Review

FOXA1: master of steroid receptor function in cancer

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FOXA transcription factors are potent, context-specific mediators of development that hold specialized functions in hormone-dependent tissues. Over the last several years, FOXA1 has emerged as a critical mediator of nuclear steroid receptor signalling, manifest at least in part through regulation of androgen receptor and oestrogen receptor activity. Recent findings point towards a major role for FOXA1 in modulating nuclear steroid receptor activity in breast and prostate cancer, and suggest that FOXA1 may significantly contribute to pro-tumourigenic phenotypes. The present review article will focus on the mechanisms, consequence, and clinical relevance of FOXA1-mediated steroid nuclear receptor signalling in human malignancy.

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FOXA transcription factors: overview

Wings of discovery

The diverse and complex genetic programs that control cell fate and organogenesis remain incompletely defined, despite significant advances over the past two centuries. However, recent discoveries illuminate the disparate and elegant means through which tissue-specific differentiation is controlled. The Hepatocyte Nuclear Factor (HNF) superfamily of transcriptional regulators represents a unique subclass of DNA binding proteins that play a critical role in this process across multiple species (Weigel *et al*, 1989; Kalb *et al*, 1998; Pohl and Knochel, 2005; Hannehalli and Kaestner, 2009; Kaestner, 2010). Initially

cloned and purified from mammalian liver, the HNF superfamily was identified as sharing a highly conserved central motif with that of the *Drosophila melanogaster* forkhead protein (*fkh*), known to bind DNA and regulate processes necessary for early fly development (Weigel *et al*, 1989; Weigel and Jackle, 1990; Lai *et al*, 1991; Costa *et al*, 2003). On the heels of this discovery, extensive genome-wide analyses in multiple systems uncovered hundreds of forkhead-related genes, across a vast array of species (Kaestner *et al*, 2000). To date, the mammalian genome alone contains >19 subclasses of these transcriptional regulators, with over 40 known to be expressed in mammalian systems (Hannehalli and Kaestner, 2009). To bridge nomenclature, the vertebrate HNF family was renamed 'FOX' for Forkhead Box, with a letter representing the subclass (Kaestner *et al*, 2000). Among the most well characterized of these subclasses is the FOXA family of transcriptional regulators (FOXA1, FOXA2 and FOXA3), which play pivotal roles in mammalian development (Kaestner, 2010).

The central forkhead box domain, requisite for DNA binding and subsequent transcriptional regulation, consists of 100 amino acids in *Drosophila* and shares a high degree of similarity to the box domain of the mammalian FOXA subclass. Crystal structure analyses demonstrated that this DNA binding domain folds into a variation of a helix-turn-helix (HTH) structure, with two flanking loops reminiscent of wings; granting the name the 'winged helix' (Brennan, 1993; Clark *et al*, 1993). The central HTH makes direct contact with the major groove of the DNA in a base-specific manner, crucial for target site recognition (Clark *et al*, 1993; Cirillo and Zaret, 2007). Each wing is postulated to interact with minor grooves adjacent to the target sequence, potentially stabilizing interactions with DNA, in a class and DNA sequence-specific manner (Cirillo and Zaret, 2007). FOXA proteins are known to bind DNA as an early event in the sequence of transcriptional regulatory programs associated with developmental processes, and are thought to serve as 'pioneering factors'. Upon chromatin binding, FOXA proteins induce nucleosomal rearrangement, often resulting in an open chromatin structure, which facilitates binding of other transcriptional regulators (e.g., sex steroid hormone nuclear receptors), with subsequent initiation of tissue-specific transcriptional programs (Cirillo and Zaret, 1999; Zaret, 1999; Cirillo *et al*, 2002; Carroll *et al*, 2005; Laganieri *et al*, 2005b). As such, members of the FOXA subclass have become an attractive subject to study in terms of their role in both developmental processes and disease progression.

Pleiotropic roles of FOXA1 in development and differentiation

Initial studies aimed at elucidating the contribution of FOXA1 in tissue development utilized targeted gene deletion to

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generate FOXA1-specific knockout mice. FOXA1 null offspring are born in expected Mendelian ratios and are of normal weight (Kaestner *et al*, 1999; Shih *et al*, 1999). However, 2–14 days postpartum, pups succumb to neonatal death attributed to defects in pancreatic function (Kaestner *et al*, 1999; Shih *et al*, 1999). Subsequent investigation demonstrated that FOXA1^{−/−} animals suffer from impaired pancreatic development, leading to persistent hypoglycaemia, low plasma insulin levels and inappropriately high glucagon levels (Kaestner *et al*, 1999; Shih *et al*, 1999; Lantz and Kaestner, 2005). Interestingly, pancreatic β cell-specific ablation of FOXA1 alone had no effect on normal pancreatic function, while a double knockout of FOXA1 and FOXA2 restored the pathological pancreatic phenotype (Gao *et al*, 2010). Conditional FOXA2 deletion in isolated β islet cells resulted in deficiencies of insulin secretion, implicating FOXA2 as a major regulator of developmental processes in this cell type (Lantz *et al*, 2004). These findings collectively indicate that the impact of systemic FOXA1 ablation on pancreatic phenotypes is not manifest solely through β cell development, and that FOXA2 can compensate for FOXA1 function in a cell type-specific manner.

The compensatory roles demonstrated between FOXA1 and FOXA2 are not isolated to pancreatic β -cell function and have been observed in a variety of highly disparate systems. For example, conditional deletion of FOXA1, or introduction of an inactivating mutation of FOXA2 alone in developing respiratory cells yielded little effect on overall histoarchitecture of the lung; however, combinatorial deletion of FOXA1 and FOXA2 suppressed respiratory cell proliferation and differentiation (Wan *et al*, 2005). Similar studies conducted using conditional deletion in hepatocytes revealed that combinatorial loss of FOXA1 and FOXA2 (but not individual genetic deletions) resulted in hyperplasia of bile ducts attributed to enhanced IL6 signalling (Li *et al*, 2009). These effects proved sensitive to the timing of development, as these phenotypes are apparent only in the developing fetal liver, whereas no effect of combined deletion was observed in the mature liver (Li *et al*, 2009). Parallel studies further demonstrated that simultaneous deletion of FOXA1 and FOXA2 in the foregut endoderm completely blocked hepatic differentiation, thus reinforcing the concept that FOXA family members play critical, overlapping roles in liver development (Lee *et al*, 2005). Such requirements are mirrored in the dopaminergic neurons of the midbrain (the neurons lost during the onset of Parkinson's disease), wherein FOXA1 or FOXA2 serve redundant functions essential to promote neuronal differentiation and function (Ferri *et al*, 2007; Mavromatakis *et al*, 2010). Thus, an abundance of literature supports the contention that FOXA1 and FOXA2 display overlapping roles in development and differentiation of the pancreas, liver and neurological system.

While the functions of FOXA1/2 appear to be compensatory in a variety of systems, striking examples exist wherein FOXA1 alone appears to be the master regulator of tissue-specific differentiation and function. These are mainly associated with tissues dependent on sex hormone signalling, such as the breast and prostate glands. Proper postnatal development of the murine mammary gland is dependent on oestrogen action, manifest through the oestrogen receptor α (ER α ; Briskin and O'Malley, 2010). Upon hormone stimulation at puberty, ER α induces a transcriptional program that

governs proliferation of the mammary primordium, ductal elongation, bifurcation and invasion into fat pads (Briskin and O'Malley, 2010). ER α is also required for subsequent alveologenesis (Briskin and O'Malley, 2010). In the developing mammary gland, FOXA1 and ER α are co-expressed within luminal epithelial cells, with strong expression observed in the terminal end buds (Bernardo *et al*, 2010). Remarkably, FOXA1 deletion results in a mammary phenotype that mirrors that of the ER α null phenotype in terms of ductal morphogenesis but not alveologenesis (Bernardo *et al*, 2010). The influence of FOXA1 deletion on mammary ductal development is due to loss of ER α expression in the luminal progenitor cells that give rise to the ductal lineage (Bernardo *et al*, 2010). Hence, FOXA1 appears to be necessary for acquisition of ER α expression in the mammary ducts. Since the stunted ducts in the FOXA1^{−/−} mammary gland are capable of undergoing terminal differentiation, and the FOXA1^{+/−} gland exhibits increased alveolar development compared with wild-type glands, FOXA1 may play a role in sustaining the luminal epithelium in an undifferentiated state. Complementary studies in the murine prostate uncovered a cell type and temporal-specific distribution of the FOXA proteins, wherein FOXA1 remains throughout development and adulthood in epithelial compartments, while FOXA2 expression appears to be confined to the budding prostatic basal epithelia and is lost upon terminal differentiation (Gao *et al*, 2005; Mirosevich *et al*, 2005). The consequence of systemic FOXA1 deletion in the prostatic luminal epithelia is dramatic, as FOXA1^{−/−} prostates demonstrate defects in ductal development resulting in immature luminal cells surrounded by abnormally thick stromal layers, with no effect on androgen receptor (AR) expression or distribution (Gao *et al*, 2005). Importantly, expression of FOXA1 in the prostate epithelium positively correlates with AR, whose expression and activity are requisite for prostate development, survival and function (Gao *et al*, 2005; Mirosevich *et al*, 2005; Knudsen and Penning, 2010). As it has been shown that FOXA1 and AR interact in cells of prostatic origin to promote expression of AR target genes, it is likely that the interplay between these two transcription factors drive prostate development, though this posit has yet to be tested in a dual null system (Gao *et al*, 2003). On balance, a clear link exists between FOXA1, sex steroid hormone nuclear receptors and hormone-sensitive tissue development. Combined, these findings not only identify FOXA1 as a critical effector of hormone-sensitive tissue development, but also imply a potential role for FOXA1 in hormone-dependent cancers.

FOXA1 expression in human malignancy

The role of FOXA1 in human malignancy remains incompletely defined, as both pro- and anti-tumourigenic functions have been uncovered. In pancreatic cancer, a putative protective function for FOXA1 and FOXA2 has been proposed. Clinical analyses suggest that FOXA1 and FOXA2 expression inversely correlates with disease progression and/or aggressiveness; for example, FOXA1 and FOXA2 are readily detected in normal epithelium and precancerous lesions, but expression is commonly lost in poorly differentiated disease (an event associated with EMT-like phenotypes; Song *et al*, 2010). Conversely, studies in thyroid cancer identified FOXA1 as a potential oncogene, reported to be amplified and/or

overexpressed in ~70% of cases (Nucera *et al*, 2009). Nuclear FOXA1 staining was associated with a high proliferative index and was detected mainly in poorly differentiated thyroid tumours (Nucera *et al*, 2009). FOXA1 was also reported as being overexpressed or amplified in both lung and oesophageal cancers, respectively (Lin *et al*, 2002), but larger clinical cohorts are needed to confirm the expression and relevance of FOXA1 in these tumour types.

To date, the largest collective evidence linking FOXA1 function to human malignancies relates to observations in hormone-dependent cancers, primarily of the prostate and breast. Metastatic prostate cancer specimens demonstrated high nuclear FOXA1 staining in 89% of tissues as compared with 19% of patient-matched primary tumour samples (Jain *et al*, 2011). FOXA1 colocalized with AR in all samples and FOXA1 levels were positively correlated with tumour size, extraprostatic extension and lymph node metastasis (Jain *et al*, 2011). Independent studies confirmed FOXA1 enrichment in metastatic tissue, and identified the 14q21.1 chromosomal locus, harbouring the *FOXA1* gene, as being amplified in a subset of clinical samples (Robbins *et al*, 2010). Such data implicate FOXA1 as strongly associated with metastatic disease in prostatic adenocarcinoma. In breast cancer, high FOXA1 expression positively correlates with outcome, but the potential impact of such expression is variant, dependent on ER α status and tumour molecular subtype (Thorat *et al*, 2008). Clinical studies involving over 3500 primary invasive ductal carcinomas demonstrated positive FOXA1 staining in ~86% of all specimens, and expression was positively correlated with favourable prognosis (Mehta *et al*, 2011). Accordingly, low FOXA1 correlated with established markers of poor prognosis including high grade, increased tumour size, basal tumours and nodal metastasis (Mehta *et al*, 2011). As in normal breast tissues, there is a strong co-expression of FOXA1 and ER α in luminal breast cancer, and like ER α expression, high FOXA1 staining is associated with favourable prognosis in luminal disease (Thorat *et al*, 2008). Recent studies have uncovered an unexpected role for FOXA1 in a subtype of ER α -negative disease. As will be discussed in subsequent sections, FOXA1 can differentially regulate nuclear receptor activity in different cancer contexts.

FOXA1 is a pioneer factor for nuclear receptor activity

As initially determined by Carroll *et al* (2005), a dynamic and intricate molecular relationship exists between FOXA1 and nuclear receptor function. Using the MCF7 breast cancer cell model system, oestrogen-dependent ER α binding was initially mapped to specific sites on chromosomes 21 and 22. These original studies not only brought forth the knowledge that ER α predominantly occupies enhancer-like regions near oestrogen responsive genes, but interrogation of neighbouring DNA sequences uncovered forkhead motifs enriched in 56% of ER α -bound chromatin. Further analyses revealed that FOXA1 occupies these regions even in the basal state (i.e., prior to ER α recruitment), and serves as a pioneer factor for ER α , facilitating an open chromatin structure in the absence of hormone (Carroll *et al*, 2005; Laganieri *et al*, 2005a; Eeckhoutte *et al*, 2006; Lupien and Brown, 2009). Upon oestrogen stimulation, ER α is recruited to oestrogen-response

element (ERE)-like sequences in the vicinity of FOXA1-bound chromatin and thereby promotes transcriptional regulation of oestrogen responsive genes (Eeckhoutte *et al*, 2006; Lupien and Brown, 2009; Hurtado *et al*, 2010). Subsequent studies in prostate cancer cells similarly identified FOXA1 as capable of facilitating AR/chromatin interactions at regulatory loci near androgen responsive genes (Wang *et al*, 2009). Such events result in cofactor recruitment and transcriptional initiation, similar to that of ER α in breast cancer cells (Eeckhoutte *et al*, 2006; Lupien *et al*, 2008). Collectively, these studies re-defined how nuclear receptor binding to DNA is topographically regulated, adding a new layer of complexity to gene regulation in hormone-dependent cancers.

Since FOXA1 is recruited to DNA prior to hormone-dependent nuclear receptor binding, significant overlap between the FOXA1 cistrome of prostate and breast cancer cells might be expected. Intriguingly, genome-wide interrogation of FOXA1 binding between the two cell types identified a large percentage of lineage-specific binding sites (Lupien *et al*, 2008; Hurtado *et al*, 2010; Serandour *et al*, 2011). Due to its ability to actively recruit either AR or ER α to sites of transcriptional regulation, the tissue-specific programs induced by these nuclear receptors are therefore postulated to be attributed, in part, to cell type-specific binding of FOXA1. In efforts to identify the mechanisms that underpin this process, mapping of the FOXA1-associated chromatin landscape revealed that specific methylation events which define transcriptionally active enhancers (mono- and di-methylation at histone H3 lysine 4, H3K4me1 and H3K4me2) are enriched at sites of FOXA1 occupancy in breast and prostate cancer cells (Lupien *et al*, 2008; Lupien and Brown, 2009; Serandour *et al*, 2011). Reversal of histone methylation at these sites (e.g., as can be achieved via overexpression of the lysine demethylase KDM1/LSD1) inhibits both FOXA1 occupancy and hormone-induced AR or ER α recruitment (Lupien *et al*, 2008; Lupien and Brown, 2009). Conversely, sites deficient in H3K4me1 and/or H3K4me2 are enriched for transcriptionally repressive marks like histone 3 lysine 9 methylation (H3K9me2) and are generally devoid of both nuclear receptor and FOXA1 binding events (Lupien *et al*, 2008; Lupien and Brown, 2009). These observations are of potentially strong translational relevance, as the AR-directed programs unique to advanced, castration-resistant prostate cancer (CRPC) appear to be dependent on the H3K4me1/2 signature (Wang *et al*, 2009). Based on these findings, a concerted effort has been made to define the role of FOXA1 in fostering AR- and ER α -dependent cancer development and progression.

FOXA1-nuclear receptor interplay in breast cancer

ER α -positive disease

The standard of care for luminal breast cancer involves aromatase inhibitors to prevent systemic and intratumoural oestrogen biosynthesis, or selective antagonists directed against ER α (e.g., Tamoxifen; Criscitiello *et al*, 2010). Tamoxifen induces receptor association with chromatin but suppresses gene expression from oestrogen-stimulated gene loci (Shang *et al*, 2000). Critically, FOXA1 proved essential for the cellular response to Tamoxifen (Hurtado *et al*, 2010). Deep sequencing of ER α -bound DNA (ChIP-seq) confirmed that ER α occupies similar regions of DNA in the presence of both

agonist (estradiol) and a selective antagonist (Tamoxifen), with opposing actions on transcriptional initiation of ER α -regulated genes. In the presence of estradiol, which stimulates ER α -dependent cellular proliferation in breast cancer cells, a concomitant reduction of cell doubling resulted after FOXA1 silencing (Hurtado *et al*, 2010). Further studies using MCF7-derived cell lines resistant to Tamoxifen showed that FOXA1 silencing not only suppressed ligand-independent ER α binding to chromatin, but also inhibited cellular proliferation without affecting ER α protein levels (Hurtado *et al*, 2010). Such results implicate FOXA1 as a key determinant of the response to anti-estrogens in ER α -positive cells, even in those that have transitioned to Tamoxifen resistance. These observations may therefore provide a molecular explanation for the correlation of FOXA1 with favourable prognosis in ER α -positive disease, and suggest that FOXA1 may modulate the response of sex hormone activated nuclear receptors to endogenous ligands and antagonists.

ER α -negative disease

While the role of FOXA1 in ER α -positive breast cancer has been the predominant focus of investigation in this tumour type, the role of FOXA1 in ER α -negative disease is only just emerging. Recent molecular profiling studies have identified a subtype of ER α -negative breast cancer dubbed 'molecular apocrine' due to enrichment of apocrine-like histological features, which possess a gene signature that is positively associated with oestrogen signalling despite lack of ER α and likely ER β (Farmer *et al*, 2005). Similar to normal apocrine glands and metaplastic lesions, molecular apocrine breast cancers harbour high AR expression and activity (Farmer *et al*, 2005; Doane *et al*, 2006), and FOXA1 expression is another signature feature of this tumour subtype. While HER2 amplification is enriched in molecular apocrine tumours, it does not sufficiently define the phenotype. In many ways, this type of breast cancer has features reminiscent of prostate cancer, and the molecular apocrine gene signature is significantly associated with the gene signature in LNCaP prostate cancer cells (Doane *et al*, 2006). In the clinical setting, apocrine carcinoma defined by histological markers is considered a rare type of breast cancer, occurring in ~1–4% of all breast cancer cases (O'Malley and Bane, 2008); however, molecular apocrine tumours as defined by gene profiling are estimated to be more common, constituting ~8–12% of breast cancer cases (Perou *et al*, 2000; Farmer *et al*, 2005). As there are few treatment options for ER-negative disease outside of chemotherapy (and anti-HER2 therapies for HER2+ disease), targeting AR signalling has emerged as a potential therapeutic strategy for this cancer subtype. Supporting this view, at least one clinical trial using the AR antagonist bicalutamide for treatment of AR-positive, ER α -negative metastatic breast cancer has been approved (identified in <http://clinicaltrials.gov> as NCT00468715). As will be discussed herein, new studies investigating the impact of FOXA1 in molecular apocrine breast cancer may provide additional support for this therapeutic approach.

Molecular apocrine breast cancer, part 1: FOXA1 drives AR to mimic ER α activity

Based on the established interplay between FOXA1 and AR in prostate cancer, the potential interaction between these transcription factors in molecular apocrine breast cancer has

been recently investigated. Not only do these studies further implicate FOXA1 as a modulator of nuclear receptor function in hormone-dependent cancers, but the study by Robinson *et al* (2011) puts forth the staggeringly unexpected hypothesis that, in the molecular apocrine subtype of breast cancer, FOXA1 may direct AR to sites normally occupied by ER α in luminal breast cancer, inducing an oestrogen-like gene program that stimulates proliferation.

Model systems to assess molecular apocrine disease are limited, but the MDA-MB-453 breast cancer cell line recapitulates salient features of this breast cancer subtype, including high AR expression in the absence of ER α or ER β and the presence of FOXA1 (Doane *et al*, 2006). Molecular profiling of multiple ER α -negative breast cancer cell lines showed that MDA-MB-453 cells are characterized by a gene signature similar to that of molecular apocrine breast cancers (Doane *et al*, 2006). Moreover, MDA-MB-453 cells express several genes that are normally regulated by ER α in luminal cancers, thus suggesting retention of 'ER α -like' signalling in the absence of oestrogen receptors. While the role of AR in molecular apocrine tumour cells had not been well defined, it had been previously shown that MDA-MB-453 cells are growth stimulated by androgens in an AR-dependent manner and exhibit an anti-proliferative response to selected AR antagonists (Birrell *et al*, 1995). Strikingly, Robinson and colleagues identified AR as a potential surrogate for ER α , capable of promoting an ER α -like gene expression program (Figure 1). Initial experiments validated the influence of AR signalling on growth of MDA-MB-453 cells; in this context, AR signalling acted in a pro-proliferative capacity, reminiscent of ER α signalling in ER α -positive breast cancer cells, and similar to the role of AR in prostate cancer. Investigation of potential mechanisms through which AR signalling may promote a luminal-like phenotype uncovered a subset of classically regulated ER α target genes under the control of AR, many of which overlap with the gene signature that is a hallmark of molecular apocrine breast tumours. These findings provide support for the provocative hypothesis that in molecular apocrine tumour cells, AR is 're-programmed' to serve in place of the absent ER α .

Exploring this concept further, genome-wide AR binding events were mapped in MDA-MB-453 and LNCaP (PCa) cells and compared with ER α binding in MCF7 cells. In these studies, the AR cistrome in MDA-MB-453 cells closely correlated with that of the ER α cistrome in MCF7 cells (50.9% overlap), and proved less similar to the AR cistrome in prostate cancer cells (29.3% overlap). These observations provide robust genome-wide evidence to support the hypothesis that AR can mimic ER α action in molecular apocrine breast cancer. Surprisingly, comparative motif analyses among cell lines identified an enrichment of ERE-like sequences at AR-occupied regions in MDA-MB-453 cells, suggesting that AR may utilize similar molecular and transcriptional regulatory processes to mimic ER α -regulated biological processes. Consonantly, FOXA1 consensus sites were found to be enriched at sites of AR occupancy, providing a putative means through which AR could occupy ER α regulated loci. ChIP-Seq analyses to map sites of FOXA1 binding confirmed this posit, as AR occupancy was almost invariably coincident with FOXA1 binding (98.1% overlap of AR binding with FOXA1). Likewise, FOXA1 sites also correlated significantly with AR binding sites in prostate cancer

Figure 1 Converging pathways of FOXA1 on AR-regulated growth of MDA-MB-453. The mechanisms through which AR regulates growth of molecular apocrine cancer cells described by Ni *et al* and Robinson *et al* are summarized. Both studies utilized gene expression arrays coupled to AR/FOXA1 ChIP sequencing to identify novel mechanisms through which AR functions in this cell type. Briefly, Robinson *et al* (2011) uncovered an ERα-like signature regulated by AR. Only in the presence of FOXA1 was AR able to bind to oestrogen-regulated genes (ERG), and induce a gene set classically regulated by ERα, responsible for growth. Concurrent studies by Ni *et al* (2011) identified a FOXA1/AR-dependent induction of the WNT7B gene. Subsequent signalling through the Wnt pathway induced nuclear accumulation of β-catenin, association with AR, and localization to the HER3 regulatory gene locus. Here, they describe the growth effects of AR signalling as dependent on HER3 induction and signalling through the AKT pathway.

cells (82% overlap), thus representing an even greater degree of concordance than seen between FOXA1 and ERα in breast cancer cells (~50% overlap). AR/FOXA1 re-ChIP experiments in MDA-MB-453 cells confirmed that these factors co-

occupy chromatin in an androgen-dependent manner, and that depletion of FOXA1 dramatically reduced AR occupancy at the sites identified in molecular apocrine cells. Combined, these data suggest that AR binding in molecular apocrine tumours is almost entirely dependent on FOXA1 occupancy.

Aiming to tie the genome-wide mapping events to the proposed biological role of AR in molecular apocrine tumours, gene expression arrays were performed in the presence of endogenous and diminished FOXA1 levels. A total of 730 transcripts were identified as being regulated by FOXA1 knockdown, which include a number of known ERα target genes (e.g., *TFF1*, *XBPI*, and *RARA*). Ontological analyses revealed enrichment for signalling networks involved in cell cycle, development and cytoskeleton remodelling, hinting to a potential role in AR-driven phenotypes. Importantly, the biological implications of FOXA1 knockdown were similar to those observed upon AR inhibition, and resulted in decreased tumour cell growth in 3D culture. Most critically, 91% of the gene signature associated with clinical molecular apocrine tumours was altered by FOXA1 knockdown, highlighting the translational relevance of the AR/FOXA1 signalling axis. While it will be of interest to determine whether these effects are shared upon ablation of AR signalling, these novel findings clearly identify AR as a factor capable of supplanting ERα function, reveal FOXA1 as a master regulator of AR activity in this subtype and illuminate the impact of FOXA1-driven AR activity as an influential effector of molecular apocrine tumour cell phenotypes.

Molecular apocrine breast cancer, part 2: impact of FOXA1 and Wnt on AR-dependent HER2/HER3 activity

While Robinson *et al* (2011) identified AR as promoting ERα-like gene signatures and resultant molecular apocrine breast cancer cell growth, a study published concurrently from Ni *et al* (2011) proposed an alternative and potentially complementary mechanism of AR-driven tumour progression in this tumour type (Figure 1). High levels of AR expression were found to be enriched in the HER2 high/ERα-negative tumour subgroup, which contains the apocrine-like tumour subset, consistent with previous findings (Farmer *et al*, 2005). Consistent with previous observations (Birrell *et al*, 1995), addition of an AR antagonist to MDA-MB-453 cells induced cell-cycle arrest and a modest apoptotic phenotype. Profiling the ligand-dependent AR cistrome in MDA-MB-453 cells by Ni *et al* (2011) identified only 10% of the total number of binding sites as reported by Robinson *et al* (2011). While the location of these sites remained consistent (mainly associated with enhancer-like regions) between studies, there was minimal overlap with the ERα cistrome in MCF7 cells generated by Ni and colleagues, and it was concluded that a large majority of AR-occupied regions are likely to be cell type specific. Subsequent motif analyses of AR-occupied regions uncovered both androgen response elements (AREs) as well as FOXA1 consensus sites, and are thus more reminiscent of AR cistromes in prostate cancer cells. Despite these differences, FOXA1 and AR cistromes were found to overlap significantly (37% of FOXA1 binding sites), and were found to be near transcriptionally active genes, validating FOXA1 as a crucial mediator of AR transcription in this cell type.

As the majority of the AR cistrome was determined to be unique in the Ni *et al* (2011) study, parallel gene expression arrays were used to define potentially relevant biological

targets. The Wnt signalling pathway was influenced by DHT stimulation, and the gene encoding the Wnt ligand *WNT7B* emerged as an AR target of critical importance. Mining of the AR cistrome illuminated an AR binding site ~1.5 kb from the transcriptional start site of the *WNT7B* gene locus, which was validated as a *bone fide* AR-occupied region by ChIP-qPCR analyses in response to DHT. AR binding was also associated with elevated *WNT7B* mRNA levels, and was further validated in two other ER α -negative/AR-positive breast cancer cell lines. Importantly, *WNT7B* correlated with AR status in clinical samples, and was shown *in vivo* to maintain mammary epithelial cells in a non-committed state through activation of β -catenin-dependent signalling cascades. In line with previous reports, β -catenin shuttled to the nucleus in response to DHT, an event that was abrogated if either AR or *WNT7B* was silenced. Interestingly, active β -catenin is known to interact with ligand bound AR (Truica *et al*, 2000; Song and Gelmann, 2005), which could therefore dictate binding to unique sites. Indeed, the AR cistromes defined at 4 and 16 h poststimulation proved markedly distinct, the difference being attributed to the presence of nuclear β -catenin at the later time point. Of note, FOXA1 was highly associated with AR occupancy at all time points, indicating that while FOXA1 is likely necessary for initial AR/chromatin interactions, other factors influence the temporal and kinetic components of AR binding. Ontological analysis of genes neighbouring AR-occupied regions identified *HER3*, a co-receptor of *HER2*, as a potential target, thus implicating the pro-proliferative role of β -catenin in this system as AR mediated and dependent. Biochemical interrogation of the *HER3* locus confirmed the occupancy of AR, FOXA1 and β -catenin only after long-term DHT treatment (16 h). Additionally, chromatin binding at this time point correlated with an increase in *HER3* expression and hyperactivation of *HER2* and downstream targets (e.g., *AKT*), all of which are intimately linked to the activity of Wnt/ β -catenin and AR signalling cascades. Notably, the pro-proliferative effects induced by DHT were abrogated by AR or Wnt/ β -catenin antagonists and tyrosine kinase inhibitors, indicating that androgen-induced growth in this system is exquisitely dependent on both pathways. Parallel studies in MDA-MB-453 xenografts confirmed this postulate, as the AR antagonist bicalutamide effectively suppressed tumour growth *in vivo*, concomitant with decreased *HER3* expression and phospho-*AKT* levels. On balance, these findings provide strong evidence for AR as a FOXA1 and β -catenin-dependent effector of *HER2/HER3* activity in molecular apocrine disease.

It is of obvious relevance to determine whether the ability of FOXA1 to drive AR-mediated ER-like or *HER2/HER3* activity acts in a synergistic fashion, and to resolve disparity in the respective genome-wide analyses. Discrepancies in methodology and timing of analyses exist between the two studies and may underlie the variation observed. For example, differences in AR ligands (DHT versus R1881) as well as disparity in algorithms utilized to define transcription factor cistromes may, in part, explain differences in overall AR binding sites reported. Indeed, Ni and colleagues found ~10-fold fewer FOXA1 and AR binding sites in MDA-MB-453 cells than did the Robinson *et al* study using similar time points. Such distinctions could be due to the fact that DHT is more readily metabolized than the synthetic ligand R1881; thus, R1881 potentially enhances AR stability and chromatin

occupancy (in this system) (Ji *et al*, 2007). Moreover, R1881 is known to bind the glucocorticoid receptor as well, with unknown consequences for downstream biology and/or AR occupancy on chromatin (Cleutjens *et al*, 1997). Additionally, while similar computational programs were utilized to identify AR binding sites, disparity in statistical analyses could explain the differentially observed peak enrichment profiles. Standardization of both ligand and methodology could facilitate comparison across multiple data sets. Separate from these issues, it will be of interest to determine the level of conservation for AR binding sites identified as vital for DHT-induced growth (e.g., *WNT7B* regulatory loci), between the data sets. Finally, it will be of significant clinical relevance to determine if the AR cistromes are maintained in human tissue samples, and the impact of the variant AR functions in human biology. Recent studies in prostate cancer have begun to address this issue, delineating several of the AR binding sites associated with tumour growth in clinical specimens (Massie *et al*, 2011). Despite the need for ongoing investigation, the Robinson and Ni studies clearly demonstrate that FOXA1-driven AR can utilize multiple mechanisms to promote pro-tumourigenic phenotypes in molecular apocrine breast cancer, thus identifying a potentially critical role for FOXA1 in this tumour subtype.

FOXA1 and AR control in prostate cancer

While the role of AR in breast cancer is dependent on relative steroid nuclear receptor expression, molecular subtype and cellular context, the function of AR in prostate cancer appears to be more straightforward. Current clinical evidence and modelling of disease demonstrate that prostatic adenocarcinomas are exquisitely dependent on AR signalling at all stages of disease, and that in the case of metastatic/advanced disease, resurgent AR activity is the major cause of relapse after hormone therapy, referred to as development of CRPC (Chen *et al*, 2008; Knudsen and Scher, 2009; Knudsen and Penning, 2010). In both early stage disease and CRPC, AR governs transcriptional programs responsible for growth, survival and tumour progression (Wang *et al*, 2009; Knudsen and Penning, 2010). Of importance, FOXA1 activity is obligatory for AR function in response to ligand, and is essential for AR-mediated expression of pro-proliferative/pro-survival genes required for CRPC growth; as such, FOXA1 is thought to contribute significantly to prostate cancer progression (Wang *et al*, 2009). These functions of FOXA1 were attributed not only to the ability of FOXA1 to directly interact with AR, but also to serve as a pioneer factor for AR at sites of transcriptional regulation. Based on these collective findings, FOXA1 was postulated to support the ability of AR to promote disease development, survival and progression.

A provocative study by Sahu and colleagues (Sahu *et al*, 2011) directly challenged this hypothesis, and discovered surprisingly divergent roles for FOXA1 in AR regulation. Genome-wide elucidation of the AR cistrome in the AR-positive, ligand-dependent LNCaP cell line, confirmed previous reports which described a high degree of overlap between both AR and FOXA1 binding sites (~70% of AR binding sites). Interestingly, only about 25% of the FOXA1 cistrome coincided with AR binding sites, implicating a potentially broad role for FOXA1-mediated transcriptional

regulation in prostate cancer cells. Most excitingly (and contrary to what might be anticipated), silencing FOXA1 resulted in the appearance of additional AR-occupied regions, increasing the number of binding events by 2.5-fold. Though a large number of binding sites were consistent with parental cells, 43% were lost, and over 13 000 new sites were identified. Thus, these findings indicate that FOXA1 both promotes and suppresses AR function, and suggest that AR binding sites can be subclassified into those that (i) require FOXA1 as a pioneer factor; (ii) occur irrespective of FOXA1 status; and (iii) are unmasked in the absence of FOXA1 (Figure 2). The relevance of these distinct binding classes was addressed through gene expression profiling after FOXA1 silencing or in FOXA1-replete isogenic cells. The total number of AR responsive genes notably increased with FOXA1 depletion, further suggestive of the novel hypothesis that FOXA1 can both assist and thwart AR activity, dependent on cellular context. Consistent with these data, genes whose expression are unmasked by FOXA1 silencing harbour regulatory regions that are enriched for canonical AREs but lack FOXA1 consensus sites. While canonical FOXA1 consensus sequences were absent, other *cis*-elements were enriched, including those of the ETS family, suggesting a potential role for these proteins in guiding AR to these sites. Such evidence indicates that FOXA1 can induce repressive features on chromatin to mask a large percentage of AR binding sites, suggesting a critical role for FOXA1 in the spatial and temporal regulation of AR-mediated gene regulation.

Determining the underlying mechanisms by which FOXA1 governs AR binding will be essential for ascertaining the relative impact of FOXA1 on AR-dependent transcriptional regulation. FOXA1 is typically recruited to histone modifications associated with active chromatin, which facilitates subsequent recruitment of nuclear receptors and transcriptional activation (Lupien and Brown, 2009). Accordingly, 70% of all shared FOXA1/AR binding sites contained this signature in parental cell lines, and these marks were present at 70% of sites either requiring or dispensable for FOXA1. A majority of the AR binding sites induced by FOXA1 depletion lacked H3K4me2, suggesting that other factors likely control AR recruitment to these sites. It will be of interest to determine the status of mono-methylation (H3K4me1) in this

data set, as this modification is also known to colocalize with pioneer factors such as FOXA1. Congruent with these findings, a majority of all FOXA1 binding site classes correlated with DNase hypersensitive sites, while loss of FOXA1 introduced novel hypersensitive sites (16% of all AR binding sites in this context), indicating that site-specific recruitment of chromatin remodelling factors can occur in an androgen-dependent manner. Parallel studies further validated the postulate that FOXA1 depletion induces the three classes of AR binding events after hormone stimulation, and implicate the mediator protein Med12 and histone acetyl transferase p300 as requisite for initiation of AR transcriptional programs in the absence of FOXA1 (Wang *et al*, 2011). Motif analyses demonstrated that FOXA1-dependent AR binding sites were significantly less enriched for AREs than either the dispensable or novel sites, providing a potential basis for the observation that AR is dependent on FOXA1 at these sites (Wang *et al*, 2011). These collective observations not only illustrate the divergent and context-specific impact of pioneer factors on sex hormone-dependent nuclear receptor function, but also elegantly demonstrate the importance of utilizing genome-wide analyses to assess the effects of nuclear receptor modulators on molecular and cellular mechanisms underpinning biological processes.

With regard to clinical relevance, the observations of Sahu *et al* strongly suggest that the levels of FOXA1 in cancer cells may significantly alter AR function and resultant tumour cell phenotypes. Thus, it is tempting to postulate that there may be a critical threshold of FOXA1 expression that dictates the divergent roles of AR during prostate development (pro-differentiative) versus prostate cancer (pro-proliferative/pro-survival). Investigation of clinical samples identified FOXA1 as elevated in primary prostate adenocarcinoma (Sahu *et al*, 2011), suggestive that disease-promoting AR programs may require higher levels of FOXA1 (compared with AR-regulated programs during development). Importantly, FOXA1 nuclear staining correlated with poor prognosis, as defined by increased Gleason grade and a reduced time to prostate cancer-specific mortality. Together with the investigation of FOXA1 function in model systems, this study supports a new paradigm in nuclear receptor signalling, wherein the level of FOXA1 governs global AR binding events, downstream transcriptional regulation and tumour behaviour.

Figure 2 Three categories of AR binding sites in prostate cancer cells. The three categories of AR binding sites that have been described by Wang *et al* (2011) and Sahu *et al* (2011) in the LNCaP cell line are highlighted. The presence of high FOXA1 directs AR to sites that are enriched for both forkhead motifs as well as AREs, while low levels of FOXA1 reveal novel AR binding sites that lack neighbouring FKH motifs but maintain ARE enrichment. Additionally, sites exist in the genome where FOXA1 is dispensable for AR binding and transcriptional induction. The number of binding sites indicated below each class are representative of the Sahu data set.

Conclusions and future directions

While the role of sex steroid nuclear receptors in promoting disease development and progression has been long appreciated, recent findings and the studies described herein increasingly paint the image of these receptors as powerfully influenced by FOXA1 status and function. In the vast majority of prostate cancers, it is clear that AR function is essential for tumour development and progression, that these activities are supported by FOXA1, and that resurgent AR activity after hormone therapy (a hallmark of the transition to lethal disease) requires FOXA1 activity. The findings described herein refine our understanding of this process, reveal that not all AR functions are FOXA1 sensitive, and identify a potentially important and intriguing subclass of AR activity that occurs only in the presence of FOXA1 depletion. Potential clinical relevance was brought to light, in that increased FOXA1 was associated with factors linked to poor prognosis and high AR activity. In the case of molecular apocrine breast cancer, equally fascinating and unexpected observations illuminate a novel role for FOXA1 in controlling AR activity and tumour growth. The impact of FOXA1 on AR activity appears multi-fold, and includes the ability of FOXA1-controlled AR to invoke HER2/HER3 activity (by way of collaboration with Wnt and β -catenin) and to serve as a surrogate for induction of ER α -like gene expression programs. Thus, in the context of both prostate cancer and molecular apocrine breast cancer, these collective findings provide the foundation for discerning the impact of FOXA1 on sex steroid hormone receptor signalling, and stimulate consideration of FOXA1 as a therapeutic target.

However, significant gaps in basic knowledge and barriers towards translational development remain that should be considered. *First, what are the molecular mechanisms that underlie gene-specific responses to FOXA1?* Significant disparity in FOXA1 requirement for AR and ER α cistromes has been observed between cell lines and studies alike. Standardization of algorithms, hormonal manipulation and timing would allow for additional clarity in comparing data sets, and would allow for potential development of a FOXA1 'signature' across model systems. *Second, what of the other FOXA family members (i.e., FOXA2 and FOXA3)?* These factors bind similar consensus sites, and the relative impact on AR and ER α function, as well as on downstream tumour phenotypes, has been only scantily considered. Preliminary studies in AR-negative PCa cells demonstrated FOXA2 but not FOXA1 can induce AR target gene expression in the absence of ligand bound AR, implicating FOXA2 induction as a potential mechanism to promote CRPC (Mirosevich, 2006). Examination of additional FOXA family members in the context of transcriptional regulation, tumour growth, and clinical correlates will figure prominently into potential

development of FOXA1 as a therapeutic target in human disease. *Third, what is the role of the other pioneer factors in governing AR activity in tumour cells?* For example, GATA2 and OCT1 have been identified as capable of serving pioneer factor roles for AR, and increased levels of GATA2 have been implicated in promoting aberrant AR activity in PCa (Perez-Stable *et al*, 2000; Wang *et al*, 2007; Jia *et al*, 2008; Bohm *et al*, 2009). Thus, it is essential to determine the relative contribution of these factors to nuclear receptor activity. *Fourth, what are the implications of the AR binding sites unmasked by FOXA1 depletion?* A rich understanding of the mechanisms that support acquisition of novel binding sites and the consequence of the associated gene expression programs requires careful consideration. It will be beneficial to determine if these sites are composed of unique AR/cofactor complexes, which drive transcriptional processes relevant to development or disease. Coupling ChIP-Seq to proteomics would help to unveil such complexes and illuminate new avenues of potential therapeutic intervention. Of note, recent advances in several small molecule technologies have led to nucleotide-based inhibitors which have been shown to antagonize transcriptional programs either by directly inhibiting transcription factor/DNA interactions (Chenoweth and Dervan, 2009) or by effectively silencing transcription factor expression (Chi *et al*, 2001; Gleave and Chi, 2005; Gleave *et al*, 2005) each of which could hold great therapeutic value in this context. *Finally, what controls FOXA1 levels?* Identification of the factors that influence FOXA1 expression and/or accumulation is of fundamental importance in both prostate cancer and molecular apocrine breast cancer. On balance, while the roles of FOXA1 appear to be context, concentration and tissue specific, it is clear that FOXA1 plays a major role in the progression and development of breast and prostate cancer subtypes; crucial next steps will be to determine how best to translate these molecular understandings and 'out-fox' this influential transcriptional regulator in the clinical setting.

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Conflict of interest

The authors declare that they have no conflict of interest.

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