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

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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.

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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

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; *Runx2*, runt related transcription factor 2; IC_{50}, 50% inhibition of growth; ID_{50}, 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; *Dppa2*, developmental pluripotency associated 2; *Pou5f1*, POU domain, class 5, transcription factor 1; *Sox2*, sex determining region Y-box 2.

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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% (ID_{50}) 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), non-essential 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% CO_2.

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.