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Abstract

Myogenesis involves expression of muscle-specific transcription factors such as myogenin and myocyte enhancer factor 2 (MEF2), and is essentially regulated by fluctuations of cytosolic Ca(2+) concentration. Recently we demonstrated that molecular players of store-operated Ca(2+) entry (SOCE), stromal interacting molecule (STIM) and Orai, were fundamental in the differentiation process of post-natal human myoblasts. Besides STIM and Orai proteins, the family of transient receptor potential canonical (TRPC) channels was shown to be part of SOCE in several cellular systems. In the present study, we investigated the role of TRPC channels in the human myogenesis process. We demonstrate, using an siRNA strategy or dominant negative TRPC overexpression, that TRPC1 and TRPC4 participate in SOCE, are necessary for MEF2 expression, and allow the fusion process to generate myotubes of normal size. Conversely, the overexpression of STIM1 with TRPC4 or TRPC1 increased SOCE, accelerated myoblast fusion, and produced hypertrophic myotubes. Interestingly, in cells depleted of TRPC1 or TRPC4, the normalization of SOCE by increasing the [...]

Reference


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During post-natal human myogenesis, normal myotube size requires TRPC1- and TRPC4-mediated Ca\textsuperscript{2+} entry

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Summary

Myogenesis involves expression of muscle-specific transcription factors such as myogenin and myocyte enhancer factor 2 (MEF2), and is essentially regulated by fluctuations of cytosolic Ca\textsuperscript{2+} concentration. Recently we demonstrated that molecular players of store-operated Ca\textsuperscript{2+} entry (SOCE), stromal interacting molecule (STIM) and Orai, were fundamental in the differentiation process of post-natal human myoblasts. Besides STIM and Orai proteins, the family of transient receptor potential canonical (TRPC) channels was shown to be part of SOCE in several cellular systems. In the present study, we investigated the role of TRPC channels in the human myogenesis process. We demonstrate, using an siRNA strategy or dominant negative TRPC overexpression, that TRPC1 and TRPC4 participate in SOCE, are necessary for MEF2 expression, and allow the fusion process to generate myotubes of normal size. Conversely, the overexpression of STIM1 with TRPC4 or TRPC1 increased SOCE, accelerated myoblast fusion, and produced hypertrophic myotubes. Interestingly, in cells depleted of TRPC1 or TRPC4, the normalization of SOCE by increasing the extracellular calcium concentration or by overexpressing STIM1 or Orai1 was not sufficient to restore normal fusion process. A normal differentiation occurred only when TRPC channel was re-expressed. These findings indicate that Ca\textsuperscript{2+} entry mediated specifically by TRPC1 and TRPC4 allow the formation of normal-sized myotubes.

Key words: TRPC channels, SOCE, Myogenesis, Ca\textsuperscript{2+} signalling, STIM1/Orai1

Introduction

Upon skeletal muscle injury, myogenic stem cells (called satellite cells) proliferate as myoblasts, migrate and align in the lesion sites where they differentiate, fuse to form multinucleated myotubes, and eventually mature into new muscle fibers (Bernheim and Bader, 2002). Ca\textsuperscript{2+} signals are known to be essential for the initiation and the maintenance of the differentiation process (Bernheim and Bader, 2002), e.g. an increase of cytosolic Ca\textsuperscript{2+} concentration is required for the activation of myogenic transcription factors such as myogenin and myocyte enhancer factor 2 (MEF2) (Molkentin and Olson, 1996; Black and Olson, 1998). Classically, Ca\textsuperscript{2+} signal is generated by Ca\textsuperscript{2+} release from the internal stores and/or by Ca\textsuperscript{2+} entry from the extracellular compartment through Ca\textsuperscript{2+} or cationic channels. A particular type of Ca\textsuperscript{2+} entry, called store-operated Ca\textsuperscript{2+} entry (SOCE), is activated by Ca\textsuperscript{2+} store depletion. It involves the stromal interacting molecule 1 (STIM1), which is a single-spanned transmembrane protein that senses the ER/SR Ca\textsuperscript{2+} concentration (Liou et al., 2005; Roos et al., 2005; Zhang et al., 2005), and a Ca\textsuperscript{2+} channel of the Orai family (Feske et al., 2005; Vig et al., 2006; Yeromin et al., 2006). STIM1 has a homolog STIM2, while the Orai family comprises three isoforms, Orai1, Orai2 and Orai3. SOCE is mostly studied in non-excitable cells, but it was recently demonstrated to be important for excitable cell physiology, such as in skeletal muscle fibers (Stiber and Rosenberg, 2011). In addition, alteration of SOCE is associated with defect of skeletal muscle differentiation in mouse (Stiber et al., 2008), a finding in line with the phenotype of human patients bearing Orai1 or STIM1 mutations that suffer from congenital myopathy together with a sever immunodeficiency (McCarl et al., 2009; Picard et al., 2009; Feske et al., 2010). Recently, we demonstrated in primary human myoblasts that STIM1, STIM2 and Orai1 molecules are essential for post-natal myoblast differentiation (Darbellay et al., 2009; Darbellay et al., 2010). In STIM1, STIM2 or Orai1 silenced myoblasts, SOCE amplitude was severely reduced (>60–70%), and this reduction was closely correlated with a decreased expression of the two early markers of myoblast differentiation, MEF2 and myogenin (Darbellay et al., 2009; Darbellay et al., 2010).

Skeletal muscle also expressed another family of non-voltage gated channels, namely the transient receptor potential canonical (TRPC) channels, which are non-selective cation channels. Signalling pathways leading to TRPC activation is still a debated issue, but it was shown that TRPC1, TRPC4 and TRPC5 participate to SOCE in several cell types (Ambudkar, 2007; Worley et al., 2007; Yuan et al., 2007; Salido et al., 2011), including primary mouse myotubes and C2C12 mouse muscle cell line (Vandebrouck et al., 2007; Louis et al., 2008; Sabourin et al., 2009). In addition, TRPC1 was shown to play an important role to sustain skeletal muscle contraction (Zanou et al., 2010) and, in C2C12 cell line, TRPC1 silencing decreased SOCE and
the fusion process (Louis et al., 2008; Formigli et al., 2009; Meacci et al., 2010), hence impairing muscle formation. Another study reported a role of TRPC3 in myogenesis, but using cells that also lack the dihydropyridine receptor (Woo et al., 2010). To our knowledge, these are the only studies evaluating the role of TRPC in muscle differentiation, and so far no data are available in human muscle. The aim of the present study was thus to investigate, in human primary myoblasts, the role of TRPC channels in SOCE, differentiation and fusion processes.

**Results**

**TRPC channel expression in human myoblasts**

Using western blot analysis, we showed that TRPC1, TRPC4 (Fig. 1A), TRPC3 and TRPC6 (supplementary material Fig. S1A) are expressed in proliferating myoblasts (GM) and during myoblast differentiation (DM). TRPC5 and TRPC7 were not studied as (unlike TRPC1, TRPC3, TRPC4 and TRPC6) mRNAs coding for these channels were not detected by RT-PCR in human myoblasts (data not shown). Interestingly, TRPC1 and TRPC4 channels presented a peak of expression about 16 hours after the initiation of differentiation (Fig. 1A,B), while the expression of TRPC3 was relatively stable, and the expression of the TRPC6 isoform increased at the end of the differentiation process, suggesting a possible role for TRPC6 isoform on mature myotubes (supplementary material Fig. S1A,B).

**TRPC1 or TRPC4 knockdown inhibits early step of human myoblast differentiation**

To evaluate a putative role of TRPC channels during human myoblast differentiation, myoblasts were transfected with specific siRNA against each TRPC isoform, and expression of two early markers of muscle differentiation, MEF2 and myogenin, was assessed. Transfected myoblasts were kept 2 days in growth medium and then transferred for 2 additional days in differentiation medium to induce myoblast differentiation. After siRNA treatment, the amount of TRPC proteins was reduced by 80% and 60%, for TRPC1 and TRPC4, respectively (Fig. 1C,D). The effect on TRPC3 and TRPC6 protein level is reported in supplementary material Fig. S1C,D. Silencing TRPC1 or TRPC4 reduced MEF2 expression (the antibody recognized different isoforms of MEF2) by 40% and 60%, respectively (Fig. 1E,F). On the other hand, myogenin expression was not significantly affected.

![Fig. 1. TRPC1 and TRPC4 expression during early human myoblast differentiation.](image-url)
affected by siTRPC1 treatment while siTRPC4 reduced myogenin expression by 25% (Fig. 1E,G). Similar effects were obtained using different siRNA against TRPC1 and TRPC4 (supplementary material Fig. S2A–C). The knockdown of both TRPC1 and TRPC4 led to a more pronounced effect on MEF2 expression while it did not further decrease myogenin expression (Fig. 1E–G). In contrast, as shown in supplementary material Fig. S1E, human myoblast differentiation was not affected by TRPC3 or TRPC6 knockdown.

TRPC1 or TRPC4 knockdown reduces the size of myotubes

Clusters of nuclei that can be observed in TRPC1 or TRPC4 silenced myoblasts (Fig. 1E, see white arrows) suggest that myotubes, although smaller than normal, are still formed in these conditions. The mean number of nuclei per myotube was used to evaluate the size of myotubes after siTRPC treatment. This number decreased by more than 60% in TRPC1 or TRPC4 silenced conditions (Fig. 2A,B), and the formation of myotubes was almost completely abolished in myoblasts silenced for both TRPC1 and TRPC4 (Fig. 2A–C). As expected, siTRPC3 or siTRPC6 treatment, which affected neither MEF2 nor myogenin expression, had no impact on this parameter (supplementary material Fig. S1F). We also evaluated the fusion index (number of nuclei in myotubes/total number of nuclei) in presence of the various siRNA. Only TRPC4 silencing slightly reduced the fusion index (Fig. 2C; supplementary material Fig. S1F). Hence, our results showed that TRPC3 and TRPC6 are not required for myoblast differentiation and fusion, while the presence of TRPC1 and TRPC4 channels are essential to establish a normal differentiation and fusion process. We thus focused our study on the role of the TRPC1 and TRPC4 isoforms.

Involvement of TRPC1 and TRPC4 isoforms in myoblast SOCE

To investigate the role of TRPC1 and TRPC4 channels in human myoblast SOCE, internal Ca\(^{2+}\) stores were depleted by application of 1 μM thapsigargin (Tg, an irreversible SERCA pump inhibitor) in Ca\(^{2+}\)-free solution, and the Tg-induced Ca\(^{2+}\) entry (SOCE) measured after re-addition of 2 mM external Ca\(^{2+}\). SOCE, quantified by measuring the peak Ca\(^{2+}\) elevation after Ca\(^{2+}\) re-addition, was clearly reduced after either TRPC1 or TRPC4 silencing. The peak SOCE decreased by 30% in myoblasts kept for 2 days in proliferation conditions after siRNA transfection (Fig. 3A,B), and by 45% in myoblasts kept for an additional day in differentiation conditions (data not shown). Furthermore, the combination of siRNA against TRPC1 and TRPC4 reduced Ca\(^{2+}\) entry by 60% (Fig. 3A,B). On the other hand, Fig. 3C shows that Ca\(^{2+}\) released from intracellular stores by Tg was not reduced by the different siRNA treatments. Silencing of TRPC1 or TRPC4 did not impact on basal Ca\(^{2+}\) entry (data not shown). After double siTRPC1 and siTRPC4 treatment, the efficiency of TRPC1 and TRPC4 silencing was between 50 and 55% at the protein level (supplementary material Fig. S4B). We obtained similar results with another siTRPC1 and another siTRPC4 (supplementary material Fig. S2E). To eliminate the possibility that siTRPC1 or siTRPC4 impacted on other targets related to Ca\(^{2+}\) entry, we verified by western blot the expression of STIM1, STIM2 and Orai1, following TRPC1 or

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Fig. 2. TRPC1 or TRPC4 knockdown affects human myoblast fusion.

(A) Human myoblasts were transfected with siTRPC1 or siTRPC4 (or both siRNAs), kept for 2 days in growth medium, and for 2 more days in differentiation medium (DM). Myotubes were stained using an antibody against myosin heavy chain (MF20 antibody), and nuclei in blue (DAPI). Scale bars: 20 μm. (B) Quantification of the number of nuclei per myotubes after siTRPC1 or siTRPC4 treatment (n>9 clones for each condition). The number of nuclei per myotube obtained in control condition was normalized to 1.
TRPC4 knockdown. No reduction of STIM or Orai1 was observed after TRPC channels silencing (supplementary material Fig. S4C,D), confirming the specificity of siRNA treatment on human primary myoblasts SOCE.

To confirm the results obtained with siRNA, we performed similar experiments (quantification of myoblast differentiation and of SOCE amplitude) in human myoblasts transiently transfected with two different TRPC mutants, TRPC4 F562A and TRPC1 F562A (Zeng et al., 2008; Kim et al., 2009). Overexpression of each mutant reduced SOCE amplitude by ~40% (Fig. 3D), validating the mutants as a dominant negative (DN) of the corresponding wild-type TRPC channel. We then investigated the impact of these dominant negative mutants on myoblast differentiation. Overexpression of DN-TRPC1 or DN-TRPC4 reduced MEF2 expression by 40 and 56%, respectively, while double DN overexpression reduced MEF2 expression by 65% (Fig. 3E). Myogenin expression was not significantly affected by DN-TRPC1 overexpression, but slightly decreased by DN-TRPC4 overexpression (Fig. 3F). Finally, when both mutated channels were overexpressed at the same time, myogenin expression was reduced by more than 70%. Hence, results obtained with a dominant negative strategy were very similar to those obtained with the siRNA approach and confirms the key role played by TRPC1 and TRPC4 in SOCE and human myoblast differentiation. To better understand the mechanism of MEF2 and myogenin downregulation after decreased of Ca2+ entry via TRPC channels, we quantified the mRNA level of three MEF2 transcription factors (MEF2a, MEF2c and MEF2d) and myogenin after overexpression of DN-TRPC channels. As shown in supplementary material Fig. S3, the transcription of MEF2 isoforms and myogenin are decreased, MEF2c being the most affected one. As a control, we also confirmed that the mRNA level of all transcription factors increased during differentiation.

We finally used Luciferase reporter gene to assess the activity of MEF2 and myogenin that was severely reduced upon overexpression of DN-TRPC channels (supplementary material Fig. S3C).

Fig. 3. TRPC1 and TRPC4 contribute to SOCE in human myoblasts. (A) Cytosolic Ca2+ was assessed using Fura-2 in proliferating myoblasts transfected with siTRPC1 or siTRPC4 (or both siRNAs). SOCE was triggered by internal store depletion with 1 μM thapsigargin (Tg) in the absence of external Ca2+ (1 mM EGTA, Ca2+ free), and measured during re-addition of 2 mM external Ca2+. Each trace represents the mean of a representative coverslip. (B) Quantification of the effects of siTRPC1 or siTRPC4 (or both siRNAs) on SOCE amplitude (>168 cells and >5 different clones in each condition). (C) Quantification of Tg-induced Ca2+ release in each condition (same cells as in B). (D) SOCE amplitude in dominant-negative TRPC1 F562A (DN-TRPC1), in dominant-negative TRPC4 F562A (DN-TRPC4) and in DN-TRPC1 + DN-TRPC4 overexpressing myoblasts (>35 cells and >3 different clones in each condition). (E) MEF2 positive nuclei in DN-TRPC1, in DN-TRPC4 and in DN-TRPC1 + DN-TRPC4 overexpressing myoblasts (>3 different clones in each condition). (F) Myogenin positive nuclei in DN-TRPC1, in DN-TRPC4 and in DN-TRPC1 + DN-TRPC4 overexpressing myoblasts (>3 different clones in each condition).

STIM1-TRPC4 or -TRPC4 overexpression accelerate myoblast differentiation

As differentiation is impaired in TRPC1- or in TRPC4-silenced myoblasts, we evaluated the impact of TRPC1 and TRPC4 overexpression on the differentiation process. Fig. 4A,B shows that, although mTRPC4 overexpression only slightly increased SOCE amplitude, the simultaneous overexpression of mTRPC4 and STIM1 increased SOCE by 2.2 fold. Fig. 4A,B also shows that STIM1 overexpression was on its own able to increase SOCE by 1.8 fold. In order to quantify the effect of TRPC/STIM1 overexpression on myoblast differentiation, MEF2 and the myotube size were assessed at the beginning of the fusion process, i.e. after one day in differentiation medium. As shown in Fig. 4C,D, MEF2 expression was increased in myoblasts overexpressing both mTRPC4 and STIM1, but not in myoblasts overexpressing mTRPC4 or STIM1 alone. Fig. 4E illustrates that the number of nuclei per myotube was doubled after simultaneous overexpression of mTRPC4 and STIM1, whereas the overexpression of mTRPC4 or STIM1 alone did not affect the size of myotubes. Similar experiments using hTRPC1 overexpression were performed. hTRPC1 overexpression alone slightly increased SOCE amplitude, whereas the simultaneous overexpression of both hTRPC1 and STIM1 increased SOCE by 2.1 fold (Fig. 4B). MEF2 expression was increased in myoblasts overexpressing both STIM1 and hTRPC1, but surprisingly also in myoblasts overexpressing hTRPC1 alone. Consistently, the size of nascent myotubes almost doubled in myoblasts overexpressing both STIM1 and hTRPC1 or hTRPC1 alone (Fig. 4E).
Hence, the large increase of SOCE following STIM1 overexpression (1.8 fold) was not able to affect neither differentiation nor fusion, whereas a slightly higher SOCE increase (around 2.3 fold) following simultaneous mTRPC4 and STIM1 (or hTRPC1 and STIM1) overexpression accelerates MEF2 expression and myoblast fusion. Surprisingly, hTRPC1 overexpression alone clearly accelerates myoblast differentiation while the effect on SOCE was minimal.

We next wondered whether Ca\(^{2+}\) entering myoblasts through TRPC channels could be substituted by Ca\(^{2+}\) entering through other Ca\(^{2+}\) channel types and could rescue the impaired differentiation of TRPC1- or TRPC4-silenced myoblasts. As shown in Fig. 5A,B, an increase of external Ca\(^{2+}\) from 2 mM to 4 mM fully normalized the Tg-mediated Ca\(^{2+}\) influx in TRPC1- and TRPC4-silenced myoblasts. However, despite normalization of SOCE amplitude, myoblast differentiation remained severely impaired in these conditions. Indeed, in myoblasts silenced for TRPC1 and TRPC4 but maintained in a differentiation medium containing 4 mM Ca\(^{2+}\), MEF2 expression and myotube size were similar to what was observed in 2 mM Ca\(^{2+}\) containing differentiation medium. Thus, although overall Ca\(^{2+}\) influx through SOCE was normalized by increasing external Ca\(^{2+}\) concentration, myoblasts silenced for TRPC1 or TRPC4 channels did not differentiate as control cells. This indicates that normalizing global Ca\(^{2+}\) influx is not sufficient to rescue myoblast differentiation of siTRPC1 or siTRPC4 treated myoblasts.

We then attempted to rescue SOCE and differentiation of TRPC4 silenced myoblasts by transfecting vectors coding for Orai1, STIM1 or mouse TRPC4 (mTRPC4). Overexpression of hTRPC1 (human) in TRPC1-silenced myoblasts was not performed, as our siTRPC1s would inhibit hTRPC1 expression. Fig. 5D shows that overexpression of each protein normalized SOCE, whereas only mTRPC4 overexpression was able to rescue myoblast differentiation (Fig. 5E). Accordingly, neither Orai1 nor STIM1 overexpression in TRPC4- or in TRPC1-silenced myoblasts restored normal myotube size, while only mTRPC4 overexpression was able to re-establish a normal number of nuclei per myotubes in TRPC4 knockdown conditions (Fig. 5F).

We further tried to restore normal fusion in TRPC1 and TRPC4 silenced myoblasts by overexpressing MEF2 and myogenin. Supplementary material Fig. S5 illustrates that the overexpression of both MEF2c and myogenin in TRPC1- and TRPC4-silenced myoblasts was not able to restore normal fusion.

TRPC, interleukin 13 and myoblast fusion
In human myoblast, it was shown that interleukin 13 (IL-13) had positive effect on myoblast fusion as it stimulates the recruitment of reserve cells to increase myotube size (Jacquemin et al., 2007). We first confirmed that exogenous IL-13 increased dose-dependently the number of nuclei per myotube (Fig. 6A). Then we compared IL-13 expression between cells in proliferation and in differentiation conditions, and found that IL-13 mRNA expression was almost doubled 48 h after the initiation of the differentiation process (data not shown). We tested the effect of IL-13 on SOCE and found that in control condition, IL-13 enhanced SOCE by about 30% while after knockdown of both TRPC1 and TRPC4, the increase of SOCE was only 11% (data not shown). The addition of IL-13 (50 ng/ml) during 48 hours did not modify the expression of TRPC1-, TRPC4 or STIM1 (supplementary material Fig. S4E,F). We then explored whether IL-13 was able to restore normal fusion in TRPC1- or TRPC4-silenced myoblasts. Fig. 6B illustrates that 50 ng/ml IL-13 restored normal myotube size when either TRPC1 or TRPC4 was still present, but that IL-13 was unable to restore normal myotube size when both channels were absent. These results suggest that IL-13 requires either TRPC1 or TRPC4 expression to increase myotube size.
In the present work, we assessed the role of TRPC channels in human primary skeletal myoblast differentiation. Particularly, we described for the first time in human the key roles played by TRPC1 and TRPC4 during post-natal myoblast fusion. Using siRNA strategy against TRPC1 or TRPC4, or overexpression of dominant negative TRPC1 or TRPC4, we observed (i) a decrease in SOCE amplitude, (ii) an important defect of myoblast differentiation and (iii) a strong reduction of late fusion events. Ca\(^{2+}\) influx is well known to be important for triggering myoblast differentiation and fusion. The involvement of SOCE during myogenesis was first described in human in 2006 (Arnaudeau et al., 2006), and recently we and other further demonstrated that STIM1, STIM2 and Orai1 are keys players on the initiation of in vitro myoblast differentiation (Stiber et al., 2008; Darbellay et al., 2009; Darbellay et al., 2010; Stiber and Rosenberg, 2011; Li et al., 2012). Besides these ‘prototypical’ SOCE molecules, TRPC channels were reported in several cellular systems to be also implicated in SOCE (Ambudkar, 2007; Zeng et al., 2008; Cheng et al., 2011). Hence, our aim was to determine whether TRPC channels are involved in human myoblasts SOCE, and whether they play a role during human myogenesis. We found that human primary myoblasts expressed TRPC1, TRPC3, TRPC4 and TRPC6 isoforms, an expression profile similar to what was reported in mouse skeletal muscle cells (Brinkmeier, 2011). Using siRNA strategy or dominant
negative constructs, we showed that TRPC1 and TRPC4 isoforms participated to SOCE in human primary myoblasts. The implication of TRPC1 in SOCE was already reported in mouse myoblasts (C2C12 cells; Louis et al., 2008) and myotubes (Sabourin et al., 2009), while the same channel was shown to be store-independent in adult mouse fibers (Zanou et al., 2010). Few studies analyzed the implication of TRPC4 in muscle cells, except one reporting that TRPC4 functions as a SOCE channel in myotubes (Sabourin et al., 2009).

TRPC1 channel was the first member of the TRPC found in the sarcosoma of skeletal muscle cells (Vandebrouck et al., 2002). It was subsequently shown that this channel is necessary for mouse skeletal muscle function (Zanou et al., 2010; Zanou et al., 2012). In C2C12 mouse muscle cell line, it was proposed that TRPC1 participate to the induction of differentiation (Louis et al., 2008; Formigli et al., 2009) via an activation of the Ca²⁺-dependent calpain (Louis et al., 2008). In the present study, we demonstrated that TRPC1 and TRPC4 silencing significantly reduced MEF2 positive cells, while only modestly affected myogenin expression. The major effect of TRPC1 and TRPC4 knockdown was a high reduction of myotube width (data not shown) and number of nuclei per myotubes (Fig. 2). This is in agreement with results obtained in TRPC1−/− mice, which reported a progressive loss of force during tonic stimulation associated with smaller fibers containing less myofibrillar proteins compared to control mice (Zanou et al., 2010). The results we obtained with siTRPC1 or DN-TRPC1 are in line with the paper of Zanou et al. (Zanou et al., 2010), and we further revealed that TRPC4 is also a key player for human myogenesis.

To the best of our knowledge, no information is available regarding putative defects of skeletal muscle development or function in TRPC4−/− (Freichel et al., 2001; Tsivlovsky et al., 2009) or double TRPC1−/−/TRPC4−/− mice models (Phelan et al., 2012; Phelan et al., 2013).

An interesting finding of our work is the specificity of Ca²⁺ entry though TRPC channels. Indeed, when TRPC1 or TRPC4 expression was reduced, the normalization of SOCE amplitude by increasing extracellular Ca²⁺ concentration was not sufficient to restore a normal differentiation process (Fig. 5). In addition, in TRPC1 and TRPC4 knockout cells, STIM1 or Orai1 overexpression restored SOCE but not the differentiation process. It was only when TRPC4 was re-expressed in TRPC4 knockout myoblasts, that the differentiation process was normalized. These results highlighted the essential role of TRPC channels during human myogenesis whose function cannot be compensated by any other type of Ca²⁺ entry. As well, the overexpression of MEF2c and myogenin could not rescue a normal fusion process. It should be noticed that, unlike in TRPC1 and TRPC4 knockdown (present data), in STIM1 knockdown conditions, increasing external Ca²⁺ concentration restore neither SOCE nor the differentiation process (Darbellay et al., 2009), showing that manipulating the expression level of TRPC channels affected myoblast differentiation in a more subtle way than reducing the expression of STIM1. Indeed, upon siSTIM1 treatment, almost no myotubes were formed 48 hours after differentiation (Darbellay et al., 2009). The stronger effect of STIM1 knockdown compared to TRPC knockdown is not surprising considering the stronger reduction of Ca²⁺ entry after siSTIM1 treatment (Darbellay et al., 2009), and the fact that STIM1 interacts also with TRPC (Yuan et al., 2007; Zeng et al., 2008; Yuan et al., 2009) and thus impacts both on Orai-dependent Ca²⁺ entry and on TRPC-dependent Ca²⁺ entry. Cell fusion is a very complex process that includes many different players and that is still not well understood. In in vitro muscle development, fusion is characterized by a two-steps process: the initial fusion between individual myoblasts, and the fusion between myoblasts and nascent myotubes (Rochlin et al., 2010). Fusion index assessments suggest that the initial fusion process was minimally affected by TRPC knockdown (Fig. 2C), while the strong decrease of the number of nuclei per myotubes (Fig. 2B) showed that the second fusion event was severely impaired by siTRPC treatments.

Horsley and co-workers (Horsley et al., 2003) showed in mouse that nascent myotubes produced IL-4 that in turn promoted the recruitment and fusion of mononucleated myoblasts to myotubes. The role of IL-4 in the fusion of myoblasts to nascent myotubes was confirmed in human, and further extended to a role of promoting myoblast migration (Lafranierie et al., 2006). Using a model of IGF-1-mediated human myotube hypertrophy, the group of Mouly showed that IL-13, but not IL-4, favors reserve cells activation, and their subsequent recruitment and fusion to myotubes (Jacquemin et al., 2007). An in vivo study further pointed to a role of IL-4 and IL-13 in exercise-induced human muscle hypertrophy (Prokopchuk et al., 2007). In this study, we showed a significant increase of IL-13 expression during human myoblast differentiation, and we confirmed that exogenous addition of IL-13 increases human myoblast fusion (Fig. 6). In addition, IL-13 enhanced SOCE in control conditions, while the effect was much less pronounced when TRPC1 and TRPC4 were downregulated. Indeed, it was recently shown that IL-13 enhanced Tg-induced Ca²⁺ entry, even though the target of IL-13 was not determined (Gao et al., 2010). Our data suggested a preferential effect of IL-13 on TRPC channels. Remarkably, the addition of exogenous IL-13 almost completely restored the fusion capacity of myoblasts in cells treated with either siTRPC1 or siTRPC4. However, when both channels were knocked down, IL-13 was not able to restore a normal fusion process. We thus proposed that IL-13 promotes the fusion between myoblasts and myotubes by enhancing Ca²⁺ influx through TRPC channels. Additional experiments would be required to further investigate the interplay between IL-13 and TRPC channels on myoblast fusion.

In summary, we have shown that TRPC1 and TRPC4 represent an essential component of SOCE in human myoblasts and that their expression is modulated during human skeletal myogenesis. We demonstrated that TRPC1- or TRPC4-mediated Ca²⁺ influx plays a crucial role during differentiation, more specifically acting on the second step of post-natal human myoblasts fusion.

Materials and Methods

Materials
Thapsigargin was obtained from Sigma. Acetoxyethylmethyl ester form of Fura-2 (Fura-2-AM) was from Molecular Probes Europe (Leiden, The Netherlands). Recombinant human IL-13 was from Peprotech. Plasmids: YFP-STIM1 from Addgene 19754 (Prakriya et al., 2006), Orai1-YFP from Addgene 19756 (Prakriya et al., 2006) Ha-mTRPC4 from K. Groschner lab (Poteser et al., 2006), Ha-TRPC1(Δ324-488Δ562) (Zeng et al., 2008), Ha-TRPC1(Δ562) (Kim et al., 2009) the laboratory of Prof. S. Mualem, and 8TRPC1-YFP the laboratory of Prof. I. Ambudkar (Cheng et al., 2011).

Cell culture and transfection
Muscle samples, cell dissociation, and clonal culture from satellite cells were prepared as previously described (Barofio et al., 1996; Arnaudeau et al., 2006). Human muscle samples were obtained from children (operated for clubfoot and

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less than 4 years old) without any known neuromuscular disease. All work on human subjects was carried out in accordance with the Declaration of Helsinki. Human samples were obtained with informed consent, as approved by the University Hospital of Geneva Research Committee on the use of human as experimental subjects (Protocol 05-078). Differentiation was induced by changing from a serum-rich medium (GM, growth medium) to a serum-free medium (DM, differentiation medium). Differentiation was assessed by quantification of nuclear expression of the myogenic transcription factor MEF2 and myogenin (Konig et al., 2006). The different constructs (2 µg of plasmid DNA) were transfected by electroporation with Amaxa Nucleofector II device (Lonza). The transfection efficiency, assessed by fluorescence-activated cell sorting for YFP-STIM1 was 83±7% (six independent experiments).

siRNA knockdown

Myoblasts were transfected in suspension by incubating 4×105 cells in a solution containing 500 µl of Opti-MEM, 3 µl of Lipofectamine RNAiMax (Invitrogen), and 100 nM of a specific siRNA (Invitrogen or Qiagen) according to manufacturer protocols (Invitrogen). The transfection efficiency assessed by Block-b-Act Fluor Red Fluorescent Oligo (Invitrogen) measurements was ~90%. Sequences of the different siRNA are summarized in Antigny et al. (Antigny et al., 2011). The siRNA AllStar from Qiagen was used as a negative control. For double siRNA/DNA construct experiments, siRNA was transfected 24 h before plasmid electroporation. TRPC1/TRPC4 knockdown experiments, the siTRPC1 was transfected 24 h before siTRPC4 (72 h and 48 h post-transfection for TRPC1 and TRPC4, respectively).

Immunostaining

Myoblasts were fixed and stained with the appropriate fluorescent markers as previously described (Aramandeau et al., 2006). Anti-MEF2 antibody (1:300; sc-313, Santa Cruz Biotechnology, Heidelberg, Germany), anti-myogenin antibody (1:600; clone F5D, BD Biosciences), anti-myosin heavy chain antibody (1:1000; MF20 antibody from hybridoma bank) were used. Secondary antibodies were Alexa 488-labeled goat anti-mouse IgG (1:1000; Molecular Probes, Eugene, OR) and Alexa 546-labeled goat anti-rabbit IgG (1:1000; Molecular Probes). DAPI (100 ng/mL Sigma) was used to localize nuclei. Images were acquired using a Zeiss Axioskop 2 microscope. 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 MetaMorph software (Molecular Devices Corp., Visitron Systems GmbH, Puchheim, Germany). A cluster of labeled nuclei inside a myotube that clearly express MEF2 or myogenin was used to define the threshold above which nuclei were considered as positive for the expression of MEF2 or myogenin (10 random fields for each clone).

Cytosolic calcium measurements

For Ca2+ imaging, human myoblasts were plated on 30 mm glass coverslips. The changes in cytosolic Ca2+ concentration were measured with Fura-2. Cells were loaded with 2 µM Fura-2/AM plus 1 µM pluronic acid for 30 min in the dark at room temperature in a medium containing (in mM): 135 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 Hepes, 10 glucose, pH adjusted at 7.45 with NaOH. Myoblasts were washed twice and equilibrated for 10–15 min in the same buffer to allow de-esterification. Ratiometric images of Ca2+ signals were obtained using a microscope (Axio Observer, Zeiss) equipped with a Lambda DG4 illumination system (Sutter Instrument Company, Novato, CA, USA), which rapidly changed the excitation wavelengths between 340 nm (340AF15; Omega Optical) and 380 nm (380AF15; Omega Optical). Emission was collected through a 415DCLP dichroic mirror, and a 510BW40 filter (Omega Optical), by a cooled, 12-bit CCD camera (CoolSnap HQ, Ropper Scientific, Trenton, NJ, USA). Image acquisition and analysis were performed with the Metafluor 6.3 software (Universal Imaging, West Chester, PA, USA). Experiments were performed at room temperature in Hepes-buffered solution containing (in mM): 135 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 Hepes and 10 glucose, pH adjusted to 7.45 with NaOH. The Ca2+-free solution contained 1 mM EGTA instead of 2 mM CaCl2.

Western blots

Western blots were performed as previously described (Antigny et al., 2011). Briefly, myoblasts were lysed using modified NP40 cell lysis buffer (Invitrogen), 50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4 and 1% Nonidet P40. Total proteins were separated on 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 anti-TRPC1 polyclonal antibody (1:500, Santa Cruz Biotechnology), rabbit anti-TRPC4 polyclonal antibody (1:200, Alomone), rabbit monoclonal anti-MEF2c (1:1000, Cell Signaling Technology), mouse monoclonal anti-myogenin (1:1500, BD Pharmingen) 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 1:10,000 (BioRad) or with HRP-conjugated goat anti-rabbit diluted 1:10,000 (BioRad), respectively. Antibodies were revealed using ECL reagents and hyperfilm ECL (Amersham Biosciences). ImageJ Software was used to quantify the level of protein expression.

Real-time PCR

After total RNA isolation from cells, 1 µg of total RNA was reverse-transcribed with the TAKARA (Bio Company) 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 three endogenous control genes (glucuronidase B, GAPDH and EE-EF1) as described by Vandesompele et al. (Vandesompele et al., 2002). Primers used for the real-time PCR are described in supplementary material Table S1.

Statistical analyses

Ca2+ measurements are expressed as mean±s.e.m. of n observations and were compared with a Student’s t-test. For all other experiments (western blot analysis and immunostaining quantification), the significance was assessed with one-way analysis of variance ANOVA. Differences were considered statistically significant when P<0.05, ns: non-significant difference, *P<0.05, **P<0.01, ***P<0.001. All statistical tests were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software).

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Author contributions

F.A. designed and performed experiments, interpreted data and wrote the manuscript, S.K. performed experiments and interpreted data, L.B. interpreted data and wrote the manuscript and M.F. designed experiments, interpreted data and wrote the manuscript.

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References

TRPC-mediated Ca\(^{2+}\) influx in myogenesis

2533


Heteromeric canonical transient receptor potential 1 and 4 channels play a critical role in dystrophic (mdx) mouse skeletal muscle fibers. *J. Cell Biol.* 158, 1089-1096.


Fig. S1. TRPC3 and TRPC6 knockdown have no impact on human myoblast differentiation. A. Western blot analysis of TRPC3 and TRPC6 protein expression during the first 48h after initiation of human myoblast differentiation (DM). α-tubulin was used as loading control. B. Quantification of Western blots from 3 to 4 different experiments. C. Human myoblasts were transfected with siRNA against TRPC3 or TRPC6. Knockdown efficiency was assessed by Western blot analysis, 48h after transfection with specific siRNA. α-tubulin was used as a loading control. D. Quantification of Western blots from 3 different clones. E. Human myoblasts were transfected with siRNA against TRPC3 or TRPC6, kept for 2 days in growth medium, and then kept for 2 more days in differentiation medium. Left panel represents the percentage of MEF2 positive nuclei in TRPC3 or TRPC6 knockdown conditions, 3 different clones. Right panel represents the percentage of myogenin positive nuclei in TRPC3 or TRPC6 knockdown conditions, 3 different clones. F. Left panel represents the fusion index, same conditions as in E. Nuclei were stained with DAPI and myotubes were delimited using a staining against myosin heavy chain. Right panel represents the number of nuclei per myotubes in control, same conditions as in E.
**Fig. S2. Efficiency of different siRNA against TRPC1, TRPC4, impact on myoblast differentiation and SOCE amplitude.**

A. Human myoblasts were transfected with siRNA against TRPC1 (siTRPC1b) or TRPC4 (siTRPC4b), kept for 2 days in growth medium, and then for 2 more days in differentiation medium. MEF2 and myogenin are shown in red and green, and nuclei in blue (DAPI). Scale bar represents 20 µm. B. Quantification of MEF2 positive nuclei in TRPC1 or TRPC4 knockdown conditions (3 different clones). C. Quantification of myogenin positive nuclei in TRPC1 or TRPC4 knockdown conditions (3 different clones). D. Quantitative RT-PCR was used to assess mRNA level, 48 h after transfection (2–4 independent experiments). Control was normalized to one, and each mRNA level expressed as compared to its own control. E. SOCE amplitude in control conditions and after siRNA treatment (>114 cells, >5 clones).
Fig. S3. Effect of DN-TRPC1 and DN-TRPC4 expression on MEF2 and myogenin mRNA level and activity. A. mRNA levels of MEF2a, MEF2c, MEF2d and myogenin were assessed by quantitative RT-PCR in proliferation condition (GM) and 48h after myoblasts differentiation (DM; 4 different clones). B. Impact of DN-TRPC1, DN-TRPC4 or DN-TRPC1 + DN-TRPC4 overexpression on mRNA level of MEF2a, MEF2c, MEF2d and myogenin 48h after myoblasts differentiation (3 different clones). C. Luciferase extracts were prepared from proliferating myoblasts (GM) and from myoblasts maintained for 3 days in differentiation medium (DM). Left panels: the activity of MEF and myogenin strongly increased in differentiation conditions. Right panels: DN-TRPC1 or DN-TRPC4 or DN-TRPC1 + DN-TRPC4 expression strongly decrease the activity of MEF2 and myogenin transcription factors 3 days in DM. Myogenin and MEF2 activity was assessed using 4RE-luc and 3MEF2-luc plasmids, respectively (>3 different clones).
Fig. S4. Absence of effect on STIM1, STIM2 and Orai1 following TRPC1 or TRPC4 knockdown. A. TRPC1 expression in siTRPC4-treated myoblasts (left panel), and TRPC4 expression in siTRPC1-treated myoblasts (right panel). B. Western blot analysis showing the decrease of TRPC1 or TRPC4 expression after simultaneous TRPC1 and TRPC4 knockdown. C. Western blot analysis of STIM1, STIM2 and Orai1 expression after TRPC1 or TRPC4 knockdown. α-tubulin was used as loading control. D. Quantification of Western blots (4 different experiments). E. Western blot analysis of STIM1, TRPC1 and TRPC4 expression after IL-13 (50ng/ml) incubation during 48h in differentiation medium (DM). α-tubulin was used as loading control. F. Quantification of the Western blots (4 different experiments).
Fig. S5. MEF2c and myogenin overexpression was not able to restore a normal fusion. A. In TRPC1 or TRPC4 silenced myoblasts, the over expression of MEF2c + myogenin was not able to restore normal myoblast fusion (2 or 3 different clones in each condition). B. MEF2c + myogenin co-transfection efficiency was assessed by Western blot analysis. α-tubulin was used as a loading control.
<table>
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<tr>
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All sequences are oriented 5’ - 3’

Table 1: Sequence of primers used for the real-time PCR