G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation

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After skeletal muscle injury, neutrophils, monocytes, and macrophages infiltrate the damaged area; this is followed by rapid proliferation of myoblasts derived from muscle stem cells (also called satellite cells). Although it is known that inflammation triggers skeletal muscle regeneration, the underlying molecular mechanisms remain incompletely understood. In this study, we show that granulocyte colony–stimulating factor (G-CSF) receptor (G-CSFR) is expressed in developing somites. G-CSFR and G-CSF were expressed in myoblasts of mouse embryos during the midgestational stage but not in mature myocytes. Furthermore, G-CSFR was specifically but transiently expressed in regenerating myocytes present in injured adult mouse skeletal muscle. Neutralization of endogenous G-CSF with a blocking antibody impaired the regeneration process, whereas exogenous G-CSF supported muscle regeneration by promoting the proliferation of regenerating myoblasts. Furthermore, muscle regeneration was markedly impaired in G-CSFR–knockout mice. These findings indicate that G-CSF is crucial for skeletal myocyte development and regeneration and demonstrate the importance of inflammation-mediated induction of muscle regeneration.

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Adult skeletal muscle has resident stem cells, called satellite cells, which are responsible for generating new muscle under both physiological and pathophysiologic conditions. Although these muscles have the capacity to regenerate, this capacity has some limitations (Le Grand and Rudnicki, 2007). There are several skeletal muscle diseases such as skeletal muscle dystrophy, myopathy, severe injury, and disuse syndrome for which there are no effective treatments (Shi and Garry, 2006). Although several studies have identified various growth factors and cytokines that regulate skeletal muscle development and regeneration, effective control of regeneration hasn't been achieved using these factors in the clinical setting (Buckingham and Montarras, 2008). Therefore, it is worth elucidating the mechanisms of skeletal muscle regeneration and developing novel regeneration therapies.

After injury to skeletal muscle, neutrophils, monocytes, and macrophages infiltrate the damaged area. Concomitantly, satellite cells differentiate into transient-amplifying myoblasts, which rapidly proliferate, fuse with one another, and regenerate skeletal myotubes. During these processes, inflammation and regeneration are tightly linked. Therefore, it is reasonable to assume that some factors expressed during the inflammatory process influence skeletal muscle regeneration. However, the precise mechanisms remain unknown.

Previously, when we looked for potent differentiation-promoting factors during embryonic stem cell differentiation (Yuasa et al., 2005, 2010), we noted a marked elevation in the expression of G-CSF receptor (G-CSFR; encoded

by *csf3r*) in developing cardiomyocytes (Shimoji et al., 2010). Interestingly, we also found a marked increase in G-CSFR expression in developing somites. G-CSF was initially identified as a hematopoietic cytokine and has been used in both basic research studies and in the clinic for the mobilization of hematopoietic stem cells (Demetri and Griffin, 1991; Welte et al., 1996; Metcalf, 2008). However, recently, studies suggest that G-CSF also plays roles in cell differentiation, proliferation, and survival (Avalos, 1996; Harada et al., 2005; Zaruba et al., 2009). These findings encouraged us to investigate the involvement of G-CSF and G-CSFR in skeletal myocyte development and regeneration and to examine the link between inflammation and regeneration.

In this study, we show that skeletal myoblasts express G-CSF/G-CSFR and proliferate in an autocrine fashion in skeletal myocyte development. We also show that both infiltrating inflammatory cell–derived G-CSF and externally administered G-CSF enhance skeletal myoblast proliferation and facilitate skeletal muscle regeneration.

RESULTS
csf3r is expressed in the developing somite
Initially, we investigated the *csf3r* expression in the developing mouse embryo. Whole-mount in situ hybridization revealed that *csf3r* was expressed in the somite of the embryonic day (E) 9.5 mouse embryo. To localize *csf3r* expression within the somites, we used several markers of skeletal myocyte differentiation (Fig. 1 a). The *c-met* gene, which encodes a receptor for hepatocyte growth factor, is expressed in the dermomyotome and is essential for the delamination/migration of muscle progenitor cells (Yang et al., 1996). The expression of *c-met* was restricted to the ventral portion of the somite, and the expression pattern of *csf3r* wasn't similar to that of *c-met*. Skeletal myocyte development is finely regulated by myogenic transcription factors. *pax3* is first expressed in the presomitic mesoderm and is expressed in the somitic epithelium of the dermomyotome (Jostes et al., 1990; Bober et al., 1994). *pax3* is repressed as dermomyotome-derived cells activate myogenic transcription factors. The expression pattern of *pax3* was different from that of *csf3r*. The myogenic bHLH (basic helix-loop-helix) genes also show unique expression patterns in different skeletal muscle developmental stages. *myoD* and *myf5* are expressed in undifferentiated proliferating myoblasts (Tapscott et al., 1988; Venters et al., 1999), whereas *mrf4* isn't expressed until a late stage in the differentiation program (Rhodes and Konieczny, 1989; Bober et al., 1991). Compared with these marker expression patterns, the *csf3r* expression pattern resembled those of *myf5* and *myoD*. The expression pattern of the late differentiation marker *mrf4* wasn't identical to that of *csf3r*.

Immunofluorescence staining of sections of embryos of different developing stages demonstrated that G-CSFR expression in the somite was restricted to the E9.5–10.5 period; before E9.5, G-CSFR wasn't observed in the somite, and by E11.5, G-CSFR expression had disappeared (Fig. 1 b). These results indicate that G-CSF is involved in the development of undifferentiated proliferating myoblasts.

G-CSF and G-CSFR are expressed in differentiating skeletal myocytes
Immunostaining for markers of several differentiation stages revealed the stage at which skeletal myocytes expressed the G-CSFR. Skeletal muscle progenitor cells arise in the central part of the dermomyotome, coexpress Pax3 and Pax7, and can differentiate into skeletal muscle fibers during embryogenesis (Messina and Cossu, 2009). Pax3 and Pax7 have partially overlapping and partially distinct functions in myogenic progenitor cells and are both down-regulated during myogenic differentiation, after myogenic regulatory factor (MRF) expression. The Pax3- and Pax7-expressing myogenic progenitor cells didn't express G-CSFR (Fig. 1 c). However, the cells with declining levels of Pax3 and Pax7, which started to express MyoD and myogenin, showed G-CSFR expression (Fig. 1 d). In agreement with a previous study on the G-CSFR expression pattern, the immunoreactivity for G-CSFR was localized to the cell membrane and cytoplasm under steady-state conditions (Aarts et al., 2004). These cells also expressed desmin, which is an intermediate filament expressed in skeletal muscle (Fig. 1 d).

G-CSF expression was also examined by immunostaining. G-CSF expression wasn't detected in the Pax3- and Pax7-expressing myogenic progenitor cells (Fig. 1 e). As seen for the G-CSFR–expressing cells, the cells with declining levels of Pax3 and Pax7, which started to express MyoD and myogenin, showed G-CSF expression (Fig. 1 f). Double immunostaining for G-CSF and G-CSFR revealed that the G-CSFR–expressing cells also expressed G-CSF. These results indicate that early skeletal myocyte differentiating cells undergo autocrine G-CSF signaling in the developing myoblasts.

G-CSF promotes myoblast proliferation in vitro
To elucidate the role of G-CSF in myogenic cells, myoblast cells were analyzed in vitro. The C2C12 cell line is a subclone of C2 cells, which were established from the regenerating thigh muscle of an adult mouse and which are widely used as a myoblast cell line (Blau et al., 1983). In low-serum conditions, C2C12 cells differentiate and fuse with each other to form multinucleated myotubes (Fig. 2 a). Immunostaining for G-CSFR and α-actinin revealed that the premature C2C12 cells expressed G-CSFR but not actinin, whereas the mature fused myotubes clearly expressed α-actinin, and the α-actinin–positive cells never expressed G-CSFR. Western blot analysis confirmed that as differentiation proceeded, α-actinin expression gradually increased, and G-CSFR expression decreased (Fig. 2 b).

To clarify the effect of G-CSF on myocytes, G-CSF was administered to C2C12 cells that expressed the G-CSFR. G-CSF administration significantly increased the number of C2C12 cells in a dose-dependent manner (Fig. 2 c). BrdU incorporation analysis revealed that the increased cell number was the result of cell proliferation induced by G-CSF (Fig. 2 d). An anti–G-CSF neutralizing antibody inhibited the serum-dependent proliferation of C2C12 cells (Fig. 2 e). We also examined whether G-CSF may affect the myogenic cell differentiation.
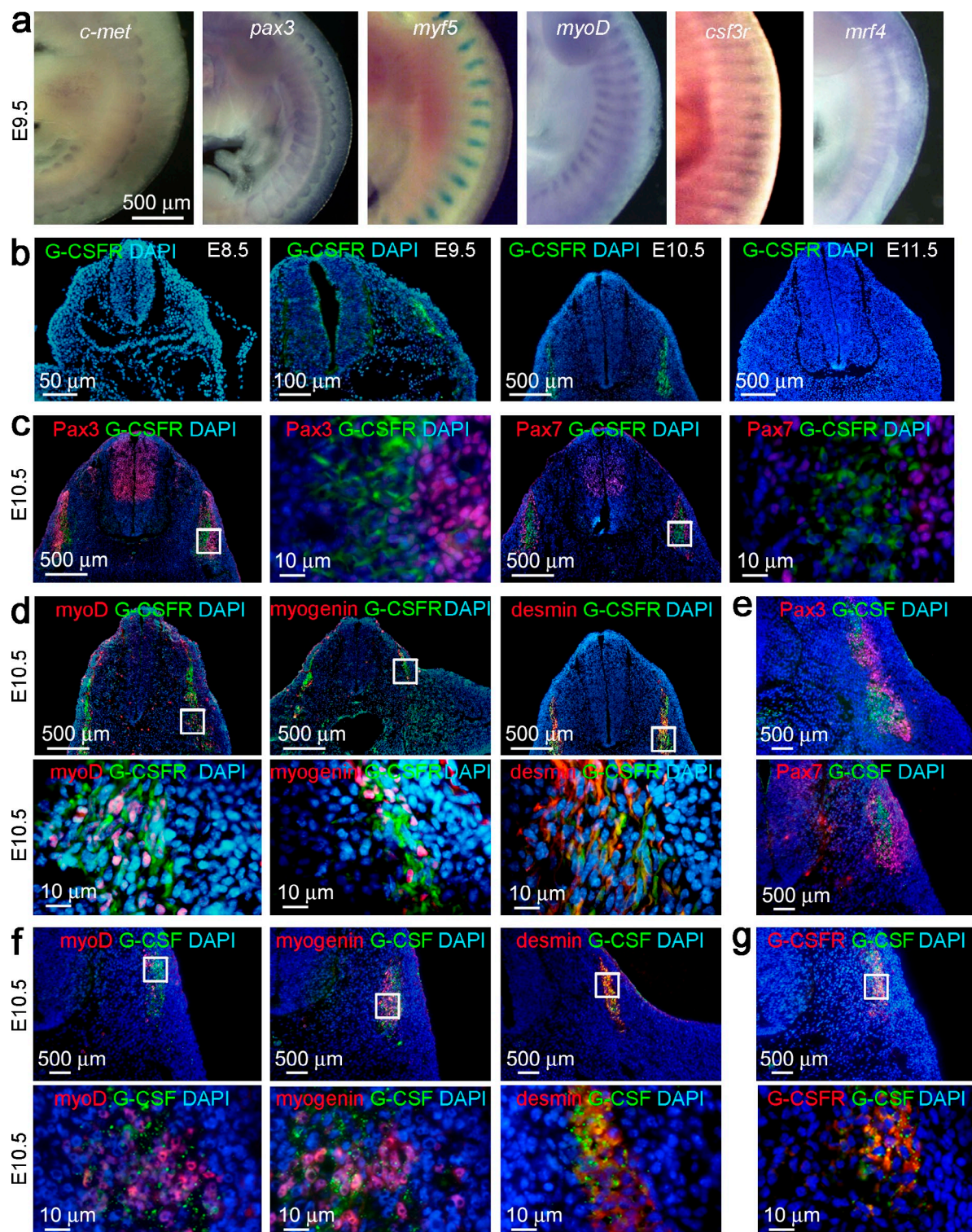
Figure 1. G–CSFR and G–CSF are expressed in developing somites after the midgestation stage. (a) Whole-mount in situ hybridization for *c-met*, *pax3*, *myoD*, *csf3r*, and *mrf4* in E9.5 embryos. The β-galactosidase staining for *myf5* nLacZ knockin mice in E9.5 embryo is also shown. (b) Immunostaining for G-CSFR and nuclei (DAPI) in E8.5, E9.5, E10.5, and E11.5 mouse embryos. (c) Triple immunofluorescence staining for Pax3, Pax7, and G-CSFR in an E10.5 embryo. DAPI indicates nuclear stain. (d) Triple immunofluorescence staining for MyoD, myogenin, desmin, G-CSFR, and nuclei (DAPI) in an E10.5 embryo. (e) Triple immunostaining for G-CSF, Pax3, Pax7, and nuclei (DAPI) in an E10.5 embryo. (f) Triple immunofluorescence staining for MyoD, myogenin, desmin, G-CSF, and nuclei (DAPI) in an E10.5 embryo. (g) Triple immunostaining for G-CSFR, G-CSF, and nuclei (DAPI) in an E10.5 embryo. (c, d, f, and g) Boxed areas are shown at higher magnification in the images to the right (c) or below (d, f, and g). Representative photographs in a are from three independent experiments with 10 embryos. Results in b–g are from five independent experiments.
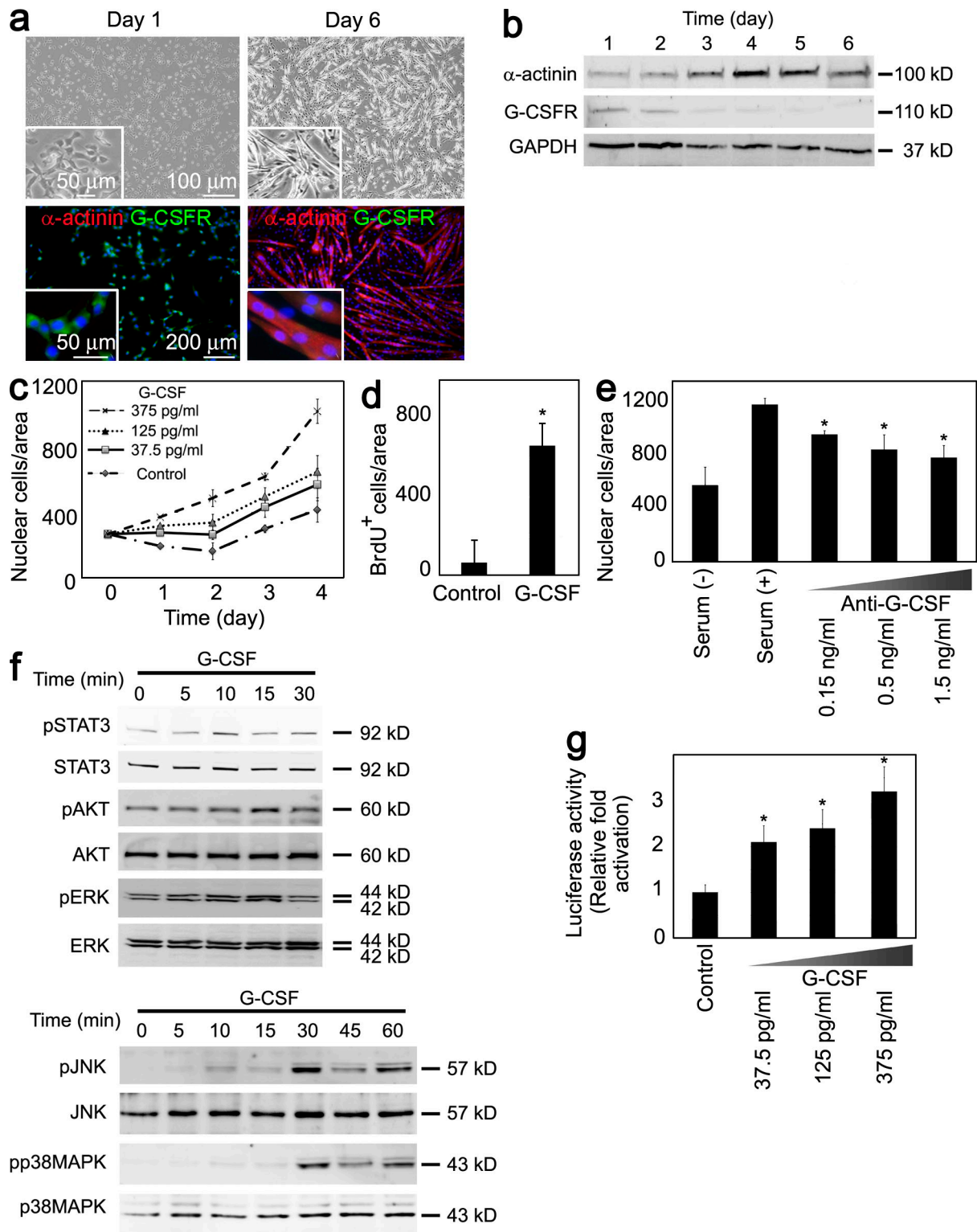
Figure 2.   G-CSF increases myoblast proliferation. (a) Phase-contrast micrography (top) and immunofluorescence (bottom) imaging of G-CSFR and α-actinin in C2C12 myoblast cell line before (day 1) and during (day 6) differentiation induced by low-serum conditions. Inset images are shown at higher magnification. (b) G-CSFR and α-actinin expression was analyzed by Western blot in differentiating C2C12 cells. GAPDH was a loading control. (c) C2C12 cells were cultured with or without the indicated concentrations of G-CSF in low-serum conditions. Cells were counted at the indicated time points. (d) C2C12 cells were cultured with or without G-CSF in low-serum conditions and were pulsed with BrdU. BrdU incorporation was measured at day 3 of differentiation. (e) C2C12 cells were incubated without serum or with serum and the indicated concentrations of G-CSF neutralizing antibody. Cells were counted on day 5 of culture. (f) C2C12 cells were cultured with or without G-CSF for the indicated time points, and phosphorylated and total

G-CSF was administered during C2C12 differentiation at different time points (Fig. S1 a), and myocyte differentiated marker expression was examined. Although G-CSF significantly increases the number of myocytes, G-CSF didn't affect the myocyte differentiated marker expression (Fig. S1 b). Thus, G-CSF plays an essential role in C2C12 cell proliferation.

The binding of G-CSF to its receptor activates various signals, including extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38MAPK, AKT, and STAT, in hematopoietic cells (Avalos, 1996). We confirmed that G-CSF activated STAT3, AKT, ERK, JNK, and p38MAPK in C2C12 cells in a time-dependent manner (Fig. 2 f). Of these factors, STAT3 has been reported to contribute to the proliferation of myocyte precursor cells (Megeney et al., 1996; Serrano et al., 2008). G-CSF addition to C2C12 cell cultures increased the activity of acute phase response element (APRE) luciferase, which responds to STAT3 activation (Fig. 2 g). These results indicate that G-CSF promotes the proliferation of C2C12 myoblasts through G-CSFR.

The G–CSFR is transiently expressed in regenerating skeletal myocytes

In general, the regeneration process resembles the mechanism of physiological development. Based on the finding that G-CSFR was transiently expressed in the developing somite, we expected that regenerating skeletal muscle would express G-CSFR and examined whether it was expressed in regenerating skeletal myocytes after injury. Cardiotoxin damages the myofiber plasma membrane but leaves the basal lamina, satellite cells, and nerves intact, allowing rapid and reproducible muscle regeneration (Hosaka et al., 2002). We injected cardiotoxin directly into the femoral muscles and performed a serial histological analysis up to day 28 after injury. After cardiotoxin injection, spontaneous regeneration of the injured muscle was observed (Fig. 3 a and Fig. S2). From day 1 to 2, several inflammatory cells infiltrated the injured muscle, and the injured myotubes were absorbed. The number of satellite cells or transient-amplifying cells began to increase from day 3, and regenerating myocytes that have centrally located nuclei were clearly identified from day 5 (Yan et al., 2003; Shi and Garry, 2006; Clever et al., 2010). These cells fused and rapidly increased in diameter thereafter. The injured area was filled with the regenerated myotubes, which had centrally located nuclei and smaller diameters than the matured myotubes from day 7. On day 28, the regenerated myotubes had almost the same diameter as the noninjured myotubes, although they had centered nuclei.

Triple immunostaining for laminin, G-CSFR, and DAPI revealed the absence of G-CSFR–positive cells in the noninjured skeletal muscle (Fig. 3 b). In contrast, G-CSFR was clearly expressed in the regenerating myocytes on day 5 after cardiotoxin injection (Fig. 3 c). The G-CSFR–positive cells were larger than the infiltrated inflammatory cells, round-shaped with centrally located nuclei, and completely surrounded by laminin. Thus, these cells were identified as regenerating early myocytes that expressed G-CSFR. Serial immuno-fluorescence staining analyses showed that the G-CSFR–expressing cells appeared only from day 3 to 8 after injury (Fig. 3, d and e).

Muscle repair is characterized by discrete stages of regeneration. In this time period, skeletal muscle regeneration involves the activation of satellite cell or transient-amplifying cell proliferation, differentiation, and maturation (Shi and Garry, 2006). The G-CSFR–expressing day corresponds to the skeletal muscle progenitor cell proliferation day.

Exogenous G-CSF augments skeletal muscle regeneration

To determine whether external administration of G-CSF facilitates skeletal myocyte regeneration, G-CSF was injected after skeletal muscle injury. G-CSF was administered i.v. or was injected i.m. into the injured muscle on day 4 and 6, at which time point G-CSFR was strongly expressed, and skeletal muscle regeneration was observed on day 7. For higher G-CSF dosages, i.v. administration was more effective for skeletal muscle regeneration than PBS administration. For lower G-CSF dosages, i.m. administration was more effective than i.v. (Fig. 4 a). The number of regenerating myocytes was significantly increased by G-CSF administration, and G-CSF administered i.m. significantly augmented skeletal muscle regeneration (Fig. 4 b). G-CSF administration also significantly increased the diameter of the regenerated muscle. The diameter of the rectus femoris was increased to a greater extent by G-CSF administered i.m. than i.v. (Fig. 4 c). Functional recovery was assessed by measuring handgrip strength after cardiotoxin injection into forearm muscles. G-CSF treatment significantly improved functional recovery on 5 and 7 d after skeletal muscle injury (Fig. 4 d).

To investigate whether innate G-CSF signaling is necessary for skeletal myocyte regeneration, an anti–G-CSF neutralizing antibody was administered after injury. This antibody reduced spontaneous skeletal myocyte regeneration in a dose-dependent manner (Fig. 4 e). The number of regenerating myocytes was drastically deceased by treatment with the anti–G-CSF antibody (Fig. 4 f). The diameter of the injured muscle was also significantly decreased by treatment with the anti–G-CSF antibody (Fig. 4 g). Individual skeletal myocyte areas in G-CSF treatment and anti–G-CSF neutralizing antibody addition were measured at day 7 after injury. At day 7, there was a substantial amount of regenerating myocytes, which were small compared with uninjured myocytes. So, the mean of

proteins were measured by Western blot. p, phospho. (g) C2C12 cells were transfected with a STAT3-responsive APRE luciferase reporter construct and were cultured with or without the indicated concentrations of G-CSF. Luciferase activity (relative to control) was measured on day 2 of culture. (c–e and g) Error bars present mean ± SD (*, P < 0.05). Micrographs in a are representative of five independent experiments. Results in b and f are from three independent experiments. Results in c–e and g are from five independent experiments.
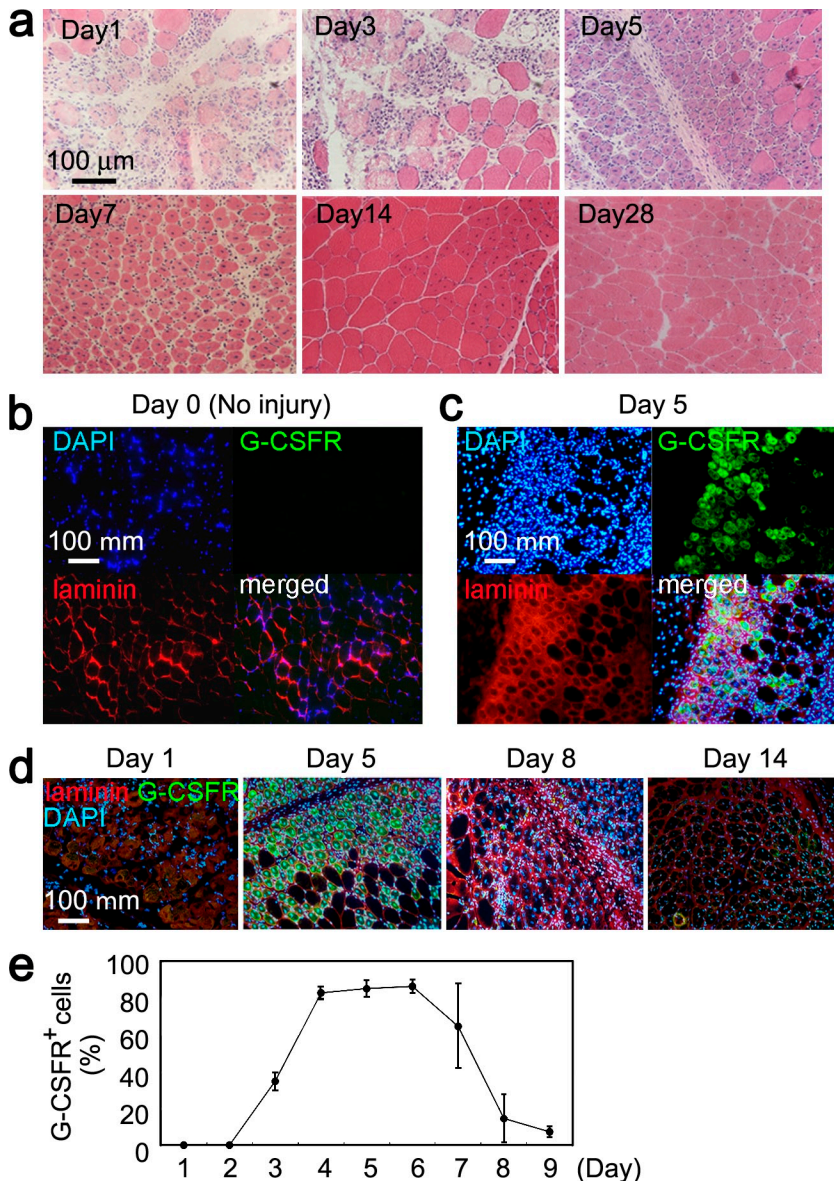
Figure 3. The G-CSFR is expressed in adult regenerating skeletal myocytes. (a) Histological analysis of cardiotoxin-injured skeletal muscle. Hematoxylin and eosin staining of the rectus femoris. (b and c) Triple immunostaining of noninjured (b; day 0) and injured (c; day 5) skeletal muscles for the detection of G-CSFR, laminin, and nuclei (DAPI). (d) Time course of G-CSFR expression in regenerating skeletal myocytes. Immunofluorescently stained injured skeletal muscles on days 1, 5, 8, and 14 are shown. (e) Percentages of G-CSFR–positive cells. The percentages of G-CSFR–positive regenerating skeletal muscle cells were assessed on days 1–9 after injury. Error bars present mean ± SD. Representative photomicrographs in a are from three independent experiments. Results in b–e are from five independent experiments.

approximately half as many as that of wild-type ($csf3r^{+/+}$) mice. Normally, delivered $csf3r^{-/-}$ mice showed no significant differences in appearance. When fully grown, the body size of the $csf3r^{-/-}$ mouse was slightly but significantly smaller than that of the $csf3r^{+/+}$ mouse. The initial histological analysis of the skeletal muscle of the $csf3r^{-/-}$ mouse revealed no significant difference compared with that of the $csf3r^{+/+}$ mouse (Fig. 5 a). However, in the sections of skeletal muscles, the myocytes were slightly but significantly larger in the $csf3r^{-/-}$ mice than in the $csf3r^{+/+}$ mice (Fig. 5 b). Moreover, the diameter of the rectus femoris was significantly smaller in the $csf3r^{-/-}$ mouse than in the wild-type mouse (Fig. 5 c). Although skeletal myocyte proliferation is correlated with hypertrophy in some situations, the molecular pathway of skeletal myocyte proliferation is an independent event of skeletal muscle hypertrophy (Rantanen et al., 1995; Adams et al., 1999; Armand et al., 2005; Philippou et al., 2007). And more, skeletal muscle hypertrophy is an adaptation process for physiological requirements (Sakuma et al., 2000; Solomon and Bouloux, 2006). These findings suggest that in the $csf3r^{-/-}$ mouse, skeletal muscle proliferation is reduced during development, and, as a consequence, the skeletal myocytes are adaptively hypertrophic.

To investigate whether innate G-CSFR is necessary for skeletal myocyte regeneration, $csf3r^{-/-}$ mice were subjected to cardiotoxin-induced skeletal muscle injury. The $csf3r^{-/-}$ mice showed deterioration of skeletal muscle regeneration on day 7 and 14 after injury in the rectus femoris muscles (Fig. 5 d). The number of regenerating myocytes in the regenerating skeletal muscle was significantly decreased in the $csf3r^{-/-}$ mice (Fig. 5 e), which suggests the G-CSFR is essential for skeletal muscle regeneration. To confirm that the observed effect of G-CSF occurred through the G-CSFR, we administrated

individual skeletal myocyte areas is inversely correlated with regeneration in G-CSF treatment and anti–G-CSF neutralizing antibody administration (Fig. 4 h). However, at day 14, regenerated myocytes grew up to uninjured muscle, and there were no significant differences among those groups (unpublished data). These results indicate that exogenous G-CSF augments skeletal myocyte regeneration and that physiological G-CSF signaling plays an essential role in innate skeletal myocyte regeneration.

The $csf3r^{-/-}$ mouse shows impaired skeletal muscle development and regeneration

To clarify the roles of G-CSF and G-CSFR signaling in skeletal myocytes, G-CSFR–knockout ($csf3r^{-/-}$) mice were used. To date, $csf3r^{-/-}$ mice have been used mainly in hematologic studies. The number of delivered $csf3r^{-/-}$ mice was
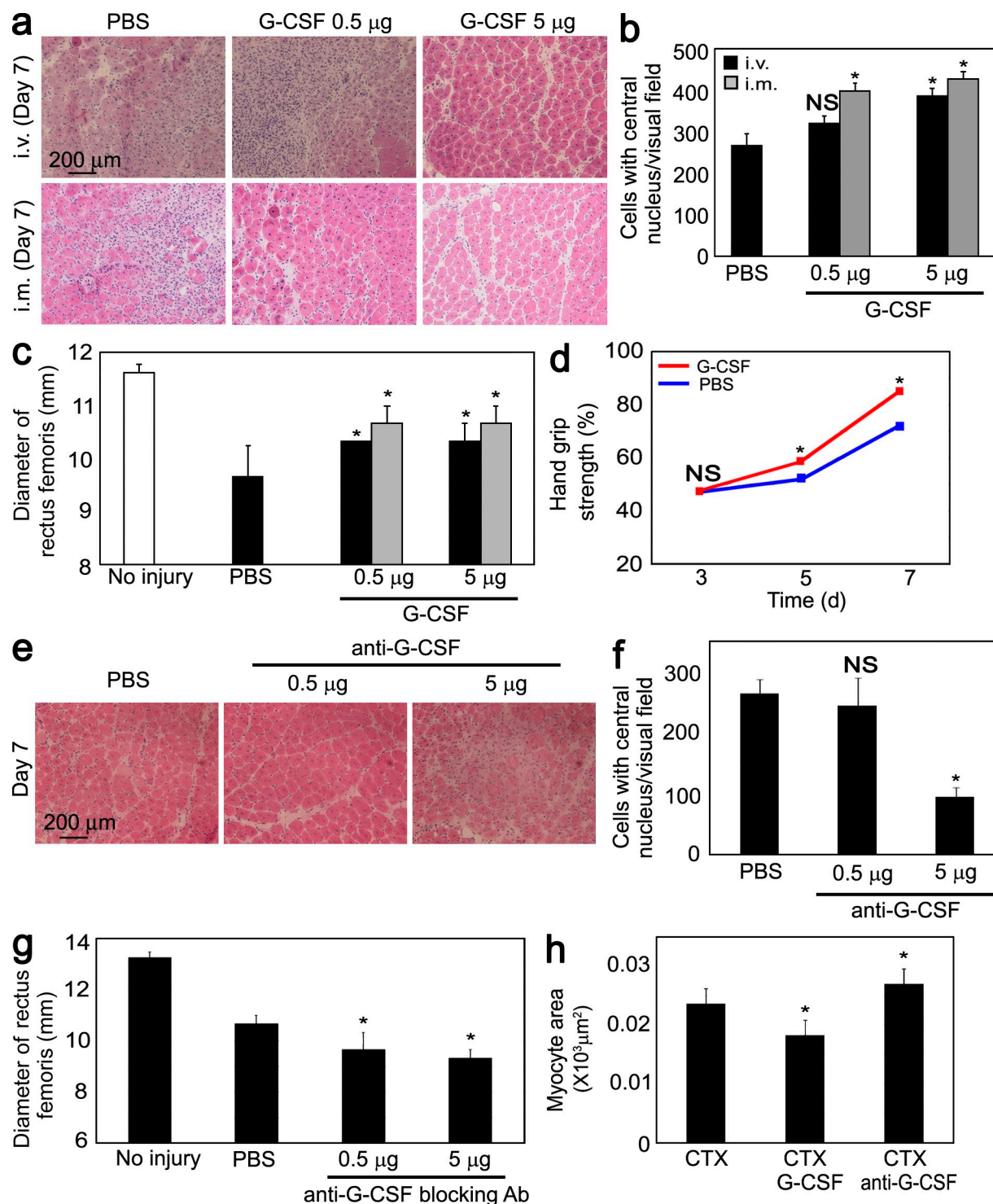
Figure 4. Both intrinsic and extrinsic G–CSF augment skeletal muscle regeneration. (a) Effect of i.v. or i.m. administration of G-CSF on cardiotoxin-induced skeletal muscle injury. Hematoxylin and eosin staining of injured rectus femoris 7 d after cardiotoxin injection. (b) Numbers of regenerating myocytes that have centrally located nuclei. 20 visual fields per individual mice were observed in the rectus femoris at 7 d after cardiotoxin injection. (c) Diameter of the regenerated rectus femoris at 7 d after cardiotoxin injection. (d) Handgrip strength on day 3–7 after cardiotoxin injury. (e) Role of the intrinsic G–CSF signal in skeletal muscle regeneration. Hematoxylin and eosin staining of an injured rectus femoris on day 7 is shown. (f) Numbers of regenerating myocytes that have centrally located nuclei. 20 visual fields per individual mice were observed in the rectus femoris at 7 d after cardiotoxin injection. (g) The diameter of the injured rectus femoris is shown with or without the anti–G-CSF neutralizing antibody (Ab) at 7 d after cardiotoxin injection. (h) Quantitative analysis of the areas of the skeletal myocyte sections. CTX, cardiotoxin. (b–d and f–h) Error bars present mean ± SD (*, P < 0.05). Results in a–h are from eight independent experiments.

G-CSF to the *csf3r*^{−/−} mice. If G-CSF functions through other receptors, the addition of G-CSF should still improve the skeletal muscle regeneration of *csf3r*^{−/−} mice. Exogenous G-CSF administration didn't improve skeletal muscle regeneration (Fig. 5 f). The numbers of regenerating myocytes in the regenerating skeletal muscles were measured. G-CSF administration significantly increased the numbers of regenerating myocytes in the *csf3r*^{+/+} mice but not in the *csf3r*^{−/−} mice (Fig. 5 g). Functional recovery was assessed by measuring handgrip strength after cardiotoxin injection into forearm muscles.
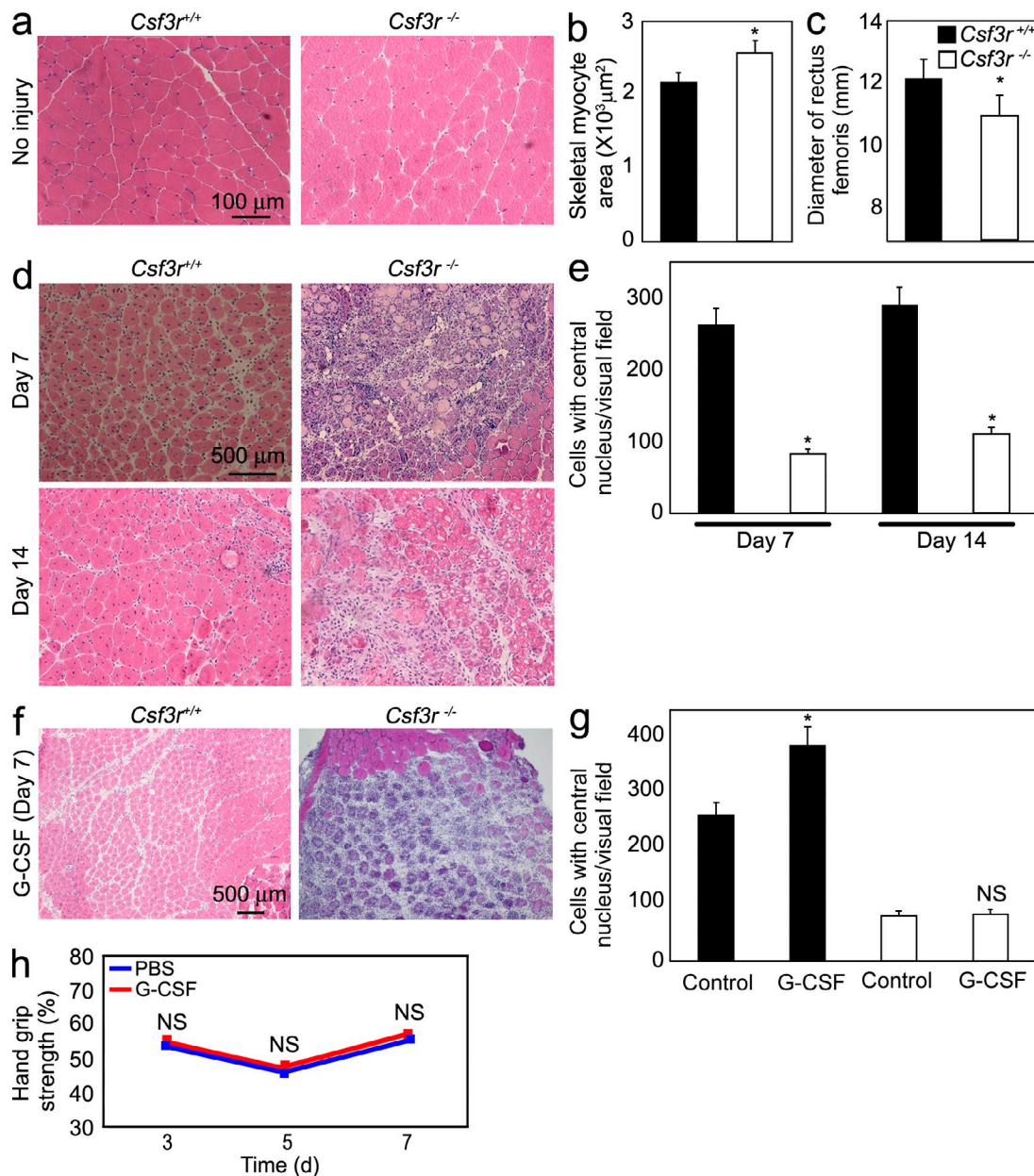

Figure 5. The *csf3r*^{−/−} mouse shows impaired skeletal muscle development and regeneration. (a) Hematoxylin and eosin staining of the rectus femoris of a wild-type mouse and a *csf3r*^{−/−} mouse. (b) Quantitative analysis of the areas of the skeletal myocyte sections in the wild-type and *csf3r*^{−/−} mice. (c) The diameter of the rectus femoris is shown. (d) Hematoxylin and eosin staining of the cardiotoxin-injured skeletal muscles of the wild-type and *csf3r*^{−/−} mice at 7 and 14 d after injury. (e) Numbers of regenerating myocytes that have centrally located nuclei on days 7 and 14 after injury in the regenerating skeletal muscles of the wild-type and *csf3r*^{−/−} mice. 20 visual fields per individual mice were observed in the rectus femoris. (f) Effects of extrinsic G-CSF administration on cardiotoxin-induced muscle injury in the wild-type and *csf3r*^{−/−} mice. Hematoxylin and eosin staining of injured skeletal muscle on day 7 after cardiotoxin injection is shown. (g) Effect of extrinsic G-CSF administration on cardiotoxin-induced skeletal myocyte injury, as assessed by the numbers of regenerating myocytes. 20 visual fields per individual mice were observed in the rectus femoris at 14 d after cardiotoxin injection. (b, c, e, and g) Error bars present mean ± SD (*, P < 0.05). (h) Handgrip strengths of cardiotoxin-injected *csf3r*^{−/−} mice with or without G-CSF treatment. Results in a–h are from eight independent experiments.

G–CSF administration didn't confer functional recovery on day 5 or 7 after injury (Fig. 5 h). To elucidate precise myoblast function, we also examined the proliferation ability of $csf3r^{-/-}$ myoblasts in vitro. The $csf3r^{-/-}$ myoblasts showed significant decreased proliferation ability (Fig. S1 c). However, the expression of myocyte differentiation marker was not altered, which indicates that myocyte differentiation ability was not impaired in $csf3r^{-/-}$ myoblasts (Fig. S1 d).

G–CSFR–expressing BM cells do not recover skeletal muscle regeneration in the $csf3r^{-/-}$ mouse

To clarify the involvement of hematopoietic cells or BM cells in the impairment of skeletal muscle regeneration, we transplanted the BM cells from $csf3r^{+/+}$ mice, which constitutively expressed GFP, to the $csf3r^{-/-}$ mice (Fig. 6 a) 60 d before cardiotoxin–induced injury. In all the mice, the BM cells stably engrafted, and chimerism was >80%, as assessed by FACS (Fig. S3 a). After cardiotoxin injection into forearm muscles, the $csf3r^{-/-}$ mice that were transplanted with BM cells from $csf3r^{+/+}$ mice didn't show any improvement in gross morphology, the number of central cells, and handgrip strength after G–CSF treatment (Fig. 6, b–d). Moreover, the diameter of rectus femoris in these mice wasn't improved by G–CSF treatment after cardiotoxin injection into the rectus femoris muscles (Fig. 6 e). These mice showed no significant improvement in the regeneration by G–CSF treatment, and myocyte area was not altered by G–CSF treatment either (Fig. S3 b).

Next, we performed the BM transplantation experiment in reverse; the BM cells from $csf3r^{-/-}$ mice were transplanted into $csf3r^{+/+}$ mice. In these mice, skeletal muscle injury was generated, and regeneration was induced with G–CSF (Fig. 6 f). G–CSF treatment markedly improved gross morphology, the number of central cells, and handgrip strength after cardiotoxin injection into forearm muscles (Fig. 6, g–i) and increased the diameter of the rectus femoris after cardiotoxin injection into the rectus femoris muscles (Fig. 6 j). These mice showed more regeneration, and mean myocyte area was decreased by G–CSF treatment (Fig. S3 c). These results indicate that G–CSF promotion of skeletal muscle regeneration is a direct effect on skeletal muscle and isn't mediated by BM cells.

DISCUSSION

This study demonstrates that G–CSF and G–CSFR play pivotal roles in skeletal myocyte development and regeneration. Interestingly, this mechanism about G–CSF and G–CSFR is conserved between embryonic skeletal myocyte development and adult skeletal myocyte regeneration. G–CSFR is transiently but strongly expressed in myoblasts during development. The total mass of skeletal muscle is lower in $csf3r^{-/-}$ mice than in $csf3r^{+/+}$ mice, which means that G–CSF and G–CSFR signaling are essential for proper skeletal muscle development. G–CSFR is also expressed in the regenerating adult myocyte. G–CSF stimulates these G–CSFR–expressing myoblasts and promotes skeletal muscle regeneration after injury. The $csf3r^{-/-}$ mice showed drastic impairment of skeletal

muscle regeneration, which suggests that G–CSF is critical for skeletal muscle regeneration.

During development, early muscle progenitor cells are characterized by Pax3 and Pax7 expression. Pax3 and Pax7 cooperatively specify the muscle progenitor pool because in mice deficient for both Pax3 and Pax7, all muscle progenitor cells are absent (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Once specified, muscle progenitor cells either proliferate or exit the cell cycle to undergo terminal differentiation. The latter process requires the activation of MRFs (Sabourin and Rudnicki, 2000). G–CSFR was expressed in cells that expressed MRFs but not in early muscle progenitor cells. Therefore, we speculate that rather than inducing early progenitor cells to increase the skeletal muscle stem cell pool, G–CSF causes late progenitor cells to adopt muscle mass requirement. In adult skeletal muscle, myogenic progenitor cells, which are characterized by the expression of MyoD, Myf5, or MRF4, and myoblasts, which are characterized by MyoD and Myf5 expression, are known as transient-amplifying cells (Weintraub, 1993; Shi and Garry, 2006; Kuang and Rudnicki, 2008; Biressi and Rando, 2010). We found that in the adult stage, G–CSFR was expressed in myoblasts, and G–CSF increased myocyte proliferation.

G–CSF is a hematopoietic cytokine that recruits hematopoietic cells (Cottler-Fox et al., 2003). The contribution of BM cells to muscle regeneration has been documented (Ferrari et al., 1998; Gussoni et al., 1999; LaBarge and Blau, 2002). To exclude the possibility that hematopoietic cells and BM mesenchymal stem cells affect skeletal muscle regeneration in response to G–CSF, we transferred wild-type BM cells to $csf3r^{-/-}$ mice. In these mice, the skeletal myocytes didn't express G–CSFR, whereas the BM cells expressed G–CSFR. If BM cells contributed to skeletal muscle regeneration, these mice would show normal or improved regeneration abilities. However, they didn't show skeletal muscle regeneration in response to G–CSF. This finding is consistent with a report that stromal progenitor cells are mobilized by vascular endothelial growth factor but not by G–CSF (Pitchford et al., 2009). We assume that the contribution of BM cells to G–CSF–mediated skeletal muscle regeneration is negligible.

Skeletal muscle regeneration is a complex process that remains to be fully understood. After muscle injury, disruption of the myofiber plasma membrane initiates an influx of extracellular calcium, leading to calcium-dependent proteolysis, which results in necrosis and degeneration of the myofibers. Several signals released from the degenerating myocytes attract and activate inflammatory cells, which secrete cytokines. Neutrophils are the first inflammatory cells to reach the injured myofibers, followed by macrophages, which phagocyte the degenerating muscle fibers (Chargé and Rudnicki, 2004). Satellite cells and macrophages interact to amplify chemotaxis and enhance inflammation. Monocytes and macrophages may support satellite cell survival by cell–cell contacts and the release of soluble factors (Chazaud et al., 2003). In addition, monocyte and macrophage infiltration leads to increased satellite cell proliferation and differentiation (Lescaudron et al., 1999).

Based on our results, we speculate that macrophages are not only important for the resolution of necrosis but also involved in the induction of muscle regeneration. These leukocytes secrete G-CSF in the presence of appropriate stimuli (Hareng and Hartung, 2002). Although previous studies showed that G-CSF seems to have some positive effects on skeletal muscle regeneration, it's not clear how G-CSF affects skeletal muscle regeneration, and especially the involvement
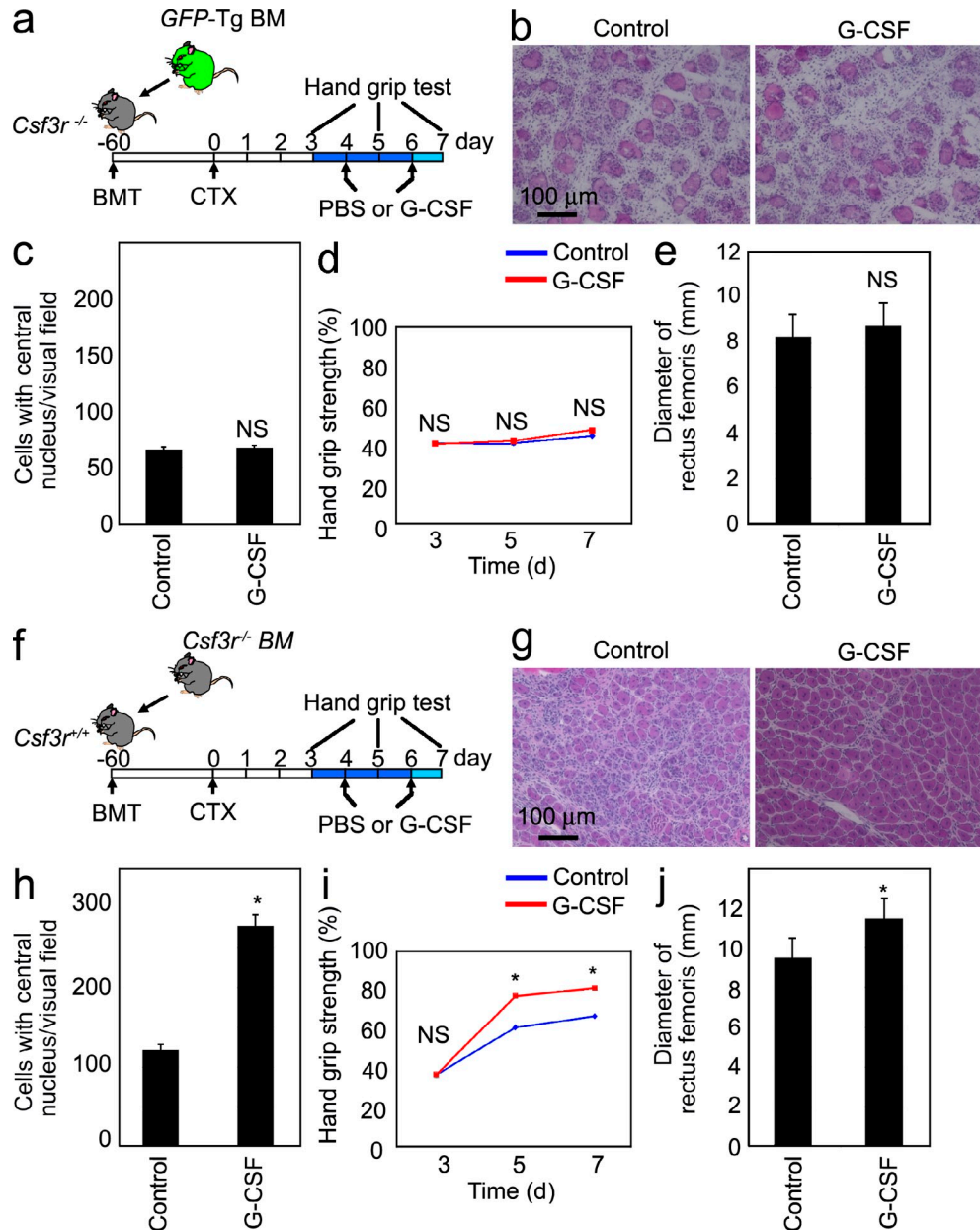

Figure 6. Effect of transplanted G-CSFR–expressing BM cells on skeletal muscle regeneration. (a) Experimental model of BM transplantation 1. BM cells were isolated from GFP-transgenic (Tg) mice and transplanted into the $csf3r^{-/-}$ mice. Cardiotoxin was injected into the rectus femoris, and G-CSF was administered on days 4 and 6. (b–e) Effects of G-CSF on skeletal muscle regeneration of $csf3r^{-/-}$ mice subjected to cardiotoxin-induced skeletal myocyte injury and transplanted with wild-type BM (from GFP-transgenic mice). (b) Hematoxylin and eosin staining of the cardiotoxin-injured skeletal muscles at 7 d after injury. (c) Effect of extrinsic G-CSF administration on cardiotoxin-induced skeletal myocyte injury, as assessed by the numbers of regenerating myocytes. 20 visual fields per individual mice were observed in the rectus femoris at 14 d after cardiotoxin injection. (d and e) Effects of G-CSF on the handgrip strength (d) and rectus femoris diameter at 14 d (e) are negligible. (f) Experimental model of BM transplantation 2. BM cells were isolated from $csf3r^{-/-}$ mice and transplanted into the wild-type ($csf3r^{+/+}$) mice. (g) Hematoxylin and eosin staining of the cardiotoxin-injured skeletal muscles at 7 d after injury. (h) Effect of extrinsic G-CSF administration on cardiotoxin-induced skeletal myocyte injury, as assessed by the numbers of regenerating myocytes. 20 visual fields per individual mice were observed in the rectus femoris at 14 d after cardiotoxin injection. (i and j) Effects of G-CSF on the handgrip strength (i) and rectus femoris diameter at 14 d (j). (c, e, and h–j) Error bars present mean $\pm$ SD (*, P < 0.05). Results in b–e and g–j are from eight independent experiments.

of G-CSFR is not well understood (Stratos et al., 2007; Naito et al., 2009). We proved that BM-derived cells were not directly involved in skeletal muscle regeneration by G-CSF; however, BM-derived cells expressing G-CSF ligand can stimulate skeletal muscle proliferation through myoblast-specific expression of G-CSFR. This study demonstrates for the first time that the factors involved in the inflammatory process switch on the process of skeletal muscle regeneration.

Clinically, G-CSF is used to treat patients with neutropenia resulting from immunosuppressive chemotherapy, severe congenital neutropenia, life-threatening infections, and stem cell harvesting (Hammond et al., 1989; Molineux et al., 1990; Welte et al., 1996). Interestingly, myalgia is one of the main side effects of G-CSF administration in humans (Taylor et al., 1989). We may speculate that innate skeletal muscle regenerates itself to some extent to adapt the physiological turn over, that G-CSF injection stimulates small population of these skeletal myoblasts, and that the burst of skeletal myocyte proliferation gives rise to myalgia. The safety and side effects of G-CSF have been studied in several clinical settings (Anderlini and Champlin, 2008). Therefore, a clinical trial of G-CSF for human skeletal muscle injury may be warranted. The results of this study underline the importance of G-CSF in skeletal muscle development and regeneration and strengthen the case for using G-CSF as a skeletal muscle regeneration therapy.

MATERIALS AND METHODS

Whole-mount in situ hybridization. Mouse embryos were removed from wild-type Institute of Cancer Research pregnant mice on E10.5. Whole-mount in situ hybridization was performed as described previously (Yuasa et al., 2005). The full-length cDNAs for mouse *c-met*, *pax3*, *myoD*, and *mrf4* (available from GenBank/EMBL/DDBJ under accession numbers NM_008591, NM_001159520, NM_010866, and NM_008657 [listed as *myf6*], respectively) were provided by M.E. Buckingham (Pasteur Institute, Paris, France). The full-length cDNA for mouse *csf3r* (GenBank accession number NM_007782) was provided by S. Nagata (Osaka University, Suita, Osaka, Japan; Fukunaga et al., 1990). The probes were generated using T3 or T7 RNA polymerase.

Animals. The *myf5 nlacZ* mice were a gift from S. Tajbakhsh (Pasteur Institute; Tajbakhsh et al., 1996). The *csf3r*$^{-/-}$ mice were a gift from D.C. Link (Washington University School of Medicine, St. Louis, MO; Richards et al., 2003). All the experimental procedures and protocols were approved by the Animal Care and Use Committee of Keio University and conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Immunofluorescence. Mouse embryos on E8.5, E9.5, E10.5, and E11.5 were fixed in 4% paraformaldehyde for 3 h and embedded in Tissue-Tek OCT (Sakura) for frozen sectioning. The samples were incubated with Triton X-100 for 5 min at room temperature, washed, and incubated with the following primary antibodies: anti–G-CSFR (1:50; Santa Cruz Biotechnology, Inc.), anti-Pax3 (1:200; American Type Culture Collection), anti-Pax7 (1:50; R&D Systems), anti-MyoD (1:50; Dako), antimyogenin (1:50; Santa Cruz Biotechnology, Inc.), antidesmin (Dako), anti–G-CSF (1:50; Santa Cruz Biotechnology, Inc.), anti–α-actinin (1:1,000; Sigma-Aldrich), and anti-GAPDH (1:200; Santa Cruz Biotechnology, Inc.). After overnight incubation, bound antibodies were visualized with a secondary antibody conjugated to Alexa Fluor 488 or 546 (Invitrogen). Nuclei were stained with DAPI (Invitrogen). For BrdU staining, a BrdU labeling kit (Roche) was used. After antigen

retrieval using HistoVT One (L6F9587; Nacalai Tesque) and blocking, BrdU staining was performed as described in the manufacturer's protocol.

Myoblast culturing. C2C12 mouse myoblasts (American Type Culture Collection) were cultured in DME/10% FBS (Invitrogen). The medium was replaced with DME/2% horse serum (Invitrogen) to induce differentiation. Recombinant mouse G-CSF (R&D Systems) was added on the indicated days. Inhibition of G-CSF signaling was analyzed by administering an anti–G-CSFR neutralizing antibody (R&D Systems).

Western blotting. C2C12 cells were treated with G-CSF. Cell extracts were prepared at 0, 5, 10, 15, 30, 45, and 60 min after G-CSF stimulation. Protein lysates were resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane, followed by immunoblotting with anti–phospho-STAT3, anti–phospho-AKT, anti–phospho-ERK, anti–phospho-JNK, and anti–phospho-p38MAPK antibodies (all from Cell Signaling Technology) and horseradish peroxidase–conjugated anti IgG, followed by development with the SuperSignal West Pico Chemiluminescent reagent (Thermo Fisher Scientific). The same membrane was retrieved and reblotted with anti-STAT3, anti-AKT, anti-ERK, anti-JNK, and anti-p38MAPK antibodies (all from Cell Signaling Technology), respectively.

Luciferase analysis. C2C12 cells plated in DME were transfected with Lipofectamine (Invitrogen) according to the manufacturer's instructions. The APRE luciferase plasmid was provided by A. Yoshimura (Keio University, Shinjuku, Tokyo, Japan) and used at a dosage of 100 ng. The administered dosages of G-CSF were 37.5, 125.0, and 375.0 pg/ml. CMV-*Renilla* luciferase was used as an internal control to normalize for variations in transfection efficiency. All of the proteins were expressed at similar levels, as confirmed by Western blotting.

Skeletal muscle injury model. 10 µM cardiotoxin (*Naja mossambica mossambica*; Sigma-Aldrich) diluted in 100 µl PBS was injected into the rectus femoris muscles of BL6/J mice using a 27-gauge needle and a 1-ml syringe. The needle was inserted deep into the rectus femoris longitudinally to the knee. Cardiotoxin was injected along the length of the muscle. The mice in the control group were injected with 100 µl PBS. Mice (treated and control groups) were sacrificed at various time points after cardiotoxin injection, and blood samples (1.0–1.5 ml from each mouse) were collected in heparin-rinsed syringes.

Handgrip strength testing. 10 µM cardiotoxin (Sigma-Aldrich) diluted in 100 µl PBS was injected into forearm muscles of BL6/J mice. Five training sessions were performed during which the animals were held, facing the bar of the grip strength meter (Muromachi Kikai), while the forearm was gently restrained by the experimenter. When the unrestrained forepaw is brought into contact with the bar of the grip strength meter, the animal grasps the bar, after which the animal is gently pulled away from the device. The grip strength meter measures the maximal force applied before the animal released the bar.

BM transplantation. BM cells were harvested from 8-wk-old enhanced GFP (EGFP)–transgenic mice. After irradiation with a single dose of 9.0 Gy, the unfractionated EGFP$^+$ BM cells (1 × 10^6 cells) were injected via the tail vein, as described previously (Kawada et al., 2006). To assess chimerism, peripheral blood cells were collected from the recipient mice 60 d after BM transplantation, and the frequency of EGFP$^+$ cells in the population of peripheral nucleated blood cells was determined in a FACS sorter (BD) after hemolysis was induced with ammonium chloride to eliminate erythrocytes.

Statistical analysis. The data were analyzed using the StatView J-4.5 software (SAS Institute, Inc.). Values are reported as means ± SD. Comparisons among groups were performed by one-way analysis of variance. Scheffe's F test was used to determine the level of significance. The probability level accepted for significance was P < 0.05.

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