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Signalling through FOXP3 as an X-linked Tumor Suppressor

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Abstract

The *FOXP3* (*forkhead box P3*) gene is a member of forkhead winged helix family transcription factors and functions as both a transcriptional activator and a repressor. FOXP3 dysfunction is responsible for an X-linked autoimmune syndrome: immune dysregulation, polyendopathy, enteropathy, X-linked syndrome. In addition to its role as an essential transcription factor in regulatory T cells, the *FOXP3* gene is an epithelial cell-intrinsic tumor suppressor for breast and prostate cancers. We will focus on the FOXP3 signalling pathway in epithelial cells and discuss how genetic and/or epigenetic inactivation of the *FOXP3* contributes to the malignant transformation of cells.

Keywords

FOXP3; epithelial cell; X-linked tumor suppressor gene; breast cancer; prostate cancer

1. Introduction

The *FOXP3* gene is a member of the forkhead winged helix family transcription factors and is essential for the development and function of CD4⁺CD25⁺ regulatory T cells (Treg) (reviewed in (Liu et al., 2010)). In addition, *FOXP3* is expressed in epithelial cells from various organs such as breast, thymus, prostate and lung (Chang et al., 2005; Chen et al., 2008). Importantly, mice that are heterozygous for *Foxp3* mutations spontaneously develop mammary carcinomas at a high frequency (Zuo et al., 2007b). Genetic analyses in both mice and humans revealed that *FOXP3* is an important X-linked tumor suppressor in breast cancers (Liu et al., 2009a; Zuo et al., 2007a; Zuo et al., 2007b). Moreover, we recently found that *FOXP3* also functions as a tumor suppressor in prostate cancer (Wang et al., 2009).

Despite the importance of FOXP3 in mammary and prostate carcinogenesis pathways, the signalling networks of FOXP3 in normal and/or malignant epithelial cells have not yet been fully elucidated. In this review, we will focus on the function of the FOXP3 as a tumor suppressor in epithelial cells, and discuss how its inactivation contributes to the malignant

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transformation of cells. We will also discuss how reactivation of FOXP3 in tumor samples may be explored for cancer therapy.

2. Functions and Pathologic Context

FOXP3 is a member of the FOXP family which has at least four members, FOXP1-4. All contain highly conserved c-terminal tetramerization domains composed of zinc-finger and leucine zipper domains and a DNA binding forkhead box domain (Fig. 1) (Lopes et al., 2006). FOXP3 localizes in the nucleus, and it functions as a sequence-specific transcription factor. The DNA binding forkhead domain of FOXP3 binds to specific DNA sequences in gene promoters: 5'-RYMAAYA-3' in which R=A/G, Y=C/T, M=A/C.

We observed that mice with germline *Foxp3* mutations are substantially more prone to developing mammary carcinomas, either spontaneously or carcinogen-induced (Zuo et al., 2007b). The role of the *Foxp3* gene in the mammary carcinogenesis has been supported by several lines of evidence (Fig. 1). The *Foxp3* gene is expressed in normal breast epithelia but is down-regulated in mammary cancer (Zuo et al., 2007b). Ectopic expression of *Foxp3* in variety of breast cancer cell lines resulted in cell cycle arrest; cessation of cell growth (Zuo et al., 2007b). Moreover, *Foxp3* directly regulates transcription of *ErbB2*, *Skp2* and p21 (Liu et al., 2009a; Zuo et al., 2007a; Zuo et al., 2007b). Furthermore, our analyses of clinical human breast cancers also supported that FOXP3 plays an important role in mammary carcinogenesis (Fig. 1). Frequent chromosomal deletions and somatic mutations of the *FOXP3* gene were detected in cancer samples (Zuo et al., 2007b). By immunohistochemistry (IHC) onto tissue microarrays, we found down-regulation of FOXP3 in cancer cells compared to normal breast epithelia (Liu et al., 2009a; Zuo et al., 2007a; Zuo et al., 2007b). Surprisingly, a recent study showed the expression of FOXP3 in over 60% of breast cancer (Merlo et al., 2009). However, this study included cancer cells expressing FOXP3 in its cytoplasmic form, which may well be mutant FOXP3 (Wang et al., 2009).

The *FOXP3* gene also plays an important role in prostate epithelia (Fig. 1) (Wang et al., 2009). Among human prostate cancers, we found frequent chromosomal deletions, somatic mutations and epigenetic silencing of the *FOXP3* gene (Fig. 1) (Wang et al., 2009). Since the *FOXP3* gene is located on the X chromosome, a genetic/epigenetic single-hit results in inactivation of this gene in males. IHC revealed that FOXP3 expression was significantly down-regulated in cancer cells when compared to normal prostate glands (Wang et al., 2009). Moreover, mice with prostate-specific ablations of *Foxp3*, *Foxp3^{fl/y}*;PB-Cre⁺, developed prostatic hyperplasia and prostatic intraepithelial neoplasm (PIN), putative pre-cancerous lesions of the prostate (Wang et al., 2009). In human samples, FOXP3 expression in PINs were already 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 (Wang et al., 2009). Importantly, we identified *c-MYC* to be directly repressed by FOXP3 in prostate epithelia (Wang et al., 2009).

A recent study reported that among 26 ovarian cancer samples, the expression of the FOXP3 was significantly decreased as compared to normal ovarian epithelia (Zhang and Sun, 2010). Another interesting aspect of FOXP3 abnormalities is that some types of cancers predominantly express splice variants of the FOXP3 protein. As shown in figure 1B, among breast and ovarian cancers, malignant melanomas and malignant T cells of Sezary syndrome, specific splice variants of the FOXP3, such as Δ3, Δ3-4, Δ3/8 and Δ8, were reported to be preferably expressed (Ebert et al., 2008; Krejsgaard et al., 2008; Zhang and Sun, 2010; Zuo et al., 2007b). The Δ3-4 splice variant resulted in a truncated FOXP3 with a premature stop codon, and therefore might contribute to the malignant progression of cells.

3. Cascades and Key Molecules

3.1. HER2/neu (ERBB2)

HER2 is a member of the transmembrane receptor tyrosine kinases and is involved in the regulations of various cellular functions such as cell growth and survival (Spector and Blackwell, 2009). The cytoplasmic portion of HER2 is phosphorylated at conserved tyrosine residues and these phosphorylated tyrosines can serve as binding sites for adaptors which link HER2 to its downstream pathways or targets such as PI3K-Akt and MAPK-Erk (Spector and Blackwell, 2009). Both *HER2* gene amplification and loss of nuclear FOXP3 contributed to HER2 overexpression in breast cancer samples (Zuo et al., 2007b). FOXP3 can repress the transcription of HER2 in human breast cancers by binding directly to *ERBB2* gene promoter (Fig. 2) (Zuo et al., 2007b). Since *in vitro* HER2 overexpression nullifies the ability of FOXP3 to inhibit cell growth (Zuo et al., 2007b), repression of HER2 may be critical for the tumor suppressor function of FOXP3 in the breast epithelial cells.

3.2. MYC

Overexpression of c-MYC contributes to more aggressive and poorly differentiated cancer phenotypes (Pelengaris et al., 2002). c-MYC is a sequence specific transcription factor and an important player of various cellular processes including cell cycle and apoptosis; processes which are also dysregulated in cancer cells with high level c-MYC expressions (Pelengaris et al., 2002). c-MYC directly activates *CDK4* and *CCND2* expression, while indirectly represses CDK inhibitors such as *CDKN1A* (*p21*) and *CDKN2B* (*p15*) expression (Pelengaris et al., 2002). Moreover, c-MYC directly up-regulates *eIF4E* and *eIF2 α* ; both of which are the rate-limiting effectors of cell cycle (Pelengaris et al., 2002). In prostatic epithelial cells, FOXP3 directly represses *c-MYC* gene expression (Fig. 2) (Wang et al., 2009). Importantly, mice with prostate-specific ablation of the *Foxp3* gene exhibited elevated expression of c-Myc, and in human prostate cancers mRNA expressions of FOXP3 and c-MYC showed an inverse relationship ($p=6.29\times10^{-6}$) (Wang et al., 2009).

3.3. p21 (CDKN1A, CIP1, WAF1)

p21 is a universal CDK inhibitor and plays an important role in regulating cell cycle progression, specifically at the G1-checkpoint (Abukhdeir and Park, 2008). It is frequently implicated in multiple malignancies including breast cancer (Abukhdeir and Park, 2008). Cancer cells with low levels of p21 can escape from G1 arrest, and thus cells acquire a growth advantage during tumor development. We have reported that in breast epithelial cells, FOXP3 occupies and activates the *p21* promoter (Fig. 2) (Liu et al., 2009a). In various breast cancer cell lines, p21 expression was significantly upregulated after *FOXP3* induction (Liu et al., 2009a). IHC of human breast cancer tissue microarray revealed that the protein expression of FOXP3 and p21 showed a positive correlation ($p=0.011$) (Liu et al., 2009a). Moreover, cell growth suppression by *FOXP3* transduction was rescued by shRNA silencing of the *p21* gene (Liu et al., 2009a). The data strongly suggests that the activation of *p21* contributes to FOXP3's tumor suppressor function.

3.4. SKP2

High levels of expression of SKP2 has been reported in a wide variety of cancers (Nakayama and Nakayama, 2006). SKP2 is an important player in the ubiquitin dependent degradation of *p27^{KIP1}*, a CDK inhibitor especially of Cyclin-E/CDK2 and Cyclin-A/CDK2 (Nakayama and Nakayama, 2006). SKP2 is robustly expressed during S and G2 phases of cell cycle and regulates p27 degradation. Thus SKP2 facilitates progression of the cell cycle. In human cancers, overexpression of SKP2 is frequently observed and is correlated with poor prognoses of breast cancers (Nakayama and Nakayama, 2006). We found that FOXP3 directly represses

SKP2 expression in human and mouse mammary epithelial cells (Fig. 2) (Zuo et al., 2007a). *Foxp3* occupies the *Skp2* promoter and represses promoter activity of the locus (Zuo et al., 2007a). Among human breast cancers, expression of FOXP3 negatively correlated with SKP2 levels ($p=0.0016$) (Zuo et al., 2007a). As in the case of p21, FOXP3 directly regulates key molecules of cell cycle regulation, which further supports the notion that FOXP3 is an important tumor suppressor.

3.5. Molecular mechanisms by which FOXP3 regulates the expression of its target genes

Previous reports have revealed that FOXP3 forms complexes with Rel family transcription factors NFAT and NF κ B, and FOXP3 blocks their ability to activate *Il-2* and *INF γ* transcription (Bettelli et al., 2005; Holmes et al., 2008). By making a repressive FOXP3:NFAT complex, FOXP3 inhibits NFAT:AP-1 complex at the *Il-2* promoter (Holmes et al., 2008; Wu et al., 2006). Another report showed that FOXP3 could also weaken DNA binding activity of AP-1 (Lee et al., 2008). AML1/RUNX1, which activates endogenous *Il-2* and *INF γ* expression in CD4⁺ T cells, is reported to make a complex with FOXP3 (Ono et al., 2007). AML1/RUNX1 could bind to the *Il-2* enhancer with FOXP3 and exert optimal repression of the *Il-2* in Tregs (Ono et al., 2007).

We and others have found that FOXP3 can dramatically change histone modifications at its binding loci (Liu et al., 2009a; Zheng et al., 2007). In this case, FOXP3 functions as a scaffolding molecule which recruits other co-factors to the FOXP3-bound loci. So far, a couple of histone modification enzymes such as TIP60, HDACs 7 and 9, and EOS/CtBP1 have been found to be physically associated with FOXP3 to form a repressor complex (Li et al., 2007; Pan et al., 2009). What FOXP3 complexes are involved in FOXP3-mediated gene activation remains elusive.

3.6. Regulation of FOXP3 expression

In Tregs, a couple of molecules and pathways have been identified to activate *Foxp3* gene expression. TGF β -Smad, *Il-2* and *INF γ* signals are known to activate *Foxp3* expression in the development of Tregs (Lal and Bromberg, 2009). SMAD, NFAT and Rel family molecules are known to occupy *Foxp3* enhancer and/or promoter loci and to activate *Foxp3* transcription (Ruan et al., 2009; Tone et al., 2008). In mammary epithelial cells, ATF-2 is essential for FOXP3 expression (Liu et al., 2009b). In cancer cells, doxorubicin, which induces activation of p53 via DNA damage response pathway, significantly upregulates *FOXP3* expression (Jung et al., 2010).

4. Therapeutic Implications

The *FOXP3* gene has important functions as a tumor suppressor of breast and prostate cancers. Thus an interesting issue is whether the *FOXP3* gene is also a tumor suppressor for other malignancies. Our investigation of heterozygous Scurfy mice *Foxp3*^{sf/+} revealed that they also harbored spontaneous lymphoma, hepatoma and sarcoma although their incidences were lower than that of mammary tumors (Zuo et al., 2007b). In addition, aberrant FOXP3 expression and/or splicing have also been reported in melanoma, malignant T cell, ovarian cancer and pancreatic cancer (Ebert et al., 2008; Hinz et al., 2007; Krejsgaard et al., 2008; Zhang and Sun, 2010). However, molecular biological functions of the FOXP3 in these malignancies have not been fully investigated.

As discussed in greater detail elsewhere (Liu et al., 2010), in female cancers, X-inactivation inactivates only one of the FOXP3 alleles, and therefore it might be possible to reactivate the silenced *FOXP3* gene from this allele for therapeutic purposes. Although molecular mechanisms have not been clarified yet, some agents have been reported to activate *FOXP3*

in cancer cells. We reported that anisomycin could induce the transcription of *FOXP3* in various breast cancer cell lines, and induced FOXP3 significantly repressed cell growth *in vitro* and tumor formations of implanted cancer cells *in vivo* (Liu et al., 2009b). In breast and colon cancer cell lines, *FOXP3* expression is directly regulated by p53, and doxorubicine which activates p53 dramatically activated *FOXP3* transcription *in vitro* (Jung et al., 2010). These reports further supports a promising hypothesis that the restoration of the FOXP3 function in tumor cells could have great potential to be a novel therapeutic strategy against human malignancies. Additional studies are needed to determine whether induction of FOXP3 can be explored for cancer therapy. And it is also substantially important to explore any methodologies and devices to re-activate the *FOXP3* gene specifically in malignant cells.

Signalling network Facts

1. FOXP3 is expressed not only in regulatory T cells but also in epithelial cells from various organs.
2. FOXP3 acts both as a transcriptional activator and repressor. FOXP3 directly regulates the expression of both oncogenes and tumor suppressor genes, including *ERBB2*, *SKP2*, *c-MYC*, *p21* and other important cancer-related genes.
3. Genetic and/or epigenetic inactivation of the *FOXP3* gene in breast and prostate epithelial cells plays an important role in the development and progression of cancers.
4. Because many important cancer-related signalling pathways are regulated by FOXP3, it may be a potential therapeutic target for human malignancies.

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Figure 1.
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. “Δ” 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. ^aSamples 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.

Figure 2.
A schematic view of the signalling networks of the FOXP3 in epithelial cells.