The role of OTX2 in medulloblastoma
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OTX2 directly activates cell cycle genes and inhibits differentiation in medulloblastoma cells

Abstract

The transcription factor OTX2 has been implicated as an oncogene in medulloblastoma, which is the most common malignant brain tumor in children. It is highly expressed in most medulloblastomas and amplified in a subset of them. To study the role OTX2 has in medulloblastoma we investigated the downstream pathway of OTX2. We generated D425 medulloblastoma cells in which endogenous OTX2 can be silenced by inducible shRNA. Silencing of OTX2 strongly inhibited cell proliferation and resulted in a neuronal-like differentiation. Expression profiling of time courses after silencing showed a progressive change in gene expression for many cellular processes. Downregulated genes were highly enriched for cell cycle and visual perception genes, while upregulated genes were enriched for genes involved in development and differentiation. This shift is reminiscent of expression changes described during normal cerebellum development where proliferating granule progenitor cells have high OTX2 expression, which diminishes when these cells exit the cell cycle and start to differentiate. ChIP-on-chip analyses of OTX2 in D425 cells identified cell cycle and perception genes as direct OTX2 targets, while regulation of most differentiation genes appeared to be indirect. The expression of many directly regulated genes correlated to OTX2 expression in primary tumors, suggesting the in vivo relevance of these genes and their potential as targets for therapeutic intervention. These analyses provide more insight in the molecular network of OTX2, demonstrating that OTX2 is essential in medulloblastoma and directly drives proliferation by regulation of cell cycle genes.
Introduction

Medulloblastoma, an embryonal tumor originating in the cerebellum, is the most common malignant brain tumor in children. One of the genes implicated in medulloblastoma tumorigenesis is **OTX2**, which encodes a member of the bicoid subfamily of homeodomain-containing transcription factors. **OTX2** is essential for normal brain and sensory organ development, such as the eye [1-3]. Mouse models showed that deletion of both **Otx2** alleles is lethal, whereas decreased levels of **Otx2** resulted in serious malformations of the brain, including the cerebellum [4]. **OTX2** protein is detected in progenitor cells of the external granule cell layer (EGL) during cerebellum development, but in postnatal cerebellum, when EGL cells have disappeared, **OTX2** is no longer expressed [5]. Medulloblastomas, however, have a high **OTX2** expression, as was first discovered using SAGE [6]. **OTX2** mRNA levels as well as protein levels are high in 75–80% of all medulloblastomas [5, 7-9]. Recently, we and others identified with expression profiling distinct molecular subtypes in medulloblastoma [10, 11]. Two of these subtypes are characterized by activated WNT-signaling or SHH activation, respectively. Other subtypes are more related to each other and are characterized by the expression of neuronal markers and/or photoreceptor genes. **OTX2** is highly expressed in WNT tumors and all non-WNT/non-SHH tumors, but expression levels are low or absent in SHH tumors.

High level amplifications and frequent focal gain of the **OTX2** locus in medulloblastoma suggest an oncogenic role for **OTX2** in medulloblastoma [5, 7, 8, 12]. Furthermore, medulloblastoma cell lines expressing **OTX2** are inhibited in proliferation and tumor formation when **OTX2** is silenced, indicating that **OTX2** is essential for medulloblastoma [7, 8]. Recently, we overexpressed **OTX2** in the medulloblastoma cell lines MED8A and DAOY to investigate the **OTX2** pathway [13]. The expression levels of many genes were changed after **OTX2** induction, but the **OTX2** overexpression also induced senescence in both cell lines, which limited further investigations of the precise role **OTX2** has in medulloblastoma. Therefore, knockdown in cells with endogenous **OTX2** expression is required.

In this study, we silenced the high endogenous **OTX2** expression in D425 medulloblastoma cells by doxycyclin-inducible shRNAs to identify genes and pathways under direct transcriptional control of **OTX2** in medulloblastoma. As a result, cells ceased proliferation and obtained a differentiated phenotype. Expression profiling and **OTX2** ChIP-on-chip analyses identified cell cycle genes as direct targets of **OTX2**, in line with our previous results obtained with the overexpression models [13]. Differentiation genes, which were strongly regulated after **OTX2**
silencing, are most likely indirect targets. The differentiation triggered by OTX2 silencing in D425 was reminiscent to expression changes during normal cerebellum development, while targets of OTX2 also correlated to OTX2 expression in a series of medulloblastoma tumors. The elucidation of the OTX2 downstream network therefore identified many genes that may mediate the oncogenic role of OTX2 in vivo, providing many potential targets for therapeutic intervention.

**Material en Methods**

**Cell line, constructs and transfection**
D425 medulloblastoma cells, a gift from Michael Grötzer (Zürich, Switzerland), were cultured as described [5]. Cells were authenticated by checking their morphology and array-CGH and Southern blot analysis, showing the MYC and OTX2 amplification, characteristic for this cell line [5, 14]. To generate D425 cells with inducible shRNA against OTX2, cells were transfected with pcDNA6/TR (Invitrogen). After selection with 8 μg/mL blasticidin (Invitrogen), surviving clones were tested for expression of the Tet repressor. Inducible constructs for OTX2 specific shRNA (Supplementary Table 1A) were generated using the pTER plasmid (gift from van de Wetering, NKI Amsterdam). After the constructs were transfected in a Tet repressor expressing D425 clone, cells were cultured in selective medium with 50 μg/mL zeocin (Invitrogen) to generate stable clones. To induce shRNA expression, doxycycline (MP Biochemicals, Eschwege, Germany) was added to a final concentration of 100 ng/mL.

**Western blotting and antibodies**
Western blotting and antibodies used were as previously described [13]. Other antibodies used included: GAPDH, NEUROD1, NOTCH2, ERK (Cell signaling technology, Danvers, MA), RB (BD Biosciences, San Jose, CA), CCND3 (Labvision, Fremont, CQ) and SPARC (Hematologic Techn, Essex Junction, VT).

**Growth assay and FACS analysis**
Cells were plated in 96-wells microplates at 1,500 cells per well and doxycycline was added 24 hr later. Cell viability was measured in time by MTT (Sigma-Aldrich, St. Louis, MO). FACS analysis was performed as previously described [13]. Cell cycle distributions were analyzed using FlowJo 7.2 software. The results of 3 independent experiments were analyzed using a two-tailed Student’s t-test to assess statistical significance.
**Immunofluorescence**

For immunofluorescence microscopy, cells were grown on glass. Cells were washed with PBS and fixed on glass using 4% paraformaldehyde in PBS for 15 min in an incubator. After washing twice, cells were permeabilized in 0.2% Triton X-100 in PBS for 20 min. After three wash steps using 0.04% Triton X-100 in PBS, slides were blocked in 1% BSA, 0.01% Triton X-100 and PBS, followed by incubation for 60 min with 1:100 MAP2 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1,000 NeuN (Millipore, Billerica, MA), or 1:200 SYN (Cell signaling technology) antibody in 100 μL blocking solution. Slides were washed three times with 0.01% Triton X-100 in PBS. Slides were then incubated for 15 min with 0.5 μL anti-rabbit ALEXA 594 or 488 or anti-mouse ALEXA 488 (Invitrogen) and (for the MAP2 slides only) 0.1 μg phalloidin-FITC labeled (Sigma-Aldrich) in 100 μL blocking solution, followed by three times washing with 0.01% Triton X-100 in PBS and once with H₂O. After drying, slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) with 400X diluted DAPI.

**RNA extraction and expression profiling**

At different time points after induction of OTX2 shRNA, total RNA was extracted with Trizol reagent (Invitrogen) according to manufacturer’s instructions. Northern blot analyses were as described.6 For expression profiling, RNA purification and hybridization to HG U133 Plus 2.0 Array (Affymetrix, Santa Barbara, CA) were described previously [9]. For normalization of the expression data we used the MAS5.0 algorithm of the GCOS program (Affymetrix). Target intensity was set to 100 ($\alpha_1 = 0.04$ and $\alpha_2 = 0.06$). Detection p values were assigned to each probe set using the MAS5.0 algorithm (trimmed mean 96 = 100). Quality of the arrays was ensured by inspection of the beta-actin and GAPDH 5’-3’ ratios as well as the percentage of present calls generated by the MAS5.0 algorithm (Affymetrix). In case of multiple probe sets for one gene, the probe set with the highest expression and correct mapping was used. Expression data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE22875.

**Data analyses**

Affymetrix data showed for 13313 genes a present call in at least one of the samples. Regulated genes were defined as having $2^{\log}$ fold change of $>1$ with a minimal expression change of 50 in all three D425-shOTX2 experiments. Cluster analyses of expression data of regulated genes were performed using the TMEV program [15]. Gene ontology analyses were performed using the DAVID tool (http://david.abcc.ncifcrf.gov/) [16] All plots of regulated genes and the correlation with OTX2 expression in non-WNT/non-SHH tumors were analyzed using the R2 microarray analysis and visualization platform (http://r2.amc.nl). SHH tumors were excluded in these correlation analyses, because they lack OTX2.
expression. Inclusion would drive correlation to differentially expressed genes between this group compared to the others. WNT tumors were excluded, as activated CTNNB1 can influence the gene regulation by OTX2 [17].

Chromatin immunoprecipitation
Chromatin immunoprecipitation, using OTX2 antibody (Millipore) or FLAG antibody (Stratagene, La Jolla, CA), and amplification and labeling of recovered DNA were carried out as described previously [13]. Labeling of the material, hybridization to the 2.1M Deluxe Promoter Array, scanning of the arrays and peak calling were performed by Nimblegen (Madison, WI). OTX2 binding was defined when a called peak overlapped with the transcription start site (±250 bp). Differences in percentage of OTX2 binding between sets of genes were tested with a Pearson’s chi-square test. OTX2 binding was validated by qPCR as described previously [13]. Primers are listed in Supplementary Tables 1B and 1C. The program DME (v2.0) was employed using the hybrid mode to identify enriched sequence motifs of length 6 in the peaks when compared to a background of peak-flanking sequences [18].

Results

OTX2 silencing inhibits cell proliferation and induces differentiation
To study the downstream pathway of OTX2 we selected the D425 medulloblastoma cell line, which has high OTX2 expression due to amplification of the OTX2 gene. Based on this amplification, which is most typical for non-WNT/non-SHH tumors, the expression of retinal genes and the presence of a MYC amplification, it is most likely that the cell line is derived from a Type E/Group C tumor [7, 9]. We used the D425 cell line to silence OTX2 expression with doxycycline (DOX) inducible shRNAs. We generated two clones of D425 with different inducible shRNAs targeting OTX2 (D425-shOTX2). Induction of shRNA1 or shRNA2 rapidly and strongly reduced both OTX2 mRNA and protein levels in each clone. In control cells without shRNA but treated with DOX or in noninduced cells OTX2 expression did not change (Figure 1 and Supplementary Figure S1). Silencing of OTX2 resulted in reduced cell proliferation, which even completely halted after 3–4 days (Figure 2A). FACS analyses at 48 hr after DOX induction showed that the reduced proliferation was accompanied by a strong arrest in G0-G1 phase (Figure 2B and Supplementary Figure S2). We found no evidence for increased apoptosis after OTX2 silencing, neither with FACS analysis nor with protein analyses of cleaved PARP or CASP3 (Supplementary Figure S2 and S3). However, within 72 hr after OTX2 silencing, D425-shOTX2 cells displayed neurite-like outgrowths instead of the normal round
shape, indicating a more differentiated phenotype (Figure 2C and Supplementary Figure S4). These results show that OTX2 is essential for proliferation and inhibits differentiation of medulloblastoma cells.

mRNA profiling identifies regulated genes after OTX2 silencing

To investigate the effect of OTX2 silencing on the cellular transcriptome in D425-shOTX2 cells, we generated mRNA expression profiles at 0, 8, 16, 24, 48 and 96 hr after DOX treatment, in triplicate for cells in which shRNA1 was induced, as well as for an identical time series of control cells. Of the 13,313 genes that were significantly expressed in any of the 24 profiles,

![Figure 1. OTX2 silencing in D425 medulloblastoma cells.](image)
2,202 (16.5%) were consistently regulated in all three time courses (log fold regulation of >1; minimal expression change 50, see Materials and Methods). There were 1,172 genes upregulated and 1,030 genes downregulated (Supplementary Tables 2–4). Most of these genes (>93%) were not regulated in the control time course.

Gene Ontology analysis of regulated genes

To distinguish different kinetic patterns of regulated genes we used k-means clustering. The expression patterns of the identified gene sets are shown in Figure 3A, ordered from top to bottom according to the onset of their up- or downregulation. Figure 3B shows the expression data of a representative gene for each gene set, with the triplicate experiments where OTX2 was silenced in blue and the control experiment in black. Supplementary Figure S5 shows the expression patterns of all gene sets for all three time courses after OTX2
Figure 3. Cluster and Gene Ontology analyses of mRNA expression profiles after OTX2 silencing. (A) The 2,202 genes regulated within the three independent OTX2 silencing experiments were grouped into 11 gene sets according to the onset of their up- or downregulation. Heat map shows the expression data of these regulated genes in one of the knockdown experiments where OTX2 was silenced. (B) For each gene set indicated in (A) the mRNA expression data of a representative gene are shown for all three knockdown experiments (blue lines) and the control (black line). (C) Gene Ontology analyses for up- and downregulated genes.
silencing and in the control cells.

We performed Gene Ontology (GO) analysis for all up- or downregulated genes to identify biological processes that were most affected by OTX2 silencing (Figure 3C and Supplementary Table 5A). Downregulated genes were strongly enriched for cell cycle genes (156 genes), in particular those involved in mitosis (86 genes, including AURKA, AURKB, CDK2, CENPE, CDKN3). They were mainly found in gene sets 10 and 11, indicating that their expression started to be downregulated around 24 hr after induction of OTX2 silencing. Genes in gene sets 8 and 9, which have an earlier onset of downregulation, were more enriched for genes involved in visual perception (36 genes, including ABCA4, TULP1, GNGT2, RP1, PDE6H), but also included several cell cycle genes (E2F2/3, CCND3 and MCM genes). Other processes significantly enriched among the downregulated genes included DNA repair and chromosome segregation (e.g., MSH2, HMGB2 and BRCA2). Major GO categories identified for upregulated genes included nervous system development (159 genes, including STMN2, DCX, BDNF and NAV1 in gene sets 5 and 6), cell communication (239 genes, including CXXC4, PSEN2 and SSTR2 in gene set 4) and transcription (191 genes, including RUNX1, ZNF238 and NR2F1 in gene sets 3 and 6). Supplementary Table 5B displays the GO results for all gene sets.

**OTX2 silencing blocks G1-S cell cycle transition**

As OTX2 silencing resulted in a strong G0-G1 growth arrest (Figure 2B) and downregulation of cell cycle genes (Figure 3B), we validated these data for several cell cycle genes at the protein level by Western blot analysis. CCND3 (Cyclin D3) is required for G1-S cell cycle transition and was indeed strongly downregulated, whereas CDKN1A (P21), an inhibitor of G1 cell cycle progression was upregulated after OTX2 silencing, confirming their regulation at mRNA level (Figure 1B-C and Supplementary Figure S1A). Interestingly, MYC (c-MYC), highly expressed in D425 cells and important for G1-S transition was also strongly downregulated at the protein level, even much stronger than observed at mRNA level. At 96 hr after OTX2 silencing MYC protein almost completely disappeared, whereas MYC mRNA levels were decreased but remained high (Figure 1B-C and Supplementary Figure S1). Finally, we analyzed the phosphorylation status of RB1, a marker for G1-S transition. RB1 phosphorylation indeed decreased after OTX2 silencing, whereas levels of the nonphosphorylated form of RB1 increased over time (Figure 1B and Supplementary Figure S1A). Protein analyses thus confirm the G0-G1 arrest induced by OTX2 silencing.

**Differentiation after OTX2 silencing resembles granule cell development**

OTX2 is expressed in proliferating progenitor cells of the external granule cell layer (EGL)
during normal cerebellum development. The expression attenuates when cells become postmitotic and start their migration toward the internal granule cell layer [5]. OTX2 expression is completely gone when granule cells are fully differentiated. These data suggest that OTX2-positive tumors may arise from EGL cells. Accordingly, D425 cells express several markers for granule cell lineage, including ZSCAN21 (ZIPRO), ZIC1, MEIS1 and PDE1C (Supplementary Figure S6) [19, 20]. In contrast, markers for Purkinje cells, like LHX1, LHX5, CALB1 or GAD1 or markers for Bergmann glia, like GFAP or FABP7, are not expressed or only at very low levels. We therefore wondered how expression profiles of normal D425 cells and D425 cells differentiated by OTX2 silencing, compare with developing granule cells.

Expression profiles of differentiation stages of EGL cells in vivo have not been published. However, many genes have been identified, both on mRNA and protein level, that are markers for EGL development in vivo [19-22]. We used these genes as surrogate markers to compare the D425 cell line model with normal cerebellum development. Genes associated with proliferation of progenitor cells in the EGL, such as NOTCH2, HMGB2, UHRF1, FOXM1, RELN and PROM1 are expressed in D425 cells, but their expression decreased after OTX2 silencing (Figure 4 and Supplementary Figure S1B-C) [21, 23]. Genes associated with later stages of granule cell development and neuronal differentiation increased their expression after OTX2 silencing. NEUROD1, BDNF, NR2F1, NFIA, MAP2, CNTN2 (TAG1), GPR56, SEMA6A, NHLH, THBS3, SPARC, SSTR2, SLC17A7, L1CAM and GRIA2, for example, were all upregulated (Figure 4 and Supplementary Figure S1B-C) [21, 22, 24-26]. Increased immunofluorescent staining of cells for neuronal markers MAP2, NeuN and SYN1 indeed confirmed the observed neuronal differentiation after OTX2 silencing (Figure 4D and Supplementary Figure S1C). Altogether, these data suggest that expression profiles of D425 cells, differentiated after OTX2 silencing, mimic normal granule cell development. Moreover, the data strongly suggest that OTX2-positive medulloblastoma arise from the EGL and that OTX2 functions to maintain the proliferative state of these cells and prevent their premature differentiation.

Chromatin immunoprecipitation identifies cell cycle genes as direct targets of OTX2

To discriminate among the regulated genes between direct and indirect targets of OTX2, we performed chromatin immunoprecipitation (ChIP) in D425 cells with an OTX2 antibody and hybridized the precipitated DNA to Nimblegen promoter arrays (ChIP-on-chip analysis). Control ChIP-on-chip experiments were performed either with a FLAG antibody or without antibody. qPCR was performed to validate binding (Supplementary Table 6). To assess the specificity of the ChIP-on-chip experiments, we performed de novo motif finding on all genomic sequences bound by OTX2 using the discriminating motif enumerator (DME) analysis tool [18]. These analyses identified TAATCC and TAATCC-related motifs as the most
Figure 4. Genes regulated after OTX2 silencing involved in granule cell layer development. (A) XY plot shows the average expression values of regulated genes at 0 and 96 hr after OTX2 silencing. Genes associated with granule progenitor cells were downregulated in time (purple), whereas genes associated with later stages of granule cell development increased their expression (black). (B) mRNA expression data represented for all three knockdown experiments (blue lines) and the control (black line) are shown for NOTCH2, NEUROD1, SPARC and MAP2. (C) Western blot analyses of D425 cells treated with or without DOX confirm the regulation of NOTCH2, NEUROD1 and SPARC proteins after OTX2 silencing. (D) Immunofluorescence analyses. Panels to the left show parental cells without OTX2 silencing. These cells are round with little cytoplasm (phalloidin in green) surrounding the nucleus (DAPI in blue). After OTX2 silencing (panels in the middle and to the right), cells displayed neurite-like outgrowths (phalloidin in green) and showed increased expression of the neuronal markers MAP2 (red), NeuN (green) and SYN1 (green).
Figure 5. Regulated genes are enriched for OTX2 binding and show more often a correlation in gene expression with OTX2 in primary tumors. (A) De novo motif finding on all genomic sequences bound by OTX2 identified TAATCC and TAATCC related motifs. (B) Up- and downregulated genes after OTX2 silencing are enriched for OTX2 binding near their transcription start sites in comparison to all other genes. Significant changes were tested in a Pearson’s chi-square test and a significance of p < 0.001 is depicted with **. (C) Gene ontology analyses on the OTX2 regulated genes with and without OTX2-binding. (D) Downregulated genes show more often a positive correlation in gene expression with OTX2 in tumors, whereas upregulated genes show more often a negative correlation in gene expression with OTX2 in tumors when compared to nonregulated genes. Significant changes were tested in a Pearson’s chi square test and a significance of p < 0.001 is depicted with **.
enriched sequences, present in 62.6% of all binding peaks (Figure 5A). These motifs form the core of all published OTX2-binding sequences and thus confirm the specificity of the OTX2 ChIP-on-chip experiment.

Genome wide analyses identified one or more significant OTX2-binding peaks in the region around the transcriptional start site (TSS) for 33.1% of all genes present on the Nimblegen array. This percentage is comparable to what has been found for other transcription factors, like TCF4, FOXA2, or E2F [27-29]. However, the frequency of OTX2 binding was significantly higher for genes regulated after OTX2 silencing (p < 0.001 in Pearson’s chi-square test). Of the 2,202 regulated genes, 2,103 were represented on the Nimblegen array and 44.5% of them bound OTX2 in the TSS region (Supplementary Table 3). The highest frequency of OTX2-binding (47.8%) was found for downregulated genes. Upregulated genes showed a lower frequency of OTX2-binding to the promoter regions (40.9%) (Figure 5B).

To discriminate between biological processes that are directly or indirectly controlled by OTX2, we performed GO analysis for the regulated genes with or without OTX2 binding. The most striking finding was that OTX2 bound to many of the cell cycle genes that showed a reduced expression after OTX2 silencing (Figure 5C and Supplementary Table 7A). This suggests that these cell cycle genes form an important group of genes directly induced by OTX2. Other biological processes enriched among the genes bound by OTX2 and with an abrogated expression after OTX2 silencing included chromosome segregation, DNA replication and visual perception. Genes downregulated after OTX2 silencing and without OTX2 binding were not enriched for any GO category. Genes upregulated after OTX2 silencing and with OTX2 binding were enriched for genes involved in regulation of gene expression. Interestingly, many genes involved in nervous system development, cell communication, or cell adhesion, upregulated after OTX2 silencing, did not bind OTX2. These analyses suggest that OTX2 directly controls cell cycle progression in medulloblastoma, but that differentiation-associated genes are indirectly regulated by OTX2.

**OTX2 pathway genes may provide candidate drug targets**

Our results show that OTX2 is essential for medulloblastoma cell proliferation and suppression of differentiation. Direct targets of OTX2 may have an important role in mediating this function. As OTX2 is a transcription factor and thereby a difficult target for therapy, downstream targets may represent better therapeutic targets. Silencing of OTX2 in cell lines and ChIP-on-chip analyses represents one of the few methods to dissect the OTX2 pathway in human cells. However, the genes identified in this *in vitro* system do not necessarily have a similar regulation in medulloblastoma *in vivo*. To test which genes are also
in vivo functioning in the OTX2 pathway, we analyzed whether their expression correlated with OTX2 in a series of primary tumors. The percentage of genes downregulated after OTX2 silencing that did show a positive correlation with OTX2 expression in primary tumors was almost twice as high (13.4%) as that of the genes not regulated (7.4%) or upregulated (7.7%). The same was true for genes upregulated after OTX2 silencing. Expression of these genes more often negatively correlated with OTX2 expression (12.1%) compared to nonregulated (6.0%) or downregulated genes (5.8%) (Figure 5C and Supplementary Table 7B).

To identify the most promising downstream targets of OTX2 for therapy, we selected for OTX2-bound genes downregulated after OTX2 silencing with a positive correlation with OTX2 in tumors (Figure 6A and Supplementary Tables 8 and 9). This stringent selection identified 69 genes (Supplementary Table 8B). Many important cell cycle genes and genes involved in chromosome segregation, including several drug targets, were present in this list (for example CDKN3, CENPA, CCNB1, MELK, C13orf34 (BORA), HMGB2, MSH2, CDC7 and HAT1). The list included also several genes involved in visual perception like RP1, RBP3, TULP1, ABCA4 and GNGT2 (Tables 1B and 1C). Figure 6B shows for four examples (RP1, ABCA4, CDKN3 and HMGB2) how these genes are regulated after OTX2 silencing, how OTX2 binds in their promoter regions, and how their expression correlates with the expression of OTX2 in primary tumors.

These selected genes seem to be valid targets of OTX2, as they were also bound by OTX2 in MED8A and DAOY cells with induced OTX2 (Supplementary Figure S7) [13]. Furthermore, most of these genes are, like OTX2, highly overexpressed in medulloblastoma in comparison to normal cerebellum or other normal brain tissues (data not shown), suggesting that they might be important for tumorigenesis. Identification of these downstream targets of OTX2 may help to develop new therapeutic strategies in the treatment of medulloblastoma patients.

Discussion

In this article we show that silencing of OTX2 in medulloblastoma cells not only inhibits cell proliferation, in line with previous data [7, 8], but also induces neuronal differentiation. Moreover, expression profiling performed in time after OTX2 knockdown, together with ChIP-on-chip analyses showed that OTX2 directly controls the expression of many cell cycle genes, especially those involved in mitosis. Expression of differentiation genes, strongly
Identification of cell cycle genes as direct targets of OTX2 is in line with our previous data from other cell lines with inducible OTX2 overexpression [13]. Indeed, many cell cycle genes, including *AURKA*, *CENPE* and *CDC25C*, were upregulated in these cell lines shortly after the OTX2 induction [13]. Moreover, 43% of genes regulated and bound by OTX2 in both cell systems (OTX2 induction and OTX2 silencing) are cell cycle genes (Supplementary Figure S8).

In general, most of the downregulated genes with direct OTX2 binding in D425 cells were also bound by OTX2 in MED8A and DAOY cells (Supplementary Figure S7). However, not all these genes were upregulated in the overexpression models. For instance, retinal genes like *RP1*, *RBP3* or *ABCA4* remained unexpressed in MED8A cells after OTX2 induction. A reason for this could be that cofactors, necessary to regulate the expression of these genes together with OTX2, are not expressed in these cells [30].

Recently, Adamson *et al.* suggested upregulation of MYC by OTX2 as a potential mechanism how OTX2 may promote proliferation [7]. Indeed, MYC protein levels strongly decrease after OTX2 silencing in D425 cells, but interestingly, in our study we observed that the decrease at the mRNA level was only limited (Figure 1B and Supplementary Figure S1). Although these results do not exclude a role for OTX2 in the transcriptional control of MYC, they strongly suggest that OTX2 might be more important in controlling the protein levels of this key oncogene. As many OTX2-positive medulloblastomas do not express MYC [9], upregulation of MYC by OTX2 might not represent a general mechanism in the control of cell proliferation by OTX2.

OTX2 as a driver of proliferation fits in the context of its role in cerebellum development. OTX2 is highly expressed in the proliferating granule progenitor cells (GPCs) in the external granule layer of the cerebellum, which is considered as the cell of origin for medulloblastoma.
When these GPCs migrate inward to form the internal granule layer, OTX2 levels decreased [5]. Moreover, conditional Otx2 knock-out mice showed that lowering OTX2 levels in GPCs decreased proliferation of these cells resulting in an abnormal development of the cerebellum [4, 32]. Thus, as OTX2 is required for proliferation of progenitor cells, maintaining high levels of OTX2 could be a critical step in medulloblastoma pathogenesis. The neuronal differentiation observed after OTX2 silencing in D425 cells is reminiscent to granule cell maturation. Moreover, expression profiling showed downregulation of genes associated with proliferating progenitor cells and upregulation of genes associated with granule cell differentiation.

Differentiation induced after OTX2 silencing might either be directly regulated by OTX2 or might be an indirect consequence of the decreased proliferation. The promoter regions of most neuronal differentiation genes, upregulated after OTX2 silencing, were not bound by OTX2, indicating that they are not direct targets of OTX2. However, the transcription factors NEUROD1 and NR2F1, involved in regulation of neuronal differentiation, are upregulated already early after OTX2 silencing and are direct targets of OTX2, as ChIP-on-chip experiments showed OTX2 binding to their promoters [21]. Therefore, OTX2 might control differentiation through regulating the expression of these transcription factors.

An interesting group of direct OTX2 targets are genes involved in visual perception. OTX2 plays an important role in normal retinal development by regulating many of the photoreceptor-specific genes [2, 33, 34]. Expression of retinal genes in medulloblastoma has been reported as early as 1987, but their role in cerebellum or medulloblastoma development remains unclear. However, we recently showed that about half of the OTX2 expressing tumors (subtypes D and E) are marked by expression of these photoreceptor genes [9].

As OTX2 is expressed in most medulloblastomas, but not in normal brain and other normal tissues, it provides an interesting target for therapy. Silencing of OTX2 in mice grafted with D425 cells, significantly prolonged survival of these animals [7]. Retinoic acid has been shown to inhibit the proliferation of OTX2 expressing cell lines [1, 8, 35]. As a result OTX2 expression is reduced and cells differentiate, similar to what we observe in D425 cells after OTX2 silencing by shRNAs. However, retinoic acid does not only target OTX2 and therefore more specific drugs are required for the optimal treatment of medulloblastoma patients. Downstream targets of OTX2, including several mitotic cell cycle genes, like AURKA, MELK or CENPE might be good candidates for therapy [36-38]. Many antimitotic drugs have been developed and investigated in clinical trials for various human malignancies, with an AURKA inhibitor already been shown to be effective in medulloblastoma xenografts [39, 40]. Some
of these drugs might be explored for novel therapeutic strategies for OTX2 expressing tumors, which comprise 80% of all medulloblastoma.

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Note: Supplementary Tables for this chapter are available online at International Journal of Cancer.
Reference List


Supplementary Figure S1. Confirmation of protein level changes in D425-shOTX2 cells with induction of shRNA2.

(A) Similar to cells with induced shRNA1 (Figure 1B), OTX2 protein levels start to decrease within 16 hr after adding doxycycline (DOX). Regulation of cell cycle genes CDKN1A, CCND3, MYC, and RB after OTX2 silencing was also similar to the experiment with induced shRNA1. (B) In time after induction of shRNA2, NOTCH2 protein levels decreased while NEUROD1 and SPARC protein levels increased, as they did for shRNA 1 (Figure 3C and 4C). (C) Neuronal markers MAP2, NeuN and SYN1 showed increased staining after OTX2 silencing (Fig. 4D).
Supplementary Figure S2. Triplicates of cell cycle distribution analyses by FACS for D425-shOTX2 cells with either induction of shRNA1 or shRNA2 or for D425-control cells. Analyses were performed at 48 hr after adding doxycycline.
Supplementary Figure S3. Silencing of OTX2 does not result in increased apoptosis. Western blot analysis of cleaved PARP and CASP3 for D425-shOTX2 shRNA1 shows no significant increase in apoptosis when OTX2 is silenced.

Supplementary Figure S4. Similar to D425-shOTX2 shRNA1 (Figure 2D), cells with shRNA2 showed neuronal differentiation phenotype at 72 hr after adding doxycycline.
Supplementary Figure S5. Regulated genes after OTX2 silencing. Heat map of k-mean clustering for all regulated genes. For these genes z-scores were calculated separately for the three independent OTX2 silencing experiments and control.
Supplementary Figure S6. D425 cells express markers for granule cell lineage, but not markers for Purkinje cells Bergmann glia. Depicted expression in based on time point zero of the three experiments and the control.
Supplementary Figure S7. Examples of OTX2 binding to candidate direct targets in other ChIP-on-chip experiments. For 15 genes from Supplementary Table S7B, which were all downregulated after OTX2 silencing and bound by OTX2 and which showed a positive correlation in gene expression with OTX2 in tumors, the ChIP-on-chip data are depicted from other experiments. In all OTX2 ChIP experiments binding signals are detected on the same location as in D425, whereas the controls do not show these peak signals. OTX2_1 and D425_control_1 represent data from an unpublished individual D425 experiment, which was hybridized to an older array version of Nimblegen. D425_OTX2_2 and D425_control_2 represent the data acquired in this paper. The data for MED8A and DAOY cells were described in Bunt et al., 2010 [14].
Supplementary Figure S8. Cell cycle genes as direct targets of OTX2 in both silencing (D425-shOTX2) and overexpression (MED8A-OTX2 and DAOY-OTX2) models. For 6 cell cycle genes, ChIP-on-chip data and expression data are shown. The data for MED8A and DAOY cells were described in Bunt et al., 2010 [14].