The Senescence-associated Secretory Phenotype Mediates Oncogene-induced Senescence in Pediatric Pilocytic Astrocytoma

Juliane L. Buhl1,2,3, Florian Selt1,3,4, Thomas Hielscher5, Romain Guih6, Jonas Ecker1,3,4, Felix Sahm7,8, Johannes Ridinger1,2,3, Dennis Riehl9, Diren Usta1,3, Britta Ismer1,2,10, Alexander C. Sommerkamp1,2,10, J.P. Martinez-Barbera6, Annika K. Wefers7,8, Marc Remke11,12,13, Daniel Picard11,12,13, Stefan Pusch7,8, Jan Gronych14, Ina Oehme1,3, Cornelis M. van Tilburg1,3,4, Marcel Kool15, Daniela Kuhn1,3, David Capper7,8,16, Andreas von Deimling7,8, Martin U. Schuhmann17, Christel Herold-Mende18, Andrey Korshunov7,8, Tilman Brummer19, Stefan M. Pfister1,4,15, David T.W. Jones1,10, Olaf Witt1,3,4, and Till Milde1,3,4

Abstract

Purpose: Pilocytic astrocytoma is the most common childhood brain tumor, characterized by constitutive MAPK activation. MAPK signaling induces oncogene-induced senescence (OIS), which may cause unpredictable growth behavior of pilocytic astrocytomas. The senescence-associated secretory phenotype (SASP) has been shown to regulate OIS, but its role in pilocytic astrocytoma remains unknown.

Experimental Design: The patient-derived pilocytic astrocytoma cell culture model, DKEZ-BT66, was used to demonstrate presence of the SASP and analyze its impact on OIS in pilocytic astrocytoma. The model allows for doxycycline-inducible switching between proliferation and OIS. Both states were studied using gene expression profiling (GEP), Western blot, ELISA, and cell viability testing. Primary pilocytic astrocytoma tumors were analyzed by GEP and multiplex assay.

Results: SASP factors were upregulated in primary human and murine pilocytic astrocytoma and during OIS in DKEZ-BT66 cells. Conditioned medium induced growth arrest of proliferating pilocytic astrocytoma cells. The SASP factors IL1B and IL6 were upregulated in primary pilocytic astrocytoma, and both pathways were regulated during OIS in DKEZ-BT66. Stimulation with rIL1B but not rIL6 reduced growth of DKEZ-BT66 cells and induced the SASP. Anti-inflammatory treatment with dexamethasone induced regrowth of senescent cells and inhibited the SASP. Senescent DKEZ-BT66 cells responded to senolytic BCL2 inhibitors. High IL1B and SASP expression in pilocytic astrocytoma tumors was associated with favorable progression-free survival.

Conclusions: We provide evidence for the SASP regulating OIS in pediatric pilocytic astrocytoma, with IL1B as a relevant mediator. SASP expression could enable prediction of progression in patients with pilocytic astrocytoma. Further investigation of the SASP driving the unpredictable growth of pilocytic astrocytomas, and its possible therapeutic application, is warranted.

1Hopp Children’s Cancer Center Heidelberg (KITZ), Heidelberg, Germany. 2Faculty of Biosciences, Heidelberg University, Heidelberg, Germany. 3Clinical Cooperation Unit Pediatric Oncology, German Cancer Research Center (DKFZ) and German Consortium for Translational Cancer Research (DKTK), Heidelberg, Germany. 4KITZ Clinical Trial Unit (ZIPO), Department of Pediatric Hematology and Oncology, Heidelberg University Hospital, Heidelberg, Germany. 5Division of Biostatistics, German Cancer Research Center (DKFZ) and German Consortium for Translational Cancer Research (DKTK), Heidelberg, Germany. 6Division of Developmental Biology and Cancer Programme, Birth Defects Research Centre, Great Ormond Street Institute of Child Health, University College London, London, UK. 7Department of Neurology, Heidelberg University Hospital, Heidelberg, Germany. 8Division of Pediatric Neuro-Oncogenomics, German Cancer Research Center (DKFZ), Heidelberg, Germany. 9Department of Pediatric Hematology, University Hospital Tübingen, Tübingen, Germany. 10Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ) and German Consortium for Translational Cancer Research (DKTK), Heidelberg, Germany. 11Department of Pediatric Neuro-Oncology, Heidelberg University Hospital, Heidelberg, Germany. 12Department of Pediatrics, Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Department of Neuropathology, Berlin, Germany. 13Division of Pediatric Neurosurgery, University Medicine Göttingen, Göttingen, Germany. 14Division of Pediatric Neurosurgery, Heidelberg University Hospital, Heidelberg, Germany. 15Institute of Molecular Medicine and Cell Research (IM2C), Faculty of Medicine, University of Freiburg, Freiburg, Germany. 16Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Department of Neuropathology, Berlin, Germany. 17Division of Pediatric Neurosurgery, University Hospital Tübingen, Tübingen, Germany. 18Division of Molecular Medicine and Cell Research, University Hospital Düsseldorf, Düsseldorf, Germany. 19Division of Molecular Genetics, Department of Pediatrics, University Hospital Düsseldorf, Düsseldorf, Germany. 20Division of Pediatric Neuro-Oncogenomics, German Cancer Research Center (DKFZ), Heidelberg, Germany. 21Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 22Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 23Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 24Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 25Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 26Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 27Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 28Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 29Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 30Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 31Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 32Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 33Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 34Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 35Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 36Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 37Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 38Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany. 39Division of Pediatric Neuro-Oncology, German Cancer Research Center (DKFZ), Heidelberg, Germany.

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Corresponding Author: Till Milde, Clinical Cooperation Unit Pediatric Oncology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg 69120, Germany. Phone: 49-62-214-3387; Fax: 49-62-214-3579; E-mail: tmilde@kitz-heidelberg.de


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nearly all cases (2, 3). Activating kinase (MAPK) signaling by genetic aberration is detectable in pediatric brain tumor (1). Activation of mitogen-activated protein astrocytomas (60% most common alterations of the MAPK pathway in pilocytic astrocytoma was established by transduction of either growth arrest (10). Another murine glioma model of pilocytic astrocytoma and postulate that the SASP is a clinically relevant factor for stable disease in pilocytic astrocytoma. The identification of factors regulating OIS in pilocytic astrocytoma could help understand the mechanisms controlling the unpredictable growth behavior of pilocytic astrocytoma tumors. This knowledge could enable both prediction of the clinical course as well as possible therapeutic interference with specific factors or pathways. Here we provide evidence of mediation of OIS by the SASP and one of its factors, IL1B, in pediatric pilocytic astrocytoma.

Materials and Methods

Cell culture

DKFZ-BT66 cells (passages 8–17), containing the doxycycline-inducible SV40 large T antigen, were cultured and viability and cell counts were measured as described in ref. 14. The identity of the cell line was confirmed by Multiplex Cell Line Authentication (MCA) test and it was proven to be free of Mycoplasma or viral contamination by Multiplex cell Contamination Test (McCT) assay (http://www.multiplexion.de; ref. 24). After generation of the cell line in our laboratory, the cells were tested for contamination (October 2015), aliquoted, and frozen. From these stocks, cells in culture were tested with VenorGeM Classic (catalog no. 11-1250, Minerva Biolabs) for Mycoplasma once a month. Normal human astrocyte (NHA) primary cells (passage 2;
obtained June 2017, catalog no. CC-2565, Lonza) were cultured in AGM Astrocyte Growth Medium BulletKit (catalog no. CC-3186, Lonza) and tested for the Mycoplasma contamination monthly with the MycoAlert Mycoplasma Detection Assay (catalog no. L07-318, Lonza). DF-1 cells were obtained from ATCC (February 2012, catalog no. CRL-12203; RRID: CVCL_0570). DF-1 cells were cultured in DMEM high glucose (catalog no. D5796, Sigma-Aldrich) with 10% FBS (catalog no. S 0115, Biochrom). 1% Penicillin–Streptomycin (catalog no. 15140122, Thermo Fisher Scientific) and 1% l-glutamine (200 mmol/L) catalog no. 25030081, Thermo Fisher Scientific). Cells from the original stock were expanded for two passages and cryopreserved in aliquots for each experiment, an aliquot was thawed and used for a maximum of four additional passages. Because of the short period of use, no additional Mycoplasma testing or cell authentication regimen was applied.

Drugs and cytokines

Water-soluble dexamethasone (catalog no. D2915, Sigma-Aldrich) was dissolved in sterile water and stored at −20°C. Anakirin (Kineret 150 mg/mL, Sobi) and tocilizumab (RoActemra 20 mg/mL, Hoffmann-LaRoche), both dissolved in sterile PBS containing 0.1% BSA and stored at −80°C. Navitoclax (ART-263, catalog no. 11500, Cayman Chemical), ABT-737 (catalog no. ab141336, Abcam), dasatinib (catalog no. S1021, Selleckchem), quercetin (catalog no. S2391, Selleckchem), and trametinib (GSK1120212, catalog no. A3018, ApexBio) were dissolved in DMSO. Carboplatin (catalog no. S1215, Selleckchem) was dissolved in sterile water. All drugs were dissolved as described and stored in aliquots at −80°C. Drugs and cytokines were diluted in cell culture medium and added to the cells for the durations and concentrations indicated.

Gene expression profiles

Generation of gene expression profiles (GEP) was performed as described previously (3). Briefly, GEP of patient samples as well as the DKFZ-BT66 cell line was done on Affymetrix U133 Plus 2.0 expression arrays, while GEP of the murine pilocytic astrocytoma model was done on Affymetrix Mouse Genome 430 2.0 arrays. Expression values from patient data was MAS5 normalized. In case of multiple probe-sets per gene, the probe-set with highest expression values was selected. All GEP datasets are publicly available on R2 (http://r2.amc.nl). GEP datasets used to identify OIS-controlling candidate genes were E-NCMF-12 (human fibroblasts/BRAFV600E, n = 20; ref. 18), E-NCMF-13 (human fibroblasts/BRAFV600E, n = 16; ref. 18), GSE54402 (human fibroblasts/H RasG12V, n = 10; ref. 25), GSE46801 (primary human melanocytes/BRAFV600E, n = 9; ref. 26), GSE41318 (human fibroblasts/RAS, n = 6; Acosta and colleagues, unpublished), and GSE60652 (human fibroblasts/RAS, n = 4; ref. 27).

In vivo mouse model

Murine pilocytic astrocytoma tumors were induced using the RCAS/Tv-a system as described previously (12). Briefly, DF-1 cells expressing RCAS/BRAFV600E were injected into the cerebral hemisphere of male and female neonatal Nestin Tv-a (Ntv-a) mice [strain: STOCK Tg(NES-TVA)J12Ech/f] from Jackson Laboratories, RRID: MGI:2663944]. Age-matched noninjected Ntv-a mice were used as controls. Ntv-a mice were maintained on a mixed background and only mice homozygous for Ntv-a were used for experiments. Mice were sacrificed 5–6 weeks after injection (weight: 15–25 g) and tumor tissue was isolated. All animal procedures were performed according to protocols approved by the German authorities (Regierungspräsidium Karlsruhe; G-69/13, DKFZ342).

Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase (SA-β-Gal) staining was performed as described previously (14).

Conditioned medium

For conditioning of media, DKFZ-BT66 cells were seeded with or without doxycycline. Cell numbers for seeding were adjusted to end up with similar numbers (n = 2 × 10^6) after five days of culturing accounting for proliferating versus senescent cells. The cell number was chosen according to ELISA results for an estimated secretion of at least 100 pg/mL of IL1B at day 5. Medium was changed on day 3 and collected on day 5, resulting in two days of conditioning. Collected medium was centrifuged and filtered (0.22 μm). Freshly collected conditioned medium (CM) was supplemented with doxycycline and recycled every second day to DKFZ-BT66 cells.

Microscopy

Bright-field pictures were taken as described previously (28).

RNA isolation, cDNA synthesis, and qRT–qPCR

RNA extraction, cDNA synthesis, and qRT–PCR were conducted as described previously (14). Quantitect primers used are given in Supplementary Table S1.

ELISA

Cytokine concentrations were measured via ELISA Kits for IL1B (catalog no. DLB50, R&D Systems) and IL6 (catalog no. D6050, R&D Systems). ELISA experiments were performed according to manufacturer’s instructions. Supernatant was centrifuged before use and stored at −80°C. Cytokine concentrations (pg/mL) were normalized to cell counts in each well (cell number/mL) resulting in cytokine concentration (cell (pg/cell)).

Western blot analysis

Western blots were performed as described previously (28). Antibodies and corresponding membranes are given in Supplementary Table S1. Depicted blots are always representative of a least three replicates.

Cytokine measurement in primary tumors by multiplex assay (Luminex)

Fresh frozen tumors and one normal fetal brain sample (1–3 mm³) were lysed by Bio-Plex Cell Lysis Kit (catalog no. 171304011, Bio-Rad) according to the manufacturer’s protocol. Total protein concentrations were determined by Pierce BCA Protein Assay Kit (catalog no. 23225, Thermo Fisher Scientific) and normalized to 1 mg/mL. A total of 27 different cytokines/chemokines (consisting of n = 14 SASP factors and n = 13 non-SASP factors) were analyzed in one sample according to Bio-Plex Pro Human Cytokine 27-plex Assay (catalog no. M500KCAFOY,
DKFZ-BT66 cells were seeded following manufacturer’s instructions. Of treatment using CellTiter-Glo One Solution assay (catalog no. G8461, Promega) and 8,000 cells/well without doxycycline. NHA cells were seeded 24 hours before treatment with 4,000 cells/well. Luminescence was detected by FLUOstar OPTIMA automated plate reader (BMG Labtech).

Statistics and bioinformatics

For the generation of the OIS-controlling candidate gene list, six publicly available gene expression datasets (GEO: GSE54402, GSE46801, GSE60652, GSE41318, ArrayExpress: E-NCMF12, E-NCMF13) were used. For each dataset separately, upregulated genes in the OIS condition were ranked according to their moderated t-statistic based on the empirical Bayes approach (29) as implemented in the Bioconductor package limma (30). Genes were represented by multiple probes, the probe with the strongest effect was selected. Consistently upregulated genes across datasets (n = 332) were identified with the rank-product approach (31), which is the geometrical mean of ranks. This analysis was based on the overlap of genes measured in each dataset. Significance of rank-product was tested according to ref. 32. P-values were adjusted to control the false discovery rate using Benjamini–Hochberg correction.

The limma approach (30) was used to test for differential gene expression. Gene-set enrichment analysis (GSEA) for SASP genes (17) was performed using the camera test (33). For GSEA, the most specific probe set per gene was selected using the jetset algorithm (34). All analyses were performed with statistical software R 3.4.

Ingenuity pathway analysis was conducted for the 323 genes according to the user manual. A list of the correlating genes for each pathway was generated (n = 36 genes in total). The expression level of the 323 genes selected from public GEPs, the IPA pathway genes, and the SASP genes were analyzed in pediatric and adult pilocytic astrocytoma patient samples [n = 182; Supplementary Table S4; refs. 3, 35], GEO: GSE16011 (36), GSE5675 (37)] and compared with the corresponding expression level in unmatched normal fetal cerebellum samples (n = 5) from nonpatients (GEO: GSE44971; ref. 38) using the R2 web-based genomics analysis and visualization platform (http://r2.amc.nl). The dataset is publicly available on R2 (http://r2.amc.nl). Finally, only genes were considered, which could be targeted by a small-molecule inhibitor or antibody and overlapped in all three candidate gene lists.

In vitro experiments were performed in a minimum of three biological replicates. All data are presented as mean ± SD. Testing for statistical significance of differences between two groups was done by unpaired Student t Test with Welch correction, with P values below 0.05 considered as significant. Graphs were generated using GraphPad Prism version 5.01 and Microsoft Powerpoint 2010 for Windows. PFS was defined as time from diagnosis to recurrence or death, whichever occurred first. Distribution of PFS was estimated with the method of Kaplan and Meier and compared between groups with the log-rank test. Univariate and multivariate Cox regression models were used to estimate the HR and corresponding 95% confidence interval of prognostic factors. IL1B log, expression and SASP sum score were standardized to give the HR per one SD increase. Groups were based on median cutoff (IL1B) or tertiles (SASP).

Results

SASP factors are upregulated in primary pilocytic astrocytoma, during OIS in pilocytic astrocytoma cells and induce growth arrest

We analyzed mRNA expression of the SASP factors in human pilocytic astrocytoma patient samples (n = 182) and compared it with normal brain (n = 5) by means of gene expression microarrays. The SASP gene set, as defined by Coppé (17), was significantly upregulated in human pilocytic astrocytomas (Fig. 1A). Likewise, the SASP gene set was significantly upregulated in a murine in vivo pilocytic astrocytoma model expressing a BRAFV600E mutation (12; n = 8) compared with healthy mice of the same age (n = 8; Fig. 1B). Using the same approach, we analyzed the regulation of the SASP gene set in OIS versus proliferation in our pilocytic astrocytoma cell line, DKFZ-BT66. The OIS state in DKFZ-BT66 was defined at five days after doxycycline withdrawal, coinciding with enlarged cell morphology, SV40 large T antigen protein degradation, cell-cycle arrest, and upregulation of markers of senescence such as CDKN1A as described previously (14). Comparison of the SASP gene set in OIS versus proliferation showed a significant upregulation of the SASP gene set overall (Fig. 1C), with 38 of 62 (61%) of the SASP factors significantly upregulated (Fig. 1D; Supplementary Table S3). To determine the secretory nature and the functional relevance of the SASP for the induction of OIS in pilocytic astrocytoma, conditioned medium (CM) experiments were conducted. CM of either senescent or proliferating DKFZ-BT66 cells was administered to proliferating DKFZ-BT66 cells together with doxycycline for continuous induction of SV40 large T antigen expression. While cells treated with CM from proliferating pilocytic astrocytoma cells continued to grow, treatment with CM from senescent cells induced growth arrest (Fig. 1E) as well as an enhanced cell morphology characteristic for senescence (ref. 11; Fig. 1F). Taken together, these data suggest that the SASP is upregulated in human and murine pilocytic astrocytoma as well as in our pilocytic astrocytoma cell line, is secreted during OIS in DKFZ-BT66 cells and is sufficient to induce growth arrest of proliferating cells.

Identification of the OIS-controlling candidate genes IL1B and IL6 in pediatric pilocytic astrocytoma

As the composition of SASP factors differs depending on tissue and cell type [16], we next investigated which SASP factors as well as other OIS-controlling genes are expressed in pediatric human pilocytic astrocytomas. To identify specific OIS-controlling genes for pediatric pilocytic astrocytoma, we collected and generated three candidate gene lists of relevant OIS and SASP genes (step 1), investigated the expression of these genes in primary human pilocytic astrocytoma samples (step 2), and generated a final candidate gene list after accounting for consensus in the three candidate gene lists as well as druggability (step 3; Fig. 2A).

The first candidate gene list ("SASP genes") consists of the SASP genes published by Coppé (n = 62 genes; ref. 17), accounting for
Figure 1.
Upregulated SASP factors in pediatric pilocytic astrocytoma cells induce growth arrest. A, Barcode plot of gene-set enrichment analysis (GSEA) depicting significant upregulation of the SASP genes in pilocytic astrocytoma (PA) patient samples (n = 182) versus fetal normal brain (n = 5). B, Barcode plot of GSEA depicting significant upregulation of the SASP genes in the murine pilocytic astrocytoma model (n = 8) versus normal brain of healthy mice of the same age (n = 8). C, Barcode plot of GSEA depicting significant upregulation of the SASP genes in DKFZ-BT66 cells in OIS versus proliferation. D, Volcano plot depicting all probe sets regulated in DKFZ-BT66 during OIS, as compared with proliferation. E, Cell count of proliferating DKFZ-BT66 cells treated with CM every second day from DKFZ-BT66 cells in proliferation (blue) or OIS (red), supplemented with doxycycline (1 μg/mL) for 20 days. Depicted are mean ± SD of three independent experiments. Significant differences are indicated as *, P < 0.05 (Student t test). F, Light microscopic comparison of DKFZ-BT66 cells grown under CM from proliferating or senescent (OIS) DKFZ-BT66 cells (as in E) at day 20.
the published SASP genes. The second candidate gene list ("Published OIS genes") was generated by screening publicly available GEP datasets from six human OIS models (including RAS or BRAFV600E–transduced fibroblasts and primary melanocytes) for genes upregulated in all 6 datasets (n = 332 genes). This list accounts for genes upregulated in independent general OIS...
models. The third gene list ("Top IPA pathway genes") was generated by identifying the top signaling pathways upregulated in the "Published OIS genes" set performing IPA and including all genes related to the respective pathways (n = 36 genes). This list additionally adds all genes related to the pathways upregulated in OIS, which may not have been included on a single gene basis. In step 2 the expression of all three candidate gene lists ("SASP genes," "Published OIS genes," "Top IPA pathway genes") was analyzed in n = 182 pilocytic astrocytoma samples (Supplementary Table S4) and compared with n = 5 normal brain samples, to identify the candidate genes relevant to pilocytic astrocytoma. In a third step, we filtered for consensus candidate genes (n = 5), by omitting all genes that did not appear in all three candidate gene lists. Finally, for experimental as well as therapeutic purposes, only genes which could be targeted by a small-molecule inhibitor or antibody were considered, resulting in a pilocytic astrocytoma-specific OIS-controlling candidate gene list of n = 3 genes (IL1B, IL6, TNFRSF1B; Fig. 2A and B). The candidates were validated in the murine in vivo pilocytic astrocytoma model (12) and found to be upregulated compared with healthy control animals (Fig. 2C). The pilocytic astrocytoma tumors induced by BRAFV600E expression in neural progenitor cells showed upregulated expression of CDKN2A on mRNA level compared with healthy controls (Supplementary Fig. S1A), and cells derived from the pilocytic astrocytoma tumors were positive for SA-ß-Gal staining (Supplementary Fig. S1B). These data indicate that the murine pilocytic astrocytoma model indeed displays markers of senescence and SASP expression.

While the increased mRNA expression was validated by gene expression profiling in DKFZ-BT66 in OIS for all three OIS-controlling candidate genes (Fig. 2D), qRT-PCR during OIS induction could confirm the mRNA increase only for IL1B and IL6 but not for TNFRSF1B (Fig. 2E). IL1B and IL6 are both secreted into the supernatant, as detected by ELISA (Fig. 2F). Upregulation of IL1A/B and IL6 has previously been described in other senescence models in fibroblasts (18, 39–41), human epithelial cells (16), BRAFV600E–transduced human melanocytes (18), and prostate epithelial cancer cells (16), suggesting that IL1B and IL6 are important regulators of OIS. We therefore focused on IL1B and IL6 as OIS-controlling candidate genes in pilocytic astrocytoma in the further analyses.

IL1B and IL6 signaling pathways are present and regulated during OIS in pilocytic astrocytoma

As we detected upregulation and secretion of IL1B and IL6 during induction of OIS, we next aimed at elucidation of their function during OIS in pilocytic astrocytoma. In a first step, the expression and activation of the IL1B and IL6 signaling cascades were determined in DKFZ-BT66 and in primary pediatric pilocytic astrocytoma tumors. The IL1R1 receptor is expressed on mRNA and protein level in DKFZ-BT66 cells (Fig. 3A and B), but no significant regulation was detected. While the activity of IL1B signaling is regulated by a variety of mechanisms, its receptor is indeed rarely upregulated in disease models (42). To determine activation of the IL1 pathway, protein expression of the downstream targets IRAK1 and phosphorylation of RELA was investigated. IRAK1, the interleukin-1 receptor-associated kinase 1, is phosphorylated and degraded upon activation of the IL1 pathway (43). Degradation of IRAK1 was indeed observed after induction of senescence and subsequent growth arrest in DKFZ-BT66 cells (Fig. 3B). NFkB, a transcription factor highly associated with OIS, has been shown to be an important regulator of the SASP (44, 45). Activation of NFkB was assessed by phosphorylation status of RELA, which increased after OIS induction in DKFZ-BT66 cells (Fig. 3B). In conclusion, the IL1 pathway is active during OIS in the DKFZ-BT66 cell model.

The IL6Ra receptor is expressed on mRNA and protein level in DKFZ-BT66 cells (Fig. 3C and D). While no significant changes were observed on mRNA level (Fig. 3C), the protein levels decreased during OIS induction (Fig. 3D). It has been described that long-term activation of the IL6 pathway initiates a negative feedback loop, leading to internalization and degradation of IL6Ra (46). In parallel, we detected reduction of IL6 signaling during OIS induction in DKFZ-BT66 cells as measured by reduced phosphorylation of the downstream target STAT3 (p-STAT3) in comparison with initial levels (Fig. 3D). Activation of the IL6 pathway leads to phosphorylation of STAT3 within minutes (47), while long-term activation induces negative feedback and leads to downregulation of p-STAT3 (48). We conclude that the initial activity of the IL6 pathway is downregulated in our pilocytic astrocytoma cell culture model, possibly due to continuous IL6 secretion during induction of OIS and subsequent growth arrest.

Expression of IL1B and IL6 protein was analyzed in n = 22 pediatric pilocytic astrocytoma samples and one normal brain sample by multiplex assay and both cytokines were detected in every sample (Fig. 3E).

Taken together, these data indicate that the IL1 as well as the IL6 pathway is regulated during OIS in our pilocytic astrocytoma model, but only the IL1 pathways remains activated while a negative feedback regulation of the IL6 pathway is observed. Both cytokines are present in primary pediatric pilocytic astrocytoma.

IL1B, but not IL6 signaling, reduces proliferation of pilocytic astrocytoma cells and induces SASP expression

After observing elevated IL1B and IL6 levels during OIS, we next determined the role of both cytokines for OIS induction. Increasing concentrations of recombinant cytokines rIL1B and rIL6 were used to actively stimulate both pathways in proliferating DKFZ-BT66 cells.

Treatment with rIL1B resulted in significant reduction of proliferation of DKFZ-BT66 cells in a concentration-dependent manner, without affecting cell viability (Fig. 4A; Supplementary Fig. S2). Increasing concentrations of rIL1B above 100 pg/mL could not maximize the resulting growth reduction. The IL1 pathway was activated by rIL1B treatment, evident by degradation of IRAK1 as well as elevated protein levels of the precursor form of IL1B (31 kDa; Fig. 4B). Elevation of IL1B due to de novo translation after positive feedback activation has been described previously (49). Of note, the maximum reduction of both proliferation and of IRAK1 protein was seen at the same concentration of rIL1B (100 pg/mL), indicating a strong correlation between IL1 pathway activity and cell proliferation. On the contrary, no significant effect on proliferation of DKFZ-BT66 cells was detected upon treatment with rIL6 (Fig. 4C). The IL6 pathway was indeed activated after short-term treatment with rIL6, as observed by elevated phosphorylation of STAT3 in a concentration-dependent manner, excluding unsuccessful stimulation of the pathway (Fig. 4D).

As the SASP is a complex mixture of inflammatory signaling molecules acting in a concerted fashion, we tested whether combination treatment of rIL1B and rIL6 has an additional or synergistic effect on cell proliferation. Cotreatment resulted in a
Figure 3. Functional validation of the IL1 and IL6 signaling pathways during OIS. 

A, Fold change of IL1R1 transcript levels were measured by qRT-PCR in DKFZ-BT66 upon OIS induction relative to levels during proliferation (day 0). Depicted are mean ± SD of three independent experiments. 

B, IL1 pathway activity was determined by protein levels of IL1R1, IRAK1, phospho-RELA, and RELA in DKFZ-BT66 upon OIS induction measured by Western blot analysis. Actin serves as loading control. 

C, Fold change of IL6Ra transcript levels as measured by qRT-PCR in DKFZ-BT66 upon OIS induction relative to levels during proliferation (day 0). Depicted are mean ± SD of three independent experiments. 

D, IL6 pathway activity was determined by protein levels of IL6Ra and pSTAT3/STAT3 upon OIS induction measured by Western blot analysis. Actin serves as loading control. 

E, Presence of IL1B and IL6 protein was detected by multiplex assay in all studied fresh frozen primary pilocytic astrocytoma (PA) samples (black; n = 22) and in normal fetal brain (gray; n = 1). Depicted is mean ± SD. Dots indicate values of individual samples.
Figure 4.
IL1B signaling contributes to reduced pilocytic astrocytoma (PA) cell proliferation and induces expression of SASP factor. A, Cell count of proliferating DKFZ-BT66 cells under rIL1B treatment in concentrations indicated for 20 days. Depicted are mean ± SD of three independent experiments. Significant differences are indicated as *, P < 0.05 (Student t test). B, Protein levels of IRAK1 and pro-IL1B were determined by Western blot analysis under stimulation with rIL1B in the depicted concentrations for 4 hours in DKFZ-BT66 cells in proliferation. Actin serves as loading control. C, Cell count of proliferating DKFZ-BT66 cells under rIL6 treatment in concentrations indicated for 20 days. Depicted are mean ± SD of three independent experiments. D, Protein levels of pSTAT3 and STAT3 were determined by Western blot analysis under stimulation with rIL6 in the depicted concentrations for 15 minutes in DKFZ-BT66 cells in proliferation. Actin serves as loading control. E, Cell count under combination treatment with rIL1B and rIL6 in the depicted concentrations for 20 days. Depicted are mean ± SD of three independent experiments. Significant differences are indicated as *, P < 0.05 (Student t test). F, Protein levels of IRAK1, CDKN1A, and SV40 measured by Western blot analysis after long-term treatment with rIL1B (500 pg/mL) (+) for 0, 5, 10, and 20 days versus solvent control (−) treatment in DKFZ-BT66 in proliferation. Actin serves as loading control. G, Light microscopic comparison of DKFZ-BT66 cells grown under treatment with 500 pg/mL rIL1B versus solvent control at day 20. H, Barcode plot of GSEA depicting significant upregulation of the SASP genes in DKFZ-BT66 cells under treatment with 500 pg/mL rIL1B and doxycycline for five days versus DKFZ-BT66 cells in proliferation.
significant downregulation of cell growth in comparison with solvent control, however, depending only on the concentration of rIL1B (Fig. 4E). Addition of rIL6 in increasing concentrations did not result in additional reduction in cell growth.

The IL1 pathway was active for the entire duration (up to day 20) of the experiment as detected by IRAK1 degradation (Fig. 5F). Therefore, the inhibitory effect of IL1B on cell proliferation is not based on alterations in SV40 large T antigen and subsequent CDKN1A expression. As previously described, SA-β-Gal cannot be used as senescence marker in DKFZ-BT66 cells (14). However, enlarged cellular morphology characteristic for senescence was observed after treatment with rIL1B (Fig. 4G). In addition, significant upregulation of the SASP factors was determined by GEP under treatment with rIL1B (Fig. 4H).

In summary, IL1B, but not IL6, induces growth arrest of proliferating pilocytic astrocytoma cells, and IL1B induces senescent morphology and upregulation of the SASP factors. We conclude that IL1B plays an important role in induction of SASP-mediated OIS in pilocytic astrocytoma.

Interference with inflammatory signaling, but not with IL1 signaling alone, leads to suppression of the SASP and growth of senescent pilocytic astrocytoma cells

Having identified IL1B as important for senescence induction, we next assessed the role of IL1B for OIS maintenance. Senescent DKFZ-BT66 cells were treated with anakinra, an antagonist of the IL1 receptor. OIS-induced growth arrest could not be circumvented by treatment with anakinra alone, as determined by cell counts over the course of 20 days (Fig. 5A). IL1B signaling was inhibited, as shown by rescued IRAK1, pro-IL1B, and p-RELA levels (Fig. 5B). Pharmacologic inhibition of the IL1 signaling pathway alone therefore cannot bypass OIS in our model, similar to reports in other OIS models (18).

As inhibition of single SASP factors did not suffice to overcome growth arrest of pilocytic astrocytoma cells in OIS, we attempted to bypass OIS by treatment with dexamethasone, a broad anti-inflammatory drug, known to inhibit the SASP (50, 51). Treatment of senescent DKFZ-BT66 cells with 100 nmol/L dexamethasone for 20 days resulted in a significant increase in cell proliferation compared with solvent control (Fig. 5C). Exemplary for SASP factors, dexamethasone strongly inhibited the IL1 pathway as determined by rescued protein levels of IRAK1 (Fig. 5D). Treatment of DKFZ-BT66 cells in OIS with dexamethasone for five days led to a significant reduction in expression of SASP factors as determined by GEP, illustrated in the GSEA (Fig. 5E). IPA analysis of the GEPs revealed IL1B as the top upstream regulator, predicted to be inhibited under dexamethasone treatment (Supplementary Table S5). Importantly, of the top five upstream regulators identified by IPA in either senescent DKFZ-BT66 cells treated with dexamethasone or in proliferating DKFZ-BT66 cells treated with rIL1B, 4 of 5 were identical (IL1B, TREM1, TNF, NFKB complex) and regulated in an opposite fashion (inhibition vs. activation; Fig. 5F). This suggests similar SASP pathways to be involved in IL1B-induced senescence and reversal of senescence by dexamethasone, in an opposite manner. In summary, these data indicate that bypass of SASP-mediated OIS-induced growth arrest is possible by inhibiting multiple SASP pathways with an anti-inflammatory drug in pilocytic astrocytoma cells.

II1B and the SASP are expressed in primary pilocytic astrocytomas and predict favorable progression-free survival independent of tumor resection status

The SASP as well as IL1B play a significant role in the regulation of OIS in our pilocytic astrocytoma cell model and could mediate the variable growth behavior of pediatric pilocytic astrocytomas observed clinically. To assess the clinical relevance of our findings we analyzed the expression of IL1B and the SASP in primary pediatric pilocytic astrocytoma samples. Using a multiplex assay, all n = 14 SASP factors present in the assay were detected in primary pilocytic astrocytomas on protein level (Supplementary Fig. S3). To test for a correlation between SASP factor mRNA expression and clinical outcome in pilocytic astrocytoma patients, the ICGC PedBrain pilocytic astrocytoma cohort [n = 112 patients; survival data available for 110/112 patients (98.2%); ref. 3], for annotations see Supplementary Table S4] was analyzed for expression of IL1B as well as by means of a SASP sum score (SASP score), which sums up the expression levels of all SASP factors for one patient. Increased expression of IL1B alone (both as continuous or grouped variable according to median cutoff) predicted favorable prognosis (e.g., continuous variable: HR = 0.4, 95% CI, 0.24–0.69, P = 0.008; n = 110 patients, Supplementary Table S6). IL1B remained a factor for good prognosis in a multivariate analysis after accounting for other significant prognostic factors such as extent of tumor resection (HR = 0.37, 95% CI, 0.18–0.75, P = 0.0056; n = 90 patients, Supplementary Table S6) or radiotherapy (HR = 0.35, 95% CI, 0.15–0.77, P = 0.0079; n = 75 patients, Supplementary Table S6). Five-year progression-free survival (PFS) was 85% in the “IL1B high” group versus 46% in the “IL1B low” group, and survival differed significantly (log-rank test: Fig. 6A). Similarly, patients with a higher SASP score (continuous variable) had a significantly more favorable PFS (HR = 0.56, 95% CI, 0.34–0.93, P = 0.026, n = 110 patients; Supplementary Table S6). The prognostic effect of the SASP score remained significant in a multivariate analysis after accounting for other significant prognostic factors such as extent of resection (HR = 0.36, 95% CI, 0.16–0.82, P = 0.01; n = 90, Supplementary Table S6) or radiotherapy (HR = 0.19, 95% CI, 0.06–0.52, P = 0.0006; n = 75 patients, Supplementary Table S6), or both (HR = 0.19, 95% CI, 0.03–0.72, P = 0.019; n = 58 patients, Supplementary Table S6). When patients were grouped into three groups according to SASP score tertiles, the 5-year PFS was 48% for the “SASP low” group, 61% for the “SASP intermediate” group, and 90% for the “SASP high” group, and survival differed significantly (log-rank test; Fig. 6B). Because resection status is a strong prognostic factor, we analyzed PFS separately for either gross total resection (GTR) or subtotal resection (STR). The 5-year PFS was 100% for patients with GTR in the “SASP high” and “SASP intermediate” group versus 63.3% in the “SASP low” group, and survival differed significantly (log-rank test; Fig. 6C). On the contrary, patients with STR in the “SASP low” group showed a particularly poor 2- and 3-year PFS of 52% and 0%, respectively (Fig. 6D).

In conclusion, SASP factors are expressed on mRNA and protein level in primary pediatric pilocytic astrocytomas. Elevated mRNA expression of SASP factors correlates with a high probability to remain progression-free, and low expression of SASP factors correlates with a high risk of recurrence, especially in subtotally resected cases.
Figure 5.
Inhibition of inflammatory signaling during OIS, but not IL1B alone, suppresses the SASP and leads to bypass of OIS. A, Cell count of senescent DKFZ-BT66 cells under anakinra treatment in the depicted concentrations for 20 days. Cells were cultured without doxycycline 5 days prior to treatment as well as throughout the duration of the entire experiment. Shown are mean ± SD of three independent experiments. B, Protein levels of IRAK1, pro-IL1B, p-RELA, and RELA determined by Western blot analysis in DKFZ-BT66 cells in OIS treated with rIL1B (500 pg/mL) + anakinra (20 µg/mL) for 4 hours. Actin serves as loading control. C, Cell count of senescent DKFZ-BT66 cells treated with 100 nmol/L dexamethasone (Dexa) or solvent control (0 nmol/L Dexa) for 20 days. Cells were cultured without doxycycline 5 days prior to treatment as well as throughout the duration of the entire experiment. Depicted are mean ± SD of three independent experiments. Significant differences are indicated as *, P < 0.05; **, P < 0.001 (Student t test). D, Protein levels of IRAK1 determined by Western blot analysis in DKFZ-BT66 cells in OIS treated with 100 nmol/L dexamethasone (Dexa) (+) or solvent control (−) for 0, 5, 10, and 20 days. Actin serves as loading control. E, Barcode plot of GSEA reveals significant downregulation of the SASP genes in DKFZ-BT66 cells in OIS under treatment with dexamethasone (Dexa) for five days. F, IPA analysis of GEPs of DKFZ-BT66 cells in OIS treated with 100 nmol/L dexamethasone (Dexa) (+) or solvent control (−) for proliferating DKFZ-BT66 cells (n = 3 replicates; red, OIS + Dexa), or DKFZ-BT66 cells in proliferation treated with 500 pg/mL rIL1B (n = 3 replicates) compared with DKFZ-BT66 cells in OIS (n = 3 replicates; blue, proliferation + rIL1B) for five days. The 4 common genes of the top 5 upstream regulators of each condition are displayed. The z-score predicts the activation status of the upstream regulator, positivity indicates activation, negativity indicates inhibition. Color indicates from which condition the z-score was calculated. White bar: z-score for TREM1 in the OIS + Dexa condition did not pass the threshold to indicate its inhibition/activation.
Senescent DKFZ-BT66 cells are more responsive to senolytic BCL2 family inhibitors than proliferating cells

The low proliferation index observed in pilocytic astrocytomas (15) suggests that most of the tumor cells persist in a senescent state and thus are unlikely to respond to standard chemotherapeutic agents relying on cell division for their effect. To investigate the targetability of senescence in pilocytic astrocytoma, we performed a drug screen in DKFZ-BT66 cells in OIS and proliferation testing single agents and combination treatments of several senolytic agents, standard-of-care chemotherapeutics, and a MEK inhibitor. Senescent DKFZ-BT66 cells showed increased sensitivity to navitoclax and ABT-737 (both BCL2 family inhibitors) in comparison with proliferating cells. We did not observe elevated sensitivity to the combination of dasatinib and quercetin (52), as has been previously described for other senescent cells (Fig. 7A–C). Primary human astrocytes, which served as control, responded at high concentrations only (Supplementary Fig. S4A–S4G). No significant response to carboplatin, vincristine, or trametinib was detected in senescent DKFZ-BT66 cells (Fig. 7D–F). In contrast to carboplatin, vincristine reduced metabolic activity in proliferating DKFZ-BT66 cells. As the SV40 large T antigen inhibits TP53 signaling in proliferating DKFZ-BT66 cells, carboplatin cannot induce TP53-dependent apoptosis. Therefore, the lack of effect is expected and due to the specifics

Figure 6.
SASP factor expression predicts PFS independent of resection status implying a crucial role of inflammatory signaling for pilocytic astrocytoma (PA) tumor growth behavior. A, Kaplan–Meier analysis of pilocytic astrocytoma patients (n = 110) depicting superior progression-free survival (PFS) in the IL1B mRNA high expression group ("IL1B high"; P = 0.006, log-rank). B, Kaplan–Meier analysis with pilocytic astrocytoma patients (n = 110) grouped into three groups according to SASP score tertiles, depicting significantly different PFS (P = 0.02, log-rank). C, Kaplan–Meier analysis, only patients with pilocytic astrocytoma with gross total resection (GTR; n = 61) are shown depicting significantly different PFS (P = 0.047, log-rank). Both "SASP high" and "SASP intermediate" have no events and curves are fully overlapping. D, Kaplan–Meier analysis, only patients with subtotal resection (STR; n = 32) are shown. Panel A–D all depict patients from the same pilocytic astrocytoma cohort from ICGC PedBrain (PFS available for n = 110/112, 98.2%). Information on resection status was available for n = 93/110 (84.5%) patients of the ICGC pilocytic astrocytoma cohort.
of this model, but not indicative of resistance. An increase in metabolic activity in both proliferating and senescent DKFZ-BT66 cells was observed under trametinib treatment, as we have described previously (14).

Navitoclax is currently being tested in several clinical trials and was evaluated as safe and well tolerated with dose-dependent thrombocytopenia as major adverse event (53). We therefore investigated the combination of navitoclax with standard-of-care treatment for patients with pilocytic astrocytoma. In our screen, we observed additive effects in the combination treatments compared with carboplatin, vincristine, or trametinib alone. No antagonistic effect was detected (Fig. 7G–I). Similar results were observed for ABT-737 in combination treatments (Supplementary Fig. S4H–S4J). In conclusion, addition of a BCL2 family inhibitor to the standard-of-care treatment regimen for patients with pilocytic astrocytoma could help eradicate senescent pilocytic astrocytoma cells, which do not respond to chemotherapy and might be the source of tumor recurrence or progression at a later time point.

**Discussion**

We here present data showing that the SASP mediates OIS in pediatric pilocytic astrocytoma, and that one of the SASP factors, IL1B, significantly contributes to OIS induction. In line with our hypothesis that SASP mediates OIS and modulation of OIS plays a
role in the growth dynamics of primary pilocytic astrocytoma tumors, we provide evidence that expression of IL1B alone as well as overall expression of SASP factors defines pilocytic astrocytoma patient cohorts with highly differing outcome. This data supports and extends upon our previous findings showing that high expression of an OIS gene signature predicts good prognosis in patients with pilocytic astrocytoma (14).

In this study, SASP factors were found to be upregulated in primary human as well as murine pilocytic astrocytoma tumors. Upon induction of OIS in the cell line DKFZ-BT66, we detected significant upregulation of the SASP factors. The SASP genes have indeed been described to be regulated on a transcriptional level (54). Our approach revealed two cytokines, IL1B and IL6, as candidate SASP factors specific for OIS regulation in pilocytic astrocytoma. Indeed, upon induction and throughout OIS, the IL1 pathway was activated, while OIS induction led to increased IL6 expression, which in short-term leads to activation of the IL6 signaling pathway. However, increased IL6 expression was followed by downregulation of IL6 signaling over time in OIS, in line with known mechanisms of negative feedback for IL6 (46).

Both cytokines have already been described to play an important role in OIS (18, 39). The activation of NFκB downstream of IL1B leads to the transcription of many SASP factors, including IL1B itself, reinforcing OIS (44, 55). We were able to recapitulate the IL1 autocrine feedback activation in our model during OIS and upon rIL1B treatment. While stimulation of the IL1 pathway alone significantly reduced growth of proliferating DKFZ-BT66 cells and induced SASP gene expression as well as the OIS-characteristic cell morphology, treatment with IL1B had no effect on cell proliferation. Conversely, inhibition of the IL1 signaling pathway using the receptor antagonist anakinra was not sufficient to circumvent growth arrest in our pilocytic astrocytoma model. Indeed, it has previously been shown in fibroblast OIS models that pharmacologic inhibition of single inflammatory pathways did not lead to bypass of OIS (18). Our results underline the importance of the IL1 pathway as part of the SASP for the induction of OIS in pilocytic astrocytoma cells, but also indicate that IL1B acts in concert with other SASP factors, as inhibition of a single SASP factor in the presence of all other SASP factors was not sufficient to overcome OIS. Treatment with the broad anti-inflammatory drug dexamethasone, however, induced proliferation, exemplary inhibition of the IL1 pathway, and suppression of the SASP in DKFZ-BT66 cells in OIS. These results are in line with previously published data indicating that the SASP is inhibited by glucocorticoids (50, 51). As dexamethasone is a drug commonly used in pediatric clinical practice, this result is potentially of high clinical relevance. Taken together, these results demonstrate that the SASP plays a significant role in induction and maintenance of OIS. While induction of a senescent phenotype can be mediated by single factors, such as IL1B, in the absence of other SASP factors, the effect of the full SASP on OIS cannot be reversed by affecting single pathways, but only by treatment with, for example, broad anti-inflammatory drugs such as dexamethasone. Follow-up studies are warranted to assess the clinical impact of the use of anti-inflammatory (and immunosuppressive) drugs such as, for example, dexamethasone in pediatric patients with pilocytic astrocytoma.

Analysis of mRNA expression of the SASP in primary pilocytic astrocytoma revealed an improved PFS for patients with a high IL1B as well as high SASP factor expression, independent of extent of tumor resection and radiation. This was particularly striking in two patient populations: (i) none of the patients with high and intermediate SASP factor expression and GTR had a tumor progression, (ii) patients with STR and low SASP expression always progressed. These results could potentially have an impact on the therapeutic management and follow-up (FU) of these patient groups: (i) patients with a high or intermediate SASP sum score and GTR could potentially have longer FU intervals or even a shorter FU period overall; (ii) patients with initially subtotal resection and low or intermediate SASP sum score could benefit from a complete re-resection or possibly adjuvant therapy, to improve their PFS. The prognostic significance of the SASP sum score of course needs to be validated first in a prospective manner in upcoming clinical trials.

An intriguing aspect only partially explored in this study is the prospect of therapeutic exploitation of the SASP and OIS. Senolytic agents showed activity specifically in senescent pilocytic astrocytoma cells, while having no relevant effect on normal cells. This avenue could be exploited to specifically target dormant senescent pilocytic astrocytoma cells not amenable to conventional chemotherapy.

Although our data is characterized in the DKFZ-BT66 cell line, and at least partially validated in primary human and murine pilocytic astrocytoma tumors, it would be highly desirable to validate the cell line data in further pilocytic astrocytoma cell lines with either BRAF fusions or other MAPK alterations. The current lack of appropriate models beyond DKFZ-BT66 prevents reproduction in a second cell line, and as such represents a limitation of this study, highlighting the urgent need for new additional models at the same time.

In summary, our data demonstrate presence of the SASP in pilocytic astrocytoma, and its relevance as a strong regulator of pilocytic astrocytoma tumor growth. The SASP can induce growth arrest in proliferating pilocytic astrocytoma cells, while suppression of the SASP by anti-inflammatory treatment leads to bypass of growth arrest. The SASP factor IL1B is an important but not the singular mediator of OIS induction. The clinical relevance of the SASP is demonstrated by outcome prediction by the SASP sum score, as well as IL1B expression alone, independent of resection status.

Disclosure of Potential Conflicts of Interest
F. Sahm reports receiving speakers bureau honoraria from Agilent and Medac, and is a consultant/advisory board member for AbbVie. C.M. van Tilburg is a consultant/advisory board member for Novartis and Bayer. A. von Deimling holds ownership interest (including patents) in Antibody VE1 (BRAF-V600E) and Antibody H09 (IDH1-R132H). O. Witt is a consultant/advisory board member for Astra Zeneca and Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: J.L. Buhl, F. Selt, J.P. Martinez-Babera, I. Oehme, T. Brummer, O. Witt, T. Milde
Development of methodology: F. Sahm, J.P. Martinez-Babera, S. Pusch
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Pasch, J. Gronych, D. Kuhn, D. Capper, A. von Deimling, C. Herold-Mende, T. Milde.

Study supervision: O. Witt, T. Milde

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Buhl et al.


The Senescence-associated Secretory Phenotype Mediates Oncogene-induced Senescence in Pediatric Pilocytic Astrocytoma

Juliane L. Buhl, Florian Selt, Thomas Hielscher, et al.


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