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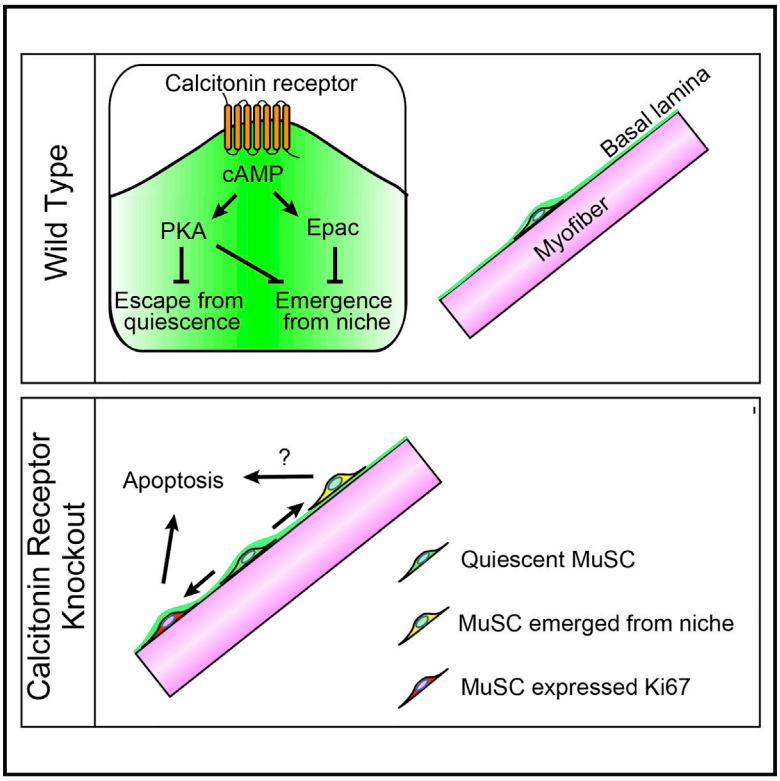
Calcitonin Receptor Signaling Inhibits Muscle Stem Cells from Escaping the Quiescent State and the Niche

Graphical Abstract



Highlights

- The calcitonin receptor (Calcr) maintains the adult MuSC pool
- The Calcr-cAMP-PKA pathway keeps MuSCs in their quiescent state
- Loss of Calcr allows emergence of MuSCs from their niche
- Calcr-cAMP-PKA and -Epac signaling inhibits emergence of MuSCs from their niche

Authors

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In Brief

Muscle stem cells express calcitonin receptor, a hormone receptor, but its physiological role is unclear. Yamaguchi et al. demonstrate that the calcitonin receptor is important in maintaining muscle stem cells in the quiescent state and in their niche.

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Calcitonin Receptor Signaling Inhibits Muscle Stem Cells from Escaping the Quiescent State and the Niche

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SUMMARY

Calcitonin receptor (Calcr) is expressed in adult muscle stem cells (muscle satellite cells [MuSCs]). To elucidate the role of Calcr, we conditionally depleted Calcr from adult MuSCs and found that impaired regeneration after muscle injury correlated with the decreased number of MuSCs in Calcr-conditional knockout (cKO) mice. Calcr signaling maintained MuSC dormancy via the cAMP-PKA pathway but had no impact on myogenic differentiation of MuSCs in an undifferentiated state. The abnormal quiescent state in Calcr-cKO mice resulted in a reduction of the MuSC pool by apoptosis. Furthermore, MuSCs were found outside their niche in Calcr-cKO mice, demonstrating cell relocation. This emergence from the sublaminar niche was prevented by the Calcr-cAMP-PKA and Calcr-cAMP-Epac pathways downstream of Calcr. Altogether, the findings demonstrated that Calcr exerts its effect specifically by keeping MuSCs in a quiescent state and in their location, maintaining the MuSC pool.

INTRODUCTION

Adult stem cells are maintained in a reversible quiescent state (or G0) and reside in a specific microenvironment referred to as a niche. Quiescent adult stem cells show low levels of transcriptional activity and energy production (Freter et al., 2010; Rodgers et al., 2014). In culture, serum deprivation forces many types of cells to enter a G0-like dormancy. Given that the quiescent state is a transient state that results from escaping the cell cycle due to the absence of growth factor, it had been assumed that the quiescent state is a "passive," rather than an "active," state. However, recent evidence suggests that quiescence is not merely a passive state (Cheung and Rando, 2013).

Quiescent adult stem cells specifically or highly express coding and non-coding genes in a G0-specific manner not observed in activated or proliferating progenitors (Cheung et al., 2012; Codega et al., 2014; Fukada et al., 2007; Tumbar et al., 2004; Venezia et al., 2004). In addition, a recent study showed that many kinds of G-protein-coupled receptors (GPCRs) are expressed more abundantly in quiescent neural stem cells (NSCs) than in activated NSCs: two ligands (sphingosine-1-phosphate [S1P] and prostaglandin D_2 [PGD_2]) of these GPCRs inhibit activation of quiescent NSCs ex vivo (Codega et al., 2014). On the other hand, two secondary messengers of GPCR, Gsα and Gq/11α, are required in stem-cell-like chondrocytes to maintain the quiescent or undifferentiated state (Chagin et al., 2014). Thus, GPCR-mediated signaling may be a common pathway to keep some types of adult stem cells from escaping the quiescent and undifferentiated state. However, their cellular receptors have not been fully identified in adult stem cells.

Adult muscle stem cells, also known as muscle satellite cells (MuSCs), are responsible for skeletal muscle regeneration after injury to the muscle. During postnatal development, MuSCs enter the G0-phase quiescent state and are maintained in the niche between the basal lamina and sarcolemma (the cell membrane of myofibers). By utilizing highly purified quiescent MuSCs and microarray analyses, we previously identified a GPCR receptor, *calcitonin receptor* (*Calcr*), as a "quiescence" gene in MuSCs (Fukada et al., 2007). Calcr is one of seven transmembrane-domain receptors known to regulate homeostasis of the

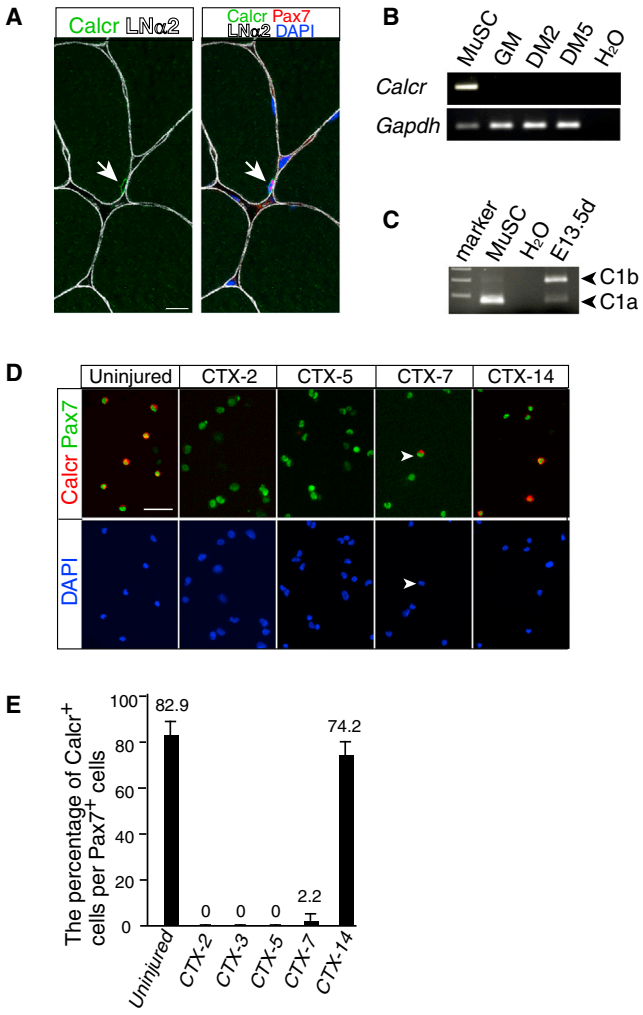

Figure 1. Expression of Calcitonin Receptor in Muscle Stem Cells

(A) Calcr (green) expression was detected on Pax7$^+$ (red) cells locating beneath the basal lamina (white; LNα2 laminin α2). Arrow indicates a Calcr$^+$Pax7$^+$ MuSC.

(B) *Calcr* mRNA was detected in freshly isolated MuSCs, but not in MuSCs cultured in growth medium for 3 days (GM) or cells additionally cultured in differentiation medium for 2 (DM2) or 5 days (DM5). As a negative control, the cDNA template was omitted from the reaction (H_2O).

(C) The PCR product of C1b mRNA is 111 bp longer than that of C1a. Ladder size markers (100 bp) are shown (marker). Mouse embryo (E13.5) or H_2O were used as positive and negative controls, respectively.

(D) Mononuclear cells were prepared from uninjured, regenerating muscle 2, 5, 7, or 14 days after CTX injection, and sorted myogenic cells on glass slides were immediately stained with anti-Calcr (red), Pax7 (green), and DAPI (blue).

(E) The graph indicates percentages of Calcr$^+$ cells in Pax7$^+$ cells during regeneration. Arrowheads indicate Calcr and Pax7 double-positive cells on the seventh day.

Scale bars, 10 μm (A) and 50 μm (D).

calcium level in blood (Becker et al., 2002). Osteoclasts abundantly express Calcr receptor, and the Calcr ligand, calcitonin, inhibits osteoclast-mediated bone resorption by disrupting actin organization (Suzuki et al., 1996). Although the relevance of Calcr in the bone system is well studied, the roles of Calcr in other locomotive tissues, including skeletal muscle, have not been investigated. In the present study, we aimed to identify the physiological consequences of Calcr depletion in MuSCs using *Calcr* conditional knockout mice and MuSC-specific Cre recombinase-expressing mice.

RESULTS

Calcitonin Receptor Is Expressed Exclusively in Quiescent Muscle Stem Cells

In our previous study, we compared the gene expression profile of quiescent MuSCs with those of activated and proliferating MuSCs (myoblasts) and non-myogenic cells (Fukada et al., 2007). We found 63 MuSC-specific quiescence genes in skeletal muscle, and *calcitonin receptor (Calcr)* was one of them. As shown in Figure 1A, we confirmed that MuSCs (stained positive for paired box 7 [Pax7]) expressed Calcr at the protein level (Figure 1A). Myogenic cells cultured in a proliferating or differentiating condition, on the other hand, did not show any detectable *Calcr* mRNA (Figure 1B). In rodents, two known major isoforms of *Calcr* mRNA, C1a (osteoclast type, lacking exon 10) and C1b (found in brain), are generated by alternative splicing (Sexton et al., 1993). Here, the MuSCs expressed the C1a isoform (Figure 1C).

Next, we examined the level of Calcr protein expression during regeneration after injury to the muscle by cardiotoxin (CTX) injection. We directly isolated myogenic cells from uninjured and regenerating muscle at six recovery time points. Consistent with our previous study using tissue sections (Fukada et al., 2007), approximately 80% of Pax7$^+$ cells (indicating MuSCs) from uninjured muscle expressed Calcr (Figures 1D and 1E). However, while Pax7$^+$ cells from regenerating muscle 2–5 days after CTX injection were completely negative for Calcr, we observed Pax7$^+$/Calcr$^+$ double-positive cells on day 7 post CTX injury (Figure 1D). Approximately 2% of Pax7$^+$ cells expressed Calcr on day CTX-7, and the percentage had increased significantly to 74.2% on the 14th day after CTX injection (the time point by which most cellular regeneration is presumed to have taken place) (Figure 1E).

Depleting Calcitonin Receptor Reduces Muscle Stem Cell Pool

We generated *Calcr*-floxed mice (Figures S1A and S1B) and crossed them with *Pax7*$^{CreERT2/+}$ mice (Figure 2A) to generate *Pax7*$^{CreERT2/+}$*::Calcr*$^{flox/flox}$ mice (cKO). In these mice, genetic inactivation of *Calcr* was induced by tamoxifen (Tm) administration. Because Pax7 is specifically expressed in MuSCs of adult skeletal muscle, the effect of depletion of Calcr by the Tm injection was limited to MuSCs. Two or 5 weeks after the Tm injection into older than 8-week-old cKO mice, less than 1% of the cells expressed Calcr in cKO-MuSCs (Figure 2B; Cont > 80% versus cKO < 1%). mRNA transcriptional depletion was also confirmed in these mice (Figure S1C).

We then investigated the role of Calcr in MuSCs. First, we detected a decreased number of MuSCs after Tm treatment in cKO mice using fluorescence-activated cell sorting (FACS) (Figures 2C and 2D). At 16 weeks after Tm treatment, the MuSC fraction was decreased approximately 35%–80% in

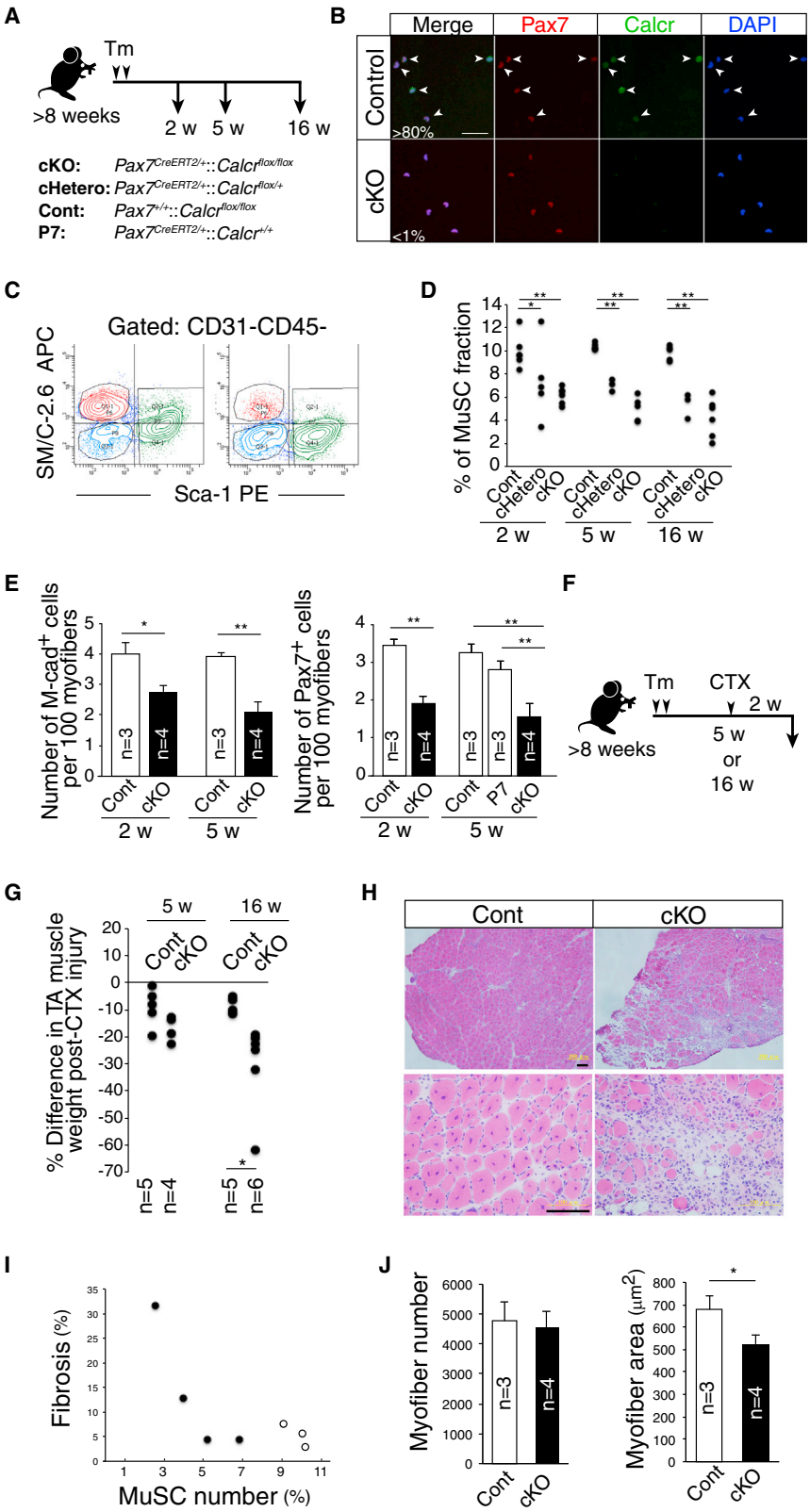

Figure 2. Calcitonin Receptor Is Necessary to Maintain Adult MuSC Pool

(A) The cartoon indicates the time course for analysis of mice after tamoxifen (Tm) injection. *Calcr*$^{flox/flox}$ (Cont), *Pax7*$^{CreERT2/+}$::*Calcr*$^{+/+}$ (P7), *Pax7*$^{CreERT2/+}$::*Calcr*$^{flox/+}$ (cHetero), and *Pax7*$^{CreERT2/+}$::*Calcr*$^{flox/flox}$ (cKO) mice were used in the following studies. All mice were treated with Tm.

(B) Freshly isolated MuSCs were stained with antibodies against Pax7 (red) and Calcr (green). Arrowheads indicate cells positive for both Calcr and Pax7.

(C) FACS profiles of mononuclear cells derived from Cont (left) or cKO (right) muscles 16 weeks after Tm injection. The profiles were gated for CD31^{-}CD45^{-} fractions.

(D) The y axis shows the percentages of Cont, cHetero, and cKO-MuSC fractions after Tm injection as indicated in the x axis. Each black circle indicates the results of one mouse.

(E) The y axis indicates the numbers of M-cadherin^{+} or Pax7^{+} cells per 100 cross-sectional TA myofibers in Cont, P7, and cKO after Tm injection at indicated weeks in the x axis.

(F) Time course for analysis of cKO mice after Tm or CTX injection.

(G) The y axis shows the difference in TA muscle weight after regeneration per opposite uninjured muscle. Each black circle indicates the result of one mouse.

(H) H&E staining of TA muscles of Cont or cKO mice treated with Tm after 16 weeks.

(I) Correlation between fibrosis area and MuSC number. Open or closed circles indicate Cont or cKO mice, respectively.

(J) The y axis indicates the number or area of myofibers.

The number of mice used is shown in each graph (E, G, and J). *$p < 0.05$, **$p < 0.01$ (D, E, and G). Scale bars, 50 μm (B) and 100 μm (H).

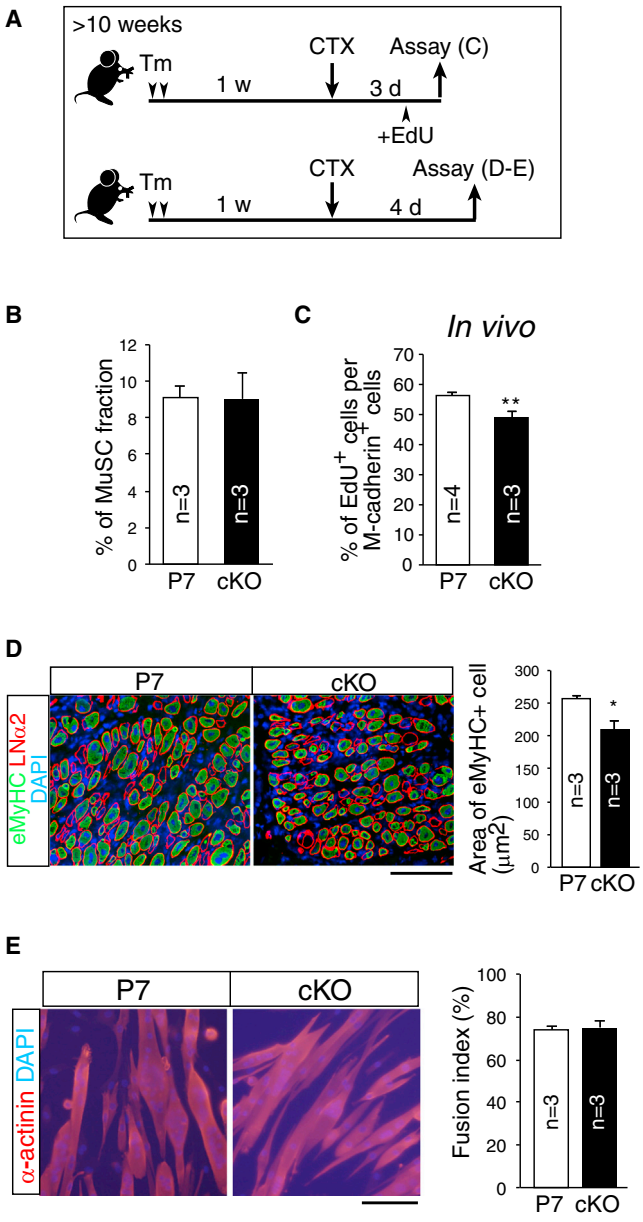

Figure 3. Loss of Calcr Secondarily Affects MuSC Proliferation

(A) Time course for analysis of cKO mice after Tm or CTX injection.

(B) The y axis indicates the percentage of the MuSC fraction.

(C) The y axis shows the mean percentage of EdU$^+$ cells in M-cadherin$^+$ cells.

(D) Immunostaining of embryonic myosin heavy chain (eMyHC, green), laminin α2 (red), and DAPI (blue) in injured muscle 4 days after CTX injection. The y axis shows the eMyHC$^+$ area.

(E) Myotubes were stained with anti-sarcomeric α-actinin antibody (red) and DAPI (blue). The y axis shows the percentage of fusion index.

Scale bars, 100 μm (D and E).

cKO mice compared with the control mice. Heterozygous mice (*Pax7*$^{CreERT2/+}$::*Calcr*$^{flox/+}$, cHetero) also showed a decrease in the MuSC fraction, although the decrease in the MuSC number was less than that of cKO mice (Figure 2D). Moreover, we quantified the number of MuSCs by immunohistochemical studies, providing further evidence that the quantity of MuSCs decreased in cKO mice (Figure 2E).

The significant decrease in the MuSC pool affected the skeletal muscle weight following injury (Fukada et al., 2011). We measured the muscle weight 2 weeks after CTX injection (Figure 2F). Five weeks after Tm injection, cKO mice demonstrated only a tendency toward a smaller muscle weight recovery (Figure 2G). The cKO group 16 weeks post Tm injection exhibited a significantly smaller muscle weight recovery, though there was individual variation. For example, one mouse recovered only 40% of the muscle weight on the injured side compared to the contralateral side, but another recovered 70% (Figure 2G). The reduction in MuSC numbers correlated with the weight loss and increased fibrotic area (Figures 2H and 2I; Figure S2). When the number of MuSCs retained was greater than 5% of all muscle-derived mononuclear cells in cKO mice, as analyzed by FACS, the mice did not exhibit an increase in fibrotic area nor a decrease in myofiber number. The myofiber area, on the other hand, consistently decreased in all cKO mice, regardless of the individual variance in muscle weight (Figure 2J).

Loss of Calcitonin Receptor Affects Proliferation of Muscle Stem Cells

As shown in Figure 1, proliferating myoblasts did not express Calcr. However, it is possible that the absence of Calcr in MuSCs exerts a secondary effect on the regenerative processes. In order to further examine the effect of Calcr loss in MuSCs after injury, we investigated mice 1 week after Tm injection because these mice still have a similar number of MuSCs as the control mice, while Calcr mRNA was significantly depleted (Figures 3A and 3B; Figure S1C).

The proliferation of MuSC-derived myoblasts in cKO mice, visualized using EdU and M-cadherin antibodies, was inferior to that of control myoblasts (Figure 3C). The area of newly generated myotubes, as shown in Figure 3D by immunostaining, also decreased in cKO samples. We then tested the cellular capacity to form myotubes ex vivo by staining and found, in contrast, that there was no morphological difference between the control group and the cKO group (Figure 3E). The results suggest that the presence of Calcr in MuSCs is secondarily necessary for muscle regeneration following injury.

Calcitonin Receptor Signaling Maintains Quiescent State via cAMP/PKA Pathway

Both quiescent and undifferentiated states are characteristic of adult stem cells, and the impairment of these states results in exhaustion of the adult stem cell pool, including MuSCs (Bjornson et al., 2012; Cheung et al., 2012; Imayoshi et al., 2010; Mourikis et al., 2012). In addition, consistent with our previous data (Fukada et al., 2007), Calcr ligands (elcatonin and calcitonin) inhibited the activation of quiescent MuSCs ex vivo (Figure 4A; Figure S3A). This effect was Calcr dependent because the inhibition was not observed in cKO-MuSCs (Figure 4A). It could be speculated that the impairment of the quiescent or undifferentiated condition in cKO-MuSCs was attributable to the loss of the MuSC pool and reduced proliferation. Hence, we studied the in vivo dormancy of cKO-MuSCs by quantifying the expression level of Ki67 (a non-quiescence marker) in freshly isolated

MuSCs. As shown in Figure 4B, the expression of *Ki67* mRNA was higher in the order of cKO, cHetero, and control MuSCs 2 weeks after the Tm injection. Expression of non-quiescence marker Ki67 protein was higher in cKO-MuSCs compared to control cells relatively soon after the Tm injection (Figure 4C). The expression levels of several other cell-cycle-related genes, *Ccna2*, *Ccnd1*, and *Skp2*, were also elevated in cKO-MuSCs 2 weeks post Tm injection. (Figure 4D).

The increase in expression of Ki67/cell-cycle-related genes (Figure 4D) and in vivo decrease in proliferation (Figure 3C) may seem contradictory. In cKO mice, about 60% of Ki67$^+$ cells were positive for EdU (Figures 4E and 4F), and at early analyses (24-hr culture), the number of EdU$^+$ cells in cKO-MuSC was higher than that of the control mice (Figure 4G). However, consistent with in vivo results (Figure 3C), after 72-hr culture, the number of proliferating EdU$^+$ cells in cKO mice had decreased (Figure 4H). Collectively, it can be suggested that MuSCs escaped dormancy in the absence of Calcr and that the loss of the Calcr-mediated mechanism secondarily affects the proliferation of MuSCs-derived daughter cells, myoblasts.

Next, we sought to reveal the downstream pathway of Calcr. Calcr is a GPCR and uses Gsα protein, which increases the intracellular level of cAMP in osteoclasts (Suzuki et al., 1996). We confirmed that when Calcr was retrovirally expressed in myoblasts, the ligand raised the intracellular cAMP levels in Calcr-expressing C2C12 cells (Figure 4I). To further clarify the effects of cAMP as a downstream initiator of Calcr signaling, we tested two cAMP activators, dibutyryl-cAMP ([dbcAMP] an analog of cAMP) and forskolin (an activator of adenylate cyclase), and both cAMP activators were found to suppress the activation of quiescent MuSCs ex vivo (Figure 4J; Figure S3B). In addition, the expressions of cell-cycle-related genes were suppressed in 24-hr-cultured MuSCs treated with elcatonin or dbcAMP (Figure 4K).

To further study the downstream effects on cAMP, either a protein kinase A (PKA)-specific cAMP analog (6-bnz-cAMP) or an exchange protein directly activated by cAMP (Epac)-specific cAMP analog (8-CPT-cAMP) targets of cAMP (de Rooij et al., 1998) was applied to freshly isolated MuSCs. As shown in Figure 4J, only the PKA-specific analog inhibited the activation (EdU uptake) of MuSCs. Pretreatment with a PKA-specific inhibitor negated the effect of Calcr signaling in freshly isolated MuSCs cultured with elcatonin, a Calcr ligand (Figure 4L). Hence, these results suggest that the Calcr-cAMP-PKA pathway maintains the quiescent state of MuSCs in adult skeletal muscle.

Calcitonin Receptor Signaling Has No Impact on Myogenic Differentiation

We next examined the effect of Calcr signaling on myogenic differentiation. In contrast to the elevated Ki67 expression level, freshly isolated cKO-MuSCs exhibited no significant elevation of myogenic genes (Pax7, Myf5, MyoD, or myogenin) compared to the control MuSCs (Figure 5A). In vivo and ex vivo protein expressions of MyoD also did not differ between control and cKO-MuSCs (Figure 5B; Figure S4A). Furthermore, although a ligand-dependent suppression of proliferation was observed in retrovirally Calcr-expressing primary myoblasts (Figure 5C), neither MyoD expression nor myotube formation was inhibited by Calcr signaling in the primary myoblasts (Figures 5D and 5E) and in a C2C12 cell line (Figures S4B and S4C). Moreover, no evidence of accelerated myogenic differentiation was observed in cKO mice carrying a Cre-inducible yellow fluorescent protein (YFP) reporter gene (cKO-YFP; *Pax7*$^{CreERT2/+}$:*Calcr*$^{flox/flox}$:*Rosa26R*$^{YFP/YFP\ or\ YFP/+}$). In this model, YFP$^+$ myofibers increase when a YFP-labeled MuSC differentiates and fuses with a myofiber. We found that the number of YFP$^+$ myofibers in cKO-YFP mice was similar to that of the control mice (YFP; *Pax7*$^{CreERT2/+}$:*Calcr*$^{+/+}$:*Rosa26R*$^{YFP/YFP\ or\ YFP/+}$) (Figure 5F).

If the number of cells in the MuSC pool was decreased by a mechanism other than premature myogenic differentiation, it can be assumed that are two other ways by which the cKO-MuSC pool decreases. One is transdifferentiation of MuSCs into non-myogenic cells, and the other is cell death. As shown in Figure 5G, although almost all YFP$^+$ mononuclear cells in control and cKO-YFP mice expressed Pax7, apoptotic cells could be detected more frequently in cKO mice (Figure 5H). We also observed apoptosis in Ki67$^+$ cells only in cKO-MuSCs (Figure 5I). Thus, Calcr signaling is necessary to maintain the quiescent state of adult MuSCs, but it is not necessary to maintain the undifferentiated state (Figure 5J).

Calcitonin Receptor Signaling Inhibits Emergence of Muscle Stem Cells from Niche

During the study using the YFP-reporter mice, MuSCs residing within the interstitial areas were unexpectedly observed in cKO-YFP mice 5 weeks after Tm injection, though in small numbers (approximately 4%) (Figure 6). Such cells had not been reported previously in normal mice. Since MuSCs were originally defined by their location of residence (Mauro, 1961), this result indicates that MuSCs moved from their niches to the interstitial areas in a steady state when Calcr is absent.

To examine the effect of Calcr signaling on the location change of MuSCs, we conducted a single-myofiber culture that allowed observation of the MuSCs locating beneath the basal lamina. At the beginning of culture, all MuSCs were located beneath the basal lamina (not shown), but approximately half of the MuSCs had emerged from their niche and located outside of the basal lamina within 24 hr of culture. Although cell division of the relocated MuSCs was not observed within the 24 hr, adding Calcr ligand significantly suppressed their movement to the outside of the basal lamina from the surface of myofibers (Figures 7A–7C).

We then similarly examined the effects of Calcr signaling on MuSC relocation. Although PKA and Epac activators exerted different effects on MuSC activation in the EdU-uptake assay (Figure 4J), dbcAMP, a PKA activator, and an Epac activator exerted effects similar to that of elcatonin (Figure 7C). To further study the contribution of PKA or Epac to the Calcr signaling effects, isolated single myofibers were pretreated with each inhibitor and then pulsed with elcatonin for 1 hr. After removing reagents, single myofibers were cultured for 24 hr (Figure 7D). The elcatonin treatment significantly inhibited the emergence of MuSCs from their niches (Figures 7E and 7F). Both PKA and Epac inhibitors rescued the suppressive effect of elcatonin

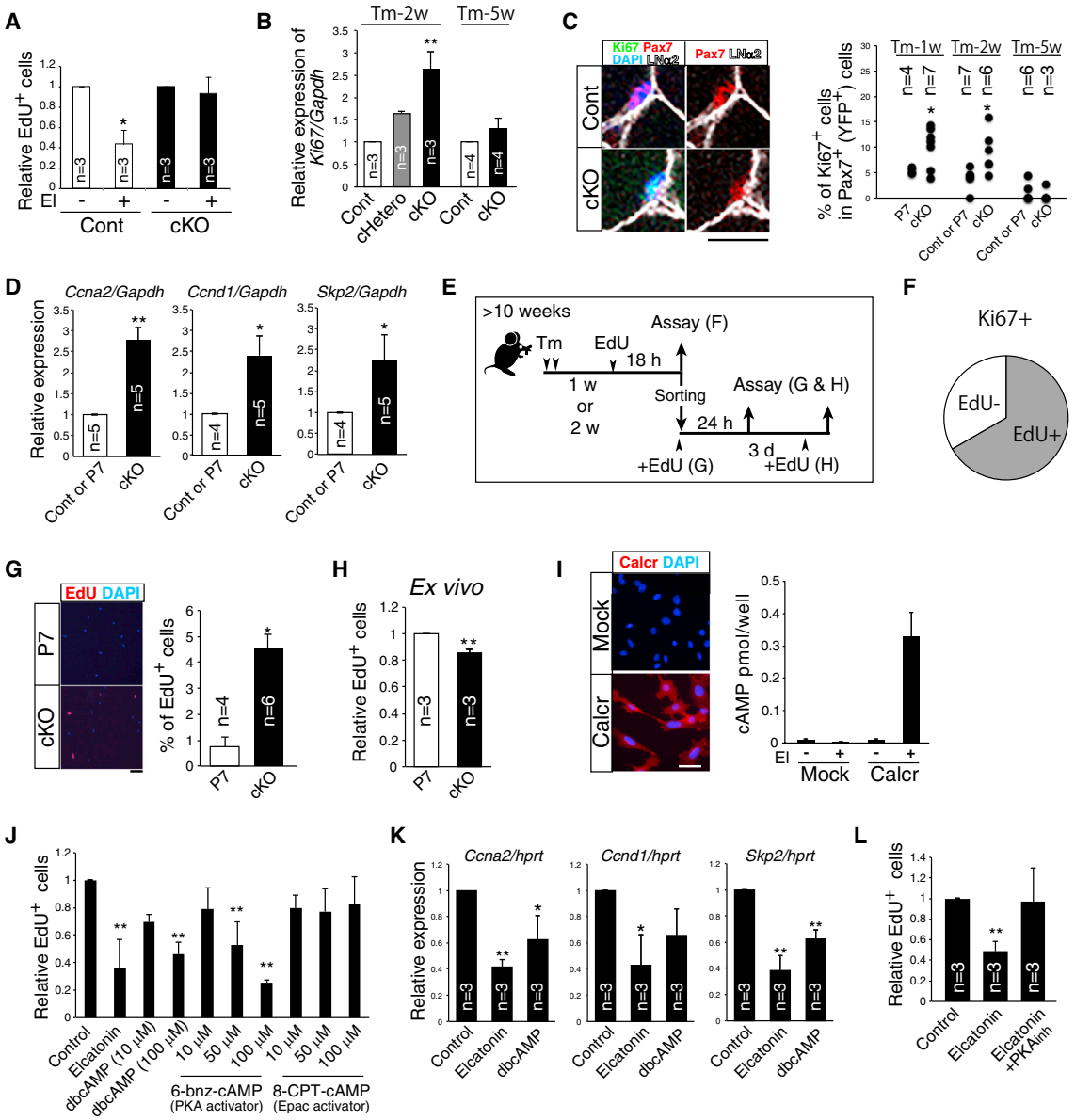

Figure 4. Calcitonin Receptor Signaling Regulates Quiescence of Muscle Stem Cells via cAMP/PKA Pathway

(A) Relative percentage of EdU$^+$ cells in freshly isolated MuSCs cultured for 2 days with or without elcatonin (El). The cells were prepared from Cont (white) or cKO (black) mice 2 weeks after Tm injection.

(B) Relative expression of *Ki67* mRNA in freshly isolated MuSCs derived from Cont (white), cHetero (gray), or cKO (black) mice 2 weeks or 5 weeks after Tm injection.

(C) TA muscle sections were stained with antibodies against Ki67 (green), Pax7 (red), and laminin α2 (white; LNα2). The graph represents the proportion of Ki67$^+$ cells in Pax7$^+$ cells in Cont, P7, or cKO mice 1–5 weeks after Tm injection.

(D) Relative expressions of *Ccna2*, *Ccnd1*, and *Skp2* mRNA in freshly isolated MuSCs were compared in Cont, P7 (white), or cKO (black) mice 2 weeks after Tm injection.

(E) Time course for analysis of cKO mice after Tm injection.

(F) Percentage of EdU$^+$ cell in Ki67$^+$ cells in cKO mice 1 week after Tm injection.

(G) Percentage of EdU$^+$ cells after culturing ex vivo for 24 hr.

(H) Percentage of EdU$^+$ cells after culturing ex vivo for 3 days.

(I) C2C12 cells were infected with Calcr-carrying (Calcr) or control (Mock) retrovirus, and the infected cells were sorted by GFP fluorescence and then stained with antibody against Calcr (red). The graph indicates the amount of cAMP in control or Calcr-expressing C2C12 cells treated or untreated with elcatonin (El); n = 3 in each group.

(J) Relative numbers of EdU$^+$ cells in freshly isolated MuSC cultured for 2 days in the absence or presence of elcatonin, dbcAMP, 6-bnz-cAMP (PKA activator), or 8-CPT-cAMP (Epac activator) at indicated concentrations; n = 3–4 in each group.

(legend continued on next page)

(Figures 7E and 7F). Furthermore, a larger number of cKO-MuSCs emerged from the niche than control MuSCs (Figure 7G shows the ratio to the control).

In this myofiber culture model, MuSCs are said to escape from the niche and then start to proliferate (Siegel et al., 2009). We did find, however, that Ki67$^+$ cells in cKO-MuSCs remained beneath the basal lamina (Figure 4C). We believe that the escapes from the niche and from quiescence are not sequential events. Our present study demonstrated that Calcr signaling has two separate roles: Calcr signaling inhibits the escape of MuSCs from quiescence by the cAMP-PKA pathway, and Calcr signaling inhibits the emergence of MuSCs from the niche by the cAMP-PKA and cAMP-Epac pathway (Figure 7H).

DISCUSSION

Our current study highlights the roles of calcitonin receptors (Calcrs) in MuSCs. Our results indicate that Calcr signaling holds the MuSCs in a quiescent state and in the niche. Calcr signaling, on the other hand, exerted no effect in the undifferentiated state of MuSCs. Further, apoptotic cells were observed in Ki67$^+$ cKO-MuSCs. As a result, the number of MuSCs decreased, followed by a reduction of muscle weight after post-injury regeneration. There was a discrepancy between the upregulated cell cycle genes and a decrease in the MuSC pool. Similar results for *Dicer*-mutated or Notch-signaling-mutated mice have been reported, however (Bjornson et al., 2012; Cheung et al., 2012; Fukada et al., 2011; Mourikis et al., 2012). For example, Mourikis et al. (2012) indicated that *Rbp-J* knockout MuSCs expressed Ki67, but the majority of them terminally differentiated without passing through the cell cycle S-phase. In *Dicer*-mutated MuSCs, the impaired quiescence resulted in cell death by apoptosis. Collectively, these abnormal quiescent states might affect subsequent MuSC survival.

We propose that Calcr-cAMP-PKA or Epac signaling is an "active" signaling pathway for maintaining the quiescence of MuSCs. Our results strongly suggest that G0-quiescent adult stem cells require active signaling pathways to maintain their pool. Although it is well known that increasing the cAMP concentration inside a cell results in growth inhibition in many types of cells (Stork and Schmitt, 2002) or arrest of the reversible cell cycle in a few types of cells (Dransfield et al., 2001; L'Allemain et al., 1997; Stambrook and Velez, 1976), the relationship between the quiescent state of adult stem cells and the cAMP pathway has largely been unknown. Two recent studies imply that the GPCR-cAMP pathway may be a common signaling pathway to maintain some types of adult stem cells. Specifically, Chagin et al. (2014) reported Gsα and Gq/11α proteins play an essential role in maintaining quiescent stem-like chondrocytes via parathyroid hormone-related protein receptors (PPRs). Gsα is a heterotrimeric subunit that activates adenylate cyclase and is activated by GPCRs like PPRs and Calcr that increase cAMP. Another study demonstrated that the abundant expression of GPCRs in quiescent NSCs and their activation can be inhibited by S1P and PGD_2 (Codega et al., 2014). Both S1P and PGD_2 have the potential to increase intracellular cAMP levels (Davaille et al., 2000; Regan et al., 1994); thus, GPCR-mediated cAMP accumulation may be a common mechanism to regulate the dormancy of adult stem cells in some tissues.

Recently, Sato et al. reported that both miR-195 and miR-497 induce cell cycle arrest by targeting the cell cycle genes *Cdc25* and *Ccnd* (Sato et al., 2014). As shown in the present study, the Calcr-cAMP pathway inhibited the expression of *Ccna2*, *Ccnd1*, and *Skp2*. *Ccna2*, a gene with the CREB-binding sequence (Kamiya et al., 2007), is commonly suppressed in adult stem cells (Cheung and Rando, 2013). CREB is the major target of PKA, and Calcr signaling activates CREB transcriptional activity. *Ccnd1* is also a well-known target of CREB. On the other hand, the expressions of cyclin-dependent kinase inhibitors (*p21*, *p27*, and *p57*) and *Spry1* (one critical regulator for maintaining MuSCs) did not change in cKO-MuSC (Figure S5). Although we did not specifically show that suppressing cyclin-related gene expression is required to maintain stem cells in a quiescent state, expression of Calcr/cAMP/PKA-mediated cyclin-related genes (*Ccna2*, *Ccnd1*, *Skp2*, etc.) is a potential mechanism by which MuSCs are maintained in a quiescent state.

One of our unexpected results was the observation of MuSCs in the interstitial space. Because we used a lineage-tracing model, the result clearly indicated that MuSCs had emerged from their niche. The small numbers of interstitial MuSCs implies that they either undergo cell death or return to sublaminal niches. While both phenomena are possible, the precise mechanism is still unknown. Notably, the discovery of the unusual residence of MuSCs suggests the existence of active signaling pathways involved in keeping MuSCs in their niches. Intriguingly, Jockusch and Voigt (2003) stated that MuSCs can exit from their niche in undamaged muscle. They transplanted intact skeletal muscle tissue by differentially labeling the graft, host skeletal myofibers, and MuSCs (which they refer to as myogenic precursor cells) and observed that donor-derived MuSCs migrated toward and fused with the host myofibers and vice versa (Jockusch and Voigt, 2003), although it took more than 7 weeks before the fused myofibers were observed. Further, we did not find interstitial YFP$^+$ cells in *Pax7*$^{CreERT2/+}$:*Calcr*$^{+/+}$:*Rosa26R*^{YFP} mice in high quantity, demonstrating that very few MuSCs move from one myofiber to another in a physiological condition. Our results demonstrate the necessity of external active signaling pressure to prevent the exodus of MuSCs from their niche.

Calcitonin is the best-characterized ligand of Calcr, but there is a difference between Calcr null and calcitonin null mice. The

(K) Relative expressions of *Ccna2*, *Ccnd1*, and *Skp2* mRNA were compared among non-, elcatonin-, or dbcAMP-treated MuSCs freshly isolated after culturing for 24 hr.

(L) Relative numbers of EdU$^+$ cells in freshly isolated MuSCs cultured for 2 days in the absence or presence of elcatonin and PKA inhibitor (20 μM). The y axis indicates means with ± SE (n = 3).

Scale bars, 10 μm (C) and 50 μm (G and I). The number of mice used is shown in each graph (A–D, G, H, K, and L). *p < 0.05, **p < 0.01 (A–D, G, H, and J–L).

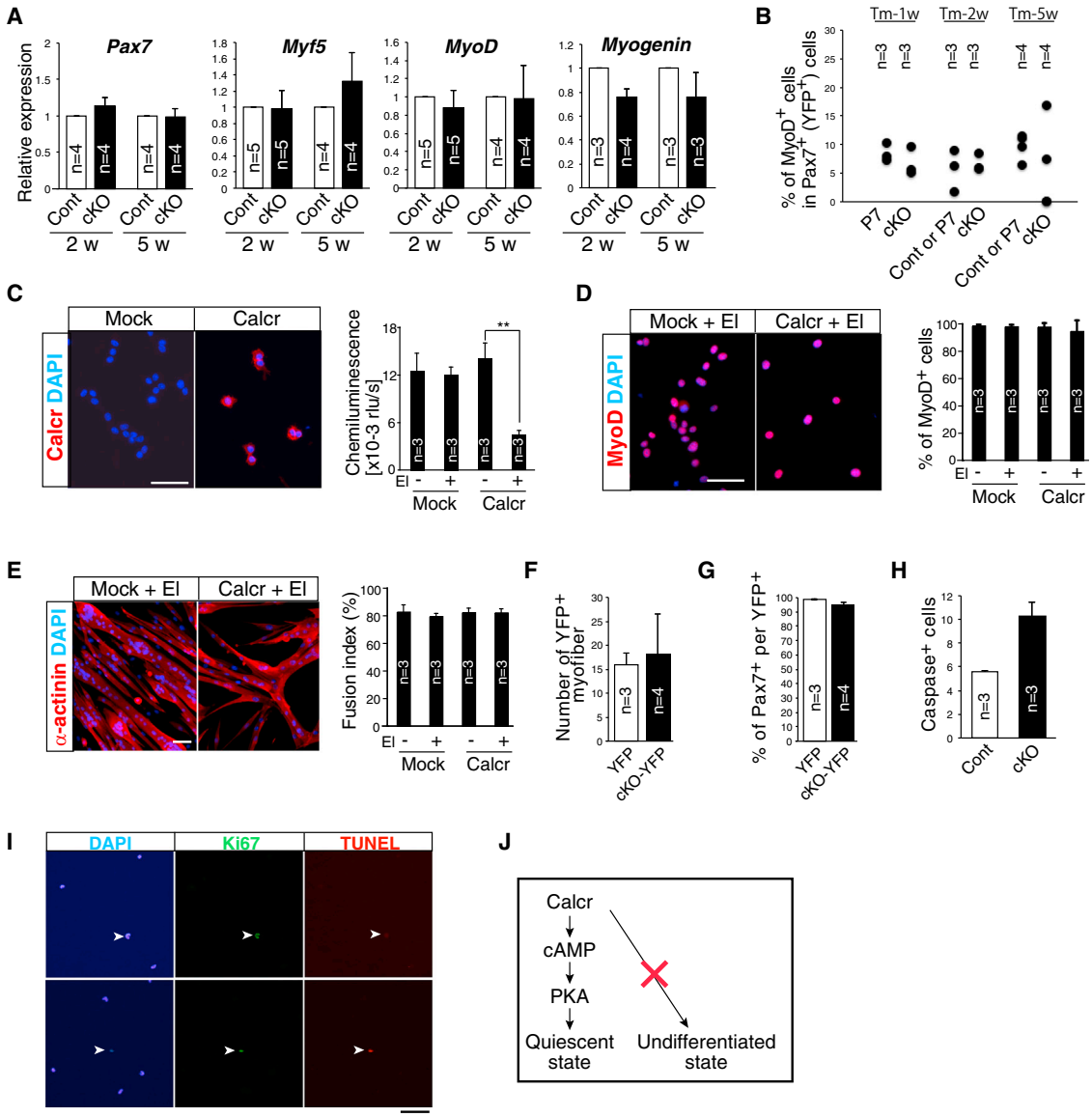

Figure 5. Calcitonin Receptor Signaling Has No Impact on Expression and Differentiation of Myogenic Genes

(A) Relative expressions of *Pax7*, *Myf5*, *MyoD*, and *myogenin* mRNA in freshly isolated MuSCs derived from Cont (white) or cKO (black) 2 or 5 weeks after Tm injection.

(B) The proportion of MyoD$^+$ cells in Pax7$^+$ (or YFP$^+$) cells in Cont, P7, or cKO mice 1–5 weeks after Tm injection.

(C) The expression of Calcr (red) in control primary myoblasts (Mock) or Calcr vector-infected cells (Calcr). Nuclei were stained with DAPI (blue). The graph shows the frequency of BrdU$^+$ cells when each type of cell was cultured with or without elcatonin (EI) for 24 hr. **$p < 0.01$.

(D and E) The frequency of MyoD$^+$ cells (D) or fusion index (E) in control (Mock) or Calcr vector-infected cells (Calcr) treated with or without elcatonin (EI). The graph shows the percentages of MyoD$^+$ cells or multinuclear cell per total nuclei. Myotubes were stained with anti-α-actinin (red).

(F) Numbers of YFP$^+$ myofibers in YFP (white) or cKO-YFP (black) mice 5 weeks after Tm injection.

(G) Frequency of Pax7$^+$ cells per YFP$^+$ mononuclear cells in YFP (white) or cKO-YFP (black) mice 5 weeks after Tm injection.

(H) Apoptotic cells (caspase$^+$) were measured by FACS in freshly isolated MuSCs derived from Cont (white) or cKO (black) 2 weeks after Tm injection.

(I) Apoptotic cells (TUNEL$^+$) in Ki67$^+$ cells of cKO-MuSC 2 weeks after Tm injection. Arrowheads indicate Ki67$^+$TUNEL$^+$ cells.

(J) A model of calcitonin receptor signaling in a quiescent or undifferentiated state in MuSCs. The number of mice used is shown in each graph.

Scale bars, 50 μm (C–E and I). Values are means ± SE.

Calcr null condition is embryonically lethal (Dacquin et al., 2004), but calcitonin null mice are viable (Hoff et al., 2002), indicating that Calcr has other ligands besides calcitonin. In fact, calcitonin receptor and receptor-activity-modifying proteins can serve as receptors for amylin, a member of the calcitonin family co-secreted with insulin by pancreatic beta cells (Christopoulos et al., 1999). Amylin inhibits the activation of quiescent MuSCs ex vivo just like calcitonin (Figure S6). Moreover, Katafuchi

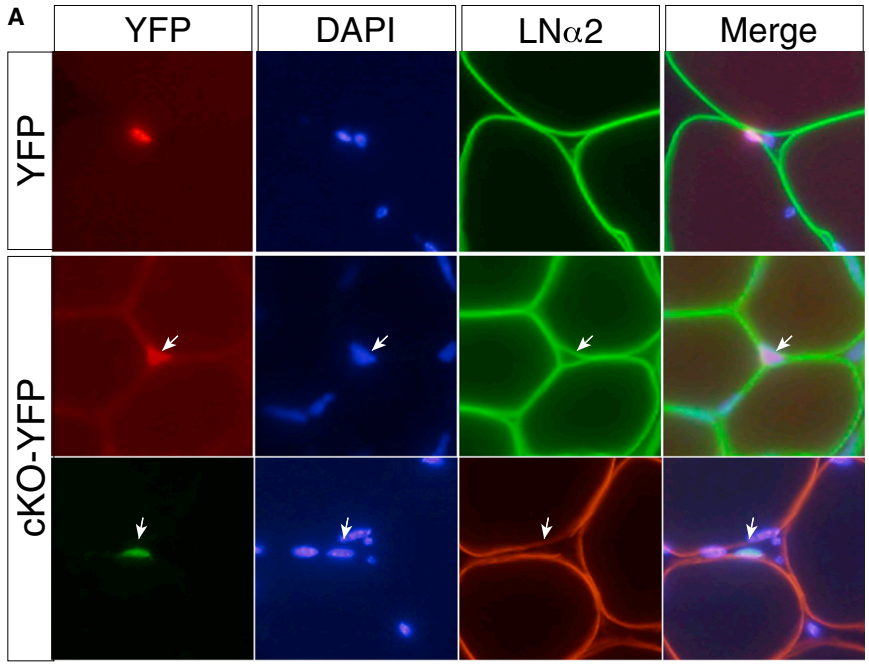

Figure 6. Detection of Interstitial Muscle Stem Cells in Calcr-Depleted Mice

(A) All YFP$^+$ cells were detected beneath the basal lamina in control (YFP) mice, but cKO-YFP mice have YFP$^+$ cells located outside the basal lamina 5 weeks after Tm injection. Arrow indicates interstitial YFP$^+$ cells. Scale bar, 10 μm.

(B) Percentage of interstitial YFP$^+$ cells 1–5 weeks after Tm injection. The number of mice used is shown in the graph.

et al. (2003) have identified another calcitonin receptor ligand named calcitonin receptor-stimulating peptide (CRSP) in the pig. Although a gene corresponding to CRSP has not been found in humans and mice, it is possible there is still an unidentified Calcr ligand that functions in MuSCs.

In addition to coding RNA, non-coding RNA plays essential roles in stem cells. One non-coding RNA, miRNA, requires Dicer for its biogenesis. By utilizing conditional *Dicer* mutant mice, Cheung et al. (2012) found the loss of the MuSC pool in *Pax7*$^{CreERT2/+}$*::Dicer*$^{flox/flox}$ mice. They also identified a quiescent MuSC-specific miRNA, mir-489. Notably, mir-489 is located in intron 4 of the mouse *calcitonin receptor* gene, which was not affected in our mouse construct. Along with mRNA expression of *Calcr*, mir-489 also decreased when MuSCs are activated. Furthermore, Cheung et al. (2012) also identified the target of mir-489, *Dek*. In our analyses, mir-489 expression in Calcr cKO-MuSCs was approximately 40% of that in control MuSCs, though *Dek* expression was not changed significantly (Figure S7). Calcr signaling directly inhibited activation and emergence of MuSCs from niches ex vivo. Thus, the phenotype of cKO most likely did not depend on mir-489. Because both Calcr and mir-489 are critical regulators, studies on the transcriptional regulation locus of *Calcr* genes will lead to in-depth understanding of molecular mechanisms in the quiescence, activation, and self-renewal of MuSCs.

In conclusion, we showed that Calcr plays two roles in sustaining the muscle stem cell pool, specifically, the regulation of dormancy and the location of stem cells. Although there still may be other Calcr ligands to be discovered, the blood level of the most potent ligand known, calcitonin, decreases during aging (Shamonki et al., 1980). The expression of Calcr on osteocytes also disappears with age (Gooi et al., 2014). In the elderly population, it is relatively common to observe sarcopenia, a significant loss of skeletal muscle mass. Age-related loss of bone density is also well-known, specifically in postmenopausal woman, and Calcr is a treatment target for osteoporosis. Calcr itself and its signaling pathway could become a shared molecular target in preventing age-related functional decline in the musculoskeletal system.

EXPERIMENTAL PROCEDURES

Generation of *Calcr*^{flox} Mice

A conditional knockout vector for *Calcitonin receptor* (PRPG00131 B F08) was purchased from KOMP (Knockout Mouse Project: https://www.komp.org/). ENSMUSE00000136685 (exon 6) and ENSMUSE00000136688 (exon 7) were floxed by the loxP sequence (Figure S1). The linearized targeting vector was introduced into embryonic stem cells (Fujihara et al., 2013). Colonies that had undergone homologous recombination were detected by PCR analysis with specific primers provided by KOMP. Mice with the *calcitonin receptor flox* allele were generated in our facility. The neomycin cassette was removed by crossing with CAG-Flip transgenic mice (Rodríguez et al., 2000).

Mice

Pax7$^{CreERT2/+}$ (Lepper et al., 2009) and *Rosa26*$^{EYFP/+}$ (Srinivas et al., 2001) mice were obtained from Jackson Laboratories. *Pax7*$^{CreERT2/+}$ mice were injected twice (24 hr apart) intraperitoneally with 200–300 μl Tm (20 mg/ml; Sigma-Aldrich #T5648) dissolved in sunflower seed oil 5% ethanol (Mourikis et al., 2012). Control mice were also treated with Tm in this study. All procedures for experimental animals were approved by the Experimental Animal Care

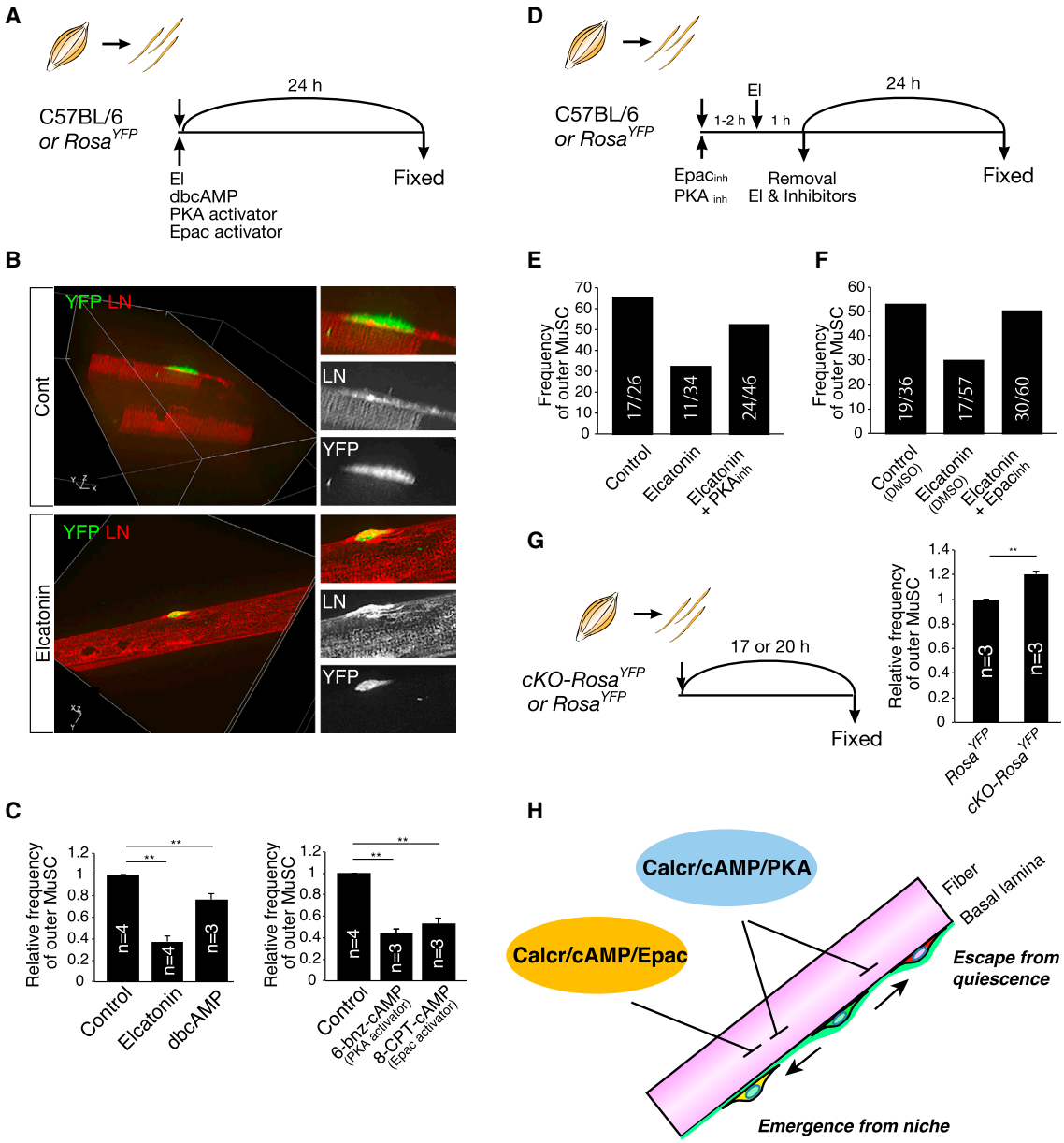

Figure 7. Calcitonin Receptor Signaling Suppresses Emergence of Muscle Stem Cell from the Niche

(A and B) Isolated single myofibers were cultured with or without elcatonin or each activator (100 μM) for 24 hr in GM (A) and then fixed and stained with anti-YFP (*Rosa26R*^{YFP}), Pax7 (C57BL/6), and LN (laminin) (B).

(C) Relative numbers of MuSCs locating outside the LN after culturing with or without elcatonin, dbcAMP (100 μM), 6-Bnz-cAMP (100 μM), or 8-CPT-cAMP (100 μM) for 24 hr in GM.

(D) Isolated single myofibers were pretreated with or without PKA inhibitor or Epac inhibitor for 1–2 hr. The single myofibers were additionally cultured with or without elcatonin for 1 hr. After removing each inhibitor and elcatonin, all myofibers were cultured in GM for 24 hr and then fixed and stained with anti-YFP (*Rosa26R*^{YFP}), Pax7 (C57BL/6), and LN antibodies.

(E and F) Frequency of MuSCs locating outside the laminin after culturing with or without elcatonin, PKA inhibitor (E; 20 μM), or Epac inhibitor (F; 10 μM) for 24 hr in GM. Similar results were obtained by independent experiments.

(G) Isolated single myofibers derived from control (*Rosa26R*^{YFP}) and cKO (cKO-*Rosa26R*^{YFP}) mice were cultured in GM for 17–20 hr and then fixed and stained with anti-YFP and -LN antibodies. The graph indicates the frequency of MuSCs locating outside the laminin in control (*Rosa26R*^{YFP}) and cKO (cKO-*Rosa26R*^{YFP}) mice.

(H) Putative roles of Calcr in muscle stem cells.

The number of mice (C and G) or MuSCs (E and F) analyzed is shown in the graph. **$p < 0.01$ (C and G).

and Use Committee at Osaka University. PCR-based genotyping was performed with the DNA using the primers listed in Table S1.

Muscle Injury

Tibialis anterior muscles were damaged by injecting 2.5 μl per g of mouse body weight of 10 μM CTX (Wako Pure Chemical Industries) in saline. When mononuclear cells were prepared from injured muscles, CTX was injected into tibialis anterior (50 μl), gastrocnemius (150 μl), and quadriceps femoris (100 μl) muscles.

Real-Time PCR

Total RNA was extracted from sorted or cultured cells using a QIAGEN RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN) and then reverse-transcribed into cDNA using a QuantiTect Reverse Transcription Kit (QIAGEN). Specific forward and reverse primers for optimal amplification in real-time PCR of reverse transcribed cDNAs are listed in Table S1.

Muscle Fixation and Histological Analysis

Isolated tibialis anterior muscles were frozen in liquid nitrogen-cooled isopentane (Wako Pure Chemicals Industries). In order to prevent YFP protein leaking, these muscles were fixed in 4% paraformaldehyde (PFA) for 30 min, immersed sequentially in 10% and 20% sucrose/PBS, and then frozen in isopentane cooled with liquid nitrogen. Transverse cryosections (10 μm) were stained with H&E.

Immunohistochemistry

For immunohistochemical studies, transverse cryosections (6 μm) were fixed with 4% PFA for 10 min. After blocking with 5% skim milk, sections were stained with primary antibodies. Detailed information on antibodies used in this study is listed in Table S2. In order to block endogenous mouse IgG, a M.O.M. kit (Vector Laboratories) was used for Pax7 or eMyHC staining. After the first staining at 4°C overnight, sections were reacted with secondary antibodies (Molecular Probes). The signals were recorded photographically using a confocal laser scanning microscope system TCS-SP5 (Leica Microsystems) or a fluorescence microscope BX51 (Olympus) equipped with a DP70 CCD camera (Olympus).

Preparation and FACS Analyses of Skeletal-Muscle-Derived Mononuclear Cells

Mononuclear cells from uninjured limb muscles were prepared using 0.2% collagenase type II (Worthington Biochemical). Mononuclear cells derived from skeletal muscle were stained with FITC-conjugated anti-CD31, -CD45, PE-conjugated anti-Sca-1, and biotinylated-SM/C-2.6 (Fukada et al., 2004) antibodies. Cells were then incubated with streptavidin-labeled allophycocyanin (BD Biosciences) on ice for 30 min and resuspended in PBS containing 2% FBS and 2 μg/ml propidium iodide. Cell sorting was performed using a FACS Aria II flow cytometer (BD Immunocytometry Systems). In the case of *Rosa26*^{EYFP} mice, MuSCs were isolated by YFP fluorescence without any staining.

Cytospin and Immunocytochemistry

FACS-sorted cells were collected on glass slides by Cytospin (Thermo Fisher Scientific) and then fixed with 4% PFA for 10 min. Cultured cells were also fixed with 4% PFA for 10 min. After permeabilization by 0.25% Triton X-100 and blocking with 5% skim milk, the cells were stained with primary antibodies at 4°C overnight and then reacted with secondary antibodies (Molecular Probes).

Cell Culture

MuSCs were cultured in a growth medium (GM) of high-glucose DMEM ([DMEM-HG] Sigma-Aldrich) containing 20% FBS (Cell Culture Bioscience, Nichirei Biosciences), 2.5 ng/ml bFGF (PeproTech), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (Gibco BRL) on culture dishes coated with Matrigel (BD Biosciences). Differentiation was induced in differentiation medium (DM) containing DMEM-HG, 5% horse serum, and penicillin-streptomycin for 3–4 days.

EdU-Uptake Assay

EdU was detected following the protocol supplied by the manufacturer (Molecular Probes). Detailed information on reagents used in this study is described in Supplemental Experimental Procedures.

Single Myofiber Culture and Staining

Single myofibers were isolated from extensor digitorum longus muscles following the previously described protocol (Rosenblatt et al., 1995) and cultured for 24 hr in GM without attaching to the culture dish. Fixation and immunostaining followed described protocols (Collins-Hooper et al., 2012; Shinin et al., 2009). To capture the 3D structure in single myofiber studies, the stained myofibers were visualized using a BZ-X700fluorescence microscope (Keyence).

Detection of Apoptotic Cells

For FACS analyses of apoptotic cells, a Vybrant FAM Caspase-3 and -7 Assay Kit was used by following the provided protocol (Molecular Probes). Apoptotic cells were detected immunohistochemically by rhodamine fluorescence using an ApopTag Red In Situ Apoptosis Detection Kit (Chemicon).

Retroviral Vector Preparation

A full-length C1a-type Calcr cDNA was amplified by real-time PCR, and the PCR product was sequenced and cloned into a bicistronic retrovirus construct, pMXs-IRES/GFP (Nosaka et al., 1999), which contains IRES and therefore simultaneously expresses Calcr and GFP. The viral particles were prepared as described (Morita et al., 2000).

Quantitation of cAMP

After infection with retroviral vectors, GFP^{+} C2C12 cells were sorted and plated on 96-well dishes at a density of 2,000–3,000 cells and cultured for 1 day. Cells were then stimulated with elcatonin (0.1 U/ml) in GM for 3 hr, and the amount of cAMP in the cells was quantified using a cAMP-Screen System (Applied Biosystems).

Statistics

Values were expressed as means ± SE. Statistical significance was assessed by Student's t test. In comparisons of more than two groups, non-repeated-measures analysis of variance (ANOVA) followed by the Bonferroni test (versus control) or SNK test (multiple comparisons) were used. A probability of less than 5% ($p < 0.05$) or 1% ($p < 0.01$) was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.083.

AUTHOR CONTRIBUTIONS

S.F. was responsible for designing and performing the experiments, analysing the data, and writing the manuscript; M.Y., M.I, K.T., H.Y., and S.F. contributed to generation of *Calcr*^{flox} mice. M.Y., Y.W., T.O., T.S., and S.F. performed the analyses of *Pax7*^{CreERT2}:*Calcr*^{flox} or *Pax7*^{CreERT2}:*Calcr*^{flox}:*Rosa26R*^{YFP} mice. Y.W. and S.F. did ex vivo analyses of BrdU- or EdU-uptake. N.M. designed experiments for using PKA or Epac activator or inhibitor. T.O. and M.N. did single myofiber and regeneration experiments. A.U., K.T., M.H., Y.M.-S., and S.T. provided reagents and materials.

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