

FM19G11, a new HIF modulator, affects stem cell differentiation status

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Running title: Sox2, a new target gene of HIF2 α

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The biology of the alpha 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 paper show the discovery of FM19G11, a novel chemical entity that inhibits the HIF α proteins that repress the target genes of the two α subunits, in various tumor cell lines as well as in adult and embryonic stem cell (ESC) 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 ESC. FM19G11 activity occurs in ependymal progenitor stem cells from rats (epSPC) - a cell model reported for spinal cord regeneration. That it allows the progression of oligodendrocyte cell differentiation in a hypoxic environment has created interest in its characterization for pharmacological research. Experiments using siRNA showed a significant depletion in Sox2 protein only in the case of HIF2 α silencing, but not in HIF1 α -mediated ablation. Moreover, ChIP data, together with the significant presence of functional hypoxia response element consensus sequences (HREs) 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 acetyl transferase 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.

INTRODUCTION

Hypoxia-inducible transcription factors (HIFs) 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 (1-4)). More recently, HIF biology has stretched 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, the 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 BHLH-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 hypoxia-responsive elements (HREs) acting as negative feedback regulators (13). HIF activity is mainly regulated at protein level, due to the hydroxylation of the key proline residues present in the oxygen-dependent degradation domain (ODD) of the α subunits by the prolyl-hydroxylases (PHDs) triggering polyubiquitination and rapid degradation of the HIF α proteins 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 the self-renewal and the 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 papers have shown how pluripotency can be acquired through only a few genetic modifications. Interestingly, the

experiments of Takahashi et al. showed that somatic cells can be reprogrammed into pluripotent stem cells by transduction of four defined transcription factors, c-Myc, KLF4, Sox2 and Oct4 (19,20), 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 the 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. MO Landazuri. This plasmid containing the neomycin resistance gene was used to generate a stably transduced HeLa cell line (HeLa-9x). Plasmids pCMV-TRE 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 mouse Sox2 promoter sequence -392/-1725 upstream of start codon (pGL3-mpSox2). 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 (mpSox2Δ) using the following primers: -1320 HRE FW: CCTATTTGTAACGGAAATGGGGCTGTGGCTC, RV_5' GAGCCACAGCCCCATTTCCGTTACAAATAGG; -725 HRE FW_5' GAATTAGGGGTTGAGGACAAATGCTGCGGTTCTTGAGC and RV_5' GCTCAAGGAACCGCAGCATTGTCTCAACCCCTAATTC. **Luciferase reporter activity assays**

10⁵ HEK293T, HeLa-9x, 293-TRE or 293-CRE cells per well were seeded onto white 96-well plates in quadruplicate 24 hours prior to assay. For transient over-expression, 0.05 ug/well in 96-well plates of each plasmid, ATF-2, JunB, c-jun, c-fos, PGL3-basic, pGL3-mpSox2, pGL3 and mpSox2Δ, were transfected with FuGene6 HD (3:6) 24 hours before stimulation. Serial dilutions of FM19G11 from 0 (containing DMSO as a control) up to 1 μM were added immediately before hypoxic stimulation in 1% O₂ atmospheres created by the *In vivo*₂ 400 chamber (Ruskin Life Sciences, USA). 6 hrs after stimulation, luciferase activity was quantified by addition of an equal volume of Bright-Glo Luciferase Reagent (Promega, USA) and detected in the VICTOR³ luminometer (Perkin Elmer, USA).

Cytotoxicity assay

Cell viability was measured by following the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay instructions (Promega, USA). 5x10⁴ HeLa cells per well were seeded onto 96-well plates 24 hours before assay. Serial dilutions of FM19G11 from 0 (containing DMSO as a control) up to 100 μM were used to stimulate the cells for 72 hours under standard oxygen conditions (~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 *Supplementary 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 media (GRM): DMEM:F12, B27 supplement (Invitrogen, CA), 25 μg/ml insulin, 6.3 ng/ml progesterone, 10 μg/ml putrescine, 50 ng/ml sodium selenite, 50 μg/ml holotransferrin, 40 ng/ml tri-iodo-thyroidin, supplemented with 4 ng/ml bFGF and 10 ng/ml EGF (Sigma, MO) for one day. Subsequently, cells were incubated with 20 ng/ml EGF and 10 μM of all-trans retinoic acid (ATRA) for 1 week. ATRA was then removed and the cells were exposed to GRM supplemented with 20 ng/ml 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 GRM supplemented with 20ng/ml EGF. For terminal differentiation, at day 35, oligodendrocyte precursor cells (OPCs) were seeded on poly-L-lysine and human laminin (Sigma, MO) 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 hrs with 500 nM FM19G11 or its DMSO vehicle as control. Then, the cells were harvested for total RNA or immunocytochemical staining.

hESC culture

Primary hESC colonies from the H9 line (WiCell Inc., Madison, USA) 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, USA), inactivated by mitomycin C in ES medium containing Knockout-DMEM, 1 mM L-glutamine, 100 mM non-essential amino acids, 20% serum replacement, 1% penicillin/Streptomycin, 8 ng/ml bFGF (Invitrogen, USA) and 100 mM β-mercaptoethanol (Sigma, USA). 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 RT-PCR

One microgram of total RNA, extracted by using the RNeasy Mini-kit (Qiagen, Germany), was reverse-transcribed (RT) 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 *Supplementary 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, CA, USA; *Supplementary data II*). As template, 40 ng of cDNA from target and housekeeping gene (*GAPDH*) were prepared in separate tubes for each primer master mix reaction. The comparative threshold cycle (C_T) method was used to calculate the relative expression (25).

DNA micro-array Analysis

epSPC isolation and DNA micro-array 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 PBS. Total cell protein extracts were isolated by use of 2% SDS-TrisCl lysis buffer plus proteinase inhibitors. Sub-cellular 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 GFAP (Chemicon, USA) at 1:1000 dilution. β -actin at 1:5000 dilution (Sigma, St. Louis, MO, USA) 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, USA), α -Hif-1 α (BD, USA), and rabbit ones, α -NG2 (Chemicon, USA) and α -Sox2 (Abcam, UK). For detection, Texas Red dye conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, USA) and Oregon Green 488 goat antimouse IgG at 1:400 (Invitrogen, CA, USA) 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 (Acc. No. NM_0243591.1) and HIF2 α (Acc. No. NM_023090.1) corresponded to the Cat. Nos. 4390816_s131713 and 4390816_s131443, respectively (Applied Biosystems, UK). 500 nM of siRNA were used for transfection.

ChIP analysis

Chromatin immunoprecipitation analysis used LowCell ChIP Kit (Diagenode, USA), in line with manufacturer's instructions, as described elsewhere (27). Samples were incubated with 10 μ g of anti-HIF2 α or AcH3 antibodies (Abcam, UK). An isotype-matched antibody was used as control for non-specific binding. Rat Sox2 promoter region was analyzed *in silico* by 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 HIF pathway, we used a stable luciferase reporter gene-based screen containing 9 repetitions of the HIF Responsive Element (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 ± 5 nM concentration (Figure 1A, *inset*). FM19G11 showed significant relative luciferase unit (RLU) inhibition from 30 nM, with steady reduction in a characteristic dose-dependent manner (Figure 1A) and reaching 80% inhibition at 1 μM (Figure 1B). To test the specificity of FM19G11 for HRE binding sites, we reproduced the luciferase-reporter gene assay by stable over-expression of CRE or TRE reporter constructs. ATF2 and JunB transient over-expression was used to induce CRE-mediated reporter gene activity; and c-fos and c-jun over-expression, for TRE induction (Figure 1B, *inset*). No significant RLU inhibition of TRE or CRE transcriptional activity was found in the presence of any tested concentration of FM19G11 (Figure 1B). It is worth mentioning that no cytotoxicity for concentrations of FM19G11 lower than 30 μM, in standard oxygen tension, or 50 μM, under hypoxic conditions, was observed on the HeLa cell line (Supplementary Figure 1).

Chemical synthesis of FM19G11

The FM19G11 compound was chemically re-synthesized for extensive evaluation. As shown in Figure 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 HIFα proteins in human tumor cell lines

We evaluated the effect of FM19G11 on total protein levels and on nuclear and cytoplasm fractionated extracts of HIF1α and 2α (Figure 1D and F), as well as the effect on the expression of their target genes PHD3 (Figure 1D) and VEGF (Figure 1E) in the HeLa cell line. Simultaneous incubation at 300 nM of FM19G11 from 1 to 12 hours of hypoxia

exposure (1% O₂) prevented HIF1α and HIF2α accumulation (this effect was extended to 24 h for HIF1α accumulation). Although most HIFα protein was found in the nucleus in all cases, a detectable amount of both isoforms (1 and 2α) 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 sub-cellular location of HIFα proteins were observed at any other tested time (Figure 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 (Figure 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 (Figure 1E). Because HIF promoter region contains multiple HRE, indicating a self-regulating mechanism (31), we also analyzed both HIFα isoforms at the mRNA levels in the presence of FM19G11. The hypoxia-dependent induction of HIF1 and 2α mRNA was significantly lower in the presence of FM19G11 after 6 hours 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 hours) or longer (9 or 12h) hypoxic exposition times (data not shown).

To investigate whether HIF1α 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. Interestingly, MG132 did not affect any isoform, HIF1 or 2α accumulation by FM19G11, suggesting a proteasome-independent mechanism on HIFα inhibition (data not shown). The action of FM19G11 on HIFα proteins was not restricted to HeLa cells, since 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 ependymal stem/progenitor cells (epSPC) and on human embryonic stem cells (hESC). Figure 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 (Figure 1H) and in hESC (data not shown) treated with 500 nM of FM19G11 for 48 hrs under hypoxia.

FM19G11 regulates Oct4 and Sox2 pluripotency markers

HIF2 α transcription factor directly regulates the expression of Oct4, indicating a function of this HIF protein in the self-renewal and differentiation of stem cell properties (8). We observed by real-time PCR that FM19G11 abrogates the increment associated with hypoxia of the transcriptional expression of Oct4 in both tested stem cell types, rat epSPC (Figure 2A and B) and human ESC (Figure 2C). In confirmation of recently published results (32), this transcriptional inhibition of Oct4 by FM19G11 was observed in parallel with the down-regulation in both stem cell types of Sox2, another important player in stemness maintenance (33) (Figure 2). In addition, epSPC treated with FM19G11 under hypoxia showed lower mRNA levels of TGF α and Nanog, target genes of Oct4 and Sox2, respectively (Figure 2 A, *lower panel*). The regulation of 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 of FM19G11 after 48 hrs in 1% O₂ (Figure 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 (Figure 2B 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*, *Nanog*, which are tightly regulated by an enhancer containing Oct4 and Sox2 binding motifs (18). Oct4 are reported to be a direct target of HIF2 α (8). Recently McCord et al., using the siRNA approach, reported for the first time experimental evidence indicating that Sox2 have HIF-dependent regulation (32). Here, we validated these results, by using specific siRNA oligos, the knock-down expression of HIF2 α , but not of HIF1 α , that blocks hypoxia-induced expression of both Oct4 and Sox2 in epSPC (Figure 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 rodent Sox2 gene. The *in silico*-predicted occupancy of HIF for HRE binding sites, over 5kb 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 ; Figure 3B). Finally, to disclose a specific role for HIF2 α in the Sox2 promoter activity depending on HRE activation, a chromatin immunoprecipitation assay (ChIP) 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 (Figure 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 Figure 3D or in Supplementary Figure 2 (for more details), two main groups after biological function clustering were over-represented 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 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 the 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 acetyl transferase (Figure 3E). These results were obtained in close association with the repression of HIF targets, including the 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 (Figure 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 till day 42, in line with our earlier research (23), by following the steps described in Figure 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 day 3 and from day 35 to day 37, respectively (Figure 4). At both stages, the hypoxic conditions blocked the differentiation process, according to the 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) (Figure 4B; and 4C for RIP expression). In all cases, the addition of FM19G11 rescued the expression of the above-mentioned cell markers (Figure 4B; and 4C 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 (Figure 4B). At day 37, the cells were also harvested for RT-PCR and Western blot analysis (Figure 4C). From day 35 of the differentiation protocol, the precursors were forced to a definitive maturation by culturing in a laminin matrix (Figure 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 (Figure 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 (Figure 4C), but in the presence of FM19G11 the cells recovered the low expression levels of these transcription factors at late differentiation stage. The expression of the astrocytic marker GFAP diminished from day 3 of the differentiation protocol after ATRA addition (23). Hypoxia significantly induced the expression of GFAP, indicating lower oligodendrocyte specification in the

epSPC culture. This induction was abolished by FM19G11 treatment (Figure 4C). Considering all above, the hypoxia-induced delay in directed oligodendrocyte differentiation was aborted by FM19G11 treatment.

DISCUSSION

Over the last 20 years, major efforts have gone into the search for HIF α inhibitors for use in new drugs (35). Though 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 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 elegantly hypothesized that Sox2 and Klf4 might also be HIF targets (36) and recently McCord et al. validated this hypothesis based on the inhibition by a siRNA of the 2 α isoform (32). However McCord et al. 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 chromatin immunoprecipitation (ChIP) experiment and the use of siRNAi experiments leading to HIF2 α -knock-down 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 the ChIP experiments carried out in the presence of the inhibitor FM19G11. All the above clearly pointed 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 of the key genetic factors that govern pluripotency. Microenvironment influence on chromatin assembly and on the accessibility and/or dynamic interplay of certain transcription factors determines 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 immuno-precipitation showed direct involvement of the acetylation mechanism in hypoxia and FM19G11 regulation over Sox2 transcriptional activity. Although p300 immunoprecipitation experiments proved a Sox2 interaction (unpublished data), 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, Nanog and TGF-alpha opened up new approaches to its use in cell reprogramming experiments with neural progenitor cells for the SCI regeneration model in rat. Therefore, loss of myelinating oligodendrocytes or oligodendrocyte progenitor cells (OPC) is a feature of many CNS 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 the 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.

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Key words: HIF, stem cell, differentiation, Sox2, ependymal cell, hESC oligodendrocyte

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FIGURE LEGENDS

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 6h. FM19G11 inhibits in a dose-responsive way HIF-translational activity in the luciferase reporter assay. *Inset*: Calculation of CI₅₀ by converting the RLU (relative luciferase units) into % of RLU inhibition. B) 293-TRE or 293-CRE, transiently over-expressing c-fos and c-jun or ATF2 and JunB, respectively, were treated with FM19G11 0.3 μ M 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 FM19G11 300 nM (+) or its vehicle (-). 24 h exposition at 20% O₂ in the absence of FM19G11 (-) was taken as 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 20% or 1% 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 FM19G11 300 nM (+) or its vehicle (-) for 4-9 h at 20% or 1% O₂. *Right panel*: Immunostaining for HIF1 α of HeLa cells treated with FM19G11 or its vehicle (DMSO) under hypoxia for 6 hs. G) epSPC: *upper panel*: Representative experiment of FM19G11 dose-response, 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 hours 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. * p <0.05 versus 20% O₂, § p <0.05 versus vehicle at 1% O₂ determined by Student's t test.

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, Tgfa 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 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 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 basal control. Results were obtained from three independent experiments. * p <0.05 versus 20% O₂, § p <0.05 versus vehicle at 1% O₂, determined by Student's t test.

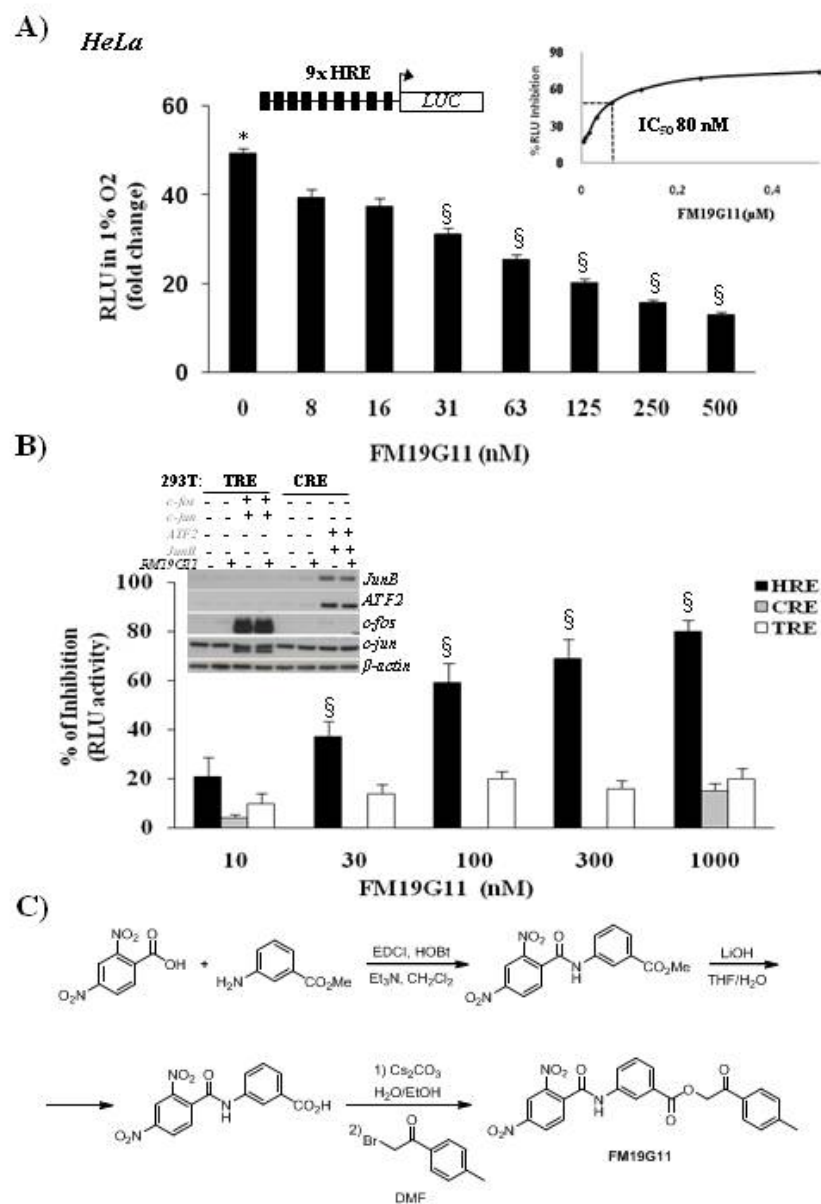
Figure 3. Hif2 α regulates Sox2 expression and influences the epigenetic mechanisms. A) epSPC were exposed to 20% O₂ or 1% O₂ for 48 h. 500 nM of each siRNA duplex, scramble (*Scr*, non-specific probe), HIF1 α or HIF2 α specific rat probes were transfected 24 h before oxygen-dependent stimulation. Western blot assay showed that only HIF2 α knock-down 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 rat Sox2 promoter. There were a significantly higher number of copies of 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 hs before hypoxic stimulus. D) FatiGO analysis of epSPC treated with 500 nM FM19G11 was compared with vehicle alone for 48h in hypoxia. Two biological functional groups were over-represented in FM19G11-treated sample after hierarchical clustering. E) *left panel*: Representative Western blot assay for epSPC treated (+) or not (-) with FM19G11 500 nM, exposed for 48 h at 20% or 1% O₂; *right panel*: densitometry analysis of three independent experiments. F) ChIP analysis within rat Sox2 promoter by using AcH3 antibody for chromatin immunoprecipitation. * (vs 20% O₂), § (vs vehicle at 1% O₂), $p < 0.05$ was determined by Student's t test.

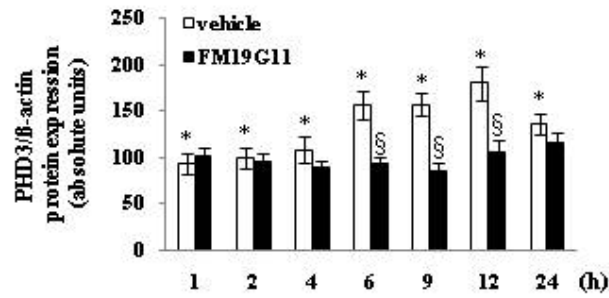
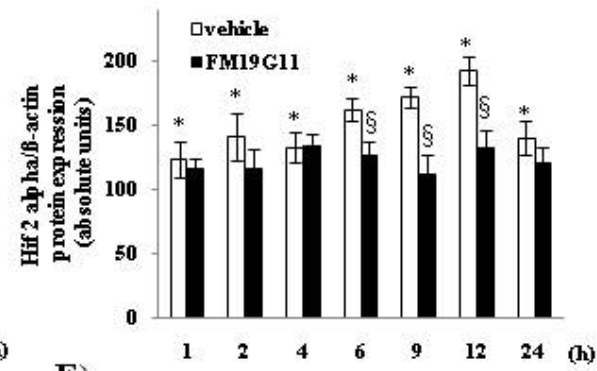
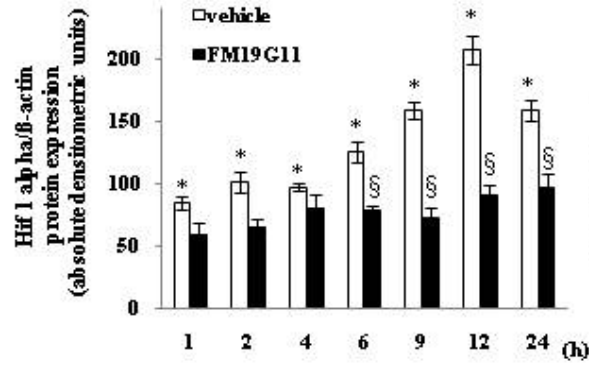
Figure 4. FM19G11 favors oligodendrocyte cell differentiation of epSPC under hypoxia.

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 day 3 of the differentiation protocol; *lower panels*, cells treated during day 35 to day 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 FM19G11 500 nM (+) and vehicle alone (-). The undifferentiated stage (Oct4, Sox2, Olig2, Nkx2.2, Notch1, Nestin, 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.

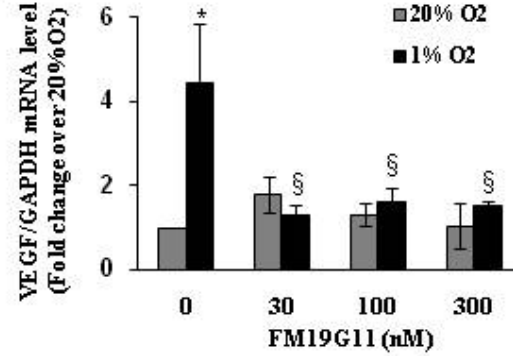
Figure 1



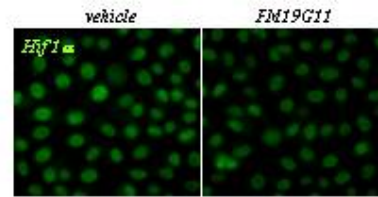
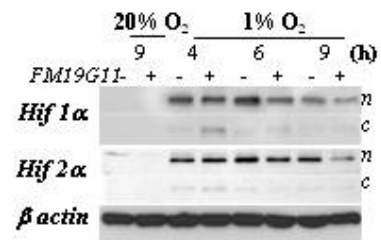
D) *HeLa*



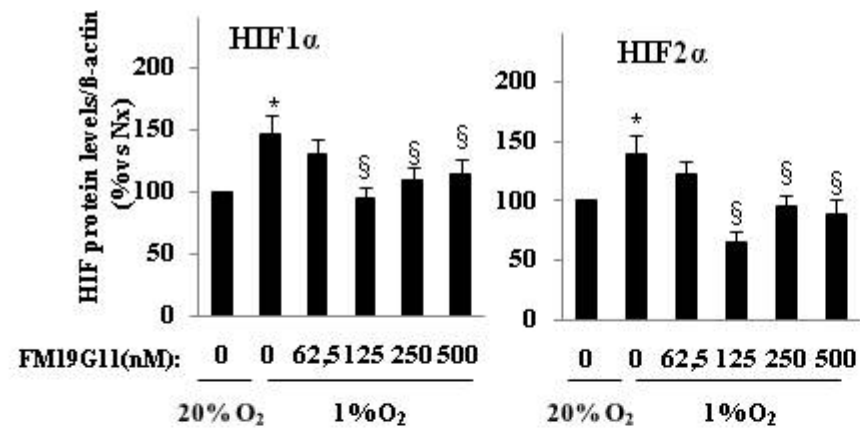
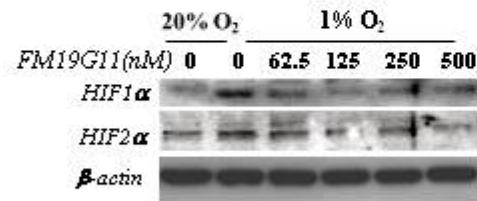
E)



F)



G) *epSPC*



H)

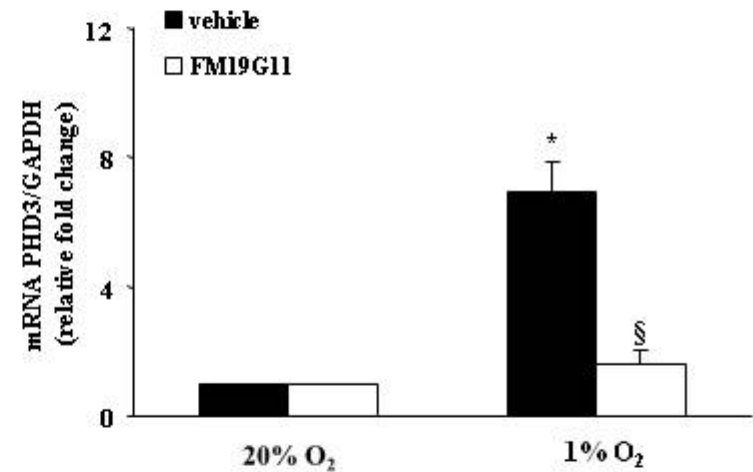
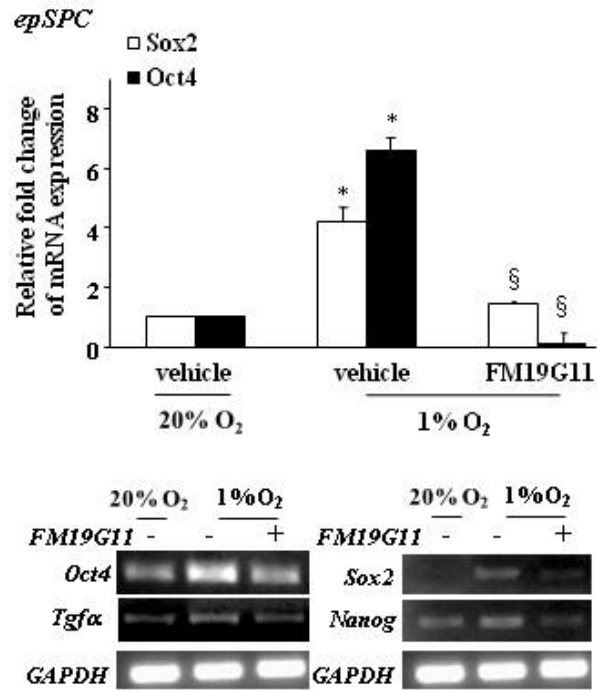
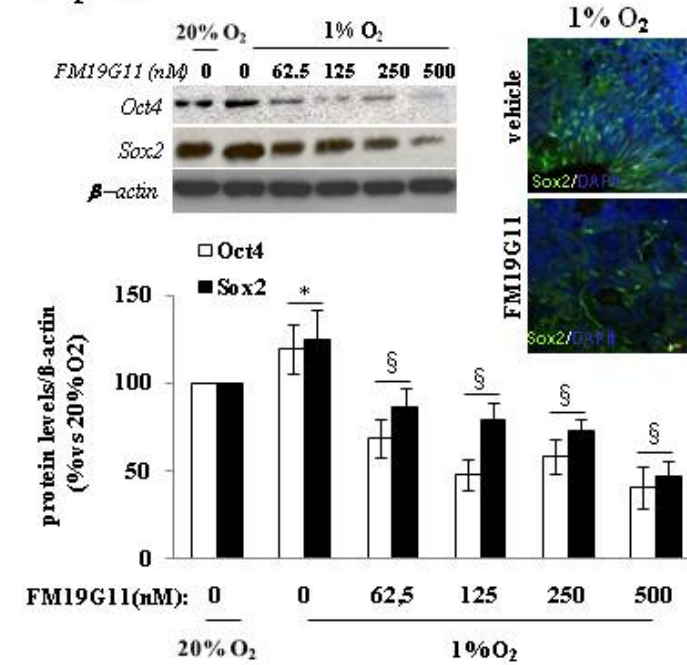


Figure 2

A)



B)



C)

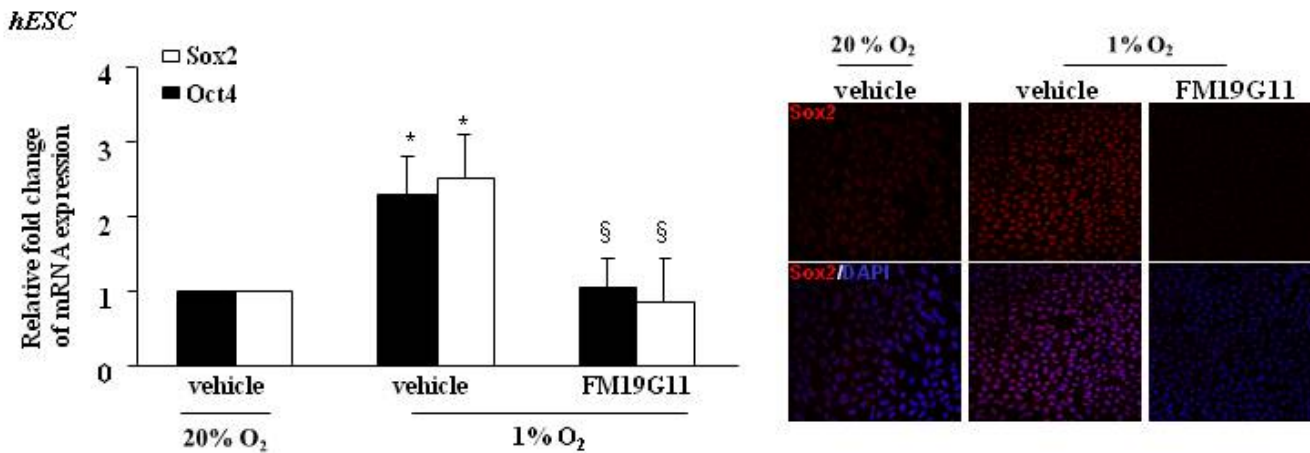
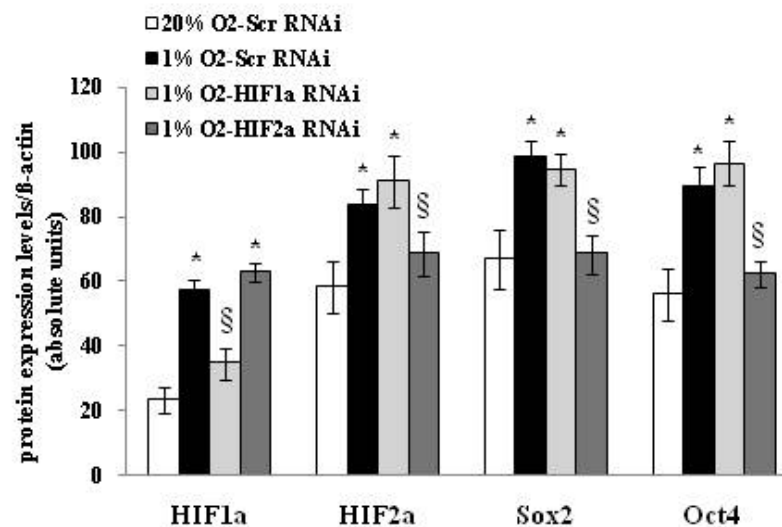
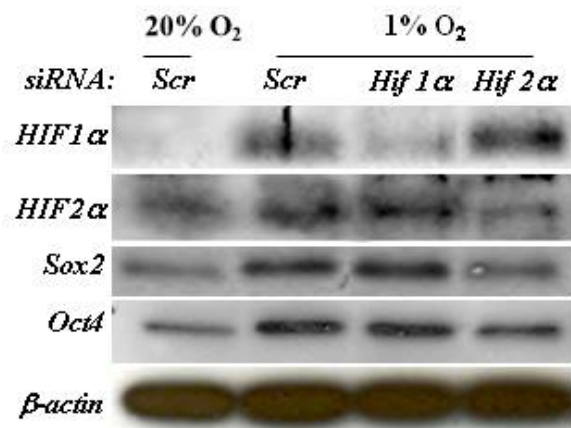
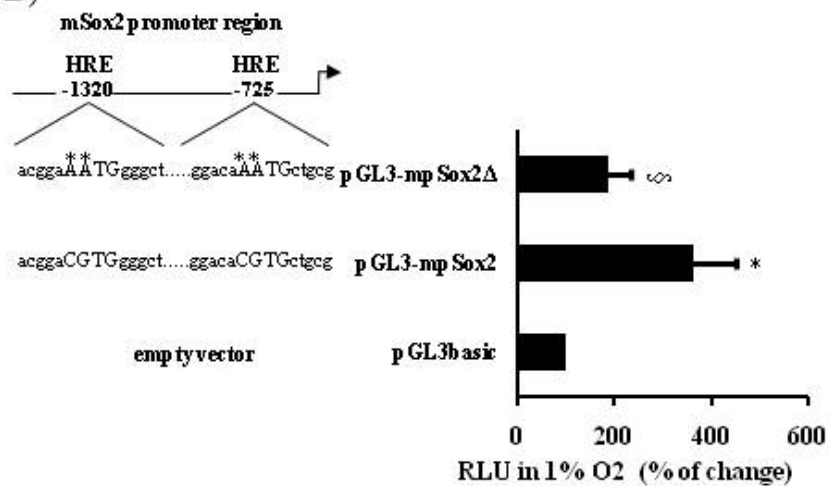


Figure 3

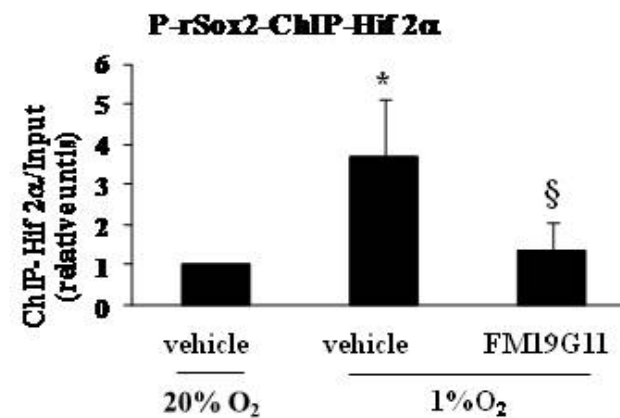
A)



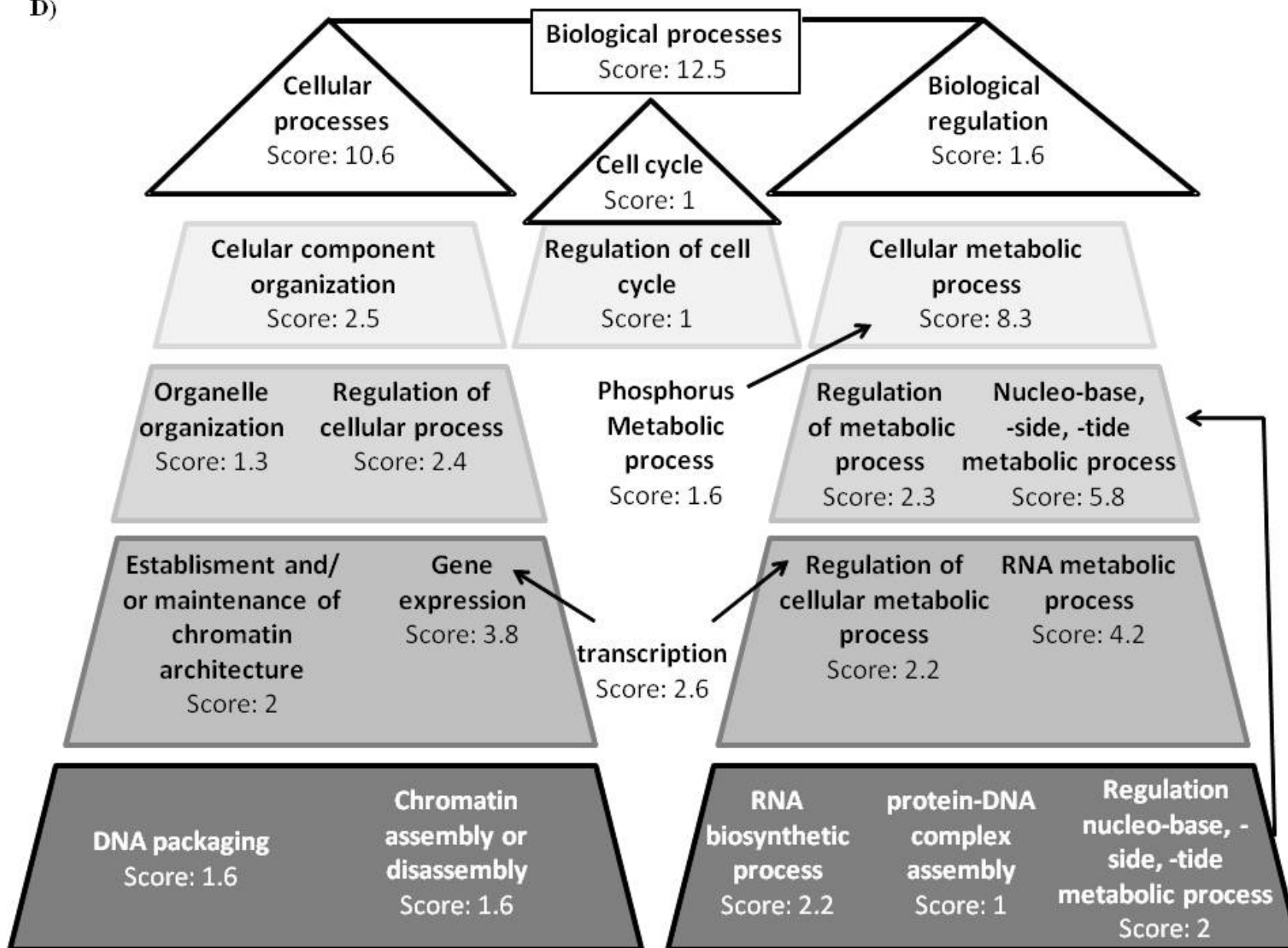
B)



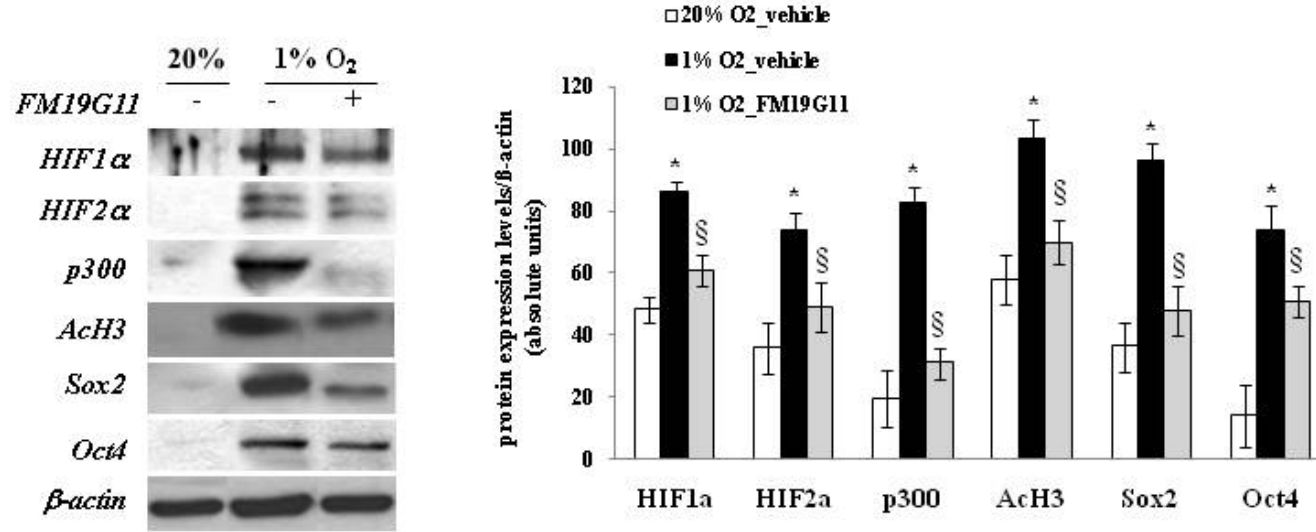
C)



D)



E)



F)

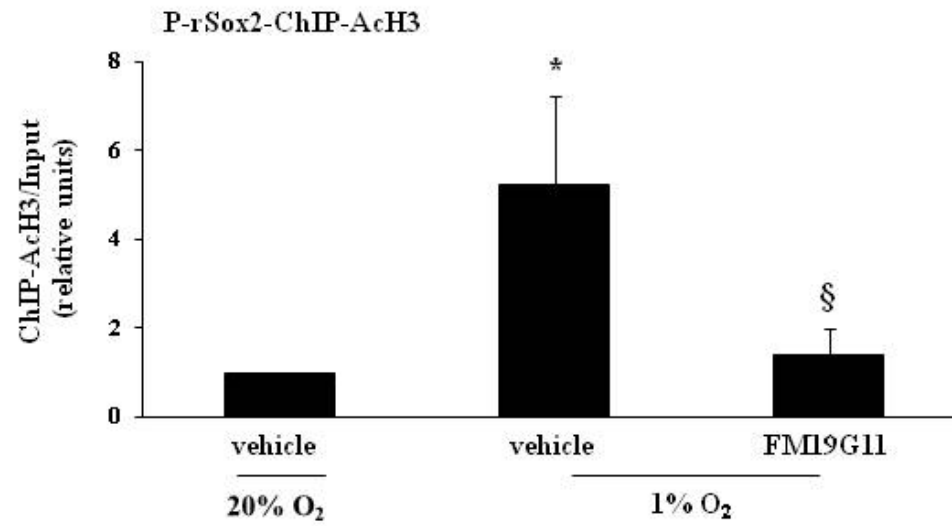
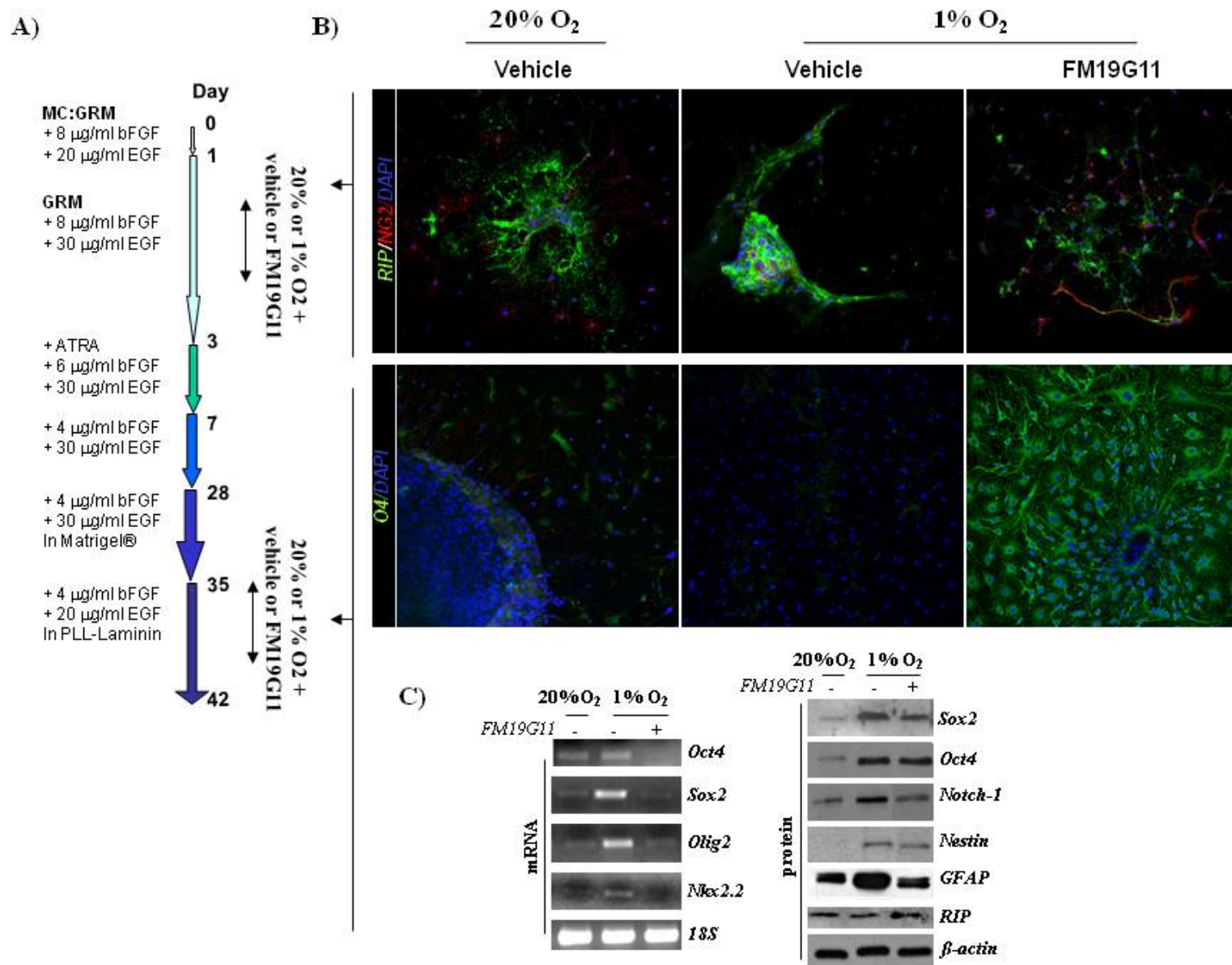


Figure 4



FM19G11, a new HIF modulator, affects stem cell differentiation status
Victoria Moreno-Manzano, Francisco Javier Rodriguez-Jimenez, Jose Luis Acena-Bonilla, Santos Fustero-Lardies, Slaven Erceg, Joaquin Dopazo, David Montaner, Miodrag Stojkovic and Jose Maria Sanchez-Puelles

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