Identification of select glucocorticoids as Smoothened agonists: Potential utility for regenerative medicine

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Regenerative medicine holds the promise of replacing damaged tissues largely by stem cell activation. Hedgehog signaling through the plasma membrane receptor Smoothened (Smo) is an important process for regulating stem cell proliferation. The development of Hedgehog-related therapies has been impeded by a lack of US Food and Drug Administration (FDA)-approved Smo agonists. Using a high-content screen with cells expressing Smo receptors and a β-arrestin2-GFP reporter, we identified four FDA-approved drugs, halcinonide, fluticasone, clobetasol, and fluocinonide, as Smo agonists that activate Hedgehog signaling. These drugs demonstrated an ability to bind Smo, promote Smo internalization, activate Gli, and stimulate the proliferation of primary neuronal precursor cells alone and synergistically in the presence of Sonic Hedgehog protein. Halcinonide, fluticasone, clobetasol, and fluocinonide provide an unprecedented opportunity to develop unique clinical strategies to treat Hedgehog-dependent illnesses.

steroids | Hedgehog signaling | Gli | stem cell proliferation | arrestin

The Hedgehog signaling pathway, mediated by the Smoothened (Smo) receptor, has been shown to regulate stem cells and is a fundamental regulator of organogenesis in developing embryos and tissue integrity in mature organisms (1–9). Smo agonists have been proposed as desired therapeutics for restoring tissue function in diseases associated with heart failure, neuronal injury/degeneration, wound repair, and retinal damage, where they could reactivate or stimulate repair mechanisms in situations in which normal regenerative capacity is compromised (10–14). However, for therapeutic modalities to be acceptable for a previously undescribed use in humans, clinical safety and efficacy must be demonstrated to gain U.S. Food and Drug Administration (FDA) approval. Some tool compounds of Smo, agonists such as purmorphamine, have demonstrated an ability to promote human embryonic stem cell differentiation (15), but the preclinical development of such small-molecule Smo agonists has lagged.

The interaction of Hedgehog ligand with the membrane protein Patched (Ptc) enables the seven-transmembrane receptor Smo to activate downstream Gli transcription factors (1). Activated Smo shares important behaviors with canonical G protein-coupled receptors (GPCRs), including an ability to undergo GPCR kinase phosphorylation and to recruit β-arrestin2 (βarr2) proteins for endocytosis, as shown in our previous study (16). Cyclopamine, a naturally occurring steroid alkaloid, inhibits the constitutive activity of Smo via direct antagonism, preventing its phosphorylation and interaction with βarr2. We exploited this observation to construct high-throughput high-content screens for Smo ligands, with a goal of accelerating the development of Hedgehog agonist drugs that could potentially have a role in tissue regeneration or be employed as tool compounds to study stem cell proliferation. We have identified four fluorinated glucocorticoids, halcinonide, fluticasone, clobetasol, and fluocinonide (Fig. 1*A*), all FDA-approved compounds, as Smo agonists that activate Hedgehog signaling and promote the proliferation of primary neuronal stem/precursor cells alone and synergistically in the presence of Sonic Hedgehog (Shh).

Results

Fluorinated Glucocorticoid Smo Agonist (FGSA) Drugs Promote Smo Intracellular Aggregation with βarr2-GFP and Promote Smo Internalization. Included among the chemical libraries we screened using a 384-multiwell format was the Prestwick Chemicals Library containing FDA-approved drugs. It contains 68 glucocorticoids or structurally related steroid compounds, including cortisone and dexamethasone. The primary screening assay employed U2OS cells, chosen for adherence, flatness, and stable expression of βarr2-GFP, and a tail substitution mutant of Smo, Smo-633, which provided better sensitivity than WT Smo (16, 17). Images of Smo-633/βarr2-GFP complexes were obtained at the rate of 5,000 per day using an automated confocal-based plate reader (ImageXpress Ultra; Molecular Devices). A read-out of compound activity for each well was provided by analyzing the corresponding image for changes in βarr2-GFP distribution that occurred as a result of compound addition.

When expressed in cells without the addition of exogenous Smo, βarr2-GFP is distributed homogenously throughout the cytoplasm (Fig. 1*B*). The overexpression of Smo (16) or Smo-633 caused a redistribution of βarr2-GFP to intracellular vesicles/aggregates (Fig. 1*C*). At a concentration of 100 nM or greater, the Smo antagonist cyclopamine (18) reverses this effect and forces βarr2-GFP back into a homogeneous distribution (Fig. 1*D*). Intravesicular aggregation of βarr2-GFP can be restored in the presence of 100 nM cyclopamine with 5 μM Smo agonist (SAG) (19) or purmorphamine (20, 21), both of which are known small-molecule Smo agonists (Fig. 1 *E* and *F*). In our primary assay, a Smo agonist is identified by its ability to aggregate βarr2-GFP in the presence of 100 nM cyclopamine in the steady-state model.

Similar to the positive control, each hit compound at 5 μM overcame the inhibition by 100 nM cyclopamine to produce intracellular βarr2-GFP aggregates (Fig. 1 *G–J*). Hit compound agonist activities in U2OS cells were also confirmed using a βarr2-GFP assay with WT Smo (16) (Fig. S1). As assessed using the primary assay, the EC_{50}s for halcinonide, fluticasone, clobetasol, and fluocinonide are 1.1 ± 0.1 μM, 99 ± 1.4 nM, 1.5 ± 0.1 μM, and >5 μM, respectively, whereas the EC_{50}s for the positive control agonists SAG and purmorphamine are 0.9 ± 0.1 nM and >5 μM, respectively (Fig. 1*K* and Table 1). In com-

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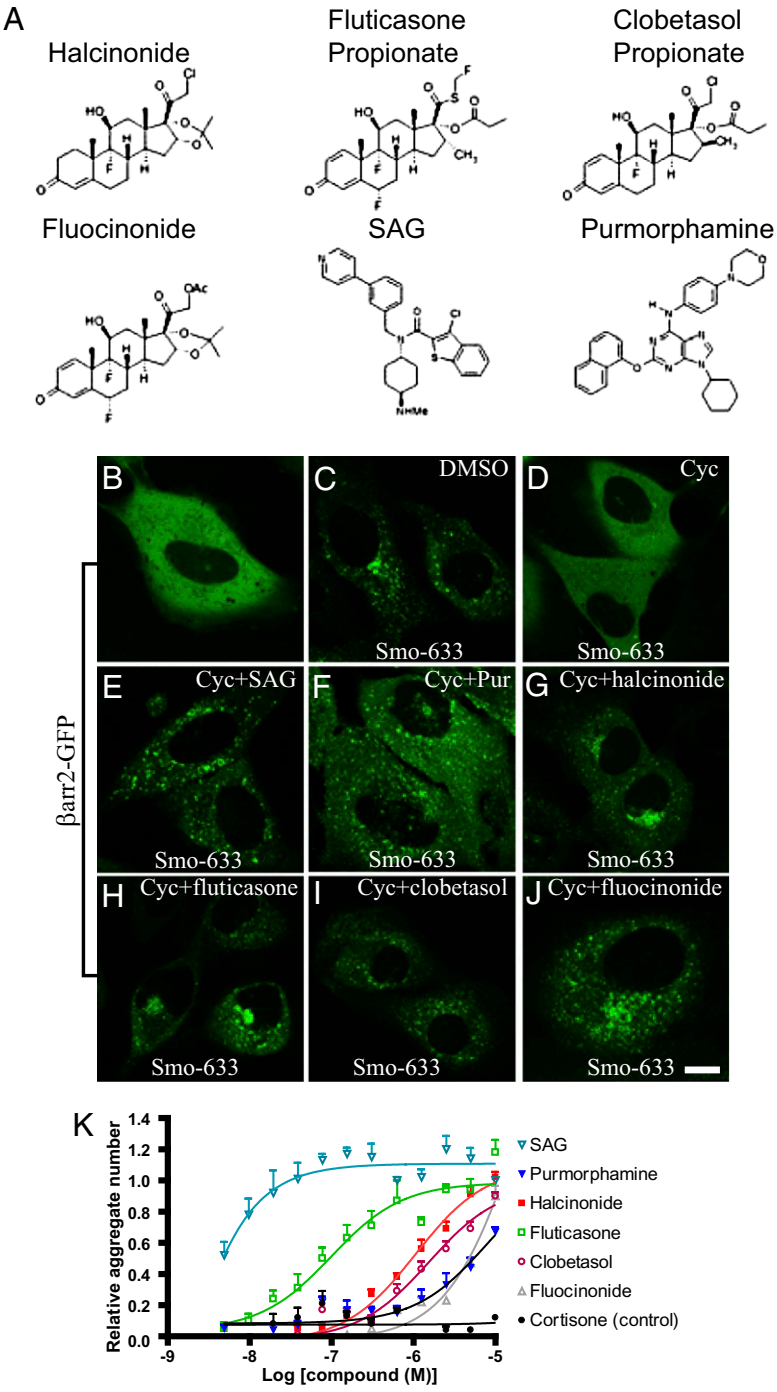

Fig. 1. FGSA drugs halcinonide, fluticasone, clobetasol, and fluocinonide as well as cyclopamine, SAG, and purmorphamine regulate the intracellular distribution of βarr2-GFP in cells stably overexpressing Smo-633 and βarr2-GFP. (*A*) Structures of the glucocorticoid drugs, SAG and purmorphamine. Confocal images of βarr2-GFP expressed alone (*B*) or stably with Smo-633 in U2OS cells (*C–J*). Cells were treated with DMSO (*C*), 100 nM cyclopamine (*D*), 100 nM cyclopamine and 5 μM SAG (*E*), 100 nM cyclopamine and 5 μM purmorphamine (*F*), 100 nM cyclopamine and 5 μM halcinonide (*G*), 100 nM cyclopamine and 5 μM fluticasone (*H*), 100 nM cyclopamine and 5 μM clobetasol (*I*), and 100 nM cyclopamine and 5 μM fluocinonide (*J*) for 2 h at 37 °C. Representative images of three independent experiments are shown. (Scale bar: 10 μm.) Cyc, cyclopamine; Pur, purmorphamine. (*K*) Concentration response profile of Smo/βarr2-GFP aggregate formation. U2OS cells stably expressing Smo-633 and βarr2-GFP were pretreated with 100 nM cyclopamine overnight in 384-well screening plates. The cells were then treated with compounds over a range of concentrations from 0–10 μM for 2 h. Tiff images of cell responses acquired on an ImageXpress Ultra were analyzed by the platform-accompanying software Transfluor HT (Molecular Devices) to quantify the aggregates produced by the compounds. The data were analyzed by nonlinear regression and fit to a sigmoid dose–response using GraphPad Prism (GraphPad Software, Inc.). Data were acquired in triplicate from three independent experiments and are presented as the mean ± SEM.

parison to SAG, which has an efficacy of 1.00 ± 0.08 in the primary assay, the efficacies for halcinonide, fluticasone, and clobetasol are 0.99 ± 0.05, 0.89 ± 0.05, and 0.87 ± 0.05, respectively, whereas the efficacies for purmorphamine and fluocinonide, although greater than 0.5, could not be determined at 10 μM because of the absence of plateaus for the fitted curves (Fig. 1*K* and Table 1). On the basis of our findings that some steroids are Smo activators, we screened a biased steroid library from Sigma containing 1,658 compounds; however, no additional hits were identified. Additionally, in a test of specificity, neither SAG nor the four hits induced βarr2-GFP aggregation with three control seven-transmembrane receptors, including the human vasopressin type 2 receptor (V2R).

We previously demonstrated that SAG induces Smo internalization (16). Fig. 2 *A–D* shows that in HEK293 cells, 2 μM SAG and 5 μM purmorphamine each stimulate Smo-YFP to internalize. All four primary assay hit compounds similarly induce Smo internalization, consistent with their roles as Smo agonists. Fig. 2 *E–H* shows representative results for halcinonide and fluticasone. In contrast, in control endocytosis experiments in HEK293 cells testing specificity for Smo, neither SAG nor fluticasone produced V2R internalization (Fig. S2).

Fluorinated Glucocorticoid Smo Agonist Drugs Displace Bodipy-Cyclopamine from Smo-Overexpressing Cells. Bodipy-cyclopamine has been used to assess ligand binding to Smo (19). We measured by saturation binding in HEK293 cells stably expressing

Table 1. Potency and efficacy data of Smo agonists

Compound	Primary assay		Bodipy-cyclopamine binding		Gli-luciferase assay	
	EC_{50}, nM	Efficacy	EC_{50}, nM	Efficacy	EC_{50}, nM	Efficacy
SAG	0.9 ± 0.1	1.00 ± 0.08	11 ± 0.5	1.00 ± 0.01	27 ± 2.5	1.00 ± 0.14
Purmorphamine	>5,000	NA	>5,000	NA	>5,000	NA
Halcinonide	1,100 ± 100	0.99 ± 0.05	78 ± 2.1	0.24 ± 0.02	1.8 ± 0.13	0.74 ± 0.08
Fluticasone	99 ± 1.4	0.89 ± 0.05	58 ± 1.2	0.34 ± 0.01	0.3 ± 0.02	0.45 ± 0.05
Clobetasol	1,500 ± 100	0.87 ± 0.05	57 ± 2.6	0.24 ± 0.02	0.2 ± 0.02	0.51 ± 0.06
Fluocinonide	>5,000	NA	1,000 ± 300	0.30 ± 0.01	0.3 ± 0.02	0.61 ± 0.07

Results are presented as the mean ± SEM of at least three experiments. NA, not able to be determined.

WT Smo that the affinity (K_d) of bodipy-cyclopamine for Smo is 3.5 ± 0.8 nM (Fig. S3). In competition binding using the same cell line, we observed that SAG completely displaced 5 nM bodipy-cyclopamine from Smo (defined as an efficacy of 1.00), with an EC_{50} of 11 ± 0.5 nM (Fig. 3 and Table 1). As opposed to cortisone, which is unable to displace bodipy-cyclopamine from Smo up to 10 μM (0 efficacy), the EC_{50}s and efficacy for displacement for halcinonide, fluticasone, clobetasol, and fluocinonide are, respectively, 78 ± 2.1 nM, 0.24 ± 0.02; 58 ± 1.2 nM, 0.34 ± 0.01; 57 ± 2.6 nM, 0.24 ± 0.02; and 1,000 ± 300 nM, 0.30 ± 0.01 (Fig. 3 and Table 1). We also observed that the known Smo agonist purmorphamine displaced bodipy-cyclopamine as a weak inhibitor, as previously described (22), with an EC_{50} >5 μM, which is less than that observed for the four steroid agonists.

Fluorinated Glucocorticoid Smo Agonist Drugs Activate Gli-Luciferase Reporter. Shh binding to Ptc relieves Ptc inhibition of Smo and results in activation of the Gli transcription factor (18), making Gli-luciferase reporter assays important indicators of activity downstream of Smo. SAG was discovered using such a Gli reporter assay (19). In a Gli assay performed in Shh-LIGHT2 cells and using only the endogenous Hedgehog signaling machinery, the four fluorinated steroids activated the Gli-luciferase reporter in a dose-dependent manner (Fig. 4*A* and Table 1). As expected, the negative control cortisone had no Gli activity in Shh-LIGHT2 cells. In addition, there does not appear to be a non-Smo-mediated pathway that would produce the same type of response (21) (Fig. S4).

We also investigated whether Shh activity from conditioned media could be potentiated by the steroid Smo agonists in Shh-LIGHT2 cells. Gli-luciferase activity from compound treatment was measured relative to a DMSO control (activity defined as 1). Shh alone at 0.5% produced a 3.8-fold increase in Gli response. We found that Gli-luciferase activity attributable to the combination of Shh (0.5%) and 5 μM halcinonide, fluocinonide, clobetasol, or fluticasone was increased compared with Shh or compound treatment alone (Fig. 4*B*). Interestingly, the combination of 5 μM SAG or purmorphamine plus 0.5% Shh did not result in significant activity change compared with either SAG or purmorphamine treatment alone.

Halcinonide, Fluticasone, and Clobetasol Promote Mouse Cerebellar Granule Cell Precursor Proliferation. Cerebellar granular cell precursors (GCPs) differentiate into distinct types of mature neurons that comprise the most abundant neurons in the brain (3, 23, 24), and the expansion in vivo of these granule precursor cells requires Hedgehog/Smo pathway signaling (23). We used a mouse GCP proliferation assay to test the growth-promoting effects of Hedgehog agonist compounds. GCPs were treated for 48 h with one of the Smo agonists, purmorphamine or SAG (positive controls); the lead compounds halcinonide, fluticasone, clobetasol, and fluocinonide; or the negative control compound cortisone. SAG had approximately a 2-fold greater efficacy than purmorphamine in promoting a GCP proliferative response. Relative to DMSO vehicle, the treatment by halcinonide resulted in a 40–50-fold increase in GCP proliferation that was similar to the maximal response produced by purmorphamine (Fig. 5*A*). Fluticasone and clobetasol had a 5–6-fold stimulatory effect, while fluocinonide or cortisone had no effect (Fig. 5*A*, *Inset*). Experiments repeated in the presence of 5 μM mifepristone (RU-486), a glucocorticoid nuclear receptor antagonist, gave similar results (Fig. S5).

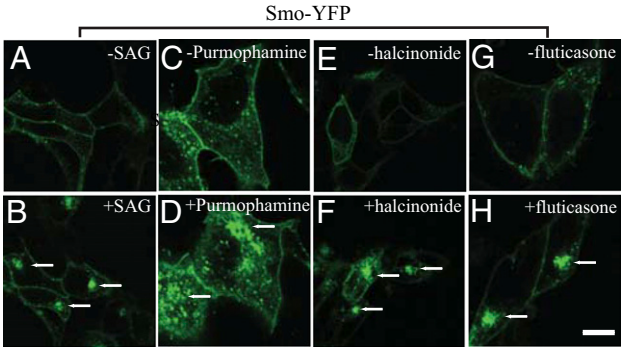

Fig. 2. Smo agonists induce Smo-YFP internalization. Effects of SAG, purmorphamine, halcinonide, and fluticasone on Smo-YFP internalization are shown. Confocal images of Smo-YFP expressing HEK293 cells left untreated (*A*, *C*, *E*, and *G*) and treated with 2 μM SAG (*B*), 5 μM purmorphamine (*D*), 2 μM halcinonide (*F*), and 2 μM fluticasone (*H*) for 30–40 min at 37 °C. Arrows indicate internalized Smo-YFP. Representative images from three independent experiments are shown. (Scale bar: 10 μm.)

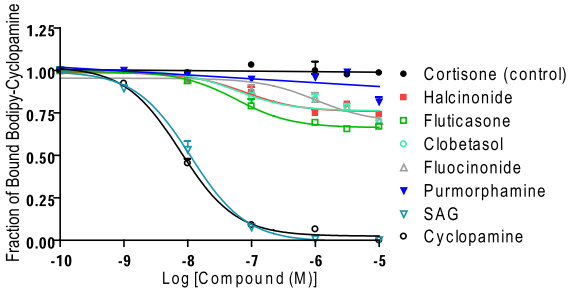

Fig. 3. Smo agonist competitively replaces bodipy-cyclopamine binding to Smo. Competitive binding of bodipy-cyclopamine with Smo agonists was performed in HEK293 cells, as described in *Materials and Methods*. Data were normalized to the maximal binding of bodipy-cyclopamine over baseline. Competition curves for each compound were initially analyzed by linear regression, and those compounds that generated a line with a slope not significantly different from zero (cortisone, $P = 0.59$; purmorphamine, $P = 0.12$; $n = 3$; $\alpha = 0.05$) were considered not able to compete with bodipy-cyclopamine for Smo binding. The displacement data of the remaining compounds were analyzed by fitting to a one-site competition curve using GraphPad Prism (GraphPad Software, Inc.). Data were acquired in triplicate from three independent experiments and are presented as the mean ± SEM.

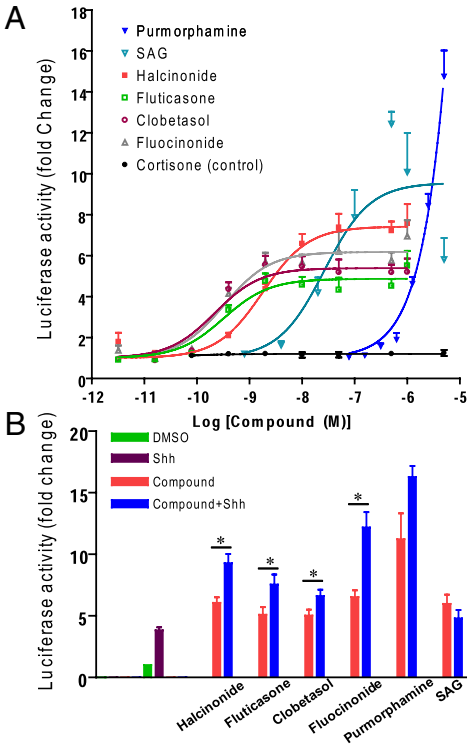

Fig. 4. Gli-luciferase response in Shh-LIGHT2 cells treated with Smo ligands. (*A*) Gli-luciferase reporter activity in Shh-LIGHT2 cells in response to Smo agonists. Shh-LIGHT2 cells cultured to confluence were individually treated for 30 h with the following compounds: halcinonide, fluticasone, clobetasol, fluocinonide, the positive controls purmorphamine and SAG, and the negative control cortisone. Results are presented as the mean ± SEM from multiple individual experiments ($n > 3$) performed in triplicate. (*B*) Effects of Shh-conditioned media (Shh) on Smo agonists. Shh-LIGHT2 cells were cultured to confluence and treated for 30 h with DMSO, 2% Shh, 5 μM of the indicated compounds, or 5 μM of the indicated compounds in the presence of 2% Shh. Results are presented as the mean ± SEM from multiple individual experiments ($n \geq 3$) performed in triplicate. The statistical significance was analyzed by a two-tailed Student's *t* test, with $^*P < 0.05$ ($\alpha = 0.05$) defined as significant.

Using [^{3}H]thymidine incorporation, we further investigated the relationship between Shh and the Smo agonists on GCP proliferation. Shh (2%) induced a 17-fold increase of GCP proliferation as opposed to the marginal GCP proliferation response to 5 μM halcinonide, fluticasone, clobetasol, or fluocinonide; 0.073 μM purmorphamine; or 0.008 μM SAG (Fig. 5*B*). Treatment of the GCP cells with 2% Shh and an agonist compound resulted in increased GCP proliferation ranging from 30-fold (fluocinonide) to 95-fold (SAG), indicating strong synergism (Fig. 5*B*). Interestingly, the glucocorticoid receptor (GR) agonist dexamethasone had a tendency to inhibit the activity of 2% Shh (Fig. 5*B*), as previously described (25). To verify further the opposite effects that the Smo agonists have on proliferation compared to dexamethasone, we treated GCP cells with Shh (2% and 20%) and various concentrations of halcinonide or dexamethasone in the absence or presence of 2% Shh. Synergism between 2% Shh and halcinonide was observed in the proliferation assay; such responses were equal to or greater than the stimulatory effects produced by 20% Shh (Fig. 5*C*, *Left*). In comparison, dexamethasone inhibited Shh-activated GCP proliferation in a dose-dependent manner (Fig. 5*C*, *Right*).

It has been reported that Hedgehog signaling promotes GCP proliferation through up-regulation of cyclin D2 and inhibition of proteasomal degradation of caspase-3, whereas dexamethasone and several other GR agonists have the opposite effect by inhibiting GCP proliferation and not inhibiting GCP apoptosis (25). Although all the fluorinated glucocorticoid smoothened agonists from our study possess the ability, like dexamethasone, to activate GR as assessed by a GR-GFP nuclear translocation assay (Fig. S6), GCP treatment with Shh; purmorphamine; SAG; or fluorinated halcinonide, clobetasol, and fluticasone (but not the weaker Smo agonist fluocinonide) increased endogenous cyclin D2 protein expression and inhibited caspase-3 degradation (Fig. 6). No such growth-enabling responses were observed in GCPs treated with cortisone, dexamethasone, prednisolone, or corticosterone, observations consistent with recent reports (25–27) (Fig. 6). The opposite response of dexamethasone in the GCP proliferation assay and its similar response in the nuclear receptor assay compared to the FGSAs suggest that the signal for the GCP proliferative response is independent of glucocorticoid nuclear receptor signaling and is most probably attributable directly to activation of Smo.

In summary, the drugs halcinonide, fluticasone, clobetasol, and fluocinonide function as Smo agonists, having an ability to bind Smo, promote Smo internalization, activate Gli, and synergistically stimulate the proliferation of primary neuronal precursor cells.

Discussion

Regenerative medicine is an emerging frontier of medical therapy that holds the promise of curing currently untreatable diseases by harnessing the body's ability to replace damaged tissues (28). Such therapies might prompt autonomous tissue repair or facilitate the implantation of engineered tissue derived from progenitors or stem cells (28, 29). Hedgehog activators are prime candidates for therapeutics to initiate or modulate tissue self-repair. In this study, we used neuronal precursor/progenitor cells derived from mouse cerebella and modulated their proliferation using these Hedgehog activators. Even though the Hedgehog pathway was identified over two decades ago, there are still only a few available research compounds that can modulate it, such as SAG and purmorphamine, and, for a variety of reasons, no clinically available agonist drugs targeted to Hedgehog-related diseases exist.

Despite our findings of FGSAs that activate Smo, a search of PubMed indicates they have no apparent association with topical cancers. One of the four hit FGSAs active in the Hedgehog assays was fluticasone. It is reported on the GlaxoSmithKline drug label that fluticasone is well tolerated orally and can be administered i.v. in humans and that its s.c. and oral median lethal doses in mice and rats are greater than 1,000 mg/kg (30).

Glucocorticoids, including the fluorinated glucocorticoids, are used clinically for the treatment of asthma, inflammation, and skin disease or injury. Other glucocorticoids, including dexamethasone, prednisone, cortisone, and corticosterone, are used to treat premature infants and have been observed to cause neuronal apoptosis and to inhibit neuronal precursors of the cerebellar granule neuronal lineage in a mouse model (25, 26). On the basis that Shh exposure cannot overcome the effects of dexamethasone but can antagonize the effects of hydrocortisone, Heine and Rowitch (25) recommend that hydrocortisone be used as a replacement for dexamethasone in infants because of the reduced potential for neurotoxicity. We have demonstrated in vitro that fluticasone and the other fluorinated steroids can be used to expand neuronal precursor cell populations and potentiate the ability of 2% Shh stimulation. Our results with FGSAs suggest that some glucocorticoids may even be neuronally protective, but this requires further clinical investigation in this particular case. For regenerative medicine, we propose that FSGAs could be used immediately orally or i.v. on an acute or chronic basis for testing in disease models in which an increase in Gli signaling and consequent Hedgehog-mediated repair are desirable, such as neovascularization after myocardial infarctions

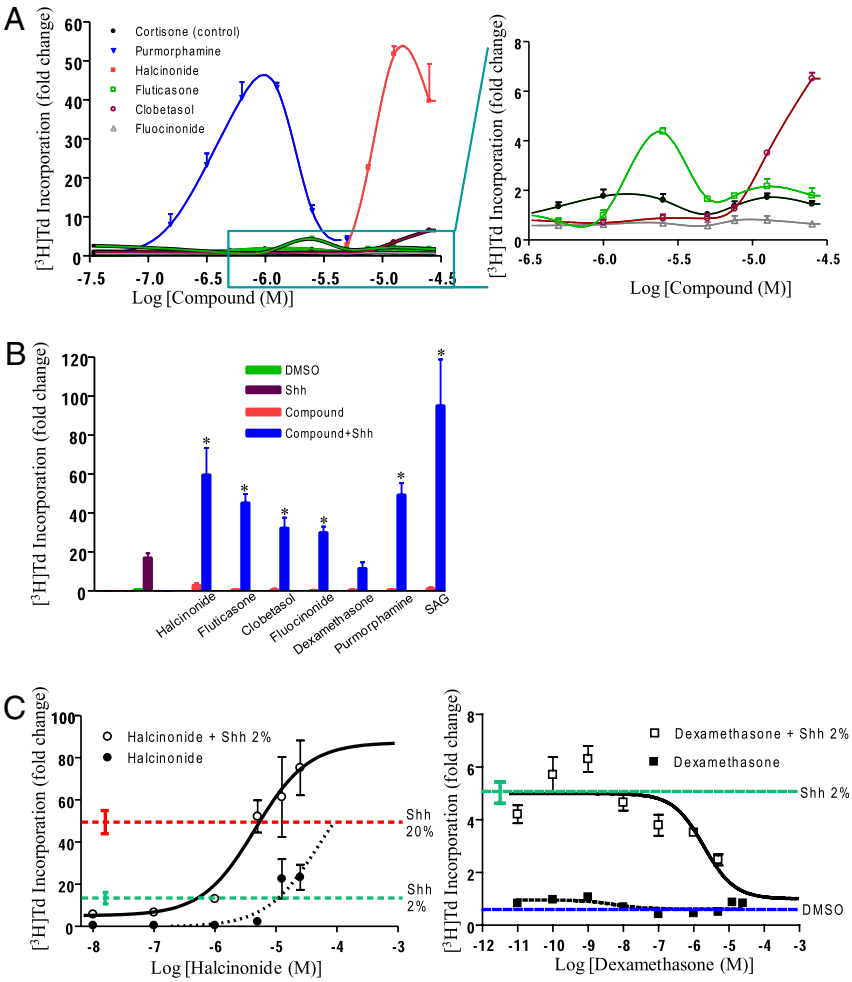

Fig. 5. Effects of FGSAs and Shh on primary neuronal GCP proliferation. (*A*) Primary neuronal GCP proliferation data of Smo agonists. Expanded version (*Right*) of the boxed region (*Left*). Cells were treated with compounds for 48 h and then pulsed with [^{3}H] thymidine ([^{3}H]Td) and cultured for 16 h before being measured for [^{3}H]Td incorporation. Cubic splines were fit to the data points using GraphPad Prism (GraphPad Software, Inc.) to highlight the responses. Data were acquired in triplicate from three independent experiments and are presented as the mean ± SEM. (*B*) Shh modulation of primary neuronal GCP proliferation in response to Smo agonists. The cells were treated with DMSO or 2% Shh alone or in the absence or presence of 2% Shh with one of the following compounds: 5 μM halcinonide, fluticasone clobetasol, or fluocinonide; 5 μM dexamethasone; and the positive control SAG (0.008 μM) or purmorphamine (0.073 μM). The [^{3}H]Td incorporation data are presented as fold change vs. DMSO treatment, which was defined as 1. Triplicate data are presented as the mean ± SEM (n = 3). The statistical significance was analyzed by a two-tailed Student's t test, with $^*P < 0.05$ ($\alpha = 0.05$) defined as significant (compound + Shh over Shh). (*C*) Halcinonide and dexamethasone have opposite effects on primary neuronal GCP proliferation. Cells were treated with DMSO, 2% Shh, 20% Shh, or halcinonide in the presence or absence of 2% Shh (*Left*) and with dexamethasone in the presence or absence of 2% Shh (*Right*; the minor change in responsiveness between experiments to 2% Shh treatment, reflected as a decrease in GCP proliferation, may result from batch-to-batch variability in Shh). Dashed lines indicate the cell responses to DMSO vehicle, 2% Shh, and 20% Shh. Data acquired in triplicate are presented as the mean ± SEM (n = 3).

(10), wound healing in diabetes (13), or neuronal regeneration after spinal cord injury (12). In summary, the well-known pharmacokinetic and pharmacodynamic properties of these FDA-approved steroid Smo agonists provide a significant jumpstart in the process of beginning human studies on their potential therapeutic applications in regenerative medicine.

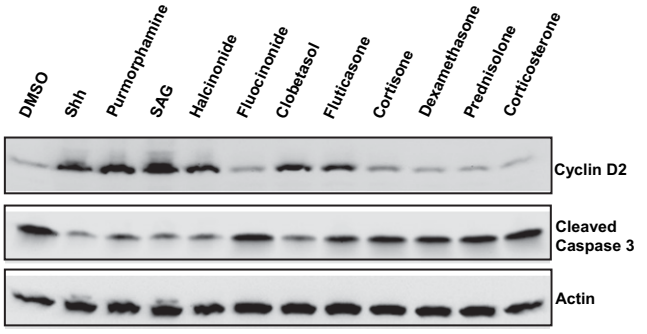

Fig. 6. Halcinonide, fluticasone, clobetasol, fluocinonide, and other glucocorticoids regulate cyclin D2 expression and caspase-3 degradation in primary neuronal GCPs. Primary neuronal GCPs derived from 4-day-old mice were individually treated for 64 h with DMSO, 2% Shh, 0.625 μM purmorphamine, 0.5 μM SAG, 2.5 μM fluticasone, and the remaining compounds at 25 μM. Cells were harvested in SDS sample buffer, protein samples were resolved on SDS/PAGE gels, and the corresponding immunoblots were probed by antibodies against cyclin D2, cleaved caspase-3, and actin (n = 3). A representative immunoblot is shown.

Materials and Methods

Materials. Details are described in *SI Materials and Methods*.

Transfection and Plasmids. Cells were transfected using either Fugene 6 (Roche) or Nucleofector (Amaxa). Details are provided in *SI Materials and Methods*.

Primary Assay-Automated High-Throughput Screening. We made multiple Smo mutants to identify the best location to attach the V2R tail (17), the addition of which, when phosphorylated and precisely located, causes βarr2-GFP to bind to Smo more strongly. Details are provided in *SI Materials and Methods*.

Bodipy-Cyclopamine Binding Analysis of Smo Agonists in Smo-Overexpressing HEK293 Cells. HEK293 cells stably expressing WT Smo were split at 166,000 cells per well in the center well (glass bottom, 10-mm diameter) of collagen-coated dishes (MatTek), followed by overnight incubation. Details are provided in *SI Materials and Methods*.

Gli-Luciferase Reporter Assay. The reporter assay using Shh-LIGHT2 cells, Smo$^{-/-}$ mouse embryo fibroblasts (MEFs), or NIH 3T3 cells was performed as described (21).

[^{3}H]Thymidine Proliferation Assay and Western Blots of Primary Neuronal GCPs. Primary GCPs were isolated from 4- or 8-day postnatal WT C57BL/6 mice, as previously described (23, 25). Details are provided in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Materials. Fluticasone propionate, halcinonide, and clobetasol propionate were independently supplied by Prestwick Chemicals and Sigma. Fluocinonide was repurchased from Prestwick Chemicals. SAG was purchased from Axxora. Purmorphamine was purchased from Calbiochem. Cortisone, dexamethasone, prednisolone, and corticosterone were purchased from Sigma. Cyclopamine and bodipy-cyclopamine were purchased from Toronto Research Chemicals.

Transfection and Plasmids. Expression vectors for βarr2-GFP, WT Smo, Smo-YFP, and Gli-luciferase reporter have been described (1–3). V2R-GFP was made in a similar manner as β2-adrenergic receptor-GFP (4). GR-GFP was provided by Terry Hinds, Jr. (University of Toledo, Toledo, Ohio). Shh conditional medium (Shh) was prepared by collecting the medium in which HEK293 cells were transfected with Shh-N plasmid (provided by Philip Beachy, Stanford University, Stanford, CA). Smo-633 was constructed by swapping the 154 amino acids of the Smo C terminus (Pro-634 to Phe-787) with the 29 amino acids of the V2R C terminus (Ala-343 to Ser-371).

Primary Assay-Automated High-Throughput Screening. Smo-633 was used in this assay because it produces a stronger signal in the U2OS Smo/βarr2-GFP assay than WT Smo, but it is otherwise pharmacologically similar. A single 15-cm plate of U2OS cells stably expressing Smo-633 and βarr2-GFP at 95–100% cell confluency was split into two glass-bottom 384-well plates (MGB101-1-2-LG; Matrical) using a Multidrop 384 dispenser (Titertek Instruments). Each well contained 25-μL aliquots of 6,000 cells in MEM [10% (vol/vol) FBS, 50 U/mL Pen/Strep] with 100 nM cyclopamine. Plates were incubated overnight at 37 °C in 5% (vol/vol) CO_2. On the following day, compounds (5 mM in DMSO) from the Prestwick Chemicals Library and the positive control (5 mM SAG) as well as the vehicle control DMSO were diluted 1:200 in MEM, and 6.25 μL of the diluted compound, SAG, or DMSO was added to each of the corresponding wells using a Biomek FX liquid handler (Beckman Coulter), for an overall 1:1,000 dilution and final concentration of 5 μM. The plates were returned to the incubator for 2 h. The media was then removed, and the cells were fixed in 30 μL of PBS containing 0.5% paraformaldehyde and a 1:50,000 dilution of DRAQ5 dye (Biostatus) to visualize the nuclei. Plates were stored at 4 °C until analysis on an ImageXpress Ultra (Molecular Devices) equipped with a 488-nM argon laser for imaging GFP and a 568-nM krypton laser for imaging DRAQ5. Plates were screened in duplicate at the rate of 5,000 wells per day and analyzed using the associated software Transfluor HT (Molecular Devices), and results were visually confirmed.

Receptor Internalization Assay. Smo-YFP and V2R-GFP internalization assays were performed as previously described (1).

Bodipy-Cyclopamine Binding Analysis of Smo Agonists in Smo-Overexpressing HEK293 Cells. Cells overexpressing wild-type Smo were washed and fixed with 4% (vol/vol) formaldehyde/PBS for 20 min at room temperature. After removing the formaldehyde buffer, the cells were incubated for 2 h at room temperature in binding buffer (HBSS without Ca^{2+} and Mg^{2+}) containing 0–50 nM bodipy-cyclopamine for saturation binding assay or 5 nM bodipy-cyclopamine and compounds over a range of concentrations from 0–10 μM for competitive binding assay. Following incubation, the cells were washed three times with the binding buffer and imaged using a Zeiss ×40 oil N.A. 1.3 plain apo objective and 488-nm excitation laser on a Zeiss LSM-510 confocal microscope. The average fluorescence intensity corresponding to each image (i.e., concentration) was determined using the accompanying histogram software. The specific binding data over baseline for compounds were normalized to the maximal binding of bodipy-cyclopamine. The data were analyzed by GraphPad Prism (GraphPad Software, Inc.) using linear and nonlinear regression.

[^{3}H]Thymidine Proliferation Assay and Western Blots of Primary Neuronal GCPs. Briefly, cerebella were removed from 4- or 8-day-old mice, minced, and digested at 37 °C in papain before being triturated in buffer containing trypsin inhibitor and BSA. Cells were then pelleted, resuspended in BSA/PBS, and strained to remove debris. Strained cells were recentrifuged and resuspended in supplemented neurobasal medium. The cells from 8-day-old mice were cultured for 48 h in the presence of compounds with or without Shh and were then pulsed with [^{3}H]thymidine and cultured for an additional 16 h before being measured for [^{3}H]thymidine incorporation.

The cells from 4-day-old mice were cultured for 64 h in the presence of DMSO vehicle, compounds, or 2% Shh. The cells were harvested in the SDS sample buffer. The protein samples were subjected to SDS/PAGE and transferred to nitrocellulose membranes, followed by incubation with three primary antibodies: cyclin D2 and cleaved caspase 3 (Cell Signaling) and actin (Santa Cruz Biotechnology, Inc.). Subsequently, immunoblots were developed using HRP-conjugated antibodies.

1. Chen W, et al. (2004) Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. *Science* 306:2257–2260.
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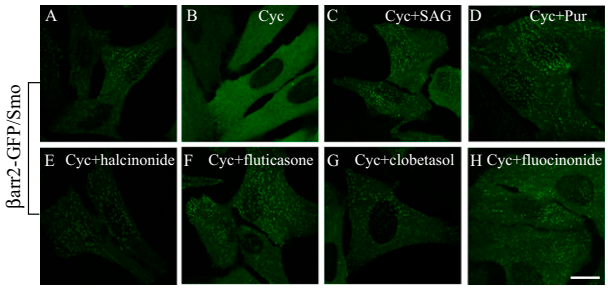

Fig. S1. Fluorinated glucocorticoid Smoothened agonist (FGSA) drugs halcinonide, fluticasone, clobetasol, and fluocinonide as well as cyclopamine, SAG, and purmorphamine regulate the membrane distribution of βarr2-GFP in cells stably overexpressing WT Smo and βarr2-GFP. (*A*–*H*) Confocal images of βarr2-GFP stably expressing Smo and βarr2-GFP in U2OS cells. Cells were left untreated (*A*) or treated with 100 nM cyclopamine (*B*), 100 nM cyclopamine and 5 μM SAG (*C*), 100 nM cyclopamine and 5 μM purmorphamine (*D*), 100 nM cyclopamine and 5 μM halcinonide (*E*), 100 nM cyclopamine and 5 μM fluticasone (*F*), 100 nM cyclopamine and 5 μM clobetasol (*G*), or 100 nM cyclopamine and 5 μM fluocinonide (*H*) for 2 h at 37 °C. In contrast to HEK293 cells, which are thicker and show aggregates at the periphery in confocal images [Chen W, et al. (2004) Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. Science 306:2257–2260], the flatter U2OS cells show aggregates over the whole surface. Representative images of three independent experiments are shown. Cyc, cyclopamine; Pur, purmorphamine. (Scale bar: 10 μm.)

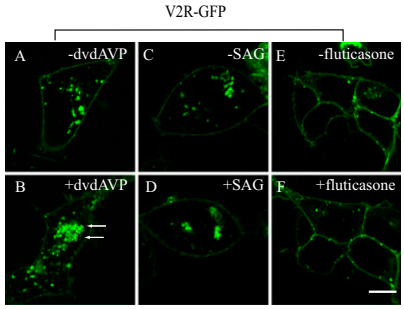

Fig. S2. SAG and fluticasone have no effects on V2R-GFP internalization. Confocal images of V2R-GFP expressed in HEK293 cells left untreated (*A*, *C*, and *E*) or treated with 2 μM V2R agonist 1-deamine-4-valine-D-arginine vasopressin (dvd AVP) (*B*), 2 μM SAG (*D*), and 2 μM fluticasone (*F*) for 30 min at 37 °C. Representative images of three independent experiments are shown. Arrows indicate internalized V2R-GFP. (Scale bar: 10 μm.)

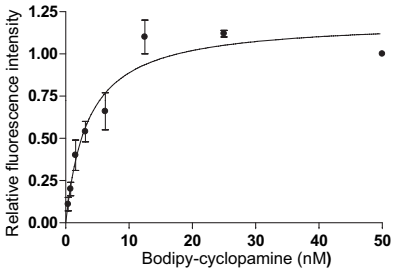

Fig. S3. Bodipy-cyclopamine saturation binding to overexpressed Smo in HEK293 cells. Binding assays were performed as described in *Materials and Methods*. The affinity (K_d) of cyclopamine for Smo was determined as 3.5 ± 0.8 nM using the program GraphPad Prism (GraphPad Software, Inc.). Data were acquired in triplicate from three independent experiments and are presented as the mean ± SEM.

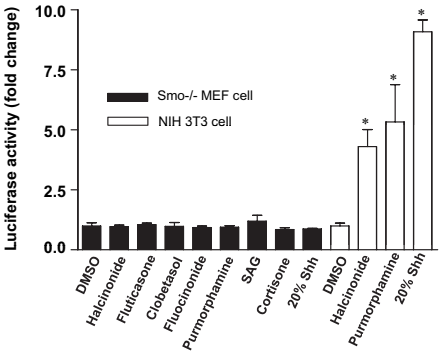

Fig. S4. Signaling of Smo agonists in Smo$^{-/-}$ mouse embryo fibroblast (MEF) cells and in NIH 3T3 cells. Smo$^{-/-}$ MEF cells or NIH 3T3 cells were transfected with Gli-luciferase reporter and control renilla pRL-TK (Promega) plasmids. The cells were cultured to confluence and treated with DMSO, 5 μM of the indicated compounds, or 20% Shh for 30 h. In Smo$^{-/-}$ MEF cells, none of the treatments resulted in a Gli-luciferase response over baseline. In NIH 3T3 cells, Gli-luciferase activities were induced by halcinonide, purmorphamine, or 20% Shh. Data are from three individual experiments performed in triplicate and presented as the mean ± SEM. The statistical significance was analyzed by a two-tailed Student's *t* test, with $*P < 0.05$ ($\alpha = 0.05$) defined as significant (over DMSO).

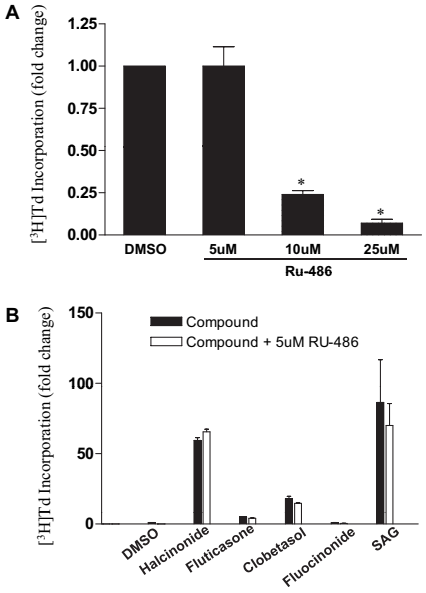

Fig. S5. GCP proliferative response to Smo agonists in the presence of mifepristone (RU-486). (*A*) RU-486 alone and GCP proliferation are shown. Primary neuronal GCPs were treated for 48 h with DMSO or RU-486 (5, 10, and 25 μM), pulsed with [^{3}H]thymidine ([^{3}H]Td), cultured for 16 h, and then assessed for [^{3}H]Td incorporation. RU-486 inhibited GCP proliferation in a dose-dependent manner. RU-486 at 5 μM had no effect on GCP proliferation. (*B*) Effect of RU-486 on Smo agonist-stimulated GCP proliferation. Primary neuronal GCPs were treated with DMSO, 12.5 μM halcinonide, 2.5 μM fluticasone, 25 μM clobetasol, 25 μM fluocinonide, or 0.5 μM SAG in the presence and absence of 5 μM RU-486. RU-486 at 5 μM had no effect on Smo agonists tested. Data obtained in triplicate are presented as the mean ± SEM ($n = 3$). The statistical significance was analyzed by a two-tailed Student's *t* test, with $*P < 0.05$ ($\alpha = 0.05$) defined as significant (over DMSO).

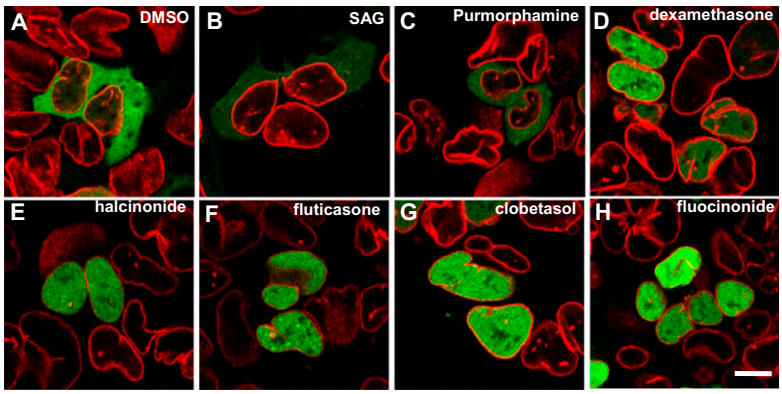

Fig. S6. Halcinonide, fluticasone, clobetasol, fluocinonide, and other glucocorticoids activate glucocorticoid receptor-GFP (GR-GFP) as assessed by GR-GFP nuclear translocation. GR-GFP-transfected HEK293 cells were treated with the indicated compound at a dose of 1 μM for 1 h, followed by immunostaining of endogenous Lamin B1 protein (red) to visualize nuclear membrane. DMSO, SAG, or purmorphamine treatment did not cause translocation of GR-GFP from the cytosol to the nucleus. Halcinonide, fluticasone, clobetasol, or fluocinonide, as well as dexamethasone, induced translocation of GR-GFP from the cytosol to the nucleus. (Scale bar: 5 μm.)