis to determine phosphorylation status of Akt on the Ser⁴⁷³ residue (upper panel) as well as total Akt protein levels (lower panel). Representative blots from three experiments are presented.

Sequentially mimics the biochemical events seen in senescent WT cells. Our findings therefore substantiate a causal role for p27Kip1 in the regulation of critical molecules at the G₁/S boundary, necessitating its down-regulation for cell cycle progression in satellite cells.

**Decreased MAP Kinase and PI 3′-Kinase Activities in Late-passage WT Satellite Cells—**Late-passage IGF-I Tg satellite cells had an 8–10-fold increased phosphorylation of both p44 (ERK1) and p42 (ERK2) MAPK isoforms, as well as ~5-fold increase in total MAPK activity in whole cell extracts, relative to late-passage WT cells (Fig. 6A, upper and lower panels). Total ERK1 and ERK2 proteins in WT and IGF-I Tg cells were similar (Fig. 6A, middle panel), indicating increased phosphorylation per unit of protein. As shown in Fig. 6B (upper panel), there was a significant accumulation of the phosphorylated lipid product, phosphatidylinositol phosphate in late-passage IGF-I Tg satellite cells indicating a greater PI 3′-kinase activity in these cells, relative to the corresponding WT cells. Similar results were obtained when whole cell lysates were immunoprecipitated with anti-p85 antibody (data not shown). Late-passage IGF-I Tg immunoprecipitates demonstrated a significant increase (6–7-fold) in the amount of tyrosine-phosphorylated p85 (Fig. 6B, middle panel), relative to the WT cells, indicating that IGF-I-mediated accumulation of the PIP lipid product was likely due to an increased activation of the p85 regulatory subunit of PI 3′-kinase, as the total amount of p85 protein by itself was similar in both groups (Fig. 6B, lower panel). In addition, late-passage IGF-I Tg cells, relative to WT cells demonstrated ~3-fold increase in the phosphorylation of the Ser⁴⁷³ residue of Akt (phosphorylation of which is critical for Akt kinase activity (36)), whereas no difference was noted in total Akt protein between the two groups (Fig. 6C). Therefore, IGF-I overexpression allows transgenic satellite cells to maintain MAPK and PI 3′-kinase activities, as well as activation of Akt even in late-passage, when corresponding WT cells have a deficiency in these signaling molecules.

**Pharmacological Inhibition of PI 3′-Kinase, but Not MAPK, Leads to an Up-regulation of p27⁰Kip1 and G₁ Cell Cycle Arrest in IGF-I Tg Satellite Cells—**In order to first establish that each pharmacological inhibitor was specific for its respective pathway, growing late-passage IGF-I Tg satellite cell cultures were treated for 24 h with 5–100 μM concentrations of either PD98059 (PD), a synthetic inhibitor of the MAPK-activating enzyme (MEK) (37) or LY294002 (LY, inhibits PI 3′-kinase by binding to the ATP-binding sites of the PI 3′-kinase p110 catalytic subunit (38)), or both. Control cultures were treated with an equivalent volume of Me2SO. Pilot experiments showed that maximum inhibitory effects for PD and LY were 50 and 30 μM, respectively (data not shown). The effects of each inhibitor individually (Fig. 7A, lanes 2 and 3) was highly specific for the inhibition of its corresponding pathway (i.e. PD reduced ERK 1 and 2 phosphorylation (first panel), but did not affect Akt phosphorylation (third panel), while LY inhibited Akt phosphorylation, but did not affect ERK1 and 2 phosphorylation), and these compounds in combination (Fig. 7A, lane 4) did not result in any further significant inhibition of phosphorylation, than either one alone. Additionally, at the doses used, there was no effect of these inhibitors on total protein levels of ERK1/ERK2 (second panel) or Akt (fourth panel).

We next determined if the lack of cell cycle progression in late-passage WT satellite cells was a consequence of a decrease in the activities of MAPK, PI 3′-kinase, or both. Treatment of growing, late-passage IGF-I Tg satellite cells with 50 μM PD alone resulted in essentially no change in the cell cycle profile compared with that of Me2SO-treated control cells (~15% increase in the %G₂/M cells for PD from control) (Fig. 7B).
However, treatment of IGF-I Tg cells with 30 μM LY resulted in an almost doubling of the proportion of cells in G1, coupled with a 5-fold reduction in the percentage of S-phase cells, compared with control (Me2SO), and nearing the value for the percentage of S-phase cells seen in late-passage WT satellite cells (Fig. 2A).

PD in combination with LY did not have a further significant increase in the percentage of G1 cells in IGF-I Tg cultures (Fig. 7B).

To explore further the effect of this LY-induced cell cycle arrest on p27<sup>Kip1</sup> protein, we investigated its expression level in LY-treated IGF-I Tg satellite cells. The Western blotting results not only showed a marked increase of 2–4-fold in p27<sup>Kip1</sup> protein (Fig. 7C, panel 1, lane 2), but also demonstrated a similar decrease in pRb levels leading to its overall hypophosphorylation (panel 2), decreased cyclin A expression (panel 3), and resulted in no changes in cyclin D1 levels (panel 4). In contrast, there was no effect of PD alone (Fig. 7C, lane 3) on these intracellular mediators, except for cyclin D1 (a modest decrease of ~30%), from that seen in Me<sub>2</sub>SO-treated control cultures (lane 1). Therefore, these results support the notion that it is a deficiency in the PI 3'-kinase activity (rather than MAPK) that results in the G1/S arrest noted in senescent late-passage WT cells, and signaling through this pathway selectively regulates p27<sup>Kip1</sup>, pRb, and cyclin A proteins in satellite cells.

**DISCUSSION**

After 76 days in culture, satellite cells from 1-month-old IGF-I Tg mice continued to proliferate. In contrast, satellite cells from WT littermates at this time point had exhausted their proliferative reserves, were refractive to exogenous LR3-IGF-I, and demonstrated negligible amounts of sarcomeric myosin staining, suggesting that this loss in proliferative capacity was likely due to cellular senescence. Thus, IGF-I overexpression in satellite cells for a period of ~4 months (~1-month in the animal + 2.5-months in culture) delayed cellular senescence by extending their in vitro replicative life span. The cumulative number of in vitro population doublings attained by IGF-I Tg satellite cells was ~5 additional rounds of divisions (i.e. on the average each satellite cell could replicate to 32 more cells), above the maximum attained by corresponding late-passage WT satellite cells derived from FVB littermates. This dramatic increase in proliferation potential seen in IGF-I Tg satellite cells was IGF-I-dependent since the IGF-I receptor antibody blocked this proliferation. These observations raised the critical question of how IGF-I Tg satellite cells were able to...
sustain their proliferative ability even in late-passages to ultimately result in the observed extension of their in vitro replicative life span.

Analysis of the molecular profile of late-passage IGF-I Tg satellite cells demonstrated characteristics of cycling cells, whereas that of the corresponding WT cells was consistent with a G1/S arrest characteristic of senescent cells. Upon release from serum starvation, the IGF-I Tg cells demonstrated a time-dependent accumulation of S-phase cells (coincident with the induction of cyclin D1 and E proteins), phosphorylated pRb, and up-regulated cyclin A protein. In addition, IGF-I overexpression in late-passage satellite cells down-regulated p27Kip1 protein levels, leading to an associated increase in the cyclin E/cdk2 kinase activity, relative to WT cells (Fig. 8). Cdk2 is one of the critical kinases required to phosphorylate pRb (39), which in turn has been suggested to be a rate-limiting step controlling cell cycle progression past the R-point to synthesize late-G1 genes necessary for S phase entry (29, 31). plywood et al. (40) suggested that p27Kip1 could affect both the activation and kinase activity of cdk2, since the binding of p27Kip1 may interfere with phosphorylation of cdk2s activating site (Thr-160). Others have suggested that p27Kip1 could act as a threshold device controlling cdk2 kinase activity, increased cyclin D1/cdk4 accumulation sequesters free p27Kip1, decreasing p27Kip1/cdk2 association, and consequently allowing for cdk2 activation (41). Consistent with this notion, we demonstrated an increased abundance of the cyclin D1/cdk4 complex in late-passage IGF-I Tg cells, which we speculate may also help sequester p27Kip1, effectively decreasing the p27Kip1 protein that is available to bind and inactivate cdk2.

Remarkably, adenoviral-mediated ectopic overexpression of p27Kip1 in exponentially growing cultures of late-passage IGF-I Tg satellite cells down-regulated cdk2 kinase activity, hypophosphorylated pRb, decreased cyclin A protein, and in vitro cell proliferation, increased cell doubling time by ~2.5-fold, and induced a G1 arrest, thereby mimicking the changes observed in senesced cultures of late-passage WT satellite cells. Previous studies have shown that p27Kip1 overexpression (27, 42) repressed transcription from the cyclin A promoter regions that contain an E2F-binding site required for cell cycle-regulated cyclin A gene expression, indicating that p27Kip1 can regulate transcription of late G1 genes. Furthermore, recent findings of Busse et al. (25) showed that antisense p27Kip1 essentially reversed both p27Kip1 up-regulation and the G1 arrest in A431 cells treated with the tyrosine kinase inhibitor AG-1478. The role of p27Kip1 as a critical regulator of cellular proliferation is further illustrated by p27Kip1 knockout mouse models, which exhibits gigantism, organomegaly, and enhanced spontaneous tumor formation (43–45). Given that cdk2 kinase activity, phosphorylation of pRb, and up-regulation of cyclin A are critical for cell cycle progression (12, 13), and the fact that all of these regulatory factors are inhibited by p27Kip1 overexpression in growing late-passage IGF-I Tg cells, it suggests that p27Kip1 is a critical mediator through which IGF-I delays G1/S arrest in these cells. These results also support the possibility that p27Kip1 could be an initiator of a cascade of G1/S cell cycle events, to consequently regulate replicative senescence (Fig. 8).

Interruption of PI 3'-kinase activity in IGF-I Tg cells by LY294002 mimicked the molecular events seen in senesced late-passage WT satellite cells, i.e. increased p27Kip1, decreased pRb phosphorylation and cyclin A protein, and caused a G1/S cell cycle arrest. In contrast, blockade of the activated MAPK by PD098059 resulted in neither an appreciable accumulation of cells in G1, nor an increase in p27Kip1 protein levels. These observations in IGF-I Tg satellite cells extend the previous findings of Collado et al. (46), who showed that inhibition of PI 3'-kinase pathway induced a senescent-like arrest that was mediated by p27Kip1 in mouse embryo fibroblasts. We therefore infer that PI 3'-kinase activation induced by IGF-I overexpression in late-passage IGF-I Tg satellite cells are necessary and sufficient for the down-regulation of p27Kip1, and consequently for the enhanced G1 to S-phase cell cycle progression, resulting in the increased in vitro doubling potential of these cells (Fig. 8).

Thus, it is probable that Akt itself or other molecules activated by PI 3'-kinase signaling pathway is essential for G1 cell cycle progression in skeletal muscle satellite cells from IGF-I Tg mice. Indeed, a requirement of an intact PI 3'-kinase signaling pathway (independent of MAPK activity in many of these cells), for G1 to S cell cycle progression has previously been reported in other cell types (20, 24, 25, 47–50). Our results are also consistent with Milasincic et al. (19) who had previously shown that the mitogenic response of IGF-I was mediated via the activation of the PI 3'-kinase pathway, independent of MAPK in C2C12 murine cell line, but they had not examined cell cycle markers. However, our results contradict the report in rat L6A1 immortalized myoblasts, which had demonstrated that pharmacological inhibition of MAPK activation attenuated the proliferative response by LR3-IGF-I in these cells (18). Thus, it appears that the pluripotent effects of factors such as IGF-I and their consequent signaling may depend on the cell context in which its receptor is activated.

Clearly, processes as complex as cellular proliferation and replicative senescence involve multiple mediators and cross-talk mechanisms. The activation of PI 3'-kinase/Akt to signal the down-regulation of p27Kip1 is a crucial phenomenon that leads to an enhanced proliferative potential, resulting in extension of the in vitro replicative life span of IGF-I Tg satellite cells. However, this need not be the exclusive mechanism. IGF-I-evoked modulation of other regulatory pathways such as the inhibition of the pro-apoptotic factor BAD via phosphorylation by Akt/PKB to enhance survival (51), up-regulation of telomerase activity (52), and/or changes in IGF-I-binding protein concentrations (53) are some of the mechanisms that need to be further explored.
While previous studies have shown that the number of replications can be extended in other cell types by other means (15, 23, 54–56), this is the first demonstration of extending the replicative life span of skeletal muscle stem cells with a growth factor. Given that satellite cells are absolutely required for postnatal muscle growth and repair (2), our data provide novel insights into some of the regulatory events which may serve as attractive tools for potential ex vivo manipulation of stem cell autografts to extend the replicative life span of old satellite cells for gene therapy in muscle wasting conditions.

Acknowledgments—We thank Dr. Jeanie McMillin for critically reading the manuscript and helpful suggestions, Louise Barnett for assistance with flow cytometry, Samantha Cristofalo and Sandra Higam for assistance with cell culture. The mouse monoclonal antibodies D3 and MF20 developed by Danto and Fischman (57) and Bader et al. (58), respectively, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences.

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