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Transcriptomic analysis of cultured whale skin cells exposed to hexavalent chromium [Cr(VI)]

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ABSTRACT

Hexavalent chromium Cr(VI) is known to produce cytotoxic effects in humans and is a highly toxic environmental contaminant. Interestingly, it has been shown that free ranging sperm whales (*Physeter macrocephalus*) may have exceedingly high levels of Cr in their skin. Also, it has been demonstrated that skin cells from whales appear more resistant to both cytotoxicity and clastogenicity upon Cr exposure compared to human cells. However, the molecular genetic mechanisms employed in whale skin cells that might lead to Cr tolerance are unknown.

In an effort to understand the underlying mechanisms of Cr(VI) tolerance and to illuminate global gene expression patterns modulated by Cr, we exposed whale skin cells in culture to varying levels of Cr(VI) (i.e., 0.0, 0.5, 1.0 and 5.0 $\mu g/cm^2$) followed by short read (100 bp) next generation RNA sequencing (RNA-seq). RNA-seq reads from all exposures (≈280 million reads) were pooled to generate a de novo reference transcriptome assembly. The resulting whale reference assembly had 11 K contigs and an N50 of 2954 bp.

Using the reads from each dose (0.0, 0.5, 1.0 and 5.0 $\mu g/cm^2$) we performed RNA-seq based gene expression analysis that identified 35 up-regulated genes and 19 down-regulated genes. The experimental results suggest that low dose exposure to Cr (1.0 $\mu g/cm^2$) serves to induce up-regulation of oxidative stress response genes, DNA repair genes and cell cycle regulator genes. However, at higher doses (5.0 $\mu g/cm^2$) the DNA repair genes appeared down-regulated while other genes that were induced suggest the initiation of cytotoxicity.

The set of genes identified that show regulatory modulation at different Cr doses provide specific candidates for further studies aimed at determination of how whales exhibit resistance to Cr toxicity and what role(s) reactive oxygen species (ROS) may play in this process.

1. Introduction

Environmental contamination from heavy metals has been well documented and represents a global concern for both ecosystems and human health (US Department of Health and Human Services, 1993). Among heavy metals, Cr [as Cr(VI)] is considered a human health concern and most of the Cr released into the environment stems from human commercial and industrial activities (e.g., fossil fuel consumption, tanning, textiles, and electroplating) (Cohen et al., 1993; Salnikow and Zhitkovich, 2008). It is known that Cr(VI) easily enters cells and upon crossing the cell membrane becomes rapidly reduced to Cr(III). The reduced Cr(VI) may bind to DNA and or induce DNA damage and it has been documented that Cr exposure is carcinogenic (O'Brien et al., 2003; Wise et al., 2008). For example, DNA double strand breaks are thought to result from ternary adducts caused by Cr stalling replication forks (Wise et al., 2008). It was found that Cr(VI) exposures decreased clonogenic survival in a dose-dependent manner (from 8 to 83%) and a strong response of cell cycle arrest, DNA double strand breaks and chromosome damage. In these studies less than 7% of the cells underwent apoptosis, but had increased activation of ATM and SMC1 (Xie et al., 2005).

A recent global assessment of Cr pollution in marine ecosystems measured in biopsies taken from free ranging whales across the globe revealed that many whales had skin Cr levels roughly 28

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fold higher than those obtained from human skin samples (Wise et al., 2009). The levels found in whale biopsies correlated with human samples previously observed in occupationally exposed humans with lung cancer (Tsuneta et al., 1980). Complementing the observation of high Cr levels in whales are several reports detailing Cr induced cyto- and genotoxicity using cultured whale and human fibroblast cells exposed to various levels of Cr (Li Chen et al., 2012; Wise, 2012). Cumulatively, these reports indicate that whale cells are able to tolerate higher levels of intercellular Cr, and exhibit lesser cytogenetic damage than human cells. The source and manner in which whales accumulate Cr in the deep ocean is not understood, nor are the genetic mechanisms that allow whale cells to exhibit Cr resistance.

In an effort to better understand the underlying mechanisms of Cr induced cyto- and genotoxicity, and how whale cells ameliorate Cr induced damage, we performed RNA-seq analysis on sperm whale skin cells after exposure to varying levels of Cr (e.g., 0.0, 0.5, 1.0, 5.0 μg/cm^2 Cr(VI)). Herein, we report assembly of a de novo sperm whale reference transcriptome based upon next generation sequencing reads (Illumina HiSeq, 100 bp paired-end; PE) from fibroblasts (cell culture). This reference transcriptome was utilized to assess global gene expression and identify genes exhibiting modulated expression upon the exposure to varying levels of Cr.

2. Methods

2.1. Cell culture and chromium exposure

Sperm whale skin fibroblasts were maintained as adherent subconfluent monolayers, fed at least twice a week, and subcultured at least once a week as described previously (Wise et al., 2011). All experiments were conducted on logarithmically growing cells cultured in DMEM/F-12 supplemented with 15% Cosmic calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM sodium pyruvate. Cells were grown in a 33 °C, humidified incubator with 5% CO_2 and routinely tested for *Mycoplasma* contamination.

Lead chromate (CAS# 7758-97-6, ACS reagent minimum 98% purity), was used as a representative particulate Cr(VI) compound and was administered as a suspension in water as previously described (Wise et al., 2002). Because lead chromate is an insoluble compound that only partially dissolves, treatments are expressed as weight per surface area (μg/cm^2). Lead chromate was chosen because it represents particulate Cr(VI) compounds, which is the more potent carcinogenic form of Cr(VI). Studies show that the genotoxicity of lead chromate is all due to the chromate anion with the lead cation having no role (Wise et al., 1994, 2005; Holmes et al., 2005).

2.2. RNA isolation and next generation sequencing

Logarithmically growing cells were seeded in 100 mm tissue culture plates and treated with 0.0–5.0 μg/cm^2 lead chromate for 24 h and then their RNA was isolated. RNA samples were sequenced using the Illumina HiSeq platform as 100 bp, PE reads. All short read data was converted to FASTQ files for filteration. The raw sequence reads were filtered using in-house software that utilized the following protocol: (1) adapter sequences from the PE FASTQ reads were removed, (2) raw PE reads with stretches of bad quality scores (score of 2, encoded in FASTQ format as 'B') were then trimmed, being untrustworthy, (3) bases with quality scores below 10 along with positions having a mean quality score of less than 20 for a window of size 3 centered on them were trimmed, (4) only the longest fragment from the remaining reads were then retained, (5) PE reads were ordered and the overlapping reads were merged, and (6) lastly the reads that had lost their PE mates were kept as single end reads (Garcia et al., 2012). After the above filtration, we had approximately 52 GB of data representing a total of ≈250 million reads, paired and single end combined.

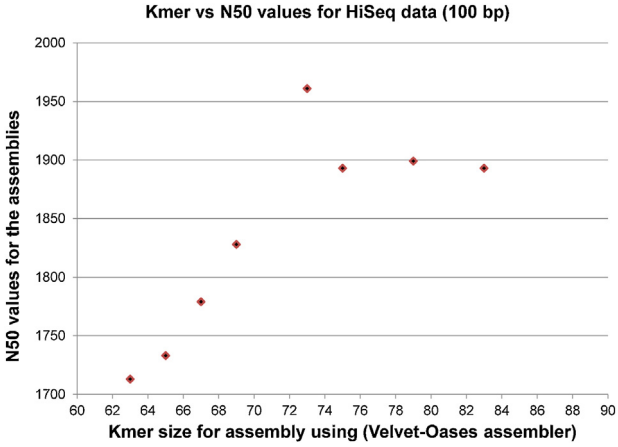

Fig. 1. Plot showing K-mer sizes and N50 values for assemblies generated using Velvet–Oases assembler. Note: the highest N50 value is associated with K-mer size of 73 bp.

A transcriptome assembly was systematically built after using different K-mer values for Velvet assembler (v1.2.03) along with its extension, Oases (v0.2.06) (Zerbino and Birney, 2008). Assemblies were analyzed for K-mer sizes between 63 and 83 bp and N50 values were calculated for each assembly. Based on the N50 plot over the range of K-mer values (Fig. 1), we used the assembly constructed from K-mer size of 73 bp, comprised of 193,644 contigs with N50 of 1961 bp and average length of 915 bp, for further assessment.

To annotate the 73-mer reference assembly, we performed BLASTX comparisons with bovine (Bos_taurus.UMD3.1.68.pep.all.fa.gz) and dolphin (Tursiops_truncatus.turTru1.68.pep.all.fa.gz) protein sequences downloaded from the Ensemble database (Flicek et al., 2012). These BLASTX searches were performed at an *e*-value cutoff of e^{-10} (Altschul et al., 1990). Comparison of the 73-mer assembled transcripts versus the bovine protein sequences resulted in ≈60% of the whale transcript contigs containing a known peptide motif. Transcripts with the highest coverage in respect to the bovine database were kept (i.e., the transcripts with the longest sequences and highest sequence identity versus bovine protein sequences) and used as the final whale reference assembly. The whale reference assembly so developed is comprised of 11,286 contigs with an N50 value of 2954 bp.

Filtered RNA-seq reads derived from each of the cell cultures representing different Cr exposures were mapped back onto the reference transcriptome assembly. Mapping of the reads was performed using the Bowtie software package (v0.12.7) employing default options, except for the – M 1 (to increase the number of reads mapped), for picking one random best hit for mapping against the assembly (Langmead et al., 2009). Approximately 25% of the filtered Illumina reads from all Cr exposed cell culture datasets were able to map onto the reference assembly. Counting of mapped reads was performed using SAMtools function Idxstats (Li et al., 2009), which were further used to assess differential gene expression.

2.3. Gene expression analysis

To identify genes differentially regulated in response to Cr(VI) exposure we employed the DESeq software package [from R