New mechanisms of transcriptional regulation of the folate receptor and other genes by steroid Receptors

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FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Biomedical Sciences

New Mechanisms of Transcriptional
Regulation of the Folate Receptor and other
genes by steroid Receptors

Submitted by:
Aymen Shatnawi

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Sciences

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Date of Defense: December 10, 2007
New Mechanisms of Transcriptional Regulation of the Folate Receptor and Other Genes by Steroid Receptors

Aymen Shatnawi

University of Toledo/ College of Medicine

2007
DEDICATION

To the soul of my father, to my mother, my wife and lovely kids. God bless all of them
ACKNOWLWDGMENT

I would like to thank my major advisor, Dr. Manohar Ratnam for his time, effort and guidance during my Ph.D study. I would like to thank all my advisory committee: Dr. Sonia Najjar, Dr. Robert Trumbly, Dr. Han-Fei Ding, Dr. David Giovannucci and Dr Douglas Pittman for their support and suggestions during my training.

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The folate receptor (FR) or Folate Binding Protein (FBP), is a glycopolypeptide, that has a high binding affinity for folic acid ($K_d < 10^{-9}$) and its circulating metabolite, $N_\text{5}'$-methyltetrahydrofolate (Antony et al., 1981; Antony, 1996). The expression of FR is tissue specific and limited to the apical surface of epithelial cells where it is inaccessible through the circulation. Three isoforms of FR have been isolated and characterized; type $\alpha$ (Elwood et al., 1986; Sadasivan and Rothenberg, 1988; Elwood, 1989; Lacey et al., 1989; Sadasivan and Rothenberg, 1989; Elwood et al., 1997), type $\beta$ (Ratnam et al., 1989) and type $\gamma$ (Shen et al., 1994; Shen et al., 1995). The cell membrane associated forms of FR, FR-\(\alpha\) and FR-\(\beta\) share 73% amino acid sequence identity and are attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Lacey et al., 1989; Yan and Ratnam, 1995). In addition, FR-\(\alpha\) and FR-\(\beta\) are localized in special membrane microdomains known as lipid rafts that serve as platforms for FR recycling (Wang et al., 2002a). On the other hand, FR-\(\gamma\) as well as its truncated form FR-\(\gamma^\prime\) are constitutively secreted due to the absence of a signal peptide for GPI attachment (Shen et al., 1994; Shen et al., 1995).

FR-\(\alpha\) is capable of transporting folate into the cell but other pathways such as the reduced folate carrier (RFC) are principally used for up-take of folate compounds in most adult tissues. The restricted expression of FR-\(\alpha\) at the luminal surface of epithelial cells in several normal tissues results in its physical isolation from the blood stream preventing access to circulating folate. The physiological significance of FR in those tissues is not fully understood. However, FR-\(\alpha\) is required for trans-placental folate uptake by the fetus (Antony, 1992; Henderson et al., 1995; Antony, 1996) and for re-absorption of folate in

Transcription of the FR-α gene is regulated by two distinct TATA-less promoters, which are designated as P1 and P4. The P4 promoter, which is located upstream of exon-4, is primarily directed by a cluster of three non-canonical G/C-rich regions, each of which contributes to promoter activity by cooperation with the downstream initiator region (Saikawa et al., 1995). The presences of alternative promoters as well as alternative splicing involving exons 1-4, generate several FR-α transcripts. All of them have the same open reading frame (ORF) and 3′-untranslated region (3′ UTR) sequence but differ in their 5′-untransalted region (5′ UTR). In FR-α positive malignant tissues, the P4 promoter drives most of the FR-α mRNA transcription. Moreover, P1 promoter transcripts have been shown to be translated less efficiently than P4 transcripts although they are predominant in some normal tissues such as kidney and testis (Elwood et al., 1997; Roberts et al., 1997).

Pre-clinical and clinical studies have shown that FR-α is a promising tumor target for a wide variety of therapeutic agents and as a tumor/serum marker for the following reasons; (I) FR-α has high binding affinity for folate, anti-folates and folate conjugated drugs and can mediate the internalization of those agents by recycling between the cell surface and intracellular compartments [reviewed in (Kamen and Smith, 2004)], (II) In proliferating normal tissues the expression of FR-α is restricted to the luminal surface of certain epithelial cells and typically isolated from the blood stream. In contrast, in certain
epithelial cell derived malignant tissues, FR-α is highly expressed, and accessible through the circulation. Many innovative therapeutic and diagnostic approaches have been successfully used for targeting FR in solid tumors and leukemic cells including, folate conjugates of cytotoxics, radiopharmaceuticals and pro-drugs (Leamon and Low, 1991; Leamon and Low, 2001), folate–coated liposomes containing therapeutic drugs, genes or antisense oligonucleotides and folate-attached nanoparticle carriers for cytotoxic agents (Leamon and Low, 1994; Lu et al., 1999; Andersson et al., 2000; Drummond et al., 2000; Sudimack and Lee, 2000; Ward, 2000; Atkinson et al., 2001; Lu and Low, 2002; Ratnam et al., 2003). Conjugation of macromolecules and nanoparticles does not affect folate binding affinity or drug bioavailability.

A major limitation of FR-targeted therapies is the frequently low expression of FR in tumor tissues. Previous studies from this laboratory have demonstrated that nuclear receptors regulate FR genes and could be modulated to elevate FR expression selectively in various tumors. FR-α is negatively regulated by the estrogen receptor (ER) and FR-β is positively regulated by the retinoic acid receptor (RAR). As an extension of those studies, this thesis investigates regulation of FR-α by glucocorticoid and progesterone receptors. In mammals, steroid receptors such as glucocorticoid receptor (GR) and progesterone receptor (PR) regulate distinct and essential biological processes in a tissue selective manner. GR, which is expressed in all tissues and induced by Glucocorticoids (stress induced hormones) (Sapolsky et al., 2000), is one of the most vital receptors that regulates essential biological process including growth, metabolism, behavior and apoptosis in both males and females. On the other hand, the progesterone receptor plays
an important role in development and reproduction in the female (Conneely and Lydon, 2000; Conneely et al., 2000; Graham and Clarke, 2002). Two isoforms of PR are well characterized, PR-A (94kD) and PR-B (120kD); they are differentially expressed in a tissue specific manner (Graham et al., 1996; Giangrande and McDonnell, 1999; Giangrande et al., 2000; Graham and Clarke, 2002).

Pure PR antagonists such as ZK98299 (onapristone) completely abrogate the transcriptional activity of PR whereas selective progesterone receptor modulators (SPRMs) such as RU486 (mifepristone) have mixed effects acting as either PR agonists or antagonists in different cell and target gene contexts. Interestingly, the A and B subtypes of PR respond differently to RU486 that binds to PR-B to function as a partial agonist under certain conditions; in contrast, RU486 can only act as an antagonist of PR-A (Meyer et al., 1990; Tung et al., 1993).

Functional analysis of the FR-α promoter did not show the presence of classical hormone response elements (HREs). The Endogenous FR-α gene and its promoter activity are negatively regulated (repressed) by the estrogen receptor (ER) (Kelley et al., 2003) and this repression is increased by estrogen treatment and reversed (de-repressed) by anti-estrogenic compounds such as Tamoxifen (Kelley et al., 2003). These findings were consistent with an earlier observation of negative correlation between FR-α expression and ER expression in primary breast cancer cells (Rochman et al., 1985). Detailed mechanistic studies underscored the importance of the initiator and the flanking sequence within the P4 core promoter as a direct target for ER repression through formation of a complex with co-repressors SMRT and/or NCOR (Hao et al., 2007). Conversely, in FR-α positive cell lines, unpublished studies from this laboratory have shown that androgens
such as testosterone and R1881 enhance FR-\(\alpha\) gene expression in a dose and time dependent manner. This regulation involves direct action of AR by interaction with C/EBP\(\alpha\) bound to a CCAAT element. The mechanisms by which ER and AR modulate the FR-\(\alpha\) gene represent novel mechanisms by which the steroid receptors regulate global gene expression.

In this study, we demonstrate that both GR and PR activate the FR-\(\alpha\) gene in a manner that is distinct from ER and AR. The FR-\(\alpha\) mRNA and protein and FR-\(\alpha\) promoter activity are up-regulated by GR and PR isoforms both in tissue cell culture and in tumor xenograft models (Tran et al., 2005; Shatnawi et al., 2007). Mechanistic studies showed that GR, PR-A and PR-B up-regulate FR-\(\alpha\) indirectly through the protein product of upstream target gene(s). Promoter analysis of FR-\(\alpha\) gene showed the involvement of the G/C-rich Sp1 binding sites of the P4 promoter in GR and PR action and an optimal initiator context. We also showed that a clinically well tolerated HDAC inhibitor such as valporic acid (VPA) enhances FR-\(\alpha\) induction by dexamethasone. Thus, the ubiquitous GR could be modulated by clinically acceptable agents (eg., dexamethasone and valproic acid) to selectively induce FR-\(\alpha\) tumor targeting or early detection. Also, the results support the concept of increasing FR-\(\alpha\) expression selectively in the FR-\(\alpha\)-positive tumors by brief treatment with nontoxic doses of GR and PR agonist, alone or in combination with a well-tolerated HDAC inhibitor, to increase the efficacy of various FR-alpha-dependent therapeutic and diagnostic applications. Interestingly, the classical PR antagonist RU486 also activated the FR-promoter but only through PR-B.

The studies were extended to the regulation of other important Sp1-dependent genes such as p21, p27 and thymidine kinase by RU486. The agonistic action of RU486 on those
genes was attributed to its agonistic activity on upstream direct target of PR, the gene(s) encoding a putative Sp1 co-activator(s). Moreover, our findings contradict the current view of Sp1-dependent gene regulation by PR and point to the existence of one or more PR target genes whose promoter and cell context(s) must be key determinants of the agonistic activity of RU486 on a large group of important Sp1-dependent downstream target genes. The upstream target gene(s) is likely encode an Sp1 co-activator(s).
LITERATURE SURVEY

Folate receptor:

Folate receptor (FR) or Folate Binding Protein (FBP), is a glycopolypeptide, that has a high binding affinity for folic acid (Kd<10^{-9}) and its circulating metabolite, N^5-methyltetrahydrofolate (Antony et al., 1981; Antony, 1996). Most normal adult tissues lack FR and its expression is tissue specific and limited to the apical (luminal) surface of epithelial cells where it is isolated from the blood stream. FR was originally reported in placenta, proximal kidney tubules, choroid plexus and others (Selhub and Rosenberg, 1978; Antony et al., 1981; Suleiman and Spector, 1981; Suleiman et al., 1981; Selhub and Franklin, 1984; Antony, 1996). In addition, certain extra-cellular fluids (milk, amniotic fluid, urine and cerebrospinal fluid) showed significant levels of soluble folate binding protein (sFBP) originally derived from a membrane-attached precursor due to membrane or intracellular action of proteases and phospholipases (da Costa and Sharon, 1980; Antony et al., 1982; Elwood et al., 1986). In contrast, FR is completely absent in normal serum and was found to be highly expressed in certain types of major malignant tumors (Wu et al., 1999; Elnakat and Ratnam, 2004).

From cDNA cloning, three isoforms of the folate receptor were isolated and characterized, type α (Elwood et al., 1986; Sadasivan and Rothenberg, 1988; Elwood, 1989; Lacey et al., 1989; Sadasivan and Rothenberg, 1989; Elwood et al., 1997), type β (Ratnam et al., 1989) and type γ (Shen et al., 1994; Shen et al., 1995). The cell membrane associated forms of FR, FR-α and FR- β, share 73% amino acid sequence identity and are attached to the cell surface membrane by a glycosyl-phosphatidylinositol (GPI)
anchor (Lacey et al., 1989; Yan and Ratnam, 1995) through serine 234 and asparagine
230 at their c-terminal end. FR-α and FR-β are localized in special membrane
microdomains known as lipid rafts that serve as platforms for FR recycling (Wang et al.,
2002a).

FR-γ as well as it is truncated form FR-γ́ are constitutively secreted type due to the
absence of the signal peptide for GPI attachment (Shen et al., 1994; Shen et al., 1995).
FR-γ was mainly detected in normal hematopoietic tissues including bone marrow, spleen
and thymus as well as in certain leukemia. A fourth isoform was recently discovered by
human and mouse genome data base mining and was designated as FR-δ (Spiegelstein et
al., 2000). In mice, the expression of FR-δ has been found to occur in spleen and thymus.
Conversely, in humans, the tissue expression of FR-δ and its folate binding ability have
not yet been characterized and very little is known about it (Spiegelstein et al., 2000;
Yamaguchi et al., 2007).

Folate receptors α and β play a crucial role in mediating the internalization of folate and
its analogs by its ability to shuttle back and forth between the cell membrane and the
cytoplasmic compartment (Leamon and Low, 1991; Turek et al., 1993). The structural
difference between α and β isoforms involving Leu-49 in FR-β and Ala-49, Val-104, and
Glu-166 in FR-α has resulted in different affinities and stereospecificities for folate and
antifolates compounds in the range of 2 fold to 100 fold (Wang et al., 1992; Shen et al.,
1997; Maziarz et al., 1999).
Physiological significance FR-α:
FR-α is capable of transporting folate into the cell but other pathways in adult tissues are principally used for up-take of folate compounds eg., the reduced folate carrier (RFC). The significant of the restricted expression of FR-α at the apical surface of epithelial cells in normal tissues where it is not accessible to the circulation is not entirely clear. However, in placenta, FR-α is required for trans-placental folate uptake by the fetus (Antony et al., 1981; Henderson et al., 1995; Antony, 1996). In kidney, FR-α could be essential for urinary clearance of folate (da Costa et al., 2000). Moreover, the presence of sFBP in milk may have a role in intestinal absorption of folate in infants (Colman et al., 1981; Mason and Selhub, 1988). Gene knock-out studies have shown that FR-α is essential for nerve tube development when dietary folate is limited and that deletion of the FR-α gene results in developmental defects of the neural tube due to impaired folate transport (Piedrahita et al., 1999; da Costa et al., 2003).

Clinical significance FR-α:
Pre-clinical and clinical studies have shown that FR-α is a promising target for a wide variety of tumor therapeutic agents and as a tumor/serum marker, for several reasons. (I) FR has high binding affinity for folate, anti-folates and folate conjugated drugs; (II) FR can mediate the internalization of FR-α bound folate and folate conjugates by recycling between the cell surface and the intracellular compartments [reviewed in (Kamen and Smith, 2004)]; (III) The physical isolation of FR-α from the blood stream in normal tissues. In proliferating normal tissues, the expression of FR-α is restricted to the luminal surface of certain epithelial cells where it is isolated from the blood stream. In contrast, in
certain epithelial derived malignant tissues, FR-α is found to be highly expressed, and it is accessible to the circulation. FR-α has been found to be highly expressed in ovarian cancers derived from ovarian epithelial cells, endometrial adenocarcinoma, testicular choriocarcinoma, ependymomas and meningiomas of the brain, and in some colon, kidney and breast cancers (Weitman et al., 1992; Ross et al., 1994; Weitman et al., 1994; Wu et al., 1999). Moreover, it has been found that in ovarian cancer FR-α expression level is higher in high grade, undifferentiated or metastatic cancer cells than the localized counterpart low grade type (Toffoli et al., 1997; Toffoli et al., 1998; Toffoli and Tumolo, 1999).

Folate receptor targeted therapy has been reported to be highly specific and effective. However, the effectiveness of such therapies is highly dependent on the FR-α expression levels in the tumor cells. Unfortunately, the expression of the FR-α varies among tumors from different individuals. In addition, the expression of FR-α has been shown to be heterogeneous within the tumor tissues (Wu et al., 1999). Optimal expression of FR in malignant cells is necessary for effective tumor targeted therapies.

Many innovative therapeutic and diagnostic approaches have been successfully used for targeting FR in solid tumors as well as leukemic cells. Such experimental approaches includes (I) Folate conjugates of cytotoxics, radiopharmaceuticals and pro-drugs (Leamon and Low, 1994; Leamon and Low, 2001); (II) Folate–coated liposomes containing therapeutic drugs, genes or antisense oligonucleotides and (III) Folate-attached nanoparticle carriers for cytotoxic drugs (Leamon and Low, 1994; Lu et al., 1999; Andersson et al., 2000; Drummond et al., 2000; Sudimack and Lee, 2000; Ward,
2000; Atkinson et al., 2001; Lu and Low, 2002; Ratnam et al., 2003). In fact, none of these conjugations has been found to impede folate binding or drug bioavailability.

**FR-α Gene structure:**

The FR-α gene chromosomal localization has been determined to be 11q13.3-q13.5 (Ragoussis et al., 1992). In addition, the organization and the basal promoter of the FR-α gene has also been characterized (Elwood et al., 1986; Sadasivan and Rothenberg, 1988; Elwood, 1989; Sadasivan and Rothenberg, 1989; Sadasivan et al., 1992; Saikawa et al., 1995; Elwood et al., 1997). The FR-α gene is approximately 7 kb in length consisting of 7 exons and 6 introns (Figure-1).

FR-α gene transcription is regulated by two distinct TATA-less promoters, which interact with different transcription factors and designated as P1 and P4. The P4 promoter which is located upstream of exon-4, is primarily directed by a cluster of three functional G/C–rich regions that are non-canonical Sp1 binding sites, each of which contributes to P4 promoter activity (Saikawa et al., 1995). On the other hand, the P1 promoter, which is upstream of exon 1, has not yet been fully characterized, but it most likely does not contain a functional TATA or GC boxes. Instead, 3′-AATAATT-5′ NP3/4 binding element spanning +27nt to +33nt has been found required for P1 promoter optimal activity (Tomassetti et al., 2003). Due to alternative promoter usage as well as alternative splicing involving exons 1-4, multiple FR-α transcripts have been isolated and characterized.
Figure (1): Folate receptor-α gene organization

- P1, P4: promoters
- +1: transcription start site for P4
- 1-7: exons
- A-F: introns
All of them have an identical open reading frame and 3’ UTR sequence but differ in their 5’ UTR. The P4 promoter drives most of the FR-α mRNA transcription in malignant tissues. P1 promoter transcripts are translated less efficiently than the P4 transcript and are present in some normal tissues including kidney and testis (Elwood et al., 1997; Roberts et al., 1997).

**Regulation of FR-α Expression:**

The narrow and distinct tissue specificity of FR expression has been reported to be governed at both transcriptional and posttranscriptional levels (Elwood et al., 1997; Roberts et al., 1997; Zheng et al., 2003). Selective instability and degradation of FR-α transcripts in the nuclear compartment of FR-α negative but not FR-α positive cell lines has been related to the presence of a unique 60 base mRNA region within FR-α open reading frame (ORF) (Elwood et al., 1997; Roberts et al., 1997; Zheng et al., 2003).

In vitro, the extra-cellular concentrations of folate have been found to modulate FR-α protein expression (McHugh and Cheng, 1979; Kane et al., 1988). FR-α positive cell lines cultured in low folate medium, showed a rapid induction of FR-α expression. Addition of folic acid or reduced folate coenzymes reversed FR-α expression. Regulation of FR-α expression by folate at the translational level or by influencing mRNA stability has also been observed (Hsueh and Dolnick, 1993; Sun and Antony, 1996). Folate dependent up-regulation of FR-α expression in cervical carcinoma cells was found occur by interaction of a 46 kDa cytosolic protein hnRNP E1 with an 18 base cis elements in the 5’ UTR region of the FR-α transcript (Sun and Antony, 1996).

Functional analysis of the FR-α promoter did not show the presence of classical hormone response elements (HREs). The Endogenous FR-α gene and its promoter activity are
negatively regulated (repressed) by the estrogen receptor (ER) (Kelley et al., 2003) and this repression is increased by estrogen treatment and reversed (de-repressed) by anti-estrogenic compounds such as Tamoxifen (Kelley et al., 2003). These findings were consistent with an earlier observation of negative correlation between FR-α expression and ER expression in primary breast cancer cells (Rochman et al., 1985). Detailed mechanistic studies underscored the importance of the initiator and the flanking sequence within the P4 core promoter as a direct target for ER repression through formation of a complex with co-repressors SMRT and or NCOR (Hao et al., 2007). Conversely, in FR-α positive cell lines, unpublished studies from this laboratory have shown that androgens such as testosterone and R1881 have enhanced FR-α gene expression in a dose and time dependent manner. This regulation involves direct action of AR by interaction with C/EBPα bound to a CCAAT element.

**Nuclear receptors: structure and transcriptional mechanism:**

In mammals, at least fifty members of the nuclear receptor (NR) super-family transcription factors have been characterized and classified into three main classes according to their cellular localization and mode of action (Escriva et al., 2004). All of them play crucial roles in cell growth, development and in physiological and cellular homeostasis (Tsai and O'Malley, 1994; Enmark and Gustafsson, 2001; Ribot et al., 2001; Mohan and Heyman, 2003). Class-I NRs or steroid receptors includes estrogen (ER), progesterone (PR), testosterone /androgen (AR), glucocorticoid (GR) and mineralcorticoid (MR) hormone receptors. In the absence of their ligands, they are mainly localized in the cytoplasm associated with a multiple protein complex consisting
of heat shock proteins and immunophilins that inhibit their transcriptional activity. Upon ligand binding, the steroid receptors dissociate from the complex, homo or hetero dimerize with other NRs, translocate to the nucleus, bind to their DNA response elements (HREs) and modulate gene transcription (DeMarzo et al., 1991; Pratt and Toft, 1997; Pratt and Toft, 2003). The class-II NRs include thyroid hormone receptor (TR), retinoic acid receptor (RAR), retinoid X receptor (RXR), vitamin D3 receptor (VDR) and many others. These receptors reside mainly in the nucleus, constitutively bound to their DNA binding elements and associated with co-repressor complexes that repress their gene activation. In the presence of their ligands, the co-repressors dissociate from the receptor and are replaced by co-activators leading to trans-activation (Glass et al., 1997). Orphan nuclear receptors or class III is the largest group of NRs, most of which were identified before identifying their natural ligands. Like class II, class III NRs are constitutively bound to their DNA response elements and associated with co-repressors complex in a ligand-independent manner. Moreover, their mechanisms of gene regulation have also been found to be similar to class II. Unlike class I and class II nuclear receptors, orphan receptors can bind as monomers to their HRE half sites with a relatively high affinity, inducing gene transcription. Some examples of this group of nuclear receptors include peroxisome proliferator activator receptor (PPAR), liver X receptor (LXR) and farnesoid X receptor (FXR) that are involved in lipid and bile acid metabolism (Mohan and Heyman, 2003; Willy et al., 2004). In addition, the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have been found to function as xenobiotic sensors (Mohan and Heyman, 2003; Willy et al., 2004). Besides the normal physiology of the nuclear receptors, they have been implicated in many pathological conditions, such as
cancer, asthma, rheumatoid arthritis, cancer, diabetes, autoimmune and others (Shao and Brown, 2004; Cutolo et al., 2007; Finck and Kelly, 2007; Jacobsen et al., 2007; Jiang et al., 2007; Lonard et al., 2007; Martinez et al., 2007; Paola and Cuzzocrea, 2007; Park et al., 2007). Nuclear receptors are fundamentally transcription factors that interact with diverse groups of transcriptional co-regulators, produce complex patterns of gene expression in response to ligands or extracellular signals (McKenna et al., 1998; McKenna et al., 1999a; McKenna et al., 1999b; McKenna and O'Malley, 2000; McKenna and O'Malley, 2001; McKenna and O'Malley, 2002b; McKenna and O'Malley, 2002a). Nuclear receptors share a common structural organization (Figure-2). Typically, NRs are composed of four major domains. First, the N-terminal domain (A/B region), is the most variable region in size and sequence among nuclear receptors or within individual nuclear receptor isoforms and contains most of the phosphorylation sites. The A/B region also contains the activation function region 1 (AF-1) that is responsible for ligand independent trans-activation of NRs by cross talking with other intracellular signaling pathways or by serving as platform for certain co-regulators interaction in promoter and cell specific manner (Rochette-Egly et al., 1991; Rochette-Egly et al., 1997; Taneja et al., 1997). For example, in vitro, estrogen receptor N-terminal domain has been found phosphorylated following treatment of cells with growth factors through activation Ras-MAPK cascade. The phosphorylation of serine or threonine residue within A/B region enhances estrogen transcriptional activity (Kato et al., 1995; Patrone et al., 1996; Kato et al., 1998). Second, The DNA binding domain (DBD) or C region is the most conserved domain of the nuclear receptors and determine the ability of the receptor to bind its hormone response elements (HREs) in its target gene (Figure-2). DBD domain comprises two zinc fingers
that span approximately 60 to 70 amino acids and a COOH-terminal extension (CTE) that contains A and T boxes. Each finger contains four cysteines residues interact tetrahedrically with one zinc ion. Amino acids required for discrimination of the core DNA motifs are present within the first zinc finger and forming “P box” While the second zinc finger is involved in the receptor dimerization through formation what is called “D box” (Luisi et al., 1991; Fairall et al., 1993; Lee et al., 1993; Schwabe et al., 1993a; Schwabe et al., 1993b). Third, the hinge region or D domain, serve as a hinge between the LBD and DBD and facilitate free rotation of the DBD. Moreover, the D domain contains nuclear localization signal (NLS) required for receptor shuttling as well as amino acid residue for co-repressor interaction with nuclear receptor (Figure-2).

Fourth, the C-terminal or Ligand Binding Domain (LBD) (E/F region) is a multifunctional region that contains amino acids involved in ligand binding, NRs homo or hetero dimerization, interaction with heat-shock proteins and ligand dependent co-regulator recruitment through activation function-2 (AF-2) (Figure-2). The (AF-2) region within LBD consists of eight amino acids that serve as a surface for receptor/co-regulators interaction (Wurtz et al., 1996). The LBD from different NRs has been crystallized and found to be consisted of 12 α helical regions that are folded to form complex turns and layers creating a cavity for ligand binding (Rochel et al., 2000). The spatial orientation changes of the LBD have also been found to be dependent on ligand nature (i.e agonist or antagonist) (Beato et al., 1995; Tanenbaum et al., 1998). In addition, it has been found that the LBD also contains specific tyrosine phosphorylation sites involved in ligand-independent activation of the NRs and may be a target for several signaling pathways (White et al., 1997). In the absence of hormones, the steroid receptor
stays transcriptionally inactive and associated with a large complex consisting of heat shock proteins including hsp90, hsp70, p23 and immunophillins. Hormone binding initiates conformational changes in the receptor that leads to dissociation of the NRs from heat shock proteins, dimerization, and binding to its hormone responsive element (HRE) in its target genes. The HREs mainly present in the 5′ flanking region of the target genes close to the core promoter, and in some cases they are located in the enhancer region several kilo bases upstream of the transcription start site (Cleutjens et al., 1997). The consensus sequence (hexameric motif) 5′-AGAACA-3′ is preferentially recognized by steroid receptors whereas 5′-AGG/TTCA-3′ serve as the recognition motifs for the rest of the nuclear receptors and specific variations within the consensus sequence are tolerated (Beato et al., 1995). Even though some receptors can bind a single hexameric motif as monomers, most of the nuclear receptors bind as homo or hetero-dimers to HREs composed normally of two-core hexameric motifs separated by three nucleotides spacer.

Although most of the attention has been focused on binding of the nuclear receptor to positive HREs as a mechanism of transcriptional activation, nuclear receptors such as thyroid and glucocorticoid receptors, can also bind negative HREs (nHREs) as monomer or dimers and mediate negative regulation by the ligand indicating the important of these binding sites in feedback mechanism. nHREs are generally located close to the transcription start sites or downstream of the TATA box or even within the 3′ untranslated regions (Bigler and Eisenman, 1995; Belandia et al., 1998).
Figure (2): Nuclear receptor protein structure
The DNA bound receptor interact directly or indirectly via co-regulators with the transcriptional machinery and mediate chromatin remodeling leading to gene activation or repression (Groudine et al., 1983; Weintraub, 1983; Smith et al., 1997; Lemon and Freedman, 1999; McKenna et al., 1999a; Nie et al., 2000; Robyr et al., 2000; Deroo and Archer, 2001b; Deroo and Archer, 2001a; Grange et al., 2001; Urnov and Wolffe, 2001c; Urnov and Wolffe, 2001a; Heinlein and Chang, 2002; Hsia and Shi, 2002; Hsiao et al., 2002; Smith et al., 2002; Villagra et al., 2002; Hsia et al., 2003). Two groups of chromatin remodeling have been identified and required for NRs gene regulation. The first group, includes histone acetylases, histone deacetylases, methylases and kinases (Spencer et al., 1997; Wang et al., 1997; Mizzen and Allis, 1998; Mizzen et al., 1999; Sterner and Berger, 2000; Sasaki and Yoshida, 2006; Sadaie and Nakayama, 2007), and they covalently modify the nucleoprotein structure. The second group is ATP-dependent chromatin remodeling complexes that require ATP for chromatin disruption and sliding of histone octamer. This group includes three subgroups, SWI/SNF, ISWI and Mi-2 that are classified according to the identity of the core ATPase subunits that form the major component of the complex (Phelan et al., 1999; Guyon et al., 2001; Becker and Horz, 2002; Narlikar et al., 2002). DNA binding is the primary mechanism of gene regulation by NRs. However, NRs were also found to be associated with their target genes by interaction with other DNA-bound transcription factors such as, AP-1, Sp1, Sp3, NF-κB, GATA, STAT, CREB, KLFs and others rather than their hormone response elements (HRE) in a DNA-independent binding manner. The protein-protein interaction of the NRs with other DNA bound co-regulators can increase or decrease the transcriptional activity of the target genes is a receptor-specific and cell or promoter context dependent manner.
NRs also control the biological processes by interacting with other cytoplasmic signaling pathways rather than DNA binding (non-genomic pathway). The non-genomic pathway, which is most notably seen in class I NRs, has been recently discovered as alternative pathway that regulates NRs activity in response to extra cellular signals (Boonyaratanakornkit et al., 2001). Kinases such as Src, MAPK, PI3K/AKT pathways have been reported to play a central role in AR, ER, GR and PR gene regulation in normal and cancerous cells lines (Haynes et al., 2000; Russell et al., 2000; Simoncini et al., 2000; Haynes et al., 2003; Kousteni et al., 2003; Manolagas et al., 2004; Vertino et al., 2005).

**Glucocorticoid receptor (GR):**

In mammals, GR is one of the most vital steroid receptors that regulates essential biological process including growth, metabolism, behavior and apoptosis (Barnes, 1998; Sapolsky et al., 2000). GR is mainly localized in the cytoplasm. hGR gene is located in chromosome 5 q31-32 and composed of nine exons that generate its open reading frame (ORF). Two alternative spliced transcripts for hGR have been characterized, hGRα and hGRβ and both of them are product of alternative splicing of the ninth exon. Both GR isoforms are identical up to amino acid 727. hGRβ is shorter than hGRα and generated by replacing the 50 carboxy terminal amino acids of hGRα by 15 non homologous amino acids (Figure-3). Moreover, the presence of several translation initiation sites within exon number 2 (Pujols et al., 2002; Schaaf and Cidlowski, 2002; Yudt and Cidlowski, 2002) proposed the existence of additional GR isoforms ending up with eight isoforms for each
of hGRα and hGRβ (Figur-3). In addition, the expression of each individual isoform is highly regulated in tissue and cell specific manner. Interestingly, recombinant cells that express each of hGRα isoforms, treated with glucocorticoid and subjected to cDNA microarray analysis reveal that, among the 2000 hGR target genes, only 189 genes were regulated (activated or repressed) by all hGRα isoforms. Thus, depending on the relative expression of GR isoforms in different tissues, the GR may differentially modulate gene expression and pointed the importance of alternative translation initiation as a mechanism of gene regulation (Lu and Cidlowski, 2004; Lu and Cidlowski, 2005; Lu and Cidlowski, 2006).

The expression of hGRα is ubiquitous and is functional when activated by glucocorticoids (Hollenberg et al., 1985). In addition, the expression levels of hGRα is also controlled by several physiological and pathological conditions (Hollenberg et al., 1985). hGRs promoter regions contain neither TATA box nor CCAAT binding site (Zong et al., 1990; Encio and Detera-Wadleigh, 1991). Instead, all of them contain CpG islands and binding site for transcription factors AP-1, AP-2, Yin Yang 1 (YY1), NF-κB, Sp1 and CREB (Nobukuni et al., 1995; Breslin et al., 2001).

The primary action of GR is gene activation. Conversely, the major anti-inflammatory effect of glucocorticoids is through repression of the inflammatory and immune genes. Functional analysis of those genes showed that few of them contain either positive or negative GRE, suggests that the inhibitory effect is mainly due to the interaction of the activated GR with other transcription factors by protein-protein interaction rather than direct DNA binding. such factors include AP-1 and NF-κB a strong mediators of inflammatory and immune genes (Karin, 1998). Unlike hGRα, hGRβ isoform is
constitutively localized in the nucleus, unable to bind glucocorticoids and transcriptionally inactive (Oakley et al., 1997). In addition, hGRβ expression is tissue selective and its level of expression is much lower than hGRα (Oakley et al., 1997; Lu and Cidlowski, 2004).

In vitro studies showed that over-expression of hGRβ act as dominant negative inhibitor on hGRα mediated gene transactivation suggesting the role of hGRβ in regulation of tissue sensitivity to glucocorticoids possibly through the formation of GRα/GRβ heterodimer. Interestingly, the tissue specific expression and the inhibitory function of hGRβ have been identified as contributing factors in several pathological conditions such as rheumatoid arthritis, allergic rhinitis, ulcerative colitis, systemic lupus erythematosus and glucocorticoid- insensitive asthma (Chrousos, 1995; Webster and Cidlowski, 1999; Sousa et al., 2000). The changes in hGRβ expression levels relative to hGRα leads to steroid resistance (Krett et al., 1995; Beger et al., 2003).

**Physiology of Glucocorticoid receptor:**

GRs are widely conserved throughout different species (Encio and Detera-Wadleigh, 1991) and they have profound effect on tissue development and differentiation and induced by Glucocorticoids (stress induced hormones). The synthesis of glucocortiocoids in the adrenal cortex is typically controlled by the hypothalamic-pituitary- adrenal (HPA) axis (Sapolsky et al., 2000). Stress factors such as pathogen, trauma and toxins induce a signal from the hypothalamus to the pituitary gland to release adrenocortocotrophic hormone (ACTH) into the blood stream.
Figure (3): Glucocorticoid receptor gene structure and protein splicing (Lu and Cidlowski, 2006)
ACTH binds with specific receptors in the adrenal gland leading to production and releasing of glucocorticoids. Due to glucocorticoids high lipid solubility, virtually they can reach each single cell within the human body including the brain through passive diffusion. Inside the cell, glucocorticoids activate GR to mediate the activity and the direction of a myriad of cell-tissue and organ-specific functions including maintaining of vascular tone, immunity and inflammatory response, intermediary metabolism such as protein; lipid and carbohydrate metabolism, tissue development and programmed cell death as well as the effects on the central nervous system normal physiology (Barnes, 1998; Chrousos, 2004; Smoak and Cidlowski, 2004; Chrousos and Kino, 2005; Rhen and Cidlowski, 2005).

GR knock out mice model showed sever developmental abnormalities and several physiological functions defects and mice died within few hours after birth (Cole et al., 1993; Cole et al., 1995). On the other hand, the knock-in mice model characterized by GR DNA-binding and dimerization defect showed that the mice were able to survive despite the impairment of several physiological functions by glucocorticoids. The later observations support the fact that GR can also interact with other proteins or nuclear receptors as well as other signaling pathways to activate or repress gene transcription as an alternative pathway of gene regulation (Reichardt et al., 1998).

**Mechanism of gene regulation by Glucocorticoid receptor:**

In the absence of ligand, the cytosolic GR sequesters a multi-protein complex consisting of several proteins including hsp90, hsp70, FKBPs, Cyp-40, p23, immunophilin and few others that inhibit its transcriptional activity (Pratt and Toft, 1997; Pratt and Toft, 2003;
The interaction of GR with two hsp90 is essential for keeping the ligand-binding pocket in its optimal and high affinity configuration. Steroids such as dexamethasone can freely diffuse across the plasma membrane and bind to GR in the cytoplasm. However, despite the fact that GR is mainly located in the cytoplasm, it is believed now that there is a rapid active cycling of GR between the cytoplasm and the nucleus (Hache et al., 1999; Kumar et al., 2004; Kumar et al., 2006). Ligand binding induces conformational changes of GR leading to receptor dissociation from its chaperone proteins and localization to the nucleus by the interaction of the nuclear localization sequence-1 (NLS1) which is located within the hinge region of the receptor with importing proteins termed as importins. Importin α, 7 and 8 are playing a major role in GR nuclear localization. On the other hand, the nuclear localization sequence-2 (NLS2), which resides within the ligand-binding domain, is less effective in GR localization to the nucleus. (Picard and Yamamoto, 1987; Savory et al., 1999; Chook and Blobel, 2001; Lee et al., 2006). Within the nucleus, a single GR can form a dimer with another GR and binds directly to the consensus DNA binding sites on its target genes, known as glucocorticoid response elements (GRE) (GGTACA\textasciitilde\textasciitilde\textasciitilde\textasciitilde\textasciitilde\textasciitilde\textasciitilde\textasciitilde\textasciitilde) (Barnes, 1998). The association of GR homodimer with its GREs on its target genes mediates GR interaction with a complex of transcription factors including SRC-1, CBP, TIF-2, p300/CBP, GRIP-1, CREB-binding protein and others to enhance gene activation (Karin, 1998; Ito et al., 2000; Kagoshima et al., 2001; Li and O'Malley, 2003; Li et al., 2003). Co-activator such as CBP and CREB-binding have intrinsic histone acetyltransferase activity and such interaction resulting in acetylation of lysine 5 and 16 residues of H4 resulting in subsequent events that lead to the releasing of the tightly
wound DNA and allowing recruitment of further transcription factors and auxiliary proteins to enhance gene transcription (Urnov and Wolffe, 2001b; Urnov, 2003).

The primary action of GR is gene activation. In contrast, GR/GRE binding can also decrease the expression of certain genes such as prolactin and osteocalcin in a ligand dependent manner. In A549 lung epithelial cell lines treated with cyclohexamide to prevent the secondary effect of six hours treatment with dexamethasone showed that, at least seventy-three of GR direct genes are down regulated, some of them have been found involved in cell proliferation, inflammation, apoptosis, and surfactant synthesis (Wang et al., 2004a). In addition, the direction and the magnitude of the transcriptional response to glucocorticoids is highly dependent on the number of GREs, the position of the GREs relative to the transcriptional start site as well as the type of the GR ligand (Wang et al., 2004a).

Interestingly, GR can bind DNA as a hetero-dimer with other transcription factors such as STAT and ETS family distinct from GR association with STAT or ETS in DNA independent-manner (Stocklin et al., 1996; Biola et al., 2000; Mullick et al., 2001). The GR heterodimer can recruit either co-activator (e.g., GRIP-1) or co-repressors (e.g., RIP140 or HDAC) and either activate or repress gene transcription in cell specific manner (Stevens et al., 2003; Barnes et al., 2004; Garside et al., 2004).

The ligand-bound GR can also repress the gene activation by interaction with negative GRE (nGRE). Glucocorticoids are widely used as anti-inflammatory agents through acting as inflammatory and immune genes suppressors. Functional promoter analysis on a large number of inflammatory and immune genes reveal that few of them have either positive or negative GREs, discount the possibility of GR/GRE binding as a fundamental
mechanism of inflammatory gene suppression. Conversely, down-regulation of these genes has been found mainly mediated by a direct interaction of GR with potent inflammatory mediators mainly NF-κB and AP-1 in DNA independent manner (Karin, 1998). The fact that why not all NF-κB target genes are repressed by active GR within the same cell type still unclear and need to be investigated and clarified. In addition, the precise mechanism by which GR inhibit NF-κB or AP-1 target genes is not fully understood and several mechanisms have been proposed to explain this repression action by one or more of the following: (I) Recruitment of certain co-repressor with HDAC activity such as NCoR (Nissen and Yamamoto, 2000; Rosenfeld and Glass, 2001; Luecke and Yamamoto, 2005); (II) Hypo-phosphorylation of RNA polymerase II enzyme (Nissen and Yamamoto, 2000; Luecke and Yamamoto, 2005), (III) Decrease immune and inflammatory genes mRNA stability by either increasing the levels of cell ribonucleases and the mRNA destabilizing proteins or by inducing MAP-1 phosphatase activity (Meyer et al., 2004; Fan et al., 2005), (IV) Direct repression of co-activator complex (Nissen and Yamamoto, 2000; Luecke and Yamamoto, 2005; Pascual et al., 2005), (V) Direct increase in the expression of NF-κB inhibitor IκB α or AP-1 inhibitor (GLIZ) in cell specific manner (Heck et al., 1997), (VI) Binding as a monomer to AP-1 and NF-κB (Heck et al., 1994) and finally, (VII) repression the action of the non genomic pathways of some extra-cellular signaling pathways such as ERK, Akt, PI3K and JNK (Gonzalez et al., 2000; Bruna et al., 2003).

A number of intracellular signaling pathways can activate GR in a non-genomic pathway. G-protein-coupled receptor and some other kinases pathways have been reported to be involved in GR non-genomic pathway of gene regulation. The presence of kinases and
phosphatases associated with the inactive hsp90-GR complex support the fact that these enzyme are involved in a rapid induction of tyrosine kinase that has been observed in certain cells types by glucocorticoids (Powell et al., 1999; Croxtall et al., 2000; Croxtall et al., 2002; Norman et al., 2004).

**Post-translational modification of GR and their roles in GR action**

Post-translational modification including, GR phosphorylation (Hoeck et al., 1989; Webster et al., 1997; Rogatsky et al., 1998; Wang et al., 2002b), ubiquitination (Rogers et al., 1986; Wallace and Cidlowski, 2001), SUMOylation (Jin et al., 2001; Tian et al., 2002), acetylation and methylation of either GR or one of its chaperone proteins (Jin et al., 2001; Aoyagi and Archer, 2005; Lee et al., 2005; Murphy et al., 2005; Aoyagi and Archer, 2007) can negatively or positively regulate GR action.

Phosphorylation of GR: Five phosphorylation sites have been identified and mainly located in the N-terminal domain of GR, serine 113, 141, 203, 211, and serine 226 (Hoeck et al., 1989; Hoeck and Groner, 1990; Ismaili and Garabedian, 2004). All of them can be phosphorylated in ligand dependent and independent manner. Interestingly, phosphorylation of each residue has distinct effect on GR sub-cellular localization, receptor stability as well as gene activation (Webster et al., 1997; Wang et al., 2002b; Zhou and Cidlowski, 2005). Moreover, kinases such as Mitogen protein kinase (MAPK), cyclin-dependent kinase CDK, glycogen synthase kinase-3 (GSK-3) and c-Jun N-terminal kinase (JNK) have distinct specificities for potential phosphorylation residues and could be reversed by phosphatases such as PP1, PP2a and PP5 (Krstic et al., 1997; Bodwell et al., 1998).
GR Ubiquitination: the ubiquitin-proteasomal degradational pathway can also regulate GR signaling pathway. The Covalent attachment of ubiquitin to mouse GR and it is role in GR destabilization and degradation by proteasome has been reported (Wallace and Cidlowski, 2001). Interestingly, the consensus binding site for ubiquitin PEST (Pro, Glu, Ser, an Thr) is located at Ser 412, the same target site for GR phosphorylation (Rogers et al., 1986; Rechsteiner and Rogers, 1996; Kinyamu et al., 2005) suggesting that phosphorylation of GR is prerequisite for its proteasomal-degradation. Further investigations showed that, mutation of the entire phosphorylation target sites in GR increased the receptor half-life and abolished GR down regulation. Conversely, treating the cells with GR antagonist RU486 has no effect on the phosphorylation status of GR but the protein down-regulation is maintained. These conflicting findings suggest that, phosphorylation of GR is important but other pathways may exist to target GR for degradation and highlight the need for further investigation on GR degradation mediated by ubiquitin-proteasomal pathway.

SUMOylation, addition of small ubiquitin related modifier (SUMO-1) a 98 amino acid protein to GR can also regulate its function in both ligand dependent and independent manner (Jin et al., 2001; Le Drean et al., 2002). Three consensus-binding sites for SUMO-1 attachment on GR were identified. K277 and K293 are located within the N-terminal domain while the third one K703 is located within the ligand-binding domain of GR. Unlike ubiquitinilation, sumoylation regulate protein stability, receptor localization and the activity of transcriptional regulators rather than directing the receptor to proteasomal degradation. The mechanism by which sumoylation regulates gene
transcription activity of GR have been shown to be dependent on the cell type, promoter context and the state of the environment such as stress (Le Drean et al., 2002).

Despite the putative acetylation motif (KXKK/RXKK) where X any amino acid within GR has been identified (amino acids 492-495), there is no evidence that GR is directly regulated by acetylation (Kovacs et al., 2005; Murphy et al., 2005). Conversely, the GR acetylation is an indirect effect of the hsp90 interaction with histone deacetylase 6 (HDAC6). Knocking down HDAC6 or treating the cells with HDAC inhibitor such as trichostatin (TSA) lead to hyper-acetylation of the hsp90, disrupt hsp90 interaction with GR and impairment of ligand binding, nuclear localization and gene trans-activation (Kovacs et al., 2005; Murphy et al., 2005).

Methylation of GR interacting proteins but not GR play an important role in GR transcriptional activity (Wang et al., 2004b). It has been found that, methylation of p300 leads to disrupt the interaction of p300 /GRIP1 a GR co-activator complex and modulate GR signaling pathway (Wang et al., 2004b; Lee et al., 2005).

**Progesterone Receptor (PR):**

Progesterone receptor is a member of class I nuclear receptors that regulates distinct biological processes in a broad range of tissues and plays an important role in development and reproduction in female (Conneely and Lydon, 2000; Conneely et al., 2000; Graham and Clarke, 2002). Two isoforms of progesterone receptors have been isolated and well characterized PR-A (94kD) and PR-B (120kD) (Figure-4). The expression of PR isoforms is highly regulated in a tissue specific manner (Graham et al., 1996; Giangrande and McDonnell, 1999). PR isoforms are generated from a single gene
in both rodent and human (Kastner et al., 1990; Kraus et al., 1993). Structurally, PR-A is a truncated form of PR-B, lacking 164 N-terminal amino acids resulted from the presence of two alternative promoters and distinct translation initiation start codons (AUG) (Kastner et al., 1990; Li and O'Malley, 2003) (Figure-4). PR-A and PR-B have multiple activation function domains (AF) that enhance PR transcriptional activity and an active inhibitory domain within PR-A (a.a 165-305) that represses its transcriptional activity through direct association with certain co-repressors complex (Figure-4) (McKenna and O'Malley, 2001; McKenna and O'Malley, 2002b). In addition, both isoforms have receptor specific effects on regulation of progesterone responsive genes.

A 60-kDa N-terminally truncated progesterone isoform PR-C was identified in certain tissues and its expression is primarily restricted to the cytosolic fraction (Wei et al., 1990; Wei et al., 1996; Wei et al., 1997). PR-C contains sequences for hormone binding, dimerization as well as nuclear localization, but lacks the first zinc finger of the DNA binding domain (Figure 4). The exact function of PR-C still unknown but few reports indicated that PR-C function is highly cell and tissue specific. PR-C could interact with PRB to enhance PR-B transcriptional activity in T47D cell line (Wei et al., 1990; Wei et al., 1996; Wei et al., 1997). In contrast, PR-C expression in uterus has inhibitory effect on PR-B transactivation leading to a loss of uterine quiescence and the onset of labor (Condon et al., 2006). The expression of PRC and it is function in mammary gland normal physiology and in breast cancer development still unclear and need to be investigated.
Figure (4): Progesterone receptors isoforms structure (Li and O'Malley, 2003)
Physiology of progesterone receptor:

The primary target of progesterone biological action is the female reproductive tract. In normal reproductive tissue, the expression of hPR in mammary glands is induced by estrogen and reduced by progestins (Janne et al., 1975; Thi et al., 1975; Alexander et al., 1989; Clarke, 1990; Savouret et al., 1994). Moreover, progesterone can either stimulate or inhibit proliferation and induce differentiation depending on the cell or tissue context. The PR knock-out mice model suggest that PR-A and PR-B function as distinct transcription factors. PR-A is the key for ovarian and uterine development while PR-B is necessary for mammary gland development. Mice lacking PR-A have normal mammary gland and thymus development but severe defect in uterine development and ovarian abnormalities (Mulac-Jericevic et al., 2000; Mulac-Jericevic et al., 2003). On the other hand, PR-B knock out mice model showed that the ovaries and the uterus in these mice were able to response to progesterone action through PR-A, but the mice suffer from a major defect in mammary gland morphogenesis (Conneely et al., 2002). As a result, PR-A is necessary to elicit progesterone dependent reproduction response while PR-B is required for progesterone proliferative action in mammary glands. In addition, PR-B over-expression leads to premature ductal growth arrest and insufficient lobuloalveolar differentiation (Shyamala et al., 2002).

The ratio of PR-A to PR-B expression is also important and critical in controlling the physiological direction of PR response to its ligands. Transgenic mice that have 3:1 (PR-A/PR-B) develop all features associated with neoplasia including epithelial hyperplasia;
excessive ductal branching and errors in basement membrane organization (Mulac-Jericevic et al., 2000; Mulac-Jericevic et al., 2003). Mice models lacking both PR isoforms showed sever pleiotropic female reproductive abnormalities, including impaired neuroendocrine and ovary function, uterine hyperplasia and inflammation, sever defects of mammary glands as well as impaired thymic function and sexual behavior (Lydon et al., 1995; Chappell et al., 1997).

**Mechanism of gene regulation by progesterone receptor:**

Like any other steroid receptor. In the absence of ligand the newly synthesized PR mainly resides in the cytoplasm as an inactive protein by association with immunophilin and chaperone molecules including hsp90, hsp70, hsp40. Upon ligand binding chaperone molecules dissociate from the complex, activate receptor nuclear localization and forming either homo or heterodimers with three possible classes A:A, A:B, B:B (Conneely and Lydon, 2000). PR dimerization allows the receptor to bind to its specific progesterone response elements (PRE) within its promoter target genes and mediates the recruitment of specific co-regulators and other general transcription factors to start gene activation (Moore et al., 1997).

It is well known that whenever PR-A is transcriptionally inactive it acts as an inhibitor of PR-B gene activation under the effect of both progesterone agonists and antagonists. The inhibitory effect is related to the presence of an active inhibitory domain (ID) within the N-terminal region of PR-A. in addition, Human PR-A but not PR-B is able to inhibit glucocorticoid, androgen, and mineralcorticoid receptor mediated gene transcription (Vegeto et al., 1993; McDonnell et al., 1994; Giangrande and McDonnell, 1999;
Conneely and Lydon, 2000; Giangrande et al., 2000). In general, on the classical PRE driven genes PR-B is much stronger activator than PR-A. The stronger activation property has been aimed at the presence of a third activation (AF-3) domain in the first extra 164 amino acids of the PR-B N-terminal domain that counteract the inhibitory domain. However, PR-A could be a strong activator, but this action is cell specific and highly dependent on target gene context (Giangrande and McDonnell, 1999; Giangrande et al., 2000; Rosenfeld and Glass, 2001). In fact, analysis the mechanism by which the progesterone receptor agonist or antagonist actions occur on each PR isoform target genes has provided insight into how the cell recognizes and responds to PR ligands.

PR can regulate target promoters including the natural promoters for the p21, p27, glycodelin, and thymidine kinase (TK) genes in the absence of a functional classical response element [progesterone response element (PRE)/glucocorticoid response element (GRE)] through G/C-rich (Sp1 binding) elements (Thomson et al., 1990; Tung et al., 1993; Owen et al., 1998; Gao et al., 2001; Gizard et al., 2005). Some observations appeared to suggest that this regulation occurs by direct association of PR with DNA-bound Sp1 (Owen et al., 1998; Gizard et al., 2005). Additional support for this suggestion is derived from the well-established role of Sp proteins in mediating promoter activation by the estrogen receptor (ER) by a nonclassical mechanism; it has been demonstrated that ER is recruited by Sp1, Sp3, or Sp4 in the absence of classical estrogen response elements to exert its genotrophic effects in a variety of gene promoters in the physiologic context (Safe and Kim, 2004; Stoner et al., 2004; Higgins et al., 2006). Moreover, PR has been found to interact with NF-κβ, STAT5 and C/EBPβ transcription factors and regulates gene expression (Bamberger et al., 1996; Kalkhoven et al., 1996; Edwards et al., 2000;
Christian et al., 2002). Interaction of PR with NF-κB is believed to be responsible for immunosuppressive action of progesterone during pregnancy. While, interaction of PR with AP-1, C/EBPβ and STAT5 has been found related to the proliferative and differentiation actions of progesterone in mammary gland and uterus. In addition, trans-activation by PR independent of a PRE is of particular interest because this may underlie the agonistic activity of RU486 on promoter activation by PR-B and the importance of this mechanism in profiling RU486 biological activity (Tung et al., 1993).

Besides the genomic gene action of PR, In vitro studies using the yeast two hybrid and GST pull down assays showed that PR-A and PR-B contain Src/SH3 interaction motif within their N-terminal domains (Boonyaratanakornkit et al., 2001). The presence of this interaction motif mediates a non genomic action of PR and stimulates Src/Ras-Raf/MEK/MAPK downstream signaling pathway which regulates several cellular processes including cell proliferation and differentiation (Boonyaratanakornkit et al., 2001).

**Post-translational modification of PR and their roles in PR action:**

The transcriptional activity of PR is mainly governed by ligand binding. Recently more studies showed that post-translational modification also play additional part in PR activity. The presence of extra six phosphorylation sites in the N-terminal domain of PR-B compared to the truncated form PR-A suggesting a differential regulation of the PR activity isoforms by kinases such as MAPK, CDK2 and casein kinase II (Lange et al., 2000; Qiu and Lange, 2003; Lange, 2004). Phosphorylation of PR can increase or decrease its transcriptional activity and this action is highly dependent on the
phosphorylation site. Phosphorylation of S294 was found to be important for receptor cross talk with other growth factors, subcellular localization as well as receptor degradation (Lange, 2004). In contrast, S400 phosphorylation by CDK2 lead to receptor stabilization (Shen et al., 2001). Despite the existence of a conserved KXKK at position 638-641 amino acids within the hinge region, progesterone receptor regulation by acetylation has not yet been confirmed. Parallel to this, in pull-down assay TAF-Iβ and p32 which are involved in de-acetylation process, were found to be associated with PR (Loven et al., 2003; Loven et al., 2004). The PR is also a target for ubiquitin/proteasomal degradation pathway in ligand dependent manner (Lange et al., 2000; Qiu and Lange, 2003). Ubiquitylation of the PR was also found to be dependent on phosphorylation of S298. Point mutation of S298 showed that the receptor did not degrade by the proteasomal pathway following progesterone treatment (Imhof and McDonnell, 1996; Verma et al., 2004). PR SUMOylation is another post-translational modification that takes place on PR and affects its activity. The site of PR sumoylation has been found located in the N-terminal moiety at position K388. Mutation of this site resulted in blocking of auto-inhibition in both PR isoforms (Abdel-Hafiz et al., 2002; Chauchereau et al., 2003; Takimoto et al., 2003; Jones et al., 2006).

**Progesterone Receptor Agonists, antagonists and selective modulators (SPRMs):**

Three groups of ligands can modulate progesterone receptor activity and have wide therapeutic applications in female health care (Cadepond et al., 1997; Mahajan and London, 1997; Spitz and Chwalisz, 2000; Schindler et al., 2003; Spitz, 2006). (I) Pure
agonist that binds to PR and enhances its transcriptional activity such as progesterone, R5020, and cyproterone, (II) Pure antagonist that fully abrogate PR function like onapristone (ZK98299) and (III) Compounds that bind to PR with different affinity and have mixed agonist and antagonist activity on PR action including mifepristone (RU486) and J-867. The third group is well known as selective progesterone receptor modulators (SPRMs) and it is action is cell and promoter specific. PR action is predominantly mediated by ligand binding leading to sequential events that direct its transcriptional action. Interestingly, early observations from in vitro studies showed that in PR positive cell line T47D cultured in stripped serum condition, both PR agonist (progesterone) and PR antagonist (RU486) were able to induce cell proliferation suggesting that RU486 could have agonist activity (Bowden et al., 1989; Hissom et al., 1989; Moore et al., 1991; Sartorius et al., 1993). Agonists and antagonists of PR produce profound physiological effects, principally by altering global gene expression profiles in the target tissues. The altered gene expression profiles presumably not only reflect changes in the activities of the direct target genes of PR but also a host of downstream genes whose expression is altered as a consequence of the changes in expression of the direct PR target genes. The physiologic relevance of this indirect gene regulation by PR is clearly exemplified in both phases of the reported (Groshong et al., 1997) biphasic response of breast cancer cells to progesterone in which there is first a stimulation of the cell cycle accompanied by an induction of TK1 followed by a delayed induction of p21 and p27 associated with G1 phase arrest.

SPRMs do not affect homodimerization of PR or its binding to its cognate response element in the target gene (DeMarzo et al., 1991; Skafar, 1993) but produce
conformational changes in the AF-2 region of PR to block the association of co-activators and promote co-repressor recruitment [reviewed in (Edwards et al., 2000; Glass and Rosenfeld, 2000; Leo and Chen, 2000)]. The A and B subtypes of PR respond differently to RU486, which binds to PR-B to function as a partial agonist under certain conditions; in contrast, RU486 can only act as an antagonist of PR-A (Meyer et al., 1990; Tung et al., 1993). The molecular mechanism of RU486 partial agonistic activity through PR-B is not fully understood. However, it has been proposed that the agonistic activity of RU486 is highly dependent on the presence of an intact AF-1 region in PR-B receptor (Meyer et al., 1990) as well as the N-terminal domain (Wardell et al., 2002) that may required for recruitment of unknown co-activators. In addition, treating cells with the protein kinase A (PKA) activator 8-bromo-cAMP converted RU486 from an antagonist into full agonist suggesting that either the receptor or its coregulators are phosphorylated leading to a loss of PR association with co-repressors NCoR and SMRT (Beck et al., 1993; Sartorius et al., 1993; Kahmann et al., 1998; Wagner et al., 1998). The ratio of co-activator to co-repressor in the cell was also found to influence the partial-agonistic activity of RU486 (Liu et al., 2002).

**Sp/KLF family and there role in regulating gene transcription:**

Sp/Krupple-like factor proteins are considered the largest transcription factor family that regulates mammalian and viral gene transcription. Specificity protein 1 (Sp1) was originally identified as a transcription factor that binds to the GC-box in the simian virus (SV40) and thymidine kinase (TK) promoters and activate their gene transcription
Sp1 protein was the first Sp/KLF family member to be cloned and characterized from HeLa cells by Kadogana and coworkers (Kadonaga et al., 1987). By 2005, twenty-five members of the Sp/KLF family had been identified in the mammalian genome. These were designated as Sp1-Sp9, KLF1-KLF16 and categorized by their similar modular structures (Figure-6) (Lania et al., 1997; Philipsen and Suske, 1999; Suske, 1999; Bouwman and Philipsen, 2002; Safe and Kim, 2004). Based on their crystal structures, all Sp/KLF family members bind to GC (GGGGCGGGG) and GT (GGTGTGGGG) rich promoter regions through a single unit consisting of three zinc finger motifs. Without exception, the three-zinc finger unit consists of 81 amino acids and is located at the C-terminal domain of the Sp/KLF protein (Kadonaga et al., 1988; Bucher, 1990; Pavletich and Pabo, 1991; Narayan et al., 1997). In addition, the Sp1-Sp4 subgroup contains several distinct overlapping regions that all contain glutamine (Q-rich) N-terminal activation domains (AD), serine/threonine rich central domain and C-terminal DNA binding domain (DBD) with three-zinc finger motif as mentioned before required for sequence-specific DNA binding. Additionally, Sp3 contains an inhibitory domain (ID) that is involved in its gene suppression activity in cell and promoter specific manner (De Luca et al., 1996; Majello et al., 1997). The remaining members of the Sp/KLF family are generally lower in molecular weight compared to Sp1-Sp4 subgroup and lack the Q-rich activation domain. Sp1, Sp3, and Sp4 bind to the classical Sp1-binding elements (GC or GT boxes) with identical affinity (Hagen et al., 1992; Hagen et al., 1995; Majello et al., 1997). On the other hand, Sp2 protein binds only to GT but not to GC rich elements in the T-cell receptor gene (Kingsley and Winoto, 1992). The role of Sp proteins in gene regulation has been extensively investigated. The
function of each individual Sp/KLF protein is highly dependent on tissue specificity, promoter, and cellular context. The specific physiological functions of Sp proteins have not yet been determined, but the knockout phenotypes of some Sp/KLF genes have shown critical and distinct cellular functions. Sp1 knockout mouse models demonstrate embryological growth retardation and die within the first 11 days of gestation. Sp4 knockout mice die shortly after birth due to similar developmental deficits (Supp et al., 1996; Marin et al., 1997; Gollner et al., 2001). The Sp7 knockout phenotype exhibited blocked bone formation. The Sp3 knockout phenotype demonstrated impaired ossification, growth retardation, and late tooth formation that resulted in death after birth (Bouwman et al., 2000; Gollner et al., 2001; Nakashima et al., 2002; Van Loo et al., 2003). Conversely, the Sp5 knockout mouse model did not show abnormal phenotype (Harrison et al., 2000). Sp/KLF family members differ in their ability to activate or repress gene transcription. It has also been shown that in certain cell types, disease states, and physiological conditions, the co-expressed Sp proteins compete for binding sites leading to activation or repression of gene transcription (Apt et al., 1996; Discher et al., 1998; Hata et al., 1998).
Figure (6): the shared structure of Sp/KLF family (Suske et al., 2005)
Sp/KLF family transcriptional activity was also found to be regulated by phosphorylation, acetylation and glycosylation (Jackson et al., 1990; Roos et al., 1997; Black et al., 1999; Braun et al., 2001; Ammanamanchi et al., 2003; Huang et al., 2005). The phosphorylation of Sp1 by various kinases has been reported to be important for activation of some Sp1 dependent genes. Several studies reveal that, phosphorylation of Sp1 by phosphoinositol kinase (PI3-k), mitogen-activated protein kinase (MAPK), protein kinase A (PKA) or protein kinase C (PKC) leads to an increase in Sp1 binding affinity to GC-rich regions and enhances its transcriptional activity on several genes such as VEGF and p27 (Fojas de Borja et al., 2001; Haidweger et al., 2001; Onishi et al., 2001; Rafty and Khachigian, 2001; Milanini-Mongiat et al., 2002; Lee et al., 2003; Banchio et al., 2004; Pore et al., 2004). Conversely, during differentiation and regeneration of liver cells, phosphorylation of Sp1 by Casein kinase II (CKII) decreases Sp1 binding affinity for DNA and reduces target gene expression including the gene for CKII. This results in a mechanism for CKII autoregulation (Leggett et al., 1995; Armstrong et al., 1997). Acetylation of Sp1 and Sp3 was also found to increase trans-activation of their target genes. (Braun et al., 2001; Ammanamanchi et al., 2003; Huang et al., 2005). Sp1 glycosylation with concurrent phosphorylation has been shown to positively or negatively alter Sp1 self-association and Sp1 interaction with basal transcription factors. post-translational modifications also modulates Sp1 degradation in a tissue specific manner (Han and Kudlow, 1997; Roos et al., 1997; Haltiwanger et al., 1998). Glycosylation is also affected by the limitation of nutrients and growth factors such as glucagon and insulin (Keembiyehetty et al., 2002). Growth of cells in glucose
free medium decreases Sp1 gene activation by increasing proteasomal degradation (Han and Kudlow, 1997; Su et al., 1999).

The presence of an activation domain within Sp1-Sp4 proteins implies that transcriptional activation involves interaction of this domain with other nuclear co-regulatory factors. TATA-binding proteins (TBP) and TATA-binding protein-associated factors (TAFs) include TFIID, dTAF110, hTAF130, TAFII55, and TAFII250 have been found to associate directly with trans-activation domains of Sp1 leading to gene trans-activation (Pugh and Tjian, 1990; Smale et al., 1990; Emili et al., 1994; Gill et al., 1994; Emami et al., 1998; Saluja et al., 1998; Rojo-Niersbach et al., 1999).

Sp1 gene trans-activation is also related to a multiple protein nuclear complex designated as Cofactors Required for Sp1 co-activation (CRSP) (Ryu and Tjian, 1999; Ryu et al., 1999; Naar et al., 2002; Taatjes et al., 2002). The 700 kD isolated CRSP complex contains at least nine proteins required for Sp1 transcription, including CRSP150, CRSP200, CRSP130, CRSP100, CRSP85, CRSP77, CRSP70, CRSP34 and CRSP30.

The Sp/KLF family regulates basal/constitutive expression of genes involved in cell proliferation, DNA synthesis, and nucleotide metabolism in both normal and cancerous tissue (Lin et al., 1996). The primary mechanism of gene regulation by Sp/KLF proteins involves initial DNA-dependent binding to GC rich sequences within the promoter of target genes. DNA-independent gene regulation by Sp/KLF through protein-protein interaction has also been reported. More than fifty co-regulators, which interact directly with the C-terminal domain of Sp proteins enhancing or decreasing Sp/KLF gene expression, have been identified. These include c-myc, Ah receptor, NF-YA, VHL tumor suppressor, promyelocytic leukemia protein (PML), helix like transcription factor (HTLF),
p50, p52, p53, Ap-2, hepatocyte nuclear factor 3 (HNF3), HNF4, myocyte enhancer factor (mef2c), VHL, cyclin A, Rb, ZBP-89, NFAT-1, NFAT-2, TGFβ, SMAD1, SMAD-2, SMAD-3, SMAD-4, GATA-1, GATA-2, GATA-3, GATA-4, NF-YA, MyOD, HDAC1, PML, MDM2, MsX1, HTLF, E2F1, YY1, c-jun, NFAT-1, BRCA1, TGFβ and others were [reviewed in (Safe and Kim, 2004)].

In addition, nuclear receptors such as estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), retinoic acid receptor (RAR), thyroid receptor (TR), vitamin D receptor (VDR), peroxisome proliferator-activated receptor gamma (PPARγ) and certain orphan receptors have also been shown to interact directly with Sp1 protein and modulate gene transcription [reviewed in (Safe and Kim, 2004)]. The biphasic action of progesterone in breast cancer cell line T47D (i.e growth stimulation followed by inhibition) has been found due to stimulation of the cell cycle activator genes such as c-myc in the first phase followed by p21 and p27 induction in the later phase. The co-immunoprecipitation result showed that the induction of p21 and p27 is due to progesterone receptor direct association with the GC elements within these gene promoters in a ligand dependent manner (Owen et al., 1998; Gizard et al., 2005).

**General mechanism by which co-regulators control transcriptional activity of NRs:**

In normal tissues, co-regulators play a central role in regulation of transcriptional activity by NRs (Rosenfeld and Glass, 2001). NRs Co-regulators have also been found over expressed and amplified in certain tumors including breast, colon, and lung cancer and underscore their roles in cancer therapy. Since the first co-regulator SRC1 was cloned, at least, 200 co-regulators have been identified and cloned within a short period of time.
NRs co-regulators are divided into two groups, co-activator such as steroid receptor co-activator (SRC)/P160 family, DRIPs, PGC-1 and TRAP and co-repressor including SMRT and NCOR. The ability of co-regulators to associate with nuclear receptors in a ligand dependent manner and their role in modulation of NRs transcriptional activity was initially described by O’Malley and Brown groups (Halachmi et al., 1994; Baniahmad et al., 1995; O’Malley, 2006; Lonard and O’Malley B, 2007). In this mechanism, the NRs box motifs LXXLL motif, which is found within many co-regulators (where X any amino acid), can be recruited to the NRs AF-2 region that is located within NRs C-terminal domain (LBD) only in the presence of the ligand (Heery et al., 1997; McInerney et al., 1998; Heery et al., 2001; Coulthard et al., 2003). However, co-regulators are able to interact with NRs in ligand independent manner by interaction with AF-1 region within the N-terminal domain of the NRs and modulate gene transcription. Co-regulators are able to control the transcriptional activity of NRs by five different mechanisms: (I) Acetylation of histones and transcription factors (Sterner and Berger, 2000), (II) Methylation of histones and transcription factors (Zhang and Reinberg, 2001), (III) Direct interaction with the basal transcription machinery to modulate recruitment of RNA-polymerase II, (IV) Platform or scaffolding function to recruit histone modifying enzymes, (V) ubiquitination and proteolytic activity (Zhang et al., 1998; Lonard et al., 2000). In addition, phosphorylation of the nuclear receptor or the associated co-regulators by extracellular signaling pathways.
such as MAPK is also crucial for NRs transcriptional activity (Bai et al., 1994; Lannigan, 2003).

**HDAC inhibitor and their role in cancer therapy:**

DNA is acidic and negatively charged. Association of positively charged (basic) histone proteins (H2A, H2B, H3 and H4) with negatively charged DNA maintains the DNA electrically neutral and compact (Luger et al., 1997a; Luger et al., 1997b). Acetylation of histones by histone acetyl transferase (HAT) and deacetylation by histone deacetylase (HDAC) enzymes have been subjected to extensive studies. In this mechanism, the co-regulators with HAT activity such as CBP/p300, CREB-binding protein, TAF250 and SRC/p160 family are recruited to the transcriptional machinery in a ligand dependent manner, resulting in hyper-acetylation and neutralization of the lysine residues of histones, releasing the tightly wound DNA, facilitating the access of the transcriptional machinery and other accessory proteins to DNA and leading to activate gene transcription (Figure-7) (Davie and Spencer, 1999; Kouzarides, 1999; Sterner and Berger, 2000; Strahl and Allis, 2000). Conversely, co-regulators that are associated with HDAC activity are recruited in both a ligand dependent and independent manner leading to hypo-acetylation of histones and increase the winding of DNA, resulting in a dense chromatin structure and gene trans-repression by reducing the access of transcription factors (Figure-7). In mammals, 18 HDACs have been identified and classified into three major classes according to their homology with yeast proteins (Taunton et al., 1996; Marks et al., 2003). Class I HDACs includes HDAC1, 2, 3 and 8, have been found to share certain homology to the yeast Rpd3 protein. Without exception, the expression of class I
members have been found to be ubiquitous in many cell lines and tissues and generally localized in the nucleus (Dangond et al., 2001; Gray and Ekstrom, 2001). Class II HDACs including HDAC4, 5, 6, 7, 9 and 10 were found to be homologous to yeast Hda1 and can shuttle between the nucleus and the cytoplasm (Verdin et al., 2003). In addition, class II were found to regulate the acetylation status of non histone proteins such as α-tubulin, p53 and Hsp90 (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003; Kovacs et al., 2005). Class III HDAC or siruntins (SIRT1-7) are homologous to yeast Sir2 family (Blander and Guarente, 2004). Deacetylation by Both class I and class II require zinc for their catalytic activity while class III is NAD+ dependent (Imai et al., 2000a; Imai et al., 2000b; Tanner et al., 2000). Unlike class I and class III, class II members have been found lager in size (120-135 kD). Many evidences indicate that hypo-acetylation of histones induces repression of tumor suppressor genes expression (figure 7). Thus, restraining HDAC de-acetylation activity by using HDAC inhibitors may enhance hyper-acetylation of histones, promote the tumor suppressor gene expression, and exert their effect on tumor curing (Figure-7) (Marks et al., 2001; Marks et al., 2004). HDAC inhibitors with negligible toxicity showed significant activity and promising results against a wide spectrum of hematological and solid tumors both in culture and animal models as well as in clinical trials (Jung, 2001; Marks et al., 2000; Marks et al., 2001). In addition, HDAC inhibitors either isolated from natural sources or from synthetic products have been divided into six groups depending on their molecular structures (Hess-Stumpp, 2005). each of which has different affinity and specificity for each HDAC class (Phiel et al., 2001). HDAC inhibitors are polar and bind to the zinc ion of HDAC catalytic pocket and blocking the substrate interaction (Finnin et al., 1999).
Moreover, Hydroxamic derived compound including trichostatin (TSA) and SAHA, short chain fatty acids such as valproic acid (VPA) have been reported to induce growth arrest, inhibition of cell differentiation and apoptosis in vitro and in clinical trial phase I and phase II (Liu et al., 2006). The induction of p21 protein expression, down-regulation of anti apoptotic Bcl2 protein, up-regulation of pro-apoptotic Bax proteins and induction of caspase 3 and caspase 9 are the common characteristics of HDAC inhibitors actions in cancer cells line (Strait et al., 2002; Archer et al., 2005; Peltonen et al., 2005; Imre et al., 2006).
Figuer (7): molecular mechanism of HDAC inhibitors as anticancer agents (Bi and Jiang, 2006)
Manuscript One:

Enhancement of Folate Receptor α Expression in Tumor Cells Through the Glucocorticoid Receptor: A Promising Means to Improved Tumor Detection and Targeting®* 

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® The findings in this report are covered by pending patents. 

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ABSTRACT

The utility of the folate receptor (FR) type α, in a broad range of targeted therapies and as a diagnostic serum marker in cancer, is confounded by its variable tumor expression levels. FR-α, its mRNA and its promoter activity were coordinately up-regulated by the glucocorticoid receptor (GR) agonist, dexamethasone (Dex). Optimal promoter activation occurred at < 50 nM Dex, was inhibited by the GR antagonist, RU486, and was enhanced by co-activators, supporting GR mediation of the Dex effect. The Dex response of the FR-α promoter progressed even after Dex was withdrawn but this delayed effect required prior de novo protein synthesis indicating an indirect regulation. The Dex effect was mediated by the G/C-rich (Sp1 binding) element in the core P4 promoter and was optimal in the proper initiator context without associated changes in the complement of major Sp family proteins. Histone deacetylase (HDAC) inhibitors potentiated Dex induction of FR-α independent of changes in GR levels. Dex/HDAC inhibitor treatment, did not cause de novo FR-α expression in a variety of receptor-negative cells. In a murine HeLa cell tumor xenograft model, Dex treatment increased both tumor associated and serum FR-α. The results support the concept of increasing FR-α expression selectively in the receptor-positive tumors by brief treatment with a non-toxic dose of a GR agonist, alone or in combination with a well-tolerated HDAC inhibitor, to increase the efficacy of various FR-α-dependent therapeutic and diagnostic applications. They also offer a new paradigm for cancer diagnosis and combination therapy that includes altering a marker or a target protein expression using general transcription modulators.
INTRODUCTION

In recent years, the glycosyl-phosphatidylinositol (GPI)-anchored folate receptor (FR) type α has served as a model target for tumor specific delivery of a broad range of pharmacological and immunological experimental therapies, for the following reasons: (i) FR-α is expressed in several cancers such as non-mucinous adenocarcinomas of the ovary and uterus, malignant pleural mesothelioma, testicular choriocarcinoma, ependymal brain tumors, non-functioning pituitary adenoma and variably in breast, colon and renal carcinoma [reviewed in (Elnakat and Ratnam, 2004)]; (ii) FR-α expression in proliferating normal tissues [reviewed in (Elnakat and Ratnam, 2004)] is restricted to the luminal surface of certain epithelial cells where it is inaccessible to the circulation whereas the receptor expressed in tumors is accessible via the circulation; FR-α-targeted low molecular weight agents that may filter through the glomerulus and bind to the receptor in proximal kidney tubules appear to be transcytosed and reabsorbed, avoiding nephrotoxicity (Gibbs et al., 2005); (iii) other FR isoforms are either expressed in a non-functional manner in mature hematopoietic cells (FR-β) (Ross et al., 1999; Pan et al., 2002) or poorly expressed and constitutively secreted (FR-γ/γ′) (Shen et al., 1994; Shen et al., 1995) and (iv) FR-α quantitatively recycles between the cell surface and intracellular compartments [reviewed in (Kamen and Smith, 2004)], effectively internalizing receptor-bound folate/antifolate compounds and folate conjugates (Lu and Low, 2002; Theti et al., 2003). Various FR-α-targeted therapeutics [reviewed in (Leamon and Low, 2001; Ratnam et al., 2003; Gabizon et al., 2004; Jackman et al., 2004; Leamon and Reddy, 2004; Lu et al., 2004; Roy et al., 2004; Zhao and Lee, 2004)] and imaging agents
[reviewed in (Ke et al., 2004)] have shown promise in pre-clinical models and in early clinical trials. These agents include radiopharmaceutical and cytotoxic conjugates of folate including prodrugs, prodrug activating enzymes, nanoparticles and liposomal drugs as well as potent novel antifolates that are dependent on FR-α for cellular uptake. The FR-α-targeted immunological therapies include bifunctional antibodies and antibody-interleukin chimeras, peptide and DNA vaccines and more innovative agents such as dual specific T cells and folate-hapten conjugates. A portion of the FR-α expressed on the cell surface is released in a soluble form by the combined action of a membrane-associated protease and GPI-specific phospholipase (Antony et al., 1989; Luhrs and Slomiany, 1989; Elwood et al., 1991; Mantovani et al., 1994; Yang et al., 1996). Soluble FR-α is low or undetected in normal human sera and therefore, the protein shed into the circulation is a potential serum marker for FR-α-positive tumors (Mantovani et al., 1994).

Even though major subtypes of malignant tissues show consistent patterns of FR-α expression there is a considerable variability and heterogeneity in tumor expression levels of the receptor covering a range of almost two orders of magnitude (Toffoli et al., 1997; Wu et al., 1999). The successful experimental FR-targeted therapies in animal models have used xenografts of human tumor cells (e.g., KB cells) that express the receptor uniformly and at levels closer to the high end of this range, underscoring the importance of developing molecular methods of up-regulating the FR gene selectively in malignant cells. Increased FR-α expression by the tumors may be expected to enhance the efficacy of the receptor-targeted therapies and whole body imaging and increase the levels of soluble FR-α for early detection as a diagnostic serum marker.
The FR-α gene has 7 exons and 6 introns with multiple transcripts resulting from the use of alternative promoters as well as alternative splicing involving exons 1-4 (Saikawa et al., 1995; Elwood et al., 1997; Roberts et al., 1997; Kelley et al., 2003). The FR-α gene contains two promoters, named P1 and P4, located upstream of exons 1 and 4, respectively. Transcripts generated by both promoters encode identical proteins, but the P4 promoter activity appears to be predominant in malignant cells (Kelley et al., 2003) and further, P1 promoter-driven transcripts appear to be translated several-fold less efficiently than the P4 promoter-driven transcript (Roberts et al., 1997). The basal TATA-less P4 promoter activity is initiated by a cluster of three G/C-rich sequences that are non-canonical Sp1 binding sites, each of which contributes to promoter activity (Saikawa et al., 1995).

We have previously reported that the FR-α gene is directly and negatively regulated by the estrogen receptor (Kelley et al., 2003). Here we report that the FR-α gene is indirectly and positively regulated at the transcriptional (P4 promoter) level by the glucocorticoid receptor (GR) agonist, dexamethasone (Dex) and that this profound regulation is further potentiated by inhibiting histone deacetylase (HDAC). The selectivity of this regulation for FR-α-positive tissues, the innocuous nature of the modulating agents and the ubiquitous expression of GR present a potential means of greatly improving the effectiveness of all available FR-α-targeted therapeutic and diagnostic strategies by the inclusion of GR modulators. The findings also illustrate the potential utility of general transcription modulators in optimizing the expression of genes encoding marker proteins.
and drug targets that are selectively expressed in tumor tissues. These considerations provided the impetus for the present study of the nature and mechanism of GR regulation of FR-α \textit{in vitro} and for examining the effect of GR modulation on tumor and serum levels of the protein \textit{in vivo}. 
MATERIALS AND METHODS

**Chemicals and Reagents:** DMEM, RPMI and penicillin/streptomycin/L-glutamine stock mix were purchased from Life Technologies Inc. Fetal bovine serum (FBS) was purchased from Irvine Scientific. FuGENE 6 was purchased from Roche Diagnostics, luciferase assay reagents from Promega, dexamethasone (Dex) from Sigma, Dex and placebo pellets from Innovative Research of America (Sarasota FL), Trichostatin A (TSA), valproic acid (VPA) and cycloheximide (CHX) from Sigma. Affinity-purified rabbit anti-human Sp1, anti-human Sp3, and anti-human Sp4 antibodies and rabbit polyclonal IgG against GR were purchased from Santa Cruz Biotechnology. Mouse anti-α tubulin clone B-5-1-2 antibody was from Sigma. Vent DNA polymerase was purchased from New England Biolabs and custom oligonucleotide primers from Life Technologies, Inc. The reagents for real time RT-PCR were from Applied Biosystems.

**DNA constructs and expression plasmids:** Construct design made use of either natural restriction sites or restriction sites created by the addition of appropriate restriction sites to upstream and downstream PCR primers. The PCR products were first digested at both ends with the appropriate restriction enzymes and cloned into the PGL3-basic plasmid (Promega) or subcloned into the FR-α-promoter construct (-3394 nt to +33 nt, relative to the transcription initiation site at +1 nt) in the PGL3 basic plasmid. The FRα -3394nt to -47nt/SV40(GC)₆ and the FR-α -3394nt to -32nt/SV40(INR) constructs are described elsewhere (Kelley et al., 2003). The 5’ deletion constructs of the FR-α promoter, i.e., -272nt to +33nt, -116nt to +33nt and –49nt to +33nt were all constructed by PCR using the appropriate primers and subcloned at Mlu1 (upstream) and Xho1 (downstream) sites.
in the pGL3 basic plasmid. In addition the G/C-rich sequence, -49nt to –35nt within the FR-α promoter (-49nt to +33nt)-luciferase construct was replaced by a TATA-box element (5’-AATAATTAA-3’) using PCR. The recombinant plasmids were amplified in E.coli strain XL1Blue and purified using the Qiagen plasmid kit (Qiagen). The entire cloned DNA sequence was verified by sequencing.

The expression plasmids for hSRC-1, hSRC-2 and hpCAF, the corresponding vector plasmid pCR 3.1 and the GRE_e1b promoter-luciferase plasmid were provided by Dr. Brian Rowan (Medical College of Ohio, Ohio). The expression plasmids for Sp1 and Sp3 were provided by Dr. Sumudra Periyasamy (Medical College of Ohio). The expression plasmid for Sp4 was provided by Dr. Guntram Suske (Institut für Molekularbiologie und Tumorforschung Philipps-Universität Marburg).

**Cell Culture and Transfection:** All of the cell lines were purchased from American Type Culture Collection (Rockville, MD) except for NB4 and KCL-22 cells, which were provided by Dr. Philip Koeffler (University of California, Los Angeles) and Ishikawa cells provided by Dr. Brian Rowan (Medical College of Ohio). Cells growing as monolayers were grown in 10cm tissue culture plates at 37°C in 5% CO₂ in the appropriate cell-culture media supplemented with FBS (10%), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Caki-1, HeLa, MG63, MCF-7, JEG, JAR, Ishikawa, SKOV-3 and SVG cells were routinely cultured in DMEM. NB4, KCL-22, K562, and KG-1 cells were grown in RPMI-1640. For treatment with various agents (Dex, VPA and TSA) and for transfection, cells were grown in phenol-red free media supplemented with charcoal-stripped FBS (5% v/v), penicillin (100 units/ml),
streptomycin (100 mg/ml), L-glutamine (2 mM), insulin (2 µg/ml), and transferrin (40 µg/ml).

Transfections with various constructs were carried out in 6-well plates (Corning) using FuGENE 6 (Roche Diagnostics), according to the manufacturer’s suggested protocol. The amount of plasmid DNAs used for the transfections varied as indicated in the appropriate figure legends. Uniformity in transfection efficiencies was ascertained from measurements of β-galactosidase activity after co-transfection with an expression plasmid for this enzyme.

**Preparation of cell lysates and luciferase assay:** Cells in each well of a 6-well tissue culture plate were washed once with phosphate buffered saline (PBS) (pH 7.5) (2 mM KH$_2$PO$_4$, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 137 mM NaCl) and lysed in 400 µl of reporter lysis buffer provided with the luciferase assay system (Promega). The samples were centrifuged at 12,000xg for 2 min at room temperature. The supernatant was assayed for luciferase activity in a luminometer (Lumat LB9501; Berthold) using the luciferase substrate from Promega.

**Real-time RT-PCR analysis:** Total RNA for real time RT-PCR from various cell lines was prepared using the Rneasy Mini kit purchased from Qiagen. Real-time RT-PCR was used to measure endogenous mRNAs for FR-α as well as GAPDH in the same samples. The reverse transcription step was carried out following standard procedure. Essentially, 200 ng of total RNA was mixed with random hexamer primers (5 x 10$^{-4}$ optical density
units/μl), Rnase inhibitor (1 U/μl), Moloney murine leukemia virus reverse transcriptase (5 U/μl), and deoxynucleoside triphosphates (1.0 mM each) in reverse transcriptase buffer (50 mM potassium chloride and 10 mM Tris HCl, pH 8.3). The 10-μl reaction mixture was first incubated at 25°C for 10 min, then at 42°C for 15 min, and finally at 99°C for 6.5 min. The subsequent real time PCR step for FR-α was carried out in the presence of 12.5 μl of PCR Mastermix (Applied Biosystems, Branchburg, N.J.), 0.5 μl each of the forward primer (AAGTGCGAGTGGAGCT) and reverse primer (CATTGCACAGAACAGTGAGCT), and 0.5 μl of the Taqman probe (6FAM-CCTGCAACCTTTCCATTTCACTTCCCTCC-TAMRA). The primers and the Taqman probe for the control GAPDH gene were purchased from Applied Biosystems. The PCR conditions were 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 15s each at 95°C and finally 1 min at 60°C. Fluorescence data generated were monitored and recorded by the Gene Amp 5700 sequence detection system (Applied Biosystems). All samples were set up in triplicates and normalized to GAPDH values.

Measurement of cell surface binding of pteroyl lysine-fluorescein (PLF): The fluorescent folate analog, PLF, was kindly provided by Dr. John Hynes. HeLa cells grown in 6-well plates and subjected to appropriate treatments were washed with PBS, detached from the plate by treatment with 2mM EDTA and re-suspended in cold PBS. The cells were then washed briefly with isotonic acid buffer (10mM sodium acetate buffer, pH 3.5/150 mM NaCl) to remove endogenous bound folate, washed again in PBS and resuspended in PBS containing PLF (10nM) and incubated on ice for 30 minutes with intermittent gentle agitation. The fluorescence on the surface of cells due to PLF binding was measured in an EPICS Elite cytometer (Beckman Coulter). Background
fluorescence on the cell surface due to non-specific binding of PLF was determined by pre-incubating the cells with unlabeled folic acid (1 μM) for 10 minutes before the addition of PLF.

[^3]H|Folic acid binding assay for serum FR: 20μL of mouse serum was diluted into 0.5ml of assay buffer (10mM sodium phosphate buffer, pH7.5/150 mM NaCl/1% Triton X-100) in a 1.5 ml Eppendorf tube. To each assay tube, 2 pmol of[^3]H|folic acid (Moraveck) was added and after incubation for 1 h at 37°C, the protein bound[^3]H|folic acid was measured by a charcoal binding method as described (6). Non-specific binding of[^3]H|folic acid was determined by carrying out the assay as above but after pre-incubating the diluted serum in the assay tube for 10 minutes with a 100-fold excess of unlabeled folic acid (200 pmol).

PI-PLC treatment: HeLa cells grown in 6-well plates and subjected to the appropriate treatments were treated with PI-PLC (0.1u/ml) by adding the enzyme directly into the culture medium followed by incubation for 3h at 37°C. The cell lysates, prepared by lysis in PBS containing 1% Triton X-100 were subjected to western blot analysis.

Preparation of nuclear extracts: HeLa cells subjected to the appropriate treatments were washed twice with PBS, scraped off the 6-well plates, snap frozen in liquid nitrogen and stored at -80°C until the next step. Nuclear extracts were prepared as described (28), except that the cytoplasmic fractions were retained after lysis of cells for subsequent western blot analysis. The nuclear extracts were desalted using G-25 Sephadex columns
(Roche Diagnostics) following the supplier’s protocol. The protein concentrations were
determined by the Bradford assay (BioRad, Hercules, CA).

**Western blot analysis:** Protein samples (10-20 μg) were resolved by electrophoresis on
8% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and electrophoretically
transferred to nitrocellulose filters. The blots were probed with the appropriate primary
rabbit antibodies followed by goat anti-rabbit immunoglobulin G (IgG) conjugated to
horseradish peroxidase (HRP) and visualized using the enhanced chemiluminescence
method. The same membrane was then similarly re-probed with a primary mouse anti-α
tubulin antibody and secondary goat-anti-mouse IgG conjugated to HRP and also
subjected to Coomassie blue staining to ensure uniform sample loading.

**Murine tumor xenograft model:** Fox chase out-bred SCID female mice (29-35 day old)
purchased from Charles River Laboratories were maintained under controlled conditions
and fed with folate-free rodent chow ad libidum during the course of the experiments.
After a period of acclimation, the mice were injected with 5 x 10^6 HeLa cells sub-
cutaneously into the interscapular region. Dex pellets (0.001 mg/pellet for a 21 day
release schedule) or placebo pellets were implanted sub-cutaneously when the tumor
became palpable and grew to a diameter of approximately 0.5 cm. Five days after
implanting the pellets, the mice were euthanized and the blood and tumor tissue
collected. The tumors were snap-frozen in liquid nitrogen and stored at -80°C. The
frozen tumor tissue was ground using mortar and pestle, lysed in PBS containing 1%
Triton X-100 and centrifuged for separation of insoluble cell debris. The supernatant was
used for western blot analysis.
RESULTS

Effect of Dex on FR-α expression in HeLa cells: Treatment of HeLa cells with Dex (100nM) resulted in a progressive increase in the expression of both endogenous FR-α mRNA, measured by real-time PCR and FR-α protein, detected by western blot using an FR-specific rabbit antiserum (Fig.1A). This induction of FR-α began between 24h and 48h and reached up to 7-fold elevation at 96h (Fig. 1A). In HeLa cells transfected with a full-length (-3394nt to +33nt) FR-α promoter-luciferase reporter construct, Dex caused a dose-dependent increase in the promoter activity, reaching optimal activity between 5nM and 50nM Dex (Fig.1B) and a corresponding Dex dose-dependent increase in endogenous FR-α expression (Fig. 1B). These results provide evidence for positive regulation of FR-α by Dex at the transcriptional level.

The induction of FR-α in HeLa cells by Dex did not reflect a global increase in gene expression since, under these conditions, the expression level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Fig. 1A) as well as tubulin and Sp family proteins (Fig. 1A and Fig. 5B, discussed in a later section) were unaltered. The typical plasma membrane localization and GPI membrane anchor attachment known for FR-α was confirmed for the receptor synthesized de novo following Dex treatment since, as seen by western blot, the Dex-induced FR was quantitatively cleaved from the cell surface upon treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), which is a diagnostic characteristic of proteins with a GPI plasma membrane anchor (Fig. 1C). Further, the Dex-induced FR-α protein retained its ability to bind ligand on the cell
surface, evident from an increase in the binding of the fluorescent folate analog, pteroyl lysine-fluorescein (PLF) on the surface of the treated cells (Fig. 1D). Thus, the subcellular localization and function of FR-α was unaltered by Dex induction.

**GR ligand-specificity of FR-α induction:** RU-486, a specific antagonist of GR that competes with Dex for binding to GR, inhibited the induction of FR-α promoter-luciferase reporter in transfected HeLa cells in a dose-dependent manner (Fig. 2A). RU-486 similarly inhibited Dex activation of the control GRE$_2$e1b promoter-luciferase reporter construct in transfected HeLa cells (Fig. 2B). The GRE$_2$e1b promoter contains a glucocorticoid response element (GRE) and is a classical target of Dex activation through GR. Under similar conditions, the dose response of inhibition of the FR-α promoter activity by RU-486 paralleled that for the GRE$_2$e1b promoter, suggesting that the regulation of the FR-α promoter by Dex is, at some level, mediated by GR.

**Response time and reversibility of Dex induction of the FR-α promoter:** HeLa cells were transiently transfected with either GRE$_2$e1b promoter-luciferase or FR-α promoter-luciferase and treatment of the cells with either Dex or vehicle begun at 12h post-transfection (Fig. 3). In the transiently transfected cells, close to maximal activation of the GRE$_2$e1b promoter-luciferase by Dex occurred at 3h, reached the maximum value at 6h and declined between 24h and 48h (Fig. 3A). In contrast, Dex activation of transiently transfected FR-α promoter-luciferase was relatively low at 3h and progressed gradually, reaching its maximum value at 24h and was sustained up to 48h (Fig. 3B). Further, when Dex was withdrawn at 6h, 12h or 24h, the activity of the GRE$_2$e1b promoter measured at
48h was greatly reduced compared to the values measured at 6h, 12h and 24h, respectively (Fig. 3A). In contrast, after withdrawal of Dex at 6h, 12h or 24h, the activity of the FR-α promoter further increased as seen at 48h (Fig. 3B). This observation explains why contrary to the GRE2e1b promoter, the activity of the FR-α promoter was sustained in the later stage of transient transfection (Fig. 3A and B). This delayed activation of the FR-α promoter by Dex indicates that the Dex response is likely mediated indirectly through a product(s) of Dex/GR action on an upstream target gene(s) of Dex and that the mode of action of Dex on the FR-α promoter is thus likely fundamentally different from its activation of a GRE-driven promoter.

**Effect of cycloheximide on Dex induction of the FR-α promoter:** The possibility that the action of Dex on the FR-α promoter requires intermediate synthesis of a protein factor(s), was tested using cycloheximide (CHX) to inhibit *de novo* protein synthesis during the early stage (0-12h) of Dex treatment (Fig. 3C). The delayed induction of FR-α promoter activity observed at 72h after only a 12h treatment with Dex was abrogated when CHX was included during the Dex treatment (Fig. 3C). This result indicates that in Dex induction of the FR-α promoter an early action of Dex is to induce *de novo* synthesis of some other protein(s).

**Effect of co-activators on FR-α promoter activity and its induction by Dex:** The effect of the nuclear receptor/GR co-activators, SRC-1, SRC-2 and pCAF on promoter activity as well as its activation by Dex was measured in HeLa cells co-transfected with expression plasmids for the individual co-activators and FR-α promoter-luciferase
Each of the co-activators enhanced the basal FR-α promoter activity. However, each of the co-activators also potentiated Dex induction of the FR-α promoter. This result lends further support to GR mediation of the Dex effect on FR-α gene expression and the view that this regulation, albeit indirect, is transcriptional.

**Identification of the target site of Dex action in the FR-α promoter:** The FR-α promoter-luciferase reporter construct used in the above studies included the FR-α gene sequence, -3394nt to +33nt, spanning both the P1 and the P4 promoters. 5’ deleted versions of this promoter construct, i.e., the –272nt to +33nt and the –116nt to +33nt fragments of the promoter, retained the Dex responsiveness of the full length construct in transfected HeLa cells (Fig. 4A). The time course of the Dex response was also unaltered for the truncated promoter fragments (Fig. 4A). The only functional cis elements known to occur between the initiator sequence and –116nt are the G/C-rich Sp1 binding sites of the P4 promoter, indicated in Fig.4B. A further 5’ deletion of FR-α promoter luciferase in which all of the promoter sequence upstream of the most proximal Sp1 element was deleted retained the Dex response (Fig. 4C). Since the single Sp1 element in the FR-α(-49nt to +33nt) construct is necessary for basal promoter activity (27), a possible role for this G/C-rich element in mediating the Dex effect was tested by replacing this sequence (-49nt to –35nt) with a TATA-box element (AATAATTAA) to retain basal promoter activity (Fig. 4C). The chimeric promoter was unresponsive to Dex treatment (Fig. 4C), implicating the Sp1 element as a mediator of the Dex effect.
To determine whether another Sp1-dependent promoter, similar to the FR-α promoter, would respond to Dex in a similar manner as the FR-α P4 promoter, the effect of Dex was tested on the Sp1-dependent SV40 promoter-luciferase reporter. In transfected HeLa cells Dex enhanced the activity of the SV40 promoter, with a time course that was similar to that for the FR-α promoter (Fig. 4D). It may be noted that the G/C-rich region of the SV40 promoter is a stronger Sp1 element than that of the FR-α P4 promoter since it contains six repeat Sp1 elements. To determine the relative roles of the initiator (and the flanking) region and the Sp1 elements in the Dex response of the P4 promoter, either the P4 initiator region (-28nt to +33nt) or the entire G/C-rich region of the P4 promoter in the full length FR-α promoter-luciferase construct was replaced by the corresponding regions of the SV40 promoter (Fig. 4D). Both the chimeric promoter constructs were responsive to Dex with a time course similar to that of the FR-α promoter; however, the magnitude of the Dex response was much greater for the FR-α promoter chimera containing the G/C-rich region of the SV40 promoter (Fig. 4D). This suggests that whereas a G/C-rich sequence element mediates and may determine the magnitude of the delayed Dex response of a promoter, for optimal response to occur, there is a preferred initiator context.

The action of Sp family proteins on the FR-α promoter and the effect of Dex on their expression levels: A well known mechanism of gene regulation through G/C-rich cis elements involves changes in differential expression and transcriptional activities of their cognate trans factors, i.e., Sp family proteins. To test this possibility, the action of major Sp family members including Sp1, Sp3 and Sp4 on the FR-α promoter activity was
tested by co-transfection of HeLa cells with FR-α promoter-luciferase and expression plasmids for the Sp proteins, individually and in combination. All of the Sp proteins were equipotent activators of the FR-α promoter (Fig. 5A). Further, Dex treatment of HeLa cells, up to 96h, did not result in any obvious substantive changes in the expression levels of endogenous Sp1, Sp3 or Sp4 or their apparent molecular weights, under conditions in which endogenous FR-α was up-regulated (Fig. 5B). These results exclude changes in the relative expression levels or phosphorylation levels of Sp1, Sp3 or Sp4 as a mechanism mediating the Dex effect on the FR-α gene and suggest that Dex regulates the interaction(s) of some other transcription factor(s) with the core transcription initiation complex of the P4 promoter.

The action of inhibitors of histone deacetylase on Dex induction of FR-α gene expression: Since the transcriptional activity of nuclear receptors entails modulation of histone acetylation, it was of interest to examine the effects of histone deacetylase (HDAC) inhibitors on Dex induction of FR-α gene transcription. The well-tolerated drug valproic acid (VPA) and the well-characterized laboratory reagent, trichostatin A (TSA) were chosen as the HDAC inhibitors for these experiments. In HeLa cells transfected with FR-α promoter-luciferase, both VPA (Fig. 6A) and TSA (Fig.6B) independently increased the promoter activity to some extent within their pharmacological dose ranges but they both greatly potentiated Dex induction of the FR-α promoter. VPA also potentiated Dex induction of the endogenous FR-α in HeLa cells, in a dose-dependent manner (Fig.6C). Finally, the potentiation of the Dex induction of the transfected FR-α promoter-luciferase in HeLa cells by VPA occurred both during the early (0-24h) and
later (24-72h) stages of Dex treatment (Fig. 6D). Under the conditions of the above treatments, the HDAC inhibitors did not affect the viability or the growth of HeLa cells (data not shown).

Effect of Dex treatment and HDAC inhibition on endogenous FR-α gene expression in FR-α-positive vs. FR-α-negative cell lines: In order to test whether Dex increased FR-α gene expression in other FR-α-positive cell lines and to examine whether Dex could alter the tissue expression pattern of FR-α by producing de novo expression of the receptor in FR-α-negative cells, a variety of cell types were treated with Dex, TSA or a combination of Dex and TSA. Cells in which FR-α mRNA was determined by real time RT-PCR to be < 1/1000 of that in HeLa cells and in which the FR-α protein was undetectable by western blot were considered to be FR-α-negative. In human hematopoietic cells (KG-1, Kcl-22, K-562,NB-4), fibroblasts (MG-63, SVG) and epithelial (Caki-1) cell lines that were FR-α-negative, there was no detectable increase in the receptor mRNA expression upon Dex/TSA treatments (Table.2); however, in JAR, Ishikawa and SKOV-3 cells, that are FR-α-positive, Dex, alone or in combination with TSA, increased FR-α expression (Table.2).

Effect of Dex/TSA on GR expression: GR is known to be down-regulated in cells treated with Dex. The effect of Dex/TSA on GR expression in HeLa cells was tested in order to explore the possibility that modulation of GR expression plays a role in the potentiation of Dex induction of the FR-α promoter by HDAC inhibition (Fig. 7). The western blot data in Fig. 7 shows that over a period of 96h of Dex treatment, GR was
progressively down-regulated by Dex, alone and also by Dex in the presence of TSA. Although TSA by itself did not alter the GR level, it did appear to decrease the extent of down-regulation of GR by Dex. However, this did not appear to be a significant factor in the synergy produced by TSA at least in the early (up to 48h) stage because the GR levels were comparable between the two treatments up to 48h.

**Up-regulation of tumor and serum FR-α by Dex in a murine tumor xenograft model:** A murine tumor xenograft model was used to test whether the *in vitro* observations of FR-α induction by Dex would extend to the regulation of the FR-α gene in the physiologic milieu. In Fig. 8A, two groups of three SCID female mice bearing subcutaneous HeLa cell tumors (~ 0.5 cm diameter) were tested. Either low dose slow release Dex pellets (to achieve a circulating concentration of 0.24 μM Dex) or placebo pellets were implanted subcutaneously in the mice for a duration of 5 days before sacrificing the mice to harvest the tumors. As expected, the Dex treatment did not cause a significant difference between the treated and placebo groups in terms of body weight and activity (data not shown). Dex treatment caused a substantial increase in FR-α protein in the tumors as seen by western blot analysis of the tumor cell lysate (Fig. 8A).

In Fig. 8B, the effect of Dex on the level of soluble FR in the serum was tested in the mouse HeLa cell tumor xenograft model and compared with control mice that did not bear the tumor. Groups of 5 mice were used in this experiment and serum FR was estimated by using [³H]folic acid binding assay. Subcutaneous inoculation of HeLa cells and treatment with Dex or placebo pellets were carried out as described above for Fig. 8B.
and at the end of the treatment blood samples were collected from the sacrificed animals. In mice bearing the HeLa cell tumors and treated with placebo, serum FR was slightly elevated compared to the mice that did not bear tumors (p<0.1) (Fig. 8B). Administration of Dex to the tumor bearing mice further increased their serum FR substantially (p<0.01). These results demonstrate that Dex induction of FR-α in a solid tumor in vivo will cause an increase in serum FR and that such a response to Dex may be considered to be indicative of the presence of an FR-α-rich tumor.
DISCUSSION

In view of the large number of pre-clinical and clinical studies that demonstrate the considerable potential for the utility of FR as a target for tumor selective delivery of a broad range of experimental therapies, there is a pressing need to address the problem of the variable and frequently limiting expression of FR in the target tumors. Recently published (Wang et al., 2000; Hao et al., 2003; Kelley et al., 2003) and unpublished studies in our laboratory have shown that the FR gene family is regulated by nuclear receptors. The specific regulatory mechanisms, however, are quite varied but none involve classical response elements. Thus, ER acts by directly interacting with the proximal P4 promoter of the FR-α gene to repress it and this repressive effect is enhanced by estrogen; antiestrogens will bind to ER and de-repress FR-α transcription (Kelley et al., 2003). Retinoid compounds act through each of the three retinoic acid receptors (RARs α, β and γ) in distinct ways, directly interacting with the FR-β gene to up-regulate its expression (Wang et al., 2000; Hao et al., 2003). Other, unpublished studies indicate positive regulation of FR-α by the androgen receptor by direct interaction with proteins bound to an enhancer element in the FR-α gene as well as indirect regulation of FR-α by the progesterone receptor. In this context, the regulation of FR-α by GR, as reported here, is particularly interesting because, unlike other steroid hormone receptors, GR is almost ubiquitously expressed (Adcock and Caramori, 2001). Further, the many clinical applications of Dex and the observation that variations in endogenous cortisol levels do not impact the physiological effects of Dex (Boonyaratankornkit et al., 2001; DeRijk et al., 2002) also indicate that gene activation by endogenous glucocorticoids is suboptimal.
The results of this study clearly demonstrate that Dex up-regulates the expression of the endogenous FR-α gene in cell lines in which the gene is transcriptionally active but not in a variety of cell types that are FR-α-negative; under these conditions other active genes such as those encoding GAPDH, tubulin and Sp family proteins were not regulated by Dex. These observations are consistent with the nature of FR regulation by other nuclear receptors and are further supported by the lack of de novo FR expression in various FR-negative tissues in mice following Dex treatment (data not shown). The Dex-induced FR-α retained the desired characteristics of the receptor as a tumor target and a releasable tumor marker i.e., it’s anchoring to the membrane by GPI and its ability to bind ligand. A substantial induction of FR-α by Dex was also observed in a murine tumor xenograft model, both within the tumor and in the serum, confirming the relevance of this regulation to the physiologic setting.

We undertook a study of the molecular processes involved in the action of GR on the FR-α gene, in the context of current knowledge of nuclear receptor functions, to provide a rational approach to designing and optimizing the use of GR ligands in FR-α targeting and FR-dependent diagnostics and to help to understand, anticipate and address associated problems. FR-α up-regulation in response to Dex treatment was relatively slow and progressed even after Dex was withdrawn as early as 6h of treatment. This suggests that Dex does not act directly on the FR-α gene and that an early sequence of events involving Dex action on some other target(s) precedes and is necessary for FR-α up-regulation. Further evidence that the expression of an intermediary protein factor in
response to Dex treatment is required to mediate the action of Dex on the FR-α promoter is provided by the observation that blocking de novo protein synthesis in the early lag phase of Dex action abolished the delayed activation of the promoter by Dex. The Dex action must be mediated by GR based on (i) the Dex dose-dependence; (ii) the inhibition of its action by the GR antagonist, RU486 and (iii) potentiation of the Dex effect by co-activators. Whereas the identity of the critical immediate (direct) target(s) of Dex/GR action is unclear, the evidence points to the transcriptional nature of these early regulatory events as opposed to the more recently discovered non-genomic actions of steroid receptors (Marks et al., 2000; Simoncini et al., 2000; Boonyaratanakornkit et al., 2001). This is evident from the GR co-activator dependence of the Dex effect as well as potentiation of Dex induction of FR-α by HDAC inhibition in the early (within 24h) as well as late (after 24h) phases of Dex action.

Among the many protein modifications that occur as intermediate steps in transcriptional activation by nuclear receptors, histone acetylation is not only a key event for nuclear receptor function but is reversible, acutely regulated and may be specifically modulated by drugs that have negligible toxicity (Struhl, 1998; Marks et al., 2000; Sterner and Berger, 2000). All of the GR co-activators that were shown in this study to synergize with Dex to enhance FR-α promoter activity i.e., SRC-1(NcoA-1), SRC-2(GRIP1/TIF2/NcoA-2) and pCAF directly or indirectly promote histone acetylation (Robyr et al., 2000). Nuclear receptor co-repressors generate a transcriptionally repressed state by recruiting class II HDACs (Kao et al., 2000; Li et al., 2000; Underhill et al., 2000; Wen et al., 2000; Jansen et al., 2004). Indeed, short chain fatty acids have been shown to sensitize cells to steroid hormones in vitro and in vivo both by activating the
MAP kinase pathway and by inhibiting HDAC (Jansen et al., 2004). Therefore it was logical to test the effect of HDAC inhibitors on Dex induction of FR-α. The short chain fatty acid, VPA (an antiepileptic and antineoplastic drug), and the hydroxamic acid, TSA (a well characterized laboratory reagent), are both inhibitors of class I and II HDACs (Marks et al., 2000; Gottlicher et al., 2001; Marks et al., 2001a). VPA and TSA both potentiated Dex induction of FR-α at the transcriptional level, in their pharmacologically effective milimolar and nanomolar concentration ranges, respectively (Marks et al., 2000; Marks et al., 2001a; Marks et al., 2001b). Despite the broad role of histone acetylation in gene regulation, HDAC inhibitors do not have global effects on gene expression. It has been established that these inhibitors alter the expression of only ~ 2 percent of actively transcribed genes and that most of them have either minimal toxicity or no toxicity at their effective doses (Marks et al., 2000). The profound effects of HDAC inhibitors on Dex induction of FR-α may be used to advantage in the receptor targeted therapies and FR-dependent diagnostics in view of the fact that a variety of HDAC inhibitors that have acceptable toxicity profiles, that produce a sustained increase in the level of acetylated histones within hours (Marks et al., 2001a) and that act on HDACs functionally associated with nuclear receptors are currently available (Marks et al., 2000; Gottlicher et al., 2001; Jung, 2001; Kramer et al., 2003).

The results also demonstrate that the ultimate downstream site of action of Dex in the FR-α gene is the proximal P4 promoter, more specifically, the G/C-rich Sp1 elements and the initiator and flanking region. Devoid of other regulatory elements, this portion of the FR-α promoter region represents the essential elements of a basal TATA-less promoter. The results of this study demonstrated that a (Sp1 binding) G/C-rich region is
essential for the Dex response. The magnitude of this response not only correlated with the strength of the Sp1 element but also depended on the context of the initiator region. A common mechanism of gene regulation through G/C-rich elements involves differential levels and effects of Sp family proteins (Apt et al., 1996) but such a mechanism for the action of Dex was ruled out because in HeLa cells, the major Sp family proteins, Sp1, Sp3 and Sp4 all regulated the FR-\(\alpha\) promoter in a similar manner and further, Dex did not alter their expression levels or their apparent molecular weights that are influenced by their phosphorylation state. It thus appears that the ultimate action of Dex in relation to FR-\(\alpha\) regulation involves modulation of some component(s) of the transcription initiation complex, whose exact composition is dictated by both the G/C-rich and initiator regions. Such a supposition is reasonable in light of the emerging view that the recruitment of several components of the pre-initiation complex is dictated by the basal promoter context (Smale and Kadonaga, 2003).

The FR-\(\alpha\) gene thus belongs to a class of indirect target genes of Dex/GR that lack a glucocorticoid response element. Based on the foregoing studies, it is reasonable to anticipate that in individuals bearing an FR-\(\alpha\)-positive tumor, brief treatment with the combination of innocuous doses of a GR agonist such as Dex and a HDAC inhibitor will boost FR-\(\alpha\) expression in the tumor and will, in addition, increase the serum level of FR. Such a treatment should greatly improve the outcome of FR-targeted therapies. GR modulation of FR-\(\alpha\) expression also offers a means of utilizing circulating FR as a serum marker to detect and follow the treatment response of major subtypes of ovarian, endometrial and other cancers. Indeed, elevation of serum FR in response to Dex/HDAC inhibitor treatment may by itself serve as a diagnostic test for the presence of an FR-
positive tumor. Following detection of the induced FR-α in the serum during initial screening, the FR-positive tumors may be detected by FR-targeted whole body imaging. Similar principles of using general transcription modulators to induce or even down-regulate gene expression, selectively in tumors, concomitant with the administration of therapeutic agents whose action is dependent on the expression level of those genes, may be of value as a general concept in combination therapies. Such an approach is also applicable to the discovery of new tumor/serum markers.
References


Table 1. Effect of co-activators ± Dex on FR-α promoter activity$^a$.

<table>
<thead>
<tr>
<th>Co-regulator</th>
<th>Promoter activity$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
</tr>
<tr>
<td>None</td>
<td>1.0 +/- 0.0</td>
</tr>
<tr>
<td>SRC-1</td>
<td>2.9 +/- 0.9</td>
</tr>
<tr>
<td>SRC-2</td>
<td>3.2 +/- 0.2</td>
</tr>
<tr>
<td>pCAF</td>
<td>4.6 +/- 0.7</td>
</tr>
</tbody>
</table>

$^a$ The promoter activity was determined by measuring luciferase reporter activity. The values are expressed as the ratios to that for the vehicle treated control in the absence of co-transfected co-regulator.

$^b$ HeLa cells ($10^6$) were transfected with FR-α promoter-luciferase (0.5 μg plasmid) and co-transfected with an expression plasmid for SRC-1, SRC-2 or pCAF or with the empty plasmid (0.5 μg plasmid).

$^c$ The transfected cells were treated with either vehicle or Dex (1μM) for a period of 48h post-transfection before harvesting them to measure luciferase activity.
Table 2. Effect of Dex ± TSA on endogenous FR expression in various cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>Endogenous FR-α</th>
<th>Vehicle</th>
<th>Dex</th>
<th>TSA</th>
<th>Dex +TSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG-1</td>
<td>Acute myelogeneous leukemia</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kcl-22</td>
<td>Myeloblastic leukemia</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K-562</td>
<td>Erythroleukemia</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NB-4</td>
<td>Acute promyelocytic leukemia</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MG-63</td>
<td>Osteosarcoma</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SVG</td>
<td>Transformed fibroblast</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caki-1</td>
<td>Kidney carcinoma</td>
<td>Negative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JAR</td>
<td>Choriocarcinoma</td>
<td>Positive</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ishikawa</td>
<td>Uterine adenocarcinoma</td>
<td>Positive</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Skov-3</td>
<td>Ovarian carcinoma</td>
<td>Positive</td>
<td>-</td>
<td>+</td>
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</tr>
</tbody>
</table>

* FR-α mRNA levels in the FR-α-negative cell lines were at least 1000-fold less than that in HeLa cells as assessed by real time RT-PCR and the protein could not be detected by Western blot.

* The '-' sign indicates that there was no increase in FR-α mRNA as assessed by real time RT-PCR.

* The '+' sign indicates that there was an increase in FR-α mRNA as assessed by real time RT-PCR.

* Treatment of the cells with Dex (0.1 μM) and/or TSA (25ng/mL) was carried out for 96h.
FIGURE LEGENDS

**Figure 1: Induction of FR-α by Dex.** A: HeLa cells were treated with either vehicle alone or Dex (100 nM) for the indicated periods at the end of which cells harvested from one triplicate set of wells for purification of total RNA and another set for preparation of cell lysates for western blot. mRNA for FR-α and GAPDH were measured in the RNA samples by real time RT-PCR (top panel). The western blots were probed with rabbit anti-FR antibody (bottom panel). The same blots were re-probed with a mouse anti-tubulin antibody (bottom panel). B: HeLa cells grown in 6-well plates were transfected with the full length FR-α promoter-luciferase reporter plasmid (0.5μg) followed by treatment either with vehicle alone or with the indicated concentrations of Dex for 96h. The harvested cells were divided into two portions, one of which was used to measure luciferase activity (top panel) and the other subjected to western blot analysis using rabbit anti-FR antibody (bottom panel). C: HeLa cells in triplicate 6-well plates were treated with the indicated concentrations of Dex for 96h at the end of which they were incubated for a further 3h, either in the absence or in the presence of PI-PLC. The cell lysates were then subjected to western blot analysis using rabbit anti-FR antibody. Uniformity of sample loading in the blots was confirmed by Coomassie blue staining. D: HeLa cells in 6-well plates were treated with either vehicle or Dex for 96h at the end of which the cells were harvested for flow cytometric analysis of the binding of PLF. In negative controls, the cells were pre-incubated with a 1000-fold excess of unlabeled folic acid. The fluorescence shift in the negative control samples was <1 percent of the signal due to unblocked FR.
Figure 2: Effect of RU486 on Dex induction of promoter activity. HeLa cells were transfected with either FR-α promoter-luciferase plasmid (A) or with a control GRE2e1b promoter-luciferase plasmid (B), and grown for a further 72h either in the absence or in the presence of Dex (100nM) in combination with the indicated concentrations of RU-486. The cells were then harvested to assay luciferase activity in the lysates. The fold-induction of promoter activity is plotted in each case, considering the promoter activity of the vehicle-treated control as unity.

Figure 3: Time course and reversibility of Dex induction of FR-α promoter activity and the effect of cycloheximide. HeLa cells were transfected with either the control GRE2e1b promoter-luciferase plasmid (Panel A) or FR-α promoter-luciferase plasmid (Panel B). 12 h post-transfection, the cells were treated for the indicated periods with either Dex (1μM) or vehicle alone and harvested after the indicated periods for measurement of luciferase activity in the cell lysates. In cases where Dex treatment was terminated before harvest, Dex was removed by aspirating the media and washing the cells with Dex-free media before replenishing the cells with media that did not contain Dex. Panel C: HeLa cells were pre-treated with either cycloheximide (10μM) or vehicle for 2h followed by the introduction of Dex (1μM) or vehicle as indicated. 12h later, Dex/CHX was removed by aspirating the media and washing the cells with Dex/CHX-free media before replenishing the cells with media that did not contain Dex/CHX. The cells were harvested 60h later and total RNA extracted for quantification of FR-α mRNA by real time RT-PCR.
Figure 4: Mapping the target site of Dex action in the FR-α promoter. A: HeLa cells were transfected with FR-α promoter-luciferase reporter constructs containing either the entire P1/P4 promoter region (-3394nt to +33nt) or its 5’ deleted fragments (-272nt to +33nt or -116nt to +33nt). 12h post-transfection, the cells were treated with Dex (1μM) for the indicated periods before harvesting for luciferase assay. Promoter activity is plotted as fold increase over that in cells that were treated with vehicle alone. B: DNA sequence of the FR-α promoter fragment -116nt to +33nt. The numbers represent the positions of the nucleotides relative to the position of the transcription start site (+1nt). The Sp1 elements are indicated in bold letters. C: HeLa cells were transfected with a 5’ deleted FR-α promoter-luciferase reporter constructs containing the promoter fragment -49nt to +33nt. A chimeric promoter construct in which a TATA-box element was attached upstream of the FR-α promoter fragment –35nt to +33nt was also used in the transfection. 12h post-transfection, the cells were treated with Dex (1μM) for the indicated periods before harvesting for luciferase assay. Promoter activity is plotted as fold increase over that in cells that were treated with vehicle alone. D: HeLa cells were transfected with FR-α or SV40 promoter-luciferase constructs containing the indicated promoter fragments of FR-α, SV40 or FR-α/SV40 promoter chimeras. 12h post-transfection, the cells were treated with Dex (1μM) for the indicated periods before harvesting for luciferase assay. Promoter activity is plotted as fold increase over that in cells that were treated with vehicle alone. The pGL3 plasmid (Promega) was used for the SV40 promoter-luciferase contained in it. The construct, FR-α -3394nt to -32nt/SV40(INR) is a chimera in which the initiator region of the SV40 early promoter is
substituted for that of the P4 promoter. The construct FR-α -3394nt to -47nt/SV40(GC)_6 is a chimera in which the cluster of 6 Sp1 elements of the SV40 early promoter is substituted for Sp1 elements of the P4 promoter.

**Figure 5:** Transactivation of the FR-α promoter by Sp family proteins and the effect of Dex on their expression levels. A: HeLa cells were co-transfected with FR-α promoter-luciferase (0.5 μg) and an expression plasmid for Sp1, Sp3 or Sp4 alone or in combination as indicated. As a negative control, cells were co-transfected with the pRc vector. The cells were harvested for luciferase assay 72h post-transfection. B: HeLa cells were grown for 96h in the presence of vehicle alone or with the addition of Dex (1μM) for either the entire 96h or for the last 24h. The cells were then harvested for preparation of nuclear extracts and cytoplasmic fractions. The cytoplasmic fractions were probed by western blot with a rabbit anti-FR-α antibody or with a mouse antibody to tubulin (bottom panels). The nuclear extracts were probed by western blot using antibodies specific for Sp1, Sp3 or Sp4.

**Figure 6: Effect of HDAC inhibitors on FR-α gene regulation by Dex.** A: HeLa cells were transfected with FR-α promoter-luciferase and treated for 72h with different concentrations of VPA either in the absence or in the presence of Dex (100nM) and then harvested for luciferase assay. B: HeLa cells were transfected with FR-α promoter-luciferase and treated for 72h with different concentrations of TSA either in the absence or in the presence of Dex (100nM) and then harvested for luciferase assay. C: HeLa cells that were either untreated or treated with Dex (100nM) and/or different concentrations of
VPA for 96h were harvested and the cell lysates probed by western blot using either a rabbit anti-FR antibody or a mouse antibody to tubulin. D: FR–α promoter-luciferase and then treated for 72h with vehicle alone or with Dex (100nM). VPA (1mM) was introduced for the entire 72h, the first 24h, or for the period between 24h and 72h of Dex treatment. The cells were harvested for luciferase assay at the end of 72h.

**Figure 7: Effect of Dex and TSA on GR expression.** HeLa cells were treated with vehicle, Dex (1μM) or TSA (4ng/ml) or with a combination of Dex and TSA for the indicated periods. The cells were harvested and the cell lysates subjected to western blot analysis using antibody to GR, FR or tubulin.

**Figure 8: Effect of Dex on tumor and serum FR-α levels in a murine tumor xenograft model.** A: Two groups of 3 SCID female mice were inoculated subcutaneously at a single site with HeLa cells (5x10⁶). When the tumors had grown to approximately 0.5cm diameter, the mice were implanted subcutaneously with slow release Dex pellets or placebo pellets. After 5 days the mice were sacrificed and cell lysates were prepared from the tumors and probed by western blot using rabbit anti-FR antibody or a mouse antibody to tubulin. B: Groups of 5 mice bearing subcutaneous HeLa cell tumors or without the tumors were implanted subcutaneously with slow release Dex pellets or placebo pellets. After 5 days, the mice were sacrificed and the blood collected. The serum folate binding protein (serum FR) was measured by a [³H]folic acid binding assay. The normal groups represent mice that did not bear tumors. The data show mean ± SEM for each group. Statistical differences between groups were determined.
using Student’s $t$ test. The level of significance for the difference between the normal groups and the placebo-treated tumor group was $p<0.1$ and between the Dex and placebo groups for the tumor-bearing mice was $p<0.01$. 
Figure 1

**A**

Fold increase in

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**B**

FRα Promoter activity (RLU x 10⁶)

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**C**

FRα

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**D**

Cell surface bound PLF (Relative fluorescence)

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Folic acid pre-incubation

- +
- -
Figure 2

A

B

GRE2 e1b promoter activity (fold increase)

Vehicle

Dex (100nM)

RU-486 (x10^{-9} M)

GRE2 e1b promoter activity (fold increase)

Vehicle

Dex (100nM)

RU-486 (x10^{-9} M)
Figure 3

**A**

GRE\(_2\)e1b promoter

**B**

FR-\(\alpha\) promoter

**C**

Fold increase in FR-\(\alpha\) mRNA
Figure 4

A

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B

Promoter activity (fold increase)

C

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Figure 5

A

B
Figure 6

A

Valproic acid (mM)

Relative luciferase units (x10^6)

0.00 0.25 0.50 1.00

Dex (0 nM)  Dex (100 nM)

B

TSA (ng/ml)

Promoter activity (fold increase)

0 1 2 3 4

Dex (0 nM)  Dex (100 nM)

C

FRα

Tubulin

Dex (nM)  0 100 0 100 0 100

VPA (mM)  0 0 0.25 0.25 0.5 0.5

Relative luciferase units (x10^6)

0 5 10 15 20

Dex (0 nM)  Dex (100 nM)

D

VPA treatment (h)  0 0-24 24-72 0-72

Time of harvest (h)  72 72 72 72

VPA (mM)  0 1 1 1
Figure 7

[Image of gel showing protein expression over time for different treatments: Untreated, Vehicle, Dex, TSA, Dex+TSA. Time points include 12h, 24h, 48h, 72h, and 96h. Proteins GR, Tubulin, and FR-α are expressed over time.]
Figure 8

A

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Tubuli

Dex               Placebo

B

[^3]H Folic acid binding (nmol/L)

Treatment Groups

Normal         Normal         Tumor         Tumor
Manuscript Two:

R5020 and RU486 Act as Progesterone Receptor Agonists to Enhance Sp1/Sp4-Dependent Gene Transcription by an Indirect Mechanism

Aymen Shatnawi, Thuyet Tran and Manohar Ratnam*

Department of Biochemistry and Cancer Biology
Medical University of Ohio at Toledo, 3035 Arlington Avenue, Toledo, OH 43614

Running Title: Gene Activation by Progesterone Receptor Ligands

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Tel. 419-383-3862; Fax. 419-383-6228; E-Mail: mratnam@meduohio.edu

Key words: Folate Receptor, Thymidine Kinase 1, p27, p21, Progesterone Receptor

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ABSTRACT

It has been suggested that ligand-dependent gene activation by the progesterone receptor (PR) can result from recruitment of PR by the promoter bound Sp1. A detailed investigation of the Sp1-dependent agonistic activity of RU486 and R5020 on the folate receptor (FR) type α, p27, thymidine kinase 1 (TK1) and p21 genes reveals a different mechanism. The FR-α P4 promoter and the endogenous FR-α gene were up-regulated by the PR agonist R5020 through either PR-A or PR-B. The classical antagonist RU486 also activated the promoter but only through PR-B. The most proximal (essential) G/C-rich (Sp1 binding) element and the initiator region constituted the minimal promoter responsive to PR regulation; substitution with a stronger cluster of G/C-rich elements enhanced the magnitude of the PR response. In contrast, substitution of the G/C-rich element with a TATA box resulted in the loss of regulation by PR. Over-expression of Sp1 and Sp4 but not Sp3 enhanced activation of the FR-α promoter by PR; knocking down Sp1 decreased the activation in a manner that was reversed by ectopic Sp1 or Sp4. The ligand-dependent action of PR on the promoter was delayed compared to its activation of a classical GRE-driven promoter and activation of both the promoter and the endogenous FR-α gene by PR required new protein synthesis. Activation by PR paralleled RNA polymerase II recruitment but was not accompanied by either association of PR or a change in the association of Sp1 with the endogenous FR-α P4 promoter. Similar observations were made for PR regulation of the genes encoding p27, TK1 and p21. The results contradict the current view of Sp1-dependent gene regulation by PR and point to the existence of a PR target gene(s) whose promoter and cell context(s) must thus
be key determinants of the agonistic activity of RU486 on a large group of important Sp1-dependent downstream target genes.
INTRODUCTION

The physiological effects of progesterone and its antagonists are principally mediated by the transcriptional activity of the progesterone receptor (PR) [reviewed in (Graham and Clarke, 1997)]. The two subtypes of PR, PR-A (94kD) and PR-B (120kD) are differentially expressed in a tissue specific manner (Rao and Wiest, 1974; Rao et al., 1974; Lessey et al., 1983; Attia et al., 2000). PR-A is a truncated form of PR-B lacking its N-terminal 164 amino acids (Kastner et al., 1990; Giangrande and McDonnell, 1999). The PR isoforms differentially regulate progesterone responsive genes and their transcriptional activation of a given promoter could be unequal (Tora et al., 1988; Meyer et al., 1990; Meyer et al., 1992; Vegeto et al., 1993; Giangrande et al., 1997; Conneely and Lydon, 2000; Richer et al., 2002).

Ligands that modulate PR action have clinical application in treating endocrine disorders, as anticancer agents and in terminating pregnancy [reviewed in (Cadepond et al., 1997; Mahajan and London, 1997; Schindler et al., 2003; Spitz, 2003)]. Pure PR antagonists such as ZK98299 (onapristone) completely abrogate the transcriptional activity of PR whereas selective progesterone receptor modulators (SPRMs) such as RU486 (mifepristone) have mixed effects acting as either PR agonists or antagonists in different cell and target gene contexts. SPRMs do not affect homodimerization of PR or its binding to its cognate response element in the target gene (DeMarzo et al., 1992; Skafar, 1993) but produce conformational changes in the AF-2 region of PR to block the association of co-activators and promote co-repressor recruitment [reviewed in (Edwards, 2000; Glass and Rosenfeld, 2000; Leo and Chen, 2000)]. The A and B subtypes of PR respond
differently to RU486 which binds to PR-B to function as a partial agonist under certain conditions; in contrast, RU486 can only act as an antagonist of PR-A (Meyer et al., 1990; Tung et al., 1993). It has been proposed that the partial agonistic activity of RU486 is dependent on the presence of an intact AF-1 region in its receptor (Meyer et al., 1990) as well as the N-terminal domain of PR-B (Wardell et al., 2002). The molecular mechanism by which RU486 works as agonist in not fully understood. Treating cells with the PKA activator 8-bromo-cAMP converted RU486 from an antagonist into an agonist (Beck et al., 1993; Sartorius et al., 1993; Kahmann et al., 1998). The ratio of co-activator to co-repressor in the cell was also found to influence the partial-agonistic activity of RU486 (Liu et al., 2002).

PR can regulate target promoters including the natural promoters for the p21, p27, glycodelin and thymidine kinase (TK) genes in the absence of a functional classical response element (PRE/GRE) through G/C-rich (Sp1 binding) elements (Thomson et al., 1990; Tung et al., 1993; Owen et al., 1998; Gao et al., 2001; Gizard et al., 2005). Some observations appeared to suggest that this regulation occurs by direct association of PR with DNA-bound Sp1 (Owen et al., 1998; Gizard et al., 2005). Additional support for this suggestion is derived from the well established role of Sp proteins in mediating promoter activation by the estrogen receptor (ER) by a non-classical mechanism; it has been demonstrated that ER is recruited by Sp1, Sp3 or Sp4 in the absence of classical estrogen response elements to exert its genotropic effects in a variety of gene promoters in the physiologic context (Safe and Kim, 2004; Higgins et al., 2006). Transactivation by PR
independent of a PRE is of particular interest because this may underlie the agonistic activity of RU486 on promoter activation by PR-B (Tung et al., 1993).

Our initial interest in discovering and studying the regulation of the folate receptor (FR) type α gene by PR ligands was based on the well established potential of FR-α as a tumor target in gynecological cancers for the selective delivery of therapeutic agents and the possibility of using innocuous PR ligands to selectively induce the expression of FR-α in the tumors (reviewed in (Elnakat and Ratnam, 2004; Elnakat and Ratnam, 2006). We discovered that the FR-α gene does not contain a functional hormone response element for PR and that the PR action is mediated by Sp1/Sp4 and the essential G/C-rich (Sp binding) elements within the TATA-less basal P4 promoter of the FR-α gene. The detailed mechanistic studies described in this report for the FR-α gene were extended to the genes encoding p27, thymidine kinase1 and p21. These studies contradict the prevalent view that genes regulated by PR through their Sp1 elements are direct targets of PR action and demonstrate that they are in fact downstream targets of PR. It follows that the key event in the agonistic activity of both R5020 and RU486 on this important and potentially large class of PR target genes is an unusual regulation by PR of an upstream target gene(s) whose product(s) activates other genes through Sp1 or Sp4.

RESULTS

The FR-α promoter and the endogenous FR-α gene are activated by PR in a ligand-dependent manner: In HeLa cells transiently transfected with PR-A or PR-B expression vectors, the potent PR agonist R5020 (promegestone) increased FR-α promoter-luciferase
reporter activity in a dose-dependent manner. The maximal activation produced by PR-A was comparable to that produced by PR-B (Fig.1A). Under similar conditions, the control GRE$_{2e1b}$-luciferase that is driven by the classical progesterone/glucocorticoid response element (GRE) was preferentially activated by PR-B (Fig.1B). Under the conditions of transfection and treatment, the expression levels of PR-A and PR-B remained constant (Fig.1C). The results demonstrate specific ligand and receptor-mediated activation of the FR-α promoter by PR which differs from that of a classical PR target promoter in receptor subtype specificity.

HeLa cells transiently transfected with PR-B showed an increase in FR-α promoter-luciferase activity upon treatment with RU486 either alone or in combination with R5020 (Fig.1D). Under similar conditions RU486 acted as an antagonist of PR-A/R5020 on the FR-α promoter (Fig.1D). Both PR-A/R5020 and PR-B/R5020 failed to activate RU486-treated cells when transfected with the GRE$_{2e1b}$-luciferase reporter construct (Fig.1D). The result shows selective induction of the FR-α promoter by RU486 under conditions in which it antagonizes ligand-dependent activation of a classical GRE-dependent promoter.

In FR-α - positive recombinant T47D breast cancer cells that express either PR-A (T47D-A cells) or PR-B (T47D-B cells), the transfected FR-α promoter-luc (Fig.2A) was up-regulated by R5020. In contrast, RU486 acted as an antagonist of R5020 in T47D-A cells but as an agonist in T47D-B cells in regulating the FR-α promoter activity (Fig.2A). Consistent with these observations, R5020 increased endogenous FR-α mRNA in both T47D-A and T47D-B cells and RU486 increased the FR-α mRNA in T47D-B cells alone
(Fig.2B). Thus transcription of the FR-α gene is positively regulated by R5020 through both PR-A and PR-B whereas RU486 antagonizes the activation of the FR-α gene by R5020/PR-A but positively regulates the gene through PR-B.

The target site of PR action is the G/C-rich element in the minimal P4 promoter:  Fig. 3A shows a variety of deletion and chimeric constructs of the FR-α promoter region that were used to identify the target site for the agonistic action of R5020/PRA, R5020/PR-B and RU486/PR-B in the FR-α gene. The full length promoter construct spanning both the P1 and P4 promoters (-3,394nt to +33nt) could be deleted from the 5’ end up to the most proximal and essential G/C-rich (non-canonical Sp1 binding) element of the P4 promoter without the loss of its typical PR-A (Fig.3B) or PR-B (Fig.3C) dependent regulation by R5020 or RU486 albeit to a lesser magnitude; however, the magnitude of the PR ligand effects of the full length promoter construct was restored if the additional non-canonical Sp1 elements of the P4 promoter were included (Fig.3B and Fig.3C). To test the requirement for a G/C-rich element in mediating the PR effects, these elements in the P4 promoter (-272nt to -35nt) were replaced with a TATA box; the chimeric TATA box-driven promoter lost the regulation by both R5020 and RU486 through either PR-A (Fig.3B) or PR-B (Fig.3C) suggesting the requirement for at least one G/C-rich element. A role for G/C-rich elements in the regulation of the P4 promoter by PR ligands was also evident from the observation that replacing the non-canonical Sp1 elements in the P4 promoter with a strong cluster of six canonical Sp1 elements derived from the SV40 promoter significantly increased the magnitude of the induction of the promoter by R5020 or RU486 through PR-A (Fig.3B) or PR-B (Fig.3C). The above results
demonstrate that PR ligands regulate the FR-α gene through its minimal P4 promoter and also collectively confirm the role of Sp1 binding G/C-rich elements in mediating the ligand-dependent trans-activation of the promoter by PR.

**Certain Sp family proteins support the regulation of the FR-α promoter by PR:** Since functional G/C-rich *cis* elements are known to bind the Sp family transcription factors, the potential roles of the Sp family proteins, Sp1, Sp3 and Sp4, in mediating PR regulation of the FR-α promoter was examined. Over-expression of Sp1 and Sp4 but not Sp3 enhanced the agonistic action of R5020/PR-A, R5020/PR-B and RU486/PR-B on the promoter (Fig.4A and Fig.4B). Over-expression of each Sp protein was confirmed by western blot (data not shown). The functionality of all three over-expressed proteins was evident from the fact that the basal P4 promoter activity was increased by the transfected Sp1, Sp3 and Sp4 by 35, 26 and 103 percent, respectively (not shown in Fig. 4).

When the endogenous Sp1 in T47D-A (Fig. 4C) or T47D-B (Fig. 4D) cells was knocked down, the activation of the FR-α promoter by R5020 or RU486 was greatly decreased. The activation by PR ligands in both cell lines was restored upon simultaneous ectopic expression of either Sp1 or Sp4 (Fig. 4C and Fig. 4D). In this experiment, knockdown or overexpression of Sp proteins was confirmed by western blot (Fig. 4E).

The results show that both Sp1 and Sp4 but not Sp3 must mediate the regulation of the FR-α gene through the G/C-rich elements of the P4 promoter.
PR ligand action on the FR-α promoter is delayed: The time course of action of PR ligands on the promoter was determined in relation to the GRE2e1b promoter, a known direct target of PR action. Fig.5A shows that the GRE2e1b promoter was activated by R5020/PR-B close to the optimal level within 3h-6h. In contrast, under identical conditions, activation of the FR-α promoter by R5020/PR-A (Fig.5B), R5020/PR-B (Fig.5C) was only discernible at 12h and showed a progressive increase up to 48h. This delayed response of the FR-α promoter suggested that this gene may not be a direct target of PR action.

New protein synthesis is required for the regulation of the FR-α promoter by PR: The relatively slow time course of activation of the FR-α promoter by PR noted above suggested that the promoter may be an indirect or downstream target of PR action and that the protein product of one or more upstream or direct target genes of PR may be required to mediate this indirect regulation. To test this possibility, the effect of blocking de novo protein synthesis using cycloheximide (CHX) during a 12h period of treatment with PR ligands on the activity of the FR-α promoter was examined (Fig.6). In HeLa cells expressing PR-A, CHX effectively blocked the R5020-induced increase in the reporter mRNA generated from the FR-α P4 promoter plasmid (Fig. 6A). Similarly CHX also blocked the promoter activity induced by either R5020 or RU486 in cells expressing PR-B (Fig.6B). In contrast, CHX did not affect the activity of the control GRE2e1b promoter, a known direct target promoter of PR (Fig.6C). In the presence of CHX, there was no significant increase in luciferase enzyme activity, indicating complete inhibition of
protein synthesis (data not shown). The results demonstrate that the ligand-dependent action of PR on the FR-α promoter is indirect and requires intermediate protein synthesis.

*The PR ligand effects on the endogenous genes for FR-α, thymidine kinase-1 (TK1) and p27 are similar and also require new protein synthesis:* The preceding experiments indicate that the regulation of the FR-α promoter by PR is indirect and that this is mediated by G/C-rich rich cis-elements in the promoter and their cognate trans-factors Sp1 or Sp4. It was therefore of interest to examine whether such an indirect mechanism was relevant to PR regulation of the endogenous FR-α gene and as well other target genes of PR including TK1 and p27 in which it has been previously shown that Sp1 elements rather than classical response elements mediate induction by progestin.

Unlike TK1, the optimal increase in p27 reportedly occurs in the second phase of the cell’s biphasic response to progestin (Groshong et al., 1997). After 48h of treatment, both R5020 and RU486 substantially (by > 5-fold) up-regulated the endogenous mRNA for p27 in T47D-B cells (Fig.7); R5020 also increased the p27 mRNA by ~8-fold in T47D-A cells but RU486 was an antagonist of this effect (Fig.7).

In both T47D-A and T47D-B cells, treatment with CHX under the experimental conditions used in Fig.8 virtually completely blocked protein synthesis as seen by the loss of the p21 protein either in the absence or in the presence of R5020 or RU486 (Fig.8A). In both T47D-A and T47D-B cells (Fig.8B) a 12h treatment with the appropriate PR ligand resulted in an increase in the endogenous FR-α mRNA in cells harvested 12h after
the treatment. However, treatment with CHX beginning 2h prior to and during the 12h treatment with PR ligands blocked this increase in the FR-α mRNA (Fig.8B). A similar experiment was performed for TK1 with the exception that since TK1 is down-regulated in the later phase of the progestin response, the mRNA was measured at the end of the 12h treatment with ligand (8C); CHX blocked the induction of the TK1 mRNA by R5020 or RU486. Similar results were obtained for the endogenous mRNA for p27 (Fig.8D). In Fig.8, the c-myc gene which is a known direct target for PR was used as a control. CHX did not block the induction of c-myc mRNA by R5020 (Fig.8E). It is thus evident that the endogenous genes for FRα, TK1 and p27 are all regulated by PR indirectly in a manner that requires new protein synthesis.

**PR does not associate with its target elements in the endogenous FR-α, TK1 or p27 genes either basally or in response to ligand:** The preceding experiments suggested that in contrast to the c-myc gene, the genes encoding FR-α, TK1 and p27 were regulated by PR ligands though the product(s) of an upstream direct target gene(s). ChIP assays were designed to further test whether PR was recruited to the promoters in the endogenous genes for FR-α, TK1 and p27 either in the absence or in response to treatment of cells with PR ligands (Fig.9). Quantitative analysis by Real Time PCR of ChIP signals from immunoprecipitated chromatin fragments using antibody to PR showed strong ligand-induced signals for the PRE region in the c-myc gene that was well above the background signal obtained from PCR amplification of a distal sequence from a coding exon chosen as the irrelevant target (Fig. 9). In contrast, the ChIP signals obtained for the G/C-rich promoter regions in the FR-α, p27 and TK1 genes corresponded to the background levels.
for their respective irrelevant targets chosen within distal coding exons (Fig. 9). Non-immune IgG (negative control) did not give a significant ChIP signal above the background in any case (data not shown). It was separately confirmed that all of the Real Time PCR primers and probes used in this experiment were capable of efficiently amplifying their target DNA sequences (Table 2). The results indicate that PR does not specifically associate with the promoter regions of the endogenous genes encoding FR-α, p27 or TK1 in contrast to c-myc promoter either in the absence or in the presence of ligand.

**PR ligands neither alter the expression of Sp proteins or change their association with the endogenous genes encoding FR-α, TK1 and p27:** Since Sp1 and Sp4 mediated the induction of the FR-α promoter by PR, the possibility that PR ligands increased either the expression of Sp1/Sp4 or their association with the promoters for FR-α, TK1 or p27 was tested. Treating T47D-A or T47D-B cells with R5020 or RU486 did not significantly alter the expression of either Sp1 or Sp4 (data not shown). ChIP assays designed to detect a possible change in the association of Sp1 or Sp4 with the functional G/C-rich elements within the basal promoters for the endogenous genes encoding FR-α, p27 and TK1 showed a specific association of both these proteins with the promoters; however, the Sp1/Sp4 interactions with the promoters were not significantly different in groups treated for 30min, 90min or 180min with vehicle, R5020 or RU486 (data not shown). This result indicates that PR ligands can neither increase the association of Sp1 or Sp4 with their cognate functional cis-elements in the FR-α, p27 or TK1 genes nor induce any other DNA binding protein that could displace them.
**The delayed effect of R5020 on the expression of FR-α, p27, TK1 and p21 genes is temporally related to recruitment of RNA polymerase II to the G/C-rich regions in the basal promoters of the endogenous genes:** To confirm the delayed activation of transcription by PR agonist, ChIP assays were used to measure the association of RNA polymerase II (Pol II) with the basal promoters of the endogenous genes for FR-α, p27, TK1 and p21 as a function of time of treatment with R5020 (Fig. 10). The C-myc gene promoter which is a direct target of PR regulation was used as a positive control. In T47D-B cells, there was a basal association of Pol II with the promoter region and R5020 greatly increased the specific association of Pol II within 1 hour (Fig. 10A). In contrast, in the FR-α (Fig. 10B), p27 (Fig. 10C) and TK1 (Fig. 10D) gene promoters, ligand-induced association of Pol II only occurred between 6 and 12 hours of treatment. The p21 gene which also lacks a classical response element for PR and is dependent on G/C-rich elements for PR regulation was also analyzed in this manner (Fig. 10E). Although R5020-induced association of Pol II with the p21 gene promoter was delayed compared to the C-myc gene promoter, it occurred earlier (by 6 hours) than in the FR-α, p27 and TK1 gene promoters (Fig. 10E). This result suggests that whereas the PR ligand-dependent induction of the p21 gene may be indirect, cis-elements and trans-factors unique to the p21 gene may also play a role in mediating regulation by PR ligands.
DISCUSSION

Agonists and antagonists of PR produce profound physiological effects, principally by altering global gene expression profiles in the target tissues. The altered gene expression profiles presumably not only reflect changes in the activities of the direct target genes of PR but also a host of downstream genes whose expression is altered as a consequence of the changes in expression of the direct PR target genes. A clinically significant aspect of PR action is that agents such as RU486 that are used to antagonize PR action have the undesirable quality of acting as PR agonists in several situations (Bowden et al., 1989). In addition to understanding the means by which PR agonists influence global gene expression profiles it is therefore also important to fully understand the underlying principles in the agonistic activity of RU486.

One aspect of significance in the present study is that the FR-α gene whose product is potentially of great value in targeted drug delivery in cancer and as a diagnostic and imaging marker may be up-regulated in the tumors using innocuous PR ligands. Inducing FR-α in this manner is potentially of value in combination therapies using the targeted drugs and in enhancing the sensitivity of the diagnostic and imaging agents (Elnakat and Ratnam, 2004; Elnakat and Ratnam, 2006). It may also be noted that genes such as FR-α that are dependent on the binding of Sp family transcription factors for their basal promoter activities represent a large class of genes, several important members of which have been shown to be regulated by PR ligands (Groshong et al., 1997; Owen et al., 1998; Gao et al., 2001; Tang et al., 2002; Gizard et al., 2005). The nature of RU486 action and PR subtype specificity of Sp1-dependent gene regulation by PR are both distinct from
those of classical PRE-mediated regulation. It is believed that this unique Sp1-dependent
gene regulation by PR occurs by direct interaction of PR with Sp1 in the target
promoters. Therefore the broader significance of the mechanistic studies of FR-α
regulation by PR is demonstrated here by extension to the TK1, p27 and p21 genes
leading to a more accurate understanding of how PR ligands regulate genes through Sp1
and the agonistic action of RU486 on this regulation.

In contrast to a typical GRE-driven promoter that is activated by R5020 predominantly
through PR-B, both PR-A and PR-B showed a comparable ability to mediate activation of
the FR-α P4 promoter and the endogenous FR-α gene by R5020. RU486 on the other
hand behaved as an antagonist of the activation of the FR-α promoter and the FR-α gene
by R5020/PR-A but as an agonist through PR-B in contrast to its antagonistic activity on
a GRE-driven promoter both through PR-A and PR-B. These observations also apply to
the induction of the genes encoding TK1, p27 and p21 that do not have the classical
response elements for PR but whose basal promoters are Sp1-dependent. The requirement
for Sp1 or Sp4 and their cognate G/C-rich cis-elements in mediating PR regulation of the
FR-α promoter is clear from the following observations: (i) the entire DNA sequence
upstream of the functional Sp1 (G/C-rich) elements in the core P4 promoter could be
deleted without affecting the nature or magnitude of PR regulation; (ii) Each of the three
non-canonical Sp1 elements in the P4 promoter contributed to the magnitude of the PR
effect; (iii) substituting the natural Sp1 elements of the P4 promoter with a stronger
cluster of six Sp1 elements derived from the SV40 promoter increased the magnitude of
the PR effect; (iv) substituting the Sp1 elements of the P4 promoter with a TATA-box
element abrogated the regulation by PR (v) over-expression of Sp1 or Sp4 but not Sp3 increased the magnitude of the PR effect and conversely, knocking down Sp1 decreased the PR response in a manner that was reversed by ectopic over-expression of either Sp1 or Sp4.

This study undertook a reexamination of the literature data suggesting that the distinctive activation of several Sp1-dependent genes by R5020 as well as RU486 could be mediated by ligand-dependent association of PR with Sp1 in their basal promoters. Complementary lines of evidence used in this study indicate that the regulation of the natural FR-α promoter by PR is actually indirect. They include the observation that the action of PR ligands on the promoter including the increase in RNA polymerase II recruitment is considerably delayed in comparison with their action on a GRE-driven promoter and that intermediate new protein synthesis is required for the delayed induction of the promoter or of endogenous FR-α mRNA. The inability of PR to associate with the endogenous promoter examined by ChIP either in the absence or in the presence of PR ligands in contrast to the c-myc promoter further confirms that the action of PR on the FR-α gene is indirect. This study has also extended this conclusion to the TK1, p27 and p21 genes. The physiologic relevance of this indirect gene regulation by PR is clearly exemplified in both phases of the reported (Groshong et al., 1997) biphasic response of breast cancer cells to progesterone in which there is first a stimulation of the cell cycle accompanied by an induction of TK1 followed by a delayed induction of p27 associated with G1 phase arrest. The time period for the delayed response of the p21 gene, however was shorter
than that of the TK1 or the p27 genes, suggesting a possible influence of additional target gene-specific factors in the PR ligand response.

The detailed studies reported here contradict the suggestion supported by limited observations (Owen et al., 1998; Gizard et al., 2005) that Sp1 can directly recruit PR to its target promoters to mediate ligand-dependent trans-activation. Besides the role of Sp1 binding DNA elements in promoter regulation by PR, the basis for the suggestion included in vitro co-immunoprecipitation of PR and Sp1 (Owen et al., 1998) an observation whose relevance could be limited to physiologic situations that would mimic the over-expression systems used. The second study (Gizard et al., 2005) used ChIP assays to show an apparent association of PR with the target promoter but did not establish ligand-dependence for this association, was not quantitative and was not supported by complementary data. The more extensive studies in the present report of the delayed Sp-dependent response of several target promoters to PR ligands showed that in no case did PR associate even basally with the promoter.

The Sp-mediated regulation of genes by PR is clearly distinct from the non-classical mechanisms by which transcription factors such as Sp proteins (Safe and Kim, 2004) and AP-1 (Jakacka et al., 2001) mediate direct transcriptional regulation by ER independent of a classical estrogen-response element. Sp1, Sp3 and Sp4 are known to recruit ER to G/C-rich elements by interacting with specific ER domains to transactivate genes in a cell type, ER subtype and promoter context-dependent manner (Castro-Rivera and Safe, 2003; Kim et al., 2003; Safe and Kim, 2004; Stoner et al., 2004; Higgins et al., 2006).
Indeed, ER also regulates the FR-α gene; however this regulation is repressive consistent with the frequently opposing physiologic effects of estrogen and progesterone (Kelley et al., 2003).

PR ligands altered neither the expression levels of several Sp proteins nor the association of Sp1 or Sp4 with the promoter regions of the endogenous FR-α, TK1 or p27 genes observed by ChIP. Therefore it is unlikely that they induced some other DNA binding transcription factor that could bind to G/C-rich elements to increase transcriptional activity. The simplest explanation consistent with the observed nature of the indirect Sp1-dependent regulation of genes by PR is that the action of PR ligands results in the generation of a co-activator of Sp1-dependent trans-activation of the target genes. The gene encoding the putative co-activator may itself be either a direct or an indirect target of PR. In any event, the promoter and cell context of the direct target gene of PR in this pathway must allow RU486 to act as a PR agonist and its activation by RU486/PR-B must be the key step in the positive regulation of a variety of Sp1-dependent genes by RU486. This direct target gene of PR must also mediate the indirect Sp1-dependent gene regulation by R5020. The broad applicability of the results in this study to a variety of indirect target genes of PR that are important in regulating cell growth and physiology presents a compelling case to attempt the identification of the putative Sp1 co-activator and as well the relevant direct target gene of PR in future studies.
MATERIALS AND METHODS

Chemicals and reagents.

DMEM, MEM, LipofectAMINE 2000, Opti-MEM reduced serum medium, Geneticin and the penicillin/streptomycin/L-glutamine stock mix were purchased from Life Technologies, Inc. (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, CA). FuGENE 6 was purchased from Roche Diagnostics (Indianapolis, IN). Promegestone (R5020) was from Berkin Elmer (Shelton, CT). Luciferase assay reagents were from Promega (Madison, WI). Mifepristone (RU486), 100X non essential amino acids, cycloheximide (CHX) and mouse anti-tubulin clone B-5-1-2 antibody were from Sigma (St. Louis, Mo). Protein A coated magnetic beads and Vent DNA polymerase were from New England Biolabs (Beverly, MA). Affinity-purified rabbit anti-human antibodies to RNA polymerase II [Pol II (H-224)], Sp1 (PEP2), Sp4 (V-20), rabbit polyclonal IgG against PR (C-20) and mouse anti-human antibody to GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Custom oligonucleotide primers were from Life Technologies (Carlsbad, CA). The reagents for real-time Reverse Transcription-PCR were from Applied Biosystems (Branchburg, NJ).

Cell culture and transfection.

Hela cells were purchased from American Type Culture Collection (Rockville, MD). Recombinant T47D cells that express either PR-A (T47D-A) or PR-B (T47D-B) were a generous gift from Dr Kathryn B. Horwitz (University of Colorado). Cells were grown in
10 cm tissue culture plates at 37°C in 5% CO2 in the appropriate cell culture media. HeLa cells were routinely cultured in DMEM supplemented with FBS (10%), 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine. T47D-A and T47D-B cells were cultured in MEM supplemented with 5% FBS, 1X of non essential amino acids, 6ng/ml insulin and 200µg/ml Geneticin. For treatment with various agents (R5020, RU486, CHX) and for transfection, cells were grown in phenol red–free DMEM supplemented with charcoal-stripped FBS (5% v/v), penicillin (100 units/mL), streptomycin (100 mg/mL), L-glutamine (2 mmol/L), insulin (2 µg/mL), and transferrin (40 µg/mL). Transfections with various plasmid constructs were carried out in six-well plates (Corning, New York, NY) using FuGENE 6 (Roche Diagnostics) for HeLa cells and lipofectAMINE 2000 for T47D cells. The transfection method followed the manufacturers’ suggested protocols. Uniformity of transfection with promoter constructs attached to the firefly luciferase reporter was ensured by co-transfection with a renilla luciferase expression plasmid and monitoring the activity of renilla luciferase.

Promoter constructs, expression plasmids and Sp1 knockdown:

Construct design made use of either natural restriction sites or restriction sites created by the addition of the appropriate sequences to upstream and downstream PCR primers. The PCR products were first digested at both ends with the appropriate restriction enzymes and cloned into the PGL3-basic plasmid (Promega) or subcloned into the FR-α promoter construct (-3394nt to +33nt, relative to the transcription initiation site at +1nt) in the PGL3 basic plasmid. Construction of the FR-α -3394nt to -47nt/SV40(GC)6 construct is described elsewhere (Tran et al., 2005). The 5’ deletion constructs of the FR-α promoter,
i.e., -272nt to +33nt, -116nt to +33nt, and -49nt to +33nt were all constructed by PCR using the appropriate primers and subcloned at MluI (upstream) and XhoI (downstream) sites in the pGL3 basic plasmid. In the TATA+ FR-α -35nt to +33nt construct, a TATA-box element (5'-AATAATTAA-3') was placed upstream of the most proximal Sp1 element in the FR-α promoter, i.e., upstream of FR-α -35nt to +33nt by the PCR method. The recombinant plasmids were amplified in E. coli strain XL1Blue and purified using the Qiagen plasmid kit (Qiagen, Chatsworth, CA). The entire cloned DNA sequence in each construct was verified by automated DNA sequence analysis.

The GRE_2e1b promoter-luciferase plasmid and the expression plasmids for PR-A and PR-B were kindly provided by Dr. Brian Rowan (Tulane University Health Sciences Center). The expression plasmids for Sp1 and Sp3 were provided by Dr. Sumudra Periyasamy (Medical University of Ohio). The expression plasmid for Sp4 was provided by Dr. Guntram Suske (Institut für Molekularbiologie und Tumorforschung Philipps-Universität Marburg). The shRNA expression plasmid for knocking down Sp1 was purchased from Sigma (St. Louis, Mo). For the knockdown experiments the Amaxa Nucleofection System (Amaxa Biosystems) was used to deliver the shRNA to T47D cells according to the vendor’s protocol.

**Preparation of cell lysates and luciferase assay.**

Cells in each well of a six-well tissue culture plate were washed once with phosphate buffered saline (PBS) of pH 7.5 (2 mmol/L KH₂PO₄, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 137 mmol/L NaCl) and lysed in 400 μL of reporter lysis buffer provided with
the luciferase assay system (Promega). The samples were centrifuged at 12,000 x g for 2 min at room temperature. The supernatant was assayed for luciferase activity in a luminometer (Lumat LB9501, Berthold, Wildbad, Germany) using the luciferase substrate from Promega. All luciferase experiments were done at least in triplicate.

**Real-time RT-PCR and real time PCR analyses.**

Total RNA for Real-time Reverse Transcription-PCR (RT-PCR) was prepared using the RNeasy Mini kit purchased from Qiagen. Real-time RT-PCR was used to measure the mRNA for luciferase, FR-α, TK1, p27 or c-myc. The mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also measured by real time RT-PCR in the same samples. For the real time RT-PCR, the reverse transcription step was carried out following standard procedures. Essentially, 200 ng of total RNA was mixed with random hexamer primers (5 x 10^-4 absorbance units/µL), RNase inhibitor (1 unit/µL), Moloney murine leukemia virus reverse transcriptase (5 units/µL), and deoxynucleoside triphosphates (1.0 mmol/L each) in reverse transcriptase buffer [50 mmol/L potassium chloride and 10 mmol/L Tris-HCl (pH 8.3)]. The 10 µL reaction mixture was first incubated at 25°C for 10 min, then at 42°C for 15 min and finally at 99°C for 6.5 min. The subsequent real-time PCR step was carried out in the presence of 12.5 µL of PCR Mastermix (Applied Biosystems). 0.5 µL each of the forward primer and reverse primer and 0.5 µL of the TaqMan probe were used in the reaction. The sequences of the forward and reverse probes and the appropriate TaqMan probe for each target are listed in Table 1. The primers and the TaqMan probe for the control GAPDH gene were purchased from Applied Biosystems. The PCR conditions were 2 min at 50°C, then 10 min at 95°C,
followed by 40 cycles each of 15 seconds at 90°C and 1 min at 60°C. Fluorescence data generated were monitored and recorded on a 7500 Real Time PCR sequence detection system (Applied Biosystems). All samples were assayed in triplicate and normalized to GAPDH values.

**Western blot analysis.**

Cells were harvested after washing two times with cold PBS. The cells were lysed with High Salt buffer (400 mM NaCl; 10 mM Tris, pH 8.0; 1 mM EDTA; 1 mM EGTA; b-mercaptoethanol; 0.1% Triton X-100) containing a protease inhibitor cocktail (1 mM phenyl methyl sulphonyl fluoride and 5 µg/mL each of aprotinin, leupeptin, and pepstatinA). Protein samples (20-50 µg) were resolved by electrophoresis on 8% sodium dodecylsulfate-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The blots were probed with the appropriate primary antibodies followed by goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase and visualized using the enhanced chemiluminescence method. The same membrane was then similarly re-probed with a primary mouse anti-tubulin antibody and secondary goat anti-mouse IgG conjugated to horseradish peroxidase. The membranes were also stained using Coomassie blue to ensure uniform sample loading.

**Quantitative chromatin immunoprecipitation (ChIP) assay.**

T47D-A cells or T47D-B were grown in 10-mm plates and treated as appropriate for each experiment. For the ChIP assays, the cells were then washed twice with ice-cold PBS and fixed with formaldehyde (1% final concentration) for 15 min at room temperature. Then
the cells were washed with ice-cold PBS twice and collected in 100 mM Tris-HCl (pH 9.0) and 10 mM dithiothreitol. After a 15 min incubation at 30°C the cells were washed twice with ice-cold PBS and lysed in ChIP lysis buffer [1% sodium dodecylsulfate, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1) plus protease inhibitor cocktail (1 mM PMSF; and 5 µg/mL each of aprotinin, leupeptin, and pepstatinA)] and incubated on ice. 10 min later the samples were sonicated on ice with a sonic dismembrator (Fisher Scientific Company, Pittsburgh, PA) at output 3 for 10 seconds pulse-on time followed by 40 seconds pulse-off time, and this procedure was repeated 3 times resulting in chromatin fragmented to an average length of about 500 bp. The samples were then centrifuged at 16000 x g for 10 min. The supernatant was diluted 10- fold in ChIP dilution buffer (1% Triton X-100; 2 mM EDTA; 150 mM NaCl; 20 mM Tris-HCl pH 8.1; plus the protease inhibitor cocktail) and pre-cleared with 2 µg of normal rabbit IgG and 5 µl of Protein-A coated magnetic beads. The beads were then separated on a magnetic rack. 10% of the supernatant was set aside for input measurements. 2 µg of the appropriate antibody [anti-Sp1 (sc-59), anti-PR (sc-539X), anti Pol II (sc-9001) or normal rabbit IgG from Santa Cruz Biotechnology] was added to the supernatant and incubated overnight on a rotary shaker at 4°C. Then the samples were mixed with sonicated salmon sperm DNA (100 µg/mL) and 5 µL of Protein A magnetic beads for a 6h incubation at 4°C. The beads were then washed for 5 min each, first with Low Salt Buffer (0.1 % SDS; 1% Triton X-100; 2mM EDTA; 20 mM Tris-HCl, pH 8.1; 150 NaCl) followed by High Salt buffer (0.1 % SDS; 1% Triton X-100; 2mM EDTA; 20 mM Tris-HCl, pH 8.1; 500 mM NaCl) and finally with LiCl buffer (0.25 M LiCl; 1% Nonidet P-40; 1% deoxycholate; 1mM EDTA; 10 mM Tris-HCl, pH 8.1). The beads were washed with TE buffer (10 mM Tris-HCl pH
8.0; 1 mM EDTA) three times for 5 min each time. The immunoprecipitated chromatin complexes were removed from the beads and de-cross-linked by incubation for 6 h at 65°C in 100 µl of 1% SDS/ 0.1 M NaHCO3 with intermittent vortexing. DNA was purified from the samples using the QIAquick Spin Kit (QIAGEN, Chatsworth, CA) according to the manufacturer’s protocol. 10 µl of the extracted DNA was used in real time PCR assays to measure the appropriate ChIP target DNA. All of the forward and reverse primers and TaqMan probes used for the various target sequences in the ChIP assays are shown in Table 1. The ability of the various PCR primers and TaqMan probes to amplify the target sequences is shown in Table 2. Every sample was assayed in triplicate.

Statistical analysis:
Results are presented as mean +/- standard error. The significance of statistical differences (P values) between the indicated groups in each experiment was determined using ANOVA.

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to progesterone response elements and are dominantly inhibited by A-receptors.


**Figure legend:**

**Figure 1: Ligand-dependent regulation of the FR-α promoter by PR:** Hela cells grown to 80% confluence were transfected with (A) full length FR-α promoter-luc or (B) GRE\textsubscript{2e1b} promoter-luc and with expression plasmid for either PR-A or PR-B or with empty vector; the cells were treated with the range of concentrations of R5020 or with vehicle and harvested 48 h later to measure luciferase activity. The values represent fold increase relative to the vehicle treated control. (C) Western blots of total cell lysates from the HeLa cells transfected in A with either PR-A or PR-B probed with antibody to PR-A (top panels) and PR-B (bottom panels) respectively and re-probed with antibody to tubulin. (D) Hela cell cells were grown to 80% confluence and transfected with either FR-α promoter-luc or GRE\textsubscript{2e1b} promoter-luc and co-transfected with PR-A or PR-B; the cells were treated as indicated with R5020 and/or RU486 and/or vehicle and harvested to measure luciferase activity at 48h. The values are represented as percent of maximum activity of either FR-α promoter-luc or GRE\textsubscript{2e1b} promoter-luc. All of the determinations were made in triplicate and the standard deviations are indicated. In panel A, $P<0.0001$ for a vs c, e, g or i; b vs d, f, h or j. In panel B, $P<0.001$ for c vs. a or d; b vs d; f vs e; h vs g and j vs i. In panel C, $P<0.0001$ for g vs a, b, d or f; c vs e or h.

**Figure 2: Regulation of the FR-α promoter and induction of endogenous FR-α mRNA in T47D-A and T47D-B cells by PR ligands:** (A) T47D-A or T47D-B cells were transfected with FR-α promoter-luc and treated with 50 nM of each ligand as indicated. After 48h the cells were lysed to measure luciferase activity. The values
represent the relative luciferase activity unit (B) T47D-A or T47D-B cells were treated with vehicle or with 50nM of either R5020 or RU486 and 48h later the cells were harvested and total RNA extracted. The RNA was subjected to Real Time RT-PCR to measure mRNA for FR-α or GAPDH. The values represent fold induction of FR-α mRNA compared to vehicle treated controls and normalized to GAPDH. In panel A, 
\[ P<0.0001 \] for a vs b; c vs d, e or f. In panel B, \[ P<0.0001 \] for a vs b; c vs d or e.

**Figure 3: Mapping and characterizing the target site of PR action in the FR-α promoter:** (A) Schematic representation of various promoter-luciferase reporter constructs. The figure shows the full-length FR-α promoter including both the P1 and P4 basal promoters (FRα-3394nt to +33 nt) and its 5’ deleted versions (FRα -272nt to +33nt) , (FRα -116nt to +33nt) and (FRα -49nt to +33nt). The [FRα-3394nt to -47nt/ SV40(GC)6] construct represents a chimeric FR-α/SV40 promoter in which the G/C-rich region of the FR-α promoter was replaced with six tandem G/C-rich elements from the SV40 promoter. In (TATA- FRα -35nt to +33nt) the Sp1 binding site in construct (FRα -49nt to +33nt) is replaced by a TATA box element. In B and C, Hela cells were transfected with the various promoter-luciferase constructs and co-transfected with PR-A (B) or with PR-B (C) and treated as indicated with vehicle, 50nM R5020 or 50 nM RU486 alone or in combination. After 48h of treatment the cells were lysed to measure luciferase activity. The values for luciferase activity are expressed as the ratio to that for the corresponding vehicle treated control. In panel B, \[ P< 0.001 \] for b vs a or c; d vs e or e. In panel C, \[ P< 0.001 \] for d vs a or g; g vs m; e vs b or h; k vs h or n; f vs c or i; l vs i or o.
Figure 4: Effect of Sp family proteins on ligand-dependent activation of the FR-α P4 promoter by PR: HeLa cells were co-transfected with FRα -272nt to +33nt -luc and expression plasmid for Sp1, Sp3 or Sp4 or with the empty vector and with expression plasmid for PR-A (A) or PR-B (B). The cells were then treated with vehicle or with R5020 (50nM) or RU486 (50nM) alone or in combination as indicated. The cells were harvested to measure luciferase activity 48 h post-transfection. The data represent fold increase in the luciferase activity compared to the corresponding vehicle control. T47D-A cells (C) and T47D-B cells (D) were nucleofected with FRα -272nt to +33nt -luc and expression plasmid for Sp1, Sp4 or siRNA for Sp1 in the indicated combinations. 12 h later the cells were treated with vehicle, R5020 (50nM) or RU486 (50nM). After 48h of treatment the cells were harvested and assayed for luciferase activity. The data represent relative luciferase units. Panel E shows a western blot of total cell lysates from the T47D-B cells nucleofected with expression vectors for Sp1, Sp4, Sp1 siRNA or a scrambled siRNA control and probed with antibody to Sp1 (top panel) and Sp4 (middle panel) and re-probed with antibody to GAPDH (bottom panel). In panel A, P < 0.0001 for a vs b or c. In panel B, P< 0.0001 for a vs d or g; b vs e or h; c vs f or i. In panel C, P< 0.0001 for a vs c, e, or g; d vs b, f or h. In panel D, P< 0.0001 for a vs d, g, or j; b vs e; c vs f; e vs h or k; f vs i or l.

Figure 5: Time course of R5020-dependent promoter activation by PR: Hela cells were transfected with GRE2e1b promoter-luc (A) or FRα -272nt to +33nt-luc (B and C). The cells were co-transfected with expression plasmid for PR-A (B) or PR-B (A and C). 24h after transfection, the cells were treated with either vehicle or R5020 (50nM) and
harvested at the indicated times. The luciferase activities measured in the cell lysates are expressed as ratios to the corresponding vehicle treated controls. In panel A, $P<0.001$ for b vs a, c or d; d vs f. In panel B, $P<0.01$ for b vs a or c; c vs d. In panel C, $P<0.001$ for b vs a or c; c vs d.

**Figure 6: Effect of cycloheximide on activation of the FR-α promoter by PR ligands:**
Hela cells were transfected with (A) FRα -272nt to +33nt-luc (B) FRα -272nt to +33nt-luc and (C) GRE2ε1b- promoter-luc. The cells were co-transfected with PR-A (A) or PR-B (B and C). 48 h later the cells were pre-treated with either vehicle or cycloheximide (10 μM) for 2h. At the end of the 2h pre-treatment, R5020 (50nM) or RU486 (50nM) was introduced alone or in combination with cycloheximide and the incubation continued for 12 h. The cells were then harvested and total RNA was extracted. The mRNAs for luciferase and GAPDH were measured by Real Time RT-PCR. The values for the reporter were normalized to GAPDH. In panel A, $P<0.0001$ for b vs a or c. In panel B, $P<0.0001$ for a vs b or c; b vs d; c vs e. In panel C, $P<0.0001$ for a vs b or c.

**Figure 7: Induction of endogenous p27 in T47D-A and T47D-B cells by PR ligands:**
T47D-A or T47D-B cells were treated with vehicle, R5020 (50nM) or RU486 (50nM). 48 h later the cells were harvested and the total RNA was extracted from the cells and subjected to Real Time RT-PCR to measure the mRNAs for p27 and GAPDH. The values for p27 mRNA are normalized to those for GAPDH. $P<0.0001$ for a vs b; c vs d or e.
Figure 8: Effect of cycloheximide on activation of the endogenous genes for FR-α, TK1 and p27: (A) T47D-A (left panel) or T47D-B cells (right panel) were pre-treated with vehicle or with CHX (20 µmol/L) for 2h followed by the inclusion of vehicle, R5020 (50nM) or RU486 (50nM) as indicated and incubated for a further 12h. The cells were then harvested and the lysates subjected to western blot analyses to probe for p21 and the blots re-probed for tubulin. In B-E, T47D-A or T47D-B were pre-treated with either cycloheximide (CHX) (20 µmol/L) or vehicle (V) for 2h followed by the addition of vehicle, R5020 (50 nM) (R) or RU486 (50 nM) (RU) as indicated. Where indicated, 12h later after the treatment, CHX, R5020 and RU486 were removed by replacing with fresh media. The cells were harvested at the indicated times and total RNA extracted. The mRNAs for FR-α (B), TK1 (C), p27 (D) or c-myc (E) were measured by Real-Time RT-PCR together with the mRNA for GAPDH. The mRNA values are normalized to those for GAPDH. In panel B, $P<0.001$ for b vs a or c; e vs d or f. In panel C, $P<0.001$ for a vs c; b vs d or e; d vs g; c vs f; e vs h. In panel D, $P<0.0005$ for a vs b or c; b vs d; c vs e. In panel E, $P<0.0001$ for a vs b or c.

Figure 9: Quantitative chromatin immunoprecipitation assays to detect association of PR with endogenous genes: T47D-B cells were treated with vehicle, R5020 (50nM) or RU486 (50nM) for 1.5h. The cells were subjected to ChIP assays as described under Experimental Procedures using antibody against PR or normal IgG (negative control). The target sequences for measurement of ChIP signals by Real Time PCR include the PRE region in the c-myc promoter, Sp1 elements in the basal promoters of the genes encoding FR-α, p27 and TK1 and as well sequences (irrelevant targets) distal to the
promoters of the c-myc, FR-α, p27 and TK1 genes (i.e., within their coding exons). The ChIP signals are expressed as fold difference in relation to the corresponding irrelevant targets in the vehicle treated controls. The normal IgG control did not produce ChIP signals (data not shown). $P<0.0001$ for b vs a, c or d.

**Figure 10:** Time course of induction of RNA polymerase II recruitment to various gene promoters by R5020: T47D-B cells were treated with vehicle or R5020 (50nM) for 1h, 3h, 6h, 12h, and 24h. The cells were subjected to ChIP assays as described under Experimental Procedures using antibody against Pol II or normal IgG (negative control). The target sequences for measurement of ChIP signals by real time PCR include the PRE elements in the basal promoter of the c-myc gene (A) or Sp1 elements in the basal promoters of the genes encoding FR-α (B), p27 (C), TK1 (D) and p21 (E) and as well coding exon sequences (irrelevant targets) distal to the promoters of the c-myc, FR-α, p27, TK1 and p21 genes. The ChIP signals are expressed as percent of the signal for the corresponding input. In panel A, $P<0.0001$ for b vs a or c; d vs c or e; e vs f. In panel B, $P<0.0001$ for a vs d or e; b vs d or e; c vs d. In panel C, $P<0.0001$ for a vs d or e; b vs d or e; c vs d. In panel D, $P<0.0001$ for d vs a, b or c. In panel E, $P<0.0001$ for a vs d,e or f; b vs d, e or f; c vs d.
Figure 1

A

FR-α promoter-Luciferase

Vector  PR-A  PR-B

Luciferase activity (fold increase)

R5020(nM) 0 0.1 0.5 1 5

B

GRE2e1b promoter-Luciferase

Vector  PR-A  PR-B

Luciferase activity (fold increase)

R5020(nM) 0 0.1 0.5 1 5

C

Transfection

Empty Vector  Expression plasmid

R5020(nM) 0 0 0.1 0.5 1 5

PR-A

Tubulin

PR-B

Tubulin

D

FR-α promoter-Luc +PR-A  FR-α promoter-Luc +PR-B  GRE2e1b promoter-Luc +PR-A  GRE2e1b promoter-Luc +PR-B

Luciferase activity (Percent of maximum)

R5020 (50nM)  ----  +++  ----  +++

RU486 (50nM)  ----  ----  +++  +++
Figure 2

A

![Bar graph showing relative luciferase units for T47D-A and T47D-B cells with different treatments (Vehicle, R5020, Ru486, R5020+Ru486).]

B

![Bar graph showing FR-α mRNA expression for T47D-A and T47D-B cells with different treatments (Vehicle, R5020, RU486).]
Figure 3

A

Fold increase in promoter activity

PR-A

FR-α -3394 nt to +33 nt
FR-α -3394 nt to -47 nt/SV40(GC)6
FR-α -272 nt to +33 nt
FR-α -116 nt to +33 nt
FR-α -49 nt to +33 nt
TATA+FR-α -35 nt to +33 nt

B

Fold increase in promoter activity

Vehicle
R5020
Ru486
R5020+Ru486

FRα -3394 nt to +33 nt
FRα -272 nt to +33 nt
FRα -116 nt to +33 nt
FRα -49 nt to +33 nt
FR-α -3394 nt to -47 nt/SV40(GC)6
TATA+FRα -35 nt to +33 nt

C

Fold increase in promoter activity

Vehicle
R5020
Ru486
R5020+Ru486

FRα -3394 nt to +33 nt
FRα -272 nt to +33 nt
FRα -116 nt to +33 nt
FRα -49 nt to +33 nt
FR-α -3394 nt to -47 nt/SV40(GC)6
TATA+FRα -35 nt to +33 nt
Figure 5

A

FR-α promoter-luc + PR-B

Fold increase in luciferase activity

Time (h) 0 3 6 12 24 48

0 100 200 300 400

GRE2e1b promoter-luc + PR-B

B

FR-α promoter-luc + PR-A

Fold increase in luciferase activity

Time (h) 0 3 6 12 24 48

0 5 10 15 20 25 30

C

FR-α promoter-luc + PR-B

Fold increase in luciferase activity

Time (h) 0 3 6 12 24 48

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150
Figure 6

A. FR-α P4 promoter-luc + PR-A

B. FR-α P4 promoter-luc + PR-B

C. GRE2e1b promoter-luc + PR-B
Figure 7

![Bar chart showing p27 mRNA levels in T47D-A and T47D-B cells treated with Vehicle, R5020, and RU486.

- T47D-A cells:
  - Vehicle: Control
  - R5020: 8x vehicle control
  - RU486: 2x vehicle control

- T47D-B cells:
  - Vehicle: Control
  - R5020: 6x vehicle control
  - RU486: 4x vehicle control

The chart indicates significant upregulation of p27 mRNA levels across different treatments and cell lines.]
Figure 8

A

T47D-A cells

Vehicle  R5020  RU486  CHX  CHX+R5020  CHX+RU486

T47D-B cells

Vehicle  R5020  RU486  CHX  CHX+R5020  CHX+RU486

B

FR-α

T47D-A  T47D-B

Fold increase in mRNA

C

TK1

T47D-A  T47D-B

Fold increase in mRNA

D

p27/T47D-B

Fold increase in mRNA

E

Cmyc/T47D-B

Fold increase in mRNA
Figure 9

Fold increase in ChIP signal

- **C-MYC FR p27 TK1**
- **Cmyc p27 TK1 FR-**

- **Irrelevant target /vehicle**
- **Irrelevant target /R5020**
- **Irrelevant target /RU486**

- **Promoter target /vehicle**
- **Promoter target /R5020**
- **Promoter target /RU486**

- **Figure 9**

- **Cmyc**
- **FR-α**
- **p27**
- **TK1**

- **Fold increase in ChIP signal**

- **Irrelevant target /vehicle**
- **Irrelevant target /R5020**
- **Irrelevant target /RU486**

- **Promoter target /vehicle**
- **Promoter target /R5020**
- **Promoter target /RU486**
Figure 10

- Normal IgG/promoter target (vehicle)
- Normal IgG/promoter target (R5020)
- Anti Pol II/Irrelevant target (vehicle)
- Anti Pol II/Irrelevant target (R5020)
- Anti Pol II/Promoter target (vehicle)
- Anti Pol II/Promoter target (R5020)

A

C-myc gene

B

FR-α gene

C

P27 gene

D

TK1 gene

E

p21 gene
<table>
<thead>
<tr>
<th>Gene</th>
<th>Target sequence</th>
<th>Forward primer 5’-3’</th>
<th>TaqMan Probe 5’-3’</th>
<th>Reverse primer 5’-3’</th>
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<tr>
<td><strong>FR-α</strong></td>
<td>Coding exon</td>
<td>AGGTGCGCAGTGGGAGC</td>
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<td>Sp1 promoter element</td>
<td></td>
<td>GCT</td>
<td>FAM-TGGTGCTAATCTACCTTTT CATTTGA-TAMRA</td>
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<tr>
<td><strong>p27</strong></td>
<td>Coding exon</td>
<td>CGGGGAGCCAGAGGGAGTTAA</td>
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<td>Sp1 promoter element</td>
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<td>GGTAA</td>
<td>FAM-TCCCTCTGTTTATTAATG AGACTCCGCTGTC-TAMRA</td>
<td>TCCGCCACACAGTTTA AAG</td>
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<td><strong>Thymidine Kinase 1</strong></td>
<td>Coding exon</td>
<td>GGCAGGCTGTGTTGCA</td>
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<td>Sp1 promoter element</td>
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<td><strong>p21</strong></td>
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<td>CTGGAGACTTCTCGGGA</td>
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<td>Sp1 promoter element</td>
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<td><strong>C-myc</strong></td>
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<td>CTGTCGCCACAGTCCAACACATCA GCACAA</td>
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<td>PRE promoter region</td>
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<td>CTGTCTTCTTCGGAAC</td>
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<td>CAGGCCGTTCCTTTAAAA CAAGT</td>
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<td><strong>Luciferase</strong></td>
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<td>TTCCGTGCTTCAAAACA ACA</td>
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<tr>
<td><strong>GAPDH</strong></td>
<td>Coding exon</td>
<td>CAACGGATTGGCTCGGTA</td>
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<td>GCAACAAATATCCACTTT ACCAGAGT</td>
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Table 1: DNA Sequences of Primers and TaqMan Probes for Real Time PCR Assays
Table 2: Amplification of Target Genomic DNA by the Real Time PCR Primers and TaqMan Probes\textsuperscript{a}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fr-α</th>
<th>p27</th>
<th>TK1</th>
<th>p21</th>
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<td>region</td>
</tr>
<tr>
<td>𝐶\textsubscript{t} Value\textsuperscript{b}</td>
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<td>18.0±0.1</td>
<td>13.3±0.1</td>
<td>17.8±0.1</td>
<td>18.1±0.1</td>
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</table>

\textsuperscript{a} Genomic DNA from untreated T47D-B cells was prepared as described for ChIP assays under Material and Methods and subjected to Real Time PCR as described under Material and Methods.

\textsuperscript{b} The 𝐶\textsubscript{t} values are the average of triplicate measurements. The 𝐶\textsubscript{t} values for negative controls (without target DNA sequence) were > 35.
**SUMMARY**

This study showed that the endogenous FR-α gene expression is up-regulated by GR and PR nuclear receptors through a unique mechanism and has laid the basis for using steroids such as dexamethasone and R5020 for improving the efficiency of tumor targeting as well as for screening of certain cancers alone or in combination with a well tolerated HDAC inhibitors. The study also added new insight into the partial agonist activity of RU486 and uncovered a large set of important genes that could be regulated in the same manner as FR-α. The results also clearly showed that the ultimate action of GR and PR on those genes requires the activation of intermediate (up-stream) target genes that are components of the transcription initiation complex acting as co-activators of Sp1.
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ABSTRACT

The folate receptor α (FR-α) is a cell surface protein that expressed in tissue selective manner and is capable of transporting folate, antifolates and folate conjugated molecules and nanoparticles into the cells. In contrast to the normal tissues where its expression is limited to the apical surface of epithelial tissues and isolated from the bloodstream, FR-α is over-expressed in certain gynecological tumors such as ovarian cancer where it is highly accessible via the circulation. Therefore, FR-α is a promising means for selective tumor targeting.

We have shown that the glucocorticoid (GR) and progesterone (PR) receptors positively regulate FR-α gene expression in cell culture and in tumor xenograft models. The expression of FR-α is enhanced further by combination of GR agonist with well-tolerated HDAC inhibitors such as valproic acid (VPA). Time course experiments and the use of protein synthesis inhibitor cycloheximide, showed that GR, PR-A and PR-B up-regulate FR-α expression indirectly in a ligand dependent manner. The promoter analysis of the FR-α gene showed the involvement of the G/C-rich Sp1 binding sites of the P4 promoter for the action of GR and PR and regulation by both receptors was optimal in the proper initiator context. Interestingly, the classical GR and PR receptor antagonist RU486 also activates FR-α expression but only through PR-B. Similar observations were made for PR regulation of the genes encoding p27, thymidine kinase 1, and p21. The results support the concept of increasing FR-alpha expression selectively in the receptor-positive tumors by brief treatment with a nontoxic dose of a GR and PR agonist, alone or in combination with a well-tolerated HDAC inhibitor, to increase the efficacy of various FR-alpha-dependent therapeutic and diagnostic applications. In addition, our findings contradict the
current view of Sp1-dependent gene regulation by PR and point to the existence of one or more PR target genes whose promoter and cell context(s) must be key determinants of the agonistic activity of RU486 on a large group of important Sp1-dependent downstream target genes.