NTNU – Trondheim
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Epigenetics mechanisms in toxicity and carcinogenicity of diesel exhaust particles

Particles induced variations in gene and microRNA expression in human bronchial epithelial cells

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Dedicated To

Nature , it's Creator

&

My Parents

ACKNOWLEDGMENT

In the Name of Almighty ALLAH to whom my all praises are. He is the one who gave me courage to perform this work. I am pleased to pay credit to Statens arbeidsmiljøinstitutt (STAMI) for offering each possible facility in making my effort triumphant.

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Oslo, 25.06.2015

Author

Abstract

Lung cancer is the leading cause of cancer related deaths worldwide. Several lines of evidence are confirming that air pollution significantly increase the risk of lung cancer. Exhaust from diesel engines is a component of air pollution and categorized as a human carcinogen by IARC. Diesel exhaust particles and the associated complex mixture of organic pollutants, such as PAHs and their derivatives are major components of diesel emissions, have been under surveillance to exert adverse effects on human health.

The aim of the thesis was to identify DEP induced changes on the expression of genes and miRNAs over short period of time and with increasing concentrations of DEP (SRM 2975). An *in vitro* human bronchial epithelial cell (HBEC3-KT) model was set up and cells were exposed for a short span of time (6-72 h) with 100 µg/ml of DEP or with different concentrations of DEP (25-400 µg/ml) for a fixed time of forty eight hours. Additionally, variations in gene/miRNA expression when the HBEC3-KT cells were exposed for long term (27 weeks) to DEP (100 µg/ml) and for short term (6-72 h) exposure to DEP & B[a]P (100 µg/ml and 3µM respectively) were analyzed. Gene expression analysis was carried out on selected candidate genes and miRNAs, which are known to be involved in lung cancer and/or environmentally induced carcinogenesis. The analyses included *CYP1A1, COX2, CDH1, IL1B, HO1, NRF2, miR17, miR21, miR27, miR31, miR146a* and *miR146b*, respectively. In addition, secretion of IL-1-β to the cell culture medium was analyzed. Expression of genes and miRNA's was measured by RT-qPCR, while secretion of IL-1-β was analyzed by ELISA.

CYP1A1, which is a phase 1 metabolizing enzyme involved in biotransformation of xenobiotic compounds, was found to be induced by the exposure of DEP both in the time course experiments (maximum at 48 h) and over a wide range of DEP concentrations. Heme oxygenase 1 (encoded by *HO1*) is involved in many biological processes such as regulation of angiogenesis, cell death, response to oxidative stress and many others. We found that *HO1* expression might be up-regulated when HBEC3-KT was exposed to DEP (and benzo[*a*]pyrene; B[*a*]P), which gives indications of oxidative stress on the cells. DEP has the ability to induce inflammatory response in cells. Interleukin-1beta (IL-1-β) is a key pro-inflammatory cytokine, which regulates the expression of several genes involved in inflammation. We found significant induction of *IL1B* by

the exposure to DEP and B[*a*]P. Similarly, IL-1-β secretion from the cells was stimulated by DEP (and B[*a*]P) exposure, but no clear time or concentration patterns were observed. COX2 is a key enzyme involved in prostaglandin production and prostaglandin function as attractant of inflammatory cells. The *COX2* gene was possibly induced by the DEP exposure to HBEC3-KT cells. In contrast, expression of *CDH1*, *NRF2* and the tested miRNAs were found not to be significantly altered by the exposure to DEP.

In conclusion, these data indicate that exposure of HBEC3-KT to DEP may affect genes involved in xenobiotic metabolism, induction of inflammatory response, and response to oxidative stress. However, further studies are needed for solid conclusions regarding several of the gene/miRNAs.

TABLE OF CONTENTS

Abbreviation

AF-media	Anti freezing media
ApoE	Apolipoprotein E
AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocation protein
BaA	benzo[*a*]anthracene
B[*a*]P	Benzo[*a*]pyrene
BPDE	B[*a*]P-trans-7,8-dihydrodiol-9,10-epoxide
CDK4	Cyclin-dependent kinase
CAMs	Calcium adhesion molecules
CYP	Cytochrome P450
Cq	Quantification cycle
CSC	Cigarette Smoke Condensate
cDNA	Complementary DNA
cAMP	Cyclic adenosine monophosphate
DMSO	Di-methyl sulfoxide
DE	Diesel Exhaust
DEP	Diesel Exhaust Particles
DNA	Deoxyribonucleic acid
DRE	Dioxin responsive element
EMT	Epithelial to mesenchymal transition
E-cadherin	Epithelial cadherin

ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FOXA	Fork-head box A
GFs	Growth factors
HBEC	Human bronchial epithelial cells
hTERT	Human telomerase reverse transcriptase
HAHs	Halogenated aromatic hydrocarbons
IARC	International Agency for research on cancer
KEAP1	Kelch-like ECH-associated protein 1
miRNA	microRNA
mRNA	Messenger RNA
MET	Mesenchymal to epithelial transition
MAPK	Mitogen-activated protein kinase
NHBECs	Normal human bronchial epithelial cells
NSCLC	Non-small cell lung cancer
NNK	4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone
NNN	3-(1-Nitrosopyrrolidin-2-yl) pyridine
N-cadherin	Neural cadherin
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffer saline
PKA	Protein kinase A
PS	Penicillin and streptomycin
PCR	Polymerase chain reaction

qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RTE	Reverse transcriptase enzyme
RT-qPCR	Reverse transcription - quantitative polymerase chain reaction
SD	Standard deviation
SE	Standard error
STAMI	Statens arbeidsmiljøinstitutt / National Institute of Occupational Health
SCLC	Small-cell lung cancer
TP*53*	Tumor protein p53
TE	Tris(hydroxymethyl)-aminomethane)-EDTA (Ethylenediaminetetraacetic acid)
TF	Transcription factors
TCDD	2, 3, 7, 8-tetrachlorodibenzo- p-dioxin
XRE	Xenobiotic Response Element

Introduction

Lung cancer kills about 1.6 million people around the world every year, and is responsible for the largest number of cancer deaths worldwide. In the past decade, it has been recognized that as many as 25% of lung cancers occur in people who have never smoked [9]. Large-scale studies are confirming suspicions that air pollution significantly increases the risk of lung cancer [10, 11]. With every breath, people living in polluted areas may inhale carcinogens and increase their chances to become a victim of cancer. Outdoor air pollution contamination from transportation, power generation, industrial and agricultural emissions, and heating and cooking are elevating the health risks. One of the fuel uses to run especially heavy duty engines is diesel, and exhaust generated by the burning of diesel fuel had serious health impacts on human health. The International Agency for Research on Cancer (IARC) classified diesel exhaust (DE) as human carcinogen of (Group1) in 2013 [12]. A lot of studies have been devoted for determining the diesel exhaust particles (DEP) exposure effects on human health. Workers who work in close proximity of diesel engine exhaust, such as underground miners, tunnel construction workers and also inhabitants near to industry and busy streets are highly exposed to DE.

1.1 Cancer

Cancer is a class of diseases that is characterized by cellular mutation, increased proliferation and aberrant cell growth [13]. Cells accumulate genetic and epigenetic changes that can lead to changes in gene activity and alter the phenotype. Over time, a cell population that ignores normal controls of proliferation may turn into cancer [14].

Cancer is caused by mutations in the genes that control proliferation or apoptosis. The vast majority arises as a consequence of chemical damage to DNA, and is referred to as genetic modifications. After DNA replication and subsequent cell division this damage is converted into a heritable change in DNA. Mutations giving cancer cells the ability to evade normal homeostatic mechanisms are the ones that are found in successful cancer cells [15]. In the later years there has been emerging evidence of epigenetic events in initiation and progression of cancer. **Epigenetics** is defined as heritable changes in the activity of gene expression without altering the DNA sequence itself. Key mechanisms in epigenetics are DNA methylation, histone modifications, nucleosome positioning and regulation by non-coding RNA [16, 17].

Two important families of genes that play a role in development of cancer are proto-oncogenes and tumour-suppressor genes. They encode several proteins involved in controlling cell growth and proliferation. Proto-oncogenes encode proteins that have functions in regulation of cell growth, apoptosis and differentiation. They can be activated to oncogenes by structural alterations, which can lead to cancer. Loss of function of tumor suppressor genes will contribute on the road to cancer, due to their role in inhibiting cell division and promote apoptosis when damage to cells cannot be repaired [14, 18, 19].

1.1.1 Carcinogenesis

Carcinogens are exogenous agents, physical or chemical, which may cause neoplasia (autonomous growth of tissue). They can be genotoxic and can damage DNA structure or non-genotoxic (epigenetic), which modify gene expression without damaging DNA [15, 19]. Tumourigenesis is a multistep process characterised by accumulation of mutations resulting in defects in regulatory circuits that govern normal cell proliferation and homeostasis, and, ultimately, leads to invasive malignancy [13, 20]. Carcinogenesis involves a series of three definable stages, known as initiation, promotion and progression as described in **Fig.1-1.**

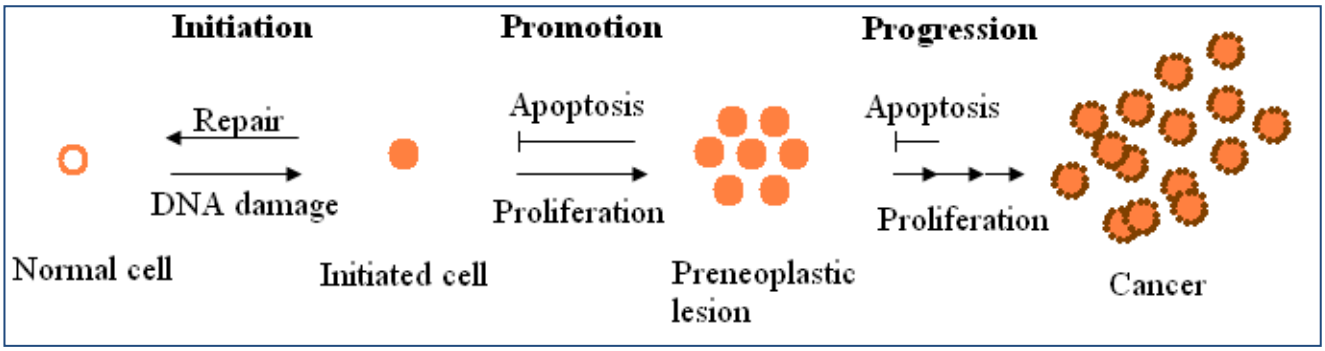


Figure 1-1 The process of carcinogenesis [1]

Induced lesions can be repaired or can be manifested as mutations during replication. Such initiated cells may undergo selective clonal expansion and produce a preneoplastic lesion, a stage known as promotion. Tumour promoter works through different mechanism, where changes in gene expression results in increased proliferation with inhibition of apoptosis. This stage can be reversed if the tumor

promoter(s) are removed. Finally, the pre-neoplastic lesion may progressively develop and change into a malignant cancer (the progression stage) which is not reversible [19].

1.1.2 Hallmarks of cancer

A set of hallmarks have been defined to provide a solid foundation for understanding the biology of cancer [1]. The hallmarks of cancer are six biological capabilities cells acquire during the multistep development of human cancer, such as. 1) Sustaining angiogenesis, 2) Evasion of apoptosis, 3) Self sufficiency in growth signals, 4) Insensitivity to antigrowth signals, 5) Tissue invasion and metastasis and 6) Limitless replication potential (**Fig. 1-2)**.

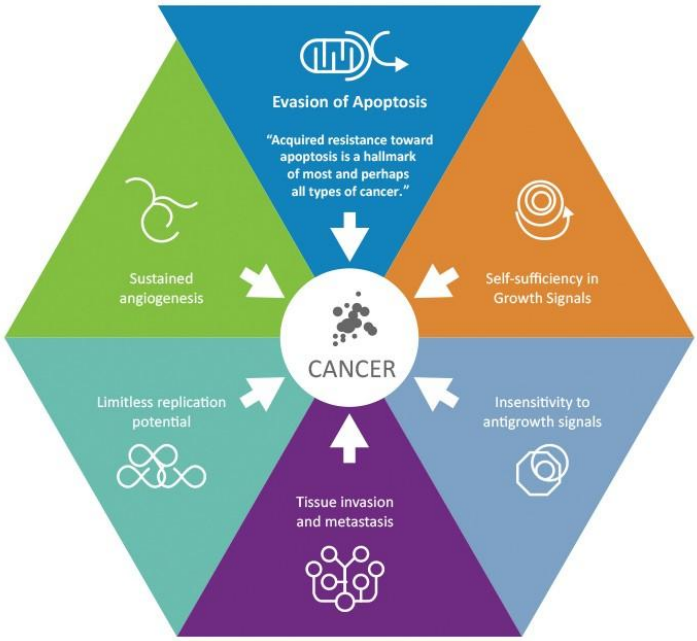


Figure 1-2 The six hallmarks of cancer [13].

Cancer involves dynamic changes in genome. Mutation may results in production of oncogenes and the loss of function of tumor suppressor genes. The tumour suppressor protein p53 is central in regulatory circuits for control of proliferation and promoting senescence and apoptosis. The p53 protein arrests cells with damaged DNA in Gap 1 (G1) phase of the cell cycle. Cancer cells circumvent apoptosis by the loss of *TP53*, which is seen in more than 50 % of human cancers [1, 13, 19].

Hallmarks of cancer each describe anticancer defense mechanisms that function to avoid and eliminate abnormalities, and cancer cells needs to prevail these safety mechanism to gain malignancy

[13]. Inter-individual differences in susceptibility to cancer can be due to single nucleotide polymorphisms (SNPs) in genes involved in any of these defense mechanisms, including DNA repair genes, telomerase genes, cell cycle regulating genes, or genes involved in inflammation [21, 22].

Genomic instability mainly results in further mutation in the DNA during chromosomal reshuffles and inflammatory reactions at the site of pre-malignant and malignant lesions, and thereby facilitating tumorgenesis. Thus, the immune system is playing a dual role in disease, both to combat and promote tumor growth. During inflammation several mediators and factors are released, including survival factors, growth factors, enzymes, and EMT inducing signals [1]. Two emerging hallmarks of cancer are evading destruction from the immune system (evading apoptosis) and re-programming energy metabolism (self sufficiency in growth signals). The immune system can also act as a barrier to tumour formation and progression, and by evading the system cancer cells. Cancer cells have a metabolic switch where they limit their energy production mostly to glycolysis, known as the "Warburg-effect". Cancer cells are typically rapidly dividing cells requiring a lot of energy, which they obtain from glycolysis [1, 23].

1.2 Lung cancer

Lung cancer, also known as carcinoma of the lung or pulmonary carcinoma, is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung. In the **Fig. 1-3** describes types of lung cancer along with their relative abundance and prognosis.

Lung cancer can be divided into two main types: Small cell lung cancer (SCLC) and non-small cell

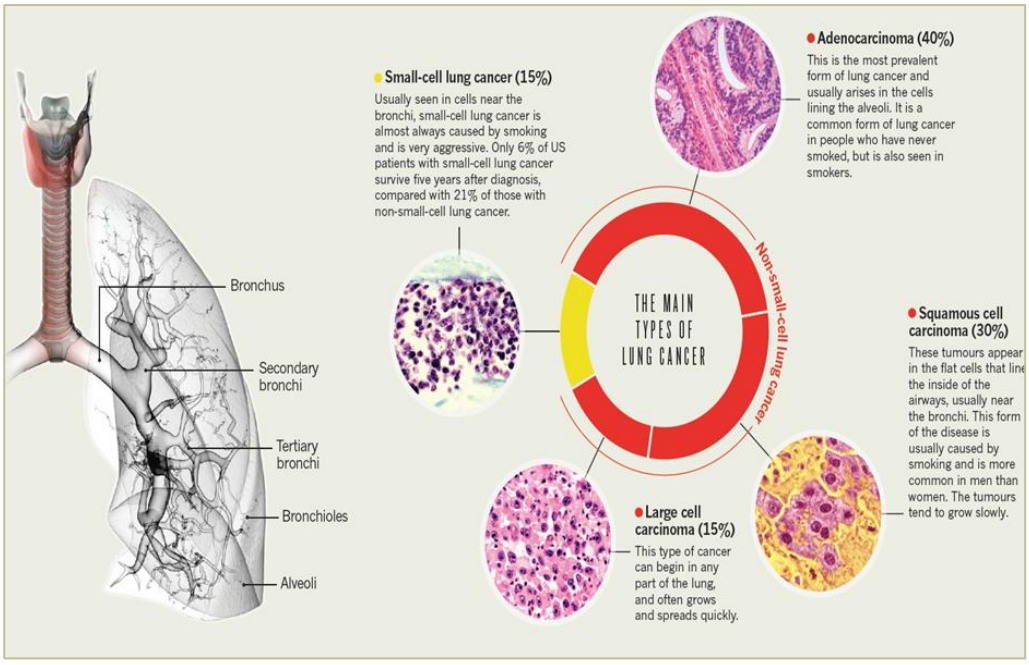


Figure 1-3 Lung cancer and sub-types, relative abundance and prognosis [4].

lung cancer (NSCLC). SCLCs consist of poorly differentiated cells. They are the most aggressive forms of the disease and have a greater potential to metastasize than other type of lung cancer. Non-small-cell lung carcinoma (NSCLC) is a type of epithelial lung cancer and is relatively insensitive to chemotherapy as compared to small cell carcinoma. NSCLCs are including squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large-cell carcinoma (LCC) [24].

1.3 Air pollution and lung cancer

Jonathan Samet, an epidemiologist at the University of Southern California in Los Angeles and the chair of the IARC working group, co-authored a review calling the epidemiological evidence for a link between air pollution and lung cancer "equivocal". But that uncertainty is dissipating rapidly, quoted from[25]. Air pollution in general is tricky to study. Air pollution comprises a complicated cocktail of chemicals and particles that waft from many different sources including industry, traffic, and domestic oven burning. Assessing adverse health effects of air pollution is tricky because air quality differs between countries, cities and even neighborhoods. A growing number of epidemiological studies established a strong link between polluted air and lung cancer [26, 27]. The International Agency for Research on Cancer (IARC) branded [12] outdoor air pollution contamination from transportation, power generation, industrial and agricultural emissions, and heating and cooking as carcinogenic in 2013. Indoor and outdoor air pollutants impair children's growing lungs, and increase the risk of respiratory infections [26]. Particulate matter (PM) air pollution in urban areas is a major public health concern. Burning solid fuels indoors for heating, light and cooking, and burning liquid fuels outdoors to power vehicle engines, results in a complex mix of gases and particulate matter (PM). The most convincing evidence for adverse health effects exists for PM [12]. Lung cancer is one of the most common causes of deaths worldwide in both males and females. 1.59 million people died of lung cancer in the year 2012 [8].

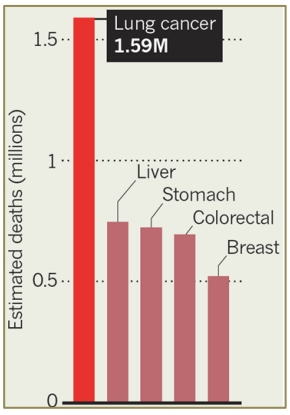


Figure 1-4 Estimated worldwide cancer mortality in the year 2012[8]

Lung cancer is one of the most common cancers and has a poor prognosis. Active smoking is the main cause, but occupational exposures, residential radon, and environmental tobacco smoke are also established risk factors. Air in urban and industrial areas may be polluted with lots of carcinogens and with every breath we inhale; the more carcinogens enter in our lungs.

Carcinogens can be generated from various sources but for this study carcinogen generated from diesel engine is under spotlight. Diesel exhaust is a human carcinogen as classified by IARC in 2013. Diesel engines have a wide range of industrial applications, including on and off-road equipment used, for example, in the mining, railroad, construction, and transportation industries. The use and application of diesel engines in industrial processes is widespread. Estimated 3 million workers were exposed to DE in the 15 countries of the European Union in 1990s [28]. Underground mining and construction workers were among the most highly exposed to DE [29].

1.4 Diesel exhaust particles (DEP)

Diesel exhaust and its particles (DEP) have been under scrutiny for adverse health effects in human. Diesel motor emission is a complex mixture of hundreds of constituents in either gas or particle form. Diesel particulate matter (DPM) is composed of a center core of elemental carbon and adsorbed organic compounds including PAHs and nitro-PAHs, and small amounts of sulfate, nitrate, metals, and other trace elements [30]. DEP represent a complex mixture of hydrocarbons, organic and in-organic elements. Organic PAHs include; Naphthalene, Acenafetileno, Fluorene, Anthracene, Pyrene, Benzo[*a*]anthracene, Benzo[*a*]fluoranthene, Benzo[k]fluoranthene and Benzo[*a*]pyrene. While DEP

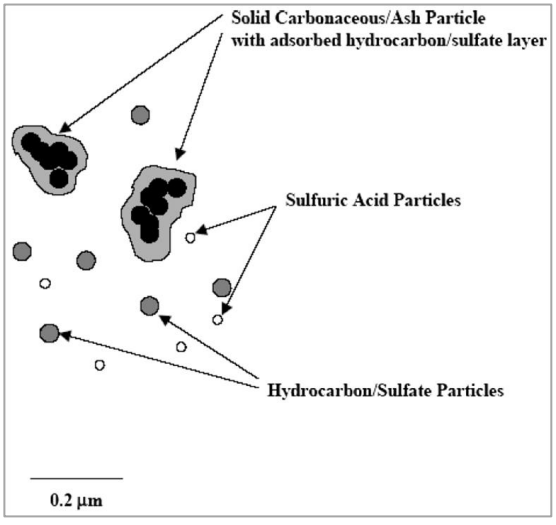


Figure 1-5 Schematic diagram of diesel engine exhaust particles [2].

may also contain metals like; Nickel (Ni), Sulfur (S), Iron (Fe), Vanadium (V), Lead (Pb), Cadmium (Cd), Chrome (Cr) and Copper (Cu). The concentration of organic and inorganic elements and compounds vary in DEP [31]. DEP single particle have small size (< 0.1 µm diameter) and large surface area, which makes them highly respirable and to reach the deep lungs and even blood circulation [2]. The chemical composition and the particles found in DEPs vary according to engine type, operating conditions, fuel formulation (high-/low-sulfur fuel), and the duration of vehicle operation [30]. DEPs from vehicles are a major contributor to metals in air pollution, and their levels could be higher than those in gasoline exhausts [32].

1.4.1 Polycyclic aromatic hydrocarbon (PAHs)

Polycyclic aromatic hydrocarbons (PAHs), a group of genotoxic (and non-genotoxic) carcinogens consisting of over 100 different chemicals that are formed during the incomplete burning of coal, oil and gas, garbage, or other organic substances like tobacco or charbroiled meat. Many PAHs require metabolic activation to DNA-reactive intermediates as epoxides. Such epoxides are mutagenic and carcinogenic, and may react easily with DNA and form DNA-adducts. The model PAH benzo[*a*]pyrene (B[*a*]P) is transformed by cytochrome p450 (CYP) multi-gene family of enzymes to the B[*a*]P-trans-7, 8-dihydrodiol-9,10-epoxide(anti) (+) (BPDE), the metabolite that most strongly interacts with DNA to form B[*a*]P-DNA adducts [7, 33]. DNA-adducts can cause errors in replication, and the bulky aromatic adducts may form stable intercalation between bases and block replication. Adducts may also induce conformational changes in the DNA [34]. And these changes can lead to mutation.

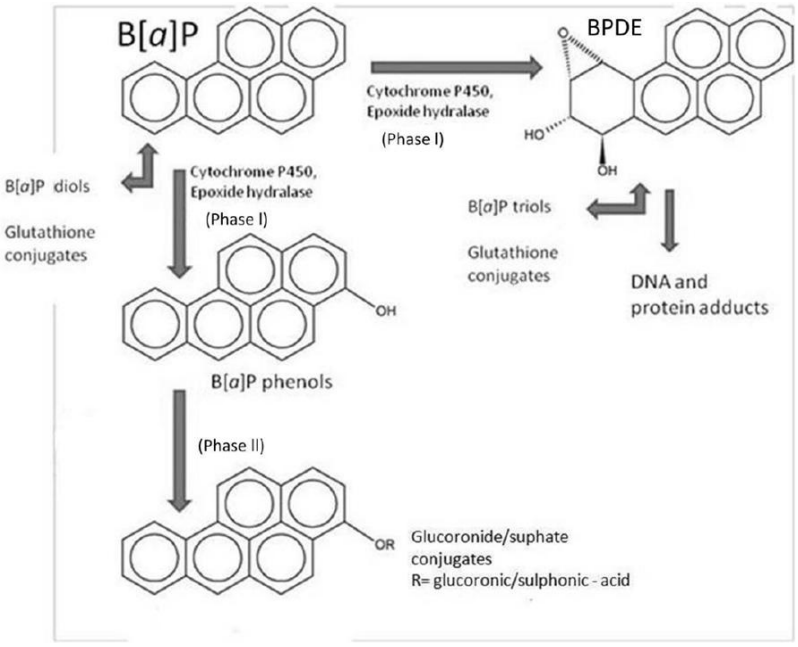


Figure 1-6 Metabolism of B[*a*]P. Horizontal row shows the formation of active carcinogen while vertical row shows detoxification reaction [7]

1.5 DEP induced health effects

Exposure to DEP is associated with human health hazards. **Acute (Short-Term Exposure)** to DEP can cause irritation (e.g. eye throat bronchial) neurophysiologic symptoms (e.g. light headaches, nausea), respiratory symptoms (cough, phlegm) and allergic reactions [35, 36]. While **Chronic (Long-Term Exposure)** causes inflammation and histopathological changes in lungs which cause lung function impairment and also cause cardio vascular and cardiopulmonary diseases. Long-term exposure can be carcinogenic and can cause lung cancer [37, 38].

1.5.1 Intra-cellular effects of DEP

Molecules (e.g. PAHs) in DEP extract can trigger various cell signaling pathways that may lead to the release of inflammatory markers directly or indirectly by causing cell death. Inflammation is considered a key step in the development of health effects associated with PM exposure [39, 40]. Activated immune cells including neutrophils and macrophages can release cytokines, reactive oxygen species (ROS), lipid mediators, and toxic proteases, which may further amplify and contribute to any DEP-induced epithelial damage. A further increased release of pro-inflammatory mediators from the epithelium and/or induced epithelial cell death may augment as well as prolong the inflammatory reactions, and ultimately result in chronic inflammation if the exposure persists. Furthermore, the increased oxidative stress caused by activated immune cells, may also contribute to DEP-induced DNA damage and mutations [41]. Notably, chronic inflammation is also a central part of cancer development [42]. DEP extracts appear to comprise components that are able to activate various membrane and cytosolic receptors.

1.5.2 Aryl hydrocarbon receptor (AHR)

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that is best known for mediating the toxicity and tumor-promoting properties of the carcinogen [5]. Numerous chemicals exhibit high-affinity for binding to AHR, altering its activity in a ligand-dependent manner. Many xenobiotics exhibit strong agonist activity, such as persistent planar halogenated polycyclic

hydrocarbons (for example, many dioxins, dibenzofurans and biphenyls) and polycyclic aromatic hydrocarbons (for example, benzo[*a*]pyrene and benzanthracene).

The unliganded aryl hydrocarbon receptor (AHR) resides in the cytoplasm of a cell. On binding an agonist, the AHR complex translocates to the nucleus and AHR nuclear translocator (ARNT) leading to AHR–ARNT heterodimer formation. Both AHR and ARNT can recruit co-activators, leading to the transcription of a wide variety of genes. The AHR target gene cytochrome P450 1A1 (*CYP1A1*) is

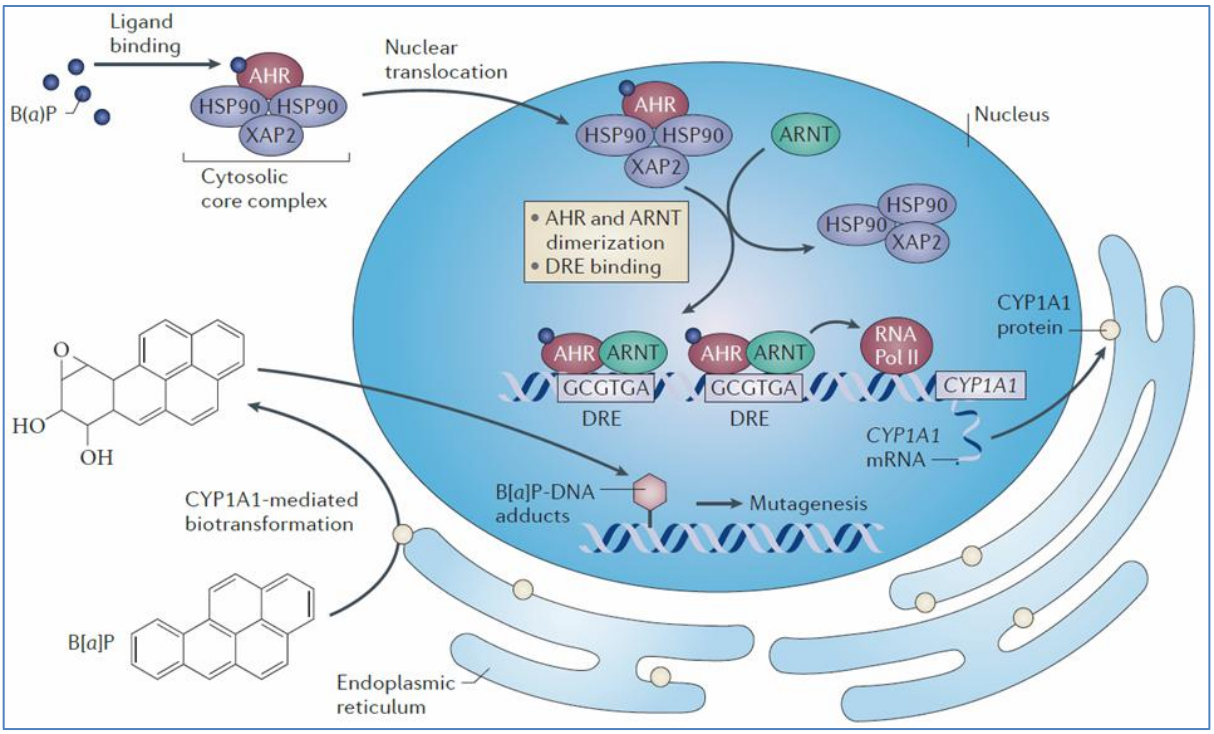


Figure 1-7 B[*a*]P (agonist) mediated activation of AHR [5].

almost totally dependent on AHR activity for expression and is highly induced by AHR activation *CYP1A1* metabolizes a number of pro-carcinogens, such as benzo[*a*]pyrene (B[*a*]P), to intermediates that can react with DNA to form adducts, resulting in mutagenesis [43, 44]. Thus, PAHs released from DEP induce the AHR, which in turn induce *CYP1A1* to biotransform the PAHs. Therefore induction of *CYP1A1* may be considered a measure of AHR activating potential of DEP.

Environmental toxicants like PAHs, ozone particulates, cigarette smoke and peroxyacetyl nitrate can increase the burden of Reactive Oxygen Species (ROS) in human tissues, as well as the upregulation of antioxidant-selective genes [45]. Excess ROS causes oxidative damage to cellular DNA, lipids, and proteins; genetic changes and/or epigenetic alterations can lead to the dysregulation of oncogenes and tumor suppressor genes, ultimately contributing to the pathogenesis of cancer [46, 47]. To alleviate this oxidative stress, there are several antioxidative stress responses, many regulated by *NRF2*. *NRF2* expression is abundant in tissues where detoxification reactions occur, including the lung [48] and under normal physiological conditions it interacts with its own negative regulator, Kelch-like ECH-associated protein 1 (KEAP1) [49]. In times of oxidative stress, selected KEAP1 cysteines become oxidized leading to a disruption of the KEAP1-*NRF2* complex and the release of the *NRF2* peptide. *NRF2* then translocates to the nucleus to transcribe genes encoding various antioxidant proteins and metabolic enzymes collectively known as phase II detoxifying enzymes [50]. Alternative pathways for *NRF2* activation are through the phosphorylation of *NRF2* by protein kinase C (PKC) or RNA-dependent protein kinase R- (PKR-) like endoplasmic reticulum kinase (PERK), resulting in the release of *NRF2* from KEAP1 [51]. In addition, analyses of tumor tissue from a variety of cancers, including lung, display overexpression of the phase II antioxidant enzymes regulated by *NRF2*, such as glutathione-S-transferase (GST) and NADP(H): quinone oxidoreductase 1 (NQO1),which are both known to facilitate the elimination of reactive, oxidized metabolites [52] along with that *NRF2* is also a transcription factor of *HO1*, which is induced by oxidative stress [53]. *HO1* produces heme oxygenase, which is an enzyme that catalyzes the degradation of heme [54]. While studies indicate that there is ample evidence to support the involvement of *NRF2* in cancer biology [33, 55], the dominant focus in human research are somatic mutations in *NRF2* and/or its repressor protein KEAP1, that confer either enhanced tumor escape from apoptosis or resistance to a variety of cancer chemotherapeutics [56].

1.5.3 Inflammation related response by DEP

Inflammation is the immune system's response to infection and injury and has been implicated in the pathogeneses of arthritis, cancer and stroke, as well as in neurodegenerative and cardiovascular disease. Inflammation is an intrinsically beneficial event that leads to removal of offending factors and restoration of tissue structure and physiological function. Prostaglandins play a key role in the

generation of the inflammatory response. Their biosynthesis is significantly increased in inflamed tissue and they contribute to the development of the cardinal signs of acute inflammation. They are generated from arachidonate by the action of cyclooxygenase (COX) isoenzymes [57]. Cyclooxygenase (COX) isoenzymes, exist as distinct isoforms referred to as COX1 and COX2 [58]. COX1, expressed constitutively in most cells, is the dominant source of prostanoids [59]. COX2, induced by inflammatory stimuli, hormones and growth factors, is the more important source of prostanoid formation in inflammation and in proliferative diseases, such as cancer [60].

1.5.4 Cytokines and DEP

Cytokines are a group of proteins which are involved in cell signaling pathway which are released by a broad range of cells e.g. immune cells endothelial cells, fibroblasts etc. They are found to be linked with inflammation and cancer. Inflammatory reactions *in vivo* involve the production and release of a range of signaling molecules such as cytokines, chemokines, and leukotrienes/prostaglandins and adhesion molecules [39, 61]. At a cellular level, the onset of pro-inflammatory reactions tends to start by release of early-responding cytokines such as interleukin (IL)-1α and-β and tumor necrosis factor (TNF)-α. IL-1α/-β and TNF-*α* tend to be expressed as inactive proforms in resting cells and may be rapidly cleaved and released without requiring activation of the transcriptional machinery. This enables an immediate response upon encounters with inhaled pathogens or xenobiotics [62]. IL-1-β, which is encoded by *IL1B,* was found to be upregulated in many cancers [63, 64].

1.5.5 Epithelial to mesenchymal transition (EMT)

The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells; these are multipotent and can differentiate into a variety of cell types.

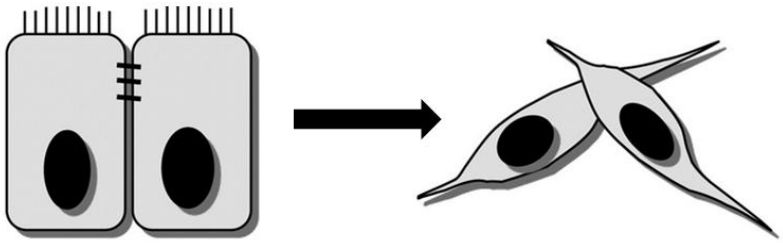


Figure 1-8 Epithelial to mesenchymal transition. Modified from [3]

Cadherins belongs to a super family of proteins that functions in cell recognition, tissue morphogenesis and tumor progression. Epithelial cells structure is stabilized by the prototypical E-cadherin. Other classical cadherins include type I, P-, N- and R-cadherins and type II cadherins (cadherins 5-12) [65]. E-cadherin is encoded by the *CDH1* (cadherin-1), which is defined as a tumor suppressor gene. Loss of E-cadherin expression is associated with a decrease in intercellular adhesion. The neural cadherin (N-cadherin), encoded by the *CDH2*, is found primarily in neural tissue and fibroblasts. A less stable and more dynamic adhesion of cells characterizes the linkages mediated by N-cadherin. N-cadherin undergoes a switch in expression and it is up regulated in invasive tumor cell lines [65-67]. Changes in cadherin expression is referred as cadherin switch and it is crucial event in tumor progression by switching from E-cadherin to N-cadherin, a signaling program is activated that promotes invasive, motility and survival capacities of tumor cells and this phenomenon is epithelial to mesenchymal transition; in which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells.

1.5.6 Epigenetics in focus to miRNA

Epigenetic effect and non-coding gene products have gained research focus over the last two decades because protein coding genes cannot account for all observed genomic effect. Epigenetics comprise a number of mechanisms, including DNA-methylation, histone modification, and microRNAs (miRNAs). Epigenetic changes can be inherited mitotically in somatic cells, which indicate that environmental effects on the epigenome can result in long-term effects on gene expression [68].

1.5.7 MicroRNA

MicroRNAs are endogenous single stranded, short non-coding RNA sequences (approximately 22 nucleotides) that regulate gene expression at post-transcriptional level. MiRNA biogenesis and mechanism of action is shown in **Fig. 1-9**.

The canonical maturation of a miRNA includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III (Pol II/III) and cleavage of the pri-miRNA by the

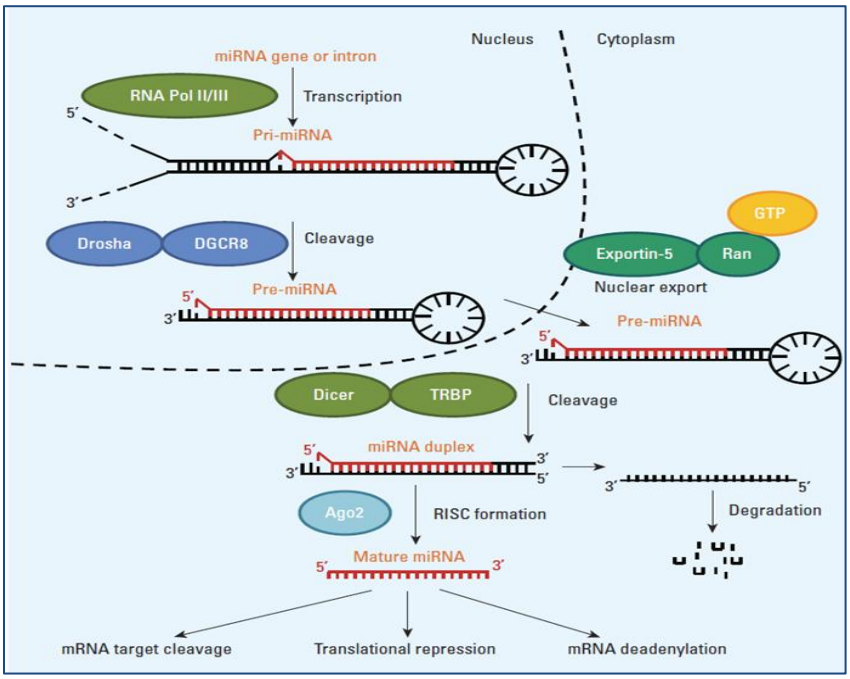


Figure 1-9 MiRNA biogenesis [6].

microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression, or de-adenylation, whereas the passenger strand [6].

1.5.7.1 MiRNA and lung cancer

MiRNAs prove useful in the prognosis, diagnosis, and staging of cancers as non-invasive biomarkers. Profiling has been exploited to identify miRNA as disease fingerprints [69]. The mutated or aberrantly expressed microRNAs and proteins, as well as other compounds [70, 71] and cancer cells [72], could be detected months or years prior to clinical diagnosis and be able to act as cancer biomarkers. A great number of groups are working on the identification of tumor-associated miRNAs, both silence and

over expression of miRNAs may be involved in the development of cancer, while the over expressed miRNAs are more suitable for early diagnosis or prognosis, which have the potential to revolutionize present clinical management [73-75].

These mutated or aberrantly expressed microRNAs and proteins, as well as other compounds [70, 71] *and cancer cells* [72, 76], *could be detected months or years prior to clinical diagnosis and be able to act as cancer biomarkers.*

1.6 Human Bronchial Epithelial Cells

For this thesis, human bronchial epithelial cells (HBEC3-KT) was used, which was established from a normal 65 year old lady and have been immortalized by insertion of cyclin-dependent kinase 4 (Cdk-4) and human telomerase reverse transcriptase (hTERT) [77]. Cdks are heterodimeric complexes composed of both catalytic (kinase) and regulatory (cyclin) subunits. Cdk-4 is a core component of the cell cycle machinery. It phosphorylates retinoblastoma protein (Rb) and further facilitates G1 to S (synthesis) transition in the cell cycle. In a hypophosphorylated state, Rb prevents cells to progress into S phase. Cyclin-dependent kinase inhibitor 2A (p16) is also a regulator of G1 progression, and belongs to INK4 family of cell cycle inhibitors. They act as inhibitors of Cdk-4 and 6. As cells age, p16 accumulates and associates with Cdk-4 and 6 to induce release of D-type cyclins, and then further arrest cells in G1. By over expression of Cdk-4, this arrest is prevented and cells continue in the cell cycle [77, 78].Telomerase is an enzyme that adds DNA sequence repetitions at the ends of chromosomes; the telomeres, and by this prevents progressive shortening of the ends. By expression of hTERT, telomere-dependent senescence can be bypassed and in combination with over expression of Cdk-4 continuously replicating, immortal cell lines is generated [77].

1.7 Aim of thesis

DEP have been under scrutiny for adverse health effects in humans, although main DEP mediators have still not been identified due to ever changing fuel and engine technology which continuously alter exhaust composition. Identification of potential DEP biomarkers or changes in gene and miRNA expression patterns before the onset of disease (e.g. lung cancer), would aid both in the mechanistic understanding of DEP toxicity and possibly early prevention.

The aim of this thesis was to identify changes in expression of genes and miRNAs after short (and long) term exposure to DEP (and B[*a*]P). Genes (*CYP1A1, COX2, CDH1, IL1B, HO1and NRF2*) and miRNAs (*miR-17, miR-21, miR-27, miR-31, miR-146a and miR-146b*) to be studied were selected based on known/suspected roles in lung carcinogenesis and/or involvement in DEP (and B[*a*]P) toxicity. Especially we wanted to:

- Identify changes in target genes/miRNAs after exposure to DEP and B[a]P for short period of time (i.e. 6 to 72 hours)
- Identify changes in the expression of genes by the increasing concentration of DEP.
- Investigate effects of long (27 weeks) and short (6 to 72 hours) term exposure to DEP, by investigating the changes in the expression of mRNA and miRNAs.

Chapter 2

Materials and methods

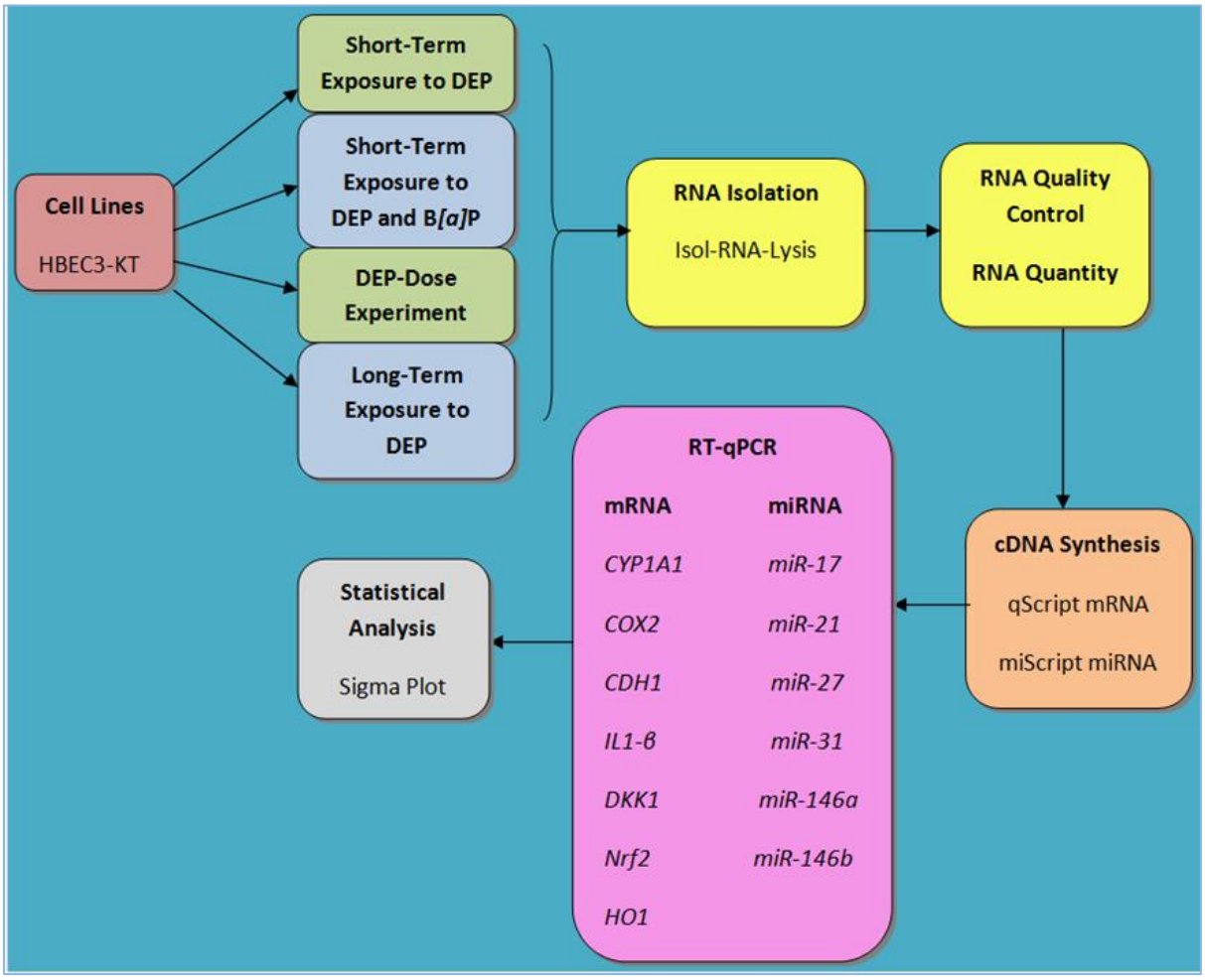


Figure 2-1 Flow chart describing the work flow for identification of genes and miRNA altered by the exposure of DEP

In order to study *in vitro*, effects of DEP on human bronchial epithelial (HBEC3-KT) cells, experiments were conducted in four different ways named as: **Short-Term exposure to DEP**, **Short-Term exposure to DEP and B[*a*]P**, **DEP-Dose Experiment** and **Long-Term Exposure to DEP**. These experimental setups were as follows:

Short-term exposure to DEP: HBEC3-KT cells were exposed to 100 µg/ml of DEP in three parallels for four different time point i.e. (6 h, 24 h, 48 h & 72 h).

Short-term exposure to DEP and B[*a*]P: HBEC3-KT cells were exposed to DEP and B[*a*]P for four different time points i.e. (6 h, 24 h, 48 h & 72 h). Exposure concentration of DEP and B[*a*]P were 100 µg/ml and 3 µM respectively.

DEP-Dose Experiment: HBEC3-KT cells were exposed to different concentrations of DEP i.e. (25, 50, 100, 200 & 400 µg/ml) for 48 hours.

Long-Term Exposure to DEP: HBEC3-KT cells were exposed to DEP (100 µg/ml) for 27 weeks.

Note: Long-Term Exposure to DEP and Short-Term Exposure to DEP and B[*a*]P, cell exposure and RNA isolations were performed by PhD student Iselin Rynning but analysis of gene and miRNA expression was carried out in this thesis. All experiments had three parallels along with three parallels of controls. DEP was diluted in sterile H_2O and control cells were exposed to cell culture medium only (details on cell culturing and exposure are described in **Section 2.1 and 2.4**) Furthermore short-term exposure to DEP was repeated twice while dose response experiment had been done just once due to time shortage.

Total RNA from cells was isolated and cDNA along with miRNA cDNA were synthesized after quantification and quality control of isolated RNA. PCR assays and primers were designed by PhD student Iselin Rynning. Then qPCR was carried out for all the genes and miRNAs to be tested. Expression levels were calculated and the Sigma Plot (v.13) software was used for statistical analysis. List of all the materials used in the experiments is provided in Appendix A. Figure 2-1 shows the work flow for the experiments performed for this thesis.

2.1 Cell Culture

See appendix B for information of general cell culture work. Human bronchial epithelial cells were taken from STAMIs cell bank, where they were stored in liquid nitrogen. The HBEC3-KT was established from normal bronchial epithelial cells from a 65 year old lady who was a smoker with no signs of lung cancer. The cell line was immortalized by the stable introduction of hTERT and CDH4 gene (as explained in **Section 1.6**). Cells were cultured in LHC-9 plus RPMI-1640 media (1:1),

supplemented with 5% fetal bovine serum (FBS) & 1% penicillin and streptomycin. All cells were grown on collagen coated dishes (see appendix B). Incubation conditions were 37°C humidified atmosphere and 5% CO_2. All cell culture work was performed under sterile conditions in OAF LAF benches.

2.2 Seeding cells for DEP exposure experiment

Cells seeded for exposure experiment were cultured in LHC-9 plus RPMI-1640 media with 5% FBS and 1% P.S. Starting material was always approximately 80% confluent cell culture.

1. The media over the cells was removed.
2. The Petri dish was washed twice with PBS (2 x 10 ml).
3. Trypsin solution (1 ml, Invitrogen) was added to the dish, and the cells were incubated (37°C) until they had loosened from the dish. This was checked in the microscope.
4. 5ml medium was added to the dish and the cell suspension was pipetted to a centrifuge tube and centrifuged (1000 rpm, 4 minutes).
5. The supernatant was removed.
6. The pellet was re-suspended in media. Amount of medium used depended on the desired dilution of the cells.
7. Cells were counted using hemocytometer
8. $3x10^5$ cells were seeded in 1.5 ml of media in each 35 mm well of a six well plate.
9. Cells were incubated for 24 hours.

2.3 Sonication of DEP

Diesel exhaust particles were purchased from National Institute of Standards and Technology (NIST) with Standard Reference Material (SRM) no. 2975. As DEP contain mixture of polar and nonpolar compounds, so diesel exhaust particles were sonicated twice (after weighing and before adding to culture media), to ensures even distribution of DEP in to the media and minimal agglomeration of DEP during exposure.

2.3.1 Weighing DEP

Work was performed in STAMI's Nano-Lab, under guidance & supervision of Iselin Rynning.

1. Approximately 5 mg of DEP were weighed out and added to a 1.5ml eppendorf tube.
2. Equivalent to the weight of particles, MQ water in ml was added to the eppendorf tubes to attain a concentration of 10 mg/ml.
3. Tubes were vortexed and spinned down and then put on ice and later on subjected to two step sonication processes.

2.3.2 Sonication of pre-weighed DEP

DEP were sonicated for 0.5 cycles at 100 amplitude for 15 minute and 4°C temperature, using UP200St with Vial Tweeter from Hielscher Ultra Sound Technology, to make a core suspension of DEP. This sonication also helps in making aliquots.

2.3.3 Sonication before adding to media for the exposure

To make a fine suspension, pre-sonicated particles from coarse sonicated eppendorf tubes were transferred to 130 µl Covaris sonication vials and further sonicated using Covaris® M220 Focused-ultrasonicator™. Settings used for sonication were as follow:

Peak power	Duty factor	Cycle/burst	Time	Temperature
75.0	5.0	200	120 sec	6

This treatment resulted in homogeneously distributed particles suspension ready for exposure to the cell.

2.4 Exposure to DEP

24 hours after seeding, cells were exposed to DEP in accordance with the experimental setups.

1. A master mix for DEP was made using pre-sonicated DEP and media.
2. Media of the cells was removed.
3. Master mix was added in accordance with the setup of experiments.
4. Figure 2.1 shows the setup of 6-well plates for short-term exposure experiment and dose response experiment.

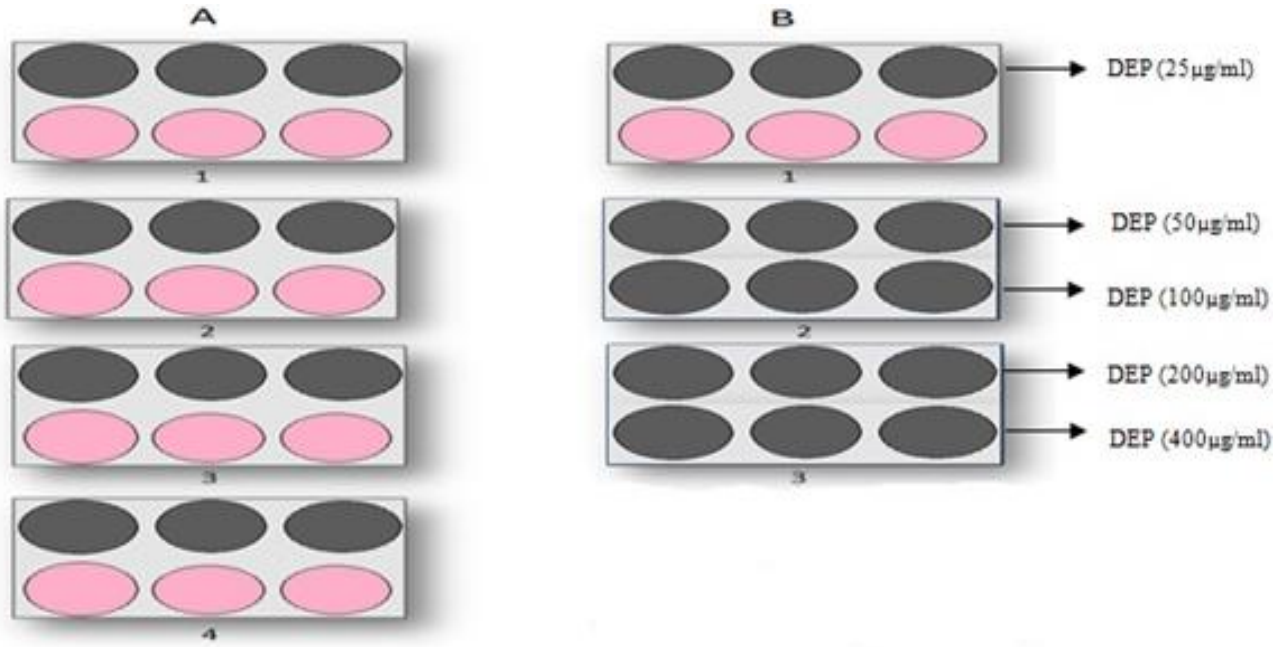


Figure 2-2 well plate setup for exposure, (A 1-4) 6-well plate setup for Short-Term Exposure to DEP, for 6 h, 24 h, 48 h & 72 h respectively. (B 1-3) 6-wel plate setup for DEP-Dose Experiment, concentration of DEP used in DEP-Dose Experiment is mentioned on right corner of the figure. While pink and grey color represent control and exposed to DEP wells.

2.4.1 Stopping exposure

Exposures were stopped on time in accordance with the setup of experiments.

1. 6 well plate containing cells were brought on ice.
2. Media was removed
3. Cells were washed twice with a solution of PBS+5%FBS.
4. Cells were washed twice with only PBS afterwards.
5. Plates were stored at -80°C.

2.5 RNA isolation

RNA was isolated from cell cultures using Isol-RNA Lysis Reagent, a monophasic solution of phenol and guanidine thiocyanate that disrupt and homogenize cells and tissues and inhibits RNases. Isol-RNA isolates total RNA and is thus usable for further analysis of both mRNA and miRNA. Chloroform is added to the TRIzol-RNA homogenate and the solution is centrifuged, which leads to the separation of three phases. The aqueous (upper) phase contains RNA, the interphase contains DNA, and the organic (lower) phase contains proteins. The RNA is precipitated with isopropanol. The pellet is washed with ethanol and resuspended in nuclease-free water.

1. The 6-well multi-dishes were taken from the freezer and placed on ice. Isol-RNA Lysis Reagent (1 mL/35mm well) was added.
2. A cell scraper was used to loosen the cells from the plate. The lysate from each well was transferred to a twist top vial and incubated (room temperature, 5 min).
3. Chloroform (0.2 mL) was added to each vial, and the vials were shaken by hand for 15 seconds before incubated (room temperature, 2-3 minutes).
4. The vials were centrifuged (12000g, 4°C, 15 min).
5. The upper, aqueous phase was transferred to a new twist top vial, added iso-propanol (0.5 mL) and incubated (room temperature, 10min). The lower, organic phase was discarded.
6. The vials were centrifuged (12000g, 4°C, 15 min).
7. The supernatant was aspirated and discarded.
8. The pellet was washed with ethanol (75%, 1mL) and vortexed.
9. The vials were centrifuged (12000g, 4°C, 5 min).

10. The supernatant was discarded; the pellet was dried (15-20 min) in the vial without the cap on.
11. The pellet was re-suspended in nuclease free water (10-15 µL, depending on the pellet size) and put on ice.
12. The vials were incubated (65°C, 10 min), spun down, mixed and put on ice.
13. The vials were put in freezer (-80°C) for minimum one night.
14. The RNA-samples were incubated (65°C, 10 min).
15. RNA was stored at -80°C.

2.6 Quantity of RNA

RNA-concentration was determined by OD measurement (260 nm/280 nm) in an Eppendorf Biophotometer (knowing the concentration of RNA is important to obtain correct dilution in further work). Nucleic acids have absorption maximum at 260 nm. 260 absorption of 1.0 is equivalent to 40.0 µg/ml RNA (and 50.0 µg/ml dsDNA). Pure RNA has a 260/280 ratio of 2.0, while pure DNA has a ratio of ~1.8. Each sample was diluted 70x (1µl RNA sample +69µl T.E. buffer) before OD measurement in a quartz cuvette. After measuring the RNA concentration samples were diluted to 1µg/µl and stored at -80°C until further use.

2.7 cDNA synthesis from mRNA

Complementary DNA is synthesized from RNA by a Reverse Transcriptase (RT) enzyme. For cDNA synthesis from mRNA the qScript cDNA Synthesis Kit (Quanta) was used. This kit contains a combination of oligo-dT and random primers, a MMLV RT enzyme, and a ribonuclease inhibitor protein.

1. A master mix was made of qScript RT-enzyme (1µL per sample), qScript cDNA Super Mix (4µL per sample), and DEPC water (14.0µl per sample). An eppendorf tube for each RNA-sample was added master mix (19µl) and 1µg RNA (1µL). The tubes were vortexed.
2. The eppendorf tubes were incubated in the thermal cycler at the following program:
    - Primer annealing, 22°C, 5 minutes.
    - Synthesis step, 42°C, 30minutes.

- Inactivation of reverse transcriptase, 85°C, 5 minutes.
- Incubation, 4°C, minimum 5minutes.

Following cDNA synthesis, 80µl of TE buffer was added to each nascent cDNA, to have a desired concentration of cDNA 10ng/µl. The cDNA was stored at -20°C.

2.8 cDNA synthesis of miRNA

For cDNA synthesis of miRNAs, miScript kit (Qiagen) was used. Mature miRNAs are not poly-adenylated, and consequently oligo-dT primers cannot be used directly for cDNA synthesis. Therefore, poly (A) polymerase is added to the mix to synthesize a poly (A) tail on all non-coding RNAs. Oligo-dT and random primers can then be used for cDNA synthesis of non-coding RNAs (as well as mRNAs). The oligo-dT primers in the miScript kit have a universal tag sequence on the 5' end, which is utilized in qPCR of miRNAs.

2.8.1 Poly (A) tailing reaction

1. All components were thawed (except enzyme), mixed thoroughly, centrifuged briefly and kept on ice before use.
2. The following components were added accordingly:

Component	Volume
Poly (A) Tailing Buffer (5X)	2µl
Nuclease-Free Water	6µl
Poly (A) Polymerase	1µl
RNA Sample (1µg/µl)	1µl
Final volume	10µl

3. Components were mixed by gentle vortexing and centrifuged briefly.
4. The poly(A) tailing mix was incubated by for 60 minutes at 37°C followed by 5 minutes at 70°C. The mix was then centrifuged briefly and kept on ice before cDNA synthesis.

2.8.2 cDNA synthesis reaction

5. The cDNA synthesis reaction was setup as follow:

Component	Volume
Poly (A) Tailing Reaction (from step 4 above)	10µl
microRNA cDNA Reaction Mix	9µl
qScript Reverse Transcriptase	1µl
Final volume	20µl

6. The cDNA synthesis solution was mixed by gentle vortexing and then briefly centrifuged.
7. The mixture was incubated for 20 minutes at 42°C followed by 5 minutes at 85°C.

After this reaction 80µl of TE buffer was added to each nascent miRNAcDNA, to have a desired concentration of cDNA 10ng/µl. The miRNAcDNA was stored at -20°C.

2.9 RT-qPCR

The polymerase chain reaction (PCR) is a method for amplification of DNA-sequences using repeated cycles of altering high and low temperatures. Reverse transcriptase quantitative PCR (RT-qPCR) is a much-used method for examining gene expression. RT-qPCR uses a fluorescent reporter molecule that binds the PCR product and reports its presence by fluorescence. For the present thesis, SYBR® was used as florescent molecule.

2.9.1 Thermal cycling

Working principle of PCR is based on "thermal cycling" in which DNA-fragments go through alternatively heating and cooling stages designed in the instrument. The main phases during the PCR process are:

Denaturation phase: In this phase, activation of the DNA polymerase enzyme and denaturation of DNA fragments is performed at 95^0C for 2 minutes which results in single stranded DNA molecules.

Annealing phase: After denaturation the temperature is reduced to 60^0C for 30 seconds this lowering in temperature favors the attachment of primers to DNA to produce products.

Extension phase: Primers synthesized DNA strands which act as DNA template in next step and this process goes on and produce much more copies of required DNA sequence.

2.9.2 Cq value

In optimal qPCR reaction the amount of PCR product will be doubled during every amplification cycle. Based on this, the PCR instrument (Step-One) will calculate the concentration of DNA template present at the start of experiment by using a quantification cycle value (Cq), is the (fraction of a) cycle at which the fluorescence level from amplification of a particular sample reaches a predefined level above the background. A lower Cq correlates with higher target expression in a sample.

2.9.3 Reference gene/ RNA

When analyzing gene expression one often uses a "normalization" gene. This gene is introduced to compensate for possible differences in RNA input and level of RNA degradation in cDNA synthesis, as well as cDNA synthesis efficiency. Ideally, the normalization (or reference) gene should be unaffected by different experimental settings. For this thesis, *β-actin* (mRNA analysis) and *RNU6b* & *SNORD44* (miRNA analysis) were used as references for expression measurements, respectively.

2.9.4 Standard curve

For standard curves, series of dilutions are prepared for both reference gene and other genes of interest. These provide information about the linearity, efficiency, sensitivity and reproducibility of the results. They are designed to covers the whole range of expected expression levels of genes included in the experiment. If the PCR reaction is 100% effective then slope value of standard curve will be -3.33, while a range between -3.1 to -3.6 is acceptable. However, one should always ensure that the curves for the different genes are parallel, which means that the normalization gene and target genes have similar efficacy. For this thesis, five points of dilution series in two replicates were prepared and the dilution factor between each point was 1:4.

2.9.5 Melting curve analysis

To control the specificity of the reaction melting curve analysis was performed at the end of each PCR reaction. Specific PCR products will show a distinct peak in the melting curve analysis, while products from primer dimers and hair pin like structure will result in shorter PCR products, which in most cases will show lower melting temperatures.

2.9.6 Primer designing and testing

Primers for *CDH1, COX2, CYP1A1, IL1-B, NRF2, IL8, HO1, miR-17, miR-21, miR-27, mir-31, miR-146a and miR-146b* were designed by using the NCBI primer BLAST in collaboration with PhD student Iselin Rynning. The primers should optimally have a GC-content between 40-60% and the amplicon should be between 50-150 bases. List of all the primers along with their sequences are provided in **Appendix A.**

Primers were tested before gene expression analysis was performed. Standard curves were made for each gene. These standard curves also gives the idea of the expression levels of genes and then also how much cDNA to use for qPCR.

1. Before setting up the reaction, the PCR run program was designed using the Step-One v2.3 software.
2. Primers stock solution were diluted (25 pmol/µl).
3. A mastermix was made for each gene, containing primers, PerfeCTa Syber Green Fastmix and nuclease free water.
4. A 4× dilution of series was made to generate a standard curve.
5. 36µl mastermix was added to PCR tube strips and 10µl of each of the steps in dilution series was added to this.
6. All steps were run in two parallels of 20µl reaction on a 96-well PCR plate.
7. After covering with a plastic film, the plate was centrifuged at 2100 rpm for 30 sec.
8. The PCR plate was inserted into the Step-One instrument and the predesigned program for the gene accordingly was run.

2.9.7 mRNA-qPCR

To obtain optimal Cq values between20-30 cycles for all genes, the cDNA was diluted to appropriate concentrations for each gene. *β-actin* was used as a reference gene for normalization of gene expression. This gene is highly and stably expressed in most cells, and therefore, a cDNA amount equivalent to 5ng RNA input in the cDNA synthesis, was used in the qPCR reaction. The amount of *CYP1A1* cDNA was 100 ng since this gene has a low expression. For all other genes 50 ng were used. Concentration of all primers was 25pmol/µl. For mRNA qPCR PerfeCTa SYBR Green FastMix, ROX (Quanta) was used. This kit contains AccuFast™Taq DNA polymerase, SYBR Green I dye, ROX as an internal fluorescence reference, along with buffer and stabilizers. qPCR of mRNA was setup manually in 96-well plates. A mastermix of 36µl per sample was made:

Component	**Volume**
dH2O	11.88µl
2x PerfeCTa SYBR Green	23µl
Primer up (25pmol/µl)	0.56µl
Primer lo (25pmol/µl)	0.56µl
Final volume	36µl

A 4x dilution series was used to generate a standard curve. In PCR tube strips, 36µl of mastermix was added cDNA (10µl), dH2O (10µl) for non-template control (NTC) or cDNA from 4x dilution series (10µl). All samples were run in 2 parallels of 20µl reactions on a 96-well plate. Cycling conditions are shown in **Table 2.1**.

PCR step	Temperature (°C)	Time	
Accu Fast Taqpolymerase activation	95	3min	
Denaturation of cDNA	95	3 sec	40 Cycles
Primer annealing and extension	60	30sec	

Table 2-1 Cycling conditions for mRNA qPCR with Perfecta kit (Quanta)

Melting curve analysis was performed at the end of each qPCR to control for check for correct PCR product.

2.9.8 miRNA qPCR

RT-qPCR detection of miRNAs is performed by using two primers, one that is miRNA specific (this is normally the entire mature miRNA sequence) and the other is a universal primer that is complementary to the tag sequence added in the cDNA synthesis step. In the analysis, *RNU6B* and *SNORD44* were used as reference RNA in normalization of relative miRNA expression.

Real-time SYBR Green qPCR was performed using 200 nM of specific pre-designed primer for miRNA and PerfeCTa Universal PCR Primer was used along with the appropriate PerfeCTa SYBR Green SuperMix.

Setup of the miRNA qPCR reactions were as follows:

Component	**Volume**
PerfeCTa SYBR Green SuperMix (2X)	23µl
PerfeCTa microRNA Assay Primer (10 µM)	1µl
PerfeCTa Universal PCR Primer (10 µM)	1µl
miRNA cDNA (1ng)	10µl
Nuclease-Free Water	11µl
Final volume	46µl

A 4x dilution series was used to generate a standard curve. In PCR tube strips, 36µl of mastermix was added cDNA (10 µl), dH2O (10 µl) for non-template control (NTC) or cDNA from 4x dilution series (10 µl). All samples were run in 2 parallels of 20µl reactions on a 96-well plate. Cycling conditions are shown in table 2.2.

PCR step	Temperature (°C)	Time	
Pre-incubation/activation	95	2min	
Denaturation of cDNA	95	10 sec	40 Cycles
Primer annealing and extension	60	30sec	

Table 2-2Cycling conditions for miRNA qPCR with Perfecta kit (Quanta)

2.9.9 Normalization of expression data from cell line experiments

Gene expression was quantified by calculating relative changes in mRNA or miRNA level between samples. The Cq value for each gene of interest (GOI) was normalized to the Cq value of a reference gene. Changes in the PCR efficiencies are caused by RT and PCR inhibitors or enhancers, and by variations in the RNA pattern extracted. Even small efficiency differences between GOI and reference gene generate false expression ratio. Variation within measured gene expression data may be due to a combination of biological and technical variations. To reduce the influence of the technical differences the gene expression data should be normalized towards one or more references, such as reference genes that ideally do not vary between the different samples in the individual experiment. The purpose of normalization is to reduce the technical variation within a dataset, enabling a better determination of the biological variation. There are several different ways to normalize high-throughput RT-qPCR data, and no one method has so far become standard. The most used approach is normalization to a reference gene. In miRNA expression analysis, stably expressed miRNAs or other short RNAs, such as short-nucleolar RNA (snoRNA) or short nuclear RNA (snRNA, for exampleRNU6b) are often used for normalization. No universal stably expressed miRNAs has been established. However, it is common to use the microRNAs that are most stably expressed across the

samples in each experiment for normalization. For this thesis *β-Actin* (mRNA) and *RNU6b & SNORD44* (miRNA) were used to as reference for expression analysis respectively. Normalization of the expression was done in two ways:

Normalization to reference gene

The comparative Ct method; **2[delta] [delta] Ct** = [delta] Ct sample - [delta] Ct reference) ×1000. Here, [delta]Ct, sample is the Ct value for sample and [delta]Ct, reference is the Ct value for the calibrator also normalized to the endogenous housekeeping gene and Ct values were generated by qPCR using StepOnePlus Real-Time PCR system.

Note: Statistical comparison (as shown in **Results**), between control and sample (exposed) were carried out using the values (2[delta] [delta] Ct) calculated by this normalization method in order to increase the robustness of the conclusion.

Normalization to control

2[delta] [delta] Ct (Experimental) ÷ 2[delta] [delta] Ct (Control); in this type of normalization of gene expression data the 2 [delta] [delta] Ct value of exposed or experimental is being divided by the 2 [delta] [delta] Ct value of the control. 2 [delta] [delta] Ct values are not multiplied by the factor of 1000 as compared to above normalization method. The calculated values after this method of normalization are referred as **Relative Expression** in **Results.**

2.10 Data analysis and statistical methods

Data and statistical analysis were performed using Microsoft Excel and Sigma Plot 13. Value of $p < 0.05$ was accepted as statically significant.

The Shapiro-Wilk test for small data sets ($n \leq 2000$), was used to test for normal distribution of data. In this test the null hypothesis is stating that data are normally distributed. For 90 percent confidence interval (CI) the p value must be 0.5 or higher for data to be normally distributed. In addition to

Shapiro-Wilk test, Equal Variance Test (Brown-Forsythe) was also performed to test for equality in variance in the data.

For normally distributed data Student *t*-test was used to analyze for significant difference between two groups i.e. (exposed v/s control) and for comparison of multiple groups one-way ANOVA followed by Dunn's test was used.

Non-parametric tests were used for data which were not normally distributed. Mann Whitney U-test is a non-parametric test that compares median value of two groups i.e. (exposed v/s control) of data and finds if they are significantly different from each other. With 95% CI, a p-value of 0.05 or lower will reject the null hypothesis and the two groups are concluded to be statistically significant different. One-way ANOVA on Ranks followed by Dunn's test was used for comparison of multiple groups on data which were not normally distributed.

In the **Results** figures, statistically significant p-values are marked by: ***** ($p<0.05$), ****** ($p<0.001$) and ******* ($p<0.0001$). In case of non-significant differences, no marks were given.

Chapter 3

Results

The cell lines used for this study are human bronchial epithelial cells (HBEC3-KT). Cells were exposed to different concentrations of DEP and B[*a*]P for different time periods in order to find the possible variations in the mRNA and miRNA expression in comparison to controls. Cell exposures were stopped at their respective time points, followed by isolation of RNA and cDNA synthesis. Gene and miRNA expression was then evaluated by qPCR.

3.1 Human bronchial epithelial cells (HBEC3-KT)

Immortalized (HBEC3-KT) cells were taken out from the STAMI's liquid nitrogen-freezer and were sub cultured twice in 100 cm^2 petri plates before seeding for exposure in 6 well petri dishes (10 cm^2, area per well). For details see **Materials and Methods Section 2.1.**

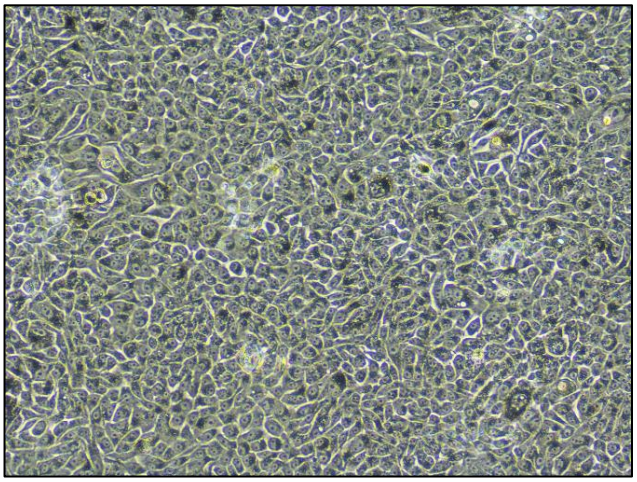


Figure 3-1 HBEC3-KT Cells, after 48 h of sub culturing

3.2 Sonication of DEP

Prior to this thesis several optimization experiments regarding preparation of properly dispergation of DEP in cell culture medium were carried out. Based on previous experimental work, DEP were sonicated twice to ensure equal distribution of particles in the media during the exposure to the cells. For details see **Section 2.3**.

3.3 Gene expression analysis

Exposure was stopped at their respective time point in accordance with the requirement of experiment, followed by RNA isolation and DNA synthesis. Expression of *CYP1A1, COX-2, CDH-1, IL1-B, HO1* and *NRF2*, *miR-17, miR-21, miR-31, miR146a* and *miR-146b* were analyzed by qPCR on a StepOnePlus Real-Time PCR system. Relative expression was calculated by normalization of 2–[delta] [delta] Ct values exposed to their respective 2–[delta] [delta] Ct values of controls. Whereas 2–[delta] [delta] Ct values were evaluated using Comparative Ct method, which is also known as the 2–[delta] [delta] Ct method (see Material and Methods). To compare the statistical significant difference between exposed cells and their respective controls, statistical analysis was performed on data before normalization to control by using their 2–[delta] [delta] Ct values. Statistical tests included parametric *t*-test and one way ANOVA, to compare two and multiple groups respectively. While non-parametric tests included Mann-Whitney U test and ANOVA on Ranks for comparison between two groups and multiple groups respectively. Whereas $p < 0.05$ was accepted as statistically significant.

3.3.1 Reference gene for gene expression analysis

Reference or (housekeeping) gene used for mRNA expression analysis, was *β-Actin*. As proposed by many other studies, *β-Actin* has shown to be a stable gene for such studies. No evident systematic variation of *β-Actin* expression with different exposures and time points were observed in this study. However two types of reference genes were tested for normalization of miRNA expression *RNU6b* and *SNORD44* (see section 3.14).

Results presented in this section are the gene and miRNA relative expression, analyzed by four different experiments. In each case (A) represents **Short-Term Exposure to DEP,** in which HBEC3-KT cells were exposed to 100 µg/ml of DEP for four different time points (6, 24, 48 & 72 hours), (B) represents **Short-Term Exposure to DEP and B[a]P,** in which HBEC3-KT cells were exposed to DEP (100 µg/ml) and B[a]P (3 mM) for four different time points (6, 24, 48 & 72 hours), (C) represents **DEP-Dose Experiment**, in which HBEC3-KT cells were exposed to 5 different concentrations (25, 50, 100, 200 & 400 µg/ml) of DEP for 48 hours, and (D) represents **Long-Term Exposure to DEP**, in which HBEC3-KT cells were exposed to DEP (100 µg/ml) for 27 weeks.

*Long-Term Exposure to DEP is also represented as (C) in miRNA results part. Results from Short-Term Exposure to DEP Experiment no.1 were presented in **Appendix C.**

In the presentation these experiments, gene and miRNA expression levels were normalized to unexposed controls (for details see **Material and methods**).

3.4 Diesel exhaust particles exposure

Fig. 3-2 was taken after 48 hours of exposure of HBEC3-KT cells to 100 µg/ml of DEP. A small portion of the picture was magnified, presented on the right side of the **Fig.3-2**. The zoomed portion indicates the presence of DEP inside the cells, staining the cytoplasm but not the nuclei. This indicates that DEP might penetrate cells through cell membrane. At higher concentrations, DEP can also be agglomerated on top of the cells, as can be seen in the **Fig. 3-4**.

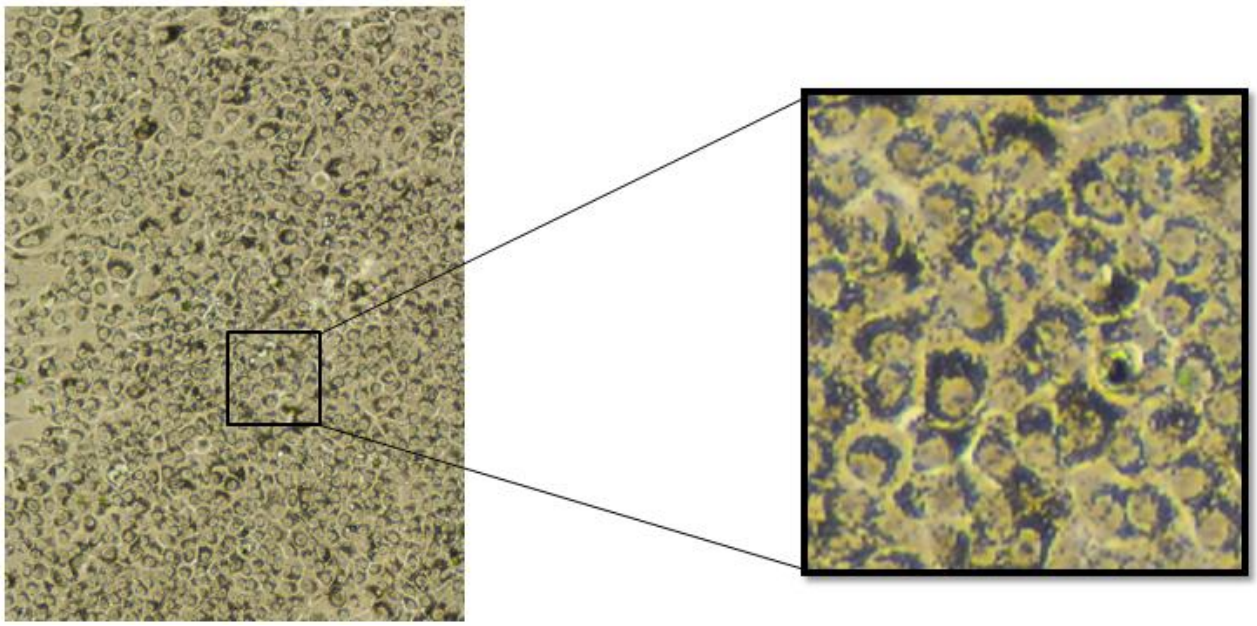


Figure 3-2 HBEC3-KT calls, when exposed to DEP (100µg/ml) for 48 hours.

3.5 DEP-Dose Experiment

Figure 3.3 and **3.4** shows HBEC3-KT cells in a comparative way, before and after exposure of 48 hours with different concentrations of DEP exposure. These figures also indicate the less growth of cells in comparison to the cells (controls) which are not exposed to DEP. **Fig. 3.3** indicates more density of cells after 48 hours in controls as compared to the rest.

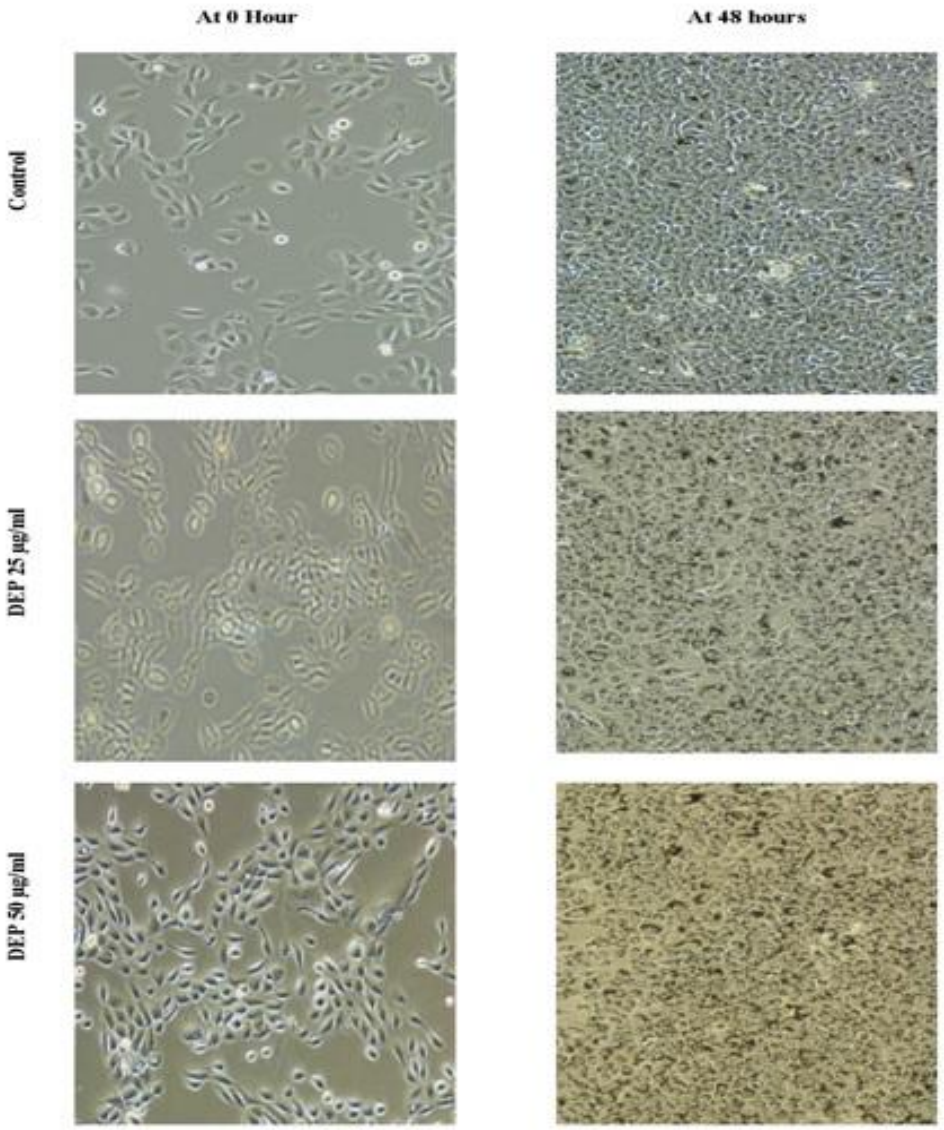


Figure 3-3 Snaps of HBEC3-KT cells from the DEP-Dose Experiment experiemnt, showing comaprison between different concentraions of DEP exposure, before and after 48 hours.

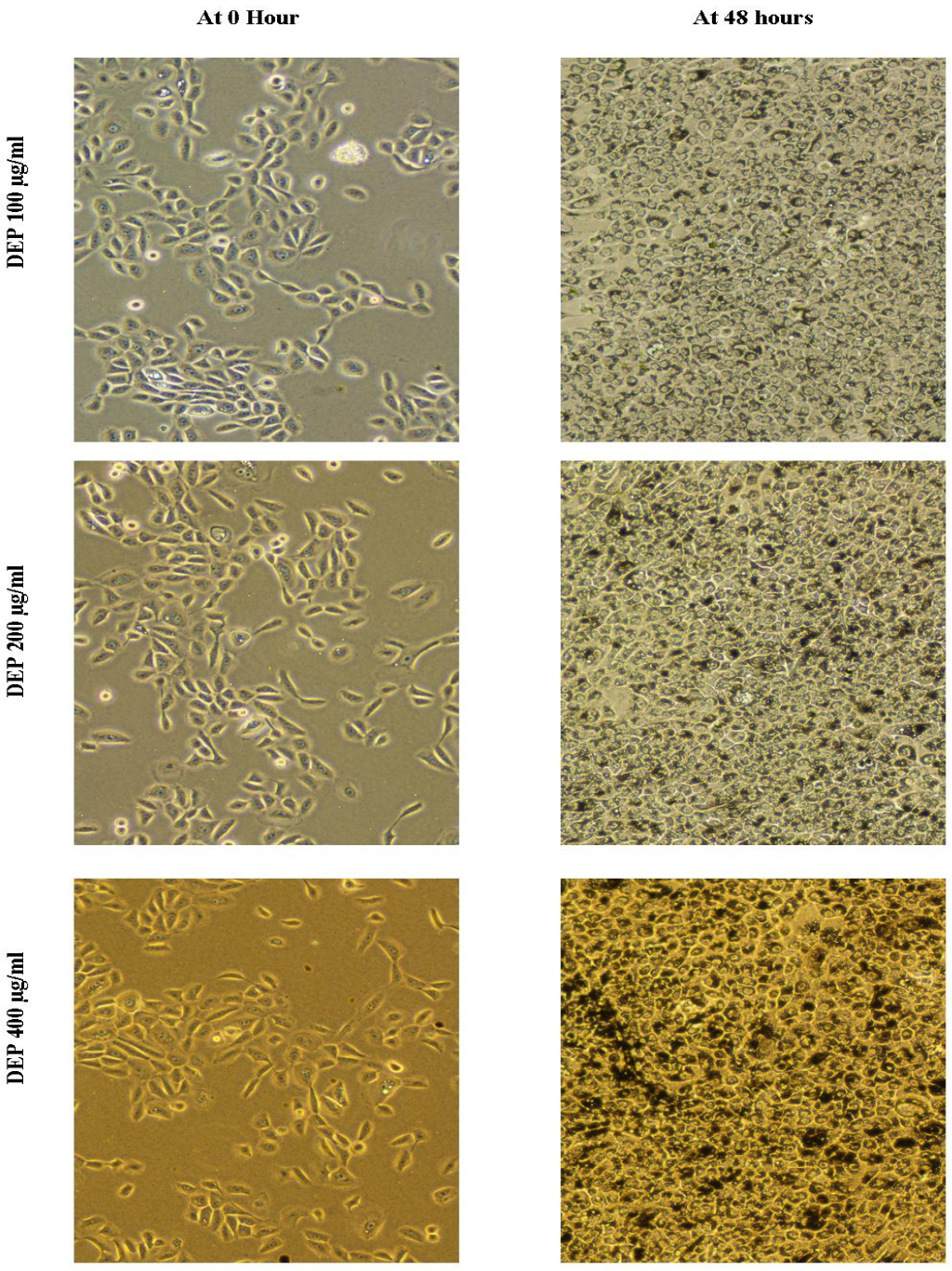


Figure 3-4 Snaps of HBEC3-KT cells from the DEP-Dose Experiment experiemnt, showing comaprison between different concentraions of DEP exposure, before and after 48 hours.

3.6 *CYP1A1*

Figure 3.5 shows variation in expression of *CYP1A1* in HBEC3-KT, exposed to DEP or B[*a*]P, as described below.

Short-Term Exposure to DEP (Fig. 3-5.A) For the time points 48 h and 72 h, *CYP1A1* expression is induced significantly when the cells exposed to 100µg/ml of DEP with a maximum of approximately 9 times after 48 h.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-5.B) Also in this experiment CYP1A1 expression was significantly up-regulated by DEP (100µg/ml) and by B[*a*]P (3mM) after 48h and 72.

In both cases maximum induction (DEP 100µg/ml and B[*a*]P 3 mM) was observed after 48 hours; which made the bases for DEP-Dose Experiment. At 72 h in case of B[*a*]P exposure the expression of CYP1A1 is decreased in comparison to 48 h time point. This effect is comparable to observations from previous experiments in our laboratory (see **Discussion**).

DEP-Dose Experiment (Fig. 3-5.C) There was a significant increase in the expre0ssion of *CYP1A1* expression when the cells were exposed to different concentrations of DEP ranging from 25 µg/ml to 400 µg/ml for 48 h. Induction compared to control cells ranged between 3 to 9 times approximately, but no dose-relationship was apparent with increased DEP concentration.

From these results combined, it appears that *CYP1A1* expression may be up-regulated by the exposure of human lung cells (HBEC3-KT) to DEP and B[*a*]P. Maximum induction was seen after 48 h and the gene was induced over a wide range of DEP concentrations.

CYP1A1

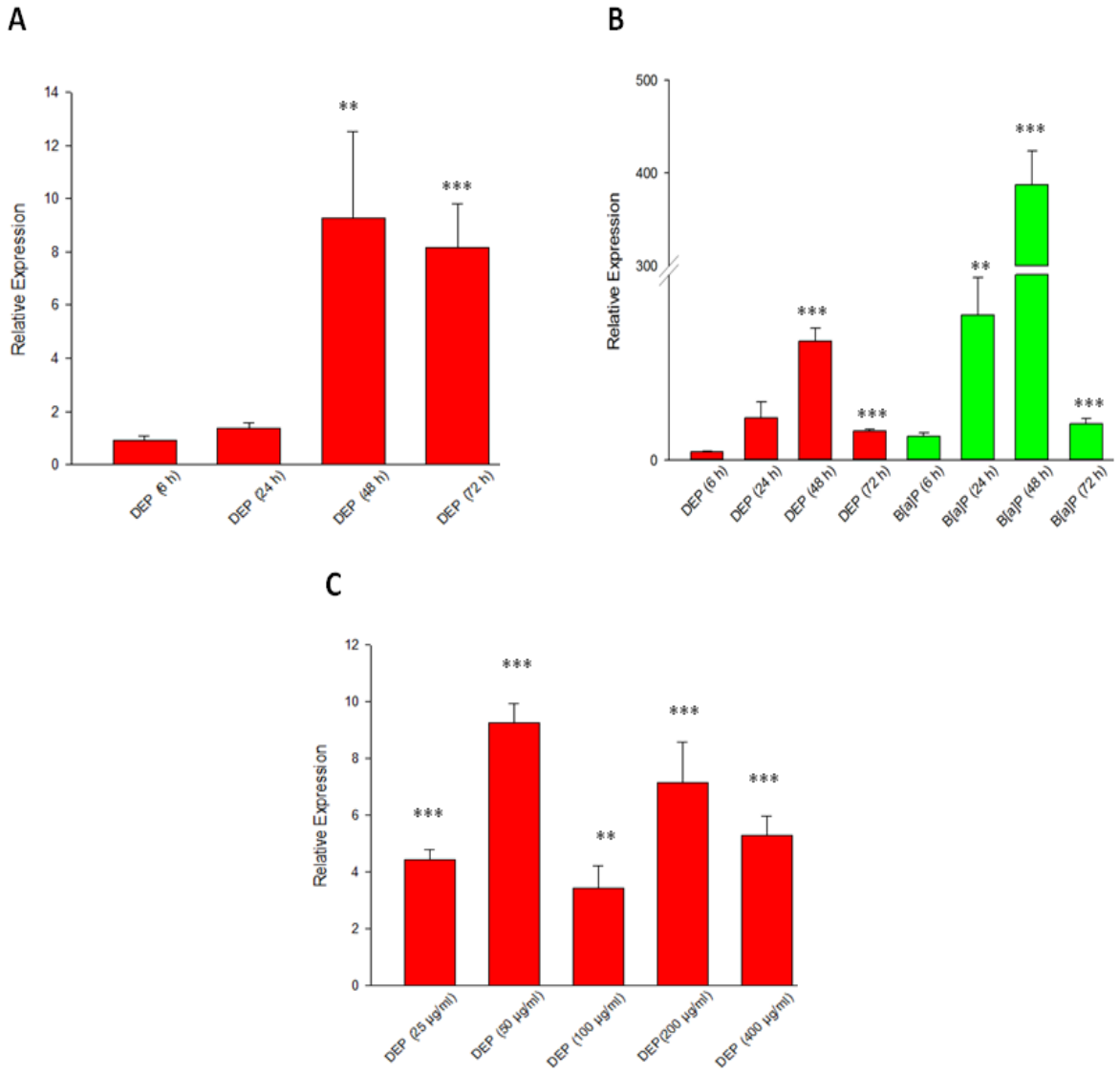


Figure 3-5 Variation in *CYP1A1* expression by the exposure of DEP and B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) DEP-Dose Experiment . Results are normalized to reference gene (*β-Actin*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. *p < 0.05, **p < 0.01, ***p < 0.0001, for comparison between control and exposed cells. For (A and B) statistical test was Student's *t*-test and for (C) it was one-way ANOVA.

3.7 *COX2*

Figure 3.6 shows variation in expression of *COX2* after exposure of HBEC3-KT cells to DEP and B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-6.A) At 24h, *COX2* expression was found to be induced significantly 1.6 times compared to the controls ($p = 0.001$), when the cells were exposed to 100µg/ml of DEP. At 48 and 72 h, a decrease in expression (approximately 50%) may be observed (statistically significant after 48 h).

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-6.B) *COX2* expression for **c**ells which were exposed to DEP (100µg/ml), was found to be significantly up-regulated in the time period of 24 – 72 h, most notably at 24 and 72 h (maximum of 2 times at 72 h). For B[*a*]P exposure only small differences from controls were observed (although significant reduction was indicated after 6 h and increase after 48 h).

DEP-Dose Experiment (Fig. 3-6.C) At the highest concentrations (200µg/ml & 400µg/ml) of DEP, *COX-2* expression was significantly up-regulated (approximately 1.6 times).

In conclusion *COX-2* expression may be up-regulated by 100 µg/ml DEP at 24 h (and possibly at later time points also). At 48 h, significant induction may be reached by the higher concentration of DEP.

COX2

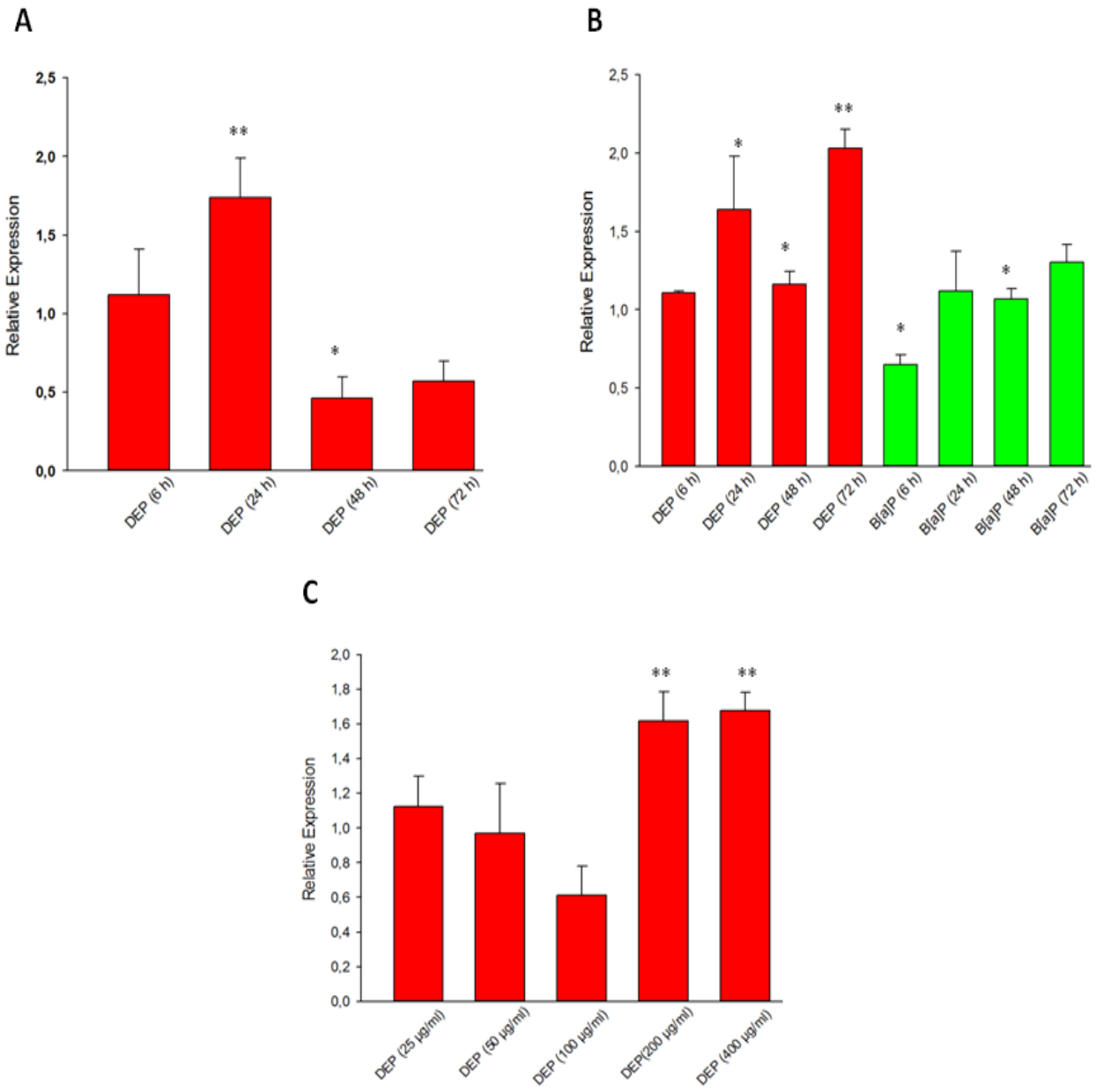


Figure 3-6 Variation in *COX2* expression by the exposure of DEP and B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) DEP-Dose Experiment . Results are normalized to reference gene (*β-Actin*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. *p < 0.05, **p < 0.01, for comparison between control and exposed cells. For (A and B) statistical test was Student's *t*-test and for (C) it was one-way ANOVA.

3.8 *CDH1*

Figure 3-7 shows variation in expression of *CDH1* after exposure of HBEC3-KT DEP and B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-7.A) *CDH1* expression showed a significant (1.9 times) increase at 72 h.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-7.B) Exposure to DEP or B[*a*]P did not alter the expression of *CDH1*to a large extent in this experiment. Only a significant, but minor (0.75 times) down regulation was observed after 48 h exposure to DEP.

DEP-Dose Experiment (Fig. 3-7.C) Significantly increased (1.5 times) and reduced (0.5 times) expression was observed after exposure to 50 and 100 µg/ml, respectively. However, there were no indications of trends in variation of *CDH1* indicated by the graph.

Long-Term Exposure to DEP (Fig. 3-7.D) No change in *CDH1* expression was observed when the cells were exposed to DEP (100 µg/ml) for 27 weeks.

Due to variations in the data between the experiments, no clear conclusion could be drawn regarding expression of *CDH1*. In all cases, observed changes were small.

CDH1

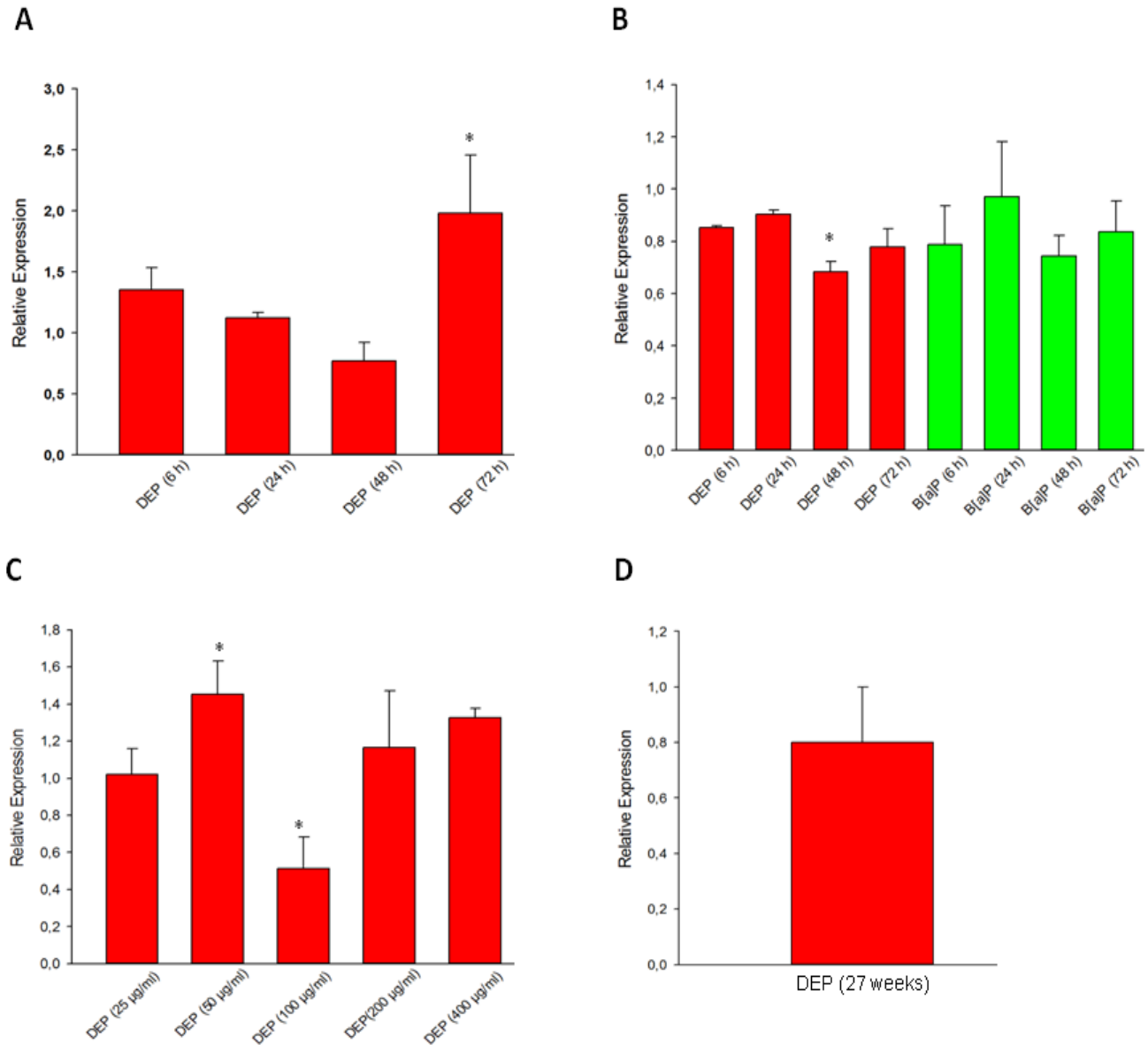


Figure 3-7 Variation in *CDH1* expression by the exposure of DEP and B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) DEP-Dose Experiment , (D) Long-Term Exposure to DEP. Results are normalized to reference gene (*β-Actin*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. *p < 0.05, for comparison between control and exposed cells. For (A and B) statistical test was Student's *t*-test and for (C) it was one-way ANOVA.

3.9 *IL1-B*

Figure 3-8 shows variation in expression of *IL1-B* after exposure of HBEC3-KT to DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-8.A) Expression of *IL1-B* seems to increase by the exposure time as indicated by the results. At time points (24 and 72 hours) the up-regulation of *IL1-B* was significant (maximum induction of approximately 4 times at 72 h).

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-8.B) As seen in previous results *IL1-B* was significantly up-regulated by the exposure of cells to DEP (100 µg/ml) by the time of exposure (maximum induction of approximately 7 times at 72 h). In case of exposure of cells to B[*a*]P, *IL1-B* was induced with a maximum induction (9 times) after 48 h. At 72 h exposure to B[*a*]P, still a significant but smaller induction (5 times) of *IL1-B* was found.

DEP-Dose Experiment (Fig. 3-8.C) *IL1-B* appeared to be up-regulated as the DEP concentration increases. For DEP (200 and 400 µg/ml) the induction was significant (approximately 5 and 7 respectively).

Long-Term Exposure to DEP (Fig. 3-8 D) No change in *IL1-B* expression was observed when the cells were exposed to DEP (100 µg/ml) for 27 weeks.

In conclusion *IL1-B* might be induced as the time of exposure increases for 6 to 72 h as well as by the increasing concentration of carcinogen (DEP).

IL1-B

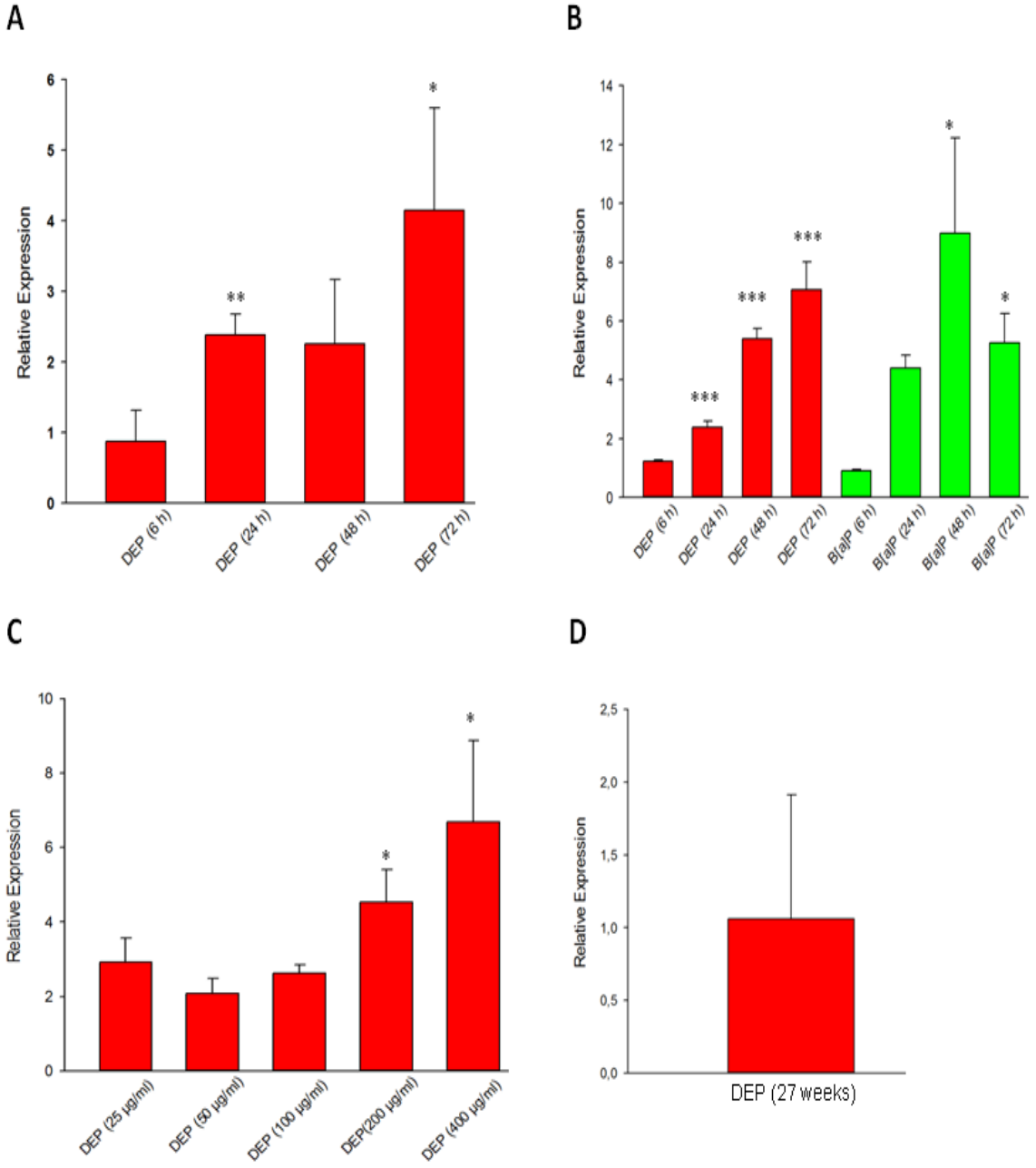


Figure 3-8 Variation in *IL1-B* expression by the exposure of DEP and B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) DEP-Dose Experiment , (D) Long-Term Exposure to DEP. Results are normalized to reference gene (*β-Actin*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. *p < 0.05, **p < 0.01, ***p < 0.0001, for comparison between control and exposed cells. For (A and B) statistical test was Student's *t*-test and for (C) it was one-way ANOVA.

3.10 IL-1-β protein

Since cytokines may be excreted from cells to the cell culture medium, analysis of IL-1-β protein excretion fromHBEC3-KT was carried out. IL-1-β protein was measured from DEP- Dose Experiment and Short-Term Exposure to DEP and B[*a*]P, experiment samples. Cell culture media (approximately 1.5 ml/35 mm well) were collected after respective time of exposure and analyzed by ELISA in collaboration with Dr. Jørn Holme (Nasjonalt folkehelseinstitutt). Due to lack of availability of samples, IL-1-β protein was not determined from Short-Term Exposure to DEP and Long-Term Exposure to DEP experiments.

Figure 3-10 shows the variation in excretion of IL-1-β protein, when the HBEC3-KT cells were exposed to DEP and B[*a*]P.

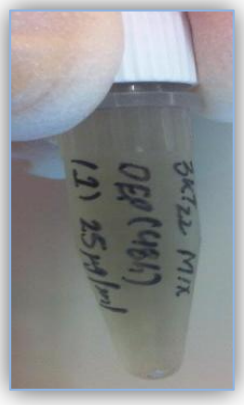


Figure 3-9 Vials containing DEP exposed media

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-10.B) In comparison to controls, IL-1-β protein excretion after exposure to DEP and B[a]P may be increased over time. However due to large variations between replicates (large error bars), it was not possible to conclude on these results and none of the values were statistically significantly different from controls.

DEP-Dose Experiment (Fig. 3-10. C) Significant increase in IL-1-β protein excretion was detected at 25 and 100 µg/ml of DEP, which indicates increased production of IL-1-β protein in comparison to controls. This coincides with the *IL1B* gene expression (**Section 3.9, Figure 3-8. C**), indicating induction of *IL1B* gene by DEP exposure. However protein excretion didn't follow the pattern as did induction of the gene.

Overall based on the results, DEP and B[*a*]P might have the potential to alter the IL-1-β protein excretion in the HBEC3-KT cells with the increasing concentration (of DEP and B[*a*]P) and time.

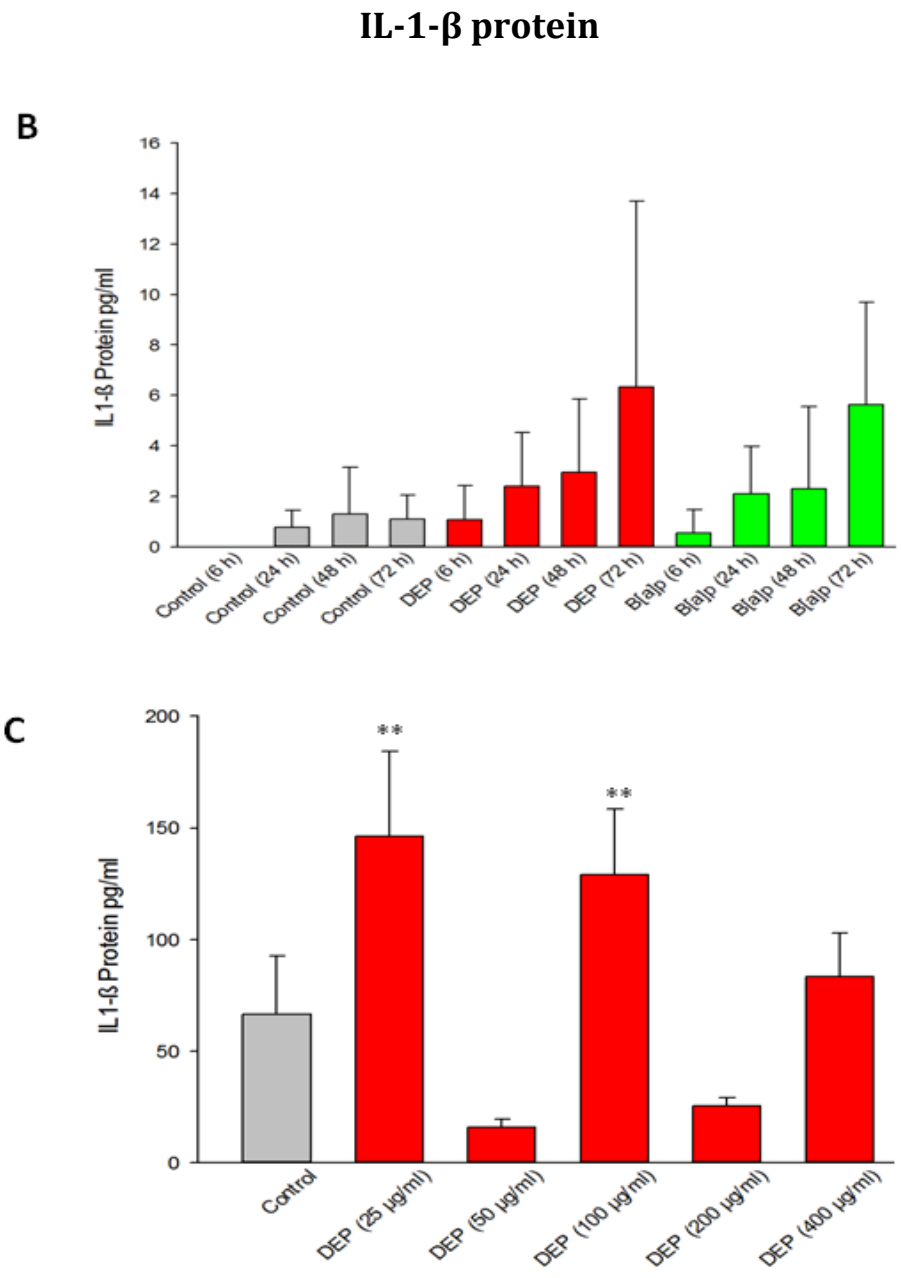


Figure 3-10 Variation in the excretion of IL-1-β protein in the cell culture media, when the cells exposed to DEP and B[*a*]P. (B) Short-Term Exposure to DEP and B[*a*]P, (C) DEP-Dose Experiment. Values are mean ± S.D. of three replicates. **p < 0.01, for comparison between control and exposed cells. For (C) statistical test was one-way ANOVA.

3.11 *HO1*

Figure 3-11 shows variation in expression of *HO1* after exposure of HBEC3-KT to DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-11.A) Expression of *HO1,* showed signs of being up-regulated by the time of exposure to DEP (100 µg/ml) exposure; especially at 72 h where there was an indication of an approximate 6 folds induction. In none of the cases, however the differences were significant.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-11.B) At 6 and 24 hours the expression level might be increased by the DEP exposure, out of which 24 h result was significant (approximately 1.5 times induction). At 72 h there was significant down-regulation (approximately 0.8 times), which was in contrast to the results in Fig. 3-11 A. While in case of B[*a*]P exposure, *HO1* expression might significantly increase at 24 and 48 h (approximately 3.5 and 2.8 times, respectively).

DEP-Dose Experiment (Fig. 3-11.C) *HO1* might be induced at higher doses of DEP. As shown in result, expression of *HO1* was up-regulated by significantly at 200µg/ml and 400 µg/ml of DEP.

From these results it might be interpreted that DEP can possibly induce *HO1* expression when cells exposed to higher concentrations of carcinogen (DEP), but it might be difficult to conclude, that DEP (100 µg/ml) induces *HO1,* as the results were mostly insignificant. However B[*a*]P (3 µM) can possibly induce *HO1* expression.

HO1

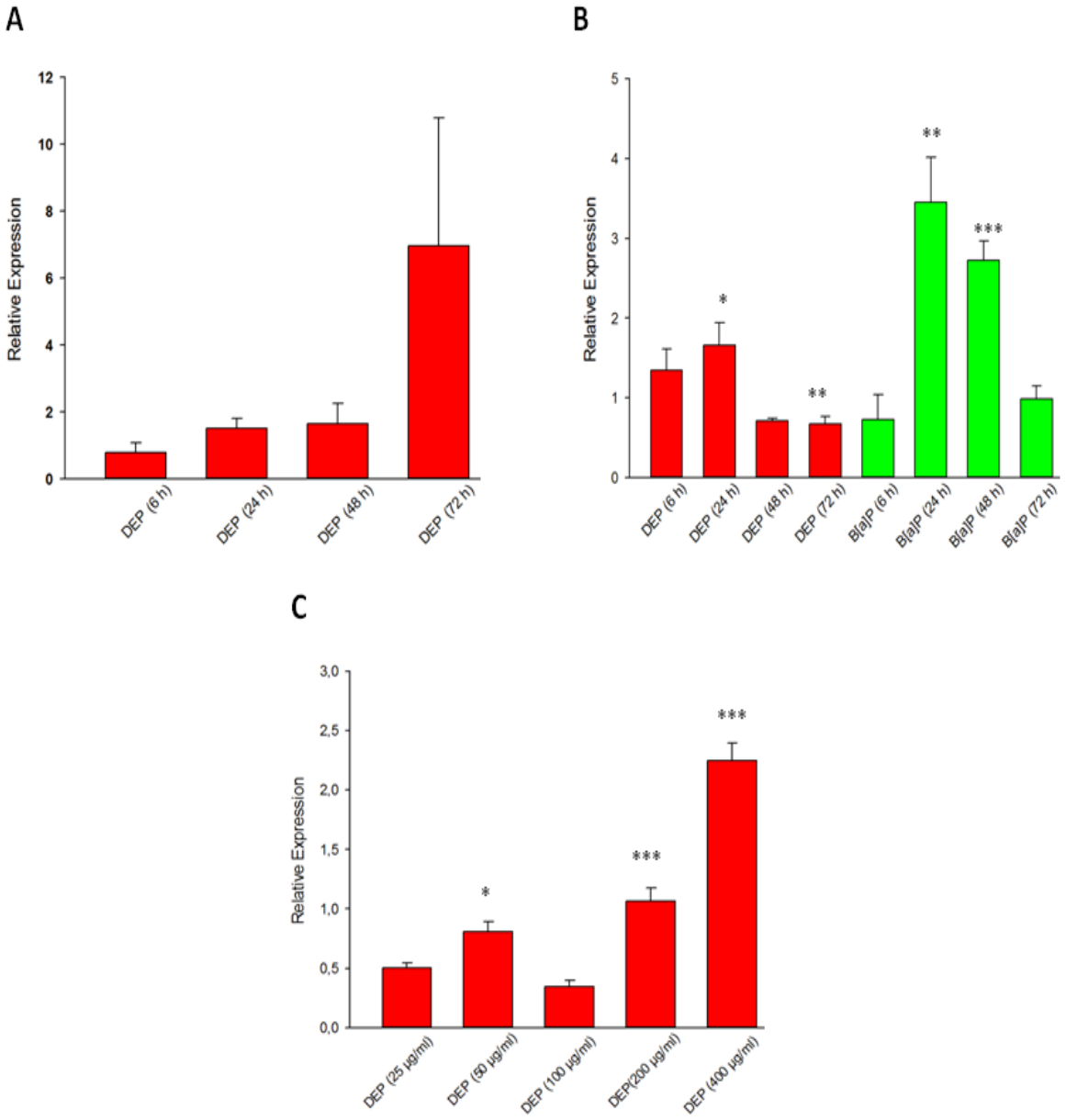


Figure 3-11 Variation in *HO1* expression by the exposure of DEP and B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) DEP-Dose Experiment . Results are normalized to reference gene (*β-Actin*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. *p < 0.05, **p < 0.01, ***p < 0.0001, for comparison between control and exposed cells. For (B) statistical test was Student's *t*-test and for (C) it was one-way ANOVA.

3.12 *NRF2*

Figure 3-12 shows variation in expression of *NRF2* after exposure of HBEC3-KT cells DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-12.A) no apparent significant variation in the *NRF2* expression was indicated by the results.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-12.B) as in previous result no major up or down-regulation was indicated by the DEP or B[a]P exposure. However in case of B[*a*]P, at 24 h and 72 h, the expression of *NRF2* was, significantly up (1.3 times approximately) and down-regulated (0.5 times approximately) respectively.

DEP-Dose Experiment (Fig. 3-12.C) no significant variations in gene expression of *NRF2* was observed with increasing exposure concentrations of DEP.

From the above results it can be concluded, that *NRF2* expression might not be affected substantially by the exposure of DEP or B[*a*]P under these experimental conditions.

NRF2

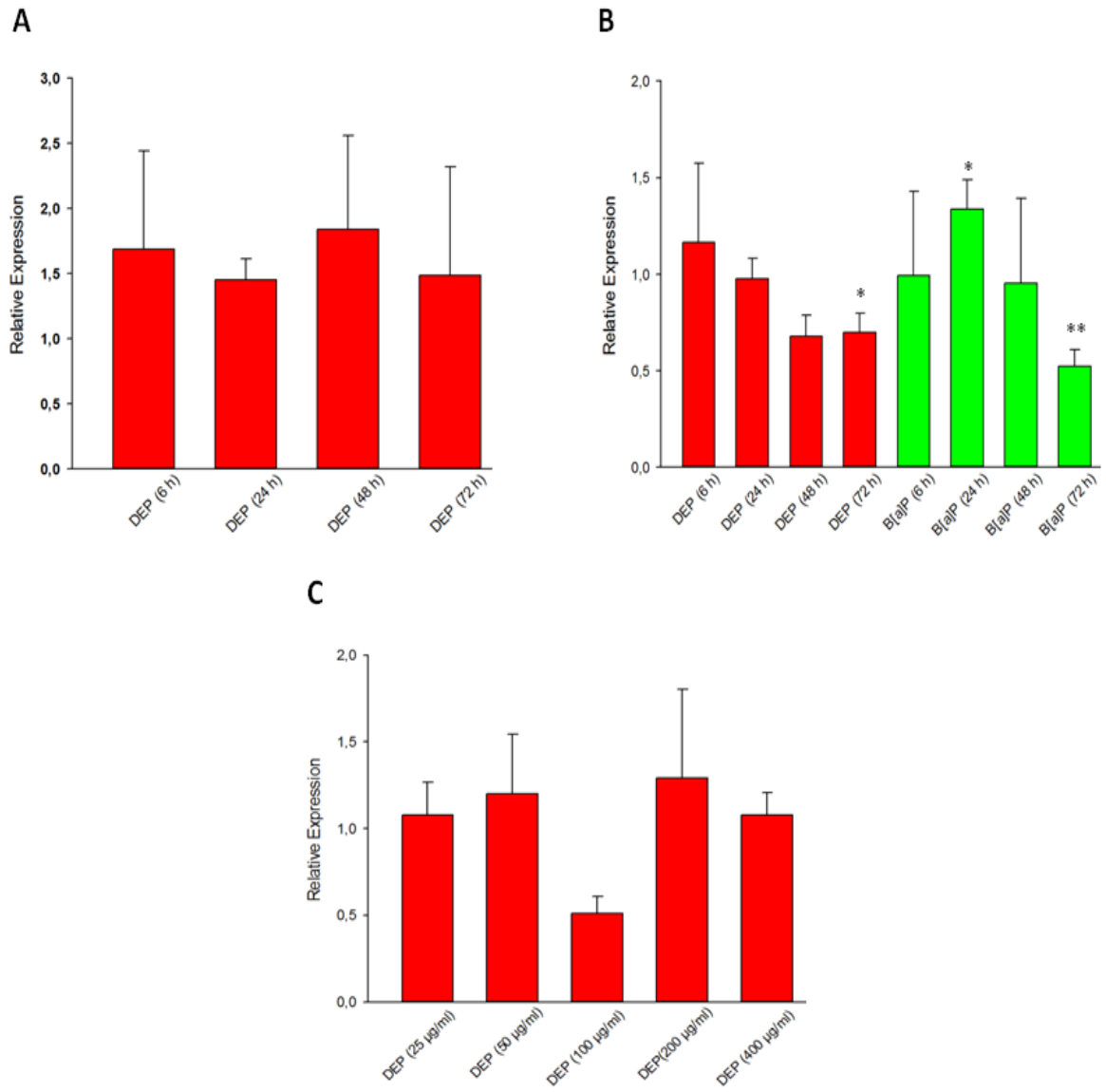


Figure 3-12 Variation in *NRF2* expression by the exposure of DEP and B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) DEP-Dose Experiment . Results are normalized to reference gene (*β-Actin*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. *p < 0.05, **p < 0.01, for comparison between control and exposed cells. For (B) statistical test was Student's *t*-test.

3.13 Micro-RNA

RT-qPCR was used to analyze the expression of miRNAs. However, since different previous studies used different reference RNA's to normalize miRNA expression we first wanted to test the variability of two frequently used candidates: *SNORD44* and *RNU6b.*

To compare variability in *SNORD44* and *RNU6b*, qPCR for both RNAs was done under similar set of conditions (mentioned in S**ection 2.9.8**) and the generated Ct values were plotted in a bar graph as shown in **Fig. 3-13**.

In **Fig. 3-13.A-F** are shown the results of measuring *RNU6b* and *SNORD44* on cDNA's from the Short-Term Exposure to DEP, Short-Term Exposure to DEP and B[*a*]P, and Long-Term Exposure to DEP experiments.

Ct-values of *RNU6b* (Fig. 3-13. A, C & E) Variations in Ct-values of *RNU6b* in Short-Term Exposure to DEP (A), Short-Term Exposure to DEP and B[*a*]P (B) and Long-Term Exposure to DEP (C). In case of Short-Term Exposure to DEP and Short-Term Exposure to DEP and B[*a*]P, the *RNU6b* Ct-value varied to quite some extent within the samples (**A**; 19.8 – 17.2 cycles, **C**; 20 – 16 cycles, and **E**, 21.5 – 20.5 cycles).

Ct-values of *SNORD44* (Fig. 3-13. B, D & F) variations in Ct-values of *SNORD44* in Short-Term Exposure to DEP (B), Short-Term Exposure to DEP and B[*a*]P (D) and Long-Term Exposure to DEP (F). Variation of *SNORD44* appeared to be much smaller compared to that of *RNU6b* (**B**; 25 – 22.5 cycles, **D**; 20.5 – 19.5 cycles, and **F**, 21.8 – 22.5 cycles).

Based on these results, *SNORD44* was selected as reference RNA to normalize miRNA expression in the following analyses as it showed the smallest variation.

Variation of reference gene for miRNA expression

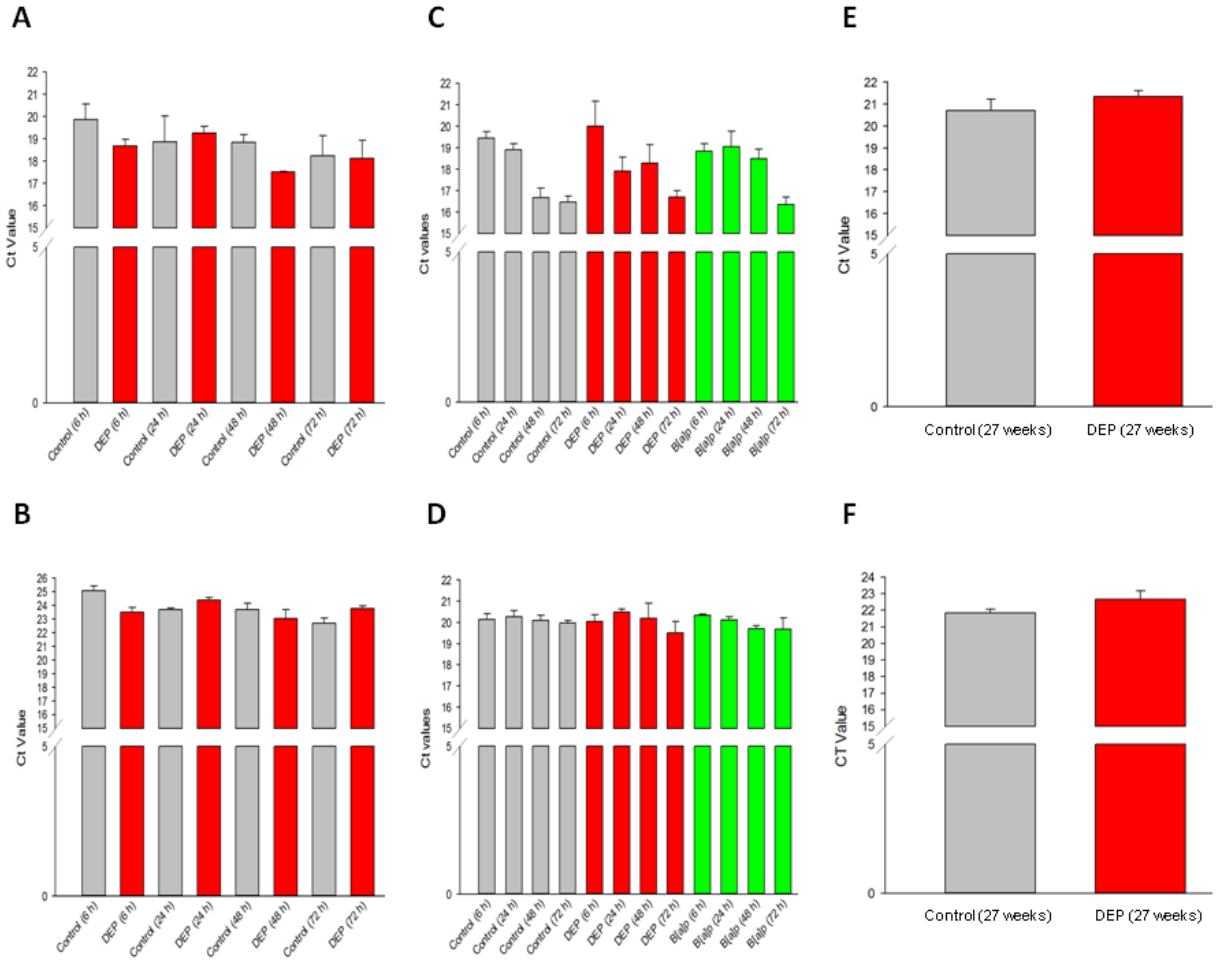


Figure 3-13 Comparison of variations in Ct-values of two reference genes (*RNU6b* & *SNORD44)* in controls and exposed cells of all experiments conducted. Whereas A, C and E represents Ct values of *RNU6b*, while B, D and E represent the Ct values of *SNORD44.*

3.14 *miR-17*

Figure 3-14 shows variation in expression of *miR-17* after exposure of HBEC3-KT to DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-14.A) Although *miR-17* expression at 6 h showed approximately 50% expression level compared to the controls, this difference was not significant. No other apparent changes in expression of *miR-17* were indicated by the exposure to DEP (100 µg/ml)

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-14.B) Similarly, no indication of up or down-regulation observed by the exposure of to DEP or B[a]P.

Long-Term Exposure to DEP (Fig. 3-14.C) When the HBEC3-KT exposed for 27 weeks to 100 µg/ml of DEP, a slight and non-significant induction of *miR-17* possibly was observed.

In conclusion, the data give no indications about up or down-regulation of *miR-17* by DEP or B[*a*]P under these experimental conditions.

miR-17

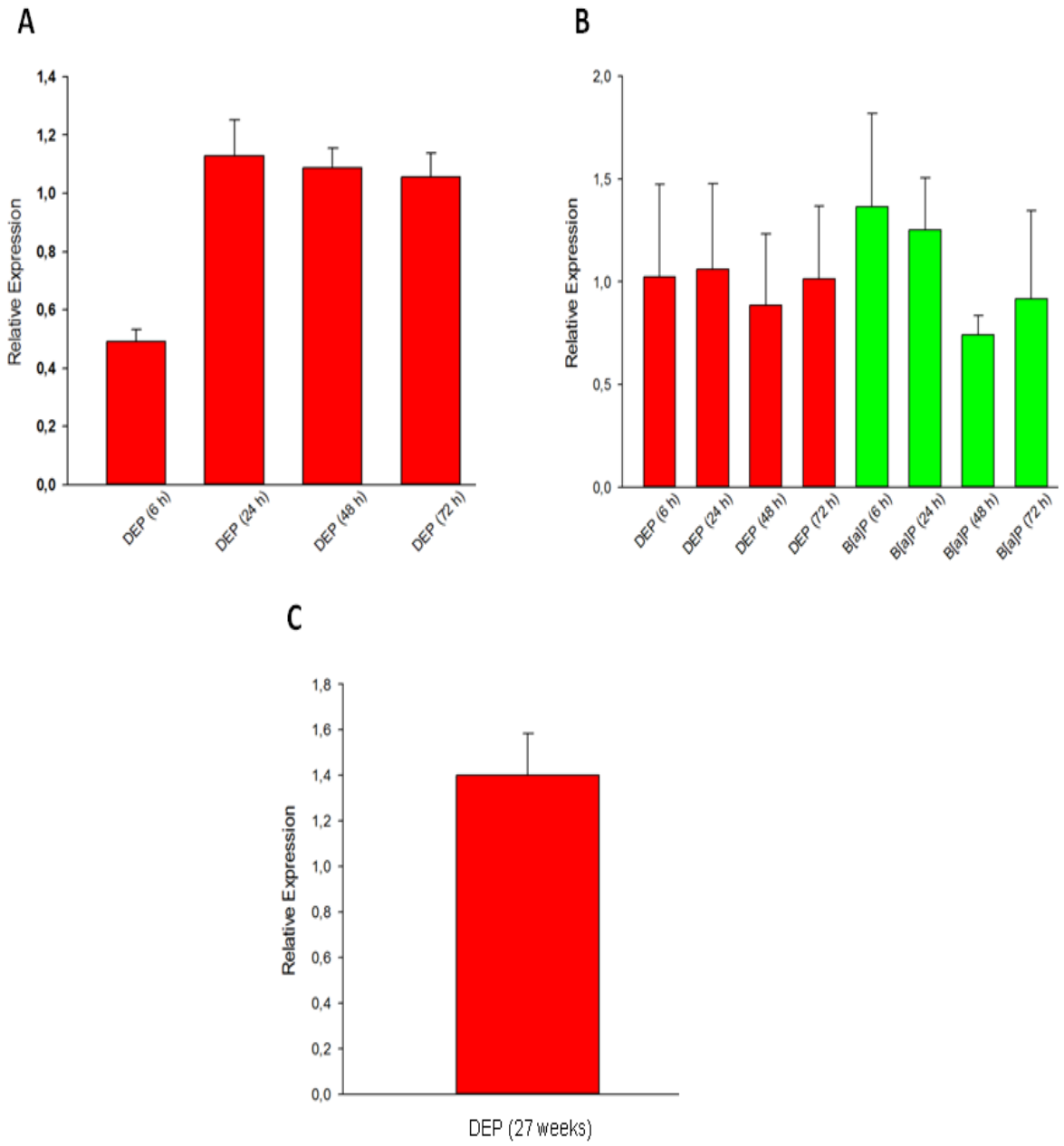


Figure 3-14 Variation in *miR-17* expression by the exposure of HBEC3-KT to DEP or B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) Long-Term exposure to DEP. Results are normalized to reference gene (*SNORD44*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates.

3.15 miR-21

Figure 3-15 shows variation in expression of *miR-21* after exposure of HBEC3-KT to DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-15.A) No observable changes in *miR-21* expression, except at 72 h, at which *miR-21* possibly induced by DEP (100 µg/ml) exposure but the result was statistically non-significant in comparison to control.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-15.B) No changes in expression of *miR-21* was observed either by exposure to DEP or B[*a*]P, with the exception of B[*a*]P exposure for 24 h where a significant reduction in expression was observed.

Long-Term Exposure to DEP (Fig. 3-15.C) A slight, but non-significant induction of *miR-21* expression in contrast to control was observed when the cells were exposed to DEP (100 µg/ml) for 27 weeks.

Due to the variation between the experiments, it was not possible to conclude about effects of DEP or B[*a*]P on *miR-21* expression under these conditions.

miR-21

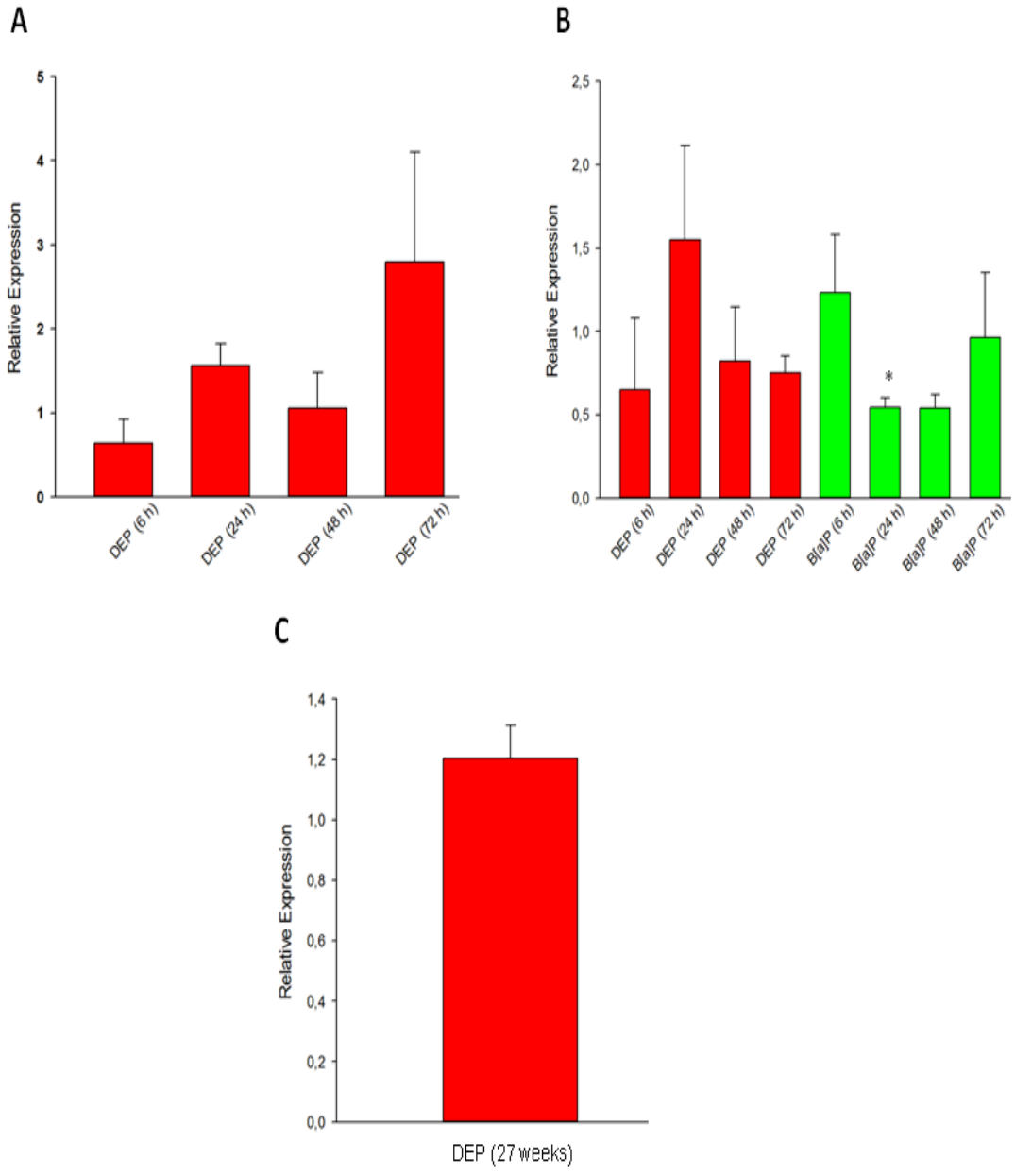


Figure 3-15 Variation in *miR-21* expression by the exposure of HBEC3-KT to DEP or B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) Long-Term exposure to DEP. Results are normalized to reference gene (*SNORD44*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. *p < 0.05, for comparison between control and exposed cells. For (B) statistical test was Student's *t*-test.

3.16 *miR-27*

Figure 3-16 shows variation in expression of *miR-27* after exposure of HBEC3-KT to DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-16.A) Possibly, a small gradual induction of *miR-27* as the time of exposure increased, starting from 24 hour. At 6 h, expression of *miR*-27 appeared to be reduced (0.8 times), but none of the result were significant.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-16.B) When the cells were exposed to 100 µg/ml of DEP no statistically significant changes in expression of *miR-27* were observed. At 24 hour an induction could be indicated, but for this data point the replicates showed large variation (non-significant). When the cells were exposed to B[*a*]P no apparent change in expression of *miR-27* was observed.

Long-Term Exposure to DEP (Fig. 3-16.C) No apparent change in the expression of *miR*-27 was observed after long duration of DEP (100 µg/ml) exposure.

Based on these data, *miR-27* appears not to be affected by the exposure to DEP or B[*a*]P in this study. Although some indications of induction were observed, when cells exposed to DEP (100 µg/ml) for a short-span (3-17.A), the results were non-significant and no conclusions can be made.

miR-27

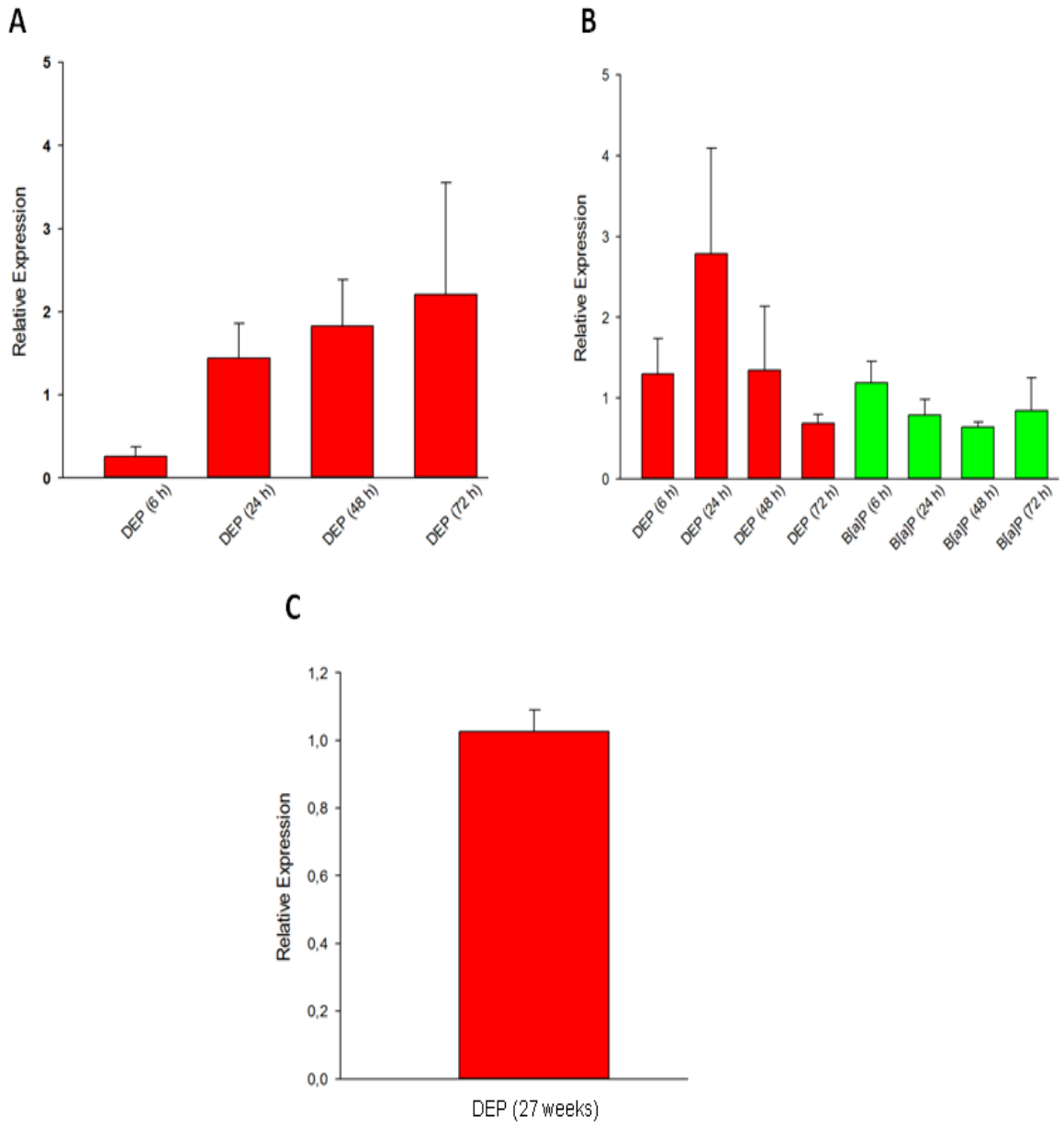


Figure 3-16 Variation in *miR-27* expression by the exposure of HBEC3-KT to DEP or B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) Long-Term exposure to DEP. Results are normalized to reference gene (*SNORD44*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates.

3.17 *miR-31*

Figure 3-17 shows variation in expression of *miR-17* after exposure of HBEC3-KT to DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-17.A) at 48 hours of exposure to DEP (100 µg/ml), a statistically significant induction (approximately 1.5 times) of *miR-31* was indicated. At the other time points, no indications of changes in the expression of *miR-31* were observed.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-17.B) No statistically significant changes in *miR-31* expression were observed by exposure to either DEP or B[*a*]P between 6 and 72 h.

Long-Term Exposure to DEP (Fig. 3-17.C) after 27 weeks of exposure of DEP (100 µg/ml) to HBEC3-KT cells, *miR-31* a minor up-regulation in comparison to control could be indicated, but the results were insignificant statistically.

Overall, based on these data there were no clear indication on effects of DEP or B[*a*]P exposure on *miR-31* expression; the possible exception being for 24 h in the Short-Term Exposure to DEP experiment.

miR-31

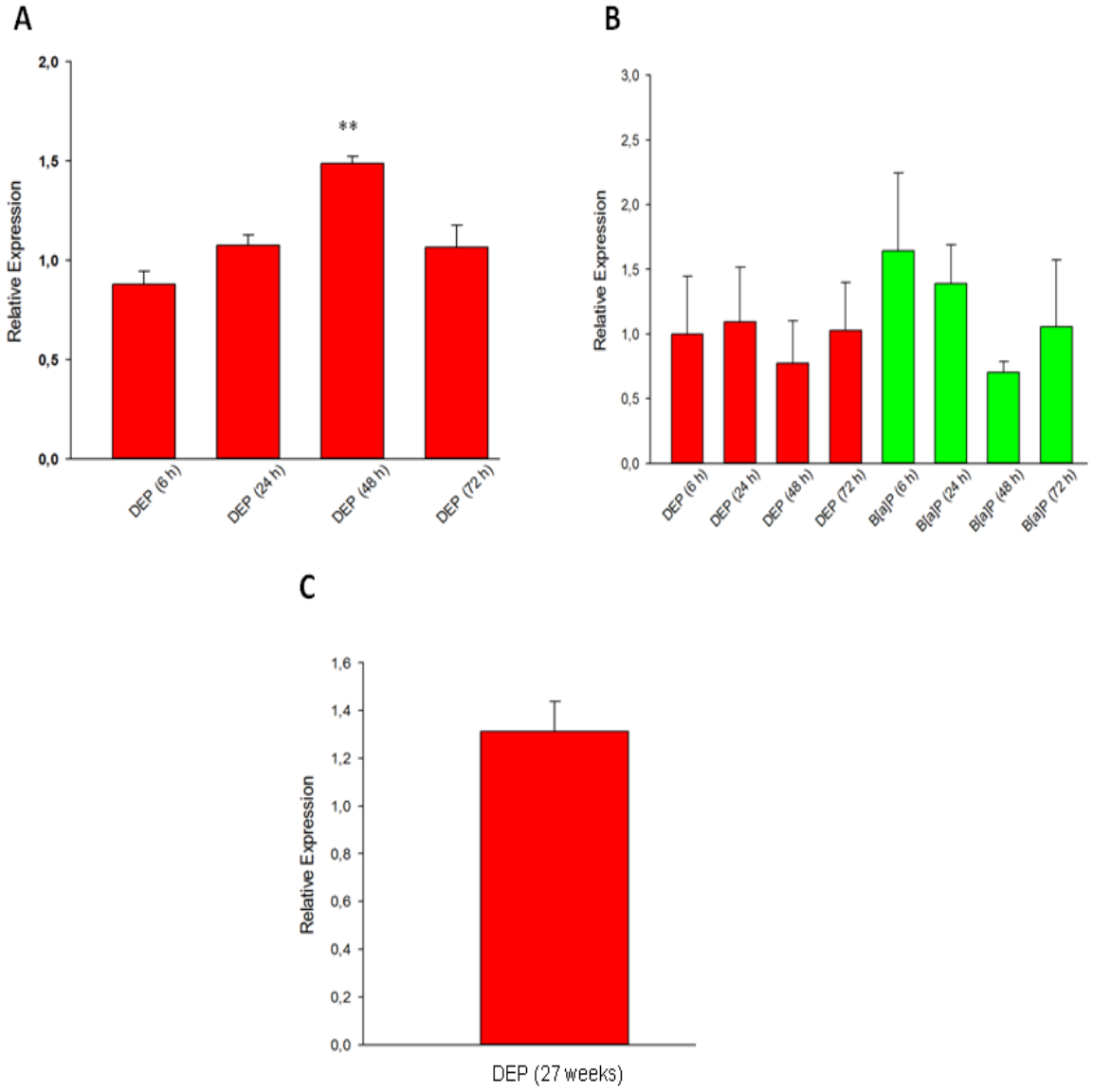


Figure 3-17 Variation in *miR-31* expression by the exposure of HBEC3-KT to DEP or B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) Long-Term exposure to DEP. Results are normalized to reference gene (*SNORD44*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. **p < 0.01, for comparison between control and exposed cells. For (A) statistical test was Student's *t*-test.

3.18 *miR-146a*

Figure 3-18 shows variation in expression of *miR-146a* after exposure of HBEC3-KT to DEP or B B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-18.A) No noticeable change in the expression of *miR-146a* was observed in accordance with the experiment conducted (in all cases non-significant).

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-18.B) Similarly, no apparent changes in the expression of *miR-146a* were observed when cells exposed to 100 µg/ml of DEP. After 6 hour of B[*a*]P exposure *miR-146a* expression was non-significantly higher than the controls, and at 48 hours it was significantly reduced (0.5 times approximately), but there were no clear pattern associated with this change.

Long-Term Exposure to DEP (Fig. 3-18.C) Long exposure of DEP did not appear to alter the expression of *miR-146a*.

From these results it is no possible to conclude about whether *miR-146a* expression in HBEC3-KT may be influenced by the exposure to DEP and B[*a*]P under these experimental conditions.

miR-146a

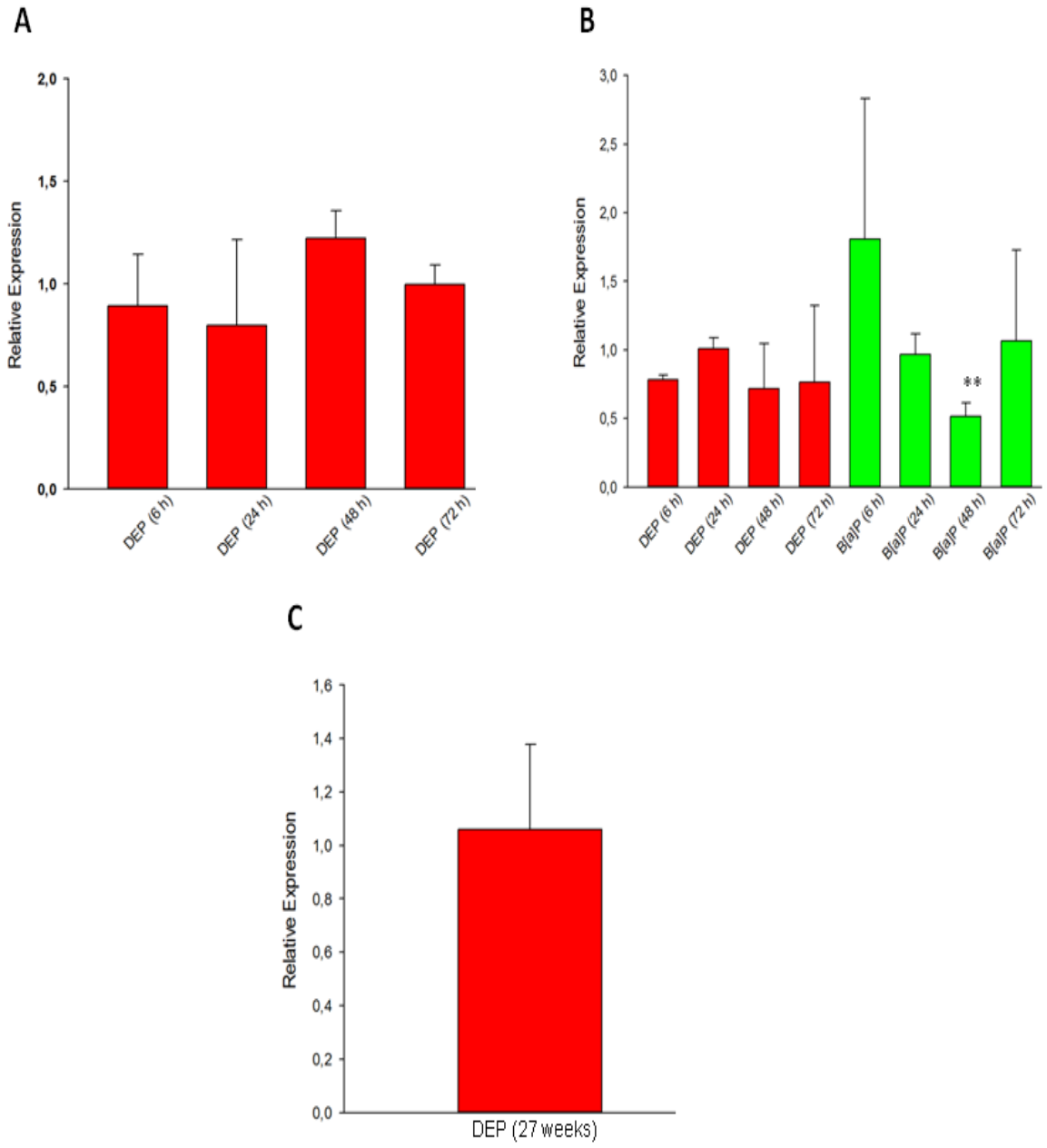


Figure 3-18 Variation in *miR-146a* expression by the exposure of HBEC3-KT to DEP or B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) Long-Term exposure to DEP. Results are normalized to reference gene (*SNORD44*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. **p < 0.01, for comparison between control and exposed cells. For (B) statistical test was Student's *t*-test.

3.19 *miR-146b*

Figure 3-19 shows variation in expression of *miR-146b* after exposure of HBEC3-KT to DEP or B[*a*]P.

Short-Term Exposure to DEP (Fig. 3-19.A) *miR-146b* expression at 6 and 48 h was indicated to be up-regulated at 24 h the expression it was down-regulated compared to the controls, although the results were non-significant.

Short-Term Exposure to DEP & B[*a*]P (Fig. 3-19.B) no apparent changes in the expression of *miR-146b* were observed when cells exposed to 100 µg/ml of DEP. However, at 6 h, *miR-146b* expression might be induced but the difference compared to the controls was statistically non-significant. At 48 h, *miR-146b* expression was significantly reduced, but there was no time related pattern in the change(s).

Long-Term Exposure to DEP (Fig. 3-19.C) long exposure of DEP, also didn't appear to alter the expression of *miR-146b* as indicated by the results.

From these results it can be concluded that *miR-146b* expression in HBEC3-KT cells might not be influenced significantly by the exposure to DEP and B[*a*]P under these experimental conditions. Interestingly, *miR-146a* and *miR-146b* showed quite similar patterns of expression, especially in the **Short-Term Exposure to DEP and B[*a*]P** experiment.

miR-146b

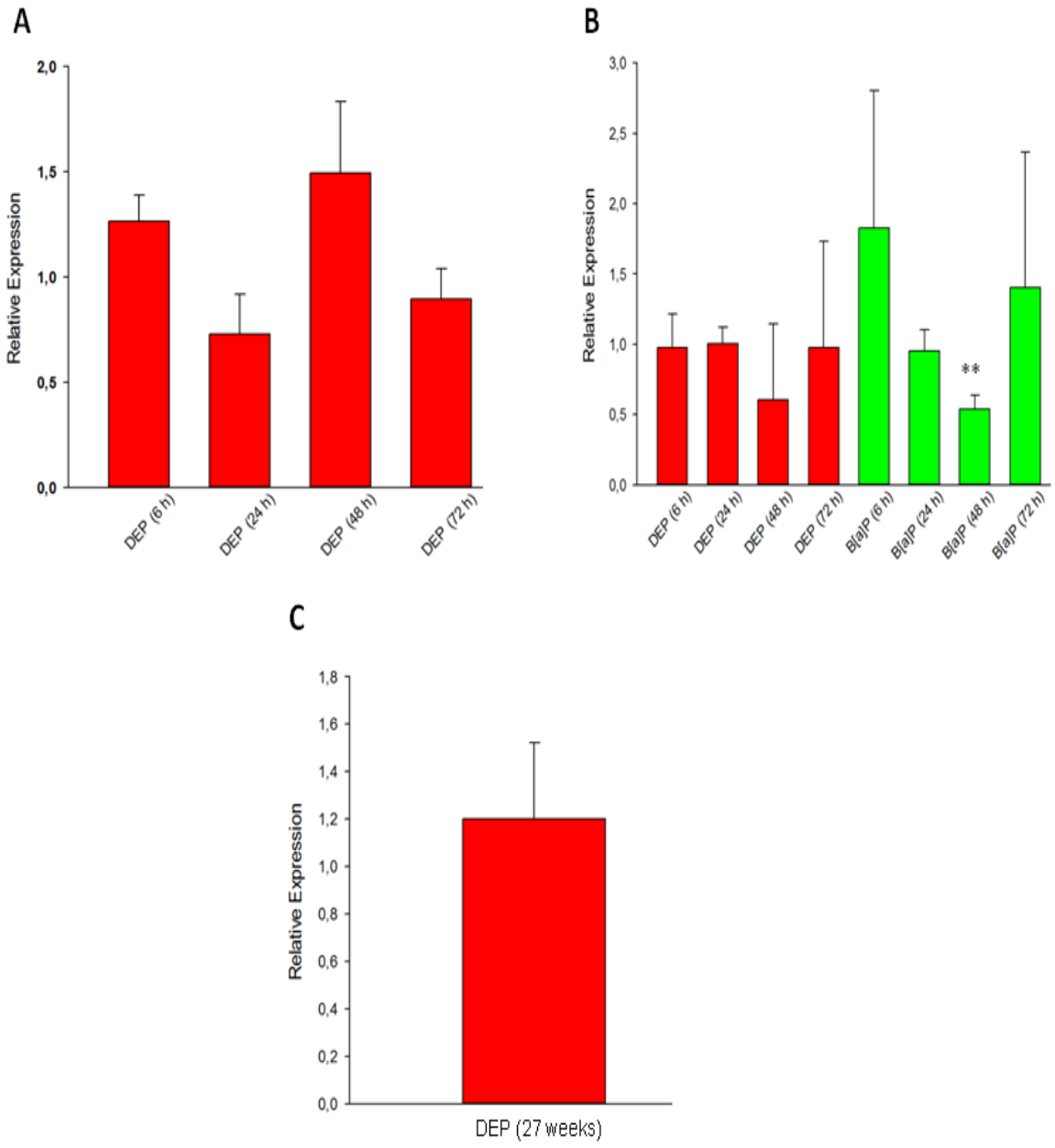


Figure 3-19 Variation in *miR-146b* expression by the exposure of HBEC3-KT to DEP or B[*a*]P. (A) Short-Term Exposure to DEP, (B) Short-Term Exposure to DEP and B[*a*]P, (C) Long-Term exposure to DEP. Results are normalized to reference gene (*SNORD44*), while statistical analysis was carried out on data before normalization to controls, as mentioned in Materials & Methods. Values are mean ± S.D. of three replicates. **p < 0.01, for comparison between control and exposed cells. For (B) statistical test was Student's *t*-test.

Chapter 4

Discussion

The aim of the present study was to identify changes in the expression of genes and miRNAs after exposure to DEP (and B[*a*]P) for short (and long term) using the immortalized human bronchial epithelial cell line (HBEC3-KT). DEP is a potential human carcinogen, and many *in vitro* studies suggest that both soluble extracts of DEP and whole particles may impact on the expression and release of proinflammatory markers [62]. Exhaust generated from the combustion of diesel fuel contains many chemical components in the gaseous and particulate forms [79-82]. DEP are composed of a center core of elemental carbon and adsorbed organic components, including aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), aldehydes, nitrogen oxides, and traces of metallic compounds [83]. Particulate matter from diesel engines is small enough to reach the lower respiratory system [82, 84]. Exposure to diesel exhaust can provoke immediate adverse health effects such as nausea, coughs, and headaches [35, 36]. Long-term exposure has negative effects on both the cardiovascular and the respiratory systems.

In vitro studies are important tools for investigating DEP induced effects, even though they have several limitations, such as genetic instability, selective growth of sub-population during culturing, lack of *in vivo* interactions and importantly the exposure concentrations that are on the high side compared to the real world situations [85]. The exact levels to which cells are likely to be exposed to *in vivo* are difficult to estimate, based on complexity of the deposition pattern [62]. Furthermore main DEP mediators have still not been identified due to ever changing fuel and engine technology which in turn alters the characteristics of the emission. Nonetheless, *in vitro* studies represent a fast and convenient system which may have implications for toxicological understanding and technology development [62]. Thus, *in vitro* models have been used to characterize toxic effects of DEP, providing information about the involved mechanisms that hopefully can be extrapolated to DEP-induced health effects *in vivo*. *In vitro* models can potentially also help to identify biomarkers related

to DEP-induced health effects, which in turn could provide help in identification of cancer at its early stages.

For this study the immortalized HBEC3-KT cell line was used, which was established from a normal 65 year old lady and they were immortalized by overexpression of *CDK4* and *hTERT* [77]. HBEC3-KT has been reported to be cultured for more than 240 populations without losing the normal phenotype, demonstrating its stability over many division cycles [85]. Furthermore HBEC3-KT provides a well suited model for *in vitro* toxicity studies and has been used for *in vitro* transformation studies with diverse carcinogenic compounds (B[*a*]P, CSC, DEP etc.) at the STAMI lab (Bersaas, Sjøberg, and Rynning, unpublished data). In this present study the cells were exposed to DEP (Standard Reference Number 2975). DEP were diluted in water to attain a concentration of 10 mg/ml and then sonicated. Diluted DEP were again sonicated before adding to the cell culture media for exposure. As DEP contain mixture of polar and nonpolar compounds, this double sonication ensures even distribution of DEP in to the media and minimal agglomeration of DEP during exposure. HBEC3-KT cells were grown in media containing 5% serum (FBS). Although these cells can also grow without the aid of serum, serum was added to make the particles properly dispersed and available to cells. Previous studies with different exposure media at STAMI suggested that addition of minimal 5% serum ensured the best dispergation of DEP in media.

Four different experimental setups have been used in this thesis namely; Short-Term Exposure to DEP, Short-Term Exposure to DEP and B[*a*]P, Long term Exposure to DEP and DEP Dose Experiment. In case of Short-Term Exposure to DEP, Short-Term Exposure to DEP and B[*a*]P and Long term Exposure to DEP, the particular focus was to find the effect of DEP (and B[*a*]P) on genes and miRNAs expression, within a short (6-72 h) and long term exposure (27 weeks, conducted for DEP only) time periods and also to compare the short and long term exposure effects of DEP. B[*a*]P is a well studied PAH and several carcinogenic studies related to B[*a*]P have been conducted in the STAMI lab [7, 86, 87]. Thus, B[*a*]P was included in the present study to compare the results as well and providing a positive control and to broaden the spectrum of the study. A concentration of DEP at 100 µg/ml was chosen for Short Term Exposure to DEP, based on previous work performed in our lab which suggested that 100 µg/ml is a safe sub-toxic concentration of DEP to the cells both for short term toxicity and long term transformation studies. It has been important to establish exposure

conditions that would be well tolerated for long-term transformation studies for which cells have to continue grow for extended time periods in the presence of carcinogen (DEP) in the cell culturing media. Also, this concentration of DEP is in the range of what has been used in other studies [88-90]. While in case of the DEP Dose Experiment, the particular aim was to find the effect of DEP on gene expression, with increasing concentration of DEP for a fixed time point (48 h). Forty-eight hours exposure time was selected on the basis of results with expression of *CYP1A1* which show maximum induction of the gene at this time point. This is in line with what has been found in previous studies in our lab. (unpublished observations). Furthermore, the Short-Term Exposure to DEP and B[a]P experiments demonstrated that *IL1-B* was also significantly induced at 48 hours by 100 µg/ml of DEP. While the concentrations of DEP for DEP Dose Experiment were below and above the used concentration of DEP in Short-Term Exposure to DEP and Short-Term Exposure to DEP and B[a]P experiments i.e. (25, 50, 100, 200 & 400µg/ml). For this thesis, cells showed no signs of toxicity at 400 µg/ml when exposed for 48 hours (**Fig. 3-4**), even though the proliferation rate was low but still most of the cells were viable. This has been confirmed in previous studies in our lab. However, toxic effects for this concentration for long term exposure cannot be ruled out. Higher than 400 µg/ml of DEP would be difficult to study because of more chances of agglomeration of DEP, and thus the particles will not be homogeneously distributed in exposure media.

The genes and miRNAs expression level investigated in this study were: *CYP1A1, COX2, CDH1, IL1B, HO1, NRF2, miR17, miR21, miR27, miR31, miR146a* and *miR146b*, respectively. These genes and miRNAs were selected based on literature searches for candidates that have proven to show links with lung cancer and/or environmental/particle toxicity.

4.1 Variation in expression of genes by the exposure of DEP and B[*a*]P

DEP have been shown to contain varying amounts of PAHs depending on the diesel source and the engine technology [62]. *CYP1A1* helps to converts PAH into reactive metabolites which can form DNA adduct and result into carcinogenesis [43]. *CYP1A1* is induced by PAHs in human lungs and found to be associated with increased risk of lung cancer [91, 92]. In our studies when the HBEC3-KT cells were exposed to DEP and B[a]P the gene expression of *CYP1A1* was significantly induced with maximum induction at 48 hours while it was also significantly induced over a wide range of DEP

concentrations. However there was no conclusive trend between increasing time and dose in correspondence to induction of expression of *CYP1A1*. A reduction in expression of gene with increasing concentration in comparison to DEP 50 µg/ml could be due to the fact that the phase 1 enzyme *CYP1A1* production was greatly reduced at higher concentrations, when inflammatory cytokines and chemokines become prominent [90]. Stimulation of the expressions of phase 2 detoxification enzymes by DEP extracts was reported to reduce the cytokine response [93]. A study indicates a reciprocal relationship between AhR/Arnt- dependent *CYP1A1* induction and cytokine production, apparently because AhR has inhibitory role in control of inflammation [94]. That could be a possible explanation for the reduction of expression of *CYP1A1* after 48 hours of exposure and at higher concentrations of DEP. PAHs on DEP may in turn combine with AhR and induce the expression of *CYP1A1*. As reported in one study, a number of compounds associated with DEP activate aryl hydrocarbon receptor (Ahr)-dependent gene expression and AhR activation is an important toxic mode of action of complex mixtures of PAHs [95-97]. Nonetheless, the contribution of specific groups of compounds or individual polyaromatic contaminants to the AhR activity of DEP still remains only partly characterized [98].

Studies indicate that DEP induces expression of phase 1 and 2 xenobiotic metabolizing enzymes through activation of AhR and Nrf2 transcription factor respectively [53, 90, 99, 100]. To alleviate oxidative stress possibly caused by DEP exposure to the cells, antioxidative stress responses come in to play. Most of these are regulated by *NRF2* and its expression is abundant in the lungs. Several studies indicate that there is ample evidence to support the involvement of *NRF2* in cancer biology [101-103]. In our studies *NRF2* expression might not affected evidently by the exposure of DEP or B[a]P. General remarks on the lack of effects is presented at the end of this chapter (**Page number 84 2nd paragraph**)

Overexpression of phase 2 antioxidant enzymes which are regulated by Nrf2 were found in lung tumor tissues [48]. Nrf2 is a transcription factor of *HO1* [45]. DEP have high content of organic compounds which can lead to highly electrophilic metabolites and the formation of ROS through redox cascade reactions [104]. Several studies report that DEP can induce the expression of antioxidant genes such as heme oxygenase (*HO1*) in endothelial cells [105] and macrophages [100]. Another study reports the up regulation of the *HO1* in the liver of ApoE null mice by ultra fine particles of DEP. In this study we observed a significant induction of *HO1* at higher concentrations of

DEP (200 and 400 µg/ml). However, for 100 µg/ml of DEP the induction was insignificant so it is difficult to conclude that *HO1* is induced with increasing time also. However in case of B[*a*]P exposure *HO1* was found to be significantly induced at 24 and 48 hours, which gives indication of oxidative stress on the cells by the exposure of carcinogens (DEP and B[*a*]P). As it has been reported in many studies that *HO1* expression exert cytoprotective, anti-oxidant, anti-apoptotic, anti-inflammatory role in protection against atherogenesis in vascular cells [106].

Exposure of humans to diesel exhaust leds to an increase in respiratory symptoms, decrease in lung function parameters, while inducing levels of inflammatory mediators in airway secretions such as interleukins [107]. Prostaglandins functions as attractant of inflammatory cells and many studies reported that DEP induces COX2 which is a key enzyme in prostaglandin production [90]. Links between cancer and *COX2* overexpression have also been found [108, 109]. A study reports that *COX2* is, induced by inflammatory stimuli and that it is an important source of prostanoid formation in inflammation and in proliferative diseases, such as cancer [60]. In another study it is reported that *COX2* was upregulated by the DEP (50-100 µg/ml) on BEAS-2B (bronchial epithelial cells) [110]. We found that *COX2* expression was upregulated by DEP (100 µg/ml) at 24 hours while significant induction was evident at higher concentrations of DEP (200-400 µg/ml). However, the results were inconclusive and no trend could be drawn between expression and increasing concentration of DEP.

Interleukin IL-1-β, is a key cytokine produced and secreted by many cell types after activation by biological or chemical agents. Many cell types including lung epithelial cells produce and secrete IL-1-β upon exposure to chemicals and other environmental agents [111]. It has also been shown that the *IL1B* gene may be induced by cigarette smoke in human cells *in vitro* [112]. Cigarette smoke also contains PAHs [113]. Interleukin-1beta (IL-1 β) is a key pro-inflammatory cytokine, which regulates the expression of several genes involved in inflammation [114] and it also play important role in carcinogenic process. They are secreted immediately to encounter inhaled pathogens or xenobiotics, as they are tend to be expressed as inactive proforms and may rapidly be cleaved and released without requiring activation of transcriptional machinery [62]. We also found, a significant induction of *IL1B* gene expression with increasing time and concentration during the exposure of HBEC3-KT cells to DEP. A significant induction of *IL1B* expression was found in case of B[*a*]P exposure as well at 48 and 72 h. However for long term exposure no induction in expression was found. It might be

speculated that during long-term exposure the cell could adapt to the chronic exposure situation in a way that makes the cells tolerate the oxidative and other stresses posed by the particles to the cells.

Cytokines can be excreted from cells to the cell culture media, therefore we hypothesized that IL-1-β might be induced in the same way as what was found for *IL1B* gene expression. A significant induction of IL-1-β protein was found in cell culture media from the DEP–Dose Experiment, but the results were in consistent and it's difficult to find any scientific logic behind the variations in the results. Although at 25 and 100 µg/ml we found significant induction of protein in comparison to control but not at other concentrations. Therefore we decided to repeat the experiment but unfortunately results from collaborating partner were not available for this thesis. Nonetheless in case of Short-Term Exposure to DEP and B[*a*]P the IL-1-β protein excretion might possibly be increased with the passage of time but due to large variations and non significant results it is difficult to conclude that protein excretion of IL-1-β increases over time.

Epithelial cells can form tight monolayer that presents a barrier to prevent the external agents from entering the circulation. While a study reports that DEP increase transepithelial electrical conductance and loosening of tight junctions, resulting in increased possibility of leakage of proteins and perhaps particles to vascular system [115]. Low levels of E-cadherin which is encoded by *CDH1*, were reported in many studies as a link to lung cancer [65-67]. Alterations in E-cadherin are most widely observed modifications in cell-to-environment interactions in cancer [3, 116]. Down-regulation of *CDH1* (encoding E-cadherin) and up-regulation of *CDH2* (encoding N-cadherin), known as the cadherin switch, has been considered a molecular hallmark of EMT [13]. In the present study, no clear conclusions regarding effects of DEP (or B[*a*]P) on *CDH1* could be drawn because as no clear trends were apparent and only non-significant changes were observed.

4.2 Variation in expression of miRNAs by the exposure of DEP and B[*a*]P

Aberrant expression of miRNAs has been observed in different types of cancer, including lung cancer [71, 117]. In one study comparison of normal lung tissues and cancerous lung tissues revealed significant differences in miRNA profiles and suggested the involvement of many miRNAs in tumor formation [118]. Several studies suggest that miRNA expression can be regulated by environmental

factors [74], but how environmental factors influence miRNA expression is still ambiguous. However one study reports the involvement of DEP exposure to induce aberrant miRNA expression in roughly 63% of the 313 detectable miRNA studied in human bronchial epithelial cells [75]. This study also suggest that inflammatory pathways are potentially regulated by increasing expression of some miRNAs while decreasing the expression of others, from which it can be concluded that environmental factors may influence miRNAs expression and miRNA play important role in modulating cellular response [75]. Therefore we decided to study selected a set of miRNAs to measure possible alterations in their expression by the exposure of DEP (or B[a]P). Such signature changes in the miRNA expression could be a useful tool in early detection of cancer and have potential to revolutionize present clinical management [71].

We investigated the expression of 6 miRNAs from three experiments: Short-Term Exposure to DEP, Short-Term Exposure to DEP and B[a]P and Long-Term Exposure to DEP. At first, variations (Ct values) in the two often used reference RNAs *RNU6b* and *SNORD44* were measured. We found out that in accordance to our experimental setups *SNORD44* was showing the least variation (**Fig. 3-15**). Based on this, miRNA expression data of the miRNAs were normalized to *SNORD44* in this thesis.

In perspective of our experiments we didn't find any evident variations in the miRNA expression, most of the results were statistically insignificant and in some cases some large variations in the results made the results inconclusive. Although the study of miRNAs in disease is still in its infancy, it is evident that miRNAs play a pivotal role both in lung development and in maintaining the lung in a disease-free state [119]. MiRNA knockout studies have shown that loss of function of the miR-17-92 cluster leads to hypoplastic lung. Furthermore, the miR-17-92 cluster is found to be overexpressed in lung cancers in several studies [120, 121]. Regarding our results from *miR-17* we didn't found any up or down regulation in the expression. MiR-21 has a well-documented in correlation to lung cancer and its elevated levels were found to be associated with lowered survival rate [122, 123]. *Mir-21* over expression enhances tumorgenesis and inhibit apoptosis [124]. According to our findings there are some indications of over expression of *miR-21* by the exposure of DEP but the results were insignificant and showed large variations. A study showed the down regulation of *miR-27a* by the exposure of DEP (10 µg/cm^2) [125]. For our exposure experiments we found no statistically significant changes in the expression of *miR-27*, although there were some indications that the expression was induced by the exposure of DEP over the time but the results were insignificant. *miR31* was over expressed in malignant lung tissues from humans and found to target the tumor

suppressor genes according to a study [126]. In another study it was reported that by knocking down *miR-31* repressed lung cancer cell growth and tumorigenicity in a dose-dependent manner [127]. These findings suggest that miR-31 act as oncogenic miRNA by targeting specific tumor suppressors for repression. Several studies have reported the down regulation of *miR-31* after the exposure of DEP, in various types of cancer [128-130]. However, no clear indications of up or down regulation was observed in our results. MiR-146a and miR-146b are negative regulators of inflammatory gene expression in lung fibroblasts, epithelial cells, monocytes, and endothelial cells. The abundance of cyclooxygenase2 (COX2) and IL-1-β is negatively regulated by the miR-146 family, suggesting miR-146a and/or miR-146b might modulate inflammatory mediator expression [131]. *MiR-146a/b* were also selected on the basis of their involvement in various types of cancer including lung carcinoma [132]. Although from our results for *miR-146a* and *miR-146b* it was not possible to conclude, their expression was influenced by DEP or B[*a*]P exposure under the present experimental conditions.

No evidence of changes after exposure to DEP was observed in the expression of *NRF2, CDH1* and *miRNA* expression, this could be due to the possible fact that there will be no effects of DEP on these genes in the HBEC3-KT line. However, it could also be that different experimental set-ups would be needed to reveal such effects (other time point or concentration, other preparations of exposure media, or other cell lines).

In light of the conducted experimental work/setups and obtained results, there is a need of repetition of some experiments, especially DEP-Dose Experiment which had been conducted just once and need to be repeated to draw solid conclusion. Also miRNA expression analysis regarding DEP-Dose Experiment needed to be done, which was not conducted due to shortage of time. It would be interesting to see study if miRNA expression is modified by the increasing concentration of DEP to HBEC3-KT cells. Regarding Long-Term Exposure to DEP, we have only looked at *IL1B* and *CDH1*, while it would also be interesting to look at other genes which we have missed due to time shortage. After the long-term exposure experiment, transformed cell lines (selected by ability to form colonies in soft-agar) from HBEC3-KT are in the process of being established (Rynning *et al*., unpublished data). For further studies, it would be interesting to characterize stable changes in gene and miRNA expression in transformed cells and compare with short term effects and changes observed in cells subjected to long-term exposure (pre-transformed). Another interesting thing would be to investigate the DNA adducts made by *CYP1A1*.

Chapter 5

Conclusion

The aim of the thesis was to investigate the possible changes in gene expression, when the HBEC3-KT cells *in vitro*, were exposed to 100 µg/ml of DEP (and 3 µM of B[a]P) over a short period of time (6-72 h) and over different concentrations of DEP (25-400 µg/ml).

Variations in the expression of genes and miRNAs (*CYP1A1, COX2, CDH1, IL1B, HO1, NRF2, miR17, miR21, miR27, miR31, miR146a* and *miR146b*, respectively) were evaluated using RT-qPCR. Gene expression of *CYP1A1, COX2, IL1B* and *HO1* were found to be possibly induced by the exposure of DEP. In contrast, no significant evidence of changes in the expression of *NRF2, CDH1* and the selected miRNAs was found by the exposure of DEP. Excreted IL-1-β protein was detected by ELISA, with indications of inductions were found but overall these analyses were inconclusive. There is need of repetition of some of the experimental work along with a need for further studies to establish the link between DEP posing oxidative stress and inducing inflammation along with xenobiotic metabolizing enzymes.

Bibliography

1. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* cell, 2011. **144**(5): p. 646-674.
2. Ris, C., *US EPA health assessment for diesel engine exhaust: a review.* Inhalation Toxicology, 2007. **19**(sup1): p. 229-239.
3. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* The Journal of clinical investigation, 2009. **119**(6): p. 1420.
4. Howlader, N., et al., *SEER Cancer Statistics Review, 1975–2011. Bethesda, MD: National Cancer Institute*. 2014.
5. Murray, I.A., A.D. Patterson, and G.H. Perdew, *Aryl hydrocarbon receptor ligands in cancer: friend and foe.* Nature Reviews Cancer, 2014. **14**(12): p. 801-814.
6. Vrijens, K., V. Bollati, and T.S. Nawrot, *MicroRNAs as Potential Signatures of Environmental Exposure or Effect: A Systematic Review.* Environmental health perspectives, 2015. **123**(5): p. 399.
7. Sagredo, C., et al., *Quantitative analysis of benzo [a] pyrene biotransformation and adduct formation in Ahr knockout mice.* Toxicology letters, 2006. **167**(3): p. 173-182.
8. Valent, P., et al., *Cancer stem cell definitions and terminology: the devil is in the details.* Nature Reviews Cancer, 2012. **12**(11): p. 767-775.
9. Samet, J.M., et al., *Lung cancer in never smokers: clinical epidemiology and environmental risk factors.* Clinical Cancer Research, 2009. **15**(18): p. 5626-5645.
10. Stieb, D.M., et al., *Approximations for Estimating Change in Life Expectancy Attributable to Air Pollution in Relation to Multiple Causes of Death Using a Cause Modified Life Table.* Risk Analysis, 2015.
11. Burnett, R.T., et al., *An integrated risk function for estimating the global burden of disease attributable to ambient fine particulate matter exposure.* 2014.
12. Loomis, D., et al., *The carcinogenicity of outdoor air pollution.* The Lancet Oncology, 2013. **14**(13): p. 1262-1263.
13. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* cell, 2000. **100**(1): p. 57-70.
14. Ponder, B.A., *Cancer genetics.* Nature, 2001. **411**(6835): p. 336-341.
15. Goodman, M.T., et al., *CYP1A1, GSTM1, and GSTT1 polymorphisms and the risk of cervical squamous intraepithelial lesions in a multiethnic population.* Gynecologic oncology, 2001. **81**(2): p. 263-269.
16. Jones, P.A. and S.B. Baylin, *The fundamental role of epigenetic events in cancer.* Nature reviews genetics, 2002. **3**(6): p. 415-428.

17. Kanwal, R. and S. Gupta, *Epigenetics and cancer.* Journal of Applied Physiology, 2010. **109**(2): p. 598-605.

18. Croce, C.M., *Oncogenes and cancer.* New England Journal of Medicine, 2008. **358**(5): p. 502-511.

19. Watkins, J.B., C.D. Klaassen, and D. Acosta, *Casarett & Doull's essentials of toxicology*. 2010: McGraw-Hill.

20. Subramanian, J. and R. Govindan, *Lung cancer in never smokers: a review.* Journal of Clinical Oncology, 2007. **25**(5): p. 561-570.

21. Brennan, P., P. Hainaut, and P. Boffetta, *Genetics of lung-cancer susceptibility.* The lancet oncology, 2011. **12**(4): p. 399-408.

22. Kang, J.U., et al., *Identification of novel candidate target genes, including EPHB3, MASP1 and SST at 3q26. 2–q29 in squamous cell carcinoma of the lung.* BMC cancer, 2009. **9**(1): p. 237.

23. Lunt, S.Y. and M.G. Vander Heiden, *Aerobic glycolysis: meeting the metabolic requirements of cell proliferation.* Annual review of cell and developmental biology, 2011. **27**: p. 441-464.

24. Youlden, D.R., S.M. Cramb, and P.D. Baade, *The international epidemiology of lung cancer: geographical distribution and secular trends.* Journal of Thoracic Oncology, 2008. **3**(8): p. 819-831.

25. Watson, T., *Environment: Breathing trouble.* Nature, 2014. **513**(7517): p. S14-S15.

26. Raaschou-Nielsen, O., et al., *Air pollution and lung cancer incidence in 17 European cohorts: prospective analyses from the European Study of Cohorts for Air Pollution Effects (ESCAPE).* The lancet oncology, 2013. **14**(9): p. 813-822.

27. Turner, M.C., et al., *Long-term ambient fine particulate matter air pollution and lung cancer in a large cohort of never-smokers.* American journal of respiratory and critical care medicine, 2011. **184**(12): p. 1374-1381.

28. Kauppinen, T., et al., *Occupational exposure to carcinogens in the European Union.* Occup Environ Med, 2000. **57**(1): p. 10-8.

29. Pronk, A., J. Coble, and P. Stewart, *Occupational exposure to diesel engine exhaust: A literature review.* Journal of exposure science & environmental epidemiology, 2009. **19**(5): p. 443-457.

30. Wichmann, H.-E., *Diesel exhaust particles.* Inhalation Toxicology, 2007. **19**(sup1): p. 241-244.

31. Seriani, R., et al., *Enriched inorganic compounds in diesel exhaust particles induce mitogen-activated protein kinase activation, cytoskeleton instability, and cytotoxicity in human bronchial epithelial cells.* Experimental and Toxicologic Pathology, 2015. **67**(4): p. 323-329.

32. Lim, Y.-H., et al., *Air pollution and symptoms of depression in elderly adults.* Environmental health perspectives, 2012. **120**(7): p. 1023.

33. Boström, C.-E., et al., *Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air.* Environmental health perspectives, 2002. **110**(Suppl 3): p. 451.

34. Penning, T.M., *Chemical carcinogenesis*. 2011: Springer Science & Business Media.

35. Reger, R., et al., *Coal miners exposed to diesel exhaust emissions.* Annals of Occupational Hygiene, 1982. **26**(8): p. 799-815.

36. Rudell, B., et al., *Bronchoalveolar inflammation after exposure to diesel exhaust: comparison between unfiltered and particle trap filtered exhaust.* Occupational and environmental medicine, 1999. **56**(8): p. 527-534.

37. Langrish, J.P., et al., *Reducing personal exposure to particulate air pollution improves cardiovascular health in patients with coronary heart disease.* Environmental health perspectives, 2012. **120**(3): p. 367-372.

38. Riedl, M. and D. Diaz-Sanchez, *Biology of diesel exhaust effects on respiratory function.* Journal of Allergy and Clinical Immunology, 2005. **115**(2): p. 221-228.

39. Salvi, S. and S. Holgate, *Mechanisms of particulate matter toxicity.* Clinical and experimental allergy, 1999. **29**(9): p. 1187-1194.

40. Ristovski, Z.D., et al., *Respiratory health effects of diesel particulate matter.* Respirology, 2012. **17**(2): p. 201-212.

41. Emmendoerffer, A., et al., *Role of inflammation in chemical-induced lung cancer.* Toxicology letters, 2000. **112**: p. 185-191.

42. Bartsch, H. and J. Nair, *Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair.* Langenbeck's Archives of Surgery, 2006. **391**(5): p. 499-510.

43. Uppstad, H., et al., *Importance of CYP1A1 and CYP1B1 in bioactivation of benzo [a] pyrene in human lung cell lines.* Toxicology letters, 2010. **192**(2): p. 221-228.

44. Pfeifer, G.P., et al., *Tobacco smoke carcinogens, DNA damage and p 53 mutations in smoking-associated cancers.* Oncogene, 2002. **21**(48): p. 7435-7451.

45. Hackett, N.R., et al., *Variability of antioxidant-related gene expression in the airway epithelium of cigarette smokers.* American journal of respiratory cell and molecular biology, 2003. **29**(3): p. 331-343.

46. Lee, J.H., et al., *Dietary phytochemicals and cancer prevention: Nrf2 signaling, epigenetics, and cell death mechanisms in blocking cancer initiation and progression.* Pharmacology & therapeutics, 2013. **137**(2): p. 153-171.

47. Kwak, M.-K. and T.W. Kensler, *Targeting NRF2 signaling for cancer chemoprevention.* Toxicology and applied pharmacology, 2010. **244**(1): p. 66-76.

48. Cho, H.-Y. and S.R. Kleeberger, *Nrf2 protects against airway disorders.* Toxicology and applied pharmacology, 2010. **244**(1): p. 43-56.

49. Sporn, M.B. and K.T. Liby, *NRF2 and cancer: the good, the bad and the importance of context.* Nature Reviews Cancer, 2012. **12**(8): p. 564-571.

50. Rotblat, B., G. Melino, and R.A. Knight, *NRF2 and p53: Januses in cancer?* Oncotarget, 2012. **3**(11): p. 1272-1283.

51. Jaiswal, A.K., *Nrf2 signaling in coordinated activation of antioxidant gene expression.* Free Radical Biology and Medicine, 2004. **36**(10): p. 1199-1207.

52. Siegel, D. and D. Ross, *Immunodetection of NAD (P) H: quinone oxidoreductase 1 (NQO1) in human tissues.* Free Radical Biology and Medicine, 2000. **29**(3): p. 246-253.

53. Li, N., et al., *Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element.* The Journal of Immunology, 2000. **165**(6): p. 3393-3401.

54. Kikuchi, G., T. Yoshida, and M. Noguchi, *Heme oxygenase and heme degradation.* Biochem Biophys Res Commun, 2005. **338**(1): p. 558-67.

55. Solis, L.M., et al., *Nrf2 and Keap1 abnormalities in non–small cell lung carcinoma and association with clinicopathologic features.* Clinical Cancer Research, 2010. **16**(14): p. 3743-3753.

56. Hayes, J.D. and M. McMahon, *NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer.* Trends in biochemical sciences, 2009. **34**(4): p. 176-188.

57. Ricciotti, E. and G.A. FitzGerald, *Prostaglandins and Inflammation.* Arteriosclerosis, thrombosis, and vascular biology, 2011. **31**(5): p. 986-1000.

58. Smith, W.L., D.L. DeWitt, and R.M. Garavito, *Cyclooxygenases: structural, cellular, and molecular biology.* Annual review of biochemistry, 2000. **69**(1): p. 145-182.

59. Dubois, R.N., et al., *Cyclooxygenase in biology and disease.* The FASEB journal, 1998. **12**(12): p. 1063-1073.

60. Warner, T.D. and J.A. Mitchell, *Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic.* The FASEB Journal, 2004. **18**(7): p. 790-804.

61. Davies, D. and S. Holgate, *Asthma: the importance of epithelial mesenchymal communication in pathogenesis: inflammation and the airway epithelium in asthma.* The international journal of biochemistry & cell biology, 2002. **34**(12): p. 1520-1526.

62. Schwarze, P.E., et al., *Inflammation-related effects of diesel engine exhaust particles: studies on lung cells in vitro.* Biomed Res Int, 2013. **2013**: p. 685142.

63. Li, C. and C. Wang, *Current evidences on IL1B polymorphisms and lung cancer susceptibility: a meta-analysis.* Tumour Biol, 2013. **34**(6): p. 3477-82.

64. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* The lancet, 2001. **357**(9255): p. 539-545.

65. Hazan, R.B., et al., *Cadherin switch in tumor progression.* Annals of the New York Academy of Sciences, 2004. **1014**(1): p. 155-163.

66. Gravdal, K., et al., *A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer.* Clinical Cancer Research, 2007. **13**(23): p. 7003-7011.

67. Hazan, R.B., et al., *Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis.* The Journal of cell biology, 2000. **148**(4): p. 779-790.

68. Jirtle, R.L. and M.K. Skinner, *Environmental epigenomics and disease susceptibility.* Nature reviews genetics, 2007. **8**(4): p. 253-262.

69. Hu, Z., et al., *Association between microrna polymorphisms, expressions, lung cancer development and prognosis.* Biomedicine & Pharmacotherapy, 2009. **63**(5): p. 322.

70. Tomizaki, K.y., K. Usui, and H. Mihara, *Protein-Detecting Microarrays: Current Accomplishments and Requirements.* ChemBioChem, 2005. **6**(5): p. 782-799.

71. Calin, G.A. and C.M. Croce, *MicroRNA signatures in human cancers.* Nature Reviews Cancer, 2006. **6**(11): p. 857-866.

72. Peng, G., et al., *Detection of lung, breast, colorectal, and prostate cancers from exhaled breath using a single array of nanosensors.* British journal of cancer, 2010. **103**(4): p. 542-551.

73. Vrijens, K., V. Bollati, and T.S. Nawrot, *MicroRNAs as Potential Signatures of Environmental Exposure or Effect: A Systematic Review.* Environmental Health Perspectives, 2015. **123**(5): p. 399-411.

74. Jardim, M.J., *microRNAs: implications for air pollution research.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2011. **717**(1): p. 38-45.

75. Jardim, M.J., et al., *Disruption of microRNA expression in human airway cells by diesel exhaust particles is linked to tumorigenesis-associated pathways.* Environ Health Perspect, 2009. **117**(11): p. 1745-1751.

76. Nowsheen, S., et al., *Molecular markers for cancer prognosis and treatment: Have we struck gold?* Cancer letters, 2012. **327**(1): p. 142-152.

77. Ramirez, R.D., et al., *Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins.* Cancer research, 2004. **64**(24): p. 9027-9034.

78. Shapiro, G.I., *Cyclin-dependent kinase pathways as targets for cancer treatment.* Journal of Clinical Oncology, 2006. **24**(11): p. 1770-1783.

79. Groves, J. and J.R. Cain, *A survey of exposure to diesel engine exhaust emissions in the workplace.* Ann Occup Hyg, 2000. **44**(6): p. 435-47.

80. Sydbom, A., et al., *Health effects of diesel exhaust emissions.* European Respiratory Journal, 2001. **17**(4): p. 733-746.

81. Kagawa, J., *Health effects of diesel exhaust emissions—a mixture of air pollutants of worldwide concern.* Toxicology, 2002. **181–182**(0): p. 349-353.

82. Penconek, A., P. Drążyk, and A. Moskal, *Penetration of diesel exhaust particles through commercially available dust half masks.* Annals of occupational hygiene, 2013. **57**(3): p. 360-373.

83. Morgenstern, V., et al., *Respiratory health and individual estimated exposure to traffic-related air pollutants in a cohort of young children.* Occupational and environmental medicine, 2007. **64**(1): p. 8-16.

84. Steenland, K., J. Deddens, and L. Stayner, *Diesel exhaust and lung cancer in the trucking industry: exposure-response analyses and risk assessment.* American Journal of Industrial Medicine, 1998. **34**(3): p. 220-228.

85. Gazdar, A.F., B. Gao, and J.D. Minna, *Lung cancer cell lines: Useless artifacts or invaluable tools for medical science?* Lung Cancer, 2010. **68**(3): p. 309-318.

86. Sagredo, C., et al., *Biotransformation of benzo [a] pyrene in Ahr knockout mice is dependent on time and route of exposure.* Chemical research in toxicology, 2009. **22**(3): p. 584-591.

87. Solhaug, A., et al., *Role of cell signaling in B [a] P-induced apoptosis: characterization of unspecific effects of cell signaling inhibitors and apoptotic effects of B [a] P metabolites.* Chemico-biological interactions, 2005. **151**(2): p. 101-119.

88. Hiura, T.S., et al., *Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages.* The Journal of Immunology, 1999. **163**(10): p. 5582-5591.

89. Løvik, M., et al., *Diesel exhaust particles and carbon black have adjuvant activity on the local lymph node response and systemic IgE production to ovalbumin.* Toxicology, 1997. **121**(2): p. 165-178.

90. Totlandsdal, A.I., et al., *Diesel exhaust particles induce CYP1A1 and pro-inflammatory responses via differential pathways in human bronchial epithelial cells.* Part Fibre Toxicol, 2010. **7**(41): p. 114.

91. Stücker, I., et al., *Relation between inducibility of CYP1A1, GSTM1 and lung cancer in a French population.* Pharmacogenetics and Genomics, 2000. **10**(7): p. 617-627.

92. McLemore, T.L., et al., *Expression of CYP1A1 gene in patients with lung cancer: evidence for cigarette smoke-induced gene expression in normal lung tissue and for altered gene regulation in primary pulmonary carcinomas.* Journal of the National Cancer Institute, 1990. **82**(16): p. 1333-1339.

93. Ritz, S.A., J. Wan, and D. Diaz-Sanchez, *Sulforaphane-stimulated phase II enzyme induction inhibits cytokine production by airway epithelial cells stimulated with diesel extract.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2007. **292**(1): p. L33-L39.

94. Monteleone, I., et al., *The aryl hydrocarbon receptor in inflammatory bowel disease: linking the environment to disease pathogenesis.* Current opinion in gastroenterology, 2012. **28**(4): p. 310-313.

95. Meek, M.D., *Ah Receptor and Estrogen Receptor-Dependent Modulation of Gene Expression by Extracts of Diesel Exhaust Particles.* Environmental Research, 1998. **79**(2): p. 114-121.

96. Jacob, A., et al., *Aryl hydrocarbon receptor-dependent upregulation of Cyp1b1 by TCDD and diesel exhaust particles in rat brain microvessels.* Fluids Barriers CNS, 2011. **8**: p. 23.

97. Andrysík, Z., et al., *Activation of the aryl hydrocarbon receptor is the major toxic mode of action of an organic extract of a reference urban dust particulate matter mixture: The role of polycyclic aromatic hydrocarbons.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2011. **714**(1–2): p. 53-62.

98. Pálková, L., et al., *The aryl hydrocarbon receptor-mediated and genotoxic effects of fractionated extract of standard reference diesel exhaust particle material in pulmonary, liver and prostate cells.* Toxicology in Vitro, 2015. **29**(3): p. 438-448.

99. Baulig, A., et al., *Involvement of reactive oxygen species in the metabolic pathways triggered by diesel exhaust particles in human airway epithelial cells.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2003. **285**(3): p. L671-L679.

100. Li, N., et al., *Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals.* The Journal of Immunology, 2004. **173**(5): p. 3467-3481.

101. Bauer, A.K., et al., *Targeted deletion of Nrf2 reduces urethane-induced lung tumor development in mice.* PLoS One, 2011. **6**(10): p. e26590.

102. Singh, A., et al., *Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer.* PLoS Med, 2006. **3**(10): p. e420.

103. Motohashi, H. and M. Yamamoto, *Carcinogenesis and transcriptional regulation through Maf recognition elements.* Cancer science, 2007. **98**(2): p. 135-139.

104. Iwamoto, N., et al., *Chemical knockdown of protein-tyrosine phosphatase 1B by 1, 2-naphthoquinone through covalent modification causes persistent transactivation of epidermal growth factor receptor.* Journal of Biological Chemistry, 2007. **282**(46): p. 33396-33404.

105. Gong, K.W., et al., *Air-pollutant chemicals and oxidized lipids exhibit genome-wide synergistic effects on endothelial cells.* Genome Biol, 2007. **8**(7): p. R149.

106. Araujo, J.A., et al., *Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress.* Circulation research, 2008. **102**(5): p. 589-596.

107. Hesterberg, T., W. Bunn, and C. Lapin, *An evaluation of criteria for selecting vehicles fueled with diesel or compressed natural gas.* Sustainability: Science, Practice, and Policy, 2009. **5**(1): p. 20-30.

108. Kawata, R., et al., *Expression of cyclooxygenase-2 and microsomal prostagalandin E synthase-1 in head and neck squamous cell carcinoma.* Auris Nasus Larynx, 2010. **37**(4): p. 482-487.

109. Hida, T., et al., *Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas.* Cancer research, 1998. **58**(17): p. 3761-3764.

110. Totlandsdal, A.I., et al., *Differential effects of the particle core and organic extract of diesel exhaust particles.* Toxicology letters, 2012. **208**(3): p. 262-268.

111. Apte, R.N., et al., *Effects of micro-environment-and malignant cell-derived interleukin-1 in carcinogenesis, tumour invasiveness and tumour–host interactions.* European journal of cancer, 2006. **42**(6): p. 751-759.

112. Hellermann, G.R., et al., *Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells.* Respiratory research, 2002. **3**(1): p. 22.

113. Stedman, R.L., *Chemical composition of tobacco and tobacco smoke.* Chemical Reviews, 1968. **68**(2): p. 153-207.

114. Dinarello, C., *The IL-1 family and inflammatory diseases.* Clinical and experimental rheumatology, 2002. **20**(5; SUPP/27): p. S1-S13.

115. Caraballo, J.C., et al., *Ambient particulate matter affects occludin distribution and increases alveolar transepithelial electrical conductance.* Respirology, 2011. **16**(2): p. 340-349.

116. Thomson, S., et al., *Epithelial to mesenchymal transition is a determinant of sensitivity of non–small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition.* Cancer research, 2005. **65**(20): p. 9455-9462.

117. Lin, P., S. Yu, and P. Yang, *MicroRNA in lung cancer.* British journal of cancer, 2010. **103**(8): p. 1144-1148.

118. Zhang, B., et al., *microRNAs as oncogenes and tumor suppressors.* Developmental biology, 2007. **302**(1): p. 1-12.

119. Nana-Sinkam, S.P., et al., *Integrating the MicroRNome into the study of lung disease.* American journal of respiratory and critical care medicine, 2009. **179**(1): p. 4-10.

120. Hayashita, Y., et al., *A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation.* Cancer research, 2005. **65**(21): p. 9628-9632.

121. Matsubara, H., et al., *Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92.* Oncogene, 2007. **26**(41): p. 6099-6105.

122. Liu, Z.-L., et al., *MicroRNA-21 (miR-21) expression promotes growth, metastasis, and chemo- or radioresistance in non-small cell lung cancer cells by targeting PTEN.* Molecular and cellular biochemistry, 2013. **372**(1-2): p. 35-45.

123. Saito, M., et al., *The association of microRNA expression with prognosis and progression in early-stage, non–small cell lung adenocarcinoma: a retrospective analysis of three cohorts.* Clinical cancer research, 2011. **17**(7): p. 1875-1882.

124. Hatley, M.E., et al., *Modulation of K-Ras-dependent lung tumorigenesis by MicroRNA-21.* Cancer cell, 2010. **18**(3): p. 282-293.

125. Zhu, H., et al., *Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells.* Biochemical pharmacology, 2008. **76**(5): p. 582-588.

126. Joshi, P., et al., *MicroRNAs in lung cancer.* World Journal of Methodology, 2014. **4**(2): p. 59-72.

127. Liu, X., et al., *MicroRNA-31 functions as an oncogenic microRNA in mouse and human lung cancer cells by repressing specific tumor suppressors.* The Journal of clinical investigation, 2010. **120**(4): p. 1298.

128. Slaby, O., et al., *Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer.* Oncology, 2007. **72**(5-6): p. 397-402.

129. Ferretti, E., et al., *MicroRNA profiling in human medulloblastoma.* International Journal of Cancer, 2009. **124**(3): p. 568-577.

130. Yamagishi, M., et al., *Polycomb-mediated loss of miR-31 activates NIK-dependent NF-κB pathway in adult T cell leukemia and other cancers.* Cancer cell, 2012. **21**(1): p. 121-135.

131. Comer, B.S., et al., *MicroRNA-146a and microRNA-146b expression and anti-inflammatory function in human airway smooth muscle.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2014. **307**(9): p. L727-L734.

132. Chen, G., et al., *miR-146a inhibits cell growth, cell migration and induces apoptosis in non-small cell lung cancer cells.* PLoS One, 2013. **8**(3): p. e60317.

Appendix

A. Materials

In the following tables is information regarding instruments (A.1), cell culture media (A.2), chemicals (A.3), solutions (A.4), kits (A.5) and computer software (A.6) used during the work of this thesis.

Instruments

Instrument	Name/Label
Microscope	Nikon Eclipse TS100
Centrifuges	Eppendorf Centrifuge 5702 & Eppendorf Centrifuge 5417R
Heating block	Grand instruments QBT2
Incubator	Thermo Scientific Steri Cycle CO_2 incubator
qPCR instrument	StepOnePlus™ Real-Time PCR Systems
Spectrophotometer	Eppendorf Biophotometer
Thermal cycler	Perkin Elmer Cetus DNA Cycler 480

Cell culture media

Medium	Additives	Manufacturer	Catalogue no.
GIBCO® LHC-9 Serum free medium	L-glutamine, Phenol red	Invitrogen	12680-3
RPMI-1640	25mM HEPES, L-Glutamine	GE Health Care Life Science	SH302255-01

Chemicals

Chemicals	Manufacturers	Chemicals	Manufacturers
Chloroform	Sigma Aldrich	Penicillin-streptomycin	Gibco
Collagen	Sigma Aldrich	Isol- RNA Lysis reagent	5’ Prime
Ethanol	Kemethyl	Tryphan Blue	Invitrogen
Fetal bovine serum (FBS)	Gibco Biotium Inc	Trypsin	Sigma Aldrich

Solutions

Solution	Content
AF-media	76% L-15 medium, 2% 1M HEPES, 2% PS, 20% FBS
BSA 60 mg/ml stock	60 mg BSA, 1 ml PBS
Collagen	1% collagen solution, 3.13 mg/ml, 99% HBS
DEPC water	0.1% DEPC in ddH2O
DMSO for cell culture storage	50% L-15 medium, 2% 1M HEPES, 8% DMSO, 40% FBS
Ethanol in DEPC water (75%)	12.5 ml DEPC H2O, Absolute ethanol to 50 ml
Fetal Bovine Serum (FBS)	Heat inactivated at 56°C for 45 min.
Phosphate Buffered Saline (PBS)	7.07g NaCl, 0.20g KCl, 1.94g NaHPO4*H2O, H2O to 1L.
Tris-acetate-EDTA (TAE) buffer	242 g Tris base, 57.1 ml glacial acetic acid, 100ml 0.5M EDTA pH8.
Trypsin 1%	50 mg trypsin, 50 ml PBS.

Kits

Process	Kit name	Manufacturer (catalogue no.)
mRNA cDNA synthesis	qScript™ cDNA Synthesis Kit	Quanta (95047-500)
mRNA qPCR	PerfeCTa SYBR Green Fast Mix, ROX™	Quanta (95073)
miRNA cDNA synthesis	miScript Reverse Transcription Kit	Qiagen (218061)
miRNA qPCR	miScript SYBR Green PCR Kit	Qiagen (218073)

PCR Primers

Gene	Sequence (5'-3')	Manufacturer
Actin Up	5' GCGAGAAGATGACCCAGATCA	DNA Technology A/S
Actin Lo	5' GATAGCACAGCCTGGATAGCAA	DNA Technology A/S
CYP1A1 Up	5' CATCCCCCACAGCACAACA	DNA Technology A/S
CYP1A1 Lo	5' CATCCCCCACAGCACAACA	DNA Technology A/S
CDH1 Up	5`ACGCCGAGAGCTACACGTTCA	DNA Technology A/S
CDH1 Lo	5`TCCTTTGTCGACCGGTGCAATC	DNA Technology A/S
COX2 Up	5' ATCACAGGCTTCCATTGACC	Already designed for thesis work.
COX2 Lo	5' CAGGATACAGCTCCACAGCA	Already designed for thesis work.
HO1 Up	5' TGACCCATGACACCAAGGAC	Already designed for thesis work.
HO1 Lo	5' GTGTTGAGTGGGGGCTTCC	Already designed for thesis work.
NRF2 Up	5' ACACACGGTCCACAGCTCAT	Already designed for thesis work.
NRF2 Lo	5' CCGTCGCTGACTGAAGTCAAAT	Already designed for thesis work.
IL1B Up	5'GCT GAT GGC CCT AAA CAG ATG	Already designed for thesis work.
IL1B Lo	5' AGT GGT GGT CGG AGA TTC GTA	Already designed for thesis work.
miR-17 Up	5' GCAAAGTGCTTACAGTGCAG	Already designed for thesis work.
miR-27 Up	5' UUCACAGUGGCUAAGUUCCGC	Qiagen (MS00003766)

miR-31 Up	5' GCAGAGGCAAGATGCTG	Already designed for thesis work.
miR-146a Up	5' TGAGAACTGAATTCCATGGG	Already designed for thesis work.
miR-146 b Up	5' GCAGTGAGAACTGAATTCCA	Already designed for thesis work.

Data Software

- Microsoft Office 2011 Excel (Microsoft Corporation, Redmond, WA)
- Sigma Plot v13 (San Jose, California, USA)
- Step One plus v2.3 (Carlsbad, California, USA)

General cell culture work

Thawing Cells

1. Ampoule containing cells were taken out from nitrogen tank and quickly thawed in water bath (37°C). 2.
2. The cell suspension was transferred to a centrifuge tube and 5ml LHC-9 growth medium was added. The cells were centrifuged for 4 minutes at 1000 rpm.
3. The supernatant was removed and the pellet was re-suspended in growth medium (3ml).
4. The cell suspension was then transferred to a 100mm Petri dish and growth medium (5ml) was added to make up final volume of 8 ml.
5. The Petri dish was kept in an incubator at 37°c temperature and humidified CO_2 environment (5%).

Passaging cells

1. The starting point was always approximately 80% confluent cell cultures.
2. Medium was removed from the cells.
3. Cells were washed with 10ml PBS twice.
4. Trypsin solution (1ml) was added to each plate.
5. Plates were kept in the incubator until the cells became detached from the surface of plates. This was checked by using a light microscope.

6. Growth medium (5ml) was added to each plate and cells were washed up and down in a 10ml serological pipette for separation of cells.
7. The cell suspension was then transferred to a centrifuge tube and centrifuged at 1000 rpm for 4 minutes.
8. The supernatant was discarded and pellet was re-suspended in medium (volume depended upon desired dilution).
9. The cell suspension was transferred to a 100 mm Petri dish and medium was added to a final volume of 8ml.
10. Plates were placed in the incubator at 37°C.

Freezing Cells

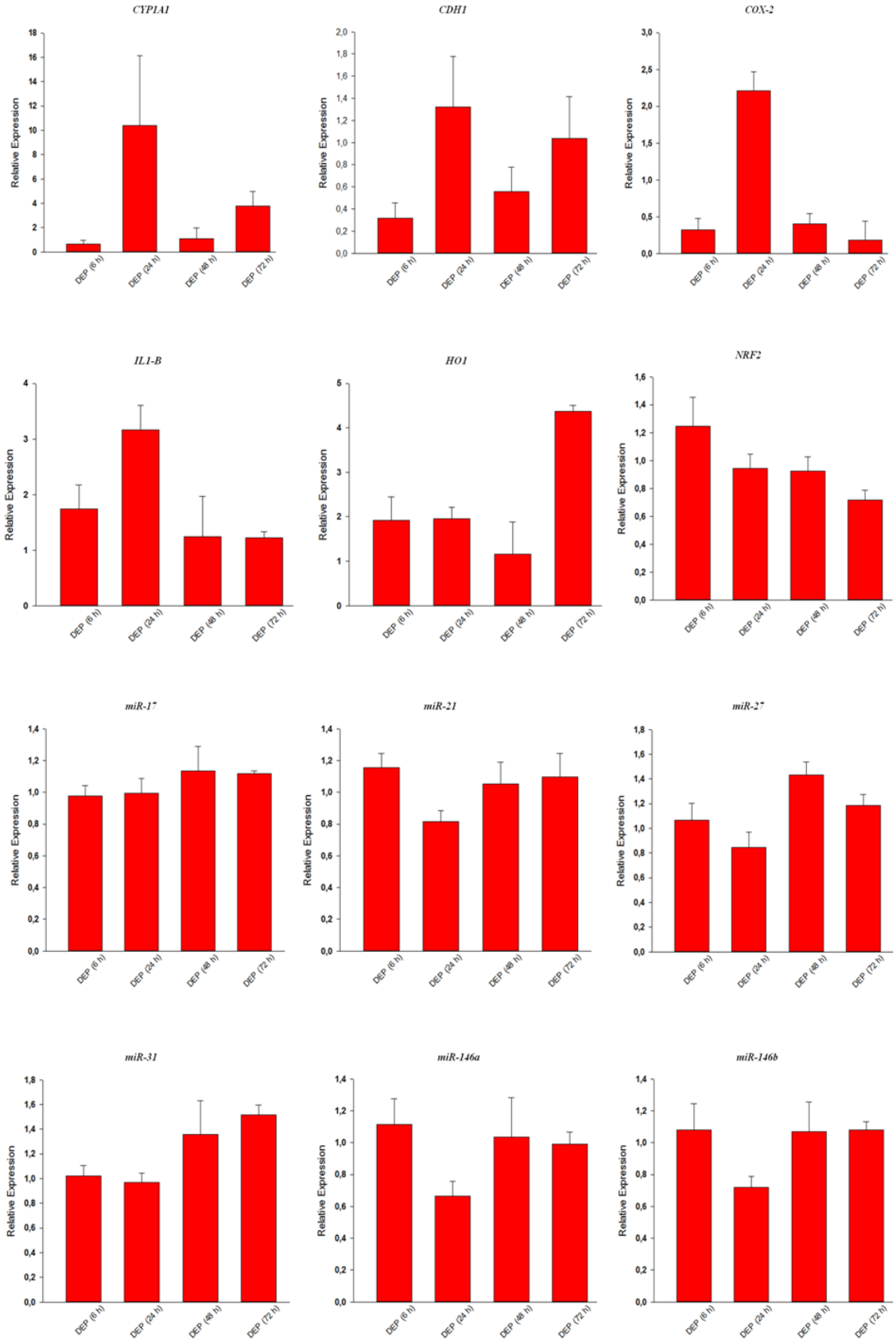
Cells were trypsinized as mentioned under the heading of passaging cells, point 1-7,

1. The cell pellet was re-suspended in AF medium (500µl) and transferred to a twist top vial.
2. Freezing medium DMSO (500µl) was added to the above tube.
3. Vials were placed in an insulated box at -80°c for 4-6 hours or overnight (to ensure gentle freezing of the cells).
4. The vials were then transferred to liquid nitrogen tank for long-term storage.

Collagen coating of petri dishes

1. PureCol® was diluted in PBS to get final concentration of 0.03mg/ml
2. The collagen solution was added to Petri dishes (100ml) and 6-well plates in 3ml and 1 ml/well, respectively, to completely cover the bottom of the dishes and wells.
3. Dishes and plates were kept in LAF-bench (at room temperature) for 2-3 hours.
4. The collagen solution was removed and dishes/plates were washed with PBS twice.
5. Dishes and plates were stored at -20°C.

C. Results from Short-Term Exposure Experiment No.1



D. Standard Curve from ELISA