Tetracycline Regulator Expression Alters the Transcriptional Program of Mammalian Cells

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Abstract

**Background:** Tetracycline regulated ectopic gene expression is a widely used tool to study gene function. However, the tetracycline regulator (tetR) itself has been reported to cause certain phenotypic changes in mammalian cells. We, therefore, asked whether human myeloid U937 cells expressing the tetR in an autoregulated manner would exhibit alterations in gene expression upon removal of tetracycline.

**Methodology/Principal Findings:** Microarray analyses revealed that 172 and 774 unique genes were significantly differentially expressed by at least 2- or 1.5-fold, respectively, when tetR expressing U937 cells were maintained in media with or without the antibiotic.

**Conclusions/Significance:** These alterations in gene expression are likely to contribute to the phenotypic consequences of tetR expression. In addition, they need to be taken into consideration when using the tetR system for the identification of target genes of transcription factors or other genes of interest.

Introduction

Inducible ectopic expression is a widely used tool to study gene function. Its advantages compared to constitutive approaches are that early consequences of the presence of the investigated gene product can be studied, and that it facilitates the analysis also of growth inhibitory or cytotoxic gene products. Initial methods for inducible gene expression were based on mammalian promoters that could be activated, e.g., by metal ions. In the early 1990s, the adaptation of the bacterial tetracycline repressor/operator system for use in mammalian cells provided, for the first time, the possibility to regulate the expression of exogenous genes through a small molecule ligand that per se was expected not to affect the physiology of mammalian cells due to their lack of endogenous receptors for it [1]. In fact, mammalian cells need to be made responsive to tetracycline by introduction of one of several modified versions of the bacterial tetracycline regulator (tetR). The transcriptional activity of some of these derivatives is responsive to tetracycline by introduction of one of several modified versions of the bacterial tetracycline repressor/operator system 

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Results

Microarray analysis of U937 cells containing an autoregulated tetR

The cell line U937T_pUHD10S contains a plasmid coding for an autoregulated tetracycline repressor as well as the empty pUHD10S vector, which drives tet-regulated expression of its cDNA inserts. It was generated as a control for cell lines expressing a gene of interest from pUHD10S. Three replicate cultures of U937T_pUHD10S cells were transferred to media with or without tetracycline. 48 h later RNA was extracted and used for hybridization of Affymetrix U133 Plus 2.0 arrays. For statistical evaluation, different levels of stringency were applied so as to reflect stringency levels commonly found in the literature [8,14,16]. 1191 probe sets (774 unique genes) were deregulated at least 1.5-fold at p<0.05 upon removal of tetracycline from U937T_pUHD10S cells (Tables 1 and S1A). If the threshold for differential expression was increased to twofold and only genes with a false discovery rate (FDR) <10% after multiple hypothesis testing were considered, 90 probe sets (99 unique genes) resulted as deregulated (Tables 1 and S1B).

Confirmation of the regulation of selected genes by real time quantitative (RTQ-) RT-PCR

Of the genes deregulated in response to tetracycline withdrawal, CD36 (NM_000072; coding for the thrombospondin receptor) and ITGAL (NM_000072; coding for alpha L integrin) were selected for confirmation by RTQ-RT-PCR. Both genes form part of several gene ontology categories that were significantly enriched among genes induced by tetracycline removal (see below). RTQ-RT-PCR indeed corroborated the upregulation of these genes 48 h after removal of tetracycline from the culture media of U937T_pUHD10S cells. Both genes were also induced upon withdrawal of tetracycline from U937T cells, which contain the tetR, but not pUHD10S (Fig. 1A,B). A similar, albeit smaller and less reproducible, effect was already present 24 h after transfer of the cells to tetracycline free media (data not shown). To ask whether the changes in the expression of these genes were related to the induction of the autoregulated tetR, or represented an endogenous response of mammalian cells to altered tetracycline concentrations, native (i.e., untransfected) U937 cells were cultured in the presence of tetracycline for one week. Then they were washed and transferred to media with or without tetracycline in the same manner as U937T_pUHD10S cells. Neither CD36 nor ITGAL was induced upon transfer of native U937 cells to tetracycline free media (Fig. 1C,D).

Gene ontology analysis

Gene ontology (GO) analysis revealed the terms “hemopoiesis”, “myeloid cell differentiation”, “leukocyte activation”, “phagocytosis”, “response to stress”, “defense response”, “immune system process”, “inflammatory response”, “innate immune response”, “cell activation”, “signal transduction”, “chemotaxis”, as well as several others, to be significantly enriched among the genes upregulated at least 1.5-fold at an FDR <10% in response to tetracycline withdrawal (Fig. 2, Table S2).

Discussion

Because we and others have found that tetR expressing cells show phenotypic changes in response to altered concentrations of antibiotic even if they do not contain a tetracycline regulated gene of interest [5,6,10,12,15], we asked whether these changes may correspond to altered gene expression patterns. Indeed, using microarray analysis we found that the levels of several hundred mRNAs were altered significantly after transfer of U937T_pUHD10S cells to tetracycline free media. The mechanism through which tetR affects gene expression is unclear and may or may not involve direct interactions with DNA. Irrespective of it, it is plausible that the alterations in gene expression patterns in tetR expressing U937 cells contribute to the phenotypic changes observed in these cells after tetracycline withdrawal. Moreover, even though tetR certainly does not play a physiological role in human myeloid cells like U937, several of the gene ontology terms enriched among the genes induced upon tetracycline removal correspond to biological responses that might be expected as a consequence of the experimental expression of a physiologically or pathologically relevant gene. Similarly, altered gene expression patterns may well contribute to recently reported phenotypic consequences of tetR expression in transgenic mice, which were observed even in the absence of any exogenous gene of interest regulated by it [17].

Our results also have practical implications for studies aimed at the identification of target genes of transcription factors or other genes of interest. Because of the still considerable cost of transcriptome analyses, some authors restrict their experiments to the comparison of gene of interest-containing cells maintained in the presence or absence of tetracycline. Factors of 1.5- or 2-fold are often defined as thresholds for differential gene expression, and rigorous statistical analyses including corrections for multiple hypothesis testing are frequently not applied [8,14,16] (and according to our experience would in fact prevent further consideration of genes whose differential expression can be confirmed by RTQ-RT-PCR). However, even after multiple

| Table 1. Numbers of unique genes and probe sets deregulated in U937T_pUHD10S cells upon removal of tetracycline. |
|---------------------------------------------------------------|
| **FC ≥ 1.5** | **p < 0.05** | **FC ≥ 2** | **p < 0.05** | **FC ≥ 1.5** | **FDR < 10%** | **FC ≥ 2** | **FDR < 10%** |
| unique genes | upregulated | 465 | 150 | 115 | 87 |
| downregulated | 309 | 22 | 46 | 12 |
| total | 774 | 172 | 161 | 99 |
| probe sets | upregulated | 729 | 188 | 122 | 82 |
| downregulated | 462 | 31 | 40 | 8 |
| total | 1191 | 219 | 162 | 90 |

FC, fold change; FDR, false discovery rate according to Benjamin and Hochberg [20]. Because fewer hypotheses were tested when only unique genes rather than all probe sets were considered, more unique genes than probe sets may pass the multiple hypothesis test in some cases.

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hypothesis testing, 99 unique genes were deregulated more than twofold in U937T_pUHD10S cells transferred to tetracycline free media. While this number may appear small at first sight, it is large enough to significantly confound the results of target gene analyses, especially if the investigated gene of interest has few true biological targets. Furthermore, the 774 genes that are deregulated $1.5$-fold at $p\leq 0.05$ may also lead to erroneous conclusions, and may do so even in analyses in which higher stringency levels are applied: a gene that would be regulated slightly less than twofold in control cells and slightly above this threshold in gene of interest expressing cells may still not be a relevant biological target of the investigated gene. Therefore, our findings need to be taken into consideration when performing gene expression experiments using the tetR system.

Materials and Methods

Cell lines and cell culture

Human U937 histiocytic lymphoma cells were cultured in a humidified incubator at 37°C and 5% CO$_2$ in RPMI 1640 medium (Invitrogen) containing 10% FBS (Invitrogen). To study the effects of tetracycline removal, 1 μg/ml tetracycline (Sigma) was added to the culture media for one week, and cells were then processed as described below.

U937T cells have been derived from U937 cells by stable transfection with a construct driving tetracycline-regulable expression of the tetVP16 fusion protein [5]. They were cultured in a humidified incubator at 37°C and 5% CO$_2$ in RPMI 1640 medium containing 10% FBS, 0.5 μg/ml puromycin (Sigma), and 1 μg/ml tetracycline. To obtain U937T_pUHD10S cells, U937T cells were electroporated (0.17 kV, 950 μF) with the vector pUHD10S, which contains seven copies of the tetracycline operator, thus facilitating tetracycline regulable expression of its cDNA inserts [18]. Transfectants were selected, cloned, and propagated in RPMI 1640 medium supplemented with 10% FBS, 0.5 μg/ml puromycin, 1 μg/ml tetracycline, and 500 μg/ml hygromycin (PAA). To remove tetracycline from the culture media, exponentially growing cells were washed 3 times with phosphate-buffered saline (PBS) and suspended in growth media without tetracycline. Control cultures were washed in the same manner but suspended in media with tetracycline.

Microarray analyses

RNA for microarray analyses was extracted from three independent replicate cultures of U937T_pUHD10S cells using the RNeasy Plus mini kit (Qiagen) according to the manufacturer's instructions. Quality control of the isolated RNA samples, labelling, and hybridization to U133 Plus 2.0 arrays (Affymetrix) were performed at the Center of Excellence for Fluorescent Bioanalytics (KFB) in Regensburg, Germany, which is an Affymetrix Service Provider and Core Facility. Sample preparation was carried out in accordance with the Affymetrix GeneChip Expression Analysis Technical Manual (one replicate) or the Affymetrix GeneChip IVT Express Kit Technical Manual (two
Figure 2. ClueGO analysis [22] of genes upregulated ≥1.5-fold at an FDR <10% in U937T_pUHD10S cells upon tetracycline withdrawal. Nodes (circles) represent gene ontology terms. Connections between two nodes (edges) indicate that two gene ontology terms share genes from the considered dataset (agreement measure kappa >0.3). The calculated kappa score is also used for defining functional groups, which are indicated by the same color. The most prominent gene ontology term for each group is highlighted.

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Tables and figures

Table 2. Primers used for RTQ-RT-PCR.

| Primer name | Primer sequence (5'-3') |
|-------------|------------------------|
| cyclophilinD | ATATTGGTAAAGTGGAAGTGAAGG |
| cyclophilinD | TGCCGAGGACCCTTTTGG |
| CD36       | TGAACAGCAAGCAACCTCAA |
| CD36 rev   | GCTGGACGAAAGGACTCTG |
| ITGAL      | CAGGAAATGTACAGGGCAA |
| ITGAL rev  | AACAGCGAAACACTGGTACG |

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Real time quantitative reverse transcriptase polymerase chain reaction (RTQ-RT-PCR)

Total RNA for RTQ-RT-PCR was extracted using Trizol (Invitrogen) and reverse transcribed using random hexamer primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. RTQ-RT-PCR primers are shown in Table 2. All assays were carried out in triplicate. Expression values for the genes of interest relative to the housekeeping gene cyclophilinD and to a reference value were determined using the ΔΔCT method [19].

Data evaluation and in silico analyses

Only probe sets with present calls in all six samples were considered for statistical analyses, which were performed on log2-transformed intensity values using the R/Bioconductor package limma employing a moderated t-test. Probe sets whose average transformed intensity values using the R/Bioconductor package limma employing a moderated t-test. Probe sets whose average transformed intensity values using the R/Bioconductor package limma employing a moderated t-test. Probe sets whose average transformed intensity values using the R/Bioconductor package limma employing a moderated t-test. Probe sets whose average transformed intensity values using the R/Bioconductor package limma employing a moderated t-test.

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