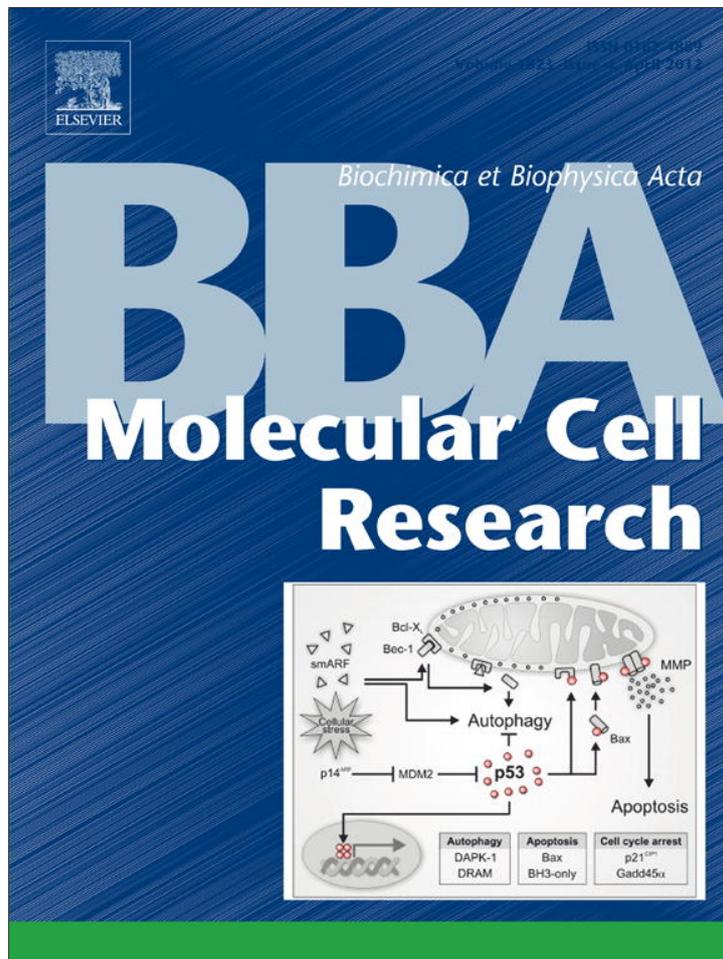


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Orai3 silencing alters cell proliferation and cell cycle progression via c-myc pathway in breast cancer cells

Malika Faouzi^{a,b}, Philippe Kischel^a, Frédéric Hague^a, Ahmed Ahidouch^{a,c,*}, Nazim Benzerdjeb^{a,d}, Henri Sevestre^{a,d}, Reinhold Penner^b, Halima Ouadid-Ahidouch^{a,**}

^a University of Picardie Jules Verne, UFR of Sciences, Laboratory of Cellular and Molecular Physiology EA 4667, SFR CAP-SANTE (FED 4231), Amiens, France

^b Queen's Center for Biomedical Research, The Queen's Medical Center, 1356 Lusitana St., UH Tower 8th Floor, Honolulu, HI 96813, USA

^c University of Ibn-Zohr, UFR Sciences, Biology Department, Agadir, Morocco

^d University of Picardie Jules Verne, Amiens Hospital, Anatomy and Pathology Department, Tumor Bank of Picardie, Amiens, France

ARTICLE INFO

Article history:

Received 16 July 2012

Received in revised form 12 December 2012

Accepted 14 December 2012

Available online 22 December 2012

Keywords:

Orai3

Breast cancer

Cell cycle

Cell proliferation

c-myc pathway

ABSTRACT

Members of the Orai family are highly selective calcium ion channels that play an important role in store-operated calcium entry. Among the three known Orai isoforms, Orai3 has gained increased attention, notably for its emerging role in cancer. We recently demonstrated that Orai3 channels are over-expressed in breast cancer (BC) biopsies, and involved specifically in proliferation, cell cycle progression and survival of MCF-7 BC cells. Here, we investigate the downstream signaling mechanisms affected by Orai3 silencing, leading to the subsequent functional impact specifically seen in MCF-7 cancer cells. We report a correlation between Orai3 and c-myc expression in tumor tissues and in the MCF-7 cancer cell line by demonstrating that Orai3 down-regulation reduces both expression and activity of the proto-oncogene c-myc. This is likely mediated through the MAP Kinase pathway, as we observed decreased pERK1/2 levels and cell-cycle arrest in G1 phase after Orai3 silencing. Our results provide strong evidence that the c-myc proto-oncogene is influenced by the store-operated calcium entry channel Orai3 through the MAP kinase pathway. This connection provides new clues in the downstream mechanism linking Orai3 channels and proliferation, cell cycle progression and survival of MCF-7 BC cells.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

The recent discovery of the Orai channels as the pore forming units of the Ca²⁺ selective CRAC channels opened a new era in the field of store-operated calcium entry (SOCE) [1]. Three distinct Orai isoforms have been described to date (Orai1, Orai2 and Orai3), with Orai3 being “special” in this family, notably because of its exclusive presence in mammals [2] and its receptivity to pharmacological modulation [3]. CRAC channels are known for their physio-pathological roles [1,4–6], but their involvement in BC is only emerging [7–9]. For instance, we have recently reported that Orai3 channels are over-expressed in BC biopsies, and have also shown that these channels are involved in proliferation, cell cycle progression and survival of BC cells by regulating the G1 phase and G1/S transition regulator proteins [9]. Moreover, these effects are specific to cancer cells, since down-regulation of Orai3 channels does not affect either cell proliferation or cell survival of normal

breast cells [9]. How Orai3 impacts such essential processes of the cancer cells' life remains elusive, and we therefore aimed at deciphering, at least in part, the mechanisms linking Orai3 and the above described cellular effects.

The ubiquitous SOCE pathway is not only necessary to refill internal calcium stores, but also for activating downstream signaling cascades [10,11]. Thus, on the assumption that at least one transcription factor was involved, we directed our interest to the c-myc pathway that has been implicated, just like Orai3, in processes controlling cell cycle progression, proliferation and apoptosis [12,13]. The proto-oncogene c-myc encodes a transcription factor of the helix–loop–helix/leucine zipper protein family, and is known to be regulated by calcium [14] and several calcium-dependent signaling pathways such as the MAP kinase and the calcineurin/NFAT pathways [15–17].

Most human cancers display enhanced c-myc expression and/or deregulated c-myc activity [18–20]. In BC, several studies have shown that between 50% and 100% of BC cases display increased c-myc protein levels (see [21] for review). Over-expression of c-myc is associated with reduced relapse-free and overall survival in BC patients [22,23]. Interestingly, down-regulation of c-myc in BC cells induces cell cycle arrest and apoptosis [24,25]. C-myc has been shown to be the key factor in G1 progression and G1/S transition phases in many cell types [26,27]. In fact, c-myc positively regulates the expression and/or activity of

Abbreviations: BC, Breast cancer; SOCE, Store-operated calcium entry; TMA, Tissue microarrays

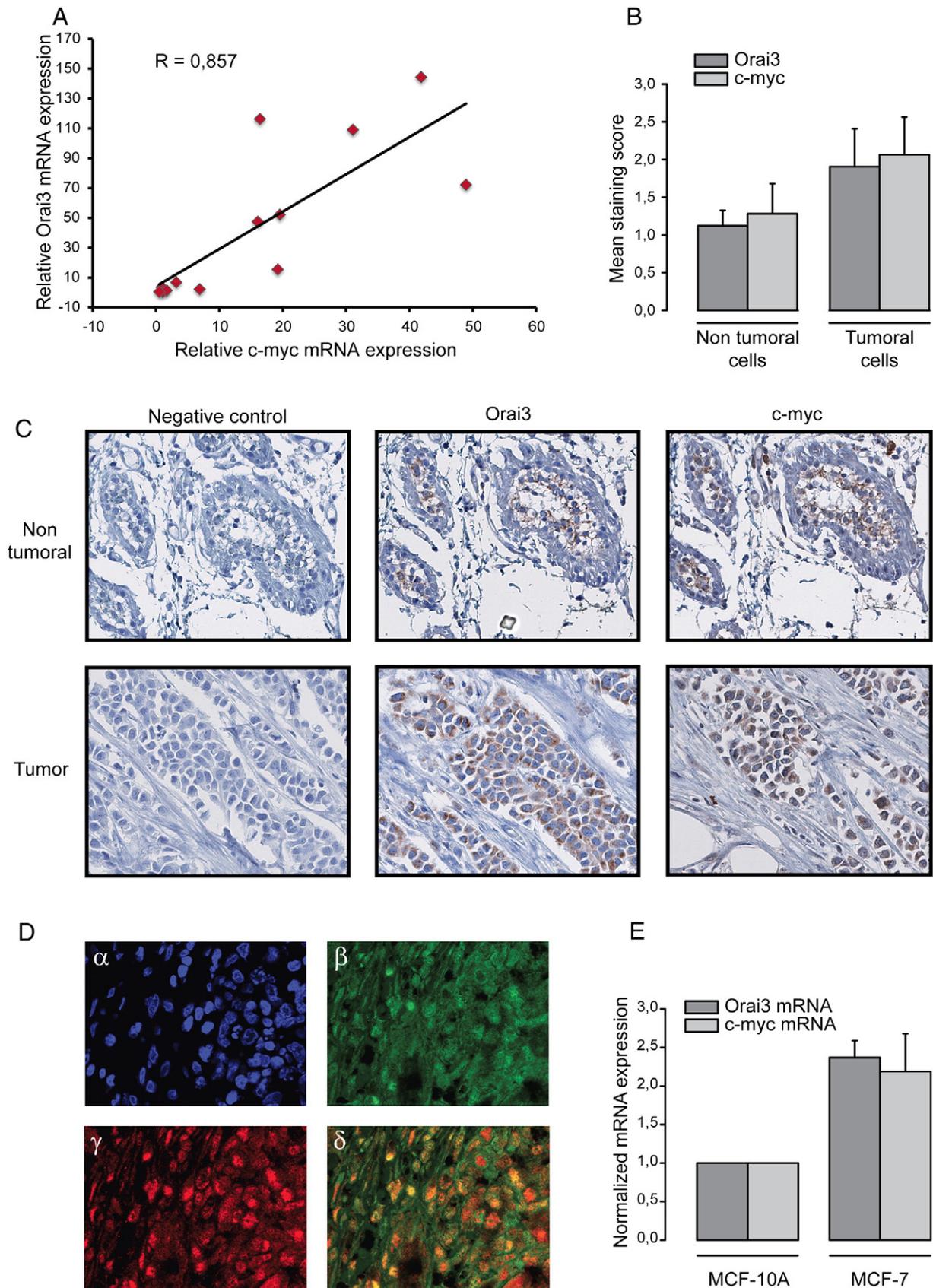
* Corresponding author. Tel.: +33 322827642; fax: +33 322827644.

** Corresponding author. Tel.: +33 322827646; fax: +33 322827644.

E-mail addresses: ahmed.ahidouch@u-picardie.fr (A. Ahidouch), ha-sciences@u-picardie.fr (H. Ouadid-Ahidouch).

cyclins (D1, D2, E, A), cyclin-dependent kinases (CDKs) 4 and 2 [28], and additionally suppresses cyclin-dependent kinase inhibitors (CDKIs) such as p15, p21 and p27 [13]. These effects are strikingly similar to

the effects seen with Orai3 down-regulation, which also alters cyclins D1 and E, CDKs 4 and 2, the cyclin-dependent kinase inhibitor p21^{Waf1/Cip1}, and the tumor-suppressing protein p53 [9].



Therefore, we hypothesized that Orai3 channels might be an upstream regulator of the c-myc pathway, and we investigated the role of c-myc in the Orai3-induced differential effect on proliferation and cell cycle progression in BC cells and normal breast cells. Using qRT-PCR and tissue microarray, we found that expression levels of Orai3 and c-myc are positively correlated. We observed that Orai3 down-regulation induced cell cycle arrest in G1 phase through the c-myc pathway. We also found that simultaneous down-regulation of both Orai3 and c-myc proteins had no additive or synergic effects on either BC cell proliferation or survival. Finally, since the MAP Kinase pathway is known to regulate c-myc protein expression and activity, we investigated the effect of Orai3 down-regulation on ERK1/2 phosphorylation and found that the phosphorylation level of ERK1/2 significantly decreased after silencing Orai3 channels.

Our results therefore suggest that Orai3 channels are amongst upstream effectors of the oncogenic c-myc pathway and constitute key players in BC cells such as the MCF-7 cell line. Importantly, based on the differential effect seen between normal and BC cells, Orai3 could represent a selective target for breast cancer treatment.

2. Materials and methods

2.1. Tissue microarrays

Cancerous breast tissue was obtained from fresh surgical specimens. Consent forms (approved by the University Hospital of Amiens) were signed by the patients before surgery to allow the use of a portion of tissue samples for research purposes. Samples of breast adenocarcinoma, as well as non-tumoral tissues from the same patient were obtained from women having undergone operations at the Amiens hospitals.

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded (FFPE) blocks with a Roche Ultra immunostainer, using antibodies against Orai3 (1:100, HPA015022, Sigma Prestige Antibody) and c-myc (1:100, N-262, Santa-Cruz BioTechnologies). This was followed by the avidin–biotin–peroxidase complex technique. Reactions were developed using a chromogenic reaction in DAB (diamino-3,3'-benzidine tetrahydrochloride) substrate solution (DAB, Sigma Fast). Counterstaining was carried out with hematoxylin solution. Micrograph acquisitions were performed using a digital camera connected to a Zeiss microscope.

Immunostaining in the tumor tissue was determined by subjective visual scoring of the brown stain. Two operators independently evaluated antigen expression. Scoring of the intensity of the staining was performed according to an arbitrary scale with steps of 0, 1, 2, and 3 where “0” was considered to be absence of staining, “1” considered weak staining, “2” was considered as moderately positive staining, and “3” was considered to be strong staining. A negative control was performed using the same technique without primary antibody.

The expression of Orai3 and c-myc was also assessed in 30 additional human invasive ductal adenocarcinoma specimens using Tissue Microarray (TMAs). Briefly, four-micrometer-thick sections of formalin-fixed paraffin-embedded tissue samples were taken from the FFPE block.

2.2. Immunofluorescence

Immunofluorescent analyses were performed on tissues following a published protocol [29]. Antibodies used were anti-Orai3 (1:100,

HPA015022, Sigma Prestige Antibody), anti-c-myc (9E10, 1:500, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), goat anti rabbit IgG DyLight 549 conjugated (Thermo, Rockford, IL, USA), and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole, 1.43 μ M). Images were obtained using a Zeiss Axiovert 200 microscope equipped with ApoTome system (Zeiss, Le Pecq, France).

2.3. Cell culture

MCF-7 BC cells were grown in Eagle's Minimum Essential Medium supplemented with 5% fetal calf serum (Lonza, Levallois-Perret, France), 2 mM L-glutamine, and 0.06% HEPES. The immortalized human mammary epithelial cell line MCF-10A was grown in DMEM/F12 medium, composed of Dulbecco's modified Eagle's medium/nutrient mixture F12 supplemented with 5% fetal calf serum, 20 ng/ml epidermal growth factor, 10 mg/ml⁻¹ insulin, 0.5 mg/ml hydrocortisone, and 100 ng/ml cholera toxin (Sigma-Aldrich, St-Quentin Fallavier, France). All cell lines were grown in a 5% CO₂-humidified incubator at 37 °C.

2.4. Transfection

Transfection of cells was performed using the nucleofection technology (Amaxa, Köln, Germany), as previously described [30]. Cells (1×10^6) were transfected either with 5 μ g scrambled siRNA as a control (siGENOME non-targeting siRNA, Dharmacon Research Inc., Chicago, IL, referred in the text to as “si-CTL”), or with siRNAs directed against an Orai isoform (Orai1 or Orai3), or with siRNA directed against c-myc, or simultaneously with both si-Orai and si-c-myc. To this end, siRNA were combined and nucleofected according to the manufacturer's protocol.

2.5. Real-time quantitative PCR

Extraction of total RNA from cell lines or biopsies and real-time PCR were performed as previously described [9]. The relative amount of Orai3 and c-myc mRNAs in breast cancer cells were normalized to the endogenous control (β -actin) and compared to the reference sample (normal breast cells) using the Pfaffl method [31]:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{ct}} \text{target}(\text{normal cells} - \text{cancer cells})}{(E_{\text{ref}})^{\Delta\text{ct}} \text{ref}(\text{normal cells} - \text{cancer cells})}$$

2.6. Western blot analyses

Whole-cell lysates were prepared with RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated by denaturing SDS-PAGE, and transferred onto nitrocellulose membranes. The primary antibodies used were: anti-Orai3 (1:100, HPA015022, Sigma Prestige Antibody), anti-c-myc (N-262, 1:500, Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-ERK1/2 and anti-pERK1/2 (1:1000, Cell Signaling Tech., Danvers, MA). Secondary antibodies were coupled to horseradish peroxidase. Actin antibody (1/2000, Santa Cruz Biotechnology) was used for loading control experiments. Bound antibodies were visualized using ECL chemiluminescent substrate (GE Healthcare, Saclay, France) and quantified using the

Fig. 1. Correlation between Orai3 and c-myc genes expression in breast cancer. A: Spearman's correlation between Orai3 mRNA and c-myc mRNA extracted from 13 patients: the R coefficient is equal to 0.857. B: Mean score of Orai3 and c-myc staining obtained on the additional cohort of 30 patients represented on the TMA. C: Representative examples of Orai3 and c-myc expression in cancerous and matched non-tumoral human breast cancer tissues, as assessed by immunohistochemistry. Paraffin sections of human breast tissues were subjected to immunoperoxidase staining, as described in **Materials and methods**. Original magnification: $\times 400$. D: Immunofluorescent staining of c-myc (β) and Orai3 (γ). Merging of both Orai3 and c-myc staining is shown in δ , and DAPI colored nuclei are shown in α . E: Relative transcript levels of Orai3 and c-myc in MCF-7 cells, with the normal breast cell line MCF-10A taken as reference for both genes ($n = 4$). mRNAs are normalized to β -actin, as described in **Materials and methods**.

Bio-Rad image acquisition software (Quantity One) associated with the ChemiDoc XRS imager system (Bio-Rad Laboratories).

2.7. *c-myc* activity measurement

Nuclear extracts were obtained using the Marligen Biosciences Kit. Briefly 72 h after transfection, cells were collected by centrifugation and washed in PBS. Cells were then allowed to swell in Complete Hypotonic Cell Lysis Buffer, and lysis was facilitated by the addition of the Detergent Solution. The cell nuclei were collected by gentle centrifugation, and the cytoplasm was removed. The nuclear pellet was washed twice in Complete Nuclear Wash Buffer and extracted by addition of Complete Extraction Buffers. The nuclear extract was clarified by centrifugation, and the protein concentration was determined using a Bradford assay. Direct quantitation of *c-myc* binding activity was then performed on a 96-well plate using Myc-Max activity measurement Kit (Marligen Biosciences).

2.8. Cell viability and mortality

Cell viability and mortality were measured 72 h post-transfection using Trypan Blue assay. After transfection with siRNAs directed against *Orai3* or *c-myc* or with non-targeting siRNA, MCF-7 cells were grown in 35 mm Petri dishes at a density of 1×10^5 cells for 72 h. Cell count was assessed using the standard Malassez cell method. Briefly, cells were removed by trypsinization and diluted in Trypan Blue solution. Cell counts were performed six times and the results were expressed as the percentage of viable or dead cells normalized to control.

2.9. Cell-cycle analysis

For the measurement of cellular DNA content, only adherent cells were collected at 72 h post-transfection. Cells were pelleted, re-suspended in PBS/EDTA, treated with 20 mg/ml RNaseA (Sigma-Aldrich) and stained with 50 mg/ml of propidium iodide (Sigma-Aldrich). Samples were then analyzed on an Elite Beckman Coulter flow cytometer. The percentage of cells in different phases was calculated using WinMDI 2.8 and Cylchred software.

2.10. Determination of p42/p44 MAP Kinase/extracellular signal regulated kinase (ERK1/2) by western blotting

The level of phosphorylation of ERK1/2 treated with either si-CTL or si-*Orai3* was examined in MCF-7 cells. Forty-eight hours after transfection, MCF-7 cells were deprived of FCS for 24 h. The medium was then changed to a fresh medium, either without FCS or with 5% FCS. Cells were incubated for 20 minutes in this medium before protein extraction.

2.11. Statistical analyses

Values are expressed as mean \pm SEM. Statistical analysis of the data was performed using appropriate ANOVA, Mann–Whitney or paired *t*-tests. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Correlation between *Orai3* and *c-myc* expression in tumor tissues and in BC cell lines

In our previous study, we showed that *Orai3* is over-expressed in 77% of the studied tumor samples [9]. Because of this, and the fact that *c-myc* expression is frequently amplified in breast cancer, we assessed the expression levels of *c-myc* mRNA in the same BC samples previously used for the evaluation of *Orai3* expression. We

found that *c-myc* was over-expressed (over-expression being defined as a two-fold or greater mRNA level when compared to the expression levels found in normal adjacent breast tissue) in 9 cases out of 13 (69.2%, Supplementary Fig. 1). Statistical analysis based on the Spearman's correlation coefficient (Fig. 1A) indicated a positive correlation between *Orai3* and *c-myc* mRNA expression ($R = 0.857$). The expression of *Orai3* and *c-myc* was also evaluated in 30 additional human invasive adenocarcinoma specimens using TMAs (Fig. 1B). Here too, considering over-expression is at least a two-fold higher expression, *Orai3* and *c-myc* were over-expressed in 70% (21 out of 30 cases) and 80% (24 out of 30 cases) cases respectively ($p < 0.001$ by Mann–Whitney Rank Sum Test). Moreover, of the 21 samples exhibiting high expression of *Orai3* (score $\geq 2+$), a high *c-myc* staining (score $\geq 2+$) was found in all samples (100%). Statistical analysis based on the Spearman's correlation coefficient indicated again a positive correlation between *Orai3* and *c-myc* protein expression ($R = 0.895$). A representative immuno-histochemistry is shown in Fig. 1C. Double immuno-fluorescence was also performed to assess co-overexpression of *Orai3* and *c-myc* in FFPE tissues from the BC patients. A representative immuno-fluorescence is shown in Fig. 1D. Although some cells express either predominantly *c-myc* or *Orai3*, a majority of cells show concomitant over-expression of both proteins.

Together, these results indicate that *Orai3* and *c-myc* are strongly correlated in BC tissues.

To test the involvement of *c-myc* in *Orai3*-dependent cell proliferation and/or survival, we first studied *c-myc* mRNA expression levels in both normal and cancer breast cells. As shown in Fig. 1E, expression of *c-myc*, as assessed by RT-qPCR, is higher in the MCF-7 cancer cell line than in the non-cancerous MCF-10A cell line. Remarkably, this over-expression (2.2 ± 0.5 fold over control) is very similar to the *Orai3* over-expression seen in these cancer cells (2.4 ± 0.2 fold over control).

3.2. *Orai3* down-regulation reduces *c-myc* expression and activity

We next silenced *Orai3* channels with small interfering RNAs in both cell lines. As can be seen in Fig. 2A, siRNAs against *c-myc* were effective in both normal and cancer cell lines. Moreover, si-*Orai3* was able to down-regulate *c-myc* mRNA expression only in MCF-7 cancerous cells. Therefore, we assessed the extent of down-regulation at the protein level, again at 72 h post-transfection, using Western blotting. The *c-myc* expression is shown in Fig. 2B and densitometric analyses of *c-myc* bands are presented in Fig. 2C. The results at the protein level are consistent with those obtained at the mRNA level, *c-myc* protein levels being strongly reduced only in MCF-7 cells transfected with si-*Orai3* ($43.1 \pm 8.7\%$ of the control value, $p < 0.05$). Interestingly, we observed no significant difference in *c-myc* expression levels between si-*Orai3* treated MCF-10A cells and MCF-10A control cells.

We next sought to evaluate the *c-myc*/Max activity in normal and cancer cells. Indeed, it is known that *c-myc* belongs to the Myc/Max/Mad network that is composed of a group of transcription factors, whose interactions result in transcriptional activation or repression of their target genes [12,32]. Max is the protein partner of *c-myc*, whose heterodimerization allows binding to specific DNA sequences located in *c-myc* target genes and permits the recruitment of transcriptional co-activators. This process results in the activation of the *c-myc* downstream genes, particularly those involved in cell cycle progression and proliferation. In this context, we measured the Myc/Max complex DNA-binding, which reflects *c-myc* activity. In BC cells, *Orai3* silencing resulted in a highly significant decrease of *c-myc* activity ($3.6 \pm 0.5\%$ vs. $100 \pm 30.3\%$ in the control, $p < 0.001$, Fig. 2D). In contrast, the effect observed in MCF-10A cells was not statistically significant ($54 \pm 21\%$ vs. $100 \pm 37.7\%$ in control, Fig. 2D).

To know whether reduction in *c-myc* expression and activity are really specific to *Orai3* down-regulation or could also be observed with silencing of another member of the *Orai* protein family, we choose to down-regulate *Orai1*, also present in MCF-7 cells [8]. The results are

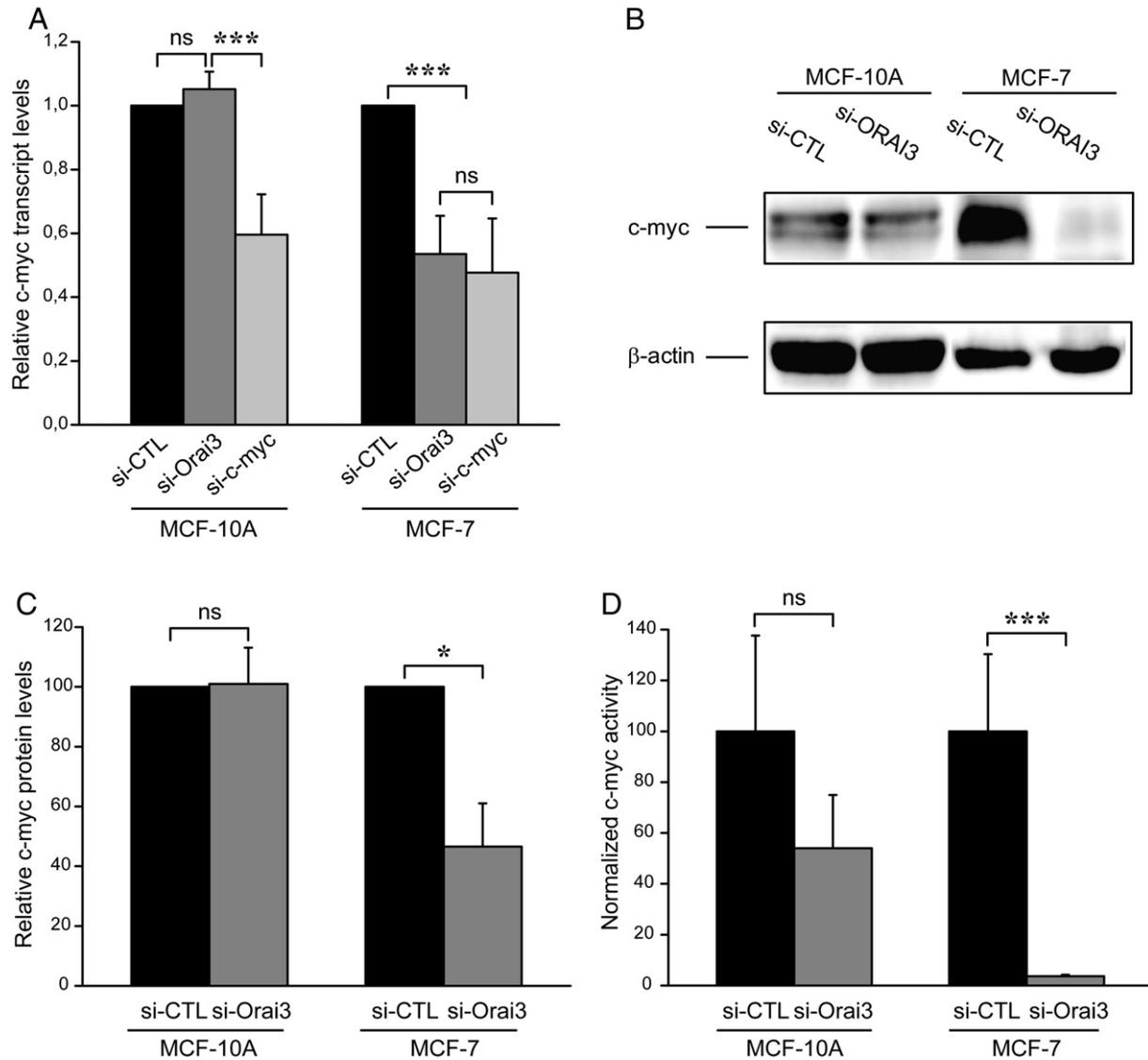


Fig. 2. Orai3 down-regulation affects c-myc protein expression and activity. A: Relative transcript levels of c-myc in MCF-10A and MCF-7 cells after transfection with either si-c-myc or si-Orai3, with a non-targeting si-RNA (si-CTL) taken as reference for both genes ($n=3$). B: Representative western blot showing the effects of cell transfection with si-control (si-CTL) or si-Orai3 on the expression of c-myc in both MCF-7 and MCF-10A cell lines. C: Quantification of c-myc levels using densitometric analyses of c-myc protein expression shown in B ($n=4$). D: c-myc activity measured in cells transfected with si-Orai3, normalized to c-myc activity measured under control condition in MCF-7 cells ($n=9$) and in MCF-10A cells ($n=6$). Values are reported as mean \pm SD, * $p<0.05$; *** $p<0.001$ vs. control; ns: not significant.

illustrated in Supplementary Fig. 2. Using a reporter plasmid, enhanced c-myc promoter activity was found when Orai1 was down-regulated ($226 \pm 11.17\%$ vs. si-CTL, data not shown). Consistently, si-Orai1 induced an upregulation of c-myc mRNA ($180 \pm 35.7\%$, Supplementary Fig. 2A). Quantitation of c-myc binding activity was also performed using the Myc–Max activity measurement kit: again, the activity of si-Orai1 treated cells was increased to $183.4 \pm 5.3\%$ when compared to the si-CTL condition (Supplementary Fig. 2B).

Taken together, these results showed that Orai3 down-regulation reduced both c-myc protein expression and activity levels exclusively in BC cells, since no statistically significant effect was detected in normal cells. While, silencing Orai1 led to the opposite effect in BC cells (increase of c-myc mRNA expression and activity).

3.3. c-myc-dependent proliferation and survival are regulated by Orai3 in BC cells

Given the results obtained for c-myc expression and activity, we next investigated the contribution of c-myc to Orai3-dependent

proliferation and survival. Cells were transfected with different siRNAs independently (siCTL and siRNAs directed against Orai3 or c-myc) or combined (si-Orai3 + si-c-myc). At 72 h post-transfection, a Trypan Blue assay was performed to assess the number of viable cells and the percentage of cell mortality for each condition. As expected, Orai3 and c-myc silencing significantly decreased BC cell viability to $34.6 \pm 4.1\%$ and $34.7 \pm 2.9\%$ of the control, respectively ($p<0.001$, Fig. 3A) and increased cell mortality ($16.5 \pm 1.3\%$ in si-Orai3 transfected cells, $16.1 \pm 0.6\%$ in si-c-myc transfected cells vs. $6.3 \pm 0.5\%$ in control cells, $p<0.001$, Fig. 3B). Interestingly, the effects of si-Orai3 and si-c-myc on cell viability and mortality were not additive when cells were simultaneously treated with both siRNAs (Fig. 3A and B). We also checked the percentage of apoptotic cells after siRNA treatments. As shown in Supplementary Fig. 3, apoptosis increased significantly in si-Orai3, si-c-myc and both si-Orai3 and si-c-myc conditions. Interestingly, values for apoptosis in si-Orai3, as well as in si-c-myc treated cells (either alone or combined with si-Orai3) were not statistically different.

In MCF-10A cells, Orai3 down-regulation had no effect on either cell viability or cell mortality. In these cells, c-myc silencing significantly

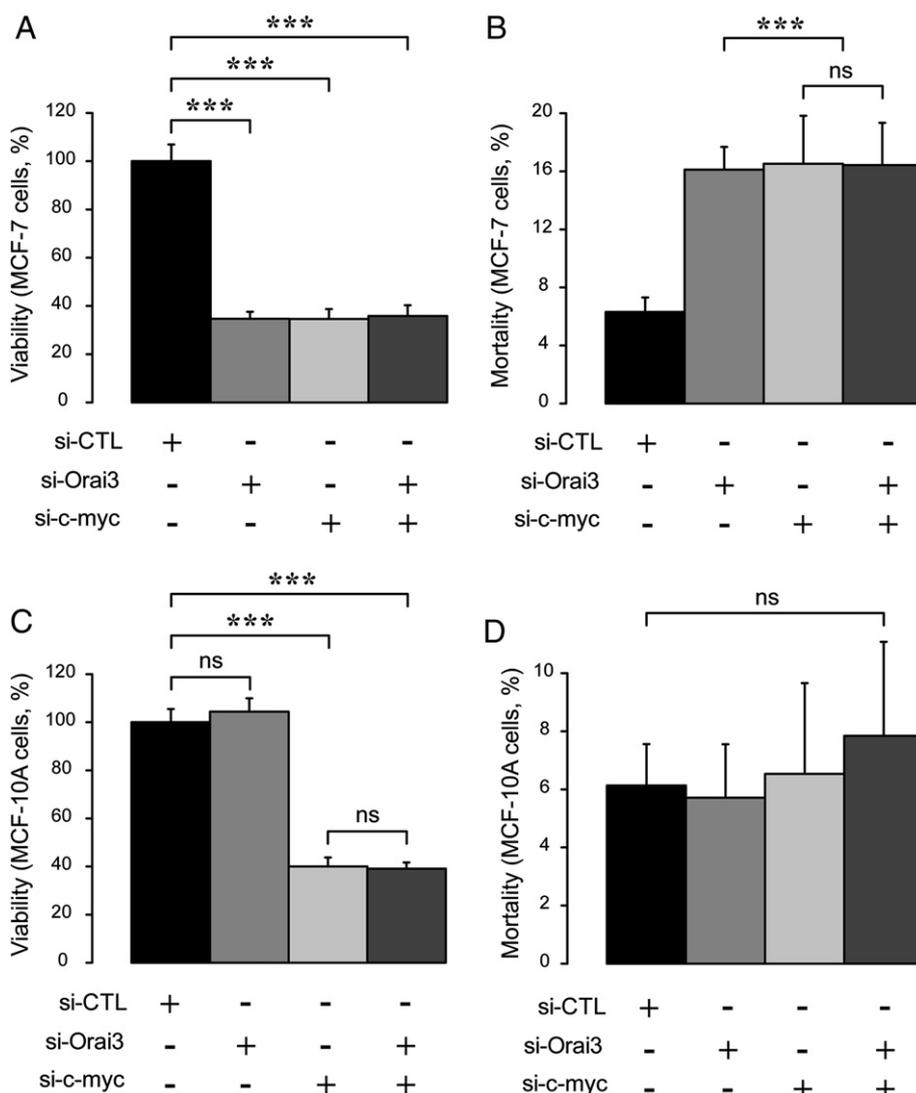


Fig. 3. c-myc involvement in Orai3-dependent proliferation and survival. A: Effects of Orai3 and c-myc silencing on MCF-7 cell viability. The cell viability was measured 72 h post transfection, and all values were normalized as percentage of si-CTL. B: Percentages of cell mortality obtained in MCF-7 cells transfected with si-Orai3 and/or si-c-myc for 72 h. C: Effects of Orai3 and c-myc silencing on MCF-10A cell viability at 72 h post-transfection. Values were normalized as percentage of siCTL. D: Effects observed on MCF-10A mortality after transfection with si-Orai3 and/or si-c-myc for 72 h. Values are reported as mean \pm SEM of triplicate experiments, *** p <0.001 vs. control; ns: not significant.

decreased the cell viability to $40 \pm 3.8\%$ of the control ($p < 0.001$, Fig. 3C), but had no effect on cell mortality (Fig. 3D). The effects of combined si-Orai3 and si-c-myc treatments were not statistically different from those given by si-c-myc alone on MCF-10A with respect to viability or mortality (Fig. 3C and D). These results are further evidence of the involvement of c-myc in the Orai3-dependent cell proliferation and survival of breast cancer cells.

3.4. Orai3 down-regulation induced cell arrest in G1 phase through the c-myc pathway

We have previously shown that Orai3 silencing induced cell cycle arrest in the G1 phase [9]. To determine the contribution of c-myc to this effect, we investigated the cell cycle distribution of cells transfected with siRNAs against Orai3 and/or c-myc. At 72 h post-transfection, cell cycle analysis was performed by flow cytometry. Fig. 4A shows the cell cycle distribution of MCF-7 BC cells. Consistent with previous observations [9], Orai3 silencing led to cell cycle arrest, with a significant accumulation of cells in the G0/G1 phase ($76.8 \pm 3.1\%$ vs. $55.6 \pm 1\%$ for the control condition, $p < 0.01$). There

was a decrease in the cell percentage in both S ($16 \pm 2.8\%$ vs. $31 \pm 0.8\%$ for the control condition) and G2/M ($7.3 \pm 0.3\%$ vs. $13.5 \pm 0.3\%$ for the control condition) phases. When c-myc was down-regulated, we observed a significant increase of cells in the G0/G1 phase ($69 \pm 0.7\%$ vs. $55.6 \pm 1\%$ for the control condition, $p < 0.001$), a significant decrease in the cell percentage in S phase ($18.4 \pm 0.7\%$ vs. $31 \pm 0.8\%$ for the control condition) and no significant effect on G2/M phase ($12.6 \pm 0.1\%$ vs. $13.5 \pm 0.3\%$ for the control condition). Cells co-transfected with both siRNAs showed no significant additive effect, and the cell distribution under this condition was similar to that observed in cells transfected with si-Orai3 alone. As expected, cell cycle distribution of MCF-10A cells was unchanged in cells with down-regulated Orai3 when compared to the control condition (Fig. 4B). In contrast, c-myc silencing resulted in cell cycle arrest, with a significant accumulation of cells in the G0/G1 phase ($69.5 \pm 4.6\%$ vs. $59.5 \pm 2.9\%$ for the control condition, $p < 0.05$), and a decrease in the cell percentage in G2/M phase ($17.4 \pm 3.9\%$ vs. $24.1 \pm 3.3\%$ for the control condition, $p < 0.05$), while no significant effect was detected in S phase. When combined, si-Orai3 and si-c-myc induced the same effects as si-c-myc alone.

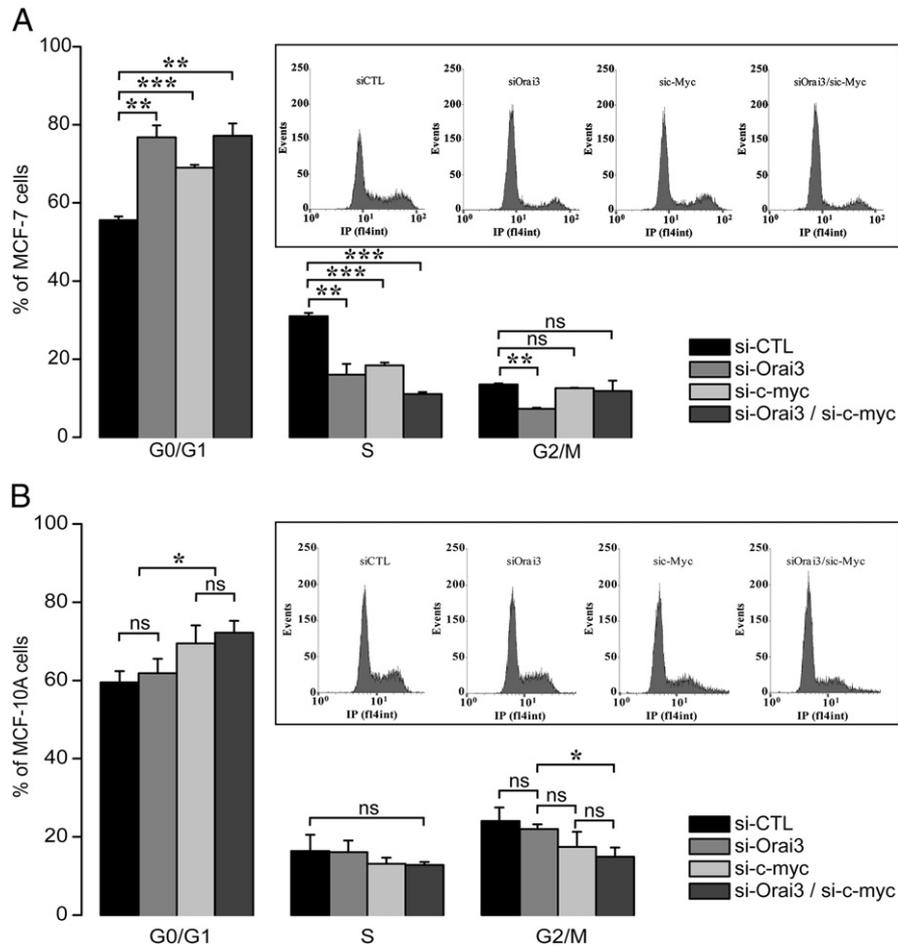


Fig. 4. c-myc involvement in Orai3-dependent cell cycle progression. A: Cell cycle distribution of MCF-7 cells transfected with siRNAs against Orai3 (si-Orai3) and/or c-myc (si-c-myc). B: Cell cycle analysis of MCF-10A cells in the same experimental conditions. The cell cycle analysis was performed at 72 h post-transfection. Insets show raw data from the FACS acquisition software. Values are reported as mean \pm SEM of triplicate experiments, * p <0.05; ** p <0.01; *** p <0.001 vs. control; ns: not significant.

3.5. Orai3 down-regulation affects the c-myc pathway, likely via the MAP Kinase pathway

We evaluated c-myc protein expression levels after either individual or concomitant down-regulation of Orai3 and c-myc (Fig. 5A). While separate transfections of either si-Orai3 or si-c-myc induced a decrease of c-myc protein expression to a similar extent (approximately 40% decrease, p <0.01), the combined transfection of both siRNAs had no additive effects (ca. 50% down-regulation, without statistical significance with respect to individual siRNAs).

We sought to determine which pathway was most likely involved in the c-myc down-regulation induced by Orai3 silencing. The MAP Kinase pathway is known to control the c-myc promoter. We therefore investigated the level of phosphorylation of ERK1/2 in MCF-7 cells treated with either si-control (si-CTL) or si-Orai3. Stimulation with 5% serum induced approximately a 8.8 fold increase of the ERK1/2 phosphorylation in cells treated with si-CTL (Fig. 5B). In si-Orai3 treated cells, this phosphorylation level was enhanced only by a factor 4, hence showing 51% decrease of the phosphorylation level of ERK1/2 when compared to si-CTL treated cells (Fig. 5B). Moreover, the ~40% decrease of the c-myc promoter activity observed in the presence of the si-Orai3 was almost fully restored by application of EGF (100 nM, Supplementary Fig. 4). These results provide supportive evidence that Orai3 specifically affects the c-myc pathway, most likely via the MAP Kinase pathway.

4. Discussion

The present study was prompted by the recent demonstration that, in MCF-7 cancer cells, SOCE was mediated by Orai3 [8]. We now provide compelling evidence that the early-response gene c-myc, which is known for its implication in cell cycle progression, proliferation and survival [12,13], is involved in the cell cycle arrest and apoptosis seen in MCF-7 cancer cells following down-regulation of Orai3. We also demonstrate that Orai3 and c-myc are both up-regulated in BC tissues.

We have previously shown that Orai3 was over-expressed in 77% of the studied cases [9]. We now show that c-myc is also over-expressed in 9 out of these 13 cases, being in good agreement with previously reported studies (see [21] for review). Importantly, using TMA on 30 BC tissues, we found that for each cancer tissue showing c-myc over-expression, there is a concomitant up-regulation of Orai3. This up-regulation is also found in the cancer cell line MCF-7 when compared to the normal cell line MCF-10A.

We also established that Orai3 down-regulation specifically reduces c-myc expression and activity, Orai1 down-regulation having opposite effects. These opposite effects could be due either to different subcellular localization of the channels, or interaction with different protein complexes involved in c-myc regulation. Reduction of c-myc activity by Orai3 silencing can be due either to the obvious subsequent reduction of c-myc expression levels demonstrated in the

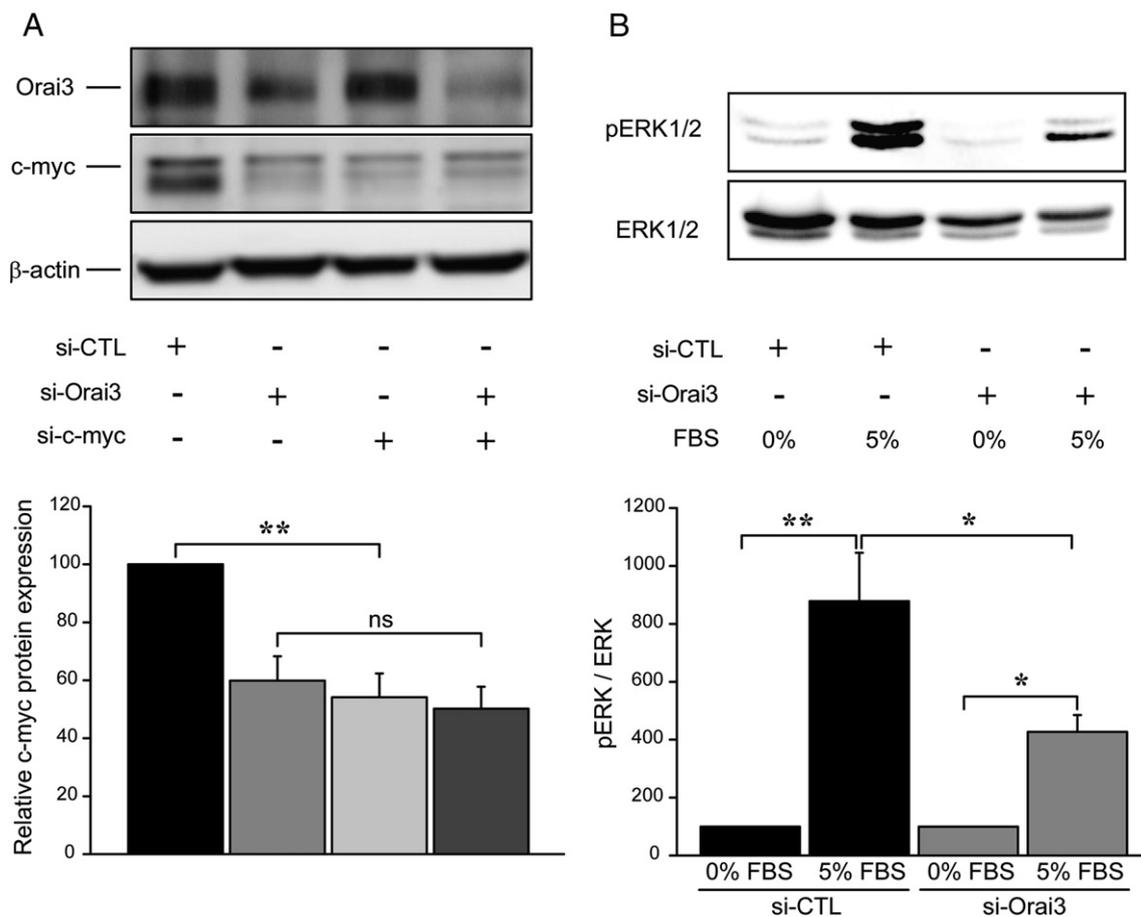


Fig. 5. Effect of si-Orai3 and si-c-myc on Orai3 and c-myc protein expression levels. A: Representative western blot showing the effect of cell transfection with either si-CTL, si-Orai3 and/or si-c-myc on the expression of Orai3 and c-myc in MCF-7 cells. The quantification of both proteins using densitometric analyses is shown in the lower part ($n=3$, $p<0.01$; ns: not significant). B: Representative western blot of pERK1/2 and ERK1/2 proteins in MCF-7 cells transfected with either si-CTL or si-Orai3. Each siRNA was tested in 0% and 5% serum. The ratio pERK/ERK shown in the lower part corresponds to the densitometric analysis of four western blots.

present study, or reduced functional activity of the protein. These two possibilities might occur concomitantly, since it is known that the MAP kinase pathway can control c-myc function and expression [15,33]. Orai3 down-regulation induced an approximate 51% decrease of the phosphorylation level of ERK1/2, suggesting that Orai3 affects the c-myc pathway most likely via the MAP Kinase pathway. Consistent with this hypothesis, MAP kinase activation via EGF treatment was able to suppress the inhibitory effect due to Orai3 downregulation on c-myc promoter activity. MAPKs can, for instance, modulate the activity of c-Jun and c-Fos proteins, known to dimerize in order to form AP-1 complexes, which are able to bind the promoter region of c-myc. Newly synthesized c-myc can, in turn, be phosphorylated by ERK on Ser 62 to induce the stabilization of c-myc (and hence c-myc accumulation), resulting in increased expression levels and enhanced transcriptional activity (see [34] for review). However, the ERK pathway might not be the only one involved in c-myc activation. Indeed, other calcium-regulated transcription factors have been shown to enhance c-myc transcription, NFAT for instance [17].

We found that si-RNAs against Orai3 and c-myc induce similar reductions in BC cell viability and similar increases in BC cell mortality. Interestingly, the effects of both si-RNAs are non-additive, suggesting that Orai3 and c-myc might be acting in series along the same signaling pathways. Furthermore, Orai3 failed to arrest normal MCF-10A cell proliferation, most probably because of the normal c-myc expression and activity in these cells. This further confirms the participation of c-myc in the Orai3-dependent cell proliferation and survival of BC cells.

Several studies have reported that c-myc expression is Ca^{2+} -dependent [35,36], and is rapidly induced upon serum stimulation [37]. On the other hand, Orai3 constitutes the main SOCE player in MCF-7 BC cells but not in the normal MCF-10A cells [8], and hence controls MCF-7 cell proliferation and survival in a Ca^{2+} -dependent manner, but fails to affect MCF-10A cell proliferation and survival [9]. Therefore, it would appear that Orai3 controls c-myc expression and activity via a Ca^{2+} -dependent pathway in MCF-7 BC cells (Supplementary Fig. 5) but not in normal MCF-10A cells.

How real activation of Orai3 occurs in MCF-7 breast cancer cells remains to be determined. It can be hypothesized that growth factors (e.g. EGF) and/or hormones play a role in Orai3 activation in cancer cells. Estrogens for instance are known cancer-causing agents, which are able to induce calcium release from the endoplasmic reticulum [38,39]. This calcium release would be able in turn to activate Orai3 channels [8,9]. Furthermore, it was recently reported that the activation of estrogen receptor alpha increased Orai3 expression and SOCE in MCF-7 cells [40].

The connection between a calcium entry player and the proto-oncogene c-myc through the calcium-dependent MAP kinase pathway provides new clues to the downstream mechanism linking Orai3 channels to MCF-7 BC cell proliferation, cell cycle progression and survival. Although some questions remain, we provide a link between Orai3 channels and a transcription factor in electrically non-excitable cancer cells. For this reason, Orai3 could represent a valid therapeutic target, owing to the fact that its down-regulation affects proliferation, cell cycle progression and survival only in the

MCF-7 BC cells. Although it is generally accepted that ion channels represent attractive targets for human cancer therapy, either for therapeutic or diagnostic purposes, efficient molecules have yet to be designed to target ion channels in breast cancer [41]. Since Ca^{2+} channels are easily and directly accessible via the bloodstream, Orai3 could have potential for antibody-targeted therapeutic strategies, as recently proposed for TRP ion channels [42] or voltage-gated potassium channels [43].

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2012.12.009>.

Acknowledgments

This study was supported by the Region Picardie, and by the Ministère de l'Éducation Nationale (France) and la Ligue Nationale Contre le Cancer (SEPTENTRION). We thank Alexandre Patrice from the Laboratory of Pr. Pattou F. (Thérapie Cellulaire du Diabète, Inserm U859, Lille) for technical assistance with the Luminex plate reader.

References

- [1] S. Feske, Y. Gwack, M. Prakriya, S. Srikanth, S.-H. Puppel, B. Tanasa, P.G. Hogan, R.S. Lewis, M. Daly, A. Rao, A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function, *Nature* 441 (2006) 179–185.
- [2] T.J. Shuttleworth, Orai3 – the ‘exceptional’ Orai? *J. Physiol.* 590 (2012) 241–257.
- [3] R. Schindl, J. Bergsmann, I. Frischauf, I. Derler, M. Fahrner, M. Muik, R. Fritsch, K. Groschner, C. Romanin, 2-Aminoethoxydiphenyl borate alters selectivity of Orai3 channels by increasing their pore size, *J. Biol. Chem.* 283 (2008) 20261–20267.
- [4] I.F. Abdullaev, J.M. Bisailon, M. Potier, J.C. Gonzalez, R.K. Motiani, M. Trebak, Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation, *Circ. Res.* 103 (2008) 1289–1299.
- [5] C. El Boustany, M. Katsogiannou, P. Delcourt, E. Dewailly, N. Prevarskaya, A.-S. Borowiec, T. Capiod, Differential roles of STIM1, STIM2 and Orai1 in the control of cell proliferation and SOCE amplitude in HEK293 cells, *Cell Calcium* 47 (2010) 350–359.
- [6] J.M. Bisailon, R.K. Motiani, J.C. Gonzalez-Cobos, M. Potier, K.E. Halligan, W.F. Alzawhra, M. Barroso, H.A. Singer, D. Jourdeheuil, M. Trebak, Essential role for STIM1/Orai1-mediated calcium influx in PDGF-induced smooth muscle migration, *Am. J. Physiol. Cell Physiol.* 298 (2010) C993–C1005.
- [7] S. Yang, J.J. Zhang, X.-Y. Huang, Orai1 and STIM1 are critical for breast tumor cell migration and metastasis, *Cancer Cell* 15 (2009) 124–134.
- [8] R.K. Motiani, I.F. Abdullaev, M. Trebak, A novel native store-operated calcium channel encoded by Orai3: selective requirement of Orai3 versus Orai1 in estrogen receptor-positive versus estrogen receptor-negative breast cancer cells, *J. Biol. Chem.* 285 (2010) 19173–19183.
- [9] M. Faouzi, F. Hague, M. Potier, A. Ahidouch, H. Sevestre, H. Ouadid-Ahidouch, Down-regulation of Orai3 arrests cell-cycle progression and induces apoptosis in breast cancer cells but not in normal breast epithelial cells, *J. Cell. Physiol.* 226 (2011) 542–551.
- [10] S. Feske, Calcium signalling in lymphocyte activation and disease, *Nat. Rev. Immunol.* 7 (2007) 690–702.
- [11] J.W. Putney, Capacitative calcium entry: from concept to molecules, *Immunol. Rev.* 231 (2009) 10–22.
- [12] S. Pelengaris, M. Khan, G. Evan, c-MYC: more than just a matter of life and death, *Nat. Rev. Cancer* 2 (2002) 764–776.
- [13] S.K. Oster, C.S.W. Ho, E.L. Soucie, L.Z. Penn, The myc Oncogene: Marvelously Complex, in: *Advances in Cancer Research*, volume 84, Academic Press, 2002, pp. 81–154.
- [14] H.S. Cross, W. Hulla, W.-M. Tong, M. Peterlik, Growth regulation of human colon adenocarcinoma-derived cells by calcium, vitamin D and epidermal growth factor, *J. Nutr.* 125 (1995) 2004S–2008S.
- [15] I. Wierstra, J. Alves, The c-myc Promoter: Still MysterY and Challenge, in: F.V.W. George, K. George (Eds.), *Advances in Cancer Research*, volume 99, Academic Press, 2008, pp. 113–333.
- [16] A. Köenig, T. Linhart, K. Schlegelmann, K. Reutlinger, J. Wegele, G. Adler, G. Singh, L. Hofmann, S. Kunsch, T. Büch, E. Schäfer, T.M. Gress, M.E. Fernandez-Zapico, V. Ellenrieder, NFAT-induced histone acetylation relay switch promotes c-myc-dependent growth in pancreatic cancer cells, *Gastroenterology* 138 (2010) 1189–1199.
- [17] M. Buchholz, A. Schatz, M. Wagner, P. Michl, T. Linhart, G. Adler, T.M. Gress, V. Ellenrieder, Overexpression of c-myc in pancreatic cancer caused by ectopic activation of NFATc1 and the Ca^{2+} /calciurein signaling pathway, *EMBO J.* 25 (2006) 3714–3724.
- [18] J.A. Nilsson, J.L. Cleveland, Myc pathways provoking cell suicide and cancer, *Oncogene* 22 (2003) 9007–9021.
- [19] A. Albiñh, J.L. Johnsen, M. Arsenian Henriksson, MYC in Oncogenesis and as a Target for Cancer Therapies, in: F.V.W. George, K. George (Eds.), *Advances in Cancer Research*, volume 107, Academic Press, 2010, pp. 163–224.
- [20] R. Ponzelli, S. Katz, D. Barsyte-Lovejoy, L.Z. Penn, Cancer therapeutics: targeting the dark side of Myc, *Eur. J. Cancer* 41 (2005) 2485–2501.
- [21] D.J. Liao, R.B. Dickson, c-Myc in breast cancer, *Endocr. Relat. Cancer* 7 (2000) 143–164.
- [22] E.M.J.J. Berns, J.G.M. Klijn, W.L.J. van Putten, I.L. van Staveren, H. Portengen, J.A. Foekens, c-myc Amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer, *Cancer Res.* 52 (1992) 1107–1113.
- [23] C. Schlotter, U. Vogt, U. Bosse, B. Mersch, K. Wabetamann, C-myc, not HER-2/neu, can predict recurrence and mortality of patients with node-negative breast cancer, *Breast Cancer Res.* 5 (2003) R30–R36.
- [24] P.H. Watson, R.T. Pon, R.P.C. Shiu, Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer, *Cancer Res.* 51 (1991) 3996–4000.
- [25] R. Bergan, F. Hakim, G. Schwartz, E. Kyle, R. Cepada, J. Szabo, D. Fowler, R. Gress, L. Neckers, Electroporation of synthetic oligodeoxynucleotides: a novel technique for ex vivo bone marrow purging, *Blood* 88 (1996) 731–741.
- [26] C. Schorl, J.M. Sedivy, Loss of protooncogene c-Myc function impedes G1 phase progression both before and after the restriction point, *Mol. Biol. Cell* 14 (2003) 823–835.
- [27] M.-J. Huang, Y.-c. Cheng, C.-R. Liu, S. Lin, H.E. Liu, A small-molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia, *Exp. Hematol.* 34 (2006) 1480–1489.
- [28] B. Amati, K. Alevizopoulos, J. Vlach, Myc and the cell cycle, *Front. Biosci.* 3 (1998) d250–d268.
- [29] D. Robertson, K. Savage, J.S. Reis-Filho, C.M. Isacke, Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue, *BMC Cell Biol.* 9 (2008) 13.
- [30] A.S. Borowiec, F. Hague, N. Harir, S. Guenin, F. Guerin, F. Gouilleux, M. Roudbaraki, K. Lassoued, H. Ouadid-Ahidouch, IGF-1 activates hEAG K(+) channels through an Akt-dependent signaling pathway in breast cancer cells: role in cell proliferation, *J. Cell. Physiol.* 212 (2007) 690–701.
- [31] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Res.* 29 (2001) e45.
- [32] C. Grandori, S.M. Cowley, L.P. James, R.N. Eisenman, The Myc/Max/Mad network and the transcriptional control of cell behavior, *Annu. Rev. Cell Dev. Biol.* 16 (2000) 653–699.
- [33] R. Sears, G. Leone, J. DeGregori, J.R. Nevins, Ras enhances myc protein stability, *Mol. Cell* 3 (1999) 169–179.
- [34] A.G. Turjanski, J.P. Vaque, J.S. Gutkind, MAP kinases and the control of nuclear events, *Oncogene* 26 (2007) 3240–3253.
- [35] I. Quesada, J.M. Rovira, F. Martin, E. Roche, A. Nadal, B. Soria, Nuclear KATP channels trigger nuclear Ca^{2+} transients that modulate nuclear function, *Proc. Natl. Acad. Sci.* 99 (2002) 9544–9549.
- [36] M.G. Todorova, E. Fuentes, B. Soria, A. Nadal, I. Quesada, Lysophosphatidic acid induces Ca^{2+} mobilization and c-Myc expression in mouse embryonic stem cells via the phospholipase C pathway, *Cell. Signal.* 21 (2009) 523–528.
- [37] M. Dean, R.A. Levine, W. Ran, M.S. Kindy, G.E. Sonenshein, J. Campisi, Regulation of c-myc transcription and mRNA abundance by serum growth factors and cell contact, *J. Biol. Chem.* 261 (1986) 9161–9166.
- [38] C. Szatkowski, J. Parys, H. Ouadid-Ahidouch, F. Matifat, Inositol 1,4,5-trisphosphate-induced Ca^{2+} signalling is involved in estradiol-induced breast cancer epithelial cell growth, *Mol. Cancer* 9 (2010) 156.
- [39] H.T. Chang, S.S. Hsu, J.S. Chen, K.L. Lin, J.L. Wang, H.H. Cheng, Y.C. Lu, B.P. Jiann, C.P. Liu, J.K. Huang, J.H. Yeh, A.J. Chiang, W.C. Chen, C.R. Jan, Effect of diethylstilbestrol on Ca^{2+} handling and cell viability in human breast cancer cells, *Chin. J. Physiol.* 46 (2003) 187–192.
- [40] R.K. Motiani, X. Zhang, K.E. Harmon, R.S. Keller, K. Matrougui, J.A. Bennett, M. Trebak, Orai3 is an estrogen receptor α -regulated Ca^{2+} channel that promotes tumorigenesis, *FASEB J.* (in press), <http://dx.doi.org/10.1096/fj.12-213801> (September 19, 2012).
- [41] G.R. Monteith, D. McAndrew, H.M. Faddy, S.J. Roberts-Thomson, Calcium and cancer: targeting Ca^{2+} transport, *Nat. Rev. Cancer* 7 (2007) 519–530.
- [42] A.F. Pla, D. Avanzato, L. Munaron, I.S. Ambudkar, Ion channels and transporters in cancer. 6. Vascularizing the tumor: TRP channels as molecular targets, *Am. J. Physiol. Cell Physiol.* 302 (2012) C9–C15.
- [43] H. Wulff, N.A. Castle, L.A. Pardo, Voltage-gated potassium channels as therapeutic targets, *Nat. Rev. Drug Discov.* 8 (2009) 982–1001.