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A Inibição do Fator Induzido por Hipóxia-1α (HIF-1α) prejudica a diferenciação induzida por ácido retinóico em células de neuroblastoma humano da linhagem SH-SY5Y

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Trabalho de Conclusão de curso apresentado como requisito parcial para obtenção do título de Bacharel em Ciências Biológicas na Universidade Federal do Rio Grande do Sul.

Orientador(a): Prof. Dr. Daniel Pens Gelain

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Resumo

Estudos indicam que a hipóxia tem um papel relevante na diferenciação de células tronco neurais. Baixas concentrações de oxigênio aumentam a taxa de sobrevivência, proliferação e de diferenciação dopaminérgica em células precursoras neurais em relação a culturas em concentrações normais de oxigênio. A hipóxia leva a acumulação do fator de transcrição induzido por hipóxia 1α (HIF-1α), estudos *in vitro* correlacionam o aumento de sua expressão com o aumento de marcadores de diferenciação neuronal, sendo assim, este fator de transcrição emerge como um possível ponto de regulação da diferenciação dopaminérgica. O objetivo deste trabalho foi investigar os efeitos da inibição seletiva deste fator de transcrição na diferenciação induzida por ácido retinóico em células da linhagem SH-SY5Y de neuroblastoma humano com o propósito de elucidar o papel do HIF-1α na diferenciação dopaminérgica. Nossos resultados indicam que a inibição do HIF-1α foi capaz de reduzir a morfologia dopaminérgica, assim como o imunoconteúdo e a expressão de marcadores de diferenciação neuronal, indicando um possível papel regulatório na diferenciação dopaminérgica por parte deste fator de transcrição.

Palavras-chave: Hipóxia, Diferenciação dopaminérgica, ácido retinóico, HIF-1α, neuroblastoma, SH-SY5Y

Resultados

Este trabalho será apresentado no formato de artigo científico, intitulado: **Hypoxia inducible factor-1α (HIF-1α) inhibition impairs dopaminergic differentiation induced by retinoic acid in SH-SY5Y cells.** Este foi redigido nos formatos do periódico Neurochemistry International.

Hypoxia inducible factor-1α (HIF-1α) inhibition impairs dopaminergic differentiation induced by retinoic acid in SH-SY5Y cells.

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Abstract: Several studies indicate hypoxia as a key player in neuronal stem cell differentiation and proliferation. Low oxygen concentration increases the survival and proliferation rate of neuronal precursors as well as the differentiation to a dopaminergic phenotype when compared to normal oxygen concentrations. Hypoxia leads to the accumulation of the hypoxia inducible factor-1α (HIF-1α). As *in vitro* studies have shown, there is a correlation between its up regulation and the increase of neuronal markers. Therefore, HIF-1α emerges as a possible regulatory step of dopaminergic differentiation. This work aims to investigate the effects of the selective inhibition of HIF-1α on the differentiation induced by retinoic acid in human neuroblastoma cells from the SH-SY5Y lineage with the purpose of elucidating its role in the dopaminergic differentiation. Our results indicate that HIF-1α inhibition is capable of reducing the neuron-like phenotype, the immunocontent, and the expression of neuronal markers indicating the regulatory role of this transcription factor in neuronal differentiation.

Keywords: Hypoxia, neural differentiation, dopaminergic differentiation, retinoic acid, HIF-1α, neuroblastoma, SH-SY5Y

1. Introduction

Neurodegenerative pathologies such as Parkinson's Disease (PD) represent a key area of focus. Between 7 and 10 million people have been diagnosed with PD, the second most common neurodegenerative disease associated with age, behind Alzheimer's disease (12). The traditional approaches to treating PD include the administration of drugs with the purpose of dopamine replacement, such as Levodopa (19). This treatment aims to ease the symptoms of the disease; however, as the neurodegeneration progresses and side effects improve, treatment lose effectiveness. This pattern leads the search for new approaches (20). For instance, cell transplantation (21,22) and the induction of endogenous stem cell differentiation (23) have become promising approaches for neuronal function replacement. Nonetheless, the results of alternative strategies vary and more studies exploring the mechanisms of neuronal dopaminergic differentiation are still needed in order to establish viable options.

Several studies have shown hypoxia as an important factor in neuronal dopaminergic differentiation regulation (1, 4, 13, 14). Studer *et al* (14) have shown that low oxygen levels promote proliferation, survival and dopaminergic differentiation in central nervous system (CNS) precursors cells when compared to normal oxygen levels. Wang *et al* (13) have shown the effects of hypoxia in mesenchymal stem cell transplantation therapies in a parkinsonian rat model of intracranial 6-OHDA administration.Under their study the animals which received hypoxia-exposed cells had long-lasting locomotor improvements when compared to normoxia-exposed cells.

The hypoxia inducible factor-1 (HIF-1) emerges as a possible regulatory agent due to its high activity during hypoxia, targeting several genes related to cell fate. HIF-1 is a

heterodimer composed by its $\boldsymbol{\alpha}$ and β subunits. The $\boldsymbol{\alpha}$ subunit has its stability, cellular location and transcriptional potency directly related to oxygen levels. In contrasts, the β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed and is not oxygen-sensible (10). Once it dimerizes HIF-1 binds to hypoxia responsive elements (HRE) and promotes gene expression related to angiogenesis, eritropoiesis, metabolism and cell proliferation and survival. Besides its fine regulation by oxygen levels, few other factors seem to stabilize HIF-1$\boldsymbol{\alpha}$ such as proinflammatory stimuli, including lipopolysaccharide (LPS) through toll-like receptors activation and succinate provenient the citric acid cycle. Zhang *et al* (17) have shown that retinoic acid administration in leukemia cell lines leads to HIF-1$\boldsymbol{\alpha}$ accumulation. The exact mechanisms by which it occurs are still unclear.

SH-SY5Y human neuroblastoma cells are an effective model to study differentiation due to its easy handling when compared to neuron primary culture and the ethical dilemma that surrounds the use of embryonic stem cells. SH-SY5Y express proliferative markers such as the immature neuronal marker nestin and the proliferating cell nuclear antigen (PCNA) (24). When treated with retinoic acid (RA), this cells exhibits extensive projections, cell-cycle arrest, and the expression of neuronal markers such as enolase 2 (*ENO2*), synaptophysin (*SYP*) and the microtubule-associated protein tau (*TAU*). SH-SY5Y are known for a catecholaminergic phenotype upon differentiation, expressing dopaminergic markers such as tyrosine hydroxylase (*TH*), dopamine transporter (*DAT*) and exhibit moderate levels of dopamine-β-hydroxylase (DßH) activity (5, 27). RA-treated SH-SY5Y cultures lack the expression of glial markers such as the glial fibrillary protein (GFAP) (24). The purpose of this study is to determine HIF-1$\boldsymbol{\alpha}$'s role in retinoic acid induced dopaminergic differentiation using SH-SY5Y cells. Our results show a partial but significant reduction of the neuronal

phenotype due to HIF-1α inhibition.

2. Methods

2.1 Cell Culture and Differentiation

Human neuroblastoma SH-SY5Y were obtained from the ECACC (European Collection of Cell Culture) and were grown in DMEM/F12 (Sigma-Aldrich - D8900) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO2 and 95% air in an humidified atmosphere. For differentiation cells were plated at 10^3 cells/cm² confluency and treated with retinoic acid at 10μM concentration in DMEM/F12 1% FBS for 7 days with retinoic acid pulses at days 0, 3 and 6.

2.2 siRNA knockdown

HIF-1α small interference RNA Silencer ® Select at 5nM (Ambion - 4392420, ID: n336610) and siRNA scramble (Ambion - AM4635) as control at 30 nM were transfected utilizing the reverse transfection protocol with siPORT™ NeoFX™ Transfection Agent (Ambion®, Applied Biosystems Inc.) and Opti-MEM following manufacturer's instructions. Cells were transfected in DMEM/F12 medium with 10% FBS without antibiotics. Knockdown efficiency was evaluated through RT-qPCR and Western Blot. Cells were transfected 24 hours prior to retinoic acid treatment (day -1).

2.3 Western Blot

For western blot analysis cells were lysed in 4X Laemmli buffer (250 mM Tris, 8% SDS, 40% glycerol and 0.002% bromophenol blue, pH 6.7), and then vigorously vortexed and boiled for ten minutes at 100°C. Samples were loaded and separated in 10% polyacrylamide gel and then electro-blotted to nitrocellulose membranes. Protein loading and

electroblotting efficiency were verified through Ponceau S staining. Membranes were washed in Tris-buffered saline Tween-20 (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20) and blocked in TBS-T with 5% BSA. Membranes were washed three times post-blocked and then incubated overnight at 4°C with primary antibodies (all primary antibodies were used at 1:1000 dilution) for HIF-1$\boldsymbol{\alpha}$ (Cell Signaling - 141795) and $\boldsymbol{\beta}$-actin (Sigma-Aldrich - A1978) and subsequent incubation with the corresponding species-specific secondary antibody (all secondary antibodies were used at 1:2000 dilution) coupled to peroxidase (Sigma-Aldrich - AP132P; AP124P) following chemiluminescence detection utilizing the Westar Nova 2.0 kit (Cyanagen - XLS071,0250) and the GE® ImageQuant LAS 4000 CCD camera to obtain images.

2.4 Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature followed by a 10 minute permeabilization utilizing ice-cold 0.1% Triton-PBS. In order to block nonspecific binding, cells were incubated with 1% albumin, 22.52 mg/mL glycine in T-PBS (PBS + Tween 20 0,1%) for 1 hour at room temperature. Cells were then incubated overnight with primary antibodies for glial fibrillary protein (GFAP) (Sigma-Aldrich - G6171) and Neurofilament-L (NEFL) (Cell Signaling - 2837) (all primary antibodies were used at 1:500 dilution), followed by an 1 hour incubation with its specie-specific corresponding secondary antibody coupled with Alexa Fluor® staining (488 nm or 555 nm) from Cell Signaling Technology® at room temperature (all secondary antibodies were used at 1:500 dilution). Cells were then incubated for 5 minutes with DAPI for nucleic acid staining (1:1000; D9542 - Sigma-Aldrich®). Between all steps cells were washed 3 times for 5 min in ice-cold PBS. Images were obtained through a Microscopy EVOS® FL Auto Imaging System

(AMAFD1000 - Thermo Fisher Scientific®). Immunocontent was measured as fluorescence intensity and was quantified utilizing the software ImageJ measuring the pixels of images with 100 μm of magnification. Total number of neurites per cell and average size of neurites were obtained with NeuronJ, an ImageJ plugin with 400 μm magnification.

2.5 Real Time-qPCR

Cells were collected utilizing the TRIzol reagent (Thermo Fisher Scientific). RNA extractions was conducted following manufacturer's instructions. cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit from Applied Biosystems. Real time polymerase chain reaction was carried employing SYBR™ Green PCR Master Mix kit, 100 ng of cDNA and 100μM of each primer: *ENO2* (fwd.: 5' CTGACAAAGTCCTGGTAGAGTG 3' rev.: 5' GATCGTTATTGGCATGGATGTTG 3'), *SYP* (fwd.: 5' AGACAGGGAACACATGCAAG 3' rev.: 5' TCTCCTTAAACACGAACCACAG 3'), *TAU* (fwd.: 5' GACAGAGTCCAGTCGAAGATTG 3' rev.: 5' AGGAGACATTGCTGAGATGC 3'), *HIF1A* (fwd.: 5' TGATGCTTTAACTTTGCTGGC 3' rev .: 5' TTTCAGCGGTGGGTAATGG 3') and the housekeeping genes *GNB2L* (fwd.: 5' GAGTGTGGCCTTCTCCTCTG 3' rev.: 5' GCTTGCAGTTAGCCAGGTTC 3') and *B2M* (fwd .: 5' TGCTGTCTCCATGTTTGAT 3' rev.: 5' TCTCCGCTCCCCACCTCTA 3'). Results were normalized in relation to the housekeeping genes (ΔCt) and for each case the most stable housekeeping gene was applied. Results were expressed using the $2^{-\Delta\Delta CT}$ method.

2.6 Statistical Analysis

Statistical analysis were performed with the GraphPad Prism software version 7.0 (GraphPad Software Inc., San Diego, USA). Data were evaluated by one-way ANOVA

(GraphPad Software Inc., San Diego, USA). Data were evaluated by one-way ANOVA followed by Tukey's Multiple Comparison *post-hoc* test or unpaired Student's t test when suitable. Differences were considered significant when $p<0.05$.

3. Results

3.1 SH-SY5Y cells HIF-1α silencing and retinoic acid induced differentiation

Cells were maintained in DMEM/F12 10% FBS and on day -1 cells were trypsinized for reverse transfection. Cells were seeded at a confluency $10^3/cm^2$. 24 hours after transfection cells were either collected for western blot or RT-qPCR or RA treatment was started. RA pulses were at days 0, 3 and 6. Every 24 hours after RA pulses pictures were taken by phase microscopy on days 1, 4 and 7. On day 7 cells were either fixed for immunofluorescence or collected for RT-qPCR. Protocol timeline is shown on Figure 1.

As shown in Figure 2, mRNA levels (2D) and HIF-1α immunocontent (2E) decreased when cells were transfected with 5 nM HIF-1α siRNA select (sHIF) in comparison to 30 nM scramble siRNA transfected cells (sSCR) without any morphological alterations (Figure 2A-C) that could indicate changes in cell viability. The incubation time used for all experiments was 24 hours since it showed greater effectiveness of inhibition when compared to other incubation times (data not shown). After we validated the siRNA transfection we proceeded with the differentiation protocol. Transfected cells were treated with 10μM retinoic acid for 7 days. Pictures were taken at days 1, 4 and 7 (Fig 3A-F). Retinoic acid treated + siRNA HIF-1α cells (sHIF + RA) cells showed apparent morphological alterations such as shorter neurites in comparison to retinoic acid treated + scramble cells (sSCR + RA).

3.2 *Downregulation of neuronal markers in response to HIF-1α inhibition*

In order to assess whether HIF-1α inhibition was capable of altering the expression of

classical neuronal markers related to the retinoic acid treatment we performed RT-qPCR for *TAU, ENO2* and *SYP.* As we can see in Fig. 3G-I the expression of such markers was reduced in sHIF + RA cells when compared to sSCR + RA cells (*TAU*: p=0.0148 ; *ENO2*: p=0.0390 ; *SYP*: p=0.0295) even though this difference wasn't as marked as the difference between the undifferentiated proliferative cells (undiff.) and sSCR + RA cells (*TAU*: p=0.0001 ; *ENO2*: p=0.0096 ; *SYP*: p=0.0049).

3.3 *Inhibition of HIF-1α leads to morphological alterations and diminished immunoreactivity of neuronal markers*

Differentiated cells were prepared for immunofluorescence staining in order to analyze the immunocontent of NEFL as a neuronal marker and GFAP as proliferative marker. As we can observe in fig. 4A-F there where morphological changes between undiff., sSCR + RA and sHIF + RA cells. The undiff. group showed a high proliferative phenotype with a strong GFAP signal, no projections and larger cell body area (fig. 4A-B). sSCR + RA cells demonstrated a highly differentiated phenotype with extensive projections and strong reactivity to NEFL antibody (fig. 4C-D). sHIF + RA showed reduced NEFL signal and shorter neurites, as well as a less differentiated state (fig. 4E-F). GFAP signal showed no significant difference between all groups (fig. 4G). sSCR + RA cells demonstrated larger neurites in comparison to both sHIF + RA (p=0.0044) and undiff. (p<0.0001) (fig. 4I). sSCR + RA showed a higher total amount of neurites/cell in comparison to undifferentiated (p=0.0094) cells but showed no significant difference when compared to sHIF + RA cells (fig. 4J).

4. Discussion

Several studies demonstrated the effects of hypoxia and hypoxia mimetic compounds

in neuronal differentiation (1, 4, 13, 14). Due to HIF-1***α***'s upregulation in such environments it has emerged as a possible modulator of cell differentiation. Lim et al (2) have shown that SH-SY5Y cells may also be differentiated by the administration of hypoxia-mimetizing chemicals such as deferoxamine (DFO) and that this differentiation is HIF-1***α*** dependent. Ceci *et al* (18) have shown that nickel, another hypoxia mimetic agent, is able to alter the expression of neuronal markers in NT2 cells. Several other studies have shown that HIF-1***α*** stabilization is able to promote dopaminergic differentiation (4, 13).

As shown by Zhang *et al* (17) retinoic acid itself is capable of rapidly increasing the HIF-1***α*** content in myeloid leukemic U937 cells and the suppression of HIF-1***α*** expression partially impaired the differentiation induced by retinoic acid in these cells. On the other hand, the upregulation of HIF-1***α*** induced by the hypoxia mimetic agent $CoCl_2$ combined with retinoic acid led to an enhanced cell differentiation. Due to its hypoxia-independent accumulation in presence of retinoic acid, HIF-1***α*** emerges as a strong candidate as regulator of cell differentiation induced by retinoic acid in a normoxic environment. The authors have also shown that HIF-1***α*** interaction with other transcription factors such as C/EBPα, Runx1 and PU.1, might be the reason why it has such an important role in RA-induced differentiation.

By RT-qPCR we demonstrated that neuronal markers suchs as *SYP*, *TAU* and *ENO2* were downregulated in sHIF + RA cells, even though, it was still higher than in undifferentiated cells. Our results complement the findings of Cimmino *et al* (3), who have shown that the inhibition of HIF-1***α*** leads to a reduced expression of neuronal markers *TUJ-1* and *NEFL* in retinoic acid-treated SH-SY5Y cells with lower neurite outgrowth and neurite average length. Even though we weren't able to see a significant difference on total neurite/cell between sSCR + RA and sHIF + RA cells, it is possible to observe an upward

tendency in the number of neurites/cell in the sSCR + RA group.

Even though the transcription factors involved in leukemic cells differentiation are not the same as in neuroblastoma cells, there are studies showing the interaction between transcription factors known to be important regulators in RA-induced differentiation in SH-SY5Y cells and HIF-1$\boldsymbol{\alpha}$. These interactions between HIF-1$\boldsymbol{\alpha}$ and other transcription factors may provide ground to explain our results. For instance, Korecka *et al* (25) demonstrated by microarray assay that the *ID1*, *ID2* and *ID3* genes were upregulated in RA treated SH-SY5Y cells while in other study Löfstedt *et al* (26) have shown by northern blot and by RT-qPCR that both *ID1* and *ID2* have a hypoxia-dependent expression. In the same study, Löfstedt and his colleagues were able to show that HIF-1 has a binding site within *ID2* regulatory sequence. HIF-1 might be able to interact with several other transcription factors involved in dopaminergic differentiation as positive or negative regulators and therefore being a key modulator of this process, leading to this diminished expression of mature neuronal markers and altered morphology.

Havrda et al (28) have shown Id2 function in adult dopaminergic neurogenesis. Using Id2 $^{-/-}$ mice they demonstrated that animals lacking such transcription factor have alterations in adult olfactory neurogenesis presenting a reduced number of dopaminergic neurons in the olfactory bulb. The authors demonstrate that Id2 is essential for dopaminergic specification in adult neurogenesis. On our work, we demonstrated that HIF-1$\boldsymbol{\alpha}$ has too an important role in dopaminergic differentiation. These findings complement previous works whereon authors have shown a stronger dopaminergic phenotype in response to either hypoxia or hypoxia-mimetic agents and subsequent HIF-1$\boldsymbol{\alpha}$ upregulation (1, 4, 13, 14). Gathering this informations and that ID-2 has an hypoxia-dependent regulation step in its expression (26), the interactions between both transcription factors turn in to a target for further studies in

dopaminergic differentiation.

By immunofluorescence microscopy we were able to show that the inhibition of HIF-1α was able to partially impair the differentiation induced by retinoic acid showing morphological alterations such as a reduced average length of neurites combined with a lower NEFL signal. We could not observe a significant difference in GFAP levels even though there is a clear reduction in its content in sSCR + RA cells. Cimmino *et al* (3) proposed that HIF-1α inhibition leads to an enhanced glial transdifferentiation in SH-SY5Y cells treated with RA. Nevertheless, SH-SY5Y cultures are composed of two different cell-types, the N-type and the less abundant S-type. The N-type cells are neuroblastic and responsive to RA, such that this population will differentiate, whereas the S-type cells maintain the phenotype. Encinas *et al* (7) demonstrated that a long-term RA treatment with high serum levels and without BDNF addition (as it was conducted by Cimmino *et al*) generates a mixed population culture. This occurs due to the fact that the RA will keep N-type cells in cell-cycle arrest while S-type cells will proliferate. This enhanced GFAP signal might be caused by the growing S-type population, given that we were not able to observe the same results in a short-period low-serum treatment whereupon the S-type population was not as significant. Further studies comparing the amount of immature/mature neuron markers might be able to explain if there is a diminished cell differentiation to a neuronal phenotype or as proposed by Cimmino *et al*, an enhanced glial transdifferentiation.

5. Conclusion

In conclusion, we show that, HIF-1α inhibition partially inhibits retinoic acid induced differentiation in SH-SY5Y cells. HIF-1α silencing was able to significantly change the morphology of retinoic acid treated cells as well as to reduce the expression of mature

neuronal markers. These findings suggest that HIF-1$\boldsymbol{\alpha}$ is involved in retinoic acid induced differentiation regulation and may provide ground for further studies about this transcription factor role in dopaminergic differentiation.

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7. Figure Legends

Figure 1: Treatment timeline. Protocols were performed on a 9-day time span, from day -1 to day 7. On day -1 cells were trypsinized and seeded for reverse transfection. On day 0, cells were either collected for RT-qPCR and western blot or retinoic acid (RA) treatment was initiated on the concentration of 10 μM. On days 3 and 6 cells received RA pulses. On day 7 cells were either fixed for immunofluorescence microscopy or collected for RT-qPCR.

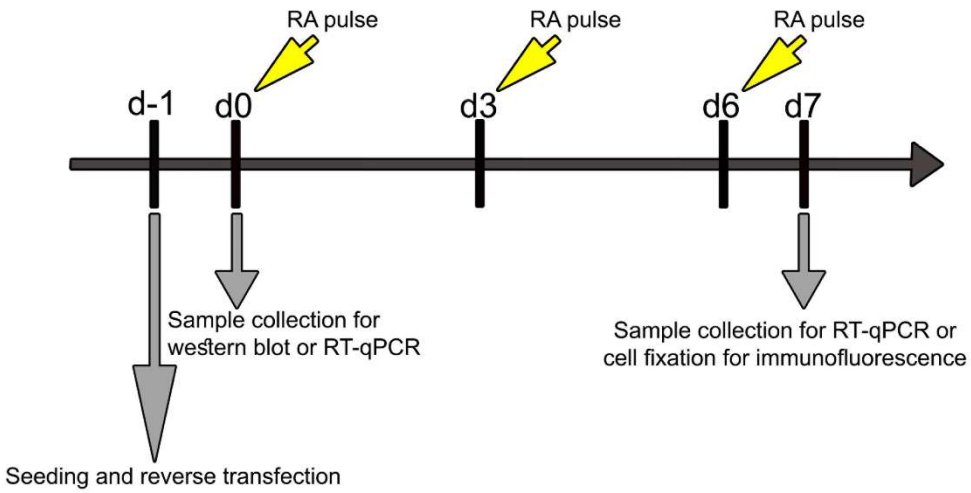
Figure 2: HIF-1$\boldsymbol{\alpha}$ siRNA transfection lead to a decrease in protein and mRNA levels without morphological alterations that could indicate changes in cell viability. A) Phase microscopy showing cell morphology in growth conditions prior to plating and siRNA transfection. B) Phase microscopy showing cell morphology after siRNA transfection of both HIF-1$\boldsymbol{\alpha}$ siRNA (sHIF) and C) scramble siRNA (sSCR). In D) HIF-1$\boldsymbol{\alpha}$ mRNA levels and E) HIF-1$\boldsymbol{\alpha}$ protein levels after transfection. (unpaired Student's t test *p<0.01, **p<0.005).

Figure 3: Cells transfected with scramble siRNA and HIF-1$\boldsymbol{\alpha}$ siRNA were treated for 7 days with retinoic acid. A) to F) show the morphology alterations due to retinoic acid treatment. Photos were taken 24h every RA pulse. On d7 cells were collected for RNA extraction and further cDNA synthesis in order to analyze neuronal markers expression through RT-qPCR. G) *ENO2*, H) *SYP*, I) *TAU* (one-way ANOVA followed by Tukey's Multiple Comparison *post-hoc* test *p<0.05, **p<0.01 ***p<0.001).

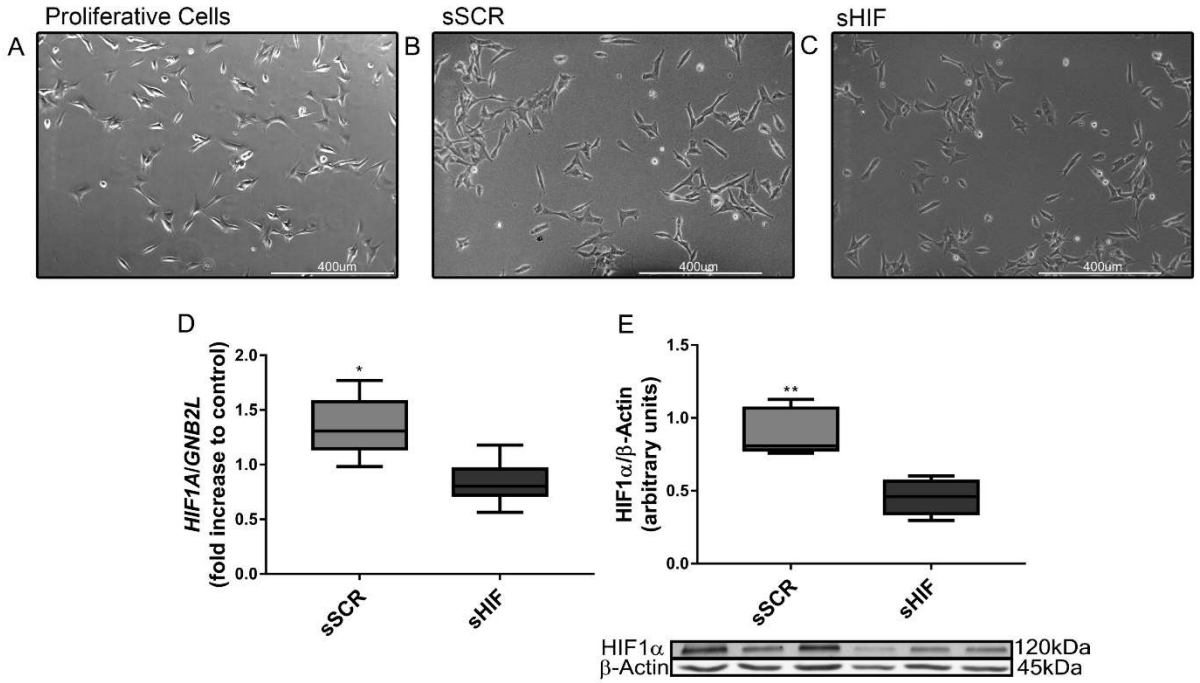
Figure 4: HIF-1α inhibition led to diminished levels of NEFL and a lower average neurite length. Cells were stained for immunofluorescence with anti-NEFL antibody (red) as a neuronal marker and anti-GFAP antibody (green) as a proliferative marker. Neurite length and total amount/cell were quantified. A) and B) show the undifferentiated (undiff.) cell morphology, C) and D) show sSCR + RA cells, E) and F) correspond to sHIF + RA cells. G) and H) show the fluorescence intensity of GFAP and NEFL respectively. Neurite average length and total amount of neurites/cell are shown in I) and J) respectively (one-way ANOVA followed by Tukey's Multiple Comparison *post-hoc* test *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001).

8. Figures

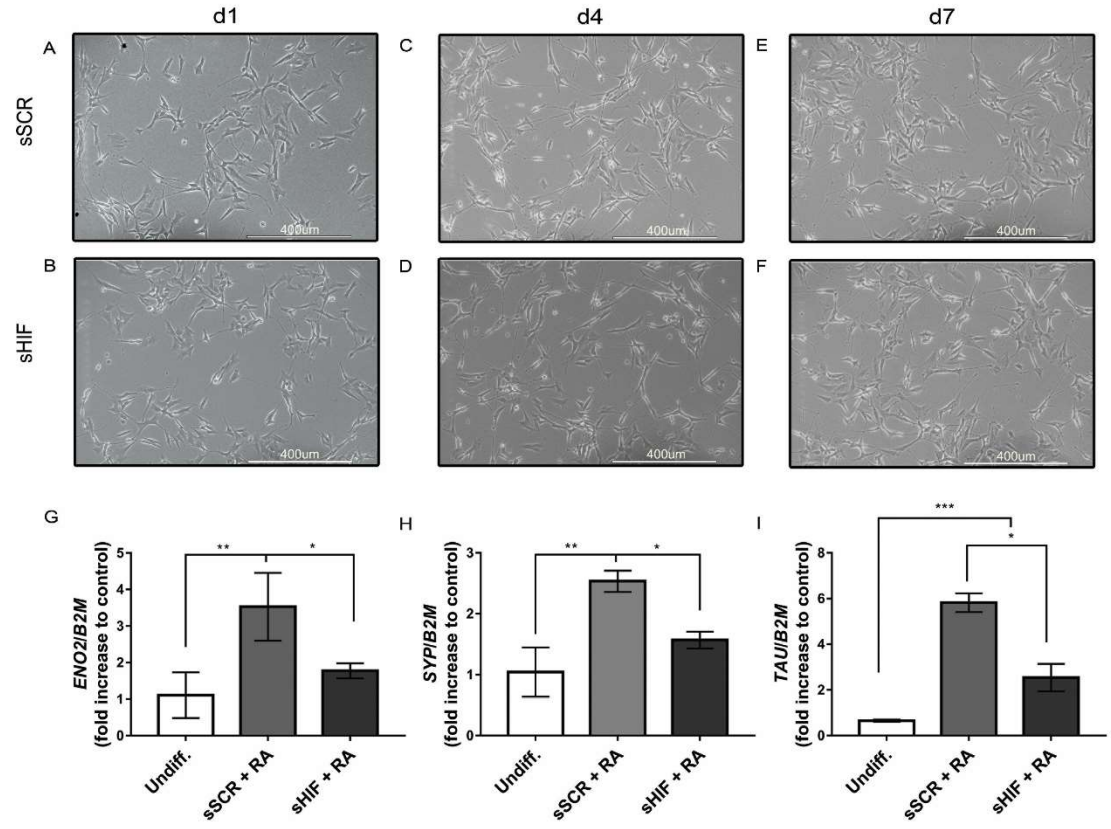
8.1 Figure 1:



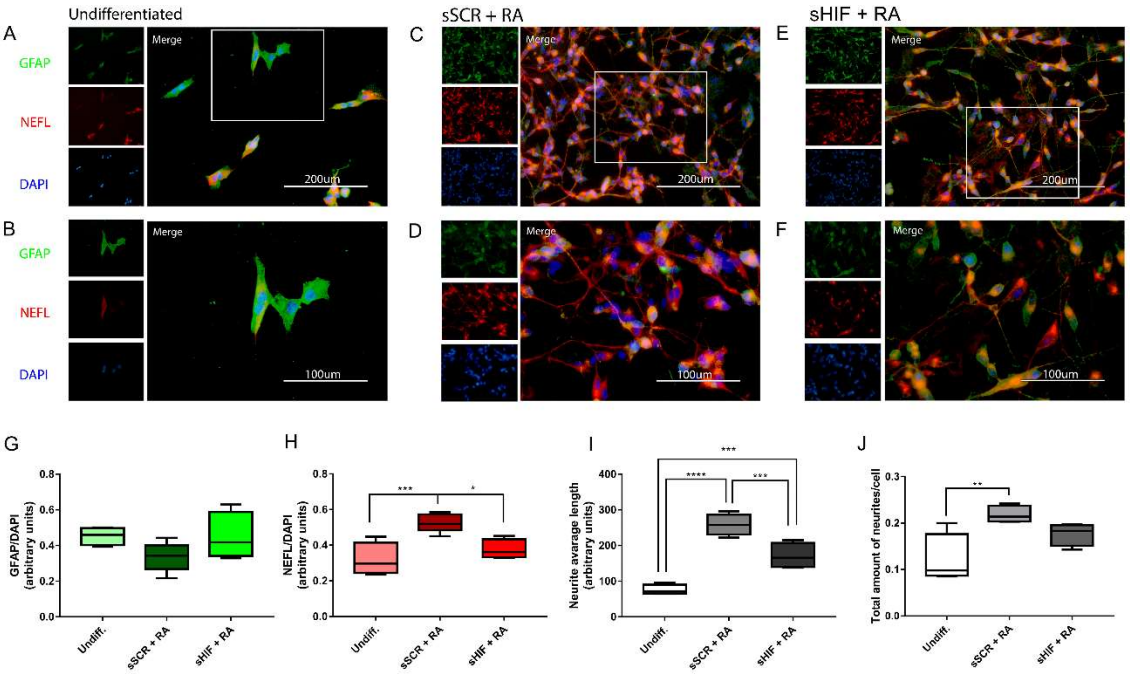
8.2 Figure 2:



8.3 Figure 3:



8.4 Figure 4:


Undifferentiated
sSCR + RA
sHIF + RA
A
B
C
D
E
F
GFAP
NEFL
DAPI
Merge
200um
100um
G
H
I
J
GFAP/DAPI (arbitrary units)
NEFL/DAPI (arbitrary units)
Neurite avarage length (arbitrary units)
Total amount of neurites/cell
Undiff.
sSCR + RA
sHIF + RA