Increased Wnt Signaling During Aging Alters Muscle Stem Cell Fate and Increases Fibrosis

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The regenerative potential of skeletal muscle declines with age, and this impairment is associated with an increase in tissue fibrosis. We show that muscle stem cells (satellite cells) from aged mice tend to convert from a myogenic to a fibrogenic lineage as they begin to proliferate and that this conversion is mediated by factors in the systemic environment of the old animals. We also show that this lineage conversion is associated with an activation of the canonical Wnt signaling pathway in aged myogenic progenitors and can be suppressed by Wnt inhibitors. Furthermore, components of serum from aged mice that bind to the Frizzled family of proteins, which are Wnt receptors, may account for the elevated Wnt signaling in aged cells. These results indicate that the Wnt signaling pathway may play a critical role in tissue-specific stem cell aging and an increase in tissue fibrosis with age.

Aging of skeletal muscle is characterized by an increase in fibrous connective tissue (*1*) and an impairment of muscle regenerative potential (*2*, *3*), manifested by a replacement of muscle by fibrous connective tissue and adipose tissue (fig. S1). In the muscular dystrophies, there is also progressive muscle fibrosis with age (*4*). We examined the cellular and molecular mechanism of this age-dependent increase in skeletal muscle fibrosis.

The regeneration of aged muscle can be enhanced by exposure to a youthful systemic environment, an effect mediated at least in part by restoration of normal signaling of the Delta-Notch pathway (*5*, *6*). Therefore, we tested whether exposure of old tissue to a youthful systemic environment, established by parabiotic pairings of old animals to young animals (heterochronic pairings) (*7*), might also reduce the fibrotic response of old muscle. Indeed, there was a reduction of collagen deposition in regenerating areas in muscles of aged mice in heterochronic pairings compared with that of aged mice in isochronic pairings (pairings of mice of the same age), and this was accompanied by enhanced proliferation of myogenic progenitors (Fig. 1, A and B). Conversely, collagen deposition was increased and progenitor proliferation was reduced in young partners of heterochronic pairings compared with young partners in isochronic pairings. Thus, systemic influences that change with age are important in mediating the fibrotic responses of aged tissues.

We examined cells derived from muscles of young (~6-month-old) and aged (~24-month-old) mice for any characteristics that might account for the age-related fibrotic response. In single-fiber cultures, we found 99% of the fiber-associated mononucleated cells appeared to be myogenic as defined by expression of combinations of myogenic markers (Pax7, MyoD, and Desmin) 2 days after fiber isolation from young animals, and 98% of the cells from fibers from aged animals were myogenic (Fig. 2A). After another 1½ days in culture, the percentage of nonmyogenic cells from the young cultures remained ~1%, but the percentage from the aged fibers had increased to ~17%, and they exhibited morphological changes characteristic of a fibroblastic lineage (Fig. 2B). No loss of cells due to detachment or apoptosis was detected, and the proliferation of nonmyogenic cells from aged muscle was, if anything, lower than that from young muscle (fig. S2). Therefore, the increase in the percentage of fibrogenic cells in cultures from aged mice most likely arose by conversion of previously myogenic cells into nonmyogenic cells.

With age, there is a decline in satellite cell functionality (*5*). That aged muscle regeneration can be enhanced by direct activation of the Notch pathway (*5*) or by exposure to a youthful systemic environment (*6*) indicates that those functional changes are largely reversible. To test for reversibility of the age-related myogenic-to-fibrogenic conversion, we examined satellite cell progeny from young and aged mice in heterochronic parabiotic pairings (*6*). Aged tissues exposed to this heterochronic systemic environment exhibited a reduction in the myogenic-to-fibrogenic conversion (from 17 to 10%), whereas the younger tissues exhibited an increase (from ~1 to 9%) (Fig. 2C). We also exposed young and aged cells in vitro to serum from young or aged animals ("young serum" and "aged serum," respectively). Aged serum increased the myogenic-to-fibrogenic conversion of young cells, whereas

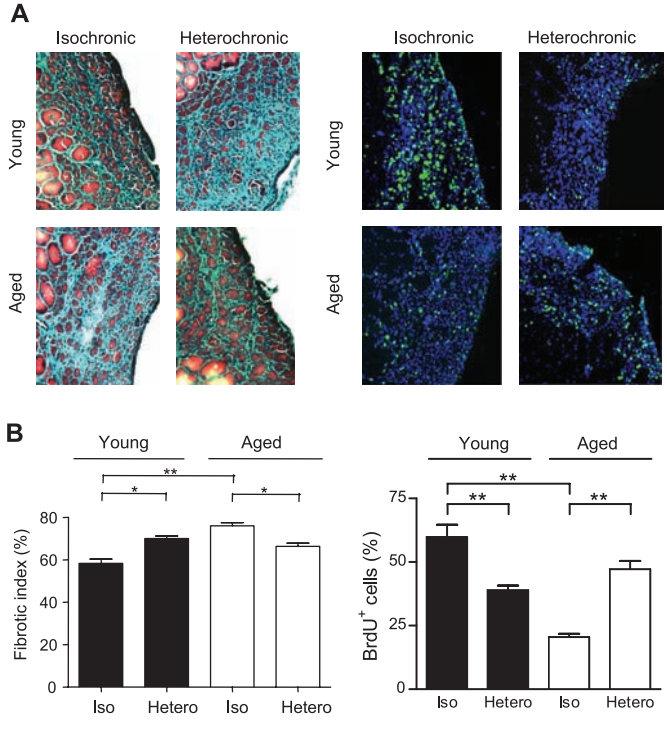

Fig. 1. Prevention of age-related increase in fibrogenesis during muscle regeneration by heterochronic parabiosis. (**A**) Isochronic or heterochronic parabiotic pairs were established for 4 weeks, and hind limb muscles were subjected to freeze injuries. Bromodeoxyuridine (BrdU) was injected intraperitoneally 2 days after injury. Three days later, muscles were sectioned and (left) stained with Gomori trichrome (red, muscle fibers; green, connective tissue) or (right) immunostained for BrdU (green). 4′,6′-Diamidino-2-phenylindole (DAPI) (blue) labels all nuclei. (**B**) Quantitative analyses of histologic studies as in (A). (Left) Fibrotic index (percentage of the injury area occupied by connective tissue in Gomori-stained sections). (Right) Proliferative response (percentage of $DAPI^+$ mononucleated cells that were also $BrdU^+$). "Young" and "aged" refer to young and aged mice, respectively; "iso" and "hetero" to isochronic and heterochronic pairings, respectively. (*$P < 0.05$; **$P < 0.01$.)

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young serum had the opposite effect on aged cells (Fig. 2D). Nearly 100% of young cells previously maintained in young serum expressed myosin heavy chain under differentiation conditions, but that declined to ~75% in cultures of young cells previously maintained in aged serum (fig. S3). Together, these results suggest that myogenic progenitors tend to deviate from their myogenic lineage in the aged environment.

We used genetic lineage tracing to confirm that the aged systemic environment promotes a myogenic-to-fibrogenic conversion. To identify fibrogenic cells, we used an antibody to a fibroblast-specific marker (ER-TR7) that was highly specific (0% of $Pax7^+$ or $MyoD^+$ cells were $ER\text{-}TR7^+$) and highly sensitive [93% of nonmyogenic cells ($Pax7^-$ and $MyoD^-$) were $ER\text{-}TR7^+$] (fig. S4). For myogenic lineage studies, we used Pax7.Cre-ER.ROSA26 mice, a strain in which tamoxifen administration leads to permanent β-galactosidase (β-gal) expression only in myogenic cells in the adult (fig. S5). When myogenic progenitor cultures from these tamoxifen-treated mice were incubated in young serum, all $\beta\text{-gal}^+$ cells had a myogenic phenotype (either $MyoD^+$ or $Pax7^+$, and $ER\text{-}TR7^-$). However, in aged serum, 18% of $\beta\text{-gal}^+$ cells were $Pax7^-$ and $MyoD^-$, and 10% were $ER\text{-}TR7^+$ (Fig. 2, E and F), which confirmed that the aged environment promotes a myogenic-to-fibrogenic conversion.

Activation of the Wnt pathway can lead to fibrogenic conversion of cells in other lineages (*8*, *9*). Thus, we examined markers of the steady-state activation of the Wnt pathway in muscle and purified satellite cells (fig. S6) from young and aged mice, as well as the effects of modulating Wnt signaling on myogenic-to-fibrogenic conversion and the fibrotic response.

We analyzed a direct downstream target of Wnt signaling, Axin2 (*10*), in uninjured muscle from young and aged mice. Axin2 transcript levels were increased in aged muscle (fig. S7A). Furthermore, purified satellite cells from aged muscle expressed more Axin2 than did such cells from young muscle (Fig. 3A). In addition, analysis of TOPGAL mice [in which β-gal expression is a read-out of Wnt signaling (*11*)] revealed a progressive increase in Wnt signaling in myogenic cells during aging (Fig. 3B).

We also analyzed other components of the canonical Wnt signaling cascade, glycogen synthase kinase 3β (GSK3β) and its substrate β-catenin. In aged satellite cells, fluorescence-activated cell sorting (FACS) analysis demonstrated that the amounts of active GSK3β decreased and active β-catenin increased (Fig. 3C), both changes being indicative of active Wnt signaling (*12*, *13*). Furthermore, these changes were due to Wnt signaling, because systemic adenoviral-mediated expression of Dickkopf-1 (DKK1) (*14*), a Wnt antagonist, decreased the percentage of myogenic progenitors expressing active β-catenin (fig. S7B).

To analyze the Wnt signaling cascade in myogenic progenitors during muscle regenera-

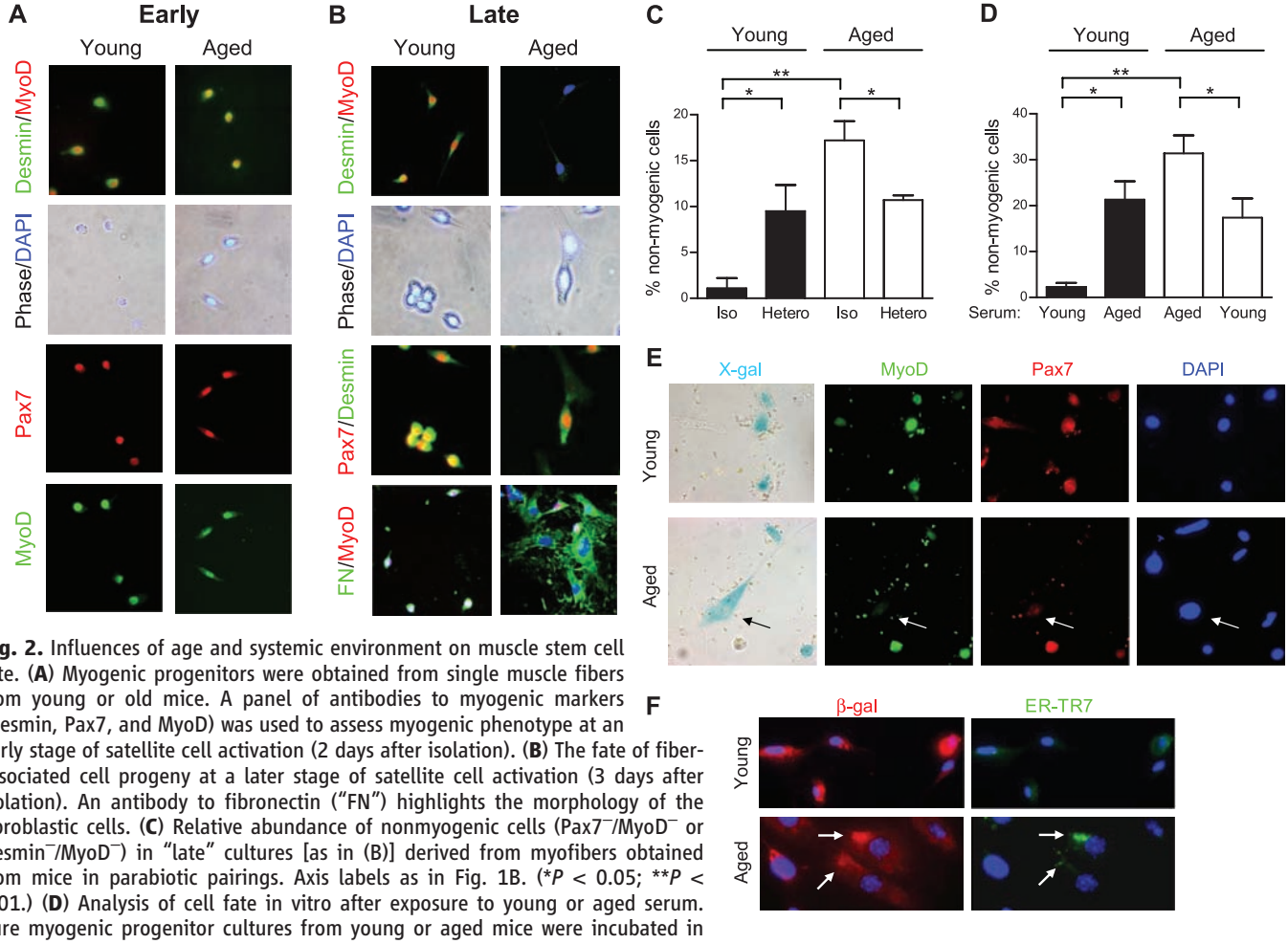

Fig. 2. Influences of age and systemic environment on muscle stem cell fate. (**A**) Myogenic progenitors were obtained from single muscle fibers from young or old mice. A panel of antibodies to myogenic markers (Desmin, Pax7, and MyoD) was used to assess myogenic phenotype at an early stage of satellite cell activation (2 days after isolation). (**B**) The fate of fiber-associated cell progeny at a later stage of satellite cell activation (3 days after isolation). An antibody to fibronectin ("FN") highlights the morphology of the fibroblastic cells. (**C**) Relative abundance of nonmyogenic cells ($Pax7^-/MyoD^-$ or $Desmin^-/MyoD^-$) in "late" cultures [as in (B)] derived from myofibers obtained from mice in parabiotic pairings. Axis labels as in Fig. 1B. (*$P < 0.05$; **$P < 0.01$.) (**D**) Analysis of cell fate in vitro after exposure to young or aged serum. Pure myogenic progenitor cultures from young or aged mice were incubated in plating media for 1½ days, exposed to young or aged serum for 1½ days, and analyzed for the percentage of nonmyogenic cells. (*$P < 0.05$; **$P < 0.01$.) (**E**) Effect of young or aged serum on myogenic stem cell fate. Myogenic progenitors isolated from Pax7.Cre-ER.ROSA26 mice were incubated in young or aged serum for 2 days. Cells were stained for β-gal with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, blue) and with antibodies to MyoD (green) and Pax7 (red). Arrow indicates a cell that has undergone myogenic-to-fibrogenic conversion. (**F**) Myogenic progenitors isolated from Pax7.Cre-ER.ROSA26 mice were incubated in young or aged serum for 2 days. Cells were stained with an antibody to β-gal (red) or to a fibroblast-specific marker, ER-TR7 (green). White arrows in the bottom panel indicate previously myogenic cells showing early fibrogenic phenotype.

tion in vivo, we isolated progenitors 2 days after injury of young and aged muscle and analyzed them by FACS. The percentage of progenitors with detectable levels of active β-catenin was increased in aged myogenic progenitors (fig. S7C). Increased β-gal activity was observed in injured areas of muscle and also in isolated muscle progenitors from aged TOPGAL mice (Fig. 3D and fig. S7, D and E). When we incubated myogenic progenitors from TOPGAL mice in young or aged serum (Fig. 3E), Wnt signaling was increased in progenitors exposed to aged serum, and this increase was prevented by a soluble Wnt inhibitor, Frizzled-related protein 3 (sFRP3).

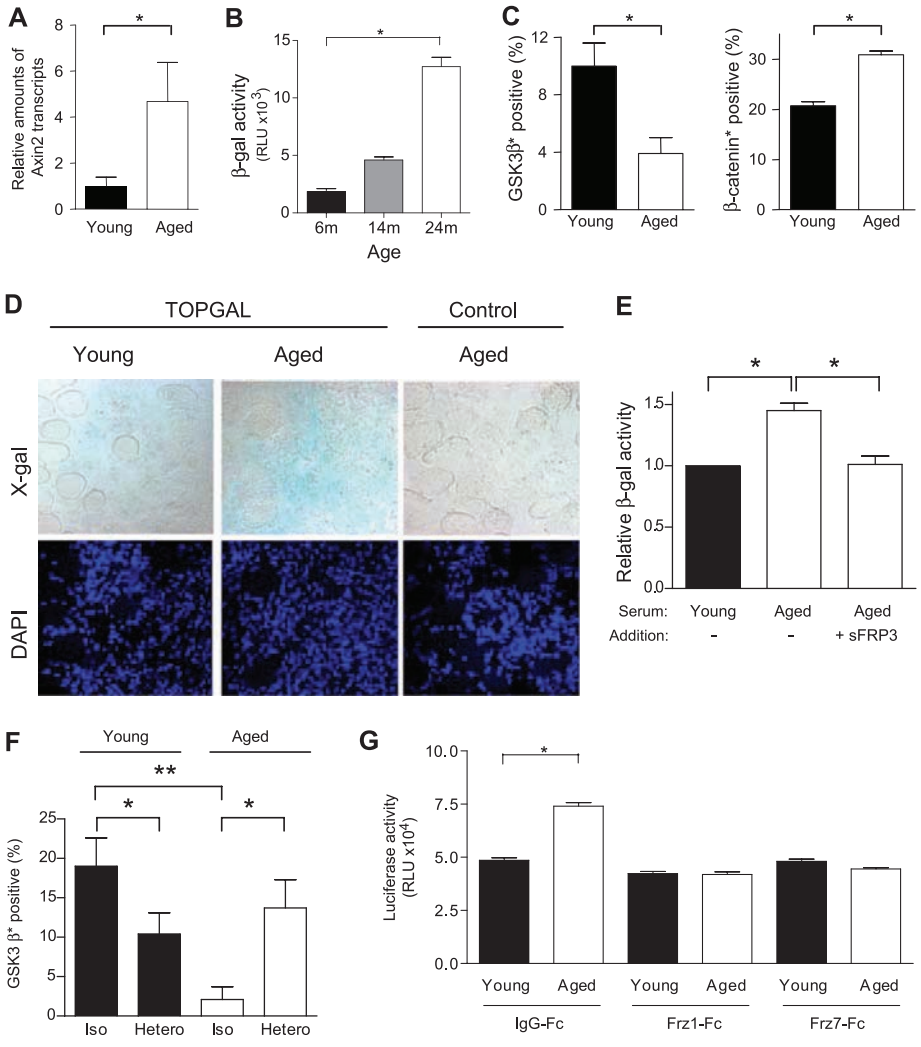

Fig. 3. Enhanced Wnt signaling in aged muscle and in myogenic progenitors exposed to aged serum. (**A**) Axin2 transcript levels assessed by real-time reverse transcription polymerase chain reaction from satellite cells obtained by FACS-sorted Syndecan4$^+$ cells (fig. S6) from uninjured muscles of young and aged mice. (**B**) β-Gal activity (normalized to DNA content) in fiber-associated cells isolated from uninjured muscle of TOPGAL mice of different ages. β-Gal activity of aged-matched wild-type controls was subtracted to normalize for any endogenous activity. (*$P < 0.05$.) (**C**) FACS analysis of active GSK3β and β-catenin (GSK3β* and β-catenin*, respectively) in myogenic progenitors isolated from myofibers cultures from young and aged animals. Graphs show the percentage of all myogenic progenitors (Syn-4$^+$) that were also positive for GSK3β* or β-catenin*. (*$P < 0.05$.) (**D**) Muscles of young and aged TOPGAL mice were injured, and the muscles were analyzed 2 days later. Cryosections were incubated with a β-gal substrate (X-gal) that is blue after enzymatic conversion (top) and DAPI (bottom). X-gal staining in aged, wild-type littermate showed negligible endogenous β-gal activity. (**E**) Analysis of Wnt signaling activity in myogenic progenitors derived from muscle fibers of TOPGAL mice. Cells were incubated in young or aged serum for 17 hours in the presence or absence of a soluble Wnt inhibitor, sFRP3. The β-gal activity was quantified and normalized to DNA content. The data are presented relative to the levels of β-gal activity in cells exposed to young serum. (*$P < 0.05$.) (**F**) Analysis of GSK3β* in myogenic progenitors isolated from parabiotic pairs. Labeling as in Fig. 1B. (*$P < 0.05$; **$P < 0.01$.) (**G**) Wnt reporter gene expression in LSL cells exposed to serum obtained from young and aged mice and incubated with agarose beads conjugated with chimeric Frizzled receptors (Frz1-Fc, Frz7-Fc) or IgG control. Cells were incubated for 24 hours, and luciferase activity was quantified and normalized to β-gal activity. (*$P < 0.05$.)

We tested whether the observed heterochronic parabiotic effects (Fig. 1) were associated with corresponding changes in the Wnt signaling pathway. Indeed, in heterochronic pairings, cells from the aged partners exhibited decreased Wnt signaling, and cells from the young partners showed increased Wnt signaling compared with that seen in cells in isochronic controls (Fig. 3F). Thus, circulating factors in aged animals appear to convey signals that result in enhanced Wnt signaling.

To test for the presence of components of serum capable of activating Wnt signaling by binding to Frizzled receptors, we used a chimeric Wnt receptor fusion protein, Frizzled-Fc, to deplete the serum of such activity (fig. S8). Serum was incubated with conjugated Frizzled-Fc or immunoglobulin IgG-Fc control and subsequently tested for its effects on a cell line (LSL) in which luciferase expression is dependent on activation of the Wnt pathway (*15*). When incubated with Frizzled-Fc, the activity in aged serum that promoted Wnt signaling decreased; there was no change in the activity of young serum subjected to the same treatment (Fig. 3G).

We altered Wnt signaling experimentally to test directly for its effects on cell fate and muscle regeneration. Addition of Wnt3A protein to young serum resulted in an increased myogenic-to-fibrogenic conversion of young progenitors in vitro. Conversely, the myogenic-to-fibrogenic conversion by aged serum was abrogated by Wnt inhibitors (Fig. 4A).

In vivo, the injection of Wnt3A into young regenerating muscle 1 day after injury resulted in increased connective tissue deposition (Fig. 4B), phenotypically similar to regenerating aged muscle (fig. S1). Exogenous Wnt also reduced cellular proliferation in young regenerating muscles (fig. S9A). We therefore tested whether inhibiting Wnt signaling in aged muscle would reduce fibrosis and enhance muscle regeneration. Indeed, there was reduced fibrosis in aged regenerating muscle injected with DKK1, whereas no change was observed in young muscle similarly treated (Fig. 4C). Injection of sFRP3 also enhanced myogenic progenitor proliferation in aged muscle (fig. S9, B and C).

These findings demonstrate that, with age, the systemic environment is less effective in maintaining the myogenic fate of muscle stem cells and, instead, facilitates conversion to a fibrogenic fate. In vivo, this is associated with impaired muscle regeneration and an enhanced fibrotic response. These effects are associated with increased Wnt signaling in the myogenic progenitors, possibly resulting from increased amounts of Wnt or Wnt-like molecules in the serum of aged animals. This generalized role of Wnt signaling in promoting an aging phenotype is consistent with the findings of Liu *et al*. from studies of the role of Klotho in tissue aging (*16*). It is clear that Wnts have multiple actions both developmen-

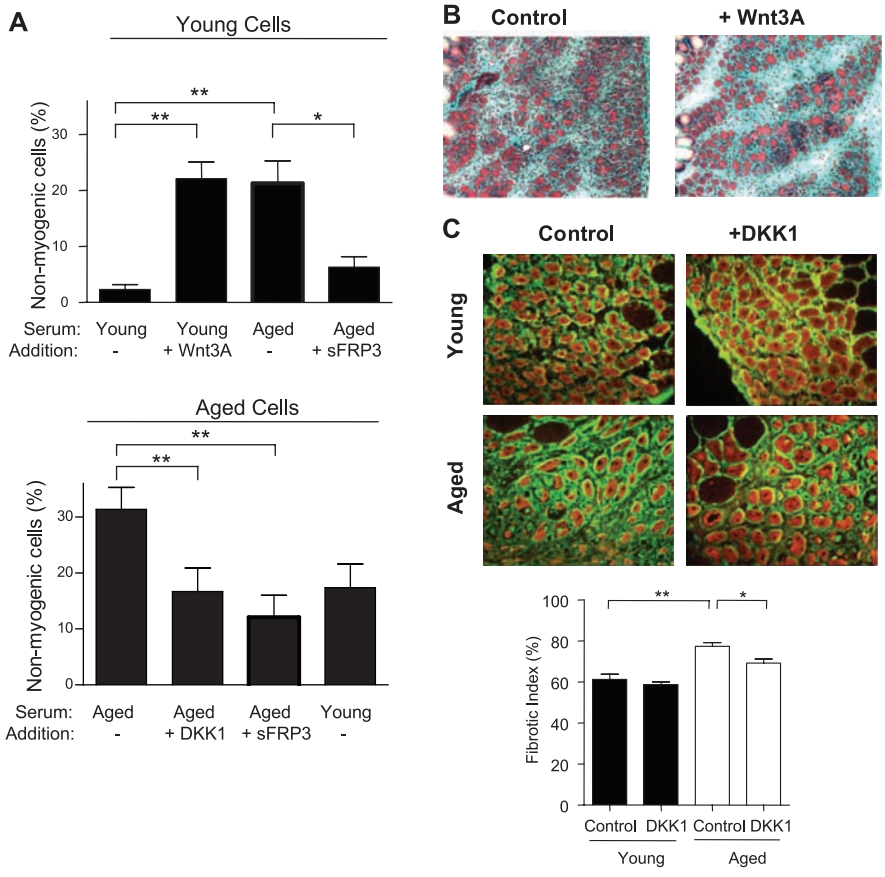

Fig. 4. The effects of the aged environment on myogenic progenitor cell fate and muscle regeneration are mediated by the Wnt signaling pathway. (**A**) (Top) The fate of myogenic progenitors from young mice incubated in young serum, with or without exogenous Wnt3A, or incubated in aged serum, with or without the Wnt inhibitor sFRP3, for 1½ days. (Bottom) Fate of myogenic progenitors from aged mice incubated in young serum or in aged serum, with or without sFRP3 or DKK1, for 1½ days. The percentages of cells that acquired a nonmyogenic cell fate were analyzed morphologically and immunohistochemically as described in Fig. 2. (*$P < 0.05$; **$P < 0.01$.) (**B**) Effects of exogenous Wnt on muscle regeneration. Muscles of young mice were injured, and either Wnt3A (200 ng/10 μl) or control solution [10 μl of 0.1% bovine serum albumin (BSA)] was injected into regenerating tissues 1 day after injury. Cryosections were stained with Gomori stain. (**C**) Effects of Wnt inhibition on fibrosis in regenerating muscle. Muscles of young and aged mice were injured, and either DKK1 (500 ng/10 μl) or control solution (10 μl of 0.1% BSA) was injected into regenerating tissues 1 day after injury. Muscles were analyzed 5 days later. Cryosections were stained with antibodies against collagen VI (green) and embryonic myosin heavy chain (red). The histogram represents the fibrotic index (as in Fig. 1B). (**$P < 0.01$; *$P < 0.05$.)

tally and postnatally (*17*). In contrast to the inhibition of myogenesis reported here, Wnt signaling may promote myogenic lineage progression during development (*18*). Such pleiotropic effects may relate to differences in the timing of Wnt signaling with regard to the state of cellular differentiation or to changes in other interacting signaling pathways during development and aging. Our results may provide a strategy to improve tissue repair, particularly under conditions in which regeneration is impaired and fibrosis is favored, such as in aging and muscular dystrophies.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/317/5839/807/DC1
Materials and Methods
Figs. S1 to S9
References

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International Conservation Policy Delivers Benefits for Birds in Europe

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Conservation of the planet's biodiversity will depend on international policy intervention, yet evidence-based assessment of the success of such intervention is lacking. Poor understanding of the effectiveness of international policy instruments exposes them to criticism or abandonment and reduces opportunities to improve them. Comparative analyses of population trends provide strong evidence for a positive impact of one such instrument, the European Union's Birds Directive, and we identify positive associations between the rate of provision of certain conservation measures through the directive and the response of bird populations. The results suggest that supranational conservation policy can bring measurable conservation benefits, although future assessments will require the setting of quantitative objectives and an increase in the availability of data from monitoring schemes.

Because global threats to biodiversity are largely anthropogenic, already considerable in scale, and accelerating rapidly (*1*), their solutions will depend largely on international policy intervention. This was recognized in the formulation of international agreements