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Review

Forkhead transcription factors in immunology

H. Jonsson[a] and S. L. Peng[a,b,*]

[a] Division of Rheumatology, Department of Internal Medicine, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, Missouri 63110 (USA)
[b] Department of Pathology and Immunology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8045, St. Louis, Missouri 63110 (USA), Fax: +1 314 454 1091, e-mail: speng@im.wustl.edu

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Abstract. The forkhead (Fox) gene family comprises a diverse group of 'winged-helix' transcription factors that play important roles in development, metabolism, cancer and aging. Recently, several forkhead genes have been demonstrated to play critical roles in lymphocyte development and effector function, including Foxp3 in the development of regulatory T cells, Foxj1 and Foxo3a in the regulation of $CD4^+$ T cell tolerance, and Foxn1 in thymic development. Roles for other forkhead genes have also been proposed, including Foxp1 in macrophage differentiation, Foxq1 in natural killer cell effector function and Foxd2 in T cell activation. Thus, forkhead genes promise insight into the mechanisms of immunoregulation in several immune cell lineages, and their dysregulation likely contributes to the pathogenesis of several immunological disorders, suggesting that their study will lead to the development of novel therapeutic agents.

Key words. Transcription factors; lymphocytes; macrophages; gene regulation; development; autoimmunity.

Introduction

The forkhead (Fox) gene family comprises a large and diverse group of transcription factors that share a 'winged helix' DNA binding domain, first defined in 1990, consisting of three alpha helices flanked by two 'wings' of beta strands and loops (reviewed [in 1]). Over 100 proteins with forkhead domains have been found, comprising at least 17 subclasses, FoxA through FoxQ [2], which play critical roles in multiple biological processes, including development, metabolism, aging and cancer (reviewed in [1, 3]). Recently, accumulating evidence has demonstrated critical roles for several forkhead family members in immunoregulation. This review presents a current understanding of these factors and their mechanisms of action; for a general discussion of the forkhead gene family, as well as the roles of forkhead genes in other biological systems, the reader is directed to several excellent recent reviews [1, 2, 4].

* Corresponding author.

Foxp3

Foxp3 (scurfin, sf, JM2) remains currently the most intensely studied forkhead family member in immunology, largely because of its association with $CD4^+$ $CD25^+$ regulatory T (T_{reg}) cells, which downregulate the reactivity of conventional $CD25^-$ $CD4^+$ helper T cells (reviewed in [5, 6]) (fig. 1, table 1). As such, defective T_{reg} function and/or Foxp3 activity have been heavily linked to the pathogenesis of autoimmunity and other immunological conditions which result in, or are caused by, dysregulated or dysfunctional T cell activity [5, 7, 8].

Expression pattern

Foxp3 is expressed at high levels in T_{reg} cells in both mice and humans [8–10], but little is known about the environmental and intracellular signals that regulate it. Recent studies have demonstrated the ability of transforming growth factor (TGF)-β to induce Foxp3 and T_{reg} activity

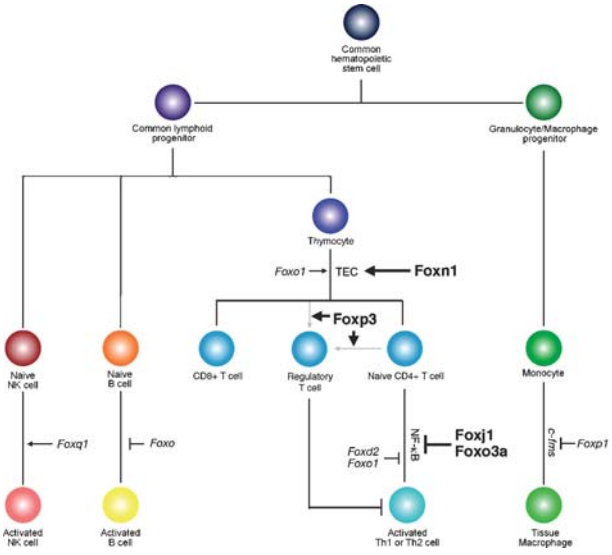

Figure 1. Overview of forkhead genes in the immune system. Shown is a schema of immune cell development and differentiation, with sites of involvement of individual forkhead genes indicated. Bold typeface indicates experimental support from multiple and/or knockout studies; italic typeface indicates experimental support from in vitro or isolated in vivo studies. Foxn1 promotes thymic epithelial cell (TEC) development, while Foxj1 and Foxo3a inhibit spontaneous CD4+ T cell activation by inhibiting NF-κB. Foxp3 is required for regulatory T cell development, but the precise ontogeny of these cells remains unknown. See text for details.

in conventional T cells [11–14], but the ability of TGF-β-deficient mice to produce functionally immunosuppressive T_{reg} cells indicates that this cytokine is not required for their development in vivo [15]. In addition, unlike murine conventional T cells, activation of conventional human $CD4^+$ $CD25^-$ T cells can induce Foxp3 and T_{reg} activity [16], suggesting potential species differences in its regulation. Finally, Foxp3 is also expressed at low levels in a $CD45RB^{low}$ subpopulation of $CD4^+$ $CD25^-$ T cells [8, 9], which may comprise a second population of regulatory cells, at least in mice [17, 18]. Thus, multiple, likely not-yet-identified, environmental signals lead to Foxp3 induction and/or activation.

One popular model of T_{reg} development proposes that Foxp3 expression is upregulated in response to high-avidity T-cell-receptor (TCR) binding in developing single-positive $CD4^+$ T cells, such that T_{reg} cells are in fact generated under unique conditions during thymic selection [6, 9]. Alternatively, Foxp3 may be induced in anergic peripheral T cells in response to antigen encounter [9], or in non-anergic T cells in response to tolerizing signals, appropriate or inappropriate, in the periphery – e.g. as might occur during the response to therapeutic immunosuppression as part of the clinical management of autoimmune diseases [19–21] or transplantation [22]; during the development of cancer [23, 24]; or during normal pregnancy [25]. Such signals may be delivered by endothelial cells [26] or dendritic cells [27]. Formal proof of these concepts in vivo, however, remains largely lacking, and the molecular pathways that mediate Foxp3 induction under such conditions remain unexplored. Thus, although Foxp3 is clearly associated with T_{reg} cells and their immunosuppressive capabilities, the mechanisms of its developmental regulation remain to be determined.

Molecular interactions and targets

Limited evidence indicates that Foxp3 functions predominantly as a transcriptional repressor. In the Jurkat T cell leukemia cell line, it inhibits transcription mediated by the nuclear factor of activated T cells (NF-AT) transcription factors, requiring the forkhead domain for both nuclear localization and DNA binding [28]. Consequently, since NF-AT target genes include several inflammatory cytokines, including interleukin (IL)-2, IL-4, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [29], Foxp3 has been proposed to suppress T-cell-mediated inflammation via the repression of inflammatory gene promoters, and in fact, ectopic Foxp3 expression in primary murine T cells represses IL-2 expression [8]. However, since Foxp3-transduced T cells also upregulate several T_{reg}-associated cell surface molecules, such as CD25 and CD152, it remains unclear if it directly or indirectly activates or suppresses the expression of these genes.

Immunological functions

Despite the lack of knowledge regarding Foxp3's molecular regulation, functional characterizations, both in vitro and in vivo, have convincingly demonstrated its immunosuppressive role via its ability to regulate the activity and number of T_{reg} cells. For instance, Foxp3 transgenic mice have increased numbers of T_{reg} cells that more strongly suppress the proliferation of antigen-stimulated, but not anti-CD3-stimulated, $CD4^+$ T cells, perhaps due to a resistance of Foxp3-transgenic T_{reg} cells to activate in response to anti-CD3/anti-CD28 co-stimulation [10, 30]. Foxp3-transgenic mice also have 25–50% fewer $CD4^+$ cells and 50–75% fewer $CD8^+$ cells than wild-type mice, despite grossly normal thymi, with smaller peripheral lymphoid organs that lack germinal centers, presumably due to the immunosuppressive effect of elevated T_{reg} activity. Indeed, transgenic animals have significantly reduced levels of immunoglobulin (Ig) G1 and IgG2a, but not IgM, compared to non-transgenic counterparts, and are unable to mount a normal humoral response against T-dependent antigens, as measured by antigen-specific levels of IgM, IgG1 and IgG2a [31]. Transgenic $CD4^+$ and $CD8^+$ cells are hyporesponsive to activation unless exogenous IL-2 is provided, and transgenic $CD4^+$ $CD25^-$ T cells express certain T_{reg} markers, such as glucocorticoid-induced TNF receptor (GITR, TNFRSF18), and

Table 1. Forkhead genes with implicated or demonstrated immunological functions

Gene	Location	Non-immunological function(s)	Immunological function(s)	Mutant mouse phenotype	Known human disease associations
Foxd2	hu 1p32-p34 ms 4 56.5 cM	kidney development	modulates cAMP sensitivity	N/A	N/A
Foxj1	hu 17q22-q25 ms 11 78.0 cM	development of cilia in the lung, choroid plexus and oviduct	suppresses spontaneous T cell activation and autoimmunity	spontaneous T cell activation, Th1 skewing, cellular autoimmunity	N/A
Foxn1	hu 17q11-q12 ms 11 45.0 cM	hair development	thymic epithelial cell development	athymia and T cell deficiency	mutations cause severe combined immunodeficiency
Foxo1	hu 13q14.1 ms 3 22.5 cM	regulates insulin signaling, adipocyte differentiation and angiogenesis	suppresses proliferation and/or induces apoptosis in vitro	N/A	fusion proteins with Pax3 associated with rhabdomyosarcoma
Foxo3a	hu 6q21 ms 10 30.0 cM	ovarian follicle development	suppresses spontaneous T cell activation and autoimmunity; suppresses proliferation and/or induces apoptosis in vitro	spontaneous T cell activation, lymphoproliferation, cellular autoimmunity	fusion proteins with MLL associated with acute lymphoblastic leukemia
Foxp1	hu 3p14.1 ms 6 D3	N/A	inihibits macrophage differentiation in vitro	N/A	implicated as a tumor suppressor by expression studies
Foxp3	hu Xp11.23 ms X 2.1 cM	possibly multiple, but likely attributable to the immunological function	regulatory T cell development	excessive T cell activation and autoimmunity, diabetes, eczema, allergy, early lethality	mutations cause immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (IPEX) syndrome
Foxq1	hu 6p25 ms 13 17.0 cM	hair development	promotes/modulates natural killer cell function	N/A	N/A

Location reflects chromosomal map coordinates for human (hu) and murine (ms) homolog. N/A, not available and/or unkown.

exhibit suppressor activity. Ectopic expression of Foxp3 in CD152 (CTLA4)-deficient mice delayed the onset of the hyperproliferative autoimmune syndrome normally observed in those animals, associated with the restoration of defective T_{reg} activity and an increase in the average life span of the mice from less than 4 weeks to over 20 weeks [10], and retroviral transduction of Foxp3 can convert effector T cells to a regulatory phenotype, inhibiting inflammatory cytokine secretion and preventing inflammatory bowel disease and autoimmune gas tritis [8]. Thus, Foxp3 expression confers an anti-activation, T_{reg} phenotype upon T cells, independent of CD152, resulting in a significant impairment of helper T cell function in vivo.

Conversely, mice with defective Foxp3 activity have demonstrated that it is absolutely critical for the production of functional T_{reg} cells and the maintenance of tolerance. The *scurfy* mutation, which results in a truncated Foxp3 protein [32–35], results in a lethal, multi-system autoimmune syndrome, including diabetes, eczema, food allergy, eosinophilic inflammation and lymphoproliferation associated with excessive hyperactivation and autoreactivity of helper CD^{4+} T cells [36, 37]. Similarly, targeted deletion of Foxp3 in mice results in spontaneous autoimmune inflammation, T cell activation and lymphoproliferation nearly indistinguishable from *scurfy* mice [9]. Although both types of mutant animals develop $CD4^+$ $CD25^+$ T cells, they appear to lack regulatory capacity entirely, since these cells are unable exert immunosuppression in vivo, and adoptive transfer of wild-type T_{reg} cells into *scurfy* or Foxp3-deficient mice abrogates the autoimmune syndrome. Thus, Foxp3 is required intrinsically in T cells to produce T_{reg} cells that are mandatory for organ-specific T cell tolerance [9].

Foxp3 in human diseases

Human mutations in Foxp3 result in the immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance [IPEX, XLAAD; Mendelian inheritance in man (MIM) #304790] disorder, a fatal condition consisting of neonatal insulin-dependent diabetes mellitus (IDDM), chronic diarrhea, food allergies and hypergammaglobulinemia (reviewed in [38]). Mutations have generally affected the coding region, such as non-conservative changes in the forkhead DNA binding domain, or changes leading to premature termination, frameshifts or messenger RNA (mRNA) instability [32, 33, 35]. On the other hand, polymorphisms in the promoter of Foxp3 have been associated with type 1 diabetes in some clinical populations [39, 40]. Thus, genetically determined differences in Foxp3 expression levels and function likely modulate the clinical expression of autoimmunity.

It is interesting to note that increased Foxp3 expression and T_{reg} activity is associated beneficially with successful immunosuppressive treatment in some autoimmune diseases [19–21], but is associated detrimentally with spontaneous local immunosuppression and carcinogene-

sis [23, 24]. Foxp3 is therefore not only a therapeutic gain-of-function target in conditions of hyperactive immune systems, such as autoimmunity, asthma, allergy and transplantation, but also a loss-of-function target in conditions of apparently insufficient immunity, such as cancer or chronic infection [24, 41].

The Foxo subfamily

The Foxo transcription factors are the mammalian homologues of the *Caenorhabditis elegans* dauer formation mutant 16 (DAF-16) gene, which regulates insulin signaling and metabolism, as well as the control of organismal life span and fertility [42, 43]. This family, which includes at least Foxo1 (FKHR, forkhead in rhabdomyosarcoma), Foxo3a (FKHRL1, FKHR-like 1), Foxo4 [AFX, mixed lineage-leukemia (trithorax homolog) translocated to 7 homolog, Mllt7] and Foxo6 [44], have been heavily investigated in multiple non-immunological contexts in the regulation of apoptosis, cell cycle, metabolism and resistance to oxidative stress (reviewed in [3, 4, 45]). Gene-targeting experiments in mice have demonstrated that Foxo1 regulates insulin sensitivity [46, 47], adipocyte differentiation [47] and angiogenesis [48], while Foxo3a regulates ovarian development and fertility [49–51] and Foxo4 appears to be largely dispensable for gross organismal homeostasis [50]. In the mammalian immune system, members of this family have been proposed to regulate leukocyte homeostasis, with much of their molecular mechanisms extrapolated from studies in other cell types and/or organisms [52] (fig. 1 and table 1).

Expression pattern

Of the mammalian Foxo genes, Foxo3a appears to be the dominant isoform expressed in lymphocytes, at least at the RNA level [44, 53–55]. Still, all the Foxo family members are detectable in peripheral lymphocytes, where their activity is presumed to be regulated by post-translational modifications as elucidated in other cell types [45] (fig. 2): in resting cells, unphosphorylated forms of the Foxo's are localized in the nucleus, where they are transcriptionally active. In the most well-described model of Foxo regulation, cellular stimulation, such as by mitogens or cellular stress, leads to activation of phosphatidyl-inositol-3-kinase (PI3K), activating protein kinase B (PKB, Akt), which then phosphorylates the Foxo's and renders them susceptible to 14-3-3-mediated nuclear export [56–62] and/or proteasome-mediated degradation [63, 64]. This model correlates with studies demonstrating the PI3K- and/or Akt-related phosphorylation and inactivation of Foxo factors in response to the bacterial chemoattractant formyl-Met-Leu-Phe (fMLP) in neutrophils [65]; B cell receptor

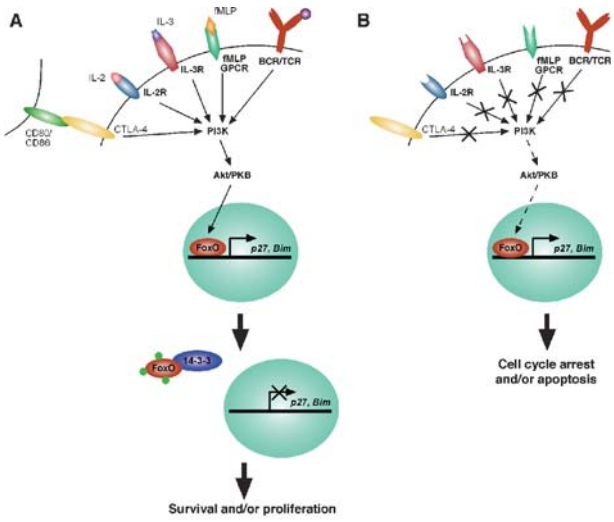

Figure 2. Regulation of Foxo transcription factor activity in lymphocytes. (*A*) Cellular stimulation activates the phosphatidyl-inositol-3-kinase (PI3K)/activating protein kinase B (PKB, Akt) pathway, which leads to phosphorylation of nuclear Foxo. Phosphorylated Foxo is then exported into the cytoplasm and sequestered there by 14-3-3. Transcription of Foxo-dependent anti-proliferative and/or pro-apoptotic genes such as Cdkn1b (p27) and/or Bim ceases, resulting in cell survival and/or proliferation. The receptors shown may also act through other pathways that are PI3K- and/or Akt-independent (see text). (*B*) Withdrawal or deprivation of stimulation results in dephosphorylation of the Foxos, allowing their nuclear localization and transcription of target genes. Dashed arrows indicate signaling pathways that have been blocked upstream. BCR, B cell receptor; fMLP, formyl-Met-Leu-Phe; GPCR, G-protein-coupled receptor; IL-2R, IL-2 receptor; IL-3R, IL-3 receptor; TCR, T cell receptor.

(BCR) stimulation, intercellular adhesion molecule ligation or IL-3 exposure in B cells [62, 66, 67]; and TCR stimulation, CD152 ligation or IL-2 exposure in T cells [55, 60, 68, 69]. Thus, in immune cells Foxo activity is inversely correlated with cellular activation.

Molecular interactions and targets

In actuality, though, an ever-growing number of molecular regulators are being described for the Foxo proteins. For instance, Foxos can be regulated by other PI3K-regulated kinases, such as serum and glucocorticoid-inducible kinase (SGK) [70, 71], or kinases in other signaling pathways, such as Ras-Ral [58, 72], the inhibitor of nuclear factor κB (IκB) kinases of the nuclear factor κB (NF-κB) cascade [73], p90-kDa ribosomal S6 kinase-2 (Rsk-2) [65], or dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1a) [74]. In addition, acetylation, such as by cyclic AMP (cAMP) -response element-binding protein (CREB)-binding protein (CBP), may inactivate the Foxos, with their activation by deacetylases such as silent information regulator 2 (Sir2, SIRT1) [75–77], leading to the promotion of Foxo-induced cell cycle arrest and resistance to oxidative stress, but the inhibition of

Foxo-induced cell death [75, 76, 78, 79]. Also, Foxo inactivation may also occur via even additional pathways, such as caspase-3-like proteolysis in response to CD95- or CD195 ligation [80]; and finally, the transcriptional activity and specificity of the Foxo proteins likely depends also upon interactions with other transcriptional regulators, such as c-myc, Smad proteins and other forkhead genes, like Foxg1 [81–83]. As such, the Foxo family likely links multiple, complex cellular networks, still unelucidated, involved in metabolism, quiescence, proliferation and differentiation.

Nonetheless, the Foxo genes have clearly been demonstrated as transcription factors, with consensus binding sites consisting of a core DAF-16 family protein-binding element (DBE), TTGTTTAC [53, 54]. Their target genes include cell cycle proteins, such as cyclin D1 [84] and cyclin G2 [85, 86], Cdkn1a (p21, Wip1, Cip1, Waf1) [83], Cdkn1b (p27, Kip1) [66, 82, 87, 88]; DNA repair proteins such as GAAD45 [89]; apoptotic genes such as TNF-related apoptosis-inducing ligand (TRAIL, TNFSF) [10, 90], FLICE-inhibitory protein (FLIP) [91] or Bim [60]; and atrophy-inducing genes like atrogin-1 [92, 93]. Although the specific details of interactions between environmental signals, signaling pathways, Foxo's and these target genes have begun to be elucidated – e.g., the ability of the TGF-β-activated Smad proteins to cooperate with Foxo to regulate Cdkn1a [83] – the environmental- and cell-specific mechanisms and contexts which link the Foxos to these distinct biological targets remain still largely unknown, particularly in immunity.

Immunological functions

In cultured and/or transformed cells, several findings have implicated the Foxo members in the regulation of lymphocyte cell death and proliferation [52]. In the human leukemia T cell line Jurkat, Foxo1 overexpression suppresses proliferation [88], while Foxo3a overexpression induces apoptosis [56]. Similarly, Foxo3a overexpression in the murine pre-B cell line Ba/F3 can also induce apoptosis [94], and in the murine CTLL-2 T cell line, IL-2, via PI3K, leads to the inhibition of Foxo family members, which regulate Cdkn1b, Bim [60] and the anti-apoptotic gene glucocorticoid-induced leucine zipper [GILZ, 95]. These findings correlate with the known effects of cytokine withdrawal: reduced PI3K but increased Foxo3a activity, as well as the induction of Cdkn1b and/or Bim, all correlate with IL-3 withdrawal in Ba/F3 pre-B cells [66, 87], and with IL-2 withdrawal in CTLL-2 T cells [60]. Such observations have prompted the proposal that Foxo's regulate lymphocyte quiescence [96, 97] (fig. 2).

Despite these many in vitro studies with Foxo genes in lymphocytes, relatively few immunological studies on Foxo have been performed in vivo. In one transgenic study, expression of a dominant-negative Foxo1 resulted in globally diminished thymocyte numbers, reflecting impaired proliferation [98]. Interestingly, in vitro selection of an optimal Foxo1 binding site yielded (G/C/A)(T/C/A)AAA(T/C)A, which was found in the CD4 proximal and CD8 single-positive enhancers, suggesting that Foxo1 may regulate thymocyte development by modulating lineage commitment, cell survival and/or cycling.

One study in vivo, however, has strongly indicated a requirement for at least the Foxo3a isoform in the maintenance of helper T cell tolerance and quiescence [55]. Foxo3a-deficient mice do not show any outward signs of disease for several months, but close histological investigation in aging animals revealed both splenomegaly and lymphadenopathy, as well as lymphoid infiltrates and inflammation, consisting predominantly of T cells, in several organs, particularly the salivary gland, lung and kidney. These findings correlated with strikingly hyperactive helper T cells expressing surface receptor patterns consistent with increased activation (increased CD44, diminished CD45RB), proliferating more vigorously in response to TCR ligation, and secreting increased quantities of both Th1 and Th2 cytokines, compared to their Foxo3a-sufficient counterparts. Interestingly, Foxo3a-deficient T cells did not display any defects in apoptosis or activation-induced cell death, but required only IL-2 for proliferation and responded briskly in autologous mixed lymphocyte reactions (AMLRs), indicating the presence of endogenously activated, autoreactive T cells. These findings correlated with the ability of Foxo3a to regulate the activity of the NF-κB transcription factor(s), which play critical roles in immediate-early helper T cell responses to antigen receptor stimulation [99], likely via the inhibitory IκB factors: Foxo3a-deficient T cells possessed increased spontaneous NF-κB activity and were relatively deficient in the IκBβ and IκBε subunits. Alternatively, this effect may be indirect, perhaps reflecting a requirement for Foxo3a in the resistance of lymphocytes to oxidative stress [100]. Nonetheless, such findings indicate that Foxo3a enforces T cell tolerance and quiescence by inhibiting spontaneous T cell activation. Whether or not Foxo3a has similar roles in other immune cells, and whether or not the other Foxo genes possess similar functions to Foxo3a in immune cells in vivo, though, awaits further investigation.

Foxos in human diseases

To date, neither elevated nor deficient Foxo activity has been documented in human immunological diseases, but since mice predisposed to develop the autoimmune syndrome lupus possess significantly diminished Foxo activity in T cells, a connection between the Foxo genes and inflammation in humans remains a distinct possibil-

ity [55]. Instead, the most well described associations of Foxo gene dysregulation in humans are in cancer, such as the generation of a paired box 3 (Pax3) transcription factor-Foxo1 fusion protein in alveolar rhabdomyosarcoma, a predominantly pediatric soft tissue cancer [101–103], where the Foxo portion of the fusion protein likely interferes with normal Pax3 function, promoting cellular transformation [104]. Similarly, mixed lineage leukemia (MLL) transcription factor fusion proteins with both Foxo3a and Foxo4 have been described in acute lymphoblastic leukemia [105, 106], but here the oncogenic effect correlates with the resultant dominant-negative effect of the fusion protein on Foxo activity. Thus disruption of cellular homeostasis and tumor progression may be induced by Foxo proteins by either interfering with the normal developmental effects of other transcription factors or the Foxo genes themselves. Investigation of the Foxo proteins in immunological diseases may therefore hopefully yield analogous insights.

Foxn1

Foxn1 (hepatocyte nuclear factor/forkhead homolog-11, Hfh-11, FKHL20, Whn, nude, nu) plays critical roles in epithelial cell development. As such, it has been the focus of both cutaneous and thymic epithelial investigations [107–109], and is most well known immunol ogically for its association with the rodent and human *nude* (*nu*) mutations, which result in defective hair and T cell development [110, 111] (fig. 1 and table 1).

Expression pattern and molecular characteristics

Embryonic expression of Foxn1 appears to initiate in the common primoridum of the third pharyngeal pouch, which gives rise to both the thymus and parathyroid glands [112, 113]. During embryogenesis it is also found in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney and urinary tract, but is largely limited to epithelial cells of the intestine, spermatocytes of the testis and thymus in adults [114]. Its expression is likely regulated by local environmental morphogens, such as wingless (Wnt) glycoproteins [115] or bone morphogenic protein (BMP)-4 [116], each of which may function in both autocrine and paracrine fashions. Foxn1 contains at least two functional domains, including the forkhead DNA binding domain and an N-terminal transactivating domain [108]. Putative Foxn1 targets include the chemokines CXCL12 (SDF-1) and CCL25 (TECK), which can elicit chemotaxis of immature thymocytes [117]; the fibroblast growth factor receptor R2-IIIb [116], which is required for thymic development [118]; and B7-H1 (programmed cell death 1 ligand 1, PD-L1) [117].

Immunological function

Mutations in Foxn1, eliminating the DNA binding domain, are responsible for the hair and thymic developmental phenotype of *nude* mice and rats, which possess defective keratinization of the hair shaft and the differentiation of epithelial progenitor cells in the thymus [108, 110, 112]. Consequently, lymphoid progenitors fail to be attracted to the thymic anlage, perhaps due to a requirement for Foxn1 in the expression of chemotactic chemokines like CXCL12 (SDF-1) and CCL25 (TECK) [119], resulting in defective T cell development. Formation of the thymic epithelial primordium is intact in Foxn1 mutant mice, but subsequent differentiation into subcapsular, cortical and medullary epithelial cells is defective. Interestingly, one study has also suggested an intrinsic role for Foxn1 in early T cell precursors, since *nude* T cells demonstrate a developmental arrest, failing to express the pre-T cell receptor pTα or develop into mature T cells, in adoptive euthymic hosts [120]. Nonetheless, the *nude* phenotype is importantly rescued in vivo by transgenes encoding wild-type Foxn1 [121, 122], and independent null mutations in Foxn1 recapitulate the *nude* phenotype [112], demonstrating that Foxn1 indeed accounts for the developmental abnormalities of the *nude* rodents.

Interestingly, the Foxn1 N-terminal domain, which lacks DNA binding activity, is required for thymic development, as demonstrated by mice with a targeted deletion of that region [123]. These animals developed normal hair, and possessed thymi with developing thymocytes, in contrast to Foxn1 null mutations, but their total thymocyte numbers were still significantly diminished. Thymic epithelial cells were capable of forming a rudimentary reticular network, but no organized cortical or medullary regions were detected, associated with defects in double-positive (DP) thymocyte production, perhaps reflecting defective cross-talk between thymic epithelial cells and other epithelial cells and/or thymocytes. Thus, Foxn1 mediates the initiation and progression of thymic epithelial cell differentiation as two distinct functions in vivo; however, the molecular mechanisms that account for this dichotomy remain unknown.

Foxn1 in human disease

A nonsense mutation in Foxn1 accounts for the human nude/severe combined immunodeficiency (SCID) syndrome, consisting of T cell deficiency, congenital alopecia and nail dystrophy (MIM #601705) [111, 124]. As might be predicted from the rodent studies, bone marrow transplantation in this syndrome is unable to reconstitute the T cell compartment fully, resulting in normal naïve CD8+ but not CD4+ T cells. Thus Foxn1 critically regulates T cell development via a lymphocyte-extrinsic role in thymic epithelial cells in both rodents and man.

Foxj1

The Foxj1 (hepatocyte nuclear factor/forkhead homolog-4, HNF-4, FKHL-13) transcription factor has been most studied for its role in the development of ciliated epithelium, such as in the lung, choroids plexus and reproductive tract [125–128]. Foxj1-deficient mice are devoid of cilia, and consequently suffer from significant developmental abnormalities including heterotaxy and hydrocephalus, resulting in lethality in utero or soon after birth [125, 127]. Immunological attention to this gene was drawn during microarray studies to identify novel transcription factors in autoimmunity [129].

Expression pattern and molecular characteristics

Like the Foxo transcription factors, Foxj1 is expressed predominantly in naïve T cells and is rapidly downregulated upon activation, such as during TCR ligation and/or IL-2 exposure [129]. However, it is not known whether or not Foxj1 is post-translationally modified and/or regulated in a similar fashion as well. Foxj1 is presumed to function in vivo as a transcription factor because of its possession of a forkhead DNA binding domain, which confers DNA binding to a TGTTGTT core sequence [130]; however, the only transcriptional target suggested to date in vivo is the IκBβ inhibitory subunit of the NF-κB family [129]. In contrast, Foxj1 is required for the development of proper microanatomical features in ciliated cells, such as the apical localization of ezrin [131] and the anchorage of basal bodies to the cytoskeleton [132], but whether these reflect direct interactions between Foxj1 and these proteins, independent of transcription, versus indirect effects of still-unknown Foxj1 target genes, remains unclear. Thus the biochemistry, molecular biology and genetics of Foxj1 remain largely unknown.

Immunological functions

One study has demonstrated a critical role for Foxj1 in helper T cell tolerance in vivo [129]. In animals with Foxj1-deficient lymphoid systems, generated by fetal liver chimerization in recombinase activating gene (Rag)-deficient animals, helper T cells spontaneously activate, resulting in multi-system inflammation, particularly of the lung, liver, kidney, and salivary gland, and a moribund appearance 12–16 weeks post-reconstitution. Foxj1-deficient T cells required only IL-2 for proliferation, in the absence of TCR or CD28 ligation, and responded vigorously in AMLRs, indicating the presence of endogenously activated autoreactive T cells. Interestingly, Foxj1-deficient T cells made disproportionately higher amounts of Th1 cytokines, associated with increased expression of the Th1 transcription factor T-bet. These findings resulted from a requirement for Foxj1 to suppress NF-κB activation in vivo via induction of the IκBβ inhibitory subunit, since Foxj1-deficient T cells had elevated levels of spontaneous NF-κB activity, diminished levels of IκBβ, and antisense knockdown of the RELA subunit of NF-κB abrogated the hyperactivated T cell phenotype in vitro. Thus, like Foxo3a, Foxj1 is required in vivo to modulate NF-κB activity and maintain T cell tolerance – but unlike Foxo3a deficiency, Foxj1 deficiency appears to be much more severe, affects a different spectrum of end organs and skews towards Th1 cytokine production. Thus these two forkhead members play somewhat overlapping yet clearly distinct roles in helper T cells, the differences of which remain to be fully elucidated.

Foxj1 in human diseases

Few studies have investigated the role of Foxj1 in human diseases. One study has described a lack of mutations in Foxj1 in a cohort of patients with primary ciliary diskinesia (PCD), a disorder of cilia development consisting of bronchiectasis, chronic sinusitis and situs inversus – but those analyses were limited to the two coding exons of Foxj1, and do not preclude the possible contributions of polymorphisms in non-coding regions, which may regulate expression levels of Foxj1 [133]. Indeed, given the immunological phenotype of Foxj1 deficiency, and the relative Foxj1 deficiency observed in autoimmune versus non-autoimmune mice [129], abnormalities in Foxj1 gene expression, function and/or metabolic pathway may predispose to human autoimmunity and/or other inflammatory conditions.

Other forkhead transcription factors in immunology

Foxd2

Foxd2 (MF2, FKHL17, FREAC9) is expressed in multiple mesodermal lineages, particularly correlating with expression of the sonic hedgehog (Shh) morphogen [134]. Initial assessments presumed that it would play a critical role in kidney development, as suggested by the lethal renal defects in mice deficient in the related Foxd1 (BF-2, Hfh10, FREAC4, FKHL8) gene [135]; however, Foxd2-deficient animals demonstrate only mild and inconsistent renal abnormalities [136]. Thus, its dominant biological function may yet be undiscovered.

In lymphocytes, limited data indicates that Foxd2 is expressed in T cells and monocytes, but not B cells [137]. Interestingly, Foxd2-deficient T cells are mildly less sensitive to cAMP-mediated inhibition of proliferation, correlating with a mild deficiency in the RIα subunit of the cAMP-dependent protein kinase type I (PKA type I).

Indeed, Foxd2 can cooperate with Akt to transactivate the RIα1b promoter, suggesting that Foxd2 modulates T cell activation by fine-tuning sensitivity to cAMP [137]. Interestingly, elevated PKA activity may account for the anergy and hyporesponsiveness of T cells in HIV infection, and of B cells in common variable immunodeficiency (CVI) [138, 139], while defective PKA activity may underlie T cell hyperactivity in lupus [140]. Thus, Foxd2 and/or its molecular pathways may yet account for the phenotypes of multiple immunological diseases.

Foxp1

Although widely expressed, Foxp1 (QRF1, MFH) has been largely studied in the context of pulmonary and nervous system development [141–145], where interestingly it is capable of forming both homo- and heterodimers with other forkhead family members [145, 146]. Expression studies in some cancers have implicated it as a tumor suppressor [147, 148].

A recent study has suggested that Foxp1 regulates tissue macrophage differentiation [149]: in THP-1 and HL60 monocyte cell lines, Mac-1 (CD11b/CD18) receptor ligation correlates with macrophage differentiation and Foxp1 downregulation, while ectopic Foxp1 expression inhibited CD11b expression, cell adhesiveness, phagocytosis, and expression of the *c-fms* locus, which encodes the M-CSF receptor required for macrophage differentiation. Future studies will hopefully confirm and further elucidate the role of this gene in vivo.

Foxq1

As one of the first forkhead genes studied, Foxq1 (HFH1, satin) has been relatively well-studied biochemically, with clear definitions of the core DNA binding domain and flanking wings which contribute to sequence specificity [150]. However, despite its widespread expression, a role for it has only clearly been demonstrated in hair differentiation, where a mutation in Foxq1 accounts for the spontaneously-arising *satin* (*sa)* mutation [151]. in vitro, Foxq1 can repress smooth muscle differentiation genes, such as telokin and SM22α [152], but whether these findings apply in vivo is not known definitively. Interestingly, the *sa* mutation confers a deficiency in natural killer cell activity but not numbers in vivo, an effect synergistic with the *beige (bg, Lyst)* mutation [153]. However, this effect may be strain specific [151] and has been studied predominantly in the presence of the *bg* mutation [153]. Thus, further investigation is warranted to determine the precise functions and mechanisms of Foxq1, not only in the immune system, but also in mammalian development and differentiation in general.

Conclusions

Existing evidence clearly indicates that the forkhead genes play critical roles in the effector differentiation and/or function of multiple immune cell lineages (fig. 1 and table 1): Foxn1 plays a critical role in T cell development by promoting the differentiation of the thymic stroma. Foxp3 enforces tolerance upon autoreactive T cells in an extrinsic fashion by promoting the development of T_{reg} cells, while Foxj1 and Foxo transcription factors enforce tolerance and suppress spontaneous activation intrinsically in naive T cells. Foxd2 may participate in fine-tuning this latter process, while Foxq1 and Foxp1 may modulate natural killer and monocyte activity and/or differentiation, respectively.

Nonetheless, much remains to be learned about the forkhead transcription factors in immunology. With the exception of the Foxo subfamily, the mechanisms by which they are regulated are largely unknown; and for many forkhead genes, immunological functions have been implicated in vitro but not yet tested in vivo. Continued investigation of the forkhead genes is therefore likely to provide key insights into the mechanisms of immunoregulation, as well as disorders of inflammation and/or immunosuppression, and hopefully will culminate in novel yet specific strategies for the therapeutic modulation of disease.

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