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1, 25 Dihydroxyvitamin D3 Uptake is Localized at Caveolae and Requires Caveolar Function

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Abstract

Vitamin D3 is an essential vitamin that has been extensively studied due to its potential role as therapeutic for many diseases, including breast cancer. Previous research has indicated that calcitriol, the active form of Vitamin D3 has a negative effect on the metastatic ability of Inflammatory Breast Cancer (IBC) cells however the mechanism is not fully understood. The effect of calcitriol on IBC cells starting from cellular uptake must be investigated in order to understand these therapeutic effects. Calcitriol bound Quantum Dots (CalQDs) are a novel nanoparticle conjugated probe that can be used to directly examine the distribution, uptake, and signaling of calcitriol in live cells. Therefore we used these conjugated probes to directly investigate the uptake of calcitriol into live IBC cells. Interestingly, calcitriol uptake was observed to decrease when caveolae mediated endocytosis is disrupted. A luciferase assay confirmed that caveolae function is necessary; since calcitriol mediated activity decreases when caveolae mediated endocytosis is disrupted in IBC cells. *In vitro* examination of the localization of the probe indicated colocalization between caveolae and CalQDs. Additionally, Vitamin D Receptor (VDR) colocalization was observed with caveolae and calcitriol. This study demonstrates that in IBC cells calcitriol enters cells via caveolae mediated endocytosis and that caveolae are required for calcitriol to be uptaken at the increased rate.

Keywords

Calcitriol; Vitamin D; Quantum Dots; Nanoparticles; Caveolae

INTRODUCTION

Calcitriol is a seco-steroid that is the active form of Vitamin D3 essential in many biological processes. Low serum levels of Vitamin D have been linked to increased risk of developing diseases such as cancer, diabetes, hypertension, multiple sclerosis, and osteoporosis, along with increased mortality in colon cancer and cardiovascular disease.³⁻⁵ Inflammatory Breast Cancer (IBC) is arguably the most aggressive form of epithelial breast cancer.⁸ Even with multimodal treatment < 40% of those treated with combined chemotherapy, surgery and radiation survive past five years.⁸ Due to its potential as a therapeutic in other breast cancers, calcitriol was explored by Hillyer, et al. as a possible treatment against IBC.^{6,7} The data

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obtained demonstrate significant effects against IBC cell invasion and metastasis both *in vitro* and *in vivo*.⁷ However, the mechanism of calcitriol's action on IBC cells must be explored. In the current study we begin exploring the mechanism of calcitriol effect on SUM149 cells, particularly how calcitriol is taken up by the cells.

It is generally thought that as a hydrophobic molecule, calcitriol enters the cell by diffusing through the membrane and that the free form in serum is the active form, which is taken up. This is known as the free hormone hypothesis.¹⁵ However, in circulation, calcitriol is mostly bound to the hydrophilic vitamin D binding protein (DBP).¹⁵ Potential receptors have been identified for DBP on macrophages, neutrophils, and trophoblasts.¹⁵ This indicates that the DBP-bound calcitriol binds to receptors on the membrane of these cells potentially through an endocytotic mechanism.

Caveolae are invaginations in the plasma membrane containing three structural proteins, caveolin-1, -2, and -3, which are involved with endocytosis and cell signaling.^{20,21} They participate in cell signaling by acting as a scaffolding domain in the cytoplasm.²² Caveolin-1 (CAV-1) is essential to the structure and function of caveolae, so down-regulation via siRNA specific for CAV-1 disrupts the formation of caveolae.²³ Similarly, chelation of cholesterol through methyl- β cyclodextrin also disrupts caveolae.²⁴

The vitamin D receptor (VDR) colocalizes with caveolae when CAV-1 is stimulated with calcitriol in skeletal muscle cells.^{25,26} Since Calcitriol may be a ligand for CAV-1 and triggers VDR translocation to the caveolae, it is possible that it could be internalized through receptor mediated endocytosis when localized at the caveolae. Changes in CAV-1 expression are associated with progression of several cancers.^{27–30} In conventional breast cancer, CAV-1 expression decreases with progression to metastasis.^{31,32} Conversely, CAV-1 overexpression is associated with IBC progression.³³

In the current study we explore cellular uptake by caveolae using calcitriol-bound QDs as an imaging probe.¹⁰ Here we demonstrate that disruption of caveolae significantly decreases calcitriol uptake in IBC cells and activation of the Vitamin D response element (VDRE). Additionally, we demonstrate an increased co-localization between VDR, CAV-1, and calcitriol over time using real time imaging. We demonstrate that in IBC cells calcitriol enters cells via caveolae-mediated endocytosis and that caveolae are required for calcitriol to be taken up at the increased rate.

MATERIALS AND METHODS

Cell Culture

SUM149 IBC cell lines are grown as previously described in Ham's F12 media with 5% FBS, 1% L-Glutamine (Fisher Scientific, Pittsburg, PA), 0.01% Hydrocortisone (Sigma-Aldrich, St. Louis, MO), 1% Antibiotic/ Antimycotic (Gemini Bio-products, West Sacramento, CA), 1% ITS Cocktail (Thermo Scientific HyClone, South Logan, UT), and 1% Penicillin/Streptomycin (100 IU/ml Penicillin, 100 μ g/ml Streptomycin, Fisher Scientific, Pittsburg, PA) at 37 °C and 95% air: 5% CO₂.⁷

Conjugation of Calcitriol to Quantum Dots

Qdot® 655 ITK™ carboxyl quantum dots (QDs) were purchased from invitrogen, Carlsbad, CA, 8 μ M. Calcitriol was obtained from (Cayman Chemical Co., Ann Arbor, MI) dissolved in dimethylsulfoxide (DMSO, Fisher Scientific, Pittsburg, PA) to yield a 5 mM stock solution. *N,N'*-Dicyclohexylcarbodiimide (DCC, AnaSpec, Inc., Freemont, CA), was prepared in DMSO fresh as a 17 mM stock solution.

In order to conjugate calcitriol to QDs, a 800 nM solution of QDs in PBS (pH 7.2) and 100 μ M Calcitriol were mixed in a total volume of 50 μ l of DMSO. 340 μ M of DCC in DMSO was added to initiate the coupling process. As a control, ConQDs were synthesized by mixing unbound QDs, Calcitriol and DCC in the absence of Calcitriol. The reaction was carried out at room temperature for 30 min and excess DCC was removed by adding 100 μ l of PBS. Separation of calcitriol linked QDs from unspecific reactants was achieved by size exclusion chromatography using Sephadex G-50 (Pharmacia Fine Chemicals St. Louis, MO) and sterile water.

Transfections

Cells were grown to 95% confluence before replacing medium with serum-free medium. After 18 h serum starvation, the media was changed to normal media and they were transfected or mock transfected. Cells are transfected using Turbofect (Fermentas, Glen Burnie, ML) according to the manufacturers protocol with 1.5 ml media per dish. After 4–6 h of transfection, the media is changed.

Immunoblot Analysis

Proteins were harvested from SUM149 IBC cells using radioimmunoprecipitation assay (RIPA) buffer (1X PBS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) mixed with 5 μ l of protease inhibitor cocktail (Calbiochem, Gibbstown, NJ). Protein concentrations were evaluated using BCA protein assay kit (Pierce Scientific, Rockford, IL). 30 μ g of protein were mixed with Laemmli buffer and denatured for 5 min in boiling water. Denatured proteins were separated by SDS-PAGE on Criterion pre-cast 4–20% Tris-HCl gels (BioRad, Hercules, CA). Proteins were transferred onto the nitrocellulose membrane and were probed with anti-caveolin-1 (BD Transduction Laboratories, San Jose CA) and anti β -actin (Cell signaling, Beverly MA) antibodies. Antibody signal was detected using ECL detection reagent.

Reporter Gene Assay

Cells are co-transfected with 2 μ g of VDRE-luc (Promega, Madison, Wisconsin) and PRL-luc (Promega, Madison, Wisconsin). The siRNACAV-1 treated group is additionally transfected with 10 μ l siRNA against caveolin-1 (siRNACAV-1) (Santa Cruz Biotechnology, Santa Cruz, CA). After 4–6 h of transfection, the media is changed. The M β CD treated group is then treated with 5 nM methyl- β cyclodextrin (M β CD) dissolved in water. After 24 h after transfection, cells were stimulated with 10 nM calcitriol (Cayman Chemical Co. Ann Arbor, MI). After 24 h after stimulation, cells were lysed with 1 $\times$ Passive Lysis Buffer (Biotium, Hayward, CA). The reporter gene assay was performed using the Firefly and Renilla Luciferase Assay Kit (30005–2) (Biotium, Hayward, CA). Luciferase activity was

measured in triplicate using the MicroLumat LB 96P plate reader. Luminosity values were calculated by averaging the three trials with the three triplicates, and normalized to a non-stimulated control.

Live Cell Imaging

Cells were plated onto glass bottom dishes and grown to 95% confluence at 37 °C and 95% air: 5% CO₂ for two days. For most imaging experiments, cells are transfected with 2 µg of caveolin-1βGFP (CAV-1GFP) and the siRNACAV-1 treated group is additionally transfected with 10 µl siRNA against caveolin-1 (siRNACAV-1) (Santa Cruz Biotechnology, Santa Cruz, CA). Cells are mock transfected (NT) with only turbofect as a control. For correlation and colocalization experiments they are cotransfected with VDR-GFP and caveolin-1αRFP (CAV-1RFP). After 4–6 h of transfection, the media is changed. The MβCD group is then treated with 5 nM methyl-β cyclodextrin (MβCD) dissolved in water.

Cells were treated with 1 nM calcitriol bound QDs (CalQDs) 2 h before imaging, prepared as stated previously. As a control, QDs not coated with calcitriol were used (ConQDs). Untreated were used as a blank comparison control. At 20 min before imaging, cells were stained with 20 µM of Hoechst 33342 (Thermo Scientific, West Palm Beach, FL) fluorescent nucleus stain. Images were taken using lasers of 405 nm wavelength for the Hoechst stain, 488 nm for green CAV-1GFP or VDR-GFP, or 560 nm for the 655 nm Infrared QDs and CAV-1RFP. Cells were imaged on the Zeiss LSM 510 Live5 Confocal Microscope and z-stacks of healthy cells were performed. Cells with good membrane and nucleus stains that best represent what was seen for each treatment were selected.

3D Imaging and Measurements

In LSM Image Examiner, selected z-stacks were made into projection images with all image slices put together into one image. Image J was used to create image slices of the selected z-stacks and to adjust the brightness and contrast of the images. A plug-in for Image J called Volume Viewer was used to create vertical image slices showing the side views of selected cells. The maximum and minimum displayed values of the channels were altered to better visualize the cells. The image slices were formatted in Coral PHOTO-PAINT X4 with an adjustment to the tone curve to enhance the brightness and contrast of the channels. The channel displaying QDs was set at a level to better display the difference between ConQD and CalQD treatments, so that ConQDs were set to show a very low intensity. The channel adjustments were kept constant for each image.

The z-stacks were put into Velocity 5.5, where they were made into three dimensional projection images with all image slices put together into one image. Velocity 5.5 was used to measure the mean pixel intensity of the CalQDs in the cytoplasm, which was calculated by subtracting the darkfield from the mean intensities. Cytoplasmic and nuclear autofluorescence was subtracted from the green intensity values with the green 525 nm QDs (Invitrogen, Carlsbad, CA) and cytoplasmic and nuclear cross-talk from the green channel was subtracted from the red channel with the red 655 nm QDs (Invitrogen, Carlsbad, CA). The control treatments with no QDs were set at zero by subtracting their mean intensity from each treatment.

Correlation and Colocalization was measured using the colocalization program in Velocity 5.5. Cells were selected and automatic thresholding was performed using the technique developed by Costes, Daelemans, Cho, Dobbin, Pavlakis, and Lockett.³⁴ Pearson's Correlation and Overlap Coefficient according to Mander's (M1 and M2) coefficients were measured. Background correlation and colocalization was subtracted from the measurements between VDR-GFP and CAV-1RFP.

Statistical Analysis

Single factor ANOVA, followed by Fisher's LSD post-hoc test was used to analyze all data presented. * and ** denotes a significant difference ($p < 0.05$ and $p < 0.01$ respectively). All experiments were repeated three or more times. Three or more cells were analyzed per experiment. All data was normalized to control in each experiment as noted. Outliers were removed using Dixon's *Q* test. Error bars are represented as a standard error of the mean (SEM). The Pearson's Correlation Coefficient and the Overlap Coefficient according to Mander's were used to examine correlation and co-localization between two different molecules. The Pearson's Correlation Coefficient is a measure of the strength of the linear dependence between two variables. The value can range from -1 , which is a perfect negative linear correlation to 1 , which is a perfect positive linear correlation. Absence of a linear relationship results in a value near 0 . An increased Pearson's Correlation Coefficient in fluorescent images shows that there is a higher linear correlation between the pixel intensity distributions between channels, indicating increased correlation and a direct linear relationship between two different types of substances. The Overlap Coefficient according to Mander's measures the proportion of the intensity in each channel that coincides with the presence of some signal, regardless of intensity, in the other channel that is above the threshold value. The Overlap Coefficient according to Mander's ranges from 0 , which is no overlap, to 1 , which is a complete overlap of all the intensity in a channel overlapping with some of the other channel. It can also be displayed as a proportion or as a percent overlap. It is displayed in this paper as a percent overlap from a possible range of 0 to 100% . An increase in Overlap Coefficient according to Mander's demonstrates that there is a higher percentage of a channel's signal that coincides with some of the other channel, indicating that a high percentage of one type of substance colocalizes with the other type of substance.

RESULTS

Expression of CAV-1GFP in SUM149 Cells is Decreased When Treated with siRNA Against Caveolin-1

We began by using two different treatments to disrupt caveolae in SUM149 cells transfected with CAV-1GFP plasmids. Caveolae are specifically disrupted with the transfection of a plasmid encoding a specific siRNA against caveolin-1 (siRNACAV-1), since caveolin-1 (CAV-1) is a crucial part of caveolae and its knockdown by siRNA was shown to disrupt caveolae formation.²³ Caveolae are alternatively disrupted by the addition of methyl β cyclodextrin (M β CD) to the cell media, which causes the chelation and removal of cholesterol from the membrane of cells.²⁴

When siRNACAV-1 was co-transfected with CAV-1GFP, the mean intensity of CAV-1GFP was significantly less in SUM149 cells than the control cells transfected with CAV-1 alone (Fig. 1(A)). This demonstrates that the siRNACAV-1 treatment is successful in decreasing the expression of CAV-1. The $M\beta CD$ treatment is known to disrupt the structure of caveolae, leading to a dispersion of caveolin-1 rather than down-regulation.²⁴ Therefore, when CAV-1GFP transfected SUM149 cells are treated with $M\beta CD$, the treatment did not show a significant difference in mean CAV-1 intensity compared to the control cells transfected with CAV-1 alone (Fig. 1(B)).

Calcitriol Bound QD Uptake in SUM149 Cells is Decreased After Disruption of Caveolae by siRNA or $M\beta CD$

We next examined the role of caveolae in calcitriol uptake. Our unique probe for calcitriol allows us to directly measure the uptake of CalQDs into the cytoplasm and nucleus of SUM149 cells after disruption of caveolae mediated endocytosis.

To successfully observe and measure calcitriol uptake in SUM149 cells, there must be significant visual and quantitative difference in uptake of CalQDs compared to control ConQDs that lack calcitriol. When SUM149 cells transfected with a plasmid CAV-1GFP were treated with CalQDs, there was a significantly higher mean intensity of CalQDs (red channel) in the cytoplasm and nucleus compared to the intensity of cells treated with ConQDs (Fig. 2). The CalQDs are also taken up without CAV-1GFP transfection, since when the cells were only mock transfected, there was a significantly higher mean intensity of CalQDs in the cytoplasm and nucleus as compared to the intensity of cells treated with ConQDs (Fig. 2). These results indicate that CalQDs are significantly taken up more than ConQDs in SUM149 cells mock transfected or transfected with CAV-1GFP. The CalQDs were taken up at a significantly greater intensity in the cytoplasm of CAV-1GFP transfected cells when compared to mock transfected (NT) cells, while no significant difference was seen in the nucleus of these cells (Fig. 2(C)). Overall, these results suggest that CalQDs are specifically taken up by the cells when compared to the behavior of the ConQDs and that CalQDs have a greater uptake into the cytoplasm when CAV-1 is up-regulated via the CAV-1GFP transfection.

We next investigated CalQD uptake into cells when caveolae mediated endocytosis is disrupted by siRNACAV-1 or $M\beta CD$. While siRNA for caveolin-1 leads to a decrease in caveolae formation. $M\beta CD$ changes the structure of caveolae, disrupting its function.^{23,24} When SUM149 cells transfected with CAV-1GFP were co-transfected with siRNA against CAV-1 and treated with CalQDs, there was a significantly lower mean intensity of CalQDs in the cytoplasm and nucleus of the cells when compared to the intensity in the cells of the CAV-1GFP CalQD treated positive control group (Fig. 3). This indicates that treatment with siRNACAV-1 decreases the uptake of CalQDs in the cytoplasm and nucleus. When SUM149 cells transfected with CAV-1GFP were additionally treated with $M\beta CD$ and CalQDs, there was a significantly lower mean intensity of CalQDs in the cytoplasm and nucleus of the cells when compared to the intensity in the cells of the CAV-1GFP CalQD treated positive control group (Fig. 4). This suggests that treatment with $M\beta CD$ also decreases the uptake of

CalQDs in the cytoplasm and nucleus. Therefore, the results suggest that caveolae function is necessary for CalQD uptake into the cytoplasm and nucleus.

With the two treatments against caveolae, there was no significant difference in QD intensity between the treated groups with CalQD treatment and the untreated groups with ConQD treatment (Figs. 3–4). This suggests that the treatments are strong enough to decrease the CalQD uptake levels down to the level that QDs alone would be taken up.

Calcitriol Biological Activity in SUM149 Cells After Treatments Against Caveolae is Decreased

The role of endocytosis in calcitriol uptake was further examined by determining the biological activity of calcitriol after disruption of caveolae mediated endocytosis. If CalQD uptake is prevented without caveolae-mediated endocytosis, then less calcitriol uptake resulting from disrupted endocytosis would decrease the amount of calcitriol in the nucleus interacting with VDRE, resulting in decreased transcription of the luciferase reporter.

When the biological activity of calcitriol was investigated using a reporter gene assay, calcitriol stimulated SUM149 cells co-transfected with VDRE and PRL-luc as a control reporter had significantly less VDRE luciferase reporter activity when additionally treated to disrupt caveolae with co-transfection of a specific siRNA against CAV-1 (Fig. 5). This indicates that functional caveolae are necessary for calcitriol induced biological activity. Also, calcitriol stimulated SUM149 cells co-transfected with VDRE and PRL-luc had significantly less VDRF luciferase reporter activity when additionally treated with M β CD (Fig. 5). However, there was significantly greater VDRE luciferase activity when VDRE and PRL-luc transfected cells were treated with M β CD as compared to transfection with siRNACAV-1 (Fig. 5). This indicates that the M β CD may not be as effective at decreasing activity, but there is still a significant drop in activity when compared to the positive control of untreated calcitriol stimulated VDRE and PRL-luc co-transfected cells. Therefore, the results of the M β CD treatment further indicate that functional caveolae are necessary for calcitriol induced biological activity. The results demonstrate that functional caveolae are essential for normal levels of VDRE induced biological activity, which could indicate that calcitriol uses caveolae-mediated endocytosis to enter the cell.

Increased Colocalization Between VDR, Caveolin-1, and Calcitriol Over Time

Our calQD probe can additionally function to directly examine the localization of calcitriol in live cells, allowing colocalization of calcitriol with other substances to be investigated in real time. Colocalization was examined between calcitriol, VDR, and CAV-1 by using 3 color colocalization with 3 channels using real time confocal microscopy imaging of live SUM149 cells. Caveolae mediated endocytosis of calcitriol in SUM149 cells is only possible if it colocalizes with caveolae *in vitro*. The colocalization was determined by examining the images of SUM149 cells co-transfected with plasmids for the expression of VDRGFP and CAV-IRFP for a 2 hour time lapse after the addition of our CalQD probe. Colocalization was determined using the 2 way comparison equations Pearson's Correlation Coefficient and the Overlap Coefficient according to Mander's between two of the three substances. The three

possible 2 way comparisons were examined for colocalization. The localization of 3 channels in the same position in an image was an indication of 3 way colocalization.

When CalQDs were added to SUM149 cells cotransfected with VDR-GFP and CAV-IRFP, there was an apparent increased correlation and colocalization between all three substances. This is visible with the appearance of white clusters approaching the nucleus over 2 h as seen in the image slices. The white color is formed by the overlay of the red (caveolin-1), blue (calcitriol), and green (VDR) channels. There was additionally an increase in the Pearson's Correlation Coefficient and an increase in the Overlap Coefficient according to Mander's in each of the two way comparisons. This indicates that when calcitriol is added, calcitriol colocalizes with caveolae and VDR. Therefore, this data suggests that calcitriol is being taken up at the caveolae in SUM149 cells.

DISCUSSION

Direct imaging of calcitriol has been problematic due to the lack of successful fluorescent labeling or antibodies specifically recognizing calcitriol. For this reason, calcitriol bound QDs (CalQDs) were developed to image calcitriol *in vitro* and *in vivo*, allowing Calcitriol uptake and localization to be determined in this study.¹⁰ Calcitriol is crucial to human health, so it is important to perform further research to examine its large variety of essential signaling pathways. Since extensive research on calcitriol has demonstrated that a deficiency can lead to many diseases, such as certain cancers and osteoporosis, it is essential to understand how this molecule is taken up by cells.³ The role of calcitriol as a potential therapeutic against breast cancer has been demonstrated in clinical trials and it appears to have an effect against the metastatic ability of Inflammatory Breast Cancer (IBC) cells.^{6,7} Calcitriol's potential as a therapeutic against IBC, a disease that has high mortality even with prompt treatment, needs to be further explored.⁷ In this study, CalQDs were used as a probe to better understand calcitriol entry into IBC cells.

When endocytosis is prevented, decreased uptake of CalQDs indicates that calcitriol does not enter the cell without endocytosis. The free hormone hypothesis states that since calcitriol is hydrophobic, it must enter the cell via diffusion through the plasma membrane.¹⁵ However, calcitriol is hydrophilic in serum when bound to the Vitamin D binding protein, and associates closely with caveolae.^{2,25,26} Caveolae are over-expressed in IBC cells, so if caveolae function is essential to calcitriol uptake, then the ability of calcitriol to treat IBC cells would depend on the caveolae expression.³³ The over-expression may even contribute to calcitriol's ability to impact the metastatic properties of SUM149 cells, but not significantly affect the non-inflammatory MDA-MB-231 cells.⁷

In order to examine this possible method of entry, caveolae mediated endocytosis was disrupted in SUM149 IBC cells. Calcitriol bound QDs were shown to have decreased uptake in the cytoplasm and nucleus of SUM149 cells when caveolae was disrupted. This is confirmed by a decrease in calcitriol mediated biological response in cells with disrupted caveolae. Caveolae appear to be necessary for normal levels of calcitriol uptake into the cell, so it is possible that Calcitriol enters the cell via a caveolae mediated endocytotic pathway.

Calcitriol must colocalize with the structures to be endocytosed. In skeletal muscle cells, VDR colocalizes with caveolae when calcitriol is a ligand for caveolae.^{25,26} This is an indication that calcitriol entry is possible at the caveolae. Without colocalization, it's more likely that the membrane structures are involved in upstream signaling necessary for uptake rather than in the uptake itself. Colocalization between VDR, caveolae, and calcitriol was examined in SUM149 cells. Like the skeletal muscle cells, there was an apparent increase in correlation and colocalization between VDR and caveolae after calcitriol was added. There was also a possible correlation and colocalization between caveolae and calcitriol and between VDR and calcitriol indicating a colocalization between all three after calcitriol enters the cell at the caveolae.

With the indicated colocalization between calcitriol and caveolae and the dependence on CAV-1 expression for uptake it is likely that calcitriol is uptaken via caveolae. However, since caveolae acts as a signaling scaffold, it is also likely that its role as a scaffold is what allows Calcitriol to be taken up, such as the interaction of CAV-1 with VDR in skeletal muscle cells.^{25,26} The free hormone hypothesis in the case of Calcitriol cannot be refuted with this study alone, but further research should be performed on the role of caveolae in the uptake of calcitriol, since this research has demonstrated that the function of caveolae is necessary for calcitriol uptake.¹⁵

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Figure 1.

Mean Intensity of CAV-1GFP in SUM149 cells. Selected z-stack images were taken using lasers of 405 nm wavelength for the Hoechst stain and 488 nm for the CAV-1GFP transfection. The mean pixel intensity of the CAV-1GFP in the cytoplasm of SUM149 cells is displayed. (A) Cells were transfected with CAV-1GFP, CAV-1GFP and siRNA against caveolin-1 (siRNACAV-1). (B) CAV-1GFP transfected cells were also treated with Methyl- β -cyclodextrin (CAV-1 + M β CD). Mock transfected cells (Non-Transfected) were examined as a control. The error bars display the S.E.M. * $p < .05$, ** $p < .01$ as determined by single factor ANOVA followed by Fisher's LSD post hoc test.

Figure 2.

Mean Intensity of Infrared Calcitriol bound QDs in SUM149 cells. Cells were transfected with CAV-1GFP (CAV-1) ((A.1) and (A.2)) or a mock transfection (NT) ((A.3) and (A.4)). CAV-1 and mock transfected groups were left untreated before imaging. The remaining groups were treated with 1 nM Infrared 655 nm Control QDs (ConQDs) ((A.2) and (A.4)) or CalQDs (CalQDs) ((A.1) and (A.3)) for 2 hours before imaging. Selected z-stack images were taken using lasers of 405 nm wavelength for the Hoechst stain, 488 nm for the CAV-1GFP transfection, and 561 nm for the 655 nm QDs. (A) The red channel displays the QDs, the green channel displays the CAV-1GFP, and the blue channel displays the nucleus. On the left are projection images, while displayed on the right are slices taken vertically through the cell, displaying a side-view of the cell. (B) Mean pixel intensity of the CalQDs

in the cytoplasm (B.1) and nucleus (B.2) of SUM149 cells. (C) Mean pixel intensity of the NT and CAV-1 transfected cells treated with CalQDs in the cytoplasm (C.1) and nucleus (C. 2) of SUM149 cells. The error bars display the S.E.M. * $p < .05$, ** $p < .01$ as determined by single factor ANOVA followed by Fisher's LSD post hoc test. Arrows display large clusters of CalQDs.

Figure 3.

Mean Intensity of Infrared Calcitriol bound QDs in SUM149 cells after Downregulation of CAV-1. Cells were transfected with CAV-1GFP (CAV-1), or CAV-1GFP and siRNA against caveolin-1 (CAV-1 + siRNACAV-1). Cells were left untreated or treated with 1 nM Infrared 655 nm Control QDs (ConQDs) or calcitriol bound QDs (CalQDs) for 2 hours before imaging. Selected 2-stack images were taken using lasers of 405 nm wavelength for the Hoechst stain, 488 nm for the CAV-1GFP transfection, and 561 nm for the 655 nm QDs. (A) The red channel displays the QDs, the green channel displays the CAV-1GFP, and the blue channel displays the nucleus. On the top are projection images, while displayed on the bottom are slices taken vertically through the cell, displaying a side-view of the cell. (A.1) Displays the CAV-1 + CalQDs treatment. (A.2) Displays the CAV-1 + siRNACAV-1 + CalQDs treatment. The green CAV-GFP channel was adjusted to be 5X brighter so that the caveolae structure would be visible. (B) The mean pixel intensity of the CalQDs in the cytoplasm (B.1) and nucleus (B.2) of SUM149 cells. The error bars display the S.E.M. * $p < .05$, ** $p < .01$ as determined by single factor ANOVA followed by Fisher's LSD post hoc test. Arrows display large clusters of CalQDs.

Figure 4.

Mean Intensity of Infrared Calcitriol bound QDs in SUM149 cells after Disruption of Caveolae. Cells were transfected with CAV-1GFP (CAV-1), or additionally treated with methyl- β -cyclodextrin (CAV-1 + M β CD). The cells were left untreated or treated with 1 nM Infrared 655 nm Control QDs (ConQDs) or calcitriol bound QDs (CalQDs) for 2 hours before imaging. Selected z-stack images were taken using lasers of 405 nm wavelength for the Hoechst stain, 488 nm for the CAV-1GFP transfection, and 561 nm for the 655 nm QDs. (A) The red channel displays the QDs, the green channel displays the CAV-1GFP, and the blue channel displays the nucleus. On the top are projection images, while displayed on the bottom are slices taken vertically through the cell, displaying a side-view of the cell. (A.1) Displays the CAV-1 + CalQDs treatment. (A.2) Displays the CAV-1 + M β CD+ CalQDs treatment. B. The mean pixel intensity of the CalQDs in the cytoplasm (B.1) and nucleus (B.2) of SUM149 cells. The error bars display the S.E.M. * $p < .05$, ** $p < .01$ as determined by single factor ANOVA followed by Fisher's LSD post hoc test. Arrows display large clusters of CalQDs

Figure 5.

Reporter gene assay showing biological activity of 10 nM Calcitriol in cells treated for disruption of caveolae. Cells were transfected with VDRE and PRL-luc and one group additionally with siRNA against caveolin-1 (siRNACAV-1). One group of VDRE and PRL-luc transfected cells were also treated with Methyl- β -cyclodextrin (M β CD). The Control was transfected with only VDRE and PRL-luc with no additional treatment. The normalized reporter values of luciferase activity are shown with the S.E.M. * $p < .05$, ** $p < .01$ as determined by single factor ANOVA followed by Fisher's LSD post hoc test.