Forkhead Box Proteins in AML

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Forkhead box (FOX) proteins are a group of transcriptional factors implicated in different cellular functions such as differentiation, proliferation and senescence. A growing number of studies have focused on the relationship between FOX proteins and cancers, particularly hematological neoplasms such as acute myeloid leukemia (AML). FOX proteins are widely involved in AML biology, including leukemogenesis, relapse and drug sensitivity. Here we explore the role of FOX transcription factors in the major AML entities, according to "The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia", and in the context of the most recurrent gene mutations identified in this heterogeneous disease.

forkhead box proteins   acute myeloid leukemia   chemoresistance

1. Introduction

Forkhead box (FOX) proteins are an extended group of transcriptional factors characterized by the presence of an evolutionary conserved DNA-binding domain (DBD) named "winged-helix" or "fork-head". The family name "fork-head" derives by the first gene discovered in *Drosophila Melanogaster* (*forkhead*, fkh) by Weigel et al. [1] in 1989 and was inspired by the fork-headed appearance of the mutated insect embryos, whereas the "winged-helix" name of the characteristic DBD present in all family members was suggested by the butterfly-like appearance of its three-dimensional structure. The prototypical DBD consists of about one hundred amino acids that under physiological conditions give rise to three α-helices, three β-sheets and two 'wing' regions that flank the third β-sheet [2].

FOX family members are now categorized, on the basis of sequence homology, into nineteen subgroups, from FOXA to FOXS, and reach at today the number of at least 50 genes distributed almost on all human chromosomes, **Figure 1** [3][4].
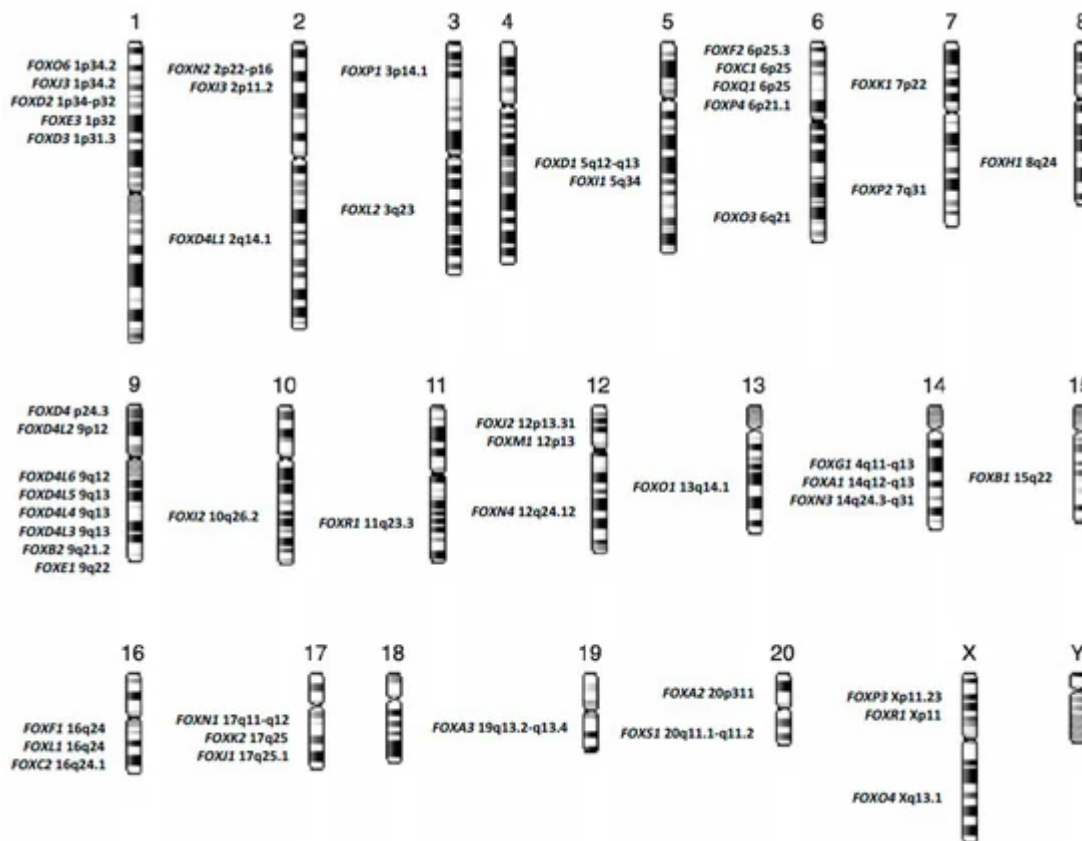
Figure 1. Chromosomal distribution of forkhead box (*FOX)* genes in the human genome.

Transcription factors are sequence-specific DNA-binding proteins (DBP) that control the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequences (promoters and/or enhancers). As transcription factors, FOX proteins are responsible for the fine-tuning of gene expression during all stages of embryonic development and are guardians of the homeostasis in adult tissues. FOX proteins have been reported as active regulators of several networks, the main of which are: development, differentiation, maintenance of multipotency, proliferation, metabolism, DNA repair, cell cycle progression, migration, senescence, survival and apoptosis [5][6][7][8][9][10][11][12][13]. Despite the high sequence conservation of the *forkhead* domain, FOX proteins may exert different roles in the fine regulation of downstream genes, acting as repressors or activators of gene expression [14].

The mechanisms of gene expression regulation controlled by FOX proteins are, in some cases, so intricate that some FOX proteins are themselves the target of other members of the same gene family, as shown by Karadedou et al. that described the mechanisms by which FOXO3A and FOXM1 antagonize the activity of one another by regulating the transcription of downstream target genes [15]. The fine regulation of gene expression performed by FOX proteins is not only due to the tissue and/or cell-specific expression, but is also due to the post-translational modifications that mainly include phosphorylation, acetylation, ubiquitylation and sumoylation [16][17]. Post-translational modifications play a central role in cellular localization and activity of FOX factors. Mainly, FOX proteins act as transcriptional regulators in the nucleus, while they are prevalently inactive in the cytoplasm where they are subjected to proteasomal degradation.

The ability of FOX proteins to contribute to the control of several fundamental signaling pathways and of all the aspects of development and cell fate allows this superfamily of transcription factors to be heavily implicated in cancer initiation and progression. Indeed, FOX factors have been shown to play a role as either oncogenes or tumour suppressors, as well as active regulators of cellular resistance to chemotherapy and actionable targets in cancer therapy.

Myeloid neoplasms are a complex and heterogeneous group of hematopoietic diseases characterized by uncontrolled proliferation and/or blockage of differentiation of abnormal myeloid progenitor cells, and variable prognosis. "The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia" categorizes myeloid malignancies into five primary types: myeloproliferative neoplasms (MPN), myeloid/lymphoid neoplasms with eosinophilia and rearrangement of PDGFRA (platelet derived growth factor receptor alpha), PDGFRB (platelet derived growth factor receptor beta), or FGFR1 (fibroblast growth factor receptor 1), or with PCM1-JAK2 (pericentriolar material 1-Janus kinase 2), myelodysplastic/myeloproliferative neoplasms (MDS/MPN), myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) and related neoplasms [18].

Accumulating evidence suggests that FOX proteins are profoundly involved in the maintenance of multipotency of hematopoietic stem cells (HSC) and in critical mechanism driving aberrant self-renewal in preleukemic cells [19].

2. Forkhead Box Proteins in RUNX1-RUNX1T1 Acute Myeloid Leukemia

One of the most frequent initiating alterations in AML is the AML1-ETO translocation t(8;21), accounting for about 10% of total AML [20]. Although, according to "The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia" the t(8;21)(q22;q22.1), RUNX1-RUNX1T1 represents a specific subgroup of "AML with recurrent genetic abnormalities", several authors have shown that the expression of RUNX1-RUNXT1 transcript in human hematopoietic stem progenitor cells (HSPC) causes deregulated differentiation and increased self-renewal of CD34+ cells without inducing AML [19][21]. Although, FOXO genes (FOXO1, FOXO3, FOXO4 and FOXO6) are frequently reported as tumour suppressors in several cancers [22][23][24][25][26][27][28][29][30], Lin et al. recently highlighted a new role of FOXO1 as an oncogene, clearly showing that the role of Forkhead box proteins depends on cellular context [19]. In this line, the Authors showed that the up-regulation of FOXO1 is required to sustain the growth of RUNX1-RUNXT1 cells, promoting the self-renewal and inhibiting the differentiation of human CD34+ HSPCs (**Table 1**). In particular, in RUNX1-RUNX1T1 CD34+ cells, increased levels of FOXO1 promote preleukemia transition and clonogenicity. Moreover, Lin et al. suggested the genetic and pharmacological ablation of FOXO1 as a therapeutic strategy for the elimination of preleukemic and leukemic t(8:21) HSPCs [19]. Indeed, an interesting therapeutic approach proposed for Acute Leukemia has been the interference with FOXO1 subcellular localization to improve blast cell sensitivity to antineoplastic drugs. Phosphorylation is the main cause of the 14-3-3 protein-mediated export of FOXO1 from an active nuclear form into the cytoplasm, where it results inactive [31][32]. Besides post-translational modifications, miRNA are emerging actors in regulating FOXOs levels and consequently AML blasts characteristics, such as drug sensitivity [33]. Together with FOXO3 activation, FOXO1 overexpression

via miRNA interactions is involved in upregulation of ABCB1 gene, coding for P-glycoprotein, well known responsible for decreased drug accumulation in multidrug-resistant cancer cells [34].

Table 1. Forkhead box factors deregulation according to acute myeloid leukemia (AML) recurrent abnormalities and their involvement in biological processes.

Recurrent Abnormalities in Acute Myeloid Leukemia	FOX Family Member	Biological Process	References
t(8;21); RUNX1-RUNX1T1	FOXO1	Self-renewal and Differentiation	Lin et al. [19]
PML-RARA	FOXO3A	Apoptosis and Granulocytic Differentiation	Sakoe et al. [35]
	FOXC1	Granulocytic Differentiation and Epigenetic Regulation	Somerville et al. [36] and Fabiani et al. [37]
NPM1	FOXM1	Cell Proliferation, Division and Chemoresistance	Laoukili et al. [38] Nakamura et al. [39] Khan et al. [40]
FLT3 ITD	FOXO3A	Apoptosis, Survival and Proliferation	Scheijen et al. [41]
	FOXO1	Cell growth, Apoptosis and antioxidant defences	Seedhouse et al. [42]
	FOXM1	Survival, Apoptosis and Chemoresistance	Liu et al. [43]
IDH 1-2	FOXOs	Cellular Differentiation and Tumor Suppression/Progression and Epigenetic Instability	Charitou et al. [44]

Leukemia/Retinoic Acid Receptor-Alpha) Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is another subgroup of acute myeloid leukemia characterized by a unique t(15;17) translocation generating the PML/RARA fusion gene.

The PML/RARA oncoprotein synthesis is the key pathogenetic event of APL specifically targeted by all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), two non-chemotherapeutic agents that synergistically act inducing oncoprotein degradation. Although it is well known that this fusion protein blocks granulocytic differentiation by direct transcriptional inhibition of retinoic acid target genes [45], the detailed mechanisms and the complete list of genes involved in APL transformation are not fully understood. Although, the ATRA-related apoptosis of APL blasts via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression has been previously reported, the pivotal transcription factor has not been yet identified [46]. Sakoe et al. have shown that activation of FOXO3A is an essential event for ATRA-induced cellular response in human t(15:17) cell line NB4 [35]. Nuclear FOXO3A tunes HSC maintenance and, if phosphorylated, it is exported to the cytoplasm and interacts with 14-3-3 proteins, hence losing its function [47]. The phosphorylation of FOXO3A, and its consequent loss of function, is regulated by AKT

pathway aberrantly activated in case of AML driver mutations such as FLT3-ITD or BCR-ABL [48], (**Figure 2**). In the APL setting, Sakoe et al. showed that ATRA treatment was able to reduce FOXO3A phosphorylation and to induce the translocation of the transcription factor in the nucleus, where the protein leads to apoptosis trough TRAIL upregulation [35]. Notably, FOXO3A silencing through shRNA inhibited ATRA-induced response in NB4 cells, as such as in ATRA resistant NB4/RA cells. ATRA treatment was unable to induce FOXO3A phosphorylation, TRAIL upregulation, apoptosis and granulocytic differentiation; whereas forced expression of active FOXO3A in the nucleus induced TRAIL production and apoptosis in NB4/RA cells [35] (**Table 1**). Thus, in APL, a member of FOXOs family acts as tumor suppressor gene differently from the previously reported role of FOXO1 in RUNX1-RUNX1T1 acute myeloid leukemia. These results highlight a possible role for FOXO3A as a potential therapeutic target in APL to overcome ATRA resistance.
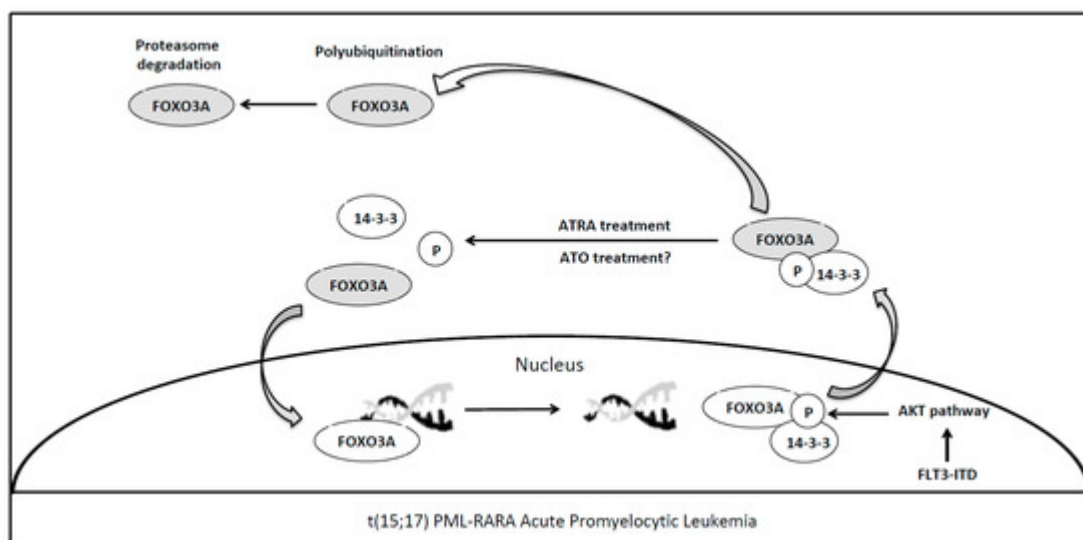
Figure 2. ATRA mediated reactivation of FOXO3A in t(15;17) PML-RARA acute promyelocytic leukemia. Nuclear FOXO3A (active form), once phosphorylated, interacting with 14-3-3 protein is exported to the cytoplasm losing its function [47]. The phosphorylation of FOXO3A, and its consequent loss of function, may be regulated by AKT pathway aberrantly activated in case of AML driver mutations such as FLT3-ITD [48]. All-trans retinoic acid (ATRA) treatment is able to reduce FOXO3A phosphorylation and to induce the relocation of the transcription factor into the nucleus, where the protein leads to blast apoptosis [35]. Arsenic trioxide (ATO) treatment in this cellular context needs to be better assessed. Light grey shows the FOXO3A inactive form.

Arsenic trioxide in combination with ATRA is currently considered the standard of care for adults with low-to-intermediate-risk APL, with a complete remission rate near to 100% [49]. Interestingly, Zhang et al. have recently shown that ATO treatment of gastric cancer cells induced the upregulation of FOXO3A expression in the nucleus and that FOXO3A knockdown attenuated the effect of ATO treatment in gastric cancer cells and in mouse models [50]. Moreover, they also demonstrate that ATO-related nuclear upregulation of active FOXO3A is the result of its phosphorylation via the aforementioned AKT pathway, leading to inhibition of cell migration. This new insight may be useful for further investigations about the role of FOXO3A in treatment response to ATO in APL patients. In this line, additional studies to identify novel therapeutic agents enabled to restore FOXO3A function may overcome

ATO resistance even in patients harboring PML-A216V mutation, accounting for about 30% of ATO-resistant cases [51].

FOXO subfamily members are not the solely forkhead transcription factors involved in APL pathogenesis. In 2015, Somerville et al. reported the overexpression of FOXC1 in nearly 20% of primary non-APL AML samples, showing its involvement in the monocyte/macrophage differentiation block and in the increased clonogenic potential of AML cells [36]. FOXC1 is deregulated in different types of cancers and is often associated with poor prognosis in AML, cooperating with HOXA/B and consequently repressing the monocyte transcriptional regulator KLF4 [36]. More recently, our group showed that FOXC1 mRNA and protein levels were significantly lower in primary marrow samples from APL patients, as compared to samples obtained from patients with other AML subtypes, and normal CD34+ hematopoietic cells [37]. Moreover, we demonstrated that FOXC1 expression was significantly increased in APL samples following consolidation treatment and that ATRA treatment unlocked FOXC1 expression in NB4, but not in NB4-R4 ATRA-resistant cells. Of note, using chromatin immune precipitation assay (ChIP), we identified functional binding sites of ATRA in the FOXC1 promoter region. In addition, we showed that in diagnostic APL samples and in NB4 cells, reduced FOXC1 expression was associated to DNA hypermethylation of the +354 to +568 FOXC1 region and that hypomethylating treatment with decitabine of NB4 upregulated FOXC1 expression [37] (**Table 1**). Our findings indicate a dual repression model of FOXC1 expression in APL giving the rationale for a potential role of hypomethylating treatment (HMT) in advanced and/or resistant APL. HMT is anecdotally reported for the treatment of resistant APL cases and the rationale for their use has been postulated by the study of Soncini et al. where they show TRAIL-dependent apoptosis of APL and AML blasts [52]. In this work Soncini et al. identify a TRAIL promoter region, affected by DNA hypermethylation and whose function is restored after decitabine administration, demonstrating in APL a novel therapeutic approach other than PML-RARA degradation (ATRA-ATO scheme), using epigenetic drugs [52]. This strategy may be helpful to overcome resistance to differentiating treatment in case of mutations in PML or RARA.

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