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BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect

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The mechanisms of retinoid activity in tumors remain largely unknown. Here we establish that retinoids cause extensive apoptosis of medulloblastoma cells. In a xenograft model, retinoids largely abrogated tumor growth. Using receptor-specific retinoid agonists, we defined a subset of mRNAs that were induced by all active retinoids in retinoid-sensitive cell lines. We also identified bone morphogenetic protein-2 (BMP-2) as a candidate mediator of retinoid activity. BMP-2 protein induced medulloblastoma cell apoptosis, whereas the BMP-2 antagonist noggin blocked both retinoid and BMP-2-induced apoptosis. BMP-2 also induced p38 mitogen-activated protein kinase (MAPK), which is necessary for BMP-2- and retinoid-induced apoptosis. Retinoid-resistant medulloblastoma cells underwent apoptosis when treated with BMP-2 or when cultured with retinoid-sensitive medulloblastoma cells. Retinoid-induced expression of BMP-2 is thus necessary and sufficient for apoptosis of retinoid-responsive cells, and expression of BMP-2 by retinoid-sensitive cells is sufficient to induce apoptosis in surrounding retinoid-resistant cells.

Although retinoids induce differentiation and apoptosis in numerous cancers, their pleiotropic effects have largely confounded previous attempts to characterize specific proteins that mediate retinoid-induced cell death[1–4]. To identify the mediator(s) of retinoid activity in tumor cells, we focused on the most common malignant childhood brain tumor, medulloblastoma[5]. These primitive neuroectodermal tumors typically arise from the cerebellar vermis and show variable degrees of arrest of neural differentiation. Endogenous retinoids are secreted by radial glia within the cerebellum and by the choroid plexus adjacent to the developing cerebellum, which depends on retinoid signaling for proper control of apoptosis and differentiation[6].

Retinoids act through families of nuclear hormone receptors (RARs and RXRs) that include alpha, beta and gamma subtypes[7]. The most widely accepted model of retinoid-mediated transcriptional activity holds that in the unliganded state, dimerized retinoic acid receptors bind nuclear corepressive elements and inhibit transcription of retinoid-responsive genes. Upon ligand binding, the corepressive elements are released and other coactivating factors are recruited, resulting in gene transcription. Retinoids convert embryonic carcinoma and neuroblastoma cells into differentiated, cell cycle–arrested neurons[1,8]. Recently, clinical trials revealed that 13-*cis* retinoic acid improved 3-year event-free survival for children with high-risk neuroblastoma in remission from 29% to 45%, representing the single greatest improvement in survival for this cohort of children[2].

In this study, we show that retinoids induce extensive cell death in medulloblastomas and neural differentiation in a fraction of surviving cells. Freshly resected human medulloblastoma cells were highly sensitive to retinoids. Medulloblastoma xenografts showed abrogation of tumor growth when treated with retinoids. Using specific retinoid receptor agonists and gene expression arrays, we identified BMP-2 as a mediator of this response. BMP-2 was both necessary and sufficient to induce cell death in primary and cultured medulloblastoma cells. These results indicate a new mechanism for retinoid-mediated tumor cell death and justify further investigation of retinoids as therapeutic agents for medulloblastoma.

RESULTS

Retinoids induce apoptosis of medulloblastoma cells

To determine the activity of retinoids on primary medulloblastoma cells, surgically derived medulloblastoma specimens were placed in tissue culture media within 20 min of resection from their blood supply, mechanically triturated into near single-cell suspensions and treated separately with all-*trans* retinoic acid (ATRA), an RAR-α agonist (AGN195183; ref. 9), an RAR-β/γ agonist (tazarotene[10]) or vehicle. Seven of nine primary medulloblastoma specimens and a supratentorial primitive neuroectodermal tumor (PNET) showed significantly increased cell death in response to each of these retinoids (**Fig. 1a**). Only 32 ± 5.2% (mean ± s.e.m.) of cells remained viable after 48 h of ATRA exposure, compared with 70 ± 5.5% of vehicle-treated cells ($P = 0.0006$). Cell viability of receptor-subtype agonist-treated cells was also significantly reduced compared with vehicle (44 ± 5.6% for RAR-α agonist, $P = 0.017$; 36 ± 7.3% for RAR-β/γ agonist, $P = 0.007$). The two tumors that did not undergo apoptosis were

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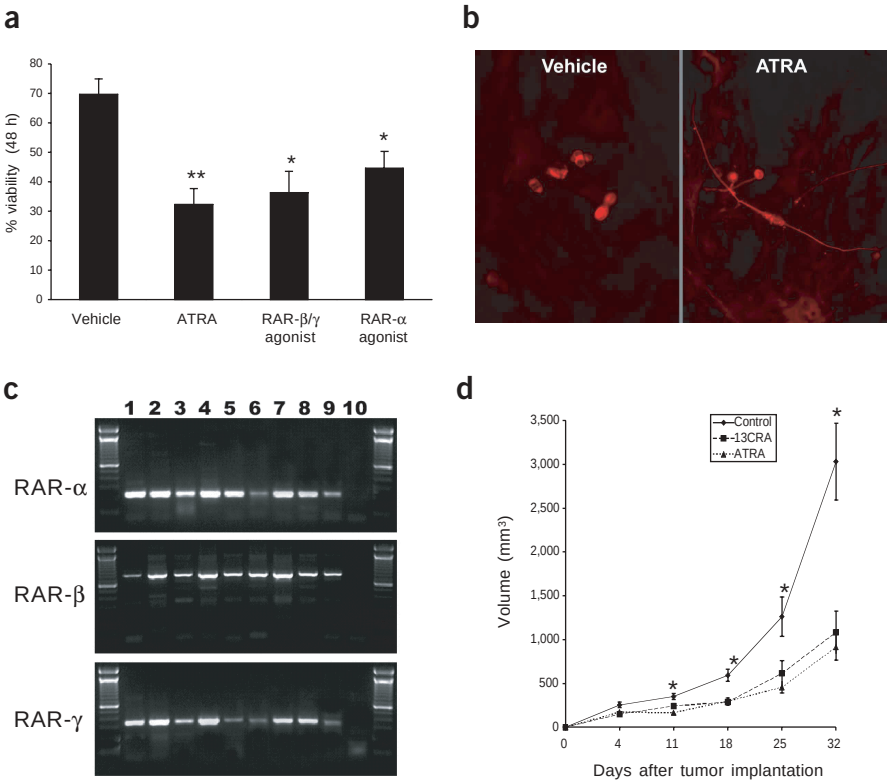

Figure 1 Retinoid activity in primary tumors and D283 xenografts. (**a**) Viability after 48 h of treatment. Data are shown as mean ± s.e.m.; $n = 10$ independent experiments. *, $P < 0.05$; **, $P < 0.005$. (**b**) Neurogenic differentiation after 7 d of culture; cells shown were immunostained with antibody to β–tubulin. (**c**) RT-PCR assay of RAR receptors in cultured and primary medulloblastoma cells. Lane 1, NMB cells known to express RARs; lane 2, D283 cells; lane 3, D341 cells; lane 4, DAOY cells; lane 5, UW228 cells; lane 6, primary medulloblastoma case R220; lane 7, primary supratentorial PNET case R228; lane 8, primary medulloblastoma R184; lane 9 primary medulloblastoma R189; lane 10, PCR negative control. (**d**) Growth of flank D283 medulloblastoma xenografts in athymic nude mice treated with 13-*cis* retinoic acid (13CRA; $n = 12$), ATRA ($n = 12$) or controls ($n = 24$). Graph shows average tumor volumes; error bars indicate s.e.m. *, $P < 0.01$.

large-cell anaplastic medulloblastomas, a less common subtype associated with poor response to therapy[11]. A subset of the surviving primary medulloblastoma cells differentiated in response to retinoids, with two- to fourfold increases in the numbers of cells showing neuronal morphology and pan-neuronal marker staining (**Fig. 1b**). Both primary medulloblastoma cells and established cell lines showed evidence of RAR expression, as assayed by RT-PCR (**Fig. 1c**)

To test the potential efficacy of retinoids as therapeutic agents *in vivo*, we established subcutaneous flank xenograft tumors in athymic nude mice with D283 cells[12]. Treatment was started 24 h after cell implantation, as retinoids have only shown therapeutic efficacy for treatment of solid tumors when used for microscopic residual disease[2]. All inoculated animals grew tumors within 4 d of injection, with decreased size and growth of tumors in retinoid-treated mice at all time points measured (**Fig. 1d**). Those treated with all-*trans* or 13-*cis* retinoic acid had tumors that were 30 ± 5% and 36 ± 8% of the volume of tumors in untreated mice after 32 d.

Because of limited amounts of primary tumor tissue, we used human medulloblastoma cell lines to elucidate the mechanism of retinoid-induced apoptosis. Two medulloblastoma cell lines, DAOY and UW228, showed no evidence of cell death in response to retinoids (retinoid-resistant lines). In contrast, ATRA induced apoptosis in the medulloblastoma lines D283 and D341 in a dose- and time-dependent fashion (retinoid-sensitive lines; **Fig. 2a**,**b**). Annexin V and propidium iodide staining for apoptosis showed that 79 ± 4% of D283 cells and 67 ± 4% of D341 cells were apoptotic after 6–8 d of retinoid treatment (**Fig. 2b**). Similar results were obtained after treatment with 13-*cis* retinoic acid. The annexin V assay was confirmed with TUNEL staining (data not shown). A pan-RAR antagonist, AGN194310 (ref. 13), prevented retinoid-induced apoptosis in D283 and D341 cells ($P < 0.001$), confirming that RAR receptors mediated the apoptotic signaling (**Fig. 2c**). Receptor-specific RAR-α (AGN195183) and RAR-β/γ (tazarotene) agonists also caused significant levels of apoptosis (**Fig. 2d**).

As seen in surgical specimens, a subset of the D283 cells that did not undergo apoptosis after treatment with retinoic acid agonists developed neuritic extensions that expressed the pan-neuronal proteins, α-internexin and medium-chain neurofilament (**Fig. 2e**). Ten percent of ATRA-treated D283 cells underwent significant neuronal differentiation after 1 week. Similarly, 13-*cis* retinoic acid and the RAR-specific agonists caused differentiation of a subset of surviving D283 cells. No differentiation was seen in vehicle-treated control cultures. Thus, in both primary cultures and established cell lines, a subset of medulloblastoma cells that do not undergo retinoid-induced apoptosis instead undergo cell cycle arrest and neuronal differentiation.

Retinoid-induced gene expression changes

The finding that both pan-retinoid and receptor-specific RAR-α and RAR-β/γ agonists were effective in inducing medulloblastoma cell death raised the possibility that mRNA induced or suppressed by all three agents was responsible. To explore this possibility, we assayed the gene expression profiles of the medulloblastoma cell lines after treatment with each drug for 24 h using 5,800 gene microarrays. Notably, ATRA induced more than three times as many genes as any of the receptor subtype–specific agents (**Fig. 3a**). Retinoids induced a similar number of transcripts in retinoid-resistant and retinoid-sensitive cell lines (data not shown), suggesting that the difference between responsive and resistant cells is due to differential induction or suppression of specific downstream effectors.

Expression of a small set of genes was induced by all RAR agonists in retinoid-sensitive but not retinoid-resistant cells (**Fig. 3b**). Three of these genes have a known role in apoptosis: ERF-2, the human homolog of Tis11d, a zinc finger protein that causes apoptosis when continuously expressed[14,15]; DDIT1 or gadd45, a downstream effector of p53 that increases when cells undergo apoptosis[16]; and BMP-2, a mediator of retinoid-induced apoptosis in several developmental paradigms[17–19]. BMP-2 induces apoptosis

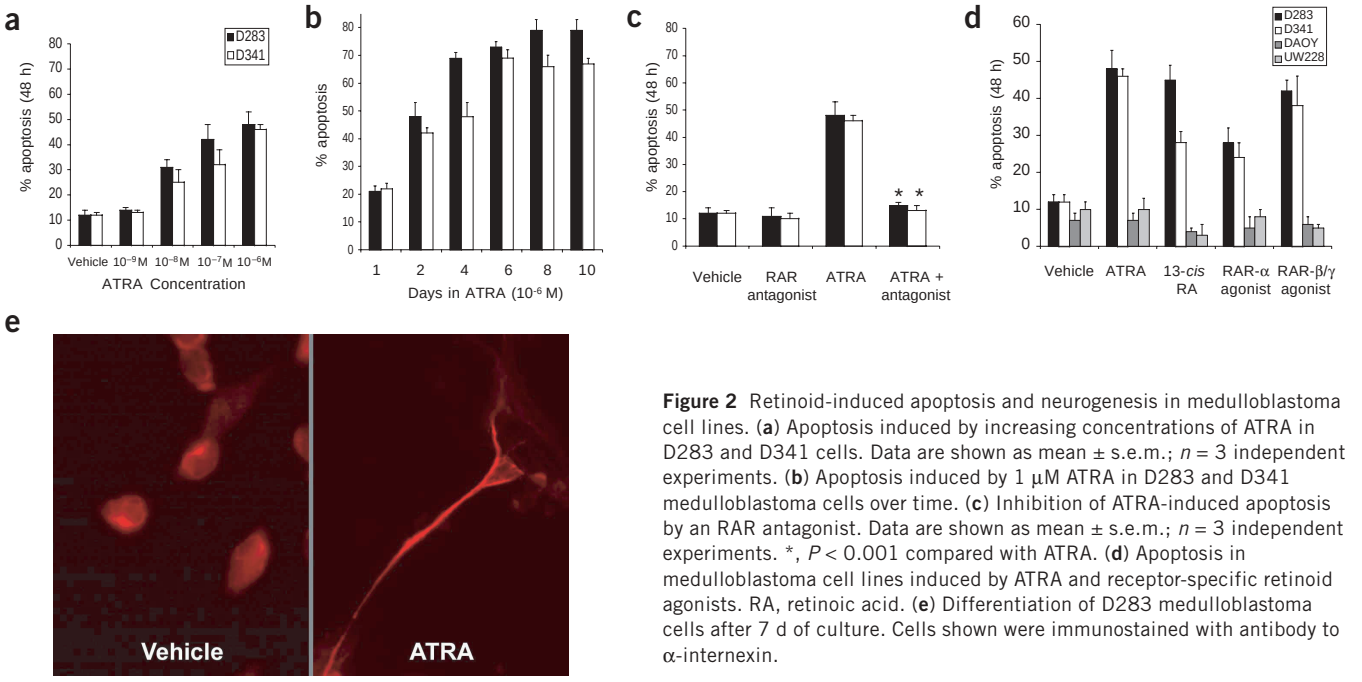

Figure 2 Retinoid-induced apoptosis and neurogenesis in medulloblastoma cell lines. (**a**) Apoptosis induced by increasing concentrations of ATRA in D283 and D341 cells. Data are shown as mean ± s.e.m.; $n = 3$ independent experiments. (**b**) Apoptosis induced by 1 μM ATRA in D283 and D341 medulloblastoma cells over time. (**c**) Inhibition of ATRA-induced apoptosis by an RAR antagonist. Data are shown as mean ± s.e.m.; $n = 3$ independent experiments. *, $P < 0.001$ compared with ATRA. (**d**) Apoptosis in medulloblastoma cell lines induced by ATRA and receptor-specific retinoid agonists. RA, retinoic acid. (**e**) Differentiation of D283 medulloblastoma cells after 7 d of culture. Cells shown were immunostained with antibody to α-internexin.

in myeloma cells[20] and also has opposing activity to sonic hedgehog in neural stem cells, a pathway critical for the development and growth of medulloblastomas[21,22]. The changes in BMP-2 expression were confirmed by RT-PCR (**Fig. 3c**), and we further explored its role in mediating retinoid-induced apoptosis in medulloblastoma.

BMP-2 mediates retinoid-induced apoptosis

To evaluate the role of BMP-2 in primary tumors, we established short-term cultures of a retinoid-sensitive primary medulloblastoma and a supratentorial PNET. Treatment with ATRA resulted in a 4.5 ± 0.6-fold and a 29.8 ± 5.4-fold increase in BMP-2 expression in the medulloblastoma and supratentorial PNET cells, respectively, as measured by

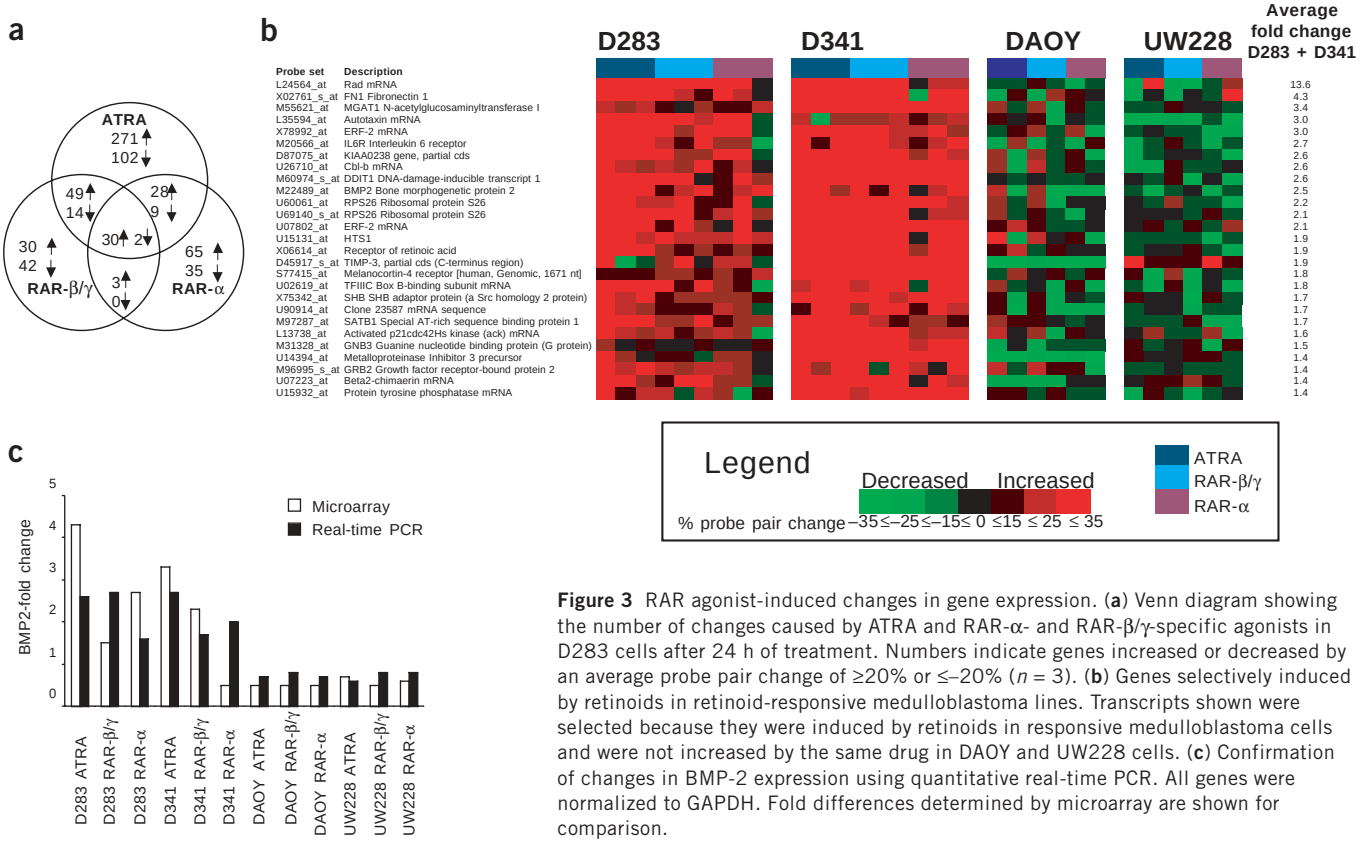

Figure 3 RAR agonist-induced changes in gene expression. (**a**) Venn diagram showing the number of changes caused by ATRA and RAR-α- and RAR-β/γ-specific agonists in D283 cells after 24 h of treatment. Numbers indicate genes increased or decreased by an average probe pair change of ≥20% or ≤–20% ($n = 3$). (**b**) Genes selectively induced by retinoids in retinoid-responsive medulloblastoma lines. Transcripts shown were selected because they were induced by retinoids in responsive medulloblastoma cells and were not increased by the same drug in DAOY and UW228 cells. (**c**) Confirmation of changes in BMP-2 expression using quantitative real-time PCR. All genes were normalized to GAPDH. Fold differences determined by microarray are shown for comparison.

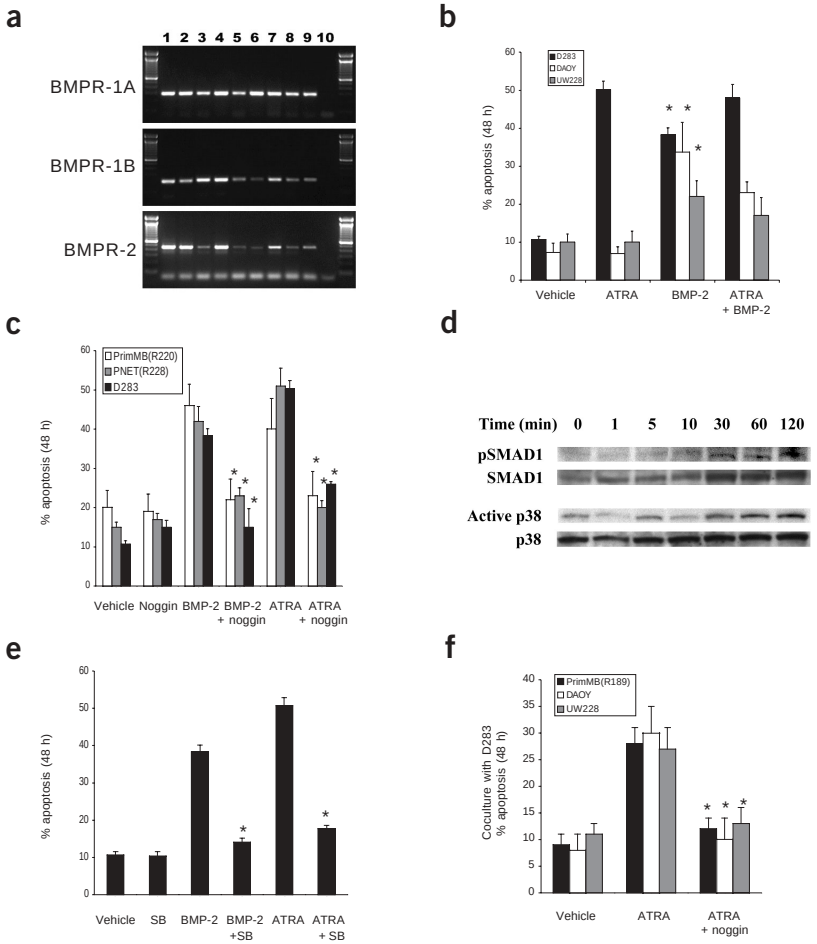

Figure 4 BMP-2 activity on medulloblastoma cells. (**a**) RT-PCR assay of BMP receptors (BMPR). Lane 1, MCF-7 cells known to express BMP receptors; lane 2, D283 cells; lane 3, D341 cells; lane 4, DAOY cells; lane 5, UW228 cells; lane 6, primary medulloblastoma case R220; lane 7, primary supratentorial PNET case R228; lane 8, primary medulloblastoma R184; lane 9 primary medulloblastoma R189; lane 10, PCR negative control. (**b**) BMP-2 induced apoptosis in retinoid-sensitive (D283) and retinoid-resistant (DAOY, UW228) cells. Data are shown as mean ± s.e.m.; $n = 3$. *, $P < 0.01$. In D283 cells, BMP-2-induced apoptosis was 76 ± 2% of ATRA-induced apoptosis (*, $P < 0.05$). (**c**) Noggin inhibition of BMP-2- and ATRA-induced apoptosis in primary human and D283 cells. Data are shown as mean ± s.e.m.; $n = 3$. *, $P < 0.05$ when compared with treatment with BMP-2 or ATRA alone. (**d**) Changes in phosphorylated SMAD1 (pSMAD1) and phosphorylated (active) p38 in response to BMP-2 in D283 cells. Quantification of band density indicated a threefold increase in pSMAD1 when corrected for nonphosphorylated SMAD; likewise, a fourfold increase in phosphorylated p38 was seen by 120 min. (**e**) Specific inhibition of p38 MAPK activity with SB203580 (SB) abrogates BMP-2- and ATRA-induced apoptosis of D283 cells. Data are shown as mean ± s.e.m.; $n = 3$. *, $P < 0.01$. (**f**) Apoptosis was induced in a retinoid-resistant primary medulloblastoma (PrimMB(R189)), DAOY and UW228 cells when cocultured with retinoid-sensitive D283 cells. Data are shown as mean ± s.e.m.; $n = 3$. *, $P < 0.01$.

RT-PCR. Medulloblastoma cell lines and primary medulloblastoma cells expressed the receptors for BMP-2 (**Fig. 4a**). Addition of purified BMP-2 protein resulted in significant apoptosis of human primary medulloblastoma cells as well as retinoid-sensitive and retinoid-resistant cell lines ($P < 0.01$; **Fig. 4b**,**c**). This response was dependent on both time and dose, and did not increase the amount of apoptosis caused by ATRA, supporting the hypothesis that BMP-2 is mediating retinoid-induced apoptosis. Although BMP-2 had previously been reported to inhibit apoptosis in DAOY cells[23], this conflicted with its proapoptotic activity in multiple developmental paradigms[17,24,25]. We were unable to replicate this earlier study. In contrast, BMP-2 induced apoptosis at doses ranging from 10 to 100 ng/ml. To antagonize BMP-2 activity, we added noggin protein. Noggin directly binds to BMP-2, thereby preventing its interaction with BMP receptors[26]. This highly specific activity has been observed in multiple models[27–30]. Addition of purified noggin protein did not alter cell viability and abrogated BMP-2 induced apoptosis as predicted (**Fig. 4c**). Retinoid-induced apoptosis of primary tumor cells was completely inhibited by noggin and was reduced by 55 ± 1% in D283 cells (**Fig. 4c**). BMP-2 did not cause neural differentiation of D283 cells. Thus, BMP-2 signaling is necessary and sufficient to mediate retinoid-induced apoptosis of both primary and cultured medulloblastoma cells.

Signaling by BMP-2 and other members of the transforming growth factor-β (TGFβ) superfamily is mediated by heteromeric complexes of two types of transmembrane serine/threonine kinase receptors expressed on the cell surface[31,32]. Ligand binding induces phosphorylation of SMAD proteins and MAPKs[33,34]. To assess these downstream signal pathways, western blotting was done on cell lysates after stimulation with BMP-2 protein. This revealed an increase in both phosphorylated SMAD1 and the phosphorylated form of p38 (**Fig. 4d**). As p38 MAPK activation has been associated with TGFβ and BMP-induced apoptosis[35,36], we evaluated the necessity of this by pharmacologic inhibition of p38 activation using the highly specific antagonist SB203580 (ref. 37). SB203580 abrogated apoptosis induced by retinoic acid and BMP-2, indicating that activation of p38 MAPK is required for apoptosis of medulloblastoma cells (**Fig. 4e**).

BMP-2 is a secreted protein, and exogenous BMP-2 induced apoptosis in retinoid-resistant cells. Therefore, we tested whether the BMP-2 expressed by retinoid-sensitive cells would induce apoptosis in cells that were retinoid-resistant in isolation. Retinoid-sensitive D283 cells were cultured with a retinoid-resistant primary tumor, as well as with DAOY and UW228 cells, separated by a semipermeable membrane (**Fig. 4f**). The previously retinoid-resistant cell lines showed increased levels of apoptosis when cultured with ATRA-treated D283 cells. This effect was completely inhibited by the addition of noggin protein. Thus, retinoid-induced BMP-2 expression in retinoid-sensitive cells can induce apoptosis in retinoid-resistant cells through a paracrine effect, indicating that retinoid-based therapies might be effective against tumors that contain mixed populations of resistant and sensitive cells.

DISCUSSION

The use of gene expression arrays to identify the transcriptional target(s) that mediate drug or signal transduction activity is often prob-

lematic because of the large number of 'hits' obtained. Within 24 h, ATRA altered expression of approximately 7% of mRNA evaluated, through RAR and RXR receptors and perhaps through interference with other transcriptional programs such as AP-1 (refs. 4, 38). In this study, the availability of multiple retinoid agonists and cell lines that were either retinoid-sensitive or retinoid-resistant permitted reduction of the candidate list from 378 mRNAs that were induced by ATRA in D283 cells to 26 mRNAs that were induced by all three agonists in sensitive, but not resistant, cells (**Fig. 4b**). The arrays do not measure the expression of all genes, and other important transcriptional changes may be occurring. BMP-2 caused apoptosis but not neural differentiation, indicating that RAR agonists activate additional biologically significant signaling pathways.

There is a pressing need for effective, low-toxicity therapies for children with medulloblastoma. Investigation of retinoids in medulloblastoma has been limited to studies of single cell lines, in which high doses of all-*trans* retinoic acid caused growth inhibition and chemosensitization to cisplatin[39–41]. Together with the large amount of clinical data from the use of retinoids in treating neuroblastoma[2], our study provides a strong rationale for further investigation of the efficacy of retinoids in the treatment of medulloblastoma. With the exception of the induction of TRAIL (apo-2L) in acute promyelocytic leukemia, identification of mediators of retinoid-induced cell death has been elusive[42]. We have shown for the first time that a secreted protein, BMP-2, mediates apoptosis in medulloblastoma cells that express or do not express BMP-2. This combination of cell-autonomous and nonautonomous activity should increase the efficacy of retinoid therapy in tumors.

METHODS

Primary medulloblastoma specimens and cell culture. Primary brain tumor tissue was obtained from patients undergoing surgical resection at Seattle Children's Hospital and Regional Medical Center, after informed consent, and processed as described[43]. These studies had prior approval from the Institutional Review Board of the Hospital. Cells were cultured in media containing 1 μM ATRA (Sigma), tazarotene (Allergan), AGN195183 (Allergan) or ethanol vehicle (1:1,000). Cell survival in human primary brain tumor cell cultures was measured at 48 h using annexin V and propidium iodide[43]. Short-term cultures of a primary human medulloblastoma and a primary supratentorial PNET were established using DMEM-F12 with 10% bovine calf serum (Hyclone). Neural origin of the cultured cells was confirmed by immunostaining for neural cell adhesion molecule (Becton Dickinson). Cells were tested between passages 2 and 6. Medulloblastoma cell lines were grown as previously described[44]. Cell viability and apoptosis assays were done as described for primary tumors. The RAR antagonist AGN194310 (Allergan) was used at a final concentration of 1 μM. In coculture experiments, 1×10^6 D283 medulloblastoma cells were cultured with the same number of DAOY or UW228 cells separated by a cell-culture membrane insert (Becton Dickinson), with ATRA or noggin protein or both added as indicated. Recombinant mouse noggin (R&D Systems) and human BMP-2 (Research Diagnostics) had been tested for specific biologic activity. They were used at concentrations established to give >90% of their specific biologic activities (3 μg/ml for noggin and 100 ng/ml for BMP-2). The specific p38 MAPK inhibitor SB203580 (Calbiochem) was used at 6 μM, the half-maximal inhibitory concentration of this agent. The average cell viability for each condition was compared to that of the vehicle-treated cells using an unpaired Student's *t*-test with a Bonferroni correction for multiple comparisons where appropriate.

Immunochemistry. Primary medulloblastomas or cell lines were grown in suspension on untreated six-well Falcon plates at 10^6 cells per well for 24 h in retinoids, and then grown on 3.5-cm poly-L-lysine-coated tissue culture plates (Becton Dickinson). Media and retinoids were replaced every 2 d during growth. Cells were immunostained as previously described[45]. Primary antibodies against α-internexin (1:500; Chemicon), medium-chain neurofilament (1:1,000; Chemicon) or β-tubulin III (TUJ-1; 1:1,000; Covance) were used. Rhodamine-conjugated secondary antibodies (1:1,000; Jackson Laboratories) were then added. The cells were assessed for neuronal staining using fluorescence microscopy.

Western blotting. D283 cells treated with BMP-2 (100 ng/ml) were harvested, protein was extracted and western blots were run as described[46]. Primary antibodies against active p38 (1:500; Promega), phosphorylated SMAD1 (1:200; Calbiochem), p38 MAPK (1:500; Cell Signaling) and SMAD1 (1:500; Upstate Biotechnology) were used to probe the blots.

Xenograft studies. Animal experiments were done according to the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by our Institutional Animal Care and Use Committee (IR #1573). Four- to six-week-old female athymic Balb/c *nu/nu* mice (Jackson Laboratories) were maintained at three per cage. Amber lights were used to ensure retinoid stability. All mice were fed powdered irradiated mouse chow and water *ad libitum*. To establish tumors 1×10^6 cells in MEM-α were injected subcutaneously in the flank, together with an equal volume of Matrigel (Becton Dickinson). The day after injection, mice were treated with 13-*cis* retinoic acid (200 mg per kg per d) or all-*trans* retinoic acid (25 mg per kg per d) mixed in feed. These had been previously established as the maximum tolerated doses of these agents in nude mice[47,48]. Tumors were measured in two perpendicular dimensions using calipers. Volumes were estimated using the formula $(\alpha^2 \times \beta) \div 2$, where α is the shorter of the two dimensions[49]. Values shown represent mean ± s.e.m. Statistical analysis was done using an unpaired Student's *t* test with two-tailed comparison[48].

Gene expression. Cells were seeded on Falcon 10-cm petri dishes (not tissue culture–treated) at a density of 3×10^6 cells/plate and aggregated in retinoids (1 μM) or vehicle (ethanol; 1:1,000) for 24 h. Cells were harvested in media and RNA was extracted and labeled according to the protocol recommended by Affymetrix [50]. Each sample was hybridized to an individual HuGeneFL oligonucleotide array and scanned according to the manufacturer's recommendations using a GeneChip Fluidics Station 400 (Affymetrix). Scanned image files were analyzed with GeneChip 4.0 software (Affymetrix).

RT-PCR. Total RNA (200 ng) and oligo-dT primers were used to synthesize single-stranded cDNA using the TaqMan Reverse Transcription kit (Applied Biosystems). The resulting cDNA was purified and PCR was set up using SYBR green Master Mix (Applied Biosystems), 1 μl cDNA and gene-specific primers at a final concentration of 0.3 μM (**Supplementary Table 1** online). Thermal cycling was carried out on the Applied Biosystems GeneAmp 5700 Sequence Detector and SYBR green dye intensity was analyzed using GeneAmp 5700 SDS software.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the Nature Medicine website for details).

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