RESEARCH ARTICLE

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Assessment of ORAI1-mediated basal calcium influx in mammary epithelial cells

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Abstract

Background: The entry of calcium ions into mammary gland epithelial cells is one of the least well-understood processes in the transport of calcium into milk during lactation. The store-operated calcium entry channel ORAI1, has been suggested as a potential mechanism for the entry of Ca^{2+} into mammary gland epithelial cells from the maternal blood supply during lactation. The down regulation of the canonical ORAI1 activator STIM1 during lactation suggests that other known ORAI activators such as STIM2 and SPCA2 may be important during lactation.

Results: Differentiation of HC11 mammary gland epithelial cells was associated with enhanced basal Ca^{2+} influx. Silencing of *Orai1* abolished this enhancement of Ca^{2+} influx. *Stim2* had a modest effect on Ca^{2+} influx in this *in vitro* model of lactation, whereas *Stim1* and *Spc2* silencing had no effect. Despite pronounced increases in *Spc2* mRNA during lactation there was no change in the generation of the alternative splice product generated by Mist1, which increases during lactation.

Conclusions: These studies support the hypothesis that lactation is associated with a remodelling of Ca^{2+} influx and this is associated with enhancement of basal Ca^{2+} influx. This enhanced Ca^{2+} influx appears to occur through the calcium channel *Orai1*.

Background

Lactation is the result of the finely orchestrated differentiation of mammary epithelial cells that gives them the ability to secrete milk. Mammary epithelial cells are unique in their ability to differentiate into lactogenic phenotypes and then dedifferentiate back to a quiescent form, in response to steroid and peptide hormones (reviewed by [1]). Milk provides an energy source, proteins and essential nutrients for the neonate, one of the key components of which is calcium (Ca^{2+}). The rapid growth of the neonate, particularly the calcification of bones and teeth, places a high demand for Ca^{2+} in milk. Depending on the species, the concentration of total Ca^{2+} in milk ranges from 8 to 60 mM [2], a level well above the maternal blood level of total Ca^{2+} . The secretory pathway and the apical plasma membrane play important roles in the transport of Ca^{2+} into milk [2-4].

Despite the importance of Ca^{2+} enrichment of milk, only recently have the Ca^{2+} transporters responsible for the

accumulation of Ca^{2+} into milk begun to be identified. The best-characterized protein involved in the enrichment of milk with Ca^{2+} is the plasma membrane Ca^{2+} ATPase isoform 2 (PMCA2). This calcium efflux pump has a very restricted tissue expression and is present in specific parts of the brain and the inner ear [5-8]. PMCA2 is markedly up regulated during lactation, particularly splice variant PMCA2bw [5-7,9], which localizes to the apical membrane of secretory cells [10,11]. PMCA2 null mice show a 60% reduction in milk Ca^{2+} content, providing direct evidence for the role of PMCA2 in the apical transport of Ca^{2+} into milk during lactation [12].

The sequestration of Ca^{2+} into the secretory pathway during lactation appears to occur via the Golgi localized pump - secretory pathway Ca^{2+} -ATPase isoform 2 (SPCA2). Like PMCA2, SPCA2 has a restricted tissue distribution [13] and is significantly up regulated during lactation [14]. SPCA2 may also have a dual role in lactation due to its Mn^{2+} pumping ability [15]. Both Ca^{2+} and Mn^{2+} are essential for enzymes necessary for the correct post-translational modification of milk proteins and lactose production [16].

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Several different Ca^{2+} permeable ion channels are proposed as the mechanism by which Ca^{2+} enters the mammary epithelial cell from the maternal blood supply during lactation. Calcium channels suggested as involved in this pathway include TRPV5 and TRPV6 [17,18]. However, recent studies suggest that the Orai1 calcium channel may be responsible for calcium influx into the mammary epithelial cell during lactation, since *Orai1* mRNA levels increase in the mouse mammary gland during lactation [19]. Indeed, Orai1 is at the basolateral membrane in mammary epithelial cells [20]. ORAI1 is the canonical mechanism for store operated calcium entry (SOCE). SOCE is the activation of calcium influx into the cell upon the depletion of intracellular stores of Ca^{2+} . Such a mechanism could be a powerful feedback loop to balance demand (the transport of Ca^{2+} into milk) with supply (the influx of Ca^{2+} into the mammary gland epithelial cell). Endoplasmic Ca^{2+} store level depletion is detected by STIM proteins; upon Endoplasmic Reticulum (ER) Ca^{2+} depletion, STIM proteins oligomerize and localize to ER-plasma membrane positions where they activate ORAI channels and promote SOCE [21-26]. The Orai1 isoform of ORAI channels is up regulated in mammary gland tissue samples taken from mice at lactation [19]. However, levels of the canonical Orai1 activator Stim1 decline during lactation. The related isoform Stim2 is suggested as the possible mechanism of activation of Orai1 during lactation as this isoform does not decrease during lactation and is linked to the regulation of basal Ca^{2+} influx [21]. Studies identifying the carboxyl terminal of SPCA2 as an activator of ORAI1 and the interaction between SPCA2 and ORAI1 in MCF-7 breast cancer cells, suggests that the up regulation of SPCA2 may not only serve to promote Ca^{2+} secretion during lactation, but also as an activator of Ca^{2+} influx. However, this has not been assessed in models of lactation. Another aspect of SPCA2 that has not yet been assessed in lactation is the role of the transcription factor MIST1, which is important in mammary gland development [27]. A novel, truncated form of Spca2 was identified in *Mist1*^{-/-} mice, but the presence of this form of Spca2 and its potential role in the regulation of Ca^{2+} transport during lactation has not been assessed. In these studies we used the HC11 model to further explore Ca^{2+} influx in mammary gland epithelial cells and to define the potential role of MIST1 regulation of Spca2 splicing during mammary gland development.

Results

Basal Ca^{2+} influx is increased in differentiated HC11 cells

β -Casein is a known marker of lactogenesis in mammary epithelial cells necessary for the binding of calcium into micelles [2] and is a reliable marker in HC11 cells to show differentiation. HC11 cells were treated to induce differentiation to a lactogenic phenotype (as described in

methods), which was assessed by the expression of β -casein at the mRNA level. Figure 1A demonstrates the increase in β -casein mRNA through real-time RT-PCR in differentiated cells in comparison to cells kept in maintenance media in a proliferative non-differentiated state.

Calcium assays were conducted to assess changes in basal Ca^{2+} influx in response to differentiation of HC11

Figure 1 HC11 cell differentiation and Ca^{2+} influx. Total mRNA was isolated and assessed for an increase in β -casein mRNA to confirm differentiation using real-time RT-PCR. Calcium assays were conducted at the same time to measure changes in basal Ca^{2+} influx in response to differentiation. **A** β -Casein mRNA levels increased in differentiated HC11 cells in comparison to proliferating cells. Data were normalized to *Ppib* and *Actb* mRNA, and are shown as relative fold expression to differentiated HC11 cells ($n = 4$; mean $\pm$ SD, $*P < 0.05$). **B** Basal Ca^{2+} influx is shown as a trace of mean fluorescence relative to baseline in proliferating and differentiated HC11 cells. **C** Rate of basal Ca^{2+} influx, calculated as the slope between 0–5 s after 2 mM Ca^{2+} addition, in differentiated and proliferating HC11 cells. Rate is expressed as a percentage relative to differentiated cells treated with siNT and CPA ($n = 4$; mean $\pm$ SEM, $*P < 0.05$).

cells. Basal Ca^{2+} influx (assessed by the rate of Ca^{2+} increase upon addition of extracellular Ca^{2+}) significantly increased in differentiated cells (Figure 1B and C) indicating that an increase in basal Ca^{2+} influx accompanies β -casein induction and may be a characterizing feature of the changes associated with lactation. We then sought to determine the role of Orai1 in this enhancement of Ca^{2+} influx.

Enhanced basal Ca^{2+} influx in differentiated HC11 cells is abolished by siRNA for Orai1

The effect of siRNA to Orai1 (siOrai1) on basal Ca^{2+} influx in proliferative and differentiated HC11 cells was assessed. *Orai1* silencing eliminated the augmentation of basal Ca^{2+} influx associated with differentiation (Figure 2A and B) suggesting that Orai1 mediates this augmentation in differentiated HC11 cells.

Stim1 silencing did not affect the augmented basal Ca^{2+} influx in differentiated HC11 cells (Figure 3A and B). Treatment of HC11 cells with siStim2 did not produce a significant inhibition of Ca^{2+} influx in differentiated cells (Figure 4A and B), however the significant increase in Ca^{2+} influx between differentiated and proliferating HC11 cells was abolished when cells were treated with siStim2 (Figure 4B). These results suggest a major role for Orai1

in Ca^{2+} influx in the HC11 model, with a modest role for Stim2.

Spca2 in HC11 cells and mouse mammary tissue samples

Truncated and full length *Spca2* transcripts [28] were assessed in proliferating and differentiated HC11 cells by comparing mRNA levels measured at exons 15–16 versus exons 26–27, (Figure 5A) and also in mouse mammary tissue samples isolated from virgin, mid pregnancy and lactation stages (Figure 5C). *Spca2* mRNA did not increase in the *in vitro* HC11 model and there was no change in the proportion of truncated versus full length transcripts (Figure 5A). However, *Spca2* mRNA did increase during lactation, although there was no significant difference between the different exon spanning regions of *Spca2* indicating that no truncated *Spca2* was present in any of the samples. We also assessed the levels of the transcription factor *Mist1*, which is speculated to regulate *Spca2* expression in pancreatic acinar cells [28], and found that *Mist1* levels did not significantly change in differentiated HC11 cells (Figure 5B). However, there was a trend of increased *Mist1* levels in mammary gland tissue samples from the lactating mice (Figure 5D) as previously described [27]. *Mist1* has been detected in differentiated SCp2 cells [27]. *Spca2* silencing had no

Figure 2 Effect of Orai1 silencing on basal Ca^{2+} in HC11 cells. Calcium assays were conducted to assess basal Ca^{2+} influx in proliferating and differentiated HC11 cells treated with siNT or siOrai1. **A** Basal Ca^{2+} influx is shown as a trace of mean fluorescence relative to baseline in proliferating and differentiated HC11 cells treated with siNT and siOrai1. **B** Rate of basal Ca^{2+} influx, calculated as the slope between 0–5 s after 2 mM Ca^{2+} addition, in differentiated and proliferating HC11 cells with siNT or siOrai1. Rate is expressed as a percentage relative to differentiated cells treated with siNT and CPA ($n = 4$; mean $\pm$ SEM, $*P < 0.05$).

Figure 3 Effect of Stim1 silencing on basal Ca^{2+} in HC11 cells. Calcium assays were conducted to assess the basal Ca^{2+} influx in proliferating and differentiated HC11 cells treated with siNT or siStim1. **A** Basal Ca^{2+} influx is shown as a trace of mean fluorescence relative to baseline in proliferating and differentiated HC11 cells treated with siNT and siStim1. **B** Rate of basal Ca^{2+} influx, calculated as the slope between 0–5 s after 2 mM Ca^{2+} addition, in differentiated and proliferating HC11 cells with siNT or siStim1. Rate is expressed as a percentage relative to differentiated cells treated with siNT and CPA (n = 4; mean $\pm$ SEM, * P < 0.05).

Figure 4 Effect of Stim2 silencing on basal Ca^{2+} in HC11 cells. Calcium assays were conducted to assess the basal Ca^{2+} influx in proliferating and differentiated HC11 cells treated with siNT or siStim2. **A** Basal Ca^{2+} influx is shown as a trace of mean fluorescence relative to baseline in proliferating and differentiated HC11 cells treated with siNT and siStim2. **B** Rate of basal Ca^{2+} influx, calculated as the slope between 0–5 s after 2 mM Ca^{2+} addition, in differentiated and proliferating HC11 cells with siNT or siStim2. Rate is expressed as a percentage relative to differentiated cells treated with siNT and CPA (n = 4; mean $\pm$ SEM, * P < 0.05).

Figure 5 SPCA2 mRNA levels and Mist1-mediated truncated SPCA2 in lactation. Proliferating and differentiated HC11 cells were assessed for; **A.** *SPCA2* mRNA spanning exons 15–16 and 26–27 and **B.** *Mist1* mRNA. Mouse mammary tissues isolated from virgin, mid pregnancy and lactation samples were assessed for; **C.** *SPCA2* mRNA spanning exons 15–16 and 26–27 and **D.** *Mist1* mRNA. For all panels (n = 4; mean ± SD, ns denotes no significant difference $P > 0.05$).

significant effect on basal Ca^{2+} influx (Figure 6A and B) suggesting that in HC11 cells *SPCA2* does not play a role in Ca^{2+} influx regulation as suggested in other cell types [29].

β -Casein levels are unchanged with *Orai1* silencing

To determine if the decrease in basal Ca^{2+} influx was an indirect result of siOrai1 inhibition of differentiation, β -casein mRNA levels were measured in siOrai1 treated differentiated HC11 cells. Real-time RT-PCR clearly showed that β -casein expression was not inhibited in siOrai1 treated cells (Figure 7). Therefore, although *Orai1* silencing abolishes the induction of increases in Ca^{2+} influx, it does not inhibit the induction of differentiation.

Orai1 mRNA levels do not change with differentiation

Given the up regulation of *Orai1*-mediated Ca^{2+} influx associated with differentiation in HC11 cells, we assessed changes in the levels of *Orai1* and its known activators. *Orai1* (Figure 8A) mRNA did not significantly change in

response to differentiation. However, differentiation was associated with a modest up regulation in *Stim1* mRNA (Figure 8B), and a modest down regulation of *Stim2* (Figure 8C) and *SPCA2* (Figure 8D) mRNA levels.

Discussion

The enrichment of milk with calcium is essential for the growing neonate. Our understanding of the specific calcium channels and pumps that are involved in this process is gradually evolving. However, the area that is least understood in the transport of Ca^{2+} into milk is the mechanism by which Ca^{2+} flows from the maternal blood supply into the mammary epithelial cell. *In vivo* studies implicate store operated Ca^{2+} entry as the calcium influx pathway involved, with *Orai1* mRNA levels increasing in mouse mammary glands during lactation [19]. Although *Orai1* silencing reduces Ca^{2+} influx in human breast cancer cell lines [19], no studies have directly assessed calcium influx mediated by this pathway in *in vitro* models of lactation. The possible roles of the *Orai1* activators *Stim1*, *Stim2* and *SPCA2* have also not been assessed through direct assessment of Ca^{2+}

Figure 6 Effect of Spca2 silencing on basal Ca^{2+} in HC11 cells. Calcium assays were conducted to assess the basal Ca^{2+} influx in proliferating and differentiated HC11 cells treated with siNT or siSpca2. **A** Basal Ca^{2+} influx is shown as a trace of mean fluorescence relative to baseline in proliferating and differentiated HC11 cells treated with siNT and siSPCA2. **B** Rate of basal Ca^{2+} influx, calculated as the slope between 0–5 s after 2 mM Ca^{2+} addition, in differentiated and proliferating HC11 cells with siNT or siSpca2. Rate is expressed as a percentage relative to differentiated cells treated with siNT and CPA ($n = 4$; mean $\pm$ SD, $*P < 0.05$).

influx in mammary gland epithelial cells. These questions were addressed in this study.

HC11 cells are a mouse mammary epithelial cell line derived from COMMA-1D cells, isolated from mid-pregnant Balb/c mice mammary glands [30,31]. Unlike many other mammary cell models, HC11 cells do not need to be co-cultured with other cell types or with extracellular matrix proteins to help induce lactogenic differentiation [30,32–34], making it suitable for the calcium influx assays used in this study. The expression of β -casein is essential for Ca^{2+} accumulation into milk, as a significant amount of calcium is present in casein micelles [2,35]. Moreover, β -casein is a marker of differentiation in HC11 cells [36,37].

Differentiation of HC11 cells with lactogenic hormones resulted in a statistically significant increase in basal Ca^{2+} influx compared to undifferentiated controls. This result is consistent with the hypothesis that lactation is associated with the remodelling of mammary gland epithelial cells to a more Ca^{2+} permeable phenotype associated with elevated basal Ca^{2+} influx. This increase in Ca^{2+} influx is likely to be required to meet the demands of the secretion and efflux of Ca^{2+} from the mammary gland epithelial cell into milk.

Silencing of *Orai1* demonstrated that this augmented Ca^{2+} influx is via *Orai1*, since the difference in Ca^{2+}

influx between differentiated and undifferentiated HC11 cells was abolished with the silencing of this calcium channel. Hence, consistent with elevated *Orai1* mRNA levels during lactation, *Orai1* appears to be a major contributor of the enhanced basal Ca^{2+} influx in mammary gland epithelial cells from a lactating host. *Orai1* along with its activator *Stim2* are regulators of basal Ca^{2+} levels [21]. Our studies now suggest that this basal influx pathway is dynamic and may be up regulated during lactation. Future studies should assess how reported regulators of calcium transport during lactation, such as the calcium-sensing receptor, affect *Orai1*-mediated calcium influx in this and other models [38]).

McAndrews *et al.* compared 2 day (undifferentiated) and 8 day (with lactogenic hormones – differentiated) HC11 cultures and found elevated *Orai1* mRNA in HC11 cells at 8 days of differentiation [19]. These studies comparing cultures at day 8 with and without lactogenic hormones, suggest that it may be days in culture and/or confluence, which produces an increase in *Orai1* mRNA levels. Lactogenic hormones may be responsible for the enhancement of *Orai1* basal activity in HC11 cells. *Orai1* does not appear to be a key pathway in the differentiation of HC11 cells given that *Orai1* silencing had no effect on β -casein levels, this is in contrast to

SCp2 cells where mammosphere formation was abolished with shOrai1 treatment [20]. The lack of effects of *Orai1* silencing on β -casein levels in HC11 cells indicates that the decrease in basal Ca^{2+} influx observed when *Orai1* was silenced was not simply a consequence of the inhibition of HC11 differentiation.

ORAI1 is activated via a number of different mechanisms including the canonical ORAI1 activator and calcium store sensor STIM1, its related isoform STIM2, and SPCA2. STIM2 is proposed as an ORAI1 activator in lactation due to its important role in basal influx in HeLa, HUVEC and HEK293T cells [21]. The maintenance of *Stim2* mRNA levels during lactation [19] and reduction in basal $[\text{Ca}^{2+}]_{\text{CYT}}$ in differentiated HC11 cells with *Stim2* silencing also support this role. Other mechanisms of ORAI1 activation during lactation have also been proposed, such as calcium store independent activation by SPCA2 [29]. Specific domains of SPCA2 protein activate ORAI1 [29], and *Spca2* mRNA levels are pronouncedly increased during lactation [14]. Activation

of ORAI1 by SPCA2 may allow the demand for Ca^{2+} sequestration via this secretory pathway Ca^{2+} pump to promote the supply of Ca^{2+} through augmentation of Ca^{2+} influx. However, in these studies, and in contrast to *Orai1* silencing, neither *Stim1* nor *Spca2* silencing abolished the augmentation of Ca^{2+} influx induced by differentiation of HC11 mammary epithelial cells. It could be that some *in vitro* systems have calcium influx pathways that are more sensitive to *spca2* dependent modulation of Orai1, such has recently been reported in Scp2 cells [20]. The ability of *Stim2* silencing to abolish the enhanced Ca^{2+} influx associated with differentiation of HC11 cells, supports previous suggestions that *Stim2* is a modulator of Orai1 during lactation, however, the inability of *Stim2* silencing to replicate the magnitude of *Orai1* silencing suggest other Orai1 activation mechanisms. Compensatory mechanisms amongst the Orai1 activators *Stim1*, *Stim2* and *Spca2* in this model and/or other factors such as the absence of coordinated polarization of mammary gland epithelial cell may be responsible for the results reported here. Further studies using 3D culture models in multiple mammary cell lines models, such as those recently published by Cross et al. in Spc2 cells [20], and *in vivo* studies with knock-out animals are required to ultimately define the specific roles of *Stim1*, *Stim2* and *Spca2* and the activation of Orai1 in lactation.

Conclusions

The influx of Ca^{2+} across mammary gland epithelial cells is a key step in the supply of Ca^{2+} to the growing neonate during lactation. These studies using HC11 mammary gland epithelial cells are consistent with recent *in vivo* studies of mRNA levels during mammary gland development suggesting that Orai1 is an important pathway of Ca^{2+} influx during lactation.

Methods

Cell culture

Mouse mammary epithelial cells (HC11) were propagated in maintenance media containing Roswell Park Memorial Institute (RPMI)-1640 Medium (R8757, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) and 10 mg/mL bovine insulin, as previously described [19]. Cells were maintained in a 37°C, humidified 5% CO_2 , 95% air incubator. Cell lines were routinely tested for mycoplasma contamination.

HC11 cell differentiation and cell culture treatments

HC11 cells were seeded at 3500 cells/well into 96 well plates in maintenance media containing 10 ng/mL murine EGF. Fresh maintenance media with EGF was added at 24 h and siRNA treatment performed at 48 h in the presence of EGF and insulin. Following 24 h siRNA treatment

Figure 8 Effect of differentiation in HC11 cells on mRNA expression of Orai1, Stim1, Stim2 and SPCA2. HC11 cells were treated to induce differentiation or kept in maintenance media to keep them proliferating. Total mRNA was isolated 144 h post plating and assessed for *Orai1* (A), *Stim1* (B), *Stim2* (C) and *Spca2* (D) levels. Data were normalized to *Ppib* and *Actb* mRNA, and are shown as relative fold expression to differentiated HC11 cells. (n = 4; mean $\pm$ SD, * P < 0.05, ns denotes no significant difference).

media was replaced with maintenance media for 24 h. After 48 h siRNA treatment, media was replaced with either maintenance media supplemented with 1 μ M dexamethasone and 5 μ g/mL ovine prolactin (L6520, Sigma Aldrich) to differentiate the cells, or with maintenance media for proliferating non-differentiated cells. Fresh maintenance media was added at 24 h with or without dexamethasone and prolactin, as appropriate. Calcium assays were performed 144 h post plating. RNA was isolated from cells after calcium measurements were taken. Experiments were conducted in triplicate wells and all experiments were performed independently on four occasions.

siRNA transient transfection

siRNA transfection was performed using Dharmacon ON-TARGETplus SMARTpool™ siRNA (100 nM), comprising a pool of four siRNA sequences rationally designed with dual strand modification and use of an algorithm to reduce seed region matches. DharmaFECT1 transfection reagent (catalogue number T-2001-01) was used (0.4 μ L/well) as per the manufacturer's instructions. The following Dharmacon On-TARGETplus SMARTpool™ mouse siRNAs were used in this study: non-targeting (D-001810-10-05), *Orai1* (L-056431-02-005), *Stim1* (L-062376-00-0005), *Stim2* (L-055069-01-0005) and *Spca2* (L-065820-01-005). siRNA knockdown was checked using real-time RT-PCR for each experimental plate 24 h post treatment (Additional file 1).

Calcium assays

Calcium assays were performed using a fluorometric imaging plate reader (FLIPR^{TETRA}, Molecular Devices Corporation) using the no-wash cytosolic free calcium PBX Ca²⁺ Assay Kit (BD Biosciences) as previously described [39]. Cells were seeded in 96 well black-walled plates (Corning). Intracellular Ca²⁺ measurements were performed with an excitation intensity of 470–495 nm and a 515–575 nm emission filter. Fluorescent values were normalized to the starting fluorescence and were expressed as relative [Ca²⁺]_{CYT}. The slope of the curve was calculated based on points at 0 to 5 s post Ca²⁺ addition and were expressed as a percentage relative to the average readings for differentiated cells treated with non-targeting siRNA with maximum *Orai1*-mediated Ca²⁺ influx induced by 10 μ M cyclopiazonic acid (CPA).

Real-time RT-PCR

Total RNA was isolated using the RNeasy Plus mini kit (Qiagen) as per the manufacturer's instructions, and mRNA was quantitated using real time RT-PCR and a 7500 real time PCR system (Applied Biosystems). Mouse mammary tissues were obtained as previously described [19]. Mouse β -casein (Mm00839664_m1),

Orai1 (Mm00774349_m1), *Spca2* (Mm01242899_m1), *Spca2* exon 15–16 (Mm01242904_m1), *Spca2* exon 26–27 (Mm01242916_m1), *Mist1* (Mm00487695_m1), *Stim1* (Mm00486423_m1), and *Stim2* (Mm01223102_m1) were amplified using the TaqMan® gene expression assays, and the data normalized to *Ppib* (Mm00478295_m1) and *Actb* (Mm01205647_g1). Data were analyzed using the comparative C_t method as described previously [40]. All data were normalized to proliferating HC11 cells treated with non-targeting siRNA. Data are shown as mean plus SD (n = 4).

Statistical analysis

All experimental treatments were conducted as three wells per experimental plate in quadruplicate. Results for the slope are presented as standard error of the mean (SEM) and statistical comparisons were performed using a two-way RM ANOVA matching by rows with a Bonferroni multiple comparison post-test. Real-time RT-PCR results are presented as standard deviation (SD) and were analyzed for significance using a paired t-test. All statistical analyses were conducted using Prism Graph Pad, Version 5.04, Berkeley, CA, and significance was demonstrated at P < 0.05 where appropriate.

Additional file

Additional file 1: HC11 cells were treated with siRNA for *Orai1* (A.), *Stim1* (B.), *Stim2* (C.) and *Spca2* (D.). Total mRNA was isolated 24 h after siRNA treatment before real-time RT-PCR for assessment of gene silencing. Data were normalised to *Ppib* and *Actb* mRNA, and are shown as relative fold expression to siNT treated HC11 cells (n=4; mean $\pm$ SD, *P < 0.05).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DR helped design and performed experiments and data analysis and wrote the paper. CS contributed to the writing of the paper and preparation of lactation samples. IA contributed to the writing and revision of the paper. SRT designed experiments and contributed to the data analysis and writing of the paper. GM designed experiments, and contributed to analysis of the data and wrote the paper. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Prof. Melissa Brown from the University of Queensland, School of Chemistry and Molecular Biosciences, for providing the mammary tissue samples used in the project. This work was supported by National Health and Medical Research Council Grant 631347.

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Received: 20 November 2013 Accepted: 9 December 2013

Published: 20 December 2013

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doi:10.1186/1471-2121-14-57

Cite this article as: Ross et al.: Assessment of Orai1-mediated basal calcium influx in mammary epithelial cells. *BMC Cell Biology* 2013 **14**:57.