Accepted Manuscript

Accepted Manuscript (Uncorrected Proof)

Title: The Effects of Exogenous IGF-I and MGF on Neural Stem Cells Proliferation in Hypoxic Environments in Vitro

Authors: Tuğba Aydıntuğ Gürbüz[1,2], Fatih Toprak[3], Selin Fulya Toprak[1,2], Selcuk Sozer[1,*]

1. *Department of Genetics, Aziz Sancar Research Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey.*
2. *Institute of Health Sciences, Istanbul University, Istanbul, Turkey.*
3. *Department of Neurosurgery, Haydarpaşa Numune Training and Research Hospital, Istanbul, Turkey.*

***Corresponding Author**: Selcuk Sozer, Department of Genetics, Aziz Sancar Research Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey. Email: ssozer@istanbul.edu.tr

To appear in: **Basic and Clinical Neuroscience**

Received date: 2022/02/08

Revised date: 2022/09/14

Accepted date: 2022/09/21

Accepted Manuscript (Uncorrected Proof)

This is a “Just Accepted” manuscript, which has been examined by the peer-review process and has been accepted for publication. A “Just Accepted” manuscript is published online shortly after its acceptance, which is prior to technical editing and formatting and author proofing. *Basic and Clinical Neuroscience* provides “Just Accepted” as an optional and free service which allows authors to make their results available to the research community as soon as possible after acceptance. After a manuscript has been technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as a published article. Please note that technical editing may introduce minor changes to the manuscript text and/or graphics which may affect the content, and all legal disclaimers that apply to the journal pertain.

Please cite this article as:

Aydıntuğ Gürbüz, T., Toprak, F., Fulya Toprak, S., Sozer, S. (In Press). The Effects of Exogenous IGF-I and MGF on Neural Stem Cells Proliferation in Hypoxic Environments in Vitro. Basic and Clinical Neuroscience. Just Accepted publication Jul. 10, 2022. Doi: http://dx.doi.org/10.32598/bcn.2022.3981.1

DOI: http://dx.doi.org/10.32598/bcn.2022.3981.1

Accepted Manuscript (Uncorrected Proof)

ABSTRACT

Ischemic stroke has high morbidity and mortality rates worldwide. Low oxygen (O_2) levels detected in such conditions create a vulnerable environment for neural stem cells (NSC), altering neuronal function and leading to neuronal injury or death. There are still no effective treatments for such consequences. We have investigated the molecular and functional effects of growth factors, IGF-I and MGF in NSC exposed to low O_2 levels. An *in vitro* ischemia model was created by rat hippocampal NSC grown in culture that were exposed to varying oxygen levels including 0%, 3%, and 20 % for the representation of anoxic, hypoxic, and normoxic conditions, respectively, during the 24 hours. NSC was investigated for *IGF-I, MGF,* and *HIF1-Alpha* (*HIF-1α*) gene expressions by real-time RT-PCR. The effects of external administration of growth factors, IGF-I and MGF on NSC proliferation in such conditions were explored. Increased *IGF-I* and *MGF* gene expressions were detected in the samples exposed to low O_2. Anoxia was the highest stimulant for *IGF-I* and *MGF* expressions. *HIF1*-α that encodes hypoxia-inducible factor-1α revealed downregulation in relative gene expression fold change with IGF-I application in all conditions, whereas MGF application upregulated its change in an anoxic environment. Furthermore, MGF-induced NSC had more proliferation- migration rate in all oxygen conditions. IGF-I induced significant NSC proliferation in 0% and 20% O_2. These findings suggest that *IGF-I* and *MGF* expressions were increased to reduce the damage in NSC exposed to low oxygen, and exogenous MGF and IGF-I application increased NSC proliferation at the time of injury. The results might imply the role of exogenous MGF and IGF-I in the treatment of ischemia for relieving the effect of neuronal damage due to their neuroprotective and proliferative effects.

Key Words: Neural Stem Cell, Hypoxia, Growth Factors, MGF, IGF-I

Accepted Manuscript (Uncorrected Proof)

INTRODUCTION

An ischemic stroke results from insufficient blood flow and causes cells in the tissue to be exposed to low oxygen (O_2) (hypoxia). The effect of such changes and impairment of the normal cellular function of the tissue inevitably progresses to severe diseases, including stroke and death in humans (1, 2). The ischemic tissue damage has a complex pathophysiology that can affect many different cells and tissues. Such neurons require a constant supply of O_2 to maintain ionic gradients across their membrane for impulse conduction and are vulnerable to hypoxic changes (3). The brain consumes 25% of the O_2 requirement of the whole body, revealing the O_2 as one of the most critical factors for the brain (4). The deficit in the vascular flow causes a decrease in the intracellular ATP levels that initiates apoptosis within 2-3 minutes after energy deprivation (5). The death of oligodendrocytes, astrocytes, endothelial cells, and various neuron types leads to the loss of regional tissue in the brain (6). Therefore, neuronal damage triggered by cerebral ischemia/reperfusion injury is the primary cause of the neurological disorder. In such cases, supporting neurogenesis is an essential factor for the success of the recovery of cerebral function. Neural Stem Cells (NSC) could regenerate the central nervous system (CNS) with the ability to differentiate into astrocytes, neurons, and oligodendrocytes(7). Neurogenesis occurs in the specialized microenvironment of NSCs. The NSC niche, thus, plays an essential role in nerve regeneration and repair of damaged brain tissue (8). It is well known that stem cells migrate to damaged areas (9). Recent studies revealed that both endogenous and transplanted NSC could be activated by cerebral ischemia and have a role in neural regeneration (10). Therefore, understanding the cellular biology of NSCs in the hypoxic environment in an *in vitro* ischemia model may provide new opportunities for controlling the expansion and proliferation of NSC, which could lead to improving new therapies after ischemic stroke.

The O_2 concentration of the niche is estimated to be 3% (11), whereas, in alveolar lung cells, it is 20% (12), and in arterial blood flow is 10.5-13% (13). In some studies, low oxygen levels (~1% O_2) have been shown to lead to cellular damage and death of the quiescent NSC as detected in brain ischemia (14). However, some findings suggest that reduction of O_2 levels in the microenvironment, such as 3-5% regulates the biological properties of NSCs *in vitro* and initiates their proliferation and differentiation ability. The stroke studies performed in rodents and primates have increased neurogenesis in their brain's sub-ventricular and sub-granular regions (15, 16).

Accepted Manuscript (Uncorrected Proof)

Insulin-Like Growth Factor-1 (IGF-I) is a neurotrophic factor for the repair and development of neurons, and numerous *in vivo* and *in vitro* studies revealed its effect on NSCs development (17). IGF-I promotes cell proliferation, differentiation, and survival (18). IGF-I creates different mRNA variants due to alternative splicing in many tissues, especially in skeletal and cardiac muscle, liver, and brain (19). At least three alternative splice variants of IGF-1 have been demonstrated. IGF-1 Ea, which acts systemically, IGF-1 Eb, and IGF-1 Ec, activated with mechanical damage and expressed in a mechano-sensitive manner, was named a mechano-growth factor (MGF) (20, 21). MGF stimulates muscle stem cells to re-enter the cell cycle, initiate proliferation, and further support new muscle cells (22, 23). MGF expression has been detected in damaged tissue, including the brain and heart, after various stress conditions such as ischemia (20), (24), particularly in the damage-resistant region. The endogenous MGF expression was also detected in proliferating cells in neurogenic niches and also hypoxic neuroblastoma cells in *in vitro* ischemic stroke model (24-26), which all were determined to have a role in neuroprotection (24, 27, 28). Furthermore, studies have shown that exogenous administration of MGF and IGF-1 reduces ischemic brain damage (29).

Transcription factor hypoxia-inducible factor-1 (HIF-1) is essential in regulating many hypoxia-activated genes across many different types of cells (30). HIF- 1 binds to hypoxia-sensitive elements (HRE) located in the promoter region of target genes and is thus a pleiotropic transcription factor that controls their transcription (31). HIF-1 consists of HIF-1α and HIF-1β subunits. The mRNAs of HIF-1α and HIF-1β are constitutively expressed in cells (32). However, HIF-1α protein expression is tightly regulated by changes in cellular oxygen and growth factors, including IGF-I, IGF-II, and angiotensin II (33). It is primarily regulated at the level of protein stability (34). In normal O_2 levels (normoxia), HIF-1α is rapidly degraded via targeted ubiquitination and followed by its subsequent degradation by the proteasome(35). In response to hypoxia, HIF-1α becomes rapidly stabilized, localized to the nucleus, and forms the HIF-1 complex with HIF-1β (33, 36). Recent studies have shown that, especially in tumors, the hypoxic cells exhibit resistance to apoptosis and these tumor cells were more aggressive, probably due to the overexpression of HIF-1α (37). Therefore, there has been an increase in the strategies to develop modalities that target HIF-1 activity and might become an alternative treatment strategy in cancerogenesis within recent years (38, 39).

Although these studies showed a neuroprotective role for IGF-I and MGF, the function of IGF-I and MGF under varying oxygen levels is still not well understood. In this study, growth factors, IGF-I and MGF, were investigated on NSC under varying oxygen levels. By doing so, an *in vitro* ischemia model was created by exposing adult hippocampal rat NSC to varying

levels of O_2, representing anoxia, hypoxia, and normoxia conditions. The effects of varying O_2 levels on NSC gene expressions, specifically *IGF-I*, *MGF*, and *HIF-1α*, were investigated. The external administration of growth factors, IGF-I and MGF, to NSC exposed to such conditions were performed, and their effects on gene expressions and NSC proliferation were examined. Our study is the first to reveal the effect of MGF and IGF-I in a hypoxic environment and further inquiries about their possible therapeutic application in such conditions.

Accepted Manuscript (Uncorrected Proof)

Materials and Methods

Neural Stem Cell Culture

Adult rat hippocampal neural stem cells line (Sigma-Aldrich, Merck, Germany) were cultured at a density of 6×10^5 cell/ml and 300 µl/well culture media in Poly-L-Ornithine (PLO) (Sigma-Aldrich) (10 mg/ml) and Laminin (Sigma-Aldrich) (6 µg/ml) coated cell culture plate. NSC culture media contained B-27 supplement (Invitrogen), DMEM/F12 (Biochrom), 100 µg/ml streptomycin (1%), l-glutamine (200mM) and FGF-2 (20 ng/ml) (PeproTech). FGF-2 was added fresh to the medium every time. NSCs were incubated at 37°C in 5% CO_2. When NSCs reached 70-80% confluence in the flask, the cells were transferred to the plates for the following experiments.

Oxygen Exposure of NSC

The Cell ASIC ONIX Microfluidic System (Merck) was applied to investigate the different oxygen (O2) levels, including 0 %, 3%, and 20%, and growth factors including IGF-I and MGF on NSC *in vitro, as* summarized in Figure 1. M04S-03 Microfluidic Plates for Cell ASIC ONIX (Merck) were coated with PLO (Sigma) and Laminin (Sigma) as described previously (40). This system provides the ability to control the environment of individual plates and provides simultaneous analysis of the living cells *in vitro*. Since it is designed to provide a dynamic cellular microenvironment, namely niche control, it is suitable for studies investigating extracellular factors' effects on cells. NSCs were seeded at $1x10^4$ cells per well, and the manufacturer applied a gravity-driven perfusion culture protocol. After 24 hours of incubation, the stabilized cells were treated with growth factors following the experiment design. DMEM / F12 solution containing were referred to as untreated. In external administration of growth factors, IGF-I 0.2µg/ml or MGF 0.2µg/ml were prepared and added to the relevant wells. The

O_2 levels were adjusted according to the experiment design: Anoxia with 0% O_2, hypoxia with 3% O_2, and normoxia with 20% O_2. Culture plates were connected to the Cell ASIC device with the manifold's help, following the manufacturer's instructions (40). The experiments were set to 24 hours in the software available on the computer. Readouts were analyzed with the program integrated into the software.

Cell Proliferation Assay

NSC exposed growth factors, IGF-I and MGF, were analyzed after 24 hours. The image program was used to measure cell proliferation. Phase-contrast images of NSCs were taken from the culture plate under an inverted microscope (Olympus CKX41) equipped with a digital camera with a 10X objective before starting the experiment (0 hours) and after the experiment was concluded (24 hours). The images were analyzed with the ImageJ program (developed by W.S. Rasband, NIH). Since the culture time for NSC in defined conditions was estimated to be 24 hours in this study, instead of counts of neurospheres, the effect of O_2 levels and growth factors on NSC in the migration-proliferation without disturbing such O_2 levels in culture conditions, the total area of the seeded cells were measured and compared. The analysis was performed on the images that could provide information for the functions of both migration-proliferation of NSC. By doing so, the area of the cells was measured with ImageJ. The calculations were performed using the formula: Area of the cultured cells after 24 hours / Area of the cultured cells in 0 hours in defined conditions.

RNA isolation, cDNA synthesis, and Real-time PCR

Total RNA isolation was performed using a total RNA purification kit (Jena Bioscience) in NSC. SCRIPT cDNA Synthesis Kit (Jena Bioscience) was used for cDNA synthesis from 50 ng/ µl RNA. *IGF-1Ea*'s forward primer was 5'-GCT TGC TCA CCT TTA CCA GC-3,' and the reverse primer was 5'-AAG TGT ACT TCC TTC TGA GTC T-3' with 130 base pairs (bp) in length. *MGF*'s forward primer was 5'-GCT TGC TCA CCT TTA CCA GC-3,' and the reverse primer was 5'-AAG TGT ACT TCC TTT CCT TCT C-3'(130bp). *HIF1-a* forward primer was 5' TGC TTG GTG CTG ATT TGT GA 3'and the reverse primer was 5'-GGT CAG ATG ATC AGA GTC CA-3'(131bp). *GAPDH* was applied as a housekeeping control. *GAPDH*'s forward primer was 5'GGT GTG AAC GGA TTT GGC CGT AT-3'and reverse primer 5'CTC AGC ACC AGC GTC ACC CCA TT3'(129bp).

Accepted Manuscript (Uncorrected Proof)

Changes in each gene expression were detected with Sensi FAST SYBR No-ROX Kit (Bioline, UK) by the Real-Time Quantitative RT- PCR (Light cycler 480, Roche Diagnostics). These specific primers were applied for amplification. The fluorescence emitted by dye above the baseline signal was detected using the software in real-time, recorded, and represented as the cycle threshold (C_T). The arithmetic mean values of C_Ts, which were performed twice, were calculated for the statistical analysis. All samples were studied in duplicate. Samples were not treated with the growth factor, but the media were evaluated as the control sample. RNA samples directly isolated from a rat hippocampal region were also applied as positive controls in RT-PCR analysis.

Statistical Analysis

The ΔΔCT method was used to determine the gene expression fold change with the results obtained from RT-PCR. CT values of target mRNAs were normalized according to the housekeeping *GAPDH* gene ($\Delta CT = CT_{Target} - CT_{GAPDH}$). Some analysis for the target gene expression changes was performed by the ($2^{-\Delta CT}$ x100) formula. For the fold change of target gene expressions analysis, the values were normalized to the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$), and $2^{-\Delta\Delta CT}$ formula was applied. Variables were used as mean ± standard deviation and percentage and frequency values. *p <0.05 and **p <0.01 levels were considered statistically significant.

RESULT

The initiation of neurogenesis after ischemia is the essential recovery opportunity for treatment success, and NSC is a crucial element in such post-ischemic repair. In this study, the external administration of the IGF-I and MGF were performed on NSC, exposed to various oxygen concentrations, including normoxia, hypoxia, and anoxia, with 20%, 3%, 0%, respectively, as summarized in Table 1. During 24 hours, their effects on the molecular function of NSC employing *IGF-I* and *MGF* and HIF-1α gene expression changes and NSC proliferation- migration rates were investigated (Figure 1).

Accepted Manuscript (Uncorrected Proof)

Low levels of oxygen induce *IGF-I* and *MGF* gene expressions

The quantitative analysis of real-time RT-PCR was performed to determine the changes in *IGF-I* and *MGF* expressions during 24 hours of NSC culture exposed to different O_2 concentrations with growth factors.

There was a significant change in *IGF-I* expression relative to oxygen levels. The highest rate that induced *IGF-I* expression was determined with the 0% O_2 following hypoxic and normoxic concentrations, 0.8, 0.3, and 0.1, respectively. The external administration of IGF-I reduced the *IGF-I* expression to 0% and 20% O_2 levels ($p < 0.05$). The IGF-I application at the hypoxic level did not reveal any significant change in *IGF-I* expression. (Figure 2A).

The *MGF* expression was determined to be affected by O_2. The highest rate of *MGF* expression was determined with the 0% O_2 following 3% and 20% O_2 levels, 0.4, 0.1, and 0.01, respectively. There was a statistically significant difference in *MGF* expression between 0% and 20% O_2 levels ($p < 0.05$). The induction of *MGF* expression with lower O_2 levels was even more significant after administration of MGF in NSC culture in such conditions. (Figure 2B).

The analysis of *HIF1-α* expression in such conditions surprisingly revealed that application of IGF-I lowered its relative fold change expression in all O_2 levels. The application of MGF achieved this downregulation with 20% and 3% O_2 levels. Contrarily, MGF application induces upregulation in the *HIF1-α* relative fold change expression at the anoxic level. (Figure 2C).

IGF-I and MGF Promotes NSC Proliferation-Migration in varying levels of oxygen

In physiological O_2 concentrations (3-5%) of the brain, the cultured NSC is known to increase the proliferation of and modulated to differentiate into neurons. Regarding the effect of oxygen levels on NSC proliferation –migration, we have applied a culture method by measuring the area of the cultured NSC from images at 0 and 24 hours. (Figure 3A-B). Comparing the area between time intervals provided evidence for the NSC's proliferation–migration under such conditions. Although it was not statistically significant, there was a slight increase in proliferation-migration of NSC in untreated NSC within 24 hours of culture at a 3% O_2 level. IGF-I is known to support neuronal survival during development as well as neuronal damage. Surprisingly, IGF-I-treated NSC in anoxic and normoxic conditions revealed a statistically significant increase in proliferation-migration. Hypoxia, however, reduced this rate (Figure 3C). It is known in the literature that MGF is a mitogen for neural cells and has neuroprotective effects in the ischemic brain model. Although it is not statistically significant,

Accepted Manuscript (Uncorrected Proof)

we have found that MGF induces NSC proliferation-migration in all conditions within 24 hours compared to untreated NSC. (Figure 3C).

Discussion

In mammalian CNS, oxygen homeostasis plays an essential role in the proliferation and differentiation of NSCs. Even small changes in the O_2 level might affect dynamic balance and disturb cellular physiology and survival by affecting signal transduction pathways that control cell proliferation, fate and survival, and tissue and organ morphogenesis and regeneration (41, 42). Although low O_2 levels are generally associated with pathological conditions in various tissues, in the hippocampus, where NSCs are commonly located, the estimated O_2 level is around 3-4% from early embryogenesis. Surprisingly, NSC maintains its stability in a hypoxic environment and this physiological hypoxia promotes the growth, survival, and maintenance of the multipotent properties of NSC (43-45). Although the consequence of the ischemia is detrimental to homeostasis, the studies on the role of NSC in ischemic conditions are still insufficient in the literature.

Furthermore, the role of growth factors in a hypoxic environment on NSC remains elusive. It has been shown that intrathecal administration of IGF-I had a protective effect on neurons in rats after hypoxic ischemia (46). MGF blocks the apoptosis of damaged myocytes and stem cells and can protect myocytes and neurons from hypoxia (47, 48). It has also been reported that MGF is markedly more effective than IGF-I (27). Under these consequences, it might be relevant to consider NSC conditioned with growth factors that have stimulant effects as an effective modality in stroke treatment. Therefore, it is essential to clarify the role of varying O_2 environments on NSC proliferation and the effects of growth factors. Based on these discoveries, *IGF-I* and *MGF* gene expressions in NSCs exposed to varying O_2 levels could provide essential evidence regarding IGF-I and MGF growth factor roles in NSC and whether external application of IGF-I and MGF might provide any effect. Including NSC proliferation that would benefit the treatment modalities of ischemic stroke.

There was a significant upregulation in *IGF-I* expression relative to oxygen levels. The 0% O_2 levels induce the *IGF-I* expression at the highest compared to 3% O_2 levels and 20% O_2. This result is concordant with the literature, where the effect of IGF-I has also been shown to be induced in damaged brain regions after cerebral ischemia (46). The external application of IGF-

Accepted Manuscript (Uncorrected Proof)

I has lowered its expression in 0% and 20% O_2 conditions but saved the 3% O_2. The proliferation-migration data supported this result by revealing the highest proliferation-migration rate within IGF-I application in 0% and 20% O_2 conditions. Mild hypoxia enhances the proliferation of human neural stem cells (49). These findings suggest that the 3% O_2, the average oxygen level in CNS, does not induce NSC proliferation, retain the cells in a state of quiescence, and IGF-I is ineffective in such an environment. Contrarily, reduced oxygen levels such as 0% O_2 occur in neuronal disorders like cerebral ischemia, transiently leading to NSC proliferation with induction of *IGF-I* expressions. The *MGF* expression relative to oxygen levels was similar to *IGF-I,* where the highest MGF expression was detected in the 0% O_2, compared to 3% O_2 and 20% O_2 levels.

On the other hand, the MGF administration dramatically increased the *MGF* expression in 0% and 3% O_2. The proliferation-migration rate with MGF external application revealed not significant, but the upregulated rate of NSC. These results were concordant with the literature where *MGF* overexpression increases the number of neural progenitor cells and promotes neurogenesis in transgenic mice that constitutively overexpresses MGF from birth (50). It has also been previously found that exogenous MGF and IGF-I increase NSCs proliferation during damage. Although MGF is known for its regenerative capability in skeletal muscle, mononucleated progenitors, and its neuroprotective effect *in vivo* and *in vitro* (28) (23, 51), This study is the first finding revealing the MGF and IGF-I effect on varying O_2 levels. At the same time, when MGF and IGF-I were administered externally to NSC exposed to anoxia and hypoxia, it was determined that these two growth factors positively affect the proliferation of NSC. The data might all provide a significant advantage and a valuable tool for growth hormones, IGF-I, and MGF-mediated NSC therapy in ischemic stroke as well as for neurodegenerative diseases like Parkinson's disease, multiple sclerosis, and Alzheimer's disease.

Furthermore, *HIF-1-α* induction correlated with changes in cellular oxygen and growth factors, including IGF-I, was also confirmed in this study, where relative HIF-1-α gene expression fold change was suppressed with IGF-I administration in varying O_2 levels. Surprisingly, the *MGF* gene expression was increased by 0% O_2. This study is the first evidence indicating the role of MGF in an anoxic environment. It might suggest that targeting HIF-1 as novel small molecule inhibitors might be an attractive strategy for therapeutic development. This study was designed to search for the environmental effects within 24 hours. Prolonged and /or intermittent exposure to hypoxia might reveal diverse findings since more complex pathways will be expected to enroll in such chronic conditions (52).

Accepted Manuscript (Uncorrected Proof)

Overall, this study shed light on using exogenous MGF and IGF-I administration for their neuroprotective and proliferative effects on NSC for the harmful effects of ischemic stroke. More *in vitro* and *in vivo* intensive studies are essential to understand the role of growth factors for such treatment modalities in ischemia.

DECLARATIONS

- Ethics approval and consent to participate:
  - No ethics approval is required for this study
- Consent for publication:
  - Yes
- Availability of data and material
  - Not needed
- Competing interests
  - The authors have no conflict of interest to declare
- Funding
  - This work was supported by the Scientific Research Projects Coordination Unit of Istanbul University (Project number: 58165).
- Author Contributions: Concept – TAG, SS; Supervision - SS..; Materials – TAG, SFT, SS..; Data Collection and/or Processing – TAG, SFT, SS..; Analysis and/or Interpretation- FT, SFT, SS..; Literature Search - TAG, FT, SFT, Writing - FT, SFT, SS; Critical Reviews- SS., FT

ACKNOWLEDGEMENTS

In honor of Kenan Ates, MD, Ph.D. Rest in peace.

Accepted Manuscript (Uncorrected Proof)

REFERENCES

1. Ostergaard L, Engedal TS, Moreton F, Hansen MB, Wardlaw JM, Dalkara T, et al. Cerebral small vessel disease: Capillary pathways to stroke and cognitive decline. J Cereb Blood Flow Metab. 2016;36(2):302-25.
2. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol. 2012;298:229-317.
3. Chamorro A, Dirnagl U, Urra X, Planas AM. Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. Lancet Neurol. 2016;15(8):869-81.
4. Steiner P. Brain Fuel Utilization in the Developing Brain. Ann Nutr Metab. 2019;75 Suppl 1:8-18.
5. Lee JM, Grabb MC, Zipfel GJ, Choi DW. Brain tissue responses to ischemia. J Clin Invest. 2000;106(6):723-31.
6. Xu S, Lu J, Shao A, Zhang JH, Zhang J. Glial Cells: Role of the Immune Response in Ischemic Stroke. Front Immunol. 2020;11:294.
7. Kriegstein A, Alvarez-Buylla A. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci. 2009;32:149-84.
8. Decimo I, Bifari F, Krampera M, Fumagalli G. Neural stem cell niches in health and diseases. Curr Pharm Des. 2012;18(13):1755-83.
9. Boltze J, Didwischus N, Li S, Jolkkonen J, Taguchi A. Increased migratory and homing abilities of neural and mesenchymal stem cell populations by transient cell modifications: Preclinical progress and clinical relevance. EBioMedicine. 2020;60:103022.
10. Zhang GL, Zhu ZH, Wang YZ. Neural stem cell transplantation therapy for brain ischemic stroke: Review and perspectives. World J Stem Cells. 2019;11(10):817-30.
11. Sharee Ghourichaee S, Leach JB. The effect of hypoxia and laminin-rich substrates on the proliferative behavior of human neural stem cells. J Mater Chem B. 2016;4(20):3509-14.
12. Yee M, Gelein R, Mariani TJ, Lawrence BP, O'Reilly MA. The Oxygen Environment at Birth Specifies the Population of Alveolar Epithelial Stem Cells in the Adult Lung. Stem Cells. 2016;34(5):1396-406.
13. Williams AJ. ABC of oxygen: assessing and interpreting arterial blood gases and acid-base balance. BMJ. 1998;317(7167):1213-6.
14. Zhang K, Zhu L, Fan M. Oxygen, a Key Factor Regulating Cell Behavior during Neurogenesis and Cerebral Diseases. Front Mol Neurosci. 2011;4:5.
15. Ceanga M, Dahab M, Witte OW, Keiner S. Adult Neurogenesis and Stroke: A Tale of Two Neurogenic Niches. Front Neurosci. 2021;15:700297.
16. Paredes MF, Sorrells SF, Garcia-Verdugo JM, Alvarez-Buylla A. Brain size and limits to adult neurogenesis. J Comp Neurol. 2016;524(3):646-64.
17. Nieto-Estevez V, Defterali C, Vicario-Abejon C. IGF-I: A Key Growth Factor that Regulates Neurogenesis and Synaptogenesis from Embryonic to Adult Stages of the Brain. Front Neurosci. 2016;10:52.
18. Tunç BS, Toprak F, Toprak SF, Sozer S. In vitro investigation of growth factors including MGF and IGF-1 in neural stem cell activation, proliferation, and migration. Brain Res. 2021;1759:147366.
19. Oberbauer AM. The Regulation of IGF-1 Gene Transcription and Splicing during Development and Aging. Front Endocrinol (Lausanne). 2013;4:39.
20. Bailes J, Soloviev M. Insulin-Like Growth Factor-1 (IGF-1) and Its Monitoring in Medical Diagnostic and in Sports. Biomolecules. 2021;11(2).
21. Goldspink G. Research on mechano growth factor: its potential for optimising physical training as well as misuse in doping. British Journal of Sports Medicine. 2005;39(11):787-8.

Accepted Manuscript (Uncorrected Proof)

22. Matheny RW, Jr., Nindl BC, Adamo ML. Minireview: Mechano-growth factor: a putative product of IGF-I gene expression involved in tissue repair and regeneration. Endocrinology. 2010;151(3):865-75.
23. Ates K, Yang SY, Orrell RW, Sinanan AC, Simons P, Solomon A, et al. The IGF-I splice variant MGF increases progenitor cells in ALS, dystrophic, and normal muscle. FEBS Lett. 2007;581(14):2727-32.
24. Dluzniewska J, Sarnowska A, Beresewicz M, Johnson I, Srai SK, Ramesh B, et al. A strong neuroprotective effect of the autonomous C-terminal peptide of IGF-1 Ec (MGF) in brain ischemia. FASEB J. 2005;19(13):1896-8.
25. Canazza A, Minati L, Boffano C, Parati E, Binks S. Experimental models of brain ischemia: a review of techniques, magnetic resonance imaging, and investigational cell-based therapies. Front Neurol. 2014;5:19.
26. Tang JJ, Podratz JL, Lange M, Scrable HJ, Jang MH, Windebank AJ. Mechano growth factor, a splice variant of IGF-1, promotes neurogenesis in the aging mouse brain. Mol Brain. 2017;10(1):23.
27. Aperghis M, Johnson IP, Cannon J, Yang S-Y, Goldspink G. Different levels of neuroprotection by two insulin-like growth factor-I splice variants. Brain Research. 2004;1009(1):213-8.
28. Dłużniewska J, Sarnowska A, Beręsewicz M, Johnson I, Srai SKS, Ramesh B, et al. A strong neuroprotective effect of the autonomous C-terminal peptide of IGF-1 Ec (MGF) in brain ischemia. The FASEB Journal. 2005;19(13):1896-8.
29. Joanna Dłużniewska AS, †, Małgorzata Beręsewicz IJ, ‡, Surjit K. S. Srai, Bala Ramesh, Geoffrey Goldspink DCG, ¶, and, et al. A strong neuroprotective effect of the autonomous Cterminal peptide of IGF-1 Ec (MGF) in brain ischemia The FASEB Journal. 2005.
30. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, et al. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J. 2003;17(2):271-3.
31. Baddela VS, Sharma A, Michaelis M, Vanselow J. HIF1 driven transcriptional activity regulates steroidogenesis and proliferation of bovine granulosa cells. Sci Rep. 2020;10(1):3906.
32. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem. 1995;270(3):1230-7.
33. Masoud GN, Li W. HIF-1alpha pathway: role, regulation and intervention for cancer therapy. Acta Pharm Sin B. 2015;5(5):378-89.
34. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem. 1996;271(50):32253-9.
35. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem. 1997;272(36):22642-7.
36. Ziello JE, Jovin IS, Huang Y. Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. Yale J Biol Med. 2007;80(2):51-60.
37. Jing X, Yang F, Shao C, Wei K, Xie M, Shen H, et al. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. Mol Cancer. 2019;18(1):157.
38. Chau N-M, Rogers P, Aherne W, Carroll V, Collins I, McDonald E, et al. Identification of Novel Small Molecule Inhibitors of Hypoxia-Inducible Factor-1 That Differentially Block Hypoxia-Inducible Factor-1 Activity and Hypoxia-Inducible Factor-1α Induction in Response to Hypoxic Stress and Growth Factors. Cancer Research. 2005;65(11):4918-28.

Accepted Manuscript (Uncorrected Proof)

39. Ma Z, Xiang X, Li S, Xie P, Gong Q, Goh BC, et al. Targeting hypoxia-inducible factor-1, for cancer treatment: Recent advances in developing small-molecule inhibitors from natural compounds. Semin Cancer Biol. 2020.
40. Croushore CA, Sweedler JV. Microfluidic systems for studying neurotransmitters and neurotransmission. Lab Chip. 2013;13(9):1666-76.
41. Fathollahipour S, Patil PS, Leipzig ND. Oxygen Regulation in Development: Lessons from Embryogenesis towards Tissue Engineering. Cells Tissues Organs. 2018;205(5-6):350-71.
42. Zhou D, Shao L, Spitz DR. Reactive oxygen species in normal and tumor stem cells. Adv Cancer Res. 2014;122:1-67.
43. Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG. Oxygen-regulated gene expression in bovine blastocysts. Biol Reprod. 2004;71(4):1108-19.
44. Studer L, Csete M, Lee SH, Kabbani N, Walikonis J, Wold B, et al. Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J Neurosci. 2000;20(19):7377-83.
45. Morrison SJ, Csete M, Groves AK, Melega W, Wold B, Anderson DJ. Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. J Neurosci. 2000;20(19):7370-6.
46. O'Kusky J, Ye P. Neurodevelopmental effects of insulin-like growth factor signaling. Front Neuroendocrinol. 2012;33(3):230-51.
47. Yoshida T, Delafontaine P. Mechanisms of IGF-1-Mediated Regulation of Skeletal Muscle Hypertrophy and Atrophy. Cells. 2020;9(9).
48. Chiong M, Wang ZV, Pedrozo Z, Cao DJ, Troncoso R, Ibacache M, et al. Cardiomyocyte death: mechanisms and translational implications. Cell Death Dis. 2011;2:e244.
49. Santilli G, Lamorte G, Carlessi L, Ferrari D, Rota Nodari L, Binda E, et al. Mild hypoxia enhances proliferation and multipotency of human neural stem cells. PLoS One. 2010;5(1):e8575.
50. Tang JJ, Podratz JL, Lange M, Scrable HJ, Jang M-H, Windebank AJ. Mechano growth factor, a splice variant of IGF-1, promotes neurogenesis in the aging mouse brain. Molecular Brain. 2017;10(1):23.
51. Quesada A, Micevych P, Handforth A. C-terminal mechano growth factor protects dopamine neurons: a novel peptide that induces heme oxygenase-1. Exp Neurol. 2009;220(2):255-66.
52. Khuu MA, Pagan CM, Nallamothu T, Hevner RF, Hodge RD, Ramirez J-M, et al. Intermittent Hypoxia Disrupts Adult Neurogenesis and Synaptic Plasticity in the Dentate Gyrus. The Journal of Neuroscience. 2019;39(7):1320-31.

Accepted Manuscript (Uncorrected Proof)

FIGURE LEGENDS:

Figure 1: The illustration of experimental design. The rat hippocampal-derived neural stem cells (NSC) were cultured *in vitro* on flasks coated with pol-L ornithine and laminin. After 70-80% confluency, the NSCs were divided into cell culture plates for three conditions of Oxygen level, either with 20%, 3%, and 0%, each representing normoxia, hypoxia, and anoxia, respectively. The growth factors (0.2µg/ml), including IGF-I and MGF, were administered to the cultured NSC for each condition.

Nine samples were investigated during 24-hour culture-time under such oxygen levels and growth factors. Afterward, the RNA isolations were performed for the gene expressional fold change analysis of *MGF* and *IGF-I* by RT-PCR analysis. The proliferation analysis was performed with the ImageJ program.

Figure 2: NSC's relative gene expression fold changes with a hypoxic environment.

The expression of *IGF-I* (A), *MGF* (B), and *HIF-1α* (C) in NSC was treated with growth factors under 20%, 3%, and 0% O_2. The study was performed with the addition of growth factors IGF-I and MGF alone to the NSC culture under 20%, 3%, and 0% O_2 and analyzing the expressional changes after 24 hours culture period by real-time RT-PCR method. The stacked proportion bar chart is sorted by increasing relative gene expressions based on the oxygen environment for individual treatments. HIF-1α was represented as the gene expression fold change where the analysis was performed relative to the untreated NSC for each condition (*: $p < 0.05$).

Accepted Manuscript (Uncorrected Proof)

Figure 3: The proliferation- migration of NSC in a hypoxic environment.

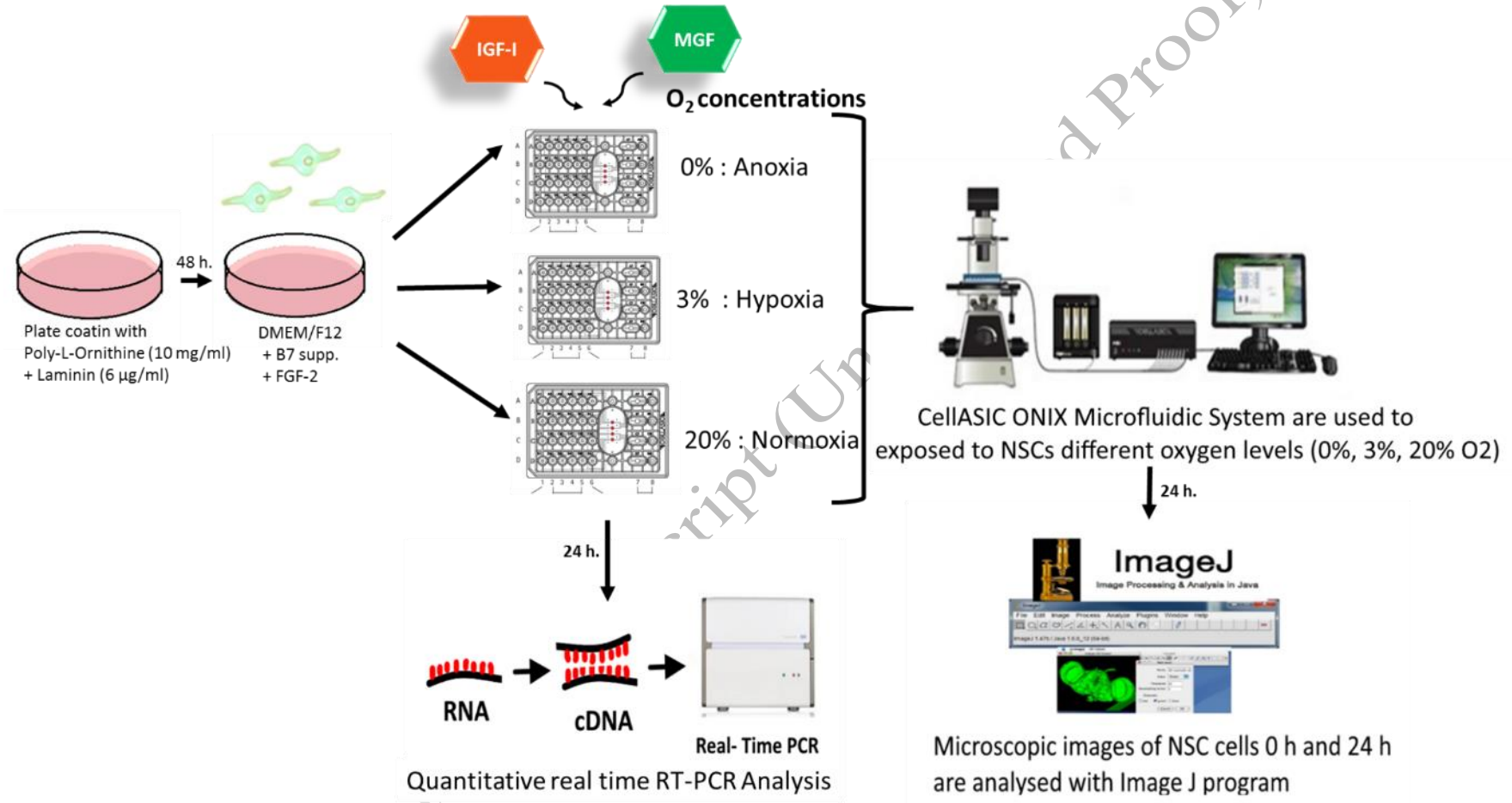
A. The images were taken from the 0 Hours and 24 hours of the culture for NSC proliferation- migration analysis under 20%, 3%, and 0% O_2 levels performed with the ImageJ program. The red color was employed by the program.

B. The representative figure of the culture chamber where the images were taken.

C. The figure of cell proliferation – migration rate (Area of the cultured cells after 24 hours / Area of the cultured cells in 0 hours in defined conditions) of each condition. (*: $p < 0.05$).

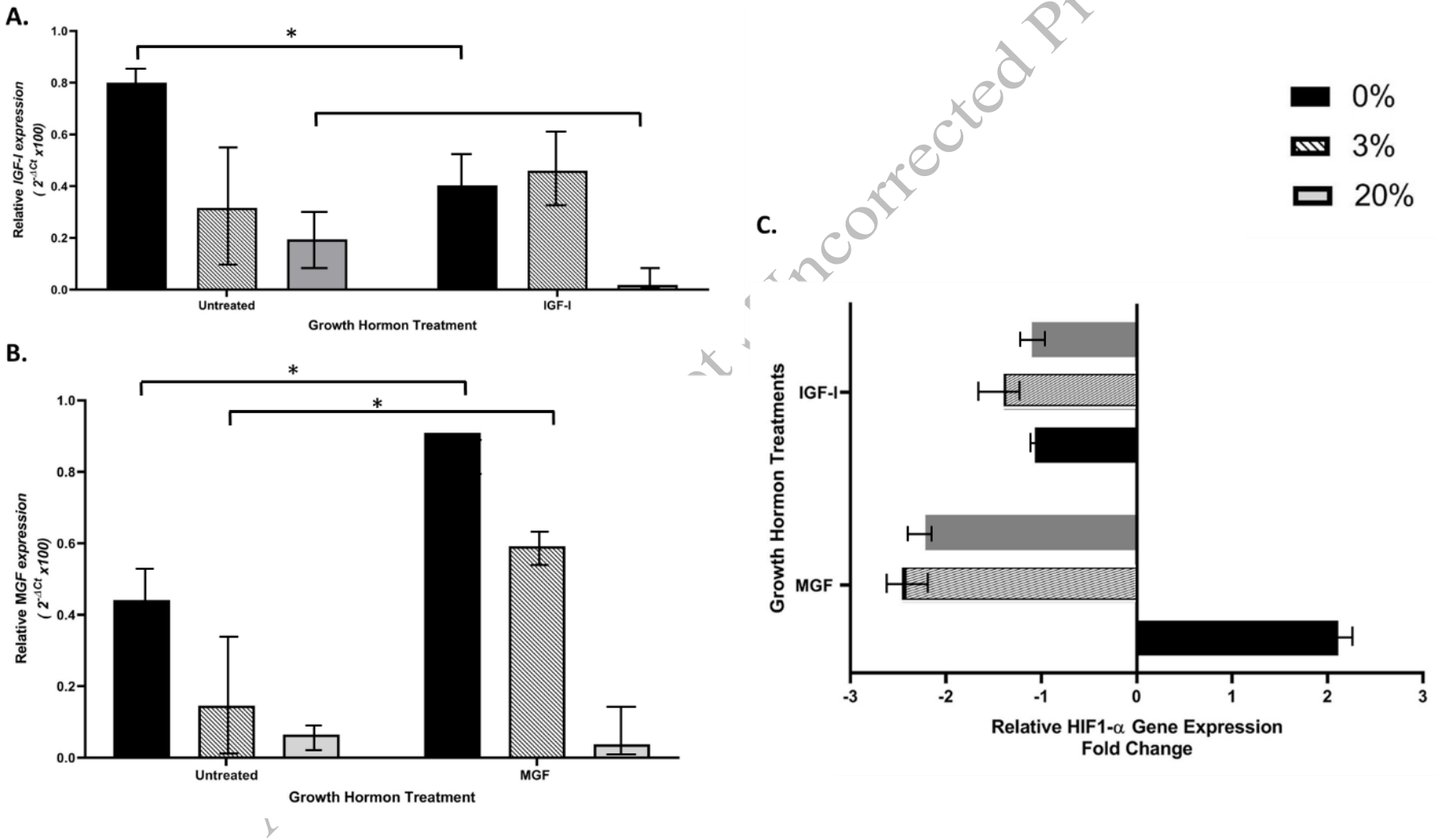
Accepted Manuscript (Uncorrected Proof)

Figure 1:



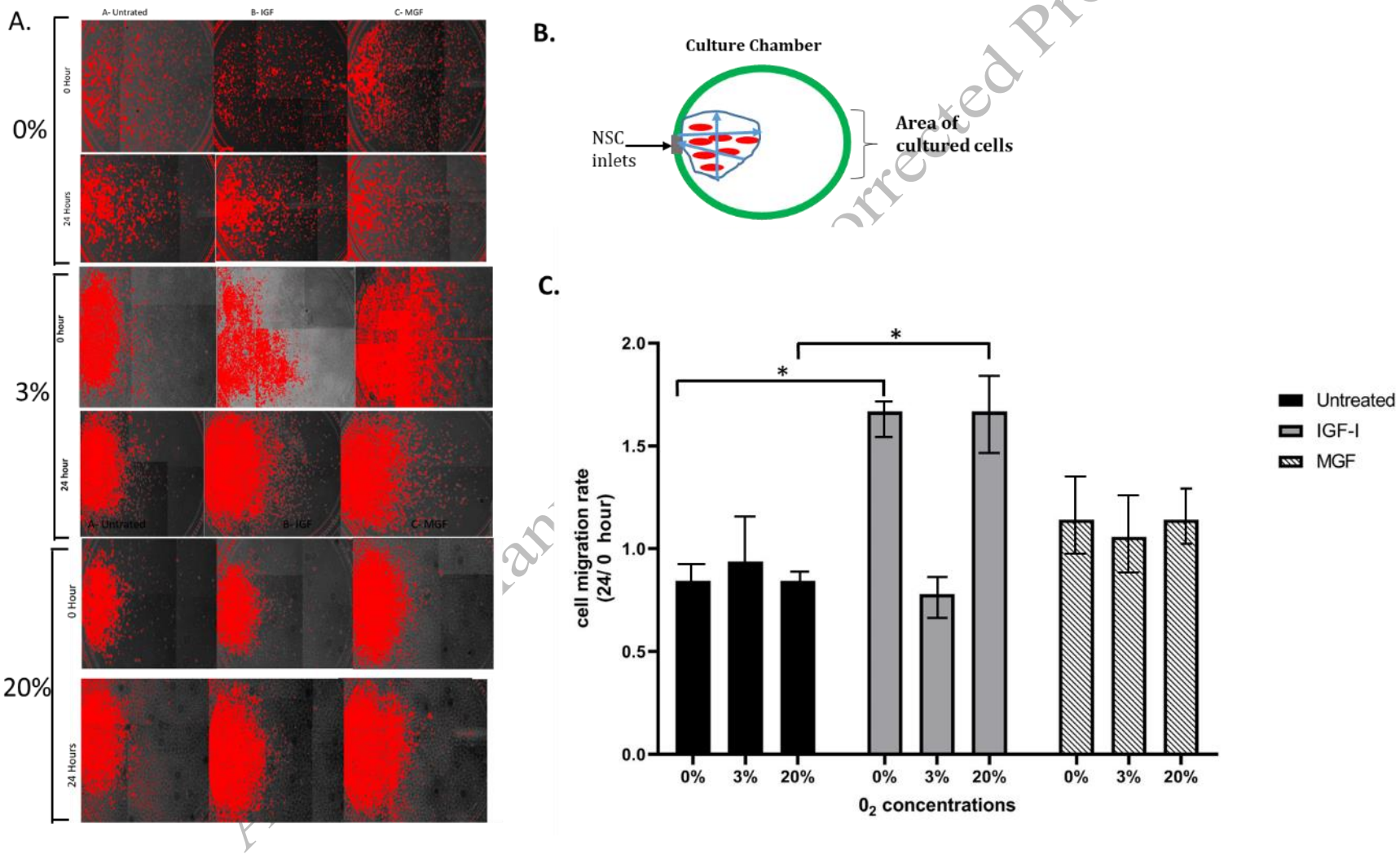
Accepted Manuscript (Uncorrected Proof)

Figure 2:



Uncorrected Proof)

Figure 3:



Accepted Manuscript (Uncorrected Proof)

TABLE 1: Cell Culture Medium Compositions For Rat Hippocampal-Derived Neural Stem Cells.

NSC Culture Compositions	
O_2 Concentrations	**Culture Media**
Normoxia (20% O_2)	**Untreated**
	MGF
	IGF-I
Hypoxia (3% O_2)	**Untreated**
	MGF
	IGF-I
Anoxia (0% O_2)	**Untreated**
	MGF
	IGF-I

Accepted Manuscript (Uncorrected Proof)