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The FoxA Factors in Organogenesis and Differentiation

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Summary

The genetic analysis of the *Foxa* genes in both total and conditional mutant mice has clearly established that organogenesis of multiple systems is controlled by this subfamily of winged helix transcription factors. These discoveries followed the establishment of the conceptual framework of the mechanism of action of the FoxA proteins as “pioneer factors” that can engage chromatin before other transcription factors. Recent molecular and genomic studies have also shown that FoxA proteins can facilitate binding of several nuclear receptors to their respective targets in a context-dependent manner, greatly increasing the range and importance of FoxA factors in biology.

Introduction

The late 1980’s were the heyday for the discovery of tissue-specific, or at least tissue-enriched, transcriptional regulators. Using the recently invented technologies of promoter-reporter assays and DNA-affinity chromatography and the rat liver as abundant and relatively homogeneous source of biomaterial, in short succession multiple Hepatocyte Nuclear Factors, or HNF’s, were purified and cloned [1]. Among these, the HNF-3 proteins, discovered by Robert Costa and Eseng Lai in James Darnell’s group, constituted a whole new class of DNA binding proteins, as they did not contain any of the DNA contact motifs, such as Zinc-fingers, known at the time [2,3]. In a wonderful example of convergence of distant scientific disciplines, at the same time the mutation responsible for the homeotic transformations in the *Drosophila* mutant *forkhead* was cloned, and was discovered to share a central, highly conserved motif with the HNF-3 proteins [4,5].

This 100 amino acid so-called forkhead box was shown by X-ray crystallography to form a variant of the helix-loop-helix fold, with two large loops, giving it the appearance of a “winged helix” [6]. This structural information, and the striking similarity with the DNA binding motif of linker histones, led to elegant studies by the Zaret lab, which demonstrated that HNF-3alpha can replace linker histones and affect chromatin structure directly, a rare feat among DNA binding proteins [7]. This property of the HNF-3 proteins is crucial to their proposed role as pioneer factors in the initiation of organ-specific transcriptional programs, which will be discussed below.

Subsequent to the discovery of the HNF-3 proteins as homologues of *Drosophila forkhead*, hundreds of related genes were cloned in species ranging from yeast to human [8]. Mammalian

genomes contain more than forty winged helix transcription factors, which have been classified into 19 subclasses or clades, based on sequence conservation [9]. In the year 2000, the rather confusing nomenclature of the vertebrate winged helix genes was streamlined, with all genes renamed as *Fox*, for *Forkhead Box*, with a letter indicating the subclass [8]. Thus, the unlinked HNF-3 genes are now named *Foxa1*, *Foxa2* and *Foxa3*.

Foxa2: First in axial patterning

From invertebrates to vertebrates, the genome was duplicated and reduplicated. This is exemplified best by the four homeobox gene clusters in vertebrate genomes, all derived from one ancient cluster. Thus, one would expect four *Foxa* genes as orthologues of the *Drosophila forkhead*; however, the putative '*Foxa4*' gene was lost in evolution. So how is it that duplicated genes retain their relevance and contribute to fitness? One common mechanism is via attainment of new expression domains, and thus new functions, by one of the recently duplicated genes [10,11].

Foxa2 is, for a short developmental time window, the only *Foxa* gene active in the early gastrula embryo. The gene is activated on day 6.5 of gestation in the mouse in the primitive streak and node, from which axial mesoderm, i.e. the notochord, originates [12–14]. Embryos null for *Foxa2* lack foregut endoderm and notochord, an essential source of sonic hedgehog signaling to pattern the neural tube [15,16]. Without this signaling source, the neural tube stays radially symmetric and unpatterned. Due to these defects and impaired extraembryonic structures, *FoxA2*-deficient embryos die around day 9 of gestation. Although never proven experimentally, it appears likely that the closely related *FoxA1* protein, which overlaps in functions and targets with *FoxA2* (see below), should be able to rescue the phenotype of the *Foxa2* null mice, if it were expressed at the right time and place.

Foxa1 and Foxa2: Cooperating in organogenesis

The cooperation of *Foxa1* and *Foxa2* in organ development is striking, and apparent in every system that has been studied genetically thus far. When the two genes are missing from the foregut endoderm, hepatic specification is blocked completely [17,18]. This finding was the first genetic validation of the competency model of liver development proposed by Zaret and colleagues who had shown that *FoxA* proteins can open chromatinized DNA templates *in vitro*, and enable subsequent access for other transcription factors [7,19–21]. In fact, even when *Foxa1/Foxa2* deficient endoderm was placed in culture with Fibroblast Growth Factor 2 as inducer of the hepatogenic program [22], activation of liver genes failed to occur [18]. In contrast, embryos retaining either *FoxA1* or *FoxA2* protein in foregut endoderm specified the liver normally, indicating overlapping functions and targets for the two genes.

In short succession, similar compensatory roles for *Foxa1* and *Foxa2* were demonstrated in the development of other organ systems. Thus, branching morphogenesis in the lung is blocked when both factors are missing, but not by ablation of either factor alone [23]. A second important example is the differentiation of dopaminergic neurons in the midbrain. These are the neurons that are lost in Parkinson's disease, causing loss of control of body movements. Here, Ang and colleagues showed that *Foxa1* and *Foxa2* control several phases of neuronal development, from the earliest specification of progenitors via activation of the transcription factors *Ngn2* and *Lmx1a/b*, to the expression of tyrosine hydroxylase an enzyme required for the conversion of tyrosine to dopamine, a function of the mature dopaminergic neuron [24, 25]. Strikingly, mice heterozygous for a *Foxa2* null allele show age-dependent motor behavior abnormalities, which correlate with progressive loss of dopaminergic neurons [26].

This function of the *FoxA* factors at multiple stages of development of the same cell type is echoed in the development of the pancreas. Using conditional alleles for both *Foxa1* and

Foxa2, and a Cre recombinase active at the earliest stage of pancreatogenesis, it was shown recently that *Foxa1/Foxa2* are obligatory activators of the master gene of pancreas development *Pdx1* (pancreas duodenum homeobox gene, [27])(see Figure 1). In addition, in the mature β -cell, the two factors cooperate to control insulin secretion and to repress the neuronal transcriptional program via an as yet unknown mechanism [28]. Again, just like in the examples cited above, single ablation of either gene had little effect on early pancreatic development. Thus, this and other genetic models have shown that the FoxA proteins act in the pancreas during organ specification, lineage differentiation, and mature function [29–32]. An important question arises: how can these transcription factors regulate different targets in a stage-specific manner?

Cell-type and stage-specific gene regulation by Foxa factors

A clue to this question came from chromatin immunoprecipitation assays with chromatin isolated from different stages of pancreatic development [27]. These data showed that the occupancy of different *cis*-regulatory elements by FoxA is stage-dependent. Current work is aiming to determine the molecular basis for this phenomenon using global location analysis. ChIP-on-Chip studies, that is chromatin immunoprecipitation followed by hybridization of the transcription factor bound DNA to tiled arrays representing part of the genome, for FOXA1 in breast and prostate cancer cell lines suggested cell-type specific target binding, although the degree to which this occurs is still not certain [33,34].

One simple and attractive model for stage-specific gene regulation has been shown to be operative in *C. elegans*, where the single *Foxa* homologue, PHA-4, is thought to bind only to high affinity sites early in development, when PHA-4 levels are low, and later to lower affinity sites, once PHA-4 levels have risen sufficiently [35]. It is likely, however, that additional mechanisms contribute to gene regulation in vertebrates, such as stage- or condition-specific post-translational modifications, and interactions with other DNA binding proteins and co-factors. One such modification is the phosphorylation and nuclear exclusion of FoxA2, but not FoxA1, by AKT [36]. However, at present this pathway is still controversial [37,38]. Very recently, in an example of complexity added through protein-protein interactions, it was shown that during earliest endoderm development, the FoxA proteins are co-expressed with *groucho*-related transcriptional repressors, and that lentiviral expression of Grg3 in liver explants inhibits the activation of the hepatic gene expression program [39], mimicking the phenotype of *Foxa1/Foxa2* deficiency introduced above [18].

FoxA factors and nuclear receptors

Over the past few years, genomic approaches have supported and expanded the concept of cooperation of hormone or signal-dependent transcription factors, such as nuclear receptors, and cell-type specific factors, in ensuring that hormone dependent gene activation occurs only in the intended cell type. As discussed below, the FoxA factors play a major role in gene activation by the glucocorticoid, androgen, and estrogen receptors.

Almost twenty years ago, Schütz and colleagues asked the question why the activation of gluconeogenic genes by glucocorticoids in response to fasting occurs only in liver and kidney, even though the glucocorticoid receptor (GR) is expressed and the ligand distributed ubiquitously. Through molecular analysis of the *cis*-regulatory elements of a fasting-activated gene, *tyrosine aminotransferase (Tat)*, they developed the model that cell-type specific activation of the gene was achieved through juxtaposition of binding sites for FoxA and GR [40]. They showed that mutation of the FoxA binding site in a promoter-reporter construct severely attenuated gene activation by glucocorticoids. A similar situation was shown to exist for another fasting-activated gene, *Pck1*, by Daryl Granner's group [41–43].

However, because these studies only mutated binding sites, they could not prove that in fact FoxA factors mediate this effect, as the same sites are also bound by other winged helix proteins, chiefly of the FoxO class, activity of which is regulated by insulin signaling [44]. Indeed, *Foxo1* is required for full activation of gluconeogenic genes. However, conditional gene ablation of *Foxa2* in the liver demonstrated that this factor regulates the expression of the fasting-induced genes *Tat*, *Pck1*, and *Igfbp1* *in vivo*. Furthermore, Zhang and colleagues showed by chromatin immunoprecipitation that GR binding to its targets *in vivo* is indeed *Foxa2*-dependent [38]. Interestingly, the targets regulated by FoxA2 in the liver depend on the physiological state of the organism, as shown by genome-wide location analysis [45]. Thus, when mice are exposed to high cholic acid levels, the genes regulated by FoxA2 are different than those controlled in the chow-fed situation. Thus, gene regulation by FoxA2 is context-dependent.

An analogous relationship exists between FoxA1 and the sex hormone receptors, AR (androgen receptor) and ER (estrogen receptor). Matusik and colleagues, while studying the regulation of prostate-specific gene expression, discovered that AR activation depended on the presence of FoxA1 at nearby binding sites for several genes, thus establishing this new paradigm [46]. These findings were recently extended to the genome-wide level by ChIP-on-Chip experiments, demonstrating that this cooperation between FoxA1 and AR is a wide-spread phenomenon [34].

In fact, this close relationship of nuclear receptors and FoxA factors appears to be a general principle (Figure 2). Brown and colleagues, determining the binding sites of the estrogen receptor to three chromosomes in human breast cancer cell lines, made the striking discovery using computational tools that ER binding sites are very frequently paired with FoxA sites [47]. In fact, when FoxA1 expression was suppressed by shRNA, ER-mediated gene activation was blunted. Similar conclusions were reached independently by Giguere and colleagues [48]. A very recent genetic study proved that *Foxa1* is indispensable for mammary gland development, specifically the estrogen-induced mammary duct expansion [49]. So how does Foxa1 serve two masters, ER and AR in a cell-type specific fashion? Brown and colleagues have begun to address this question, comparing FoxA1 occupancy in breast and prostate cancer cell lines. They found only partial overlap between the two sets of binding sites, suggesting again that there is no universal set of FoxA binding sites [34]. Overall it is clear, however, that the FoxA factors have greatly expanded their range of action by cooperation with nuclear receptors in conditional gene activation.

Conclusions

The genetic analysis of the *Foxa* genes using both total and conditional alleles has clearly established that organogenesis of multiple systems is controlled by the this small subfamily of winged helix transcription factors. These discoveries followed the establishment of the conceptional framework of the mechanism of action of these proteins by the Zaret lab, who had used biochemical tools to provide evidence for the function of the FoxA factors as 'pioneer factors'. Recent genome-wide location analysis, combined with prior gene-specific studies, have let do the model that several nuclear receptors (PR, GR, and ER) bind near FoxA factors, and that in fact nuclear receptor binding is FoxA-dependent.

The genetic data described above raise some important questions, however: Why is that *Foxa1* and *Foxa2* cooperate in gene regulation by controlling overlapping sets of target genes, while the closely related *Foxa3* cannot compensate, even in tissues such as pancreas and hepatogenic endoderm where it is coexpressed? How is it that *Foxa1* has been maintained in evolution, even though the gene is expressed in a largely overlapping pattern with *Foxa2*, and is activated later in ontogeny? One possibility is that in addition to many common targets,

which control organogenesis of liver, lung, pancreas, prostate and midbrain, *Foxa1* and *Foxa2* might also regulate small sets of unique targets that cannot be compensated by the other factor. With the advent of genome-wide location analysis, especially ChIP-Seq, the answer to this question is within reach.

Acknowledgments

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Figure 1.

Pancreas development is dependent on joint action of Foxa1 and Foxa2 to activate the pancreatic master regulator Pdx1. Similarly, the two factors cooperate to guide liver, midbrain, and lung development. (A) In wild type mice, the pancreatic bud develops into a complex organ with a ductal tree, acinar tissue for synthesis of digestive enzymes, and embedded islets of Langerhans that consists of endocrine cells. In the absence of Foxa1 and Foxa2, only a rudimentary ductal system remains, resembling the phenotype of Pdx1 ablation. From Gao et al., *Genes & Development* 2008, with permission. (B) Foxa binding to the Pdx1 enhancers is stage dependent, with the promoter-proximal enhancer only occupied in the mature pancreatic islet. This is one example of many that Foxa sites are used conditionally.

Figure 2.

Coordinate gene regulation by Foxa factors and nuclear receptors. Depending on the cell type, Foxa1 and/or Foxa2 facilitate binding of the androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR). Foxa1 also activates expression of the estrogen receptor itself.