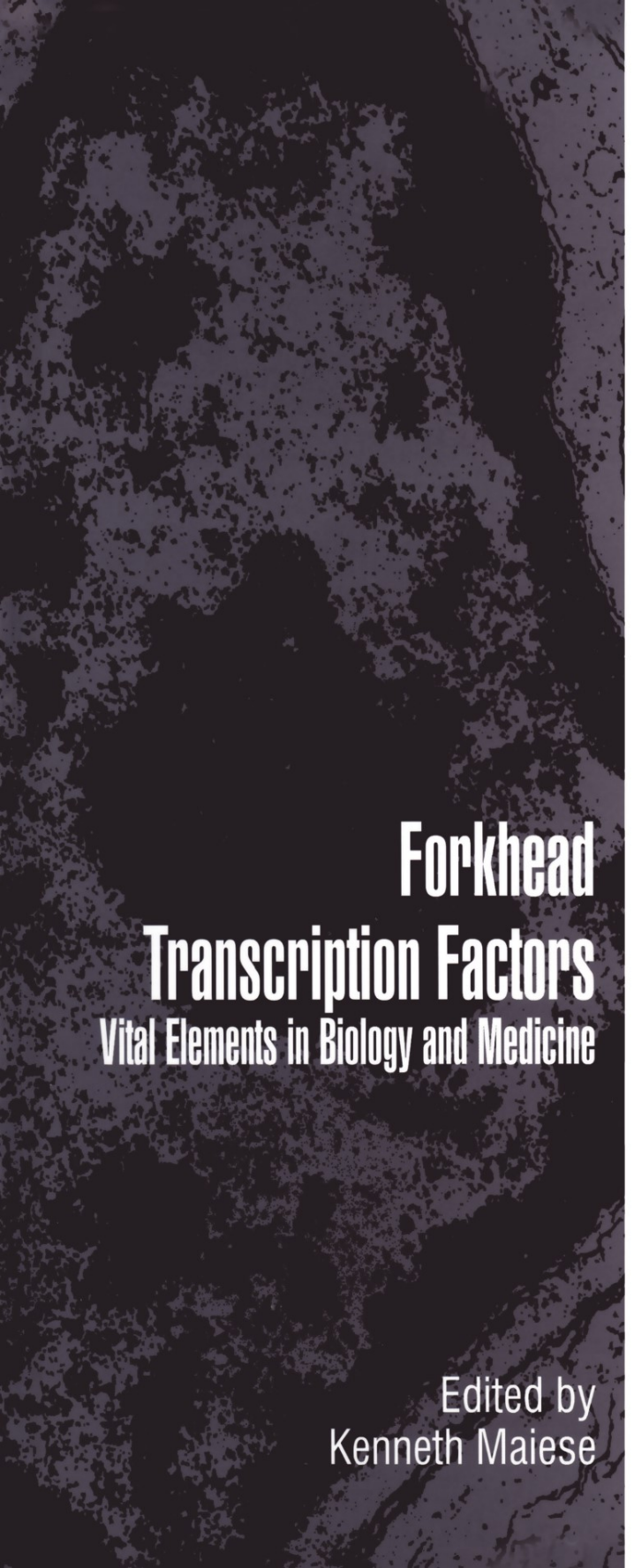

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 665

Forkhead Transcription Factors

Vital Elements in Biology and Medicine

Edited by
Kenneth Maiese

Forkhead Transcription Factors

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PREFACE

Disorders that involve a broad array of conditions, such as atherosclerosis, cancer, cardiovascular disease, diabetes, neurodegeneration, infertility, or autoimmune disease, affect a wide spectrum of the world's population leading to significant disability or untimely demise. For example, metabolic disorders that include diabetes mellitus occur in greater than 16 million individuals in the United States and more than 165 million individuals worldwide. In addition, by the year 2030, it is predicted that more than 360 million individuals will be afflicted with diabetes mellitus and its debilitating conditions. Successful care and treatment for any one of these disease entities can therefore rely heavily upon the modulation of novel cellular pathways that integrate cellular proliferation, metabolism, inflammation, and longevity.

If history serves as a potential guide for the sophisticated basic and clinical research enterprises that exist today, one can look back upon early pioneers in medicine such as Ernest Starling who astutely recognized that systems within the body were involved in constant communication that could affect numerous bodily functions. In his second Croonian lecture to the Royal College of Surgeons in 1905, Starling states that "these chemical messengers, however, or hormones, as we might call them, have to be carried from the organ where they are produced to the organ which they affect by means of the blood stream and the continually recurring physiological needs of the organism must determine their repeated production and circulation throughout the body". With this presentation, Starling invoked the term "hormones" that was initially derived from the Greek term "excite" or "arouse". It is believed in his conversations with William Hardy and the Greek poet scholar W.T. Vesey that he may have desired to portray a more integrated approach to address disease processes that involves multiple organs.

Although prior to this time the use of the term "hormone" in the scientific literature was extremely limited, work during the mid-nineteenth century, such as by Claude Bernard, described additional processes responsible for the internal secretion of chemicals. With his studies that focused upon the release of glucose from glycogen in the liver, Bernard illustrated the importance to understand the intimate relationship that existed among the systems of the body. During this period, other pioneers such as Arnold Adolphe Berthold also spoke of the interaction and communication between

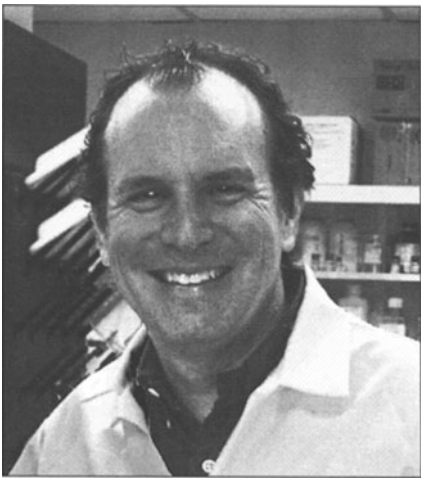
the different organs in the body. As these concepts became more accepted, physicians later in the nineteenth century reported the use of extracts of animal thyroid, pancreas, and even adrenal glands to treat patients suspected of suffering from the loss of circulating chemicals. Eventually, subsequent investigations in disciplines such as endocrinology have fostered the understanding of disease processes in numerous other fields that involve cardiovascular biology, neuroscience, renal physiology, genetics, metabolomics, organ development, cancer, and immunology. Clinically, the advances from these fields that rely upon the basic premise that cells, tissues, and organ systems are tightly integrated during normal physiology and disease have led to remarkable strides in areas such as diabetes, cardiovascular medicine, neurodegeneration, and reproductive medicine.

In the present time, the ability to "excite" or "arouse" appears to have been captured by a family of proteins known as forkhead transcription factors. Forkhead proteins function as transcription factors to either inhibit or activate target gene expression. As a result, these proteins bind to DNA through the forkhead domain that consists of three α-helices, three β-sheets, and two loops that are referred to as the wings. On X-ray crystallography or nuclear magnetic resonance imaging, the forkhead domain is described as a "winged helix" as a result of a butterfly-like appearance. Forkhead transcription factors are extremely compelling to consider as potential clinical targets for multiple disorders since they control processes associated with angiogenesis, stem cell proliferation, cardiovascular injury, neurodegeneration, metabolism, cancer, immune surveillance, aging, and cell longevity. More than 100 forkhead genes and 19 human subgroups that range from *FOXA* to *FOXS* are now known to exist since the initial discovery of the fly *Drosophila melanogaster gene forkhead.*

As a result, *Forkhead Transcription Factors: Vital Elements in Biology and Medicine* provides a unique platform for the presentation of novel work and new insights into the vital role that forkhead transcription factors play in multiple systems throughout the body. Leading international authorities provide their knowledge and insights to offer a novel perspective for translational medicine that highlights the role of forkhead genes and proteins that may have the greatest impact for the development of new strategies for a broad array of disorders. Equally important, *Forkhead Transcription Factors: Vital Elements in Biology and Medicine* clearly sets a precedent for the necessity to understand the diverse and complex nature of forkhead proteins since this family of transcription factors can limit as well as foster disease progression depending upon the cellular environment. The presentation and discussion of innovative studies and especially those that examine previously unexplored pathways that may influence clinical survival and longevity offer an exciting approach to address the potential of forkhead transcription factors for new therapeutic avenues in multiple disciplines.

Kenneth Maiese, MD

ABOUT THE EDITOR...



KENNETH MAIESE is a physician-scientist whose interests focus on the basic and clinical mechanisms that control neuronal and vascular longevity and immune system function. He presently is the Chief of the Division of Cellular and Molecular Cerebral Ischemia and is a Professor in the Departments of Neurology and Anatomy and Cell Biology, the Center for Molecular Medicine and Genetics, the Barbara Ann Karmanos Cancer Institute, and the National Institute of Environmental Health Sciences at Wayne State University School of Medicine.

Dr. Maiese graduated from the University of Pennsylvania Suma cum Laude with Distinction and received his medical degree as a Teagle and Grupe Foundation Scholar from Weill Medical College of Cornell University. He obtained his internship and residency at The New York Hospital-Cornell Medical Center and subsequently completed his clinical and basic science postdoctoral training at Cornell and the National Institute of Aging.

Dr. Maiese has been fortunate to receive recognition with outstanding teaching awards and election to America's Top Physicians and The Best of U.S. Physicians. His investigations are designed to translate basic science into successful therapeutic treatments for conditions such as cancer, metabolic disorders, cardiovascular disease, diabetes, stroke, and Alzheimer's disease. His work has received the distinction of "High Impact Research and Potential Public Health Benefit" by the National Institutes of Health with continuous funding from sources that include the American Diabetes Association, the American Heart Association, the Bugher Foundation, a Johnson and Johnson Focused Giving Award, and the National Institutes of Health. He chairs national grant committees and is a chartered panel member or consultant for several national and international foundations as well as multiple study sections and special emphasis panels for the National Institutes of Health.

Dr. Maiese serves as the Editor-in-Chief for two international journals as well an Associate Editor or a member of the editorial board for several journals, executive committees, technology transfer panels, and scientific advisory councils. Given the broad applications of his work, Dr. Maiese is frequently honored as the chairperson and/or the plenary speaker for a number of international symposiums in a range of disciplines that include cell biology, neuroscience, vascular biology, cardiac disease, molecular oncology, and renal physiology.

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Section I

Insights into Immunity, Infection and Cancer

CHAPTER 1

FOXO1, T-Cell Trafficking and Immune Responses

Florent Carrette, Stéphanie Fabre and Georges Bismuth*

Abstract

Efficient T-cell adaptive immune response require a faultless coordination between migration of naive T-cells into secondary lymphoid organs and critical biological outcomes driven by antigen such as cell division and cell differentiation into effector and memory cells. Recent works have shown that the phosphoinositide 3-kinase (PI3K) pathway could govern several of these processes. In this control, transcriptional factors of the Forkhead box O (FoxO) family, in particular FOXO1, a downstream effector of PI3K, appears to play a major role by coordinating both cellular proliferation of T-cells after antigen recognition and expression of homing molecules essential for their trafficking in the body.

Introduction

Efficient immune surveillance requires that naive T-lymphocytes circulate permanently between the blood stream, secondary lymphoid organs and lymphatic vessels. It has been estimated that at a given time the pool of T-cells in the blood represents only 5% of the total T-cell count, 70% of them being localized in lymph nodes and approximately 20% in the spleen. In normal conditions, a T-cell usually stays less than 30 minutes in the blood circulation, making repeated visits of several hours in secondary lymphoid organs. Because of the limited number of specific T-lymphocytes for a given antigen, this trafficking is fundamental to increase the probability for a T-cell to encounter the antigen and thereby warrant immunological surveillance of the body. Moreover, antigen-presenting cells (APC), especially dendritic cells (DCs) (the only APC that can activate naive T-cells), migrate preferentially into lymph nodes after the capture of foreign antigens in tissues at the periphery. Thus, lymph nodes occupy a strategic position at the crossroads of the blood and lymphatic vessels to bring together in an adapted microenvironment these different actors of the immune response. This trafficking of T-cells, the so called homing process, is tightly controlled by a set of coordinated mechanisms, notably involving cell surface molecules and soluble signals such as chemokines. We will describe in this chapter the mechanisms by which this spatiotemporal control can be exercised at the different steps of a T-cell response and consider very recent discoveries showing that the Forkhead box O transcriptional factor FOXO1, one major downstream effectors of the PI3K pathway, has an unanticipated role in these regulations.

Mechanisms of T-Cell Homing into Lymph Nodes

T-lymphocytes migrate into lymph nodes by crossing the vascular endothelium at the level of specialized postcapillary vessels, termed high endothelial venules (HEV). This passage is based on a hierarchical sequence of interactions between lymphocytes and endothelial cells,

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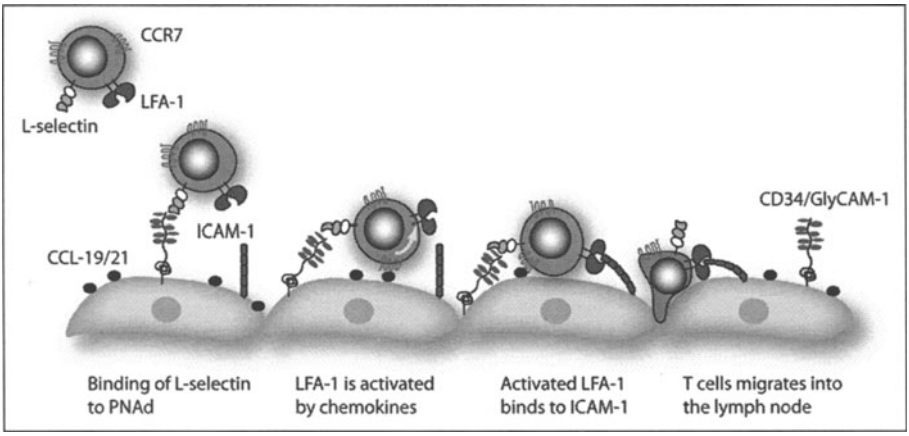

Figure 1. The different steps of T-cell transmigration. In HEV L-selectin expressed at tips of T-cell microvilli can interact with peripheral node addressins (PNAd) like CD34 or GlyCAM-1 decorated with carbohydrates. A rolling movement along the endothelium that slows down the cell and allows its firm adhesion follows this initial anchoring. The integrin LFA-1 (Leukocyte Function-associated Antigen-1) and the adhesion molecule ICAM-1 (Intercellular Adhesion Molecule-1) expressed on T-cells and endothelial cells, respectively, are the two key receptors involved during this phase. However, in resting T-cells, the avidity of LFA-1 for its ligand is weak and the T-cell needs supplementary signals to transmigrate at the level of the HEV. These signals are triggered by chemokines present on endothelial cells (such as CCL19 or CCL21) that interact with CCR7 on T-cells and strongly increase the avidity of LFA-1 for ICAM-1.

governed by adhesion molecules, such as selectins and integrins and signals given by chemokines (Fig. 1). L-selectin, a Type C lectin of ~90 kDa also named CD62-L or LAM-1 (Leukocyte Activated Molecule-1), is a key player in this process. It was discovered nearly two decades ago as a glycoprotein involved in the interaction of leukocytes with the endothelium of lymph nodes and inflamed tissues.[1-3] L-selectin is expressed at the end of plasma membrane microvilli and specifically interacts with sialylated oligosaccharides carried by molecules like CD34 and glycosylation-dependant cell adhesion molecule-1 (GlyCAM-1) (also called PNAd for peripheral node addressins), constitutively expressed on the surface of HEV endothelial cells. Invalidation of the L-selectin gene in the mouse has demonstrated the essential role played by this molecule in the migration of leukocytes into secondary lymphoid organs in vivo.[4] Deficient mice show a severe reduction of the number of T-lymphocytes in lymph nodes, as well as a defect of primary immune responses to antigen. Lymphocytes from these mice cannot adhere to the HEV anymore, indicating that the interaction between L-selectin and its vascular ligands is essential for later stages of transendothelial migration.

According to this central role played by L-selectin in the migration of T-cells within lymph nodes, the mechanisms regulating its membrane expression were the subject of many studies. L-selectin expression is rapidly down-modulated (in a few tens of minutes) after T-cell receptor (TCR) triggering by antigen[5] and activated T-cells always express reduced levels of L-selectin.[6] Our knowledge of the molecular mechanisms controlling this rapid decrease, early after activation, is still partial but the use of pharmacological inhibitors has revealed the involvement of metalloproteases from the extracellular matrix,[7,8] with as a potential, but not exclusive candidate, the ADAM17 metalloprotease (also known as CD156b or TNF-α converting enzyme).[9] The contribution of L-selectin proteolysis in T-cell homing was unambiguously demonstrated by the study of transgenic mice expressing a shedding-resistant L-selectin molecule.[10,11] While these mice do not present abnormalities in the cellular composition of secondary lymphoid organs, suggesting that the cleavage of L- selectin is not required for the migration of naive T-lymphocytes into

these organs, activated T-cells harboring this L-selectin mutant still migrate into lymph nodes after adoptive transfer.

However, in addition to proteolytic cleavage, transcriptional mechanisms are also at work in T-lymphocytes to regulate the membrane expression of L-selectin. Indeed, analyses in murine T-cells have revealed three distinct phases after activation in vitro: a fast decrease of L-selectin, linked to the action of metalloproteases, arises within the first four hours; then L-selectin levels are restored during 48 hours, a process likely related to a greater stability of its mRNA (this phase is not clearly distinguishable in human T-cells); finally, a prolonged decrease is observed from day-3 to day-4 after stimulation, corresponding to a decline in the rate of transcription of the L-selectin gene.[12] Consistently, transgenic T-lymphocytes expressing a mutant L-selectin molecule resistant to proteolysis also show after a few days of stimulation in vitro a similar decrease in membrane expression of L-selectin, suggesting the implementation of a late transcriptional control.[11]

As already mentioned, chemokines also play a key role in T-cell homing within lymph nodes besides other effects like leukocyte maturation, organization of lymphoid organs and recruitment of effector T-cells at the sites of inflammation.[13] The main chemokines involved in T-cell homing within lymph nodes are CCL19 and CCL21, whose common receptor is CCR7.[14,15] CCR7 is expressed in naive T-cells, but it is down-regulated after activation. The essential role of these chemokines in the migration of T-cells at the level of the HEV in vivo was demonstrated by the study of *plt* (paucity of lymph node T-cells) mice carrying a spontaneous alteration of CCL19 and CCL21 expression in lymphoid organs accompanied by a defective homing T-cells into lymph nodes.[16-18] After adoptive transfer of normal T-lymphocytes in *plt* mice the firm adhesion of the T-cells to the HEV of these mice is largely compromised.[19,20] Identical results have been obtained with mice deficient in CCR7.[21] By stimulating CCR7, CCL21 and CCL19 allow LFA-1-dependent interactions of naive T-lymphocytes with ICAM-1 (intercellular adhesion molecule-1), expressed by endothelial cells, by an inside-out activation mechanism.[22-24] CCL21 is produced by stromal cells in the T-cell zone of the lymph node and by endothelial cells of the HEV.[25,26] CCL19 is only produced by stromal cells and diffuses by transcytosis to the luminal face of the HEV.[27] Chemokines can be immobilized on the luminal surface of the HEV either directly by glycosamoniglycans and proteoglycans with heparan-sulfate of the extracellular matrix,[28] or indirectly via glycoproteins susceptible to modulate their function and their bioavailability.[29]

FOXO1, L-Selectin and T-Cell Homing

Until very recently no experimental data were available suggesting that the PI3K/FOXO1 pathway is involved in the homing of T-cells. This gap was recently filled by studies conducted in the human Jurkat leukemic T-cell line deficient in PTEN (phosphatase and tensin homolog deleted on chromosome 10), the master lipid phosphatase that regulates cellular phosphatidylinositol-3,4,5-P_3 (PIP_3) levels.[30] These cells show a constitutive activation of the PI3K pathway and only residual activity of FOXO1 after its phosphorylation by the serine/threonine kinase Akt, the main downstream effector of PI3K, and its nuclear eviction as illustrated in Figure 2. Unexpectedly, it was found that in this cell line an active FOXO1 molecule, mutated on its three phosphorylation sites by Akt (T24/S256/S319), strongly increases transcription of the *SELL* gene coding for L-selectin.[31] Strikingly, CCR7 as well as the sphingosine-1-phosphate (S1P) endothelial differentiation gene-1 (EDG-1) receptor which controls T-cell egress from secondary lymphoid organs (see below) are also up-regulated. Restoration of nuclear localization of FOXO1 in these cells after PIP_3 hydrolysis by PTEN (see Fig. 2) has a similar effect. Finally, FOXO1 also blocks the prolonged decrease of L-selectin at the plasma membrane observed in normal human T-cells after activation. Accordingly, activated murine T-cells transduced with the same active FOXO1 mutant and adoptively transferred into recipient mice show a persistent ability to home into lymph nodes as compared to control cells (Fig. 3). Surprisingly, they also migrate better into the spleen, though L-selectin is usually not required for T-cell homing into this specific organ. Thus, apparently, T-lymphocytes make use of several routes to regulate L-selectin levels: firstly, a rapid

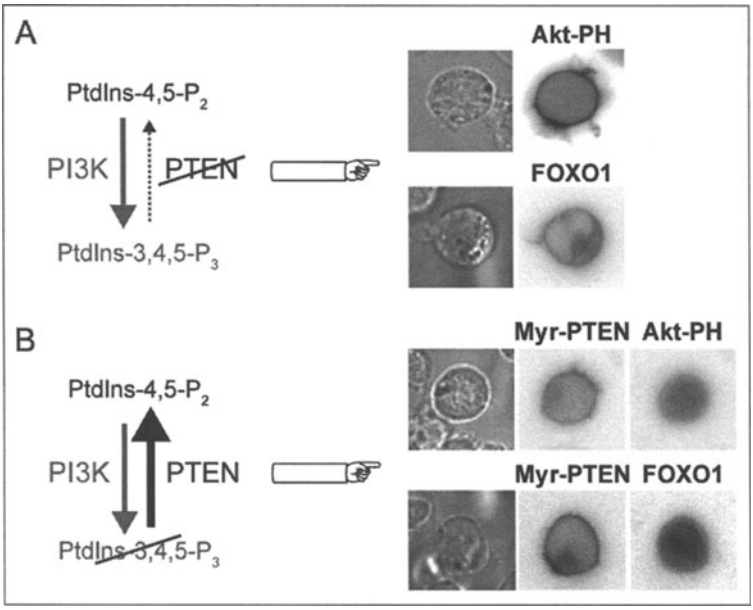

Figure 2. PTEN opposes PI3K to sequester FOXO1 outside the nucleus in T-cells. A) In Jurkat T-cells that lack the lipid phosphatase PTEN, the PH domain of Akt (Akt-PH), which binds the PI3K product PtdIns-3,4,5-P3 and expressed as a GFP-tagged molecule, is heavily recruited to the plasma membrane. In these cells, a FOXO1-GFP molecule is sequestered into the cytoplasm and inactive. B) Restoration of PTEN activity and hydrolysis of PtdIns-3,4,5-P3 by expressing of a membrane targeted form of PTEN (myristoylated-PTEN; myr-PTEN) has the opposite effect.

proteolytic cleavage, early after T-cell activation and secondly a transcriptional arrest, after the nuclear exclusion of FOXO1 triggered via the PI3K/Akt pathway. Captivatingly, another recent report has shown that the rapid shedding of L-selectin after TCR stimulation is inhibited in murine $CD8^+$ T-cells expressing a catalytically inactive form of p110δ, the predominant PI3K catalytic subunit activated downstream of antigen recognition in T-cells.[32] Thus both cleavage and transcriptional regulation of L-selectin might be under the control of PI3K, though preliminary results do not show a clear inhibition of L-selectin shedding in human T-cells treated with pharmalogical inhibitors of PI3K (F.C., Marianne Mangeney and G.B., unpublished). Interestingly enough, in the mouse, comparative transcriptome analyses of thymocytes with conditional loss of all three FoxO alleles (*FoxO1*, *FoxO3* and *FoxO4*) also point toward *SELL* as a candidate gene for FoxOs in this cell type.[33] Finally, a recent work has reported homing defects together with alteration of L-selectin levels in murine B-cells lacking FoxO1, showing that it might be a common transcriptional activator of the *SELL* gene in lymphoid tissues.[34] One challenge now, using these gene invalidation models, will be to see how loss of all three or individual *FoxO* genes could alter L-selectin expression and other homing molecules in mature peripheral T-cells in homeostatic conditions or during immune response; concomitantly, these mouse models would also help clarify functional redundancy among these closely related transcription factors to regulate T-cell trafficking in vivo.

Mechanistically, it is not yet clear whether FOXO1 needs to bind the promoter of L-selectin directly to exercise its transcriptional control in human T-cells. Favoring this hypothesis, a FOXO1 molecule mutated on the residue that binds DNA (H215R mutation in the helix 3 of the so-called winged-helix domain of FOXO1 to bind DNA) does not trigger this effect anymore. Analysis between species of the promoter of the *SELL* gene, coding for L-selectin, does not show typical

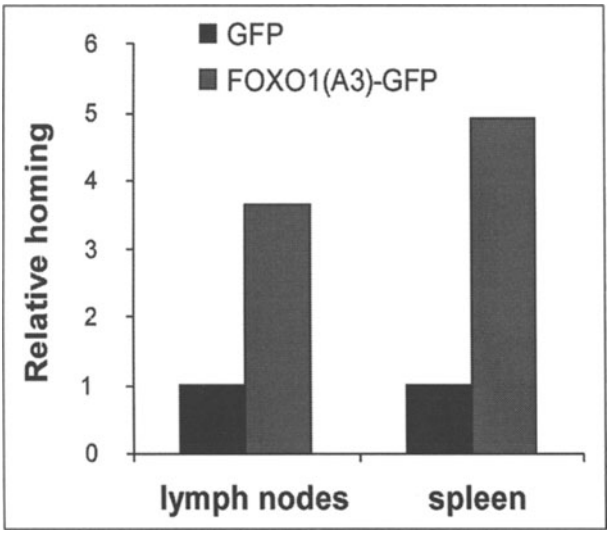

Figure 3. Active FOXO1-transduced T-cells show a better ability to home into lymph nodes. T-lymphocytes were purified from C57Bl/6 mice and stimulated in vitro with anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) monoclonal antibodies coated on 24 well-plate in RPMI 1640 medium supplemented with 10% FCS and IL2 (30 u/ml). At 1 day poststimulation, cells were transduced with retroviral constructs encoding either GFP alone or a constitutively active FOXO1 molecule (T24A/S256A/S319A) fused to GFP (FOXO1-(A3)-GFP). 48 hours later cells were adoptively transferred into recipient mice during 4 hours. Lymph nodes (axillaries, brachials and inguinals) and spleen were harvested and analyzed by flow cytometry for GFP expression. Histogram represents fold change compared to GFP alone.

IRS (insulin response sequence) FoxO binding motifs (5′-TTGTTTAC-3′), which represents the conserved consensus core recognition motif. Nevertheless, several IRS-like sequences are present in the promoter, indicating that SELL might be a true direct target of FoxOs in T-cells. However, unexpectedly, we have observed that this mutant has also a strong dominant negative effect on L-selectin membrane levels when it is expressed in primary resting T-cells. This result suggests that the transcriptional control by FOXO1 is permanent and sufficient to maintain high levels of L-selectin in unstimulated T-cells; but it is also indicative that L-selectin regulation by FOXO1 might be indirect, as demonstrated for many other genes regulated by FOXOs. For example, FoxO transcription factors cooperate with delta EF1 to activate growth suppressive genes in B-lymphocytes.[35] In fact, various interactions with various transcription factor protein partners have been reported to help control the expression of FoxO target genes in different cell systems; it has also been proposed that a "FoxO" code may exist, dictated by the posttranslational modifications of FoxOs and interpreted by protein partners to direct the activity of FoxOs within cells.[36] Thus, it is possible that the FOXO1 mutant competes with the endogenous wild-type molecule to make these partnerships fruitless in the control of L-selectin.

FOXO1 and T-Cell Proliferation

Intravital microscopy analyses of lymph nodes have considerably increased our knowledge of the in situ behavior of T-lymphocytes. In the absence of antigen, naive T-cells migrate in the paracortex, then move rapidly and continually within this zone along privileged axes formed by a network of follicular reticular cells.[37-42] During this course, they establish very transient and unceasing interactions with DCs to scan their surface. This very dynamic behavior, helped by the multiple extensions of DCs inside the lymphoid tissue, is highly efficient to detect antigen and it

is estimated that a DC can contact between 500 and 5000 T-cells per hour.[38,43] Chemokines plays a key role in supporting this performance of T-cells within lymph nodes, in particular CCL19 and CCL21, the two ligands of CCR7.[44-46] However, after antigen recognition a "stop" signal is given to the T-cell and a stable contact is established with the DC, which can last 24 hours.[38,39,47] Later on, a new phase occurs during which T-cells start to divide actively and show concomitantly loosen contacts with DCs.

The PI3K pathway is a key regulator of cell growth and proliferation and frequent alterations of its members are involved in a broad range of cancers. Several effectors of this pathway are affected downstream of PI3K that may explain this trait and particularly FoxOs. FoxOs are important regulators of cell quiescence in different cell systems,[48] including in T-cells. Numerous studies have shown that Akt phosphorylates these transcription factors, promoting their nuclear exclusion and their inactivation.[49-51] This explains why the introduction of FOXO3 or FOXO4 constitutively active molecules in Jurkat leukemic T-cells leads to apoptosis and cell cycle arrest;[52,53] consistently, the nuclear exclusion of FOXO1 also regulates Il-2-dependent cell growth of the murine CTLL-2 T-cell line.[54] We previously showed that conjugate formation of a T-cell with an APC in the presence of antigen in vitro leads to a dramatic increase of PIP_3 levels.[55] Hence, we raised the possibility that a comparable process of FoxOs exclusion was running during the antigenic stimulation of primary T-cells to support their clonal expansion.

Confirming this hypothesis, we have shown that FOXO1 is excluded from the nucleus of human T-cells activated by antigen, after its phosphorylation by Akt. Live imaging experiments reveal that this process is not immediate after the formation of the conjugate, but starts ~5 minutes after the initial accumulation of PIP_3 into the plasma membrane.[56] Our results also indicate that this exclusion is very stable, lasting for hours and requires a permanent activation of PI3K, as revealed by the rapid movement of FOXO1 back into the nucleus after addition of PI3K inhibitors. Importantly, a constitutively active FOXO1 mutant has a strong inhibitory effect on T-cell blastogenesis, indicating that this prolonged inactivation of FOXO1 is essential to permit cell cycle entry of resting T-cells; similar results have been obtained with stimulated primary murine B-cells which are also retained into quiescence by FoxOs.[57] Consistently, another study has shown that the PI3K/Akt/FOXO1 pathway is directly controlled by Vav1 in murine T-cells and that the inhibition of the proliferation observed in Vav1-deficient T-cells is related to a defect in nuclear exclusion of FOXO1 and a persistent expression of the cell cycle inhibitor p27kip1, a distinctive target of FOXO1 in various tissues.[58] It is to note here that the H215R mutation of FOXO1 uncouples the control of cell growth and homing since an active FOXO1 molecule harboring this mutation in the DNA binding site has the same potential to inhibit T-cell growth induced by antigen. Interestingly enough, in PTEN-deficient tumoral cell lines also, inhibition of tumoral cell growth does not require direct binding of FOXO1 to DNA.[59] These findings suggest that distinct mechanisms support the biological functions of FOXO1 in human T-lymphocytes. Mainly they show that like in typical oncogenic situations with deregulated PI3K activity, one major functional consequence of the massive production of PIP_3 in T-cell contacting APCs might be to securely sequester FoxO1 out of the nucleus and neutralize its negative regulation on the cell cycle. As in many other cellular systems, PI3K appears therefore as a major molecular switch for T-cell commitment into cell growth and cell division. In agreement, mice expressing catalytically inactive p110δ PI3K, show a defect in their T-cell proliferative responses to antigen stimulation.[60,61] For the same reason, it is also attractive to speculate here that one rationale for the T-cell/APC conjugate to last for hours in the lymph node is to keep up this process of FoxOs inactivation effective, as long as cell divisions continue. However this speculation is still hazardous since we do not know in vivo how inactivation of FoxOs and T-cell division progress in parallel, at which stage of the immune response FoxOs are again within the nucleus and active and how all these changes are correlated with PI3K activity.

It is obvious that these results do not exclude other molecular requirement in the control of T-cell proliferation. Indeed, we know that cell quiescence is actively controlled by various

molecular actors probably acting in a cooperative way on multiple targets;[62] moreover the fact that the surexpression of an active FOXO1 molecule blocks the proliferation of T-cells is only indicative that the inactivation of the endogenous forms is necessary to allow the clonal expansion after antigen exposure in physiological situation. It is also a rather simplistic view to liken nuclear localization of FOXO1 and transcriptional activity, as far as supplementary mechanisms independent from PI3K and Akt can regulate the activity of this factor within the nucleus. Various previously identified partners of FoxOs, such as p53, Smad, δEF-1, nuclear receptors or β-catenin could be involved in this suppressive effect[35,63-66] and any modification of such interactions during the stimulation of T-cells could also contribute to alter the transcriptional control exercised by FoxOs. Thus, a review of every facet of FoxO metabolism in T-cells is now needed to understand their specific contribution. However, it remains clear that the loss of the control exercised by FoxOs may have dramatic consequences on the survival and the proliferation of T-cells as attested by studies demonstrating that mice deficient in FoxO3 present a spontaneous inflammatory syndrome and T-cell lymphoproliferative disorders.[67] The aforementioned triple *FoxO1/FoxO3/Foxo4* null mice also show signs of lymphoid development abnormalities that can culminate with thymic lymphomas after a few weeks of age.[33] So, we can hope that further studies using these models will help deciphering the real position of FoxOs in the network of molecules involved in T-cell clonal expansion.

New Potential Targets of FOXO1 to Impose T-Cell Fate

It is now well-admitted that the activity of FoxOs is largely dependent on the cellular context, though, regardless of the cellular system, prototypical targets regulating cell survival and cell proliferation are usually found. Curiously, despite the strong antiproliferative and proapoptotic effects of an active form of FOXO1 in Jurkat T-cells, only a few of them were identified in our screen. For example, Bim and FasL, two typical FOXO3 targets in T-cells[54] were poorly induced. The increase of the cell cycle inhibitor p27kip1 was also modest (~2x), but it has been established that the induction of this gene is not required to block the proliferation of PTEN-deficient cells.[59] In these cells, FOXO1 seems rather to regulate the proliferation via the inhibition of cyclin D and the increase of p130Rb2 proteins, but both were also not significantly induced in our model. We nevertheless found other potential targets of FOXO1 susceptible to explain the inhibitory effect of this factor on the survival and the proliferation of T-cells. This is the case of the pro-apoptotique gene BBC3 (Bcl-2-binding component), also named PUMA (p53-upregulated modulator of apoptosis). Initially identified as a target of p53, PUMA can promote Bax (Bcl-2 associated X protein) multimerisation at the mitochondrial membrane, releasing cytochrome c and inducing pro-apoptotic cascades. Interestingly, PUMA has been described as a direct target of FOXO3 involved in the apoptosis of activated T-lymphocytes following cytokine or growth factor withdrawal.[68]

Noticeably, a marked inhibition of several genes of the early growth response (EGR) family of transcription factors was also observed. The most strongly repressed molecule was EGR1, initially described as a positive regulator of the G0/G1 transition in human lymphocytes.[69] EGR1 is frequently overexpressed in prostatic cancer cells and stimulates their proliferation by inducing cyclin D1 and by repressing cell cycle inhibitors such as p19, Rb2 and cyclin G2.[70,71] Thus, FOXO1 may control the expression of these molecules indirectly via EGR1 to regulate T-cell proliferation. However, the functional effects of EGR1 are very complex and it can have an antiproliferative role by positively regulating tumor suppressors like PTEN, p53 or TGF-β in some tumor models. Amongst the other genes strongly induced by FOXO1, interleukin-16, the CD52 antigen or the CD10 endopeptidase are other candidates to be cited, since all of them can exert antiproliferative functions in T-cells.[72-74] It is to note that CD10, which is also a marker of apoptotic T-lymphocytes,[75] is a tumor suppressor apparently involved in the membrane recruitment of PTEN.[76] Thus, via the induction of CD10, FOXO1 could also contribute to the negative regulation of PIP_3 levels to maintain its own activity. Would this also hold true for normal T-cells during clonal expansion remains however to be established.

FOXO1, T-Cell Egress and Peripheral Homing

Once proliferating, activated T-cells differentiate to several types of memory cells: central memory T-cells (T_{CM}) and effector memory T-cells (T_{EM}), which need to return to peripheral blood circulation and infected organs. To this end, they migrate from the lymph node paracortex to the cortical sinusoids, directly linked to efferent lymphatic. Efferent lymphatic then return to blood circulation via the thoracic duct.[77] Lymphocytes that did not encounter their specific antigen presented on APC also exit lymph nodes through this way, but once back in blood re-enter and explore other lymphoid organs. Only T_{CM} that express L-selectin and CCR7 at similar levels than naive T-cells are found in secondary lymphoid organs, such as lymph nodes and spleen, to give faster and stronger immune responses after a new challenging with the same antigen. In contrast, effector memory T-cells never recover a normal expression of CCR7 and usually show altered levels of L-selectin, a phenotype functionally important to circumvent any recirculation into secondary lymphoid organs.[10,11] These cells also usually express a new set of chemokine receptors (like CCR1, CCR3 and CCR5) to gain access to sites of infection and inflammation in nonlymphoid tissues.[78-80]

We are still ignorant of the mechanisms involved in this striking difference between T_{CM} and T_{EM}. In fact, the activation status of T-cells when they leave the lymph nodes is largely unknown. One could expect that activation, including of PI3K, is rapidly shut off in circulating T_{CM} and T_{EM} with a restoration of membrane levels of L-selectin and CCR7. In agreement, it has been observed that the totality of activated $CD4^+$ T-cells detected in the blood circulation after immunization is L-selectin high.[81] In this case the loss of expression of L-selectin in T_{EM} would arise secondarily, in the periphery. However it has been reported unexpectedly that T_{CM} in human have higher phospho-FOXO3 levels than T_{EM}.[82] Thus, the phosphorylation status of FOXO3 in T_{CM} does not seem to be in accordance with high CD62L expression at their cell surface. We still do not know whether FOXO3 is as effective as FOXO1 to trigger L-selectin expression and if FOXO1 is also phosphorylated in these cells, but these findings might be suggestive of a persistent activation of PI3K in T_{CM}. Another possibility to explain the difference between T_{CM} and T_{EM} is that the transcriptional activity of FoxOs is unproductive in this latter subset. Indeed, many other regulatory mechanisms control the activity of FoxOs. We know for example that, beside phosphorylation, the nature of the genes regulated by FoxOs in various cellular systems can be strongly influenced by other posttraductional modifications; thus, acetylation by CBP/p300 and deacetylation by Sirt1, as well as molecular partnerships with transcriptional coactivators like β-catenin or PGC-1α can strongly alter FOXO activity positively or negatively.[83-86] In agreement, we have observed that L-selectin expression in T-cells is strongly affected by mutation of critical residues controlling FOXO1 acetylation (Marianne Mangeney and G.B., unpublished). Overall, these regulations have been however poorly documented in T-cells and an interesting challenge would be to investigate these molecular aspects in effector and memory T-cells at the various stages of the immune response.

As already discussed, during differentiation, T-cells profoundly reshape their pattern of cell surface receptors in order to express molecules involved in lymphoid organs egress and homing to inflamed tissues. The first characterisation of molecular process involved in egress has been obtained by using of a immunosuppressive and allograft rejection preventing drug, FTY720.[87] FTY720 greatly decreases the number of lymphocytes in periphery by sequestrating them in lymph nodes, but not spleen. Further experiments showed that FTY720 is structurally similar to S1P.[88,89] The S1P receptor S1P1, also called EDG1, is a 7-transmembrane receptor belonging to the G-protein-coupled family of receptor; it is sensitive to S1P concentration gradient, which increases between the lymph node to the efferent lymphatic. Recent studies have demonstrated that EDG1 levels in murine T-cells are controlled by kruppel-like factor 2 (KLF2) directly at the transcriptional level. KLF2 belongs to the zinc-finger transcription factor family. It was firstly described to control quiescence of T-lymphocytes,[90] but has recently been reported as playing a role in T-cell trafficking.[91,92] KLF2-deficient T-cells recapitulate part of the behaviour of T-cells lacking S1P1, with partial retention in the thymus. However, these two knockout models also

display unique functions. Thus KLF2-deficient T-cells in periphery are found neither in blood nor in lymph nodes, but only in spleen, whereas EDG1-knockout T-lymphocytes are sequestrated into lymph nodes. This discrepancy is related to the fact that KLF2-deficient T-cells abnormally express chemokines receptors like CCR1 and CCR5, allowing them to massively home to nonlymphoid organs, such as liver, colon and skeletal muscle. The mode of regulation of KLF2 is still poorly understood. KLF2 mRNA and protein are dramatically repressed very early after T-cell activation, but its was not suspected that PI3K has to do with KLF2 expression until a recent study proved that KLF2 mRNA levels are restored in activated $CD8^+$ cytotoxic murine T-lymphocytes treated with drugs inhibiting the PI3K and the mTOR pathways.[32] Collectively, these results point to KLF2 as a critical target gene to control T-cell trafficking, an assumption further consolidated by the striking observation that restoration of significant KLF2 protein levels in activated T-cells also up regulates L-selectin.[31,93]

Remarkably, we found in our array study in T-cells that, together with L-selectin mRNA, FOXO1 also strongly induces those of EDG1 and KLF2, arguing for a FOXO1 KLF2 EDG1/L-selectin functional link in T-cell trafficking. In this cascade, the role of FOXO1 may be limited to the control of KLF2. However, some independent regulation by the two transcriptional factors is also possible since in Jurkat cells, in contrast to normal T-cells, KLF2 overexpression up-regulates EDG1 but not L-selectin (FC, Marianne Mangeney and GB, unpublished). A similar result was reported in an independent study analyzing genes directly activated by KLF-2 in this cell line.[94] This intriguing result needs further investigations, but it may indicate that the two transcription factors can stand-alone to regulate genes, like *SELL*. Yet, as some evidences of epigenetic changes affecting the chromatin state are mediated by FOXO1 in the nucleus,[95] the promoter of L-selectin might also be less accessible to KLF2 depending of the activation status of the cell, specifically in leukemic cells like Jurkat that no more express nuclear FOXO1. Analysis of FOXO1 effects in KLF2-deficient cells may help to clarify this important issue. It is worth mentioning here that expression of the FOXO1 H215R DNA binding mutant in primary T-lymphocytes induces the activation membrane marker CD69.[31] CD69 is one of the earliest inducible cell surface glycoprotein acquired during lymphoid activation. This observation is probably not fortuitous since previous results have demonstrated that CD69 inhibits EDG1 and lymphocyte egress from lymphoid organs caused by a mutual negative regulation of their membrane expression levels in T-lymphocytes.[96] Moreover, in Jurkat T-cells, active FOXO1 reduces CD69 mRNA levels. Thus, the opposite modification of CD69 and EDG1 seen in activated T-cells might be controlled by FOXO1. Interestingly, KLF2-deficient T-cells in lymphoid organs presents a comparable $EDG1^{low}$, $CD69^{high}$ phenotype, further establishing the proposed FOXO1/KLF2 network in the regulation of essential T-cell trafficking molecules.

Conclusion

Be in the right place and at the right time and get the signal as soon as it becomes available to clonally expand: this is the credo of a naive T-cell. To fulfill this mission the cell needs first to home into secondary lymphoid organs to meet APCs and then cycle very actively after antigen recognition. Convergent studies described in this chapter show now that these two essential biological processes of the adaptive immunity are probably coregulated in vivo by a network of signaling molecules and transcriptional factors, such as FOXO1, controlling expression of various molecules involved in both T-cell proliferation and trafficking (Table 1). This spatio-temporal control makes sense as it would seem important that mechanisms regulating expression of membrane receptors that guide T-cells within secondary lymphoid organs stay constantly "on" in naive T-cells. The discovery that one molecule controlling this traffic is FOXO1 (and possibly other FoxOs), a well-known and important switch to maintain cell quiescence downstream of PI3K, further suggests that the two missions of a naive T-cell, "travel and stay at rest", are in fact the two sides of the same medal (Fig. 4). Thus, it is probably not accidental that the exact opposite orders, "stay here and cycle", are also simultaneously given to the T-cell when this control is "off" after antigen recognition in the lymph node. This scenario

Table 1. FOXO1 candidate target molecules in T-cell trafficking and proliferation

Molecule Name	Function	Text References[a]
L-selectin/CCR7/KLF2	Homing	31-33,91-93
KLF2/EDG1/CD69	Egress	31,91-94
p27kip1/KLF2/EGR1/IL-16/CD52	Cell proliferation	31,54,56,58,90,94

[a]selected text references are shown suggesting, as described in the chapter, some direct or indirect control by FOXO1 of the listed molecules and corresponding functions.

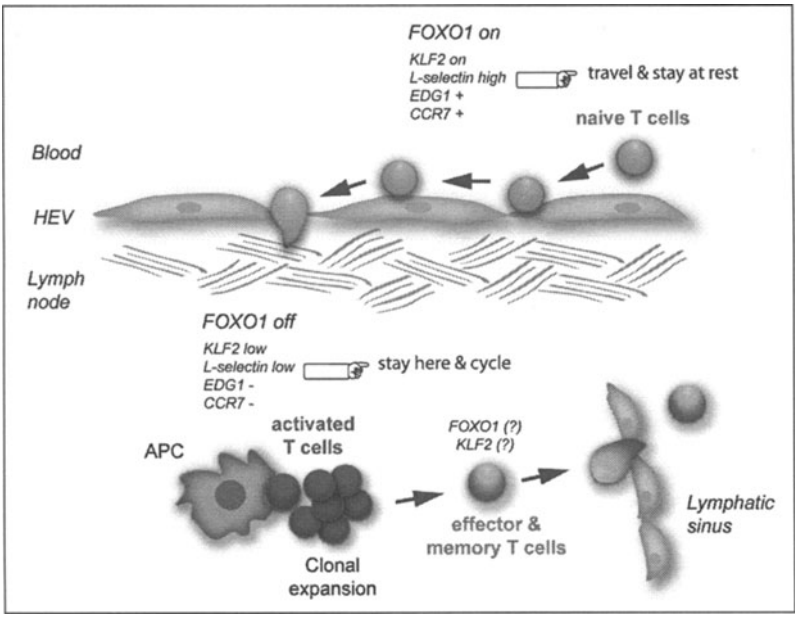

Figure 4. An hypothetical model of regulation by FOXO1 of T-cell homing and egress. The following scenario can be proposed: in naive T-cells the transcriptional control exerted by FOXO1 contributes to the preservation of the membrane levels of L-selectin, EDG-1 and CCR7 receptors, possibly via KLF2 (at least for EDG1) whose expression is also controlled by FOXO1. As long as no antigen is detected within lymph nodes, this control allows the cells to maintain their homing and egress capacities unaffected. After antigen recognition, steady activation of PI3K at the immunological synapse leads to prolonged inactivation of FOXO1 together with rapid decrease of KLF2 protein. As a result, membrane levels of L-selectin, CCR7 and EDG-1 are down regulated. This may help sequestering activated T-cells in LN during their clonal expansion, before their differentiation into effector and memory cells. One central question here will be to understand the mechanisms that make this captivity reversible at this stage. In particular, we do not know whether in these cells the transcriptional control exercised by FOXO1 is restored together with normal levels of KLF2, keeping in mind that EDG-1 is a direct transcriptional target of KLF2 and is required for T-cell egress in the lymphatic sinus.

is probably oversimplified as many other parameters such as the strength of the signal, which is an essential parameter controlling the T-cell fate after antigen encounter, may modify this behavior. However, these new insights into T-cell physiology showing that PI3K/Akt signaling

has a key role in this choreography are of invaluable interest as they give us new potential targets to control this critical phase of the adaptive immune response. Indeed, extensive drug discovery activities have been directed over the past few years to identify compounds that effectively and specifically disrupt the intracellular components of this pathway in cancer cells, which could represent now novel immunomodulatory therapies.

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CHAPTER 2

FOXP3 and Its Role in the Immune System

Chang H. Kim*

Abstract

FOXP3 is a member of the forkhead transcription factor family. Unlike other members, it is mainly expressed in a subset of $CD4^+$ T-cells that play a suppressive role in the immune system. A function of FOXP3 is to suppress the function of NFAT and NFκB and this leads to suppression of expression of many genes including IL-2 and effector T-cell cytokines. FOXP3 acts also as a transcription activator for many genes including *CD25*, *Cytotoxic T-Lymphocyte Antigen 4 (CTLA4)*, *glucocorticoid-induced TNF receptor family gene (GITR)* and *folate receptor 4*. $FOXP3^+$ T-cells are made in the thymus and periphery. The $FOXP3^+$ T-cells made in the thymus migrate to secondary lymphoid tissues and suppress antigen priming of lymphocytes. Antigen priming of naïve $FOXP3^+$ T-cells and naïve $FOXP3^-$ T-cells leads to generation of memory $FOXP3^+$ T-cells which are efficient in migration to nonlymphoid tissues. Memory $FOXP3^+$ T-cells are, therefore, effective in suppression of effector T-cell function, while naïve $FOXP3^+$ T-cells are adept at suppressing the early immune responses in lymphoid tissues. Both naïve and memory $FOXP3^+$ T-cells are required for effective maintenance of tolerance and prevention of autoimmune diseases throughout the body. Many factors such as cytokines and noncytokine factors regulate the generation of $FOXP3^+$ T-cells. For example, retinoic acid, produced by the dendritic cells and epithelial cells in the intestine, works together with TGF-β1 and promotes generation of small intestine-homing $FOXP3^+$ T-cells by upregulating the expression of FOXP3 and gut homing receptors. $FOXP3^+$ T-cells can be produced in vitro from autologous naïve T-cells and, therefore, have great therapeutic potentials in treating a number of inflammatory diseases and graft rejection.

Introduction

FOXP3 is one of the most extensively studied members of the FOX family which is defined by a common DNA-binding domain (DBD) termed the forkhead box or winged helix domain.[1] FOXP3 receives a lot of attention because of its clear role in generation of immune suppressor T-cells. The function of FOXP3 in programming the gene expression to make suppressor T-cells is attributed to its transcription regulation activity.[2] Its major targets include NFAT and NFκB, key transcription factors that mediate antigen receptor signals. FOXP3 suppresses the function of these transcription factors but induces expression of many other genes through mechanisms that are incompletely understood at this stage. Our body is making $FOXP3^+$ T-cells in both the thymus and periphery. $FOXP3^+$ T-cells play important roles in limiting the activation of immune cells in response to infection.[3-6] They play important roles also in prevention of autoimmune diseases. It appears that some pathogens and cancer cells have been evolved to utilize $FOXP3^+$ T-cells to avoid

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immune responses because FOXP3+ T-cells would effectively suppress antipathogen and anticancer cell-specific immune responses.[7,8] While FOXP3 is mainly expressed by T-cells, it is also expressed by epithelial cells in certain organs such as thymus and mammary glands.[9] In these organs, FOXP3 seems to play completely different roles.[9,10] This monograph is to provide general information on the structure and function of the FOXP3 gene and protein and on the immunological roles of the cells expressing FOXP3.

Structure and Function of FOXP3

The human FOXP3 gene is composed of 11 exons and is present in the p arm of the X chromosome (Xp11.23, Fig. 1).[11,12] The translation of the FOXP3 protein starts from the middle of the second exon. The mouse gene is at X 2.1 cM, a location comparable to that of the human gene. It is also called JM2 (human) or scurfin (mouse). FOXP3 is a 48 kD protein composed of 431 amino acids. The FOXP3 protein has four distinctive domains: forkhead (FH) domain, leucine zipper, zinc-finger and the proline-rich repressor domain.[4] The C-terminal forkhead domain consists of ~100 amino acids and forms a DNA binding domain. FOXP3 binds genes containing the forkhead binding motif.[13,14] The forkhead domain is required also for nuclear localization of FOXP3. The role of the zinc-finger domain is unknown. The leucine zipper domain is thought to mediate dimerization or tetramerization of the transcription factor.[15] The N-terminal repressor domain (amino acids 1-193) is composed of two subdomains. The first subdomain (amino acids 1-105) is

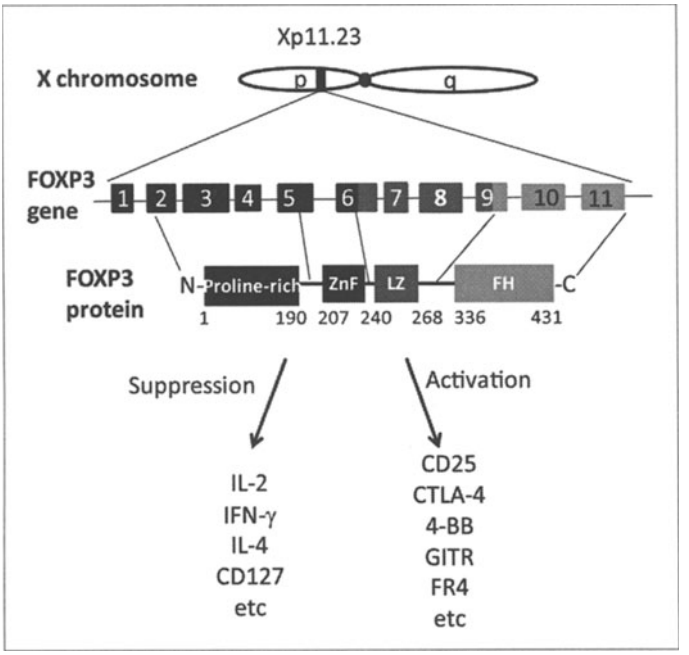

Figure 1. The structure of the FOXP3 gene and protein. The human FOXP3 gene is located on the p arm of chromosome X (Xp11.23). The FOXP3 gene is composed of 11 exons. There are four identifiable domains in the FOXP3 protein. The N-terminal proline-rich domain is involved in suppression of NFκB and NFAT. The leucine zipper domain is required for dimerization or tetramerization. The C-terminal forkhead domain has a nuclear localization sequence and a DNA binding domain. FOXP3 functions to induce the expression of many genes such as CTLA-4, FR4 (folate receptor 4),[142] GITR and CD25 and to suppress the expression of other genes such as IL-2,IL-4 and IFN-γ. It has been reported that expression of 700-1000 genes is regulated by FOXP3 either directly or indirectly.

involved in general transcriptional repression by FOXP3 and the second subdomain (N-terminal 106- to 190-aa proline-rich region) is involved in suppression of NFAT and NFκB-mediated transcription.[15,16] The second half of the domain mediates the association of the FOXP3 protein with key transcriptional regulators such as Tat-interactive protein 60 kDa (TIP60)[17] and class II histone deacetylases (HDAC7).[18] Mutations have been found in the forkhead domain, leucine zipper domain and repressor domain of the FOXP3 gene of human IPEX patients.[19-21]

FOXP3 can bind forkhead DNA binding elements in many genes including IL-2, CTLA4, GITR and CD25.[22] FOXP3 decreases IL-2 expression but increases the expression of CTLA4, GITR and CD25. Thus, FOXP3 acts as a transcriptional activator and repressor. Studies using Chip-on-Chip (chromatin immunoprecipitation) revealed that 700-1100 genes are regulated either positively or negatively by FOXP3.[23,24] Most of the genes would be indirectly regulated as the result of T-cell differentiation rather than as the consequence of direct FOXP3 binding. For suppression of NFAT, the N-terminal repressor domain is required.[16] FOXP3 and NFAT cooperatively bind to the antigen receptor response element (AREE2) within the IL-2 promoter in a manner similar to the binding of AP-1 and NFAT.[25] Some amino acid residues in the forkhead domain are important for this interaction. FOXP3 also interacts with NFκB and suppresses its activity.[16]

Expression of FOXP3

FOXP3 is most highly expressed by a subset of $CD4^+$ T-cells, commonly called $CD4^+CD25^+$ regulatory T-cells.[5,26] Expression of the FOXP3 gene is more tightly regulated in mouse T-cells compared to human T-cells.[27] In human T-cells, simple T-cell activation induces FOXP3 at a low but detectable level. In addition, some $CD8^+$ T-cells also express FOXP3 and function as regulatory T-cells.[28] In the mouse thymus, FOXP3 expression is detected on a small subset of CD4 and CD8 double positive T-cells and CD4 single positive T-cells.[29-31] In humans, however, small numbers of double negative thymocytes also express FOXP3.[32] Expression of FOXP3 is important for T-cells to gain the suppressive function. In mice, FOXP3 over-expression by retroviral gene transfer was sufficient to generate suppressive T-cells.[33,34] Again, there is a species difference in this regard that enforced FOXP3 expression in human T-cells by itself was not sufficient to turn regular T-cells into suppressor T-cells.[35]

While normal T-cell receptor (TCR) activation would not efficiently induce $FOXP3^+$ T-cells, premature termination of TCR signaling and inhibition of phosphatidyl inositol 3-kinase (PI3K) p110a, p110d, protein kinase B (Akt), or mammalian target of rapamycin (mTOR) effectively induced FOXP3 expression.[36] FOXP3 expression is regulated at both genetic and epigenetic levels. NK-cells, for example, don't express FOXP3 but do express it when they are treated with 5-aza-2'-deoxycytidine, a DNA methylation inhibitor.[37] Complete demethylation of CpG motifs as well as histone modifications are found on the conserved region of the FOXP3 promoter in $FOXP3^+$ cells but not $FOXP3^-$ T-cells.[38] Methylation at the FOXP3 promoter can block the binding of transcription factors such as cyclic-AMP response element binding protein (CREB)/activating transcription factor (ATF) which are involved in activation of the FOXP3 promoter.[39] TGF-β1 induces FOXP3 expression in T-cells undergoing T-cell receptor activation.[40,41] The transcription factors Smad3 which mediates TGF-β1 signaling and NFAT which mediates the T-cell receptor activation signal are required to induce FOXP3 expression.[42] Interestingly, these TGF-β1-induced $FOXP3^+$ T-cells are not heavily methylated on their FOXP3 promoter locus compared to natural $FOXP3^+$ T-cells.[43] However, another group reported more complete methylation at the FOXP3 promoter locus in induced $FOXP3^+$ T-cells, suggesting that the methylation in the in vitro-induced $FOXP3^+$ T-cells varies depending on the culture condition.[44] Induced $FOXP3^+$ cells, generated in vivo from naïve T-cells, exhibited more complete methylation on the FOXP3 locus and thus natural and fully differentiated-induced $FOXP3^+$ T-cells are indistinguishable in methylation at the FOXP3 locus.[45]

In addition to TGF-β1, IL-2 promotes the generation of induced $FOXP3^+$ T-cells. Consistently, the intracellular signaling mediators of IL-2 such as STAT5a and STAT5b play positive roles in

expression of FOXP3.[37,46] It has been reported that IL-4 suppressed, while STAT6 (a mediator of IL-4 signaling) gene deletion enhanced, the TGFβ1-induced expression of FOXP3.[47] TGF-β1 can enhance FOXP3 expression but suppress the expression of FOXP3 when IL-6 is present. Indeed, TGF-β1 and IL-6 induce different effector T-cells called "Th17 cells", which can characteristically produce IL-17.[48,49] In line with this, TGF-β1 treatment increases acetylated FOXP3 on the chromatin but IL-6 down-regulates FOXP3 binding to the chromatin in the presence of TGF-β1.[50]

Ontogeny and Migration of FOXP3$^+$ Cells

FOXP3$^+$ T-cells are generated in thymus as naïve FOXP3$^+$ cells and in periphery as induced FOXP3$^+$ cells (Fig. 2). The naïve FOXP3$^+$ T-cells, generated in thymus, express CD62L and CCR7 and migrate to secondary lymphoid tissues.[31] CD62L would mediate rolling and CCR7 triggers integrin-mediated firm adhesion on endothelial cells.[51-56] This trafficking receptor phenotype is retained as long as the FOXP3$^+$ T-cells do not encounter antigens in the secondary lymphoid tissues.[31] Unlike the naïve FOXP3$^+$ T-cells, the induced FOXP3$^+$ T-cells have heterogeneous memory/effector type trafficking receptors.[31] It is thought that memory FOXP3$^+$ T-cells and induced FOXP3$^+$ T-cells are similar to each other in trafficking receptor phenotype and suppressive function. Some induced FOXP3$^+$ T-cells express gut homing receptors such as CCR9 and α4β7.[31] These receptors allow the migration of the T-cells into the small intestine.[57-63] Some FOXP3$^+$ T-cells express CXCR5 and migrate into B-cell follicles including germinal centers.[64]

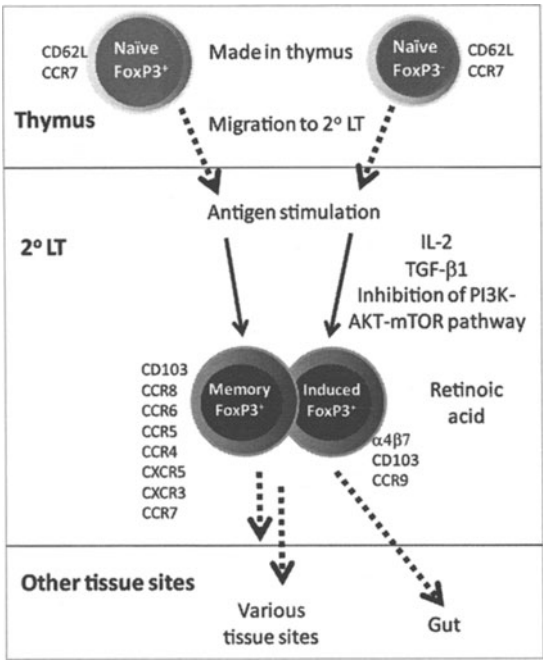

Figure 2. Generation and trafficking of FOXP3$^+$ T-cells. FOXP3$^+$ T-cells are made in the thymus at the double negative stage (human), double positive stage and single positive stage (human and mice). The thymus emigrating FOXP3$^+$ T-cells have the naïve T-cell phenotype in trafficking behavior and migrate to secondary lymphoid tissues. The migration of natural FOXP3$^+$ T-cells into secondary lymphoid tissues is to regulate the antigen priming of lymphocytes and to undergo antigen priming themselves. Antigenic stimulation of naïve FOXP3$^+$ T-cells changes their homing behavior for migration to various nonlymphoid tissues. Antigen priming of naïve T-cells also drives the conversion of FOXP3$^-$ naïve T-cells into FOXP3$^+$ T-cells. Certain factors such as TGF-β1 and retinoic acid play important roles in promotion of this event.

Some other FOXP3$^+$ T-cells express CCR8, a skin-homing related receptor.[31] In general, induced or memory FOXP3$^+$ T-cells highly express CD103, CCR4, CCR6, CXCR3, CXCR4 and CXCR6.[31,65] CCR4 is required for successful suppression of inflammation by FOXP3$^+$ T-cells. In a heart transplantation model, recruitment of FOXP3$^+$ cells to the allograft tissue is dependent on CCR4.[66] The CCR4-dependent recruitment of FOXP3$^+$ T-cells is required for effective induction of tolerance with tolerizing strategies such as CD154 mAb therapy. Scurfy mice reconstituted with CCR4-deficient FOXP3$^+$ cells develop severe inflammatory diseases in the skin and lungs.[67] Another chemokine receptor CCR7 appears to be important for FOXP3$^+$ T-cell migration to the T-cell area of lymphoid tissues. CCR7-deficient FOXP3$^+$ cells fail to migrate into the lymph nodes and suppress antigen-induced T-cell responses.[68]

The induction mechanism of gut homing FOXP3$^+$ T-cells has been elucidated. In 2007, six groups reported that retinoic acid has the function of triggering the expression of FOXP3 in T-cells undergoing activation.[69-76] Retinoic acid induces chromatin reorganization by inducing histone acetylation in the FOXP3 promoter. Retinoic acid alone can generate human FOXP3$^+$ T-cells but TGF-β1 is required at least at a suboptimal level to induce retinoid-induced mouse FOXP3$^+$ T-cells.[77] Retinoic acid is produced from retinol by dendritic cells and epithelial cells in the intestine.[78] Therefore, the intestinal microenvironment provides the signal to induce gut homing FOXP3$^+$ T-cells. This role of retinoic acid is thought to be important for inducing tolerance in the gut by generating FOXP3$^+$ T-cells that would suppress potentially harmful immune responses in the intestine. It has been well-established that immune responses to commensals can cause inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.[79,80] It is thought that retinoic acid functions to prevent inflammatory bowel diseases by promoting the immune tolerance in the intestine. Another function of retinoic acid is to suppress the differentiation of naïve T-cells into Th17 cells in vitro. This could also promote the immune tolerance in the intestine by suppressing Th17 cells.[69,73,74,81] However, the function of retinoic acid in suppression of Th17 cells has not been confirmed in vivo. This may be because of the fact that retinoic acid production in vivo is tightly regulated that retinoic acid would not be available at the high concentrations (100-1000 nM) used in vitro in demonstration of the suppression of Th17 cells.

Another trafficking receptor that is potentially important for FOXP3$^+$ regulatory T-cells is CD103.[65,82,83] CD103 is the alpha subunit of the integrin αEβ7, which serves as the ligand for E-cadherin. It is unclear how CD103 functions in terms of the suppressive function of FOXP3$^+$ T-cells. It was proposed that CD103$^+$ FOXP3$^+$ T-cells are memory cells and they are more efficient in suppression of inflammation in the joints.[65] One caveat with this is that there are many CD103$^-$ memory FOXP3$^+$ T-cells as well. Thus, CD103 is not a universal marker for all memory FOXP3$^+$ T-cells. In suppression of graft-versus-host disease (GVHD), however, CD62L$^+$ FOXP3$^+$ T-cells are more efficient than CD62L$^-$ memory FOXP3$^+$ T-cells.[84,85] Therefore, it is not memory FOXP3$^+$ T-cells that are always more efficient than naïve FOXP3$^+$ T-cells in suppression of inflammation. Whether a FOXP3$^+$ T-cell subset is effective or not effective in suppression of inflammation in a certain tissue would be determined by the migration ability of the FOXP3$^+$ T-cells to the major tissue site of initiation or amplification of the inflammation. In other words, naïve FOXP3$^+$ T-cells or their migration into lymphoid tissues is important if initiation and amplification of the inflammatory disease occur in the lymphoid tissues. Otherwise, memory FOXP3$^+$ T-cells or their migration to effector sites would be important for suppression of inflammation at effector sites (Fig. 3).

Mechanisms of Suppression Mediated by FOXP3$^+$ T-Cells

TGF-β1 is implicated in the suppressive function of FOXP3$^+$ T-cells. Nakamura et al reported that spleen CD4$^+$CD25$^+$ T-cells produce soluble TGF-β1.[86] In their study, the TGF-β1 concentration in the culture supernatant of CD4$^+$CD25$^+$ T-cells reached ~2 ng/ml, which is a concentration sufficient to suppress target T-cells. Moreover, CD4$^+$CD25$^+$ T-cells expressed surface TGF-β1 as the latency associated protein. Neutralizing anti-TGF-β1 abrogated the suppressive activity of CD4$^+$CD25$^+$ T-cells. Piccirillo et al, however, reported that neutralization of TGF-β1 was not

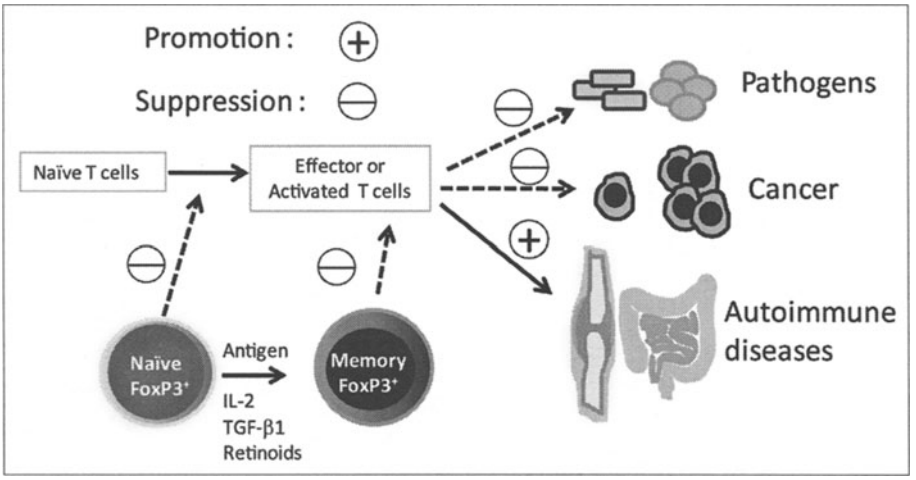

Figure 3. Immune regulatory functions of FOXP3+ T-cells. Naïve FOXP3+ T-cells have the tissue tropism for secondary lymphoid tissues while memory FOXP3+ T-cells have diverse tissue tropisms for nonlymphoid tissues (e.g., gut versus other tissues). Therefore, naïve T-cells are designed to suppress the immune responses in secondary lymphoid tissues perhaps to limit the activation of various immune cells. This would be important to prevent the generation of autoimmune effector T- and B-cells. Memory FOXP3+ T-cells can migrate to nonlymphoid tissues. Depending on the site of antigen priming, some can migrate to the gut, while others migrate to different tissue sites. Therefore, memory FOXP3+ T-cells can suppress the potentially inflammatory activity of effector lymphocytes in diverse peripheral tissues. It is thought that FOXP3+ T-cells can suppress harmful autoimmune responses but can be utilized by tumors and pathogens to delay beneficial immune responses.

able to abrogate the suppressive effect of $CD4^+CD25^+$ T-cells.[87] Similarly, Smad3 (−/−) T-cells and the T-cells that cannot receive the TGF-β1signaling were suppressed by $CD4^+CD25^+$ T-cells. In their study, TGF-β1 (−/−) $CD4^+CD25^+$ T-cells were able to suppress target T-cells. This group also performed an in vivo study through which they found that the suppression of autoimmune gastritis by $CD4^+CD25^+$ T-cells was not reversed by anti-TGF-β1. A caveat with this study is that in vivo neutralization would not always work and thus this data does not prove lack of a role for TGF-β1 in vivo. Mamura et al provides evidence that compromises the results of the two reports.[88] Adoptive transfer of TGF-β1 (−/−) splenocytes into TGF-β1 (+/+) Rag2 (−/−) mice induced an autoimmune inflammatory disease and cotransfer of TGF-β1 (−/−) $CD4^+CD25^+$ T-cells partially ameliorated the disease. However, this suppression was weaker compared to that by wild type $CD4^+CD25^+$ T-cells, suggesting that $CD4^+CD25^+$ cells may suppress target T-cells in both TGF-β1-dependent and independent manners. Using a dextran sodium sulfate (DSS)-induced colitis mouse model in conjunction with a model with impaired TGF-β1-signaling by overexpressing a truncated version of the TGF-β Type II receptor in T-cells, Huber et al reported that transfer of wild-type but not transgenic $CD4^+CD25^+$ T-cells was found to suppress colitis in wild-type mice.[89] Unlike $CD4^+CD25^+$ T-cells from wild type mice, $CD4^+CD25^+$ T-cells from TGF-β1(−/−) mice did not protect recipient mice from colitis in T-cell induced SCID mice.[90] In contrast, a different group reported that $CD4^+CD25^+$ cells from either TGF-β1 (+/+) or TGF-β1 (−/−) mice can suppress the incidence and severity of colitis.[91] It was notable, however, that $CD4^+CD25^+$ cells from TGF-β1 (+/+) mice were always more efficient than the $CD4^+$ $CD25^+$ cells from TGF-β1 (−/−) mice in suppression of inflammation. These authors observed that anti-TGF-β1 neutralization exacerbated effector-T cell induced colitis and claimed that $CD4^+CD25^+$ T-cells are able to suppress intestinal inflammation by a mechanism not requiring Treg cell-derived TGF-β1. One

problem with this claim is that FOXP3$^+$ T-cells can be induced following naïve T-cell transfer. Overall, it appears that TGF-β1 has certain roles in the suppressive function of FOXP3$^+$ T-cells but the degree of contribution may depend on the type of disease and immune responses. This implies that there are TGF-β1-independent mechanisms of suppression.

Indeed, there are a number of candidate mechanisms that could mediate the suppressive function of FOXP3$^+$ T-cells. FOXP3$^+$ T-cells highly express CTLA-4 and CTLA-4 can suppress antigen presenting cells through the cognate CTLA4-B7 interaction.[92,93] The CTLA4-B7 interaction triggers the expression of indoleamine 2, 3-dioxygenase (IDO). IDO converts tryptophan to kynurenine, 3-hydroxyanthranilic acid, picolinic acid and quinolinic acid and thus is an enzyme that depletes tryptophan required for proliferation and function of immune cells.[94] Certain regulatory T-cells express cytotoxic molecules such as granzyme A and granzyme B, which can kill target cells in perforin-dependent and independent mechanisms.[95-97] FOXP3$^+$ T-cells express also heme oxygenase (HO)-1, an enzyme that produces carbon monoxide.[98,99] The suppressive function of human CD4$^+$CD25$^+$ T-cells was blocked in the presence of an HO-1 inhibitor, suggesting a role of carbon monoxide in the suppressive function of FOXP3$^+$ T-cells.[98]

Role of FOXP3$^+$ T-Cells in Suppression of Diseases

Immune dysregulation, polyadenopathy, enteropathy and X-linked inheritance (IPEX) patients develop various clinical symptoms. Most patients suffer from systemic autoimmune diseases evidenced by severe acute enteritis, Type I diabetes, elevated serum IgE and eczema.[19-21] The patients variably have also hypothyroidism, anemia, thrombocytopenia, neutropenia and autoantibodies. The exact phenotype is thought to be determined by the type of mutations in the FOXP3 gene because partially functional FOXP3 can be made with certain types of mutations. Also, other factors such as genetics and environmental factors can affect the progression of the disease. Scurfy mice are a mouse version of human IPEX.[100,101] Male scurfy mice with the scurfy mutation in the X chromosome develop runting, exfoliative dermatitis, hypergammaglobulinemia and severe anemia.[100,101] In a manner similar to IPEX patients, scurfy mice die young at around 3 weeks of age. The phenotypes of IPEX patients and scurfy mice clearly show that autoimmune responses play central roles in developing the disease. FOXP3 is mainly expressed by CD4$^+$ T-cells and therefore, this suggests that FOXP3$^+$ T-cells play important roles in prevention of the autoimmune disease. The scurfy symptom can be prevented by adoptive transfer of FOXP3$^+$ T-cells,[102,103] further supporting the role of these cells in prevention of the disease.

Because FOXP3$^+$ T-cells can suppress many types of immune cell such as CD4$^+$ T-cells, CD8$^+$ T-cells, CD1d-restricted NKT cells, monocytes/macrophages, naïve/memory B-cells, dendritic cells and NK cells,[104-110] they have the potential to suppress a wide spectrum of immunological diseases. This is indeed true in animal models that FOXP3$^+$ T-cells can either prevent or suppress existing immunological diseases such as experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (IBD), diabetes, collagen-induced arthritis, lupus, autoimmune gastritis and allergy.[111-118] Similarly, FOXP3$^+$ T-cells can effectively suppress allogeneic immune responses leading to graft rejection and graft-versus-host disease.[119-121] Infection is a type of diseases that are different from autoimmune diseases and the suppressive function of FOXP3$^+$ T-cells may be disadvantageous for the hosts during infection. In infection, some pathogens can suppress immune responses by expanding FOXP3$^+$ T-cells.[122-125] FOXP3$^+$ T-cells are perhaps required to terminate immune responses and prevent over-active immune responses which could lead to autoimmune diseases. However, excessive expansion of FOXP3$^+$ T-cells could deter clearance of pathogens by the immune system. Cancer is yet another class of diseases. Most tumor types including colorectal cancer, head and neck cancer, hepatocellular carcinoma, breast cancer, pancreas adeno caricinoma, melanoma, cervical carcinoma, gastrointestinal tract cancer, lung cancer, ovarian cancer, leukemia and lymphoma have increased numbers of tumor-infiltrating FOXP3$^+$ T-cells.[126-137] It is unclear if these cells are induced within the tumor or immigrated into tumors. What seems clear is that these tumor-associated FOXP3$^+$ T-cells have the potential to suppress antitumor immune responses.

Functions of FOXP3 in Nonhematopoietic Cells

FOXP3 appears to have a role in thymic epithelial cells. The scurfy mutation in the *FOXP3* gene causes diminished proliferation of double negative thymocytes and thymic atrophy.[9] Interestingly, FOXP3 is expressed also by nonimmune cells such as epithelial cells in mammary glands, prostate and lungs.[10,138] The function of FOXP3 in the epithelial cells is largely unknown but FOXP3-deficient mammary gland cells are more prone to become cancerous.[10] It is possible that FOXP3 would regulate the expression of certain oncogenes in these cells. HER-2/ErbB2 oncogene and S-phase kinase-associated protein 2 (SKP2, a component of the E3 ubiquitin ligase SKP1-Cul1-Fbox complex) are such oncogenes that are implicated in FOXP3-mediated suppression of cell proliferation in mammary gland cells.[10,139] FOXP3 functions to down-regulate the expression of ERB2 and SKP2.[10] Therefore, FOXP3 appears to play a potentially important role in regulation of the proliferation of epithelial cells in certain organs. Although the role is unclear, FOXP3 is expressed also by some tumor cells.[140,141]

Concluding Remarks

The significance of FOXP3 in regulation of the immune system is well-established. FOXP3 functions as a transcription activator and suppressor and programs the gene expression program in T-cells in a direction to promote immune tolerance. The detailed mechanisms for the gene expression regulation by FOXP3 remain to be determined but it appears to modulate the function of major transcription factors and to change the chromosomal conformation. A plethora of information is available regarding the immune regulatory function of FOXP3$^+$ T-cells. The data clearly support the clinical application potential of FOXP3$^+$ T-cells in suppression of inflammation and prevention of immunological diseases. Control of immunological diseases can be achieved either through increasing the numbers of FOXP3$^+$ T-cells for suppression of immune cells or decreasing the numbers for promoting immune responses. Autoimmune diseases can be treated by utilizing the former method, while cancer and control of infection can be achieved by adopting the latter method. FOXP3$^+$ T-cells can be prepared in vitro by culturing naïve CD4$^+$ T-cells in the presence of TGF-β1 and IL-2 or various other agents that can turn on the expression of FOXP3. The migratory and functional properties of FOXP3$^+$ T-cells can be altered by using homing receptor inducers such as retinoic acid or by gene therapy. This would make them more efficient in migration to target tissues and to control diseases. It is expected that FOXP3-based therapies would be actively utilized in treating human patients in the near future.

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CHAPTER 3

Molecular Regulation of Cellular Immunity by FOXP3

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Abstract

The immune system is responsible for not only eliminating threats to the body, but also for protecting the body from its own immune responses that would cause harm if left unchecked. Forkhead box protein 3 (FOXP3) is a forkhead family member with an important role in the development and function of a type of $CD4^+$ T cell called T regulatory cells that is fundamental for maintaining immune tolerance to self. This chapter reviews the structure of FOXP3 and how its role in the immune system was discovered. Studies of patients with mutations in FOXP3 who suffer from a syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked) are also discussed. Investigation into how expression of FOXP3 is regulated and how it interacts with other proteins have recently provided considerable insight into mechanisms by which the lack of this protein could cause disease. We also discuss how FOXP3 is involved in the reciprocal development of T regulatory cells and proinflammatory T-cells that produce IL-17. A better understanding of how FOXP3 is regulated and the molecular basis for its function will ultimately contribute to the development of T regulatory cell-based cellular therapies that could be used to restore dysregulated immune responses.

Introduction

The immune system is designed to maintain a balance between destroying threats to the body and protecting self from immune-mediated damage caused by inflammation and autoimmunity. One of the mechanisms by which this balance is controlled is immune suppression by specialized T-cells known as T regulatory cells. Although many different types of T regulatory cells have been identified, those that are $CD4^+$ and express the forkhead box protein P3 (FOXP3) transcription factor (hereafter Treg cells) have been the most intensely studied due to the compelling evidence for their therapeutic potential to prevent and even reverse many immune-mediated diseases.[1] Although the mechanisms by which Treg cells suppress the activation and effector functions of $CD4^+$ and $CD8^+$ T-cells, antigen presenting cells and B cells remain to be fully elucidated, what is clear is that FOXP3 is of central importance to both the development and function of Treg cells.[2-7] Beyond its role in immunity, FOXP3 also has a newly recognized role as a tumour suppressor gene and is also expressed in epithelial cells of multiple organs.[8,9] Since this is an emerging field and relatively little is known about how FOXP3 functions in nonimmune cells, in this chapter we will focus on its immunological functions. We will begin by summarizing what is known about the structure of FOXP3 and how its role in Treg-cell genesis was discovered. We will also discuss how the study of T-cells from patients who suffer from IPEX (immune dysregulation, polyendocrinopathy,

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enteropathy, x-linked) due to mutations in FOXP3 have helped us to understand the role of this protein in immunity. Since understanding disease mechanisms and developing strategies to target Treg cells therapeutically rely on understanding how FOXP3 acts at the molecular level, we will also discuss what is known about different isoforms of FOXP3, how it interacts with other proteins, and how epigenetic changes control its expression. Finally, the functional consequences of interactions between FOXP3 and other transcription factors will be illustrated by reviewing its role in the reciprocal development of Treg cells and a newly-described proinflammatory subset of T-cells called Th17 cells.

Discovery of FOXP3 and Key Structural Features

FOXP3 is an X-linked gene (located in Xp11.23) whose fundamental importance in immune regulation was first recognized in 2001 when three parallel studies identified it as the gene mutated in the *scurfy* mouse[10] and in humans with IPEX.[11,12] Scurfy mice arose as a spontaneous mutation at the Oak Ridge National Laboratory in 1949 and suffer from an X-linked, rapidly fatal autoimmune disease mediated by $CD4^+$ T-cells.[13] The similar phenotype of IPEX patients will be discussed in more detail below. Sequence analysis of the mutated gene revealed a protein with a classical forkhead (FKH) domain and ultimately recognition of a novel member of the FKH family of transcriptional factors.[10] Early structure-function studies confirmed that FOXP3 was able to bind the consensus FKH binding sites in the transthyretin and immunoglobulin variable regions V1 (V1P) promoters.[14] Although FOXP3 was originally thought to exclusively repress transcription since it inhibited luciferase activity driven by an SV40 promoter appended to three V1P forkhead consensus sites,[14] more recent studies have revealed that it can also act as an activator of transcription.[15-17]

In humans FOXP3 is a 431 amino acid (aa) protein consisting of an amino terminal proline-rich region (aa 1-193), a C2H2 zinc finger domain (aa 200-223), a leucine zipper motif (aa 240-261) and a carboxyl-terminal forkhead domain (aa 338-421) (Fig. 1).[18] Its FKH and zinc finger/leucine zipper sequences are 73-80% and 44-51% identical to FOXP1, FOXP2 and FOXP4, respectively, while the remainder of the sequence is divergent.[19] Specifically, the proline-rich amino terminal region of FOXP3 contrasts significantly with the glutamine-rich amino termini of the other FOXP members.[20] Furthermore, FOXP3 lacks the C terminal binding protein 1 (CtBP1) transcriptional repressor domain present between the leucine zipper and FKH domains in FOXP1 and FOXP2, suggesting that the mechanisms by which it represses transcription differ from other FOXP family members.[20] The amino-terminal 106 to 190 aas have recently been identified as necessary, but not sufficient for the repressive activity of FOXP3.[21,22] As discussed in more detail below, this region is also responsible for binding to the histone acetyltransferase TIP60 and histone deacetylase HDAC7.[22] The leucine zipper region is essential for homo- and hetero-dimerization[21,23,24] while

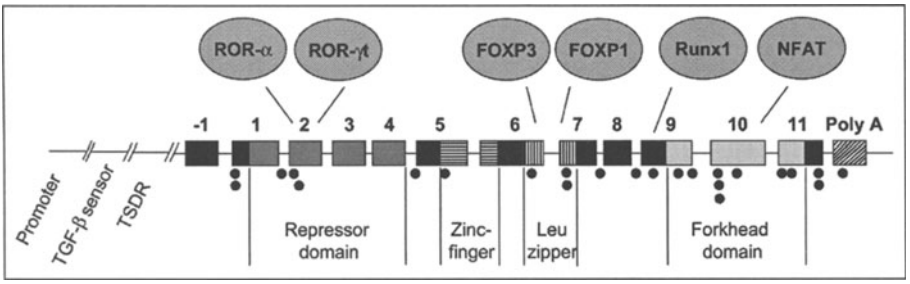

Figure 1. Schematic diagram of the exon/intron structure of FOXP3 and its known functional domains. Black shading indicates uncharacterized regions and locations of mutations found in IPEX patients are indicated by black dots. Regions encoding sites of known protein-protein interactions are shown. Regions known to be subject to epigenetic regulation include the promoter, TGF-β sensor and Treg-cell-specific demethylated region (TSDR).

the zinc finger does not seem to be essential for either homodimerization or repressive activity.[21] As for all FKH proteins, the FKH domain of FOXP3 contains a DNA-binding sequence, as well as a nuclear localization sequence.[21]

Phenotype and Function of FOXP3$^+$ Treg Cells

In the immune system, FOXP3 is expressed by a population of CD4$^+$ Treg cells[25-27] which were originally identified based on high and constitutive expression of the high affinity IL-2Rα chain (CD25) and functional capacity to suppress immune responses in vitro and in vivo.[28,29] Since FOXP3 is a nuclear protein, live FOXP3$^+$ cells cannot be isolated except from reporter mice;[30-32] thus, the majority of studies rely on expression of CD25 to isolate Treg cells and investigate their biological properties. Beyond expression of CD25, some other notable characteristics of FOXP3$^+$ Treg cells include their inability to produce typically T-cell-derived cytokines such as IL-2, IFN-γ or TNF-α and their in vitro hyporesponsiveness to T-cell receptor (TCR) activation.[1,33,34] They do, however, retain the ability to produce immunosuppressive cytokines such as IL-10, TGF-β and the recently described IL-35.[35-41] The cell-surface proteins expressed by Treg cells are generally characteristic of activated T-cells, and in addition to CD25, include cytotoxic T-lymphocyte-accociated antigen 4 (CTLA-4) and glucocorticoid-induced tumour-necrosis factor receptor family-related gene (GITR)[42-44] but not CD127 (IL-7 receptor, alpha chain).[45,46] Other potentially more Treg-specific proteins include folate receptor 4[47] and neuropilin-1,[48] but further research is required to clarify the specificity and utility of these molecules. The pros and cons of various cell surface markers proposed to be useful for isolating Treg cells have been reviewed by others.[33,49,50]

There is extensive evidence from animal models that insufficient Treg cell numbers and/or function can lead directly to autoimmunity and allergy, whereas an over abundance of these cells can suppress antitumor and antipathogen immunity. The role of Treg cells in disease has been extensively reviewed[1,34,42,49,51] and thus we will not focus on this topic here. Briefly, evidence that adoptive transfer of Treg cells can not only prevent autoimmunity in *scurfy* mice[27] but can also prevent and/or reverse other pathologies such as Type 1 diabetes,[52] inflammatory bowel disease,[53] graft versus host disease[54,55] and rejection of transplanted organs[56,57] has lead to widespread interest in developing similar cell therapy-based approaches in humans. Indeed, in humans, a reduction in the number or function of Treg cells is often, but not always, associated with various autoimmune diseases including myasthenia gravis, autoimmune polyglandular syndrome Type II, ulcerative colitis and multiple sclerosis,[43,58-63] as well as graft-versus host disease and allograft rejection.[64,65]

Role of FOXP3 in the Development of Treg Cells

Naturally Occurring versus Induced Treg Cells

Treg cells may either arise directly in the thymus or be induced in the periphery when naive CD4$^+$ T-cells encounter their antigen in a tolerogenic environment. Currently, there are no known phenotypic markers that distinguish thymically-derived or naturally-occurring (n) Treg from peripherally-induced (i) Treg cells. There is evidence, however, that they likely differ in antigen-specificity since nTreg cells tend to recognize self antigens[66,67] and are selected when the strength of TCR signalling is above that of classical positive selection but below that of negative selection.[68] In contrast, iTreg cells can be specific for any antigen presented in the context of tolerogenic dendritic cells,[69] or immunosuppressive cytokines such as TGF-β, as discussed in more detail below.

There is little doubt that FOXP3 is required for the normal function of Treg cells since the lack of this gene results in autoimmunity, but there is debate on the precise role of FOXP3 in the thymic differentiation of nTreg cells. Several years ago mixed bone marrow chimera experiments revealed that stem cells from mice genetically deficient for FOXP3 were unable to give rise to CD4$^+$CD25$^+$ T-cells,[27] leading to the conclusion that FOXP3 was necessary for this process. In more recent experiments, however, it was found that although expression of FOXP3 is required for suppressive activity, cells expressing a Treg-associated gene signature and cell surface molecules

still developed in the absence of FOXP3.[31,70] These findings suggest that the role of FOXP3 may to be amplify and stabilize the predetermined Treg lineage rather than to define the lineage itself. In support of this idea, it has been reported that many aspects of the Treg cell signature are not directly controlled by FOXP3 since it contains gene clusters that are co-activated with, rather than trans-activated by, FOXP3.[71]

When naive peripheral T-cells are activated in the presence of TGF-β and IL-2, expression of FOXP3 is induced and the resulting iTreg cells are suppressive in vitro[72-74] and in vivo.[75-77] Interestingly, addition of all-trans retinoic acid, a vitamin A metabolite, in vitro can enhance this conversion and it has recently been shown that the source of a similar vitamin A signal could be from gut derived CD103$^+$ dendritic cells.[78-81] TGF-β may also be involved in the development of nTreg cells since it has recently been shown that conditional deletion of TGF-βRI blocks the appearance of Foxp3$^+$ thymocytes.[82] An outstanding question is whether these TGF-β-induced Tregs are stable and persistent FOXP3$^+$ cells. Floess and colleagues observed that when TGF-β is removed, FOXP3 expression is rapidly reduced,[83] suggesting that exposure to TGF-β alone is not sufficient to generate iTreg cells. Indeed, beyond TGF-β there is also clearly a requirement for IL-2 in this conversion.[84] Evidence that expression of FOXP3 in TGF-β-induced Tregs is stabilized in inflamed environments in vivo suggests that cytokine signals in addition to IL-2 may be required for the stability, survival and/or expansion of iTeg cells.[76,77]

Reprogramming CD4$^+$ T-Cells into Treg Cells by Ectopic Expression of FOXP3

The finding that retrovirus-mediated over-expression of FOXP3 in naive CD4$^+$ T-cells from mice is sufficient to recapitulate all of the known features of Treg cells, including suppression of autoimmunity and rejection of transplanted grafts in vivo,[26,27,85] led to the idea that similar gene-and-cell therapy based approaches should be possible in humans. We first began to investigate this question in 2003, following the original reports from the groups of Sakaguchi, Rudensky and Ramsdell.[25-27] We initially found, however, that retrovirus mediated over-expression of FOXP3 in human CD4$^+$ T-cells was not sufficient to generate a population of cells with potent in vitro suppressive capacity.[86] Further investigation into why there was an apparent discrepancy between the ability of FOXP3 to reprogram mouse and human cells into Treg cells led to the finding that in order for human CD4$^+$ T-cells to be efficiently converted into Tregs, they must express not only high, but also stable levels of FOXP3.[87]

We developed a lentivirus-based method to ectopically express FOXP3 under control of the elongation factor one alpha promoter, the activity of which does not depend on the activation state of the cell. When FOXP3 was over-expressed in naive or memory CD4$^+$ T-cells from adult peripheral blood using this method, we were able to generate a population of T-cells that was phenotypically and functionally identical to ex vivo Treg cells based on in vitro assays of proliferation, cytokine production, cell surface marker expression and suppressive capacity. These data indicate that the ability of FOXP3 to convert conventional CD4$^+$ T-cells into Tregs is more tightly regulated in humans than in mice, possibly due to the fact that FOXP3 can also be expressed transiently in activated T effector (Teff) cells[88] as discussed below. The ability to generate and efficiently expand stable populations of suppressive Treg cells in vitro is an important advance in the development of therapeutic applications to translate the immunosuppressive powers of these cells to the clinic.

We also used the lentivirus-based system to investigate the dose- and time-dependent requirements for expression of FOXP3 to mediate efficient conversion into Treg cells. Using a version of FOXP3 fused to the hormone binding domain of the estrogen receptor (ER), we created an inducible form of the transcription factor, the activity of which can be pharmacologically controlled by the estrogen analog 4-hydroxytamoxifen (4HT).[89] In the absence of 4HT, the FOXP3-ER fusion protein is sequestered in the cytoplasm and inactive. Upon addition of 4HT, which causes protein-refolding, stimulates nuclear translocation and increases protein stability, we found that the relative suppressive capacity is correlated with the concentrations of 4HT and presumably higher levels of FOXP3 activity. Moreover, the suppressive capacity is maximal when cells are kept in 4HT for 7-12 days. Since 4HT-induced nuclear translocation of FOXP3 happens within

a few hours, these data suggest that significant changes in gene expression are necessary before the functional effects are manifested. Notably, the suppressive capacity is rapidly reversed when 4HT is withdrawn from the FOXP3-ER-expressing cells, lending support to the notion that FOXP3 does more than define the Treg lineage. The conclusion that FOXP3 is required for the suppression function of Treg cells is also supported by evidence in mouse models.[31,70,90] This conditionally active form of FOXP3 will also be a valuable tool to further study the cellular and molecular phenotype of Tregs and indeed, it was recently shown that Foxp3-ER expressing cells can be used to suppress collagen-induced arthritis "on demand" upon in vivo administration of 4HT.[91]

Role of FOXP3 in Conventional T-Cells

A unique property of conventional $CD4^+$ T-cells in humans is that they also express FOXP3 transiently when they are activated.[92-96] It has been suggested that this is exclusively a TGF-β-mediated process,[93] but we were not able to confirm this (unpublished data). We found that activated Teff cells express FOXP3 maximally three days after TCR-stimulation and that under conditions of strong activation, almost 100% of Teff cells become $FOXP3^+$.[94] However, a key difference between activated Teff cells and Treg cells remains evident: although the intensity of FOXP3 expression of activated Teff cells reaches that of resting Treg cells, it remains significantly lower than that in similarly-activated Tregs cells.[94] These data correlate with our finding that only stable and high expression of FOXP3 can convert human $CD4^+$ T-cells into Tregs[87,89] and further support the conclusion that Treg cell development and function does not simply rely on the presence or absence of FOXP3, but rather on the magnitude and stability of its expression.

A consistent finding is that the transient expression of FOXP3 in activated Teff cells is not sufficient to suppress cell division or cytokine production.[92,94,95] These data indicate that the molecular activity of FOXP3 in Treg and Teff cells is fundamentally different, likely as a consequence of other yet-to-be-defined interactions with other Treg-specific proteins. One point of controversy that has arisen from these studies is whether $FOXP3^+$ Teff cells transiently acquire suppressive activity. Some reports indicate that $FOXP3^+$ Teff cells temporarily become suppressive[97,98] whereas we and others could not confirm this finding.[92-94,96] It should be noted that it is very difficult to make meaningful conclusions from in vitro suppression assays performed with activated Teff cells as they are not only susceptible to activation-induced-cell death but also have fundamentally different kinetics of proliferation. Thus, it is possible that "suppression" of thymidine incorporation could be due to either induction of cell death or that the peak of proliferation was missed, as we previously showed in comparisons of T-cells activated by immature or mature dendritic cells,[99] rather than true suppression. Nevertheless, further investigation into the role of FOXP3 in Teff cells is clearly warranted.

Molecular and Cellular Biology of IPEX

The main features of IPEX are entheropathy, characterized by refractory diarrhoea, multiple endocrine organs autoimmunity, such as Type I insulin-dependent diabetes and thyroiditis, hyper IgE and eczema (www.ipexconsortium.org).[11,12,100-102] The onset of the disease is often in early infancy and the course can be rapidly lethal. The disease is rare, but retrospective data on clinical cases of early autoimmune enteritis associated with Type I diabetes or of neonatal diabetes of unknown origin suggests that the actual frequency of the disease may be underestimated.[102] In addition to supportive care including parenteral nutrition, blood transfusions and treatment of diabetes, immunosuppressive drugs, such as high-dose steroids, cyclosporin A, tacrolimus, methotrexate, infliximab and rituximab, are commonly used to treat IPEX patients.[101] Unfortunately, immunosuppression is usually only partially effective and the dose is limited by infectious complications and toxicity. More recently, sirolimus (rapamycin) has been used to treat IPEX patients[103,104] since it can specifically suppress Teff cells and spare, or even promote, Treg cell expansion.[105,106]

Currently, the only curative treatment for IPEX patients is bone marrow transplantation,[102,107] but this approach is limited by toxicity and availability of HLA-compatible donors. Thus, we are

also exploring whether a gene-therapy based approach to correct genetic defects in FOXP3 in T-cells would be a novel treatment option. In healthy females who are heterozygous for different types of *FOXP3* mutations, we found that their peripheral blood mononuclear cells and $CD4^+$ naïve, effector and memory T-cells have a random pattern of X-chromosome inactivation (Di Nunzio et al, submitted). In contrast, circulating Treg cells in these women exclusively express the wildtype allele of *FOXP3*, suggesting either that wildtype FOXP3 is necessary for the development of Treg cells, or that in vivo it gives a selective advantage for the normal homeostasis of Treg cells. The latter possibility would be in line with the notion that FOXP3 is required for the "competitive fitness" of Treg cells.[70] The fact that Treg cells expressing wildtype FOXP3 can maintain peripheral tolerance in subjects whose other haematopoietic cells are mosaics of wildtype and mutant FOXP3 supports the rationale for gene or cellular-based therapeutic approaches to restore Treg cell function in IPEX patients.

To date, more than 30 different types of *FOXP3* mutations have been described, including mis-sense mutations, splice site alterations and deletions.[101] Importantly, the type of mutation does not necessarily correlate with clinical manifestations. We recently studied 14 unrelated affected males to investigate whether *FOXP3* mutations and changes in protein expression correlated with molecular and clinical data. Notably, the only known mutations that completely prevent transcription and expression of FOXP3 protein are those that occur in the ATG start codon and these IPEX patients have very severe disease.[102] All patients and particularly those carrying mutations in known functional domains of FOXP3 or mutations that alter protein expression, have enteropathy, generally associated with endocrinopathy and eczema. Similar genotypes, however, do not always result in similar disease presentation and severity. These data indicate that beyond FOXP3, other genetic and environmental factors contribute to the development of IPEX.[102]

In order to understand how FOXP3 regulates autoimmunity in humans, we also investigated whether IPEX patients have numerical or functional defects in Treg cells. We found that depending on the type of mutation, surprisingly many IPEX patients have normal numbers of circulating $FOXP3^+$ T-cells.[102,108] Moreover, only Treg cells from patients with mutations in the ATG start codon, which completely abrogates expression of FOXP3, completely lack suppressive activity in vitro.[108] In contrast, patients who have mutations in the FKH domain of FOXP3 have more subtle changes in Treg cell function that depend on the strength of TCR activation and whether the targets of suppression are allogeneic or autologous. Thus normal DNA-binding activity of FOXP3 is not essential for Treg development, and moreover, IPEX is not simply a result of a global defect in Treg cells. Studies are currently on-going to better define how different types of mutations affect Treg cell development at both the cellular and molecular level.

Another surprising finding from our studies on IPEX patients is that their conventional $CD4^+CD25^-$ T-cells display a defect in production of IL-2 and IFN-γ, suggesting that IPEX could be due not only to impaired Treg cell function but also to a parallel defect in Teff cell function.[108] These data are in direct contrast to the finding that in FOXP3-deficient mice Teff cells are hyper-activated and produce increased levels of pro-inflammatory cytokines.[14] They do, however, correlate with the fact that in humans FOXP3 is also expressed transiently in activated T-cells,[92-97] suggesting that there is a functional role for FOXP3 outside of Treg cells that has yet to be defined.

Isoforms of FOXP3 in Humans

In humans, T-cells co-express two isoforms of FOXP3: FOXP3a is the full-length transcript that is exclusively expressed in mice, whereas FOXP3b is a splice variant that lacks exon 2.[86,109] We investigated whether FOXP3a and b differ in their ability to drive Treg development and found that although FOXP3b suppresses transcription of a luciferase reporter under control of the IL-2 promoter, ectopic expression of FOXP3b in T-cells is less effective than FOXP3a at suppressing IL-2.[86] In contrast, Smith et al reported that FOXP3b is equally effective as FOXP3a at suppressing IL-2 production.[110] Functional differences between FOXP3a and b have recently been attributed to differential interactions between another transcription factor involved in T-cell development:

retinoic acid-related orphan receptor (ROR)-α. Specifically, FOXP3 and ROR-α interact via an LxxLL motif in exon 2 of FOXP3, resulting in inhibition of ROR-α-mediated transcriptional activation.[111] In addition, it has been reported that while FOXP3a is distributed both in the nucleus and cytoplasm, FOXP3b is primarily located in the nucleus, possibly due to the lack of a lysine-rich region encoding a typical nuclear export signal present in exon 2.[19]

Another splice form of FOXP3 that lacks both exon 2 and exon 7, which contains part of the leucine zipper domain, has also been identified and is referred to as FOXP3 Δ2Δ7.[110] Recently, preferential expression of FOXP3b and Δ2Δ7 was observed in a subgroup of patients with Sezary syndrome (SS), a form of cutaneous T-cell lymphoma (CTCL) characterized by lymphocytes with atypical cerebriform nuclei (Sezary cells) in the skin, lymph nodes and blood.[112] Interestingly, both the FOXP3b and Δ2Δ7 isoforms are defective at repressing an NFκB-driven luciferase reporter.[112] Although CTCL cell lines from SS patients were suppressive, this function was mediated by IL-10 and independent of FOXP3. These data suggest that the alternative splice forms of FOXP3 may mediate malignant Treg cell differentiation but not their suppressive action.

FOXP3 Protein Interactions

Homo-Oligomerization

Increasing evidence indicates that protein-protein interactions are a key for the normal function of FOXP3. For example, FOXP3 forms homotetramers and homooligomers,[24] and one of the mutations that causes IPEX is a deletion of aa 251 in the leucine zipper domain that abrogates homotetramerization and association with FOXP1.[24] This mutant is defective at suppressing transcription and cannot bind to the NFAT/forkhead site of the IL-2 promoter, suggesting that homo- or hetero-associations are important for the ability of FOXP3 to act as a transcriptional regulator.[21,24] Indeed, knockdown of FOXP1 in FOXP3-expressing Jurkat T-cells partially inhibits FOXP3-mediated repression of IL-2 production.[24] Furthermore, a homologous deletion of aa 250 in mouse Foxp3 decreases its transcriptional repressor activity and when expressed in Treg cells interferes with their ability to suppress inflammatory bowel disease.[23] Notably, mouse cells expressing Foxp3 Δ250 retain their ability to suppress IFN-γ intrinsically and from other $CD4^+CD25^-$ T-cells, but not from fully differentiated Th1 cells.[23] Thus, although leucine zipper-mediated interactions are necessary for many of the functions of FOXP3, some aspects of its activity are independent from this domain.

Interactions with NFAT

As its name suggests, the nuclear factor of activated T-cells (NFAT) transcription factor is fundamental for the transcription of activation-associated genes in T-cells.[113] Interestingly, many of the genes regulated by NFAT are also regulated by FOXP3, including IL-2, IL-4, CD25 and CTLA-4.[15,26,114] Recently, it was found that FOXP3 and NFAT form a cooperative complex that regulates NFAT-mediated transcription of promoters with NFAT:AP1 binding sites such as IL-2, CD25 and CTLA-4.[15,115] A mutant form of Foxp3 that is unable to interact with NFAT is defective at inhibiting IL-2 and upregulating CTLA-4 and CD25, and moreover, when ectopically expressed in T-cells, is less able to suppress autoimmunity in vivo.[15] Since FOXP3 occupies the AP1 binding site of the NFAT:AP1 consensus, it has been suggested that NFAT may preferentially bind to FOXP3 in Treg cells and AP1 in Teff cells. In line with this hypothesis, a constitutively active form of NFAT which cannot bind to AP1 induces T-cell anergy[116] and cooperates effectively with FOXP3.[15]

Interactions with AP1

FOXP3 also directly interacts with AP1, which is a heterodimer of C-FOS and C-JUN. Forced expression of FOXP3 blocks the ability of C-JUN to bind to the AP1 promoter and inhibits C-JUN-driven AP1 transcriptional activity in cotransfected HEK 293 cells or activated Treg cells.[117] The amino terminal region of FOXP3, but not the forkhead domain, is sufficient for association with c-jun and suppression of AP1 transcription activity.[117] Interestingly, ectopic

expression of the amino terminal region of FOXP3 alone is sufficient to promote the in vitro anergic characteristic of Treg cells but not to confer suppressive capacity,[117] indicating that these two biological events are molecularly distinct. Phosphorylation of C-JUN by JNK is required for AP1 transcriptional activity[118,119] and evidence that pharmacological inhibition of JNK interferes with the association between FOXP3 and C-JUN indicates that phosphorylation of C-JUN is also essential for this interaction.[117]

Interactions with NFκB

FOXP3 has also been reported to associate with NFκB in nonimmune cells and inhibit NFκB-mediated transcription,[115,120] partly via inhibiting translocation of NFκB to the nucleus.[120] Normally, in the absence of T-cell activation, nuclear translocation of NFκB is inhibited by IκB. Expression of FOXP3 however, increases the stability of IκB, thereby reducing NFκB translocation and the activity of NFκB-responsive genes.[115] It has yet to be shown whether FOXP3 interacts with NFκB in immune cells and thus whether direct interaction between these two proteins is important for normal Treg cell function remains unclear.

Interactions with Runx1

FOXP3 also binds to the runt-related transcription factors Runx1, Runx2 and Runx3. Most investigations are focussed on Runx1 since it is expressed in Treg and Teff cells and regulates expression from the IL-2 and IFN-γ promoters.[121] Indeed, FOXP3 suppresses Runx1-stimulated IL-2 production in Teff and Treg cells.[121] The Runx1-binding domain of FOXP3 is located within aa 278-336, a region between the leucine zipper and FKH domain. A mutant form of FOXP3 unable to bind to Runx1 fails to suppress IL-2 production, demonstrating that interaction is critical for this function.[121] The importance of Runx1 to Treg cell function is further illustrated by the fact that mouse $CD4^+$ T-cells transduced with a mutant FOXP3 that cannot bind to Runx1 are less suppressive than cells transduced with wild type FOXP3. Moreover knockdown of Runx1 in human Treg cells attenuates their suppressive capacity.[121] Recent data indicate that interactions between Runx1 and Foxp3 are also necessary for Foxp3-mediated inhibition of IL-17-producing T-cells.[122] Of note, one of the known point mutations of FOXP3 that causes IPEX occurs in the Runx1-binding region of FOXP3 and causes a late-onset, mild and spontaneously remitting disease.[108] Further investigation will be required to define if altered interactions between FOXP3 and Runx1 may underlie autoimmunity in this patient.

Interaction with ROR-α

ROR-α was first shown to interact with the amino terminal region of FOXP3 in a yeast-two hybrid screen and the interaction was confirmed in coimmunoprecipitation studies.[111] FOXP3 suppresses ROR-α-mediated transcription, but unlike repression of NFAT-mediated transcription, this function does not depend on the FKH domain, but rather on a region in exon 2, as discussed above. Recently the association between Foxp3 and ROR-α was also found to occur in mouse cells.[123] Since ROR-α has a newly recognized role in the development of Th17 cells, it will be of interest to investigate how FOXP3 may affect this process.

Interaction with ROR-γt

After finding that TGF-β-induced Foxp3 expression represses expression of ROR-γt-driven IL-17 production, it was of interest to investigate whether ROR-γt and FOXP3 interact. Indeed, the two proteins co-immunoprecipitate in transfected 293T cells via a mechanism that is DNA-independent.[124] Notably, cotransduction of $CD4^+$ T-cells with ROR-γt and FOXP3 suppresses IL-17 production, but there are conflicting data on the domains of FOXP3 required for this effect. Zhou et al found that a FKH deletion mutant of FOXP3 and a form containing a point mutation (R397W) in the FKH domain from an IPEX patient, lost their ability to suppress IL-17.[124] On the other hand, Yang et al found that mutant forms of FOXP3 that either lacked the FKH domain or had a mutant leucine zipper retained their ability suppress IL-17 and Th17 cell differentiation.[123] Notably, the interaction involves a region encoded by exon 2.[124] Further

studies will be required to fully elucidate the molecular interactions between these two proteins. Interestingly, the inhibitory effects of FOXP3 on ROR-γt-induced IL-17 production can be reversed by IL-6 or IL-21, suggesting that these cytokines may cause inhibitory or stimulatory posttranslational modifications of FOXP3 or ROR-γt, respectively.[124]

The FOXP3-TIP60-HDAC7 Complex

FOXP3 interacts with the histone acetyltransferase (HAT) protein HIV-1 TAT-interactive protein, 60kDa (TIP60) via an amino terminal domain (106-190) of FOXP3.[22] Since this region of FOXP3 is also required for transcription repression,[21] Li et al investigated whether TIP60 is required for this function. Indeed, when expression of TIP60 is knocked down FOXP3-mediated repression of reporter activity is reduced.[22] Moreover, a HAT-deficient form of TIP60 reduces the ability of FOPX3 to repress transcription, suggesting that the HAT activity of TIP60 is important for the repressive activity of FOXP3.[22] One hypothesis is that the HAT activity of TIP60 may be directly involved in acetylation of FOXP3. Since acetylated FOXP3 binds to chromatin preferentially,[125] TIP60 may function to enhance the ability of FOXP3 to bind to promoters. Since TIP60 is known to recruit Class II histone deacetylase 7 (HDAC7) in other transcriptional repressor complexes, the possibility that FOXP3 also interacts with this protein was investigated. Indeed, co-immmunprecipitation studies demonstrated that FOXP3 associates with HDAC7 in human Treg cells. As for TIP60, the HDAC7-association domain is in the amino terminal 1-190 aa.[22] Thus, the amino terminal 106-190 aas are key for the ability of FOXP3 to repress transcription via a mechanism that depends on a tri-molecular complex of FOXP3, TIP60 and HDAC7.

Interaction with HDAC9

Beyond associations with HDAC7, depending on the state of activation, FOXP3 also interacts with HDAC9 in Treg cells. In resting Treg cells HDAC9 is primarily located in the nucleus, but upon stimulation it is transported out of the nucleus, suggesting that it only associates with FOXP3 in the resting state and that release of this interaction is required for suppression.[126] Further evidence for the role of HDAC9 as a negative regulator of FOXP3 function comes from analysis of $Hdac9^{-/-}$ mice. These mice not only have a 50% increase in Treg cells in lymphoid tissues, but their Treg cells are also three to fourfold more suppressive than control cells.[126] Inhibiting the catalytic activity of HDAC9 has a similar effect on Treg cells as the absence of the protein: treatment of mice with the HDAC-inhibitor trichostatin A (TSA) increases Foxp3 expression in Treg cells and Treg cells from TSA treated mice are more suppressive in vitro and in vivo than the cells from control-treated mice.[126] HDAC9 may inhibit Treg cell activity by deacetylating FOXP3, thereby decreasing its ability to bind DNA. Consistent with this notion, treatment with TSA increases the amount of acetylated FOXP3 in Treg cells and binding to the IL-2 promoter.[126]

Post-Translational Modifications of FOXP3

FOXP3 is subject to posttranslational acetylation of lysines and phosphorylation of tyrosines, serines and threonines. The significance of phosphorylation has yet to be reported, thus here we will discuss acetylation. Focussing on lysine residues conserved between mouse and humans in the FKH domain, Toa et al found that mutation of Lys383 and Lys393 significantly reduced the capacity of FOXP3 to suppress IL-2 and confer suppressive capacity to naive $CD4^+$ T-cells.[126] Thus, acetylation of Lys383 and Lys393 is important for enhancing the association of FOXP3 with target genes. Notably, the possibility of pharmacological inhibition of HDACs to enhance FOXP3 acetylation and improve Treg function has important clinical implications. Work in animal models has shown that TSA-treated mice have increased numbers of $Foxp3^+$ Treg cells in lymphoid tissues and reduced disease severity in the mouse model of colitis induced by dextran sulphate sodium. Furthermore, mice transplanted with MHC-mismatched cardiac and islet grafts and treated with TSA have a small survival advantage that is greatly enhanced by the combined treatment of TSA and rapamycin to inhibit the proliferation of alloreactive T-cells.[126]

Epigenetic Regulation of FOXP3 and Its Target Genes

Epigenetics of Genes Regulated by FOXP3

One way to promote or repress transcription is to modify chromatin structure at target genes. Since FOXP3 associates with histone-modifying proteins such as TIP60 and HDACs 7 and 9, this may be fundamental to its mechanisms of action. Usually, acetylation of histones on lysine, particularly on histone 3, is associated with an open chromatin structure conducive to gene transcription, while methylation of histone 3 at lysine 9 marks closed chromatin and repressed gene transcription.[127] Interestingly, binding of FOXP3 to promoters it represses (e.g., IL-2 and IFN-γ) results in histone 3 deacetylation at these promoters. In contrast, for promoters it transactivates (e.g., GITR, CD25 and CTLA-4), Foxp3 binding is correlated with increased histone acteylation.[128] Further evidence that FOXP3 and its associated proteins modify chromatin structure comes from studies on the effect of FOXP3 on the IL-4 promoter and a cis-regulatory element when it is over-expressed in Th2 cells. The normally high levels of acetylated histone 3 in the regulatory elements of the IL-4 promoter in Th2 cells are significantly reduced in the presence of FOXP3. Moreover, levels of repressive methylated histone 3 at lysine 9 are low in Th2 cells expressing IL-4, but enhanced when FOXP3 is overexpressed.[120] As this is an emerging area of interest, further research will be required to better define how FOXP3 directly alters chromatin structure.

Epigenetic Regulation of FOXP3 Expression

In general, expression of FOXP3 is induced by short and weak TCR stimulation and we and others have shown that pharmacological treatment with inhibitors of the phosphatidylinositol 3' kinase (PI3K) pathway favours FOXP3 expression.[105,129-131] Activation of STAT5 downstream of IL-2 is also key for induction and maintenance of FOXP3 in both nTreg and iTreg cells.[132,133] Moreover, as discussed above, TGF-β can directly stimulate de novo expression of FOXP3 and contribute to the development of iTreg cells.[84] Ultimately, these factors all contribute to the epigenetic changes that determine the stability and magnitude of FOXP3 expression. Since stable and high levels of expression are necessary for Treg lineage-commitment and function,[87,89,90] understanding how epigenetic changes contribute to FOXP3 expression is fundamental for the development of cell-therapy based applications.

Currently, three main regions of FOXP3, which are highly conserved in mice and humans, are known to be subject to epigenetic modifications that impact transcriptional activity of the locus: the promoter, the so-called TGF-β-sensor region, and the Treg-cell-specific demethylated region (TSDR).[134] Bisulphite sequencing to analyze the methylation state of CpG motifs in these regions revealed that they were highly methylated (indicative of inactive chromatin) in Teff cells but almost completely demethylated in Treg cells.[83,135] Further examination of the histone modifications associated with these regions by chromatin immunoprecipitation demonstrated that in Treg cells these regions also have increased acetylation of histone 3 and trimethylation of lysine 4 on histone 3 compared to Teff cells, indicating an open chromatin structure for Treg cells and a condensed structure for Teff cells.[136]

There is a great deal of interest in determining how the epigenetic changes in these three regions act as on/off switches and whether they also determine the stability and magnitude of FOXP3 expression. For example, evidence that the degree of demethylation in the TSDR is less in thymic than peripheral Tregs cells, suggests that expression of FOXP3 may be stablized in the periphery.[83] Moreover, when the effects of TGF-β on epigenetic changes in these loci were investigated, it was found that although there is a certain amount of demethylation of the locus, it is not to the same extent and is transient in comparison to ex vivo Treg cells.[83] The fact that TGF-β alone may not be sufficient to irreversibly open the FOXP3 locus could underlie the variable reports on the capacity of TGF-β to induce suppressive Treg cells.[84] Epigenetic analysis also provided further evidence that the expression of FOXP3 upon TCR activation of Teff cells is transient.[137] Importantly, the stability of FOXP3 expression can be manipulated pharmacologically using compounds that alter epigenetic changes. For example, blocking maintenance DNA methylation induces stable activation-dependent FOXP3 expression in Teff cells and also confers stability to TGF-β-mediated

induction of Foxp3 expression.[138] Thus, the development of successful cell therapies based on generating iTreg cells in vitro may rely on the use of strategies to ensure the epigenetic changes in the FOXP3 locus are stable.

Role of FOXP3 in Treg versus Th17 Cell Development

When naive CD4+ T-cells encounter their antigen they differentiate into subsets defined by differences in cytokine production and effector function. For example, Th1 cells develop in the presence of IL-12 and secrete IFN-γ thereby promoting cellular immunity and elimination of intracellular pathogens. By contrast, IL-6, IL-1β and TGF-β promote the development of Th17 cells which contribute to host defence against pathogens that require robust tissue inflammation to be cleared.[139] Pathologically, both Th1 and Th17 cells can also mediate autoimmunity. The fact that TGF-β seems to be required, either directly or indirectly, for the development of pro-inflammatory Th17 cells is at odds with the parallel role for this cytokine in the development of anti-inflammatory Treg cells. This has lead to a great deal of interest in defining the molecular basis for the developmental relationship between Th17 cells and Treg cells, since therapeutic strategies to reduce autoimmunity must not be confounded by parallel promotion of Th17 cells.

As discussed above, ROR-α, ROR-γt and Runx1 are critical transcription factors in Th17 development and function and there is much interest in defining how their interactions with FOXP3 define the Treg versus Th17 cell lineage.[122,139,140] It is thought that in the presence of TGF-β alone, expression of FOXP3 is induced and that it interacts with Runx1 and ROR-γt to inhibit expression of IL-17, either directly via a Foxp3/Runx1 complex or indirectly by preventing Runx1 from enhancing ROR-γ t-mediated IL-17 transcription.[122] Conversely, in Th17 polarizing conditions, signals from TGF-β and IL-6 are thought to combine to decrease FOXP3, allowing Runx1 to preferentially bind to ROR-γt and enhance IL-17 expression.

In addition to direct interactions with ROR-γt and Runx1, interactions between Foxp3 and the TIP60/HDAC7 complex also contribute to repression of ROR-γt-mediated transcription and thus IL-17 production. Analysis of a mutant form of Foxp3 (Δ105-190) that cannot bind to TIP60 or HDAC7 revealed that its ability to suppress ROR-α or ROR-γt mediated-transcription was attenuated.[22] Mutation of both the ROR-α-interacting domain (the LxxLL motif) and the TIP60/HDAC7 binding domain completely abolished the ability of Foxp3 to suppress ROR-α and ROR-γt-mediated reporter activity. Thus Foxp3 cooperates with both TIP60/HDAC7 and the ROR transcription factors to repress ROR-mediated transcription and Th17 cell differentiation.

Because FOXP3 inhibits Th17 differentiation, it was of interest to determine if in the absence of FOXP3 TGF-β is sufficient to induce Th17 cells. However, when CD4+ T-cells from *scurfy* mice are stimulated with TGF-β they do not produce IL-17, indicating that the role of IL-6 in Th17 cell differentiation is not simply to inhibit Foxp3.[123] Moreover, when Foxp3- T-cells are stimulated with TGF-β and IL-6, they have reduced IL-17 and enhanced IFN-γ production compared to wild type cells. Further investigation revealed that although FOXP3 is not directly required for Th17 cell development, it is indirectly required via its ability to suppress Th1 cell development.[123] These data lend further support to the paradox that FOXP3 has a dual role in immune homeostasis: beyond defining the Treg lineage it can also potentially contribute to the development of Th17 cells. Since CD4+ T-cells which co-express FOXP3 and ROR-rt are suppressive,[141] Treg development may be dominant in this process.

A further complexity in the relationship between FOXP3, Treg and Th17 cells is that several groups have recently shown that Treg cells can be reprogrammed into Th17 cells.[123,142-144] When Treg cells are activated in the presence of pro-inflammatory cytokines such as IL-6 or IL-1β, expression of FOXP3 is downregulated and there is a parallel increase in IL-17 expression and loss of suppressive capacity.[123] This conversion is blocked by inhibition of histone deacetylases, suggesting that the differentiation of Treg cells into IL-17 producing cells depends on epigenetic modifications.[144] Evidence that this conversion can also occur in vivo comes from studies in which CD45.2+ GFP+ Treg cells from Foxp3 reporter mice were mixed with congenic CD45.1+ Teff cells and transferred into $Rag1^{-/-}$ recipients. When experimental autoimmune encephalomyelitis

is induced in these mice, the CD45.2$^+$ cells, which were originally Treg cells, downregulate Foxp3 and start to secrete IL-17.[123] Thus, there appears to be a considerable amount of plasticity in the Treg cell lineage and further research into how expression of FOXP3 contributes to this process will be key to understanding how this process may go awry and lead to autoimmunity.

Conclusion

Studies of how the structure of FOXP3 relates to its function have provided significant insight into how this FKH transcription factor regulates the development and function of Treg cells. Originally it was thought that the DNA-binding FKH was the major effector region of the protein, but it is now clear that many of the critical activities of FOXP3 are mediated not by direct DNA-binding, but rather by interactions with proteins such as histone acetyltransferases and deacetylases and transcription factors such as NFAT, ROR-α, ROR-γt and Runx1. Many of these interactions directly impact the balance between the development of the immunosuppressive Treg program and the pro-inflammatory Th17 program. It will be of considerable interest to define the molecular basis for how mutations in FOXP3 cause IPEX and tip immune responses towards pathological inflammation.

A major outstanding question is what is the role of FOXP3 in human Teff cells? Evidence that Teff cells from IPEX patients have reduced cytokine production suggests that FOXP3's role in cytokine production in conventional T-cells may be the opposite from that in Treg cells.[108] The finding that in activated Teff cells essentially all of the cytokine-producing cells are also FOXP3$^+$[94] lends indirect support for this hypothesis. Another possibility is that in IPEX patients the mutant forms of FOXP3 could act as dominant negatives and interfere with the normal function of transcription factors, such as NFAT and AP-1, which regulate cytokine production. If this is true, then the normal function of FOXP3 in Teff cells could be to act as part of a negative feedback pathway that controls effector function. Since to date there is no evidence for a similar role of FOXP3 in Teff cells in mice, studies to investigate these various possibilities must be performed with human cells. An additional consideration in human T-cells is the co-expression of two isoforms of FOXP3, one of which cannot interact with ROR-α or ROR-γt. As Treg-based therapies for autoimmunity are translated to the clinic, studies to define how expression of FOXP3b may lead to human-specific molecular events that impact Th17 cell development will be key.

Mouse models have illustrated the power of Treg-cell based therapies to prevent or even cure autoimmunity and to restore antitumour immunity. It is clear that the further development of therapeutic strategies to perform similar Treg-based manipulations in humans will require a more detailed understanding of what regulates stable and high expression of FOXP3 and the molecular basis for its function. The recent finding that pharmacological-manipulations of epigenetic changes can stabilize the Treg lineage represents a major advance in this direction.[134] Intense research into the underlying mechanisms for the newly recognized plasticity of the Treg lineage and the biological relevance of their ability to turn into Th17 cells will shed light onto the potential dangers of Treg-based cell therapies and hopefully also point to strategies to ensure that effective tolerogenic therapies become available to humans in the future.

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CHAPTER 4

The Biology of FoxP3:
A Key Player in Immune Suppression during Infections, Autoimmune Diseases and Cancer

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Abstract

The Transcription factor FoxP3 belongs to the forkhead/winged-helix family of transcriptional regulators and shares general structural features with other FoxP family members. FoxP3 functions as a master of transcription for the development of regulatory T-cells (Treg cells) both in humans and in mice. Natural genetic mutations of FoxP3 that disrupt its function in humans result in an autoimmune syndrome called Immune Polyendocrinopathy, Enteropathy, X-linked (IPEX) and in mice, its deletion causes the Scurfy phenotype, with similar pathology. The finding that FoxP3 is required for the development and function of Tregs has led to an explosion of research in determining its regulation and function in the immune system. Understanding the biological properties of FoxP3 has a wide range of implications for immune tolerance, autoimmune disorders, inflammation and immune response to infectious diseases and cancer.

Introduction

The Immune system has evolved sophisticated mechanisms to mount effective protective immune responses and to limit damage to the host by tightly regulating its potentially harmful side effects. A specialized cell type within the immune system called regulatory T-cells (Tregs) is instrumental in preventing immune responses against self-antigens and dampening immune activation to nonself antigens. These regulatory T-cells were initially defined by high expression of the IL-2 receptor alpha chain (CD25) and were found to be part of the $CD4^+$ helper T-cell subset. Treg cells were then shown to express and require the transcription factor FoxP3, which also became a defining factor for their biology.

The Discovery of FoxP3

The forkhead family transcription factor Foxp3 was shown to be critically important for the development and function of regulatory T-cells.[1,2] FoxP3 was first identified as the culprit mutant gene responsible for the spontaneous scurfy mutation in mice and the human syndrome called Immunedysregulation Polyendocrinopathy Enteropathy, X-linked, or IPEX.[3,4] Both of these genetic defects resulted in death of animals and humans.

In 2003 it was discovered that FoxP3 is expressed in 5-10% of peripheral $CD4^+$ T-cells in mice and 1-5% in humans. FoxP3 expression was shown to be sufficient for murine Treg cell development and function as revealed by studies using ectopic expression of FoxP3 in otherwise conventional T-cells.[2] In humans, ectopic overexpression of FoxP3 in naïve T-cells was also shown to differentiate

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these cells into Treg mimics in vitro,[5,6] although the role of FoxP3 turned out to be more complex in the human system, as discussed below.

Tregs are functional only when activated though their T-cell receptors (TCR) and are derived from the thymus, where they are selected based on their positive affinity for self antigens.[7] Thus, Tregs can recognize self antigens to suppress self-reactive T-cells once they migrate to the periphery. Although Tregs require antigenic stimulation for their suppressive function, they are hyporesponsive to in vitro TCR activation.[8,9] Upon stimulation, Treg cells fail to efficiently flux calcium, display impaired proliferative capacity and produce reduced levels of proinflammatory cytokines, such as IL-2 and IFNγ, when compared to effector T-cells.[7,9,10]

How Tregs exert their suppressive function is not fully characterized; however a number of mechanisms have been identified or proposed.[11] Treg cells constitutively express several surface markers, including CD25, GITR and CTLA-4.[7] However these molecules are also present on activated conventional T-cells. The discovery of FoxP3 was monumental in this regard, as it served to define Treg cells both genetically and phenotypically through protein expression.

Functional and Structural Features of FoxP3

In humans, FoxP3 maps to the Xp11.23-Xq13.3 locus.[4] FoxP3 has 11 exons, which encode a 431 amino acid protein.[12] Murine FoxP3 is 86% similar to the human protein.[12] FoxP3 shares a structural scaffold with FoxP1, FoxP2 and FoxP4. It has the greatest percent homology with FoxP1.[13,14] Similar to other members of the family, FoxP3 has a forkhead domain at the C-terminus, which is responsible for DNA binding, a leucine zipper like domain, which mediates oligomerization and a zinc finger motif with unknown function.[13,15] At the N-terminus, FoxP3 contains a proline rich region, while other FoxP proteins have a glutamate rich poly Q region.[13] The N-terminus is thought to be the repressor domain.[13] Most of the IPEX mutations map to the Forkhead domain of FoxP3. The proline rich repressor domain and the leucine zipper domain are also mutated in several IPEX patients, albeit at a lower frequency.[15] Missense mutations within the Forkhead, leucine zipper and repressor domains also cause IPEX sydnrome,[15,16] suggesting the necessity of all three domains for proper function of this transcription factor. Other Foxp3 mutations in IPEX patients include C-terminal elongation due to loss of a stop codon and a point mutation in the polyadenylation site, which affects mRNA stability.[16] The latter mutation in the polyA site is interesting because it was identified in a multigenerational family in which some affected males lived well into the first decade and one even into the third decade of life,[12] indicating an intermediate IPEX severity due to low levels of mRNA translation. Also, the deaths associated with these 'intermediate IPEX patients' occurred after infection or immunization[16] highlighting the importance of intact Treg function during an immune response. A similar phenotype is seen in 'FILIG' mice, which have attenuated expression of FoxP3. These mice display uncontrolled lymphocyte proliferation, but the disease severity is lower in FILIG mice compared to scurfy mice,[17] which completely lack FoxP3 expression due to a frameshift mutation.[18]

Forkhead Domain

The FoxP family of proteins is unique in that the Forkhead domain lies at the C-terminal end, whereas the other Fox family members have an N-terminal Forkhead domain.[16] The Forkhead domains of the 4 FoxP family members share a >90% similarity.[14] In FoxP3, this domain extends from exon 9 to exon 11.[16] The Forkhead domain contains a putative nuclear localization sequence (NLS) at the C terminal end.[16] It is also responsible for binding the DNA targets of FoxP3 and for binding Nuclear Factor of Activated T-cells (NFAT).[19,20]

Cofractionation experiments in FoxP3 transfected and activated T-cells found that FoxP3 associates with both a high molecular weight and a low molecular weight complex. The former contains chromatin remodeling factors, while the latter is associated with FoxP1 and NFAT.[13] Although the interaction with FoxP1 has not been characterized, the interaction with NFAT has been localized to the Forkhead domain.[19]

NFAT is a transcription factor activated by the calcium flux that occurs when all T-cells are activated. Together with another protein called AP-1, the NFAT complex binds to promoters of cell activation genes, such as IL-2 and CD25. The FoxP3 Forkhead domain binds to NFAT, as well as the AP-1 target DNA sequence. Thus FoxP3 effectively blocks AP-1 activity by stealing its binding partner and by occupying its position on the DNA. Using a ChIP assay, the NFAT-FoxP3 complex was shown to bind to the promoters of IL-2, CD25 and CTLA-4.[19] Interestingly, acetylation of FoxP3 in the Forkhead region was also shown to enhance FoxP3 binding to the IL-2 promoter,[21] suggesting that the Forkhead domain can undergo posttranslational modification to modulate its function.

The Forkhead domain of FoxP3 also has numerous DNA binding sites. A genome wide analysis using microarray on the nuclear fraction from mouse $CD4^+CD25^+$ cells and a CHIP assay found that FoxP3 binds at 1,276 regions throughout the mouse genome.[22] FoxP3 binding sites were substantially enriched within 10 kb of the 5′ untranslated region of genes, correlating with the position of promoter regions, as would be expected from a transcription factor. The list of FoxP3 binding targets that are up or downregulated in $FoxP3^+$ cells confirms that FoxP3 can act as both an activator and a repressor.[22] Histone H3 modifications are common at FoxP3 binding sites, indicating that chromatin remodeling occurs during FoxP3 activity. This is probably a result of the ability of the N-terminal region of FoxP3 to recruit chromatin-remodeling factors. It was also revealed that FoxP3 bound genes were mostly involved in TCR signaling, cell communication and transcriptional regulation. These profiles support the notion that FoxP3 is involved in regulating TCR mediated signals intracellularly, can promote the expression of genes with intercellular effector functions and contributes to genetic programming and cell development.[22]

Leucine Zipper Domain

Leucine zipper and zinc finger domains are both traditionally known as protein-protein interaction domains, which have the potential to bind DNA.[16] The leucine zipper is known to be indispensable for FoxP3 function based on two IPEX patient missense mutations. Although the function of the zinc finger domain of FoxP3 is not currently established, the leucine zipper is responsible for oligomer formation. FoxP3 can form homo-oligomers and can also form a heterodimer with FoxP1. In fractionation experiments, FoxP1 was found in the low molecular weight complex with FoxP3 and NFAT.[13] In addition, recombinant FoxP3 raised in either bacterial or mammalian cells, forms homotetramers. The IPEX E251 mutation of FoxP3 eluted as a monomer, indicating that compromising the oligomer formation could be disrupting protein function.[16]

Forkhead- Leucine Zipper Linker Region

The region that bridges the Forkhead and leucine Zipper domains in FoxP3 (aa 278-336) binds to the Acute Myeloid Leukeamia-1 (AML-1)/Runt Related transcription factor (RUNX-1) protein, specifically, in the C-terminal repressor domain. AML-1 binds upstream of the IL-2 gene, acting as a promoter enhancer. FoxP3 is shown to block this enhancement and FoxP3 mutations that attenuate binding to AML-1, result in increased IL-2 production. Furthermore, these mutations impair the expression of Treg phenotype markers and some Treg functions.[23]

N-Terminal Proline Rich Repressor Domain

Analysis of ChIP and microarray experiments show that FoxP3 directly binds only 6% of the genes that it regulates.[22] This could be because FoxP3 binds to the promoters of genes that in turn control other genes or because DNA binding is not the only mechanism by which FoxP3 alters gene expression. Indeed, DNA binding activity alone probably does not account for the indispensable activity of FoxP3 in regulatory T-cells, as Tregs also express FoxP1, which has 90% similarity to FoxP3 in the Forkhead domain.[14] In other studies, it was noted that the N-terminus of the protein is also important in interaction of FoxP3 with NFAT and its function.[19] Thus it is conceivable that the N-terminal domain of FoxP3 is a major distinguishing factor between the function of FoxP3 and the other members of the family.[13]

The N-terminal proline rich region has crucial function in binding to chromatin remodeling factors that are necessary for FoxP3 transcriptional activity. As mentioned above, fractionation experiments with FoxP3 overexpressing cells showed that FoxP3 associates with both a high and a low molecular weight complex in cells.[13] The high molecular weight complex is composed of chromatin remodeling factors.[13] Specifically, it was found that TIP60, a histone acetyltransferase, binds to the N-terminal proline rich region of FoxP3.[24] TIP60 acetylates FoxP3 in Tregs and a TIP60 mutant, deficient in the ability to acetylate (HAT domain mutated) cannot promote transcriptional repression. This interaction was thought to be necessary for repression of FoxP3 target genes as assessed through IL-2 production, because repression of IL-2 does not occur in TIP60 knockdown cells.[6] In addition, TIP60 recruits a histone deacetylase called HDAC7.[13] Histone deacetylases remove acetyl groups from histone tails, which in turn encourages high-affinity binding of histones to DNA. Therefore, HDAC7 could be preventing transcriptional access, consistent with a model of FoxP3 mediated repression of some target genes. Indeed, HDAC7 is also found in complex with FoxP3 during coimmunoprecipitation experiments. Mutating the N terminal proline rich region abolishes the coimmunoprecipitation of FoxP3 and HDAC7 and abolishes the transcriptional repressor function of FoxP3.[13] However, it was also shown that treating Treg cells with a broad based HDAC inhibitor increased their suppressive function.[21] This effect however, may be the result of HDAC regulation of the FoxP3 gene itself, as HDAC inhibitor treatment also resulted in increased expression of FoxP3 in the cells. In addition, FoxP3 binding to the promoters of cytokines IL-2 and IFNγ was shown to deacetylate histone H3, inhibiting chromatin remodeling and effectively blocking transcription.[25]

Multiple Isoforms and Subcellular Localization

In contrast to the murine version, human FoxP3 has two isoforms, which are called FoxP3a and FoxP3b. FoxP3a is full-length protein and FoxP3b is a splice variant lacking exon 2. Interestingly, in activated $CD4^+CD25^+$ cells, FoxP3a can be found in both the nucleus and the cytoplasm, FoxP3b is only found in the nucleus.[14] Exon 2 has a nuclear export signal (NES), thus FoxP3b is not properly exported to the cytoplasm after activation due to lack of an NES.[14] The implications of a cytoplasmic export in human cells is not clear since mouse FoxP3 appears to be only localized to the nucleus.[26]

It was also reported that expression of full length FoxP3a results in a more unresponsive T-cell phenotype as compared to the FoxP3b isoform. Human cells expressing only FoxP3b have an intermediate Treg phenotype in terms of curbed proliferative capacity and dampened cytokine secretion.[27] However, in other reports, both isoforms were shown to possess a similar capacity to induce Tregs and to suppress T-cell activation.[6,28,29] The region encoded by exon 2 is also thought to be critical for the association of Foxp3 with transcription factors retinoic acid related orphan receptor alpha RORα[30] and RORγt,[28] which are master transcription factors for development of a proinflammatory T-cell subset called Th17.

FoxP3 Regulation and Function

Role of FoxP3 in Development and Function of Tregs

It is now well-established that FoxP3 is required for development of Treg cells both in humans and mice. However, it is not fully clear whether FoxP3 expression alone is sufficient to program conventional T-cells into bona fide Tregs, especially in the human system. Ectopic expression of FoxP3 in $CD4^+CD25^-$ non-Treg cells produced a regulatory phenotype, as these cells exhibited suppressive activity in vitro and also protected the host mice from autoimmune diseases in several adoptive transfer models.[1,2,31] In humans, ectopic overexpression of FoxP3 in naïve T-cells was also shown to differentiate these cells into Treg mimics in vitro.[5,6] However in microarray experiments the gene expression profile between natural Tregs and FoxP3 ectopically expressing cells in mice were found to be different;[32] specifically, there are genes upregulated in Tregs that are not under the control of FoxP3. In experiments utilizing FoxP3 knock-out/GFP knock-in mice, it was found that some Treg characteristics and marker genes are present even in the absence of FoxP3.[33,34] Taken

together, these results suggest there may be other important factors required along with FoxP3, in the development of Treg lineage cells.

Cell Extrinsic Regulation of FoxP3

The cytokine TGFβ induces FoxP3 expression in $CD4^+CD25^-$ cells.[41] In mice, TGFβ induced FoxP3 programs cells with Treg characteristics and the ability suppress T-cell activation, these cells are sometimes referred as iTreg, or inducible Treg.[42] Peripheral, but not thymic Tregs were found to be reduced in eight to ten day old TGFβ1−/− mice and Tregs deficient in TGFβ Receptor II were also poorly maintained in the periphery, suggesting TGFβ's critical role in peripheral Treg maintenance.[43,44] Human $CD4^+CD25^-$ T-cells upregulate FoxP3 upon activation in the presence of TGFβ.[41] However, in human cells, such induction does not confer suppressive function.[37,38] It is possible that FoxP3 has a second role in human cells, in mediating hyporesponsiveness of $CD4^+CD25^-$ T-cells in vivo.[45] Recently, a molecule called GARP was shown to be specifically expressed on Tregs and can potentially be used to differentiate between $FoxP3^+$ bona fide Tregs and TGFβ-induced FoxP3 expressing cells.[46]

The downstream signaling cascade leading to FoxP3 induction is not yet clearly established; however several key players have been identified. In keeping with conventional TGFβ signaling, Smad3 has been identified as necessary for FoxP3 induction.[47] Stat5, which functions downstream of IL-2 signaling, binds the FoxP3 promoter similarly to NFAT, which is activated after TCR triggering.[48,49] These findings are consistent with the requirement of IL-2 and TCR activation for Treg function. Signaling through the Notch receptor/trancription factor pathway may also be involved in FoxP3 expression, as pharmacological inhibition of Notch1 blocks FoxP3 induction.[47] Another signaling protein important in cellular survival called Akt has been established as a repressor of novel FoxP3 induction, although it cannot reverse already established FoxP3 expression.[50] Phosphoinositide 3-kinase and downstream signaling molecule mTOR can also antagonize FoxP3 expression;[51] in fact, the mTOR inhibitor Rapamycin promotes FoxP3 expression both in vitro and in vivo and has been used therapeutically in IPEX patients.[52-54]

It was recently reported that the Vitamin A metabolite retinoic acid (RA) could promote FoxP3 expression in T-cells.[55] RA is present in the gut and produced by antigen-presenting cells such as macrophages, which have the necessary metabolic enzymes.[55,56] It is possible that RA may play a role in establishing oral tolerance to ingested food and to the vast microbiome that inhabits the human gut. In fact, dietary vitamin A has been known for over twenty years to protect against autoimmunity in mice.[57] It was also suggested that RA enhances stability of FoxP3 induced by TGFβ.[58]

Epigenetic and Posttranslational Regulation of Foxp3

As discussed in the structural section, FoxP3 is subject to posttranslational modification in its N-terminal repressor domain by TIP60. FoxP3 can also be acetylated in the Forkhead domain and optimal Treg repressor function is dependent on this acetylation, as it allows binding to the IL-2 promoter.[21] The administration of HDAC inhibitors therefore positively regulates FoxP3 activity.[21]

Evidence also exists that FoxP3 may regulate itself through positive feedback. During analysis of mice genetically modified to replace FoxP3 with GFP at the FoxP3 locus ($FoxP3^-GFP$ knock-in mice), $FoxP3^-GFP^+$ T-cells downregulated GFP over time, while the majority of the $FoxP^+/GFP^-$ cells maintained FoxP3 expression,[33,41] indicating that FoxP3 presence promotes further transcription at the FoxP3 locus. A positive feedback loop for FoxP3 expression is also supported by the findings that FoxP3 obstructs development of other helper T-cell subsets.[17] Recent research has suggested a role for epigenetic chromatin patterning in this process. Specifically, demethylation occurs near the FoxP3 promoter in naturally occurring Tregs.[59] Methylation of DNA is a mechanism to limit access to transcriptional proteins and demethylation would be predicted to relieve this restriction. Using azacytidine, a DNA methyl transferase inhibitor, FoxP3 expression was induced stably in cells that do not physiologically express it, including conventional T-cells.[60] Furthermore, demethylation at the FoxP3 locus was a faithful marker of natural Tregs and neither transiently FoxP3 expressing cells nor TGFβ induced $FoxP3^+$ cells were demethylated at this locus.[59]

The stable expression that demethylation at the FoxP3 gene locus confers may also contribute to a positive feedback mechanism in which FoxP3 promotes its own synthesis, thus maintaining abundant and sustained levels in the cell.

Recent studies have confirmed the link of chromatin remodeling to the regulation of FoxP3 and have provided mechanistic insight into cell extrinsic mechanisms in this process. An enhancer region upstream of the FoxP3 gene together with Smad3 and NFAT are required for histone acetylation at the enhancer, thus opening up the region for transcription.[61] As several Smads are involved in TGFβ signaling, this may also help to explain the TFGβ-mediated induction of FoxP3 expression.[61] The T-cell cytokine IL-4 was also found to inhibit FoxP3 induction, through transcription factor STAT6, which was shown to bind to the silencer region in the vicinity of FoxP3 and inhibit chromatin remodeling at the locus.[62] Interestingly, RA reduced STAT6 binding to the silencer region, relieving the inhibition and enhancing histone acetylation.[62] Another cytokine, IL-6, can promote methylation at the FoxP3 locus, silencing its transcription.[63] Epigenetic control of the FoxP3 locus may therefore be critical in understanding complex regulation of FoxP3 gene expression.

Role of FoxP3 in Cancer

As FoxP3$^+$ Tregs mainly function to eliminate self-reactive lymphocytes, they can be potentially detrimental to the immune response against tumors. Because most tumor-associated antigens are recognized as self, they are more likely to activate Tregs rather than effector T-cells capable of mounting an immune response. In addition, tumor cells often acquire the ability to secrete cytokines such as TGFβ, which induces FoxP3 expression in T-cells. Indeed, high levels of FoxP3$^+$ cells have been detected in the tumor environments of many cancers and strategies to eliminate them to block their tumor protective effects are in development.

Foxp3$^+$ T-cells are also actively recruited to tumor sites. In a model of human ovarian cancer, it was found that a chemokine called CCL22 is released by cells in the tumor microenvironment and specifically recruits Tregs.[64] Several groups have shown that in various tumor models in mice and man, natural Tregs are present and proliferating in the tumor tissue.[65-68] TGFβ, which is often produced by tumor cells,[70] is favored to be the inducer Treg proliferation in these tumor microenvironments.[69]

It is also known that tumors can induce expression of FoxP3 in conventional T-cells. In addition to TGFβ, indoleamine 2,3-dioxygenase (IDO) can contribute to this induction. An IDO inhibitor abolishes conversion of conventional CD4$^+$ cells to Treg in the A20 lymphoma model[71] and IDO expression by human leukemia cells correlates with the number of FoxP3$^+$ cells in the blood. Tumor resident antigen-presening cells such as plasmacytoid dendritic cells can also produce IDO.[72] Both TGFβ and IDO inhibitors are under investigation to override tumor mediated immune suppression.[69]

FoxP3 expression by non-T-cells may also have an important role in development of certain malignancies such as breast cancer. For example, mice that are heterozygous for FoxP3, have increased incidence of breast cancer development. Furthermore, human breast cancer cells that express the HER/*neu* markers of aggressive malignancy, downregulate FoxP3 in breast tissue.[73] In fact, FoxP3 was found to repress transcription of SKP2, a breast cancer oncogene.[74] Loss of FoxP3 in non-T-cells therefore may lead to more aggressive tumor growth. Thus Foxp3 expression is a double-edged sword in cancer.

FoxP3 in Infectious Diseases

Parasitic Infections

Recent observations have further demonstrated that FoxP3$^+$ Tregs may influence the immune response to many microbes. One of the first observations on the role of Tregs during infection was made with the parasitic pathogen *Leishmania major*.[75,76] When Tregs were removed from the site of infection, the animals could better discard the infection.[75] However, further studies showed that in certain strains of mice Tregs actually held the cutaneous infection in check, which otherwise would result in progressive lesions.[76] A similar picture was observed in adoptive transfer of Treg depleted

cells into SCID mice, which developed more severe infections than those that also received the Treg subset. From these studies it is clear that Tregs could play a useful role in Leishmania pathogenesis, although too much Treg response also diminishes the immunity to the pathogen resulting in chronic disease. Similarly, in Malaria, increased Tregs were detected in the peripheral blood, where *Plasmodium falciparum* resides on red blood cells. A positive correlation between FoxP3$^+$ T-cells and growth rate of the parasite was observed.[77]

Viral Infections

Several viral infections, especially those that persist, may perturb the immune response,[78,79] which can result in increased susceptibility to other infections, tumors or even autoimmunity.[80] Tregs have recently been implicated in mediating functional impairment of CD8$^+$ T-cells during persistent retroviral infection.[81] Other instances of viral infections wherein the Treg response acts to the detriment of the host are recognized. For example, in HSV infection of mice, the magnitude of both CD8 and CD4 responses against the virus were elevated two to three fold if mice were depleted of Treg cells prior to the infection.[82] In chronic hepatitis C infection, Tregs can curb liver damage.[77] Tregs are also expanded in mice persistently infected with Friend retrovirus, suggesting that they may contribute to immunosuppression in the absence of T-cell depletion in chronic viral infections.[83]

HIV Infection

Another viral infection where FoxP3$^+$ T-cells may have a critical dual role is HIV infection. The ability of HIV to establish a persistent infection is critically dependent on T-cell activation signals.[84] Indeed, a chronic state of hyperactivation is a hallmark of HIV infection.[85] Consequently, this state of chronic immune activation combined with the direct destruction of CD4$^+$ T-cells by HIV leads to a profound immunodeficiency characterized by progressive deterioration in immune function.[86] FoxP3$^+$ T-cells were found to be highly susceptible to HIV infection both in vitro[5] and in vivo.[87] It is possible that the loss of FoxP3$^+$ T-cells in turn could potentially result in hyperactivity of conventional T-cells due to the lack of regulation by Tregs, thereby creating more T-cell targets for HIV. In a mouse model reconstituted with a human immune system to study HIV pathogenesis, FoxP3$^+$ Treg cells were preferentially infected and depleted.[88] When these mice were depleted of their Tregs during acute infection, HIV infection was reduced.[88] Conversely, if Tregs are specifically activated by HIV during the earlier stages of infection, this could have a suppressive effect on the protective immune response against the virus.[89-91]

Foxp3 may also play a direct role in facilitating HIV transcription in infected T-cells. HIV gene transcription is dependant on endogenous host cell factors such as NFAT and NFκB.[92] FoxP3 was shown to enhance NFκB binding the HIV LTR, increasing HIV-transcription in these cells.[93] Abrogating FoxP3 binding to NFκB prevented this enhancement. However, other groups found that FoxP3 suppressed gene expression from the HIV LTR,[94,95] FoxP3 and FoxP3$^+$ T-cells thus play a multifaceted role during HIV infection.

Foxp3 in Transplantation Tolerance

FoxP3$^+$ Tregs are partly responsible for maintaining peripheral tolerance to self in the body and could be invoked to suppress immune responses to foreign antigens. This would be particularly important in a not fully matched organ transplantion, which can result either in rejection of the transplanted tissue or an immune response by the donor called graft-versus-host disease (GVHD). It is conceivable for example to educate donor Tregs to recognize allogeneic antigens from the transplated host and transfer these along with the transplant tissue. This would presumably suppress donor effector T-cells from attacking the host, thus preventing GVHD. Patients with chronic GVHD indeed show diminished FoxP3$^+$ T-cell numbers and low dose IL-2 therapy is currently being explored as an approach to induce FoxP3 and promote Treg survival in these patients.[96]

Alternatively, if FoxP3 expression can be induced by host cells after transplantation, this could also help to establish tolerance and complement immune-suppressive therapies. There is evidence to support that the Treg response does not need to be specific to transplant tissue and can prevent

immune activation by bystander suppression.[97] Indeed, higher levels of FoxP3 mRNA detected in the urine of renal transplant patients and higher Treg cells correlated with reduced graft rejection.[97] In this regard, the immune-suppressive drug rapamycin could have dual function both by dampening immune responses and by selectively inducing FoxP3$^+$ Tregs.[97]

FoxP3 in Autoimmune Diseases

Disruption of FoxP3 function leads to severe autoimmunity both in humans and mice, highlighting the critical importance of this transcription factor in preventing unwanted immune response against self. Here we will review some of the experimental autoimmune models in mice, where FoxP3$^+$ Tregs were shown to play a crucial role.

Multiple Sclerosis

Experimental autoimmune encephalomyelitis (EAE) is a syndrome of inflammation of the Central Nervous System (CNS), which is used as a mouse model for human multiple sclerosis (MS) disease, also caused by autoimmune response to myelin.[98] EAE is typically induced by myelin injection or by transferring myelin-reactive CD4$^+$ cells to susceptible mice. Early experiments done before the discovery of FoxP3 showed that CD4$^+$CD25$^+$ T-cells transferred from healthy mice could protect susceptible mice against EAE.[99] It was then determined that FoxP3$^+$ Treg cells were responsible for this protection in an antigen (myelin) specific or bystander fashion.[98] In humans, analysis of blood samples and spinal fluid from MS patients also shows evidence of Treg perturbation.[98]

Inflammatory Bowel Diseases

The murine colitis model is used to gain insight into ways to control human autoimmune diseases of the intestine, such as ulcerative colitis and Crohn's disease. In this model, immune deficient mice are populated with naïve CD4$^+$ T-cells, which causes severe intestinal inflammation. Mice that receive CD4$^+$FoxP3$^+$ T-cells are cured of the disease within weeks and it was shown that Treg cells migrated to the colon, which is the site of inflammation.[98]

Type I Diabetes

Type I diabetes, or diabetes mellitus, is an autoimmune syndrome in which the insulin producing beta cells in the pancreas are attacked by the immune system. Neonatal diabetes mellitus is characteristic of IPEX patients with FoxP3 mutations. A broad study with Type 1 diabetes patients showed that (GT)n microsatellite polymorphisms in the FoxP3 gene were also associated with the disease.[100] Another study correlated a lower FoxP3 mRNA level with Type I diabetes patients.[101] In a mouse model of Type I diabetes called nonobese diabetic (NOD), FoxP3$^+$ T-cells decreased as the disease progressed.[102] The main culprit in this mouse model appears to be increased beta cell specific effector T-cells that are also resistant to suppression by FoxP3$^+$ T-cells;[103,104] there is no defect in the generation or maintenance of Tregs, indicating that FoxP3 function is intact.[105,106] However, when beta cell specific Tregs from diabetic mice were expanded in vitro and transferred back to diseased mice, the diabetes regressed.[107] In alternative experiments T-cells specific to pancreatic beta cells were genetically manipulated to express FoxP3, which also caused regression of disease when transferred to diabetic mice.[107]

Emerging and Potential Therapeutic Intervention

Foxp3$^+$Tregs or Foxp3-programmed T-cells have a vast array of functions and roles in human diseases (Table 1). Thus, Foxp3 is potentially a significant target for therapeutic approaches against these diseases. On the one hand enhancing FoxP3$^+$ Tregs could be useful in the treatment of autoimmune syndromes, inflammatory disorders, transplantation and complications from chronic infections. On the other hand attenuating the FoxP3$^+$ Treg responses would be beneficial in enhancing antitumor immunity, responses to acute infections and boosting the potency of vaccines.

Although the prospect of targeting a transcription factor is generally avoided because of the widespread and often unforeseen activities of transcriptional regulators, FoxP3 has been shown to be relatively specific to the immune system and associated primarily with immune activation. Several

Table 1. Function and role of Foxp3 in diseases

Disease	Role of Foxp3	References
IPEX syndrome	Natural genetic mutations in Foxp3 causes autoimmune syndrome in humans	3,4
Ovarian cancer	Foxp3$^+$ Tregs recruited to the site of tumor	64
Breast cancer	Breast cancer cells that express the HER/*neu* markers of aggressive malignancy, downregulate FoxP3 in breast tissue	73
Breast cancer	FoxP3 represses transcription of SKP2, a breast cancer oncogene	74
Malaria	A positive correlation between FoxP3$^+$ T-cells and the growth rate of *plasmodium falciparum* was observed	77
HSV infection	The magnitude of immune responses against the virus elevated if mice were depleted of Foxp3$^+$ T-cells prior to the infection	82
Hepatitis C infection	Foxp3 expressing Tregs can curb liver damage	77
HIV infection	FoxP3$^+$ T-cells were found to be highly susceptible to HIV infection	87,88
HIV infection	FoxP3 shown to enhance NFκB binding the HIV LTR, increasing HIV-transcription in infected cells	93
GVHD	In chronic GVHD FoxP3$^+$ T-cell numbers are reduced, they could potentially be protective against the disease	96
Renal transplant rejection	Higher levels of FoxP3 mRNA detected in the urine of renal transplant patients correlated with reduced graft rejection	97
Multiple sclerosis	FoxP3$^+$ Treg cells are responsible for protection of mice against EAE, the model of multiple sclerosis in mice	98
Type I diabetes	Polymorphism in the FoxP3 gene is associated with the disease, lower FoxP3 mRNA level in Type I diabetes patients	100,101

questions remain to be answered in order to manipulate FoxP3 or FoxP3 expressing cells during human diseases. First, how can we induce FoxP3 in specific cell types? It is possible that the signaling pathways used by TGFβ to induce FoxP3 can be exploited to develop pharmacological agonists to induce FoxP3 expression. Conversely, in conditions such as cancer or acute infectious diseases it may be desirable to dampen FoxP3 expression to amplify the immune response.

Second, how can we generate antigen-specific FoxP3$^+$ Tregs and direct them to the sites of inflammation? It may be possible to identify certain epitopes of antigens that preferentially stimulate Tregs versus effector T-cells. Reverse approaches to exclude these epitopes in vaccines would boost immune response to antigens. Migration of T-cells to tissues is largely dependent on their chemokine receptor expression profiles. Increased knowledge in this field has revealed various biological agents such as cytokines that can program cells to express given chemokine receptors and target them to sites of infection or inflammation. Future approaches to genetically manipulate T-cells to ectopically express FoxP3, forced expression of TCRs specific to antigens of interest or specific chemokine receptors on bona fide Treg could also be powerful cellular treatment options in controlling chronic immune activation or inflammation.

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Section II

Elucidating the Components Responsible for Vascular Development and Disease in the Cardiovascular System

CHAPTER 5

The Cooperative Roles of Foxc1 and Foxc2 in Cardiovascular Development

Tsutomu Kume*

Abstract

Foxc1 and Foxc2 are closely related members of the Forkhead/Fox transcription factor family. The two *Foxc* genes have overlapping expression patterns in mesodermal and neural crest derivatives during development, as well as similar functions of gene regulation. Consistently, mouse mutants for each gene have similar abnormalities in multiple embryonic tissues, including the eye, kidney and cardiovascular system. Analysis of compound *Foxc1; Foxc2* mutant embryos reveals that the two *Foxc* genes have dose-dependent, cooperative roles in development. In particular, recent studies demonstrate that Foxc1 and Foxc2 are essential for arterial cell specification, lymphatic vessel formation, angiogenesis and cardiac outflow tract development. This chapter will summarize and discuss current knowledge about the function of Foxc1 and Foxc2 in cardiovascular development.

Introduction

The cardiovascular system is the first functional unit to form in the developing vertebrate embryo. The generation of the vasculature and heart requires a complicated series of morphogenetic interactions involving cells of several embryonic origins. Indeed, congenital cardiovascular defects represent the most common group of human birth defects, but the molecular mechanisms underlying the different anomalies still remain largely unknown. In particular, many studies must be completed to fully understand gene regulation associated with critical signaling pathways during cardiovascular development. This chapter will discuss recent findings concerning the cooperative and overlapping roles of Foxc1 and Foxc2 transcription factors in this complex developmental process.

FoxC1 and FoxC2 Proteins

Murine *Foxc1* (formerly *Mf1)* and *Foxc2* (formerly *Mfh1)* encode proteins with virtually identical DNA binding domains (97% identity; 99% similarity), while the N- and C- terminal flanking regions are somewhat diverse (56% and 30% homology, respectively). A duplication of the ancestral *FoxC* gene is likely to have taken place in deuterostomes,[1] as vertebrate species including frog, chicken, mouse and human, possess the two *FoxC* genes. Human *FOXC1* and *FOXC2* genes are located on chromosomes 6 (6p25) and 16 (16q22-q24), respectively, while mouse *Foxc1* and

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Foxc2 genes are located on chromosomes 13 and 8 in regions of conserved synteny between human and mouse, respectively (Mouse Genome Informatics; http://www.informatics.jax.org). In addition to in vitro DNA binding assays to determine the consensus DNA binding sequence for FOXC1,[2] NMR structural analysis suggests that the two FOXC proteins act as monomers and have the same binding specificity to target sequences.[3] Therefore, it is likely that the two proteins regulate the same downstream target(s) where they are co-expressed in the same cells.

Overlapping Expression of *Foxc1* and *Foxc2* during Development

While mouse *Foxc1* and *Foxc2* are not transcribed in the axial-mesoderm and its derivatives such as the notochord, they show largely overlapping domains of expression in many embryonic tissues that are derived from the nonaxial mesoderm, including the cardiovascular system.[4-13] For instance, expression of *Foxc1* and *Foxc2* is detected in endothelial and mesenchymal cells of the developing heart and blood vessels, second heart field (SHF) progenitors and their derivatives, as well as the proepicardium. Foxc1 and Foxc2 are expressed in arterial and venous endothelial cells of the developing blood vessels.[10] In addition, *Foxc1* and *Foxc2* are co-expressed in neural crest derivatives, including cells populated in the pharyngeal arches and the endocardial cushions of the cardiac outflow tract.[13,14] There are some regions in which expression domains of *Foxc1* and *Foxc2* do not overlap during development. Although transcripts of both *Foxc* genes are detected in neural crest-derived periocular mesenchyme surrounding the developing eye,[15,16] *Foxc1*, but not *Foxc2*, is expressed in mesoderm-derived periocular mesenchyme.[15] The onset of expression of the *Xenopus* homologue of *Foxc2* before gastrulation is earlier than that of the *Xenopus* homologue of *Foxc1*.[17]

Developmental Defects in *Foxc* Mutant Mouse Embryos

While this chapter focuses primarily on the functions of Foxc1 and Foxc2 in cardiovascular development, it should be noted that single mouse mutants for each gene similarly exhibit numerous developmental abnormalities in accordance with their broad expression patterns. A comprehensive summary of the phenotypes of single *Foxc* mutant mice, as well as compound *Foxc1; Foxc2* mutant mice, is given in Table 1.

Mice that are homozygous for either a spontaneous mutation in *Foxc1* (*congenital hydrocephalus*, $Foxc1^{ch}$) or an engineered null mutation ($Foxc1^{lacZ}$) die prenatally or perinatally with identical phenotypes.[7,18,19] These phenotypes include hemorrhagic hydrocephalus and multiple skeletal, ocular, genitourinary and cardiovascular defects, including the interruption or coarctation of the aortic arch, ventricular septal defects (VSD) and aortic and pulmonary valve dysplasia.[6-8,16,20-22] *Foxc1* mutant mice also lack the frontal bones of the skull vault, derivatives of cranial neural crest cells.[23] Endothelial-specific *Foxc1* mutant mice have recently been generated and survive into adulthood, but these conditional *Foxc1* mutant mice have defects in the postnatal microvasculature (Table 1).[24,25] In addition, a forward genetic screen using ethylnitrosurea (ENU) mutagenesis has recently identified a hypomorphic mouse allele for *Foxc1* (*hole-in-the-head*, $Foxc1^{hith}$) that survives into adulthood.[26] A missense mutation in the $Foxc1^{hith}$ allele results in a Phe-to-Leu substitution at amino acid 107 within the second helix of the DNA binding domain, leading to destabilization of the protein. Analysis of $Foxc1^{hith}$ has revealed that Foxc1 plays a role in meaningeal differentiation, thereby regulating cortical development.

Foxc2 null mutants also die pre or perinatally with skeletal, genitourinary and cardiovascular defects similar to those seen in *Foxc1* homozygous mutants.[5,6,8,12,13,27] It is of interest to note that Foxc2 has been implicated in lymphatic vessel development. Heterozygous *Foxc2* mutant mice have hyperplasia of lymphatic vessels,[28] while homozygous *Foxc2* mutant mice show defective lymphatic valves and abnormal pericyte recruitment of lymphatic vessels.[29] These abnormalities underlie congenital defects caused by *FOXC2* mutations in humans (see below). Additionally, endothelial cells isolated from heterozygous *Foxc2* mutant mice exhibit impaired formation of microvessels.[30] Together, these findings demonstrate that Foxc1 and Foxc2 are required for mammalian embryonic development, including the cardiovascular system.

Table 1. Developmental defects found in Foxc mouse mutants

Mutant	Phenotype	References
Foxc1$^{+/-}$ (*Foxc1*$^{ch/+}$)	Delayed calvarial formation; Anterior segment abnormalities in the eye	19,21,22
Foxc1$^{-/-}$ (*Foxc1*$^{ch/ch}$)	Hydrocephalus; Malformations in the cranial and axial skeleton; Duplex kidneys and double ureters; Impaired gonad development; Anterior segment abnormalities in the eye	6,7,16,18, 19,20,21,76
Foxc1$^{hith/hith}$	Hydrocephalus; Incomplete skull closure; Cortical dysplasia; Microphthalmia	26
Conditional *Foxc1* (Endothelial specific)	Reduced migration of endothelial cells; Reduced expression of *CXCR4* and *Hey2*	24,25
Foxc2$^{+/-}$	Hyperplastic lymphatic vessels; Extra eyelashes; Anterior segment abnormalities in the eye; Impaired functions of microvessels	22,28,30
Foxc2$^{-/-}$	Malformations in the cranial and axial skeleton; Hypoplastic kidneys; Abnormal aortic arch patterning; VSD; Increased pericyte investment and agenesis of valves of lymphatic vessels; Abnormal glomerular development	5,6,12,27, 29,76
Foxc1$^{+/-}$; *Foxc2*$^{+/-}$	Hypoplastic kidneys and a single hydroureter; Abnormal aortic arch patterning; VSD; Anterior segment abnormalities in the eye	6,13,22
Foxc1$^{-/-}$; *Foxc2*$^{+/-}$	Die at around E12.5; Etiology of lethality is not determined	8,10,11,37
Foxc1$^{+/-}$; *Foxc2*$^{-/-}$	Small somites; expansion of intermediate mesoderm; Impaired remodeling of blood vessels; Hypoplastic OFT; Apoptotic neural crest; Abnormal epicardium	8,10,11,37
Foxc1$^{-/-}$; *Foxc2*$^{-/-}$	No somites formed; Expansion of intermediate mesoderm; Impaired remodeling of blood vessels; Disrupted arterial cell specification; Absence of the OFT; Apoptotic neural crest	8,10,11,37

Mutations in *FOXC1* and *FOXC2* Genes Associated with Developmental Disorders in Humans

Consistent with the importance of *Foxc* genes in murine development, mutations of human *FOXC* genes have been found in individuals with congenital anomalies. Mutations of the human *FOXC1* are associated with the dominantly inherited Axenfeld-Rieger anomaly (ARA), characterized by anterior chamber dysgenesis in the eye and congenital glaucoma [Online Mendelian Inheritance in Man (OMIM) no. 601090].[31,32] *Foxc1*+/– mice exhibit ocular abnormalities similar to those seen in human ARA patients, but these *Foxc1* heterozygous mice do not show increased intraocular pressure, the most important risk factor for glaucoma.[22] Some humans heterozygous for mutations in *FOXC1* have congenital heart defects such as mitral valve dysplasia and atrial septal defects.[13,31,33]

Mutations in human *FOXC2* are responsible for the autosomal dominant syndrome, Lymphedema-distichiasis (LD), characterized by the obstruction of lymphatic drainage of the limbs and the growth of an extra set of eyelashes (OMIM no. 602402).[34] In addition to lymphatic valve failure, mutations of human *FOXC2* are also associated with venous valve failure,[35] while

~15% of these humans exhibit conotruncal cardiac defects.[34] Although *Foxc2*+/– mice have similar ocular abnormalities as those seen in *Foxc1*+/– mice,[22] LD patients with *FOXC2* mutations present mild anterior chamber abnormalities unassociated with glaucoma.[36] Mutations of human *FOXC1* or *FOXC2* are likely to lead to haploinsufficiency and no individuals that are homozygous for a mutation of either *FOXC1* or *FOXC2* have been found. Elucidation of overlapping functions of the two *FoxC* genes required genetic analysis of compound *Foxc1; Foxc2* mutant mice.

Cooperative Roles of Foxc1 and Foxc2 in Cardiovascular Development

To determine functional interactions between Foxc1 and Foxc2, the generation of compound *Foxc1; Foxc2* mutant mice was performed. It is remarkable that the majority of compound *Foxc1; Foxc2* heterozygous mice from crosses of *Foxc1*+/– and *Foxc2*+/– mice die pre or perinatally, whereas only a small percent of the compound heterozygotes survive into adulthood. Most compound heterozygotes show a similar spectrum of cardiovascular, genitourinary and eye abnormalities like those seen in each single homozygous null mutant.[6,13,22] These include interruption/coarctation of the aortic arch, VSD, dysplasia of the aortic and pulmonary valves and an abnormally thin myocardium. Thus, these findings demonstrate that the two *Foxc* genes functionally overlap and cooperate with each other in vivo and that they play dose-dependent roles in many aspects of embryonic development.

From crossing fertile compound *Foxc1; Foxc2* heterozygotes, compound homozygous and hetero/homozygous embryos have been obtained and analyzed.[8,10,11,37] While compound *Foxc1*+/–; *Foxc2*–/– and *Foxc1*–/–; *Foxc2*+/– mutants die at embryonic day 12.0-12.5 (E12.0-12.5), compound homozygous embryos die around E9.5 with a phenotype that is much more severe than that of a single homozygote, a compound heterozygote, or a compound hetero/homozygous mutant (Table 1). As described below, compound homozygous embryos show disrupted arterial specification.[10] In addition, compound *Foxc1*+/–; *Foxc2*–/– mutants have a reduction in the number of Prox1-positive lymphatic endothelial cells sprouting from the cardinal vein.[10] Compound *Foxc1; Foxc2* mutants also have a wide spectrum of early cardiac abnormalities in a dose-dependent manner.[11] These observations further reinforce the idea of gene-dosage effects of the two *Foxc* genes during cardiovascular development. Of interest, zebrafish has only one *foxC* gene, *foxC1*, although, due to genome duplication, there are two zebrafish homologues, *foxC1.1* and *foxC1.2*.[38] Given that *foxC2* appears to be absent from the zebrafish genome,[39,40] knockdown of *foxC1.1*, but not *FoxC1.2*, in zebrafish results in the lack of segmented somites,[41] a phenotype similar to that seen in compound *Foxc1; Foxc2* homozygous mouse mutants (Table 1).[8] It is therefore likely that the cooperative roles of the *FoxC* genes in development are conserved in vertebrates.

Foxc Function in Arterial Specification

During vascular development, angioblasts, which are multipotent endothelial progenitors originating from the mesoderm, coalesce and undergo vasculogenesis to form the primitive capillary plexus. Angiogenesis, the subsequent process of vascular remodeling, which gives rise to a mature network of blood vessels including arteries and veins, is regulated in part by hemodynamic forces. However, recent studies in zebrafish and mice clearly demonstrate that in the developing embryo, arterial and venous identity is established by genetic mechanisms before circulation begins.[42,43] For arterial specification (Fig. 1), vascular endothelial growth factor (VEGF) induces expression of Notch signaling genes, including *Notch1* and its ligand, *Delta-like 4* (*Dll4*) and also triggers a positive-feedback loop by inducing expression of *Neuropilin 1* (*Nrp1*), an arterial-specific coreceptor for VEGF. Upon activation of Notch signaling, the Notch effector genes, *Hey1/2* in mice or *gridlock* in zebrafish, further promote arterial differentiation. In contrast, the orphan nuclear receptor, COUP-TFII, is a determinant factor for venous specification by inhibiting expression of arterial specific genes, including *Nrp1* and *Notch/Dll4* (Fig. 1).[44]

Compound *Foxc1; Foxc2* homozygous mouse mutants show defective vascular remodeling of primitive blood vessels and abnormal vascular connections between arteries and veins (so called arteriovenous malformations).[8,10] Arteriovenous malformations similarly develop in endothelial

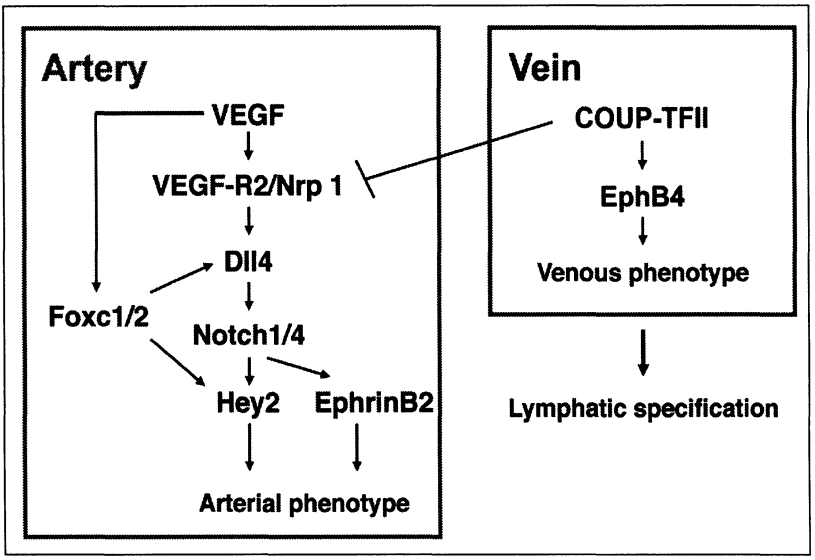

Figure 1. Genetic program of arterial-venous specification during vascular development. The VEGF and Notch pathways control the specification of arterial endothelial cells, while COUP-TF-II regulates venous cell fate. Foxc1 and Foxc2 interact with VEGF and Notch signaling and thereby induce arterial-specific genes, Dll4 and Hey2. Bi-directional Ephrin B2 and EphB4 signaling is induced arterial and venous endothelial cells, respectively and is involved in interactions between arteries and veins.

cells of mutant mice and zebrafish in which Notch signaling is defective.[45-49] Endothelial cells of compound *Foxc1; Foxc2* homozygous mutants fail to express arterial-specific genes such as *Nrp1* as well as Notch signaling molecules including *Notch1*, *Dll4* and *Hey2*, whereas venous markers such as COUP-TFII and EphB4 are normally expressed in compound homozygotes.[10] Most significantly, Foxc1 and Foxc2 can directly activate the *Dll4* promoter via a Foxc-binding element (FBE). Together, Foxc1 and Foxc2 act upstream of Notch signaling in arterial cell specification (Fig. 1).[10] This observation is consistent with the role of *Foxc* genes in regulation of Notch signaling events during the formation of the somites.[8]

In addition to *Dll4*, a recent study has demonstrated that Foxc1 and Foxc2 directly regulate expression of the Notch target gene, *Hey2* (also called *HRT2, HERP1, CHF1* and *Hesr–2*), by activating its promoter in endothelial cells.[25] Consistently, *Foxc*-mutant endothelial cells isolated from adult lungs of either endothelial-specific *Foxc1* mutant mice or *Foxc2*+/– mice show reduced expression of *Hey2*. The *Hey2* promoter includes two FBEs that are adjacent to a binding site for Suppressor of Hairless [Su (H)]. Upon activation of Notch signaling leading to a proteolytic cleavage to release the Notch intracellular domain (NICD) into the cytoplasm, Su(H) interacts with translocated NICD in the nucleus and is critical for Notch-mediated *Hey2* induction. When Foxc2 is combined with NICD, the *Hey2* promoter is synergistically activated as compared to either Foxc2 or NICD. In contrast, Foxc1 shows no synergistic effects on NICD-induced promoter activity. These data, together with the fact that Foxc2, but not Foxc1, directly binds to Su(H) and forms a protein complex with Su(H) and NICD, suggest that Foxc2 functionally interacts with Notch signaling to induce *Hey2* expression in endothelial cells.[25]

Foxc-induced promoter activity of *Dll4* and *Hey2* is significantly enhanced by VEGF in endothelial cells (Fig. 1).[25] In in vitro mammalian cell studies, the VEGF-mediated phosphoinositide 3-kinase (PI3K) pathway induces the transcription of *Notch1, Dll4* and *Hey2*.[25,50] Interestingly, modulation of Foxc activity by VEGF is enhanced by the PI3K pathway or inhibited by the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway. This

suggests that Foxc1 and Foxc2 interact with VEGF signaling in arterial gene expression. However, in the zebrafish embryo, the VEGF-activated PI3K pathway inhibits the stimulation of the ERK signaling cascade, leading to suppression of arterial differentiation.[51] Although reasons for the discrepancy between the in vitro and in vivo results remain unclear, one possible explanation is that these in vitro experiments were not conducted in uncommitted endothelial progenitor cells.[25,50] Since Foxc1 and Foxc2 are expressed in both arteries and veins in the mouse embryo,[10] it is possible that VEGF-mediated posttranslational modifications, such as phosphorylation, are critical for the activation of Foxc proteins in the induction of arterial-specific genes. Another interesting aspect of Foxc function in VEGF signaling is enhanced expression of *VEGF* in compound *Foxc1; Foxc2* mutants compared with the wild-type,[10] suggesting upregulation of a feedback response to impaired VEGF signaling.

There is now compelling evidence that arterial-venous cell fate determination is regulated by the multi-step regulatory system associated with the VEGF and Notch pathways.[42] A critical step is the induction of *Neuropilin 1* (*Nrp1*), coreceptor for VEGF and VEGF signaling promotes arterial differentiation as a positive feedback loop.[52] *Nrp1* expression is regulated by Foxc2 in endothelial cells.[25] Since COUP-TFII suppresses an arterial cell fate by inhibiting expression of Nrp1,[44] it will be important to determine whether Foxc proteins functionally counteract with COUP-TFII in the positive feedback loop of VEGF signaling during arterial-venous specification. These observations suggest that Foxc transcriptional factors control multiple steps of the VEGF-Notch/Dll4-Hey2 molecular cascade, thereby reinforcing arterial cell determination.

Foxc Function in Lymphatic Vessel Development

After arterial and venous endothelial cells differentiate, a subpopulation of venous endothelial cells is thought to become competent to acquire a lymphatic cell fate by progressively expressing the transcription factors Sox18 and Prox1 to differentiate into lymphatic endothelial cells.[53,54] The mammalian lymphatic vascular system originates solely from the venous endothelial cells.[55] VEGF-C, a VEGF receptor 3 (VEGFR-3) ligand, is expressed mainly in mesenchymal cells surrounding embryonic veins.[56] Prox1/VEGFR-3-positive lymphatic endothelial progenitors subsequently sprout from the veins via paracrine VEGF-C/VEGF-R3 signaling, leading to the formation of the lymphatic network, a process called (developmental) lymphangiogenesis.

Compound *Foxc1+/–; Foxc2–/–* mutant embryos show a significant reduction in the number of Prox1+ lymphatic endothelial cells sprouting from the cardinal vein (Fig. 2).[10] Importantly, $Sox18^{RaOP}$ mutants have similar defects in lymphatic vessel formation and Sox18 can induce *Prox1* expression in the cardinal vein.[54] These data indicate that Sox18 directly acts upstream of Prox1 in the specification of lymphatic cell fate. Although *Foxc* genes and *Sox18* are co-expressed in lymphatic endothelial progenitors in the cardinal veins, the nature of functional interactions between Foxc proteins and Sox18 in lymphatic specification remains to be elucidated. On the other hand, expression domains of *Foxc1* and *Foxc2* overlap with those of *VEGF–C* in the mesenchyme surrounding the cardinal vein. Since compound *Foxc1+/–; Foxc2–/–* mutant embryos exhibit significant reduction in *VEGF-C* expression,[10] it is possible that Foxc1 and Foxc2 regulate the paracrine signal of VEGF-C in lymphatic vasculature development. This idea is supported by the finding that Foxc-dependent regulation of potent angiogenic factors, including Angiopoietin-2, in adipocytes influences vascular formation in a paracrine manner.[57]

Foxc Function in Angiogenesis

Angiogenesis is a critical process to grow new blood vessels from pre-existing vessels and involves endothelial cell proliferation, sprouting, migration and vascular tube formation. Angiogenesis is a necessary process in development, while pathological angiogenesis is involved in cancer and other ischemic diseases. Although angiogenic factors such as VEGF are known to control various processes of angiogenesis, the mechanistic basis for the regulation of endothelial gene expression is largely unknown. Recent studies have demonstrated that Foxc1 and Foxc2 control the process of angiogenesis by directly regulating the expression of two cell surface proteins in endothelial

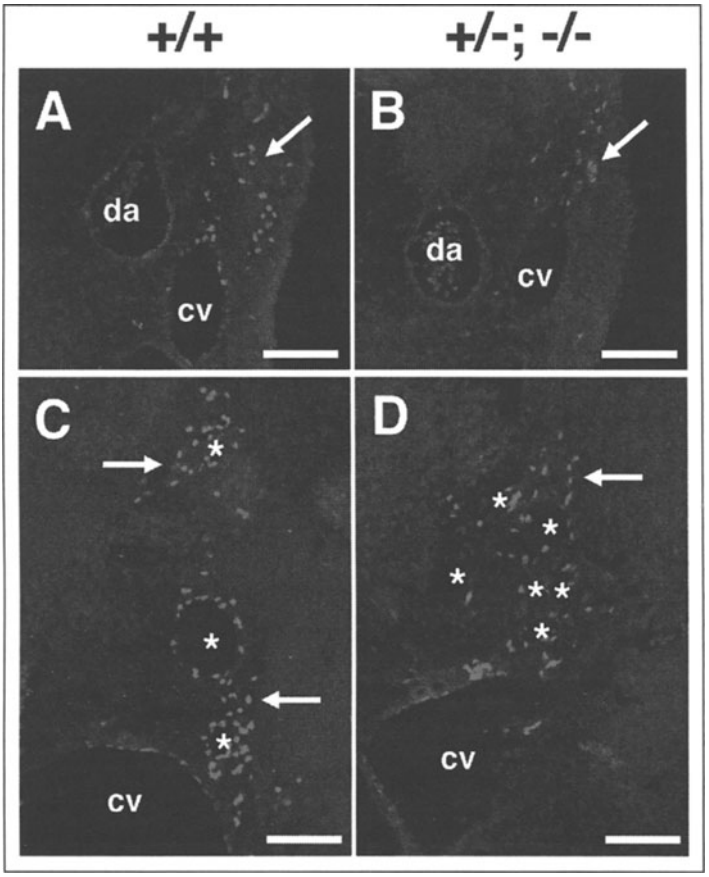

Figure 2. Compound *Foxc1+/-; Foxc2-/-* mutants have defective lymphatic vessel development. A-D) Immunohistochemical analysis to detect a lymphatic endothelial cell marker, Prox1, using transverse sections at the level of the heart at E10.5 (A,B) and E11.5 (C,D). A,B) Compound *Foxc1+/-; Foxc2-/-* mutant embryo (B) shows a reduction in the number of Prox1-positive lymphatic endothelial cells (arrows) from the cardinal vein (cv), compared to the wild type (A). C,D) At E11.5, the wild-type embryo (C) has well-formed lymph sacs (asterisks) and the sprouting of lymphatic endothelial cells (arrows). By contrast, abnormal formation of the lymph sacs and the reduced sprouting of lymphatic endothelial cells are observed in compound *Foxc1+/-; Foxc2-/-* mutant D. da, dorsal aorta. Scale bars, 50 μm. Adapted from Seo et al,[10] ©2006 with permission from Elsevier.

cells, the chemokine receptor CXCR4 and integrin β3,[24,30] which are essential for endothelial cell migration.[58,59] Upon binding of the CXCL12 ligand, CXCR4 activates downstream components to induce cell migration. The integrin β3 subunit forms a heterodimeric complex with the integrin αv subunit to allow interaction with extracellular matrix components. The integrin β3 subunit also functionally interacts with VEGF receptor 2 (VEGFR-2) in endothelial cells. Although Foxc2 does not enhance endothelial proliferation, Foxc2 increases endothelial cell migration, as well as sprouting and microvessel formation in aortic ring assay (Fig. 3).[30] In contrast, microvascular endothelial cells isolated from either endothelial-specific *Foxc1* mutant mice or *Foxc2*+/− mice show reduced cell migration.[24,30] These results indicate that Foxc transcription factors directly regulate angiogenesis via induction of integrin β3 and CXCR4.[60] Consistent with these findings,

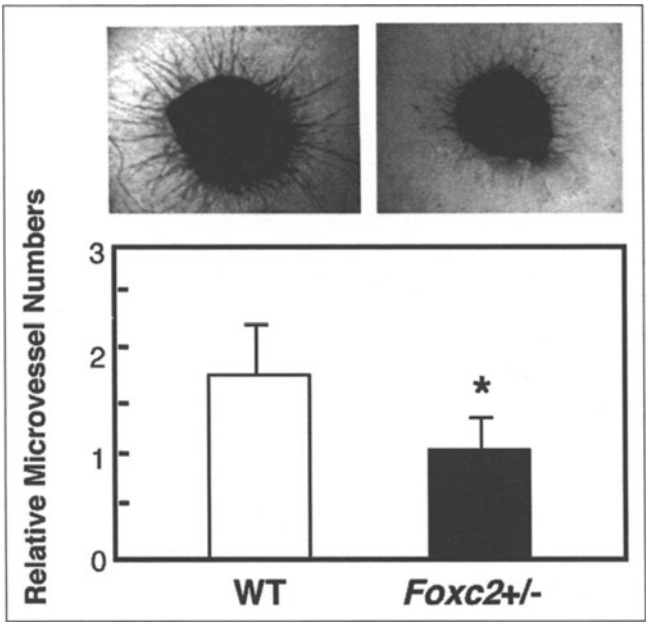

Figure 3. Foxc2 regulates angiogenesis. Aortic ring assay using adult aortas of wild-type and *Foxc2*+/– mice. Data are presented as the relative number of microvessels sprouting from aortic rings. Results are presented as the means ± S.D. (n = 9 or more). P values were determined by the corresponding sample indicated using Student's t test. *, $P < 0.05$ versus the corresponding control. Adapted from Hayashi et al,[30] ©2008 with permission from The American Society for Biological Chemistry.

Foxc2 has also been shown to enhance the migration of Maden-Darby canine kidney (MDCK) epithelial cells by upregulating matrix metalloproteinase (MMP)-2, 9.[61]

The Notch-Dll4 pathway is also critical for angiogenesis.[62] VEGF induces vascular sprouting through the filopodia of endothelial tip cells at the beginning of angiogenesis. On the other hand, Dll4 is induced in the tip cells by VEGF and precisely controls vessel branching. However, several important questions about this process need to be answered. For instance, it is currently unknown whether endothelial tip cells are positive for integrin $\alpha v\beta 3$ during vascular sprouting. Similarly, it remains to be elucidated whether the expression and activity of Foxc1 and Foxc2 are localized in the tip cells during angiogenesis.

Foxc Function in the Second Heart Field

The population of cardiac progenitors, derived from the anterior lateral mesoderm and located symmetrically in the cardiac crescent, is known as the first heart field (FHF) and gives rise to the left ventricle of the mammalian heart. The second heart field (SHF), which is originally located dorsal and medial to the FHF in the mesoderm, gives rise to cells that form the right ventricle, the outflow tract (OFT) and portions of the inflow tract.[63] The recent identification of genes involved in SHF progenitors, including *Islet 1* (*Isl1*), *Fox* genes (*Foxa2*, *Foxc1/c2* and *Foxh1*) and *Tbx1*, has provided the genetic and molecular basis for transcriptional regulation during the formation and development of the FHF and SHF.[63-66]

Although the FHF and SHF lineages derive from a common progenitor probably before the cardiac crescent stage (at the onset of gastrulation),[67] the two lineages diverge with distinct gene expression patterns. Whereas *Nkx2.5* and *Mef2c* are expressed in both lineages at the cardiac crescent stage, other genes appear to be restricted to either lineage. For instance, *Isl1* expression is

primarily detected in the SHF at the cardiac crescent stage and is downregulated as SHF progenitors migrate into the heart tube, although recent studies show that Isl1 is detected in both FHF and SHF regions at the cardiac crescent stage.[68] Together with the GATA4 transcription factor, Isl1 regulates the SHF-specific enhancer of *Mef2c,*[69] whereas activity of another SHF-specific enhancer of *Mef2c* requires the combination of Nkx2.5, Foxh1 and Smad.[70] Like *Mef2c* expression in the SHF, Isl1 can also cooperate with GATA4 as well as Tbx20 to activate the SHF enhancer of *Nkx2.5.*[71] *Isl1* mutant embryos lack the OFT and right ventricle and Isl1 is required for the proliferation and survival of SHF cells as well as transcriptional regulation of other SHF genes and signaling molecules such as bone morphogenetic proteins (BMP) and fibroblast growth factors (FGF).[72] Therefore, Isl1 is a key early regulator for the molecular hierarchy in SHF progenitors. Another key molecule is *Tbx1,* which is lost in 22q11.2 deletion syndrome in humans and is required for the alignment and separation of the OFT.[73] Of note, Fox transcription factors (Foxa2, Foxc1 and Foxc2) have been shown to activate an enhancer sufficient to drive *Tbx1* expression in the SHF,[74] while Tbx1, in turn, regulates an enhancer of *Fgf8* in the pharyngeal mesoderm.[75]

Compound *Foxc1; Foxc2* mutant embryos have a wide spectrum of early cardiac abnormalities. These include hypoplasia or lack of the OFT, right ventricle and the inflow tract as well as abnormal formation of the epicardium in a dose dependent manner (Fig. 4).[11] In SHP progenitors and their derivatives in compound *Foxc1; Foxc2* mutants, expression of *Tbx1* and *Fgf8/10* is significantly

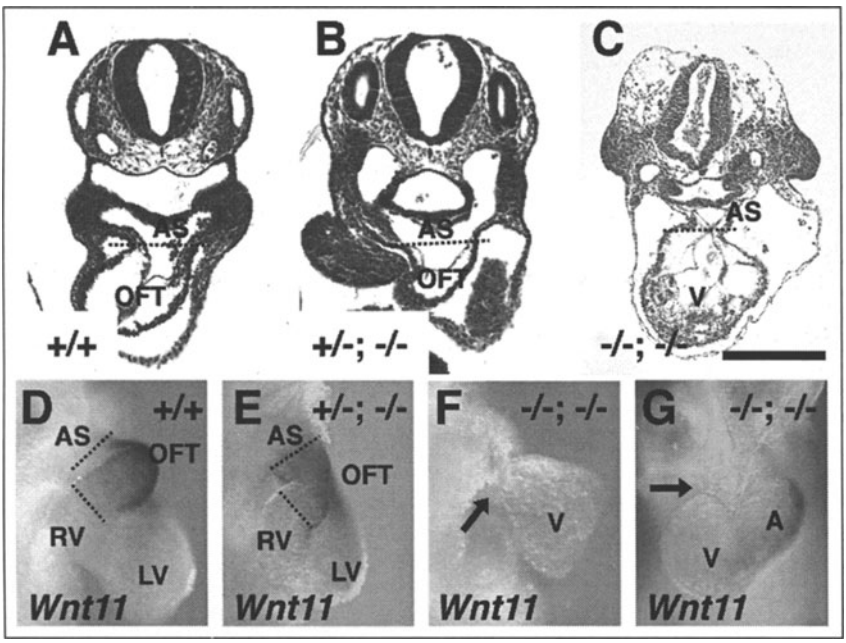

Figure 4. Compound *Foxc1; Foxc2* mutant embryos have cardiac abnormalities. A-C) Histological analysis of wild-type (A) and compound *Foxc1+/–; Foxc2–/–* (B) and *Foxc1–/–; Foxc2–/–* mutant (C) embryos at E9.0 at the levels of the heart. Compound *Foxc1; Foxc2* mutant embryos show hypoplasia (B) or lack (C) of the OFT in a dose-dependent manner. Note the direct connection of the disorganized aortic sac (AS) to the ventricle (V) in compound *Foxc1–/–; Foxc2–/–* mutant (C). Dotted lines indicate the boundary between the aortic sac (AS) and outflow tract (OFT). D-G) Whole-mount in situ hybridization at E9.0 to detect *Wnt11* expression in the OFT. The expression domain of *Wnt11* is reduced in compound *Foxc1+/–; Foxc2–/–* mutant (E, right view), while it is not detected in compound *Foxc1–/–; Foxc2–/–* mutant (arrows) (F, right view; G, front view). Dotted lines demarcate the expression domain of *Wnt11* in the OFT. A, atrium; LV, left ventricle; RV, right ventricle; V, ventricle. Scale bar, 100 μm. Adapted from Seo and Kume,[11] ©2006 with permission from Elsevier.

downregulated, whereas *Isl1* expression is slightly reduced but still remains. This observation suggests that Foxc1 and Foxc2 function upstream of the Tbx1-FGF cascade during the morphogenesis of the OFT. Since it is unknown whether Foxc proteins and Isl1 functionally interact with each other in SHP progenitors, it remains to be determined whether a reduction in cell proliferation in the SHF of compound *Foxc1; Foxc2* mutants is due to a failure of the expansion of the Isl1-positive SHP lineage. Foxc1 and Foxc2 can directly regulate *Tbx1* expression in the SHF as well as head mesenchyme by binding to multiple FBEs on its enhancers.[74,76] Since compound *Foxc1; Foxc2* mutants have much more severe OFT defects than *Tbx1* mutants, it is plausible that in addition to controlling *Tbx1* expression, Foxc1 and Foxc2 are required for regulating additional genes/pathways in SHF development.

Foxc Function in Cardiac Neural Crest Cells

Cardiac neural crest cells are a nonmesodermally derived cell population that significantly contributes to the developing heart. They arise from the caudal hindbrain (rhombomeres 6-8), migrate through the branchial arch 3, 4 and 6 and invade the aortic arch and OFT of the heart.[77] This cell population subsequently participates in OFT septation and differentiates into the cardiac ganglia and the tunica media of the great vessels. Disruption of the cardiac neural crest before migration in chick embryos leads to a variety of malformations such as interruption or coarctation of the aortic arch, VSD and persistent truncus arteriosus (PTA).[77,78] Ablation of the cardiac neural crest is also associated with aplastic or hypoplastic thymus, parathyroid and thyroid glands. These abnormalities are frequently seen in human congenital syndromes, including DiGeorge syndrome, which in most cases results from chromosome 22q11.2 deletion (*del22q11*). Moreover, cardiac neural crest cells influence cardiac development in a non-cell autonomous manner. Ablation of the cardiac neural crest in the chick results in a failure to elongate the SHF-derived OFT myocardium.[79] Although the precise effects of the cardiac neural crest on SHF development remain unknown, this cell population alters the availability of FGF8 in the caudal pharynx.[80,81]

Besides the broad expression of *Foxc1* and *Foxc2* in the mesoderm and it derivatives, the two genes are also expressed in the neural crest cell lineage. As described above, single *Foxc* mutant mice exhibit abnormal aortic arch patterning, suggesting that Foxc1 and Foxc2 in the neural crest play a role in remodeling aortic arch arteries.[5,13] Furthermore, cardiac neural crest cells of compound *Foxc1; Foxc2* mutant embryos undergo abnormal apoptosis during migration, leading to a failure of the OFT septation (PTA), a characteristic phenotype of the ablation of this cell lineage in the chick.[11] This observation indicates that Foxc1 and Foxc2 are required for the survival for cardiac neural crest cells. Despite extensive apoptosis, *Foxc*-mutant cardiac neural crest cells are able to differentiate into smooth muscle. Compound *Foxc1+/–; Foxc2–/–* mutants also show lack of the 2nd pharyngeal arch, suggesting defects in cranial neural crest cells.[8] Although the indirect effects of cardiac neural crest cells on the addition of SHF-derived myocardial cells to the developing heart have been suggested,[78,82] autocrine FGF signaling in the SHF is primarily required for OFT morphogenesis.[83] Thus, given the fact that reduced expression of SHF markers such as *Tbx1* and *Fgf8/10* is already observed in compound *Foxc1; Foxc2* mutant embryos at E8.5 before the onset of cardiac neural crest migration, the OFT abnormalities in these *Foxc* mutants are likely attributable to defective SHF progenitors in a cell-autonomous manner.

Foxc Function in Epicardial-Derived Cells

Another tissue that is critical to form the heart is the epicardium, which is the epithelial cell layer that covers the surface of the heart. The epicardium originates from a specific population of mesothelial cells from the proepicardium (PE). The epicardium plays an essential role in coronary vessel development by providing a source of vascular smooth muscle and endothelial cells after epithelial to mesenchymal transformation (EMT).[84,85] Importantly, multipotent epicardial progenitor cells that differentiate into cardiac myocytes have recently been identified.[86,87]

Both *Foxc1* and *Foxc2* are expressed in a subset of cells in the PE before the formation of the epicardium, while transcripts of the *Foxc* genes appear to be downregulated in the developing epicardium.[11] Compound *Foxc1*+/–; *Foxc2*–/– mutant embryos have abnormal formation of the epicardium, which is detached from the underlying myocardium, while *Foxc* single mutants or compound *Foxc1*–/–; *Foxc2*+/– mutants normally form the epicardium. Although the epicardial-derived mesenchymal cells are generated over the entire surface of the heart through EMT, the majority of these cells are produced at the atrioventricular junction.[88] Intriguingly, compound *Foxc1*+/–; *Foxc2*–/– mutants show abnormal accumulation of mesenchymal cells that are particularly localized at the subepicardial space of the conoventricular region and some of these cells are differentiated into either smooth muscle or endothelial lineage.[11] It is, however, unclear whether the mesenchymal cells at the subepicardial space of compound *Foxc1; Foxc2* mutants are solely derived from the epicardium.

Signal(s) from the myocardium are important for the initiation of EMT in the epicardium[89] and several molecular signals such as VEGF, FGF and TGFβ are critical for the regulation of epicardial EMT.[84,90] Since compound *Foxc1*+/–; *Foxc2*–/– mutants have the above-mentioned defects in the OFT, the abnormal mesenchymal phenotype at the subepicardial space may result from dysregulation of myocardially-derived signaling molecules. Another, nonexclusive possibility is that compound *Foxc1; Foxc2* mutants may lack proper cell-cell interactions between the myocardium and epicardium. Further experiments are needed to clarify the nature of epicardial defects in compound *Foxc1; Foxc2* mutants.

Future Directions

I have presented a summary of the current understanding of the cooperative roles of Foxc1 and Foxc2 in cardiovascular development. Evidently, they are key transcriptional regulators controlling multiple processes in this system. However, many important questions about the function of the *Foxc* genes in cardiovascular development remain to be answered. At present, much of our knowledge about Foxc function has been obtained from the studies of conventional *Foxc* mutant mice. Given their broad expression in mesodermal and neural crest derivatives, tissue- and/or time-specific ablation of *Foxc* genes will provide further invaluable information on the direct involvement of *Foxc* genes in the development of the cardiovascular system. For example, it has recently been shown that cardiac neural crest cells and SHF-derived cells reciprocally interact with each other during OFT morphogenesis.[83] In addition, although the two *Foxc* genes have overlapping expression patterns in the cardiovascular system, an unsolved question is whether they have similar but distinct functions. It is important to note that Foxc2, but not Foxc1, can functionally interact with Notch-mediated transcription in endothelial cells.[25] Moreover, given evidence that other *Fox* genes are also expressed during cardiovascular development, functional redundancy of the *Fox* gene family must be considered. For instance, Foxh1 is essential for the development of the SHF,[70] while Foxo1 mutant embryos have impaired angiogenesis.[91,92] A new aspect of the mechanism of Foxc function in vascular development is the combinatorial activity of Foxc2 and the Ets transcription factor Etv2 in regulating endothelial-specific gene expression during early development.[93] Further studies are needed to reveal the mechanisms of transcriptional regulation involving Foxc and other transcription factors/cofactors.

Although knockout and transgenic approaches in mice and other species have facilitated cardiovascular research over the past decade, the utility of stem cell-based research on cardiac and endothelial cell differentiation is likely to lead to significant progress in deciphering complex networks of transcriptional events associated with multiple signaling pathways. In particular, recent studies have shown that signaling pathways such as FGF, BMP and Wnt are critical for the induction and expansion of cardiac progenitor cells.[94] Therefore, it is anticipated that future studies using cell-based approaches will contribute to understanding the molecular mechanisms that control the genetic program associated with the critical pathways and Foxc-mediated transcriptional regulation in cardiovascular progenitors.

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CHAPTER 6

FoxO Proteins and Cardiac Pathology

Albert Wong and Elizabeth A. Woodcock*

Abstract

The FoxO family of transcription factors mediate a wide range of cellular responses from cell death to cell survival, growth inhibition and glucose utilization. This complex array of responses is regulated by an equally complex regulatory system, involving phosphorylation, ubiquitinization and acetylation, in addition to interactions with other transcription factors and transcriptional modifiers. In heart, FoxO proteins have been shown to be involved in development, in limiting hypertrophic growth responses and in cardioprotection provided by silent information regulator 1 (Sirt1). However, the range of responses mediated by FoxO proteins and the clear evidence for involvement of FoxO regulators in cardiac pathology, suggest that further pathological actions of FoxO family members remain to be elucidated.

The FoxO Family

FoxO proteins are members of the forkhead family of transcription factors characterized by the presence of a forkhead box or Fox, which binds DNA at GTAAACA consensus sequences.[1,2] Genes encoding the FoxO proteins were initially identified at chromosome break points in tumour cells and shown to be homologues of the *Caenohabditis elegans* DAF 16 protein that regulates longevity.[3] Thus, from their initial discovery, the FoxO proteins have been associated with cell survival and cell death responses. There are currently four FoxO proteins known to be expressed in mammalian tissues; FoxO1, FoxO3, FoxO4 and the more recently described FoxO6.[4,5] The first 3 FoxO proteins are expressed in heart,[6] show strong sequence similarity and are regulated similarly. FoxO6 is expressed only in the central nervous system and will not be discussed further here. While FoxOs 1, 3 and 4 are expressed in cardiomyocytes, there is relatively little information about their functional roles in the heart. However, a number of factors that regulate FoxO activity have been shown to have major roles in protecting the heart under pathological conditions or in some cases in causing cardiac damage. This chapter will examine the evidence for an involvement of FoxO proteins in cardiac pathology and will also examine the roles of known FoxO effectors and suggest ways in which their cardiac responses may be mediated by FoxO transcription factors.

The Spectrum of Transcriptional Responses Mediated by FoxO Family Members

FoxO proteins are transcription factors that mediate a bewildering range of cellular responses, which in some cases appear to be opposing. In worms and flies the FoxO homologues, DAF 16 and dFoxO respectively, extend longevity by promoting resistance to stressors, including infectious agents and oxidative stress.[7] The functions of the FoxOs are more complex in mammalian tissues. FoxO 1 and 3 are widely expressed in mammalian cell types and responses observed depend to an extent on the cell type studied. Expression of FoxO4 is more restricted, but it is expressed in the myocardium

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along with FoxOs 1 and 3. Even within the one cell type, responses generally differ between the FoxO subtypes.[8,9] Furthermore, there is evidence that FoxOs can have different functions depending on the nature or the magnitude of the stimulus, as well as on the presence other transcriptional effectors.[9-14] In various mammalian cell types, FoxOs can promote resistance to oxidative stress by transcriptional activation of catalase and MnSOD that serve to remove reactive oxygen species.[15,16] However, FoxO members are also able to initiate apoptosis via transcriptional increases in apoptotic effectors such as Fas-L and Bim.[14,17-19] FoxO proteins increase DNA repair via growth arrest and DNA damage-inducible 45 (GADD 45) and damaged DNA binding protein 1 (DDB1),[20] cause cell cycle arrest via p21, p27, p130 cyclin-dependent kinase inhibitors, as well as cyclin G2[21-23] and regulate glucose and energy homeostasis via glucose 6-phosphatase, phosphoenolpyruvate carboxy kinase, Agouty related peptide (AgRP) and neuropeptide Y (NPY).[24-27]

Regulation of FoxO Proteins

Phosphorylation

In keeping with the array of responses associated with FoxO transcription factors, their regulation, both positive and negative, involves multiple mechanisms. Initially FoxO proteins were shown to be phosphorylated by the protein kinase Akt (or protein kinase B, PKB).[28] Akt itself is activated by phosphorylation subsequent to phosphatidylinositol 3-kinase (PI 3-kinase) activation following stimulation with growth factors or G protein coupled receptor agonists.[29] Akt phosphorylates FoxOs 1, 3 and 4 at three specific sites, as outlined in Figure 1. This phosphorylation results in nuclear exclusion and association with 14-3-3 proteins[30] and thereby inhibits FoxO from functioning as a transcription factor. As shown in Figure 1, one of the Akt phosphorylation sites is in the DNA binding domain and the nuclear localization sequence. Phosphorylation of this site (S^{253} in mouse FoxO1) generally occurs prior to phosphorylation occurring at the other two sites.[31] It is now also clear that phosphorylation of S^{253} displaces DNA-bound FoxO and thereby directly inhibits transcriptional activity,[32] in addition to facilitating exclusion from the nucleus. Thus, there is a defined hierarchy between the Akt phosphorylation sites in FoxO. Removal of phosphorylated FoxO from the nucleus is a complex process involving both the nuclear exclusion sequence (NES) exposed following phosphorylation and the 14-3-3 association.[28,33] In heart,

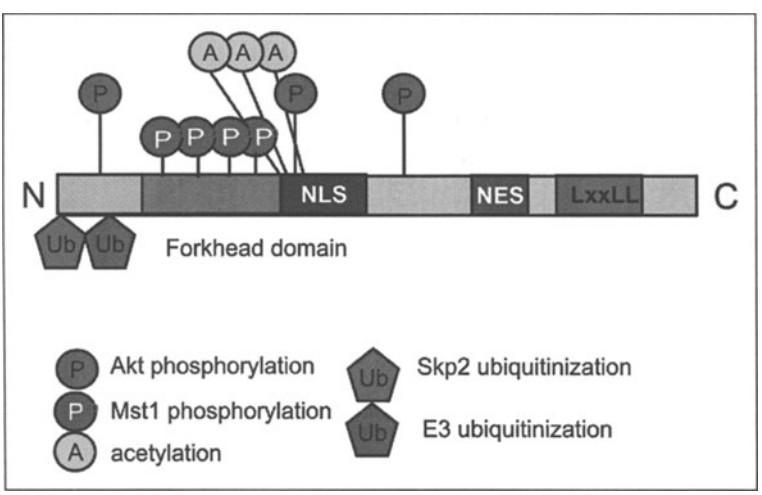

Figure 1. The structure of FoxO family members. FoxO1, FoxO3 and FoxO4 follow a similar pattern of phosphorylation, ubiquitinization and acetylation sites. NLS, nuclear localization sequence; NES, nuclear exclusion sequence; LxxLL is s sequence associated with binding of nuclear hormone receptors.

phosphorylation of FoxO by Akt has been demonstrated[6] and, under basal conditions, FoxO proteins are largely cytoplasmic.

In marked contrast, more recent studies have revealed that FoxO family members can also be activated by phosphorylation, although on different sites from those phosphorylated by Akt, as shown in Figure 1. Activating phosphorylation is mediated by mammalian sterile 20 like kinase 1 (Mst1) a homologue of the *Drosophila* sterile 20 kinase.[15] Mst1 is a Ser, Thr directed protein kinase activated by stressors, including oxidative stress.[15,34] The biology of Mst1 remains to be fully investigated. Mst1 is activated by phosphorylation downstream of K-Ras signalling[35] and K-Ras can be activated downstream of NADPH oxidase 1 (Nox1).[36] Mst1 can also be activated following cleavage by caspase 3.[37] Under this scenario, activation by caspase 3 cleavage could serve to perpetuate Mst1 activation and consequent cellular damage following initiation of apoptosis.[38] Mst1 phosphorylates FoxO family members on Ser/Thr residues within the DNA-binding forkhead domain to enhance DNA binding and therefore FoxO transcriptional activity.[39] Mst1 phosphorylation also disrupts association with 14-3-3 proteins and thus facilitates nuclear retention.

Ubiquitinization

FoxO proteins, phosphorylated by Akt and shunted into the cytoplasm, are subsequently polyubiquitinated and degraded via the proteosome system,[40] by a process that absolutely requires phosphorylation of Akt phosphorylation sites. The ligase most prominent in this response is Skp2 and overexpression of Skp2 can reverse FoxO mediated responses.[41] Thus, polyubiquitinization and proteosomal destruction is the end point of Akt initiated FoxO inhibition (Fig. 2).

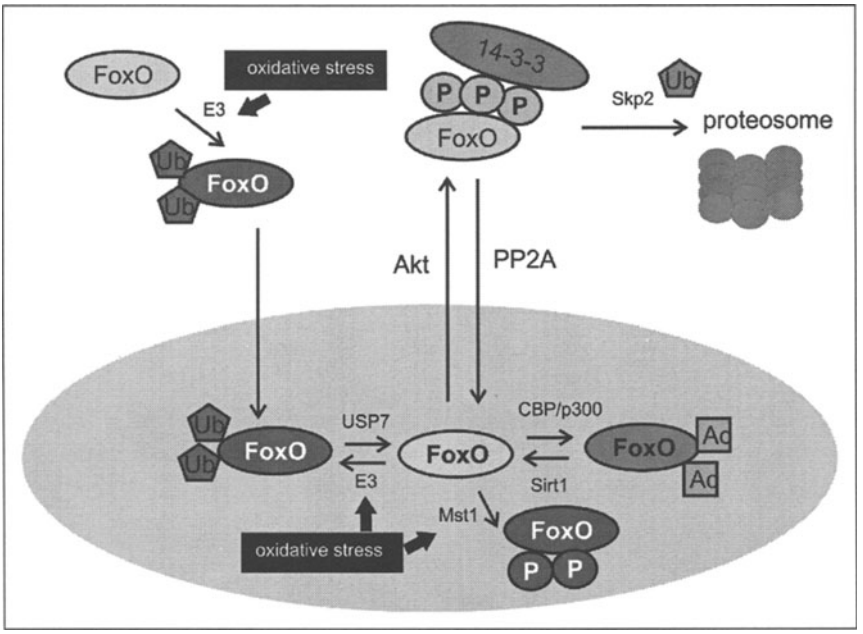

Figure 2. Regulation of FoxO family members under conditions of ischemia and reperfusion. FoxO proteins are phosphorylated by Akt and dephosphorylated by protein phosphatase 2A (PP2A). Skp2 ubiquitinates Akt-phosphorylated, 14-3-3-bound FoxO and targets it for degradation. E3 ubiquitin ligase activates FoxO and this is reversed by herpes virus-associated ubiquitin specific protease (HAUSP or USP7). Mst1 phosphorylation activates transcriptional activity of FoxO. Additionally, FoxO activity is regulated by acetylation by cAMP response element binding protein (CBP) and p300 histone acetyltransferase. Deacetylation is achieved by silent information regulator 1 (Sirt1).

In addition to this polyubiquitinization that is required for proteosomal degradation, FoxO proteins can also be selectively ligated with monomers of ubiquitin. This mono-ubiquitinization, (on K^{63} in mouse FoxO1) mediated by ligases such as E3-ubiquitin ligase, enhances nuclear localization and transcriptional activity.[42] This increases nuclear localization and enhances transcriptional activity of FoxO.[43] Removal of these ubiquitin residues is achieved by herpes virus-associated ubiquitin specific protease (HAUSP). The balance between mono-ubiquinylated and nonubiquinylated FoxO determines transcriptional activity and this balance is regulated by reactive oxygen species.

Acetylation

In addition to regulation by phosphorylation and ubiquitinization, FoxO proteins are regulated by acetylation. Acetylation is mediated by histone acetyl transferases including p300 and the cAMP response element binding protein (CBP) and involves critical lysine residues (K^{242}, K^{245}, K^{262}, in mouse FoxO1).[44] Such acetylation reduces the positive charge on the FoxO protein reducing DNA binding and thereby reducing transcriptional activity.[39] Acetylation also facilitates Akt phosphorylation of S^{253}, further limiting FoxO functioning, as described above.[44] Deacetylation is achieved primarily by class III histone deacetylases, particularly silent information regulator 1 (Sirt1), a homologue of Sir2 in *C. elegans*. Inhibition, rather than activation, of FoxO activity by deacetylation has also been reported.[45,46] The reason for the apparently opposing effects of FoxO acetylation is not known. Given that acetylation of positively charged lysine residues inhibits DNA binding, it is possible that increased transcriptional responses reflect FoxO acting as a transcriptional partner rather than a direct DNA binding transcription factor at those particular promoters.

Transcriptional Partners of FoxO

FoxO family members have direct transcriptional activity by binding forkhead consensus sequences, but in addition these proteins also interact with other transcription factors and transcription modifiers to regulate transcription. There are a number of different ways in which this can be accomplished. In some cases, FoxO and its transcriptional partner both bind their respective DNA sequences, as occurs for the interaction between FoxO and Smads.[47] In other cases FoxO-DNA interaction is not involved. This mechanism is exemplified by the inhibitory interaction with myocardin, where FoxO4 reduces the association between myocardin and serum response factor.[48] There are also examples of FoxO functioning by simply soaking up a transcriptional cofactor and reducing its availability. The interaction between FoxO and β-catenin removes β-catenin from another transcription factor (TCF) and reduces its activity[49] (Fig. 3).

FoxOs interact strongly with nuclear hormone receptors via its LxxLL domain (Fig. 1), resulting in altered activity of both proteins.[9] Among these, interactions with peroxisome proliferator-activated receptors (PPAR), PPARα and PPARγ are likely to be important in heart, where these transcription factors are protective.[50,51] FoxO proteins also interact with the PPARγ co-activator PGC-1α; in this case the interaction enhances FoxO activity.[52] PGC-1α is deacetylated by Sirt1. This suggests a complex relationship between PPAR and FoxO family members.

The transcriptional modifier, myocardin, is active only in smooth and cardiac muscle where it plays critical roles in development and in postnatal growth, via association with serum response factor.[53] Myocardin interacts with FoxO4 in a mutually inhibitory manner,[48] but this interaction has not been reported in heart, to date.

The Smad family of transcription factors is activated by phosphorylation downstream of transforming growth factor β (TGFβ) receptors. Phosphorylated Smad3 translocates to the nucleus where it associates with Smad4 and the Smad3/4 complex is transcriptionally active. The Smad3/4 complex can form a larger complex with FoxO family members and this heightens responses to both FoxO and Smad transcription factors.[9,54] As shown in Figure 3, both FoxO and Smad bind to their respective consensus sequences, but with heightened activity. Thus some of the responses initiated by FoxO family members may result from interaction with Smads.

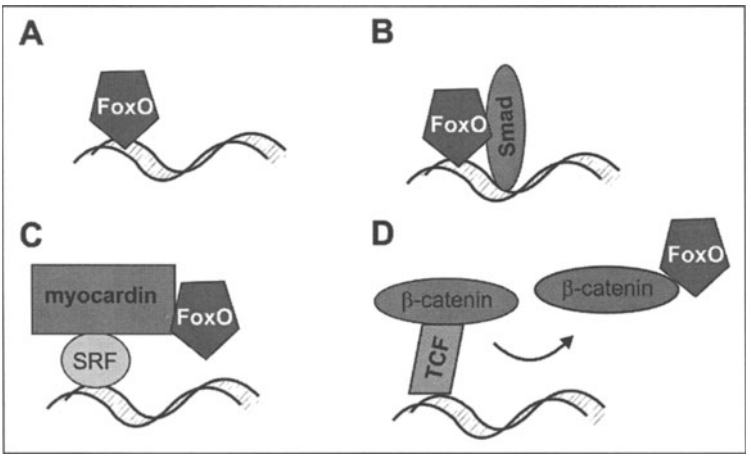

Figure 3. Transcriptional activity of FoxO transcription factors. A) FoxO family members can bind DNA consensus sequences to activate transcription. B) FoxO proteins can associate with other transcription factors, with both factors binding their respective consensus sequences, as shown for the Smad family of transcription factors. C) FoxO proteins can interact with transcriptional enhancers to reduce their activity, as shown for myocardin enhancement of serum response factor (SRF) activity. D) FoxO can bind other transcriptional activators removing them from other effectors, as shown for β-catenin activation of TCF responses.

β-catenin is another factor that associates with FoxO family members, in this case in a mutually inhibitory fashion.[49] Under conditions of cell stress, β-catenin translocates from the plasma membrane to the nucleus and initiates cell survival responses by binding its partner, the transcription factor TCF. FoxO inhibits these responses by sequestering β-catenin away from TCF[55] (Fig. 3).

FoxO Transcription Factors in Cardiac Pathology

Myocardial Ischemia and Post-Ischemic Reperfusion

By definition, myocardial ischemia involves a critical reduction in the blood supply to the myocardium. Clinically, this generally involves blockage of the coronary arteries supplying the ventricle. In the experimental situation, isolated hearts are subjected to reduced flow of perfusate delivering oxygen and nutrients,[56] or isolated cardiomyocytes are subjected to low oxygen together with changes in media composition.[57] Reperfusion is achieved by re-instating blood flow, or in the experimental situation, by re-introducing oxygen.[56,58] While reperfusion is essential to prevent irreversible tissue damage, it introduces further damage to the myocardium, mediated, in part, by the generation of reactive oxygen species (ROS). Ischemia and postischemic reperfusion are major initiators of cardiac pathology. Acutely, ischemic episodes cause arrhythmia often leading to sudden cardiac death.[59,60] Ischemic episodes that are not immediately fatal cause myocardial infarction that is followed by compensatory cardiomyocyte hypertrophy, leading eventually to heart failure.[61] Damage to cardiomyocytes during ischemia and subsequent reperfusion involves both necrotic and apoptotic cell death[62] and at least in animal models, reduction in cardiomyocyte apoptosis improves functional outcomes.[63]

In model systems, ischemic injury can be ameliorated by activation of PI 3-kinase and subsequent activation of the protein kinase Akt, a FoxO inhibitor described above.[64,65] Akt protection results in reduced infarct size, indicating improved cell survival, as well as improved functional recovery.[64,65] Akt phosphorylates a number of targets including pro-apoptotic effectors. Important among these are the pro-apoptotic proteins BAD, Mst1, caspase 9[66] and all of the FoxO proteins expressed in heart.[30] FoxO proteins promote apoptosis via transcriptional activation of apoptotic effectors such as tumor necrosis factor related apoptosis inducing ligand (TRAIL), Bim and Fas ligand.[67-69] Cardiomyocytes

are relatively resistant to apoptosis and, in particular, the extrinsic pathway of apoptosis is though to be only minimally involved in cardiomyocyte damage.[70,71] However, there have been reports of Fas activation and its possible involvement in ischemic injury.[72,73] Cardiomyocyte apoptosis most commonly involves the mitochondrial, intrinsic pathways.[74] Bim, a proapoptotic BcL-2 protein, is a transcriptional target of FoxO proteins and thus increased Bim expression, subsequent to FoxO activation, is a possible contributor to Akt-reversible cardiomyocyte cell death. This, of course, leaves open the question of how FoxOs would be activated by ischemia/reperfusion. This question is discussed further below.

One possible mechanism by which FoxO proteins might be activated under conditions of ischemia/reperfusion involves the activating protein kinase Mst1, alluded to earlier. Mst1 is activated by stressors, including oxidative stress,[15,34] as occurs under conditions of ischemia/reperfusion.[75] As noted above, Mst1 phosphorylates FoxO family members within the forkhead domain to enhance DNA binding and therefore FoxO transcriptional activity.[39] Thus, Mst1 phosphorylation of FoxO acts in opposition to the inhibitory phosphorylation mediated by Akt. Recently, Ste20/oxidant stress response kinase-1 (SOK1), a close relative of Mst1, has been shown to be directly activated by interaction with reactive oxygen species.[34] However, there are no similar reports of direct activation by ROS for Mst1 itself. Overexpression of Mst1 in heart in vivo (Tg-Mst1) causes severe dilated cardiomyopathy,[75] by inhibiting hypertrophy and autophagy while activating apoptosis. Importantly, inhibiting Mst1 activity by expressing a dominant negative Mst1 mutant in heart reduced apoptosis and dysfunction following myocardial infarction.[76] This suggests that Mst1 is an important contributor to heart failure following ischemic insult.

However, it is less clear that FoxO family members are the mediators of Mst1-induced cardiac pathology. Mst1 inhibits hypertrophy and autophagy, while increasing apoptosis. Inhibition of hypertrophy by FoxO3 has been demonstrated in heart[77] and initiation of apoptosis by FoxO family members has been described in other tissues[67,69,78] FoxO3, however, is associated with increased autophagy in the myocardium,[79,80] seemingly opposite to responses initiated by Mst1. However, it is clear that transcriptional responses mediated by the FoxOs vary depending on the cell type, the nature of the stimulus and the intensity of the stimulus. Therefore, it is possible that FoxO members mediate the apoptotic and antihypertrophic actions of Mst1, but not the inhibition of autophagy. The question of FoxO mediation of Mst1-induced cardiac pathology will only be answered satisfactorily, with FoxO knock-out animals, or by expressing dominant negative FoxO.

As discussed earlier, FoxO family members are subject to acetylation/deacetylation reactions mediated by histone acetyl transferases (HATs) and class III histone deacetylases (HDACs), respectively. In heart, FoxO3 is acetylated by cAMP response element binding protein (CBP)[81] and p300 acetylase[39] and deacetylated by silent information regulator-1 (Sirt1, a homologue of yeast Sir2).[82-84] In mammalian heart, Sirt1 is a cardioprotective factor activated following oxidative stress.[10,16] Moderate increases in Sirt1 expression in heart are protective under conditions of pathological growth or under ischemic challenge and this is related to increased expression of detoxifying enzymes such as catalase and manganese superoxide dismutase (MnSOD). This protective response was prevented by dominant negative FoxO, pointing to a role for FoxO in ischemic protection.[10] As dominant negative FoxO inhibits the activity of all members of the FoxO family, the FoxO subtype responsible for this response was not identified in this study. This apparently protective action of FoxO members is opposite to what would be expected based on effects of Akt and Mst1. However, in addition to its transcriptional activation of potentially apoptotic factors, FoxO proteins increase transcription of catalase and MnSOD,[10] factors that aid in removing ROS and would be expected to ameliorate ischemic damage. Higher levels of expression of Sirt1 in heart caused rapid development of hypertrophy, followed by heart failure.[10] It is not clear whether this deleterious response to Sirt1 was also mediated by FoxO family members.

From these data, it appears that FoxO proteins can have both advantageous and disadvantageous effects on the heart during ischemic episodes. It should be noted that there are also reports that deacetylation by Sirt1 can inhibit FoxO1 and specifically can reduce FoxO-mediated apoptosis, although these observations were not made in cardiomyocytes.[16,46,84,85]

FoxO proteins are regulated positively and negatively by ubiquitination. Ubiquitinization by Skp2 is essentially the end point of Akt mediated FoxO inhibition, by targeting FoxO for proteosomal degradation.[41] On the other hand, ubiquitinization is also a mechanism of FoxO activation and this process is enhanced under ischemic conditions.[86] By this E3 ligase mediated mechanism, FoxO proteins are ubiquitinated on K^{63} in mouse FoxO1,[42] enhancing nuclear localization and transcriptional activity.[43] Removal of these ubiquitin residues is achieved by herpes virus-associated ubiquitin specific protease (HAUSP). The balance between mono-ubiquinitated and nonubiquinitated FoxO determines transcriptional activity and this balance is regulated by reactive oxygen species generated under conditions of ischemia/reperfusion.[43]

FoxO proteins interact with a number of critical factors, often in a mutually inhibitory fashion. Important among these is β-catenin.[49] β-catenin translocates from the sarcolemma to the nucleus of cardiomyocytes under ischemic conditions and protects from cardiomyocyte apoptosis.[87] Expression of β-catenin reduces infarct size following myocardial infarction and furthermore, inhibition of β-catenin by cardiac-targeted knock-out or by expression of a dominant negative mutant leads to growth failure in response to challenge and thus precipitates heart failure.[87,88] The interaction between β-catenin and FoxO is heightened under ischemic conditions.[89] As this is a mutually inhibitory association, FoxO would be expected to reduce the beneficial effects of β-catenin. However, there are also reports that β-catenin is required for adaptive cardiac hypertrophy,[88] but it is not certain that this involves FoxO family members.

Hypertrophy

Cardiomyocytes are terminally differentiated and do not undergo cell division to any significant extent after birth. However, heart size can be induced to increase by a process of hypertrophy whereby the size of the individual cells increases without their undergoing mitosis. The heart undergoes hypertrophic growth in response to increased work demand on the cardiomyocytes. Essentially there are two apparently distinct types of hypertrophy; physiological hypertrophy that accompanies exercise and pathological hypertrophy. Physiological hypertrophy results in a larger more powerful heart that does not degenerate into heart failure.[90] Pathological hypertrophy, on the other hand, is initially a compensatory response to produce a larger more powerful heart, but in this scenario, increased growth is associated with arrhythmia and sudden death and in the longer term degenerates into heart failure.[91,92] Pathological hypertrophy follows loss of myocytes due to infarction, as mentioned above, or when there is pressure or volume overload exerted on the heart, e.g., by increased blood pressure or renal impairment, respectively. FoxO transcription factors are associated with inhibition of growth in many cell types. This involves transcriptional activation of the cell cycle regulators, p21 and p27, as well as other intermediates and this maintains cells in the G_0 state.[7] In terminally differentiated cardiomyocytes, FoxO3 has been shown to inhibit hypertrophic growth. FoxO3 induces transcription of atrogin-1,[77] a muscle F-box protein. Atrogin-1 associates with calcineurin promoting its degradation via the proteosome,[93] thereby inhibiting the calcineurin/nuclear factor of activated T-cells (NFAT) response pathway that is pivotal in pathological hypertrophy. In addition, atrogin-1 and E3 ubiquitin ligase cause ubiquitinization on K^{63} of FoxO1 and FoxO3, promoting nuclear localization and transcriptional activity. This ubiquitinization serves to oppose the actions of Akt and by this mechanism FoxO members can limit physiological hypertrophy,[86,93,94] that depends on PI 3-kinase and Akt activation.[95]

Other antihypertrophic mechanisms involving FoxO have been reported also. Statins, cholesterol-lowering drugs that inhibit HMG CoA reductase, have a direct action to limit cardiac hypertrophy, in addition to their lipid lowering activity. Studies by Hauck et al (2007)[96] show that statins facilitate the recruitment of FoxO3 to the p21 promoter and thereby initiate growth-suppression via p21 signaling pathways.

FoxO4 interacts in a mutually inhibitory manner with myocardin[48] and myocardin is a powerful activator of cardiac hypertrophy.[97] However, the interaction between FoxO4 and myocardin has not been demonstrated in heart as yet.

Development

Unlike post natal growth, the fetal development of the heart requires cell growth and division and FoxO family proteins are involved in this process. This was demonstrated in studies where FoxO1, FoxO3 and FoxO4 were expressed under a β-myosin heavy chain promoter to initiate expression during fetal development.[98] Overexpression of FoxO3 caused death at embryonic day 18 due to restricted mitosis, whereas embryonic overexpression of FoxO1 was lethal by 10.5. FoxO4 overexpression was not lethal during prenatal growth. Knockout of the FoxO1 gene is embryonic lethal at E 10.5 due to restricted vascular development. Deletion of either FoxO3 or FoxO4 was not lethal during development.[99]

As noted earlier, the transcriptional partner of serum response factor, myocardin, is a critical regulator of heart specification.[100] In addition to being negatively regulated by FoxO4, myocardin is a transcriptional target of FoxO, which, in this case, acts together with myocyte enhancer factor 2 (Mef2) to activate myocardin gene transcription.[101] This being the case, it is unclear why deletion of FoxO does not prevent early heart development. The answer may reside in functional redundancy between family members. This possibility will only be addressed by expressing a dominant negative mutant FoxO in early embryos to interfere with the transcriptional activity of all family members.

Conclusion

The FoxO family of transcription factors clearly mediate a wide range of cellular responses and this is achieved by an even more complex regulatory network responsible for FoxO activity. To date, the only cardiac effects definitively ascribed to FoxO are developmental regulation and growth inhibition. However, given the number of cardiac effectors that are FoxO regulators, it seems inevitable that further functions will be described for FoxO family members in the myocardium.

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CHAPTER 7

The Forkhead Transcription Factors Play Important Roles in Vascular Pathology and Immunology

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Abstract

Transcription factor families are a small number of upstream master genes in "higher hierarchy" that control the expression of a large number of downstream genes. These transcription factors have been found to integrate the signaling pathways underlying the pathogenesis of cardiovascular diseases with or without autoimmune inflammatory mechanisms. In this chapter, we organize our analysis of recent progress in characterization of forkhead (FOX) transcription factor family members in vascular pathology and immune regulation into the following sections: (1) Introduction of the FOX transcription factor superfamily; (2) FOX transcription factors and endothelial cell pathology; (3) FOX transcription factors and vascular smooth muscle cells; and (4) FOX transcription factors, inflammation and immune system. Advances in these areas suggest that the FOX transcription factor family is important in regulating vascular development and the pathogenesis of autoimmune inflammatory cardiovascular diseases.

Introduction

The forkhead (FOX) transcription factor family modulates the expression of a large number of major regulatory genes that expansively regulate still more signaling pathways, integration points and pathological processes. Given such a position atop a regulatory hierarchy, it is easy to see why studies of FOX transcription factors have grown exponentially since their discovery. While we cannot propose to detail every known aspect of FOX transcription factors within the scope of a single chapter, we can relate the roles of FOX transcription factors in well-worn fields of medical research that could derive beneficial therapeutic strategies.

Atherosclerosis is characterized by focal arterial lesions containing cholesterol, fibrosis, intense immunological activity, inflammatory cell infiltrates and cell death.[1] Several risk factors have been identified for the atherogenic process including hyperlipidemia, low density lipoprotein (LDL), cigarette smoking, diabetes, hypertension, obesity[2] and excessive quiescence.[3] Wang's laboratory and others have confirmed that hyperhomocysteinemia (HHcy) also acts as an independent risk factor in accelerating atherosclerosis.[4-6] In addition, atherosclerosis is positively correlated with the endotoxin load in patients' plasma.[7] These risk factors independently or synergistically lead to chronic vascular inflammation, which is an essential requirement for the progression of atherosclerosis in patients.[8] Most recently, Yang's laboratory and others have shown that transcription

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factor Foxp3-controlled $CD4^+CD25^{high}$ regulatory T-cells[9] suppress vascular inflammation,[10,11] diabetes[12] and atherosclerosis.[13,14] Despite significant advances in elucidating atherosclerotic pathology, atherosclerosis remains as the leading cause of morbidity and mortality in industrialized society. Therefore, continuous improvement of our understanding of the atherogenesis and vascular inflammation initiated and promoted by risk factors will lead to the future development of novel therapeutics for ischemic stroke, myocardial infarction and other cardiovascular diseases.

In understanding the molecular signals underlying vascular inflammation and metabolic stress, the expression profiles of numerous genes in vascular cells in response to inflammation and metabolic stress stimuli have been identified.[15,16] The question remains whether there are a small number of upstream master genes in "higher hierarchy" that controls the expression of a large number of downstream genes and integrates the signaling pathways underlying vascular development and the pathogenesis of cardiovascular diseases. Among these master genes are several transcription factor families, notch signaling components and epigenetic machinery. Recent reviews more specifically detail FOX transcription factors,[17] myocardin related transcription factors,[18] notch signaling,[19] Kruppel-like transcription factors,[20] E26 transforming-specific sequence (ETS) transcription factors[21] and epigenetic regulatory mode.[22] The results obtained in the studies of transcriptional genomics using microarrays associate several FOX transcription factors (FOXC1, C2, P1, P4 and O1A) with human heart failure, suggesting the pathophysiological significance of this gene family.[23] In this chapter, we focus on analyzing the roles of FOX transcription factors identified in vascular pathology, diabetes and immunology. We apologize for not being able to include many valuable articles and reviews due to limited space.

Introduction of the FOX Transcription Factor Superfamily

Structure

Since the discovery of Drosophila transcription factor fork head[24] and subsequent identification of the forkhead DNA-binding domain in the 1990s,[25] more than 100 FOX genes and 19 human subgroups have been identified (FOXA to FOXS).[26] Subgroups are designated by a letter and within each subgroup proteins are given a number.[27] The FOX transcription factors are termed using the following convention: all capital letters for human (e.g., FOXA1); only the first letter capitalized for mouse (e.g., Foxa1); and the first letter and subgroup capitalized for all other chordates (e.g., FoxA1).[27] Members of this family have three α helices and two large loops or butterfly-like 'wings'.[27] Therefore, the forkhead domain with approximately 100 amino acids is sometimes referred to as the forkhead/winged helix domain.[27] The FoxO subgroup has received the most attention because of its recently discovered roles in reactive oxygen species (ROS) detoxification,[28,29] cell cycle progression,[30] apoptosis,[31,32] cell size,[17,33-35] DNA repair,[30] glucose metabolism[36-38] and vascular homeostasis.[39] Given such extensive characterization, the FoxO subgroup will serve as a basis for introduction to the FOX transcription factor family.

Expression

FoxO protein expression has been reported in the ovary, prostate, skeletal muscle, brain, heart, lung, liver, pancreas, spleen, thymus and testis. However, in different cell types or organs, the expression levels of those FoxOs can differ considerably.[27] FoxO1 is highly expressed in adipose tissue, whereas FoxO4 is highly expressed in muscle and FoxO3a in liver.[40] FoxO6 expression appears to be restricted to brain.[41] FOXO1, FOXO2, FOXO3a and FOXO4 are identified in fusion genes derived from chromosomal translocations in human soft tissue tumors and leukemias. FOXO1 is known as forkhead in rhabdomyosarcoma (FKHR), FOXO3a is termed as FKHR like protein 1 (FKHRL1), FOXO4 is known as AFK, an acute leukemia fusion gene located in chromosome X, and a fusion between FOXO2 and MLL occurs in some cases of acute myeloid leukemia.[26]

Functional Modes of FOX Transcription Factors

FoxO transcription factors must bind to DNA to either activate or repress target gene expression. They preferentially bind to DNA at the "FoxO-recognized element," which has the core consensus

sequence 5′-T/C-G/A-A-A-A-C-A-A-3′. Fourteen protein-DNA contacts occur in the forkhead domain with the primary recognition site located at α-helix H3.[26] Both the first and second loops of FoxO proteins make contact with DNA, but it is the second loop that can enhance the specificity and stability of the binding.[26] The mechanisms underlying the binding of FoxO proteins to DNA have not been completely defined. FOX transcription factors may interact with a variety of cofactors such as CBP/p300, Smad (a class of transcription factors that modulate the activity of transforming growth factor (TGF)-β ligands), STAT (the Signal Transducers and Activators of Transcription protein), PPAR (the peroxisome proliferator-activated receptors), Runx (a transcription factor that controls the timing of gene activation/inactivation), p53 (a tumor suppressor), other FOX transcription factors and nuclear receptors for androgens, glucocorticoids, thyroid hormone and retinoic acid. Such interactions may change the FOX transcription factors' DNA binding ability, thereby affecting their ability to promote or repress target gene expression.[26,42] FoxO transcription factors contribute to cardiac muscle remodeling and insulin signaling and link insulin resistance with maladaptive heart hypertrophy. Calcineurin is an important phosphatase that activates a cascade of gene regulation through the nuclear factor of activated T-cell 1 (NFAT1) transcription factors. The heterotrimeric protein phosphatase 2 (PP2, formerly PP2A) is a ubiquitous and conserved serine/threonine phosphatase with broad substrate specificity and diverse cellular functions. Sustained activation of FoxO1 or FoxO3 in cardiomyocytes selectively enhances the activity of protein kinase B (Akt) and reduces insulin signaling through inhibition of calcineurin and PP2.[43,44]

Combinatorial Regulation of Gene Expression by FOX Transcription Factors and Other Transcription Factors

In addition to working with other cofactors, FOX transcription factors often fulfill their function in regulating gene expression in combination with other transcription factors. Vascular development begins when mesodermal cells differentiate into endothelial cells, which then form primitive vessels. A 44 bp transcriptional enhancer in the genes' promoter regions is sufficient to direct gene expression specifically and exclusively to the developing vascular endothelium. This enhancer is regulated by a composite cis-acting element, the FOX:ETS motif, which is bound and synergistically activated by FOX and ETS transcription factors. Coexpression of FoxC2 and the ETS protein Etv2 induces ectopic expression of vascular genes in Xenopus embryos. Combinatorial knockdown of the orthologous genes in zebrafish embryos disrupts vascular development. Finally, FOX:ETS nucleotide sequence motifs are present in many known endothelial-specific enhancers, indicating that this motif is an efficient predictor of endothelial enhancers in the human genome.[45] The interaction of FoxO with other transcription factors such as nuclear factor-κB (NF-κB) likely contributes to the complexity of the synergy between vascular endothelial growth factor (VEGF) signaling and FoxO in the upregulation of numerous genes including matrix metalloproteinase-10 (MMP-10), vascular endothelial cell adhesion molecule-1 (VCAM-1), endothelial-specific molecule-1 (ESM-1), bone morphogenetic protein-2 (BMP-2) and CBP-interacting transactivator-2 (CITED-2).[46] HOXA13 is a transcription factor that plays a role on placental formation.[47] FoxF1 promoters bind to HOXA13 and can use these bound promoter regions to direct gene expression, providing a functional vascular endothelial labyrinth necessary for embryonic growth and survival.[47]

Post-Translational Modification

In addition to being regulated at transcriptional and translational levels, the activities of the FOX transcription factor family are also regulated posttranslationally via phosphoryaltion by Akt, a serine/threonine kinase.[48] FOXO1 has phosphorylation sites at residues Thr24, Ser256 and Ser319.[48] FOXO4 has phosphorylation sites at residues Thr28, Ser193 and Ser258.[48] These phosphorylation sites are not equally modified. Among the three phosphorylation sites of FOXO3a (Thr32, Ser253, Ser315), Akt preferentially phosphorylates Ser253.[32] Mutation of the Akt phosphoacceptor amino acids to alanine residues on FOXO can render it resistant to Akt phosphorylation and enhances the transcriptional activity of FOXO, suggesting that phosphorylation by Akt inhibits the transcriptional activity of FOXO.[49] After activation, Akt translocates to the nucleus

and phosphorylates the FOXO transcription factor, which results in the export of FOXO into the cytosol.[48] Alternatively, the phosphorylation of FOXO proteins results in their inactivation through cytoplasmic retention.[48] In the absence of Akt activity or following the mutation of FOXO phosphorylation sites, FOXO is exclusively localized to the nucleus.[48] The translocation of FOXO following Akt phosphorylation is associated with the protein 14-3-3.[48] The 14-3-3 family of proteins functions by binding to its protein ligands in a phosphorylation-dependent manner. Two binding motifs of 14-3-3 proteins have been identified, namely RSXpSXP and RXY/FXpSXP,[50] which are present in nearly all known 14-3-3 proteins. Akt phosphorylation of FOXO3a results in the association of FOXO3a with 14-3-3 protein and retention of FOXO3a in the cytoplasm, rendering it ineffective to target genes in the nucleus and thus blocking its pro-apoptotic role.[48] In addition, translocation of FOXO can also occur in response to a cellular insult, such as oxygen or glucose deprivation.[48] FoxO factors can also be phosphorylated by other cellular kinases including NF-κB inhibitor (IκB) kinase, serum-glucocorticoid-regulated kinase, casein kinase 1, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A, cyclin-dependent kinase 2 and mammalian Ste20-like kinase.[42]

Some phosphorylation events can also regulate FoxO activity independent of cytosol-nuclear shuttling. For example, the phosphorylation mediated by Ras-related GTPase 9Ral-Jun kinase, cyclin-dependent kinase[51] and other mitogen-activated protein kinase pathways[42] modulate FoxO activity. In addition to phosphorylation, FOXO factors are also tightly regulated by other posttranslational modifications including cAMP response element-binding protein (CBP)/p300-mediated acetylation,[42] ubiquitin ligase Skp2-mediated ubiquitination[52] and deubiquitinase USP7-mediated deubiquitination.[42]

In addition to being regulated by Akt, FoxO3a can also regulate the activation of Akt and extracellular signal-regulated kinases (ERK) as a feedback pathway. Foxo3a-deficient mice develop marked neutrophilia with age or during hematopoietic recovery after myelosuppressive stress induced by 5-fluorouracil (5-FU), an antitumor chemotherapeutic drug, in a cell-autonomous manner. Akt and ERK activation are evident in hematopoietic stem cells (HSCs) of 5-FU-treated or aged Foxo3a$^{-/-}$-deficient mice. FoxO3a$^{-/-}$-deficient cells are hyperresponsive to cytokine stimulation, a phenotype effectively reversed by treatment with inhibitors of the Akt-mammalian target of rapamycin (mTOR) pathway or the MEK (mitogen-activated protein kinase kinase)-ERK pathway, indicating that FoxO3a plays a pivotal role in maintenance, integrity and stress resistance of HSCs through negative feedback pathways for proliferation.[53]

FOX Transcription Factors and Endothelial Cell Pathology

The work of Wang's laboratory suggests that the roles of risk factor(s) in atherogenesis are related to endothelial damage caused by the risk factor(s).[5,6] It is seen that endothelial damage is a main mechanism underlying the pathogenesis of atherosclerosis, restenosis and posttransplant graft atherosclerosis in addition to infiltration of inflammatory cells and proliferation of smooth muscle cells. Blocked apoptosis of endothelial cells results in significant reduction of intimal hyperplasia in vivo.[54] FOX transcription factors fit into this paradigm according to Figure 1, which will serve as a useful reference for the ensuing discussion.

Endothelial Apoptosis

In vascular endothelial cells, Foxo1 and Foxo3a are pro-apoptotic proteins regulated by Akt.[55] Upon activation by VEGF or insulin/insulin-like growth factor-1 (IGF1), Akt mediates prosurvival and anti-apoptotic signaling in part via the phosphorylation and inactivation of FoxO transcription factors.[32,56-57] Akt-regulated FOXO3a controls endothelial cell viability through modulation of the expression of caspase-8 inhibitor FLIP (FLICE-inhibitory protein).[58] FLIP is a homologue of caspase-8 that lacks catalytic activity and has been shown to be important in protecting endothelial cells from apoptosis.[58] Akt promotes FLIP expression in endothelial and tumor cells.[58] Transduction of a nonphosphorylatable, constitutively active mutant of FOXO3a (TM-FOXO3a) down-regulates FLIP, increases caspase-8 activity and promotes apoptosis in endothelial cells.[58]

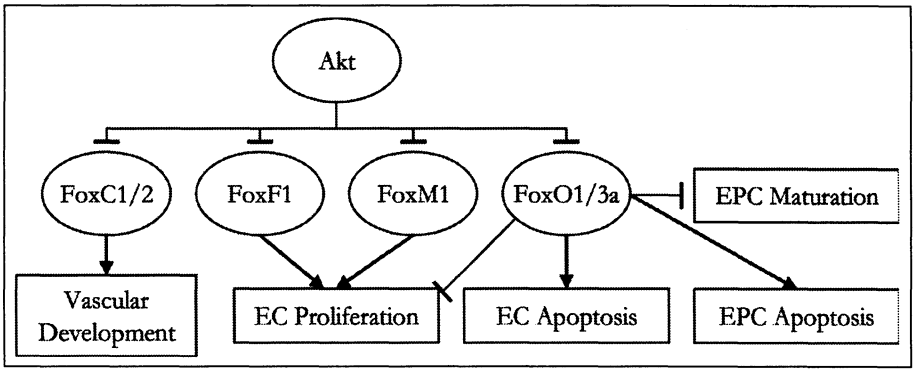

Figure 1. FOX transcription factors in endothelial cells. Various FOX transcription factors affect the growth and apoptosis of endothelial cells (EC) and endothelial progenitor cells (EPC).

Conversely, transduction of a dominant-negative mutant of FOXO3a up-regulates FLIP levels and protects endothelial cells from apoptosis induced by serum deprivation conditions.[58] Restoration of intracellular FLIP blocks caspase-8 activation and inhibits apoptosis in TM-FOXO3a-transduced cells.[58] This establishes a convincing mechanism for FOX-mediated endothelial cell apoptosis.

EPC Apoptosis and Maturation

FOXO may contribute to endothelial progenitor cell (EPC) apoptosis. EPCs are present in the systemic circulation and home in on sites of ischemic injury.[59,60] Circulating EPC levels are an indicator of cardiovascular health.[61,62] Phosphorylation and therefore inactivation of FOXO4 by statins prevents EPC apoptosis.[63] In addition, statins reduce the expression of the pro-apoptotic FOX-regulated protein Bim in a phosphatidylinositol 3-kinase (PI3K)-dependent manner.[63] Similarly, scleroderma serum-induced EPC apoptosis is mainly mediated by the Akt-FOXO3a-Bim pathway, which may account, at least in part, for the decreased circulating EPC levels in scleroderma patients.[64] Moreover, Akt expression is attenuated in the early stages of differentiation and is gradually upregulated during EPC maturation. FOXO3a, an Akt downstream target, is down-regulated through phosphorylation in the late stages of EPC differentiation. Adenovirus-mediated overexpression of activated FOXO3a in peripheral blood mononuclear cells markedly increases the number of cell foci but reduces the number of Di-acetyl LDL-expressing EPCs that appear at later time points. These data suggest that Akt/FOXO3a signaling is an important regulator of EPC maturation.[65]

Endothelial Cell Proliferation

FOX transcription factors modulate the migration and proliferation of aortic endothelial cells, which are critical processes involved in atherosclerosis and postangioplasty restenosis.[57] Inhibition of FOXO factors promotes endothelial proliferation by down-regulating p27Kip1, a member of the universal cyclin-dependent kinase inhibitor family.[66] Transfection of endothelial cells with constitutively active TM-FOXO3a up-regulates p27Kip1, whereas transfection with a constitutively active Akt decreases p27Kip1 expression.[66] Reducing FOXO expression using RNA interference attenuates p27Kip1 expression and stimulates endothelial cell proliferation.[66] In contrast, targeted inactivation of FoxF1 leads to loss of vasculature.[67] FoxF1 is crucial for assembly of endothelial cells into simple tubes from clusters of mesodermal angioblasts.[67] Similarly, endothelial cell-restricted disruption of FoxM1 impairs endothelial repair following bacterial toxin lipopolysaccharide (LPS)-induced vascular injury.[68] Endothelial cells isolated from endothelial cell-restricted Foxm1-deficient mouse lungs fail to proliferate and small interfering RNA (siRNA)-mediated suppression of FOXM1 in human endothelial cells results in defective cell cycle progression.[68] These data suggest differential roles of FOX proteins in endothelial cell proliferation.

Another mode by which FOX transcription factors may regulate endothelial cell proliferation is downstream of homocysteine (Hcy)-induced effects. Wang's laboratory and others confirmed that HHcy acts as an independent risk factor in accelerating atherosclerosis.[4-6] High levels of Hcy induce a sustained injury of arterial endothelial cells, which accelerates the development of thrombosis and atherosclerosis. In addition, Hcy specifically inhibits the growth of endothelial cells. Hcy induces dephosphorylation of Akt and FOXO3a and upregulates p27Kip1 in a time- and dose-dependent manner. PI3K activator peroxovanadate (PV) and PP2 inhibitor okadaic acid can reverse the Hcy inhibition of endothelial growth. Pretreatment with PV and okadaic acid prevents Hcy-induced cell cycle G1 phase arrest. Transfection with specific antisense oligonucleotides to Akt further supports these observations. These results suggest a new pathogenic mechanism underlying HHcy as an independent risk factor for cardiovascular diseases.[69]

Roles of FOX Transcription Factors in VEGF Signaling

FoxC1 and FoxC2 are essential for arterial cell specification during development. In the developing embryo, arterial and venous identity is established by genetic mechanisms before circulation begins. VEGF signaling and its downstream Notch pathway play critical roles in arterial cell fate determination. FoxC1 and FoxC2 directly induce the transcription of Delta-like 4 (Dll4), a ligand for Notch receptors. FoxC2 physically and functionally interacts with a Notch transcriptional activation complex containing Su(H) and Notch intracellular domains to induce Hey2 promoter activity. FoxC transcriptional factors interact with VEGF and Notch signaling to regulate arterial gene expression in multiple steps of the VEGF-Dll4-Notch-Hey2 signaling pathway.[70] In addition, VEGF is a direct transcriptional target of FoxM1b. In glioma cells, FoxM1b overexpression increases VEGF expression, whereas blockade of FoxM1b expression suppresses VEGF expression.[71]

Incubation of human coronary artery endothelial cells with hepatocyte growth factor (HGF) induces prolonged PI3K/Akt-dependent phosphorylation and nuclear exclusion of FOXO1. HGF-mediated inhibition of FOXO1 activity results in secondary attenuation of VEGF-induced expression of FOXO1-dependent genes including VCAM-1, manganese superoxide dismutase (MnSOD), ESM-1, CBP/p300 interacting transactivator with ED-rich tail-2, BMP-2, MMP-10 and MGC5618.[72] Foxo1-deficient mice have also been ascribed to have an insufficient endothelial response to VEGF. FoxO1-deficient yolk sacs show reduced expression of essential endothelial gap junction connexins 37 and 40 and ephrin-B2 (a ligand for the Eph receptor responsible for vascular patterning and identity).[17] Postnatal deletion of the FoxO1 gene by the transient activation of Cre recombinase through the Mx1 promoter results in the appearance of hemangiomas, but deficiencies of FoxO3 and/or FoxO4 do not recreate the tumor phenotype observed in Foxo1 deficient mice. These results suggest that in endothelial cells, FoxO1 is the dominant factor in suppressing tumor formation.[17]

Endothelial Responses to Stress

FOX transcription factors have specific responses under conditions of stress. Prolonged shear stress (18 hours) leads to a significant (50%) decrease in hydroxyl-methylglutaryl coenzyme A reductase (HCR) mRNA expression via the phosphorylation and degradation of FoxO1a.[73] HCR is the rate-limiting enzyme for cholesterol synthesis.[73] Correspondingly, the downregulation of FoxO with siRNA decreases HCR expression.[73] In addition, angiotensin II (Ang II) is a powerful accelerator of atherosclerosis and modulates the expression of endothelial nitric oxide synthase (eNOS).[74] Exposure of human umbilical vein endothelial cells to Ang II elicits a rapid phosphorylation of Akt and FoxO1.[74] Constitutively active Akt inhibits the promoter activity of a scavenger receptor of the BI class (hSR-BI/CLA-1), whereas a dominant-negative mutant of Akt or mutagenesis of a FoxO1 response element in hSR-BI/CLA-1 abolishes the ability of Ang II to suppress promoter activity.[74] Thus FoxO1 mediates two distinctive outcomes in response to stress.

Neovascularization

eNOS, which is essential for postnatal neovascularization, is regulated by FoxO1 and FoxO3a.[75] EPCs promote neovascularization in sites of ischemic injury.[59,60] Constitutively active FoxO1 and FoxO3a repress eNOS expression by binding to the eNOS promoter.[75] In vivo, FoxO3a deficiency increases eNOS expression and enhances postnatal vessel formation and maturation.[75] BMPs are involved in embryonic and adult blood vessel formation in health and disease. BMPER (BMP endothelial cell precursor-derived regulator) is a differentially expressed protein in embryonic endothelial precursor cells. BMPER is a downstream target of FoxO3a and consistently exerts activating effects on endothelial cell sprouting and migration in vitro and in vivo.[76] Intercellular junctions mediate adhesion and communication between adjoining cells. Although formed by different molecules, tight junctions (TJs) and adherens junctions (AJs) are functionally and structurally linked. Vascular endothelial (VE)-cadherin at AJs upregulates TJ adhesive protein claudin-5. This regulation requires alleviation from inhibition by FoxO1 and the T-cell factor (Tcf)-4-β-catenin transcriptional repressor complex. VE-cadherin acts by inducing the phosphorylation of FoxO1 through Akt activation and by limiting the translocation of β-catenin to the nucleus.[77] Collectively, the studies of FOX transcription factors in endothelial cells show a myriad of distinct roles for development, proliferation and cell death as summarized in Figure 1.

FOX Transcription Factors and Vascular Smooth Muscle Cells (VSMCs)

VSMC Apoptosis

FoxO transcription factors have roles in regulating VSMC apoptosis. VSMCs are essential for the structural integrity and contractile responses of the arterial vessel wall.[78] During the early phase of atherogenesis, the proliferation of VSMCs in response to inflammatory stimuli dominantes over VSMC apoptosis.[16] As with endothelial cells, apoptosis of VSMCs is an important regulator of the stability of atherosclerotic plaques.[78] Blockage of phosphorylation of FoxO3 correlates with increased VSMC apoptosis.[79] FoxO transcription factors can modulate VSMC cell surface expression of CD95 (Fas) ligand (FasL), which is an important determinant for cell death.[80] Ectopic expression of FoxO3 in VSMCs induces FasL expression and DNA fragmentation, which is partially dependent on the activity of caspase-8.[80] Brunet et al. identified three putative overlapping FoxO3 response elements in the FasL promoter, two of which are found to bind FoxO3.[32]

VSMC Proliferation

FoxO transcription factors also regulate VSMC proliferation. VSMC proliferation and survival are implicated in vascular diseases such as restenosis following angioplasty or stenting. Inactivation of FOX transcription factors can lead to transcriptional down-regulation of p27Kip.[81-83] Down-regulation of p27Kip1 is associated with increased cell cycle entry.[82] FOX transcription factor inactivation and p27Kip1 downregulation are prevented by one of the following approaches: (1) inhibition of PI3K with wortmannin or LY294002; (2) overexpression of a constitutively inactive form of Akt; or (3) overexpression of constitutively active forms of FOX transcription factors.[81-83] The antiproliferative effect of TM-FOXO3 can also be partially reversed by siRNA against p27Kip1.[81] In the carotid artery balloon injury model, TM-FOXO3 delivered by adenovirus to arteries decreases the proliferation of VSMCs and reduces the intima/media ratio with an accompanying increase of p27Kip.[81,83] Recent evidence also suggests that the upregulation of p27Kip may not be the only mechanism by which FoxO inhibits VSMC proliferation.[17] The cysteine-rich protein 61 (CYR61, CCN1), an immediate early gene and a potent angiogenic factor rapidly expressed and secreted from VSMCs after angioplasty or Ang II stimulation. CYR61 has been shown to be negatively regulated by FoxO in VSMCs.[84] CYR61 is an extracellular matrix-associated protein that can interact with integrins to promote VSMC migration and adhesion. CYR61 has been implicated in processes such as atherosclerosis and vascular resenosis.[85-87] A functional association between FoxO and CYR61 is

first noted after identification of a forkhead binding element in the promoter of the CYR61 gene.[17] Adenoviral delivery of TM-FOXO3 suppresses CYR61 expression, inhibits proliferation and reduces cell viability.[78,84] This repression seems to work via a direct effect because FOXO3a is detected at the CYR61 promoter by chromatin immunoprecipitation.[78] Moreover, a reporter assay shows that deletion of the FOXO binding site in the CYR61 promoter abrogates the repression of CYR61 expression by TM-FOXO3a.[78] Conversely, concomitant delivery of adenoviruses expressing CYR61 and TM-FOXO3a reverses the intima-sparing effect of TM-FOXO3, inhibits FOXO3a-induced cellular detachment and reduces viability in vitro without affecting the proportion of cells in the sub-G1 (presumably apoptotic) population identified by propidium iodide apoptotic cell staining.[78,84] Epidermal growth factor ligand, such as betacellulin (BTC), induces the phosphorylation of FoxO1 and FoxO4, in a dose- and time-dependent manner.[88] FoxO4 localization in the nuclei of cultured aortic smooth muscle cells is associated with reduced expression of myogenic markers.[17] Myogenic differentiation-specific genes that are likely to be repressed by nuclear FoxO4 include SMα-actin, SM-calponin and SM-22α.[17] In agreement with this model, the nuclear translocation of FoxO4 is found to occur in proliferating VSMCs after in vivo vascular injury.[89] Cyclin D1 is a key regulator of cell proliferation that promotes the progression of G1 phase. Recently, a correlation between FoxO phosphorylation and cyclin D1 expression has been found in the regulation of VSMC proliferation.[88] BTC, which increases the expression of cyclin D1 in VSMC, induces the phosphorylation of FoxO via the PI3K/Akt signaling pathway. This indicates that the up-regulation of cyclin D1 induced by BTC in VSMC may be caused by releasing the repression of FoxO factors.

FoxO in VSMC Differentiation

FoxO4 has been shown to modulate the transition of VSMCs from a contractile to a more proliferative phenotype.[90] Myogenic differentiation-specific genes are repressed by nuclear FoxO4. These genes are under positive regulation of myocardin,[91] a transcriptional coactivator that is essential for the induction of differentiation in VSMCs.[92,93] The interaction of FoxO4 with myocardin in cultured aortic smooth muscle cells was shown to repress the differentiation of VSMCs initiated by myocardin.[89] The nuclear translocation of FoxO4 was found to occur in proliferating VSMCs after in vivo vascular injury.[89] This suggests that nuclear prevalence of FoxO4 is necessary to suppress the differentiation program and promote a dedifferentiated and more proliferative phenotype.

FoxO in Aging VSMCs

In VSMCs of old rats, phosphorylated FoxO3a is increased.[94] MnSOD is one of the major cellular antioxidant defense systems and a recent study showed that both MnSOD protein expression and activity are reduced in VSMCs from old animals as compared to that from young animals. FoxO3 interacts with the promoter of the rat MnSOD gene and inhibition of FoxO3a transcription leads to reduction of MnSOD expression.

One of Akt's VSMC protective effects has been identified as its inhibition of FoxO3a or glycogen synthase kinase-3 (GSK3) by phosphorylation. Activation of IGF1R, which is increased in the VSMCs of old animals, leads to the activation of Akt and FoxO3a. Genes for p27Kip, catalase and MnSOD, which play important roles in the control of cell cycle arrest and stress resistance, are found to be FOXO3a targets. IGF1R signaling modulates these genes through activation of the Akt/FOXO3a pathway.[95] The deregulation of the Akt-FoxO3a-GSK3 pathway, due to a reduction of IGF1R signaling, promotes apoptosis in atherosclerosis.[96]

Roles of Other FOX Transcription Factors in VSMCs

In addition to FoxO, other forkhead transcription factors are also involved in VSMC survival. Lungs of Foxm1$^{-/-}$ mice exhibit severe hypertrophy of arteriolar smooth muscle cells and defects in the formation of peripheral pulmonary capillaries as evidenced by significant reduction in the staining of capillary-marker platelet endothelial cell adhesion molecule 1 in the distal lung.[97] Premature expression of the FoxM1b transgene protein accelerates proliferation of different lung cell types, including endothelial cells of pulmonary capillaries and arteries.[98] Thus FOX transcription factors have important roles in multiple types of cardiopulmonary smooth muscle.

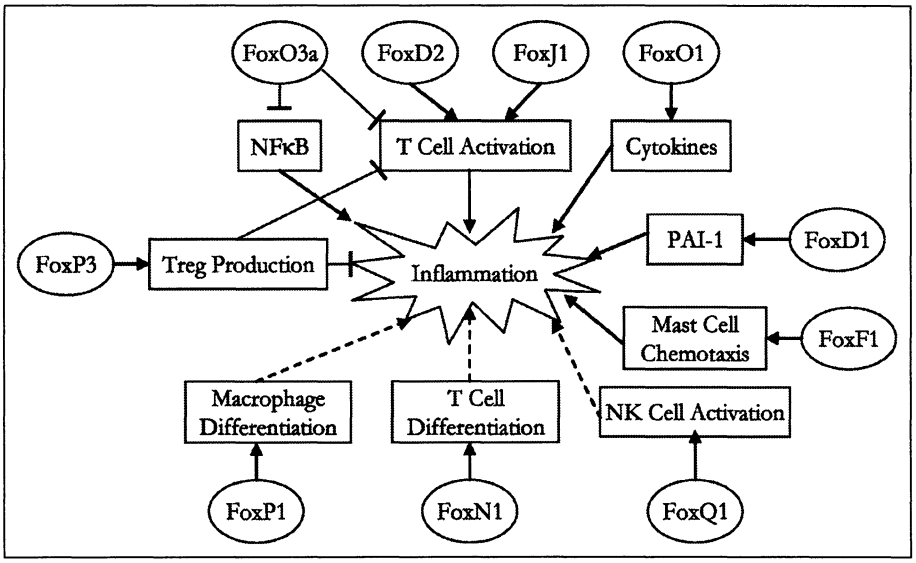

Figure 2. FOX transcription factors in immune responses and inflammation. Various FOX transcription factors mediate immune responses that regulate inflammation. Dashed lines represent pathways that do not necessarily produce inflammation. PAI-1, Plasminogen Activator Inhibitor-1; NF-κB, Nuclear Factor-κB.

FOX Transcription Factors, Inflammation and Immune System

Recent studies performed on hypercholesterolemic mice deficient in different components of the immune system uniformly suggest that the net effect of immune activation is pro-atherogenic and that atherosclerosis, at least to some extent, should be regarded as an autoimmune disease.[3,16,99,100] Therefore, some of the roles of FOX transcription factors in the pathogenesis of cardiovascular diseases are associated with their roles in the regulation of immune responses and inflammation. In addition, it becomes clear that these FOX transcription factors also play crucial roles in various aspects of immune regulation. Several members of the FOX transcription factor family, for example FOXF1, FOXP3, FOXN1, FOXO1 and FOXO3, have been shown to execute diverse functions in regulating inflammation and adaptive immune response.[9,42,101,102] Figure 2 will serve as a reference for our following discussion.

FOX Transcription Factors and Inflammation

FOX transcription factors play important roles in vascular inflammation. FoxF1 (previously known as HFH-8 or Freac-1) is expressed in endothelial and smooth muscle cells in the embryonic and adult lung. Haploinsufficient Foxf1$^{+/-}$ mice develop severe airway obstruction and bronchial edema associated with increased numbers of pulmonary mast cells and increased mast cell degranulation after injury. Pulmonary inflammation in Foxf1$^{+/-}$ mice is associated with diminished expression of Foxf1, increased mast cell tryptase and increased expression of CXCL12, the latter being essential for mast cell migration and chemotaxis. Foxf1 haploinsufficiency causes pulmonary mastocytosis and enhanced pulmonary inflammation after chemically-induced or allergen-mediated lung injury, indicating that Foxf1 plays an important role in inhibiting the pathogenesis of pulmonary inflammatory responses via suppressing mast cell migration and chemotaxis.[103]

FoxD1 has a role in the induction of plasminogen activator inhibitor-1 (PAI-1), a serpin class protease inhibitor that plays a central role in the regulation of vascular function and tissue remodeling by modulating thrombosis, inflammation and the extracellular matrix. A central mediator in controlling PAI-1 expression is immunosuppressive cytokine TGF-β, which induces PAI-1 expression and promotes fibrosis. Overexpression of Smad6s (an endothelial splice variant) from

the Smad family of signal transduction molecules in endothelial cells increases PAI-1 promoter activity and secretion, whereas antisense Smad6s suppresses the induction of PAI-1 by TGF-β. The levels of Smad6s can alter the levels of TGF-β and the subsequent induction of PAI-1 via a FoxD1 transcription site. Further data suggests that this process, which is up-regulated in diseased vessels, can be modulated by the inhibition of protein kinase C (PKC)-β.[104]

FoxO proteins may have a role in propagating the obesity-associated low-grade inflammation in adipose tissue that results from increased production of pro-inflammatory cytokines. Subsequently, increased proinflammatory cytokines can contribute to the development of insulin resistance. Tumor necrosis factor (TNF)-α treatment attenuates Akt-dependent phosphorylation of FoxO1 and enhances transcriptional activity of FoxO1. FoxO1 increases the expression of CCAAT/enhancer binding protein (C/EBPβ, a positive regulator of monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-6 genes) through directly binding to its promoter. These findings suggest that activation of FoxO1 triggered by TNF-α up-regulates the expression of C/EBPβ in 3T3-L1 adipocytes, thereby leading to an increased production of pro-inflammatory cytokines, MCP-1 and IL-6.[105] However, unlike TNF-α, bacterial endotoxin LPS utilizes the PI3K pathway to inhibit FoxO3a. Inhibition of PI3K attenuates LPS-induced production of proinflammatory cytokine IL-8. LPS-induced IL-8 is increased in HT-29 cells with silenced FOXO3a. Moreover, in HT-29 cells with silenced FOXO3a, the expression level of IκBα, an NF-κB inhibitor, is decreased. Thus, LPS and bacterial infection inactivate FoxO3a in intestinal epithelia via the PI3K pathway and further suppresses IκBα, leading to the activation of NF-κB and subsequent upregulation of IL-8.[106] Currently, it remains unclear whether the roles of FOX factors in regulating proinflammatory cytokines either positively or negatively are cell-specific, cytokine specific, or inflammation phase specific.

FoxP3, Regulatory T-Cells and Immune Suppression

FoxP3 is by far the most intensely studied forkhead family member in immune regulation due largely to its roles in differentiation, homeostasis and suppression of $CD4^+CD25^{high}$ regulatory T-cells (Tregs).[9] Tregs, characterized by high expression of CD25 (an IL-2 receptor α-chain), comprise 5-10% of the total population of $CD4^+$ T-cells in mice. Tregs downregulate the reactivity of $CD4^+CD25-$ T helper cells (Th cells) and play crucial roles in the suppression of inflammation, antitumor immune responses, autoimmune diseases and transplant rejection.[9,107-109] FoxP3 is highly expressed in Tregs in both humans and in mice.[110,111] The Scurfy mice have been identified to have defective Foxp3 genes. The function of Foxp3 is essential for normal immune homeostasis.[112] The phenotypes in the Scurfy mice include lethality of hemizygous males 16-25 days after birth and overproliferation of activated $CD4^+$ T-cells with multi-organ infiltration.[113] Interestingly, adoptive transfer of wild-type lymphocytes can control the T-cell activation in the Scurfy mice and prevents autoimmune disease development,[113] which indicates that the Scurfy mice lack a certain lymphoid compartment that can repress the activity of activated T-cells. A variety of studies on patients' families also indicate that FOXP3 mutation leads to the immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans.[114] Patients with IPEX have massive T-cell infiltration into the skin and gastrointestinal tract as well as high levels of autoantibodies in serum. Foxp3 transgenic mice have increased numbers of Tregs, which strongly suppress the proliferation of antigen-stimulated $CD4^+$ T-cells. Transgenic $CD4^+$ and $CD8^+$ cells are hyporesponsive to activation. All of the disease manifestations and animal models support the crucial role of FoxP3 in Tregs' development. Most recently, Yang's laboratory and others showed that the survival/apoptosis pathway of Foxp3-controlled $CD4^+CD25^{high}$ Tregs modulate vascular inflammation,[10,11] diabetes[12] and atherosclerosis.[13] These studies clearly suggest that Foxp3, through its function in differentiation and promotion of Tregs' homeostasis and suppressive function, suppresses inflammation and inhibits certain inflammatory cardiovascular diseases like atherosclerosis and hyperlipidemia. Tregs represent a safe and efficient source for therapy and they could become an important weapon in the fight against immune mediated pathology.[115]

FoxN1 and Thymocyte Development

FoxN1 has an important role in epithelial cell development. FoxN1 mediates thymic epithelial cell differentiation both in mice and human. Thymic epithelial cells create a proper microenvironment in the thymus stoma for the development and selection of thymocytes (precursors of T-cells).[116] The role of FoxN1 in thymic epithelial development makes it a focus for immunology research.[117] Mouse nude mutations, eliminating the DNA binding domain of FoxN1, lead to defective differentiation of epithelial progenitor cells in the thymus.[118] Consequently, these defective epithelial cells fail to attract lymphoid progenitors to the thymic anlage and finally results in defective thymocyte/T cell development.[119] Human nude/severe combined immunodeficiency (SCID) syndrome, consisting of T-cell deficiency, congenital alopecia and nail dystrophy, is caused by a nonsense mutation in FOXN1.[120] Even bone marrow transplantation cannot restore normal levels of $CD4^+$ T-cells in this syndrome, suggesting that defects resulting from FOXN1 mutation are thymus-derived but not bone marrow-derived.

FOX Transcription Factors and T-Cell Activation

Although the FoxO transcription factors have been widely studied for their metabolic and homeostatic roles, the immunological role of FoxO3a in suppressing spontaneous T-cell activation and autoimmunity makes it a widely studied target in immunology. Interferon-γ driven tryptophan catabolism by cytotoxic T-lymphocyte antigen 4 (CTLA-4) might activate FoxO3a to protect dendritic cells from injury in nonobese diabetic mice.[121] At the transcriptional level, FoxO3a is the dominant isoform expressed in lymphocytes. $Foxo3a^{-/-}$ mice develop lymphoproliferative disease with multi-organ infiltrates, resulting in multisystem inflammation,enlarged spleen and lymph nodes due to the increased lymphocyte proliferation.[122] Foxo3a deficient mice have spontaneous, autoreactive helper T-cell activation. $FoxO3a^{-/-}$ deficient T-cells possess increased spontaneous NF-κB activity and are relatively deficient in the NF-κB inhibitors, IκBβ and IκBε subunits.[101] FoxO3a can also regulate cell proliferation and apoptosis, both of which contribute to lymphocyte homeostasis. For example, FOXO3a has been shown to regulate cell division through a cyclin G2-dependent mechanism.[123] Cyclin G2 has been shown to maintain the quiescent state of differentiated cells and negatively regulates lymphocyte proliferation. Activated FOXO3a has been shown to control the expression of some proapoptotic genes, for example, FasL. No human immunological diseases caused by FOXO3a defects have been clearly identified. However, there is FoxO3a dysregulation in cancers in which anticancer immunosurveillance is weakened. For example, mixed lineage leukemia transcription factor fusion proteins with FoxO3a have been identified in acute lymphoblastic leukemia.[124] Many other forkhead transcription factors also play important role in the regulation of a variety of immunologic functions. FoxJ1 suppresses spontaneous T-cell activation and autoimmunity.[125] In animals with FoxJ1 (hepatocyte nuclear factor/forkhead homolog 4, HNF-4, FKHL-13) deficient lymphoid systems, Th spontaneously activate, resulting in multi-system inflammation, particularly of the lung, liver, kidney and salivary glands. Unlike FoxO3a deficiency, FoxJ1 deficiency appears to be much more severe, affecting a different spectrum of organs and skewing toward cytokine production by Type I Th (Th1).[101] Moreover, recent reports show that FoxQ1 promotes natural killer cell function.[126] FoxP1 regulates tissue macrophage differentiation.[101] FoxD2 modulates T-cell activation by fine-tuning sensitivity to cAMP.[101] Thus it is seen that numerous Fox proteins have numerous roles in regulating immunological activity as summarized in Figure 2.

Conclusion

Mammalian FOX transcription factors have increasingly become recognized as important targets for disorders of the cardiovascular system and in the immunoregulation of cardiovascular disease pathogenesis. Knowledge of FOX transcription factors will continue to lay the foundation for the successful translation of these transcription factors into novel and robust clinical therapies.[127]

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CHAPTER 8

Regulatory T-Cells, FoxP3 and Atherosclerosis

Michal Entin-Meer, Arnon Afek and Jacob George*

Abstract

Innate immune responses follow accumulation of modified lipids within the arterial wall thereby influencing atherosclerotic plaque progression. One of the mechanisms evolved in maintaining immunologic self-tolerance involves upregulation of regulatory T-cells, among which the $CD4^+CD25^+$ $FoxP3^+$ regulatory T-cells (Treg) are best characterized. The putative important role of Treg in the initiation of atherosclerotic lesions as well as in the progression towards unstable plaques leading to ischemic events, supported by human studies and, indirectly, by murine models. Herein, we summarize the experimental approaches taken in order to study the possible mechanisms of Treg involvement in atherosclerosis as well as the beneficial clinical potential of Treg in stabilizing atherosclerotic plaques.

Atherosclerosis, Inflammation and Autoimmunity

The immune system plays a pivotal role in the pathogenesis of atherosclerosis, the underlying cause of many cardiovascular diseases, including myocardial infarction, stroke and ischemic gangrene.[1,2] Atherosclerosis involves the innate immune responses with the recruitment and activation of monocytes/macrophages that respond to the accumulation of modified lipids, mainly the oxidatively modified LDL (OxLDL) within the arterial wall. These events are possibly followed by adoptive immune responses comprising differential antigen-specific T-lymphocytes. Most of the effector T-lymphocytes in atherosclerotic lesions are $CD4^+$ T-helper cells with the phenotype characteristic of a proinflammatory T-helper 1 (Th1) subset.[3-5] Most of the T-cells bear T-cell receptors (TCR)[6,7] and are often found in clusters in shoulder regions of the lesion.[8,9] These cells specifically recognize antigens that are produced in relative abundance in hypercholesterolemic individuals or in plaques, including Ox-LDL and HSP 60/65 in the form of antigen-presenting cells(APC) such as macrophages or dendritic cells.[10] The accumulation of inflammatory cells within the arterial wall leads to local production of chemokines, interleukines and proteases that enhance the influx of monocytes and lymphocytes, among which are IFN-gamma, tumor necrosis factor (TNF)-alpha and membrane CD40 ligand, thereby amplifying the immune response and promoting progression of atherosclerotic lesions.

Regulatory T-Cells, Developmental and Functional Aspects

Many mechanisms have evolved to maintain immunologic self-tolerance and to limit responses to foreign antigens.[13] One of these mechanisms involves regulatory T-cells that actively suppress responses of effector T-cells, via homing in on peripheral tissues in order to maintain self-tolerance and to prevent autoimmunity by inhibiting pathogenic lymphocytes. Several types of regulatory

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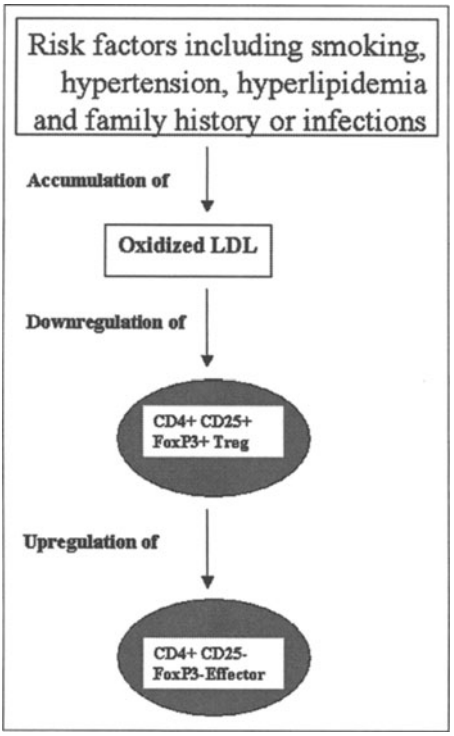

Figure 1. Potential involvement of Treg in atherosclerosis.

T-cells have been identified, including IL-10-producing Type 1 regulatory T-cells (Tr1), transforming growth factor beta (TGF beta)-producing Th3 cells[14,15] and the $CD4^+CD25^+$ (interleukin-2 receptor-α chain) $FoxP3^+$ regulatory T-cells (Treg) which are best ones characterized. Tregs are natural regulatory T-cells that mature in the thymus and comprise 5% to 10% of the peripheral $CD4^+$ T-cells.[16] FoxP3, a forkhead family transcription factor, is a lineage-specific factor for Treg, which plays a crucial role in their suppressive function as outlined in Figure 1. Whereas initial studies characterized these cells by their co-expression of CD4 and CD25 surface markers, subsequent reports identified expression of other surface markers including CTLA-4 (Cytotoxic T-Lymphocye Antigen 4 also known as CD152) and GITR (Glucocorticoid-Induced TNF Receptor)[17,18] as well as CD103, CD62L, lymphocyte activation gene 3 protein (LAG 3), C-C chemokine receptor Type 5 (CCR5) and neurophilin, and the concomitant absence of certain markers such as CD127 (the alpha chain of the IL-7 receptor).[18-21] Major progress in the understanding of the homeostasis of naturally occurring Tregs was made with the identification of FoxP3 as a requisite factor for the development of Tregs and for their suppressive functions, as will be described in detail in the section below.

Natural Treg are generated during thymic development, but are also induced in peripheral tissues during immune responses[16] and atherosclerosis(Fig. 2). Treg express antigen receptors typical of effector T-cells and are presumably activated by peptide antigens presented by APCs. They also acquire interleukin (IL)-2 receptor for development and survival. In this context, two populations of potential Treg have been described: those that originate from a committed lineage of FoxP3-expressing cells in the thymus and those that convert from mature $CD4^+$ cells in the periphery.[22] The basic characteristics of natural Treg, and adaptive Treg versus effector T-cells are summarized in Table 1. Three general models of suppression have been proposed to explain the inhibitory actions of Treg cells on activated T-cells, none of which have been completely elucidated: 1. Cell contact-dependant suppression

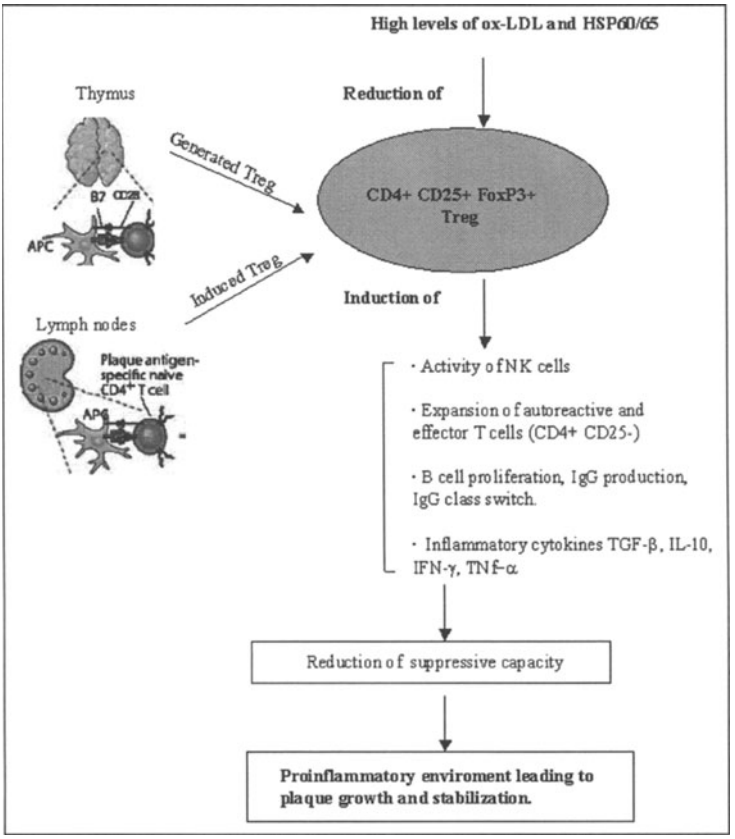

Figure 2. Treg and atherosclerosis. Natural Treg develop in the thymus and may be induced in peripheral tissues. Naïve T-cells specific for plaque antigens (such as oxidized LDL and HSP60) are activated by APCs and differentiate into Th1 effector cells, which migrate into atherosclerotic lesions, reactivated by lesional APCs, secrete IFN-gamma and promote disease. The reduced numbers and functional impairment of Tregs could promote atherosclerosis by several mechanisms as described.

mediated by engagement of CTLA-4 (expressed on Treg cells) with CD80 molecules (expressed on effector T-cells) or interaction of CTLA-4 with CD80/CD86 on APCs.[23] 2. Consumption and limitation of growth factors such as IL-2. Effector T-cells secrete IL-2 upon activation, which binds to CD25 on Treg cells, thus maintaining and activating Treg cell genes such as FoxP3, which in turn down-regulate IL-2 secretion in a feedback loop. This action results in deprivation of effector T-cells from the essential growth factor IL-2. This feedback process might therefore induce apoptosis of activated T-cells in vitro and in vivo.[24] 3. Production of inhibitory cytokines, including IL-10, TGF-β or IL-35.[25] Production of these cytokines may induce deactivation of dendritic cells, leading to a loss of ability to activate effector T-cells with distinct antigen specificity to Treg cells, a mechanism called 'bystander immune suppression'. In addition, TGF-β inhibits the proliferation, activation and differentiation of T-cells towards Th1 and Th2.[26,27]

FoxP3 (Forkhead Box Protein P3), a member of the forkhead winged helix protein family of transcription factors, was demonstrated to govern mouse $CD4^+CD25^+$ Treg function.[28,29] Loss of function mutations of FoxP3 were shown to eliminate $CD25^+$ Treg and result in lethal lymphoproliferative autoimmune syndrome in mice associated with extremely enlarged spleens

Table 1. A basic comparison between naturally-occurring Tregs, induced Tregs and effector T cells

	Natural Treg	Induced Treg	Effector T cells
Generation site	Thymus	Peripheral lymph nodes	Thymus
CD25 expression	High	Variable	No
FoxP3 expression	Yes	Yes	No
IL-2 dependency	Yes	Yes	No
Specificity	Self	Self and foreign	Foreign

and lymph nodes and lymphocytic infiltrates in multiple organs, associated with deficiency or malfunction of Treg.[30] This finding was strongly supported by the observation that patients with the rare immune system dysregulation, polyendocrinopathy, enterophathy and X-linked inheritance (IPEX), have a severe inflammatory disease accompanied by a mutation in the FOXP3 gene.[31] The requirement of FoxP3 in $CD4^+CD25^+$ regulatory T-cell development was demonstrated upon generation of a mixed bone marrow (BM) chimeric mice in which lethally- irradiated C57BL/6 (B6) $Thy1.1^+$ congenic mice were reconstituted with T-cell-depleted BM from congenic B6 $Ly5.1^+$ mice mixed at a 1:1 ratio with BM from either $FoxP3^-$ or $FoxP3^+$ mice. The $CD4^+CD25^+$ regulatory T-cell population in the ($Ly5.1^+B6^+FoxP3^-$) chimeras was solely of $Ly5.1^+B6$ origin in both the thymus and lymph nodes, whereas, both $Ly5.1^+B6$ and $FoxP3^+$ BMs contributed equally to the $CD4^+CD25^+$ regulatory T-cell compartment in the ($Ly5.1B6^+$ $FoxP3^{+/+}$) chimeras.[28] Moreover, ectopic FoxP3 expression was found to be sufficient to activate a program of suppressor function in peripheral $CD4^+CD25^-$,[28,29] pointing to FoxP3 as a unique marker of $CD4^+CD25^+$ Treg, distinguishing them from activated $CD4^+CD25^-$ T-cells and as a master transcriptional regulator of Treg homeostasis. Therefore, in contrast to other molecular markers used to identify regulatory T-cells, such as GITR, CTLA-4 and CD25, FoxP3 is not upregulated by activated $CD4^+CD25^-$ T-cells.

Recently, several monoclonal antibodies specific for human Foxp3 became available for detection of endogenous human FoxP3 by flow cytometry and immunohistochemistry.[32] Similar to murine FoxP3, the majority of human FoxP3 was also expressed by the majority of the $CD4^+CD25^{high}$ T-cells in peripheral blood, enabling investigation of human FoxP3 for clinical use. FoxP3-GFP knock-in mice[33] as well as FoxP3-GFP-hCre bacterial artificial chromosome transgenic mouse[34] were recently created. Those mouse strains may pave the way for better characterization of the different $FoxP3^+$Treg subpopulations and thus provide a better analytic tool to identify the subpopulation mostly involved in atherosclerosis progression.

FoxP3 in Experimental Models of Autoimmunity and Atherosclerosis

In recent decades, the role of the immune system in atherosclerosis development has received considerable attention.[1,2] The general belief is that risk factors such as hypertension, hyperlipidemia, family history of premature atherosclerosis as well as infectious pathogens could promote LDL oxidation within the vessel wall and in the circulation. These downregulate the numbers and functions of FoxP3-expressing Treg (Fig. 1).[35] In the last decade experimental approaches successfully used in other disease model, have been employed to test the importance of autoimmunity in the development of atherosclerosis. Initial studies have identified putative autoantigens within atherosclerotic plaques, including heat shock proteins, oxidized LDL and β2-glycoprotein.[36-38] Several studies, some of which were performed in our laboratory, demonstrated that adoptive transfer of antigen-responsive lymphocytes or alternatively passive transfer of antibodies, significantly enhance development of atherosclerosis in experimental models.[39-41] Furthermore, induction of immune tolerance to plaque-associated components, such as OxLDL, attenuated the progression of atherosclerosis in mice.[42-44] Several studies were later conducted in an attempt to elucidate the potential role of the

$CD4^+CD25^+FoxP3^+$ Treg cell repertoire in the control of atherosclerotic plaque development. Since CD25-deficient (IL2rα−/−) mice die prematurely from severe autoimmune disease with cachexia and malabsorption,[45] they are not suitable for the study of the effect of Treg cell deficiency on atherosclerosis. Therefore, two alternative transgenic atherosclerosis-prone mice strains have been studied for assessment of the development of atherosclerotic plaques: 1. The apolipoprotein E-deficient (ApoE−/−) mice. These mice develop complex atherosclerotic lesions that result from plasma accumulation of cholesterol-rich lipoproteins.[46] The number and functional properties of Treg were found to be compromised in ApoE−/− mice compared with those in wild-type C57BL/6 littermates.[47,48] 2. The low-density lipoprotein receptor-deficient (Ldlr−/−) mice, known to be susceptible to development of atherosclerosis when fed a high-fat, high- cholesterol diet.[49] The experimental design included depletion of Treg cells by either genetic or antibody-mediated means[50] and by enrichment of Treg by adoptive transfer, as reported by our research group and others.[50,51] Using these approaches, a direct effect of Treg on atherosclerosis was demonstrated. Our group has recently shown that compared with controls, ApoE−/− mice exhibit reduced Treg numbers and compromised Treg function.[51] Interestingly, proatherogenic Ox LDL triggered a more robust depletion in the splenic Treg population than in the effector T-cell population, and the ApoE−/− mice were more susceptible to this attenuation than control animals. Moreover, Treg deficiency related to genetic ablation of the B71/2-CD28 costimulatory pathway in the hematopoietic compartment was shown to enhance atherosclerotic lesion development in Ldlr−/− mice.[52] Treg depletion using an anti-CD25 antibody also enhanced atherosclerosis in ApoE−/− mice.[50] Deficiency of the T-cell costimulatory molecule ICOS resulted in enhanced atherosclerosis in Ldlr−/− mice, which can be attributed to an impaired Treg development and function.[53] Interestingly, Treg depletion did not influence lesion size or inflammatory phenotype when a host effector T-cell population was genetically engineered to be insensitive to TGFβ.[50] This finding together with a previous work showing markedly enhanced atherosclerosis in ApoE −/− mice with TGF-β resistant T-cells,[50] suggests that TGF-β is required for the atheroprotective effects of Treg. Reduction in atherosclerosis in Apo E −/− mice has also been achieved through adoptive transfer of $CD4^+$ $CD25^+$ regulatory T-cells,[50,51] possibly through expression of distinct forms of TNF-alpha in ApoE (−/−)mice[54] or via induction of oral tolerance to HSP60 in Ldlr (−/−) mice.[44] A recent study performed in our laboratory demonstrated an association between hypoxia and the homeostasis of Treg mediated by upregulation of HIF-1α (hypoxia-inducing factor alpha), pointing to the additional potential mechanisms of vasculo-protective effects of Treg.[55] In vivo expression of HIF-1α achieved by hydrodynamic injection of HIF-1α expressing vector induced an increase in FoxP3 expression and an increase in the number of functionally active $FoxP3^+CD4^+CD25^+$ Treg. We therefore assume that hypoxic sites (tumoral, ischemic, inflammatory) may downregulate local early Th1-mediated inflammatory response by inducing expression of HIF-1α within local lymphocytes with consequent upregulation of the Treg pool.

Foxp3, Regulatory T-Cells and Atherosclerosis in Humans

When comparing the data from human studies investigating the potential involvement of Treg in atherosclerosis with data from murine studies, it is important to keep in mind one crucial factor. Whereas most murine studies test plaque burden as determined by lipid accumulation, in humans it is practically impossible to quantitatively evaluate the extent of atherosclerotic vasculatur.[56] In humans, a more realistic marker for assessing atherosclerosis may be the clinical syndrome, namely the presence of plaque rupture as evidenced by the occurrence of acute coronary syndromes (ACS). It is now recognized that most plaques that cause ACS exhibit angiographic obstruction of less than 70%[57,58] and that the onset of ACS is mainly associated with changes in the inflammatory response in these lesions, including a shift in the phenotype of intraplaque T-cells.[59,60] The majority of ACS-related atheromas are caused by rupture of plaques consisting of a large, thrombogenic core of lipid and necrotic debris, including foci of macrophages, T-cells, old haemorrhage, angiogenesis and calcium. The factors that govern the transition of the plaque from a stable to a rupture-prone lesion are not entirely understood. However, accumulating evidence supports the role of immune system dysregulation, including reduction and impaired function of the pool of the naturally

occurring $CD4^+CD25^+$ $FoxP3^+$ Tregs in the alteration of the plaque phenotype. Indeed, in two independent studies, one of which was performed in our laboratory, Treg purified from peripheral blood of patients with ACS exhibited a significantly reduced expression of FoxP3 compared to blood from patients with stable angina or from normal coronary arteries.[61,62] An additional in situ study pioneered by De Boer et al[63] demonstrated significantly reduced mean numbers of intimal as well as adventitias FoxP3 and GITR in atherosclerotic lesions compared to inflammatory skin lesions,[63] as opposed to normal vessel fragments in which T-cells were virtually absent. This novel finding may account for the chronic inflammatory process that takes place throughout the long-standing course of atherosclerosis. In addition, high-risk lesions contained significantly-increased numbers of Treg compared to early lesions. Similarly, the frequency of $FoxP3^+$ cells in high-risk lesions was somewhat higher compared to the stable ones. Similar to Treg, the frequency of activated T-lymphocytes is reported to be significantly increased in unstable lesions[59,60] and the onset of ACS was shown to be associated with the antigen-driven proliferation of certain T-cell subpopulations.[64] It appears that the overall increase in T-cell-mediated inflammatory activity within the unstable plaque environment may account for the subsequent increased frequency of Treg in these unstable lesions. De Boer et al[63] speculated that the reason for this low frequency of Treg in atherosclerosis may rise from local inhibition by oxidized lipids already present in the intima or from the direct contact with plaque-derived lipoproteins transported via microvessels to the adventitia. The mechanisms involved in Treg suppression of proatherogenic immune responses, however, have yet to be resolved. The mechanisms may involve contact-dependant or cytokine-dependant suppression as some studies would suggest.[44,50,53] However, when interpreting the data, caution should be taken when extrapolating findings from animal models to humans. One should keep in mind that unlike mice, in which most $CD4^+CD25^+$ Treg express FoxP3, this master transcription factor is less abundant in humans in an equivalent population.[65] Moreover, in humans, T-cell receptor engagement is sufficient to stimulate a notable expression of FoxP3, whereas this is not evident in $CD25^-$ cells from mice.

Our laboratory has recently demonstrated that several statins (HMG-CoA reductase inhibitors), which are in widespread use due to their LDL-reducing properties and concomitant improvement of clinical outcome in patients with and without preexisting atherosclerosis, induce expansion of functionally active $CD4^+CD25^+Foxp3^+$ Treg in humans in vitro and in vivo.[66] Increased numbers of Treg cells by statins in the atherosclerotic lesion may be beneficial by reducing the pathogenic responses mediated by the effector T-cells in the atheroma and thus possibly enhance the stability of the atheromatous plaque. Altogether, those studies shed light on the encouraging beneficial clinical potential of Treg in stabilizing atherosclerotic plaques.

Treg and Atherosclerosis: Prospects

The last decade has witnessed very important progress in our understanding of the pathophysiology of atherosclerosis. The discovery of endogenous counter-regulators of the pathogenic immune response in atherosclerosis led to the identification of an important role for Treg cells in the control of lesion development and/or progression. FoxP3 was demonstrated to be a "master regulator" gene for this subset of T-cells. Data gathered from in vivo data in general, and in particular data demonstrating that increasing the numbers of Tregs in the atherosclerosis prone (ApoE−/−) mice by means of adoptive transfer leads to smaller atherosclerotic lesions, suggests that the Treg population appears to be capable of modifying plaque burden in vivo. Reduced numbers of functionally active intraplaque Treg as well as in peripheral blood in patients with ACS compared to bood from patients with stable plaques or blood from healthy individuals further supports the perception of Treg involvement in immunomodulatory reactions protecting from coronary diseases. Although the data reviewed here suggest that Treg function has a central role in the regulation of the proatherogenic T-cell response, much effort should be directed towards the delineation of the major determinants of the regulatory response and to the molecular mechanisms involved in their survival, homing and suppressive function. The potential for treating atherosclerosis by manipulating Treg responses will require a better characterization

of the antigens that proatherogenic T-cells recognize and the antigens that drive development of peripheral induction of Treg which migrate into atherosclerotic lesions. Identification of such antigens might pave the way for vaccination-like strategies using such antigens to promote a disease-specific regulatory response and reduce atherosclerosis development. In addition, greater knowledge about the long-term behavior of Treg after transfer to humans is also essential in order to establish treatment protocols. Thus preliminary human trials of adoptive Treg transfer may provide further insights into the use of Treg- modulating strategies in the treatment of patients with atherosclerosis and ACS. Lastly, although the expression of FoxP3 is now accepted as the gold standard for defining either thymic-derived Treg cells or Treg cells that might be generated in the periphery, one must consider the potential role of subpopulations of $FoxP3^+$ Treg with different functional properties, especially in humans in whom the CD25 expression levels might vary among the $CD4^+FoxP3^+$ T-cell expressors.

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Section III

Dissecting the Pathways of Neuronal Integration and Plasticity in the Nervous System

CHAPTER 9

FOXP Genes, Neural Development, Speech and Language Disorders

Hiroshi Takahashi,* Kaoru Takahashi and Fu-Chin Liu

Abstract

Foxp subfamily genes were recently recognized to be members of the *Fox* gene family. *Foxp* subfamily members contain a zinc finger domain and a leucine zipper motif in addition to a forkhead domain and their DNA binding capacities and transcriptional activities are regulated by homo- and heterodimerization via a zinc finger and a leucine zipper motif. Three Foxp subfamily members are abundantly expressed in developing brains. The expression patterns of these genes are overlapping, but they are distinctly expressed in some regions. Thus these genes appear to be involved in the development control of the central nervous system. Recently, *FOXP2*, a member of the *Foxp* subfamily, was identified as the first gene to be linked to an inherited form of language and speech disorder. The discovery of a mutation in *FOXP2* in a family with a speech and language disorder opened a new window to understanding the genetic cascades and neural circuits that underlie speech and language via molecular approaches. The spatiotemporal *FOXP2* mRNA expression pattern suggests that the basic neural network that underlies speech and language may include motor-related circuits, including frontostriatal and/or frontocerebellar circuits. This assumption is supported by brain imaging data obtained by using fMRI and PET on the *FOXP2*-mutated patients and also by analysis of Foxp2 mutant mice.

Introduction

The *Fox* gene family encodes a large group of transcription factors that share a common DNA binding domain of sequences called the forkhead or winged helix motif after the founding member of this gene family, *forkhead* in *Drosophila*.[1] Many Fox family members are involved in embryonic morphogenesis and mutations in *Fox* genes have been implicated in a range of human developmental disorders.[2] *Foxp* subfamily genes were recently recognized to be members of the *Fox* gene family. Members of the *Foxp* subfamily contain a zinc finger domain and a leucine zipper motif in addition to a forkhead domain.[3] Recent studies have revealed that three *Foxp* subfamily members are abundantly expressed in developing brains and that the expression patterns of these genes are overlapping, but distinctly in some regions. Thus these genes appear to be involved in development of the central nervous system. Recently, *FOXP2*, a member of the *Foxp* subfamily, was identified. It is the first gene to be linked to an inherited form of language and speech disorder.[4] The discovery of a mutation in *FOXP2* in a family with a speech and language disorder opens a new window to understanding of the genetic cascades and neural circuits that underlie speech and language via molecular approaches. In this chapter, we focus on the neural expression of *FOXP2* as a 'Language Gene' as well as the expression patterns of other *Foxp* subfamily members and their correlation with anatomical and functional abnormalities in the brains of *FOXP2*-mutated patients.

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The Foxp Subfamily

The Foxp subfamily, which consists of four members, Foxp1, Foxp2, Foxp3 and Foxp4, is characterized on the basis of its members containing a C2H2-type zinc finger domain and a leucine zipper motif in addition to a forkhead domain at the C-terminus.[3-6] C-terminal location of the forkhead domain is an atypical feature in the Foxp subfamily, as most other Fox family members have this domain in N-terminal portion. Among the subfamily members, Foxp1, Foxp2 and Foxp4 are highly homologous (showing more than 60% identity at the amino acid level); in particular, their forkhead domains show approximately 80% identity at the amino acid level. Also, Foxp1 and Foxp2, but not Foxp4, have polyglutamine tracts at the N-terminus and these may be involved in protein-protein interactions.

Members of the Fox family of proteins have been demonstrated to bind to target DNA as monomers. By contrast, Foxp1, Foxp2 and Foxp4 proteins require dimerization for DNA binding and their transcriptional activities are regulated by homo- and heterodimerization.[7] The dimerizations are dependent on the conserved stretch of sequence, containing a zinc finger and a leucine zipper motif.

Although one might suspect that the *FOXP2* gene, being linked to an inherited language and speech disorder, might be a human-specific gene, because speech and language is unique to humans, orthologs exist in many species. Comparison of the *Foxp2* genes of many organisms has revealed that the Foxp2 protein is rather extraordinarily conserved (among the 5% most conserved proteins) among mammals.[8] There are only two amino acids different (out of 715 amino acid residues) between humans and chimpanzees and three different between humans and mice. Surprisingly, the amino acid sequence of the forkhead domain is completely identical among rodents, nonhuman primates and humans. Recently, Krause and colleagues[9] reported that the Neanderthals carried a FOXP2 protein that was identical to that of modern humans in the two positions that differed between humans and chimpanzees.

Discovery of *FOXP2* as a 'Language Gene'

Speech and language disorders are common in childhood. Although twin studies have shown that genetic factors play an important role in the etiology of such disorder, a gene that predisposes individuals to speech and language disorders had not been identified until *FOXP2* was discovered.

In 1990, Hurst and colleagues reported a unique case of a large three-generation pedigree (called the KE family), half of whose members have a developmental verbal dyspraxia that is inherited in a pattern consistent with an autosomal dominant penetrance.[10] (Details of the language impairments of the KE family will be addressed later). Using standard positional cloning techniques in combination with bioinformatics, Fisher and colleagues[11] performed a genome-wide search for the candidate gene underlying the speech and language disorders in this family. They mapped the gene locus to the long arm of chromosome 7. In 2001, they finally identified *FOXP2* as the gene responsible for this speech and language disorder by further analyzing the breaking point of the genome of a patient, CS, who had similar symptoms to the affected members of the KE family and a translocation between chromosomes 5 and 7.[4]

The one point mutation in the *FOXP2* gene of the affected members of the KE family is predicted to result in an arginine-to-histidine substitution (R553H) in the forkhead domain of the FOXP2 protein. R553 is invariant among all FOX proteins in species ranging from yeast to humans. This mutation occurred in every affected KE family member, but not in unaffected members, nor in unrelated control subjects. The translocation breakpoint in CS disrupted the gene structure of FOXP2. Furthermore, a nonsense mutation at arginine 328 (R328X) in the *FOXP2* gene was found in a family, whose affected members had orofacial dyspraxia.[12] Therefore, it is likely that the amino acid substitution in FOXP2 protein leads to a loss of function of one copy of the FOXP2 gene and that the remaining copy is insufficient for *FOXP2* function (haploinsufficiency). There are several examples of human disease states regarded to be s consequence of haploinsufficiency of FOX proteins: mutations in *FOXC1, FOXC2, FOXE1* and *FOXL2* in humans are associated with

congenital hereditary glaucoma, hereditary lymphedema-distichiasis syndrome, thyroid agenesis and ovarian failure with craniofacial anomalies (blephalophimosis/ptosis/epicanthus inversus syndrome) with autosomal dominant inheritance.[13-16]

FOXP2 and Specific Language Impairment (SLI) and Autism

Although the phenotype in the KE family characterized by verbal dyspraxia does not duplicate the language abnormalities of autism and common forms of specific language impairment, chromosome 7q31, in which FOXP2 is located, has been considered to be a potential susceptibility locus for the language deficits in specific language impairment (SLI) and autism. Therefore, association and mutation screening analyses on *FOXP2* gene have been performed in these disease groups.

Chromosome 7q31 has been implicated in SLI.[17,18] No mutations were found in exon 14 (where the KE family mutation exists) of the *FOXP2* gene, but a strong association to genetic markers adjacent to *FOXP2* was found. However, no mutation or association with *FOXP2* within SLI patients was found in two studies.[19,20] Thus it is still unclear whether the role of FOXP2 in speech and language disorders is generalized to more common and genetically complex forms of language impairment.

Chromosome 7q31 has been repeatedly linked to autism, suggesting that this chromosomal region is likely to harbor a susceptibility gene for autism. Therefore, association studies with *FOXP2* and autism were conducted. Although two genetic association studies in Japanese and Chinese subjects showed a positive association,[21,22] the results of the majority of association studies of *FOXP2* and autism have been negative.[23-25] The *FOXP2* gene is very large in size (>600 Mb) and novel exons have recently been found.[26] Further genetic studies on the relationship between *FOXP2* and SLI or autism will be necessary.

Expression of Foxp Subfamily Members in the Brain

The tissue distributions of *Foxp* subfamily genes and proteins have been investigated in many species, such as zebrafish, mice, rats, songbirds, nonhuman primates and humans. *Foxp1, Foxp2* and *Foxp4* are expressed in the brain, whereas *Foxp3* is not. *Foxp3* is exclusively expressed in the immune system.[27] However, the brain is not the only region where *Foxp1, Foxp2* and *Foxp4* are expressed: their expression is also seen in other organs, including the lung, heart and gut.

Since this chapter is focused on the relationship between *Foxp* subfamily members and the nervous system, we will first describe the expression patterns of *Foxp1* and *Foxp4* in other organs briefly, because their mutant mice have some phenotypes in tissues outside the brain.

Murine *Foxp1* is expressed in the developing brain, heart, lung and gut. *Foxp1* null embryos have severe defects in the cardiovascular system, including defects in ventricular and outflow tract separation, endocardial cushion development and cardiac myocyte proliferation and maturation.[28] Because *Foxp1* null embryos die at E14.5, the role of *Foxp1* in the later stages of brain development has not been fully clarified. *Foxp1* null embryos showed abnormalities in motor neuronal identity in the spinal cord.[29,30] In addition, *FOXP1* has received considerable attention in the field of cancer research, as discussed in another chapter.[31]

It has been revealed that *Foxp4* is essential for cardiac morphogenesis: mouse *Foxp4* null mice developed abnormally, with two complete hearts and died in the embryonic stage.[32] Although *Foxp4* is expressed in developing rodent brains as described below, the role of *Foxp4* in neural development has not yet been fully elucidated, because of early embryonic death.

Several groups have demonstrated the expression patterns of *Foxp2* mRNA or protein in rodent, nonhuman primate and human brains.[33-38] The expression patterns of *Foxp2* in fetal mouse, rat, nonhuman primate and human brains show striking similarities at comparative developmental stages. Therefore, we describe the data on the expression pattern in rodents, unless otherwise commented in this section.

Foxp2 is expressed in several structures of the central nervous system during development, including the cerebral cortex, striatum, thalamus, cerebellum and spinal cord. There are many overlaps between the expression patterns of *Foxp2* and those of its paralogs *Foxp1* and *Foxp4*,

although detailed analysis revealed a distinct pattern of expression for each member in some neuronal cell types, even though they are expressed in the same anatomical regions. Given that homo- or heterodimerization of Foxp1, Foxp2 and Foxp4 proteins is required for DNA binding and their transcriptional activities,[7] the precise combination of homodimers and heterodimers of different Foxp proteins in the same neurons may regulate the transcription of downstream target genes during brain development and, thus control the patterning of brain structures.

Basal Ganglia

Foxp1, Foxp2 and *Foxp4* share partially overlapping and yet differentially regulated expression patterns in the striatum during development.[33-37,39,40] During development in rodents, these three *Foxp* genes are expressed in the striatal primordia (lateral ganglionic eminence, LGE). *Foxp2* and *Foxp1* are persistently expressed in adulthood, whereas expression of *Foxp4* is developmentally down-regulated in the postnatal stage.

Although these three genes have a common character in that all three *Foxp* genes are expressed only in the LGE, but not in the MGE (medial ganglionic eminence), there was a subtle difference among the expression patterns of these three genes within the LGE. Recent studies have suggested that the LGE can be divided into a large ventral domain (ventral LGE) giving rise to the striatum and a smaller dorsal domain (dorsal LGE) suggested to give rise to interneurons that migrate in the rostral migratory stream to populate the olfactory bulb.[41] Both *Foxp2* and *Foxp4* are expressed in the subventricular zone (SVZ) and the mantle zone of the dorsal and ventral LGE, while *Foxp1* is only expressed in the SVZ and the mantle zone of the ventral LGE, but not in the dorsal LGE.[36]

The ontogeny of *Foxp* expression is also distinct in the striatal compartments.[35,36] The striatum comprises two distinct neurochemical compartments, striosomes (or patch) and the matrix.[42-44] Neurons in these two compartments differ in terms of the expression levels of various neurochemical molecules, neurogenesis and neural connectivity.

Expression of *Foxp1* starts later than that of *Foxp2* and *Foxp4* in the early embryonic stage. *Foxp1* expression is detected in both striosomal and matrix compartments until adulthood. Although expression of *Foxp2* and *Foxp4* in the striatum starts at the same early embryonic stage and similar and homogeneous expression patterns continue until the late embryonic stage, *Foxp2* expression becomes restricted to the striosomal compartment and continues until adulthood, while *Foxp4* expression declines in compartmental order: first in the striosomes and later in the matrix, from the late embryonic to the early postnatal stages. Thus in a certain time window, the striosomes are $Foxp2^{high}/Foxp4^{low}$, whereas the matrix is $Foxp2^{negative\sim low}/Foxp4^{high}$. The differential expression of *Foxp1, Foxp2* and *Foxp4* might play an important role in establishing specific types of neuron in each compartment of the striatum.

In the fetal human brain, *FOXP2 and FOXP1* mRNAs are also expressed in the striatal primordia.[34,38] The expression of *FOXP2* is developmentally regulated: the expression is quite low in the adult according to northern blot analysis.[4]

In developing nonhuman primates, *FoxP2* is selectively expressed in the striosomal compartment of the basal ganglia in the perinatal period.[37] Thus the *Foxp2/FOXP2* expression pattern in the basal ganglia seems to be conserved in rodents and primates. Nevertheless, there are several aspects in which *Foxp2/FoxP2* striatal expression differs in monkey and rodent brains. First, the striosomal *FoxP2* expression pattern in the monkey striatum is only detected during the perinatal and early postnatal periods and expression declines during postnatal development. By contrast, *Foxp2* striosomal expression in the rodent striatum persists into adulthood. Second, regional differences in *FoxP2* mRNA expression exist within the striatum. The monkey striatum comprises the caudate nucleus and the putamen. In the monkey brain, the *FoxP2* mRNA expression level in the striosomal compartment is differentially regulated in the caudate nucleus and putamen; specifically, *FoxP2* expression in the caudate nucleus is higher than that in the putamen in the perinatal stage and expression is barely detectable in the putamen in the postnatal period (Fig. 1).

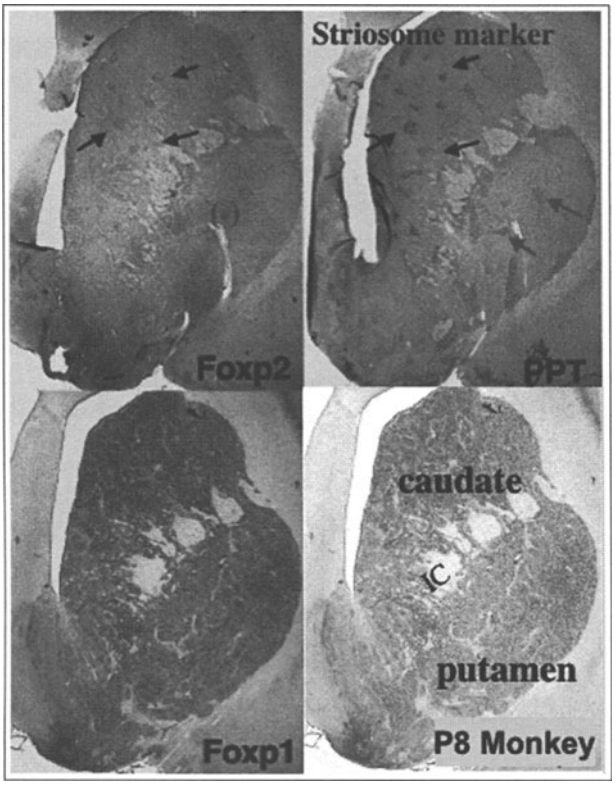

Figure 1. Expression of *FoxP2 and FoxP1* in postnatal monkey striatum. *FoxP2*-positive patches were aligned with *PPT (preprotachykinin)*-positive striosomes in the monkey caudate nucleus. In contrast, *FoxP1* was expressed homogeneously in the caudate nucleus and the putamen.

In contrast to the striosomal *FoxP2* expression pattern, *FoxP1* mRNA expression is homogeneous in the caudate nucleus and putamen in the prenatal and postnatal periods in monkeys.[37] Then, *FoxP1* expression decreases both in the caudate nucleus and the putamen.

Cerebral Cortex and Hippocampus

Foxp family genes are also differentially expressed in the developing cerebral cortex. The expression patterns of *Foxp1, Foxp2 and Foxp4* genes show characteristic medio-lateral differences and layer specificity.[33-36]

In the early embryonic stage, *Foxp4* has a mediolateral graded expression in the cerebral cortex: high expression in the medial cortex, low expression in the lateral cortex. By contrast, the cortical expression pattern of *Foxp2* mRNA is very different from that of *Foxp4*. *Foxp2* is only expressed in the lateral telencephalon without any gradient. *Foxp1* expression starts a little later than expression of *Foxp2* and *Foxp4* and becomes apparent in the medial telencephalon.

In the embryonic and postnatal periods, *Foxp4*, *Foxp2* and *Foxp1* genes are expressed in specific layers of the cerebral cortex. During the early developmental stages in the cerebral cortex, *Foxp4* is expressed in proliferating cells in the ventricular zone/subventricular zone (VZ/SVZ) and migrating neurons in the intermediate zone (IZ). Expression of *Foxp1* mRNA is observed in the upper half of the cortical plate (CP), while that of *Foxp2* mRNA is observed in the lower part of the cortical plate (CP). Thus the expression pattern of *Foxp2* appears complementary to that of *Foxp1*.

During the late developmental stages of corticogenesis, *Foxp2* is expressed in the lower cortical layers, where early-born neurons reside and *Foxp1* is expressed in the upper cortical layers, where late-born neurons reside. On the other hand, *Foxp4* is expressed in the entire cortical layers, including the subplate (SP). This complex pattern of expression may suggest the possibility that a layer-specific identity of cortical neurons may be defined, at least in part, by combinatorial codes of *Foxp* genes.

In the postnatal cerebral cortex, expression of each *Foxp* gene shows regional specificity. *Foxp1* is expressed in the dorsal and medial cortex, while *Foxp2* is not expressed in the dorsal or medial cortex, but is expressed in the lateral cortex, such as in the insular cortex. In addition, both *Foxp2* and *Foxp1* are expressed in the olfactory tubercle, but not in the piriform cortex, although the expression patterns of these two genes are different within the olfactory tubercle. By contrast, the regional expression of *Foxp4* is different from that of *Foxp1* or *Foxp2. Foxp4* is expressed in the piriform cortex, but not in the olfactory tubercle. These expression patterns of *Foxp1* and *Foxp2* in the cerebral cortex persist until adulthood, while *Foxp4* expression declines by adulthood.

In addition to the developing cerebral cortex, *Foxp1, Foxp2* and *Foxp4* are differentially expressed in the developing hippocampus. *Foxp4* expression is first observed in the medial telencephalon, including the hippocampal anlage, but is absent from the most medial part called the cortical hem. During this developmental stage, there is no expression of *Foxp2* or *Foxp1* in the hippocampal anlage.

In the postnatal hippocampus, *Foxp4* is expressed in the hilar region and from CA3 to CA1, while *Foxp1* is expressed mainly in CA1. There is no *Foxp2* expression in the hippocampus throughout development. *Foxp4* expression declines in the mature hippocampus, while *Foxp1* expression persists until adulthood. In human fetal cortex, *FOXP1* is expressed in more superficial layers than *FOXP2*, as in rodents. The expression of *FOXP1* and *FOXP2* in the cerebral cortex is not asymmetrical.

In the developing monkey cerebral cortex, differential expression of FoxP1 and FoxP2 is evident. *FoxP2* is expressed in the deeper cortical layers, whereas *FoxP1* is highly expressed in the more superficial layers. The layer-specific expression of these two *FoxP* genes is similar to that in mouse, rat and human brain.[33,35,38] *FoxP2* is widely expressed throughout the cortical areas, including frontal, parietal, temporal, insular and occipital cortices, although expression is faint in the cingulate cortex. By contrast, moderate *FoxP1* expression is detected in all areas examined, including the cingulate cortex. *Foxp2/FoxP2* expression in the developing cerebral cortex appears to be conserved in rodents and primates.

Hippocampal *FoxP2* and *FoxP1* expression patterns in the nonhuman primate are also similar to those in the rat:[35,37] that is, low *FoxP2* expression is detected in the hippocampus, whereas *FoxP1* is expressed in the CA1-CA3 region of the hippocampus. On the whole, regional *Foxp2/FoxP2* expression is very similar in rats and nonhuman primates, although the temporal expression pattern of *Foxp2/FOXP2* is different.

Thalamus

In the developing diencephalon, *Foxp1, Foxp2* and *Foxp4* are expressed in the epithalamus, the dorsal thalamus (DT), the ventral thalamus and the hypothalamus. However, the distributions and expression levels of these family members in each region are distinct; for example, *Foxp4* is highly expressed in the proliferating cells in the DT, whereas *Foxp2* expression is high in differentiated cells located in the lateral part of the DT, but low in proliferating cells. Thus expression pattern of *Foxp2* is complementary to that of *Foxp4*. The expression of *Foxp1* in differentiated cells is similar to that of *Foxp2*, although the level of *Foxp1* expression is much lower than that of *Foxp2*.

Human *FOXP1* and *FOXP2* expression overlaps in the developing thalamus.[38] *FOXP2* is highly expressed in the centromedian nucleus and mediodorsal nucleus of the thalamus. More moderate expression of *FOXP2* is observed in the anterior nucleus and parafascicular nucleus. In the neonatal nonhuman primate brains, expression of *FoxP1* and *FoxP2* is quite similar to that in human counterparts.

Cerebellum

Foxp1, Foxp2 and *Foxp4* are all expressed in the cerebellar primordia. Expression of these members is detected in Purkinje cells, cerebellar nuclear neurons, but not in granular neurons or cerebellar interneurons. They are also expressed in the inferior olive. The expression of these members in cerebellum and inferior olive declines postnatally and diminished in the adulthood.

Spinal Cord

Foxp1 is expressed in most of the motor neurons in the brachial spinal cord, while *Foxp4* is expressed in a subset of motor neurons and a subset of spinal interneurons.[29] *Foxp2* is not expressed in motor neurons, but is expressed in a large number of interneurons.

Language Impairments in the Affected Members of the KE Family

Despite the extensive behavioral analyses of the KE family, there have been inconsistencies in the analyses and it is still unclear how many core deficits there are. However, there is at least one core deficit, verbal and orofacial dyspraxia underlying the speech and language disorders, in the affected members of the KE family. The verbal and orofacial apraxia in the affected members strikingly resembles 'Broca's aphasia', which is usually seen in patients suffering from brain damage in the 'Broca's language center' located in the left frontal lobe, often caused by cerebrovascular diseases. There KE patients have no hearing loss or neurological deficits that affect swallowing or limb movements. Nor do they have abnormality in other organs. In that sense, the disease observed in the affected family members in the KE family is really regarded as distinctive speech and language disorders.

Imaging Studies on the KE Family

Although there is still much to be learned about neuropathology, there is no reported autopsy of an affected member of the KE family. The structural and functional abnormalities in the brains of the affected members of the KE family have been investigated using MRI.[45,46] The structural brain abnormalities in the affected members of the KE family were investigated using voxel-based morphometric (VBM) methods of MRI analysis. The VBM analyses showed bilateral abnormalities in the caudate nucleus, the inferior frontal gyrus (Broca's area), the precentral gyrus, temporal pole and the cerebellum (lobules VIIB and VIIIB) in the affected members, compared with unaffected members and age-matched controls, who did not differ from each other. The abnormality in the caudate nucleus was of particular interest, because functional abnormality was also found in a related positron emission tomography (PET) study. Moreover, the reduction in volume was significantly correlated with the performance of the affected members on several language tasks.

The affected members of the KE family showed highly atypical fMRI brain activation when performing both covert (silent) and overt (spoken) verb generation tasks, as well as word repetition tasks.[47] The unaffected family members showed a typical left-dominant activation in the inferior frontal gyrus, including Broca's area, in both generation tasks and a more bilateral activation in the repetition tasks, whereas the affected members showed significant underactivation of Broca's area and its right homolog, as well as language-related cortical regions and the putamen. Also, in affected cases, paradoxical activation was observed in cortical regions that are not usually involved in language tasks.

The underactivation of Broca's area in affected members reminds us of patients suffering from motor aphasia caused by cerebrovascular brain damage. The language deficits in both cases are very similar. The functional abnormality in the putamen suggests dysfunction of the striatum.

The *FOXP2* Expression Pattern in the Brain and Its Relation to the Cognitive Functions of Speech and Language

FOXP2 is extensively expressed in the developing brain and its expression is down-regulated in the adult. This fact suggests the possibilities that the speech and language impairments found in the affected members of the KE family are due to the developmental defects of the neural

network critically involved in speech and language function and that formation of this network is dependent upon a gene network via FOXP2. The spatiotemporal *FOXP2* mRNA expression pattern suggests that the basic neural network that underlies speech and language may include motor-related circuits, including corticostriatal and/or corticocerebellar circuits.

This assumption is supported by brain imaging studies on *FOXP2*-mutated KE family members. Morphometrical analysis using MRI and a functional anatomical study, using PET and fMRI, revealed a bilateral abnormality in the inferior frontal cortex, caudate-putamen and cerebellum.[46-48] In particular, *FOXP2* expression in the striosomes of the caudate nucleus might have important implications for brain abnormalities induced by *FOXP2* mutations in KE family patients. Graybiel and colleagues have shown that striosomes with patches containing low levels of acetylcholinesterase activity are more prominent in the caudate nucleus than in the putamen,[43,49] suggesting that striosomes may fully engage in the neural circuits running through the caudate nucleus. If *FOXP2* expression levels in the caudate nucleus of the human brain are truly higher than those in the putamen, the *FOXP2* mutation may result in a stronger phenotype in the caudate nucleus, which is observed in the brains of affected KE family members.

The affected members of the KE family show impaired movement of mouth, lips and tongue during speech. A study using monkeys has shown that a striosome-dominant activation, as marked by immediate-early gene expression, could occur under conditions in which repetitive movements are induced by dopamine agonists.[50] Moreover, dopamine agonist-induced dyskinesia of repetitive movements is present in experimental parkinsonism.[51,52] Therefore, we hypothesize that the symptoms of orofacial dyspraxia in KE family patients may be related to dysfunction of the striosomal system in the striatum.

As described above, language impairments in the affected members of the KE family resemble those in Broca's aphasia, which usually involves the inferior frontal lobe. Although the basal ganglia are not generally considered to be necessary for language acquisition, several recent reports suggest that the caudate nucleus, in particular, is involved in language processing.[53-55] Damasio and Damasio[56] hypothesize that the basal ganglia circuitry contributes to grammatical rule processing in conjunction with the frontal lobe. Ullman and colleagues[54,55] found that grammatical mistakes occurred in patients suffering from Parkinson's disease or Huntington's disease and developed a declarative/procedural model of language. According to the model, the mental grammar involves procedural memory-like skills and habits and is rooted in the frontal lobe-basal ganglia, whereas the mental lexicon depends on declarative memory and is rooted in the temporal lobe.

The role of the cerebellum in language function is partially understood.[57] It is not certain whether the speech and language disturbances in the KE family are caused by defects of cortico-striatal or cortico-cerebellar circuits, or both.[58]

Phenotype in Foxp2 Mutant Mice

Foxp2 mutant mice have been generated and analyzed by three groups.[59-61] Homozygotes deficient for both *Foxp2* alleles (null mutant) showed severe motor impairment (delayed righting-reflex maturation), premature death and an absence of ultrasonic vocalization when pups were isolated from their mothers. Shu and colleagues[61] reported abnormalities in the cerebella of homozygotes. Specifically, alignment of Purkinje cells was irregular and the external granular layer (EGL), which should not be retained at the comparative age, was retained. Heterozygotes also showed a modest developmental delay, cerebellar abnormalities and a significant change in ultrasonic vocalization in response to isolation.

Other groups[59,60] generated knock-in mice with a point mutation in the *Foxp2* gene to give rise to a R552H mutation (corresponding to the human FOXP2 R553H mutation). Homozygous and heterozygous R552H mice reported by Fujita and colleagues[59] showed largely similar phenotypes to the KO mice reported by Shu.[61] Of particular interest, in the homozygous R552H mutants, some neurons had nuclear aggregates of Foxp2 protein. In addition to the immature cerebellar development, the nuclear aggregates might further compromise the function of Purkinje cells and cerebral neurons, resulting in their death.

There were some different phenotypes and interpretations in another line of mutant mice with the same R552H mutation.[60] Homozygous R552H mice showed severe reductions in cerebellar growth and postnatal weight gain, but were able to produce complex ultrasonic vocalization in response to isolation. Heterozygous R552H mice were overtly normal in brain structure and development. The most interesting findings in their study were that heterozygous R552H mice show significant deficits in motor-skill learning and abnormal synaptic plasticity is observed in striatal and cerebellar brain slices by electrophysiological analysis.

Thus findings in these mutant mice seem to support a role for Foxp2 in the development and function of the striatum and cerebellum and a possible involvement of corticostriatal and/or corticocerebellar circuits in the brains of the *FOXP2*-mutated KE family members.

Transcriptional Activity of the FOXP2 Protein

Protein expression, subcellular localization, DNA-binding and transactivation properties of disease-causing mutations in FOXP2 have been studied using cultured cell models.[62,63]

Wild-type FOXP2 protein expressed in human cell lines is localized mainly in the nucleus. This intracellular localization is disrupted in the mutants: FOXP2 with a R553H mutation is localized in both nucleus and cytoplasm, whereas FOXP2 with R328X localized predominantly in the cytoplasm. In addition, R328X yields an unstable protein product possibly by nonsense mediated RNA decay.

The DNA-binding properties of wild-type and mutant forms of FOXP2 were investigated via electrophoretic mobility shift assays (EMSAs) using an oligonucleotide probe bound to mouse Foxp1. Wild-type FOXP2 protein possesses DNA-binding capacity, while neither R553H nor R328X mutants bound to the target DNA.

It was reported that mouse Foxp1 and Foxp2 proteins strongly repress transcription from the SV40 promoter, via binding to a naturally occurring target site in the promoter sequence.[64] The transcription capacities of FOXP2 variants were determined by luciferase reporter gene assays. Wild-type FOXP2, FOXP1 and FOXP4 function as transcriptional repressor for SV40 promoter, whereas R528H and R328X mutants lose the repressor activity.[62]

In sum, FOXP2 disease-causing mutations disrupt normal subcellular localization, DNA-binding or transactivation capacities in mammalian cell model systems. Thus similar functional changes caused by the mutations are expected to occur in vivo in affected humans.

Foxp2 Upstream and Downstream Genes (Fig. 2)

FOXP2 gene mutation is so far the only known cause of developmental speech and language disorders in humans. Identifying the molecular network of this gene and its encoded protein will provide a unique window into neural processes involved in speech and language. The upstream regulatory mechanisms that control *FOXP2* expression and the downstream molecular events that are regulated by the *FOXP2* gene are being investigated by several approaches as follows.

Since FOXP2 is a transcription factor, its potential transcriptional targets can be identified by using the technique of chromatin immunoprecipitation followed by the microarray analysis of promoter regions (ChIP-chip assay) and the functional regulation of targets by FOXP2 can be validated in vitro and in vivo. Two groups have identified targets of FOXP2 in vivo in two brain regions (basal ganglia and inferior frontal cortex) of the human fetal brain and also in a human neuronal cell models.[65,66] Interestingly, half of the target genes identified by the these studies are overlapped. FOXP2 protein bound to the promoters of genes involved in diverse biological functions, including cell signaling, synaptic transmission, neural development, ion transport and axon guidance in fetal human brain and living neuron-like cell lines. The expression of a majority of the target genes (such as ANK1, KCNJ15 and LBR) was repressed by FOXP2 in cell culture models, while expression of a minority of the targets (such as TAGLN and CALCRL) was activated by FOXP2. Thus FOXP2 can act as both a repressor and an activator under certain circumstances, possibly dependent upon FOXP2 cofactors (such as Foxp1, Foxp4 and CtBP1) or its posttranslational modifications.

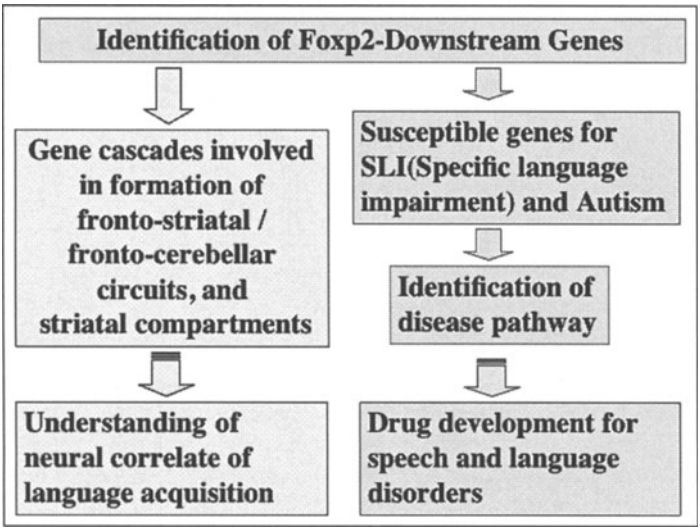

Figure 2. Perspectives. Identification of Foxp2-downstream genes may provide insights into the molecular mechanisms underlying the neural development such as striatal compartmentalization and the neural network formation of frontostriatal and frontocerebellar circuits, potentially related to language acquisition. The identification of the Foxp2-downstream genes might also lead to the discovery of the susceptible genes for SLI (specific language Impairment) and autism. Further understanding the gene network may be valuable for development of the therapeutics for SLI and autism.

It has been reported that Foxp1 works as a cofactor for Hox protein in establishing spinal motor neuronal identity.[29,30] A number of transcription factors have been shown to be preferentially and highly expressed in the developing striatum.[67] A gene expression study using in situ hybridization revealed that two transcription factor genes, *Pbx3* and *Meis2*, belonging to the TALE (three amino acid loop extension) superclass of the homeobox gene family,[68] as well as *Foxp2*, to be preferentially expressed in the striosomes of the developing rat and nonhuman primate striatum.[35,37] Because Pbx and Meis proteins act as cofactors for various transcription factors, such as Hox proteins and bHLH proteins,[69,70] and *Foxp2*, *Pbx3* and *Meis2* are co-expressed in the developing striatum, a direct or indirect interaction between Foxp2 and Pbx/Meis proteins is expected.

There is so far only one report on the upstream regulation of the *Foxp2* gene.[71] There are six Lef1 binding sites common between zebrafish, mouse and humans in the *Foxp2* genomic region and expressions of *Lef1* and *Foxp2* is overlapped in the zebrafish brain during development. Lef1 is a transcription factor activated by the canonical Wnt/β-catenin signaling pathway involved in body patterning, neuronal cell specification and axon pathfinding. Knockdown of Lef1 using siRNA caused loss of Foxp2 expression in a restricted part of the brain in zebrafish experiments. Also, a ChIP experiment confirmed that Lef1 binds to sites in the *Foxp2* enhancer region. Thus Lef1 may also regulate the expression of *Foxp2* in humans.

Perspectives

Molecular network analysis regarding FOXP2 will provide at least two important opportunities in the field of cognitive and behavioral neurology. First, uncovering the gene and protein networks related to *FOXP2*/FOXP2 will aid elucidation of the molecular mechanisms underlying neural development potentially involved in language acquisition (especially in the striatal compartments, cerebral and cerebellar structures). Second, understanding the genes and pathways that are regulated by FOXP2 might lead to discovery of candidate genes for SLI (specific language Impairment) and

autism. Finally, the progresses that we make in the 'neurobiology of language' will give us hints in developing pharmacological tools not only for treating speech and language disorders, but also for potentiating or improving speech and language skills.

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CHAPTER 10

Pathophysiological Relevance of Forkhead Transcription Factors in Brain Ischemia

Kohji Fukunaga* and Norifumi Shioda

Abstract

Forkhead box transcription factor, class O (FOXO) is a mammalian homologue of DAF-16, which is known to regulate the lifespan of *Caenorhabditis elegans* and includes subfamilies of forkhead transcription factors such as FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX) and FOXO6. All these FOXO members are expressed in the brain with different spatial patterns. FOXO1 is phosphorylated on three sites (Thr-24, Ser-256 and Ser-319) in phosphatidylinositol 3-kinase (PI3-K)/Akt-dependent manner, thereby inhibiting apoptosis signals. We here documented dephosphorylation of FOXO1, FOXO3 and FOXO4 following transient forebrain ischemia with its concomitant translocation into the nucleus in neurons in the gerbil and mouse brains. The dephosphorylation of FOXO1 following brain ischemia is in part mediated by constitutively active calcineurin in the mouse hippocampus. The activation of FOXOs preceded delayed neuronal death in the vulnerable hippocampal regions following ischemic brain injury. The FOXO1 activation is accompanied by an increase in DNA binding activity for FOXO1-responsive element on the Fas ligand promoter. Thus, downstream targets induced by FOXO1 include Fas ligand and Bcl-2-interacting mediator of cell death (Bim) in the brain ischemia. Accumulating evidence documented how FOXO activation is involved in the mechanisms of ischemic cell death. In this chapter, we document the activation mechanism of FOXO factors following brain ischemia and define their downstream targets underlying neuronal death. The pathophysiological relevance of crosstalk between FOXOs and calcineurin pathways is also discussed. Finally, we propose therapeutic perspectives to rescue neurons from delayed neuronal death by promoting the Akt signaling. Vanadium compounds, protein tyrosine phosphatase inhibitor, up-regulates Akt activity in the brain and thereby rescues neurons from delayed neuronal death by inhibiting FOXO-dependent and -independent death signals in neurons.

FOXO Phosphorylation Regulating Shuttling between Nucleus and Cytoplasm

The forkhead box transcription factor, class O (FOXO) is mammalian homologue of DAF-16, which is known to regulate life span of *Caenorhabditis elegans*[1] and includes subfamilies of forkhead transcription regulators such as FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX) and FOXO6. The FOXO factors share DNA-binding specificity to a core consensus site named as Forkhead-responsive element[2] and downstream targets of diverse protein kinases stimulated

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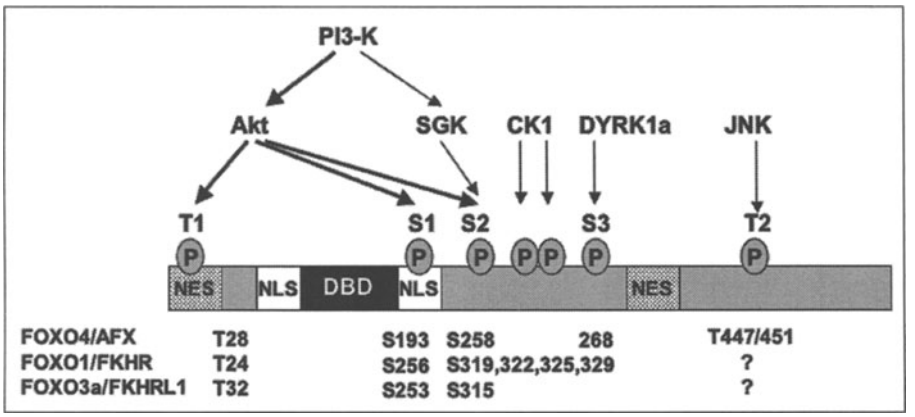

Figure 1. Possible phosphorylation sites for Akt and other possible kinases in FOXOs. The FOXO members are phosphorylated by several protein kinases. Phosphorylation of T1, S1 and S2 by Akt activity through PI3-K underling growth factor signaling is crucial step of the FOXO function. Serum- and glucocorticoid-inducible kinase (SGK) underlying PI3-K pathway also phosphorylates T1 and S2. The S2 phosphorylation by Akt is required for subsequent phosphorylation of Ser 322 and Ser325 in FOXO1 by casein kinase 1 (CK1). Together with these phosphorylation, the S3 phosphorylation by the dual-specific tyrosine-regulated kinase 1a (DYRK1a) may contribute the nuclear export of FOXO. In addition, T2 in the C terminal regions is also phosphorylated by JNK underlying the Ras-Ral pathways as described in the text.

by various cellular stresses such as DNA damage, nutrient deprivation, cytokines and hypoxia. FOXO factors are phosphorylated in vivo on multiple threonine and serine residues (T1, T2, S1, S2 and S3) as shown in Figure 1. Protein kinase B (Akt) in response to growth factor and insulin stimulation directly phosphorylate FOXO1 at three specific sites (Thr-24, Ser-256 and Ser-319, labeled T1, S1 and S2, respectively).[3] Serum- and glucocorticoid-inducible kinase (SGK) underlying PI3-K pathways also phosphorylates T1 and S2 of FOXO3.[4] The functions of FOXO factors negatively regulated by Akt- and SGK-dependent phosphorylation on these sites, thereby promoting maintenance of cell survival. Akt phosphorylates Ser-256 and SGK phosphorylates Ser-319 preferentially. Reporter assays for transcriptional activity and mutational analysis of the phosphorylation sites T1, S1 and S2 show that Akt-induced phosphorylation inhibits the transcriptional activity of FOXO factors. The Akt/SGK-dependent phosphorylation regulates the shuttling of FOXO factors between the nucleus and the cytoplasm. Homologous sequences for nuclear localization signal (NLS) and nuclear export signal (NES) have been identified within the FOXO factors as shown in Figure 2. The shuttling of FOXOs between nucleus and cytoplasm is regulated by accessory proteins such as importins or exportins (Crm1). The phosphorylation of murine FOXO1 at Ser-253 (corresponding to Ser-256 in human FOXO1) is required for phosphorylation of the other two sites for Akt.[5] In the case of FOXO1, one of the NLS lies near the Akt-dependent phosphorylation site S1. Upon phosphorylation on S1 by Akt, the NLS in FOXOs is inactivated. The 14-3-3 protein possibly recognized the S1 phosphorylated form of FOXO1 and export it to the cytoplasm by masking the NLS and/or by promoting nuclear export. However the importin that binds to the NLS remains unidentified. The function of additional Akt-dependent phosphorylation sites of T1 and S2 also remains unclear. Since nuclear export of FOXOs is inhibited by leptomycin B treatment, implying involvement of Crm1 in the nuclear export. Zhao et al[6] proposed two putative NES in the C terminal region and one NES near phosphorylation site S3. Although exportin (Crm1) may not directly bind to the NES, the phosphorylation of T1 and S3 possibly affects the Crm1 binding to the NES. The phosphorylation of T1 and S2 dose not affect the binding between FOXOs and Crm1. The association between FOXO and exportins is also regulated by the small GTPase Ran. Phosphorylation of Ser-322 and Ser-325 by casein kinase 1

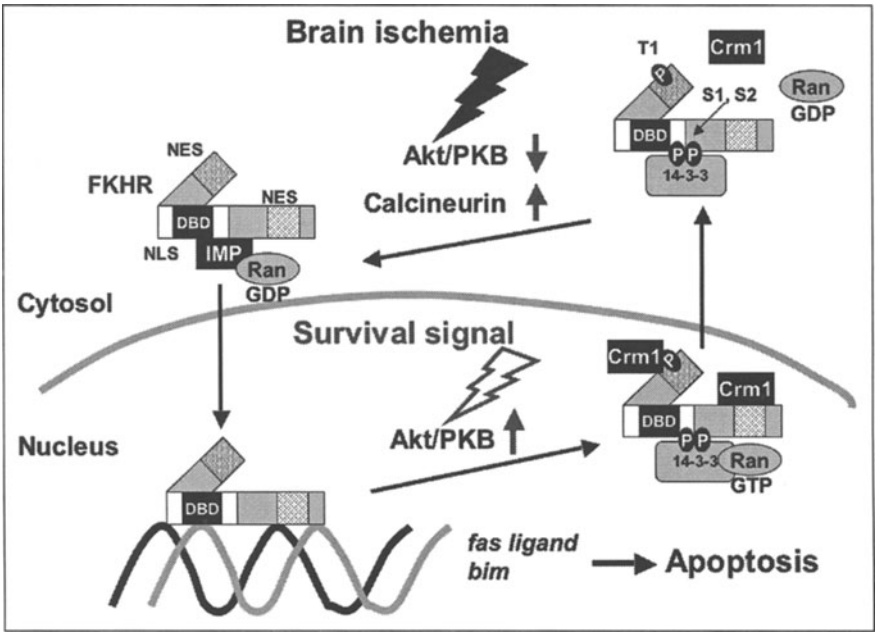

Figure 2. Functional relevance of FOXO phosphorylation in the shuttling between cytoplasm and nucleus. FOXO1 in the neurons normally stays in the cytoplasm because of Akt-dependent phosphorylation. Once Akt activity is reduced by growth factor depletion or ischemic conditions, FOXO1 is dephosphorylated by protein phosphate 2A and calcineurin in neurons. Although importins remains undefined, the dephosphorylation contributes its nuclear import though nuclear localization signal (NLS). DNA-binding domain is required for activation of the promoters of *fas-ligand* and *bim* genes. Thus FOXO1-inducible proteins are involved in the apoptosis. Neurotrophic factors and their mimetic compounds such as orthovanadate activate Akt pathways, thereby rescuing neurons from apoptosis though phosphorylation and inactivation of FOXO1. An exportin, Crm1 together with 14-3-3 protein and Ran are involved in the nuclear export though nuclear export signal in FOXO1 molecule.

(CK1) may also contribute to promote FOXO1 relocalization to cytoplasm by increasing the binding to exportins.[7] Likewise phosphorylation of Ser-329 by dual-specific tyrosine-phosphorylated and -regulated kinase 1a (DYRK1a) also accelerates nuclear export and inhibition of FOXOs' transcriptional activity.[8]

FOXO Phosphorylation in Response to Oxidative and Ischemic Stresses

Generally, once survival factors are depleted, FOXOs are dephosphorylated and translocate into the nucleus.[5,9-12] In the nucleus, FOXOs are activated by other protein kinases which are integrated to cellular stresses such as oxidative stress, DNA damage, cytokines and ischemia. Oxidative stress induced by H_2O_2 treatment triggers activation of the small GTPase Ral. The Ral activation results in Jun N-terminal kinase (JNK)-dependent phosphorylation of FOXO4 on Thr-447 and Thr-451 in mouse NIH3T3 cells.[13] The phosphorylation of these residues is critical to FOXO4 activation on its transcriptional activity. Stimulation with tumor necrosis factor α (TNFα) also induced FOXO4 activation with the similar mechanism. The H_2O_2-induced JNK activation dose not affect Akt-dependent phosphorylation of FOXO4. The mild activation of FOXO4 by H_2O_2 or TNFα likely mediates induction of manganese superoxide dismutase (MnSOD) and catalase to reduce the level of cellular oxidative stress.[13]

By contrast, the mammalian Ste20-like kinase 1 (MST1)-mediated FOXO3 phosphorylation in response to oxidative stress induced apoptosis in cultured cerebellar granule neurons.[14] The MST1-induced phosphorylation at Ser-207 disrupts FOXO3's interaction with 14-3-3 proteins and promotes its translocation to nucleus. Thus, the MST1-induced activation of FOXO3 mediates cell death in neurons. Unlike JNK-induced FOXO4 phosphorylation, neuronal Akt pathways is highly sensitive to oxidative stress. In culture cerebellar granule neurons, H_2O_2 inhibits Akt activity through downregulation of PI3-K pathways by p38 MAPK.[15] Concomitant with reduced Akt-dependent phosphorylation of FOXO3, its phosphorylation by JNK triggers neuronal death by oxidative stress.

DNA damage also reduces FOXO1 phosphorylation at Ser-249 through inhibition of cyclin-dependent kinase 2 (CDK2) and thereby induces apoptosis in prostate cancer cells.[16] The CDK2 inhibition in part mediated by the protein kinases Chk1 and Chk2 activated by DNA damage. CDK2-induced FOXO1 phosphorylation at Ser-249 is critical to its trafficking from the nucleus to the cytoplasm. However, the pathological relevance of FOXO1 phosphorylation by CDK2 in neuronal apoptosis remains unclear.

The diverse functions of FOXOs are also mediated by phosphorylation through the energy sensor AMP-activated protein kinase (AMPK) in neurons. An elevation of AMP levels or an increase in the AMP/ATP ratio triggers activation of AMPK, thereby regulating cellular metabolism and gene expression to restore ATP levels.[17,18] The reduced ATP level is critical to brain ischemic damage. Thus, a persistent activation of AMPK is documented in the brain ischemia.[19] Notably, the pharmacological inhibition of AMPK rescues neurons from ischemic damage. However, the downstream targets for AMPK underlying deterioration of neuronal damage have not been defined. AMPK directly phosphorylates human FOXO3 at Ser-413, Ser-588 and Ser-626 in vivo.[20] Nutrient deprivation induced these phosphorylation in mammalian cultured cells. The AMPK phosphorylation of FOXO3 does not affect its cellular localization but enhances the ability of the transcription factor to upregulate the specific target genes.[20] The decreased Akt activity is more critical to lead to neuronal death following brain ischemia compared to the AMPK activation.[21]

Decreased Akt Activity following Brain Ischemia Triggers Activation of Proapoptotic Proteins

Transient forebrain ischemia results in delayed neuronal death of pyramidal neurons in the hippocampal CA1 region. Although molecular mechanisms underlying the pathogenesis of delayed neuronal death is unclear at present, histological and biochemical evidences demonstrated involvement of apoptosis in dying cells after ischemia. Activation of Akt has been demonstrated to protect cells from apoptosis.[22,23] We recently reported that a decreased Akt activity is involved in ischemic-induced cell death[21,24-26] and that an increased Akt activity account for neuroprotection in ischemic tolerance in gerbil hippocampal neurons.[27] Akt is phosphorylated on the two residues Thr-308 and Ser-473 prior to it activation.[28] Plasma membrane translocation is also an essential step in the Akt activation. Thereafter, the activated Akt detaches from plasma membrane and translocates to the nucleus. Several potential substrates for Akt related to cell survival include Bad, caspase 9 in the cytoplasm and cAMP-responsive element binding protein (CREB), NF-kappaB and FOXOs in the nucleus.[9,29-36]

Dephosphorylation and Activation of FOXOs following Brain Ischemia

Because Akt phosphorylates FOXOs such as FOXO1 and FOXO3 as downstream target in cell survival signaling,[4,9,29,34] we first focused on FOXO1 phosphorylation as well as Akt phosphorylation in the gerbil hippocampus after transient forebrain ischemia.[24] The transient forebrain ischemia induced dephosphorylation of FOXO1-Ser-256 in the hippocampal CA1 region immediately, 0.5 and 1 h after reperfusion without changes in the FOXO1 protein levels. The FOXO1-Ser-256 phosphorylation returned to the basal level within 2 h after reperfusion. The FOXO1-Ser-256 dephosphorylation was closely correlated with the dephosphorylation of Akt-Ser-473. The dephosphorylation of FOXO1 leads to translocation into the nucleus in the hippocampal CA1 regions.

In sham-operated animals, phosphorylated FOXO1 predominantly localized in the cytoplasm of the pyramidal neurons in the CA1 regions. The transient ischemia caused apparent translocation of FOXO1 into the nuclei of the CA1 pyramidal neurons.

However, severe brain ischemia in mouse transient middle cerebral artery occlusion (MCAO) model, dephosphorylation and subsequent activation of FOXO1, FOXO3 persisted for more than 6 hours in the ischemic hemisphere (Fig. 3).[37] On the other hand, FOXO4 phosphorylation unchanged at lease until 24 h after occlusion. Consistent with dephosphorylation of FOXO1 and FOXO3, DNA binding activities to forkhead-responsive element with in the Fas-ligand promoter elevated until 6 hours. The DNA binding complexes following brain ischemia mainly included FOXO1 and FOXO3 and faintly FOXO4 in the hippocampal nuclear extracts. To ensure the involvement of decreased Akt activity in the FOXO dephosphorylation, a novel PI3-K/Akt activator, vanadyl compound VO(OPT) tested on the ischemia-induced dephosphorylation of FOXO1 and FOXO3. VO(OPT) treatment stimulated the decreased phosphorylation of Akt (Ser-473) observed 1 and 2 hours after MCAO.[37] Likewise, the prolonged dephosphorylation of FOXO1 and FOXO3 restored to the levels comparable to sham-operated animals (Fig. 3).

Crosstalk between Akt and Calcineurin Signaling in Neuronal Death

Notably, the dephosphorylation of Akt following MCAO was transient and returned to the basal phosphorylation within 6 hours, whereas the dephosphorylation of FOXO1 and FOXO3 after MCAO was prolonged up to 6 hours. The protein levels of FOXOs unchanged at least until 6 hours after MCAO. Specific protein phosphatases may account for the dephosphorylation of FOXOs. Calcineurin (CaN) is compound of A and B subunits with 60-kDa and 19-kDa, respectively. CaN A subunit contains the catalytic, CaN B binding, calmodulin binding and autoinhibitory domains, while CaN B subunit has an intrinsic Ca^{2+} binding domain. The enzyme becomes active when calmodulin (CaM) binds to CaN A subunit by releasing the autoinhibitory domain from the catalytic active site.[38,39] In addition to Ca^{2+}/CaM-dependent activation, CaN is converted in vitro to a constitutively active by cleaving out the autoinhibitory domain with calpain treatment.[38,40] Previous studies have shown that artificial overexpression of the constitutively active CaN induces apoptosis in neuronal and nonneuronal cells.[41-43] In the recent in vivo studies, generation of constitutively active CaN activity was evident in neuronal insults including kinate-induced neurotoxicity in rat brain and Alzheimer's disease in human brain.[44,45] The generation of constitutively active CaN by calpain and the calpain-induced CaN activation mediates delayed neuronal death in brain ischemia.[46,47] Interestingly, phosphorylated Ser-256 of FOXO1 is dephosphorylated by constitutively active CaN. FOXO1 forms a complex with CaN and thereafter the complex is translocated into nuclei in the hippocampal CA1 neurons after MCAO (Fig. 4). Since the complex following MCAO is not affected with treatment FK506, a specific CaN inhibitor, implying that the catalytic activity is not required to make the complex. CaN also mediates Akt dephosphorylation in the ischemic retinal cells.[48] The interaction between CaN and Akt also evident after the ischemic retina. In this case, the formation of CaN-Akt complex is inhibited by FK506 treatment, suggesting that Akt is recognized as a substrate by CaN during ischemia. The CaN-induced Akt dephosphorylation preceded the apoptotic neuronal death in ischemic rat retina.[48]

Synergistic Activation of Fas-Ligand Promoter by NFATs and FOXOs

Nuclear factor of activated T-cells (NFAT), a downstream target for CaN is an attractive candidate underlying its detrimental effects. Five members of the NFAT family including NFAT1 (NFATp or NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3 or NFATx) and NFAT5 have been identified.[49,50] The translocation of NFATs into the nucleus in response to an increase of intracellular Ca^{2+} is led by the dephosphorylation by CaN. Following dephosphorylation of NFAT by CaN, NFAT makes a complex with CaN and in turn leads to translocation of CaN with NFAT from the cytoplasm into the nucleus in U2OS cells.[51] We also found in vivo that activation of constitutively active CaN led to translocation of NFATc4 into the nuclei following ischemic brain.[46] NFAT and FOXO1 dephosphorylated by constitutively active CaN activate

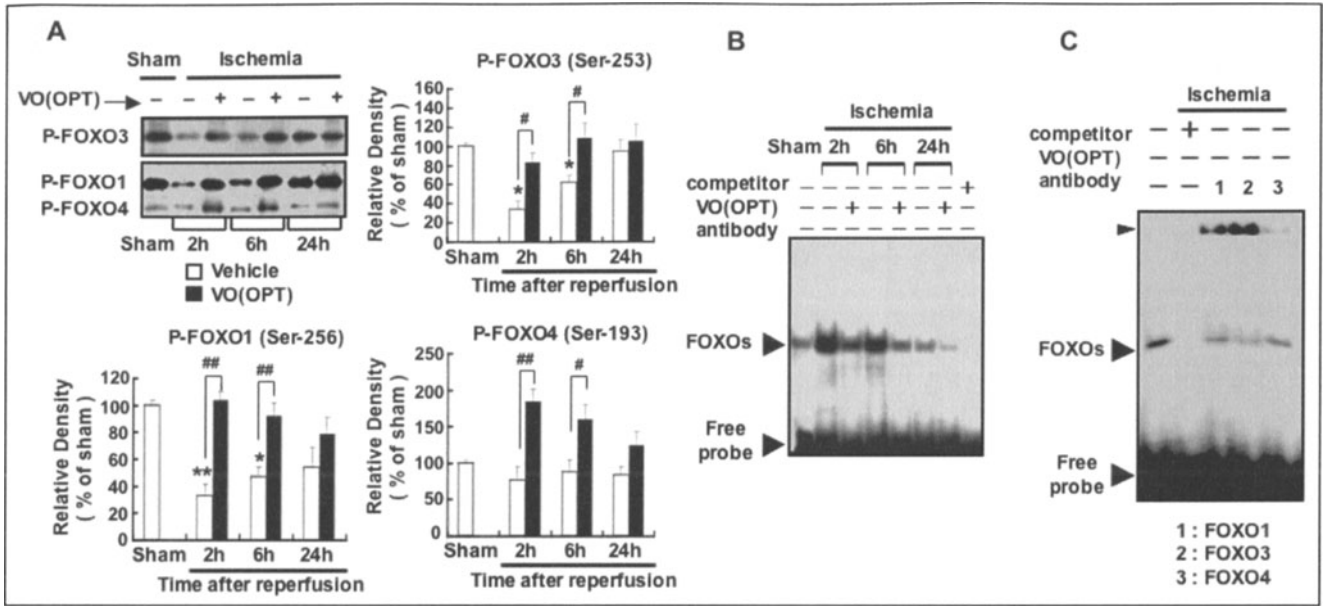

Figure 3. Dephosphorylation of FOXOs following brain ischemia and enhanced FOXO binding to Fas-ligand promoter. A) Ischemia and reperfusion in middle cerebral artery occlusion induces dephosphorylation of phospho (P)-FOXO1 (Ser-256), (P)-FOXO3 (Ser-253), or P-FOXO4 (Ser-193) in the ischemic brain regions. Quantitative analyses of P-FOXO1, P-FOXO3 and P-FOXO4 levels performed by densitometry are shown in the each panel. Data are expressed as percentage of value of sham-operated animals. The VO(OPT) treatment increased FOXO1, FOXO3 and FOXO4 phosphorylation 2-6 h after ischemia/reperfusion. Each bar represents the mean ± S.E.M. *P <0.05, **P < 0.01 versus the sham-operated animals and # P < 0.05, ## P < 0.01 versus the vehicle group (n = 5, in each group). B) Gel mobility shift assay using the forkhead-responsive element in the promoter of the Fas-ligand gene. Gel mobility shift analysis showing the nuclear protein binding to the consensus element for FOXOs within the promoter of the Fas-ligand gene increased after cerebral ischemia. The increased binding to forkhead-responsive element suppressed by treatment with VO(OPT) in 2-6 h after ischemia. The excess amount of nonlabeled competitor was added during incubation. C) For supershift experiments, antibodies to FOXO1, FOXO3, or FOXO4 were added to the extracts after adding the ^{32}P-labeled probe and incubated for 30 min at room temperature. Arrowhead shows the band of each protein-antibody complex supershifted. Modified from Shioda N et al,[37] ©2007 with permission from Elsevier.

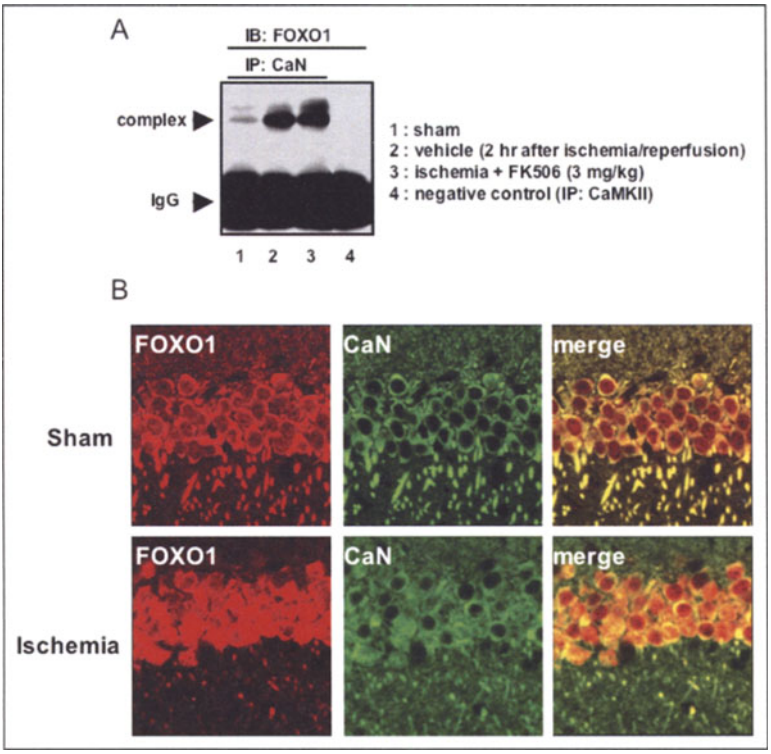

Figure 4. Calcineurin(CaN) makes complex with FOXO1 and the complex translocates into the nuclei after brain ischemia. A) Extracts obtained from the ipsilateral hemisphere from sham-operated (lane. 1), subjected to MCAO at 2 h after ischemia/reperfusion (vehicles) (lane. 2), subjected to MCAO at 2 h after ischemia/reperfusion pretreated FK506 (lane. 3) underwent immunoprecipitation (IP) with anti-CaN antibody and the immunoprecipitates were then analyzed by immunoblotting (IB) with anti-FOXO1 antibody. The negative control underwent immunoprecipitation with anticalcium/calmodulin-dependent protein kinase II (CaMKII) antibody (lane. 4). B) Hippocampal slices from sham-operated and ischemic animals at 2 h after ischemia/reperfusion were double stained with FOXO1 and CaN antibodies. In sham-operated animals, the immunoreactivities for FOXO1 and CaN predominantly localize in the cytoplasm, whereas both immunoreactivities translocated to the nuclei in some neurons in the hippocampal CA1 region. Modified from Shioda et al. J Neurochem 2007; 102:1506-1517;[47] ©2007 with permission from Wiley-Blackwell.

Fas-ligand promoter activity and synergistically promote Fas-ligand expression. FOXO1-indeuced Fas-ligand expression is also correlated with neuronal death in gerbil hippocampal CA1 after global ischemia.[25] The DNA-binding activity of FOXO1 and NFAT within the Fas-ligand promoter following MCAO are inhibited by FK506 treatment.[46]

Downstream Targets for FOXO1 in Delayed Neuronal Death

Fas (also called CD95, APO-1), a member of TNF receptor family and Fas ligand (also called CD95-L, APO-1L) play the important role in apoptosis.[52] Activation of Fas leads to formation of death-inducing signaling complex composed with Fas-associated death domain and pro-caspase 8. Pro-caspase 8 is proteolytically cleaved and consequently activates caspase pathways and thereby cells are led to apoptosis. Fas/Fas ligand system, which was first documented in the immune system, is also important for pathophysiology in the neurodegenerative disorders such as Alzheimer's disease,

multiple sclerosis, trauma and ischemia. Recently Fas/Fas ligand system is involved in the mitochondrial apoptotic pathway by cleaving the Bcl-2 family member, Bid.[53,54] FOXOs are known to induce the expression of apoptosis-related genes such as Fas-ligand and BH3-only member of the Bcl-2 family (Bim).[9,55] Expression of Fas ligand in the ischemic brain region was elevated 2 days after ischemia/reperfusion.[47] Vanadium compound treatments prevented the ischemia-induced expression of Fas ligand.[37] Likewise, Fas ligand mRNA expression increased in the cerebral cortex following permanent MCAO in adult rats[56] and Fas ligand mRNA and its protein were expressed in the cerebral cortex after reversible MCAO in adult rats.[57-59] Furthermore *lpr* mice expressing mutant Fas without its function had significantly smaller infarct size after MCAO as compared to the wild type mice.[59]

Activation of FOXOs also accounts for expression of another apoptotic protein, Bim.[55] Developing sympathetic neurons die by apoptosis when derived of nerve growth factor (NGF). Bim is induced after NGF deprivation in which FOXO3 is involved in the Bim expression.[60] FOXO binding sites are required for activation of the *bim* promoter by FOXO3 and NGF withdrawal. Induction of Bim is also associated with vulnerability of hippocampal neurons after experimental seizures and in hippocampi from patients with intractable temporal lobe epilepsy.[61] To address the relevance of activation FOXO1 in the Bim induction in ischemic brain injury, expression of Bim was also investigated in mouse MCAO. Bim protein level markedly increased 24 h after ischemia/reperfusion.[37,62] FOXOs are required for Bim promoter activation as seen in cultured human BV173 and mouse BaF3/Bcr-Abl-expressing cells.[63] Bim originally plays an important role in the apoptosis of hematopoietic cells.[64] Bim is also present in the adult central nervous system[65] and plays a pathogenic role in ischemic brain.[66] For example, increased mitochondrial localization of Bim coincides with a marked release of cytochrome *c* from mitochondria, resulting in Caspase-9 activation.[66] Furthermore, active Caspase-9 cleaves the executioner of apoptosis, Caspase-3, which leads to apoptosis in brain ischemia.[67] Both Bim expression and Caspase-3 activation following MCAO were inhibited by vanadium compound stimulating Akt activity.[37]

Regulation of FOXO Pathways by SIRT1

In addition to phosphorylation of FOXOs, deacetylation of FOXOs by SIRT1 is known to regulate their transcriptional activities. SIRT1, the mammalian homolog of silence information regulator 2 (Sir2) in *Saccharomyces cerevisiae*, is an NAD-dependent deacetylase implicated in regulation of lifespan. The in vivo deacetylation targets of mammalian SIRT1 include nuclear transcription factors such as p53,[68] FOXO[69,70] and nuclear factor (NF)-kappaB[71]. In cerebellar granule cells, fibroblast and embryonic stem cells, apoptosis triggered by FOXO3 acetylation in response to oxidative stress and DNA damage is inhibited by SIRT1.[69,72] Indeed, the acetylation of FOXO1 and FOXO4 by cAMP-response element-binding protein (CREB)-binding protein (CBP) inhibits their transcriptional activities.[73,74] Conversely, deacetylation of FOXO1 by SIRT1 activates its transcriptional activity.[75] The acetylation of FOXO1 also increases its phosphorylation at Ser-253 by Akt. Thus, the consequence of SIRT1 mediated deacetylation of FOXOs is rather complex and elicits diverse effects depending on promoter of target genes. Taken together, SIRT1 enhances the expression of FOXO target genes that are involved in the cell arrest and resistance to oxidative stress, but inhibits the expression of FOXO genes associated with cell death induction.[76]

Therapeutic Perspectives

The characterization of Akt/FOXO pathway which is critical for decision of cell death following the ischemic brain opens the potential strategy for clinical treatments not only in ischemic insult but also in neurodegenerative disorders. Neurotrophic factors including IGF-1, basic FGF and brain derived neurotrophic factor (BDNF) are potentially important therapeutic agents for neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. The benefits of these neurotrophic treatments include activation of PI3-K/Akt pathways concomitant with extracellular signal-regulated kinase (ERK) activation, both of which have pivotal role in upregulation of survival signaling. However, like most proteins, neurotrophins have poor medical properties, including limited ability of penetration into brain tissue and poor chemical stability in blood circulation. Our goal is to

develop stable, small molecules having neurotrophin mimetic functions to prevent neuronal loss and to maintain neuronal connection. We have introduced a novel approach for creating Akt stimulants as a small compound such as sodium orthovanadate, vanadyl sulfate and bis(1-oxy-2-pyridinethiolato) oxovanadium(IV) [VO(OPT)]. Indeed, the phosphatidylinositol 3-kinase (PI3-K)/Akt activation by sodium orthovanadate (Na_3VO_4) rescue neurons from transient forebrain ischemia in gerbils and rats.[24,25-27,77] The inhibition of FOXO-induced Fas-ligand and Bim expression in part mediated the neuroprotective action of orthovanadate and VO(OPT).[25,37] Importantly, in vivo treatments with orthovanadate and VO(OPT) markedly enhanced neurogenesis following brain ischemia in the subventricular zone of the frontal cortex and the subgranular zone of the hippocampus.[78,79] Like the protective effects of vanadium compounds in brain ischemia, vanadyl sulfate and VO(OPT) elicits cardioprotection in myocardial ischemia/reperfusion-induced injury and thereby markedly promoted the functional recovery of heart constriction.[80-83] The VO(OPT)-induced cardioprotection was mediated by increased FLICE-inhibitory protein (FLIP) expression and decreased Fas ligand and Bim expression via Akt activation.

Possible mechanism underlying neuroprotective mechanism of VO(OPT) on brain ischemic neuronal cell death was shown in Figure 5. Binding of trophic/survival factors to tyrosine kinase

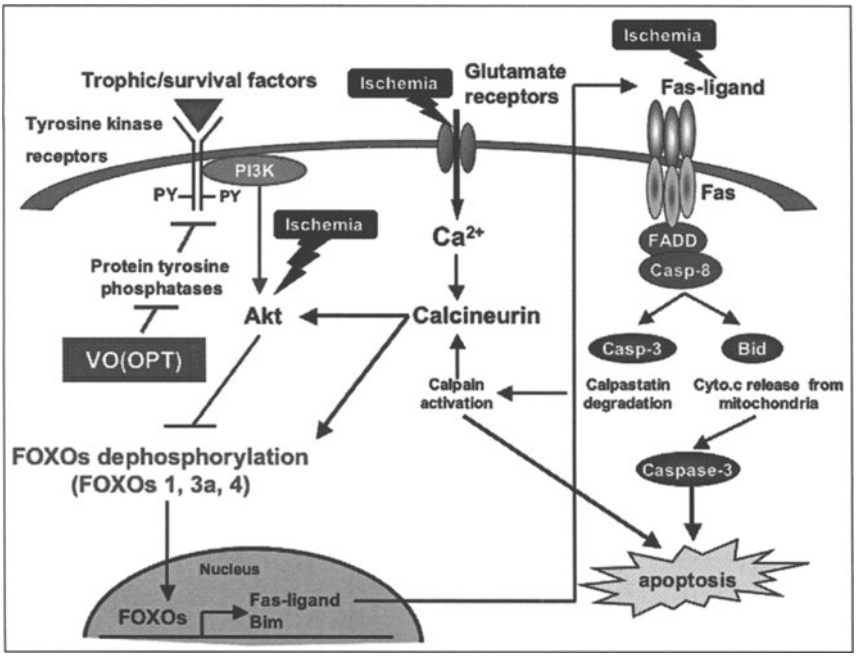

Figure 5. Possible mechanisms underlying Fas-ligand expression and neuroprotective action of VO(OPT) in ischemic brain insults. Binding of trophic/survival factors to tyrosine kinase receptors activate Akt through PI3K activation. Akt inhibits FOXO function by phosphorylation. Brain ischemia causes a transient inactivation of Akt via dephosphorylation by protein phosphatase 2A and calcineurin, thereby promoting apoptosis through dephosphorylation of FOXOs. Calcineurin activating by Ca^{2+} elevation and calpain-induced limited proteolysis also accounts for dephosphorylation of FOXOs both in the cytoplasm and nucleus. VO(OPT) activates Akt activity through inhibition of protein tyrosine phosphatases, thereby preventing dephosphorylation of FOXOs. Finally, the VO(OPT) treatment preferentially inhibits expression of Bim and Fas-ligand through inactivation of FOXOs. The inhibition of Bim and Fas-ligand expression leads to prevention of apoptosis induction. Caspase-3 activation triggers degradation of calpastatin, an endogenous calpain inhibitor, promotes aberrant calpain activation, thereby generating constitutively active calcineurin. The calcineurin activation also promotes apoptotic neuronal death.

receptors activates Akt through PI3-K activation. The Akt inhibits functions of FOXOs by phosphorylation. The ischemia/reperfusion causes dephosphorylation and inactivation of Akt, thereby decreasing phosphorylation of FOXOs. Calcineurin activation is likely involved both in dephosphorylation of Akt and FOXOs. Once FOXOs are dephosphorylated, the factors translocate into nucleus and in turn elicit apoptosis by inducing Bim and Fas-ligand. Fas is a tumor necrosis factor (TNF) receptor family member and is activated by Fas-ligand binding. Activation of Fas-ligand/Fas signaling leads to caspase-8 activation through a death-inducing signaling complex with FADD. The mitochondrial-dependent and -independent apoptosis is induced following caspase-8 activation. In the mitochondrial-dependent pathway, caspase-8 cleaves Bid, a "BH3-only" pro-apoptosis Bcl-2 family protein, thereby promoting cytochrome *c* release. The released cytochrome *c* binds to apoptotic protease-activating factor-1 (Apaf-1) and in turn triggers caspase-9 activation. In the mitochondrial-independent pathway, caspase-8 activation directly leads to caspase-3 activation. Thus, VO(OPT) treatment inhibit protein tyrosine phosphatases, thereby stimulating receptor tyrosine kinases activated by growth and trophic factors. Akt activation through PI3-K in turn restores the phosphorylation of FOXO phosphorylation, thereby inhibiting FOXO-mediated Fas-ligand and Bim expression. The inhibition of Fas ligand and Bim expression rescues neurons from the mitochondrial-dependent and –independent apoptosis.

In addition to Akt signaling pathway, beneficial effects of caloric restriction on longevity are mediated by SIRT1. Resveratol (trans-3, 5, 4"-trihydrovystibene), a naturally occurring polyphenol produced by a wide variety o plants, activates SIRT1. SIRT1 is located in the nucleus and deacetylates p53, NF-kappaB and FOXOs. Ischemic preconditioning is protective against lethal ischemic indults by deactivating p53 in the heart and brain.[84,85] SIRT1 inhibitor sirtinol abolished resveratol-mediated hippocampal neuroprotection in oxygen glucose deprivation-induced cell death in the organotipic hippocampal slices.[86] Resveratol also protect PC12 cell injury against Aβ-induced reactive oxygen species (ROS) production and DNA damage.[87] Since SIRT1 enhances expression of an antioxidant manganese superoxide dismutase (MnSOD) in NF-kappaB- and FOXO-dependent manner, the upregulation of MnSOD likely mediates the ROS-induced cell damage.[76] Likewise, caloric restriction is potential clinical therapy of Alzheimer's disease by increasing expression of neurotrophic factor.[88] Taken together, regulation of FOXO function by Akt and SIRT1 is attractive therapeutic strategy for the neuroprotection against ischemic insults and neurodegenerative disorders.

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CHAPTER 11

New Insights for FOXO and Cell-Fate Decision in HIV Infection and HIV Associated Neurocognitive Disorder

Min Cui, Yunlong Huang, Yong Zhao and Jialin Zheng*

Abstract

Human immunodeficiency virus Type 1 (HIV-1) infection and associated diseases continue to represent major health problem worldwide. FOXO transcriptional factors play an important role in the regulation of cell apoptosis, cell cycle arrest, stress resistance, metabolism and differentiation. This chapter will discuss the diverse functions of FOXO in different cell types including T-cells, macrophages, neurons and astrocytes within the context of HIV-1 infection. Given the overwhelming evidence that FOXO proteins influence the cell fate of immune cells and involve in the homeostasis of the central nervous system (CNS), we will also discuss the potential role of FOXO factors in HIV-1-associated neurological disorders.

Introduction

The Forkhead Box O (FOXO) transcription factor family, the mammalian orthologs of *Caenorhabditis elegans* "forkhead protein" DAF-16, is characterized by a conserved DNA binding domain commonly known as a "forkhead box" or a "winged helix".[1-4] FOXO proteins play an essential role in the crosstalk between many signaling pathways, including cell cycle, metabolism, apoptosis and cell survival. Regulation of FOXO transcription factors is carried out by a complex interplay of phosphorylation, acetylation, ubiquitination and interaction with other protein partners. These posttranslational modifications of FOXO proteins work in concert to determine the role of FOXO in diverse cellular processes, which may be dependent on the microenvironmental cues and the appropriate downstream signals. Recently accumulating evidence shows that FOXO proteins play a critical role in the pathogenesis of HIV-1-infection. This chapter provides an overview of FOXO proteins and their potential roles in various pathological conditions such as HIV-1 infection and its associated neurological complications. Understanding the involvement of FOXO proteins in the pathogenesis of HIV-1 infection and other CNS-associated diseases may provide therapeutic targets for the treatment of HIV-1 infection and its associated neurodegenerative disorders and neuronal injury.

FOXO Family Members and General Function

Four FOXO isoforms, namely FOXO1, FOXO3a, FOXO4 and FOXO6, have been identified in mammalian cells to date.[1-5] The expression and function of FOXO isoforms have been investigated in great detail. Unlike other members of the family, FOXO6 is only detected in the developing brain and has different posttranslational regulation mechanisms due to the lack

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of conserved Akt-1 phosphorylation motif.[5] FOXO1, FOXO3a and FOXO4 are ubiquitously expressed and participate in diverse physiologic processes, including cell cycle regulation, differentiation, apoptosis, stress resistance and metabolism. Notably, the cellular outputs and the cell fate decisions of FOXO are determined by a host of downstream factors that appear to be cell type- and microenvironment-specific. One important aspect of the FOXO family is that the gene expression pattern overlaps during development and in the adulthood, implying that FOXO proteins may bind to and regulate the same target genes. Thereby, FOXO isoforms display functional redundancy and compensation in vivo, whilst keeping isoform-specific function in some cell linages and tissues.

Regulation of FOXO Protein Activity

Multiple posttranslational modifications of FOXO proteins affect FOXO transcription activity, including phosphorylation, acetylation, ubiquitination and protein-protein interactions. Phosphorylation is the most critical modification as it essentially regulates the translocation of all FOXO proteins between the nucleus and cytoplasm. An exception to this is FOXO6, which is a nuclear factor and does not translocate out of the nucleus. FOXO phosphorylation primarily inhibits FOXO function with rare exceptions. Phosphatidylinositol 3-kinase (PI3K), which responds to growth factors and cytokines, has been known to regulate FOXO function.[6-9] Akt-1 phosphorylates FOXO3a at three residues and retains FOXO3a in the cytoplasm by promoting its association with adaptor molecule14-3-3,[8,10,11] subsequent deactivation and degradation of FOXO3a. In the absence of Akt-1 phosphorylation, FOXO3a translocates to the nucleus and controls cell cycle, apoptosis and other processions through transcription of target genes.[12-14] In addition to Akt-1, many kinases also phosphorylate FOXO. These include serum and glucocorticoid-regulated kinase (SGK), c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), dual specificity tyrosine-phosphorylated and regulated kinase (DYRK1A), mammalian Ste20-like kinase-1 (MST1) and IκB kinase (IKK) (for summary, see Table 1).[8,11,15] Though phosphorylation usually prevents activation of FOXO through cytoplasmic translocation, there are two exceptions to this rule. In response to stress stimuli, JNK and Mst1 phosphorylate FOXO at a distinct set of threonine residues and promote nuclear translocation leading to transcriptional activation (see Table 1). Commonly, following cytoplasmic translocation, FOXO phosphorylation also results in FOXO ubiquitination and proteasomal degradation, further reducing the transcriptional activity of FOXO.[10,16-18]

Another intriguing regulation method of FOXO is acetylation and/or deacetylation. Due to its influence on phosphorylation, acetylation can also affect FOXO subcellular localization. FOXO can be acetylated by the calcium response element-binding (CREB)-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF); whereas FOXO can be deacetylated by histone deacetylases (HDACs) and NAD-dependent deacetylases.[33-36] Acetylation may suppress FOXO protein activity and serve as a negative feedback signal during FOXO activation. Acetylation-defective FOXO1 mutants have higher transcription than wild-type, but the mutant FOXO1 tends to be rapidly ubiquitinated and degraded. However, mutations that mimic the acetylated state of FOXO increase stability but impair FOXO1 transcription.[37] More specifically, acetylation of FOXO by CBP and/or p300 prevents the binding of FOXO to its target DNA, reduces the stability of the FOXO-DNA complex and increases the phosphorylation of the nonessential Akt-1 phosphorylation site.[38,39] Deacetylation, on the other hand, may enhance the transcription activity of FOXO. During oxidative stress, SIRT2 expression increases and results in the acetylation level of FOXO3a reduced. As a consequence, FOXO transcriptional activity increases along with its target genes, p27Kip1, manganese superoxide dismutase (MnSOD) and Bcl-2-interacting mediator of cell death (Bim), which work on oxidative stress mediated apoptosis.[40] Interestingly, in mouse pancreatic β cells, acetylation and deacetylation seems to reach equilibrium to maintain FOXO3a transcription activity. FOXO1 protects pancreatic β cells against oxidative stress by forming a complex with promyelocytic leukemia protein (Pml) and deacetylase SIRT1, subsequently activating the

Table 1. Summary of upstream kinases, phosphorylation sites and the cellular outcomes for FOXO members

	FOXO1	FOXO3a	FOXO4	Cellular Outcome	References
Akt-1	T24, S256, S319	T32*, S253*, S315	T28, S193, S258	Inactivation, cytoplasmic translocation	19-22
SGK	T24, S256, S319	T32#, S253, S315#	T28, S193, S258	Inactivation, cytoplasmic translocation	11,23-25
CK1	S322, S325	S318, S321	S261, S264		26,27
CDK2	S249				28
MST1	S212	S207		Activation, interact with JNK pathway	12
DYRK1	S329	S325	S268		29
ERK		S344, S294, S425		Inactivation, cytoplasmic translocation	30
JNK			T447, T451	Activation, nuclear translocation	31
IKKβ		S644		Cytoplasmic translocation and ubiquitination	32

* indicates the phosphorylation preference of Akt-1, and # indicates the phosphorylation preference of SGK.

expression of NeuroD and MafA, two Insulin2 (Ins2) gene transcription factors that are known to alleviate oxidative stress.

Ubiquitination of FOXO proteins provide another avenue to regulate their transcriptional activities. Notably, poly- and mono-ubiquitination result in different cellular outcomes for FOXO. Phosphorylation by Akt-1, ERK, or SGK not only retains FOXO in the cytoplasm but also facilitates the polyubiquitination and degradation of FOXO. This polyubiquitination provides a potential negative feedback regulation to properly control FOXO activity in response to growth factor signaling.[17,30,41-43] Phosphorylation of FOXO3a C-terminal residue Ser-644 by IKK also promotes polyubiquitination and degradation of FOXO3a through the proteasome pathway. Residue Ser-644 is exclusive to FOXO3a and is absent in the other FOXO proteins.[44] Though polyubiquitination promotes degradation of FOXO,[41,45] monoubiquitination of FOXO in response to cellular oxidative stress leads to the nuclear translocation and transcriptional activation of FOXO proteins.[46-48] Monoubiquitination of FOXO is counteracted by USP7, a deubiquitinating enzyme that binds to FOXO proteins and represses FOXO activity. Surprisingly, neither monoubiquitination nor USP7-mediated deubiquitination affects FOXO protein stability.[48]

One crucial protein in the regulation of subcellular localization of FOXO is the chaperone protein 14-3-3, which plays a direct role in the phosphorylation and acetylation of FOXO. The 14-3-3 protein has a U-shaped structure that serves as a dock for the phosphorylated serine or threonine residues of FOXO. The 14-3-3 protein isoforms are also able to form stable homo- or heterodimers and thus could bind two ligands simultaneously.[49,50] Many of the phosphorylated serine or threonine residues are located in the FOXO nuclear localization sequence (NLS). Binding of 14-3-3 proteins to FOXO therefore masks or obscures the NLS and subsequently prevents FOXO protein translocation into the nucleus.[11,12,49,51] In addition to binding to the

phosphorylated residues, the 14-3-3 proteins seem to be required to bridge FOXO3a and SIRT1 together to facilitate the deacetylation process.

The fine-tuned regulation of FOXO transcriptional activity typically involves the interaction of FOXO and other partners. Depending on the cell type and cues from the microenvironment, these protein-protein interactions can either foster or suppress FOXO transcriptional activity and affect the subsequent cellular response. FOXO proteins play key roles in cell cycle control through *p21Cip1*, *p27Kip1* and other genes by directly binding to their promoters. Furthermore, in the case of transforming growth factor beta (TGFβ) stimulation, FOXO requires additional proteins, namely Smads and FOXG1, to control the expression of the growth inhibitory gene *p21Cip1*. Smad2 and Smad3 hetero-oligomerize with Smad4, translocate to the nucleus and form a complex with FOXO. This complex can bind to the promoter of *p21Cip1* and turn on its expression. In contrast, FOXG1, a protein from a distinct FOX subfamily, inhibits *p21Cip1* expression through binding FOXO/Smad complexes. These interactions enable the TGFβ/Smad pathway and the FOXG1 to delicately regulate the expression of downstream factors of FOXO.[52-56] In addition, p15INK4b, another downstream factor of FOXO, requires CCAAT/enhancer binding protein β (C/EBPβ) and the FOXO-Smad complex to properly respond to TGFβ.[52] RUNX3, a runt domain-containing transcription factor, is also required by FOXO to induce the expression of Bim. The *Bim* promoter contains one FOXO binding site and two RUNX3 binding sites in close proximity and the interaction of FOXO and RUNX3 coordinately upregulates Bim expression and promotes apoptosis in gastric cancer cells.[57]

The interaction of FOXO proteins and their partners may release the transcriptional repressor from the promoter of target genes and this removal leads to the expression of these genes. In this case, FOXO proteins serve as co-activators rather than specific transcription factors. For example, the tumor suppressor p53 normally inhibits *Sirt1* expression by binding to two sites of *Sirt1* promoter. Under nutrient deprivation, FOXO binds to p53 and releases p53 from the *Sirt1* promoter, therefore activating *Sirt1* expression. This interaction between FOXO and p53 is independent of the binding between FOXO and the *Sirt1* promoter.[58] Similarly, in muscle cells, the binding of FOXO with the transcriptional repressor Csl also releases Csl from *Hes1* promoter and induces *Hes1* expression. In addition, FOXO may interact with other proteins and inhibit FOXO transcription activity in return. For example, FOXG1 binds to FOXO-Smad complex and leads to FOXO target gene suppression. Furthermore, the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) may inhibit FOXO activity transcription through the interacting with FOXO.[59]

FOXO in HIV-1 Infection and HIV-1 Associated Neurocognitive Disorders

In HIV-1 pathogenesis, depletion of T-cells and other immune cells is the most fundamental pathophysiological consequence. Notably, the immune activation may induce T-cells and other cell loss in different stages of HIV-1-infection.[60,61] HIV-1 can directly kill the infected $CD4^+$ T-cells and destroy the uninfected and bystander cells simultaneously. In addition to the disruption of the peripheral immune system, HIV-1 also leads to a spectrum of viral-induced neurocognitive disorders. Although the mechanisms have yet to be fully elucidated, HIV-1-mediated brain inflammation including overproduction of cytokines, chemokines, glutamate and others factors have been shown to play significant roles in disease progression.[62-64] HIV-1 enters the brain shortly after initial infection, crossing the blood brain barrier via peripherally infected monocytes.[65] Brain macrophages and microglia, unlike other cellular residents in the CNS, are able to sustain a productive HIV-1 infection within the brain.[66] Therefore, while the rest of the body in general experiences a rise of viral load followed by a gradual decline, the isolated CNS maintains a low, but persistent level of HIV-1-infection. Although neurons are not infected by HIV-1, a dementia specific to HIV-1 has been described as HIV-1-associated dementia (HAD).[67,68]

HAD is the clinical consequence of neuronal injury and dropout. The pathologic correlate to HAD, HIV encephalitis (HIVE), is characterized by HIV-1-infected and -activated macrophages

and microglia, damage of neuronal dendrites and axons and apoptotic neurons. As a result of HIV-1 infection, the immune cells are recruited to the proximate site and produce an array of factors including cytokines, proteases and other factors, yet they are unable to clear the infection. The difficulty in eradicating HIV-1 infection prolongs the immune response leading to a chronic inflammatory state. Chronic inflammation has both detrimental and beneficial effects. On one hand, these responses are essential in limiting viral spread; yet on the other hand, excessive inflammation is detrimental to resident cells such as neurons or neural stem cells that are important for the neuronal repair process.

HIV-1 infection and its consequence in the brain, macrophage/microglia infection and activation and its associated neuronal injury and/or loss, and the changes in neuronal repair process have been the focus of much investigation. As introduced previously, FOXO is a key factor in the determination of cell fate in different environments. In the following sections, we will discuss the potential roles of FOXO in HIV-1 infection, its associated immunodeficiency and the long-term consequences on the host central nerve system during HIV-1 infection.

FOXO in T-Cell Depletion

Potential Signaling Pathway of FOXO3a in T-Cell Depletion

HIV-1 Infection leads to progressive $CD4^+$ T-cell depletion, resulting in AIDS (Acquired Immune Deficiency syndrome) development. The mechanisms that trigger the $CD4^+$ T-cell death are not fully understood, but data indicate that apoptosis plays a major role in this cell loss. Both infected and uninfected $CD4^+$ T-cells die during HIV-1 infection by different cell death pathways and bystander $CD4^+$ T-cell loss is now recognized as essential to the immunodeficiency.[69-71]

The general roles of FOXO proteins in the immune system might be relevant to immune homeostasis.[72-74] Normally, FOXO inactivation is indispensable to maintain T-cell survival and proliferation. Once FOXO is activated, FOXO triggers apoptosis in T-cells by regulating the expression of several pro-apoptotic genes, such as *FasL* (Fas ligand, also known as CD95 ligand), *Bcl-6* (B-cell lymphoma 6), *Bim* and *Puma* (p53 upregulated modulator of apoptosis).[75-79] In HIV-1-induced T-cell apoptosis, FOXO may also play an important role. Accumulating evidence suggests FOXO members participate in HIV-1-induced T-cell apoptosis, directly or indirectly, through a differential regulation of apoptosis. Specifically, HIV-1-infection can trigger both intrinsic and extrinsic apoptotic pathways that are regulated via FOXO in infected and uninfected T-cells during HIV-1 infection.

Several HIV-1 proteins have been shown to interfere with cellular proteins implicated in the control of cell cycle and apoptosis, particularly, cell cycle G2 arrest.[80] HIV-1 protein Vpr induces cell cycle G2 arrest and blocks infected cells from proliferating.[80] Vpr blocks cell cycle progression by activating the ATR (ataxia-telangiectasia and Rad3-related) complex (including ATR, Rad17 and Rad9-Hus1-Rad1), leading to Cdc25c functional suppression, Cdk1 and cyclin B downregulation. At the same time, activation of ATR complex also induces Gadd45a expression. Both G2 arrest and Gadd45a expression result in Bax activation, which induces apoptosis via the mitochondrial pathway.[81-85] Interestingly, FOXO3a has been mechanistically linked to Vpr-induced cell cycle arrest and apoptosis.[84,86-88] Vpr may modulate FOXO function through two ways. First, Vpr is able to interfere with the association of FOXO3a with 14-3-3 and subsequently impede the shuttling of FOXO3a from the nucleus to the cytoplasm. Second, Vpr inhibits insulin/PI3K/Akt-1 signaling pathway, leading to FOXO3a activation and translocation to the nucleus.[89,90] The activation of FOXO may also induce G2/M cell cycle arrest through the upregulation of Gadd45a and cyclin G2.[91,92] In addition, FOXO may facilitate cell cycle arrest through the inhibition of the FOXM, which is known to positively drive G2/M phase transition.[29,93]

FOXO is also involved in HIV-1 protein Tat-induced $CD4^+$ T-cell apoptosis. Tat triggers Egr1-PTEN-Akt-1 (early growth response-1/phosphate and tensin homolog deleted on chromosome 10/Akt-1) and p53 pathways, which converge on the regulation of FOXO3a transcription factor and result in FOXO3a activation. The FOXO3a target genes, *FasL* and *TRAIL* (Tumor

necrosis factor(TNF)-related apoptosis-inducing ligand), are the primary TNF family members that engage in the extrinsic apoptotic pathway; while *Puma*, *Noxa* and *Bim* are members of BCL-2 family that participate in the intrinsic apoptotic pathway. Therefore, HIV-1 protein Tat could induce apoptosis in $CD4^+$ T-cells through multiple pro-apoptotic target genes associated with FOXO3a.[94] As Tat can be secreted by infected cells and taken up by uninfected cells, Tat-induced apoptosis could potentially affect both infected cells and uninfected cells.[95,96]

As reviewed by Selliah, other HIV-1 proteins have been implicated in aspects of apoptosis, including Nef, Vpu, Env and protease.[80] Limited evidence is available to confirm the involvement of FOXO in apoptosis induced by those aforementioned proteins. However, the potential function of FOXO members through their target genes (such as *FasL*, *TRAIL* and *Bim*) and the participation of these genes in the HIV-1-induced cell death, indicates that FOXO might contribute to the cell death induced by HIV-1-infection or HIV-1 encoded proteins.

FOXO3a Commits to the Survival of Central Memory $CD4^+$ T-Cell in HIV Infection

$CD4^+$ central memory (TCM) and effector memory (TEM) T-cells are two distinct populations of memory T-cells that can recognize foreign invaders such as bacteria or viruses upon second encounter. TCM and TEM have different properties regarding proliferation, apoptosis and persistence. TCM cells are more resistant to apoptosis and have an increased capacity to proliferate or survive than TEM cells in vitro. These fundamental functional differences of TCM and TEM are conferred by the activation and phosphorylation status of two transcription factors, STAT5 and FOXO3a.[97] In response to proliferative signals, TCM cells showed a significant increase in the levels of STAT5 phosphorylation compared with TEM cells; moreover, ex vivo TCM cells express higher levels of the inactive phosphorylated forms of FOXO3a and lower levels of the pro-apoptotic FOXO3a target protein Bim. The high level of active STAT5 and inactive FOXO3a ensure the TCM cell longevity and survival, which are critical in immunological memory. In HIV/AIDS, the persistence of TCM cells is critical to maintain proper immunological functions, as the rate of TCM cell decline predicts HIV disease progression. TCM and TEM cells from HIV+ elite controller (EC) subjects, who naturally control viral replication, are less susceptible to FasL-mediated apoptosis and survive longer after multiple rounds of T-cell receptor activation when compared to TCM and TEM cells from successfully treated aviremic subjects or from HIV-1 seronegative donors. The persistence of TCM cells from EC subjects is a direct consequence of inactivation of the FOXO3a pathway. Silencing the FOXO3a by small interfering RNA or introducing a FOXO3a dominant-negative form extends the long-term survival of TCM cells from successfully treated subjects to a length of time similar to that of TCM cells from EC subjects. Therefore, inactivation of FOXO3a in both TCM and TEM cells of HIV patients may benefit the immune response specifically to HIV-1 and protect T-cells from apoptosis. The crucial role of FOXO3a in the persistence of memory T-cells provides a new prospect of therapeutic avenue to control the HIV-1 persistence.[97,98]

FOXO in Macrophage/Monocyte Pathology of HIV-1 Infection

Macrophage represents early cellular target and a reservoir of HIV-1 in its natural host. Compared to T-cells, macrophage/monocytes are more resistant to cytopathic effects of virus and sustain long-term productive infection throughout the disease course. Although the virus follows similar life cycle in macrophages and T-lymphocytes, the infected macrophages are prone to evade the immunological attack, which results in the establishment of long-term reservoirs in macrophages and subsequently disseminates the virus to various tissues such as the brain and lung. The investigation of these tissue macrophages is often difficult because of their limited accessibility and inefficient recovery. Therefore, many in vitro studies of infection utilize monocyte-derived macrophages (MDMs), which provide a unique model for effective laboratory and primary HIV-1 infection. With this cellular model, we have found that FOXO3a contributes to HIV-1-mediated cell death of macrophages during productive infection. Similar role of FOXO3a has been identified

in lymphocyte apoptosis. However, significant question remains to be answered regarding the exact role of FOXO3a in human macrophage in vivo. Whether the in vitro cellular model could fully recapitulate the complexity of the viral replication and transmission in vivo? The complex nature of viral infection requires an integration of viral proteins, RNA and a range of host cellular factors. Therefore, multiple metabolic or cellular signaling pathways may participate and interfere with the regulation of transcription factors such as FOXO, which often affect the data interpretation and variable results may co-exist in the literature.[99]

The Potential Relationship of FOXO Proteins and Viral Replication in Macrophage

HIV-1 accessory protein Vpr, which arrests T-cells in cell cycle G2 phase, has been found to disrupt the interaction between adaptor molecule 14-3-3 and FOXO3a. The transcriptional activity of FOXO3a is normally suppressed by insulin-induced phosphorylation. Vpr inhibited insulin-mediated Akt-1 phosphorylation and may change the subcellular localization of FOXO3a. Vpr may also interfere with insulin-induced coprecipitation of 14-3-3 and FOXO3a and antagonize the negative effect of insulin on FOXO3a transactivation on FOXO-responsive promoter. These results indicate that Vpr has the potential to activate FOXO3a and cause cell cycle arrest of HIV-1-infected cells.[90] In contrast to $CD4^+$ T-cells, HIV-1-infected macrophages, which are terminally differentiated cell, typically resist cell death, support viral replication and consequently may facilitate HIV-1 transmission. There is evidence that shows HIV-1 accessory protein Vpr may also affect viral replication in macrophages through transcription factor FOXO3a. HIV-1 accessory protein Vpr has been found to regulate cyclin-dependent kinase inhibitor 1A (p21Cip1), which is significantly upregulated during HIV-1 replication.[100] The signaling pathway involved in Vpr-mediated p21 increase is unknown. It has been reported that transcription factor FOXO3a binds to p21 promoter and triggers p21 expression. Therefore, it is possible that Vpr activates FOXO3a through interfering with the 14-3-3-FOXO3a interaction and leads to p21 protein upregulation and contributing to viral replication.[100]

Another potential player interacting with FOXO3a in macrophage during HIV-1 infection is NF- κB. FOXO3a could inhibit NF-κB activity, because Foxo3a-deficient mice show increased NF-κB activation.[74] In HIV-1 infection, FOXO3a may also play a similar role in the inhibition of macrophage NF-κB activation. It has been known that HIV-1 replication in macrophage requires NF- κB activity. In the early stage of HIV-1-infection, NF-κB is activated by upstream kinases; and FOXO is functionally inhibited by its upstream kinases such as Akt-1 or ERKs. This functional inhibition is important for NF-κB activation, which would promote HIV-1 replication and inflammatory cytokine production in HIV-1-infected macrophages.[101] In the later stage of HIV-1-infection, productive HIV-1 infection attenuates PI3k/Akt-1 pathway, which lead to the activation of FOXO and translocation of FOXO into the nucleus. Activated FOXO may further inhibit NF-κB activity, preventing its pro-survival function as demonstrated in infected macrophages.

The Dual Role of FOXO3a in HIV Infection in Macrophages

The exact molecular changes of protein profile in macrophages during HIV-1-infection in vivo remain to be fully understood. Studies have shown that HIV-1-encoded proteins are able to manipulate cellular pathways, modifying the apoptotic machinery that regulates host cell death in either a pro- or anti-apoptotic manner. This is critical during early stage of HIV-1 infection, in which proper cell survival is needed for viral replication. Akt-1 and NF-κB, important for macrophage survival, are activated, so that cells are highly resistant to cell death compared with other tissue cell types. With infection progresses, RNA transcription in productively infected macrophages indicates a conflicted state where pro-apoptotic and anti-apoptotic cascades are modified as the cells respond to HIV-1. Death factors such as TRAIL, TNF and Fas are upregulated and the anti-apoptotic factors Bcl-2, NAIP (neuronal apoptosis inhibitory protein) and Akt-3 are significantly downregulated, but survival factors including XIAP (X chromosome

linked inhibitor of apoptosis protein), MDM2 (murine double minute 2) and SOD2 (superoxide dismutase 2) are upregulated as well (N. Erdmann et al manuscript in preparation). These data suggest that HIV-1 infection in macrophages is quite dynamic and HIV-1 may modulate the survival-apoptotic equilibrium in favor of optimal viral replication. Disruption of the equilibrium in either way during viral life cycle has been proved to be detrimental. The role that FOXO3a plays in the HIV-1 infection may also be bidirectional. First, during the early stage of infection, phosphorylation of Akt-1 and FOXO3a is increased and this benefit cell survival and HIV-1 replication.[102,103] HIV-1 proteins, such as Tat, gp120 and Nef, have been shown to activate the PI3K/Akt-1-dependent survival pathway, which facilitates HIV-1 replication and viral particle production.[102,104-106] Second, PI3K/Akt-1 pathway is important for the resistance to cell death of HIV-1-infected macrophages. As the inhibition or attenuation of Akt-1 activity dramatically reduces the viability of long-living virus-infected macrophages. Alternatively, inhibition or attenuation of Akt-1 may sensitize infected macrophages to stresses or extracellular stimuli, which would otherwise not cause cell death of macrophages.[103] Indeed, both the phosphorylation of Akt-1 and FOXO3a decreased once productive infection established.[107,108] These evidences suggest that PI3K/Akt-1 activation contributes to viral replication and macrophage resistant to cell death in the early stage of infection. As a main downstream factor of PI3K/Akt-1 pathway, FOXO is regulated through phosphorylation on T32, S253 and S315 (FOXO3a) or on homologous sites (other members). The detailed mechanism of how Akt-1 activation leads to FOXO3a inhibition and subsequent apoptosis-resistance has been well-documented. However, the exact role of this signaling pathway during HIV infection has remained to be fully elucidated. HIV-1 does not induce significant apoptosis during early replication. Once the productive infection established, HIV-1 increases the activity of transcription factor FOXO3a by translocation to nucleus. Adenoviral delivery of constitutively active FOXO3a, which contains three mutated phosphorylation sites maintaining a transcriptional active FOXO3a was found to induce DNA fragmentation with decreased cell viability in MDM. Moreover, a dominant-negative mutant of FOXO3a, or small interfering RNA for FOXO3a in HIV-1-infected MDM decreased DNA fragmentation and protected macrophages from cell death, which suggests elevated FOXO3a activity promotes HIV-1-infected macrophage cell death.[108] In addition, overexpression of constitutive active Akt-1 is sufficient to induce FOXO3a phosphorylation, suggesting that FOXO3a is a downstream of Akt-1 in macrophage.[108] Comparison of primary HIV-1 isolates with laboratory strains also indicates that a similar infection course and cell loss during productive infection. The infection levels and cell loss are associated with the phosphorylation status of Akt-1 and FOXO3a, suggesting Akt-1/FOXO3a pathway plays an important role in HIV-1-induced cell death of human macrophage.

Based on the studies described above, we propose that FOXO3a may play a dual role in HIV-1-infected macrophages (Fig. 1). In the early stage of infection, PI3K/Akt-1 are activated leading to FOXO3a inactivation and subsequent resistance to cell death; with the virus replication and accumulation in macrophages, the PI3K/Akt-1 pathway is gradually downregulated and leads to FOXO3a activation. As a consequence of FOXO3a activation, cell death and apoptosis signaling pathways are triggered that result in macrophage cell death. Further investigation of this proposed model and the elucidation of the PI3K/Akt-1/FOXO3a pathway and its role in macrophages during HIV-1 infection should continue, as it will bring further understanding of HIV-1 pathogenesis.

FOXO and HIV-1 Mediated Central Nervous System Damage

HIV-1-infected monocytes or macrophages infiltrate into the CNS and may serve as a viral reservoir for persistent replication. Currently, about 40% to 70% of people infected with HIV-1 develop CNS disorders and neurological complications.[109,110] More serious neurological symptoms typically present in patients with high HIV loads, when a person has advanced AIDS. HIV-associated neurocognitive disorder (HAND), which includes HIV-1-associated mild neurocognitive disorders and HIV-1 associated dementia (HAD), is frequently accompanied by

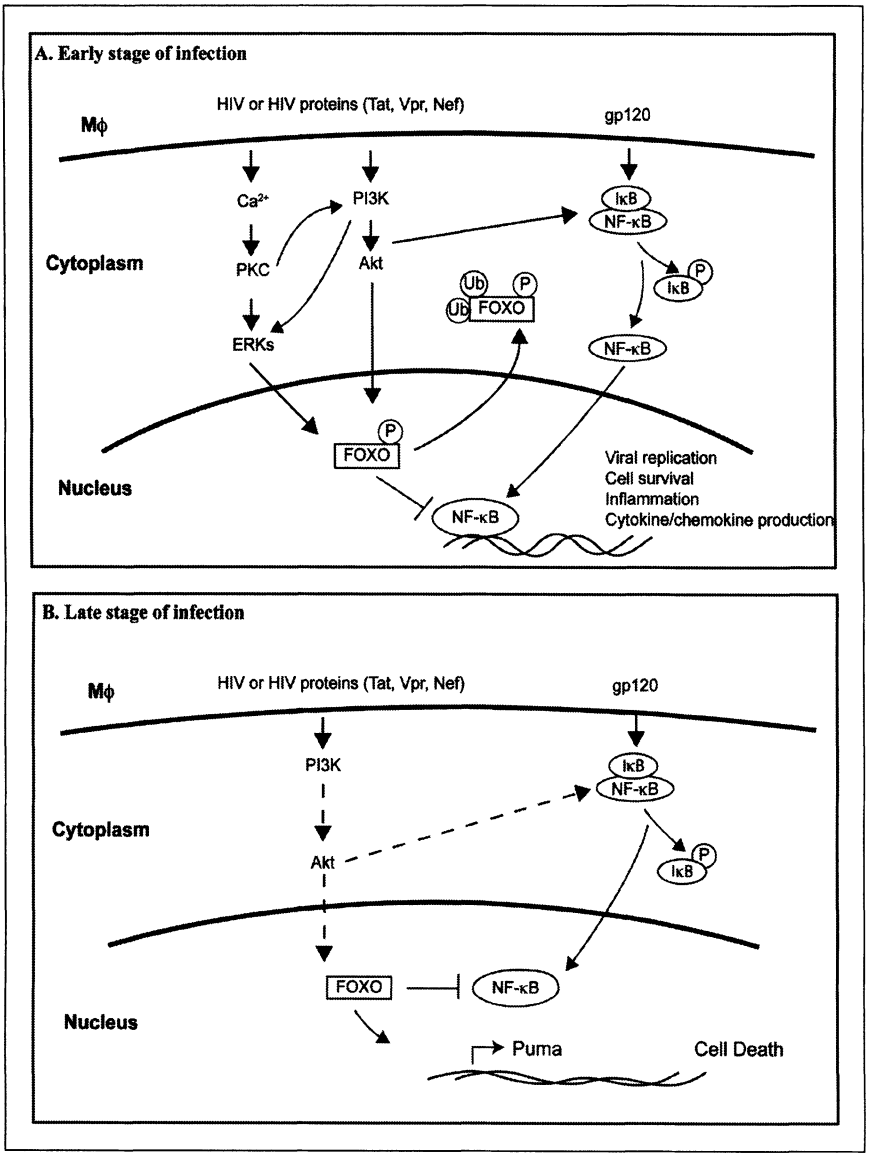

Figure 1. Proposed mechanisms for how FOXO affects macrophage function during early and late stage of HIV-1 infection. A) In the early stage of HIV infection, the binding of HIV-1 or HIV-1 proteins with macrophage cell surface receptors induces intracellular signaling cascades such as Akt-1, ERKs and NF-κB pathways. Activated Akt-1 and ERKs may inhibit FOXO function by phosphorylation. Subsequently, phosphorylated FOXO translocates to the cytoplasm and facilitates ubiquitination and degradation. As a consequence, inhibitory effect of FOXO to NF-κB was removed, which leads to enhanced NF-κB activation that promote viral replication, cell survival, inflammation and cytokine/chemokines production. B) In the late stage of HIV infection, it has been suggested that productive HIV-1 infection compromises PI3k/Akt-1 pathway, which lead to the activation of FOXO and translocation of FOXO in the nucleus. Activated FOXO triggers apoptosis pathways through increased expression of apoptotic proteins such as Puma. Activated FOXO can also inhibit NF-κB, preventing its prosurvival function. Dashed line indicates signal attenuation.

neuronal injury and loss of neuronal subpopulations in the neocortex, limbic system and basal ganglia in association with synaptic and dendritic damage, astrogliosis and formation of microglial nodules and multinucleated giant cells.[111] Although HIV cannot infect neurons, cell of the nervous system, such as astrocytes and microglia, as well as monocytes/macrophages that have migrated to the brain, can be infected with the virus. HIV-1-infected cells release proinflammatory cytokines, chemokines and some toxic products and deliver aberrant signals, leading to neuronal toxicity and neuronal damage in the brain.

In this section, we will further discuss the role of FOXO members in HIV-1-induced neurological disorders. It is acknowledged that HIV-1-induced neurological disorders share some similar molecular mechanisms with other neurodegenerative diseases as it decreases neuronal survival, changes in neural stem/progenitor cells function and causes astrogliosis. Thus, we will expand our topic to the potential function of FOXO in general neuronal stem/progenitor cell homeostasis, neuronal apoptosis and astrogliosis.

FOXO Proteins in the Regulation of Stem Cell Homeostasis in the Nervous System

FOXO proteins are homologous to *C. elegans* Daf-16, which determines metabolic insulin signaling and leads to lifespan extension. It is known that insulin-like signaling is essential for growth and metabolism in *C. elegans*. Inhibition of insulin-like signaling leads to Daf-16 activation and increase of stress resistance and longevity.[112] Restored insulin-like pathway in neurons is sufficient to reinstate a wild-type life span.[112] Further investigation revealed that Daf-16 is the key factor downstream of insulin-like pathways that controls the life span of *C. elegans* and regulates the expression of free radical-scavenging enzymes, catalase and SOD. In mammals, it has also been reported that FOXO proteins are important to maintain the stem cell pool. In Foxo3a-deficient mice, the proliferation and differentiation of hematopoietic progenitors were normal, but the number of colony formation cells was reduced. The ability of Foxo3a−/− hematopoietic stem cells (HSCs) to support long-term reconstitution of hematopoiesis was also impaired and was coupled to an elevation of reactive oxygen species (ROS), defective maintenance of quiescence and hypersensitive to cell-cycle-specific myelotoxic injury. Consequently, HSC frequencies were significantly decreased in aged Foxo3a-deficient mice.[113] Considering the redundancy and compensability of three FOXO members, another group use conditional knockout of all FoxO1, FoxO3 and FoxO4 in the adult mouse hematopoietic system. FoxO-deficient mice exhibited an expansion of both myeloid and lymphoid lineages accompanied with cell cycle progression of the long-term hematopoietic stem cells, suggesting that FoxO preoteins are important in maintaining HSCs in the quiescent state. The FoxO-deficient HSCs also display an increased level of apoptosis further contributing to the aberrant decrease in cell number.[114] All these observations demonstrated that FOXO proteins are important in the maintenance of stem/progenitor cell homeostasis via cell cycle regulation and functional resistance to oxidative stress.

How FOXO regulates the function of neural stem cells or progenitor cells remains unclear, but evidence shows that FOXO proteins also play a role in the regulation of neuronal precursor cells. Erythropoietin (EPO), the traditional mediator of erythroid maturation, was found to modulate neural stem cell in the cellular protection and angiogenesis during development. EPO significantly increased neural progenitor cell proliferation and promoted neural progenitor cell differentiation into neurons, while it also functioned as a protective and an anti-inflammatory factor during oxidative stress.[115] Further signaling studies demonstrate that EPO can activate Akt-1, JAK2 and negatively regulate downstream transcription factor FOXO3a.[115-117] This study indicates that FOXO may play a similar role in neuronal stem cells and in hematopoietic stem cells as both share common properties of all stem cells. Based on this understanding and our observation on neuronal stem cells, we propose the following hypotheses (Fig. 2). First, FOXO proteins play a role in the maintenance of neuronal stem/progenitor cell homeostasis. FOXOs control the cell cycle of stem cells and maintain the majority of stem cells in a quiescent state while a subset of them enter the cell cycle for self-renewal or differentiation. Second, FOXO proteins prevent neuronal stem/progenitor cells from oxidative stress-induced cell damage through scavenging free radicals,

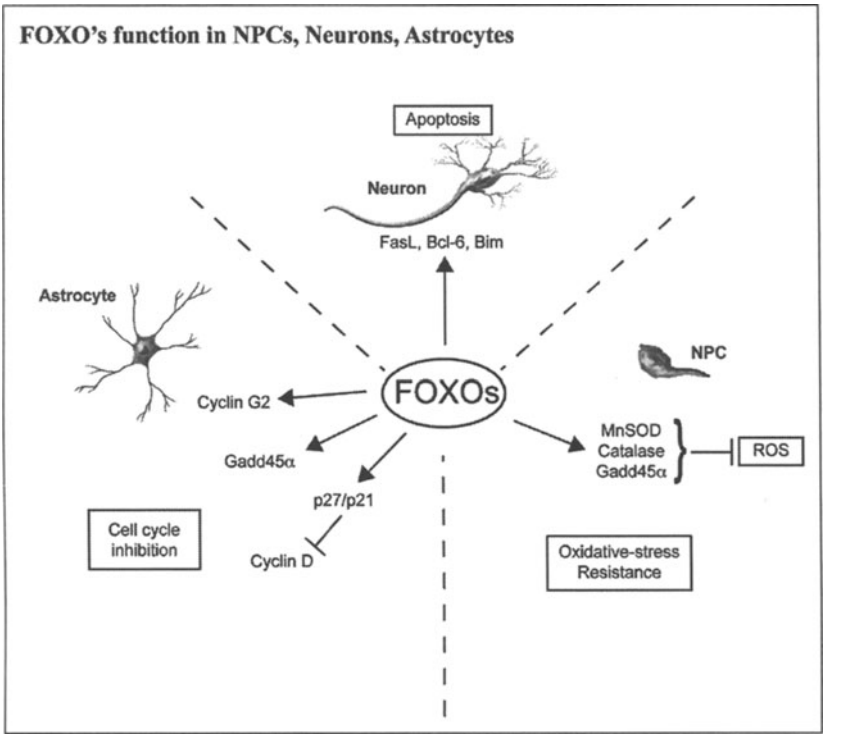

Figure 2. Proposed mechanism for how FOXO influence neurons, astrocytes and neuronal progenitor cell function. In response to oxidative stress or starvation, FOXO–dependent transcription in neurons serves to trigger apoptosis by inducing gene expression of *FasL*, *BcL-6*, *Bim*, etc. In astrocyte, FOXO suppress cell proliferation by inducing cell cycle regulatory proteins cyclin G2, Gadd45a and p27/p21. FOXO also play a crucial role in the homeostatic maintenance of neuronal progenitor cells by coordinating quiescence, stress resistance and/or terminal differentiation.

regulating SOD expression. Note that many aspects of this model have been derived from studies in nonneuronal stem cells and have been extrapolated to neuronal stem/progenitor cells here.

FOXO Proteins Are Pivotal Factors in Neuronal Apoptosis

In the nervous system, aberrant neuronal death is a feature of neurodegenerative diseases. Compared with other cell types, neurons are more sensitive to stress or other apoptotic stimuli other cells of the brain. Indeed, oxidative stress-induced neuronal death is involved in Alzheimer's disease, HIV-associated dementia and other neuronal disease.[118-120] In response to stress stimuli, FOXO-triggered expression of downstream factors Bim, FasL, Puma and TRAIL, may contribute to neuronal death. A recent study showed that oxidative stress elicits neuronal death through activation of FOXO by a dual process that involves timed activation of stress kinases and abrogation of IGF-I neuroprotection. On the one side, ROS induces activation of p38 MAPK, which inhibits IGF-I signaling by interfering IGF-I receptor/IRS-1 interactions through phosphorylation of IRS-1. This leads to abrogate the inhibition of FOXO. On the other side, ROS recruits JNK2 to activate FOXO. These two pathways are independently in response to ROS; but both pathways inhibit FOXO3a trafficking from the nucleus to the cytoplasm and result in FOXO3a transcriptional activation and downstream pro-apoptotic factor Bim expression.[121] Similarly, epileptic brain injury in rats leads to FoxO1 and FoxO3a activation in hippocampal neurons followed by Bim upregulation and neuronal

apoptosis.[122] Many other studies also demonstrated that FOXO transcriptional regulators provide an important link between stress signaling pathways and the neuronal cell death.[122-125]

FOXO3a in Proinflammatory Cytokine-Induced Astrogliosis

Reactive astrogliosis, including astrocyte proliferation and activation, is one of the hallmarks of neurodegenerative diseases. Astrocytes proliferation in response to abnormal stimuli contributes to astrogliosis during brain disorders. The cell cycle inhibitors (cyclin-dependent kinase inhibitors), including flavopiridol, roscovitine and olomoucine inhibit cell cycle progression at the G1/S and G2/M phases and reduce reactive astrogliosis initiated by ischemia or traumatic brain injury.[126,127] The PI3k/Akt-1 pathway seems to be important in the cell proliferation and cell cycle regulation in astrocytes. It has been reported that PI3K/Akt-pathway is involved in the process of injury-induced astroglial proliferation and anti-apoptosis in vivo. p-Akt1/2/3 increased in immunostaining in temporal correlation with the mechanical damage. The phosphorylated Akt-positive-cells were often found colabeled with GFAP around the stab wound.[128] This indicated that the brain injury could activate Akt in astrocytes and subsequent astrocyte proliferation and result in astrogliosis.

Neurodegeneration is often found to be accompanied with an increase of proinflammatory cytokines, such as IL-1β, TNF-α and IL-6 and these proinflammatory cytokines have been showed to mediate astrogliosis; however, the mechanisms by which this process occurs are not well-defined. The investigation about the role of FOXO3a in inflammatory factor-mediated astrocyte proliferation has suggested that FOXO3a interfere astrogliosis via cell cycle regulation (Cui et al unpublished observation). IL-1β and TNF-α induced a significant increase of astrocyte proliferation as determined by Ki67 immunostaining. Cyclin D1, which marks the cell cycle progression, was also increased. FOXO3a, the main upstream regulator of cyclin D1, was phosphorylated and translocated from the nucleus to the cytoplasm with IL-1β and TNF-α stimulation. Wild-type FOXO3a (WT-FOXO3a) overexpression significantly upregulated downstream factor p2, therefore inhibited cyclin D1 expression, which affects on G1 phase; WT-FOXO3a also upregulated Gadd45α expression, which can arrest cell cycle in G2 phase. In contrast, dominant-negative FOXO3a (DN-FOXO3a) decreased p27 and Gadd45α while upregulated cyclin D1. Consequently, WT-FOXO3a inhibited astrocyte proliferation. All these results demonstrated that FOXO3a is a pivotal transcriptional factor in proinflammatory cytokine-induced astrogliosis (Cui et al unpublished observation).

Summary and Future Directions

The regulation and function of the FOXO family have been extensively studied in the last decade and substantial progress has been made in understanding the signaling pathways and mechanisms involved in different cell types and systems. However, many questions remain to be answered about FOXO in HIV-diseases. FOXO appears to be important in cell survival and apoptosis in both HIV-1-infected cells and noninfected cells. Unraveling the multifaceted aspects of FOXO regulation will provide important insights to the processes including T-cell and macrophage apoptosis/survival in HIV-1 infection, neuronal apoptosis and astrogliosis in HIV-neurological diseases. Thus, a detailed understanding of FOXO proteins and their biology will provide new opportunities for developing more effective therapeutic approaches to treat HIV-diseases.

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Section IV

Translating Vital Cellular Mechanisms into Successful Clinical Care

CHAPTER 12

FOXP3$^+$ Regulatory T-Cells in Chronic Kidney Disease:
Molecular Pathways and Clinical Implications

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Abstract

CD4$^+$/FOXP3$^+$ regulatory T-cells (Tregs) are essential for the maintenance of self-tolerance and Tregs deficiency results in spontaneous autoimmunity in both mice and humans. The forkhead box P3 (FOXP3) expression is required for both survival of Tregs precursors as well as their function. This suggests that Tregs may use multiple mechanisms to limit autoimmunity and may reflect functional heterogeneity among Tregs subsets that localize to distinct tissue environments. Both cell contact- and cytokine-based immunosuppressive mechanisms would require that Tregs be in close proximity to their targets. The fundamental regulatory activity that can be consistently demonstrated by Tregs in vivo and in vitro has stimulated great interest in developing novel strategies for treating ongoing inflammatory conditions. Patients with end-stage kidney disease (ESKD) are known to display a cellular immune dysfunction. Uremic solutes that accumulate during ESKD may be involved in these processes. In these patients, oxidative stress induced by oxLDL may increase Tregs sensitivity to Fas-mediated apoptosis in part as a consequence of 26S proteasome activation. The 26S proteasome, an ATP-dependent multisubunit protease complex found in the cytoplasm and in the nucleus of all eukaryotic cells, constitutes the central proteolytic machinery of the ubiquitin/proteasome system. Considering the effect of uremia and oxLDL, Tregs from patients with ESKD exhibit early cell-cycle arrest and become apoptotic. These phenomena are the consequence of the oxLDL inhibited proteasome proteolytic activity of $p27^{Kip1}$ and Bax proteins in Tregs. This may be one mechanistic explanation of the cellular immune dysfunction in patients with ESKD and may have important implications in clinics, since this response could contribute to the micro-inflammation and atherogenesis encountered in this population.

Introduction

Alterations of the immune system in patients with end-stage kidney disease (ESKD) represent a complex issue. On one hand, hypercytokinemia is a typical feature of uremia, likely due to accumulation of pro-inflammatory cytokines as a consequence of decreased renal elimination and/or increased generation following induction by uremic toxins, oxidative stress and volume overload.[1,2] On the other hand, uremia is associated with immunosuppresion due to the impact of the uremic milieu and a variety of associated disorders exerted on immunocompetent cells.

The increased rate of infections, together with an impaired response to vaccination and a common failure of tuberculin skin test to diagnose latent tuberculosis indicate that the adaptive immunity is weakened in the ESKD population.[3] Studies performed in vitro show that T-cell proliferation is decreased in the uremic milieu.[4,5] As mentioned, T helper lymphocytes (Th) play a

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crucial role in controlling the immune response. Th1 cells produce several proinflammatory cytokines, notably tumor necrosing factor (TNF)-α, interleukin (IL)-12 and interferon (IFN)-γ[2]. Th2 cells, in turn, produce mainly IL-4 and IL-5. By producing different cytokines, they have a diverse impact on the immune response.[6] Th1 lymphocytes activate macrophages and neutrophils, whereas Th2 cells are involved in promoting humoral immunity. In patients with ESKD, the maturation of both subsets of Th cells is impaired compared with controls. Although the maturation of Th lymphocytes is sustained, ESKD patients present with significantly elevated Th1 levels, leading to an increased Th1/Th2 ratio.[6,7]

The spectrum of $CD4^+$/$FOXP3^+$ regulatory T-cells (Tregs) capable of mediating dominant suppression is expanding to include both naturally arising and inducible subsets. In addition to their expression of CD4 and CD25, these cells are anergic to proliferative responses in vitro and do not express key cytokines, including IL-2 or IFN-γ in response to stimulation.[8,9] Functionally, they are characterized by the capacity to suppress proliferation of effector T-cells in vitro in a process requiring activation and cell contact but not IL-4, IL-10, or transforming growth factor-β (TGF-β).[10]

Patients with ESKD chronically hemodialyzed present changes not only in T-cell immunity but also in lipid profile.[11,12] Apart from their immune function, circulating T-cells may actively participate to atherogenesis and treatments aimed at reducing T-cell activation and apoptosis in patients with ESKD reduce the risk of developing cardiovascular disease.[13] Evidence exists that HD patients are exposed to enhanced oxidative stress that is initiated by the generation of oxygen free radicals, mainly in tissue and probably in the circulation. The most potent O_2-generating proteins are oxidatively modified lipoproteins, mainly oxidized low-density lipoproteins (oxLDL).[14] The pathophysiological relevance of oxLDL-induced Tregs immunodeficiency and pro-atherogenic effect in HD patients remains uncertain, but may be one explanation of the immune dysfunction, illustrated by the high rate of bacterial infections and impaired vaccine response seen in these patients and may be suggested as a contributor of the micro-inflammation seen in the atherogenesis frequently encountered in patients with ESKD and especially in chronic HD patients.[15]

Pathobiology of $CD4^+$/$FOXP3^+$ Regulatory T-Cells

$CD4^+$/$FOXP3^+$ regulatory T-cells seem to represent the resurrection of the old suppressor T-cells. While most basic knowledge about these cells is derived from animal studies, the recent identification of these cells in humans has further attributed to their characterisation by in vitro analysis. Results obtained have led to broad speculations about therapeutic potential by interference with these regulatory T-cells. These cells are characterized by the expression of CD25 and the forkhead-family transcription factor FOXP3 (forkhead box P3) and they have the capacity to suppress the activation of other T-cells in a contact-dependent manner.[16-18] Furthermore, FOXP3 can inhibit activation-induced expression of *IL2* by T-cells. However, FOXP3 can target genes other than cytokine genes or genes that are regulated by nuclear factor of activated T-cells (NFAT).

$CD4^+$/$FOXP3^+$ regulatory T-cells constitute approximately 7-10% of peripheral $CD4^+$ T-cells in humans and mice and can suppress T-cell function both in vitro and in vivo. They appear to influence immune responses to self antigens, tumors and pathogenic organisms. Research mainly from in vitro studies has revealed that Tregs can exert suppressive effects against multiple cell types involved in immunity and inflammation.[19] These include the induction as well as the effector and memory function of $CD4^+$ and $CD8^+$ T-cells, the inhibition of proliferation, immunoglobulin production and class switching of B-cells, the inhibition of NK and NK T-cell cytotoxicity, the function and maturation of dendritic cells, as well as effects on the function and survival of neutrophils.

$CD4^+$/$FOXP3^+$ regulatory T-cells have a specific response to T-cell receptor (TCR) engagement. Several studies have shown that Tregs isolated both from humans and from rodents do not proliferate when appropriately activated.[20,21] They also do not produce cytokines such as IL-2, IL-4 and IFN-γ, as well as other effector molecules such as TNF and TNF-receptor-family members. However, Tregs are not completely unresponsive to TCR-mediated signals, as TCR engagement is required for their ability to suppress the activation of responder T-cells.

In addition to making a start at identifying FOXP3-target genes, the precise role of FOXP3 in establishing the Treg-cell differentiation programme is also being worked out. Several recent papers have addressed the role of FOXP3 in Tregs development and function in the thymus and in the periphery. Two groups, Gavin et al and Lin et al, generated knock-in mice in which the gene encoding green fluorescent protein (GFP) or enhanced GFP (EGFP) was fused in frame into the *Foxp3* endogenous locus resulting in a fluorescent nonfunctional FOXP3 protein (referred to here as *Foxp3*^{GFP} knock-in mice).[22,23] These studies indicate that FOXP3 is required for the suppressive functions of Tregs, as well as for their anergic state, but there are other factors that have important roles in Tregs development.

Forkhead Box P3 and T-Cell Receptor Signaling in Tregs

$CD4^+$ effector T-cells undergo a stereotypical activation programme after engagement of their TCR and appropriate costimulation. This programme consists of the activation of specific signaling pathways that result in the induction of effector functions, including the production of IL-2.[24,25] It is suggested that generation of Tregs may require higher affinity interaction between the agonist peptides/MHC II and TCR within the thymus in contrast to the process of conventional $CD4^+$ T-cells production.[26] Support for this notion has been provided by analyzing Tregs development in mice expressing a transgenic TCR and its cognate ligand in the thymus. Recent studies show that TCR transgenic $CD4^+$ T-cells can adopt the regulatory cell phenotype with a higher frequency when they encounter their cognate antigen in the thymus. Based on these observations, engagement of transgenic TCR by a high-affinity self-ligand is expected to initiate signaling cascades that ultimately induce FOXP3 expression and commit thymocytes to Tregs lineage.

Costimulatory Signaling Pathways and FOXP3 Expression

It is widely accepted that T-cell activation requires at least two signals. The first one is specific and initiated by the interaction between TCR and the MHC-peptide complex; the engagement of CD28 by B7 molecules on the antigen presenting cells provides the nonspecific costimulatory signals essential for T-cell full activation and function.[27] Similarly, FOXP3 expression and the production of Tregs both in the thymus and in the periphery critically depend on the costimulatory signaling. $CD28^{-/-}$ and B7-$1^{-/-}$/B7-$2^{-/-}$ ($CD80^{-/-}$/$CD86^{-/-}$) mice show significantly reduced numbers of Tregs in the thymus and the periphery, indicating that CD28:B7 interaction is required for the development and maintenance of Tregs. $CD4^+$/$FOXP3^+$ regulatory T-cells constitutively express the intracellular and surface CTLA-4.[28] CTLA-4 deficient mice display the phenotypes critically resembling the Foxp3 mutant ones, indicating a close link may exist between CTLA-4 and Tregs.[29] In addition, other costimulatory molecules may also contribute to FOXP3 expression and Tregs development and function. Blockade of the programmed death 1 (PD1)-PD-L pathway with the anti-PD1 mAb significantly interrupted the vascular endothelium-induced FOXP3 expression and the conversion into the Tregs from $CD4^+$/$CD25^-$ T-cells, indicating that PD1-PD-L interaction seems to be critical for Foxp3 expression and the conversion into Tregs in mice.[30] OX40-OX40 ligand (OX40-L) interaction may be important for the development and homeostasis of Tregs, as the significantly reduced number of Tregs in OX40-deficient mice and the increased Tregs in constitutively active OX40-L expressing mice were observed.[31]

Interleukin-2 and FOXP3 Expression

Interleukin-2 has long been known as a potent T-cell growth factor essential for T-cell proliferation and function. Interleukin-2 signaling has been associated with Tregs development, homeostasis and function. It has been proposed that IL-2 has an essential, nonredundant function in the development of Tregs in the thymus.[32] It has also been proposed that in the absence of IL-2, Tregs cannot survive or expand their numbers in the thymus or in the periphery.[33,34] Finally, studies have suggested that IL-2 is directly required for Tregs function and in its absence, Tregs fail to suppress T-cell proliferation.[35,36] Although those proposed functions for IL-2 are not mutually exclusive, in many cases the results and conclusions of those studies have been contradictory.

Impaired CD4$^+$/FOXP3$^+$ Regulatory T-Cell Function in Patients with ESKD

In chronic kidney disease, as in other chronic inflammatory diseases, monocytes/macrophages and their mediators make an important contribution to the inflammatory process. Previous findings suggested that in patients with ESKD, a significantly high percentage of activated T-cells ultimately did not proliferate but became apoptotic.[37,38] The induction of activated Tregs apoptosis from hemodialyzed (HD) patients is dependent on Fas/FasL expression, which leads to a cell contact form of circulating CD4$^+$ T-cell self-injury.[39] Furthermore, activated Tregs from HD patients fail to respond adequately to exogenous IL-2. This is due to the down-modulation of surface IL-2 receptor (IL-2R) α-subunits (IL-2Rα) expression, impaired IL-2 signal transduction in CD4$^+$ T-cells and/or increased serum levels of soluble IL-2R (sIL-2R).[11] Decreased proliferative capacity of Tregs from subjects with normal renal function incubated with serum from chronic HD patients and its restoration by normal serum strongly suggests that mediators induced by HD affect transduction mechanisms in the IL-2/IL-2R pathway. Finally, IL-2 seems to inhibit the apoptotic process at many stages by interacting with various proteins.[40]

The clinical consequences of the Tregs dysfunction in patients with ESKD are numerous including immune dysregulation, micro-inflammation and atherogenesis.[11,15,41]

Place of oxLDL in Tregs Apoptosis in Patients with ESKD

In HD patients, oxLDL may play a dual role in Tregs. On the one hand, oxLDL activates Tregs and induces Fas expression, thereby initiating a cascade of substrate-specific pro-apoptotic caspases leading to cell cycle arrest.[15] On the other hand, oxLDL alters IL-2/IL-2R pathway and sensitizes activated Tregs from HD patients to exogenous IL-2 explaining the reported Tregs apoptosis in these patients.[11] In activated Tregs from uremic patients and more particularly in those from HD patients that oxLDL induce Fas expression on the cell surface, which corresponds to the early phase of cell apoptosis. The evaluation of intracellular Fas synthesis and DNA fragmentation confirms Fas-mediated apoptosis in Tregs in response to oxLDL. The percentage of apoptotic cells is related to the copper mildly oxidized LDL concentration. Fas activation induces the recruitment of procaspase-8 to the Fas receptor and this association triggers the caspase cascade that leads to apoptosis.[42] Overexpression of Fas sensitizes cells to Fas-induced apoptosis, suggesting that increased clustering of Fas on the plasma membrane results in a stronger ability to recruit procaspase-8, which would overcome the sequestering of procaspase-8 by Bcl-2 and could influence the inhibitory function of Bcl-2 or Bcl-xL on Fas-induced apoptosis.[43] Moreover, experiments with blocking antibodies to Fas suggest that mildly oxidized LDL acts mainly by up-regulating expression of Fas.[9] Activation of the Fas pathway results in oligomerization of Fas and recruitment of Fas-associated death domain (FADD) and FADD homologous, interleukin-1β-converting enzyme (ICE)-like protease (FLICE), which then activate caspases. The observation that the FLICE inhibitory protein is down-regulated by oxLDL further supports the involvement of the Fas pathway in oxLDL-induced apoptosis.[44-46] Finally, mildly oxidized LDL causes an overexpression of Fas at the Tregs surface. The stimulation of Fas expression seems to be a key element of this process. Indeed, activation of Tregs induces transient expression of FasL triggering Fas-dependent apoptosis.

Place of 26S Proteasome in Tregs Apoptosis

Proliferation and division of cells implies two basic steps finally yielding to a proper genome duplication: (1) the replication of chromosomal DNA and (2) the separation and division of sister chromosomes. Both of these steps have to ensure a proper distribution of the entire genome into two new cells:

1. During the tightly regulated G1/S-phase, the sister chromatids are separated and complemental DNA synthesis and replication takes place. The proper custodial regulation of DNA replication generally refers to the timely ordered progression from G1- to S-phase that constitutes the strict initiation and completion of only one round of DNA replication in each cell cycle. This duplication relies on the coordinated activities of positive regulators, such

as cyclins, cyclin-dependent kinases (CDK), CDK-cyclin complexes, E2F and Cdc6 and negative regulators, such as CDK inhibitors (CKI) of the Cip/Kip and INK4 families. The coordinated timely presence and action of these positive and negative regulators is governed by inactivation as a result of proteasomal degradation.[47]

2. During the G2/M-phase, the doubled chromosome set is separated along kinetochore microtubules and divided into two new cells. The ordered progression of the S- and M-phase also highly depends on the spatial and temporal control of cell cycle regulatory proteins by proteasomal degradation that finally ensures proper cell cycle transitions and adequate frequencies of cell division.[48]

Proteasomal degradation of the CKI $p27^{Kip1}$ is thought to be required for G1-to-S-phase progression and mainly occurs at the early onset of S-phase), although $p27^{Kip1}$ degradation also can take place at the G0-to-G1-phase transition. Consequently, $p27^{kip1}$ protein is abundant in G0 and G1 cells and is down-regulated in proliferating and S-phase cells. Moreover, ectopic overexpression of mutant $p27^{Kip1}$, but not of wild-type $p27^{Kip1}$, results in cell cycle arrest in the S-phase strongly suggesting that proteasomal degradation of $p27^{Kip1}$ is essential for the entry into S-phase.[49] However, inactivation of $p27^{Kip1}$ function may not only occur by proteasomal degradation, but also via alternative pathways such as proteolytic processing.

One candidate mechanism of how proteasomal activity promotes apoptosis at an upstream point of apoptotic signal transduction has been uncovered recently in primary mouse thymocytes: XIAP and c-IAP1, members of the highly conserved family of inhibitors of apoptosis proteins (IAPs) that exert their anti-apoptotic activity, at least in part, by inhibiting the activation and enzymatic activity of caspases and by ubiquitination and targeting of caspase-3 for proteasomal degradation, are autoubiquitinated and subsequently degraded by the 26S proteasome in response to various apoptotic stimuli.[50-52]

Another candidate mechanism of providing proapoptotic signals by proteasomal activity has been demonstrated in HUVECs induced to undergo apoptosis by treatment with TNF-α. Early after the initiation of TNF-α treatment of HUVECs, Bcl-2, a mitochondrial membrane-anchored protein capable of blocking apoptosis induced by diverse stimuli, was shown to be specifically degraded by the 26S proteasome.[53,54] This event was demonstrated to be operative in inducing apoptosis, because pretreatment of HUVECs with specific proteasome inhibitors reversed both TNF-α-induced Bcl-2 degradation and induction of apoptosis.

$FOXP3^+$ Regulatory T-Cells Apoptosis in Uremia: Role of the 26S Proteasome

As recently demonstrated, oxLDL inhibit proteasome enzymatic activity of the CKI $p27^{Kip1}$ and the pro-apoptotic molecule Bax.[15] The consequences result in the increased accumulation of these key regulatory proteins in Tregs from HD patients. The mechanisms by which oxLDL modify Tregs proteasome activity in uremic patients remain poorly understood. However, besides enhancing the oxidative damage of proteins such as $p27^{kip1}$ and Bax, oxLDL may lead to accumulation of ubiquitinated proteins via inhibition of proteasome enzymatic activity (Fig. 1). It can be speculated that the oxLDL-related protein damage is responsible for the Tregs cycle arrest at G_1 phase and their apoptosis. Indeed, oxLDL produce a rapid decay of proteasomal proteolysis in inducing derivatization of cell proteins by 4-hydroxynonenal (4-HNE) resulting in an inhibition of the 19S. This is because 4-HNE cross-linked proteins are resistant to proteolysis and are able to inhibit the 26S proteasome and because 26S proteasome is less resistant to H_2O_2-induced oxidative stress than the 20S proteolytic core. The second step (i.e., inhibition of the 20S core) may be a result of the progressing intracellular oxidative stress induced by oxLDL. At this stage, when the proteasome is completely inhibited, Tregs are rapidly dying.

On the other hand, oxLDL affect the anti-apoptotic protein Bcl-xL degradation by increasing its removal in parallel with the activation of the 20S and 26S proteasome in phytohemagglutinin (PHA)-stimulated Tregs from healthy subjects treated with various concentrations of oxLDL or cultured with uremic serum from HD patients.

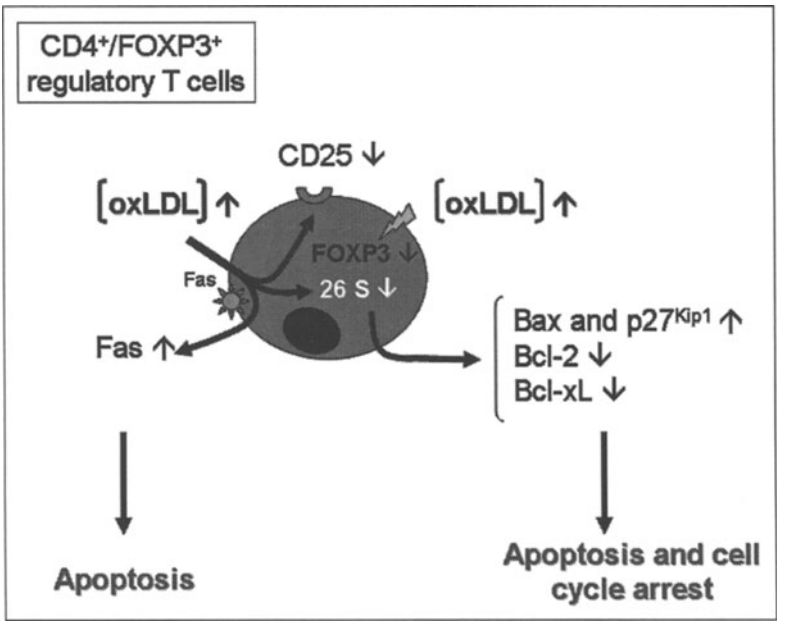

Figure 1. Model for 26 S proteasome-mediated regulation of FOXP3+ regulatory T-cells apoptosis in uremia.

Conclusion

Accumulating evidence suggests an important role for Tregs in the control of immunity and some inflammatory diseases development and/or progression. Future studies should aim at the delineation of the critical subtypes of Tregs responsible for these protective effects, the factors and molecular mechanisms involved in their survival, migration and suppressive function especially in patients with ESKD. Indeed, the modified Tregs number and function seen in this patient population are the consequences of the $p27^{Kip1}$ and Bax accumulation in these $CD4^+$ T-cell subtypes, which was due to oxLDL proteasome activity alteration. Proteolytic degradation of cell proteins by the 26S proteasome is a highly complex and tightly regulated process that plays pivotal roles in the regulation of basic cellular processes, including differentiation, proliferation, cell cycling, apoptosis, gene expression and signal transduction. From a mechanistic view, the 26S proteasome is capable of governing strictly opposite biologic features that crucially determine the fate of a cell, proliferation and apoptosis. Because proteasomal protein degradation is a highly ordered and elaborated process, it is obvious that this process also can underlie deregulation as observed in several human diseases that exhibit an imbalance of proliferation and apoptosis as a fundamental pathogenetic feature as encountered in patients with ESKD. A fact which may have important implications in clinics, since this response could contribute to the $CD4^+$ T-cell immune dysfunction in patients with ESKD including micro-inflammation and atherogenesis.

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CHAPTER 13

Transcriptional Role of FOXO1 in Drug Resistance through Antioxidant Defense Systems

Tomoko Goto* and Masashi Takano

Abstract

FOXO transcription factors promote cell cycle arrest, apoptosis, DNA damage repair and detoxification of reactive oxygen species by regulating specific gene setting. As FOXO possess diverse functions, which partly seemed opposing, the multiple mechanisms, including transcriptional activity and subcellular localization, are differentially regulated according to various types or intensities of cellular stress responses. Since FOXO transcription factors are critical mediators of apoptosis in cytotoxicity inducing drugs, its involvement in the development of drug resistance is an important issue in cancer therapy. Indeed, FOXO1 expression was distinctively upregulated in paclitaxel resistant cell line and enhanced by exposure to paclitaxel with subcellular translocation. In addition, FOXO1 overexpression, predominantly in cytosol, was frequently observed in cancer tissue samples from chemoresistant patients compared to chemosensitive patients. FOXO1 silencing in paclitaxel resistant cell line decreased its resistance through modulation of downstream targets of FOXO1 involving oxidative stress. Alteration of oxidative stress by cotreatment with pharmacologic modulators of reactive oxygen species also attenuated cytotoxicity of paclitaxel. Furthermore, FOXO1 silencing attenuated intracellular reactive oxygen species levels, which collectively suggest one of possible explanations in transcriptional role of FOXO1 as redox mechanism leading to drug resistance through its downstream target involving defence mechanism against oxidative stress.

Introduction

The mammalian FOXO family of Forkhead transcription factors, consisting of FOXO1, FOXO3a and FOXO4, is a direct downstream target of the PI3K/Akt pathway.[1,2] Posttranslational modification of FOXO proteins is an important mechanism that regulates the ability of different transcription factors to activate distinct gene sets, involved in cell cycle inhibition,[3] apoptosis,[4] defense against oxidative stress and DNA repair.[5,6] FOXO-induced cell cycle arrest and apoptosis is critical as antiproliferative effect for tumors, whereas FOXO proteins play an important role in protection of cells against genotoxic and environmental stresses, such as FOXO-dependent resistance to oxidative stress for longevity. As FOXO possess diverse functions, which partly seemed opposing, the multiple mechanisms, including transcriptional activity and subcellular localization, are differentially regulated according to various types or intensities of cellular stress responses. Since FOXO proteins were reported to be critical mediators of apoptosis in cytotoxicity inducing

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drugs in many cells,[4,7,8] we postulated that FOXO expression or transcriptional activity could be important event in the drug sensitivity in cancer cells. The consequence of FOXO1 expression correlating with paclitaxel cytotoxicity and sensitivity was examined in ovarian cancer cell lines using parent cells and paclitaxel resistant derivative cells and its expression was also confirmed in clinical samples from chemosensitive and resistant patients. Furthermore, the possible underling mechanism in involvement of FOXO1 with paclitaxel resistance was explored and discussed.

Differential Expression of FOXO1 in Cancer Cells

To examine the role of FOXO transcriptional factor, screening of FOXO protein expression was performed in three representative ovarian cancer cell lines, parent cells KF28, cisplatin resistant derivative and paclitaxel resistant derivative cells, KFr13 and KFr13Tx.[9,10] Among these cells, KFr13Tx, paclitaxel resistant cell line, only showed marked FOXO1 expression in transcript level and protein level (Fig. 1A). Comparing to FOXO1, FOXO3a and FOXO4 did not show much difference among these cell lines. As speculated, PI3K/Akt activity was considerably lower in KFr13Tx, as reflected by the phosphorylated Akt levels. The same observasion was reported in the previous study for endometrial cancer cell lines, paclitaxel sensitive Ishikawa cells and resistant HEC-1B cells (Fig. 1C). The results from two different cancers strongly suggested that overexpression of FOXO1 correlates especially with the mechanism of paclitaxel resistance. To investigate whether in vitro data is relevant to clinical practice, immunohistochemical reactivities of FOXO1 in ovarian cancer samples, obtained at surgery prior to chemotherapy, with different chemotherapeutic response to paclitaxel-based chemotherapy, were examined. Representative immunohistological staining of responder and nonresponder are shown in Figure 1E. FOXO1 overexpression with strong cytoplasmic staining was predominantly observed in nonresponders, whereas it was less frequently detected in responders.

Induction and Translocation of FOXO1 by Paclitaxel in Cancer Cell Lines

To further investigate the correlation of FOXO1 and paclitaxel, FOXO1 expression was examined in parent KF28 cells and paclitaxel resistant KFr13Tx cells treated with paclitaxel at the increased concentrations for 24 hours. Strong induction of FOXO1 expression was observed in KFr13Tx cells by paclitaxel treatment, whereas its induction was very weak in KF28 cells (Fig. 2A). Conversely, cleaved PARP expression as apoptosis marker was distinctively induced in KF28 cells, whereas its expression was almost undetectable in KFr13Tx cells. FOXO1 mRNA expression was also induced in both cells, which were peaked after 24 hours, especially marked in KFr13Tx cells. For further analysis, translocation of FOXO1 was also investigated using protein fraction. Nuclear translocation of FOXO1 was clearly observed in both cells, which were again peaked after 24 hours treatment (Fig. 2B). The nuclear decrease after 48 hours correlates with increase in phosphorylated (Ser256) FOXO1 levels in cytosol. The same observasions were also reported in previous study for endometrial cancer cell lines, paclitaxel sensitive Ishikawa cells and resistant HEC-1B cells (Fig. 2C,D). Notably, cytoplasmic FOXO1, which is likely to be inactive and should have no affect on expression of target genes in stress response, was strongly expressed in resistant cells both in ovarian cancer cell lines and clinical samples. In contrast, induction and nuclear FOXO1 was markedly induced by 24 hours exposure of paclitaxel in both sensitive and resistant ovarian cancer cells. It is likely that acute exposure to paclitaxel leads to FOXO1-dependent activation of a pro-apoptotic gene program and that prolonged or chronic exposure promotes selection of cells with another transcriptionally activated gene settings by FOXO1, which are involved in cellular survival and drug resistance. The major mechanism how FOXO transcription factors are regulated in response to external stimuli is by changes in subcellular localization, which are rapidly reversible. Acquiring intracellular mechanisms during response to the tumor environment, if localization of a nuclear protein into the cytoplasm may render it ineffective as a target for chemotherapy, it is possible that blocking nuclear export reverse drug resistance.

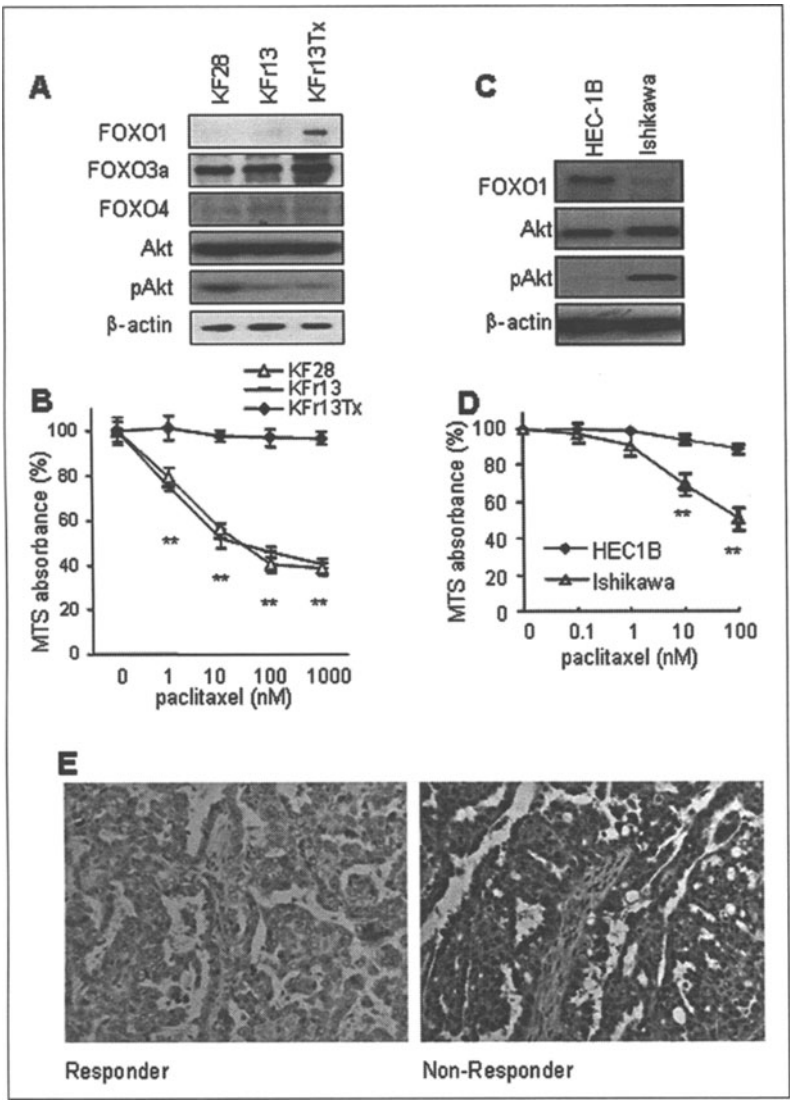

Figure 1. Differential expression of FOXO1 and sensitivity to paclitaxel in drug sensitive and resistant cancer cell lines and ovarian cancer samples. A) Comparative analysis of FOXO1, FOXO3a, FOXO4, total and phosphorylated Akt (pAkt) expression in KF28, KFr13 and KFr13Tx cells by Western blot analysis. β-actin served as a loading control. B) KF28, KFr13 and KFr13Tx were incubated with the indicated concentrations of paclitaxel and cell viability was determined by MTS assay 24 h later. The results show mean ± SD of triplicate measurements and **denotes $P < 0.001$. C) Comparative analysis of FOXO1, total and phosphorylated Akt (pAkt) expression in HEC-1B and Ishikawa cells by Western blot analysis. β-actin served as a loading control. D) HEC-1B and Ishikawa cells were incubated with the indicated concentrations of paclitaxel and cell viability was determined by MTS assay 24 h later. The results show mean ± SD of triplicate measurements and **denotes $P < 0.001$. E) The representative immunohistological staining of FOXO1 of a chemotherapy-responder and nonresponder is shown. Reprinted with permission from: Goto T. Br J Cancer 2008; 98:1070-1071[16] (Parts A,B,E); and Goto T. Oncogene 2008; 27:10-12[8] (Parts C,D).

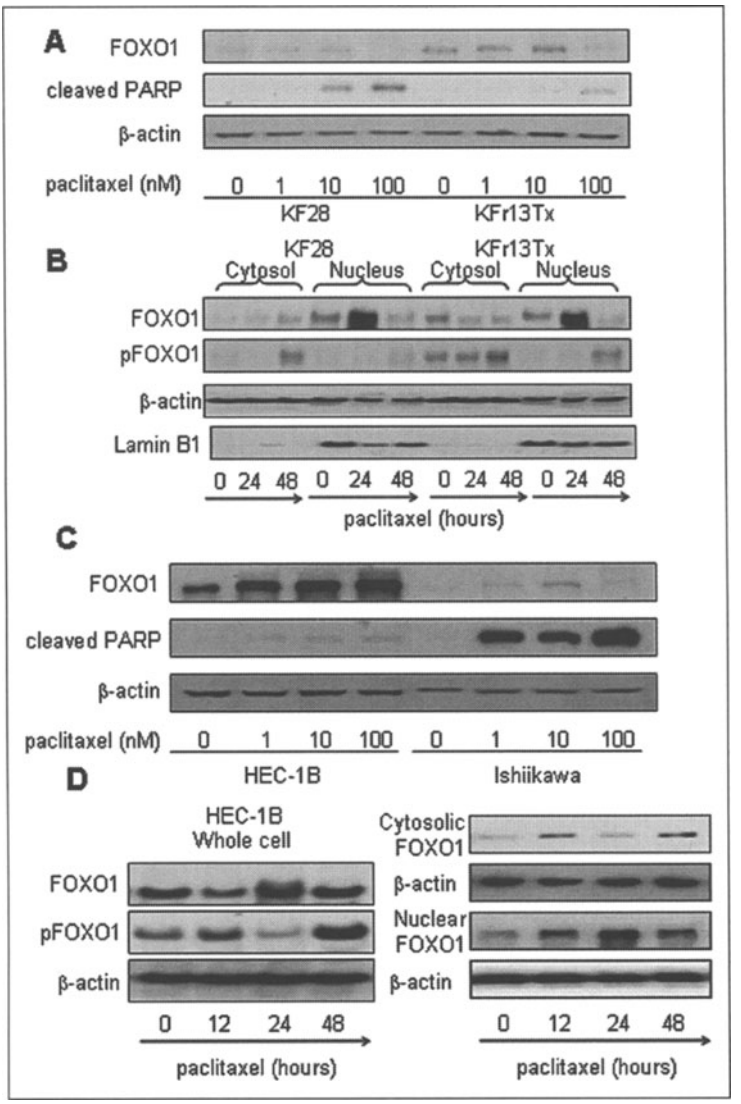

Figure 2. Induction of FOXO1 by paclitaxel in ovarian cancer cell lines. A) KF28 and KFr13Tx cells were incubated with the indicated concentrations of paclitaxel for 24 h and whole cell lysates were probed for FOXO1 and cleaved PARP expression by Western blot analysis. β-actin served as a loading control. B) KF28 and KFr13Tx cells treated with 10 nM paclitaxel were harvested at the indicated time points and the cytosolic and nuclear protein fractions were probed for FOXO1 and phospho-FOXO1 expression. β-actin and Lamin B1 served as a loading control. C) HEC-1B and Ishikawa cells were incubated with the indicated concentrations of paclitaxel for 24 h and whole cell lysates were probed for FOXO1 and cleaved PARP expression by Western blot analysis. β-actin served as a loading control. D) HEC-1B cells treated with 10 nM paclitaxel were harvested at the indicated time points and the whole cell lysates were probed for FOXO1 and phospho-FOXO1 expression and cytosolic and nuclear protein fractions were also probed for FOXO1 expression. β-actin served as a loading control. Reprinted with permission from: Goto T. Br J Cancer 2008; 98:1071[16] (Parts A,B); and Goto T. Oncogene 2008; 27:12[8] (Parts C,D).

FOXO1 Attenuates Sensitivity to Paclitaxel-Induced Cell Death in Paclitaxel Resistant Cell Lines

To clarify the role of FOXO1 in ovarian cancer cells, gene silencing experiment prior to paclitaxel treatment was performed in KFr13Tx cells. FOXO1 siRNA considerably increased the sensitivity to paclitaxel as determined by MTS assay (Fig. 3A). These findings were confirmed by FACS analysis using PI-staining for 24 hours treatment of paclitaxel (Fig. 3B) The same observasions were reported in the previous endometrial cancer study for paclitaxel resistant HEC-1B cells (Figs. 3C and D). Although drug resistance in cancer should be multifactorial, it is well-recognized that a slower growth rate represents one component of drug resistance. After the same silencing experiment in KFr13Tx cells by transfection with FOXO1 siRNA, cellular proliferation was monitored by MTS assay. FOXO1 siRNA slightly promoted cellular proliferation, whose effect was not quite remark-

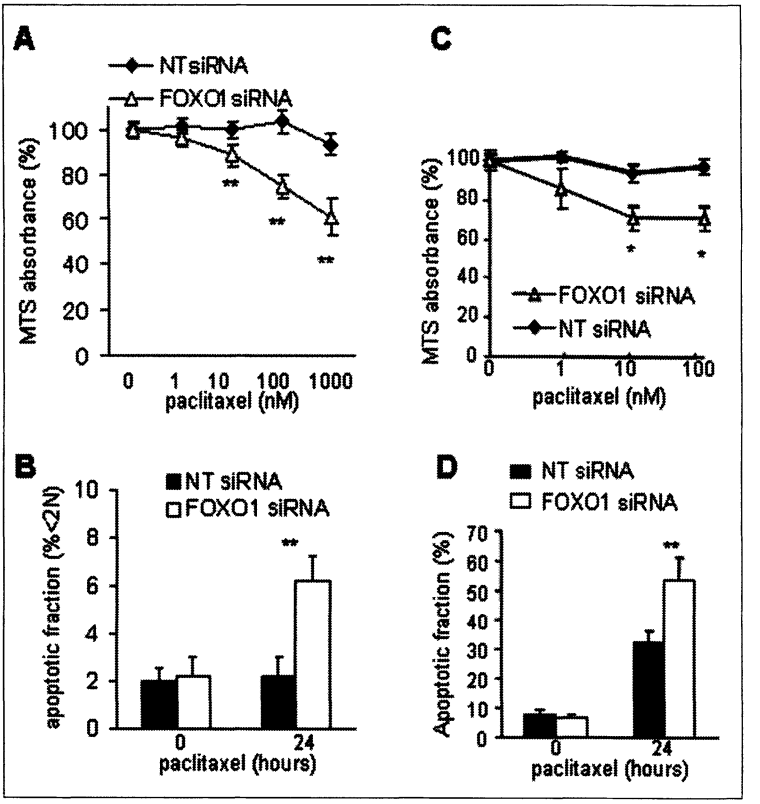

Figure 3. FOXO1 attenuates proliferation and sensitivity to paclitaxel-induced cell death in paclitaxel resistant cell lines. A) Loss of cell viability was determined by MTS assay in KFr13Tx cells first transfected with nontargeting (NT) siRNA or FOXO1 siRNA and treated later with increasing doses of paclitaxel for 24 h. B) In parallel experiments, KFr13Tx cells transfected with nontargeting (NT) siRNA or FOXO1 siRNA were treated with 10 nM paclitaxel for 24 h and the apoptotic cell fraction was determined by flow cytometry. C) Loss of cell viability was determined by MTS assay in HEC-1B cells first transfected with nontargeting (NT) siRNA or FOXO1 siRNA and treated later with increasing doses of paclitaxel for 24 h. D) In parallel experiments, HEC-1B cells transfected with nontargeting (NT) siRNA or FOXO1 siRNA were treated with 10 nM paclitaxel for 24 h and the apoptotic cell fraction was determined by flow cytometry. The results show mean ± SD of triplicate measurements and **denotes $P < 0.001$ and *denotes $P < 0.05$. Reprinted with permission from: Goto T. Br J Cancer 2008; 98:1072[16] (Parts A,B); and Goto T. Oncogene 2008; 27:13[8] (Parts C,D).

able. Indeed, FOXO1 silencing decreased expression level of p27Kip1 in these cells (see Fig. 5B), which is one FOXO1 target gene involving cell cycle inhibition.[3] However, cellular proliferation was not actually attenuated, which suggests more critical event other than cell growth retardation is involved in paclitaxel sensitivity.

Attenuation of Oxidative Stress by Paclitaxel and FOXO1 in Ovarian Cancer Cell Lines

Reactive oxygen spices (ROS) levels are increased in cells exposed to various stress agents, including paclitaxel and other anticancer drugs.[11,12] To investigate the possible underlying mechanism that FOXO1 attenuates paclitaxel sensitivity in ovarian cancer cells, intracellular ROS induced by paclitaxel was first measured in KF28 cells and KFr13Tx cells. As assessed by C-H2DCFDA fluorescence, intracellular H_2O_2 levels were increased in KF28 cells when exposed for 4 hours to increasing concentratios of paclitaxel or H_2O_2 as indicated, whereas those changes were not marked in KFr13Tx cells exposed with paclitaxel (Fig. 4A). Agents that decrease ROS can suppress taxol-induced cytotoxicity, while increase of ROS levels by inhibition of SOD or glutamylcysteine synthase can enhance taxol-induced cytotoxicity in cancer cell lines.[12] To further study the role of ROS accumulation in paclitaxel cytotoxicity, the effects of co-incubation of paclitaxel or H_2O_2 with antioxidant, N-acetylcysteine (NAC), H_2O_2 scavenger, or NaN_3, inhibitor of catalase, were investigated in both cells. Cotreatment with NAC or NaN_3 in KF28 cells significantly decreased or increased paclitaxel or H_2O_2 induced cell death, whereas cotreatment with NaN_3 in KFr13Tx cells also increased paclitaxel or H_2O_2 induced cell death (Fig. 4B). The cellular responses to paclitaxel involve activation of MAPK pathways.[13] Higher ROS levels and SAPK (stress-activated protein kinases) JNK activity were measured in tumour cells that were sensitive to anticancer agents than in those that were drug-resistant, suggesting that ROS-mediated JNK and p38 activation played a key role in the sensitization to stress signals and to anticancer drugs.[14,15] Thus, control of endogenous ROS level and the regulation of MAPK pathway may involve in proliferation and sensitivity to stress stimuli including anticancer drugs in cancer cells. Whether FOXO1 attenuates paclitaxel-induced cytotoxicity through oxidative stress was studied again by ROS measurement in KFr13Tx cells using silencing experiment. As shown in Figure 4C, intracellular H_2O_2 levels were increased in KFr13Tx cells transfected with FOXO1 siRNA when exposed for 4 hours to increasing concentratios of paclitaxel. Thus, modifying intracellular ROS level by pharmacologic modulators as well as FOXO1 silencing could indeed attenuate paclitaxel sensitivity in these cells.

MnSOD Expression in Paclitaxel Sensitive and Resistant Ovarian Cancer Cell Lines and Ovarian Cancer Samples

FOXO enhances the expression of the several key enzymes in the antioxidant defense system, including DNA repair enzyme GADD45α, mitochondrial MnSOD (manganese superoxide dismutase) and catalase, which are scavengers of oxygen-free radicals.[5] Cells lacking MnSOD have greatly reduced oxidant damage protection, demonstrating the critical role in the oxidative stress defence pathway. In human endometrial stroma cells, FOXO1 induces the expression of MnSOD upon differentiation with heightened defence mechanisms against oxidative stress.[7] To further determine the relevance of putative FOXO1 target genes in ovarian cancer cells, the protein levels of p27Kip1, MnSOD, catalase and GADD45α expression in KF28, KFr13 and KFr13Tx cells were compared. As shown in Figure 5A, p27Kip1 and MnSOD were strongly expressed especially in paclitaxel resistant cell line, whereas GADD45α expression was also comparably obsereved in cisplatin resistant KFr13 cells and catalase expressions were almost the same among these three cell lines. To further investigate the possibility whether FOXO1 attenuates paclitaxel-induced cytotoxicity through oxidative stress, FOXO1 silencing followed by paclitaxel treatment in KFr13Tx cells was again performed and the same FOXO target genes were examined. Transfection with FOXO1 siRNA decreased expression levels of these target genes, especially in p27Kip1 and MnSOD, regardless of paclitaxel treatment (Fig. 5B). Notably, cleaved PARP was detectable by paclitalxel treatment only in FOXO1 silencing cells, supporting the previous results (Fig. 3A). Thus, FOXO1 silencing not only attenuated intracellular H_2O_2 levels but also decreased expression of its downstream target gene, MnSOD, simultaneously

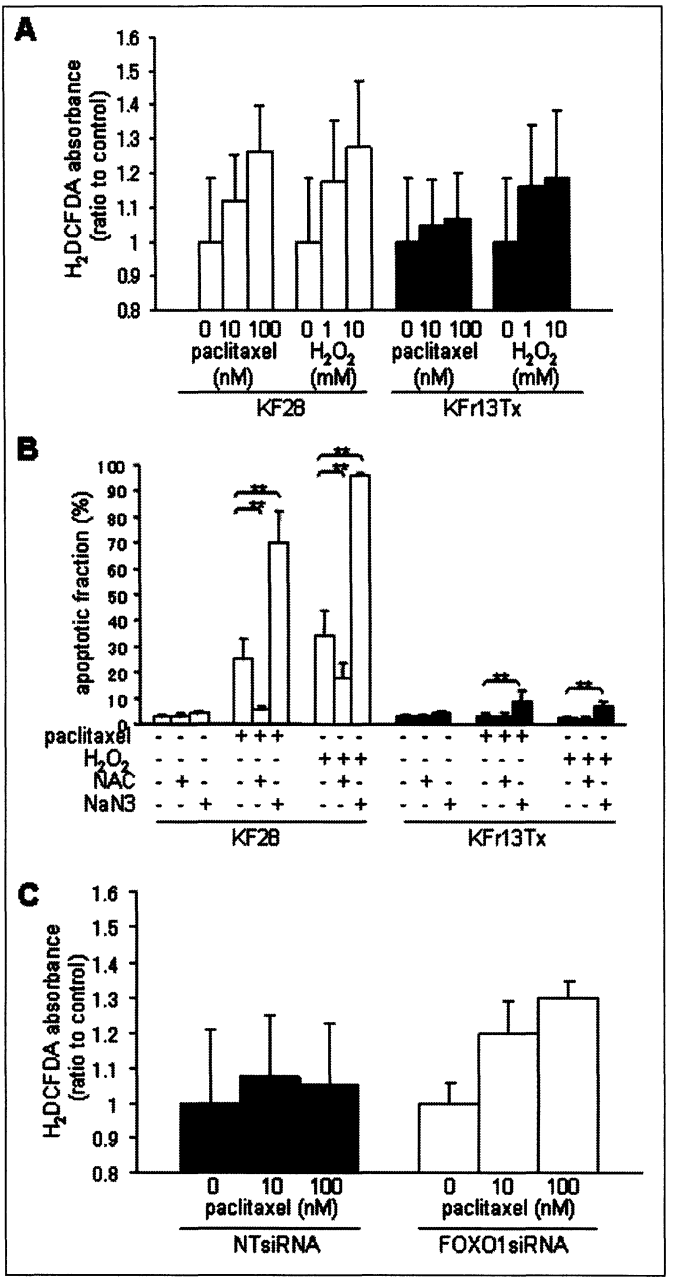

Figure 4. Paclitaxel attenuates oxidative stress in ovarian cancer cell lines. A) After exposure of KF28 and KFr13Tx cells to paclitaxel or H_2O_2, a concentration-dependent intracellular increase in H_2O_2 levels were detected by spectrofluorometry. B) Concomitant treatment with N-acetylcysteine (NAC), H_2O_2 scavenger, or NaN_3, catalase inhibitor, decreased or increased paclitaxel or H_2O_2-induced cell death. The results show mean ± SD of triplicate measurements and **denotes $P < 0.001$. C) KFr13Tx cells transfected with nontargeting (NT) siRNA or FOXO1 siRNA were exposed to paclitaxel and intracellular increase in H_2O_2 levels were detected by spectrofluorometry. Reprinted with permission from: Goto T. Br J Cancer 2008; 98:1073.[16]

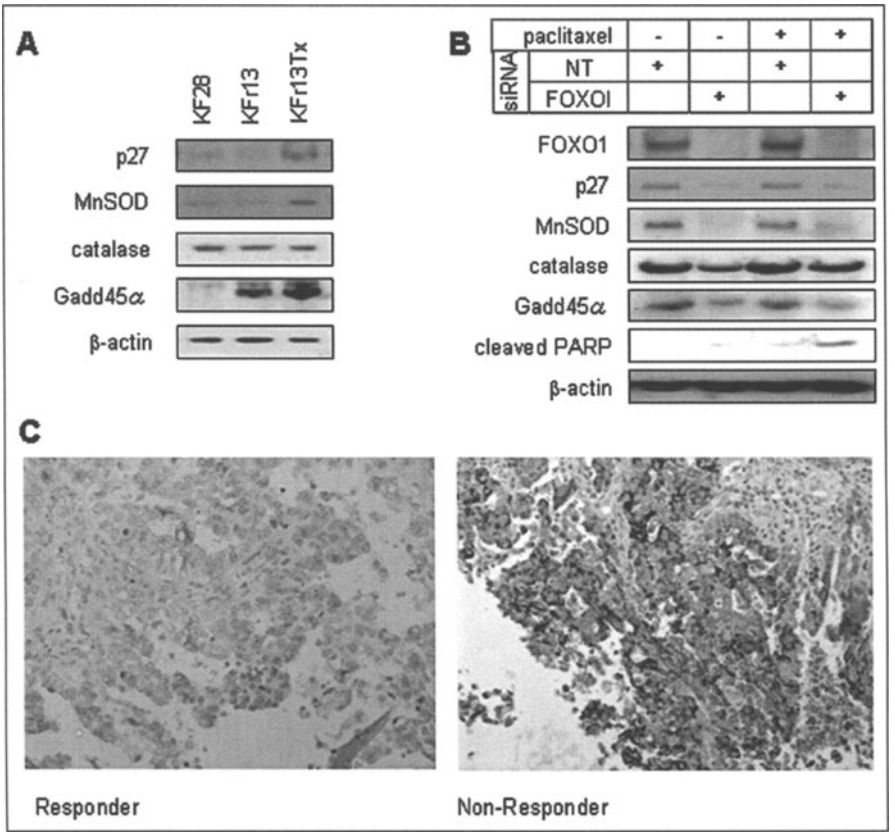

Figure 5. Differential expression of MnSOD in drug sensitive and resistant ovarian cancer cell lines and ovarian cancer samples. A) Comparative analysis of p27Kip1, MnSOD, catalase, GADD45α expression in KF28, KFr13 and KFr13Tx cells by Western blot analysis. β-actin served as a loading control. B) Whole cell protein lysates of KFr13Tx cells transfected with nontargeting (NT) siRNA or FOXO1 siRNA and treated with or without 10 nM paclitaxel for 24 h were analysed by Western blot for FOXO1, p27Kip1, MnSOD, catalase, GADD45α and cleaved PARP expression. β-actin served as a loading control. C) The representative immunohistological staining of MnSOD of a chemotherapy-responder and nonresponder is shown. Reprinted with permission from: Goto T. Br J Cancer 2008; 98:1072-1074.[16]

showing increased paclitaxel-induced cytotoxicity, which collectively suggest one of possible explanation in transcriptional role of FOXO1 as redox mechanism to cytotoxic stimuli such as paclitaxel in these cells. Again to investigate whether in vitro data is relevant to clinical practice, immunohistochemical reactivities of MnSOD in the same ovarian cancer samples were examined. Representative immunohistological staining of responder and nonresponder are shown in Figure 5C. MnSOD overexpression with strong cytoplasmic staining was significantly observed in nonresponders, whereas it was less frequently detected in responders. Furthermore, the cases with overexpression of FOXO1 also showed MnSOD overexpression in nonresponder patients. Drug resistance still remains a major problem and understanding its mechanism is necessary for developing effective cancer therapy. More research needs to be done to elucidate far more mechanisms how FOXO switch its control from pro-apoptotic to pro-survival target genes in response to a variety of environmental stimuli though (Fig. 6), FOXO1 is likely to be the candidate to predict the chemotherapeutic response and could be a molecular target for the treatment of drug resistant cancers.

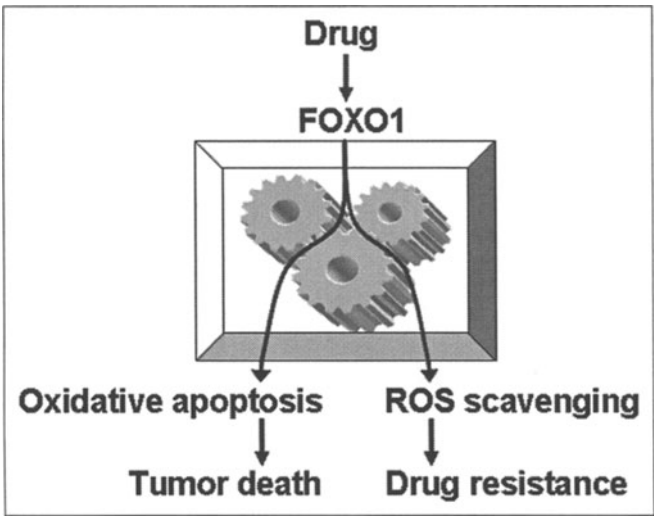

Figure 6. A profound mechanism according to the response against the tumor environmental stimuli leading to apoptosis or survival in cancer cells.

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Chapter 14

Foxp3 Expressing Regulatory T-Cells in Allergic Disease

Kayhan T. Nouri-Aria*

Abstract

Allergic diseases such as asthma, rhinitis and eczema are increasing in prevalence worldwide, in particular in industrialised countries affecting up to 20% of the population. Regulatory T-cells (Tregs) have been shown to be critical in T-cell homeostasis and in the maintenance of immune responses, such as prevention of autoimmunity and hampering allergic diseases. The so-called 'natural' $CD4^+CD25^+$ Tregs and/or IL-10-producing Tr1 cells have been shown to be responsible for the protection of immune tolerance and intact immune reactions following exposure to allergens such as aeroallergens or food allergens. In this regard, both cell-cell contact (through membrane bound TGF-β or via suppressive molecules such as CLTA-4) and soluble cytokine-(TGF-β and IL-10) dependent mechanisms have been shown to contribute to the ability of Tregs to operate effectively. The transcription factor Foxp3, a member of the forkhead-winged helix family, appears to be critical in the suppressive abilities of regulatory T-cells. Adoptive transfer of $CD4^+CD25^+$ Tregs from healthy to diseased animals corroborated and provided further evidence of the vital role of these populations in the prevention or cure of certain autoimmune conditions. Clinical improvement seen after allergen immunotherapy for allergic diseases such as rhinitis and asthma has also been associated with the induction of IL-10 and TGF-β producing Tr1 cells as well as Foxp3 expressing $CD4^+CD25^+$ T-cells, resulting in the suppression of Th2 cytokine milieu. Activation and expansion of antigen-specific $CD4^+CD25^+$ Tregs in vivo using adjuvants such as IL-10 or pharmacological agents such as low dose steroids or vitamin D3 could represent novel approaches to induce antigen-specific tolerance in immune-mediated conditions such as allergic asthma, autoimmune disease and the rejection of transplanted organs in man.

Introduction

The pursuit of regulatory T-cells was revived in the 1990s following the observation that athymic nude mice injected with $CD25^+$ -depleted $CD4^+$ cells developed multiorgan autoimmune diseases[1,2] and that these autoimmune incidents could be reversed by the adoptive transfer of $CD4^+CD25^+$ T-cells from healthy mice to the nude mice. This landmark observation provided robust evidence for the putative regulatory role of $CD4^+CD25^+$ T-cells in the control of immune responses and generated considerable interest in all aspects of basic and clinical immunology. The discovery of $CD4^+CD25^+$ Tregs in the peripheral blood and lymphoid tissues in mice was followed by similar findings in man.[3-6] Numerous in vitro studies on $CD4^+CD25^+$ T-cells revealed a critical role for these T populations as the regulator of immune responses in clinical settings including autoimmunity, tumour and microbial infection, transplantation and allergic diseases.

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Background

Natural regulatory T-cells (Tregs) constitutively express the high-affinity interleukin-2 (IL-2) receptor α-chain (CD25), a receptor which is crucial for IL-2 signalling and events leading to maintenance, homeostasis and function of Tregs cells in vivo. However, the main source of IL-2 for the survival of $CD4^+CD25^+$ Treg cells seems to be T-cells other than Tregs themselves present in close proximity to $CD4^+CD25^+$ Tregs within lymphoid organs.[7,8] Natural $CD4^+CD25^+$ Tregs are also equipped with a variety of cell surface molecules including cytotoxic T-lymphocyte antigen-4 (CTLA-4, or CD152) and glucocorticoid-induced tumour necrosis factor receptor (GITR), OX40 and programmed death-1 (PD-1) antigen.[9-11] Unlike CD25 which is upregulated on both newly activated and regulatory T-cells, the intensity of CD127 antigen on natural Tregs has been reported to inversely correlate with their suppressive ability, making CD127 unique amongst cell surface markers expressed on Tregs.[12] $CD4^+CD25^+$ Tregs also express high levels of LAG-3 (a CD4-related molecule that binds MHC class II) upon activation (Fig. 1). Tregs may also express CCR4 and CCR6, lymphoid homing receptors CD103 and CD62L and molecules such as perforin and granzyme A.[13] However, none of these surface markers are unique to natural Tregs, but combinations of these molecules would make useful surrogate markes for the functional ability of Tregs.

Thymic selection of $CD4^+CD25^+$ Tregs is regulated by the transcription factor *foxp3*,[13-15] a gene with a pivotal role in the development of functional Tregs. Foxp3 is a member of the forkhead-winged helix family of transcription regulators located on chromosome Xp11.23. Foxp3 full-length protein is encoded by 11 exons and contains a forkhead DNA-binding domain at the C terminus which can bind to the IL-2 promoter and repress IL-2 mRNA transcription.[16] Foxp3 is constitutively expressed at high levels on natural Tregs in both man and mice.[17] Foxp3 can activate or stifle other transcription factors e.g., T-bet (Th1) and STAT6 and GATA3 (Th2) (Table 1), signalling pathways or membrane expression of certain molecules in the periphery. *Scurfy* mice lacking Foxp3 are deficient in $CD4^+CD25^+$ Tregs and develop severe lymphoproliferative and autoimmune disease.[18]

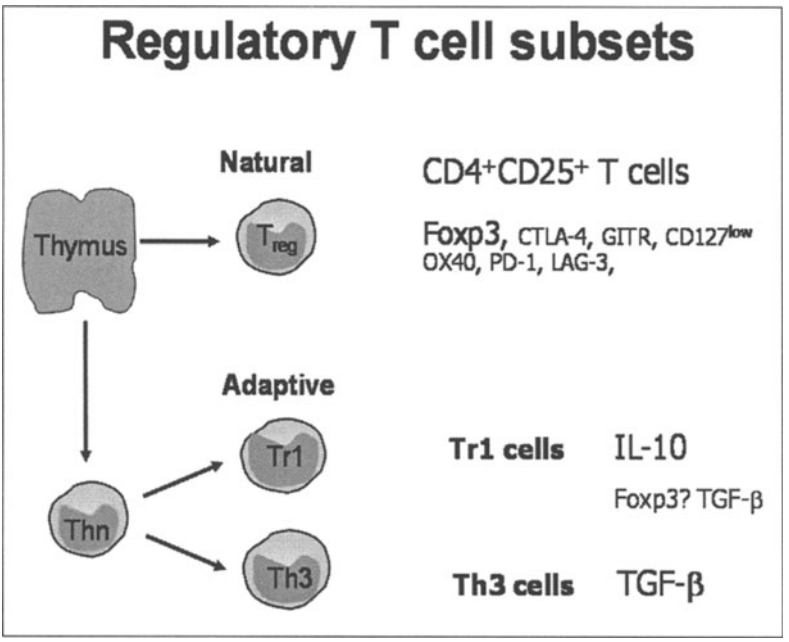

Figure 1. Regulatory T-cells (natural and adaptive-Tr1 and Th3). Cell surface markers, the transcription factor Foxp3 and their cytokines (IL-10 and TGF-β) are depicted. Reproduced with permission from Nouri-Aria KT, Durham Sr. Inflammation & Allergy-Drugs Targets, 2008; 7:237-252.

Table 1. T-cell subsets, their transcription factors and cytokines

Subset	Transcription Factor	Cytokines
Th1 (Autoimmunity)	T-bet, STAT1, STAT4	IFN-γ, IL-2, TNF-α
Th2 (Allergy and helminith infection)	GATA3, STAT6	IL-4, IL-5, IL-9, IL-13
Th17 (Inflammation and autoimmunity)	RORgt (mice), RORC2 (man)	IL-17, IL-25, IL-21, IL-22
Treg (Immunetolerance)	Foxp3 (mice), FOXP3 (man)	IL-10, TGF-β

Mutations in the *foxp3* gene in man result in IPEX syndrome, a condition with the spontaneous development of allergic airways inflammation, hyper IgE and eosinophilia which predominantly affect male offspring.[19] Conversely, the ectopic expression of Foxp3 phenotypically and functionally convert effector T-cells to Tregs with full regulatory function.[20] In mice, Foxp3 expression has been shown to be both necessary and sufficient for Tregs development.[21] Whereas in man there is increasing evidence demonstrating that Foxp3 is also transiently expressed in activated T-cells.[20,21] The observation that Foxp3 mRNA expression in newly activated $CD4^+CD25^+$ cells lacking regulatory function[22] may suggest that in humans Foxp3 alone is not sufficient to command regulatory activity of $CD4^+CD25^+$ cells.

The molecular mechanisms involved in the regulation of Foxp3 expression remain poorly understood. However, it has been established that the transcriptional activity of Foxp3 promoter is dependent on TCR signalling and several AP1- and NFAT-binding sites within the promoter.[23] The binding of STAT5 and STAT3 to conserved binding sites of the Foxp3 locus have also been implicated in the regulation of Foxp3 expression in man.[24] In both peripheral blood and tissues $Foxp3^+CD4^+CD25^+$ Tregs can be induced from $CD4^+CD25^-$ T-cells in the presence of TGF-β and interleukin-2 (IL-2).[25] Demethylation of DNA plays a critical role in the conversion of $CD4^+CD25^-$ T-cells into $Foxp3^+$ Tregs.[26] The mechanism of suppression used by Tregs remains contentious, nonetheless it has been postulated that TGF-β 1 may mediate the immunosuppressive activity of Tregs. In a recent report the in vitro induction of Foxp3 failed to upregulate *EBI3, p35* mRNA, or IL-35 secretion,[27] refuting the earlier suggestion that IL-35 contribute to the suppressive mechanism of human Treg.[28,29]

Regulatory T-Cells in Health and Allergic Conditions

In the last few decades allergic diseases such as asthma, atopic dermatitis and rhinitis have been increasing in prevalence worldwide and in particular in the industrialised countries. The prominent role of natural Tregs in preventing autoimmune diseases, rejection of solid organ transplants and uncontrolled tumour growth collectively suggest $CD4^+CD25^+$ Tregs may also play a critical role in hampering allergic diseases. A low proportion of Tregs and/or a defect in the ability of allergen specific Tregs may be responsible for the increase in the number of individuals with allergy seen in the past 30 years. Thus, better understanding of mechanisms of Tregs controlling Th2 responses, the characteristic feature of allergic conditions, may help in developing more effective therapeutic strategies for treatment of these diseases.

The role of Th2-driven immune responses has been decisively established in the development of allergic diseases. Nevertheless environmental factors and the genetic predisposition of allergic individuals are also believed to contribute, as cofactors, in the severity of these Th2 diseases.[30] Conversely, the lack of responses to allergens in nonatopic healthy individuals and the mechanisms by which such immune tolerance is induced and regulated are poorly understood. This chapter summarises in vitro and in vivo evidence for the functional abilities of regulatory T-cells in controlling effector T-cell responses (cell proliferation and cytokine production) in health and in a number of allergic disorders including asthma, allergic rhinitis and atopic dermatitis as well as in experimental models of allergies. It also discusses the

effects of treatment (glucocorticoids and allergen immunotherapy) on modification of Tregs and the possibility of manipulating these T-cell populations for treatment of allergic conditions.

Health

Despite constant exposure to aeroallergens or food allergens, healthy subjects maintain stable immune tolerance in their gastrointestinal and respiratory systems. The role of $CD4^+CD25^+$ Tregs in controlling immune responses to allergens in man was established[31] by the lack of proliferative responses to allergens and a trend towards cytokine profile of Tr1 cells in healthy volunteers. This was in sharp contrast to T-cell recognition of allergens resulting in elevated Th2 cytokine production (IL-4, IL-5 and IL-13) and heightened proliferative T-cell responses to allergens in atopic individuals.[32] These findings may imply that active immune surveillance by Treg populations operates in healthy nonatopic individual and is possibly absent in atopic subjects. The majority of allergen-specific T-cells in these healthy individuals were of IL-10-secreting Tr1 type. These observations support the notion that an impairment in the ability of Tregs to control exaggerated Th2 responses rather than an imbalance between Th2:Th1 responses, may exist in atopy. It would also provide a plausible explanation as to why no immunological responses are seen in nonatopic healthy volunteers following allergen exposure.

In line with these findings was the lack of proliferative T-cell responses to cows' milk antigen in healthy children. Depletion of $CD4^+CD25^+$ T-cells from PBMCs reversed T-cell recognition of cow's milk antigen, implying that the food tolerance to dietary antigens may be an active process imposed by $CD4^+CD25^+$ Tregs.[33] Contrary to these data, children who had outgrown cows' milk allergy revealed increases in the frequency of circulating $CD4^+CD25^+$ T-cells and decreased in vitro proliferative responses to bovine β-lactoglobulin (a milk protein) when compared with children who remained clinically milk sensitive.[33] A similar scenario of IL-10-associated T-cell-induced anergy has been reported in hyperimmune individuals i.e., bee keepers, who had received multiple bee stings. The intracytoplasmic $IL\text{-}10^+$ cells from these individuals were colocalized to $CD4^+CD25^+$ T-cells with specificity for bee venom antigens.[34]

Experimental Models

The strategic role of $CD25$ in the induction of $CD4^+$ Tregs in a murine model of allergic conjunctivitis was identified following the injection of anti-CD25 in thymectomized mice immunized with ragweed pollen. Subsequent allergen challenge in these mice resulted in severe allergic conjunctivitis as judged by conjunctival eosinophil numbers, ragweed-specific IgE and IgG1 levels, an increased proliferative response and Th2 cytokine production by splenocytes to ragweed allergen, confirming that thymus-derived $CD25^+$ T-cells are involved in the development and the regulation of allergic conjuctivitis. The adoptive transfer of $CD4^+CD25^+Foxp3^+$ T-cells from healthy, naïve mice into ragweed-sensitized mice, suppressed the development of allergic conjuctivitis, reinforcing the perception that Foxp3 expressing Tregs play a pivotal role in the regulation of allergic conditions.[35]

Foxp3 mutant mice, generated by means of knock-in mutagenesis developed an intense multiorgan inflammatory response associated with allergic airway inflammation, hyperimmunoglobulinemia E, eosinophilia and dysregulated Th1 and Th2 cytokine production in the absence of overt Th2 skewing, is consistent with the striking influence of Foxp3 in the development of allergic inflammation.[36]

The influence of IL-10 producing Tr1 cells governing Th2 type responses in experimental model of OVA challenged mice was established by the adoptive transfer of $IL\text{-}10^+CD4^+CD25^+$ cells from naive mice to OVA sensitized mice, resulting in the resolution of inflammatory responses in the bronchial mucosa.[37] This observation is consistent with the low concentration of IL-10 in the bronchoalveolar lavage fluid of adult asthmatic patients compared with healthy controls.[38] Neutralization studies using anti-IL-10 almost abolished the suppressive effect of Tr1 cells in vitro and further strengthened the role of this immunesuppressive cytokine in the control of Th2 responses and in the maintenance of T-cell homeostasis.

Similarly to IL-10, TGF-β is also involved in the control of atopic conditions. Thus in TGF-β knockout mice a higher susceptibility to bronchial hyperreactivity and bronchial inflammation resembling asthmatic reactions in man has been observed.[39] In a mouse model of food allergy, a significant reduction of secreted IgA antibodies, a class of immunoglobulin which is tightly regulated by TGF-β has been demonstrated in the gut. Subsequent recovery from food allergy in these mice was associated with local production of TGF-β.[40]

A failure of immune tolerance rather than a defective in Th1 immunity appears to underlie the immunobiology of Th2-driven allergen-induced airway disease in a mouse model of asthma. The reduction in the bronchial inflammation appears to be the consequence of regulatory processes involving dendritic cell-T-cell interactions. In contrast to OVA-induced murine models of asthma in which mice developed Th2-driven airway disease, the prolonged/three week OVA exposures resulted in the suppression of airway hypersensitivity. The mechanism underlying tolerance by chronic repetitive allergen (OVA) exposure in this rodent model included the recruitment of considerable numbers of Tregs expressing $CD4^+CD25^+Foxp3^+$ plus enhanced TGF-β1 production in the airways, despite tissue eosinophilia and high serum levels of, OVA-specific IgE and IgG_1. The resolution of airway hyperresponsiveness, tissue eosinophilia and Th2 cytokine profile were associated with the accumulation of Foxp3 expressing regulatory T-cells in local draining lymph nodes.[41,42] Understanding of mechanisms involved in airway tolerance to inhaled allergens could potentially help to improve treatment for allergic diseases and asthma.

In another study of murine model, immune tolerance was induced by repeated low-dose aerosolized OVA exposure. In this model, $CD4^+$ T-cells with regulatory effects expressed both cell surface and the soluble form of TGF-β and inhibited the development of an allergic phenotype when these cells were administrated to naive recipient mice challenged with the allergen. Although the blockade of TGF-β particularly interfered with immunosuppression, the severity of suppression was profound when $CD4^+$ T-cells were obtained from the tolerized mice expressing high levels of Foxp3.[43] These findings suggest that the cell surface expression of TGF-β rather than the secreted form of this immunosuppressive cytokine may be responsible for the effective inhibition and blunting the development of allergic responses. The study also suggested antigen-induced tolerance requires cell-cell contact with Tregs expressing Foxp3 and the expression of membrane bound TGF-β as the dominant component in the mechanisms of suppression of allergic responses.[43]

Allergic Rhinitis and Asthma

Allergic rhinitis, a debilitating allergic condition affecting up to 30% of populations in northern Europe and in the USA, is a Th2 predominant disease. The factors driving such Th2 responses have not been fully resolved and pathological mechanisms are unclear. It is however, conceivable that reductions in absolute numbers or a defect in function of the $Foxp3^+CD4^+CD25^+$ in circulation and/or in the nasal mucosa of allergic rhinitics[44] could, in parts, be responsible for the clinical manifestation and the high prevalence of this condition in the western world. In several in vitro studies, $CD4^+CD25^+$ T-cells from aeroallergen sensitive individuals (e.g., grass and birch pollens, cat and house dust mite) were found to be defective resulting in high proliferative responses and IL-5 secretion when $CD4^+CD25^+$ T-cells were cocultured with autologous allergen-stimulated $CD4^+CD25^-$ T-cells.[45-46] The dysregulation of $CD4^+CD25^+$ Tregs in the atopics controlling IL-13 and IL-5 production[32] was most pronounced during the peak of the birch and grass pollen seasons. In contrast, $CD4^+CD25^-$ T-cells from both allergic and nonallergic individuals were efficiently able to suppress T-cell proliferation and Th2 cytokine production to allergens outside of the pollen season with significant levels of Foxp3.

The frequencies of the $Foxp3^+$, $CD4^+CD25^+$ and $Foxp3^+CD4^+CD25^+$ populations were found to be more abundant in the nasal mucosa of healthy controls than in hay fever sufferers, whereas the concentration of IL-2 and IFN-γ secreted by PBMC in hay fever sufferers was significantly greater than in the control group, consistent with a defect in regulatory pathways in this clinical condition. The results further indicate that $CD4^+CD25^+$ Tregs as well as Foxp3 expressing cells may play a crucial

role in immunological imbalance in hay fever, suggesting that Foxp3$^+$CD4$^+$CD25$^+$ T-cells have the potential to act as a new therapeutic target for the treatment of this allergic disorder.[47]

Children with allergic disease have been shown to have fewer CD4$^+$CD25^{hi} T-cells than control subjects. Surprisingly, numbers of CD4$^+$CD25$^+$ and CD4$^+$CD25^{hi} T-lymphocytes were higher in children with persistent allergic rhinitis and/or moderate-severe bronchial asthma than in those with respective milder disease and the frequency of these cells were correlated with total serum immunoglobulin E level.[48] Foxp3 expression of CD4$^+$CD25$^+$ T-cells was elevated in moderate-severe versus mild asthma. Similarly, patients with moderate-severe bronchial asthma had increased expression of IL-10 compared with patients with mild asthma. The suppressive capability of Tregs from patients with more severe asthma appeared to be intact in vitro.[48] On balance, decreased numbers of Tregs in children with allergic airway disease may also represent a defect of the Treg function. The unexpected findings may however represent the recruitment of Foxp3$^+$IL-10$^+$ Tregs from the lymphoid organs to the target organ and/or circulation to combat and dampen of the ongoing allergic inflammation in the lungs of severe asthmatics.

The antigen specificity of Tregs and its influence in the induction and maintenance of tolerance remain contentious. Studies on Tregs from nonallergic healthy subjects demonstrated a profound inhibition of proliferation of effector cells stimulated with influenza antigen as well as birch pollen allergen. This was in sharp contrast with CD4$^+$CD25$^+$ cells from allergic rhinitis patients which were capable of dampening proliferative responses of T-cells to influenza antigen, but not birch pollen. Similarly, the regulation of Th2, but not Th1 cytokine production by CD4$^+$CD25$^+$ cells was impaired in allergic patients, upon stimulation with birch pollen extract. Neutralization of IL-10 led to increased production of IFN-γ and TNF-α in the nonatopic controls, substantiating a dysregulation of allergen-specific Foxp3 expressing CD4$^+$CD25$^+$ T-cells in atopic subjects. The elevated concentration of TNF-α following neutralization of IL-10, however suggests a pro-inflammatory role for this cytokine and that the IL-10 produced by Tregs is possibly involved in promoting tolerance.[49]

Further evidence for the existence of antigen specificity of Foxp3 expressing Tregs was provided by studies of allergen (Der-p 1)- specific and streptokinase (SK) specific-CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the peripheral blood of atopic individuals.[50] CD4$^+$CD25$^+$Foxp3$^+$ T-cells from Der p 1- sensitive atopic individuals when cultured with DCs activated with Der p-1, but not those cultured with either unloaded or SK-loaded DCs, suppressed the proliferative responses of autologous CD4$^+$CD25$^-$ phenotype to Der p 1 or SK respectively.[50] These findings may also imply that the pool of human circulating CD4$^+$CD25^{high}Foxp3$^+$ T-cells consist of Treg populations specialised in the recognition of antigens with different specificities. It has been postulated that natural Tregs with a large repertoire for self-specific T-cell receptors suppress immune responses via contact-dependent mechanisms, whereas the inducible Tregs consist of both self- and nonself-specific cells recognising autoantigens as well as foreign antigens and the latter populations are capable of suppressing a wide range of immune cells via high concentrations of TGF-β 1 (possibly Th3 cells) or IL-10/TGF-β 1 producing Tr1 cells.[51] Although antigen contact is required by Tregs to induce a suppressive mechanism, once they are set in motion, subsequent suppression may not require antigens and can inhibit both in antigen specific and antigen nonspecific fashion.

Mucosal System

The induction of mucosal tolerance through the recruitment of Foxp3 expressing cells has been proposed as an alternative approach for the treatment of respiratory allergy. Long-term efficacy and mechanisms of mucosal tolerance induction were investigated by the means of an experimental model of birch pollen allergy. Two structurally diverse products of Bet v1 allergen i.e., unmodified native three-dimensional major BP allergen Bet v 1 and nonconformational hypoallergenic fragment were applied intranasally before- (prophylactic) or after sensitization (therapeutic) with birch pollen allergen.[52] Both native- and the modified fragment Bet v 1 allergen showed prophylactic and therapeutic effects. The immune tolerance induced with the native Bet v 1 allergen however was associated with the enhanced expression of TGF-β, IL-10 and Foxp3 expressing CD4$^+$ T-cells. These observations provide further evidence on antigen specificity of Tregs and suggest that the native and

conformational structure of antigens rather than modified fragment is an important component in the induction of appropriate immune regulatory effects by Tregs.[52] In this scenario Foxp3 seem to be the dominant molecule for the long-term efficacy of immunosuppression and in the dampening of immunopathology of birch allergy.

Epidemiological studies have indicated that infection with helminth parasites may counteract allergies, possibly by generating Tregs and suppression of the Th2 limb of immunity. To address whether the gastrointestinal nematode Heligmosomoides polygyrus was capable of down-regulating allergic reactions, a rodent model of OVA and house dust mite allergen—Der p 1-induced asthma was used.[53] The administration of the parasite induced suppression of inflammatory cell infiltrates in the lung, but was reversed if mice were treated with anti-CD25. The inhibition of bronchial hyperresponsiveness and airway inflammation was transferable with mesenteric lymph node cells (MLNC) from helminth infected animals to uninfected allergen sensitized mice. MLNC from infected animals showed significant numbers of $CD4^+CD25^+Foxp3^+$ T-cells, high expression of TGF-β and strong interleukin IL-10 responses to parasite antigen. Unexpectedly, MLNC from IL-10-deficient animals also transferred suppression to sensitized hosts, indicating that IL-10 per se may not be the primary suppressor of the allergic response.[52] These data support the contention that helminth infections can elicit a regulatory T-cell population capable of down-regulating allergen- induced lung pathology in vivo. Intervention studies with hookworm in parasite-naïve allergic individuals are currently ongoing in the United Kingdom to test these hypotheses further.[54]

Atopy

Maternal atopic status and the adaptive immune responses to microbial exposure at an early stage in life may decide the outcome of developing allergic disease or atopy during the childhood. Using cord blood mononuclear cells from 50 healthy neonates (31 nonatopic and 19 atopic mothers) and the innate TLR2 agonist peptidoglycan (Ppg) or the adaptive allergen house dust mite Dermatophagoides farinae (Der f 1)[55] as stimuli demonstrated that peptidoglycan was more able to induce high levels of IL-10, IFN-γ, IL-13 and TNF-α cytokine secretion and lymphocyte proliferation than Der f 1. Foxp3 and GITR expression of cord blood mononuclear cells (CBMC) and IL-10 production were also greater in CBMC from neonates without maternal atopy than those with maternal atopy. IL-10 production was highly correlated with the increased expression of Foxp3, GITR and CTLA4, independent of maternal atopy. The increased IL-10 and Foxp3 induction in cord blood mononuclear cells of nonatopic compared to atopic mothers and the induction of IL-10 producing Tregs via TLR2, suggest possible intrinsic defect in the induction of adaptive responses to microbial stimuli which may be associated with atopy.[55]

In a group of children with egg allergy, the ability of $CD4^+CD25^+CD127^{low}$ Treg cells in suppression of IFN-γ production by autologous $CD4^+$ effector T-cells in responses to staphylococcal endotoxin B revealed significantly less Treg cell-associated suppression in the allergic group compared with nonallergic children, although the proportion of circulating $CD4^+CD25^+CD127^{low}$ Treg and Foxp3 expressing cells were similar in both groups.[56]

Maternal atopy is also considered as a strong candidate predicting the development of childhood allergic diseases. Cord blood from offspring of atopic mothers showed fewer Lipid A peptidoglycan-induced $CD4^+CD25^{high}$ Treg cells, lower expression of GITR and Foxp3 and decreased IL-10 and IFN-γ secretion. In contrast IL-17 response to Lipid A was independent of maternal atopy and highly correlated with IL-13 secretion.[57] Similarly, mitogen-induced suppression of T effector cells in cord blood of offspring from atopic mothers was also impaired.[57] These findings imply that impairments in Foxp3 expressing Treg numbers and/or function may be a predisposing factor in the development of atopic diseases in childhood.

Atopic Dermatitis

The high levels of serum IgE in patients with atopic dermatitis was hypothesized to derive from a dysregulation of Tregs controlling IgE synthesis.[58] The frequency of circulating $Foxp3^+CCR4^+CLA^+$ cells was found to be greater in atopic dermatitis with highly elevated

serum IgE compared with low IgE levels with a strong association between Foxp3 expressing cells and the disease severity. $CD25^{hi}$ T-cells appeared to consist of two subsets based on the differential expression of the chemokine receptor CCR6. Although the ratio of $CCR6^+$ and $CCR6^-$ subtypes within the $CD25^{hi}$ subset were similar in atopic dermatitis, the intensity of CCR6 expression was strongly correlated with the suppressive ability of Tregs. $CCR6^-$ populations, in contrast, demonstrated functional characteristics of Th2 effector cells and synthesized large quantities of IL-5 in response to Staphylococcus aureus superantigen derived from the skin colonizing organism, possibly indicating the expression of CD25 on these populations merely represent cell activation.[57] Moreover, the $CCR6^+Foxp3^+CLA^+$ cells had greater suppressive abilities controlling proliferation of effector cells than $CCR6^-$ populations.

Studies on the cutaneous coexpression of Foxp3 and GITR on a panel of different inflammatory skin diseases using dual immunohistochemical staining revealed that Foxp3 and GITR were almost exclusively present on T-cells that express both CD4 and CD25 and were more prevalent in the inflammatory skin conditions than in healthy skin.[58] Similar findings were identified using peripheral blood $CD4^+$ T-cells co-expressing Foxp3 and GITR. In contrast to healthy volunteers whose biopsies showed low numbers of $Foxp3^+GITR^+$ T-cells, cutaneous $Foxp3^+$ T-cells in patients with spongiotic dermatitis, psoriasis and lichen planus showed a frequency of 25-29% and in patients with leishmaniasis this was ~15%.[58] These observations at a glance were suggestive of mechanisms of suppression via molecules of Foxp3 and GITR may be intact in these skin conditions. However, the recruitment of $Foxp3^+GITR^+$ T-cells in the inflamed skin may play a central role in the disease recovery, cessation of immune responses to invasive pathogens and the establishment of immunologic tolerance.

Analysis of Treg cells infiltrated in the skin conditions such as atopic dermatitis and psoriasis showed that $CD25^+$ cells were present in the perivascular and papillar dermis of all lesional specimens and $FoxP3^+$ cells were distributed in the perivascular and interstitial atopic dermatitis dermis. In atopic dermatitis and psoriatic skin, $CD4^+CD25^+FoxP3^+$ T-cells were absent in the lesional region and in the atopy patch test areas of the skin, despite the abundant expression of IL-10 and TGF-β as well as receptors for these cytokines in the dermis.[59] In contrast skin biopsies from healthy volunteers despite having few $Foxp3^+$ T-cells showed an even distribution through the dermis. Double immunostaining demonstrated that $CD25^+FoxP3^+$ cells were distributed in the perivascular, interstitial and periadnexal dermis, in contrast healthy skin specimens featured few $CD25^+$ $FoxP3^+$ cells scattered throughout the dermis. These findings suggest an impaired regulatory T-cell function rather than the absolute numbers of Tregs in the cutaneous lesions may play a key role in the immunopathology of atopic dermatitis.

Modulation of T_{Regs} with Treatment

Glucocorticoids

The administration of glucocorticoids, inhaled or systemic, has been reported to increase Foxp3 and IL-10 expression in the bronchial mucosa in patients with severe asthma. $Foxp3^+$ T-cells were tightly correlated with IL-10 expressing cells but not with the expression of TGF-β1, possibly suggesting the Tr1 nature of the Treg population. The frequency of $CD4^+CD25^+$ T-cells in circulation and the Foxp3 expression by $CD4^+$ T-cells was transient, but significantly greater in patients who received systemic glucocorticoid treatment. In vitro cultures of $CD4^+$ T-cells with corticosteroid induced upregulation of IL-10 and Foxp3 expression on these cells,[60-61] corroborates that glucocorticoids are not only potent immunosuppressor agents with anti-inflammatory effects. They are also capable of inducing the differentiation of $CD4^+$ T-cells towards a Foxp3 expressing Tr1 phenotype with suppressive consequences.

In asthmatic children, inhaled corticosteroid treatment was also associated with an increased proportion of $CD4^+CD25^{hi}$ T-cells in both peripheral blood and bronchial alveolar lavage fluid (BALF) and an improvement in suppression of proliferation and cytokine/chemokine production by $CD4^+CD25^-$ responder T-cells.[62] The role of immunosuppressive drugs, vitamin D3

and dexamethasone, in the induction of IL-10 producing regulatory T-cells from naive $CD4^+$ T-cells have been reported in man and mouse. The newly induced Tregs failed to synthesize IL-5 or IFN-γ, despite retaining strong proliferative capacity. The inhibition nuclear factor (NF)-κ B and activator protein (AP)-1 activities confirmed the influence of these two immunosuppressive agents in the development of Tregs.[60]

The population of $CD4^+CD25^{hi}$ Tregs and Foxp3 mRNA levels in peripheral blood and in the BALF of asthmatic children were found to be lower than in children with chronic cough and healthy control children.[63] Increased percentages of $CD4^+CD25^{hi}$ T-cells in peripheral blood and BALF were identified in asthmatic children after inhaled corticosteroid.[62] In contrast to the asthmatic group, isolated BALF and peripheral blood $CD4^+CD25^{hi}$ T-cells from non-asthmatic subjects suppressed the proliferation and cytokine as well as chemokine production by $CD4^+CD25^-$ responder T-cells. Corticosteroid treatment restored the regulatory activities of $CD4^+CD25^{hi}$ T-cells after inhalation, suggesting that the lung pathology seen in paediatric asthma, as with adult asthma, may stem from impaired regulatory T-cell control of Th2 responses. Pulmonary Tregs may also represent a therapeutic target in paediatric asthma.[63]

Specific Allergen Immunotherapy

The efficacy of allergen specific immunotherapy (SIT) in treatment of selected patients with IgE mediated diseases has been established.[64,65] SIT was initially described about 100 years ago[66] and involves subcutaneous administration of small but increasing doses of allergen using a relatively crude allergen extract.

To date SIT is the only treatment that can alter the natural course of allergic rhinitis, conjunctivitis and allergic reactions to stinging insects.[67] Conventional subcutaneous allergen immunotherapy prevents further allergen sensitisations and the development of asthma in patients with allergic rhinitis.[68] The clinical improvement following allergen immunotherapy is sustained for years after discontinuation, thus SIT is believed to modify the underlying immunological mechanisms of allergic responses.[69] The mechanisms by which allergen immunotherapy reduces allergic symptoms have been studied for decades. Induction of blocking antibody i.e., IgG4, a shift from Th2 to IFN-γ producing Th1 cytokine profile[70,71] and reductions in the numbers of effector cells such as eosinophils, mast cell and basophils in the target organ are amongst the immunological changes observed following successful SIT.

More recently induction of functional $CD4^+CD25^+$ Tregs[72] capable of attenuating allergen-induced proliferation of Th2 cells and their cytokine pattern have been reported with successful SIT. The intracellular IL-10 positive T-cells from patients who had completed a course of allergen immunotherapy was almost exclusively localized to $CD4^+CD25^+$ cells. IL-10-producing $CD4^+CD25^+$ regulatory T-cells have therefore emerged as potential mediators of immune tolerance following grass pollen immunotherapy.[72]

Two subsets of regulatory T-cells, IL-10-producing Type 1 regulatory T-cells and the natural $CD4^+CD25^+Foxp3^+$ Treg cells have been reported to play important roles in the control of allergic inflammation. Successful SIT dampens allergen-specific effector T-cells and activates uncommitted $CD4^+CD25^-$ phenotypes to possibly IL-10 secreting Tr1 populations. Tr1 cells suppress Th2 cells and effector cells of allergic inflammation, such as eosinophils, mast cells, basophils, through IL-10 and possibly TGF-β. Understanding of the mechanisms of $IL\text{-}10^+$ Tr1 cells may be helpful in developing new strategies for treatment of allergic diseases.[73,74]

The effect of house dust mite (HDM) specific immunotherapy on the induction of Tregs expressing markers such as Foxp3, CTLA4, IL-10 and TGF-β was studied using peripheral blood $CD4^+$ T-cells from both HDM sensitive asthmatic- and nonatopic children. This revealed a temporary increase in CTLA-4 at three months after SIT with no significant changes in IL-10 production or in the expression $Foxp3^+$ Tregs. Contrary to these findings were significant increases in TGF-β and Foxp3 expression by $CD4^+CD25^+$ Tregs and the associated clinical improvement following the completion of SIT at one year, suggesting that conventional SIT requires high concentrations of allergens to induce an effective clinical and immunological

tolerance, with TGF-β and Foxp3 as the two sensitive biomarkers for monitoring the response to immunotherapy.[75]

Using house dust mite-sensitive mice, peptide IT increased the number of $CD4^+CD25^+$ Tregs in the peripheral blood and the adoptive transfer of $CD4^+CD25^+$ Tregs precluded the induction of experimental allergic encephalomyelitis.[76] Although these $CD4^+CD25^+$ Tregs showed suppressive capabilities in vitro, their effects in vivo depended on the induction of antigen-specific IL-10 producing Tr-1 cells. As indicated above, Tr1 cells are likely to play a crucial role in the control of allergic disease with major effector mediator cytokines such as IL-10 and TGF-β.

The suppressive mechanisms of Tr1 cells not only involve cytokines IL-10 and TGF-β, but also molecules such as CTLA-4 and PD-1 antigens. IL-10 inhibits CD28 tyrosine phosphorylation and prevents the binding of phosphatidylinositol 3-kinase p85, hence reducing the costimulatory CD28 signalling pathway.[77] Induction of antigen-specific Tr1 cells can thus redirect inappropriate immune responses against allergens using a broad range of suppressor mechanisms.

The autocrine action of IL-10 and TGF-β is important the induction of peripheral T-cell tolerance and plays a crucial role in the mechanisms of allergen-SIT.[78,79] Reactivation of T-cells tolerized by IL-10 and TGF-β can result in the distinct pattern of either Th1 or Th2 cytokine profiles depending on the cytokine milieu in the target organ. Peptide presentation to the anergic T-cells was, however, fully restored in the presence of IL-2 or IL-15 as documented by the secretion of IFN-γ, but no IL-4 could be detected in this system suggesting that the suppression induced by Tregs are reversible.[79] Both IL-10 and TGF-β expressing cells have been reported to increase in the nasal mucosa of grass pollen IT treated patients, with a strong association between TGF-β expressed in the nasal mucosa and the levels of secretory IgA2 produced in the circulation, implying that the immunological changes are not only systemic, but occur in the target organ, i.e., nasal mucosa. Thus, in addition to their cellular inhibitory influence, IL-10 and TGF-β are the critical factors in switching from an inflammatory immunoglobulin E (IgE) to the noninflammatory isotypes IgG4 and IgA respectively, the two classes of Ig with significant values in the outcome of SIT treatment and protection of mucosal surfaces.[78,79]

Another crucial change seen after successful allergen-SIT is a shift in the balance from IgE to IgG, in particular to IgG4 subclass, the latter being under the regulation of IL-10. Two independent studies have reported that the increased IL-10- and TGF-β production by Tregs in vivo may endorse that high and increasing doses of allergens administrated during the course of grass pollen IT are responsible for the proliferation and activation of Tr1 populations. Jutel et al reported the suppression induced by $CD4^+CD25^+Tr1^+$ cells was partially blocked by neutralization of antibodies against secreted forms or membrane-bound IL-10 and TGF-β.[80]

Regulatory T-cells are thought to play an important role in allergic diseases and tolerance induction during specific immunotherapy. In a recent publication, significant numbers of $CD4^+CD25^+$Foxp3 expressing cells were identified in the nasal mucosa of allergic rhinitics who had completed a successful course of SIT. Seasonal increases in $CD4^+Foxp3^+$ and $CD25^+Foxp3^+$ cells in these patients were accompanied by suppression of local allergic inflammation, indicative of the development and differentiation of a regulatory T-cell phenotype post immunotherapy. $CD25^+IL\text{-}10^+$ T-cells were both Foxp3 positive and Foxp3 negative and co-existed in a close microenvironment within the nasal mucosa, providing evidence for the emergence of phenotypically and functionally distinct populations of regulatory cells i.e., "adaptive" Foxp3 expressing Tregs and IL-10 expressing "Tr1" cells following SIT.[81,45] Similar observations have been reported in patients with inflammatory bowel disease, in whom IL-10 producing $Foxp3^+CD4^+CD25^+$ cells were present at increased density in the colon and the presence of IL-10 expressing Tregs were associated with amelioration of colitis.[82] These findings encourage strategies which augment numerically and/or functionally Tregs locally which would be beneficial in the treatment of allergic rhinitis.

Venom Immunotherapy

Venom immunotherapy (VIT) induces long-lasting immune tolerance to hymenoptera venom antigens; however, the underlying mechanisms have yet to be clarified. In a longitudinal

study VIT induced a significant progressive increase in percentage and absolute numbers of $CD25^{bright}Foxp3^{+}$ $CD4^{+}$ regulatory T-cells, with the particular effect on Foxp3 confirmed both at mRNA and protein levels.[83] These changes were unrelated to alterations in the expression of activation markers or imbalances in the naïve/memory T-cell compartments. Interestingly, the increase in the circulating $Foxp3^{+}$Tregs were correlated with a shift from the venom-specific IgE to IgG4, corroborating the findings in grass pollen IT in allergic rhinitis. VIT is also associated with a progressive expansion of circulating regulatory T-cells as defined by high expression of CD25 and/or $CD4^{+}Foxp3^{+}$ T-cells, supporting a role for these cells in the induction of tolerance and unresponsiveness to subsequent venom exposure similar to the phenomenon of natural sting tolerance in bee keepers. Wasp venom- or phospholipase A2-pulsed dendritic cell stimulation of $CD4^{+}CD25^{-}$ T-cells from healthy donors resulted in inhibition of proliferation and Th2 cytokine production by Tregs at 10-fold lower than the optimal concentration. In contrast, IFN-γ production was inhibited at all concentrations, suggesting that the threshold of response is different between allergic and nonallergic individuals.[84]

Less is known about the underlying molecular mechanisms of TGF-β-mediated suppression in allergen immunotherapy. Blocking of CTLA-4 was associated with decreased TGF-β levels within the bronchoalveolar lavage fluid of a murine model of allergic inflammation.[85] TGF-β has been recognized to deviate antibody response from an IgE to an IgA-dominated response in man and in mice post IT.

Based on these observations and the decreased Treg cell populations as possibly one major cause of allergic diseases, the upregulation of Tregs numerically or functionally gives promise of therapeutic potential in the treatment of allergic diseases. Novel strategies should be adopted to improve the clinical efficacy of IT using adjuvants such as IL-10, vitamin D3, or TLR agonist such as CpG. Mycobacterium induced allergen-specific Tregs producing IL-10 and TGF-β protected against airway inflammation in mice.[86-89] The application of adenoviral vectors encoding IL-10 also resulted in a longer suppressive effect, with a very limited half-life of IL-10, hence with less or no side effects. Alternatively immunostimulatory CpG motifs, an agonist of TLR9, may improve clinical efficacy when combined with pollen immunotherapy.

Conclusion

Natural $CD4^{+}CD25^{+}$ Tregs play a critical role in the control of peripheral tolerance to self-antigens and in prevention of allergic diseases including rhinitis, atopic dermatitis and asthma. $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs and IL-10 producing Tr1 cells capable of suppressing Th2 responses to allergens seem to be defective in those who develop allergic sensitization. Significant progress has been made in understanding specific mechanisms resulting in allergic inflammation and IL-4 may be a key factor in preventing the de novo induction of Treg cells and re-induction of allergen tolerance. However the exact mechanism of suppression remains controversial. Better understanding of regulatory mechanisms involved in the development of allergic sensitization and the manipulation of Treg cells holds the promise of effective treatment strategies to prevent and treat allergic diseases. Allergen immunotherapy modifies T-cell responses to allergen and may do so through induction of adaptive Tregs, e.g., IL-10—producing Tr1 cells contributing to the clinical efficacy of the treatment in aeroallergen sensitive individuals. Allergen immunotherapy may enhance the development of allergen-specific Tregs and provide safe, specific and long-term control of allergic diseases and asthma. Co-administration of specific allergen immunotherapy with drugs such as corticosteroids and vitamin D_3, adjuvants, for example IL-10 or CpG, are promising candidates for enhancing and generating antigen specific regulatory responses.

Adoptive transfer of Tregs may represent an effective, donor-specific therapeutic approach, although it can also be cost effective. In vivo induction and/or expansion of Tregs in patients remain an attractive option. This may present a more realistic line to improve allergen specific Tregs and provide significant benefit to allergic rhinitis and asthmatic patients.

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CHAPTER 15

Human Clinical Phenotype Associated with *FOXN1* Mutations

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Abstract

In *humans*, a proper immune response relies on the innate immunity, characterized by a rapid and nonspecific initial response to infections and later on the adaptive immunity, characterized by a specific response to a particular antigen. Disruption of any part of the orchestrated immune response results in the inability to control infections and, subsequently, in illness. An impairment of both effector arms of the specific immunity characterizes the clinical phenotype, known as severe combined immunodeficiency (SCID), which represents a heterogeneous group of inherited disorders due to abnormalities of T, B and NK cells. The first congenital SCID was described as spontaneous immunodeficiency in 1966 in mice and referred as Nude/SCID, based on the association of athymia with complete hairless. In 1996, the human equivalent of the murine Nude/SCID phenotype (MIM #601705) was reported. As in mice, also in *humans* this form is characterized by an intrinsic defect of the thymus, congenital alopecia and nail dystrophy and is due to mutations of the *FOXN1* gene, as well. FOXN1 is mainly expressed in the thymus and skin epithelial cells, where it plays a critical role in differentiation and survival. *FOXN1* belongs to the forkhead box (FOX) gene family that comprises a diverse group of 'winged helix' transcription factors involved in development, metabolism, cancer and aging. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions. In immune system, alterations of FOXN1 result in a thymus anlage that lacks the capacity to generate mature and functional thymocytes. Because the significant expression levels of FOXN1 in skin elements, keratinocytes have been successfully used to support a full process of human T-cell development in vitro, resulting in the generation of mature T-cells from hematopoietic precursor cells (HPCs). This finding would imply a role for skin as a primary lymphoid organ. Thus, the present chapter will focus on the information that came out from the original description of the human Nude/SCID phenotype and on the role of FOXN1 and of the other members of FOX subfamilies in those immunological disorders characterized by abnormal T-cell development or abnormal T-cell regulatory homeostasis.

Introduction: Severe Combined Immunodeficiencies

Primary immunodeficiency (PID) diseases are heritable disorders of immune system.[1] Disruption of any part of the orchestrated immune response can result in an inability to control infections and subsequent illness. Apart from physical barriers, the immune response is composed from a diverse network of defenses, including cellular components and soluble mediators. A proper immune response relies on the innate immunity, characterized by a rapid and nonspecific initial

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Table 1. Different genotypic forms of SCID classified on the basis of the immunological phenotype

Lymphocyte Phenotype	Form of SCID
$T^-B^+NK^-$	X-linked (deficit of γc)
	Deficit of Jak 3
	Deficit of CD45
$T^-B^+NK^+$	Deficit of IL-7Rα chain
	Deficit of CD3δ chain
$T^-B^-NK^-$	Deficit of Adenosine Deaminase
$T^-B^-NK^+$	Deficit of RAG1 or RAG2
	Deficit of Artemis
$T^{low}B^+NK^+$	Deficit of FOXN1

response to infections and later on the adaptive immunity, characterized by a specific response to a particular antigen. The innate immune response involves three major cell types: phagocytic cells, such as neutrophils and macrophages, natural killer (NK) cells and antigen presenting cells, which are also involved in the induction of an adaptive immune response. The adaptive immune system includes T and B lymphocytes responsible for cellular and humoral responses, respectively. However, these components of immune system to maintain a normal resistance to infections act in a well-orchestrated and integrated unique system.

In the last 5 decades, since the first human genetic defect was identified more than 200 PID syndromes have been described. PIDs can be divided into subgroups based on the component of the immune system that is predominantly affected, including T, B, NK lymphocytes, phagocytic cells and complement proteins (Table 1). The antibody deficiencies (B-cell or humoral immunodeficiencies) are characterized by a genetic lesion, that selectively affects antibody production, but a normal cell-mediated immunity. In the cellular deficiencies, cellular effector mechanisms are compromised, whereas antibody production is largely normal in that B-cell intrinsic machinery is intact. The combined immunodeficiencies are characterized by an impairment of both effector arms of the specific immunity, which results in a more severe clinical phenotype. However, since an efficient B-cell antibody response also depends on T-cell activation of B lymphocytes, defects in either cell type have the potential to affect both cellular and humoral immunity to varying degrees.

Of note, most of the diseases within the last category are due to genetically determined blocks in the T-lymphocyte differentiation program. In the absence of mature T-cells, adaptive immunity is abrogated, thus resulting in a broad-spectrum susceptibility to multiple pathogens also including opportunistic micro-organisms. Overall, unrespectively of the pathogenic mechanism of the individual form of severe combined immunodeficiencies (SCIDs), a common hallmark of these diseases is the feature that bacterial, viral and fungal infections are often overwhelming.

The discovery of a so wide number of distinct clinical entities which differ in either the genetic cause or the altered immunological function led to an uncomparable increase in the knowledge of the intimate mechanism by which a proper immune response is generated. Intriguingly, most of the genes whose alterations underlie PID are selectively expressed in hematopoietic cells with a few exception as, for example, ataxia telangiectasia mutated (ATM) gene, also expressed in Purkinje cells and adenosine deaminase (ADA) which is ubiquitous. This dogma, however, led to underestimate those novel immunodeficiencies, which have different features involving other nonhematopoietic tissues.

In 1996, a novel form of SCID (MIM 601705; Pignata guarino syndrome) was described and referred as the human equivalent of the well-known murine phenotype named Nude/SCID.[2] This disease is the first example of SCID not primarily related to an hematopoietic cell abnormality, but rather to an intrinsic thymic epithelial cell defect .[3]

The Nude/SCID Phenotype

In 1966, S.P. Flanagan identified a new mouse phenotype that spontaneously appeared in the Virus Laboratory of Ruchill Hospital, Glasgow, UK, characterized by loss of the hair. This mouse showed an abnormal keratinization in hair fibers, with follicular infundibulum unable to enter the epidermis.[4] The mice also showed an inborn dysgenesis of the thymus[5] resulting in a compromised immune system lacking T-cells.

Subsequently, the molecular nature of the nude defect was characterized and attributed to a genetic alteration of the transcription factor FOXN1 (also called WHN or HFH11), mainly expressed in thymus and skin.[6-8] The analysis of the genomic sequence of the nude mouse revealed the presence of a single base pair deletion in exon 3, absent in the wild-type allele. This deletion led to a frameshift that resulted in an aberrant protein prematurely terminating in exon 6 and the loss of the postulated DNA binding domain.

The mouse nude mutation led to an abnormal development of the skin and thymus[4,9] and a severe alteration of the nails.[10] Later studies demonstrated that both defects, as lack of fur development and agenesis of the thymus, are pleiotropic effects of the same gene.[11] In particular, the skin of the nude mouse contains the same number of hair follicles as a wild-type control, but these follicles result in an uncomplete hair, that could not enter skin surface.[4,10] Flanagan analyzed carefully nude mouse skin and observed that at birth the hair follicles were normal, but by six days after birth the hair started to twist and coil, failing to penetrate the epidermis.[4] This hairless condition could be reverted by oral administration of cyclosporine A or recombinant keratinocyte growth factor (KGF or FGF-7), that influence the number of hair follicles or the cyclic hair growth.[12,13] Furthermore, the nude mouse epidermis shows failure in differentiation and a reduced number of tonofilaments are observed in spinous, granular and basal layers.[10] The nude *Foxn1* gene doesn't affect the growth of hair follicles, but the epidermal differentiation process, regulating the balance between proliferation and differentiation of keratinocytes in the hair follicle.[14,15] In addition to these cutaneous abnormalities, nude animals develop an abnormal thymus, resulting in a severe T-cell deficiency and an overall severely impaired immune system. In fact, thymus morphogenesis is stopped at the first stages of development with no subcapsular, cortical and medullary regions formation, that characterizes a normal mature organ.[16] In addition, the observation that thymus restoration doesn't lead to hair growth demonstrated that the lack of the hair and the athymia were not related one to each other.[16,17]

Furthermore, the nude phenotype is characterized by nail malformations and poor fertility. The first condition is attributed to an abnormal production of filaggrin protein in nail matrix and nail plate, subsequent to a loss of keratin 1 protein. Differently, the second condition may be the result of changes in hormonal status, as demonstrated by the altered serum levels of estradiol, progesterone and thyroxine.[10]

For many years the human counterpart of nude mouse phenotype has been erroneously considered the DiGeorge syndrome, which occurs spontaneously and is mainly characterized by thymic hypoplasia or aplasia. However, several lines of evidence argue against the analogy between these two disorders. In fact, DiGeorge syndrome is often associated with neonatal tetany and major anomalies of great vessels. These defects are due to malformation of the parathyroid and heart, derived from a major embryologic defect in the third and fourth pharyngeal pouch from which the thymus primordium emerged. In addition, in this syndrome hairlessness is missing and gross abnormalities in skin annexa are not found. Children with DiGeorge syndrome also have lymphopenia, with a reduction of T-cells, that are poorly responsive to common mitogens.[18]

The discovery of the human phenotype completely equivalent to the nude mouse phenotype began with the identification of two sisters, whose clinical phenotype was characterized by congenital alopecia, eyebrows, eyelashes, nail dystrophy, as shown in Figure 1 and several T-cell immunodeficiencies, illustrated in Table 2.[2] The two patients were born from consanguineous parents who originated from a small community of South of Italy that may be considered geographically and genetically isolated, as below detailed. This led to consider the syndrome as inherited as an autosomal recessive disorder. The T-cell defect was characterized by a severe functional impairment, as shown by the lack of proliferative response to mitogens.

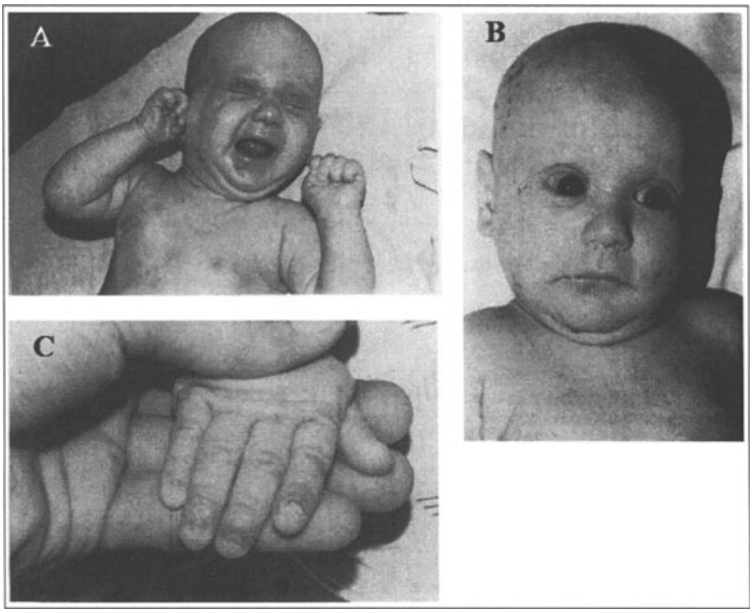

Figure 1. A,B) Alopecia of scalp, eyebrows and eyelashes in two sisters in whom the human Nude/SCID phenotype was first described. C) Nail dystrophy in human Nude/SCID. Reprinted with permission from: Pignata C, Fiore M, Guzzetta V et al. Congenital Alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. Am J Med Genet 1996; 65:167-170.

Alopecia and nail dystrophy are also found in other syndromes, such as dyskeratosis congenita (DC).[19,20] However, this novel syndrome profoundly differed from DC, in that major signs, such as abnormal pigmentation of the skin and mucosal leucoplakia, were lacking in the Nude/SCID. Moreover, the immunological abnormalities were different from those reported in patients with DC in either the severity of clinical course or type of alterations.[21,22] Both Nude/SCID patients showed alopecia at birth and in one sib it still persisted after a bone marrow transplantation, thus ruling out that it was secondary to an acquired skin damage. This finding suggested that the alopecia in this patient was primitive in nature.[2] Furthermore, these features were similar to those reported in athymic mice, that completely lack body hair and in which restoration of a thymus did not lead to hair growth.[11] Taken together, these observations suggested that the association between alopecia and the immunodeficiency reported in the two sisters were linked to a single gene defect.[2]

Due to the similarities between the human clinical features and the mouse Nude/SCID phenotype, a molecular analysis of the *FOXN1* gene was performed in these patients and revealed the presence of a C-to-T shift at 792 nucleotide position in the cDNA sequence. This mutation leads to a nonsense mutation R255X in exon 5, with a complete absence of a functional protein[23] similar to the previously described rat and mouse *Foxn1* mutations.[24-26] In *humans*, *FOXN1* is located on chromosome 17[23] and encodes a transcription factor mainly expressed in the epithelial cells of the skin and thymus, where it maintains the balance between growth and differentiation.

Since the first description of these Nude/SCID patients, other patients with a similar phenotype were identified. In particular, a Nude/SCID patient was diagnosed in Portugal. The newborn presented with alopecia and nail dystrophy associated with severe infections. The screening for R255X mutation of *FOXN1* gene revealed that the patient was homozygous for the mutation. It should be noted that the patient was born to consanguineous parents, both from Lisbon (communicated to European Society for Immunodeficiencies, 2006).

Table 2. Major clinical and immunological features of the first identified human Nude/SCID patients. For more details see reference 3.

	Patient 1	Patient 2
Clinical features		
Alopecia	+	+
Nail dystrophy	+	+
Growth failure	+	+
Omen-like syndrome	+	-
Severe interstitial pneumopathy	?	+
Immunological features		
Percentage of positive cells		
T-cells (CD3)	32	25
B-cells (CD19)	63	37
NK cells (CD56)	23	25
Proliferative response to mitogens	Absent	Absent
Serum immunoglobulins		
IgG (g/L)	4.94	6.10
IgA (g/L)	0.49	0.43
IgM (g/L)	0.80	1.25
IgE (KU/L)	N.T.	2760
Specific antibody response		
Tetanus toxoid	Absent	Absent
Allohemoagglutinins	Absent	Low
HbsAg	Absent	Absent
WHN mutation	R255X	R255X

Reprinted with permission from: Pignata C. A lesson to unraveling complex aspects of novel immunodeficiencies from the human equivalent of the nude/SCID phenotype. J Hematother Stem Cell Res 2002; 11:409-414.

In the village where the patients originated, additional patients of previous generations were affected with congenital alopecia and died early in childhood because of severe infections.[27]

A population study aimed to identify an ancestral founder effect for this phenotype was conducted in the village and in particular a genetic screening for the presence of the R255X mutation was performed. The study led to identify 55 subjects, corresponding to 6.52% of the studied population, who carried the mutation in heterozygosity.[27] The identification of the haplotype for the *FOXN1* locus, by analysing 47 chromosomes carrying the mutation R255X, led to identify the single ancestral event that underlies the human Nude/SCID phenotype. All the affected cases belonged to an extended seven-generational-pedigree, founded by a single ancestral couple born at the beginning of the 19th century from which four family groups originated. The pedigree analysis revealed that 33.3% of heterozygotes inherited the mutant allele from their mother, whereas 66.7% from their father.

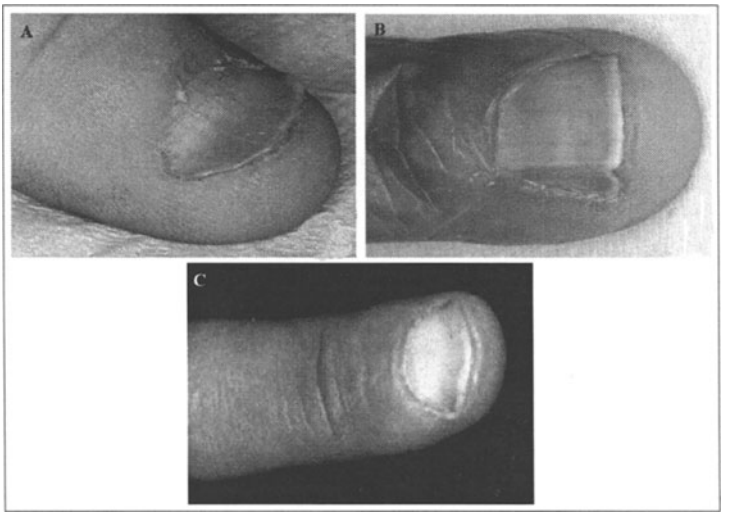

Figure 2. Nail dystrophy patterns in subjects heterozygous for the *FOXN1* mutation: A) koilonychia; B) canaliform dystrophy; and C) leukonychia. Reprinted with permission from: Auricchio L, Adriani M, Frank J et al. Nail distrophy associated with a heterozygous mutation of the Nude/SCID human *FOXN1* (*WHN*) gene. Arch Dermatol 2005; 141:647-648; ©2005 American Medical Association. All rights reserved.

Moreover, this pedigree was also characterized by a high rate of consanguineous matings, typical of a small community. In fact, 14 of 151 marriages were between consanguineous subjects.[27]

Subsequently, the identified heterozygous subjects were examined with a particular regard to ectodermal alterations, namely of hair and nails, in order to define whether the heterozygosity was associated with mild clinical signs. The examination revealed no association between gross alteration of the hair and heterozygosity, while 39 of the 55 heterozygous subjects showed a nail dystrophy.[28] Of note, this alteration was not found in other control subjects and was not related to an acquired form of nail dystrophy. The most frequent phenotypic alteration affecting the nails was koilonychia ("spoon nail"), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself. Less frequently, a canaliform dystrophy and a transverse groove of the nail plate (Beau line) was also observed (Fig. 2).[28] However, the most specific phenotypic alteration was leukonychia, characterized by a typical arciform pattern resembled to a half moon and involving the proximal part of the nail plate. These alterations of digits and nails were also reported in a few strains of nude mice.[15] *FOXN1* is known to be selectively expressed in the nail matrix where the nail plate originates, thus confirming that this transcription factor is involved in the maturation process of nails and suggesting nail dystrophy as an indicative sign of heterozygosity for this molecular alteration.[28]

As this form of SCID is severe due to the absence of the thymus and the blockage of T-cell development, a screening program for prenatal diagnosis in this population was conducted for the identification of fetuses carrying the mutation. The genetic counselling offered to couples at risk led to identify two affected female fetuses during the first trimester of pregnancy, thus indicating the importance of this effort. Both fetuses were homozygous for the R255X mutation and the autoptical examination revealed the absence of the thymus and a grossly abnormal skin which was tighter than usual and which showed basal hyperplasia and dysmaturity, suggestive of an impaired differentiation program. Of note, one of the two fetuses also showed multiple-site neural tube defects, including anencephaly and spina bifida that could explain the high rate of mortality in utero observed in the described population. Intriguingly, the other forms of SCID become clinically evident in postnatal life, when the protection of the newborn by the mother immune system declines. In the community where the Italian patients originated, a high rate of prenatal mortality was observed. Moreover, there

was an evidence that the mouse *Foxn1* gene is also expressed in epithelial cells of the developing choroids plexus, a structure filling the lateral, third and fourth ventricles of the embryonic brain.[29] Even though no formal demonstration is available, a possible explanation for the prenatal mortality could be that *FOXN1* genetical alteration is also implicated in more severe development defects at least in the conditions of highest clinical expressivity. This could also explained the surprising long interval of time that elapsed between the description of mouse and human diseases.

Fox Family Members and Immune System

The forkhead box (FOX) gene family comprises a diverse group of 'winged helix' transcription factors that are involved in development, metabolism, cancer and aging. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions.[30] They were first recognized in *Drosophila*, but later they were also identified in other organisms, from *yeasts* to *humans*. The term FOX is now used to refer to all chordate forkhead transcription factors. A phylogenetic analysis led to classify all known FOX proteins in at least 15 subfamilies (named from A to Q)[31] on the basis of their structure; in each subfamily (or class), an individual gene is identified by a number. The crystal structure of the forkhead DNA binding domain is a 'winged helix' motif, consisting of three α helices flanked by two 'wings' of β strands and loops.[32] The structure and the amino acids sequence are highly conserved within species and family members.

The functional effect of all FOX proteins can be either the activation (transactivation) or the inhibition of gene transcription[33] in a wide range of context. *Fox* gene mutations can be associated with diverse phenotypes as cranio-pharyngeal developmental defect (*FOXE1*), speech and language abnormalities (*Foxp2*) and hearing loss (*Foxj1*).[34] Moreover, most of these winged helix proteins play crucial roles in several aspects of immune regulation. In particular, genetic alterations of at least four FOX family members, FOXP3, FOXJ1, members of the FOXO subfamily and FOXN1, result in paradigmatic immune disorders and well-defined novel clinical entities.[33]

FOXP3 (scurfin, sf, JM2) is the most studied forkhead family member in immunology, because of its role in the pathogenesis of autoimmunity associated with immunological functional disorders.[35-37] FOXP3 was found to be expressed in $CD4^+$ $CD25^+$ regulatory T-cells (Treg), that represent a subset of $CD4^+$ T-cells bearing high levels of CD25 (the IL-2 receptor α-chain), whose role is to maintain self-tolerance by downregulating the reactivity of conventional $CD25^-$ $CD4^+$ helper T-cells.[35,38] *Foxp3* is also expressed in lymphoid organs, such as spleen and thymus, where it plays an essential role during development, allowing the differentiation of the Treg population. The study of scurfy mice (mice with X-linked recessive mutation in *Foxp3*) revealed an overproliferation of activated $CD4^+$ T-cells, resulting in dysregulation of lymphocyte activity.[39,40] The lack of DNA binding domain of the protein leads to death of hemizygous males at 16-25 days after birth[39,40] and in the surviving mice in a great exacerbation of the autoimmune phenotype.[41]

The corresponding human disorder is represented by immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX; also known as X-linked autoimmunity and allergic dysregulation syndrome, XLAAD). This fatal recessive disorder is due to truncated protein or inhibition of DNA binding domain. It develops in early childhood and is associated with protracted diarrhoea, thyroiditis, dermatitis, allergic manifestations, insulin-dependent-type 1 diabetes and anaemia, besides massive T-cell infiltration into the skin and gastrointestinal tract and high serum levels of autoantibodies, as a sign of autoreaction.

Recent studies have shown that the expression of FOXP3 and the subsequent conversion of human and mouse peripheral naïve T-cells in Treg is induced by transforming growth factor-β (TGF-β).[42,43] Most probably, this event is mediated by activation of small mothers against decapentaplegic (SMAD) transcription factors. Generally, the inhibition of TGF-β-mediated signaling involves SMAD7 in an autoregulatory loop, but it was also shown that FOXP3 can inhibit it, as well. The induction of *FOXP3* expression by Treg results in a prolongation of TGF-β-mediated signaling, perhaps allowing the stabilization or expansion of the Treg pool.[33]

FOXOs are the mammalian homologues of the *Caenorhabditis elegans* dauer formation mutant 16 (DAF-16) and, in this organism, they seem to be involved in longevity regulation. 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] are the most studied members of this family for their implication in the regulation of apoptosis, cell cycle, metabolism and resistance to oxidative stress.[45-47] Gene targeting experiments in mice have demonstrated that FOXO1 regulates insulin sensitivity,[48,49] adipocyte differentiation[49] and angiogenesis,[50] while FOXO3A regulates ovarian development and fertility[51-53] and FOXO4 appears to be largely dispensable for gross organismal homeostasis.[52] FOXO proteins are ubiquitously expressed, even if there is a tissue specific expression for the diverse isoforms. While FOXO1 is ubiquitous, FOXO3A is expressed in lymphocytes and it appears the dominant isoform of the mammalian family. Cellular stimulation by mitogens or cellular stress, leads to activation of several intracellular kinases such as phosphatidyl inositol 3 kinase (PI3K), serum/glucocorticoid-regulated kinase (SGK) and protein kinase B (PKB, Akt), resulting in the phosphorylation of the FOXOs. This makes FOXO unable to bind DNA and renders it susceptible to 14-3-3-mediated nuclear export[54-59] and/or proteasome mediated degradation (IkB kinase (IKK))[60,61] thereby preventing FOXO-mediated transcription. In resting cells, unphosphorylated forms of the FOXOs are localized in the nucleus, where they are transcriptionally active and regulate several biologic processes, including proliferation, apoptosis and response to cellular stress.

To date, there is no evidence that an alterated FOXO activity is associated with a human immunological disease. However, in mice a significant diminished FOXO activity in T-cells is associated with autoimmune lupus syndrome, thus leading to hypothesize a possible relationship between the *FOXO* genes and inflammation in *humans*.[62] Differently, *FOXO* gene dysregulation has been well-documented in human cancer.

The FOXJ1 (hepatocyte nuclear factor/forkhead homolog-4, HNF-4, FKHL-13) transcription factor plays an important role in the development of ciliated epithelia.[63-66] Thus, FOXJ1 is expressed in all structures containing ciliated cells, such as the lungs, spermatids, oviducts and choroid plexus.[67] The loss of FOXJ1 results in lethality in utero or soon after birth as demonstrated by observations of *Foxj1*$^{-/-}$ mice that die during embryonic development.[63,65] In fact, *Foxj1*-deficient mice are characterized by absence of cilia and, subsequently, suffer from significant developmental abnormalities including heterotaxy and hydrocephalus.[33] Besides its role in the differentiation of ciliated cells, recently a new role for FOXJ1 has been discovered in the differentiation of other cell types. It was observed that *Foxj1* is downregulated in lymphocytes isolated from mice affected with systemic lupus erythematosus (SLE); this evidence suggests that FOXJ1 might prevent autoimmune reactions.[68]

FOXJ1 is expressed in naïve T-cells and its downregulation occurs after interleukin-2 (IL-2) and/or T-cell receptor (TCR) stimulation.[68] FOXJ1, similarly to FOXO3A, is required in vivo to modulate NF-κB activity, upregulating IkBβ and maintains T-cell tolerance, but unlike FOXO3A deficiency, FOXJ1 deficiency is much more severe, affecting a different spectrum of organs and Th1 cytokine production. Thus, these two forkhead members play either overlapping or clearly distinct roles in helper T-cells, even though the intimate mechanisms remain to be elucidated.[69]

Foxn1, a highly conserved transcription factor, has been previously extensively mentioned. It exerts its function after activation through phosphorylation, that promotes its nuclear translocation.[54-56] Into the nuclei it interacts with DNA as a monomer through its forkhead box, but the target genes and the specific biochemical mechanism of interaction with the promoter regions remain to be elucidated.[32,70] FOXN1 expression is strongly regulated by wingless (Wnt) proteins[71] and bone morphogenetic proteins (BMPs)[72] in both autocrine and paracrine fashions[69] and its expression is restricted to epithelial cells in the skin[14] and in the thymus. There are no data available on FOXN1 mRNA expression in liver, spleen, testis, lung, heart and brain, but murine choroid plexus.[29] During embryogenesis, FOXN1 is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney and urinary tract. In adult life, its expression is limited to epithelial cells of the intestine, spermatocytes of the testis and thymus.[73] In particular, on the basis of the observation that nude mice keratinocytes do not differentiate in a normal fashion, FOXN1 could be considered as a key regulator of the balance between keratinocytes growth

and differentiation. It suppresses the involucrin and locrin expression, both components of the cornified envelope and the profilaggrin, involved in the aggregation of the intermediate filaments. Other factors have been identified as Foxn1 target. In fact, recent studies have shown that Foxn1 is linked to Akt (PKB) expression,[74] thus giving a possible explanation of FOXN1 involvement in epidermal layer regulation. FOXN1 also controls follicular formation, influencing the expression of two hair keratins, mHa3 and mHb5.[75]

Moreover, FOXN1 transcription factor regulates thymus epithelial cells differentiation. Null mutation of this protein led to an immature thymus, but the molecular mechanism used by FOXN1 in this context remains still unclear.

The human and mouse clinical phenotype associated with FOXN1 genetical alteration has been extensively described above.

FOXN1 Skin Specific Expression and T-Cell Development

So far, the thymic tissue has been considered the only organ with a unique capacity to support the generation of a functional population of human mature T-cells, thus expressing a diverse repertoire of antigen receptors.[76,77] In particular, within the mature and functional thymus, mature T-lymphocytes derive from the interaction between the thymic epithelial cells, that are the main component of the stroma and the T-cell precursors originated in the bone marrow.[78,79] Thymic epithelial cells are implicated in either thymus organogenesis or in most stages of maturation of thymocytes.[78,79] The absence of FOXN1, as in Nude/SCID phenotype, results, as previously extensively mentioned, in a thymus anlage that lacks the capacity to interact with the hematopoietic progenitor cells, thus precluding the maturation of thymocytes[80,81] and leading to the immunodeficiency.[16]

FOXN1 gene spans about 30 kilo bases (kb)[5] and it is composed of nine exons.[70] Interesting, an extensive screening of cDNA clones obtained from skin cells revealed the presence of two different first exons which are noncoding,[70] the exons 1a and 1b, that undergo to alternative splicing to either of two splice acceptor sites of the exon 2, located upstream of the initiation codon. This suggests the presence of two distinct promoters of exons 1a and 1b.[5] The alternative usage of the exon 1a or 1b seems to be tissue specific,[70] in that promoter 1a is active in thymus and skin, while promoter 1b is active only in skin.

In the interfollicular epidermis, FOXN1 expression parallels the onset of terminal differentiation. It is primarily expressed in the first suprabasal layer that contains keratinocytes in the early stages of differentiation, that have left the cell cycle and initiated terminal differentiation.[15] In the hair follicle, FOXN1 expression is restricted to a specific compartment, the supramatrical region,[15] where the cells stop to proliferate and begin terminal differentiation.[82] On the basis of these observations, FOXN1 could be considered a marker of transition from proliferation to a postmitotic state and an important regulator of the initiation of terminal differentiation.[5]

Of note, significant expression levels of FOXN1 were found in cultures containing skin cells along with hematopoietic precursor cells (HPCs), suggesting a role of human skin in supporting a full process of human T-cell development.[83] Although thymus and skin are different in their three-dimensional structure, experiments performed with keratinocytes and fibroblasts of the skin and HPCs obtained from bone marrow, reconfigured in a different three-dimensional arrangement, demonstrated the capacity of this "surrogate" organ to generate mature and functional T-cells from precursors.[83] Of note, these cells show the same characteristics of recent thymic emigrants such as the T-cell surface markers, including the CD3/TCR complex[84] and the TCR rearrangement excision circles (TRECs), derived from the recombination of TCR genes. These cells also possess a diverse TCR repertoire and can be considered mature and functional because they have full capacity to proliferate, express the activation antigen CD69 and produce cytokines in response to TCR/CD3 stimulation.[83] Thus, it is conceivable that skin and bone marrow derived cells can be potentially used to generate de novo mature, functional, diverse and self-tolerant T-cells. These data would imply their potential future therapeutic usage in patients with immunological disorders.[83]

The present chapter contains information of the recent works that came out from the original description of Nude/SCID phenotype. For the first time, only recently, a careful description of

clinical manifestations associated with an alteration of the *FOXN1* gene has been provided, thus leading to identify the human equivalent of the well-studied spontaneous murine Nude/SCID immunodeficiency. In this context, alterations of FOXN1 and of other members of FOX subfamilies are now emerging as intriguing causes of immunological disorders mainly characterized by abnormal T-cell development or abnormal T-cell regulatory homeostasis.

Eventually, it should be underlined that the Nude/SCID phenotype is the only form of SCID associated with an alteration of a gene that is not expressed in the hematopoietic cell.

Novel knowledge in this field would be very helpful in the comprehension of the intimate mechanisms underlying T-cell ontogeny process in *humans* and in discovering novel clinical entities related to abnormalities of the process.

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CHAPTER 16

FOXL2:
At the Crossroads of Female Sex Determination and Ovarian Function

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Abstract

The gene *FOXL2* encodes a forkhead transcription factor whose mutations are responsible for the blepharophimosis ptosis epicanthus-inversus syndrome. This genetic disorder is characterized by eyelid and mild craniofacial abnormalities often in association with premature ovarian failure. FOXL2 orthologs are found throughout the animal phylum and its sequence is highly conserved in vertebrates. FOXL2 is one of the earliest ovarian markers and it offers, along with its targets, a model to study ovarian development and function. In this chapter, we review recent data concerning its mutations, targets, regulation and functions. Studies of the cellular consequences of FOXL2 mutations seem to indicate that aggregation is a common pathogenic mechanism. However, no reliable genotype/phenotype correlation has been established to predict the exact impact of point mutations in the coding region of *FOXL2*. FOXL2 has been suggested to be involved in the regulation of cholesterol homeostasis, steroid metabolism, apoptosis, reactive oxygen species detoxification and inflammation processes. Interestingly, all these processes are not equally affected by *FOXL2* mutations. The elucidation of the impact of the FOXL2 function in the ovary will allow a better understanding of normal ovarian development and function as well as the pathogenic mechanisms underlying BPES.

Introduction

The gene *FOXL2,* previously known as *PFrk,* encodes a transcription factor (MIM 605597), whose mutations are responsible for the blepharophimosis ptosis epicanthus-inversus syndrome (BPES; MIM 110100). BPES is a rare genetic disease, mainly characterized by severe eyelid malformations. Patients present with small palpebral fissures, epicanthus-inversus (fold curving in the mediolateral direction, inferior to the inner canthus), ptosis of the eyelids and a flat nasal bridge.[1] Vignes (1889) was probably the first to describe this entity, as an eyelid dysplasia.[2] In 1976, Moraine and collaborators suggested that female infertility was a pleiotropic effect of mutations in the same locus.[3] This link between BPES and female infertility was confirmed afterwards, with a report on a family in which all affected females had Premature Ovarian Failure (POF).[4] More recently, Zlotogora and collaborators defined 2 forms of BPES: Type I (with POF) and Type II (isolated BPES, without POF).[5] BPES was long considered as an exclusively autosomal dominant disease, but a case of recessive BPES in a large consanguineous Indian family has recently been described.[6]

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FOXL2 is a member of the superfamily of winged helix/Forkhead transcription factors and contains a characteristic conserved DNA-binding domain, the Forkhead domain, spanning about 110-amino acids.[7] Forkhead transcription factors are involved in many different developmental and metabolic processes and their mutations are responsible for several human diseases.[8-10] FOXL2 also contains a polyAlanine tract (polyAla), whose precise role (if any) still remains elusive. However, length variations of the polyAla domain have been associated with the BPES phenotype.[6,11] FoxL2 has been shown to localize to the nucleus of cells where it is expressed.[12] This is compatible with the fact that two distinct Nuclear Localization Sequences (NLS) have been mapped to the C-terminus of its Forkhead Domain.[13]

FoxL2 Protein Sequence Is Highly Conserved

FOXL2 orthologs are found quite widely throughout the animal phylum and more particularly in chordates. Indeed, FoxL2 orthologs have been confirmed in mammals, birds, teleostean fishes (i.e., rainbow trout, tilapia, medaka), chondrichtyans (i.e., dogfish), amphibians (i.e., African clawed frog, Japanese wrinkled frog), reptiles (i.e., turtle) and even in the Urochordate Ciona intestinalis (NCBI ID: AB210441).[7,14-20] In all the species where FoxL2 function has been investigated, it has been found to be involved to some extent in ovarian determination/differentiation.

The ratio dN/dS (where dS is the rate of synonymous/silent substitutions. and dN the rate of nonsynonymous substitutions between pairs of homologous sequences), gives a good indication of the selective pressure at the protein level.[21] Such an analysis has been performed for FoxL2 proteins and all pairwise comparisons involving mammalian sequences result in low dN/dS ratios, indicating a strong purifying selection.[22] Interestingly, the multiple alignment of FoxL2 protein sequences shows that homopolymeric runs of amino acids (polyAla, polyPro, or PolyGly) are not conserved among species, in contrast with the strong conservation of the rest of the protein sequence.[22] Indeed, in all eutherian mammals whose sequences are available, the length of the polyAlanine region is strictly conserved (i.e., 14 residues), whereas in fishes and in the chicken, this domain is completely absent. This suggests that, whereas the polyAla tract length in eutherian mammals is under strong evolutive constraints limiting its length, it is a dispensable element in other species (see discussion below).

The N-terminal region of FOXL2 is less conserved than the C-terminal region. Thus, it might be responsible for functional differences among species, or could have a less important role in FOXL2 function. On the contrary, the high conservation of the C-terminal region indicates that it should contain functionally relevant domains, yet to be mapped.

Expression of FOXL2

The pattern of expression of FoxL2 has been studied at the mRNA and/or protein levels in several species. In human, mouse and goat, FOXL2 has mainly been detected in the developing eyelids as well as in fetal and adult ovaries, in agreement with the BPES phenotype.[7,12,23] Interestingly, FoxL2 expression at the protein level is always absent in testes at any stage of development analyzed thus far.

In human developing eyelids, FOXL2 is expressed in a wide region of the primordial mesenchyma. Consistently, atrophy or hypotrophy of the eyelid superior levator muscle in BPES patients was found through magnetic resonance imaging (MRI).[24] This led the authors to suggest that FOXL2 could be involved in the development of this muscle. However, the much wider expression domain of the protein in the peri-ocular region, in the primordial mesenchyma, could also suggest a role in the development of other tissues.[12]

In mammals, FOXL2 ovarian expression begins early in development during the period of ovarian determination, well before the onset of folliculogenesis and is maintained until adulthood. The expression of FOXL2 seems restricted to the somatic compartment, with granulosa cells displaying a strong protein expression and the stromal cells a more diffuse one.[12,25] However, staining of mouse stromal cells is barely detectable, in line with RNA in situ hybridization results.[7] Interestingly, *Foxl2* mRNA has been observed in both granulosa cells and some oocytes of fetal

and adult mouse ovaries.[15] In the chicken, FoxL2 protein is expressed in a sex-dimorphic manner and is mainly found in granulosa cells and at lower levels in aromatase-positive thecal cells.[16] In the turtle, a species whose sex determination is temperature-dependent, *FoxL2* is more strongly expressed in the developing gonads at female-promoting temperatures.[15] Interestingly, weak levels of *Foxl2* mRNA have been detected in goat, mouse and chicken testis, but the proteins have been undetectable in those tissues.[12,16,23,25] Either the levels of mRNA are too weak to be relevant, or FoxL2 expression could undergo some kind of negative translational regulation. This may also explain the presence of *Foxl2* transcript in some mouse oocytes and in chicken oocytes, whereas the protein is undetectable.[16] FoxL2 is one of the earliest known sex-dimorphic marker of ovarian determination/differentiation. The conservation of its sequence and expression pattern suggests that it should be a key gene in the early development and in the maintenance of the ovary.

FoxL2 expression is not restricted to eyelids and gonads. Indeed, *Foxl2* is expressed ventrally in the developing pituitary (in the invaginating Rathke's pouch) and is expected to participate in its organogenesis.[26,27] *Foxl2* expression is maintained in the adult pituitary gland, where it is found essentially in gonadotrope and thyrotrope cells as well as in some prolactin-containing cells during pregnancy.[28]

Even if FoxL2 expression pattern has not been characterized extensively outside of the craniofacial and gonadal regions, RNA expression studies suggest its expression pattern may be wider. Indeed, an exploration of the Gene expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/sites/entrez?db = geo), suggests that FoxL2 is expressed in the heart (GDS2614), in macrophages (GDS2686; GDS2041), in circulating blood reticulocytes (GDS2655), in the colon (GDS756; GDS3226; GDS1780), in hepatocytes (GDS1729; GDS2766; GDS2239), in bronchial muscle cells (GDS2628), etc. The relevance of FoxL2 expression in these cells/tissues remains to be investigated.

Disease-Causing *FOXL2* Mutations in Humans

The mutations identified in the *FOXL2* locus affect either the structure of the protein, its function (intragenic mutations) or its expression/regulation (extragenic mutations). Such mutations has been found in 83% of BPES patients (http://medgen.igent.be/foxl2/).[29] BPES is found both as sporadic and familial cases.

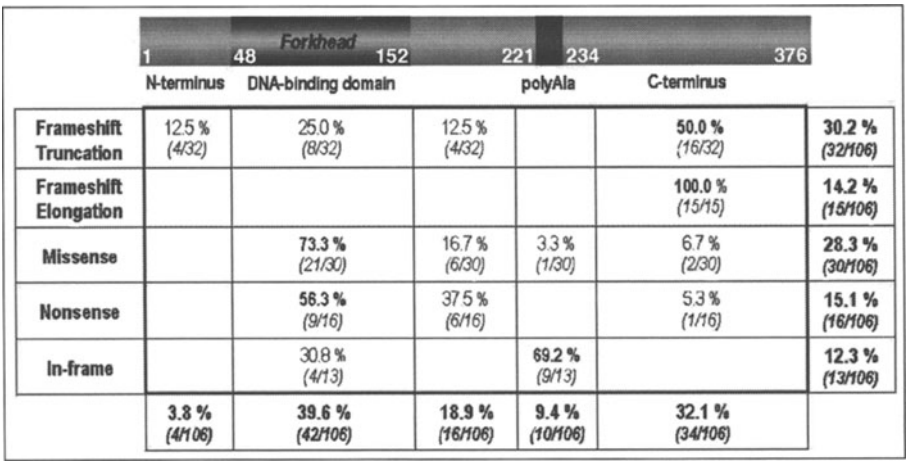
Intragenic FOXL2 *Mutations*

Intragenic mutations represent 70% of identified FOXL2 mutations. They can be sorted in any of 4 types: (i) Missense mutations, (ii) Nonsense mutations or early-stop codon- inducing Frameshift mutations, leading to the synthesis of a truncated protein, (iii) Frameshift mutations leading to elongated proteins and (iv) polyAlanine expansions. A detailed analysis of FOXL2 mutations as a function of its domains can be found in Figure 1.

Missense Mutations

The analysis of missense mutations associated with BPES indicates that most of them lie within the Forkhead domain. As expected, the consequences of the mutations are dependent on the affected amino acid. Functional assays of missense mutations in vitro (cultured cell) indicate that most of them can induce nuclear and cytoplasmic aggregation of FOXL2, along with altered transcriptional activity.[30,31]

A structural 3D-model of FOXL2 Forkhead domain was established using the FoxP2 crystal structure as a model in order to identify interactions (protein/protein or protein/DNA) which were compromised by known amino-acid mutations.[31] As expected, the majority of mutations present in the third helix of the Forkhead domain, such as R103C, H104R or N105S, were proposed to alter DNA-binding, whereas mutations in the first or second helices, such as I63T, A66V or E69L, were suggested to interfere with protein/protein interactions.[31] Nevertheless this distinction is not absolute, as mutations in the second helix might also interfere with DNA-binding (L77P) and mutations in the third helix could alter protein/protein interactions (W98R, L106F).[31] Interestingly, the residues R103, H104 and N109, which were found mutated in BPES patients, were predicted



Frameshift Truncation	12.5 % (4/32)	25.0 % (8/32)	12.5 % (4/32)		**50.0 %** (16/32)	**30.2 %** (32/106)
Frameshift Elongation					**100.0 %** (15/15)	**14.2 %** (15/106)
Missense		**73.3 %** (21/30)	16.7 % (6/30)	3.3 % (1/30)	6.7 % (2/30)	**28.3 %** (30/106)
Nonsense		**56.3 %** (9/16)	37.5 % (6/16)		5.3 % (1/16)	**15.1 %** (16/106)
In-frame		30.8 % (4/13)		**69.2 %** (9/13)		**12.3 %** (13/106)
	3.8 % (4/106)	**39.6 %** (42/106)	**18.9 %** (16/106)	**9.4 %** (10/106)	**32.1 %** (34/106)	

Figure 1. Schematic representation of FOXL2 and analysis of 106 different described mutations according to their types and their localization. Domains of FOXL2 are drawn to scale. Amino acids positions of FOXL2 domains are written in white. Mutations are analyzed according to their type (frameshift truncation or elongation, missense, nonsense and in-frame mutations), values are given in percentage and in brackets as absolutes. Values represent the numbers of a particular type of mutation in each domain of FOXL2. In bold, percentage value for the main domain where each type of mutation occurs. The last line of the table gives the number of mutations for each domain of FOXL2 and the last column the number of each type of mutations among all studied FOXL2 mutations.

to mediate at least in part the interaction of the Forkhead with its target DNA sequence, by analogy with conserved interactions previously demonstrated for other Forkhead factors.[32] Thus far, it is difficult to predict the type of BPES induced by *FOXL2* missense mutations. Interestingly, a missense mutation in the ORF of *FOXL2,* leading to the amino-acid substitution Y258N, was identified in a nonsyndromic POF patient.[33]

Nonsense or Early Stop Codon-Inducing Frameshift Mutations

The phenotypic consequences of these mutations are more or less severe depending on the position of the mutation. If the truncation occurs before the Forkhead DNA-binding domain, the mutation is likely to lead to a loss of function and therefore to complete haploinsufficiency. This is the case for the BPES Type I-associated mutation Q53X,[34] which was shown to lead to the absence of FOXL2 synthesis from this allele in COS-7 cells.[35] Stop mutations within the Forkhead domain should lead to the synthesis of inactive proteins and have also been described in association with BPES, as for instance Y83X, W98X and Q99X.[24,36,37] *FOXL2* mutated alleles leading to the synthesis of a truncated protein with a complete Forkhead domain, but lacking the polyAlanine tract have also been described, such as F167X, G196X or S203X.[7,37,38] They might compete with the normal protein for DNA binding. However, our preliminary evidence suggest this potential competition does not lead to a dominant negative effect. Interestingly, mutations leading to truncated FOXL2 without a polyAlanine domain always induce Type I BPES.[11,36] Mutations leading to the synthesis of FOXL2 proteins truncated after the polyAlanine domain (lacking the C-terminus), such as A253fs or Y274X, have also been reported.[11,31] Interestingly, the Y274X mutation can lead to both types of BPES (i.e., with or without ovarian dysfunction).[11] This indicates that the polyAlanine domain and, to a lesser extent, the very C-terminus of FOXL2, have an important role in the function of FOXL2 in the ovary. The phenotypic variability associated with FOXL2 mutations also suggests the existence of other sources of genetic variability that can modulate the severity/impact of FOXL2 mutations, such as polymorphisms in target gene promoters or protein partners.

Frameshift Mutations Leading to Elongated Proteins

This kind of mutations in the *FOXL2* ORF is supposed to induce the synthesis of (mostly) aberrant proteins. For instance, the mutation L376fs (FOXL2 mutation database identifier: FOXL2_00240), leading to an out of frame extension, has been associated with BPES of an unknown type. Again, the position of mutation in the ORF is crucial, as the closer the mutation is to the initiating ATG, the more severe the loss of function is predicted to be. Not only this kind of aberrant proteins cannot assume their function, but also their presence could be toxic. The molecular consequences of this type of mutations inducing protein elongation are still unknown.

PolyAlanine Length Variations

Mutations leading to expanded polyAlanine domains in FOXL2 are found in 30% of BPES patients.[11,36] The most frequent expansion leads to a +10 Alanine residues in the polyAlanine domain (FOXL2-Ala24) and is found most often in Type II BPES patients (without ovarian dysfunction).[7,36] A +12 expansion (Ala26) has been described in a Type I BPES patient with secondary amenorrhea and an enormous ovarian cyst.[39] Interestingly, a +5 expansion (Ala19) was also recently described in association with BPES.[6] Whereas the Ala24 and Ala26 alleles are associated with a classic dominant transmission of BPES, the Ala19 variant was found to induce a BPES Type I phenotype only when at a homozygous state and following a recessive transmission pattern in an Indian consanguine family.[6] Moreover, a deletion of 10 Alanines of the polyAlanine domain (Ala4) has been identified in a patient presenting with isolated POF.[33,40]

The effects polyAlanine length alterations on its subcellular localization and to a lesser extent on its function as a transcription factor, has been assessed in cellular models. Interestingly, the frequent FOXL2-Ala24 mutant induces strong cytoplasmic delocalization, as well as nuclear and cytoplasmic aggregation and is able to interfere with the availability of the synexpressed wild-type protein.[41] Indeed, a potential promoter-specific dominant-negative effect of the FOXL2-Ala24 protein on the WT protein has recently been reported.[32] Moumné and collaborators have generated and studied the properties of an allelic series of FOXL2 variants presenting with increasingly expanded polyAlanine domains, including the naturally-occuring Ala19 and Ala24.[42] They have shown that the longer the polyAlanine domain of FOXL2, the more it is delocalized to the cytoplasm and that its solubility and transactivation ability decreases with the length of its polyAlanine.[42] This length-dependent loss of transactivation has been shown to be more or less severe for each variant depending on the number of FOXL2 binding sites and of their affinity in the target promoters.[32,42] The Ala24 variant seems to retain a partial transactivation ability on high-affinity promoters, which was suggested to be the explanation why it is most often associated with Type II BPES.[32,42] Interestingly, the complete deletion of FOXL2 polyAlanine domain (Ala0) seems to induce nuclear aggregation in 20% of cells when expressed in COS-7 cells.[35] This suggests that contractions of the polyAlanine domain, such as was found in the isolated POF patient described by Harris and collaborators in 2002,[33] could also somehow compromise protein solubility and FOXL2 interaction with its partners. Considering the high conservation of the length of FOXL2 polyAlanine in mammals and the isolated POF phenotype induced by its contraction, we can surmise that the polyAlanine domain could be essential for FOXL2 function in the ovary.[22,33] As shown above, poly-Ala tracts display a threshold length beyond which deleterious effects appear. Thus, polyAla runs might serve a general function, such as species-dependent regulation of the intranuclear concentrations of active factor by tuning the equilibrium between inactive/aggregated and active forms.[22,43]

The variety of mutations associated with BPES or isolated POF in the ORF of *FOXL2* indicates that its structure, localization and function are very sensitive to even slight alterations of the protein sequence.

Chromosomal Rearrangement and Extragenic Mutations Leading to BPES

Chromosomal rearrangements represent 16% of molecular defects found in BPES patients. Partial or total deletion of the *FOXL2* ORF conducing to the total loss of function of FOXL2 has

been observed.[44] Moreover, a minimal region of 126kb mapping at around 230kb upstream of the *FOXL2* transcription unit (the Shortest Region of Overlap; SRO) has been delimited through the comparative analysis of deletions leading to BPES.[44] Interestingly, these deletions always lead to Type II BPES, which suggests that this region could mostly regulate palpebral expression. Interestingly, balanced translocations at 170kb upstream to *FOXL2*, with a chromosomal breakpoint in the same region than the deletions, have also been identified in association with BPES, presumably inducing altered FOXL2 expression through the disruption of long-range regulatory elements or owing to position effects.[7,36,45,46] The regulatory implications of these chromosomal rearrangements outside of the FOXL2 transcription unit and the conserved mechanisms they suggest, will be discussed in details afterwards.

A Genotype/Phenotype Correlation?

Type I BPES is supposed to stem from loss of function mutations, while Type II BPES is supposed to be the consequence of hypomorphy in the ovary (partial function, sufficient for correct ovarian function, but not for palpebral morphogenesis). The first studies of *FOXL2* mutations, performed in 2001, reported a genotype-phenotype correlation.[36] Indeed, it seemed that a mutation leading the production of a truncated protein led to a Type I BPES (with a premature ovarian failure), whereas a mutation leading to the production of an elongated protein led to a Type II BPES (without ovarian dysfunction). However, predictions cannot be made for missense mutations in the Forkhead domain, as some lead to Type II BPES, such as N109K or N105S, while others lead to Type I BPES, such as I80T or I84S. Moreover, exceptions to the genotype/phenotype correlation have been found for nonsense mutants (such as Y274X, see before), with the same mutation leading to both types of BPES in different family members (or in different families). Therefore, it seems very difficult to predict the type of BPES in a child with palpebral defects just from her genotype. Indeed, nowadays, the diagnostic of the type of BPES is made a posteriori based on the presence or not of POF. Furthermore, POF in itself is a rather frequent condition (1-3%) and can have multiple origins, such as genetic, environmental or iatrogenic, which could explain some of the observed phenotypic variability. It would therefore be most valuable to obtain a diagnostic tool to diagnose the potential BPES type from only the detected mutation.

Learning from Mice Models

In 2004, two independent *Foxl2* knock-out (KO) mice models were developed.[47,48] Interestingly, neither of these two KO models present with a striking phenotype at the heterozygous state. This is in line with the fact that mice are less sensitive to gene-dosage effects.

During the first week following birth, homozygous KO mice display more than 50% perinatal lethality.[48] *Foxl2* KO mice are characterized by a small size (less than 85% of the wild type's size).[48] This was proposed to result from the reduction of 60% of plasmatic Igf1 (Insulin-Like Growth Factor 1) concentration, which could be linked to a pituitary defect.[48] Moreover, they display severe craniofacial abnormalities, including a severe eyelid hypoplasia and they are born with opened eyes, which is consistent with the BPES phenotype in humans.[48]

There are no defects in the testis development of male *Foxl2*$^{-/-}$ mice, which have a normal fertility.[47,48] XX *Foxl2*$^{-/-}$ mice are sterile, but phenotypically female.[47,48] Ovaries of KO mice are small, severely disorganized and primary follicles are not formed.[47,48] Surprisingly, the defect resulting in the absence of primary follicles differs a little between the two KO models. Schmidt and collaborators observe a normal formation of primordial follicles. However, in their model, the squamous to cuboidal morphological transition of granulosa cells does not occur (the cells stay 'flattened' and primary follicles do not form).[47] Uda and collaborators describe an earlier defect in the formation of the follicular pool. Indeed, their KO female mice do not even form correct primordial follicles, presumably because of a severe impairment of the granulosa-cell differentiation program.[48] Indeed, the main reported defect involves a faulty intercalation of oocyte clusters by pregranulosa cells (fragmentation of sex cords).[48] The differences between the onset of the follicular formation defects could be explained by the differences in the genetic backgrounds of the two *Foxl2* KO models (129S6/SvEvTac versus 129/BlackSwiss/CD1 backgrounds).[47,48]

Interestingly, germ cells of neither *Foxl2* KO mice models seem affected during the first stages of folliculogenesis.[47,48] Immediately after birth, the number of oocytes are similar between mutant and wild-type mice.[47,48] However, folliculogenesis is stopped by a massive follicular atresia, leading to a severe depletion of the follicular stock, to a significant decrease in the ovarian size and to premature infertility.[47,48] Interestingly, KO mice for another Forkhead transcription factor *Foxo3a*, also display a premature infertility phenotype, consequent to global follicular activation leading to oocyte death and early depletion of functional follicles.[49,50] Although the onset of infertility occurs later than in *Foxl2*$^{-/-}$ mice, these two Forkhead factors could indicate a potential cooperation in the regulation of female fertility.

Further analysis of *Foxl2* KO mice models has shown that their granulosa cells acquire Sertoli cell-specific characteristics, including a high expression of *Sox9*, *Amh*, as well as other genes of the testicular differentiation program.[51] This partial transdifferentiation occurs late in gonad formation, after oocyte determination and therefore is independent of oocyte loss.[51] In the absence of *Foxl2*, the genital tract remains morphologically female.[51] *Foxl2* surexpression in Sertoli cells of XY transgenic mice lead to a disorganization of seminiferous tubules and to the development of an ovotestis-like gonad.[52] All these observations argue in the favour of an 'anti-testis' action of *Foxl2*.

A late phenotypic reversion of granulosa cells to Sertoli cells is also observed in *Wnt4* KO mice.[53] *Wnt4*$^{-/-}$ XX mice have male genitalia and testes, but do not develop seminiferous tubules or express the testis differentiation gene *Sox9*.[53] Contrary to *Foxl2* KO XX mice, granulosa-to-Sertoli transdifferentiation only appears after oocyte loss.[53] Double-mutants *Wnt4*$^{-/-}$*Foxl2*$^{-/-}$ mice present a complete female-to-male sex-reversion phenotype, which suggests that these two genes and the signaling cascades they are part of, have a complementary role in ovarian formation and testis repression.[52]

The analysis of *Foxl2* KO models highlights the crucial role of *Foxl2* in ovarian development and differentiation, partly through granulosa cell differentiation and repression of the testis developmental program. However, maintenance of *Foxl2* expression in the ovary after ovarian development points towards a function of *Foxl2* in the ovary throughout female fertile life. Generating KO models inducible after ovarian development will be a powerful tool to study *Foxl2* function in the adult ovary.

Regulation of the Expression and Activity of FOXL2

In goats, the polled intersex syndrome (PIS) is characterized by hornlessness and XX recessive female-to-male sex-reversal.[54] The mutation underlying PIS has been mapped to a 11.7-kb deletion located in the goat 1q43 band, which is homologous to the human 3q23 band and whose DNA sequence contains mainly repetitive elements.[23] The PIS mutation has been shown to induce extinction of *FoxL2* expression, presumably through a cis-regulation effect, from about 200kb away from the transcriptional start site of *FoxL2*, suggesting the presence of a long-range regulation element of the locus.[23] Nongenic sequences deleted in the PIS mutation were found to be conserved between the goat, mouse and human locus.[44,45] Genetic evidence suggests that the long-range regulation of *FoxL2* expression by the PIS locus is somehow conserved in humans. Indeed, as mentioned above, breakpoints or deletions encompassing the PIS locus orthologous sequences in humans can induce a BPES, without direct disruption of the sequence of the *FOXL2* coding sequence (see before).[44,45] The disruption of *FoxL2* expression in PIS mutant goats and in patients carrying rearrangements or deletions of the orthologous region suggest that these nongenic sequences contain distant cis-elements affecting the global state of the chromatin around the *FOXL2* locus and imposing long-range regulation of FOXL2 expression.[23,44,45] The precise mechanistic details of this conserved long-range regulation of *FoxL2* in mammals remain to be explored.

FOXL2 expression levels are also regulated through a more direct modulation of the activity of its promoter. Indeed, it was recently shown through luciferase assay experiments that FOXL2 is able to up-regulate the activity of its own promoter, thus giving rise to a positive feedback loop.[42] This is consistent with the observation that transcription from the *Foxl2* promoter was compromised and decreased significantly to very low levels until 16 weeks of life in homozygous *Foxl2*^{lacZ} mutant ovaries.[47] This positive feedback loop could explain why, once activated, *FoxL2*

expression is maintained throughout life.[12] Recent evidence shows that this positive feedback can be counterbalanced by a negative feedback through the NAD-dependent deacetylase SIRT1 (details below).[55] In vitro transactivation assays indicate that the balance between the activation of these positive and negative feedback loops can be disturbed by pathogenic mutations, thus providing a potential molecular pathogenesis mechanism, through altered cellular dosage of FOXL2.[55] Interestingly, disruption of this negative feedback loop through supplementation of cultured cells with nicotinamide, the active form of Vitamin B3 and a noncompetitive inhibitor of SIRT1, was shown to upregulate FOXL2 expression/activity and this action was also seen in whole mice ovaries after intraperitoneal injections.[55] Since many BPES-causing mutations induce haploinsufficiency (see before), nicotinamide supplementation might be a promising therapeutic lead to treat the infertility associated with BPES.

Interestingly, yet another positive feedback regulation, though indirect, has been suggested for FoxL2, through the regulation of one of its targets, the CYP19A1 aromatase, which catalyzes the synthesis of estrogens (see below). Indeed, treatment of caryotypically male Rainbow trouts with estrogens induces *FoxL2* expression[56] and treatment of female chicken embryos with aromatase inhibitors downregulates *FoxL2* in the gonads.[57] However, no such phenomenon could be observed in the Medaka fish.[18] This potential discrepancy could indicate that this feedback loop is not strictly conserved among vertebrates, or was at least lost secondarily in the Medaka.

Many homeobox containing transcription factors are required for ovarian development and function.[58] Moreover, joint regulation of targets between directly interacting HOX and FOX factors has been previously suggested as a potential highly conserved mechanism of gene regulation.[59] Interestingly, the FoxL2 caprine promoter-driven luciferase reporter pFoxL2-luc[60] was found to be activated in consequence to the overexpression of the homeobox transcription factor Hoxd13 in human granulosa tumor KGN cells [61] (Fig. 2). An analysis of transcriptome in KGN cells shows that 48 transcription factors from the superfamily of homeobox factors are expressed, among which, interestingly, is found HOXD13[62] (ArrayExpress accession number E-MEXP-985). The role of homeobox genes in sex differentiation and development, processes which are both known to involve FOXL2, has remained relatively unexplored. Thus, the potential regulation of FOXL2 expression early in the gonad development by homeobox transcription factors, through not necessarily exclusively by HOXD13, deserves further investigation.

The *FoxL2* promoter has been shown to have a bidirectional activity in goats and humans.[60] *PFOXic*, for promoter *FoxL2* inverse complementary, is transcribed in the same sex-dimorphic manner as *FoxL2* and is also under the regulation of the PIS locus in goats.[60] Interestingly, in heterozygous PIS mutants, which do not display the characteristic PIS phenotype, *FoxL2* expression levels are identical to those of wild-type goats, whereas their *PFOXic* expression is dramatically decreased.[60] Pannetier and colleagues have thus proposed that *FoxL2* transcription is regulated through a negative feedback mechanism, which, once a certain *FoxL2* expression threshold is reached, induces the upregulation of *PFOXic* transcription, redirecting the "transcriptional overflow" on the other gene regulated by the bidirectional promoter.[60]

Although no definite posttranscriptional regulation mechanisms have been fully demonstrated in the case of *FoxL2*, experimental clues seem to indicate that they could exist. In rodents, *Foxl2* has been shown to possess 2 distinct polyadenylation sites separated by about 400bp, giving rise to 2 alternative transcripts with different 3'-UTRs (untranslated regions), which are synexpressed, though they could have distinct stability properties.[63] Even if the role of these *Foxl2* alternative transcripts is not clear, in other instances, the use of alternative UTRs has been shown to participate in the regulation of gene expression, by influencing mRNA stability as well as translational efficiency.[64] Moreover, the existence of an antisense RNA in rodents, *Foxl2OS* (for *Foxl2* Opposite Strand), spanning the *Foxl2* transcription unit and the 3'-UTR of the short *Foxl2* polyadenylation isoform was also demonstrated.[63] Antisense transcripts have been proposed to regulate the stability status of the corresponding sense transcript by masking parts of their UTRs through a RNA duplex formation, as was shown in the case of *bcl-2*.[65] Interestingly, sequencing of RT-PCR reactions specific of the reverse strand of FOXL2 have shown that antisense RNAs are produced

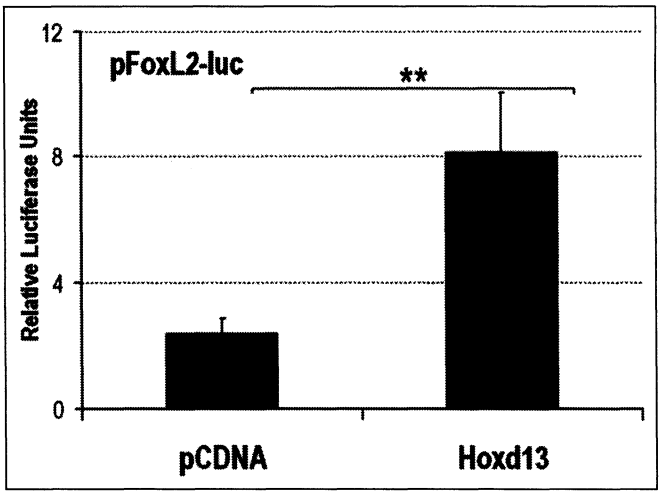


Figure 2. Activation of the pFoxL2-luc promoter reporter by HOXD13 overexpression in granulosa-like KGN cells Transfections were carried out in five biological replicates. pFoxL2-luc was described previously.[60] The Hoxd13 expression vector was a kind gift from Dr. S. Mundlos. Error bars represent the standard deviation. **: $p < 0.01$ in a Student *t*-test.

in human cells, some of which cover the complete coding region of *FOXL2*. The structure of these antisense RNAs, which seems to exist in numerous isoforms, is different from the murine *Foxl2OS* transcripts previously described, but some splicing sites are conserved (J. Cocquet, unpublished observations). These results indicate the conservation of the expression of FoxL2 antisense transcripts in mammals and suggest that they could play a regulatory function.

Another potential level of regulation of FOXL2, through posttranslational modification (PTM), was uncovered recently.[66] Indeed, FOXL2 was shown to possess a rich pattern of PTM isoforms both in human granulosa-like KGN cells and in mice whole ovaries through 2D-Western Blot experiments.[66] FOXL2 modification isoforms are contained in two distinct trains of modification, a basic poorly modified train and a more acidic hypermodified train, separated by a pI (isoeletric point) leap, with a remarkable absence of modification intermediates.[66] Perturbations of FOXL2 2D migration profile through forced dephosphorylation or forced acetylation, coupled with a bioinformatics pI prediction analysis, have revealed that FOXL2 is a highly posttranslationally modified protein, with multiple potential acetylations and phosphorylations.[66] This analysis also suggested that FOXL2 undergoes parallel processive/concerted modifications, leading to the existence of several distinct 'mature' forms. The absence of modification intermediates is compatible with the recruitment of poorly modified FOXL2 isoforms in a posttranslational 'modification factory'.[66] The exact position of the residues actually modified in protein sequence of FOXL2 have not been mapped yet but, interestingly, the sequence of FOXL2 contains many modifiable residues, whose positions and sequence contexts are conserved in a multiple sequence alignment between *Gallus gallus*, *Xenopus tropicalis*, *Dario rerio*, *Takifugu rubripes*, *Mus musculus* and *Homo sapiens* FoxL2 proteins (Fig. 3A,B). Moreover, several BPES-causing *FOXL2* mutations and one described in an isolated POF case alter potentially modifiable residues (Fig. 3C). When investigated, these mutations induced at least a partial alteration of transactivation, aggregation and/or cytoplasmic delocalization, thus indicating the potential importance of the posttranslational modification level of regulation in the control of FOXL2 function and subcellular localization.[30] The study of FOXL2 regulation through PTM is particularly relevant, as modulation by distinct combinations of phosphorylations and acetylations have been proven crucial in the regulation of the subcellular localization and activity of other forkhead family members. Indeed, FOXO factors family members are inactivated and relocated to the cytoplasm through phophorylation by Akt/PKB[67] and their

action is proposed to shift from apoptosis induction to survival promotion depending on their acetylation status.[68] In contrast, FOXM1 phosphorylation by the Raf-MEK-ERK signaling cascade is necessary to its relocation to the nucleus and FOXP3 acetylation by TIP60 is crucial to its correct function.[10] Interestingly, PTM has been shown to alter the DNA-binding specificity and affinity of the Forkhead transcription family member FOXO1.[69] More generally, the existence of 'PTM codes' on transcription factors is emerging as a common and powerful tool for swift adaptation of cellular effects to environmental cues through modulation of binding partners and specificity of targets.[70,71] Interestingly, the action of the SIRT1 deacetylase on FOXL2 does not alter FOXL2 ability to regulate its targets uniformly, as many targets are regulated less efficiently by deacetylated FOXL2 and at least one target is regulated more efficiently by deacetylated FOXL2.[55] Distinct hypermodified mature forms of FOXL2, with distinct sets of PTM, could thus differ in their preferred protein partners or in their DNA binding specificity, allowing distinct FOXL2 pools to regulate distinct cellular processes according to different input signals.

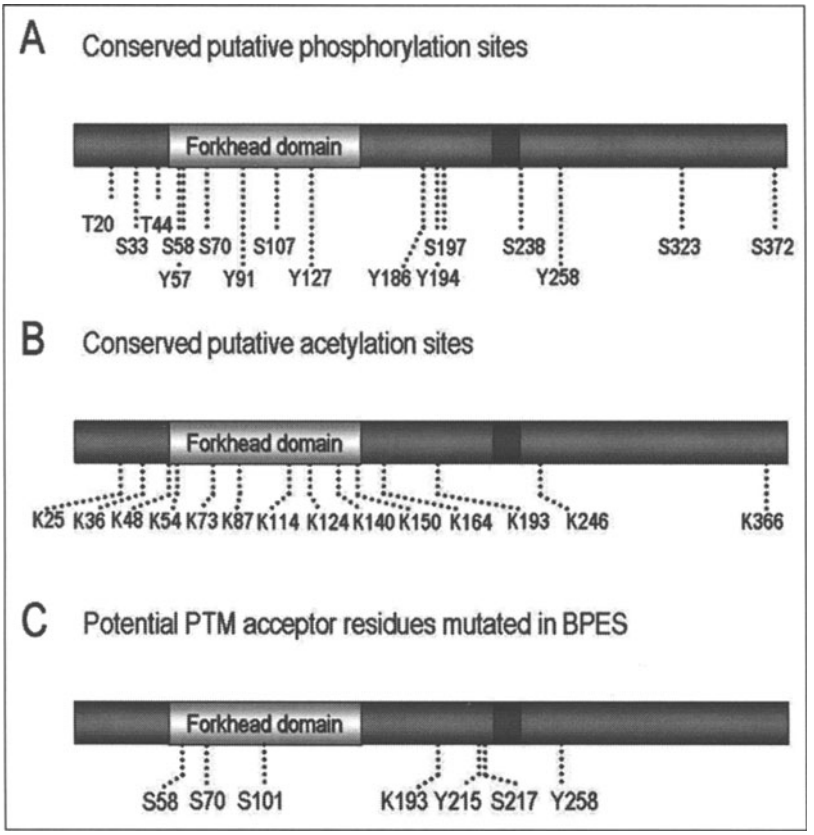


Figure 3. Map of potential posttranslational modification acceptor sites in human FOXL2 Distances are drawn to scale. Conservation measured in a ClustalW alignment between *Gallus gallus, Xenopus tropicalis, Dario rerio, Takifugu rubripes, Mus musculus* and *Homo sapiens* FoxL2 protein sequences (Alignment not shown). A) Map of conserved phophorylatable residues in human FOXL2. Predictions using the NetPhosK 1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK/) and the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/). B) Map of conserved lysines in human FOXL2. C) Map of BPES- and nonsyndromic POF-causing point mutations on potentially modifiable residues in human FOXL2. Mutations from the FOXL2 mutation database (http://medgen.ugent.be/foxl2/) and POF mutant Y258N.[33]

Regulation of Target Genes and of Cellular Functions by FOXL2

In spite of the crucial importance of FOXL2 in ovarian development, ovarian maintenance and testis repression, as illustrated by the phenotypes of PIS goats, KO and transgenic mice and BPES Type I patients, only few of its direct or indirect targets have been identified and confirmed thus far (Table 1).

FOXL2 Targets in the Pituitary

Expression of *Foxl2* immediately precedes the differentiation of gonadotrope and thyreotrope cells in the developing anterior pituitary.[26] This expression is detected in the progenitors of gonadotrope and thyreotrope cells during the pituitary gland development and is maintained through adulthood.[27,28] *Foxl2* seems to play a crucial role in the pituitary organogenesis and function.[26-28] Two pituitary specific Foxl2 targets have been described until now, the gonadotropin-releasing hormone receptor (GnRHR) and the glycoprotein hormone α-subunit (α-GSU).[28,72] In gonadotrope cells, the secretion of gonadropins is regulated by the binding of the gonadotropin-releasing hormone (GnRH) secreted by hypothalamic neurons to its receptor, the GnRHR. The secretory response is a function of both the amount of secreted GnRH and of expressed GnRHR at the plasma membrane of gonadotrope cells. GnRHR expression is regulated through a composite regulatory sequence, the GnRHR activating sequence (GRAS) and composed of partially overlapping binding sites for Smad3, AP-1 and Foxl2.[72] Each transcription factor of the complex interacts directly with its target subsequence inside the GRAS element and seems necessary for the correct regulation of GnRHR expression.[72] In participating to the regulation of the GnRHR expression, Foxl2 seems to play a crucial role in gonadotropin secretion regulation. This critical involvement is further supported by recent experimental evidence. Indeed, in the pituitary thyreotrope and gonadotrope cells, the expression of *Foxl2* precedes the expression of the α-GSU (the common subunit to pituitary glycoprotein hormones LH, FSH and TSH), though colocalization of expression patterns is achieved secondarily.[28] Stimulation of α-GSU expression by Foxl2 has been demonstrated in both a cellular model and in transgenic mice.[28]

FOXL2 Targets in the Ovary

In homozygous mutant PIS XX goats, which display a female-to-male sex reversal and have an impaired expression of FoxL2, genes normally upregulated only in males, such as SOX9 and AMH, are found upregulated.[73] Later in the development, WNT4 is downregulated in sex-reversed gonads, in a male-specific expression.[73] This female-to-male sex-reversal is reminiscent of the phenotype of the *Wnt4*$^{-/-}$*Foxl2*$^{-/-}$ double KO mice.[52] Upregulation of Sox9 and Amh is observed in *Foxl2* mice KO.[51] Consistently, Amh is downregulated in the ovotestis-like gonads of *Foxl2* transgenic mice.[52] Since *Foxl2* KO female mice and BPES XX patients are phenotypically female, sex-reversal in PIS goats should be the result of the deregulation of another key sex-determining gene in addition to *FoxL2*. Recent evidence has excluded the role of PISRT1, another gene cis-regulated by the PIS region.[74] However, these observations suggest that FOXL2 represses directly or indirectly the expression of the key male-determining genes SOX9 and AMH in gonads, thus inhibiting ectopic activation of the male sexual differentiation program in females.

Homozygous PIS mutant goats also present with a strong decrease in expression of the CYP19A1 aromatase in their ovaries as early as 36 days postcoitum, which points towards a potential regulatory relationship.[73] FoxL2 has also been suggested to regulate aromatase expression in numerous other vertebrate species, such as the chicken, the Rainbow trout, the Nile Tilapia and the Japanese wrinkled frog.[16,18,20,56] The demonstration of the direct up-regulation of aromatase expression by FOXL2 was done in human and goat granulosa cells[75] and in the Medaka fish.[76] The steroidogenesis acute response (StAR) is an enzyme that catalyzes the limiting step of steroidogenesis, i.e., the translocation of the cholesterol from the outer to the inner mitochondrial membrane, where it undergoes transformation into pregnenolone and other derived steroid hormones.[77] Pisarska and colleagues have shown that FOXL2 interacts with a Forkhead family response element found in the *StAR* promoter and that FOXL2 can repress robustly the transcriptional activity of the *StAR* promoter.[78] *StAR* expression is a marker of late differentiation of granulosa cells of pre-ovulating

Table 1. Functions of direct and indirect targets genes of FoxL2

Function/Process	Gene	Function	Regulation in Cells or Animals	Interaction with Promoter	Regulation of Promoter	Species	References
Pituitary hormones secretion regulation	***GnRHR***	GnRH receptor		EMSA	+	M	72
	αGSU	Alpha subunit of pituitary glycoprotein hormones (LH, FSH, TSH ...)	+		+	M	28
Sexual differentiation	***SOX9***	SRY (sex-determining region y)-box 9	_			M	51
	AMH	Anti Müllerian Hormone	_			M; H	51,52,62
Cholesterol and steroids metabolism	***CYP19A1 (Aromatase)***	Catalyzes the aromatization of androgens to produce estrogens	+	ChIP; EMSA	+	G; H; T; F	20,75,76
	StAR	Catalyzes the transport of cholesterol from the outer to inner mitochondrial membrane (rate-limiting step of steroidogenesis)		EMSA	_	M	78
	PPARGC1A	Gluconeogenesis, steroidogenesis, ROS metabolism	+			H	62
	NR5A2	Cholesterol homeostasis, anti-apoptosis	+			H	62
	CH25H	Cholesterol 25-hydroxylase, pro-apoptotic	+	ChIP		H	55,62
Inflammation (ovulation?)	***CCL3, 20, 3L1, 3L3***	Inflammatory chemokine ligands	+			H	62
	CXCL2, 3	Inflammatory chemokine ligands	+			H	62
	IL11, 29	Pro-inflammatory cytokines	+			H	62
	PTGS2	Synthesis of prostaglandins	+			H	62

continued on next page

Table 1. Continued

Function/Process	Gene	Function	Regulation in Cells or Animals	Interaction with Promoter	Regulation of Promoter	Species	References
Apoptosis regulation	***BCL2A1***	BCL2-related protein A1	+	ChIP		H	55,62
	IER3	Immediate early response 3	+	ChIP	+	H	55,62 Figure 4
	TNFAIP3	Tumor necrosis factor-alpha-induced protein 3	+	ChIP		H	55,62
	FOS	Apoptosis	+			H	62
	ATF3	Activating transcription factor 3, anti- or pro-apoptotic according to its isoform	+	ChIP		H	55,62
Stress response and aging	***MnSOD***	Manganese superoxide dismutase	+	ChIP	+	H	55,62
	SIRT1	NAD-dependant deacetylase		ChIP	+	H	55

"Regulation in cells or animals" details whether FoxL2 upregulates (+) or downregulates (–) target gene expression in cultured cells or transgenic animals at the mRNA or protein level. Direct interaction of FoxL2 with the target promoter as assessed by Chromatin Immunoprecipitation (ChIP), or Electromobility Shift Assay (EMSA). Regulation of promoters tested by the ability of FoxL2 to up-regulate (+) or downregulate (–) expression from a target promoter-driven luciferase reporter. Species in which the regulatory demonstration was performed; M: Mouse; H: Human; G: Goat; F: Frog; T: Tilapia.

follicles and the inhibition of its expression by FOXL2 was proposed to allow the maintenance of immature follicles in a quiescent state.[78] This repression was found to be mediated by the Proline- and Alanine-rich C-terminal region of FOXL2.[78] The role of FOXL2 in the regulation of steroid metabolism in the ovary was further illustrated by a recent transcriptome perturbation study in granulosa-like KGN cells through overexpression of FOXL2.[62] Indeed, the expression levels of other actors of steroid metabolism, such as *PPARGC1A*, *NR5A2* and *CH25H*, were found to be upregulated by FOXL2.[62] The involvement of FoxL2 in the regulation of gonadotropin secretion in the pituitary and in the steroid hormone production in the ovary makes it a key regulator of hypothalamus-pituitary-ovarian axis, whose deregulation could explain the POF phenotype associated with some of its mutations.

Other molecular and cellular pathways have been recently proposed to be regulated by FOXL2. Indeed, FOXL2 overexpression in KGN tumor cells upregulated several inflammatory chemokine ligands of the CCL and CXCL families, of pro-inflammatory cytokines, as well as of the PTGS2 (Prostaglandin synthase 2).[62] Interestingly, in *Ptgs2* KO mice, ovulation is severely compromised, even under hyperstimulation, despite an apparently normal folliculogenesis.[79] Moreover, treating rats with a PTGS2 inhibitor reduces dramatically the rate of induced ovulation.[80] This is compatible with the hypothesis according to which ovulation is an inflammatory-like process[80] and is in line with a role of FOXL2 throughout follicular maturation, up to ovulation.

The massive follicular atresia occurring in the ovaries of *Foxl2* KO mice first suggested that FoxL2 acted as an anti-apoptotic agent.[47,48] This was in accordance with the clinical BPES data, with pathogenic FOXL2 mutations inducing POF in BPES Type I patients. In contrast, FOXL2 was also proposed to be pro-apopototic, using the DEAD-box RNA helicase DDX20 (DP103) as a co-activator.[81] The involvement of FOXL2 in apoptosis regulation, as well as its ambivalent behavior, were further illustrated by its ability to up-regulate the expression of several apoptosis regulators in KGN cells, such as *BCL2A1*, *IER3*, *TNFAIP3*, *FOS*, *ATF3* and *CH25H*.[62] Direct binding of FOXL2 to the promoters of *CH25H*, *TNFAIP3*, *BCL2A1* and *ATF3* after induction of oxidative stress has been proven.[55] The potential ambivalence of a Forkhead transcription factor with regard to apoptosis is not restricted to FOXL2. Indeed, FOXO factors can promote cell survival or cell death according to the signaling input and cellular contexts.[67,82]

The transcriptome perturbation study by Batista and colleagues revealed another cellular function/process that could potentially be regulated by FOXL2 in the granulosa: the metabolism of reactive oxygen species (ROS).[62] Indeed, FOXL2 overexpression induced robust transcriptional activation of the Manganese mitochondrial Superoxide Dismutase (*MnSOD*), the Peroxisome Proliferative Activated Receptor γ Co-activator 1α (*PPARGC1A*) and the Immediate Early Response 3 gene (*IER3*).[62] Regulation if the promoter of IER3 has been confirmed in luciferase assays in human granulosa-like KGN cells (Fig. 4). The direct involvement of FOXL2 in the cell stress response was confirmed recently.[55] Indeed, *FOXL2* expression was found to be upregulated in consequence to oxidative and heat stress in KGN cells.[55] FOXL2 was also hyperacetylated in response to oxidative stress, which was associated with an increased recruitment to stress-response target promoters as assessed by Chromatin Immunoprecipitation (including *IER3* and *MnSOD*) and an increased transactivation ability.[55] As already mentioned, the NAD-dependant deacetylase SIRT1, whose involvement in the cell stress response and the regulation of aging is conserved from yeasts to mammals, inhibited robustly FOXL2 activity on chosen target promoters. SIRT1 was also found to be a direct transcriptional target of FOXL2.[55] Interestingly, SIRT1 action enhanced FOXL2 ability to regulate the SIRT1 promoter.[55] This negative feedback loop could serve as a molecular brake, helping the return to "normal" FOXL2 activity after the end of a stress. Theories of aging suggest that accumulation of oxidative damages in cells over time is part of the aging process at the cellular level and that the control of oxygen free radicals production and detoxification is crucial for cell survival and protection against age-related diseases, such as cancer.[83] The issue of the regulation of the oxidative stress response is even more topical in the ovary, as ovulations are accompanied by massive ROS production.[84]

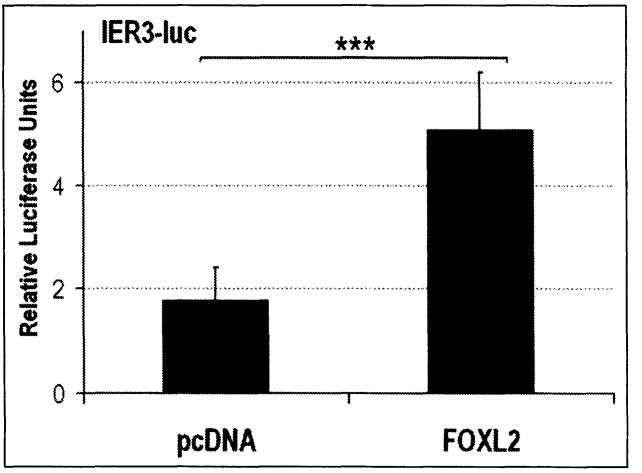


Figure 4. Activation of the IER3-luc promoter reporter by FOXL2 in human granulosa-like KGN cells Transfections were carried out in five biological replicates. IER3-luc (IEX1-luc) was described previously[94] and was a kind gift from Dr. F. Porteu. Error bars represent the standard deviation. ***: $p < 0.001$ in a Student *t*-test.

Interestingly, a recent study of FOXL2 expression in ovarian granulosa cell tumors in children revealed that FOXL2 expression was switched off, or markedly tuned down, in the most aggressive tumors.[85] Interestingly, granulosa tumors with reduced or absent FOXL2 expression displayed a higher mitotic activity and a more advanced oncologic staging than others.[85] This suggests that FOXL2 could act as a potential tumor-suppressor in the granulosa and deserves further molecular exploration. A role of FOXL2 in the regulation of cell cycle would not be unprecendented among Forkhead factors, as other members have been involved in oncogenesis or tumor suppression.[10]

The FOXL2 Response Element (FLRE) and Its Specificity

Members of the Forkhead superfamily of transcription factors are involved in many cellular and developmental processes.[9,10] These include, not exhaustively, eye organogenesis (FoxC1-2), language acquisition (FoxP2), thymus organogenesis (FoxN1), stress response and aging regulation (FoxO1,3,4), as well as ovarian determination and female fertility (FoxL2).[8,9,47,48,86] The Forkhead DNA-binding domain is highly conserved among members of the superfamily.[87] However, since Forkhead transcription factors are involved in this variety of processes, regulating a potential great diversity of target genes, it is expected that some target recognition specificity should exist to achieve the regulation of distinct targets by distinct members. This can be achieved through three nonexclusive mechanisms: (i) differences in expression patterns (for instance, the high hepatic expression of FOXA3,[88] versus the high ovarian, periocular and pituitarian expression of FOXL2[22,28]), allowing access to a differentially open chromatin in expressing cells, (ii) existence of cofactors specific to some family members through tissue-specificity or specific protein/protein interaction domains and (iii) a specific DNA target binding sequence.

Interestingly, whereas the 12 other described Forkhead transcription factor high-affinity binding sites are highly similar and correspond to the general consensus 5′-(G/A)(T/C)(A/C)AA(C/T)A-3′, FOXL2 possesses a divergent high-affinity response element, the FLRE (FoxL2 Response Element), whose consensus is 5′-GT(C/G)AAGG-3′.[32] Interestingly, it is specifically bound and regulated by FOXL2 and other FOX factors seem unable to properly activate it.[32] The gonadal primordium is unique among all organ primordia because of its bipotential nature, which allows it to develop into one of two distinct organs, a testis or an ovary.[89] Given that FOXL2 is essential for ovarian development and testis repression,[47,48,51,52] the strong target specificity that can be achieved through the FLRE is therefore not unexpected. Indeed, to ensure proper testicular development

and prevent unscheduled ovarian differentiation in males, a specific regulation of FOXL2 ovarian differentiation targets should be mandatory on evolutionary grounds.

Conclusion

As already stated, FOXL2 is a very well-conserved transcription factor and its orthologs are found in a wide range of species across evolution. Historically, FOXL2 was identified independently as an actor of pituitary organogenesis in mice and as an actor of eye morphogenesis and female fertility actor through the positional cloning of the BPES locus.[7,26] Consequently, its involvement in eye morphogenesis, in the pituitary function and in ovarian determination/development has been more particularly investigated fruitfully until now. However, FoxL2 expression and role outside these tissues remains largely inexplored and deserve further investigation.

Recent evidence has suggested that FOXL2 is also involved in other processes, such as the ovarian oxidative stress response.[55,62] Current theories of aging propose that oxidative damage accumulation is an important part of the aging process at the cellular level and that control of oxygen free radicals levels and detoxification is necessary to ensure cell survival, stem cell pool conservation and protection against age-related diseases.[83] The importance of the stress response in the control of aging, coupled with the premature ovarian aging seen observed in BPES Type I women and *Foxl2* KO mice, suggests that FOXL2 belongs to the complex networks that orchestrate the onset and the rate of aging in mammals, at least at the ovarian level. Other than infertility, ovarian aging has deleterious consequences on postmenopausal women, such as increased prevalence of osteoporosis,[90] cardio-vascular diseases[91] and neurodegenerative diseases.[92,93] Therefore, the in-depth study of FOXL2 as a potential regulator of ovarian aging is of high relevance nowadays.

Finally, even if FOXL2 mutations lead to BPES, the precise molecular and developmental pathogenesis mechanisms are still poorly understood. For instance, no reliable genotype/phenotype correlation has been established to predict the exact phenotypic impact of point mutations in ORF of *FOXL2* (Type I BPES, Type II BPES, or isolated POF). The development of a diagnostic tool could be useful in the case of female prepubertal patients, as some therapeutic options, including ovary cryo-preservation or hormonal therapy, have to be considered early in order to improve their outcome. Interestingly, studies of the cellular consequences of FOXL2 mutations seem to indicate that aggregation is a common pathogenic mechanism.[30,31,35,42] The use of anti-aggregation molecules to resolubilize at least partially FOXL2 could therefore be a seducing therapeutic avenue for BPES patients and deserves further exploration. However, the aspect of FOXL2 aggregates vary greatly between mutants.[30,35,42] This suggests the existence of specific protein partners in the aggregates, which, if uncovered, could be targeted more finely in patients' ovaries to restore an adequate FOXL2—and, hopefully, ovarian—function.

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CHAPTER 17

FOXO Transcription Factors:
From Cell Fate Decisions to Regulation of Human Female Reproduction

Jan J. Brosens,* Miranda S. C. Wilson and Eric W.-F. Lam

Abstract

All key reproductive events in the human ovary and uterus, including follicle activation, ovulation, implantation, decidualization, luteolysis and menstruation, are dependent upon profound tissue remodelling, characterised by cyclical waves of cell proliferation, differentiation, apoptosis, tissue breakdown and regeneration. FOXO transcription factors, an evolutionarily conserved subfamily of the forkhead transcription factors, have emerged as master regulators of cell fate decision capable of integrating a variety of stress, growth factor and cytokine signaling pathways with the transcription machinery. The ability of FOXOs to regulate seemingly opposing cellular responses, ranging from cell cycle arrest and oxidative stress responses to differentiation and apoptosis, renders these transcription factors indispensable for cyclic tissue remodelling in female reproduction. Conversely, perturbations in the expression or activity of FOXO transcription factors are increasingly linked to common reproductive disorders, such as pregnancy loss, endometriosis, endometrial cancer and primary ovarian insufficiency.

Introduction

The first forkhead protein to be described was *Fork head* in 1989,[1] named after the mutant *Drosophila*'s "forked" head.[2] Since then, more than 150 forkhead transcription factors have been characterised, including over 40 in humans.[3,4] All share a conserved forkhead domain/forkhead box containing a "winged helix" type structure, making forkheads a superfamily of helix-turn-helix proteins.[5] Initial work on this diverse family provided an equally diverse set of names, but the number of forkhead proteins being discovered meant that a unified nomenclature became necessary to avoid confusion.[6] The proteins became known as FOXs (forkhead box). In vertebrates, FOX proteins have been classified alphabetically into 19 subgroups, FOXA to FOXS. The agreed nomenclature also distinguishes between different systems: for all forkheads, capitalising all letters (such as for FOXO1) is reserved for human proteins. Mouse proteins have a capital F only (e.g., Foxo1), while other chordates are named with capitals for the group letter as well as the initial letter (e.g., FoxO1).

Some of these 19 FOX subgroups have attracted more interest than others, perhaps none more so than the FOXO subfamily of tumor suppressors. In humans, this group contains four members: FOXO1 (previously known as FKHR), FOXO3 (FKHRL1), FOXO4 (AFX or Mllt7) and FOXO6. In addition, a number of poorly characterised splice variants have been described.[7] With the exception of FOXO6, which reportedly is only present in adult brain tissue, mammalian FOXOs are ubiquitously expressed in a variety of tissues throughout the body.[8] However, there is

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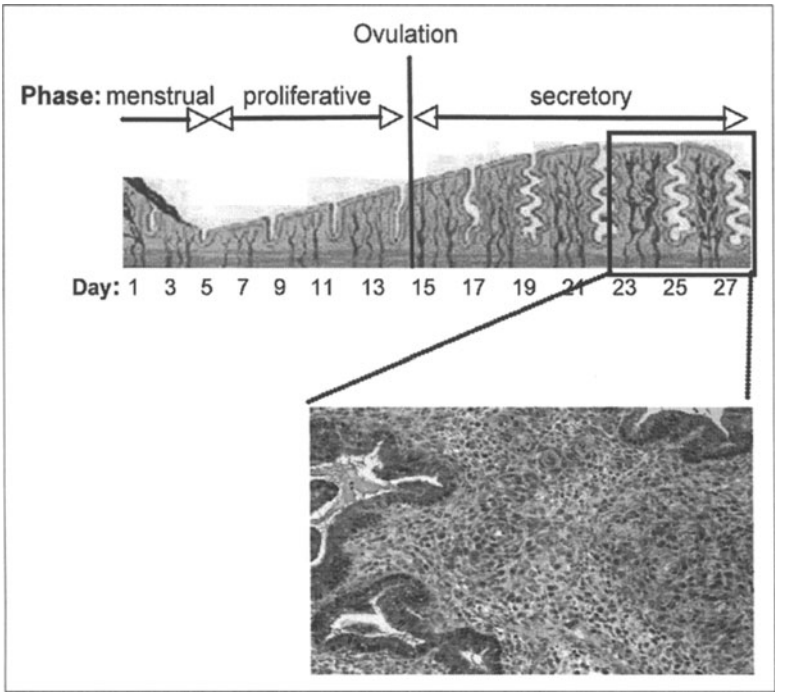

Figure 1. Expression of FOXO1 in human endometrium. The uterine mucosa undergoes cyclical waves of proliferation, secretory transformation, menstrual shedding and regeneration. FOXO1 is constitutively expressed in endometrial epithelial cells. In the stroma, FOXO1 expression is confined to decidualizing cells during the latter half of the secretory phase of the cycle.

significant differential expression, depending on tissue type and developmental stage.[9] For example, mouse Foxo3a is most highly expressed in the developing liver but more abundant in the brain and spleen in adult animals. Similarly, Foxo4 is expressed highly in skeletal muscle during development but also in cardiac muscle and adipose tissue in adulthood. The highest levels of Foxo1 are found in adipose tissue but also in the uterus and ovaries of cycling female animals. In the human uterus, FOXO1 is constitutively expressed in the endometrial epithelium throughout the menstrual cycle.[10-12] It is also expressed in the stroma, but only upon differentiation of endometrial fibroblast into decidual cells during the mid-secretory phase of the cycle (Fig. 1). In contrast to the induction of FOXO1, decidual transformation of endometrial stromal cells is associated with repression of FOXO3a expression.[13] Although FOXO4 transcripts are present in human endometrium, evidence of expression at protein level is as yet lacking. Foxos—and more specifically Foxo3a—have emerged as master regulators of ovarian function, at least in rodents.[14-19] Although FOXO transcripts are present in the human ovary,[20] somewhat surprisingly their expression profile at protein levels has not yet been studied.

In this chapter, we discuss the mechanisms that control FOXO activity, highlight the role of these transcription factors in cell fate decisions and focus on their role in normal and abnormal uterine and ovarian function.

Regulation of FOXO Activity

The forkhead box DNA binding domain recognises the consensus sequence 5′-TTGTTTAC-3′.[21] FOXO family members are highly homologous within the forkhead box (about 40% identity overall, but up to 70-90% within forkhead box),[8,22,23] often show functional redundancy and can bind

to the same promoter sequences. As transcription factors with many diverse targets and apparently contradictory activities, ranging from promoting differentiation to inducing gene networks involved in cell cycle arrest and apoptosis, FOXOs must be tightly regulated for correct cellular function. This occurs mainly by posttranslational modifications, which can affect FOXO stability, subcellular localisation, or DNA binding activity. Perhaps the most prominent regulator of FOXOs is the serine/threonine kinase Akt (also called PKB), which acts downstream of phosphoinositol-3-kinase (PI3K) in the insulin/growth factor signaling pathway (Fig. 2). Akt phosphorylates FOXO at several hierarchical conserved sites (Thr24, Ser256 and Ser319 in FOXO1).[24,25] The middle site, within the forkhead box, is modified first, resulting in a conformational change, which exposes the other sites. Next, the N-terminal residue is phosphorylated, allowing the inhibitory 14-3-3 complex to bind. Finally, phosphorylation of the N-terminal site brings about nuclear export and thus transcriptional inactivation of FOXOs. FOXO6 is an exception to this rule as it lacks the third Akt phosphorylation site,[8] which renders it constitutively nuclear although not constitutively active.[26] Compelling evidence has emerged indicating that FOXOs engage in a feedback mechanism with the PI3K/Akt pathway by trans-activating *PIK3CA,* which encodes the catalytic p110α subunit of PI3K (class 1A)[27] or by repressing the expression of the Akt inhibitor tribble 3.[28] Thus, while activation of the PI3K/Akt pathway antagonises FOXO activity, FOXOs in turn are capable of amplifying PI3K/Akt signaling. This homeostatic mechanism may be of particular relevance for the survival of cells under adverse conditions.

In addition to Akt, other kinases, such as SGK1 (serum- and glucocorticoid-inducible kinase 1), IKKβ (inhibitor of NFκB kinase β), CK1 (casein kinase 1) and DYRK1A (dual-specificity tyrosine-phosphorylated and regulated 1A), have also been implicated in FOXO phosphorylation and nuclear export.[21] Once in the cytoplasm, FOXO is sequestered and tagged for degradation by the proteasome by polyubiquitination by the E3 ubiquitin ligase SCF^{Skp2} complex.[29] Conversely, kinases such c-Jun N-terminal kinase (JNK) and MST1 (mammalian Sterile 20-like kinase 1) have been shown to enhance FOXO transcriptional activity.[24,30] JNK phosphorylates Thr447 and Thr451 in FOXO4 and presumably similar residues in FOXO1 and FOXO3a.[31] JNK is also known to phosphorylate 14-3-3 directly and represses Akt activity indirectly. MST1 can phosphorylate FOXO1 and FOXO3 (at Ser207, Ser213, Ser229 and Ser230 in FOXO3),[30] but its role in enhancing FOXO activation is complicated by the fact that these protein modifications actually reduce FOXO1 DNA binding.[32] MST1 may therefore increase FOXO activity primarily by disrupting 14-3-3 binding, allowing FOXO to enter the nucleus before other proteins remove the inhibitory phosphate groups. Moreover, MST1 is also able to increase JNK activity.[33]

Other posttranslational modifications also affect FOXO activity, either directly or indirectly. For example, methylation of arginines within a consensus motif for Akt phosphorylation in FOXO1 (Arg248 and Arg250) by PRMT1 (protein arginine methyltransferase 1) antagonises Akt phosphorylation, thereby promoting nuclear localisation and transcriptional activity of FOXOs.[34] Acetylation by proteins such as the histone acetylases p300 and CBP increases FOXO1 stability by reducing polyubiquitination yet also enhances inhibitory phosphorylation by Akt and reduces FOXO1 DNA binding activity.[20] Conversely, the nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases Sirtuins (also called SIRTs) are capable of reversing FOXO acetylation, which enhances oxidative and genotoxic stress defences and attenuates cell death responses (Fig. 2).[35,36]

FOXO and Cell Fate

As alluded to, FOXOs are capable of promoting differentiation, survival as well as cell death, depending on the cellular environment and the nature of upstream signaling pathways. Consequently, FOXOs are important determinants of the function of specific organs, as discussed later for the uterus and ovary, but also for the fate of the entire organism. Invertebrates, such as *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster*, have been particularly informative in delineating the role of FoxO proteins within the context of the whole organism, especially as essential determinants of organismal stress responses, metabolism and lifespan in response to nutritional and environmental cues.[28,37]

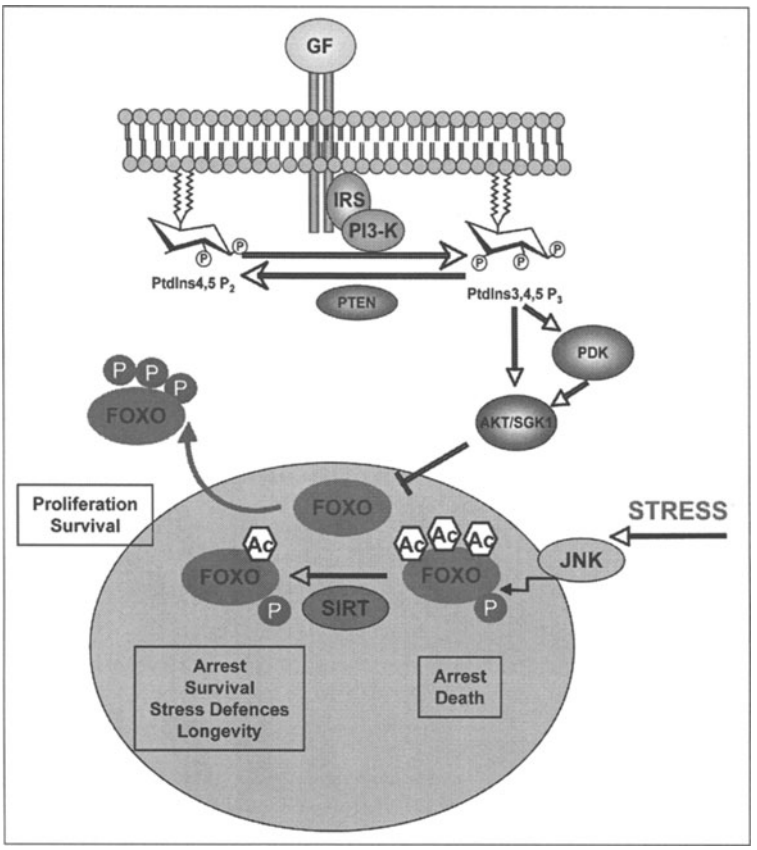

Figure 2. FOXO proteins are key regulator of cell fate and regulated by insulin/IGF signaling pathway. Insulin or IGF stimulation leads to PI3K activation, which in turn results in Akt/PKB induction. Akt/PKB phosphorylates and inactivates FOXOs by promoting translocation to the cytoplasm, which promotes proliferation. The transcriptional programs regulated by FOXOs are influenced by SIRT- and JNK-dependent modifications. Phosphorylation and acetylation are denoted by P and AC, respectively.

Cell Cycle Progression, Checkpoint Control and Apoptosis

FOXO proteins are important for correct progression through the cell cycle, a tightly regulated process divided into four phases: an S (DNA-synthesis) phase and M (mitosis) phase, which are separated by two intervals, G1 (gap 1-between M and S) and G2 (gap 2-between S and M) phases. In G1, cyclin Ds are upregulated and bind to CDK4 (cyclin-dependent kinase 4) and CDK6.[38] These active cyclin D-CDK4/6 complexes are necessary to pass the restriction point (R), a checkpoint near the end of the G1 phase and enter S phase, by phosphorylating and activating pRB family proteins and by upregulating the transcription factor E2F.[39] Constitutively active FOXO is able to cause cell cycle arrest in G1 by downregulating both CDK4 activity and expression of D-type cyclins, possibly via downregulation of cyclin D1 and upregulation of the known FOXO target Bcl-6, a *cyclin D2* transcriptional repressor.[40,41] Cyclin-CDK complex activity is also downregulated by CKIs (cyclin-dependent kinase inhibitors). FOXOs can upregulate at least two Cip/Kip class CKIs, $p21^{Cip1}$ and $p27^{Kip1}$, as well as two INK4 class CKIs, $p15^{INK4b}$ and $p19^{INK4d}$.[38] Furthermore, the regulation of the pRB-related protein p130 h by FOXO is believed to have a role in cell cycle exit.[42]

Assuming that cells make it through into S phase, the next FOXO-regulated hurdle is the G2/M DNA damage checkpoint. FOXO1 reduces expression of important G2/M proteins such as cyclins B1 and B2.[43] Cyclin G2, a cyclin B-CDK1 inhibitor, is also a target induced by FOXO.[44] In the absence of DNA damage, CDK1 and 2 help regulate the G2/M transition by phosphorylating and inhibiting FOXO (at Ser249 in FOXO1).[21,45] When damage is present, it is recognised by damage sensors such as the MRN (Mre11-Rad50-Nbs1) complex and ATR (ataxia telangiectasia mutated (ATM) and Rad3 related), which induce repair as well as aiding cell cycle arrest.[46] ATR has in fact been identified as a positive regulator of FoxO in a screen for FoxO-regulating kinases and phosphatases in *Drosophila* as well as in another proteomic screen of proteins phosphorylated in response to DNA damage.[47,48] FOXO3a may also upregulate ATM expression and activity.[49,50] Another FOXO target, GADD45a, is also capable of causing cell cycle arrest at G2/M and aid in DNA damage repair.[51-53]

If the damage is too great to repair, FOXO can trigger the expression of several pro-apoptotic proteins, including the BH3-only Bcl-2 family members Bim and bNIP3, as well as TRAIL and FasL,[54] which induce extrinsic or mitochondria-independent apoptosis upon binding to death receptors. Bcl-6 expression also aids apoptosis by repressing Bcl-xL.[55] FOXO3a-induced apoptosis has been reported to be dependent on induction of PUMA and its downstream mediator Bax.[56] Interestingly, FoxO3a has been shown to bind and promote the nuclear export of p53, the "guardian of the genome".[57] Like FOXO proteins, p53 is an important transcription factor in several linked pathways that controls various cellular responses, ranging from metabolism to apoptosis and senescence. This tumor suppressor is well-known to induce proapoptotic genes, such as PUMA and $p21^{Cip1}$, but can also elicit apoptosis by inducing the expression of Bax which binds to and inhibits Bcl-2 and Bcl-xL at the mitochondria.[56]

Cellular Stress Response and Longevity

In addition to DNA damage, FOXO factors are regulated by a variety of other stress stimuli, including nutrient deprivation, hypoxia and reactive oxygen species (ROS). In *C. elegans*, dauer denotes an alternative developmental stage of the larva, characterised by low metabolism, resistance to harsh conditions and increased longevity. Dauer formation is induced by food deprivation, oxidative stress or other environmental stress.[58] In fact, many longevity mutants in *C. elegans* are resistant to oxidative stress.[59] Dauer formation requires the activation of the nematode FoxO homologue Daf-16 to induce G1 cell cycle arrest, mediated at least partially through the induction of the nematode Cip/Kip inhibitor, Cki-1.[60] Expression of Cki-1 can induce premature cell cycle arrest in G1, whereas silencing of Cki-1 activity by RNA interference (RNAi) promotes S phase entry.[60] Consequently, Daf-16 silencing by RNAi reduces the resistance of worms to oxidative stress.[61,62] However, Daf-16 is normally inactivated by the insulin/insulin-like growth factor (IGF) signaling (IIS) pathway in conditions of high nutrient availability and low environmental stress.[21,63] Similarly, in the fruit fly *Drosophila*,[23] when nutrients are scarce the IIS pathway is suppressed, dFoxO activation promotes G1 cell cycle arrest and reduces the metabolic rate but lengthens lifespan.[64]

The stress-activated kinase JNK negatively controls metabolism and lifespan in both *Drosophila* and *C. elegans* by antagonising the IIS pathway through phosphorylation of FoxO.[16,29] At molecular level, JNK-phosphorylation leads to nuclear translocation and acetylation of FoxO.[25] In this context, sirtuin regulation of FoxO is of particular interest because the yeast homolog of deacetylase SIRT1, Sir2, is associated with increased lifespan in yeast and required for the longevity response to caloric restriction (CR), an extremely conserved mechanism for increasing lifespan.[65] Overexpression of Sir2 homologs in Drosophila or in *C. elegans* also suffices to extend lifespan.[64] Consistent with the role of FoxO in controlling oxidative stress response and lifespan, FOXO3a knockout in human fibroblasts increases intracellular ROS levels and induces senescence-like morphological changes.[66] Thus, in multicellular organisms, from worms to flies to mammals, FOXO are modulators of lifespan and stress responses, capable of causing cell cycle arrest, lowering metabolism and thereby counteracting aging.

Tumor Suppression

FOXO proteins are known tumor suppressors, which regulate and are regulated by other tumor suppressors (e.g., p53) and oncogenes (e.g., β-catenin). Cellular senescence is an important antitumorigenic mechanism that prevents precancerous cells from proliferating. Loss of PTEN activity, Akt hyperactivity and deregulation of downstream targets such as FOXOs are prevalent features in cancer that interfere with normal senescence.[67] In breast cancer, downregulation or loss of FOXO activity is associated clinically with a poor prognosis.[68] In glioblastoma, an aggressive brain tumor, increased activity of Akt reduces FOXO binding to the $p21^{Cip1}$ promoter, thereby disabling cell cycle arrest.[54] RUNX, a tumor suppressor often lost in gastric cancer, mediates apoptosis by enhancing FOXO-mediated transcription of *BIM*.[69] Moreover, FOXO proteins have been implicated in the mechanism of action for many chemotherapeutic drugs, including taxol, doxorubicin, cisplatin and gefitinib.[70]

Hormone-dependent malignancies are particularly relevant, due to the interactions between FOXO factors and various nuclear steroid receptors, including the androgen, estrogen and progesterone receptors (AR, ER and PR, respectively). For example androgens control proliferation of normal prostate cells by activating AR, which then regulates transcription of various target genes.[71] In early stage cancer of the prostate (CaP) androgens are still required for proliferation and androgen ablation therapy is an effective first-line treatment. As well as increasing proliferation by regulating cell cycle proteins, such as CDKs androgens confer protection against apoptosis.[72] Activated AR binds to FOXO1 and probably FOXO3a, reduces its DNA binding activity and thereby attenuating the expression of proapoptotic proteins, such as FasL.[72] PTEN is known to inhibit AR activity, which is mediated by FOXOs, possibly via antagonising the interactions between the AR and its coactivators and/or by interfering with the ability of the receptor to adopt an active conformation upon androgen binding.[74] Cross-talk between these transcription factors is further exemplified by the observation that *AR* is a direct FOXO3a target gene.[75] CaP cells eventually develop the ability to proliferate in the absence of androgens. Androgen insensitivity has been linked to a reduction in the activity and expression of FOXO3a, the most abundant family member in prostate cells.[76] Deregulation of the interactions between steroid receptors and PI3K/AKT/FOXO pathway plays an important role in other endocrine cancers. For example, in MCF-7 breast cancer cells, FOXO3a not only binds and inhibits the ER but also represses the expression of this nuclear receptor. Expression of FOXO3a in the ER-positive MCF-7 cells decreases the expression of several ER-regulated genes and upregulates expression of the cyclin-dependent kinase inhibitors $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$.[79] Together, these observations highlight the importance of functional interactions between FOXO3a and nuclear hormone receptors in suppressing steroid-dependent cancer cell growth and tumorigenesis. The role of FOXO1 in mediating the antiproliferative effects of progesterone in the endometrium will be discussed later.

FOXO proteins are also involved in the response to chemotherapy as well as the emergence of treatment resistance, which often occurs in late stage or recurring cancers. For example, FOXO3a mediates the cytotoxic effects of cisplatin in colon cancer cells and inhibition of Akt-dependent phosphorylation of FOXO3a activity resensitises resistant ovarian cancer cells to cisplatin.[80,81] Although activation of FOXOs by anticancer drugs may induce cycle arrest and programmed cell death, chronic FOXO activity may in fact contribute to development of drug resistance in some cancer cells, for example by inducing the expression of multi-drug resistance transporter genes such as *MDR1 (ABCB1)*.[82,83] Using the K562 chronic myelogenous leukaemia (CML) cell line and the doxorubicin-resistant derivatives lines KD30 and KD225 as models, we recently observed that enhanced PI3K/Akt activity and acquisition of chemoresistance paradoxically correlated with increased expression and nuclear accumulation of FOXO3a. Moreover, knockdown of endogenous FOXO3a expression reduced PI3K/Akt activity and sensitised these cells to doxorubicin.[27] The FOXO target DUSP6, mentioned above, also modulates responses to therapy. For example, increased DUSP6 expression levels in breast cancer cells is associated with resistance to the anti-estrogen tamoxifen,[84] while loss of this MAPK inhibitor in ovarian cancer plays a role in cisplatin resistance.[85] Although the underlying mechanisms remain to be further explored, it is

increasingly clear that FOXO proteins have a very ambivalent role in drug treatment of cancers; on the one hand conferring the desirable cytotoxic effects and, on the other, contributing to the emergence of drug resistance.

FOXO and the Endometrium

The endometrium is the mucosa, characterised by glands and stroma, which lines the lumen of the uterus. During the reproductive phase, ovarian estradiol induces the ordered growth of the endometrium whereas the postovulatory rise in progesterone levels controls differentiation of this tissue in preparation for an implanting embryo. This differentiation process is characterised first by secretory transformation of the endometrial glands, followed by influx of various bone-marrow immune cells, predominantly uterine natural killer cells, angiogenesis and transformation of endometrial stromal cells into specialized epitheloid decidual cells, a process known as 'decidualization'.[86] Both in vitro and in vivo, initiation of the decidual process requires elevated intracellular cAMP levels and sustained activation of the protein kinase A but maintenance of the decidual phenotype is strictly dependent upon elevated progesterone levels.[87,88] In the absence of pregnancy, falling sex hormone levels induce a switch in the secretory repertoire of decidual stromal cells, now characterised by expression of pro-inflammatory cytokines, chemokines and matrix metalloproteinases, which triggers a sequence of events leading to menstrual shedding of the superficial endometrial layer.[89]

Endometrial Differentiation and Menstrual Shedding

FOXO1 has emerged as a major regulator of progesterone-dependent differentiation of the human endometrium and subsequent menstrual shedding (Fig. 3). It was first identified in this tissue as a transcriptional regulator of *PRL* (*prolactin*), a major differentiation marker of decidualizing endometrial stromal cells.[12] Subsequent studies demonstrated FOXO1 expression is not only induced in endometrial stromal cells in response to cAMP signaling but also that its expression in vivo during the mid-secretory phase of the cycle coincides with that of several other transcriptional regulators of the decidual process, including wild-type p53, Sp1, STAT5 (signal transducers and activators of transcription 5) and C/EBPβ(CCAAT/enhancer-binding protein β).[12,90,91] Moreover, many of these factors are capable of physically interacting with the progesterone receptor (PR) and regulate PRL expression by binding to a discrete region in the proximal decidua-specific promoter that contains overlapping FOXO and C/EBPβ DNA binding sites as well as PR response element half-sites. These observations have lead to the suggestion that the activated PR serves as a platform for binding of cAMP-induced transcription factors, including FOXO1, and that these multimeric complexes regulate the expression of decidual gene networks.[87,88] In a recent study, FOXO1 knockdown using small interfering RNA was shown to perturb the expression of 507 genes regulated upon decidualization, which accounted for 15% of all regulated transcripts.[43] Several genes that encode for major secretory products of decidual cells (e.g., *IGFBP1*, *PRL*, *LEFTY2* and *WNT4*) are FOXO1-dependent. Moreover, FOXO1 regulates the induction of *CDKN1C* (*$p57^{Kip2}$*), a cyclin-dependent inhibitor involved in G1 arrest and simultaneously represses several genes important for either DNA replication/S phase (e.g., *MCM5*), G2/M transition (e.g., *CCNB1*, *CCNB2*, *CDC2*, *BIRC5* and *BRIP1*) or mitosis (e.g., *PRC1*, *NUSAP1*, *CENPF*, *SPBC25* and *ASPM*). As mentioned, FOXO transcription factors integrate various signal transduction pathways by binding to Smad3 and Smad4 in response to TGF-β signaling or to β-catenin upon activation of the canonical WNT pathway. In differentiating endometrial cells, FOXO1 in turn regulates the expression of several genes capable of modulating the activities of these upstream regulatory pathways. For instance, FOXO1 induces the expression of *INSR* (insulin receptor), a major activator of the PI3K pathway, as well as *WNT4* and *BAMBI* (*BMP and activin membrane-bound inhibitor*).[43] BAMBI is a transmembrane glycoprotein related to TGF-β family Type I receptors. However, it lacks an intracellular kinase domain and serves as a potent inhibitor of BMP, activin and TGF-β signalling.[92] Thus, the profiling of FOXO1 target genes in decidualizing endometrial cells further points towards the existence of complex feedback mechanisms that determine the preference and fine-tune the activity of upstream regulatory pathways.

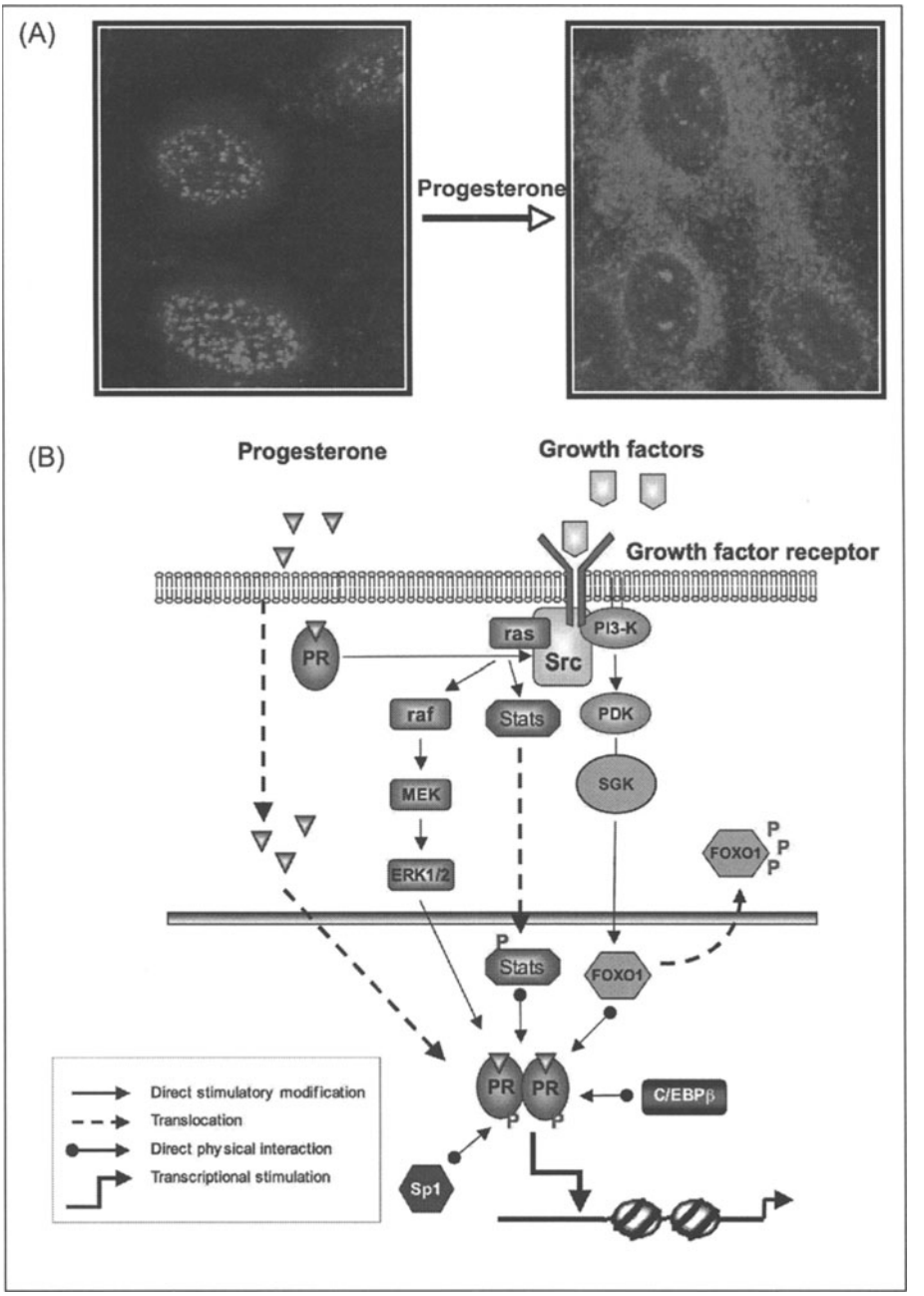

Figure 3. Regulation of FOXO1 by progesterone signalling in decidualizing endometrial stromal cells. A) progesterone triggers a partial translocation of FOXO1 from the nucleus to the cytoplasm. B) the progesterone receptor (PR) serves as platform for interaction with several decidua-specific transcription factors, including FOXO1.

Although cAMP is the primary signal for induction and nuclear accumulation of FOXO1 in decidualizing endometrial cells, its transcriptional output in these cells is tightly regulated by progesterone through multiple mechanisms. First, progesterone enhances SGK1 expression and activity, which in turn causes a partial translocation of FOXO1 to the cytoplasm.[93] Second, the progesterone inhibits the expression of Skp2, which directs FOXO1 ubiquitination and proteasome degradation.[29] Consequently, in addition to nuclear FOXO1, decidual cells also accumulate a pool of inactive FOXO1 in the cytoplasm upon continuous progesterone signaling. Withdrawal of progesterone from differentiated cultures results in nuclear re-accumulation of FOXO1, enhanced BIM expression and cell death, a response that likely contributes to menstrual shedding of the endometrium in the absence of pregnancy.[94]

The Decidua of Pregnancy

Human pregnancy has been described as a hyper-inflammatory process, characterised by influx of macrophages and specialised natural killer cells, profound vascular remodelling and deep invasion of the fetal trophoblast into the maternal deciduas and underlying inner myometrium.[95] The ability of the maternal decidua to cope with proinflammatory and oxidative stress signals is key to safeguarding the integrity of the feto-maternal interface during the process of deep trophoblast invasion. There are several components to the mechanism that confers this resistance to environmental insults. First, decidualization is associated with the induction of various free radical scavengers, most notably mitochondrial superoxide dismutase 2 (SOD2), monoamine oxidases A and B, thioredoxin, glutaredoxin, peroxiredoxin and glutathione peroxidase 3 (GPx3), a secreted enzyme with potent extracellular antioxidant activity.[43] Second, and most importantly, ROS strongly induce FOXO3a expression in undifferentiated but not in decidualizing endometrial cells. Notably, expression of a constitutively active FOXO3a mutant triggers apoptosis in decidualized cells whereas silencing of endogenous FOXO3a expression in undifferentiated cells abrogates apoptosis induced by free radicals.[13] Finally, JNK activation in response to ROS is firmly silenced upon decidual transformation of the endometrium, which is accounted for, at least in part, by the strong induction of the MAPK phosphatase MKP1 (also called DUSP1) (Brosens J, unpublished observations). Thus, decidual cells not only possess heightened ROS defences and repair capacity but the simultaneous repression of JNK signaling and FOXO3a expression also disables the pathway responsible for oxidative cell death. Oxidative cell death at the maternal-fetal interface is a feature of a variety of common pregnancy disorders, ranging from recurrent pregnancy loss to fetal growth restriction and preeclampsia.[96] While as yet untested, it appears likely that impaired decidualization and FOXO3a silencing underpin these obstetric complications.

Endometriosis and Endometrial Cancer

These cyclical waves of endometrial proliferation, differentiation, shedding and regeneration are unparalleled in any other tissue of the body and occur on average 400 times during reproductive life. Not surprisingly, endometrial cancer is the most common malignancy of the female reproductive tract and its incidence is increasing in North America and Europe. Endometrial carcinomas are divided into two groups; endometrioid (Type I) and nonendometrioid (Type II) endometrial cancers.[97,98] The common Type I, which accounts for 80% of all endometrial carcinomas, is estrogen-related, low-grade, affects pre and peri-menopausal women and is often preceded by complex atypical endometrial hyperplasia. In contrast, Type II tumors are not estrogen-driven and mostly develop in atrophic endometrium.

In addition to its key role in regulating normal endometrial cyclicity, several strands of evidence indicated that loss of FOXO1 activity would be an integral event in neoplastic transformation of the endometrium, especially in endometrioid endometrial cancer (EEC). First, loss or mutation of the tumor-suppressor gene *PTEN* is the earliest detectable genetic defect in EECs and leads to unrestrained PI3K/Akt signaling,[99] which in turn causes phosphorylation, inactivation and cytoplasmic sequestration of FOXO transcription factors. Second, Skp2 is overexpressed

in EEC[78,100] suggesting increased FOXO1 degradation. Finally, unopposed estrogen signaling is a well-recognised risk factor for EEC and has been shown to enhance PI3K/Akt activity, for instance through binding of ER to the p85α regulatory subunit of PI3K.[101] In agreement, several studies have now reported a marked decrease or complete loss of FOXO1 expression in both low- and high-grade EECs, which in turn has been functionally linked to uncontrolled cell proliferation, increased susceptibility to genotoxic insults and decreased responsiveness to progestin treatment.[11,78,102] However, loss of FOXO1 expression in EECs is not merely the consequence of PI3K/Akt hyperactivity and proteasomal degradation but reflects a marked decrease in transcript levels. A recent study demonstrated methylation of the FOXO1 promoter in 9 out of 10 primary EECs, although the degree varied considerably between tumor samples. On the other hand, comparative analysis of two widely used EEC lines, HEC-1B and Ishikawa cells, suggested that loss of FOXO1 expression is the consequence of increased decay of its mRNA.[11] It is interesting to note that microRNAs complementary to FOXO1 have been identified in several species, including humans (http://microrna.sanger.ac.uk/). Furthermore, FOXO1 mRNA has an extended 3′-UTR that contains several AUUA pentamers, indicating potential regulation by RNA-binding proteins. Thus, loss of FOXO1 in ECCs may involve several mechanisms, including promoter inhibition and increased mRNA instability.

Interestingly, FOXO1 mRNA levels are also lower in eutopic endometrium of patients with endometriosis,[103,104] although this is not attributable to methylation of the FOXO1 promoter (Brosens and Lam, unpublished observation). Endometriosis is a prevalent disorder, affecting 5-10% of women during the reproductive years and a major cause of pelvic pain and infertility. It is characterised by the presence of endometrial lesions at ectopic sites, predominantly the pelvic cavity and ovaries. There is overwhelming evidence indicating that retrograde menstruation and implantation of viable endometrial cells at ectopic sites is the primary cause of endometriosis. How attenuated FOXO1 expression in eutopic endometrium contributes to the pathogenesis of this debilitating disease is unknown but likely to involve impaired apoptotic responses prior to menstruation, leading to an increased load of viable cells in the menstrual effluent. Although somewhat controversial, several studies indeed reported that apoptosis is markedly reduced in premenstrual endometrium of endometriosis patients as well as in the endometriotic lesions.[105,106] Whatever the mechanism, it is clear that FOXO1 is indispensable for safeguarding endometrial homeostasis during the rapid waves of proliferation, differentiation, apoptosis, menstrual shedding and regeneration.

FOXO and the Ovary

Follicular Development and Ovulation

While FOXO1 is a master regulator of endometrial differentiation, FOXO3a orchestrates ovarian follicular development. In recent years, several elegant mouse models illustrated the critical role of this transcription factor in ovarian follicle activation, a term that refers to the recruitment and development of primary follicles from the primordial pool. Castrillon and colleagues were the first to show that Foxo3a$^{-/-}$ female mice exhibit a distinctive ovarian phenotype of global follicular activation, leading to oocyte death, early depletion of functional ovarian follicles and secondary infertility; an observation confirmed by others.[17,18] More recently, Liu et al generated transgenic mice in which a constitutively active Foxo3a mutant is selectively expressed in oocytes under the control of the zona pellucida glycoprotein 3 gene promoter.[107] Interestingly, these mice were also infertile, caused by retarded oocyte growth and follicular development and anovulation. Unrestrained Foxo3a activity enhanced nuclear localization of p27^{kip1} in oocytes and caused a dramatic reduction in the expression of bone morphogenic protein 15, connexin 37 and 43, which are important for paracrine signaling and gap junction communications in follicles. While perhaps not entirely conclusive, these observations indicate that intra-oocyte Foxo3a activity plays a decisive role in follicular development.

Two other mouse models provided additional evidence for the central role of Foxo3a in follicle activation. The first was designed to explore the role of intra-ovarian PRL signaling.[108] After ovulation PRL is important for maintenance of corpus luteum formation and continuous progesterone production after ovulation. It mediates its effect through binding and activation of the PRL receptor (PRLR), a member of the class 1 cytokine receptor superfamily. Alternative splicing, both in humans and rodents, generates a long PRLR form and several shorter forms that differ in their intracellular domains. PRLR null female mice are infertile because of premature corpus luteum involution and insufficient progesterone expression. Interestingly, selective expression of a short receptor isoform, termed PR-1, in *PRLR*$^{-/-}$ mice leads to a dramatic down-regulation of Foxo3a expression, premature follicular development, followed by massive follicular cell death and premature ovarian failure.[108] More recently it was shown that selective ablation of *Pten* in mouse oocytes results in activation of the entire primordial follicle pool and complete depletion by early adulthood.[109] While the role of FOXO3a is beyond dispute, FOXO1a may also be important for normal ovarian function. For example, induction, phosphorylation and inactivation of FoxO1 in response to follicle-stimulating hormone have been implicated in differentiation of primary rat granulosa cells.[19,110]

Primary Ovarian Insufficiency

Primary ovarian insufficiency, a designation now preferred over terms like 'premature ovarian failure' or 'hypergonadotropic hypogonadism', is defined as a primary ovarian defect characterised by absent menarche (primary amenorrhea) or by premature depletion of ovarian follicles or arrested folliculogenesis before the age of 40 years (secondary amenorrhea).[111] Primary ovarian insufficiency is a multifactorial disorder that affects approximately 1% of women. In addition to iatrogenic (e.g., radiotherapy), endocrine, environmental, infectious and immunological causes, a number of single gene defects (e.g., *FMR1*, *FSHR*, *GALT*, *EIF2B* and *FOXL2*) have been implicated in familial primary ovarian insufficiency,[111-116] although the majority of cases remain unexplained. In view of the premature reproductive senescence phenotype of *Foxo3a*$^{-/-}$ female mice, a recent study examined the prevalence of heterozygous or homozygous polymorphisms or mutations in FOXO3a in a cohort of women with primary ovarian insufficiency.[117] Although 8 single-nucleotide polymorphisms were identified, none seem to affect FOXO3a activity or were associated with ovarian insufficiency. Notably, primary ovarian insufficiency is a dynamic disorder and spontaneous remissions, leading to pregnancy, are not uncommon although often transient. Therefore, rather than genetic mutations, perturbation in the mechanisms that control FOXO3a expression or activity are more likely implicated in this distressing disorder.

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CHAPTER 18

The "O" Class:
Crafting Clinical Care with FoxO Transcription Factors

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Abstract

Forkhead Transcription Factors: Vital Elements in Biology and Medicine provides a unique platform for the presentation of novel work and new insights into the vital role that forkhead transcription factors play in both cellular physiology as well as clinical medicine. Internationally recognized investigators provide their insights and perspectives for a number of forkhead genes and proteins that may have the greatest impact for the development of new strategies for a broad array of disorders that can involve aging, cancer, cardiac function, neurovascular integrity, fertility, stem cell differentiation, cellular metabolism, and immune system regulation. Yet, the work clearly sets a precedent for the necessity to understand the cellular and molecular function of forkhead proteins since this family of transcription factors can limit as well as foster disease progression depending upon the cellular environment.

With this in mind, our concluding chapter for *Forkhead Transcription Factors: Vital Elements in Biology and Medicine* offers to highlight both the diversity and complexity of the forkhead transcription family by focusing upon the mammalian forkhead transcription factors of the O class (FoxOs) that include FoxO1, FoxO3, FoxO4, and FoxO6. FoxO proteins are increasingly considered to represent unique cellular targets that can control numerous processes such as angiogenesis, cardiovascular development, vascular tone, oxidative stress, stem cell proliferation, fertility, and immune surveillance. Furthermore, FoxO transcription factors are exciting considerations for disorders such as cancer in light of their pro-apoptotic and inhibitory cell cycle effects as well as diabetes mellitus given the close association FoxOs hold with cellular metabolism. In addition, these transcription factors are closely integrated with several novel signal transduction pathways, such as erythropoietin and Wnt proteins, that may influence the ability of FoxOs to lead to cell survival or cell injury. Further understanding of both the function and intricate nature of the forkhead transcription factor family, and in particular the FoxO proteins, should allow selective regulation of cellular development or cellular demise for the generation of successful future clinical strategies and patient well-being.

Abbreviations

Aβ, β-amyloid; Akt, protein kinase B; AFX, acute leukemia fusion gene located in chromosome X; AGC, protein kinase A/protein kinase G/protein kinase C; CBP, CREB-binding protein; DAF-16, DAuer Formation-16; DM, diabetes mellitus; EPO, erythropoietin; FKHR, forkhead in rhabdomyosarcoma; FKHRL1, forkhead in rhabdomyosarcoma like protein 1; IKK, IκB kinase; IGF-1, insulin-like growth factor-1; IGFBP1, insulin-like growth factor binding protein-1; IRS, insulin-responsive sequence; JNK, Jun N-terminal kinase; NF-κB, nuclear factor-κB; PCP, planar cell polarity; PS, phosphatidylserine; PTEN, tumor suppressor phosphatase and tensin homolog deleted on chromosome ten; PSR, phosphatidylserine receptor; ROS, reactive oxygen species; *Wg, Drosophila Wingless.*

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Introduction

Clinical care for many disease entities requires new therapeutic strategies that focus upon a number of pathways and systems in the body to modulate cellular proliferation, metabolism, inflammation, and longevity. In this respect, members of the mammalian forkhead transcription factors of the O class (FoxOs) that include FoxO1, FoxO3, FoxO4, and FoxO6 have been identified as important regulators of cellular proliferation, function, and demise. These transcription factors are increasing considered as potential clinical targets for multiple disorders since they control processes associated with angiogenesis, stem cell proliferation, cardiovascular injury, neurodegeneration, tumorigenesis, and cell longevity. More than 100 forkhead genes and 19 human subgroups that range from FOXA to FOXS are now known to exist since the initial discovery of the fly *Drosophila melanogaster gene forkhead*.[1] The prior nomenclature for these proteins, such as forkhead in rhabdomyosarcoma (*FKHR*), the Drosophila gene fork head (*fkh*), and Forkhead RElated ACtivator (FREAC)-1 and -2, has been replaced. The current nomenclature for human Fox proteins places all letters in uppercase, otherwise only the initial letter is listed as uppercase for the mouse, and for all other chordates the initial and subclass letters are in uppercase.[2] Initially, the FoxOs were first reported in fusion genes in human soft-tissue tumors and leukemias. FOXO1, termed forkhead in rhabdomyosarcoma (FKHR), and FOXO3a, also known as FKHRL1 (forkhead in rhabdomyosarcoma like protein 1), and their genes were identified through chromosomal translocations in alveolar rhabdomyosarcoma tumors.[3] The acute leukemia fusion gene located in chromosome X (*AFX*), also known as the *FOXO4* gene, was described as a gene that fused to MLL transcription factor as a result of the *t(X; 11)* chromosomal translocation in acute lymphoblastic leukemia.[4] A fusion between FOXO2 and MLL also occurs in some cases of acute myeloid leukemia that also is believed to be identical to FOXO3a.[5]

FoxO Protein Expression

FoxO proteins are found throughout the body and are expressed in tissues of the reproductive system of males and females, skeletal muscle, the cardiovascular system, lung, liver, pancreas, spleen, thymus, and the nervous system.[6-11] Since FoxO proteins are not equally expressed in all tissues, it is possible that individual FoxO proteins may have specificity in regards to cellular function. For example, FoxO6 expression is found in several regions of the brain that play a significant role in cognitive function and emotion, such as the hippocampus, the amygdala, and the nucleus accumbens.[9] In contrast, FoxO1 may be more suited for the control of motor function and memory formation, since the expression of this protein is primarily in the striatum and sub-regions of the hippocampus.[9] In addition, FoxO3 is more diffusely represented in the hippocampus, cortex, and cerebellum, suggesting a complementary role for this FoxO protein to control cognitive and motor function. FoxO expression can be variable in other tissues. Although studies in mice have shown that the mRNA distribution of Foxo1, Foxo3a, and Foxo4 is similar in the embryo and adult,[7] Foxo1 expression was highest in adipose tissue, Foxo3a expression was greatest in the liver, and Foxo4 expression was strongest in muscle.[7] Subsequent work in mice has described Foxo1 expression in all tissues with high levels in the ovaries.[12] Foxo3a also was found to be expressed in all tissues and Foxo4 expression was considered to be more tissue specific in skeletal muscle.[12]

FoxO Protein Structure and Function as Transcription Factors

Forkhead proteins function as transcription factors to either inhibit or activate target gene expression.[13] As a result, these proteins must bind to DNA through the forkhead domain that relies upon fourteen protein-DNA contacts. The forkhead domain in Fox proteins consists of three α-helices, three β-sheets, and two loops that are referred to as the wings,[14] but not all winged helix domains are considered to be Fox proteins.[15] On X-ray crystallography[14] or nuclear magnetic resonance imaging,[16] the forkhead domain is described as a "winged helix" as a result of a butterfly-like appearance. High sequence homology is present in the α-helices and β-sheets with variations described in either absent β-sheets and loops or additional α-helices. Although

both the first and second loops make contact with DNA, it is the second loop that can influence the stability of DNA binding. In addition, posttranslational modification of FoxO proteins, such as phosphorylation or acetylation that block FoxO activity, alter the binding of the C-terminal basic region to DNA to prevent transcriptional activity.[17] However, other mechanisms may influence DNA binding of forkhead proteins, such as variations in the N-terminal region of the DNA recognition helix, changes in electrostatic distribution, and the ability of forkhead proteins to be shuttled to the cell nucleus.[10,18]

FoxO Proteins, Posttranslational Modulation, Novel Signal Transduction Pathways, and Cell Cycle Regulation

Posttranslational modulation of FoxO proteins involves pathways associated with phosphorylation, acetylation, and ubiquitylation (Fig. 1).[3,10,19-21] The serine-threonine kinase protein kinase B (Akt) is a primary mediator of phosphorylation of FoxO1, FoxO3a, and FoxO4 that can block activity of these proteins.[3,22] Activation of Akt is usually cytoprotective, such as during hyperglycemia,[23] hypoxia,[24] β-amyloid (Aβ) toxicity,[25] cardiomyopathy,[26] cellular aging,[27] and oxidative stress.[28-30] Akt can prevent cellular apoptosis through the phosphorylation of FoxO proteins.[31] Posttranslational phosphorylation of FoxO proteins will maintain FoxO transcription factors in the cytoplasm by association with 14-3-3 proteins and prevent the transcription of pro-apoptotic target genes.[32,33] An exception to these observations involving the subcellular trafficking of FoxO proteins involves FoxO6. This FoxO protein usually resides in the nucleus of cells and is phosphorylated by Akt in the nucleus. FoxO6 does not contain a conserved C-terminal Akt motif which limits nuclear shuttling of this protein, but FoxO6 transcriptional activity can be blocked by growth factors independent of shuttling to the cytosol through a FoxO6 N-terminal Akt site.[34]

Modulation of Akt activity also oversees apoptotic pathways of caspases that may offer an alternative mechanism to regulate FoxO proteins (Fig. 1).[35] Caspases are a family of cysteine proteases that are synthesized as inactive zymogens that are proteolytically cleaved into subunits at the onset of apoptosis.[36-38] The caspases 1 and 3 have been linked to the apoptotic pathways of genomic DNA cleavage, cellular membrane PS exposure, and activation of inflammatory cells.[39-41] Caspase pathways may be tied to the forkhead transcription factor FoxO3a since increased activity of FoxO3a can result in cytochrome c release and caspase-induced apoptotic death.[32,42-44] Pathways that can inhibit caspase 3 activity appear to offer a unique regulatory mechanism. For example, cell death pathways that rely upon FoxO3a also appear to involve caspase 3 activation. Prior studies suggest that not only does FoxO3a activity promote caspase-induced apoptotic death,[32,42-44] but also demonstrate that inhibition of caspase 3 has been shown to maintain the phosphorylated "inactive" state of FoxO3a to prevent cell injury.[32,42,43] Other work has shown that caspase 3 activity and cleavage is promoted during transfection of a triple mutant FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of FoxO3a.[45] Recent work adds further insight to these studies by illustrating that FoxO3a may control early activation and subsequent apoptotic injury in microglia during amyloid (Aβ) exposure through caspase 3 (Fig. 2).[46] Since Aβ exposure can facilitate the cellular trafficking of FoxO3a from the cytoplasm to the cell nucleus to potentially lead to "pro-apoptotic" programs by this transcription factor,[46] one program in particular that may be vital for apoptotic injury appears to involve the activation of caspase 3. Aβ exposure leads to a rapid and significant increases in caspase 3 activity with 6 hours following Aβ administration, but that this induction of caspase 3 activity by Aβ requires FoxO3a, since loss of FoxO3a through gene silencing prevents the induction of caspase 3 activity by Aβ.

Posttranslational modulation of FoxO proteins also requires pathways associated with ubiquitylation and acetylation.[47,48] Akt phosphorylation of FoxO proteins not only retains these transcription factors in the cytoplasm, but also leads to ubiquitination and degradation through the 26S proteasome 19, 48. In the absence of Akt, IκB kinase (IKK) also can directly phosphorylate and block the activity of FoxO proteins, such as FoxO3a.[3,10] This leads to the proteolysis of FoxO3a via the Ub-dependent proteasome pathway.[3,10,19-21] The serum- and

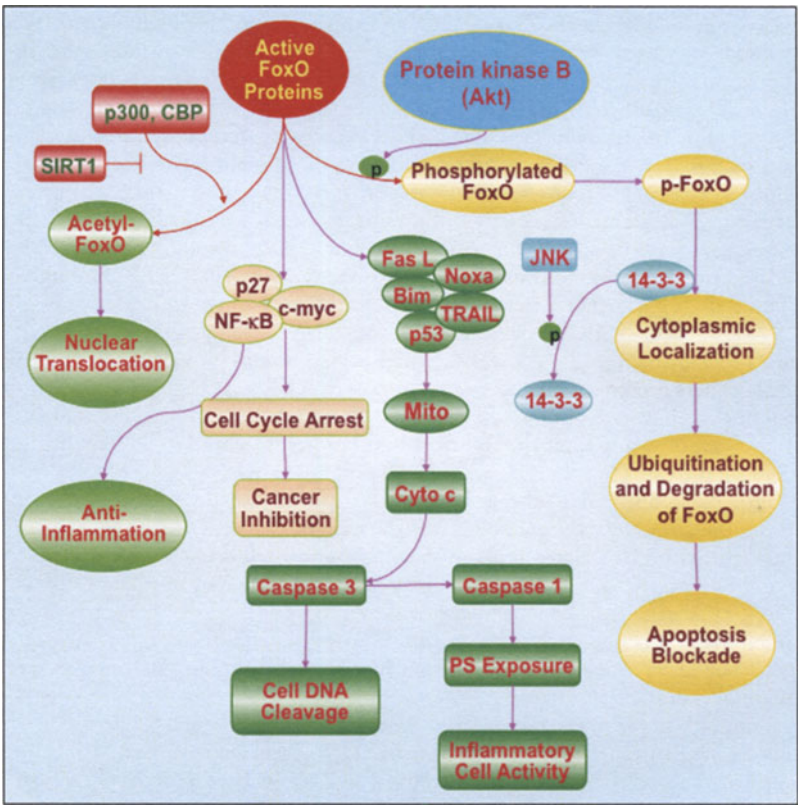
glucocorticoid-inducible protein kinase (Sgk), a member of a family of kinases termed AGC (protein kinase A/protein kinase G/protein kinase C) kinases which includes Akt, also can phosphorylate and retain FoxO3a in the cytoplasm.[49] Knowledge that Sgk and Akt can phosphorylate FoxO3a at different sites may offer new opportunities to more effectively prevent apoptotic cell injury that may be mediated by FoxO3a activity. Yet, phosphorylation of FoxO proteins does not always lead to negative regulation. The protein kinase mammalian sterile 20-like kinase-1 also can phosphorylate FoxO proteins directly and lead to their activation.[50] The ability of sterile 20-like kinase-1 to activate FoxO proteins may be linked to c-Jun N-terminal kinase (JNK), since sterile 20-like kinase-1 can increase JNK activation.[51] FoxO proteins also are acetylated by histone acetyltransferases that include p300, the CREB-binding protein (CBP), and the CBP-associated factor and are deacetylated by histone deacetylases, such as Sirt1, a NAD+-dependent deacetylase and the mammalian ortholog of the silent information regulator 2 (Sir2) protein (Fig. 1).[10] Acetylation of FoxO proteins provides another avenue for the control of these proteins. Once acetylated such as by CBP, FoxO proteins may translocate to the cell nucleus but have diminished activity since acetylation of lysine residues on FoxO proteins has been shown to limit the ability of FoxO proteins to bind to DNA.[52] In addition, acetylation can increase phosphorylation of FoxO proteins by Akt.[52]

FoxO proteins are also tied to other unique signal transduction pathways that involve proteins derived from the *Drosophila Wingless* (*Wg*) and the mouse *Int-1* genes.[20] The Wnt proteins are secreted cysteine-rich glycosylated proteins that can control cell proliferation, differentiation, survival, and tumorigenesis.[53,54] More than eighty target genes of Wnt signaling pathways have been demonstrated in human, mouse, *Drosophila*, Xenopus, and zebrafish. These genes are present in several cellular populations, such as neurons, cardiomyocytes, endothelial cells, cancer cells, and pre-adipocytes.[55] At least nineteen of twenty-four Wnt genes that express Wnt proteins have been identified in the human.[53,54,56]

The canonical Wnt pathway controls target gene transcription through β-catenin.[53,54] It is the β-catenin pathway that appears to tie FoxO proteins and Wnt signaling together.[57] For example, in relation to Alzheimer's disease, Aβ is toxic to cells,[25,58] and is associated with the phosphorylation of FoxO1 and FoxO3a that can be blocked with ROS scavengers.[59] A common denominator in the pathways linked to Ab toxicity involves Wnt signaling through β-catenin. β-catenin may increase *FoxO* transcriptional activity and competitively limit β-catenin interaction with members of the lymphoid enhancer factor/T cell factor family[60] and β-catenin also has been demonstrated to be necessary for protection against Aβ toxicity in neuronal cells.[58]

Additional shared signal transduction pathways between Wnt and FoxO proteins involve Akt. Processes that involve cellular proliferation, injury, and immune system modulation with FoxO proteins[61] also have parallel cellular pathways with Wnt and Akt. For example, Wnt relies upon Akt for the proliferation and differentiation of cardiomyocytes.[62] In addition, reduction in tissue injury during pressure overload cardiac hypertrophy and the cytoprotective benefits of cardiac ischemic preconditioning also appear to depend upon Akt.[53,54] Furthermore, Wnt over-expression can independently increase the phosphorylation and the activation of Akt to promote cellular protection and control microglial activation.[58]

Yet, other members of the forkhead family in addition to FoxOs also rely upon Wnt signaling in several scenarios that involve regulated as well as unchecked cell proliferation.[53,54,63] For example, FoxD3 is activated by the Wnt pathway to control neural plate development[64] and Foxl1 activates the Wnt/β-catenin pathway to increase extracellular proteoglycans, promote gastrointestinal cell proliferation, and possibly foster carcinogenesis.[65] The Wnt pathway also utilizes forkhead members to modulate endocrine activity and can activate Foxn1 for regulatory control of thymic function.[66] In other examples of cell development, Wnt signaling has been shown to rely upon Foxf1 and Foxf2 during intestinal maturation in murine models.[67] In addition, Foxa2 in mice may be a significant component in early anterior-posterior axis polarization.[68] Deregulation of Wnt alone also promotes activation of β-catenin that has been associated with the proliferation of medulloblastoma tumors.[69] In addition, reduced expression of inhibitors of the Wnt pathway, such as axin, may



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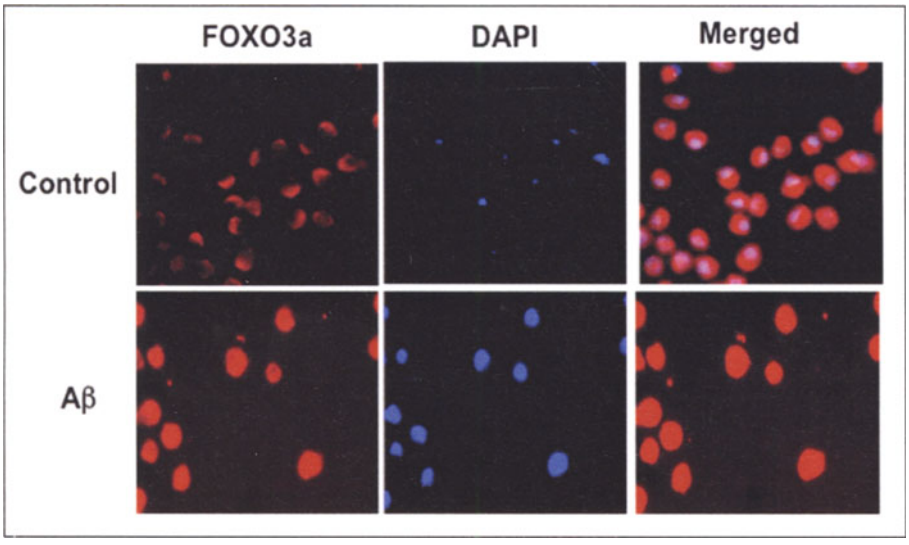

Figure 2. Please see figure legend on following page.

Figure 1, viewed on previous page. Posttranslational modulation of FoxO proteins is associated with intricate cellular signal transduction pathways. Posttranslational modulation of FoxO proteins involves pathways associated with phosphorylation, acetylation, and ubiquitylation. Protein kinase B (Akt) can prevent cellular apoptosis through the phosphorylation of FoxO proteins and phosphorylation (p) of FoxO proteins will inhibit FoxO transcription factors through cytoplasmic localization by association with 14-3-3 proteins and prevent the transcription of target genes that lead to apoptosis. If activated, FoxOs can prevent inflammatory cell activation through the inhibition of nuclear factor-κB (NF-κB) and may control inflammatory cell activation through membrane phosphatidylserine (PS) externalization. FoxO proteins can lead to apoptotic death pathways that involve mitochondrial (Mito) release of cytochrome c (Cyto c) and caspase activation through a Fas-mediated ligand (Fas L) death pathway, tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), BH3-only proteins Noxa and Bim, or p53. Cell cycle inhibition that blocks tumor growth through FoxO protein activation may require c-myc, p27, and NF-κB.

foster lung cancer cell invasion.[70] Multiple other studies also point to the activation of the Wnt pathway during gastric cancer. For example, Wnt5a expression has been correlated with advanced gastric cancer stages and a poor prognosis[71] while experimental activation of the β-catenin pathway leads to the development of gastric tumors.[72] In conjunction with forkhead proteins, loss of Foxl1 that can regulate the Wnt pathway and prevent β-catenin nuclear accumulation is believed to be a significant etiology for gastrointestinal tumorigenesis.[65]

FoxO proteins also appear to be ideal to cellular proliferation not only through Wnt mediated pathways, but also through the blockade of cell cycle progression. For example, FoxO3a and FoxO4 can promote cell cycle arrest in mouse myoblastic cell lines through modulation of growth-arrest and DNA-damage-response protein 45.[10,73] Treatment of chronic myelogenous leukemia cell lines with the Bcr-Abl tyrosine kinase inhibitor imatinib requires FoxO3a activation to antagonize cell proliferation and promote apoptotic cell death through increased TRAIL production.[74] In addition, the transcription factor E2F-1 that controls the induction of the cell cycle has been reported in cell lines to increase the endogenous expression of FoxO1 and FoxO3a to lead to cell cycle arrest.[75] In contrast, the loss of FoxO3a activity in association with c-myc, p27, and nuclear factor-κB (NF-κB) can result in cell cycle induction and malignant transformation of mouse cells in the presence of oncogene activation (Fig. 1).[3,10] Other work suggests that FoxO proteins utilize the p53 upstream regulator p19(Arf) through myc to block cell cycle induction and lymphoma progression.[76]

FoxO Proteins, Apoptosis, and Oxidative Stress

Although genes linked to apoptosis sometimes foster cellular proliferation rather than cell death, cellular apoptosis can become a significant component for pathology in diseases such as neurodegenerative disease, diabetes mellitus (DM), and cardiovascular injury.[77] More importantly, regulation of apoptotic pathways appears to serve a critical juncture for the control of tumor growth and unregulated cell proliferation.[10,57] Apoptotic cell death is considered to be a dynamic process that involves both early and late events. Membrane phosphatidylserine (PS) externalization is an early event during cell apoptosis that assists microglia to target cells for phagocytosis.[29,78] This process occurs with the expression of the phosphatidylserine receptor (PSR) on microglia during oxidative stress,[79-81] since blockade of PSR function in microglia prevents the activation of microglia.[30,40] As an example, externalization of membrane PS residues occur in cells during periods of oxidative stress that involve anoxia,[58] reactive oxygen species (ROS) exposure,[82] and with agents that produce ROS, such as 6-hydroxydopamine.[83] In contrast to cells with PS exposure,

Figure 2, viewed on previous page. During amyloid ($A\beta_{1-42}$) exposure in inflammatory microglial cells, FoxO3a translocates to the cell nucleus to govern an initial activation and proliferation of microglial cells. Microglia were followed at 6 hours after $A\beta_{1-42}$ (10 μM) (Aβ) administration with immunofluorescent staining for FoxO3a (Texas-red). Nuclei of microglia were counterstained with DAPI. In merged images, control cells have readily visible nuclei (white in color) that illustrate absence of FoxO3a in the nucleus. In contrast, merged images after $A\beta_{1-42}$ (10 μM) exposure are not visible (red in color) and demonstrate translocation of FoxO3a to the nucleus. Control = untreated microglia.

the cleavage of genomic DNA into fragments is considered to be a later event during apoptotic injury.[77,84] Endonucleases responsible for DNA degradation have been identified and include the acidic, cation independent endonuclease (DNase II), cyclophilins, and the 97 kDa magnesium—dependent endonuclease. In the nervous system, endonucleases include a constitutive acidic cation-independent endonuclease, a constitutive calcium/magnesium-dependent endonuclease, and an inducible magnesium-dependent endonuclease.[77,84]

Interestingly, the induction of apoptosis in cells through FoxO proteins may require pathways aligned with oxidative stress. Oxidative stress is a result of the release of reactive oxygen species (ROS) that consist of oxygen free radicals and other chemical entities. Oxygen free radicals and mitochondrial DNA mutations have become associated with tissue injury, aging, and accumulated toxicity for an organism.[77] ROS include superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide, and peroxynitrite.[84] Most reactive species are produced at low levels during normal physiological conditions and are scavenged by endogenous antioxidant systems that include superoxide dismutase, glutathione peroxidase, catalase, and small molecules, such as vitamins C, E, D3 and nicotinamide, the amide form of niacin or vitamin B3.[78,85,86] During periods of oxidative stress, FoxO transcription factors can lead to apoptosis,[31] since forkhead transcription factors such as FoxO1 and FoxO3a must be present for oxidative stress to result in apoptotic cell injury.[87] Under other conditions of oxidative stress, FoxO3a in conjunction with JNK have been shown to modulate an apoptotic ligand activating a Fas-mediated death pathway in cultured motoneurons,[88] to lead to apoptosis through tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) and BH3-only proteins Noxa and Bim in neuroblastoma cells,[44] and to promote pro-apoptotic activity of p53.[89] Additional work shows that loss of FoxO expression during oxidative stress is protective to cells. For example, protein inhibition or gene knockdown of FoxO1 or FoxO3a can lead to reduction in ischemic infarct size in the brain,[90] mediate protection of metabotropic glutamate receptors during vascular injury,[42] enhance pancreatic β-cell or neuronal survival through NAD+ precursors during oxidative stress,[43] and provide trophic factor protection with erythropoietin (EPO)[32] and neurotrophins.[91] Yet, it should be noted that some studies suggest that the loss of FoxO1, FoxO3a, and FoxO4 protein expression may actually lead to an increase in free radical release that can be responsible for oxidative stress.[92] In addition, FoxO proteins may be protective during aging and exercise, since FoxO3a activity may enhance vascular smooth muscle antioxidant properties in aged animals and be beneficial to the cardiovascular system during physical exertion.[93]

FoxO Proteins, Metabolism and Cell Longevity

Clinical and experimental studies highlight the role of FoxO proteins during cellular metabolism and cellular longevity. When one considers DM, this disorder is a significant health concern for both young and older populations.[94,95] Approximately 16 million individuals in the United States and more than 165 million individuals worldwide suffer from DM. By the year 2030, it is predicted that more than 360 million individuals will be afflicted with DM and its debilitating conditions. Type 2 DM represents at least 80% of all diabetics and is dramatically increasing in incidence as a result of changes in human behavior and increased body mass index.[85,94] Type 1 insulin-dependent DM is present in 5-10% of all diabetics, but is increasing in adolescent minority groups.[85,94] Furthermore, the incidence of undiagnosed diabetes and impaired glucose tolerance in the population raises additional concerns.

Patients with DM can develop significant neurodegenerative[55,85,96] and cardiovascular disease.[85,97] Interestingly, the development of insulin resistance and the complications of DM can be the result of cellular oxidative stress.[85,94] Hyperglycemia can lead to increased production of ROS in endothelial cells, liver cells, and pancreatic β-cells.[85,94,95] Recent clinical correlates support these experimental studies to show that elevated levels of ceruloplasmin are suggestive of increased ROS.[85,94,95] Furthermore, acute glucose swings in addition to chronic hyperglycemia can trigger oxidative stress mechanisms, illustrating the importance for therapeutic interventions during acute and sustained hyperglycemic episodes.[85,94]

Early work with FoxO proteins has shown that metabolic signaling with these transcription factors is conserved among multiple species including *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals. FoxO proteins are homologous to the transcription factor DAuer Formation-16 (DAF-16) in the worm *Caenorhabditis elegans* that can determine metabolic insulin signaling and lead to lifespan extension,[98,99] suggesting a significant role for FoxO proteins in relation to mammalian cell function.[3,10] In fact, FoxO proteins can stimulate the insulin-like growth factor binding protein-1 (IGFBP1) promoter by binding to the insulin-responsive sequence (IRS).[100] Both insulin and insulin-like growth factor-1 (IGF-1) can suppress this activity through activation of Akt.[100,101]

In clinical studies, analysis of the genetic variance in *FOXO1a* and *FOXO3a* on metabolic profiles, age-related diseases, fertility, fecundity, and mortality have observed higher HbA_{1c} levels and increased mortality risk associated with specific haplotypes of *FOXO1a*.[102] These clinical observations may coincide with the demonstration in human endothelial progenitor cells that elevated glucose levels can reduce posttranslational phosphorylation of FOXO1, FOXO3a, and FOXO4 and allow for the nuclear translocation of these proteins to initiate an apoptotic program in endothelial progenitor cells.[103] In experimental models, FoxO proteins may prevent the toxic effects of high serum glucose levels. Interferon-gamma driven expression of tryptophan catabolism by cytotoxic T lymphocyte antigen 4 may activate Foxo3a to protect dendritic cells from injury in nonobese diabetic mice.[104] Additional studies have demonstrated that adipose tissue-specific expression of Foxo1 in mice improved glucose tolerance and sensitivity to insulin during an elevated fat diet.[105] FoxO proteins also may protect against diminished mitochondrial energy levels known to occur during insulin resistance such as in the elderly populations.[85,94,95] In caloric restricted mice that have decreased energy reserves, Foxo1, Foxo3a, and Foxo4 mRNA levels were noted to progressively increase over a two-year course.[8] These observations complement studies in *Drosophila* and mammalian cells that demonstrate an increase in insulin signaling to regulate cellular metabolism during the up-regulation of FoxO1 expression.[106]

However, the ability for FoxO proteins to maintain proper physiologic controls over cellular metabolism may be limited and occur only during specific circumstances. For example, mice with a constitutively active Foxo1 transgene have increased microsomal triglyceride transfer protein and elevated plasma triglyceride levels.[107] Studies in cardiomyocytes also suggest detrimental results with enhanced FoxO activity. Increased transcriptional activity of FoxO1, such as by the Sirt1 activator resveratrol, can diminish insulin mediated glucose uptake and result in insulin resistance.[108] In addition, over-expression of Foxo1 in skeletal muscles of mice can lead to reduced skeletal muscle mass and poor glycemic control,[109] illustrating that activation of FoxO proteins also may impair cellular energy reserves. Additional investigations that block the expression of Foxo1 in normal and cachectic mice[110] or reduce FoxO3 expression[111] show the reverse with an increase in skeletal muscle mass or resistance to muscle atrophy. These results become especially relevant in patients with cancer and cachexia, since FoxO protein expression may further muscle wasting for these individuals. Given these concerns, one potential agent to consider for the maintenance of cellular metabolism in cancer patients is nicotinamide,[36,78] an agent that also can inhibit FoxO protein activity.[43] In patients with DM, oral nicotinamide protects β-cell function, prevents clinical disease in islet-cell antibody-positive first-degree relatives of Type-1 DM, and can reduce HbA1c levels.[36,78,94] Nicotinamide, which is closely linked to cell longevity pathways,[112,113] may derive its protective capacity through two separate mechanisms of posttranslational modification of FoxO3a. Nicotinamide not only can maintain phosphorylation of FoxO3a and inhibit its activity, but also can preserve the integrity of the FoxO3a protein to block FoxO3a proteolysis that can yield pro-apoptotic amino-terminal fragments.[43]

As an extension to the work with cellular metabolism, FoxO proteins also have been linked to cell longevity and aging as shown by early studies linking DAF-16 in *Caenorhabditis elegans* to increased longevity.[3,19,21,114] However, the relationship between FoxO transcription factors and proteins that increased cellular lifespan has been met with controversy. Sirt1 is a NAD^+-dependent deacetylase and the mammalian ortholog of the silent information regulator

2 (Sir2) protein associated with increased lifespan in yeast. Some studies suggest that stimulation of Sirt1 during starvation is dependent upon FoxO3a activity as well as p53.[115] In contrast, other work has shown in cell culture that Sirt1 may repress the activity of FoxO1, FoxO3a, and FoxO4, suggesting that cellular longevity may benefit from reduction in FoxO protein generated apoptosis.[116] Additional studies offer alternative views to illustrate that Sirt1 binds to FoxO proteins, such as FoxO4, to catalyze its deacetylation and enhance FoxO4 activity while acetylation of FoxO4 by cyclic-AMP responsive element binding (CREB)-binding protein serves to inhibit FoxO4 transcriptional activity.[3,19,21,114]

FoxO proteins also may be protective during aging, cell senescence, and exercise. In cultured human dermal fibroblasts, gene silencing of FoxO3a protein results in cell morphology consistent with cell senescence, cell population doubling times, and the generation of ROS, suggesting that FoxO protein activity may be required to extend cell longevity and limit oxidative stress.[117] Additional work in animal models of aging demonstrates a reduction in Sirt1 in the heart, but no significant change in FoxO3a expression with advanced age. However, during exercise training, an up-regulation of FoxO3a and Sirt1 activity is observed in the heart,[93] suggesting that the benefits of physical activity for the cardiovascular system may be associated with FoxO proteins. Interestingly, increased levels of Sirt1 less than 7.5-fold can be associated with expression of catalase, an anti-oxidant that is controlled by FoxO1a to possibly reduce cell injury during oxidative stress. Yet, elevated levels of Sirt1 at 12.5-fold can result in cardiomyocyte apoptosis and decreased cardiac function.[118] In addition, FoxO proteins may be protective during aging, since loss of FoxO3a activity in explanted vascular smooth muscle of aged animals may limit tissue antioxidant properties through decreased manganese-superoxide dismutase and lead to enhanced cell injury with aging.[119] Extension of cellular lifespan that depends upon the prevention of cell senescence at least in primary human cultured vascular cells also may require the negative regulation of Akt to allow for the activation of FoxO3a.[120]

FoxO Proteins, Stem Cells and Cardiovascular Development

FoxO proteins represent important targets for several disorders, but high on the list may be therapies to block cancer growth since FoxO proteins can modulate stem cell proliferation and new vessel growth. The initial identification of FoxO proteins in soft-tissue tumors and leukemias, neoplasms now believed to harbor cancer stem cells for tumor self-renewal,[69] suggests that FoxO proteins may be closely associated with the oversight of stem cell proliferation and differentiation. For example, either simultaneous deletion of *Foxo1*, *Foxo3a*, and *Foxo4* or single deletion of *Foxo3a* in mice prevents the repopulation of hematopoietic stem cells and leads to apoptosis in these stem cell populations.[92,121] Furthermore, vascular cytoprotective agents, such as the growth factor EPO,[33,122,123] also may be required to modulate FoxO protein activity such as during erythroid progenitor cell development,[73,124] suggesting that current clinical use of agents such as EPO during anemia or cancer may have less defined treatment implications for patients than originally anticipated.[33,124] In cell culture and animal studies, EPO is cytoprotective in vascular cells and can stimulate postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow.[73,124,125] Interestingly, the ability of EPO to foster eythroid progenitor cell development is dependent upon the inhibition of FoxO3a activity,[33,124] but also may require regulation of specific gene expression through an EPO-FoxO3a association to promote erythropoiesis in cultured cells.[126] In relation to the reproductive potential of an organism, deletion of the *FoxO3a* gene results in the depletion of oocytes and subsequent infertility.[127] Other work using a mouse model of FoxO3a over-expression in oocytes further suggests that FoxO3a retards oocyte growth and follicular development and leads to anovulation and luteinization of unruptured follicles.[128] These studies may suggest a role for FoxO proteins, and specifically FoxO3a, in relation to not only the development of cancer stem cell niches, but also in regards to oocyte and follicular cell maturation. For example, in a small percentage of women who suffer from premature ovarian failure mutations in *FOXO3a* and *FOXO1a* have been observed.[129]

In addition to the modulation of stem cell development, FoxO proteins play a significant role to govern new vessel growth that can impact upon tumor cell growth and dispersion. New capillary formation from pre-existing vessels into an avascular area is a process known as angiogenesis that is present during embryogenesis, during menstruation, and during pathological processes that involve wound healing, chronic inflammation, and tumor growth.[54,124] FoxO proteins are intimately involved in endothelial cell development and angiogenesis. For example, *Foxo3a -/-* and *Foxo4 -/-* mice develop without incidence and are indistinguishable from control littermates. However, mice that are singly deficient in *Foxo1* die by embryonic day eleven and lack development of the vascular system.[130] Additional studies illustrate that endothelial cell colonies in *Foxo1*-deficient mice fail to respond to vascular endothelial growth factor in a manner similar to wild-type endothelial cells,[131] suggesting that FoxOs are necessary for the development of vascular cells as well as for the biological response to cellular mediators.

During cardiac development, FoxO proteins also appear to be necessary to modulate cardiomyocyte proliferation. Both FoxO1 and FoxO3 are expressed during embryonic through prenatal stages in the developing myocardium. The expression of these FoxO proteins is believed to negatively regulate cardiomyocyte growth, since overexpression of FoxO1 blocks cardiomyocyte proliferation but expression of dominant negative FoxO1 leads to enhanced cardiomyocyte growth.[132] These observations may provide clues into the roles of FoxO proteins during cardiac hypertrophy. Atrogin-1, a protein that can block cardiac hypertrophy, may rely upon the up-regulation of Foxo1 and Foxo3a to disrupt cardiac hypertrophy, since mice lacking atrogin-1 are susceptible to cardiac hypertrophy and do not yield increased expression of Foxo1 and Foxo3a.[133]

In regards to smooth muscle cell growth, Foxo3a has been demonstrated to block vascular smooth muscle proliferation and may lessen the effects from disorders such as atherosclerosis and hypertension. In a rat balloon carotid arterial injury model, gene transfer of FoxO3a can inhibit neointimal hyperplasia through the prevention of vascular smooth muscle growth.[134] However, not all FoxO proteins may exert an inhibitory effect upon vascular smooth muscle cells. FoxO4 may inhibit smooth muscle cell differentiation through the repression of the transcriptional coactivator of smooth muscle genes myocardin,[135] but other work suggests that FoxO4 also can increase matrix metalloproteinase 9 expression to promote vascular smooth muscle migration and foster neointimal hyperplasia.[136]

In light of the ability of FoxO proteins to regulate vascular smooth muscle cell proliferation, these transcription factors may have a significant clinical role in regards to disorders that involve hypertension and cardiac failure. Vascular smooth muscle cells are vital for the regulation of vascular tone and systemic arterial blood pressure. For example, high flow states in vessels can reduce FoxO1 activity, resulting in the potential proliferation of vascular smooth muscle cells, vascular neointimal hyperplasia, and subsequent pathological states such as hypertension.[137] In fact, α1-adrenergic agonists that increase systemic blood pressure can have the reverse effect and stimulate the expression of FoxO1 and its nuclear translocation that ultimately may lead to apoptotic endothelial cell injury.[138] In addition, more than moderate levels of vessel cyclic stretch that can occur during hypertension may lead to the phosphorylation and inhibition of Foxo1 and Foxo3a in smooth muscle cells to further contribute to pathological smooth muscle cell proliferation.[139] Furthermore, in human as well as murine models of cardiac failure, increased expression of Fox transcription factors, such as FoxO1a, have been observed to suggest a potential association of FoxO proteins with imminent cardiac failure.[140]

FoxO Proteins and the Immune System

Forkhead transcription factors have a vital role in maintaining immune system function. For example, the forkhead family member FoxP3 can control the development and function of thymic-derived CD4(+)CD25(+) regulatory T cells (Treg) that impart autoimmunity. Loss of FoxP3 can result in autoimmune disorders.[141] In addition, recent work identifies the expression of FoxP3 in tumor cells, such as melanoma,[142] as well as in Tregs which may significantly affect patient

mortality since the increased presence of Tregs in cancer patients combined with FoxP3 expression in tumors may impair antitumor autoimmune responses and lead to high mortality.[143]

In regards to FoxO proteins, these forkhead transcription factors also may impact upon neoplastic progression since they lead to the induction of apoptotic pathways and may influence early apoptotic membrane PS externalization (Fig. 1). The ability to regulate early apoptotic membrane PS exposure[40] and inflammatory cell activity[29] can ultimately impact upon cell survival since activated immune cells can lead to the phagocytic removal of tumor cells.[79,84] Inflammatory cells, such as macrophages or microglia, require the activation of intracellular cytoprotective pathways to proliferate and remove injured cells.[80,144] At times, this can be a beneficial process and form a barrier for the removal of foreign micro-organisms and promote tissue repair during cell injury.[73,85] However, inflammatory cells also may lead to cellular damage through the generation of ROS and through the production of cytokines.[73] Interestingly, in mice deficient for *Foxo3a*, lymphoproliferation, organ inflammation of the salivary glands, lung, and kidney, and increased activity of helper T cells results, supporting an important role for FoxO3a in preventing T cell hyperactivity.[145] FoxO3a also appears to be necessary for neutrophil activity, since *Foxo3a* null mice are resistant to models of neutrophilic inflammation that involve immune complex-mediated inflammatory arthritis.[146]

In clinical studies, patients with rheumatoid arthritis and osteoarthritis show phosphorylation of FOXO3a in T lymphocytes as well as FOXO1 and FOXO4 in synovial macrophages, suggesting that loss of functional FOXO family members may lead to inflammatory cell activation in these disorders.[147] *FOXO1* gene transcript levels also are down-regulated in peripheral blood mononuclear cells of patients with systemic lupus erythematosus and rheumatoid arthritis,[148] illustrating a potential etiology through the loss of functional FOXO proteins for these disorders and possibly providing a biomarker of disease activity. Other work has demonstrated that FOXO1 protein regulates L-selectin expression that can regulate human T lymphocyte trafficking.[149] More importantly, studies suggest a relationship between the regulation of immune system activity and the induction of apoptotic pathways that are dependent upon FoxO proteins. Prevention of inflammatory activation and apoptosis in the nervous system such as in systemic lupus erythematosus in animal models may require the up-regulation of different Fox proteins, such as FoxJ1 and FoxO3a, that can block NF-κB activation and interferon-gamma secretion.[150] FoxO proteins also may work in concert with Fas signaling to clear activated T cells following a decrease in cytokine stimulation in patients with autoimmune lymphoproliferative syndromes,[151] suggesting that activation of specific FoxO proteins may be beneficial for autoimmune disorders but may impair treatments designed to target tumor cells through immune mediated pathways.

FoxO Proteins and Cancer

As previously mentioned, one of the most important treatment possibilities for FoxO proteins involves strategies designed to control human cancer progression in light of the ability of FoxO proteins to lead to apoptosis and block cell cycle progression. For example, studies with prostate cancer have shown that the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is mutated in approximately 80% of tumors with the loss of FOXO1 and FOXO3a activity. In cell cultures, over-expression of FoxO1 and FoxO3a in prostrate tumor cell lines also leads to apoptosis, suggesting that FoxO1 and FoxO3a are necessary for limiting prostate cell tumor growth.[11] In addition, it has been shown that inhibition of FoxO3a activity can result in enhanced prostate tumor cell growth[152] while agents that increase FoxO3a activity in both androgen sensitive and androgen insensitive prostate cell lines prevent prostate cancer cell progression.[153] Furthermore, therapeutic strategies that rely upon the over-expression of a nonphosphorylatable form of FoxO3a that cannot be inactivated can sensitize prostate cancer cells to androgen-withdrawal-induced apoptosis.[154] Yet, it should be noted that in prostate cell lines FoxO3a can be a positive regulator of androgen receptor expression and therefore may play a complex role in prostate cancer cell proliferation and growth inhibition.[155] Other factors that control FoxO protein function also may play a role during prostate tumor progression. In prostate

cancer cells, cyclin-dependent kinase 1 (CDK1) can become over-expressed and subsequently phosphorylate FOXO1 to block its transcriptional activity and contribute to prostate tumorigenesis.[156] In a similar manner, it has been shown that astrocyte-elevated gene-1 (AEG-1) can be upregulated in clinical prostate cancer,[157] possibly lead to activation of Akt that suppresses FOXO3a[158] and apoptosis in prostate tumor cells.

Initial investigations of FOXO3a in clinical breast cancer suggested that activation of FOXO3a was associated with lymph nodal metastasis and a poor prognosis.[159] In contrast to these observations, other studies reported that FOXO3a was inactivated by IKK and that inactivation of FOXO3a was associated with a poor prognosis in breast cancer,[160] suggesting that FOXO3a sub-cellular localization and pathways that enhance its activity could be used not only as prognostic assays but also as therapeutic targets. Other work in breast cancer cells demonstrate the tumor repressive ability of FoxOs by illustrating that increased activity of FoxO3a in association with JNK in breast cancer cell lines[161] or in association with cyclin-dependent kinase inhibitor p27 in isolated human breast cancer cells can prevent breast cancer growth.[162] In addition, FoxO proteins may be able to modulate estrogen function and indirectly block breast cancer growth. Over-expression of FoxO3a in breast cancer cell lines can decrease the expression of estrogen receptor regulated genes and inhibits 17beta-estradiol (E2)-dependent breast cancer growth.[163]

In addition to the ability to inhibit prostate and breast tumor growth, FoxO proteins may represent a viable option to control tumor progression in other tissues. FoxO proteins can function as redundant repressors of tumor growth. For example, somatic deletion in mice of *Foxo1*, *Foxo3a*, and *Foxo4* results in the growth of thymic lymphomas and hemangiomas.[164] Other work illustrates that FoxO3a activation in colon carcinoma cell lines prevents tumor proliferation through Myc target genes that involve the Mad/Mxd family of transcriptional repressors.[165] In addition, the loss of FoxO3a activity may participate in oncogenic transformation in B-chronic lymphocytic leukemia[166] and in the progression of chronic myelogenous leukemia cell lines.[74] Furthermore, studies suggest that some proteins, such as the Kaposi's sarcoma-associated herpes virus latent protein LANA2, may specifically block the transcriptional activity of FoxO3a to lead to tumor growth.[167] In cell models of endometrial cancer, presensitization of cells to block Akt activation and foster transcription activity of FoxO1 enhances the effect of chemotherapy to limit tumor growth.[168]

Conclusions and Future Perspectives for Clinical Care

The potential translation of FoxO proteins and their signal transduction pathways into viable therapeutic strategies offer exciting prospects for the future. FoxO proteins control several vital cellular pathways in relation to cell proliferation, metabolism, inflammation, and survival. For example, the known mutations in FoxO proteins that exist in several disease entities may provide novel insights for therapeutic strategies that can address a broad range of disorders. Further analysis in larger populations of patients with premature ovarian failure, diabetes, or stroke could enhance our understanding of the role of FoxO proteins in these disorders. When one considers the role of FoxO proteins at the cellular level such as in cardiac and endothelial cells, targeting the activity of FoxO1, FoxO3a, or FoxO4 may prevent the onset of pathological cardiac hypertrophy and neointimal hyperplasia that may result in atherosclerosis. Interestingly, new work suggests that the utilization and combination of multiple biomarkers may improve risk assessment for patients suffering from cardiovascular disorders.[169] These studies illustrate that FoxO proteins may serve as biomarkers of disease activity such as in individuals with imminent cardiac failure.[140]

In regards to potential treatments directed against cancer, the ability of FoxO proteins to control cell cycle progression and promote apoptosis highlights the potential of FoxOs to become an important component for new strategies directed against tumorigenesis. For example, use of triple mutant FoxO1 or FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of this protein has been proposed as a potential therapeutic agent against melanoma tumors[45] and endometrial cancer.[170] Other work also offers additional support for the use of FoxO proteins as biomarkers of cancer progression. As an example, down regulation of the phosphatidylinositol 3 kinase and Akt pathways have been associated with increased transcript

levels for FOXO1a and FOXO3a in clinical prostate cancer samples and may indicate the onset of precancerous changes or the progression of on-going tumor growth.[171] Although loss of Akt activity in prostate cancer cells can result in enhanced FoxO3a activity and subsequent apoptosis of tumor cells,[157] it is conceivable that early stages of cancer may lead to reduced Akt activity with insufficient levels of active forkhead transcription factors to limit tumor progression. In addition, the early and persistent expression of phosphorylated FOXO1a in gastric tumors may not only indicate the onset of cancer, but also suggest an improved prognosis for patients.[172]

Despite the presently known attributes of FoxO proteins to potentially treat a number of disorders, FoxO transcription factors also may limit clinical utility. Further investigations are required since FoxO protein inhibition of cell cycle progression may not consistently lead to apoptotic cell death. Some investigations suggest that during oxidative stress, FoxO3a activation in association with Sirt1 can lead to cell cycle arrest, but not result in apoptotic cell injury.[173] Furthermore, during hypoxic stress, forkhead transcription factors, such as FOXO3a, may potentiate anti-apoptotic pathways in breast cancer cells to further tumor growth.[174] FoxO proteins also have been linked to potential chemotherapy drug resistance. Increased expression of MDR1 (P-glycoprotein) has been associated with chemotherapy drug resistance in breast cancer cells and recent work shows that FoxO1 can stimulate the transcriptional activity of MDR1 that may promote increased tolerance of tumor cells.[175] In addition, the common pathways shared between Wnt and forkhead proteins may have another side that impacts upon the ability to control tumor growth.[53,63] FoxO proteins may assist with β-catenin activation in the Wnt pathway and lead to tumor cell proliferation.[54] In the presence of Wnt deregulation and increased β-catenin activity, tumorigenesis may ensue, such as with the proliferation of medulloblastoma tumors.[69] Therefore, prediction of biological outcomes during FoxO protein involvement may be uncertain and may be influenced by a host of factors such as tissue characteristics, cellular metabolic state, and the age of an individual. Given these circumstances, further basic and clinical investigations will be required to continue to elucidate the immense potential of FoxO proteins as well as to understand the potential limits of these transcription factors.

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