Our previous work on human myoblasts suggested that a hyperpolarization followed by a rise in $[\text{Ca}^{2+}]_{\text{in}}$ involving store-operated $\text{Ca}^{2+}$ entry (SOCE) channels induced myoblast differentiation. Advances in the understanding of the SOCE pathway led us to examine more precisely its role in post-natal human myoblast differentiation. We found that SOCE orchestrated by STIM1, the endoplasmic reticulum $\text{Ca}^{2+}$ sensor activating Orai $\text{Ca}^{2+}$ channels, is crucial. Silencing STIM1, Orai1, or Orai3 reduced SOCE amplitude and myoblast differentiation, whereas Orai2 knockdown had no effect. Conversely, overexpression of STIM1 with Orai1 increased SOCE and accelerated myoblast differentiation. STIM1 or Orai1 silencing decreased resting $[\text{Ca}^{2+}]_{\text{in}}$ and intracellular $\text{Ca}^{2+}$ store content, but correction of these parameters did not rescue myoblast differentiation. Remarkably, SOCE amplitude correlated linearly with the expression of two early markers of myoblast differentiation, MEF2 and myogenin, regardless of the STIM or Orai isoform that was silenced. Unexpectedly, we found that the hyperpolarization also depends on SOCE, placing SOCE upstream of K$^+$ channel activation in the signaling cascade that controls myoblast differentiation. These findings indicate that STIM1 and Orai1 are key molecules for the induction of human myoblast differentiation.

Muscle regeneration relies on satellite cells, myogenic stem cells able to proliferate and differentiate after strenuous physical workout (muscle hypertrophy) or following a muscle lesion (muscle repair). This myogenesis process involves muscle-specific transcription factors such as myogenin and MEF2, whose expression is regulated by increases in the cytosolic $\text{Ca}^{2+}$ concentration (1−4). Human myoblasts can generate $\text{Ca}^{2+}$ signals via voltage-gated channels, such as T-type $\text{Ca}^{2+}$ channels (5), and by $\text{Ca}^{2+}$ release from inositol 1,4,5-trisphosphate-sensitive $\text{Ca}^{2+}$ stores followed by $\text{Ca}^{2+}$ entry through SOCE$^2$ channels (1).

The molecular machinery that orchestrates SOCE has been recently discovered, improving our understanding of the cellular events connecting $\text{Ca}^{2+}$ store depletion to SOCE activation (6). Two major SOCE components have been identified; the stromal interaction molecules (STIM) and the Orai family of channels (also known as CRACM). STIM1 is the transmembrane ER $\text{Ca}^{2+}$ sensor that activates SOCE upon $\text{Ca}^{2+}$ store depletion (7, 8), whereas its homologue STIM2 seems more implicated in the control of basal cytosolic and ER $\text{Ca}^{2+}$ levels (9). Both molecules function as ER $\text{Ca}^{2+}$ sensors and are predominantly located in the ER or in ER subcompartments (7, 10, 11). Both STIM1 and STIM2 possess an EF-hand domain located in the ER lumen whose affinity for $\text{Ca}^{2+}$ is in the range of $\text{Ca}^{2+}$ values measured in the lumen of the ER (12, 13), and mutations that reduce STIM1 affinity for $\text{Ca}^{2+}$ induce a permanent SOCE (7). Upon $\text{Ca}^{2+}$ store depletion, STIM1 redistributes to plasma membrane clusters (7, 11), where it co-localizes with the Orai1 channel to form the elementary subunit of SOCE (14). The close apposition of the ER and plasma membrane at the STIM1-Orai1 clusters enables sarco/endoplasmic $\text{Ca}^{2+}$ ATPases (SERCA) pumps on the juxtaposed ER membrane to rapidly and efficiently refill the ER $\text{Ca}^{2+}$ stores (15).

The channel component, Orai1, and its two homologues Orai2 and Orai3 were identified almost simultaneously by three laboratories (Refs. 16−18; for review, see Refs. 19−21). Orai1 was since demonstrated to be the pore subunit of the prototypic store-operated CRAC channel of blood cells (17, 22, 23). Co-expression of STIM1 and Orai1 generates robust CRAC currents in a number of expression systems (24), indicating that expression of these two molecules is sufficient to recapitulate SOCE. These genetic studies generated indirect evidence that point to a role for SOCE in the growth, repair, or survival of skeletal muscle. In mice, Orai1 and STIM1 are expressed at high levels in skeletal tissue, and genetic ablation of Orai1 or STIM1 causes a severe growth defect, the Orai1 or STIM1 null mice being much smaller than its wild-type littermates until maturity (25−27). Furthermore, in the pioneering studies by Feske et al. (16, 28) that revealed the key mutation in the CRAC channel Orai leading to immune deficiency, the two severe

The abbreviations used are: SOCE, store-operated $\text{Ca}^{2+}$ entry; STIM, stromal interaction molecule; ER, endoplasmic reticulum; DAPI, 4',6-diamidino-2-phenylindole; DM, differentiation medium; siRNA, small interference RNA; Tg, thyssagargin; RT, reverse transcription; wt, wild type.
combined immunodeficiency syndrome patients also suffered from congenital non-progressive myopathy. These facts led us to re-examine with new tools the role of the SOCE pathway in primary culture of human myoblasts.

In this study, we used gene silencing and overexpression to investigate the role of STIM1 and Orai channels during the differentiation process of cultured myoblasts derived from human muscle biopsies. We show that silencing of STIM1, Orai1, or Orai3 greatly reduces SOCE and impairs myoblast differentiation, whereas Orai2 knockdown had no effects. Strikingly, the amplitude of SOCE correlated with the degree of differentiation regardless of the molecule that was depleted. Conversely, enforced expression of STIM1 together with Orai1 increased SOCE and accelerated the differentiation of myoblasts. STIM1 and Orai1 were up-regulated during the first 2 days of myoblast differentiation, but their depletion at this stage neither delayed the differentiation process nor impaired late myogenic events such as cell fusion, indicating that the molecules were only limiting during the initial phase of differentiation. Accordingly, the plasma membrane hyperpolarization that we previously described as the earliest known event in myoblast differentiation (3) was impaired in cells depleted for STIM1 or Orai1, confirming that SOCE regulates the very early steps (<6 h) of the differentiation process. Together, our data indicate that STIM1 and Orai1 are key signaling molecules that control the initiation of the differentiation process of human myoblasts that eventually leads to contractile myotubes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Muscle samples, cell dissociation, and clonal culture from satellite cells were prepared as previously described (1, 29, 30). Human muscle samples were obtained from 10 children (younger than 4 years old) without any known neuromuscular disease. 21 different clones were used in this study. The level of differentiation was assessed by quantification of the nuclear expression of the myogenic transcription factor MEF2 and myogenin (2). Plasmids (human Orai1-myc from Addgene plasmid 12199 and STIM1-wt from ORIGENE Technologies) were transfected by electroporation as previously described (31). Transfection efficiency assessed for STIM1-YFP by FACS was of 83 ± 7% (n = 12).

**Small Interference RNA (siRNA) Knockdown**—Myoblasts were transfected in suspension by incubating 4 × 10⁶ cells in a solution containing 500 μl of Opti-MEM, 3 μl of Lipofectamine RNAiMax (Invitrogen), and 20 pmol of a specific siRNA (Ambion or Qiagen) according to manufacturer protocols (Invitrogen). The transfection efficiency assessed by Block-iT Alexa Fluor Red Fluorescent Oligo (Invitrogen) measurements was approximately 90%. Optimal quantities of Lipofectamine and siRNA were defined by evaluating the strongest effect of siRNA STIM1 on SOCE (see below) and were constantly used thereafter. This optimal effect was obtained 36 to 48 h post-transfection. Sequences of the different siRNA are summarized on Table 1. The siRNA Med Universal Control-siMed from Invitrogen was used as a negative control.

**Real-time PCR**—Total RNA was isolated from cells using the QIAshredder and the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer’s instructions. One microgram of total RNA was reverse-transcribed with the Superscript RT II (Invitrogen) according to the manufacturer’s instructions. Real-time experiments were performed at the Genomics Platform of the NCCR Frontiers in Genetics (Geneva). For each PCR reaction, 1/20th of the cDNA template was PCR-amplified in a 7900HT SDS System using Power SYBR Green PCR master mix (both from Applied Biosystems, Foster City, CA). We obtained raw threshold-cycle (Ct) values using SDS 2.0 software (Applied Biosystems). A mean quantity was calculated from triplicate PCR reactions for each sample, and this quantity was normalized to the average of four endogenous control genes (2-microglobulin and EE-EF1) as described by Vandesompele et al. (32). Primers used for the real-time PCR are described in Table 1.

**Immunostaining**—Myoblasts were fixed and stained with the appropriate fluorescent markers as described previously (1). Anti-MEF2 antibody (1:200; sc-313, Santa Cruz Biotechnology Inc., Heidelberg, Germany) and anti-myogenin antibody (1:900; clone F5D, BD Biosciences) were used together with anti-GOK/STIM1 antibody (1:500; BD Biosciences). Secondary antibodies used were Alexa 488-labeled goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR) and Alexa 546-labeled

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**TABLE 1**

Sequences of the siRNA and primers used for the real-time RT-PCR

| siRNA         | Sense*    | Antisense* |
|---------------|-----------|------------|
| STIM1         | GCCUCUGUGUAGACUUGUUCGTGTTT | GAUCACUGUGUAUCGACGCTGTTT |
| STIM1         | GCCUCUGUGUAGACUUGUUCGTGTTT | GAUCACUGUGUAUCGACGCTGTTT |
| Orai1         | CUGCACAGAUCUCACACACAGTT | CUGCACAGAUCUCACACACAGTT |
| Orai2         | GGACUGUGUAUGGUGUGGTTGCTT | GGACUGUGUAUGGUGUGGTTGCTT |
| Orai3         | UUGAAGCUGUGACCAUACAGTT | UUGAAGCUGUGACCAUACAGTT |
| β2-Microglobulin | TGGCTGCTGCTCCTGTT | TGGCTGCTGCTCCTGTT |
| glcuronidase B | CCACAGGAGCCACCATCCTAAT | CCACAGGAGCCACCATCCTAAT |
| EEF1A1        | AGCAAACTGACCCACAGTT | AGCAAACTGACCCACAGTT |
| GAPDH         | GCCACAAGGAGGAAGAGGACC | GCCACAAGGAGGAAGAGGACC |

* All sequences are oriented 5′–3′.

* siRNA from Qiagen.
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goat anti-rabbit IgG (1:500; Molecular Probes). DAPI (100 ng/ml; Sigma) was used to localize nuclei. Images were acquired using a Zeiss Axiovert S100 TV Microscope equipped with a Cairn Optoscan monochromator and a motorized stage. The DAPI, Alexa 488, and Alexa 546 fluorescence were acquired by changing the excitation wavelengths with the monochromator using a triple band filter set (XF2050 dichroic mirror and XF3063 emission filter from Omega Optical Inc., Brattleboro, VT). The motorized stage was used to automatically acquire 10 random fields in each condition (200–400 cells/condition). Analysis to count the labeled nuclei was carried out using the Metamorph 6.3r7 software (Molecular Devices Corp., Visitron Systems GmbH, Puchheim, Germany). A cluster of labeled nuclei inside a myotube that clearly express MF2 or myogenin was used to define the threshold above which nuclei were considered as positive for the expression of MF2 or myogenin (10 random fields for each clone).

Western Blots—Western blots were performed as previously described (2). Briefly, myoblasts were lysed using PhosphoSafe Extraction Buffer (Novagen). Total proteins were separated on a SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in T-TBS (0.1% Tween 20, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl) and 5% nonfat milk. Blots were incubated with the primary antibodies diluted in T-TBS and nonfat milk as follows: mouse monoclonal antibody anti-GOK/STIM1 1:500 (BD Biosciences) or rabbit polyclonal anti-STIM2 antibody (1:500; ProSci Inc.) and mouse monoclonal antibody against α-tubulin (clone DM1A, Sigma) 1:10,000. Blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse diluted 1:6000 (Bio-Rad) or with HRP-conjugated goat anti-rabbit diluted 1:6000 (Bio-Rad), respectively. Antibodies were revealed using ECL reagents and Hyperfilm ECL (Amersham Biosciences). Optiquant 03.00 (Packard instrument Co.) program was used to quantify the level of protein expression.

Cytosolic Calcium Measurements—Myoblasts grown on coverslips were loaded 30 min at room temperature with the cell-permeant fluorescent Ca\(^{2+}\) indicator Fura-2-AM (Biotium Inc., Chemie Brunschwig AG, Basel, Switzerland). Fura-2-AM preparation was done as described previously (1). Ratimometric images of Ca\(^{2+}\) signals were obtained using a Zeiss Axiovert S100 TV microscope (Zeiss AG, Feldbach, Switzerland) equipped with a Cairn Optoscan monochromator (Cairn Research Ltd., Faversham, UK) which rapidly changed the excitation wavelengths between 340 and 380 nm. Fluorescence emissions were captured through a 510WB40 filter (Omega Optical Inc.) using a CoolSnap HQ camera (Photometrics-Ropper Scientific, Tucson, AZ). Image acquisition and analysis were carried out with the Metafluor 6.3r7 software (Molecular Devices, Visitron Systems GmbH, Puchheim, Germany). Rmax was evaluated in all conditions (using a solution containing 4 μM ionomycin and 5 mM Ca\(^{2+}\)), ruling out any possible non-linearity between data obtained in myoblasts transfected with different plasmids or siRNA. No significant difference between the various Rmax was found.

Confocal Monitoring of Membrane Potential—As described previously in Konig et al. (2), confocal image acquisition of the fluorescent probe for membrane potential bis(1,3-dibarbituric acid)-trimethine oxonol (DiBAC\(_4\)(3); Molecular Probes) was performed using a spinning disc confocal microscope. The incident laser beam (488 nm) (Innova 70C spectrum, Coherent, Inc.) was injected into a Yokogawa spinning disc confocal scan head (QLC100, Visitec International) mounted on an inverted microscope (Zeiss Axiovert 200 M). Fluorescence images (520-nm long-pass filter) were captured with a Cascade II 16-bit cooled EMCCD frame transfer camera (Photometrics-RopperScientific). Images were acquired using the Metafluor 6.3r7 software (Molecular Devices). Cells were plated on glass coverslips and continuously superfused with differentiation medium (DM). Calibration was made by adding gramicidin to isotonic DM containing 5.36 mM K\(^+\) and varying ratios of Na\(^+\) and N-methyl-D-glucamine to maintain [Na\(^+\)] + [N-methyl-D-glucamine] = 136.3 mM. The membrane potential (Em) was then calculated as shown in Equation 1,

\[
Em = \frac{RT\ln(\frac{[Na^+] + [K^+]}{[Na^+] + [K^+]})}{\alpha F}
\]  

with [Na\(^+\)] + [K\(^+\)], assumed to be 150 mM, T = 310 K, Z is the number of elementary charges of the ion, and F is the Faraday constant.

RESULTS

STIM1 Silencing Impairs Myoblast Differentiation and Ca\(^{2+}\) Handling—To test the role of SOCE in muscle differentiation, we manipulated the levels of the SOCE regulatory protein STIM1 in cultured myoblasts derived from human satellite cells. Myoblasts were incubated for 5 h with a siRNA designed against STIM1 (siSTIM1a) and 42 h later switched from a proliferation to a differentiation medium to induce their differentiation into multinucleated myotubes. As shown in Fig. 1A, incubation of myoblasts with siSTIM1a fully prevented the formation of nuclei clusters, a distinctive marker of cell fusion revealed with DAPI staining (top), as well as the appearance of the transcription factor MEF2, an early marker of myoblast differentiation (2) detected by immunofluorescence (bottom). Quantification of the MEF2/DAPI ratio yielded an index of 7 ± 5% (n = 7) instead of the control-siMed index of 60 ± 18% (n = 7), indicating that myoblast differentiation was severely impaired by the genetic invalidation of STIM1. Similar results were obtained with myogenin, another marker of myoblast differentiation (control-siMed = 67 ± 9%, siSTIM1a = 8 ± 3%, n = 3; see Fig. 3, A and C). The amount of STIM1 protein measured by Western blot at the onset of differentiation was reduced by 80% in cells treated with siSTIM1a (Fig. 1B), confirming that STIM1 silencing was quite effective. STIM1 silencing was used to define the threshold above which nuclei were considered as positive for the expression of MF2 or myogenin (10 random fields for each clone).

Because STIM1 is known to control the activation of SOCE, we then evaluated the impact of STIM1 silencing on cell Ca\(^{2+}\) handling using the fluorescent Ca\(^{2+}\) indicator Fura-2. As shown in Fig. 1C, STIM1 silencing altered three main components of the Ca\(^{2+}\) signaling machinery: the basal Ca\(^{2+}\) levels, the amount of Ca\(^{2+}\) released from intracellular stores by thap-
The effect of STIM1 knockdown on the amount of Ca\(^{2+}\) released from intracellular stores by thapsigargin was confirmed by using 1 \(\mu\)M ionomycin in low external Ca\(^{2+}\) (supplemental Fig. S2, A and B). We also verified that re-addition of 1.8 mM Ca\(^{2+}\) does not on its own elicit any Ca\(^{2+}\) transients (supplemental Fig. S2C). Interestingly, reducing external Ca\(^{2+}\) to 250 nM decreased resting Ca\(^{2+}\) levels in control myoblasts but not in siSTIM1a-treated myoblasts (see the dotted lines in supplemental Fig. S2C), suggesting that STIM1 is involved in the regulation of basal Ca\(^{2+}\) entry in myoblasts.

To test whether we could rescue the impaired differentiation of STIM1-silenced cells, we attempted to normalize the defective Ca\(^{2+}\) phenotype of these cells by growing them for 24–30 h in medium supplemented with 6 mM Ca\(^{2+}\). As shown in Fig. 1E, this procedure normalized the resting Ca\(^{2+}\) levels as well as the store content, but not SOCE, which remained inhibited by 73 ± 5% (n = 4). Despite the rescue of two of three visible Ca\(^{2+}\) defects, the differentiation of myoblasts remained severely impaired in Ca\(^{2+}\)-rich medium, as assessed by MEF2 immunofluorescence, which suggests that SOCE was the critical factor required for differentiation.

We then attempted to rescue the differentiation process at the genetic level by re-expressing STIM1 in myoblasts treated with siSTIM1 (Fig. 1F). For this, we designed a new siRNA against STIM1 (siSTIM1b) that targeted the 3'-untranslated non-coding region of the STIM1 mRNA. This siRNA was less efficient than the original siSTIM1a as cells transfected with siSTIM1b (at 1.5-fold the usual concentration of siSTIM1a) showed an inhibition of SOCE and of MEF2 expression of 52 ± 14 and 60 ± 15%, respectively, compared with 87 ± 4% and 90 ± 6% for STIM1a (n = 4, Fig. 1F; see also Fig. 4A for a correlation of SOCE and MEF2 staining; note that at higher doses siSTIM1b was as efficient as siSTIM1a in reducing resting Ca\(^{2+}\), Tg, and SOCE responses). Both SOCE and MEF2 expression could be fully rescued by co-transfecting cells with a
Interestingly, each siOrai impacted differently on cell Ca\(^{2+}\) homeostasis; Orai1 silencing significantly decreased both the resting cytoplasmic Ca\(^{2+}\) levels as well as SOCE (Fig. 2A), whereas Orai2 knockdown had no visible effects on Ca\(^{2+}\) handling (Fig. 2B), and Orai3 siRNA only diminished SOCE without altering resting Ca\(^{2+}\) levels (Fig. 2C). Quantification of the peak SOCE response confirmed that Orai2 knockdown did not alter Ca\(^{2+}\) influx and showed that Orai1 silencing was more efficient than Orai3 silencing in reducing SOCE (57 ± 1 \textit{versus} 40 ± 12% inhibition, \(p < 2 \times 10^{-4}\), Fig. 2F). We verified by quantitative RT-PCR analysis that the level of Orai1 mRNA was preserved in cells treated with siOrai3 (data not shown). Notably, none of the Orai siRNA fully recapitulated the effect of STIM1 silencing, which not only reduced resting Ca\(^{2+}\) levels and SOCE but also blunted Tg-induced Ca\(^{2+}\) release.

We then assessed the effects of the three Orai siRNA on myoblast differentiation. As shown in Fig. 3, A and B, MEF2 immunolabeling was normal in Orai2-silenced myoblasts and strongly decreased in Orai1- and Orai3-depleted cells (from 60 ± 18 to 13 ± 7 and 16 ± 12%, respectively, \(p < 5 \times 10^{-4}\) for both conditions, \(n = 6\)), approaching the very low levels observed with STIM1 silencing. An analysis of variance test confirmed that all the siRNA except siOrai2 significantly prevented MEF2 expression (F ratio = 30, \(p = 5 \times 10^{-8}\)). Similar results were obtained with the expression of myogenin (control-siMed = 67 ± 9\%, siOrai1 = 11 ± 9\%, siOrai2 = 69 ± 15\%, siOrai3 = 19 ± 14\%; Fig. 3, A and C). Thus, Orai1 and Orai3 silencing mimic the effects of STIM1 silencing in reducing SOCE and in preventing the initiation of the differentiation process.

Analogous results were obtained with a second set of siRNA designed against Orai1 and Orai3, confirming that the effects were due to the depletion of these two Orai isoforms. The alternative Orai siRNAs were slightly less efficient in decreasing SOCE and MEF2 expression than the original set, but quantitative analysis of the different siRNAs effects provided further support for a causal link between SOCE and myoblast differentiation. As shown in Fig. 4A, MEF2 expression varied linearly as a function of the residual SOCE activity for all siRNAs used, from the most efficient (siSTIM1a) to the less efficient (siOrai2).
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To test whether this increased SOCE activity was required for late differentiation to proceed, we silenced STIM1 shortly (5 h) before initiating the differentiation. In these conditions SOCE was preserved at the onset of differentiation but was strongly inhibited after 14 h in DM and maximally inhibited after 34 h in DM (by 65 ± 10 and 80 ± 4%, respectively, p < 10⁻³), and a strong SOCE inhibition was also observed in multinucleated cells (Fig. 5B, left panel, black bars). Despite this strong late inhibition of SOCE, MEF2 expression and myotubes formation proceeded normally (Fig. 5B, right panel, black diamonds). Thus, myoblast differentiation proceeded unabatedly when SOCE was decreased during the differentiation process but was preserved at the onset of differentiation.

In contrast, when STIM1 was silenced 48 h before switching cells to differentiation medium, myoblast did not differentiate for more than 3 days, and MEF2 expression reappeared only at later time points, likely reflecting the exhaustion of the siRNA. Indeed, both STIM1 protein as well as SOCE began to re-appear after 2 days in differentiation medium, i.e. 1–2 days before the recovery of differentiation (supplemental Fig. S3).

These results clearly discriminate two successive roles of SOCE during post-natal myogenesis. Although SOCE is required for the initiation of myoblast differentiation, its up-regulation after 34 h in DM is not required for differentiation to proceed and is, thus, probably involved in a specific function that rely on Ca²⁺ in adult muscle cells.

A more accurate timing cue was obtained using a potentiometric fluorescent probe, DIBAC₄(3), to measure the changes in plasma membrane potential during myoblast differentiation. Myoblasts hyperpolarize to −70 mV within the first 6 h of differentiation due to the activation of ether-à-go-go and Kir2.1 K⁺ channels (33–35), and this hyperpolarization favors Ca²⁺ entry by increasing the driving force on Ca²⁺ ions (2, 3). As shown in Fig. 5, C and D, the resting membrane potential averaged −70 mV in control-siMed cells placed 36–48 h in DM but only −47 mV in STIM1-depleted cells and −37 mV in Orai1-silenced cells. Similar results were obtained with siSTIM1b (supplemental Fig. S4) which reduced SOCE at half the efficiency of siSTIM1a without altering resting Ca²⁺ levels, indicating that the failure of knockdown cells to hyperpolarize were related to the defect in SOCE. These results indicate that STIM1 and Orai1 are required for the hyperpolarization of

and throughout the whole range of siRNAs with intermediate activity (siOrai1a > siOrai3a = siSTIM1b > siOrai1b = siOrai3b > Control-siMed). Thus, MEF2 expression was linearly related to the activity of SOCE, independently of the signaling molecule that was knocked down. A similar relationship was found for myogenin expression as shown in Fig. 4B. These results indicate that the amplitude of SOCE critically determines a differentiation signal that drives the early differentiation of myoblasts.

**FIGURE 3. Orai1, Orai3, and STIM1 knockdown impact on differentiation.** A, myoblasts were transfected with a siRNA against STIM1 (siSTIM1), Orai1, Orai2, or Orai3, kept for 2 days in proliferation medium, and then kept for 2 more days in DM. MEF2 is stained in red, myogenin in green, and nuclei in blue (DAPI). B, effects of siRNA against STIM1, Orai1, Orai2, and Orai3 on MEF2 expression. Colored marks show the fraction of MEF2 kept for 2 more days in DM. MEF2 is stained in siSTIM1 with a siRNA against STIM1 (siSTIM1b/H11022), Orai1, Orai2, or Orai3, kept for 2 days in proliferation medium, and then throughout the whole range of siRNAs with intermediate activity (siOrai1a/siOrai3a = siSTIM1b > siOrai1b = siOrai3b > Control-siMed). Thus, MEF2 expression was linearly related to the activity of SOCE, independently of the signaling molecule that was knocked down. A similar relationship was found for myogenin expression as shown in Fig. 4B. These results indicate that the amplitude of SOCE critically determines a differentiation signal that drives the early differentiation of myoblasts.

**STIM1 Is Required for the Initiation of Myoblast Differentiation**—To define more precisely the timing of the differentiation events that relies on SOCE, we measured the relative changes in mRNA of the SOCE signaling molecules during the differentiation process. As shown in Fig. 5A, the levels of STIM1 and Orai1 mRNA measured by quantitative RT-PCR 34 h after inducing myoblast differentiation were up-regulated by ~3 and ~6-fold, respectively. The increase in STIM1 and Orai1 correlated with an increase in SOCE which was predominately detected in multinucleated myotubes, i.e. differentiated cells that appeared 34 h after the induction of differentiation (Fig. 5B, left panel, open bars). At this stage, STIM1 immunoreactivity was also detected essentially in multinucleated myotubes (data not shown), suggesting that an increased SOCE might be required for events occurring later in the differentiation process.

To test whether this increased SOCE activity was required for late differentiation to proceed, we silenced STIM1 shortly (5 h) before initiating the differentiation. In these conditions SOCE was preserved at the onset of differentiation but was strongly inhibited after 14 h in DM and maximally inhibited after 34 h in DM (by 65 ± 10 and 80 ± 4%, respectively, p < 10⁻³), and a strong SOCE inhibition was also observed in multinucleated cells (Fig. 5B, left panel, black bars). Despite this strong late inhibition of SOCE, MEF2 expression and myotubes formation proceeded normally (Fig. 5B, right panel, black diamonds). Thus, myoblast differentiation proceeded unabatedly when SOCE was decreased during the differentiation process but was preserved at the onset of differentiation.

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STIM1 and Orai1 Control Myoblast Differentiation

A

![Diagram A](image)

B

![Diagram B](image)

**FIGURE 4.** **SOCE is a crucial Ca\(^{2+}\) source for myoblasts differentiation.**

A, MEF2-positive nuclei are represented as a function of SOCE (filled squares = means ± S.E.). For each experiment MEF2 and SOCE are normalized to their respective control-siMed. A linear regression was fitted independently of the SOCE partner knocked down. The linear fit was forced neither through (0; 0) nor through (1; 1); correlation factor = 0.95. B, Myogenin-positive nuclei are represented as a function of SOCE (mean ± S.E.). MEF2 and SOCE are normalized to their respective control-siMed. The linear fit was forced neither through (0; 0) nor through (1; 1).

myoblasts, placing SOCE as one of the earliest event (<6 h) of the differentiation process.

To ensure that the SOCE increase detected in multinucleated myotubes (Fig. 5B) is truly related to an up-regulation of STIM1-Orai1 channels (and does not only reflect an increase in driving force on Ca\(^{2+}\) due to the Kir2.1-linked membrane hyperpolarization), we evaluated the thapsigargin-induced SOCE in low (5 mM) and high KCl (58 mM) conditions (supplemental Fig. S5). An external solution containing 58 mM KCl will clamp the membrane potential around −20 mV. This protocol was applied to proliferating and differentiated myoblasts (34 h in differentiation medium) and to small myotubes (also 34 h in differentiation medium). As expected, altering the external K\(^{+}\) does not affect SOCE amplitude in proliferating myoblasts lacking Kir2.1 channels (and, thus, depolarized). In contrast, reducing the external K\(^{+}\) from 58 to 5 mM increased SOCE by 50% in differentiated myoblasts and by 400% in small multinucleated myotubes. Because both differentiated myoblasts and multinucleated myotubes are hyperpolarized, the massive SOCE increase observed in myotubes (400 versus 50%) likely reflects the up-regulation of STIM1–Orai1 channels rather than an increased driving force on Ca\(^{2+}\).

**DISCUSSION**

In this study we reveal a central role for SOCE during postnatal human myoblast differentiation. Using siRNA silencing, we show that the levels of the ER Ca\(^{2+}\) sensor STIM1 and of its

**STIM1 and Orai1 Overexpression Accelerate Myoblast Differentiation**—Given the severe effect of STIM1 and Orai1 knockdown on the initiation of myoblast differentiation, we tested whether overexpression of these two signaling molecules could also alter the differentiation process. For this, myoblasts were electroporated 72 h before initiating differentiation with plasmids encoding for STIM1 and Orai1 either alone or in combination. As shown in Fig. 6A, SOCE was increased in myoblasts overexpressing STIM1 or the combination of STIM1 and Orai1. In both cases the peak responses upon Ca\(^{2+}\) readdition to Tg-treated cells increased by ~4-fold (p < 0.01). Interestingly, the duration of the Ca\(^{2+}\) influx phase was markedly prolonged in myoblasts overexpressing the STIM1 + Orai1 combination. In cells overexpressing STIM1 alone, SOCE inactivated rapidly, and the duration of the Ca\(^{2+}\) influx phase was similar to control cells, whereas Ca\(^{2+}\) influx failed to inactivate in cells co-expressing STIM1 and Orai1. Overexpression of Orai1 alone did not affect SOCE (data not shown), suggesting that STIM1 is a limiting factor for SOCE activation by Orai1 in human myoblasts.

To confirm that Orai1 is the dominant partner of STIM1, we silenced the different Orai channels in myoblasts overexpressing STIM1. As showed in Fig. 6B, the increased SOCE conferred by STIM1 overexpression in myoblasts was reversed by Orai1 silencing but not by Orai2 or Orai3 silencing. Thus, STIM1 requires Orai1 to activate SOCE in human myoblasts.

We then assessed the effects of STIM1 and Orai1 overexpression on myoblast differentiation. As shown in Fig. 6C, MEF2 expression assessed after 18−24 h of differentiation was increased in myoblasts co-expressing STIM1 and Orai1 but not in myoblasts overexpressing STIM1 alone (40 ± 4 versus 16 ± 4% MEF2 positive nuclei, respectively, p < 0.0001, n = 4). No differences were observed when MEF2 expression was assessed after 42−50 h of differentiation (Fig. 6D), indicating that the increased SOCE conferred by STIM1 and Orai1 overexpression affected the initiation rather than the progression of differentiation. Thus, as predicted from the correlation between SOCE amplitude and MEF2 expression levels derived from our validation studies (Fig. 4, A and B), increasing SOCE by overexpression of STIM1 and Orai1 accelerated the differentiation of myoblasts into myotubes.
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channel partners Orai1 and Orai3 regulate the amplitude of SOCE in proliferating myoblasts. The SOCE amplitude in turn determined the fate of myoblasts upon induction of the differentiation program. The effects of the siRNA were very robust and were observed in all clones tested regardless of the time spent in culture before the genetic invalidation. Failure to differentiate was not due to toxic deregulation of the Ca^{2+} homeostatic machinery or nonspecific effects of the siRNAs, as STIM1 knockdown cells grew normally and differentiated into myotubes upon re-expression of the STIM1 protein. Remarkably, the SOCE levels measured at the onset of differentiation were linearly related to the amount of expression of transcription factors MEF2 and myogenin, two early markers of differentiation. The straight correlation between SOCE amplitude and factors MEF2 and myogenin, two early markers of differentiation, indicates that SOCE is the limiting factor in the signaling cascade that controls the fate of myoblasts. In an earlier study we showed that pharmacological inhibition of SOCE prevented myoblasts differentiation but only in a fraction of the clones tested (1). The stringent dependence on self-replicate but do not differentiate, stringently rely on SOCE for their Ca^{2+} homeostasis. This is not unexpected, as myoblasts at this stage, although they have sodium channels (36), are probably essentially non-excitable cells that cannot yet rely on voltage-gated Ca^{2+} channels for signaling. Strikingly, however, SOCE inhibition did not limit the ability of non-excitable myoblasts to proliferate but specifically impaired their ability to differentiate into excitable myotubes. This defect persisted when both resting Ca^{2+} and Ca^{2+} store content were corrected with SiOrai1, (C) or siOrai1a at 48 h have an average resting potential around −40 mV, whereas differentiated myoblasts hyperpolarize to around −70 mV (siSTIM1 n = 3 clones, siOrai1 n = 4 clones).

SOCE revealed by our invalidation study suggests that some clones could escape the pharmacological blockade, which is not surprising regarding the poor selectivity of the available agents. Our new findings clearly indicate that for post-natal myoblast clones adequate levels of SOCE are definitely required to initiate the differentiation process.

In human myoblasts a decrease in SOCE amplitude impaired several parameters of the cellular Ca^{2+} homeostatic machinery. Cells with markedly decreased SOCE had an abnormally low resting Ca^{2+} concentration as well as a decreased amount of Ca^{2+} releasable from intracellular stores. A minor decrease in the basal Ca^{2+} concentration was previously reported in HeLa cells depleted of STIM1 (9), although this study identified STIM2 as the major molecule that regulates resting cytosolic and ER Ca^{2+} levels. We verified by Western blot that a decrease in STIM2 expression did not occurred concurrently with STIM1 knockdown (supplemental Fig. S6). The decreased basal Ca^{2+} levels observed in proliferating myoblasts were, thus, not caused by a decrease in STIM2 levels but instead correlated with the degree of SOCE inhibition. The marked alterations in Ca^{2+} signaling caused by SOCE inhibition indicates that proliferating myoblasts, i.e. myoblasts that

FIGURE 5. STIM1 is required for the initiation of myoblast differentiation but not for cell fusion. A, evaluation of mRNA levels shows that STIM1 and Orai1 transcription is up-regulated after 34 h in DM (mRNA levels were normalized to 1 in growth medium (GM); mean ± S.D., n = 3 clones). B, SOCE is up-regulated in multinucleated myotubes after 34 h in DM and strongly inhibited by transfecting siSTIM1 shortly (−5 h) before the initiation of differentiation (mean ± S.E., n = 3 clones). SOCE was preserved at t = 0 in cells transfected at −5 h (left panel). Myoblasts transfected with siSTIM1 at −5 h differentiated normally (right panel, black diamonds; mean ± S.D. on 3 clones). C and D, bar graph showing the distribution of membrane potential in a population of differentiating myoblasts. Myoblasts transfected with siSTIM1 (C) or siOrai1a at 5 h differentiated normally (right panel, black diamonds; mean ± S.D. on 3 clones). D, transfected at 5 h (left panel). Myoblasts transfected with siSTIM1 at −5 h differentiated normally (right panel, black diamonds; mean ± S.D. on 3 clones).
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FIGURE 6. STIM1-Orai1 overexpression accelerates differentiation. A, effect of STIM1-wt overexpression either alone or together with Orai1 on SOCE measured by Fura-2 in proliferating myoblasts. SOCE was revealed by re-addition of 1.8 mM Ca$^{2+}$ after store depletion with 1 μM thapsigargin in a medium containing 250 nM Ca$^{2+}$ (mean ± S.E., STIM1-wt n = 3 clones, STIM1-wt + Orai1 n = 4 clones, experiments were all independent). B, the increased cytoplasmic Ca$^{2+}$ response conferred by STIM1-wt overexpression was specifically reversed by Orai1 silencing (mean ± S.E. on 15 cells). SOCE was revealed as in A. CEF2 expression at the onset of differentiation (18–24 h) in myoblasts transfected either with STIM1-wt alone or with STIM1-wt and Orai1 (mean ± S.E., n = 3 clones). D, MEF2 expression after 42–50 h in DM in myoblasts transfected with STIM1-wt and Orai1 (mean ± S.D., n = 3 clones). E, scheme illustrating the central function of the SOCE in the initiation of myoblast differentiation. Ca$^{2+}$ entry through STIM1-Orai1 channels initiates the differentiation by generating Ca$^{2+}$ signals that drive the hyperpolarization of myoblasts. The hyperpolarization increases the driving force for Ca$^{2+}$ entry, enabling SOCE channels to generate larger Ca$^{2+}$ signals that activate calcineurin, thereby irreversibly committing myoblasts toward differentiation into multinucleated myotubes. SOCE requirement decreases gradually during this signaling cascade because of the built in amplification conferred by the hyperpolarization.

gene in a whole organism often has little effect because of compensatory mechanisms developed during evolution, like genetic redundancy, that act as safeguards (37, 38). Consistent with a possible in vivo compensatory mechanism for STIM1, we could rescue in vitro normal Ca$^{2+}$ handling in siSTIM1-treated myoblasts by overexpressing STIM2 (data not shown). According to the linear relationships between differentiation and SOCE (Fig. 4), this result clearly suggests that an increased expression of STIM2 can substitute for STIM1 knockdown and could on its own explain why STIM1 null mice are viable (27). Another adaptive intracellular response that could explain the phenotype of STIM1 and Orai1 KO mice is that other Ca$^{2+}$ sources, such as voltage-gated T-type Ca$^{2+}$ channels, may be recruited to allow myogenesis (1). We should, however, emphasize that, although such compensatory mechanisms probably occur in STIM1 and Orai1 null mice, they do not allow normal muscle differentiation. The majority of STIM1 and Orai1 KO littermates die within hours after birth due to unknown muscular defects leading to reduced muscle mass (25, 26, 39, 40). This strongly suggests defects in myoblast differentiation and/or proliferation, the two major determinants of muscle mass at birth. Finally, substantial disparities exist between mice and human in the reliance on SOCE components. The severe combined immunodeficiency syndrome is a hallmark of the Orai1$^{-/-}$ human phenotype (28) but is not phenocopied by Orai1$^{-/-}$ mice, although murine B and T cell activation has been shown to depend on Orai1 (39). Muscle differentiation might also rely differently on STIM1 and Orai1 in human and mice. Altogether these results provide a rational explanation for the observation that STIM1 and Orai1 KO mice have functional but much reduced muscle mass.

We have previously shown that within the first 6 h of the differentiation process, human myoblasts up-regulate two K$^{+}$ channels, the ether-à-go-go and the Kir2.1 inward rectifier K$^{+}$ channels (2, 30, 33–35, 41). The role of these channels is to hyperpolarize the resting membrane potential to approximately −70 mV which, by increasing the driving force on Ca$^{2+}$ ions, will amplify all Ca$^{2+}$ influxes. Thus, as proposed in Fig. 6E, Ca$^{2+}$ entry through the STIM-Orai machinery could promote its own amplification by activating these K$^{+}$ channels. In the present work we found that myoblasts with reduced expression of STIM1, Orai1, or Orai3 could not hyperpolarize to −70 mV when triggered to differentiate. This suggests that SOCE is indeed required to induce the early developmentally linked K$^{+}$ channel activation and confirms that SOCE activity is crucial in the very first steps of the differentiation process. In addition, two siRNA that insufficiently decreased SOCE channel activity and confereed by the hyperpolarization, indicating that SOCE rather than resting cytoplasmic Ca$^{2+}$ or intracellular Ca$^{2+}$ store levels is required for the early K$^{+}$ channel activation. These observations are in
agreement with the fact that store-operated Ca\(^{2+}\) channels are already present in proliferating myoblasts, i.e. at the very beginning of the differentiation process, before K\(^{+}\) channel activation.

In blood cells SOCE channel activity is essential for both rapid responses such as mast cell degranulation and long term responses that rely on gene transcription, such as the proliferation and production of cytokines by T cells (42). After the discovery by Feske et al. (16) of the Orai1 mutation that abrogates SOCE in T cells from patients with severe combined immunodeficiency, several animal models have confirmed the central role of SOCE molecules in blood cells. Orai1 null mice have mast cell defects and cannot mount allergic reactions (25), whereas T cell-specific ablation of either STIM1 or STIM2 selectively decreases the number of thymus-derived regulatory T cells (27), and mice with a mutation that constitutively activates STIM1 suffer from thrombocytopenia (43). The STIM and Orai proteins are, thus, essential for the development and function of blood cells, but these studies have also shown that genetic ablation of either STIM1, STIM2, or Orai1 caused severe growth defects in mice (25, 27), hinting at a role for SOCE in muscle development. Accordingly, two severe combined immunodeficiency syndrome patients with mutations in the Orai1 gene developed severe non-progressive myopathy (16), and the Orai1 channel protein is highly expressed in skeletal muscle (25). Our study now demonstrates that the ubiquitous SOCE Ca\(^{2+}\) influx pathway critically controls the regeneration of human skeletal muscle, consistent with the recent studies of Stiber et al. (26), and that the two major molecules that orchestrate the SOCE machinery in myoblasts are STIM1 and Orai1.

Our results also suggest that Orai3 contributes to SOCE in human myoblasts, consistent with studies in other cell types (44–46). However, the effect of Orai3 knockdown on SOCE and differentiation varied considerably among different clones, indicating that the expression of Orai3 is more variable than that of Orai1. In myoblasts overexpressing STIM1, Orai3 silencing had no effect, but Orai1 silencing was still fully effective (Fig. 6), suggesting that Orai1 is expressed in excess compared with Orai3. Moreover, the residual Ca\(^{2+}\) entry observed upon Orai1 silencing was 2-aminoethoxydiphenyl borate (2-APB) sensitive (supplemental Fig. S7) and, thus, cannot be attributed to Orai3, as 2-APB stimulates rather than inhibits Orai3 (45, 46). Taken together, these findings suggest that Orai3 plays a minor role in human myoblasts and probably depends on Orai1 to form a functional store-operated Ca\(^{2+}\) channel.

The identical SOCE reliance of blood cells and myoblasts points to similarities in the signaling cascades that control the development of lymphocytes and of skeletal muscle cells. In lymphocytes, Ca\(^{2+}\) signals are frequency-encoded, and Ca\(^{2+}\) influx determines the frequency of the cytosolic Ca\(^{2+}\) oscillations that control the activation of specific genes (42). For instance, in T cells NF-AT is optimally activated by rhythmic Ca\(^{2+}\) oscillations occurring with a period of 6 min, whereas NF-κB is also activated by much slower oscillations (47). The rhythmic Ca\(^{2+}\) signals activate the Ca\(^{2+}\)-dependent phosphatase calcineurin, which dephosphorylates NF-AT, inducing its nuclear translocation (42). Much less is known about the signaling cascade that controls early muscle cell differentiation. As in T cells, the calcineurin pathway is used to control gene transcription, and inhibition of calcineurin with cyclosporin A and FK506 abrogates myoblast differentiation (3). Calcineurin synergizes with Ca\(^{2+}\)-calmodulin kinase II, another Ca\(^{2+}\)-dependent enzyme, to activate the muscle-specific transcription factors myogenin and MEF2 that drive the differentiation of myoblasts into myotubes (3). Whether NF-AT participates in the activation of these muscle-specific genes is not clear (48). Ca\(^{2+}\) oscillations have not been reported during myoblast differentiation, and we do not know whether the Ca\(^{2+}\) signals that induce muscle differentiation are encoded in time, space, frequency, or amplitude. Two types of Ca\(^{2+}\) signals have been postulated, as calcineurin activation, but not Ca\(^{2+}\)-calmodulin kinase II activation, was prevented by inhibition of the hyperpolarization that occurs during early differentiation (3). By impacting on the calcineurin pathway, depolarization largely prevented the differentiation. However, a much more dramatic effect was observed here with STIM1 silencing, suggesting that both the hyperpolarization-dependent Ca\(^{2+}\) signal that activates calcineurin as well as the hyperpolarization-independent Ca\(^{2+}\) signal that activates Ca\(^{2+}\)-calmodulin kinase II are fueled by SOCE.

The mysterious Ca\(^{2+}\) signals that drive the differentiation process have so far not been visualized, but this is probably due to technical limitations, as the differentiation process takes days, whereas cytosolic Ca\(^{2+}\) measurements with dyes are limited to a maximum of a few hours. Although genotypically encoded Ca\(^{2+}\) indicators can greatly prolong the duration of Ca\(^{2+}\) recordings (49), the visualization of the specific Ca\(^{2+}\) signals that trigger the differentiation remains very difficult as the timing of their occurrence during the differentiation process was so far unknown. Our study indicates that SOCE is required at the onset of differentiation, and the Ca\(^{2+}\)-dependent process occurs within the first 6h of differentiation, thus pinpointing the precise timing of the elusive Ca\(^{2+}\) signals. Further studies should focus on this early phase and might benefit from the increased SOCE conferred by STIM1 and Orai1 co-expression to better visualize the Ca\(^{2+}\) signals that control myogenesis.

In summary, our study demonstrates a central role of STIM1 and Orai1 in the induction of the differentiation process of post-natal human myoblasts (Fig. 6E). SOCE not only controls the fate and function of blood cells but in our model determines the transition from non-excitatory proliferating myoblasts to excitatory skeletal muscle cells. These findings could have potential applications in the treatment of genetic myopathies, as drugs modulating SOCE activity could promote cell transplantation efficiency either by keeping engrafted cells in a prolonged proliferating state or by forcing engrafted cells to initiate their differentiation.

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