

FM19G11, a New Hypoxia-inducible Factor (HIF) Modulator, Affects Stem Cell Differentiation Status^{*S}

Received for publication, April 28, 2009, and in revised form, October 13, 2009 Published, JBC Papers in Press, November 6, 2009, DOI 10.1074/jbc.M109.008326

Victoria Moreno-Manzano^{†1}, Francisco J. Rodríguez-Jiménez^{†1}, Jose L. Aceña-Bonilla[‡], Santos Fustero-Lardies^{‡§}, Slaven Erceg[‡], Joaquin Dopazo[‡], David Montaner[‡], Miodrag Stojkovic[‡], and Jose M. Sánchez-Puelles^{†¶12}

From the [†]F.V. Centro de Investigación Príncipe Felipe, E-46012 Valencia, the [§]Universidad de Valencia, E-46071 Valencia, and the [¶]Centro de Investigaciones Biológicas, CSIC, E-28040 Madrid, Spain

The biology of the α subunits of hypoxia-inducible factors (HIF α) has expanded from their role in angiogenesis to their current position in the self-renewal and differentiation of stem cells. The results reported in this article show the discovery of FM19G11, a novel chemical entity that inhibits HIF α proteins that repress target genes of the two α subunits, in various tumor cell lines as well as in adult and embryonic stem cell models from rodents and humans, respectively. FM19G11 inhibits at nanomolar range the transcriptional and protein expression of *Oct4*, *Sox2*, *Nanog*, and *Tgf- α* undifferentiating factors, in adult rat and human embryonic stem cells, FM19G11 activity occurs in ependymal progenitor stem cells from rats (epSPC), a cell model reported for spinal cord regeneration, which allows the progression of oligodendrocyte cell differentiation in a hypoxic environment, has created interest in its characterization for pharmacological research. Experiments using small interfering RNA showed a significant depletion in Sox2 protein only in the case of HIF2 α silencing, but not in HIF1 α -mediated ablation. Moreover, chromatin immunoprecipitation data, together with the significant presence of functional hypoxia response element consensus sequences in the promoter region of *Sox2*, strongly validated that this factor behaves as a target gene of HIF2 α in epSPCs. FM19G11 causes a reduction of overall histone acetylation with significant repression of p300, a histone acetyltransferase required as a co-factor for HIF-transcription activation. Arrays carried out in the presence and absence of the inhibitor showed the predominant involvement of epigenetic-associated events mediated by the drug.

Hypoxia-inducible transcription factors (HIFs)³ have been the subject of numerous research studies, as they are the key

regulators of cell reaction to the lack of cell oxygen. They are widely referred to, in the context of pathological processes of cancer, inflammation, cardiovascular, and neurodegenerative diseases and, in general, all the angiogenic pathologies (reviewed in Refs. 1–4). More recently, HIF biology has progressed due to its interactions with cell pathways that regulate stem cell self-renewal and differentiation, suggesting a new mechanism whereby HIF proteins may drive tumor growth through the generation of tumor-initiating cells or cancer stem cells (5, 6). HIF α proteins, a hallmark of different tumor types, were the focus of many drug discovery efforts, but most inhibitors did not comply with the pharmacological properties required for approval of the drug by the regulatory agencies. Thus, even after more than 20 years of research, there is still room for intervention with novel small molecules that modulate HIF. Strategies for HIF inhibitors include the wide area of angiogenic pathologies and, within the field of regenerative medicine, promising treatments for degenerative diseases and/or the pre-conditioning of the stem cells used for cell transplantation therapies.

HIF is a heterodimer consisting of an oxygen-regulated α subunit (1 α , 2 α , or 3 α) and a constitutively expressed β subunit, or ARNT. HIF proteins are members of the basic helix loop helix-PAS family and bind to canonical DNA sequences (hypoxia-regulated elements or HREs) in the promoters or enhancers of target genes. Despite the existing similarities, α subunits trigger overlapping and specific genes and are therefore involved in different molecular pathways with different physiological consequences for the cells with non-redundant or compensatory function (7–9). Briefly, HIF1 α , but not HIF2 α , induces genes involved in the glycolysis process (10), whereas HIF2 α regulates the angiogenic route, even in the absence of hypoxia (11). HIF2 α is also seen as the physiological regulator of Epo production in adult mice (12). HIF3 α , however, forms an abortive transcriptional complex with HIF-2 α and prevents the engagement of HIF-2 with the HREs acting as negative feedback regulators (13). HIF activity is mainly regulated at the protein level, due to the hydroxylation of key proline residues present in the oxygen-dependent degradation domain of the α subunits by the prolyl-hydroxylases (PHDs) triggering polyubiquitination and rapid degradation of the HIF α proteins

* This work was supported by the Fondo de Investigaciones Sanitarias, the Instituto de Salud Carlos III (Spain), the Ministerio de Educación, Ciencia y Tecnología, and Generalitat Valenciana (Spain) Projects PI051973 RD06/0010/1006, SAF2007-63714, and GVRE/2008/254.

^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3, Tables S1–S3, and data I and II.

¹ Both authors contributed equally to this study.

² To whom correspondence should be addressed: Centro de Investigaciones Biológicas, CSIC, 28040 Madrid, Spain. Tel.: 348373112; Fax: 34915360432; E-mail: jmspuelles@cib.csic.es.

³ The abbreviations used are: HIF, hypoxia-inducible transcription factors; HRE, hypoxia-responsive element; PHD, prolyl-hydroxylase; ChIP, chromatin immunoprecipitation; RLU, relative luciferase unit; CRE, cAMP-response element; GO, gene ontology; Ach3, histone 3-acetylated form; DMSO, dimethyl sulfoxide; epSPC, ependymal progenitor stem cell; SCI, spinal cord injury; EGF, epi-

dermal growth factor; hESC, human embryonic stem cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; GFAP, glial fibrillary acidic protein.

Sox2, a New Target Gene of HIF2 α

through an E3 ubiquitin ligase complex (14). Depletion of oxygen prevents destruction, leading to stabilized α proteins that interact with ARNT in the nucleus, recognize the HRE sequences in the DNA, and activate the transcription mediated by the p300-CBP complexes.

HIF α proteins affect self-renewal and differentiation processes of stem cells by specific regulation of relevant genes and the key transcription factors involved in these processes. It is now known that lowered oxygen concentration enhances neurogenesis and delays certain differentiation processes (15, 16). For instance, HIF1 α interacts with Notch1 to maintain undifferentiated cell states (5), whereas HIF2 α binds to the marker of the undifferentiated state *Oct4* promoter, inducing its expression and transcriptional activity (8). Sox2 controls pluripotency by direct modulation of Oct4 levels in embryonic stem cells of mice (17, 18). Recent articles have shown how pluripotency can be acquired through only a few genetic modifications. Interestingly, the experiments of Takahashi *et al.* (19, 20) showed that somatic cells can be reprogrammed into pluripotent stem cells by transduction of four defined transcription factors, c-MYC, KLF4, SOX2, and OCT4, two of which (OCT4 and c-MYC) are directly activated by HIF2 α (8, 21).

One of the most challenging objectives in cell therapy is to restore neurological function after spinal cord injury (SCI). After SCI, there is a significant cell proliferation of ependymal-derived stem/progenitor cells (epSPC) (22). It is possible to restore locomotor activity when epSPC, activated by the injured tissue (epSPCi), are ectopically transplanted (23). Altering the fate of engrafted or endogenous epSPCi, to restrict differentiation to oligodendrocytes or a neuronal lineage, would replace the loss of functional units and would delay the demyelination process (23). In the present study, we identified and characterized a new chemical entity, FM19G11, which inhibits the expression and transcriptional activity of HIF α isoforms and their corresponding target genes, including the HIF2 α -mediated regulation of *Sox2*, newly characterized here. The specific inhibition of HIF α proteins by FM19G11 reduces the transcriptional activation of the expression of pluripotency markers Sox2 and Oct4 and the corresponding target genes *Tgf- α* and *Nanog* in epSPC, thus driving cell differentiation to oligodendrocytes in a process that may favor the design of pharmacological strategies for spinal cord regeneration.

EXPERIMENTAL PROCEDURES

Plasmids and Treatments—The plasmid 9x-HRE-Luc with a luciferase reporter gene was kindly provided by Dr. M. O. Landazuri. This plasmid containing the neomycin resistance gene was used to generate a stably transduced HeLa cell line (HeLa-9x). Plasmids pCMV-TRE (12-*O*-tetradecanoylphorbol-13-acetate-responsive element) and pGL2-CRE containing a firefly luciferase gene, and the plasmids containing the cDNAs of *ATF2*, *JunB*, *c-FOS*, and *c-JUN*, kindly provided by Dr. R. Farrás, were used for stable and transient transfection in the HEK293T cell line (the 293-TRE and 293-CRE cell lines were created).

For *Sox2* promoter transcriptional activity analysis, a reporter construct was created in pGL3-basic, including the region of the mouse *Sox2* promoter sequence $-392/-1725$

upstream of start codon (pGL3-mp*Sox2*). The *in silico* search of HRE sequences in the mouse sequence showed the presence of two sites, -725 and -1320 , and both were point-mutated by PCR (mp*Sox2* Δ) using the following primers: -1320 HRE FW: CCTATTTGTAACGGAAATGGGGCTGTGGCTC, RV_5'-GAGCCACAGCCCCATTTCCGTTACAAATAGG; -725 HRE FW_5'-GAATTAGGGGTTGAGGACAAATGCTGCG-GTTCCTTGAGC and RV_GCTCAAGGAACCGCAGCAT-TTGTCTCAACCCCTAATTC.

Luciferase Reporter Activity Assays— 10^5 HEK293T, HeLa-9x, 293-TRE, or 293-CRE cells per well were seeded onto white 96-well plates in quadruplicate 24 h prior to assay. For transient overexpression, 0.05 μ g/well in 96-well plates of each plasmid, *ATF2*, *JunB*, *c-JUN*, *c-FOS*, PGL3-basic, pGL3-mp*Sox2*, pGL3, and mp*Sox2* Δ were transfected with FuGENE6 HD (3:6) 24 h before stimulation. Serial dilutions of FM19G11 from 0 (containing DMSO as a control) up to 1 μ M were added immediately before hypoxic stimulation in 1% O₂ atmospheres created by the *In vivo*₂ 400 chamber (Ruskin Life Sciences). 6 h after stimulation, luciferase activity was quantified by addition of an equal volume of Bright-Glo Luciferase Reagent (Promega) and detected in the VICTOR³ luminometer (PerkinElmer Life Sciences).

Cytotoxicity Assay—Cell viability was measured by following the CellTiter 96[®] Aqueous Non-radioactive Cell Proliferation Assay instructions (Promega). 5×10^4 HeLa cells per well were seeded onto 96-well plates 24 h before assay. Serial dilutions of FM19G11 from 0 (containing DMSO as a control) up to 100 μ M were used to stimulate the cells for 72 h under standard oxygen conditions ($\sim 20\%$ O₂) or hypoxic atmosphere (1% O₂).

Chemical Synthesis of FM19G11—The detailed protocols for the synthesis of FM19G11 and its precursors are included in [supplemental data I](#).

Ependymal/Progenitor Cell Isolation and Culture—epSPC were harvested from adult female Sprague-Dawley rats (~ 200 g), isolated, and cultured as described elsewhere (23).

Oligodendrocyte-directed Differentiation—Differentiation was performed as previously described (23). Briefly, epSPCs were cultured with glial restriction medium: Dulbecco's modified Eagle's medium, F-12, B27 supplement (Invitrogen), 25 μ g/ml of insulin, 6.3 ng/ml of progesterone, 10 μ g/ml of putrescine, 50 ng/ml of sodium selenite, 50 μ g/ml of holotransferrin, 40 ng/ml of tri-iodothyroidin, supplemented with 4 ng/ml of basic fibroblast growth factor and 10 ng/ml of EGF (Sigma) for 1 day. Subsequently, cells were incubated with 20 ng/ml of EGF and 10 μ M of all-*trans*-retinoic acid for 1 week. All-*trans*-retinoic acid was then removed and the cells were exposed to glial restriction medium supplemented with 20 ng/ml of EGF for 25 days. At day 28, the spheres were plated in Petri dishes coated with 1:30 Matrigel for 1 week and cultured on glial restriction medium supplemented with 20 ng/ml of EGF. For terminal differentiation, at day 35, oligodendrocyte precursor cells were seeded on poly-L-lysine and human laminin (Sigma)-coated slides. At days 0 and 35, the cells were incubated under hypoxic conditions (1% O₂) in the *In vivo*₂ 400 chamber (Ruskin Life Sciences) for 72 h with 500 nM FM19G11 or its DMSO vehicle as control. Then, the cells were harvested for total RNA or immunocytochemical staining.

hESC Culture—Primary human embryonic stem cell (hESC) colonies from the H9 line (WiCell Inc., Madison, WI) were cultured as described elsewhere (24). Briefly, hESC were mechanically dispersed into several small clumps and cultured on fresh, commercially available human foreskin fibroblasts (American Type Culture Collection, Manassas, VA), inactivated by mitomycin C in ES medium containing knock-out Dulbecco's modified Eagle's medium, 1 mM L-glutamine, 100 mM non-essential amino acids, 20% serum replacement, 1% penicillin/streptomycin, 8 ng/ml of basic fibroblast growth factor (Invitrogen), and 100 mM β -mercaptoethanol (Sigma). ES medium was changed every second day. Human embryonic stem cells were passaged by mechanical dissociation and then removed to a freshly prepared human foreskin fibroblast layer.

RNA Isolation, Semi- and Quantitative Reverse Transcription-PCR—One microgram of total RNA, extracted by using the RNeasy Mini-kit (Qiagen, Germany), was reverse transcribed in a total reaction volume of 50 μ l by means of incubation at 42 °C for 30 min using random hexamer primers. The primer sequences for semiquantitative PCR are detailed in [supplemental data II](#). The target gene value was normalized to the expression of an endogenous reference (*GAPDH*).

For quantitative PCR, mRNAs were amplified and quantified by SYBR Green or TaqMan probes (Applied Biosystems) ([supplemental data II](#)). As template, 40 ng of cDNA from the target and housekeeping gene (*GAPDH*) were prepared in separate tubes for each primer master mixture reaction. The comparative threshold cycle (C_T) method was used to calculate the relative expression (25).

DNA Microarray Analysis—epSPC isolation and DNA microarray hybridization was performed as described elsewhere (23). The gene profile was sorted by differential expression levels between the two experimental conditions (epSPC 48 h in hypoxia with FM19G11 *versus* DMSO) and clustered into biological functional profiles by FatiGO application (26).

Western Blot Analysis—Cells were collected and washed with cold phosphate-buffered saline. Total cell protein extracts were isolated by use of 2% SDS Tris-Cl lysis buffer plus proteinase inhibitors. Subcellular fractionation was performed in two steps, by using hypotonic and hypertonic buffers for cytoplasm and nuclear fraction isolation, respectively. SDS-PAGE and hybridization steps were carried out as previously described (23), with antibodies against HIF1 α (a kind gift from Dr. Berra), HIF2 α , PHD3, Sox2, Oct4, Notch1 (Abcam, UK), RIP, NG2, Nestin, and glial fibrillary acidic protein (GFAP) (Chemicon) at 1:1000 dilution. β -Actin at 1:5000 dilution (Sigma) was used as loading control. The resulting bands were densitometrically analyzed by ImageJ software.

Immunocytochemistry—Fixed and permeabilized cells (0.05% Triton X-100), after blocking (1% fetal bovine serum), were incubated overnight at 4 °C with the primary mouse antibodies (1:200), α -RIP, α -O4 (Chemicon), α -HIF-1 α (BD Bioscience), and rabbit antibodies, α -NG2 (Chemicon) and α -Sox2 (Abcam). For detection, Texas Red dye-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and Oregon Green 488 goat anti-mouse IgG at 1:400 (Invitrogen) were used. Signals were viewed by confocal microscopy (Leica).

RNA Interference by Small Interfering RNA (siRNA) Duplex Transfection for HIF1 α and HIF2 α —Annealed siRNA duplexes were purchased from Applied Biosystems. The siRNA sequences targeting rat *HIF1 α* (accession number NM_0243591.1) and *HIF2 α* (accession number NM_023090.1) corresponded to catalog numbers 4390816_s131713 and 4390816_s131443, respectively (Applied Biosystems). 500 nM siRNA were used for transfection.

ChIP Analysis—Chromatin immunoprecipitation analysis used the LowCell ChIP kit (Diagenode), following the manufacturer's instructions, as described elsewhere (27). Samples were incubated with 10 μ g of anti-HIF2 α or AcH3 antibodies (Abcam). An isotype-matched antibody was used as control for nonspecific binding. The rat *Sox2* promoter region was analyzed *in silico* using the Genomatix bioinformatics software portal.

Statistical Analysis—Statistical comparisons were assessed by the Student's *t* test. All *p* values were derived from a two-tailed statistical test using the SPSS 11.5 software. A *p* value <0.05 was considered statistically significant.

RESULTS

FM19G11, a New HIF α Inhibitor—To identify novel molecules targeting the HIF pathway, we used a stable luciferase reporter gene-based screen containing 9 repetitions of the HRE 5' upstream of the start codon in the active promoter region constitutively expressed in the HeLa cell line (HeLa-9x-HRE-Luc). These cells displayed more than 100-fold higher luciferase activity after incubation in hypoxia (1% O₂). We tested the HeLa-9x-HRE-Luc screen against a compound bank, containing more than 12,000 compounds, chosen as representatives of the total chemical space. FM19G11 reduced hypoxia-induced luciferase activity by 50% (IC₅₀) at 80 \pm 5 nM concentration (Fig. 1A, *inset*). FM19G11 showed significant relative luciferase unit (RLU) inhibition from 30 nM, with steady reduction in a characteristic dose-dependent manner (Fig. 1A) and reaching 80% inhibition at 1 μ M (Fig. 1B). To test the specificity of FM19G11 for HRE binding sites, we reproduced the luciferase-reporter gene assay by stable overexpression of CRE or TRE reporter constructs. ATF2 and JunB transient overexpression was used to induce CRE-mediated reporter gene activity, and *c-fos* and *c-jun* overexpression, for TRE induction (Fig. 1B, *inset*). No significant RLU inhibition of TRE or CRE transcriptional activity was found in the presence of any tested concentration of FM19G11 (Fig. 1B). It is worth mentioning that no cytotoxicity for concentrations of FM19G11 lower than 30 μ M, in standard oxygen tension, or 50 μ M, under hypoxic conditions, was observed on the HeLa cell line ([supplemental Fig. S1](#)).

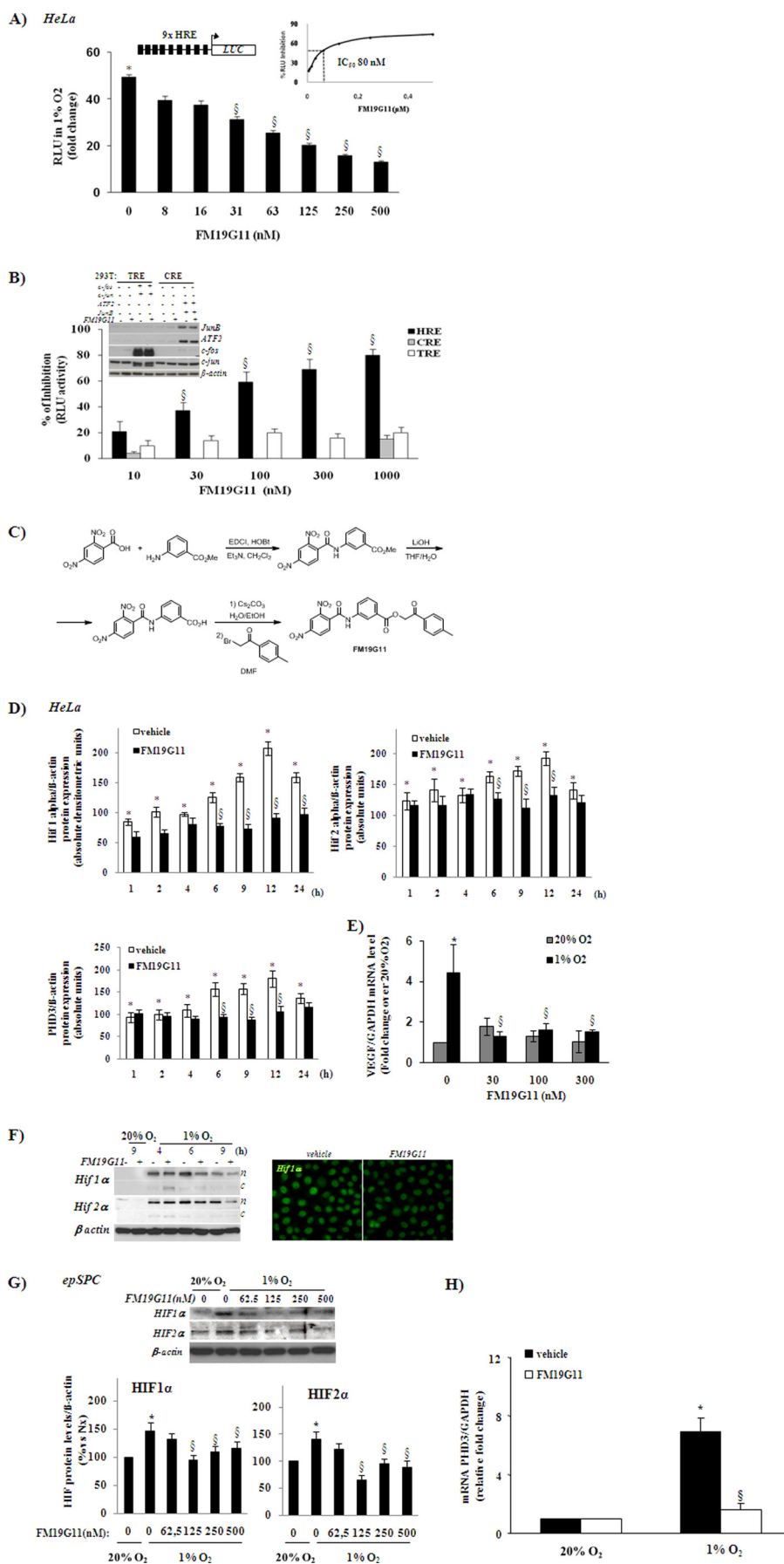
Chemical Synthesis of FM19G11—The FM19G11 compound was chemically re-synthesized for extensive evaluation. As shown in Fig. 1C, reaction of 2,4-dinitrobenzoic acid with methyl 3-aminobenzoate was followed by methyl ester hydrolysis and coupling with (bromomethyl)*p*-tolylketone to afford the target compound. FM19G11 was purified by flash column chromatography.

FM19G11 Inhibits HIF α Proteins in Human Tumor Cell Lines—We evaluated the effect of FM19G11 on total protein levels and on nuclear- and cytoplasm-fractionated extracts of

Sox2, a New Target Gene of HIF2 α

HIF1 α and -2 α (Fig. 1, D and F), as well as the effect on the expression of their target genes *PHD3* (Fig. 1D) and *VEGF* (Fig. 1E) in the HeLa cell line. Simultaneous incubation at 300 nM FM19G11 from 1 to 12 h of hypoxia exposure (1% O₂) prevented HIF1 α and HIF2 α accumulation (this effect was extended to 24 h for HIF1 α accumulation). Although most HIF α protein was found in the nucleus in all cases, a detectable amount of both isoforms (1 and 2 α) was found in the cytosolic fraction in the presence of FM19G11 after 4 h of incubation at 1% O₂. However, no significant changes in the subcellular location of HIF α proteins were observed at any other tested time (Fig. 1F). The prolyl hydroxylases (PHDs/EGLNs) are the central regulators of the molecular responses to oxygen availability (28) and PHD3 is also directly regulated by both α proteins (29). FM19G11 significantly inhibited PHD3 protein levels (Fig. 1D). In addition, the hypoxic transcriptional induction of VEGF, a well known target gene of HIF α proteins (30), was significantly blocked by all tested doses of FM19G11 (Fig. 1E). Because the HIF promoter region contains multiple HRE, indicating a self-regulating mechanism (31), we also analyzed both HIF α isoforms at the mRNA levels in the presence of FM19G11. The hypoxia-dependent induction of HIF1 and 2 α mRNA was significantly lower in the presence of FM19G11 after 6 h of incubation (data not shown), coinciding with the significant reduction of the protein levels induced by the compound. However, no significant changes in protein levels, in comparison with vehicle-treated cells, were obtained at shorter (1 or 3 h) or longer (9 or 12 h) hypoxic exposition times (data not shown).

To investigate whether HIF1 α protein inhibition by FM19G11 was mediated by promoting the activation of the proteasomal system, we performed an experiment in the presence of the proteasomal inhibitor MG132 in normoxia. Interest-



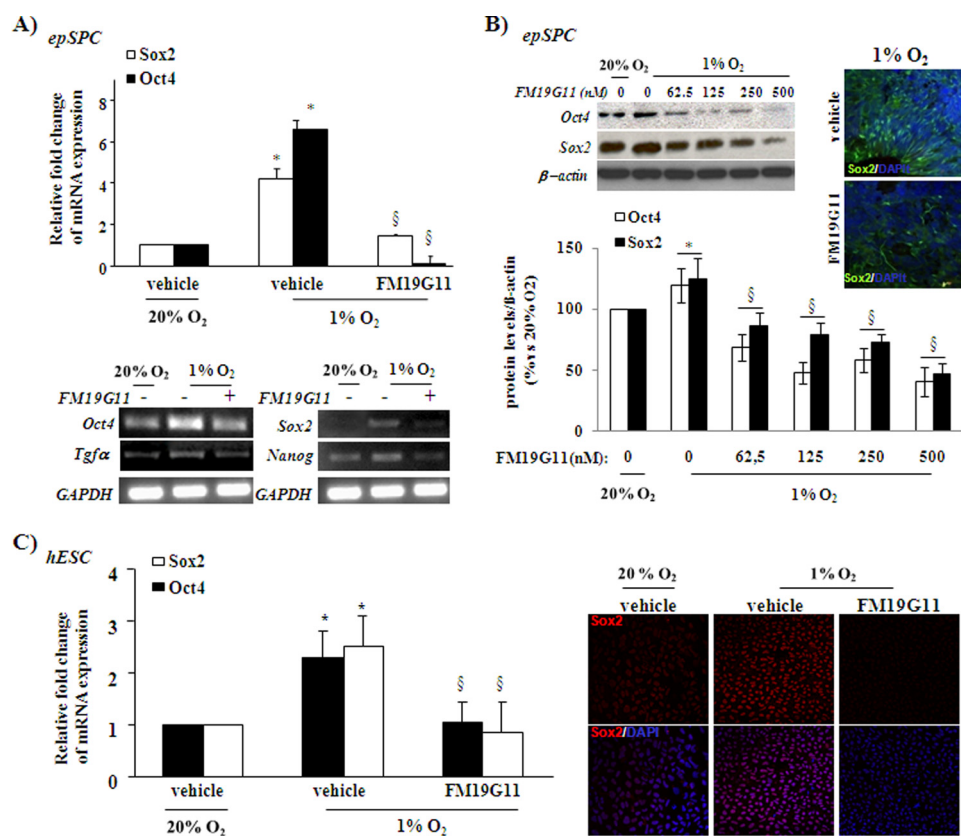


FIGURE 2. FM19G11 regulates Oct4 and Sox2 pluripotency markers in rodent and human stem cells. epSPC (A and B) and hESC (C): A, upper panel: real time SYBR Green PCR analysis of rat Sox2 and Oct4 relative expression levels. Lower panel, semi-quantitative PCR of Oct4, Sox2, and their direct target genes, Tgf α and Nanog, respectively. Cells were incubated with 500 nM FM19G11 (+) or its vehicle (-) for 48 h under hypoxia (1% O₂). 20% O₂ was taken as the basal condition. GAPDH served as a loading control. B, left panels: representative Western blot (upper panels) and densitometry analysis of three independent experiments (lower panels). FM19G11 dose-dependent effect on Oct4 and Sox2 protein expression after 48 h of hypoxia exposure. Values are shown as a percentage of the control (20% O₂). Right panels: representative immunostaining of the Sox2 protein in undifferentiated neurospheres treated for 48 h in 1% O₂ with vehicle or 500 nM FM19G11. C, left panel: TaqMan real time-PCR analysis of human Sox2 and Oct4 relative expression levels in hESC treated with 500 nM FM19G11 or its vehicle; right panel: qualitative immunostaining analysis of human Sox2 protein expression in undifferentiated hESC colonies treated for 48 h in 1% O₂ with DMSO (vehicle) or 500 nM FM19G11. 20% O₂ condition served as the basal control. Results were obtained from three independent experiments. Error bars represent S.D. *, $p < 0.05$ versus 20% O₂; §, $p < 0.05$ versus vehicle at 1% O₂, determined by Student's *t* test.

ingly, MG132 did not affect any isoform, HIF1, or 2 α accumulation by FM19G11, suggesting a proteasome-independent mechanism on HIF α inhibition (data not shown). The action of FM19G11 on HIF α proteins was not restricted to HeLa cells, because it was also observed in adult human cell lines derived

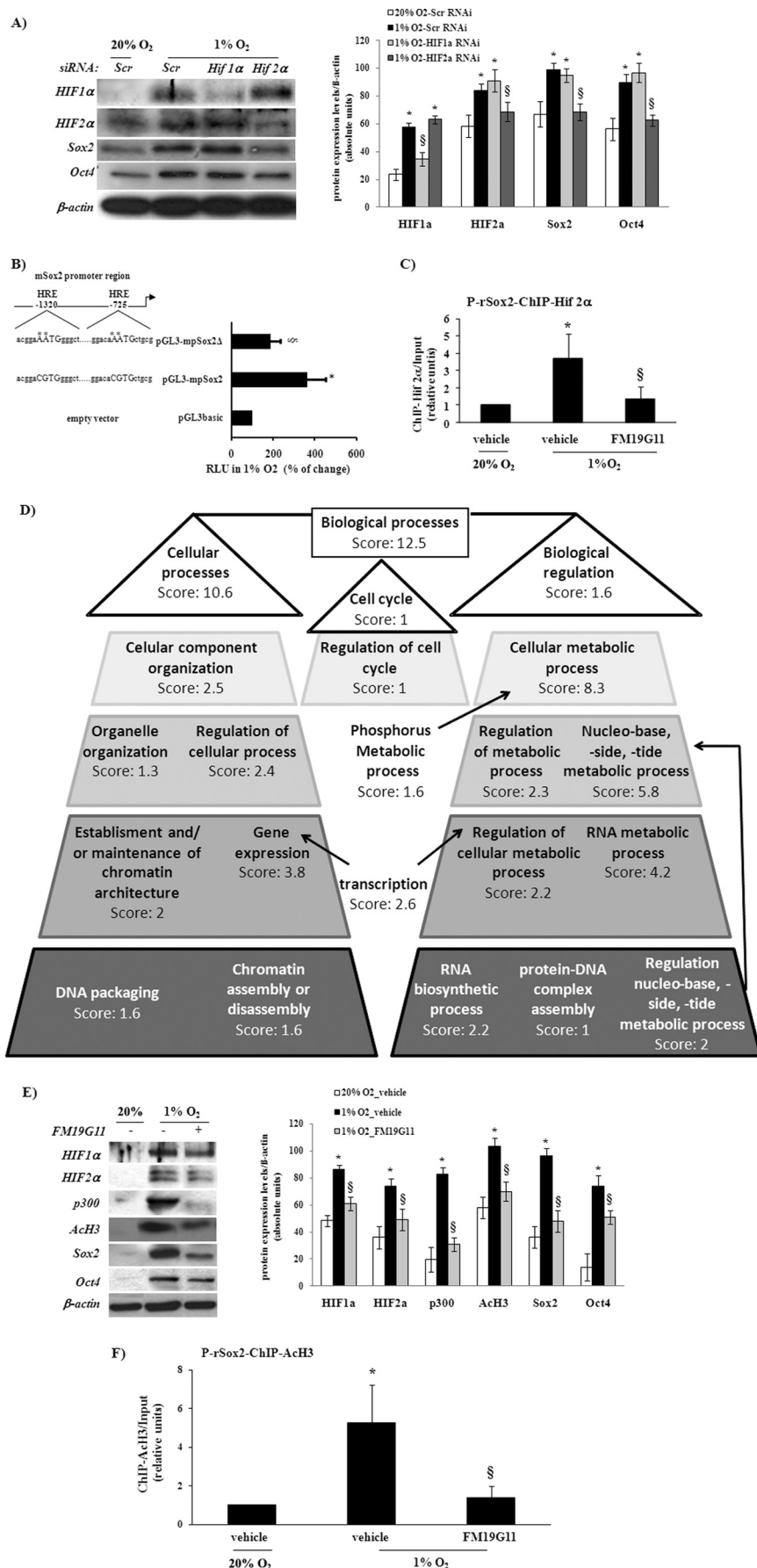
from various tissue types, including colon HT-29 and the breast cancer cell line MDA-MB-435-S (data not shown).
FM19G11 Inhibits HIF α Protein Accumulation in Adult and Embryonic Stem Cells from Rodents and Humans, Respectively—Interestingly, the compound FM19G11 had a similar effect on HIF regulation in stem cells. We analyzed the effect of the compound on adult rat epSPC and on hESC. Fig. 1G shows the dose-dependent inhibitory effect of FM19G11 on both HIF α proteins in epSPC. Transcriptional repression of *Phd3* was also observed in the rat epSPC (Fig. 1H) and hESC (data not shown) treated with 500 nM FM19G11 for 48 h under hypoxia.

FIGURE 1. Synthesis and analysis of FM19G11 activity in HIF α proteins and their target genes under hypoxia. A, HeLa-9x-HRE-Luc cells treated with different concentrations of FM19G11 (0–0.5 μ M) were exposed to 1% O₂ for 6 h. FM19G11 inhibits in a dose-responsive way the HIF-translational activity in the luciferase reporter assay. Inset, calculation of IC₅₀ by converting the RLU into % of RLU inhibition. B, 293-TRE or 293-CRE, transiently overexpressing *c-fos* and *c-jun* or ATF2 and JunB, respectively, were treated with 0.3 μ M FM19G11 for Western blot analysis (inset) or with 0–1 μ M for 6 h in the luciferase reporter assay. Luciferase activity in vehicle-treated cells was taken as 0% of inhibition. C, chemical synthesis of FM19G11 in three steps. The reaction of 2,4-dinitrobenzoic acid with methyl 3-aminobenzoate was followed by methyl ester hydrolysis and coupling with (bromomethyl)*p*-tolylketone to form the target compound. D, intracellular levels of HIF1 and -2 α and PHD3 proteins were assayed after HeLa exposition to 1% O₂ for 1–24 h with 300 nM FM19G11 (+) or its vehicle (-). 24 h exposition at 20% O₂ in the absence of FM19G11 (-) was taken as the basal condition. β -Actin served as loading control. The graphs represent the mean of absolute densitometry values of each condition from three independent experiments. E, TaqMan[®] real time-PCR analysis of VEGF in HeLa cells treated for 6 h with FM19G11 (0–300 nM) at 1 or 20% O₂. F, left panel, representative example of nuclear (n) and cytoplasm (c) fractionated cell proteins assayed for HIF α protein expression. HeLa cells were treated with 300 nM FM19G11 (+) or its vehicle (-) for 4–9 h at 1 or 20% O₂. Right panel, immunostaining for HIF1 α of HeLa cells treated with FM19G11 or its vehicle (DMSO) under hypoxia for 6 h. G, epSPC, upper panel: representative experiment of the dose-response of FM19G11, showing the effect on HIF α protein expression after 48 h in hypoxia (1% O₂). Lower panel, mean of densitometry values of HIF α protein expression analysis. Values are shown as a percentage of the control (20% O₂). H, epSPC: TaqMan real time-PCR analysis of PHD3 relative expression levels with vehicle or FM19G11 (500 nM) treated for 48 h under normoxia (20% O₂) or hypoxia (1% O₂). Results were standardized by the housekeeping gene GAPDH. mRNA levels were calculated by the 2^{- $\Delta\Delta$ CT} method. Results were obtained from three independent experiments. Error bars represent S.D. *, $p < 0.05$ versus 20% O₂; §, $p < 0.05$ versus vehicle at 1% O₂ determined by Student's *t* test.

Sox2, a New Target Gene of HIF2 α

the two proteins, Oct4 and Sox2, by the compound showed a dose-dependent inhibitory pattern. The time and dose-response experiments carried out indicated that the hypoxia-induced expression of Oct4 and Sox2 was more efficiently reduced at 500 nM FM19G11 after 48 h in 1% O₂ (Fig. 2B, upper and left panel). The immunocytochemical studies confirmed the diminished expression of Sox2 in cells treated with FM19G11, with no apparent alterations in protein location (Fig. 2, B and C) in relation to the controls.

HIF2 α Regulates Sox2 Expression—The role of Sox2 in pluripotency is mostly based on its function of maintaining Oct4 levels and the consequent expression of many pluripotency associated genes, e.g. *Fgf4*, *Lefty1*, and *Nanog*, which are tightly regulated by an enhancer containing Oct4 and Sox2 binding motifs (18). *Oct4* was reported to be a direct target of HIF2 α (8). Recently McCord *et al.* (32), using the siRNA approach, reported for the first time experimental evidence indicating that Sox2 have HIF-dependent regulation. Here, we validated these results, by using specific siRNA oligos, and the knockdown expression of HIF2 α , but not of HIF1 α , which blocks hypoxia-induced expression of both Oct4 and Sox2 in epSPC (Fig. 3A). These results strongly indicate the direct involvement of HIF2 α in the positive regulation of both pluripotency markers, Oct4 and Sox2. However, to provide further and new data that may indicate the direct connection between HIF2 α and Sox2 regulation, we searched for putative HRE-binding sequences within the promoter region of the rodent *Sox2* gene. The *in silico*-predicted occupancy of HIF for HRE binding sites, over the 5-kb promoter region immediately upstream of the transcription start signal of *Sox2*, was first analyzed by a reporter-based screen including the promoter sequence containing two HRE sites (pGL3-mpSox2) and after the performance of point



mutation (pGL3-mpSox2 Δ) to inactivate both sites. Transcriptional activation under hypoxia, by using the wild-type sequence (pGL3-mpSox2) rather than the empty vector, was significantly abolished by using the mutated sequence (pGL3-mpSox2 Δ ; Fig. 3B). Finally, to disclose a specific role for HIF2 α in Sox2 promoter activity depending on HRE activation, a ChIP assay was performed. The specific ChIP signals obtained after immunoprecipitation with anti-HIF2 α antibody showed, first, more amplified immunoprecipitated sequences after hypoxia stimulation and, then, reduced binding of HIF2 α within the Sox2 promoter when epSPC were treated with FM19G11 (Fig. 3C).

Epigenetic Influences on FM19G11-dependent Sox2 Regulation—To reveal the extended mechanism involved in FM19G11-dependent regulation of Sox2 expression, we performed a DNA microarray analysis, comparing the gene expression profile of epSPC when treated under hypoxia with the compound FM19G11 or its vehicle. The differentially expressed genes were organized according to gene ontology (GO) by using the corresponding gene-GO association table to obtain FatiGO-implemented analysis. As shown in Figs. 3D or supplemental S2 (for more details), two main groups after biological function clustering were overrepresented in the presence of FM19G11. The first group was related to chromatin assembly; and the second, to transcriptional regulation. Taken together, they may indicate that FM19G11 activity in transcriptional regulation is mediated throughout alteration of epigenetic events by chromatin modifications. Stabilized HIF α proteins bind with the ARNT subunit and recruit the p300-CBP complex, two coactivators with histone acetyltransferase activity (34). Based on the transcription profile obtained in the presence of FM19G11, we tested whether FM19G11 would behave as a histone deacetylase inhibitor, but no positive results were obtained (data not shown). However, the presence of FM19G11 inhibited the hypoxia-induced expression levels of the histone 3-acetylated form (AcH3), as well as the levels of total p300, which act as acetyltransferase (Fig. 3E). These results were obtained in close association with the repression of HIF targets, including pluripotency markers. Furthermore, the ChIP signals obtained after immunoprecipitation with anti-AcH3 antibody revealed a rich acetylated region in the Sox2 promoter when the epSPC were exposed to hypoxia. In contrast, a significant reduction in AcH3 signals was seen in epSPC maintained under hypoxic conditions when FM19G11 was present (Fig. 3F).

FM19G11 Promotes Oligodendrocyte Differentiation under Hypoxia—Hypoxia blocks stem cell differentiation; and HIF expression is widely accepted to be associated with stemness. Here, we showed the effect of FM19G11, an HIF α inhibitor, on oligodendrocyte differentiation under hypoxia. An oligodendrocyte differentiation protocol from undifferentiated epSPC was induced until day 42, in line with our earlier research (23), by following the steps described in Fig. 4A. The process of differentiation with epSPC occurred in parallel, in the presence and absence of FM19G11, in an atmosphere of 1% O₂, and for early and late differentiation stages from day 1 to 3 and from day 35 to 37, respectively (Fig. 4). At both stages, the hypoxic conditions blocked the differentiation process, according to cell markers RIP and NG2 at early stage (day 1–3) and the mature oligodendrocyte markers O4 and RIP at late stage (day 35–37) (Fig. 4, B and C, for RIP expression). In all cases, the addition of FM19G11 rescued the expression of the above mentioned cell markers (Fig. 4, B and C, for RIP expression). It is also important to mention the poor migration from the epSPC neurospheres into the matrix, under low oxygen concentration and at an early stage of the differentiation protocol (Fig. 4B). At day 37, the cells were also harvested for reverse transcription-PCR and Western blot analysis (Fig. 4C). From day 35 of the differentiation protocol, the precursors were forced to a definitive maturation by culturing in a laminin matrix (Fig. 4A). Sox2, Oct4, Notch1, and Nestin, typically expressed in undifferentiated progenitor cells, were at this late stage up-regulated in hypoxia, in comparison with normoxic conditions (Fig. 4C). Olig2 and Nkx2.2, homeodomain transcription factors, are linked to oligodendrocyte early specification during spinal cord development, gradually reducing in mature cells. The exposure to low oxygen concentration induced the expression of these early specific oligodendrocyte markers, Olig2 and Nkx2.2 (Fig. 4C), but in the presence of FM19G11 the cells recovered the low expression levels of these transcription factors at late differentiation stages. The expression of the astrocytic marker GFAP diminished from day 3 of the differentiation protocol after all-*trans*-retinoic acid addition (23). Hypoxia significantly induced the expression of GFAP, indicating lower oligodendrocyte specification in the epSPC culture. This induction was abolished by FM19G11 treatment (Fig. 4C). Considering all of the above, the hypoxia-induced delay in directed oligodendrocyte differentiation was aborted by FM19G11 treatment.

FIGURE 3. Hif2 α regulates Sox2 expression and influences the epigenetic mechanisms. A, epSPC were exposed to 1 or 20% O₂ for 48 h. 500 nm of each siRNA duplex, scramble (Scr, nonspecific probe), HIF1 α , or HIF2 α -specific rat probes were transfected 24 h before oxygen-dependent stimulation. The Western blot assay showed that only HIF2 α knockdown also reduced the protein levels of Sox2 and Oct4. β -Actin served as a loading control. *, when compared with scramble at 20% O₂; §, when compared with scramble at 1% O₂; $p < 0.05$ was determined by Student's *t* test. B, ChIP analysis within the rat Sox2 promoter. There were a significantly higher number of copies of the Sox2 promoter by real time-PCR amplification after chromatin immunoprecipitation assays using specific antibody for HIF2 α . The presence of FM19G11 significantly inhibited hypoxia-dependent induction. *, $p < 0.05$ versus 20% O₂; §, $p < 0.05$ versus vehicle at 1% O₂ determined by Student's *t* test. C, luciferase reporter assay under hypoxia for 6 h. HEK293T cells transiently transfected with pGL3-basic, empty vector, including the wild-type mouse Sox2 promoter sequence (pGL3-mpSox2) or point mutated at both HRE sites (pGL3-mpSox2 Δ) (see diagram on the right) 24 h before hypoxic stimulus. D, FatiGO analysis of epSPC treated with 500 nm FM19G11 was compared with vehicle alone for 48 h in hypoxia. Two biological functional groups were overrepresented in the FM19G11-treated sample after hierarchical clustering. E, left panel: representative Western blot assay for epSPC treated (+) or not (–) with 500 nm FM19G11, exposed for 48 h at 1 or 20% O₂; right panel: densitometry analysis of three independent experiments. F, ChIP analysis within the rat Sox2 promoter by using AcH3 antibody for chromatin immunoprecipitation. Error bars represent S.D. *, versus 20% O₂; §, versus vehicle at 1% O₂; $p < 0.05$ was determined by Student's *t* test.

Sox2, a New Target Gene of HIF2 α

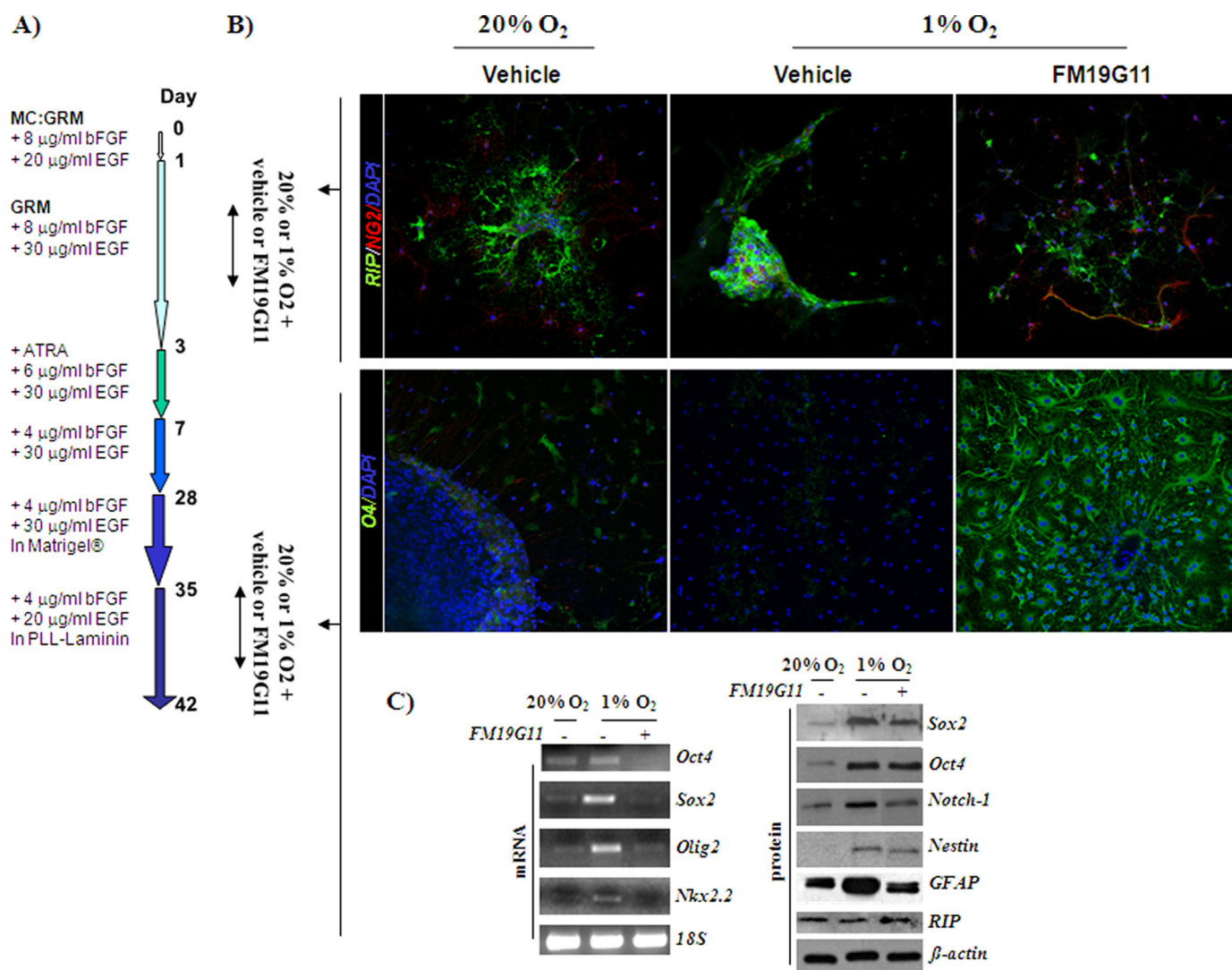


FIGURE 4. **FM19G11 favors oligodendrocyte cell differentiation of epSPC under hypoxia.** *A*, diagram of differentiation protocol. *B*, immunostaining assay for oligodendrocyte cell markers: RIP and NG2 (upper panels) and O4 (lower panels). Upper panels, cells treated with 500 nM FM19G11 or DMSO (vehicle) from day 1 to 3 of the differentiation protocol; lower panels, cells treated during day 35 to 37 of the differentiation protocol under both normoxic (20% O₂) and hypoxic (1% O₂) conditions. *C*, extended analysis at day 37 of the differentiation process. epSPC were cultured under normoxic (20% O₂) and hypoxic (1% O₂) atmospheres and treated with 500 nM FM19G11 (+) and vehicle alone (-). The undifferentiated stage (Oct4, Sox2, Olig2, Nkx2.2, Notch1, Nestin, and GFAP) and the oligodendrocyte-specific fate cell marker (RIP) were assayed by Western blot (right panel) and/or PCR (left panel). 18S and β -actin expression served as loading controls for PCR and Western blot, respectively.

DISCUSSION

Over the last 20 years, major efforts have gone into the search for HIF α inhibitors for use in new drugs (35). Although a wide range of diverse molecules have been found to inhibit the HIF pathway, these molecules often have other actions that indirectly cause lower HIF protein levels. At present, none of the reported HIF inhibitors have met the pharmacokinetic requirements for human therapeutic use. Here we demonstrate that the new chemical entity FM19G11 acts as a potent inhibitor of HIF α proteins in hypoxia, giving high selectivity against other transcription factors of the AP-1 complex used during the screening campaign. Furthermore, we show that this molecule represses the target genes of both HIF proteins, 1 α and 2 α , in cancer cell lines of various tissues showing lower transcript and protein levels in rat epSPC and human ESC, which suggests a steady mechanism of action for this new drug. The complete eradication of what are known as cancer stem cells might be

crucial in curing cancer; reduction of HIF activity may promote their differentiation and decrease their ability to repopulate tumors after chemo- and radiotherapy (6, 36). The low toxicity of this small molecule, no cytotoxicity was observed at concentrations a thousand times higher than the IC₅₀ even in a hypoxic atmosphere, permitted its safe use in a wide variety of live-cell assays, including immuno-based determinations and long-lasting experiments in stem cell differentiation.

Although hypoxia is widely linked to many pathological procedures (1–3), it is also a controller of major physiological processes, such as differentiation status during embryogenesis and in adulthood (1, 6, 8). Hypoxia is associated with the undifferentiated status of stem cells; and the function of HIF α proteins in maintaining multipotency was only found quite recently. The real mechanisms by which the HIF pathway interacts with other pathways to keep stemness are still largely unknown, despite a great many publications in the last few years (6, 36).

First, HIF1 α was shown to block neuronal and myogenic differentiation in a Notch-dependent manner (5) and, more recently, *OCT4* was identified as a HIF2 α -specific target gene (8) controlled by Sox2 (18). First of all, Keith and Simon (36) elegantly hypothesized that *SOX2* and *KLF4* might also be HIF targets and recently McCord *et al.* (32) validated this hypothesis based on the inhibition by a siRNA of the 2 α isoform. However, McCord *et al.* (32) do not show whether *Sox2* is solely under the control of HIF2 α or if there is an overlap with the 1 α isoform. A reporter assay, based on the promoter region of *Sox2* containing two HRE sites, a HIF2 α ChIP experiment, and the use of siRNA experiments leading to HIF2 α knockdown cells strongly demonstrated that *Sox2* is a direct target of the HIF α proteins, and, in particular, that its regulation resides specifically in the 2 α isoform. Complementary information that reinforces the role of HIF2 α in the direct control of *Sox2* was provided by ChIP experiments carried out in the presence of the inhibitor FM19G11. All of the above clearly point to the utility of this small molecule, at present seen just as a tool compound, to clarify the hierarchy of HIF2 α in the control of two key genetic factors that govern pluripotency.

Microenvironment influence on chromatin assembly and accessibility and/or dynamic interplay of certain transcription factors determines the stem cell differentiating status (27, 37–39). In fact, the Oct4 locus adopts a closed conformation in differentiating embryonic somatic cells, making it refractory to regulation by HIF2 α (8). Here, we confirmed the direct association between the HIF2 α -positive transcriptional regulation of *Sox2* and the open chromatin conformation of its promoter. FM19G11 prevented the general H3 acetylation induced by hypoxia in epSPC and reduced the expression of p300, the main co-activator for transcriptional activation of HIF α proteins with histone acetyltransferase activity. ChIP analysis by AcH3 immunoprecipitation showed direct involvement of the acetylation mechanism in hypoxia and FM19G11 regulation over the *Sox2* transcriptional activity. Although p300 immunoprecipitation experiments proved a *Sox2* interaction,⁴ no evidence linking *Sox2* and p300 transcriptional regulation on maintaining the undifferentiated stage was found, as was previously described in the case of Notch1 (40). The inhibitory activity of FM19G11 on Oct4 and *Sox2*, Notch, and Nanog and transforming growth factor- α opened up new approaches to its use in cell reprogramming experiments with neural progenitor cells for the SCI regeneration model in the rat. Therefore, loss of myelinating oligodendrocytes or oligodendrocyte progenitor cells is a feature of many central nervous system injury and disease states. Moreover, due to secondary damage after SCI, the ischemic environment does not allow re-myelination, partly because there is an arrest of oligodendrocyte lineage maturation (41). Indeed, when undifferentiated progenitors are transplanted into an ischemic environment, no significant cell differentiation occurs (23, 42). The cell fate modulation of transplanted or endogenous stem cells by forcing the generation of oligodendrocytes to re-myelinate spared axons in the vicinity of the lesion would be a powerful therapeutic approach

for SCI regeneration (23, 43, 44). As mentioned above, FM19G11 did repress a variety of key genes involved in stemness, and our reprogramming experiments showed that the inhibitor favors oligodendrocyte differentiation, possibly through modulation of Sox2 and Oct4 expression and by allowing neural stem and/or precursor cells to differentiate. Sox-2 was shown to be the key player in cell fate control, regulating Oct4 and, combined with a few other factors (c-myc and/or Klf4), confers ES-like properties on mature murine fibroblasts (20). However, given the results reported here, it should be emphasized that HIF2 α is now positioned in the upper hierarchy of cell fate. All in all, the low toxicity profile of this drug favors pharmacological approaches and enables it to act on SCI regeneration in rigorously defined models.

Acknowledgments—We are especially grateful to Dr. M. O. Landazuri and Dr. R. Farras for the HeLa-9x cell line and AP1 related plasmid, respectively. We gratefully acknowledge Dr. Maria Teresa Calvo and Raquel Garijo Fernández for excellent technical support. We also thank the Confocal Microscopy and Genomic Services of the Centro de Investigación Príncipe Felipe (Valencia, Spain).

REFERENCES

1. Folkman, J. (2007) *Nat. Rev. Drug Discov.* **6**, 273–286
2. Pouyssegur, J., Dayan, F., and Mazure, N. M. (2006) *Nature* **441**, 437–443
3. Bertout, J. A., Patel, S. A., and Simon, M. C. (2008) *Nat. Rev. Cancer* **8**, 967–975
4. Rankin, E. B., and Giaccia, A. J. (2008) *Cell Death Differ.* **15**, 678–685
5. Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U., and Bondesson, M. (2005) *Dev. Cell* **9**, 617–628
6. Simon, M. C., and Keith, B. (2008) *Nat. Rev. Mol. Cell Biol.* **9**, 285–296
7. Raval, R. R., Lau, K. W., Tran, M. G., Sowter, H. M., Mandriota, S. J., Li, J. L., Pugh, C. W., Maxwell, P. H., Harris, A. L., and Ratcliffe, P. J. (2005) *Mol. Cell Biol.* **25**, 5675–5686
8. Covello, K. L., Kehler, J., Yu, H., Gordan, J. D., Arsham, A. M., Hu, C. J., Labosky, P. A., Simon, M. C., and Keith, B. (2006) *Genes Dev.* **20**, 557–570
9. Covello, K. L., Simon, M. C., and Keith, B. (2005) *Cancer Res.* **65**, 2277–2286
10. Hu, Y., Leaver, S. G., Plant, G. W., Hendriks, W. T., Niclou, S. P., Verhaagen, J., Harvey, A. R., and Cui, Q. (2005) *Mol. Ther.* **11**, 906–915
11. Dutta, D., Ray, S., Vivian, J. L., and Paul, S. (2008) *J. Biol. Chem.* **283**, 25404–25413
12. Gruber, M., Hu, C. J., Johnson, R. S., Brown, E. J., Keith, B., and Simon, M. C. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104**, 2301–2306
13. Maynard, M. A., Evans, A. J., Shi, W., Kim, W. Y., Liu, F. F., and Ohh, M. (2007) *Cell Cycle* **6**, 2810–2816
14. Metzén, E., Zhou, J., Jelkmann, W., Fandrey, J., and Brüne, B. (2003) *Mol. Biol. Cell* **14**, 3470–3481
15. Studer, L., Csete, M., Lee, S. H., Kabbani, N., Walikonis, J., Wold, B., and McKay, R. (2000) *J. Neurosci.* **20**, 7377–7383
16. Zhang, C. P., Zhu, L. L., Zhao, T., Zhao, H., Huang, X., Ma, X., Wang, H., and Fan, M. (2006) *Neurosignals* **15**, 259–265
17. Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003) *Genes Dev.* **17**, 126–140
18. Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A. A., Ko, M. S., and Niwa, H. (2007) *Nat. Cell Biol.* **9**, 625–635
19. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) *Cell* **131**, 861–872
20. Takahashi, K., and Yamanaka, S. (2006) *Cell* **126**, 663–676
21. Gordan, J. D., Bertout, J. A., Hu, C. J., Diehl, J. A., and Simon, M. C. (2007) *Cancer Cell* **11**, 335–347

⁴V. Moreno-Manzano, F. J. Rodríguez-Jiménez, and J. M. Sánchez-Puelles, unpublished data.

Sox2, a New Target Gene of HIF2 α

22. Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisén, J. (1999) *Cell* **96**, 25–34
23. Moreno-Manzano, V., Rodríguez-Jiménez, F. J., García-Roselló, M., Láinez, S., Erceg, S., Calvo, M. T., Ronaghi, M., Lloret, M., Planells-Cases, R., Sánchez-Puelles, J. M., and Stojkovic, M. (2009) *Stem Cells* **27**, 733–743
24. Erceg, S., Láinez, S., Ronaghi, M., Stojkovic, P., Pérez-Aragó, M. A., Moreno-Manzano, V., Moreno-Palanques, R., Planells-Cases, R., and Stojkovic, M. (2008) *PLoS ONE* **3**, e2122
25. Livak, K. J., and Schmittgen, T. D. (2001) *Methods* **25**, 402–408
26. Al-Shahrour, F., Minguez, P., Vaquerizas, J. M., Conde, L., and Dopazo, J. (2005) *Nucleic Acids Res.* **33**, W460–W464
27. Rodríguez-Jiménez, F. J., Moreno-Manzano, V., Lucas-Dominguez, R., and Sánchez-Puelles, J. M. (2008) *Stem Cells* **26**, 2052–2062
28. Appelhoff, R. J., Tian, Y. M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) *J. Biol. Chem.* **279**, 38458–38465
29. Pescador, N., Cuevas, Y., Naranjo, S., Alcaide, M., Villar, D., Landázuri, M. O., and Del Peso, L. (2005) *Biochem. J.* **390**, 189–197
30. Fitz, L. J., Morris, J. C., Towler, P., Long, A., Burgess, P., Greco, R., Wang, J., Gassaway, R., Nickbarg, E., Kovacic, S., Ciarletta, A., Giannotti, J., Finnerty, H., Zollner, R., Beier, D. R., Leak, L. V., Turner, K. J., and Wood, C. R. (1997) *Oncogene* **15**, 613–618
31. Koh, M. Y., Spivak-Kroizman, T., Venturini, S., Welsh, S., Williams, R. R., Kirkpatrick, D. L., and Powis, G. (2008) *Mol. Cancer Ther.* **7**, 90–100
32. McCord, A. M., Jamal, M., Shankavaram, U. T., Lang, F. F., Camphausen, K., and Tofilon, P. J. (2009) *Mol. Cancer Res.* **7**, 489–497
33. Kim, J. B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Araúz-Bravo, M. J., Ruau, D., Han, D. W., Zenke, M., and Schöler, H. R. (2008) *Nature* **454**, 646–650
34. Fedele, A. O., Whitelaw, M. L., and Peet, D. J. (2002) *Mol. Interv.* **2**, 229–243
35. Giaccia, A., Siim, B. G., and Johnson, R. S. (2003) *Nat. Rev. Drug Discov.* **2**, 803–811
36. Keith, B., and Simon, M. C. (2007) *Cell* **129**, 465–472
37. Ruau, D., Ensenat-Waser, R., Dinger, T. C., Vallabhapurapu, D. S., Rolletschek, A., Hacker, C., Hieronymus, T., Wobus, A. M., Müller, A. M., and Zenke, M. (2008) *Stem Cells* **26**, 920–926
38. Maltepe, E., Krampitz, G. W., Okazaki, K. M., Red-Horse, K., Mak, W., Simon, M. C., and Fisher, S. J. (2005) *Development* **132**, 3393–3403
39. Lyssiotis, C. A., Walker, J., Wu, C., Kondo, T., Schultz, P. G., and Wu, X. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104**, 14982–14987
40. Wallberg, A. E., Pedersen, K., Lendahl, U., and Roeder, R. G. (2002) *Mol. Cell. Biol.* **22**, 7812–7819
41. Segovia, K. N., McClure, M., Moravec, M., Luo, N. L., Wan, Y., Gong, X., Riddle, A., Craig, A., Struve, J., Sherman, L. S., and Back, S. A. (2008) *Ann. Neurol.* **63**, 520–530
42. Parr, A. M., Kulbatski, I., Zahir, T., Wang, X., Yue, C., Keating, A., and Tator, C. H. (2008) *Neuroscience* **155**, 760–770
43. Meletis, K., Barnabé-Heider, F., Carlén, M., Evergren, E., Tomilin, N., Shupliakov, O., and Frisén, J. (2008) *PLoS Biol.* **6**, e182
44. Ohori, Y., Yamamoto, S., Nagao, M., Sugimori, M., Yamamoto, N., Nakamura, K., and Nakafuku, M. (2006) *J. Neurosci.* **26**, 11948–11960