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.

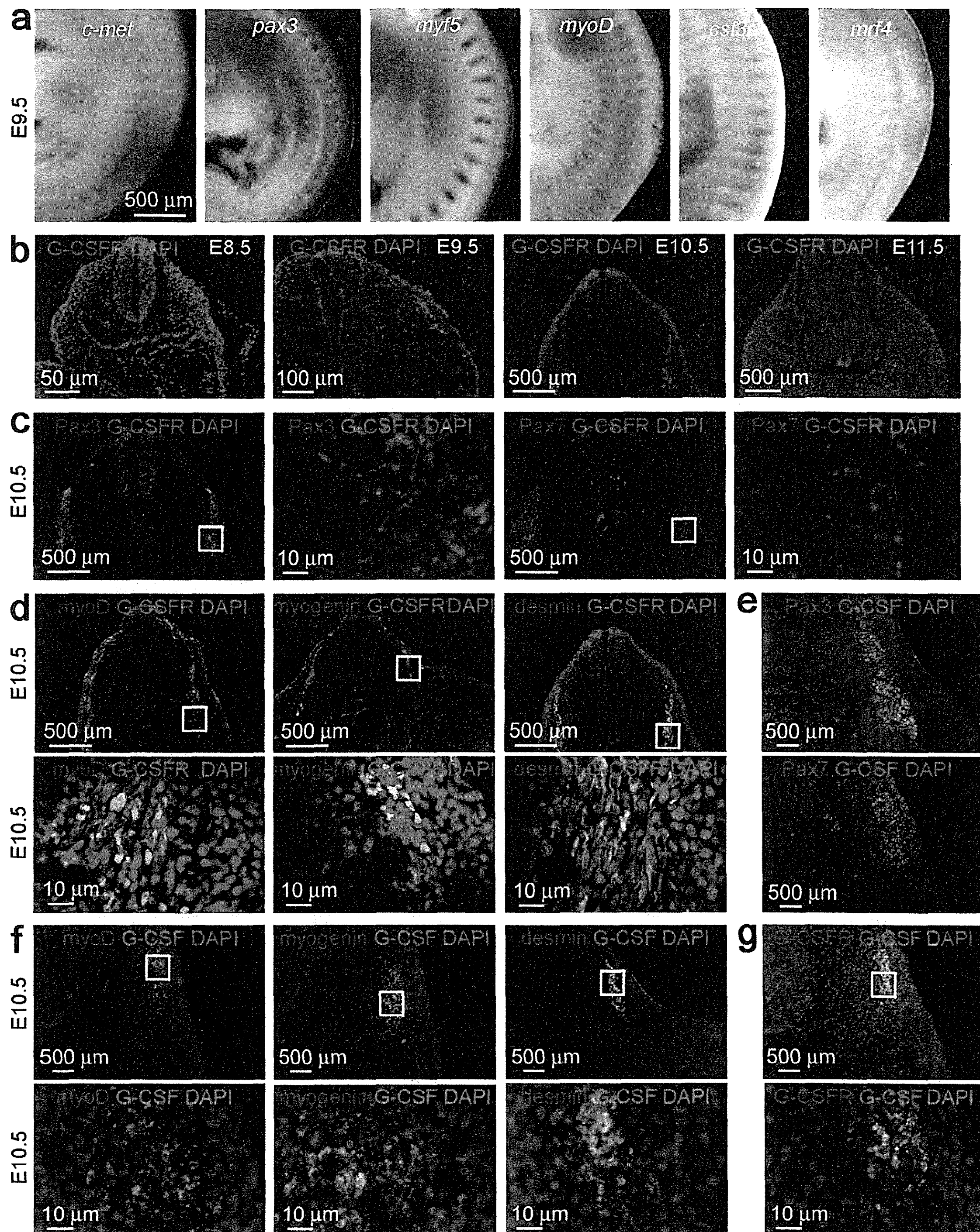

Figure 1. G-CSFR and G-CSF are expressed in developing somites after the midgestation stage. (a) Whole-mount in situ hybridization for *c-met*, *pax3*, *myoD*, *csf3r*, and *mrf4* in E9.5 embryos. The β-galactosidase staining for *myf5* nLacZ knockin mice in E9.5 embryo is also shown. (b) Immunostaining 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.

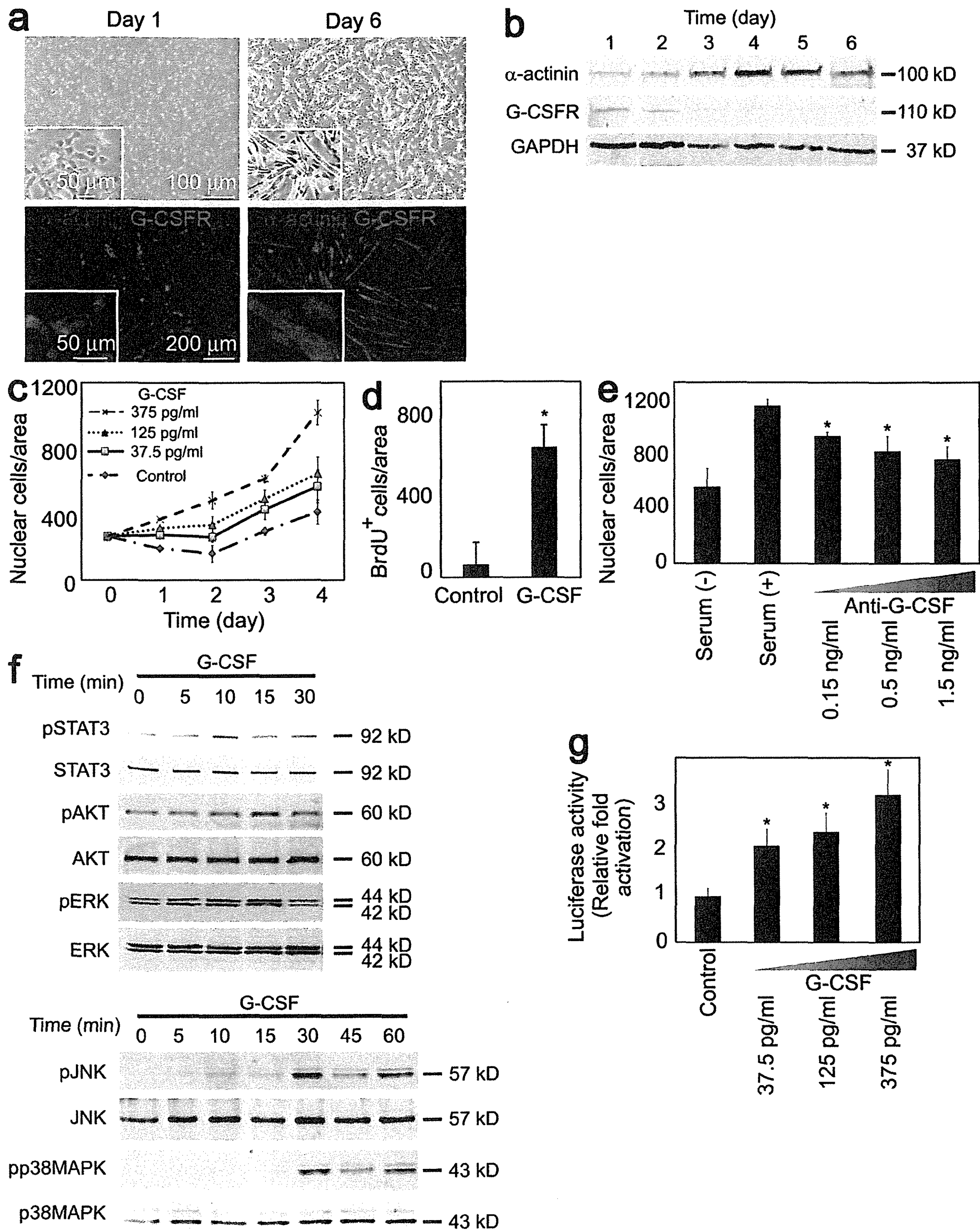

Figure 2. G-CSF increases myoblast proliferation. (a) Phase-contrast micrography (top) and immunofluorescence (bottom) imaging of G-CSFR and α-actinin in C2C12 myoblast cell line before (day 1) and during (day 6) differentiation induced by low-serum conditions. Inset images are shown at higher magnification. (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 immunofluorescence 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.

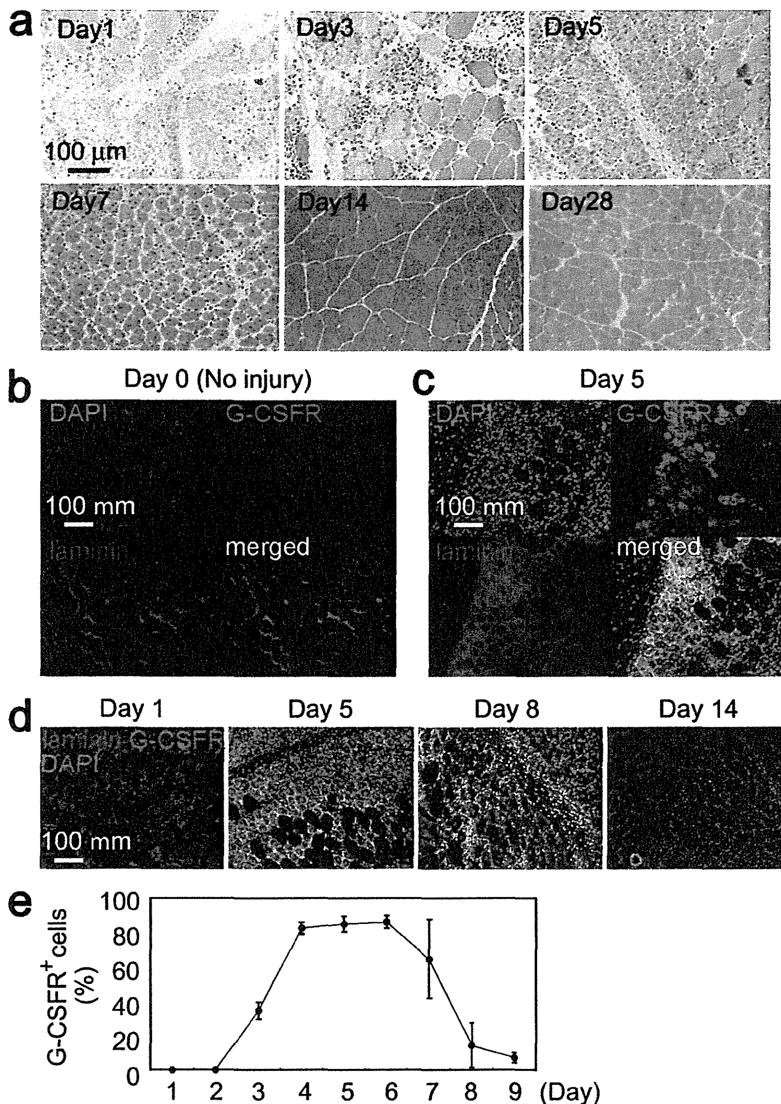

Figure 3. The G-CSFR is expressed in adult regenerating skeletal myocytes. (a) Histological analysis of cardiotoxin-injured skeletal muscle. Hematoxylin and eosin staining of the rectus femoris. (b and c) Triple immunostaining of noninjured (b; day 0) and injured (c; day 5) skeletal muscles for the detection 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.

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 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

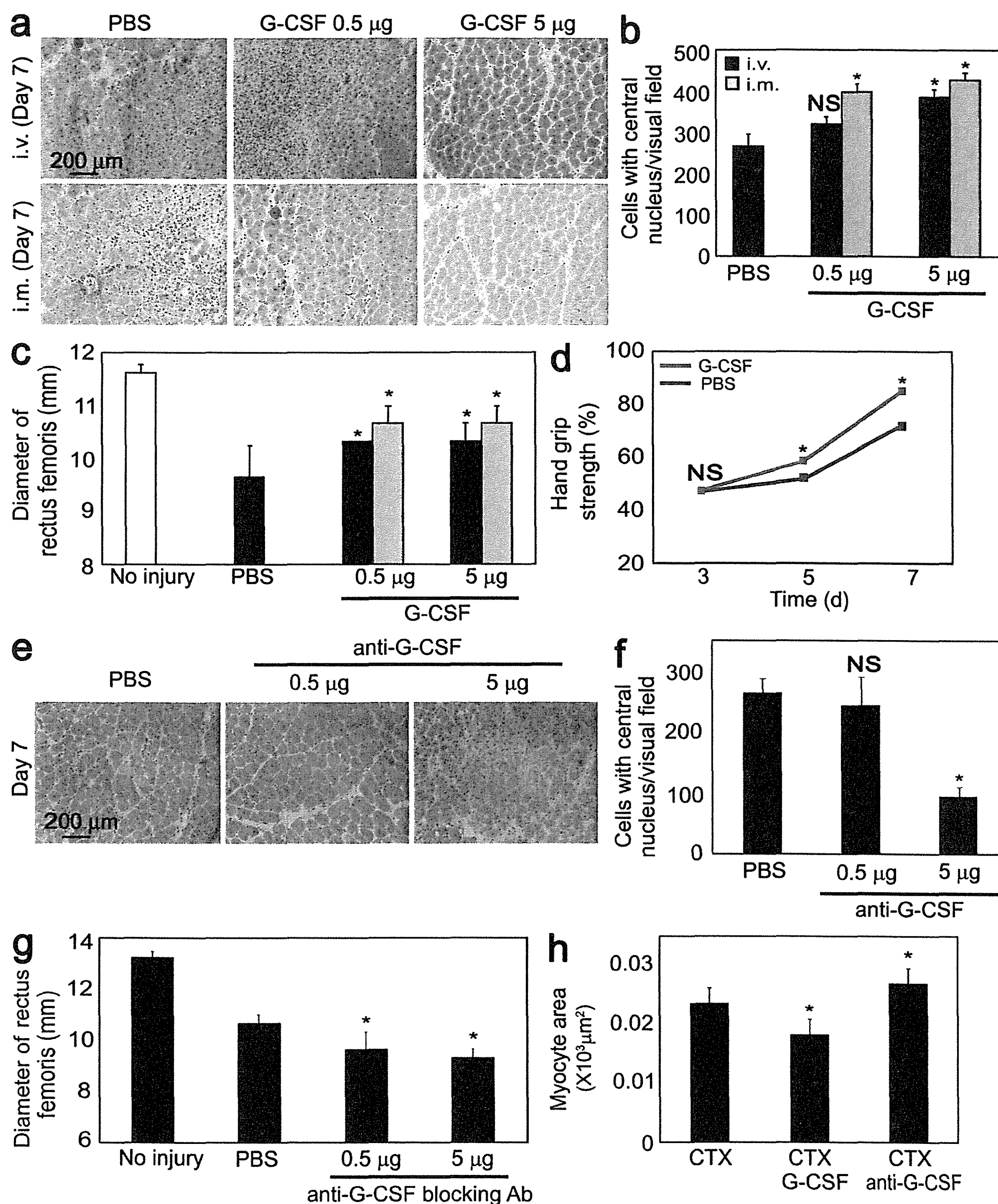

Figure 4. Both intrinsic and extrinsic G-CSF augment skeletal muscle regeneration. (a) Effect of i.v. or i.m. administration of G-CSF on cardiotoxin-induced skeletal muscle injury. Hematoxylin and eosin staining of injured rectus femoris 7 d after cardiotoxin injection. (b) Numbers of regenerating myocytes 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.

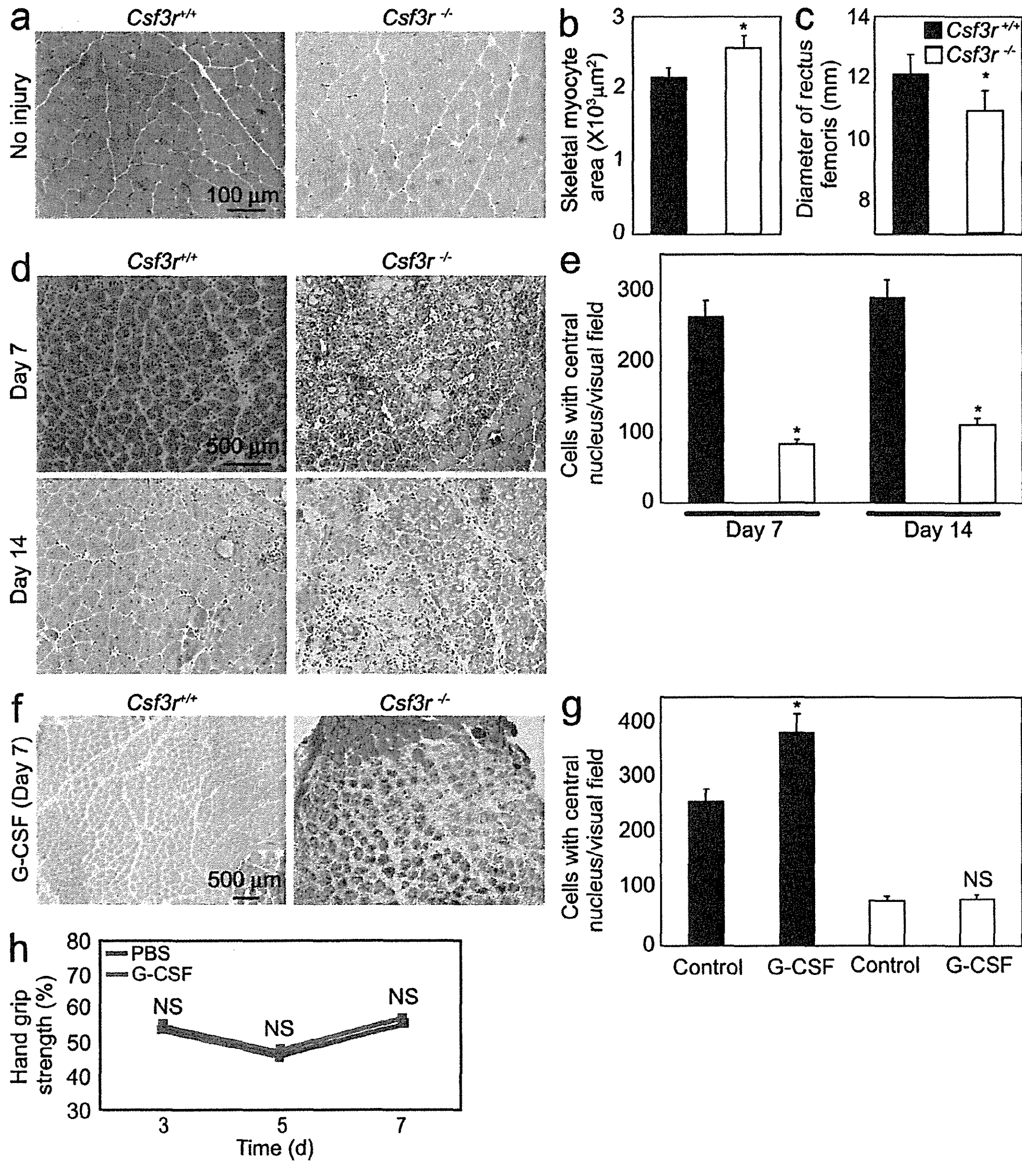

Figure 5. The $csf3r^{-/-}$ mouse shows impaired skeletal muscle development and regeneration. (a) Hematoxylin and eosin staining of the rectus femoris of a wild-type mouse and a $csf3r^{-/-}$ mouse. (b) Quantitative analysis of the areas of the skeletal myocyte sections in the wild-type and $csf3r^{-/-}$ 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

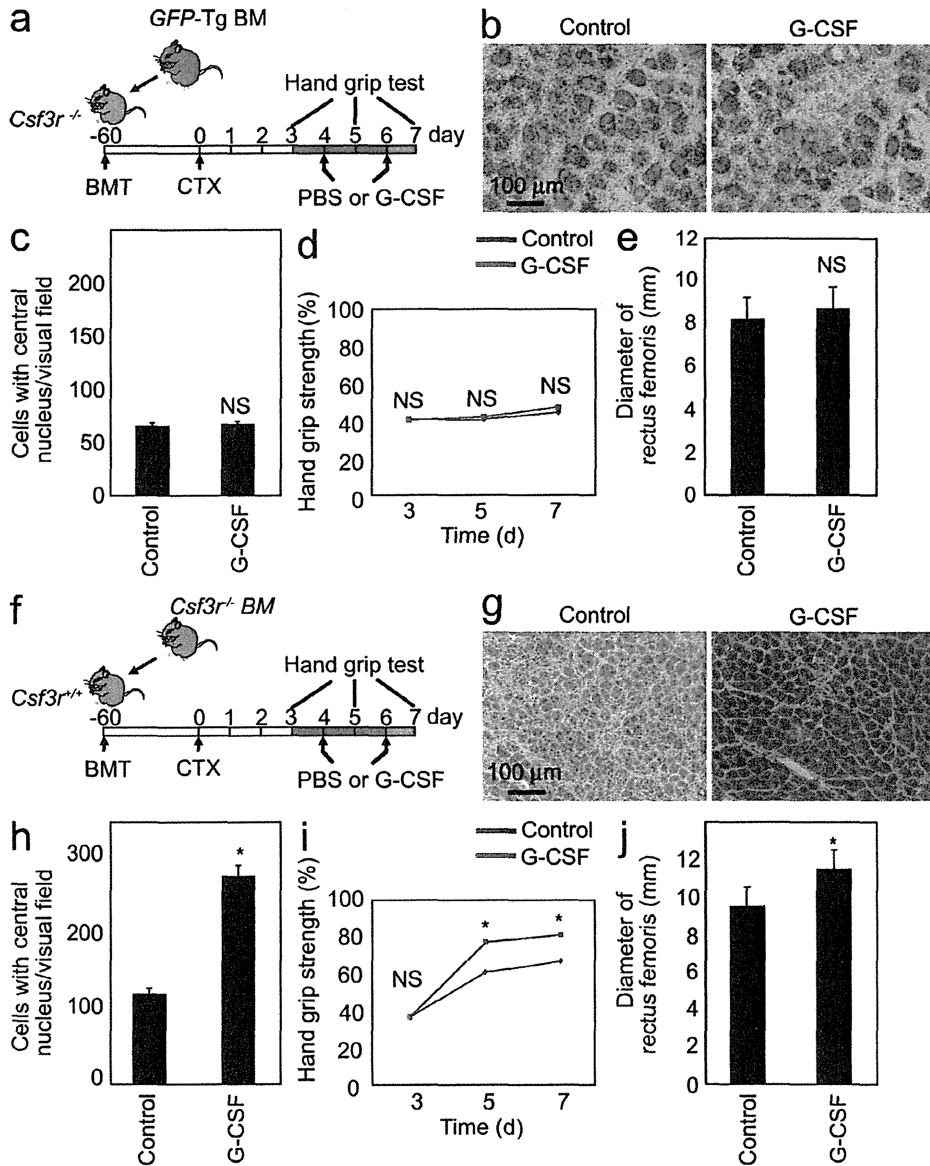

Figure 6. Effect of transplanted G-CSFR–expressing BM cells on skeletal muscle regeneration. (a) Experimental model of BM transplantation 1. BM cells were isolated from GFP-transgenic (Tg) mice and transplanted into the *csf3r*$^{-/-}$ mice. Cardiotoxin was injected into the rectus femoris, and G-CSF 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 ± 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 $P < 0.05$.

Online supplemental material. Fig. S1 shows the effect of G-CSF on myoblast differentiation in C2C12 cells and myoblasts harvested from *csf3r*$^{+/+}$ and wild-type mice. Fig. S2 shows histological analysis of cardiotoxin-injured skeletal muscle from day 1 to 28. Fig. S3 shows the chimerism of hematopoietic cells before and after BM cell transplantation and quantitative analysis of the areas of the skeletal myocyte sections in the *csf3r*$^{-/-}$ and *csf3r*$^{+/+}$ mice with BM transplantation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101059/DC1.

This study was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation.

The authors have no conflicting financial interests.

Submitted: 26 May 2010
Accepted: 11 February 2011

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血管医学

Vascular Biology & Medicine

Vol.14/No.1 2013 3 別刷

iPS細胞を用いた心臓再生医療の現状と未来

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KEY WORDS
- iPS細胞
- 心筋細胞
- 心不全

ヒトiPS細胞が初めて報告され5年が経過したが，ものすごい速さで基礎研究が進んでいる．ヒトiPS細胞の応用としては，大きく分けて再生医療と疾患モデルの構築であり，それらを両輪として論文発表も相次いでいる．後者は病態解明と創薬を目的としており，前者は直接的な治療法として考えられている．心臓病に対する再生医療を実現させるためには，心筋細胞分化，心筋細胞純化そして心筋細胞移植方法などのさまざまな基盤技術が必要になり，それらの開発が進んできており，いよいよ現実味を帯びてきた．本稿では，特に再生医療に対する，これまでの取り組みについて概説していく．

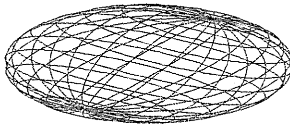
はじめに

循環器領域において，現代の内科的・外科的治療では治癒不能な予後不良疾患が依然として数多くある．特に問題となるのは，心筋梗塞や心筋症などのさまざまな循環器疾患の終末像である末期心不全である．末期心不全は内科的治療にきわめて抵抗性を示し，根本的治療方法は心臓移植しかない．しかしながら，本邦においては臓器移植のための臓器提供が大幅に不足しており，また移植後に免疫抑制薬を終生内服する必要があることなど多くの問題点があり，満足のいく治療法とは言えない．

近年の基礎医学研究の進歩により，これまで治療不能とされてきたさまざまな疾患が再生医療により治療可能となるのではないかと期待されている．再生医療とは，臓器機能不全に陥った際に機能細胞を再生し，臓器の機能を回復しようとする医療である．すなわち，体のすべての臓器・細胞を対象にした新規医療であり，さまざまな基礎研究で成功を収めてきた．しかし現状では，再生医療を一般臨床医療として実現するには，まださまざまな障壁が存在している．本稿では心筋再生医療の変遷と未来像について概説する．

再生医療のために注目される幹細胞の変遷

幹細胞とは，「自己増殖能と多分化能を有する細胞」と定義される．幹細胞は発生早期に多く存在し，組織・臓器・個体形成に貢献しており，成長に伴い減少していくことが広く知られている．また成体の中で，さまざまな臓器においても体性幹細胞として存在し，組織の修復と維持に関与していることが知られてきた．基礎研究の進歩に伴い幹細胞に対する理解が進み，近年は体性幹細胞を分離・選別し，体外で増殖・分化させた後に，病的臓器に移植する再生・細胞移植医療を確



立させようとする試みが世界中で活発になされている．

元来，成人の心筋細胞は終末分化しており，増殖能は無いとされていた．一方で近年の研究により，さまざまな実験動物，さらにヒトにおいても，低い頻度ではあっても成体心筋細胞が増殖能を示すことが示されてきた[1)]．また，成体の心臓の中に自己増殖能と多分化能を有する心筋幹細胞が存在する可能性が示されてきた．これらの心筋幹細胞を体外で増殖させて，病気の心臓に元気な心筋細胞を移植することにより心不全の治療ができるのではないかと考えることができる．これらに関しては，日本，米国を含めたさまざまな国で小規模な臨床研究が行われているのが現状である．しかしながら，一方では「心筋幹細胞は増殖能が限られており，ヒトの末期心不全を治療し効果を得るために十分な量の心筋細胞を得ることは難しい」とも考えられている．これらの効果に対する結論を出すのは早急であり，現在もしくは今後行われていく臨床研究の結果を待ち，安全性と効果を比較検討していく必要がある．

このように，体性幹細胞は成体に存在する幹細胞であるために比較的得るのが容易であり，倫理的問題も少ないという利点があり，すでに臨床研究も行われている．一方で，体性幹細胞は存在する割合が低い，増殖能や分化能が低いといった多数の問題点が存在している．そこで，胎児由来の幹細胞である胚性幹細胞(embryonic stem cell：ES細胞)が再生医療の細胞ソースとして注目されていた．マウスやヒトにおける初期胚である胚盤胞の内部細胞塊に由来するES細胞は，増殖能や多分化能が，体性幹細胞に比べてきわめて優れていることが知られていた．1998年にヒトES細胞が初めて樹立され，いよいよ再生医療へ応用可能になるのではないかという期待が高まってきた[2)]．しかしながら，ES細胞は幹細胞としての性質は非常に秀でているが，他人由来の細胞であるために，問題点としては移植医療に用いた場合には拒絶反応を防ぐために免疫抑制薬を用いる必要がある．また，ヒトES細胞の樹立には初期胚を破壊する必要があるために，ヒトES細胞の樹立および使用自体に関して倫理的・法的問題が解決されていないのが現状である．

しかしながら，そのような問題を打破する研究が報告された．2006年に本邦において，マウス体細胞から人工多能性幹細胞(induced pluripotent stem cell：iPS細胞)の樹立が初めて報告された[3)]．iPS細胞は，ES細胞に多く発現する転写因子を線維芽細胞に遺伝子導入することにより樹立される人工多能性幹細胞である．iPS細胞はES細胞とほぼ同様の能力を有しており，成体の線維芽細胞から作成可能であるために自己体細胞由来の幹細胞を作ることが可能となり，世界中に強い衝撃を与えた．翌1997年には，ヒト皮膚線維芽細胞からのヒトiPS細胞の樹立が初めて報告され，さらにヒト末梢血などからの樹立方法の開発などを経て，ますます臨床応用へ期待されるようになってきた[4)-7)]．iPS細胞は無限に増殖可能で，心筋細胞を含めたあらゆる細胞に分化可能であり，心筋細胞移植医療への魅力的な細胞ソースである．

多能性幹細胞からの心筋細胞分化

iPS細胞は開発されてからの歴史は浅いが，ES細胞とほぼ同等の性質を有することより，心筋細胞分化誘導などに関してはES細胞を用いた研究に関する知見を応用することができる．マウスES細胞の樹立が報告されてから約4年後の1985年には，Doetschman TCらにより，ES細胞から心筋細胞が分化可能であることが報告された[8)]．ES細胞は初期胚に由来しており，ES細胞分化は正常の発生機構を模倣している．正常の心臓発生機構を詳細に解明し，ES/iPS細胞から心筋細胞への分化誘導に応用することにより，効率的に心筋細胞を分化誘導する方法が開発されてきた[9)]．また，1998年にヒトES細胞の樹立が報告され，2001年にはヒトES細胞もまた心筋細胞に分化可能であることが報告された．その頃から，ES細胞由来心筋細胞を心不全モデルの心臓に移植することにより，

末期心不全が治療できる可能性があることが期待されてきた．ES細胞を用いた再生医療を開発・実現化するために，心筋細胞分化誘導効率を高めるためのさまざまな基礎研究が行われ，さまざまな方法が提唱されてきた[10][11]．

2006年にマウスiPS細胞の発明後まもなく，相次いでマウスiPS細胞から心筋細胞への分化とマウスiPS細胞由来心筋細胞の解析が報告された[12]．これらの報告によると，それまでの心筋細胞以外の神経細胞や血液細胞などのさまざまな報告と同様に，心筋細胞に関してもマウスES細胞と同様の分化能も示していることが判明した．ヒトiPS細胞に関してもヒトES細胞と同様に心筋細胞に分化可能であり，その機能も同様であることが示されている[13][14]．

多能性幹細胞を用いた心筋再生療法の開発

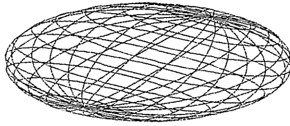
1990年代中盤に入ると，ES細胞から心筋細胞へ分化誘導後に細胞移植を行うことを念頭に研究が進められていくこととなった[15]．ES細胞，iPS細胞から心筋細胞を分化誘導し移植する際に，最初に問題となるのは，心筋細胞以外の細胞を排除する必要があることである．ES/iPS細胞から心筋細胞を分化誘導する際に，すべての細胞を心筋細胞に分化誘導する方法は開発されておらず，必ずほかの細胞と一緒に心筋細胞が得られることになる．移植に際して，心臓に心筋細胞以外のさまざまな細胞を移植することは理想ではなく，心筋細胞だけを純化しようと古くからさまざまな方法が開発されてきた．当初の試みとしては，心筋細胞だけに薬剤耐性遺伝子が発現するような遺伝子操作をES細胞に行い，分化誘導後に薬剤選択を行い心筋細胞だけを純化するというものであった．ヒト多能性幹細胞を用いた再生医療の開発を念頭に置いた場合には，遺伝子導入をすることは理想ではなく，臨床応用には向かない方法である．また，遺伝子操作を行わない心筋細胞純化方法として，心筋／心筋前駆細胞特異的な細胞表明マーカーを用いた心筋細胞の選択や，心筋細胞の特性を利用したミトコンドリア色素を用いた選択方法や，心筋細胞代謝特性を応用した心筋細胞純化培地の開発を行い[16][17]，ヒトへの臨床応用を念頭に置いた方法が考案されつつある．これらの技術を用いて，心筋細胞のみを選択することが可能となり，より安全で効果的な治療方法を開発することが可能となる．

次に考えなくてはいけない問題として，移植方法がある．さまざまな臓器を対象にした細胞移植の際に常に問題となり，病的組織に細胞を注射するだけでは十分な効果が得られにくいと言われている．そこで組織工学技術を用いることにより，心筋細胞シートを作製したり，心筋細胞塊を作製した後に移植することにより，移植心筋細胞の長期生存と心機能に対する効果を期待した試みがなされている．

これらの技術を順次使うことにより，ヒトを対象にした再生医療が現実味を帯びてくる．末期心不全の患者さんから末梢血を採取し，遺伝子導入することにより，患者さん特有のiPS細胞を樹立することが可能である．このiPS細胞から心筋細胞へ分化誘導することにより，若くて元気な心筋細胞が大量に得られ，それらを心不全患者さんの心臓へ移植することにより心機能が改善する．これまでの動物実験の結果からは，ES細胞やiPS細胞由来の心筋細胞を心不全モデル動物に移植することにより，心機能の改善，生命予後の改善が確認されている．現在は世界中で，ヒトiPS細胞からの効率的で安定的な心筋細胞分化誘導方法の開発，効果的な移植技術の開発さらには腫瘍形成の有無などの安全性を検証している．これらの問題が順次解決されていくことにより，将来の再生医療が実現すると考えられる[18]．

おわりに

循環器疾患における幹細胞を用いた再生医療においては，間葉系幹細胞，血管内皮前駆細胞，骨格筋幹細



胞，心筋幹細胞などの体性幹細胞が主役であった．しかしながら，ヒトiPS細胞の樹立に伴い，循環器再生医療の主役はiPS細胞に変わりつつある．これまでも，圧倒的な自己増殖能と多分化能を有するES細胞は将来の再生医療を担うと期待されていたが，自己の細胞でないことと倫理的問題から中心的存在にはなり得ていなかった．iPS細胞はES細胞とほぼ同等の性質を有しており，これまでのES細胞を用いた基礎研究をそのまま応用することができる．心筋再生医療を実現するために，iPS細胞から安定的に大量の心筋細胞を分化誘導する必要があり，そのためにはこれまでのES細胞を用いた基礎研究が有用である．また，心不全モデルへのES細胞由来心筋細胞移植医療の研究は多数なされており，ヒトiPS細胞を用いた再生医療の開発を早期に実現するには，これらの知見が有用である．これらをもとに，さらなる効果の向上と安全性の検証を早期に行い，ヒト臨床研究へ移行することが期待されている．今後は，ヒトiPS細胞から心筋細胞を工場で生産するように生み出し，安定した細胞移植・再生医療の開発へつなげていく必要がある．

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iPS 細胞を用いた循環器診療はどこまで進んだか？

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POINT

1. 2006 年にマウス iPS 細胞の樹立が初めて報告され，翌 2007 年にヒト iPS 細胞の樹立が報告された．ヒト多能性幹細胞が患者から容易に作成することが可能であることより，すぐに世界中で iPS 細胞を用いたさまざまな研究が始まった．
2. 研究の流れは，iPS 細胞樹立が報告された直後の基礎的な iPS 細胞解析や新規樹立方法の開発から，近年は臨床により近い研究に変わってきている．臨床応用に向けて最も期待されているものは，再生医療への応用である．末期心不全などの傷害された臓器に対して iPS 細胞由来の元気な心筋細胞を移植することによる再生医療である．
3. 一方で，遺伝性疾患の病態解明と新規治療方法の開発に向けた疾患モデル作製としての iPS 細胞研究も盛んに行われている．iPS 細胞はゲノムにコードされたすべての遺伝情報を受け継いでおり，遺伝性疾患の原因遺伝子も受け継いでいる．心筋症患者などから iPS 細胞を樹立し心筋細胞を分化誘導することにより，未解決だった病気の原因解明や，同細胞を用いたドラッグスクリーニングなどにより新規治療方法の開発ができるのではないかと考えられる．これらの研究結果をもとに，革新的な新規治療方法の開発が待たれる．

はじめに

2006 年にマウス人工多能性幹細胞（induced pluripotent stem cell：iPS cell）の樹立が京都大学山中伸弥教授らにより初めて報告された[1]．最初の報告はマウス線維芽細胞を用いたものであり，ヒト iPS 細胞が樹立されるようになるまでにはもう少し時間がかかるのではないかと考えられていた．しかしながら翌2007年にはヒトiPS細胞の樹立が報告され[2]，間もなく世界中で爆発的に iPS 細胞研究が進

み始めた．iPS細胞は体細胞から樹立可能な多能性幹細胞であり無限に増殖可能で，体内のどのような細胞にも分化することが可能である．患者体細胞からiPS細胞を樹立した場合には，自己の多能性幹細胞が得られることを意味しており，患者の遺伝情報をすべて有している細胞であるので，移植した際に免疫拒絶されることはないとされている．当初の世界的なiPS細胞研究の流れは，基礎的なiPS細胞の解析や新規樹立方法の開発に重点が置かれていた[3]．しかしながら臨床の場においてもiPS細胞は多くの期待を寄せられており，特に再生医療への応用へ向けた基礎研究が世界中で進められている．さまざまな疾患によって臓器障害に陥った際には，各臓器で機能細胞が減ってくることが知られている．そのような傷害された臓器に対してiPS細胞由来の元気な分化細胞を移植することにより，再生医療を開発しようと試みられている．現在のヒトiPS細胞を用いた再生医療開発の取り組みとしては，長期的な安全性の検証と効果的な細胞移植医療の開発が活発に試みられている．

一方でiPS細胞が患者の遺伝情報を受け継いでいることより，遺伝性疾患の病態解明と新規治療方法の開発に向けた疾患モデル作製としてのiPS細胞研究が行われ始めている．すなわち遺伝性疾患患者より体細胞を得て，同体細胞に遺伝子導入を行うことでiPS細胞の樹立を行う．このようにして樹立されたiPS細胞は患者のゲノムにコードされたすべての遺伝情報を受け継いでいるために，遺伝性疾患の原因遺伝子やさまざまな多型等も受け継いでいる．例えば遺伝性心筋症などの患者からiPS細胞を樹立し，培養皿上で心筋細胞を分化誘導することにより，生きたヒト心筋症心筋細胞がin vitroで容易に作ることが可能である．この病気のヒト心筋細胞を解析することにより，未解決だった病気の原因解明や，同細胞を用いたドラッグスクリーニングなどで新規治療方法の開発ができるのではないかと期待されている．

column

初心者のための用語解説

疾患iPS細胞

さまざまな遺伝性疾患を有する患者から，樹立されたiPS細胞のことである．ヒトにおける遺伝性疾患に関して，原因遺伝子は大きな家系を用いた連鎖解析で決定されることが多かった．しかしながら同遺伝子変異を有している者においても，同疾患が発症しないことも多く，浸透度や多型等が原因であろうとされていた．それを検証するためにこれまでに用いられていたのが遺伝子改変マウスなどの実験動物であるが，実験動物で再現されないヒト疾患が数多く存在し，病気の原因がわからず，治療方法の開発が進んでいない疾患が多数ある．すでに疾患が発症している患者からiPS細胞を作成することにより，多型等も含めたすべての遺伝情報を引き継いだ幹細胞が入手可能で，分化誘導することにより生きた疾患細胞が無限に得られるようになった．同細胞を解析することにより，病態解明やドラッグスクリーニングが可能となる

ヒト末梢血よりiPS細胞樹立方法の確立

このようにさまざまな可能性を有するiPS細胞だが，実際に臨床応用を念頭に研究を始めると，問題点が幾つか残されていることに気づく．その一つは，初期に開発されたヒトiPS細胞の樹立方法は，皮膚生検を行い皮膚組織から線維芽細胞を樹立する必要があることである．皮膚生検は，消毒，麻酔，生検，縫糸，感染予防，抜糸が順次行われていく．皮膚生検といえども手術に準じたものであり，患者の痛み，わずかながらのリスク，そして傷跡の問題がある．特に子どもや女性では痛みや傷跡の問題は避けなければならないものである．iPS細胞を広く臨床応用するためには，侵襲性の低い患者検体からiPS細胞を樹立する方法を開発する必要があると考えられる．末梢血は日常的に血液検査で得ることが可能であり，最も侵襲性が少なく得られる患者検体の一つである．われわれはさまざまな検討の結果，ヒト末梢血中に存在する最終分化Tリンパ球からiPS細胞が効率的に樹立し得ることを見出した．特にわれわれは，センダイウイルスという遺伝子の運び屋を用いることとした．センダイウイルスは広く用いられているレトロウイルスと異なりゲノムへ挿入されることなく，目的の細胞で遺伝子を発現させることが可能なウイルスである．センダイウイルスと少量末梢血から得られる活性化T細胞を組み合わせて用いることにより，ごく少量の血液サンプルからも，ゲノムに外来遺伝子が挿入されずにヒトiPS細胞が効率的に樹立可能であることを見出した[4]．非侵襲的な簡便・迅速・高効率なiPS細胞樹立方法を開発することにより，臨床応用を強く推進できると考えている．

循環器疾患特異的iPS細胞の開発

iPS細胞を再生医療に用いようとする試みは，概念としてはわかりやすく，世界中で多くの試みが成されているが，一方で疾患iPS細胞研究に関しても徐々に研究が進んできている．前述したように，患者由来iPS細胞は患者のゲノムにコードされた全遺伝情報を有しているために，現在治療法がない遺伝性疾患などの病態解明と新規治療方法の開発という観点から，循環器領域においても疾患iPS細胞研究が進んでいる．さらに現在は同モデルを用いた薬剤スクリーニング系の開発と新規薬剤候補が提示されてきており，実臨床に役立つ研究に近づいてきていることが実感される．本稿では，2010年末頃より相次いで報告されている循環器疾患特異的iPS細胞を用いた解析について概説する．

遺伝性QT延長症候群特異的iPS細胞

循環器疾患特異的iPS細胞研究の中でも最も早く報告されてきたものに遺伝性QT延長症候群がある．特に2010年末頃から相次いで報告された．それらはQT延長症候群のtype1，2，8に関して，それぞれiPS細胞の作製とiPS細胞由来心筋細胞の機能解析を行っている．心筋細胞が適切に収縮と拡張を繰り返すためには，脱分極と再分極を適切に繰り返し，電気的恒常性を保つ必要があるが，それらは心筋細胞内のイオン濃度により調節されており，心筋細胞膜に発現するナトリウムチャネル，カリウムチャネルやカルシウムチャネルが重要な役割をしている．遺伝性QT延長症候群の多くは，単一遺伝子変異による疾患であり，細胞膜にあるイオンチャネルの異常により生じる．QT延長症候群は心臓突然死の多くの原因，特に若年突然死の多くの原因になっていると言われているが，根本的な治療方法はないのが現状である．現実には，ある程度の効果が期待される薬物療法と植込み型除細動器を組み合わせて治療することになる．適切な動物モデルもないのが現状であり，これまでは基礎研究の進展も期待されてはいなかった．ヒトiPS細胞を用いることでQT延長症候群の詳細な分子生物学的・電気生理学的な解析と根