Different Stimuli for Inference of Gene Regulatory Network in Rheumatoid Arthritis

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Abstract: Since genetic and epigenetic factors are known to be involved in the pathogenesis of rheumatoid arthritis the search for key players in this disease is one of the most important challenges. For this purpose gene regulatory networks are one possibility to reveal underlying interactions for different stimuli. In this study we analyzed the cellular response of synovial fibroblasts to 4 different stimuli. We infered a gene regulatory network that is able to explain the observed data for stimulation by TNF-α, TGF-β1, IL-1 and PDGF-D simultaneously.

1 INTRODUCTION

Unveiling the dynamic and interlaced nature of gene regulation is one of the most important aims in systems biology. The activity of functional gene products is on the one hand influenced by transcription factors (TFs) and co-factors that influence transcription, on the other hand by post-translational modification of proteins as well as by the degradation of proteins and transcripts. Gene regulatory networks (GRNs) are a possibility to capture relations between molecular entities. Networks are usually represented as graphs consisting of nodes (representing genes and/or proteins) and edges (representing molecular interactions such as protein-protein and protein-DNA interactions). In this publication we present a GRN that integrates data of 4 different stimuli acting on synovial fibroblasts (SFBs) of rheumatoid arthritis (RA) patients. RA is a multifactorial polygenic disease with inflammatory impact of synovial joints. The inflammatory processes are triggered by cytokines and other immune system-related genes. Several cytokines play a critical role as mediators of immune regulation but the precise molecular mechanisms are still unclear. To investigate the therapeutic effects, cytokines like TNF-α, TGF-β1, IL-1 and PDGF-D are used in clinical practice. GRNs which describe the cellular response to the individual stimulus are helpful in the investigation of the effects of cytokines. However, simultaneous investigations of multiple stimuli offer the possibility to investigate the cellular actions from multiple perspectives and therefore provide more information and better understanding.

2 MATERIALS & METHODS

2.1 Data

Synovial membrane samples were obtained following tissue excision upon joint replacement/synovectomy from RA patients (n = 6; all Caucasian) at the Clinic of Orthopedics, Waldkrankenhaus 'Rudolf Elle' (Eisenberg, Germany) as outlined in Kupfer et al. (Kupfer et al., 2012). Synovial fibroblasts were stimulated with TNF-α, TGF-β1, IL-1 or PDGF-D for 0, 1, 2, 4, or 12 hours. By using U133 Plus 2.0 RNA microarrays (Affymetrix, Santa Clara, CA, USA; total of 60 microarrays) the analysis of gene expression was performed (for details see Kupfer et al. (Kupfer et al., 2012)).

To resolve the problem of choosing reliable and non-contradictory probesets for each transcript, the alternative Chip Definition File (CDF) of Ferrari et al. was used for annotating the genes (Ferrari et al.,

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2007). The microarray data were preprocessed using RMA in the default configuration (Irizarry et al., 2003). Concerning the present study where a combination of different data sets was used we corrected the data regarding the creation date were the microarrays were generated with a modified version of ComBat (Kupfer et al., 2012). The Sample Information File was created as described in the ComBat manual. The creation date of the microarrays was tagged as 'batch effect' and the parameters time point (total of 5), disease group (RA), and stimulation (TNF-α, TGF-β1, IL-1 and PDGF-D) were marked as covariates for every array.

To detect differentially expressed genes arisen from microarray experiments, the R-package Limma was used (Smyth, 2005). By using the expression data, the contrast and the design matrix, differentially expressed genes (DEGs) were obtained (Kupfer et al., 2012). A combination of fold-change and p-value was recommended by Shi et al. (Shi et al., 2008).

For testing the association between a given gene list and Gene Ontology (GO) terms the Bioconductor package *GOstats* was used (Falcon and Gentleman, 2007).

2.2 Network Inference

The transcription factor binding site integrating LARS (TILAR) (Hecker et al., 2009) was used to infer the gene regulatory network shown in Figure 1. The algorithm was modified to support time series data. The TILAR concept of modeling permits gene regulation only via TFs for which the regulated gene has a transcription factor binding site (TFBS) in its promoter region and the regulating gene has not. The advantage of this concept is that a initial prior-knowledge network template of TF-to-gene relations is constructed that automatically groups genes with identical binding sites. This can decrease the number of possible edges in the network and therefore lowers the complexity of the inference problem. The information of the binding sites was extracted from database Transfac (Matys, 2006). The least angle regression (LARS) algorithm is then used for simultaneous selection of the gene-to-TF relations and optimization of their weights. If such a relation is supported by prior-knowledge (expert knowledge or text mining programs such as PathwayStudio) the TILAR algorithm allows its soft integration. This results in sparse network models with a high content of biological prior-knowledge. Optimization of the network template is performed by a stepwise forward selection procedure. In that, the algorithm starts with a network template containing no TF-to-gene relation, creates the model and calculates the residual sums of squares (RSS) of the predicted to the measured expression values. For each TF-to-gene relation, the new model is created according to the network template, LARS is applied and the RSS is calculated. The TF-to-gene relation that led to the lowest RSS is fixed. This process is iteratively repeated until the RSS of the new model is not lower then the RSS of the previous model.

2.3 Transfac

To extract TFBSs within the promoter regions of the obtained differentially expressed genes Transfac can be used (Matys, 2006). One advantage Transfac is that the database contains experimentally validated TFBSs.

2.4 PathwayStudio

For the extraction of validation knowledge PathwayStudio 9.0 was used with the integrated Mammalian database (Nikitin et al., 2003). With PathwayStudio it is possible to obtain literature knowledge about the genes, the TFs and the corresponding associations among each other.

3 RESULTS

3.1 Data Pre-processing

Starting with RMA-normalized data of the 60 arrays for the time points 0, 1, 2, 4 and 12 hours, standard hierarchical clustering dendrograms (using the R function hclust with Euclidean distances) were employed to monitor possible batch effects. Furthermore the expression values were reviewed regarding differences in their levels. To correct system biased differences ComBat was applied.

3.2 Extracting Differentially Expressed Genes

Limma was used to obtain differentially expressed genes (DEGs; filtering by the conditions: $\geqslant$ 2-fold-change; p-value of $\leqslant 10^{-10}$) for the question concerning a genetic difference between time point 0 and the later time points within every single stimulus (TGF-β1: 507 genes; TNF-α: 582 genes; IL-1: 333 genes and PDGF-D: 534 genes). Creating the union of the 4 obtained gene lists provided a gene list containing 1448 genes. For the key question concerning RA

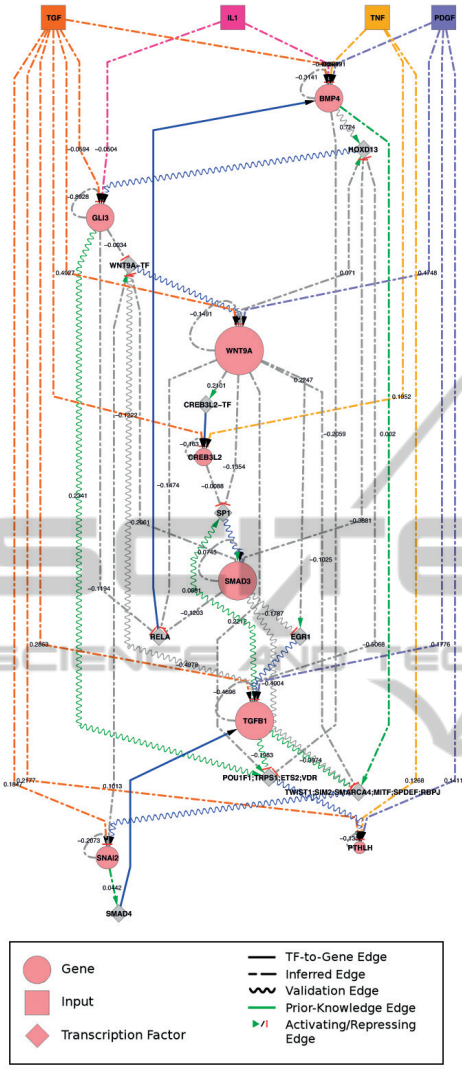

Figure 1: GRN describing the cellular response of 4 applied stimuli.

and osteoarthritis (OA) a previous and published gene list was used which contains genes differentially expressed between both diseases for the time point 0 (Kupfer et al., 2012). Using this list and the union of the differentially expressed genes for all 4 stimuli the intersection of 541 genes was extracted. This constitutes a genetic difference between RA and OA. For the resulting list a gene enrichment analysis was done with GOstats (p-value ≤ 0.05). As shown in Table 1 the highest ranked GO term was cartilage development with a p-value of 1.42^{-07} and 18/134 genes. With regard to the computational complexity of network modeling we have chosen 8 out of the 18 obtained genes of this GO term as highlighted in Table 2.

Table 1: Overrepresented GO categories in the set of DEGs and the obtained p-values.

TermID	Count	Size	Pvalue	Term
GO:0051216	18	134	1.42e-07	cartilage development
GO:0002062	12	58	1.49e-07	chondrocyte differentiation
GO:0048518	133	2898	1.87e-07	positive regulation of biological process
GO:0009888	61	1016	2.76e-07	tissue development
GO:0048522	122	2649	6.73e-07	positive regulation of cellular process
GO:0048705	18	150	7.87e-07	skeletal system morphogenesis
GO:0001503	23	243	1.70e-06	ossification
GO:0006357	56	964	2.56e-06	regulation of transcription from RNA polymerase II promoter
GO:0001649	15	116	2.56e-06	osteoblast differentiation
GO:0008284	38	551	2.61e-06	positive regulation of cell proliferation

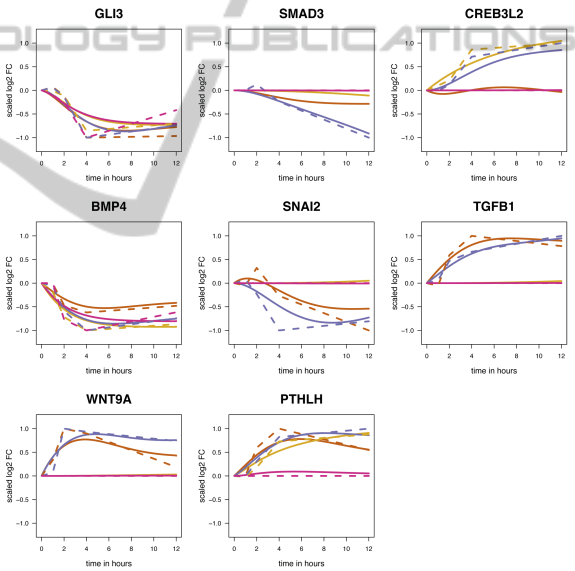

Figure 2: Measured and simulated expression profiles. Dashed lines represent the measured $\log_2$-FC of the 4 stimuli. The simulated results are shown with solid lines.

3.3 Knowlege Extraction

By using Transfac we extracted all experimental validated TFBSs for the obtained genes for the following network modeling. In the TILAR concept of modelling, these TF-to-gene relations are used as a network structure template. Furthermore, we extracted literature knowledge for the genes, the TFs and the extracted TFBSs by using *Pathway Studio 9.0*. Therefore, we used the genes to be modeled, the obtained regulating TFs and collected prior knowledge about

Table 2: Differentially expressed genes of the top-ranked GO category (GO:0051216 cartilage development). Genes used for network inference are highlighted in bold.

SYMBOL	ENTREZ	GENENAME	UNIPROT
OSR1	130497	odd-skipped related 1	Q8TAX0
COMP	1311	cartilage oligomeric matrix protein	P49747
DLX2	1746	distal-less homeobox 2	Q07687
FGF2	2247	fibroblast growth factor 2	P09038
GHR	2690	growth hormone receptor	P10912
GLI2	2736	GLI family zinc finger 2	P10070
GLI3	**2737**	**GLI family zinc finger** 3	**P10071**
SMAD3	**4088**	**SMAD family member 3**	**P84022**
CHST11	50515	carbohydrate (chondroitin 4) sulfotransferase 11	Q9NPF2
PTHLH	**5744**	**parathyroid hormone-like hormone**	**P12272**
CREB3L2	**64764**	**cAMP responsive element binding protein 3-like 2**	**Q70SY1**
BMP4	**652**	**bone morphogenetic protein 4**	**P12644**
BMP6	654	bone morphogenetic protein 6	P22004
SNAI2	**6591**	**snail homolog 2**	**O43623**
SNAI1	6615	snail homolog 1	O95863
TGFB1	**7040**	**transforming growth factor, beta 1**	**P01137**
WNT9A	**7483**	**wingless-type MMTV integration site family, member 9A**	**O14904**
FGF18	8817	fibroblast growth factor 18	O76093

edges by Pathway Studio. The extracted knowledge was verified manually.

3.4 Network Inference

Prior to the network inference, the gene expression data of the three different experiments (TGF-β1 & TNF-α, PDGF-D and IL-1) was scaled to a maximum of 1. This was done to remove the experiment-specific bias due to separate pre-processing and analysis. Subsequently, for each gene which did not exceed a absolute $\log_2$- fold change of 0.6 in the expression profile of a treatment, all values of the corresponding profile were substituted by 0. This way, small changes in the expression which are likely to resemble noise were removed preventing the algorithm from modeling artificial rather then the true biological signal. Linear interpolation was used to obtain measurements at equidistant time points. We used a constant input function to simulate the input of cell stimulation. TILAR was then used for network inference. Identification of the best algorithm parameters was performed in a parameter study optimizing the number of integrated prior-knowledge edges with respect to the error between the observed and the predicted $\log_2$- fold-changes. The final network (Figure 1) was visualized in Cytoscape (Smoot et al., 2011) and is composed of 21 nodes (8 modeled genes, 9 bridging TFs and 4 input perturbations) and 51 edges out of which 42 were inferred by the algorithm (26 gene-to-TF edges and 16 input-to-gene edges). The remaining edges are prior-knowledge TF-to-gene edges. Furthermore, 8 known direct gene-to-gene relations which were not used by the algorithm during the inference were identified in the constructed network. The node size of the modeled genes resembles their out-degree as visualized in Figure 1. Together with the highest input-to-gene edge weights, this property identifies especially WNT9A as a signal distributing gene. The only gene which is strongly affected by all four inputs is BMP4. Regarding the fit of the simulated data compared to the measured once we got akin dynamics as shown in Figure 2.

3.5 Discussion

To our knowledge, this is the first network model simulating the initial regulatory steps in SFBs during stimulation with four different cytokines/growth factors. An early event in fibroblast activation by TNF-α, TGF-β1, IL-1, and PDGF-D is the expression of genes coding for additional secreted factors also modulating cellular responses. In this context, a variety of factors is synergistically induced in response to two or more stimuli. For instance, both TGF-β1 and PDGF-D are able to induce the expression of members of the TGF family (i.e., BMP4 and TGFB1), WNT9A (also known as WNT14), and PTHLH (also known as PTHRP). PTHLH may also be driven by TNF, whereas BMP4 could be induced by all applied stimuli. Our model confirms other studies reporting TGF- or TGF signalling-dependent TGFB1, PTHLH, and WNT9A expression (Bascom et al., 1989) (Kiriyama et al., 1993) (Spagnoli et al., 2007) and TNF-dependent PTHLH or BMP4 expression (Funk et al., 1998) (Horiguchi et al., 2000) in several cell types.

BMP4, WNT9A, and PTHLH are secreted factors involved in tissue development, especially cartilage and bone formation (Bramlage et al., 2006) (Hartmann and Tabin, 2001) (Karaplis et al., 1994). In addition, PTHLH has been shown to mediate antiproliferative effects and to induce matrix-degrading enzymes (Maioli et al., 2002) thus potentially influencing matrix and bone remodelling. The stimulatory effects of these factors may trigger cellular characteristics of SFB and other cell types in the joint such as chondrocytes or osteoblasts (Tsumaki et al., 2002)(Guo et al., 2004)(Amizuka et al., 2000)(Ikegame et al., 2001). This cascade reflects

the influence of activated SFB on development, function, and maintenance of the joints or, pathophysiologically, on joint destruction, synovitis, and fibrosis, e.g., in the course of rheumatoid arthritis (Karouzakis et al., 2006)(Huber et al., 2006). Another set of stimulation-dependent genes consists of selected TFs, e.g., TGF- and IL-1-inducuble GLI3 and TGF-inducible SNAI2 (also known as SLUG) which are involved in regulating a variety of developmental processes (Johnson and Tabin, 1997) (Nieto et al., 1994) or TGF- and TNF-inducible CREB3L2 (also known as BBF2H7) which participates in regulating cell survival and chondrogenesis (Sheng et al., 2010)(Saito et al., 2009). In part, these findings are in good agreement to the literature, since it has already been shown that SNAI2 may be induced by TGF (Aomatsu et al., 2011). Further TFs are predominately induced in response to secondarily secreted factors, e.g., BMP4-induced HOXD13 which is also a regulator of tissue/organ development (Goodman, 2002) or WNT9A-induced EGR1 which contributes to basic processes such as tissue repair, (Braddock, 2001), cellular growth regulation, and apoptosis (Liu et al., 1998). Here, our model provides new insights into the intricate successive regulation of TF-induction, since indirect activation pathways are still inadequately characterized in the literature. Following expression and activation, these TFs mediate the regulation of further target genes (which are not included in Figure 1) defining the superordinate cellular response of SFB to the (combination of) different stimuli. However, in the presented network, negative regulatory (feedback) mechanisms occurring during TNF/TGF/IL-1/PDGF stimulation are also predicted. They are mediated either directly in response to the primary stimuli (e.g., inhibition of WNT9A TF, RelA, and GLI3 by TGF- and IL-1-inducible GLI3) or indirectly in response to secondarily secreted factors (e.g., POUF1, TRPS1, ETS2, and VDR or TWIST1, SIM2, SMARCA4, MITF, SPDEF, and RBPJ in response to WNT9A). In consequence, a variety of genes is regulated via a complex network of positively or negatively regulated TFs representing the interplay between activating and deactivating features during stimulation with several cytokines/growth factors.

4 CONCLUSIONS

In this study we were able to present a single network model that describes TGF-β1, TNF-α, IL-1 and PDGF-D stimulation simultaneously. The fit to all expression profiles of genes included was excellent and the robustness analysis showed that the obtained network is reliable. Moreover, the biological meaning of the infered GRN shows new insights like the intricate successive regulation of TF-induction as well as already published results regarding the single stimuli. These results are now combined in one network.

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