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Transcriptomics in toxicology

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Abstract

Xenobiotics, of which many are toxic, may enter the human body through multiple routes. Excessive human exposure to xenobiotics may exceed the body's capacity to defend against the xenobiotic-induced toxicity and result in potentially fatal adverse health effects. Prevention of the adverse health effects, potentially associated with human exposure to the xenobiotics, may be achieved by detecting the toxic effects at an early, reversible and, therefore, preventable stage. Additionally, an understanding of the molecular mechanisms underlying the toxicity may be helpful in preventing and/or managing the ensuing adverse health effects. Human exposures to a large number of xenobiotics are associated with hepatotoxicity or pulmonary toxicity. Global gene expression changes taking place in biological systems, in response to exposure to xenobiotics, may represent the early and mechanistically relevant cellular events contributing to the onset and progression of xenobiotic-induced adverse health outcomes. Hepatotoxicity and pulmonary toxicity resulting from exposure to xenobiotics are discussed as specific examples to demonstrate the potential application of transcriptomics or global gene expression analysis in the prevention of adverse health effects associated with exposure to xenobiotics.

Keywords

Transcriptomics; Hepatotoxicity; Pulmonary toxicity; Mechanisms; Biomarker

1. Introduction

The term "xenobiotic" refers to agents that are foreign to the body or a biological system. Drugs constitute a major class among xenobiotics that are being developed, manufactured, and marketed to treat diseases. The pharmaceutical industry is under constant demand to develop drugs that are therapeutically more effective and safe. This can be achieved by designing and developing entirely novel drugs. Alternatively, existing drug molecules can be modified to improve their therapeutic efficacy. The resulting new drug molecules may

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present unknown and/or unexpected safety concerns and, therefore, it becomes essential to determine the toxicity or safety of these molecules prior to their intended use.

Another very important class of xenobiotics are agents that are being manufactured or synthesized and marketed for multiple applications. The number of new chemicals introduced for various applications has steadily increased over the years. For example, since the establishment of the Toxic Substances Control Act (TSCA) Inventory in 1976, more than 40,000 new chemical submissions (Premanufacture Notices or PMNs) have been reviewed by the United States Environmental Protection Agency (EPA). This is in addition to the approximately 15,000 PMN exemptions. In addition to classic chemicals, other agents with significant applications are being regularly introduced. For example, a large number of materials, collectively referred as nanomaterials, have been introduced in recent years. Products enabled by nanotechnology have a wide range of applications in healthcare, consumer products, energy production, and the automobile industry. Currently, the global nanotechnology market is $39.2 billion and is anticipated to reach $90.5 billion by 2021 at a compound annual growth rate of 18.2%.

In addition to the synthetic or manufactured xenobiotics described above, there are many naturally occurring xenobiotics present in the earth’s crust. Some of these xenobiotics or materials that contain them have significant commercial or industrial application, and significant exposure to such xenobiotics can take place through occupation-related activities. Crystalline silica, asbestos and various metals are examples of such naturally occurring xenobiotics that are causative agents for various adverse health effects following significant human exposure to them (Kim et al., 2015; Sauve, 2015; Gilham et al., 2016).

While xenobiotics, either manufactured or naturally occurring, are of immense benefit to mankind, some of them, including several commonly used ones are potentially toxic. Significant quantities of potentially toxic xenobiotics have been detected in air, water, and food. Exposure to xenobiotics, therefore, is almost unavoidable and may pose a significant threat to human health. Virtually every organ/tissue in the body has been identified as a target for toxicity and adverse health effects resulting from unintended human exposure to various xenobiotics. Exposure to xenobiotics, for example, has been identified as a major etiological factor for several adverse health outcomes such as cardiovascular effects (Lippmann, 2014), renal effects (Mascarenhas et al., 2017), pulmonary effects (Peretz et al., 2006), neurological effects (Ognjenovic and Perry, 2005) reproductive effects (Cavallini et al., 2016), diseases affecting the immune system (Pollard, 2016), and cancer (Lamm et al., 2015).

As described above, human exposure to xenobiotics is almost unavoidable. In addition, an association is known to exist between exposure to xenobiotics and the incidence of adverse health effects in humans (Peretz et al., 2006; Lamm et al., 2015). Therefore, it is very important to develop effective strategies to prevent the onset and progression of such life-threatening adverse health effects associated with human exposure to xenobiotics. Prevention of the xenobiotic-induced toxicity and the resulting adverse health effects may be achieved by detecting the toxicity at an early, reversible or preventable stage. In addition, a proper understanding of the mechanisms underlying the xenobiotic-induced toxicity is

instrumental in the prevention of the life-threatening adverse health effects associated with human exposure to toxic xenobiotics. Such an understanding, if possible, should not be confined to a single tissue or organ in the body, but rather to all the toxic effects potentially taking place in all organs and organ systems in the body. This involves the identification of the molecular targets of the toxicity, the interaction of the xenobiotics with the molecular targets, perturbations of the cellular networks and pathways, the onset and progression of toxicity, and structural and functional impairment in target organs/organ systems leading to potential life-threatening adverse health effects.

A systems toxicology approach, with integration of classical toxicology with quantitative analysis of large networks of molecular and functional changes occurring across multiple levels of biological organization, is promising in studying the full spectrum of toxicity of xenobiotics (Sturla et al., 2014). A systems toxicology approach includes studying and understanding anything and everything that happens in a biological system in response to its exposure to a xenobiotic. The major elements of systems toxicology are:

1. Absorption and distribution of the xenobiotic within the biological system.
2. Transformation, for example metabolism, of the xenobiotic resulting in the generation of toxic and/or non-toxic intermediates.
3. Interaction of the parent xenobiotic and/or its products with the cellular targets of toxicity.
4. Alterations in the cellular molecules including genes, proteins, and lipids.
5. Structural manifestations of target organ toxicity, for example histological changes.
6. Functional manifestations of toxicity, for example impairment of critical functions in target organ(s)/organ system(s), and
7. Elimination of the xenobiotic and/or the transformation intermediates from the biological system.

Systems toxicology is based on the premise that manifestations of xenobiotic-induced toxicity, structural and/or functional changes, are caused mainly by changes in cellular molecules such as genes, proteins, lipids, metabolites, etc. at cellular, tissue, organ, or organism level. Systems toxicology requires powerful and revolutionary techniques capable of capturing the “global” changes taking place in a biological system in response to the xenobiotic exposure. Recent developments in biology, especially molecular biology, computer science, mathematics, and bioinformatics have contributed enormously to the genesis of various “omics” techniques which play an important role in systems toxicology. The major “-omics” techniques comprise genomics (study of the genome or DNA), transcriptomics (study of the transcriptome or mRNA), proteomics (study of the proteome or proteins), lipidomics (study of the lipidome or lipids), metabolomics or metabonomics (study of the metabolome or metabolites or small molecules), adductomics (study of DNA adducts due to xenobiotic exposure), and epigenomics (study of the epigenome). The “-omics” technologies enable the simultaneous measurement of global or all definable entities of an “-ome”. Transcriptomics, for example, enables the simultaneous detection and analysis

of all the transcripts or mRNAs expressed in a biological system at a given time. Whereas the various "-omics" techniques are helpful and important to understand the system or total toxicity of a xenobiotic, the scope of this review article is restricted to transcriptomics in toxicology.

In general, xenobiotics influence global gene expression profile by genetic and/or epigenetic mechanisms. Genetic mechanisms involve a direct interaction between a xenobiotic and DNA, the genetic material. The direct interaction between the xenobiotic and DNA may result in DNA damage (Poirier, 2016) such as DNA adduct formation and/or mutation capable of resulting in a change in the DNA sequence. The DNA damage resulting from the interaction between a xenobiotic and DNA may influence the transcription machinery resulting in altered gene expression profile (Labib et al., 2016). Epigenetic mechanisms, on the other hand, do not involve a direct interaction of the xenobiotic with DNA. The effect of the xenobiotic on the DNA is indirectly and mediated through the epigenome resulting in gene expression changes. The major epigenetic mechanisms affecting gene expression profile are DNA methylation (Bird, 2011), histone modification (Mudbhary and Sadler, 2011), and interaction with non-coding RNA such as microRNA (Costa, 2008). The various epigenetic mechanisms may alter chromatin structure resulting in gene expression changes (Probst et al., 2009). The role of epigenome in regulating gene expression has been well recognized under a variety of toxicity conditions (Zhang et al., 2010; Bernal and Jirtle, 2010; Vanhees et al., 2011; Engstrom et al., 2013; Williams et al., 2014; Thomson et al., 2014). Data derived from *in vitro*, *in vivo*, and epidemiological studies have demonstrated the potential of various xenobiotics to result in gene expression changes that are mediated through their effect on the epigenome (Paul et al., 2011; Herceg and Vaissiere, 2011; Baccarelli and Bollati, 2009).

The potential applications of transcriptomics in toxicology, with a special emphasis on the prevention of adverse health effects resulting from human exposure to xenobiotics, will be described in this review article. Special emphasis will be on the early detection of xenobiotic-induced toxicity and the molecular mechanisms of target organ toxicity with reference to the prevention of adverse health effects resulting from exposure to hepatotoxic and pulmonary toxic xenobiotics.

2. Transcriptomics in toxicology – technical aspects

The techniques currently available to determine changes in gene expression profiles in biological samples in response to their exposure to a xenobiotic include northern hybridization (Kendall and Riley, 2000), quantitative real time PCR (QRT-PCR) (Song and Freedman, 2005), subtractive hybridization (Volz et al., 2005), serial analysis of gene expression (Taulan et al., 2004), differential display (Tomita et al., 2002), microarray analysis (Sellamuthu et al., 2011b), and next generation sequencing (Klaper et al., 2014). Depending on the objective of the study, one or more of the abovementioned techniques may be employed to determine either partial or global expression profiles of the transcripts present in a biological sample. However, the most popular gene expression profiling techniques currently employed are QRT-PCR, microarray analysis, and next generation sequencing. If the objective is to determine the expression profile of a single or a limited

number of transcripts expressed in a biological sample, QRT-PCR analysis is most often the method of choice. QRT-PCR analysis is a relatively simple technique that involves the reverse transcription of mRNAs followed by PCR amplification of the resulting cDNAs using oligo-nucleotide primers specific for the gene of interest as well as one or more house-keeping genes and quantitating the amount of the PCR amplified gene products. While QRT-PCR is able to accurately determine the expression of an individual gene transcript or those of a limited number of transcripts, from a system toxicology perspective, the technique has some limitations. Analyzing the expression profiles of thousands of transcripts that comprise the entire transcriptome in an organism by QRT-PCR is neither practical nor economical. Therefore, in order to study the systems-level toxicity of a xenobiotic, it becomes necessary to develop high throughput techniques capable of determining expression levels of the entire transcriptome. Microarray and next generation sequencing are the two most popular high throughput global transcriptome analysis techniques available currently.

The microarray technique represents a major breakthrough among the high throughput global transcriptome analysis techniques developed in the post-human genome sequencing era. Similar to Southern hybridization technique (Southern, 1975), the microarray technique takes advantage of the ability of DNA molecules to bind or hybridize in a nucleotide sequence-specific manner. Microarray can be considered as a high throughput northern blot hybridization technique in which the expression levels of thousands of transcripts are detected simultaneously. Due to the advances made in microarray technology, it has become possible to detect the expression of almost all the genes that are expressed in a biological sample at a given time Canales et al., 2006. Therefore, the microarray technique enables the detection of differences in the global gene expression profiles in a biological sample in response to exposure to xenobiotic(s). The microarray technique is rather complex and involves the isolation of high quality RNA from biological samples, reverse transcription of the mRNA to synthesize cDNA, cRNA synthesis and labeling to generate “probes”, hybridization of the “probes” with “targets” present on the microarray, detection of the signal intensity of the hybridized probe-target complex, and analysis of the resulting data to determine the expression profiles of the genes present in the biological samples employed in the study (Joseph, 2011). Through the adoption of standards for presenting and exchanging microarray data, it has become possible to easily reproduce, interpret, and verify the results obtained from studies analyzing global gene expression pro-file by employing microarray (Brazma et al., 2001). Microarray-based transcriptome analysis has been applied in fields such as environmental toxicology (Dix et al., 2006; Williams et al., 2014), drug development (Frueh, 2006; Shi et al., 2010; Cheng et al., 2011; Zhang et al., 2012; Chen M. et al., 2014; Chen S. et al., 2014; Otava et al., 2015; Kohonen et al., 2017), and occupational toxicology (Nikota et al., 2016). The differential gene expression data obtained from a microarray analysis provides a snapshot of all the genes whose expression levels are affected in a biological sample in response to xenobiotic exposure.

Next generation sequencing (NGS) is the most recently developed global transcriptome analysis technique that can be applied to determine a global gene expression profile in RNA obtained from biological samples (Finotello and Di Camillo, 2015). The various steps involved in NGS are RNA isolation, removal of abundant RNA molecules such as ribosomal RNA and globin RNA to eliminate their interference in sequencing and detection of the less

abundant transcripts, preparation of sequencing libraries, PCR amplification, and sequencing of the libraries. The resulting data is analyzed and the abundance of the individual transcripts is determined. The reagents and equipments required for NGS are available from several vendors and over the years the cost associated with NGS has substantially decreased enabling more wide-spread use of NGS in global transcriptome profiling.

3. Application of transcriptomics in the prevention of adverse health effects potentially associated with xenobiotic exposure

The ultimate goal of any toxicity study is to prevent the toxicity and the associated adverse health effects potentially resulting from human exposure to xenobiotics. Detection of target organ toxicity, prior to the appearance of permanent or irreversible damage, has significant benefit in preventing the adverse health effects associated with human exposure to xenobiotic(s). Similarly, a thorough understanding of the mechanisms underlying the xenobiotic-induced toxicity is helpful to prevent or manage the potential adverse health effects. The transcriptome, unlike the genome, is highly dynamic in nature and often responds sensitively to xenobiotic exposure in a biological system. Therefore, expression profiling of the entire transcriptome in a biological system, following its exposure to a xenobiotic and the identification of the genes whose expression levels are significantly affected because of the xenobiotic exposure, may be considered a sensitive, early indicator of toxicity resulting from exposure to the xenobiotic agent. The differentially expressed genes and/or their products, following appropriate validation, may be employed as biomarkers for detection of exposure and/or toxicity of the xenobiotic being investigated. Similarly, diligent bioinformatic analysis of the significantly differentially expressed transcripts detected in a biological system, in response to a xenobiotic exposure, provides valuable insight into the mechanism(s) underlying the toxicity of the agent. Therefore, global gene expression profiling, as supported by the results of several studies (Waring et al., 2001; Hamadeh et al., 2002a, b; Amin et al., 2004; Heinloth et al., 2004; Sellamuthu et al., 2012, 2013; Otava et al., 2015), is often considered a sensitive and mechanistically relevant approach to detect and understand target organ toxicity associated with exposure to xenobiotics. Past studies have demonstrated the application of global transcriptomics in early detection of the xenobiotic-induced target organ toxicity as well as in elucidating the molecular mechanisms underlying the toxicity-key requirements for the prevention of adverse health effects resulting from human exposure to xenobiotics. Xenobiotic-induced hepatotoxicity and pulmonary toxicity will be discussed in this article as specific examples to illustrate the application of transcriptome analysis in the early detection of toxicity and determination of the molecular mechanisms underlying target organ toxicity. Results of studies conducted using acetaminophen, a representative hepatotoxic xenobiotic, are described mostly to demonstrate the application of transcriptomics in studying hepatotoxicity. Similarly, results of the studies conducted using crystalline silica, an inhalable xenobiotic present in many workplaces, are described in this article to demonstrate the application of transcriptomics in pulmonary toxicity.

4. Transcriptome profiling to detect hepatotoxicity

The application of high throughput transcriptome profiling in toxicity studies is illustrated best in the case of hepatotoxicants or xenobiotics that target liver as their primary target organ for toxicity. In fact, many of the pioneering studies that investigated the application of high throughput transcriptome profiling in toxicology were conducted by employing xenobiotics that were primarily hepatotoxic in nature (Bulera et al., 2001; Hamadeh et al., 2002a, b; Waring et al., 2001). In their study, Waring et al. (2001) administered 15 different hepatotoxic chemicals to rats. The various chemicals selected in this study are all known to result in hepatotoxicity such as necrosis, DNA damage, cirrhosis, hypertrophy and hepatic carcinoma. Hepatotoxicity was determined by histopathology in the liver and clinical chemistry evaluation of the blood. In addition, global gene expression profiles in the liver of the rats were determined by microarray analysis. Overall, a good correlation was noticed between histopathology, clinical chemistry, and gene expression profiles induced by the hepatotoxic agents employed. The authors also attempted to cluster the various hepatotoxic compounds employed in the study based on their hepatotoxicity (histopathology and clinical chemistry results) and liver gene expression profiles. The results demonstrated that the various hepatotoxic compounds employed in the study clustered together in fairly good agreement based on the observed changes in clinical chemistry, histopathology and gene expression profile. In some cases, liver gene expression profiles outperformed the traditional hepatotoxicity endpoints (histopathology of liver and blood clinical chemistry) in clustering the hepatotoxic chemicals. The authors, based on the results of their study, concluded that global gene expression profiling could be employed as a highly sensitive and reliable technique to identify hepatotoxic chemicals.

4.1. Mechanisms of hepatotoxicity revealed by transcriptome profiling

Bioinformatic analysis of the gene expression data obtained from a toxicity study is expected to provide information regarding the molecular mechanisms underlying the toxicity of the xenobiotic under investigation. Several investigators have conducted studies investigating the mechanisms of hepatotoxicity based on global gene expression changes taking place in the liver in response to xenobiotic exposure. Hamadeh et al. (2002b) employed global gene expression profiling to classify hepatotoxic chemicals that differed in their mechanisms of toxicity. Rats were administered either peroxisome proliferators (clofibrate, Wyeth 14,643 and gemfibrozil) or an enzyme inducer (phenobarbital) to result in hepatotoxicity. Hepatotoxicity and liver gene expression profiles of the rats were determined by histopathology and microarray analysis, respectively. The microarray data were further subjected to several computational analyses to determine whether the hepatotoxic chemicals exhibit gene expression patterns distinguishable based on their mechanisms of toxicity. Results of this study demonstrated that the peroxisome proliferators exhibited a gene expression pattern that was clearly distinguishable from the enzyme inducer suggesting the potential application of global gene expression profiling to classify hepatotoxic chemicals based on their mechanism(s) of toxicity. The results of a study carried out by Huang et al. (2008) further supported the findings that gene expression profiling can be employed to predict hepatotoxicity as well as understand the mechanisms of xenobiotic toxicity in the liver. Rats were administered one of seven hepatotoxic chemicals that are known to induce

necrosis. Microarray analysis of the global gene expression profile in the rats identified several differentially expressed genes in the chemical treated rats compared with the controls. Using a Random Forrest classifier with feature selection, a signature consisting of 21 genes was identified as a predictor of necrosis induced by the hepatotoxic chemicals. The selected predictor genes were able to predict necrosis induced by the model hepatotoxic chemicals such as acetaminophen, carbon tetrachloride and allyl alcohol with an accuracy of 90%, 80% and 60%, respectively. Pathway and network analysis of the necrosis predictor genes identified inflammation and apoptosis as major biological processes responsible for necrosis induced by the selected hepatotoxic chemicals.

4.2. Early detection of hepatotoxicity by transcriptome profiling

Early detection of hepatotoxicity is important in the prevention of serious and potentially life-threatening adverse health effects associated with exposure to hepatotoxic xenobiotics. Obviously, this will require technique(s) with superior sensitivity capable of detecting the earliest and preventable stage in the development of liver toxicity. A major advantage of gene expression profiling in toxicity studies is its superior sensitivity to detect target organ toxicity compared with the traditional toxicity endpoints such as histology and clinical chemistry. Several studies have indicated that gene expression changes indicative of hepatotoxicity precede the onset of clinical or histopathological changes associated with hepatotoxicity. In a study conducted by Heinloth et al. (2004) rats were administered either sub-toxic (50 and 150 mg/kg b.w.) or toxic (1500 mg/kg b.w.) doses of acetaminophen. At time intervals of 6, 24 and 48-h following administration of the chemical, groups of rats were euthanized and histopathology and clinical chemistry in blood were analyzed to assess the induction of hepatotoxicity. At each time interval, RNA was isolated from the liver and global gene expression profiles were determined by microarray analysis. The rats administered the sub-toxic doses of acetaminophen exhibited normal histology and clinical chemistry at all time intervals. This may suggest either the absence of hepatotoxicity in the rats or the inability of these traditional hepatotoxicity endpoints, because of their poor sensitivity, to detect subtle toxicity induced by acetaminophen. Similarly, rats administered the overtly toxic dose of acetaminophen (1500 mg/kg b.w.) did not exhibit alterations in either histology or clinical chemistry at the earliest time interval of 6 h following administration of the chemical. However, significant hepatotoxicity, as indicated by alterations in liver histology and blood clinical chemistry, was noticed in the rats administered 1500 mg acetaminophen/kg b.w. at later time intervals of 24 and 48 h. Microarray analysis of global gene expression profiling identified significant differential expression of several genes including those involved in energy metabolism and stress response in the liver of the rats administered 1500 mg acetaminophen/kg b.w. for 24 and 48-h. Interestingly, many of the genes which were found differentially expressed at time intervals of 24- and 48-h in the rats administered the overtly toxic dose of 1500 mg/kg b.w., and are therefore considered as indicators of acetaminophen-induced hepatotoxicity, were also found differentially expressed in the rats administered the sub-toxic doses of the chemical. It is worth mentioning that the rats administered the sub-toxic doses of acetaminophen did not exhibit any histological or clinical changes indicative of toxicity. Taken together, these results demonstrated the superior sensitivity of gene expression changes to detect hepatotoxicity compared with the traditional histological and clinical

markers of hepatotoxicity. The superior sensitivity of gene expression profiling compared with the traditional hepatotoxicity end points, in detecting hepatotoxicity has also been reported by other investigators (Hamadeh et al., 2002b).

4.3. Transcriptome profiling to develop biomarkers for hepatotoxicity detection

As described in detail above, global analysis of the transcriptome is certainly a viable and attractive approach to detect the target organ toxicity, including hepatotoxicity, of xenobiotics in animal models. However, a major limitation in determining the toxicity of xenobiotics in human beings is the difficulty in obtaining samples of target organs. For example, due to ethical and/or other practical issues such as the health and safety of the individuals involved, it is not possible to obtain samples of liver tissues from human beings to determine hepatotoxicity. A practical solution to this problem is the potential use of surrogate tissues to study target organ toxicity including hepatotoxicity (Burczynski and Dorner, 2006). Of all the surrogate tissues employed to study target organ toxicity, blood is not only the one most frequently used but also the most suitable one (Rockett, 2006). Blood may be considered as the internal environment for the various organs and tissues in the body that are often the targets for xenobiotic toxicity. Alterations in the activity of specific enzymes or the absolute amount of certain biochemical molecules or even the amount of the parent compound and/or its metabolite(s) present in the circulating blood or blood cells may be considered as markers for xenobiotic-induced target organ toxicity. For example, activities of transaminases (aspartate and alanine aminotransferase) in the blood are routinely employed as reliable surrogate markers of hepatotoxicity (Heinloth et al., 2004; Beyer et al., 2007; Bushel et al., 2007).

While the above mentioned surrogate toxicity markers have been helpful to determine the target organ toxicity of xenobiotics, all or most of them suffer from significant short-comings. These include, but are not limited to, the non-specificity and poor sensitivity of these surrogate biomarkers to detect target organ toxicity. For example, significant structural damage, such as necrosis, to the hepatocytes is necessary for transaminases to be released from the liver to the peripheral blood and then measured as markers of hepatotoxicity. This contributes to poor sensitivity of blood transaminases as surrogate markers of hepatotoxicity. Furthermore, the serum level of transaminases may be affected by conditions that affect organs other than the liver. For example, significant alterations in serum transaminase levels have been reported under chronic kidney disease conditions (Sette and Almeida Lopes, 2014) complicating the use of serum levels of transaminases as diagnostic indicators of hepatotoxicity.

Gene expression changes taking place in target organs, for example liver, are known for their superior sensitivity to detect toxicity compared with the traditional biochemical and histological toxicity markers (Heinloth et al., 2004). Therefore, it is reasonable to investigate whether gene expression profiling to determine target organ toxicity can be performed in easily available surrogate tissues such as blood. In a rat study, Bushel et al. (2007) investigated the application of blood gene expression profiles as sensitive indicators of hepatotoxicity induced by the model hepatotoxic drug e acetaminophen. Rats were given either a sub-toxic (150 mg/kg b.w.) or one of two overtly toxic (1500 and 2000 mg/kg b.w.)

doses of acetaminophen. Blood and liver obtained from the control and acetaminophen administered rats were analyzed to determine hepatotoxicity induced by the drug. The parameters employed to assess toxicity included liver histology, various hematological parameters, blood level of transaminases and global gene expression profiling in blood. The results of this study demonstrated that blood gene expression profiles exhibited superior sensitivity as hepatotoxicity markers compared with the traditional toxicity endpoints: histological, hematological, and biochemical changes. The authors, based on the results of their study, concluded that blood gene expression profiles may be employed as sensitive surrogate markers to detect hepatotoxicity induced by acetaminophen.

Results of a study carried out in our laboratory (Umbright et al., 2010) further supported the findings of the study by Bushel et al. (2007) suggesting that blood gene expression profiling may be employed as a sensitive, alternate approach to detect hepatotoxicity. Rats were administered a single, acute dose of the hepatotoxicant, acetaminophen. Administration of acetaminophen resulted in hepatotoxicity as evidenced from significant elevation in blood transaminase activities. Microarray analysis of the global gene expression profile of leukocytes obtained from blood samples identified a distinct gene expression profile in association with the acetaminophen-induced hepatotoxicity in rats. The blood gene expression markers of hepatotoxicity were detectable earlier than the appearance of the commonly used clinical markers of hepatotoxicity, further supporting the superior sensitivity of blood gene expression markers compared to traditional hepatotoxicity endpoints (Umbright et al., 2010). The ability of the marker genes to detect hepatotoxicity was further confirmed using the blood samples obtained from rats administered additional model hepatotoxic chemicals (thioacetamide, carbon tetrachloride, and dimethylnitrosamine).

The potential application of blood transcriptomics as suitable surrogate markers of hepatotoxicity was also demonstrated by the results of another comprehensive study (Lobenhofer et al., 2008). In their study, Lobenhofer and colleagues employed a series of eight well characterized hepatotoxic chemicals. The hepatotoxic chemicals employed in the study are known to exhibit similarities and differences in the type and location of necrosis induced in liver. The study was designed to provide a means to generate mechanistic and predictive measures of toxicity by integrating multiple data streams obtained from liver (target organ) and blood (surrogate tissue). The hepatotoxic chemicals selected were administered to rats at three doses (low, medium and high) for three time intervals (6-, 24- and 48-h) to result in sub-toxic to severe hepatotoxicity (necrosis). Toxicity was determined based on established histo-logical, biochemical, and hematological parameters in liver (the target organ) and blood (surrogate tissue). In addition, global gene expression profiling of liver and blood was determined by micro-array analysis and a support vector machine (SVM) approach was employed to identify hepatotoxicity-specific classifiers for the target organ and the surrogate tissue. The classifiers thus obtained were used to group the samples based on principal component analysis and hierarchical clustering to determine whether the animals exposed to each of the hepatotoxicants could be classified into different compound groups. Results of the study demonstrated that the classifiers derived from both liver and blood transcriptomic data were able to accurately classify samples on the basis of the hepatotoxic chemical administered. However, the classifiers generated from the blood data outperformed those generated from the liver data in classifying the hepatotoxicants. These

results, therefore, in agreement with the findings of others (Bushel et al., 2007; Umbright et al., 2010), confirmed the superior sensitivity of blood gene expression profiles as surrogate markers of hepatotoxicity.

It is quite encouraging to see that the findings of blood transcriptomics studies conducted in animal models to detect hepatotoxicity have been extended to human subjects. The potential application of peripheral blood gene expression profiling in the detection of hepatic response to the administration of the model hepatotoxic drug, acetaminophen, in human subjects has been demonstrated in subsequent studies (Fannin et al., 2010, 2016; Bushel et al., 2017). Recently, Bushel et al. (2017) employed whole blood as a surrogate biospecimen to identify gene expression signatures as potential early markers of acetaminophen-induced hepatotoxicity in human subjects. Healthy human subjects were administered acetaminophen or a placebo daily for 7 days. Alanine aminotransferase (ALT) and a gene expression signature consisting of 12 genes were evaluated in the blood of the placebo or acetaminophen administered subjects for detection of hepatotoxicity, if any. Significant elevation in blood level of ALT was detected in the acetaminophen administered subjects, compared to those administered the placebo, between 4 and 9 days subsequent to the first day of the drug administration. In contrast, expression profiles of the blood marker genes were significantly altered within 24 h of administration of the first daily dose of acetaminophen. The early responsive genes for hepatotoxicity in blood separated the subjects by class and dose period. In addition, the genes clustered the acetaminophen-administered patients apart from the placebo controls. Furthermore, the blood gene expression signature predicted the exposure classifications with 100% accuracy. These findings supported the potential application of blood gene expression signatures as sensitive, early indicators of hepatotoxicity in humans in agreement with the findings of animal studies (Bushel et al., 2007; Lobenhofer et al., 2008; and Umbright et al., 2010).

4.4. Transcriptomics – applications in predicting drug-induced liver injury

Drug-induced liver injury (DILI) is the major reason for failure of a large number of candidate drug molecules both in the pre-clinical and clinical stages of drug development (Shi et al., 2010). Additionally, DILI continues to be the major reason for the withdrawal of drugs that are already approved and marketed for human use (Lee, 2008). It has been estimated that 10% of the drugs documented in human clinical practice are associated with DILI (Hussaini and Farrington, 2007) and DILI is the number one etiology for acute liver failure in Europe and the USA (Lee, 2008). The underlying causes of DILI include toxic effects caused by reactive metabolites, reactive oxygen species, induction of inflammation, mitochondrial dysfunction, and imbalances between cellular damage and protective mechanisms (Kaplowitz, 2005; Jaeschke et al., 2012; Ju and Reilly, 2012; Yuan and Kaplowitz, 2013; Chen M. et al., 2014; Chen S. et al., 2014).

In vivo rodent models are employed traditionally to investigate and predict the potential of a drug to cause liver injury in humans (Dixit and Boelsterli, 2007). Elevations in blood levels of hepatotoxicity markers, viz: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bilirubin (TBIL), and γ-glutamyltranspeptidase (GTP) are generally accepted as preclinical markers of DILI

(Giannini et al., 2005; Tsubouchi et al., 2010). Liver histological changes are also very often considered as reliable indicators of liver injury in rodents (Lobenhofer et al., 2008). In spite of the wide-spread use of animal models to predict DILI in human, approximately 40% of DILI cases are not detected in the pre-clinical studies (Zhang et al., 2012). The animal tests to detect DILI are costly and time consuming and, therefore, not suitable to screen a large number of drug candidates. There is also the constant demand to reduce, refine, and replace the use of animal models in preclinical studies designed to determine the safety of drug molecules. Such concerns have resulted in the exploration of alternate in silico or *in vitro* cell culture models that can efficiently and accurately predict DILI during the early stages of drug development.

In vitro cell culture models are attractive alternatives to *in vivo* animal models to evaluate the potential of drug candidates to result in DILI. Cell culture models are cheaper, faster, and more convenient to screen a large number of drug molecules for liver toxicity (Yang et al., 2004). Several investigators have attempted to integrate transcriptome analysis in cell culture models as an alternate approach to the use of expensive *in vivo* models for early detection of liver toxicity. Substantial differences have been noticed in the gene expression profiles between *in vitro* and *in vivo* toxicogenomics models that are exposed to the same xenobiotic (Otava et al., 2015) questioning the relevance of *in vitro* cell culture models in detecting early liver toxicity. Pathway analysis have demonstrated that most of the disconnected genes identified between the *in vitro* and *in vivo* drug-induced hepatotoxicity models belonged to known pathways such as drug metabolism and oxidative stress due to reactive metabolites, bilirubin increase, glutathione depletion and phospholipidosis. It has been suggested that pathway analysis of the disconnected genes is likely to improve our understanding of uncertainties in the mechanisms of toxicity of drug candidates in humans (Otava et al., 2015).

Based on the results obtained from an *in vivo* rodent study that involved the use of six heterogenous hepatotoxic compounds, a multi-gene expression signature to detect and predict hepatotoxicity was developed by Cheng et al. (2011). The multi-gene predictor signature, consisting of 32 genes, obtained from the *in vivo* rodent study was independently validated and employed to predict hepatotoxicity in an *in vitro* human liver cell culture model consisting of human liver cells. The predictor signature distinguished the hepatotoxic compounds from non-hepatotoxic compounds. In addition, the predictor signature distinguished between toxic and non-toxic doses of the same drug molecule in the hepatocytes. Based on the results obtained from the study, the authors concluded that their predictive expression signature model involving *in vitro* human liver cells could potentially be used to detect liver toxicity in early phases of drug discovery. Similar studies, investigating the potential use of transcriptomics-based approach in *in vitro* cell culture models, to predict DILI have been reported by several investigators (Jennen et al., 2014; Kohonen et al., 2017; Chen M. et al., 2014; Chen S. et al., 2014; Liu et al., 2017). The consensus of the findings of the various studies is that animal testing systems are still considered as the standard way to assess drug safety and detect potential safety concerns. However, the putative hepatotoxicity of a relatively large number of molecules can be screened easily using the *in vitro* model facilitating the selection of candidates for further *in vivo* toxicity studies. Therefore, the expression profiling of candidate signature genes for

hepatotoxicity in *in vitro* cell culture systems seems to complement, not replace, the animal-based *in vivo* liver injury models to predict DILI in human subjects.

5. Transcriptome profiling to detect pulmonary response to xenobiotic exposure

Crystalline silica is a classic example of a xenobiotic that targets the lungs for toxicity resulting in potentially life-threatening adverse health effects in humans. Because of its extremely common natural occurrence and the wide range of materials and products that contain it, occupational exposures to respirable crystalline silica occur in a variety of industries and occupations. Millions of workers in the U.S (OSHA, 2016). and worldwide (WHO, 2007) are exposed to crystalline silica annually. The NIOSH Recommended Exposure Limit (REL) for crystalline silica is 0.05 mg/m^3 as a time weighted average (TWA) for up to a 10-h workday during a 40-h workweek (NIOSH, 2002). However, workers in several U.S. industries are occupationally exposed to silica at much higher levels than the NIOSH REL (Linch et al., 1998; Esswein et al., 2013; Pavilonis and Mirer, 2017) posing a significant risk among such workers for adverse health effects associated with the exposure.

Occupational exposures to respirable silica increase the risk of developing diseases such as lung cancer (IARC, 1997), tuberculosis (ATS, 1997), autoimmune disorders (Cowie, 1987; Rafnsson et al., 1998), and chronic renal disease (Steenland et al., 1990; Calvert et al., 1997). Silicosis, an incurable but preventable and potentially fatal disease, is one of the most serious adverse health effects associated with exposure to silica (Steenland and Brown, 1995aa,b; WHO, 2007). Past epidemiologic studies have demonstrated that workers have a significant risk of developing silicosis when they are exposed to respirable silica over a working lifetime at the current Occupational Safety and Health Administration (OSHA) Permissible Exposure Limit (PEL), the Mine Safety and Health Administration (MSHA) PEL, or the NIOSH REL (Hnizdo and Sluis-Cremer, 1993; Steenland and Brown, 1995b; Kreiss and Zhen, 1996). In the U.S., at least 100 people die annually due to complications arising from silicosis (Mazurek et al., 2015).

Prevention and/or elimination of silicosis and silica-related diseases in the U.S. are priorities of NIOSH, OSHA, MSHA, and the American Lung Association. However, the inability to detect this incurable disease in the early, preventable stage remains a major obstacle in the prevention and/or elimination of silicosis. In view of this, NIOSH has recommended developing highly sensitive and non-invasive or minimally invasive technique(s) for early detection and prevention of silicosis (NIOSH, 2002). Our laboratory is currently engaged in research investigating the molecular mechanisms underlying silica-induced pulmonary toxicity and developing blood gene expression signature(s) as sensitive surrogate biomarker(s) for early detection of silicosis. A brief description of the major findings of our research project is provided in the following sections, and additional details of the findings can be found in our original publications (Sellamuthu et al., 2011a, 2011b; 2012, 2013 and 2017).

5.1. Transcriptomics changes in response to crystalline silica exposure

Whether a relationship exists between crystalline silica-induced pulmonary toxicity and global gene expression changes taking place in biological samples was investigated using cell culture and animal models. It has been fairly well established that crystalline silica, compared to amorphous silica, is more biologically active and toxic (Warheit et al., 1995; Johnston et al., 2000; Fubini and Hubbard, 2003). *In vitro* cell culture models were employed to investigate whether global gene expression changes would reflect the differences in the toxicity potential reported between amorphous and crystalline silica. The effect of crystalline silica on the transcriptome, compared with amorphous silica, was more profound in human bronchial epithelial cell line (BEAS2B) and primary human bronchial epithelial cells (NHBE) (Perkins et al., 2012). The number of significantly differentially expressed genes and the changes in gene expression were more in the cells treated with crystalline silica compared with amorphous silica. Similarly, a cell culture study was conducted in our laboratory (Sellamuthu et al., 2011a), investigating the relationship, if any, between cytotoxicity and global gene expression changes in response to crystalline silica exposure in A549, human lung epithelial cell line. A549 cells were treated with Min-U-Sil-5 crystalline silica at final concentrations of 15, 30, 60, 120, and 240 μg/cm^2 cultured area for 6-h or 60 μg/cm^2 cultured area for time intervals of 2-, 6-, and 24-h and cytotoxicity was determined. RNA samples were isolated from the control and crystalline silica exposed cells and microarray analysis was conducted to determine global gene expression profiles. The crystalline silica-induced cytotoxicity and the number of significantly differentially expressed genes (SDEGs) detected in the cells correlated very well (Sellamuthu et al., 2011a) suggesting the potential application of global gene expression profiling in detecting the pulmonary response to silica exposure.

Further evidence to support the existence of a relationship between crystalline silica-induced pulmonary toxicity and global gene expression changes is obtained from the results of animal experiments. In a study conducted in our laboratory, rats were exposed to Min-U-Sil-5 crystalline silica by whole body inhalation (15 mg/m^3, 6 h/day, 5 days) and pulmonary damage and global gene expression profiles were determined in the lungs at post-silica exposure time intervals of 0, 1, 2, 4, 8, 16, and 32 weeks (Sellamuthu et al., 2011b, 2012; 2013). Pulmonary damage was assessed in the bronchoalveolar lavage fluid (BALF) on the basis of LDH activity and concentrations of albumin, total protein, and the pro-inflammatory cytokine, macrophage chemoattractant protein 1 (MCP1). Global gene expression profiles in the lungs of the control and silica exposed rats were determined by microarray analysis during the post-exposure time intervals. A very strong positive correlation was seen between the progression of silica-induced pulmonary toxicity and the number of SDEGs detected in the lungs of the silica exposed rats (Sellamuthu et al., 2013). These results, in agreement with the findings of the *in vitro* cell culture studies (Sellamuthu et al., 2011a), reaffirmed the potential application of transcriptome analysis in detecting silica-induced pulmonary toxicity.

5.2. Transcriptomics studies to determine molecular mechanisms of silica-induced pulmonary toxicity

Crystalline silica particles, following their entry into the lungs, are phagocytosed by the alveolar macrophages (AMs) for their detoxification and elimination. In the absence of efficient elimination from the lungs, such as under conditions of excessive crystalline silica exposure, interaction with the inhaled silica particles activates the AMs resulting in their death and release of the engulfed silica particles and various signaling molecules within the lungs. The crystalline silica particles as well as the signaling molecules released into the lungs may interact with the alveolar epithelium to initiate a cascade of pulmonary events. The net result is the release of toxic reactive oxygen and nitrogen species, recruitment of inflammatory cells into the lungs and the induction of inflammation, and fibrosis potentially resulting in the development of silicosis. The crystalline silica particles as well as the various signaling molecules released from activated AMs may cause alterations in the gene expression profile resulting in the functional disruption of the corresponding biological functions, pathways and networks that may be vital for normal cell/tissue/organ function. Therefore, global gene expression profiling of biological samples that are exposed to crystalline silica and functional analysis of the differentially expressed genes may provide valuable information with respect to the molecular mechanisms underlying the pulmonary toxicity and adverse health effects associated with silica exposure. In spite of the reports that excessive silica exposure is a risk factor for several diseases (for example, cancer, autoimmune disease, and renal disease), the scope of this article is limited to the molecular mechanisms potentially underlying silicosis. Further, the discussion of the gene expression data is limited to support the involvement of only oxidative stress, inflammation, and fibrosis in silicosis.

5.3. Crystalline silica and oxidative stress

The finding that silicotic lungs are in a state of oxidative stress (Vallyathan and Shi, 1997) has led to the belief that oxidant-mediated lung damage may play a role in the development of silicosis. Crystalline silica exposure generates reactive oxygen species (ROS) potentially capable of resulting in oxidative stress and the resulting toxicity. Induction of oxidative stress by crystalline silica can be either the direct effect of the particles (Vallyathan et al., 1995) or mediated indirectly through interaction of the particles with cellular processes (Vallyathan et al., 1992).

Transcriptome analysis results supported the generation of ROS in biological samples in response to crystalline silica exposure. A significant and crystalline silica concentration-dependent overexpression of several oxidative stress responsive genes belonging to the nuclear factor kappa B (NFkB) and activator protein-1 (AP-1) family has been observed in the A549 cells (Sellamuthu et al., 2011a). Superoxide anion, a reactive oxygen species generated in response to crystalline silica exposure, undergoes dismutation catalyzed by superoxide dismutase (SOD) to generate hydrogen peroxide (H_2O_2) (Liochev and Fridovich, 2007). H_2O_2 is also generated during the metabolism of spermine catalyzed by spermine oxidase (SMOX) (Murray-Stewart et al., 2008). The toxic and reactive H_2O_2 generated is detoxified, predominantly by catalase, in order to prevent its interaction with intracellular target(s) potentially leading to toxicity. The net result of the significant and crystalline silica

concentration-dependent over-expression of SOD and SMOX with simultaneous decrease in the expression of catalase, as noticed in the crystalline silica exposed A549 cells (Sellamuthu et al., 2011a), is the cellular accumulation of toxic and reactive H_2O_2 contributing to crystalline silica-induced oxidative stress and toxicity.

Several genes involved in the generation of ROS as well as in the cellular response to oxidative stress, viz: SOD2, heme oxygenase 1 (HMOX1), metallothionein 1A (MT1A), neutrophil cytosolic factor 1 (NCF1), lipocalin 2 (LCN2), arginase 1 (ARG1), lactoperoxidase (LPO), and NADPH oxidase organizer 1 (NOXO1), were significantly overexpressed in the lungs of the crystalline silica-exposed rats suggesting the induction of oxidative stress under conditions that resulted in pulmonary toxicity (Sellamuthu et al., 2012, 2017). The NOXO1 gene codes a protein that is an activator of the superoxide-generating gene NADPH oxidase (NOX1) (Banfi et al., 2003), and its significant overexpression in the crystalline silica exposed rat lungs (Sellamuthu et al., 2012, 2017) may, therefore, imply the generation of superoxide anion capable of resulting in oxidative stress. The significant overexpression of SOD2 with no corresponding increase in the expression of H_2O_2 detoxifying genes, catalase and glutathione peroxidase/reductase (Gaetani et al., 1994), as seen in the crystalline silica exposed A549 cells (Sellamuthu et al., 2011a) and rat lungs (Sellamuthu et al., 2012) should favor the excessive generation and accumulation of reactive and toxic H_2O_2, contributing to oxidative stress and toxicity. This argument is further supported by the significant overexpression of LPO, an H_2O_2-responsive gene (Davies et al., 2008), seen in the crystalline silica-exposed rat lungs (Sellamuthu et al., 2012).

5.4. Crystalline silica and inflammation

Crystalline silica exposure results in the induction of inflammation (Chen et al., 1999; Fubini and Hubbard, 2003; Porter et al., 2004), and a central role for inflammation in silicosis is well known (Castranova, 2004). Microarray analysis of global gene expression profiles in the lungs of the crystalline silica exposed rat lungs, at various post-silica exposure time intervals, supported the induction and progression of pulmonary inflammation and toxicity seen in the crystalline silica exposed rats (Sellamuthu et al., 2013). Inflammatory response, inflammatory diseases, and cellular movement were three of the top ranking Ingenuity Pathway Analysis (IPA) biological functions identified as being significantly enriched by crystalline silica exposure in the rat lungs (Sellamuthu et al., 2012, 2017). In addition, multiple canonical pathways and molecular networks involved in the induction of inflammation were significantly and progressively enriched during the post-silica exposure time intervals in our rat silicosis model (Sellamuthu et al., 2012, 2017). Interestingly, the number of inflammation related biological functions, pathways, and networks that were significantly enriched by crystalline silica exposure in the lungs also steadily increased along with the progression of crystalline silica-induced pulmonary toxicity in the rats. These observations suggested the existence of a possible relationship between crystalline silica-induced differential expression of genes involved in inflammation and the toxicity progression seen in the rat lungs (Sellamuthu et al., 2013).

Several inflammatory response genes that code inflammatory cytokines/chemokines were significantly overexpressed in crystalline silica exposed rat lungs, and the magnitude of their over-expression steadily increased along with the progression of silica-induced pulmonary toxicity (Sellamuthu et al., 2012, 2013). Many of these pro-inflammatory cytokines/chemokines function as chemoattractants and recruit inflammatory cells, especially PMNs, into the lungs in response to pulmonary damage (Olson and Ley, 2002). This may, therefore, account, at least in part, for the significant increase in the number of PMNs detected in the lungs resulting in the induction and progression of pulmonary inflammation and toxicity in the crystalline silica exposed rat lungs (Sellamuthu et al., 2011b, 2012). In addition to the genes encoding inflammatory cytokines/chemokines, significant overexpression of other genes known to play prominent roles in the induction of inflammation such as S100A8 (Ryckman et al., 2003), *RETNLA* (Holcomb et al., 2000), *TREM1* and *TREM2* (Ford and McVicar, 2009), *LCN2* (Zhang et al., 2008), *CHI3L1* (Eurich et al., 2009), secreted phosphoprotein 1 (*SPP1*) (Sabo-Attwood et al., 2011), and several members of the complement system (Li et al., 2007) and acute phase response (Whicher et al., 1999) were found in the crystalline silica exposed rat lungs (Sellamuthu et al., 2012). It is noteworthy that over-expression of these inflammatory response genes steadily increased along with the progression of crystalline silica-induced pulmonary inflammation and toxicity in the rats during the post-exposure time intervals analyzed, further supporting their involvement in the progression of pulmonary inflammation and toxicity.

5.5. Crystalline silica and pulmonary fibrosis

Pulmonary fibrosis is a major component of silicosis (Ng and Chan, 1991). Crystalline silica-induced fibrosis includes the release of fibrogenic factors from alveolar macrophages, proliferation of the fibroblasts and increased production of collagen by pulmonary fibroblasts culminating in fibrosis. Thickening of the alveolar epithelium and positive staining of the lung sections to Mason's trichrome stain suggested lung fibrosis in response to inhalation exposure to crystalline silica in our rat silicosis model (Sellamuthu et al., 2012, 2017).

Global gene expression profiling and bioinformatic analysis of the gene expression data in the lungs of the crystalline silica exposed rats, as compared to the control rats, identified significant differential expression of several genes involved in tissue remodeling and fibrosis (Sellamuthu et al., 2013, 2017). Matrix metlloproteinases (MMPs) are a family of proteins that participate in many homeostatic biological processes as well as in pathological processes including fibrotic lung diseases (Nagase and Woessner, 1999). MMPs, because of their involvement in extracellular matrix degradation (Scabilloni et al., 2005), have been implicated in airway remodeling and granuloma formation. Of the *MMPs* that were significantly overexpressed in the crystalline silica exposed rat lungs, *MMP12* overexpression was most significant (Sellamuthu et al., 2013, 2017). A definite role for *MMP12* in the induction of pulmonary fibrosis has been demonstrated previously in mice carrying a targeted deletion of the *MMP12* gene (Matute-Bello et al., 2007). Osteopontin, one of the key components of extracellular matrix, mediates the migration, adhesion, and proliferation of fibroblasts culminating in pulmonary fibrosis (Takahashi et al., 2001). The profibrotic gene *SPP1*, which codes osteopontin protein, was significantly overexpressed in

the crystalline silica exposed rat lungs, especially at the late 32-week post-silica exposure time interval (Sellamuthu et al., 2012). A definite role for SPP1 in fibrosis is suggested based on decreased expression levels of type 1 collagen in $SPP1^{-/-}$ mice (Berman et al., 2004). The *ARG1* gene which was significantly and progressively overexpressed in the silica exposed lung samples (Sellamuthu et al., 2012) has been associated with bleomycin-induced pulmonary fibrosis in mice (Endo et al., 2003). The significant overexpression of the profibrotic chemokines *CCl2* (Mercer et al., 2009) and *CCl7* (Moore and Hogaboam, 2008) observed in the crystalline silica-exposed rat lungs (Sellamuthu et al., 2012) may indicate their involvement in silica-induced pulmonary fibrosis. This view is further supported by the significant overexpression of these chemokines in tuberculosis, a human infectious fibrotic lung disease (Nau et al., 1997). The involvement of RETNLA in the induction of pulmonary fibrosis by promoting the differentiation of myoblasts that mediate collagen deposition has been suggested (Liu et al., 2004). The *RETNLA* gene was highly overexpressed in the silica exposed rat lungs (Sellamuthu et al., 2012, 2013 and 2017). Since a definite relationship is known to exist between unresolved pulmonary inflammation and fibrosis (Reynolds, 2005), it is reasonable to assume that the significant overexpression of the multiple pro-inflammatory genes detected in the rat lungs facilitating the unresolved pulmonary inflammation contributed to the crystalline silica-induced pulmonary fibrosis. The magnitude of overexpression of the genes that are known to be involved in tissue remodeling and fibrosis steadily increased in parallel with the progression of crystalline silica-induced pulmonary toxicity in the rats suggesting their role in crystalline silica-induced pulmonary fibrosis and possibly resulting in silicosis (Sellamuthu et al., 2012, 2017).

5.6. Novel mechanisms of crystalline silica-induced pulmonary toxicity

A unique feature of global gene expression profiling is the potential to screen all the cellular targets and processes that are potentially involved in the response of a cell or an organism to exposure to a toxic agent. Therefore, whole transcriptome expression profiling may facilitate the identification of novel target(s) and/or mechanism(s) of toxicity that may not be achieved by conventional toxicity studies.

A significant overexpression of several members of the solute carrier (SLC) family of genes was found in the crystalline silica exposed rat lungs at all post-exposure time intervals. In parallel with the progression of pulmonary toxicity noticed in the crystalline silica exposed rats (Sellamuthu et al., 2013, 2017), several members of the SLC family of genes exhibited a steady increase in their overexpression. This may suggest the potential involvement of the SLC genes in the initiation as well as the progression of crystalline silica-induced pulmonary toxicity which remains to be investigated.

The SLC gene that was most significantly overexpressed in the lungs of the crystalline silica exposed rats was *SLC26A4* (Sellamuthu et al., 2013, 2017) and several lines of evidence suggest a potential role for this gene in crystalline silica-induced pulmonary toxicity. The *SLC26A4* gene codes the protein pendrin, which is responsible for excessive mucus production by airway epithelial cells (Nakao et al., 2008). A relationship is known to exist between excessive mucus production by airway epithelial cells and morbidity and mortality from certain respiratory diseases (Rogers, 2004; Rose and Voynow, 2006). The steady

increase in the overexpression of the *SLC26A4* gene noticed in the crystalline silica exposed rats may also account, at least in part, for the progression of pulmonary inflammation noticed in them. It has been reported previously that forced overexpression of the *SLC26A4* gene, by yet to be identified mechanism(s), resulted in the activation of the CXCl1 and CXCl2 chemoattractants and facilitated the infiltration of neutrophils into lungs resulting in the induction of pulmonary inflammation (Nakao et al., 2008). In this regard, it is important to note that both the *CXCl1* and *CXCl2* genes were significantly and progressively over-expressed and a significant increase in the number of infiltrating PMNs and induction of inflammation occurred in rats in response to their exposure to crystalline silica (Sellamuthu et al., 2011a, b; 2012). Collectively, the findings of our study and those reported previously (Nakao et al., 2008) may suggest the involvement of the *SLC26A4* gene in crystalline silica-induced pulmonary inflammation and toxicity in the rats. Future investigations by employing a SLC26A4 transgenic mouse model (Lu et al., 2011) may facilitate the understanding of and confirmation of the role of this gene, if any, in crystalline silica-induced pulmonary toxicity and silicosis.

5.7. Prediction of crystalline silica exposure/toxicity using transcriptomics

An interesting finding of the global gene expression analysis, with respect to crystalline silica-induced pulmonary fibrosis, was the superior sensitivity of the fibrosis marker genes, compared with Masson's trichrome staining of lung tissue, a conventional fibrosis detection approach, in predicting fibrosis outcome. In our rat model, the earliest indication of crystalline silica-induced pulmonary fibrosis, as detectable by positive trichrome staining of lung tissues, was detected at the 32-week post-silica exposure time interval (Sellamuthu et al., 2012). However, significant overexpression of several genes involved in tissue remodeling and fibrosis were detectable in the lungs of crystalline silica exposed rats as early as one week following the termination of crystalline silica exposure (Sellamuthu et al., 2013). Furthermore, over-expression of the genes involved in fibrosis steadily increased during the late post-silica exposure time intervals. The post-silica exposure time interval with the highest overexpression of the fibrosis-related genes matched with the onset of fibrosis, as revealed by the results of Masson's trichrome staining of the lung tissues in the rats (Sellamuthu et al., 2012). These results, therefore, suggested the superior sensitivity of gene expression changes as potential markers of lung fibrosis, a key feature of silicosis.

The superior sensitivity of gene expression changes, compared with the traditional approaches, to detect the pulmonary effects associated with crystalline silica exposure lead to studies investigating the identification of blood gene expression signatures as sensitive and minimally invasive surrogate biomarkers for silica toxicity. Typically, occupational exposure to crystalline silica among workers takes place at very low concentrations over a prolonged period of time. In addition, there is a latency period between occupational exposure to crystalline silica and the onset of pulmonary diseases, especially silicosis, among exposed workers. Therefore, the adverse health effects associated with crystalline silica exposure may not be detectable immediately. Similar to any other adverse health effect, the key to prevent silicosis and any other health effects associated with occupational exposure to crystalline silica is the early detection of the potential adverse health effects. This is because the adverse health effects may still be reversible at the early stage and,

therefore, preventable by the application of appropriate preventative and therapeutic approaches. The observation that blood gene expression changes are superior in sensitivity to traditional markers of target organ toxicity (Bushel et al., 2007; Lobenhofer et al., 2008; Umbright et al., 2010) prompted us to investigate whether exposure to crystalline silica at a very low sub-toxic concentration (concentration that does not result in pulmonary toxicity detectable by conventional toxicity detection methods such as biochemical and histological changes) could be detected by employing a blood gene expression signature.

Blood gene expression data obtained from rats exposed to crystalline silica at a toxic dose of 15 mg/m^3, 6 h/day for 5 days (0-week post-exposure time interval) or filtered air (control) were used as the training set data to develop gene expression signatures for crystalline silica exposure and/or toxicity. One of the blood gene expression signatures consisting of 7 genes (Sellamuthu et al., 2011b) was tested in the rats that were exposed to lower concentrations of crystalline silica (1 or 2 mg/m^3, 6 h/day, 5 days). Exposure of rats to crystalline silica at 2 mg/m^3, 6 h/day for 5 days resulted in mild pulmonary toxicity as evidenced from the observation of a slight, but statistically significant, elevation in BALF parameters of pulmonary toxicity (LDH activity, albumin and protein content). In contrast, exposure of rats to crystalline silica at 1 mg/m^3, 6 h/day for 5 days did not result in any detectable pulmonary toxicity as evidenced from normal LDH activity and protein and albumin contents in their BALF (Sellamuthu et al., 2011b). The predictive blood gene expression signature developed for crystalline silica exposure and toxicity correctly identified seven out of 8 rats (87.5%) that were exposed to crystalline silica at 2 mg/m^3, 6 h/day for 5 days (Sellamuthu et al., 2011b). Six out of eight rats (75%) that were exposed to crystalline silica at 1 mg/m^3, 6 h/day, 5 days and did not result in any detectable pulmonary toxicity were correctly identified as crystalline silica exposed rats by the predictive blood gene expression signature (Sellamuthu et al., 2011b). These results, in addition to confirming the superior sensitivity of blood gene expression signatures to detect pulmonary toxicity, suggested the potential application of blood gene expression profiling to develop highly sensitive and minimally invasive surrogate biomarkers for early detection of the adverse health effects associated with crystalline silica exposure. Following appropriate validation studies in human subjects, these biomarkers may be employed in routine monitoring of workers for early detection of silicosis. This is in line with the NIOSH recommendation to develop highly sensitive and non-invasive or minimally invasive biomarkers for early detection and prevention of silicosis (NIOSH, 2002).

6. Summary

The field of transcriptomics has evolved substantially over the past few decades and made remarkable contributions in biology and toxicology. Mainly because of the discovery of high throughput transcriptomics techniques such as microarray and NGS, it has become possible to determine the expression profile of the entire set of genes present in a biological system. The gene expression data determined in a biological system, following its exposure to xenobiotics, may have implications in the prevention of toxicity and the adverse health effects potentially associated with the exposure. Gene expression changes precede classical toxicity endpoints such as biochemical and histological changes. This has facilitated the identification of gene expression signatures as predictive bio-markers for the detection of

early target organ toxicity. Similarly, bioinformatic analysis of the gene expression data provides valuable information about the mechanisms underlying the toxicity of xenobiotics in a biological system. As discussed in this review article, global gene expression profiling has been applied successfully in the identification of biomarkers for early detection of hepatotoxicity and pulmonary toxicity as well as understanding the mechanisms underlying the toxicity. These findings, following appropriate validations in human subjects, may be employed to prevent the adverse health effects potentially associated with human exposure to toxic xenobiotics.

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