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Functional and genomic analyses of FOXP3-transduced Jurkat-T cells as regulatory T (Treg)-like cells ☆

Joon-Young Kim [a,*], Han-Jong Kim [a], Elaine M. Hurt [a], Xin Chen [b], O.M. Zack Howard [c], William L. Farrar [a,*]

[a] *Cancer Stem Cell Section, Laboratory of Cancer Prevention, NCI-Frederick, 1050 Boyles Street, Building 560, Room 21-78, Frederick, MD 21702, USA*
[b] *Basic Research Program, SAIC-Frederick, Inc., NCI-Frederick, MD 21702 USA*
[c] *Laboratory of Molecular Immunoregulation, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702, USA*

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Abstract

FOXP3, a forkhead transcription factor is essential for the development and function of $CD4^+CD25^+$ regulatory T cells (Tregs). In contrast to conversion of murine naive T cells to Tregs by transduction of Foxp3, it is controversial whether ectopic expression of FOXP3 in human $CD4^+$ T cells is sufficient for acquisition of suppressive activity. Here, we show that retroviral transduction of FOXP3 induces a Treg phenotype in human leukemic $CD4^+$ Jurkat-T cells, evidenced by increased expression of Treg-associated cell surface markers as well as inhibition of cytokine production. Furthermore, FOXP3-transduced Jurkat-T cells suppress the proliferation of human $CD4^+CD25^-$ T cells. Additionally, DNA microarray analysis identifies Treg-related genes regulated by FOXP3. Our study demonstrates that enforced expression of FOXP3 confers Treg-like properties on Jurkat-T cells, which can be a convenient and efficient Treg-like cell model for further study to identify Treg cell surface markers and target genes regulated by FOXP3.

Keywords: FOXP3; Treg; Transduction; Jurkat-T cells

The maintenance of immunological self-tolerance involves the deletion of immature self-reactive T lymphocytes in the thymus (central tolerance) and suppression of the activation of potentially self-reactive effector T (Teff) lymphocytes in the periphery (peripheral tolerance) [1]. $CD4^+CD25^+$ regulatory T cells, a naturally arising regulatory T (nTreg) cells have emerged as a unique subset of T cells that are critical for the maintenance of peripheral tolerance [2–4]. Deficiency of Treg development and function can cause various autoimmune and inflammatory disorders (e.g., immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome [IPEX] [5], multiple sclerosis [6], and type 1 diabetes [7]). Typically, nTregs are anergic upon T-cell receptor (TCR) stimulation and suppress the proliferation and cytokine production of Teff cells *in vitro* in a cell–cell contact dependent manner [4,8,9]. In addition to CD25, a number of Treg-associated cell surface molecules have been identified, including glucocorticoid-induced tumor necrosis factor receptor (GITR), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), and inducible T-cell co-stimulator (ICOS). However, no unique Treg-specific surface markers have been identified thus far, as these molecules are also upregulated in activated T cells.

FOXP3, a forkhead transcriptional factor is exclusively expressed in $CD4^+CD25^+$ Tregs and essential for the development and function of nTregs [10,11]. Mutations in the *FoxP3* gene lead to fatal autoimmune or inflammatory diseases (Scurfy disease in mice [12] and IPEX in humans [5]) due to the deficiency or malfunction of nTregs. Furthermore, ectopic expression of Foxp3 converts naïve murine T cells towards Treg-like cells [10,11]. Transduction of

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* Corresponding authors. Fax: +1 301 846 6104.
E-mail addresses: jykim@ncifcrf.gov (J.-Y. Kim), farrar@mail.ncifcrf.gov (W.L. Farrar).

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Foxp3 in naïve murine T cells results in hyporesponsiveness to TCR stimulation, inhibition of cytokine production, up-regulation of cell surface markers such as CD25, CTLA-4 and GITR, and confers an *in vivo* and *in vitro* suppressive activity on naïve T cells. However, FOXP3 expression has been shown to be induced by TCR activation in human but not in murine $CD4^+CD25^-$ T cells, although the suppressive activity of activated $FOXP3^+$ T cells is controversial [13–15]. Contrary to a previous report [16], a recent study showed that ectopic expression of FOXP3 in human $CD4^+$ T cells does not result in acquisition of suppressive activity [17]. It has been suggested that a sustained high expression of FOXP3 is required for full suppressive activity of human nTregs as well as FOXP3-induced Treg-like cells, although FOXP3-independent mechanisms may be required to obtain full suppressive function of nTregs.

Here, we show that transduction of FOXP3 can induce a Treg phenotype in Jurkat-T cells evidenced by increased expression of Treg-associated cell surface markers and inhibition of cytokine production following PMA/PHA stimulation, as well as repression of NF-κB and NFAT activity. Furthermore, FOXP3-transduced Jurkat-T cells acquire suppressive activity *in vitro*, demonstrating that transduction of FOXP3 can confer Treg properties on Jurkat-T cells. Using microarray, we also identify Treg-related genes regulated by FOXP3. Therefore, our study provides a convenient and efficient Treg-like cell model for further study to identify Treg-specific cell surface markers and target genes regulated by FOXP3.

Materials and methods

Antibodies and chemical reagents. Anti-CD3 (OKT3), anti-CD28, and anti-GITR-PE antibodies were obtained from eBioscience (San Diego, CA); anti-CD4-PE and anti-CD25-APC antibodies from Invitrogen (Carlsbad, CA); CTLA-4-APC antibody from BD Pharmingen (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin-P (PHA), and anti-Flag antibody were purchased from Sigma–Aldrich (St. Louis, MO).

Retroviral constructs and transduction. Vxy/Flag-FOXP3 was cloned by inserting PCR product of FOXP3 with an NH2-terminal Flag-epitope tag into bicistronic retroviral vector Vxypuro [18] containing the puromycin resistant gene (Puro). Details can be found in Supplemental Materials and Methods. Jurkat-T cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin transductions were performed as described previously [18] and transduced Jurkat-T cells were selected with puromycin (5 μg/ml) for 1 week. Expression of FOXP3 was determined by Western blot using anti-Flag antibody.

Semiquantitative RT-PCR. First-strand cDNA was synthesized from 2 μg of total RNAs from FOXP3 or Vxypuro-transduced Jurkat-T cells and then amplified to measure mRNA levels of FOXP3 and β-actin by 25 cycles of PCR using specific primers. The primer sequences used for PCR are as follows; FOXP3 forward, 5′-TGCCTCCTCTTCTTCCTTGA-3′ and reverse, 5′-CCACTTGCAGACACCATTTG-3′, and β-actin forward, 5′-GGACTT CGAGCAAGCGATGG-3′ and reverse, 5′-AGCACTG TGTTGGCGTACAG-3′.

Flow cytometric analysis. FOXP3 or Vxypuro-transduced Jurkat-T cells were washed in FACS buffer (PBS containing 2% BSA and 5 mM EDTA) and then stained with anti-CD25-APC, anti-GITR-PE or isotype control antibodies. For intracellular CTLA-4 staining, cells were fixed and permeabilized with fixation and permeabilization buffers (eBioscience) according to the manufacturer's instruction, and then incubated at 4 °C for 20 min with CTLA-4-APC or isotype control antibodies. Cell fluorescence was measured using a FACSCalibur flow cytometer, and data were analyzed using FCS Express (De Novo Software, Ontario, Canada).

Cytokine production. FOXP3 or Vxypuro-transduced Jurkat-T cells in 96-well plates (2×10^5 cells/well) were stimulated with either PMA (50 ng/ml) plus PHA (2 μg/ml), or immobilized anti-CD3 mAb (5 μg/ml) plus soluble anti-CD28 mAb (2 μg/ml) for 24 h. Supernatant from activated cells in triplicate was collected to measure IL-2 and IFN-γ production by ELISA.

Transient transfection assays. FOXP3 or Vxypuro-transduced Jurkat-T cells were split in 24-well plates (1×10^5 cells/well) in duplicate and transfected using FuGene6 (Roche, Indianapolis, IN) with the luciferase reporters, NFAT-Luc and NF-κB-Luc. After 24 h, cells were stimulated with PMA (50 ng/ml) and PHA (1 μg/ml) for 6 h. Cells were harvested to measure luciferase and β-galactosidase activities and luciferase activity were normalized to β-gal activity.

In vitro suppression assay. FACS sorted $CD4^+CD25^-$ T cells (50,000 cells/well as a responder) in triplicate were labeled with carboxyfluoroscein succinimidyl ester (CFSE, 2.5 μM; Invitrogen), stimulated with anti-CD3 mAbs (1 μg/ml) and autologous or allogenic APCs (CD3-depleted PBMCs, irradiated at 30 Gy, 50,000 cells/well) and cultured in 96-well plate with or without FOXP3 or Vxypuro-transduced Jurkat-T cells at a 1:2 ratio (responder:transduced Jurkat-T). Freshly isolated peripheral $CD4^+CD25^+$ Tregs were used as a positive control. After 96 h, the amount of CFSE dye dilution was analyzed to assess the suppression activity.

DNA microarray. DNA microarrays were performed using 20 μg of total RNA in a reverse transcription reaction and labeled with either Cy3-dUTP (Vxypuro) or Cy5-dUTP (FOXP3). The resulting cDNA was mixed and hybridized onto oligonucleotide arrays prepared by the NCI Microarray facility using Operon's Human Version 3.0 oligo set (Operon Biotechnologies, Huntsville, AL), which contains 34,580 oligos, representing approximately 25,000 unique genes. Arrays were scanned in a GenePix 4000B scanner and analyzed using GenePix Pro (Molecular Devices, Sunnyvale, CA). Data analysis was preformed using Cluster and TreeView, offered by Michael B. Eisen as freeware (http://rana.lbl.gov/EisenSoftware.htm). The ratio of Cy5/Cy3 is presented as the fold change.

Results

Ectopic expression of FOXP3 induces a Treg phenotype in human $CD4^+$ leukemic Jurkat-T cells

Contrary to the murine model [10,11], acquisition of suppressive activity by FOXP3 overexpression is controversial in human $CD4^+$ T cells [16,17]. To generate human Treg-like cell lines, $CD4^+$ human leukemic Jurkat-T cells were transduced with retroviruses expressing Flag-tagged FOXP3 (Vxy/FOXP3) or a control virus (Vxypuro) (Fig. 1A). Semiquantitative RT-PCR and Western blot analyses showed a large increase in mRNA and protein expression of FOXP3, respectively, in Jurkat-T/FOXP3 cells compared with undetectable levels of FOXP3 in Jurkat-T/Vxypuro cells (Fig. 1B).

Previous reports have shown that Tregs constitutively express Treg-associated cell surface markers such as CD25, GITR, and CTLA-4 [9,16]. To investigate whether FOXP3 can induce this Treg phenotype in Jurkat-T cells, expression of cell surface markers was analyzed by flow cytometry. Cell surface expression of CD25 and GITR was notably increased in Jurkat-T/FOXP3 compared with that of Jurkat-T/Vxypuro control cells (Fig. 1C).

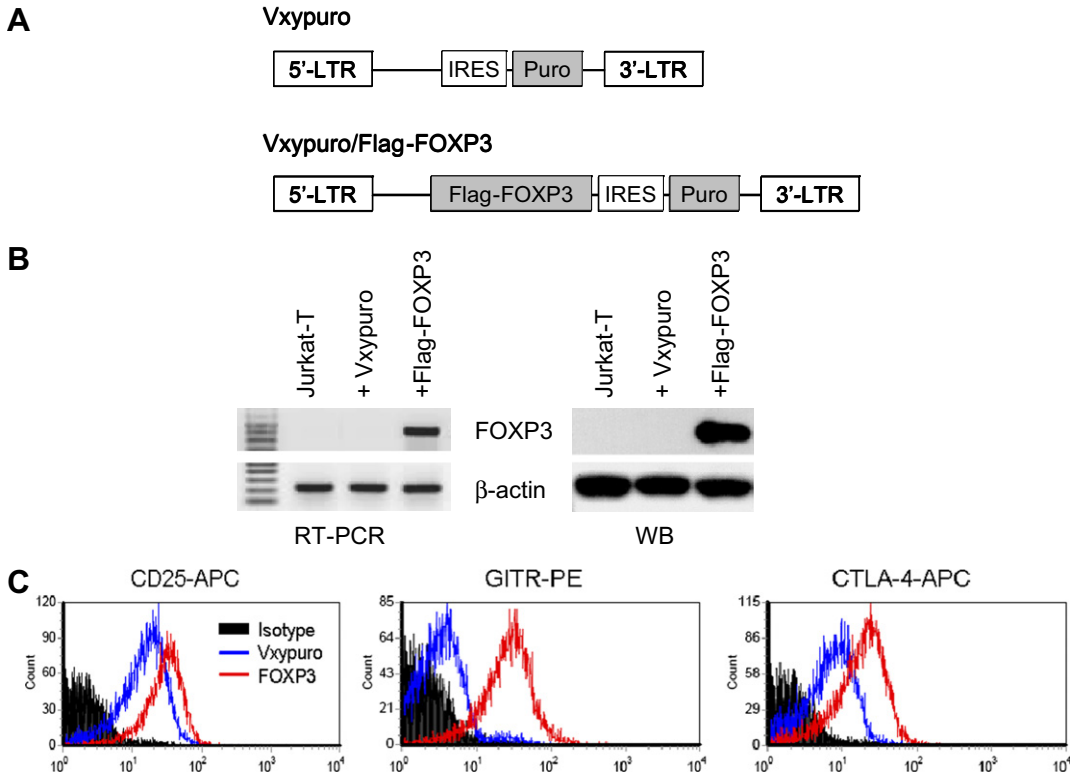


Fig. 1. Transduction of FOXP3 induces a Treg Phenotype in Jurkat-T cells. (A) Schematic representation of retroviral vector expressing Flag-FOXP3 or vector alone (Vxypuro). (B) Semiquantitative RT-PCR and Western blot analyses. Jurkat-T cells were retrovirally transduced with Vxy/Flag-FOXP3 or vector (Vxypuro) alone. (C) Surface expression of Treg-associated markers. Cell surface CD25 and GITR and intracellular CTLA-4 expression were analyzed by flow cytometry.

Intracellular CTLA-4 expression is also considerably increased by FOXP3 overexpression in Jurkat-T cells. These results suggest that ectopic expression of FOXP3 can directly induce a Treg phenotype in Jurkat-T cells.

Effect of FOXP3 overexpression in Jurkat-T cells on the cytokine production and transactivity of NF-κB and NFAT

nTregs are hyporesponsive to TCR stimulation and show an attenuated cytokine production such as IL-2 and IFN-γ [8,9]. Consistent with previous data [20], Jurkat-T/FOXP3 cells showed a decrease in proliferation compared with Jurkat-T/Vxypuro control cells (data not shown). To assess the effect of FOXP3 on cytokine production, IL-2 and IFN-γ levels were measured after stimulation with PMA/PHA or anti-CD3/anti-CD28 mAbs. Both IL-2 and IFN-γ production following PMA/PHA stimulation were significantly repressed by FOXP3 overexpression compared with that of Jurkat-T/Vxypuro control cells (Fig. 2A). In addition, IL-2 production is also reduced in Jurkat-T/FOXP3 cells stimulated with anti-CD3/anti-CD28 mAbs, whereas IFN-γ level was undetectable in either cells (data not shown).

It has been reported that FOXP3 represses the expression of NFAT and NF-κB target genes such as IL-2 and IFN-γ, potentially through the direct binding to forkhead binding sites or protein–protein interaction with NFAT or NF-κB [19,21,22]. To test whether FOXP3 functions as a repressor for NFAT or NF-κB activity, reporter assays were performed. Stimulation with PMA/PHA results in a dramatic activation of the reporters in Jurkat-T/Vxypuro cells, whereas both basal and PMA/PHA-induced NFAT and NF-κB luciferase activity was significantly decreased in Jurkat-T/FOXP3 cells (Fig. 2B). These results suggest that FOXP3-transduced Jurkat-T cells are anergic to TCR stimulation similar to nTregs at least in part, by inhibition of NFAT or NF-κB activity.

Ectopic expression of FOXP3 in Jurkat-T cells results in acquisition of suppressive activity

Contrary to a previous report [16], a recent study showed that overexpression of FOXP3 is not sufficient for suppressive activity of human $CD4^+$ cells *in vitro*, although transduced cells are anergic and phenotypically similar to Tregs [17]. To investigate whether FOXP3 transduction can confer suppressive activity on Jurkat-T cells, freshly isolated human peripheral $CD4^+CD25^-$ cells were labeled with CFSE and co-cultured with Jurkat-T/FOXP3, Jurkat-T/Vxypuro control cells, or $CD4^+CD25^+$ nTregs as a positive control. Co-culture with Jurkat-T/Vxypuro cells had little effect on proliferation of $CD4^+CD25^-$ T cells stimulated with anti-CD3 mAb and autologous APCs (Fig. 3). In contrast, co-culture with Jurkat-T/FOXP3 cells

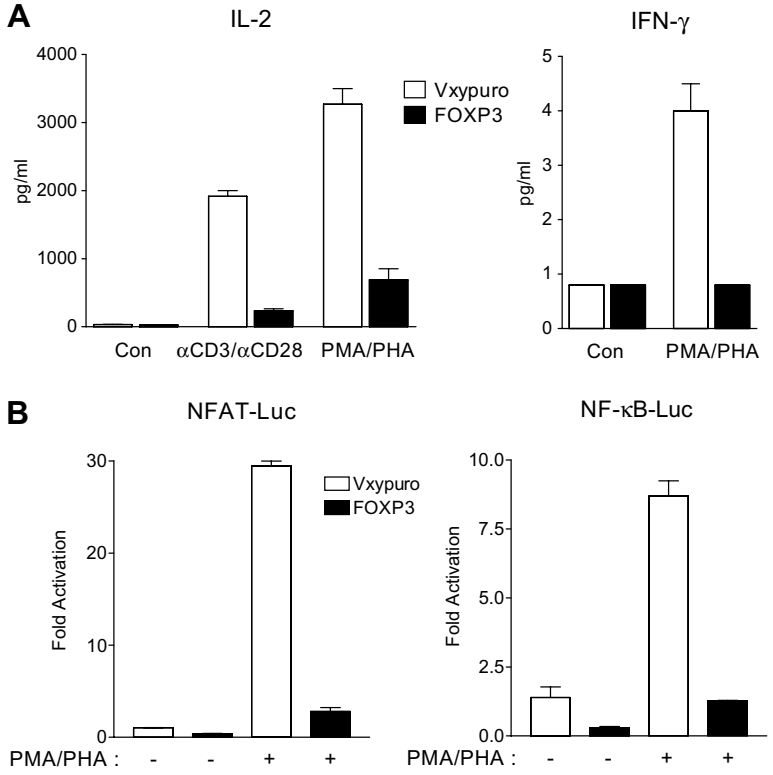
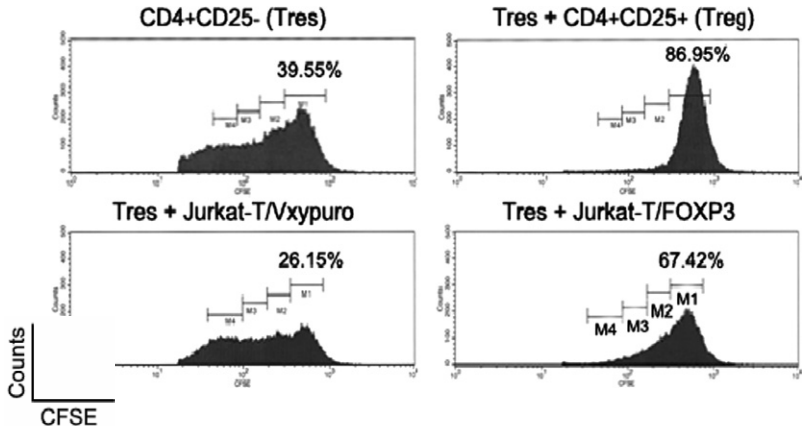


Fig. 2. Ectopic expression of FOXP3 reduces cytokine production and transactivity of NFAT and NF-κB. (A) Ectopic expression of FOXP3 decreased IL-2 and IFN-γ production following stimulation with either PMA/PHA or anti-CD3/anti-CD28 mAbs for 24 h. (B) FOXP3 repressed NFAT and NF-κB transactivity. NFAT and NF-κB luciferase reporters were transfected in Jurkat-T cells expressing FOXP3 or Vxypuro. After 24 h, transfected cells were activated with PMA/PHA for 6 h and then harvested for luciferase assay. Data represent the normalized relative luciferase fold activity compared with those of the reporters without stimulation in the Vxypuro-transduced Jurkat-T cells. The data are representative of at least three independent experiments.



	Tres	+ Treg	+ Vxy	+ FOXP3
M1 (%)	39.55	86.95	26.15	67.42
M2 (%)	20.17	3.58	23.86	18.48
M3 (%)	12.89	1.21	21.93	5.32
M4 (%)	13.3	0.67	23.67	1.89

Fig. 3. FOXP3-transduced Jurkat-T cells acquire suppressive activity. CFSE-labeled $CD4^{+}CD25^{-}$ T responder (Tres) cells were stimulated with anti-CD3 mAbs and APCs, and mixed with Vxypuro- or FOXP3-transduced Jurkat-T cells, or human $CD4^{+}CD25^{+}$ T (nTreg) cells as a positive control at a 1:2 ratio (responder cells/transduced cells). Proliferation of CFSE-labeled $CD4^{+}CD25^{-}$ T cells was analyzed by flow cytometry after 96 h. The percentage of undivided Tres cells is indicated. The table shows the percentage of the cells with the corresponding CFSE dilution.

strongly suppressed proliferation of $CD4^+CD25^-$ T cells, although the suppressive activity of Jurkat-T/FOXP3 cells was weaker than that of nTregs, suggesting that FOXP3-independent pathways may be involved in the suppressive function of nTregs or that the expression level of FOXP3 may be lower in Jurkat-T cells than that of nTregs. This result indicates that ectopic expression of FOXP3 can confer suppressive activity on Jurkat-T cells.

Genes regulated by FOXP3 in Jurkat-T cells

To screen for the genes specifically controlled by FOXP3, we performed DNA microarray analysis and compared gene expression profile of Jurkat-T/FOXP3 and Jurkat-T/Vxypuro control cells. Among 272 genes with at least two-fold regulation by FOXP3 (Supplemental Table 1 and 2) are 29 selected genes up- or down-regulated by FOXP3 shown in Fig. 4A, and they are newly identified as FOXP3 target genes. Among the 151 up-regulated genes is Caspase 10 (CASP10), which is associated with apoptosis and autoimmune lymphoproliferative syndrome, type II [23]. FOXP3 also up-regulated ICOS expression which is constitutively expressed in Tregs and involved in function of Tregs, as well as activated T cells [24,25]. Among the 121 genes decreased by FOXP3 overexpression are genes involved in proliferation (MAPK1 and MKNK1) and T cell activation and differentiation (IRF4, CD44, CD69, and MAL).

To further examine the functions of these genes, we analyzed the 272 2-flod regulated genes using Ingenuity's Pathway Assist software (Ingenuity, Redwood City, CA). Of the 272 genes, 122 genes were identified in the database (called focus genes). These genes are mapped to 21 different networks, which are defined as the sum of the interactions between the proteins based on literature results. There were 9 networks that contained 9 or more focus genes (Supplemental Table 3), while the remaining groups contained only 1 focus gene. Of these 9 networks, FOXP3 was identified in network 3 (Fig. 4B), containing genes classified by either hematological system development and function, or immune and lymphatic system development and function. Based on this analysis, further study can illuminate the functional correlation between FOXP3 and other cellular molecules in Tregs. Taken together, these results suggest that FOXP3 may exert its multiple effects on Treg-associated function and phenotype in part by regulation of gene expression, although further study needs to elucidate their involvement in Treg function and phenotype.

Discussion

Foxp3 is exclusively expressed in $CD4^+CD25^+$ nTregs and necessary for development and maintenance of nTregs, and neither naïve nor activated T cells express Foxp3 in mice. Retroviral transduction of *Foxp3* is sufficient to convert conventional murine $CD4^+$ T cells into Tregs. However, in human, FOXP3 can be up-regulated in activated T cells, although the expression is transient and lower than that of nTregs and the suppressive function of activated T cells is controversial [13–16]. Furthermore, a recent study showed that continued expression of FOXP3 is required for maintenance of suppressive activity of mature Tregs [26]. In this study, we showed that ectopic expression of FOXP3 confers both suppressive function on Jurkat-T cells and Treg phenotype, although the suppressive activity was less potent than that of $CD4^+CD25^+$ nTregs (Fig. 3). Additionally, we observed that Jurkat-T cell clones expressing low levels of FOXP3 showed modest suppressive activity

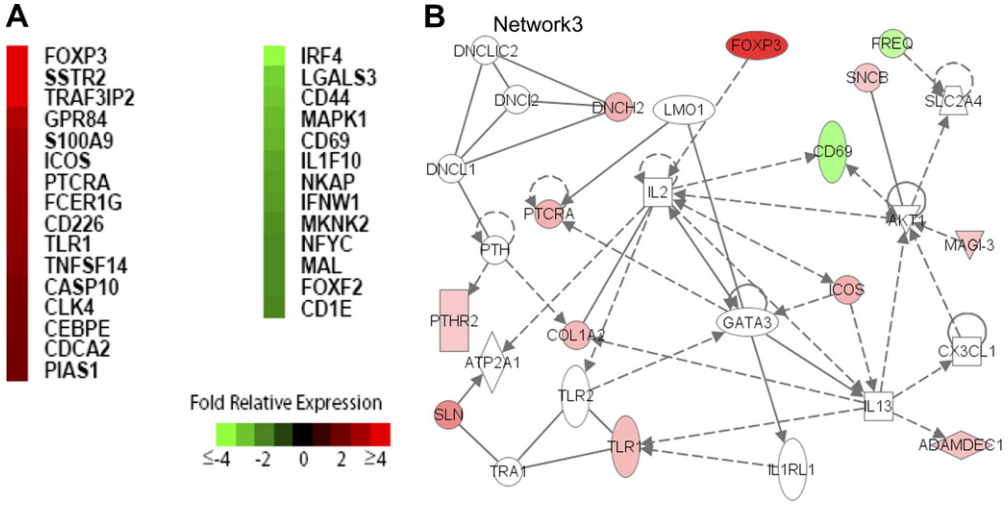


Fig. 4. Gene expression changes in Jurkat-T cells transduced with FOXP3. (A) Gene expression changes are shown by the color bar where red represents an increase (⩾ 2-fold) and green represents a decrease (⩽ 2-fold) compared with Vxypuro-transduced Jurkat-T cells. (B) The network 3 of FOXP3-regulated genes identified using Ingenuity's Pathway Assist software. Red represents increase (⩾ 2-fold) genes and green represents decrease (⩽ 2-fold) gene by FOXP3 overexpression in Jurkat-T cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(data not shown). Previous studies and our observations suggest that sustained high expression of FOXP3 may be required for full suppressive activity of human nTregs as well as FOXP3-induced Treg-like cells, although FOXP3-independent mechanisms may be involved in obtaining the full suppressive function of nTregs.

In addition to the crucial role of FOXP3 in Tregs, induction of FOXP3 following TCR stimulation shuts off T cell activation in activated T cells [14,15]. Global TCR-mediated signaling is attenuated in Tregs, and expression of Foxp3 in $CD4^+$ T cells leads to impairment of TCR signaling [27,28]. These studies suggest that activation-induced FOXP3 plays critical roles in effector T (Teff) cell function via multiple mechanisms, distinguishing this aspect of FOXP3 function from its role in Tregs. FOXP3 may regulate $CD4^+$ T cell activation in part, by inhibiting NFAT and NF-κB-mediated transcription which mediates T cell activation signaling [19,21,22]. In a support of this, NFAT and NF-κB transactivity was decreased in FOXP3-transduced Jurkat-T cells (Fig. 2B). Furthermore, we showed that CD44 and CD69, T cell activation markers are down-regulated by FOXP3 (Fig. 4A), suggesting that activation-induced FOXP3 may regulate Teff cell function in part by regulating the expression of CD69 and CD44. FOXP3 also up-regulates ICOS, a T cell activation marker that is also constitutively expressed in Tregs, confirming the crucial roles of ICOS in Treg function [24,25]. This result also suggests a link between FOXP3 and ICOS for their regulatory effects on Tregs as well as activated T cells. Additionally, FOXP3 may control some cytokine signaling pathways in Tregs and Teff cells via induction of PIAS, an inhibitor of the JAK/STAT pathway that is a downstream signaling of cytokines, and inhibition of IRF4 expression, which is implicated in T cell activation and IL-4 production.

Tregs are considered to be novel, promising therapeutic tool for the treatment of various autoimmune diseases, allergies, graft versus host disease (GVHD), rheumatoid arthritis, and cancers. However, there remain several questions to be answered including the basic biology of the Tregs, Treg-specific cell surface markers, the suppression mechanisms, and the exact role of FOXP3 in these processes. In addition to the function in Tregs, FOXP3 plays essential roles in control of T cell activation in Teff cells. We have identified many genes that are regulated by FOXP3 in Jurkat-T cells. These genes include some with known roles in the Treg or Teff function (e.g., ICOS, CD44, CD69, and IRF4), while others have not been ascribed to Treg or Teff function (e.g., CASP10, MAL, SSTR2, and PIAS1). Therefore, further study is needed to elucidate the expression, function, and action mechanism of these genes regulated by FOXP3 in Tregs or Teff cells. In this context, our study suggests that FOXP3-transduced Jurkat-T cells can be used as convenient and efficient Treg-like cell model for identification of Treg-specific markers and target genes regulated by FOXP3. Furthermore, FOXP3 transduced Jurkat-T cells can be an attractive *in vitro* cell model system to study Teff cell biology.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.187.

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