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Inferring statin-induced gene regulatory relationships in primary human hepatocytes

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ABSTRACT

Motivation: Statins are the most widely used cholesterol-lowering drugs. The primary target of statins is HMG-CoA reductase, a key enzyme in cholesterol synthesis. However, statins elicit pleitropic responses including beneficial as well as adverse effects in the liver or other organs. Today, the regulatory mechanisms that cause these pleiotropic effects are not sufficiently understood.

Results: In this work, genome-wide RNA expression changes in primary human hepatocytes of six individuals were measured at up to six time points upon atorvastatin treatment. A computational analysis workflow was applied to reconstruct regulatory mechanisms based on these drug–response data and available knowledge about transcription factor (TF) binding specificities and protein–drug interactions. Several previously unknown TFs were predicted to be involved in atorvastatin-responsive gene expression. The novel relationships of nuclear receptors NR2C2 and PPARA on CYP3A4 were successfully validated in wet-lab experiments.

Availability: Microarray data are available at the Gene Expression Omnibus (GEO) database at www.ncbi.nlm.nih.gov/geo/, under accession number GSE29868.

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1 INTRODUCTION

Statins are widely used cholesterol-lowering drugs that inhibit HMG-CoA reductase, a key enzyme in cholesterol synthesis. Clinical trials, however, indicate that statins additionally cause cholesterol-independent or pleiotropic effects (Liao and Laufs, 2005). These pleitropic effects may either be beneficial (e.g. decrease of oxidative stress and inflammation) or toxic (e.g. statin-induced liver injury; Cash *et al.* 2008). Currently, the specific regulatory mechanisms that cause these adverse effects are not sufficiently understood. Transcriptional regulators, including statin-responsive transcription factors (TFs) and regulatory co-factors, are suspected to be involved in these mechanisms (Hanai *et al.*, 2007).

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Genome-wide mRNA expression profiling techniques are widely used tools to monitor large-scale regulatory effects in response to drug treatments. For this purpose, gene expression profiles are first clustered into sets of co-expressed genes, which are assumed to be co-regulated. Putative TFs are then inferred by mapping position frequency matrices (PFMs), which are widely used models for TF binding motifs, to the promoter sequences of putative target genes (Wingender *et al.*, 1997). According to the *futility theorem* (Wasserman and Sandelin, 2004), however, the grand majority of predicted transcription factor binding sites (TFBSs) is non-functional, which is partly due to the degeneracy of TFBSs and the low specificity of PFMs. One common approach to overcome this problem and increase the specificity of TFBS detection is to search for common combinations of TFBSs in sets of co-expressed genes, referred to as *cis*-regulatory module (CRM) detection. CRM detection methods rely on the assumption that co-regulated genes are controlled by specific combinations of TFs, which bind in physical proximity to each other (Loo and Marynen, 2009). The specificity of CRM detection can be further increased by taking additional sources of evidence into account, such as correlations between regulators and putative target genes on the gene expression level (Wrzodek *et al.*, 2010). In this work, a multiobjective CRM detection approach was used to infer new statin-induced gene regulatory relationships based on microarray measurements, transcription factor binding sites and protein–drug interaction data.

2 APPROACH

Primary human hepatocytes were used as model system to investigate genome-wide regulatory effects upon statin treatment. Microarray profiling was performed for six different individuals at up to six time points (i.e. 0 , 6 , 12, 24, 48 and 72 h) after stimulation with atorvastatin. A total of 1 429 probes were identified to be differentially expressed (>1.7-fold), leastwise at one time point after atorvastatin treatment compared with the control.

2.1 Clustering

In contrast to cell lines, primary tissue material is obtained from individual donors and is characterized by considerable inter-subject variability. In order to account for this variability, the Extended Dimension Iterative Signature Algorithm (EDISA) was applied to

find sets of genes with highly correlated expression profiles over time. The 3D clustering algorithm EDISA (Supper *et al.*, 2007) is able to deal with multicondition time-resolved datasets. EDISA mines for independent response clusters, which are sets of genes that show highly correlated profiles within each condition, but different expression profiles between the conditions. Furthermore, EDISA is a fuzzy clustering algorithm. Thus, one gene can occur in multiple clusters. This is biologically evident, since one gene can be part of various regulatory programs. Gene expression profiles of different donors were not pooled, but perceived as different experimental conditions. By this means, as depicted in Figure 1a, inter-subject variability was accounted for. In total, 13 different sets of co-expressed genes were identified (File S1 in Supplementary Material). As depicted in Figure 1a, expression profiles of the same genes differ between the six individuals, but strongly correlate within each individual. In order to assess if sets of co-expressed genes are functionally related, Gene Ontology (GO) enrichment analysis was applied. To this end, for each cluster of genes a hypergeometric test was performed to check if certain GO categories are overrepresented. As depicted in File S1 of Supplementary Material, statistically significant enrichments were detected in nine clusters. Genes of steroid biosynthesis were overrepresented most frequently, and also genes of fatty acid and drug metabolism were found to be co-expressed and thus putatively co-regulated. Interestingly, coordinated induction of several cytochrome P450 enzymes following atorvastatin treatment of primary human hepatocytes within a similar time-frame has recently been observed in Feidt *et al.* (2010).

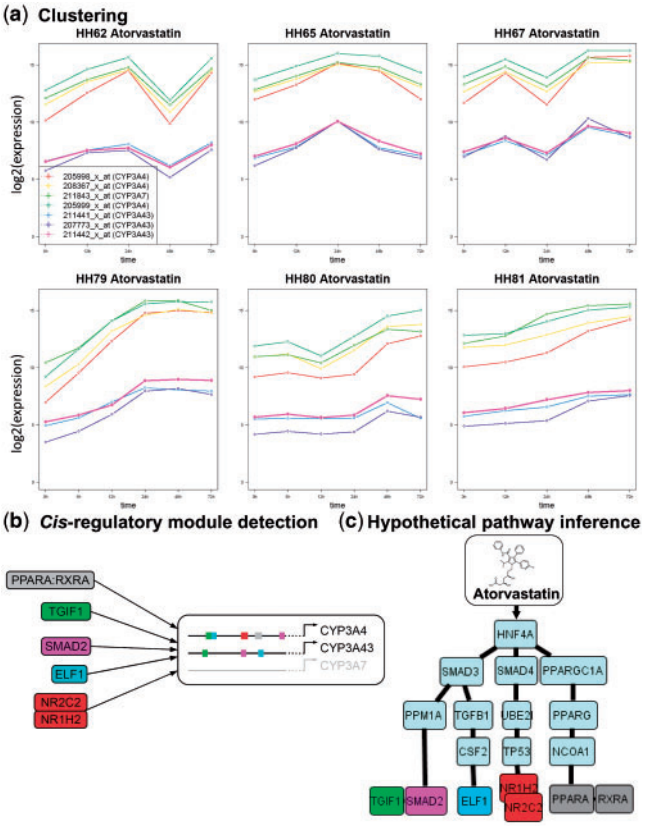

Fig. 1. Transcriptional and hypothetical signaling network inference of atorvastatin cluster 4. (**a**) Depicted are the expression profiles of seven Affymetrix probes, which map to three different cytochrome P450 monooxygenases (CYP3A4, CYP3A43, CYP3A7). The expression profiles are highly correlated within each individual, but slightly diverse between the individuals. (**b**) Six different TFs have been identified by *cis*-regulatory module detection to be involved in the atorvastatin-responsive regulation of CYP3A4 and CYP3A43. (**c**) Inferred hypothetical pathway connection between the six identified TFs and atorvastatin, based on protein/drug interaction data. Among the discovered TFs are different nuclear hormone receptors (NRs), like the PPARA:RXR heterodimer, NR2C2 and NR1H2 (LXR). The latter two NRs are associated to the same binding site and may be involved in regulatory cross talk on the binding site level. The regulatory influence of LXR on CYP3A4 was previously published in Duniec-Dmuchowski *et al.* (2007). The regulatory relationship between PPARA:RXR as well as NR2C2 an CYP3A4 was not known before and could successfully be validated in wet-lab experiments (see Section 2.3).

2.2 *Cis*-regulatory module detection

CRM detection was applied next, to identify specific combinations of TFs for each cluster of putatively co-regulated genes. CRM approaches usually tackle the problem of low motif specificity in promoter sequences by mining for patterns of co-occurring TFBSs in proximal promoter sequences of co-expressed genes (Loo and Marynen, 2009). The identification of such patterns of TFBSs is computationally a complex combinatorial optimization problem. One possible approach to solve this problem is to explore the search space of possible solutions, i.e. possible combinations of TFBSs, by a heuristic search procedure. For this purpose, Aerts *et al.* (2004) propose an evolutionary algorithm based on a new CRM scoring function. Multiobjective CRM detection algorithms further increase the specificity of TFBS modules by integrating additional knowledge about the respective experimental conditions into the search procedure, as introduced by the ModuleMaster algorithm (Wrzodek *et al.*, 2010). ModuleMaster is based on a multiobjective genetic algorithm (MOGA) that samples the search space by generating candidate solutions, which are iteratively recombined, mutated and evaluated according to the following objective functions: (i) the module score evaluates the quality of a single CRM according to the single TFBS scores within all promoter sequences; (ii) correlation score that quantifies the linear relationship of expression profiles of candidate TFs and the cluster centroid; and (iii) associations between TFs and a certain stimulus, based on protein–protein and protein–drug interaction data. The third objective function was applied to further increase the specificity of the derived CRMs with respect to the given experimental condition (i.e. atorvastatin treatment). To this end, interaction networks from the protein–protein interaction database STRING (Jensen *et al.*, 2009) and the protein–drug interaction database STITCH (Kuhn *et al.*, 2010) were integrated. These networks contain confidence values between 0 and 1 for all interactions depending on their reliability. Confidence scores between a candidate set of TFs and the atorvastatin stimulus were calculated based on shortest path algorithms (Supper *et al.*, 2009). Thus, the third objective function makes sure that the TFs of a candidate solution are likely to be downstream targets of the atorvastatin stimulus. A schematic flowchart of the implemented analysis workflow is depicted in Supplementary Figure S3.

Application of this multiobjective CRM detection method revealed several interesting combinations of TFs, which are putatively involved in atorvastatin-responsive gene regulation. As depicted in File S1 of Supplementary Material, among the most frequent TFs in atorvastatin-treated primary human hepatocytes are Krüppel-like factors, i.e. KLF4 and KLF11. KLFs have previously been published in the context of statins (Tuomisto *et al.*, 2008) and were recently found to be involved in the adipogenesis pathway (Brey *et al.*, 2009). The hypoxia-inducible factor 1 (HIF1A) was previously found to be involved in the regulation of the ATP-binding cassette (ABCA1) and plays a role in hypoxia-mediated inhibition of cholesterol synthesis (Ugocsai *et al.*, 2010). The retinoid X receptor (RXR) in combination with various other TFs of the nuclear receptor (NR) family were found to be involved in the regulation of three different atorvastatin clusters (see Cluster 1, 4 and 5 in File S1 of Supplementary Material). The heterodimer RARA:RXR, for instance, is known to regulate the expression of bile acid transporters and also influences the expression of HMG-CoA (Dawson *et al.*, 2009). Further known regulators in the context of statins are hepatocyte nuclear factor 4 (HNF4; Vock *et al.* 2008), SP3 (SP1) transcription factor (Lin *et al.*, 2008) and POU class 2 homeobox TF 1 (OCT1), which was shown elsewhere to get activated upon statin treatment (Ortego *et al.*, 2002). The CRM detection results of Cluster 4 (Fig. 1b) suggest that CYP3A4, which is one of the best characterized enzymes of atorvastatin detoxification (Jacobsen *et al.*, 2000), is regulated by several TFs of the NR family [i.e. the PPARA:RXR heterodimer, NR1H2 (LXR), NR2C2] and three further TFs (i.e. TGIF1, SMAD2 and ELF1). The NR NR2C2 as well as NR1H2 bind to the same xenobiotic response element within the CYP3A4 promoter. This indicates that these TFs may be involved in regulatory cross-talk on the binding site level. The regulatory influence of liver X receptor (NR1H2) on CYP3A4 has previously been shown in Duniec-Dmuchowski *et al.* (2007), but the putative influence of NR2C2 was unknown so far. This regulatory relationship was successfully validated in wet lab experiments as described below. The heterodimer PXR:RXR (see Cluster 5 in File S1 of Supplementary Material) was previously shown to be activated by vitamin E (Traber, 2004) and additionally regulates the expression of transporters as SLCO1B1 (contained in Cluster 5), which is able to transport statins (Rodrigues *et al.*, 2009). The main target gene of statins, the HMG-CoA reductase, which is contained in Cluster 7, has previously been shown to be regulated in the context of hypoxia by HIF1A (Pallottini *et al.*, 2008).

2.3 Experimental validation

Putative NR2C2 binding sites in the CYP3A4 promoter region at −1.1 kb was identified by computer-aided search for consensus motifs. Electrophoretic mobility shift assay demonstrated NR2C2 binding to the predicted sequence within CYP3A4 promoter (Supplementary Fig. S1). The proposed association between PPARA and CYP3A4 was validated by shRNA silencing experiments using lentiviral shRNA infection in three independent hepatocyte cultures. All cultures were treated with two different PPARA targeting shRNAs. PPARA-silenced hepatocyte cultures showed >50 % decreased CYP3A4 expression levels, as determined by quantitative PCR, compared with control cultures infected by non-silencing shRNA (data not shown). Additionally, as depicted in Supplementary Figure S2, the treatment of primary human hepatocytes with an PPARA agonist (WY14,643) results in an increased expression of PPARA mRNA itself, paralleled by an induction of mRNA expression of its known target gene HMGCR as well as CYP3A4. The treatment with a chemical antagonist of PPARA (MK886) demonstrates that the inhibition of PPARA leads to the downregulation of HMGCR and CYP3A4 mRNA expressions.

3 METHODS

3.1 Microarray experiments and preprocessing

Whole-genome Affymetrix U133 plus 2.0 (Affymetrix, Santa Clara, CA, USA) microarray measurements were conducted using samples of primary human hepatocyte cultures from six individuals. Each sample was treated with atorvastatin (60 μM) and dimethylsulfoxide (DMSO), which was used as control solvent. Microarray measurements were performed at five time points (0, 6, 12, 24, 48 and 72 h) after the drug stimulus. Low-level microarray preprocessing, i.e. normalization and background correction was conducted using the GC-RMA algorithm implemented in R Bioconductor (www.bioconductor.org). Differentially expressed genes were determined using a fold change filter of 1.7.

3.2 Clustering

The *Extended Dimension Iterative Signature Algorithm (EDISA)* was used in this work to detect sets of co-expressed genes (Supper *et al.*, 2007). EDISA is especially designed for time-series datasets that are observed under several experimental conditions. The algorithm iteratively screens for groups of genes and conditions, whereas the same genes are highly correlated according to Pearson's correlation coefficient. EDISA expects the two correlation thresholds τ_G and τ_C as input parameters. τ_G specifies how well each gene has to be aligned with the average trajectory of the cluster, and τ_C specifies how well each condition has to be aligned with the average trajectory of the cluster. Low values of τ_G and τ_C will produce clusters with few highly correlated gene expression profiles. Increasing the values of τ_G and τ_C will result in clusters with a high number of genes that show a reduced correlation. In this work, $\tau_G = 0.05$ and $\tau_C = 0.25$ were determined by a grid search, as described in File S1 of Supplementary Material. GO enrichment analyses were performed using hypergeometric testing (Hahne *et al.*, 2008). To this end, probe set identifiers were mapped to their annotated gene names. To avoid the accumulation of type I errors, subsequently, the Sidak equation was applied to correct for multiple testing (Ludbrook, 1998). The Sidak equation is, similar to the widely used Bonferroni method, a conservative multiple testing correction strategy, which is additionally characterized by a low false positive rate. Since all successive calculations are performed on the gene level, the expression profiles of probe sets belonging to the same gene were averaged.

3.3 Mapping TFBSs

PFMs were mapped to the promoter sequences according to Aerts *et al.* (2004) by calculating a match score for all subsequences in the promoter. These scores indicate how likely it is that the considered subsequence is generated by the motif model with respect to the background (Aerts *et al.*, 2004). As background model, a fourth-order hidden Markov model derived from coding sequences was used. Promoter sequences (1000 bp upstream from TSS) were retrieved from the Ensembl database (www.ensembl.org). In order to decide if a certain match score should be counted as putative binding site or not, cutoff levels need to be defined. Individual cutoff values were precalculated for each PFM individually, rather than choosing a global cutoff score for all PFMs. To this end, a conservative cutoff strategy is implemented, which is based on scanning non-regulatory (i.e. exonic) sequences and calibrating the cutoff score such that no hits are found on non-regulatory sequences (Kel *et al.*, 2003).

Sources of PFMs : PFMs from JASPAR (jaspar.genereg.net) and TRANSFAC™ (Biobase International, Wolfenbüttel, Germany) were used to scan for TFBSs. Until now, PFMs have been experimentally determined for a small fraction of all human TFs only. Thus, highly accurate PFM predictions were additionally included into the analysis workflow. These predictions were provided by a machine learning approach based on similarities of various sequence derived features (Schröder *et al.*, 2010).

3.4 Multiobjective CRM detection

In this work, CRMs were detected by the ModuleMaster algorithm using three objective functions. The first objective function sums up PFM match scores of the candidate solutions within all promoter sequences. ModuleMaster expects a predefined module size (i.e. the number of TFBSs) as input parameter, which was set to 5. Penalty terms guarantee that the TFBSs of a candidate solution within each promoter sequence are arranged within a certain window size, which is set to 200 bp. Furthermore, two single binding sites are not allowed to overlap, which is also ensured by a penalty term. The second objective function evaluates the linear relationship of expression profiles between candidate TFs and the cluster centroide by multivariate linear regression, which is quantified by the coefficient of determination (Zou *et al.*, 2003). More details about the implementation of these functions can be found in Wrzodek *et al.* (2010). The third objective function is based on the BowTieBuilder algorithm (Supper *et al.*, 2009), a heuristic pathway inference algorithm that identifies the most confident pathway between two given sets of proteins. To this end, interaction networks from the protein–protein interaction database STRING (Jensen *et al.*, 2009) and the protein–drug interaction database STITCH (Kuhn *et al.*, 2010) were merged. Low-confidence interactions (<0.7) were removed. As mentioned above, each interaction is associated with a confidence score between 0 and 1 depending on the reliability of the information sources. BowTieBuilder (Supper *et al.*, 2009) optimizes this confidence score during pathway inference in order to find the most confident connection between atorvastatin and a given set of candidate TFs. The confidence of a path is calculated by multiplying the confidence scores of each interaction. Details about this scoring scheme are given in File S1 of Supplementary Material. The ModuleMaster algorithm was run with 30000 fitness evaluations, mutation rate of 0.1 and a crossover rate of 0.7.

3.5 Experimental validation

Human NR2C2 protein was synthesized *in vitro* using the expression plasmid pcDNA3 1-His-TAK1 (Yan *et al.*, 1998), which was kindly provided by A.M. Jetten (NIEHS, Research Triangle Park, NC, USA), and the TNT T7 Quick Coupled transcription/translation system (Promega). Nuclear receptor response elements were generated by annealing 1 nmol each of two complementary oligonucleotides in 25 mM Tris–Cl, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$ in a total volume of 200 µl. Radioactive labeling was performed by incubating 10 pmol of the annealed double-stranded oligonucleotide with 2 U of Klenow fragment and 25 µCi -32 P dCTP in 50 mM Tris–Cl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dTTP in a total volume of 50 µl at 37˚C for 60 min. The labeled double-stranded oligonucleotides were purified through ProbeQuant™ sephadex G-50 micro columns (GE Healthcare). Binding reactions and gel electrophoresis were performed as described elsewhere (Geick *et al.*, 2001). Retarded complexes were quantified with the BAS1800 II phosphor-storage scanner (Fuji, Kanagawa, Japan) and AIDA software (Raytest, Staubenhardt, Germany). For chemical treatment, two batches of human primary hepatocytes from different donors were cultured in Williams E Medium as described previously (Feidt *et al.*, 2010). Treatment was performed using 10 µM WY14643 (Sigma, C7081) and 10 µM MK886 (Sigma, M2692) for 48 h. For quantitative RT–PCR analysis, cDNA was synthesized using 500 ng of total RNA as described elsewhere (Feidt *et al.*, 2010). The self-designed forward and reverse primers as well as probes for the detection of PPARA and CYP3A4 are available upon request. For detection of HMGCR, Taqman Gene Expression Assay was purchased (HS00168352m1) from Applied Biosystems (Foster City, USA).

4 DISCUSSION AND CONCLUSION

Microarray time-series experiments in primary human hepatocytes were conducted in order to investigate genome-wide regulatory effects upon atorvastatin treatment. We applied a novel computational analysis workflow to reconstruct transcriptional regulators that are involved in atorvastatin-responsive regulation of drug metabolism and other metabolic processes. As stated by the *futility theorem* (Wasserman and Sandelin, 2004), conventional sequence based methods to infer regulatory relationships fail to find specific results. To this end, a multiobjective CRM detection method was applied here that integrates knowledge about the experimental condition and drives the search toward regulators that are highly specific with respect to the drug treatment. Several new regulatory relationships in atorvastatin-treated hepatocytes were discovered. Binding of NR2C2 to the promoter of certain cytochrome P450 family (CYP) genes was successfully validated using gel-shift assays. Furthermore, the inferred regulatory influence of PPARA on CYP3A4 was successfully validated using specific agonist and antagonist experiments in primary human hepatocytes. These transcriptional regulators constitute promising candidates for future studies. First, because of their relationship to the expression of CYP3A4, which is the most important enzyme for atorvastatin biotransformation (Neuvonen *et al.*, 2006), and second due to their involvement in adverse drug reactions and other pleiotropic statin effects (Pascussi *et al.*, 2008). PPARA, for instance, one of the identified transcription factors, is well known to influence fatty acid profiles as well as inflammatory processes, both of which are known to be beneficially influenced by statins.

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