NOX4 REGULATES TGFβ-INDUCED PROLIFERATION AND SELF-RENEWAL IN GLIOBLASTOMA STEM CELLS

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Background

Glioblastoma (GBM) is the most aggressive and common glioma subtype with a median survival of 15 months after diagnosis. Current treatments have limited therapeutic efficacy, thus more effective approaches are needed. The glioblastoma tumoral mass is characterized by a small cellular subpopulation, the glioblastoma stem cells (GSCs), which has been held responsible for initiation, invasion, proliferation, relapse and resistance to chemo- and radiotherapy. Targeted therapies against GSCs are crucial, and so is the understanding of the molecular mechanisms that govern the GSCs. Transforming growth factor β (TGFβ) signaling and Reactive Oxygen Species (ROS) production are known to govern and regulate cancer-stem cell biology. One of the main ROS producers are the NADPH oxidases (NOX). In particular, NOX4 is constitutively active and its function in GBM has been related to cell growth, survival, invasion and therapeutic resistance by hypoxia-induced radiation.

NOX4 is upregulated by TGFβ in GBM

A

	FC_U3031MG	FC_U3034MG
LIF	19.55	2.79
ODAPH	10.93	5.44
TNFAIP6	10.34	3.46
IL11	9.18	3.19
ID1	9.06	36.88
NOX4	7.71	2.55
SKIL	7.18	4.32
FOXS1	5.32	2.18
MSC	4.63	3.34
PMEPA1	4.24	3.79

B

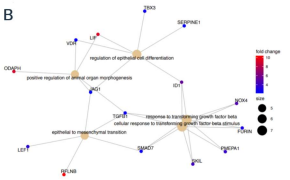


Figure 1. Table indicating the fold-change values of the common top 10 upregulated genes by TGFβ1 in U3031MG cells and U3034MG cells after 24 hours of treatment (**A**) and the Gene-concept network depicts the linkages between the common upregulated genes by TGFβ1 in both cell lines and the biological concepts as network; the size of the GO term stands for the number of upregulated genes in the TGFβ1 treated cells that are annotated based on the term. Color scale of the gene name stands for the mean foldchange in gene expression of the TGFβ1 treated cells compared to the untreated cells (**B**)

NOX4 expression is higher in GSCs vs differentiated cells

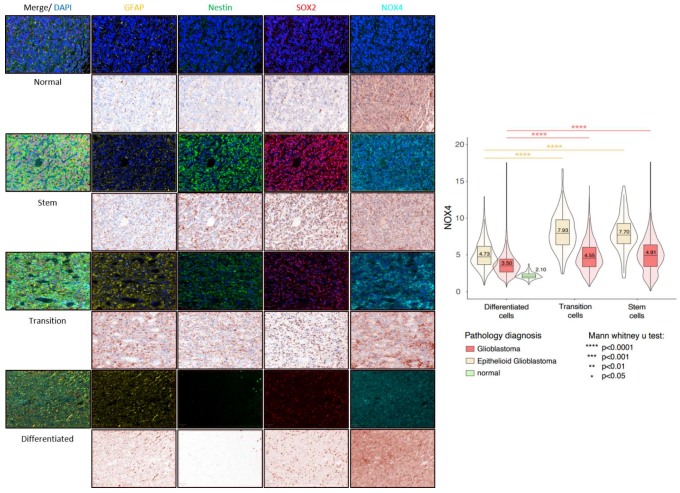


Figure 2. Multiplex staining of GBM TMA with samples from normal, glioblastoma and epitheloid glioblastoma patients; colour-coded (from Biomax.us GL806f). **Left:** Representative images displaying staining of the four proteins, NOX4/Nestin/SOX2/GFAP, in normal brain and glioblastoma tissue samples. **Right:** NOX4 expression level per cell; cells were classified and plotted as a function of the three marker proteins expression in the same cell into three groups: GBM-diff (GFAP^{high}/Nestin^{low}/SOX2^{low}), GBM-transition (GFAP^{medium}/Nestin^{medium}/SOX2^{medium}) and GBM-Stem (GFAP^{low}/Nestin^{high}/SOX2^{high}).

TGFβ1 increases ROS in a NOX4-dependent manner

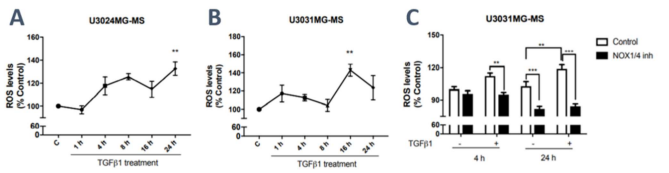


Figure 3. Time course of ROS production upon TGFβ1 treatment in U3024MG cells (**A**) and U3031MG cells (**B**). ROS production upon TGFβ1 stimulation in the presence or absence of NOX1/4 inhibitor (GKT137831, 20 µM) in U3031MG cells after 4 and 24 hours of treatment (**C**). Statistical comparison indicates *p < 0.05, **p < 0.01, ***p < 0.001.

TGFβ1 increases cell proliferation in a NOX4-dependent manner

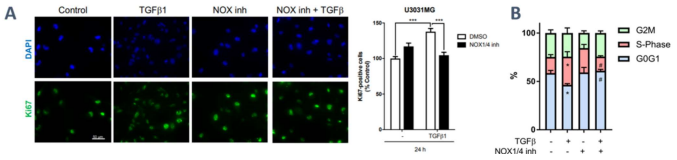


Figure 4. U3031MG cells were stimulated with TGFβ1 for 24h, in the presence or absence of the NOX1/4 inhibitor (GKT137831, 20 µM). Representative microscopic images of immunofluorescence of Ki67 (green) and DAPI (blue) (scale bar, 50 µm and quantification of Ki67 positive cells with respect to the control. Statistical comparison indicates ***p < 0.001 (**A**). Distribution of cell cycle for U3031MG cells after treatment. *p < 0.05 indicates a significant difference vs the control group. #p < 0.05 indicates a significant difference vs the TGFβ1 group (**B**).

NOX4 regulates GSC self-renewal capacity

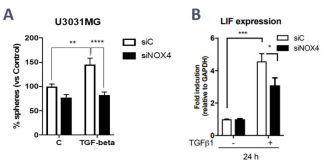


Figure 5. U3031MG cells were transiently transfected with control (siControl) or NOX4 (siNOX4) siRNAs and stimulated with TGFβ1 for 24h. 1000 cells/ml were seeded in an ulta-low attachment 24 multiwell plate and 7 days after being seeded, spheres bigger than 50 µm were counted and represented in the graph as percentage vs control (**A**). LIF mRNA expression levels were analyzed by qPCR (**B**). Statistical comparison indicates * p < 0.05, ** p < 0.01, *** p < 0.001.

NOX4 regulates GSCs metabolism

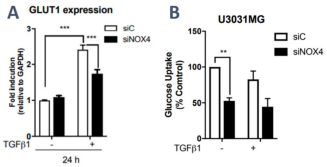


Figure 6. NOX4 regulates GLUT1 expression downstream of TGFβ, and has an impact in GSC glucose uptake. GLUT1 mRNA expression levels analyzed by qPCR in U3031MG cells transiently transfected with control (siControl) or NOX4 (siNOX4) siRNAs and stimulated with TGFβ1 for 24h (**A**). Glucose uptake in U3031MG cells were transiently transfected with control (siControl) or NOX4 (siNOX4) siRNAs and stimulated with TGFβ1 for 24h (**B**). Statistical comparison indicates * p < 0.05, ** p < 0.01, *** p < 0.001.

NOX4 overexpression mimics TGFβ effects in GSCs

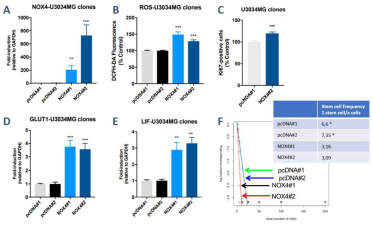
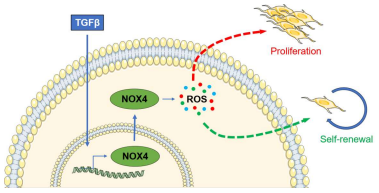


Figure 7. U3034MG cells stably overexpressing the empty vector pcDNA or pcDNA-V5-NOX4, pool1 and pool2 were analyzed. NOX4, LIF and GLUT1 mRNA expression levels analyzed by qPCR (**A, D, E**). Basal ROS production in the different indicated clones after 48h of being seeded (**B**). Quantification of Ki67-positive cells with respect to the control with lower and upper confidence intervals (**C**). Limiting dilution neurosphere assay was performed in U3034MG clones for 6 days, analyzed by ELDA, the tables show the stem cell frequency and the statistical differences between both groups (**F**). Statistical comparison indicates * p < 0.05, ** p < 0.01, *** p < 0.001.

Conclusion

Our data revealed that the TGF pathway depends on NOX4-derived ROS to regulate proliferation, self-renewal and glucose metabolism in GSCs, as well as demonstrating that NOX4 alone is a key regulator of stemness in glioblastoma. This work functionally establishes NOX4 as a key mediator of GSC biology.



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