MECHANISM OF HIC-5/ARA55 ACTION, A NOVEL STROMAL-SPECIFIC NUCLEAR RECEPTOR COACTIVATOR

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Hydrogen peroxide inducible clone-5/Androgen Receptor Activator 55 (Hic-5/ARA55) is a group III LIM domain protein that functions at focal adhesion complexes as well as in the nucleus as a nuclear receptor coactivator. Because the interaction of the androgen receptor (AR) with Hic-5/ARA55 results in enhanced androgen-induced transcription, we analyzed Hic-5/ARA55 expression in prostate tissue sections from normal human donors and prostate cancer patients. In each sample, Hic-5/ARA55 expression was confined to the stromal compartment of the prostate. Furthermore, in a human prostate stromal cell line (i.e. WPMY-1 cells) Hic-5/ARA55 was localized both at focal adhesion complexes and within the soluble cytoplasmic compartment. The ability of Hic-5/ARA55 to shuttle between the nuclear and cytoplasmic compartments within WPMY-1 cells was revealed upon inhibition of nuclear export with leptomycin B (LMB). siRNA ablation experiments established endogenous Hic-5/ARA55 as a coactivator for both viral and endogenous cellular AR-regulated genes. Furthermore, chromatin immunoprecipitation (ChIP) analysis showed androgen-dependent recruitment of Hic-/ARA55 to the promoter of the stromal androgen-responsive KGF gene. Using the A1-2 derivative of T47D

breast cancer cells, we examined the mechanism by which Hic-5/ARA55 potentiates nuclear receptor transactivation. Hic-5/ARA55 was found to be an important component of glucocorticoid receptor (GR)-coactivator complexes in A1-2 cells since ablation of Hic-5/ARA55 expression by RNA interference-mediated silencing reduced GR transactivation. As shown by ChIP assays, Hic-5/ARA55 is recruited to glucocorticoid-responsive promoters of the MMTV, *c-fos*, and p21 genes in response to glucocorticoid treatment. Results from sequential ChIP assays established that Hic-5/ARA55 associates with the corepressor, NCoR, in the absence of glucocorticoids. However, upon glucocorticoid stimulation, Hic-5/ARA55 interacts with GR-coactivator containing complexes at these promoters. Ablation of Hic-5/ARA55 expression resulted in reduction of both TIF-2 and p300 recruitment to glucocorticoid-responsive promoters. These data provide the first demonstration of a stromal-specific AR coactivator that has an impact on an androgen regulated growth factor that is essential for stromal/epithelial cell communication in the prostate. Furthermore, these results suggest that Hic-5/ARA55 is required for optimal GR-mediated gene expression possibly by providing a scaffold that organizes or stabilizes coactivator complexes at some hormone-responsive promoters.

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PREFACE

I would first like to thank my Lord for providing strength and patience as well as many extraordinary people whose culmination of inspiring words and deeds without which this thesis would not exist.

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the impossible just takes a little longer. My siblings, Aaron C. Heitzer, Tammy L. Burkett, and Tracy G. Walborn, have continued to encourage me, and I thank them all very much for supporting me even at the expense of time spent together. Finally, I want to thank my G. Mbella Ekema for moving to Pittsburgh to continue our life together. Mbella, through the multitude of your talents, you have taught me that the only restraints that impede us are those we place on ourselves. You have exemplified your love and support for my dreams by not settling for second best in your endeavors. I look forward to our fully post-graduate life with much anticipation.

CHAPTER 1 INTRODUCTION

Prostate Function

The prostate gland secretes the main protein components found in seminal fluid. Structurally, the prostate is composed of both a glandular epithelium and a fibromuscular stroma, which are separated by a basement membrane. The epithelial compartment is composed of two types of epithelium, basal and secretory. Basal epithelial cells secrete proteins found in the basement membrane, and some may constitute secretory epithelial stem cells (4, 5). The secretory epithelial compartment is responsible for production and secretion of enzymes, such as human prostate acid phosphatase (hPAP) and prostate specific antigen (PSA), which are found in seminal fluid. The stromal compartment is composed of smooth muscle cells, fibroblasts, endothelial cells, nerve cells, and lymphocytes. The stroma secretes growth factors and cytokines responsible for normal growth and development of the epithelial compartment (6).

Epithelial-Stromal Interactions in the Prostate

Communication between the epithelial and stromal compartments of the prostate, mediated by growth factors and cytokines, is crucial for the maintenance of prostate growth and function (Figure 1, 7, 8). Examples of such growth factors in the prostate include transforming growth factor beta (TGF-β), transforming growth factor alpha (TGF-α), epidermal growth factor (EGF), keratinocyte growth factor (KGF), fibroblast

growth factor-2 (FGF-2), and insulin-like growth factor (IGF) (9, 10). Most of these growth factors enhance both growth and development of the prostate, although TGF-β may negatively affect prostate growth (2).

During prostate development, the urogenital sinus mesenchyme (UGM) stimulates epithelial differentiation, ductal branching, and proliferation (11). Conversely, signals derived from the urogenital epithelium (UGE) promote mesenchymal differentiation (12). Along with epithelial-stromal communication, androgen signaling through AR is crucial for prostate development (13). Reconstitution experiments with isolated stromal and epithelial cells performed by Cunha and Lang illustrated the necessity for functional AR in the UGM for the development of a functional prostate (14). In these studies, prostate tissue was derived from either normal or testicular feminized (TFM) mice that have a loss-of-function mutation in the AR gene. Whereas combining TFM UGE and normal UGM produced normal prostate organ formation in the presence of androgen, analogous tissue reconstitutions with normal UGE and TFM UGM did not support prostate organogenesis. This correlates with AR expression patterning seen in the developing prostate. Specifically, before and during prostatic bud formation, AR expression is restricted to the UGM (13). These data suggest that AR controls the expression of a soluble factor in the UGM that is necessary for proper epithelial differentiation and thus prostate development.

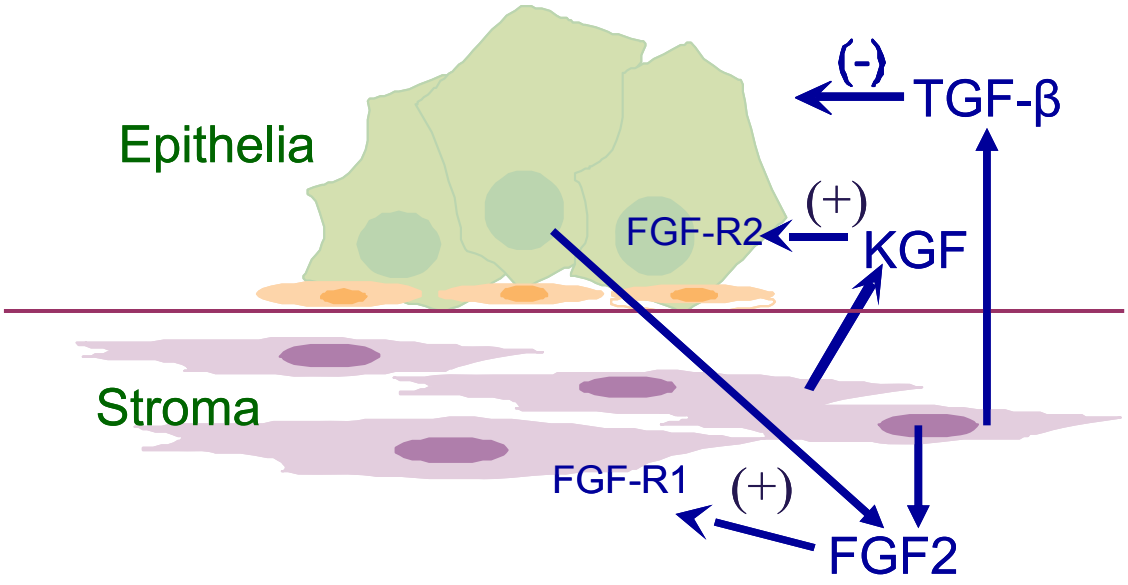


Figure 1. Stromal-epithelial interactions in the prostate. Communication between the stromal and epithelial compartments of the prostate via growth factor and cytokines can either induce or repress cell growth. KGF, a paracrine mediator of epithelial-stromal interactions, is produced in the stromal compartment while its receptor is expressed solely in the epithelial compartment, resulting in epithelial proliferation (1). Alternatively, TGF-β is produced by both the epithelial and stromal compartment and functions to negatively influence epithelial cell growth by inhibiting cell division as well as inducing apoptosis (2). Finally, FGF2 is produced by the both the epithelial and stromal compartment, acting in a paracrine and autocrine fashion respectively, positively influencing stromal cell growth (3).

Androgens and Prostate Function

Circulating androgens derived primarily from the testes also contribute to normal growth and development of the prostate gland (15). Testicular Leydig cells secrete testosterone (T) that is converted in the prostate to dihydrotestosterone (DHT) by 5α-reductase (16). Androgen action in both stromal and epithelial compartments of the prostate is mediated through AR (5). In the epithelial compartment, androgens are required for both proliferative and secretory functions of the prostate (12). For example, androgens induce the expression of prostate-specific secretory proteins, including human prostate acid phosphatase (hPAP), prostate specific antigen (PSA), and prostate-specific kallikrein (hK2) (17, 18). In the stromal compartment, AR regulates the expression of various growth factors such as KGF (FGF-7), a member of the fibroblast growth factor family (14, 19-21). KGF is exclusively expressed in the stromal compartment of the prostate while its receptor, a spliced variant of the FGF type 2 receptor (FGFR2IIIb), is expressed solely in the epithelial compartment (3). These characteristics suggest that KGF is a paracrine mediator of signaling from the stroma to the epithelial compartment that can influence epithelial cell growth (1, 22).

Nuclear Receptors

The nuclear receptors comprise a large family of ligand-dependent transcription factors that regulate target gene expression through a variety of mechanisms. Receptors for steroid hormones include estrogen receptor (ER), androgen receptor (AR), progesterone

receptor (PR), and glucocorticoid receptor (GR). In the absence of hormone, steroid receptors are associated with heat shock proteins and immunophilins (23). Once bound by ligand, steroid receptors undergo a conformational change, permitting homo-dimerization and translocation into the nucleus. Upon entering the nucleus, steroid receptors induce gene expression by binding hormone response elements (HREs) located in the promoter regions of target genes. Alternatively, nonsteroidal nuclear receptors including thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR), and peroxisome proliferators activated receptor (PPAR) are nuclear proteins regardless of the presence or absence of ligand. In the absence of ligand, these receptors form heterodimers with retinoid X receptor (RXR), binding HREs in association with a repressor complex and preventing target gene expression. Upon ligand binding, the repressor complex disassociates and gene expression ensues (24). Along with steroid and nonsteroidal nuclear receptors, orphan receptors such as constitutive androstane receptor (CAR), liver X receptor (LXR), pregnane X receptor (PXR), farnesoid X receptor (FXR) and hepatocyte nuclear factor (HNF) are also part of the nuclear receptor family of transcription factors. Orphan receptors were first grouped together as proteins that resemble nuclear receptors and most of their physiological ligands were not known. However, in recent years, a diverse range of endogenous and xenobiotic lipophilic compounds have been identified as ligands for some of the orphan receptors. For example, bile acids activate FXR-mediated gene expression while LXR is activated by cholesterol (reviewed (25).

Structurally, nuclear receptors are modular proteins composed of an amino terminal domain (NTD), a central DNA binding domain (DBD), and a carboxyl terminal ligand binding domain (LBD) (Figure 2). Both the DBD and LBD are highly conserved while the NTD is more variable. Both the NTD and LDB contain activation function domains, AF-1 and AF-2 respectively, which potently activate transcription of nuclear receptor target genes when expressed. However, AF-1 activates nuclear receptor-mediated gene expression independent of ligand whereas AF-2 activation requires ligand (26).

Once in the nucleus, ligand-bound nuclear receptors are recruited to target gene promoters either through direct binding to HREs or association with other promoter-bound transcription factors to positively or negatively affect gene expression (27). For example, prostate specific antigen (PSA) and phosphoenolpyruvate carboxykinase (PEPCK) are both positively regulated by AR and GR, respectively (28, 29). Alternatively, GR interacts with chromatin-associated NF-κB as well as AP-1 transcription factors, repressing their transcriptional activity in a ligand dependent manner, termed transrepression. Initially, it was hypothesized that GR binds NF-κB, occluding other protein interactions or recruiting histone deacetylase (HDAC) complexes, thus preventing NF-$_K$B-mediated gene expression. Additionally, in A549 cells, GR inhibits NF-κB-mediated gene expression by preventing phosphorylation of serine 2 of the C terminal domain of pol II, thereby preventing pol II-mediated transcription (30). Along with binding other transcription factors, ligand-bound nuclear receptors promote the recruitment of coactivator complexes that stimulate gene expression by remodeling

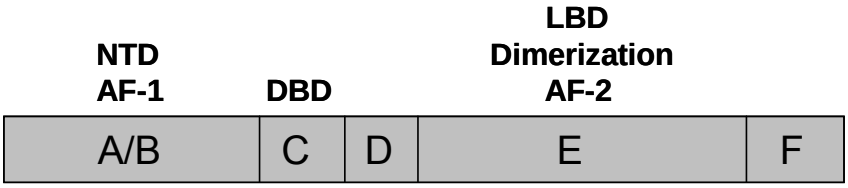


Figure 2. Modular structure of the nuclear receptor family of transcription factors. Nuclear receptors are composed of three major domains: the amino terminal domain (NTD), which contains the AF-1 transactivation domain (TAD), the DNA binding domain (DBD), and the ligand binding domain (LBD), which contains the AF-2 TAD as well as a domain required for receptor dimerization.

chromatin or contacting the basal transcription machinery, thereby stabilizing the transcription initiation complex (31).

Nuclear Receptor Coactivators

Nuclear receptor coactivators enhance nuclear receptor-dependent gene expression through a variety of mechanisms (32). Coactivator complexes are assembled onto receptor-bound promoters and stimulate nuclear receptor-mediated transcription either through direct interactions with the basal transcription machinery or by inducing local chromatin remodeling, including histone acetylation or methylation (31-36). Some coactivators possess enzymatic activity such as histone acetyltransferase (HAT) and methyltransferase activities that post-translationally modify histone proteins to affect

chromatin structure, while others that lack such activities function to recruit chromatin modifying enzymes to active promoters.

Once a potential coactivator has been isolated, it must fulfill three main criteria to be defined as a "classical" nuclear receptor coactivator. For example, coactivators must enhance nuclear receptor-mediated gene expression without altering the basal expression of a target gene. Furthermore, coactivators must reverse squelching effects observed by the addition of other, competing nuclear receptors. Finally, coactivators must contain intrinsic activation domains, capable of activating target gene expression when fused to a DNA binding domain. Although these criteria were originally used to differentiate coactivators from other nuclear receptor-interacting proteins, this definition may be too limiting as it ignores other potentially novel contributions of nuclear receptor interacting proteins to transactivation.

Most coactivators interact with the ligand binding domain of a variety of nuclear receptors including GR and AR via nuclear receptor (NR) boxes such as LXXLL motifs (37, 38). However, differences in binding affinities between nuclear receptors and their coactivators may play a role in determining the specificity of hormonal responses. For example, AR can bind glucocorticoid receptor interacting protein (GRIP/TIF-2/SRC-2) with a higher affinity then steroid receptor coactivator-1 (SRC-1) (39). Although both AR and GR interact with similar coactivators, it is postulated that unique coactivator complexes may be responsible for specific cellular and gene responses to each hormone. Furthermore, each nuclear receptor associates with specific coactivator complexes at the

same promoter (40). For example, using ChIP analysis of the mouse mammary tumor virus (MMTV) promoter, which contains a common hormone response element capable of binding AR, GR, or the progesterone receptor (PR), Li et al. isolated a specific coactivator complex associated with PR that differed from the coactivator complex that bound to GR (14). Specifically, SRC-1, RAC3, CBP, and p300 interacted with PR whereas TIF-2, RAC3, pCAF, and p300 interacted with GR. Additionally, these alternative coactivator complexes resulted in alternative posttranslational modifications of histones. Histone 4 is acetylated at lysine 5 after progesterone stimulation, and histone 3 is phosphorylated at serine 10 as well as acetylated at lysine 14 upon glucocorticoid treatment (40).

While many coactivators are likely to be redundant in their effects on nuclear receptor function in cultured cell lines, recent *in vivo* studies suggest that different physiological outcomes may result from variations in coactivator expression (35-37). For example, TIF-2 and RAC3 null mice display reduced reproductive capability (41, 42). TIF-2 null male mice have defective spermiogenesis and display testicular degeneration, whereas females display poor placental development, resulting in embryonic growth retardation (41). RAC3 -/- mice have decreased mammary gland growth, ovulatory capacity, and litter size (42). Alternatively, whereas SRC-1 null mice are fertile, they exhibit partial hormone resistance (43). Specifically, SRC-1 -/- mice have decreased growth and development of the uterus, mammary gland, prostate, and testes in response to steroid hormones (43).

Recently, many coactivators have been identified and classified into sub-families consisting of similar members. For example, the p160 family of coactivators, designated nuclear receptor coactivators (SRCs) consists of SRC-1 (or NcoA1), SRC-2 (or TIF-2, GRIP1), and SRC-3 (or p/CIP, RAC3, ACTR, or AIB1) (24-30). These coactivators share conserved sequence regions termed nuclear receptor interaction domains that permit interactions with a broad range of nuclear receptors. Other coactivators that are distinct from the p160 family are components of large complexes, such as the vitamin D interacting proteins (DRIPs) and thyroid receptor associated proteins (TRAPs) (31, 32). Finally, many other proteins have been identified as androgen receptor activators (ARAs) that may utilize unique mechanisms to impact receptor transactivation that remain largely undefined (33, 34). For example, ARA70, an AR-specific coactivator, may play a role in uncovering the agonist activity of certain anti-androgens to activate AR activity (44). Furthermore, a LIM domain containing protein that associates with focal adhesions, hydrogen peroxide-inducible clone-5 (Hic-5/ARA55) was identified as a nuclear receptor coactivator (45, 46).

LIM Domain-Containing Proteins

LIM domains are cysteine-rich motifs that may provide critical surfaces for protein-protein interactions (47). LIM domain proteins are grouped into three broad families based on their subcellular localization and function (48). Group I LIM proteins such as LIM only 2 (LMO) are nuclear proteins that influence gene expression by interacting with transcription factors (49). Group II LIM proteins are localized primarily in the

cytoplasm and function in the organization of the actin cytoskeleton (50). Finally, group III LIM proteins were initially found to localize to the cytoplasm often associated with focal adhesion complexes, but recently, our lab as well as others have revealed distinct pools of group III LIM domain proteins including Hic-5/ARA55, paxillin, and Trip6 in the nucleus (51-53).

A variety of LIM domain-containing proteins have been described that either positively or negatively influence gene expression (54). For example, thyroid interacting protein partner (Trip6), a group III LIM domain protein, is able to both enhance and repress AP-1 and NF-κB-regulated promoters by assembling different complexes in response to differential cellular signals (52). Upon TPA or TNF-α treatment, Trip6 is recruited to the collagen I or interleukin-8 (ColI or IL-8) promoters, enhancing AP-1 and NF-κB-mediated gene expression respectively. However, upon glucocorticoid treatment, Trip6 represses AP-1 and NF-κB action by tethering GR to specific promoters (52). This indicates that specific signaling events can determine if Trip6 activates or represses specific gene expression. Additionally, Four and a half LIM-only protein (FHL2) is also a LIM-domain containing protein that can both enhance or repress gene expression. FHL2 was initially identified as an AR-specific coactivator whose expression in the prostate overlaps that of AR and was later reported to influence β-catenin-mediated gene expression (55, 56). The mechanism by which FHL2 coactivates β-catenin-mediated transcription is due in part to a physical interaction with CBP/p300, resulting in increased acetylation of β-catenin by CBP/p300 (55). Acetylation of β-catenin may enhance its interaction with TCF4, thereby increasing β-catenin-mediated gene expression (57).

Interestingly, FHL2 represses β-catenin-mediated gene expression in muscle cells, resulting in myogenic differentiation (58).

As mentioned previously, group III LIM domain containing proteins, including Hic-5/ARA55, are predominantly localized at focal adhesions. However, some group III LIM domain proteins such as zyxin, lipoma preferred partner (LPP), Trip6, paxillin, and Hic-5/ARA55 can also be found in the nucleus (45, 51, 52, 59, 60). In fact, distinct pools of Hic-5/ARA55 were found in the nucleus associated with the nuclear matrix (45). Although many LIM domain containing proteins have been detected in the cytoplasm and at focal adhesions and their nuclear export sequences have been identified, the precise signals that induce their translocation are mostly unknown (reviewed in (54). For example, both LPP and Trip6 are detected in the nucleus after LMB treatment, indicating residual shuttling between the compartments (60, 61). However, FHL2 translocates to the nucleus in response to activation of Rho, a small GTPase, while mechanical forces applied to vascular smooth muscle cells lead to nuclear accumulation of zyxin (62, 63). Furthermore, it has been reported that nuclear accumulation of Hic-5/ARA55 occurs in response to oxidants such as H_2O_2 (64). However, in A1-2 cells, increased nuclear receptor-mediated gene expression in the presence of both ligand and H_2O_2 not only resulted in reduced nuclear receptor-mediated gene expression (unpublished results). Subsequent analysis of GR and Hic-5/ARA55 promoter recruitment to the MMTV promoter in the presence of dexamethasone (Dex) and H_2O_2 by ChIP analysis revealed less GR and Hic-5/ARA55 promoter localization in the presence of H_2O_2 (unpublished results).

In addition to nuclear receptors, Hic-5/ARA55 functions as a coactivator for Sp1, enhancing p21 expression (65). In this case, Hic-5/ARA55, forced into the nucleus with an added heterologous nuclear localization signal (NLS), influences p21 expression by interacting directly with Smad3 and indirectly with Sp1 and p300 (65). Although a direct interaction between Sp1 and Hic-5/ARA55 has not been detected, Hic-5/ARA55 has been found to associate with Smad3. Also, a LIM 4 deletion mutant of Hic-5/ARA55 interfered with the coactivation properties of p300, suggesting a functional interaction between these coactivators.

Along with AR and GR, Hic-5/ARA55 serves as a coactivator for PPARγ (66). Specifically, Hic-5/ARA55 enhances PPARγ-mediated induction of genes such as keratin 20, a cytokeratin that is expressed in mature intestinal epithelium as well as uroepithelium, L-FABP, a fatty acid binding protein found in differentiated intestinal epithelium, and KLF, a zinc finger transcription factor that is also expressed in intestinal epithelial cells (67-69). Furthermore, siRNA ablation of Hic-5/ARA55 expression reduced the expression of PPARγ-regulated genes. Finally, a role for Hic-5/ARA55 in epithelial differentiation induced by PPARγ was reported. Forced expression of Hic-5/ARA55 in preadipocytes not only inhibited adipocyte differentiation but also induced epithelial gene expression in these mesenchymal cells (66).

Hic-5/ARA55 was isolated by yeast two hybrid analyses as a nuclear receptor coactivator, specifically binding the τ2 transactivation domain of GR located in its hinge

region (45). However, the Hic-5/ARA55 interacting domain for AR has only been broadly localized to its ligand binding domain (46). Although the association of Hic-5/ARA55 with AR and GR has been well established, the nature of their interaction and specifically in which compartment it occurs and how it affects nuclear-receptor-mediated gene expression is not clear. Furthermore, redistribution of Hic-5/ARA55 to the nucleus in response to hormone treatment has not been detected (unpublished results).

Nuclear Receptor Corepressors

Along with coactivators, another set of nuclear receptor interacting proteins has recently been identified termed corepressors, including both nuclear receptor corepressor (NCoR) and silencing mediator or retinoid and thyroid receptors (SMRT) (70, 71). Corepressors repress nuclear receptor-mediated gene expression in the absence of ligand or presence of antagonist by interacting with histone deacetylases (HDACs) that in turn modify chromatin by removing acetyl groups on histone tails thereby promoting a closed chromatin structure, repressing transcription (24).

Individually, coactivators induce and corepressors repress nuclear receptor-mediated gene expression (Figure 3). However, most cells express a combination of both corepressors and coactivators that also interact with each other. For example, RAC3 interacts with NCoR, modifying thyroid hormone receptor (TR) regulated transcription (72). An equilibrium model hypothesizes that it is not the absolute amount of coregulator expression but the ratio of corepressors versus coactivators that determines the extent of

nuclear receptor-mediated gene expression (73). For example, overexpression of SMRT antagonizes TIF-2 coactivation of GR-mediated gene expression (74). Because coactivators and corepressors, collectively referred to as coregulators, alter nuclear receptor-mediated gene expression in endocrine target tissues, their activity and expression along with various nuclear receptors, including AR and ER, in prostate as well as breast cancer has been an area of intense investigation.

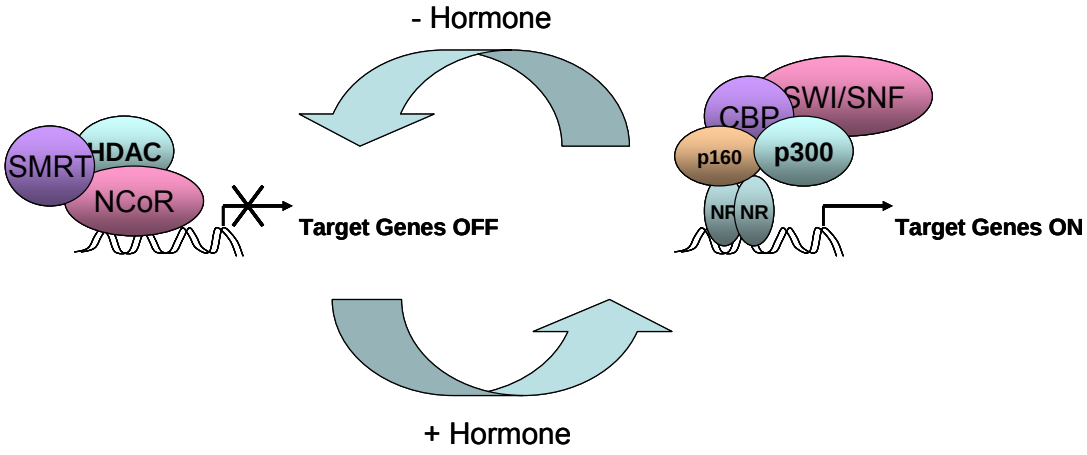


Figure 3. Coregulator exchange on nuclear receptor-regulated genes.

In the absence of hormone, a corepressor complex is associated with hormone responsive promoters, maintaining a closed chromatin state. Upon hormone stimulation, the corepressor complex is replaced with ligand-bound nuclear receptors as well as a coactivator complex and target gene transcription ensues.

AR and Prostate Tumor Development

Changes in androgen function are linked to the development and progression of prostate cancer (PCa). In fact, androgen-regulated gene products, such as PSA, are used as a marker of tumor progression and responsiveness to therapy (75, 76). As previously mentioned PSA is produced by the epithelial cells and secreted into the seminal fluid. Normally, the basement membrane acts as a barrier to block PSA from escaping into systemic circulation. However, disruption of the basement membrane in PCa, results in a significant increase in serum PSA.

Due to the androgenic dependence of prostate growth and survival, surgical and chemical castration has been used for more than sixty years as treatment for PCa. Huggins et al. reported that castration significantly reduced levels of prostate acid phosphatase, an androgen-regulated gene product, also used as a tumor marker in patients with PCa, indicating the androgenic dependence of these tumors (77). Since then, androgen ablation therapy including, orchiectomy and flutamide or bicalutamide anti-androgen treatment, which interfere with androgen binding to AR, has been the primary treatment for metastatic PCa (78, 79). Androgen ablation therapy leads to temporary remission of most PCa, but most tumors eventually regress into an androgen independent or hormone refractory tissue, capable of metastasis (80).

Reactive Stroma

Although the epithelia compartment is the source of tumor cells, growth factor and cytokine secretion from both compartments of the prostate is altered in tumor development. In fact, development of an altered stromal microenvironment, termed reactive stroma, carcinoma associated fibroblasts (CAF) or tumor stroma, enhances tumor cell survival, cellular proliferation, and migration (Figure 4, 81, 82). Reactive stroma is characterized by the presence of undifferentiated myofibroblast cells that express both smooth muscle cell and fibroblasts markers (83). Myofibroblasts are commonly found at areas undergoing tissue remodeling, producing ECM such as collagen I and fibronectin as well as proteases, including matrix metalloproteinases (MMPs) (84-86). Although the process by which normal stroma converts to reactive stroma phenotype is unknown, differential epithelial-stromal interactions during carcinogenesis may result in the inability of neoplastic epithelium to maintain a differentiated stromal compartment (87). Furthermore, TGF-β may be involved in the progression to a reactive stroma phenotype by inducing myofibroblast formation (83).

Data indicate that normal stroma cannot support tumor growth. Using the Dunning rat prostate andenocarcinoma model, Hayashi and Cunha grafted fragments of tumorigenic tissue into normal mesenchyme located under the renal capsule (11). After one month, not only did the tumor tissue's growth rate decrease, but the cells also adopted the normal morphology of tall columnar secretory epithelium. Alternatively, reactive stroma can

induce initiated, non-tumorigenic epithelial cells to form prostate tumors whereas normal stromal cells failed to promote tumor formation (88).

To date, the exact mechanism by which reactive stroma influences the development of prostate tumors is unclear. However, data indicate altered gene expression of growth factors such as KGF, FGF-2, TGF-β, and IL-6, matrix metalloproteinases (MMPs), and extracellular matrix in reactive stromal cells (22, 89-92). The differential gene expression pattern induced by the reactive stroma may confer resistance to cell cycle inhibitors or metastatic properties to neighboring cells.

Extracellular Matrix Remodeling in PCa

Along with changes in reactive stromal-associated cells, alterations in the composition of the ECM associated with prostate tumors occur also. For example, myofibroblasts produce ECM such as collagen I and fibronectin, resulting in increased expression of these ECM components in reactive stromal microenvironment (83, 85, 86, 93). In addition to differential ECM production, myofibroblasts also synthesize various proteinases that aid in ECM remodeling within reactive stroma (84).

ECM remodeling in PCa may disrupt cell adhesion as well as promote cellular invasion. For example, addition of fibronectin, which mediates cell adhesion, induced invasion of DU145 prostate cancer cells through basement membranes (94). Also, a MMP inhibitor,

A-177430, reduced tumor growth, angiogenesis, and metastasis, indicating that ECM remodeling may be necessary for tumor progression (95).

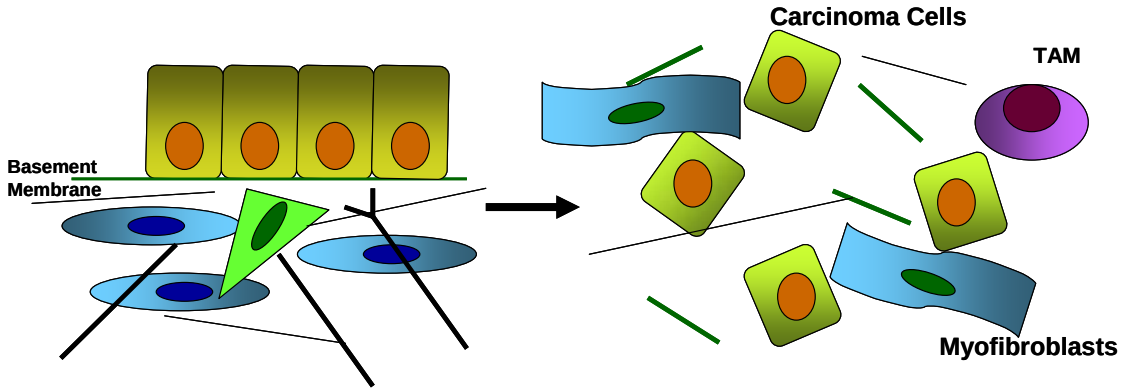


Figure 4. Transition of normal epithelial-stromal homeostasis to carcinoma-reactive stroma interactions in PCa. Normal epithelial-stromal interactions are disrupted during PCa progression. While the epithelial compartment, the source of the carcinoma cells, undergoes significant changes in PCa, the stromal compartment, termed reactive stroma, as well as the surrounding ECM changes in PCa as well. Normal stroma is mainly comprised of fibroblasts as well as smooth muscle cells. The presence of myofibroblasts, which express both smooth muscle cell and fibroblasts markers, as well as tumor associated macrophages (TAM) results in differential growth factor, cytokine, and ECM expression in reactive stroma.

Hormone Refractory Prostate Cancer

Despite the initial remission of most PCa upon androgen ablation therapy, most tumors eventually regress into an androgen independent or hormone refractory tissue, capable of metastasis (80). The mechanism by which PCa develops into an androgen independent state is not known. However, AR expression is maintained in androgen independent tissue and may play a role in continued prostate survival during the androgen-independent state (96). For example, disrupting AR activity by addition of an AR binding antibody or an AR mRNA hammerhead ribozyme effectively inhibited proliferation of androgen-independent prostate cancer cells (97).

Although the mechanisms by which AR is activated post androgen ablation therapy in androgen independent prostate cancer is unclear, several theories have emerged (1). The prostate may develop a capacity to increase its sensitivity to low levels of androgen through amplification of AR gene expression, increased AR stability, or enhanced AR nuclear localization (98, 99). For example, elevated AR expression may be necessary for PCa progression to an androgen-independent state (100). Furthermore, increased AR expression correlates with a higher probability of recurrence (101). (2) In some cases, the AR is mutated within its LBD, resulting in its activation by other steroid hormones, such as estrogen, progesterone, and glucocorticoids (102). However, the frequency of this mutation in patients is rather low (103). (3) Altered secretion of autocrine or paracrine factors may account for prostate cellular proliferation and AR activity in the absence of androgens. Altered growth factor secretion has not only been implicated in the reactive

stroma phenotype and subsequent tumor support, but also has been reported to increase AR activity in a ligand independent manner, promoting androgen independent prostate cancer growth. For example, IGF-1, EGF, KGF and IL-6 treatment of LNCaP cells induced PSA expression by AR in the absence of androgen (104, 105). (4) Altered expression of coactivators may enhance ligand-independent AR activity or increase androgen responsiveness in androgen independent prostate cancer. Furthermore, transient overexpression of TIF-2 in CV-1 cells enhanced AR-mediated MMTV reporter activity in response to estradiol as well as progesterone (106).

AIB 1 (amplified in breast 1)/ACTR/SRC-3/RAC3 is a nuclear receptor coactivator whose expression is amplified in 10% of primary breast tumor biopsies (107). Many investigators have subsequently examined whether altered expression, activity, or mutation of other nuclear receptor coactivators are associated with tumors of different stages (107-109). Analysis of coactivator expression in prostate cancer has yielded variable results. For example, Gregory *et al.* report increased SRC-1 and TIF-2 expression in prostate tumors whereas Fujimoto *et al.* report similar SRC-1 expression in benign, intermediate, and high grade cancers (106, 110). Interestingly, Hic-5/ARA55 expression was reduced in tumor as compared to normal tissue and Hic-5/ARA55 mRNA levels were lower in hormone-refractory PCa than that of previously untreated PCa (111,112)). However, the effect of decreased Hic-5/ARA55 expression on AR-mediated gene expression as well as PCa progression is not known.

SPECIFIC AIMS AND RATIONALE

Aim 1. Determining the mechanism by which Hic-5/ARA55 functions as a coactivator for nuclear receptors.

Because Hic-5/ARA55 does not possess an obvious catalytic domain that is responsible for its coactivation properties, its mechanism of coregulator function has remained largely undefined. At focal adhesion complexes, Hic-5/ARA55 serves as an adapter protein, coordinating multiple protein-protein interactions. Therefore, it may serve as an adaptor molecule, either recruiting or stabilizing promoter-specific protein complexes by interacting with multiple coactivator proteins, coordinating their association with nuclear receptors at hormone responsive promoters.

The overall goal of this aim was to determine if Hic-5/ARA55 interacts with nuclear receptors as well as other coregulators in a chromatin context. Interaction studies preformed thus far have used yeast two hybrid analyses and in vitro pull down assays (45, 46). Here, we attempted to isolate GR and Hic-5/ARA55 in association with a known GR-responsive promoter, the mouse mammary tumor virus (MMTV) as well as endogenous p21 and *c-fos* gene promoters, using chromatin immunoprecipitation (ChIP) analysis (Figure 5). Furthermore, the requirement of Hic-5/ARA55 for GR activity and promoter recruitment of coactivators was analyzed using RNA interference (RNAi) analysis. The results from this aim indicate that Hic-5/ARA55 is associated to hormone-responsive promoters in the absence and presence of glucocorticoids along with other

well-characterized coregulator proteins. Furthermore, reduced expression of Hic-5/ARA55 results in altered coactivator recruitment to target promoters.

Aim 2. Determining Hic-5/ARA55 expression and function in the prostate.

In the prostate, AR activity appears critical for tumor development and/or progression (96, 97, 106). Because Hic-5/ARA55 potentiates androgen receptor (AR)-mediated gene expression, its expression patterns in prostate cancer cell lines and tissue has been examined. In normal prostates, Hic-5/ARA55 expression was restricted to the stromal compartment, and Hic-5/ARA55 mRNA levels were lower in hormone-refractory PCa than that of previously untreated PCa (111). Because most analyses of coactivator expression in PCa progression use quantitative PCR, it is difficult to ascertain whether quantitative PCR results of Hic-5/ARA55 expression represents reduced expression of Hic-5/ARA55 within individual cells or alterations in the balance between stromal/epithelial cell content in tumors.

The overall goal of this aim was to determine Hic-5/ARA55 expression levels in normal and tumor-derived prostates along with examining Hic-5/ARA55 coactivator function in the prostate. Using immunohistochemistry analysis, Hic-5/ARA55 expression was found to be confined primarily to the stromal compartment in the prostate. I therefore employed a prostate stromal cell line, WPMY-1 cells, and determined that Hic-5/ARA55 was recruited along with AR to the androgen-responsive KGF promoter. Furthermore, the responsiveness of KGF to androgen stimulation was lost after Hic-5/ARA55

expression was partially silenced using RNAi. These results indicate that Hic-5/ARA55 serves as a stromal-specific AR coactivator in the prostate.

Aim 3. **Determining the effects of extracellular matrix signaling on GR-mediated transcription.**

Integrins, a family of membrane receptors located at focal adhesion complexes, bind specific ECM components, activating intracellular signaling cascades that influence cell migration, proliferation, and apoptosis. Furthermore, ECM also influences nuclear receptor activity, resulting in differential cell growth and/or gene expression. Malignant mammary cells grown on a laminin-rich basement membrane displayed greater ERα activity and express higher levels of ERα (6). Although cross-talk between ER and ECM signaling has been reported, the mechanism by which ECM influences nuclear receptor-mediated gene expression is unclear (6).

Because Hic-5/ARA55 modulates focal adhesion complex signaling as well as functioning as a nuclear receptor coactivator, I analyzed the role of Hic-5/ARA55 in mediating GR responses to various ECM. In this aim, I first determined the effects of ECM signaling on GR-mediated gene expression. Matrigel decreased GR activity by decreasing GR protein levels. Alternatively, fibronectin enhanced GR-mediated gene expression without effecting GR and Hic-5/ARA55 expression, subcellular localization, and DNA binding. These results indicate that ECM does influence GR activity although the exact mechanisms by which those signals are transmitted are unclear.

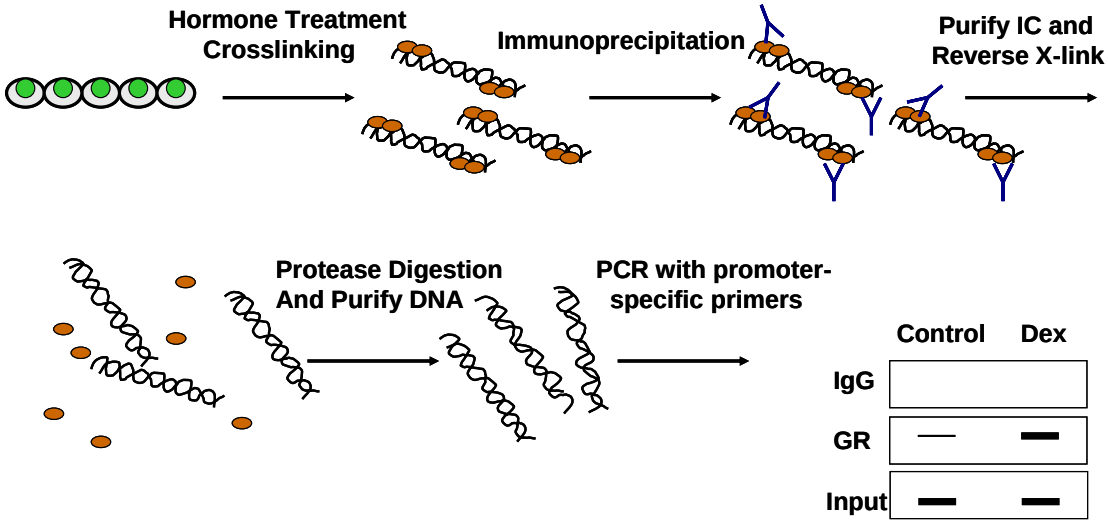


Figure 5. Chromatin Immunoprecipitation (ChIP) Assay. After the cells are stimulated for 1 hour with hormone to allow nuclear receptor translocation to the nucleus and promoter recruitment, the cells are crosslinked with formaldehyde to preserve nuclear receptor-DNA interactions. After shearing the chromatin to 500-2000 base pair fragments, the resulting chromatin is immunoprecipitated using nuclear receptor specific antibodies as well as non-specific antibody controls (IgG). After the immune complex is isolated using protein A and G sepharose, the crosslinks are reversed and the proteins are removed by protease digestion. The DNA is purified and subjected to PCR analysis, using promoter-specific primers.

CHAPTER 2 MATERIALS AND METHODS

Antibodies, Plasmids, and Reagents – Antibodies used in this study included: anti-GR (Affinity Bioreagents, Inc., Golden, CO); anti-Hic-5/ARA55 (BD Transduction Labs, Los Angeles, CA); anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH CSA-335 Stressgen Victoria, British Columbia, Canada); anti-NCoR (06-892 Upstate, Charlottesville, VA); anti-GR (H-300); anti-AR (N-20); anti-p300 (C-20); anti-SRC-1 (M 341); anti-TIF-2 (M 343) (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho AKT; anti-AKT; anti-phosphoERK; anti-ERK (all from Cell Signaling Technology, Beverly, MA). Plasmids used in this study included: an androgen responsive MMTV-luciferase reporter, pLC-Luc, as well as the Renilla-luciferase control. Cell signaling inhibitors used in this study include: U0126 and LY294002 (both from CalBiochem, SanDiego, CA)

Cell Culture and Transient Transfection- The A1-2 cells were derived from the T47D human mammary carcinoma cells line by stable transfection with pGRneo and MMTV-LTR-luc plasmids as previously described (113). Thus, these cells contain exogenous copies of stably integrated rat GR and a glucocorticoid-responsive luciferase reporter. The A1-2 cells were routinely maintained in modified Eagle's medium at 37 C under 5% CO_2. The media was supplemented with 100 μg/ml penicillin-streptomycin, 10% FBS, 10 mM HEPES, 2 mM glutamine, and 160 μg/ml G418. WPMY-1 cells were routinely maintained in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) supplemented with 100 μg/ml penicillin-streptomycin and 5% fetal bovine serum (FBS)

at 37 C under 5% CO_2. The WPMY-1 cells were isolated from a normal human prostate specimen and immortalized using SV40 large-T-antigen (114). A1-2 cells were seeded on 12-well cell culture dishes at a density of 1.5×10^5 cells/ well for 24 h prior to transfection. WPMY-1 cells were seeded on 12-well cell culture dishes at a density of 7.5×10^4 cells/ well for 24 h prior to transfection. Transfections were performed using Opti-MEM (Gibco, Grand Island, NY) and Lipofectamine transfection reagent (Invitrogen Technology, Carlsbad, CA) according to manufacturer's instructions. After 3 h, fresh medium was added to the cells and hormone treatments were initiated where relevant.

Extracellular matrix application to tissue culture plates – Cell culture plates were first coated with nitrocellulose that was dissolved in methanol (115). After the nitrocellulose was applied, various ECM was diluted in PBS and incubated at room temperature for 1 hour. The ECM examined included: vitronectin (Gibco BRL Life Technology, Carlsbad, CA), fibronectin (BD Biosciences, Bedford, MA), laminin mix (kind gift from Marcia Lewis, University of Pittsburgh), and growth factor reduced matrigel (BD Biosciences, Bedford, MA). After the plates were washed with PBS, the A1-2 cells were plated.

Western Blot Analysis- Cell lysates were collected in RIPA buffer (10 mM Tris, pH 8, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 1% Triton X-100, 0.1% Deoxycholic acid, 0.1% SDS, 1 mM PMSF, and protease inhibitors) and were boiled in Sample Buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol, 0.001% Bromophenol Blue) for 5 min. Proteins were then separated on 7.5% SDS-PAGE and transferred to

PVDF transfer membrane (Bio-Rad, Hercules, CA) in transfer buffer (20% methanol, 48 mM Tris, 39 mM glycine, and 1.3 mM SDS) at 15 V for 30 min. Membranes were then incubated in blocking buffer (5% dry milk in Tris-buffered saline, pH 7.4) for 2 h to overnight. Next, the membranes were incubated with the indicated antibodies diluted 1:1000 in blocking solution for 2 h at room temperature. After extensive washing, the membranes were then probed with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in blocking solution for 1 h. Finally, the membranes were washed and developed using the Renaissance Western Bolt Chemiluminescence Reagent (PerkinElmer, Wellesley, MA) according to the manufacturer's instructions. The membranes were stripped with Re-Blot Plus Strong Solution following the manufacturer's instructions (Chemicon International, Temecula, CA) and reprobed for GAPDH as a loading control. Where indicated, quantification of scanned images was performed using the NIH Image software.

RNAi- RNAi plasmids were constructed following the manufacturer's guidelines (pSilencer 2.1-U6 hygro, Ambion, Austin, TX). The DNA template sequences for human Hic-5/ARA55 were RNAi-A 5'-GGAGGACCAGTCTGAAGAT-3', RNAi-B 5'-GAAAAGACCCAGCCTCCCT-3', RNAi-C 5'-GCATCACAGATGAAATCAT-3'and RNAi-D 5'-GTGGATTGCACACAGACAA-3'. The oligos (2μg each) were annealed to form double-stranded DNA after incubation in DNA annealing solution (pSilencer kit) for 3 minutes at 90 C followed by 1 hour at 37 C. The resulting double stranded DNA was then inserted into the pSilencer 2.1-U6 hygro plasmid using T4 DNA ligase (New England Biolabs, Ipswich, MA) for 4 hours at 25 C. Positive clones were identified after

enzymatic digestion by HindIII and BamHI (New England Biolabs, Ipswich, MA), releasing the 65 base pair insert. The GFP template sequence was used as a positive control during the plasmid construction and a negative control for transfection analyses.

Luciferase Assays - Luciferase activity in cell-free lysates was measured using a Victor 3 1420 Multilabel plate reader (PerkinElmer, Wellesley, MA). Cells were washed with PBS and lysed in Reporter Lysis Buffer (Promega, Madison, WI) followed by a freeze and thaw incubation to ensure proper cell lysis. The lysate (25 µl) was incubated with luciferase assay reagent followed by a 10 sec relative luciferase unit measurement. Luciferase activity was normalized to Renilla luciferase activity according to manufacturer's instructions. All experiments were performed three or more times.

Chromatin Immunoprecipitation – A1-2 or WPMY-1 cells were grown on 15 cm plates to 80-90% confluence in medium supplemented with charcoal dextran treated FBS for 24 hours. The cells were treated with EtOH-vehicle, 100 nM Dex, or 10 nM Mibilerone (Mib) for 1 h and crosslinked by addition of formaldehyde (1% final) to the medium for 30 min at room temperature. After the crosslinking reaction was stopped by addition of 0.1 volume of 1.4 M glycine, the cells were washed with PBS, scraped into ice-cold PBS, and centrifuged at 3000 RPM for 5 minutes at 4 C. The cells were lysed and the nuclei collected by resuspending the pellet in 2 ml of cold ChroIP Buffer (50mM HEPES, KOH pH 8, 1mM EDTA, pH 8, 0.5mM EGTA, pH 8, 140mM NaCl, 10% glycerol, 0.5% IGEPAL, 0.25% Triton X-100, and 1mM PMSF) for 10 minutes and centrifuged at 3000 RPM for 5 minutes at 4 C. The pellet was resuspended in 2 ml of

cold Wash Buffer (10mM Tris-HCl, pH 8, 1mM EDTA, pH 8, 0.5mM EGTA, pH 8, 200mM NaCl, and 1mM PMSF) for 10 minutes at 4 C followed by centrifugation at 3000 RPM for 5 minutes at 4 C. The pellet was resuspended in 2 ml of ice-cold RIPA Buffer (10mM Tris-HCl, pH 8, 1mM EDTA, pH 8, 0.5mM EGTA, pH 8, 140mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 1mM PMSF, and protease inhibitor cocktail) for 10 minutes at 4 C followed by centrifugation at 3000 RPM for 5 minutes. The isolated nuclei were then sonicated using a water bath sonicator for three minutes (Misonix Sonicator with Cuphorn, Farmingdale, NY, Output at 6, Processing time 3 minutes, Pulse on 10 sec, Pulse off 15 sec) to produce fragments 500-2000 base pair in size as previously described (30). Chromatin fragments were precleared for 1-4 h at 4C using 50 µl of protein A and G sepharose containing salmon sperm DNA (Upstate, Lake Placid, NY) to reduce nonspecific binding followed by centrifugation at 3000 RPM for 5 minutes at 4 C. Next, 1-10% of the sheared chromatin was removed to use as input samples later. The chromatin fragments were diluted with RIPA buffer (1 ml/IP), separated into 2 to 4 IPs, and immunoprecipitated with specific antibodies (1 µg/ IP) overnight at 4 C. After immunoprecipitation, 30 µl of protein A and G sepharose (Upstate, Lake Placid, NY) was added and the incubation was continued for 1 h. The IPs were washed twice with RIPA buffer, twice with RIPA buffer +500 mM NaCl, and once with LiCl buffer (10mM Tris-HCl, pH 8, 1mM EDTA, pH 8, 0.5mM EGTA, pH 8, 250mM LiCl, 1% Triton X-100, 1% Na-deoxycholate, and 1mM PMSF). The precipitates were eluted with 100 µl of freshly made reverse cross-linking buffer (1% SDS and 0.1 M $NaHCO_3$), adding 4 µl of 5 M NaCl at 65 C for 4 h. The Input samples were reversed by diluting 10µl Input into 190 µl ChIP Dilution Buffer (0.01% SDS 1.1%

Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH8, and 167mM NaCl) adding 8 µl of 5M NaCl/tube also for 4 hr at 65 C. The proteins were removed by addition of proteinase K (2 µl/sample, Roche, Mannheim, Germany, 20 mg/ml) for 1 h at 45 C. DNA was isolated using the QIAquick PCR purification kit (Quiagen, Valencia, CA). Thermocycling conditions involved an initial denaturation step at 95 C for 12 min followed by 28-32 cycles at 95 C for 30 sec and 56 C for 30 sec and 72 C for 30 sec. Primers for the MMTV promoter were: forward primer 5'-GCGGTTCCCAGGGCTTAAGT-3' and reverse primer 5'-CCATTTTACCAACAGTACCG-3'. Primers for the *c-fos* promoter were: forward primer 5'-TCCCAGCAGTCGAGGTATTC-3' and reverse primer 5'-GGTCAGTTCGGGATGACAAG-3'. Primers for the p21 promoter were: forward primer 5'-GGTGTCTAGGTGCTCCAGGT-3' and reverse primer 5'-GCACTCTCCAGGAGGACACA-3'. Primers for the KGF promoter were: forward primer 5'-CCCTTTCCCCTTCTAACTGC-3' and reverse primer 5'-ACCTTTGCTGACCTCATTGG-3'. Primers for the luciferase gene were: forward primer 5'-CCAGGGATTTCAGTCGATGT-3' and reverse primer 5'-AATCTGACGCAGGCAGTTCT-3'. PCR products were resolved on a 12% polyacrylamide gel and visualized with ethidium bromide. Semi-quantitation was done using densitometric analysis of the resolved gels using the Kodak Imaging System. Data points were subtracted for background and normalized to the Input data.

For sequential ChIPs, complexes immunoprecipitated with either anti-GR or anti-Hic-5/ARA55 antibodies (3 µg/ IP) were eluted by incubation with 100 µl of 10 mM DTT for

30 min at 37 C. After centrifuging the eluted IPs for 10 minutes at 3000 RPM 4C, they were diluted 1:50 in ChIP Dilution Buffer (20 mM Tris-HCl, pH 8.1, 2mM EDTA, 150 mM NaCl, 1% Triton X-100), followed by reimmunoprecipitation with either isotype control Ab, anti-Hic-5, or various coactivators or corepressor antibodies (1 μg/ IP).

Reverse Transcriptase PCR - Total RNA was isolated from A1-2 and WPMY-1 cells using the RNAqueous RNA isolation kit (Ambion, Austin, TX) following the manufacturer's instructions. For RT-PCR, 1 μg of RNA was incubated with 100 μl of reaction mix containing 25 mM $MgCl_2$, 25 mM dNTPs (PerkinElmer), 10X PCR II Buffer (Gibco BRL), 40 U/μl RNAsin RNase inhibitor (Promega), 45 μM random hexamers (IDT, Coralville, IA), 200 U/μl Superscript reverse transcriptase (Gibco), and nuclease free water (Ambion). Parallel reactions were performed without reverse transcriptase to control for the presence of contaminant DNA. The samples were incubated at 25 C for 10 min, at 48 C for 30 min, and at 95 C for 5 min followed by 4 C for 5 min to inactivate the reverse transcriptase.

For amplification, a PCR reaction containing a cDNA aliquot along with AmpliTaq Gold DNA polymerase in a volume of 25 μl was used according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Primers used for gene expression analysis were: *c-fos*: forward primer 5'-TGCCAACTTCATTCCCACGGT-3' and reverse primer 5'-TAGTTGGTCTGTCTCCGCTTG-3'; p21: forward primer 5'-GCGACTGTGATGCGCTAATGG-3' and reverse primer 5'-TCCCAACTCATCCCGGCCTC-3'; KGF: forward primer 5'-

CAATCTAGAATTCACAGATAGGAGGAGGC -3' and reverse primer 5'-ACAATTCCAACTGCCACTGTCCTGATTTC-3; GAPDH: forward primer 5'-CATCACCATCTTCCAGGAGCGAGA-3' and reverse primer 5'-GTCTTCTGGGTGGCAGTGATGG-3'. Thermocycling conditions involved an initial denaturation step at 95 C for 12 min followed by 28-30 cycles at 95 C for 30 sec and 56 C for 30 sec and 72 C for 30 sec. Specific PCR amplification products were separated on a 12% PAGE and detected by EtBr staining. Experiments were performed with duplicates for each data point.

Immunofluorescence – WPMY-1 cells were cultured on glass coverslips treated with EtOH-vehicle or 100 nM Leptomysin B (LC Laboratory, Woburn, MA) for 1 hour, fixed with 4% paraformaldehyde in PBS for 30 minutes at 4°C, washed with PBS and permeabilized in PBS containing 1% BSA and 0.5% Triton X-100 for 5 minutes at room temperature. The cells were incubated overnight at 4 C with anti-Hic-5/ARA55 specific antibodies diluted 1:1000 in PBS. After extensive washing with PBS, the cells were incubated 2 hours at room temperature with anti-mouse IgG-Cy2 conjugated antibodies (Molecular Probes, Portland, OR) diluted 1:300 in PBS. Nuclei were stained with DAPI (10 μg/ml, Molecular Probes, Carlsbad, CA) according to manufacturer's instructions.

Immunohistochemistry – Paraffin-embedded prostate tissue sections were obtained from the Shadyside Hospital Tissue Bank. Tissue sections were deparaffinized, rehydrated, and antigens retrieved by boiling in 10 mM citrate buffer, pH 5.5 for 10 minutes. The sections were incubated overnight at 4 C in a humid chamber with anti-Hic-5/ARA55 antibodies diluted 1:2000 in PBS. Immunostaining was performed using a

Vecta stain kit (Vector Laboratories Inc., Burlingame, CA) following manufacturer's instructions. Normal mouse IgG was used as controls.

Statistics

Comparisons of two mean values were performed using a paired *t* test. p values of < 0.05 were taken to be significant and all data were analyzed using GraphPad Prism 3.00 (Graph Pad Software, San Diego, CA).

CHAPTER 3 Determining the mechanism by which Hic-5/ARA55 functions as a coactivator for nuclear receptors.

HYPOTHESIS:

Because Hic-5/ARA serves as an adapter protein at focal adhesion complexes coordinating multiple protein-protein interactions, it serves as nuclear receptor coactivator by interacting with multiple coactivator proteins, coordinating their association with nuclear receptors at hormone responsive promoters.

Introduction

Hic-5/ARA55 is associated primarily with focal adhesion complexes as well as localized within the nucleus (53). At focal adhesion complexes, Hic-5/ARA55 as well as other group III LIM domain proteins link various intracellular signaling modules to plasma membrane receptors that respond to various extracellular signals including growth factors and the extracellular matrix (116, 117). Furthermore, Hic-5/ARA55 was recently isolated as a nuclear receptor interacting protein that functions as a coactivator for nuclear receptor-mediated gene expression (45, 46, 66). Reduced expression of Hic-5/ARA55 resulted in decreased AR and PPARγ-mediated transactivation (46, 66).

Because Hic-5/ARA55 does not possess an obvious catalytic domain that is responsible for its coactivation properties, its mechanism of coregulator function has remained undefined. However, it may serve as an adaptor molecule, either recruiting or stabilizing

promoter-specific protein complexes. LIM proteins are well recognized for their roles as molecular adaptors, functioning in stabilizing higher order protein complexes at focal adhesion complexes. Within focal adhesion complexes, Hic-5/ARA55 as well as other group III LIM domain proteins link various intracellular signaling modules to plasma membrane receptors that respond to extracellular signals including growth factors and the extracellular matrix (116, 117). For example, Hic-5/ARA55 and paxillin interact with multiple focal adhesion-associated proteins such as vinculin and focal adhesion kinase (FAK) (118).

Here, we examined the mechanism of Hic-5/ARA55 action as a coactivator, focusing exclusively on endogenous Hic-5/ARA55 in the A1-2 derivative of T47D breast cancer cells and not relying on artificial enhancement of its nuclear localization. Single and sequential ChIP assays revealed an association of Hic-5/ARA55 with GR and various coactivators on viral and cellular glucocorticoid-responsive promoters. Furthermore, siRNA-mediated ablation experiments established Hic-5/ARA55 in maintaining the assembly of coactivator complexes required for efficient glucocorticoid-induced transcription. Thus, Hic-5/ARA55 may function as a nuclear receptor coactivator as an adaptor protein, recruiting or stabilizing histone acetyltransferase-containing complexes at steroid responsive promoters.

Results

Hic-5/ARA55 is localized to a glucocorticoid-responsive promoter.

While an interaction between Hic-5/ARA55 and GR has been revealed in yeast two hybrid assays, the relevance of this association to the coactivator activity of Hic-5/ARA55 towards GR is unknown (45, 46). In order to determine the mechanism by which Hic-5/ARA55 serves as a GR coactivator, we assessed whether Hic-5/ARA55 was bound to the glucocorticoid responsive mouse mammary tumor virus (MMTV) promoter, using chromatin immunoprecipitation (ChIP) assays. A1-2 cells, a T47D cell derivative that contains an integrated MMTV-luciferase gene, were grown in medium containing steroid depleted serum for 2 days before initiating hormone treatments. Following a 1 h ethanol-vehicle or Dex (100nM) treatment, GR and Hic-5/ARA55 recruitment to the MMTV promoter was analyzed by ChIP analysis using antibodies specific for each (Figure 6). As expected, there was a Dex-dependent localization of GR to the MMTV promoter, but not the coding region of the luciferase gene. Hic-5/ARA55 was also recruited to the MMTV promoter in the presence of Dex. These results provide the first demonstration of endogenous Hic-5/ARA55 binding to a specific promoter in vivo and the hormone-dependent recruitment of Hic-5/ARA55 to a nuclear receptor responsive promoter.

GR and Hic-5/ARA55 co-occupy a glucocorticoid-responsive promoter in vivo. To determine if GR and Hic-5/ARA55 are contained within a stable complex at GR-

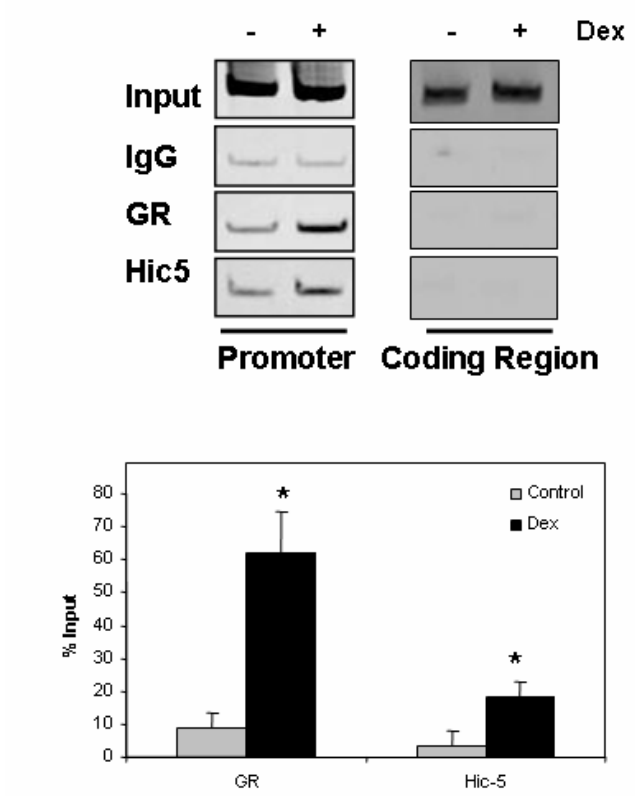


Figure 6. A. Binding of Hic-5/ARA55 to the MMTV promoter. Soluble chromatin was prepared from A1-2 cells treated with EtOH-vehicle or Dex (100 nM) for 1 h. Protein-bound DNA complexes were immunoprecipitated with antibodies against GR or Hic-5/ARA55. After crosslink reversal, purified DNA was amplified with primers specific for the MMTV promoter (left panels) or the coding region of the luciferase gene (right panels). PCR products in the Input panel were amplified using diluted chromatin that was not immunoprecipitated. A rabbit IgG was used to detect any nonspecific immunoprecipitated DNA. Gel shown of PCR products, resulting from 28 rounds of PCR amplification, is representative of three separate experiments. B. Relative changes in GR and Hic-5/ARA55 recruitment to the MMTV promoter were calculated based on semi-quantitative results from the original images. Values represent percent of sample input after subtraction of IgG control ± SD for three separate experiments. *, $P < 0.05$, significantly different from the mean value of ethanol controls; bars, SD.

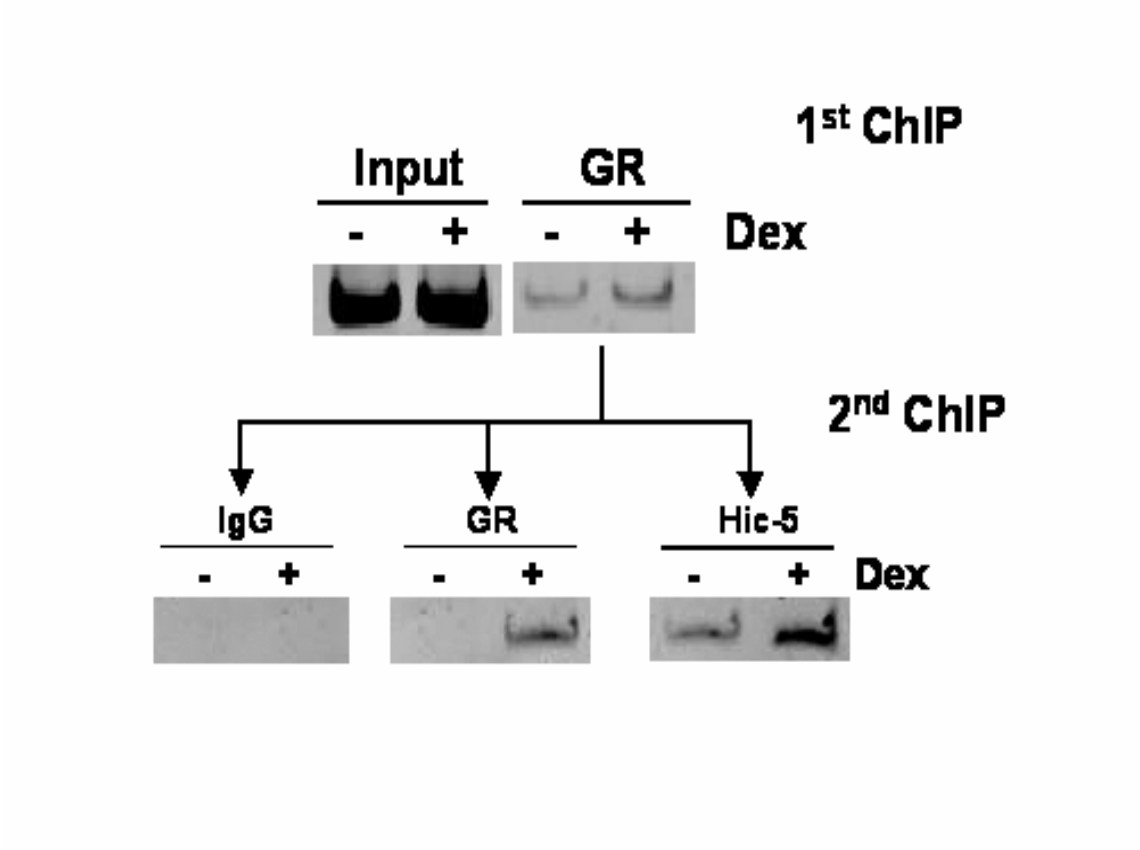


Figure 7. Co-occupancy of GR and Hic-5/ARA55 on the MMTV promoter. Soluble chromatin was prepared as in Figure 1. GR-bound DNA complexes were immunoprecipitated (indicated as 1st ChIP), eluted, and re-immunoprecipitated with GR and Hic-5/ARA55 specific antibodies. After crosslink reversal, purified DNA was amplified with primers specific for the MMTV promoter. PCR products in the Input panel were amplified using diluted chromatin that was not immunoprecipitated. A rabbit IgG was used to detect any nonspecific immunoprecipitated DNA. Gel shown of PCR products, resulting from 32 rounds of PCR amplification, is representative of two separate experiments.

responsive promoters, we performed sequential immunoprecipitations followed by ChIP analysis (Figure 7). A1-2 cells were grown and treated as in Figure 6 and the diluted chromatin was first immunoprecipitated using antibodies specific for GR. After extensive washes, the precipitates were reimmunoprecipitated using antibodies specific for Hic-5/ARA55. As shown in Figure 7, GR containing chromatin segments of the MMTV promoter also contain Hic-5/ARA55. These data demonstrate that GR and Hic-5/ARA55 can co-occupy the MMTV promoter in vivo and that this association is enhanced upon Dex treatment. The sequential immunoprecipitation reduced the detection of GR in chromatin isolated from cells not treated with hormone. However, Hic-5/ARA55 was still detected in MMTV chromatin isolated from untreated cells. Thus, Hic-5/ARA55 may play a role in both basal and hormone-regulated transcription from the MMTV promoter.

GR and Hic-5/ARA55 associate with endogenous glucocorticoid-responsive promoters in vivo.

To establish whether both GR and Hic-5/ARA55 are recruited to endogenous promoters, we initially performed RT-PCR analysis to identify endogenous glucocorticoid responsive genes in A1-2 cells. Glucocorticoid induction of p21 expression has been demonstrated in A1-2 cells (119). Although the mechanism responsible for hormone effects on p21 expression is not known, p21 may be a target of Hic-5/ARA55 action as overexpression or forced nuclear retention of Hic-5/ARA55 resulted in increased p21 and *c-fos* expression in human immortalized fibroblasts (120, 121). To test whether Hic-

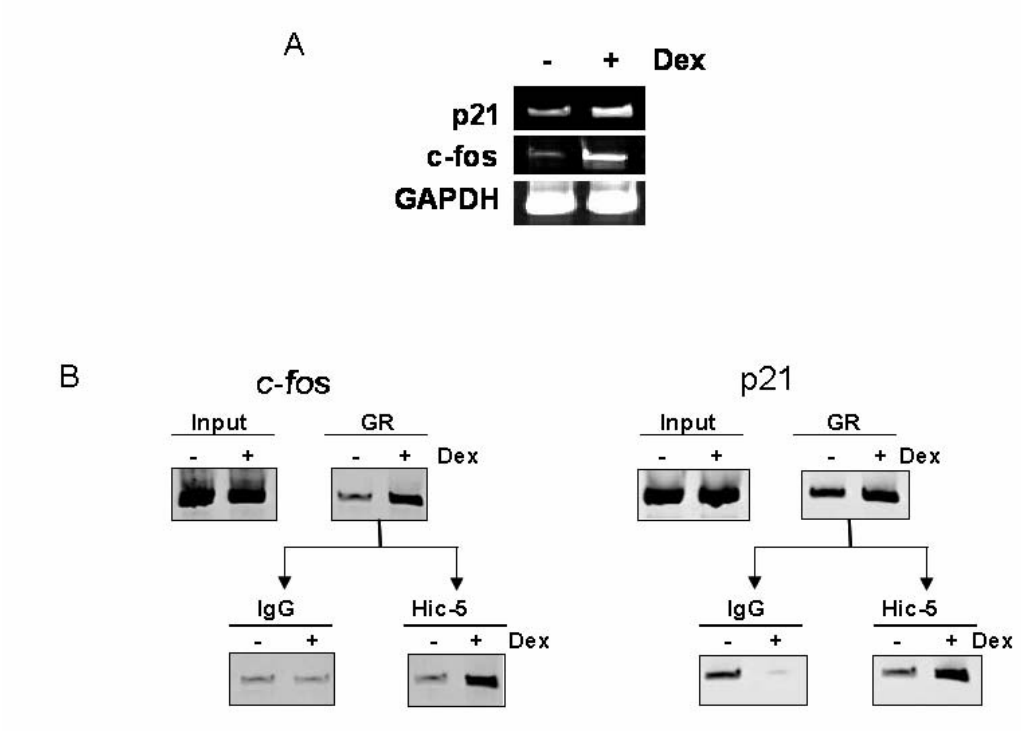


Figure 8. Association of GR and Hic-5/ARA55 with the chromatin of endogenous promoters *in vivo*. A. Glucocorticoid responsive gene expression in A1-2 cells was analyzed by RT-PCR. A1-2 cells were treated with EtOH-vehicle or Dex (100nM) for 10 h. RNA from A1-2 cells was isolated to determine the relative expression of p21, c-fos, and GAPDH mRNAs. The reverse transcriptase reaction was carried out with 0.5 μg total RNA. Parallel reactions performed without reverse transcriptase to control for the presence of contaminant DNA did not generate specific PCR products for any primers (data not shown). The gel shown of PCR products, resulting from 28 rounds of PCR amplification, is representative of two separate experiments. B. Chromatin Re-IP analysis was performed as in Figure 2 using *c-fos* or p21 promoter-specific primers. The gels shown of PCR products, resulting from 32 rounds of PCR amplification, are representative of two separate experiments.

5/ARA55 played a role in glucocorticoid induction of these genes in A1-2 cells, we used ChIP assays to assess promoter occupancy of the p21 and *c-fos* genes.

A1-2 cells were grown in medium containing charcoal dextran stripped FBS. After 2 days, the cells were treated with ethanol or 100nM Dex for 10 h followed by RT-PCR analysis. As shown in Figure 8A, we confirmed using RT-PCR that both p21 and *c-fos* mRNAs were induced by Dex in A1-2 cells. Furthermore, GR association with the *c-fos* and p21 promoters was enhanced by Dex treatment (Figure 8B). Importantly, sequential ChIP experiments demonstrated that Hic-5/ARA55 was also a component of GR-containing chromatin at these endogenous promoters. Thus, endogenous Hic-5/ARA55 is included within GR complexes at the promoter of endogenous genes whose transcription is regulated by glucocorticoids.

Reduced expression of Hic-5/ARA55 results in decreased GR transactivation.

It has been established that overexpression of Hic-5/ARA55 increases GR-mediated transcription (45). However, these types of experiments do not reveal whether endogenous Hic-5/ARA55 is necessary for GR activity. Thus, we used a siRNA approach to ablate Hic-5/ARA55 expression in A1-2 cells and assess the impact on GR transactivation. A1-2 cells were analyzed for Hic-5/ARA55 expression in A1-2 cells by Western blot analysis following transfection with either a control GFP siRNA or Hic-5/ARA55 siRNA. Densitometric analysis revealed that there was approximately 60% less Hic-5/ARA55 in cells transfected with the Hic-5/ARA55 siRNA as compared to control (Figure 9A). Two other distinct Hic-5/ARA55 siRNAs tested were less effective

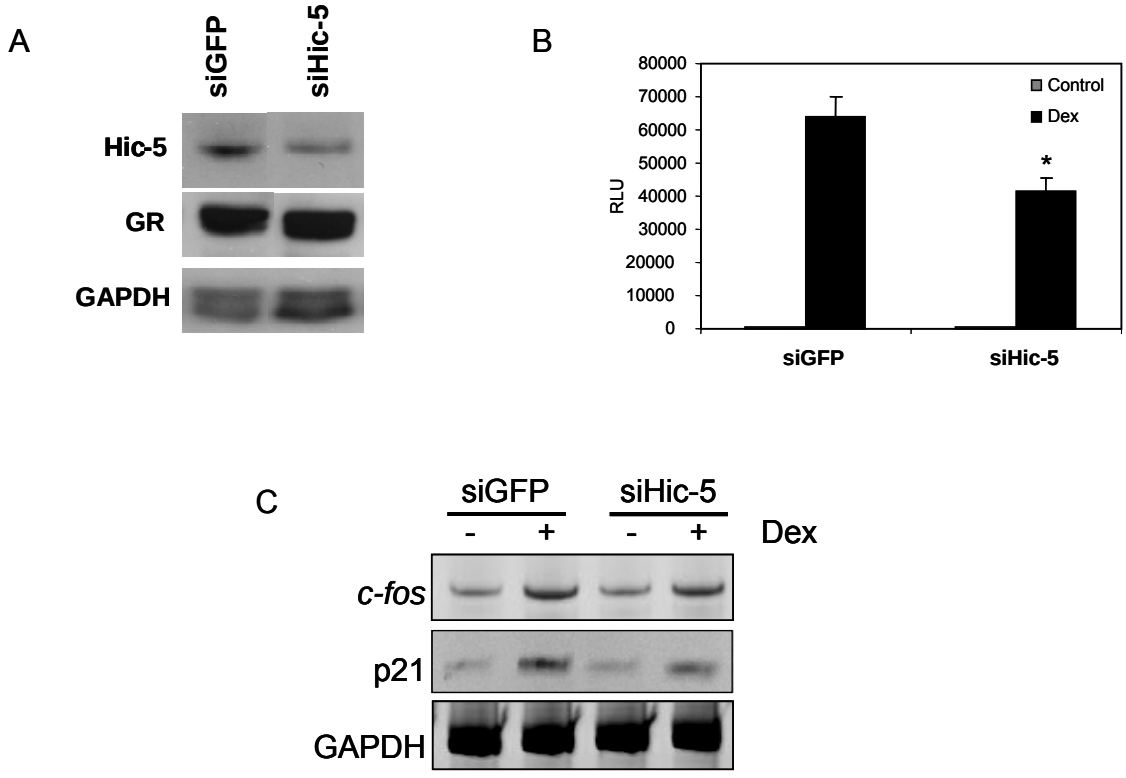


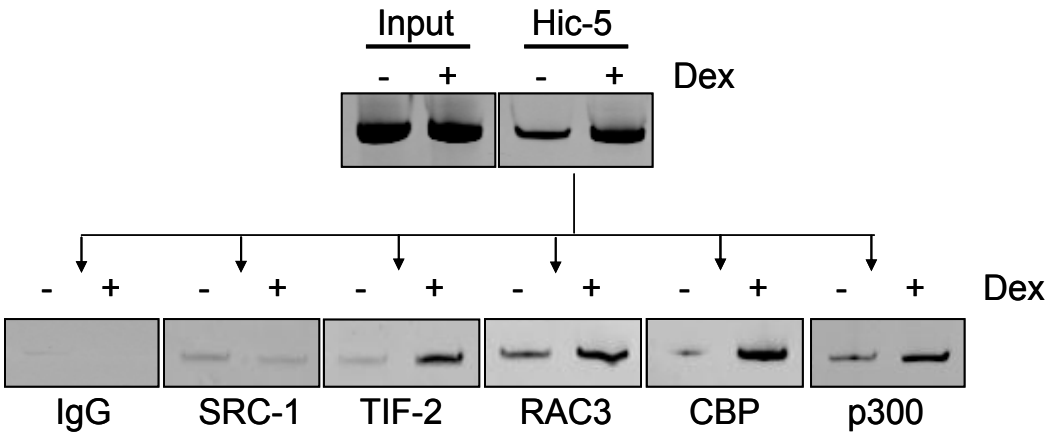
Figure 9. Effects of RNAi-mediated silencing of Hic-5/ARA55 on GR transactivation. A. A1-2 cells, cultured in 6 well plates, were transfected with 2 μg of GFP (negative control) or 2 μg human Hic-5/ARA55 RNAi constructs. After 48 h, cells were harvested, lysed, and analyzed for Hic-5/ARA55, GR, and GAPDH protein levels by Western blotting. B. A1-2 cells were transfected with RNAi constructs as in A. After 48 h, the cells were treated with EtOH-vehicle or 1nM Dex for 24 h after which luciferase activity was measured. Data shown are mean of three separate experiments. C. A1-2 cells were transfected as in A. After 48 h, the cells were treated with 1nM Dex for 8 h. RT-PCR reactions were then performed as in Fig. 3A. The gel shown of PCR products, resulting from 28 rounds of PCR amplification, is representative of three separate experiments. *, $P < 0.005$, significantly different from the mean value of siGFP controls; bars, SD.

at reducing endogenous Hic-5/ARA55 expression. Nonetheless, the reduction in Hic-5/ARA55 expression in A1-2 cells by the most effective siRNA was sufficient to generate reduced GR transactivation, as measured by ligand-dependent luciferase expression (Figure 9B). Furthermore, this reduction in GR activity was not due to decreased GR expression in siHic-5/ARA55-transfected cells (Figure 9A). We also analyzed glucocorticoid-induced *c-fos* and p21 mRNA expression after transfection with Hic-5/ARA55 siRNA (Figure 9C). Although there was no reduction in *c-fos* expression, there was a reduction in p21 expression upon silencing of Hic-5/ARA55. Because the silencing of Hic-5/ARA55 expression was not completely effective, we can not conclude that Hic-5/ARA55 is essential for GR activity. However, these results indicate that endogenous Hic-5/ARA55 contributes to optimal GR transactivation from a subset of promoters in A1-2 cells.

Hic-5/ARA55 interaction with coactivators on GR responsive promoters.

Because Hic-5/ARA55 does not possess HAT or methyltransferase activity, it may not modify histones directly. However, Hic-5/ARA55 may be involved in recruiting other chromatin modifying coactivators. To determine if Hic-5/ARA55 interacts with other coactivator proteins at glucocorticoid responsive promoters, we performed sequential ChIPs, first using antibodies specific for Hic-5/ARA55 followed by re-immunoprecipitation with anti-SRC-1, anti-TIF-2, anti-RAC3, anti-CBP or anti-p300 antibodies. As shown in Figure 10, TIF-2, RAC3, CBP, and p300, but not SRC-1 were associated within Hic-5/ARA55-containing chromatin at the MMTV promoter in glucocorticoid treated A1-2 cells. Interestingly, while TIF-2, RAC3, and CBP were

MMTV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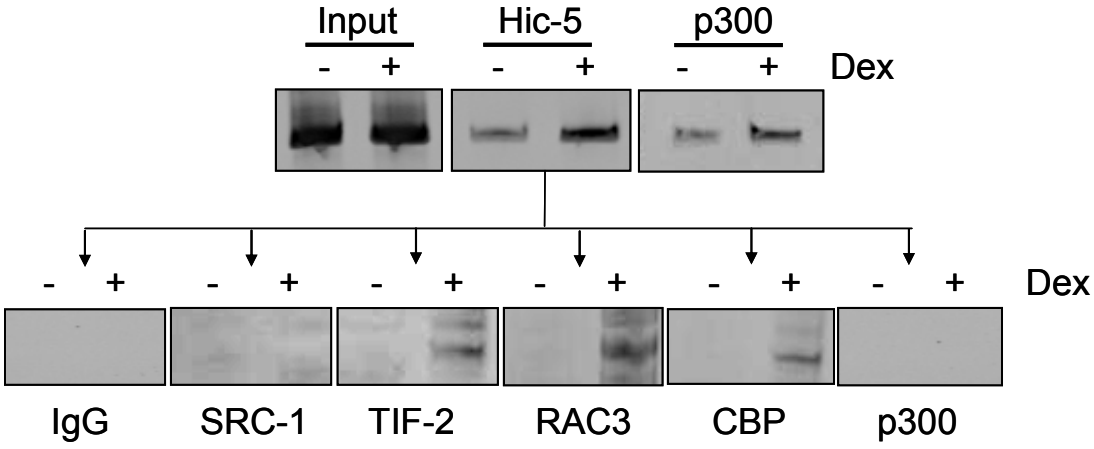
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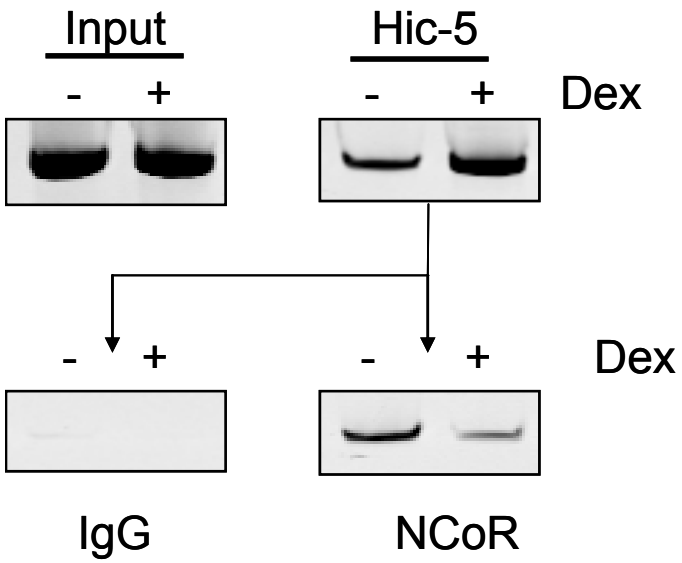

Figure 10. Co-occupancy of Hic-5/ARA55 and coregulators on the MMTV and *c-fos* promoters *in vivo*. Soluble chromatin was prepared as in Figure 1. Hic-5-bound DNA complexes were immunoprecipitated, eluted, and re-immunoprecipitated with *A and B*, SRC-1, TIF-2, RAC3, CBP, p300, or *C*, NCoR specific antibodies. After crosslink reversal, purified DNA was amplified using MMTV (A and C) or *c-fos* (B)-specific primers. PCR products in the Input panel were amplified using diluted chromatin that was not immunoprecipitated. The gels shown of PCR products, resulting from 28-32 rounds of PCR amplification, are representative of three separate experiments.

detected within Hic-5/ARA55-containing chromatin complexes at the *c-fos* promoter, neither p300 nor SRC-1 was detected in Hic-5/ARA55 chromatin complexes (Figure 10B). p300 is recruited to the *c-fos* promoter in response to glucocorticoids (Figure 10B), but must exist in a separate coactivator complex that does not include stably associated Hic-5/ARA55. These results indicate that Hic-5/ARA55 is located within a complex of coactivator proteins at glucocorticoid responsive promoters and that recruitment of p300 to Hic-5/ARA55-associated chromatin complexes may be promoter specific.

Because Hic-5/ARA55 was present on the chromatin of glucocorticoid-responsive promoters in the absence of hormone stimulation, we analyzed its possible interaction with corepressor-containing complexes. Using sequential ChIP, we first immunoprecipitated Hic-5/ARA55-containing complexes followed by re-immunoprecipitation with NCoR-specific antibodies. As shown in Figure 10C, the association of NCoR with Hic-5/ARA55 at the MMTV promoter is higher in cells not exposed to hormone. Thus, Hic-5/ARA55 can be contained within both coactivator and corepressor complexes at an individual promoter.

Reduced promoter recruitment of TIF-2 and p300 after silencing Hic-5/ARA55 expression.

Because Hic-5/ARA55 is made up of multiple protein interaction motifs, it may function as an adaptor protein at hormone-responsive promoters, interacting with multiple

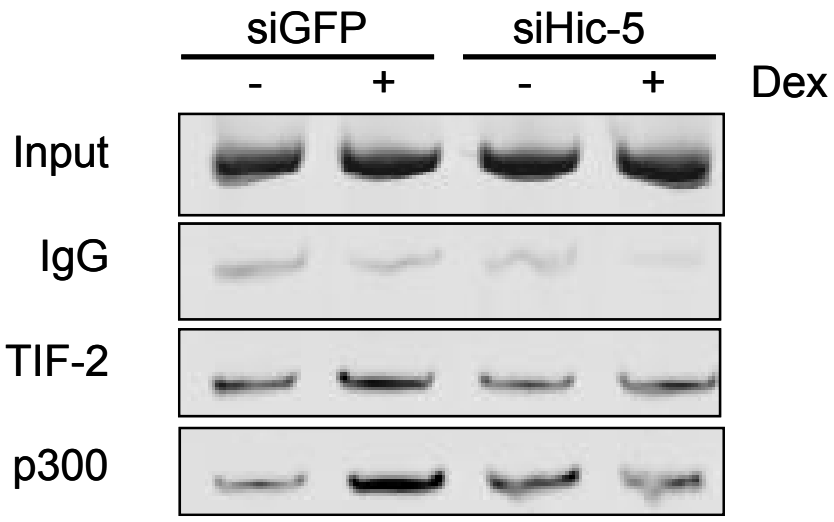

Figure 11. Coactivator recruitment on the MMTV promoter following ablation of Hic-5/ARA55. A1-2 cells were transfected as in Fig. 4. with vectors expressing siRNA for Hic-5/ARA55 or GFP. After 72 hours, ChIP analysis was performed as in Fig.1. After crosslink reversal, purified DNA was amplified with primers specific for the MMTV promoter. Gel shown of PCR products, resulting from 28 rounds of PCR amplification, is representative of two separate experiments.

coregulator proteins or stabilizing the coactivator complex. In order to assess the necessity of Hic-5/ARA55 in coactivator recruitment to glucocorticoid-responsive genes, we performed ChIP assays in cells after silencing Hic-5/ARA55 expression. As shown in Figure 11, after ablation of Hic-5/ARA55 expression, TIF-2 and p300 recruitment to the MMTV promoter in response to glucocorticoids was reduced. Interestingly, there was increased p300 recruitment in the absence of hormone upon silencing Hic-5/ARA55 expression. The corresponding reduction of GR transactivation (Figure 9) and coactivator recruitment (Figure 11) that results from partial Hic-5/ARA55 ablation demonstrates the critical role of Hic-5/ARA55 in maintaining the assembly of coactivator complexes required to bring about efficient glucocorticoid-induced transcription.

Discussion

Here, we showed that endogenous Hic-5/ARA55 is recruited to both stably integrated viral and endogenous glucocorticoid-responsive promoters in response to hormone treatment. Furthermore, GR and Hic-5/ARA55 co-occupied these promoters within stable complexes that can be recovered by sequential ChIPs. We also demonstrated an association between Hic-5/ARA55 and coactivator-containing complexes at glucocorticoid-responsive promoters using sequential ChIPs. Using gel shift assays and co-immunoprecipitations, Shibanuma *et al.* showed that Hic-5/ARA55 containing a heterologous nuclear localization signal (NLS) regulated p21 expression by interacting with Smad3, but not p300 or Sp1 directly in transfected cells (65). While these results

imply that Hic-5/ARA55 association with p300 may be cell type- or promoter-specific, it may not be appropriate to compare the function of endogenous Hic-5/ARA55 in our case with transfected, overexpressed Hic-5/ARA55 that contains a heterologous NLS to force robust nuclear retention. However, promoter-specific recruitment of p300 to endogenous Hic-5/ARA55 chromatin complexes was observed in A1-2 cells, corroborating the results obtained with exogenously introduced NLS-Hic-5/ARA55 constructs.

Interestingly, for the promoter in which p300 in not a component of Hic-5/ARA55 complexes (i.e. the *c-fos* promoter), partial ablation of Hic-5/ARA55 was not sufficient to impact glucocorticoid-regulated transcription. While we cannot rule out the possibility that more complete ablation of Hic-5/ARA55 would hinder glucocorticoid-induced transcription from the *c-fos* promoter, Hic-5/ARA55 may only exert an essential function to maintain the stability of distinct subsets of coactivator complexes (i.e. p300-containing). Partial Hic-5/ARA55 ablation also blocked PPARγ-induced expression of a select set of epithelial-specific genes (66). While the differential recruitment of coactivators was not examined in this case, these results highlight the importance of Hic-5/ARA55 in impacting gene-specific effects of nuclear receptors.

Although there have been no reports indicating a repressive function for Hic-5/ARA55 on nuclear receptor-mediated gene expression, ChIP analysis consistently revealed Hic-5/ARA55 binding to the MMTV promoter in the absence of hormone. The enhanced association of NCoR with Hic-5/ARA55 complexes at the MMTV promoter in non-hormone treated cells suggests that Hic-5/ARA55 may in fact participate in maintaining

low basal levels of transcription from this promoter. However, Hic-5/ARA55 was not essential for limiting MMTV transcription in the absence of hormone as its partial ablation does not lead to enhanced transcription. Whereas we have observed enhanced basal transcription from a steroid hormone-regulated gene upon Hic-5/ARA55 ablation in a prostate cell line, definitive demonstration of Hic-5/ARA55 participation in transcriptional repression will require more thorough analysis.

Finally, siRNA ablation experiments reported here establish that Hic-5/ARA55 is required for the stable association of p300 and TIF-2 with the MMTV promoter. Thus, Hic-5/ARA55 may stabilize select protein complex formation at GR-responsive promoters by serving as an adaptor molecule.

CHAPTER 4 Determining Hic-5/ARA55 expression and function in the prostate.

Hypothesis 2

Because Hic-5/ARA55 expression in the prostate is restricted to the stromal compartment, it modulates AR-mediated gene expression, products of which are required for epithelial-stromal interactions in the prostate.

Introduction

As previously mentioned, communication between the epithelial and stromal compartments of the prostate is crucial for the maintenance of prostate growth and function (7, 8). Additionally, androgens along with functional AR expression in the stromal compartment are necessary for prostate development (14). In the stromal compartment, AR regulates the expression of various growth factors such as KGF (FGF-7), a member of the fibroblast growth factor family (14, 19-21). KGF is exclusively expressed in the stromal compartment of the prostate while its receptor, a spliced variant of the FGF type 2 receptor (FGFR2IIIb), is expressed solely in the epithelial compartment (3). These characteristics suggest that KGF is a paracrine mediator of signaling from the stroma to the epithelial compartment that can influence epithelial cell growth (1, 22).

Along with prostate development, androgens are critical for PCa progression and may be involved in the transition to hormone refractory PCa (97). AR activation in the absence

of circulating androgens in hormone refractory PCa is an area of intense investigation. Some reports suggest that signaling events issued by cytokines or growth factors such as, IGF-1, EGF, KGF and IL-6, can induce AR-mediated gene expression in the absence of androgens (104, 105). Furthermore, KGF expression is significantly increased in prostate cancer (22). In addition to androgen independent activation of AR, increased expression of coactivators in PCa cell lines have enhanced AR responsiveness to low levels of androgen, anti-androgens, and other steroid hormones such as estrogen or progesterone (44, 106).

In the prostate, Hic-5/ARA55 expression was restricted to the stromal compartment (111). Interestingly, Hic-5/ARA55 expression was reduced in tumor as compared to normal tissue (112). Furthermore, Hic-5 mRNA levels were lower in hormone-refractory PC than that of previously untreated PCa, and in recurrent hormone refractory PCa, higher Hic-5/ARA55 expression correlated with unfavorable recurrence-free survival as well as overall survival (122). Because most analyses of coactivator expression in PCa uses quantitative PCR, it is difficult to ascertain whether this represents reduced expression of Hic-5/ARA55 within individual cells or alterations in the balance between stromal/epithelial cell content in tumors.

In this study, we examined Hic-5/ARA55 expression and activity in the prostate. In both normal and tumor derived prostates, Hic-5/ARA55 expression is confined to the stromal compartment. We therefore focused our analysis of Hic-5/ARA55 function in a prostate stromal, myofibroblast cell line, WPMY-1 cells. We show here that Hic-5/ARA55

functions as an AR coactivator in WPMY-1 cells and is necessary for effective androgen induction of the stromal paracrine factor, KGF.

Results

Regional expression of Hic-5/ARA55 in human prostate tissue.

Given Hic-5/ARA55 effects on AR and its role in hormone-dependent growth of prostate cancer (PCa) cell lines, a number of studies have examined its expression in prostate tissue. For example, Hic-5/ARA55 mRNA expression, as quantified using PCR, is reduced in hormone refractory prostate cancer tissue (HRPC) as compared to normal or benign prostatic hypertrophy (BPH) tissue (112, 122). Since these tissue samples contained a heterogeneous population of cells, potential cell type specific differences in Hic-5/ARA55 expression would not be revealed. Therefore, we analyzed prostate tissue from both normal human donors and prostate cancer patients for Hic-5/ARA55 expression using immunohistochemistry. As shown in Figure 12 (top panels), Hic-5/ARA55 expression is confined to the stromal compartment of the prostate. The epithelial compartment was consistently negative for Hic-5/ARA55 expression. This profile of Hic-5/ARA55 expression in the prostate is consistent with those previously published using normal human prostates (111). In tumor-derived prostate tissue, Hic-5/ARA55 was also restricted to stromal cells and not detected in the prostate tumor cells (Figure 12, bottom panels). In both cases, the Hic-5/ARA55 staining appeared diffuse and its precise subcellular compartmentalization was not apparent.

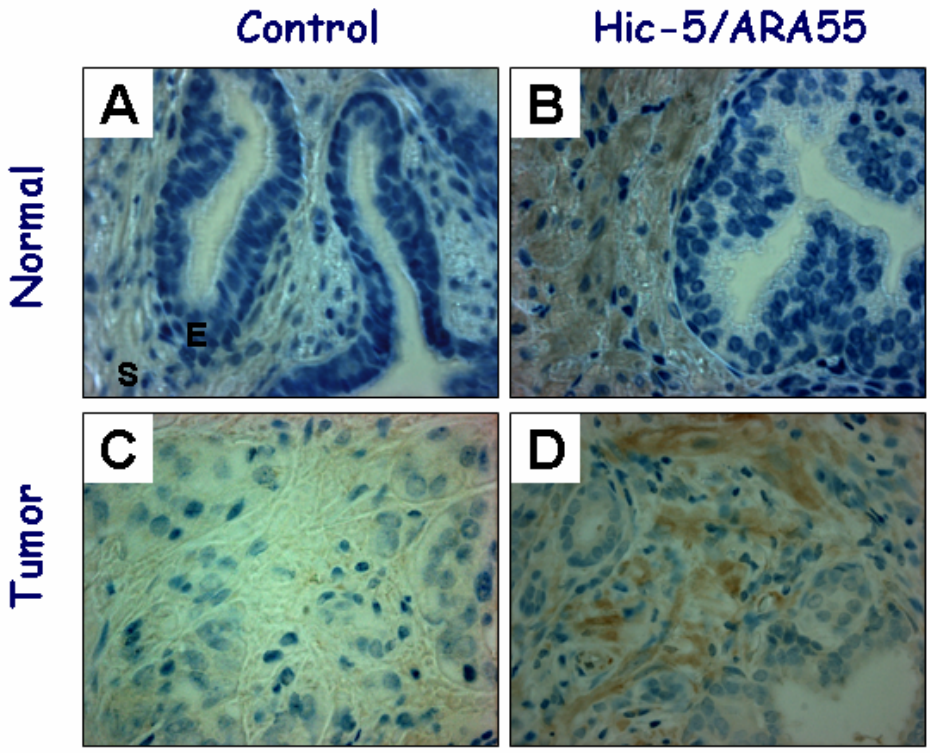

Figure 12. Hic-5/ARA55 expression in human prostate tissue sections. Normal human prostate tissue sections from donor prostates (A and B) or from PCa patients (C and D) were stained with a non-immune antibody control (A and C) or an anti-Hic-5/ARA55-specific antibody (B and D). Original magnification X66. Compartments of the prostate are distinguished in A, **E**, epithelial compartment and **S**, stromal compartment.

Nucleocytoplasmic shuttling of Hic-5/ARA55 in the WPMY-1 prostate stromal cell line.

Hic-5/ARA55 coactivator function has been analyzed in a variety of epithelial-derived PCa cell lines, including LNCaP, PC3, and DU145 cells (46). However, because its expression is confined to the stromal compartment of the prostate, PCa cell lines may not provide a suitable model system with which to analyze Hic-5/ARA55 activity. Given the apparent stromal expression of Hic-5/ARA55, we considered it more appropriate to examine Hic-5/ARA55 effects on AR in stromal cells rather than PCa-derived epithelial cells. We therefore employed WPMY-1 cells, a prostate myofibroblastic cell line that was derived from a human prostate primary stromal culture that was immortalized using the SV40 large-T-antigen (114). Hic-5/ARA55 expression in WPMY-1 cells was confirmed by Western blot analysis (Figure 13A). Immunofluorescence analysis revealed that most Hic-5/ARA55 is localized within the cytoplasm and at focal adhesions (Figure 13B, top panels). Although the extent of Hic-5/ARA55 expression within the nucleus of WPMY-1 cells was not apparent from the immunofluorescence images, Leptomycin B (LMB) treatment of the cells resulted in robust nuclear accumulation of Hic-5/ARA55 (Figure 13B, bottom panels). LMB is an inhibitor of a major nuclear export receptor and has been used to reveal the nucleocytoplasmic shuttling properties of a variety of proteins, including Hic-5/ARA55 as well as the Hic-5/ARA55 family member, zyxin (64, 123). The effects of LMB on Hic-5/ARA55 localization in WPMY-1 cells establish that Hic-5/ARA55 is indeed capable of shuttling between the cytoplasmic and nuclear compartments and must, therefore, have some residence time within the nucleus. Furthermore, Hic-5/ARA55 staining was not depleted from focal adhesions

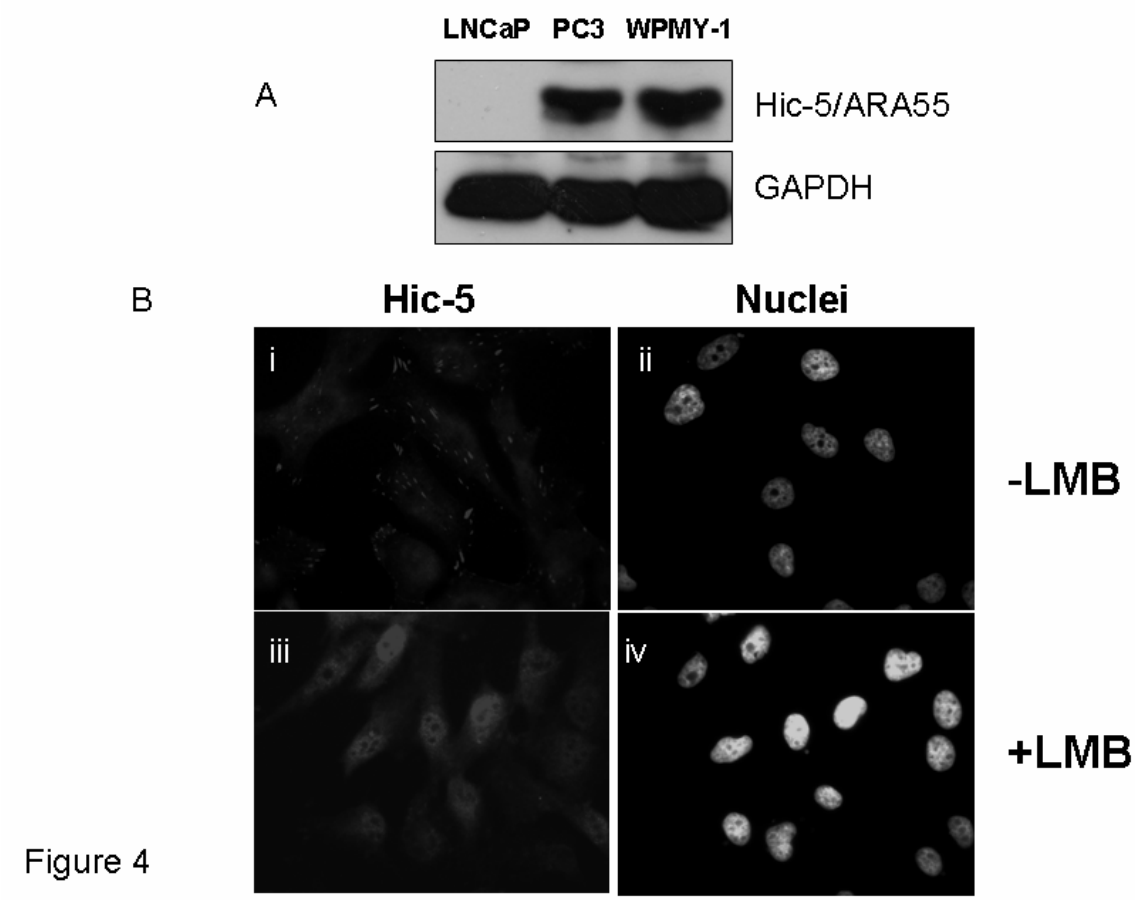

Figure 4

Figure 13. Expression and nucleocytoplasmic shuttling of Hic-5/ARA55 in the WMPY1 prostate stromal cell line. A, Hic-5/ARA55 expression was analyzed in two prostate epithelial cell lines, LNCaP and PC3, as well as a prostate stromal cell line, WPMY-1, by Western blot analysis. Equivalence in protein loading was confirmed by reprobing stripped blots with an anti-GAPDH antibody. B, Immunostaining of WMPY1 cells treated with vehicle alone (i and ii) or 100 nM Leptomysin B (LMB) (iii and iv) for 3 h and stained with an α-Hic-5/ARA55-specific antibody (i and iii) or DAPI (ii and iv).

upon LMB treatment (data not shown), indicating that shuttling of Hic-5/ARA55 most likely occurs between the cytoplasmic and nuclear compartments.

AR and Hic-5/ARA55 recruitment to the KGF promoter.

Because Hic-5/ARA55 enhances AR-mediated gene expression in transient transfection assays, we set out to reveal whether Hic-5/ARA55 was localized to endogenous androgen-responsive promoters in WPMY-1 cells. KGF is a well established marker of androgen induction in prostate stromal cells (19, 20). As shown in Figures 14A and 14B, KGF mRNA expression is induced 2-fold in response to treatment with various androgens. In primary stromal cell cultures, KGF expression is similarly induced approximately 2-fold by androgen treatment (20). Furthermore, androgen induction of KGF mRNA expression is blocked by the AR antagonist, hydroxyflutamide, indicating that androgen induction of KGF mRNA in WPMY-1 cells is indeed mediated by AR.

Because Hic-5/ARA55 has been found to directly interact with AR, we sought to determine if Hic-5/ARA55 is bound to the KGF promoter using ChIP assays and whether this binding is affected by androgen treatment. Detailed analysis of the human KGF promoter revealed that promoter proximal sequences located between -225 to +190 were necessary for basal KGF expression (124). Although a canonical hormone response element is not located within this region, there is a half-palindromic glucocorticoid response element located between positions -178 to -183 around which we designed our primers for ChIP analysis (124). After stimulation with androgens for 1 hour, both AR and Hic-5/ARA55 were localized to the KGF promoter (Figure 15). Given the high

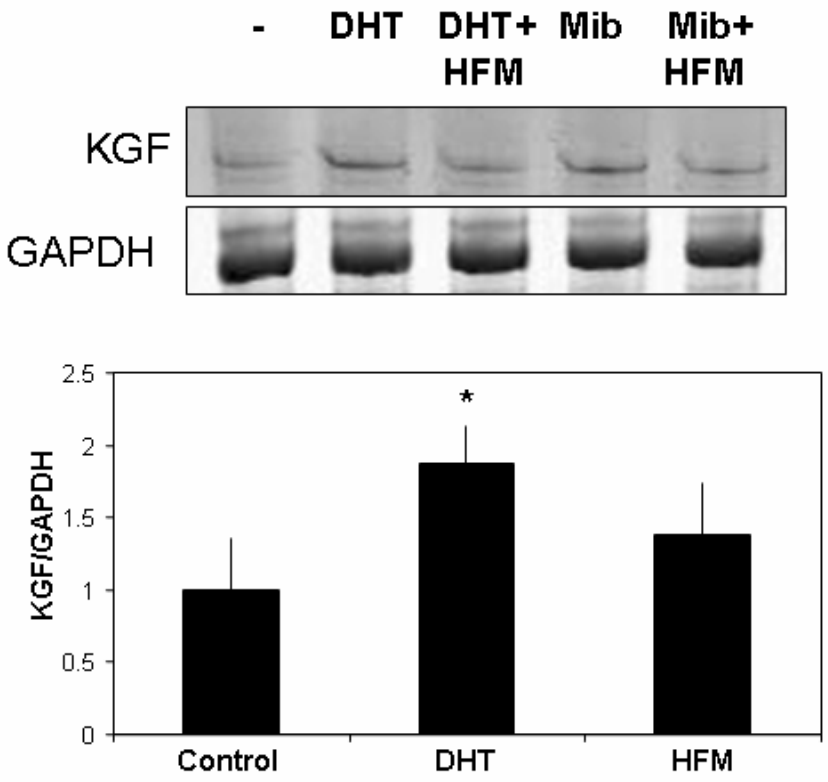

Figure 14. Androgen-induced KGF expression in WPMY-1 cells. A, Androgen effects on KGF mRNA expression in WPMY-1 cells was analyzed by RT-PCR. Gel shown of PCR products, resulting from 32 rounds of PCR amplification, following 8 h treatment with indicated agents is representative of three separate experiments. B, Relative changes KGF expression were calculated based on semi-quantitative results from scanned images as in A. Values represent KGF expression after normalization with GAPDH ± SD for three separate experiments. *, $P < 0.05$, significantly different from the mean value of ethanol controls; bars, SD. DHT, dihydroxytestosterone; HFM, hydroxyflutamide; Mib, mibilerone.

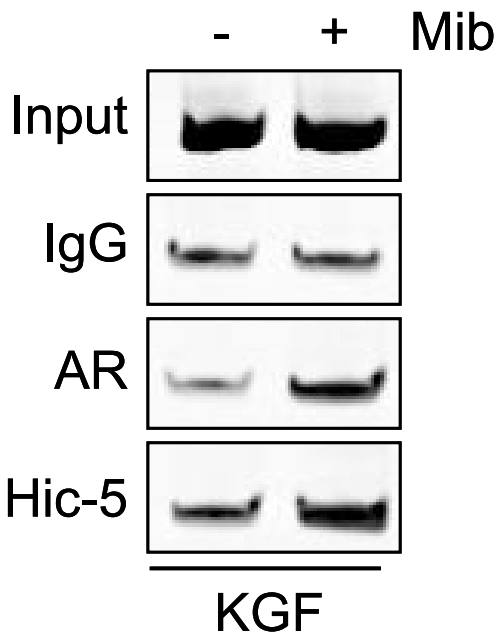

Figure 15. Association of AR and Hic-5/ARA55 with the chromatin of the KGF promoter *in vivo*. ChIP analysis of AR and Hic-5/ARA55 recruitment to the KGF promoter in WPMY-1 cells in response to 1 h Mib treatment. PCR products generated following precipitation of isolated chromatin fragments with non-immune IgG, AR or Hic-5/ARA55 antibodies are shown. PCR products in the Input panel were amplified using diluted chromatin that was not immunoprecipitated. Gel shown of PCR products, resulting from 32 rounds of PCR amplification, is representative of three separate experiments. Mib, mibilerone.

degree of background obtained in ChIP assays with non-immune IgG, it is difficult to assess the extent of AR and/or Hic-5/ARA55 binding to the KGF promoter in the absence of androgen treatment. Nonetheless, the ChIP results from hormone treated cells suggest that Hic-5/ARA55-mediated coactivation of AR activity in WPMY-1 cells may be mediated in part by their recruitment to endogenous androgen-responsive promoters.

Ablation of Hic-5/ARA55 expression results in decreased AR transactivation.

Given the localization of Hic-5/ARA55 to the KGF promoter in response to androgen treatment, we ascertained whether Hic-5/ARA55 was necessary for androgen induction of KGF expression using a siRNA approach to ablate Hic-5/ARA55 expression in WPMY-1 cells. WPMY-1 cells were analyzed for Hic-5/ARA55 expression by Western blot analysis following transfection with either a control GFP siRNA or Hic-5/ARA55 siRNA. Densitometric analysis revealed that there was approximately 60% less Hic-5/ARA55 in cells transfected with the Hic-5/ARA55 siRNA oligo D as compared to control (Figure 16A). Although this was the maximal amount of Hic-5/ARA55 ablation that could be attained with Hic-5/ARA55 siRNA transfections, it was sufficient to reduce androgen induction of the transfected androgen-inducible mouse mammary tumor virus (MMTV) promoter (Figure 16B) and of endogenous KGF mRNA expression (Figure 16C). Basal levels of KGF expression were reproducibly higher upon Hic-5/ARA55 ablation, suggesting that Hic-5/ARA55 may negatively regulate basal KGF promoter activity. Irrespective of these effects of Hic-5/ARA55 on basal KGF promoter activity,

the siRNA ablation experiments establish a role for Hic-5/ARA55 in androgen regulation of both a viral androgen-responsive promoter and an endogenous growth factor gene.

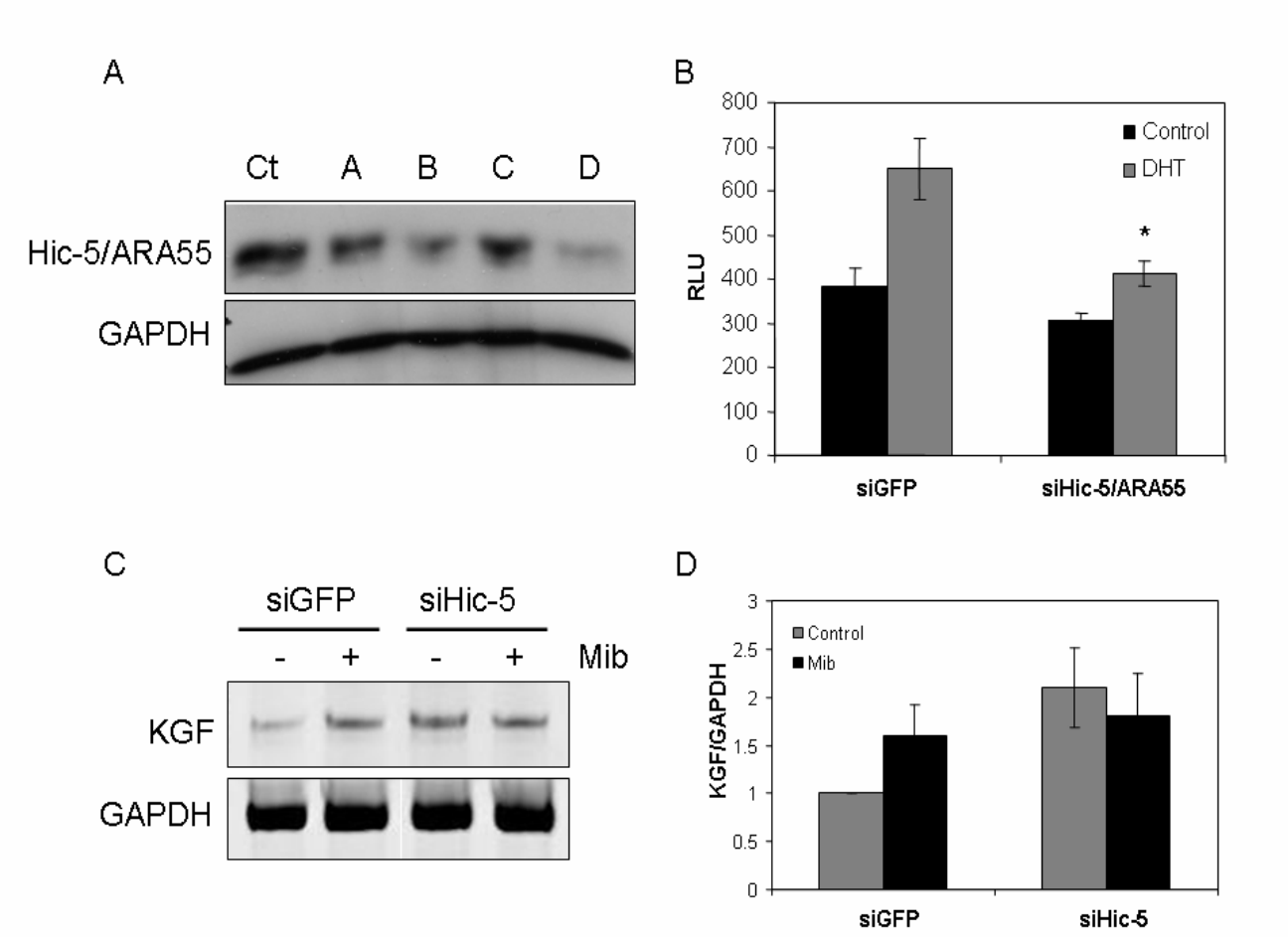

Figure 16. Effects of RNAi-mediated silencing of Hic-5/ARA55 on AR transactivation. A, WPMY-1 cells were transfected with 2 μg GFP (Ct, negative control) or 2 μg human Hic-5/ARA55 RNAi constructs (A-D). After 72 h, cells were harvested, lysed, and analyzed for Hic-5/ARA55 and GAPDH protein levels by Western blotting. B, WPMY-1

cells were transfected with RNAi constructs (GFP and D) as in A as well as the MMTV promoter-linked reporter. After 48 h, the cells were treated with EtOH-vehicle or DHT (10^{-8} M) for 24 h after which luciferase activity was measured. Data shown are mean of three separate experiments. *, $P < 0.05$, significantly different from the mean value of siGFP controls; bars, SD. C, Androgen-induced KGF expression was analyzed after silencing Hic-5/ARA55. Cells were transfected as in A. After 72 h, the cells were stimulated with Mib (10^{-8} M) for 8 h and KGF expression was analyzed by RT-PCR and normalized to GAPDH expression. D, Relative changes KGF expression were calculated based on semi-quantitative results from scanned images as in C. Values represent KGF expression after normalization with GAPDH ± SD for three separate experiments.

Discussion

Our results identify Hic-5/ARA55 as a compartment-specific AR coactivator that has an impact on androgen regulation of a paracrine growth factor, KGF, in the stroma. The importance of KGF in prostate gland development has been revealed by various approaches. For example, KGF neutralizing antibodies inhibit prostate epithelial growth as well as ductal branching (1). Furthermore, exogenous KGF was sufficient to produce epithelial growth as well as ductal branching of the prostate in the absence of androgen (1).

Analysis of Hic-5/ARA55 expression had revealed its limited tissue distribution. For example, immunohistochemical analysis was used to reveal selective expression of Hic-5/ARA55 in smooth muscle and myoepithelial cells (125). Hic-5/ARA55 was not detected in epithelial cells of the tissues examined, including the stomach, colon, liver, skin, and mammary gland (125). Additionally, Hic-5/ARA55 is expressed in the stromal, but not epithelial compartment of the prostate (111). Here, we confirmed the stromal-specific expression of Hic-5/ARA55 in normal prostate. Furthermore, Hic-5/ARA55 expression was also restricted to the stromal compartment in prostate tumor tissue.

The stromal-specific expression of Hic-5/ARA55 suggests that this AR coactivator is unlikely to contribute directly to the development or progression of PCa. However, Hic-5/ARA55 may play a role in stromal/epithelial cell communication in the prostate since androgen induction of a stromal-selective paracine factor (i.e. KGF) is lost upon partial ablation of Hic-5/ARA55. Furthermore, the increase in basal KGF expression upon Hic-5/ARA55 ablation suggests that this coactivator may interact with other transcription factors or corepressors to negatively regulate KGF expression in the absence of androgens (65). Thus, by serving opposing roles to positively or negatively regulate KGF gene transcription, Hic-5/ARA55 may be critical in sculpting tissue organization in the prostate, responding to various hormonal and paracrine cues that serve to maintain the appropriate balance between distinct stromal and epithelial cell layers. Furthermore, given the role of stromal-derived KGF on the growth, transformation, and invasive properties of PCa cells, coactivators such as Hic-5/ARA55 may influence tumor development and progression through its impact on either basal and/or androgen-induced

expression of KGF in stroma during both androgen-dependent and androgen-independent phases (126, 127).

Detailed sequence analysis of the human KGF promoter revealed a half-palindromic steroid hormone response element between positions -178 to -183, implicating AR regulation of KGF transcription (124). ChIP analysis of the KGF promoter revealed both AR and Hic-5/ARA55 recruitment to this region of the KGF promoter in response to androgens. Although most Hic-5/ARA55 is found at focal adhesions and the cytoplasm in WPMY-1 cells, Hic-5/ARA55 shuttles between the cytoplasm and the nucleus. Interestingly, Hic-5/ARA55 was localized to the KGF promoter in the absence of LMB. This indicates that there may be a small pool of Hic-5/ARA55 resident in the nucleus that may not be apparent by immunofluorescence analysis, but which nonetheless participates in transcriptional regulation of androgen-regulated promoters.

In endocrine target tissues, nuclear receptor activity appears critical for tumor development and/or progression (128). For example, elevated AR expression may be necessary for PCa progression to an androgen-independent state (100). Furthermore, increased AR expression correlates with a higher probability of recurrence (101). In response to elevated expression of AR, mutations in AR, or altered expression of coactivators, the activity of nuclear receptors within individual cells may exhibit a heightened response to non-steroidal signals. Our work suggests the existence of another mechanism for coactivator effects on prostate cancer that does not function in a cell

autonomous context. AR action in cells that support the tumor, located in the stromal compartment, may also be affected by stromal-specific coactivators.

Chapter 5 Determining the effects of extracellular matrix (ECM) signaling on GR-mediated transcription in A1-2 cells.

Hypothesis: Because Hic-5/ARA55 associates with focal adhesion proteins as well as nuclear receptors, Hic-5/ARA55 may relay extracellular matrix signals to nuclear receptors, affecting their transactivation properties.

Introduction

Intracellular signaling originating from the extracellular matrix (ECM) is mediated by a well-established group of receptors known as integrins. Integrins form heterodimer receptors between members of the α and β families, which are specific for an ECM component. For example, α5β6 binds fibronectin primarily, whereas α1β1 binds both laminin and collagen (reviewed in (129). Resulting signals following integrin-ligand interactions include modulation of cellular migration, proliferation, survival, and apoptosis. However, the exact mechanism by which ECM signaling influences nuclear receptor-mediated gene expression in normal as well as tumorigenic tissue is less clear.

Cross-talk between estrogen receptor (ER) and ECM signaling, resulting in differential cell growth and/or gene expression, has been well studied. Specifically, malignant mammary cells grown on a collagen IV or laminin-1 basement membrane express higher levels of ERα, resulting in increased ER-mediated gene expression irrespective of the

presence of estradiol (130). Furthermore, analysis of the ERα promoter revealed STAT5 binding sites located within the region that was responsive to laminin-rich basement membranes, indicating that upregulation of ERα expression may be mediated by STAT5 (131). However, Woodward *et al.* report that laminin inhibits ERα activity, namely negatively influencing estrogen-mediated progesterone receptor (PR) expression and proliferation of breast cancer cells (132). These discrepancies are most likely due to minor differences in the application of the ECM, culturing conditions, and/or the differential responsiveness to ECM between different cell lines.

Along with breast cancer cell lines, primary breast epithelial cells also respond to ECM signaling. Epithelial cells derived from nulliparous mice also exhibit increased responsiveness to progesterone stimulation when plated on fibronectin or collagen IV (133). After plating the cells on various ECM, increased proliferation in response to progesterone treatment was detected in nulliparous-derived but not pregnancy-derived breast epithelial cells (133). These results indicate that mammary gland responsiveness to both steroid hormones as well as ECM signaling is dependent on mammary gland differentiation.

Although the effects of certain ECM components such as collagen IV and laminin-1 on ERα-mediated gene expression have been reported, the exact mechanism by which ECM enhances ERα expression and/or activity is unclear. For example, activation of integrin signaling pathways in response to various ECM components may influence nuclear receptor phosphorylation, effecting its subcellular localization, coactivator interaction, or

protein stability. Alternatively, ECM may influence nuclear receptor-mediated gene expression in a more direct manner, via interaction of focal adhesion-associated proteins such as Hic-5/ARA55 with the nuclear receptors, affecting their gene activation.

Along with a possible role for Hic-5/ARA55 in transmitting ECM signals, which in turn affect nuclear receptor-mediated gene expression, Hic-5/ARA55 also modulates ECM expression. Using cells that overexpress Hic-5/ARA55, Shibanuma et al. report that Hic-5/ARA55 expression may influence ECM expression directly (121). Both collagen and fibronectin expression correlated with Hic-5/ARA55 expression (121). Furthermore, overexpression of Hic-5/ARA55 led to alternative splicing of fibronectin, which is also associated with the senescence process (121). The modulation of ECM expression by Hic-5/ARA55 may provide a feedback loop, serving to ameliorate or alter ECM signaling events.

Here, we examined GR-mediated gene expression in response to various ECM as well as Hic-5/ARA55's role in transmitting ECM-derived signals. Both fibronectin and matrigel influenced GR transactivation in opposing ways. Matrigel repressed GR-mediated gene expression by reducing the levels of GR protein. Fibronectin did not influence either GR or Hic-5/ARA55 expression, subcellular localization or promoter recruitment in the presence of ligand, suggesting that other mechanisms by which fibronectin may influence GR transactivation, such as post translational modifications or GR-coactivator interactions.

Results

Effects of ECM on GR-mediated transcription

To determine the effects of ECM signaling on GR-mediated gene expression, we took advantage of the integrated MMTV promoter in the A-12 cells. Because T47D cells, the parental cell line of A-12 cells, express α1, α3, α4 and α5 integrin subunits, the extracellular matrices that were analyzed include: laminin, collagen, fibronectin as well as a growth factor reduced matrigel (136). Because growth factors such as EGF and IGF-1 have been reported to activate nuclear receptors-mediated gene expression in the absence of ligand, we used a growth factor reduced matrigel to analyze the effects of basement membrane components on GR activity (104). After coating cell culture plates with the appropriate ECM, A-12 cells were treated with dex (100 nM) for 18 hours after which luciferase reporter activity was measured. Whereas fibronectin enhanced GR-mediated luciferase expression in A1-2 cells, matrigel reduced it (Figure 17). However, neither laminin nor vitronectin affected GR transactivation properties. Furthermore, there was no change in GR-mediated gene expression when cells were plated on a nonspecific poly-$_L$-lysine substratum, indicating that the responses of GR activity on both fibronectin and matrigel were specific and may be mediated by integrin signaling.

Because the effect of fibronectin on GR-mediated gene expression was modest at 100nM Dex, we analyzed the effect of fibronectin at lower concentrations of ligand. Again, fibronectin enhanced GR-mediated gene expression at all concentrations examined

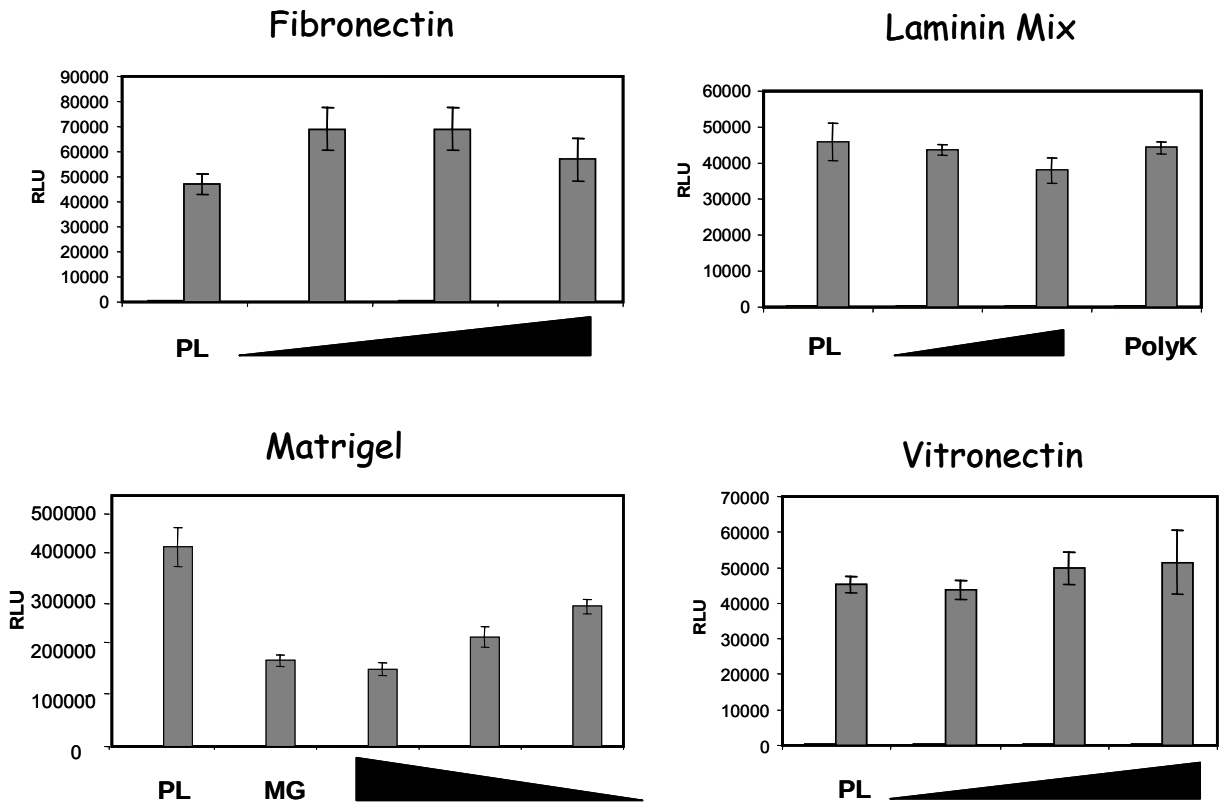

Figure 17. Effects of extracellular matrices on GR-mediated transcription. Cell culture plates were coated with fibronectin (0.5, 1, and 2.5 μg/cm$^{2)}$, laminin (0.625 and 1.3 μg/cm^2), growth factor reduced matrigel (undiluted, MG, to diluted 1:1, 1:2 and 1;3), or vitronectin (50, 100, 250 ng/ cm^2). A1-2 cells were cultured on plastic or fibronectin, laminin, growth factor reduced matrigel, or vitronectin. After 24 hours, the cells were stimulated with either ethanol-vehicle or 100 nM Dex for 18 hours after which luciferase activity was measured. Relative luciferase activities (RLU) were normalized to protein concentration by Bradford analysis. *, $P < 0.05$, significantly different from the mean value of the plastic controls; bars, SD. PL, plastic; poly K, poly lysine; MG, matrigel.

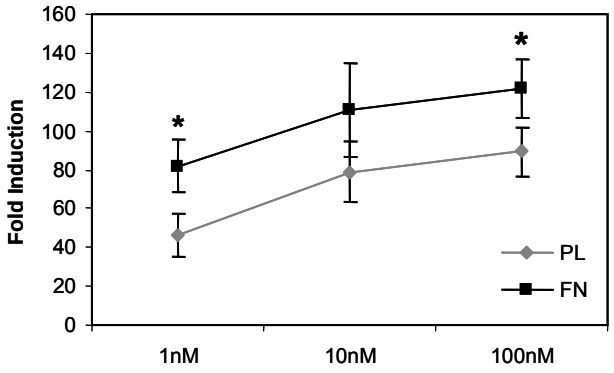

Figure 18. Effects of fibronectin on various concentrations of Dex. A1-2 cells were cultured on plastic or fibronectin-coated (1 μg/cm^2) plates. After 24 hours, the cells were stimulated with either ethanol-vehicle or 1-100 nM Dex for 18 hours after which luciferase activity was measured. Relative luciferase activities (RLU) were normalized to protein concentration by Bradford analysis. Data shown are the mean of three separate experiments. *, $P < 0.05$, significantly different from the mean value of the plastic controls; bars, SD. PL, plastic and FN, fibronectin.

(Figure 18). Furthermore, at 1nM Dex, fibronectin induced a 2-fold increase in GR activity.

Effects of ECM on GR expression

Because both matrigel and fibronectin influence GR-mediated gene expression in A1-2 cells, we wanted to determine if this was due to altered GR expression and/or protein stability. After A1-2 cells were plated on the indicated ECM as in Figure 17, cell lysates were analyzed for GR expression. As shown in Figure 19, GR expression was not affected when A1-2 cells were plated on fibronectin. However, plating A1-2 cells on matrigel reduced GR levels, thereby, which could account for the repressive effects of matrigel on GR-mediated gene expression. Because Hic-5/ARA55 is localized at focal adhesion complexes and also known to influence nuclear receptor-mediated gene expression, we also analyzed its expression in A1-2 cells plated on fibronectin or matrigel. Hic-5/ARA55 expression was also reduced by matrigel (data not shown). Similar down-regulation of focal adhesion complex proteins including paxillin by reduction in protein synthesis along with protease activation has been reported (137). This down-regulation was due to inhibition of focal adhesion complex formation when the cells were cultured in the presence of collagen gel.

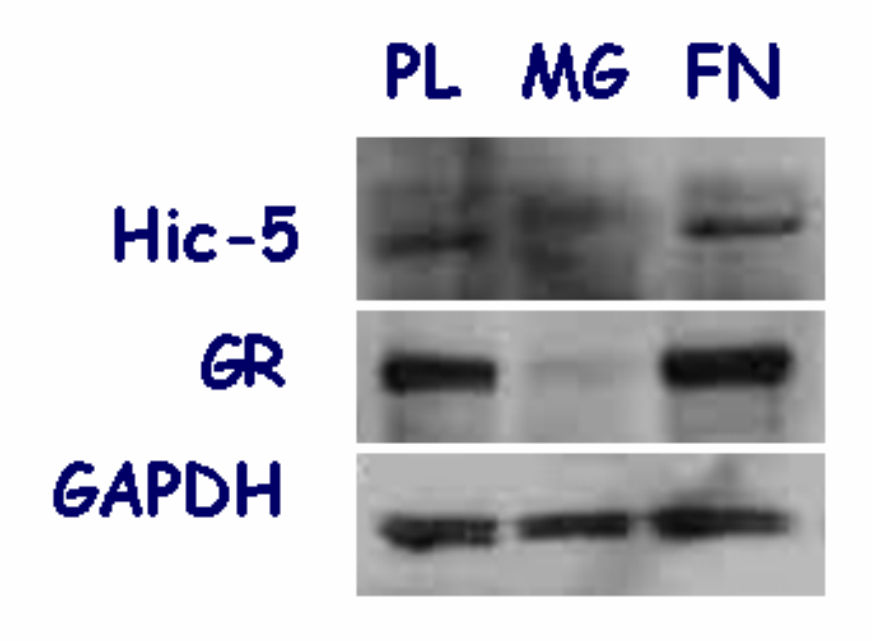

Figure 19. Effects of Matrigel and Fibronectin on GR and Hic-5/ARA55 expression. Cells were cultured on plastic, matrigel, fibronectin as in Figure 16. A1-2 cell lysates were analyzed for GR and Hic-5/ARA55 expression by Western blot analysis using specific antibodies. The membranes were stripped and reprobed with GAPDH-specific antibodies as a loading control. PL, plastic; MG, matrigel; FN, fibronectin.

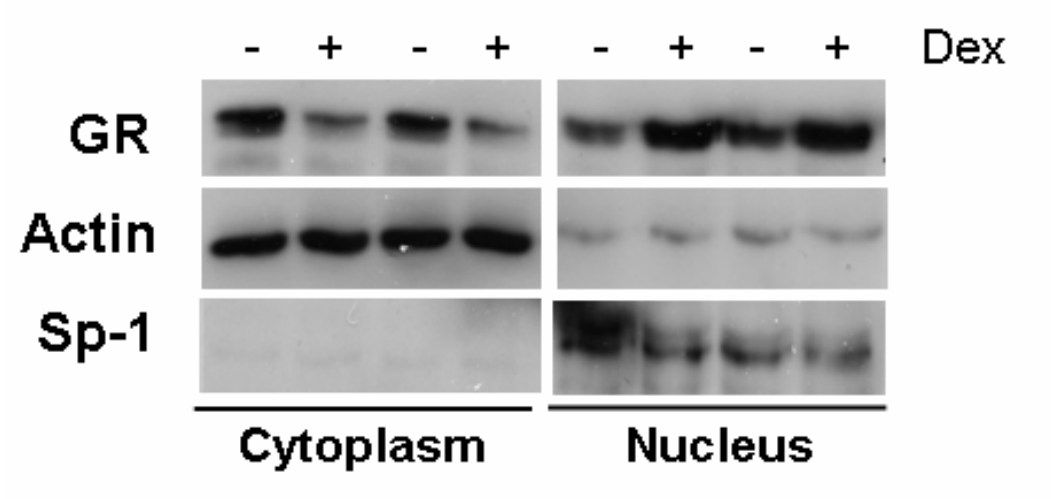

Figure 20. Effects of fibronectin on nucleocytoplasmic shuttling of GR. Cells were cultured on plastic or fibronectin in Figure 16. After treatment with vehicle (-) Dex (+, 100nM) for 1 h, A1-2 cells were harvested and fractionated in order to separate the nuclear and cytoplasmic compartments. Lysates were analyzed for GR expression by Western blot analysis using an anti-GR-specific antibody. Purity of each fractionation was analyzed using cytoplasmic or nuclear markers, actin and Sp-1 respectively.

Effects of fibronectin on nucleocytoplasmic shuttling of GR

Because fibronectin enhances GR-mediated gene expression, but neither GR nor Hic-5/ARA55 protein levels, we analyzed GR and Hic-5/ARA55 shuttling in the presence and absence of glucocorticoid stimulation. Ogawa, M et al. report enhanced nuclear localization of paxillin in cells plated in vinculin (138). This altered subcellular localization of paxillin in the presence of an extracellular matrix component was dependent on MAP Kinase (MAPK) activity, leading to differential phosphorylation of paxillin. In the absence of hormone, most GR is located in the cytoplasm in cells grown on plastic or fibronectin (Figure 20). Upon stimulation with glucocorticoids, GR transits to the nucleus with similar efficiency regardless of whether the cells were grown on plastic or fibronectin. Furthermore, there is no change in Hic-5/ARA55 localization upon glucocorticoid stimulation in cells plated on either plastic or fibronectin (data not shown).

Effects of cell signaling inhibitors on fibronectin-enhanced GR activity

To determine the mechanism by which fibronectin stimulates GR-mediated transcription in A1-2 cells, we analyzed potential signaling pathways that may act through integrin receptors, affecting GR activity. Both ERK as well as PKB signaling occurs at focal adhesions in response to stimulation by extracellular matrices via integrin receptors (139). Using pharmacological inhibitors, U0126 and LY294002, to inhibit ERK and PKB signaling respectively, we analyzed GR-mediated gene expression in cells plated on both plastic and fibronectin (Figure 21). Neither U0126 not LY294002 abrogated the effect of fibronectin on GR activity. To ensure that both compounds were active, we analyzed cell lysates for phosphorylated ERK and AKT after treatment with U0126 and

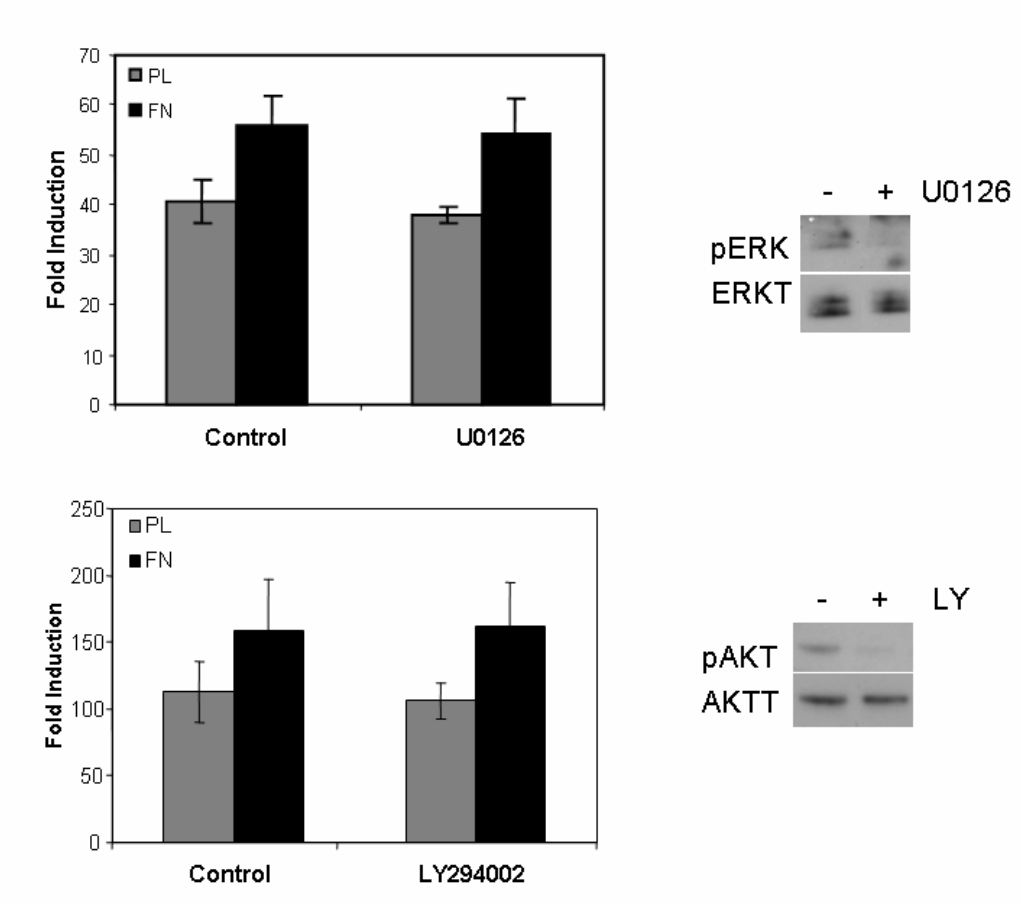

Figure 21. Effects of cell signaling inhibitors on fibronectin-enhanced GR activity. Cells were cultured on plastic or fibronectin as in Figure 16. After 24 hours, the cells were stimulated with ethanol-vehicle or Dex (100nM) in the presence or absence of A, ERK inhibitor, U0126 (10 μM), or B, PKB inhibitor, LY294002 (10 μM). Cellular lysates were analyzed for phosphorylated ERK or AKT (pERK and pAKT, right panels) by western blot analysis in the presence of absence of specified inhibitors. Total ERK (ERKT) or AKT (AKTT) was determined by western blot analysis as a loading control.

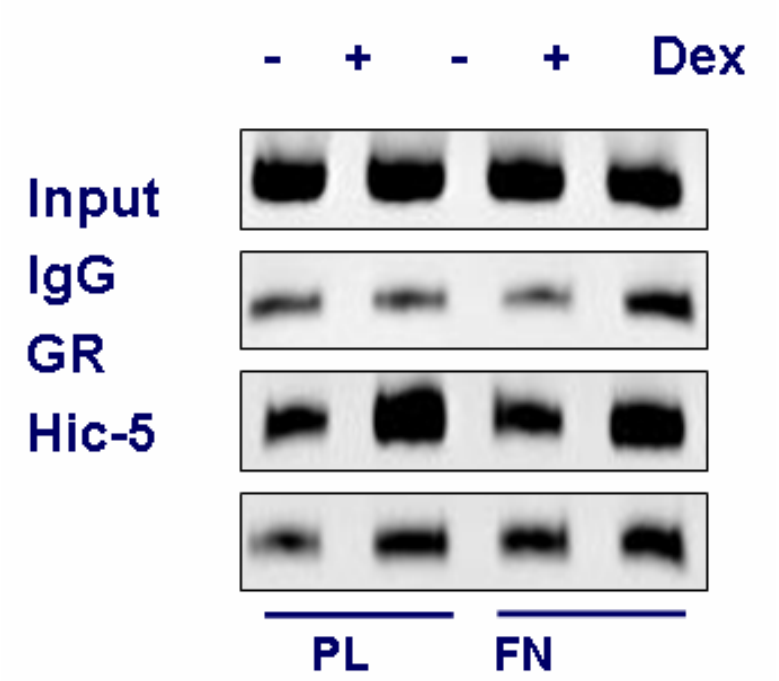

Figure 22. Effects of fibronectin on GR and Hic-5/ARA55 promoter recruitment in response to glucocorticoids. The cells were plated on plastic (left lanes) or fibronectin (right lanes) as before and soluble chromatin was prepared from A1-2 cells treated with EtOH-vehicle or Dex (100 nM) for 1 h. Protein-bound DNA complexes were immunoprecipitated with antibodies against GR or Hic-5/ARA55. After crosslink reversal, purified DNA was amplified with primers specific for the MMTV promoter. PCR products, resulting from 28 rounds of PCR amplification, in the Input panel were amplified using diluted chromatin that was not immunoprecipitated. A rabbit IgG was used to detect any nonspecific immunoprecipitated DNA.

LY294002 respectively. Both compounds efficiently inhibited phosphorylation of the appropriate signaling molecule, indicating that neither ERK nor PKB signaling is involved in the effect of fibronectin on GR transactivation.

Effects of fibronectin on GR and Hic-5/ARA55 promoter recruitment in response to glucocorticoids

Because both GR and Hic-5/ARA55 are recruited to glucocorticoid-responsive promoters in the presence of hormones, we analyzed the effect of fibronectin on GR and Hic-5/ARA55 promoter recruitment. ChIP analysis of the MMTV promoter in A1-2 cells plated on plastic or fibronectin revealed that both GR and Hic-5/ARA55 are recruited to the promoter upon glucocorticoid stimulation (Figure 22). Although there was no detectable difference in either GR or Hic-5/ARA55 MMTV promoter recruitment in the presence of Dex, there may be a slight increase in Hic-5/ARA55 promoter binding in the absence of hormone in cells plated on fibronectin. However, this result is preliminary and based upon a limited number of experiments.

Discussion

Recent work by various labs has highlighted the responsiveness of nuclear receptors to ECM components. Specifically, Novaro et al. reported that basement membrane components such as collagen IV or laminin-1 upregulated ER-mediated gene expression

by increasing ERα expression (130, 131). Furthermore, Sisci et al. described increased ERα nuclear localization and ligand-independent activation of gene expression in cells plated on either fibronectin or collagen IV (140). In fact, increased ERα activity in both MCF-7 and MDA-MB-231 breast cancer cells was mediated by increased c-Src activation after cellular attachment to ECM, thereby affecting cellular mobility (140). Interestingly, increased expression and activity of c-Src has been reported in various tumors, including breast cancer, possibly increasing their malignant capabilities (141, 142).

Using A1-2 breast cancer cells, we analyzed various ECM for their ability to modulate GR-mediated transactivation. Furthermore, because Hic-5/ARA55 is a focal adhesion complex-associated protein that influences gene expression, it was postulated that it may transmit signaling events from the ECM to the nucleus. As mentioned previously, it has been reported that nuclear accumulation of Hic-5/ARA55 occurs in response to oxidants such as H_2O_2 (64). Furthermore, using LMB for 1 h in WPMY-1 cells, we were able to detect most Hic-5/ARA55 in the nucleus, indicating that Hic-5/ARA55 rapidly transits between cytoplasmic and nuclear compartments (Figure 8).

After analyzing various ECM, we determined that both fibronectin and growth factor reduced matrigel influenced GR transactivation in A1-2 cells by different mechanisms. Matrigel negatively influenced GR-mediated gene expression by reducing GR as well as Hic-5/ARA55 expression levels. Although the exact by which matrigel reduces GR and Hic-5/ARA55 expression levels is not known, Wang et al. report a similar reduction of

focal adhesion complex proteins such as focal adhesion kinase (FAK) and paxillin by matrigel (137). Both a reduction in protein synthesis as well as activation of calpain proteinases resulted in decreased focal adhesion protein levels and was mediated by α2β1 integrin activity (137). Following collagen binding, α2β1 integrin signaling may be involved in focal adhesion complex formation or stabilization by inducing cytoskeletal rearrangements, ultimately affecting cell migration.

Along with matrigel, fibronectin altered GR-mediated gene expression in A1-2 cells, increasing GR transactivation. However, fibronectin failed to modulate GR expression, subcellular trafficking, or promoter recruitment. Hic-5/ARA55 expression and activity were also unaffected by fibronectin. Furthermore, inhibitors of both ERK and PKB signaling pathways failed to repress fibronectin-induced GR activation in A1-2 cells. However, it is possible that integrin signaling through other proteins may be involved, possibly through c-Src. Furthermore, because the effect of fibronectin on GR-mediated gene expression was modest, induction of slight changes in GR expression, subcellular trafficking or promoter recruitment may be undetectable. Alternatively, because ECM stimulates numerous intracellular signaling events, fibronectin may promote alternative GR phosphorylation, resulting in enhance GR-mediated gene expression. GR is a phosphoprotein, capable of being phosphorylated at multiple sites, some of which induce greater GR nuclear localization and activity (143). A detailed analysis of the phosphorylation state of GR in cells plated on fibronectin will determine if that is the mechanism by which fibronectin enhances GR-mediated gene expression.

CHAPTER 6 DISCUSSION

The aim of this project was to determine the mechanism by which Hic-5/ARA55 serves as a nuclear receptor coactivator as well as determining its potential involvement in PCa progression. Tumorigenesis is a multi-step process which involves changes in not only the malignant cells but also the surrounding microenvironment of that tumor, including stromal cells and ECM. Because Hic-5/ARA55 is a nuclear receptor coactivator that is involved in cellular differentiation as well as growth, analysis of its expression and activity may illuminate certain aspects of prostate cancer initiation or progression. Our results identify Hic-5/ARA55 as a compartment-specific AR coactivator that has an impact on androgen regulation of a paracrine growth factor, KGF, in the stroma.

Hic-5/ARA55-coregulator interactions

In recent years, numerous nuclear receptor-interacting proteins have been identified that modify chromatin, thereby influencing nuclear receptor-mediated gene expression. Because assays using reporter gene analysis to determine nuclear receptor activity revealed that overexpression of most coactivators enhanced the activity of many nuclear receptors, coactivator specificity was questioned. Hence, it was postulated that coactivators function in a cell type or promoter-specific manner. Using the MMTV promoter, Li et al. isolated a specific coactivator complex that associated with PR that differed to the coactivator complex which bound to GR (14). These results indicate that combinations of coactivators rather than individual coactivators may be responsible for

promoter or hormone-specific gene expression. Furthermore, coactivator knock out analysis provided evidence that at least in part, coactivator function is not totally redundant. For example, TIF-2 and RAC3 null mice display reduced reproductive capability while SRC-1 null mice are exhibit partial hormone resistance (41, 42).

Along with coactivators that modify chromatin, other coactivators such as Hic-5/ARA55 have been discovered whose mechanism of action is largely unknown. Because Hic-5/ARA55 does not possess a catalytic domain that is responsible for its coactivation properties, its mechanism of coregulator function has remained undefined. However, it may serve as an adaptor molecule, either recruiting or stabilizing promoter-specific protein complexes. LIM proteins are well recognized for their roles as molecular adaptors, functioning in stabilizing higher order protein complexes at either focal adhesion complexes or promoter sequences. siRNA ablation experiments reported here establish that Hic-5/ARA55 is required for the stable association of p300 and TIF-2 with the MMTV promoter (Figure 6). Thus, Hic-5/ARA55 may stabilize select protein complex formation at GR-responsive promoters by serving as an adaptor molecule.

Not only does Hic-5/ARA55 interact with various coactivator complexes, but it also associates with NCoR corepressor complexes in the absence of hormone at nuclear receptor-responsive promoters (Figure 5C). This suggests that Hic-5/ARA55 is capable of interacting with other coregulators directly not necessarily via nuclear receptors. Furthermore, because Hic-5/ARA55 is present on GR-responsive promoters in the

absence and presence of glucocorticoids, it may function in coordinating corepressor release and coactivator recruitment upon glucocorticoid stimulation.

Recently, transducin β-like 1 (TBL1), an adaptor-like protein, has been reported to mediate the exchange of corepressors for coactivators on nuclear receptor-responsive promoters in response to ligand (144). TBL1 was initially isolated as part of the corepressor complex (145). ChIP analysis of nuclear receptor target promoters revealed prolonged TBL1 promoter association in the presence of ligand (144). Furthermore, TBL1 recruited proteosome machinery to nuclear receptor target promoters, leading to degradation of the corepressor complex followed by association of the coactivator complex (144). Although the possible interaction of Hic-5/ARA55 with components of the proteosome machinery has not been analyzed, it may provide a mechanism by which Hic-5/ARA55 interacts with both corepressors and coactivators, culminating in enhanced nuclear receptor-mediated gene expression.

Although Hic-5/ARA55 was localized to GR-responsive promoters in the absence of ligand and detectable promoter-bound GR, the mechanism by which Hic-5/ARA55 is bound in the absence of receptor is unclear. In addition to receptor-independent binding of Hic-5/ARA55 to the MMTV promoter, we also isolated receptor-independent localization of NCoR to the MMTV promoter in the absence of ligand. Direct binding of either Hic-5/ARA55 or NCoR to the MMTV promoter has not been analyzed. Interestingly, Hic-5/ARA55 does display zinc-dependent DNA binding (146). However, a specific DNA sequence for which Hic-5/ARA55 is capable of binding has yet to be

identified. Instead, it is thought that Hic-5/ARA55 may be capable of binding DNA in a more non-specific manner. If Hic-5/ARA55 is capable of directly binding the MMTV promoter, it may function in tethering the NCoR-containing complex to the MMTV promoter in the absence of ligand. However, it is also possible that both Hic-5/ARA55 and NCoR are binding the MMTV promoter through another, yet unidentified DNA binding protein.

Although Hic-5/ARA55 interactions with other coactivators as well as NCoR were observed in the A1-2 cells, we anticipate that similar interactions would be found in the WMPY-1 stromal cells. Specifically, we detected Hic-5/ARA55 bound to the KGF promoter in the absence of androgens (Figure 15). Ligand independent promoter localization of Hic-5/ARA55 was also observed in the A1-2 cells. Furthermore, addition of ligand enhanced the amount of Hic-5/ARA55 promoter recruitment in both cell lines. Because Hic-5/ARA55 is found within coactivator as well as corepressor complexes in A1-2 cells, we anticipate that similar interactions may occur in WPMY-1 cells, possibly in a promoter-specific manner.

Coactivators and PCa

Because some steroid-responsive nuclear receptors such as AR and ER have been associated with cancers derived from endocrine target tissues, their expression as well as coactivator expression and activity has been analyzed in breast as well as PCa. As previously mentioned, treatment of PCa with anti-androgen therapy initially thwarts

tumor growth. However, most PCa eventually progresses into a hormone refractory, androgen-independent PCa, capable of growth in the presence of anti-androgens. Although the exact mechanism by which PCa becomes androgen-independent is unclear, coactivator expression and/or activity may be involved. As previously mentioned, altered expression of coactivators may enhance ligand-independent AR activity in androgen independent prostate cancer. For example, increased TIF-2 expression enhanced AR-mediated gene expression in response to estradiol as well as progesterone (106). Interestingly, Hic-5/ARA55 expression was reduced in tumor as compared to normal tissue (112). Furthermore, Hic-5 mRNA levels were lower in hormone-refractory PCa than that of previously untreated PCa. In recurrent hormone refractory PCa, higher Hic-5/ARA55 expression correlated with unfavorable recurrence-free survival as well as overall survival (122). Because most analyses of coactivator expression in PCa progression use quantitative PCR analysis of whole prostate tissue sections, it is difficult to ascertain whether this represents reduced expression of Hic-5/ARA55 within individual cells or alterations in the balance between stromal/epithelial cell content in tumors. Subsequent analysis of Hic-5/ARA55 expression in primary prostate stromal cells derived from normal or tumor prostates revealed reduced Hic-5/ARA55 expression in tumor stromal cells (unpublished data). However, analysis of Hic-5/ARA55 expression in prostate tissue sections indicated that there was some continued expression of Hic-5/ARA55 in prostate tumors (Figure 12).

Because partially silencing Hic-5/ARA55 expression in WMPY-1 cells resulted in increased basal KGF expression, we anticipate that reduced Hic-5/ARA55 expression in

reactive stromal cells may also result in increased KGF expression. In fact, KGF expression is significantly increased in prostate cancer (22). Enhanced KGF expression possibly due to reduced Hic-5/ARA55 expression may result in androgen-independent activation of AR or increased tumor epithelial proliferation, thereby promoting androgen-independent PCa progression.

Reactive Stromal Transition

Along with changes in the epithelia compartment, which is the source of tumor cells, development of an altered stromal microenvironment, termed reactive stroma, carcinoma associated fibroblasts (CAF) or tumor stroma, enhances tumor cell survival, cellular proliferation, and migration (81, 82). The presence of undifferentiated myofibroblast cells which express both smooth muscle cell and fibroblasts markers induces changes in ECM expression as well (83). Myofibroblasts are commonly found at areas undergoing tissue remodeling, producing ECM such as collagen I and fibronectin as well as proteases, including matrix metalloproteinases (MMPs) (84-86). Along with altered ECM expression by reactive stromal cells, data indicate altered gene expression of growth factors such as KGF, FGF-2, TGF-β, and IL-6, and matrix metalloproteinases in reactive stromal cells (22, 89-92). The differential gene expression pattern induced by the reactive stroma may confer resistance to cell cycle inhibitors or metastatic properties to the malignant cells. As mentioned previously, TGF-β may be involved in the progression to a reactive stroma phenotype by inducing myofibroblast formation (83). Interestingly, TGF-β induces Hic-5/ARA55 expression in fibroblasts although it is

unclear whether its expression is upregulated in reactive stromal cells. Because Hic-5/ARA55 is involved in both ECM and KGF expression in normal prostate stromal cells, upregulation of Hic-5/ARA55 expression resulting from increased TGF- β stimulation could result in differential ECM as well as KGF production.

Hic-5/ARA55 and development

During tissue development, progenitor cells terminally differentiate whereby they usually exit the cell cycle as well as phenotypically differentiate. Hic-5/ARA55 expression has been shown to both induce and repress cellular differentiation in a cell type-specific manner. For example, rat calvarial bone cells (RCT-1) differentiate into osteoblasts after retinoic acid treatment, which decreases cell growth and induces alkaline phosphatase and α I pro-collagen expression (147). During RCT-1 differentiation, Hic-5/ARA55 expression is upregulated (147). Furthermore, overexpression of Hic-5/ARA55 in RCT-1 cells leads to decreased cellular proliferation as well as increased αI pro-collagen synthesis, both indicative of osteoblast differentiation (147). Alternatively, during myogenesis, Hic-5/ARA55 expression correlates with apoptotic myoblasts (148). The process of myogenesis is characterized by myoblast fusion and induction of muscle-specific gene expression, culminating in myotubule formation. Myoblasts that fail to differentiate undergo apoptosis, and Hic-5/ARA55 expression not only blocks myogenesis but also induces apoptosis in mouse C_2C_{12} myoblasts (148).

Along with either promoting or inhibiting cellular differentiation, Hic-5/ARA55 may also be involved cell type-specific differentiation programs. For example, Hic-5/ARA55 is expressed in gut epithelial cells during development (66). Furthermore, it serves as a coactivator for PPARγ-mediated gene expression in intestinal epithelial cells. Partial ablation of Hic-5/ARA55 expression repressed PPARγ-mediated expression of keratin 20, L-FABP, and KLF. Finally, expression of Hic-5/ARA55 in preadipocytes not only inhibits adipogenesis but also induces epithelial gene expression in these mesenchymal cells, suggesting that Hic-5/ARA55 regulates epithelial gene expression in the gut (66). Interestingly, TBL1, another nuclear receptor associated- adaptor protein, expression is necessary for PPARγ-induced adipogenesis (144).

We show here a role for Hic-5/ARA55 in KGF expression in WPMY-1 human stromal cells of the prostate. KGF, a crucial mediator of epithelial-stromal interactions during prostate development, is regulated by androgens. For example, KGF neutralizing antibodies inhibit prostate epithelial growth as well as ductal branching (1). Furthermore, addition of exogenous KGF to a serum-free organ culture system leads to prostate growth as well as ductal branching, overcoming the requirement for testosterone (1). As shown in Figure 10, androgen regulation of KGF expression is mediated by AR recruitment to the KGF promoter. Silencing of Hic-5/ARA55 expression led to increased basal expression of KGF in the absence of androgens (Figure 16). Furthermore, the increase in basal KGF expression upon Hic-5/ARA55 ablation suggests that this coactivator may interact with other transcription factors or corepressors to repress KGF expression in the absence of androgens (65). By partially silencing Hic-5/ARA55 expression in WPMY-1

cells, Hic-5/ARA55-mediated repression may be removed, leading to KGF expression in the absence of androgens. Furthermore, AR recruitment to the KGF promoter along with subsequent coactivator interactions in the presence of androgens may negate the repression by Hic-5/ARA55, resulting in androgen-induced KGF expression. In A1-2 cells, the enhanced association of NCoR with Hic-5/ARA55 complexes at the MMTV promoter in non-hormone treated cells suggests that Hic-5/ARA55 may, in fact, participate in maintaining low basal levels of transcription from this promoter. Thus, by serving opposing roles to positively or negatively regulate KGF gene transcription, Hic-5/ARA55 may be critical in sculpting tissue organization in the prostate, responding to various hormonal and paracrine cues that serve to maintain the appropriate balance between distinct stromal and epithelial cell layers. Indeed analysis of Hic-5/ARA55 expression during prostate development may be beneficial in determining the mechanism by which Hic-5/ARA55 and AR regulation of KGF expression may be involved in prostate development.

Hic-5/ARA55 and cell growth

As mentioned previously, Hic-5/ARA55 negatively affects cell growth in both osteoblasts and myoblasts (147, 148). In nontumorigenic, immortalized human fibroblasts, overexpression of Hic-5/ARA55 induced senescence (121). Cellular senescence or cellular aging is characterized by an irreversible growth arrest. Interestingly, p21 (Cip1/WAF1/sdi1) cyclin-cdk inhibitor, a gene that is induced during senescence, is upregulated in cells that overexpress Hic-5/ARA55 (121, 149). As shown

in Figures 3 and 4, Hic-5/ARA55 is recruited to the p21 promoter after glucocorticoid stimulation, and silencing Hic-5/ARA55 reduced glucocorticoid-induced p21 expression. It is possible that overexpression of Hic-5/ARA55 during senescence may induce p21 expression directly, thereby overcoming the need for glucocorticoid induction. Furthermore, induction of *c-fos* expression upon serum stimulation is lost in cells undergoing senescence, inhibiting cellular proliferation (150). In Hic-5/ARA55 overexpressing cells, *c-fos* induction upon TPA stimulation is lost as well (121). However, H_2O_2 treatment, inducing Hic-5/ARA nuclear localization, upregulated *c-fos* expression in TIG-7 human fibroblasts (64). In A1-2 cells, we saw no effect on *c-fos* expression upon Hic-5/ARA55 depletion, suggesting that Hic-5/ARA55 expression is not necessary for *c-fos* induction by glucocorticoids in nonsenescent cells (Figure 4).

The recent demonstration of Hic-5/ARA55 involvement in PPARγ-induced epithelial cell differentiation program illustrates the importance of "adaptor" coactivators that lack enzymatic activity in assembling functional coregulator complexes on distinct promoters. More detailed analysis of this novel family of nuclear receptor coactivators may unlock multiple nuclear receptor gene networks that utilize LIM domain-containing adaptors to organize gene-specific coactivator assemblies. In conclusion, given the role of stromal-derived KGF on the growth, transformation, and invasive properties of PCa cells, coactivators such as Hic-5/ARA55 may influence tumor development and progression through its impact on either basal and/or androgen-induced expression of KGF in stroma during both androgen-dependent and androgen-independent phases (126, 127).

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