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Dynamic changes in energy metabolism upon embryonic stem cell differentiation support developmental toxicant identification

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ABSTRACT

Embryonic stem cells (ESC) are widely used to study embryonic development and to identify developmental toxicants. Particularly, the embryonic stem cell test (EST) is well known as *in vitro* model to identify developmental toxicants. Although it is clear that energy metabolism plays a crucial role in embryonic development, the modulation of energy metabolism in *in vitro* models, such as the EST, is not yet described. The present study is among the first studies that analyses whole genome expression data to specifically characterize metabolic changes upon ESC early differentiation. Our transcriptomic analyses showed activation of glycolysis, truncated activation of the tricarboxylic acid (TCA) cycle, activation of lipid synthesis, as well as activation of glutaminolysis during the early phase of ESC differentiation. Taken together, this energy metabolism profile points towards energy metabolism reprogramming in the provision of metabolites for biosynthesis of cellular constituents. Next, we defined a gene set that describes this energy metabolism profile. We showed that this gene set could be successfully applied in the EST to identify developmental toxicants known to modulate cellular biosynthesis (5-fluorouracil and methoxyacetic acid), while other developmental toxicants or the negative control did not modulate the expression of this gene set. Our description of dynamic changes in energy metabolism during early ESC differentiation, as well as specific identification of developmental toxicants modulating energy metabolism, is an important step forward in the definition of the applicability domain of the EST.

1. Introduction

Embryonic stem cells (ESC) are derived from the inner cell mass of the pre-implementation embryo, and are considered the prototypical stem cells (Evans and Kaufman, 1981). These cells can be cultured for a prolonged period without losing their pluripotent characteristics, and can be induced to differentiate into all foetal and adult cell types. *In vitro*, it has been shown that ESC can originate a wide variety of cell types, including cardiomyocyte, neural and hepatic cells. These *in vitro* models can provide a powerful model system to study embryonic development on the level of embryonic cell differentiation. Experiments comparing gene expression profiles of *in vivo* embryonic development and *in vitro* ESC differentiation have shown significant overlap in the regulation of genes annotated to developmental processes (Hettne et al., 2013; Robinson et al., 2011).

The good correlation between modulation of processes *in vivo* and *in vitro* explains the successful initiatives of ESC differentiation-based *in vitro* approaches for identification of developmental toxic compounds (Wobus and Loser, 2011). The best known example of these approaches is the embryonic stem cell test (EST) (Spielmann et al., 1997). Initially, identification of developmental toxicants was based on disturbance of ESC differentiation into cardiomyocytes. Later, more objective molecular endpoint measures were introduced (Osman et al., 2010; Seiler et al., 2004; van Dartel et al., 2009a), which improved the efficiency, objectivity, and accuracy of the EST.

Gene expression changes upon ESC differentiation generally show enrichment of three processes: cell cycling, development and metabolism (van Dartel et al., 2010b). The first two themes have often been identified to be regulated upon ESC differentiation, whereas altered energy metabolism has been less extensively described. Recently, a *de novo* analysis of ESC differentiation

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transcriptomics data has shown a network of genes that are clearly enriched for energy metabolism (Pennings et al., 2011). This study indicated that altered energy metabolism is a common finding in ESC differentiation studies. Although this study has shown regulation of common genes involved in energy metabolism, it did not provide direct insight into the type of physiological alterations in energy metabolism that take place upon ESC differentiation.

Energy metabolism is pivotal during mammalian development. For example, during early mammalian development, embryos are adapted for glycolytic energy production, but acquire the dependence on oxidative metabolism at several times during pre- and post-implantation development (Knudsen and Green, 2004). In *in vitro* studies, this shift from anaerobic (glycolytic) metabolism to aerobic (oxidative phosphorylation) metabolism has also been observed during differentiation of stem cells (Varum et al., 2011). Moreover, energy metabolism can also be adapted for generation of building blocks for growth, a process characteristic for cells with high proliferative capacity (Lunt and Vander Heiden, 2011). So far, most studies that evaluated alterations of energy metabolism upon ESC differentiation compared mitochondrial activities of stem cells with that of fully differentiated cell types. Although these studies gave important insight into changes of metabolism-related processes upon stem cell differentiation, these studies were limited by the relatively late stages of differentiation that were studied, as well as by their predefined end point measurements. Although it is clear that energy metabolism is one of the major regulated processes during development, a detailed description of the type of changes in energy metabolism upon early ESC differentiation is currently lacking. An improved description of the biological processes that are active during ESC differentiation, including energy metabolism, will contribute to an improved description of the applicability domain of the EST, which is essential for more accurate identification of developmental toxicants.

Whole genome gene expression analysis is a technique that does not necessarily require prior mechanistic knowledge and provides an integral platform for identification of effects between groups. It is ideally suited to genome-wide describe developmental changes in detail. In this study, we have used this attractive tool to elucidate the shift in energy metabolism upon ESC differentiation during an early and broad time span. Our results show that energy metabolism is altered during early ESC differentiation to favor cell growth. Moreover, we demonstrate that altered gene expression of an energy metabolism based gene-set can successfully be used to identify developmental toxicants. This study contributes to an improved description of the applicability domain of the EST, and to mechanism-based identification of developmental toxicants using the EST.

2. Materials and methods

2.1. Data set selection

We used the largest data repository ArrayExpress (Parkinson et al., 2011), which encompasses over 50.000 MIAME-compliant experiments, to select data sets for our analyses. The search term: 'stem cell' AND 'differentiation' was used to identify potential usable data sets. Data sets that were not available when we started our analyses (August 7, 2013) were excluded. We reasoned that a study should include at least 12 samples in order to represent sufficient time points to reflect dynamic temporal changes and to obtain sufficient statistical power. Only data derived from human or mouse ESC were included. Finally, the study design of each remaining study was evaluated for inclusion.

In this work, gene expression data of our previously published work on neuronal differentiation of ES-ES-D3 ESC was used to validate the findings of the *de novo* analysis. The raw data of this study have been deposited in EBI's ArrayExpress (http://www.ebi.ac.uk/arrayexpress) and are accessible through ArrayExpress accession number E-MTAB-1108. Methods and data regarding culture conditions and gene expression profiling have been described in full detail by Theunissen et al. (Theunissen et al., 2011).

2.2. Data analysis and statistics

All selected studies for our analyses used the Affymetrix platform. Raw data were downloaded from ArrayExpress and the Affymetrix CEL files were normalized using the Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003) using RMAexpress (Bolstad et al., 2003). For probe to gene mapping, a custom Common Data Format (CDF) was performed as we described previously (van Dartel et al., 2010a). In total, 16,346 probe sets were used in our analyses.

Statistical analyses were carried out using the R statistical software environment (http://www.r-project.org) using ln-transformed values. Maximal fold ratios (FR) in individual gene expression between the experimental groups were determined by comparing the average normalized signal values per group. Genes that were significantly differentially expressed between any of the experimental groups were identified by a one-way ANOVA on the normalized data, using the cut-off criterium FDR < 0.01. Arrangement of the samples was achieved by hierarchical clustering using GeneMaths XT based on Euclidian clustering in combination with Ward linkage (Applied Maths, Sint-Martens-Latem, Belgium).

To study similarities in differentiation progression between the selected data sets we compared the gene expression profiles of a previously identified set of genes that have been previously identified to be related to pluripotency (Assou et al., 2007). Additionally, we selected marker genes for to evaluate similarities in proliferation, pluripotency and differentiation. Initially, we selected 3–4 marker genes per process based on scientific literature (all processes) and on our own historic data (differentiation). Marker genes were included in the analysis if these genes were present on all platforms used in our analyses and if these genes were present in the work data set for data analyses. For proliferation we used *Ccnd1, Ccne1,* and *Pcna (*Kanehisa and Goto, 2000; Stacey, 2003), for pluripotency we used Pou5f1 and Sox2 (Chambers and Tomlinson, 2009), and for differentiation we used Cyp26a1, Gata4, Vegfa, and Rbp4 (Hescheler et al., 2006).

To evaluate enrichment of biological process, molecular function or cellular component of the gene clusters, DAVID EASE was used (http://david.abcc.ncifcrf.gov) (Huang da et al., 2009). This annotation was performed using only terms to which maximal 300 genes are annotated, to exclude terms that are too general for functional interpretation. Terms with an enrichment score >5 were used to describe the enrichment of the gene clusters.

Gene set enrichment analysis (GSEA) was performed to discover differential expression of sets of genes that are related to energy metabolism (Subramanian et al., 2005). For the GSEA, we refined the publicly available C5 gene set collection for energy metabolism-related gene sets by only including the child terms of the term 'metabolic process'. Gene sets were considered being significantly affected if $p < 0.05$. GSEA was followed by molecular concept analysis, in which the regulated gene sets are visualized within a network based on their overlap in genes, as we described previously (van Dartel et al., 2009b).

Analysis of glucose levels in ES-ES-D3 ESC tissue culture medium

Glucose levels were analyzed in medium samples of pluripotent ES-D3 ESC and ES-D3 ESC differentiation cultures at day 3, 5 and 7, using three biological replicates. Cells were cultured as we described