Regulation of Cell Cycle Genes and Induction of Senescence by Overexpression of OTX2 in Medulloblastoma Cell Lines

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Abstract

The transcription factor orthodenticle homeobox 2 (OTX2) has been implicated in the pathogenesis of medulloblastoma, as it is often highly expressed and sometimes amplified in these tumors. Little is known of the downstream pathways regulated by OTX2. We therefore generated MED8A and DAOY medulloblastoma cell lines with doxycycline-inducible OTX2 expression. In both cell lines, OTX2 inhibited proliferation and induced a senescence-like phenotype with senescence-associated β-galactosidase activity. Expression profiles of time series after OTX2 induction in MED8A showed early upregulation of cell cycle genes related to the G2-M phase, such as AURKA, CDC25C, and CCNG2. Paradoxically, G1-S phase genes such as MYC, CDK4, CDK6, CCND1, and CCND2 were strongly downregulated, in line with the observed G1 arrest. ChIP-on-chip analyses of OTX2 binding to promoter regions in MED8A and DAOY showed a strong enrichment for binding to the G2-M genes, suggesting a direct activation. Their mRNA expression correlated with OTX2 expression in primary tumors, underscoring the in vivo relevance of this regulation. OTX2 induction activated the P53 pathway in MED8A, but not in DAOY, which carries a mutated P53 gene. In DAOY cells, senescence-associated secretory factors, such as interleukin-6 and insulin-like growth factor binding protein 7, were strongly upregulated after OTX2 induction. We hypothesize that the imbalance in cell cycle stimulation by OTX2 leads to cellular senescence either by activating the P53 pathway or through the induction of secretory factors. Our data indicate that OTX2 directly induces a series of cell cycle genes but requires cooperating genes for an oncogenic acceleration of the cell cycle. Mol Cancer Res; 8(10); 1344–57. ©2010 AACR.

Introduction

Medulloblastoma is the most common malignant brain tumor in children and accounts for ~10% of all childhood cancer deaths. Although overall survival rates are improving in recent years, current therapies are still associated with serious long-term side effects, including many cognitive defects. A better understanding of the molecular biology of medulloblastomas is needed to find novel treatment modalities and improve overall survival and quality of life.

One of the genes implicated in medulloblastoma tumorigenesis is orthodenticle homeobox 2 (OTX2). SAGE analyses initially identified OTX2 as highly expressed in medulloblastoma (1). Subsequently, OTX2 was also found to be strongly amplified in a set of primary tumors and cell lines, suggesting that this gene acts as an oncogene in medulloblastoma (2-4). The OTX2 gene encodes a member of the bicoid subfamily of homeodomain-containing transcription factors that is essential in brain and sensory organ development (5-7). Mouse models have shown that deletion of both Otx2 alleles is lethal, whereas decreased levels of Otx2 result in serious malformations of the brain, including cerebellum (5, 8). During cerebellum development, OTX2 is expressed in proliferating progenitor cells in the external granular cell layer, but expression disappears when these cells migrate to the internal granular cell layer and become fully differentiated (3). No OTX2 was detected in postnatal cerebellum. However, immunohistochemical staining of 152 primary tumors showed OTX2 protein expression in 114 (75%) of all medulloblastomas (3). The OTX2 expression strongly correlated with a classic histology, whereas desmoplastic histology mainly occurred in tumors without OTX2 expression. We recently generated mRNA profiles of 62 medulloblastomas (9). Five molecular subtypes of medulloblastoma were identified with distinct genetic profiles, pathway signatures, and clinicopathologic features. OTX2 was highly expressed in four subtypes (A, C, D, and E), but is not or only weakly expressed in subtype B. These type B tumors frequently showed desmoplastic histology.

The biological role of OTX2 in these different molecular subtypes of medulloblastoma is still unknown. Silencing OTX2 expression in medulloblastoma cell lines reduced cell proliferation and tumor formation (4, 10). These data suggest
a crucial role for OTX2 in medulloblastoma, but the transcriptional targets and pathways controlled by OTX2 remain unknown. To investigate OTX2 and downstream pathways, we made use of the MED8A and DA0Y medulloblastoma cell lines, which lack endogenous OTX2 expression. Clones with doxycycline-inducible ectopic OTX2 expression were generated. OTX2 overexpression resulted in a reduced cell proliferation and a senescent phenotype. To identify the transcriptional network of OTX2 that may explain the phenotype of OTX2-induced senescence, we performed mRNA profiling of a time series after OTX2 induction in MED8A cells as well as OTX2 chromatin immunoprecipitation followed by promoter array analysis (ChIP-on-chip). Data were validated in DA0Y cells with OTX2 induction. Finally, we combined all data with the tumor mRNA profiles to validate the functional OTX2 targets in medulloblastoma tumors.

Materials and Methods

Cell lines, constructs, and transfection procedures
Medulloblastoma cell lines were cultured in DMEM (Richter’s modification) (MED8A, U228-2, D458, D556, and D283) or MEM (DAOY, D425, and D341; Invitrogen), supplemented with 10% fetal bovine serum, 1% NaN3, and 0.5 μg/mL streptomycin (Invitrogen) at 37°C in a humified atmosphere containing 5% CO2. To generate OTX2-inducible clones of the MED8A and DA0Y medulloblastoma cell lines, cells were first transfected and plated in selective medium containing 1 or 4 μg/mL blasticidin (Invitrogen) for DAOY and MED8A, respectively. Surviving clones were tested for high expression of the Tet repressor.

An inducible expression construct of OTX2 was made by cloning the coding region of OTX2 (NM_172337) into the pcDNA4/TO/myc-HisA plasmid (Invitrogen). Sequence analysis verified the correct sequence of the construct. This pcDNA4/TO/OTX2 or the pcDNA4/TO/myc-HisA vector was transfected into MED8A and DA0Y clones expressing the Tet repressor. Transfected cells were cultured in selective medium containing 0.1× PBS containing 7.5 μg/mL zeocin (Invitrogen) for DAOY and MED8A, respectively. Surviving clones were tested for high expression of the Tet repressor.

Western blotting and antibodies
For preparation of protein extracts, ice-cold buffer containing 10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, complete protease inhibitor (Roche Applied Sciences), 0.5 μmol/L NaF, and 0.5 μmol/L Na3VO4 was added to the plates. Plates were incubated for 10 minutes on ice. DNA was sheared and lysates were cleared by centrifugation. For cytoplasmic and nuclear fractions, the ProteoExtract Subcellular Proteome Extraction Kit (EMD Chemicals Inc.) was used.

Protein was quantified using the detergent-compatible protein assay (Bio-Rad). SDS-PAGE gels were run with 5 to 30 μg of protein and electroblotted onto an Immobilon-P membrane (Millipore, Billerica). Blocking and incubation were done in 1× TBS with 0.05% Tween 20 and 5% dry milk using standard procedures. OTX2 mouse monoclonal antibody was a gift from Dr. G. Corte (Department of Translational Oncology, National Institute for Cancer Research, Genova, Italy). Other antibodies were commercially obtained: glyceraldehyde-3-phosphate dehydrogenase, phospho-53, and phospho-extracellular signal–regulated kinase (ERK)-3 (Cell Signaling Technology); histone H3 (Upstate Cell Signaling Solutions); MYC (Roche Applied Sciences); TUBA (Sigma); RB (BD Biosciences); CCND2, CDK4, CDK6, and p-RB (Santa Cruz Biotechnology); CCND1, P16, P21, P27, and P53 (Neomarkers); interleukin-6 (IL-6) and insulin-like growth factor binding protein 7 (IGFBP7; Abcam); and SPRY2 (Abnova). After incubation with a secondary sheep anti-mouse or anti-rabbit horseradish peroxidase–linked antibody (GE Healthcare), proteins were visualized by enhanced chemiluminescence (GE Healthcare).

Growth assay
Uninduced cells and 72-hour induced cells were plated in 96-well microplates. After 4 days, cell viability was measured by adding 25 μL of 3 mg/mL MTT (Sigma) and incubating for 2 to 4 hours at 37°C. Medium was discarded and the remaining precipitate was solubilized by the addition of 50 μL of DMSO. Subsequently, absorbance was measured at 570 and 650 nm (reference) on a Bio-Rad 3550 microplate reader equipped with a spectrophotometer (Bio-Rad). Cell viability was expressed as relative signal compared with uninduced cells, after correction for background absorbance.

Fluorescence-activated cell sorting analysis
For quantification of cell cycle distribution, 100 ng/mL doxycycline was added to cells 24 hours after plating. After 72 hours of induction, cells were trypsinized and washed. Pellet was resuspended in 0.1× PBS containing 7.5 μmol/L propidium iodide and 50 μg/mL RNase A. After minimal incubation time of 1 hour, cells were analyzed on a BD FACSCanto flow cytometer (BD Bioscience). Obtained data were analyzed using FlowJo 7.2 software (Tree Star, Inc.).

Senescence-associated β-galactosidase staining
To assess senescence-associated β-galactosidase activity, cells were washed with 1× PBS and fixed by incubation in 3% paraformaldehyde. After subsequent washing with 1× PBS, cells were incubated overnight in a 37°C incubator with X-gal staining solution (1 mg/mL X-gal (Invitrogen), 150 mmol/L NaCl, 2 mmol/L MgCl2, 5 mmol/L K4Fe(CN)6, 5 mmol/L K3Fe(CN)6 in phosphate buffer (pH 6)). Pictures were taken using a DMLB microscope with a DC300 camera and IM 500 software (Leica).

RNA extraction and expression profiling
For expression profiling, total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's
instructions. RNA was purified using the RNeasy Mini Kit (Qiagen). RNA quantity and quality were determined by spectrophotometry (NanoDrop) and microfluidics-based electrophoresis (Agilent 2100 Bioanalyzer, Agilent). For Affymetrix microarray analyses, fragmentation of RNA, hybridization to HG U133 Plus 2.0 Array, and scanning were all carried out according to the manufacturer’s instructions (Affymetrix, Inc.) at the Microarray Department of the Swammerdam Institute of Life Science of the University of Amsterdam.

Data analyses

All expression data were normalized with the MAS5.0 algorithm of 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 $96 = 100$). In case of multiple probe sets for one gene, the probe set with the highest expression and correct mapping was used. Publicly available HG U133 Plus 2.0 microarray expression data were obtained from the National Cancer Institute Gene Expression Omnibus database: GSE5326 contains 355 normal tissue samples from the whole body [central nervous system (CNS) and non-CNS; ref. 11]. GSE4290 (12) contains 180 samples derived from normal brain and brain tumor samples (glioblastoma, astrocytoma, and oligodendrogloma). We used only the data for the 153 tumor samples in this series, which were clearly annotated. GSM97826, GSM97836, GSM97858, and GSM97879 were therefore left out. GSE7307 contains annotated. GSM97826, GSM97836, GSM97858, and GSM97879 were therefore left out. GSE7307 contains 153 tumor samples in this series, which were clearly annotated. GSE97826, GSM97836, GSM97858, and GSM97879 were therefore left out. GSE7307 contains 153 tumor samples in this series, which were clearly annotated. GSE97826, GSM97836, GSM97858, and GSM97879 were therefore left out. GSE7307 contains 153 tumor samples in this series, which were clearly annotated. GSE97826, GSM97836, GSM97858, and GSM97879 were therefore left out. GSE7307 contains 153 tumor samples in this series, which were clearly annotated. GSE7307 contains 153 tumor samples in this series, which were clearly annotated.

All analyses were done using the in-house developed software, called R2 (http://r2.amc.nl).1

For the MED8A-OTX2 time course, the following criteria applied for genes to be considered significantly regulated. First, the probe set should have minimally 1 present call within the experiment. Second, at least one expression value should exceed an expression value of 50. Finally, compared with $T = 0$, a minimum fold change of 0.5 $\log$ with $P < 0.00005$ is required. K-means clustering was done using the TMEV program (13). Genes were annotated using the Functional Annotation Tool of DAVID (http://david.abcc.ncifcrf.gov/; ref. 14). For Gene Ontology enrichment analyses, we used all expressed genes within the experiment as background in these analyses. Enrichment was considered when the enrichment score for levels 4 and 5 in biological processes was higher than 2.5.

To identify genes that correlate with OTX2 expression in vivo, we used the mRNA profiling data of primary tumors (9). Only genes with a minimum of 1 present call were included. Data were extracted using R2 software.1

Luciferase assay

To monitor P53 transcriptional activity after OTX2 induction, MED8A-OTX2 and MED8A-control cells were transfected in a 24-well plate with 125 ng of pG13-luc or pM15-luc (kindly provided by Bert Vogelstein, Ludwig Center for Cancer Genetics and Therapeutics, Johns Hopkins Kimmel Cancer Center, Baltimore, MD) using Fugene HD (Roche Applied Sciences; ref. 15). As a normalization control, 20 ng of pGL4.74 (Promega) were included. Luciferase activity was monitored using the dual-luciferase reporter assay kit (Promega) according to the manufacturer’s protocol using the Synergy HT Multi-Mode Microplate Reader (BioTek).

P53 mutational analysis

Exons 5 to 8 of P53 were sequenced using BigDye Terminator v1.1 chemistry (Applied Biosystems; Supplementary Table S7). Sequencing was done on an ABI 3730 capillary sequencer (Applied Biosystems). Electropherograms were analyzed using Codon Code aligner.

ChIP-on-chip analysis

MED8A-OTX2 and DAOY-OTX2 cells incubated for 48 hours with (to induce OTX2) or without doxycycline were cross-linked with 1% formaldehyde for 10 minutes. After washing, cells were incubated for 5 minutes in swelling buffer (5 mmol/L PIPES, 85 mmol/L KCl, 0.5% NP40) and passed through a 23-gauge needle. Isolated nuclei were lysed for 10 minutes in 1 mol/L Tris-HCl/1% SDS/0.5 mol/L EDTA (pH 8) on ice. Lysates were sonicated on ice for 7 × 25 seconds at 30 mA. Three milliliters of sample were diluted 1:10 in 1% Triton X-100/150 mmol/L NaCl/50 mmol/L Tris-HCl/2 mmol/L EDTA and cleared for 30 minutes with 40 µL of protein A-agarose (Roche) and 125 µL of 10 mg/mL haring sperm DNA (Roche). Thirty microliters of OTX2 antibody (Millipore) with 40 µL of beads were added to cleared samples and tumbled overnight in a cold room. The next day, the beads were sequentially washed with 0.1% SDS/1% Triton X-100/5 mol/L NaCl/20 mmol/L Tris-HCl/2 mmol/L EDTA; with the same solution with 500 mmol/L NaCl; with 1% deoxycholate, 1% NP40/250 mmol/L LiCl/10 mmol/L Tris-HCl/2 mmol/L EDTA; and finally with 10 µL of 10 mg/mL DNA (Roche). Thirty microliters of OTX2 antibody (Millipore) with 40 µL of beads were added to cleared samples and tumbled overnight in a cold room. The next day, the beads were sequentially washed with 0.1% SDS/1% Triton X-100/5 mol/L NaCl/20 mmol/L Tris-HCl/2 mmol/L EDTA; with the same solution with 500 mmol/L NaCl; with 1% deoxycholate, 1% NP40/250 mmol/L LiCl/10 mmol/L Tris-HCl/2 mmol/L EDTA; and finally with 10 µL of 10 mg/mL DNA (Roche). Thirty microliters of OTX2 antibody (Millipore) with 40 µL of beads were added to cleared samples and tumbled overnight in a cold room. The next day, the beads were sequentially washed.

The recovered DNA was amplified for labeling as described previously (16). Labeling of the material, hybridization to the 2.1M Deluxe Promoter Array, scanning of the arrays, and peak calling were performed by Nimblegen, Inc. All peaks as called by the Nimblegen algorithm were assigned to transcriptional start sites (TSS). Peaks were regarded as unique when no peak was detected within a region surrounding a TSS in the control experiment. Raw data were visualized using the R2 program.1

1 J. Koster, personal communication.

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Results

**OTX2 expression is specific for medulloblastoma**

Analysis of mRNA expression data showed that OTX2 is highly expressed in medulloblastoma, but not in other brain or non-brain tumors or normal tissues (Fig. 1A). Expression profiling of medulloblastoma cell lines showed that five cell lines (D283, D341, D425, D458, and D556) also highly expressed OTX2, comparable to the levels observed in tumors (Fig. 1A). Most likely, these cell lines were derived from type E tumors, as they have gain of 17q, expression of MYC and retinal genes, and no CTNNB1 mutations (9, 17). Three cell lines (UW228-2, MED8A, and DAOY) without OTX2 expression were most likely derived from type B tumors. For instance, they all lack 17q gain, lost 9q (MED8A; ref. 17), were derived from a desmoplastic tumor (DAOY; ref. 18), and all have low *NEUROG1* expression.

**FIGURE 1. OTX2 expression in vivo and in vitro.** A, OTX2 mRNA expression in medulloblastoma compared with expression data from 8 medulloblastoma cell lines, 2,207 other tumor samples, and 857 normal tissue samples (see Materials and Methods for references). Similar to tumors, these medulloblastoma cell lines have either very low expression levels (UW228-2, MED8A, and DAOY) or very high expression levels (D458, D425, D556, D341, and D283) of OTX2. All expression values represent MAS5.0 normalized data for probe set 242128_at of the Affymetrix HG U133 Plus 2.0 Array. B, OTX2 expression in the nuclei of MED8A-OTX2 and DAOY-OTX2 cells. After addition of 100 ng/mL doxycycline (DOX), OTX2 expression is induced. Within 4 h, in both MED8A-OTX2 and DAOY-OTX2 cells, OTX2 protein can be detected. Within 16 h, OTX2 expression reaches a maximum. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. C, OTX2 expression on Western blot for both cell lines was compared with the endogenous OTX2 protein level of D425 medulloblastoma cell line in the cytoplasm and nucleus. As in the case of D425, OTX2 is only localized in the nucleus of MED8A-OTX2 and DAOY-OTX2 24 h after induction. Also, expression levels are comparable. Histone H3 and TUBA were used as loading controls.
Overexpression of OTX2 in MED8A and DAOY medulloblastoma cell lines

We selected the MED8A and DAOY cell lines (18, 19), which have no endogenous OTX2 expression, to investigate the effect of OTX2. We generated two clones, MED8A-OTX2 and DAOY-OTX2, which have doxycycline-inducible OTX2 expression (Fig. 1B). No OTX2 protein expression can be detected in uninduced cells (Fig. 1B) or MED8A and DAOY parental cells (data not shown). OTX2 protein was expressed within 4 hours after addition of doxycycline and reached maximum levels within 24 hours. The induced levels of OTX2 were comparable to the endogenous OTX2 levels in the medulloblastoma cell line D425, which has a strong amplification of the OTX2 gene (Fig. 1C; refs. 2-4). Moreover, in all three cell lines, OTX2 was detected only in the nuclear fraction (Fig. 1C). The two cell lines with inducible OTX2 therefore represent a good model for the analysis of OTX2 at physiologically relevant levels in medulloblastoma.

OTX2 induces a senescence-like phenotype

OTX2 overexpression reduced the proliferation of MED8A and DAOY cells. After 1 week of OTX2 induction, the number of viable cells was reduced by half in both cell lines compared with uninduced cells (Fig. 2A). However, time course analyses of cell proliferation performed early after OTX2 induction showed that up to 24 hours, cell numbers first slightly increased and, after 48 hours, started to decrease (Supplementary Fig. S1). For MED8A, fluorescence-activated cell sorting analyses revealed that the reduced proliferation was accompanied by a significant increase of cells in G1-phase, whereas the S and G2-M fractions decreased (Fig. 2B; Supplementary Fig. S2). In DAOY cells, however, OTX2 induction resulted in a significant increase of cells in G2-M-phase (Fig. 2B). This difference in cell cycle distribution after OTX2 induction may be related to the difference in P53 status. Sequence analysis showed that MED8A cells are wild-type for P53, but DAOY cells harbor a homozygous P53 mutation (20). We found no indications for increased apoptosis induced by OTX2 because in both cell lines the sub-G1 fractions did not increase with time, nor did poly(ADP-ribose) polymerase or caspase-3 cleavage (Supplementary Fig. S3). Furthermore, sustained induction of OTX2 in MED8A or DAOY cells resulted in clear morphologic changes. Cells became flattened and showed an increase in the amount of cytoplasm (Fig. 2C). Also, some multinucleated cells were observed. As these features are characteristic of senescent cells, we stained both cell lines for senescence-associated β-galactosidase. β-Galactosidase activity was detected in cells with OTX2 expression, but not in control cells (Fig. 2D). The presence of this β-galactosidase staining together with the decreased proliferation and change in morphology strongly suggests that OTX2 induces senescence in both medulloblastoma cell lines.

OTX2 induces strong changes in gene expression profile

Because OTX2 is a transcription factor, we were interested in the changes in gene expression after OTX2 induction. A time series analysis of MED8A-OTX2 cells was done using Affymetrix HG U133 Plus 2.0 Arrays. RNA was isolated at 0, 8, 24, and 48 hours after induction of OTX2. Expression of 12,781 genes could be detected in MED8A-OTX2 cells, of which 2,009 were regulated after OTX2 expression (2log-fold regulation >0.5 with P < 0.00005; Supplementary Table S1).

OTX2 first stimulates mitotic genes before cells go into senescence

To identify patterns in gene regulation after OTX2 induction, we performed K-means clustering on all genes regulated over time. Nine clusters with distinct regulation patterns were identified (Fig. 3). Six clusters (I, III, IV, V, VI, and IX) represent early regulated genes, that is, genes that are up- or down-regulated within 8 hours after OTX2 induction. The genes in the other three clusters are regulated at later time points (II, VII, and VIII). Gene Ontology analysis performed for each gene cluster showed enrichments in specific biological processes for several clusters (Supplementary Table S2; ref. 14). Early induction of specific functional groups was detected in clusters I, IV, and IX. Cluster IX was significantly enriched in genes functioning in mitosis and sister chromosome segregation, such as AURKA, CDC25C, CCNG2, CENP4, and CENPE. They were rapidly upregulated after OTX2 induction, but their expression decreased again after 24 hours (Fig. 4A). Clusters I and IV, consisting of early regulated genes, were enriched for genes involved in transcription. The late regulated cluster II was significantly enriched for genes involved in ribosome biogenesis and protein translation. Their downregulation may reflect the observed phenotype of reduced proliferation after induction of OTX2. The other five clusters (III, V, VI, VII, and VIII) showed no clear enrichment for biological processes. Similar results were obtained after K-means clustering of genes regulated with other cutoff levels (data not shown). These analyses show that although the overexpression of OTX2 in MED8A cells ultimately leads to growth arrest and senescence, OTX2 may initially stimulate cell cycle progression by inducing G2-M-related genes. These data are in line with the data for cell proliferation showing that early after OTX2 induction, cell numbers first slightly increased (Supplementary Fig. S1).

OTX2 expression inhibits G1-S cell cycle progression

Most G2-M genes that were induced by OTX2 overexpression in MED8A were highly expressed in all medulloblastomas (Fig. 4A; Supplementary Table S1), suggesting that OTX2 might be responsible for their high expression in tumors. Surprisingly, a group of G1-S-phase–specific genes, such as CCND1, CCND2, CDK4, CDK6, and MYC, were all strongly downregulated by OTX2 (Fig. 4B). Nevertheless, these genes are generally highly expressed in medulloblastoma (Fig. 4B; Supplementary Table S1). Strikingly, G1-S transition inhibitors such as P15 (CDKN2B), P21 (CDKN1A), P27 (CDKN1B), and P57 (CDKN1C) were all upregulated by OTX2 in MED8A (Supplementary Table S1). To validate the
paradoxical regulation of G1-S and G2-M genes, we analyzed protein lysates from time-series experiments of MED8A-OTX2. The levels of CCND1, CCND2, CDK4, CDK6, and MYC proteins were quickly downregulated after OTX2 induction (Fig. 4C). Despite the differences in cell cycle distribution between MED8A and DAOY cells after OTX2 induction, most of these proteins were also downregulated in DAOY cells (Supplementary Fig. S4). Moreover, protein levels of P21 and P27 were increased in MED8A cells. In DAOY cells, however, which are inhibited in G2-M, these proteins were not upregulated. Our results show that induction of OTX2 in MED8A cells inhibits the expression of G1-S-phase genes and, at the same time, stimulates expression of G2-M genes, suggesting that unbalanced regulation of cell cycle progression might cause oncogenic stress in the cells.
OTX2 expression results in P53 pathway activation in MED8A cells

The P53 pathway is responsive to cell cycle stress and is involved in senescence (21, 22). We therefore asked whether this pathway was activated after OTX2 induction in MED8A cells. Gene Ontology analyses (14) performed for the complete set of regulated genes in the time series identified the P53 pathway as most affected by OTX2, with 23 of its genes being regulated (Supplementary Table S2). Moreover, 32 of 129 direct transcriptional targets of P53 known from literature [as tabulated and reviewed by Riley et al. (23)] were up- or down-regulated in the expected direction when OTX2 was induced (Supplementary Table S1; ref. 23). Examples include \( P21 \), \( GADD45A \), and \( BAX \). Western blot analysis confirmed the increased expression of \( P21 \) after OTX2 induction (Fig. 4C). \( P53 \) mRNA levels did not change, but OTX2 induction resulted in increased nuclear levels of \( P53 \) protein. Also, a slight increase in \( P53 \) phosphorylation was observed, which is known to stabilize the \( P53 \) protein (Fig. 5A).

To confirm P53 activation after OTX2 induction, we transfected wild-type and mutant reporter constructs for P53 activity into MED8A-OTX2 and MED8A-control cells. The results plotted in Fig. 5B show an up to 4-fold increase in luciferase activity after OTX2 induction in MED8A-OTX2 cells, but not in MED8A-control cells or in MED8A-OTX2 cells transfected with a mutated reporter construct. These data suggest that P53 activity may play a role in the OTX2-induced senescence in MED8A, comparable to the known role of P53 in senescence induced by other oncogenes (21, 22).

OTX2 expression induces senescence-associated secretory factors in DAOY cells

As \( P53 \) and \( P16 \) (\( CDKN2A \)) are mutated in DAOY cells, the OTX2-induced senescence in these cells must involve other pathways. Senescence-associated secretory factors involved in insulin-like growth factor signaling or inflammation have been shown to play a role in oncogene-induced senescence (24-26). To investigate whether they are involved in the OTX2-induced senescence in DAOY cells, we examined the expression of several candidate secretory factors by Western blot analysis. Indeed, the expression of senescence-associated secretory factors IL-6 (27) and IGFBP7 (28) was strongly increased after OTX2 induction (Fig. 5C). As IL-6 and IGFBP7 signaling have been associated with activated RAS signaling, we also investigated whether RAS signaling was activated after OTX2 induction. Both the levels of phosphorylated ERK and SPRY2, a known target of RAS signaling, were also elevated after OTX2 induction. The levels of phosphorylated RB decreased after OTX2 induction, in line with the observed diminished cell proliferation. These data show that OTX2 induction causes senescence in both medulloblastoma cell lines, but the mechanisms involved are different.

**FIGURE 3.** Patterns of regulation after OTX2 expression. K-means clustering of the 2,009 genes that were significantly regulated after OTX2 induction in MED8A-OTX2 cell line. The genes were clustered into nine groups using the TMEV software (13). Clusters I, III, IV, V, VI, and IX represent early regulated genes. The other three clusters (II, VII, and VIII) contain genes regulated at later times.
Mitotic genes are enriched for OTX2 binding

We performed ChIP-on-chip analysis to investigate which genes and processes were likely to be directly regulated by OTX2. Of all expressed genes, 17.6% had one or more significant OTX2 binding peaks in the region around their transcriptional TSSs (250 bp upstream or downstream), but not in the control experiment where OTX2 was not present. A selection of these binding peaks was...
validated by quantitative PCR (Supplementary Table S4). Figure 6 shows the OTX2 binding patterns as obtained with the ChIP-on-chip analyses for some representative regulated genes. We analyzed whether the percentage of OTX2 binding was enriched among genes up- or downregulated after OTX2 induction. OTX2 binding was significantly enriched among regulated genes (20.7%) and even stronger among upregulated genes (22.2%) when compared with nonregulated genes (17.0%; Fig. 7A). Moreover, analysis of the nine clusters of regulated genes identified by K-means clustering (Fig. 3) showed a strong enrichment of OTX2 binding (31.8%) for genes in cluster IX, which are associated with mitosis and chromosome segregation. Similar results were obtained when we used larger or smaller regions around the TSSs or different cutoffs for OTX2 binding (data not shown). Our data suggest that OTX2 may function as a transcriptional activator and that genes regulating the mitotic cell cycle are among the direct targets of OTX2.

Gene regulation and OTX2 binding compared with the mRNA data of primary tumors

We analyzed whether genes that correlate in expression with OTX2 in tumors were enriched for OTX2 binding. We used the mRNA expression profiles of medulloblastoma tumors (9), but excluded the profiles of type A and type B tumors. Type B tumors, characterized by activated SHH signaling, have no or very low OTX2 expression. Including these tumors would result in many genes that correlate with OTX2 expression but, in fact, represent subtype-specific differences not related to OTX2. Type A tumors, characterized by activated WNT signaling, were excluded because of the very different genetic background compared with other subtypes.

FIGURE 5. Different senescence-associated pathways activated after OTX2 induction. A, cytoplasmic and nuclear fractions were isolated in MED8A-OTX2 24 h after induction. Total nuclear as well as phospho-serine 37 (p-S37) and phospho-serine 46 (p-S46) levels of P53 increased after induction of OTX2. Histone H3 and TUBA were used as loading controls. B, firefly luciferase reporters containing multiple copies of consensus (PG13-Luc) or mutant P53-binding sites (MG15-luc) along with internal control Renilla luciferase reporter were transfected into MED8A-OTX2 or MED8A-control cells. Dual luciferase assays were carried out at different time points. Firefly luciferase activity was normalized against Renilla luciferase activity. The relative luciferase activity of induced cells over uninduced cells was plotted with the basal activity at 0 h set as 1. Only the luciferase activity of PG13-Luc in MED8A-OTX2 cells increased. C, Western blot analyses of senescence-associated secretory factors in DAOY-OTX2 cells after OTX2 induction. Protein levels of IL-6 and IGFBP7 were determined for DAOY-OTX2 at different time points after induction of OTX2. Also, the RAS-ERK pathway was activated after OTX2 induction, as shown by elevated levels of p-ERK and SPRY2. Furthermore, p-RB levels decreased, indicating an impaired G1-S transition. ACTB was used as a loading control.
Of all genes expressed in C, D, and E (CDE) tumors, 17.1% showed binding of OTX2 in MED8A cells (Fig. 7B). Genes that positively correlate with OTX2 expression in CDE tumors were enriched for OTX2 binding, and this enrichment increased to >25% when we used more stringent cutoffs for correlation with OTX2 expression (Fig. 7B). In contrast, genes that negatively correlate with OTX2 expression were not enriched for OTX2 binding.

FIGURE 6. Examples of regulated genes with OTX2 binding and their correlation with OTX2 expression in tumors. CDC40 (A), CCNG2 (B), HBP1 (C), and CDKN3 (D) represent genes upregulated by OTX2 in MED8A-OTX2 (first panel), with direct OTX2 binding near the TSS (second panel), and a corresponding positive correlation with OTX2 in primary medulloblastoma (third panel). All these genes are involved in cell cycle regulation.
FIGURE 7. Relationship between genes regulated by OTX2, OTX2-bound genes, and genes that correlate with OTX2 expression in tumors. A, OTX2-regulated genes in MED8A are enriched for OTX2 binding near TSS. This enrichment is most clear among upregulated genes, especially for genes in cluster IX. B, similarly, genes with increasing positive correlation with OTX2 in primary tumors also show more OTX2 binding in the MED8A-OTX2 ChIP-on-chip experiment. C, upregulated genes in MED8A-OTX2 cells strongly overlap with genes that positively correlate with OTX2 expression in primary tumors. D, the overlap between downregulated genes and genes that negatively correlate with OTX2 expression is much smaller. E, all data sets combined. OTX2 binding near TSS is most prominent in genes upregulated in MED8A-OTX2 and with a positive correlation in primary tumors. F and G, identification of regulated genes that bind OTX2 and correlate with OTX2 expression in tumors ($R \geq 0.2$). *, $P < 0.0001$, $\chi^2$ test with Yates correction.
OTX2 Regulates Cell Cycle Genes and Induces Senescence

and even showed lower percentages of OTX2 binding. We also analyzed the overlap between genes that are regulated by OTX2 in MED8A cells and genes that correlate with OTX2 expression in tumors. The strongest overlap was found for genes that are upregulated by OTX2 in MED8A cells and that positively correlate in tumors (Figs. 6 and 7C and D; Supplementary Table S5). Finally, we combined all parameters (regulation in MED8A; binding by OTX2, and correlation in tumors; Fig. 7E). Genes upregulated in MED8A cells and with a positive correlation with OTX2 in tumors were clearly enriched for OTX2 binding (30.4%). This was not the case for downregulated genes. These data support our previous analyses of gene regulation by OTX2 in MED8A cells, suggesting that, in vivo, OTX2 also mainly functions as a transcriptional activator.

Mitotic genes are among the most promising direct targets of OTX2

To identify which of the regulated genes in MED8A are most likely direct targets of OTX2 in medulloblastoma, we combined all data (Supplementary Table S6). Thirty-one genes were downregulated and bound by OTX2 in MED8A cells, suggesting that, in vivo, OTX2 also mainly functions as a transcriptional activator.

Expression profiling of MED8A cells showed that, in reduced cell proliferation. Both cell lines displayed an oncogene-induced senescence-like phenotype characterized by increased cytoplasm, loss of shape, and senescence-associated β-galactosidase activity. The OTX2-induced senescence explains why, in the past, we failed to generate constitutively OTX2-expressing cell lines of both MED8A and DAOY cells. OTX2-induced senescence was not anticipated, as both cell lines that have no endogenous OTX2 express low levels of OTX1, which encodes a functional homologue of OTX2 (29). Medulloblastoma tumors without OTX2 expression also have OTX1 expression, although in most cases at low levels (3). However, the proliferation of MED8A and DAOY cells was apparently not compatible with high OTX2 levels. Nevertheless, we identified genes regulated and bound by OTX2 in MED8A-OTX2 cells that show a clear correlation with OTX2 expression in tumors (Fig. 7F and G; Supplementary Tables S1 and S6). Many of these genes were also regulated and bound by OTX2 in DAOY cells.

Oncogene-induced senescence was first described in cells with overexpression of constitutively activated RAS genes (30). These oncogenes are able to transform cells, but only in collaboration with other oncogenes such as MYC (31, 32). Similar results have been described for ectopic expression of other components of the RAS pathway, such as RAC1 and RAF (33, 34), as well as other oncogenes, including E2F and STAT5A (35, 36). Although, in most studies, these experiments were conducted in primary fibroblasts or other primary cell lines, oncogene-induced senescence has also been described in tumor cell lines (37, 38). The molecular mechanisms underlying oncogene-induced senescence are complex, but most signals seem to converge on the RB and P53 pathways (22), as observed in MED8A cells. Accordingly, virtually all human cancers lack functional RB and P53 pathways and thereby escape from senescence (39). Alternative mechanisms of oncogene-induced senescence involve the activation of senescence-associated secretory factors (24-26), as observed in DAOY cells after OTX2 induction for IL-6 (27) and IGBP7 (28). Interestingly, these secretory factors were not activated in MED8A cells after OTX2 induction (Supplementary Table S1), most likely because they were suppressed by P53 (40).

Expression profiling of MED8A cells showed that, among many others, mitosis-related genes were initially upregulated, followed by a decrease at later time points (cluster IX of Figs. 3 and 4A). Furthermore, these genes were enriched for OTX2 binding to the TSS region and for positive correlation with OTX2 expression in tumors (Fig. 7A and C), suggesting that they represent direct targets of OTX2. These data further hint at an initial stimulation of cell proliferation. Such an effect has been described for other oncogenes that induce senescence (35, 41, 42). We also observed a slight increase in cell proliferation shortly after OTX2 induction in MED8A and DAOY cells before the onset of senescence and reduced cell proliferation (Supplementary Fig. S1). This initial increase in proliferation in our model is modest,
which might be due to the higher basal proliferation rates of cancer cells relative to the fibroblast cells used in other studies. In addition, already early after OTX2 induction, G1-S cell cycle genes were downregulated (Fig. 4B and C). This imbalance in cell cycle regulation by OTX2 probably creates stress that leads to P53 pathway activation, such as in MED8A cells, or, when P53 is mutated, to activation of senescence-associated secretory factors, such as in DAOY cells. Expression profiling and reporter assays of MED8A cells showed activation of P53 and its downstream target genes after induction of OTX2.

Most medulloblastomas express high OTX2 levels (9). We hypothesize that inactivation of the P53 pathway is one of the potential mechanisms in medulloblastoma to prevent OTX2-induced senescence. However, P53 mutation or amplification of MDM2, which inhibits P53, has been infrequently reported for medulloblastoma (20, 43–46). However, PPMID, shown to inhibit P53 in medulloblastoma, is highly overexpressed in most medulloblastoma and even amplified in some (9, 47–49).

Furthermore, to prevent an imbalance in cell cycle regulation induced by OTX2, medulloblastomas must have found ways to drive G1-S transition. CCND1, CCND2, CDK4, CDK6, and MYC are all highly expressed in medulloblastoma (Fig. 4B; Supplementary Table S1). In some tumors, this is caused by gene amplifications (9, 49, 50). Especially MYC, but also its family members MYCN and MYCL, are frequently amplified in medulloblastomas (9, 51). If not amplified, these genes are transcriptionally upregulated as direct targets of the WNT or SHH signaling pathway, which are constitutively activated in ~40% of medulloblastomas (9, 52).

The potential role of OTX2 as a positive regulator of cell cycle progression, as suggested by the regulation of mitosis genes, fits with our observation that, in normal cerebellum, OTX2 is mainly expressed in proliferating progenitor cells, but not in differentiated neurons (3). It is also in line with the results reported by Yan and colleagues that silencing of OTX2 in D425 cells inhibited proliferation and induced differentiation (4, 10). However, ectopic expression of OTX2 alone is apparently not sufficient to drive proliferation and instead results in senescence in both MED8A and DAOY cells. Here, we present the first data about the genes and pathways regulated by OTX2. OTX2 directly and indirectly upregulates genes associated with the G2-M phase of the cell cycle. These data are confirmed in D425 cells in which we silenced OTX2 expression using inducible shRNA. Microarray and OTX2 ChiP-on-chip analyses performed for these cells also identified the mitotic cell cycle genes as one of the major categories of direct targets of OTX2. The full description of these data will be published elsewhere.2 Our results emphasize the role of OTX2 as an oncogene. In addition, our data obtained with MED8A and DAOY cells also suggest that cooperating genes are required for a balanced acceleration of the cell cycle by OTX2 in medulloblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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