A transcriptomic study of Williams-Beuren syndrome associated genes in mouse embryonic stem cells

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Williams-Beuren syndrome (WBS) is a relatively rare disease caused by the deletion of 1.5 to 1.8 Mb on chromosome 7 which contains approximately 28 genes. This multisystem disorder is mainly characterized by supravalvular aortic stenosis, mental retardation, and distinctive facial features. We generated mouse embryonic stem (ES) cells clones expressing each of the 4 human WBS genes (WBSCR1, GTF2I, GTF2IRD1 and GTF2IRD2) found in the specific delated region 7q11.23 causative of the WBS. We generated at least three stable clones for each gene with stable integration in the ROSA26 locus of a tetracycline-inducible upstream of the coding sequence of the genet tagged with a 3xFLAG epitope. Three clones for each gene were transcriptionally profiled in inducing versus non-inducing conditions for a total of 24 profiles. This small collection of human WBS-ES cell clones represents a resource to facilitate the study of the function of these genes during differentiation.

Background & Summary

Williams-Beuren Syndrome (WBS) is a neurodevelopmental disorder caused by a hemizygous deletion of 1.5 Mb segment occurring in approximately 95% of cases and a larger 1.84 Mb deletion observed in about 1 of 20 cases 1,2. Clinical main features comprise, distinctive facial features (elfin face) 3,4, supravalvular aortic stenosis, connective tissue anomalies, hypertension, infantile hypercalcemia 5, dental, kidney and thyroid abnormalities, premature ageing of the skin 6, impaired glucose tolerance and silent diabetes 2,7. The cognitive hallmark includes mental retardation, hypersensitivity to sound due to the absence of acoustic reflexes and hypersociability 8,9. While the primary cause of WBS is well understood 10, we still know little about the molecular basis of the phenotype. The first genome-wide transcription study performed in primary fibroblasts from eight individuals with WBS resulted in set of candidate pathways mis-regulated in WBS possibly involved in associated phenotypes 2.

To facilitate the study of genes involved in WBS, we generated and transcriptionally profiled of mouse embryonic stem (ES) cells 11,12 with inducible expression of the three GTF-transcription factors (GTF2IRD1, GTF2IRD2 and GTF2I) together with the translation initiation factor Eif4h (the human homolog is known as WBSCR1) 13,14. The ES properties to self-renew 15 and to differentiate in the three germ layers 16,17 have made these cells a unique in vitro system for studying the molecular mechanisms that regulate lineage specification. The three GTF-family members are all highly expressed in the brain. Mouse hemizygote models for GTF2I and GTF2IRD1 present cognitive and behavioural phenotypes associated with WBS 14, moreover GTF2I deletion is known to be associated with increased sociability while the GTF2I duplication results in increased separation anxiety 16,19. Targeted Gtf2IRD1 knockout mouse is known to cause the up-regulation of growth factors and other genes involved in brain development and cellular proliferation which may be linked with the extreme thickening of the epidermis observed in the mouse model 20. Moreover it has been reported that the transgenic expression of each of the three family members in skeletal muscle causes significant fiber type shifts 21. Finally, WBSCR1, the human homolog of Eif4h, is known to contribute to neuroanatomical WBS deficits 22: in vivo studies on knockout mice displayed

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growth retardation, a smaller brain volume, a reduction in both the number and complexity of neurons and severe impairments of fear-related associative learning and memory formation.22

In a previous study on Down Syndrome, we generated a collection of mouse ES clones capable of the inducible expression of 32 mouse genes (orthologs of human chromosome 21 genes) under the control of the tetracycline-response element (tetO)14. Here we used the same approach exploiting the ROSA-TET system23 to generate 12 mouse ES clones carrying the 4 Open Reading Frames (ORFs) of the GTF-transcription factors (GTF2IRD1, GTF2IRD2 and GTF2I) together with the translation initiation factor Eif4h (Fig. 1). Three positive clones (Supplementary Fig. 1) for each gene were selected and grown in medium deprived of tetracycline (Tc) to perform an induction time course. RNA was extracted (Supplementary Fig. 2) from each clone at the time-point of maximal expression (24 hrs, Supplementary Fig. 3) and total RNA extracted from un-induced clones used as control. Total RNA was profiled by Affimetrix microarrays (the whole set of results is available in the GEO database [GSE9670124])15,28. This analysis was performed to detect differentially expressed genes (that is, in induced versus non-induced cells, Supplementary Fig. 4) in ES cells modeling the WBS.

Methods

Generation of recombinant WBS-ES clones. The generation of recombinant WBS-ES clones started with the modification of the cell line EBRtch3 (EB3) as described in14. The cells were cultured in ES media supplemented with the leukemia inhibitory factor (LIF), at 37 °C in 5% CO2. The ES media contained DMEM high glucose (Invitrogen, Catalog No. 11995–065) supplemented with 15% fetal bovine serum defined (hyClone, Catalog No. SH30070.03), 0.1 mM non-essential aminoacids (Gibco-Brl, Catalog No. 11140–050), 0.1 mM 2-mercaptoethanol (Sigma, Catalog No. M6250) and 1,000 U/ml ESERO-LIF (Millipore, Catalog No. ESG1107). The basal expression of the transgenes in each stable clone was assured by the growth of the cells in this ES media + LIF supplemented with 1 μg/ml Tetracyclin (Tc) (Sigma, Catalog No. T7660). The selection of positive recombinant clones was assured by the growth of the cells the ES media (+ LIF and + Tc) supplemented with 1.5 μg/ml Puromycin (Puro, Sigma, Catalog No. P9620). After trypsinization (Trypsin-EDTA solution 10x, Sigma, Catalog No. T4174) the cells were plated 1 day before the nucleofection on a layer of 0.1% Gelatin (Gelatin Type 5 was modified as described in14 and the epitope 3xFLAG was designed to be in frame with Oct-3/4 vector pPthC

Cloning strategy. Each human coding sequence was cloned from the ATG to the stop codon without the 5’ and 3’ UTRs. For the 4 WBS ORFs, we cloned the longest annotated coding sequence (NM_001368300 for GTF2IRD2; NM_001199207 for GTF2IRD1; NM_032999 for GTF2I; NM_022170 for WBSCR1). The exchange of the Tet-off system as described in28. Cells to be induced were grown in medium deprived of Tc to perform a time course of induction (17, 24, 39 and 48 hours), by using the growth in Tc as control, time 0. Total RNA was extracted at each time point of the time course and at time 0 and then 1 μg of each reverse-transcribed as described in14. The levels of each transcript was measured by Real-time RT-PCR experiments by using Light Cycler 480 Syber Green I Mastermix (Roche, Catalog No. 04887352001) for cDNA amplification and in LightCycler 480 II (Roche) for signal detection. RT-PCR results were analyzed using the comparative Ct method normalized against the housekeeping gene Actin B (refer to Supplementary File 2). All primer pair sequences used for RT-PCR are available in Supplementary File 2. For the time course of induction of the GTF2I clones (named C6, B3, A1), of the GTF2IRD1 clones (named D1, D2, D4), of the GTF2IRD2 clones (named A3, A4, A5) and of the WBSCR1 clones (named A3, A4, A1) refer to Supplementary File 3. The Escherichia coli positive clones were selected by enzymatic digestions and then sequenced by using the universal M13Fw primer and, for longer sequences, internal forward primers specific to the gene of interest.

Microarray hybridization, data processing and statistical analysis. The preparation of the RNA samples for the microarray hybridization on the Affymetrix GeneChip Mouse Genome 430_2 array was described
Low-level analysis was performed by robust multiarray average (RMA) implemented using the RMA function of the Affymetrix package of the Bioconductor project in the R programming language. The low-level analysis for the BAMarray tool (v3.0) was performed using the MAS5 method as described in and implemented using the corresponding function of the same Bioconductor package. For each gene, a t-test was used on RMA normalized data to determine the differentially expressed genes (induced versus uninduced). P-value adjustment for multiple comparisons was done with the FDR of Benjamini-Hochberg (threshold FDR < 0.05, refer to Supplementary File 4 and Supplementary Fig. 4).

Accession codes. The whole set of results is available in the GEO database as "A transcriptomic study of Williams-Beuren syndrome associated genes in mouse embryonic stem", SuperSeries code GSE96701 (Supplementary File 4, Supplementary Fig. 4 and Online-only Table 1). The title of the SuprSeries is "Expression data from inducible ES stable cell line overexpressing the human GTF2IRD1, GTF2IRD2, WBSCR1, or GTF2I Fig. 1.

Data Records
The whole set of results is available in the GEO database as "A transcriptomic study of Williams-Beuren syndrome associated genes in mouse embryonic stem", SuperSeries code GSE96701.

Technical Validation
The overexpression of the 4 selected WBS genes was based on the inducible expression by means of a tetracycline-repressible promoter (tet-off system). The first validation of the system was based on the cloning of the luciferase (Luc) into the exchange vector as described in, the second was the establishment of expression of the YFP reporter gene, which is separated from the Luc gene in the recombinant locus by an IRES sequence, by detecting a comparable level of the YFP expression and protein accumulation following induction. The study of the growth properties of our mES line (EB3) compared to the parental line (E14) (data not shown) and the ability of these cells to differentiate in the three main germ layers was also performed in; in details the down-regulation of the pluripotens’ marker Oct3/4 was also confirmed in the EB3 as well as a farther induction of the mesodermal (Brachyury), ectodermal (Gfap) and endodermal (Afp) markers during mES differentiation. Collectively these data suggest that the system we chose allows the efficient and long-term overexpression of the transgene in a dose and time-dependent manner. It is therefore suitable for systematic expression of WBS cDNAs. The positive

Fig. 1 Flowchart of experimental design of this study.
clones overexpressing the 4 selected WBS genes were identified by PCR using the primer pair used in previous studies\(^1\), \(^2\), \(^3\), \(^4\), \(^5\). (Supplementary Fig. 1).

**Code availability**

Codes that were used for data processing are included in the Methods and available as supplementary material (Supplementary File 1 includes the sequences Asc1-Pac1 of the 4WBS ORFs; Supplementary File 2 the Primers Codes that were used for data processing are included in the Methods and available as supplementary material (Supplementary File 4).

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Author contributions
Collection and assembly of data, data analysis and interpretation, conception and design, manuscript writing: R.D.C. Performed the experiments: R.D.C., S.I. and A.F. Analyzed the data: R.D.C. and D.d.B. Contributed reagents/materials/analysis tools: R.D.C., S.I. and A.B. A.O.F. provided technical input with respect to cloning. R.D.C. wrote the manuscript. Conception and financial support: A.B.

Competing interests
The authors declare no competing interests.

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