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# Wnt pathway co-factors TCF1 and TCF4 control glioblastoma phenotype through an oxygen-dependent mechanism

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# SUMMARY

Glioblastoma multiforme (GBM) is one of most common and still poorly treated primary brain tumors. This pathology represents a dramatic challenge for researchers and clinicians and the biology underlying this cancer is not yet fully elucidated. So far, the existence of a subpopulation of cancer stem cells, responsible for GBM growth and aggressiveness, has been extensively demonstrated. Thus, the possibility of reprogramming this subpopulation toward less aggressive phenotypes has been rapidly growing during years. To reach this aim, however, a more complete understanding of the mechanisms preserving Glioma Stem Cells (GSCs) phenotype is needed, in particular considering the role of the microenvironmental stimuli driving GBM cell proliferation and self-renewal. In this context, hypoxia has assumed a fundamental role as a key regulator of signaling pathways involved in GSCs proliferation and tumor progression. For this reason, the characterization of the hypoxic niche where GSCs reside revealed that hypoxia, through HIF-1α transcription factor, can regulate several biological processes which are exploited by different populations of cells in the tumor mass in order to survive. The Wnt/β-catenin/TCF cascade is one of the most important hypoxia-modulated pathway implicated in tissue development and in the progression of several human cancers. Interestingly, our laboratory previously characterized a biological process that impacts on GSCs phenotype and that is regulated in synchrony by HIF-1α and the Wnt pathway. Indeed, in hypoxic conditions, Wnt pathway activation is able to induce a pro-differentiating switch of GSCs toward neurons, weakening their aggressive phenotype. This process is strictly dependent from environmental oxygenation and can be exploited to reprogram GSCs and neutralize their resistance to treatments. Here, we deepen into the mechanisms underlying this pro-differentiating process in primary GBM-derived cells and, in particular, we aim to finely characterize the role of the Wnt pathway transcriptional effectors TCF1 and TCF4 in controlling GBM phenotype. Once observed that Wnt pathway activation was able to mediate two different phenotypic effects at different oxygen levels in GBM cells, we found that, in hypoxia, the observed neuronal differentiation process of GBM cells was mediated by a molecular complex formed by TCF1, HIF-1α and β-catenin. Moreover, ChIP-sequencing experiments confirmed the capacity of the above mentioned complex to sit on promotorial sequences above the DNA and, in turn, trigger the expression of specific genes involved in the neuronal differentiation processes. As a confirmation, TCF1 knockdown in GBM cells cultured in hypoxia completely blocked the neuronal differentiation process. On the other hand, TCF4 expression resulted to be preponderant in normoxia, where we assumed

that TCF4E inhibitory isoforms could block this Wnt-dependent neuronal differentiation observed. Indeed, GBM cells cultured in normoxic conditions are characterized by an high expression of TCF4E which probably blocks the neuronal differentiation process mediated by TCF1 and HIF-1α. Moreover, in normoxic conditions ChIP-sequencing experiments suggested TCF4 as able to bind the same genomic regions controlled by the TCF1/HIF-1α molecular complex which assembles in hypoxic conditions. Further demonstrations for this hypothetical inhibitory function of TCF4 came from functional experiments in which we silenced TCF4 in normoxia. Herein, we observed an induction of the neuronal differentiation and a complete block of this process when GBM cells were forced to over-express the inhibitory isoforms TCF4E.

***Conclusions***

In this study we demonstrated that the cooperation between the Wnt pathway transcriptional co-factor TCF1 and HIF-1α is able to induce a long-lasting neuronal differentiation process in GBM cells. Indeed, Wnt pathway activation induces a switch from a stem-like phenotype towards neurons and triggers, exclusively under hypoxia, a TCF1/HIF-1α-dependent transcription of neuronal differentiation genes. This process is impaired upon standard oxygen conditions (20% $O_2$) by the binding of TCF4 to the same genes on distal sequences, thus exerting an inhibitory function on this process.

In conclusion, we unveil a tightly regulated mechanism by which the TCF1/HIF-1α transcriptional complex is able to induce a reminiscent neuronal differentiation of GSCs, which might represent a future potential strategy to therapeutically weaken their aggressiveness.

# SOMMARIO

Il Glioblastoma multiforme (GBM) è il più comune dei tumori cerebrali nell'adulto e rappresenta tuttora una patologia dalla prognosi molto infausta e senza trattamenti efficaci. Purtroppo i meccanismi biologici che portano alla formazione di questo tumore e che ne sostengono l'aggressività sono scarsamente caratterizzati. Finora molte evidenze scientifiche dimostrano come una piccola popolazione di cellule staminali tumori sia la responsabile dell'insorgenza e della crescita del GBM. Di conseguenza, la possibilità di riprogrammare queste cellule verso un fenotipo meno aggressivo e differenziato è stata valutata da molti ricercatori negli ultimi anni. Per capire in maniera più approfondita i meccanismi responsabili del mantenimento del fenotipo staminale di queste cellule diventa di fondamentale importanza studiare approfonditamente il microambiente circostante la massa tumore del GBM e caratterizzare gli stimoli che guidano la proliferazione e l'autorinnovamento delle cellule di GBM. Il microambiente tumore è costituito da un gradiente di ossigeno per cui le cellule tumori sfruttano bassi livelli di ossigeno, condizione denominata ipossia, per mantenere le loro caratteristiche staminali. Lo studio della nicchia ipossica in cui risiedono le cellule tumori di GBM ha portato alla dimostrazione che il fattore di trascrizione HIF-1α è responsabile del controllo di numerosi processi biologici responsabili del mantenimento del fenotipo scarsamente differenziato di queste cellule. Tra le molteplici vie di segnale regolate da HIF-1α troviamo il *pathway* di *Wnt/β-catenin*. Studiando le relazioni che intercorrono tra queste due vie di segnale, il nostro gruppo di ricerca ha caratterizzato un meccanismo biologico di differenziamento neuronale delle cellule di GBM indotto in sincronia dalle vie di segnale controllate da HIF-1α e Wnt. Questo processo risulta essere fortemente dipendente dai livelli di ossigeno presenti nel microambiente. Infatti, cellule di GBM coltivate in condizioni di ipossia vanno incontro a differenziamento neuronale dopo attivazione del *pathway* di Wnt. Tale processo potrebbe rappresentare uno strumento efficace per riprogrammare le cellule di GBM verso un fenotipo meno aggressivo e per questo motivo abbiamo approfondito i meccanismi che inducono tale processo nelle cellule primarie di GBM derivate da paziente. Il nostro obiettivo è stato quello di caratterizzare in maniera univoca il ruolo dei fattori trascrizionali TCF1 e TCF4, appartenenti alla via di segnale di Wnt, nel determinare il fenotipo del GBM. Abbiamo confermato che l'attivazione del *pathway* di Wnt induce due diversi effetti fenotipici nelle cellule di GBM a diverse concentrazioni di ossigeno. Successivamente abbiamo dimostrato che, in condizioni di ipossia, il differenziamento neuronale osservato nelle cellule di GBM è mediato da un complesso trascrizionale formato

da TCF1, HIF-1α e *β-catenin.* Inoltre, esperimenti di *ChIP-sequencing* hanno confermato la capacità di questo complesso trascrizionale di regolare in maniera attiva specifiche sequenze genomiche del DNA delle cellule di GBM ed attivare la trascrizione di geni implicati in processi di differenziamento neuronale. Abbiamo confermato questi risultati silenziando TCF1 nelle cellule di GBM coltivate in condizioni di ipossia ed abbiamo dimostrato che il differenziamento neuronale, altrimenti indotto in presenza di TCF1, viene completamente bloccato dopo attivazione del *pathway* di Wnt. Al contrario, abbiamo riscontrato una preponderante espressione di TCF4 ad alti livelli di ossigeno (ossigeno ambientale, 20%), in cui le cellule di GBM non subiscono il differenziamento neuronale osservato in ipossia e mantengono un fenotipo prettamente staminale. Per giustificare tale fenotipo dopo attivazione del *pathway* di Wnt, abbiamo ipotizzato che, tra le molteplici isoforme di TCF4, quelle a carattere inibitorio sulla trascrizione potessero bloccare il processo di differenziamento neuronale, altrimenti attivato dal *pathway* di Wnt in ipossia, e favorire il fenotipo non differenziato delle cellule di GBM. Per dimostrare quest'ipotesi abbiamo silenziato TCF4 in cellule di GBM coltivate in normossia ed abbiamo osservato che l'attivazione del *pathway* di Wnt induce in maniera significativa il differenziamento neuronale osservato in ipossia. Inoltre, se le cellule di GBM sono forzate ad esprimere l'isoforma inibitoria di TCF4, TCF4E, il fenotipo acquisito dalle cellule di GBM risulta essere non differenziato, confermando la capacità di inibire tale processo in cellule di GBM coltivate ad alti livelli di ossigeno.

***Conclusioni***

In questo studio abbiamo dimostrato che la cooperazione tra il *pathway* di Wnt e HIF-1α è in grado di indurre un cambiamento nel fenotipo di cellule di GBM in condizioni di ipossia. Il processo di differenziamento neuronale osservato in queste cellule dipende esclusivamente dal microambiente e, in particolare, risulta essere specifico per cellule non differenziate di GBM coltivate in ipossia. La dimostrazione che è possibile modulare il fenotipo di queste cellule in maniera specifica e dipendente dai livelli di ossigeno rappresenta una potenziale strategia futura per ridurre l'aggressività e la resistenza ai trattamenti di queste cellule.

# INTRODUCTION

## Glioblastoma multiforme

Brain tumors consist of a diverse group of neoplasms afflicting both children and adults and are among the human cancers with the poorest outcome (1). Glioblastoma multiforme (GBM) is a primary brain neoplasm, consisting of a genetically and phenotypically heterogeneous group of tumors. Ninety percent of GBM cases develop *de novo* (primary GBM) from normal glial cells by multistep tumorigenesis. The remaining 10% of gliomas are cases of secondary neoplasm, developing through progression from low-grade tumors (diffuse or anaplastic astrocytomas) (2). Although many risk factors associated the development of GBM have remained unidentified, risk factors such as exposure to ionizing radiation have proven to be detrimental for disease development in some cases. Other risk factors including cell phone use, head trauma, and pesticide exposure have yet to be confirmed as increasing risk for gliomagenesis. Symptoms of disease depend on the specific location of the tumor, and diagnosis is most commonly made following surgical resection. The prognosis for patients with GBM is often very poor (only 2% of patients aged 65 years or older, and only 30% of those under the age of 45 years at diagnosis, survive for 2 years or more), and treatments to cure this cancer have yet to be devised (3).

GBM is characterized by highly proliferative and invasive capacities in order to infiltrate surrounding tissues as fast as possible and leading to dramatic aggressiveness and progression. As a consequence, the tumor mass is not clearly distinguishable from the normal tissue and, unfortunately, the complete resection of GBM tumor mass is almost impossible, with radiotherapy not always efficient. Moreover, the blood-brain barrier makes treatment more difficult. In addition, tumor cells found in the areas of hypoxia are resistant to radiotherapy. Surgical resection to the extent feasible, followed by chemotherapy and radiotherapy, is the mainstay of GBM treatment. Surgical treatment, chemotherapy and radiotherapy prolong the survival time in young people up to 202 weeks. However, even after the maximal safe surgical resection, GBM could easily relapse and the tumor often become more aggressive (2). Unfortunately, the establishment of a new tumor mass, which occurs in almost all patients, still decrease their short survival possibilities (4).

**Neural stem cells (NSCs) and Cancer Stem Cells (CSCs)**

Treatment failure of GBM is in part due to a poor knowledge of the mechanisms regulating GBM tumor growth and aggressiveness and understanding these mechanisms is the major challenge that researchers have to face. The fundamental problems not yet resolved are which are the cells in the diverse tumor populations that initiate and maintain brain tumor growth and what are the molecular mechanisms involved in this phenomenon. In the adult brain, neural stem cells (NSCs) were observed at any stage of the development, from the embryo to the adult organism. NSCs are primarily located in the subventricular zone (SVZ) (5), in the subgranular zone (SGZ) and the dentate gyrus of the hippocampus (5). In particular, NSC have been described to reside in their specific niches around the blood vessels where they are in communication with other cells and the extracellular matrix. Different cellular types are present in these niches, such as neuroblasts and transitory amplifying progenitors, all surrounded by ependymal cells (6,7). NSCs are multipotent cells capable of multi-lineage differentiation as a result of which they lose their stem properties (8). NSCs divide symmetrically for self-renewal and generate differentiated progeny (neurons, astrocytes, oligodendrocytes) through asymmetric divisions (Figure 1A). Moreover, their proliferative capacity and the association with blood vessels stimulate NSCs to migrate and colonize surrounding tissues. While NSCs are necessary for a correct neurological development and activity, cells with aberrant NSC characteristics have been often correlated to brain tumors. Indeed, increasing evidences suggest the existence of a population of CSCs or tumor initiating cells (TICs) with high self-renewal ability, thus promoting brain tumor growth (9). In the light of the "CSC hypothesis", the transformation of NSCs or progenitors into CSCs follows the rules of the normal physiology, but with aberrant order, timing and intensity of the underlying mechanisms sustaining the disruption of the regulatory mechanisms that control self-renewal and proliferation and contributing to the formation of an aberrant disorganized tissue (Figure 1B). CSCs may originate from normal NSCs undergoing tumorigenic alterations. Differently, they can derive from more differentiated or terminally differentiated transit-amplifying neural cells being affected by multiple mutations, thus reverting to a stem phenotype. Moreover, an arrest of the normal maturation process of the NSC has been also reported, thus leading to intensive cell division and lack of differentiation. CSCs originating through these different processes are generally described as a small sub-population of dividing cells with stem cell-like properties, huge self-renewal ability, peculiar genetic alterations, tumorigenic potential, and the ability to differentiate into all different bulk tumor cells (10). During last years, the

connection between normal stem cells and cancer has emerged in many tissues, particularly, blood, mammary gland, gut, skin and brain. Thus, researchers have proposed the so called "hierarchical model" of tumor progression in which malignant tumors are initiated and maintained by a population of tumor cells that share similar biologic properties with normal adult stem cells. This model, supported by increasing experimental data, holds that only a rare subset of cells within the tumor own unlimited proliferation capacity and, in particular, the ability to self renew and differentiate into the different cellular subtypes that form the mass. This hypothesis postulates that a population of cells with stem cell-like features (CSCs) exists in tumors and it is able to generate new tumors when transplanted. This cell population would be able to give rise to the whole cellular bulk of the tumor mass, with the remainder cells representing differentiating or terminally differentiated cells originated by a stem cell precursor that sits on top of the tumor's "differentiation hierarchy". Under certain conditions, non-CSCs can become CSCs and exhibit an enhanced ability to form spheres, thereby suggesting that the CSC state may be plastic (11). Several groups studying human brain tumors identified small numbers of cells with clonogenic potential based on the neurosphere assay. In culture, these brain tumor cells form self-renewing neurosphere-like colonies, and they have the ability to differentiate into one or more neural lineages. Moreover, a growing body of evidence indicate that these rare populations of brain cancer cells, termed GBM stem cells (GSCs), are characterized by the ability to generate xenografts representing the initial tumor in immunodeficient animals and to divide asymmetrically to allow self-renewal as well as differentiation into a non-CSC population (2). GSCs expressed both neuronal and astroglial markers on differentiation, together with several key determinants of neural stem cell fate, that can be often used for their identification. CD133 (also known as prominin-1) along with nestin, SOX2, Bmi1, Musashi, CD44, CD15, or ABC transporter proteins (11). Since GBM tumors display high degree of phenotypic, cellular, genetic, and epigenetic heterogeneity, it is plausible to believe that a major problem in the unresponsiveness of GBM tumors to therapy is the existence of GSCs within the tumor (12). Unfortunately, GSCs role during initiation, progression, and recurrence of GBM has been accepted to be the primarily responsible for radiation and chemotherapy resistance and poor survival of GBM patients (13).

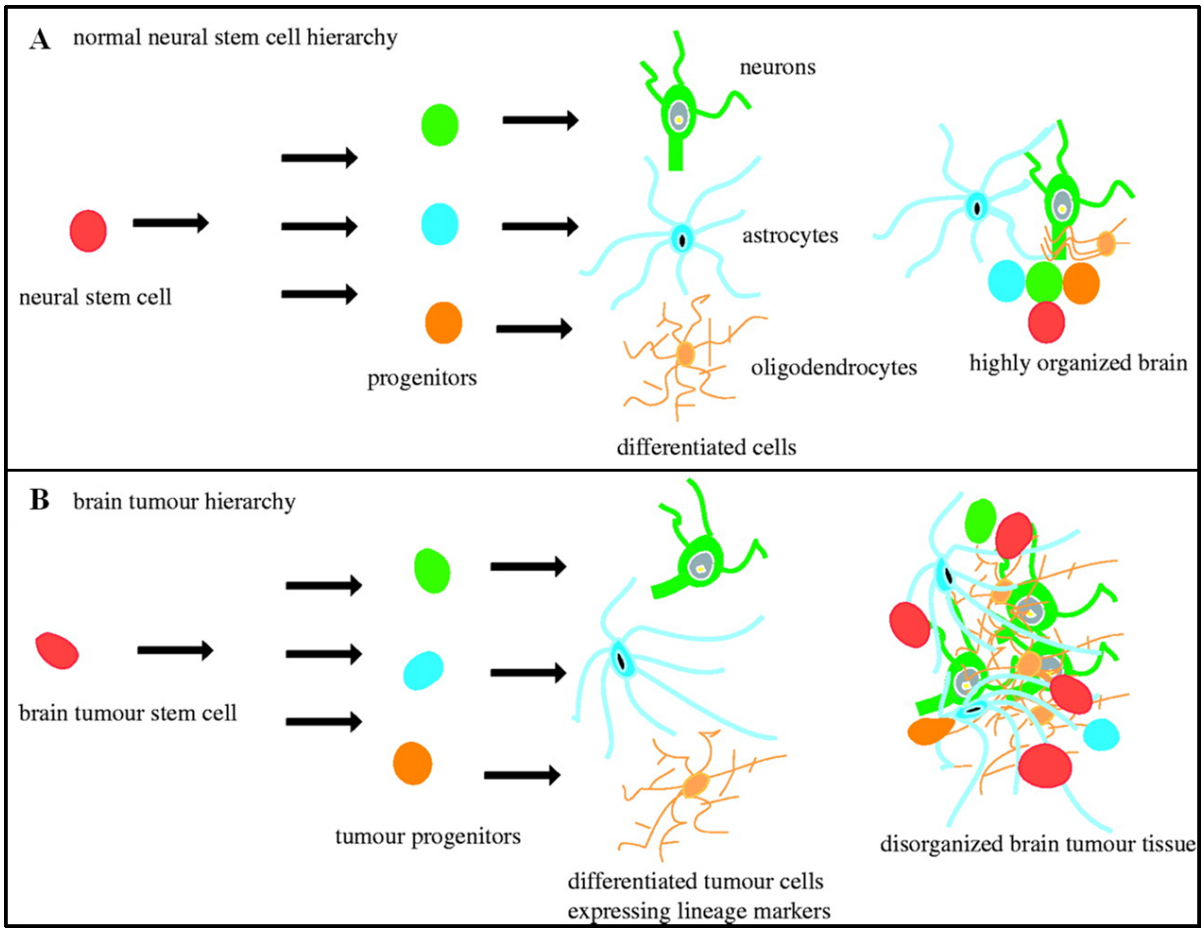


**Figure 1. Stem cell hierarchy in normal brain and brain tumours.**
**(A)** Normal brain: rare neural stem cells in the brain are able to self-renew to maintain themselves and also generate progenitors that proliferate and then differentiate into mature cells that form the highly organized brain parenchyma. **(B)** Brain tumors: cancer stem cells are dysregulated in terms of self renewal and proliferation and they aberrantly differentiate into cells that form the bulk of the disorganized cancer tissues (2).

## Tumor microenvironment and the hypoxic/vascular niches

Microenvironment represents a fundamental mediator in maintaining the aggressive behavior of GSCs and an inexhaustible source of stimuli pushing tumor progression. Indeed, GBM complexity is driven by numerous stimuli which originate from the microenvironment, suggesting its importance also for its pathogenesis and the documented resistance to therapy. It has been described that GBMs display high cellular heterogeneity (9) described a model which integrates the plethora of signals which regulate GBM plasticity. GBM cells communicate with the perivascular niche and with the hypoxic niche, by originating a "teamwork", withstanding to hierarchic rules and complex networks. The three-layers concentric model represents a clear explanation to elucidate the complexity of signals integration in GBMs, particularly deriving from microenvironment. According to the hierarchical theory for tumor progression, the "tumor-initiating cells" GSCs may originate

from the SVZ and the SGZ, which include progenitor cells able to originate multi-lineage differentiated cells. These specific niches are essential for maintaining stemness and self-renewal properties of GBM precursors, which are secondly instructed to proliferate and differentiate. The central area of the tumor mass consists of a necrotic core, highly hypoxic and enriched in GSCs, and as going to the periphery, the tumor mass includes an intermediate layer, hypoxic and rich in GSCs too. The surrounding peri-tumor zone corresponds to the peripheral layer of the "three-layer model", and it is highly vascularized and presents few GSCs and more differentiated cells. A hypoxic gradient is arranged from the core to the periphery, associated to a progressive change in the expression of specific markers, from CD133 and Nestin in the necrotic area, to differentiation markers, such as GFAP and β-III-tubulin, in the more oxygenated periphery (Figure 2).

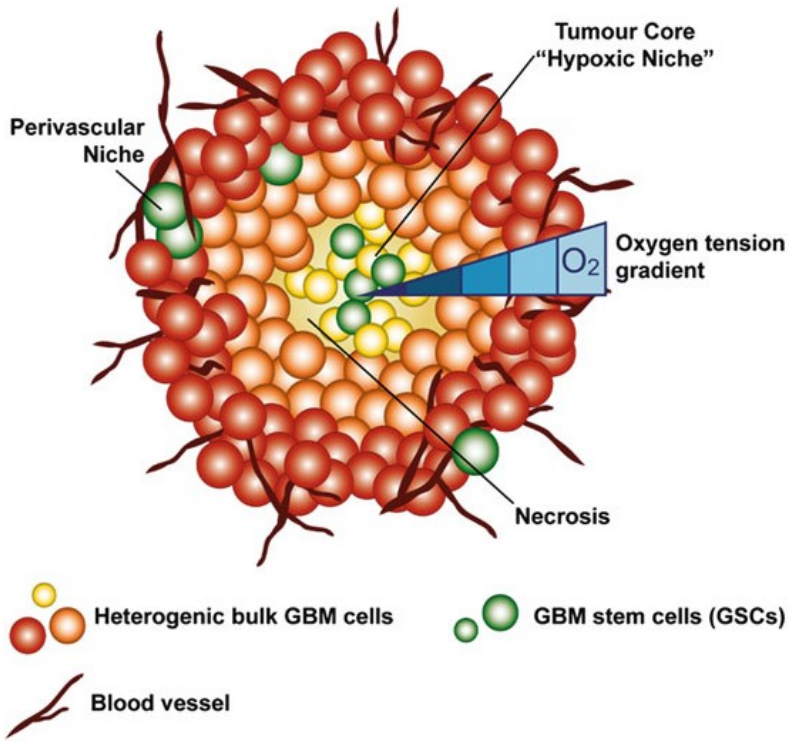


**Figure 2. The three layer model of glioblastoma.**
In this model GSCs are located along the hypoxic gradient in the tumor mass, mostly residing in the inner portions of the mass and in the so called perivascular niche. A hypoxic gradient is present from the tumor core to the periphery, associated to a progressive change in the expression of specific markers such as stemness markers, like CD133 and Nestin in the necrotic area, to differentiation markers, such as GFAP and β-III-tubulin, in the more oxygenated periphery (3).

Two main niches are detected in GBM microenvironment, the hypoxic and the peri-vascular ones. They finely regulate cellular fate by releasing numerous stimuli, which promote cell differentiation or stemness maintenance. GBMs are highly vascularized tumors, characterized by strong angiogenesis, but the blood flow is not the only key factor to play a pivotal role in contributing to the complexity of vascular microenvironment, since many cell types infiltrate the tumor mass. In particular, the peri-vascular niche consists of the surrounded area of angiogenic and tumor microvascular structures, characterized by the presence of several mature and differentiated cells (endothelial cells, fibroblasts, astrocytes, macrophages or microglia) which orchestrate intercellular crosstalk. Endothelial cells are the principal components of the vascular niche, and they differ from endothelial cells which constitute vessel walls. Blood flow is necessary to provide oxygen and nutrients to GBM cells, particularly to CSCs, nevertheless many non-structural endothelial cells exist, and they remain separate from tumor capillaries, without increasing the tumor microvascular density. They have the task of releasing a lot of diffusible factors to maintain the self-renewal ability of neural stem cells and neurogenesis. On the other hand, GBM cells release pro-angiogenic stimuli like VEGF to recruit endothelial cells which proliferate and give rise to new capillaries. Moreover, other pro-angiogenic mechanisms were described for GBM angiogenesis, such as the transdifferentiation of cancer stem cells into tumor-derived endothelial cells (TDECs), in order to continuously preserve the vascular microenvironment (14–16). Pericytes are contractile cells which are tightly associated to endothelial cells, endowed with the function of stabilizing and maintaining the integrity of the newly formed tumor vessels. They has been described to be involved in the regulation of the angio-architecture structural shape of the tumor vascular niche, and they intimately depend on endothelial cells along the vessel walls. Analogously, astrocytes are closely associated to the endothelial cells forming blood vessels, and they both maintain the integrity of the blood brain barrier, and produce neurotrophic factors which promote GBM proliferation (17). Fibroblasts reside in the peri-vascular niche, and they are responsible of GBM invasion, as reported for other cancer types. They express critical markers associated to tumor progression and malignancy, such as metalloproteases (pro-MMP2). The presence of tumor induces a physiological immune response, and GSCs showed the expression of pro-inflammatory genes, which stimulate the enrichment of microglia at the tumor peri-vascular site. Microglia are the macrophages which lie in brain tissue, and they are the principal cytokine stimulators important for tumor proliferation, migration and progression. They are located in many sites, depending on their role. They promote metastasis when arranged in the perivascular space,

cell motility and invasion when sited in the advanced tip of tumor, and their localization in the peri-necrotic area increases angiogenesis, explaining the positive correlation between macrophages infiltration and vascular density in gliomas (18,19). The combination of all these cell types results in a complex system of crosstalk between cells, which culminates in a fine balance of a plethora stimuli for GBM cells. Particularly, GSCs are strictly connected to endothelial cells, as well as other stromal cells, defining the entirely plasticity, typical of the tumor microenvironment. It has been observed that GSCs arrange themselves along the capillaries, in order to be prone to respond to signaling cues deriving from endothelium, by direct cell-to-cell contact and soluble factors. They stimulate GSCs to proliferate and self-renew, and the increase of the number of endothelial cells has been associated to an accelerated brain tumor initiation and growth. On the other hand, GSCs express elevated levels of VEGF or other pro-angiogenic factors, which in turn stimulate endothelial cells to proliferate and undergo angiogenesis. This evidence shows a bidirectional signaling and cross-talk between stem cells and vascular niche (16). A peculiar aspect of GBM microenvironment is the hypoxic niche. GBM mass is characterized by low oxygen concentrations, ranging between 0.1 % and 2.5 %, different from the healthy brain, which physiologically range between 12.5 % and 2.5 % of oxygen. GBMs are marked out by hypoxic gradients, which present areas with moderate or severe hypoxia, and necrotic zones in the tumor core. The inner layer shows a considerable expression of hypoxic markers, associated to tumor aggressiveness and GSCs maintenance (3).

Studies conducted on GBM-derived cells confirmed the importance of oxygen tension in determining the phenotype of GBM cancer cells and that oxygen is a critical parameter in interpreting standard laboratory culture conditions (21% oxygen) versus the *in vivo* microenvironment, known to be hypoxic. Increasing oxygen tension promotes activation of the pro-apoptotic intracellular pathway in GBM-derived cells and the percentage of $CD133^+$ GSCs decreased after acute exposure to environmental oxygen, with a corresponding increase in $CD133^-$ cells enriched in $GFAP^+$ astrocytes , consistent with a pro-differentiating effect. Additionally, normal brain precursors behaved in the same way, indicating that low oxygen tensions play a physiological protective role in maintaining the resident stem cells pool (11).

**GBM resistance to treatments**

GBM display high resistance to conventional radiotherapy and chemotherapy (20). Indeed, soon after their initial description, GSC resistance to treatments have been described (21,22), thus suggesting them as one the principal contributors to GBM tumor recurrence. GSCs have been demonstrated to be more resistant to radiation than the non-stem glioma cells (21). Indeed, chemotherapy with Temozolomide (TMZ) delays GBM tumor growth, but long term survivors are extremely rare and recurrence after TMZ therapy strongly indicates the presence of TMZ-resistant GSCs (23). In an *in vivo* mouse model of GBM, TMZ treatment increased tumor side population (SP), a cell population that have been described to be enriched in CSCs, suggesting that TMZ treatment could even favor tumor recurrence (24). For these reasons, it is now widely accepted that GSCs contribute to GBM recurrence after conventional therapies. Initial models of GSC regulation have been based on neural stem cell (NSC) biology, the most similar cellular surrogate. In this context, GSCs seem to be governed by pathways which are already active during brain development, including Notch, Wnt, bone morphogenetic proteins (BMP), transforming growth factor-β (TGF-β ), other RTK pathways (3,25).

**HIF-1α pathway**

To better investigate the characteristics of the exclusive niche where GSCs reside, it is fundamental to study the mechanisms responsible of the low oxygen tension maintenance. A milestone in understanding oxygen physiology was the identification by Wang and Semenza of the HIF transcriptional complex, which is the key intracellular molecular system of the hypoxic response (26). In particular, activation of HIF-1α transcription factor is the most recognized pathway adopted by hypoxic cells to survive and maintain tumor progression. HIF-1α protein activity and accumulation are regulated at different levels through its life cycle inside the cells. Independently from $O_2$ levels, HIF-1α is constitutively transcribed and synthesized through a series of signaling events involving several growth factors and other signaling molecules. Under normoxic conditions, HIF-1α protein expression is negatively regulated by proteosomal degradation and ubitiquination in a pathway involving von Hippel-Lindau protein (VHL), a tumor suppressor, and one of the recognized components of an E3 ubiquitin protein ligase. The two proline residues located in the LXXLAP amino acid motif in the Oxygen Dependent Degradation Domain (ODDD) of HIF-1α were found to be good

substrates for the action of a group of enzymes called proly1-4-hydroxylases (PHD 1,2,3). These are 2-OG-dependent dioxygenases enzymes which require oxygen for their hydroxylation action. Hence, hydroxylation of proline residues by PHDs occurs only when there is sufficient amount of oxygen. Consequently, hydroxylated HIF-1α subunits are preferably recognized by VHL and are tagged for ubiquitination and proteasomal degradation. In contrast, under hypoxic conditions, HIF-1α subunits hydroxylation does not occur and HIF-1α structure reaches a conformational stabilization in the cytoplasm. With these properties, HIF-1α is able to enter the nucleus and form a transcriptional complex with HIF-1β subunit, p300 and CBP for binding hypoxia responsive elements (HREs) and to finally trigger the activation of hypoxia target genes, implicated in the regulation of several biological processes as cell proliferation and survival (Figure 3) (27). HIFs can transcribe more than 40 target genes (26), among which the carbonic anhydrase isoform 9 (CAIX), involved in increasing the metastatic potential of GBM by acidificating the tumor microenvironment, and Notch1, which leads to NFAT activation, cell proliferation and tumor growth. Thus, hypoxia sustains GBM cells proliferation, particularly preserving the stem population in the perivascular and hypoxic niches, by up-regulating other transcription factors like Notch and Oct4, which control self-renewal and multipotency of stem cells. Moreover, it has been described that HIF counteracts the differentiating stimuli induced by BMPs (28). *In vitro* hypoxia stimulates both the expression of the stem markers CD133, Nestin, Sox2, and the formation of neurospheres, characterized by elevated plurpotency (29–31). HIF is directly engaged in angiogenesis and tumor invasion, by activating several factors such as VEGF, metalloproteases, TGF factors and CXCR4 (3).

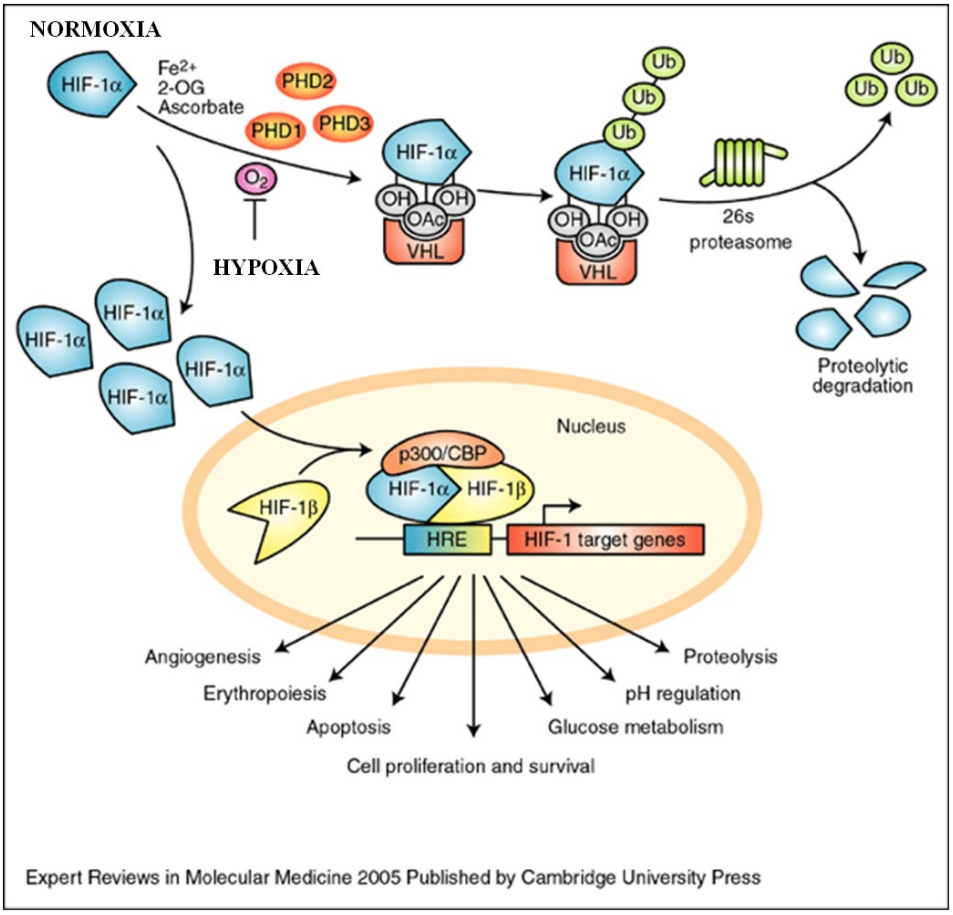


**Figure 3. HIF-1α regulation by proline hydroxylation.** In NORMOXIA, the alpha subunits of HIF (HIF-1α) are hydroxylated at conserved proline residues by HIF prolyl-hydroxylases (PHD 1,2,3), allowing their recognition and ubiquitination by the VHL E3 ubiquitin ligase, which labels them for rapid degradation by the proteasome. In HYPOXIA, PHDs are inhibited, since they utilize oxygen as a co-substrate. HIF-1α subunits reach stabilization and enter the nucleus, where they form a complex with HIF-1β subunits, p300 and CBP to finally trigger the activation of hypoxia target genes which in turn regulate several biological processes, as cell proliferation and survival.

During last years, lots of evidences have suggested that lowered oxygen tensions positively correlate with tumour aggressiveness (32,33) and over-activity of Hypoxia Inducible Factor-1α (HIF-1α) is implicated in tumour progression (34). The correlation between hypoxia and tumor aggressiveness has been causally linked to increased genomic instability (35), but it is also related to increased survival of proliferating cells by suppression of p53 and its associated cell growth control (36). Importantly, hypoxia has been shown to promote de-differentiation of neuroblastoma cells (33,37,38), suggesting that it may reinforce an environment for aggressive tumor growth. It may also prevent a pre-existing stem cell population from differentiating, which is important in light of increasing evidence that cancer is initiated by dysfunctional stem cells (39–41). Activated HIF-1α plays a crucial role also in the adaptive responses of the tumor cells to changes in oxygen through transcriptional

activation of over 100 downstream genes which regulate vital biological processes required for tumor survival and progression (27).

**Wnt/β-catenin/Tcf pathway**

Among the several pathways directly modulated by hypoxia, the Wnt/β-catenin/Tcf pathway is one of the most important, whose implication in tissue development and in the progression of several human cancers has been extensively investigated. Wnt signals play important roles during development (42), as well as in adult tissues that are refreshed and repaired by stem cells (43). It is the essential function of Wnt signaling in stem cell self-renewal and cell proliferation that links this pathway to problems of aging and disease such as cancer and diabetes (44,45). The core of the pathway is the E-cadherin cell-cell adhesion adaptor protein and the transcriptional co-regulator β-catenin (46). In the absence of WNT signaling, β-catenin levels in the cytoplasm and nucleus are low as a result of continuous phosphorylation by the serine/threonine kinases CK1 (casein kinase 1) and GSK3β (glycogen synthase kinase 3β), leading to binding of β-transducin-repeat-containing protein (βTRCP) and to ubiquitylation and degradation by the proteasome. The destruction complex is composed of CK1 and GSK3β, as well as the anchor proteins AXIN1 (axis inhibition protein 1) and APC (adenomatous polyposis coli). In the nucleus, members of the T-cell factor (TCF) family are bound by co-repressors such as GRG/TLE (Groucho/transducin-like enhancer) proteins that shut off expression of Wnt target genes. Other components of the repressor complex include CTbP (C-terminal binding protein) and HDACs (histone deacetylases). β-catenin in the nucleus is inhibited from binding TCF by ICAT (cell autonomous inhibitor of β-catenin and TCF). The Frizzled receptor complex is composed of Frizzled and LRP5 (LDL-receptor-related protein 5) or LRP6 and can also be actively inhibited by receptor-bound soluble inhibitors such as DKK1 (Dickkopf homologue) (Figure 4A). When a lipid-modified WNT protein binds to the receptor complex, a signaling cascade is initiated. LRP is phosphorylated by CK1 and GSK3β, and AXIN1 is recruited to the plasma membrane. The kinases in the β-catenin destruction complex are inactivated and β-catenin translocates to the nucleus where is able to bind TCF factors to form an active transcription factor complex. In the nucleus, co-factors such as legless (LGS; also known as BCL9) and Pygopus (PYGO), CBP/p300, Brahma and MED12 are recruited to initiate transcription of a large set of target genes (Figure 4B). To trigger the activation of these genes, the transcriptional effectors act downstream of the signaling pathway at the molecular level binding specific DNA sequences. These

transcriptional factors belong to the mammalian TCF/LEF family which encompasses LEF-1, TCF7 (also known as TCF1 but encoded by *TCF7* gene), TCF3 (encoded by *TCF7L1*), and TCF7L2 (also known as TCF4 but encoded by *TCF7L2* gene). Structural characterization studies revealed several functional domains which have been mapped on the TCF family of proteins. In particular, the high mobility group (HMG) DNA binding domain is the most highly conserved region of the TCF proteins (47,48). Like other HMG box-containing proteins, TCF/LEF proteins possess minimal transcriptional activity on their own and can efficiently affect transcription only by recruiting various binding co-factors, which, in turn, recruit chromatin modifiers to suppress or activate their target genes (46). *In vitro* studies with recombinant proteins revealed that TCF/LEF proteins recognize a core consensus sequence, the TCF/LEF DNA binding motif (49,50). Moreover, these monomers contain a domain that can interact with Armadillo repeats, which serve as the platform for β-catenin binding (51,52). Indeed, β-catenin binds TCF/LEFs through 3–10 Armadillo repeats and then uses its C terminus to interact with other co-factors, including the chromatin modifiers CBP/p300 and Brg1, which ensure the efficient transcription of their target genes (46).

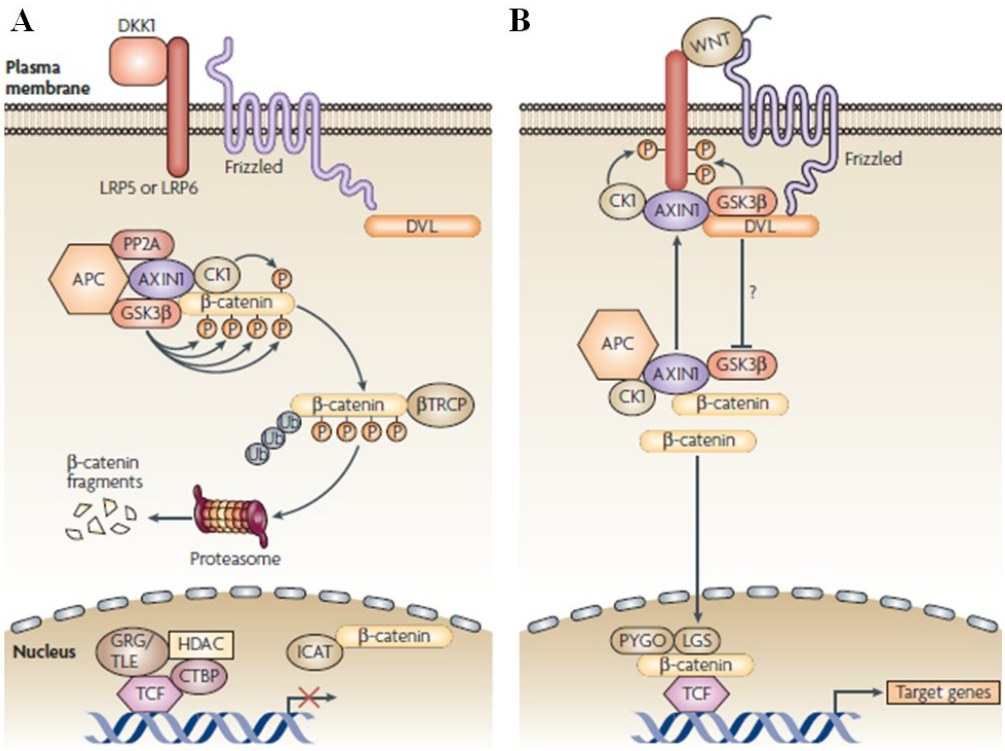


**Figure 4. Canonical or WNT–β-catenin–TCF/LEF signaling. (A)** In the absence of WNT signaling, β-catenin levels are maintained low by continuos ubiquitylation and degradation by the proteasome. In the nucleus, TCF (T-cell factor) molecules are bound by co-repressors such as GRG/TLE (Groucho/transducin-like enhancer) proteins that shut off expression of WNT target genes. **(B)** Upon binding of a lipid-modified WNT protein to the receptor complex, a signaling cascade is initiated and β-catenin translocates to the nucleus to form an active transcription factor complex with TCF, leading to transcription of a large set of target genes (53).

**WNT pathway/hypoxia correlation and pro-differentiating strategies**

Recently, several studies proposed promising approaches to mild GSCs aggressiveness by inducing neuronal differentiation. Indeed, forcing GSCs to differentiate into neuronal cells as a mean to reduce tumor growth may represent a powerful tool to overcome treatment resistance. Guichet *et al*. explored the possibility to eliminate GSCs forcing their terminal differentiation overexpressing neurogenic transcription factors involved in neurons formation processes. *In vitro* engineered GSCs encountered massive cell death, proliferation arrest and a drastic reduction of neurosphere formation. *In vivo*, GSCs were unable to form orthotopic tumors. By inducing cell death, cell cycle arrest or differentiation, it has been demonstrated that neurogenic proteins are able to oppose GSCs stem-like and non-stem-like cell growth (54).Using anticancer drugs may represents another promising approach to induce neuronal differentiation of GSCs. Indeed, it has been demonstrated that the anticancer drug taxol was able, not only to induce cell death in C6 glioma cells, but also morphological changes, including cell elongation, thinning of cellular processes, irregular shapes and fragmented nucleation or micronuclei in the survived C6 cells. These data suggested as taxol could induce the expression of neural differentiation markers and inspired that some anticancer drugs may be applied to elimination of the malignant cancer cells as well as changing proliferation and differentiation status of tumor cells (55). Another approach to reach this pro-differentiating purpose consists in exploiting the molecular pathway implicated in the regulation of CSCs phenotype. In this context, although the precise interactions between HIF-1α and the β-catenin/TCFs complex are under evaluation, some observations suggest an important role for tissue hypoxia in regulating the Wnt/β-catenin/TCF signaling cascade and, subsequently, the GSCs phenotype. Interestingly, Wnt signaling has been suggested to regulate differentiation of normal neural progenitors, promoting neurogenesis in the murine adult hippocampus (56), an hypoxic brain zone where adult neural stem cell reside in mice and humans (57). In the same context, it has been demonstrated that hypoxia can promote canonical Wnt signaling activation and that HIF-1α enhances NSC differentiation and neuronal maturation by co-operating with β-catenin activation (58). So far, it has been demonstrated that the interaction between HIF-1α and HIF-2α with β-catenin modulates TCF4-mediated transcriptional activity (59). Moreover, it has been reported that hypoxia (HIF-1α) is able to induce the over-expression of β-catenin co-factors TCF1 and LEF-1, sustaining the activation of Wnt pathways. Conversely, in high oxygen conditions, TCF1 and LEF-1 expression decreased and TCF4 transcript resulted augmented, confirming the central role of HIF-1α in the regulation of

TCF/LEF-1 levels (58,60). Despite these observations in normal brain development, the role of Wnt activation in regulating brain tumour phenotype remains controversial. Previous studies showed that lithium ($LiCl_2$) potently and specifically blocked glioma cell migration through inhibition of serine/threonine protein kinase glycogen synthase kinase-3 (GSK3), a β-catenin inhibitor (61). Moreover, the use of other GSK3 inhibitors have been reported to increase β-catenin levels, thus down-regulating stem cell markers, such as Nestin and Sox2 and increasing the fraction of cells expressing β-III-tubulin and GFAP in a cell line-dependent manner (62). A recent study demonstrated that Wnt activation promotes neuronal differentiation of GBM cancer cells under hypoxia, and that these effects are exerted by antagonizing Notch signaling, leading to up-regulation of pro-neuronal genes and inhibition of stemness-related pathways (60). In addition, Wnt activation promotes a dramatic differentiation of GBM cancer stem-like cells towards a neuronal, less aggressive phenotype (60). However, other authors reported that over-expression of Wnt in astrocytic glioma specimens promoted CSCs self renewal and proliferation (63–65). Another recent study pointed out as Wnt signaling was active in at least a subset of malignant gliomas and that inhibition of this pathway could slow tumor growth, reduce the stem-like cellular fraction, and block clonogenicity in some GBM neurosphere lines (66). Thus, the interaction between Wnt pathway and hypoxic signaling in brain tumours remain to be elucidated and needs a further deeper investigation in order to fully understand the impact of these signaling pathway in GBM tumor formation.

# MAIN AIMS OF THE STUDY

1. To characterize the role of the Wnt related transcription factors TCF1 (encoded by *TCF7* gene) and TCF4 (encoded by *TCF7L2*) in determining GBM phenotype.

2. To elucidate the interaction between TCF1/TCF4 and the GBM microenvironment by exploiting multiple approaches:

   - ✓ Evaluation of the cooperation between the hypoxia factor HIF-1α and the TCFs transcription complex and how this interaction can modulate TCFs binding to the DNA. by ChIP experiments;
   - ✓ Study of the interaction between HIF-1α and the TCFs under Wnt pathway activation;
   - ✓ Analysis of GBM derived cells gene expression profile at different oxygen concentrations for genes related to Wnt pathway and neural cells phenotype.

3. To correlate *in vitro* results in human GBM samples

# RESULTS

## Microenvironmental oxygen tension differentially regulates GBM cell fate upon Wnt pathway intracellular activation

Since the role of the secreted signaling proteins has been extensively debated for their effects on the phenotype of cancer cells, these recombinant molecules are nowadays considered as an interesting tool to be investigated for cancer therapy. Moreover, recent research point out the importance of the microenvironment in determining cancer cell phenotype (67,68). Starting from these considerations, we derived primary GBM cells directly from patient's biopsies, treated them with recombinant Wnt3a both at 2% oxygen (hypoxia, H) or either at 20% oxygen (normoxia, N) and assessed their response to Wnt pathway activation in these different microenvironmental conditions. Immunofluorescence analysis based on stemness (Nestin) and neuronal differentiation (β-III-tubulin) markers revealed that normoxia de-sensitizes GBM cells to Wnt treatment compared to Wnt-treated cells in hypoxia, where cells are subjected to a strong neuronal differentiation, and confirmed our previous finding that a Wnt-mediated neuronal differentiation of primary GBM derived cells occurs only in hypoxia (60) (Figure 5A,B). We confirmed also that Wnt3a treatment and high oxygen tension are both able to decrease nestin expression, as previously reported (28,60) (Figure. 5A,B).

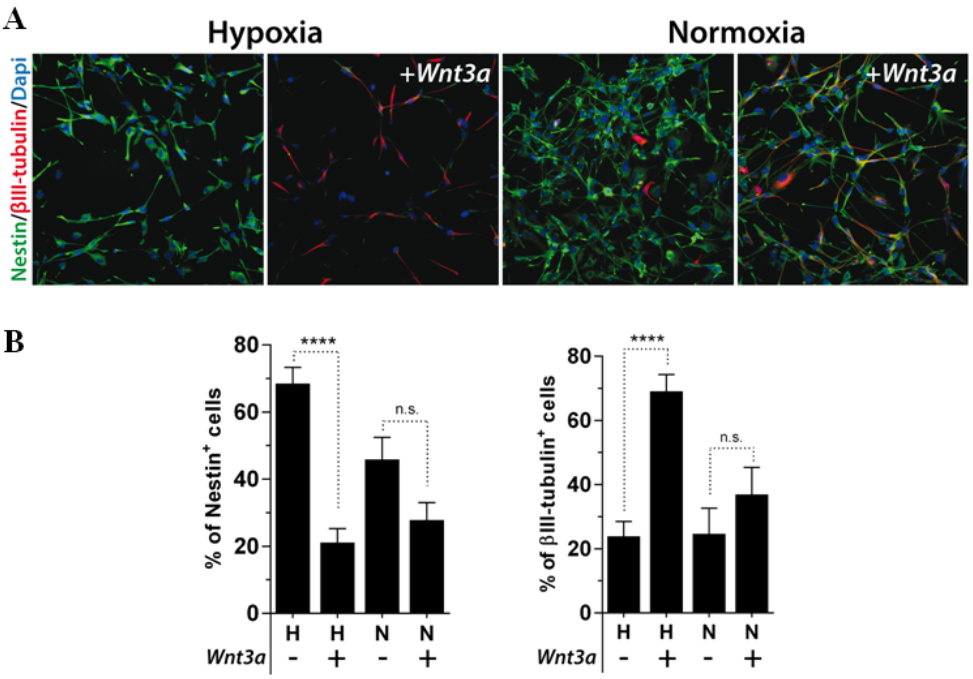


**Figure 5. Wnt pathway activation differentially modulates the phenotype of GBM cells depending on the oxygen tension.** Primary GBM cells were initially expanded in 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N) and then acutely exposed(+) or not exposed (-) to Wnt3a (30ng/mL) for 96 hours **(A)** Representative immunofluorescence images of GBM cells stained for Nestin (green)/ β-III-tubulin (red) and DAPI (blu). **(B)** Bar graph reporting relative quantification of images described in panel (A).

Once the impact of Wnt pathway activation on cell phenotype has been recorded, we investigated its role on GBM cells proliferation and self-renewal capacity. Thus, the stem-like potential was assessed by serial dissociation and re-plating of GBM neurospheres and, interestingly, we found that the number of neurosphere decreased after Wnt pathway activation only in hypoxia (Figure 6A,B). On the other hand, we observed an opposite effect in normoxia, confirming the different effect of Wnt pathway activation depending on oxygen levels (Figure 6A,B). In addition, we measured the effects of Wnt pathway activation on GBM initiating cells frequency, depending on microenvironmental conditions. Indeed, we set up limiting dilution assays (LDAs) to assess the effect of Wnt pathway activation on GBM cells cultured at different oxygen levels and treated with Wnt3a. We observed that Wnt3a treatment significantly decrease the frequency of self-renewing GBM cells at low oxygen levels with an opposite effect at high oxygen levels, where GBM stem cells frequency even increased (Figure 6C). More specifically, the frequency of GBM initiating cells was measured for all the microenvironmental conditions and we obtained these values: 1/30 for GBM cells cultured in hypoxia, 1/282 for GBM cells cultured in hypoxia and treated with Wnt3a, 1/368 for GBM cells cultured in normoxia and 1/56 for GBM cells cultured in normoxia and treated with Wnt3a. These evidences confirmed the impact of Wnt pathway activation on GBM cells cultured in hypoxia in terms of a negative regulation of self-renewal potential, and induction of a neuronal differentiation process, thus prompting us to hypothesize the existence of an opposing phenotypic effect that could be engaged by Wnt signaling activation depending on the microenvironmental oxygen.

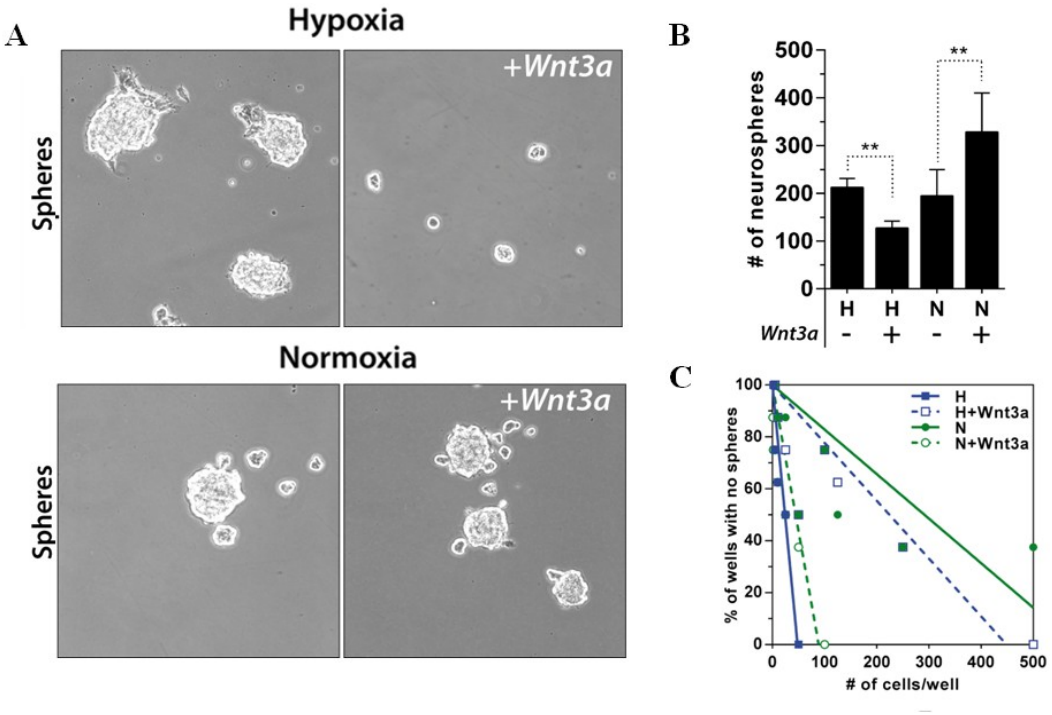


**Figure 6. Wnt pathway activation differentially affects self-renewal capacity of GBM cells depending on the microenvironment. (A)** Representative images of primary GBM cells expanded as neurospheres for 1 week in 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N) and then acutely exposed to Wnt3a (30ng/mL) for 96 hours. **(B)** Bar graphs represent the number of GBM derived neurospheres formed after dissociation and replating for 3 times. Mean of 3 tumors ± S.E.M. **(C)** Limiting dilution assay of GBM cells expanded in 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N) and then acutely exposed to Wnt3a (30ng/mL).

**Wnt pathway activation induces the formation of a TCF1/β-catenin/HIF-1α molecular complex at low oxygen tension**

Since the Wnt pathway activation was able to mediate two different phenotypic effects at different oxygen levels in GBM cells, we were interested in dissecting the molecular basis underlying the above mentioned effects. Thus, we analyzed the proteins levels of the β-catenin co-factors TCF1, TCF4 and their relationship with the known hypoxia sensor HIF-1α. Through WB analysis we observed, as expected, that TCF1 expression is strongly induced after Wnt pathway activation both in hypoxia and normoxia (60). Interestingly, we found that TCF4 expression was oxygen dependent with higher protein levels at 20% $O_2$ (Figure 7A). Moreover, WB showed that the more abundant TCF4 isoforms expressed by GBM cells are the full length (75kDa, putative TCF4E isoform) (69), whose function has been described to be inhibitory (70). Of note, the lower molecular weight TCF4 isoforms were barely detectable. To better characterize the expression data of TCF1 and TCF4, we set up immunofluorescence experiments which confirmed the results obtained with western blot analysis (Figure 7B).

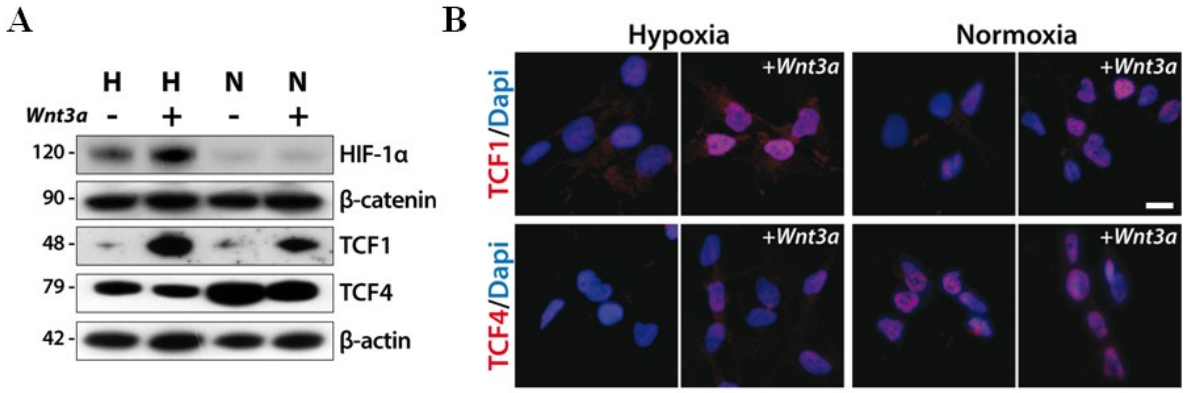


**Figure 7. Wnt pathway transcriptional effectors are differentially expressed in hypoxic and normoxic GBM cells.** Primary GBM cells were initially expanded at 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N) and then acutely exposed(+) or not exposed(-) to Wnt3a (30ng/mL) for 24 hours **(A)** Representative western blot analyses of HIF1α, β-catenin, TCF1 and TCF4 along with β-actin as loading control; 3 different GBM have been analyzed. **(B)** Representative immunofluorescence images of primary GBM cells stained for TCF1(red) and TCF4 (red) and DAPI (blu). 3 different GBM have been analyzed. bar=50μm

To unveil the molecular interactions of these transcription factors in different microenvironmental conditions, we immunoprecipitated β-catenin and HIF-1α in cells treated with Wnt3a and either maintained in hypoxia or normoxia. TCF1 and TCF4 differentially bound to β-catenin in GBM cells grown at different oxygen tensions, giving also a meaning to the variable expression of TCF1 and TCF4 depending on oxygen levels, previously reported by our group (60). In particular, we demonstrated that TCF1 bound to β-catenin at both 2% and 20%$O_2$ after Wnt3a administration (Figure 8A). while the more expressed isoform of TCF4 in GBM cells was curiously not associated to β-catenin (Figure 8A). Similarly, TCF1 immunoprecipitated with HIF1α in hypoxia only after Wnt3a treatment with TCF4 showing absence of binding also to HIF1α (Figure 8B).

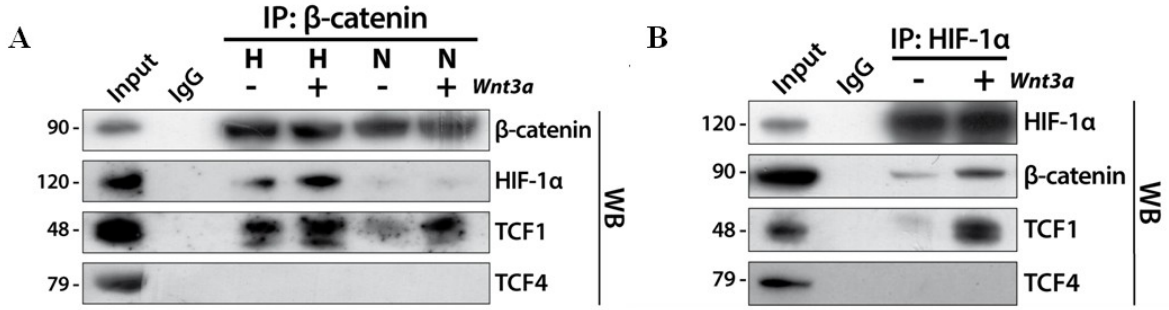


**Figure 8. Wnt pathway transcriptional effectors bind different co-factors depending on the oxygen tension.** Primary GBM cells were initially expanded in 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N) and then acutely exposed(+) or not exposed(-) to Wnt3a (30ng/mL) for 24 hours (A) Representative images of GBM cells immunoprecipitates extracted with β-catenin antibody and analyzed by western blotting with HIF-1α, TCF1 and TCF4 antibodies (numbers represent kDa). (B) Representative images of GBM cells immunoprecipitates extracted with HIF-1α antibody and analyzed by western blotting with HIF-1α, TCF1 and TCF4 antibodies. 3 different GBM have been analyzed.

With these experiments, we demonstrated the existence of a molecular complex activated by Wnt3a administration in hypoxia composed by TCF1, β-catenin and HIF-1α, and probably responsible for the neuronal differentiation process (Figure 9A). On the other hand, in response to high oxygen levels, we observed a strong increase of high MW TCF4 isoforms (TCF4E) which were not able to bind neither β-catenin nor HIF-1α. Given its reported inhibitory role on transcription and the documented lack of neuronal differentiation of GBM cells in normoxia, we hypothesized that TCF4E could repress the process of neuronal differentiation engaged by GBM cells after hypoxic Wnt activation (Figure 9B).

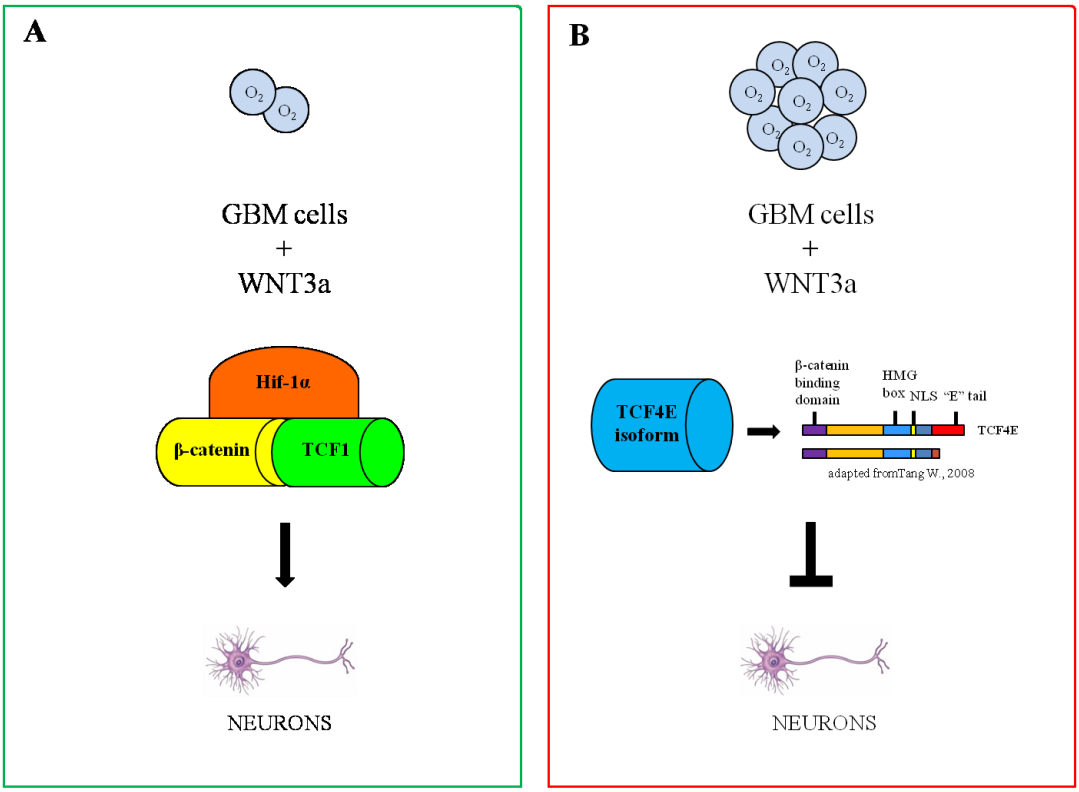


**Figure 9. Proposed molecular model of the neuronal differentiation process mediated by Wnt pathway activation in GBM. (A)** HYPOXIA: Wnt pathway activation triggers the formation of a molecular complex formed by TCF1, β-catenin and HIF-1α which induces the neuronal differentiation of GBM cells towards neurons. **(B)** NORMOXIA: neuronal differentiation of GBM cells is impaired by the inhibitory action of TCF4E isoforms which block the neuronal commitment observed at low oxygen levels in the presence of HIF-1α.

## Hypoxia cooperates with Wnt pathway to regulate the transcriptional milieu of GBM cells

A well accepted paradigm in molecular biology is that gene transcription is induced or repressed by transcription factors that, by recruiting others co-factors, form complexes on the genome to activate or inhibit target genes. Thus, it is important to know the position of these transcription factors and how they are combined on specific regions of the genome to understand their regulation on gene expression. Once demonstrated the existence of a molecular complex formed by TCF1, β-catenin and HIF-1α triggered by Wnt pathway activation, we assessed the impact of this complex on the transcriptional landscape of GBM cells. Thus, we performed ChIP-sequencing experiments in which we immunoprecipitated HIF-1α, TCF1 and TCF4 and analyzed the genomic sequences they were bound to, at different oxygen tensions.

First, we examined the general binding pattern of HIF-1α, TCF1 and TCF4 on the genome in cells maintained in hypoxia and treated for 24 hrs with Wnt3a and the general binding pattern of TCF4 on the genome in cells acutely expose to normoxia upon Wnt activation (Figure

10A). Of note, from cells acutely exposed to 20% oxygen we could not retrieve interpretable high-quality peak calls from ChIP-seq data from HIF-1α and TCF1, thus excluding these data from further analysis. In particular, heatmaps showing the chromatin features of each transcription factor revealed genomic areas of co-binding between HIF-1α and TCF co-factors. Interestingly, TCF4 showed a different genomic binding pattern in hypoxia compared to normoxia (Figure 10A). All the transcription factors analyzed bound different genomic regions and 17% of called sequences resulted to be promotorial (Figure 10B). We then examined how TCFs co-factors were arranged on the genome after Wnt activation at 2% oxygen and their mutual relationship. We confirmed that TCF1 and TCF4 localized in genomic regions spanning approximately 2kb around Transcriptional Starting Sites (TSS) (Figure 10C) of genes involved in the regulation of metabolic and transcriptional processes (Figure 10D).

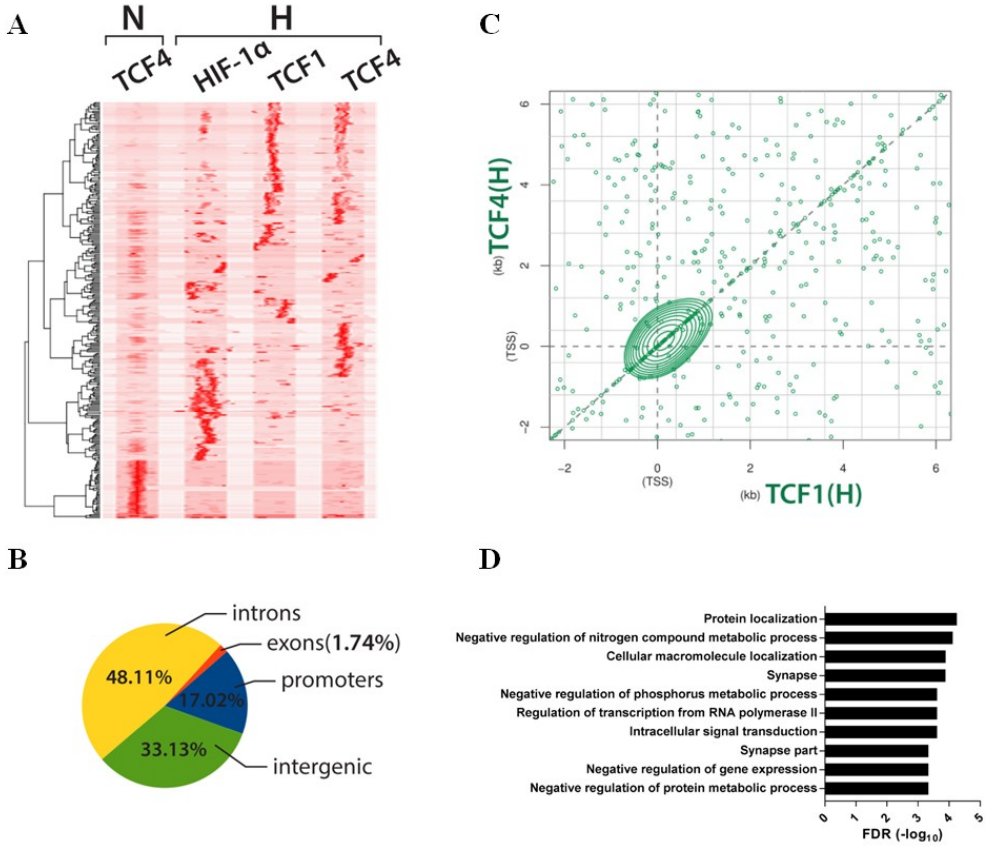


**Figure 10. The binding pattern of TCFs on the genome depends on microenvironmental conditions.**
**(A)** Heatmaps showing the chromatin features within 250 bases around the top 50 peaks for each TF; the peaks were selected for showing both the higher fold-enrichments and the lower p-values. **(B)** Molecular characterization of the genomic regions bound by the different TF found in (A). Bi-dimensional dot plot reporting the peak positions for pairs of TFs in relation to the TSS of genes on the genome of GBM cells, after WNT pathway activation; level-lines identify the regions of higher dot densities for each TF. **(C)** HYPOXIA (H): TCF1 paired with TCF4 **(D)** Gene Ontology (GO) analysis of genes in common for TCF1 and TCF4 in hypoxia.

Then, we tried to characterize the transcriptional cooperation between TCFs co-factors and HIF-1α in hypoxia. In particular, we observed that HIF-1α and TCF1 co-localized on the genome very close to TSS of genes which were also targeted by TCF4, but in different, and more distal (≈ +3kbp) sequences (Figure 11A). The gene list retrieved from this analysis, revealed that HIF-1α, TCF1 and TCF4 together, regulate genes involved in neurogenesis and neuron differentiation processes as emerged by GO analysis (Figure 11B) and corroborating our phenotypic results on hypoxic Wnt3a–treated GBM cells (Figure 5A) Surprisingly, investigating the binding pattern of TCF4 in GBM cells exposed to high oxygen levels (normoxia), we discovered that TCF4 was seated above the same genomic regions bound by HIF-1α and TCF1 in hypoxia (Figure 11C) thus originating a very similar GO analysis, showing its involvement in genes regulating the neuronal differentiation process as well (Figure 11D).

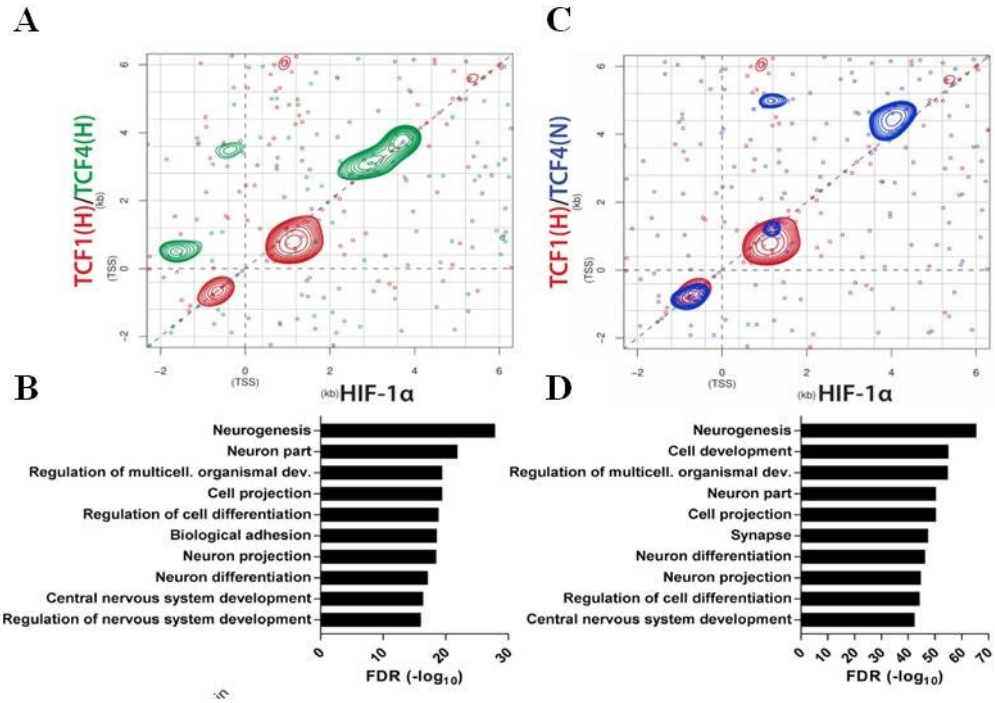


**Figure 11. Wnt pathway transcriptional effectors trigger the activation of specific genes depending on the microenvironment.** Bi-dimensional dot plot reporting the peak positions for pairs of TFs in relation to the TSS of genes on the genome of GBM cells, after WNT pathway activation; level-lines identify the regions of higher dot densitites for each TF. **(A)** HYPOXIA (H): TCF1 paired with HIF-1α (red), and TCF4 paired with HIF11-α (green) **(B)** Gene Ontology (GO) analysis of genes in common for TCF1 and HIF-1α in hypoxia **(C)** NORMOXIA (N): TCF4 paired with HIF-1α (blue). **(D)** Gene Ontology (GO) analysis of genes in common for TCF4 and TCF1/HIF-1α in normoxia.

Taken together, these data prompted us to hypothesize a molecular model of the transcriptional control exerted by TCFs co-factors depending on the microenvironmental oxygen tension in GBM cells. In particular, our data suggest that low oxygen levels (HIF-1α stabilization) together with Wnt stimulation, support the formation of a HIF-1α/TCF1 molecular complex (Figure 8B) which triggers several genes involved in the neuronal differentiation processes (Figure 11A,B). On the other hand, high oxygen levels promote HIF-1α degradation and favor the expression of TCF4 isoforms endowed with an inhibitory function to be exerted against the same neuronal differentiation genes activated by the HIF-1α/TCF1 complex in hypoxia. (Figure 11C,D). Indeed, when we limited our analysis on the genes specifically bound by HIF-1α/TCF1 in hypoxia (a 40bp genomic region containing a HIF-1α/TCF1 co-localization binding site flanked by an additional 40bp region in which TCF1 and HIF-1α are positioned in sequence spanning 10bp from each other) (Figure 12A) a significant neurogenesis/neuron development-related GO was obtained (Figure 12B), thus corroborating their central role in inducing neuronal differentiation. Moreover, when we intersected this gene list with a series of genes putatively controlled by TCF4 in normoxic conditions, we generated a gene signature (91 genes) with a peculiar expression pattern across glioma samples of different grade and thus able to distinguish them from normal brain samples in an unsupervised analysis (Figure 12C) . More importantly, we intriguingly found that genes consistently down-regulated in GBM compared to normal brain (n=54) were significantly involved in neurogenesis and neuron specification processes (Figure 12D). On the contrary, up-regulated genes were mainly related to transcriptional regulatory mechanisms (Figure 12E).

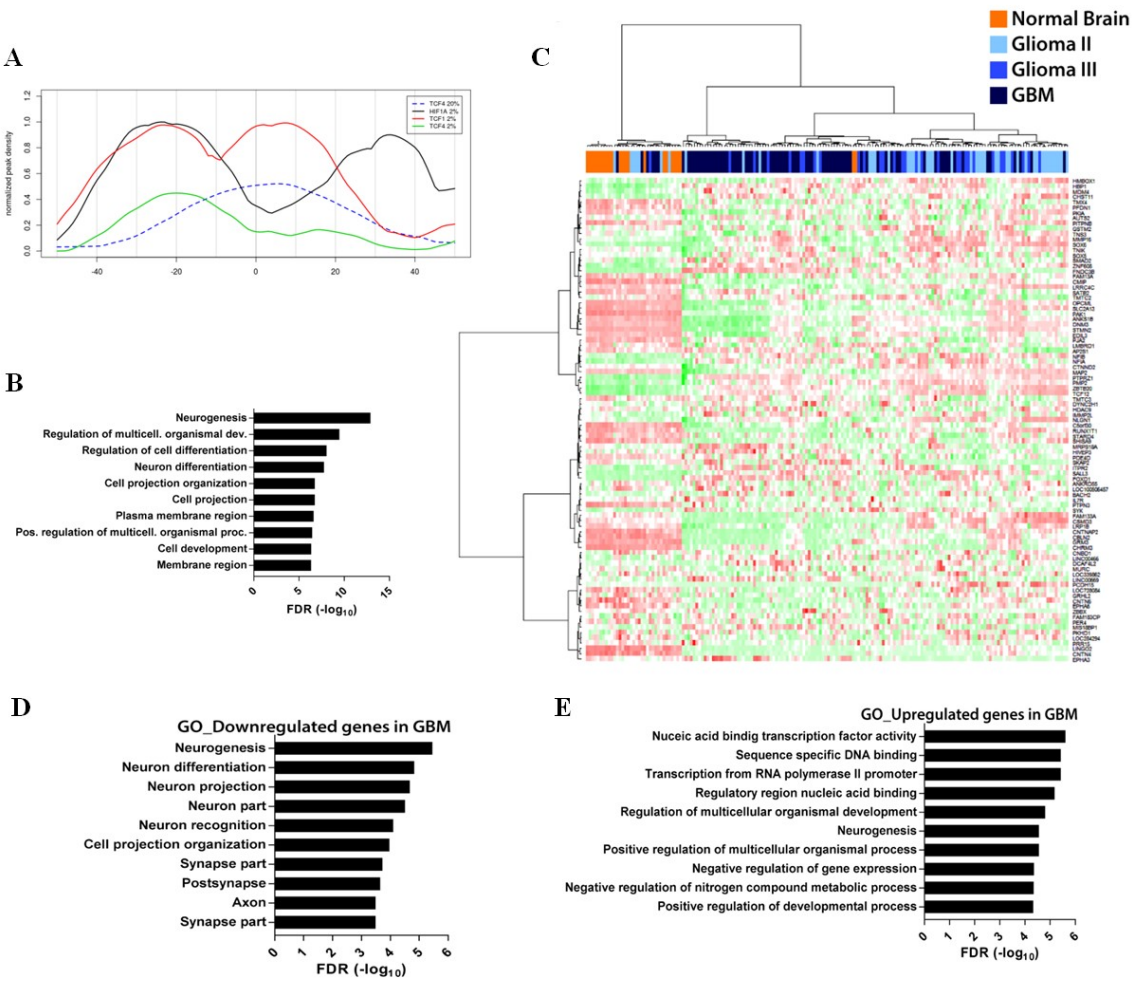


**Figure 12. Gene signature obtained from TCF1/HIF-1α co-regulated genes is diffentially expressed in gliomas of different grade. (A)** Cumulative ChIP-seq profiles of all the TFs around the peaks showing the HIF-1α /TCF1 co-localization under hypoxic conditions. (Red: TCF1 2% oxygen; Black: HIF-1α 2% oxygen; Green: TCF4 2% oxygen; Blue: TCF4 20% oxygen) **(B)** Gene Ontology (GO) analysis of genes containing a HIF-1α/TCF1 co-localization binding **(C)** Heatmap generated by un-supervised analysis of HIF-1α /TCF1 common regulated genes and bound by TCF4 in normoxia applied on the GSE4290 dataset (71) **(D)** Gene Ontology (GO) analysis of down-regulated genes derived from (C). **(E)** Gene Ontology (GO) analysis of up-regulated genes derived from (C).

In order to functionally validate these observations, we focused on the genes mainly down-regulated in GBM relative to normal samples, showing their progressive reduction of expression with increasing glioma grade (Figure 13A,B). Importantly, a series of three representative genes (*CHRM3, CMIP, LRP1B*) resulted to be dramatically up-regulated upon Wnt3a stimulation only in hypoxia, thus validating our hypothesis (Figure 13C).

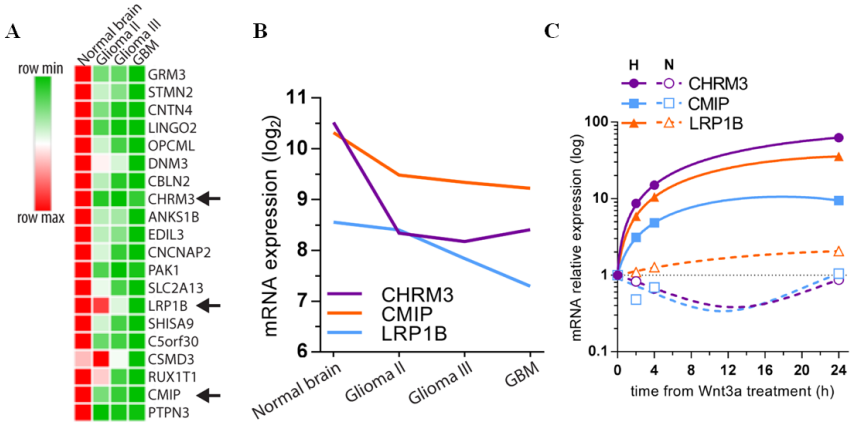


**Figure 13. TCF1/HIF-1α co-regulated genes are down-regulated in GBM, but strongly activated by hypoxic Wnt stimuli**. **(A)** Level plot representing the median fold change, normalized on normal brain, of the 20 more down regulated genes in GBM from heat map in figure 12C. **(B)** mRNA expression level of three representative genes comparing normal brain with glioma samples of different grade. **(C)** RQ-PCR analysis of the same gene as in (B) at different time points of Wnt3a treatment both in hypoxia and normoxia. 3 different GBM have been analyzed.

Our results suggest that the balance between cell differentiation and the maintenance of an undifferentiated phenotype is regulated in GBM by an oxygen-dependent mechanism which involves a fine molecular tune of TCFs co-factors and HIF-1α. (Figure 14).

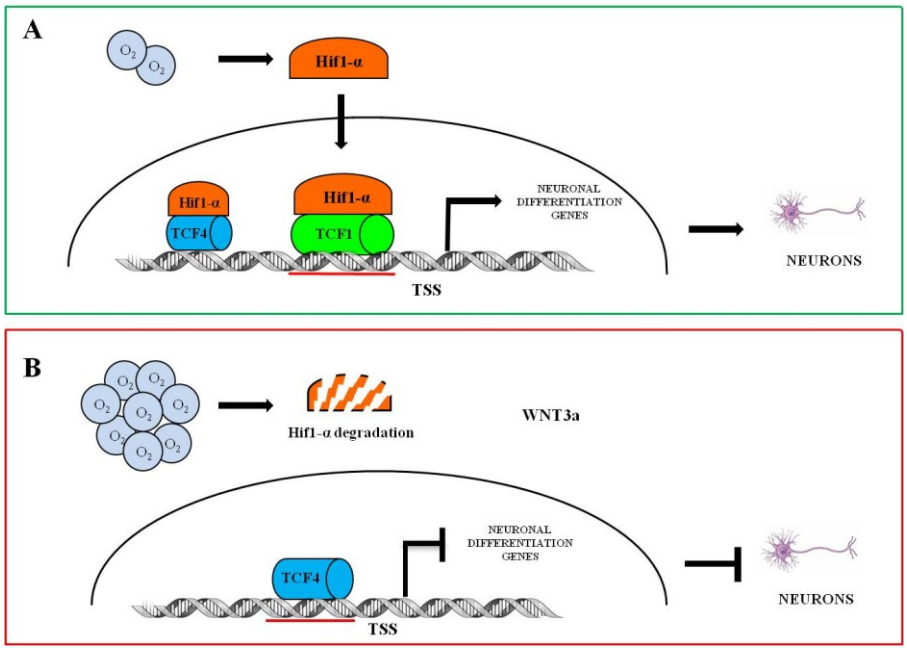


**Figura 14. Proposed model for the transcriptional regulation mediated by Wnt pathway effectors depending on microenvironmental oxygen tension. (A)** HYPOXIA: Wnt pathway activation (Wnt3a treatment) induces the switch of GBM cells toward neurons activating the expression of genes implicated in neuronal differentiation processes. Upon low oxygen levels, HIF-1α is stabilized and binds to TCF1. This complex sits on promotorial regions close to TSS and activates genes related to neuronal differentiation processes. On the other hand, TCF4 binds to HIF-1α and sits on distant regions not affecting transcription of neuronal differentiation dependent genes. **(B)** NORMOXIA: Wnt pathway activation (Wnt3a treatment) does not induce the switch of GBM cells toward neurons. Upon high oxygen levels, HIF11-α is degradated, TCF4 is free to sit on the same promotorial regions bound by the molecular complex in hypoxia and inhibit the same genes related to neuronal differentiation processes.

**TCF4 expression is increase by high oxygen levels and exerts a transcriptional inhibitory function in GBM cells**

ChIP sequencing results strongly support the hypothesis of a peculiar role of HIF-1α and TCF1 in mediating the neuronal differentiation of GBM cells observed at low oxygen levels upon Wnt stimulation. On the other hand, dissecting the molecular mechanisms underlying the behavior of GBM cells at higher oxygen levels represents a more complicated challenge. ChIP sequencing data give strength to the hypothesis that TCF4 may exert an inhibitory action against neuronal differentiation in this context. For these reasons, we needed to functionally validate these results in GBM cells. To this end, we set up knockdown experiments in which we abolished TCF1 or either TCF4 expression in primary GBM cells *in vitro*, showing that both TCF1 and TCF4 specific siRNAs were able to strongly decrease their mRNA and protein expression levels in primary GBM cells (Figure 15).

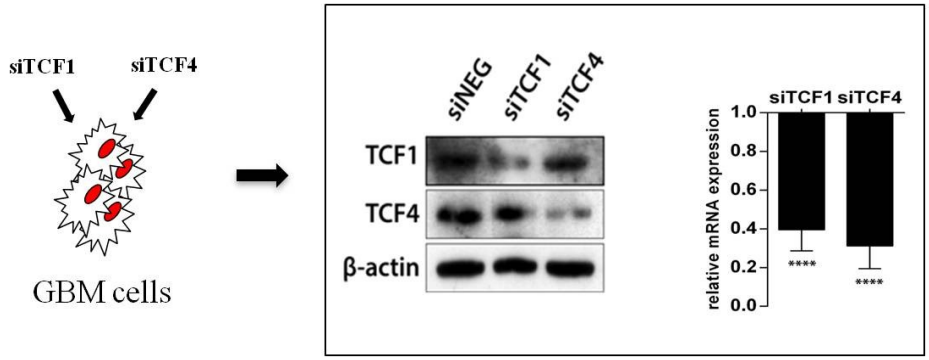


**Figura 15. TCF1 and TCF4 knockdown efficiency in GBM cells.** Representative images of protein expression and mRNA expression levels of TCF1 and TCF4 in GBM cells after treatment with specific siRNA, assessed by western blotting and RQ-PCR, respectively. siNEG was used as negative control of knockdown. β-actin was used as loading control.

Primarily, in order to verify the supposed inhibitory function of TCF4 against neuronal differentiation, we transfected TCF4-silenced GBM cells with a luciferase-based Wnt signaling reporter plasmid (BAT-lux) and assessed the impact of TCF4 levels on Wnt pathway activation. TCF4 knockdown significantly increased the responsiveness of BAT-lux upon Wnt stimulation in both hypoxia and normoxia, confirming the presence of a TCF4-mediated suppression of Wnt signaling (Figure 16A). Then, we investigated the impact of TCF4 knockdown on the phenotype of GBM cells through immunofluorescence analysis. As anticipated by previous data, TCF-silenced cells showed a potent induction of Wnt-mediated neuronal differentiation also in normoxia (Figure 16B), with a significant decrease of Nestin expression together with a strong increase of the amount of β-III-tubulin$^{+}$ cells (Figure 16C,D).

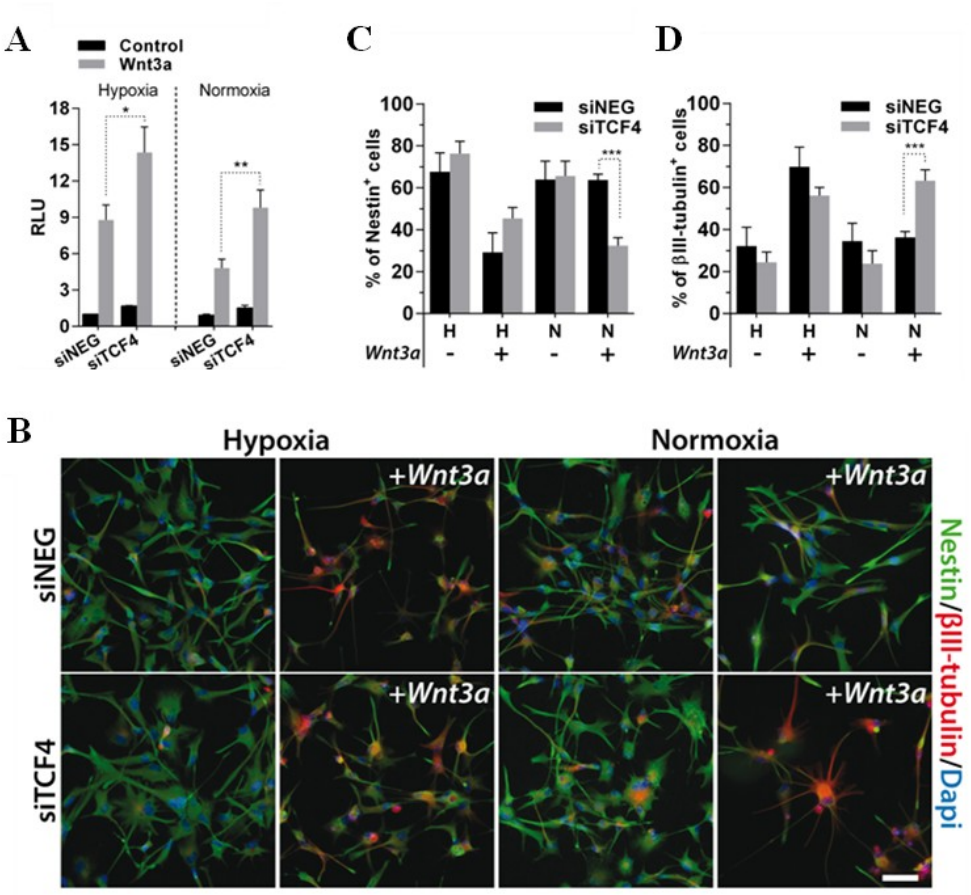


**Figure 16. TCF4 knockdown induces neuronal differentiation of GBM cells upon Wnt pathway activation at high oxygen tensions.** Primary GBM cells were initially expanded in 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N), transfected with a luciferase-based Wnt pathway reporter plasmid (BAT-lux), silenced for TCF4 and then acutely exposed(+) or not exposed(-) to Wnt3a (30ng/mL) for 24 hours **(A)** Graph bar representing the luciferase relative light units (RLU) in GBM cells. Data were normalized to the control siNEG. 3 different GBM have been analyzed. **(B)** Representative immunofluorescence images of GBM cells stained for Nestin (green)/ β-III-tubulin (red) and DAPI (blu). 3 different GBM have been analyzed and siNEG was used as control. **(C) (D)** Bar graph reporting the percentage of positive cells for Nestin and β-III-tubulin, respectively, in relation to images described in panel (B) Hypoxia(H), Normoxia(N).

We then further validated the inhibitory function of TCF4 by over-expressing a 75kDa isoform of TCF4 (TCF4E) and evaluating its ability to suppress the HIF1a/TCF1-mediated induction of neuronal differentiation in hypoxic conditions. Western blot confirmed the presence of TCF4E protein after transient plasmid transfection of GBM cells (Figure 17). Moreover, we demonstrated that TCF4E over-expression was sufficient to completely block the initiation of the neuronal differentiation process normally engaged after Wnt pathway activation in hypoxia (Figure 18A). Indeed, high levels of TCF4E brought the levels of Nestin+ and β-III-tubulin+ cells back to a control condition (hypoxic non-stimulated cells). (Figure 18B,C).

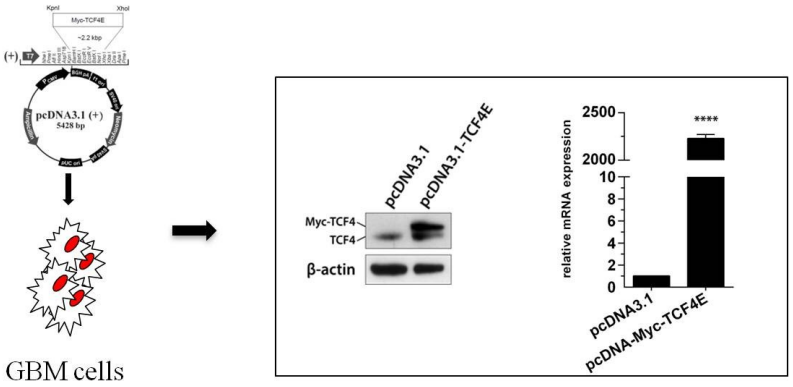


**Figure 17. Transient transfection of the inhibitory isoform TCF4E (75kDa) in GBM cells.** Representative images of protein expression and mRNA expression levels of TCF4 in GBM cells after transient transfection with the inhibitory isoform TCF4E (75kDa), assessed by western blotting and RQ-PCR, respectively. pcDNA3.1 was used as negative control of transfection. β-actin was used as loading control.

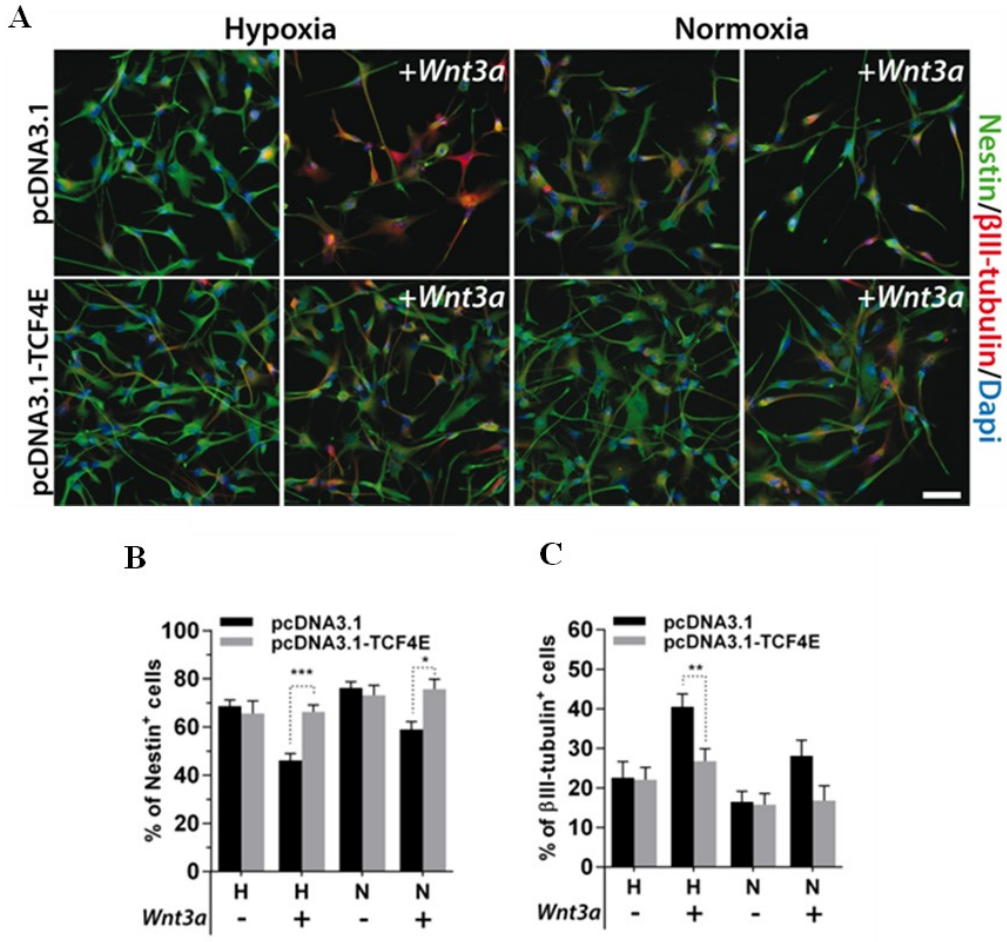


**Figure 18. TCF4E over-expression blocks the neuronal differentiation of GBM cells upon Wnt pathway activation at low oxygen levels.** Primary GBM cells were initially expanded in 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N), transfected with the inhibitory isoform TCF4E and then acutely exposed(+) or not exposed (-) to Wnt3a (30ng/mL) for 96 hours **(A)** Representative immunofluorescence images of GBM cells stained for Nestin (green)/ β-III-tubulin (red) and DAPI (blu). 3 different GBM have been analyzed and pcDNA3.1 empty vector was used as control. **(B) (C)** Bar graph reporting the percentage of positive cells for Nestin and β-III-tubulin, respectively, in relation to images described in panel (A).

Altogheter, these functional experiments confirm the hypothesis that TCF4 may exert an inhibitory function on the process of Wnt-mediated neuronal differentiation of GBM cells observed at low oxygen levels.

**HIF-1α and TCF4 display opposing functions in response to Wnt signaling activation**

So far, we demonstrated that in normoxia Wnt pathway activation is not able to switch the phenotype of GBM cells towards neurons and that this phenomenon is mainly due to a TCF4-dependent inhibition of the expression of a specific gene signature. Conversely, high levels of HIF-1α ensure a proper Wnt-dependent induction of neuronal differentiation. To deepen these opposing mechanisms involved in the control of GBM cell phenotype, we overexpressed a constitutive active form of HIF-1α (pcDNA-HIF-1αΔODD) in GBM cells cultured in normoxia, and concomitantly modulated TCF4 levels by gene silencing approaches (Figure 19).

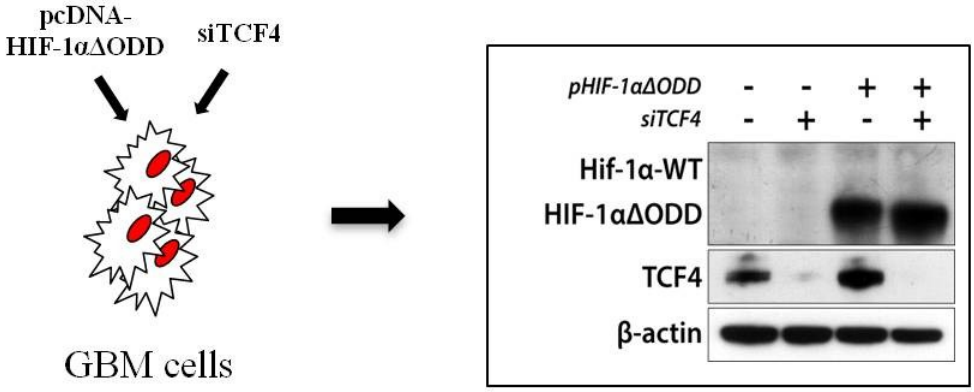


**Figure 19. Transient transfection of pcDNA-HIFΔODD as a constitutive activated form of HIF-1α together with TCF4 silencing in GBM cells.** Representative images of protein expression assessed by western blotting in GBM cells after transient transfection with pcDNA-HIF-1αΔODD (constitutive activated form of HIF-1α) and treatment with siTCF4. siNEG was used as negative control of knockdown. β-actin was used as loading control. HIFΔODD mutated protein has a lower molecular weight respect to native HIF-1α protein., due to lack of the portion which drive degradation of native protein at high oxygen levels.

In this context, we confirmed once again that TCF4 suppression re-sensitizes GBM cells to a WNT-dependent induction of differentiation even in high oxygen conditions (Figure 20A; left panels). More importantly, we show that a strong ectopic expression of HIF-1α was sufficient to partially initiate the neuronal differentiation process (Figure 20A; right panels) and that suppressing TCF4 in these cells almost 80% of GBM cells acquired a neuronal differentiated phenotype and with a dramatic loss of nestin expression (Figure 20B,C).

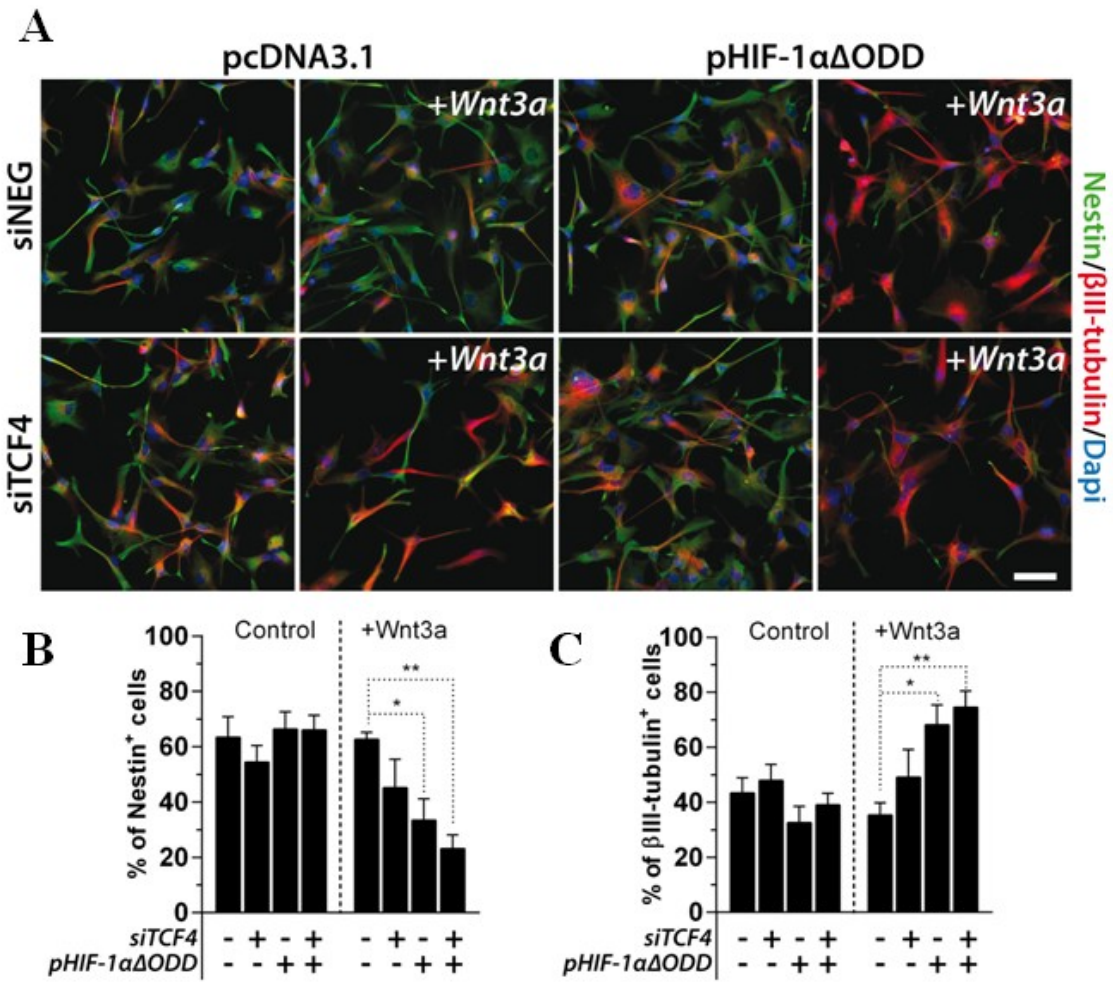


**Figure 20. TCF4 knockdown, together with HIF-1α induction, induces a strong neuronal differentiation of GBM cells at high oxygen levels.** Primary GBM cells were initially expanded in normoxia, transfected with pcDNA-HIF-1αΔODD, silenced for TCF4 and then acutely exposed(+) or not exposed(-) to Wnt3a (30ng/mL) for 96 hours **(A)** Representative immunofluorescence images of GBM cells stained for Nestin (green)/ β-III-tubulin (red) and DAPI (blu). 3 different GBM have been analyzed. pcDNA3.1 and siNEG were used as negative control of transfection and silencing, respectively. **(B) (C)** Bar graph reporting the percentage of positive cells for Nestin and β-III-tubulin, respectively, in relation to images described in panel (A).

These results depict an interesting scenario in which:

1. HIF-1α represents a potent co-factor in order that an efficient neuronal switch of GBM cells should be induced;
2. TCF4 exerts a strong inhibitory function in suppressing the Wnt-mediated pro-differentiation stimuli.

**TCF1 co-factor is the master regulator of neuronal differentiation in GBM cells**

Once characterized the role of TCF4 in governing the balance between stemness and differentiation in GBM cells we further deepened the contribute of TCF1 in triggering the activation of neuronal genes in peculiar (hypoxic) microenvironmental conditions.

In the same way as for TCF4, we confirmed that TCF1 acts as an activator of the Wnt signaling cascade by using the BAT-lux-based luciferase assay. Indeed, TCF1 knockdown significantly attenuated the Wnt-induced signaling activation in both hypoxia and normoxia (Figure 21A). Since we demonstrated that TCF1 can bind to HIF-1α in hypoxic conditions (Figure 8B) and that they are able to co-localize upon the same genomic regions (Figure 11A), we tried to understand how HIF-1α can seat to TCFs consensus sequences and the mechanism by which they trigger the activation of neuronal differentiation genes. To this end, we transfected GBM cells with the BAT-lux reporter construct (containing a 7xTCF/LEF consensus repetition), and then performed ChIP-droplet digital PCR (ChIP-ddPCR) experiments in which we immunoprecipitated HIF-1a and amplified the cross-linked plasmid sequences with specific primers flanking the Wnt-responsive promotorial region (Figure 21B). Wnt administration in hypoxia strongly promoted the binding of HIF-1α to the TCF/LEF consensus sequences compared to untreated or either normoxic cells (Figure 21C). In addition, the specific binding of HIF-1α to TCF/LEF consensus has been significantly impaired by TCF1 silencing, (Figure 21D) suggesting that HIF-1α is recruited by TCF1 in order to generate a molecular complex able to bind the TCF/LEF consensus on the genome and activate a differentiation program.

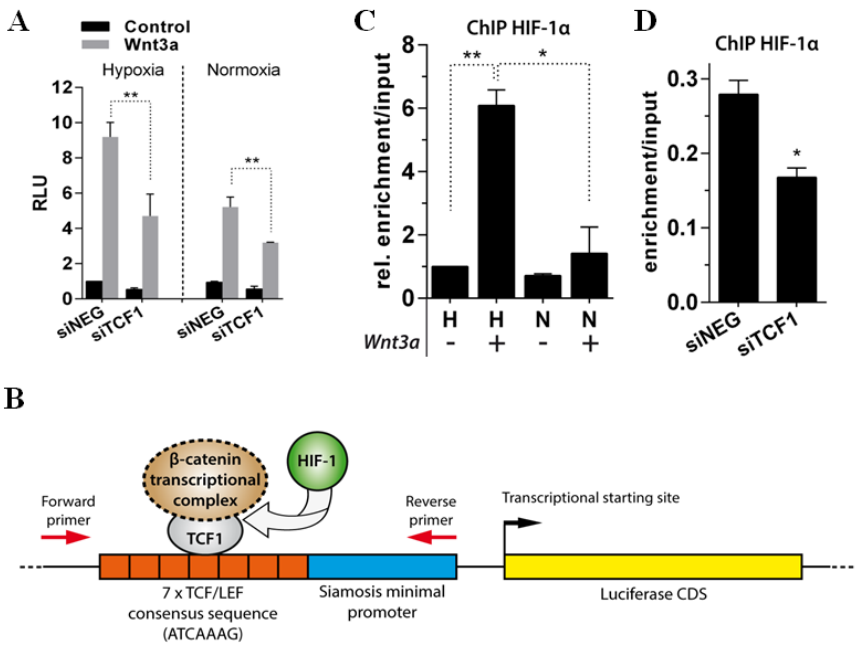


**Figure 21. TCF1 knockdown decreases Wnt signaling pathway activation and impairs HIF-1α localization onto TCF/LEF consensus sequences.** Primary GBM cells were initially expanded at 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N), transfected with a luciferase-based Wnt pathway reporter plasmid (BAT-lux), silenced for TCF1 and then acutely exposed(+) or not exposed(-) to Wnt3a (30ng/mL) for 24 hours. **(A)** Graph bar representing the luciferase relative light units (RLU) in GBM cells. Normalization of the data to the control siNEG was done. 3 different GBM have been analyzed. siNEG was used as negative control. **(B)** Graphic model of the 7X TCF/LEF binding site based BAT-lux reporter used for analyzing binding of co-factors on consensus sequence in GBM cells **(C)** Graph bar representing relative enrichment over input obtained with ddPCR from GBM cells transfected with 7X TCF/LEF binding site based BAT-lux reporter (B) and immunoprecipitated for HIF1-α. Hypoxia (H), Normoxia (N). **(D)** Graph bar representing relative enrichment over input obtained with ddPCR from GBM cells transfected with 7X TCF/LEF binding site based BAT-lux reporter, immunoprecipitated for HIF-1α and silenced for TCF1. siNEG was used as control.

Once again, we functionally validated this hypothesis by analyzing the phenotypic changes associated to TCF1 silencing in both hypoxia and in normoxia. Immunofluorescence analysis confirmed that, in hypoxia, TCF1-deficient cells were not able to differentiate toward the neuronal lineage upon hypoxic Wnt-stimulation (Figure 22A-C). Confirming previous results, at high oxygen levels, GBM cells phenotype was not affected by the modulation of TCF1 levels (Fig.22A-C).

All these data definitively sustain the fundamental role played by the microenvironment, and in particular by the oxygen tension and its sensor HIF-1α, in governing the phenotypic switch triggered by Wnt pathway activation in GBM cells. Indeed, activating Wnt pathway signaling through soluble molecules can push GBM cells to undergo phenotypic modifications, tightly regulated by the coordinated action of different transcription factors and transcriptional adaptors, which exert opposing role in balancing fundamental developmental stimuli such as stemness maintenance and neurogenic differentiation.

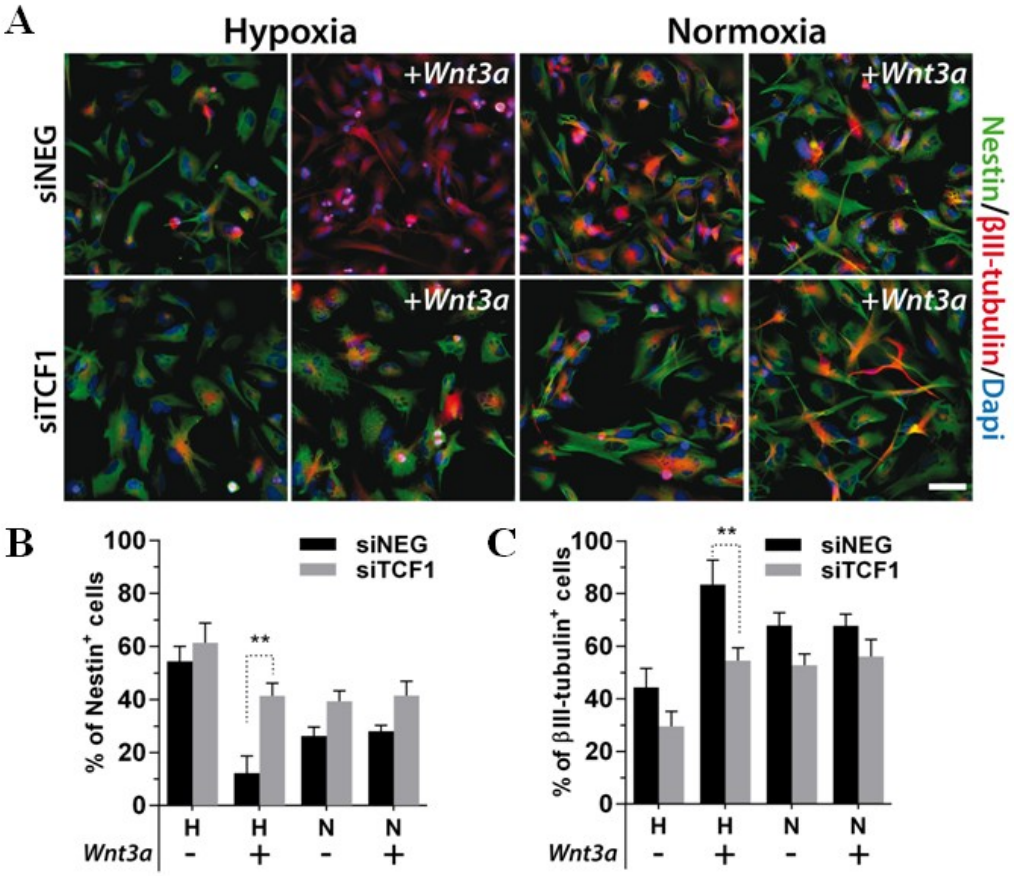


**Figure 22. TCF1 knockdown blocks neuronal differentiation of GBM cells upon Wnt pathway activation at low oxygen levels.** Primary GBM cells were initially expanded at 2% oxygen (hypoxia=H) or 20% oxygen (normoxia=N), silenced for TCF1 and then acutely exposed(+) or not exposed(-) to Wnt3a (30ng/mL) for 96 hours **(A)** Representative immunofluorescence images of GBM cells stained for Nestin (green)/ β-III-tubulin (red) and DAPI (blu). 3 different GBM have been analyzed. siNEG was used as control. **(B) (C)** Bar graph reporting the percentage of positive cells for Nestin and β-III-tubulin, respectively, in relation to images described in panel (A).

## TCF1/HIF-1α expressions positively correlate with a neuronal phenotype of GBM patients

Once demonstrated the pivotal function of TCF1 as the principal mediator of the neuronal differentiation process in hypoxic GBM cells, we sought to investigate if TCF1 expression may correlate with an increased neuronal phenotype in series human GBM tumors. We evaluated TCF1 expression by immunohistochemistry in 142 GBM samples derived from 71 different patients and correlated its levels, with the amount of HIF-1α and β-III tubulin staining in these tumors. Interestingly, showing no TCF1 protein expression also displayed very low levels of β-III tubulin and HIF-1α whereas tumors characterized by a strong expression of TCF1 demonstrated a marked staining of both β-III tubulin (Figure 23A) and HIF-1α (data not shown). Importantly, when we subdivided GBM samples based on their TCF1 expression (TCF1+ versus TCF1-) we found that the group of TCF1+ biopsies was

significantly enriched in β-III-tubulin-highly expressing samples (Figure 23B). Moreover, these TCF1+ samples positively correlated with the expression of high levels of HIF-1α (Figure 23C).

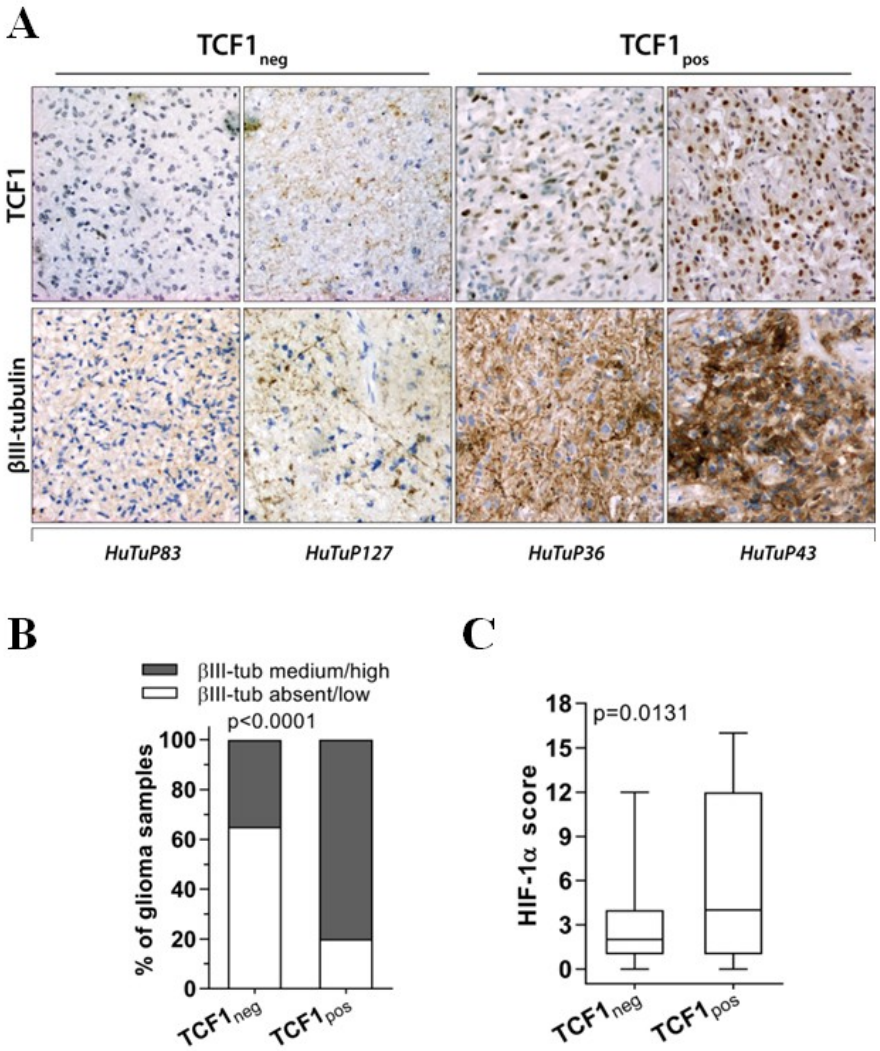


**Figure 23. TCF1 expression positively correlates with β-III tubulin and HIF-1α in a cohort of GBM patient samples. (A)** Representative immunohistochemistry images of 5µm sections of paraffin embedded GBM specimens stained for TCF1 and β-III tubulin. n= 142 brain tumors samples were analyzed. **(B)** Graph bar representing the percentage of glioma samples negative($TCF1_{neg}$) or positive($TCF1_{pos}$) for TCF1 and expressing (β-III-tub medium/high) or not (β-III tubulin absent/low) β-III-tubulin. **(C)** Graph bar representing the positivity score of HIF-1α for samples negative ($TCF1_{neg}$) or positive($TCF1_{pos}$) for TCF1.

As a confirmation, immunofluorescence experiments demonstrated that TCF1 and β-III-tubulin co-localized TCF1 expressing tumors (Figure 24A), pointing at TCF1 as a fundamental mediator of neuronal differentiation also in this less artificial context.

Finally, we tried to correlate each other all the 3 factors analyzed (TCF1, HIF-1α and β-III-tubulin) by comparing TCF1 and HIF-1α scores with β-III tubulin expression levels. Integration of IHC data disclosed that TCF1, despite being heterogeneously expressed in GBM biopsies (from score 0 to score 15), it significantly and positively correlated to both β-III tubulin expression and HIF-1α score as shown by the principal components analysis (PCA) in Figure 24 C.

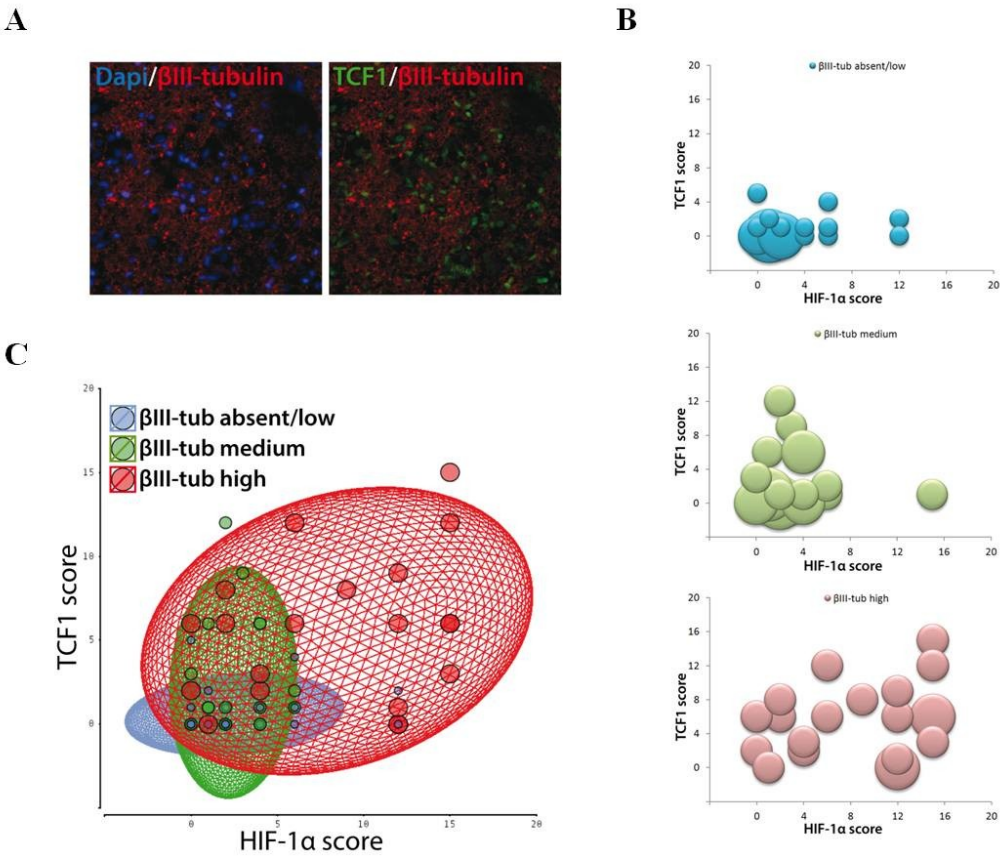


**Figure 24. TCF1/HIF-1α expressions positively correlates with β-III tubulin in a cohort of GBM patient samples. (A)** Representative immunofluorescence images of GBM patients specimens stained for β-III tubulin(red), TCF1(green) and DAPI(blu). **(B)** Graphs representing the correlation between TCF1 and HIF-1α scores in relation with β-III tubulin expression. Blu graph: samples with high HIF-1α score and low TCF1 score are correlated with a low/absent β-III tubulin expression. Green graph: samples with low HIF-1α score and high TCF1 score are correlated with a medium β-III tubulin expression. Red graph: samples with high HIF-1α score and high TCF1 score are correlated with a high β-III tubulin expression **(C)** Tridimensional model representing correlation between TCF1, HIF-1α and β-III tubulin expression in the GBM patient cohort

In particular, we faced three different scenarios:

1. samples showing absent/low expression of β-III tubulin were characterized by relatively high HIF-1α scores but low TCF1 (Figure 24B upper panel);
2. intermediate β-III tubulin expression correlated with absent/low HIF-1α but relatively high TCF1 scores (Figure 24B medium panel);
3. neuronal differentiated samples expressing high levels of β-III tubulin displayed a synchronized high positivity for both HIF-1α TCF1 (Figure 24B lower panel).

These data sustain the molecular model we characterized in vitro in which HIF-1α and TCF1 should act together and in synchrony in order to induce a neuronal phenotypic switch of GBM cells.

# DISCUSSION

GBM represents a dramatic challenge for clinicians and researchers and, despite lots of efforts in trying to understand the mechanisms underlying GBM aggressiveness, a more complete knowledge of its biology needs to be achieved. GBM is a highly proliferative and invasive tumor with a significant tendency to infiltrate surrounding normal brain parenchyma, thus frustrating the complete resection of GBM tumor mass (2). Moreover, radiotherapy and chemotherapy can often be considered only as palliative treatments, making the clinical management of these tumors extremely difficult.

GBM aggressiveness has been demonstrated to be sustained by a subpopulation of cells endowed with stem-like characteristics, cancer stem cells (CSCs) which plays a pivotal role also in treatment resistance and tumor recurrence (11). Microenvironmental conditions are peculiar to maintain the undifferentiated phenotype of CSCs and, in particular, oxygen tension is crucial to direct the fate of these cells. Indeed, it has been previously demonstrated by our group that GBM grows as an organized entity that we described as a three-layer concentric model in which the central hypoxic niche hosts CSCs, whereas the more differentiated cells reside in the external normoxic layers (9). The organized growth of GBM tumor mass is regulated by several signals deriving by cancer cells themselves and from the microenvironment. In this context, it has been previously demonstrated as hypoxia is fundamental to sustain GBM cells proliferation, particularly preserving the stem population in the perivascular and hypoxic niches, by activating specific transcription factors such as Notch1 which leads to NFAT activation and cell proliferation and tumor growth (26) or by up-regulating other transcription factors like Notch and Oct4, which control self-renewal and multipotency of stem cells. Moreover, we previously demonstrated that hypoxia cooperates with Wnt pathway to control GBM cell phenotype in terms of induction of neuronal differentiation. In particular, we underlined that Wnt pathway activation is able to induce the neuronal differentiation of GBM cells only in hypoxia, condition that maintain high levels of the hypoxia inducible factor HIF-1α. This process strictly depends on oxygen tension and is efficient in attenuating GBM cells aggressiveness (60).

In this study, we investigated a tightly regulated mechanism by which canonical Wnt pathway and hypoxia cooperate to induce a reminiscent neuronal differentiation of GBM cells. In particular, we focused on dissecting the role of β-catenin/TCF1/TCF4 transcriptional complex and its interaction with the hypoxia sensitive transcription factor HIF-1α in deciding GBM cell fate depending on microenvironmental oxygen tension. Indeed, recent literature point out

the importance of complex transcriptional machineries in determining cellular fate both in normal tissues and cancer (72–74). Of note, they exert their intracellular function by modulating gene expression profile, which in turn determined the identity of cells (75,76). Moreover, the main regulator of these machineries is the microenvironment where cells are located, from which cells receive signals through the plasmatic membrane and then transduce them to the nucleus (77,78). In GBM, the role of the canonical Wnt pathway β-catenin/TCF1/TCF4 complex has been extensively debated (60–66) but the specific interactions of different co-factors and how they respond to external microenvironmental stimuli has never been fully elucidated. Here, we demonstrate that oxygen tension has a strong impact in GBM cell response to Wnt activation. In particular, we found that 2% oxygen determine a pro-differentiative effect upon Wnt signaling activation. Conversely, 20% oxygen gives to Wnt pathway a pro-oncogenic function as emerged by the neurosphere forming and the limiting dilution assays (Figure. 6). Our attention was then attracted by the transcription factor TCF4, that, surprisingly, not only resulted to be up regulated in normoxia, compared to 2% oxygen, but also was not able to bind nor β-catenin neither HIF-1α (79). This intriguing data, prompted us to deepen the role of TCF4 in GBM biology and to hypothesize that it can exert an inhibitory function on gene transcription. It has previously been described in colorectal cancer that the higher molecular weight isoforms of TCF4 (i.e. TCF4E) contain specific DNA binding domain with an inhibitory function (70). Of note, in GBM cells are expressed almost exclusively the high molecular weight isoforms (Figure 7). Moreover, when we looked at specific genes bound by these TFs we clearly demonstrated that the HIF-1α/TCF1 complex bound to genes with a gene ontology enriched in neurogenesis and neurons maturation processes at 2% oxygen and that TCF4 in normoxia bound to the same genes, without exerting any neuronal differentiation effect on GBM cells. Indeed, these genes were significantly up-regulated upon Wnt3a treatment in hypoxia while in normoxia their expression remained totally comparable to untreated cells. Collectively these data corroborate the hypothesis of a TCF4 inhibitory function, finally confirmed by specific gene silencing experiments by which we demonstrated that TCF4 inhibition significantly activated the Wnt pathway reporter signal and allowed a prompt neuronal differentiation of GBM cells also in normoxia (Figure 16).

What is peculiar in our study is the controversial function of the hypoxic sensor HIF-1α. Historically, HIF-1α has been considered as an oncogene, especially in solid tumors which are particularly characterized by hypoxic and anoxic areas (80–83) Previous studies from our group focused on dissecting the role of hypoxia and HIF-1α in maintaining GBM cells

stemness and proliferation (84) demonstrating the relevance of the hypoxic microenvironment in supporting the survival of GBM stem cells and promoting their resistance to therapy. Physiologically, HIF-1α has a fundamental role in the neuronal differentiation process of the developing brain (58,85,86) and, notably, in neuron regeneration and axon reconstruction after brain injuries (87). For these reasons, we wondered if the same mechanism could be still active in a cancer setting. Our data disclosed that HIF-1α is a crucial player of the neuronal differentiation process by strengthening the TCF1-driven Wnt intracellular cascade. Moreover, by ChIP-ddPCR experiments we discovered that HIF-1α binds to TCF1, which is able to re-direct HIF-1α to TCFs specific sequences and suggesting that HIF-1α functions as a helper for TCF1-mediated transcription. Indeed, by functional experiments we demonstrated that the fundamental promoter of the GBM cells neuronal differentiation is TCF1 whose action is enhanced by HIF-1α in hypoxia and inhibited by TCF4 at 20% oxygen.

In the last decade, lots of efforts have been made in order to target CSCs and, in particular, their resistance to therapies and their ability to recapitulate the entire tumor mass upon surgical resection. Thus, pro-differentiating therapy has been proposed as an effective approach in attenuating CSC aggressiveness and in reducing chemo- and radioresistance in several tumors, comprising GBM (3). Knockdown of CD44 negatively impact on breast cancer stem cells, resulting in a loss of stem-like characteristics and an increase in susceptibility to chemotherapy and radiation (88) and all-trans-retinoic acid (ATRA) leads to complete remission in patients with acute promyelocytic leukemia (APL) by inducing terminal cell differentiation by disrupting the promyelocytic leukemia/retinoic acid receptor α (PML/RARα) fusion protein that arrests the maturation of myeloid cells at the promyelocytic stage (89). In glioma, retinoids (ATRA or 13-CRA) induced astrocytic differentiation of GBM cells with a down regulation of the telomerase activity, an increased sensitivity to TXL and enhance apoptosis(90). Moreover, i*n vitro* differentiation of stem-like glioma cells with ATRA containing medium induces therapy-sensitizing effects, impair the secretion of angiogenic cytokines, and disrupts motility (91).

In our study, we described a mechanism of GBM cell neuronal differentiation induced by Wnt pathway activation and characterized the principal mediators of this process by depicting a potential therapeutic strategy to be specifically exploited for reducing GBM cells aggressiveness. Combining our findings with chemo- and radiotherapy can represent a powerful improvement in the management of GBM treatment.

# CONCLUSIONS

In this study we finely characterized the role of Wnt pathway transcriptional effectors in inducing GBM phenotype depending on the microenvironmental conditions. Indeed, we definitely uncovered the role of TCF1 in pushing GBM cells to acquire a neuronal phenotype through the formation of a transcriptional complex with HIF-1α which is recruited above the TCF/LEF sequence to trigger activation of genes implicated in neuronal differentiation processes (Figure 25A). Moreover, TCF1 is essential to preserve the ability of the transcriptionally active complex in modulating GBM cell phenotype in terms of neuronal commitment induction. We demonstrated that the neuronal differentiation process is induced at the transcriptional level and impact on the phenotype of GBM cells, strictly depending on the microenvironment, and thus confirming the hypothesis that the presence of HIF-1α strengthen the action of TCF1 in achieving the neuronal switch of GBM cells. On the other hand, high oxygen levels favor the action of an inhibitory isoform of TCF4, TCF4E, whose expression is more abundant in GBM cells cultured in normoxia. In this conditions, genes involved in the neuronal differentiation processes are inhibited by TCF4 and the Wnt pathway-related switch toward neurons that we previously observed at low oxygen levels, is not induced (Figure 25B).

In conclusion, we unveil a tightly regulated mechanism by which the TCF1/HIF-1α transcriptional complex is able to induce a reminiscent neuronal differentiation of GSCs, which might represent a future potential strategy to therapeutically weaken their aggressiveness.

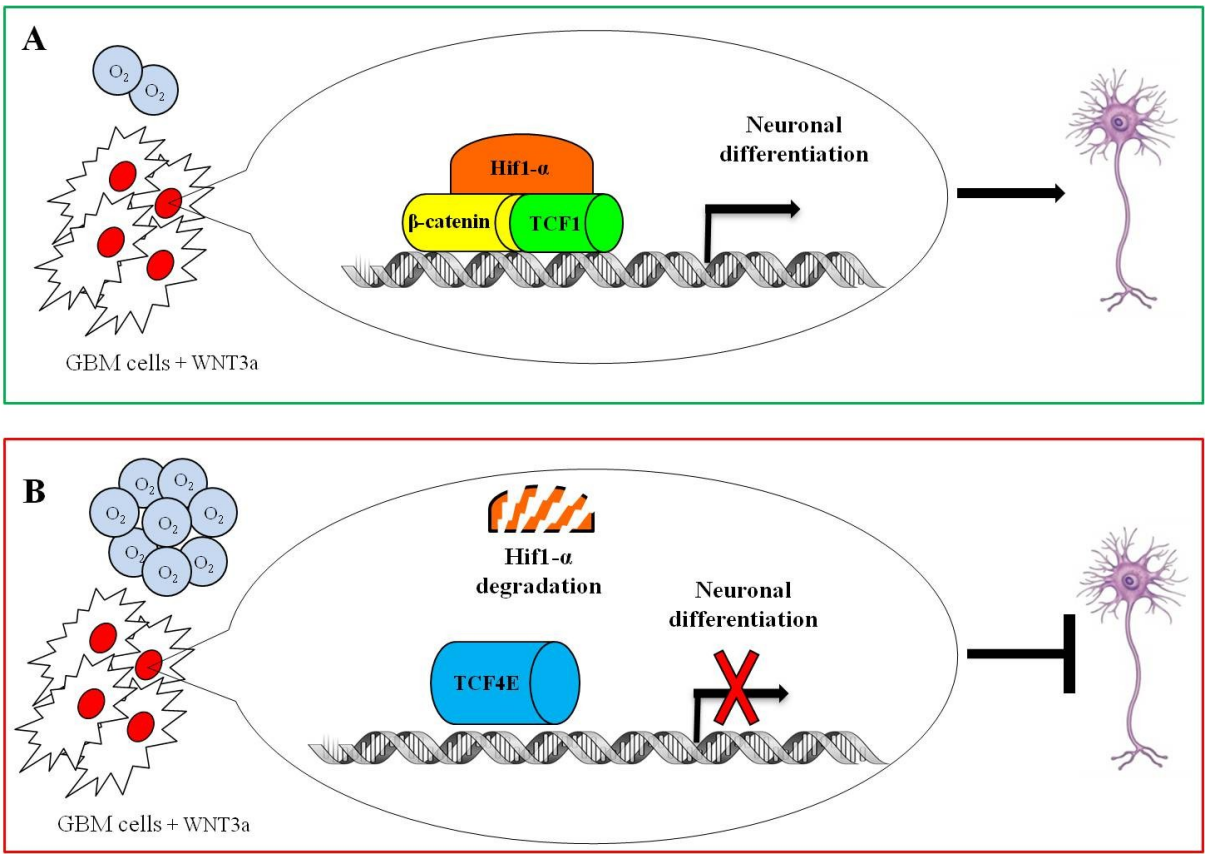


**Figure 25. Wnt pathway transcriptional effectors orchestrate the phenotypic balance of GBM cells through an oxygen-dependent mechanism. (A)** At low oxygen levels, Wnt pathway activation induces the formation of a molecular complex formed by TCF1, HIF-1α and β-catenin which triggers the expression of genes correlated with neuronal differentiation processes and induces a phenotypic change in GBM cells by inducing a switch toward neurons. **(B)** At high oxygen levels, the formation of the molecular complex is impaired upon Wnt pathway activation and, subsequently to HIF-1α , TCF4 can inhibit the activation of genes related to neuronal differentiation processes. GBM cells does not encounter a phenotypic switch toward neuron and maintain their undifferentiated phenotype.

# MATERIALS AND METHODS

**Neurosurgical sample collection, isolation and gas-controlled expansion of GBM cells**

Written informed consent for the donation of adult tumor brain tissues was obtained from patients before tissue acquisition under the auspices of the protocol for the acquisition of human brain tissues obtained from the Ethical Committee of the Padova University Hospital. All tissues were acquired following the tenets of the Declaration of Helsinki. Patients from which we derived GBM primary cultures are listed in supplementary table 1.

Primary GBM cells were isolated and maintained in culture as described previously (92). Briefly, tumor biopsies were subjected to mechanical dissociation and the resulting cell suspension was cultured on fibronectin-coated dishes in DMEM/F12 medium supplemented with BIT9500 (Stemcell Technologies Inc., Vancouver, Canada), 20ng/ml basic Fibroblast Growth Factor (bFGF; Sigma-Aldrich S.r.l., Milan, Italy) and 20ng/ml Epidermal Growth Factor (EGF; R&D Systems, Minneapolis, MN). GBM cells were maintained in an atmosphere of 2% oxygen, 5% carbon dioxide and balanced nitrogen in a Ruskinn C300 system for hypoxic cell culture (Ruskinn Technology Ltd, Bridgend, UK). Cells were not cultured for more than 8 passages *in vitro* in order to avoid long term culture related effects. To activate Wnt pathway signaling GBM cells were treated with recombinant protein Wnt3a (30ng/mL) for a time depending on experimental settings.

**Neurosphere forming assay**

Self-renewal capacity of primary GBM cells was assessed as follows: the first day $2 \times 10^5$ cells per well were seeded onto six-well plates with fibronectin-coating. At day one, cells were treated with Wnt3a (30ng/ml) under hypoxic or normoxic culture conditions (no treated cells were used as control). At day 6, cells were trypsinized and seeded onto uncoated 6-well plates at $10^4$ cells per well. After a week, neurospheres were counted and dissociated with MgCl 1M (1:1000), DNase (2µL/mL) and trypsine (TrypLE™ Express Enzyme, ThermoFischerScientific). After neurosphere disgregation, $10^4$ cells were reseeded onto uncoated 6-well plates for secondary neurosphere formation. Others two identical replating were carried out.

**Limiting dilution assay (LDA)**

To assess the GBM cancer initiating cells frequency we set up limiting dilution assays as follow: the first day we seeded GBM cells in 6 well coated plates under hypoxic and normoxic conditions. Then, we treated cells with Wnt3a (30 ng/mL) for 3 days and, at day 4, we replated serial dilutions of cells ranging from 0 to 500 cells per well in 96 well uncoated plates. The cells were cultured under low or high oxygen levels for 1 week and then were counted the wells in which there was no significant spheres formation. Percentage of wells with no spheres were plotted against the number of cells per well seeded into p96 plates.

**Western blot**

Equal amounts of proteins (20µg) extracted from primary GBM cells were resolved using a SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) Immobilon-p membrane (Merk-Millipore, Darmstadt, Germany). Membranes were blocked with I-block™ (Thermo Fisher Scientific, Waltham, MA) for at least 1 hour at room temperature and then were incubated overnight at 4°C under constant shaking with the primary antibodies listed in supplementary table 2. Membranes were next incubated with peroxidase-labeled secondary antibodies for 1 hour. All membranes were visualized using ECL Select (GE Healthcare, Catania, Italy) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

**Immunoprecipitation**

For immunoprecipitation experiments GBM cells were cultured in 10 mm dishes and treated with Wnt3a (30ng/ml) after 24 hours in hypoxic or normoxic conditions. No treated cells were used as control. After 48 hours from treatment, cells were solubilized in lysis buffer ($MgCl_2$ 1M, KCl 1M, EDTA 0,5M, TRIS-HCl pH 7.5, Chapso 1%). A small quantity of cell lysates prior to binding with antibodies (INPUT) was collected. Then, an equal amount of each protein lysate was incubated with polyclonal antibodies, listed in supplementary table 2, for 2 hours at 4°C, followed by incubation with 100 µl of protein A/G-Microbeads (µMACS$^{TM}$ MultiMACS Protein A/G kit, Miltenyi Biotec) overnight at 4°C. IgG was used as negative control. The immune complexes were analyzed by Western blot analyses with antibodies listed in supplementary table 2.

**Immunofluorescence**

GBM cells were cultured on 4-well chamber slides (BD Bioscience, San Jose, CA), treated depending on the experimental plan, fixed in cold 4% formaldehyde and stored at +4°C prior to analysis. Primary antibody used for the staining was listed in supplementary table 2. After incubation, cells were washed and incubated with species-specific secondary antibodies conjugated to Alexa dyes (Thermo Fisher Scientific, Waltham, MA). Cells were counterstained with DAPI (1:10000; Sigma-Aldrich, Milan, Italy) to evidence cell nuclei and measure total cell number. Staining was visualized by epifluorescence with a ViCo microscope (Vico, Nikon, Melville, NY).

**Chip Sequencing (ChIP-SEQ)**

For ChIP-seq experiments we immunoprecipitated HIF-1α, TCF1 and TCF4 in GBM cells treated with Wnt3a (30ng/mL) under hypoxic or normoxic conditions. ChIP-seq experiments were performed as previously described(93) with slight modifications. Primary GBM cells were crosslinked and washed. Then, cells were lysed in Lysis buffer 1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 1 × protease inhibitors) and, after centrifugation, resuspended in Lysis buffer 2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1× protease inhibitors). Cells were pelleted and resuspended in Sonication buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.05% *N*-lauroylsarcosine, 1× protease Inhibitors) and sonicated in Bioruptor sonicator. Cell lysates were added to protein G beads (Invitrogen 100-04D), previously resuspended in 250 µl of PBS, 0.5% BSA and 5 µg of each antibody, and incubated at 4°C overnight. A small quantity of cell lysates prior to addition to the beads was kept as input. Crosslinking was reversed and the DNA of cells was extracted by Phenol/Chloroform extraction. For sequence preparation, all samples were prepared with the Illumina/Solexa Genomic DNA kit (Illumina- IP-102-1001) according to the manufacturer's instructions.

**ChIP-SEQ data analysis**

ChIP-Seq datasets were aligned using Bowtie2 (version 2.1.0) (94) to the human genome (build hg19) with parameters -k 1 -m 1 -n 2. We used the MACS2 (ver. 2.0.9) (95) to find peaks and identifing regions of signal enrichment over the input DNA control, with the parameters --no-model --keep-dup=1, 'mfold' was set to 5 and 10000, 'q-value' to 0.05 and 'p-

value' to 0.0005. The heatmaps of Figure 10A were generated using the 'heatmap.plus' function of the 'heatmap.plus.package' of R statistical software.

**Gene expression profiling and gene ontology**

The unsupervised hierarchical clustering analysis and the heatmap in Figure 12C was generated using the gene list generated intersecting HIF-1α /TCF1 co localizing genes with the TCF4 gene list in normoxia and apply it to a cohort of glioma and normal brain samples (GSE4290 dataset) (71). Specifically, the clustering analysis was generated with R software using Euclidean distance as a distance measure between genes and Ward.D method.

The level plot in Figure 13A was generated by mediating the expression level of the 20 more down-regulated genes in GBM compare to normal brain in each group (normal brain, Glioma II, Glioma III and GBM) of considered data set (GSE4290). Specifically, the level plot was generated with Morpheus software (https://software.broadinstitute.org/morpheus/). Gene ontology analysis were performed by applying specific gene lists to GSEA software (http://software.broadinstitute.org/gsea/msigdb/annotate.jsp) with C5 compute overlaps and filtering the genesets for FDR q-value below 0.05.

**Reverse transcription and real-time PCR**

RNA was extracted from GBM cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions and 1-2µg of total RNA reverse-transcribed using SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA). Quantitative RT-PCR reactions were run in triplicate using Platinum SYBR Green Q-PCR Super Mix (Thermo Fisher Scientific, Waltham, MA). Fluorescent emission was recorded in real-time (Sequence Detection System 7900HT, Applied Biosystems, Foster City, CA). The specificity of primers was confirmed for every PCR run by dissociation curve analysis. Primers used are listed in supplementary table 3 and their specificity was confirmed with the software Human BLAT Search (http://genome.ucsc.edu). Relative RNA quantities were normalized to *GUSB* according to the ΔΔCt Method.

**Transfection of primary GBM cells**

To achieve a suitable gene silencing, GBM cells were transfected with 200 pmol of a small interfering RNAs (siRNAs) against *TCF7* and *TCF7L2* as well as with a non targeting siRNA (siNEG) using the Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. *Silencer® Select Custom Designed siRNAs*

have been produced by AMBION (Life Technologies LTD, Waltham, MA) and sequences are listed in supplementary table 4. Transfected cells were then cultured for 24-48 hours and the analysis of silencing specificity was achieved by WB.

**Luciferase reporter assay**

GBM cells were transfected using a protocol for transient transfection of adherent cells using TransIT®-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI) with BAT-luciferase reporter construct (BAT-lux) (Addgene plasmid # 20890) which consists of seven TCF/LEF-binding sites upstream of a 0.13-kb fragment containing the minimal promoter–TATA box of the gene *siamois* (96) driving the expression of Firefly luciferase reporter gene. To overexpress TCF4 inhibitory isoforms we used the plasmid pcDNA3.1-TCF4E (Addgene plasmid # 32738). Luciferase experiments on GBM cells were set as follows: at day 1 cells were plated at $2x10^5$ per well, at day 2 cells were transfected with an opportune quantity of pcDNA3.1-TCF4E or pcDNA3.1 according to manufacturer's instructions. At day 3, cells were transfected with BAT-luciferase reporter construct together with pMAX-GFP plasmid to control transfection efficacy and normalize luciferase detection. At day 4 cells were treated with Wnt3a (30ng/mL) and no treated cells were used as control. At day 5, cells were solubilized in passive lysis buffer (PLB, Promega) and luciferase activity was analysed. The same experimental set was used for luciferase reporter assays after *TCF7* and *TCF7L2* silencing (day2). Values, expressed in relative light units (RLUs), were normalized to the values obtained from cells.

**Chromatin immunoprecipitation (ChIP) and droplet digital PCR (ddPCR)**

To investigate the molecular binding of transcription factors to specific DNA sequences, we set up chromatin immunoprecipitations experiments as follows: first, we transfected GBM cells cultured under hypoxic or normoxic conditions with the 7X TCF/LEF binding site based BAT-lux reporter. The day after transfection, cells were treated with Wnt3a to activate the signaling pathway (no treated cells are used for negative control) and, in another experimental setting, silenced for TCF1 (siNEG was used as negative control). The day after, cells were fixed with formaldehyde, lysated in an appropriate lysis buffer and sonicated in a water bath sonicator. Then, immunoprecipitation was performed using HIF-1α antibody (IgG antibody will be used as isotype control). Purification of plasmid-DNA was performed by phenol/chloroform extraction. We also obtained an INPUT sample for subsequent data normalization. To analyze the DNA sequences bound to HIF-1α we set up Digital-PCR

(ddPCR) experiments using EvaGreen Digital PCR Supermix (Bio-Rad) and a pair of primers (listed in supplementary table 3) able to amplify the 7xTCF binding sites DNA sequence. Then, each sample was emulsionated and the obtained droplets were processed in a standard thermocycler. After the amplification, the droplets generated for each sample (INPUT, IgG as negative control, ChIP samples) were processed in the QX200 Droplet Digital PCR (ddPCR™) System. DNA quantity of each ChIP sample was measured as DNA copies/μL and was normalized on each experimental INPUT, obtaining the enrichment over input ratio plotted on the bar graphs.

**Immunohistochemistry**

TCF1, HIF-1α and β-III-tubulin immunohistochemistry was conducted on 5μm sections of paraffin embedded GBM specimens with standard procedures. Briefly, sections were re-hydrated and then antigen retrieval was performed by incubation with citrate buffer 0.01M pH6 at 95°C. After saturation with the more appropriate normal serum, slides were incubated with the primary antibody listed in supplementary table 2. After incubation, sections were washed and incubated with species-specific biotin-conjugated secondary antibodies (Vector Laboratories Inc. Burlingame, CA). TCF1, HIF-1α and β-III-tubulin expression was revealed by using the Dako Liquid DAB$^+$ Substrate Chromogen System (Dako, Glostrup, Denmark) according to manufacturer's guidelines. Tissues were counterstained with Meyer's Hematoxylin and images acquired with a Zeiss Imager M1 microscope (Carl Zeiss, Oberkochen, Germany). The specificity of each staining procedure was confirmed by replacing the primary antibodies with an Isotype control.

The expression level of TCF1 and HIF-1α was scored using a combined method accounting for both the staining intensity and the percentage of positive stained cells. The resulting combined score was calculated as the multiplication of the score accounting for the percentage of TCF1$^+$ cell nuclei or HIF-1α$^+$ cell nuclei (0-6) and the intensity score (0-3). TCF1, HIF-1α and β-III-tubulin stained slides were independently evaluated by two different pathologists.

**Statistical analyses**

Graphs and associated statistical analyses were generated using Graph Pad Prism 6.07 (GraphPad, La Jolla, CA). All data in bar graphs are presented as mean ± standard error of the mean (S.E.M.). Statistical significance was measured by one-way ANOVA with Newman–Keuls multiple comparison post test (for more than two comparisons) and paired t-test

(comparison of two groups); *p<0.05, **p<0.01, ***p<0.001. For all graphs, asterisks over brackets indicate a significant difference with another variable as indicated and asterisks over bars indicate a significant difference with the control group. Integration of IHC data has been obtained by applying Principal Component Analysis to IHC scores.

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# SUPPLEMENTARY TABLES

Supplementary table 1**: GBM patient-derived cells used in this study**

| Patient | Diagnosis | Age | Sex |
|---|---|---|---|
| HuTuP01 | Glioblastoma | 64 | Male |
| HuTuP10 | Glioblastoma | 75 | Female |
| HuTuP13 | Glioblastoma | 67 | Male |
| HuTuP36 | Glioblastoma | 49 | Female |
| HuTuP43 | Glioblastoma | 59 | Male |
| HuTuP47 | Glioblastoma | 81 | Female |
| HuTuP53 | Glioblastoma | 70 | Male |
| HutuP82 | Glioblastoma | 50 | Male |
| HuTu83 | Glioblastoma | 55 | Male |
| HuTu174 | Glioblastoma | 69 | Male |
| HuTu175 | Glioblastoma | 57 | Female |
| HuTu187 | Glioblastoma | 56 | Male |
| HuTu197 | Glioblastoma | 48 | Male |
| Brain tumours were acquired directly from surgery, dissociated and cells were expanded in culture. First neuropathological review of the tumour tissue was followed by a second independent review. Patient ages listed in years (y). | | | |

Supplementary table 2**: Antibodies used in this study**

| Antibody | MW (kDa) | Manufacturer | Host | Application |
|---|---|---|---|---|
| TCF1 | 48,50 | Cell Signaling | Rabbit | WB |
| TCF1 | / | Sigma-Aldrich | Mouse | IHC/IF |
| TCF4 | 58,79 | Cell Signaling | Rabbit | WB |
| TCF4 | / | Sigma-Aldrich | Mouse | IHC/IF |
| HIF-1α | 120 | BD Biosciences | Mouse | WB |
| HIF-1α | / | Abcam | Rabbit | IP/ChIP |
| HIF-1α | / | Sigma-Aldrich | Rabbit | IHC |
| β-actin | 45 | Sigma-Aldrich | Mouse | WB |
| β-catenin | 85 | Abcam | Rabbit | IP/WB |
| Nestin | / | Millipore | Rabbit | IF |
| β-III tubulin | / | Covance | Mouse | IF/IHC |

Supplementary table 3: **Sequence of primers used in this study**

| ***Gene*** | ***Sequence (5'-3')*** | ***Amplicon (bp)*** |
|---|---|---|
| BAT-lux plasmid forward | CGCGGGAATTCGATTAAGGAC | 291 |
| BAT-lux plasmid reverse | AACAGGGGACAAAGGGTGTG | |
| *CHRM3* forward | GCCGGGATCATCATGACCGT | 175 |
| *CHRM3* reverse | TGCATCGGAGGGGCTGTGTAT | |
| *TCF7* forward | CCTAGCAAGGAGGAGCGAGA | 143 |
| *TCF7* reverse | CCGGTTGGCAAACCAGTTGTAG | |
| *TCF7L2* forward | TTTAAGGGGCCACCGTATCC | 119 |
| *TCF7L2* reverse | TGCCGGACTGAAAATGGAG | |
| *CMIP* forward | GGGGTCTCGCACAGGTTCAG | 236 |
| *CMIP* reverse | GGGGTCTCGCACAGGTTCAG | |
| *LR1PB* forward | AGATGCTGTGGCCAAACGGT | 160 |
| *LR1PB* reverse | TGCGACAGTCCGAAAGGGTG | |
| β-glucuronidase (*GUSB*) forward | GAAAATACGTGGTTGGAGAGCTCATT | 101 |
| β-glucuronidase (*GUSB*) reverse | CCGAGTGAAGATCCCCTTTTTA | |

Supplementary table 4: **Sequence of siRNA used in this study**

| **Gene** | **Sequence** | **Manufacturer** |
|---|---|---|
| *TCF7* | 5'AUGCUAGGUUCUGGUGUACtt3' | Ambion |
| *TCF7L2* | 5'CACGCCUCUUAUCACGUACtt3' | Ambion |

# ABBREVIATIONS USED IN THE TEXT

| | |
|---|---|
| ATRA | All-trans-retinoic acid |
| bFGF | Basic fibroblast growth factor |
| BMP | Bone morphogenetic protein |
| CAIX | Carbonic anydrase 9 |
| ChIP | Chromatin immunoprecipitation |
| DAPI | 4',6-diamidin-2-fenilindolo |
| ddPCR | Droplet digital polymerase chain reaction |
| DMEM | Dulbecco's modified eagle medium |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Egtazic acid |
| EGF | Endothelial growth factor |
| FDR | False discovery rate |
| GBM | Glioblastoma multiforme |
| GFAP | Glial fibrillary acidic protein |
| GO | Gene ontology |
| GSC | Glioma stem cell |
| GSEA | Gene set enrichment |
| GSK3 | Glycogen synthase kinase3 |
| HIF | Hypoxia-inducible factor |
| HMG | High-mobility Group |
| HRE | Hypoxia-response element |
| IF | Immunofluorescence |
| IHC | Immunohistochemistry |
| IP | Immunoprecipitation |
| LDA | Limiting dilution assay |
| LEF | Lymphoid enhancer factor |
| MW | Molecular weight |
| NFAT | Nuclear factor of activated T-cells |
| NSC | Neural stem cell |
| ODDD | Oxygen-dependent degradation domain |
| PCA | Principal component analysis |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PHD | Prolyl hydroxylase domain |
| PVDF | Polyvinylidene Difluoride |
| RLU | Relative light unit |
| RTK | Receptor tyrosine kinase |
| SDS | Sodium dodecyl sulfate |
| SGZ | Subgranular zone |
| SP | Side population |
| SVZ | Subventricular zone |
| TCF1 | T-cell factor 1 |
| TCF4 | T-cell factor 4 |
| TDEC | Tumor-derived endothelial cells |
| TF | Transcription factor |
| TGF-β | Transforming growth factor beta |
| TMZ | Temozolomide |
| TSS | Transcriptional starting site |
| TXL | Taxol |
| VEGF | Vascular endothelial growth factor |
| VHL | von Hippel-Lindau disease tumor suppressor |
| WB | Western blot |
| WNT | Wingless-related integration site |