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Abbreviations and Acronyms

3D	Three-dimensional
8-OHdG	Anti-8-Hydroxyguanosine
AKR1C3	Aldo-Keto Reductase family 1, member C
ANOVA	Analysis of variance
APL	Acute promyelocytic leukaemia
As	Arsenic
ASK1	Apoptosis signal-regulating kinase 1
ATO	Arsenic trioxide
ATP	Adenosine triphosphate
ATRA	All-*trans* retinoic acid
ATSDR	Agency for Toxic Substances and Disease Registry
BCC	Basal cell carcinomas
BFT	*Bacteroides fragilis* toxin
CANSA	Cancer Association of South Africa
CDK4	Cyclin-dependent kinase 4
CK	Cytokeratins
CRDs	cysteine-rich domains
CTD	Comparative Toxicogenomics Database
DBCP	Disulphide Bonding Connectivity Pattern

DMAsV	Dimethylarsinic acid
DMEM	Dulbecco's modified Eagle's Medium
DNA	Deoxyribose nucleic acid
EC	European Commission
EFSA	European Food Safety Authority
ETBF	Enterotoxigenic Bacteroides fragilis
FBS	Fetal bovine serum
FDA	United States Food Drug Administration
GM-CSF	Granulocyte macrophage-colony stimulating factor
GO	Gene Ontology
GPR	GenePix Results
GSH	Glutathione
GSS	glutathione synthetase
GUSB	Glucuronidase
HaCaT	spontaneously immortalized human keratinocyte cell line
HAT	Histone acetyltransferases
HK	Human Keratinocyte
HMP	Human Microbiome Project
HPV	Human papillomavirus
IARC	International Agency for Research on Cancer

IGF	A insulin growth factor
IGFL1	Insulin Growth Factor-Like family member 1
IKK	Inhibitor of nuclear factor kappa-B kinase
IPA	Ingenuity Pathways Analysis
JNK	c-Jun N-terminal kinases
LACAAS	LAI's Automated Comet Assay Analysis System
LD	Lethal dose
MALT	Mucosa-associated lymphoid tissue
MCC	Merkel cell carcinoma
MCs	Merkel cells
MGB	Minor groove binder
MiMI	Michigan Molecular Interactions
miRNA	microRNA
mRNA	messenger RNA
MSMAsV	Monosodium methanearsonate
NCR	National Cancer Registry
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK	Normal human epidermal keratinocytes
NMSC	Nonmelanoma skin cancer

PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PKC	Protein kinase C
qPCR	Quantitative polymerase chain reaction
RGS2	Regulator of G-protein Signalling
RIN	*RNA* Integrity Number
RT-qPCR	Real-time polymerase chain reaction PCR
ROS	Reactive oxygen species
SCC	Squamous cell carcinomas
SLOTUs	Species-level operational taxonomic units
TBE	Tris-Borate-EDTA
TGF- α	Transforming growth factor-alpha
THD	TNF homology domain
TNF	Tumour necrosis factor
TNFSF18	Tumour necrosis factor (ligand) superfamily, member
U.S.EPA	United States Environmental Protection Agency
USA	United State of America
UVR	Ultraviolet radiation
VA	Visual analytics
WHO	World Health Organization

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Chapter 1

Introduction

1.1 Overview

Arsenic (As) is an environmental toxicant and hazardous, naturally-occurring element widely distributed in the crust of the earth (Hall et al. 2006;IARC 1987;Tchounwou et al. 2003). Due to its public health significance, arsenic is classified by the United States Environmental Protection Agency (Goering et al. 1999;U.S.EPA 2001) as a Group A carcinogen. Human health effect caused by chronic exposure to low dose of arsenic through drinking water affect millions of people in more than 70 countries (IARC 2004). The adverse effects on health due to chronic arsenic exposure varies and may be influenced by the population groups, age, gender, cumulative dose of arsenic, nutritional status, genetic factors, lifestyle, individual susceptibility, and different chemical forms of arsenic in drinking-water (Ferreccio et al. 2006;Kristiansen et al. 1997;Mitra et al. 2004). In the middle of the 19th century arsenic poisoning was a leading cause of death in South Africa (Copeman 1940). Exposure to arsenic has been strongly associated with cancer in human and especially the skin cancer (Lynn et al. 1997;Schulz 1967). Apart from Australia, South Africa has the highest incidence of skin cancer in the world with more than 700 deaths each year (CANSA 2011). Though most of the cases in South Africa are associated with exposure to ultra violet (UV) radiation from the sun, the effect of secondary contact with arsenic cannot be totally ruled out. This is because in South Africa, high levels of arsenic have been found in the soils contaminated by the abandoned cattle tick dip

operations (Okonkwo 2007). In areas where water contamination with arsenic is endemic, exposure to low-dose arsenic over a long period of time leads to increased risk of developing epithelial cancers of the skin including intraepidermal carcinomas (Bowen disease) (Col et al. 1999), squamous cell carcinomas (SCC), basal cell carcinomas (BCC) (Yu et al. 1992), Merkel cell carcinoma (MCC) (Ho et al. 2005), and head and neck cancers seen mostly in alcoholics who are also exposed to arsenic (Bao et al. 2010). ATO is a multi-site human carcinogen which targets the skin, (McNeely et al. 2006), bladder and kidney (Sasaki, Oshima, & Fujimura, 2007), liver (Liu et al. 2006), prostate and lung (Chen et al. 1996), bone marrow (myeloma), (Wang et al. 2006a), peripheral lymphocytes (Argos et al. 2006), neural tube (Wlodarczyk et al. 2006), and urogenital cells (Su et al. 2006). Epithelial cancers of internal organs most often co-exist with cutaneous diseases such as arsenical keratosis, hyperpigmentation, and multiple cutaneous malignancies (Maloney 1996;NEUBAUER 1947). Studies have shown that arsenic induces tumours of the liver, lung, ovaries and adrenal glands in other mammals as well (Waalkes et al. 2008). Aside cancers, other human diseases have been attributed to arsenic ingestion or inhalation. Such diseases include; hyperkeratosis and hyperpigmentation (Maloney 1996), black foot disease, atherosclerosis (Wang et al. 2006a), cerebral infraction (Chiou et al. 1997), hypertension (Chen et al. 1996;Rahman et al. 2001), diabetes mellitus (Tseng 2008), skin lesions, liver injury and neurologic damage (peripheral neuropathy, encephalopathy, and intellectual deficits) in children (ATSDR 2007a;Wasserman et al. 2011). Also, ventricular fibrillation could be an outcome of arsenic poisoning (St et al. 1970). Thus, public health concerns about long-term arsenic exposure have

arisen due to the increase in epidemiological reports of arsenic related cancers in different parts of the world including south-eastern Michigan, Taiwan, China, India and Bangladesh (McDonald et al. 2006;Mead 2005;Meliker et al. 2007;Tchounwou et al. 2003).

The mechanisms of arsenic toxicity and carcinogenicity have been reported on various cell types (Bae et al. 2002;Hamadeh et al. 2002;Rea et al. 2003) but the exact mechanism of arsenic-mediated carcinogenesis remains a subject of debate, with several lines of evidence supporting causes such as stimulation of cell proliferation (Chowdhury et al. 2010;Miller, Jr. 2002), alteration of DNA methylation, perturbation of signalling cascades (Chowdhury et al. 2010), oxidative stress and chromosomal aberrations (Kundu et al. 2011). There are also suggestions on disruption of transcriptional activity following arsenic exposure with extensive changes in global gene expression, and disruption of diverse regulatory mechanisms of gene expression (Das et al. 2011). Epigenetic dysregulation and arsenic metabolic activities involving human microbiome are currently stimulating the interest of researchers (Alava et al. 2012;Betts 2011;Pinyayev et al. 2011;Van de Wiele et al. 2010;Wu et al. 2011).

Other scientific evidence tend to support the view that the breakdown of arsenic by human microbiota arsenic metabolizing organisms could be a plausible mechanism of action; this may directly or indirectly enhance or reduce the carcinogenicity of arsenic. Identifying the microbes isolated in both presence/absence of arsenic induced disease could be useful biomarkers in disease diagnosis. Furthermore, understanding the genetics of human

microbiome could be achieved with metagenomics techniques (Chauhan et al. 2009;Chauhan et al. 2011;Gao et al. 2007). Such knowledge may explain why only some individuals who are exposed to environmental carcinogen, or carrying a genetic predisposition to cancer develop disease.

A promising area of research for the discovery of arsenic mechanism of action and biomarkers of arsenic toxicity is genomics. Advances in the genomics and other "-omics" technologies are providing massive amounts of datasets and tons of scientific publications that describe potential gene, protein and biological processes as potential biomarkers of adverse health effects of environmental chemicals. Toxicogenomics, a molecular technique applied in a whole-genomic capacity to investigate toxicant effects, is a recognized approach to discover potential biomarkers of toxicity and exposure biomarkers. Toxicogenomics could as well validate/quantify biomarker signatures (Afshari et al. 2011). The growth in data from toxicogenomics research has led to the development of bioinformatics databases such as the Comparative Toxicogenomics Database (CTD) for curating toxicogenomics relationships (chemical-gene, chemical-disease and gene-disease) found in scientific publications (Davis et al. 2011). As of the December 7, 2011, the CTD contained 28,413 PubMed references, 352,925 chemical-gene relationships, 6,605 unique chemicals, 20,710 unique genes and 334 unique organisms. These toxicogenomics relationships and data when combined with biological information on human genes from other bioinformatics databases can lead to knowledge building (discovering previously unknown relationships from data) on potential biomarkers. Significant over-representation (enrichment) of certain biological topics for a gene is an example of biological information from a

bioinformatics database called ConceptGen (Sartor et al. 2010). The database currently consists of ~18,000 concepts, each with 5 or more assigned genes. Further, diverse bioinformatics tools are now available to reconstruct molecular interactions from predicted and experimentally validated data (Chautard et al. 2011;Croft et al. 2011;Stark et al. 2006;Tarcea et al. 2009;Turner et al. 2010). The reconstruction of molecular pathways involving potential biomarker genes and proteins can also yield insights on how normal cellular activities are altered in different chemical exposures or disease conditions. These diverse datasets from bioinformatics databases therefore present opportunities for discovery and inferences on biological processes affected by arsenicals. It is of interest to identify genes for further research on mechanisms of toxic action and cancer initiation of arsenic on skin keratinocytes.

1.2 Motivation

Why arsenic? Arsenic is widely found in the environment and has been classified as a Group A carcinogen (U.S.EPA 2001) but there are still gaps in the precise mechanisms of arsenic-related carcinogenicity. Therefore, knowledge of the arsenic mechanism of action using gene expression models, gene networks and biological pathways perturbed by exposure to arsenic are important in terms of arsenic-related risk and exposure. Skin cancer is the most common arsenic-related cancer (Schwartz 1997;Smith et al. 1992). In the United States about 3,507,693 cases of non-melanoma skin cancer (NMSC) was reported in 2006, resulting in high morbidity and high cost of treatment (Rogers et al. 2010). Arsenic exposure has been closely linked to the

pathogenesis of multiple skin cancers (Yu et al. 2006). HaCaT keratinocyte cell line was selected for this in vitro research investigation because HaCaT is the first permanent immortalized epithelial cell line from adult human skin that exhibits normal differentiation and provides a promising tool for investigating the regulation of keratinization in human cells (Boukamp et al. 1988). The cultured HaCaT cell line was exposed to chronic low dose of arsenic in order to mimic the in vivo paradigm of chronic exposure in arsenic endemic areas (WHO 2001). This thesis research identified aberrations in gene expression and potential pathways that are part of the response after chronic exposure to ATO (after 22 passages). Also, time point cytotoxic effects of low dose chronic ATO exposure on HaCaT cells were studied to observe the patterns of toxicity after long-term exposure. Further understanding of the cytotoxic and genotoxic mechanisms of arsenic may provide more effective management of environmental exposure while also facilitating the discovery of biomarkers of arsenic induced cancer for effective diagnosis, prognosis and management of such cancers.

1.3 Research Goal, Purpose, Hypothesis and Objectives

The main goal of this research is to determine the mechanism of arsenic carcinogenesis in human skin epidermal keratinocytes. The purpose is to treat the skin cells with low dose ATO mimicking natural set up in endemic areas of arsenic poisoning through drinking water and determine the genomic changes that occur over a period of time. It is hypothesized that long-term, chronic exposure of human epidermal cells (HaCaT cells lines) to low dose arsenic will

lead to perturbation of cellular and molecular activities in keratinocytes. Knowledge of alterations in biological pathways affecting networks of the genes is crucial in understanding the mechanism of action of arsenic carcinogenesis in HaCaT cells.

The following specific objectives were designed to test the hypothesis;

1.) Determine cytotoxic and genotoxic effects of ATO on human skin keratinocytes (HaCaT cells) and Identify differentially expressed genes in HaCaT cells chronically exposed to low-dose ATO
2.) Identify specific pathways from the gene expression datasets of HaCaT exposed to ATO.

Chapter 2

2.0 Literature Review

2.1 Arsenic species

Arsenic compounds are divided into three major groups: inorganic arsenic compounds; organic arsenic compounds; and arsine gas (ATSDR 2010). They are further classified into four valence states namely; 0 oxidation state [AS(O), metalloid arsenic], trivalent, 3 oxidation state [AS(III), arsenites], pentavalent, 5 oxidation State [As(v), arsenate] and -3 oxidation state (arsine gas) (ATSDR 2007a;ATSDR 2010). The different arsenic species differ in their toxicity, biochemical and environmental behaviours (Alava et al. 2012;Gong et al. 2002). The most common arsenic species and their oxidation states are shown in Table 1. Arsenate [As (V)] is the most common environmental form of inorganic arsenic, but arsenite [As (III)] is more toxic and the most likely carcinogenic species (Bertolero et al. 1987;Lerman et al. 1983;Tinwell et al. 1991).

Table 1: Arsenic Compounds of Environmental and Human Relevance*

Trivalent oxidation state [As(III), or As(+3)]	Abbreviations	Formula	Pentavalent oxidation state [As(V), or As(+5)]	Abbreviations	Formula
ATO	As^{III}	As_2O_3	Arsenic pentoxide	As^{V}	As_2O_5
Arsenite, arsenous acid	As^{III}	$As(OH)_3$	Arsenate, arsenic acid	As^{V}	$AsO(OH)_3$
Monomethylarsonous acid	[MMA(III)]	$CH_3As(OH)_2$	Monomethylarsonic acid	MMA^{V}	$CH_3AsO(OH)_2$
Dimethylarsinous acid	DMA^{III}	$(CH_3)_2AsOH$	Dimethylarsinic acid (Cacodylic acid)	DMA^{V}	$(CH_3)_2AsO(OH)$
Trimethylarsine	TMA^{III}	$(CH_3)_3As$	Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$
Arsenic trichloride		$AsCl_3$	Monomethylmonothioarsenate	$MMMTA^{V}$	$CH_3AsO_2S^{-2}$
			Arsanilic acid (*p*-aminophenylarsonic acid	p-AsA	$H_2NC_6H_4AsO_3H_2$
			Arsenobetaine		$C_5H_{11}AsO_2$
			Lead arsenate	LA	$PbHAsO_4$
			Calcium arsenate		$Ca_3(AsO_4)_2$

* Arsenic compounds can be classified into three major groups: inorganic arsenic compounds; organic arsenic compounds

2.2 Routes of Exposure

Arsenic is released from both natural and anthropogenic sources. Environmental arsenic contamination occurs from industrial smelting of metals, power generation with coal, and applications of pesticides and herbicides (NEUBAUER 1947;USEPA 2006).

Human exposure occurs mainly through, food, water, air and soil as well as the natural accumulation of soil arsenic into grains, vegetables, fish, and meats (ATSDR 2007a;Hughes et al. 2011;Liu et al. 2010). Arsenic may enter the organism by dermal contact, inhalation, or ingestion of contaminated drinking water, foodstuffs or medication (IARC 1998). Understanding the environmental levels of arsenic that can cause a public health concern is crucial in planning mitigation strategies

.

2.2.1 Arsenic contamination of Drinking Water

The main source of high exposure of general population to arsenic compounds is water (Liu et al. 2010). The United States (U.S.) Public Health Service and the World Health Organization have set the guideline for arsenic in drinking water as 10µg/l but in endemic developing countries arsenic drinking water standards is relaxed to 50µg/l (Petrusevski et al. 2008;Smith et al. 2004). High arsenic levels have been detected in groundwater of different parts of the world, (Nordstrom 2002). Bangladesh and West Bengal (India) are the most affected areas of the world with arsenic concentration in groundwater in some area up to 3200 µg/L (Chakraborti et al. 2010;Chakraborti et al. 2011). The inorganic form of arsenic is the most common found in water. It can be stable as both arsenite and arsenate inorganic arsenicals (Khan et al. 2006;Saxe et al. 2007). Arsenite is the most prevalent species in the groundwater while arsenate species more significant in the surface water of the rivers (Pandey et al. 2006). The major source of arsenic in underground water is through the reductive dissolutions of arsenic-rich Fe (III) oxyhydroxides and/or al-hydroxides present in aquifer. Other processes that introduce arsenic into underground water may include oxidation of aquifer arsenical pyrite and other arsenic-bearing sulphide minerals, and the exchange of adsorbed arsenic with other competitive anions (phosphate, bicarbonate and silicate) (McArthur JM 2001;Nickson RT 2000;Pandey et al. 2006). In a U.S. Geological Survey (USGS) report, the median ground water concentration was estimated to be 1 µg/L or less, with much higher levels in some groundwater aquifers, particularly in the western U.S. such as Nevada which have median levels of about 8 µg/L (Focazio 1999). High natural occurring arsenic levels of up to

1,000µg/L have been reported in the U.S. in drinking water (Lewis et al. 1999;Steinmaus et al. 2003). The shallow ground water of the western United States, Arizona, Utah, Nevada, California and Washington in particular are hotspots for arsenic contamination (Twarakavi 2006). In a research conducted in Bangladesh, a range of 0.05 to 2.50 µg/ml arsenic levels in drinking water was reported (Anawar et al. 2002) and concentrations of up to 3.4 µg/ml of arsenic were recorded in drinking water source in West Bengal, India (Guha Mazumder et al. 1998).

2.2.2 Arsenic food contamination

In the mid-19th century, arsenic was intentionally added to food as a preservative prior to the discovery of its deleterious effects on human health (Hughes et al. 2011). Food especially seafood has proven to be a major source of arsenic exposure to human (Uneyama et al. 2007). In a survey of heavy metals in commercial fish in New Jersey, USA, arsenic levels in fish including Chilean sea bass, croaker, flounder, porgie, and whiting exceeded the U.S. Environmental Protection Agency (EPA) regulatory limit by 1.3 µg/ml (Burger et al. 2005). Apart from drinking water, diet is a major source of both inorganic and organic arsenic and estimates of dietary inorganic arsenic intakes vary. Arsenic could enter the food cycle by growing the crop on arsenic contaminated soil or by irrigating the farm with arsenic contaminated water (Das et al. 2004). At a rice paddy in Bangladesh, rice grain grown in soil with high arsenic concentrations resulted in rice grain samples with arsenic levels above 1.7 µg/g dry weight (Meharg et al. 2003). In the U.S., estimates indicate

that adults and children consume an average of 3.2µg/day with a range of 1-20µg/day (Cullen et al. 1995;Schoof et al. 1999). The estimated arsenic daily intake in Europe by the European Food Safety Authority is 0.13 to 0.56 µg/kg/day for average consumers and 9.1 to 39.2 µg/day for a 70-kg adult (EFSA 2009) with respect to the ratio of inorganic arsenic to total arsenic in food. In India, it was observed that cooked foods had higher levels of arsenic than raw foods. Daily dietary intakes of arsenic from the foodstuffs for adults were from 171.20 - 189.13 µg/day, while the range for children was 91.89 - 101.63 µg/day (Roychowdhury et al. 2002).

2.2.3 Arsenic from the Soil

The natural content of arsenic in soils globally ranges from 0.01 to over 600 mg/kg, with an average of about 2 to 20 mg/kg (Kabata-Pendias A 1992;Yan-Chu 1994). The major source of arsenic in soil is the parent rock from which soils are formed, and thus the lithology of parent rock materials, volcanic activity, bioactivity, weathering history, transport, sorption, and precipitation all contribute to the nature of arsenic in soil (U.S.EPA 1987). The biotransformation of arsenic species mainly occur in the soil and the three major modes of biotransformation observed include: the biosynthesis of organoarsenic compounds, redox transformation between arsenite and arsenate, and the reduction and methylation of arsenic (Andreae et al. 1983). Arable lands could be contaminated with arsenic from run-off water and the use of arsenic-rich ground water for irrigation (Meharg et al. 2003;Saha et al. 2007). A nationwide survey in the U.S. conducted in areas perceived to have

no anthropogenic sources of arsenic reported a natural background concentrations in soil ranged from < 1 to 97 mg/kg (Shacklette 1984). In South Africa, elevated total arsenic levels (1,033–1,369 µg/ml) were detected in the soils contaminated by historically cattle tick dip operations (Okonkwo 2007). The greatest arsenic value (1,369 µg/ml) was obtained at the surface, indicating that arsenic was still abundant at the surface even though the dip is no longer in operation (Moremedi 2007). Inorganic arsenic is the major form of arsenic in soil but high levels of organic forms are also seen in soils. However, pentavalent arsenic is more commonly seen in soil as trivalent arsenical are easily oxidized (Gong et al. 2002). Major contributors of arsenic to the soil are anthropogenic activities such as mining manufacturing activities, and application of arsenic-containing pesticides (Roberts et al. 2002). High soil arsenic are seen at mine tailings, smelter facilities, cattle dip sites, electric substations, wood treatment (chromated copper arsenate) sites, pesticide treatment areas, railroad rights-of-way, golf courses, and dumps (Roberts et al. 2002). Although exposure to soil arsenic could be via inhalation of soil particles blown by wind and dermal absorption, the amount of arsenic in ambient air is low; also, arsenic is poorly absorbed through the skin from the soil (U.S.EPA 2001). Thus, incidental ingestion is the main source of exposure to arsenic in soil but when compared with the other natural routes of exposure, the amount of arsenic from soil is far less than the amount from drinking water and diet (Boyce et al. 2010). This is likely because there is reduced amount of inorganic arsenic as well as reduced bioavailability of arsenic in soil compared to water (Roberts et al. 2002).

2.2.4 Arsenic from the air

Inorganic arsenic is the major form of arsenic found in air and it is very low in concentration, contributing to less than 15% of arsenic exposure when compared to arsenic exposure from food, water and soil (EC 2000;U.S.EPA 1999). The concentration of arsenic in air due to non-human activities ranges from 1 to 3 ng/m^3, and the range of the concentration in urban areas is 20 to 100 ng/m^3 (ATSDR 2007b). According to the European Commission (EC 2000) reports, the range of arsenic in air are; 0-1 ng/m^3 in remote areas, 0.2-1.5 ng/m^3 in rural areas, 0.5-3 ng/m^3 in urban areas, and up to about 50 ng/m^3 at industrial sites. Anthropogenic activities such as combustion processes introduce highly soluble arsenic oxides into the air which are circulated by the wind and returned to the earth in wet or dry deposition. Also, output gas from power plants using coal and oil which naturally contain arsenic may contaminate the atmosphere with arsenic (Pacyna JM. 1987).

2.2.5 Arsenic in medication

Although historically, arsenic was considered a poison (Gallagher 1998), its use in the treatment of various ailments dates back to more than 2400 years ago. There are references suggesting its application as medication for the treatment of many diseases such as toothaches, ulcers and abscesses (Antman 2001;Hyson, Jr. 2007;Jolliffe 1993;Riethmiller 2005;Waxman et al. 2001). Fowler's solution which consists of 1% solution of potassium arsenite was discovered in 1786 and was effectively used to treat malaria, syphilis, asthma, chorea, eczema, and psoriasis (Cuzick et al. 1992;Scheindlin 2005).

Fowler's solution was also used in leukaemia patients to lower white blood cell count (Antman 2001) and as a tonic for anaemia. It was used to treat rheumatism, dermatitis herpetiformis, Hodgkin's disease, pemphigus and pernicious anaemia (Jolliffe 1993). An organic arsenical, salvarsan was introduced in 1910 by Paul Ehrlich for treating syphilis and trypanosomiasis (Aronson 1994;Riethmiller 2005). In September 2000, United States Food Drug Administration (FDA) approved the use of ATO for the treatment of relapsed or refractory acute promyelocytic leukaemia (APL) cases resistant to all-trans retinoic acid (ATRA) (Ablain et al. 2011;Antman 2001;Rust et al. 2001;Zhang et al. 2001). A better understanding of arsenic mechanism of action has led to increase in clinical trials undertaken to determine the therapeutic effect of ATO on other types of cancers such as non-APL acute myeloid leukaemia and myelodysplastic syndromes (Murgo 2001;Sekeres 2007). There are also on-going research on the combination of ATO with other agents such as vitamin C (ascorbic acid) for the treatment of lymphoproliferative disorders (Hussein 2001) and multiple myeloma (Munshi et al. 2002). ATO was the drug of choice before the discovery of metronidazole in 1959 for the treatment of *Trichomona vaginalis* infection (trichonomiasis) which causes vaginal discharge (Forgan 1972;Jolliffe 1993). In traditional Chinese medicine, arsenic derivatives are still used to benumb the pulp of a painful tooth (Miller, Jr. et al. 2002). Organic arsenic compound, Melarsoprol, is still the drug of choice for the treatment of the protozoan parasite, trypanosomiasis (Bisser et al. 2007).

2.2.6 Occupational Arsenic Exposure

Arsenic is as an essential ingredient in the manufacturing of a wide variety of products, including wood preservatives, herbicides, insecticides, pesticides, fungicides, high-emitting diodes, and semi-conductors. This makes workplaces sources of inhalation of and dermal exposure to arsenic (ATSDR 2007b). Arsenic was first used for tick control in South Africa in 1893 and since then, over a thousand cattle dipping vats were constructed throughout the country where arsenic was applied as the sodium salt of arsenous acid (Wagner et al. 2003). High arsenic levels were found in mining environments and abandoned metal ores because as arsenic is released during the smelting process and coal burning, the resulting stack dust and flue gas contaminate the soil and water with arsenic (Bhumbla 1994). Workers at such places are exposed to arsenic mainly by inhalation of arsenic dust or from ATO vapours. The uptake of airborne arsenic could be influenced by arsenic compound characteristics, matrix composition, and particle size distribution (Yager et al. 1997). Cullen, 2005 reviewed arsenic toxicity due to inhalation of airborne arsenic from wall papers which contained arsenic dyes in the form of Paris green or copper acetoarsenite (Cullen 2005;Scheindlin 2005). Hughes, in their 2011 review article reported that lead arsenate which was used as a pesticide for apple and cherry orchards was discontinued in 1988 by the U.S. government because orchard farmers were getting sick and there was concern that fruits may also have arsenic residues (Hughes et al. 2011;Wagner et al. 2003). Studies on the orchard workers later provided a basis for understanding some of the long-term effects of occupational exposure to arsenic. However, occupational exposure through inhalation studies in the copper smelting industry was used

to establish definitive links between arsenic, (a by-product of copper smelting), and lung cancer (Enterline et al. 1995;Hughes et al. 2011;Mabuchi et al. 1980;Nelson et al. 1973;Tollestrup et al. 1995). The high lung cancer mortality observed among miners was related to the estimated average intensity of exposure to arsenic but not to the duration (Jarup et al. 1989;Pinto et al. 1977). Another research at a Smelter environment in Sweden described chromosomal aberrations in workers exposed to arsenic (Nordenson et al. 1978). Dermal and neurological effects were also increased in some of these studies. Increased vasospastic reactivity in the fingers and Raynaud's phenomenon in smelter workers seems to be due to functional alterations in the vessels caused by inhalation of arsenic (Lagerkvist et al. 1986). Lagerkvist and co-workers concluded that peripheral vascular disturbances caused by arsenic are dependent on long-term arsenic exposures and are independent of short-term fluctuations in arsenic exposure (Lagerkvist et al. 1988).

Although the use of some arsenic based pesticides such as lead arsenate has been discontinued, the vast land mass formerly used as orchards in New Jersey, Washington and Wisconsin may still be contaminated with lead arsenate (Hood 2006). Inorganic arsenic base pesticides are currently being replaced by organic arsenicals including monosodium methanearsonate (MSMAsV) and dimethylarsinic acid (DMAsV), also known as cacodylic acid because they are less toxic than inorganic arsenicals (Cohen et al. 2006;Steinmaus et al. 2010). With the association of arsenic exposure to occupational health defects, it is very necessary that the industries establish effective environmental safety measures to protect their workers and the environment.

2.3 Arsenic mechanism of action

Mechanisms of action of arsenic in humans have been described as summarized in Figure 1 and Table 2. The previously suggested mechanisms with the addition of the interaction between pre-systemic arsenic and human resident microbial flora are briefly reviewed.

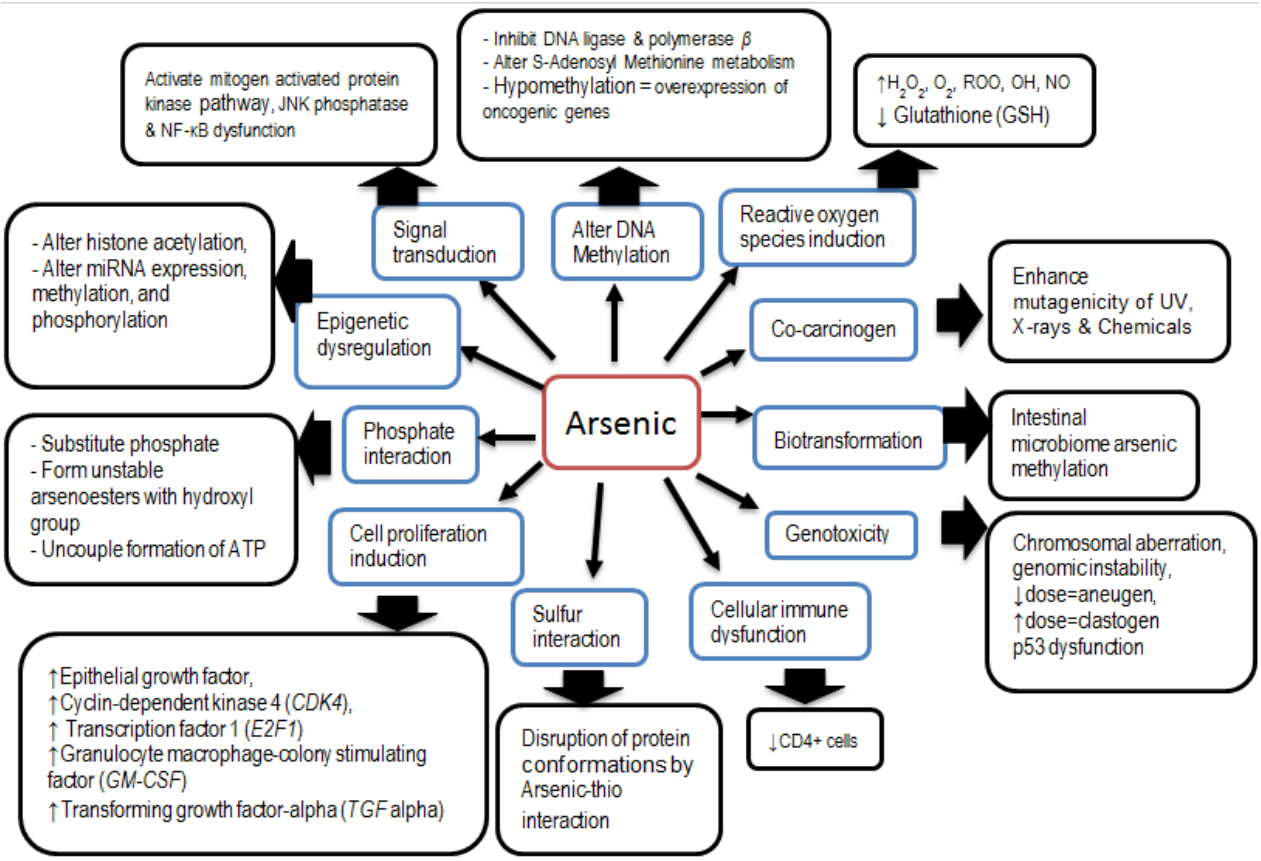

Figure 1: Proposed Modes of Action of Arsenic

Table 2: Modes of action for arsenic and associated biochemical effects

Mode of action	Biochemical effects	References - PubMed Identifiers (PMID) *
Human microbiome interaction	Intestinal microbiome arsenic methylation	20603239, 21807598, 21388151, 21905058
Perturbation of keratin expression	Cell growth, differentiation and maturation distortion,	19524636, 19776502, 19524636,1717607,5644201, 7689238, 19524636,18572023
Genotoxicity	Chromosomal aberration, genomic instability, ↓dose = aneugen, ↑dose = clastogen p53 dysfunction	21461292,21035470, 8993795,17450239, 9380733
Cellular immune dysfunction	↓CD4+ cells	16807664, 10967126,
Sulphur interaction	Arsenic-thio interaction disrupts protein conformations	1641401, 20981267
Induced Cell proliferation	Cyclin-dependent kinase 4 (CDK4), ↑ Transcription factor 1 (E2F1) ↑Granulocyte macrophage-colony stimulating factor (GM-CSF) ↑Transforming growth factor-alpha (TGF alpha)	20016248, 20654705, 8917704, 11312651
Phosphate interaction	Form unstable arsenoesters with hydroxyl group, Uncouple formation of ATP via Asenolysis	15257611, 21357385, 21266531, 19478237
Epigenetic dysregulation	Altered histone acetylation, miRNA expression, methylation, and phosphorylation	18448484, 15313424, 20682481, 14732866, 19707557, 20461219, 22072212
Signal transduction	Activation of mitogen activated protein kinase pathway, JNK phosphatase & NF-κB dysfunction	20339924, 20654705, 10967126, 19524636, 14971646, 16807664, 11312654, 12076506
Altered DNA Methylation	Inhibition of DNA ligase & polymerase *β* Altered SAM metabolism Hypomethylation = overexpression of oncogenic genes	18448484, 20682481, 14580687, 17050553, 12569548, 12377979, 9380733, 12426129
Reactive oxygen species induction	↑H_2O_2, O_2, ROO, OH, NO ↓ Glutathione (GSH)	12482246,15257611, 19203718, 16807664
Cocarcinogen	Enhanced mutagenicity of UV, X-rays & Chemicals	15276419, 17316729

* Citation for PubMed can be obtained from http://www.pubmed.gov

2.3.1 Perturbation of Keratin Expression

The expression of cytokeratins (CK) in HaCaT keratinocytes is impaired by chronic arsenic exposure. Aberrant and progressive alterations CK expression is a common feature observed in human skin lesions, including hyperkeratosis

and squamous cell carcinoma (SCC) (Markey et al. 1991;Sun et al. 2009;Tseng et al. 1968). Thus, the expression of simple epithelial keratins could be used as a biomarker for monitoring malignancy of keratinocytes and determine tumour invasion and/or changes in epithelial-mesenchymal interactions (Leigh et al. 1993;Markey et al. 1991). Keratins are the major abundant and structural proteins of the epidermal layer of epithelial cells (Coulombe et al. 2002). Keratins primarily maintain the structure of epithelial cells and protect the cells from mechanical and non-mechanical traumas that may cause cell death (Sun et al. 1983). They may also be involved in signal transduction between adjacent cells, and could serve as markers for different stages of epithelial differentiation (Markey et al. 1991;Perkins et al. 1992). Keratins are intermediate filament proteins found in the skin, nails and hair. There are two major subgroups; the epithelial cytokeratin (CK type I), and trichocyte (hair/hard, type II) keratins (Rugg et al. 2004). Cytokeratins are located in the cytoplasm and are so named to differentiate them from specialized keratins found in the nails, and hairs (Jacques et al. 2009). Tissue-specific and cell-specific differentiation of epithelial cells is closely linked with the expression of keratin intermediate filaments. Also, because the intermediate filament types are largely conserved during malignant conversion, their expression in a tumour can be used to identify its origin (Osborn et al. 1982). The suggested role of increased expression of CK in arsenic carcinogenesis of skin may be exacerbated by an increase in the expression of other proteins such as matrix metalloproteinase-9 secretion (MMP-9), an enzyme often secreted by cancer cells to help invade through the local extra-cellular matrix (Pi et al. 2008;Sun et al. 2009). The transcripts of various

keratin genes used as markers of different stages of cell differentiation are increased in arsenic- treated HaCaT cells. These include CK1, associated with hyperkeratosis, CK10, a biomarker of dermal cancer progression and always co-expressed with CK1, CK13, CK8 and CK18 (Markey et al. 1991). Other keratins affected by arsenic include differentiation marker genes in simple epithelial tissues which are the earliest keratin genes expressed during embryogenesis, filaggrin (linked to proliferation) (Winge et al. 2011), involucrin (an early differentiation marker) and loricrin (late differentiation gene), CK6 (induced in response to stressful stimuli, such as wounding), and CKs 6/16 and 7/17 (stress response, adaptive response to arsenic) (Jacques et al. 2009;Sun et al. 2009).

2.3.2 Genotoxicity

Gene expression studies are promising areas of demonstrating genotoxic effect of arsenic on exposed human cells. Because gene expression is a tightly regulated process, it could be relied on to measure the response of cells to environmental stimuli and to its changing needs. Many diseases have been linked to disruptions in gene expression (Wheeler et al. 2008). Although most cells of the body contain a full set of chromosomes and identical genes, not all the gene is involved in gene expression. Only a subset participate in the transcription of information from the DNA into messenger RNA (mRNA) molecules and translation of mRNA into proteins that perform most of the critical functions of cells (Zhou 2010). The amounts of mRNA produced by a cell have been used in gene expression studies to evaluate genomic stability

and chromosomal aberration by observing which genes are up or down regulated as a response to environmental toxicant (Kundu et al. 2011;Moore et al. 1997;Udensi et al. 2011a;Zhang et al. 2007;Zhao et al. 1997). High doses of arsenic are clastogenic, while low doses are aneugenic and induce sister chromatid exchanges in a variety of mammalian cells *in vitro* (Klein et al. 2007;Vainio et al. 1981). ATO has genotoxic effect on human colon cancer cells (Stevens et al. 2010) and in keratinocytes (Graham-Evans et al. 2004). Arsenic affects cell cycle check points proteins and its inhibition of DNA repairs and DNA damage by the production of hydroxyl radicals (H_2O_2) (Flora 2011;Shi et al. 2004) and aberrant cytokeratin (CK) expression are consistent with skin carcinogenesis process (Sun et al. 2009).

2.3.3 Aberrations in gene expression

Arsenic causes alterations of gene expression in cultured human keratinocytes (Rea et al. 2003). High-throughput gene expression technologies such as microarrays have been employed to investigate multiple mechanisms based on alterations in expression of target genes. DNA microarrays typically consist of thousands of immobilized DNA sequences present on a miniaturized surface (Li et al. 1989). Microarrays have been used to propose a mechanism of arsenic toxicity/carcinogenicity in skin (Argos et al. 2006), kidney (Sasaki et al. 2007), bone marrow (myeloma) (Wang et al. 2006a), peripheral lymphocytes (Argos et al. 2006)], neural tube (Wlodarczyk et al. 2006)], liver (Liu et al. 2006) and urogenital cells (Su et al. 2006). Other researchers have

used microarrays to investigate the effects of arsenic on keratinocytes (Bae et al. 2002;Hamadeh et al. 2002).

2.3.4 Cellular immune dysfunction

Arsenic exposure induces dysfunction of the cellular immune system by targeting the CD4+ cells with concomitant reduction in CD4+ cells in epidermal keratinocytes which may trigger arsenic induced skin cancer (Kapahi et al. 2000;Yu et al. 2006).

2.3.5 Distortion of protein structure

Sulphur is involved in disulphide bonds formation in proteins especially and is involved in maintaining stability of protein conformations (Chuang et al. 2003). These covalent bonds are the main cross-links present in proteins that are involved in folding and stabilizing their three-dimensional (3D) scaffold (Kadokura et al. 2003;Wedemeyer et al. 2000). Interaction between arsenic and thiol groups disrupts protein conformations (Isokpehi et al. 2010;Snow 1992). Targets proteins that have high cysteine content and accessible thiol groups (Snow 1992) and bind to vicinal cysteines (Isokpehi et al. 2010) and such interaction may lead to disruption of the covalent bond and protein structure. Arsenite interferes with sulfhydryl group of amino acids and disturb protein structure (Chowdhury et al. 2010)(reference). Also, arsenate, which is a phosphate analogue, can substitute for phosphate, thus affecting its uptake and the cellular processes that involve phosphate including ATP and DNA

synthesis (Catarecha et al. 2007;Chavan et al. 2011;Gregus et al. 2009;Sherwood et al. 2011).

2.3.6 Cell proliferation induction

The mechanisms by which arsenic enhances cell proliferation have been extensively investigated. The mechanisms include induction of over expression of growth factors in human keratinocytes (Bailey et al. 2010;Germolec et al. 1996;Vega et al. 2001), induction of cyclin A with increase in S phase population of cells in cell cycle (Chowdhury et al. 2010), cyclin-dependent kinase 4 (CDK4), transcription factor 1 (E2F1), granulocyte macrophage-colony stimulating factor (GM-CSF), and transforming growth factor-alpha (TGF alpha) (Bi et al. 2010;Chowdhury et al. 2010;Germolec et al. 1996;Vega et al. 2001). Low concentrations (0.5 to 1 µg/ml) of ATO stimulate keratinocyte proliferation whereas higher concentrations (> 1 µg/ml) induce cell death by activating caspase-3 and cell cycle arrest at the G2-M phase (Bi et al. 2010;Evens et al. 2004;Graham-Evans et al. 2004).

2.3.7 Epigenetic dysregulation

An epigenetic trait is an inherited phenotype that results from changes in a chromosome without alterations in the DNA sequence (Feinberg et al. 2004). Gene expression is regulated by both genetic and epigenetic mechanisms, which must be put into perspective in toxicogenomics as well as cell-transforming ability of arsenic and other toxicants capable of causing

aberrations in gene expression and cancer (Arita et al. 2009). Epigenetic processes play an important role in gene expression and its dysregulation could lead to arsenic-induced changes in gene expression and cancer in both people exposed to arsenic directly and those who may inherit the dysregulated genes (Drobna et al. 2009;Hei et al. 2004;Jensen et al. 2008;Ren et al. 2011). The three major epigenetic mechanisms reported in arsenic toxicity and carcinogenicity are; DNA methylation (Ren et al. 2011;Styblo et al. 2002;Zhao et al. 1997), histone modification (acetylation, methylation, and phosphorylation) (Barr et al. 2009;Jensen et al. 2008;Rahman et al. 2004), and microRNA (miRNA) expression (Ghaffari et al. 2012). Hypomethylation of DNA may cause overexpression of oncogenic genes (Andrew et al. 2003;Banerjee et al. 2007) and decrease in DNA repair, stress defence mechanisms and apoptosis (Ahsan et al. 2003;Andrew et al. 2003;Banerjee et al. 2007;Hamadeh et al. 2002).

2.3.8 Co-carcinogenicity

Arsenic has also been considered as a weak mutagen and may not initiate but potentiate the mutagenicity, cytotoxicity, and clastogenicity of other carcinogens such as UV radiation, X-ray and other heavy metals by acting as a tumour promoter, or co-carcinogen in skin cancer development. According to Yu, et al, 2006, Arsenic and UVB stimulated caspase pathways; caspase-9 and caspase-8 signalling respectively which induced apoptosis in keratinocytes (Rossman et al. 2004;Yu et al. 2006). Also, some studies have explored a possible interaction between arsenic exposure and smoking in the

causation of cancers of the lung, bladder and skin (Bates et al. 1995;Ferreccio et al. 2000;Knobeloch et al. 2006).

2.3.9 Signal transduction interference

Cell signalling process is a very crucial communication system between cells or within a cell in which a change in the activity of the cell is sent as a signal that may trigger a cascade of reaction for the body to respond accordingly. Cell signal transduction regulates cellular processes such as transcription and cell metabolism, and this is reportedly altered under arsenic exposure (Huang et al. 2004). The degree of perturbation of the signalling pathways may depend on the oxidation state of the arsenic species and cell characteristics (Porter et al. 1999;Shen et al. 1975). The role of altered signal transduction and cell cycling under arsenic insult has been reviewed extensively (Hughes 2002;Hughes 2006;Kitchin 2001). Some of the notable cells signal pathways perturbed by arsenic include; activation of mitogen activated protein kinase pathway, c-Jun N-terminal kinases (JNK) phosphatase and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) dysfunction (Chowdhury et al. 2010;Huang et al. 2004;Kapahi et al. 2000). NF-κB dysfunction occurs when arsenic blocks inflammatory signal transduction by inhibiting inhibitor of nuclear factor kappa-B kinase (IKK) required to activate proinflammatory transcription factor NF-κB (Kapahi et al. 2000). Any process that potentiates the activities of NF-κB encourages carcinogenesis since this transcription factor promotes proliferation and angiogenesis. Arsenite species despite their toxigenic properties also induce translocation of protein kinase C

(*PKC*) isoforms (PKCepsilon, PKCdelta, and PKCalpha) from cytosol to membranes where these enzymes interact with MAP kinase (Erks, JNKs, and p38 kinases) pathways in a way that may alleviate arsenic toxicity (Chen et al. 2000).

2.3.10 Reactive oxygen species induction

Oxidative stress may be described as the imbalance between the production of cellular oxidant species and the capability of the cells to produce antioxidants (Mates et al. 2008). Arsenic induces skin carcinogenesis by disturbing the pro/antioxidant balance namely induction of oxidative stress/ reactive oxygen species (ROS), increased transcript levels of keratinocyte growth factors, and modulation of MAPK (p38, Erk), Akt and NF-κB pathways in exposed cells and permanently activated NF-κB triggers oncogenic effects (Kitchin et al. 2010). At the cellular level, exposure to ATO can cause morphologic alterations in mitochondrial integrity that leads to inactivation of mitochondrial enzymes and loss of mitochondrial membrane potential (Pourahmad et al. 2005). Arsenite may act as a bypass for electrons from the respiratory chain, that facilitate the formation of superoxide anion radicals and generation of ROS (H_2O_2, O_2, ROO, OH, and NO,) (Nesnow et al. 2002;Pourahmad et al. 2005;Raghu et al. 2009;Shi et al. 2004). Arsenite induces a reduction in activity of an important cellular antioxidant, glutathione (gamma-glutamylcysteinylglycine, GSH) (Peng et al. 2010). Also, oxygen is reduced directly to H_2O_2 triggering formation of arsenic peroxyl radicals which are mediators of DNA damage (Shi et al. 2004;Yamanaka et al. 1990), mitosis

disrupters and apoptosis promoters (Kitchin 2001;States et al. 2002). Inorganic arsenate stimulates a rapid burst of oxidative stress in mammalian cells as a result of the repetitive reduction of pentavalent to trivalent arsenic followed by the oxidative methylation of trivalent arsenic (Aposhian 1997). Reactive oxygen species, particularly hydroxyl radicals, play an important causal role in the genotoxicity of arsenical compounds in mammalian cells (Kitchin et al. 2010;Liu et al. 2001). A concomitant occurrence of oxidative stress in cells and tissues result in increased carcinogenic risk in arsenic-exposed persons. Another important trigger of ROS productions is tumour necrosis factor (TNF). TNF signalling also involves ROS-dependent modulation of histone acetyltransferases (HAT) and deacetylases (HDAC) that causes inflammatory gene expression (Rahman et al. 2004). Although ROS trigger signals that enhance cell proliferation such as activation of transcription factor AP-1 and nuclear factor NF-κB, (Hu et al. 2002;Li et al. 2002), it also has the capacity to induce apoptosis through prolonged activation of JNK/AP-1, and SAPK/JNK signalling. SAPK/JNK signalling activates AP-1 that stimulates apoptosis (Yazdanpanah et al. 2009). AP-1 activity could be very important in explaining the discrepancy observed between the proliferative effect of low dose arsenic and the pro-inflammatory high dose. ROS can also activate apoptosis signal-regulating kinase 1 (ASK1), which causes mitochondrial cytochrome c release and activation of effector caspases (Matsukawa et al. 2004). The down-stream effects of ROS signalling depend on both cell-type and cellular condition. The implications for carcinogenesis are therefore variable as well. The detection of Anti-8-Hydroxyguanosine (8-OHdG) in urine is suggested as a reliable biomarker for oxidative stress (De Vizcaya-Ruiz et al. 2009).

2.3.11 Perturbation of Biological Pathways

Normal cellular activities are altered in different disease conditions (Roden et al. 2006). Insights into biological networks perturbed by arsenic exposure could help to discover the mechanism of action in arsenic-induced skin cancer. A variety of bioinformatics software is now available to reconstruct molecular interactions (Chautard et al. 2011;Croft et al. 2011;Stark et al. 2006;Tarcea et al. 2009;Turner et al. 2010).

2.4 Biotransformation of arsenic by human microbiome

2.4.1 Gut Microbiome

The Human Microbiome Project (HMP) has brought a new perspective on the role of human microbial habitats in maintaining normal physiology and predisposition to disease (Betts 2011;Turnbaugh et al. 2007). Microbiome is the totality of all the microbial flora of humans including bacteria, archaea, eukaryotes, and viruses (Turnbaugh et al. 2007). The information on the relationship between gut microbiota and cancer is evolving and prompting new ways of thinking about cancer prevention. However, toxicologists have linked some human cancer to microorganisms. Microorganisms are capable of initiating and sustaining chronic inflammation such as *Helicobacter pylori*, enterotoxigenic *Bacteroides fragilis* (ETBF), Hepatitis B&C, Epstein Barr virus, Human papillomavirus (HPV) (Hope et al. 2005). *Helicobacter pylori* is linked with Gastric MALT (mucosa-associated lymphoid tissue) lymphoma, and

intestinal cancer (Compare et al. 2011;Hope et al. 2005;Liu et al. 2011) with increased cancer risk in allele 2 of the interleukin-1 receptor antagonist gene (IL1RN2) genes carriers (Persson et al. 2011). Also associated with colon cancer is enterotoxigenic *Bacteroides fragilis* (ETBF) which secretes *B. fragilis* toxin (BFT) that causes persistent human inflammatory diarrhoea with high probability of progressing to colon tumour formation, and gastric cancer (Persson et al. 2011). BTF like other carcinogens activate oncogenic pathways such as activation of NF-κB, an epithelial tumourigenesis promoter and cleavage of E-cadherin, and activation of the β-catenin/Wnt pathway, seen in almost all colon cancers (Wu et al. 2009). Chronic hepatitis (*Hepatitis B virus* or *Hepatitis C virus*) is the major cause of primary liver cancer, which is the third leading cause of cancer deaths globally and the ninth leading cause of cancer deaths in the United States (El-Serag et al. 2007;Mitchell et al. 2011;Momin et al. 2011;Wang et al. 2011). Cancer results when physiologically responsive cells are transformed into autonomously replicating tumours with the ability to invade adjacent tissues and spread widely and host/microbiome interaction may yield effects that can either enhance or suppress tumourigenesis (Kong 2011).

A better understanding of the microbial components of the human genetic and metabolic sphere has revealed that metabolic processes initially attributed to human metabolism are currently observed as microbiome metabolic processes. For example, arsenic methylation and reduction under anaerobic conditions of the less toxic arsenate to the more toxic arsenite (Styblo et al. 2002). Interest in the implication of the action of microbes on arsenic has risen based on the observation that some microbes in the human gut can

metabolise, transform, and speciate arsenic (Alava et al. 2012). Since different arsenic species vary widely in their toxicity, improved knowledge on the process of pre-systemic arsenic transformation within the body would be a boost to deciphering microbiome role in arsenic metabolism. In this regards, Van deWiele and co-researchers in 2010, have made some progress. They analysed how the human intestinal bacteria metabolize inorganic arsenic from contaminated soils. Van deWiele's team's demonstrated that bacteria metabolized and transformed inorganic arsenic to methylated arsenicals and thioarsenicals including monomethylmonothioarsonic acid which is the first known metabolically derived thioarsenical (Van de Wiele et al. 2010). This is novel because thioarsenicals are not part of the conventional scheme of arsenic methylation which results in the production of methylated oxyarsenicals (Van de Wiele et al. 2010). The conventional enzymatic methylation of trivalent arsenicals (Styblo et al. 2002) is catalysed by AsIII-methyltransferases which use *S*-adenosylmethionine (AdoMet) as the methyl group donor (Aposhian et al. 2000;Lin et al. 2002;Zakharyan et al. 1995) and yield methylated oxyarsenicals but not thioarsenical. The metabolism of ingested arsenate to oxy-and thio-arsenicals before absorption across the gastrointestinal barrier could affect bioavailability, systemic distribution, and increased toxicity (Pinyayev et al. 2011;Styblo et al. 2002;Thomas et al. 2004). It is not clear if the liver enzymatic conversion is a detoxification process because methylation of inorganic arsenic activates it to more reactive, toxic and carcinogenic forms (Lin et al. 1999) whereas microbial methylation has been shown to detoxify arsenic with trimethylarsine as the end product (Cullen et al. 1984;Qin et al. 2006). Comparing the human intestinal flora and human

enzymatic metabolism of arsenic, the indigenous microbial flora inhabiting an individual metabolizes pre-systemic arsenic to species that are less carcinogenic while the human enzymatic arsenic metabolism produces tumour enhancing species. Nevertheless, it is still debatable if the products of bacterial methylation of arsenic are as carcinogenic as those of human enzymatic processes, (Styblo et al. 2002). Thus, the role of human microbiomes in arsenic metabolism and toxicity regulation cannot be overlooked anymore as the impact of intestinal bacteria may equal or exceed that of genetic polymorphisms that regulate metal transformations within the body (Betts 2011).

Aside molecular analysis of arsenic metabolic pathway in the gut microbiome, a perspective of the skin microbiome is necessary since arsenic accumulates in the skin. Consequently, the evidence of chronic arsenic exposure is manifested on the skin. The Human Microbiome Project (Fierer et al. 2008), has revealed that each body part has its own unique microbiome which starts to populate at birth with several transitions during early life development and remain relatively constant until about age 65 (Claesson et al. 2011;Dominguez-Bello et al. 2010).

2.4.2 Skin Microbiome and Arsenic Metabolism

The skin is the human body's largest organ, and it acts as the interface between the human body and its external environment, preventing loss of moisture and entry of pathogenic organisms or toxic substances (Segre 2006). The skin is home to many different types of microorganisms. The type

and location of organisms found on the skin are influenced by topographical location, endogenous host factors and exogenous environmental factors and the bacterial density may be as high as 10^7 cells per square centimetre (Fredricks 2001;Grice et al. 2011). Interpersonal variation and intrapersonal variation in bacterial community have been observed in human populations. The inter-individual differences among human microbiomes may make a significant difference in the toxicity of metals (Grice et al. 2008). The skin microbial flora consists of both pathogenic and harmless organisms that may even be beneficial to their host (Grice et al. 2011). Microbial diversity studies show that only less than 1% bacteria of total bacterial diversity can be cultured using standard culturing techniques and the remaining 99% microbial pool is represented by vast diversity of uncultured bacteria (Amann et al. 1995). The uncultured bacteria are studied using metagenomic techniques which is a culture independent strategy to explore the untapped gene pool of uncultured bacteria that may possess novel physiological pathways (Chauhan et al. 2009). The metagenomic techniques include DNA sequencing and other genome-based technologies such as NextGen sequencing, pyrosequencing (Claesson et al. 2011;Dominguez-Bello et al. 2010), rRNA genes (16S rDNA) PCR-based sequencing (Gao et al. 2008;Grice et al. 2009), TaqMan probes (designed to increase the specificity of qRT-PCR assays) (Gao et al. 2010), microRNA (miRNA) (Ahmed et al. 2011;Griffiths-Jones 2004;Pogribny et al. 2007). Epigenetic techniques have enabled studies of fastidious human microbes which were previously difficult to isolate and characterize. Such sequence-based approaches are more sensitive than traditional cultivation techniques (Gao et al. 2007), and are currently used in

identifying the diversity and variability of skin microbiome. The recent advances in human microbiome research signals a new horizon for translational research in human diseases because the new knowledge gained on the role of microbiome in human skin disorders could be used to develop novel pro-microbial and antimicrobial therapeutic approaches for their treatment and prevention (Grice et al. 2011;Kong 2011). The normal human superficial bacterial biota is highly diverse with 182 species-level operational taxonomic units (SLOTUs) belonging to eight phyla observed after examining the skin biota from the superficial volar left and right forearms in six healthy subjects using broad-range small subunit rRNA genes (Gao et al. 2007). Microbial colonization on the skin adds to the skin's defence against potentially pathogenic organisms (Roth et al. 1988). Exposure to environmental toxicants such as arsenic may have the potential to alter the skin microbiome selectively and may underlie the incidence of human skin disorders as seen in arsenic water contamination in endemic communities.

2.4.3 Bacteria arsenic metabolism

Bacteria metabolize arsenic to evade its toxic effects. The *ars* genes possessed by most organisms help to develop arsenic resistance. As shown in Table 3, the *ars* genes are organized in operons, such as *ars*RBC, *ars*RABC, and arsRDABC, but in some cases the *ars* genes appear singly (Butcher et al. 2000;Butcher et al. 2002;Diorio et al. 1995;Sato et al. 1998). The *ars* operon is conserved mostly in Gram-negative bacteria and has a functional role in arsenic detoxification (Diorio et al. 1995). The *ars* system is

an arsenic responsive mechanism observed in most living organisms and further exploration of the activities may lead to discovery biomarkers of arsenic insult. The *ars* genes perform different functions in arsenic metabolism; the Transcriptional repressor (*ars*R) encodes an As(III)-responsive transcriptional repressor, (Wu et al. 1991) that binds to target promoters and controls the expression of other *ars* genes (Rosenstein et al. 1994;Wang et al. 2009). Anion-transporting ATPase (*ars*A) as the name implies functions as an ATPase. Arsenical pump membrane protein (*ars*B) is a membrane efflux transporter. *Ars*A and *Ars*B form an oxyanion-translocating complex (Rosen 2002). And sometimes arsB function without *ars*A in arsenic extrusion (Dey et al. 1995). Arsenate reductase and related proteins are encoded by glutaredoxin family (*ars*C) and it converts arsenate to arsenite. Also, AqpS which encodes water-transport channel participates in a *ars*C-catalysed reduction to induce resistance (Yang et al. 2005). AqpS is also known to transport glycerol in plants due to their lack of aquaglyceroporins (Zardoya R 2002). Arsenical resistance operon trans-acting repressor (*ars*D) is an arsenic metallochaperone which increases the rate of arsenic extrusion by transferring As(III) to *Ars*A (Lin et al. 2006;Wu et al. 1993). *Ars*D is a homodimer and each of its subunit has three vicinal cysteine pairs, Cys12-Cys13, Cys112-Cys113 and Cys119-Cys120 (Lin et al. 2007). These vicinal cysteines are probably the binding sites of the proteins to arsenic. Another member of the *ars* gene operon, arylsulfatase family, member H (*ars*H) encodes an NADPH-flavin mononucleotide oxidoreductase which is involved in arsenic resistance in *Yersinia enterocolitica* and *Sinorhizobium meliloti* (Neyt et al. 1997). Another arsenic resistance gene *ars*M encodes an arsenite

S-adenosylmethionine methyltransferase (Wang et al. 2009) which plays a role in arsenic detoxification in bacteria. *ars*M stimulates arsenite [As(III)] resistance by catalysing the formation of a number of methylated intermediates from As(III), and production of volatile trimethylarsine (Qin et al. 2006). A putative membrane permease (*Ars*P) is also a member of the *ars* gene operon. Both *ars*D and *ars*A play crucial role in the binding and extrusion of the reduced arsenite from the bacteria and into the host environment. The *Ars*D metallochaperone delivers trivalent metalloids, As(III), to the *Ars*A ATPase, the catalytic subunit of the *Ars*AB As(III) efflux pump. Transfer of As(III) increases the affinity of *Ars*A for As(III), allowing resistance to environmental arsenic concentrations. Two different transport mechanisms are involved in the uptake of the two different arsenic oxidation states; (i.) phosphate transporters which facilitate the uptake of As(V) in the form of arsenate and (ii.) the aquaglyceroporins which transport As(III) in the form of arsenite. Other mechanisms of bacterial arsenic resistance include; the reduction of As(V) to As(III) by arsenate reductases, and extrusion or sequestration of As(III). The extrusion is mediated by efflux transporters, reduction of arsenate [As(V)] to arsenite [As(III)], which is subsequently extruded by efflux transporters and methylation of As(III) by S-adenosylmethionine methyltransferase (Qin et al. 2006;Rosen 2002;Yuan et al. 2008).

Table 3: Arsenic Responsive Genes in Bacteria

Organism	Operon	Gene	Function	References - PubMed Identifiers (PMID) *
Campylobacter jejuni	*Ars* operon	Putative membrane permease (ArsP)	Resistance to arsenite and arsenate	PMID: 9537360, PMID: 19502436
Campylobacter jejuni, *Staphylococcus xylosus* (pSX267), *Bacillus subtilis skin* element, *Staphylococcus* and *Escherichia coli*		Transcriptional repressor (ArsR)		PMID: 8121414, PMID: 1838573, PMID: 9537360, PMID: 19502436
Campylobacter jejuni , *Bacillus subtilis skin* element, *Staphylococcus* and *Escherichia coli*		Arsenate reductase (ArsC)		PMID: 19502436, PMID: 7721697
Campylobacter jejuni		Efflux protein (Acr3)		PMID: 19502436, PMID: 21299644
		arsA		PMID: 21299644
Bacillus subtilis skin element		Carrier protein (ArsB)	Arsenic transport	PMID: 10354596, PMID: 9537360
Escherichia coli		Anion-translocating ATPase (ArsAB)		PMID: 10354596, PMID: 7814328, PMID: 21299644
Salmonella enterica		*arsD,* metallochaperone		PMID: 21313837, PMID: 21188475, PMID: 21299644, PMID: 21299644
Crocosphaera watsonii		*arsH*		PMID: 19283378, PMID: 22046174
Escherichia coli		AsIII S-adenosylmethionine methyltransferase *(arsM)*		PMID: 18522094
Bacillus subtilis skin element		*ORF2*,		PMID: 9537360
Saccharomyces cerevisiae		*acr1*	Transport	PMID: 9374482
Saccharomyces cerevisiae		*acr1*	Transport	PMID: 9374482
Saccharomyces cerevisiae		*Acr3p*	Arsenite extrusion, transport	PMID: 10801893, PMID: 10220408, PMID: 9374482
Saccharomyces cerevisiae		*Ycf1p*	Transport	PMID: 10220408
Achromobacter sp. SY8 and *Pseudomonas sp.* TS44	arsenite oxidase (aox)	*aoxX-aoxS-aoxR*		PMID: 19283378
Achromobacter sp. SY8 and *Pseudomonas sp.* TS44		*aoxA-aoxB-aoxC-aoxD*		PMID: 19283378

** Citation for PubMed can be obtained from http://www.pubmed.gov

2.5 Skin Cancer overview

Skin cancer is the most common of all human cancers. Annual global incidence of non-melanoma skin cancers is currently, between 2 and 3 million, and about 132,000 for melanoma skin cancers (WHO 2012). More than 1 million people are diagnosed with skin cancer each year in the United States. There has been steady increase in the incidence of both non-melanoma and melanoma skin cancers over the past decades. Most skin cancers are benign but some are malignant and capable of metastasis. The major types of skin cancer are: basal cell carcinoma (BCC) (Zoccali et al. 2011), squamous cell carcinoma (SCC) (Kraljik et al. 2011) and melanoma. Basal cell carcinomas and squamous cell carcinomas are described as non-melanoma and are more common in older people while melanomas are more common in younger people. For example, melanoma is the most common cancer in people 25-29 years of age. Other types of non-melanoma skin cancers are; keratoacanthomas, Merkel cell carcinoma, skin lymphoma, Kaposi sarcoma, skin adnexal tumours, and sarcomas (ACS 2012;Kramkimel 2013). Most common cause of skin cancer is ultraviolet radiation (UVR) exposure, mostly from sunlight. Other important causes include; use of tanning booths, immunosuppression-impairment of the immune system, exposure to unusually high levels of x-rays, contact with certain chemicals such as arsenic, hydrocarbons in tar, oils, and soot. People most prone to skin cancer are those with fair skin that freckle, sunburn easily, or become painful in the sun, blond or red hair and blue or green eyes, certain genetic disorders that deplete skin pigment such as albinism, xeroderma pigmentosum, treated skin cancer,

numerous moles, unusual moles, or large moles that were present at birth, and those who inherit the genes from their parents.

2.5.1 Basal cell carcinoma (BCC) and Squamous cell carcinoma (SCC)

Basal cell carcinoma and Squamous cell carcinoma are the frequently encountered type of malignant skin cancer and they do not normally spread to other tissues but may disfigure the affected area if not treated promptly. The BCC affects mostly people over 50 years with children in rare occasions (Skellett et al. 2012;Zoccali et al. 2011). Additionally, SCC has a high prevalence among the elderly, 70 years and over. This maliganant cancer affects the parts of the body exposed to sunlight such as the head, neck and backs of the hands with the ratio of the photo exposed area to photo non-exposed area as 5:1 (Kraljik et al. 2011). Squamous cell cancinoma is initiated as a small nest of aberrant cells expands to dominate a tissue and form a macroscopic tumour. During early neoplastic progression, pre-invasive lesions demonstrate dysplastic foci that are initially surrounded by normal, undisturbed tissue (Farber 1996).

2.5.2 Melanoma

Malignant melanoma accounts for most of the fatalities associated with skin cancer as it is aggressive and spreads easily to other organs. Exposure to ultra violet radiation (UVR) from the sun is the major cause of melanoma worldwide. Iit is more prevalent among White populations than in Black African

and Asian populations (Armstrong et al. 1993). This is attributed to the melanin content and melanosome dispersion in people of colour compared with light-coloured skin persons. The light-coloured skin readily absorbs UV light and thus more susceptible to UV damage (Taylor 2002). Although the body most often recovers and repairs the damage caused by exposure to UV light, co-exposure to arsenic may hamper the repair mechanism leading to cancer development (Rossman et al. 2004;Taylor 2002). Exposure to UV rays increases the risk of developing non-melanoma skin cancer (Wei-Passanese et al. 2012). Melanoma begins in melanocyte (cells that make the pigment melanin) as moles in the skin and other pigmented tissues including the eye or in the intestine (Skellett et al. 2012;Zoccali et al. 2011). About 76,250 new cases are estimated to be diagnosed and 9,180 are projected to die of melanoma in the United States in 2012. The aetiology of melanoma, the most lethal form of skin cancer, is complex, involving both genetic and environmental components. MicroRNA (miRNA)-mediated epigenetic regulation of tumour suppressor genes and oncogenes has been shown to play a central role in melanomagenesis (Bonazzi et al. 2012). Most skin cancers start as a mole which undergoes dysplastic changes to become actinic keratosis, a patch of red or brown, scaly, rough skin, a precursor to SCC or melanoma.

2.5.3 Skin Cancer Treatment

Basal cell carcinoma and squamous cell carcinoma are treated by surgical removal of the lesion. Malignant melanoma is more complicated and may

require different approaches depending on the stage and organs involved. The treatment methods include surgery, radiation therapy, and chemotherapy (Bajetta et al. 2002;Burusapat et al. 2012).

2.6 Skin Cell Culture System

The mammalian skin is a complex dynamic organ composed of thin multi-layered epidermis, a thin layer of basement membrane and a thick underlying connective tissue layer dermis containing fibroblasts (Figure 2). The epidermis provides physical, chemical／biochemical (antimicrobial, innate immunity) and adaptive immunological barriers. The skin function as a physical barrier lies mostly on the stratum corneum which is the outermost layer. But it is supported by the nucleated epidermis, especially the cell–cell junctions and associated cytoskeletal proteins. The presence of lipids, acids, hydrolytic enzymes, antimicrobial peptides and macrophages also assist the skin to function as a chemical and antimicrobial and a boost to innate immunity (Proksch et al. 2008).

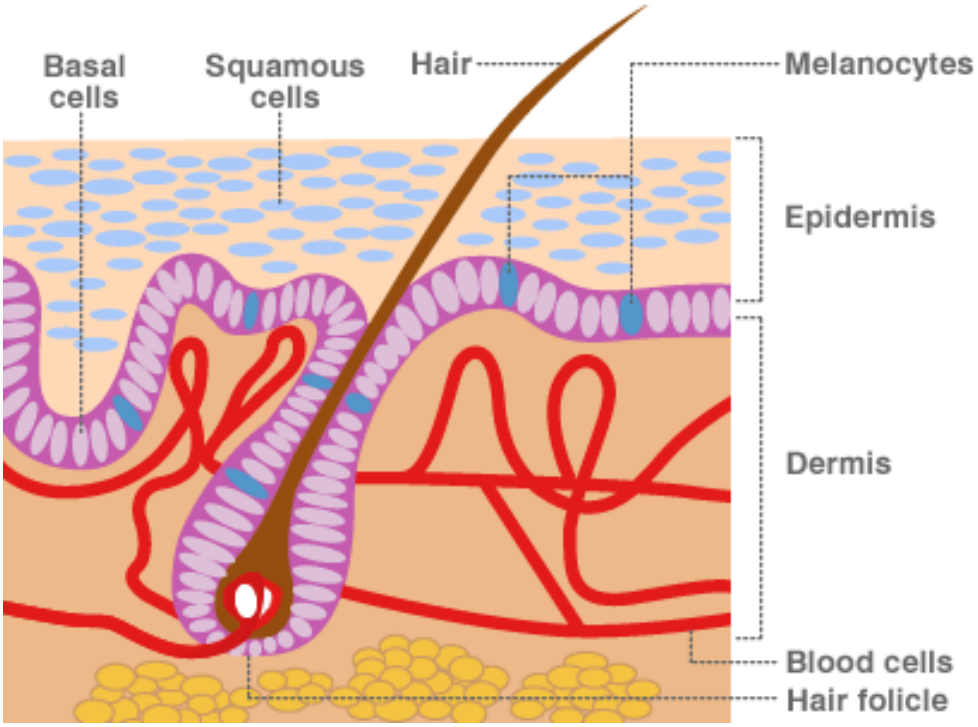

Figure 2: Human skin physiological constituents

From: http://www.ocaesthetics.org/images/pic-dermal-medical-skin-care-1.jpg (Williams 2012)

The epidermis is continually renewed and replaced throughout life (Singh et al. 2012). Sustained keratinocyte growth required both intact basement membrane and dermal fibroblasts. Different skin cells have been used to study the molecular nature of carcinogens and tumour promoters such as arsenic. Since there is wide variability between how laboratory animals and human metabolize arsenic, several skin constructs to simulate human skin system have been developed to enable *in vitro* experiments. Depending on the experimental design, keratinocytes cell model systems have been develop to answer different biological questions. Some of the models include (i.) single layer cell culture; e.g. Human Keratinocyte (HK), Normal human epidermal keratinocytes (NHEK), a spontaneously immortalized human keratinocyte cell line (HaCaT cells), 3T3 fibroblast, (ii.) organotypic keratinocytes culture and

(iii.) co-cultures system. Examples of co-culture are; keratinocytes-melanocytes coculture system, keratinocyte culture with collagen gel, keratinocyte coculture with 3T3 fibroblast, a co-culture of human keratinocytes and *Staphylococcus aureus* (Ahn et al. 2010;Ma et al. 2010;Nicolay et al. 2003;Tandara et al. 2011;Wiegand et al. 2009), collagen/fibroblast (Wu et al. 1982). Organotypic cultures may help in understanding how the basement membrane components and/or dermal fibroblasts direct the assembly and organization of structured basement membrane and the concomitant normalization of epidermal phenotype (Andriani et al. 2003;Javaherian et al. 1998;Margulis et al. 2003). A example of organotypic culture system is Merkel cells (MCs) containing epidermal sheets embedded in collagen gel. This model has been used to estimate the effects of nerve cells on the maintenance of MCs within the epidermis. Merkel cells are normally located in the epidermal basal layer, in contact with keratinocytes (Nagase et al. 2009). Organotypic cultures with HaCaT cells have been developed to investigate cell migration and differentiation (Hinitt et al. 2011). Organotypic cultures have a high level of tissue normalization with the ability to form a structured, mature basement membrane when the construct involves dermal fibroblasts grown on pre-existing basement membrane components (Andriani et al. 2003;Javaherian et al. 1998;Margulis et al. 2003).

2.7 Microarray and PCR Technologies

A microarray is a tool for analysing gene expression. DNA microarrays are basically miniaturized solid surfaces (membrane or glass slides) with

immobilized sequences from thousands of different genes stationed in a regular pattern at specific spots (Koschmieder et al. 2012). Gene expression encompasses all the processes from the transcription of the information contained within the DNA, into messenger RNA (mRNA) molecules to the translation into the proteins which perform most of the critical functions of cells. Although full set of chromosomes and identical genes may be found in every cell, not all are turned on or participate in the transcription process. Thus, only the subset that is transcribed confers unique properties to each cell type (Benson et al. 2008). The amounts of mRNA produced by a cell are studied to learn which genes are expressed, and how the cell responds to its changing needs (Benson et al. 2008). Although DNA microarrays are excellent tools in discovery-based genomic and biomedical research (Edgar et al. 2006;Sun et al. 2007), additional verification tests especially RT-PCR are needed to confirm that the genes of interest are differentially expressed. This is due to the variability that exist in microarray results from laboratory to another, user to user, and platform to platform (Larsson et al. 2006). Real-time PCR, also referred to as quantitative reverse transcription PCR (RT-qPCR), and quantitative PCR (qPCR), is a very powerful and sensitive gene analysis technique. It is used for a broad range of applications including quantitative gene expression analysis, genotyping, copy number, drug target validation, biomarker discovery, pathogen detection, and measuring RNA interference (Invitrogen 2012). The difference between Real-time PCR and traditional PCR is that Real-time measures PCR amplification as it occurs, thus making it possible to determine the starting concentration of nucleic acid. In contrast, the traditional PCR, results can only be collected after the reaction is complete,

making it impossible to determine the starting concentration of nucleic acid (Arya et al. 2005;Invitrogen 2012).

Real time PCR is able to produce the qualities lacking in microarray such as high detection sensitivity, sequence specificity, large dynamic range as well as its high precision and reproducible quantitation (Wang et al. 2006b). Thus, RT-PCR is regarded as the “gold standard” for microarray confirmation (Shippy et al. 2004). The basic principles of RT-PCR and its application in molecular diagnostics based on high-throughput, automated technology with lower turnaround times has been reviewed in detail (Arya et al. 2005). SYBR Green and TaqMan (fluorogenic 5’ nuclease chemistry) gene expression assays are the two common fluorescent reporter molecule in real-time PCR based techniques designed to evaluate microarray experiments. SYBR® Green I, is a dye that binds to double-stranded DNA, to detect PCR product as it accumulates during PCR cycles (Schneeberger et al. 1995;Xiang et al. 2012) while in Taqman a fluorogenic probe is used to monitor accumulation of a specific PCR product during PCR cycles (Life Technologies 2012;Wang et al. 2006b). The amount of fluorescence emitted from the fluorophore increases as the quantity of target amplicon increases (Arya et al. 2005;Invitrogen 2012). A schematic diagram comparing TaqMan and SYBR Green chemistries is shown in Figure 3.

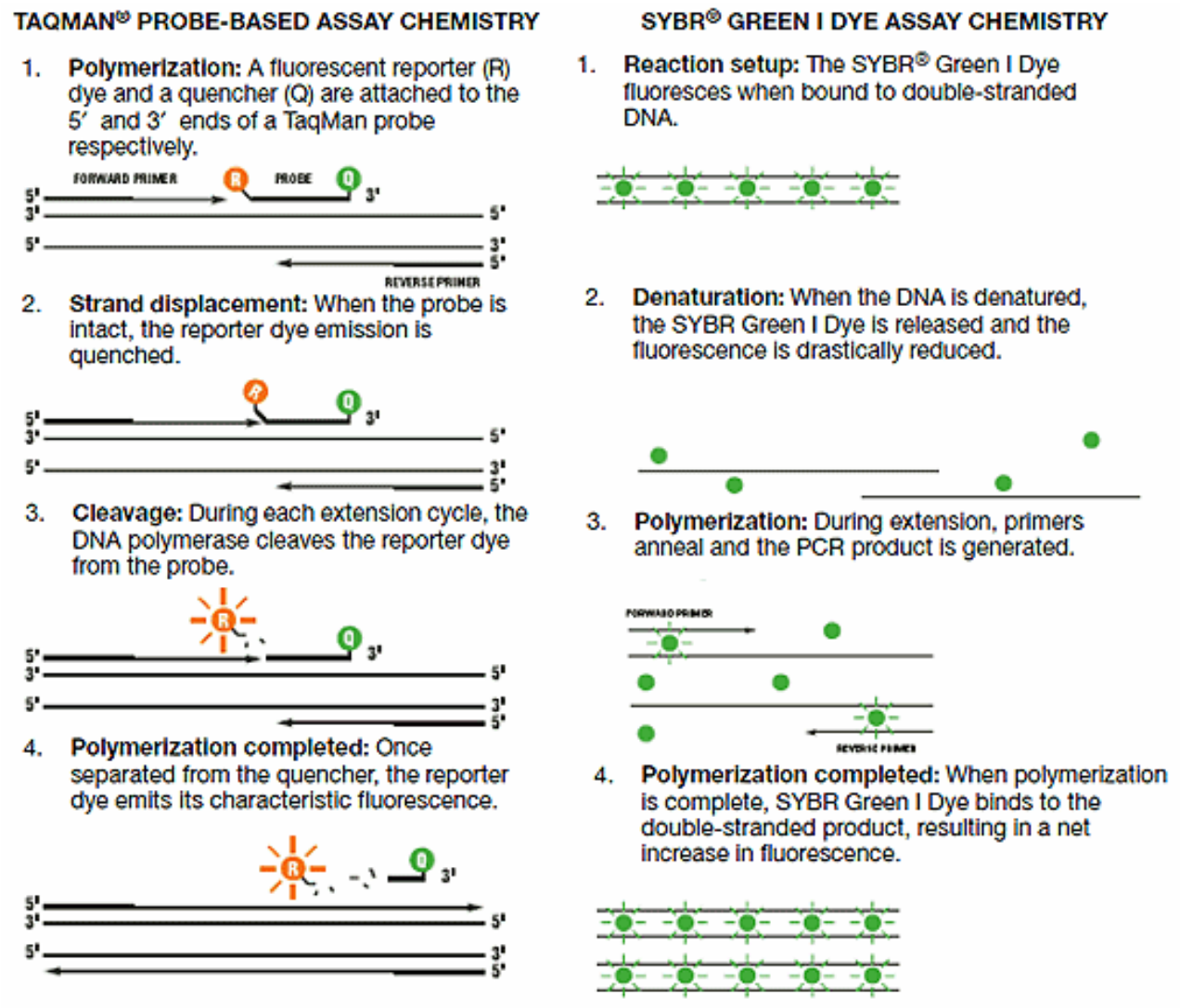

Figure 3: Gene expression assay reaction steps comparison of TaqMan and SYBR Green chemistries

Source: Invitrogen Corporation (Invitrogen 2012)

2.8 Computational Biology, Bioinformatics tools and data bases

Computational biology is the development of a technique and the validation test of the developed technique. Bioinformatics is the field of science in which biology, computer science, and information technology merge to form a single discipline (Baxevanis et al. 2005). Bioinformatics is applied in the validation, adaptation and usage of the computational biology techniques to numerous

real life problems. Bioinformatics tools and techniques have enabled the discovery of new biological insights and creation of a global perspective from which unifying principles in biology have been discerned. The bedrock of Bioinformatics is the creation and advancement of databases, algorithms, computational and statistical techniques and theory to solve formal and practical problems arising from the management and analysis of biological data (Benson et al. 2007). To maintain the database, existing data must be updated regularly with new or revised data, and designs and user friendly interfaces to grant researchers easy access (Kulikova et al. 2007;Sugawara et al. 2007).

Bioinformatics tools and techniques are currently used to analyse and interpret various types of data, including nucleotide and amino acid sequences, protein domains, and protein structures (De et al. 2006). Scientists have been able to determine how normal cellular activities are altered in different disease conditions (Roden et al. 2006).

Computational biology techniques will be applied in the course of this research analyses and interpretation of the high-throughput data that will be generated. Tools such as; Michigan Molecular Interactions (MiMI) web tool (http://mimi.ncibi.org), (Tarcea et al. 2009), Cytoscape web (Lopes et al. 2010) and Dianna web server, (http://clavius.bc.edu/~clotelab/DiANNA/) (Ferre et al. 2006) have developed algorithms and statistical methods with which to assess relationships among members of large data sets, such as methods to locate a gene within a sequence, predict protein structure and/or function, and cluster protein sequences into families of related sequences.

Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Redwood City, CA) is a software for determining models of biological pathways and networks that are significantly represented in the differentially expressed (both up and down regulated) genes. It generates a P-value for each network and canonical pathway, which is the likelihood that a given network was identified by chance. IPA assigns biological functions to each network by using annotations from scientific literature stored in their knowledge base. Fisher exact test is used to calculate the P-value for each biological function/disease or pathway being assigned by chance.

2.8.1 Comparative Toxicogenomics Database

Comparative Toxicogenomics Database (CTD http://ctd.mdibl.org/) advances understanding of the effects of environmental chemicals on human health. The interactions between environmental factors and genes that modulate important physiological processes are proposed as the underlying cause of most chronic diseases (Olden et al. 2000;Schwartz et al. 2004;Toscano et al. 2005). This conclusion is based on the observations that many complex diseases are caused by reversible behaviours or avoidable exposures. Additionally, there is relatively rare number of diseases attributed to single gene mutations (Olden et al. 2000). Many common conditions including asthma, cancer, diabetes, hypertension, immune deficiency disorders and Parkinson's disease have been linked to environmental factors. But there is an information gap on the molecular mechanisms underlying these correlations (Toscano et al. 2005). The CTD is a repository for curated data which describe cross-species

chemical–gene/protein interactions and chemical– and gene–disease relationships. The molecular mechanisms underlying variable susceptibility and environmentally influenced diseases and complex chemical–gene and protein interaction networks could be studied using the CTD.

2.9 Visual Analytics

Visual analytics is an emerging discipline that combines visualization methods with data analysis and human-computer interaction (Alako et al. 2006;Hu et al. 2004;Moll et al. 2005). Visual analytics is increasingly used to analyse multidimensional biological datasets of all sizes and presenting them in an interactive visual display with one of the purpose to identify patterns (Chabot 2009;Johnson et al. 2010;Shih et al. 2011;Udensi et al. 2011b). The purpose of visualization is to transform the invisible to visible. Visual analytics is different from other data analysis methods because it integrates visualization with data analysis. The iterative and synergistic process of visualization and analysis which is facilitated by computer hardware technology makes it easy for the user to interact with the software and gives an opportunity to manipulate the data to formulate different hypotheses based on the data analysis results (Heer et al. 2006). The visualization approach could allow the researchers to make discoveries faster. Visual analytics methods make it easy to mine, analyse and assimilate the mounds of complex data generated from high throughput screening (HTS) techniques such as flow cytometry, genotyping, DNA, RNA, RNAi sequencing, without losing crucial information patterns which can easily be lost using traditional statistical techniques (Pui

Shan et al. 2006). The Excel-based spread-sheet paradigm does not provide the level of human-computer interaction that is necessary to both understand a complex data set and inform data mining and machine learning analyses. Visualization helps the investigator to become familiarized with their high-dimensional data in a way that might not be possible with a spreadsheet or database. Application of visual analytics tools have evolved over the years. Earlier tools such as the protein-protein binding databases DIP (Xenarios et al. 2001), was used to demonstrate dynamic visualization of interaction networks. This allowed users to navigate among links in particular data sets. Recent visualization and analysis tools such as Cytoscape (Ideker et al. 2002), MintViewer (Zanzoni et al. 2002) and Osprey (Breitkreutz et al. 2003), have expanded this concept. They include features for viewing and querying larger subsets of the interactome on a more global scale. The tools typically operate from the viewpoint of physical associations among proteins, or correlated gene expression, and include information that summarizes annotated functions, such as Gene Ontology (GO) (Ashburner et al. 2000) groupings, among sub-networks of linked genes or proteins. VisANT, a protein interaction databases is a unique tool to store, in a computer readable form, the protein interaction information disseminated in the scientific literature (Hu et al. 2004). There are general purpose visual analytics which provide simple, interactive way of exploring and interpreting data that have been very useful in the analysis of scientific data. These tools were created to process and visualize categorical data such as census and survey data, inventory, weather forecast and any data that can be summed up in a cross-tabulation. Examples include the Tableau Software (Shih et al. 2011;Tableau 2012), Parallel Sets (ParSets)

(Kosara 2012), Yurbi (Yurbi 2012). These softwares are used in analysing huge business data to deliver quick business intelligence such as using the data result to develop business cases that prioritize and reliably quantify the impacts of value improvement projects, and to convert data into actionable, accessible information to drive decision making (HFMA 2012).

Chapter 3

Research Methodology

3.1 Materials/Methods

3.1.1 Chemical and Reagents

- ATO (dissolved in dilute nitric acid, 1mL = 1mg As, Reference Standard Solution, 1000µg/ml +/-1%/Certified, 99.9% purity, Fisher Scientific Suwanee, GA) dilutions were prepared using a complete DMEM medium as the diluent.
- Foetal bovine serum (FBS, Hyclone Laboratories Logan, UT),
- Dulbecco's modified Eagle's Medium (DMEM),
- Phosphate buffered solution (PBS 1x) (ATCC, Manassas VA) and Streptomycin/Penicillin antibiotics (Invitrogen, Carlsbad, CA), were purchased for culturing the HaCaT cell line.
- The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide] reagent was obtained from ATCC (Manassas VA)
- Collagen-IV from Sigma Chemical Company (St. Louis, MO) (Lot no 108K0503)
- Ambion Message Amp aRNA kit (Cat. No. 1753) or Ambion amino allyl cDNA kit (Cat. No. 1705)
- NanoDrop ND-1000 for optical density measurement
- The reactive amino group of 5-(3-aminoallyl)-UTP/5-(3-aminoallyl)-dUTP was used to conjugate the purified aRNA/cDNA with the NHS-CyDye

3.2 Experimental Design

This research has two components:

(i) Wet laboratory experiments: This include the following experiment

Cell culture
Evaluation of chronic toxicity of ATO to keratinocytes
Genotoxicity experiment
RNA extraction and gene expression studies

(ii) Bioinformatics analyses of the high throughput data generated from the wet laboratory experiments

3.3 Wet laboratory experiments

3.3.1 Cell Culture Procedure

The HaCaT keratinocytes used for this research were provided by Dr Van Wilson (Microbial & Molecular Pathogenesis, College of Medicine, Texas A&M Health Science Centre, College Station, Texas, USA). Approximately 1.5×10^5 HaCaT cells were cultured in 10 ml of complete DMEM in T-25 culture flasks and incubated in a humidified atmosphere with 5% CO_2 at 37 ºC. The culture medium was replaced every 96 hours. The cells were split after growing to 90% confluence by aspirating the culture medium and washing the cell monolayer three times with sterile phosphate buffered saline (PBS). The cell monolayer was treated with 2 mL 0.25% trypsin-EDTA per plate and incubated briefly at 37 ºC. The cells were then viewed under the microscope to ensure that cells were completely detached and were re-suspended in a complete

DMEM medium. An aliquot of the HaCaT cell suspension was stained with 4% trypan blue for 1 to 2 minutes and counted with a haemocytometer. The cells used for chronic exposure were considered chronic after undergoing 10 passages (cells trypsinised at 90% confluence, Figure 4, and sub-cultured in another cultured flask). In this research investigation, acute cells are defined as the cells that were exposed to ATO for the first time while chronic cells are cells that have been exposed continuously up to 10 passages with a very low dose 0.5 µg/ml of ATO.

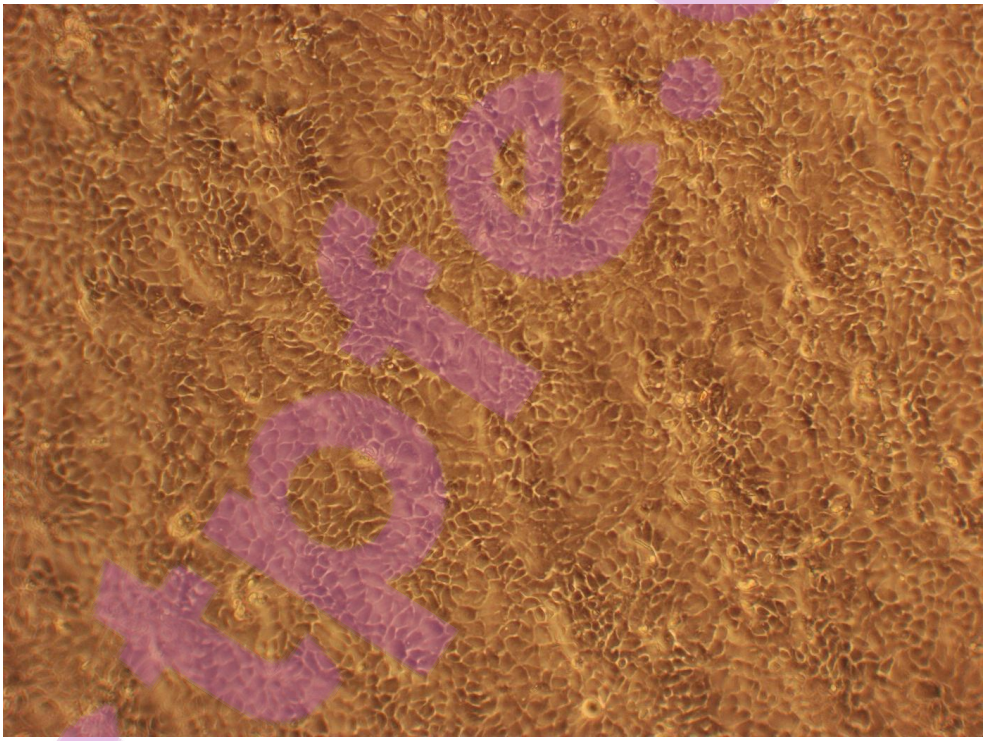

Figure 4: 100% Confluent HaCaT cell culture

3.3.1.1 Protocol for Splitting of Cells

- The media (DMEM), trypsin-EDTA and PBS were warmed up in 37^{o} C water bath

- Inside the hood (Using sterile environment), 10 ml of media were added to new T-25 culture flasks
- Old media were aspirated from cell culture flasks and washed twice with 10 ml sterile PBS
- PBS was aspirated and 2ml Trypsin-EDTA solution was added to a confluent culture flask as shown in Figure 4, and the flask tilted to mix
- The flask was incubated at 37 C for 2 minutes or until cells were observed to detach from the plate
- 5 mL of media was added to each plate to stop trypsinisation reaction and was transferred to sterile 15ml centrifuge tube.
- The cell suspension was centrifuged at 800 rpm for 5 min at room temperature
- The supernatant was aspirated, and 4 ml of fresh media added to cell pellet
- The cells were mixed by pipetting up and down gently for several times
- Cell count was performed using haemocytometer
- The cells were either used for plating or stored frozen
- One confluent T-25 flask was used to plate four T-25 flask i.e. remove 1ml of cell suspension from the trypsinised cell and transfer to a new T-25 flask with fresh media
- The media (DMEM), trypsin-EDTA bottle caps were tightly closed and returned to 4^{o} C.

3.3.1.2 Cell Count Calculation

- Four large squares (with 16 smaller squares) of the haemocytometer were counted
- Total Cells Count/ml = the average count x the dilution factor x 10^4
- Total Cells Count/ml = count in 4 large squares/4 x total volume (4ml) x 10^4
- Assuming the count from four squares= 184
- Total Cells Count/ml = 184/4 x 4 x 10^4 = 184 x 10^4 =18.4 x 10^5/ml

3.3.1.3 Determination of Concentration of Cells to Plate

Concentration of Cells to Plate = Cell concentration required / total cell count/ml x volume required

Concentration required = 1.5 x 10^5/ml

Volume required = 10ml

Total Cell Count = 18.4 x 10^5/ml

1.5 x 10^5 / 18.4 x 10^5 x 10ml = 0.8ml

Therefore 0.8ml of well mixed cell suspension will be transferred to a fresh flask containing 10 ml medium.

3.3.1.4 Cryopreservation of HaCaT Cells

The HaCaT cells at each passage were cryopreserved to avoid loss by contamination and to avoid genetic drift. The cells were checked for contamination prior to cryopreservation. A seed lot system was used in which a portion of the lot at each passage is designated as seed material and is set

aside from the working stocks. It is stored different to avoid being used until the first working stock lot has been depleted (Hay 1988). The seed lot serves as a source for subsequent working stock.

The Cryopreservation media used consist of 10% DMSO (dimethylsulfoxide)

- Under the hood (sterile environment), a 0.22 µl pore filter was attached to the tip of a 10 ml syringe with the plunger pulled out
- 9 ml of foetal bovine serum (FBS) was transferred into the barrel of the 10 ml syringe
- The plunger was put back and pushed to filter the FBS into a sterile 15 ml centrifuge tube
- 1 ml of DMSO was added to the filtered FBS
- The tube was labelled “Freezing Medium” and was placed at 4 °C
- $1x10^5$ cells/ml was added to 1ml freezing media in a 1.5 ml cryovial
- The cryovial was placed in Thermo Scientific Nalgene Mr Frosty freezing container overnight at -80 °C to achieve a uniform cooling rate close to -1 °C per minute.
- A vial of the frozen cell was tested to determine if the cells survived the freezing procedure. The cells were thawed and tested for viability and ability to establish a cell population after been frozen for 48 hours
- The vials were transferred to liquid nitrogen freezer at -150 °C

3.3.2 Cytotoxicity Assay (MTT Assay)

Cytotoxicity assay is applied to evaluate chronic and acute toxicity of ATO to skin keratinocytes. MTT Cell Proliferation Assay Kit is a basic method used to measure cell viability and proliferation in response to external stimuli such as drugs and cytotoxic agents. It can also conversely determine the reduction in cell viability due to metabolic events such as apoptosis or necrosis. The MTT assay was developed by Mossman (Mosmann 1983) and it is an indirect method using standard microplate absorbance readers. Its fluorescent or chromogenic indicators provide the most rapid and large scale assays. MTT assay involves the reduction of the water soluble yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) to an insoluble intracellular purple formazan and reducing equivalents such as NADH and NADPH by metabolically active cells and in part by the action of dehydrogenase enzymes. The formazan is then solubilized, and the concentration quantified by spectrophotometric means at optical density 570 nm. The result is a sensitive assay with a low background absorbance values in the absence of cells. Also, an excellent linear relationship between cell number (up to approximately 10^6 cells per well) and signal produced is established, which allows an accurate quantification of changes in the rate of cell proliferation or cell death.

Two MTT cytotoxicity assays were performed:

1. To determine the LD10/LD 50 and estimate the minimal cytotoxic concentration that would be used to establish a chronic cell exposure condition
2. To determine dose response and cytotoxic patterns in chronically exposed and acutely exposed skin keratinocytes to ATO

3.3.3 MTT Test 1

Dose response relationship was determined by measuring the survival rate (% viability) of cells after treatment with ATO using MTT (tetrazolium salts) assay as follows; about 3×10^4 cells suspended in 200 µl of medium were inoculated in each well of a flat-bottom 96-well culture plate. There were nine experimental groups: negative control, blank control (no cells, no arsenic), and seven arsenic treatment groups with final concentrations of ATO of: 0.0, 1.0, 5.0, 10.0, 15.0, 20.0 and 25.0 µg/ml as shown in Table 4 and Figure 5 . Each of the group was exposed to ATO for 48 hours. The cells were treated with 20 µl MTT cell proliferation assay kit (lot no: 9426FS) and incubated for 4 hours. 100 µl of detergent solution was added and incubated in the dark for 2 hours. The absorbance was measured at wavelength 570nm in a microplate reader (Fluoroskan II microplate reader Helsinki, Finland). Acute cytotoxicity experimental results were used to determine tolerated dose (LD_{10}) in all the cell lines for chronic assays as used by Trouba et.al, (Trouba et al. 1999).

Table 4: Concentration of ATO used for LD50 determination on HaCaT cells

Concentration	100 µg/ml Stock ATO	Media (µl)	Final Volume (µl)
25	500	1500	2000
20	400	1600	2000
15	300	1700	2000
10	200	1800	2000
5	100	1900	2000
1	20	1980	2000
0	0	2000	2000

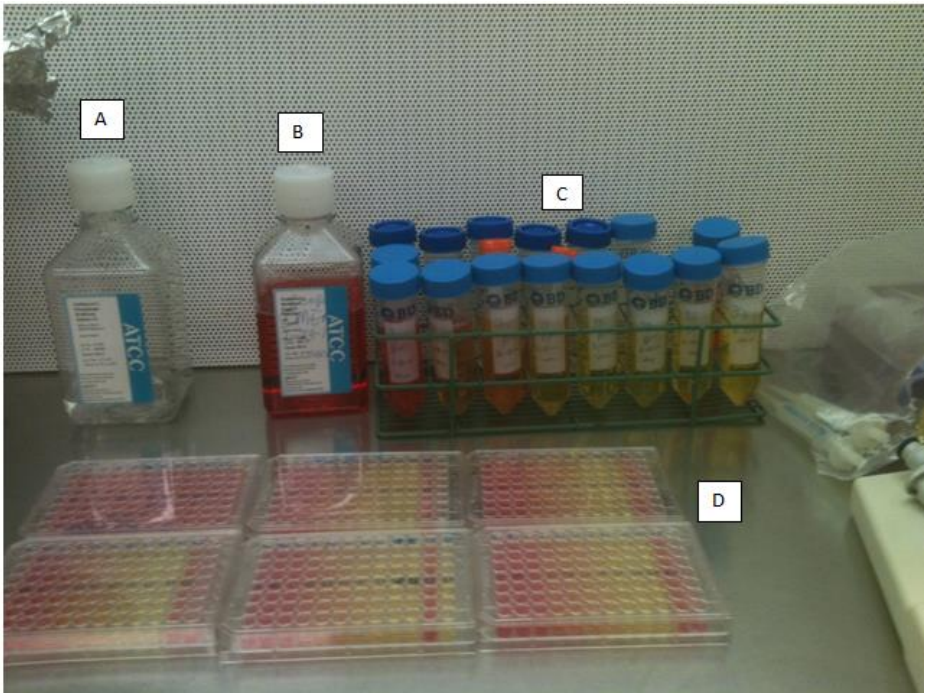

Figure 5: Culture media and arsenic dilutions used for MTT Assay.

A: phosphate buffered saline (PBS), B: culture medium (DMEM), C: different concentrations of ATO diluted with DMEM, 96 well plates set up for MTT Assay

3.3.4. Collagen Coating

Collagen IV is the major constituent of basement membrane and is used in coating cell culture surfaces to establish long-term cultures because it

provides a good physiological coating for the culture of many cell types. Collagen coating was performed using Kleinman's method (Kleinman 2001).

Protocol:

- Stock solution was thawed slowly on ice
- Culture plates and pipette tips were pre-chilled on ice for 20 minutes
- Collagen IV stock solution was diluted with pre-chilled PBS 1:30

  Concentration in 1ml stock solution of collagen IV = 0.3 mg ≡ 300 µg/ml

  Required concentration = 30 µg/ml

  Want/Have = 30/300 = 0.1

  Therefore 0.1 ml collagen IV + 0.9 ml PBS = 30 µg/ml
- 75 µl of Collagen IV (30 µg/ml) was transferred to pre-chilled wells of 96 well tissue culture plates
- The solution was evenly spread to completely cover the flat-bottom of the wells. The plates were incubated at 37°C for 2 hours and washed 3 times with sterile phosphate buffered saline.

3.3.5 MTT Assay 2

Eight different 96 well plates were used for the different time-points; 4 different 96 well plates labelled day 2+AS, day 5+AS, day 8+AS and day 14+AS for the chronic cells treatment group and additional 4 different 96 well plates labelled day 2-AS, day 5-AS, day 8-AS and day 14 -AS for the acute cells, cells

without previous arsenic exposure, (+AS = With Arsenic and –AS = Without previous arsenic exposure).

Column one of the pre-collagen coated 96 well plates was used for media alone, column two for media and cells, (cells grown in culture medium without arsenic which served as passage-matched control group), columns three to twelve for the various concentrations of ATO. Each of the concentrations was performed in triplicates. A total of 200 µL complete media only was added to the first column, then 100 µL of HaCaT cell suspension in complete media containing approximately 20,000 cells/well were added to columns two to twelve wells of the pre-collagen coated 96 well plates, 100 µL of media was added to column two and 100 µL of ATO concentrations were added to columns three to twelve ensuring that the final concentrations were 0, 1.0, 5.0, 7.5, 10, 15 µg/ml respectively. They were incubated in a humidified incubator at 37 °C in a 5% CO_2 for a period of 2 days, 5 days, 8 days and 15 days. Arsenic has a very short half-life of approximately 5 days, (Tokar et al. 2011) consequently, media and arsenic were replaced every 4 days to maintain arsenic concentration level and nutrient for the cells thus removing any confounding factor that may come from depleted nutrients and cell metabolism wastes. At each of the time points, the culture plate was removed from the incubator and 10 µl of the yellow MTT (3-(4, 5 diphenyltetrazolium bromide, a tetrazole) reagent was added to each of the wells and incubated at 37 °C for four hours until purple formazan precipitate was visible. 100µl of the detergent reagent was added to each of the wells and kept in the dark at room temperature for 2 hours. The absorbance was measured at 570 nm wave

lengths using a microtiter plate reader (Fluoroskan II microplate reader Helsinki, Finland). LD_{10} and LD_{50} were determined from dose response curve. The experiment was repeated using chronic HaCaT cells (HaCaT that have been exposed to low dose 0.5 µg/ml ATO up to 10 passages).

3.3.6 Single Gel Electrophoresis (Comet Assay)

Comet Assay is a simple and effective way of evaluating DNA damage in cells. Denatured DNA fragments migrate out of the cell under the influence of an electric field whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. The evaluation of the DNA “comet” tail shape and migration pattern is used to assess the level of DNA damage when visualized using an epiflourescence microscope. Experiment was done 4 replicates for 3 conditions; Unexposed HaCaT (Control) HaCaT exposed to 0.5µg/ml As2O3, passage 1, HaCaT exposed to 0.5µg/ml As2O3, passage 22 (chronic exposure).

The experiment was performed using a Comet assay kit (Trevigen Inc., Gaithersburg, MD). The manufacturer’s protocol was followed to conduct the experiment. About 1.5 $\times 10^5$ HaCaT cells were cultured in 7.5ml of complete DMEM in T-25 culture flask and incubated in humidified atmosphere with 5% CO_2 at 37 °C. After growing to 80% confluence the DMEM was aspirated and replaced with different concentrations of ATO diluted in complete DMEM (0, 1, 5, 7.5, 10, and 15 µg/ml) and incubated for 48 hours. The untreated HaCaT Cells (0 µg/ml) served as the DNA damage control. After ATO treatments, the

medium was removed, and the cells were washed three times with pre-chilled PBS and trypsinised with 1 mL of 0.25% trypsin-EDTA, and the cells were transferred to a centrifuge tube and were counted. The cells were centrifuged at 3000 rpm for 5 min and washed ones in ice cold PBS. The pellet was re-suspended in ice cold PBS at 1 × 10^5 cells/ml. The cells were mixed with molten LMAgarose (at 37 ºC) at a ratio of 1:10 (v/v), and 50 µL was immediately pipetted onto pre-coated CometSlide™. The slides were placed flat in a refrigerator at 4 °C in the dark for 30 minutes. The slides were immersed in a pre-chilled lysis solution and left on ice for 60 minutes. Excess buffers were drained from the slides and then immersed in a freshly prepared alkaline solution, pH > 13 (0.6 g of NaOH pellets, 250 µL of 200 mM EDTA and 49.75 mL of dH2O) for 60 minutes at room temperature. Slides were washed twice for 5 minutes with 1X TBE (Tris-Borate-EDTA neutral buffer) electrophoresis buffer. The slides were aligned equidistant from electrodes in a horizontal gel apparatus and 1X TBE was added and electrophoresed at 1 V/cm (22 V) for 10 minutes. Excess TBE was tapped out and the slides were immersed twice in distilled water for 10 minutes and in 70% ethanol for 5 minutes. Excess 70% ethanol was removed and the slides were air dried overnight and stained with 100 µL of diluted SYBR Green and were placed in the refrigerator for 5 minutes, removed, and tapped to remove SYBR Green. Slides were allowed to air dry at room temperature in the dark. The slides were examined using an Olympus Epifluorescence Microscope and the LAI's Automated Comet Assay Analysis System (LACAAS) (Loates Associates, Inc. Westminster, MD) was used to determine the extent of DNA damage by measuring the percent DNA in the Tail (the integrated tail intensity x 100 / the

total integrated cell intensity for a normalized measure of the percent of total cell DNA found in the tail), (Woodgatte 2008) comet moment (% DNA in tail X tail length) and Tail Length (the distance of DNA migration from the body of the nuclear core). Loats System is an automated comet assay analysis system which enables objective quantitative studies of DNA damage, protection and repair and studies of apoptosis inducing mechanisms and agents. The advantages of using the Loats System include increase in analytic capacity, high turnaround time, improved data accuracy, reliability and traceability. It is equipped with fluorescent microscope with computer controlled servo-driven stage and focus and a high performance computer controlled thermo-electrically cooled digital camera. And also, a robotic vacuum slide handling subsystem and windows based computer. The fully automated system version makes it possible to process unattended batch of multiple slides in a single run. Also, its proprietary extended dynamic range image acquisition feature avoids problems of camera saturation and thus providing more accurate quantitative data on cellular DNA distribution. Further, it has a locally adaptive background correction feature which eliminates detrimental effects of spatially varying non-specific fluorescence. The Loat System used in this study has a sophisticated image processing algorithms which provide automated delineation of comet head and tail and objective quantitative measures over full range of DNA damage. The Loats System is able to put all the comet images and derived measures into a database for subsequent review and statistical analysis. And it provides statistical summary reports immediately after analysis of slides. Seventy comets were randomly selected from each slide for statistical analysis and photographs were taken to show the changes

in DNA morphology due to exposure to ATO. Experiments were conducted in triplicates.

3.4 Gene Expression studies in HaCaT Keratinocytes Chronically Exposed to ATO

3.4.1 The experimental design:

Two experimental groups;

1. The treatment group: HaCaT Cell treated with 0.5 µg/ml of ATO sub-cultured up to passage 22 to establish a chronic exposure state.
2. The passage control group: untreated HaCaT cell that were also sub-cultured up to 22 passages but with no exposure to ATO.

There were 4 technical replicates with 3 replicates making a total of 8 ×3=24 samples.

3.4.2 Treatment Dose Determination:

The LD10 dose result obtained from the initial cytoxicity assay (Figure 6) was arbitrarily divided by 1/2 to get 0.5 µg/ml of ATO that was used for chronic dosage treatment.

Working Solution Preparation:

Stock solution: 1000 µg/ml (1000 µg/ml) ATO

Working solution: 0.5 µg/ml (0.5 µg/ml) ATO

To make 10 ml of 0.5 µg/ml from 1000 µg/ml

Concentration = Want/Have x Final volume

0.5 µg/ml /1000 µg/ml x 10,000uL = 5uL

1.5 $\times 10^5$ HaCaT cells were cultured in 7.5 ml of complete DMEM containing 10% Foetal Bovine Serum (FBS) and 1% penicillin, streptomycin in T-25 culture plate. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. The treatment groups were exposed to 0.5µg/mL ATO (equivalent to LC 0.5), and passaged at 90% confluent. To establish a chronic exposure the treatment groups were sub-cultured up to passage 22.

3.4.3 RNA Extraction and Gene Expression

Total RNA was extracted from 4 technical replicates of unexposed HaCaT cells and HaCaT cells chronically exposed to ATO up to passage 22 using RNA STAT-60 (TEL-TEST, INC, Friendswood, TX, USA) (Chomczynski et al. 1987). A NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE) was used to quantify the RNA by optical density reading. Also, the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used to determine the purity and quality of the extracted RNA. Only high quality RNA, having a RNA Integrity Number *(*RIN*)* of >7.0, (Schroeder et al. 2006) and an A260/280 absorbance ratio of >1.8, was utilized for microarray experiments.

3.4.4 Microarray Analysis

3.4.4.1 Microarray Platform:

The Human Whole Genome OneArray™ (Phalanx Biotech, Palo Alto, CA) platform (GEO Accession number GPL6254G) was used to perform DNA microarray analysis. The slide list as used for the experiment is illustrated in Table 5.

3.4.4.2 Label Protocol:

RNA was converted to double-stranded cDNA and amplified using in vitro transcription systems that included amino-allyl UTP, and the aRNA product was subsequently conjugated with Cy5™ NHS ester (GEH Life sciences, Pittsburgh, PA).

3.4.4.3 Hybridization Protocol:

Fragmented aRNA was hybridized at 42 °C overnight using the HybBag mixing system with 1X OneArray Hybridization Buffer (Phalanx Biotech, Palo Alto, CA), 0.01 mg/ml sheared salmon sperm DNA (Promega, Madison, WI, USA), at a concentration of 0.025 mg/ml labelled target. After hybridization, the arrays were washed according to the OneArray protocol.

3.4.4.4 Scan Protocol:

Raw intensity signals for each microarray were captured using a Molecular Dynamics™ Axon 4100A scanner, measured using GenePixPro™ Software, and stored in GenePix Results (GPR) format.

3.4.4.5 Data Processing - Normalization:

The data from all microarrays in each experimental set was then passed to Rosetta Resolver (Rosetta Biosoftware, Seattle, WA) for analysis and it computed the normalised signal intensity. Pearson correlation tables (R values) for each technical repeats were generated. R values were calculated from raw log2 intensity (R) and normalized $\log_2$ intensity (N) values for each dataset and compared to each other. Only probes with P value (detected) less than 0.05 were included in the calculation. Individual pairwise comparisons were also performed to ascertain the level of consistency and to identify the top altered genes for each major comparison.

Table 5: Microarray Experiment; ATO treated vs. Untreated HaCaT cells Slide List

Sample	Sample_Label	Chip_Barcode	Chip_Label	Note
HaCaT Cell untreated	UT	H001-0606026753	H1_H001	H1
HaCaT Cell untreated	UT	H002-0606026754	H1_H002	H1
HaCaT Cell untreated	UT	H003-0606026743	H1_H003	H1
HaCaT Cell untreated	UT	H004-0606026110	H2_H004	H2
HaCaT Cell untreated	UT	H005-0606026112	H2_H005	H2
HaCaT Cell untreated	UT	H006-0606026113	H2_H006	H2
HaCaT Cell untreated	UT	H007-0606026747	H3_H007	H3
HaCaT Cell untreated	UT	H008-0606026748	H3_H008	H3
HaCaT Cell untreated	UT	H009-0606026749	H3_H009	H3
HaCaT Cell untreated	UT	H010-0606026764	H4_H010	H4
HaCaT Cell untreated	UT	H011-0606026765	H4_H011	H4
HaCaT Cell untreated	UT	H012-0606026766	H4_H012	H4
HaCaT Cell treated	T	H013-0606026767	A1_H013	A1
HaCaT Cell treated	T	H014-0606026768	A1_H014	A1
HaCaT Cell treated	T	H015-0606026769	A1_H015	A1
HaCaT Cell treated	T	H016-0606026124	A2_H016	A2
HaCaT Cell treated	T	H017-0606026125	A2_H017	A2
HaCaT Cell treated	T	H018-0606026126	A2_H018	A2
HaCaT Cell treated	T	H019-0606026750	A3_H019	A3
HaCaT Cell treated	T	H020-0606026770	A3_H020	A3
HaCaT Cell treated	T	H021-0606026759	A3_H021	A3
HaCaT Cell treated	T	H022-0606026751	A4_H022	A4
HaCaT Cell treated	T	H022-0606026757	A4_H022.1	A4
HaCaT Cell treated	T	H023-0606026752	A4_H023	A4

Where UT = Untreated; T= Treated, HaCaT = HaCaT Cell; A= HaCaT with Arsenic

3.4.5 Two-step quantitative qRT-PCR

Relative quantitation using the comparative C_T method (Livak et al. 2001) was employed to confirm the microarray gene expression data. Sample H1 (untreated HaCaT cell) was used as calibrator and Beta glucuronidase (GUSB) as endogenous control gene for normalization. Applied Biosystems (Applied Biosystems, Carlsbad, CA) standard protocol was followed. The RNA samples were reverse-transcribed for 120 min at 37 °C with High Capacity cDNA Reverse Transcription Kit. Quantitative PCR was carried out for 10 min at 95 °C, and 40 cycles of 15 sec at 95 °C, 1 min at 60 °C using 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and 200 nM of forward and reverse primers. The primers used and the quality control results are listed in the Appendix on page 127. Triplicates of each assay were run on an Applied Biosystems 7300 Real-Time PCR system and expression fold-changes were derived using the comparative threshold (C_T) method. Each replicate cycle threshold (C_T) was normalized to the average C_T of GUSB on a per sample basis. Applied Biosystems formula was used to calculate the relative amount of the transcripts in the arsenic treated HaCaT and the untreated sample H1 (control), and both were normalized to the endogenous control (GUSB): $\Delta\Delta C_T = \Delta C_T$ (treated) - ΔC_T (control), where ΔC_T is the difference in C_T between the target gene and endogenous controls by subtracting the average C_T of controls. The fold-change for each treated sample relative to the control sample equals $2^{-\Delta\Delta C}{}_T$.

3.5 Statistical Analysis

The absorbance values obtained per treatment from the MTT assay were converted to percentages of cell viability. Statistical analysis for differences in mean levels of ATO was done using the Student's t-test for comparing two sample sets at $p < 0.05$. The data generated from the Comet assay were analysed by the Student's t-test by comparing the means of the treated and untreated control HaCaT cells, and the significance level was $p < 0.05$.

3.6 Bioinformatics analyses

The following bioinformatics tools and data bases where used in this study;

3.6.1 Visual Analysis Method

Tools available in the Tableau Public Software (http://www.tableausoftware.com/public) were used to construct interactive views of the cytotoxicity and genotoxicity data produced from the experimental results.

3.6.2 Biological Pathway Modelling

Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Redwood City, CA) was used to determine models of biological pathways and networks that were significantly represented in the differentially expressed (both up and down regulated) genes.

IPA identifies networks and pathways represented in the gene lists of interest. It generates a P-value for each network and canonical pathway, which is the likelihood that a given network was identified by chance. Networks scoring ≥ 2, which have > 99% confidence of not being generated by chance, were selected (Gerling et al. 2006).

IPA assigns biological functions to each network by using annotations from scientific literature and stored in their knowledge base. Fisher exact test was used to calculate the P-value for each biological function/disease or pathway being assigned by chance. Also, Benjamini-Hochberg corrected P ≤ 0.05 was used to select highly significant biological functions and pathways represented in the datasets analysed (Gerling et al. 2006).

3.6.3 Prediction of cysteine state and disulphide bond partners

The cysteine state and disulphide bond partners in protein sequences were predicted using DiANNA (Ferre et al. 2005;Ferre et al. 2006), DBCP (Lin et al. 2010) and DISULFIND (Ceroni et al. 2006). DiANNA is a unified software for Cysteine state and Disulphide Bond partner prediction available at http://clavius.bc.edu/~clotelab/DiANNA/. The output include the following; positions of cysteine, positions of oxidized cysteine, predicted disulphide bonds, and predicted positions of cysteine in metal binding site (Ferre et al. 2006). The Disulphide Bonding Connectivity Pattern (DBCP) provides prediction without the prior knowledge of the bonding state of cysteine. http://140.120.14.136/dbcp/ (Lin et al. 2010). The output of the DISULFIND

server (http://disulfind.dsi.unifi.it/) is a simple visualization of the assigned bonding state (with confidence degrees) and the most likely connectivity patterns (Ceroni et al. 2006).

3.6.4 Prediction of Biological Networks

The Michigan Molecular Interactions (MiMI) plugin for Cytoscape (Gao et al. 2009) was used to retrieve molecular interactions and interaction attributes in the Michigan Molecular Interactions (MiMI) for query genes and their nearest neighbours. The Cytoscape platform was used to display the biological networks with the biological entities (genes/proteins) represented as nodes and the biological interactions represented as edges between nodes (Cline et al. 2007;Smoot et al. 2011;Tang et al. 2012).

Chapter 4

Results

4.1 Cytotoxicity Assay

Cytotoxicity MTT assay showed that acute exposure of HaCaT cells to arsenic had LD10 and LD50 values of 1 µg/ml and 10 µg/ml respectively (Figure 6). The LD10 dose result obtained from the initial cytotoxicity assay (Figure 6) was arbitrarily divided by 1/2 to get 0.5 µg/ml of Arsenic that was used for chronic dosage treatment.

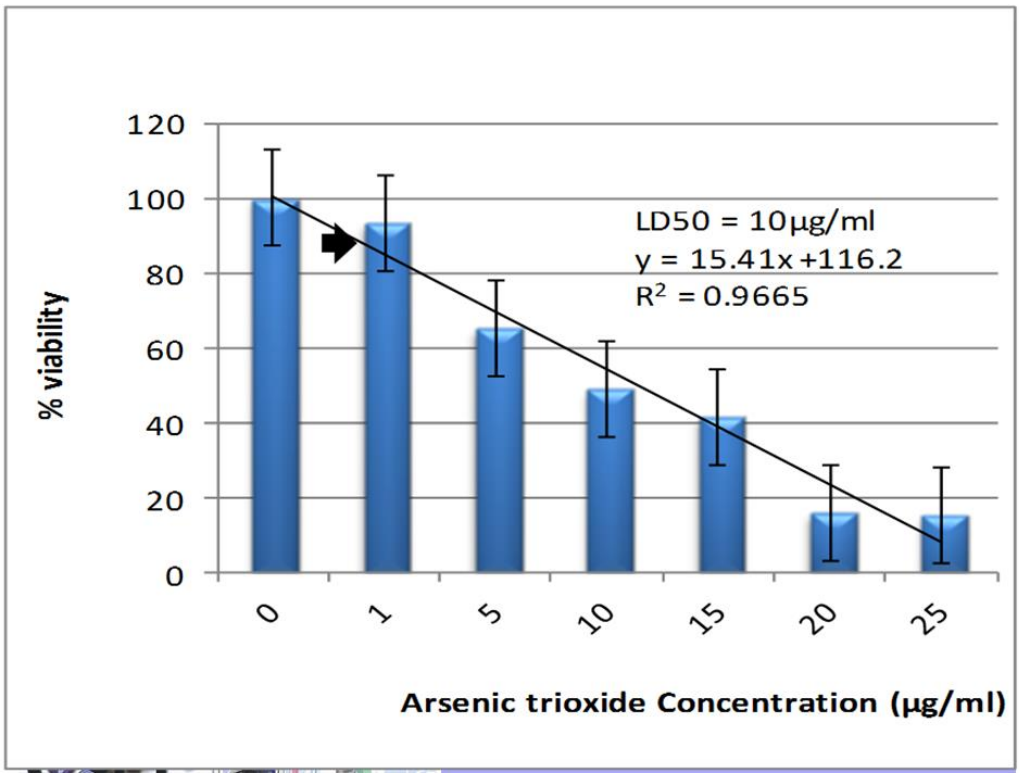

Figure 6: Cytotoxicity Assay (MTT) of HaCaT cell line showing LD50

Acute exposure of HaCaT cells to varying concentrations of ATO to determine the lowest tolerable concentration to be used for chronic exposure studies.

4.2 Cytotoxicity and Chronic Toxicity Assay

The MTT Assay II was used to evaluate cytotoxicity and chronic toxicity patterns of ATO on HaCaT keratinocytes. Long-term cultures were established by culturing HaCaT cells on collagen IV. The LD_{10} value of 1µg/ml obtained from the MTT I assay was arbitrarily divided in half to get 0.5 µg/ml dose used to establish the chronic HaCaT cells. HaCaT cells exposed to ATO up to 8 passages were considered chronic.

4.2.1 Acute Exposure

Three visual analytics representations of the cell viability data are presented in Table 6, Figure 7 and Figure 8. Time course cell viability pattern, showing acute vs. chronic exposure to ATO is illustrated in Figure 7. Chronic HaCaT cells were more tolerant to the 5 µg/ml dose compared to the acute HaCaT cells. A low dose of 1 µg/ml was observed to improve cell viability while high doses above 5 µg/ml were toxic to both chronic and acute HaCaT cells. Figure 7 presents a time course for each dose while Figure 8 presents a view comparing the exposure types for each time point and concentration. In addition, Figure 9 and Figure 10 present the cytotoxicity data as a bar chart with error bars showed. Overall in both acute and chronic exposure, the HaCaT cell viability was dose dependent. Thus an increase in dose resulted in decreased cell viability. The 1 µg/ml dose in the acute treatment cells resulted in 92.4% viability on Day 2 and cells continued to grow in the time course with viability rates of 93.4%, 107% and 115.9% for Day 5, Day 8 and Day 14 respectively (Figures 5, 6, and 7). The 5 µg/ml concentration resulted in viability rates of 30.8%, 36%, 46.7% and 32.7% for the time points.

Table 6: HaCaT/Arsenic Viability Table showing Chronic vs. Acute Exposure

Day	Concentration	Exposure	
		Acute (%)	Chronic (%)
Day 2	0 µg/ml	100	100
	1 µg/ml	92.4	92.1
	5 µg/ml	30.8	42.9
	7.5 µg/ml	23.8	27.1
	10 µg/ml	17.1	20.4
	15 µg/ml	13.4	14.7
Day 5	0 µg/ml	100	100
	1 µg/ml	93.4	98.5
	5 µg/ml	36.0	45.9
	7.5 µg/ml	23.3	30.2
	10 µg/ml	17.3	25.2
	15 µg/ml	11.8	17.3
Day 8	0 µg/ml	100	100
	1 µg/ml	107.0	114.5
	5 µg/ml	46.7	67.1
	7.5 µg/ml	15.6	20.3
	10 µg/ml	10.0	10.8
	15 µg/ml	6.3	7.2
Day 14	0 µg/ml	100	100
	1 µg/ml	115.9	120.0
	5 µg/ml	32.7	56.7
	7.5 µg/ml	15.6	20.9
	10 µg/ml	8.0	10.1
	15 µg/ml	5.8	7.4

Notes: Data in table can be downloaded at: http://public.tableausoftware.com/views/hacat_arsenic_3/viability_table

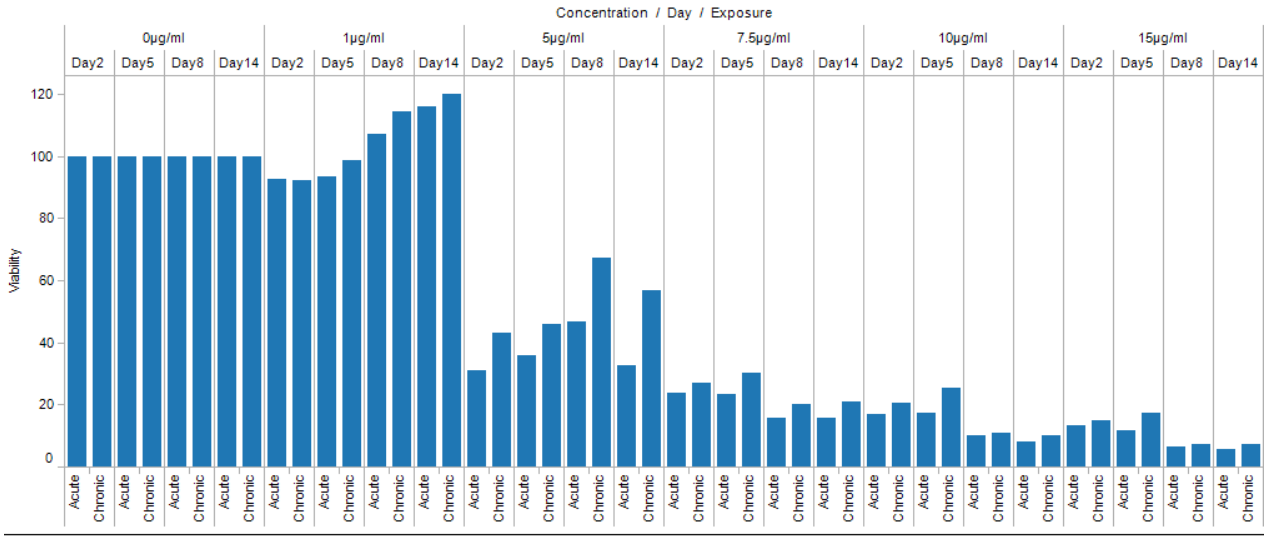

Figure 7: Dose and time dependent response to the cytotoxic effect of ATO on HaCaT keratinocytes.

The 1 µg/ml dose encouraged growth in both acute and chronic cells while higher doses caused cell death.

Interactive visual analytics resource for image available at http://public.tableausoftware.com/views/hacat_arsenic_3/viability_timecourse

4.2.2 Chronic Exposure

Treatment doses of 7.5 µg/ml, 10 µg/ml and 15 µg/ml inhibited growth of chronic cells (Figure 8). In the chronic treatment cells, the 1 µg/ml dose gave viability rates of 92.1%, 98.5%, 114.5% and 120% for days 2, 5, 8, and 14 respectively, while the 5 µg/ml gave viability rates of 42.9%, 45.9%, 67.1% and 56.7% for days 2, 5, 8, and 14 respectively (Figures 7, 8, and 10). The chronic cell had higher viability values that could resist the cytotoxic effect of the high doses of 7.5µg/ml, 10µg/ml and 15µg/ml. There was no significant difference at $p < 0.05$ when the chronic HaCaT cells were compared with the acute HaCaT cells at the various time points except for 5 µg/ml dose using

student's t-test. Additionally, there was no significant difference when the controls were compared with the 1 µg/ml dose in both acute and chronic cells at $p < 0.05$. However, there are significant differences at $p < 0.05$ on Day 2 between the controls and other treatment groups; 5µg/ml, 7.5µg/ml, 10µg/ml and 15µg/ml in both acute and chronic cells.

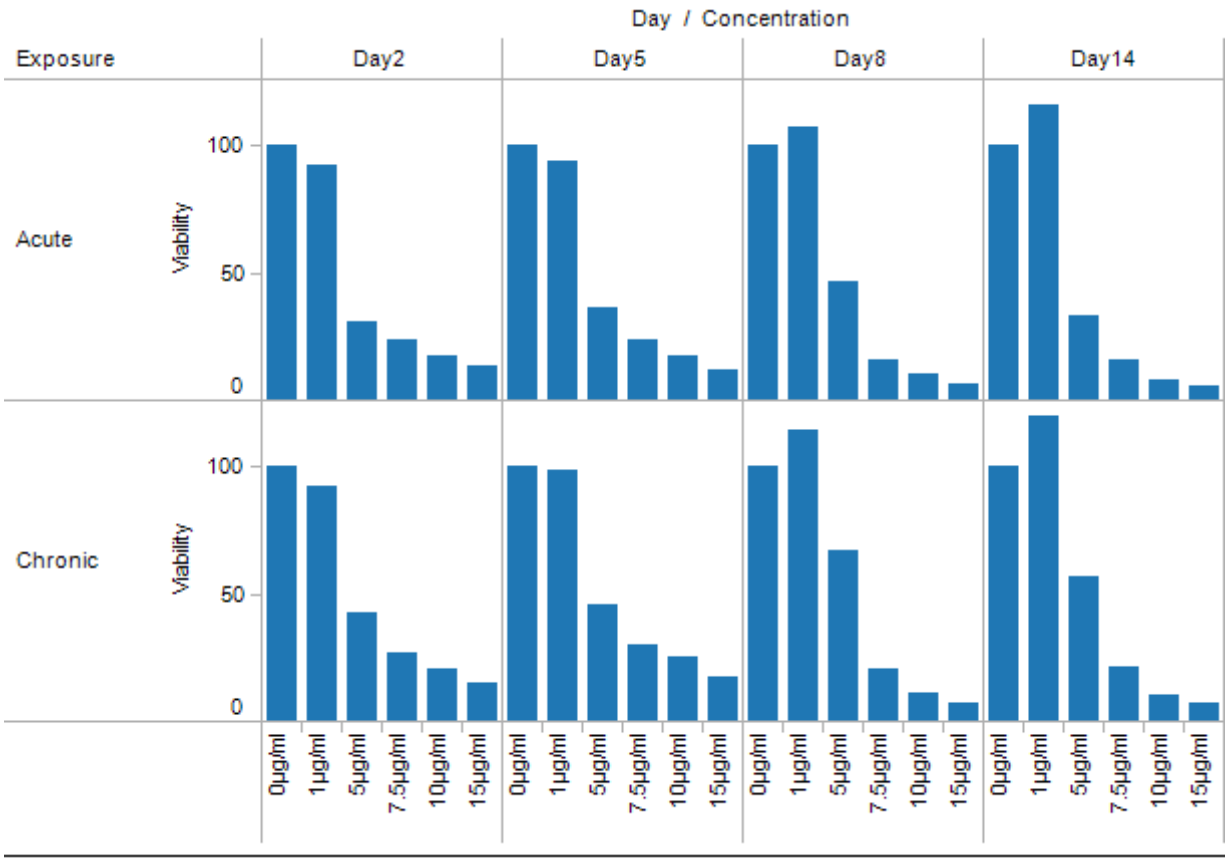

Figure 8: Time course cell viability pattern, showing acute vs. chronic exposure to ATO. Previous exposures appear to confer some level of protection to chronic cells than acute cells.

Interactive visual analytics resource for image available at http://public.tableausoftware.com/views/hacat_arsenic_3/viability_figure

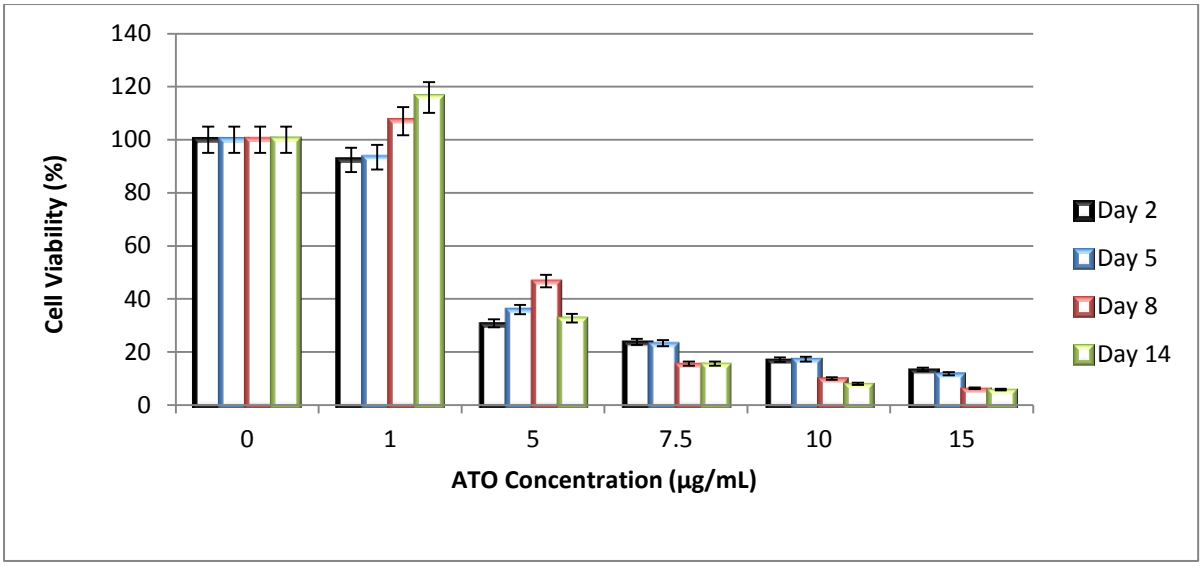

Figure 9: Dose and time dependent response of HaCaT keratinocytes without prior exposure to low dose (0.5 µg/ml) of ATO over 10 passages (static image with error bars). The 1.0 µg/m dose appears to stimulate cell growth while higher concentrations caused cell death

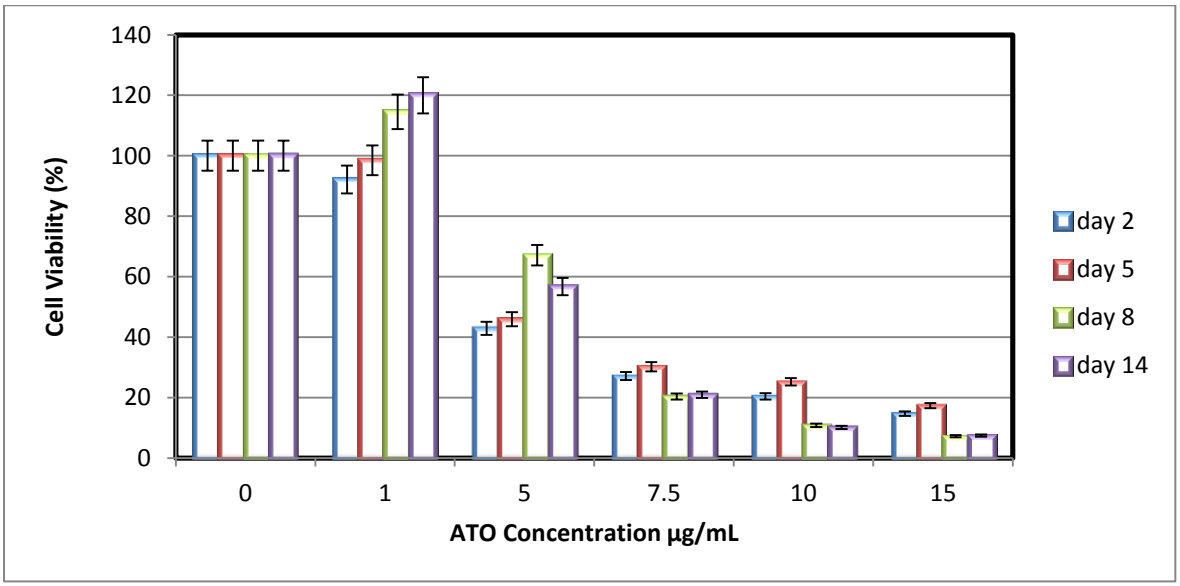

Figure 10: Dose and time dependent response of HaCaT keratinocytes with prior exposure to low dose (0.5 µg/ml) of ATO over 10 passages (static image with error bars).

4.3 DNA Damage Assay (Comet Assay)

Comet assay was performed in HaCaT cells exposed to different concentrations of ATO to determine the dose that has the highest genotoxic effect. Comet assay was done using TBE, a neutral buffer which produces elongated comet shape, stained with SYBR® Green and images generated and measured automatically on Loats System. The parameters measured in the 70 comets randomly selected per sample were the moment, comet tail length and DNA damage. There was an increase in the length and rate of DNA migration in the HaCaT cells treated with ATO when compared with the untreated control HaCaT cell. The comet assay result shows a dose dependent response to ATO exposure as shown in Figure 11. The % DNA damage results were 0.27, 3.45, 3.22, 4.0, 7.5 and 11 for the 0 µg/ml, 1 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml and 15 µg/ml respectively. There was a significant difference in DNA damage between the control and the treatment doses at ($p<0.05$) using F-Test two-sample for variance. A similar pattern was observed for comet moment and comet tail length results. Images representing the comets observed are shown in Figure 12 and values in Table 7**.** In Figure 12, Panel A represents a comet from the untreated cell control; Moment 0.0, %DNA 0.08, Length 0.0. Panel B represents 1 µg/ml; Moment 0.04, %DNA 2.1, Length 5, and Panel C; 15 µg/ml; Moment 8.18, %DNA 28.62, Length 73. A remarkable increase in the length of DNA migration and the rate of DNA migration of the HaCaT cells were observed in the chronically exposed passage 22 HaCaT dose (0.5µg/ml) ($p<0.05$). There was no significant difference ($p>0.05$) between the passage 1 and the untreated HaCaT cell (control). This was analysed using general linear model (GLM)

procedure of SAS (9.1v). Thus, long term exposure of low dose, 0.5 µg/ml ATO on HaCaT cell line is genotoxic ($p<0.05$).

Table 7: Summary of Comet Assay data showing percentage DNA damage of HaCaT cells exposed to ATO*

Concentration	% DNA	Length	Comet Moment
0.0 µg/ml	0.27	2.00	0.26
1.0 µg/ml	3.45	3.00	0.97
5.0 µg/ml	3.22	4.50	1.03
7.5 µg/ml	4.00	4.50	5.00
10.0 µg/ml	7.50	6.50	2.34
15.0 µg/ml	11.0	10.00	9.00

Notes: Data in table can be downloaded at: http://public.tableausoftware.com/views/hacat_arsenic_3/comet_table

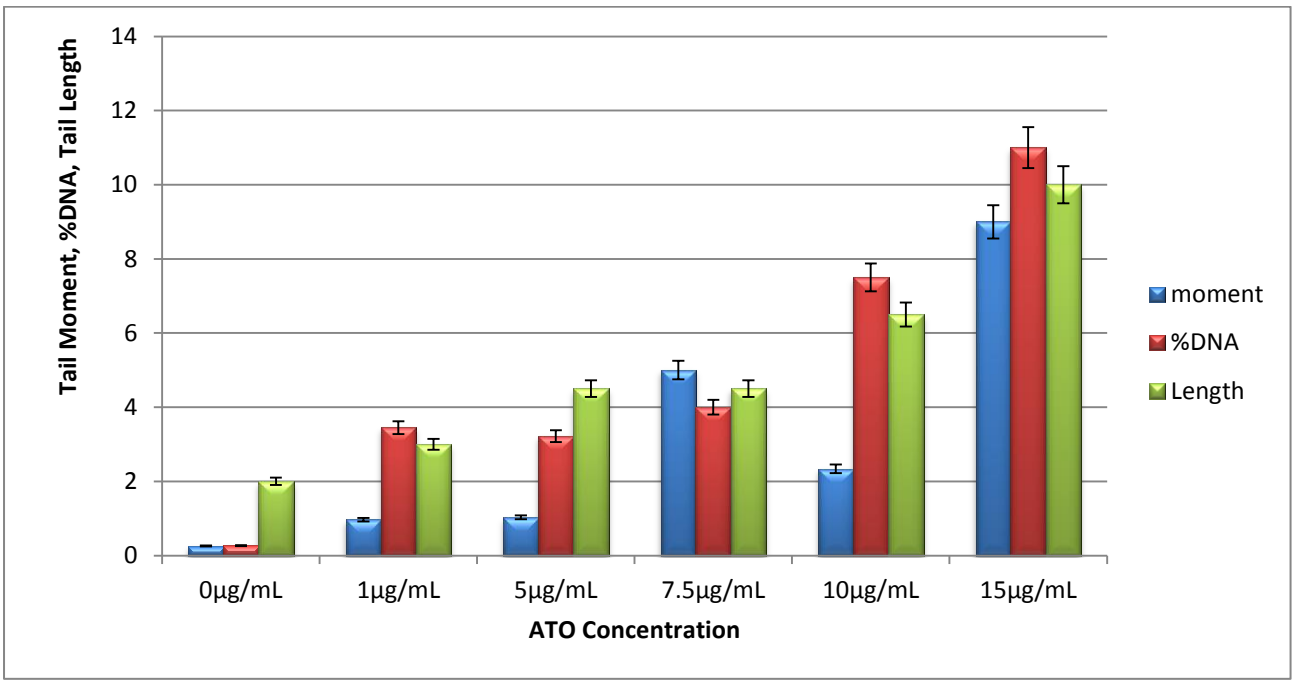

Figure 11: Genotoxic effect of ATO on HaCaT keratinocytes with Comet assay (static image with error bars). The higher the concentration of arsenic, the higher the DNA damage on HaCaT cells.

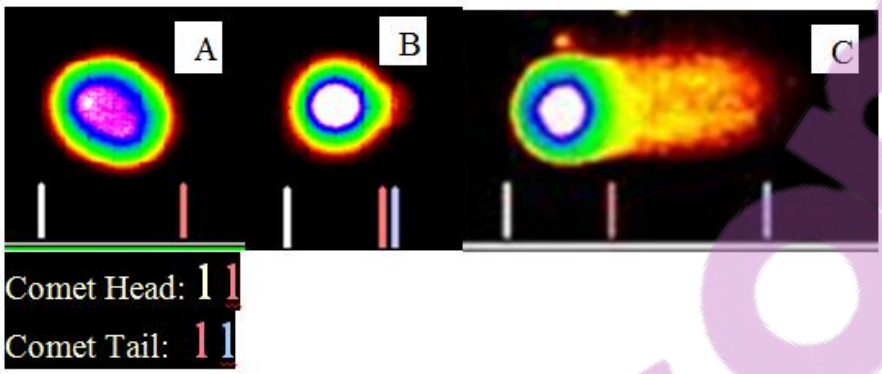

Figure 12: Representative comets from exposure of HaCaT keratinocytes to concentrations of ATO

4.4 Gene Expression of HaCaT keratinocytes

4.4.1 RNA Quality Control Results

The RNA quality control were obtained using Agilent 2100 Bioanalyzer and the Agilent 2200 Tape Station and the result analyses were displayed in form of tables, electropherograms and gel images. As shown in Table 8, total RNA per sample ranges from 35.2 µg to 90.9 µg and each of them exceeded the 6 µg to 12 µg requirements for aRNA preparation method and 50 µg for cDNA preparation method. The optical density of the total RNA was measured by NanoDrop ND-1000. The RNA purity was estimated by taking the ratio of absorbance at 260 nm and 280 nm and ratios between 1.8 and 2.2 indicate a pure sample. Also, as shown in Table 9, all the samples have 260 nm/280 nm ratios of 1.90 to 2.01. RNA Integrity Number (RIN) algorithm assigns a 1 to 10 RIN score, where level 10 RNA is completely intact. The RIN should be above 7 to get a reliable and acceptable microarray result. All the samples as shown in Table 8 have RIN of 10. RIN score was given by Agilent RNA 6000 Nano

Assay. To get a good and reliable microarray result, the labelling efficiency must be above 10. As demonstrated in Table 9, all the samples labelling efficiency values are above 10. The purified aRNA/cDNA was conjugated with the NHS-CyDye using reactive amino group of 5-(3-aminoallyl)-UTP/5-(3-aminoallyl)-dUTP. And the labelling efficiency was calculated by the concentration of CyDye and aRNA/cDNA measured by NanoDrop ND-1000. Agarose electrophoresis was performed to determine if there serious DNA contamination. Figure 13 shows that there was no DNA contamination in the samples.

Table 8: AminoAllyl aRNA Quality Control Result

Item	Sample Name	OD260/280 1.8	Total Amount (µg) 6 µg	RIN 7.0	QC Result Pass or Fall	Note
1	HaCaT Cell untreated	2.01	90.9	10.0	Pass	H1
2	HaCaT Cell untreated	1.95	71.6	10.0	Pass	H2
3	HaCaT Cell untreated	1.91	46.8	10.0	Pass	H3
4	HaCaT Cell untreated	1.92	35.2	10.0	Pass	H4
5	HaCaT Cell treated	1.92	43.4	10.0	Pass	As1
6	HaCaT Cell treated	1.90	59.4	10.0	Pass	As2
7	HaCaT Cell treated	1.93	6.4	10.0	Pass	As3
8	HaCaT Cell treated	1.94	42.9	10.0	Pass	As4

Table 9: AminoAllyl a RNA and Labelling QC Result

Item	Sample Name	OD260/280 >1.8	Labelling Efficiency Cy5 > 10	Quality Pass or Fail
1	HaCaT Cell untreated	2.03	32.7	Pass
2	HaCaT Cell untreated	2.04	44.6	Pass
3	HaCaT Cell untreated	2.02	36.2	Pass
4	HaCaT Cell untreated	2.01	36.5	Pass
5	HaCaT Cell treated	2.01	32.1	Pass
6	HaCaT Cell treated	1.98	30.7	Pass
7	HaCaT Cell treated	2.04	30.5	Pass
8	HaCaT Cell treated	2.04	30.5	Pass

Note: Labelling Efficiency: # dye molecules/per 1000 nucleotides

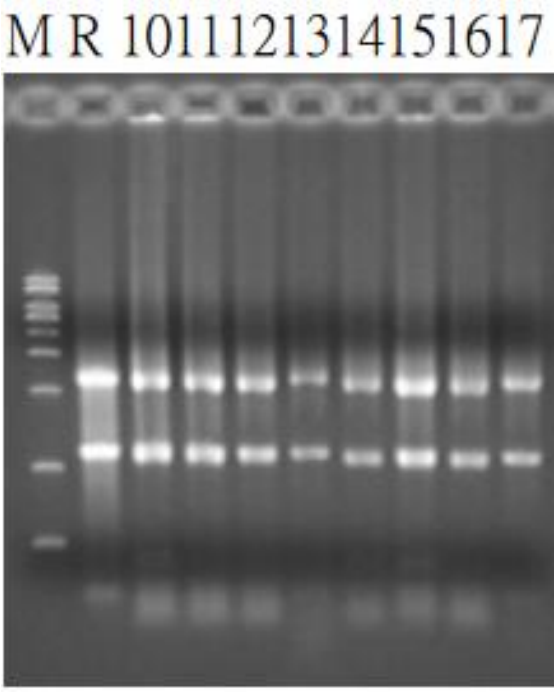

Figure 13: RNA Quality Control Gel

(M=ladder, 10,11,12,13 = Untreated HaCaT Cells; 14, 15, 16, and 17=Arsenic Treated HaCaT Cells)

The Electropherogram of total RNA (Figure 14) shows the ribosomal RNA peaks are at a ratio of about 2 (28S:18S) suggesting that the RNA integrity are or ultrahigh quality.

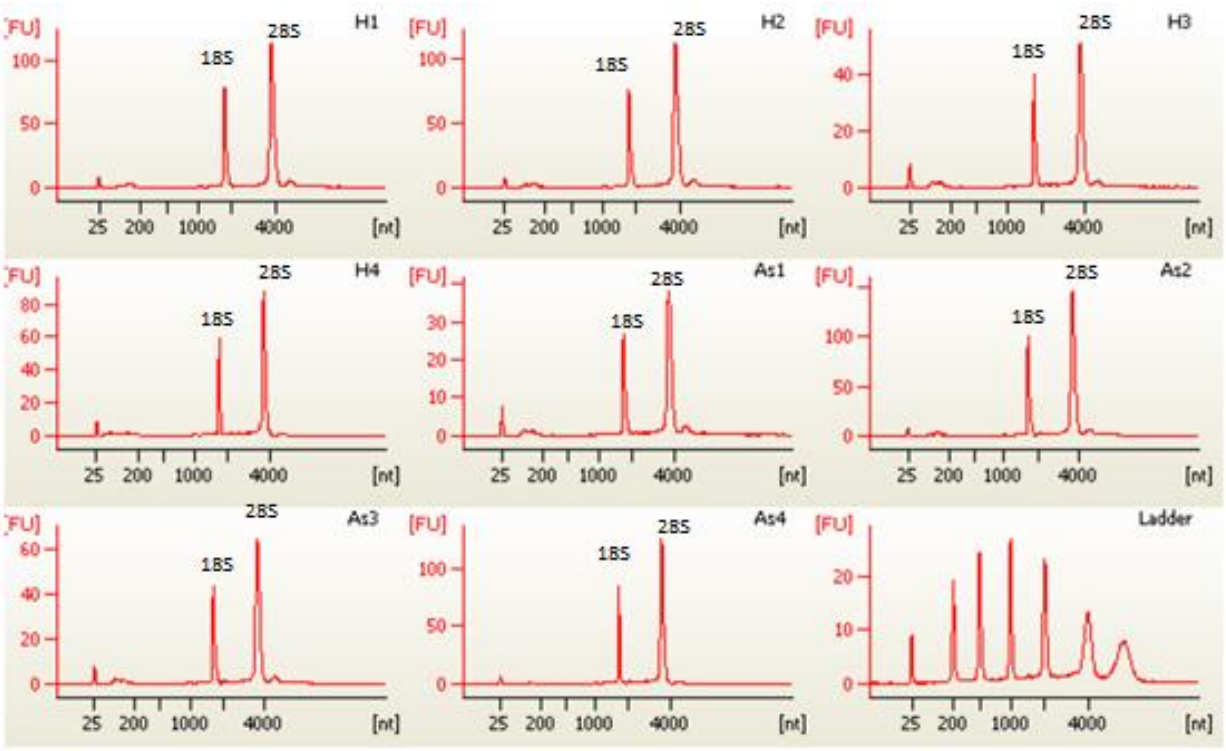

Figure 14: Electropherogram of total RNA of human Skin Keratinocytes (HaCaT) Quality Control Report (Report generated from Bioanalyzer 2100 *Agilent,* USA)

4.4.2 Global Gene Expression

The criterion for selection of differentially expressed genes (DEGs) was a fold change greater than or equal to 2. Comparison of the microarray data from untreated (control) and chronically exposed HaCaT keratinocyte cell identified 35 differentially expressed genes of interest with 14 genes up-regulated (Table 10) and 21 genes down-regulated (Table 11). Genes with ≥ 2 fold changes and P-value ≤ 0.05 were considered significantly expressed and were selected for confirmation using qRT-PCR. The functional annotations including Gene Ontology for these genes were determined using the Michigan Molecular Interactions (MiMI) web tool (Tarcea et al. 2009) (http://mimi.ncibi.org/).

Table 10: Genes up-regulated in response to chronic-dose exposure of ATO to HaCaT keratinocyte cells

Gene Symbol	Gene Description
AGPAT4	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)
AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)
C22orf42	Uncharacterized protein C22orf42
GLT6D1	glycosyltransferase 6 domain containing 1
IGFL1	IGF-like family member 1
IL1R2	interleukin 1 receptor, type II
KLHDC8A	Kelch domain-containing protein 8A
NQO2	NAD(P)H dehydrogenase, quinone 2
PCSK1	proprotein convertase subtilisin/kexin type 1
STRAP	STRAP serine/threonine kinase receptor associated protein
TMEM70	Transmembrane protein 70
TNFSF18	tumor necrosis factor (ligand) superfamily, member 18
ZFP36L1	zinc finger protein 36, C3H type-like

Table 11: Genes down-regulated in response to chronic-dose exposure of ATO to HaCaT keratinocyte cells

Gene Symbol	Gene Description
CCDC150	coiled-coil domain containing 150
CLCC1	Chloride channel CLIC-like 1
CSRP1	Cysteine and glycine-rich protein 1
FGF1	fibroblast growth factor 1 (acidic)
GCNT3	Glucosaminyl (N-acetyl) transferase 3, mucin type
GDA	Guanine deaminase
HOXA5	homeobox A5
IMP5	Signal peptide peptidase-like 2C Precursor (Protein SPP-like 2C)(Protein SPPL2c)(EC 3.4.23.) (Intramembrane protease 5)(IMP5)
MAK	male germ cell-associated kinase
MKNK1	MAP kinase interacting serine/threonine kinase 1
MRC2	mannose receptor, C type 2
NP153	nucleoporin 153kDa
NT5C	5', 3'-nucleotidase, cytosolic
PHF12	PHD finger protein 12
PPP1R13B	Apoptosis-stimulating of p53 protein, protein phosphatase 1, regulatory (inhibitor) subunit 13B
PRKAR1B	protein kinase, cAMP-dependent, regulatory, type I, beta
RASL10A	RAS-like, family 10, member A
RGS2	regulator of G-protein signalling 2, 24kDa
TM4SF4	transmembrane 4 L six family member 4
TOM1	target of myb1 (chicken)
ZNF19	zinc finger protein 19

4.4.3 Quantitative PCR confirmation of microarray data

Relative quantitation using the comparative C_T method was used to confirm the microarray gene expression data. The genes expressed for the RNA of

arsenic treated cells were compared with the reference untreated control sample. The qRT-PCR confirmed the expression of 4 up-regulated genes and 1 down-regulated gene for genes with a fold change of ≥ 2. The up-regulated genes were AKR1C3 (9.2 fold), IGFL1 (3.1), IL1R2 (5.9 fold), and TNFSF18 (167 fold) and down-regulated gene was RGS2 (2.0 fold). A visualization comparing the fold change obtained by microarray and qRT-PCR for 9 genes is presented in Figures 15.

Gene	QPCR (Fold Change)	Microarray (Fold Change)
AKR1C3 Aldo-keto reductase family 1, member C3	9.2	10.4
IGFL1 Insulin growth factor-like family member 1	3.1	4.0
IL1R2 Interleukin 1 receptor, type II	5.9	4.9
PCSK1 Proprotein convertase subtilisin/kexin type 1	-3.1	6.9
PPP1R13B Protein phosphatase 1, regulatory (inhibitor) subunit 13B	-1.4	-100.0
RGS2 Regulator of G-protein signaling 2	-2.0	-9.9
TMEM70 Transmembrane protein 70	0.9	4.0
TNFSF18 Tumor necrosis factor (ligand) superfamily, member 18	166.6	5.9
GUSB Beta glucuronidase	-1.2	-1.3

Figure 15: Comparison of fold change between microarray data and qRT-PCR data. Most of the upregulated genes in the microarray experiment were confirmed by qPCR.

(Red: up-regulated; yellow: unchanged; green: down-regulated).

4.5 Biological pathway analysis

The functional and pathway annotation analysis to identify specific pathways of interest from the microarray gene expression datasets was performed using

Ingenuity Pathway Analysis tool (IPA; Ingenuity Systems, Redwood City, CA). Figure 16 shows the pathways affected by arsenic exposure with the most affected being Liver X Receptor /Retinoid X Receptor (LXR/RXR) pathway which are involved in lipid metabolism and immune response (Christoffolete et al. 2010;Laffitte et al. 2003). IPA was also used to determine models of biological pathways and networks that are significantly represented in the differentially expressed (both up and down regulated) genes. Benjamini-Hochberg corrected $P \leq 0.05$ is used to select highly significant biological functions and pathways represented in the datasets (Borch et al. 1992). Gene functional pathways and interactions for IL1R2, TNFSF18 and AKR1C3 were generated using Ingenuity Pathway Analysis tool as presented in Figure 17, Figure 18 and Figure 19 respectively. These genes were up-regulated in response to chronic-dose exposure of ATO to HaCaT cells as documented in Table 10. Molecules in red are up-regulated while molecules in green are down-regulated in the microarray data. Figure 17 network suggests molecules that could be involved in ILR2 expression under chronic arsenic exposure. There is an interaction between IL1A, IL1B, IL1RAP and GLI1 with IL1R2.

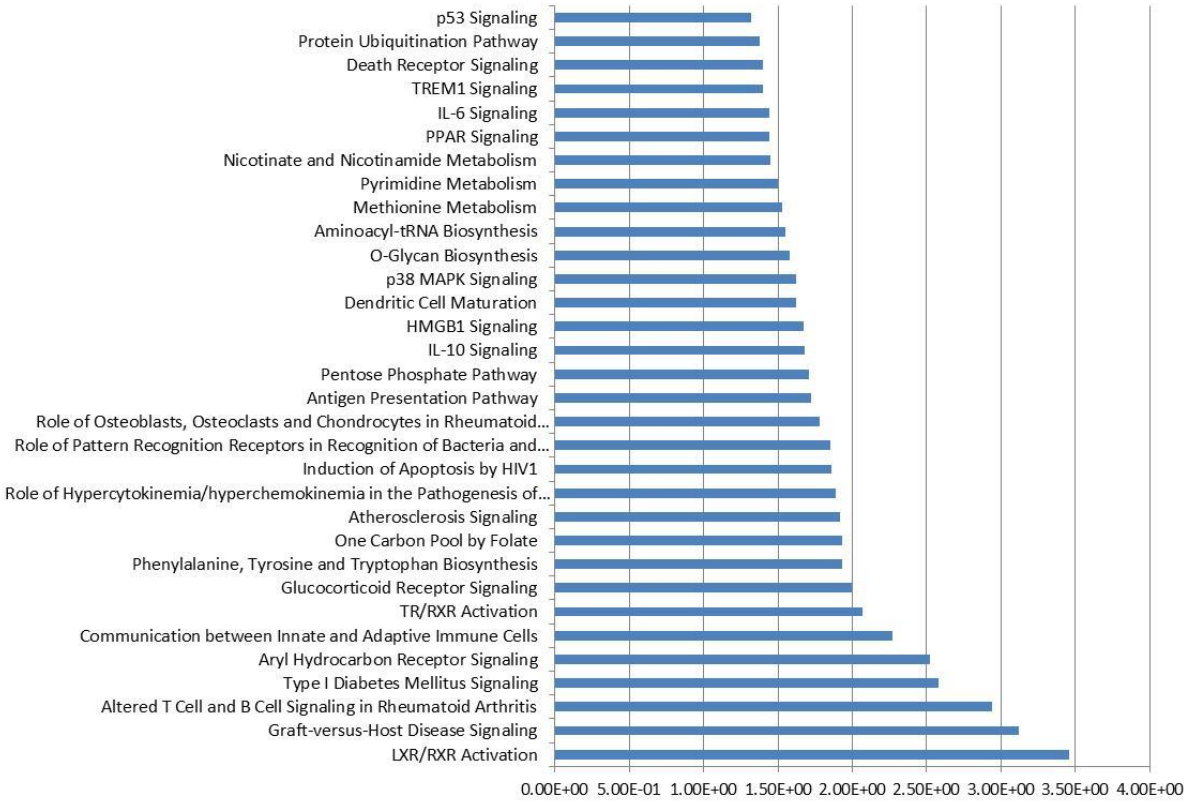

Figure 16: Canonical Pathways most affected by Arsenic exposure

The Liver X Receptor /Retinoid X Receptor (LXR/RXR) Activation is the most enriched

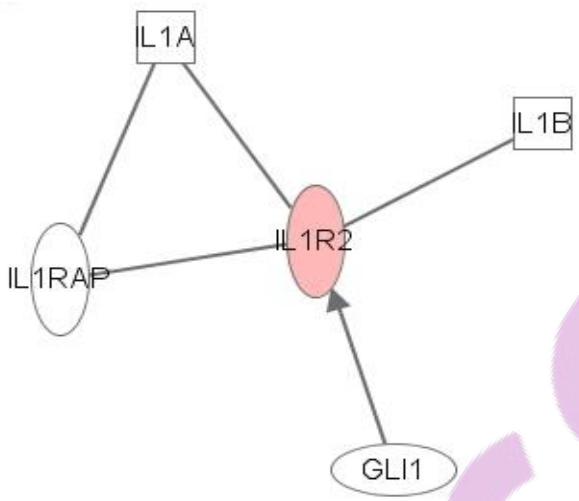

Figure 17: Interaction network of Interleukin 1 Receptor, Type II (IL1R2). IL1R2 up-regulation may activate the expression of the other molecules in its network

(Molecules in red are up-regulated while molecules in green are down-regulated in the microarray analysis data).

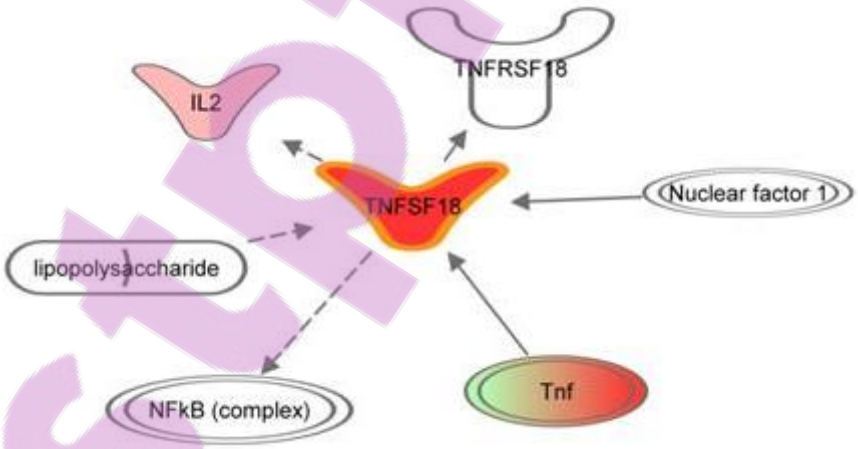

Figure 18: Interaction network for TNFSF18 interaction network. TNFSF18 up-regulation may activate the expression of the other molecules in its network

(Molecules in red are up-regulated while molecules in green are down-regulated in the microarray data)

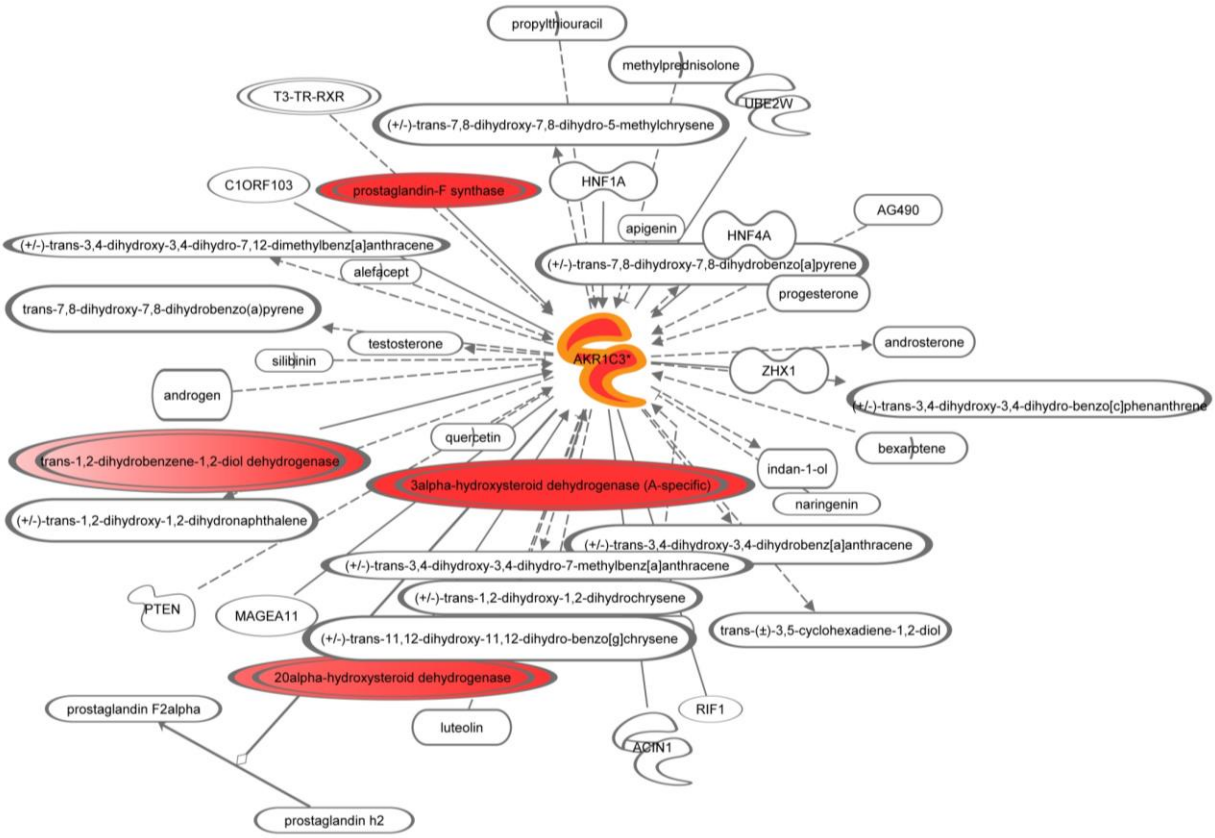

Figure 19: Interaction network for AKR1C3; AKR1C3 up-regulation may activate the expression of the other molecules in its network

(Molecules in red are up-regulated while molecules in green are down-regulated in the microarray data)

Figure 19 shows that AKR1C3 is associated with biological pathways which are involved in thyroid hormone response, lipid and carbohydrate metabolism, growth and cell proliferation. AKR1C3 is highly up-regulated, and it is obvious from its interaction network that other molecules involved in that network are also up-regulated, suggesting that AKR1C3 up-regulation may activate the expression of the other molecules in its network. For example, activation of 3-

beta hydroxysteroid dehydrogenase, 3b-hsd, (or prostaglandin F synthase) might be activating AKR1C3.

As illustrated in Figure 18, TNFSF18 networks with TNF and a transcription factor (Nuclear Factor 1) but expression of TNFSF18 could also trigger the expression of IL2 and TNF. Molecules in Green are down-regulated in the microarray data.

Figures 20 and 21 illustrate Nuclear Receptor Pathways Affected as Predicted by Ingenuity Pathway Analysis. Liver X Receptor/Retinoid X Receptor (LXR/RXR) Pathway is the most affected pathway that is involved in lipid metabolism and immune response. Thyroid Hormone/Retinoid X Receptor (TR/RXR) Pathway is highly involved in lipid metabolism, growth and cell proliferation and has been shown to be perturbed by ATO (Figure 20).

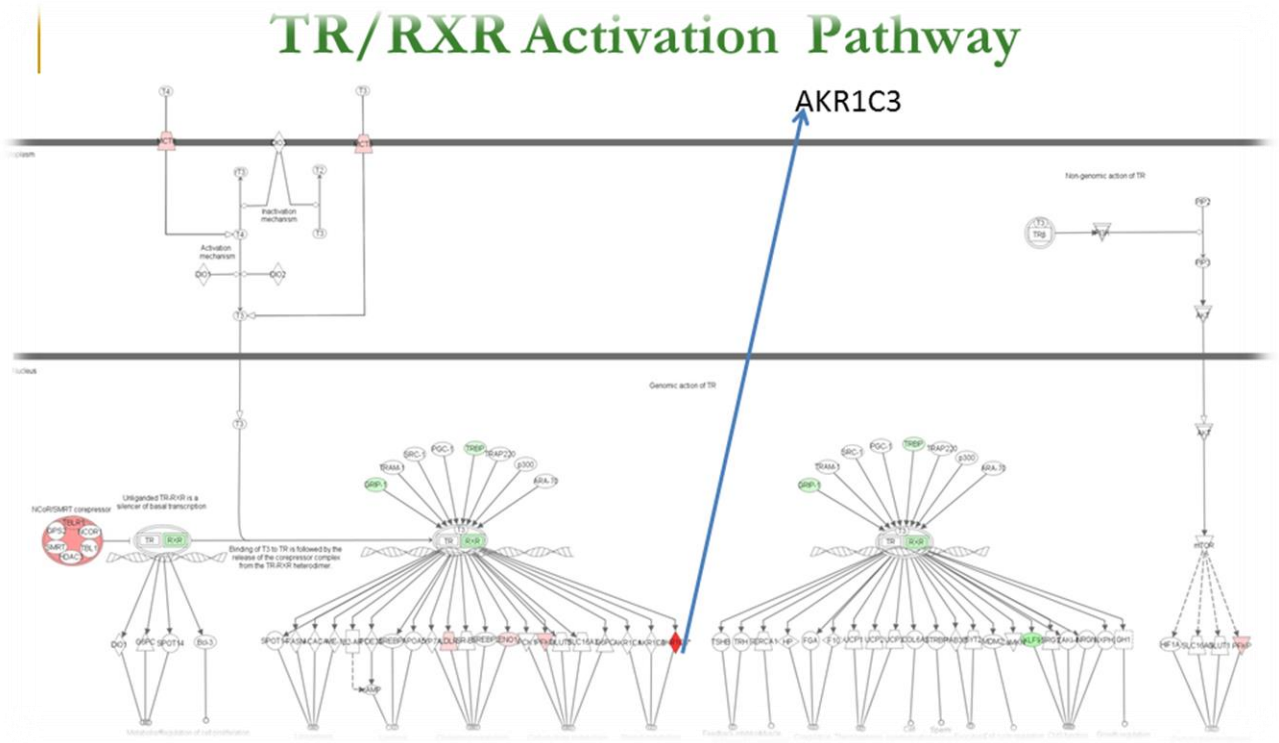

Figure 20: AKR1C3 is activated by the Thyroid Hormone/Retinoid X Receptor (TR/RXR) Activation Pathway involved in lipid metabolism (enriched by ATO exposure).

Colour genes are differentially expressed in the microarray data (red: up-regulated, green: down-regulated).

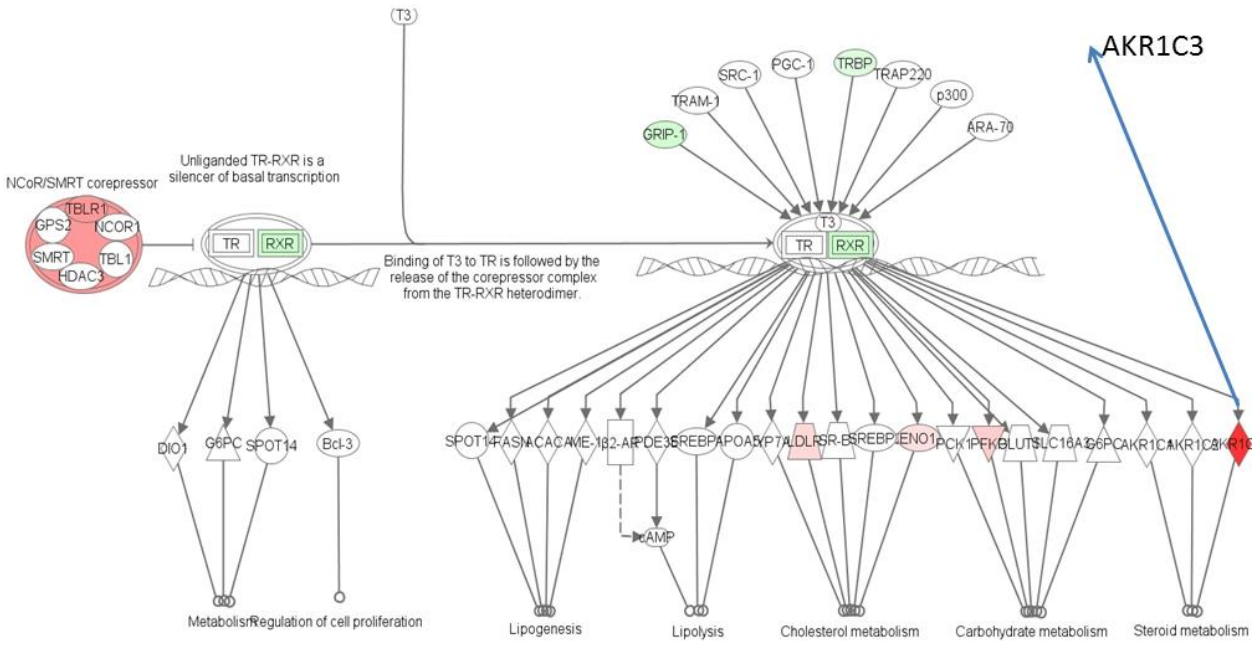

Figure 21: Close up of Figure 20, Thyroid Hormone/Retinoid X Receptor (TR/RXR) Activation Pathway highlighting the interaction with AKR1C3 which was up-regulated in response to chronic-dose exposure to ATO.

Colour genes are differentially expressed in the microarray data (red: up-regulated, green: down-regulated).

4.6 Arsenic Up-regulated Membrane Proteins

The membrane plays a role in determining the function of proteins by modulating protein function through localization with the substrate, activator, or downstream target, and activation of the protein by a conformational switch (Johnson et al. 1999). Two genes (IL1R2 and TNFSF18) from microarray gene expression results are focused on because they encode membrane proteins. The rationale for this focus is that membrane localized proteins could bind with arsenic at the cell surface and lead to subsequent changes in cellular biological pathways.

4.7 Prediction of cysteine state and disulphide bond partner

Table 12 presents results of the cysteines state and disulphide bond partner prediction using the four properties disulphide bonds, half cystine, free cysteine and metal bonded cysteines. All the tools predicted the same number of cysteines and positions of the cysteines in the sequences. IL1R2 protein sequence had 9 cysteines residues, while TNFSF18 protein sequence had 6 cysteines residues. However, the prediction software disagreed on the speciation of the cysteines. DBCP detected 2 cysteines with metal binding sites in TNFSF18. For TNFSF18, the DISULFIND predicted 6 half cystines while DiANNA predicted only 1 half cystine. Both DISULFIND and DiANNA predicted 3 disulphide bond formations whereas DBCP tool predicted no disulphide bond formation in TNFSF18. In IL1R2, the DISULFIND predicted 8 half cystine and 1 free cysteine, the DiANNA predicted 2 half cystines and 7 free cysteines while the DBCP predicted 4 half cystine and 5 free cysteines. The three bioinformatics software that predicted disulphide bond formation differed in the cysteines involved in the linkages (Table 12). Based on the agreement of software for disulphide bond prediction, we prioritized for further investigation the disulphide bond Cys152-Cys207 in IL1R2 and disulphide bond Cys24-Cys80 in TNFSF18.

Table 12: Prediction of disulphide bond partner and cysteine states for TNFSF18 and IL1R2

Software and Protein	Predicted disulphide bonds*	Half cys	Free Cys	Metal Bonded
DiANNA – TNFSF18	10-100, **24-80**, 52-61	1	5	0
DISULFIND – TNFSF18	10-61, **24-80**, 52-100	6	0	0
DBCP – TNFSF18	10-61, **24-80**, 52-100	0	4	2
DiANNA – IL1R2	28-373, 50-116, **152-207**, 258-326	2	7	0
DISULFIND – IL1R2	28-116, 50-152, 108-326, 207-258	8	1	0
DBCP– IL1R2	28-108, **152-207**	4	5	0

*****Disulphide bonds in bold are where two or three prediction software agree.

4.8 Prediction of Biological Networks

Genes for Interleukin 1 Receptor, type II (IL1R2) and Tumour Necrosis Factor (ligand) Superfamily, member 18 (TNFSF18) are part of ligand-receptor systems that encode membrane proteins. It is assumed that the predicted molecular interactions for IL1R2 will have connections to ligands IL1B and IL1A (Mantovani et al. 1998) while TNFSF18 will have connection to its receptor TNFRSF18 (Krausz et al. 2007). The human IL1R2 and TNFSF18 sequences were retrieved from UniProtKB/Swiss-Prot database with ID: IL1R2_HUMAN (P27930) and TNF18_Human (NP_005083.2) respectively. The IL1R2_HUMAN sequence was retrieved in FASTA format and used for analysis (Figure 22).

The Michigan Molecular Interactions (MiMI) plugin for Cytoscape (Gao et al. 2009) was used to retrieve molecular interactions and interaction attributes in the Michigan Molecular Interactions (MiMI) for query genes IL1B, IL1R2, IL1A,

TNFSR18 and TNFSF18 and their nearest neighbours. The Cytoscape platform was used to display the biological networks with the biological entities (genes/proteins) represented as nodes and the biological interactions represented as edges between nodes (Cline et al. 2007;Smoot et al. 2011).

4.9 Predicted Arsenic-Modulated Molecular Networks Affecting Onset of Keratinocyte Differentiation

The reconstructed molecular networks revealed interconnections to E2F4, an oncogenic transcription factor, predominantly expressed at the onset of keratinocyte differentiation. These genes can provide insights into the mode of action of arsenicals as well as discover gene and protein biomarkers of effects of arsenicals on human health. Figure 22 Illustrates Interaction Map for IL1B, IL1R2, IL1A, TNFSR18 and TNFSF18 reveals E2F4 Transcription Factor as link between sub-networks. E2F4 controls cell cycle and acts on tumour suppressor proteins. The molecular interaction map also predicted Necdin (NDN) as the connection between E2F4 and IL1A. TNF receptor associated factor 2 (TRAF2) was predicted as the connection between E2F4 and TNFSR18. The Activating Transcription Factor-2 (ATF2), a sequence-specific DNA-binding protein was predicted to interact with both E2F4 and IL1B. The reconstructed network consists of a total of 24 nodes.

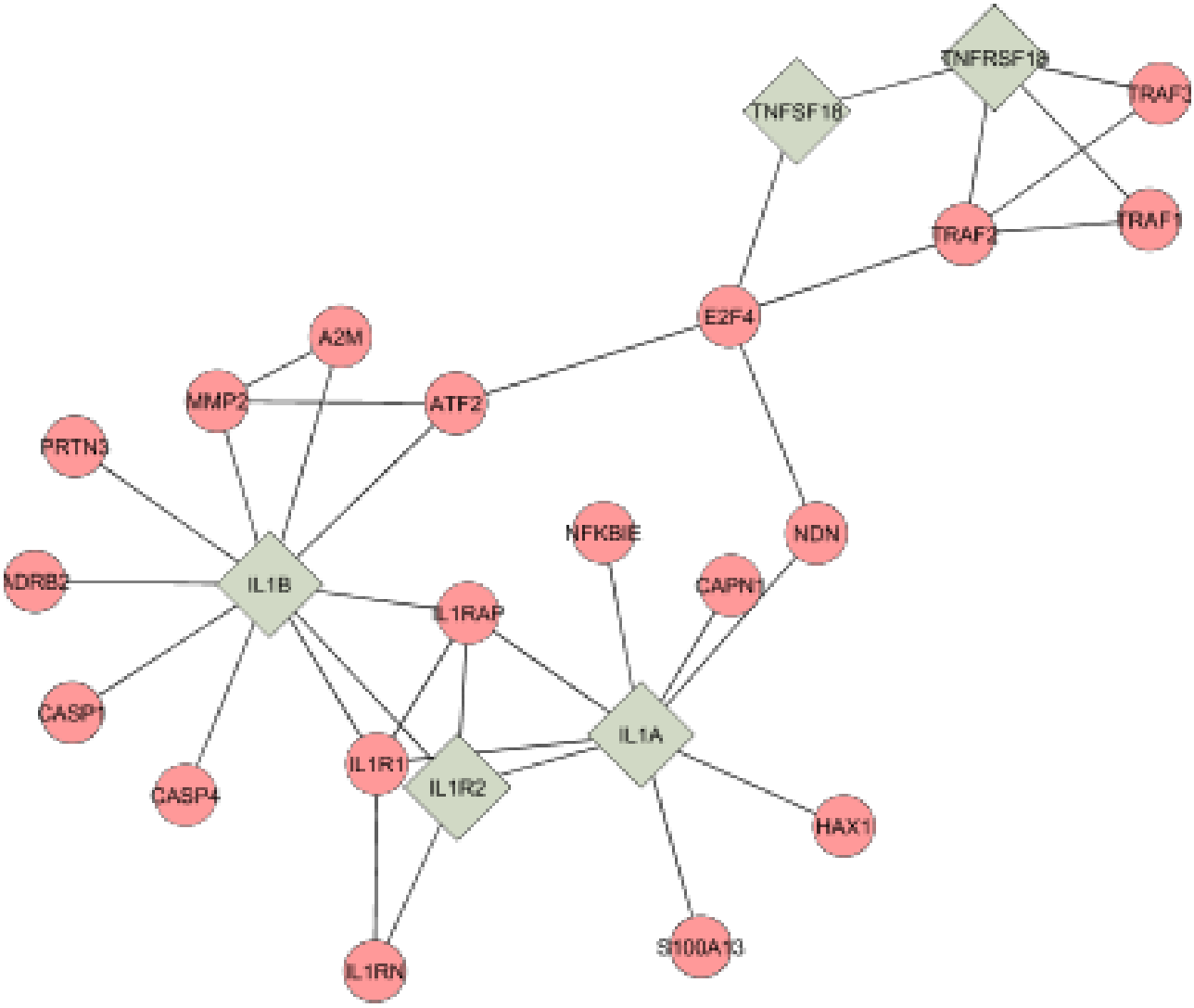

Figure 22: Interaction Map for IL1B, IL1R2, IL1A, TNFSR18 and TNFSF18.

The map reveals E2F4 Transcription Factor as link between sub-networks. E2F4 controls cell cycle and acts on tumour suppressor proteins.

Chapter 5

Discussion

Chronic cultures of HaCaT cells were established using a low dose of 0.5 µg/ml of ATO derived from cytotoxicity (MTT) Assay (Figure 6 on page 76). HaCaT cells were considered chronic after treated with 0.5 µg/ml ATO up to eight passages. The chronic HaCaT cells and untreated control HaCaT cells were subsequently exposed to higher doses of ATO. Bioinformatics tools and softwares were used to analyse the data and construct interactive visualizations of the acute and chronic exposures at the various time points (Figures 7 page 79 and Figure 8 page 80) and to demonstrate the genotoxic effect of arsenic on HaCaT cells (Figures 9 and Figure 10 on page 81). The visual analytics resources in this report unlike the standard static figures will allow for user-defined queries beyond those reported here. The visualizations could help generate hypothesis from novel insights on the patterns of arsenic cytotoxicity as the cells mature and differentiate from basal cell to squamous cells.

The cytotoxicity results reaffirm that ATO is cytotoxic to HaCaT cells (Graham-Evans et al. 2004). A biphasic response was observed at 5 µg/ml doses with cell viability peaking on Day 8 in both chronic cells and acutely exposed cells. It is obvious that a low dose of 1 µg/ml ATO enhanced HaCaT keratinocyte proliferation and doses above 7.5 µg/ml inhibited growth. This observation concurs with previous reports (Graham-Evans et al. 2004;Liao et al. 2004) but the time course profiling of ATO cytotoxicity using long-term HaCaT cell

cultures provides an approach to model the human epidermal cellular response to varying doses of ATO exposure. The behavior of the 1 µg/ml dose is similar to that previously observed with low micromolar concentrations of sodium arsenite. A very low dose of sodium arsenite maintained the proliferative potential of epidermal keratinocytes, and decreased their exit from the germinative stage under conditions that enable differentiation of untreated cells. Germinative cells are targets of tumour initiators and promoters and their persistence and proliferation after arsenic exposure suggests that arsenic could have co-carcinogenic and tumor co-promoting activities in the epidermis (Patterson 2005). Also, low concentrations of ATO may regulate the expression of cell cycling pathway genes such as cyclin-dependent kinase 4 (CDK4) and transcription factor 1 (E2F1) (Bi et al. 2010) and this may explain the observation with 1 µg/ml dose. A molecular level interpretation of the induction of growth of cells by low arsenic concentration could be linked to the up-regulation of insulin growth factor like family member 1(IGFL1) as observed in the microarray gene expression experiment (Figure 13 on page 86) in which HaCaT cells were exposed to low dose 0.5 µg/ml of ATO up to 22 passages (Udensi et al. 2011a). IGFL1, like other related insulin growth factors (IGF), is involved in cellular energy metabolism, growth and development and promotion of cell division (Emtage et al. 2006). IGFL1 is frequently up-regulated in skin conditions such as psoriasis which promote the abnormal proliferation and differentiation of epidermal keratinocytes (Lobito et al. 2011;Tonel et al. 2009). Another factor that may stimulate increase in cell proliferation at 1 µg/ml dose could be the induction of cyclin D1 transcription by low dose arsenic as reported by Hwang et al., (Hwang et al. 2006). Cyclin D1

stimulates growth by shifting the G1 growth phase into the S/G2 cell cycle compartment. A similar observation had been made with ATO on porcine aortic endothelial cells (PAEC), in which low concentrations stimulated cell proliferation with increases in superoxide and hydrogen peroxide (H_2O_2) accumulation, H_2O_2-dependent tyrosine phosphorylation, and NF-κB-dependent transcription (Barchowsky et al. 1999;Lee 2005).

Genotoxic capability of ATO on HaCaT keratinocytes was observed using the Comet Assay on section 4.3, page 82. There was a significant difference between the control cell and the 1 µg/ml dose in the Comet assay ($p<0.05$) (Figure 10, page 81). The results show that ATO has differential effects on cell growth and DNA damage depending on its concentration and corroborates with other investigations using different cell lines other than HaCaT cell line (Andrew et al. 2006;Shi et al. 2004;Stevens et al. 2010). Arsenic dose dependent effects has been observed on telomerase activity in human promyelocytic leukaemia cells (HL-60) and HaCaT cells where low doses result in an increase in activity observed as elongated telomere length and promotion of cell proliferation. At high doses of arsenic, telomerase activity was reduced with reduced telomere length and cell death (Zhang et al. 2003). Telomere homeostasis is critical in maintaining chromosome and genomic stability (Misri et al. 2008;Viscardi et al. 2005). Another cell line that proliferates under the influence of low dose arsenic exposure is normal non-transformed NHEK. Arsenite was reported to induce cell proliferation by increasing redox-related gene expression and decreasing DNA repair

(Hamadeh et al. 2002). Therefore, low concentrations of ATO can significantly enhance HaCaT keratinocyte proliferation.

Global gene expression (section 4.4 on page 84) was performed to understand ATO mechanism of action from the induction of aberrations in gene expression perspective. All the RNA samples used for this study were of high quality with RIN of 10 as shown in Table 8, page 85. A comparison of the microarray data from untreated (control) and chronically exposed HaCaT keratinocyte cell identified a total of 35 differentially expressed genes with 14 genes up-regulated (Table 10, page 88) and 21 genes down-regulated (Table 11, page 89). Genes with ≥ 2 fold changes and P-value ≤ 0.05 were considered significantly expressed and were selected for confirmation using complementary qRT-PCR approach. The global transcriptomics approach identified 167 fold over expression of TNFSF18 (tumour necrosis factor (ligand) superfamily, member 18), 5.9 fold for IL1R2 (Interleukin 1 receptor, type 2), IGFL1 (Insulin Growth Factor-Like family member 1) (3.1 fold), and AKR1C3 (Aldo-Keto Reductase family 1, member C) (9.2 fold). Also, a decreased expression of RGS2 (Regulator of G-protein Signalling 2) (2.0 fold) was confirmed (see Figure 13, page 86). This suggests that low dose arsenic exposure induces immunotoxic, anti-differentiation, growth factor promotion and anti-apoptotic effects in skin keratinocytes (Martinez et al. 2011).

IL1R2 also referred to as CD121b, IL1RB, and MGC47725 (Dale et al. 1999) plays a vital role in immune response and it is associated with the membrane

(Figure 15, page 90). IL1R2 is a decoy receptor for inflammatory interleukin 1 (IL-1). It acts by sequestering active and inactive IL1, which in turn restricts the availability of the ligand for the functional receptor and inhibits its maturation (Bossu et al. 1995;Colotta et al. 1994;Subramaniam et al. 2004;Symons et al. 1995). IL1R1 and IL1R2 are the known receptors of IL1 and cell activation that transduce the activation signal which occurs when IL1 binds to cell surface IL1R1 in conjunction with IL1R accessory protein (IL1RAP) (Dinarello 2004). IL1R2 is known as a potent, specific and natural inhibitor of IL1, but in contrast to IL1R1 it has no signalling properties when bound to IL1 (Bossu et al. 1995;Colotta et al. 1994;Subramaniam et al. 2004;Symons et al. 1995). Over expression of IL1R2 has been reported in human uroepithelial cell line (HUC-1) chronically exposed to arsenite (Chang et al. 2009). Both results show that low dose arsenic can induce expression of IL1R2 in HaCaT and HUC-1. IL1R2 may therefore be a biomarker for chronic exposure to arsenicals. In HUC-1, IL1R2 over expression is linked with enhanced expression of Smad-interacting protein 1 (SIP-1) and reduced expression of E-cadherin (Chang et al. 2009). E-cadherin is a calcium-dependent, epithelial cell adhesion molecule, whose reduced expression has been associated with tumour de-differentiation and increased lymph node metastasis in clinical studies involving several carcinomas (Siitonen et al. 1996). Furthermore, reduced expression levels of E-cadherin was associated with moderately and poorly differentiated squamous and small cell carcinoma in a limited number of patients with lung cancer (Bohm et al. 1994). IL1R2 also improves cell migration (Chang et al. 2009), which is suggestive of an oncogenic potential of IL1R2. Gene network analysis with IL1R2 gene as illustrated in Figure 15, page 90, showed that

IL1R2 interacts with IL1RAP, IL1A, IL1B and GLI1 and this agrees with previous findings (Bossu et al. 1995;Colotta et al. 1994;Subramaniam et al. 2004;Symons et al. 1995). But the difference is that this investigation is a chronic exposure experiment and the HaCaT was exposed to low concentration of ATO (0.5 µg/ml) up to 22 passages. GLI1 proteins interconnect with IL1R2 as presented in Figure 15. GLI1 protein was originally isolated from human glioblastoma (Kinzler et al. 1987;Saran 2010) and is the effector of Hedgehog (Hh) signalling which plays a critical role in carcinogenesis. Furthermore, GLI1 was reported to be upregulated in many tumours including basal cell carcinomas. Thus, IL1R2 indirectly interacts with GLI1, which is the trigger for basal cell cancer (BCC) implicating GLI1 in skin carcinogenesis. IL1R2 had been implicated in ulcerative colitis as candidate gene which can provide potential important information on the disease pathogenesis (Anderson et al. 2011). This receptor binds to two cytokines ligands (IL-1 alpha and IL-1 beta) which are active participants in the regulation of inflammation, immune responses and haematopoiesis. The crystal structures of interleukin receptor family members have been resolved. The fold consists of twelve beta-strands which form a six-stranded beta-barrel, closed on one side by three beta-hairpin loops. Cys69 and Cys116 are linked via a disulphide bond and Pro53 has been built in the cis-conformation (Schreuder et al. 1995). The IL1R2 protein was found to possess 11 regulatory points for binding of arsenic as a ligand (Kasper et al. 2006). This protein acts as a decoy receptor that inhibits the activity of its ligands. For example Interleukin 4 (IL4) antagonize the activity of interleukin 1 by inducing the expression and release of IL1R2 cytokine (Rennick et al. 1987). Also, arsenic

acts as an antagonist in certain pathways leading to cancer (Kim et al. 2010). These observations suggest that arsenic will bind on the ILR2 receptor protein putative antagonist binding site as an antagonist, thus inducing the expression and release of the cytokine gene ILR2 as seen with IL4 above. The induction of ILR2 may account for the up regulated expression of IL1R2 gene as previously reported (Rennick et al. 1987). Anti-inflammatory response is triggered upon binding of arsenic ligand to the Hedgehog (HR) receptor (Kim et al. 2010). This arsenic-receptor binding could possibly account for the inflammatory reaction seen with patients suffering from arsenic skin cancer. Pathway activation within cancer cells has been established for tumours of ectodermal or mesodermal origin, primary cancer of the skin, brain and muscle (Hahn et al. 1996;Johnson et al. 1996). Ligand dependent activation of some pathway ways, for example hedgehog (Hh) signalling, has been associated with tumourigenesis. Hence, arsenic may act similarly as a ligand on its surrounding cells to elicit secretion of factors that in turn support the growth and progression of the cancer cells (Yauch et al. 2008).

The cytokine TNFSF18, which can also be represented as glucocorticoid-induced tumour necrosis factor receptor-related ligand (GITRL), is a ligand for receptor TNFRSF18/AITR/GITR, and it modulates T lymphocyte survival in peripheral tissues (Figure 15). TNFSF18 is found in extracellular space and integral to membrane (Pruitt et al. 2007). GITRL was significantly over expressed with a fold change of more than 174. The glucocorticoid-induced tumour necrosis factor receptor-related gene (GITR) is expressed on

regulatory T-cells (Treg), which are $CD4^{+}CD25^{+}$ lymphocytes. Binding of its ligand, GITRL, leads to down-regulation of the biological function of Tregs. A defect in Tregs causes a skin condition resembling atopic dermatitis (Baumgartner-Nielsen et al. 2006). Soluble forms of GITRL (sGITRL) are released by human tumour cells (Baltz et al. 2008) with the determination of sGITRL levels might be implemented as a tumour marker in patients. Activated keratinocytes are known to engage intraepithelial T-cells through co-stimulatory molecules, keratinocytes express GITRL and through this important co-stimulatory molecule expressed by antigen-presenting cells (APCs), they have the potential to influence T-cell numbers in the skin via chemokine production and through a direct cell-cell effect on T-cell proliferation (Byrne et al. 2009). Sustained arsenic insult could have activated the HaCaT keratinocytes, which may lead to an immunotoxigenic reaction as reported by Baumgartner-Nielsen et al. (Baumgartner-Nielsen et al. 2006).

Aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) (AKR1C3) (Azzarello et al. 2009) was up-regulated up to 9.2 folds in this investigation. Human AKR1C3 is an enzyme involved in steroid metabolism as illustrated in Figure 19, page 95. Elevated levels of AKR1C3 expression are implicated in leukaemia cell differentiation, prostate cancer (in both androgen-dependent and androgen-independent prostate cancer), (Fung et al. 2006) endometrial cancer (Rizner et al. 2006) and chronic inflammation (Fung et al. 2006).

A 3.1 fold change in expression of insulin growth factor-like family member 1 (IGFL1) was observed. The IGF-like (IGFL1) gene encodes proteins that contain 11 conserved cysteine residues at fixed positions including two CC motifs (Emtage et al. 2006)]. The knowledge on the biological functions and gene interactions of IGFL1 is limited. However, the structure and sequence suggest that IGFL proteins are distantly related to the Insulin-like growth factors (IGF), a superfamily of growth factors. Both IGFL mRNAs and IGF display specific expression patterns; they are expressed in many cancers (Emtage et al. 2006).

The human skin is the critical organ of arsenic toxicity because arsenic has a strong affinity for the keratin proteins, that are rich in the sulphur containing cysteine residues (Ralph 2008) and potentially arsenic-binding proteins based on presence of vicinal cysteines (Kitchin et al. 2005). It was previously observed that proteins with abundance of vicinal cysteines could increase responsiveness to arsenic-induced keratinocyte carcinogenesis (Isokpehi et al. 2010). Since IGLF1 encodes proteins rich in cysteine residues, it could be playing a vital role in arsenic binding and responsiveness in keratinocytes. Further, IGFL1 is associated with embryonic tissue and was observed in libraries derived from carcinoma cell lines (Emtage et al. 2006). Therefore, the increase in IGFL1 might be involved in cancer development and progression and is probably a marker of chronic exposure to ATO.

The growth suppressor gene RGS2 accelerate GTPase activity of heterotrimeric G proteins, resulting in inactivation of specific signalling pathways (De et al. 2000). Down regulation of RGS2 occurred in human prostate tumour specimens (Cao et al. 2006) as well as in recurrence and metastasis-derived colorectal cancer cell lines (Jiang et al. 2010). In HaCaT keratinocyte cells, aberrant expression of RGS2 may aid in the spread of cancer or metastasis.

The functional and pathway annotation analysis to identify specific pathways of interest from the microarray gene expression datasets was performed using Ingenuity Pathway Analysis tool (IPA; Ingenuity Systems, Redwood City, CA). Figure 16, page 93 shows the pathways affected by arsenic exposure with the most affected being Liver X Receptor /Retinoid X Receptor LXR/RXR pathway which are involved in lipid metabolism and immune response (Christoffolete et al. 2010;Laffitte et al. 2003). Previously, perturbation of lipid metabolism by arsenic toxicity is accessed by the lipid peroxidation assay. The LXRs act as molecular sensors of cholesterol levels and respond by inducing processes that reduce cholesterol levels (Lehmann et al. 1997).

Ingenuity Pathways Analysis has been used to determine models of biological pathways and networks that are significantly represented in the differentially expressed (both up and down regulated) genes. Benjamini-Hochberg corrected $P \leq 0.05$ is used to select highly significant biological functions and pathways represented in the datasets. Benjamini-Hochberg is a type of

multiple hypothesis correction that adjusts the p values downwards to account for using a null hypothesis process with multiple – often thousands – of variables with only a few samples. Confidence is higher when multiple hypothesis correction is included (Borch et al. 1992).

AKR1C3 is associated with biological pathways which are involved in thyroid hormone response, lipid and carbohydrate metabolism, growth and cell proliferation (Figure 19, page 95). AKR1C3 is highly up-regulated, and it is obvious from its interaction network that other molecules involved in that network are also up-regulated, suggesting that AKR1C3 up-regulation may activate the expression of the other molecules in its network. For example, activation of 3-beta hydroxysteroid dehydrogenase (3b-hsd) (or prostaglandin F synthase) an enzyme which catalyzes the synthesis of progesterone from pregnenolone, (Cravioto et al. 1986) might be activating AKR1C3. As illustrated in Figure 18, TNFSF18 networks with TNF and a transcription factor (Nuclear Factor 1) but expression of TNFSF18 could also trigger the expression of IL2 and TNF. Molecules in Green are down-regulated in the microarray data. Figure 19, page 95 and Figure 20, page 97, illustrate Nuclear Receptor Pathways affected by arsenic exposure as Predicted by Ingenuity Pathway Analysis. Liver X Receptor/Retinoid X Receptor (LXR/RXR) Pathway is the most affected pathway that is involved in lipid metabolism and immune response. Thyroid Hormone/Retinoid X Receptor (TR/RXR) Pathway is highly involved in lipid metabolism, growth and cell proliferation and has been shown to be perturbed by ATO. Previous studies however have reported perturbation

of Lipid metabolism by arsenic toxicity which influenced the use of lipid peroxidation assay to assess arsenic toxicity. LXRs act as molecular sensors of cholesterol levels and respond by inducing processes that reduce cholesterol levels (Lehmann et al. 1997).

Further analysis of the global microarray gene expression data revealed two genes that encode membrane proteins. Interest was focused on these two genes, TNFSF18 and IL1R2, as models for studying arsenic modulated molecular networks affecting the onset of keratinocyte differentiation. The membrane plays a role in determining the function of proteins by modulating protein function through localization with the substrate, activator, or downstream target, and activation of the protein by a conformational switch (Johnson et al. 1999). The rationale for this focus is that membrane localized proteins could bind with arsenic at the cell surface and lead to subsequent changes in cellular biological pathways. TNFSF18 is a ligand for receptor TNFRSF18 and it modulates T lymphocyte survival in peripheral tissues playing a vital role in resistance to infection and cancers. TNFSF18 is found in extracellular space and integral to membrane (Pruitt et al. 2007). It belongs to the TNF ligand super family that contain uniform structural motif, the TNF homology domain (THD), which binds to cysteine-rich domains (CRDs) of TNF receptors (Bodmer et al. 2002). TNF can exert many of its effects by binding to cell membrane receptor. The members of TNF receptor superfamily possess an identical characteristic of an extra cellular domain containing two to six repeats of cysteine rich motifs (Idriss et al. 2000). It has been suggested that

the sequence homology between the CRDs and the DNA-binding 'zinc-fingers' may be used to speculate intracellular protein phosphorylation by protein kinase C (PKC) (Hommel et al. 1994). IL1R2 also referred to as IL1RB, encodes membrane bound proteins known to be crucially involved in immune response and it also acts as a decoy receptor for inflammatory interleukin 1 (IL-1) (Pruitt et al. 2007). The cysteine predictions show that IL1R2 has 9 cysteine residues, and TNFSF18, 6 cysteine residues (Table 12, page 100).

Disulphide bonds play a key structural role in stabilizing protein conformations. Prediction of disulphide bond connectivity facilitates structural and functional annotation of proteins (Huang et al. 1999;Skolnick et al. 1997). The cleavage of one or more of the disulphide bonds in a protein by arsenic or any other catalyst will affect its function (Hogg 2003). Since arsenic is known for its preference to bind to cysteine residues, predicting the C-terminal cysteine residues may play a critical role in arsenic sensing. Phenylarsine oxide (PAO) covalently binds to vicinal protein cysteine thiol groups (He et al. 2009) suggesting that the cysteines in IL1R2 and TNFSF18 could be playing a vital role in arsenic binding on HaCaT cells. Disulphide-containing proteins are attractive drug and diagnostic candidates as their interactions are potent and specific. Since arsenic has high affinity to bind to cysteine residues, detecting cysteine disulphide bonds could be an arsenic insult marker in proteins perturbed by arsenic exposure (Harvey et al. 1998;Harvey 2002). Proteins that contain disulphide bonds are heterogeneous and participate in different cellular activities such as cell-to-cell recognition, cell signalling and cell

defence and some of the proteins have direct contact with the membrane (Mas et al. 2001). This membrane binding may promote rearrangement, dissociation, or conformational changes within many protein structural domains, resulting in an activation or deactivation of their biological activity (Johnson et al. 1999;Mas et al. 2001). For some proteins including toxins, their biological effects are incited by specific interaction with diverse ion channels (Possani et al. 1999)], and cellular receptors within the membrane (Valentin et al. 2000). Hains, et al. (Hains et al. 1999) reported membrane involvement in toxins biological reaction. Thus, the membrane could also aid in the binding of arsenic with membrane localized proteins at the cell surface and help to transport arsenic into the cell to trigger of the changes in cellular biological pathways associated with arsenic exposure.

A comparison of the different disulphide bond predictions tools showed that all the software predicted the same number of cysteines and positions of the cysteines in the sequences as expected since it is based on recognition of "C" in the sequence. However, there is a discrepancy on the speciation of the cysteines residues. The terms oxidized, disulphide bonded and half cysteines can be used interchangeably while the other species are termed free cysteines or non-disulphide bonded and metal bound or ligand bonded cysteines (Ferre et al. 2006;Lippi et al. 2008;Petersen et al. 1999;Yan et al. 2009). The prediction of the cysteine bonding state (Matsumura et al. 1989;Richardson 1981;Vullo et al. 2004), structure and sequence features analysis and connectivity patterns classification are integral parts of disulphide bonds

characterization (Harrison et al. 1994;Petersen et al. 1999;Yan et al. 2009), which could be used in protein identification and classification (Lenffer et al. 2004). Based on the agreement of software for disulphide bond prediction, we prioritized for further research disulphide bond Cys152-Cys207 in IL1R2 and disulphide bond Cys24-Cys80 in TNFSF18. The Cys152 and Cys 207 residues of IL1R2 and the pair for TNFSF18 cysteines involved in this disulphide bond formation are most likely half cysteines (Petersen et al. 1999). Using X-RAY crystallography, the following disulphide bonds were confirmed for IL1R2, 28-116, 50-108, 152-207, 258-326 (Wang et al. 2010) .

The molecular interaction map for IL1B, IL1R2, IL1A, TNFSR18 and TNFSF18 revealed E2F4 transcription factor as a link between sub-networks (Figure 22, page 102). E2F4 controls cell cycle and function in the suppression of proliferation-associated genes, and its gene mutation and increased expression may be associated with human cancer (Souza et al. 1997). It is predominantly expressed at the onset of keratinocyte differentiation (Paramio et al. 2000). Further, E2F4 is a promoter of proliferation of human intestinal epithelial crypt cells and colorectal cancer cells (Garneau et al. 2009). The known predominant expression of E2F4 in the onset of keratinocyte differentiation raises the possibility that arsenic perturbation of protein-protein interactions that include E2F4 could alter keratinocyte differentiation (Paramio et al. 1998). The molecular interaction map predicted Necdin (NDN) as the link between E2F4 and IL1A, which binds with IL1R2. The NDN protein physically binds to the carboxyl-terminal transactivation domains of E2F4 (Kobayashi et

al. 2002) and the oncogenic activity of the N-terminal of pre-IL1A is attributed to the interaction between NDN and the N-terminal of pre-IL1A (Hu et al. 2003). NDN function in the suppression of proliferation-associated genes and its mutation and expression disruption may be associated with human cancer (Kawamata et al. 2003).

A member of the TNF receptor associated factor (TRAF2) adapter protein family was predicted to interact with E2F4 and TNFSR18 (Figure 22, page 102). X-ray crystallographic structure of the TRAF domain of TRAF2 revealed a trimeric assembly, with each TRAF domain monomer containing a surface crevice responsible for binding peptidyl motifs found in the cytosolic domains of the TNF family receptors to which TRAF2 is known to bind (McWhirter et al. 1999). TRAF2 is required for TNF-alpha-mediated activation of MAPK8/JNK and NF-kappaB (Pruitt et al. 2007). NF-kappaB functions in immunity however the signalling pathways that are involved in its activation have been implicated in tumour development (Karin et al. 2002). Inhibition of NF-kappa B activation by arsenite through reaction with a critical cysteine in the activation loop of Ikappa B kinase has been reported (Kapahi et al. 2000).

Activating Transcription Factor-2 (ATF2) was predicted to interact with both E2F4 and IL1B. ATF2 is a sequence-specific DNA-binding protein that belongs to the bZIP family of proteins and has diverse functions in mammalian cells. It is activated by stress kinases such as JNK and p38 and responds to stress stimuli, by activating gene targets including cyclin A, cyclin D and c-jun,

which are involved in oncogenesis in various tissue types (Vlahopoulos et al. 2008). Further, ATF2 expression has been correlated with maintenance of a cancer cell phenotype. ATF2 plays a pivotal role in melanoma development and has been implicated in transcriptional regulation of immediate early genes that regulate stress and DNA damage responses (Shah et al. 2010). Malfunction of tumour suppressor activities of ATF2 by arsenic may contribute to carcinogenesis.

Chapter 6

Conclusions, Recommendations, Future Studies

6.1 Conclusion

The main goal of this research was to determine the mechanism of arsenic carcinogenicity in human skin epidermal keratinocytes. The aim was to treat the skin cells with a low dose of ATO simulating the natural way that people in endemic areas are exposed to inorganic arsenic through drinking water; and to determine the genomic changes that occur over a period of time. Two specific objectives were used to test the hypothesis that long-term, chronic exposure of human epidermal keratinocytes cells (HaCaT cells) to low dose arsenic disrupts cellular and molecular processes in keratinocytes. Knowledge of the biological pathways and networks of the genes that are significantly expressed or altered are crucial in understanding the mechanism of action of arsenic carcinogenesis in HaCaT cells.

The first specific objective was to determine cytotoxic and genotoxic effects of ATO on HaCaT cells and identify differentially expressed genes in HaCaT cells that were chronically exposed to low-dose ATO. The cytotoxic and genotoxic effects of ATO on HaCaT cells were determined using MTT assay and Comet assays respectively. ATO was observed to be cytotoxic and genotoxic to HaCaT keratinocytes. Although such observations have been made previously by other researchers, the contrast is that this research is centred on chronic exposure, whereas the previous studies focused on acute

arsenic exposure. It was also observed that long-term exposure of HaCaT cells to a low dose ≤ 1.0 µg/ml of ATO may enhance cell proliferation, while higher doses are cytotoxic. A low dose of ATO appears to aid cell growth, but concomitantly disrupts the DNA transcription process.

The second aspect of the first specific objective one was to demonstrate differentially expressed genes in HaCaT cells chronically exposed to low-dose ATO. This objective was achieved with the microarray gene expression experiment and subsequent confirmation of the selected differentially expressed genes of interest with qRT-PCR. The expression of immune response genes such as TNFSF18 (167 fold), IGFL1 (3.1 fold), IL1R2 (5.9 fold) and AKR1C3 (9.2 fold) and the down regulation of RGS2 (2.0 fold) suggest that chronic arsenic exposure could perturb normal cellular immune responses by producing sustained levels of TNF with modulation by an IL-1 analogue resulting in chronic immunologic insult. Also, the observed decrease in growth inhibiting gene (RGS2) and increase in AKR1C3 may contribute to chronic inflammation leading to metaplasia, which may eventually lead to carcinogenicity in the skin keratinocytes. Further, increased expression of IGFL1 may trigger cancer development and progression in HaCaT keratinocytes.

The second specific objective was to identify specific pathways from the gene expression datasets of HaCaT exposed to ATO. This objective also included the use of bioinformatics tools to perform functional and pathway annotation

to identify specific pathways of interest from the gene expression datasets. High-throughput gene transcription assays such as DNA microarrays allows for the identification of biological pathways affected by arsenic that lead to initiation and progression of skin cancer. Pathway analyses of the differentially expressed genes were also performed, and qRT-PCR confirmed the gene expression results. In the functional and pathway annotation analysis, the Liver X Receptor/Retinoid X Receptor LXR/RXR pathway commonly involved in lipid metabolism and immune response was the most affected by arsenic exposure.

The disulphide bond prediction and molecular network analyses on selected up-regulated genes encoding membrane proteins were performed. The prediction identified cysteine residues that are potential markers for arsenic-binding in two up-regulated genes (IL1R2 and TNFSF18). From the arsenic protein interaction, it may be suggested that arsenic functions as a ligand in the pathway leading to skin cancer. It attaches to the antagonist binding site on the IL1R2 protein, activating its pathway. Arsenic pathogenesis may involve both pathway activation and transcription.

Further, reconstructed molecular networks revealed interconnections to E2F4, an oncogenic transcription factor, predominantly expressed at the onset of keratinocyte differentiation. This report provides insights into previously unknown gene markers that may explain the mechanisms of arsenic-induced dermal disorders including skin cancer.

A combination of microarray, gene functional annotation data and qRT-PCR has been used to identify genes that are differentially expressed in HaCaT cell in response to chronic, low dose ATO. Further research will be to find out the details on the roles of each gene perturbed by arsenic exposure in keratinocytes.

6.2 Significance of the Study

This research has generated microarray data on global alteration of gene expression in HaCaT keratinocyte in response to ATO. A set of genes has been identified for more possible focal points of interest in skin carcinogenesis especially the interactions of arsenic with encoded proteins having known roles in skin physiology. Gene lists and pathways of arsenic toxicity to HaCaT cells were generated. Also, models of interaction of arsenic with cysteine residues (regulatory residues for protein interaction with arsenic) were developed.

6.3 Recommendations

Bioinformatics analyses and visual analytics are very essential in the analysis of high troughput datasets to discover interesting patterns of potential biological significance in skin cancer induced by arsenicals. Also, investigation of the perturbation of noncoding RNAs, such as miRNA, induced by arsenic in

HaCaT may lead to more discoveries of arsenic mechanisms of action. Since abnormal pigmentation is a common sign of chronic arsenic toxicity, further investigation to identify genes enriched for melanosomes (melanin-pigment bearing organelles) could be helpful to understand the molecular basis of aberrant pigmentation.

6.4 Future Studies

Further studies are planned to validate the gene expression results using enzymatic and immunochemistry assays, since arsenic toxicification was generated. Reactive oxygen species (ROS) assays would be performed to measure NADPH oxidation, and glutathione synthetase (GSS) oxidation and reduction. Also, Western Blot against an anti ROS protein would be performed. Further, peoteomics studies will be undertaken to correlate the gene expression result with protein expression. Protein data bases and bioinformatics tools would be used for the data analysis. The adoption of a new proteome data analysis strategies and combined information from proteome and transcriptome can enhance the insights gained from either type of data alone.

A more focused research is needed on the set of genes identified in this study to determine the interactions of arsenic with the encoded proteins with known roles in skin physiology. Additional bioinformatics analyses and visual analytics will be performed on the datasets to unravel interesting patterns of potential

biological significance in skin cancer-induced by arsenicals. For instance, a molecular network construction for arsenic upregulated genes TNFSF18 and IL1R2 revealed subnetwork interconnections to E2F4, an oncogenic transcription factor, predominantly expressed at the onset of keratinocyte differentiation. Visual analytics integration of gene information sources helped identify RAC1, a GTP binding protein, and TFRC, an iron uptake protein as prioritized arsenic-perturbed protein targets for biological processes leading to skin hyperpigmentation RAC1 regulates the formation of dendrites that transfer melanin from melanocytes to neighboring keratinocytes. Increased melanocyte dendricity is correlated with hyperpigmentation. TFRC is a key determinant of the amount and location of iron in the epidermis. Aberrant TFRC expression could impair cutaneous iron metabolism leading to abnormal pigmentation seen in some humans exposed to arsenicals **(Isokpehi 2012)**. The application of miRNA technology will be necessary to study more on the interaction between arsenic, keratinocytes and melanocytes and TFRC induction in skin carcinogenesis process.

Environmental management of arsenic is also crucial. and this could benefit from determining the presence of genes known to metabolize arsenic containing compounds. Understanding arsenic metabolism by microbial communities in the environment and in human hosts could lead to novel strategies to prevent and manage arsenic-induced diseases. Determining the presence of genes known to metabolize arsenic containing compounds will contribute to the knowledge on the role of human microbial flora in arsenic

metabolism. Furthermore, revealing the identity and function of microbial communities (microbiome) in human body habitats, using DNA sequencing technologies holds promise for dynamic assessment of health status and disease management. This microbiome knowledge could further be used to characterize the relationship between genetic variation and disease susceptibility.

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Appendix

Quantitative PCR Analysis Report

Relative Quantification: using GAPDH as endogenous control

Sample: human total RNA
H1, H2, H3, H4, As1, As2, As3, As4

Target Gene:
RGS2, PPP1R13B, MKNK1, TM4SF4, AKR1C3, TMEM70, TNFSF18, IGFL1, IL1R2, PCSK1

Materials and methods:

Primer Sequence

Human RGS2-F	GAATTCTGGCTGGCCTGTGA
Human RGS2-R	ATGTTTATCTCTTTTGGAGCTTCCTT
Human PPP1R13B-F	GCCACACCACCTAAGAATTACCA
Human PPP1R13B-R	GAGAGGTTGAACCCGAAGGTAAA
Human MKNK1-F	CAACTCCTGTACCCCCATAACC
Human MKNK1-R	TGGCCTGGTCCGTGAAGA
Human TM4SF4-F	TGGGCCTGAAGAACAATGACT
Human TM4SF4-R	CAAGAATCCAACCACAGCAAATAT
Human AKR1C3-F	GGAGAAGTGTAAGGATGCAGGATT
Human AKR1C3-R	GTACTTGAGTCCTGGCTTGTTGAG
Human TMEM70-F	AAGGCATGGGATCGTTTCC
Human TMEM70-R	ACTCCTGGCTCAATACTGATGGA
Human TNFSF18-F	AGCCCTGTATGGCTAAGTTTGG
Human TNFSF18-R	GCCATTCTGAAGTATCTCCAGCTT
Human IGFL1-F	CATCGTAGCTGTCTTTGCCATT
Human IGFL1-R	TGGCTGGCACAGCATCAG
Human IL1R2-F	CACTACGCACCACAGTCAAGGA
Human IL1R2-R	ATCCATATTCCCCCCAAAACC
Human PCSK1-F	CCTGGAAGCAAACCCAAATC
Human PCSK1-R	ATCCAAATCGACTATTCACCATCA

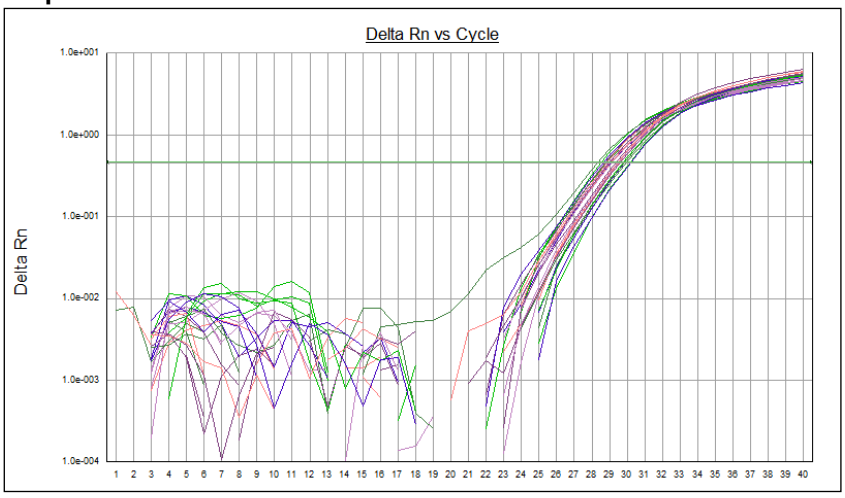
Results (H1 as calibrator):

Relative quantitation using the comparative C_T method

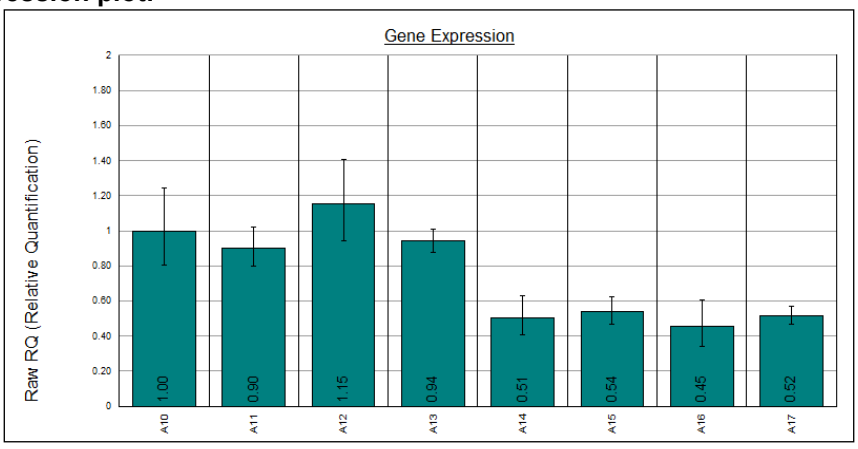
1. RGS2

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	27.78±0.11	16.24±0.02	11.53±0.11	0.00±0.11	1.00
H2	27.75±0.06	16.07±0.01	11.68±0.06	0.15±0.06	0.90
H3	27.38±0.10	16.05±0.02	11.33±0.10	-0.21±0.10	1.15
H4	27.99±0.02	16.38±0.03	11.62±0.04	0.09±0.04	0.94
As1	28.89±0.10	16.38±0.03	12.51±0.12	0.98±0.12	0.51
As2	28.36±0.06	15.94±0.04	12.42±0.07	0.89±0.07	0.54
As3	28.82±0.15	16.14±0.02	12.67±0.15	1.14±0.15	0.45
As4	28.41±0.04	15.93±0.03	12.48±0.05	0.95±0.05	0.52

Amplification plot:



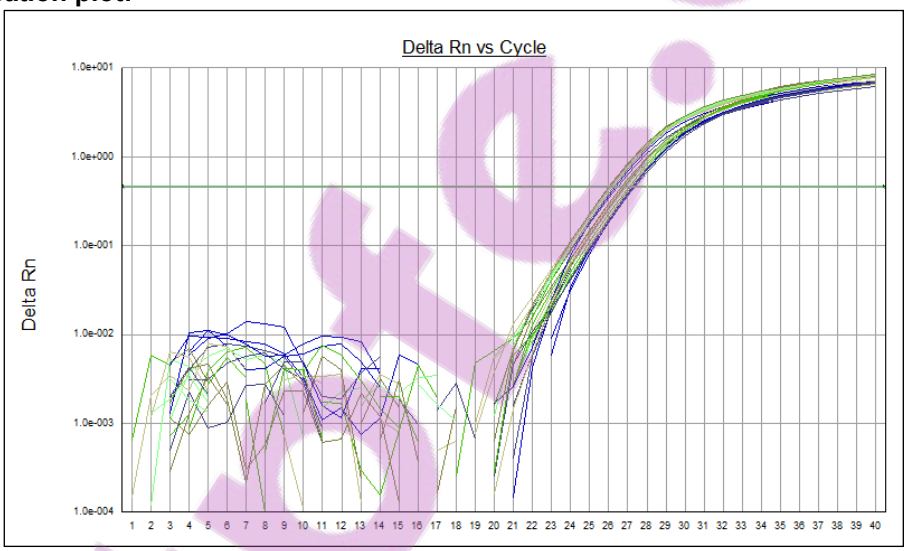
Gene expression plot:



2. PPP1R13B

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T- $\Delta C_{T,A10}$	Rel. to A10
H1	26.64±0.07	16.24±0.02	10.39±0.07	0.00±0.07	1.00
H2	26.55±0.03	16.07±0.01	10.48±0.03	0.09±0.03	0.94
H3	26.19±0.05	16.05±0.02	10.14±0.05	-0.25±0.05	1.19
H4	25.97±0.04	16.38±0.03	9.60±0.05	-0.80±0.05	1.74
As1	27.14±0.05	16.38±0.03	10.76±0.08	0.36±0.08	0.78
As2	26.70±0.03	15.94±0.04	10.76±0.05	0.37±0.05	0.77
As3	27.08±0.06	16.14±0.02	10.94±0.07	0.54±0.07	0.69
As4	25.95±0.04	15.93±0.03	10.02±0.05	=.37±0.05	1.29

Amplification plot:



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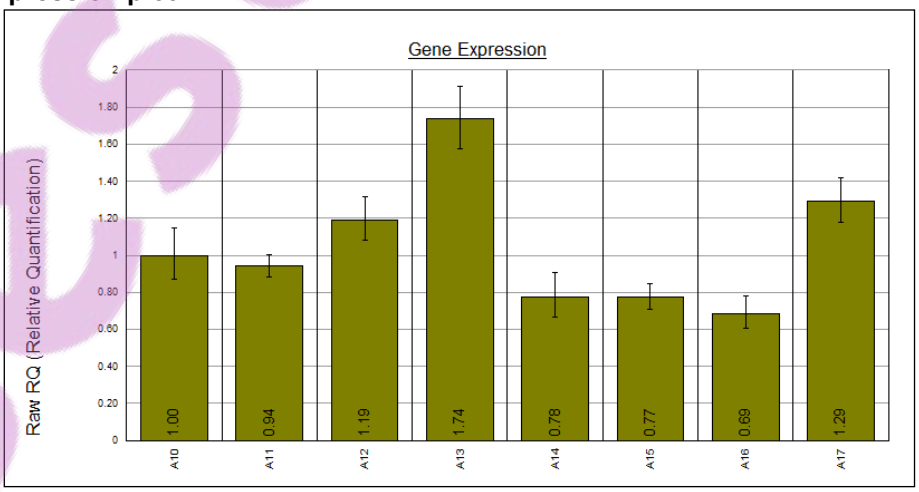
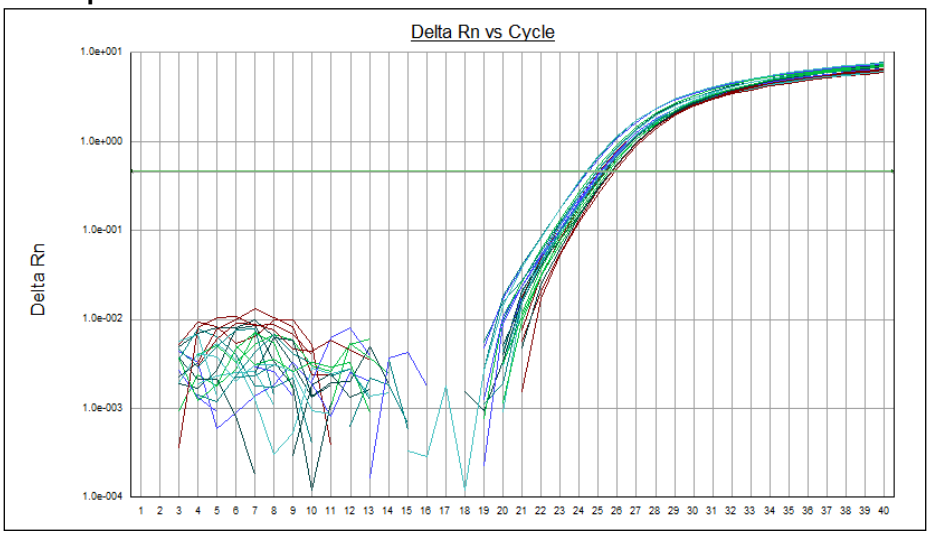


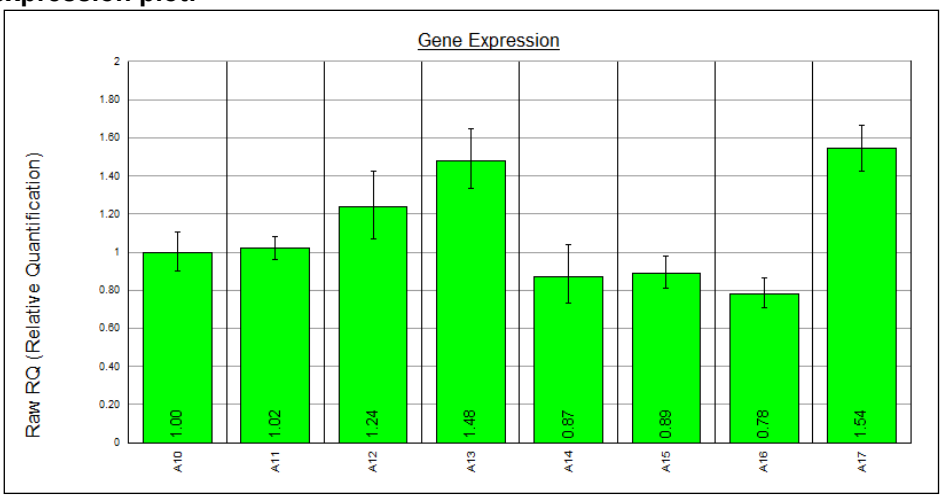
3. MKNK1

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	24.93±0.05	16.24±0.02	8.69±0.05	1.00±0.05	1.00
H2	24.73±0.03	16.07±0.01	8.66±0.03	-0.03±0.03	1.02
H3	24.43±0.07	16.05±0.02	8.38±0.07	-0.31±0.07	1.24
H4	24.49±0.05	16.38±0.03	8.12±0.06	-0.57±0.06	1.48
As1	25.26±0.06	16.38±0.03	8.88±0.09	0.20±0.09	0.87
As2	24.79±0.03	15.94±0.04	8.85±0.05	0.17±0.05	0.89
As3	25.18±0.05	16.14±0.02	9.04±0.05	0.35±0.05	0.78
As4	23.99±0.02	15.93±0.03	8.06±0.04	-0.63±0.04	1.54

Amplification plot:



Gene expression plot:



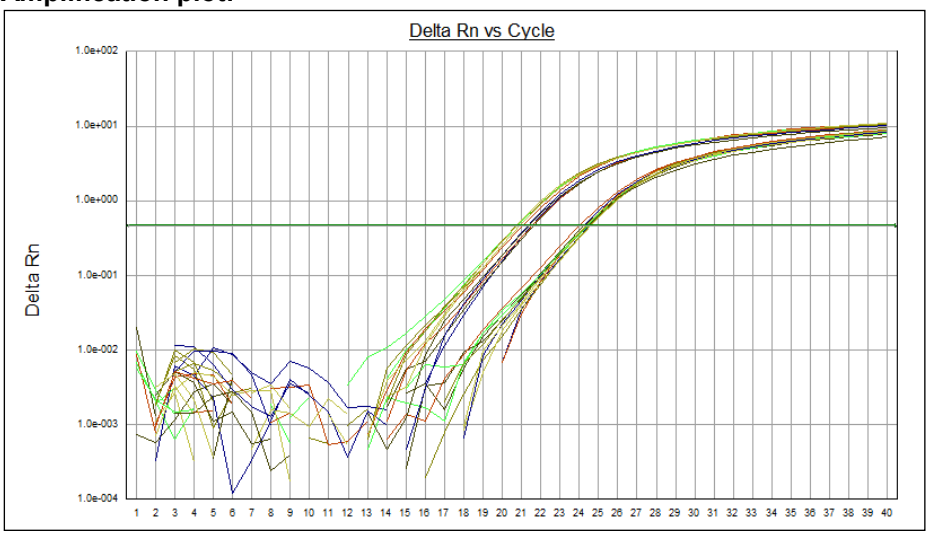
4. TM4SF4

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	Undet.	16.24±0.02	Undet	Undet	Undet
H2	35.90±0.07	16.07±0.01	19.83±0.07	Undet	Undet
H3	Undet	16.05±0.02	Undet	Undet	Undet
H4	Undet	16.38±0.03	Undet	Undet	Undet
As1	Undet	16.38±0.03	Undet	Undet	Undet
As2	Undet	15.94±0.04	Undet	Undet	Undet
As3	Undet	16.14±0.02	Undet	Undet	Undet
As4	Undet	15.93±0.03	Undet	Undet	Undet

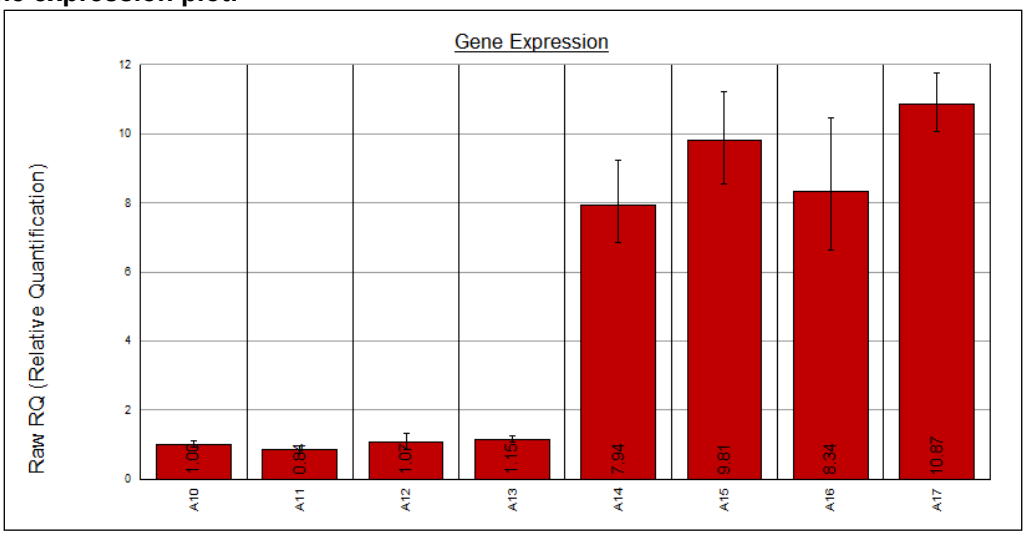
5. AKR1C3

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	23.15±0.05	16.24±0.02	6.90±0.05	0.00±0.05	1.00
H2	23.22±0.06	16.07±0.01	7.15±0.07	0.25±0.07	0.84
H3	22.86±0.11	16.05±0.02	6.81±0.11	-0.10±0.11	1.07
H4	23.08±0.03	16.38±0.03	6.70±0.04	-0.20±0.04	1.15
As1	20.29±0.04	16.38±0.03	3.91±0.08	-2.99±0.08	7.94
As2	19.55±0.06	15.94±0.04	3.61±0.07	-3.29±0.07	9.81
As3	19.99±0.12	16.14±0.02	3.84±0.12	-3.06±0.12	8.34
As4	19.39±0.02	15.93±0.03	3.46±0.04	-3.44±0.04	10.87

Amplification plot:



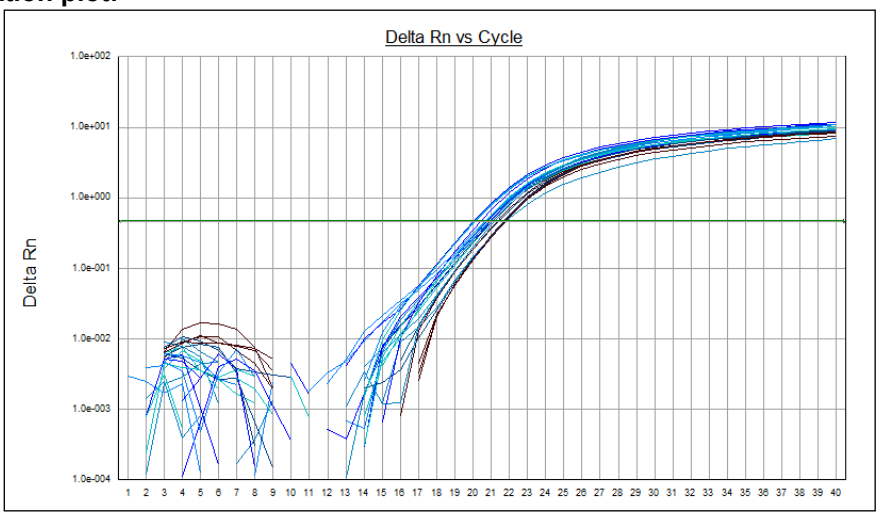
Gene expression plot:



6. TMEM70

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T- $\Delta C_{T,A10}$	Rel. to A10
H1	20.88±0.14	16.24±0.02	4.64±0.14	0.00±0.14	1.00
H2	20.21±0.04	16.07±0.01	4.14±0.05	-0.49±0.05	1.41
H3	20.30±0.12	16.05±0.02	4.25±0.12	-0.39±0.12	1.31
H4	19.95±0.06	16.38±0.03	3.57±0.07	-1.07±0.07	2.09
As1	21.00±0.01	16.38±0.03	4.62±0.07	-0.02±0.07	1.01
As2	19.85±0.10	15.94±0.04	3.91±0.11	-0.73±0.11	1.66
As3	20.74±0.32	16.14±0.02	1.60±0.32	-0.04±0.32	1.03
As4	19.29±0.03	15.93±0.03	3.37±0.04	-1.27±0.04	2.41

Amplification plot:



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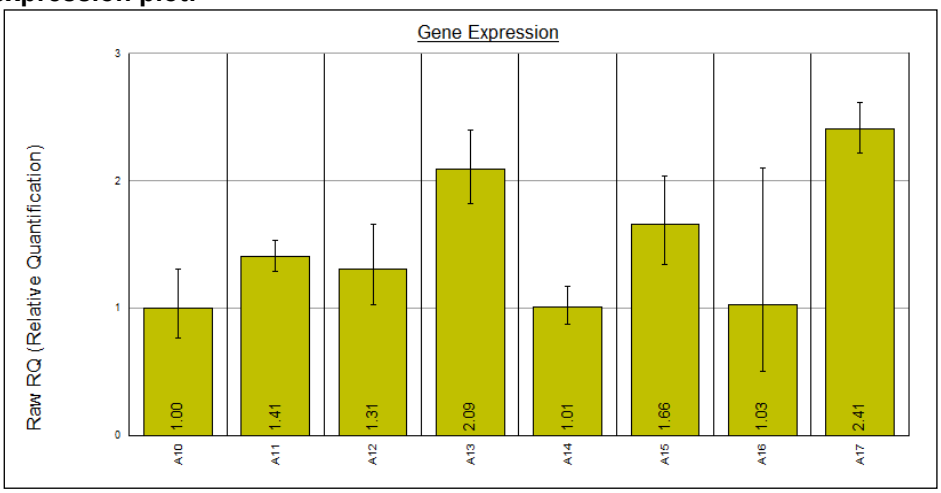
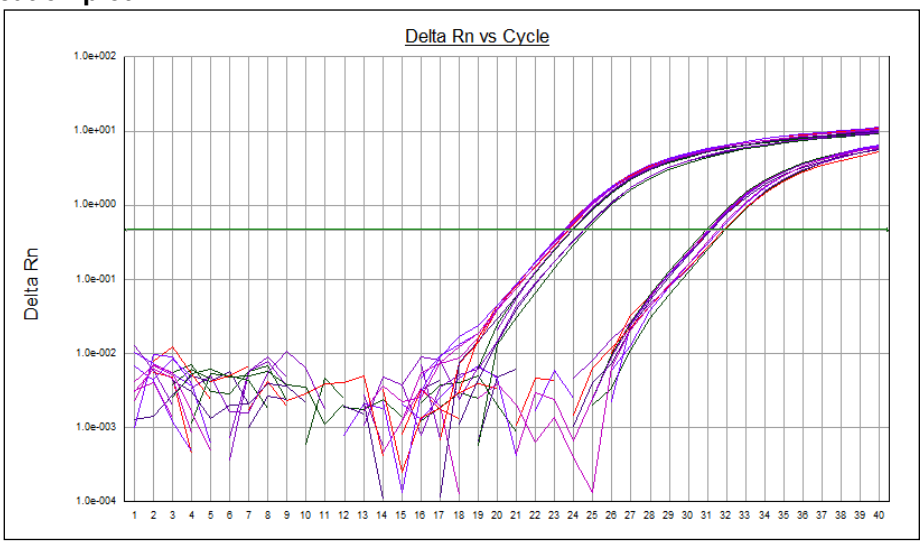


7. TNFSF18

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	31.84±0.04	16.24±0.02	15.60±0.04	0.00±0.04	1.00
H2	31.07±0.04	16.07±0.01	15.00±0.04	-0.60±0.04	1.52
H3	31.05±0.08	16.05±0.02	15.00±0.08	-0.61±0.08	1.52
H4	31.62±0.09	16.38±0.03	15.25±0.10	-0.36±0.10	1.28
As1	24.53±0.05	16.38±0.03	8.16±0.08	-7.45±0.08	174.47
As2	23.46±0.02	15.94±0.04	7.52±0.04	-8.09±0.04	271.53
As3	23.93±0.02	16.14±0.02	7.79±0.03	-7.82±0.03	225.43
As4	23.64±0.03	15.93±0.03	7.71±0.05	-7.89±0.05	237.67

Amplification plot:



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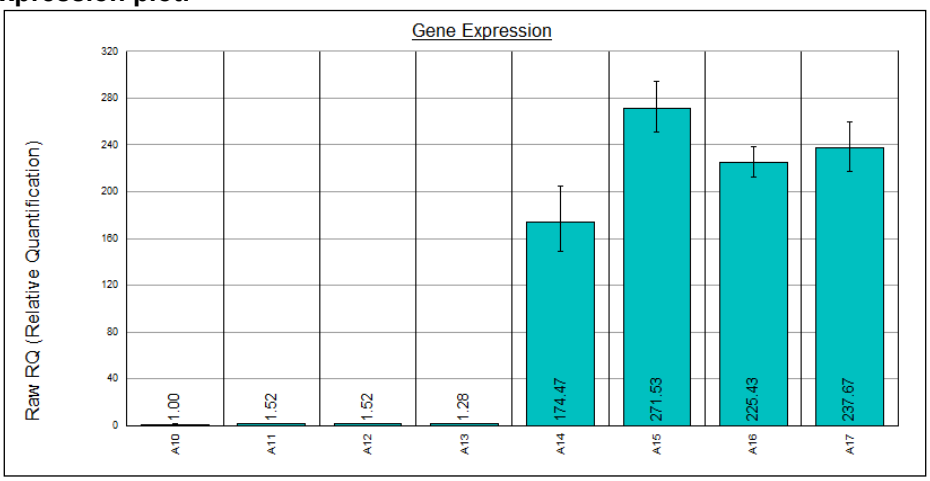
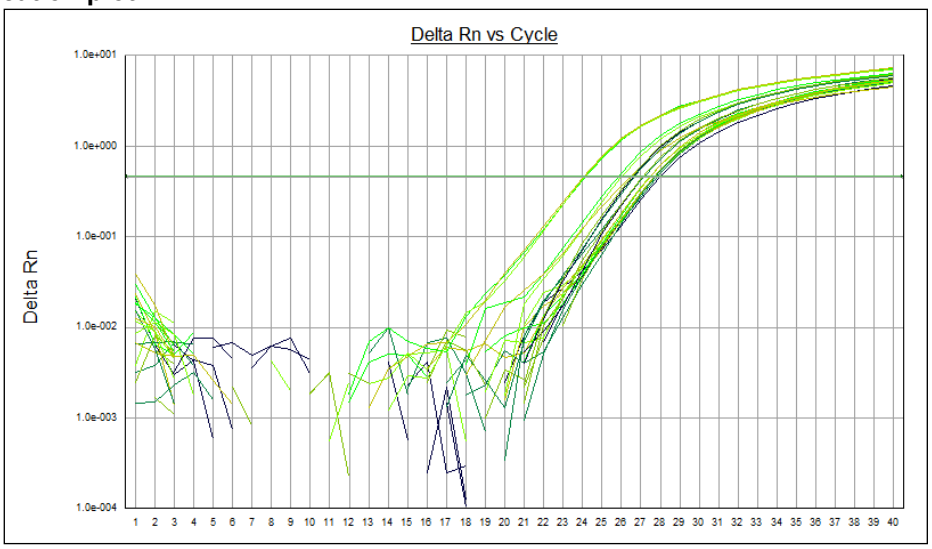


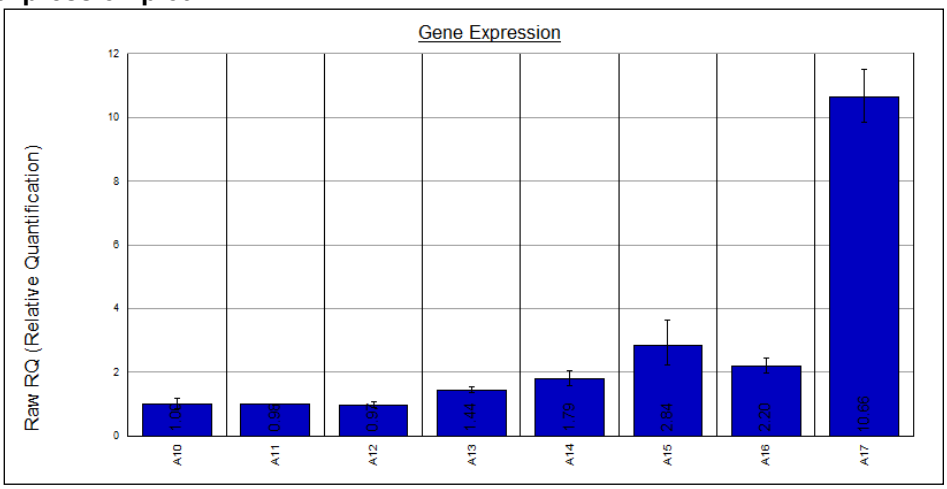
8. IGFL1

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	26.85±0.08	16.24±0.02	10.61±0.09	0.00±0.09	1.00
H2	26.70±0.01	16.07±0.01	10.63±0.02	0.02±0.02	0.99
H3	26.70±0.04	16.05±0.02	10.65±0.05	0.04±0.05	0.97
H4	26.45±0.02	16.38±0.03	10.08±0.04	-0.53±0.04	1.44
As1	26.14±0.01	16.38±0.03	9.76±0.07	-0.84±0.07	1.79
As2	25.04±0.12	15.94±0.04	9.10±0.13	-1.51±0.13	2.85
As3	25.61±0.05	16.14±0.02	9.47±0.05	-1.14±0.05	2.20
As4	23.12±0.02	15.93±0.03	7.19±0.04	-3.41±0.04	10.66

Amplification plot:



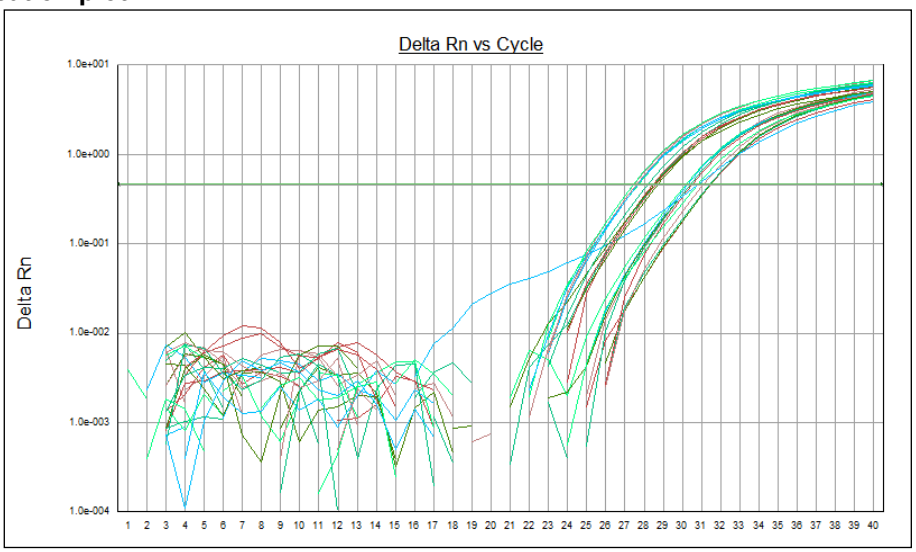
Gene expression plot:



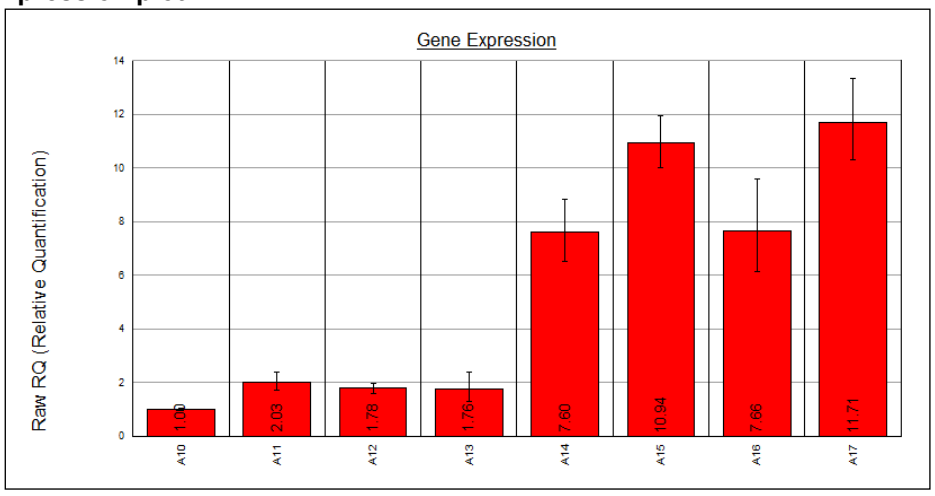
9. IL1R2

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	30.90±0.02	16.24±0.02	14.66±0.02	0.00±0.02	1.00
H2	29.71±0.09	16.07±0.01	13.64±0.09	-1.02±0.09	2.03
H3	29.88±0.05	16.05±0.02	13.83±0.05	-0.83±0.05	1.78
H4	30.21±0.16	16.38±0.03	13.84±0.16	-0.82±0.16	1.76
As1	28.11±0.04	16.38±0.03	11.73±0.08	-2.93±0.08	7.60
As2	27.14±0.03	15.94±0.04	11.21±0.05	-3.45±0.05	10.94
As3	27.86±0.12	16.14±0.02	11.72±0.12	-2.94±0.12	7.66
As4	27.03±0.06	15.93±0.03	11.11±0.07	-3.55±0.07	11.71

Amplification plot:



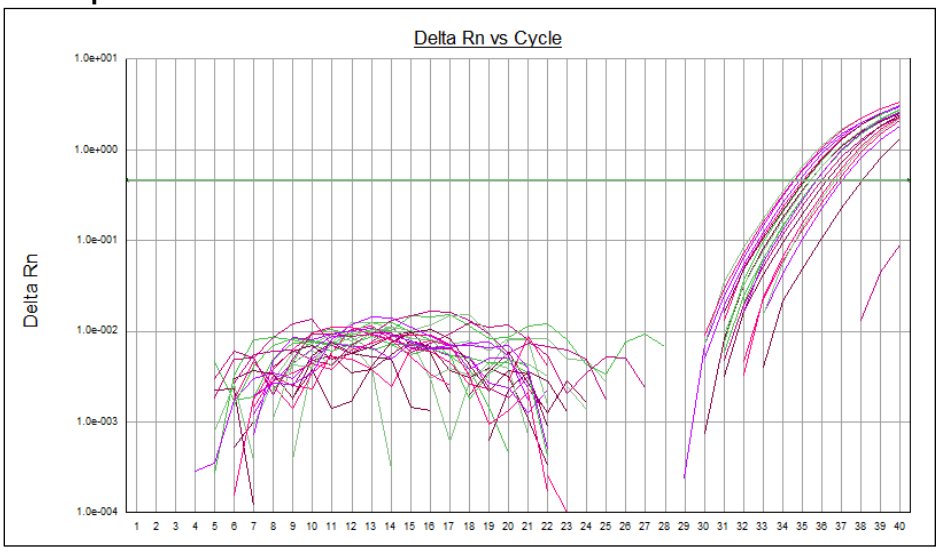
Gene expression plot:



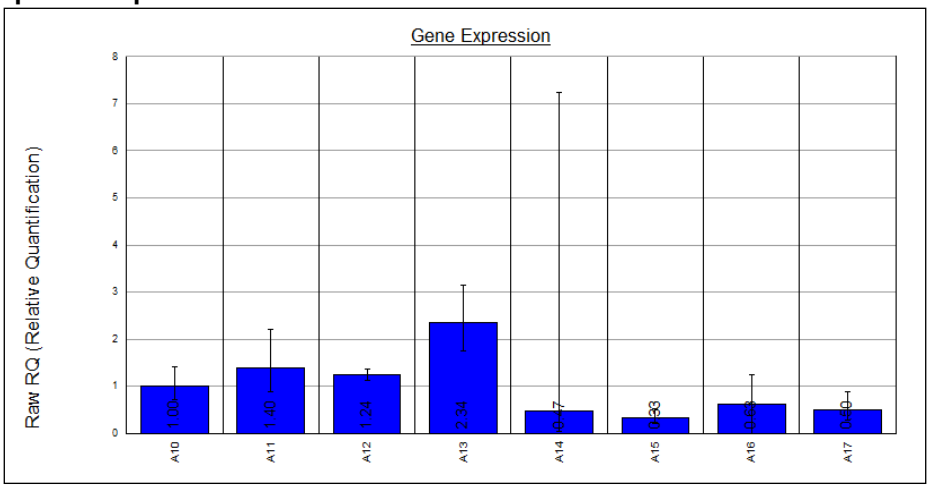
10. PCSK1

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	33.55±0.18	16.24±0.02	17.31±0.18	0.00±0.18	1.00
H2	32.90±0.24	16.07±0.01	16.83±0.24	-0.48±0.24	1.40
H3	33.05±0.04	16.05±0.02	17.00±0.04	-0.32±0.04	1.24
H4	32.46±0.15	16.38±0.03	16.08±0.15	-1.23±0.15	2.34
As1	34.77±1.23	16.38±0.03	18.39±1.24	1.08±1.24	0.47
As2	34.84±0.20	15.94±0.04	18.90±0.20	1.59±0.20	0.33
As3	34.13	16.14±0.02	17.98	0.67	0.63
As4	34.24±0.30	15.93±0.03	18.31±0.30	1.00±0.30	0.50

Amplification plot:



Gene expression plot:



Quantitative PCR Analysis Report with GUSB as Endogenous Control

Sample: human total RNA

H1, H2, H3, H4, As1, As2, As3, As4

Target Gene:

RGS2, PPP1R13B, MKNK1, TM4SF4, AKR1C3, TMEM70, TNFSF18, IGFL1,

IL1R2, PCSK1, KLK7, GUSB

- **Primer Sequence**

Human GUSB-F	TGATCGCTCACACCAAATCC
Human GUSB-R	CCCCTTGTCTGCTGCATAGTTA
Human KLK7-F	CTCATGTGCGTGGATGTCAAG
Human KLK7-R	GCGCACAGCATGGAATTTT

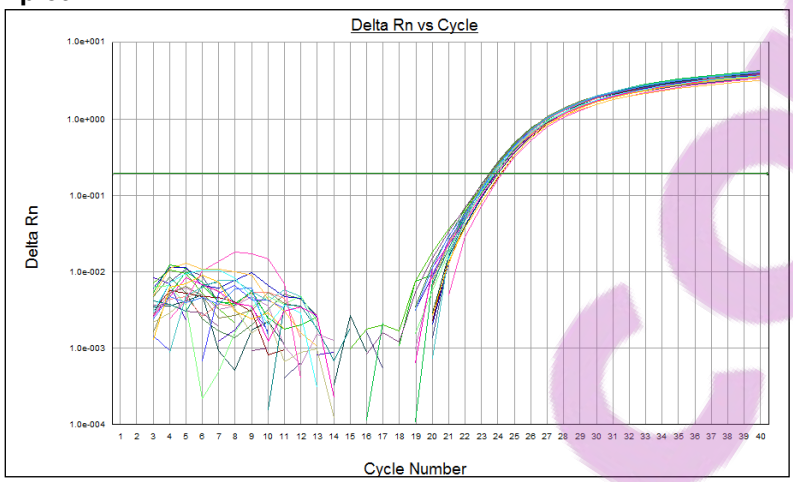
Result 1 (using **GAPDH** as endogenous control**):**

Relative quantitation using the comparative C_T method

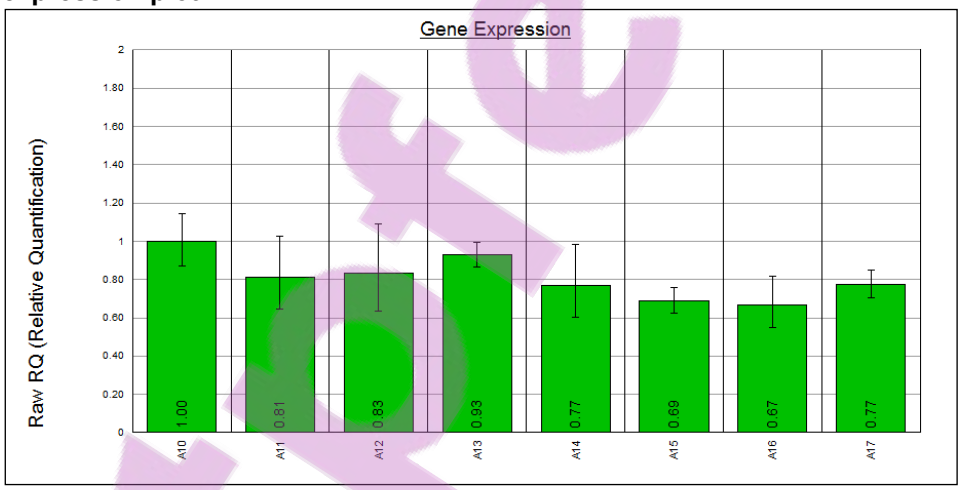
11. GUSB

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T,A10}$	Rel. to A10
H1	22.73±0.06	17.33±0.02	5.40±0.07	0.00±0.07	1.00
H2	22.85±0.11	17.14±0.02	5.70±0.12	0.29±0.12	0.81
H3	22.96±0.13	17.30±0.04	5.66±0.14	0.26±0.14	0.83
H4	22.79±0.03	17.28±0.01	5.51±0.03	0.10±0.03	0.92
As1	23.20±0.09	17.42±0.08	5.78±0.12	0.37±0.12	0.77
As2	22.87±0.01	16.93±0.04	5.94±0.05	0.53±0.05	0.68
As3	23.30±0.09	17.32±0.01	5.98±0.09	0.58±0.09	0.66
As4	22.94±0.04	17.16±0.01	5.77±0.04	0.37±0.04	0.77

Amplification plot:



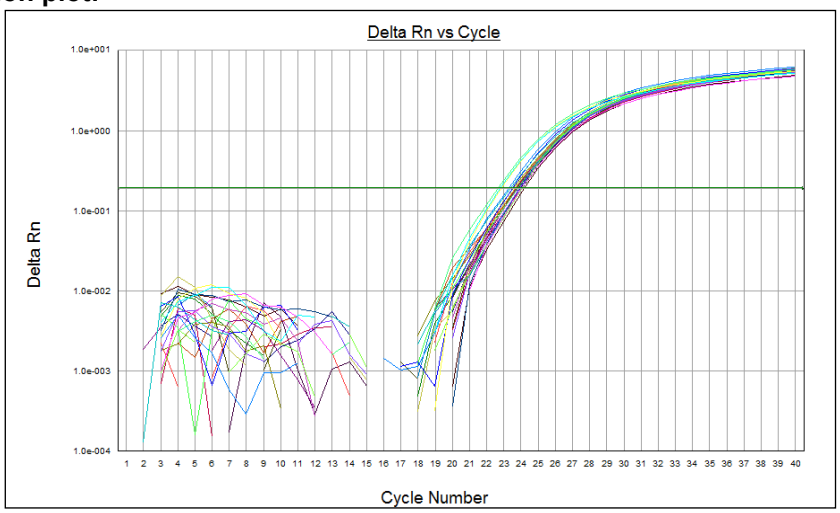
Gene expression plot:



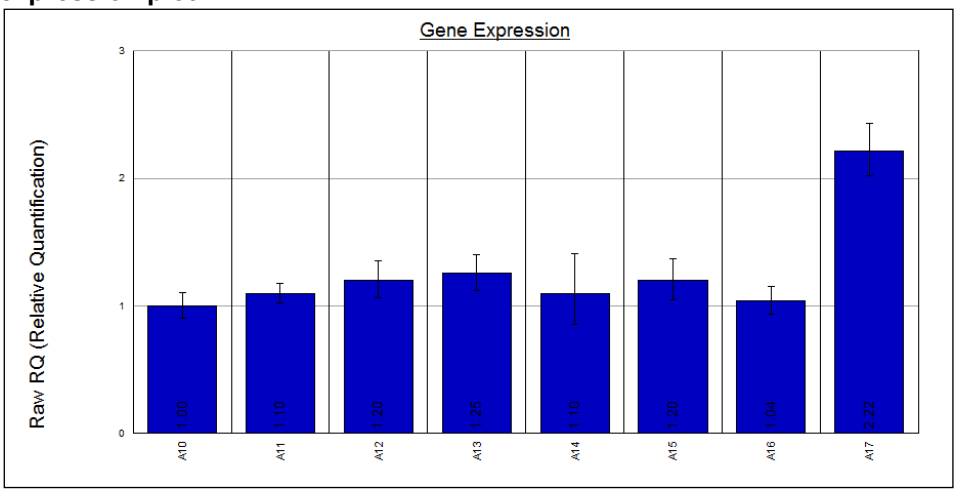
12. KLK7

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	24.86±0.04	17.33±0.02	7.52±0.05	0.00±0.05	1.00
H2	24.53±0.02	17.14±0.02	7.39±0.03	-0.13±0.03	1.09
H3	24.56±0.04	17.30±0.04	7.26±0.06	-0.26±0.06	1.19
H4	24.48±0.05	17.28±0.01	7.20±0.05	-0.32±0.05	1.25
As1	24.81±0.09	17.42±0.08	7.39±0.13	-0.13±0.13	1.09
As2	24.20±0.05	16.93±0.04	7.26±0.07	-0.26±0.07	1.19
As3	24.78±0.04	17.32±0.01	7.46±0.04	-0.06±0.04	1.04
As4	23.54±0.04	17.16±0.01	6.37±0.04	-1.15±0.04	2.21

Amplification plot:



Gene expression plot:



Result 2 (using GUSB as endogenous control):
Relative quantitation using the comparative C_T method

1. RGS2

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	27.78±0.11	16.24±0.02	11.53±0.11	0.00±0.11	1.00	1.00
H2	27.75±0.06	16.07±0.01	11.68±0.06	0.15±0.06	0.90	1.11
H3	27.38±0.10	16.05±0.02	11.33±0.10	-0.21±0.10	1.15	1.38
H4	27.99±0.02	16.38±0.03	11.62±0.04	0.09±0.04	0.94	1.02
As1	28.89±0.10	16.38±0.03	12.51±0.12	0.98±0.12	0.51	0.66
As2	28.36±0.06	15.94±0.04	12.42±0.07	0.89±0.07	0.54	0.79
As3	28.82±0.15	16.14±0.02	12.67±0.15	1.14±0.15	0.45	0.68
As4	28.41±0.04	15.93±0.03	12.48±0.05	0.95±0.05	0.52	0.67

2. PPP1R13B

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	26.64±0.07	16.24±0.02	10.39±0.07	0.00±0.07	1.00	1.00
H2	26.55±0.03	16.07±0.01	10.48±0.03	0.09±0.03	0.94	1.16
H3	26.19±0.05	16.05±0.02	10.14±0.05	-0.25±0.05	1.19	1.43
H4	25.97±0.04	16.38±0.03	9.60±0.05	-0.80±0.05	1.74	1.89
As1	27.14±0.05	16.38±0.03	10.76±0.08	0.36±0.08	0.78	1.01
As2	26.70±0.03	15.94±0.04	10.76±0.05	0.37±0.05	0.77	1.13
As3	27.08±0.06	16.14±0.02	10.94±0.07	0.54±0.07	0.69	1.04
As4	25.95±0.04	15.93±0.03	10.02±0.05	0.37±0.05	1.29	1.67

3. MKNK1

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	24.93±0.05	16.24±0.02	8.69±0.05	1.00±0.05	1.00	1.00
H2	24.73±0.03	16.07±0.01	8.66±0.03	-0.03±0.03	1.02	1.25
H3	24.43±0.07	16.05±0.02	8.38±0.07	-0.31±0.07	1.24	1.49
H4	24.49±0.05	16.38±0.03	8.12±0.06	-0.57±0.06	1.48	1.60
As1	25.26±0.06	16.38±0.03	8.88±0.09	0.20±0.09	0.87	1.12
As2	24.79±0.03	15.94±0.04	8.85±0.05	0.17±0.05	0.89	1.30
As3	25.18±0.05	16.14±0.02	9.04±0.05	0.35±0.05	0.78	1.18
As4	23.99±0.02	15.93±0.03	8.06±0.04	-0.63±0.04	1.54	2.00

4. TM4SF4

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10
H1	Undet.	16.24±0.02	Undet	Undet	Undet
H2	35.90±0.07	16.07±0.01	19.83±0.07	Undet	Undet
H3	Undet	16.05±0.02	Undet	Undet	Undet
H4	Undet	16.38±0.03	Undet	Undet	Undet
As1	Undet	16.38±0.03	Undet	Undet	Undet
As2	Undet	15.94±0.04	Undet	Undet	Undet
As3	Undet	16.14±0.02	Undet	Undet	Undet
As4	Undet	15.93±0.03	Undet	Undet	Undet

5. AKR1C3

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	23.15±0.05	16.24±0.02	6.90±0.05	0.00±0.05	1.00	1.00
H2	23.22±0.06	16.07±0.01	7.15±0.07	0.25±0.07	0.84	1.03
H3	22.86±0.11	16.05±0.02	6.81±0.11	-0.10±0.11	1.07	1.28
H4	23.08±0.03	16.38±0.03	6.70±0.04	-0.20±0.04	1.15	1.25
As1	20.29±0.04	16.38±0.03	3.91±0.08	-2.99±0.08	7.94	10.31
As2	19.55±0.06	15.94±0.04	3.61±0.07	-3.29±0.07	9.81	14.42
As3	19.99±0.12	16.14±0.02	3.84±0.12	-3.06±0.12	8.34	12.63
As4	19.39±0.02	15.93±0.03	3.46±0.04	-3.44±0.04	10.87	14.11

6. TMEM70

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	20.88±0.14	16.24±0.02	4.64±0.14	0.00±0.14	1.00	1.00
H2	20.21±0.04	16.07±0.01	4.14±0.05	-0.49±0.05	1.41	1.74
H3	20.30±0.12	16.05±0.02	4.25±0.12	-0.39±0.12	1.31	1.57
H4	19.95±0.06	16.38±0.03	3.57±0.07	-1.07±0.07	2.09	2.27
As1	21.00±0.01	16.38±0.03	4.62±0.07	-0.02±0.07	1.01	1.31
As2	19.85±0.10	15.94±0.04	3.91±0.11	-0.73±0.11	1.66	2.44
As3	20.74±0.32	16.14±0.02	1.60±0.32	-0.04±0.32	1.03	1.56
As4	19.29±0.03	15.93±0.03	3.37±0.04	-1.27±0.04	2.41	3.12

7. TNFSF18

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	31.84±0.04	16.24±0.02	15.60±0.04	0.00±0.04	1.00	1.00
H2	31.07±0.04	16.07±0.01	15.00±0.04	-0.60±0.04	1.52	1.87
H3	31.05±0.08	16.05±0.02	15.00±0.08	-0.61±0.08	1.52	1.83
H4	31.62±0.09	16.38±0.03	15.25±0.10	-0.36±0.10	1.28	1.39
As1	24.53±0.05	16.38±0.03	8.16±0.08	-7.45±0.08	174.47	226.58
As2	23.46±0.02	15.94±0.04	7.52±0.04	-8.09±0.04	271.53	399.30
As3	23.93±0.02	16.14±0.02	7.79±0.03	-7.82±0.03	225.43	341.56
As4	23.64±0.03	15.93±0.03	7.71±0.05	-7.89±0.05	237.67	308.66

8. IGFL1

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	26.85±0.08	16.24±0.02	10.61±0.09	0.00±0.09	1.00	1.00
H2	26.70±0.01	16.07±0.01	10.63±0.02	0.02±0.02	0.99	1.22
H3	26.70±0.04	16.05±0.02	10.65±0.05	0.04±0.05	0.97	1.16
H4	26.45±0.02	16.38±0.03	10.08±0.04	-0.53±0.04	1.44	1.56
As1	26.14±0.01	16.38±0.03	9.76±0.07	-0.84±0.07	1.79	2.32
As2	25.04±0.12	15.94±0.04	9.10±0.13	-1.51±0.13	2.85	4.19
As3	25.61±0.05	16.14±0.02	9.47±0.05	-1.14±0.05	2.20	3.33
As4	23.12±0.02	15.93±0.03	7.19±0.04	-3.41±0.04	10.66	13.84

9. IL1R2

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	30.90±0.02	16.24±0.02	14.66±0.02	0.00±0.02	1.00	1.00
H2	29.71±0.09	16.07±0.01	13.64±0.09	-1.02±0.09	2.03	2.50
H3	29.88±0.05	16.05±0.02	13.83±0.05	-0.83±0.05	1.78	2.14
H4	30.21±0.16	16.38±0.03	13.84±0.16	-0.82±0.16	1.76	1.91
As1	28.11±0.04	16.38±0.03	11.73±0.08	-2.93±0.08	7.60	9.87
As2	27.14±0.03	15.94±0.04	11.21±0.05	-3.45±0.05	10.94	16.08
As3	27.86±0.12	16.14±0.02	11.72±0.12	-2.94±0.12	7.66	11.60
As4	27.03±0.06	15.93±0.03	11.11±0.07	-3.55±0.07	11.71	15.20

10. PCSK1

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	33.55±0.18	16.24±0.02	17.31±0.18	0.00±0.18	1.00	1.00
H2	32.90±0.24	16.07±0.01	16.83±0.24	-0.48±0.24	1.40	1.72
H3	33.05±0.04	16.05±0.02	17.00±0.04	-0.32±0.04	1.24	1.49
H4	32.46±0.15	16.38±0.03	16.08±0.15	-1.23±0.15	2.34	2.54
As1	34.77±1.23	16.38±0.03	18.39±1.24	1.08±1.24	0.47	0.61
As2	34.84±0.20	15.94±0.04	18.90±0.20	1.59±0.20	0.33	0.48
As3	34.13	16.14±0.02	17.98	0.67	0.63	0.95
As4	34.24±0.30	15.93±0.03	18.31±0.30	1.00±0.30	0.50	0.64

11. KLK7

Sample	Target Average C_T	GAPDH Average C_T	ΔC_T Target - GAPDH	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,A10}$	Rel. to A10 (GAPDH as control)	Rel. to A10 (GUSB as control)
H1	24.86±0.04	17.33±0.02	7.52±0.05	0.00±0.05	1.00	1.00
H2	24.53±0.02	17.14±0.02	7.39±0.03	-0.13±0.03	1.09	1.34
H3	24.56±0.04	17.30±0.04	7.26±0.06	-0.26±0.06	1.19	1.43
H4	24.48±0.05	17.28±0.01	7.20±0.05	-0.32±0.05	1.25	1.35
As1	24.81±0.09	17.42±0.08	7.39±0.13	-0.13±0.13	1.09	1.41
As2	24.20±0.05	16.93±0.04	7.26±0.07	-0.26±0.07	1.19	1.75
As3	24.78±0.04	17.32±0.01	7.46±0.04	-0.06±0.04	1.04	1.57
As4	23.54±0.04	17.16±0.01	6.37±0.04	-1.15±0.04	2.21	2.87