Introduction

Hexavalent chromium [Cr(VI)] is widely used in numerous industrial processes, including chrome pigment production, chrome plating, stainless steel manufacturing, and leather tanning, etc. Epidemiological studies have reported a high incidence of lung cancer among chromium workers exposed occupationally to Cr(VI) by inhalation [1–3]. An early epidemiology study showed that 21.8% of deaths among chromium workers were due to respiratory cancer while only 1.4% of deaths could be attributed to respiratory cancer in the unexposed reference population [2]. The lung cancer risk among chromium workers was positively correlated with a longer duration of employment and with exposure to less water-soluble chromium compounds [2]. Numerous studies suggested that chromium induced DNA damage, mutation, genetic instability and epigenetic modulation of histones and DNA may contribute to its carcinogenicity, however, the molecular mechanisms of Cr(VI) induced lung cancer are not well understood.

Chromate can induce a wide variety of injuries in cells. After entering cells, Cr(VI) undergoes a series of metabolic reductions to form reactive Cr(V) and Cr(IV) intermediates as well as the final stable metabolite Cr(III) [4][5]. These reactive intermediates and final products generated from the reduction process are able to induce the formation of stable Cr-DNA ternary adducts, protein-DNA cross-links, and DNA-DNA cross-links. These modifications, in combination with reactive oxygen species (ROS), may generate DNA single or double-strand breaks, which in turn may lead to mutations, chromosomal aberrations, and microsatellite instability [6][7]. An increased frequency of microsatellite instability in Cr(VI)-induced lung tumors has been attributed to the ability of chromate to disrupt DNA mismatch repair [8][9].

In addition to DNA damage, Cr(VI) is able to induce a broad range of changes in the epigenetic machinery. Chromium exposure of G12 Chinese hamster cells increased both genome-wide and gene-specific DNA methylation and silenced the expression of a gpt transgene [10]. In human lung cells, chromium exposure modulated histone methylation in both global and gene-specific fashions. The mechanisms by which Cr(VI) induces DNA damage and how these changes contribute to its carcinogenicity remain to be elucidated.
promoter-specific manner [11]. Interestingly, Histone H3 lysine 9
dimethylation, a silencing mark, was enriched in the human DNA
mismatch repair MLH1 gene promoter following chromate
exposure and this was correlated with decreased MLH1 mRNA
expression [11]. Moreover, increased DNA methylation in the
promoter region of MLH1 gene and subsequent gene silencing
were found in chromium-induced human lung tumors [8],
suggesting epigenetic modulation as an important mechanism
mediating Cr(VI)-induced lung carcinogenesis.

Cr(VI) induced tumorigenesis is thought to be a multistep
process involving DNA damage, mutation, chromosome instabi-
lity, aneuploidy, as well as epigenetic modulation [3]. The
ultimate outcome of this process is the malignant cell phenotype
that exhibited an altered gene expression profile. Previous studies
have shown changes in gene expression following acute exposure
of human cells to chromate (1 day or less), and identified a number
of genes that were altered in response to acute chromate induced
stress [12–14]. However, due to the complex effects of chromate in
cells, changes noted in these early response genes may not play a
role in cell transformation and tumorigenesis that arises with a
latency period of at least a month. The purpose of our study was to
identify genes that are characteristic of Cr(VI) induced cell
transformation. Here, we employed a strategy to select cells for
anchorage-independent growth following chronic chromate expo-
sure. Our results showed that chronic exposure of immortalized
normal human bronchial epithelial BEAS-2B cells to low doses of
chromate promoted a high incidence of anchorage-independent
growth. Interestingly, cell lines derived from soft agar colonies
following chromate exposure exhibited altered morphology
compared to cell lines derived from the untreated small colonies
that arose spontaneously which morphologically resembled
normal BEAS-2B cells. 409 differentially expressed genes were
identified in Cr(VI) transformed cells compared to control cells,
and these changes contributed to the functional and phenotypic
differences between these two cell populations. It is of interest that
gene expression profiles in six independent cell lines derived from
Cr(VI) treated cells were very similar to each other, yet differed
greatly from gene expression profiles in control cell lines derived
from colonies that arose spontaneously in soft agar.

Results

Establishment of chromate transformed cell lines

With the exception of cells from hematopoietic and lymphoid
lineages, most normal cells rely on physical attachment to the
extracellular matrix in order to manifest normal cell growth. In
contrast, many transformed cells acquire the ability to grow and
survive without attaching themselves to the substratum, a
phenomenon commonly known as anchorage-independent
growth. Due to its high correlation with tumor progression in vivo,
anchorage independent growth has been considered a hallmark of
cell malignancy and is used as a marker for in vitro cell
transformation assays [15][16]. In order to establish Cr(VI)
transformed cell lines, we employed a strategy to select and sub-
cloned the transformed cells by taking advantage of their ability to
grow in an anchorage-independent manner (Figure 1A).

To mimic low dose and long term exposure in vivo, immortalized
human bronchial epithelial BEAS-2B cells were continuously
cultured in medium containing 0.25 or 0.5 μM of Cr(VI), doses in
which BEAS-2B cells exhibited minimal toxicity (Figure 1B).

Figure 1. Effect of Cr(VI) on colony survival of immortalized human bronchial epithelial BEAS-2B cells. (A) Schematic presentation of
the strategy to establish Cr(VI) transformed cell lines. (B) BEAS-2B cells were exposed to different doses of Cr(VI) (0.25, 0.5, 1.0, 2.0, and 4.0 μM) for
24 hours, and then subjected to colony survival assay in the absence of Cr(VI) for 2 weeks. Cell colonies were stained with Giemsa solution. The
number of colonies was counted and presented as the mean ± SD (n = 3).
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During a 4-week exposure, cells were maintained in a sub-confluent state. While Cr(VI) treated cells exhibited a slower growth rate compared to untreated cells, no obvious difference in cell morphology was observed during the treatment (data not shown). After a 4-week exposure, anchorage-independent growth was assessed in both untreated and chromate treated BEAS-2B cells. Both Cr(VI) treated and untreated control BEAS-2B cells were grown in 0.35% top agar for three weeks. While there was a low level of background growth with untreated BEAS-2B cells, both 0.25 and 0.5 μM Cr(VI) treated cells formed significantly more colonies in soft agar (Figure 2A). Cells exposed to Cr(VI) demonstrated the greatest number of colonies that were three times more abundant compared to the untreated cells (Figure 2B). These results indicated that Cr(VI) exposure was able to significantly enhance anchorage-independent growth of BEAS-2B cells. After 3 weeks of growth in soft agar, colonies reached a size that allowed them to be collected individually using a dissecting microscope. Five colonies were isolated from untreated colonies that arose spontaneously in soft agar and this group was designated as the control group. Ten colonies were selected from the 0.5 μM Cr(VI) exposure for further study. Five of these were large colonies and therefore designated as the Cr_large group, while the other five were selected to best match the size of the untreated control colonies and designated as Cr_small group. Isolated colonies were trypsinized and expanded in monolayer culture. Thus, each sub-cloned cell line contained a cell population derived from a single cell.

After expansion in monolayer culture, Cr(VI) transformed cells exhibited distinct cell morphology compared to control cells. As shown in Figure 3A, control cells were flat, diamond-shaped, and similar to their parental BEAS-2B cells, while cells in both the Cr_large and the Cr_small groups were rounder, forming a more compact “cobblestone” monolayer. In addition, these Cr(VI) transformed cells easily detached from the culture surface when trypsinized, and exhibited a slightly faster growth rate as compared to control cells (Figure 3B). Moreover, 9 out of 12 mice injected with cells from Cr_large and Cr_small group developed tumors, while the mice injected with control cells did not form tumors after 6 months (Figure 3C). These data indicated that chronic exposure to Cr(VI) resulted in malignant transformation of BEAS-2B cells.

Differential gene expression profiles in control and Cr(VI) transformed cells

Cr(VI) induced cell transformation is a multistep process in which cells go through an initial adaption to Cr(VI)-induced cell death and toxicity, then displayed anchorage-independent growth, and with time acquired the transformed phenotype. In order to characterize Cr(VI) transformed cells and identify the genes mediating Cr(VI) transformation, we analyzed the gene expression profiles in Cr(VI) transformed cell lines using microarray analysis. Three independent cell lines from each group (Cr_large, Cr_small, untreated control) were randomly chosen and subjected to microarray analysis using Affymetrix Human Gene 1.0 ST Array containing 28,869 well-annotated genes. Parental BEAS-2B cells were also included in this study.

First, we explored the microarray results by comparing gene expression profiles among Cr_large, Cr_small and untreated control groups. As shown in Figure 4A, a total of 1299 genes in the Cr_small group and 1216 genes in the Cr_large group displayed a greater than 1.3-fold difference as compared with the control group. When the cut-off threshold was increased to a 5.0-fold difference, the numbers decreased to 40 and 47 respectively (Figure 4B). Interestingly, when the Cr_small group and the Cr_large group were compared, only 21 genes whose expression changed more than 1.5-fold showed a statistically significant difference between the two groups (p<0.05, alpha level only), but none of these genes exhibited more than a 2.0-fold change, suggesting that cells from the Cr_large and the Cr_small group shared a very similar gene expression pattern. Principal Components Analysis (PCA) of the microarray data revealed a clear separation among samples from the control group and those from both Cr(VI) transformed groups (Figure 4C, square vs triangle), but not between the Cr_large and the Cr_small groups (Figure 4B, brown vs blue triangles). The size of the small colonies derived from the Cr(VI) treated cells were specifically selected to match the size of the control colonies, indicating that it is the Cr(VI) treatment and not the colony size that contributed to the observed differences in gene expression.

Identification of differentially expressed genes in Cr(VI) transformed cells

Since the gene expression pattern from the Cr_large group and the Cr_small group were quite similar, a combined gene list from
two groups was generated for further comparison with the untreated control. After elimination of the probe sets that represented unannotated genes, there were a total of 45 genes that changed more than 5-fold in 1 out of 2 groups in the Cr(VI) transformed cells compared with the control group, including 23 down-regulated and 22 up-regulated genes. The gene names and fold change of these two groups are listed in Table 1.

Among genes up-regulated in chromate transformed cells, there were two major sub-groups. The first group was related to female reproduction, including 5 pregnancy specific beta-1-glycoproteins.
Table 1. Genes with more than 5-fold changed expression in Cr(VI) transformed cells.

| Affymetrix ID | Gene Symbol | Gene Name | Fold change (Cr_large vs. control) | Fold change (Cr_small vs. control) |
|---------------|-------------|-----------|-----------------------------------|-----------------------------------|
| 8022692       | DSC3        | desmocollin 3 | 36.99                             | 40.20                             |
| 8069676       | ADAMTS1     | ADAM metallopeptidase with thrombospondin type 1 motif, 1 | 16.80                             | 28.63                             |
| 8174513       | CHRD1       | chordin-like 1 | 14.05                             | 18.21                             |
| 8037272       | PSG5        | pregnancy specific beta-1-glycoprotein 5 | 10.33                             | 9.21                              |
| 8037267       | PSG2        | pregnancy specific beta-1-glycoprotein 2 | 10.32                             | 8.06                              |
| 8072626       | TIMP3       | TIMP metallopeptidase inhibitor 3 | 9.43                              | 7.55                              |
| 8037251       | PSG7/8/4    | pregnancy specific beta-1-glycoprotein 7/8/4 | 9.41                              | 8.41                              |
| 7961142       | OLR1        | oxidized low density lipoprotein (lectin-like) receptor 1 | 9.29                              | 4.89                              |
| 8169949       | RP6-213H19.1| serine/threonine protein kinase MST4 | 8.94                              | 6.11                              |
| 8022711       | DSC2        | desmocollin 2 | 8.00                              | 7.03                              |
| 7912520       | NPPB        | natriuretic peptide precursor B | 6.80                              | 4.71                              |
| 8037231       | PSG3        | pregnancy specific beta-1-glycoprotein 3 | 6.73                              | 6.15                              |
| 8112274       | ELOVL7      | ELOVL family member 7 | 6.04                              | 4.72                              |
| 8059279       | EPHA4       | EPH receptor A4 | 5.86                              | 5.52                              |
| 8168589       | ZNF711      | zinc finger protein 711 | 5.67                              | 3.97                              |
| 8129880       | PERP        | PERP, TP53 apoptosis effector | 5.61                              | 3.11                              |
| 8015268       | KRT34       | keratin 34 | 5.36                              | 3.66                              |
| 8152506       | SAMD12      | sterile alpha motif domain containing 12 | 4.79                              | 5.96                              |
| 8063536       | TFAP2C      | transcription factor AP-2 gamma | 4.74                              | 5.10                              |
| 8058091       | SATB2       | SATB homeobox 2 | 4.68                              | 5.59                              |
| 8069689       | ADAMTS5     | ADAM metallopeptidase with thrombospondin type 1 motif, 5 | 4.52                              | 5.45                              |
| 8037240       | PSG1        | pregnancy specific beta-1-glycoprotein 1 | 3.65                              | 5.37                              |
| 7997642       | CRISPLD2    | cysteine-rich secretory protein LCCL domain containing 2 | -5.02                             | -5.93                             |
| 8176578       | USP9Y       | ubiquitin specific peptidase 9, Y-linked | -5.07                             | -5.50                             |
| 8113073       | ARRDC3      | arrestin domain containing 3 | -5.23                             | -4.66                             |
| 8092040       | CPE         | carboxypeptidase E | -5.28                             | -4.34                             |
| 8089145       | ABI3BP      | ABI gene family, member 3 (NESH) binding protein | -5.44                             | -4.74                             |
| 8029779       | IGF1        | IGF-like family member 1 | -5.47                             | -6.36                             |
| 8170648       | BGN         | biglycan | -5.58                             | -5.76                             |
| 8067233       | PMEPF1      | prostate transmembrane protein, androgen induced 1 | -5.98                             | -5.70                             |
| 8157524       | TLH4        | toll-like receptor 4 | -6.62                             | -8.58                             |
| 8059580       | DNER        | delta/notch-like EGF repeat containing | -6.76                             | -6.34                             |
| 8121277       | AIM1        | absent in melanoma 1 | -6.91                             | -6.69                             |
| 7902565       | LPHN2       | latrophilin 2 | -7.64                             | -3.69                             |
| 8051583       | CYP1B1      | cytochrome P450, family 1, subfamily B, polypeptide 1 | -7.87                             | -3.65                             |
| 8176384       | ZFY         | zinc finger protein, Y-linked | -7.95                             | -8.34                             |
| 8177222       | CD24        | CD24 molecule | -11.36                            | -9.99                             |
| 8097628       | HHIIP       | hedgehog interacting protein | -11.53                            | -12.45                            |
| 7981986       | SNRPN       | small nuclear ribonucleoprotein polypeptide N | -11.73                            | -11.80                            |
| 8104746       | NPR3        | natriuretic peptide receptor C/guanylate cyclase C | -12.13                            | -13.03                            |
| 8122150       | EYA4        | eyes absent homolog 4 (Drosophila) | -12.48                            | -12.72                            |
| 8176719       | EIF1AY      | eukaryotic translation initiation factor 1A, Y-linked | -14.55                            | -15.00                            |
| 8057620       | COL5A2      | collagen, type V, alpha 2 | -19.76                            | -18.94                            |
| 8176624       | DDX3Y       | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked | -25.71                            | -26.58                            |
| 8176375       | RP54Y1      | ribosomal protein S4, Y-linked 1 | -32.64                            | -32.95                            |

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(PSG1, 2, 3, 5, and 7) that were clustered on human chromosome 19. The second group contained three genes (DSC2, DSC3, and Perp) required for the assembly of the desmosome complex, a cell-to-cell junction important for maintaining the structural integrity of the epithelia.

In contrast to the up-regulated genes, the down-regulated gene exhibited more diversification in functional categories. Most of down-regulated genes encoded proteins that were either localized in the plasma membrane or in the extracellular space. For example, a major sub-group of the down-regulated genes encoded proteins associated with a cell surface receptor that mediated cell signaling, including CD24, DDX3Y, BGN, CPE, DNER, HHHIP, LPHN2, and TLR4. Interestingly, among the most significantly down-regulated genes, RPS4Y1, DDX3Y, EIF1AY, ZFY and USP9Y, were located on the Y chromosome. The five PSG genes increased in Cr(VI) transformed cells were also localized in a cluster on chromosome 19. It is not clear whether these changes were gene-specific or loci-specific. Cr(VI) exposure has been shown to induce chromosome instability, including both numerical and structural aberrations, which might cause similar expression changes in multiple genes clustered in a chromosomal region. It is worth noting that all six Cr(VI) transformed cell lines exhibited similar changes in these two sub-groups of genes. Further analysis of chromosome damage and aberration as well as epigenetic mapping of these Cr(VI) transformed cells will help us to understand the underlying mechanism for these gene expression changes.

Real time PCR Validation of gene expression

To validate the results obtained from our microarray study, quantitative real-time PCR was performed on a subset of 4 genes exhibiting at least a 5-fold change in gene expression. Three up-regulated genes (KRT34, DSC3, PSG2) and 1 down-regulated gene (RSP4Y1) were chosen based on their levels of expression in the microarray study. Differential expression of these genes in either Cr(VI) Large or Cr(VI) Small samples was validated using quantitative real-time PCR. As shown in Figure 5, the up- or down-regulated patterns for 4 genes obtained from real-time PCR were similar to those in the microarray study.

Functional annotation and pathway analysis of differentially expressed genes

To assess the biological relevance of the differentially expressed genes, we performed the Gene Ontology (GO) annotation and pathway analysis using the DAVID functional annotation software [17]. The combined gene list from two Cr(VI) transformed groups exhibiting more than a 2-fold change in gene expression were used to identify the functional categories that were significantly over-represented (p<0.05) in Cr(VI) transformed cells. There were a total of 409 genes, including 142 up-regulated and 267 down-regulated genes (Table S1). GO term and KEGG pathways with significant over-representation were listed in Table 2 (up-regulated genes) and Table 3 (down-regulated genes). Among 142 up-regulated genes, the largest group with respect to both degree of significance (p<0.01) and number of genes was extracellular region (n = 22) in Cellular component, followed by cell motion (n = 10) in Biological process. The major pathways associated with up-regulated genes are p53 pathways (n = 3) and PPAR pathways (n = 3). Among 267 down-regulated genes, the largest group with respect to both degree of significance (p<0.001) and number of genes was extracellular region (n = 22) in Cellular component, followed by cell motion (n = 10) in Biological process. The major pathways associated with down-regulated genes were Focal adhesion (n = 12) and ECM-receptor interaction (n = 7). The following is a summary of genes changed in several major functional categories.

Changes in genes associated with cell junction. A major group of genes altered in Cr(VI) transformed cells were genes related to cell junction, a type of specialized structure mediating the contact between cells or between cells and extracellular matrix. In epithelial cells, there are four major types of cell-cell junctions: tight junction, gap junction, adherens junction and desmosomes. The three major groups of the desmosome complex, DSC2, DSC3, and Perp, were increased approximately 8-, 40- and 5-fold, respectively, in Cr(VI) transformed cells. In addition, CDH6, a type II classical cadherin involved in adherens junction, and CLDN1, the major protein for tight junction, were also up-regulated 4- and 2-fold, respectively, in Cr(VI) transformed cells. L1CAM, the L1 cell adhesion molecule, increased 4.7-fold in Cr(VI) transformed cells. Thus, genes associated with cell junction were up-regulated in Cr(VI) transformed cells.

Changes in genes associated with cell to extracellular matrix adhesion. In contrast to up-regulation of genes related to cell-to-cell contact, many genes involved in focal adhesion, a major type of cell junction mediating cell and extracellular matrix interaction, were decreased in Cr(VI) transformed cells. Integrins, the key component family of focal adhesion, are transmembrane receptors that recognize and bind to most extracellular matrix proteins, such as collagen, fibronectin, and laminins [18]. Each integrin molecule is a heterodimer formed from 9 beta and 25 alpha subunits. Cr(VI) transformed cells exhibited decreased integrin alpha 5 (ITGA5), beta 3 (ITGB3) and beta-like 1 subunits (ITGBL1), but increased alpha 4 subunit (ITGA4). Similarly, the major component of the extracellular matrix, the collagenous subunits of the integrin receptor, were also decreased in Cr(VI) transformed cells: COLIA1, COL5A1, COL5A2, LAMB1 and LAMC2. Moreover, Fibulin-1 (FBLN1), an extracellular matrix protein often associated with fibronectin, which was able to inhibit the motility of a variety of cell types [19], decreased 3.6-fold in transformed cells. Fibulin-1, a large extracellular matrix glycoprotein that sequestered TGFβ via an interaction with latent TGFβ binding protein [20], was also down-regulated in Cr(VI) transformed cells.

Additional changes in genes associated with extracellular matrix. ADAM metallopeptidase with thrombospondin type 1 molecules, ADAMTS-1 and ADAMTS-5, were up-regulated 28- and 5.4-fold respectively in Cr(VI) transformed cells. It was reported that over-expression of ADAMTS-1 promoted pulmonary metastasis of TA3 mammary carcinoma and Lewis lung carcinoma cells [21], and forced expression of ADAMTS-5 in glioma cell lines stimulated cell invasion [22]. In addition, matrix metalloproteinase-2 (MMP-2), an enzyme which degraded type IV collagen, was decreased in Cr(VI) transformed cells. Simultaneously, its inhibitor, TIMP-3, was up-regulated more than 9-fold in the transformed cells.

Changes in genes associated with cell proliferation and growth. Despite the slightly faster growth rates observed in transformed cells, the expression of cell cycle related genes was quite similar in control and Cr(VI) transformed cells, with the exception of cyclin D1, which was increased about 2-fold in Cr(VI) transformed cells. Interestingly, dysregulation of cyclin D1 was found in 11 out of 16 (69%) chromium-induced lung SCC but only 3 out of 26 (12%) non-exposed lung SCC [23], indicating a specific connection between cyclin D1 overexpression and the lung cancer induced by chromate exposure. Thus, our findings of increased cyclin D1 in Cr(VI) transformed cells was consistent with the finding in vivo, supporting a crucial role of cyclin D1 in the development of chromate induced lung cancer.
In addition to genes directly involved in cell cycle progression, genes that regulated cell proliferation were also altered in expression. TGFβ signaling [24] and hedgehog signaling [25] are important pathways involved in regulating cell growth. We observed that there was decreased expression of TGFβ2 and TGFβR2 (2.4- and 3.5-fold respectively) in Cr(VI) transformed cells, suggesting that the loss of a cell response to TGFβ induced growth inhibition might be an early step of cellular transformation and tumorigenesis. Moreover, HHIP, a gene that antagonizes hedgehog signaling pathways, was decreased about 12-fold in Cr(VI) transformed cells.

**Changes in genes associated with cell apoptosis.** There are two major pathways controlling cell apoptosis. The extrinsic pathway involves the interaction of a death receptor including Fas and TNF receptor superfamily members and ligands, and the intrinsic pathway involves the mitochondria that operate in both p53-dependent and independent manner [26][27]. Although the molecules involved in each pathway were quite different, both pathways lead to caspase activation and apoptosis. Several direct targets of p53 were increased in Cr(VI) transformed cells, including CYFIP2, Perp, and RNF144B, which were known to mediate p53-dependent apoptosis. MRPS30, a mitochondrial ribosomal protein associated with programmed cell death, was also up-regulated in transformed cells. In contrast to up-regulated genes related to intrinsic apoptosis, genes associated with extrinsic apoptosis pathways were slightly down-regulated in transformed cells. For example, NUAK2, a gene induced by FasL or TNF-alpha [28], was down-regulated 2-fold. It was previously reported that NUAK2 protects cells from FasL mediated cell apoptosis [29]. SEMA3A and RHOB, are also associated with the TNF and Fas pathways, and they decreased 4- and 3.1-fold, respectively. Within the caspase family member, only caspase 4 was found to be increased in Cr(VI) transformed cells.

**Identification of genes commonly expressed in both Cr(VI) treated and control cells**

Both Cr(VI)-transformed and control cells were derived from colonies that grew in soft agar. These cells underwent a change in growth that may have caused an alteration in gene expression. We next compared the gene expression profiles from cells derived from the soft agar colonies to their parental BEAS-2B cells. As shown in Figure 6A, there was a total of 851 genes changed more than 2-fold in Cr(VI) transformed cells.
fold in 1 out of 3 groups compared to normal BEAS-2B cells, including 325 genes (38%) from the control group, 572 genes (67%) from the Cr_small group, and 528 genes (62%) from the Cr_large group. Similarly, within the 92 genes that changed more than 5-fold, only 13 genes (13%) are from the control group, compared to 42 (46%) and 37 (40%) genes in the Cr_small and the Cr_large groups, respectively (Figure 6B). Therefore, the differences between the Cr(VI) transformed cells and the control cells that grew in soft agar were more striking as compared to any differences caused by growth in soft agar.

Similar results can be seen by a hierarchical clustering analysis of 851 genes (Figure 6C), in which the samples were sorted based on the similarity of gene expression. The gene expression profiles of Cr(VI) treated cells were clearly separated from those in the control group as well as in parental BEAS-2B cells, however, no obvious separation can be seen among the six Cr(VI) transformed cell lines that were derived from colonies with different sizes. In contrast, control cells shared similar expression profiles with parental BEAS-2B cells and were clustered in the same group. It is of interest that the heat maps of gene expression were remarkably similar in six independently derived cell lines following Cr(VI) exposure. Additionally, heat maps of control cell lines derived from spontaneously arose clones were also remarkably similar to each other, yet very different from those derived from Cr(VI) exposed cells.

It is worth noting that there were 108 entities shared by three groups as shown by the Venn diagram (Fig. 6A). 91 out of 108 entities represented well-annotated genes with similar changes in gene expression between control and Cr(VI) treated cells, which are likely the outcome of anchorage-independent growth. Functional annotation of these genes revealed an up-regulation of genes involved in the p53 signaling pathway and angiogenesis, as well as a down-regulation of genes associated with cell adhesion and cell growth inhibition. The complete list of 91 genes including 41 up-regulated and 50 down-regulated genes as well as the functional annotation can be found in Tables S2 and S3.

Loss of TGF-β1 responsiveness in Cr(VI) transformed cells
TGFβ family members regulate a wide range of biological processes including cell proliferation, migration, differentiation, apoptosis, and extracellular matrix deposition [24]. Previous reports showed that TGF-β1 can cause growth inhibition in normal human bronchial epithelial (NHBE) [30] and BEAS-2B cells [31], but not in human lung carcinoma A549 cells [30] as well as BEAS-2B cells overexpressing mutant p53 [31]. Resistance to TGFβ mediated growth inhibition in human lung cancer may occur through the loss of type II receptor (TGFβR2) expression. For example, it was reported that 77% NSCLC exhibited a reduced level of TGFβR2 [32]. Forced expression of TGFβR2 in lung cancer cells showed reduced colony formation in a soft agar assay as well as reduced tumorigenicity when injected into athymic nude mice [32]. Since the levels of TGFβR2 were down-regulated in Cr(VI) transformed cells (Fig. 7A), we tested whether these cells retained their ability to response to TGF-β1 induced growth inhibition. Control and Cr(VI) transformed cells as well as BEAS-2B cells were exposed to 10 ng/ml of recombinant human TGF-β1 for 24 hours. DNA synthesis were measured by [3H]thymidine incorporation. Consistent with previous reports, thymidine

| Category              | GO Term                              | Number of genes | P Value   |
|-----------------------|--------------------------------------|-----------------|-----------|
| Biological Process    | female pregnancy                     | 8               | 0.0007    |
|                       | cell migration                       | 8               | 0.0020    |
|                       | cell motion                          | 12              | 0.0035    |
|                       | reproduction                         | 14              | 0.0102    |
|                       | cell adhesion                        | 11              | 0.0145    |
|                       | cell differentiation                 | 23              | 0.0158    |
|                       | cell death                           | 15              | 0.0172    |
|                       | cell surface receptor linked signal transduction | 20          | 0.0261    |
|                       | blood circulation                    | 5               | 0.0322    |
|                       | fatty acid metabolic process         | 5               | 0.0391    |
|                       | neurogenesis                         | 9               | 0.0398    |
|                       | folic acid transport                 | 2               | 0.0443    |
|                       | spermatid development                | 3               | 0.0474    |
| Molecular Function    | sugar binding                        | 5               | 0.0279    |
|                       | folic acid transporter activity      | 2               | 0.0290    |
|                       | vitamin binding                      | 4               | 0.0412    |
| Cellular Component    | desmosome                            | 3               | 0.0066    |
|                       | extracellular region                 | 22              | 0.0099    |
|                       | plasma membrane                      | 33              | 0.0279    |
|                       | cell junction                        | 8               | 0.0408    |
|                       | axon part                            | 3               | 0.0437    |
| KEGG Pathway          | p53 signaling pathway                | 3               | 0.0910    |
|                       | PPAR signaling pathway               | 3               | 0.0933    |

Table 2. Functional Annotation and pathway analysis of genes increased more than 2-fold in Cr(VI) transformed cells.

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Fold in 1 out of 3 groups compared to normal BEAS-2B cells, including 325 genes (38%) from the control group, 572 genes (67%) from the Cr_small group, and 528 genes (62%) from the Cr_large group. Similarly, within the 92 genes that changed more than 5-fold, only 13 genes (13%) are from the control group, compared to 42 (46%) and 37 (40%) genes in the Cr_small and the Cr_large groups, respectively (Figure 6B). Therefore, the differences between the Cr(VI) transformed cells and the control cells that grew in soft agar were more striking as compared to any differences caused by growth in soft agar. Similar results can be seen by a hierarchical clustering analysis of 851 genes (Figure 6C), in which the samples were sorted based on the similarity of gene expression. The gene expression profiles of Cr(VI) treated cells were clearly separated from those in the control group as well as in parental BEAS-2B cells, however, no obvious separation can be seen among the six Cr(VI) transformed cell lines that were derived from colonies with different sizes. In contrast, control cells shared similar expression profiles with parental BEAS-2B cells and were clustered in the same group. It is of interest that the heat maps of gene expression were remarkably similar in six independently derived cell lines following Cr(VI) exposure. Additionally, heat maps of control cell lines derived from spontaneously arose clones were also remarkably similar to each other, yet very different from those derived from Cr(VI) exposed cells.

It is worth noting that there were 108 entities shared by three groups as shown by the Venn diagram (Fig. 6A). 91 out of 108 entities represented well-annotated genes with similar changes in gene expression between control and Cr(VI) treated cells, which are likely the outcome of anchorage-independent growth. Functional annotation of these genes revealed an up-regulation of genes involved in the p53 signaling pathway and angiogenesis, as well as a down-regulation of genes associated with cell adhesion and cell growth inhibition. The complete list of 91 genes including 41 up-regulated and 50 down-regulated genes as well as the functional annotation can be found in Tables S2 and S3.

Loss of TGF-β1 responsiveness in Cr(VI) transformed cells
TGFβ family members regulate a wide range of biological processes including cell proliferation, migration, differentiation, apoptosis, and extracellular matrix deposition [24]. Previous reports showed that TGF-β1 can cause growth inhibition in normal human bronchial epithelial (NHBE) [30] and BEAS-2B cells [31], but not in human lung carcinoma A549 cells [30] as well as BEAS-2B cells overexpressing mutant p53 [31]. Resistance to TGFβ mediated growth inhibition in human lung cancer may occur through the loss of type II receptor (TGFβR2) expression. For example, it was reported that 77% NSCLC exhibited a reduced level of TGFβR2 [32]. Forced expression of TGFβR2 in lung cancer cells showed reduced colony formation in a soft agar assay as well as reduced tumorigenicity when injected into athymic nude mice [32]. Since the levels of TGFβR2 were down-regulated in Cr(VI) transformed cells (Fig. 7A), we tested whether these cells retained their ability to response to TGF-β1 induced growth inhibition. Control and Cr(VI) transformed cells as well as BEAS-2B cells were exposed to 10 ng/ml of recombinant human TGF-β1 for 24 hours. DNA synthesis were measured by [3H]thymidine incorporation. Consistent with previous reports, thymidine
incorporation in BEAS-2B and control cells was reduced by 30% and 40% respectively in the presence of TGF-β1. However, Cr(VI) transformed cells from both Cr_large and Cr_small colony groups exhibited an increased DNA synthesis after exposing to TGF-β1 (Fig. 7B), suggesting that these transformed cells were able to escape from TGFβ1 induced growth arrest. These results further support that the loss of a cell response to TGFβ induced growth inhibition is acquired during cellular transformation and tumorigenesis.

**Discussion**

Cr(VI) is a well established carcinogen that induced respiratory cancer and several other types of human cancer [2–3]. However, the molecular mechanism underlying Cr(VI) induced lung cancer and the down-stream genes that mediated Cr(VI) carcinogenicity are not yet completely understood. In the present study, we showed that chronic exposure of immortalized normal human bronchial epithelial BEAS-2B cells to low doses of Cr(VI) significantly enhanced their ability to grow in an anchorage-independent manner as assessed by increased number and size of soft agar colonies. In addition, the cell lines derived from the Cr(VI) exposed colonies exhibited striking changes in cell morphology and were able to form tumors when injected into nude mice, indicating that Cr(VI) was capable of inducing malignant transformation in BEAS-2B cells. Moreover, gene expression analysis revealed a change in expression profile in Cr(VI) transformed cells that may facilitate cell growth and migration. Interestingly, the gene expression profile of six Cr(VI) transformed cell lines were remarkably similar to each other, yet very different from those of the control cell lines.

**Table 3. Functional Annotation and pathway analysis of genes decreased more than 2-fold in Cr(VI) transformed cells.**

| Category              | GO Term                                      | Number of genes | P Value     |
|-----------------------|----------------------------------------------|-----------------|-------------|
| Biological Process    | wound healing                                | 12              | 0.00002     |
|                       | angiogenesis                                 | 8               | 0.00239     |
|                       | cell motion                                  | 14              | 0.00600     |
|                       | blood circulation                            | 8               | 0.00828     |
|                       | cell differentiation                         | 32              | 0.00970     |
|                       | regulation of cell growth                    | 8               | 0.01029     |
|                       | regulation of cell size                      | 8               | 0.01397     |
|                       | response to drug                             | 8               | 0.01766     |
|                       | cell adhesion                                | 16              | 0.02637     |
|                       | heterophilic cell adhesion                   | 3               | 0.02969     |
|                       | regulation of cell communication             | 21              | 0.03085     |
|                       | transforming growth factor beta receptor signaling pathway | 4 | 0.03776 |
|                       | response to stress                           | 30              | 0.03898     |
|                       | respiratory system development               | 5               | 0.04454     |
|                       | chromatin organization                       | 10              | 0.04470     |
|                       | epidermal growth factor receptor signaling pathway | 3 | 0.04632 |
|                       | elevation of cytosolic calcium ion concentration | 5 | 0.04710 |
| Molecular Function    | carbohydrate binding                         | 15              | 0.00014     |
|                       | calcium ion binding                          | 25              | 0.00044     |
|                       | metalloproteinase activity                   | 10              | 0.00047     |
|                       | metal ion binding                            | 69              | 0.00480     |
|                       | actin binding                                | 10              | 0.02074     |
|                       | integrin binding                             | 4               | 0.03687     |
|                       | heparin binding                              | 5               | 0.03940     |
|                       | GTPase binding                               | 5               | 0.04686     |
| Cellular Component    | extracellular matrix                         | 13              | 0.00076     |
|                       | plasma membrane                              | 64              | 0.00108     |
|                       | cytoplasm                                    | 106             | 0.00259     |
|                       | extracellular space                          | 18              | 0.00284     |
|                       | chromatin                                    | 7               | 0.03015     |
|                       | collagen type V                              | 2               | 0.03467     |
|                       | integrin complex                             | 3               | 0.04491     |
|                       | lamellipodium                                | 4               | 0.04870     |
| KEGG pathway          | Focal adhesion                               | 12              | 0.00041     |
|                       | EM-C receptor interaction                    | 7               | 0.00246     |

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indicating that altered gene expression was indeed the consequence of chronic Cr(VI) exposure.

Two recent studies have reported the effect of chronic Cr(VI) exposure on BEAS-2B cells. Rodrigues et al. reported the establishment of Cr(VI) transformed cells by ring-cloning BEAS-2B cell after chronic exposure of cells to 1 mM Cr(VI) [33]. These transformed cells exhibited an aneuploid phenotype, up-regulation of genes associated with malignant transformation and DNA repair, as well as the ability to form tumors in nude mice [33]. Our present study confirmed their finding that chronic exposure to Cr(VI) could result in malignant transformation of BEAS-2B cells. However, we were not able to detect any changes in genes associated with DNA repair as reported by Rodrigues et al. The difference is likely due to the strategies used to establish transformed cell lines. In Rodrigues' study, cells were continuously exposed to Cr(VI) after the transformed clones were derived [33]. In our study, Cr(VI) exposure was terminated after 30 days. Cells were then allowed to form colonies in soft agar and subsequently expanded in monolayer in the absence of Cr(VI). Therefore, cells in their study were constantly exposed to Cr(VI) that activated a response to DNA damage and expressed genes related to DNA repair. Clearly, this was not the case in our study. A lack of continuous exposure to Cr(VI) may also explain why there were very few changes in genes related to oxidative stress in our transformed cells.

Costa et al reported that chronic exposure of BEAS-2B cells to sub-cytotoxic Cr(VI) resulted in dramatic changes in cell morphology and growth patterns [34], that was in agreement with our observation. Cr(VI) treated and transformed cells were more sensitive to trypsinization, suggesting extracellular matrix adhesion is a major target of Cr(VI). The phenotypic change of transformed cells was further supported by alteration in expression of genes during Cr(VI) induced cell transformation. The genes associated with cell to matrix adhesion were significantly down-regulated in Cr(VI) transformed cells, including many genes involved in focal adhesion. Decreased gene expression of integrin receptors, ligands, and other matrix components could result in a poor connection between cells and the substratum, that in turn could have contributed to a transformation phenotype and further facilitated cell migration and invasion.

**Figure 6. Gene expression profiles of control, Cr(VI) transformed and parental BEAS-2B cells.** (A) Venn diagram showing the numbers of entities with more than 2-fold changed expression in pairwise comparison of Cr_large, Cr_small, control group and parental BEAS-2B cells. Numbers inside each compartment represent the number of entities. (B) The number of genes with more than 1.5-, 2-, and 5-fold changed expression level in pairwise comparison. (C) Hierarchical cluster analysis of genes with more than 2-fold changed expression in one out of three groups (control, Cr_small, Cr_large) compared to parental BEAS-2B cells. The color bar related color code to the expression value determined after quantile normalization and baseline transformation to the median levels of all samples.

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It was interesting that genes related to cell junction were increased in Cr(VI) transformed cells. Genes belonging to this category were normally down-regulated during tumorigenesis to free tumor cells from the surrounding cell contacts and facilitate tumor invasion and metastasis. However, dysregulation of cell junction genes were found in a number of human cancers, and were required for tumor cell motility and invasion. For example, up-regulation of CHD6 has been observed in several types of human cancer, suggesting a possible role in metastasis and invasion [35]. L1CAM was reported to be involved in the advanced stages of tumor progression, and overexpression of L1CAM in normal and tumor cells increased cell motility and metastasis [36]. Interestingly, both CHD6 and L1CAM were increased more than 4-fold in Cr(VI) transformed cells, suggesting a possible role of cell junction related genes in Cr(VI) induced cell transformation.

It is worth noting that our study demonstrated a dramatic increase in genes associated with desmosome complex in Cr(VI) transformed cells. Desmosome is a intracellular junction mediating the interaction between adjacent epithelia. Desmocollins (DSC1, DSC2 and DSC3) are members of the cadherin family, which form the transmembrane core of the desmosome through heterotypic interaction with another member of the same family, desmogleins (DSG) [37][38]. Depletion of DSC3 in mice resulted in embryonic lethality before implantation [39], while conditional depletion of DSC3 in mouse epidermis led to epidermal blistering [40]. Perp was originally known as an apoptosis-associated target of p53. Recent work on Perp null mice indicate that Perp is crucial for desmosome formation as the Perp deficient mice died postnatally with dramatic blistering similar to DSC1 null mice [41]. Recently, DSC2 and DSC3 were found increased in human lung cancer, especially in lung squamous cell carcinoma [42]. DSC3 was considered as a marker for squamous carcinoma due to its high levels in almost all lung squamous carcinoma [43]. Interestingly, the majority of lung cancers in chromium workers were squamous cell carcinoma. Our finding that DSC3 increased more than 40-fold in Cr(VI) transformed cells was consistent with its high level in tumor cells. Two other components of desmosome, DCS2 and Perp, were also up-regulated in Cr(VI) transformed cells. It is possible that these desmosome genes were direct targets of chronic Cr(VI) exposure, and their up-regulation by Cr(VI) may contribute to its carcinogenicity. In addition, genes related to cell cycle control as well as cell proliferation were also changed. The level of cyclin D1, an important cyclin expressed in early G1 phase and required for cell cycle progression, was increased in Cr(VI) transformed cells. Dysregulation of cyclin D1 was frequently found in the early stage of tumorigenesis in many different cancers, and has been reported at high levels in chromate induced lung cancers. TGFβ is an important negative regulator of lung epithelial cells, and loss of TGFβ signaling is an early event that contributes to cell growth. Cr(VI) transformed cells exhibited significantly reduced levels of TGFβ2 and TGFβR2, and were able to escape from TGFβ induced growth inhibition. These results suggested that Cr(VI) may have promoted tumor cell growth by stimulating proliferation associated genes and inhibiting anti-proliferation genes.

In summary, our studies analyzed gene expression profiles in transformed cells lines expanded from a single colony in soft agar after chronic exposure to low doses of Cr(VI). We have identified many novel changes in gene expression that were different from the immediate response genes identified from previous studies using acute exposure of Cr(VI). These genes may be involved in Cr(VI) induced malignant transformation. Thus, further analysis of these genes in vivo and dissecting the mechanism of Cr(VI) induced expression alterations will provide a better understanding of the mechanism underlying chromium carcinogenicity.

**Materials and Methods**

**Cell Culture**

Human normal bronchial epithelial BEAS-2B cells [44] were cultured in DMEM (Invitrogen) supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). The cells were cultured at 37°C in an incubator with a humidified
atmosphere containing 5% CO₂. For Cr(VI) exposure, cells were treated with 0.25 or 0.5 μM potassium chromate (K₂Cr₂O₇, J. T. Baker Chemical Co.) for 1 month. The medium was changed every other day, and cells were split in the presence of Cr(VI) every 3 days.

Colony survival assay
BEAS-2B cells were treated with various doses of Cr(VI) (0.25, 0.5, 1.0, 2.0, and 4.0 μM) for 24 hours. Control and Cr(VI) treated cells were plated at 500 cells/dish in 100-mm cell culture dishes, and cultured for two weeks. Cells were stained with Giemsa solution, and the number of colonies was counted and presented as mean ± SD (n = 3).

Cell proliferation assay
Normal BEAS-2B cells, control cells derived from spontaneously formed colonies of untreated cells, and Cr(VI) transformed cells derived from large colonies (Gr_large) or small colonies (Gr_small) were seeded at 2500 cells/well in 96-well plates. Viable cell number was determined using the MTS CellTiter 96 aqueous one solution cell proliferation assay (Promega) according to the manufacturer’s instructions.

[³H]Thymidine incorporation assay
Cells were seeded at density of 5x10⁵ cells/well in 24-well plate. On the following day, cells were treated with 10 ng/ml recombinant human TGF-β1 (R&D System) for 24 hr. Cells were then pulse labeled with 0.5 μCi of [³H]thymidine for 2 hr. The cells were washed twice with ice cold PBS, followed by precipitated with ice-cold 5% TCA twice, each with 10 minutes. The cells were then extracted with 0.5 ml of 0.5 M NaOH for 2 hours at room temperature, and neutralized with 0.25ml of 1N HCl. The cell lysate was transferred to scintillation vials with 5 ml of scintillation fluid, and the radioactivity was assessed using a Scintillation Counter.

In vivo tumorigenesis
Six-week old female athymic nude mice (NCI, Frederick) were injected subcutaneously at the left or the right flank with control cells and Cr transformed cells (5x10⁶ cells/0.1 ml/injection). Each cell line was injected into 3 mice. Six months later, the mice were photographed and sacrificed. Tumors and other tissues were collected for further analysis.

Soft-agar assays
Control and Cr(VI) treated cells were plated at 5000 cells/well in 6-well plates with culture medium containing 0.35% low-melting-point agarose over a 0.5% agarose base layer and cultured at 37°C incubator with 5% CO₂ for 3 weeks. The colonies were stained with INT/BCIP (Roche) and photographed.

Microarray hybridization and data analysis
Total RNA was extracted from each cell line using TRIzol Reagent (Invitrogen), and converted to single stranded cDNA using Superscript III (Invitrogen). Quantitative real-time PCR analysis was performed using SYBR green PCR system (Applied Biosystems) on ABI prism 7900HT system (Applied Biosystems). All PCR reactions were performed in triplicate. Relative gene expression level, normalized to 18s rRNA expression, was calculated by 2⁻DDCt. The results were presented as fold change to the level expressed in BEAS-2B cells.

Real-time quantitative PCR
Total RNA was extracted from each cell line using TRIzol Reagent (Invitrogen), and converted to single stranded cDNA using Superscript III (Invitrogen). Quantitative real-time PCR analysis was performed using SYBR green PCR system (Applied Biosystems) on ABI prism 7900HT system (Applied Biosystems). All PCR reactions were performed in triplicate. Relative gene expression level, normalized to 18s rRNA expression, was calculated by 2⁻DDCt. The results were presented as fold change to the level expressed in BEAS-2B cells.

Supporting Information
Table S1 Complete list of 409 genes differentially expressed more than 2-folds in Cr(VI) transformed cells. (XLS)
Table S2 Complete list of 91 genes shared similar expression changes in both Cr(VI) transformed and control cells. (XLS)
Table S3 Functional annotation of 91 common genes between control and chromium transformed cells. (DOC)

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Author Contributions
Conceived and designed the experiments: HS MC. Performed the experiments: HS HC TK. Analyzed the data: HS JZ. Contributed reagents/materials/analysis tools: MC JZ. Wrote the paper: HS MC.

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