Bile Acid-Mediated Transdifferentiation of Stem Cells From the Deep Esophageal Glands

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Scholarly Report submitted in partial fulfillment of the MD Degree at Harvard Medical School

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Scholarly Report Title: Bile Acid-Mediated Transdifferentiation of Stem Cells from the Deep Esophageal Glands

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Abstract:

Background

      Barrett's esophagus (BE) is a precancerous condition characterized by replacement of the normal stratified squamous epithelium by metaplastic intestine-like columnar mucosa as a result of chronic damage from acid reflux. The pathogenesis of BE is poorly understood and the cells giving rise to the metaplastic tissue remain largely unknown. Previous research from our lab suggested a role for myoepithelial cells within the submucosal gland in the initiation of metaplastic progression in response to deoxycholic acid (DCA), a secondary bile acid and major constituent of bile acid reflux in humans.

Aims

      Given the increasing body of evidence for the role of bile acids in the metaplastic transformation observed in BE, we aim to assess whether DCA alters cells from the deep esophageal gland towards columnar epithelial and glandular phenotypes, similar to that observed in BE.

Methods

      Isolated myoepithelial cells from pig esophageal glands (iPEMC), were exposed to DCA (100uM, pH 7.4) for 72 hours and harvested for flow cytometry and quantitative polymerase chain reaction (qPCR). Flow cytometry was performed on untreated (negative control) and DCA-treated iPEMC to quantify percent of cells expressing antibody markers characteristic of a squamous epithelial phenotype (cytokeratin 5, CK5), glandular epithelial phenotype (cytokeratin 7, CK7), or myoepithelial phenotype (smooth muscle actin, SMA). qPCR was performed to assess for relative expression of various stem cell markers, namely transformation-related protein 63 (TP63), NANOG, and LGR5, in both untreated and DCA-treated iPEMC. Relative gene expression of phenotypic markers, specifically CK5, CK7, and SMA, were also measured.

Results

      Flow cytometry demonstrated an increase in the number of CK5-expressing cells after DCA-treatment from 26% (untreated control cells) to 42% ($p < 0.0001$). The percentage of CK7 expressing cells also increased from 2% to 40% ($p = .001153$). SMA similarly increased from 40% to 73% following DCA exposure ($p < 0.0001$). qPCR results were non-significant, but exhibited increased CK7 and decreased expression of the stem cell markers TP63, NANOG, and LGR5 following DCA exposure, which may reflect a differentiation into columnar epithelial cells and loss of stemness.

Conclusions

The results from this study suggest that DCA, a major component of bile acid reflux and potential mediator of cell injury, also promotes the differentiation of iPEMC *in vitro*, as demonstrated by an increased expression of CK7, suggesting transdifferentiation into a glandular phenotype. Differentiation was further demonstrated by the loss of stem cell marker expression. An increase in expression of CK7 and SMA suggests that myoepithelial cells are able to differentiate into additional cell types, supporting their pluripotency and hypothetical role as adult progenitor cells of the esophageal submucosal gland. The effect of DCA, inducing cellular injury and promoting the initial steps of glandular differentiation in this cell culture model, offers a preliminary potential mechanism for the origin of esophageal metaplasia seen in BE.

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Glossary of Abbreviations

BE – Barrett's esophagus

CI – confidence interval

CK5 – cytokeratin 5, expressed in squamous epithelial and myoepithelial cells

CK7 – cytokeratin 7, expressed in glandular epithelial cells

DCA – deoxycholic acid, a bile acid constituent

dEG – deep esophageal glands

iPEMC – isolated pig esophageal myoepithelial cells

iPS – induced pluripotent stem cell

PBS – phosphate-buffered saline

PPI – proton pump inhibitors

qPCR – quantitative polymerase chain reaction

SMA – smooth muscle actin

Introduction

Barrett's esophagus (BE) is a precancerous condition characterized by columnar metaplasia of the esophageal mucosa as a result of chronic damage from acid reflux [1]. It is estimated that 5.6% of the United States adult population is affected by BE [2]. This metaplastic change is clinically silent but imparts a 30-fold increase in the risk of developing esophageal adenocarcinoma [3]. Esophageal adenocarcinoma has the notoriety of having the fastest rising incidence of any cancers, which is attributed, at least in part, to the increasing prevalence of obesity-associated gastroesophageal reflux disease [4].

The pathogenesis of BE is poorly understood and the cells giving rise to the metaplastic tissue remain unknown [5]. Previous studies have utilized animal models to investigate the origin of BE epithelium, which have resulted in various hypotheses. Studies in rats suggest that metaplasia develops from bone marrow stem cells that implant at sites of damaged epithelium [6]. In contrast, mouse models provide evidence for two possible mechanisms: that progenitor cells from the gastric cardia migrate upwards to replace damaged esophageal mucosa or that stem-like cells within the gastroesophageal junction are activated to expand and replace damaged mucosa [7, 8].

The main limitation with rodent models is that they do not have dEG as humans do and it is still unclear whether these models are truly equivalent to BE tissues. Additionally, rodent esophagi are lined with keratinized stratified squamous epithelium as opposed to non-keratinized stratified squamous

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epithelium observed in humans [9]. In contrast, a 2007 study utilizing human tissue provides strong evidence that esophageal submucosal glands play an important role in the formation of metaplastic columnar tissue in BE. In this study, human biopsy samples of both Barret's and normal esophagus were cultured and exposed to lithocholic acid (a bile acid constituent of acid reflux), and all trans retinoic acid (a powerful inducer of cell differentiation) [10]. Following 32-hours of incubation with both all trans retinoic acid and lithocholic acid, esophageal biopsy samples from healthy volunteers were observed to desquamate. By 48 hours, the entirety of the esophageal stratified squamous epithelium had sloughed off, however the submucosal glands were noted to have fused with the surface of the biopsy samples. At the sites of mucosal gland fusion, cells with columnar morphology were observed. Additionally, the submucosal glands fusing with the biopsy surface were shown to stain positive for cytokeratin 8/18, which are normally expressed in native columnar tissue [11].

This study offers significant evidence that submucosal glands are an integral part of BE pathogenesis. However, the question remains as to which cells within the submucosal glands are forming the columnar epithelium and the role of bile acid in driving these cells to differentiate and divide. To answer this question, we sought to investigate the components of the dEG and identify the adult stem cell responsible for both maintaining the dEG and the potential source of BE epithelium. Previous studies by our lab have identified and isolated a potential candidate: the myoepithelial cells within the dEG [12]. We hypothesized that myoepithelial cells of the dEG are adult progenitor stem cells capable of differentiating into other dEG cell types and potentially the esophageal epithelium. Additionally, we hypothesized that bile acid acts as a myoepithelial cell mutagen. Taken altogether, we sought to investigate whether myoepithelial cells of the dEG are progenitor cells that may be induced by bile acid to differentiate into the columnar epithelium observed in BE.

By identifying the responsible cell and molecular signaling by which BE arises, we hope to identify novel targets for therapies that may allow the prevention of BE, or target more specifically and efficiently the ablation of metaplastic cells once.

Student Role

I was involved in all aspects of the project including experimental design, data collection/analysis, and manuscript writing/editing. Specifically, I assisted with cell culture maintenance and performed RNA extraction, cDNA synthesis, qPCR set-up, gene expression analysis and statistical interpretation of data. At this time, I continue to perform qPCR and western blot analysis to confirm flow cytometry results.

Methods

Experimental Set-up

Pig dEG myoepithelial cells, isolated as described below, were grown in 12-well plates in epithelial cell medium (ScienCell Research Laboratories, Carlsbad, CA), until 60% confluent using growth conditions of 5% CO_2 at 37 ˚C. Subsequently, the cells were trypsinized using 0.05% Trypsin-EDTA and passaged into a 6-well plate. After two days in culture, three wells were designated as controls and three wells were exposed to 100 uM deoxycholic acid (DCA, a secondary bile acid), for 72 hours as previously performed by Goldman et al [13].

dEG and myoepithelial cell isolation

Characteristically, identification of stem cells utilizes a technique known as "lineage tracing". This entails instilling posited stem cells with a label-retaining or genetic marker and observing the cells' progeny [14]. Lineage tracing has been primarily performed with mice cells; however, rodents do not have dEG. Therefore, previous experiments in our lab have employed dEG cells isolated from pig esophagi and selectively expanded in conducive media to produce stem cell-like clones. Lineage tracing is not commonly performed on pig cells, but the homogeneous nature of the clonal cell culture allows for quantitative PCR (qPCR) and possibly proteomics to evaluate changes in expression of target stem cell genes and functional markers.

In order to isolate myoepithelial cells from pig dEG, excised normal adult wild-type pig esophagi were obtained from Dr. Hemant Thatte, Ph.D. after heart transplantation experiments performed at the Veterans Affairs Boston Healthcare System under sterile conditions. Esophagi were placed in ice-cold PBS (containing 25 mM HEPES), with 5X antibiotic-antimycotic solution (Thermo Fisher Scientific, Waltham, MA). The dEG were microdissected from the proximal portion of the esophagi under dissecting scope (Zeiss Stemi 2000 CS, Edmund Optics, Barrington, NJ), and placed in Dulbecco's modified Eagle medium (DMEM), containing Sodium Pyruvate (1 mM), 2% FBS, HEPES (20 mM), penicillin/streptomycin (100 U/ mL). Whole glands were placed in epithelial cell medium and cultured overnight in an incubator at 37 °C. The following day, the glands were washed with PBS, transferred into a 15 mL conical tube, and spun down (200 x g for 10 minutes). The glands were enzymatically disaggregated in epithelial culture medium containing Collagen IV (500 U/ mL), at 37°C in a T25 flask for 3 hours. Once the cells started to detach, they were washed with PBS, transferred into a 15-mL conical tube, and spun down (200 x g for 10 minutes). The supernatant was aspirated and the cells treated with TrypLE (Thermo Fisher Scientific, Waltham, MA), for 10 minutes at 37°C. The cells were spun down (200

x g for 10 minutes), the supernatant aspirated, and epithelial culture medium added. Lastly, the cells were plated on a T-25 flask previously coated with 0.1% gelatin. After three days in culture, the cells were trypsinized and plated in a 3-dimensional matrigel culture (Corning Life Sciences, Tewksbury, MA). On the third day in matrigel culture, cells were observed to form spheres that later hollowed, as if forming dEG. These cells were identified as the potential adult progenitor cells of the esophagus and found to have characteristics of myoepithelial cells based on positive staining for cytokeratin 5 (CK5), as was performed in a separate preliminary study by our group [12]. These myoepithelial cells were plated in 12-well, 2-dimensional cell culture plates and grown as needed for additional experimentation.

RNA extraction, cDNA synthesis, and qPCR

Isolated pig dEG myoepithelial cells were grown in epithelial cell medium until 60% confluent using growth conditions of 5% CO_2 at 37 ˚C. Subsequently, the cells were incubated with 5 mL trypsin solution (0.05% Trypsin-EDTA) for 5 min at 37 ˚C. Dissociated cells were collected and spun down at 600 g for 5 minutes at room temperature. Supernatant was discarded and pelleted cells resuspended in PBS solution. Cells were lysed and RNA extracted using Trizol reagent and the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using the SuperScript III Reverse Transcriptase system (Invitrogen, Carlsbad, CA). Thermo Scientific NanoDrop was used to determine yield of cDNA synthesis (Thermo Fisher Scientific, Waltham, MA). qPCR was performed on QuantStudio 6 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA), which allows for confirmation of melt curves and real-time analysis of reactions. Primers were purchased from ThermoFisher Scientific and designed to target genes known to be expressed in myoepithelial progenitor cells of the mammary glands [14-16]. Table 1 summarizes the genes targeted and their primer sequences. Additional primers were designed to target genes uniquely expressed in non-myoepithelial dEG cells, esophageal mucosal cells, and columnar epithelial cells [12]. qPCR was performed using the aforementioned primers and relative gene expression calculated using GAPDH as the reference genes. Table 2 summarizes the genes targeted, their associated protein, and in which cells they are expressed.

Flow Cytometry

Flow cytometry was performed on both DCA exposed and unexposed (control) iPEMC. Primary antibodies targeted against CK5, CK7 and SMA (Abcam, Cambridge, United Kingdom), were used to evaluate the percent of cells expressing the proteins of interest. The secondary antibody used was

fluorescein isothiocyanate goat anti-rabbit (BD Biosciences, Franklin Lakes, NJ). The flow cytometer was acquired from BD Biosciences and results were analyzed using FlowJo (FlowJo LLC, Ashland, OR).

Pig dEG myoepithelial cells were trypsinized using 0.05% Trypsin-EDTA by exposing cells for 5 minutes at 37 ˚C. The trypsinized cells were subsequently collated and spun down at 600 g for 8 minutes at room temperature. The supernatant was discarded and cells resuspended in 5 mL of ice-cold acetone and allowed to cool at -20 ˚C for 10 minutes. PBS with 0.1% triton was added to the resuspended cells and left at room temperature for 15 minutes. Cells were then filtered through a cell strainer to remove debris.

The filtered cells were incubated in primary antibody (either anti-CK5, anti-CK7, or anti-SMA) for 1 hour and subsequently washed with flow buffer (Dulbecco's phosphate-buffered saline containing 2% fetal bovine serum and 0.15% sodium azide), twice, for 10 minutes per wash. Secondary antibody was added at a 1:500 ratio and incubated overnight at 4 ˚C on a rocker. Cells were washed once using PBS with tween and resuspended in 500 uL of flow buffer.

Calculation of Relative Gene Expression from qPCR Data

Relative gene expression was calculated from qPCR data using the comparative C_t method [17]. GAPDH was used as the reference gene.

Statistical Analysis

For the flow cytometry results, the primary outcome was a statistically significant increase in the percent of cells expressing epithelial and columnar phenotype markers and a decrease in the percent of cells expressing myoepithelial cell markers following iPEMC exposure to DCA. A chi-squared test was used to calculate the significance of flow cytometry data.

For the PCR results, there were two primary outcomes. The first was a statistically significant decrease in the expression of genes associated with myoepithelial stem cells following iPEMC exposure to DCA (p-value <0.05), which would indicate induction of differentiation and loss of stemness. Specifically, TP63, NANOG, LGR5, and SOX9 [14-16]. The second outcome was a statistically significant increase in the expression of genes associated with squamous epithelial (CK5) or glandular epithelial (CK7) phenotypes and a decrease in the gene associated with a myoepithelial phenotype (SMA). A change in expression of genes uniquely associated with columnar or epithelial cell phenotypes would suggest that bile acid-induced injury initiates the process of dEG myoepithelial transdifferentiation into other cell types within the dEG or into BE epithelium.

Sample size and power were calculated using preliminary gene expression data as follows: SMA gene expression from six clonal myoepithelial cell populations exposed to ATRA yielded an average Ct value of 34.76 cycles. SMA gene expression of six clonal myoepithelial cell populations with no exposure (negative control), yielded an average Ct value of 32.52 cycles. Standard deviation of control samples was 1.59 cycles. In order to achieve a power of 0.8 and a significance value of 0.05, 8 samples were needed. Significance of experimental results were calculated using a two-tailed Student's t-test.

Results

Flow Cytometry

Flow cytometry performed on control iPEMC demonstrated 26% of cells expressing CK5 markers, 2.1% of cells expressing CK7 markers, and 40% of cells expressing SMA markers, as demonstrated in figure 1.1-1.3. Following 72-hour exposure to 100 uM DCA, iPEMC demonstrated an increase to 42% CK5 expression, 21% CK7 expression, and 72% SMA expression, as demonstrated in figure 2.1-2.3. P-value for the increase in CK5 expression following DCA exposure of iPEMC was < 0.0001 (X^2 of 1089.8021). For CK7, the p-value was .001153 (X^2 of 276.7229) and for SMA, the p-value was <0.0001 (X^2 of 2702.0496).

qPCR results comparing the expression of target genes in iPEMC following DCA exposure to control iPEMC (fig 3), demonstrated a 0.167-fold increase in CK7 expression 95% CI [-0.25, 0.58], 0.054-fold decrease in SMA expression 95% CI [-0.90, 0.79], 0.873-fold decrease in TP63 expression 95% CI [-2.34, 0.59], 1.426-fold decrease in NANOG expression 95% CI [-2.60, -0.25], and a 0.595-fold decrease in LGR5 95% CI [-2.53, 1.34]. CK5 and SOX9 results remain pending.

Discussion, Limitations, Conclusions, and Suggestions for Future Work

Discussion

After exposure of iPEMC cells to DCA, the qPCR results reflected our prediction, albeit non-significantly, that myoepithelial cells are induced by bile acid to undergo a phenotypic shift towards glandular epithelium, similar to BE. This is demonstrated by an increase in CK7 expression, which reflects an increase in the glandular epithelium phenotype, and a decrease in SMA expression, which reflects a loss of the myoepithelial phenotype. Additionally, a decrease in the expression of stem cell markers TP63, NANOG, and LGR5 reflect a loss of stemness, which would be expected in a progenitor cell undergoing differentiation. It is important to note that the qPCR results are mostly non-significant, with the 95% confidence intervals including both positive and negative values for CK7, SMA, TP63, and LGR5

gene expression. Only NANOG demonstrated a significant decrease in gene expression, with the confidence interval remaining entirely within negative ranges.

The most probable reason for non-significant confidence intervals is not obtaining enough cell samples for qPCR. The difference in gene expressions level may be minute, and require a greater number of samples in order to be detected. A 0.167-fold increase in CK7 and 0.054-fold decrease in SMA gene expression is relatively miniscule for a progenitor cell undergoing differentiation, however the flow cytometry results demonstrate that significant changes in protein expression levels are occurring. Increasing the power of the study would elucidate whether the change in gene expression is actually minor. An alternative explanation would be that the change in gene expression is minor when iPEMC are exposed only to DCA. Cells may require exposure to additional bile acid constituents in order to stimulate a synergistic effect on gene expression levels. Future experimentation should consider utilizing whole bile acid, rather than a single bile acid component. ATRA, a powerful inducer of bile acid signaling, could be given to cells together with DCA in order to observe if a greater degree of gene expression change is noted.

In contrast to the qPCR data, our flow cytometry results were significant. Similar to qPCR results, the expression of CK7 increased. However, CK5 also increased (not measured by qPCR), as did SMA. An increase in the percent of cells expressing CK5 and SMA markers would indicate that DCA exposure increased the myoepithelial phenotype of iPEMC while similarly increasing the glandular epithelial phenotype, as demonstrated by an increase in the percent of cells expressing CK7 markers. A possible reason for the increase in both squamous epithelial and glandular phenotypes is that myoepithelial cells have bipotentiality. As demonstrated in our previous study, myoepithelial cells can differentiate into components of the dEG, which include epithelial cells and glandular cells [12]. DCA, a known mediator of cell damage, is likely inducing myoepithelial cells to differentiate into dEG components as demonstrated by increased CK5 and SMA expression, in addition to the columnar epithelium expected in BE, as demonstrated by increased CK7 expression and supported by observations made in the publication by Chang et al. The reason for why the qPCR and flow cytometry data do not match remains unclear, however the qPCR data is non-significant. Repeating qPCR or increasing the number of samples may yield final results that reflect the flow cytometry data. At this time, we are planning to confirm the flow cytometry results through western blotting and to complete additional samples of qPCR, including SOX9 and CK5 gene expression levels.

A number of limitations are present with our current methods, necessitating additional experimentation. The first limitation is the inability to perform lineage tracing. Currently, lineage tracing remains the gold-standard for identification of adult progenitor stem cells. It entails instilling posited stem cells with a label-retaining marker and observing the cells' progeny [14]. Although powerful, it is traditionally completed in rodent models and resource intensive. As described earlier in the methods section, rodents do not have dEG. Therefore, previous experiments in our lab have employed dEG cells isolated from pig esophagi and selectively expanded to produce stem cell clones. Given the homogeneous nature of the clonal cell culture, qPCR demonstrating the increased expression of genes uniquely associated with epithelial and glandular phenotypes should suffice as strong evidence for the adult stem cell activity of myoepithelial cells. However, it remains uncertain if the iPEMC used for this study are of clonal origin, or rather a selective expansion of a subset of cells. This issue could be potentially addressed by isolation, growth, and treatment of single cells by limited dilation onto multi-well plates.

Moreover, this cell culture model is limited by missing the complex cell-cell interplay of neighboring stromal, immune, vascular, and neural cells of in vivo or whole tissue models, and the multitude of paracrine and cytokine mediators. Furthermore, cell culture models are susceptible to contact inhibition-induced differentiation, which can alter expected gene expression levels. However, cell culture does have the advantage of homogeneity, which enables the investigation of discrete and early cellular pathways. Therefore, a cell culture model is ideal for defining acute changes at the cellular level for short-term exposure to bile acid.

Additional limitations include using pig dEG myoepithelial cells instead of human cells. This assumes synteny and similar pathophysiological pathways. However, the porcine genome and animal model is far less characterized compared to rodent models, which lack dEGs and do not manifest intestinal metaplasia of the esophagus truly mimicking BE, even in surgical animal models [9]. We will plan for future experiments using human dEG cells to validate findings from this current project. Furthermore, bile acid is comprised of numerous substances, of which DCA is only one. The role of additional bile acid constituents and their potential synergistic role should be explored.

Conclusions

Currently, additional experimentation is needed to reconcile the differences between the qPCR and flow cytometry data. However, it appears indisputable that DCA is altering in some capacity the

expression of key genes seen in cells of the dEG and columnar epithelial cells of BE. In order to further characterize the effect of DCA on myoepithelial cells, additional samples for qPCR are needed. Additionally, CK5 and SOX9 relative gene expression levels are needed to complete the data set. Regarding the flow cytometry results, western blot analysis of CK5, CK7, and SMA protein levels are required for confirmation.

Suggestions for Future Work

Next steps to further investigate the role of bile acid on myoepithelial cells of the dEG include repeating the above study using myoepithelial cells isolated from human dEG. Previous studies have observed the effect of bile acid on whole esophageal biopsy specimens, but observing the individual role of the human myoepithelial cell would be novel [11]. As mentioned earlier, western blot analysis of CK5, CK7, and SMA protein levels should be undertaken to confirm the flow cytometry results.

Future experimentation should also consider exposing iPEMC to whole bile acid, rather than a single constituent. The various components comprising bile acid may act synergistically to accelerate myoepithelial differentiation into dEG and BE epithelium. The role of proton pump inhibitors (PPI), and their ability to alter bile acid permeability through cell membranes via protonation should also be considered, given the incredible popularity and widespread usage of PPI. Additional experimentation might involve exposing iPEMC to DCA at acidic, neutral, and alkaline pH to observe whether differences in environmental pH alters the ability of DCA to induce myoepithelial differentiation. Lastly, we have also embarked on proteomics using this cell culture model, since the homogeneity of treated and untreated sample is conducive to defined manipulation (such as exposure to DCA).

Acknowledgments

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Tables and Figures

Table 1 Primer Sequences used for qPCR

Gene	Forward	Reverse
CK5	5' -TACCAGACCAAGTACGAAG- 3'	5' -TGGATCATCCTGTTCATTTC- 3'
CK7	5' -ACAAGTTCGCCTCGTTCATC- 3'	5' -TGGTCGACTTCTGCTCCTG- 3'
SMA	5' -CAAAAGAGGAATCCTGACC- 3'	5' -CATTGTAGAAAGAGTGGTGC- 3'
SOX9	5' -CAGACCTTGAGGAGACTTAG- 3'	5' -GTTCGAGTTGCCTTTAGTG- 3'
TP63	5' -TGGACGTATTCCACTGAAC- 3'	5' -CATCACTTTGATCTGGATGG- 3'
NANOG	5' -CCTGAAGAAAATTATGCCTCC- 3'	5' -CCTTCTCTGTGCTCTTCTC- 3'
LGR5	5' -AGAACACTGACTTTGAATGG- 3'	5' -AGTTAAAGTCAGACTCTCCAG- 3'
GAPDH	5' -TTTAACTCTGGCAAAGTGG- 3'	5' -GAACATGTAGACCATGTAGTTG- 3'

Table 2 Target Genes for qPCR Evaluation of Myoepithelial Differentiation in Response to DCA

Gene	Protein	Cell Type Normally Expressed/Function
CK5	Cytokeratin 5	Basal epithelial cells, myoepithelial cells
CK7	Cytokeratin 7	Luminal epithelial cell marker, present in BE epithelium
SMA	Smooth Muscle Actin	Myoepithelial cells
SOX9	SOX9	Transcription factor expressed in adult stem cells
TP63	Tumor Protein p63	Expressed in basal stem cells and involved in nucleotide excision repair/base excision repair
NANOG	NANOG	Transcription factor involved in embryonic stem cell proliferation, renewal, and pluripotency
LGR5	Leucine Rich Repeat Containing G Protein-Coupled Receptor 5	Marker of intestinal stem cells

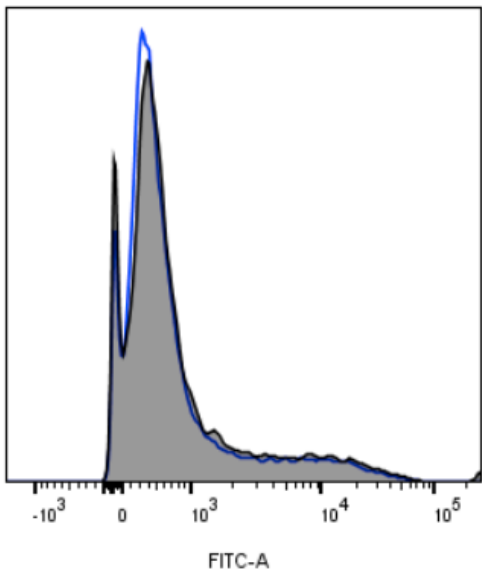**Figure 1.1** Control (Unexposed) iPEMC Flow Cytometry Results for CK5

Parameter	SE Dymax %Positive	Overton %Positive	Chi-Squared T(X)	K-S Max Difference	K-S Max at Intensity	K-S Probability
FITC-A	28.7898	26.0675	111.9060	26.1	1744.3318	> 99.9%
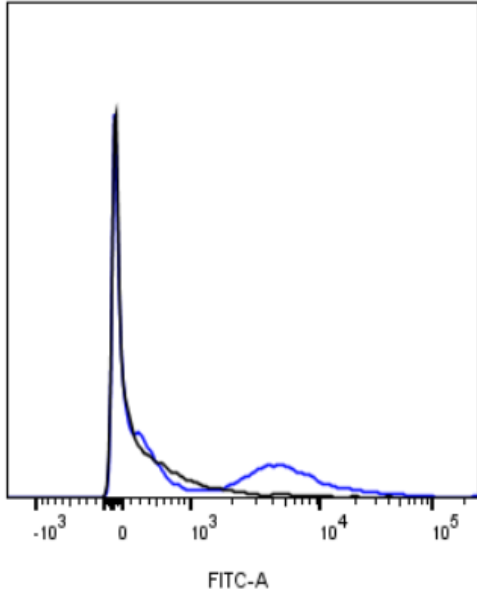
	Sample Name	Count
☐	imPGEC_ck5.fcs	n/a
☐	imPGEC_secondary only.fcs	3691

FITC-A

Figure 1.2 Control (Unexposed) iPEMC Flow Cytometry Results for CK7

Parameter	SE Dymax %Positive	Overton %Positive	Chi-Squared T(X)	K-S Max Difference	K-S Max at Intensity	K-S Probability
FITC-A	22.4629	2.1057	34.2335	4.79	424.3058	> 99.9%

	Sample Name	Count
▣	imPGEC_ck7.fcs	n/a
☐	imPGEC_secondary only.fcs	17034

FITC-A

Figure 1.3 Control (Unexposed) iPEMC Flow Cytometry Results for SMA

Parameter	SE Dymax %Positive	Overton %Positive	Chi-Squared T(X)	K-S Max Difference	K-S Max at Intensity	K-S Probability
FITC-A	44.8404	40.0704	276.0297	40.1	1546.5309	> 99.9%
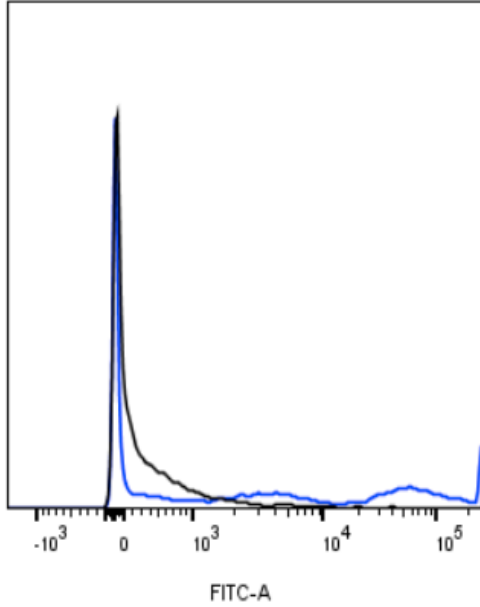
Sample Name	Count
☐ imPGEC_sma.fcs	n/a
☐ *imPGEC_secondary only.fcs*	*3691*

FITC-A

Figure 2.1 DCA Exposed iPEMC Flow Cytometry Results for CK5

Parameter	SE Dymax %Positive	Overton %Positive	Chi-Squared T(X)	K-S Max Difference	K-S Max at Intensity	K-S Probability
FITC-A	62.3799	42.1303	1089.8021	42.1	314.2880	> 99.9%
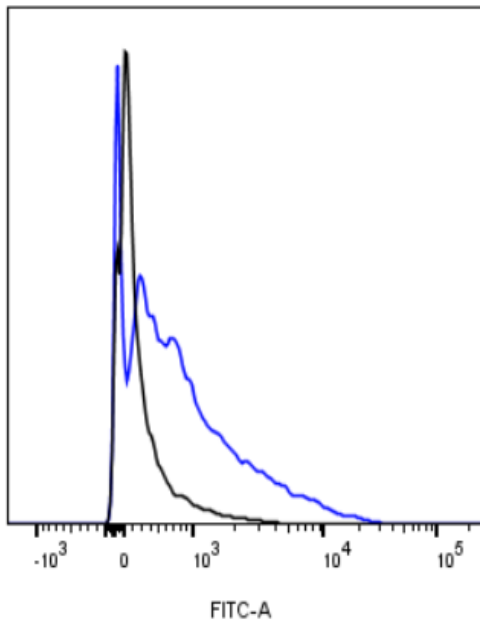
Sample Name	Count
☐ imPGEC DCA_ck5.fcs	n/a
☐ *imPGEC DCA_secondary only.fcs*	*16098*

FITC-A

Figure 2.2 DCA Exposed iPEMC Flow Cytometry Results for CK7

Parameter	SE Dymax %Positive	Overton %Positive	Chi-Squared T(X)	K-S Max Difference	K-S Max at Intensity	K-S Probability
FITC-A	43.3065	21.0308	276.7229	21.0	166.2774	> 99.9%
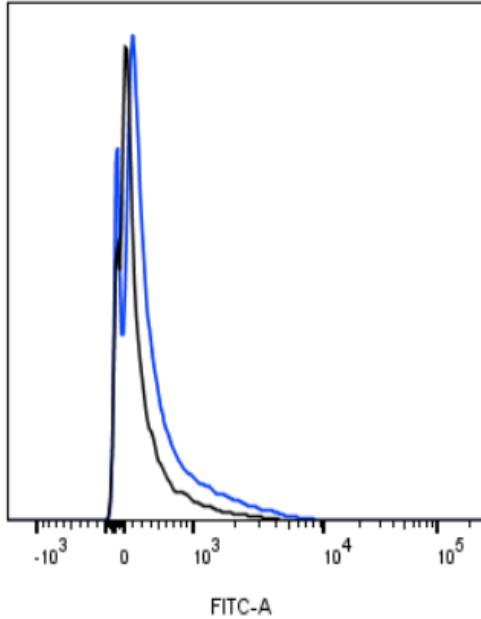
	Sample Name	Count
☐	imPGEC DCA_ck7.fcs	n/a
☐	imPGEC DCA_secondary only.fcs	16098

FITC-A

Figure 2.3 DCA Exposed iPEMC Flow Cytometry Results for SMA

Parameter	SE Dymax %Positive	Overton %Positive	Chi-Squared T(X)	K-S Max Difference	K-S Max at Intensity	K-S Probability
FITC-A	80.5071	72.6175	2702.0496	72.6	1105.6924	> 99.9%
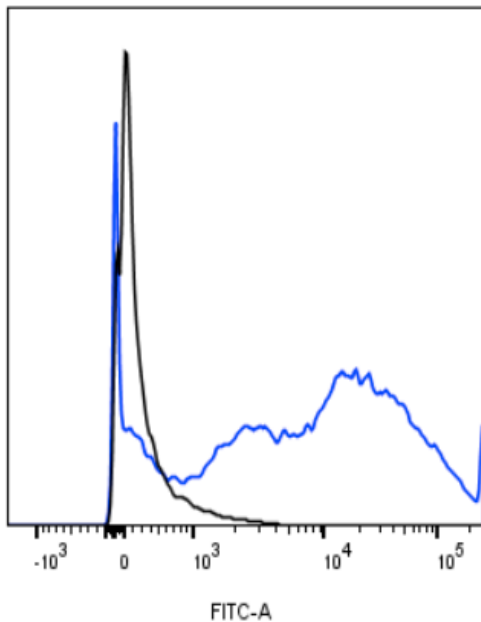
	Sample Name	Count
☐	imPGEC DCA_sma.fcs	n/a
☐	imPGEC DCA_secondary only.fcs	16098

FITC-A

Figure 3 Change in Relative Gene Expression Following iPEMC Exposure to DCA as Measured by qPCR