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IPEX Syndrome, *FOXP3* and Cancer

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Abstract

In this review, we introduce the IPEX syndrome and its relationship with germline mutations of the *FOXP3* gene. We then describe the multiple functional roles of *FOXP3* in regulatory T cells and epithelial cells as well as in IPEX syndrome and tumor progression. Potential mechanisms of *FOXP3* inactivation and transcriptional regulation are discussed with recent advances. Finally, we point out current issues and a potential *FOXP3*-mediated therapeutic strategy as well as the reactivation of *FOXP3* in patients with IPEX syndrome and cancer.

Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) Syndrome

In 1982, Powell and his colleagues first reported an X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in a family of 8 males within 3 generations [1]. Later studies identified that this X-linked syndrome is a rare genetic auto-immune disease called IPEX syndrome, characterized by the development of systemic autoimmune disorders which affect multiple organs, including the intestines, skin, and various endocrine glands/organs [2,3]. The clinical and molecular features of IPEX syndrome include severe diarrhea, diabetes, eczema, erythroderma, psoriasis, and thyroiditis, etc. Most patients with X syndrome are males, typically beginning in the first few months of life and dying within the first one to two years of life from metabolic derangements or sepsis [2,3]. IPEX syndrome is considered to be directly caused by the proliferation of autoaggressive T cells and autoantibody-producing B cells [4]. However, the molecular mechanism for IPEX syndrome has not been fully elucidated. As well as evidenced by genetic analysis, approximately 25% of males with IPEX syndrome have been identified to have germline mutations in an X-linked Forkhead box P3 (*FOXP3*) gene (Figure 1), which may cause some cases of IPEX syndrome [3,5-8].Thus, the role of *FOXP3* in the immune system is essential for understanding the molecular mechanism of IPEX syndrome.

Mutation of the FOXP3 results in the Dysfunction of Regulatory T Cells (Treg) and Leads to the IPEX Syndrome

The Human *FOXP3* gene at Xp11.23 is a member of the forkhead-box/winged-helix transcription factor family. This gene was identified during the positional cloning of *Scurfin,* and functional loss of this gene causes X-linked autoimmune diseases similar to IPEX syndrome in mice and humans [5-8]. As a transcription factor, *FOXP3* can bind to specific regions of DNA and controls the activation and repression of target genes [9-11]. *FOXP3* is essential for the maintenance of self-tolerance, the development and normal function of Treg cells, as well as the control off the immune system and the prevention of autoimmune disorders [12]. Inactivating mutations of this gene leads to the absence of Treg cells that can increase activation of T cells and immune responses to multiple self-tissues and organs, causing the multiple autoimmune disorders present in mouse as well as in patients with IPEX syndrome [5,6,8]. In males, one mutant allele of the *FOXP3* in each cell is sufficient to cause the IPEX disease. In females, IPEX is inherited in an X-linked recessive pattern. The disease only occurs in women with a mutation in both alleles. Thus, males are more prone to be affected by *FOXP3* mutations to cause X-linked IPEX syndrome, while females can be heterozygous mutant carriers to IPEX patients. In addition, although the mutations have been reported in several IPEX patients [5,13,14], some patients with IPEX syndrome who lack *FOXP3* expression do not have a detectable mutation in the *FOXP3* coding region [3] (Figure 1). Interestingly, the clinical features can also vary considerably between patients with the same mutation, suggesting that other modifying genes, epigenetic events, or environments may affect function in Treg cells [2]. Therefore, the molecular mechanism of IPEX syndrome is still not fully known.

Tumor Suppressive Function of the *FOXP3* in Epithelial Cancer Cells

FOXP3 is expressed not only in lymphocytes but also in epithelial tissues of the breast, lung, and prostate [15]. Recent studies suggested that *FOXP3* is an epithelial cell-intrinsic tumor suppressor for breast, prostate, ovary and other cancers. Aging mice with a heterozygous *Scurfy* mutation of the *FOXP3* have a high rate of spontaneous breast cancers [16]. Importantly, 40% of these mice with primary breast tumors develop lung metastases [16]. Genetic alterations of the *FOXP3* have also been identified in human breast cancer samples [16]. While the majority of wild-type *FOXP3* is expressed in the nucleus of normal epithelial cells, recent studies by our lab and others have demonstrated that *FOXP3* is localized in the cytoplasm in 60-80% of breast cancer samples 0 [16-22]. Furthermore, somatic mutations of *FOXP3* have been shown to prevent its nuclear expression [11,16,19,21,23,24], suggesting that the cytoplasmic *FOXP3* reflects an accumulation of mutant *FOXP3*.

The Xp11.22-23region has been proposed to be associated with susceptibility to prostate cancer [25-27]. X-linked *FOXP3* may contribute to susceptibility of prostate cancer. In human prostate epithelial cells, nuclear *FOXP3* is present in more than 90% of normal

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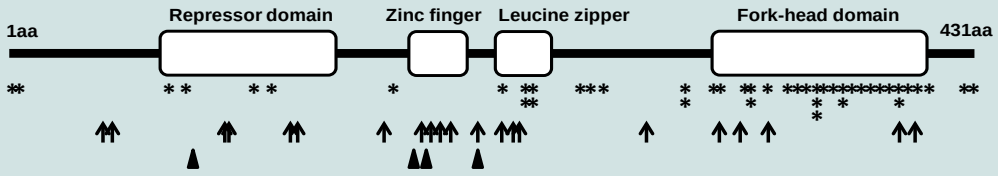

Figure 1: Functional domains and mutations in coding region of the human *FOXP3* gene. *FOXP3* transcript consists of 12 exons and the open reading frame is from exons 2 to 12. There are 4 potential functional domains (hollow boxes). Black starts indicate germline mutations identified from IPEX patients. Black arrows indicate somatic mutations from breast cancer patients, and solid triangles indicate somatic mutations from prostate cancer patients.

prostates, but only in 30% of human prostate cancers. Inactivated somatic mutation (25%) and gene deletion (14%) of the *FOXP3* have been frequently identified in prostate cancer samples (Figure 1). As supportive evidence, tumor cell growth is dramatically inhibited by *FOXP3* in prostate cancer cell lines [24]. In *in vivo* studies, *FOXP3* conditional knockout in mouse prostate epithelial cells leads to prostate hyperplasia and prostatic intraepithelial neoplasia. These data indicate that *FOXP3* is an X-linked tumor suppressor in both the mouse and human prostate, but it is likely that genetic alteration of the *FOXP3* is an early event in prostate carcinogenesis and inactivation of *FOXP3* appears to work in concert with additional genetic hits to cause prostate cancer progression.

In other cancers, *FOXP3* is weakly or not expressed in ovarian cancer cells [28]. Interestingly, *FOXP3* does not only inhibit cell proliferation, but also reduces cell migration and invasion in ovarian cancer cells [28]. In a mouse model of colon cancer, *FOXP3* treatment resulted in a significant reduction of tumor metastasis [29]. These data suggest that tumor suppressive function of *FOXP3* may also be involved in tumor invasion and metastasis.

IPEX Syndrome, Cancer, and Their Related *FOXP3* Mutation

There are few reports of IPEX patients with cancer because they die within the first one to two years. However, a case report has demonstrated that an IPEX patient with *FOXP3* mutation was managed with rapamycin and subsequently developed Epstein Barr virus induced lymphoma [30], suggesting that *FOXP3* mutation in IPEX patients may be associated with susceptibility to cancer. Although no association between *FOXP3* polymorphisms and risk of breast cancer was identified in a northern Israel population [31], it is of interest to determine whether *FOXP3* mutant carriers in females are associated with susceptibility to cancer. Interestingly, the *FOXP3* mutations in epithelial cancer cells are all somatic without any immune deficiency in the patients and there is also a significant difference in the distribution of mutations between IPEX and cancer patients (Figure 1).

FOXP3 protein contains 4 potentially functional domains including the repressor, zinc finger, leucine zipper and fork-head (FKH) domains (Figure 1). Each of these domains is related to molecular function of the *FOXP3*. Currently, there have been more than 30 germline mutations and 20 somatic mutations of this gene identified in IPEX and cancer patients [21,24,32,33], and they distribute in both the non-coding and coding regions. First, the repressor domain is localized to the N-terminal region of *FOXP3* and is required to repress NFAT-mediated transcriptional activity [33-35], which is critical for orchestrating the response of T cells to immune stimulation. *FOXP3* can also bind AP1 through its repressor domain [35,36] as same as sites with IPEX mutations such as E70H [37] and T108M [38] mutants, which may affect the maintenance of Treg cells (Figure 1). These mutations appear to be associated with milder cases of IPEX [37,38]. In breast cancer and prostate cancer, we have identified 5 nonsynonymous mutations in the repressor domain of *FOXP3* [21,24]. A replacement of codon 87 from Gly to Asp (G87D) was observed in 2 cases (Figure 1). Second, part of the sequences in exons 6 and 7 comprises a zinc finger domain that is highly conserved between mouse and human *FOXP3* [6]. Since no mutation of this domain was observed in the IPEX patients, the function of this domain in *FOXP3* has not been suggested [2]. Surprisingly, the mutations in this region appear to be most frequent as 6 nonsynonymous mutations that were observed within a stretch of less than 30 amino acids in the cancer samples [21,24] (Figure 1), suggesting that the function of this domain may be specific for tumor development. Interestingly, a K270R mutation between zinc finger and leucine zipper domains was identified in both breast cancer and prostate cancer [21,24]. Although this mutation is not located at any domains, this site may be a common area with some potential function for tumor development. Also, the IPEX mutations frequently occur in leucine zipper domain such as a ΔGlu251 mutation [2]. In cancer patients, only 3 mutations were identified in breast cancer but not prostate cancer [21,24] (Figure 1). This domain is essential for homo-oligomerization of *FOXP3*, transcriptional regulation by *FOXP3* and suppressive function by Treg cells [33,39,40]. Thus, it has been suggested that the mutations in this domain are associated with severe cases of IPEX [2]. Finally, the FKH domain is critical for both DNA binding and nuclear localization [41]. The FKH domain is the most frequent target in IPEX patients. In cancer cases, five nonsynonymous mutations target this domain in breast cancer but not one in prostate cancer (Figure 1). The FKH domain is necessary for *FOXP3* function, especially the ability of *FOXP3* to bind DNA for transcriptional regulation. Thus, mutations in this domain should have an impact on *FOXP3* transcriptional activity and on the function of Treg cells, which are related to the disease development in both IPEX and cancer.

Potential Mechanism of *FOXP3* Inactivation

Inactivation of *FOXP3* in IPEX patients is caused by germline mutations of this gene, while somatic mutations can result in *FOXP3* silencing in epithelial cancer cells. However, mutations only occur in the minority of IPEX and cancer patients [3] and the mechanism of *FOXP3* inactivation in most of cases is not fully known. In *FOXP3*-negtive $CD4^+$ T cells, a small CpG motif in intron 1 of the *FOXP3* has been reported as a Treg Cell-Specific Demethylated Region (TSDR) involved in epigenetic inactivation of the *FOXP3* in T cells [42-44]. It is of great interest to test whether the DNA methylation in this region is associated with IPEX in patients without *FOXP3* mutations.

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Males have only one X chromosome and females have two X chromosomes, but one X chromosome in each female cell is randomly inactivated throughout the lifetime. Thus, a single genetic or epigenetic hit to the active allele may be sufficient to inactivate the X-linked gene *FOXP3*. Interestingly, the majority of *FOXP3* mutations and deletions identified in breast cancer are heterozygous, and female *Scurfy* mice with a *FOXP3*-heterozygous mutation develop spontaneous breast cancer [16], suggesting that the active allele is affected. These data reaffirm the notion that a single-genetic hit is sufficient to inactivate X-linked genes in breast cancer [45]. Since mutations or gene deletions do not occur in the majority of cancer patients, it is still unexplained why there is a lack of *FOXP3* in the most of breast cancers [16]. Thus, X-linked *FOXP3* may be inactivated by multiple events such as loss of heterozygosity, mutation, bi-allelic methylation and skewed X-inactivation in breast cancer. The bi-allelic methylation has been proposed as an epigenetic mechanism of *FOXP3* inactivation. A prominent 5'CpG island was also identified in the promoter region of *FOXP3* [46,47]. We recently tested the methylation in the 5'CpG island of *FOXP3* promoter using pyrosequencing technology. Our data revealed that approximately 50% methylated in normal breast epithelial cells, which is consistent with X chromosome inactivation (unpublished data). Thus, it is of interest to test whether the inactivation of *FOXP3* is also caused by the bi-allelic methylation in breast cancer cells. Apart from TSDR and 5'CpG island, DNA demethylation at two CG sites in proximal promoter region of the *FOXP3* was identified in Treg cells. Interestingly, an increased 5-hmC levels were also detected at this promoter region in Treg cells and are consistent with the DNA demethylation levels, suggesting that the generation of 5-hmC may be part of the mechanism of demethylation [48]. A recent study revealed a substantial enrichment of 5-hmC within the TSDR from thymic Treg cells but not peripheral Treg cells, supporting the notion that TSDR demethylation is initiated and to a great extent finalized during thymic Treg development [49].

In addition, inactivation of *FOXP3* function can also be caused by disruption of intracellular localization or alternative splicing [24,45]. The histone modification [42,50] and skewed X-chromosome inactivation [51] may also contribute to inactivation of *FOXP3* function with a single-hit epigenetic mechanism, which needs to be validated by further studies. In prostate cancer, somatic mutations and gene deletion of *FOXP3* have been identified in less than 30% cancer samples [24], but approximately 70% of prostate cancer samples exhibited a loss of nuclear *FOXP3* expression [24], which also suggest potential additional epigenetic mechanisms to the *FOXP3* inactivation. Recently, Nie *et al.* discovered a new mechanisms regarding *FOXP3* transcriptional regulation [52]. Their data showed that *FOXP3* transcriptional activity and Treg cell suppressive function are regulated by TNF-α-dependent phosphorylation of the Ser418 residue in the DNA-binding domain of *FOXP3*, which reduces its DNA-binding activity and impairs the suppressive function of Treg cells [52]. However, the mechanism is still not tested in epithelial cells.

Transcriptional Regulation in the Context of *FOXP3*

FOXP3 can bind to and regulate several hundred target genes in Treg cells or epithelial cells in both mice and humans [9-11,53], thus *FOXP3* should have broad functions. *FOXP3* can directly bind more than 700 up- or down-regulated genes in *FOXP3*+ T cells, suggesting that *FOXP3* acts as both a transcriptional activator and a repressor [9,10]. *CTLA4* and *IL2Ra* are two major targets of *FOXP3*, which directly binds to the promoters and 5' regulatory regions of them to enhance their transcription, while *IL2* and *IL7Ra* as direct targets are transcriptionally inhibited by *FOXP3* [53] (Figure 2). These transcriptional regulations of *FOXP3* are required for development and function of Treg cells, and also contribute to development and progression of IPEX disease. As upstream of *FOXP3*, T cell receptor (TCR) and CD28 signaling is required for inducing *FOXP3* expression in Treg cells [41,54] (Figure 2). Interestingly, conversion of conventional CD4+ T cells into *FOXP3*+ Treg cells can be obtained after TCR activation by stimulation of exogenous TGFβ [55] (Figure 2).

In addition to its well-known function as a transcription factor in Treg cells, we have identified both activated and repressed target genes of *FOXP3* and they are epigenetically regulated by histone modifications in proximal promoter region of target genes in epithelial cells. *FOXP3* controls transcriptional activity of *SKP2*, *HER2/ErbB2*, *p21*and *LATS2* in breast cancer and *cMYC* and *LATS2* in prostate cancer [16,19,21,23,24] (Figure 2). The *SKP2*, *HER2/ErbB2* and *cMYC* are repressive targets but *p21* and *LATS2* are activated targets of *FOXP3*. Interestingly, *cMYC* in prostate epithelial cells is inversely correlated with *FOXP3* in both mRNA and protein expression in human primary prostate cancers [24]. *FOXP3*-mediated transcriptional repression of c*MYC* is a necessary mechanism for controlling cMYC levels in prostate epithelial cells, which help us to understand the widespread overexpression of *cMYC* in prostate cancer [24]. Furthermore, an up-regulation of *FOXP3* in breast and colon carcinoma cells requires p53 function. *FOXP3* expression can be induced by DNA-damaging agents in p53-positive carcinoma cells, but not in cells lacking p53 function. After knockdown of *FOXP3,* this p53-mediated growth inhibition has disappeared [56]. These results suggest that p53 is an upstream regulator of *FOXP3* expression during DNA damage responses (Figure 2). Likewise, ATF2/cJun can induce the transcription of *FOXP3* in various breast cancer cell lines, and induces *FOXP3*-mediated repression of cell growth *in vitro* and tumor formation from implanted cancer cells *in vivo* [57] (Figure 2). In addition, transfection of *FOXP3* led to decreased expression of Ki67 and CDKs, resulting in inhibition of cell proliferation, migration and invasion in ovarian cancer cells. This phenotype may be caused by either that *FOXP3* reduced expression of MMP2 and uPA or that *FOXP3* inhibits the activation of mTOR and NF-κB signaling [28] (Figure 2). Likewise, transfection of *FOXP3* into the CXCR4 expressed MDA-MB-231 breast cancer cells resulted in a decreased expression of CXCR4, that is a tumor metastatic process-associated protein to promote metastasis in breast cancer [58,59], and the chemotactic response of these cells to a CXCL12 gradient was effectively inhibited [60,61], suggesting a repressive function of tumor metastasis by *FOXP3*. Recently, we identified that *FOXP3* can directly bind the *BRCA1* to down-regulates the transcriptional activity of this gene, suggesting that *FOXP3* repress the BRCA1-mediated DNA repair program [62] (Figure 2). Therefore, *FOXP3* functions as a master regulator working in a complex network during tumor development and progression.

Despite *FOXP3* can promote or inhibit hundreds of genes, the molecular basis of *FOXP3* function has not been fully understood. In Treg cells, several mouse *FOXP3* binding partners as co- activators or repressors have been identified, including Foxp1 [39,63], Nuclear Factor of Activated T cells (NFAT) [35], Ikaros family member Eos

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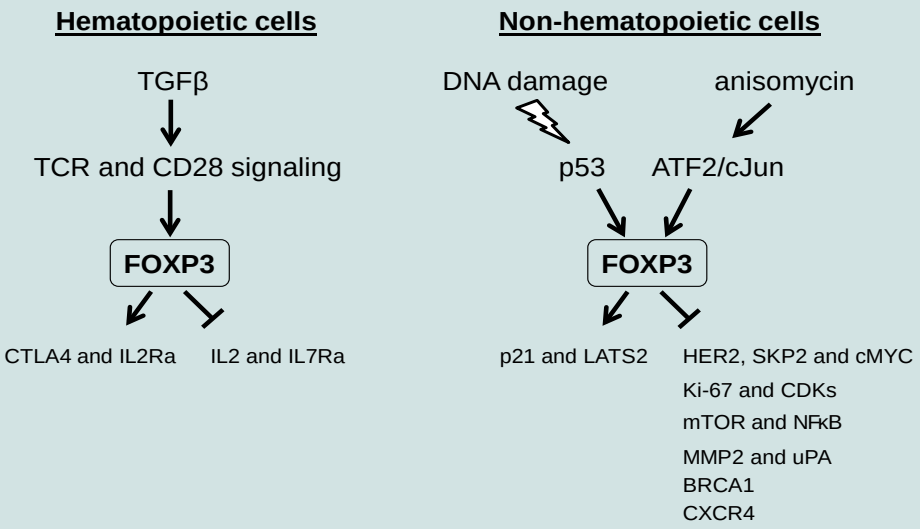

Figure 2: Functional signaling pathways in the context of *FOXP3*. *FOXP3* is highly expressed in Treg cells, but also detected in both epithelial and non-epithelial cells, including many cancer cells. Several hundred target genes of the *FOXP3* have been identified in both Treg cells and epithelial cells. TGFβ: transforming growth factor β; TCR: T cell receptor; CTLA4: cytotoxic T-lymphocyte-associated protein 4; IL2: interleukin 2; IL2Ra: IL2 receptor a; IL7Ra: IL7 receptor a; LATS2: large tumor suppressor kinase 2; ATF2: ATF2 activating transcription factor 2; HER2: v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; SKP2: S-phase kinase-associated protein 2; CDKs: cyclin-dependent kinases; mTOR: mammalian target of rapamycin; NFκB: nuclear factor κB; MMP2: matrix metalloproteinase 2; uPA: urokinase-type plasminogen activator; BRCA1: breast cancer 1, early onset; CXCR4: chemokine (C-X-C motif) receptor 4.

[64], Runx1-Cbfβ complex [65], and GATA3 [66]. The *FOXP3* binding partners are likely to assist the *FOXP3*-DNA interactions by an epigenetic mechanism. Indeed, Histone Acetyl Transferases (HATs) and Histone Deacetylases (HDACs) as epigenetic modifiers have also been implicated in *FOXP3* function and they contribute to *FOXP3*-mediated transcriptional repression byan acetylation regulation [67,68]. Interestingly, a recent study revealed that *FOXP3* forms multi-protein complexes of more than 400 kDa and they identified approximately *FOXP3*-transcriptionally associated proteins [66]. These data imply that multiple *FOXP3* partners play an important role in *FOXP3* function. In epithelial cells, the mechanism of *FOXP3*-mediated transcriptional regulation also remains largely unexplored. During investigating p21 induction by *FOXP3*, we identified that *FOXP3* specifically inhibited binding of HDAC2 and HDAC4 to the site and increased local histone H3 acetylation, which provides a epigenetic mechanism for transcription activation by *FOXP3* in cancer epithelial cells [19]. Recently, we also identified a novel *FOXP3* binding partner MOF as both co- activator and repressor in cancer epithelial cells [11]. MOF is a MYST family histone acetyltransferase and specifically acetylates histone histones H4 acetyl Lys16 [69-71]. *FOXP3*-mediated transcriptional regulation is correlated with both histones H4 acetyl Lys16 and H3 trimethyl Lys4, which epigenetic modifications are simultaneously achieved by recruiting MOF and by displacing an H3 methylated Lys4demethylase PLU1 [11]. Our data suggest a pull-push model for *FOXP3*-associated gene activation by an epigenetic mechanism.

The Variety of *FOXP3* Function and Its Clinical Implications

FOXP3 is a useful marker for Treg cells, but its expression has also been identified in non-hematopoietic cells, including epithelial cells and non-epithelial cells [15]. In the immune system, *FOXP3* is necessary for the development and function of naturally occurring Treg cells [12,72]. In epithelial cells, *FOXP3* may play dual functions [73]. Mouse model and *in vitro* studies clearly present a tumor suppressive function of the *FOXP3* in several cancers [16,24,28,29]. However, the clinical significance of *FOXP3* expression in tumor cells by immune histochemical analysis is still not concluded in human cancers, but evidence revealed a strong correlation between *FOXP3* expression and metastasis [73]. A better disease outcome and survival was related to patients with both *FOXP3*+ and HER2+ breast cancer [17] and colorectal cancer [74], and a poor disease outcome or metastasis in patients with breast cancer [75], colorectal cancer [76], non-small cell lung cancer [77], urinary bladder cancer [78], hepatocellular carcinoma [79], esophageal squamous cell carcinoma [80], tongue squamous cell carcinoma [81], melanoma [82] and gliomas [83], but no significance in patients with non-small cell lung cancer [84]. However, recent clinical observations in larger sample cohorts with more than 1,000 patients have indicated that nuclear *FOXP3* expression in tumor cells predicts better survival in breast cancer patients and they reported that essentially all *FOXP3* in breast tumor cells is cytoplasmic [17,18,20]. Since *FOXP3* localization in cells is essential for its transcriptional activity, and only nuclear *FOXP3* have a tumor suppressive function, thus nuclear *FOXP3* may be a useful marker for predicting disease outcome in human cancers. Therefore, a separate analysis by nuclear and cytoplasmic *FOXP3* is required in future studies. A recent report showed that normal breast epithelia expressed nucleus *FOXP3* and failed to express *CXCR4*, whereas diminished levels of nuclear *FOXP3* was significantly associated with higher levels of membrane *CXCR4* in breast tumors and metastases [60,61]. As supported by evidence in our gene target analysis, *FOXP3* can reduce *CXCR4* expression in MCF7 cells by more than three-fold [11]. In addition, somatic mutations, gene deletions and splicing forms are frequently identified in tumor cells [16,24] and function of *FOXP3* in tumor cells may be broken by these genetic events, which may have an impact on understanding the role of *FOXP3* expression in tumor cells. On the other hand, the dual role of the *FOXP3* in both proliferation and metastatic spread of tumor cells

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may also be explained by a putative immunosuppressive function that contribute to induce cancer progression [73].

Although the function of the *FOXP3* is not fully understood in epithelial cells, this gene is likely to play multiple roles in different cell types or tumor stages.

Future Perspectives

IPEX can be diagnosed by clinical symptoms but is required to be confirmed with the *FOXP3* mutations by DNA sequencing. Some patients with IPEX syndrome but without the *FOXP3* mutations are required to examine whether they lack *FOXP3* expression by an expression analysis. However, the reason of *FOXP3* loss in these patients still remains unclear. Further studies are needed to investigate epigenetic events of the *FOXP3*, other modifying genes, or environments involved in function of Treg cells, which may assist in the diagnosis of IPEX. Currently, immunosuppressive therapy such as tacrolimus and cyclosporine are the most effective initial therapies to control IPEX syndrome, but does not cure IPEX or extend life span even prior to initiating haematopoietic stem cell transplantation [2]. Thus, gene therapy or gene repair approaches have been proposed to treat IPEX. However, most IPEX patients are males, thus single allele of X-lined *FOXP3* with mutation in males will be a challenge for gene repair.

Previous studies have provided clear evidence for a single-hit genetic inactivation of X-linked *FOXP3* and illustrated the dramatic difference between X-linked and autosomal tumor suppressors, substantially enhancing our understanding of the X-linked tumor suppressor gene [21,24]. However, the mechanism of *FOXP3* inactivation still needs to be fully addressed. Currently, one of the most difficult challenges in cancer therapy is to restore the function of inactivated tumor suppressors. Since allele from inactivated X chromosome in a female do not appear to undergo mutational changes during carcinogenesis, it may be possible to reactivate the silenced *FOXP3* from this inactivated allele for therapeutic purposes in female cancer. Since *FOXP3* has different functions between epithelial and T cell types, this reactivation may affect immune responses. In addition, restoration of gene expression from the inactive X-chromosome may bring about unexpected or undesired results due to loss of dosage compensation of various important X-linked genes. Thus, current challenges are to develop a method for targeted reactivation of specific genes in specific tissues lead to agents with fewer side effects during reactivation of *FOXP3*.

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