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Differential gene regulation in human small airway epithelial cells grown in monoculture versus coculture with human microvascular endothelial cells following multiwalled carbon nanotube exposure

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ABSTRACT

Concurrent with rising production of carbon-based engineered nanomaterials is a potential increase in respiratory and cardiovascular diseases due to exposure to nanomaterials in the workplace atmosphere. While single-cell models of pulmonary exposure are often used to determine the potential toxicity of nanomaterials in vitro, previous studies have shown that coculture cell models better represent the cellular response and crosstalk that occurs in vivo. This study identified differential gene regulation in human small airway epithelial cells (SAECs) grown either in monoculture or in coculture with human microvascular endothelial cells following exposure of the SAECs to multiwalled carbon nanotubes (MWCNTs). SAEC genes that either changed their regulation direction from upregulated in monoculture to downregulated in coculture (or vice versa) or had a more than a two-fold changed in the same regulation direction were identified. Genes that changed regulation direction were most often involved in the processes of cellular growth and proliferation and cellular immune response and inflammation. Genes that had a more than a two-fold change in regulation in the same direction were most often involved in the inflammatory response. The direction and fold-change of this differential gene regulation suggests that toxicity testing in monoculture may exaggerate cellular responses to MWCNTs, and coculture of cells may provide a more in-depth assessment of toxicological responses.

1. Introduction

Human industrial activities have increased the manufacturing of organic, inorganic, and carbon-based nanomaterials and nanoparticles [1]. This increase potentially leads to a rise in respiratory and cardiovascular diseases in production workers due to high concentrations of nanomaterials of varying chemical compositions in the workplace atmosphere that can enter living organisms through different routes of exposure [1–4]. The increasing use of these engineered nanoparticles in consumer products likely also increases exposure to consumers [1,5–7]. While in vivo animal testing of engineered nanoparticles provides information on distribution and risk, animal models have limitations, including the ethics of animal testing and the feasibility of testing large numbers and varieties of engineered nanoparticles [5,8]. To address this issue, in vitro systems have been developed that attempt to investigate the toxicity of engineered nanoparticles across a variety of biological barriers [5,7].

Pulmonary exposure to multiwalled carbon nanotubes (MWCNTs) results in lung inflammation, pulmonary fibrosis, and lung adenocarcinoma promotion as well as extrapulmonary transport to many areas of the body [9–16]. MWCNT-induced inflammation and extrapulmonary transport have numerous effects in vivo, including cardiovascular effects, increased oxidative stress, reduced cognitive abilities in rats, and disruption of the reproductive cycle in female mice [3,15,17–20]. MWCNTs can be released into the air by industrial processes, such as transfer, mixing, and weighing. Since MWCNTs are being used to improve the performance of polymer composites, MWCNT release is also feasible during drilling, sanding, weathering, and incineration of MWCNT-enabled composites [21]. In light of such exposures, in vitro systems that attempt to mimic the interactions of

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MWCNTs with the alveolar-capillary barrier to study their pulmonary effects have been developed [22,23].

The molecular mechanisms by which MWCNTs induce pulmonary diseases remain elusive; moreover, the limited information regarding these molecular mechanisms has been mainly generated from single-cell culture models (monoculture), which do not account for cellular crosstalk between adjacent cells [24–27]. The lung is composed of many different cell types that undergo pivotal cellular communication in response to pulmonary exposures [28–30], including exposure to MWCNTs [27,31]. Cytotoxicity profiling using conventional monocultures is often markedly different from that of relevant in vivo models [32,33]. A previous study by our group used an alveolar-capillary coculture model of small airway epithelial cells (SAECs) and human microvascular endothelial cells (HMVECs) to identify cellular effects in HMVECs following exposure of SAECs to dispersed MWCNTs [22]. This study found that, while MWCNTs were engulfed by SAECs, they were not apparent in HMVEC preparations, suggesting that effects in HMVECs were caused by downstream signals, such as the release of VEGF-A, ICAM1, and VCAM1 from SAECs [22]. Exposure of SAECs to MWCNTs induced reactive oxygen species production, disrupted the endothelial barrier, and increased capillary-like formation and intracellular inflammatory signals in HMVECs [22]. This coculture system models the alveolar-capillary unit of the lower respiratory tract where MWCNTs pose a serious point of attack [34–37]. It has been well established that the injury and activation of alveolar-capillary units play major roles in the pathogenesis of pulmonary inflammation, damage, and fibrosis [38–41]. This coculture system represents a reasonable model in view of the close proximity of alveolar epithelial cells and capillary endothelial cells in vivo [42].

In a companion study from our laboratory, gene regulation in SAECs and HMVECs grown in coculture was found to be more concordant with gene regulation from mouse lungs exposed to MWCNTs than SAECs or HMVECs grown in monoculture [23]. Given this evidence, this current study was conducted to compare gene regulation from SAECs grown separately in monoculture or together with HMVECs in coculture. We aimed to identify genes that either were downregulated in monoculture versus upregulated in coculture (or vice versa) or had a more than two-fold up- or downregulation in monoculture versus coculture. The identification of genes differentially expressed between monoculture and coculture systems could reveal important signaling events involved in communication and feedback loops (both positive and negative) between lung epithelial cells and endothelial cells. This study identified 116 unique molecules that were either upregulated in SAECs in monoculture and downregulated in SAECs grown with HMVECs in coculture, or vice versa. Additionally, 91 unique molecules had more than two-fold up- or downregulation in SAECs grown in coculture with HMVECs versus SAECs grown in monoculture. Ingenuity Pathway Analysis (IPA) analysis of these unique genes determined that those involved in growth, exocytosis, and transcription were most often upregulated in SAECs in monoculture but downregulated in SAECs in coculture. Genes involved in inflammation, wound repair, and cell adhesion were most often altered in SAECs grown in both monoculture and coculture, although these changes were attenuated in coculture. While the use of in vitro systems that can closely mimic the in vivo environment is being undertaken with increasing necessity, this is the first study to our knowledge to take an in depth look at overall gene regulation changes induced by different culture conditions. The findings of this study suggest that coculture of SAECs with HMVECs attenuates inflammatory reactions induced by exposure to MWCNTs, whereas gene regulation changes following exposure to SAECs in monoculture may overestimate these responses.

2. Materials and methods

2.1. MWCNTs and preparation

The MWCNTs used in this study were provided by Mitsui-& -Company (MWCNT-7, lot # 05072001 K28). Characterization and preparation of these MWCNTs for cellular studies has been previously described [11,23].

2.2. Cell culture and RNA profiling

The SAECs and HMVECs used in this study and their culture conditions have been previously described [22,23]. Total RNA was isolated from SAECs and HMVECs and analyzed as previously described [23].

2.3. Microarray data processing and statistical analysis to determine significant genes

Genes with regulatory changes were determined from in vitro microarray data as previously described [23,43]. The mRNA microarray data from monoculture and coculture SAEC and HMVEC are available at NCBI Gene Expression Omnibus (GEO) with accession number GSE129640.

2.4. Determining up- and downregulated genes at 6 and 24 h

Significantly changed genes at 6 h and 24 h in SAECs monoculture or coculture system were identified with Statistical Analysis of Microarray (SAM) in R using a false discovery rate (FDR) < 1% and a fold change > 1.5 compared with the no treatment (DM) group. Among these significant genes, those genes showing opposite regulation patterns between coculture and monoculture cell systems were identified. In addition, genes that had at least two-fold increase or decrease in the same regulation direction were identified in the coculture system compared with the monoculture system at different treatment time points.

2.5. IPA

The two sets of genes that either changed direction or had more than a two-fold increase in regulation were uploaded to IPA (QIAGEN) and subjected to an IPA Expression Analysis, which determined the top canonical pathways in which the provided genes were involved.

Both sets of genes that either changed regulation direction or had more than a two-fold change in regulation were also input into IPA pathways. The Grow → Diseases and Functions tool was used to determine significant genes that were involved in specific cellular functions known to follow MWCNTs pulmonary exposure. Inflammatory Response, Fibrosis, Hypertension, and Adhesion of Neutrophils disease and function overlays were placed over the significant genes, and the gene lists were pared down to only those significant genes involved in those disease and functions.

3. Results

3.1. Changes in SAEC gene regulation direction following MWCNT exposure in monoculture versus coculture with HMVECs

Over all comparisons, 116 unique genes and long noncoding RNAs in SAECs were either downregulated in monoculture and upregulated in coculture or upregulated in monoculture and downregulated in coculture (Supplemental File 1). The top 10 genes with the most significant differences in fold change at either 6 h or 24 h between monoculture and coculture and their functions are listed in Table 1.

All genes that were either upregulated in monoculture and downregulated in coculture or vice versa were uploaded as a dataset into IPA

Table 1
Top 10 "changed regulation direction" SAEC genes with the biggest difference in fold change between monoculture and coculture.

Gene Name	Fold Change in Monoculture	Fold Change in Coculture	Function
ACBD3	3.53 (24 h)	−3.77 (24 h)	Maintenance of Golgi structure and function; hormonal regulation of steroid formation
DMRTA1	3.32 (6 h)	−4.15 (6 h)	Unknown
EXOC6B	2.56 (6 h)	−4.85 (6 h)	Exocytosis
FAM198B	6.63 (6 h)	−2.45 (6 h)	Unknown
GHR	3.30 (24 h)	−3.69 (24 h)	Growth
IL1R1	2.83 (24 h)	−2.91 (24 h)	Cytokine-induced immune and inflammatory responses
IRF2	2.31 (24 h)	−3.57 (24 h)	Inhibits interferon alpha and beta transcription
LONRF1	2.19 (6 h)	−4.43 (6 h)	Immune response
PARM1	2.83 (24 h)	−5.89 (24 h)	Apoptosis resistance
ZNF304	2.21 (24 h)	−3.15 (24 h)	Transcriptional repressor

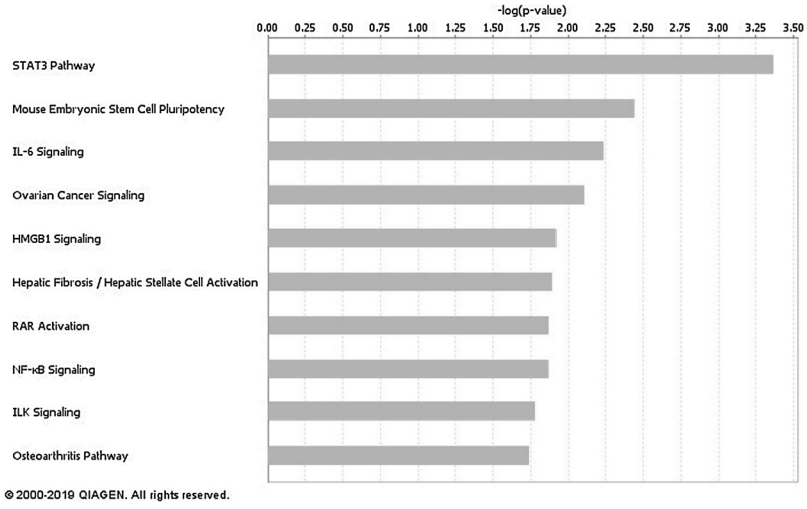

Fig. 1. Top 10 IPA pathways involving SAEC genes that changed regulation direction.

and analyzed using the Expression Analysis feature. The top 10 pathways in which these genes were involved included Mouse Embryonic Stem Cell Pluripotency, IL-6 Signaling, Ovarian Cancer Signaling, NF-kB Signaling, Hepatic Fibrosis/Hepatic Stellate Cell Activation, RAR Activation, ILK Signaling, STAT3 Pathway, Osteoarthritis Pathway, and AMPK Signaling (Fig. 1).

All 116 unique molecules were also analyzed through IPA for their involvement in diseases and functions known to be affected by MWCNT pulmonary exposure, such as inflammation, fibrosis, and cardiovascular effects (Fig. 2) [9,11,14,18,44]. Of the 116 unique molecules, seven genes that were upregulated in monoculture and downregulated in coculture (SNAI1, TNFSF4, IL1R1, CFTR, IRF2, FGFR2, TET2) were associated with fibrosis. Five genes that were upregulated in monoculture and downregulated in coculture (IL1R1, CFTR, CD9, GJA1, TNFSF4) and one gene that was downregulated in monoculture and upregulated in coculture (VEGFA) were associated with inflammation. One gene that was upregulated in monoculture and downregulated in coculture (IL1R1) and one gene that was downregulated in monoculture and upregulated in coculture (VEGFA) were associated with adhesion of neutrophils, and three genes that were upregulated in monoculture and downregulated in coculture (ACBD3, SMAD9, TNFSF4) and one gene was that downregulated in monoculture and upregulated in coculture (VEGFA) were associated with hypertension. Fold changes of the genes involved in MWCNT-induced disease states

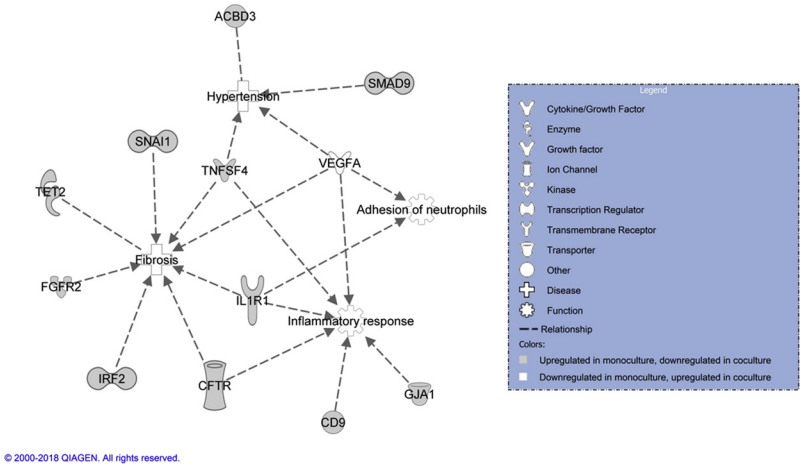

Fig. 2. IPA disease and function overlays for SAEC genes that changed regulation direction.

Table 2
Fold changes of SAEC genes that changed regulation direction between monoculture and coculture identified in IPA overlays.

Gene Name	Fold Change in Monoculture	Fold Change in Coculture
ACBD3*	3.53 (24 h)	−3.77 (24 h)
CD9*	2.47 (6 h)	−1.91 (6 h)
CFTR*	2.74 (6 h)	−3.90 (6 h)
FGFR2*	2.60 (6 h)	−2.89 (6 h)
GJA1*	2.54 (6 h)	−2.94 (6 h)
IL1R1*	2.83 (24 h)	−2.91 (24 h)
IRF2	2.31 (24 h)	−3.57 (24 h)
SMAD9*	2.54 (6 h)	−2.12 (6 h)
SNAI1*	4.58 (6 h)	−2.51 (6 h)
TET2	2.19 (24 h)	−2.79 (24 h)
TNFSF4*	2.73 (24 h)	−2.74 (24 h)
VEGFA*	−2.00 (6 h)	2.08 (6 h)

are shown in Table 2.

3.2. SAEC genes that had a greater than two-fold increase or decrease in regulation in the same direction following MWCNT exposure in monoculture or coculture with HMVECs

Over all conditions, 91 unique genes were either upregulated in both monoculture and coculture or downregulated in both monoculture and coculture with a more than two-fold difference in regulation between the two (Supplemental File 2). The top 10 genes with the biggest differences in fold change and their functions are listed in Table 3. Eight of these genes were downregulated (CXCL1, MMP3, CXCL2, TOP2A, DMTB1, SAA4, STEAP4, and PTX3), and two were upregulated (DAPL1, SPON2).

All 91 unique genes were uploaded to IPA and analyzed for pathway involvement using the Expression Analysis feature. The top 10 pathways in which these 91 genes are involved include cAMP-Mediated Signaling, Glucocorticoid Receptor Signaling, Bladder Cancer Signaling, Acute Phase Response Signaling, Granulocyte Adhesion and Diapedesis, G-Protein Coupled Receptor Signaling, Agranulocyte Adhesion and Diapedesis, tRNA Splicing, Role of IL-17A in Psoriasis, and Role of IL-17A in Arthritis (Fig. 3).

Similar to the genes that changed regulation direction between monoculture and coculture, the 91 genes that had a more than two-fold difference in the same regulation direction were also analyzed for their involvement in inflammation, fibrosis, and cardiovascular outcomes (Fig. 4). Six genes (CXCL2, CXCL3, IL1R1, PTX3, CDKN1A, SERPINE1) were involved in inflammation, and five genes (IL1R1, TLR2, PTX3, SERPINE1, SMURF2) were involved in fibrosis. Five genes (CXCL1, CXCL2, CXCL3, IL1R1, TLR2) were involved in adhesion of neutrophils, and five genes (SMURF2, TTK, SLC39A8, PDE4B, SERPINE1) were involved in hypertension. Fold changes of these genes involved in MWCNT-induced disease states are shown in Table 4.

4. Discussion

While monoculture of individual cell types has always been, and is still, the mainstay of molecular biology, multicell in vitro systems that can mimic the cellular interactions and responses of in vivo exposure are a growing need for the diverse field of toxicology. This study identified genes that were either upregulated in monoculture and downregulated in coculture (or vice versa) or up- or downregulated in both monoculture and coculture with at least a two-fold difference in regulation between the two to determine gene regulation differences between cells grown in monoculture or coculture.

The top pathways affected by genes that were either downregulated in monoculture and upregulated in coculture (or vice versa) most often involved the processes of cellular growth and proliferation and cellular immune response and inflammation. The genes with the biggest differences in fold change between monoculture and coculture (Table 1) saw upregulation of genes in monoculture and downregulation of genes in coculture. Previous work from our group has shown that cellular signals from SAECs and HMVECs can cross between the coculture membrane and affect the other cell type. This crosstalk between the SAECs and HMVECs may affect gene regulation in SAECs following MWCNT exposure. The finding that the same gene can be upregulated in monoculture while downregulated in coculture (or vice versa) suggests that this crosstalk affects how SAECs respond to MWCNT exposure.

Additionally, differentially regulated genes in coculture may have been regulated to a significantly greater or lesser degree than in monoculture. In particular, CXCL1 and CXCL2, well-known players in the inflammatory response, were greatly downregulated in monoculture compared to coculture, as were other genes involved in the acute phase response. This suggests that, while both SAECs in monoculture and SAECs in coculture appear to show activation and repression of inflammatory mediators following MWCNT exposure, these responses are, for the most part, attenuated in SAECs grown in coculture. This suggests that SAECs grown in monoculture may overestimate the cellular response to MWCNTs.

For genes that had a more than 2-fold difference in fold change in the same regulation direction, all of the genes involved in adhesion of neutrophils (CXCL1, CXCL2, CXCL3, IL1R1, and TLR2) were downregulated, with greater fold changes in CXCL1, CXCL2, CXCL3, and TLR2 in monoculture than coculture. CXCL1, CXCL2, and CXCL3 belong to the same chemokine superfamily and are involved in increasing neutrophil and endothelial cell adhesion [45–47]. TLR2 increases the expression of adhesion molecules, while loss of IL1R1 function has been associated with decreased neutrophil adhesion [48,49]. In the lungs, neutrophils are the earliest immune cells to be recruited to the site of injury or inflammation [50]. While this recruitment is intended for resolution of the offending agent, impaired recruitment has been implicated in incomplete resolution and results in tissue damage [51]. Several downregulated genes, SERPINE1, PDE4B, SLC39A8, and TTK, were also suggested by IPA to be involved in hypertension. SERPINE1

Table 3
Top 10 “same regulation direction” SAEC genes with the biggest difference in fold change between monoculture and coculture.

Gene Name	Fold Change in Monoculture	Fold Change in Coculture	Fold Change Difference	Function
CXCL1	−125.33 (6 h)	−3.43 (6 h)	121.90	Inflammation; chemoattractant for neutrophils
DAPL1	56.67 (6 h)	10.71 (6 h)	45.97	Epithelial differentiation; apoptosis
MMP3	−45.36 (6 h)	−2.83 (6 h)	42.53	Wound repair; progression of atherosclerosis; tumor initiation
CXCL2	−40.14 (6 h)	−3.04 (6 h)	37.10	Inflammation and suppression of hematopoietic progenitor cell proliferation
TOP2A	−3.06 (6 h)	−28.63 (6 h)	25.57	DNA regulation during transcription
DMTB1	−33.79 (6 h)	−13.76 (6 h)	20.03	Interaction of tumor cells and the immune system
SAA4	−18.58 (6 h)	−2.08 (6 h)	16.50	Acute phase reactant; inflammation
STEAP4	−17.23 (6 h)	−3.68 (6 h)	13.55	Inflammation
PTX3	−14.72 (6 h)	−2.53 (6 h)	12.19	Inflammation regulation; complement activation
SPON2	12.47 (6 h)	2.91 (6 h)	9.56	Cell adhesion

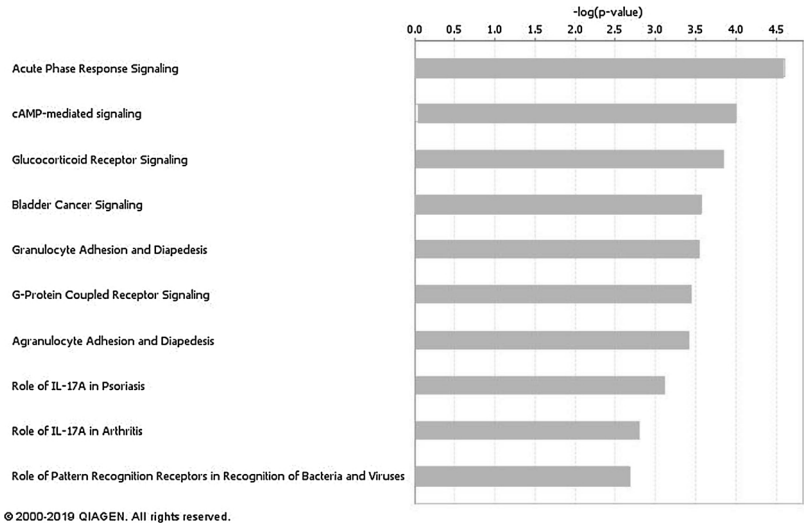

Fig. 3. Top 10 IPA pathways involving SAEC genes that had a more than 2-fold difference in the same regulation direction.

and TTK were downregulated to a greater extent in coculture, while PDE4B and SLC39A8 were downregulated to a greater extent in monoculture. SMURF2 was upregulated to a greater extent in monoculture versus coculture. Upregulation of SMURF2 and TTK have been associated with increases in hypertension and fibrosis, while decreases in SERPINE1 have been associated with decreased risk of hypertension [52–54].

In the analysis of genes that changed regulation direction between coculture and monoculture, several genes were identified that were involved in known outcomes of MWCNT in vivo exposure: inflammation, fibrosis, and cardiovascular effects. Interestingly, all genes were upregulated in monoculture and downregulated in coculture except for 1, VEGF-A. VEGF-A has been a particular target of previous studies as this gene was upregulated in coculture of SAECs and HMVECs (with the source originating from the SAECs), and HMVECs showed increased angiogenic potential when grown in coculture with SAECs exposed to MWCNTs [22]. This study again corroborates the findings that VEGF-A, which was implicated by IPA analysis to be involved in all 4 outcomes analyzed (inflammatory response, fibrosis, hypertension, and adhesion

Table 4
Fold changes of SAEC genes that had a more than 2-fold difference in the same regulation direction between monoculture and coculture identified in IPA overlays.

Gene Name	Fold Change in Monoculture	Fold Change in Coculture	Fold Change Difference
CDKN1A	−4.98 (6 h)	−2.45 (6 h)	2.53
CXCL1	−125.33 (6 h)	−3.43 (6 h)	121.91
CXCL2	−40.14 (6 h)	−3.04 (6 h)	37.10
CXCL3	−15.82 (6 h)	−6.71 (6 h)	9.11
IL1R1	−2.82 (6 h)	−5.35 (6 h)	2.53
PDE4B	−7.77 (6 h)	−5.00 (6 h)	2.77
PTX3	−14.72 (6 h)	−2.53 (6 h)	12.19
SERPINE1	−2.98 (6 h)	−5.04 (6 h)	2.06
SLC39A8	−11.80 (6 h)	−3.16 (6 h)	8.64
SMURF2	7.45 (24 h)	3.43 (24 h)	4.02
TLR2	−5.56 (6 h)	−3.10 (6 h)	2.46
TTK	−2.23 (6 h)	−5.76 (6 h)	3.51

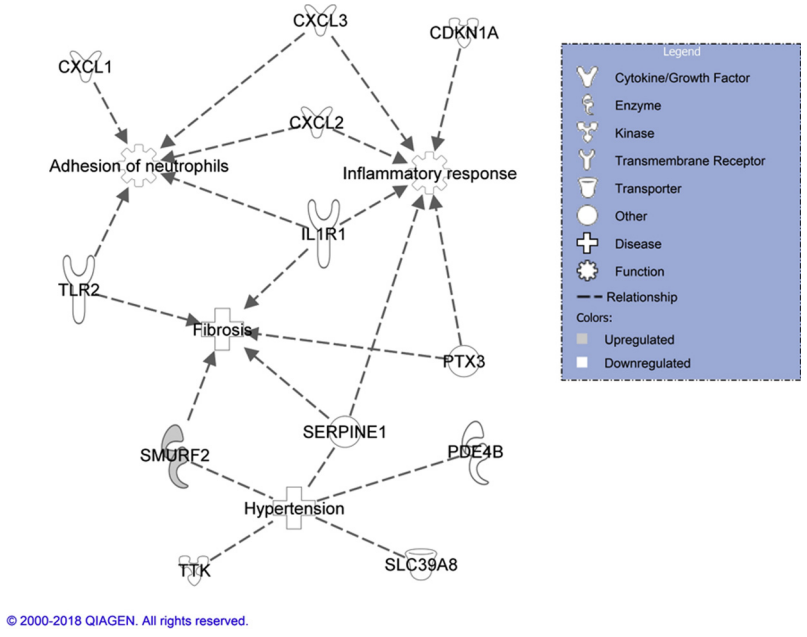

Fig. 4. IPA disease and function overlays for SAEC genes that had a more than two-fold difference in the same regulation direction.

of neutrophils), may be a major player in the response of SAECs to MWCNTs, which may affect cellular responses to cells outside of the SAECs when grown in coculture.

In addition to VEGF-A, IL1R1 was also suggested by IPA to play a role in adhesion of neutrophils. IL1R1 expression was found in both the changed regulation direction and larger fold change analyses, having been associated with a greater downregulation in comparison of the monoculture and coculture six-hour timepoints (Table 4) and continuing to be downregulated in coculture as its regulation increased in monoculture at later timepoints. (Table 2). As IL1R1 plays an important role in mediating the immune and inflammatory responses, these differences in regulation may show a difference in how SAECs grown in different types of cellular conditions respond to MWCNT exposure.

5. Conclusions

Overall, this study took an in depth look at gene regulation in SAECs grown either in monoculture or in coculture with HMVECs following exposure to MWCNTs and determined that multiple genes had either a change in gene regulation direction or were more than two-fold change in regulation. Coupled with our previous findings that global SAEC and HMVEC gene regulation is more concordant with in vivo gene regulation when the two cell types are grown in coculture versus each in monoculture alone [23], this study suggests that using monoculture of cell types to identify toxicological effects may exaggerate these responses and that coculture of multiple cell types may provide a more in-depth assessment of toxicological responses.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxrep.2019.05.010.

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