Published in final edited form as:

Reprod Toxicol. 2017 April ; 69: 75–83. doi:10.1016/j.reprotox.2017.02.001.

Transcriptomics analysis of early embryonic stem cell differentiation under osteoblast culture conditions: Applications for detection of developmental toxicity*

Xinrong Chen^{a,*}, Tao Han^a, J. Edward Fisher^b, Wafa Harrouk^b, Melissa S. Tassinari^b, Gwenn E. Merry^a, Daniel Sloper^a, James C. Fuscoe^a, Deborah K. Hansen^a, and Amy L. Inselman^a

^aDivision of Systems Biology, National Center for Toxicological Research, Food and Drug Administration, United States

^bCenter for Drug Evaluation and Research, Food and Drug Administration, United States

Abstract

The mouse embryonic stem cell test (mEST) is a promising *in vitro* assay for predicting developmental toxicity. In the current study, early differentiation of D3 mouse embryonic stem cells (mESCs) under osteoblast culture conditions and embryotoxicity of cadmium sulfate were examined. D3 mESCs were exposed to cadmium sulfate for 24, 48 or 72 h, and whole genome transcriptional profiles were determined. The results indicate a track of differentiation was identified as mESCs differentiate. Biological processes that were associated with differentiation related genes included embryonic development and, specifically, skeletal system development. Cadmium sulfate inhibited mESC differentiation at all three time points. Functional pathway analysis indicated biological pathways affected included those related to skeletal development, renal and reproductive function. In summary, our results suggest that transcriptional profiles are a sensitive indicator of early mESC differentiation. Transcriptomics may improve the predictivity of the mEST by suggesting possible modes of action for tested chemicals.

Keywords

Osteoblast; Embryonic stem cell test; Embryotoxicity; Transcriptomics

★This document has been reviewed in accordance with United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents or conclusions necessarily reflect the position or opinions of the FDA nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

*Correspondence author. xchen@cpsc.gov.

Conflict of interests

None of the authors have any competing interests in this study.

Authors' contributions

Xinrong Chen designed and carried out the cell culture experiments, designed the microarray study, analyzed both cell culture and microarray data, drafted the manuscript. Tao Han carried out the microarray experiment and participated in the microarray data analysis. J. Edward Fisher participated in the development of the protocol and helped to draft the manuscript. Wafa Harrouk participated in the development of the protocol and helped to draft the manuscript. Melissa S. Tassinari participated in the development of the protocol and helped to draft the manuscript. Gwenn E. Merry carried out the Alizarin red S staining. Daniel Sloper carried out the RT-PCR experiment. James C. Fuscoe participated in the design of the microarray experiment and data analysis of the microarray experiment. Deborah K. Hansen participated in the development of the protocol and the design of the cell culture experiment. Amy L. Inselman developed the protocol and participated in the design of the cell culture experiment.

1. Introduction

The high throughput transcriptomics screening method has been proven to have the capacity to monitor toxic responses before overt toxicity is detected [1], making it a useful tool for identifying biological events at early time points and at low doses. Recently, applicability of transcriptomics to the mouse embryonic stem cell test (mEST) has been examined by several groups. Piersma et al. used this technique to monitor the embryotoxicity of several known *in vivo* developmental toxicants in the mEST using both neural cells and cardiomyocytes as differentiation endpoints [1–3]. In their approach, the dynamics of early mESC differentiation were described by a differentiation track which was created by examining transcriptomics of unexposed controls in chronological order. Compounds which caused significant deviations from the differentiation track were predicted as potential developmental toxicants. Teratogenicity of thalidomide was evaluated by using human [4] and mouse [5] ESCs. In addition to the expected perturbation of limb and embryonic development, other biological processes dysregulated by thalidomide included heart and neural development.

Cadmium is a heavy metal that occurs in nature at low concentrations. However, due to its widespread occurrence, cadmium is detectable in the human body through exposure to various sources such as food, water, and air [6]. Cadmium is reported to induce toxicity in multiple organs and cells [7]. The best known example of cadmium intoxication is the itai-itai disease discovered in Japan after World War II; people were sickened by eating food contaminated with cadmium released by industrial waste disposal. Cadmium causes bone softening and kidney failure in the affected population, indicating bone and kidney are the primary target organs in itai-itai disease [8,9]. Cadmium is embryotoxic and affects development of multiple organ systems when exposed *in utero*. [10]. Cadmium causes neural tube defects in mice and zebrafish by impairing neurogenesis as characterized by reduced neuronal differentiation during early embryonic stages [11–13]. In clinical situations, children of women exposed to cadmium during pregnancy display lower motor and perceptual abilities with impaired intelligence and lowered school achievement [11]. Cadmium-induced limb defects in mice show differential strain sensitivity [14,15]. The period of gestation between day 7 to day 10 is the most susceptible stage for cadmium-induced limb malformations in mice [16], which has been associated with reactive oxygen species (ROS)-mediated endoplasmic reticulum stress [17]. Maternal exposure to cadmium during pregnancy perturbs cardiovascular development of rat offspring which in turn contributes to a long term increased risk of cardiovascular diseases [18]. Cadmium damages vascular tissues, induces endothelial dysfunction and promotes atherosclerosis by oxidative mechanisms [19,20]. In addition to its teratogenic effects, cadmium also causes reproductive toxicity by affecting both testis and oocyte development; increased cadmium concentrations in the ovary have been associated with failure of oocyte development and failure to ovulate [10]. Cadmium induced oxidative stress in the testis can result in reduced sperm count [21,22]. In recent years, cadmium was reported to act as an endocrine disruptor which acts by mimicking the effect of estrogen in the uterus and mammary gland through binding to estrogen receptor alpha [23, 24]. In summary, cadmium affects many different organs and

tissues where the specific effects after exposure are linked to such factors as species, dose, and exposure window.

We recently determined a quick and highly reproducible culture method to generate osteoblasts from a D3 mESC single cell suspension within 14 days of culture. For the first time, we tested the embryotoxicity of cadmium sulfate in the mEST with differentiation to both osteoblasts and cardiomyocytes. Both osteoblast and cardiomyocyte endpoints correctly classified cadmium sulfate as strongly embryotoxic [2]. Cadmium sulfate inhibited osteoblast differentiation in a dose-dependent manner as indicated by reduced mineralization and reduced expression of osteoblast lineage specific markers including *Runx2* and osteocalcin. Similarly, cadmium sulfate inhibited cardiomyocyte differentiation by reducing functional beating cardiomyocytes. The concentration of cadmium sulfate which decreased differentiation by 50% (ID₅₀) for the two differentiation endpoints were very similar with 1.3 µg/ml for osteoblasts and 1.2 µg/ml for cardiomyocytes, respectively.

The aim of the current study was to determine gene expression profiles of early mESC differentiation under osteoblast culture conditions and to assess whether treatment with cadmium sulfate over a time course of 72 h altered the gene expression pattern. Collectively, our findings suggest that transcriptomics analysis is a sensitive tool that can be used to detect perturbations early in mESC differentiation and thus may improve the prediction ability of our recently developed osteoblast mEST.

2. Materials and methods

2.1. Culture media, chemicals and reagents

The D3 mESC line (cat. no. CRL-1934, unknown passage number) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Mouse primary embryonic fibroblasts (PMEFs) (cat. no. PMEF-CFL), murine leukemia inhibitory factor (LIF) (cat. no. ESG1106) and 0.1% gelatin solution (cat. no. ES-006-B) were obtained from EMD Millipore (Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM) (cat. no. 41965), L-glutamine (cat. no. 25030-024), penicillin/streptomycin (cat. no. 15140-122), nonessential amino acids (NEAA) (cat. no. 11140-035), trypsin-EDTA (cat. no. 25300-054), β-mercaptoethanol (cat. no. 21985-023), TaqMan® Gene Expression Master Mix (cat. no. 4369016), cDNA Reverse Transcription Kit (cat. no. 4368814), and TaqMan® primers for *Gapdh* (Mm99999915_g1), *Dppa2* (Mm01343391_gH), *Pou5f1* (Mm03053917_g1), *Sox2* (Mm03053810.s1), *Hand2* (Mm00439247_m1), *Msx2* (Mm00442992_m1), *Cited1*, (Mm01235642_g1), *Gpc3* (Mm00516722_m1), *Wnt11* (Mm00437327_g1), and *Pth1r* (Mm00441046_m1) were purchased from the former Life Technologies (Grand Island, NY) now part of Thermo Fisher Scientific. Cadmium sulfate (cat. no. 383082), trypan blue (cat. no. T8154), β-glycerophosphate (cat. no. G5422), dexamethasone (cat. no. D4902), Alizarin red S (cat. no. A5533) and thiazolyl blue tetrazolium bromide (MTT) (cat. no. M5655) were obtained from Sigma Aldrich (St. Louis, MO). Ascorbic acid (cat. no. 07157) was obtained from StemCell Technology (Vancouver, Canada). The RNeasy Mini kit (cat. no. 74106) was obtained from Qiagen (Hilden, Germany). Defined ES-qualified fetal bovine serum (FBS) (cat. no. SH30070.03) and PBS (cat. no. SH30256.01) were obtained from Fisher Scientific (Pittsburgh, PA). A Zeiss microscope with AxioVision and MosaicX software systems was

obtained from Carl Zeiss (Jena, Germany). The single color (Cyanine 3 or Cy3) Agilent Whole Mouse Genome 4 × 44k Microarray gasket slides (cat. no. SH30256.01), the One-Color Quick-Amp Labeling Kit (cat. no. 5190-0442), the RNA 6000 Nano ladder (cat. no. 5067-1529), the RNA 6000 Nano kit (cat. no. 5067-1511), the Agilent 2100 Bioanalyzer and the Agilent FE software were obtained from Agilent Technologies (Santa Clara, CA). The GenePix 4000B microarray scanner was obtained from Molecular Devices (Sunnyvale, CA).

2.2. Mouse embryonic stem cell culture

The mESCs were routinely cultured according to standard protocols [25–27]. Briefly, D3 mESCs (passage number 4 within our laboratory) were co-cultured with x-ray irradiation inactivated PMEFs in DMEM medium supplemented with 15% FBS, 1000 units/ml LIF, 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 1X NEAA and 0.1 mM β-mercaptoethanol. The medium for the D3 cells was refreshed daily during the week and once during the weekends; cells were routinely sub-cultured every 2–3 days and maintained in a humidified atmosphere at 37 °C with 5% CO₂.

2.3. Osteoblast differentiation for the mEST

Osteoblast differentiation was conducted as previously described [25]. Briefly, PMEF co-cultured D3 cells were purified by replating the detached co-culture mixture onto 100 mm tissue culture dishes for two rounds to deplete the adhesive PMEFs. The recovered purified D3 cells were directly plated into 24-well plates with 20,000 mESCs per well in osteoblast medium, which consisted of DMEM supplemented with 15% FBS, 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 1X NEAA, 0.1 mM β-mercaptoethanol, 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 1 µM dexamethasone. The culture medium was refreshed every 2–3 days. Osteoblast differentiation was evaluated at the end of a 14 day culture using Alizarin red S (ALR) staining.

2.4. Cadmium sulfate treatment and cell culture for microarray analysis

For microarray analysis, mESC differentiation was initiated on day 0 using a single cell suspension of purified D3 mESCs prepared as described above. For osteoblast differentiation, the cell density (20,000 cells/well for a 24-well plate) and osteogenic differentiation media used in the osteoblast mEST (detailed above) were also used. Different from the traditional osteoblast differentiation protocol in which a tested chemical was added to the culture medium beginning on day 0 and remained in the culture throughout the experiment, cadmium sulfate was added to the culture medium on day 3 (exposure time 0) for the microarray experiments. By adding cadmium sulfate on day 3 instead of day 0, the cells were able to proliferate prior to treatment, increasing confidence that the observed effects were not simply due to cytotoxicity of the compound. The concentration of cadmium sulfate used in the microarray analysis was 1.3 µg/ml; this dose corresponds to the ID₅₀ value of *Runx2* mRNA expression in the traditional osteoblast EST, which was the most sensitive endpoint [25]. Water (0.1% final concentration, same as added volume of cadmium sulfate) was used as a solvent control for cadmium sulfate exposure. Cadmium sulfate or solvent control was added to the culture starting on day 3 (exposure time 0); cells were harvested on exposure days 3, 4, 5 or 6 (corresponding to 0, 24, 48 or 72 h exposure, respectively, 5–6 replicates per group) according to the experimental design illustrated in Fig. 1 [2, 3, 28].

Parallel cultures for cytotoxicity analysis (MTT assays) were included to verify that the dose of cadmium sulfate did not cause significant cytotoxicity to the exposed cells. To determine if osteoblast differentiation was comparable to that of our earlier study [25], four extra wells were prepared with cadmium sulfate or 0.1% water added to the culture medium from day 3 to day 14, and osteoblast differentiation was evaluated using ALR staining at the end of the 14 day culture period.

2.5. Cytotoxicity assay (MTT assay)

Cytotoxicity of cadmium sulfate was measured in an experiment run concurrently with the microarray experiments. Cadmium sulfate or 0.1% water was added to the culture medium on day 3 (exposure time 0), and the MTT assay was conducted on exposure days 4, 5 or 6 (corresponding to 24, 48 or 72 h exposure, respectively) to match the harvest time in the microarray experiments. There were four replicates in each group for the MTT assay. The cytotoxicity data is presented as a percentage of controls, and the IC₅₀ is defined as the concentration which produced a 50% decrease in cell viability.

2.6. Alizarin red S staining

ALR staining of osteoblasts was conducted at the end of the 14 day culture period as previously described [25, 29]. Briefly, cells cultured in 24-well plates were washed with PBS, fixed with 0.5 ml formaldehyde (10%) at room temperature for 15 min, washed twice with distilled water and stained with 0.5 ml ALR solution (40 mM, pH 4.2) at room temperature for 15 min. The stained cells were then extensively washed to remove nonspecific staining. Plates were air dried and images were captured using a Zeiss microscope and AxioVision software. Compiled images, representing entire individual wells of ALR-stained cells, were obtained by stitching 169 individual phase contrast images (5× objective) together using Zeiss MosaicX software.

2.7. RNA isolation

Total RNA was isolated from cells in each well (approximately 0.4 million cells with a yield of about 10 µg RNA) of the 24-well plates using the RNeasy Mini Kit according to the manufacturer's protocol; isolated RNA was eluted in a total volume of 30 µl H₂O. RNA concentrations were determined spectrophotometrically by measuring optical density at 260 nm with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE), and only RNA samples with both 260/230 and 260/280 ratios equal to or greater than 2 were used for subsequent analysis. The quality of RNA was further evaluated with the Agilent 2100 Bioanalyzer, and only samples with an RNA integrity number (RIN) equal to 10 were used in the subsequent microarray analysis. A total of seven experimental groups were used in the transcriptomics analysis with five or six samples (wells) in each group, these included control group at day 3 (0 h), control and cadmium sulfate exposed groups at day 4 (24 h), day 5 (48 h) and day 6 (72 h).

2.8. Microarray experiments

Agilent mouse gene expression microarrays (4 × 44k) with 45,220 features were used for microarray analysis. RNA labeling, hybridization and washing were performed according to

the One-Color Microarray-based Gene Expression Analysis Protocol (V6.5, Agilent Technologies). In brief, 500 ng total RNA were used in each labeling reaction. The Agilent One-Color Spike-In Kit was used as a positive control to monitor labeling efficiency across different batches. The resultant cRNA was evaluated using a NanoDrop 1000 to ensure every labeling reaction had a yield greater than 1.65 µg cRNA and a specific activity greater than 9 pmol Cy3 per µg cRNA. Subsequently, 1.65 µg Cy3 labeled cRNAs of each sample were fragmented and hybridized with the microarray slide at 65 °C for 17 h. Samples were randomized by cadmium sulfate treatment and exposure time such that biological replicates of the same experimental group were not placed on the same slide. After hybridization, slides were washed and scanned using the Agilent DNA Microarray scanner. The resulting images were analyzed using Agilent FE software (v12.0).

2.9. Microarray data analysis

The raw intensity data were uploaded to the Array Track database [30,31] and Partek Genomics Suite (Partek Inc., St. Louis, MO) for data analysis. The data were normalized using 75th-percentile scaling before downstream statistical analysis [32]. One-way ANOVA was used to identify differentially expressed genes (DEGs) with a cut-off value false discovery rate (FDR) <0.05, fold change (FC) >1.5. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were conducted to measure the similarity between different experimental groups. To visualize the differences between the different time points in the unexposed culture, a mESC differentiation track was developed using a two-dimensional PCA (2D-PCA) approach [1–3,33] utilizing Partek Genomics Suite. Functional and pathway analysis were conducted using Ingenuity Pathway Analysis (IPA) software (<https://apps.ingenuity.com/ingsso/login>) to identify biological pathways, canonical pathways and networks associated with significantly regulated genes.

2.10. RT-PCR confirmation of microarray results

mRNA expression of stem cell pluripotency genes and bone development-related genes was further evaluated using RT-PCR analysis to confirm the microarray results. The RNA sample used in the RT-PCR analysis came from the same batch as the microarray experiment. RT-PCR was performed as previously described [25] using an ABI 7900HT Fast Real-Time PCR System with 384-well plates (Applied Biosystems). The TaqMan primers used included pluripotency genes *Dppa2* (Mm01343391_gH), *Pou5f1* (Mm03053917_g1) and *Sox2* (Mm03053810_s1); bone development related *Hand2* (Mm00439247_m1), *Msx2* (Mm00442992_m1), *Cited1* (Mm01235642_g1), *Gpc3* (Mm00516722_m1), *Wnt11* (Mm00437327_g1) and *Pth1r* (Mm00441046_m1). Relative gene expression in the PCR analysis was calculated as 2^{-CT} using *Gapdh* (Mm99999915_g1) as an endogenous control.

3. Results

3.1. Expression profile of early mESC differentiation

In the current study, transcriptomics was used to improve the predictivity of the mEST assay and to develop more mechanistic information. The gene expression profiles of control cultures were compared using one-way ANOVA with a cut-off criterion of a p-value <0.01

with Bonferroni correction using Partek Genomics Suite. A total of 6308 DEGs were identified. HCA analysis of the four control groups showed a clear time dependent expression pattern (Fig. 2A). The upregulated transcripts, shown in red, at early time points were gradually down-regulated (blue color) as time progressed and vice versa. To visualize the temporal gene expression profile of the control samples, 2D-PCA was performed (Fig. 2B). On the 2D-PCA, samples within the same experimental groups all clustered together. When different experimental groups were connected using a dashed line and arranged in chronological order, a bell-like smooth curve was identified. This line represents continuous gene expression changes as the mESCs differentiate in the osteoblast medium. Similar results have been reported in the literature where a similar gene expression pattern change was described with neural and cardiomyocyte differentiation from D3 mESCs [1–3].

3.2. Biological processes modulated in the control culture

To determine the biological pathways modulated in the early differentiation of mESCs, the gene expression profile of cells treated with vehicle for 24, 48 or 72 h were compared to the 0-h control group. The total number of genes meeting the filtering criteria of FDR <0.05, FC >1.5 increased over time, with the most significant changes occurring between 24 and 48 h (Fig. 3A). A total of 1730, 6630, and 9190 genes were significantly changed after 24, 48 and 72 h of differentiation, respectively. The number of upregulated genes was very similar to the number of down-regulated genes at all three time points during early mESC differentiation (Fig. 3A). A high degree of gene overlap was observed after 24, 48, and 72 h of differentiation, among which 1189 genes were commonly expressed. As a result, more than 68% of genes at 24 h overlapped with genes at 48 and 72 h. In addition, a total of 4431 genes overlapped between 48 and 72 h (Fig. 3B).

A reduction in expression of stem cell pluripotency genes is crucial for differentiation. RT-PCR analysis showed several stem cell pluripotency related genes were down-regulated in a time dependent manner in the control culture (Fig. 3C). The expression of developmental pluripotency-associated 2 (*Dppa2*) decreased 1.9, 4.9, and 8.2 fold, *Pou5f1* decreased 1.0, 1.6, and 3.2 fold, and *Sox2* decreased 1.1, 1.2, and 1.3 fold after 24, 48 and 72 h of differentiation, respectively, indicating a progressive differentiation of the mESCs. The gene expression profile of the stem cell pluripotency genes obtained from microarray analysis was in agreement with the RT-PCR analysis indicating the accuracy of the microarray data.

Biological processes associated with the 1189 commonly responding genes in the control culture included pathways such as embryonic development, organismal development, tissue development, tissue morphology, as well as organ specific pathways such as cardiovascular, renal, and skeletal development (Fig. 3D).

In contrast to the down-regulation of stem cell pluripotency genes, the genes involved in bone development were significantly upregulated in the control cultures (Table 1). For example, the expression of *Hand2*, a gene closely related to osteoblast differentiation, limb development and bone morphogenesis [34–36] was dramatically upregulated in the control culture over time. *Hand2* expression increased 2.7, 8.7 and 25.7 fold after 24, 48 and 72 h of differentiation, respectively, as indicated by RT-PCR analysis. The microarray results corresponded well with the PCR results, which showed 3.3, 6.5 and 11.3 fold increases after

24, 48 and 72 h of differentiation, respectively. Other bone development-related genes including *Msx2* [37–39], *Cited1* [40], *Gpc3* [41], *Wnt11* [42], and *Pth1r* [43–45] showed a time dependent increase similar to that of *Hand2*.

3.3. Classical toxicological evaluation of the effects of cadmium sulfate

In our previous study [25], we have determined the most sensitive endpoint for detecting cadmium sulfate toxicity in the osteoblast mEST was *Runx2* expression. The ID₅₀ for that endpoint was 1.3 µg/ml, so a single dose of 1.3 µg/ml cadmium sulfate was selected for the gene expression profiling analysis in the current study. Cadmium sulfate had no significant effect on cell viability at any of the examined time points (Fig. 4A). This is important since it eliminates the possibility that changes in gene expression were due to cytotoxicity. To ensure that the culture conditions used for the microarray experiment generated mature osteoblasts, a concurrent 14 day culture experiment was conducted in parallel with the microarray study. Results indicate that osteoblast production was similar to that obtained previously [25] (Fig. 4B). Efficient osteoblast production was detected in the extended 14 day concurrent control plates as indicated by ALR staining; more importantly, cadmium sulfate had no significant adverse effect on osteoblast differentiation when the exposure was limited from day 3 to day 14 (Fig. 4B).

3.4. Cadmium sulfate effects on mESC differentiation

The effects of cadmium sulfate exposure on gene expression during mESC differentiation were analyzed using three-dimensional PCA (3D-PCA) with the Partek Genomics Suite; all seven experimental groups were included in the analysis (Fig. 5). On the 3D-PCA plot, replicates within the same experimental groups clustered together, while the different experimental groups separated well from each other, indicating good reliability and reproducibility of the microarray data. The control samples are shown arranged from left to right in chronological order. Noticeably, all of the cadmium sulfate-treated groups were separated from their time-matched control groups. Cadmium sulfate induced changes and shifted the gene expression patterns of the treated cells to the left of their concurrent control groups. This represents a more undifferentiated status (Fig. 5), suggesting that the compound was delaying or inhibiting differentiation.

3.5. Biological pathways affected by cadmium sulfate

The number of DEGs significantly modulated by cadmium sulfate exposure was not very different across the three time points examined; a total of 1008, 1353, or 1208 DEGs were identified after 24, 48 or 72 h, respectively, of cadmium sulfate treatment. The number of upregulated DEGs was greater than the number of down-regulated DEGs at all three time points. There were 739, 807 and 709 upregulated DEGs, and there were 269, 546 and 499 downregulated DEGs after 24, 48 and 72 h, respectively, of cadmium sulfate exposure (Fig. 6A). A Venn diagram was used to analyze the overlap of global DEGs between the three time points; a total of 407 genes, referred to as commonly responding genes, changed upon treatment with cadmium sulfate at each time point examined. In addition to the commonly responding genes, 232 DEGs overlapped between the 24- and 48-h time points; 353 DEGs overlapped between the 48- and 72-h time points, and only 44 DEGs overlapped between the 24- and 72-h time points (Fig. 6B). The biological processes that are significantly regulated

by the 407 commonly responding genes include embryonic development and organ specific pathways such as renal, reproductive, cardiovascular, nervous, endocrine, and skeletal system development and function (Fig. 6C). Consistent with the previously reported teratogenic effect of cadmium sulfate on limb development [14], bone development-related genes, including *Hand2*, *Msx2*, *Cited1*, *Gpc3*, *Wnt11* and *Pth1r*, demonstrated a clear time-dependent inhibition in the cadmium sulfate exposed cultures as indicated by both RT-PCR and microarray analysis (Table 1).

4. Discussion

The purpose of the current study was to determine if the incorporation of transcriptomics to the osteoblast mEST could improve the prediction ability of the assay. Global gene expression profiles of mESCs which spontaneously differentiate in osteoblast medium were first examined; subsequent analyses were designed to learn whether the embryotoxicity of cadmium sulfate could be detected in this system. The results show that transcriptomics could be used as a sensitive tool to evaluate early mESC differentiation and detect chemical-induced teratogenicity as well as other toxic effects.

Examination of the gene expression pattern of the stem cell marker genes, in regards to differentiation time in untreated control cultures, revealed features that are in agreement with their functions. Expression patterns for stem cells generally indicate loss of pluripotency and gain of lineage specificity during spontaneous differentiation. For instance, DPPA2 plays an important role in maintenance of the undifferentiated state; expression of *Dppa2* is high in ES cells and decreases markedly in differentiated cells [46–48]. In the present study, expression of *Dppa2* decreased dramatically in the control cultures as mESCs differentiated (Fig. 3C). The POU transcription factor OCT4 (encoded by POU5F1) is a master regulator of pluripotency in ES cells, and a critical amount of OCT4 is required to sustain stem cell self-renewal and lineage commitment [49,50]. Similar to *Dppa2*, expression of *Pou5f1* was dramatically reduced as mESCs differentiated. SOX2 (sex determining region Y-box 2) is a transcription regulator in pluripotent cells; down-regulation of SOX2 in the early embryo correlates with a commitment to differentiate [51]. Although inhibition of *Sox2* was not as dramatic as the reductions observed with *Dppa2* and *Pou5f1*, the repression was very consistent at all three time points. These stem cell pluripotency gene expression results are consistent with a previous report which also indicated expression of *DPPA2*, *POU5F1* and *LIN28A* in human ESCs were significantly down-regulated after 14 days of differentiation [4]. In summary, the inhibition of stem cell pluripotency gene expression allows the cells to differentiate and alter expression of genes in embryonic development and other pathways such as organismal development, tissue development and tissue morphology (Fig. 3D).

In contrast to the reduced expression of stem cell pluripotency genes, the expression of several lineage specific genes, specifically those involved in bone development, was significantly upregulated as mESCs differentiated. HAND2 is a transcription factor that controls both limb and cardiac development; mouse embryos homozygous for the *Hand2* deletion have underdeveloped limbs and die at mid-gestation from cardiac abnormalities [34,52]. In the present study, expression of *Hand2* increased dramatically with time in the control cultures as mESCs differentiated (Table 1). Cadmium sulfate inhibited *Hand2*

expression at all three time points. *MSX2* is a transcription factor that plays crucial roles at multiple sites during organogenesis; specifically, *Msx2* is required for both chondrogenesis and osteogenesis [37,38]. In the present study, the expression pattern of *Msx2* was very similar to that of *Hand2* which showed induction in the control cultures and inhibition upon cadmium sulfate treatment. Expression patterns of *Cited1* and *Gpc3* also showed time-dependent induction in the control cultures, and the expression of both genes was inhibited by cadmium sulfate treatment. *Cited1*, formerly melanocyte-specific gene 1 (*Mslg1*), is predominantly expressed in the nascent mesoderm and limb buds during development [53]. GPC3 (glypican-3) is a cell surface heparin sulfate proteoglycan, and mice deficient in *Gpc3* display severe skeletal abnormalities [41]. The Wnt signaling pathway is also known to play an important role in key processes of embryogenesis. During development, WNT11 is expressed in the perichondrium and in the axial skeleton and sternum [54]. WNT11 enhances BMP-induced osteoblast maturation and mineralization [42]. Consistent with the role of WNT11 involved in bone development and osteoblast differentiation, *Wnt11* expression was moderately upregulated during mESC differentiation in culture, and the induction was suppressed by cadmium sulfate treatment. Expression of *Pth1r* was noticeably upregulated in the control cultures. Cadmium sulfate, however, inhibited *Pth1r* expression to a much higher degree when compared to the other bone-related genes (Table 1). Concurrent with dysregulated embryonic development, the skeletal and muscular system pathway was one of the gene ontology pathways that was enriched under the osteoblast culture conditions (Fig. 3D). Other organ systems associated with significantly regulated genes include cardiac, renal and the reproductive system. All of the pathways shown in Fig. 6D were significantly affected by cadmium sulfate treatment; the probability scores for all pathways were less than 10^{-7} . That several pathways were significantly affected by treatment was not unexpected since mESCs have the potential to become all different types of cells, and it is difficult to obtain an absolute homogeneous cell population under the current culture conditions.

In our previous study we determined the IC_{50} and ID_{50} values of cadmium sulfate for D3 mESCs to be 0.76 and 1.3 $\mu\text{g/ml}$, respectively [25]. In the present transcriptomics study, the cells were treated with a single dose of cadmium sulfate at a final concentration of 1.3 $\mu\text{g/ml}$. The ID_{50} concentration was chosen for this study because ID_{50} concentrations have previously been shown to be effective doses for transcriptomics studies and used by other investigators [55]. Although a dose of 1.3 $\mu\text{g/ml}$ is nearly double the IC_{50} (0.76 $\mu\text{g/ml}$) concentration, the current results indicate that a single dose of 1.3 $\mu\text{g/ml}$ cadmium sulfate with an exposure time up to 72 h did not cause any significant cytotoxicity. This may, in part, be due to cadmium sulfate being added at day 3 of culture rather than day 0 as in the classic mEST assay. At day 3 of culture, the cells had already grown under unexposed culture conditions and had reached a confluent status which may have made them more resistant to chemical treatment. Another possibility for the lack of cytotoxicity is the shorter treatment time of 24, 48 or 72 h in the current transcriptomics study rather than 10 days as in the mEST from which the ID_{50} values were determined. Therefore, the current results indicate that cadmium sulfate did not cause significant cytotoxicity to the cells; and eliminates the possibility that changes observed in the transcriptomics study were due to cytotoxicity.

In contrast to the traditional mEST which classifies the embryotoxicity of tested chemicals into one of three categories, transcriptomics is a more mechanistic based screening assay. The original assay simply categorizes compounds as strongly-, weakly- or non-embryotoxic, and offers no information on pathways that might be altered by the compounds. Using cadmium sulfate as an example, the traditional mEST can only classify this chemical as strongly embryotoxic. Transcriptomics techniques, however, not only detected the expected toxic effects of cadmium on the skeletal system, but also identified gene sets involved in this process. In addition, transcriptomics also uncovered other possible developmental effects of cadmium sulfate treatment. These include possible effects on renal, reproductive, cardiac, neural, and endocrine systems (Fig. 6C). Compared with the traditional mEST, a transcriptomics based mEST has obvious advantages. First, it provides insights on pathways that might be affected. Secondly, transcriptomics can greatly reduce the time required for testing. Taking the osteoblast mEST as an example, the traditional test requires 14 days of culture, while the transcriptomics analysis requires only 6 days to detect alterations in gene expression. In support of our results, Piersma et al. was able to identify developmental toxicants 96 h after exposure in the mEST [1], while Gao et al. was able to detect thalidomide-induced effects within 24h after exposure using transcriptomics [5]. In conclusion, this study suggests that transcriptomics is a promising tool that can be a valuable addition to the drug safety evaluations toolbox.

In summary, we describe the global gene expression profile of mESCs differentiating under osteoblast culture conditions. Under the influence of osteogenic factors, pluripotency genes were downregulated, while skeletal and bone development-related genes were upregulated over time. By using multiple analytical methods, including HCA, PCA and functional pathway analysis, several biological pathways were identified that are significantly dysregulated during the early stages of mESC differentiation. Additionally, the embryotoxicity of cadmium sulfate was assessed by exposing cultured cells to a single dose of chemical. Our data contributes to a better understanding of osteoblast differentiation at early stages of *in vitro* development, as well as to the mode of action of cadmium sulfate-induced teratogenicity. Future work will be directed to demonstrate whether transcriptomics can be more widely used for developmental toxicity evaluation by including more chemicals.

Acknowledgments

This study was funded by FDA/NCTR under E073540 to A. Inselman. We greatly appreciate Carrie Moland for helping with the evaluation of RNA quality with the Agilent 2100 Bioanalyzer.

Abbreviations

mEST	mouse embryonic stem cell test
mESCs	mouse embryonic stem cells
ESCs	embryonic stem cells
ECVAM	European Centre for the Validation of Alternative Methods

PMEF	primary mouse embryonic fibroblasts
FBS	fetal bovine serum
DMEM	Dulbecco's modified eagle medium
MTT	thiazolyl blue tetrazolium bromide
ALR	alizarin red S
<i>Runx2</i>	runt related transcription factor 2
IC₅₀	50% inhibition of growth
ID₅₀	50% inhibition of differentiation
RIN	RNA integrity number
HCA	hierarchical clustering analysis
PCA	principal component analysis
DEGs	differentially expressed genes
GO	gene ontology
IPA	Ingenuity Pathway Analysis
FDR	false discovery rate
FC	fold change
Cy3	cyanine 3
<i>Dppa2</i>	developmental pluripotency associated 2
<i>Pou5f1</i>	POU domain, class5, transcription factor 1
<i>Sox2</i>	sex determining region Y-box 2

References

1. van Dartel DA, Pennings JL, van Schooten FJ, Piersma AH. Transcriptomics-based identification of developmental toxicants through their interference with cardiomyocyte differentiation of embryonic stem cells. *Toxicol Appl Pharmacol.* 2010; 243(3):420–428. [PubMed: 20036270]
2. Theunissen PT, Pennings JL, van Dartel DA, Robinson JF, Kleinjans JC, Piersma AH. Complementary detection of embryotoxic properties of substances in the neural and cardiac embryonic stem cell tests. *Toxicol Sci.* 2013; 132(1):118–130. [PubMed: 23243006]
3. Theunissen PT, Pennings JL, Robinson JF, Claessen SM, Kleinjans JC, Piersma AH. Time-response evaluation by transcriptomics of methylmercury effects on neural differentiation of murine embryonic stem cells. *Toxicol Sci.* 2011; 122(2):437–447. [PubMed: 21613230]
4. Meganathan K, Jagtap S, Wagh V, Winkler J, Gaspar JA, Hildebrand D, Trusch M, Lehmann K, Hescheler J, Schluter H, et al. Identification of thalidomide-specific transcriptomics and proteomics signatures during differentiation of human embryonic stem cells. *PLoS One.* 2012; 7(8):e44228. [PubMed: 22952932]

5. Gao X, Sprando RL, Yourick JJ. Thalidomide induced early gene expression perturbations indicative of human embryopathy in mouse embryonic stem cells. *Toxicol Appl Pharmacol.* 2015; 287(1):43–51. [PubMed: 26006729]
6. Nordberg GF. Historical perspectives on cadmium toxicology. *Toxicol Appl Pharmacol.* 2009; 238(3):192–200. [PubMed: 19341754]
7. Rani A, Kumar A, Lal A, Pant M. Cellular mechanisms of cadmium-induced toxicity: are view. *Int J Environ Health Res.* 2014; 24(4):378–399. [PubMed: 24117228]
8. Kah M, Levy L, Brown C. Potential for effects of land contamination on human health. 1. The case of cadmium. *J Toxicol Environ Health B Crit Rev.* 2012; 15(5):348–363. [PubMed: 22852814]
9. Baba H, Tsuneyama K, Kumada T, Aoshima K, Imura J. Histopathological analysis for osteomalacia and tubulopathy in itai-itai disease. *J Toxicol Sci.* 2014; 39(1):91–96. [PubMed: 24418713]
10. Thompson J, Bannigan J. Cadmium: toxic effects on the reproductive system and the embryo. *Reprod Toxicol.* 2008; 25(3):304–315. [PubMed: 18367374]
11. Chow ES, Hui MN, Lin CC, Cheng SH. Cadmium inhibits neurogenesis in zebrafish embryonic brain development. *Aquat Toxicol.* 2008; 87(3):157–169. [PubMed: 18342959]
12. Robinson JF, Yu X, Moreira EG, Hong S, Faustman EM. Arsenic- and cadmium-induced toxicogenomic response in mouse embryos undergoing neurulation. *Toxicol Appl Pharmacol.* 2011; 250(2):117–129. [PubMed: 20883709]
13. Webster WS, Messerle K. Changes in the mouse neuroepithelium associated with cadmium-induced neural tube defects. *Teratology.* 1980; 21(1):79–88. [PubMed: 6247774]
14. Layton WM Jr, Layton MW. Cadmium induced limb defects in mice: strain associated differences in sensitivity. *Teratology.* 1979; 19(2):229–235. [PubMed: 473073]
15. Kuczuk MH, Scott WJ Jr. Potentiation of acetazolamide induced ectrodactyly in SWV and C57BL/6J mice by cadmium sulfate. *Teratology.* 1984; 29(3):427–435. [PubMed: 6463905]
16. Padmanabhan R, Hameed MS. Characteristics of the limb malformations induced by maternal exposure to cadmium in the mouse. *Reprod Toxicol.* 1990; 4(4):291–304. [PubMed: 1726510]
17. Wang Z, Wang H, Xu ZM, Ji YL, Chen YH, Zhang ZH, Zhang C, Meng XH, Zhao M, Xu DX. Cadmium-induced teratogenicity: association with ROS-mediated endoplasmic reticulum stress in placenta. *Toxicol Appl Pharmacol.* 2012; 259(2):236–247. [PubMed: 22252055]
18. Ronco AM, Montenegro M, Castillo P, Urrutia M, Saez D, Hirsch S, Zepeda R, Llanos MN. Maternal exposure to cadmium during gestation perturbs the vascular system of the adult rat offspring. *Toxicol Appl Pharmacol.* 2011; 251(2):137–145. [PubMed: 21236285]
19. Chen CY, Zhang SL, Liu ZY, Tian Y, Sun Q. Cadmium toxicity induces ER stress and apoptosis via impairing energy homeostasis in cardiomyocytes. *Biosci Rep.* 2015; 35(3)
20. Messner B, Knoflach M, Seubert A, Ritsch A, Pfaller K, Henderson B, Shen YH, Zeller I, Willeit J, Laufer G, et al. Cadmium is a novel and independent risk factor for early atherosclerosis mechanisms and in vivo relevance. *Arterioscler Thromb Vasc Biol.* 2009; 29(9):1392–1398. [PubMed: 19556524]
21. Martynowicz H, Skoczynska A, Karczmarek-Wdowiak B, Andrzejak R. Effects of cadmium on testis function. *Med Pr.* 2005; 56(2):167–174. [PubMed: 20067217]
22. Siu ER, Mruk DD, Porto CS, Cheng CY. Cadmium-induced testicular injury. *Toxicol Appl Pharmacol.* 2009; 238(3):240–249. [PubMed: 19236889]
23. Stoica A, Katzenellenbogen BS, Martin MB. Activation of estrogen receptor-alpha by the heavy metal cadmium. *Mol Endocrinol.* 2000; 14(4):545–553. [PubMed: 10770491]
24. Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, Clarke R, Sholler PF, Lirio AA, Foss C, et al. Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. *Nat Med.* 2003; 9(8):1081–1084. [PubMed: 12858169]
25. Chen X, Hansen DK, Merry G, DeJarnette C, Nolen G, Sloper D, Fisher JE, Harrouk W, Tassinari MS, Inselman AL. Developing osteoblasts as an endpoint for the mouse embryonic stem cell test. *Reprod Toxicol.* 2015
26. Tremml G, Singer M, Malavara R. Culture of mouse embryonic stem cells. *Curr Protoc Stem Cell Biol.* 2008 Chapter 1: Unit 1C 4.

27. Seiler AE, Spielmann H. The validated embryonic stem cell test to predict embryotoxicity in vitro. *Nat Protoc.* 2011; 6(7):961–978. [PubMed: 21720311]
28. Schulpen SH, Robinson JF, Pennings JL, van Dartel DA, Piersma AH. Dose response analysis of monophthalates in the murine embryonic stem cell test assessed by cardiomyocyte differentiation and gene expression. *Reprod Toxicol.* 2013; 35:81–88. [PubMed: 22813628]
29. Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem.* 2004; 329(1):77–84. [PubMed: 15136169]
30. Fang H, Harris SC, Su Z, Chen M, Qian F, Shi L, Perkins R, Tong W. ArrayTrack: an FDA and public genomic tool. *Methods Mol Biol.* 2009; 563:379–398. [PubMed: 19597796]
31. Tong W, Cao X, Harris S, Sun H, Fang H, Fuscoe J, Harris A, Hong H, Xie Q, Perkins R, et al. ArrayTrack-supporting toxicogenomic research at the U. S. Food and Drug Administration National Center for Toxicological Research. *Environ Health Perspect.* 2003; 111(15):1819–1826. [PubMed: 14630514]
32. Kwekel JC, Desai VG, Moland CL, Branham WS, Fuscoe JC. Age and sex dependent changes in liver gene expression during the life cycle of the rat. *BMC Genomics.* 2010; 11:675. [PubMed: 21118493]
33. Pearson K. On lines and planes of closest fit to systems of points in space. *Philos Mag.* 1901; 2:14.
34. Charite J, McFadden DG, Olson EN. The bHLH transcription factor dHAND controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. *Development.* 2000; 127(11):2461–2470. [PubMed: 10804186]
35. McFadden DG, McAnally J, Richardson JA, Charite J, Olson EN. Misexpression of dHAND induces ectopic digits in the developing limb bud in the absence of direct DNA binding. *Development.* 2002; 129(13):3077–3088. [PubMed: 12070084]
36. Yanagisawa H, Clouthier DE, Richardson JA, Charite J, Olson EN. Targeted deletion of a branchial arch-specific enhancer reveals a role of dHAND in craniofacial development. *Development.* 2003; 130(6):1069–1078. [PubMed: 12571099]
37. Han J, Ishii M, Bringas P Jr, Maas RL, Maxson RE Jr, Chai. Concerted action of Msx1 and Msx2 in regulating cranial neural crest cell differentiation during frontal bone development. *Mech Dev.* 2007; 124(9–10):729–745. [PubMed: 17693062]
38. Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney, et al. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet.* 2000; 24(4):391–395. [PubMed: 10742104]
39. Bensoussan-Trigano V, Lallemand Y, Saint Clément C, Robert B. Msx1 and Msx2 in limb mesenchyme modulate digit number and identity. *Dev Dyn.* 2011; 240(5):1190–1202. [PubMed: 21465616]
40. Yang D, Guo J, Divieti P, Shioda T, Bringham. CBP/p300-interacting protein CITED1 modulates parathyroid hormone regulation of osteoblastic differentiation. *Endocrinology.* 2008; 149(4):1728–1735. [PubMed: 18187554]
41. Paine-Saunders S, Viviano BL, Zupicich J, Skarnes WC, Saunders. Glypican-3 controls cellular responses to Bmp4 in limb patterning and skeletal development. *Dev Biol.* 2000; 225(1):179–187. [PubMed: 10964473]
42. Friedman MS, Oyserman SM, Hankenson. Wnt11 promotes osteoblast maturation and mineralization through R-spondin 2. *J Biol Chem.* 2009; 284(21):14117–14125. [PubMed: 19213727]
43. Yang D, Guo J, Divieti P, Bringham. Parathyroid hormone activates PKC-delta and regulates osteoblastic differentiation via a PLC-independent pathway. *Bone.* 2006; 38(4):485–496. [PubMed: 16325485]
44. Datta NS, Kolailat R, Fite A, Pettway G, Abou-Samra. Distinct roles for mitogen-activated protein kinase phosphatase-1 (MKP-1) and ERK-MAPK in PTH1R signaling during osteoblast proliferation and differentiation. *Cell Signal.* 2010; 22(3):457–466. [PubMed: 19892016]
45. Standal T, Johnson RW, McGregor NE, Poulton IJ, Ho PW, Martin TJ. Sims, gp130 in late osteoblasts and osteocytes is required for PTH-induced osteoblast differentiation. *J Endocrinol.* 2014; 223(2):181–190. [PubMed: 25228504]

46. Du J, Lin G, Nie ZY, Lu. Molecular cloning and characterization analysis of HPESCRG1, a novel gene expressed specifically in human embryonic stem cell. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 2004; 21(6):542–547. [PubMed: 15583978]
47. Du J, Chen T, Zou X, Xiong B, Lu. Dppa2 knockdown-induced differentiation and repressed proliferation of mouse embryonic stem cells. *J Biochem*. 2010; 147(2):265–271. [PubMed: 19846433]
48. Maldonado-Saldivia J, van den Bergen J, Krouskos M, Gilchrist M, Lee C, Li R, Sinclair AH, Surani MA, Western. Dppa2 and Dppa4 are closely linked SAP motif genes restricted to pluripotent cells and the germ line. *Stem Cells*. 2007; 25(1):19–28. [PubMed: 16990585]
49. Niwa H, Miyazaki J, Smith. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet*. 2000; 24(4):372–376. [PubMed: 10742100]
50. Nichols J, Zevnik B, Anastasiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998; 95(3):379–391. [PubMed: 9814708]
51. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev*. 2003; 17(1):126–140. [PubMed: 12514105]
52. Srivastava D, Cserjesi P, Olson. A subclass of bHLH proteins required for cardiac morphogenesis. *Science*. 1995; 270(5244):1995–1999. [PubMed: 8533092]
53. Dunwoodie SL, Rodriguez TA, Beddington. Msg1 and Mrg1, founding members of a gene family, show distinct patterns of gene expression during mouse embryogenesis. *Mech Dev*. 1998; 72(1–2): 27–40. [PubMed: 9533950]
54. Lako M, Strachan T, Bullen P, Wilson DI, Robson SC, Lindsay. Isolation, characterisation and embryonic expression of WNT11, a gene which maps to 11q13.5 and has possible roles in the development of skeleton, kidney and lung. *Gene*. 1998; 219(1–2):101–110. [PubMed: 9757009]
55. Schulpen SH, Pennings JL, Tonk EC, Piersma AH. A statistical approach towards the derivation of predictive gene sets for potency ranking of chemicals in the mouse embryonic stem cell test. *Toxicol Lett*. 2014; 225(3):342–349. [PubMed: 24480513]

Fig. 1.

Study design for microarray analysis. Osteoblast differentiation was initiated using a single cell suspension of D3 mESCs plated in 24-well plates in osteoblast medium. Cadmium sulfate or solvent control (0.1% water) exposure began on day 3 of culture and continued for 24, 48 or 72 h for microarray, RT-PCR, and cytotoxicity analysis. To parallel the transcriptomics study with the traditional osteoblast mEST study, concurrent plates were prepared with 0.1% water or cadmium sulfate added to the culture from day 3 to day 14; osteoblast differentiation was evaluated on day 14 using ALR staining. Solvent control samples for microarray analysis were harvested at exposure times (Exp.T) of 0, 24, 48 and 72 h; cadmium sulfate exposed cultures were harvested at Exp. T of 24, 48 and 72 h. Con represents control, and CS represents cadmium sulfate.

Fig. 2.

Temporal gene expression pattern of mESC differentiation in the control culture. A: HCA of the control cultures using Partek Genomics Suite software. A total of 6308 DEGs were identified for the four control groups using one-way ANOVA with Bonferroni adjustment at $p < 0.01$. Red color or positive number indicates upregulation, and blue color or negative number indicates down-regulation. B: Two dimensional PCA (2D-PCA) of the control culture using the same set of DEGs identified in A. The dashed line connecting different experimental time points represents the differentiation track of the mESCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Biological pathways significantly altered by DEGs during mESCs differentiation to osteoblasts. D3 mESCs were maintained in osteoblast medium for 3 days, and cultures were either harvested (0 h control) or further exposed to 0.1% water for 24, 48 or 72 h. **A:** Total number of upregulated genes (red) and down-regulated genes (blue) for each time point as compared to the 0 h control using Array Track with FDR <0.05 and FC >1.5. **B:** Venn diagram analysis using Array Track shows the degree of gene overlap between different time points. The common response genes (1189) were used for downstream pathway analysis in **D**. **C:** Expression of pluripotency genes was decreased with time as indicated by both RT-PCR and microarray analysis (MA). Both RT-PCR and microarray data were expressed as relative fold changes compared to the 0 h control with negative numbers indicating down-regulation. There were three or four biological replicates for each RT-PCR analysis and five or six biological replicates for each microarray analysis. **D:** The top eight biological pathways (sorted by p values) altered by commonly responding genes using IPA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Classical toxicological evaluation of the effects of cadmium sulfate. A: Cytotoxicity of cadmium sulfate was evaluated by the MTT assay. Osteoblast differentiation was initiated with 20,000 D3 mESCs per well in 24-well plates in osteoblast medium. Cadmium sulfate or 0.1% water was added to the culture medium beginning on day 3 and remained in the medium for 24, 48 or 72 h with no medium changes. MTT assays were conducted 24, 48 or 72 h after cadmium sulfate exposure. The cytotoxicity experiment was repeated two times with four replicates (wells) per experiment. B: ALR staining and quantification. Osteoblast differentiation was initiated with 20,000 D3 mESCs per well in 24-well plates with cadmium sulfate or 0.1% water added to the culture medium from day 3 to day 14. ALR staining was performed on day 14 to evaluate osteoblast differentiation. Images for individual wells of the 24-well plates were obtained by stitching 169 individual phase contrast images (5× objective) together using Zeiss MosaicX software. ALR staining was quantified using acid extraction, and the absorbance of ALR was measured at 405 nm. The experiment was repeated two times with three replicates per treatment in each experiment. Con represents control, and CS represents cadmium sulfate.

Fig. 5.

Cadmium sulfate inhibits mESC differentiation. Three-dimensional PCA (3D-PCA) of all seven experimental groups using Partek Genomics Suite software. Global relationships between the expression patterns of individual samples were visualized using the 15,156 DEGs meeting the initial filtering criteria with FDR <0.01. Each triangle or square represents the expression from one sample (well). Samples of the same time point have the same color and the same shape. Con represents control, and CS represents cadmium sulfate.

Fig. 6.

Biological pathways significantly affected by cadmium sulfate exposure. A: Total number of upregulated genes (red) and down-regulated genes (blue) in cadmium sulfate-exposed cultures after 24, 48 or 72 h of treatment. Data were analyzed using Array Track with FDR <0.05, FC >1.5. B: Venn diagram analysis with Array Track shows the degree of overlap for DEGs between the different time points of cadmium sulfate-exposed cultures; the common response genes (407) among all three time points were used for downstream pathway analysis. C: Top seven biological pathways (sorted by p values) associated with the 407 common cadmium sulfate response genes using IPA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Expression analysis of genes involved in bone development and the effect of cadmium sulfate exposure. Gene expression changes were analyzed by both RT-PCR and microarray (MA) analysis. For the unexposed cultures (24, 48 and 72), the data are presented as relative fold changes compared to day 0 controls. For the cadmium sulfate exposed cultures (24 CS, 48 CS and 72 CS), the data are presented as relative fold changes compared to the time-matched unexposed controls. Positive numbers indicate upregulation, and negative numbers indicate down-regulation. There were three or four biological replicates for each RT-PCR analysis and five or six biological replicates for each microarray analysis.

Table 1

	<i>Hand</i>		<i>Mx2</i>		<i>Cited1</i>		<i>Gpc3</i>		<i>Wnt11</i>		<i>Pth1r</i>	
	PCR	MA	PCR	MA	PCR	MA	PCR	MA	PCR	MA	PCR	MA
24	2.7	3.3	3.5	3.4	2.9	2.8	2.4	2.1	2.3	2.2	1.3	0.8
48	8.7	6.5	6.6	6.6	6.0	5.6	5.7	4.5	2.1	2.4	2.8	1.7
72	25.7	11.3	13.5	10.9	9.8	8.6	9.2	6.7	3.7	4.1	6.1	2.9
24 CS	-1.6	-1.9	-1.1	-1.2	-2.6	-2.6	-1.5	-1.5	-1.3	-1.3	-1.9	-2.1
48 CS	-2.1	-2.2	-1.7	-1.7	-2.8	-2.9	-2.1	-1.8	-1.6	-1.6	-2.8	-2.9
72 CS	-2.5	-1.9	-1.6	-1.4	-2.7	-2.5	-2.1	-1.8	-2.0	-2.2	-4.2	-3.5