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## DIMET PhD-Translational and Molecular Medicine

# FOXP3, a novel glioblastoma oncosuppressor, affects proliferation and migration

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Tutor: **Dott. Gaetano Finocchiaro** Co-tutor: **Dott.ssa Serena Pellegatta**  To everyone who shares my passion for Science.

Day after day I hope to contribute to Science and Medicine in the best way that I can

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# Chapter 1

#### **General Introduction**

#### Glioblastoma, a deadly brain tumor

Glioblastoma (GBM) is the most common and lethal primary malignant brain tumor. The clinical hallmarks of glioblastoma are its aggressive growth and inexorable reccurence despite multimodal therapy with surgery followed by radiation and temozolomide therapy. Unfortunatly, current standard-of-care therapy results in a median survival of about 15 months [1].

Glioblastoma is the most malignant variant of diffuse gliomas: its precise histogenesis remains unclear despite considerable advances in the understanding of its basic biology. Most gliomas diffusely infiltrate surrounding brain tissue and together represent a broad diagnostic group which the World Health Organization (WHO) divides into astrocytic, oligodendroglial and mixed (oligoastrocytic) categories [2]. The presence of histological features such as nuclear atypia, increased proliferation, microvascular proliferation and necrosis typically result in higher grade classification. Additionally, although the WHO classification remains grounded in morphological criteria, relevant molecular information regarding the different tumor classes has been integrated over time.

GBM is typicaly characterized by complex chromosome abnormalities and extensive cytogenetic and histological heterogeneity. Indeed, cytogenetically related or unrelated clones coexist in different regions within the same tumor thus increasing the difficulty in targeting and possibly eradicating the tumor [3]. For example, amplification/overexpression of epidermal growth factor receptor (EGFR) and the EGFRVIII variant can be found in scattered cell populations in the same GBM specimen [4, 5]

GBMs have been subdivided into primary or secondary GBM subtypes. Primary glioblastoma typically arises *de novo* as a combination of genetic alterations that include epigenetic modifications, point mutations, translocations, amplifications, or deletions, and modify gene functions in ways that deregulate cellular signalling pathways leading to the cancer phenotype [6]. These alterations result in the activation of proto-oncogenes involved in progrowth pathways and in the inactivation or suppression of tumor suppressors such as p53 and Rb. Secondary GBM both develop from the progression of lower-grade tumors by the accumulation of molecular alterations.

#### Molecular classification of glioblastoma

The slow development of an effective treatment for glioblastoma is contrasted by the rapidly advancing research on the molecular mechanisms underlying the disease. In previous years many groups have attempted to derive a molecular subclassification of glioblastomas exploiting its extensive genomic multiplatform characterization. This is providing a high resolution picture of the molecular alteration signature of glioblastoma.

Importantly, many efforts have been considerably aided by multiinstitutional cooperative projects such as The Cancer Genome Atlas (TCGA). This effort, with GBM as the first characterized tumor type, has so far accumulated expression, CNA (Copy Number Alterations) and sequencing data from hundreds of histologically confirmed glioblastomas [7] (Figure 1).



Figure 1: From Brennan et al., Curr Neurol Neurosci Rep. 2011

Based on global transcript profiling, glioblastoma can be divided into three to four distinct subtypes (Figure 1). The recognition that glioblastoma consists of subtypes varying in molecular and biological behaviour suggests that no therapy can be universally effective.

One model of molecular classification based on gene expression analyses was proposed by Phillips et al. By selecting a set of genes associated with survival in their patient cohort enriched for long-term survivors (>2 years), they identified 3 glioblastoma subtypes with distinct molecular signatures, which they termed proneural, proliferative, and mesenchymal [8]. The proneural signature is associated with oligodendroglial morphology, younger age, the lack of phosphatase and tensin homolog on chromosome ten (PTEN), epidermal growth factor receptor (EGFR) abnormalities, activation of the Notch pathway, and better outcome. The proliferative and mesenchymal signatures are more common in older patients and are characterized by PTEN loss, Akt pathway activation and have a less favorable prognosis. They are distinguished by a preponderance of either proliferation or angiogenesis.

Verhaak et al took an unsupervised approach, extracting gene expression patterns that yielded 4 molecular signatures for glioblastoma. They were termed proneural, neural, classic, and mesenchymal subtypes, showing with signatures proposed by Phillips et al. These subtypes also segregate with characteristic mutations [9]. The proneural subtype comprises most isocitrate dehydrogenase (IDH) 1 mutations and is enriched for p53 mutations, whereas the classic subtype particularly enriches for EGFR-amplified tumors expressing also the EGFRvIII variant. The mesenchymal subtype contains most neurofibromatosis (NF)-1-mutant tumors. Hence, the

expression subtypes overlap with the major previously identified pathogenetic pathways involved in tumorigenesis. Noteably,  $O^6$ methylguanine methyltransferase (MGMT) promoter methylation is not particularly enriched in any specific subtype. It seems that patients with classic or mesenchymal glioblastoma derive more benefit from an aggressive treatment, but this requires confirmation within a prospective clinical trial.

#### **Current and future therapies**

The difficulty in treating this malignant disease lies both in its inherent complexity and numerous mechanisms of drug resistance. Furthermore, most drugs are unable to reach effective concentrations within the tumor due to the prescence of blood–brain barrier (BBB), as well as to elevated intratumoral pressure, restrictive vasculature and other limiting factors.

In cancer drug development, surface molecules, such as receptors, are relatively more accessible for drug targeting [10]. Protein kinases inhibitors, including the intracellular kinase domains of growth receptors, such as EGFR and platelet-derived growth factor receptor (PDGFR) have been extensively tested in various clinical trials, in both recurrent GBM and primary GBM in addition to the standard of care providing surgical resection and radio-chemiotherapy with TMZ [11] (Figure 2). Many therapeutic approaches are aimed at EGFR which is overexpressed (in 60% of GBMs) or amplified (in 40% of GBMs) as well as at its variant, EGFRVIII, a rearranged and constitutively activated form of EGFR [12].

Anti-angiogenic therapies have also been widely tested in clinical trials and cancer therapies. Bevacizumab, a humanized monoclonal antibody against VEGF, is approved as a second-line treatment for recurrent GBM [13] (Figure 2). Its use for treatment for initial GBM is currently undergoing Phase III trials.



**Figure 2**: Schematic overview of current molecular targeted therapies of GBM. Aberrant oncogenic RTK pathways are frequent therapeutic targets in GBM. The PI3K-AKT (green) and RAS (pink) oncogenic pathways are often targeted intracellularly with small molecule inhibitors. EGF, VEGF and PDGF, as well as their receptors, can be blocked by small molecules and monoclonal antibodies. Items in blue boxes include examples of drugs that target the respective pathways. Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinase; Topo I, topoisomerase I.

The major challenges of GBM are its intratumoral heterogeneity and the invasive growth pattern. Due to these features a more personal targeted therapy seems to be a promising method of treatment, for example, determining the O-6-methylguanine methyltransferase (MGMT) status to a predict response to TMZ [1, 14].

Iavarone and Lasorella recently uncovered a reccurent oncogenic fusion protein in a subset of GBMs (3.1%) that can directly interfere with cell division causing aneuploidy. These tumors harbor oncogenic chromosomal translocations that fuse in-frame the tyrosine kinase coding domains of fibroblast growth factor receptor (FGFR) genes (FGFR1 or FGFR3) to the transforming acidic coiled-coil (TACC) coding domains of TACC1 or TACC3, respectively. *In vivo*, FGFR inhibition prolonged the survival of mice bearing intracranial xenografts of FGFR3-TACC3-expressing astrocytes. Importantly, this data indicates that patients with glioblastoma that express FGFR-TACC fusions could benefit from targeted and personalized FGFR kinase inhibition [15].

Transcription factors can also play an important role in glioblastoma biology and be targeted therapeutically. The forkhead transcription factor FOXP3 plays an essential role in the development and function of regulatory T cells (Treg), defined as FOXP3+CD4+CD25+ T cells. Its expression was first considered specific to this cell type, but it has been observed that FOXP3 can also to be transiently expressed in Tcell antigen receptor-activated human nonregulatory T cells. Notably, recent reports demonstrate that FOXP3 is also expressed by nonlymphocytic normal or cancer cells, suggesting that FOXP3 may have a broader role in cancer than initially thought.

#### FOXP3, the marker of regulatory T cells

FOXP3 is considered the master regulator for the development and function of regulatory T lymphocytes CD4+ CD25+ that control the expression of multiple genes that mediate their regulatory activity [16]. As dedicated suppressors of diverse immune responses and inflammation, and important gatekeepers of immune homeostasis, Tregs play a pivotal role in the maintenance of peripherical tolerance [17]. Tregs were initially defined as immunosuppressive CD4+ T cells expressing constitutively the  $\alpha$ -subunit of the interleukin (IL)-2 receptor (CD25) on their surface [18]. The nuclear expression of FOXP3 is now considered as the most specific marker for these cells [18]. However, in human, FOXP3 can be expressed transiently on nonregulatory CD4+ T cells upon T-cell antigen receptor (TCR) activation.

The functional mechanisms used by Treg cells are complex and still

not completly understood. There is increasing evidence that Treg cells use multiple mechanisms to regulate immune response in lymphoid and non-lymphoid tissues [19]. Treg cells have a widespread distribution. They are constitutively present in secondary lymphoid tissues but they can also be found in non-lymphoid tissues and within tumors. Regulatory T cells in seconday lymphoid tissues use different strategies to inhibit dendritic cell (DC) function and block initiation of autoimmunity or prevent tumor clearence. The various potential suppression mechanisms can be grouped into four basic modes of action: suppression by inibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of DC (Figure3).



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Figure 3: Differing immunosuppressive mechanisms used by TReg cells in lymphoid and non-lymphoid tissues. Regulatory T (Treg) cells in secondary

lymphoid tissues use multiple mechanisms to inhibit dendritic cell (DC) function and block initiation of autoimmunity or prevent tumour clearance. TReg cell production of interleukin-10 (IL-10) is essential for immunoregulation at mucosal tissues, such as the intestines and the lungs, and in the skin. In tissue-draining lymph nodes, TReg cells can inhibit the priming of effector T cells by preventing DC maturation (through cytotoxic T lymphocyte antigen 4 (CTLA4)-dependent mechanisms) or by killing mature DCs in a perforin- and granzyme-dependent manner. The relative importance of other immunosuppressive mechanisms used by TReg cells (central box) in lymphoid and non-lymphoid tissues remains to be established. LAG3, lymphocyte activation gene 3; TCR, T cell receptor; TGF $\beta$ , transforming growth factor- $\beta$ .

The production of IL-10 by Treg is essential for immunoregulation of mucosal tissues, such as the intestines, the lungs and the skin. In tissue-draining lymph nodes, Treg cells can inhibit the priming of effector T cells by preventing DC maturation (through cytotoxic T lymphocyte antigen 4 (CTLA4)-dependent mechanisms) or by killing mature DCs in a perforin- and granzyme-dependent manner. The relative importance of other immunosuppressive mechanisms used by Treg cells in lymphoid and non-lymphoid tissues remains to be established.

#### FOXP3: gene and protein structure

FOXP3 belongs to the family of forkhead box (FOX) transcription factors which has at least four members, FOXP1–4. The forkhead gene product was initially identified in the fruit fly Drosophila melanogaster as factor required for the terminal pattern formation in the terminal regions of the embryo [20]. Several members of the FOX family have crucial roles in various aspects of immune regulation [21]. Above all, FOXP3 is considered to be a master regulator in the development and function of regulatory T cells (Treg) [22–24]. The role of FOXP3 was defined after positional cloning of *Scurfin*, as a gene responsible for X-linked autoimmune diseases in mice and humans (immune deregulation, polyendopathy, enterophathy, X-linked, IPEX) [25]. Scurfy mice bearing this lethal X-linked recessive immunodysregulation lack functional expression of Foxp3 caused by an AA insertion in exon 8 [26]. Moreover, ectopic expression of Foxp3 in conventional murine T cells endows them with the full phenotype and function of Treg. In humans, there is also a strong association between FOXP3 expression and the Treg phenotype, although the relationship is more complex than in mice [27].

The FOXP3 gene is well conserved in mammalians [27]. The human FOXP3 gene is located on the X chromosome at Xp11.23 and is submitted to X chr inactivation [28, 29]. The gene contains 11 coding exons (exons 1-11) and 3 non-translated exons [28] encoding a protein of 431 amino acids [30]. The FOXP family is characterized by highly conserved C terminus tetramerization domains. FOXP3 contains a proline-rich repressor domain required for repressing the expression of target genes, a zinc-finger, a leucine zipper motif which allows FOXP3 homo- or heterodimerization, and the conserved DNA-binding forkhead domain (FKH) with two NLS targeting FOXP3 localization to the nucleus at its C and N terminus [30].



**Figure 4**: Structure of gene and protein, and principal functions of human FOXP3. (a) Exon 3 is spliced out in the short isoform of human FOXP3 (Allan et al., 2005). The N-terminal repression domain binds to and inhibits several transcription factors, including (b) nuclear factor of activated T cells (NFAT), leading to repression of IL-2 (Rudensky et al., 2006; Wu et al., 2006) and (c) retinoic acid receptor-related orphan receptor (ROR) $\alpha$ , blocking transformation into Th17. (d) (Lopes et al., 2006). (e) FOXP3 preferentially binds DNA as a dimer, when two paired FKH domains recognize tandem copies of a core DNA element (Koh et al., 2009). (f) NLS: nuclear localization signals, located at the N- and C-terminals of the FKH domain (Lopes et al., 2006).

The FOXP3 promoter is conserved between humans and mice. Located 6.5 kb upstream of the first exon, it contains six NFAT and AP-1 binding sites and a TATA and CAAT box. In addition to the promoter the FOXP3 locus contains three proximal intronic conserved non-coding DNA sequence (CNS) elements [31]. CNS1, an intronic enhancer with TGF-b-responsive element and binding site for transcription factor such as NFAT and Smad, is involved in TGF-binduced FOXP3 expression in Tregi cells. CNS2, corresponding to the TCR-responsive enhancer, contains a CpG island and binding site for transcription factors such as CREB and STAT and it is required for FOXP3 expression in mature nTreg cells [31]. CNS3 has a prominent role in the generation of Treg cells in the thymus and the periphery [31]. Epigenetic modifications of CNS, in particular CpG methylation, regulate the transcription of FOXP3. Interestingly, the CpG island in CNS2 have been found hypo-methylated in nTreg, almost completly methylated in T cells and only incompletly demethylated in iTreg cells [32–34].

The FOXP3 protein is highly conserved [35]. FOXP3 can be part of a large molecular complexes of 600kDa, together with histone deacetylases, histone acetyltransferases and other trascriptions factors such as Runx1 [36, 37].

In contrast with mouse Treg, in which Foxp3 is expessed as a full length protein [16], in human Treg express two major isoforms of FOXP3: a full-length transcript designated FOXP3a (molecular weight: 58 kDa) that represents the ortholog of mouse Foxp3 and an alternative-splicing product lacking exon 2 designated FOXP3b (apparent molecular weight: 54 kDa) [38]. These isoforms can be differentiated from one another by their intracellular distribution. While the FOXP3a isoform appears to be distributed in equal amounts in both cytoplasmic and nuclear fractions of human CD4+CD25+ cells, the splice variant FOXP3b isoform is primarily found within the nucleus. This differential expression can be explained by the lack of this NES in the FOXP3b isoform [39].

#### The dual role of FOXP3 in human cancers

Due to the Treg lineage specification of FOXP3, its tissue expression, primarily by lymphoid tissues such as thymus, spleen and lymphnodes, is expected and has been well documented [16]. However, FOXP3 expression has been recently demonstrated in various nonhematopoietic cells and in cancer cells of non-hematopoietic origin (pancreatic carcinoma, breast cancer, melanoma, lung cancer, colon cancer) [40]

#### FOXP3<sup>+</sup> Treg and cancer

It is well established that FOXP3-expressing Tregs are more abundant in the peripherical blood of cancer-bearing patients compared to healthy subjects [41, 42]. Most human tumors are infiltrated by Treg, presenting as small and round cells with lymphocytic morphology and high FOXP3 expression. FOXP3<sup>+</sup> cells can be localized in the proximity of the tumor but predominantly in the peripherical lymphoid-enriched areas [43–45].

As FOXP3 Treg are immunosupressive cells, their abundance in tumor infiltrates is expected to be associated with an unfavorable prognosis. However, there are discrepancies in the prognostic studies relying on the presence of Treg in tumor infiltrates.

In breast carcinoma [45, 46] as well as in cervix [47], gastric [44], hepatocellular [48], ovarian [43] and pancreatic [49] carcinoma, the increased Treg infiltration in the tumor bed predicted reduced survival in cancer-bearing patients. Paradoxically, a high density of FOXP3+

T-cell infiltration was associated with improved overall survival in patients with colorectal [50], head and neck carcinoma [51] and also in patients with lymphoma [52, 53].

Experimental data demonstrate that Treg depletion with for example ciclophosphamide can slow down tumor growth and increase the efficacy of tumor immunotherapy [54].

#### FOXP3 expression in human cancer cells

FOXP3 is expressed not only in regulatory T cells but also in epithelial cells from various organs such as the breast, thymus, prostate and lung [55].

Foxp3 expression in noncancerous epithelial cells was investigated in  $Rag2^{-/-}$  mice, which are notably devoid of T lymphocytes, and in mice with the *Scurfy* mutation that deletes Foxp3 expression [56]. This study revealed expression of Foxp3 mRNA and protein in the nuclei of epithelial cells in the breast, lung and prostate, but not in the liver, kidney and intestine.

The function of FOXP3 in cancer cells can be different and somehow contradictory [40, 57]. Comparing FOXP3 expression in human cancer cells and in their normal homologs, two opposite situations have been found.

In some cancers, FOXP3 expression is restricted to the tumor cells. Normal pancreatic duct cells don't express FOXP3, but its expression was detected in human pancreatic cancer cells [58]. FOXP3 was also found to be expressed in human melanoma cells but was not detected in normal melanocytes [40]. In contrast, FOXP3 appears to be expressed in normal epithelial cells of human breast and prostate, but downregulated in the corresponding cancer cells [29, 59].

These data suggest a dual role of FOXP3, one linked to immune escape and another to tumor suppression.

FOXP3 expression in cancer cells as a novel mechanism of immune evasion

Normal pancreatic ducts cells were devoid of FOXP3 expression, but FOXP3 was detected by immunohistochemistry in human pancreatic cancer cells [58]. Cancer cell staining was cytoplasmic in most patients, whereas in others, it was predominantly nuclear. Interestingly, FOXP3-expressing pancreatic cell lines strongly inhibited the proliferation of anti-CD3/anti-CD28-stimulated T cells without impeding their activation, suggesting that FOXP3 expression in cancer cells might trigger a mechanism of immune evasion. FOXP3 was also found to be expressed by human melanoma cells but was not detected in normal melanocytes [40]. Reverse transcription-PCR confirmed the presence of FOXP3 transcripts in melanoma cells that expressed the two isoforms found in human Treg, including the fulllength variant. Immunohistochemistry with anti-FOXP3 on melanoma tissue sections showed a nuclear labeling of melanoma cells. FOXP3 was widely expressed in human melanoma cells.

#### FOXP3 as an X-linked tumor suppressor gene

FOXP3 is a multifaceted factor. Beside its role as master regulator gene of Treg, genetic analyses in both mice and humans revealed that

FOXP3 is an important X-linked tumor suppressor in breast and in prostate cancer [29, 56, 57, 59–71]. Since the FOXP3 gene is located on the X chromosome, a genetic/epigenetic single-hit results in inactivation of this gene in males, escaping the Knudson model [72].

The role of the FOXP3 gene in mammary carcinogenesis has been supported by several lines of evidence. Importantly, mice that are heterozygous for FOXP3 mutations spontaneously develop mammary carcinomas at a high frequency [56]. Mice with germline FOXP3 mutations are substantially more prone to developing both spontaneous and carcinogen-induced mammary carcinomas [71]. The FOXP3 gene is expressed in normal breast epithelia but is downregulated in mammary cancer. Ectopic expression of FOXP3 in a variety of breast cancer cell lines resulted in cell cycle arrest and cessation of cell growth [59].

FOXP3 also plays an important role in prostate epithelia. Immunohistochemistry revealed that FOXP3 expression is significantly down-regulated in cancer cells when compared to normal prostate glands [68]. Moreover, mice with prostate-specific ablations of FOXP3, (FOXP3<sup>fl/y</sup>; PB-Cre<sup>+</sup>) developed prostatic hyperplasia and prostatic intraepithelial neoplasm (PIN) that are putative precancerous lesions of the prostate [29, 73]. In human samples, FOXP3 expression in PINs is down-regulated compared to adjacent normal prostate glands, which suggests that the inactivation of the FOXP3 gene plays an important role in the initial stage of prostatic carcinogenesis [73]. The inactivation of FOXP3 in human breast (b.c.) and prostate (p.c.) cancers, is explained by the frequent chromosomal deletions (12.6% in b.c. and 13.9% in p.c.), somatic mutations (35.4% in b.c. and 20.0% in p.c.) and epigenetic silencing found in the FOXP3 gene [29, 64, 69] (Figure 5).



(C) FOXP3 abnormalities in human breast and prostate cancers									
	immunohistochemistry <sup>a</sup>			chromosomal	somatic				
		FOXP3 (+)	FOXP3 (-)	(FISH)	mutation <sup>b</sup>	reterence			
Breast cancer	Ca	58/275 (21.09%)	217/275 (78.91%)	28/223 (12.6%)	28/223 23/65 (12.6%) (35.4%	23/65	Zuo T.,		
	Nor	39/50 (78.00%)	11/50 (22.00%)			(35.4%) et 20	2007		
prostate cancer	Ca	170/554 (30.69%)	384/554 (69.31%)	23/165	5/20	Wang L.,			
	Nor	172/185 (92.97%)	13/185 (7.03%)	(13.9%) (20.0%	(13.9%) (20.0%)	2009			

Figure 5: A. Diagram of the human FOXP3 and its somatic mutations found among human breast and prostate cancers. ZF: zing finger domain. LZ: leucine-zipper

domain. FKH: forkhead domain. B. Splice variants of the FOXP3 that are predominantly expressed in human cancers. " $\Delta$ " represents any exons that is/are deleted in the variant forms of FOXP3. Breast Ca: Breast cancer. Sezary synd.: Sezary syndrome. C. FOXP3 aberrations found in human breast and prostate cancers. <sup>a</sup>Samples with nuclear FOXP3 staining were scored as positive. bSixty-five breast cancer samples and 20 prostate cancer samples were sequenced. Four out of five somatic mutations of prostate cancer were missense mutations, while the remaining one was found in an intron. Ca: cancer tissue. Nor: normal tissue. FISH: fluorescent in situ hybridization.

Also of interest is that some types of cancers such as cutaneous melanomas, breast and ovarian cancers, and malignant T cells of Sezary syndrome, predominantly express FOXP3 transcripts with exon 3, exon 4 and/or exon 8 deletion (FOXP3 $\Delta$ E3, FOXP3 $\Delta$ E3 $\Delta$ 4, FOXP3 $\Delta$ 8 and FOXP3 $\Delta$ E3 $\Delta$ 8) [74, 75] (Figure 5).

#### FOXP3 master regulator in cancer

FOXP3 acts as both a transcriptional activator and repressor. FOXP3 directly regulates the expression of both oncogenes and tumor suppressor genes, including ERBB2, SKP2; two oncogenes involved in mammary carcinogenesis [59, 71], c-MYC involved in prostate carcinogenesis (Wang et al., 2009), p21 (CDKN1A) tumor supressor in prostate cancer and other important cancer-related genes [29, 61] (Figure 6).



Figure 6: A schematic view of the signalling networks of the FOXP3 in epithelial cells.

The FKH DNA-binding domain of FOXP3 interacts with motifs in the promoter of these oncogenes.

The c-MYC oncogene has been demonstrated to be directly repressed by FOXP3 in prostate epithelia [29]. Overexpression of c-MYC contributes to more aggressive and poorly differentiated cancer phenotypes and has been involved in the biology of melanoma. c-MYC is a sequence-specific transcription factor and an important player in various cellular processes including cell cycle and apoptosis; processes which are also dysregulated in cancer cells with high c-MYC expression levels. C-MYC directly activates CDK4 and CCND2 expression, while indirectly repressing CDK inhibitors such as CDKN1A (p21) and CDKN2B (p15) expression [76, 77]. C-MYC is involved with *ras* in prostate carcinogenesis [78] and is overexpressed in 80% of the prostate cancer samples. FOXP3 contributes to HER2 overexpression in breast cancer samples [66, 79]. HER2 is a member of the transmembrane receptor tyrosine kinases and is involved in the regulation of various cellular functions such as cell growth and survival. Between 15 and 20% of invasive breast cancers overexpress HER-2 and have a worse prognosis than HER-2-negative tumors [80]. FOXP3 can repress transcription of HER2 in human breast cancers by binding directly to the ERBB2 gene promoter [71] [79].

High levels of expression of SKP2 (S-phase kinase-associated protein) have been reported in a wide variety of cancers [81, 82]. SKP2 is an important player in the ubiquitin-dependent degradation of p27<sup>KIP1</sup>, a CDK inhibitor of Cyclin-E/CDK2 and Cyclin-A/CDK2 [81, 82]. SKP2 is strongly expressed during S and G2 phases of the cell cycle. SKP2 is involved in the ubiquitination and degradation of the cdk-inhibitor p27, thus facilitating progression of the cell cycle. SKP2 is overexpressed in nearly 50% of breast carcinomas [83]. Interestingly, FOXP3 directly represses SKP2 expression in human and mouse mammary epithelial cells [59]. FOXP3 occupies the Skp2 promoter and represses promoter activity of the locus [59].

p21, as universal CDK inhibitor, plays an important role in preventing cell cycle progression by acting at the G1 checkpoint [84]. p21 is down-regulated in many types of cancer including breast cancer [84]. Cancer cells with low levels of p21 can escape from G1 arrest, and thus cells acquire a growth advantage in tumor development. Liu et al., reported that FOXP3 occupies and activates the p21 promoter in

normal breast epithelia [85]. FOXP3 specifically inhibits binding of histone deacetylase (HDAC) 2 and 4 to the site and increases local histone H3 acetylation. The lack of FOXP3 was associated with p21 down-regulation in breast cancer samples [85]. In various breast cancer cell lines, p21 expression was significantly up-regulated after FOXP3 induction [85]. IHC on human breast cancer tissue microarray revealed a positive correlation (p=0.011) between FOXP3 and p21 proteins [85].

A significant correlation was observed between FOXP3 downregulation and HER-2 and SKP2 or cMYC overexpression in breast and prostate cancer cells and downregulation of p21 in breast cancer [29, 59, 71].

FOXP3 are often heterozygous in female cancer cells [71, 86]. Since one allele of an X-linked tumor suppressor gene has not undergone selection during carcinogenesis, it may be possible to reactivate the wildtype allele for cancer therapy. Indeed, anisomycin treatment induced FOXP3 expression in both mouse and human breast cancer cell lines [87] Such induction resulted in increased apoptosis of cancer cells and reduced growth of established mouse mammary tumors [87].This observation raises the intriguing possibility that restoration of FOXP3 may have a therapeutic function in cancer cells.

#### References

1. Stupp R, Mason WP, Van Den Bent MJ, Weller M, Fisher B, Taphoorn MJB, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO: **Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.** *The New England Journal of Medicine* 2005, **352**:987– 96.

2. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK: WHO Classification of Tumours of the Central Nervous System. IARC; 2007:309.

3. Walker C, Du Plessis DG, Joyce KA, Machell Y, Thomson-Hehir J, Al Haddad SA, Broome JC, Warnke PC: **Phenotype versus genotype in gliomas displaying inter- or intratumoral histological heterogeneity.** *Clinical Cancer Research* 2003, **9**:4841–4851.

4. Nishikawa R, Sugiyama T, Narita Y, Furnari F, Cavenee WK, Matsutani M: Immunohistochemical analysis of the mutant epidermal growth factor, deltaEGFR, in glioblastoma. *Brain Tumor Pathology* 2004, 21:53–56.

5. Jeuken J, Sijben A, Alenda C, Rijntjes J, Dekkers M, Boots-Sprenger S, McLendon R, Wesseling P: Robust detection of EGFR copy number changes and EGFR variant III: technical aspects and relevance for glioma diagnostics. *Brain pathology Zurich Switzerland* 2009, **19**:661–671.

Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000, 100:57–70.

7. Cancer T, Atlas G: Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008, **455**:1061–1068.

8. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro JM, Colman H, Soroceanu L, Williams PM, Modrusan Z, Feuerstein BG, Aldape K: Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006, **9**:157–173.

9. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, et al.: Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010, 17:98–110.

10. Chico LK, Van Eldik LJ, Watterson DM: Targeting protein kinases in central nervous system disorders. *Nature Reviews Drug Discovery* 2009, **8**:892–909.

11. Hainsworth JD, Ervin T, Friedman E, Priego V, Murphy PB, Clark BL, Lamar RE: Concurrent radiotherapy and temozolomide followed by temozolomide and sorafenib in the first-line treatment of patients with glioblastoma multiforme. *Cancer* 2010, 116:3663–9.

12. Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS, Vogelstein B: Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proceedings of the National Academy of Sciences of the United States of America* 1992, **89**:2965–2969.

13. Norden AD, Young GS, Setayesh K, Muzikansky A, Klufas R, Ross GL, Ciampa AS, Ebbeling LG, Levy B, Drappatz J, Kesari S, Wen PY: Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence. *Neurology* 2009, **72**:779–787.

14. Weller M, Stupp R, Reifenberger G, Brandes AA, Van Den Bent MJ, Wick W, Hegi ME: **MGMT promoter methylation in malignant gliomas: ready for personalized medicine?** *Nature reviews Neurology* 2010, **6**:39–51.

15. Singh D, Chan JM, Zoppoli P, Niola F, Sullivan R, Castano A, Liu EM, Reichel J, Porrati P, Pellegatta S, Qiu K, Gao Z, Ceccarelli M,

Riccardi R, Brat DJ, Guha A, Aldape K, Golfinos JG, Zagzag D, Mikkelsen T, Finocchiaro G, Lasorella A, Rabadan R, Iavarone A: **Transforming Fusions of FGFR and TACC Genes in Human Glioblastoma.** *Science* 2012:3–8.

16. Fontenot JD, Rudensky AY: A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nature Immunology* 2005, 6:331–337.

17. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH: Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *The Journal of Experimental Medicine* 2001, 193:1285–1294.

18. Sakaguchi S, Sakaguchi N: Regulatory T cells in immunologic self-tolerance and autoimmune disease. *International Reviews of Immunology* 2005, 24:211–226.

19. Campbell DJ, Koch MA: Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nature Reviews Immunology* 2011, **11**:119–130.

20. Weigel D, Jürgens G, Küttner F, Seifert E, Jäckle H: The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell* 1989, **57**:645–658.

21. Coffer PJ, Burgering BMT: Forkhead-box transcription factors and their role in the immune system. *Nature Reviews Immunology* 2004, 4:889–899.

22. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF: Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *The Journal of Biological Chemistry* 2001, 276:37672–37679.

23. Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology* 2003, 4:330–336.

24. Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003, 299:1057–61.

25. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F: Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* 2001, **27**:68–73.

26. Bennett CL, Brunkow ME, Ramsdell F, O'Briant KC, Zhu Q, Fuleihan RL, Shigeoka AO, Ochs HD, Chance PF: A rare polyadenylation signal mutation of the FOXP3 gene (AAUAAA-->AAUGAA) leads to the IPEX syndrome. *Immunogenetics* 2001, 53:435–439.

27. Ziegler SF: FOXP3: of mice and men. Annual Review of Immunology 2006, 24:209–226.

28. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD: The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics* 2001, **27**:20–21.

29. Wang L, Liu R, Li W, Chen C, Katoh H, Chen G-Y, McNally B, Lin L, Zhou P, Zuo T, Cooney KA, Liu Y, Zheng P: Somatic single hits inactivate the X-linked tumor suppressor FOXP3 in the prostate. *Cancer Cell* 2009, 16:336–346.

30. Lopes JE, Torgerson TR, Schubert LA, Anover SD, Ocheltree EL, Ochs HD, Ziegler SF: Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor. *The Journal of Immunology* 2006, 177:3133–3142.

31. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY: Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* 2010, 463:808–812.

32. Baron U, Floess S, Wieczorek G, Baumann K, Grützkau A, Dong J, Thiel A, Boeld TJ, Hoffmann P, Edinger M, Türbachova I, Hamann A, Olek S, Huehn J: **DNA demethylation in the human FOXP3** locus discriminates regulatory T cells from activated FOXP3(+)

conventional T cells. European Journal of Immunology 2007, 37:2378–2389.

33. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang H-D, Bopp T, Schmitt E, Klein-Hessling S, Serfling E, Hamann A, Huehn J: **Epigenetic Control of the foxp3 Locus in Regulatory T Cells**. *PLoS Biology* 2007, **5**:10.

34. Nagar M, Vernitsky H, Cohen Y, Dominissini D, Berkun Y, Rechavi G, Amariglio N, Goldstein I: Epigenetic inheritance of DNA methylation limits activation-induced expression of FOXP3 in conventional human CD25-CD4+ T cells. International Immunology 2008, 20:1041–1055.

35. Lal G, Bromberg JS: Epigenetic mechanisms of regulation of Foxp3 expression. *Blood* 2009, 114:3727–3735.

36. Li B, Samanta A, Song X, Iacono KT, Bembas K, Tao R, Basu S, Riley JL, Hancock WW, Shen Y, Saouaf SJ, Greene MI: FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences of the United States of America* 2007, 104:4571–4576.

37. Ono M, Yaguchi H, Ohkura N, Kitabayashi I, Nagamura Y, Nomura T, Miyachi Y, Tsukada T, Sakaguchi S: Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* 2007, 446:685–689.
38. Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, Ziegler SF, Roncarolo MG, Levings MK: The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *Journal of Clinical Investigation* 2005, 115:3276–3284.

39. Li B, Samanta A, Song X, Furuuchi K, Iacono KT, Kennedy S, Katsumata M, Saouaf SJ, Greene MI: FOXP3 ensembles in T-cell regulation. *Immunological Reviews* 2006, 212:99–113.

40. Ebert LM, Tan BS, Browning J, Svobodova S, Russell SE, Kirkpatrick N, Gedye C, Moss D, Ng SP, MacGregor D, Davis ID, Cebon J, Chen W: The regulatory T cell-associated transcription factor FoxP3 is expressed by tumor cells. *Cancer Research* 2008, 68:3001–3009.

41. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH: Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Research* 2001, 61:4766–4772.

42. Liyanage UK, Moore TT, Joo H-G, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC: Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *The Journal of Immunology* 2002, 169:2756–2761.

43. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature Medicine* 2004, 10:942–949.

44. Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H, Fujii H: Localisation pattern of Foxp3+ regulatory T cells is associated with clinical behaviour in gastric cancer. *British Journal of Cancer* 2008, **98**:148–153.

45. Gobert M, Treilleux I, Bendriss-Vermare N, Bachelot T, Goddard-Leon S, Arfi V, Biota C, Doffin AC, Durand I, Olive D, Perez S, Pasqual N, Faure C, Ray-Coquard I, Puisieux A, Caux C, Blay J-Y, Ménétrier-Caux C: Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. *Cancer Research* 2009, **69**:2000–2009.

46. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, Banham AH: Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *Journal of Clinical Oncology* 2006, 24:5373–5380.

47. Jordanova ES, Gorter A, Ayachi O, Prins F, Durrant LG, Kenter GG, Van Der Burg SH, Fleuren GJ: Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-

cell ratio: which variable determines survival of cervical cancer patients? *Clinical Cancer Research* 2008, 14:2028–2035.

48. Gao Q, Qiu S-J, Fan J, Zhou J, Wang X-Y, Xiao Y-S, Xu Y, Li Y-W, Tang Z-Y: Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *Journal of Clinical Oncology* 2007, **25**:2586–2593.

49. Hiraoka N, Onozato K, Kosuge T, Hirohashi S: Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clinical Cancer Research* 2006, **12**:5423–5434.

50. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B: Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *Journal of Clinical Oncology* 2009, **27**:186–192.

51. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay NEH, Mosseri V, Laccourreye O, Bruneval P, Fridman WH, Brasnu DF, Tartour E: **Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers.** *Clinical Cancer Research* 2006, **12**:465–472.

52. Carreras J, Lopez-Guillermo A, Fox BC, Colomo L, Martinez A, Roncador G, Montserrat E, Campo E, Banham AH: **High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are**  associated with improved overall survival in follicular lymphoma. *Blood* 2006, **108**:2957–2964.

53. Alvaro T, Lejeune M, Salvadó MT, Bosch R, García JF, Jaén J, Banham AH, Roncador G, Montalbán C, Piris MA: Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. *Clinical Cancer Research* 2005, **11**:1467–1473.

54. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, Solary E, Le Cesne A, Zitvogel L, Chauffert B: *Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients.* Springer; 2007, **56**:641–648.

55. Chang X, Gao JX, Jiang Q, Wen J, Seifers N, Su L, Godfrey VL, Zuo T, Zheng P, Liu Y: The Scurfy mutation of FoxP3 in the thymus stroma leads to defective thymopoiesis 10.1084/jem.20050157. *Journal of Experimental Medicine* 2005, 202:1141–1151.

56. Chen G-Y, Chen C, Wang L, Chang X, Zheng P, Liu Y: Cutting edge: Broad expression of the FoxP3 locus in epithelial cells: a caution against early interpretation of fatal inflammatory diseases following in vivo depletion of FoxP3-expressing cells. *The Journal of Immunology* 2008, **180**:5163–5166.

57. Karanikas V, Speletas M, Zamanakou M, Kalala F, Loules G, Kerenidi T, Barda AK, Gourgoulianis KI, Germenis AE: **Foxp3** expression in human cancer cells. *Journal of Translational Medicine* 2008, **6**:19.

58. Hinz S, Pagerols-Raluy L, Oberg H-H, Ammerpohl O, Grüssel S, Sipos B, Grützmann R, Pilarsky C, Ungefroren H, Saeger H-D, Klöppel G, Kabelitz D, Kalthoff H: Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. *Cancer Research* 2007, **67**:8344–8350.

59. Zuo T, Liu R, Zhang H, Chang X, Liu Y, Wang L, Zheng P, Liu Y: FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2. *Journal of Clinical Investigation* 2007, 117:3765–3773.

60. Gupta S, Joshi K, Wig JD, Arora SK: Intratumoral FOXP3 expression in infiltrating breast carcinoma: Its association with clinicopathologic parameters and angiogenesis. *Acta oncologica Stockholm Sweden* 2007, **46**:792–797.

61. Katoh H, Zheng P, Liu Y: Signalling through FOXP3 as an Xlinked tumor suppressor. *The international journal of biochemistry cell biology* 2010, **42**:1784–1787.

62. Kiniwa Y, Miyahara Y, Wang HY, Peng W, Peng G, Wheeler TM, Thompson TC, Old LJ, Wang R-F: **CD8+ Foxp3+ regulatory T** 

**cells mediate immunosuppression in prostate cancer.** *Clinical Cancer Research* 2007, **13**:6947–6958.

63. Ladoire S, Martin F, Ghiringhelli F: **Prognostic role of FOXP3+** regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer. *Cancer immunology immunotherapy CII* 2011, 60:909–918.

64. Li W, Wang L, Katoh H, Liu R, Zheng P, Liu Y: Identification of a tumor suppressor relay between the FOXP3 and the Hippo pathways in breast and prostate cancers. *Cancer Research* 2011, 71:2162–2171.

65. Liu Y, Zheng P: FoxP3: A Life beyond Regulatory T Cells. International journal of clinical and experimental pathology 2008, 2:205–210.

66. Mahmoud SMA, Paish EC, Powe DG, Macmillan RD, Lee AHS, Ellis IO, Green AR: An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. *Breast Cancer Research and Treatment* 2011, **127**:99–108.

67. Merlo A, Casalini P, Carcangiu ML, Malventano C, Triulzi T, Mènard S, Tagliabue E, Balsari A: FOXP3 expression and overall survival in breast cancer. *Journal of Clinical Oncology* 2009, 27:1746–1752.

68. Valdman A, Jaraj SJ, Compérat E, Charlotte F, Roupret M, Pisa P, Egevad L: Distribution of Foxp3-, CD4- and CD8-positive lymphocytic cells in benign and malignant prostate tissue. *APMIS acta pathologica microbiologica et immunologica Scandinavica* 2010, 118:360–365.

69. Wang L, Liu R, Ribick M, Zheng P, Liu Y: FOXP3 as an Xlinked tumor suppressor. *Discovery Medicine* 2010, 10:322–328.

70. Yokokawa J, Cereda V, Remondo C, Gulley JL, Arlen PM, Schlom J, Tsang KY: Enhanced functionality of CD4+CD25(high)FoxP3+ regulatory T cells in the peripheral blood of patients with prostate cancer. *Clinical Cancer Research* 2008, 14:1032–1040.

71. Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W, Liu Y, Wang Y, Liu X, Chan MWY, Liu J-Q, Love R, Liu C-G, Godfrey V, Shen R, Huang TH-M, Yang T, Park BK, Wang C-Y, Zheng P, Liu Y: FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. *Cell* 2007, 129:1275–1286.

72. Spatz A, Borg C, Feunteun J: X-chromosome genetics and human cancer. *Nature Reviews Cancer* 2004, 4:617–629.

73. Ebelt K, Babaryka G, Frankenberger B, Stief CG, Eisenmenger W, Kirchner T, Schendel DJ, Noessner E: **Prostate cancer lesions are** 

surrounded by FOXP3+, PD-1+ and B7-H1+ lymphocyte clusters. *European Journal of Cancer* 2009, **45**:1664–1672.

74. Kaur G, Goodall JC, Jarvis LB, Hill Gaston JS: Characterisation of Foxp3 splice variants in human CD4+ and CD8+ T cells-identification of Foxp3 $\Delta$ 7 in human regulatory T cells. *Molecular Immunology* 2010, 48:321–332.

75. Smith EL, Finney HM, Nesbitt AM, Ramsdell F, Robinson MK: Splice variants of human FOXP3 are functional inhibitors of human CD4+ T-cell activation. *Immunology* 2006, 119:203–211.

76. Hongyan W, Huan L, Yali Q, Yubing C, Shouliang G: *The expressions of -catenin, c-myc and cyclin D1 in thymoma induced by ionizing radiation*. IEEE; 2011:257–259.

77. Wang C, Lisanti MP, Liao DJ: Reviewing once more the c-myc and Ras collaboration: Converging at the cyclin D1-CDK4 complex and challenging basic concepts of cancer biology. *Cell Cycle* 2011, 10:57–67.

78. Thompson TC, Southgate J, Kitchener G, Land H: Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted organ. *Cell* 1989, **56**:917–930.

79. Zhou Y, Cheng Z, Zhu H, Feng D, Zhao W, Ling B, Wei H, Tian Z: Stable suppression of HER-2 gene expression using siRNA

increases the lysis of human ovarian carcinoma cells mediated by NK-92 cell line. *Oncology Reports* 2008, **20**:1425–1431.

80. Burstein HJ: The distinctive nature of HER2-positive breast cancers. *The New England journal of medicine* 2005, **353**:1652–4.

81. Katagiri Y, Hozumi Y, Kondo S: Knockdown of Skp2 by siRNA inhibits melanoma cell growth in vitro and in vivo. *Journal of Dermatological Science* 2006, **42**:215–224.

82. Rose AE, Wang G, Hanniford D, Monni S, Tu T, Shapiro RL, Berman RS, Pavlick AC, Pagano M, Darvishian F, Mazumdar M, Hernando E, Osman I: Clinical relevance of SKP2 alterations in metastatic melanoma. *Pigment cell melanoma research* 2011, 24:197–206.

83. Signoretti S, Di Marcotullio L, Richardson A, Ramaswamy S, Isaac B, Rue M, Monti F, Loda M, Pagano M: Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. *Journal of Clinical Investigation* 2002, **110**:633–641.

84. Abukhdeir AM, Park BH: **P21 and p27: roles in carcinogenesis** and drug resistance. *Expert Reviews in Molecular Medicine* 2008, **10**:e19.

85. Liu R, Wang L, Chen G, Katoh H, Chen C, Liu Y, Zheng P: FOXP3 up-regulates p21 expression by site-specific inhibition of

**histone deacetylase 2/histone deacetylase 4 association to the locus.** *Cancer research* 2009, **69**:2252–9.

86. Rivera MN, Kim WJ, Wells J, Driscoll DR, Brannigan BW, Han M, Kim JC, Feinberg AP, Gerald WL, Vargas SO, Chin L, Iafrate AJ, Bell DW, Haber DA: An X chromosome gene, WTX, is commonly inactivated in Wilms tumor. *Science* 2007, **315**:642–645.

87. Liu Y, Wang Y, Li W, Zheng P, Liu Y: Activating transcription factor 2 and c-Jun-mediated induction of FoxP3 for experimental therapy of mammary tumor in the mouse. *Cancer research* 2009, **69**:5954–60.

#### Scope of the thesis

Despite advances in modern chemo- and radiotherapy, glioblastoma remains a higly vascularized, aggressive and diffusely infiltrating primary brain tumor that is rarely, if ever, cured. There is an urgent need to find a long-term therapeutic strategies that specifically target tumor cells while minimizing collateral damage to surrounding normal brain tissue and killing all residual tumor cells that infiltrate in the adjacent areas of the brain.

In preliminary data, we found the presence of Foxp3 positive cells in murine malignant gliomas derived from the GL261 cells. In particular, the expression of Foxp3 was only detectable in early stages of tumor development (10 days after the intracranial injection) and was lost during tumor progression (20 days after the intracranial injection).

Based on these observations we decided to focus our attention on the functional consequences and the signaling pathways triggered by FOXP3 expression in human glioblastoma (GBM).

The first aim was to investigate the expression level of FOXP3 in a cohort of human GBM specimens and in their corresponding gliomastem like cell lines both in vitro and in vivo; second to characterize for the first time, the FOXP3 expression in the normal brain and finally to identify potential downstream targets of FOXP3 involved in tumorigenesis.

### Chapter 2

# FOXP3, a novel glioblastoma oncosuppressor, affects proliferation and migration

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#### Introduction

The forkhead transcription factor FOXP3 plays an essential role in the development and function of regulatory T cells (Treg), defined as FOXP3+CD4+CD25+ T cells [1]. In humans, FOXP3 is present in two isoforms, referred to as a and b, but the functional differences between the two isoforms are still unclear [2, 3]. A recent report found that in melanomas, FOXP3 is also expressed by tumor-reactive CD8<sup>+</sup> T cells. These lymphocytes do not express regulatory markers and maintain early effector profiles (CD38<sup>+</sup>, T-bet<sup>+</sup>, perforin<sup>+</sup>) [4]. The expression of FOXP3, however, is not restricted to lymphoid tissues such as the thymus, spleen and lymph nodes. It was recently reported that FOXP3 is expressed in tumor cells from pancreatic carcinoma, breast cancer, melanoma, lung cancer and colon cancer [5]

FOXP3 appears to be a multifaceted factor with seemingly opposite functions in cancer biology. In pancreatic carcinoma and in melanoma, FOXP3 has a tumor-enhancing role through Tregs and their effect on tumor tolerance [6, 7]; in ovarian, breast and prostate cancer, FOXP3 has a tumor-suppressing function [8, 9]. In breast and prostate cancer, FOXP3 may modulate the expression of oncogenes or tumor suppressor genes, including ERBB2, SKP2, c-Myc, p21 and other important cancer-related genes [9-11]. Finally, adult T-cell leukemia/lymphoma cells from blood and skin tumors express FOXP3 at high levels but lack suppressor activity, suggesting that in these cells, despite their derivation from the immune system, the role of FOXP3 is unrelated to immune escape [12].

We investigated FOXP3 expression in normal brains and in gliomas, the most frequent of primary brain tumors. We focused our studies on glioblastoma (GB), the most malignant glioma, and GB stem-like cells, the GB subpopulation relevant for tumor perpetuation [13]. A growing number of data has been obtained in recent years, contributing to an improved definition of the GB genome [14]. The identification of mutations of isocytrate dehydrogenase 1 (IDH1) has been particularly relevant [15], contributing to novel efforts aimed at therapeutic targeting of the GB genome [16]. Another example is provided by the identification of increased copy number of TACC3, an Aurora-A kinase substrate [17]: we have recently collaborated to the identification of a fusion protein of TACC3 with fibroblast growth factor receptor with constitutive kinase activity, triggering aneuploidy in GB cells [18]. Here we have found that FOXP3 is involved in modulating the biological properties of GB stem-like cells, such as proliferation and migration, by activation of p21 and repression of c-MYC expression.

#### Results

#### FOXP3 is strongly down-regulated or absent in glioblastoma

In the initial experiments shown in Figure S1, we found Foxp3+ cells in murine malignant gliomas derived from the GL261 cells. Foxp3 expression was detectable in the early stages of tumor development (10 days after the intracranial injection) but disappeared with tumor progression (20 days after the intracranial injection) (Figure S1A-B). An immunofluorescence analysis confirmed that the Foxp3 + cells were not of immune origin (Figure S1C).

We then investigated the expression of FOXP3 in human GB specimens. An immunohistochemistry analysis of 35 GB showed variable expression of FOXP3 (Table S1), with most of the specimens displaying complete absence or scarcity (less than 20%) of FOXP3+ cells: only 4 of 35 GB (11%) showed moderate or strong staining for FOXP3 (Table S1 and Figure 1A and B). By histological analysis, we detected positive nuclear staining for FOXP3 not only in small lymphocytes but also in cells with neoplastic features, such as irregular hyperchromatic nuclei (Figure 1B left panel). To ascertain identity of FOXP3+ cells, the we performed combined immunostaining for FOXP3, CD3 and GFAP. FOXP3+ tumor cells were identified by GFAP expression and negativity for the CD3 marker (Figure 1B central and right panel).

We used real-time PCR to evaluate the mRNA expression of FOXP3 in 11 low-grade gliomas (LGG), 59 GB (55 primary and 4 recurrent) and 20 GB primary cell lines growing in culture as neurospheres (NS)

(Figure 1C). FOXP3 expression was significantly higher in LGG in GB (mean  $\pm$  SD: 0.4  $\pm$  0.4 fold, P < 0.0001 vs. normal brain). GB may or may not give rise to NS (GB-NS-Yes and GB-NS-No, respectively): 58% of these tumors (34/59) were GB-NS-Yes and had lower FOXP3 expression compared to GB-NS-No (mean  $\pm$  SD: 0.3  $\pm$ 0.3 vs.  $0.6 \pm 0.5$ , respectively, P = 0.03). The overall survival (OS) of patients with GB-NS-Yes and lower FOXP3 expression was significantly shorter than the OS of patients with GB-NS-No and higher FOXP3 expression (P = 0.03 by Kaplan Meier analysis; Figure 1D). These patients (n = 36) had all been treated by surgery, radiotherapy and chemotherapy with temozolomide [19]. In addition, we analysed FOXP3 expression by real-time PCR in 20 GB-NS. The mean expression compared to normal brain was  $0.4 \pm 0.3$  (P < 0.0001 vs. normal brain). Only BT165-NS and the corresponding specimen expressed FOXP3 at higher levels than normal brain (1.8 and 1.5 fold change vs. control, respectively; top dot in the plot showing NS, Figure 1C).

These data were in agreement with a western blot analysis performed on 14 NS primary cell lines, 11 cell lines derived from primary GB and 3 cell lines from recurrent GB with normal brain lysate as a control (Figure 1E). Six NS cell lines expressed higher levels of FOXP3 than the others. Of these cell lines, BT165-NS grew faster than the other five cell lines and was used for further experiments. Isoform a was weakly present or absent, as also found in immortalized and malignant mammary epithelial cell lines that preferentially express the FOXP3 isoform b (Figure S2) [20].

#### FOXP3 is differentially expressed in normal brain

FOXP3 expression has not been observed in normal brain to date. We first used the public microarray dataset GSE4290 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4290) to compare FOXP3 expression in human normal brain and in GB. After a quality control evaluation, we selected 88/105 samples; 17 outliers were excluded. An analysis with the microarray GSE4290 dataset of two probe sets corresponding to the FOXP3 gene (221334 s at and 224211 s at) confirmed that the expression of FOXP3 is significantly down-regulated in 73 glioblastomas compared to 15 normal brains (P = 0.003 for 221334 s at; P = 0.008 for 224211 s at; Figure 2A). We then investigated FOXP3 expression by immunohistochemistry in five-month-old human fetal brain. FOXP3+ cells were found in the periventricular zone (Figure 2B, left) and the cortical area (Figure 2B, right). Of note, in the periventricular zone, many cells co-expressed FOXP3 and GFAP, while in the cortical layer, most cells were only positive for FOXP3. In the adult brain, several FOXP3+ cells were found in cortical areas (Figure 2C, lower right), while the white matter was negative (Figure 2C, lower left).

We also analysed FOXP3 expression in the public GSE3526 microarray dataset

(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3526).

After quality controls, 138/151 samples were selected. FOXP3 was differentially expressed in the 18 brain areas represented in the data set and significantly up-regulated in the cerebellum and the putamen (Figure 2D).

## FOXP3 affects glioblastoma proliferation and migration both in vitro and in vivo

To study the regulation of FOXP3 expression in GB, we first tested the effects of TGF- $\beta$ , because this factor is a major regulator of FOXP3 expression in T lymphocytes [21, 22]. TGF- $\beta$ 1 and TGF- $\beta$ 2 did not influence FOXP3 mRNA and protein levels in three GB-NS (Figure 3A and B). We then investigated the biological effects of the modulation of FOXP3 expression by either over-expression by vector transfection or silencing via a lentiviral vector.

To investigate their specific role, FOXP3 isoforms a and b were separately over-expressed in GB-NS. The over-expression was confirmed by real-time PCR and western blot analysis using the empty vector as the internal control (Figure 3C and D). FOXP3-b was significantly reduced 48 h after transfection (P < 0.001, Figure 3C); a similar significant reduction was observed for FOXP3-a (Figure S3A). We then verified the impact of FOXP3 up-regulation on proliferation and migration by performing in vitro proliferation and migration assays. Reduced proliferation was observed when the FOXP3 isoform b (Figure 3E) or isoform a (Figure S3B) was over-expressed ( $P < 10^7$ from 6 to 24 h; P = 0.001 at 36 and 48 h). Differences in the proliferation rates decreased after 24 h in parallel with FOXP3 expression. Over-expression of FOXP3 b but not a was associated with a significant reduction of migration compared to the empty vector (P = 0.003) (Figure 3F and Figure S3B, respectively). The GB-NS in which isoforms a and b were overexpressed displayed

phenotypic changes. In particular, we observed rare neurospheres and single cells attached to the plate (data not shown).

FOXP3 silencing was studied in BT165-NS cell line, which expresses measurable levels of FOXP3 and has an adequate proliferation rate that allows in vitro propagation. We obtained a 59% decrease of FOXP3 protein as shown by immunoblotting (Figure 4A); decreased FOXP3 expression was also confirmed by real time-PCR (not shown). The effects of FOXP3 inhibition on proliferation and migration were then evaluated in shFOXP3-NS and in scrambled-NS as the control (Figure 4B, upper panel). Proliferation assays performed at 24 h, 48 h and 72 h showed that shFOXP3-NS proliferate significantly more than scrambled-NS (24 h P < 0.01; 48 h P = 0.02, 72 h P = 0.04). shFOXP3-NS also had a significantly higher migration capacity compared to scrambled cells (2.8 fold vs. scrambled cells, P = 0.003; Figure 4B, lower panel). In vivo, we found that mice injected with shFOXP3-NS survived significantly less than mice injected with scrambled-NS (mean  $\pm$  SD: 41.6  $\pm$  1.1 vs. 65.6  $\pm$  2.2 days, P = 0.002; Figure 4C). Histology and immunohistochemistry of the tumors revealed an absence of FOXP3+ cells in shFOXP3 tumors (P < 0.0001), higher proliferation as measured by Ki67+ cells (P < 0.01), and higher migration ability, as evaluated by the identification of cells positive for doublecortin (DCX) in tumors originated by shFOXP3-NS compared to scrambled tumors (P < 0.001; Figure 4D and E). Overall, these data demonstrate that FOXP3 negatively affects proliferation and migration.

#### FOXP3 is a transcriptional regulator of p21 and c-myc in GB-NS

FOXP3 was shown to be involved in the induction of several tumor suppressors, including p21, p18, LAT2, and ARHGAPS in breast cancer [11]. In addition, FOXP3 was reported as to be a repressor of the oncogene c-MYC in prostate cancer [9]. We have focused our validation on p21 as a negative regulator of cell growth [23] and on c-MYC, involved in regulating proliferation and survival of glioma cancer stem cells [24, 25].

In order to directly demonstrate the FOXP3-mediated induction of p21 and repression of c- MYC, we performed a ChIP assay on GB-NS. We found that FOXP3 specifically binds the p21 transcription start site (TSS). Interestingly, specific binding of FOXP3 was also demonstrated for the TSS of c-MYC (Figure S4). We then evaluated the expression levels of FOXP3, p21 and c- MYC in 7 GB-NS lines and found that the expression of FOXP3 and p21 was weak or absent in the presence of c-MYC up-regulation (Figure 5A). To investigate further the relationship of p21, c- MYC and FOXP3 expression, we analysed p21 and c-MYC levels in BT165-NS after FOXP3 silencing or over-expression. We found a significant reduction of p21 and an increase of c-MYC expression in shFOXP3 cells (P < 0.001 and P <0.0001 compared to scrambled cells, respectively; Figure 5B). On the contrary, over-expression of FOXP3 isoform b caused a significant increase of p21 and a strong down-regulation of c-MYC expression compared to empty cells (P < 0.0001; Figure 5C). We did not find differences in p21 and c-MYC levels by over-expressing isoform a (data not shown).

These data support the evidence that FOXP3 is a direct transcriptional regulator for p21 and c-MYC.

#### Discussion

Recent reports demonstrate that FOXP3 is expressed in nonlymphocytic cells, suggesting that its expression and function are not restricted to the T-cell lineage. Normal pancreatic ducts cells are devoid of FOXP3 expression, which has been detected in human pancreatic cancer cells [6]. FOXP3 is also expressed in human melanoma cells but not in normal melanocytes [7]. In contrast, in the breast and prostate, FOXP3 is expressed in normal epithelial cells but down-regulated in corresponding cancer cells [9, 10]. In the present study, we provide evidence of down-regulation of FOXP3 expression in both primary and recurrent GB specimens and in corresponding cell lines growing as neurospheres.

We found that GB-NS express both isoforms, but isoform b is more expressed than isoform a. Treg cells co-express equal amounts of the two isoforms. Moreover, equal amounts of isoform a are localized in the cytoplasm and the nucleus, while isoform b, lacking the nuclear export signal, is primarily located within the nucleus. After specific stimulation, isoform a preferentially locates into the cytoplasm of activated T-cells [26], where it can bind NFkB and prevent its localization to the nucleus following activation stimuli [27]. This is relevant, given the important role that the NFkB signalling pathway and its target genes play in GB progression [28].

The overexpression of isoform a and b separately resulted in a similar strong suppression of proliferation, with a less potent reduction of migration in the presence of isoform a. Notably, we show for the first time that FOXP3 is expressed in normal brain, supporting further research on the potential role of FOXP3 in brain development.

The major finding of this study, however, is the contribution of FOXP3 down-regulation to GB biology. We first verified that FOXP3 expression in GB-NS is not modulated by TGF-b, a factor playing a central role in the maintenance of FOXP3 expression in Treg cells [21, 29], suggesting that FOXP3 expression in GB-NS is not related to immune escape. Instead, our data, obtained by modulating FOXP3 expression, establish a role for FOXP3 in modulating the biological properties of GB stem-like cells, including proliferation, migration and in vivo aggressiveness.

Downstream FOXP3, we found that down-regulation of p21, a protein involved in stem cell differentiation and apoptosis [23], is associated with FOXP3 down-regulation, as recently reported in breast cancer samples [11]. We also confirmed that FOXP3 is a direct transcriptional regulator of c-MYC, as described in prostate cancer [9]. c-MYC plays a role in the survival and maintenance of GB stemlike cells and is considered a central gene implicated in genetic reprogramming [30, 31]. Moreover, c-MYC is one of seven genes whose expression is associated with worse prognosis in GB [32]. In one of the mouse models developed to study glioma origin [33], cmyc expression under the GFAP promoter in developing astroglia predisposes to malignant gliomas [34]. These tumors seem to originate from GFAP expressing cells in the ventricular zone, indicating that during astrocytic development, c-myc overexpression is sufficient to promote a neoplastic process by inducing the proliferation of early astroglial cells. This scenario and our results, in particular the presence of periventricular FOXP3/GFAP+ cells in normal fetal brains, support the hypothesis that during astrocytic development, and possibly during astrocytic proliferation in reactive gliosis [35], FOXP3 orchestrates the induction of astroglial terminal differentiation by preventing c-myc activation and proliferation in glial cell precursors.

Further studies are required to investigate the molecular mechanisms responsible for FOXP3 down-regulation in glioblastomas. Phosphatidylinositol 3 kinase (PI3K) signalling, necessary to stimulate glioma invasion and migration [36, 37] downregulates Foxp3 expression by sequestering FoxO1 and FoxO3a factors in the cytoplasm [38]. Thus, a thorough investigation of the role of this pathway in down regulation of Foxp3 expression in GB is of interest, also considering that FoxO3a is an important regulator of differentiantion and tumorigenicity of GB-NS [39].

Dysregulation of the epigenetic control of Foxp3 expression can also play a role in downregulation of Foxp3 expression [40]. Studies on FOXP3 promoter and surrounding chromatin in Treg reveal that histone H4 is hyperacetylated when the gene is activated suggesting that FOXP3 expression may be sensitive to regulation by HDAC inhibitors (HDACi). Polycomb histone methyltransferease can silence the Foxp3 promoter [41] through the action of its catalytic subunit EZH2, that we and others have found upregulated in GB and malignant gliomas [42], [43]. Besides it has been demonstrated that SAHA delays GBM growth in vitro through accumulation of cells in G2-M and also in vivo by slowing development of intracranial GBM thank to its capacity to cross the blood-brain-barrier (Yin D. et al., 2007 Clin Cancer Research). Because histone deacetylation of tumor suppressor genes occur in a variety of human tumor it is important to investigate the effects of HDACi such as SAHA and Trichostatin on FOXP3 expression and also on GBM growth. Furthermore, DNA methyltransferase DNMT1, involved in the maintenance and self-renewal of progenitor cells in somatic tissues [44], and DNMT3B are up-regulated in gliomas [45]. The inhibition of DNMT1 and DNMT3B in T-cells leads to FOXP3 expression, suggesting another pathway of FOXP3 regulation by epigenetic modification.

Although accumulating evidence indicates that FOXP3 activates and inhibits a large group of genes by inducing histone modification, how FOXP3 regulates the epigenetic machinery remains largely unknown.

Identification of FOXP3 as an X-linked tumor suppressor gene in both males and females raised the intriguing question of whether FOXP3 polymorphism may contribute to cancer susceptibility in humans. This issue can be tested in male cancer with evidence of non-male-to-male transmission or in female heterozygous carriers of a FOXP3 mutation.

#### **Material and Methods**

#### Tumor specimens and cell cultures

Primary glioblastoams (GB), recurrent glioblastoma (GBMR) and grade II gliomas, including oligoastrocytomas, fibrillary and gemistocytic astrocytomas (low-grade gliomas, LGG), were obtained from the department of Neurosurgery of the "Istituto Neurologico Carlo Besta" after the informed consent of the patients was obtained. Glioma specimens were frozen and/or placed in a saline solution after surgery. GB cell lines were obtained after dissociation in collagenase type I (Invitrogen-Life Technologies, Carlsbad, California, USA) and grown as neurospheres (GB-NS) in DMEM/F12 (GIBCO- Life Technologies, Carlsbad, California, USA) containing penicillinstreptomycin (1:100, EuroClone, Milan, Italy), B-27 (1:50, GIBCO-Life Technologies), human recombinant fibroblast growth factor 2 (bFGF; 20 ng/mL; Tebu-bio, Milan, Italy), and epidermal growth factor (EGF; 20 ng/mL; Tebu-bio).

#### **RNA extraction and Real-Time PCR**

Total RNA was extracted using Trizol (Life Technologies, Rockville, Maryland, USA) from human snap frozen tissues and human GB-NS. Total RNA was reverse-transcribed using a High Capacity cDNA Synthesis KIT (Applied Biosystems-Life Technologies, Carlsbad, California, USA). The expression of FOXP3 was analysed by real-time PCR TaqMan chemistry, performed on an ABI PRISM 7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The FOXP3 primer (FAM dye-labelled) was provided by an on-demand TaqMan Gene expression Assay (Hs00203958\_m1, Applied Biosystems). Beta-2-microglobulin (Hs99999907\_m1, Applied Biosystems) was chosen as the reference gene, and commercial RNA from a normal human brain (Life Technologies) was used as the calibrator for the calculation of fold expression levels with the  $\Delta\Delta$ Ct method. The RNA inputs were normalized against beta-2-microglobulin.

#### Western blot analysis and antibodies.

Membranes with transferred proteins were incubated with a primary antibody; either anti-FOXP3 antibody (1:250; eBioscience, Science Center Drive, San Diego, USA) or anti-alpha-tubulin antibody (1:5000). The primary antibody interaction was followed by incubation with peroxidase conjugated to the secondary antibody [anti-rat (1:10000), anti-mouse (1:10000) or anti-rabbit (1:10000). Chemoluminescence was detected using the ECL (enhanced chemiluminescence) Plus kit (Amersham, GE Healthcare). Human normal brain tissue lysate (GeneTex, Inc., Irvine CA) was used as the control.

#### Silencing and overexpression of FOXP3

The cells were transduced with lentiviral particles (MISSION shRNA Lentiviral Vectors, Sigma Aldrich, St. Louis, Missouri, USA) containing FOXP3 specific shRNA sequences (shFOXP3) according to the manufacturer's recommendations. Five different FOXP3specific sequences were screened, and the most efficient sequence was chosen. As a negative control, we used shRNA Lentiviral Particles encoding non-specific shRNA (scrambled cells). Four days after infection, cells were selected for puromycin resistance (1.2 mg/ml) for one week.

Two pCMV6-FOXP3 vectors (OriGene Technologies, Inc., Rockville, MD) were used to transfect GB stem-like cells for FOXP3 overexpression: variant 1 (isoform a) or variant 2 (isoform b). The pCMV6-empty vector was used as the negative control.

#### In vivo experiments

Sixteen immune-deficient CD1-nude mice received a brain injection of  $10^5$  "silenced" or "scrambled" cells (n = 5/group for survival, n = 3/group for histological studies). The stereotactic coordinates with respect to the bregma were 0.7 mm posterior, 3 mm left lateral, and 3.5 mm deep into the nucleus caudatum. The animals were monitored every day until they were euthanized, in accordance with the current directives of the Campus animal IFOM-IEO house facility, the Ethics Committee of the Institution and the Minister of Health.

#### **Proliferation and migration Assay**

The Cell Proliferation Reagent WST-1 (Roche Applied Science, Hague Road Indianapolis USA) was used to test for GB-NS proliferation and was performed by plating 5000 cells/well, as suggested by the manufacturer. Eight replicates per point were completed. In vitro migration was assayed using the Transwell-96 system (BD Bioscience,Qume Drive San Jose, CA, USA) as provided by the manufacturer. After 24 h, migrated cells were stained with crystal violet solubilised with 10% acetic acid.

#### Immunohistochemistry and immunofluorescence

Paraffin was removed with xylene and the sections were rehydrated in graded alcohol. Antigen retrieval was carried out using preheated target retrieval solution (pH 6.0), and the primary antibodies were incubated overnight. The following antibodies were used: FOXP3 (eBiosciences; 1:40), Ki67 (BD Bioscience; 1:50), and CD3 (1:100; Thermo Scientific, Wyman Street Waltham MA, USA). Single immunostains were performed using a standard immunoperoxidase protocol (Vectastain Elite ABC kit, PK-6100; Vector Laboratories, Inc., Burlingame, CA, USA) followed by a diaminobenzidine chromogen reaction (Peroxidase substrate kit, DAB, SK-4100; Vector Lab). The tumor sections were also stained with hematoxylin and eosin to assess the volume of tumor growth. Bright field combined immunostains were performed using the rat-on-mouse HRP-Polymer Kit (Biocare Medical, Pike Lane Concord, CA, USA) for the detection of FOXP3 or the MACH4 Universal AP Polymer Kit (Bio care Medical) for the detection of CD3 and GFAP. The chromogen reaction was developed by DAB, Ferranti Blue or Alkaline Phosphatase/RED, Rabbit/Mouse (DAKO), and the nuclei were counterstained with methyl green. For the double immunofluorescence analysis, tumor sections were incubated with Alexa Fluo-conjugated

antibodies for 1 h, counterstained with DAPI (4',6-diamidino-2phenylindole, Sigma), and examined using a LEICA SP2 confocal microscope.

#### Statistical analysis

Cumulative survival curves were constructed by Kaplan–Meier method (MedCalc 9.3). Statistical comparisons of data sets were performed by Student's two-tailed t-test, and the results were considered significant at P < 0.05.

#### References

1. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol. 2005; 6: 331-337.

Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol. 2006;
24: 209-226.

3. Allan SE, Passerini L, Bacchetta R, Crellin N, Dai M, Orban PC, Ziegler SF, Roncarolo MG, Levings MK. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. J Clin Invest. 2005; 115: 3276-3284.

4. Anichini A, Molla A, Vegetti C, Bersani I, Zappasodi R, Arienti F, Ravagnani F, Maurichi A, Patuzzo R, Santinami M, Pircher H, Di Nicola M, Mortarini R. Tumor-reactive CD8+ early effector T cells identified at tumor site in primary and metastatic melanoma. Cancer Res. 2010; 70: 8378-8387.

5. Karanikas V, Speletas M, Zamanakou M, Kalala F, Loules G, Kerenidi T, Barda AK, Gourgoulianis KI, Germenis AE. Foxp3 expression in human cancer cells. J Transl Med. 2008; 22: 6-19.

6. Hinz S, Pagerols-Raluy L, Oberg HH, Ammerpohl O, Grussel S, Sipos B, Grützmann R, Pilarsky C, Ungefroren H, Saeger HD, Klöppel G, Kabelitz D, Kalthoff H. Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. Cancer Res. 2007; 67: 8344-8350.

7. Ebert LM, Tan BS, Browning J, Svobodova S, Russell SE, Kirkpatrick N, Gedye C, Moss D, Ng SP, MacGregor D, Davis ID, Cebon J, Chen W. The regulatory T cell-associated transcription factor FoxP3 is expressed by tumor cells. Cancer Res. 2008; 68: 3001-3009.

8. Zhang HY, Sun H. Up-regulation of Foxp3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer. Cancer Lett. 2010; 287: 91-97.

9. Wang L, Liu R, Li W, Chen C, Katoh H, Chen GY, McNally B, Lin L, Zhou P, Zuo T, Cooney KA, Liu Y, Zheng P. Somatic single hits inactivate the X-linked tumor suppressor FOXP3 in the prostate. Cancer Cell. 2009 16: 336-346.

10. Zuo T, Liu R, Zhang H, Chang X, Liu Y, Wang L, Zheng P, Liu Y. FOXP3 is a novel transcriptional repressor for the breast cancer oncogene SKP2. J Clin Invest. 2007; 117: 3765-3773.

11. Liu R, Wang L, Chen G, Katoh H, Chen C, Liu Y, Zheng P. FOXP3 up-regulates p21 expression by site-specific inhibition of histone deacetylase 2/histone deacetylase 4 association to the locus. Cancer Res. 2009; 69: 2252-2259.

12. Shimauchi T, Kabashima K, Tokura Y. Adult T-cell leukemia/lymphoma cells from blood and skin tumors express cytotoxic T lymphocyte-associated antigen-4 and Foxp3 but lack suppressor activity toward autologous CD8+ T cells. Cancer Sci. 2008; 99: 98-106.

13. Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, Pastorino S, Purow BW, Christopher N, Zhang W, Park JK, Fine HA. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell. 2006; 9: 391-403.

14 Parsons DW. The evolving picture of the glioblastoma genome. Oncotarget. 2010; 1:237-8.

15. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, et al. An Integrated Genomic Analysis of Human Glioblastoma Multiforme. Science. 2008; 321: 1807-1812.

16. Wolf A, Agnihotri S, Guha A. Targeting metabolic remodeling in glioblastoma multiforme. Oncotarget. 2010; 1: 552-62.

17. Duncan CG, Killela PJ, Payne CA, Lampson B, Chen WC, Liu J, Solomon D, Waldman T, Towers AJ, Gregory SG, McDonald KL, McLendon RE, Bigner DD, Yan H. Integrated genomic analyses identify ERRFI1 and TACC3 as glioblastoma-targeted genes. Oncotarget. 2010; 1: 265-77.

18. Singh D, Chan JM, Zoppoli P, Niola F, Sullivan R, Castano A, Liu EM, Reichel J, Porrati P, Pellegatta S, Qiu K, Gao Z, Ceccarelli M, Riccardi R, Brat DJ, Guha A, et al. Transforming fusions of FGFR and TACC genes in human glioblastoma. Science. 2012; 337: 1231-5.

19. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005; 352: 987-996.

20. Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W, Liu Y, Wang Y, Liu X, Chan MW, Liu JQ, Love R, Liu CG, Godfrey V, Shen R, Huang TH, et al. FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. Cell. 2007; 129: 1275-1286.

21. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J Exp Med. 2005; 201: 1061-1067.

22. Coffer PJ, Burgering BM. Forkhead-box transcription factors and their role in the immune system. Nat Rev Immunol. 2004; 4: 889-899.

23. Dotto GP. p21(WAF1/Cip1): more than a break to the cell cycle? Biochim Biophys Acta. 2000; 1471: M43-56.

24. Wang J, Wang H, Li Z, Wu Q, Lathia JD, McLendon RE, Hjelmeland AB, Rich JN. c-Myc Is Required for Maintenance of Glioma Cancer Stem Cells. PlosOne. 2008; 3: 3769-3769

25. Panicker SP, Raychaudhuri B, Sharma P, Tipps R, Mazumdar T, Mal AK, Palomo JM, Vogelbaum MA, Haque SJ. p300- and Mycmediated regulation of glioblastoma multiforme cell differentiation. Oncotarget. 2010; 1: 289-303.

26. Li B, Samanta A, Song X, Furuuchi K, Iacono KT, Kennedy S, Katsumata M, Saouaf SJ, Greene MI. FOXP3 ensembles in T-cell regulation. Immunol Rev. 2006; 212: 99-113.

27. Grant C, Oh U, Fugo K, Takenouchi N, Griffith C, Yao K, Newhook TE, Ratner L, Jacobson S. Foxp3 represses retroviral transcription by targeting both NF-kappaB and CREB pathways. PLoS Pathog. 2006; 2: e33.

28. Nogueira L, Ruiz-Ontañon P, Vazquez-Barquero A, Moris F, Fernandez-Luna JL. The NFkB pathway: a therapeutic target in glioblastoma. Oncotarget. 2011; 2: 646-653.

29. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? Nat Rev Immunol. 2009; 9: 83-89.

30. Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, Perry SR, Tonon G, Chu GC, Ding Z, Stommel JM, Dunn KL, Wiedemeyer R, You MJ, Brennan C, Wang YA, et al. p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. Nature. 2008; 455: 1129-1133.

31. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature. 2007; 448: 318-324.

32. Bredel M, Scholtens DM, Harsh GR, Bredel C, Chandler JP, Renfrow JJ, Yadav AK, Vogel H, Scheck AC, Tibshirani R, Sikic BI. A network model of a cooperative genetic landscape in brain tumors. JAMA. 2009; 302: 261-275.

33. Muñoz DM, Guha A.Mouse models to interrogate the implications of the differentiation status in the ontogeny of gliomas. Oncotarget. 2011; 2: 590-598.

34. Jensen NA, Pedersen KM, Lihme F, Rask L, Nielsen JV, Rasmussen TE, Mitchelmore C. Astroglial c-Myc overexpression predisposes mice to primary malignant gliomas. J Biol Chem. 2003; 278: 8300-8308.

35. Robel S, Berninger B, Gotz M. The stem cell potential of glia: lessons from reactive gliosis. Nat Rev Neurosci. 2011; 12: 88-104.
36. Luk SK, Piekorz RP, Nürnberg B, Tony To SS. The catalytic phosphoinositol 3-kinase isoform p110δ is required for glioma cell migration and invasion. Eur J Cancer. 2012; 48: 149-57

37. Weber GL, Parat MO, Binder ZA, Gallia GL, Riggins GJ. Abrogation of PIK3CA or PIK3R1 reduces proliferation, migration, and invasion in glioblastoma multiforme cells. Oncotarget. 2011; 2: 833-849.

38. Merkenschlager M, von Boehmer H. PI3 kinase signalling blocks Foxp3 expression by sequestering Foxo factors. J Exp Med. 2010; 5: 1347-50.

39. Sunayama J, Sato A, Matsuda K, Tachibana K, Watanabe E, Seino S, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A, Kitanaka C. FoxO3a functions as a key integrator of cellular signals that control glioblastoma stem-like cell differentiation and tumorigenicity. Stem Cells. 2011; 29: 1327-1337

40. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. Blood. 2009; 114: 3727-3735.

41. Xiong Y, Khanna S, Grzenda A, Sarmento OF, Svingen PA, Lomberk GA, Urrutia RA, Faubion WA Jr. Polycomb antagonizes p300/CBP associated factor (PCAF) to silence FOXP3 in a Kruppel like factor (KLF)-dependent manner. J Biol Chem. 2012; DOI/10.1074/jbc.M111.325332 42. Orzan F, Pellegatta S, Poliani PL, Pisati F, Caldera V, Menghi F, Kapetis D, Marras C, Schiffer D, Finocchiaro G. Enhancer of Zeste 2 (EZH2) is up-regulated in malignant gliomas and in glioma stem-like cells. Neuropathol Appl Neurobiol. 2011; 37: 381-394.

43. Smits M, Nilsson J, Mir SE, van der Stoop PM, Hulleman E, Niers JM, de Witt Hamer PC, Marquez VE, Cloos J, Krichevsky AM, Noske DP, TannousBA,Würdinger T. miR-101 is down-regulated in glioblastoma resulting in EZH2-induced proliferation, migration, and angiogenesis. Oncotarget. 2010; 1: 710-720.

44. Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA. DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature. 2010; 463: 563-567.

45. Rajendran G, Shanmuganandam K, Bendre A, Mujumdar D, Goel A, Shiras A. Epigenetic regulation of DNA methyltransferases: DNMT1 and DNMT3B in gliomas. J Neurooncol. 2011; 104: 483-494.

# Figures



**Figure 1. FOXP3 is strongly down-regulated in GB specimens and in corresponding GB-NS lines.** A) Two representative GB specimens labelled with anti-human FOXP3 antibody (magnification 20X). The

section on the left shows one GB completely negative for FOXP3 reactivity. The section on the right shows one GB with FOXP3+ cells mainly concentrated around blood vessels. B) Left panel. FOXP3 staining is detected in one cell with lymphocyte morphology (small and round nucleus; arrow, brown) and in one cell with the morphologic features of tumor cells (large, irregular nucleus, brown). Central panel: Infiltrating lymphocytes identified using CD3 as the marker are localized near blood vessels (thin arrow, blue). Right panel: one FOXP3+ GFAP+ tumor cell (thick arrow, red) is located around a blood vessel together with a CD3+ lymphocyte (thin arrow) (magnification 40X). C) Real-time PCR analysis was performed on 11 LGG, 59 GB and 20 GB-NS. Normal brain was defined as 1.0. The GB specimens were divided into GB-NS-Yes (34/59) and GB-NS-No (25/59) based on their capacity to give rise to NS. GB-NS-Yes expressed less FOXP3 compared to GB-NS-No (P = 0.03). GB-NS shows FOXP3 down-regulation, with the exception of one line (BT165-NS). D) NS formation is associated with shorter survival. A Kaplan Meier survival analysis showed that the median survival of patients with GB forming NS (GB-NS-Yes; n = 18) was lower than that of other patients (GB-NS-No; n = 18) (9.2 months vs. 13.0 months; P = 0.03). E) A western blot shows FOXP3 in GB-NS lines derived from primary (11 lines) or relapsing GB (3 lines) and in normal brain (NB). FOXP3 isoform a is weakly detected in all cases.



**Figure 2. FOXP3 is expressed in human fetal and adult normal brain.** A) Boxplots represent FOXP3 expression reported on the y-axis as the log2-transformed probe set intensity in GB compared to NB (GSE4290 dataset). The probe set intensity signal represents the amount of FOXP3 mRNA. The boxes are drawn from the 25<sup>th</sup> and 75<sup>th</sup> percentiles in the distribution of FOXP3 intensity. The median FOXP3 expression is higher in NB than in GB (see also Methods in Supplementary Data). B) FOXP3 expression in a normal fetal brain. Combined FOXP3 and GFAP double staining was carried out on the periventricular zone (left) and on the cortical layer (right). C) FOXP3 immunohistochemistry was performed on adult human brain obtained by Biochain (Hayward, CA USA). The upper panel shows the brain area investigated (2.5X); the lower panels show the white matter (i) and the cortical area (ii) (20X). D) Boxplots represent the log2-

transformed, probe set intensity of FOXP3 (221333\_at probe set) categorized for 18 brain areas. The probe set intensity signal is the measure of FOXP3 mRNA. The probe set 221333\_at that targets FOXP3 was significantly up-regulated in the putamen compared to the other 10 areas (\* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.005). All P values were calculated using a t-test, except for the P values of the occipital lobe and the temporal lobe, which were obtained using a Wilcoxon test (Method in Supplementary Data).



Figure 3. Modulation of FOXP3 expression affects the proliferation and migration of GB-NS. A and B) Three GB-NS lines (BT150-NS, BT165-NS, BT168-NS) were treated with 5, 10, or 20 ng/ml of TGF- $\beta$ 1 or TGF- $\beta$ 2 for 24 h or 48 h. The FOXP3 transcript was evaluated by real-time PCR. Histograms represent the mean  $\pm$  SD of three different GB-NS lines. C) The relative expression of FOXP3-b vs. empty after transfection with pCMV6 retroviral vector shows a significant increase in FOXP3-b levels (2022.6  $\pm$  240.1 fold vs. empty vector at 24 h), decreasing significantly at 48 h (804.2  $\pm$  56.9 48 h fold vs. empty vector (P < 0.0005 vs. 24 h). D) A western blot displays a

significant increase of FOXP3 in FOXP3-*b* pCMV6 cells compared to empty cells. Alpha-tubulin was used as the control. The data for FOXP3 over-expression are based on a representative cell line; this experiment was performed using four GB-NS. E) The proliferation analysis indicates that pCMV6 FOXP3-b cells proliferate significantly less than empty cells (\* P < 0.01). F) The migration assay shows a significant decrease in pCMV6 FOXP3-b cells vs. empty cells (\*\* P < 0.001).



Figure 4. FOXP3 silencing affects migration and proliferation in vitro and in vivo. A) Western blot analysis of FOXP3 expression in BT165-NS after infection with the shFOXP3 lentiviral vector reveals a 59% reduction of FOXP3 vs. scrambled compared to scrambled; i.e., containing casual non-specific shRNA. FOXP3 expression was normalized with alpha-tubulin and measured with the ImageJ setting the scrambled FOXP3 level at 100%. Alpha-tubulin was used as the control. B) Upper panel: proliferation analysis after FOXP3 silencing. shFOXP3-NS proliferate significantly more than scrambled-NS (\* P <0.05). Lower panel: migration of shFOXP3 NS vs. scrambled NS. Significantly increased migration was observed in shFOXP3 cells vs. scrambled cells (\*\* P < 0.01). C) Kaplan Meier survival analysis of immunodeficient mice injected with 105 BT165-NS transduced with shFoxp3 lentiviral vector or scrambled lentiviral vector (n = 5 per group). D) Immunohistochemistry (evaluations on five 40X independent fields) showed that gliomas from scrambled-NS contain clusters of tumour cells with nuclear FOXP3 staining, while shFOXP3 gliomas were negative. The shFOXP3 tumors have a higher proliferation index (Ki67) and a higher positivity for DCX compared to scrambled tumors. E) Histograms represent the quantification of immunohistochemical staining for FOXP3 ( $0.0 \pm 0.0$  % cells in shFOXP3 tumour vs.  $21.8 \pm 5.9$  % in scrambled tumors), Ki67, and DCX positive cells in shFOXP3 and scrambled tumors (\* P < 0.01; \*\*\* P < 0.0001, \*\*\*\* P < 0.000001). Three mice for each group were studied, and representative images for each tumor are displayed.



Figure 5. FOXP3 is a transcriptional regulator of p21 and c-MYC. A) RT-PCR showed that c-MYC expression is high in GB-NS expressing low levels of FOXP3 and p21. B) A decrease in p21 and an increase in c-MYC expression is observed when FOXP3 is silenced. C) Induction of p21 and reduction of c-MYC expression are induced by FOXP3 over-expression. (\*\* P < 0.005, \*\*\* P < 0.0001).

Figure S1. Foxp3 expression in murine GL261 glioma during tumor development.



A) GL261 glioma 10 days after tumor implantation: Foxp3 positive cells present morphological features of tumor cells with large and irregular nuclei (magnification 40X). B) GL261 glioma 20 days after tumor implantation: Foxp3 positive cells are absent (magnification 40X).

C) Immunofluorescence of Foxp3 and CD4 staining of paraffinembedded GL261 glioma: Foxp3<sup>+</sup> cells (red) and CD4<sup>+</sup> cells (green). We detected Foxp3 positive cells into the tumor mass while CD4 positive cells are mainly disseminated along a blood-vessel. DAPI staining (blue) highlights the abnormal number of nuclei per cell, a characteristic of tumor cells. Figure S2. Characterization of FOXP3 in primary NS cell lines compared to Immortalized and Malignant Mammary Epithelia Cells



Western Blot shows FOXP3 protein level in BT165-NS compared to immortalized (MCF10A) and malignant (BT474, MCF7 and SKBr3) mammary epithelial cells. Vinculin has been used as a loading control. These cell lines preferentially express the FOXP3 isoform b, as reported by Zuo et al [1]. BT165 –NS cell line reported in Figure S2 expresses moderate levels of isoform a.

Figure S3. Effects of FOXP3 a overexpression on proliferation, migration and apoptosis.



A) Relative expression of FOXP3 after transfection of the FOXP3-a construct increased at 24h after transfection ( $P = 10^{-6}$ ). B) Left panel. Proliferation of BT165-NS cells transfected with pCMV6 FOXP3-a is slower than that of empty cells (\*\*\* P < 0.0001, \*\* P < 0.005, \* P < 0.01). Right panel. Migration assay shows that the decrease in cell migration of pCMV6 FOXP3-a cells vs empty cells is not significant (P = 0.06).

Figure S4. Binding of FOXP3 to p21and c-myc Transcription Starting Sites (TSS) in GB-NS.



A-B) Nuclear preparations from BT165-NS were fixed with paraformaldehyde. After sonication, genomic DNA associated with FOXP3 was immune-precipitated and quantified by RT-PCR. The quantity of precipitated DNA was compared with the total input of genomic DNA, amplified by p21 and c-Myc specific primers. Primers mapping on telomeric regions were used as negative control (N2 primers). Non specific binding from control IgG ChIPs was subtracted and data were represented as percent input. Standard deviations from two experimental replicates are shown.

NS were processed for qChIP as previously described [2], with few modifications. Briefly, formaldehyde (37%) was added to the culture medium to a final concentration of 1%. Cross-linking was stopped by addition of glycine (0.125 M final concentration). Fixed cells were washed twice with PBS and harvested in SDS buffer (50 mM Tris pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA, and protease inhibitors). Cells were pelleted by centrifugation and resuspended in IP buffer (100 mM Tris pH 8.6, 0.3% SDS, 1.7% Triton X-100, and 5 mM

EDTA). DNA fragments with a bulk size of 300-500 bp were obtained using a Branson digital sonifier 250 D. For each immuneprecipitation, 1 ml of diluted lysate ( $5x10^6$  cells/ml) was pre-cleared using protein A beads (50% slurry protein A-Sepharose, Amersham) and immunoprecipitated overnight at 4°C with antibody specific for FOXP3 (ChIP grade antibody from Abcam, UK), in presence of 20ul of magnetic dynabeads. Beads were washed and crosslink was reversed in 1ml of water with 10% Chelex 100 (Bio-Rad, cat. no. 142-1253), and used directly for qPCR. PCR primer sequences:

### p21 primers:

p21\_Fw AGGCACTCAGAGGAGGTGAGA; p21\_Rv CAGAAACACCTGTGAACGCA.

### N2 primers:

N2\_Fw AGCTATCTGTCGAGCAGCCAAG; N2\_Rv CATTCCCCTCTGTTAGTGGAAGG.

## c-Myc primers::

c-Myc\_Fw GAAATTGGGAACTCCGTGTG; c-Myc\_Rv CTAGGGCGAGAGGGAGGTT

### **Microarray Dataset analysis**

Microarray data Selection and accession numbers. Two microarray datasets were selected from microarray experiments performed with GeneChips Affymetrix HGU133Aplus 2.0 arrays (Dataset GSE4290; 105 samples – 22 Normal Brain and 82 Glioblastomas) [3] and HGU133Aplus 2.0 arrays (Dataset GSE3526; 151 samples – 18 regions of the central nervous system). All microarray data were available from the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress).

*Pre-processing microarray data.* All arrays were tested for statistical quality controls provided by the array Quality Metrics Bioconductor package [4]: outlier samples were excluded from the analysis. This package allows users to perform a wide variety of data quality assessment approaches and to identify outlier arrays (i.e., arrays of low quality or very different from the whole dataset), in such a way to enhance statistical and biological significance of the analysis.

The Robust Multichip Average (RMA) [5] algorithm was applied to normalize with quantile method and calculate probeset intensity. Normality of the distribution and homogeneity of variance in our datasets were tested using the Shapiro–Wilk and Bartlett's tests [6]. Differences of FOXP3 expression were studied with the t-test or Wilcoxon together with a false discovery rate correction of the p-value (Bonferroni correction). The corrected p-value threshold cut-off was less or equal to 0.05.

### References

1. Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W, Liu Y, Wang Y, Liu X, Chan MW, Liu JQ, Love R, Liu CG, Godfrey V, Shen R, Huang TH, et al. FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. Cell. 2007; 129: 1275-1286.

2. Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B. Binding of c-myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. Genes Dev. 2001; 15: 2069-2082.

3. Sun L, Hui AM, Su Q, Vortmeyer A, Kotliarov Y, Pastorino S Menon J, Walling J, Bailey R, Rosenblum M, Mikkelsen T, Fine HA. Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. Cancer Cell. 2006; 9: 287-300.

4. Kauffmann A, Gentleman R, Huber W. arrayQualityMetrics--a bioconductor package for quality assessment of microarray data. Bioinformatics. 2009; 25: 415-416.

5. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of affymetrix GeneChip probe level data. Nucleic Acids Res. 2003; 31: e15.

6. McCall MN, Irizarry RA. Thawing frozen robust multi-array analysis (fRMA). BMC Bioinformatics. 2001; 12: 369.

# Table S1. FOXP3 staining in a cohort of 35 GB

	Number of positive cells per HPF (40x)					Staining intensity of positive cells			
Tumor #	0-5	6-20	21-50	51-70	71-100	0 absent	1+ weak	2+ Moderate	3+ strong
GB1	Х					Х			
GB2		Х					Х		
GB3		Х					Х		
GB4	Х					X			
GB5	Х					X			
GB6		Х					Х		
GB7		Х						Х	
GB8		Х					Х		
GB9	Х						Х		
GB10	Х						Х		
GB11	Х					Х			
GB12	Х					Х			
GB13		Х					Х		
GB14		Х						Х	
GB15	Х					Х			
GB16			Х				Х		
GB17					Х				Х
GB18				Х			Х		
GB19		Х					Х		
GB20			Х				Х		
GB21	Х								
GB22	Х								
GB23	Х								
GB24	Х								
GB25	Х								
GB26		Х							
GB27	Х								
GB28		Х							
GB29		Х							
GB30	Х								
GB31	Х								
GB32	Х								
GB33									X
GB34	Х								
GB35	X								

# Chapter 3

## Summary

The transcription factor FOXP3, a master regulator of Treg cells has been proposed to function as a tumor suppressor in breast and prostate cancer. In the present study we provide evidence that FOXP3 is expressed in normal brain but strongly down-regulated in both primary and recurrent glioblastoma (GB) specimens and in corresponding cell lines growing in culture in the presence of mitogenic factors (mostly Epidermal Growth Factor - EGF and b-Fibroblast Growth Factor – bFGF) as neurospheres (NS).

We also found that FOXP3 expression was higher in low-grade gliomas than in GB. Neurosphere generation, a feature present in 58% of the GB that we examined, correlated with lower expression of FOXP3 and shorter patient survival.

Our main result refers to the contribution of FOXP3 expression in affecting proliferation and migration in vitro and in vivo. FOXP3 was silenced in one GB-NS expressing measurable levels of the gene. Intracranial injection of these GB-NS cells in nude mouse brains increased significantly tumor development and aggressiveness. Deriving gliomas showed a total absence of FOXP3 expression associated with a significant increase in proliferation and migration. Conversely, FOXP3 over-expression impaired GB-NS migration and proliferation in vitro. We also demonstrated using ChIP that FOXP3 is a transcriptional regulator of p21 and c-MYC supporting the idea that dysregulated expression of these factors is a major mechanism of tumorigenesis driven by the loss of FOXP3 expression in gliomas. These findings support the assertion that FOXP3 exhibits tumor suppressor activity in glioblastomas.

# Conclusions

Glioblastoma is the most frequent of primary malignant brain tumors [1]. In about 90% of cases GB appears de novo; in other cases GB derives from lower grade gliomas. A number of reports have shown that in GB and other cancers, a sub-population of cells, defined as cancer stem cells (GSC) is responsible for tumor perpetuation [2]. Recent data then demonstrated that tumor microenvironment can favor the amplification of cancer cells exploiting stem cell programs for survival: hypoxia now appears as one major driver in these processes [3]. Remarkably the switch to stem cell programs may rely on epigenetic rather than genetic changes, allowing cells to adapt faster to environmental challenges, without the need of numerous cell generations required for advantageous mutations to prevail [4]. Thus, the rigid, hierarchical model of CSC initially proposed for GB and other cancers should probably be substituted by a more flexible concept of cancer stem-like cells, a subpopulation of cells fitter than others for tumor adaptation to environmental (and possibly therapeutical) challenges thanks to the exploitation of stem cell programs. In our laboratories we isolate and culture GSC from fresh GB specimens growing in the absence of serum and in the presence of EGF and bFGF as neurospheres. We appreciate that NS may mirror much more closely than previous, serum-based glioma cell lines, the actual biology of GB. Also, they are always tumorigenic in immunodeficient hosts and the tumors they form in these hosts are much more representative of the clinical

presentation of human GB [5]. More important, the potential for GB to form NS is associated to increased aggressiveness and decreased survival in patients.

Recent progress has been made in unraveling the molecular heterogeneity of GB, pointing to three subtypes characterized by different molecular alterations: proneural, proliferative and mesenchymal [6, 7]. Furthermore, more recent data we obtained in collaboration with Carla Boccaccio and collegues suggest that NS maintain in vitro many features of their original subclassification, defined in agreement to Phillips et al and Verhaak et al [8]

In this scenario, an important goal of our studies was represented by the identification of novel oncosuppressor critically involved in the GB aggressiveness.

Gaining better insights of the genetics and of the molecular regulation of FOXP3 is essential to design new therapeutic strategies. Although molecular mechanisms down-regulating FOXP3 have not been clarified yet, some agents have been reported to increase FOXP3 expression in cancer cells. Anisomycin induces FOXP3 expression in various breast cancer cell lines, resulting in a significantly repressed cell growth in vitro and in xenograft in vivo [9]. In breast and colon cancer cell lines, FOXP3 expression is directly regulated by p53. Doxorubicin, which activates p53, dramatically activates FOXP3 transcription in vitro [10].

One of the most difficult challenges in cancer therapy is to restore the function of inactivated tumor suppressors. Conversly to autosomal tumor suppressor genes often deleted and/or mutated in both alleles, mutation of X-linked tumor suppressor genes, such as FOXP3 are often heterozygous in female cancer cells [11, 12]. Since one allele of an X-linked tumor suppressor gene has not undergone selection during carcinogenesis, it may be possible to reactivate the wildtype allele for cancer therapy.

Most importantly, recent data described that FOXP3 expression in glioma cells is induced by chemotherapeutics like Camptothecin and Temozolomide and is correlated with increased apoptosis [13].

These data support our evidence that the loss of FOXP3 drives tumorigenesis by favoring proliferation and migration of glioma cells.

Importantly, our study, although basic and fundamental, has a great potential to make an impact in the treatment of brain tumours.

# **Future Perspectives**

A wide screening on genomic DNA derived from patients belonging to multi-centric and indipendent sets is required to identify inactivating mutations and or deletions of FOXP3. Once assessed that the majority of GBM tumors have at least one wt allele of FOXP3 gene, and given the detrimental effect of FOXP3 expression on tumor cell growth, it would be of great value to develop a drug or a treatment that can reactivate the FOXP3 gene in cancer. Interestingly, preliminary data indicate that GBM neurospheres up-regulate FOXP3 expression in response to differentiation stimuli, DNA damage and cell stress induced by retinoic acid, ionizing radiation and anisomycin respectively. In order to steer this research toward clinical we propose to design an in vivo and an in vitro model aimed to simulate the standard radio-chemotherapy commonly used in patients affected by GBM. We observed that the irradiation of GL261 neurosphere (murine glioma model) as well as the irradiation of rat 9L gliomas on day 7 after injection of 9L cells, causes a significant increase of FOXP3 expression with a concomitant strong reduction of nestin and a relevant expression of differentiation marker as GFAP and bIII-tubulin. Based on these preliminary results the next step would be to determine the X-ray lethal dose of human GSCs and verify if radiations cause FOXP3 up-regulation and cell differentiation. To mimic patient chemotherapy we will treat GSCs with temozolomide and/or mitoxantrone both inducing DNA damage or with radiotherapy to study FOXP3 expression

changes. The use of Vorinostat (SAHA HDAC inhibitor), causing cell growth arrest and differentiation of human breast and prostate cancer cells, could be useful to find a relationship between tumor cell-stress and FOXP3 involvement. Overall we believe that this information provides an intriguing background for studies investigating the effects of chemotherapeutic agents and their relationship with FOXP3 induction.

Part of the contradictory functions of FOXP3 in cancer may also depend on its subcellular localization that has been poorly investigated. It is possible that malignant transformation is associated with a cytoplasmic localization. Recently it has been reported that apoptotic stimuli themselves induce translocation of FOXP3 [13]. The shuttle from nucleus to cytosol depends from complex factors [14]. However the molecular interactions occuring in the cytoplasm between FOXP3 and others partners involved in carcinogenesis are still unkown and need to be investigated.

Therefore, better understanding of FOXP3 expression in normal and cancer cells may provide new approaches for cancer therapy. Another hypothesis that could explain FOXP3 downregulation in

GBM is a negative post-transcription regulation of FOXP3 by MicroRNA. First of all we want to perform a large-scale expression profiling of miRNAs in GSCs overexpressing FOXP3 to pinpoint new microRNA that could target and negatively regulate FOXP3. In particular, it would be interesting to study the role of Mir155 in GSCs, as this is a known target of FOXP3 in Treg and is overexpressed in GBM [15, 16]. A recent study in breast cancer indicates that FOXP3 induces two miRs, miR-7 and miR-155, which specifically target the 3'-UTR of SATB1, a chromatin organizer and transcription factor, also upregulated during glioma progression [17, 18].

These observations stimulate to investigate FOXP3 downregulation as part of an epigenetic signature associated to the acquisition of stem-like features in glioblastoma.

# **References:**

1. Preusser M, De Ribaupierre S, Wöhrer A, Erridge SC, Hegi M, Weller M, Stupp R: Current concepts and management of glioblastoma. *Annals of neurology* 2011, **70**:9–21.

2. Das S, Srikanth M, Kessler J a: **Cancer stem cells and glioma.** *Nature clinical practice. Neurology* 2008, **4**:427–35.

3. Soeda a, Park M, Lee D, Mintz a, Androutsellis-Theotokis a, McKay RD, Engh J, Iwama T, Kunisada T, Kassam a B, Pollack IF, Park DM: Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. *Oncogene* 2009, **28**:3949–59.

4. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg R a: An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nature genetics* 2008, **40**:499–507.

5. Tunici P, Bissola L, Lualdi E, Pollo B, Cajola L, Broggi G, Sozzi G, Finocchiaro G: Genetic alterations and in vivo tumorigenicity of neurospheres derived from an adult glioblastoma. *Molecular cancer* 2004, **3**:25.

6. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro JM, Colman H, Soroceanu L, Williams PM, Modrusan Z, Feuerstein BG, Aldape K: **Molecular**  subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006, **9**:157–173.

7. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, et al.: Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010, 17:98–110.

 De Bacco F, Casanova E, Medico E, Pellegatta S, Orzan F, Albano R, Luraghi P, Reato G, D'Ambrosio A, Porrati P, Patanè M, Maderna E, Pollo B, Comoglio PM, Finocchiaro G, Boccaccio C: The MET oncogene is a functional marker of a glioblastoma stem cell subtype. *Cancer research* 2012, 72:4537–50.

9. Liu Y, Wang Y, Li W, Zheng P, Liu Y: Activating transcription factor 2 and c-Jun-mediated induction of FoxP3 for experimental therapy of mammary tumor in the mouse. *Cancer research* 2009, **69**:5954–60.

10. Jung D-J, Jin D-H, Hong S-W, Kim J-E, Shin J-S, Kim D, Cho B-J, Hwang Y-I, Kang J-S, Lee W-J: Foxp3 expression in

**p53-dependent DNA damage responses.** *The Journal of biological chemistry* 2010, **285**:7995–8002.

11. Rivera MN, Kim WJ, Wells J, Driscoll DR, Brannigan BW, Han M, Kim JC, Feinberg AP, Gerald WL, Vargas SO, Chin L, Iafrate AJ, Bell DW, Haber DA: **An X chromosome gene, WTX, is commonly inactivated in Wilms tumor.** *Science* 2007, **315**:642–645.

12. Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W, Liu Y, Wang Y, Liu X, Chan MWY, Liu J-Q, Love R, Liu C-G, Godfrey V, Shen R, Huang TH-M, Yang T, Park BK, Wang C-Y, Zheng P, Liu Y: FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. *Cell* 2007, 129:1275–1286.

13. Held-Feindt J, Hattermann K, Sebens S, Krautwald S, Mehdorn HM, Mentlein R: The transcription factor Forkhead box P3 (FoxP3) is expressed in glioma cells and associated with increased apoptosis. *Experimental cell research* 2012, **3**:1–9.

14. Magg T, Mannert J, Ellwart JW, Schmid I, Albert MH: Subcellular localization of FOXP3 in human regulatory and nonregulatory T cells. *European Journal of Immunology* 2012, 42:1627–38. 15. Lages E, Guttin A, El Atifi M, Ramus C, Ipas H, Dupré I, Rolland D, Salon C, Godfraind C, deFraipont F, Dhobb M, Pelletier L, Wion D, Gay E, Berger F, Issartel J-P: MicroRNA and target protein patterns reveal physiopathological features of glioma subtypes. *PloS one* 2011, 6:e20600.

16. Kohlhaas S, Garden O a, Scudamore C, Turner M, Okkenhaug K, Vigorito E: Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *Journal of immunology (Baltimore, Md. : 1950)* 2009, **182**:2578–82.

17. McInnes N, Sadlon TJ, Brown CY, Pederson S, Beyer M, Schultze JL, McColl S, Goodall GJ, Barry SC: FOXP3 and FOXP3-regulated microRNAs suppress SATB1 in breast cancer cells. *Oncogene* 2011, **31**:1045–54.

18. Chu S-H, Ma Y-B, Feng D-F, Zhang H, Zhu Z-A, Li Z-Q, Jiang P-C: Upregulation of SATB1 is associated with the development and progression of glioma. *Journal of translational medicine* 2012, **10**:149.

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