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Physiologic Oxygen Concentration Enhances the Stem-Like Properties of CD133+ Human Glioblastoma Cells *In vitro*

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Abstract

In vitro investigations of tumor stem-like cells (TSC) isolated from human glioblastoma (GB) surgical specimens have been done primarily at an atmospheric oxygen level of 20%. To determine whether an oxygen level more consistent with *in situ* conditions affects their stem cell–like characteristics, we compared GB TSCs grown under conditions of 20% and 7% oxygen. Growing $CD133^+$ cells sorted from three GB neurosphere cultures at 7% O_2 reduced their doubling time and increased the self-renewal potential as reflected by clonogenicity. Furthermore, at 7% oxygen, the cultures exhibited an enhanced capacity to differentiate along both the glial and neuronal pathways. As compared with 20%, growth at 7% oxygen resulted in an increase in the expression levels of the neural stem cell markers CD133 and nestin as well as the stem cell markers Oct4 and Sox2. In addition, whereas hypoxia inducible factor 1α was not affected in $CD133^+$ TSCs grown at 7% O_2, hypoxia-inducible factor 2α was expressed at higher levels as compared with 20% oxygen. Gene expression profiles generated by microarray analysis revealed that reducing oxygen level to 7% resulted in the up-regulation and down-regulation of a significant number of genes, with more than 140 being commonly affected among the three $CD133^+$ cultures. Furthermore, Gene Ontology categories up-regulated at 7% oxygen included those associated with stem cells or GB TSCs. Thus, the data presented indicate that growth at the more physiologically relevant oxygen level of 7% enhances the stem cell–like phenotype of $CD133^+$ GB cells.

Introduction

Survival of patients with glioblastoma (GB) remains dismal, with the vast majority succumbing to disease within 1 to 2 years of diagnosis (1). Developing more effective therapies will depend on a better understanding of fundamental GB biology. Toward this end, recent studies have begun to focus on stem-like cells isolated from GB surgical specimens and grown *in vitro* as neurosphere cultures (2–4). Such tumor cells have a number

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of properties in common with neural stem cells, including continuous self-renewal, expression of stem cell markers, and at least partial differentiation along the neuronal and/or glial pathways (2–4). Moreover, in contrast to established human GB cell lines, these tumor stem-like cells (TSC) form brain tumors in immunocompromised mice that simulate the original primary tumor histology (5). Thus, consistent with the cancer stem cell model initially developed for other tumor types (6), TSCs are considered to play a critical role in initiating and maintaining GBs (2, 5). Consequently, the *in vitro* analysis of GB TSC cultures has generated considerable interest as an experimental approach for investigating not only the fundamental aspects of GB biology (2, 7–9) but also therapeutic response (10, 11).

As for *in vitro* studies in general, investigations using GB TSC cultures to date have been primarily done under atmospheric conditions of 20% O_2. However, whereas alveolar oxygen concentration is ~14% (12), normal brain oxygen levels range from 5% to 10% (13, 14). Oxygen is a well-established mediator of a variety of signaling pathways as well as overall gene expression (15). It is also well established that O_2 levels influence such fundamental processes as cell metabolism, proliferation, and survival (16). Finally, with respect to normal stem cells *in vitro*, reduction of O_2 levels from 20% to 3–5% has been reported to enhance the survival of hematopoietic stem cells (17, 18) and neural precursors (19, 20). Thus, it would seem that O_2 concentration needs to be taken into account in the *in vitro* analyses of GB TSCs.

Toward this end, the goal of the current study was to define the TSC phenotype *in vitro* under an O_2 concentration that is representative of those that exist for the majority of GB cells *in situ*. Whereas O_2 levels within GBs *in situ* range from 0.1% to 10% (13, 14, 21), studies using the nitroimidazole hypoxia marker EF5 indicate that although there is intertumor and intratumor heterogeneity, the vast majority of GB cells exist under O_2 levels of 6% to 7% (13). Therefore, as an initial investigation into whether a more physiologically relevant O_2 level affects their *in vitro* characteristics, we grew and maintained GB TSC cultures under conditions of 7% O_2. The data presented indicate that as compared with the standard culture conditions of 20% O_2, growth of GB TSCs at 7% results in increased self-renewal capacity, multilineage differentiation potential, and expression of stem cell–related genes/proteins. These data thus suggest that culturing at the reduced O_2 concentration enhances the stem cell–like properties of GB TSCs.

Results

Enrichment of CD133$^+$ Cells at 7% O_2

MDNSC11, MDNSC20, and MDNSC23 neurosphere cultures, which were derived from surgical GB specimens, display the *in vitro* stem cell characteristics and are tumorigenic in immunodeficient mice (22). Whereas such neurosphere cultures contain a heterogeneous mixture of cell types, the critical marker denoting the stem cell–like subpopulation is considered to be CD133 (4, 11). To determine whether 7% O_2 influences the percentage of CD133$^+$ cells, neurospheres that had been grown under the standard O_2 concentration of 20% were disaggregated and placed back into culture under conditions of 7% or 20% O_2. Neurospheres reformed and, after 10 to 14 days, reached diameters of ~100 μm, at which

time the percentage of $CD133^+$ cells in each culture was determined. As shown in Fig. 1A, MDNSC23 contained >90% $CD133^+$ cells when grown at 20% O_2, which was not affected by growth at 7% O_2. However, when MDNSC11 and MDNSC20 neurospheres were grown at 7% O_2, the percentage of $CD133^+$ cells in each culture increased by ~2-fold. These results suggest that formation and growth of the neurospheres at the more physiologically relevant O_2 concentration led to an enrichment in the $CD133^+$ population. Consistent with the increase in the percentage of $CD133^+$ cells, after 5 days at 7% O_2, the neurospheres in MDNSC11 (Fig. 1B) and MDNSC20 (data not shown) cultures were larger than those grown under standard *in vitro* conditions of 20% O_2.

$CD133^+$ TSCs

Given its significance as a marker for GB TSCs (4, 11), subsequent studies focused on the $CD133^+$ cells of each neurosphere culture. Toward this end, fluorescence-activated cell sorting was used to isolate $CD133^+$ cells from MDNSC11 and MDNSC20 cultures that were grown under standard conditions of 20% O_2; this procedure was not necessary for MDNSC23, which already contained >90% $CD133^+$ cells (Fig. 1A). $CD133^+$ cells corresponding to each culture were then used to initiate neurospheres, which were maintained for 7 days at 20 or 7% O_2 before use in an experiment. It should be noted that whereas some CD133-negative cell cultures isolated from GBs have been reported to show stem cell–like qualities *in vitro* (23), in our hands the CD133-negative cells sorted from MDNSC11 and MDNSC20 cultures did not reform neurospheres or proliferate *in vitro* (data not shown).

Proliferation Rate and Self-Renewal Potential of $CD133^+$ TSCs Are Increased at 7% O_2

To determine whether O_2 level affects proliferation rate, $CD133^+$ MDNSC11, MDNSC20, and MDNSC23 neurospheres, which had been maintained at 7% and 20% O_2, were disaggregated and placed back into neurosphere-forming conditions at their respective O_2 levels. The number of cells in each culture was then determined at times out to 14 days, and the mean population doubling time calculated as described (24). As shown in Table 1, as compared with 20%, the 7% O_2 environment significantly reduced the doubling time of each of the $CD133^+$ cultures. These data indicate that the reduced O_2 level enhanced the proliferation rate of the $CD133^+$ TSCs.

A characteristic of stem or stem-like cells is continuous self-renewal (2, 4), which is reflected *in vitro* by colony forming efficiency (clonogenicity). To determine whether O_2 level influences the clonogenic potential of GB TSCs, the $CD133^+$ neurosphere cultures grown at 7% or 20% O_2 were disaggregated into single-cell suspensions and seeded onto poly-L-lysine–coated tissue culture plates at clonogenic densities (i.e., 100-1,000 cells). Plates were returned to their respective O_2 condition and the number of adherent colonies was determined after 14 to 20 days. As shown in Fig. 2, colony formation for each of the $CD133^+$ TSC lines was significantly greater at 7% O_2 as compared with 20%. It should be noted that the vast majority of cells with the colonies formed for each TSC cell line at 20% and 7% O_2 continued to express CD133 (data not shown). These results suggest that the reduced O_2 level modifies the phenotype of $CD133^+$ cells such that their self-renewal potential is enhanced.

Differentiation Pattern of TSCs

An additional characteristic of GB TSCs is the potential to differentiate along the neuronal and glial pathways. To determine whether the reduction in O_2 influences this differentiation pattern, $CD133^+$ MDNSC11, MDNSC20, and MDNSC23 neurospheres were cultured at 7% or 20% O_2 for 7 days, disaggregated, and seeded onto slides in growth factor–free medium containing 10% fetal bovine serum to induce differentiation. After an additional 7 days at the corresponding O_2 level, the percentages of cells expressing glial fibrillary acidic protein (GFAP), a glial marker, or βIII tubulin, a neuronal marker, were determined. At 20% O_2, the $CD133^+$ TSCs differentiated primarily toward the astrocyte lineage, with relatively few cells expressing the neuronal marker (Fig. 3), which is consistent with results from other GB TSCs (25). However, at 7% O_2, more cells were positive for βIII tubulin, indicating an increase in the number of cells differentiating along the neuronal lineage. Thus, as compared with 20%, the $CD133^+$ cells at 7% O_2 seem to have a more diverse differentiation potential, consistent with an enhancement of the stem cell–like properties.

Increased Expression of Stem Cell Markers in $CD133^+$ TSCs at 7% O_2

The cell level studies described above suggest that growth of $CD133^+$ TSCs under conditions of 7% O_2 enhances their stemlike phenotype. To pursue this idea at a molecular level, the effects of the reduced O_2 level on the expression of proteins previously implicated as stem cell markers were determined. As for the data described in Table 1 and Figs. 2 and 3, these analyses were done on $CD133^+$ neurosphere cultures. Whereas under both 20% and 7% O_2, >90% of cells expressed CD133, as shown in Fig. 4A, the median fluorescence intensity corresponding to CD133 expression was consistently greater for each of the TSC cultures at 7% as compared with 20% O_2, consistent with an increase in the level of CD133 expression. This increase was most apparent for the MDNSC11 and MDNSC23 lines; although relatively minor in MDNSC20, the increase in CD133 expression was consistent throughout the experiments. Immunoblot analysis was then used to determine whether 7% O_2 influences the expression of other stem cell–associated proteins (Fig. 4B). As compared with 20%, after 7 days in 7% O_2, nestin expression was increased in each of the $CD133^+$ neurosphere cultures. In contrast to the uniform increase in CD133 and nestin expression across the 3 $CD133^+$ TSC cultures, the expression of stem cell markers Oct4 and Sox2 was enhanced at 7% O_2 only in MDNSC11 and MDNSC23. In MDNSC20 culture, Oct4 protein was not detectable at either O_2 level; Sox2 protein expression was essentially the same at both O_2 levels. Thus, although there is some heterogeneity between $CD133^+$ neurosphere cultures, which is consistent with the intertumor heterogeneity of primary GB *in situ* (26), these results indicate that growth at 7% O_2 enhances the expression of proteins previously associated with a stem cell–like phenotype.

Hypoxia-Inducible Factor-2α Expression Is Enhanced at 7% O_2 and Drives the Expression of Oct4 and Sox2

Hypoxia-inducible factors (HIF-1α and HIF-2α) are critical transcription factors mediating changes in gene expression in response to hypoxia and have recently been implicated in the regulation of the stem cell phenotype (16). However, most investigations of HIF expression have focused on O_2 levels of <5%. Therefore, HIF-1α and HIF-2α protein levels were

determined in the $CD133^+$ MDNSC11, MDNSC20, and MDNSC23 neurospheres grown at 20% and 7% O_2 (Fig. 4B). HIF-1α protein was expressed at low levels in each of the $CD133^+$ cultures with no difference between the O_2 levels. In contrast, HIF-2α levels were clearly detectable in each of $CD133^+$ cultures and, moreover, were increased by growth at 7% O_2 as compared with 20%. These data suggest that at the more physiologic O_2 condition, HIF-2α may play a role in regulating gene expression in TSCs.

Given that *Oct4* has been identified as a putative HIF-2α target gene (27), we investigated whether the expression of stem cell markers that were up-regulated at 7% O_2 was dependent on HIF-2α activity. Specifically, because Oct4 and Sox2 are part of the same transcriptional network (28, 29), we hypothesized that these two proteins would be down-regulated if HIF-2α activity was inhibited. To assess the role of HIF-2α in regulating the expression of these stem cell markers, we grew $CD133^+$ MDNSC11 at 7% O_2 for 1 week, then transfected the cells with siRNA specific for the gene that codes for HIF-2α (*EPAS-1*) or a nonspecific scrambled siRNA. After 24 h, cells were assessed for knockdown of *EPAS-1*, and levels of nestin, CD133, Oct4, and Sox2 were determined by immunoblot (Fig. 4C). As expected, HIF-2α levels were reduced after si*EPAS-1* transfection. Oct4 and Sox2 protein levels were also markedly decreased after knockdown of *EPAS-1*. These data provide a putative HIF-2α–dependent pathway by which Oct4 and Sox2 are up-regulated at physiologic O_2. However, CD133 and nestin levels were not affected, suggesting that HIF-2α–independent pathways also play a role in the stem cell phenotype of TSCs.

Influence of 7% O_2 on Gene Expression Profiles Generated from $CD133^+$ Cells

The data presented indicate that the reduction in O_2 to a more physiologic level affects the phenotype of $CD133^+$ TSCs, suggesting modifications in the global gene expression pattern. Therefore, microarray analysis was done on the $CD133^+$ MDNSC11, MDNSC20, and MDNSC23 neurospheres grown at 7% and 20% O_2. To identify the genes whose expression was either up-regulated or down-regulated at 7% O_2, biological replicates were averaged for each cell line and compared with the gene expression profile generated from their corresponding 20% O_2 culture using SAM (false discovery rate of <0.1). As shown in the Venn diagrams in Fig. 5A, each of the $CD133^+$ neurosphere cultures contained a significant number of genes up-regulated and down-regulated as a result of growth at 7% O_2. Moreover, a significant number of genes were commonly affected at 7% O_2 among the three TSC lines, suggesting a general response to the more physiologic O_2 condition. To compare the gene expression response elicited by 7% O_2 to established GB cell lines, the same experiment was done using U251 and U87 cells. As shown in the Venn diagrams (Fig. 5A, *bottom*), there were considerably fewer genes whose expression was modified at 7% O_2 and fewer genes commonly affected in the two established GB cell lines. These results suggest that the TSCs are more susceptible to O_2-mediated changes in gene expression than the traditional established GB cell lines. To determine whether the genes affected by 7% O_2 in the $CD133^+$ TSCs correspond to specific biological/biochemical processes, a gene set analysis was done according to Gene Ontology (GO) category enrichment. For this analysis, to account for the heterogeneity between the $CD133^+$ TSC cultures, genes significantly up-regulated and down-regulated at 7% O_2 in any two of the three cultures were distributed into GO categories, with the enriched GO categories used to generate a heat map (Fig. 5B). The

number of enriched GO categories ($P < 0.05$) that were up-regulated and down-regulated were 39 and 57, respectively. Of interest with respect to TSCs, the enriched up-regulated GO categories included the frizzled-2 signaling pathway, Notch2, transforming growth factor β, and angiogenesis, all of which have been associated with brain tumor stem cells (30).

Oxygen-Dependent Enhancement of Stem-Like Phenotype Is Reversible

Whereas CD133 identifies TSCs, the $CD133^+$ cells most likely include a number of subpopulations (31, 32). This potential heterogeneity suggested that it was also possible that the survival data in Fig. 2 corresponded to the selection of a $CD133^+$ subpopulation with greater clonogenic potential. If such a selection process was operative, the enhanced survival induced by 7% O_2 would be expected to be irreversible. Therefore, to test the issue of reversibility, MDNSC11, MDNSC20, and MDNSC23 $CD133^+$ neurosphere cultures were initiated at 7% O_2 and, 7 days later, moved to 20% O_2 for an additional 7 days (7→20) followed by clonogenic analysis. Colony formation was also determined for accompanying cultures maintained at 7% O_2 or 20% O_2. Consistent with the results in Fig. 2, the colony formation at 7% O_2 was significantly greater than at 20% (Fig. 6A). However, when $CD133^+$ TSCs were moved from 7% to 20% O_2 (7→20), the colony formation returned to essentially the same values as for cultures maintained at 20% O_2. These data indicate that the enhancement in $CD133^+$ TSC clonogenicity induced at 7% O_2 is a reversible process. Moreover, these results are suggestive of an epigenetic modification in the TSC phenotype and not the selection of a subpopulation of $CD133^+$ cells with increased colony forming efficiency.

To determine whether the reversible effect of 7% O_2 on stem cell phenotype extended to the up-regulation of stem cell markers, the $CD133^+$ MDNSC11 cells were grown as above at 7, 20, and 7→20 oxygen conditions, after which levels of the proteins nestin, Oct4, Sox2, and HIF-2α were determined by immunoblot (Fig. 6B). After reintroduction to 20% O_2, the expression levels of nestin, HIF-2α, and Oct4 returned to control levels (20%). However, the expression of Sox2 was not reversed after 7 days at 20% O_2. The implications of reversibility of only two of the three stem cell markers are unclear and warrant further studies. The reversibility of most of the stem cell markers is consistent with the reversible increase in clonogenicity and supports the hypothesis that the 7% O_2 condition does not simply select for a subpopulation of TSCs.

Discussion

According to the cancer stem cell hypothesis, TSCs exist as a small percentage of the total cells within a GB, and yet play a significant role in tumor growth and response to therapy. Given the difficulties of *in vivo* investigations on such a minor subpopulation of cells, *in vitro* cultures provide an experimentally expedient model system essential for defining the fundamental biology of GB TSCs. Whereas interpreting data generated from any cell culture approach must be tempered by the inability to recapitulate the tumor microenvironment, there are parameters that can be modified to better simulate *in situ* conditions. One such parameter involves O_2, a critical signaling molecule that has been implicated in regulating the neoplastic phenotype (15) as well as that of normal stem and progenitor cells (18–20, 33,

34). In this report, we tested the hypothesis that a more physiologically relevant O_2 concentration modifies the *in vitro* phenotype of GB TSCs.

CD133 serves as the marker protein typically used to identify and isolate TSCs from the heterogeneous neurosphere cultures initiated from GB surgical specimens (4, 5). As few as 100 such $CD133^+$ cells have been reported to form brain tumors in immunocompromised mice (4). Platet et al. (35) reported that culturing neurosphere-forming cells from GB surgical specimens at 3% O_2 increased the percentage of $CD133^+$ cells as compared with conditions of 20% O_2. As shown here, a less severe reduction of O_2 levels to 7% also results in a significant increase in the percentage of $CD133^+$ cells within GB neurosphere cultures. This enrichment in CD133-expressing cells is consistent with subsequent analyses of $CD133^+$ cells sorted from each neurosphere culture, indicating that their doubling time was reduced and their clonogenic potential was increased at 7% O_2. At this reduced O_2 level, the capacity of the $CD133^+$ cells to differentiate along both the glial and neuronal pathways was also enhanced. A similar increase in neuronal differentiation has been reported for normal neural stem cells maintained at *in vitro* oxygen levels of 3% to 5% (19, 20, 34), as well as increased astrocytic death in primary rat cultures at 20% O_2 (36). Whether the enhancement of stem cell phenotype at 7% O_2 is a result of an increase in stem cell traits or a release of an inhibition of stem cell traits resulting from 20% O_2 remains to be determined. However, the increases in proliferation rate, clonogenicity (self-renewal), and multilineage differentiation potential of the $CD133^+$ cells are consistent with an enhancement of their stem-like properties at the more physiologic O_2 level of 7%.

Within a GB *in situ*, the O_2 level to which a given cell or group of cells are exposed is likely to be somewhat transient, varying according to not only tumor growth dynamics but also treatment response. As shown here, with respect to clonogenic potential and expression of stem cell markers, most of the changes elicited at 7% O_2 were completely reversible, suggesting that if a similar variation occurs *in vivo*, the stem cell–like properties of TSCs may also vary. Whether such an oscillation in phenotype actually occurs *in vivo* and whether it would have any treatment significance are clearly speculative. However, this *in vitro* reversibility is consistent with the plasticity generally associated with TSCs as well as normal stem cells and their ability to respond to environmental signals. These results lend support to the hypothesis that “sternness” in the context of tumor-initiating cells may be an environmentally inducible state (16).

Further support for $CD133^+$ TSCs assuming a more stem cell–like phenotype at 7% O_2 can be derived from the analyses of stem cell markers. The expression of nestin, a well-established marker for stem cells of neural origin (37), was elevated at 7% O_2, as was the absolute level of CD133. However, although established as markers, the specific function, if any, of nestin and CD133 in stem cell biology remains undefined. In contrast, reducing O_2 levels to 7% was also found to enhance the expression of the transcription factors Sox2 and Oct4, which are two of the four transgenes necessary to convert normal fibroblasts to embryonic stem-like cells (38) and play critical roles in regulating genes mediating normal stem cell behavior (28, 39, 40). HIF proteins have been implicated in the hypoxia-mediated regulation of cancer stem cells, contributing to the maintenance of their undifferentiated state (41). The mechanism was reported to involve the HIF-2α-mediated, but not the

HIF-1α-mediated, induction of Oct4 expression (27). As described here, in $CD133^+$ cells HIF-2α was expressed at a higher level than HIF-1α and its level was increased at 7% O_2. Specific knockdown of HIF-2α at 7% O_2 resulted in a reduction in Sox2 and Oct4 proteins. Although clearly requiring further investigation, these results suggest that HIF-2α may play a role in regulating the stem cell–like phenotype of $CD133^+$ GB TSCs.

To further define the influence of oxygen on the phenotype of $CD133^+$ GB TSCs, microarray analysis was used to compare the gene expression profiles generated under conditions of 7% and 20% O_2. As compared with the traditional established GB cell lines, the $CD133^+$ TSC lines were more susceptible to changes in gene expression resulting from growth at 7% O_2, consistent with the putative heightened ability of TSCs to respond to their environment. As illustrated by the gene set enrichment analysis, a number of the GO categories up-regulated in TSCs at 7% O_2 have previously been associated with brain tumor stem cells. Bao et al. (7) and Calabrese et al. (42) have suggested that TSCs play a major role in driving angiogenesis, consistent with the up-regulation of the angiogenesis GO category shown here. Notch and frizzled-2 signaling pathway genes, which have been associated with embryonic stem cells as well as TSCs (30), were up-regulated by growth at 7%. Moreover, the frizzled-2 pathway includes many of the WNT genes, which have been implicated in regulating the radioresistance of normal mammary stem cells (43). Finally, transforming growth factor β, which was up-regulated at 7% O_2, has been associated with the regulation of stem cell differentiation and interacts with other developmental pathways such as WNT and BMP (30). Thus, the changes in gene expression profiles induced by 7% O_2 are consistent with an enhanced stem cell phenotype.

The cells within a GB *in situ* can be exposed to a wide range of O_2 levels (21) from the severely hypoxic (<0.1%) to that of well-oxygenated tissue (~10%). The studies described here addressed only a single O_2 level (7%), which likely reflects the O_2 level to which the majority of GB cells are exposed. Whether lower O_2 levels affect $CD133^+$ cells in a similar or more dramatic manner remains to be determined. However, as shown, the relatively modest reduction in O_2 has a significant effect on stem cell–like characteristics of $CD133^+$ TSCs. Thus, investigations of $CD133^+$ TSCs at physiologically relevant O_2 levels may provide a model system for generating additional insight into GB biology.

Materials and Methods

Isolation and Culture of GB Tumor Stem Cells

Neurosphere-forming cultures MDNSC11, MDNSC20, and MDNSC23 were isolated from three human GB surgical specimens as described previously (2). These cells exhibit the *in vitro* stem cell characteristics of self-renewal and multipotent differentiation and were tumorigenic in immunodeficient mice (22). Neurospheres were maintained in medium consisting of DMEM/F-12 (Invitrogen), B27 supplement (0.5×; Invitrogen), and human recombinant basic fibroblast growth factor and epidermal growth factor (50 ng/mL each; R&D Systems). To dissociate neurospheres into single cells, spheres were treated with TryplE Express (Invitrogen) for 5 min at 37°C, then subjected to mechanical disaggregation and strained through a 40-μm cell strainer (BD). For the standard *in vitro* condition of 20% O_2, cultures were maintained in a Forma Series II CO_2 incubator at 5% CO_2/95% air. For

culture at 7% O_2, cultures were maintained in a Forma Series II multi-gas incubator in which CO_2 and N_2 were continuously supplied to achieve a balance of 5% CO_2/7% O_2.

Clonogenic Analysis

Clonogenicity was defined using a colony forming efficiency assay. Neurospheres were disaggregated into single-cell suspensions as described above. A specified number of cells were then seeded into poly-L-lysine–coated six-well plates, which allows for adherent colony formation, containing the serum-free growth medium noted above. After 14 to 20 d, colonies, defined as >25 cells, were fixed and stained with 0.5% crystal violet and colony forming efficiency was determined.

Flow Cytometry and Fluorescence-Activated Cell Sorting

For determination of CD133-expressing cells by flow cytometry, spheres were disaggregated into a single-cell suspension, washed twice in PBS, and incubated with a phycoerythrin-conjugated anti-CD133 (1:10; Miltenyi) or an isotype control (mouse IgG1, Miltenyi) and human Fc blocking reagent (1:10, Miltenyi). Cells were analyzed on a FACScan (BD). For sorting of $CD133^+$ cells, spheres were processed as above under sterile conditions and sorted on a FACSVantage cell sorter. Only cells positive for CD133 were collected. Purity was determined post-sort as >90% positive.

Immunoblot Analysis

Cell lysates were prepared as previously described for *in vitro* cultures (44). For immunoblot analysis, lysates (25 μg) were electrophoresed on SDS-polyacrylamide gels and electrophoretically transferred at 100 V for 2 h to Immobilon-P membranes. Membranes were blocked with 5% nonfat dry milk in 500 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), and 0.1% Tween 20 (TBST) for 1 h followed by incubation with primary antibody at the following concentrations: anti-nestin (1:2,500; R&D Systems), anti-Oct4 (1:500; Abnova), anti-Sox2 (1:1,250; Millipore), anti–HIF-1α (1:1,000; Abcam), anti–HIF-2α (1:500; Abcam), and anti–β-actin (1:5,000; Sigma). Blots were washed in TBST and incubated with horse-radish peroxidase–conjugated secondary antibody (1:2,500 dilution in 5% nonfat dry milk/TBST; Santa Cruz Biotechnology). Blots were developed using Western blotting ECL detection kit (Pierce Biotechnology) according to instructions from the manufacturer.

Differentiation Assay

To define differentiation potential, neurospheres were disaggregated into single cells, which were then seeded onto CC2-coated glass slides (Nalge Nunc Int.) in growth factor–free medium containing 10% FCS (5). After 7 d, slides were fixed in 4% paraformaldehyde in PBS for 10 min, washed with PBS, and permeabilized with 0.2% NP40. Slides were blocked in 5% goat serum in 1% bovine serum albumin for 1 h at room temperature and incubated with primary antibodies diluted in 1% bovine serum albumin [anti-GFAP, 1:200 (Millipore); anti–βIII tubulin, 1:500 (Abcam)] for 2 h, and then washed and incubated with secondary antibodies (goat anti-mouse conjugated to AlexaFluor 488, Invitrogen) for 1 h at room temperature followed by washing and mounting in anti-fade with 4′,6-diamidino-2-phenylindole (Invitrogen). Cells were analyzed on a Zeiss upright fluorescent microscope.

siRNA Transfection for Silencing of HIF-2α

MDNSC11 were seeded at subconfluency in 24-well plates and grown for 48 h. Transfections were done with Lipofectamine RNAimax (Invitrogen) according to the manufacturer's directions for forward transfection. siRNA for *EPAS-1* (Santa Cruz Biotechnology) or a scrambled control (10 or 20 pmol) was added per well. Cells were harvested after 24 h and immunoblots were done as described.

Microarray Procedure

Total RNA was isolated from neurosphere cultures using TRIzol reagent (Invitrogen) following the manufacturer's protocol and further purified using the RNeasy cleanup procedure (Qiagen, Inc.). The quality of total RNA was assessed by agarose gel electrophoresis and by analysis on the Agilent 2100 Bioanalyzer. Five micrograms of total RNA served as the mRNA source for microarray analysis. The polyadenylic acid RNA was specifically converted to cDNA and then amplified and labeled with biotin following the procedure initially described by Van Gelder et al. (45). Hybridization to U133A GeneChips (Affymetrix) was done according to the manufacturer's instructions (46).

Microarray Data Analyses

Raw data analysis was done using code written in R[4] and software from open-source Bioconductor Project (47). Preliminary data quality control assessments were done with affyQCReport package. The raw data were background adjusted, normalized, and converted to log 2 transformed expression level data by using a method implemented in the robust multiarray average method (48). Differences in gene expression between 7% and 20% O_2 culture conditions for each of stem cell line in duplicate were done on most variable genes (SD > 0.2 across samples) using the SAM algorithm (49) with the false discovery rate set to 10%. Overlap of the significant gene lists from each of the tested samples was evaluated according to Venn diagrams; genes common to any two of the three cell lines were selected for gene set analysis. Pathway analysis was done using Gene Set Enrichment Analysis (50). Gene Set Enrichment Analysis takes a list of genes and tests whether, within that queried list of genes, there is statistically significant enrichment of predefined groups of genes or "gene sets." An a priori gene set file was created from the cellular, molecular, and biological categories of Gene Ontology (GO) database. We have selected all categories with gene membership of <500 or >15 in each category. This resulted in 594 categories in total. False discovery rate and *P* value estimates were computed for each gene set based on 1,000 separate permutation distributions. Gene sets were deemed to be enriched when $P < 0.05$.

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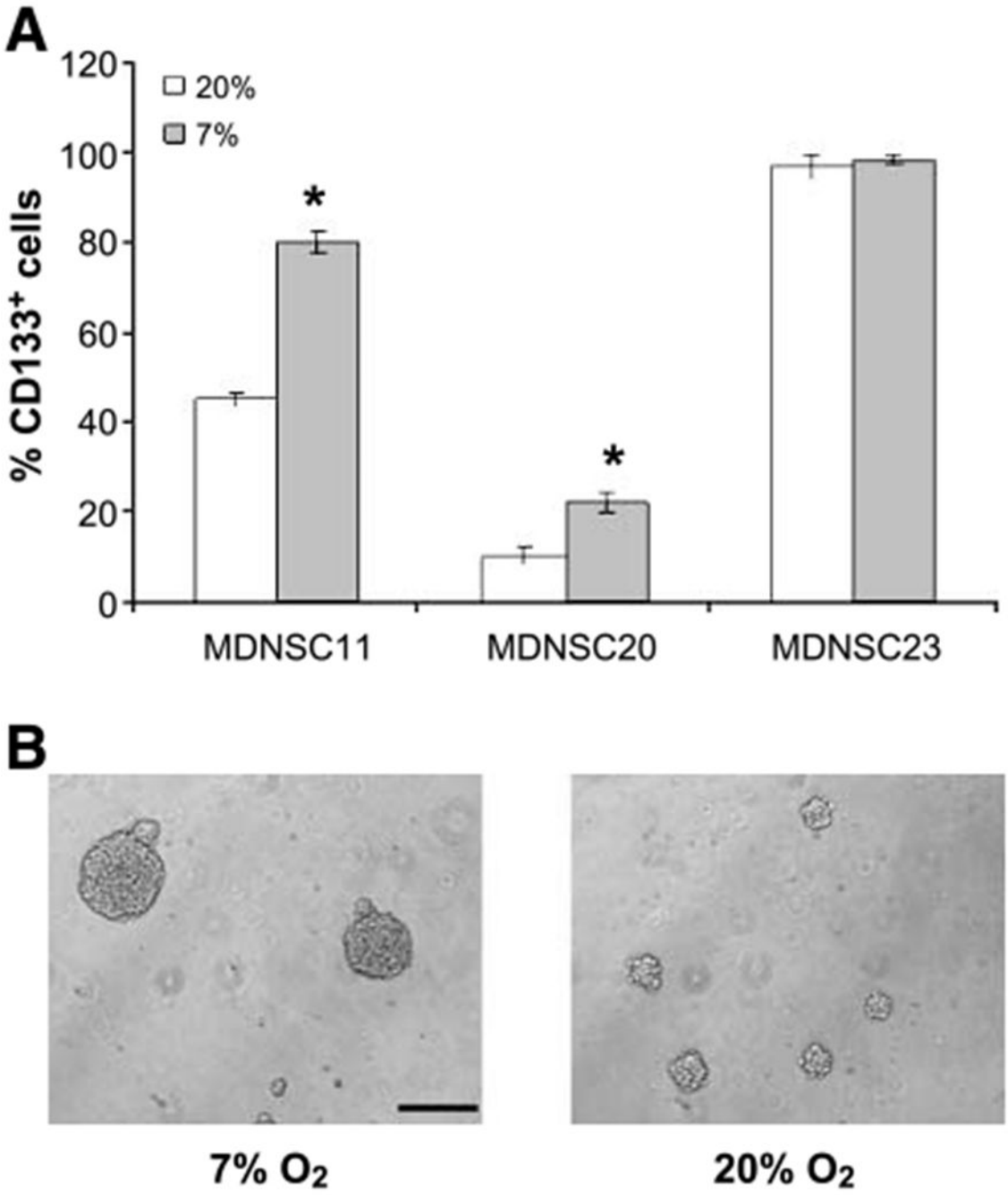

FIGURE 1.

Influence of reduced O_2 on the percentage of $CD133^+$ cells in GB neurosphere cultures. **A.** MDNSC11, MDNSC20, and MDNSC23 neurosphere cultures were grown at 7% or 20% O_2 for 10 to 14 d (~100 μm in diameter) and the percentage of $CD133^+$ cells in each culture was determined by flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.003$. **B.** Representative photomicrographs of MDNSC11 cultures after 5 d of growth at 7% or 20% O_2 (20× bar, 50 μm).

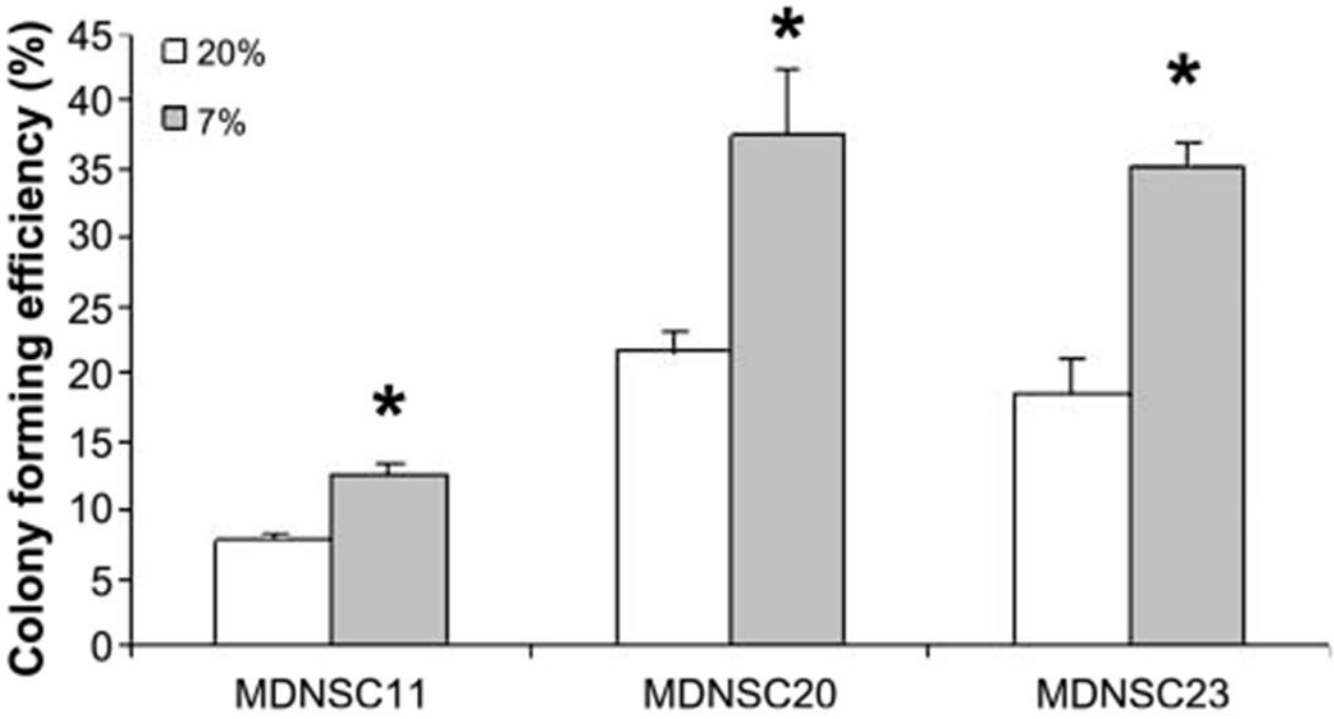

FIGURE 2.

Influence of O_2 on the clonogenicity of $CD133^+$ cells. $CD133^+$ cells from MDNSC11, MDNSC20, and MDNSC23 cultures were grown at 7% or 20% O_2 as neurospheres for 7 d and subjected to clonogenic analysis (*, $P < 0.02$).

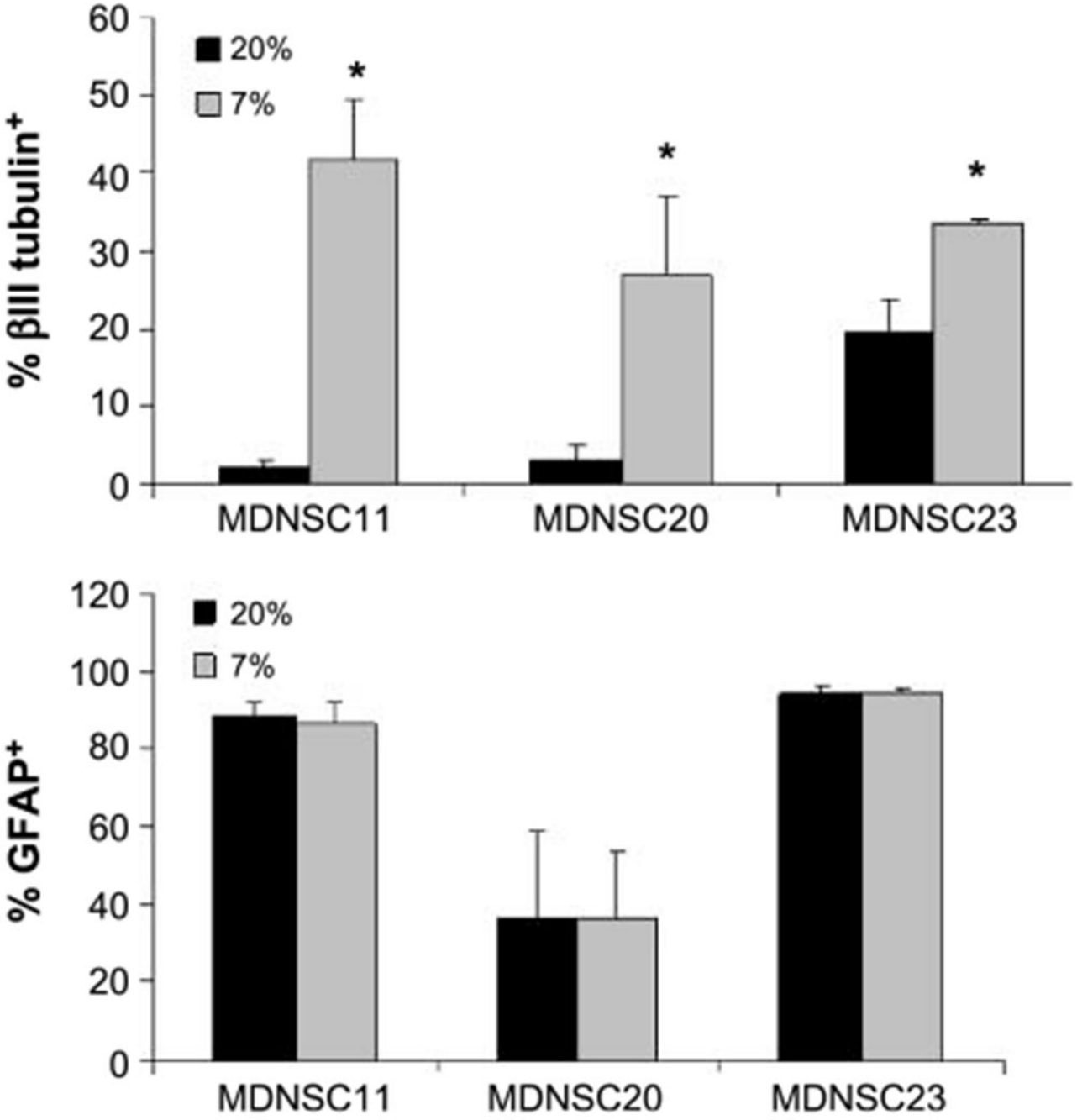

FIGURE 3.

Influence of O_2 on CD133$^+$ cell differentiation. CD133$^+$ cells from MDNSC11, MDNSC20, and MDNSC23 were grown at 7% and 20% O_2 for 7 d, then subjected to differentiation and stained for βIII tubulin or GFAP. Cells were scored as positive or negative for either marker. Columns, mean of three experiments; bars, SE.

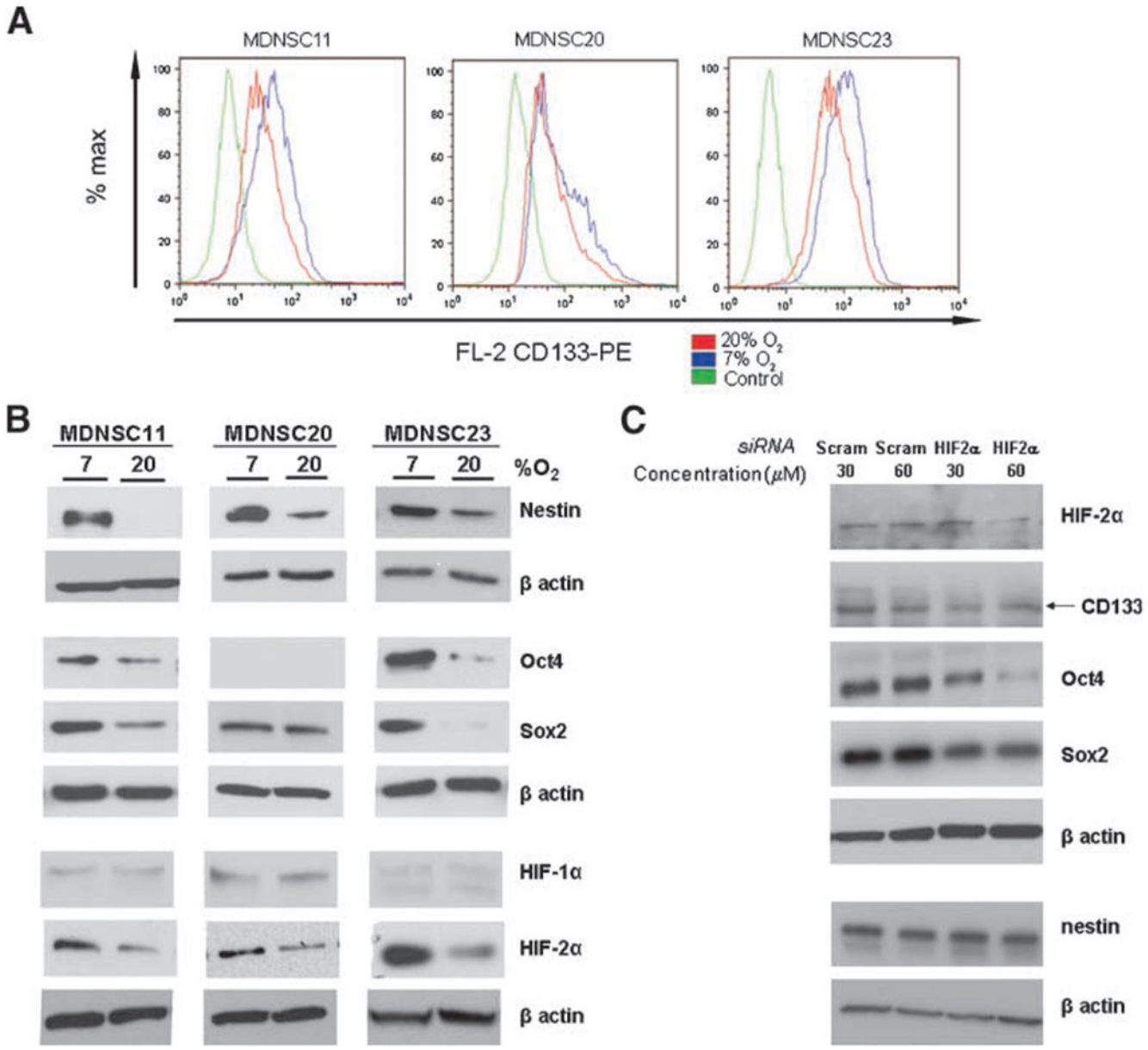

FIGURE 4.

Influence of O_2 on the expression of stem cell markers in $CD133^+$ cells. $CD133^+$ cells from MDNSC11, MDNSC20, and MDNSC23 were cultured at 7% or 20% O_2 for 7 d, then subjected to flow cytometry for CD133 expression using a mouse IgG1 as a staining control (*green*; **A**) or immunoblot analysis for stem cell–associated proteins using β-actin as a loading control (**B**). **C.** $CD133^+$ MDNSC11 were maintained at 7% O_2 and transfected with a scrambled control (*scram*) or siRNA for HIF-2α at the indicated concentration for 24 h, and immunoblots were done. Blots are representative of two independent experiments.

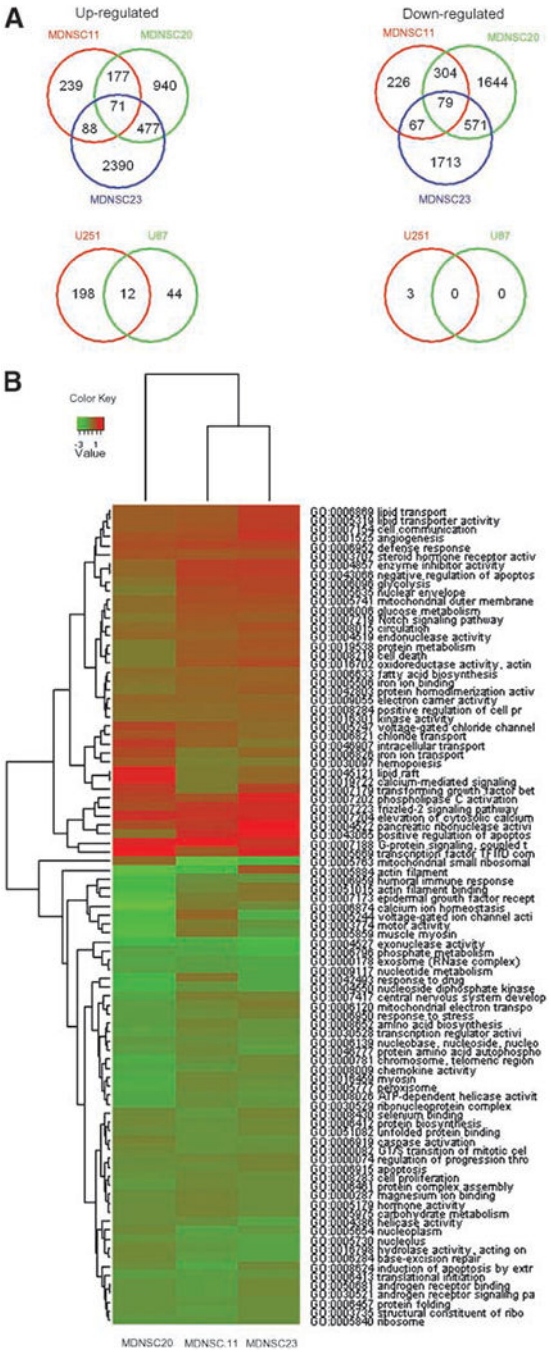

FIGURE 5.

Effects of *7%* O_2 on the gene expression profiles of $CD133^+$ cultures. $CD133^+$ cells from MDNSC11, MDNSC20, and MDNSC23 were grown at 7% and 20% O_2 for 7 d and then subjected to microarray analysis. **A.** Venn diagrams depicting the number of genes that were commonly up-regulated (*left*) or down-regulated (*right*) by growth at 7% O_2 as compared with 20% O_2. The same experiment was also done for monolayer cultures of U87 and U251 (*bottom*). For each culture, the genes whose expression was affected by 7% O_2 were defined by SAM (<0.10 false discovery rate). **B.** Gene set enrichment analysis according to GO

functional categories. Genes identified as being significantly affected by SAM in two of the three $CD133^+$ TSC cultures were submitted to enrichment analysis, with the corresponding GO categories displayed as a heat map.

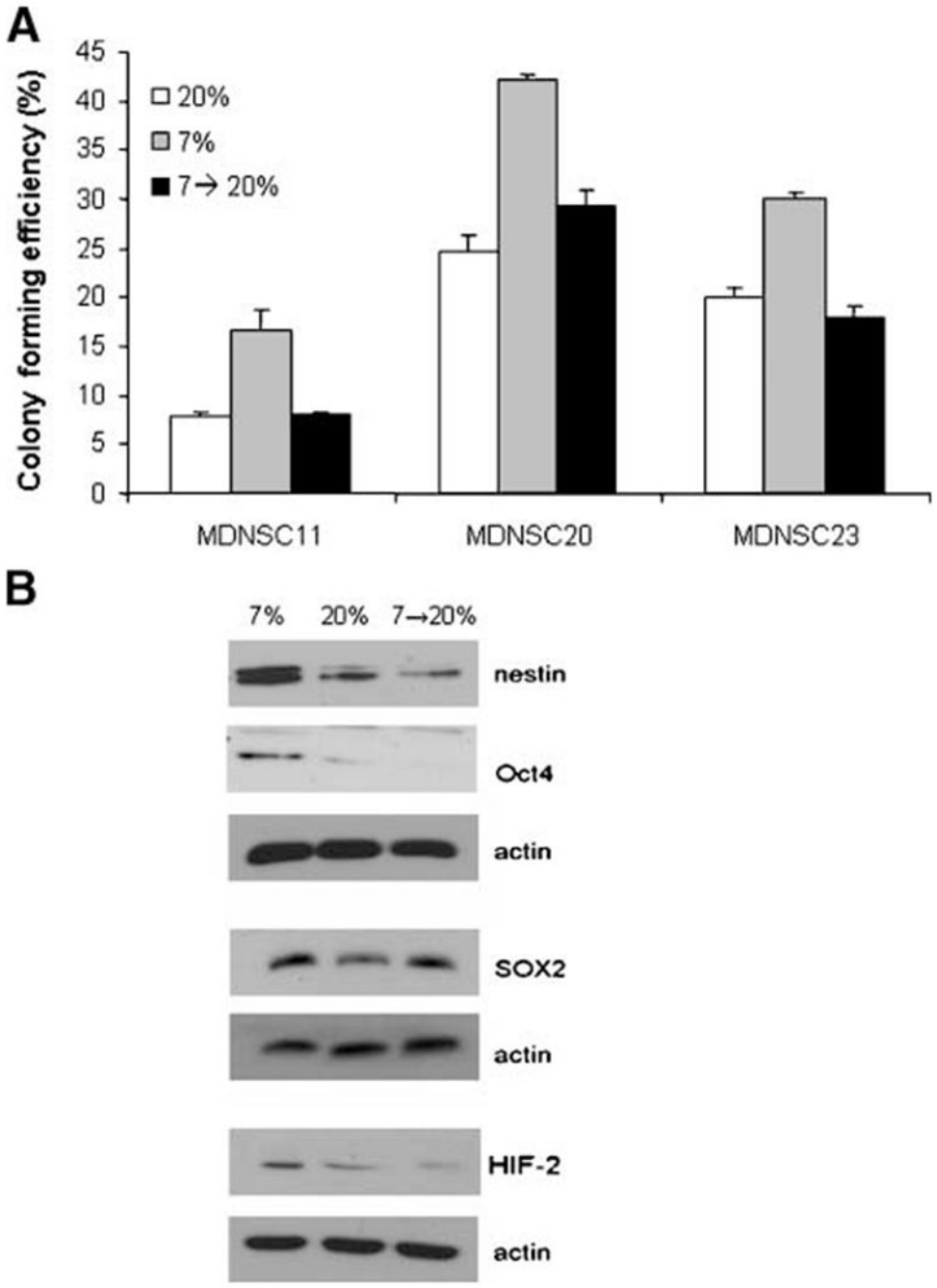

FIGURE 6.

Reversibility of 7% O_2 effects on stem cell phenotype. $CD133^+$ MDNSC11, MDNSC20, and MDNSC23 cultures were maintained at 7% O_2 for 7 d and then returned to 20% O_2 for 7 d (7→20); separate cultures were maintained at 7% or 20% for 7 d. These cells were then subjected to clonogenic analysis (**A**). Columns, mean of three independent experiments; bars, SE. *, $P < 0.02$. **B.** $CD133^+$ MDNSC11 were maintained at 7%, 20%, or (7→20) O_2

condition, then subjected to immunoblot analysis for nestin, Oct4, Sox2, and HIF-2α. Representative of three independent experiments.

Table 1.

Mean Population Doubling Time (Hours) of $CD133^+$ TSCs

% O_2	MDNSC11	MDNSC20	MDNSC23
20%	117.70 ± 4.16	141.27 ± 13.44	35.46 ± 1.91
7%	80.36 ± 6.51 *	76.07 ± 1.98 *	26.29 ± 1.65 *

NOTE: $CD133^+$ cells from MDNSC11, MDNSC20, and MDNSC23 were cultured at 7% or 20% O_2 for 7 d, and then disaggregated and cultured in T25 flasks under neurosphere-forming conditions. The neurospheres were disaggregated every 3 d, cells were counted, and mean population doubling time (hours) was determined. Values represent the mean of three independent experiments ± SE.

* $P < 0.02$.