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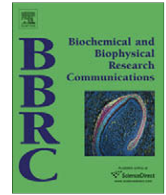

Concomitant inhibition of prolyl hydroxylases and ROCK initiates differentiation of mesenchymal stem cells and PC12 towards the neuronal lineage

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ABSTRACT

This study demonstrates that a prolyl hydroxylase inhibitor, FG-0041, is able, in combination with the ROCK inhibitor, Y-27632, to initiate differentiation of mesenchymal stem cells (MSCs) into neuron-like cells. FG-0041/Y-27632 co-treatment provokes morphological changes into neuron-like cells, increases neuronal marker expression and provokes modifications of cell cycle-related gene expression consistent with a cell cycle arrest of MSC, three events showing the engagement of MSC towards the neuronal lineage. Moreover, as we observed in our previous studies with cobalt chloride and desferroxamine, the activation of HIF-1 by this prolyl hydroxylase inhibitor is potentiated by Y-27632 which could explain at least in part the effect of this co-treatment on MSC neuronal differentiation. In addition, we show that this co-treatment enhances neurite outgrowth and tyrosine hydroxylase expression in PC12 cells. Altogether, these results evidence that concomitant inhibition of prolyl hydroxylases and ROCK represents a relevant protocol to initiate neuronal differentiation.

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In addition to a well known effect on angiogenesis, recent data have demonstrated that low oxygen tension favours neurogenesis. Indeed, hypoxia enhances proliferation, survival, and dopaminergic differentiation of neuronal precursors *in vitro* [1]. In addition, mild transient hypoxia in the newborn rat was shown to trigger substantial neurogenesis [2].

Oxygen deprivation initiates a wide range of adaptative responses and this process is largely controlled by the transcriptional factor hypoxia-inducible factor-1 (HIF-1), which consists of two subunits HIF-1α and HIF-1β. Whereas HIF-1β does not respond to changes in oxygen tension, HIF-1α is rapidly degraded in normoxia. Indeed, in the presence of O_2, Fe^{2+} and 2-oxoglutarate, HIF-1α is hydroxylated on prolines by prolyl-4-hydroxylases (PHs) which allows the binding of von Hippel–Lindau protein (pVHL) and the subsequent HIF-1α degradation through the ubiquitin–proteasome pathway. An asparaginyl-hydroxylase FIH-1 (Factor inhibiting HIF-1), requires also oxygen, iron and the co-factor 2-oxoglutarate to hydroxylate an asparagine on HIF-1α protein. This hydroxylation, under normoxic conditions, prevents the transcriptional activation of HIF-1 target genes. Therefore, hypoxia by inactivating PHs and FIH-1 stabilizes HIF-1α and allows the activation of hypoxia responsive elements (HREs) in HIF-1 target genes. As PHs and FIH require iron to be active, HIF-1 stabilization and activation can also be obtained with iron chelators (such as desferroxamine, DFX) and heavy metal ions (such as cobalt) [3]. More recently, it has been shown that HIF-1 activation can be induced also *in vitro* with PH inhibitors such as 2-oxoglutarate mimetics [4].

HIF-1α may be an essential mediator in the hypoxia-induced neurogenic effect since HIF-1α has been shown to be essential for normal brain development [5] and for the production of dopaminergic neurons [6]. In addition, EPO (erythropoietin) and VEGF (vascular endothelial growth factor), two well known HIF-1 targets, enhance neurogenesis both *in vitro* and *in vivo* [7].

In this context, we hypothesized that pharmacological activation of HIF-1 could promote neuronal differentiation. To test this hypothesis, we previously studied the effect of two HIF-1-activation mimicking agents, cobalt chloride ($CoCl_2$) and DFX, on neuronal differentiation of bone-marrow-derived mesenchymal stem cells (MSC) [8,9]. Our results indicated that HIF-1 activation, provoked by $CoCl_2$ or DFX, induced morphological and expression changes of MSC in accordance with neuronal differentiation [8,9]. Interestingly, both treatments were potentiated by Y-27632, an inhibitor of ROCK which is known to play a role in neuronal development [10]. Our hypothesis was further validated by our data showing that $CoCl_2$/Y-27632 co-treatment promoted also neuronal differentiation of neurospheres and PC12 cells [9].

Here, we used a PH inhibitor, FG-0041, which is a small molecule that has some structural features with 2-oxoglutar-

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ate. This 2-oxoglutarate mimetic induces HIF-1α stabilisation by interacting with the active site of PHs and by inhibiting their enzymatic actions [4,11]. The present study shows that PHs and ROCK inhibitions represent a potential strategy to initiate differentiation of MSC and other cell types such as PC12 cells towards the neuronal lineage.

Materials and methods

MSC purification and treatments: Mouse MSC were purified and characterized as previously described [8] and cultured in αMEM supplemented with 20% FCS and 2 mM glutamine. FG-0041 was kindly provided by Lundbeck A/S (Copenhagen, Denmark). Subconfluent MSC were treated with 50 μM FG-0041 ±30 μM Y-27632. Since FG-0041 and Y-27632 were resuspended in DMSO, control cells were also treated with 0.25% DMSO or 0.3% DMSO, respectively.

PC12 cells studies: Rat pheochromocytoma PC12 cells were maintained in Ham's F-12 medium supplemented with 15% HS and 2.5% FCS on collagen-coated 24-well plates. Cells were treated for 3 d with 10 or 25 μM FG-0041 and/or 30 μM Y-27632. Thereafter, cells were incubated with Hoechst 33342 (10 μg/ml) for 15 min. Neurite length and number were measured using ImageJ software and were then divided by the total number of cells.

Quantitative real-time RT-PCR: Total RNAs were extracted with Nucleospin® RNA II kit according to the manufacturer's protocol. One microgram of total RNAs from each sample were reverse-transcribed using Promega's RT system (RT: 42 °C for 1 h). Two or three microliters of RT product were then used for PCR amplification in a total volume of 25 μl. Forward (F) and reverse (R) primers were designed using Beacon Designer software and are summarized in Table 1. Assays were run in duplicate on the iCycler iQ™ real-time PCR detection system. The amplification profile was as follows: Hot Goldstar enzyme activation, 95 °C for 3 min; PCR 50 cycles at 95 °C, 15 s and 60 °C, 1 min. The PCR was done according to the manufacturer's protocol using the qPCR™ Core Kit Sybr™ Green I—No Rox. The amount of target was given by the formula $2^{-(\text{Ct gene of interest - Ct housekeeping gene})} \times 1000$ where Ct is the threshold cycle value. Results are expressed relative to the housekeeping gene: HPRT1 (hypoxanthine phosphoribosyltransferase 1) for MSC.

HIF-1α immunocytochemistry: Cells were fixed with 4% paraformaldehyde for 10 min and washed in PBS. Cells were then permeabilized with 0.5% Triton X-100-PBS and treated with 10% FCS-PBS for 30 min. Thereafter they were incubated overnight at 4 °C with the primary antibody (anti-HIF-1α, 0.4 μg/ml, 100-105 Abcam Novus-Biologicals) in 1% BSA–PBS. After washing in PBS, cells were then incubated 1.5 h with FITC-conjugated goat anti-mouse. Observations were performed on the stage of a Nikon Eclipse (TE2000-E) inverted confocal C1 microscope.

Table 1
Primers used for real-time RT-PCR.

Gene	Sequence	Product length (bp)
BTG2	F 5′-CCGTCATCATCGTTCTAATACAGC-3′	118
	R 5′-CCTCAGGAGACTGGAGAGGAAA-3′	
EPO	F 5′-AGCTCAGAAGGAATTGATGTCGC-3′	108
	R 5′-AGGAAGTTGGCGTAGACCCG-3′	
HPRT1	F 5′-ATTGACACTGGTAAAACAATGCAAA-3′	141
	R 5′-AATTTCAAATCCAACAAAGTCTGGC-3′	
Nestin	F 5′-GAGAAGACAGTGAGGCAGATGAGTTA-3′	113
	R 5′-GCCTCTGTTCTCCAGCTTGCT-3′	
p21	F 5′-CGAGAACGGTGGAACTTTGACTT-3′	96
	R 5′-GTAGACCTTGGGCAGCCCTAG-3′	
Tuj1	F 5′-GGCGCCTTTGGACACCTATT-3′	91
	R 5′-CCTCCGTATAGTGCCCTTTGG-3′	
VEGF-A	F 5′-AAATCACTGTGAGCCTTGTTCAG-3′	130
	R 5′-GCTGCCTCGCCTTGCA-3′	

Western blotting: After extraction with TNT buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X-100, pH 7.4 Sigma), proteins were separated on 4–12% gradient gels using the XCell Surelock Mini-Cell and the NuPAGE® MES SDS Running Buffer (Invitrogen Life technologies). Proteins were then transferred to polyvinylidene difluoride (PDVF) membranes using the XCell II Blot Module and the NuPAGE® Transfer Buffer (Invitrogen Life technologies). Membranes were blocked for 1 h in 5% non fat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (T-TBS) and incubated with the primary antibody anti-tyrosine hydroxylase (TH; 0.08 μg/ml, Chemicon) overnight at 4 °C. After washing in T-TBS, membranes were incubated for 1 h at room temperature with peroxidase-labelled secondary antibody. The immunoreactive bands were visualized by enhanced chemiluminescence.

Luciferase assay: HRE-pGL3SV40-luciferase construct was kindly provided by Pr. Soubrier (from INSERM U525, Paris, France) [12]. Using Amaxa nucleofection technology™, cells were transfected with 2 μg of luciferase construct (HRE-pGL3SV40-luciferase or pGL3SV40-luciferase) and pRL-TK plasmid. After 16 h of culture, cells were treated with 50 μM FG-0041 ±30 μM Y-27632. Cells were then lysed with passive lysis buffer. Firefly luciferase (FLS) and *Renilla* luciferase (RLS) signals were measured sequentially by using a Dual-Luciferase Reporter assay system and a luminometer.

Results

FG-0041 induces HIF-1 activation and the expression of its target genes in MSC

First, we verified the ability of FG-0041 to induce HIF-1α expression in MSC. By immunocytochemistry, we evidenced that 50 μM FG-0041 induced HIF-1α nuclear accumulation and increased its expression in MSC as early as 3 h of treatment (Fig. 1A). We studied the effect of different concentrations of FG-0041 (25, 50 and 100 μM) on MSC. Since 100 μM FG-0041 was toxic for MSC (data not shown), we chose the 50 μM concentration for the following studies on MSC. As HIF-1 binds to HREs in the enhancers of its target genes, we used a HRE-pGL3SV40 construct for a luciferase reporter assay to study whether 50 μM FG-0041 was also able to induce HIF-1 activation. As shown on Fig. 1B, treatment of MSC with FG-0041 resulted in HIF-1 activation after 36 h of treatment. In accordance with this HIF-1 activation, we observed an increase of mRNA expression for several HIF-1 target genes including EPO, VEGF and p21, 24 h after FG-0041 treatment (Fig. 1C).

Taken together, these data confirmed that FG-0041 was able to induce chemical activation of HIF-1 and the expression of its target genes in MSC.

FG-0041 and Y-27632 alone have no significant effect on MSC neuronal differentiation but FG-0041/Y-27632 co-treatment initiates this process

Seven days after treatment with FG-0041, MSC were elongated whereas they displayed a large flat morphology in control conditions (Fig. 2A). However, these morphological changes of MSC were less drastic than those observed in our previous studies with $CoCl_2$ or DFX [8,9]. Indeed, these modifications of MSC morphology only appeared after 7 d of FG-0041 treatment whereas 3 d of $CoCl_2$ or DFX treatments were sufficient to induce changes [8,9]. In addition, in accordance with these slow and slight morphological changes, we did not detect any modification of neural stem cell and neuronal marker mRNA expressions (nestin, Tuj1) after 24 h of FG-0041 treatment (Fig. 2B). However, when the ROCK inhibitor

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