Secretory pathway Ca^{2+}/Mn^{2+}-ATPase isoform 2 and lactation: specific localization of plasmalemmal and secretory pathway Ca^{2+} pump isoforms in the mammary gland

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Abstract

The supply of calcium to the developing neonate via milk is an important physiological process. Until recently the mechanism for the enrichment of milk with calcium was thought to be almost entirely mediated via the secretory pathway. However, recent studies suggest that a specific isoform of the plasma membrane calcium ATPase, PMCA2, is the primary mechanism for calcium transport into milk, highlighting a major role for apical calcium transport. We compared the expression of the recently identified secretory calcium ATPase, SPCA2, and SPCA1, in the mouse mammary gland during different stages of development. SPCA2 levels increased over 35 fold during lactation, while SPCA1 increased only a modest two fold. The potential importance of SPCA2 in lactation was also highlighted by its localization to luminal secretory cells of the mammary gland during lactation, while SPCA1 was expressed throughout the cells of the mammary gland. We also observed major differences in the localization of PMCA2 and PMCA1 during lactation. Using the SCp2 mouse mammary epithelial cell 3D culture model, differences in the sub-cellular distribution of PMCA2 and PMCA1 were clear. These studies highlight the likely specific roles of PMCA2 and SPCA2 in lactation, and link the recently characterized SPCA2 calcium pump to the supply of calcium into milk and the regulation of Golgi resident enzymes important in lactation. They also indicate that calcium transport into milk is a complex interplay between apical and secretory pathways.

Keywords (3 to 5 words that do not appear in the title)

Calcium, PMCA2, SPCA1, PMCA1, SPCA2

Introduction

Intracellular free Ca^{2+} levels are under tight regulation and control, with resting free cytosolic Ca^{2+} levels maintained at around 100 nM (3). The amplitude, location, duration and frequency of intracellular Ca^{2+} increases (sometimes into the μM range) regulate a variety of cellular processes including gene transcription, proliferation and cell death (3). This regulation is achieved by proteins responsible for the translocation of Ca^{2+} ions, which include Ca^{2+} channels, Ca^{2+} exchangers and Ca^{2+} pumps (3, 4). During lactation, the unidirectional transport of Ca^{2+} from the maternal blood supply to the milk must be achieved without causing major disturbances to cellular signaling within the epithelial cells of the mammary gland (11). Concurrent with tightly maintaining intracellular Ca^{2+} levels, a large gradient between free and total Ca^{2+} between the maternal blood supply and milk is established. Indeed the total Ca^{2+} content of blood is 3 mM (11), while that of the milk is approximately 8 mM in humans, and over 60 mM in other species such as mice (13).

Active transporters of Ca^{2+} ions include the plasma membrane Ca^{2+} ATPases (PMCAs), the sarcoplasmic endoplasmic Ca^{2+}-ATPases (SERCAs) and the secretory pathway Ca^{2+}-ATPases (SPCAs) (4, 11). Consistent with a minor role for the endoplasmic reticulum in Ca^{2+} enrichment of milk, levels of SERCA isoform transcripts expressed in the rat mammary gland (SERCA2 and SERCA3) do not significantly increase during pregnancy or during the early stages of lactation (18). In contrast, PMCA2, which has a highly restricted tissue distribution (9, 22), increases approximately 30 fold during the early stages of lactation in the rat mammary gland as compared to the almost undetectable levels in nulliparous rats (17, 18). The presence of PMCA2 protein in milk fat globule membranes suggests a predominately apical distribution for PMCA2 during lactation, and a major role in the efflux of Ca^{2+} across the apical membrane into milk (13, 17). The reduction in the Ca^{2+} content of milk in PMCA2 null mice by 60%

(19), when combined with only a comparatively modest increase (6-8 fold) in the expression of the secretory pathway pump SPCA1 (17, 18), challenges the widely cited view of little to no involvement of direct apical transport of Ca^{2+} and the major role of the Golgi apparatus during lactation (13). Although a possible role for PMCA2-mediated Ca^{2+} accumulation into the Golgi during trafficking to the membrane has been discussed (19), the recent report of PMCA2 localization to the apical membrane in mammary tissue from a lactating mouse (25) represents further evidence for its direct role in apical calcium transport.

The regulation of key Golgi enzymes involved in post-translational modification of milk proteins and lactose production by Ca^{2+} and Mn^{2+} (11, 12), both substrates for the secretory pathway Ca^{2+} pump (24), still suggests a major role for Golgi Ca^{2+} accumulation in lactation, as does the secretion of Ca^{2+} in casein micelles derived from the Golgi (13). In this study we sought to address the dichotomy arising from the modest increase in SPCA1 expression and the pronounced increase in PMCA2 during lactation, and the seemingly major role for the secretory pathway in Ca^{2+} secretion for the enrichment of milk with Ca^{2+} (13).

Subsequent to the studies discussed above regarding the expression of SPCA1 and PMCA2 in lactation, a second secretory pathway Ca^{2+} / Mn^{2+} pump isoform has been identified, named SPCA2, encoded by the ATP2C2 gene in humans (27). Similar to PMCA2, SPCA2 has a more restricted tissue distribution compared to SPCA1. SPCA2 is expressed in the brain and testis (27) as well as the cells of gastrointestinal and respiratory tract and mammary, salivary, and thyroid glands (26). Differences in the enzyme properties of SPCA1 and SPCA2, such as the higher catalytic turnover rate for Ca^{2+} of SPCA2 compared with SPCA1, when over-expressed in HEK-293 cells, are proposed as possible evidence for

specific functional roles for SPCA2, particularly in secretory cells (7). However, the possible physiological processes where SPCA2 may play a major role are still unclear. In these studies, we addressed the hypothesis that SPCA2 expression undergoes greater changes in expression than SPCA1 during pregnancy and lactation, and that PMCA isoforms and SPCAs have unique cellular distributions during lactation consistent with specific roles in Ca^{2+} homeostasis in the mammary gland and the transport of Ca^{2+} into milk.

Materials and Methods

Animals

Animal ethics approval was obtained for all studies from the University of Queensland animal ethics committee. CBA x C57Bl6 mice were fed standard rodent chow and water ad libitum. Nulliparous animals were euthanized at 14 weeks of age. For all other developmental stages, dams were mated when 12 weeks old. Animals were then sacrificed at the following stages of mammary gland development: mid-pregnancy (~ day 10); lactation (day 1); and involution (48h post-forced weaning). Pregnant animals were estimated to be day 10 based on microscopic examination of the developing fetus. The glands from lactating mice were imbedded in Optimum Cutting Temperature (OCT) compound (Tissue-Tek®, Torrance, CA) and frozen on dry ice for tissue sectioning.

RNA isolation and Real Time RT-PCR

Total RNA was isolated from whole mammary glands using the Qiagen RNeasy mini Kit incorporating an on-column DNase treatment (Qiagen, Doncaster, Vic, Australia). RNA was quantified spectrophotometrically at 260 nm. All samples were stored at -80 ˚C until required. SPCA1 and SPCA2 mRNA levels were quantified using real time RT-PCR. The mRNA was reverse transcribed using the Omniscript® RT kit (Qiagen) while the real time PCR step was performed using a TaqMan® universal PCR master mix (Applied Biosystems, Scoresby, VIC, Australia) and the following TaqMan® gene expression assays: Mm00723486_m1 (SPCA1; Applied Biosystems); and Mm01242899_m1 (SPCA2; Applied Biosystems). Expression was normalized to endogenous 18S rRNA levels and is expressed relative to nulliparous animals. Reactions were cycled in an ABI PRISM 7500 Sequence Detector (Applied Biosystems) under the following conditions: 95 ˚C for 10 min; followed by 40 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min. Threshold cycle (Ct) values were used for all analysis. Three separate PCR

reactions were performed from each RNA sample from each mouse, to assess variability in Ct responses between replicates and between mice. The mean differences between Ct responses for the different targets, and their standard errors, were estimated using a random effects model, with an additional mouse effect, allowing for the variation in Ct responses between and within mice. Standard errors of further quantities derived from these estimates were determined using the delta technique. Significance was assessed using a z-test based on a normal distribution.

Immunofluoresence (in vivo samples)

Glands were sectioned at 5 µM thickness. Sections were fixed with 4 % paraformaldehyde (ProSciTech, Thuringowa Central, QLD, Australia) in phosphate buffered saline (PBS; Sigma Aldrich, Castle Hill, NSW, Australia). Slides were incubated with primary antibody for 1 h at room temperature in a humidified chamber. Primary antibodies used included: anti PMCA1 (1:100, PA1-914; Affinity BioReagents, Golden, CO); anti-PMCA2 (1:800, PA1-915; Affinity BioReagents); affinity purified anti-SPCA1 (1:500); affinity purified anti-SPCA2 (1:100 (27)); and anti-smooth muscle actin (SMA) Cy3 Conjugate (1:400, 1A4; Sigma Aldrich). Sections were incubated with the secondary antibody Alexa Fluor® - 488 goat anti-rabbit (1:500; Molecular Probes, Mt Waverley, VIC, Australia) for 40 min, at room temperature in a humidified chamber. Cells were co-stained with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI; 2 µg/mL; Sigma Aldrich). Fluorescence images were captured with a Nikon Eclipse TE300 microscope (Nikon UK, Kingston, UK), equipped with a 40X oil immersion NA 1.3 objective lens (Nikon), and with MetaFluor® imaging software (Universal Imaging, Downington, PA). DAPI, Alexa Fluor® 488, and Cy3 were excited at 364 nm, 488 nm and 540 nm, respectively, and emitted light was collected at 454 nm, 520 nm, and 570 nm, respectively. Exposure times for the three channels were 500 ms, 1000 ms and 1000 ms, respectively. All images were analyzed with MetaMorph®, version 6.2 r5 (Universal Imaging).

Cell culture

SCp2 cells, a clonal derivative of the COMMA 1D mouse mammary epithelial cell line (5) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)/Hams F-12 (UCSF Cell Culture Facility, San Francisco, CA) supplemented with 2% (v/v) fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), gentamicin (50 µg/mL, UCSF Cell Culture Facility) and insulin (5 µg/mL; Sigma-Aldrich) (6). Cultures were maintained at 37 ˚C in a humidified 5 % CO_2/ 95 % air incubator. SCp2 were grown also as a three-dimensional (3D) culture on top of a layer of reconstituted basement membrane gel from Engelbreth-Holm-Swarm extracellular matrix extract, growth factor-reduced (Matrigel; BD Biosciences, Bedford, MA) (10). Briefly, wells of a 12 well plate (Falcon, Franklin Lakes, NJ) were coated with 80 µL of chilled Matrigel, rocked to ensure even coverage and incubated at 37 ˚C for approximately 40 min to allow the Matrigel to solidify. SCp2 cells were trypsinized as usual and seeded at a density of 7.6 x 10^4 cells/well on top of the solidified Matrigel and left to adhere for 1 h. Media were then replaced with differentiation media, which consisted of DMEM/F-12 supplemented with gentamicin (50 µg/mL), insulin (5 µg/mL), hydrocortisone (1 µg/mL; Sigma Aldrich), prolactin (3 µg/mL; Sigma Aldrich), and 2 % Matrigel. All media were refreshed every 2-3 days with fresh differentiation media for 7 days. Cells were harvested by aspirating the culture media, and scraping the Matrigel/cell suspension from the bottom of the well. The Matrigel/cell suspension (15µL) was then smeared onto a microscope slide and allowed to dry for 10 – 15 min at room temperature. The slides were either stored at -20 ˚C until required or immunostained immediately.

Immunofluorescence (SCp2 cells)

Cells grown in a monolayer (2D) to approximately 90 % confluence in 8-well chamber slides (Nalge Nunc International, Rochester, NY), or in 3D, were fixed in an ice cold methanol/acetone solution (1/1;

v/v) for 10 min on ice. Slides were blocked for 1 h with IF wash buffer (0.1% BSA, 0.2% Triton X100, 0.05% Tween 20 in PBS) plus 10 % goat serum (UCSF Cell Culture Facility) then incubated with primary antibody overnight at 4 ºC. Primary antibody dilutions were anti-PMCA1 (1:100), anti-PMCA2 (1:300) and anti–α6 integrin (1:100; MAB 1378; Chemicon International, Temecula, CA). Slides were incubated with the following secondary antibodies for 40 min at room temperature: goat anti-rat Alexa Fluor® 568 and goat anti-rabbit Alexa Fluor® 488 (all 1:500; Molecular Probes). Cells were co-stained with DAPI (2 µg/mL). Widefield fluorescence images were obtained with a Nikon Diaphot 300 microscope equipped with a Nikon PlanApo 60X (NA 1.4) objective lens and Spot RT camera and software (Diagnostic Instruments Inc., Sterling Heights, MI). Confocal analysis was performed using a Solamere Technology Group (Salt Lake City, UT) spinning disk confocal system comprised of a Zeiss Axiovert 200M inverted microscope, Yokagawa CSU10 confocal scan head and Stanford Photonics XR/Mega-10 ICCD camera, run by QED InVivo software (Media Cybernetics). Images were obtained using Zeiss LD Plan-Neofluar 40X (NA 0.6) and Zeiss Plan-Apochromat 63X (NA 1.4) objective lenses. Fluorochromes DAPI, Alexa Fluor® 488/FITC and Alexa Fluor® 568 were excited with 405 nm, 488 nm, and 568 nm light, respectively, and emitted light was collected using the following emission filters: D460/50, HQ535/50 and HQ620/60 (Chroma Technology Corp., Rockingham, VT). Optical z-sections were acquired at 0.5 µm intervals and are an average of 4 exposures. Exposure times for the three channels were 400 ms, 200 ms, and 200 ms, respectively. All images were analyzed with MetaMorph®, version 6.2 r5.

Immunoblotting

Total cell lysates were isolated from SCp2 cells grown in 2D monolayers on 12 well plates (in the presence of normal media or differentiation inducing media) to reach approximately 90 % confluence, using RIPA buffer (1% NP-40, 0.5 % sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl,

pH 7.4) plus 10 ul/mL protease inhibitor cocktail set 1 (Calbiochem®, San Diego, CA). Protein concentration was determined using Bio-Rad DC protein assay reagents (BioRad, Hercules, CA). Proteins (30 µg) were resolved using a 10% tris-glycine SDS page gel (Invitrogen, Carlsbad, CA) then transferred to a nitrocellulose membrane. Membranes were incubated for 1 h at room temperature with primary antibodies. These included: anti β-actin (1:1000, AC-15; Sigma Aldrich) and anti–β-casein (1:1000) prepared from a hybridoma kindly provided by Dr Kaetzel, Institute of Pathology, Case Western Reserve University, Cleveland, OH (23). Secondary antibodies goat anti-mouse or goat anti-rabbit IgG horseradish peroxidise-conjugated (both 1:1000; Pierce, Rockford, IL), were incubated for 40 min at room temperature. Proteins were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce) and a FluorChem™ 8900 Chemi Imager (Alpha Innotech Corp., San Leandro, CA).

Results

To ascertain which isoform, SPCA1 or SPCA2, is the predominant isoform up-regulated during lactation, their mRNA levels were compared in mammary glands isolated from nulliparous, pregnant, lactating and involuting mice. Consistent with the increase seen in SPCA1 in rats (17, 18), mouse SPCA1 levels increased modestly during pregnancy (~ 1.5 fold at day 10) and lactation (~ 2 fold at day 1) (Fig 1). In contrast, a dramatic up-regulation of SPCA2 was observed in mouse mammary glands during lactation (~ 35 fold at day 1) with an increase also seen during pregnancy (~ 8 fold at day 10) compared to nulliparous animals (Fig 1).

Consistent with its more restricted tissue distribution (26), SPCA2 was expressed predominately in luminal epithelial cells of the mouse mammary gland during lactation (Fig 2A & B; compare with myoepithelial cells identified by smooth muscle actin staining), with no expression in cells outside the acini (arrows). The staining pattern was vesicular throughout the cell interior, consistent with localization to the secretory pathway. SPCA1 staining was also punctate, however, this isoform was expressed in all cell types of the tissue section, including myoepithelial and stromal cells (Fig 2C & D).

Analogous to SPCA1 and SPCA2, PMCA1 and PMCA2 exhibited differential isoform expression in the different cell types of the mammary gland during lactation, with distinct differences in sub-cellular distribution. Fig 3A & B shows PMCA2 in the lactating mouse mammary gland. Consistent with its restricted distribution, PMCA2 expression appeared confined to the alveolus, whereas non-acinar cells did not express PMCA2. PMCA2 also exhibited an apical localization in many of the acinar structures. Such a distribution suggests that PMCA2 may have a role in the direct translocation of Ca^{2+} across the apical

membrane and is consistent with the recent work of VanHouten et al (25). In sharp contrast to PMCA2, PMCA1 appeared to be ubiquitously expressed in the mammary gland sections (Fig 3C & D) with a more basolateral distribution, consistent with a possible role in regulation of cytosolic free Ca^{2+} in cells of the mammary gland, rather than the active transport of Ca^{2+} into milk.

To confirm the distinct differences between PMCA1 and PMCA2 in sub-cellular distribution under more controlled conditions, the mouse mammary SCp2 cell line was differentiated to produce 3D acini (Fig 4A & B). These cells were polarized to form a lumen and secreted the milk protein, β-casein (Fig 4C), analogous to primary cultures (2). In this culture model, SPCA2 expression was vesicular in both 2D (Fig 4D) and 3D (Fig 4E). PMCA1 and PMCA2 exhibited pronounced differences in their sub-cellular localization. PMCA1 expression was basolateral (Fig 5), whereas PMCA2 localization was more predominant towards the apical membrane and exhibited a cytosolic punctate staining (Fig 6). These studies shed some light on the distinct roles of these calcium pumps through their differential expression and localization, sub-cellularly as well as in different cell types, of the mammary gland.

Discussion

The modest increase in SPCA1 expression compared with PMCA2 expression during lactation in the rat (17, 18), and the reduction of the Ca^{2+} content of milk in PMCA2$^{-/-}$ mice (19), has challenged the dogma of the greater importance of the Golgi compared to the apical membrane in the enrichment of milk with Ca^{2+}. The data presented here suggest that SPCA2, rather than SPCA1, is the major secretory pathway Ca^{2+} ATPase important in lactation and is consistent with a major role of the Golgi in Ca^{2+} enrichment in milk.

The role of SPCA2 in mammary gland secretory cells during lactation is likely to be two-fold: Firstly, the up-regulation of SPCA2 may function to supply the secretory pathway with Ca^{2+} ions for packaging into casein micelles for calcium secretion into milk (13). Indeed, the higher enzymatic turnover rate of SPCA2 compared to SPCA1 when over-expressed in HEK-293 cells and its high apparent affinity for Ca^{2+} (7), suggests that SPCA2 has an enhanced capability to replenish Golgi Ca^{2+} as Ca^{2+} is lost via secretion (7). Since milk has a high Ca^{2+} content, lactation is an event where loss of Ca^{2+} from the tissue would be pronounced, and our observation of increased levels of SPCA2 during lactation is suggestive that this SPCA isoform has a major role in meeting the demand for Golgi Ca^{2+} replenishment. The second role for SPCA2 in lactation may go beyond the supply of Ca^{2+} to the Golgi for secretion into milk, and relate to the control of Ca^{2+} and Mn^{2+} ions in the Golgi for the regulation of Golgi-localized Ca^{2+} and Mn^{2+} sensitive enzymes, which are critical for posttranslational modification of milk proteins and lactose production (11).

One key enzyme for which the regulation of Ca^{2+} and Mn^{2+} may be important is 1,4-β-galactosyltransferase I. This enzyme is a *trans*-Golgi enzyme (20) and is responsible for the transfer of

galactose, a key event in the formation of some glycoconjugates (8, 20). 1,4-β-Galactosyltransferase I is up-regulated during lactation, where it forms a heterodimer with α-lactalbumin to enable lactose production for secretion into milk (20). The increase in both 1,4-β-galactosyltransferase I and SPCA2 during mid-pregnancy (20), combined with the sensitivity of 1,4-β-galactosyltransferase I to Mn^{2+} (21) suggests that SPCA2 may also be important in regulating the activity of this enzyme, and subsequently the lactose and glycoprotein profile of milk. The up-regulation of SPCA2 during mid-pregnancy is likely to be hormonally regulated as SPCA2 transcription is increased by prolactin in human MCF-7 breast cancer cells (1) and prolactin levels increase during pregnancy (14).

In addition to highlighting a physiological role for SPCA2 in lactation, this study provides further evidence for the isoform specific roles of PMCA2 and PMCA1 in the mammary gland, through the demonstration of their distinct sub-cellular and cellular locations during lactation. As expected, the ubiquitously expressed PMCA1 was present in the majority of cells in the lactating mammary gland with a basolateral localization both in vivo during lactation and in 3D culture. Such a distribution of PMCA1 is consistent with a role in the regulation of calcium fluxes in mammary gland epithelial cells, rather than direct transport of calcium into milk. Conversely, PMCA2 was localized to the secretory alveolus in vivo, with an apical localization, which is consistent with its proposed role in direct apical transportation of Ca^{2+} ions into milk (19, 25). PMCA2 localization in the 3D culture model appeared more pronounced towards the apical membrane, however, a vesicular distribution was also apparent, suggestive of a high turnover due to loss of PMCA2 during lactation as previous proposed (17), or indicative of a role for PMCA2 in the accumulation of Ca^{2+} into secretory vesicles (19) during particular periods of lactation. The reduction of total milk Ca^{2+} content by 60% in PMCA2 null mice, combined with the major contribution of casein micelles in milk Ca^{2+} enrichment, could also suggest a role for PMCA2 in Golgi Ca^{2+} accumulation (19). However, the observation that PMCA2 heterozygous mice do not have reduced

milk Ca^{2+} content, despite a 38% reduction in mammary PMCA2 levels (19), and our observation of pronounced up-regulation of SPCA2, suggests that more complex reasons may exist for the pronounced reduction of Ca^{2+} milk content in PMCA2 null mice, and that this result does not imply that 60% of Ca^{2+} transport into milk is mediated via PMCA2. Although the complete lack of PMCA2 expression will certainly reduce apical Ca^{2+} transport, this also may be associated with altered homeostatic pathways of the entire animal manifested as a reduction in the Ca^{2+} content of milk, or the complete lack of PMCA2 may be associated with altered function, signaling and/or transcription in mammary gland secretory cells. Indeed SPCA1 and SERCA2 levels are increased in PMCA2 null mice (19). SPCA1-null mice have only been characterized recently and are embryonic lethal (15). SPCA2-null mice have not been characterized; however, we would predict from our studies that their phenotype may be associated with altered calcium secretion into milk, and an altered lactose content and glycoprotein profile of milk.

Despite our increasing knowledge of the transport of Ca^{2+} during lactation, important questions still remain. Our studies have, however, helped to clarify and highlight the importance of the secretory pathway ATPase, SPCA2, during pregnancy and lactation. They have also focused attention on the specific roles of the PMCA isoforms, PMCA1 and PMCA2 suggested by their different cellular and sub-cellular localization in the lactating mouse mammary gland. Given that lactose and the Ca^{2+} content of milk varies among species (13), it will be interesting to see if future studies show species differences in the degree of SPCA2 up-regulation during lactation. Since hypocalcaemia (milk fever) during lactation is associated with elevated SPCA1 expression in mammary gland biopsies from cows prior to parturition (16), assessment of SPCA2 in milk fever also appears warranted.

Acknowledgments.

We wish to thank Prof Malcolm Faddy for help with statistical analysis. This work was funded with help from an Australian Postgraduate Award (HF), a University of Queensland GSRTA (HF), a QLD Cancer Fund travel award (HF) and the University of Queensland Foundation, and by grants from the Office of Biological and Environmental Research of the US Department of Energy (DE-AC02-05CH11231 and a Distinguish Fellow Award to MJB) and the National Cancer Institute (CA57621 to MJB and Zena Werb).

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Figure Legends

FIGURE 1: SPCA2 mRNA levels increase more than SPCA1 during murine mammary gland development. SPCA2 and SPCA1 mRNA levels during murine mammary gland development (relative to nulliparous). Bars represent the mean ± SEM (n = 3-4 mice) and are representative of 2 independent real time RT-PCRs. The asterisk (*) denotes a significant difference ($P < 0.05$) compared to nulliparous tissue, using a z-test based on a normal distribution.

FIGURE 2: Immunolocalization of SPCA1 and SPCA2 in the lactating mouse mammary gland. Mammary gland sections isolated from lactating mice and immunostained for either SPCA2 or SPCA1 and SMA. A. Localisation of SPCA2. B. The same field of view as in A. showing SPCA2 (green) and SMA (red) overlaid with the DAPI (blue) stain. White arrows show cells which do not express SPCA2 outside the acini. C. Localisation of SPCA1. D. The same field of view as in C. showing SPCA1 (green) and SMA (red) overlaid with the DAPI (blue) stain. White arrows show cells not part of secretory acini that express SPCA1. Each panel contains a magnified view from the corresponding boxed area. The scale bars represent 10 μm in all panels.

FIGURE 3: Immunolocalization of PMCA1 and PMCA2 in the lactating mouse mammary gland. Mammary gland sections isolated from lactating mice and immunostained for either PMCA1 or PMCA2 and SMA. In all panels the insert represents a magnified subsection of the acini. A. Localisation of PMCA2. B. The same field of view as in A. showing PMCA2 (green) and SMA (red) overlaid with the DAPI (blue) staining pattern. C. Localisation of PMCA1. D. The same field of view as in C. showing

PMCA1 (green) and SMA (red) overlaid with the DAPI (blue) stain. Each panel contains a magnified view from the corresponding boxed area. The scale bars represent 10 μm in all panels.

FIGURE 4: SCp2 cells functionally differentiate, produce milk protein, and express SPCA2. A. SCp2 cells grown in a 2D culture. B. SCp2 cells grown in a 3D culture. C. A representative immunoblot for β-casein in SCp2 cells grown in 2D and treated with normal media (lane 1) or differentiation media (lane 2). D. SPCA2 localization in SCp2 cells grown in a 2D culture. E. SPCA2 localization in SCp2 cells grown in a 3D culture. The scale bars represent 10 μm in all panels.

FIGURE 5: Localisation of PMCA1 in SCp2 cells grown in 3D culture. A. A representative acinus showing staining for DAPI (left), PMCA1 (middle) and α6 integrin (right). B. The merged image of same acinus seen in A.; DAPI (blue), PMCA1 (green), α6 integrin (red). C. A montage of the stacks collected from the acinus described above. The plane shown in A. and B. corresponds to the image with an asterisk (*). D. The corresponding z-x plane through the acinus described above. The scale bars represent 10 μm in all panels.

FIGURE 6: Localisation of PMCA2 in SCp2 cells grown in 3D culture. A. A representative acinus showing staining for DAPI (left), PMCA2 (middle) and α6 integrin (right). B. The merged image of same acinus seen in A.; DAPI (blue), PMCA2 (green), α6 integrin (red). C. A montage of the stacks collected from the acinus described above. The plane shown in A. and B. corresponds to the image with an asterisk (*). D. The corresponding z-x plane through the acinus described above. The scale bars represent 10 μm in all panels.

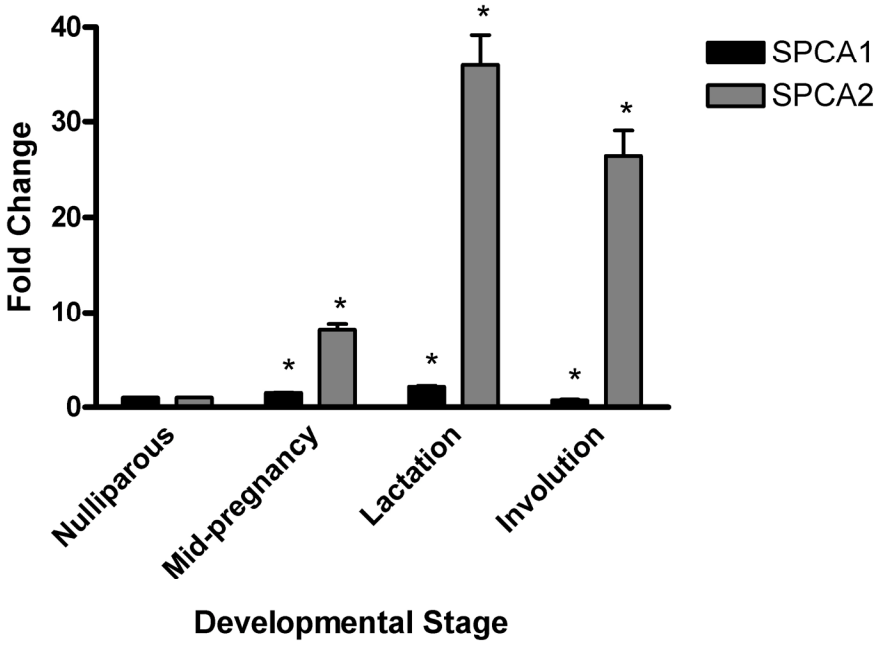
40
30
20
10
0
Fold Change
SPCA1
SPCA2
*
*
*
*
*
*
Nulliparous
Mid-pregnancy
Lactation
Involution
Developmental Stage

Fig 1

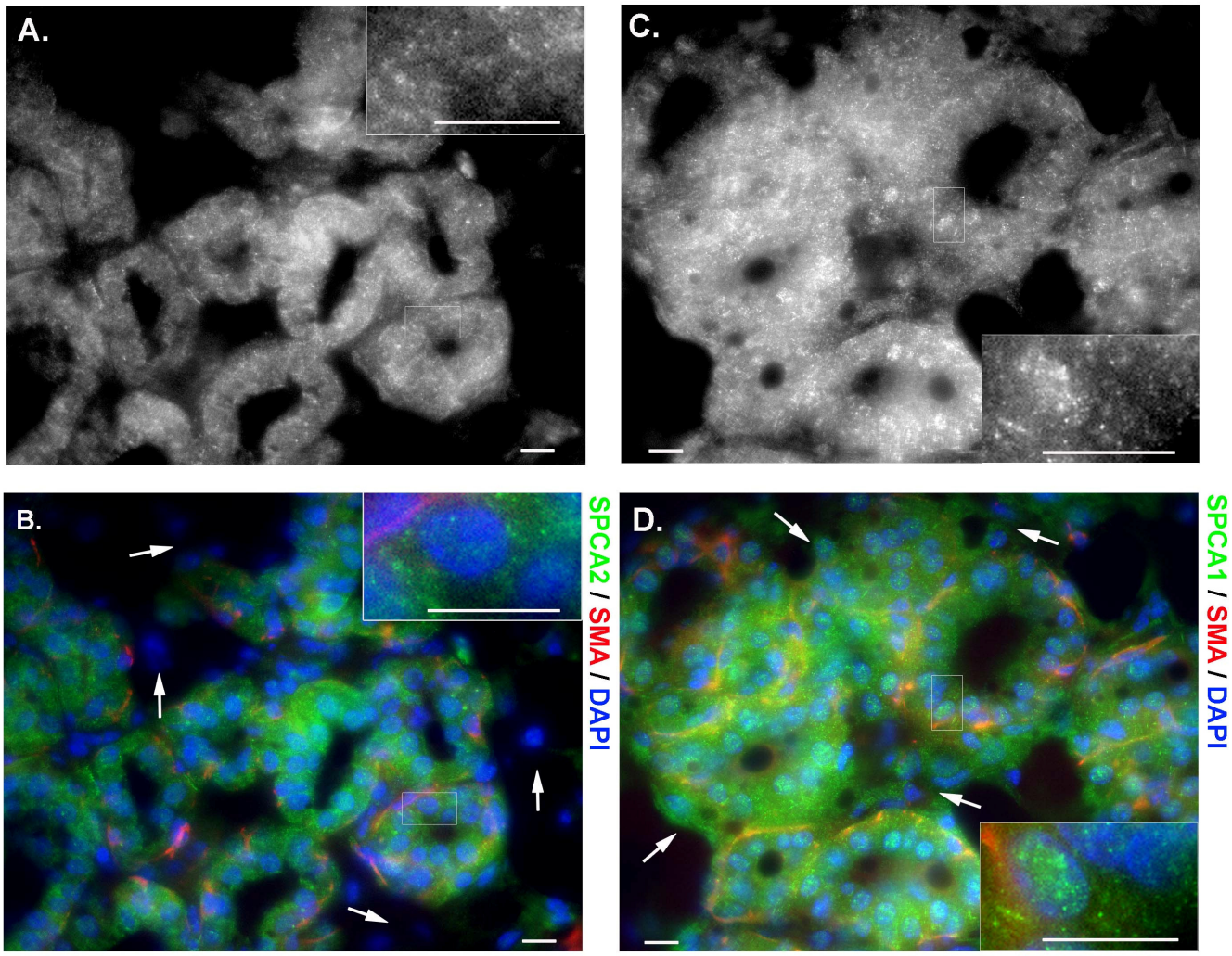

Fig 2

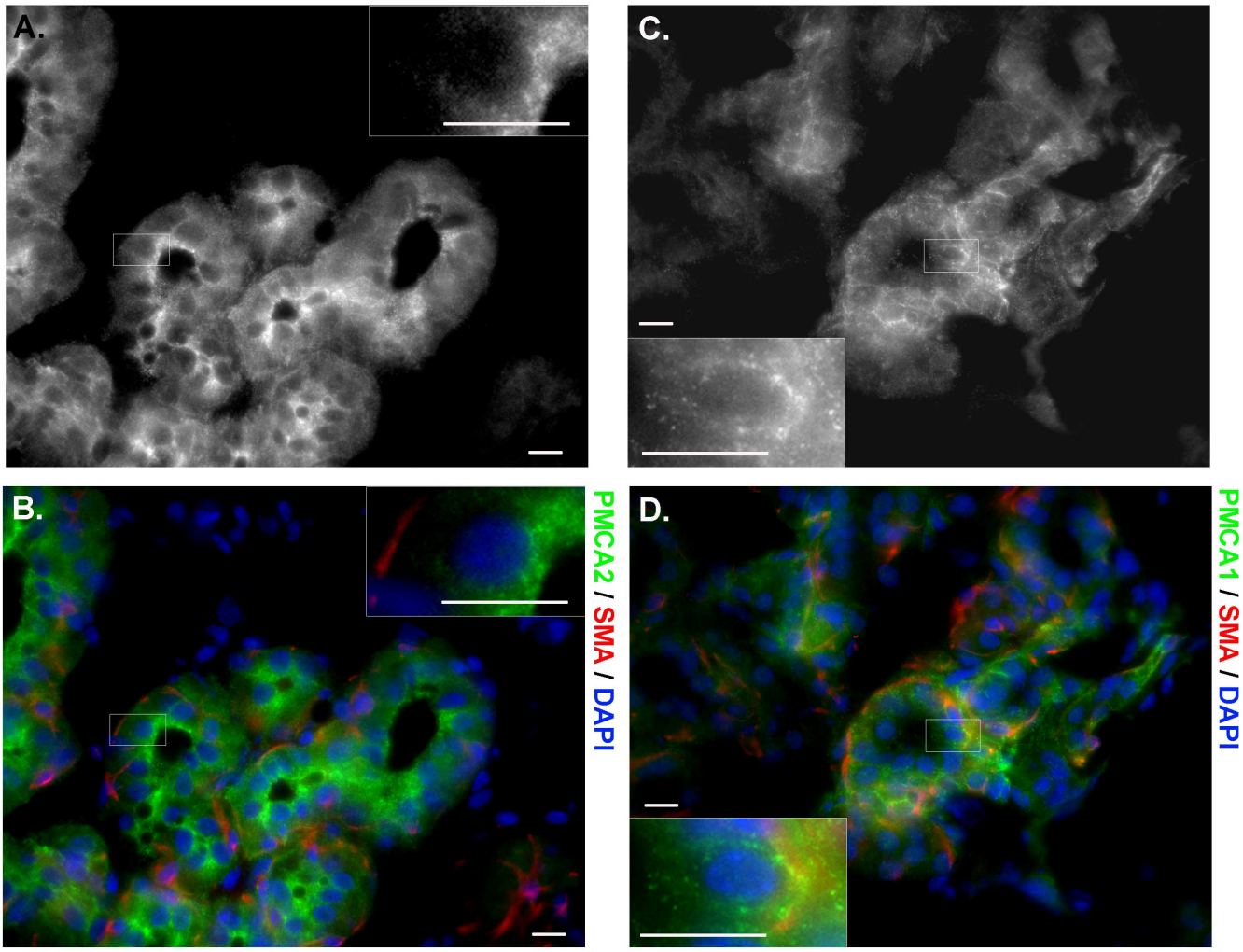

Fig 3

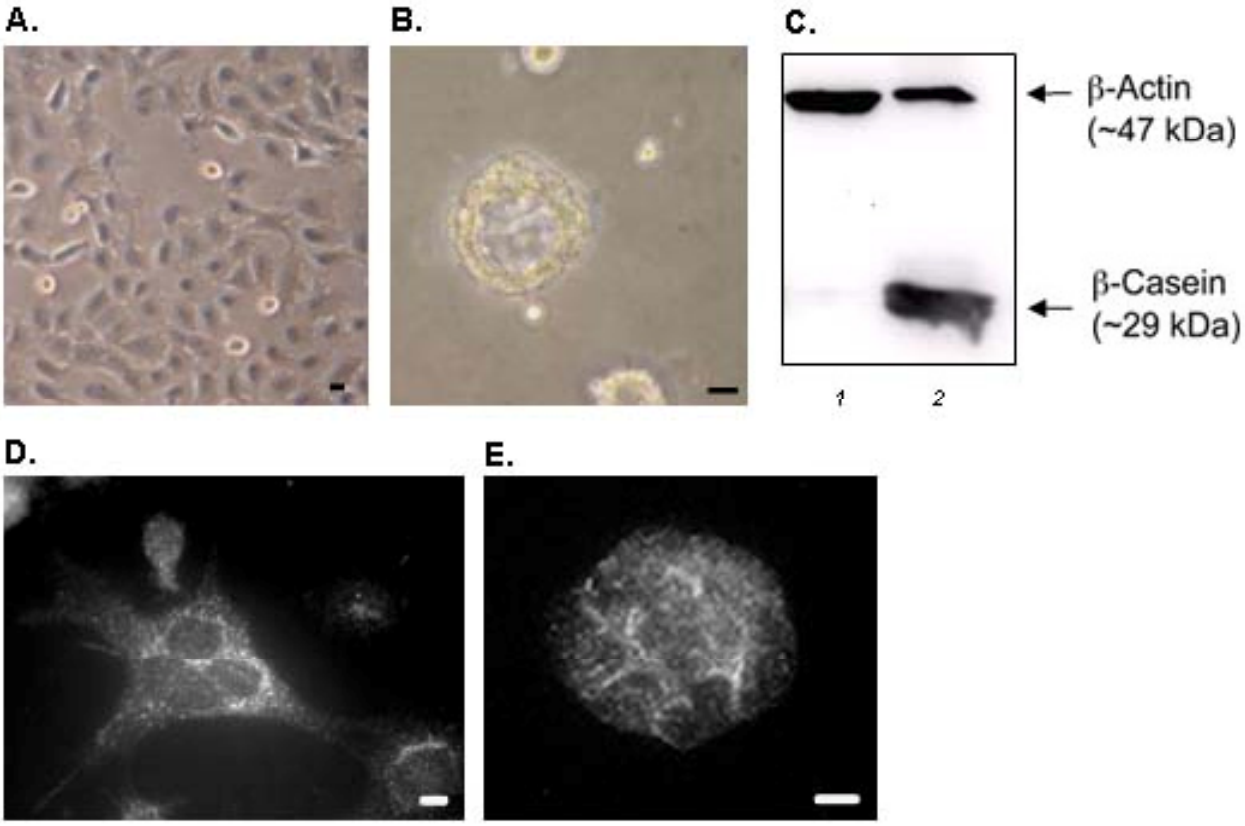

Fig 4

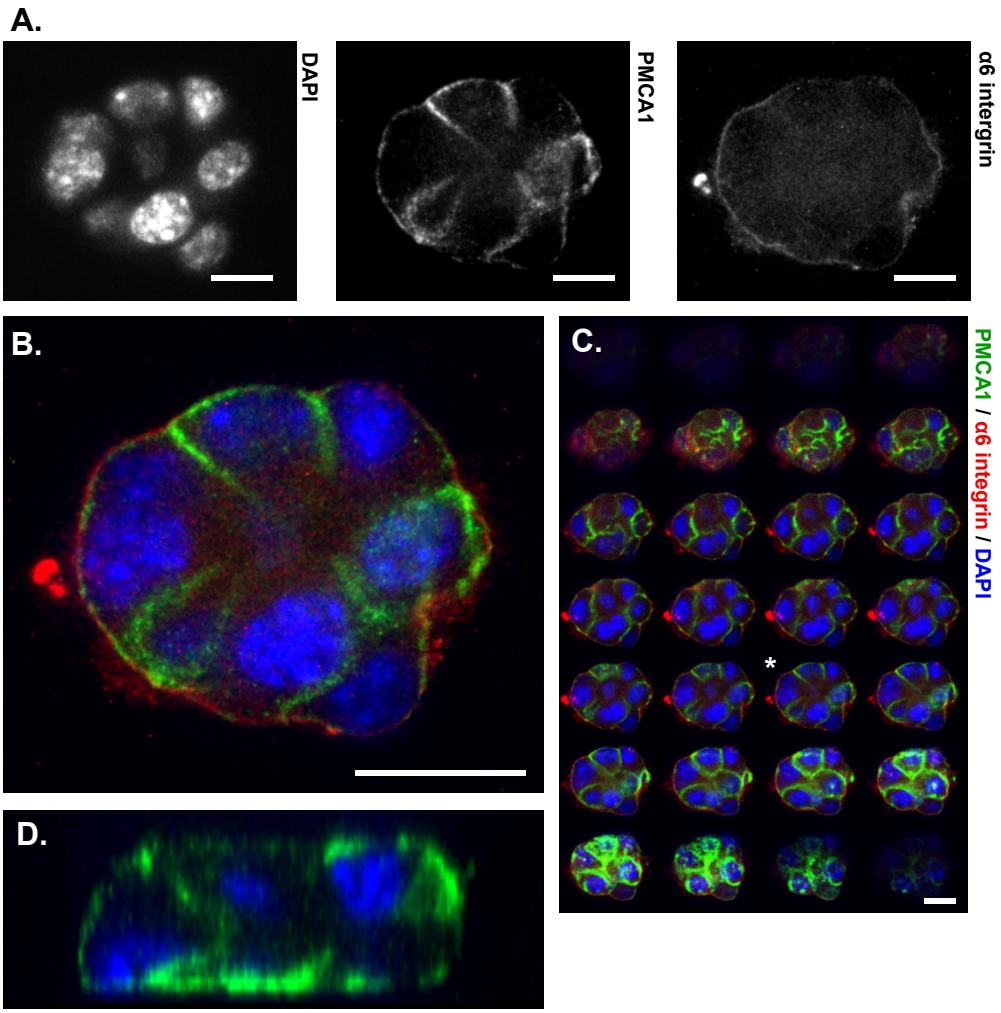

Fig 5

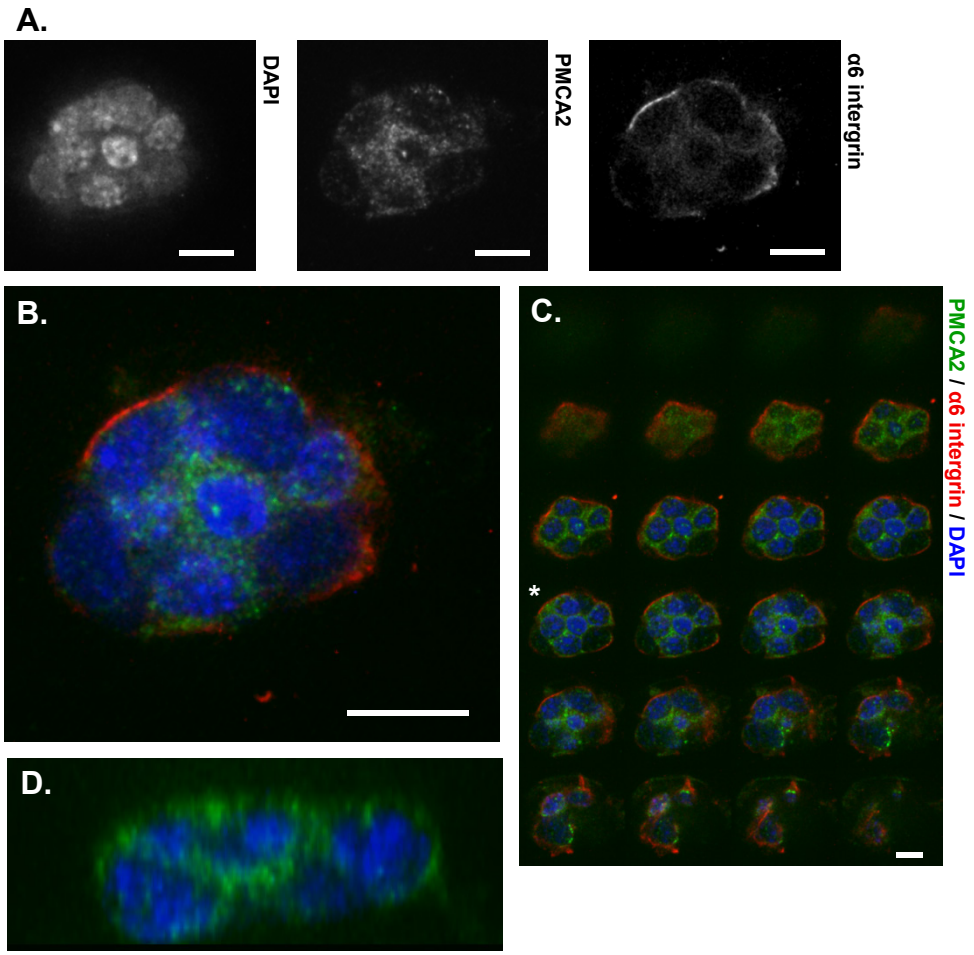

Fig 6