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CULTURE CONDITIONS PROFOUNDLY IMPACT PHENOTYPE IN BEAS-2B, A HUMAN PULMONARY EPITHELIAL MODEL

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Abstract

BEAS-2B, an immortalized, human lung epithelial cell line, has been used to model pulmonary epithelial function for over 30 years. The BEAS-2B phenotype can be modulated by culture conditions that include the presence or absence of fetal bovine serum (FBS). The popularity of BEAS-2B as a model of arsenic toxicology, and the common use of BEAS-2B cultured both with and without FBS, led us to investigate the impact of FBS on BEAS-2B in the context of arsenic toxicology. Comparison of genome-wide gene expression in BEAS-2B cultured with or without FBS revealed altered expression in several biological pathways, including those related to carcinogenesis and energy metabolism. Real-time measurements of oxygen consumption and glycolysis in BEAS-2B demonstrated that FBS culture conditions were associated with a 1.4-fold increase in total glycolytic capacity, a 1.9-fold increase in basal respiration, a 2.0-fold increase in oxygen consumed for ATP production, and a 2.8-fold increase in maximal respiration, compared to BEAS-2B cultured without FBS. Comparisons of the transcriptome changes in BEAS-2B resulting from FBS exposure to the transcriptome changes resulting from exposure to 1 μ M sodium arsenite revealed that mRNA levels of 43% of the arsenite-modulated genes were also modulated by FBS. Cytotoxicity studies revealed that BEAS-2B cells exposed to 5% FBS for 8 weeks were almost 5 times more sensitive to arsenite cytotoxicity than non-FBS-exposed BEAS-2B cells. Phenotype changes induced in BEAS-2B by FBS suggest that culture conditions should be carefully considered when using BEAS-2B as an experimental model of arsenic toxicity.

Keywords

FBS; Glycolysis; Metabolism; Arsenic; BEAS-2B; Gene Expression

INTRODUCTION

BEAS-2B, an SV40 large T-antigen-immortalized, human lung epithelial cell line, has been used extensively as an *in vitro* model of pulmonary epithelium in many experimental contexts, including toxicology testing, respiratory injury, wound healing, and neoplastic transformation. This is reflected in almost 1,200 publications referring to BEAS-2B

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currently in NCBI PubMed (Amstad *et al.*, 1988; Reddel *et al.*, 1995; Veranth *et al.*, 2004; Wang *et al.*, 2012; Garcia-Canton *et al.*, 2013). The cell line was originally isolated from the normal human bronchial epithelium of a cancer-free individual (Lechner *et al.*, 1982; Reddel *et al.*, 1988). Because it is non-malignant, extensive use has been made of BEAS-2B as an experimental model of malignant transformation. BEAS-2B can be malignantly transformed *in vitro* by overexpression of HRAS, CYP2A13, and SPR1, as well as exposure to chromium, arsenite, cadmium, cigarette smoke, and uranium (Lau *et al.*, 2000; Yang *et al.*, 2002; Zhang *et al.*, 2012; He *et al.*, 2013; Zhang *et al.*, 2014). Reports describing the use of BEAS-2B do not describe uniform culture conditions. Both defined, serum-free media as well as FBS-supplemented media are commonly used. Cell culture conditions reported for BEAS-2B include (serum free) bronchial epithelial cell growth medium (BEGM) and Dulbecco's modified Eagle's medium (DMEM) with FBS supplementation (Ding *et al.*, 2009; Sun *et al.*, 2011; Wang *et al.*, 2011; Martin *et al.*, 2012; Wang *et al.*, 2012; Zhang *et al.*, 2012; Nymark *et al.*, 2013). The use of BEAS-2B as a model of arsenic-induced toxicity and carcinogenesis includes instances of both these culture conditions. This is of interest because prior studies have demonstrated that the BEAS-2B phenotype can be influenced by the presence or absence of FBS in the culture media. Exposure of BEAS-2B to FBS is associated with squamous differentiation, alterations in cytokine secretion, and response to toxicants (Lechner *et al.*, 1984; Miyashita *et al.*, 1989; Veranth *et al.*, 2008).

The commonly reported use of BEAS-2B cultured under different conditions, as well as its use as a model in arsenic toxicology studies led us to examine the influence of FBS on BEAS-2B phenotype and response to arsenic.

MATERIALS AND METHODS

Cell culture

BEAS-2B (ATCC, Manassas, VA), was cultured in BEGM media (Lonza, Walkersville, MD), with or without 5% fetal bovine serum (FBS) for 8 weeks. All studies were performed using Beas-2B cells between passage 10 and passage 30, with passage 1 defined as the thawed cells from the supplier. Trypsin-EDTA (0.25%) was used to remove cells from culture flasks for sub-culturing. Cell cultures were incubated in humidified 95% air: 5% CO₂. Two million cells were seeded in 75 cm² culture flasks and sub-cultured at 90% confluence. BEAS-2B cells referred to in this study as "control" were cultured for appropriate passage-matched durations in BEGM without FBS and without arsenite. Cells referred to as "FBS-exposed" were cultured in BEGM, with 5% FBS, without arsenite, for 8 weeks. Cells referred to as "arsenite-exposed" were cultured in BEGM, without FBS, with 1 µM arsenite (Sigma, St. Louis, MO), for 8 weeks. The identity of the BEAS-2B cell line was confirmed using commercial forensic DNA testing based on polymorphic STR markers. Beas-2B cells were assayed for mycoplasma contamination every two weeks using the MycoAlert system (Lonza, Switzerland). No indication of mycoplasma contamination was found during the course of this study.

Antibodies and immunoblot analysis

Primary antibodies for immunoblot analysis were used at the following dilutions: E-cadherin (E-Cad) 1:250 and α -tubulin 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies included goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology), which were used at a 1:5000 dilution. Cells were washed twice with PBS, and lysed in sample buffer [10% glycerol, 100 mM DTT, 50 mM Tris-HCl (pH 6.8), 2% SDS]. Samples were then denatured at 90°C for 5 min. After sonication, protein concentration was measured using the Pierce 660 nm Protein Assay (Thermo Scientific, Rockford, IL). Equal protein masses of samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Immunoblots were visualized by addition of chemiluminescent substrate (Thermo Scientific, Rockford, IL) and quantified using a GeneGenome5 imaging system (Syngene, Frederick, MD).

Cellular energy metabolism analysis

Energy metabolism was measured using an XF⁹⁶ extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA), with the glycolysis stress test kit and the mitochondrial stress test kit (Wu *et al.*, 2007). Briefly, 30,000 cells per well were seeded in customized 96-well plates 24 hours prior to analysis. Un-buffered glycolysis stress test media was prepared according to manufacturer protocol and supplemented with 2 mM L-glutamine. Injection conditions for the glycolysis-stress test were: port-A, glucose (6 mM); port-B, oligomycin (1 μ M); port-C, 2-deoxy-D-glucose (2-DG, 100 mM). Glycolysis metabolic measurements were calculated based on measurements of extracellular acidification rate (ECAR). Basal glycolysis rate was calculated as ECAR just prior to oligomycin injection subtracted by ECAR just prior to glucose injection; total glycolytic capacity was calculated as ECAR just after oligomycin injection subtracted by ECAR measurement just before glucose injection. For the mitochondrial stress test, XF media was supplemented with 6mM glucose and 5 mM sodium pyruvate. Injection conditions were: port-A, oligomycin (1 μ M); port-B, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP, 0.25 μ M), port-C, antimycin A (1 μ M). Mitochondrial metabolic measurements were based on measurements of cellular oxygen consumption rate (OCR). Basal respiration was calculated as OCR just before oligomycin injection subtracted by OCR just after antimycin A injection. Oxygen consumed for ATP production was calculated by OCR just before oligomycin injection subtracted by OCR measurement just before FCCP injection. Maximal respiration was calculated as OCR just after FCCP injection subtracted by OCR just after antimycin A injection.

Cell viability assay and inhibitory concentration 50% (IC50) determination

Cell viability was measured using the CellTiter 96 Aqueous One Cell Proliferation Assay (MTS, Promega, Madison, WI) according to manufacturer protocols. Briefly, control and FBS-exposed cells were seeded at a cell density of 5,000 cells per well in a standard 96-well tissue culture plate. Following incubation for 72 hours in the presence of arsenite or vehicle, 20 μ L of MTS reagent was added to each well, and the plate was further incubated for 2 hours under 5% CO₂ at 37°C. Dye conversion to formazan was measured by optical absorbance at 490 nm. Data was analyzed using GraphPad Prism version 6.0 for MAC (GraphPad, La Jolla, CA). Raw absorbance data was transformed to percentage survival by

defining the dye conversion of the vehicle control group as 100% survival. Inhibitory concentration 50% (IC₅₀) values were estimated using the non-linear modeling of inhibitory dose-response module in GraphPad.

Genome-wide gene expression analysis

Microarray-based genome-wide mRNA measurement was performed in control, 8 week FBS-cultured cells, and 8 week As-exposed BEAS-2B cells. For each group, four experimental replicates were analyzed, with each replicate representing a different cell culture passage. RNA was isolated using an RNeasy Mini Kit (Qiagen). RNA quality was verified by optical absorbance (Nanodrop 2000, Thermo Scientific). mRNA was assayed using the Affymetrix GeneChip Human Gene 2.0 ST array, which measures the mRNA expression of 40,716 genome-wide RefSeq transcripts. Affymetrix CEL files were imported into GeneSpring GX 12.5 (Agilent Technologies, Santa Clara, CA) analysis software. Data were normalized using robust multichip analysis (RMA). Statistically significant expression differences were based on the moderated T-test, separately contrasting control Vs. arsenite-exposed cells, and control Vs. FBS-exposed cells. Type I error control was based on the Benjamini-Hochberg False Discover Rate (FDR). Modulated gene expression in this study was defined as those genes with expression differences between the comparison groups that were greater than 1.5-fold different, and at an FDR-adjusted $P < 0.01$.

Statistical analyses (non- microarray data)

For data with two comparison groups, unpaired t-tests were used to compare mean differences between control and treatment group at a significance threshold of $P < 0.05$, using GraphPad Prism.

RESULTS

FBS-cultured BEAS-2B sustain altered morphology and altered epithelial identity

The addition of FBS to BEAS-2B cells cultured in serum-free BEGM media resulted in a change in cell morphology (Figure 1A). BEAS-2B cells cultured in serum-free media have been reported to have a predominantly cuboidal, polygonal appearance typical of respiratory epithelial cells (Lechner and LaVeck, 1985; Ke *et al.*, 1988). BEAS-2B cells cultured with 5% FBS do not have a defined cell border and appear flattened, suggesting a more squamous appearance. The altered morphology induced by FBS exposure was accompanied by changes in epithelial identity. Immunoblot analysis established that FBS exposure resulted in a loss of E-cadherin protein (Figure 1B). Microarray analysis corroborated this change (Figure 1C), suggesting that the loss of E-cadherin expression occurred at a transcriptional level.

FBS exposure alters the BEAS-2B transcriptome, including genes in common with those modulated by arsenite exposure

Compared to control cells, FBS exposure in BEAS-2B resulted in altered mRNA expression in 3,662 genes. Pathway analysis using the Genespring GX 12.5 WikiPathways-Analysis module identified a total of 74 pathways that were significantly altered by FBS exposure (Supplemental Table 1) (Kelder *et al.*, 2009). Among the altered pathways were energy

metabolism associated cholesterol biosynthesis, oxidative phosphorylation, pentose phosphate pathway, and glycolysis/gluconeogenesis (Supplemental Table 1). Several of the altered pathways were cancer-associated, including RB in cancer, integrated breast cancer pathway, integrated pancreatic cancer pathway, gastric cancer networks, the Wnt signaling pathway, and the integrated cancer pathway. In a separate statistical contrast, we analyzed differences in gene expression between control and arsenite-exposed BEAS-2B cells. The mRNA expression of 1574 genes was altered by arsenite exposure. The 1574 genes with mRNA expression modulated by arsenic included 674 genes in which expression was also modulated by FBS (Figure 2A and 2B). The expression of three-fourths of those 674 shared genes (510 genes) was modulated in the same direction by both arsenite and FBS exposures (Figure 2B). Exemplary in this context are the genes related to cholesterol biosynthesis and utilization that were statistically up-regulated by both arsenite and by FBS. These genes included LDLR (low density lipoprotein receptor), LSS (lanosterol synthase), MSMO1 (methylsterol monooxygenase 1), MVD (mevalonate decarboxylase), DHCR7 (7-dehydrocholesterol reductase), and FDFT1 (farnesyl-diphosphate farnesyltransferase 1).

FBS exposure alters cellular energetics in BEAS-2B

Based on the genome-wide gene expression profile suggesting altered energy metabolism pathways, we analyzed cellular energetics in BEAS-2B using real-time analysis of oxygen consumption and extracellular acidification. Exposure of BEAS-2B to FBS altered both anaerobic and oxygen-dependent energy production in BEAS-2B. FBS exposure did not change basal glycolysis rate. However, FBS-exposed cells demonstrated a 1.4-fold increase in total glycolytic capacity (Figure 3A and 3B). Analysis of aerobic metabolism revealed that FBS exposure resulted in a 1.9-fold increase in basal respiration, a 2.0-fold increase in oxygen consumed for ATP production, and a 2.8-fold increase in maximal respiration (Figure 3C and 3D).

FBS-cultured BEAS-2B are more sensitive to arsenic cytotoxicity

Exposure of BEAS-2B to FBS resulted in enhanced sensitivity to arsenite-induced cytotoxicity, reflected in nearly a five-fold difference in arsenite IC₅₀ values, comparing control to FBS-exposed cells. Nearly a 50% reduction in cell survival resulting from 4 μ M arsenite (72 hours) was observed in FBS-exposed BEAS-2B, while non-FBS-exposed cells under identical arsenite exposure sustained no measurable viability reduction (Figure 4). Non-linear fitting of cytotoxicity dose-response curves resulted in arsenite IC₅₀ estimates of 21.3 μ M for non-FBS-exposed BEAS-2B (IC₅₀ 95% confidence interval 19.4 μ M, 23.3 μ M) and 4.3 μ M for FBS-exposed BEAS-2B (IC₅₀ 95% confidence interval 3.7 μ M, 4.9 μ M).

DISCUSSION

This study confirms earlier work showing that exposure of the BEAS-2B cell line to FBS induces squamous differentiation and loss of E-cadherin protein expression (Lechner *et al.*, 1984; Stewart *et al.*, 2012). Work by Veranth *et al.* demonstrated that exposure to FBS caused BEAS-2B cells to respond differently to a battery of toxicants, as measured by interleukin 6 (IL-6) secretion (Veranth *et al.*, 2008). In that study exposure to 10% FBS resulted in a 10-fold increase in IL-6 secretion. Moreover, the Veranth study demonstrated

that the IL-6 secretion, as a response to exposure to 7 toxicants, was different in magnitude and rank order between control and FBS-exposed BEAS-2B cells.

In this study we have extended the characterization of the impact of culture conditions on the cellular biology of BEAS-2B. Microscopic analysis confirmed the acquisition of squamous morphology as a consequence of FBS exposure. Gene expression analysis confirmed the FBS-induced down-regulation of E-cadherin observed in prior studies. This study builds on these reports with the novel finding that, compared to defined BEGM media alone, exposure to bovine serum results in genome-wide reprogramming of gene expression, altered cellular energetics, and enhanced sensitivity to arsenite-induced cytotoxicity.

Given the substantial morphological changes suggesting FBS-induced differentiation in BEAS-2B, it is not surprising that genome-wide gene expression is substantially modulated by FBS exposure. Biological pathways that were significantly modulated by FBS exposure represent diverse biological functions and sub-cellular compartments. Among the altered pathways, FBS exposure appeared to have an impact on cellular energetics. We pursued this lead with functional analyses of energetics, focusing on glycolysis-related extracellular acidification rate and on oxidative-phosphorylation-related oxygen consumption. The disruption of energetics detected in the microarray experiments was also seen functionally. FBS-exposed BEAS-2B had increased glycolytic capacity, basal respiration, oxygen consumed for ATP production, and maximal respiration. Our study did not identify a specific, functional consequence of FBS-altered energetics. However, the pathology of several chronic diseases have been linked to disrupted energy metabolism (Saks *et al.*, 1996; Calabrese *et al.*, 2010; Kapogiannis and Mattson, 2011; Donohoe *et al.*, 2012).

We were also interested in the extent to which the gene expression changes induced by arsenite exposure were similar to those induced by FBS exposure. Genome-wide analysis of mRNA levels revealed that, under non-cytotoxic arsenic exposure conditions, nearly half of the 1574 arsenite-modulated genes are also modulated by exposure of BEAS-2B cells to 5%. Moreover, about 75% of the 674 shared genes are altered in an identical direction by both arsenite and FBS, suggesting a commonality in the effect of the two exposures. Exemplary in this regard is the observation that both exposures induced a significant up-regulation of genes involved in cholesterol biosynthesis, suggesting that BEAS-2B cells responded to both exposures by increasing the synthesis of cholesterol. Prior studies have also reported genome-wide gene expression changes in BEAS-2B cells exposed to arsenite. Andrew *et al.* measured gene expression in confluent cultures of BEAS-2B grown in defined LHC-9 medium, using matrix (fibronectin/bovine collagen/bovine albumin)-coated flasks (Andrew *et al.*, 2003). That study used a targeted analysis of 1,200 genes in cells that had been exposed to 5 μ M or 50 μ M arsenite for 4 hours. Perhaps as a consequence of the use of different conditions for culture, arsenite exposure, and gene expression analysis, there were no modulated genes in common between that study and ours. Chilakapati *et al.* also studied gene expression in BEAS-2B cultured in defined LHC-9 medium. In that study gene expression data (analyzed using a previous generation of Affymetrix chip as that used in our study) was reported for cells exposed to 15 μ M arsenite for 24 hours. Messenger RNA levels for IL1A (Interleukin-1 alpha), IL1B (Interleukin-1 beta), NQO1(NAD(P)H dehydrogenase, quinone 1), SERPINB5 (serpin peptidase inhibitor, clade B (ovalbumin), member 5),

SERPINE1 (serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1, member 1), and TLR4 (toll-like receptor 4) were found to be up-regulated by arsenite exposure in both our study and that of Chilakapati *et al.* No genes down-regulated by arsenite were common to both studies. The differing experimental conditions of the Andrew and Chilakapati studies, compared to those described in this report, limit the ability to identify commonalities. However it is of interest that a subset of genes that was acutely up-regulated by arsenite in the Chilakapati study was also found to be up-regulated over a much more extended arsenite exposure in this study.

An intriguing correlate of the FBS-induced phenotypic disruption is the pronounced increase in sensitivity of FBS-exposed BEAS-2B to arsenite cytotoxicity. While it is possible that the presence of serum provided proteins that bound arsenic and facilitated its transport across the plasma membrane, this is not in agreement with the recognized role of aquaglyceroporins in arsenic transport (Yoshino *et al.*, 2011; Yang *et al.*, 2012; Mukhopadhyay *et al.*, 2014). Alternatively, FBS exposure may have altered the uptake or response to 1 μ M arsenite by transcriptionally altering the quantity of transport proteins or stress-response proteins.

Based on prior experimental work that characterized the ability of FBS to modify important cellular functions related to in vitro toxicology, our purpose was to determine if FBS exposure could impact BEAS-2B response to arsenite in ways that might complicate the interpretation of FBS-cultured, arsenite-exposed BEAS-2B cultures. At a minimum this study suggests that it may be problematic to compare results from different studies of arsenite-exposed BEAS-2B using different culture conditions. Given the common set of genes sustaining altered mRNA expression from both arsenite and FBS, it is possible that exposing FBS-cultured BEAS-2B cells to arsenite may augment gene expression changes in that set of genes. Some specific aspects of our culture conditions are worth noting. We did not use substrate coating (fibronectin/collagen/albumin) of culture flasks as has been reported for BEAS-2B. Successful culture of BEAS-2B without substrate coating has been reported in many studies (Liu *et al.*, 2011; Stueckle *et al.*, 2012; Zhang *et al.*, 2012; Chen *et al.*, 2013; Li *et al.*, 2014). It is also important to note that we have not fully modeled a comparison of two commonly used culture conditions, BEGM and DMEM with 5% FBS. We have modeled the impact of adding 5% FBS to BEGM. A more elaborate research design will be needed to model the effects of both basal media differences, together with the effects of the defined additives of BEGM compared to FBS as an additive.

Our objective in this study was not to define the “correct” media conditions in which to perform arsenic exposure studies. Depending on the objective of the experiment, alternative culture condition may each have distinct advantages and disadvantages in arsenic exposure studies using BEAS-2B. Nevertheless, this study adds more definition to the phenotypic differences induced by FBS, allowing a more informed choice of culture conditions for investigators using this important experimental model.

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Abbreviations

As	Arsenite
E-cad	E-cadherin
FBS	Fetal bovine serum

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Figure 1.

A) Representative micrograph of BEAS-2B morphology, 100x magnification. B) Immunoblot of E-cadherin and α -Tubulin in BEAS-2B control (-FBS) and 8 week +FBS (representative blot of 2 independent replicates). C) E-cadherin gene expression from microarray analysis, y-axis on log2 scale. * P (FDR adjusted) = 1.94×10^{-7} .

Figure 2.

A) Volcano plot of gene expression of 8 week +As vs. -As (-FBS) and 8 week +FBS vs. -FBS (-As). *Left panel:* BEAS-2B cells cultured without FBS in presence (+As) Vs. absence (-As) of 1 μ M arsenite. *Right panel:* BEAS-2B cells cultured without Arsenite in presence (+FBS) Vs. absence (-FBS) of 5% FBS. Genes significantly expressed at a $P < 0.001$ level are in the upper rectangle with no translucent overlay. B) Venn diagram of modulated genes in two contrasts: BEAS-2B 8 week +As vs. -As (-FBS) and 8 week +FBS vs. -FBS (-As). Vertical hatching represents gene modulation in the same direction, and horizontal hatching represents gene modulation in opposite directions.

Figure 3.

Energy metabolism profiling in BEAS-2B cultured with or without 8 week FBS. A) Extracellular acidification rate (ECAR) measurement, arrows indicate injection of glucose, oligomycin (Oligo), and 2-deoxy-D-glucose (2-DG) at the indicated time point. B) Calculated glycolysis and glycolytic capacity levels (Mean, S.E.M., 3 independent replicates). * $P < 0.05$. C) Oxygen consumption rate (OCR) measurement, arrows indicate injection of oligomycin (Oligo), Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and Antimycin A (Anti-A) at the indicated time point. D) Calculated basal respiration, oxygen consumed for ATP production (ATP production), and maximum respiration (Max respiration). Control vs. FBS-exposed BEAS-2B (Mean, S.E.M., 3 independent replicates), * $P < 0.05$.

Figure 4. Cell viability assay of control and FBS-exposed BEAS-2B, cultured with 0–32 μM sodium arsenite for 72 hours (Each plot point: Mean, S.E.M., n=12 from 2 experimental replicates).