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RESEARCH

In Vitro Model of Milking Pulses and Stretching Cycles: Assessing Mechanical Effects on Bovine Mammary Cells

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Abstract

Culture models of mammary glands can be significantly enhanced by simulating physical forces, including those experienced by the tissue during milking or nursing. In particular, in vitro biophysical studies of bovine mammary cells can provide novel insights into the effects of milking pulses on cellular function, which are currently not well understood. We introduce here a system for modeling uniaxial stretching cycles in culture such as those exerted by vacuum pulses during milking. Bovine mammary epithelial cells (MECs) were cultured under several conditions in stretchable silicone chambers and monitored before and after applying various protocols of stretching pulses. Image processing was conducted for detailed analysis of thousands of MECs in each condition. In the stretchable chambers and upon stretching pulses the bovine MECs maintained high viability, normal proliferation and expression of fat globules, indicating that this system is suitable for tracing functional aspects. We demonstrated usage of the stretching pulses methodology in two possible fields of interest. The first is the fundamental study of the reaction of isolated bovine MECs to stretching cycles, in which we observed cell rounding under various stretching protocols, while maintaining surface adherence and high viability. In the second branch we imitated physiological conditions of mammary epithelium under milking pulses and found that morphological homeostasis was maintained as well as high production of lipid globules with preserved size distribution. Furthermore, our analysis suggests that in collagen-coated chambers and differentiation medium, milking pulses enhance the extracellular release of fat globules. Collectively, the data in this section reflected the ability of mammary epithelium to maintain structural integrity, function effectively, and potentially enhance functionality under milking pulses.

Keywords Mammary Glands · Milking Pulses · Biomechanics · Bovine Mammary Epithelial Cells · Cell Morphology · Image Analysis · Milk Fat Globules

Introduction

During nursing, mechanical forces are applied on mammary glands due to the action of suckling. Pumping instruments are aimed to imitate these forces for efficient milk ejection [1–3]. In industrial bovine milking, machines are used for applying pumping protocols at which various parameters such as the pulsation rate, pulsation ratio and vacuum amplitude are determined [1, 4–7]. Investigating in culture the possible effects of these repeating external forces on the tissue can largely contribute to the field of mammary gland research, and specifically to studies of bovine milking.

In the last decades several studies that examined effects of bovine milking machine parameters were conducted in vivo. The main goal of these studies was to search for protocols that optimize the milking performance. Desired

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outcomes included for example minimization of the machine-on time, speeding up the milk flow, and improving the quality of the milk. It has been shown that increasing the pulsation rate, the pulsation ratio or the vacuum amplitude elevated the milking efficiency [4–7]. However, the values of these parameters should be limited as they can lead to tissue damage and increase the vulnerability of udders to infections [8, 9]. A delicate balance between high productivity, on the one hand, and prevention of tissue damage on the other hand is desired. In spite of this need, to our knowledge there is lack of culture models that can mimic the effects of milking pulses on udder cells.

In vitro models have the clear advantage of providing insight about the behavior of cells and tissues without preforming animal experiments. However, while culture systems provide controlled environments for mechanistic studies, they often fail to replicate the complexity of living tissues, which are governed by a combination of biological, structural, mechanical, and environmental factors within the broader context of organs and whole organisms. The mammary gland is particularly intricate, as its function in the adult is governed by continuous changes in the tissue's physical state and mechanical forces, unlike most somatic adult tissues that typically do not undergo remodeling [10–13].

When mammary glands prepare for lactation, processes such as epithelial cell proliferation, formation of ducts and alveoli, and structural ordering of fibrillar collagens massively occur [10–13]. Such processes are directly related to physical tissue properties like rigidity and architecture, and increase the forces within the tissue. On the contrary, during involution the tissue is depleted and the forces decline [10–13]. That is, the mammary cells within the tissue have the unique ability to remain functional and continuously adapt to the changing mechanical states of the extracellular matrix and microenvironment.

Lactation and milk flow are governed by mechanical stimuli generated by the cyclic suckling of the nursing infant, or by pumping devices that mimic these forces, such as milking machines in dairy cows. Upon periodic suckling, oxytocin stimulates contraction of myoepithelial cells, generating inward tensile stress on luminal epithelial cells and milk flow from the alveoli to the ductal system [10, 11]. During this process, the tight junctions between luminal alveolar cells remain tightly sealed to prevent paracellular milk leakage. In the absence of suckling forces, milk accumulates in the acinus and exerts outward compression on the epithelium. If this condition persists, the prolonged compressive forces weaken the tight junctions, increasing permeability and triggering tissue remodeling, leading to involution [10, 11].

It is very clear that mechanical forces play a major role in governing the function of mammary glands, and that understanding how mammary cells respond to applied forces is important not only as fundamental knowledge but also as a basis for further applied studies. In the context of dairy cows, such insights can have agricultural significance by revealing mechanisms that affect animal welfare, productivity, and health. However, in vivo studies in dairy cows are logistically challenging, ethically sensitive, and resource-intensive. Moreover, schemes for performing biomechanical measurements in live animals in a controlled manner are challenging and complex. Thus, improving culture models to account for relevant mechanical forces could enhance the reliability of in vitro studies and provide valuable input.

In the animal, mammary luminal epithelial cells are the machines that produce milk, and under appropriate conditions these cells can express milk components in culture. The expression of caseins and whey proteins is regulated by a hierarchy of ECM-dependent signals [14]. Large sets of experiments, primarily in rodents, have shown that the first stage of this hierarchy involves a shape change of MECs from flattened to round, which induces lactoferrin expression. At a higher level, laminin-specific activation of ECM elements results in β-casein expression, and at the next level, morphogenesis occurs when MECs are grown within an appropriate mechanical environment such as Matrigel, leading to the formation of alveolar structures and the expression of whey proteins [14]. In bovines, the non-tumorigenic mammary luminal epithelial cell line L1, derived by German and Barash and used in our study, formed structures on Matrigel resembling ducts and alveoli [15]. These cells expressed β-lactoglobulin on Matrigel, with the highest levels observed in the presence of prolactin, insulin, and hydrocortisone [15].

Assessments of milk protein expression are often performed using processes that predominantly involve cell harvesting or treatments such as cell fixation. However, these procedures disrupt the mechanical context of the culture. Therefore, to evaluate lactation-related phenotypes in culture while preserving the mechanical environment as much as possible, it is preferable to use methods applied to live cells without altering the culture conditions, such as microscopy detection of milk fat globules (MFGs). MFGs are of irreplaceable substantial nutritional value, largely due to their unique envelopes, the milk fat globules membranes (MFGMs), whose composition varies based on the nursling needs, and are rich in essential phospholipids and proteins [16–18]. Milk fat globules originate as triacylglycerol droplets formed within the endoplasmic reticulum (ER) membranes. In mammary epithelial cells, lipid droplets can fuse and are transported toward the apical membrane, where they become enveloped by the membrane and are released into

the lumen. Fusion events and droplet enlargement occur mainly near the apical membrane, resulting in a broad range of fat globule sizes [18, 19]. Some evidence showed intracellular clustering of the droplets prior to pinching off using transmission electron microscopy [19, 20]. In raw milk the size distribution of MFGs is wide, with reported diameters ranging from tens of nanometers to several microns, where smaller MFGs are often richer in protein compared to the larger ones [18–22]. Therefore, the expression of MFGs by MECs and their size distribution can provide insights into the lactation-related phenotypes of these cells.

In this work, we introduce an in vitro system for culturing bovine mammary epithelial cells (MECs) and subjecting them to controlled stretching cycles that can mimic the uniaxial stress pulses exerted by milking machines. By integrating cell culture, mechanical manipulation, microscopy techniques, and high-throughput image analysis of thousands of cells, we quantified cellular responses to stretching pulses under diverse conditions. Our study focused on two branches: (i) basic science studies on isolated MECs while minimizing crowding effects and cell–cell interactions, where we found that stretching pulses induced a smoother and more rounded cell morphology; and (ii) mimicking milking pulses under physiologically relevant conditions, where cell shape remained stable, lipid droplet size distribution was maintained across culture conditions, and their extracellular expression is suggested to be enhanced in differentiation medium on collagen-coated substrates.

Materials and Methods

Cell Culture and Maintenance

L1 is a spontaneously immortalized bovine MEC line derived by German and Barash [15]. These cells were routinely cultured in proliferation medium composed of Sartorius (Göttingen, Germany) DMEM/F-12 (Ham) 1:1 with HEPES, supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA), 0.1 U/mL penicillin and 1 µg/mL streptomycin (Biological Industries, Beit HaEmek, Israel), 0.05 µg/mL gentamicin (Gibco, Waltham, MA, USA), 2.50 mM L-alanyl-L-glutamine (Sartorius, Göttingen, Germany), 1 µg/mL hydrocortisone (prepared in ethanol), 5 µg/mL insulin from bovine pancreas (Sigma-Aldrich (St. Louis, MO, USA) prepared in 0.01 M HCl, and 10 ng/mL human EGF (ProSpec-Tany TechnoGene, Rehovot, Israel). L1 Cells were routinely cultured at 37 °C and 5% CO_2 in standard tissue-culture plates and were passaged twice weekly using 0.25% trypsin-EDTA.

For differentiation conditions, L1 cells were first seeded and grown in the proliferation medium for 48 h until full confluency was reached, followed by medium removal and three washes with 1× phosphate-buffered saline (PBS; Diagnovum, Greifswald, Germany). The cells were then incubated in differentiation medium for additional 48 h. The differentiation medium contained DMEM/F-12 supplemented with 0.1 U/mL penicillin and 1 µg/mL streptomycin, 0.005 µg/mL gentamicin, 2.50 mM L-alanyl-L-glutamine, 1 µg/mL hydrocortisone, 5 µg/mL insulin, 4 µg/mL ovine prolactin (PLR Ltd; stock 5 mg/mL in 4 mg/mL sodium bicarbonate), and 20 ng/mL ovine growth hormone (PLR Ltd; stock 100 µg/mL).

HEK293T cells were used as a control of non-mammary cells in (Fig. 4H). These cells were routinely cultured in complete high-glucose DMEM medium. The medium was prepared from Dulbecco's Modified Eagle Medium 1X (DMEM; Gibco, Cat. No. 41965-039) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat. No. 10101-145), 1X penicillin-streptomycin (Gibco, Cat. No. 15140-122), 2 mM L-glutamine solution (200 mM, 100X; Sartorius, Cat. No. 03-020-1B), 1 mM sodium pyruvate solution (100 mM, 100X; Sartorius, Cat. No. 03-042-1B), and HEPES buffer (1 M; Sartorius, Cat. No. 03-025-1B). The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2, and medium was replaced every 3–4 days. Cultures were passaged at approximately 70–90%, detaching with trypsin-EDTA, neutralizing with complete medium, and reseeding into fresh culture dish.

Stretching Pulses

Stretching pulses were applied on L1 cells using the Cyto-Stretcher-1 device in either 50 × 50 mm or 12 × 12 mm silicone chambers (CuriBio Ltd., Seattle, WA, USA), and data was collected and analyzed before and after the pulses, as illustrated in (Fig. 1). In the low confluency experiments approximately 150,000 cells were seeded into the chambers

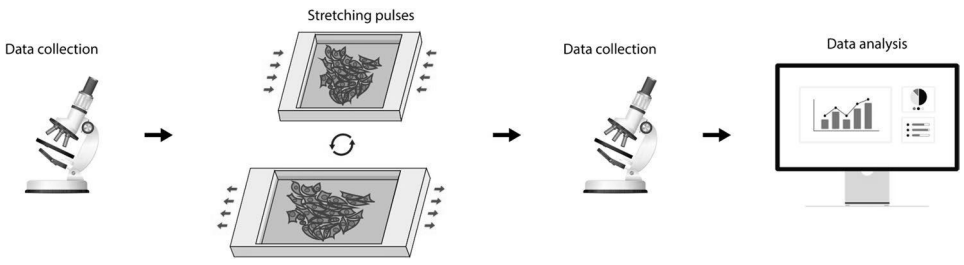

Fig. 1 Stretching pulses methodology. MECs were seeded in stretchable silicon chambers, and uniaxial stretching pulses were applied on them. Morphological and functional information was collected before and after the pulses. Python scripts were used for analyzing data of thousands of cells

and incubated for one day to allow cell adherence to the chambers. In each experiment at least three chambers underwent stretching pulses and were imaged both before and immediately after the pulses, capturing thousands of cells at each condition for the analysis. One chamber was used as control, and imaged at the same time points as the experiment group, to validate that morphological cell changes were not spontaneous and resulted from the stretching pulses (data not shown). The cells were stretched for 100 cycles, either by 10% strain at frequencies 0.22 Hz, 0.44 Hz, or 0.89 Hz, or in a fixed frequency of 0.22 Hz and varying strains of 5%, 10%, and 18%. The experiments were repeated at least three times using cells from different thawing batches and at different passages, and conducted on different days, apart from the 10% strain and 0.44 Hz condition which was performed in duplicate.

In the physiological mimicking conditions, higher density of cells was used, the stretching protocols were chosen in accordance with milking machine parameters. For a better mimic of physiological conditions, the tested parameters included both collagen-coated and non-coated substrates, as well as a comparison between proliferation and differentiation media. For the coating, Nutragen® Type I bovine collagen (Advanced BioMatrix, Carlsbad, CA, USA) was diluted in sterile double-distilled water (DDW) to a final concentration of 75 µg/mL. Six ml of the solution was added to 50 × 50 mm chambers or 0.3 ml to the 12 × 12 mm chambers, allowed to set for 2 h at room temperature, and the excess material was then aspirated prior to cell seeding. The milking mimicking pulses were conducted with 1 Hz frequency and 40% hold intervals at which no force was applied [4–7]. A strain of 6% was assumed, as in vivo data on this parameter are lacking, it cannot be directly derived from milking machine settings and varies with the specific location within the tissue. The overall duration of the pulses was of 8 min, similar to a typical milking procedure, comprising 480 cycles. The experiments were repeated at least three times using cells from different thawing batches and at different passages, and conducted on different days.

Cell Counting and Viability Assessments

To assess cell number and viability, the culture medium was first collected from the plates or chambers and transferred to a 15 mL conical tube, with at least three repetitions in each group. Adherent cells were then detached by incubating with 2 mL of 0.25% trypsin-EDTA (Gibco) for 3.5–5 min. Trypsinization was quenched by adding 6 mL of complete proliferation medium, and the entire suspension was transferred into the same tube containing the original medium. The cell suspension was centrifuged at 700 × g for 10 min, after which the supernatant was discarded and the pellet was resuspended in 1 mL of fresh proliferation medium. For cell counting, a 1:1 dilution of the cell suspension was prepared using Trypan Blue solution (Sigma Aldrich, Lot No. RNBL9248). A 10 µL of the sample was loaded onto the CellDrop device (DeNovix, Wilmington, DE, USA) using the Trypan Blue assay mode. The instrument provided total, live, and dead cell counts, as well as viability expressed as the percentage of live cells and concentrations in cells/mL.

Image Analysis

Low Confluency Image Analysis

Bright field images of L1 cells in low confluency before and after stretching pulses were analyzed for morphological knowledge using a Python script. The images were processed using functions from the *Scikit-image* library [23]. The workflow began with converting the images to grayscale and applying contrast limited adaptive histogram Equalization (CLAHE) to correct for uneven illumination. The images were then processed with a Gaussian blur, brightness and contrast adjustments, and thresholded using the Yen method to generate an initial binary mask. To refine the results, holes in the mask were filled, and small objects and noise were removed through morphological operations. The resulting mask was labeled to define distinct regions, and properties such as area, centroid, major and minor axis lengths, orientation, and perimeter were computed and stored. Size filtration was further preformed with an area between 100 and 2000 pixels for excluding objects other than individual cells. The main morphological features extracted from these regions were the aspect ratio: $AR = \frac{minor\ axis}{major\ axis}$ and the compactness $\chi = \frac{Perimeter^2}{4 \cdot \pi \cdot Area^2}$.

Monolayers Image Analysis

In cases of milking pulses in high confluency, cell segmentation was performed using the Cellpose 3 deep learning model [24] on composite images integrating brightfield, Hoechst (nuclei), and FITC fluorescence (lipid droplets) channels. This multi-channel approach, following brightness adjustments, enabled the precise identification of individual cells within densely packed regions. For lipid globule analysis, segmentation was conducted exclusively on the FITC fluorescence channel, where the contrast between the green fluorescent puncta and the dark background allowed for isolated detection of the globules. Following segmentation, the identified cells underwent the same morphological analysis as described in the low-confluency section.

Confocal Microscopy

For lipid droplet visualization, BODIPY™ 493/503 was added directly to the chamber or the tissue culture plate in a volume ratio of 1:1000. For cell nuclei visualization, two drops of NucBlue® Live reagent (Hoechst 33342) were added per 1 mL of growth media. Confocal fluorescence imaging was performed using a STELLARIS 8 FALCON FLIM system (Leica Microsystems, Wetzlar, Germany), equipped with a white light laser (WLL, 440–790 nm). BODIPY was excited at 491 nm, and fluorescence emission was collected using a HyD S detector within the 499–586 nm range, and Hoechst was detected using a DAPI filter (excitation/emission maxima: 360/460 nm). A HC PL APO C32 63×/1.2 NA water-immersion objective was used for image acquisition. Digital images were acquired at a resolution of 1024 × 1024 pixels with a pixel dwell time of 1.575 µs. Image processing of fat globules was achieved using a Python script with segmentation and area calculation as described in the Low Confluency Image Analysis section.

Live Cell Imaging

Time-lapse imaging was performed using the Olympus (Tokyo, Japan) CM30 incubation monitoring system. L1 cells plated on standard culture plate or on silicone chambers were monitored inside a CO2 incubator for several hours. Bright field images were captures every 8–10 min.

Statistical Analysis

Quantitative data were extracted from image analysis, cell counting, and viability assays and analyzed using Python-based statistical workflows. Morphological parameters, including cell area, aspect ratio (AR) and compactness (χ) were calculated for individual cells. Cell count and viability data were derived from independent chambers. Prior to statistical testing, data distributions were visually inspected using histograms and summary statistics. As most datasets did not meet assumptions of normality, non-parametric statistical tests were applied. Comparisons between two independent groups were performed using the Mann-Whitney U test. When comparing paired measurements acquired from the same chamber before and after stretching, paired non-parametric tests were used. For experiments involving more than two conditions, comparisons were conducted by applying pairwise non-parametric tests between relevant groups. Results are presented as mean ± standard deviation or median with interquartile range (IQR), depending on data distribution. For lipid globule area measurements, variability around median values was estimated using the interquartile range. In addition, confidence intervals for mean globule area were estimated using a non-parametric bootstrapping approach with 5,000 resamples (n_boot = 5000), a 95% confidence interval, and a fixed random seed (seed = 0). Statistical significance was defined as $p < 0.05$. All statistical analyses and data visualizations were performed in Python using custom scripts incorporating NumPy, Pandas, SciPy, and Matplotlib libraries.

Results

Bovine MECs Exhibit High Viability, Proliferative Capacity, and Extracellular Fat Globule Expression Under Stretchable Culture Conditions

A general sketch of the system used in the stretching pulses experiments is presented in Fig. 1, and elaborated in the Methods section. The stretching experiments were conducted in flexible silicone chambers. Figure 2 demonstrates that L1 cells exhibited normal growth patterns on flexible chambers in the presence (Fig. 2A and Movie S1) or absence (Fig. 2B and Movie S2) of collagen coating, similar to standard tissue culture plates (Fig. 2C and Movie S3). The snapshots shown in Fig. 2 are cropped frames of S1-S3 Movies. The cells adhered the chambers, showed normal morphology, were motile and proliferated (examples of cell division events are marked in circles). Moreover, application of stretching pulses on L1 cells in non-coated (Fig. 3A) or collagen-coated (Fig. 3B) silicone chambers did not affect their growth patterns and their high viability. The pulses were of 6% strain, 1 Hz frequency, and 40% hold intervals, and lasted 8 min. Cell proliferation was slower on the chambers as compared with the tissue culture plates (Fig. 3C), however the cells maintained high viability in the three types of plates, indicating that L1 cells can live well on the silicone chambers. The cell count values in Fig. 3B were normalized relative to the seeding numbers on day 0; in all cases the seeding numbers corresponded to a density of about 10,000 cells / cm^2.

In this work, for assessing the cells functionality in terms of their lactation potential, we have examined the production of lipid globules by the bovine MECs. Figure 4A-B demonstrates that L1 cells release lipid-containing globules, which were stained by BODIPY, in proliferation medium. Examples of two pinching off events are presented, captured as snapshots from Movies S4 and S5. In each example, the left images (A) show a merge of bright-field and FITC fluorescence following BODIPY staining in a tissue culture plate, while the right images (B) present a bright-field time-lapse sequence. Droplets visible in the stained images on the left were observed being released from the cells. In the

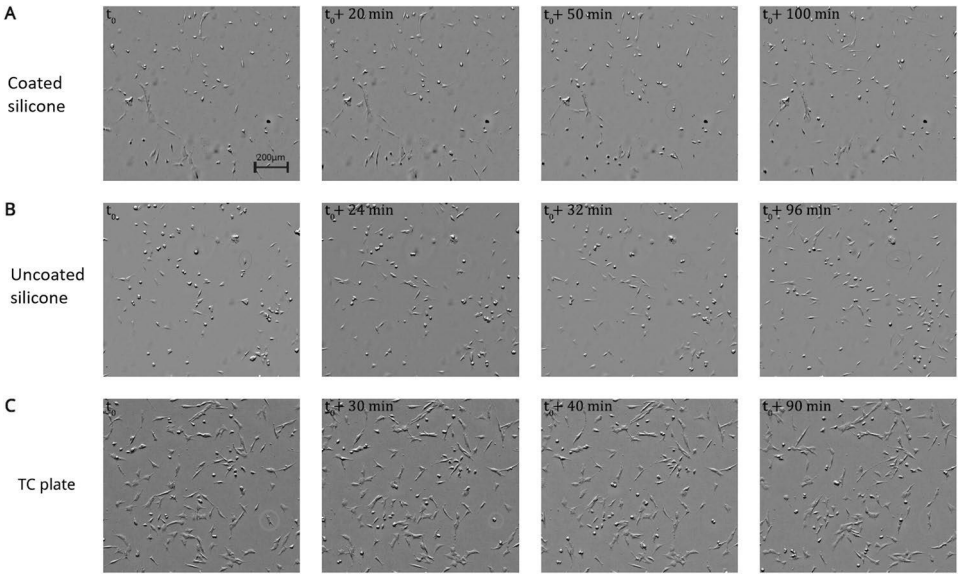

Fig. 2 Bovine MECs cultured in stretchable chambers. L1 cells incubated in collagen coated **(A)** or non-coated **(B)** stretchable silicone chambers are shown, as well as L1 cells in a standard tissue culture (TC) plate **(C)** for comparison. In all cases the cells showed normal morphology, motility and proliferation. Examples of cell division events are marked. The images presented in panels **A**, **B** and **C** are cropped snapshots of **Movies S1**, **S2** and **S3**, respectively. Scale bar: 200 µm

example shown at the bottom of the panel and in Movie S5, lipid droplets clustered near the apical membrane and subsequently released together as a single entity. The association of the droplets near the apical membrane of the MECs, and particularly their observed release from the cells, can indicate that these structures were milk fat globules. This further implies that L1 cells can produce MFGs, which are stained by BODIPY.

Moreover, lipid globules were detected by confocal imaging outside L1 cells across all stretchable culture configurations examined in this study. These included non-coated silicone chambers in proliferation medium (Fig. 4C), collagen-coated silicone chambers in proliferation medium (Fig. 4D), and in differentiation medium in both non-coated (Fig. 4E) and collagen-coated (Fig. 4F) chambers. A confocal image of L1 cells cultured under standard tissue culture conditions is shown as a reference (Fig. 4G). In contrast to L1 cells, lipid globules were not observed in cell-free areas of cultures of the non-mammary epithelial cell line HEK293T, as demonstrated in (Fig. 4H). Under most conditions, epithelial cells that are not subjected to stress are not expected to release lipid droplets, whereas mammary epithelial cells do so as part of their lactation-related function. Therefore, the presence of lipid globules in areas outside L1 cells across the various stretchable chamber conditions suggests that the cells maintain aspects of lactation-related functionality under these culture conditions.

Stretching Pulses in Various Frequencies and Strains Resulted in Cell Rounding in Low Confluencies

In this part of the work, we aimed to isolate the effects of stretching pulses on individual cells while minimizing crowding effects and cell–cell interactions. To examine the morphological effects of stretching pulses on single cells, we applied stretching cycles to L1 cells plated at low density on non-coated chambers. Thousands of cells were imaged before and after stretching pulses in each condition, following geometrical analysis of their shape. Segmentation and size filtration were performed for identification of single cells (Fig. 5A), and geometrical data of the cells was collected. We primarily observed changes in two morphological parameters: the aspect ratio (AR) and the compactness (χ) of the cells, defined in Fig. 5B. The AR measures the ratio between cell width and length, after a cell is fitted into an ellipse. AR = 1 represents a circle and lower values indicate on elongated shapes, with $0 < AR \leq 1$ (Fig. 5C). While the AR can provide good indication of cell elongation, it does not inform on geometrical roughness or protrusions. On the other hand, the compactness parameter, defined as $\chi = \frac{perimeter^2}{4\pi \bullet area}$ can reflect shape protrusions. A higher χ indicates more protrusions or irregularities in cell shape. The compactness values satisfy $\chi \geq 1$ where $\chi = 1$ corresponds to a perfect circle (Fig. 5B, C).

Figure 6A shows the effects of a range of pulse frequencies and strains on cell geometry. In all cases tested, cell rounding was observed post the stretching cycles, where cell detachment has not been observed. The images in Figure 6A demonstrate that the fraction of elongated cells decreased after application of pulses. This observation was also reflected in the analysis showing changes in the aspect ratio (Figure 6B) and the compactness (Figure 6C). Across the various frequencies and strains tested, an increase in AR and a decrease in χ were observed. This combination indicated that the pulses caused cell rounding as well as smoothing, with reduction in elongated or protruded cells.

Figure 7A shows that in all the conditions tested, before and after pulses, the compactness increased with cell area,

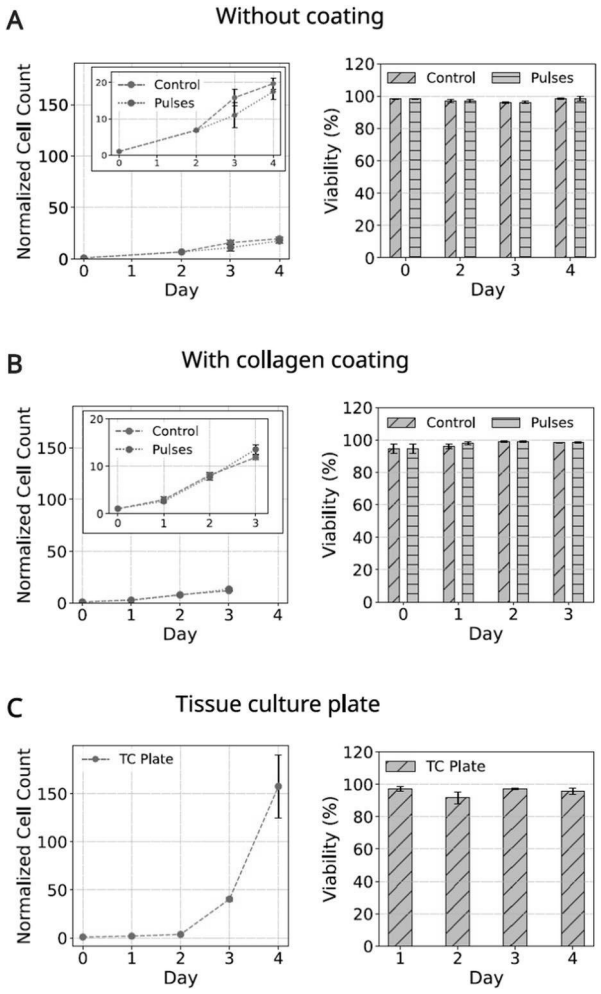

Fig. 3 The proliferation ability and high viability of bovine MECs was maintained upon daily stretching pulses. Cell count and viability are shown for L1 cells in non-coated and collagen-coated stretchable chambers **(A** and **B** respectively), and in standard tissue culture plates for comparison **(C)**. In the stretchable chambers, no differences were observed between relaxed cells and cells that underwent daily stretching pulses, and cell proliferation in these chambers was slower compared to standard tissue culture plates. Cell count values were normalized to seeding numbers; the same scale is used for chambers and tissue culture plates for comparison, with an inset for chambers showing a smaller scale. High viability was maintained under all conditions

emphasizing the correlation between cell protrusions and spreading of the cells. That is, the surface area of rounded cells was smaller than that of protruded cells, as could be predicted. Stretching pulses in all tested frequencies lowered the compactness in a manner that the slope of the linear trends reduced. That is, the stretching pulses increased the compactness of the cells with a pronounced effect on large cells (Figure 7B). Namely, cell protrusions vanished to form a round morphology, a property that in other systems was found to correlate with enhanced lactation functionality [14, 25, 26].

Our results have shown that within the range of strains and frequencies tested here, similar morphological effects were observed. This can imply that cell rounding occurs robustly due to stretching cycles in those conditions. Figure 8A further shows that the cells maintained the round and smooth configurations for at least 90 min after the pulses. The aspect ratio and compactness of L1 cells were analyzed at different time points following 100 cycles of 10% strain at 0.89 Hz, and no apparent variations were observed during this period. Furthermore, after 90 min, cell viability remained close to 100%, similar to control cells that did not undergo stretching, as shown in (Fig. 8B). This indicated that cell rounding was not associated with cell death.

Mimicking Milking Pulses Preserves Cell Morphology and Fat Globule Size While Suggesting Potential Effects on Extracellular Expression

In this part of the work we examined L1 cells in conditions that mimic milking pulses. The pulses were applied on L1 cells in high confluency, similar to the monolayers formed by epithelial cells in physiological conditions. The stretching pulses were conducted using parameters similar to those of milking machines with a frequency of 1 Hz and hold intervals of 40%, for eight minutes4-7, and a strain of 6%, as shown in (Figure 9A). For morphological analysis of the cells in monolayers, a machine learning model was used 24 (Figure 9B). In a daily examination of milking pulses over two consecutive days, where the chambers were coated with Type I bovine collagen for biological context, images were taken before and after stretching and analyzed each day (Figure 9C). The data was integrated into histograms of the aspect ratio (Figure 9D) and the compactness (Figure 9E) and included more than 8000 cells in each condition. While the distributions of compactness narrowed from day 1 to day 2, the morphological features were not affected by the pulses on either day. That is, in physiological mimicking conditions homeostasis in cell morphology has been maintained. This can indicate on cumulative interactions of the cells to keep physiological function under milking pulses. The high confluency can contribute to keeping the shape of the cells during the pulses, and the homeostasis of the epithelium, which is crucial for physiological function. This is unlike the case of diluted cells in non-physiological conditions, where direct cell-cell contacts were sparse, and the cells have changed their morphology upon stretching pulses, as discussed in the previous section.

Next, we examined how biologically relevant conditions influenced the response to the stretching pulses, including collagen coating versus no coating, as well as culture in proliferation medium versus differentiation medium. While the expression of milk proteins often requires prolactin,

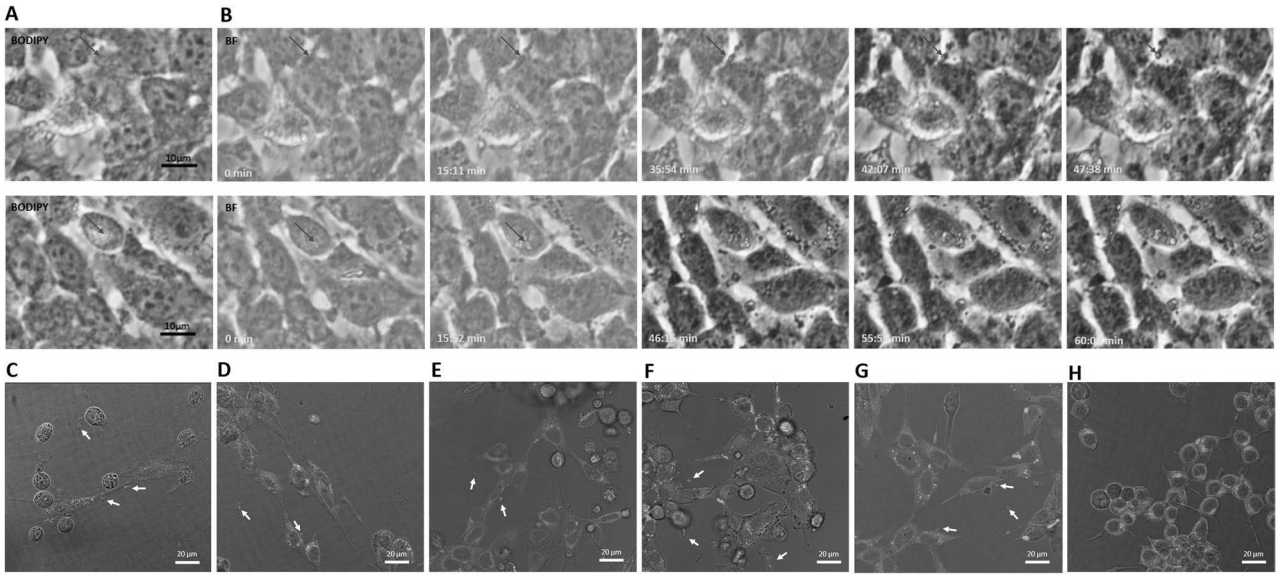

Fig. 4 L1 cells can release fat globules, and extracellular globules are observed under all culture conditions tested here. **A**–**B** Time-lapse sequences showing lipid globule release from L1 cells cultured in proliferation medium on standard tissue culture plates, also shown in Movies S4 and S5. **A** Merged FITC (BODIPY staining) and brightfield images at time zero. **B** Brightfield time-lapse images. In the lower example, several lipid droplets appear to cluster and are released from the cell as a single particle. Lipid globules were observed outside L1 cells under all culture conditions examined in this study, as demonstrated by confocal imaging: (**C**) non-coated silicone chambers in proliferation medium; (**D**) collagen-coated chambers in proliferation medium; (**E**) non-coated chambers in differentiation medium; and (**F**) collagen-coated chambers in differentiation medium. **G** Lipid globules observed outside L1 cells under standard tissue culture conditions in proliferation medium, as a reference condition. **H** In contrast, in the non-mammary epithelial cell line HEK293T, BODIPY-stained lipid globules were not observed in cell-free areas. Scale bars: (**A**–**B**) 10 µm; (**C**–**H**) 20 µm.

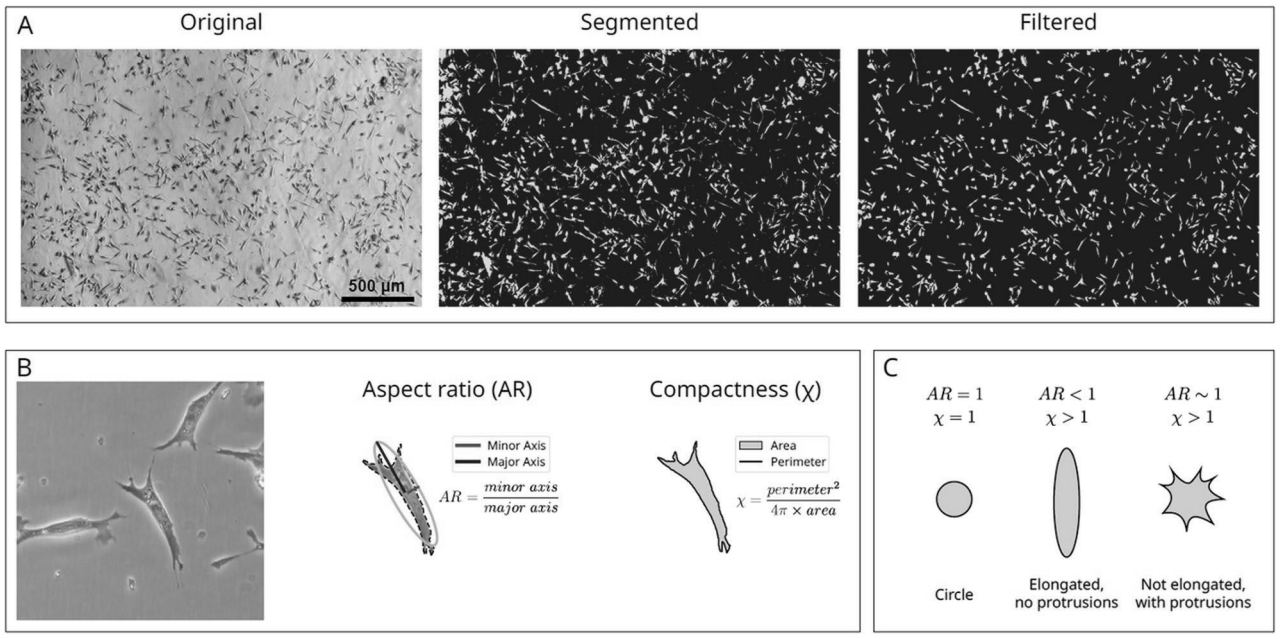

Fig. 5 Image processing and morphological analysis of cells in low confluency. **A** Segmentation and size filtration of the original images were performed for identifying isolated cells. Scale bar is 500 µm. **B** Calculations of the aspect ratio (AR) and the compactness (χ) for a segmented cell. **C** While the aspect ratio can distinguish between circular and elongated cells, it does not provide information about protrusions or smoothness of the cells. The later can be informed by the compactness values, as illustrated in this panel.

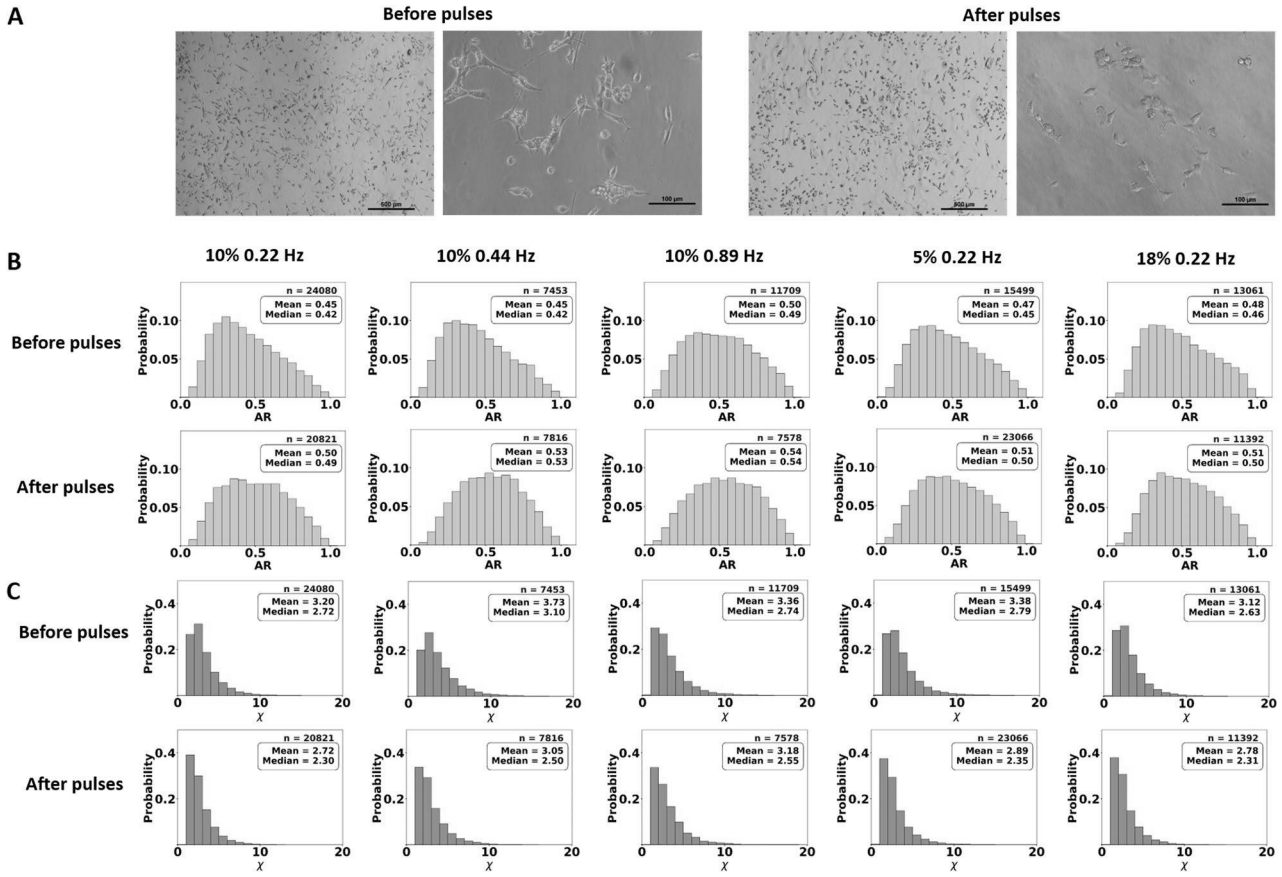

Fig. 6 Stretching pulses in various frequencies and strains resulted in rounding and smoothing of isolated bovine MECs. **A** Brightfield images of MECs before and after stretching pulses of 10% and 0.22 Hz demonstrate the decrease in the fraction of elongated cells post pulses application; Images in two magnifications are presented with scale bars of 500 µm (left) and 500 µm (right). Normalized histograms of the cells aspect ratio (**B**) and compactness (**C**) before and after stretching pulses indicated on rounding and smoothing of the cells, where an increase in AR and a decrease in χ were observed in all conditions tested.

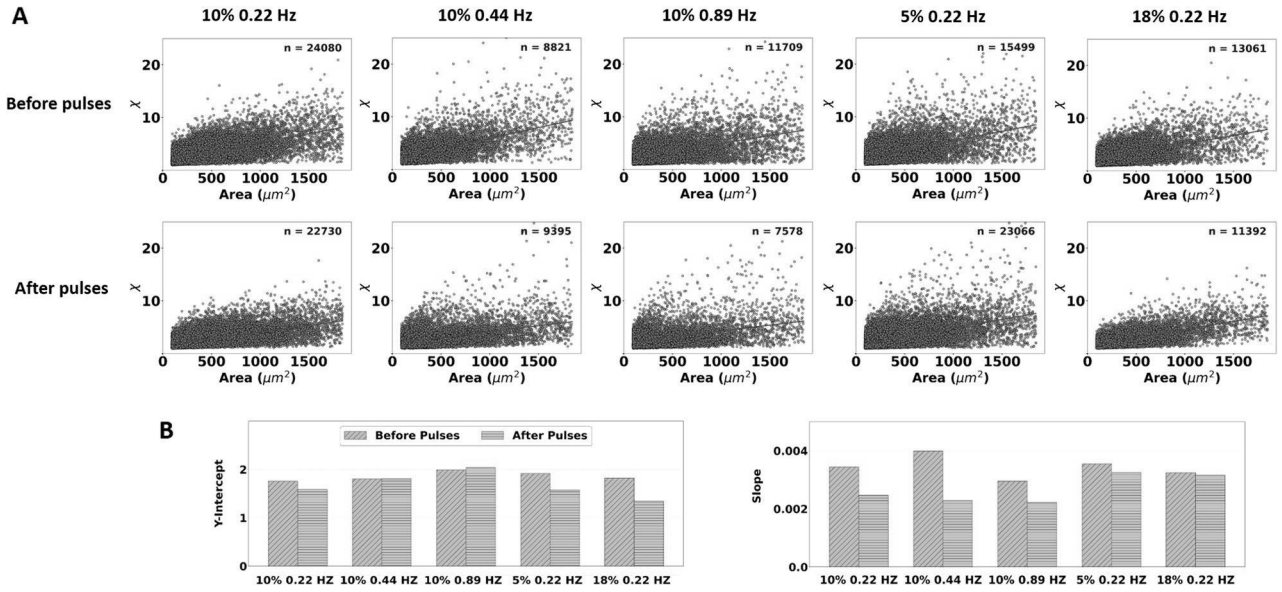

Fig. 7 Compactness (χ) vs. cell area in various frequencies and strains of the stretching pulses. **A** A linear increasing trend was observed in the compactness – area scatter plots both before and after the pulses. **B** Values of the Y-intercepts and the slopes of the linear regression. The decrease in slopes after the pulses, indicated that the rounding effect was more pronounced in the larger cells

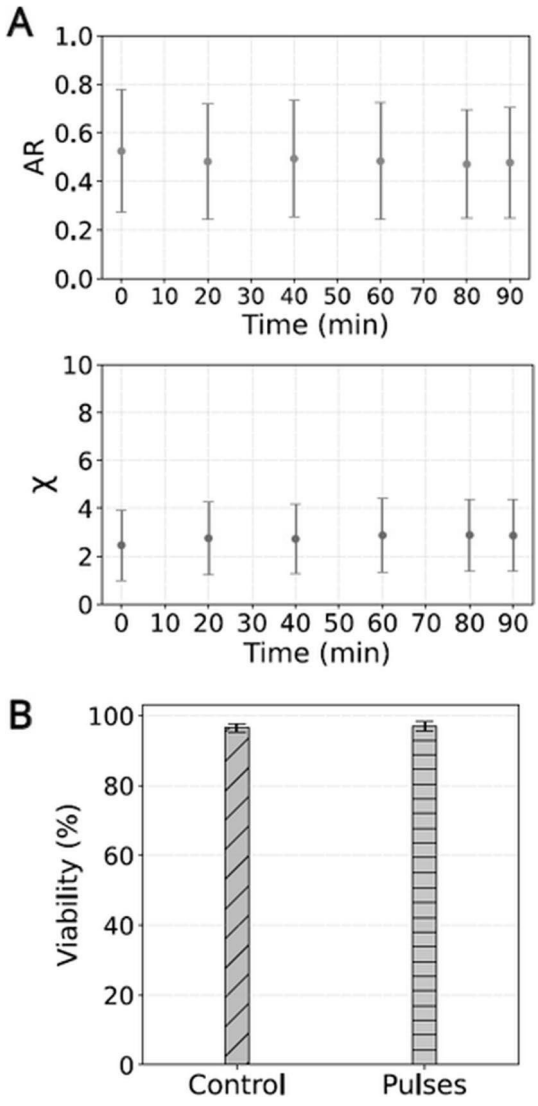

Fig. 8 L1 cells at low confluency maintained morphological characteristics and high viability following stretching pulses. **A** Aspect ratio and compactness were analyzed for 90 minutes after pulses consisting of 100 cycles at 10% strain and 0.89 Hz frequency. **B** Cell viability remained close to 100% in both stretched and control cells 90 minutes after the pulses

the production and release of fat globules can occur in the absence of prolactin, as demonstrated by the release events shown in Figure 4. Figure 10A presents confocal images of L1 cells before and after milking pulses in non-coated (A) and collagen-coated (B) chambers in proliferation medium, as well as in non-coated and collagen-coated chambers in differentiation medium (C and D, respectively). Similar to the results shown in Figure 9, no apparent changes in cell morphology were observed following the pulses under any of the tested conditions. In these images, cell nuclei were stained with Hoechst (blue), and lipid droplets with BODIPY (green). For quantifying properties related to lactation-associated functionality we focused on fat globule expression. Globule segmentation was performed on the FITC fluorescence channel, as demonstrated in (Figure 10E).

Figure 11A presents distributions of globular area projection before and after milking pulses. In the four culture conditions the pulses did not affect the size of the droplets: the area distributions before and after milking pulses were very similar. Fat globule area was relatively similar across all four conditions with average around 0.9 μm². Slightly larger globules were observed in coated chambers under proliferation conditions.

To assess whether the pulses affected the presence of extracellular fat globules, we correlated fat globule detection with regions lacking cells, as determined by the cell segmentation. Figure 11B shows the number of globules per cell-free area. Interestingly, in collagen-coated chambers cultured in differentiation medium, a condition expected to be considered the most biologically relevant, a more than twofold increase in the density of extracellular fat globules was observed following the milking pulses. These results suggest that milking pulses do not alter globule size but may promote their release from cells. Future studies using complementary, higher-throughput methods are needed to validate this finding, as globules located on top of cells may be under-detected in the present analysis.

Discussion

Mechanical environments and forces are tightly correlated with biological function. However, in the context of normal mature mammary glands, and particularly in research on bovine mammary cells, there is a need to deepen our understanding of how external forces, such as those exerted by pumping devices, affect cellular behavior. In the field of milking cows, due to the tight involvement of forces in the milk yield, several applications can be enhanced by insights related to the force response of the cells. These can include increasing the efficiency of milking, the health of the udder, the numbers of gestation cycles etc. As a consequence, the development of culture schemes that mimic physiological conditions and account for mechanical forces may, in the long term, impact agricultural and economic aspects as well as animal welfare.

Specifically, with the increasing adoption of robotic milking systems on dairy farms, there is a growing need to understand the potential effects of milking forces on mammary tissue. Unlike conventional milking, robotic systems allow cows to voluntarily choose when to be milked, and the frequency of milking is not externally controlled, resulting in variable milking patterns that can be higher than in scheduled systems [27]. This raises questions about the cumulative impact of repeated mechanical forces on udder health

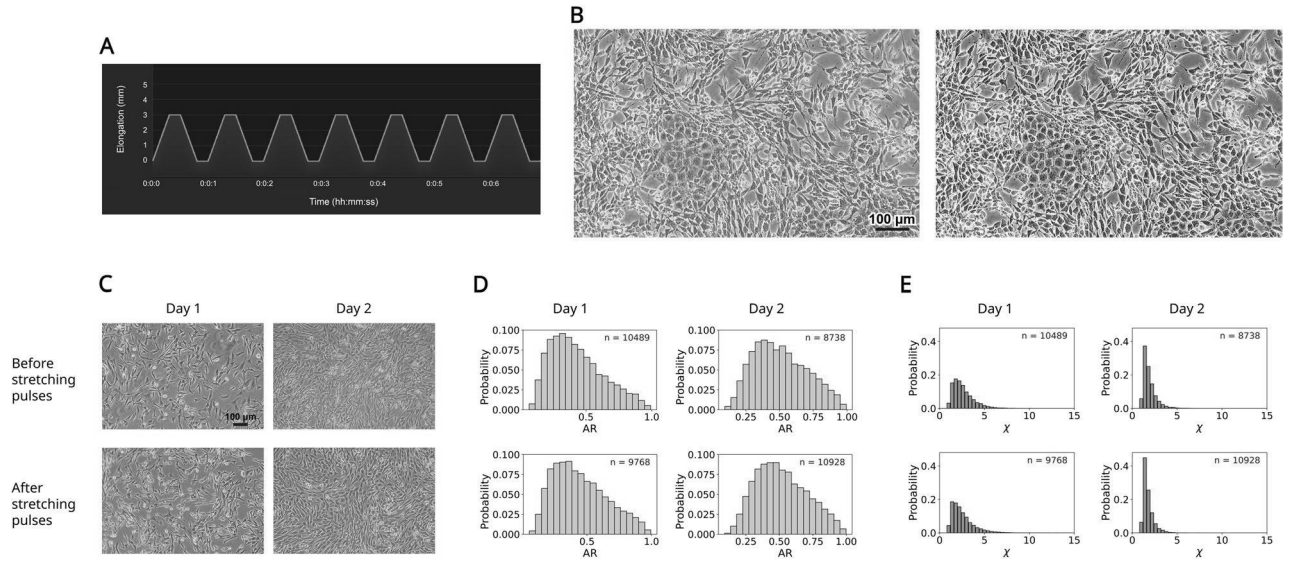

Fig. 9 Morphological homeostasis in a model of daily milking pulses. **A** Milking-like stretching profile in Cytostretcher software. **B** Cell segmentation using Cellpose3 model. Left: Brightfield image (scale bar: 100µm). Right: Segmented image with yellow contours outlining the identified cells. **C** Normalized histograms of the cells aspect ratio and compactness before and after milking pulses for two days showed that the cell morphology was maintained under the stretching cycles

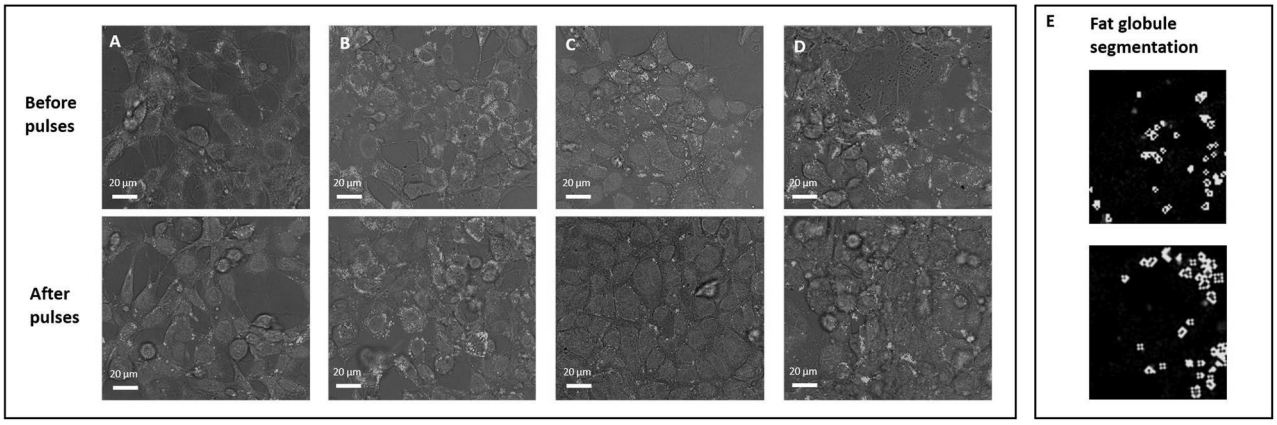

Fig. 10 Confocal images before and after milking pulses under various conditions and fat globule segmentation. L1 cells before and after milking pulses under non-coated (**A**) and collagen-coated (**B**) chambers in proliferation medium, and non-coated (**C**) and collagen-coated (**D**) chambers in differentiation medium. Scale bar = 20µm. (**E**) Fat globule segmentation was performed based on the FITC channel of the images

and tissue function. To address these issues, controlled experimental models are required to replicate and study these forces under physiologically relevant conditions.

We have shown here that bovine mammary epithelial cells can be cultured in stretchable silicone chambers, in the presence or absence of collagen coating. Moreover, the cells well withstood uniaxial stretching cycles, and maintained high viability and normal proliferation (Figs. 2, 3 and 8B, Movies S1-S3). Importantly, the cells also preserved the ability to express extracellular fat globules on the stretchable chambers both in proliferation medium and in differentiation medium, collagen-coated or non-coated chambers (Fig. 4). These finding imply that the in vitro system of stretching pulses allows to keep biological function of the mammary cells. This system can thus be utilized for many research purposes. Here we focused on two branches. One is the study of cells in low density which can provide fundamental knowledge about the reaction of single cells to the pulses, and the other is the mimic of physiological conditions and milking pulses, that is relevant for achieving in culture a model system as close as possible to udder cells under milking.

The reaction of cells to stretching forces has previously been explored in various systems, but to our knowledge not in the context of modeling mammary glands under milking or nursing. Previous studied varied from investigating

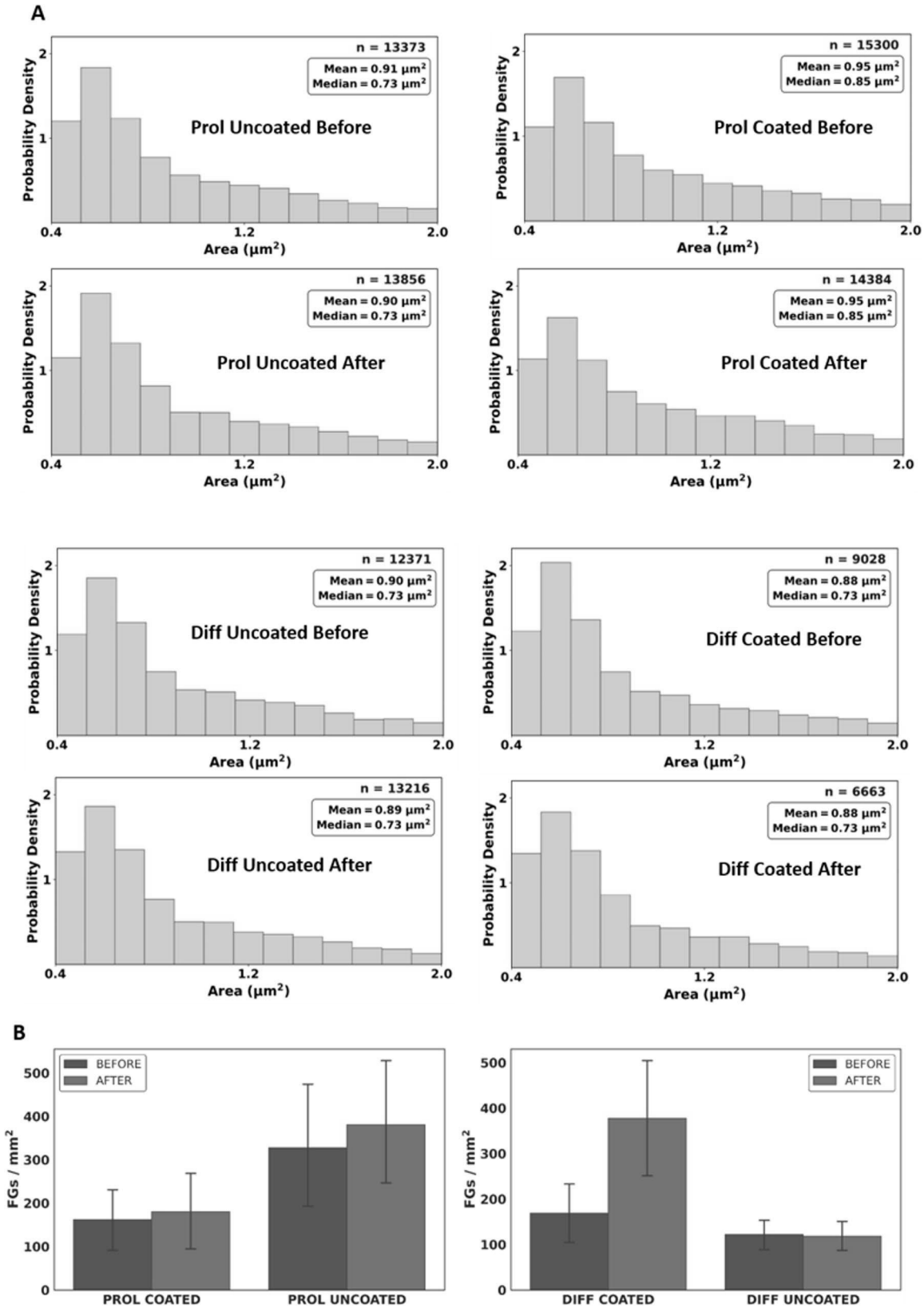

Fig. 11 Effects of milking pulses on fat globule area distribution and extracellular occurrence. **A** Distributions of globule projected area in L1 cells cultured in differentiation or proliferation media, in non-coated or collagen-coated chambers, before and after milking pulses. In all conditions the pulses did not affect globule area. **B** Density of fat globules in cell-free areas. In collagen-coated chambers cultured in differentiation medium, milking pulses may have enhanced extracellular globule expression

processes such as muscle contraction or heart beating, to explorations of fundamental biophysical mechanisms [28–30]. One point of interest in such experiments is isolating the effects of (cyclic or non-cyclic) stretching on individual cells in low confluency where crowding effects are neglected. Then, it can be desirable to have initial conditions that are as uniform as possible in terms of cell morphology. The reason is that uniform initial conditions will allow to exclude effects of the uniaxial force that can be related to arbitral geometry or orientation of cells. When cells display arbitrary orientations and a wide variety of shapes, their mechanical responses may be confounded by these pre-existing geometric differences. We have shown here that stretching cycles applied to bovine MECs in the order of minutes, and the range of 0.22–0.89 Hz frequencies and 5–18% strains increase the uniformity of isolated cells by making them round and smooth, while keeping their surface adherence and their high viability (Figs. 6, 7 and 8). This suggests that short-term stretching cycles may be used as initial steps prior to long-term experiments, for achieving uniform conditions.

Different effects on epithelial cell morphology have been previously reported in experiments of cyclic uniaxial cell stretching. Examples include cell reorientation, elongation, columnarization, and rounding [31–34]. The contradicting trends emphasize the complexity of cells, the differences between cell types, and the various factors that can govern their reaction to cyclic stretching. Moreover, there is sensitivity to experimental parameters of the stretching cycles, such as the duration, strain and frequency of the cycles, the cell density and the substrate characteristics. We found here that the dominant morphological effect on single L1 cells due to 100 stretching cycles of 0.22–0.89 Hz frequencies and 5–18% strains was cell rounding, with no apparent change in cell orientation (data not shown). In rodents, MEC rounding was previously correlated with lactoferrin expression by MECs [14, 25, 26], this may suggest that stretching pulses could be related to increasing the lactation related phenotypes of low confluency MECs in culture; however, a more thorough investigation of this hypothesis is needed.

Our experiments were conducted for relative short durations compared to previous studies, of only a few minutes, to be comparable with milking procedures. In this time scale turnover of cytoskeletal elements and of adhesion complexes can occur [35], that may reduce cell protrusions. We found that the round morphology of L1 cells persisted for at least 90 min post the stretching pulses (Fig. 8), which may suggest the involvement of non-linear and visco-elastic processes whereby cells do not immediately return to their original configuration. It can be interesting to investigate these mechanisms in a future study. Moreover, the cells remained adherent to the substrate and maintained high viability, indicating that their rounding was not related to cell death.

We found that in conditions that imitate epithelial cell monolayer under milking cycles, homeostasis in cell morphology was obtained (Fig. 9). This can be a result of cell-cell and cell-ECM interaction that lead to excluded volume effects which maintain the tissue integrity. In morphogenesis, the formation of cell junctions in a dense epithelial colony plays a crucial role in preserving the structural properties of the cellular network, and in maintaining tissue homeostasis [36]. In the context of mammary alveoli, the tight junction integrity is crucial for preventing milk leakage during lactation [10, 11].

As shown in Fig. 11A, the size distribution of lipid globules remained consistent across various culture conditions and was unaffected by the application of pulses. The range of projected globule areas was broad and asymmetrically distributed, similar to observations in raw milk [18–22]. In standard culture conditions we found here that fat globules were released from L1 cells in proliferation medium, indicating that prolactin was not required for this process (Fig. 4A, Movies S4 and S5).

Our analysis in Fig. 11B showed that the density of fat globules in cell-free area when cultured in collagen-coated chambers in differentiation medium was higher after milking pulses compared to before. These conditions (collagen coating and differentiation medium) are considered to better mimic physiological environments. This observation may suggest that milking pulses enhance the release of lipid droplets from mammary epithelial cells. However, a more detailed and statistically robust analysis is required to confirm this finding. For example, the current analysis does not account for extracellular globules located on top of cells.

In this study, we focused on cycles of lateral uniaxial tension to examine effects of the stretching pulses exerted on udder mammary cells during milking. The forces transmitted through the tissue during external pumping are however more complex and integrate multiple components. These include stresses associated with suction forces, radial compression, shear stress, and hydrodynamic forces, among others. Modeling uniaxial stretching pulses is therefore an initial step, while fully accounting for the complexity of mechanical forces in a controlled manner within tissue models remains a significant challenge and requires substantial further work. One major difficulty is the limited understanding of the magnitude of forces and how they propagate through the tissue in vivo, information that is essential for designing accurate model systems. In the stretching pulses experiments, we gained information about the frequency and hold durations needed to mimic milking, based on data regarding pulsation rate and pulsation ratio. On the other hand, the magnitude of strain within the tissue due to

external pumping varies by location and remains challenging to determine. Additionally, the diversity of cell types and the multicellular structures they form are crucial for mammary function, and these elements are expected to significantly modulate cellular responses to mechanical forces. While our focus here was on a flat epithelial monolayer, a more advanced model could incorporate complex structures of functional units, primarily alveoli and ducts, comprising larger verity of cell types, as well as additional compartments that are strongly influenced by external forces, such as the teats.

Overall, increasing awareness of the importance of mechanical force application in MEC culture studies could significantly enhance the physiological relevance of in vitro mammary gland research, for example in contexts of milking machine simulation. By modeling uniaxial milking pulses exerted on bovine MECs in culture, we found that the cellular response to force depends on cell density and that homeostasis is maintained under physiologically mimicking conditions. Further development of systems is required to enable more investigations into the effects of other relevant forces on MECs. Achieving this integration will require multidisciplinary approaches that combine cell culture models, force application, and advanced analyses, in close collaboration with in vivo research to ensure physiological relevance. Promoting such integration will help narrow the gap between culture models and the complex phenomena observed in native tissue.

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Authors' Contributions G.D.: Experimental design and analysis, pulses experiments, image processing, writing the initial draft of selected sections of the manuscript, and preparation of figures. Y. G.: Experimental design and analysis, pulses experiments, image processing, writing the initial draft of selected sections of the manuscript, and preparation of figures. L.T.: Live-cell imaging, preparation of figures and assistance with cell culture and lab maintenance. N.Z.: Assistance with cell culture and microscopy. E.B.-S.: Assistance with cell culture and lab maintenance. E.B.: Confocal imaging. Y.B.-K.: Conception, experimental design and analysis, manuscript writing, project supervision and fundraising.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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