CHAPTER 6

Identification of novel targets of the Forkhead transcription factor FKHR-L1 by gene array analysis

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In preparation

Abstract

The Forkhead/ winged helix family of transcription factors shares a structurally related domain, the Forkhead domain, but are highly divergent in their tissue distribution and their contribution to cellular functions. One subclass of this family, consisting of AFX, FKHR and FKHR-L1, have recently been described as targets of Protein Kinase B. Their transcriptional activity, resulting in cell cycle arrest, as well as induction of the apoptotic program, is inhibited by PKB. To obtain further insight into the mechanism of action of this subclass of transcription factors, we performed a gene array screen using cells stably expressing an 4-hydroxy tamoxifen (4-OHT) inducible construct of FKHR-L1. A mouse gene array was probed with cDNA generated from cells treated with or without 4-OHT for three hours. Two members of the AP-1 family of transcription factors, c-Jun and Fra-2, were found to be rapidly and specifically upregulated upon activation of FKHR-L1. Both proteins were also upregulated upon cytokine withdrawal. These findings may help to shed light on the mechanisms by which the AFX, FKHR and FKHR-L1 subclass of transcription factors can regulate proliferation and survival in a variety of cell types.

Introduction

The family of Forkhead/ winged helix transcription factors consists of over 100 members, that vary in their tissue distribution, regulation, and also in their DNA binding sequence specificity and thus also in their transcriptional targets. They all share a conserved 100 amino acid DNA-binding domain, called the Forkhead domain[1]. These transcription factors are involved in a myriad of cellular functions, including embryogenesis, cellular differentiation, mediating cell death, immune homeostasis, maintenance of ovarian follicles[2-6]. Recently, a subclass of Forkhead transcription factors was identified, comprising AFX, FKHR and FKHR-L1, of which chromosomal rearrangement has been linked to oncogenesis[7-10]. These transcription factors are highly homologous in their DNA binding domain which binds to a similar core sequence (TTGTTTAC), but their binding specificity of the surrounding sequences is distinct for each protein[11]. The tissue distribution patterns of AFX, FKHR and FKHR-L1 mRNAs overlaps in the embryo, being high in muscle, adipose tissue and liver. These patterns of expression are similar in the adult, although FKHR-L1 is then ubiquitously expressed[11,12]. These proteins have been identified as targets of PI3K-PKB signaling[6,13-15]. Phosphorylation by PKB results in nuclear exclusion and thus inhibits their activity[6,13-15]. As AFX, FKHR and FKHR-L1 have been linked to both cell cycle arrest and induction of apoptosis[6,14,16,17], chromosomal rearrangement might promote oncogenesis by interfering with their physiological activity. Transcriptional activity of AFX, FKHR and FKHR-L1 can upregulate the cell cycle inhibitor $p27^{KIP1}$(16-18). In addition, transcriptional activity of FKHR-L1 has been shown to upregulate pro-apoptotic protein Bim (Bcl-2 interacting mediator of cell death), which binds to and inactivates anti-apoptotic Bcl-2 members[19]. Furthermore, FKHR-L1

has also been proposed to regulate the expression of Fas ligand[6], which can initiate apoptosis through binding to Fas (CD95, Apo-1) reviewed by Nagata[20]. Recently, signaling by cytokines was also demonstrated to promote survival by inhibiting the activity of Forkhead transcription factor FKHR-L1 through PKB[19,21]. Upon cytokine-withdrawal FKHR-L1 is dephosphorylated[19], which is accompanied by a cell cycle arrest and induction of the apoptotic program, suggesting that these events might be mediated by FKHR-L1. In addition, the activity of this subclass of Forkhead transcription factors might also be involved in the differentiation of cells, as FKHR has recently been described in positive selection of T cells[5].

To identify novel transcriptional targets for AFX, FKHR and FKHR-L1 we made use of cells stably overexpressing an inducible FKHR-L1 mutant, FKHR-L1(A3):ER*, in which all three phosphorylation sites have been mutated. This construct is expressed constitutively in the cell, but is only active when 4-hydroxy tamoxifen (4-OHT) is added. Addition of 4-OHT results in rapid induction of FKHR-L1(A3):ER* activity, and has been proven to be an excellent tool to specifically analyze FKHR-L1 activity[17,19]. To define novel FKHR-L1 target genes we took a gene array approach. ATLAS array filters were screened with probes generated from RNA isolated from FKHR-L1(A3):ER* cells that were treated with or without 4-OHT. Using this strategy, we have identified two potential FKHR-L1 targets, the immediate-early genes c-Jun and Fra-2 (fos-related antigen 2). FKHR-L1 activity, as well as cytokine-withdrawal resulted in an elevation of c-Jun and Fra-2 protein levels. These data have consequences for the regulation of survival, proliferation and differentiation by Forkhead-related transcription factors in a variety of cell systems.

Experimental procedures

Cell culture

Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 produced in COS cells[22]. Ba/F3 cells stably expressing FKHR-L1(A3):ER* have been described previously[17] and were cultured in the presence of 500 μg/ml G418. For cytokine-withdrawal experiments, cells were washed twice with PBS and resuspendend in RPMI 1640 supplemented with 8% Hyclone.

ATLAS array screen

To perform the ATLAS array screen the Mouse Broad-Coverage ATLAS array #7741-1 was purchased (Clontech, Palo Alto, CA, U.S.A.), which was provided with reagents for all RNA treatments described below. For the isolation of RNA, FKHR-L1(A3):ER* cells (10^8) were cultured in the presence of IL-3 with or without 4-OHT (100 nM) for three hours and RNA was isolated as described previously[23]. Next, 500 μg RNA of each sample was subjected to a DNAse treatment to avoid contamination with genomic DNA. RNA was subsequently analyzed on gel to verify that the RNA was not degraded.

Efficiency of the DNAse treatment was verified by testing whether a PCR (35 cycles) on 2 μg RNA with β-actin primers did not give any visible product. Next, polyA+ RNA was isolated with magnetic beads coated with poly dT using 250 μg of total RNA and a probe of cDNA was generated using specific primers for the genes on the array to specifically generate cDNA of those genes. The reverse transcription of the RNA to generate cDNA was carried out at 54ºC by incubating in the presence of radioactive γ32P-dATP. The duplo array filters were incubated overnight with the probe generated from either untreated or 4-OHT treated cells at 72ºC, washed the next day filter and exposed to X-ray film.

Antibodies and reagents

Constructs for mouse c-Jun[24] and the c-Jun promoter[25] have been described previously. Fra-2 was obtained by PCR amplification from 4-OHT-treated FKHR-L1(A3):ER* cells, cloned into pSG5-myc and verified by sequencing. Polyclonal antibodies against c-Jun (H-79), Jun B (N17), Jun D (329)-G, Fra-2 (Q20), Fra-1 (Q20) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.); p27 and RACK1 mAb were purchased from Transduction Laboratories (Lexington, Kentucky, U.S.A.).

Western blotting

For the detection of all proteins, cells were lysed in ELB buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA together with inhibitors)[26], protein concentration was measured and equal amounts of protein were loaded. Blots were incubated overnight at 4°C with appropriate antibodies (1:1000) and after hybridization with secondary antibodies developed utilizing Enhanced Chemiluminescence (ECL, Amersham).

Northern blotting

FKHR-L1(A3):ER* cells were cultured in the presence of IL-3 with or without 4-OHT for appropriate times prior to RNA isolation. Total RNA was isolated as described previously[23] and twenty micrograms were used for Northern blotting and hybridized with a Fra-2 probe consisting of full-length Fra-2 cDNA. Equal RNA loading was verified by reprobing the blots with a 1.4 kb cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Luciferase assays

Ba/F3 cells were electroporated (0.28 kV; capacitance 960 μF) with c-Jun-CAT promoter construct together with an internal transfection control (pRL-TK; Promega). Transfections were divided over two wells and 4-OHT was added to one well 2 hours after transfection. 24 hours after transfection, cells were harvested for both CAT assays as described previously[27] to assess c-Jun promoter activity and luciferase activity to correct for transfection efficiency.

Results and discussion

The Forkhead transcription factor FKHR-L1 has previously been shown to be a potent inducer of apoptosis in both T cells, as well in an IL-3-dependent pro B cell line, Ba/F3[6,17]. In T cells, FKHR-L1 has been proposed to promote apoptosis through upregulation of Fas ligand[6]; in Ba/F3 cells, both $p27^{KIP1}$, as well as Bim (Bcl-2 interacting mediator of cell death) have been demonstrated to be upregulated and involved in FKHR-L1-mediated cell cycle arrest, as well as induction of apoptosis[17,19]. As Forkhead transcription factors are also involved in triggering differentiation[5,28,29], the subclass of Forkhead transcription factors AFX, FKHR and FKHR-L1 might also play a role in the differentiation of hematopoietic cells. Indeed, FKHR has recently been described to be involved in the negative selection of thymocytes[5]. To identify novel FKHR-L1 transcriptional targets we used Ba/F3 cells stably expressing FKHR-L1(A3):ER*. This cell line allows us to specifically analyze effects of FKHR-L1 transcriptional activity which is induced upon treatment with 4-OHT. ATLAS array filters (Mouse Broad Coverage #7741) were hybridized with probes generated from either untreated or 4-OHT-treated FKHR-L1(A3):ER* cells (Fig. 1A). $p27^{KIP1}$ was upregulated in 4-OHT-treated cells compared to untreated cells (data not shown), demonstrating that this approach is suitable to identify FKHR-L1 target genes. Of the genes whose expression was regulated on the filter hybridized with the probe from FKHR-L1(A3):ER* cells we focused on two potential FKHR-L1 targets. These proteins are c-Jun and Fra-2 (fos-related antigen 2), which are both members of the transcription factor AP-1 family (activator protein 1, reviewed in [30,31]) (Fig. 1A). AP-1 refers to the assembly of Jun:Jun or Jun:Fos/Fra proteins that bind together to specific regulatory sequences in the promoter of target genes. Jun members include c-Jun, JunB and JunD, whereas Fos members include c-Fos, FosB, Fra-1 and Fra-2. While JunB, JunD and Fos were also present on the ATLAS gene array filter, they were not upregulated following FKHR-L1 activity (data not shown). This suggests that FKHR-L1 activity promotes upregulation of specific AP-1 members.

Although c-Jun[32,33], as well as Fra-2[33] have both been proposed to be involved in proliferation, c-Jun[32,34,35], as well as Fra-2[34] have also been described to be involved in induction of apoptosis as well as differentiation[36-39], thus prompting us to further investigate these proteins. To examine whether the upregulation of c-Jun and Fra-2 was also reflected in an increase in protein levels, FKHR-L1(A3):ER* cells were cultured in the absence or presence of 4-OHT. A dramatic induction of c-Jun, as well as Fra-2 was observed after overnight treatment with 4-OHT (Fig. 1B). The extent of this induction was similar to that of $p27^{KIP1}$, a recently described FKHR-L1 target[16,17].

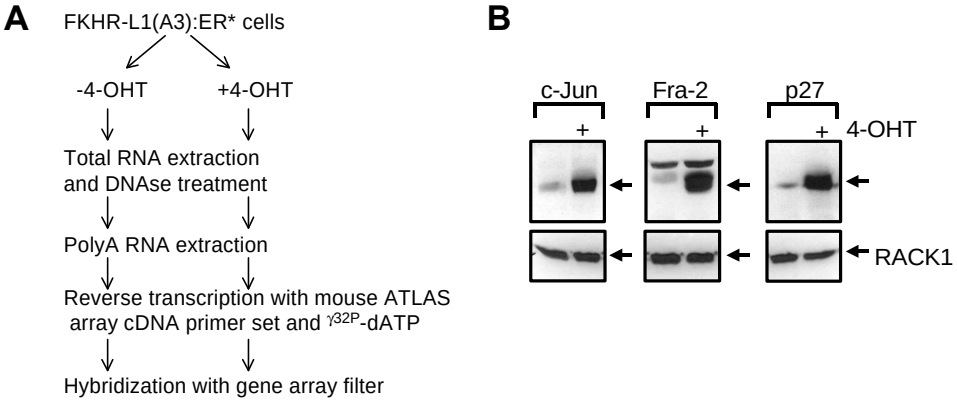

Figure 1 c-Jun, Fra-2 and p27^{KIP1} are upregulated in 4-OHT-treated FKHR-L1(A3):ER* cells.

(A) Schematic representation of the Forkhead array screen (B) FKHR-L1(A3):ER* cells were cultured with IL-3 with or without 4-OHT (100 nM) overnight, lysed and analyzed for levels of c-Jun, Fra-2 or p27^{KIP1}. Blots were reprobed with a RACK1 antibody to verify equal protein loading.

Next, we analyzed the kinetics of FKHR-L1-mediated upregulation of c-Jun and Fra-2 by analyzing FKHR-L1(A3):ER* cells that were treated with 4-OHT for the times indicated (Fig. 2). c-Jun induction was clearly visible as early as 4 hours after induction of FKHR-L1 activity, and increased over time with kinetics similar to those of FKHR-L1 target p27^{KIP1}. Fra-2 protein levels were increased as early as 2 hours after induction of FKHR-L1 activity, but were not further elevated over time (Fig. 2 and data not shown). No elevation in protein levels was observed in JunB, JunD or Fra-1 (data not shown), suggesting that induction of FKHR-L1 activity promotes upregulation of specific AP-1 members.

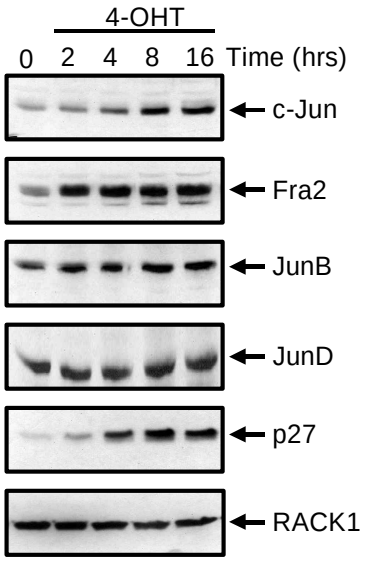

Figure 2 Time course analysis of levels of c-Jun, Fra-2, JunB and p27^{KIP1}.
FKHR-L1(A3):ER* cells were either left untreated or treated with 4-OHT (100 nM) for indicated times, lysed and analyzed for protein levels of c-Jun, Fra-2, JunB, JunD and p27^{KIP1}. Equal protein loading was verified by reprobing the blot with a RACK1 antibody. Data are representative of at least two different clonal FKHR-L1(A3):ER* cell lines.

IL-3 has previously been demonstrated to result in phosphorylation and subsequent inactivation of FKHR-L1[17]. Conversely, withdrawal of IL-3 results in the dephosporylation of FKHR-L1, which is accompanied by upregulation of FKHR-L1 targets p27^{KIP1} and Bim[17,19]. To examine whether IL-3 withdrawal also resulted in an induction of c-Jun and Fra-2, Ba/F3 cells were IL-3-starved for the times indicated and assessed for levels of c-Jun and Fra-2. c-Jun was quite rapidly upregulated following IL-3 withdrawal (Fig. 3A), with levels being highly elevated as early as one hour, which was much faster than the upregulation observed in FKHR-L1(A3):ER* cells (compare with Fig. 1B upper panel). However, levels of c-Jun decreased as early as 4 hours after cytokine withdrawal, with levels returning to basal levels after 16 hours. Similar observations were made for Fra-2 (Fig. 3B). Also in the case of Fra-2, cytokine withdrawal resulted in a much more rapid elevation of Fra-2 than observed in FKHR-L1(A3):ER* cells (Fig. 1B).

The differences in kinetics of upregulation between cytokine withdrawal and 4-OHT-treated FKHR-L1(A3):ER* cells could be explained in that experiments in 4-OHT-treated cells were carried out in the presence of IL-3. IL-3 might act to induce the activity of

a transcriptional repressor of c-Jun and Fra-2, which is rapidly degraded in the absence of IL-3. The kinetics of elevation of c-Jun and Fra-2 protein levels in FKHR-L1(A3):ER* cells might subsequently be delayed as the activity of this repressor needs to be overcome first.

Although activity of FKHR-L1 is probably sustained over time following cytokine withdrawal[17], this is not reflected by an increase in upregulation of c-Jun and Fra-2. An explanation for the sustained c-Jun and Fra-2 levels in 4-OHT-treated cells might be that IL-3 in some way increases their stability. Indeed, MAPK-mediated phosphorylation results in a reduction of ubiquitination and potential degradation of c-Jun[40]. MAPK-mediated stabilization has also been suggested for Fra-2[41].

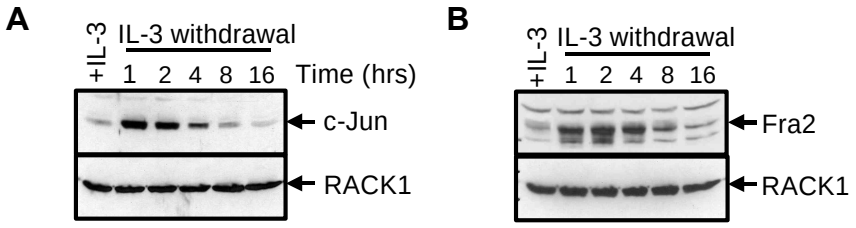

Figure 3 IL-3 withdrawal promotes a transient upregulation of c-Jun, as well as Fra-2.

(A) Ba/F3 cells were IL-3-starved for the indicated times, lysed and assessed for levels of c-Jun (top) or RACK1 (bottom) to verify equal protein loading. (B) As in (A) but probing with Fra-2 instead of c-Jun.

Thus far we only examined the effect of FKHR-L1 transcriptional activity on the elevation of protein levels of c-Jun and Fra-2. To analyze whether indeed upregulation of these protein levels is also reflected by an increase of mRNA levels or promoter activity, FKHR-L1(A3):ER* cells were cultured with or without 4-OHT for the indicated times. Fra-2 mRNA (Fig. 4A) was dramatically elevated upon 4-OHT-treatment, suggesting that indeed transcriptional activity of FKHR-L1 is involved in the elevation Fra-2 protein levels observed. Unfortunately, as pretreatment of cells with protein synthesis inhibitor cycloheximide results in a superinduction of c-Jun[42], as well as Fra-2 (data not shown), we were not able to use this approach to demonstrate that FKHR-L1 directly upregulates c-Jun and Fra-2. However, Forkhead consensus DNA binding sites were found in both the c-Jun[43] and Fra-2[44] promoter, suggesting that FKHR-L1 may indeed directly induce c-Jun or Fra-2 transcription.

Next, we examined whether FKHR-L1 activity could also promote transcriptional activation of the c-Jun promoter. Two clonal Ba/F3 cell lines stably expressing FKHR-L1(A3):ER* cells were analyzed, to exclude artefacts due to clonal selection. In both of the clones examined, 4-OHT treatment of FKHR-L1(A3):ER* cells strikingly elevated c-Jun promoter activity (Fig. 4B, left). This demonstrates FKHR-L1 regulation of both promoter activity, as well as induction of mRNA levels. Furthermore, analysis of c-Jun promoter activity in Ba/F3 cells cultured in the absence or presence of IL-3 also revealed that IL-3 withdrawal resulted in an elevation of c-Jun promoter activity (Fig. 4B, right). Taken together, this suggests that FKHR-L1 activity can result in elevation of c-Jun and Fra-2 protein levels by enhancing their transcription.

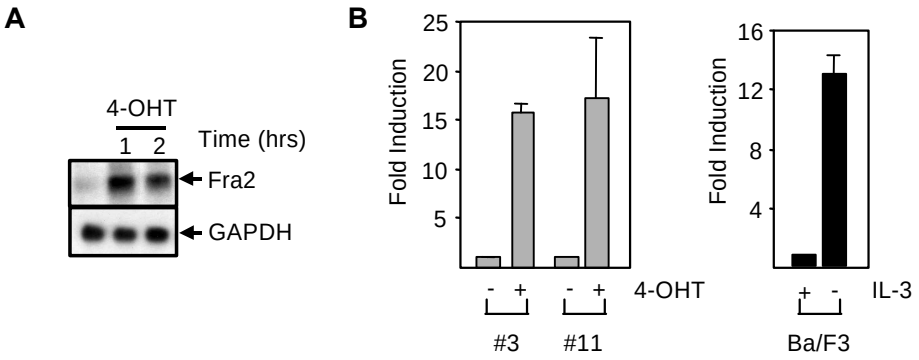

Figure 4 FKHR-L1 induces an increase of Fra-2 mRNA levels, as well as an induction of c-Jun promoter activity.

(A) IL-3-cultured FKHR-L1(A3):ER* cells were either left untreated or treated with 4-OHT (100 nM) for indicated times, 20 μg of total RNA was used for Northern blotting and hybridized with a Fra-2 probe (top). Equal RNA loading was verified by GAPDH reprobing (bottom). (B) Two different clonal Ba/F3 cell lines stably expressing FKHR-L1(A3):ER* (left) or Ba/F3 cells (right) were electroporated with 20 μg of c-Jun-CAT plasmid together with 500 ng of pRL-TK plasmid as an internal transfection control. FKHR-L1(A3):ER* cells were cultured for 24 hours with IL-3 in the absence or presence of 4-OHT (100 nM) and Ba/F3 cells in the absence of presence of IL-3 before analyzing CAT and luciferase activity as described in Materials and Methods. The data are representative of several independent experiments.

If a Forkhead transcriptional target were to be important for regulation of apoptosis or cell cycle arrest, then overexpression of this protein should be sufficient to promote apoptosis or cell cycle arrest. However, overexpression of neither c-Jun nor Fra-2 resulted in Ba/F3 cells resulted in an alteration of cell cycle distribution or decreased survival (data not shown). However, in neuronal cells, c-Jun and Fra-2 have both been demonstrated to promote induction of apoptosis. NGF withdrawal-induced apoptosis is accompanied by elevation of c-Jun mRNA and microinjection of antibodies against c-Jun, as well as Fra-2 protected NGF-deprived neurons from apoptosis[34]. Furthermore, overexpression of dominant negative c-Jun was found to protect sympathetic neurons from apoptosis following NGF-withdrawal[45] and increased c-Jun activity was sufficient to trigger apoptotic cell death in NIH 3T3 fibroblasts[35]. This suggests that activity and function of AP-1 members may differ depending on the cell type and conditions. Thus, in neuronal cells, Forkhead-related transcription factors may play a an important role in the induction of apoptosis through transcriptional induction of AP-1 family members. Importantly, the PI3K-PKB pathway has been shown to be critical in NGF-mediated survival[46].

Fra-2 and c-Jun have also been proposed to function in the differentiation of cells. Ectopic expression of c-Jun is sufficient to induce differentiation of P19 embryonal carcinoma cells[43], as well as murine myelomonocytic WEHI-3B D+ cells[47]. In addition, expression of antisense c-Jun blocked differentiation in a murine erythroleukemia cell line[48]. Fra-2 and JunD heterodimers have also been proposed to be involved in osteoblast differentiation[49] as well as with the transition of proliferating granulosa cells to terminally differentiated, non-dividing luteal cells[39]. These observations suggest that transcriptional upregulation of c-Jun and Fra-2 by FKHR-L1 or related Forkhead family members might be linked to differentiation of cells. It will be interesting to examine this in one of the cell types in which AP-1-mediated differentiation has been proposed.

It should be noted that Fra-2 has a high affinity for JunD and increases the activity of JunD, upon heterodimerization[50]. Thus, upregulation of Fra-2 may promote heterodimerization of Fra-2 to other AP-1 members. c-Jun itself is also involved in the activation of transcription factors other than members of the AP-1 family, such as PU.1[51].

These data demonstrate that FKHR-L1(A3):ER* cells are a powerful tool to obtain further insight into potential targets of Forkhead transcription factors AFX, FKHR and FKHR-L1 and their mechanism of action. While c-Jun and Fra-2 do not appear to have an apparent role in the induction of the apoptotic program in Ba/F3 cells, there may be a role in neuronal cells. A further challenge will be to identify what the consequences of Forkhead-mediated upregulation of c-Jun and Fra-2 is on the transcriptional activity of AP-1 family members and investigate how this is related to their transcriptional regulation of genes in a variety of physiological processes.

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