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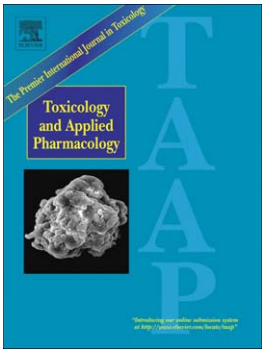

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TITLE:

Molecular basis of carcinogenicity of tungsten alloy particles

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ABSTRACT

The tungsten alloy 91% tungsten, 6% nickel and 3% cobalt (WNC 91-6-3) induces rhabdomyosarcoma when implanted into rat thigh muscle. To investigate whether this effect is species-specific human HSkMc primary muscle cells were exposed to WNC 91-6-3 particles and responses were compared with those from a rat skeletal muscle cell line (L6-C11). Toxicity was assessed by the adenylate kinase assay and microscopy, DNA damage by the Comet assay. Caspase 3 enzyme activity was measured and oligonucleotide microarrays were used for transcriptional profiling. WNC 91-6-3 particles caused toxicity in cells adjacent to the particles and also increased DNA strand breaks. Inhibition of caspase 3 by WNC 91-6-3 occurred in rat but not human cells. In both rat and human cells, the transcriptional response to WNC 91-6-3 showed repression of transcripts encoding muscle-specific proteins with induction of glycolysis, hypoxia, stress responses and transcripts associated with DNA damage and cell death. In human cells, genes encoding metallothioneins were also induced, together with genes related to angiogenesis, dysregulation of apoptosis and proliferation consistent with pre-neoplastic changes. An alloy containing iron, WNF 97-2-1, which is non-carcinogenic *in vivo* in rats, did not show these transcriptional changes *in vitro* in either species while the corresponding cobalt-containing alloy, WNC 97-2-1 elicited similar responses to WNC 91-6-3. Tungsten alloys containing both nickel and cobalt therefore have the potential to be carcinogenic in man and in vitro assays coupled with transcriptomics can be used to identify alloys, which may lead to tumour formation, by dysregulation of biochemical processes.

249 words

Keywords [6]:

Tungsten, nickel, cobalt, carcinogen, transcriptomics, rhabdomyosarcoma

BACKGROUND

Alloys of tungsten have been developed as potentially safer alternatives to depleted uranium in the manufacture of armour-piercing munitions. These materials have many attractive characteristics for this purpose including the fact that they are both dense and tough and their physical properties can be tuned by modifying their elemental composition [1]. Due to the high melting point of tungsten, these alloys are manufactured by mixing a fine tungsten powder with small quantities (typically less than 10 % w/w) of other metal powders, such as cobalt, copper, iron and nickel. The mixture is heated so that the other metals melt and some of the tungsten powder dissolves in the liquid alloy so that when the mixture cools it forms a composite of the residues of the tungsten particles bound together by an alloy enriched in the non-tungsten constituents [1]. **Figure 1** shows the structure and composition of the alloy WNC-91-6-3 which is comprised of 91% W, 6% Ni and 3% Co by mass. The composition of the binding alloy is actually 49% W; 34 % Ni; 17% Co when expressed by weight (w/w) and 23.5% W; 51% Ni; 25.5% Co when expressed by molarity. Because of this, there are safety concerns about these materials since it has long been established that metallic Ni and Co, in both powder and pellet form, are carcinogenic when implanted into rat muscles [2,3]. Furthermore, WNC 91-6-3 itself has also been investigated and, when implanted into the thigh muscles of F344 rats all animals developed aggressive rhabdomyosarcomas that metastasized to the lung within 4 to 5 months [3]. It is believed that the mechanism of toxicity of these materials is related to preferential dissolution of the binding alloy into biological fluids resulting in the release of high concentrations of nickel and cobalt ions, which are detectable in urine of rats following implantation with pellets of alloy WNC 91-6-3 [4,5]. WNF 97-2-1 pellets in contrast do not contain cobalt and also display far less corrosion in rat muscles, leading to lower concentrations of metals detectable in urine and no elevated cancer rate [5].

There is a clear need to develop robust *in vitro* screening tests to identify potentially carcinogenic alloys thereby reducing the need for expensive *in vivo* testing. We have previously described the development of a rapid *in vitro* exposure model capable of differentiating between carcinogenic and non-carcinogenic alloys using a cultured rat skeletal muscle cell line (L6-C11). On dissolution, particles of WNC-91-6-3 were found to generate reactive oxygen species, killing cells in their immediate vicinity, and induce DNA damage in the bulk population of cells in culture. Further mechanistic studies also showed that WNC 91-6-3 inhibited caspase 3 activity and altered gene expression in a manner consistent with carcinogenesis [6]. The aim of the current study was to further assess the likely hazard to human health of these materials by investigating whether human primary skeletal muscle cells (HSkMC) respond similarly to the previously studied rat cell line, and whether the properties of particles change upon long term storage, thereby altering their toxicity in a cell culture system.

RESULTS

Behaviour of particles in cell culture

The overall viability of HSkMC and L6-C11 cultures treated with alloy particles was assessed using the Toxilight® adenylate kinase assay (Lonza), revealing no significant increase in adenylate kinase release into the media. Visual analysis by microscopy, however, showed that treatment with all types and ages of WNC particles resulted in formation of regions of dead and apoptotic cells around the particles (**Figure 2**). In contrast, no damage was apparent in response to tantalum or WNF 97-2-1 particles. Unlike the L6-C11 cells [6], it was not possible to maintain the differentiated HSkMC cells in culture for long enough to follow the process until particle disintegration occurred.

Induction of DNA strand breaks by WNC particles

DNA strand breaks were determined by the Comet assay. Treatment of HSkMC cells with WNC 97-2-1 statistically significantly elevated DNA strand breaks compared with untreated controls (**Figure 3A**). Similar results were obtained for WNC 91-6-3 particles that had been stored for 7 or 67 months (**Figure 3B**) but tantalum or WNF 97-2-1 exposures resulted in no significant changes. However, treatment with WNC 91-6-3 particles that had been stored for 39 months did not significantly alter DNA strand breaks (**Figure 3A**). Similarly, when rat L6-C11 cells were exposed to the 39-month old particles no significant induction of DNA strand breaks was observed (data not shown).

Inhibition of caspase 3 by WNC particles

Caspase 3 enzyme activity was not significantly altered in human HSkMC cells following treatment with any of the particles investigated (**Figure 4 A, B**). In rat L6-C11 cells however, caspase 3 activity was significantly reduced following treatment with WNC 91-6-3 particles. Furthermore, the newest particle preparation WNC 91-6-3 (7 months) elicited a significantly lower caspase 3 activity in the rat L6-C11 cells than the oldest particle preparation WNC 91-6-3 (67 months) (**Figure 4**).

Transcriptional changes following treatment with WNC particles

Two independent transcriptomics experiments were carried out using microarrays and analysed separately. Lists of differentially expressed transcripts were generated by finding those statistically significantly (FDR<0.05) altered in expression versus the relevant untreated control group, and restricting these lists to only those transcripts displaying a 1.3-fold or higher fold change versus the controls. The fold change cut-off was performed in order to focus on those transcriptional changes that were more likely to be biologically significant. The numbers of differentially expressed transcripts discovered under these criteria are shown in **Table 1**. The tantalum control treatment resulted in differential expression of 776 transcripts in the first experiment, but this was not confirmed in the second experiment, where no significant changes were detected. WNF 97-2-1 led to minimal transcriptional changes. WNC 97-2-1 led to 827 transcriptional changes, 91% of which were also detected in one or more WNC 91-6-3 treated groups (**Figure 5A**). WNC 91-6-3 exposures led to very extensive transcriptional changes (**Table 1**), 87% of the transcripts identified as altered after WNC 91-6-3 (39) exposure were also altered by WNC 91-6-3 (7) or WNC 91-6-3 (67) exposure (**Figure 5B**). The second transcriptomics experiment appeared more sensitive, detecting additional changes in response to WNC 91-6-3. There were no significant differences in transcript expression between WNC 91-6-3 (7) and WNC 91-6-3 (67) but there was a general trend towards a greater magnitude of change with the newer WNC 91-6-3 (7) particles (**Figure 5B**). As two independent experiments had been carried out, this enabled the

identification of a verified consensus set of transcriptional changes elicited by every WNC 91-6-3 treatment. This consisted of 1556 transcriptional changes (**Table 1**; **Figure 5B**). The full list of transcriptional changes is shown in [**Additional File 1**]. Transcripts induced over 5-fold after WNC particle exposures included *TFPI2, HMOX1, ASCL2, INHBB, APLN, CABP1, SPINK1, PLIN2* and *AK4*. A number of transcripts were highly induced in the second experiment by WNC 91-6-3 particles, but were below the limit of detection in the first, these included *IL8, CA9, SLCO2B1* and *HKDC1*. *KANK4* transcript was repressed more than 5-fold following all WNC 91-6-3 exposures.

Multivariate principal components analyses (PCA) were carried out on transcriptomic data from all samples and scores plots were generated. In experiment 1, WNC 91-6-3 (39) and WNC 97-2-1 were clearly separated from all other treatment groups (**Figure 6**), WNF 97-2-1 samples separated from controls but showed high inter-individual variability, tantalum samples overlapped with controls but were less variable. In experiment 2, WNC 91-6-3 (7) and WNC 91-6-3 (67) were clearly separated from controls; controls and tantalum-treated cells overlapped (**Figure 7**). As illustrated by Figures 6 and 7, the tantalum-exposed cell cultures in the second experiment displayed greater inter-culture variability than in the first experiment. The reason for this is not clear but may be related to biological variability and the lower number of controls used in the second experiment.

Annotation enrichment analyses were performed with DAVID, resulting in detection of highly significant associations between the differentially expressed transcripts following particle exposure and a number of biological pathways, processes, functions and components. These data are shown in full in [**Additional File 2**], and enrichment scores for selected terms are shown in **Table 2**. Notably there was a high degree of consistency between all WNC 91-6-3 exposures and a comparable, though less extreme, response to WNC 97-2-1. Particle exposures induced transcripts encoding members of key biological

pathways including glycolysis, response to hypoxia, response to oxidative stress, anti-apoptosis and angiogenesis. Pathways whose members were transcriptionally repressed were particularly associated with differentiated muscle tissue, including muscle protein, contractile fibre, actin, differentiation and calcium ion binding.

Class prediction was carried out using the Support Vector Machines algorithm. The transcriptomic data from exposure of rat L6-C11 cells to alloy particles [6], limited to those genes with known rat and human orthologs that were detected in all microarray experiments, were used to train the classification algorithm, which was then applied to classify the sample groups from HSkMC experiments 1 and 2. **Table 3A** shows experiment 1 HSkMC cells exposed to WNC 91-6-3 (39) and WNC 97-2-1 were classified as rat L6-C1cells exposed to WNC 97-2-1, while HSkMC control, tantalum and WNF 97-2-1 exposures were classified as L6-C11 controls. **Table 3B** shows experiment 2 HSkMC cells exposed to WNC 91-6-3 (7) and WNC 91-6-3 (67) were classified as rat L6-C1cells exposed to WNC 97-2-1, while HSkMC control, and tantalum exposures were classified as L6-C11 controls. When the experiment 1 HSkMC data were used to train the algorithm and used to classify experiment 2 groups, both WNC 91-6-3 exposures were classified as WNC 91-6-3, while control and tantalum exposures were both classified as controls (**Table 3C**). In all cases cobalt-containing alloys could be distinguished from controls and alloys containing no cobalt. Principal components analysis was carried out on group mean expression of transcripts significantly altered in L6-C11 cells exposed to alloys (1-way ANOVA, FDR<0.05), normalized to the mean of control group values for each experiment. PCA also demonstrated a common response between the rat and human cells. As shown by the PCA scores plot (**Figure 8**), rat and human cells were separated along principal component 1 (PC1) while cobalt-containing alloys were separated from controls and cobalt-free alloys along PC2.

DISCUSSION

The responses of primary human HSkMC cells to tungsten alloys displayed considerable similarity to those of the rat L6-C11 cell line shown previously [6]. Particles of tungsten nickel cobalt alloys, but not tantalum or tungsten-nickel-iron, killed both L6-C11 and HSkMC cells in their immediate vicinity (**Figure 2**). Despite this acute local toxicity, the overall viability of the cell cultures was not reduced, as measured by adenylate kinase release. Therefore, for the majority of cells in culture, the exposure was sub-lethal, allowing mechanistic investigation. These findings were consistent with those reported previously for rat and mouse cells exposed to a re-constituted mixture of W, Ni and Co powders [7].

In common with rat L6-C11 cells, the human HSkMC cells showed no increase in DNA strand breaks with tantalum and WNF 97-2-1 treatment, but DNA damage was significantly increased by WNC 97-2-1 and by WNC 91-6-3 at 7 and 67 months post particle manufacture. The lack of detection of DNA damage with exposure to WNC 91-6-3 at 39 months (**Figure 3A**) is difficult to explain, especially as these particles did affect other measured parameters, but may be due to oxidation on the surface of the particles affecting the rate of dissolution of one or more of the metals in the binding alloy, metal ion interactions with chromatin structure [8] or high numbers of apoptotic cells [9].

The response of caspase 3 enzyme activity to alloy particle exposure was markedly different between the rat L6-C11 cell line and human HSkMC primary cells. In agreement with previous results [6] caspase 3 activity was reduced by WNC alloys in L6-C11 cells (**Figure 4 C, D**). However caspase 3 activity was unaffected in HSkMC cells (**Figure 4 A, B**). This may represent a genuine species difference or be caused by cell culture and cell type differences, as L6-C11 is an immortalized myocyte cell line while HSkMC cells are primary myocytes. There was an indication that particle age affected the caspase 3 response, as activity was significantly less in 7 month old than in 67 month old WNC 91-6-3 samples, which suggests

that the toxicological properties of these materials is at least partly determined by their surface and that particle age and storage needs to be carefully taken into account in any future testing of these and related materials.

Transcriptomic analysis demonstrated that cobalt-containing alloys caused extensive changes in gene expression in human HSkMC cells (**Figure 6**), and that this was reproducible in an independent follow-up experiment (**Figure 7; Table 3C**). Tantalum did not elicit reproducible gene expression changes, while the iron-containing alloy, WNF 97-2-1, elicited only a few. WNC 91-6-3 particles of different ages, when tested in parallel (7 months and 67 months) showed no statistically significant differences in transcription, but there was a trend towards a reduction in degree of response with the older alloy, reflected in the lower number of genes passing a 1.3-fold cut-off (**Figure 5B**). The responses of rat L6-C11 cells [6] and human HSkMC cells to treatment with WNC 91-6-3 showed broad similarities when compared by principal components analysis (**Figure 7**) and rat cell responses could be used to classify human cell sample groups (**Table 3 A, B**), again implying similarity between the molecular responses to alloy particles of human primary muscle cells and a rat muscle cell line.

To characterize the transcriptomic responses of HSkMC cells to alloy particles and compare these with the responses of rat L6-C11 cells, functional annotation analyses were performed. Many functionally related groups of genes were either induced or repressed in HSkMCs in response to WNC alloys (**Table 2**); [**Additional File 2**]. While changes in the amount of a particular mRNA transcript does not necessarily predict increased protein production or enzyme activity, the identification of consistent changes for multiple transcripts from the same pathway increases confidence in the predicted biological effects. Gene expression profiling studies previously carried out on rat L6-C11 cells exposed to WNC 91-6-3 [6] showed repression of transcripts encoding muscle-specific proteins and induction of transcripts

encoding proteins of carbohydrate metabolism, stress and DNA damage response and proteins associated with cell death. HSkMC cells similarly responded with repression of transcripts encoding muscle proteins and induction of those involved in carbohydrate metabolism, the stress response and cell death.

Human HSkMC cells showed highly statistically significant repression of transcripts encoding muscle-specific proteins, together with repression of differentiation-associated genes. These changes are consistent with the apparent de-differentiation observed in longer-term cell culture of rat L6-C11 cells with WNC 91-6-3 particles [6], the blocked differentiation of muscle cells observed *in vivo* in rhabdomyosarcoma [10] and the cobalt-induced atrophy of skeletal myotubes [11].

WNC 91-6-3 and WNC 97-2-1 particles have been shown previously to generate high levels of reactive oxygen species in cell culture media, as does cobalt, but not nickel [6,12]. Further evidence for a role of ROS in the mechanism of action of WNC particles was provided in the current study by the transcriptional induction of a cellular oxidative stress response via the NRF2 pathway, determined from microarray data. This included induction of genes encoding antioxidant enzymes such as superoxide dismutases, peroxiredoxin, sulfiredoxin and NAD(P)H dehydrogenase (*SOD1, SOD2, PRDX1, SRXN1, NQO1*) and components of the glutathione system such as glutamate-cysteine ligase, glutathione reductase, a glutathione peroxidase and glutathione-*S*-transferases (*GLCLC, GSR, GPX3, GSTO1, GSTP1*). While these changes could be adaptive to oxidative stress, there are other indications that the anti-oxidant capacity of the cells is exceeded, leading to oxidative DNA damage and an inflammatory response including transcriptional induction of cytokines (*IL1B, IL6, IL8, IL11*).

There was a highly significant induction of transcripts encoding glycolytic and pentose phosphate pathway enzymes, including aldolase, enolases, hexokinase, lactate dehydrogenase, phosphofructokinases and phosphoglycerate kinase (*ALDOC, ENO1, ENO2, HK2, LDHA, PFKFB3, PFKFB4, PFKP, PGK1*). This coordinated transcriptional induction strongly indicates an increased glycolysis pathway activity following treatment of cells with WNC alloys. Interestingly these changes are consistent with the higher rate of glycolysis typically observed in many cancer cells, known as the Warburg effect [13], suggesting that WNC particle treatment of muscle cells *in vitro* induces changes in gene expression that are consistent with changes known to be important in the development of a cancer cell phenotype *in vivo*. Similarly, in rat pneumocytes exposed to cobalt particles [14] the hexose monophosphate shunt was activated, mediated by oxidative stress and leading to higher rates of glycolysis. Genes encoding metallothioneins (*MT1A, MT1B, MT1E, MT1G, MT1H, MT1L, MT1X, MT2A, MT3*), small cysteine-rich metal binding proteins, were also induced in response to WNC alloys. The MT2A (HMTIIA) promoter region was activated when HepG2 cells were exposed to W, Ni and Co powders or a mixture of the three [15]. Metallothioneins have long been used as biomarkers of metal ion exposure and oxidative stress [16] and MT proteins are also over-expressed in certain tumours [17]. 3',5'-cyclic-AMP phosphodiesterase transcripts (*PDE4B,C,D, 7B, 8A*) were induced by WNC 91-6-3, implying interference with cAMP signaling.

There was a clear induction of other genes responsive to hypoxia, notably haem oxygenase 1 (*HMOX1*), highly induced in rat cells exposed to WNC 91-6-3, and *HILPDA* (hypoxia inducible lipid droplet-associated) consistent with the well-known hypoxia-like effects of cobalt ions [18] and nickel ions [19]. Cobalt has been shown to stabilize HIF1-alpha protein [20] independently of transcription, and in these experiments no transcriptional induction of HIF1-alpha was found, indeed it was mildly but significantly repressed by WNC 91-6-3. Carbonic anhydrase IX (*CA9*) was highly and significantly induced by WNC 91-

6-3; this transcript is induced by hypoxia and ameliorates acidosis in tumour cells [21]. Related to the hypoxia-like response was a significant induction of angiogenesis genes such as vascular endothelial growth factor A (*VEGFA*). Additional proliferative genes were also induced; *VEGFC*, epidermal growth factor receptor (*EGFR*), activating transcription factor 3 (*ATF3*), several oncogenes (*ETS1, MAFG, MYC, RELA* and *LYN*) and *JUN* proto-oncogene. Despite the lack of caspase 3 inhibition in HSkMC cells exposed to WNC alloys, microarray data predicted that other routes to evade apoptosis were activated, as genes associated with negative regulation of apoptosis were significantly induced. These included *BIRC3, BCL6, ETS1* and *MDM2*. These functional categories of genes are interlinked; particularly response to hypoxia, angiogenesis, proliferation and anti-apoptosis. DNA hypomethylation is also considered to be a key feature of tumour cells [22] and transcription of methyltransferases was significantly repressed with WNC, raising the possibility of epigenetic changes in response to alloy exposure, as has previously been reported for WNC-91-6-3 [23].

The diverse groups of functionally related genes that were differentially expressed in response to WNC alloys showed remarkable similarities with those contributing to the phenotype of cancer cells. The "hallmarks of cancer" [25,26] are biological capabilities acquired during the multi-step development of human tumours. **Table 4** shows transcriptional changes following treatment of cells with WNC 91-6-3 compared with the "hallmarks of cancer". The microarray data and biochemical assays provide evidence for pro-carcinogenic changes relevant to at least eight of the ten hallmarks. Taken together, these data strongly suggest that WNC treatment results in a cellular environment in which carcinogenesis is more likely to occur. This is consistent with the rat L6-C11 cell data [6] and with both rat and mouse *in vivo* data, where WNC pellet implantation led to rhabdomyosarcoma [3,27]. Rat tumours induced by WNC-91-6-3 similarly showed disruptions to cellular energetics and loss of muscle-specific gene expression, but also showed additional changes in gene expression related to cell cycle and cell division,

characteristic of rapidly-dividing tumour cells [5]. The responses to WNC 91-6-3 detected by microarray gene expression analysis showed similarities to those seen when human peripheral blood mononucleated cells were exposed to cobalt and tungsten carbide particles, haem oxygenase being very highly induced [28] and when human keratinocytes were exposed to tungsten carbide-cobalt nanoparticles [29]. The similarities in results of these studies to ours suggest that these mechanisms may occur in many different cell types, not just myocytes, implying toxic and carcinogenic effects in other tissues. Ballistic aerosols formed by tungsten alloy munitions can also generate cytotoxic nanoparticulates [30], raising further concerns about the inhalation hazard from tungsten alloy particles and nanoparticles [31,32].

CONCLUSIONS

We have previously shown that the cobalt-containing tungsten alloys (WNC) generate reactive oxygen species within tissue culture media [6] as does elemental cobalt, but not nickel, and this is consistent with their ability to induce DNA strand breaks and transcriptional changes indicative of cellular oxidative stress in cultured cells. In the current study we have shown that tungsten-nickel-cobalt alloys undergo changes in their ability to affect specific toxicological endpoints in cells on storage, but that these changes did not undermine the ability of transcriptomics to identify gene expression profiles consistent with pre-neoplastic change in these materials. Indeed the transcriptional response of cultured muscle cells from two species (human and rat) to these materials was remarkably stable over an extended period of time. *In vivo* rodent studies utilizing alloy pellets have demonstrated preferential dissolution of the tungsten-nickel-cobalt binding matrix in comparison with the pure tungsten particles [5]; this dissolution, attributed to galvanic corrosion, was significantly faster for WNC 91-6-3 pellets than for WNF 97-2-1 pellets. Although based on tests from a limited number of alloys, the *in vitro* results from

L6-C11 cells are predictive of the carcinogenicity seen *in vivo* when alloy pellets are implanted into rats. As both nickel and cobalt metal particles are rodent carcinogens [2,3], it is not unexpected that tungsten-nickel-cobalt alloys are also carcinogenic when implanted into rodent muscles [3,5]. The lack of comparable carcinogenicity of tungsten-nickel-iron (WNF 97-2-1) in rats requires further explanation but is indicative of the importance of cobalt in the mechanism of carcinogenicity of these materials. WNF 97-2-1 dissolves more slowly, at least from pellets [5], produces only small quantities of reactive oxygen species compared to WNC 91-6-3, [6] and results in relatively few gene expression changes in HSkMC cells. As nickel can also alter expression of many of the genes affected by cobalt, for example *HMOX1* and *VEGFA* [33,34], alloy formulations that dissolve to form higher local concentrations of nickel may also have greater effects, though to test this would require analysis of a range of different alloy formulations. Overall the results from the HSkMC cells agree with those from L6-C11 cells. It therefore seems probable that the results from HSkMC and L6-C11 cells are equally predictive of what would happen if a piece of tungsten alloy became embedded in a human. The potential carcinogenicity of metal alloys can therefore be identified by relatively rapid *in vitro* tests using a rodent cell line that is cheap and easy to culture compared to human primary cell cultures suggesting that transcriptional profiling in L6-C11 cells could therefore form the basis of an *in vitro* screen for prioritization of suitable formulations in the future, streamlining development and reducing the need for expensive *in vivo* studies.

METHODS

Materials

All chemicals were bought at the highest grade available from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) unless otherwise stated: heat-inactivated foetal bovine serum gold from PAA Ltd (Yeovil,

Somerset, UK); frozen stocks of L6-C11 rat skeletal muscle myoblasts from HPACC (Porton Down, Salisbury, UK); adult human skeletal muscle cells, growth medium, differentiation medium, subculture kits from tebu-bio (Peterborough, Cambridgeshire, UK); collagen-coated cell culture flasks from BD Biosciences (Oxford, UK); Giemsa's stain solution and DePeX mounting medium from VWR International Ltd (Poole, Dorset, UK); Jenner Stain from Raymond A. Lamb Ltd (Eastbourne, Sussex, UK); EnzChek Caspase-3 assay kit #2 from Invitrogen Ltd. (Paisley, UK). ToxiLight® BioAssay kits from Lonza (Rockland, Maine, USA). RNA preparation kits were from Qiagen (Crawley, UK) and microarrays, microarray reagents, scanner, feature extraction (FE) software and Genespring microarray data analysis software were from Agilent Technologies (Berkshire, UK).

Particles

Tungsten heavy metal alloys and tantalum chips were provided by Rheinmetall Waffe Munition GmbH. The alloy particles were cleaned and sterilized by ultrasonicating three times in acetone and dried and packaged aseptically by Dstl (Porton Down, Salisbury, UK). Particles were examined by scanning electron microscopy, dimensions of samples were measured and particle surface areas estimated. The quantities of material used in each experiment aimed to give a surface area of 207mm^2 per tissue culture flask, based on the results of Miller *et al* [35]. Two independent experiments were carried out, the first used untreated controls, tantalum, WNF 97-2-1, WNC 97-2-1 and WNC 91-6-3 at 39 months post production, denoted as WNC 91-6-3 (39). The second experiment used untreated controls, tantalum, WNC 91-6-3 at 7 months post production, denoted as WNC 91-6-3 (7) and WNC 91-6-3 at 67 months post production, denoted as WNC 91-6-3 (67). The WNC 91-6-3 (39) and (67) particles were the same batch tested at different times, while WNC 91-6-3 (7) was a new preparation.

Cells and exposures

The rat L6-C11 cell line was cultured as reported previously [6]. Human cells (HSkMC, Lot no 2527, obtained from a 37 year old male) were resuscitated and cultured in a 75 cm^2 collagen-coated tissue-culture flask using HSkMC growth medium as directed by tebu-bio. Cells were trypsinised and then stored in the vapour phase of liquid nitrogen in cryopreservation medium (HSkMC basal medium 75% v/v: foetal bovine serum 15% v/v: dimethylsulphoxide 10% v/v). These aliquots of frozen cells were used in subsequent experiments to ensure that all the cells had undergone a similar number of divisions. Cells were exposed to alloy particles suspended in media as detailed previously [6]. Briefly, HSkMC cells were grown to near confluence in 75cm^2 tissue culture flasks, the growth medium was replaced with differentiation medium and the cells allowed to differentiate for four days prior to being exposed to particle suspensions in HSkMC differentiation medium (tebu-bio) for 24 hours.

Assay Methods

Experimental procedures for cell culture, measurement of DNA damage by Comet assay and measurement of caspase 3 activity were performed as per Harris *et al.* [6]. Briefly, cell viability was measured using the Toxilight® adenylate kinase assay kit (Lonza, Rockland, Maine, USA). Caspase-3 activity was measured using the EnzChek Caspase-3 Assay Kit #2 (Invitrogen, Paisley, Scotland). The comet assay method employed was based on that devised by Dusinska and Collins [36]. Measurement of percent tail DNA was chosen to assess the extent of DNA damage as this has been shown to suffer much less from inter-run variation than other comet parameters [37].

Microarrays

HSkMC cells were grown and treated for 24 hours in 75 cm² flasks, washed in phosphate buffered saline, scrape harvested in 500 µl RNAlater (Qiagen) and then stored at –75°C. Total RNA was extracted from the cells using an RNeasy minikit (Qiagen). In order to obtain RNA of sufficient purity for labelling, the standard protocol was modified by the inclusion of an extra wash with 80% ethanol (700 µl) after the initial wash step. For the first experiment, 50 samples were prepared; untreated controls (n=26), tantalum-treated (n=6); WNF 97-2-1 (n=6); WNC 97-2-1 (n=6) and WNC 91-6-3 (39) (n=6). For the second experiment, 40 samples were prepared; comprising untreated controls (n=10); tantalum (n=6); WNC 91-6-3 (7) (n=6) and WNC 91-6-3 (67) (n=6). The isolated RNA samples were stored at –75°C until all samples had been obtained, after which they were packed in dry ice and transferred to the Functional Genomics and Proteomics Facility (School of Biosciences, University of Birmingham, UK). Here, the samples were randomised and cRNA was prepared, labelled with either Cy3 or Cy5 fluorescent dyes. An aliquot of each of the fluorescent-labelled samples was combined to produce separate Cy3-labelled and Cy5-labelled reference pools for each experiment. The fluorescent-labelled samples in each treatment group were then randomly assigned to one of two equal-sized groups in a random sequence, Cy3-labelled samples from one group were mixed with aliquots from the Cy5-labelled pool, and Cy5-labelled samples from the other group were mixed with aliquots from the Cy3-labelled pool. The resulting mixtures were hybridised to Agilent human gene expression 4×44k microarray slides, Design ID 026652, which were incubated, washed and scanned according to the manufacturer's instructions. MIAME-compliant microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession numbers E-MTAB-2991 and E-MTAB-2993.

Microarray Data Analysis

Raw microarray data were initially analysed within the Genespring software package (v 7.3.1; Agilent). One tantalum-exposed sample was removed as an outlier from the second experiment. Data were dye-swapped then quality-filtered by removing probes with low fluorescence intensity (control channel less than 20 arbitrary units) and high variability (SD >1.4). Quality-filtered data were Lowess normalised then the 'ComBat' Empirical Bayes method was applied to remove dye-bias effects [38]. ANOVA and T-tests were performed within Genespring using a Benjamini and Hochberg multiple testing correction [39] for a false discovery rate corrected P-value (FDR) of <0.05. Additional fold-change filters of 1.3-fold were employed. Principal components analysis (PCA) scores plots and hierarchical clustering (Spearman correlation) were performed within Genespring. For annotation enrichment analysis the general approach was first to generate lists of genes with significantly altered expression (FDR<0.05) between the group in question and the control group, then to filter these to find genes significantly 1.3-fold induced and genes significantly 1.3-fold repressed in the test group compared with the control group. Where required, a background list was used of all genes detected by the microarray. The programs and resources used were Genespring, DAVID (Database for Annotation, Visualisation and Integrated Discovery [40,41]), GSEA (Gene Set Enrichment Analysis [42]), CTD (Comparative Toxicogenomics Database [43]), KEGG (Kyoto Encyclopedia of Genes and Genomes), NCBI (National Centre for Biotechnology Information) and MeV (Multi Experiment Viewer [44]). Classification was carried out using the Support Vector Machines (SVM) algorithm with gene selection by Golub's method, via Genespring. Enrichment analysis of gene-chemical annotation was performed by an adaptation of the method described by Williams *et al.* [45]. Briefly, for all genes detected by microarray, "chemical to gene expression induction" relationships for any species were downloaded from CTD [43], and used to annotate each gene with its "chemical inducers". Gene expression data were then interrogated for enrichment of annotation using the GSEA software package [42].

List of abbreviations

CTD: comparative toxicology database

FE: feature extraction

GSEA: Gene Set Enrichment Analysis

HSkMC: human primary skeletal muscle cells

PCA: Principal components analyses

WNC: tungsten nickel cobalt

WNC-91-6-3: Tungsten alloy 91% tungsten, 6% nickel and 3% cobalt

WNC-97-2-1: Tungsten alloy 97% tungsten, 2% nickel and 1% cobalt

WNF 97-2-1: Tungsten alloy 97% tungsten, 2% iron and 1% cobalt

Competing Interests

The author declare that they have no competing interests

Authors' Contributions

RMH carried out all laboratory studies and helped to draft the manuscript. TDW analyzed microarray data and drafted the manuscript. RHW and NJH conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1:

Structure and composition of tungsten-nickel-cobalt alloy 91-6-3. Percentage composition of (a) entire alloy by weight, (b) and (c) binding alloy by weight and molarity respectively. Key: Tungsten dark grey, nickel green, cobalt purple, binding alloy light grey.

Figure 2:

Effect of particles on HSkMC cells. A- Unexposed control 1; B- Tantalum 1; C- WNF 97-2-1; D- WNC 97-2-1; E – WNC 91-6-3 (39); F- Unexposed control 2; G- Tantalum 2; H- WNC 91-6-3 (67); I - WNC 91-6-3 (7). Cells were grown to confluence and allowed to differentiate for four days prior to dosing with particles. Cells were fixed with methanol and stained with Jenner-Giemsa stain 24 h after dosing. Tantalum (A, G) and WNF-97–2–1 (C) showed no visible damage. Particles of the two cobalt-containing alloys WNC 97–2–1 (D) and WNC-91–6–3 (E, H, I) were surrounded by zones of dead and dying cells. Pictures were taken using a Canon EOS TD digital camera mounted on a Nikon Eclipse TS100F microscope and enhanced by processing once with the one step photo fix function of Paint Shop Pro 9.01 (Jasc Software Inc.) before converting to greyscale.

Figure 3:

DNA damage of HSkMC cells following particle exposure. Comet median % tail DNA values for 100 cells each from 3 separate experiments were normalised to the mean of control values +/- standard deviation. A: Experiment 1; B : Experiment 2. Single asterisk denotes paired T-test P-value <0.05; Double asterisk denotes paired T-test P-value <0.01

Figure 4:

Caspase 3 enzyme activity of HSkMC and L6-C11 cells following particle exposure. Mean caspase 3 enzyme activities from 3 separate experiments were normalised to the mean of control values +/- standard deviation. A: HSkMC Experiment 1; B : HSkMC Experiment 2; C : L6-C11 Experiment 1; D: L6-C1 Experiment 2. Single asterisk denotes paired T-test P-value <0.05; Double asterisk denotes paired T-test P-value <0.01

Figure 5:

Venn diagrams showing numbers of transcripts differentially expressed (FDR<0.05, 1.3-fold) between alloy particle treated and control groups. A comparison of WNC 91-6-3, WNC 97-2-1 and WNF 97-2-1 transcriptional effects. B Comparison of WNC 91-6-3 transcriptional effects elicited by particles of different ages.

Figure 6:

Principal components analysis (PCA) scores plot of transcriptomics data from experiment 1. Points are shown in cyan for cells treated with WNC 91-6-3 (39); pink for WNC 97-2-1; blue for WNF 97-2-1; yellow for tantalum; red for controls.

Figure 7:

Principal components analysis (PCA) scores plot of transcriptomics data from experiment 2. Points are shown in yellow for cells treated with WNC 91-6-3 (7); orange for WNC 91-6-3 (67); red for tantalum; cyan for controls.

Figure 8:

Principal components analysis (PCA) scores plot of transcriptomics data by mean of treatment group from rat L6-C11 cells (Harris et al., 2011) and human HSkMC cells. Groups exposed to the same formulation of particles are circled or linked with blue lines. Dashed grey lines separate rat from human groups and cobalt from non-cobalt groups.

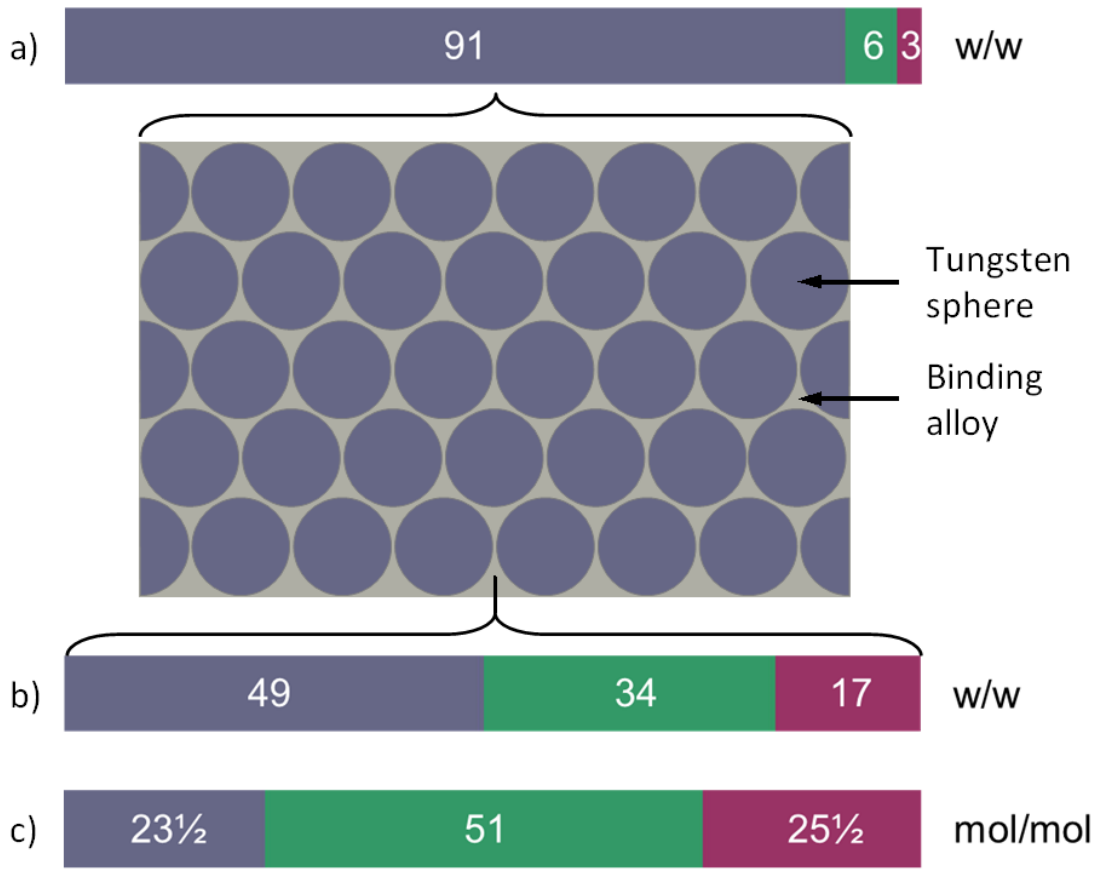


Figure 1

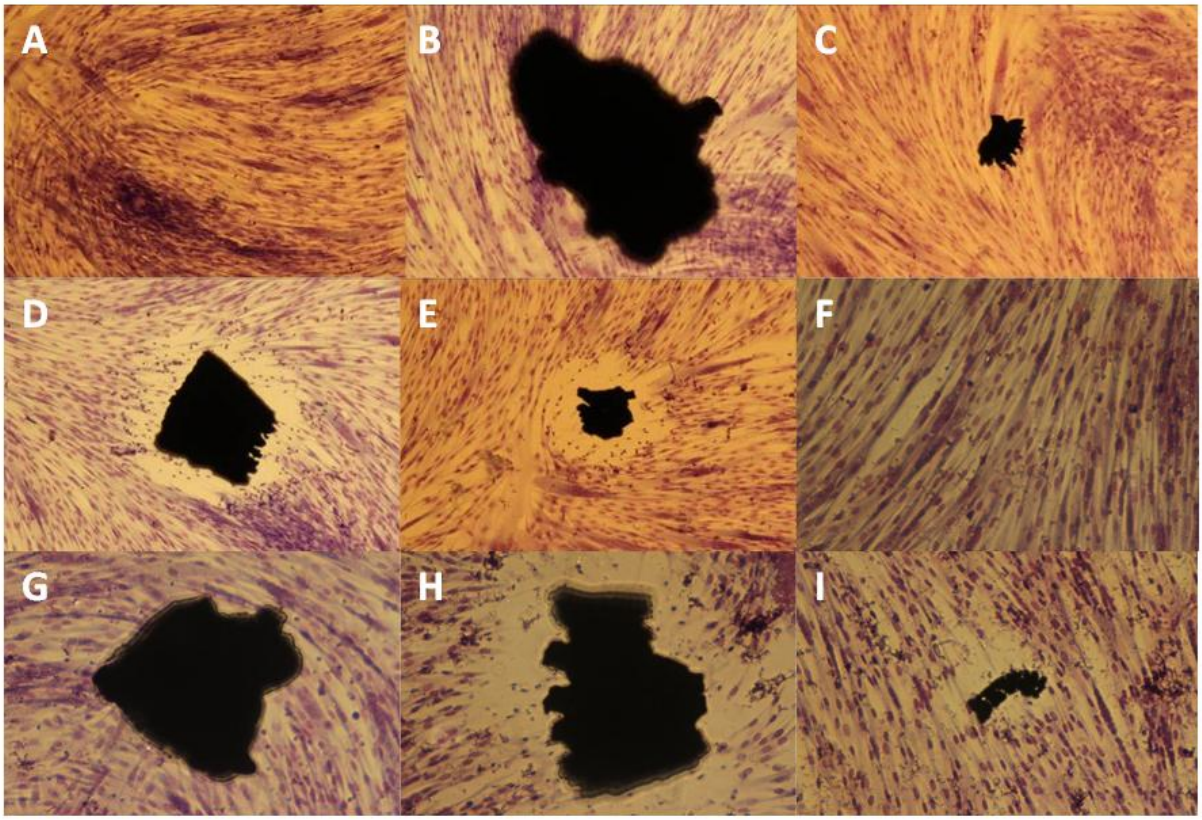
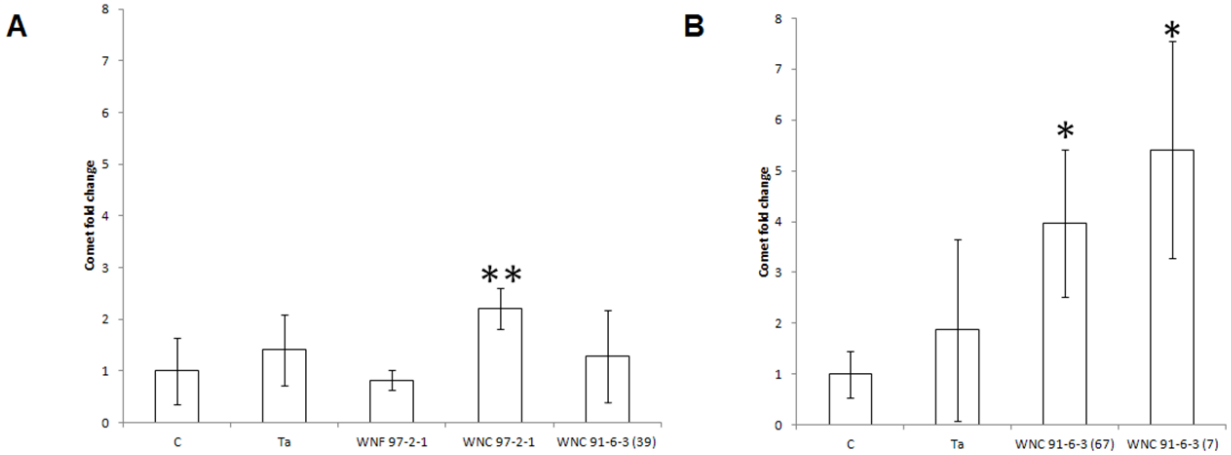


Figure 2



DNA damage of HSkMC cells following particle exposure. Comet median % tail DNA values for 100 cells each from 3 separate experiments were normalised to the mean of control values +/- standard deviation . A: Experiment 1; B : Experiment 2. Single asterisk denotes paired T-test P-value <0.05; Double asterisk denotes paired T-test P-value <0.01

Figure 3

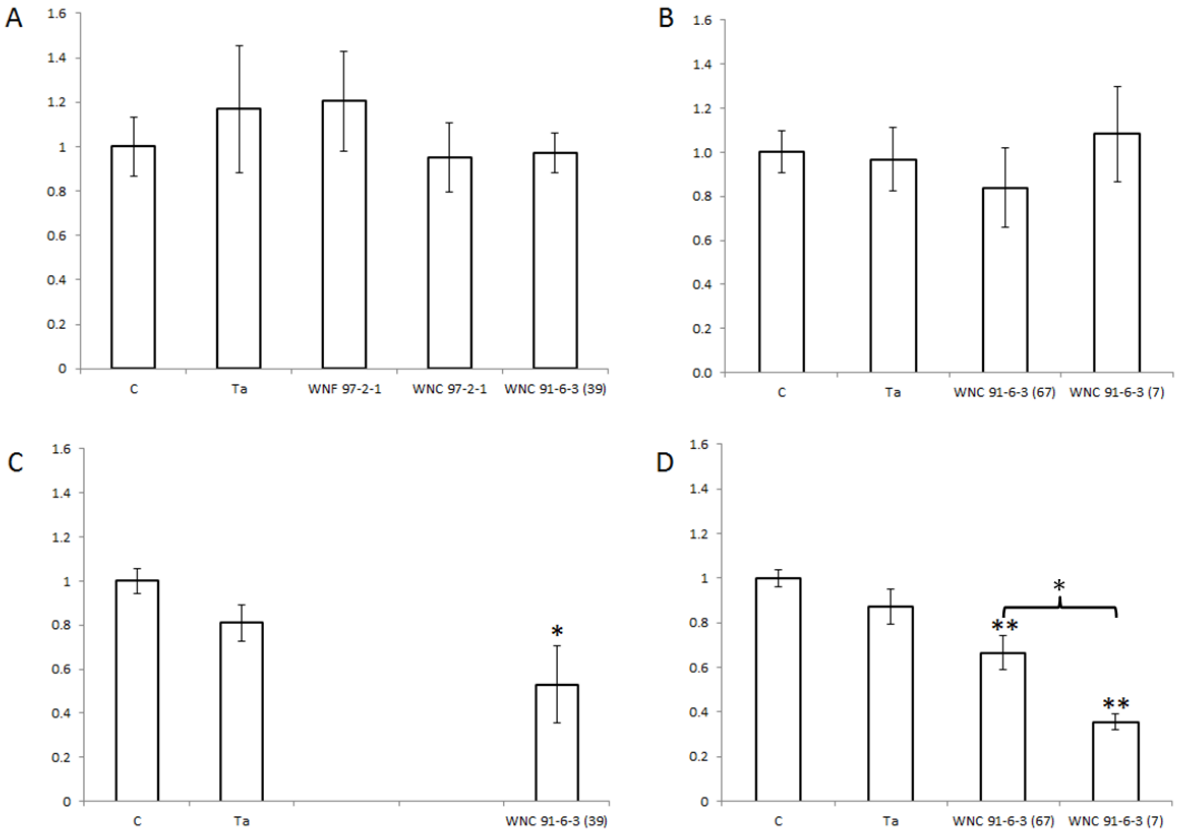


Figure 4

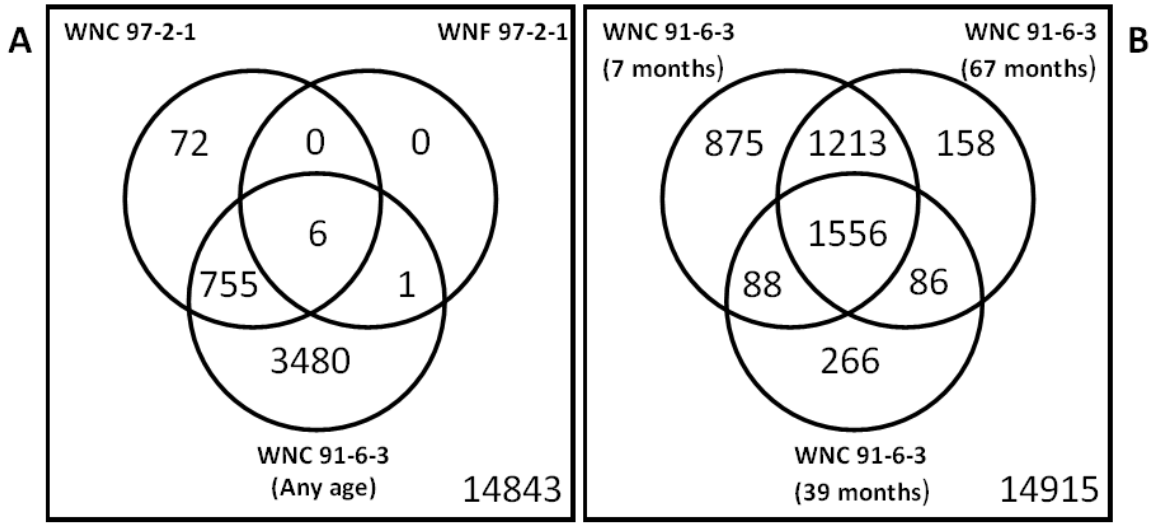


Figure 5

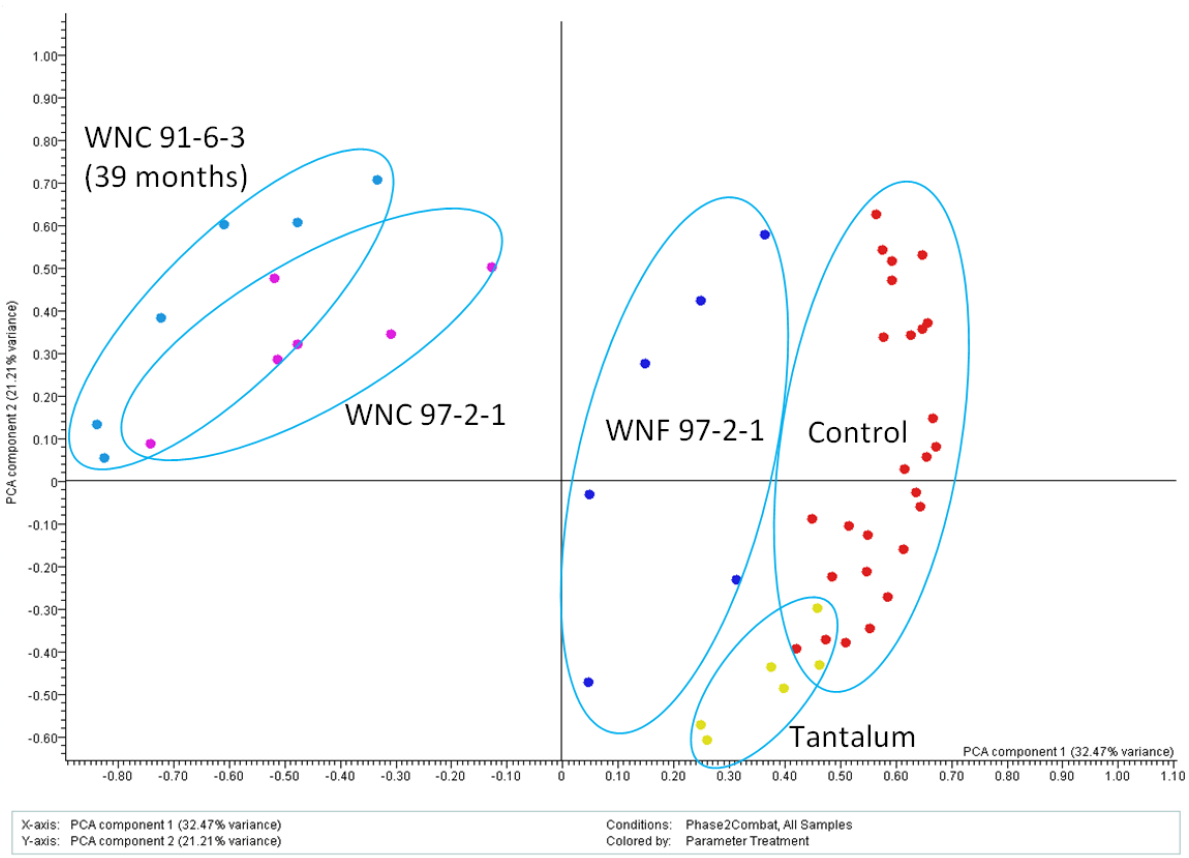


Figure 6

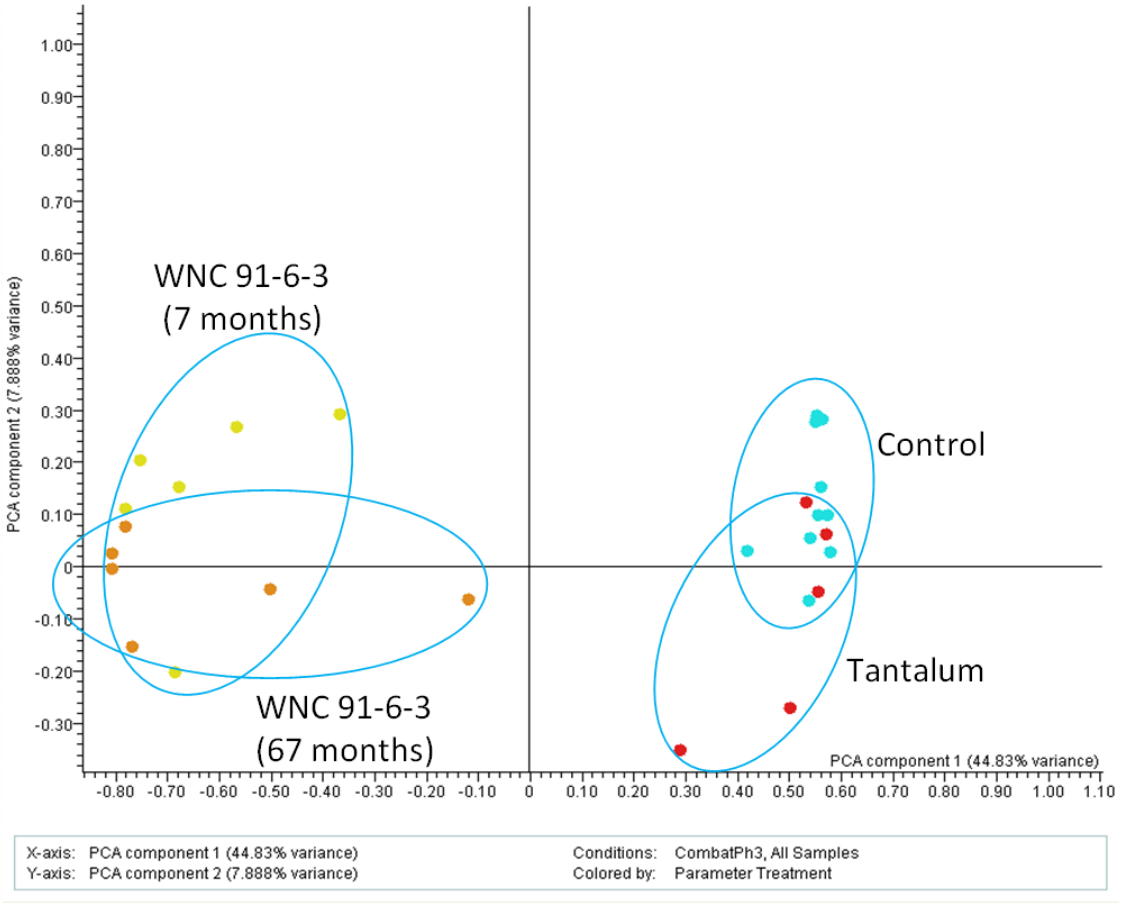


Figure 7

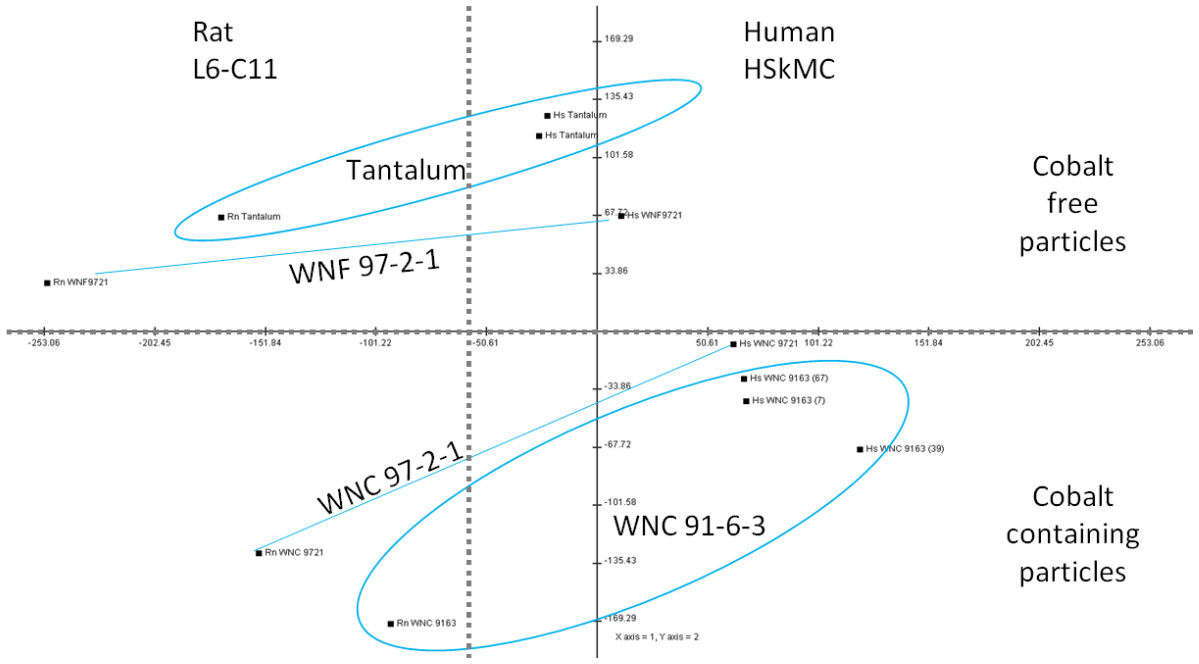


Figure 8

TABLES

Table 1: Transcriptional changes after particle exposure

Expt.	Particle Exposure	Transcripts 1.3-fold up FDR<0.05	Transcripts 1.3-fold down FDR<0.05
1	Tantalum 1	438	338
1	WNF 97-2-1	7	0
1	WNC 97-2-1	562	296
1	WNC 91-6-3 (39)	1072	958
2	Tantalum 2	0	0
2	WNC 91-6-3 (67)	1556	1646
2	WNC 91-6-3 (7)	1848	2092
1&2	WNC 91-6-3 Consensus	847	709

Table 2: Gene annotation enrichment analysis

Annotation Type	Terms Enriched for Induced Transcripts	WNC 97-2-1	WNC 91-6-3 (7 months)	WNC 91-6-3 (39 months)	WNC 91-6-3 (67 months)
GO:0004115	3',5'-cyclic-AMP phosphodiesterase activity	ne	7.33	11.28	6.76
IPR000006	metallothionein, vertebrate	ne	7.17	5.83	7.36
GO:0006096	glycolysis	11.20	4.56	7.33	5.90
GO:0006749	glutathione metabolic process	4.51	3.33	5.59	4.30
h_arenrf2Pathway	oxidative stress induced gene expression via Nrf2	4.51	3.08	4.17	3.98
SP_PIR_KEYWORDS	angiogenesis	3.57	2.90	3.28	3.41
GO:0046660	female sex differentiation	3.53	2.82	3.89	3.29
hsa04060	cytokine-cytokine receptor interaction	2.80	2.53	2.92	2.40
GO:0007596	blood coagulation	2.88	2.36	2.04	1.94
hsa04620	Toll-like receptor signaling pathway	2.66	2.35	2.09	2.04
GO:0001666	response to hypoxia	3.77	2.18	2.98	2.63
GO:0006954	inflammatory response	2.07	2.04	1.63	1.96
GO:0043066	negative regulation of apoptosis	3.01	2.00	2.14	2.10
GO:0006979	response to oxidative stress	2.48	1.86	2.20	2.00
GO:0009611	response to wounding	2.09	1.81	1.68	1.91
GO:0050801	ion homeostasis	1.80	1.72	1.40	1.78
GO:0006955	immune response	2.04	1.69	1.68	1.84
GO:0010648	negative regulation of cell communication	2.15	1.67	1.86	1.94
SP_PIR_KEYWORDS	secreted	2.62	1.60	1.80	1.72
SP_PIR_KEYWORDS	disulfide bond	2.05	1.52	1.61	1.69
GO:0042127	regulation of cell proliferation	1.88	1.41	1.65	1.53
Annotation Type	**Terms Enriched for Repressed Transcripts**	**WNC 97-2-1**	**WNC 91-6-3 (7 months)**	**WNC 91-6-3 (39 months)**	**WNC 91-6-3 (67 months)**
SP_PIR_KEYWORDS	muscle protein	3.79	5.29	3.95	5.18
GO:0043292	contractile fiber	3.52	4.24	3.07	4.18
GO:0042246	tissue regeneration	ne	3.52	ne	3.13
IPR013151	Immunoglobulin	3.38	2.57	3.35	2.71
GO:0006694	steroid biosynthetic process	ne	2.42	ne	2.52
GO:0015629	actin cytoskeleton	ne	2.10	1.71	2.21
GO:0015267	channel activity	ne	2.00	1.55	2.20
GO:0005509	calcium ion binding	ne	1.66	1.56	1.54
SP_PIR_KEYWORDS	differentiation	2.41	1.56	1.77	1.61
GO:0007155	cell adhesion	1.72	1.54	1.43	1.50

Table 2: Gene annotation enrichment analysis performed with DAVID, selected terms shown. Scores show fold enrichment in lists of genes induced or repressed in comparison with controls (FDR<0.05; Fold change >1.3) and are highlighted in red text for statistically significant induction (FDR<0.05) and in blue text for statistically significant repression (FDR<0.05). ne denotes no enrichment found.

Table 3: Class Prediction

A: SVM Classification of human HSkMC particle exposure experiment 1, trained using rat L6-C11 data						
True Value	**Prediction**	**Control**	**Tantalum**	**WNC 91-6-3**	**WNC 97-2-1**	**WNF 97-2-1**
Control	Control	**0.2**	-1.068	-1.406	-2.893	-1.013
Tantalum	Control	**-0.335**	-0.67	-1.589	-2.427	-1.045
WNC 91-6-3 (39)	WNC 97-2-1	0.213	-2.763	-0.807	**1.028**	-2.766
WNC 97-2-1	WNC 97-2-1	0.257	-3.173	-0.682	**0.343**	-1.564
WNF 97-2-1	Control	**0.0651**	-1.547	-0.589	-1.921	-1.84

B: SVM Classification of human HSkMC particle exposure experiment 2, trained using rat L6-C11 data						
True Value	**Prediction**	**Control**	**Tantalum**	**WNC 91-6-3**	**WNC 97-2-1**	**WNF 97-2-1**
Control	Control	**0.29**	-1.147	-1.61	-3.554	0.137
Tantalum	Control	**0.103**	-0.71	-1.788	-3.003	-0.343
WNC 91-6-3 (67)	WNC 97-2-1	0.0646	-2.464	-0.653	**0.944**	-2.811
WNC 91-6-3 (7)	WNC 97-2-1	-0.137	-2.306	-0.513	**0.784**	-2.951

C: SVM Classification of human HSkMC particle exposure experiment 2, trained using human HSkMC experiment 1 data						
True Value	**Prediction**	**Control**	**Tantalum**	**WNC 91-6-3 (39)**	**WNC 97-2-1**	**WNF 97-2-1**
Control	Control	**1.358**	-0.958	-1.326	-0.778	-2.64
Tantalum	Control	**1.216**	-0.719	-1.334	-1.031	-2.675
WNC 91-6-3 (67)	WNC 91-6-3 (39)	-1.054	-1.588	**5.097**	-2.558	-3.206
WNC 91-6-3 (7)	WNC 91-6-3 (39)	-1.001	-1.328	**6.18**	-3.915	-2.888

Table 3: Class prediction. Margin scores are shown for each comparison, highest margin scores are highlighted in bold text.

Table 4: Hallmarks of Cancer?

Hallmarks of Cancer (Hanahan & Weinburg 2011)	**Transcriptional Responses of HSkMC to WNC 91-6-3**
Evading growth suppressors	+ Negative regulation of cell communication - Cell communication
Sustaining proliferative signaling	+ Regulation of cell proliferation + Growth factor activity
Deregulating cellular energetics	+ Glycolysis/gluconeogenesis
Resisting cell death	+ Negative regulation of apoptosis
Genome instability and mutation	+ DNA damage (Comet assay) - DNA repair
Inducing angiogenesis	+ Angiogenesis
Activating invasion and metastasis	- Cell adhesion -Extracellular matrix
Tumour-promoting inflammation	+ Inflammation
Enabling replicative immortality	No evidence
Avoiding immune destruction	- Glycoproteins? +/- Intrinsic to plasma membrane?

Table 4: Comparison of the hallmarks of cancer identified by Hanahan and Weinburg [26] and the transcriptional responses of HSkMC cells to tungsten-nickel-cobalt alloy WNC 91-6-3.

Highlights

- Use of transcriptomics to identify likely carcinogenic tungsten alloys *in vitro*
- Cobalt containing alloys cause oxidative stress, DNA-damage and perturb apoptosis
- Presence of cobalt cause changes in gene expression consistent with carcinogenesis
- Development of an *in vitro* screening platform for tungsten alloy toxicity