Modulation of folate receptor-α by glucocorticoid receptor and progesterone receptor

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FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Medical Sciences

Modulation of Folate Receptor-α by Glucocorticoid Receptor
and Progesterone Receptor

Submitted by

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

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Modulation of Folate Receptor-α by Glucocorticoid Receptor and Progesterone Receptor

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Medical College of Ohio
2004
ACKNOWLEDGEMENTS

I would like to thank my major advisor, Dr. Manohar Ratnam for the time, effort and guidance throughout my Ph.D. training. I also would like to thank all the members of the advisory committee: Dr. Sonia Najjar, Dr. Brian Rowan, Dr. Lerim Shemshedini, and Dr. Robert Trumbly for their support during my training.

I would like to acknowledge the contribution of in vivo mouse data by Dr. Xuan Zheng for Manuscript One: Enhancement of Folate Receptor α Expression in the Receptor-Positive Tumor Cells Through the Glucocorticoid Receptor: A Means to Improve Tumor Targeting.

I would like to thank Dr. Brian Rowan (Medical College of Ohio) for the expression plasmids of hPR, SRC-1, SRC-2, pCAF and GRE/PRE-promoter luciferase construct. I would like to thank Dr. Sumudra Periyasamy (Medical College of Ohio) for the expression plasmids of Sp1 and Sp3. I would like to thank Dr. Guntram Suske (Institut fur Molekularbiologie und Tumorforschung Philipps-Universitat Marburg) for the expression plasmid of Sp4.

I also would like to thank all members of the lab: Dr. Xuan Zheng, Dr. Karen Kelly, Dr. Hong Hao, Huiling Qi, Aymen Shatnawi, Juan Zhang, Hala Elnakat, and Mariana Stoeva for all the helpful suggestions and technical contributions.
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INTRODUCTION

Folate receptor (FR) is a glycoprotein that exhibits high affinity for folic acid and circulating folate (6S)N5-Methyl tetrahydrofolate (Kd<10^{-9}M) (Antony 1996). Three isoforms of FR have been identified and characterized, FR-α (Elwood 1989; Lacey et al., 1989), FR-β (Ratnam et al., 1989) and FR-γ/FR-γ’ (Shen et al., 1994, 1995; Wang et al., 1998). The FR-γ’ is a truncated form of FR-γ due to a deletion mutation in the FR-γ gene (Shen et al., 1994; Wang et al., 1998). Folate receptors α and β can mediate the internalization of FR bound folate compounds and folate conjugates (Leamon and Low 1991; Turek et al., 1993; Wu et al., 1997).

The organization and basal promoters of FR genes have been characterized (Elwood et al., 1997; Sadasivan et al., 1994; Saikawa et al., 1995; Wang et al., 1998). Folate receptor genes’ promoters are characteristically TATA-less. Folate receptor α gene is characteristically controlled by two promoters P1 and P4 whose usage exhibits tissue specificity. The P1 promoter appears to lack a functional TATA box. The P4 promoter activity is primarily directed by a cluster of GC-rich sequences which are non-canonical Sp1 binding sites. The overall promoter activity is a result of cooperative interactions between the GC-rich sequences and a down-stream initiator region (Inr) (Saikawa et al., 1995).

The expression of FR isoforms is tissue specific. Folate receptor α is found on certain normal epithelial cells and highly expressed in various cancer tissues including non-mucinous ovarian adenocarcinomas and uterine endometrial endometrioid adenocarcinoma (Campbell et al., 1991; Ross et al., 1994; Weitman et al., 1992; Wu et
al., 1999). Folate receptor β is highly expressed in the placenta (Ratnam et al., 1989); mature neutrophils (Ross et al., 1999) where it is unable to bind folates; and in activated monocytes and macrophages (Nakashima-Matsushita et al., 1999). Also, FR-β is highly expressed in 70% of all acute myeloid leukemias (Pan et al., 2002b; Ross et al., 1999) which makes it an interesting research target for folate-mediated therapies. Folate receptor γ is undetectable in normal serum; however, it could be elevated in certain lymphoid malignancies.

In normal tissues, FR-α is restricted on the apical (luminal) aspect of the polarized epithelial cells, thus rendering it inaccessible through the circulation (Buist et al., 1995; Veggian et al., 1989; Weitman et al., 1992; Wu et al., 1999). However, it is accessible through the circulation in malignant tissues owing to the hypervascularization seen in these tissues. Folate compounds or conjugates can be effectively taken up into the cell by means of FR-α due to its ability to shuttle back and forth between the cell membrane and endosomal compartment.

Folate receptor mediated targeting has been shown to be highly specific and effective (Leamon and Low 2001; Pan et al., 2002). The degree of success of FR mediated targeting has been shown to correlate with the density of FR on the cell membrane (Leamon and Low 1994). Unfortunately, however, the expression of FR in tumors is highly variable within a given tumor type and widely heterogeneous within a tumor (Wu et al., 1999). Therefore, an optimal expression of FR in malignant tissues will help optimize the effectiveness of FR-mediated targeting. Previously, we have shown that FR expression can be manipulated by ligands of nuclear receptors. For example all trans retinoic acid (ATRA) can up-regulate FR-β in acute myeloid leukemia cells (AML)
providing a potential means to enhance delivery of folate conjugates specifically to AML cells (Pan et al., 2002b; Wang et al., 2000). Also, estrogen was found to repress FR-α promoter and this repression can be reversed by anti-estrogenic agents including tamoxifen and ICI 182 780 (Kelley et al., 2003). Here, we explore the possibility of using agonists of progesterone and glucocorticoid receptors to up-regulate FR-α expression in order to improve the effectiveness and efficacy of FR-targeting.

Glucocorticoid receptor and progesterone receptor are closely related members of type I of nuclear receptor family (Thornton 2001). They share many similar structural features and functional characteristics including association with a similar complex of molecular chaperones in the absence of hormone (Pratt and Toft 1997), dissociation from the chaperone complex and formation of dimmers upon ligand binding (Mangelsdorf et al., 1995), binding with high affinity to the same consensus GRE/PRE (glucocorticoid/progesterone response element) in the nucleus (Cato et al., 1986; Hynes et al., 1983; Lieberman et al., 1993; Payvar et al., 1983; Scheidereit et al., 1983) and recruitment of a similar set of co-activators in the presence of hormone target gene promoters to modulate transcription (McKenna et al., 1999; Westin et al., 2000). Despite all of these similarities, the cognate hormones elicit quite distinct physiological actions. (Corroyer et al., 1997; Frost et al., 1994; Rhee et al., 1995; Rogatsky et al., 1997; Sanchez et al., 1993).

Classical actions of glucocorticoids include regulation of metabolism, inhibition of inflammation and the immune system, and suppression of bone formation (Lane and Lukert 1998; Masuzaki et al., 2001; Rackoff and Rosen 1998; Rosmond et al., 1998; Schacke et al., 2002; Ziegler and Kasperk 1998). The major physiological roles of
progestins in the mammals are maintaining pregnancy, promoting lobular-alveolar development in the mammary gland, and to suppress milk protein synthesis before parturition (Graham and Clarke 1997).

We have found that both in cell culture and in a tumor xenograft mouse model, Dexamethasone (Dex) can up-regulate the FR-\(\alpha\) gene in Hela cells. This up-regulation is significantly enhanced when histone deacetylase inhibitors are used in combination with Dex. In addition, in Hela cells co-transfected with a FR-\(\alpha\) promoter luciferase reporter construct and an expression vector for either progesterone receptor A or B, the progestin R5020 treated cells show up to 10-fold increase in the expression of the luciferase reporter compared to the untreated cells. Furthermore, in non-responsive cells such as T47D, co-treatment of Dex or R5020 with trichostatin A can up-regulate the expression of the FR\(\alpha\) promoter luciferase reporter construct. The detailed studies in this thesis explain the mechanisms of FR-\(\alpha\) regulation by Dex and progestin. Our findings establish the usefulness of employing Dex, R5020 and HDAC inhibitors to up-regulate FR-\(\alpha\) in order to improve FR-\(\alpha\)-mediated targeting.
Folate Receptor

Folate Receptor Iso-forms

Folate receptor (FR) is a glycoprotein that exhibits high affinity for folic acid and circulating folate (6S)N5-methyl tetrahydrofolate (Kd<10^-9M) (Antony 1996). Most normal adult tissues lack FR. However, it is highly expressed in placenta. It is also expressed in choroid plexus, proximal kidney tubules and epidermoid carcinoma cells KB (Antony et al., 1981; Antony 1992; Elwood et al., 1986; Selhub et al., 1979; Selhub and Franklin 1984; Suleiman and Spector 1981). A soluble high affinity folate binding protein sFBP is found in milk, cerebrospinal fluid, amniotic fluid and KB cell culture media (Antony et al., 1982; da Costa and Sharon 1980; Elwood et al., 1986; Hansen et al., 1985, 1989; Holm et al., 1990; Kamen and Caston 1975). This sFBP is thought to be derived from a membrane associated precursor FR (Kane et al., 1986; Luhrs and Slomiany 1989; Verma et al., 1992). Normal serum contains no detectable sFBP while umbilical cord serum contains sFBP likely from FR in the placenta.

Three isoforms of FR have been identified and characterized, FR-α (Elwood 1989; Lacey et al., 1989), FR-β (Ratnam et al., 1989) and FR-γ (Shen et al., 1994, 1995). Folate receptors α and β are GPI-anchored membrane proteins whereas FR-γ is a secretory protein for lack of the signal for GPI attachment (Lacey et al., 1989; Shen et al., 1995; Yan and Ratnam 1995). In addition, there is a truncated form of FR-γ, the FR-γ’, generated as a consequence of a deletion mutation in the FR-γ gene, thus producing polymorphism for the FR-γ gene (Shen et al., 1994; Wang et al., 1998). Folate receptors
α and β are important in that they can mediate the internalization of FR bound folate compounds, folate conjugates and antifolate drugs (Leamon and Low 1991; Turek et al., 1993; Wu et al., 1997). The difference in the primary structure of FRα and β results in their different binding affinities and stereospecificities for folate compounds and antifolates (Maziarz et al., 1999; Shen et al., 1997; Wang et al., 1992).

The expression of FR isoforms is tissue specific. Folate receptor γ is undetectable in normal serum; however, it could be elevated in certain lymphoid malignancies. Folate receptor β is highly expressed in the placenta (Ratnam et al., 1989); mature neutrophils (Ross et al., 1999) where it is unable to bind folates; in activated monocytes and macrophages (Nakashima-Matsushita et al., 1999). Especially, FR-β is highly expressed in about 70% of all acute myeloid leukemias (Pan et al., 2002b; Ross et al., 1999) which makes it a candidate target for FR-mediated therapies.

Folate receptor α is interesting in that besides being found on certain normal epithelial cells, it is highly expressed in various cancer tissues including non-mucinous ovarian cancers, uterine endometrial endometrioid adenocarcinoma (Campbell et al., 1991; Ross et al., 1994; Weitman et al., 1992; Wu et al., 1999). It is found expressed less frequently in cervical adenocarcinoma and absent in cervical squamous cell carcinoma. Folate receptor α is also found in certain normal epithelia and in normal reproductive system including germinal epithelium of the ovary, the surface epithelium of the uterus and the columnar and glandular epithelia of the uterine cervix. In those places, FR-α is restricted on the apical (luminal) aspect of the polarized epithelial cells, thus rendering it inaccessible through the circulation (Buist et al., 1995; Veggian et al., 1989; Weitman et al., 1992; Wu et al., 1999).
**Organization of Folate Receptor Genes** (Figure 1)

The folate receptor genes are located on chromosome 11, region 11q13.3-q13.5 (Ragoussis et al., 1992). The organization and basal promoters of these genes have been characterized (Elwood et al., 1997; Sadasivan et al., 1994; Saikawa et al., 1995; Wang et al., 1998). They are approximately 5Kb-7Kb. They share similar nucleotide sequences in the protein coding regions but display distinctive 5’ flanking regions upstream of the translation initiation site. Folate receptor β and γ show closer similarity than either of them does with FR-α. Specifically, FR-β and γ possess similar intron-exon organization. They both have 5 exons, 4 introns and the protein encoding sequence starts in exon 2. Folate receptor α has 7 exons, 6 introns and multiple transcripts as the result of differential promoter usage and alternative splicing involving exon 1-4. The protein encoding sequence starts in exon 4.

Folate receptor genes’ promoters are characteristically TATA-less. In FR-α gene, P4 is primarily driven by a cluster of GC-rich sequence, which are non-canonical Sp1 binding sites. The overall promoter activity is a result of cooperative interactions between the GC-rich sequences and a down-stream initiator region (Inr) (Saikawa et al., 1995). The P1 appears to lack a functional TATA box. All the transcripts produced by promoter P1 and P4 of FR-α gene have an identical protein coding sequence, thus all can be translated into an identical FR-α protein. However, the 5’ un-translated region for each transcript is unique as a result of different alternative splicing events. Transcripts resulting from P1 and P4 exhibit comparable stability (Roberts et al., 1997). However, transcripts resulting from P1 promoter are translated less efficiently (Roberts et al., 1997).
Figure 1: FRα gene

P1, P4: promoters
+1: transcription start site for P4
1-7: exons
A-F: introns
In malignant tissues and cell lines, the most abundant transcript species are the ones resulting from P4 promoter. The preferential usage of either P1 or P4 is tissue specific (Roberts et al., 1997).

**Regulation of Folate Receptor Expression**

The expression of FR can be regulated by the extra-cellular concentration of folate in some cells (Kane et al., 1988; McHugh and Cheng 1979). The lower the folate concentrations in the medium, the more rapid is the induction of FR expression. This increased FR expression in response to low folate concentrations in the medium can be reversed by addition of folic acid or reduced folate co-enzymes to the medium. In cervical carcinoma cells, folate-dependent up-regulation of FR-α expression was found to occur at translational level (Sun and Antony 1996). In this study, folate dependent up-regulation of FR-α was found due to the interaction between a 46KDa cytosolic protein and an 18 base cis element in the 5’ un-translated region of the transcripts. In other instances, folate dependent regulation was found to occur by changing the abundance of the transcripts (Hsueh and Dolnick 1993).

The expression of FR also can be regulated by ligands of nuclear receptors. For example all trans retinoic acid (ATRA) can up-regulate FR-β in acute myeloid leukemia cells (AML) providing a potential means to enhance delivery of folate conjugates specifically to AML cells (Hao et al., 2003; Pan et al., 2002b; Wang et al., 2000). For FR-α, it was found to be down-regulated by ATRA in a squamous cell carcinoma cell line (Orr et al., 1995), up-regulated in mouse embryonic stem cells (Bolton et al., 1999). However, ATRA does not alter the expression of FR-α in many other cell lines including Hela cells (Wang et al., 2000). Also, estrogen was recently found to repress FR-
α promoter by inducing a direct interaction of estrogen receptor α (ERα) with one of the GC-rich sequences in the FR-α P4 promoter. This repression can be reversed by antiestrogenic agents including tamoxifen and ICI 182 780 (Kelley et al., 2003).

**Physiological Function of Folate Receptor**

Folate receptor functions in transport of folates into the cell even though other mechanisms can be employed in adult tissues. In the placenta, FR-α is required for transport of folates from maternal blood to fetal tissues (Antony 1992). In the kidneys, FR-α may be required for urinary reabsorption of folate (da Costa et al., 2000). It has been reported that FR-α is essential for neural tube development in the absence of very high levels of folate (Piedrahita et al., 1999). This helps explain the correlation between neural tube defects in newborns and low consumption of folate in their mothers.

**Clinical Importance of Folate Receptor**

Clinically, FR can serve as cell surface marker for folate mediated tumor targeting for a number of reasons. First, folate compounds can bind to FR with high affinity which in turn can be internalized into the cell where they will dissociate from FR molecules, freeing the FR molecules for recycling to the cell surface ready for a new round of folate uptake. This mechanism provides an efficient means for internalization of folate compounds and conjugates. Secondly, FR-α is expressed on the apical aspect of the epithelial cells in normal tissues (except in the placenta), which makes it inaccessible to the general circulation. However, it is accessible through the circulation in malignant tissues owing to the hypervascularization seen in these tissues. Folate receptor α has been found to be highly expressed in ovarian cancers derived from ovarian epithelium, endometrioid endometrial adenocarcinoma, testicular choriocarcinoma, ependynomas and
menigiomas of the brain, and in some colon, kidney and breast cancers (Mattes et al., 1990; Wu et al., 1999). Interestingly, it has been found that in ovarian cancers in which FR-α is highly expressed, the levels of FR-α expression is higher in high grade, undifferentiated or metastatic cancer cells than the low grade localized counterpart (Toffoli et al., 1997). These findings offer a potential strategy for combating low grade, metastatic ovarian cancers, which account for almost all the failed attempts in treatment of ovarian cancers, by FR mediated specific targeting of cancer cells.

In addition, FR isoforms are expressed in a tissue specific manner. Folate receptor β is expressed in hematopoietic tissues and malignancies derived from those tissues, where as FR-α is expressed in epithelia and cancers derived from epithelia such as some specific types of gynecological cancers. Also, the preferential usage of either P1 or P4 of the FR-α gene is tissue specific (Roberts et al., 1997). If one understood the molecular mechanism of this tissue specific expression of FR and the tissue specific usage of P1 and P4 at the transcriptional level of gene expression, one could apply this knowledge in designing a therapeutic gene that could be modulated and expressed in a tissue specific manner, providing a potential strategy for cancer tissue specific expression of the therapeutic gene in gene therapy.

Folate receptor targeted therapies have been shown to be highly specific. For example, folate coated liposomes entrapping Doxorubicin are highly specific for FR-β expressing cells (Pan et al., 2002b). Folate receptor α can be employed as a potential means of delivering therapeutic agents to tumor cells, and as tumor and serum marker (Leamon and Low 2001; Reddy and Low 1998; Sudimack and Lee 2000). Radionuclide conjugates of folic acid have been shown to be a promising means for noninvasive
visualization of FR positive tumors (Leamon and Low 2001). Folic acid conjugates of cytotoxics can specifically kill FR-α positive but not FR-α negative cells (Ladino et al. 1997; Leamon et al. 1993; Leamon and Low 1992, 1994). Some experimental folic acid conjugates of various radiopharmaceuticals are potential means for noninvasive diagnostic and radiotherapeutic procedures (Andersson et al., 2000; Mathias et al., 1998, 1999, 2000). One can synthesize bi-specific antibodies that can direct cytotoxic T cells or IL2 to the tumor to elicit an immune reaction against it (Canevari et al., 1995, 1997; Luiten et al., 1997; Mazzoni et al., 1996; Melani et al., 1998; Mezzzanica et al., 1991). Folate receptor can also be used as a tumor marker for delivery of various therapeutic agents including antisense oligonucleotides/cytotoxics entrapped in folate coated liposomes, folate coated adenoviral vector, folate conjugates of prodrug activating enzymes (Douglas et al., 1996; Goren et al., 2000; Lee and Low 1994; Lu et al., 1999; Wang et al., 1995). Some anti-folate drugs can be taken up by means of FR (Westerhof et al., 1995). The application of FR can be extended to making DNA and polypeptide vaccines against tumor cells (Kim et al., 1999; Neglia et al., 1999; Peoples et al., 1999; Rodolfo et al., 1998, 1999).

**Nuclear Receptors**

**Classification of Nuclear Receptors**

Nuclear receptors (NRs) are composed of a large group of nuclear transcription factors (Giguere 1999) that share remarkable structural and organizational similarities. They are grouped into three subclasses (McKenna and O'Malley 2002). The type I subclass (the steroid receptors) of NRs includes the receptors for progesterone (PR), estradiol (ER), androgen (AR), glucocorticoid (GR) and mineralocorticoid (MR).
Without the presence of ligand, they are sequestered in a multiprotein complex consisting of heat shock protein hsp90, hsp60, hsp70, p23 and immunophilins (DeMarzo et al., 1991; Pratt 1993, 1998; Pratt and Toft 1997; Pratt and Toft 2003) and do not modulate gene transcription. Steroid receptor-hsp90 heterocomplexes are formed in an ATP-dependent process by a multi-protein chaperone system (Scherrer et al., 1990; Smith et al., 1990). Upon binding to the ligand, these receptors form dimers in solution or on DNA in order to be functional (DeMarzo et al., 1991; Mangelsdorf et al., 1995). The ligand bound dimers bind to specific DNA sequences (the hormone responsive elements: HRE) located in promoter regions and modulate transcription (Bagchi et al., 1988; Renoir et al., 1990; Tsai et al., 1988). The type II subclass includes the thyroid receptor (TR), the retinoic acid receptor (RAR), the retinoid X receptor (RXR) and the vitamin D3 receptor (VDR). These receptors constitutively bind to DNA even in the absence of ligand. They interact with direct repeats of the 5’-AGGTCA-3’ core recognition sequence with variable spacer lengths (Glass 1996; Mangelsdorf et al., 1995). The type III subclass is comprised of orphan receptors for which no natural ligands have been identified. Some examples of this group of NRs include peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR) and farnesoid X receptor (FXR) that function as lipid and bile-acid sensors; and pregnane X receptor (PXR) and constitutive androstane receptor (CAR) that function as xenobiotic sensors (Mohan and Heyman 2003).

**General Organization of Nuclear Receptors** (Figure 2)

The NRs share many similar structural features. In general, a NR molecule consists of 4 principal domains: A/B, C, D, E/F in the order from N-terminus to C
Figure 2: general organization of nuclear receptor proteins

A-F: individual domains
N, C: N and C termini
NTD: N-terminus domain
DBD: DNA-binding domain
Hinge: hinge region
LBD: Ligand-binding domain
AF-1, AF-2: Activation function 1 and 2
terminus (Beato et al., 1995; Evans 1988; Krust et al., 1986). The A/B domain is the N-terminus domain (NTD). This NTD is highly variable among the NR and contains most of the phosphorylation sites in NR. It harbors the transcriptional activation function region 1 (AF-1) (Danielian et al., 1992; Lees et al., 1989; Meyer et al., 1992; Tora et al., 1989). Domain C is the most conserved among NR and is folding into two structures called zinc-fingers. It is the DNA binding domain and mediates the interaction between the receptor and DNA (Kumar and Chambon 1988). Domain D is the hinge region. Domain E/F is the ligand binding domain (LBD) and is a major interaction region for co-activators and co-repressors. It is somewhat conserved among the NR and confers specificity for ligand. The LBDs derived from several NR have been crystallized and known to fold into a three layered helical sandwich (Bourguet et al., 1995; Renaud et al., 1995; Sack et al., 2001; Wagner et al., 1995; Williams and Sigler 1998). They possess the transcriptional activation function region 2 (AF-2) which is made up of about eight amino acid residues in the C terminal of the E/F domain (Danielian et al., 1992; Lees et al., 1989). The AF-2 has been described as consisting of helices 3, 4, 5 and 12 which appear to form a protein interaction surface (Feng et al., 1998; Parker and White 1996; Wurtz et al., 1996). This AF-2 mediates ligand dependent activation of the NR (Durand et al., 1994; Jimenez-Lara and Aranda 1999; Onate et al., 1998; Sheldon et al., 1999). The 3-D structures of isolated DBDs and LBDs of various NRs have revealed conserved structural motifs that form the basis for sequence specific DNA binding and ligand binding (Bourguet et al., 2000). Receptor dimerization induces positive cooperativity in ligand binding to the receptor (Brandt and Vickery 1997; Carbajo et al., 1996). Also, DNA can influence ligand-receptor interaction even though no obvious general pattern regarding
this aspect has been identified (Aliau et al., 1995; Fritsch et al., 1992; Klinge 1999; Tyulmenkov and Klinge 2001).

**Ligand Induced Conformational Change in the NR Molecule and Related Events**

Binding of the ligand to the NR molecule induces changes in the receptor’s conformation, enabling it to interact with co-regulators and chromatin remodeling factors, leading to alterations in the chromatin structure of the promoter region and recruitment of the transcriptional machinery to initiate transcription (Bagchi et al., 1990; Bourguet et al., 2000; Dilworth and Chambon 2001; Freedman 1999; Lemon and Freedman 1999; Leo and Chen 2000; McKenna et al., 1999; Onate et al., 1995; Smith et al., 1996; Urnov and Wolfe 2001a,b; Voegel et al., 1996; Xu et al., 1999). The helix 12 of AF-2 in the ligand binding domain (LBD), through changes in its spatial orientation depending on whether the ligand is an agonist or an antagonist (Beato et al., 1995; Tanenbaum et al., 1998; Williams and Sigler 1998) influences the selective co-regulator recruitment by the NR (Wagner et al., 1998). From available crystal structures, it is inferred that the LBDs of NRs adopt distinct conformations as apo-receptors, holo-agonist and holo-antagonist bound conformations appropriate for interactions with chaperone proteins, co-activators (McInerney et al., 1998) or co-repressors (Hu and Lazar 1999), respectively. The helices 3 and 4 form a co-activator binding site that interacts with the LxxLL motif of the co-activator; and helix 12 determines the agonist and antagonist conformations of the NR (Bourguet et al., 2000; Brzozowski et al., 1997; Shiau et al., 1998). Agonistic ligands bind to the NR and induces a spatial rearrangement of helix 12 of AF-2 in a way that allows the accommodation of p160 co-activators within the hydrophobic cleft of the
receptor LBD through direct interaction with the LxxLL motifs of the co-activators (Brzozowski et al., 1997; Darimont et al., 1998; Feng et al., 1998; McInerney et al., 1998; Nolte et al., 1998; Renaud et al., 1995; Wagner et al., 1995). On the contrary, antagonists induce an alternative spatial arrangement of helix 12 that blocks p160 co-activator binding (Glass and Rosenfeld 2000; Shiau et al., 1998).

**NR-mediated Chromatin Remodeling**

Chromatin remodeling is a critical event in transcriptional modulation by NRs. Nuclear receptors have been reported to bind DNA and induce chromatin changes (Deroo and Archer 2001b; Grange et al., 2001; Heinlein and Chang 2002; Hsia and Shi 2002; Lemon and Freedman 1999; McKenna et al., 1999; Robyr et al., 2000a,b; Smith and Hager 1997; Urnov and Wolfe 2001a,b; Villagra et al., 2002; Weintraub 1983; Willis and Seyfred 1996). To date, two groups of chromatin remodeling factors have been described. The first group includes those that co-valently modify components of the nucleoprotein structure including histone acetylases and deacetylases, methylases and kinases (Mizzen and Allis 2000; Sterner and Berger 2000). The second group is comprised of ATP-dependent chromatin-remodeling complexes (CRCs) that require ATP to disrupt chromatin (Becker and Horz 2002; Kingston and Narlikar 1999; Krebs and Peterson 2000; Peterson 2000; Peterson and Workman 2000; Vignali et al., 2000; Workman and Kingston 1998).

**NR-induced CRC-mediated Chromatin Remodeling**

The CRCs are highly conserved from yeast to humans (Becker and Horz 2002; Kingston and Narlikar 1999; Krebs and Peterson 2000; Peterson 2000; Peterson and Workman 2000; Vignali et al., 2000; Workman and Kingston 1998). Three families of
CRCs, namely Swi/Snf, ISWI and Mi-2 (Becker and Horz 2002) have been described based on the core ATPase subunit contained within the complex. Among the three, the Swi/Snf complex has been shown in vivo to interact with some NRs in a ligand dependent manner (Belandia et al., 2002; Fryer and Archer 1998; Nie et al., 2000). The Swi/Snf complex was originally identified in Saccharomyces cerevisiae (Vignali et al., 2000). It exhibits certain properties that earn it the title “chromatin-remodeling complex.” Those include causing chromatin DNA to be attacked more readily by endonucleases in its presence (Logie and Peterson 1997); having the ability to slide nucleosomes in cis (to other sites on the same stretch of DNA) (Lorch et al., 1999) or to transfer histone octamers in trans conformations (to other DNA molecules) (Owen-Hughes and Workman 1996; Whitehouse et al., 2000); being able to alter the structure of a single nucleosome to an alternative stable conformation (Schnitzler et al., 1998). In Drosophila and humans, Swi/Snf-related complexes have been identified (Dingwall et al., 1995; Sudarsanam and Winston 2000). The BRG-1 (brahma-related gene 1)-associated factor (BAF) and PBAF (Polybromo, BRG-1 associated factor) complexes are human version of the Swi/Snf complex (Wang et al., 1996a,b). Each complex contains an ATPase subunit being either BRG1 or brm (brahma). The more than 70% identical BRG1 and brm ATPase subunits are highly homologous to yeast ATPase subunit Swi/Snf2 (Eisen et al., 1995) and are associated with other proteins known as BRG1-associated factors or BAFs (BAF250, BAF180, BAF170, BAF155, BAF110, BAF60, BAF57, BAF53, and BAF47) to make up the BAF and PBAF complexes. BAF250 is unique for the BAF complex and BAF180 for the PBAF complex (Nie et al., 2000). Brahma-related gene 1 (BRG1), BAF47, and BAF155/BAF170 form the minimal catalytic core that is able to remodel
mononucleosomes and nucleosomal arrays *in vitro* (Phelan et al., 1999). The involvement of BRG-1 containing complexes in NRs transcriptional activation has been expertly studied using a model consisting of GR and the MMTV (mouse mammary tumor virus) promoter (also see Mechanism of GR).

**NR-induced Chromatin Remodeling by Modification of Histones**

Chromatin remodeling as a result of histone acetylation also is a critical step in NR-mediated transactivation (Chen et al. 1999a). The degrees of histone acetylation, thus transcriptional activity of the promoter are determined by both histone acetyltransferases (HATs) and histone deacetylases (HDACs) that may be recruited to the promoter region by NR-co-regulators complex. In general, histone acetylation results in transcriptional activation and histone deacetylation in transcriptional inertness, although this may not always be true (Deckert and Struhl 2001; Khochbin et al., 2001; Kuo et al., 2000; Ura et al., 1997; Wolff 1997). Three families of HATs have been identified. They are the GCN5-related N-acetyl-transferase (GNAT, also known as SAGA [Spt-Ada-Gnc5 acetyltransferases]/GCN5 [General Control Non-derepressible-5]/pCAF [p300/CBP-associated factor]), the Moz (Monocytic leukemia Zn finger protein), Ybf2/Sas-3 (something about silencing-3, Tip60 (Tat-interaction protein-60) (MYST, also known as NuA4/Tip60), and the p300/CBP (CREB [cyclic AMP-responsive element-binding protein]-binding protein) family (Brown et al., 2000; Brownell et al., 1996; Narlikar et al., 2002; Ogryzko et al., 1996; Yang et al., 1996). Among the HDACs, two major complexes: the HDAC1/mSin-3A and the HDAC2/NURD (NUclear Remodeling and Deacetylation) complexes contain co-repressors in their composition (Khochbin et al., 2001) (also see Histone deacetylases and their inhibitors). According to current paradigm,
ligand binding to NRs alters the association of receptors with co-regulators (Blanco et al., 1998; Fondell et al., 1996; Hanstein et al., 1996; Kraus and Kadonaga 1998; Naar et al., 1999; Rachez et al., 1999). Upon ligand binding the receptor molecule undergoes conformational change in a way that facilitates its selective recruitment of co-regulators. The NR-coregulators complex then interacts with HREs located in the promoter regions of target genes. Co-regulators possess chromatin-remodeling properties through their enzymatic activities (histone acetyl transferase or deacetylase) that enable them to modulate transcription of target genes. Gene activation requires interaction with co-activators whereas gene repression requires co-repressors (Burke and Baniahd 2000). Co-activators recruited by agonist bound NR include members of the p160/SRC (160 kDa or steroid recetor co-activator) family of proteins (Cavailles et al., 1994; Halachmi et al., 1994) which are encoded by three distinct genes referred to as SRC-1 (steroid receptor co-activator-1) (Onate et al., 1995), SRC-2 (steroid receptor co-activator-2, also known as TIF-2: transcriptional intermediary factor-2, GRIP-1: glucocorticoid receptor interacting protein-1) (Hong et al., 1996, 1997; Voegel et al., 1996) and SRC-3 (steroid receptor co-activator-3, also known as ACTR: activator of the thyroid and retinoic acid receptor, AIB-1: amplified in breast cancer-1, p/CIP: p300/CBP co-integrator associated protein, RAC-3: receptor-associated co-activator-3, TRAM-1: TR activator molecule-1) (Anzick et al., 1997; Chen et al., 1997; Torchia et al., 1997). Recently, TRAP (thyroid hormone receptor-associated protein) (Fondell et al., 1996, 1999) and DRIP complexes (vitamin D3 receptor interacting protein) (Rachez et al., 1998) also have been shown to potentiate transcription by NRs. Also, they are found to be related to a number of other co-factors complexes (Ito et al., 1999; Rachez et al. 1999). The agonist bound NR binds
to and recruits the p160/SRC co-activators to target promoters (Beato et al., 1995; Chen et al., 1997; DiRenzo et al., 1997; Feng et al., 1998; Glass et al., 1997; Henttu et al., 1997; Hong et al., 1997); they in turn recruit additional proteins (Chen et al., 1997; Yao et al., 1996). The p160/SRC co-activators share a unique structural feature, namely the presence of LxxLL motifs (NR boxes) in their molecule (Heery et al., 1997; Torchia et al., 1997) that allow them to interact with NRs to potentiate trans-activation by NRs (Darimont et al., 1998; Ding et al., 1998; Heery et al., 1997; Kalkhoven et al., 1998). They function as bridging proteins that facilitate the NR’s recruitment of other types of co-activators such as p300/CBP (Chakravarti et al., 1996; Hanstein et al., 1996; Kamei et al., 1996; Torchia et al., 1997; Voegel et al., 1998), or pCAF (Blanco et al., 1998; Lee et al., 2001b); via their activating domain 1 (AD1) (Ma et al., 1999). Furthermore, SRC-1 (Hassig and Schreiber 1997; Spencer et al., 1997), SRC-3 (Chen et al., 1997), p300/CBP (Bannister and Kouzarides 1996; Ogryzko et al., 1996; Xu et al., 1999) and pCAF (Yang et al., 1996) all have intrinsic HAT activity. Acetylation of core histones by HATs alters nucleosomal packing to increase access of transacting factors and components of the basal transcriptional machinery to the local DNA, thus facilitating trans-activation (Wade et al., 1997). Ligands can regulate NR function not only by inducing recruitment of co-regulators but also by influencing the availability of those regulatory proteins. For instance, SRC-1 mRNA and protein levels were shown to be down-regulated by Dex in vitro and in vivo (Kurihara et al., 2002).

The functions of p160/SRC proteins are not simply limited to serving as co-activators for NRs, many of these co-regulatory proteins have other functions as well. Particularly, SRC-1 was shown to activate transcription via interaction with AP-1
(activation protein-1) (Lee et al., 1998), NF-κB (nuclear factor κB) (Na et al., 1998), SRF (serum response factor) (Kim et al., 1998) and p53 (Lee et al., 1999). Emerging evidence suggests that p160/SRCs play distinct roles in different biological processes (Borud et al., 2002; Gehin et al., 2002; Leers et al., 1998; McKenna et al., 1999; Xu et al., 1998).

The most studied co-repressors to this date are: nuclear receptor co-repressor (N-CoR), silencing mediator of retinoic receptors and thyroid receptors (SMRT) and Alien (Chen et al., 1996; Dressel et al., 1999; Heinzel et al., 1997; Horlein et al., 1995; Nagy et al., 1997). The N-CoR and SMRT have been shown to associate with mSin3A and HDAC complexes which are deacetylases (Hassig et al., 1997, 1998; Nomura et al., 1999; Zhang et al., 1999). Unliganded NRs are thought to associate with co-repressors resulting in tonic repression of NRs in the absence of ligands (Xu et al., 1999). However, upon binding to appropriate agonistic ligands, they give up co-repressors and bind to co-activators (Leo and Chen 2000; Mangelsdorf et al., 1995). For example, TR bound to a simple thyroid hormone response element (TRE) are associated with co-repressors N-CoR, SMRT or Alien (Chen et al., 1996; Dressel et al., 1999; Horlein et al., 1995), which in turn bind mSin3A and HDACs (Heinzel et al., 1997; Nagy et al., 1997). However, repression by steroid receptor appears to require neither HDAC activity nor co-repressors N-CoR or SMRT. This is inferred from the fact that no functional co-repressors have been identified for steroid receptors (Chen and Evans 1995; Dressel et al., 1999; Horlein et al., 1995).

**Specificity of NR Actions**

Despite sharing many common co-regulators, NRs do exhibit differential biological activities. These functional differences could in part be explained by the
preferential recruitment of different p160/co-activators by different NRs (Bramlett and Burris 2002; Mukherjee et al., 2002). For example, SRC-1 and SRC-2 have been reported to exhibit similar but not identical binding preferences to different NRs. The AR binds well to SRC-2 but poorly to SRC-1 (Ding et al., 1998). The basic for differential interactions between various p160/SRC co-activators and different NRs resides in the intricate structural differences of the LBDs of the NRs that result in preferential interactions with individual NR boxes of the p160/SRC co-activators (Darimont et al., 1998; Ding et al., 1998; McInerney et al., 1998; Voegel et al., 1998). For instance, the VDR and ERβ interact with different α helical NR boxes of SRC-3 (Leo et al., 2000); GR and TR LBDs exhibit different affinity for NR box 2 or 3 of SRC-2 (Darimont et al., 1998). These differential interactions also are influenced by different conformational changes induced by different ligands or receptor-DNA interactions (Lefstin and Yamamoto 1998). Besides structural differences in the LBDs of NRs, differences in their N-terminal regions also play a critical role in determining which gene promoters and which functional pathways are regulated by these receptors (Abdel-Hafiz et al., 2002; Bain et al., 2000, 2001; He et al., 2002; Kumar and Thompson 2003; Tung et al., 2001). In addition, since the functions of components of co-activator complexes appear to be distinct (Korzus et al., 1998), the composition of various co-activator complexes interacting with different NRs probably dictates the differential biological activities of the NRs.

**Non-transcriptional Actions of NRs**

The functions of NRs are not only limited to regulation of transcription, but also involve control of biologic processes through non-transcriptional mechanisms. This is
most notably seen in type I of NRs. Estrogen and androgen receptors can prevent bone
loss through activation of protein kinase Src and MAPK (mitogen-activated protein
kinase) (Kousteni et al. 2000, 2002). Activation of PI3K (phosphotidylinositol 3-
kinase)/Akt pathway by estrogen is ERα dependent which leads to activation of epithelial
NO synthase (eNOS) resulting in increased epithelial production of nitric oxide (NO) and
subsequent vasodilation, a vascular protective effect of estrogen (Chen et al., 1999b;
Haynes et al., 2000; Simoncini et al., 2000). Estrogen receptor β may also mediate
cellular responses through nongenomic pathways (Chambliss et al., 2002).

**Progesterone receptor:**

**Organization of progesterone receptor** (Figure 3)

Progesterone receptor (PR) is a member of type I subclass of nuclear receptors
and exists in two iso-forms PRα (97 kD) and PRβ (120 kD). They both are derived from a
single gene driven by two distinct estrogen-inducible promoters and result from
translation initiation at two alternative AUG signals (Conneely et al., 1989; Kastner et al.,
1990). PRα and PRβ are identical except that PRβ possesses an extra 164-residue
sequence at the N-terminus named B upstream segment (BUS). Some studies have
identified unique functions of this BUS in PRβ (Giangrande et al., 1997; Hovland et al.,
1998; Huse et al., 1998; Meyer et al., 1990; Sartorius et al., 1994). The structure of PR
protein is similar to that of members of the nuclear receptor family. In short, each isoform
contains four principal domains: the A/B (NTD) domain containing AF-1 which is
required for partial agonist activity of progesterone antagonists (Meyer et al. 1990), the C
(DBD) domain, the D domain or the hinge region (H), and the E (LBD) domain
containing AF-2. Also, the PRβ contains an additional activation function region (AF-3)
Figure 3: Organization of the PRa and PRb proteins

- A/B: N-terminus domain (NTD)
- C: DNA binding domain (DBD)
- D: the hinge region.
- E: Ligand binding domain (LBD)
- AF-1, 2 & 3: Activation Function 1, 2 & 3
- BUS: B Upstream Sequence
within the BUS (Sartorius et al., 1994) that contributes to differential co-regulator recruitment by PRα and PRβ (Giangrande et al., 2000) and to distinct cell and promoter specific trans-activation properties of the iso-forms (Giangrande and McDonnell 1999; Hovland et al., 1998; Tora et al., 1988). The activation function (AFs) regions do not function independently. The AF-1 and AF-2 have been shown to exhibit high degree of synergism mediated by an intra-molecular interaction between the N and C-termini facilitated by p160/SRC protein co-activators (Tetel et al., 1999). The AF-3 also synergizes with AF-1 and AF-2 (Meyer et al., 1992; Tung et al., 2001).

With the advent of crystallography, 3-D crystal structure of PR LBD has been described (Tanenbaum et al., 1998; Williams and Sigler 1998). It consists of 12 α helices and two short β-pleated sheets. The 3-D crystal structure of LBD of PR shares remarkable similarities with that of ER (Pike et al., 1999; Tanenbaum et al., 1998; Williams and Sigler 1998) and probably with those of other steroid receptors.

**Mechanism of PR Actions**

In the absence of hormone, PR isoforms are complexed with several chaperone molecules including heat shock protein hsp90, hsp70, hsp40, Hop and p23. These interactions are requisite for proper protein folding and assembly of stable steroid receptor-hsp90 hetero-complexes which in turn acquire the ability to bind ligand (Pratt 1998; Pratt and Toft 2003). Upon ligand binding, the PR molecule undergoes a conformational change, allowing it to dissociate from hsp, dimerize and become capable of interacting with specific progesterone response elements (PREs) in the promoter regions of target genes (Chalbos et al., 1987; Moore et al., 1997). The interaction between ligand and PR is reinforced upon binding to DNA. For instance, DNA has been
shown to slow the dissociation of progesterone from the PR-progesterone complex (Pokrovskaya et al., 2003). Counter-intuitively however, it has been shown there is a reverse correlation between liganded PR affinity for DNA and the agonisticity of the ligands (Shchelkunova et al., 2002). In the cell, even though PR is predominantly nuclear, it has been shown to shuttle between nucleus and cytoplasm (Guiochon-Mantel et al., 1991).

Like other NRs, PR regulates transcription by recruiting regulators to promoters of target genes. The recruitment of either co-activators or co-repressors is determined at least in part by whether the ligand is agonist or antagonist. For example, RU-486, an anti-progestin, has been shown to cause PRb to preferentially recruit co-repressors including SMRT, NCoR or an NCoR related factor (Beato et al., 1995; Wagner et al., 1998). Progesterone receptor also exhibits selective recruitment of co-regulators, leading to preferential histone modification. For instance, it preferentially recruits SRC-1 and CBP, resulting in acetylation of K5 on histone H4, while GR preferentially recruits GRIP-1 and pCAF, leading to modification of histone H3 (Li et al., 2003b).

The PR isoforms are usually co-expressed in many tissues. They can dimerize and bind DNA as AA, BB, AB forms (Conneely and Lydon 2000). Depending on cell types and promoter context, human PRa (hPRa) and human PRb (hPRb) have remarkably different transcriptional activities (Tung et al., 1993; Vegeto et al., 1993; Wen et al., 1994). Human PRa is in general a much weaker trans-activator than hPRb on PRE driven promoters in most cell lines tested. This property of hPRa has been attributed to the presence of 140 N-terminal residues in the NTD of the PR called inhibitory domain (ID) (also known as inhibitory function: IF). In hPRb, this ID/IF’s inhibitory effect is
overridden by the presence of the 164 N-terminal residues BUS (Giangrande et al., 2000). This ID/IF is found to be necessary for trans-repression of PRE by hPRa (Hovland et al., 1998). The ID/IF also contributes to higher affinity for co-repressors SMRT by hPRa isoform (Giangrande et al., 2000). Recently, the trans-repression activity of PR has been shown to involve N-terminal sumoylation in combination with intra-molecular N/C terminal communication. There is a consensus SUMO-1 binding motif IKEE within the ID/IF of PRa and PRb where sumoylation occurs and mediates trans-repression and auto-inhibitory function (Abdel-Hafiz et al., 2002). The inability of hPRa to trans-activate can also be explained by its poor ability to recruit co-activators SRC-1 and GRIP-1 (Giangrande et al., 2000). In addition, hPRa has been shown to function as a strong trans-dominant repressor of estrogen, androgen, glucocorticoid and mineralocorticoid receptors’ ligand dependent transcriptional activities (Giangrande et al., 1997; Hovland et al., 1998; McDonnell et al., 1994a,b; Tung et al., 1993; Vegeto et al., 1993; Wen et al., 1994). It is interesting to note that anti-progestins such as RU-486 can exhibit anti-estrogenic activities only in the presence of hPRa but not hPRb (McDonnell and Goldman 1994).

Even though PRa is transcriptionally weak in transient transfection experiments in most cell lines, recent study has shown that in response to progesterone, PRa and PRb regulate unique subsets of genes in T47D breast cancer cell line (Richer et al., 2002). Also some genes are regulated by PRa independent of ligands in vivo (Jacobsen et al., 2002). In addition, while both isoforms are expressed in most tissues, their levels vary with developmental and hormonal status and during carcinogenesis (Conneely et al. 2002; Graham et al., 1996; Graham and Clarke 1997). In estrogen treated primates, the
hypothalamus expresses predominantly PRb whereas the pituitary gland expresses predominantly PRa (Bethea and Widmann 1998). Also, in human endometrium, the levels and ratios between PRa and PRb vary extensively during the menstrual cycle (Mangal et al. 1997; Mote et al., 1999, 2000). In general PR expression increases during the proliferative phase and decreases during the secretory phase of the menstrual cycle (Noe et al., 1999; Snijders et al., 1992). This suggests that the relative abundance of each isoform contributes to the overall response of the tissue to progestins.

**Physiologic Importance of the PR**

Physiologic functions of the two isoforms also differ depending on the tissues. The PRa has been found to be essential for ovarian and uterine development while PRb for mammary gland development. In PRa knock-out mice, PRb mediates responses of mammary gland to progesterone stimulation resulting in normal mammary gland development. The ablation of PRa expression results in severe abnormalities of ovarian and uterine development including uterine hyperplasia and reproductive defects leading to infertility (Mulac-Jericevic et al., 2000). However, over-expression of PRb results in premature ductal growth arrest and inadequate lobuloalveolar differentiation (Shyamala et al., 2000). In PRb knock-out mice, only activation of PRa is sufficient to elicit normal ovarian and uterine responses to progesterone but results in defective mammary morphogenesis. In these mice, pregnancy-associated ductal side-branching and lobulo-alveolar development are markedly reduced due to decreased ductal and alveolar epithelial cell proliferation and survival. The defects seen in these mice are associated with an inability of PRa to activate receptor activator of NF-κB ligand (RANKL) signaling pathway in response to progesterone (Mulac-Jericevic et al., 2003). In
transgenic mice in which there is a 3:1 over-expression of PRa over PRb, all features associated with neoplasia such as epithelial hyperplasia, excessive ductal branching and disorganized basement membrane were observed (Mulac-Jericevic et al., 2000). Mice lacking both isoforms display various reproductive abnormalities including incomplete mammary gland development (Lydon et al., 1995). In mice with null mutation of the PR gene, severe pleiotropic female reproductive abnormalities are observed. Among these are impaired neuroendocrine, ovarian function, uterine hyperplasia and inflammation, severe defects of mammary glands, impaired thymic function and sexual behaviors (Chappell et al., 1997; Lydon et al., 1995).

**Clinical Relevance of the PR**

The PR may have different functions in normal versus malignant tissues. Progesterone is proliferative in normal mammary gland (Graham and Clarke 1997) but anti-proliferative in breast cancer (Groshong et al., 1997; Lin et al., 1999; Musgrove et al., 1991). In cancer tissues, PR iso-forms also display differential functions. For instance, PRa expressing T47D xenograft tumors are only half the size of PRb expressing tumors (Sartorius et al., 2003).

The PR is an important therapeutic target. Progestins have been used to treat PR positive breast cancer and endometrial hyperplasia (Howell et al., 1998; Kimmick and Muss 1998). Anti-progestin such as RU-486 is used in birth control. In breast cancer, PR is routinely measured together with ER status for aid in making treatment decision and as one of prognostic parameters (Horwitz et al., 1985; Horwitz and McGuire 1978; McGuire 1978). Progestins have been used in combined oral contraception and as a component in regimens of hormonal replacement therapy for menopausal women. Unexpectedly
however, while progestins added to hormonal replacement therapy regimens help
decrease the incidence of endometrial cancer, they increase the incidence of breast cancer
(Persson et al., 1999; Schairer et al., 2000).

In cancer tissues, particularly breast cancer, ovarian cancer and endometrial
cancer, the levels and ratios between PRa and PRb alter. In breast cancer and uterine
endometrial cancer, more than half of the tumors studied show that expression of PRa is
higher than that of PRb (Arnett-Mansfield et al., 2001; Graham et al., 1996; McGowan
and Clarke 1999). Over-expression of PRa in breast cancer cells has been shown to be
associated with loss of their adherent property (Fujimoto et al., 1997). In epithelial
ovarian cancer, PRb expression is increased while PRa expression is unchanged
compared to normal epithelium (Akahira et al., 2000; Li et al., 2003). Also, PRb is found
predominant over PRa in metastases from uterine endometrial cancer, uterine cervical
cancer and ovarian cancer (Fujimoto et al., 1997). Because PRa and PRb are functionally
distinct, it is reasonable to predict that differential expression of the two iso-forms affects
the responsiveness of the above mentioned cancers to progestins in hormone therapy.

**Non-classical Mechanisms of PR Actions**

Besides the classical trans-activation mediated by PR interaction with PRE,
progestins have been shown to regulate a number of genes whose promoters do not
contain canonical PRE. Without PRE, PR may interact with DNA through the mediation
of other transcription factors such as Sp1(Owen et al., 1998; Tang et al., 2002), c-jun, c-
fos of AP-1 (Tseng et al., 2003). Interestingly, unlike trans-activation through the
classical mechanism in which PRa is a weak trans-activator, PR trans-activation through
non-classical mechanism can be effectively mediated by PRa. For example, the
regulation of fibronectin promoter (Tseng et al., 2003) or insulin-like growth factor binding protein-1 (IGFBP-1) (Gao et al., 2000) can be elicited by both PRa and PRb in which PRa is a much stronger trans-activator.

Progesterone receptor also can mediate non-genomic action of progestins. Progestin treatment of breast cancer cells causes a rapid and transient activation of MAPK signaling which is independent of PR transcriptional activity but is PR-dependent (Boonyaratanakornkit et al., 2001; Migliaccio et al., 1998). Human PR contains a proline-rich (PxxP) motif that mediates direct binding of PR to the SH3 domains (src-homology 3) of the p60-Src kinase family in a progestin-dependent manner (Boonyaratanakornkit et al., 2001). Mutation of this PxxP motif in PR abrogates ligand-dependent PR binding to and activation of c-Src and p42/p44 MAPKs. This suggests that progestin mediated activation of MAPKs are likely c-Src-dependent (Boonyaratanakornkit et al., 2001). The non-genomic effect of PR may require involvement of other steroid receptor. It is recently shown that PR mediated progestin-dependent activation of c-Src and MAPKs depends upon the presence of unliganded ERα which interacts with PRb via two domains flanking the PxxP motif of PR (Ballare et al., 2003). Regardless what is required for the non-genomic action of PR, evidence has been gleaned to testify for the existence of PR mediated ligand-dependent activation of cytoplasmic signaling pathways. For example, transcriptional synergy between progestins and epithelial growth hormone (EGF) on certain non-PRE promoter has been reported (Richer et al., 1998). The up-regulation of cyclin D1, whose promoter lacks canonical PRE, by progestins alone or a combination of progestins and growth factors is found to be dependent on MAPK (Groshong et al., 1997; Lange et al., 1998, 1999). Although the significance of non-genomic action of PR
remains unclear, one of which may well be for optimization of the biologic effects of progestins.

**Functional Relevance of PR Phosphorylation**

Both iso-forms of hPR are phosphoproteins and phosphorylation of hPR is concentrated mainly within the N-terminus even though it can occur on serine residues throughout the molecule (Knotts et al., 2001; Takimoto et al., 1996; Weigel 1996; Zhang et al., 1994, 1995, 1997). Some sites are constitutively phosphorylated in the absence of hormone (Zhang et al., 1997), while others are phosphorylated following progestin treatment (Zhang et al., 1995). Also, selected sites have been found to be phosphorylated by specific kinases such as casein kinase (Zhang et al., 1994), MAPK (Lange et al., 2000; Shen et al., 2001) and cyclin A/CDK2 (cyclin-dependent protein kinase 2) complexes (Knotts et al., 2001; Zhang et al., 1997). Phosphorylation of a specific site can be both ligand dependent and independent (Lange et al., 2000; Nardulli and Katzenellenbogen 1988; Shen et al., 2001). Phosphorylation at different sites in the PR molecule leads to different consequences. To date, two phosphorylation sites in the hPR have been shown to influence its functions: Serine 294 and Serine 400.

Phosphorylation of Serine 294 has been demonstrated to occur both following treatment with progestins and with growth factors. Phosphorylation Ser 294 induced by progestins mediates ligand-induced receptor down-regulation via the ubiquitin-proteasome pathway (Lange et al., 2000; Nardulli and Katzenellenbogen 1988). Phosphorylation of Ser 294 induced by both progestins and growth factors is required for transcriptional synergy between progestins and growth factors as it was found that phosphorylation of PR at Ser 294 by MAPK greatly increases transcriptional activity of
liganded PR on PRE promoters (Shen et al., 2001). Activator of p42/p44 MAPKs by EGF can quickly induce Ser294 phosphorylation, resulting in rapid nuclear accumulation of PR independently of progestins (Qiu et al., 2003). Also, heregulin, an EGF family member can induce nuclear localization, DNA binding and transcriptional activity of PR in T47D cells in the absence of hormone (Labriola et al., 2003). This suggests that Ser 294 phosphorylation may play some role in ligand-independent PR action.

Unlike Ser 294, phosphorylation of Ser 400 by CDK2 is both constitutive and ligand-dependent. However, this helps stabilize PR protein (Zhang et al., 1997) potentially leading to prolonging PR actions. Over-expression of CDK2 increases PR transcriptional activity in a ligand-independent manner, suggesting that PR action may be cell cycle dependent. Mutation of Ser 400 to alanine selectively abrogates the increase in PR transcriptional activity observed in the absence but not in the presence of progestins, suggesting that CDK2 may constitutively activate PR in the absence of ligands (Lange 2004).

**Glucocorticoid Receptor**

**Organization of the GR (Figure 5)**

The structure of the GR shares the same general organization with that of other nuclear receptors (Mangelsdorf et al., 1995). Three trans-activation domains (referred to as τ1 or AF-1, τ2 and τc or AF-2 were identified in the GR (Evans and Hollenberg 1988; Giguere et al., 1986; Hollenberg et al., 1985; Hollenberg et al., 1987; Hollenberg and Evans 1988). The τ1 domain (also referred to as enh2 or AF-1: residues 77-262 in hGR or 106-318 in rGR) is found within the A/B N-terminal domain and is highly acidic and phosphorylated (Almlof et al., 1995; Bodwell et al., 1998). It is generally considered a
Figure 4: human GR genes and mRNAs

A

A-H: introns
1-9: exons
J: join region
9α/β 3’UTR: 3’untranslated region in exons 9α/β
hormone-independent constitutive trans-activation domain (Beato et al., 1995). It makes contact with proteins in the basal transcriptional apparatus, including TBP (TATA box-binding protein) (Ford et al., 1997; Henriksson et al., 1997; McEwan et al., 1993). It may undergo inter-domain interactions subsequent to DNA binding (Kumar et al., 1999). The τ2 and τc or AF-2 domains are both located in the ligand-binding domain (LBD: domain E/F) of the GR. The τ2 domain (residues 526-556 in hGR or 544-573 in rGR) possesses trans-activation potential in the context of full-length GR (Milhon et al., 1997); exhibits nuclear matrix targeting activity (Tang et al., 1998); and serves as binding domain for hsp90 (Giannoukos et al., 1999). The C-terminal τc or AF-2 domain (Boruk et al., 1998; Danielian et al., 1992) (residues 727-763 in hGR or 745-781 in rGR) forms part of the amphipathic α-helix 12 of the LBD and is responsible for the hormone-dependent interaction with co-activators of the p160 family, such as SRC-1 and GRIP-1 (Ding et al., 1998; Onate et al., 1995). Mutations of this domain disrupts the direct interaction of GR with SRC-1 (Kucera et al., 2002).

**Glucocorticoid Receptor in the Cell**

The subcellular distribution of GR in the cell is still debatable. There are studies demonstrating that un-liganded GR resides predominantly in the cytoplasm and translocates to the nucleus upon ligand binding (Htun et al., 1996; Oakley et al., 1999b; Webster et al., 1997). However, strict control of steroid concentrations in the culture media and the use of cryo-sections instead of formaldehyde fixed tissues in immunohistochemistry experiments led to the observation that un-liganded GR was predominantly in the nucleus (Brink et al., 1992). This agrees with study by some others (Gougat et al., 2002). Data reconciling these contradictory findings have been produced
by still some other investigators who show that GR exists both in the cytoplasm and the
nucleus of the cell (Albrecht et al., 2002; Dufourny and Skinner 2002a,b). A likely
explanation for these conflicting data is that many factors other than the status of ligand
binding contribute to the sub-cellular distribution of the GR. Indeed, some non-steroid
chemicals including lipopolysaccharide (LPS) (Mandrekar et al., 2002) and anti-
depressants (Okuyama-Tamura et al., 2003) are potent inducers of GR nuclearization.

The GR localization within a cell, however, is not predestined and unchanged.
There appears to be a continuous nucleo-cytoplasmic movement of both liganded GR and
un-liganded GR in hormone-free cells(Defranco et al., 1995; Madan and Defranco 1993).
Liganded GR localization to the nucleus makes use of the cytoskeleton and at least one
chaperone Hsp90 (Galigniana et al., 1998; Yang and Defranco 1996). It also is mediated
by the presence of two nuclear localization sequences (NLSs) within its molecule (Picard
and Yamamoto 1987). The first sequence NLS-1 is located between residues 478 and 500
(Cadepond et al., 1992). The second sequence NLS-2 exact position in the GR is unclear
but known to be in the LBD (Picard and Yamamoto 1987). The nuclear translocation
mediated by the NLS-2 is slower than translocation mediated by NLS-1 and is hormone
dependent (Savory et al., 1999). In the nucleus, a sub-fraction of nuclear GR is attached
to the nuclear matrix(van Steensel et al., 1995) probably through a domain designated as
nuclear matrix targeting signal (NMTS) within the GR molecule (Tang et al., 1998). This
might serve as a mechanism for GR storage in the nucleus, allowing fast supply of GR
when needed. Nuclear GR movement back to the cytoplasm is mediated by 15 residue
sequence located between the two zinc fingers in the DNA binding domain (DBD) that
confers nuclear export signal (Black et al., 2001). In addition, there exist other
mechanisms that favor cytoplasmic localization of the GR such as the calcium-calreticulin nuclear export system (Holaska et al., 2001, 2002). Recently, protein 14-3-3δ; a member of the 14-3-3 family proteins that are involved in several biologic processes including apoptosis, intracellular signaling of growth factors, insulin, and the fork-head transcription factors (Brunet et al., 1999; Craparo et al., 1997; Fantl et al., 1994; Fu et al., 1994; Holaska et al., 2001, 2002; Zha et al., 1996) was shown to facilitate the localization of GFP-hGRα in the cytoplasm in the absence of ligand and potentiate its nuclear export after ligand withdrawal (Kino et al., 2003). In the absence of ligand, GR forms a large heteromeric complex containing the Hsp90-based chaperone complex from which it dissociates upon ligand binding (Pratt and Toft 1997). The existence in a complex with Hsp90 is requisite for GR to have steroid binding activity (Bresnick et al., 1989; Dittmar et al., 1997; Dittmar and Pratt 1997; Hutchison et al., 1992; Morishima et al., 2000; Pratt 1993; Pratt and Toft 1997; Scherrer et al., 1990). Regardless of where GR is in the cell, it is activated by glucocorticoids’ binding to its LBD (Hollenberg and Evans 1988). The liganded GR then dissociates from the GR-Hsps complex (Hutchison et al., 1992; Picard et al., 1990; Scherrer et al., 1993), dimerizes, recruits co-activators such as GRIP-1 and SRC-1 for sequence-specific DNA binding and trans-activation activity (Hong et al., 1997; McKenna et al., 1999).

**Mechanisms of GR Actions**

**The Interaction Between GR and DNA**

In the nucleus, GR binds to a consensus sequence called the glucocorticoid responsive element (GRE) which is comprised of two imperfect palidromic repeats (half sites) separated by three base pair 5' GGTACAnnnTGTCT 3' (Beato et al., 1989; Beato
1989; Cato et al., 1992; Luisi et al., 1991; Payvar et al., 1981, 1982, 1983; Tsai and O'Malley 1994; Zilliacus et al., 1995). Studies have shown that GR trans-activates target genes by binding as a receptor dimer to the GRE (Dahlman-Wright et al., 1990, 1991; Tsai et al., 1988). The GRE, however, is also bound by PR, AR and MR; probably because their DBDs share a great deal of sequence similarity (Freedman 1992).

Interesting, the binding of GR to GRE is specific with high affinity and independent of the status of ligand binding (Aumais et al., 1996; Groyer et al., 1987; Pandit et al., 2002; Rusconi and Yamamoto 1987). In addition to binding to the consensus GRE, GR can elicit transcriptional modulation by interaction with non-consensus GRE (Alheim et al., 2003; Ma et al., 2000; Stafford et al., 2001; Tseng et al., 2001). Besides trans-activation of gene transcription, GR is able to trans-repress the expression of specific target genes by binding to negative GREs (nGREs) (Cairns et al., 1993; Drouin et al., 1989, 1993; Meyer et al., 1997; Morrison and Eisman 1993; Nakai et al., 1991; Sakai et al., 1988; Stromstedt et al., 1991) in which case a dimerization of the GR is not necessarily the norm because some nGREs are able to bind single to multiple monomers of GR (Drouin et al., 1993; Radoja et al., 2000). The trans-repression by GR also can be mediated by its binding to composite GREs, which are DNA elements consisting of a consensus GRE and a binding site for another transcription factor such as AP-1 as seen in the mouse proliferin gene promoter (Diamond et al., 1990; Harrison et al., 1995; Pearce and Yamamoto 1993). On this element, GR can potentiate or repress AP-1 induced trans-activation depending on the cell type and the composition of the AP-1 dimer.

In in vitro transcription assays, the consensus GRE is not receptor specific (Roche et al., 1992) since it can bind to and trans-activate not only GR but also PR, AR and MR.
This contrasts with the observation that different steroids elicit different effects in different cell types and tissues. Therefore, how a single cis-element (the consensus GRE) can mediate the induction of distinct effects by different hormones remains a puzzle. Potential mechanisms for these differential biologic activities include cell-specific regulation of receptors levels; tissue specific ligand metabolism such as the metabolism of cortisol by 11β-hydroxysteroid dehydrogenase to the inactive cortisone whose accumulation in the tissue favors its responsiveness to the MR agonist aldosterone instead of cortisol (Bonvalet et al., 1990); receptor specific interactions with transcription factors (Pearce and Yamamoto 1993). Furthermore, only the receptor and its cognate ligand are not sufficient to determine the wide range of biological effects of the receptor. The cellular context definitely plays an important role. For instance, multiple regulated surfaces of the GR respond to the cellular context to determine the spectrum of its activities as demonstrated by the ability of GR mutants generated by mutation of specific residues in the GR to distinguish its actions on the consensus GRE versus the composite GRE whose actions require cellular transcription factors other than GR (Guido et al., 1996). Also, there is evidence that the differential interaction of steroid receptors and non-receptor factors in the cell at composite elements determines the induction of differential effect. For example, under conditions where GR represses AP-1, MR is inactive on the composite GRE (Pearce and Yamamoto 1993). Recently, a novel tissue specific co-activator hPGC-1/LEM6 (ligand effect modulator 6) has been identified and was shown to markedly enhance the trans-activation by ER, MR, GR but not AR (Knutti et al., 2000). The existence of cis-element unique for a steroid receptor also helps explain the diverse effects elicited by different steroid receptors. For instance, AR has its own
androgen response elements (AREs) which are partial direct repeats of the 5'-TGTTCT-3' core recognition sequence (Schoenmakers et al., 1999, 2000; Verrijdt et al., 2000) besides sharing the consensus GRE with GR, PR and MR. The differential biologic activities of different steroid receptors can also be explained by preferential interaction of steroid receptors with variants of GRE as seen in the case of rat cytosolic aspartate aminotransferase (cAsp AT). A GRE-like sequence consisting of two overlapping imperfect GRE each comprising a conserved half-site and a poorly conserved half-site is found in the promoter of cAsp AT that is responsive to glucocorticoids but not androgens (Massaad et al., 2000).

The dimerization of GR is mediated by the D loop within the second zinc finger of the GR DBD. Disruption of this region through mutagenesis could abolish the ability of GR to dimerize and consequently inhibit glucocorticoid (GC)-mediated activation (Dahlman-Wright et al., 1991). The mechanism of transcription activity mediated by GR is not yet fully dissected, but GR interactions with co-regulatory factors such as CBP (Kamei et al., 1996; Sheppard et al., 1998), SRC-1 (Onate et al., 1995), TIF-2 (Hong et al., 1996, 1997; Voegel et al., 1996), and RIP140 (Receptor Interacting Protein 140) (Subramaniam et al., 1999) play an important role. The p160/SRC co-activators (Anzick et al., 1997; Chen et al., 1997; Hong et al., 1996, 1997; Li et al. 1997; Onate et al., 1995; Torchia et al., 1997; Voegel et al., 1996; Yao et al., 1996) interact with the AF-2 within the LBD of GR in conjunction with binding of agonist such as Dex but not antagonist such as RU-486. Several lines of evidence suggest that ligands that bind to the GR have an indirect effect on the recruitment of specific co-activators to transcriptional complexes (Bourguet et al., 2000; Xu et al., 1999). The GR-LBD structure in complex with Dex and
a co-activator peptide has been available (Bledsoe et al., 2002; Kauppi et al., 2003). Binding of RU-486 blocks the binding of co-activators at the AF-2 site while simultaneously recruiting the binding of co-repressor N-CoR or SMRT (Schulz et al., 2002). This agrees with a recent study showing that in a 3D crystallographic structure of the RU-486 bound GR-LBD, helix 12 was in a position that covers the co-activator pocket (Kauppi et al., 2003). However, RU-486 can elicit agonistic effects in the absence of co-repressors by acting through the AF-1 region (Schulz et al., 2002). Also, glucocorticoids can at least regulate their own activities by means of modulation of co-activator levels. For instance, SRC-1 mRNA and protein were shown to be down-regulated by Dex in vitro and in vivo (Kurihara et al., 2002).

The GR-DNA interaction is not static but rather a multi-step dynamic process. Foot-printing and photo-bleaching experiments uncover that the hormone bound receptor is in fast movement on and off its chromatin regulatory elements (Hager et al., 2000; Hager and Fragoso 1999; McNally et al., 2000; Rigaud et al., 1991), instead of remaining bound to DNA sites as traditionally thought. The hormone bound GR and PR have been shown to transiently bind to their response elements and leave their sites accessible to other factors including nuclear factor-1 (NF-1) (Truss et al., 1995) and hepatoma nuclear factor-5 (HNF-5) (Rigaud et al., 1991). During chromatin remodeling following GR binding to the MMTV promoter and recruitment of Swi/Snf, GR is actively displaced from the chromatin template (Fletcher et al., 2002). In-vitro assays demonstrated that in the absence of ATP, GR binds to multiple sites on the chromatin array and prevents restriction enzyme access to recognition sites. Upon addition of ATP, GR induces remodeling and leaves the DNA template resulting in increased access to restriction
enzymes sites within the DNA template (Fletcher et al., 2000). Electro-mobility shift assays also demonstrated that Dex-bound GR dissociated from DNA significantly faster than ligand free GR or RU-486 bound GR, supporting the concept of dynamic GR-DNA interaction (Pandit et al., 2002). Photo-bleaching experiments, however, have provided direct evidence that the hormone occupied GR moves rapidly between the MMTV chromatin and the nucleo-plasmic compartment (McNally et al., 2000).

**Glucocorticoid Receptor-induced Chromatin Remodeling**

In addition to the actions of co-regulators, transcription mediated by GR also is modulated by the Swi/Snf or BAF chromatin modeling proteins. Most of the studies about the role of these proteins in GR transcriptional activation have been done on the MMTV promoter. It has been shown that in-vivo, GR can recruit the BRG1 complex to the promoter via protein-protein interaction with BAF250 in a ligand dependent manner (Fryer and Archer 1998; Nie et al., 2000). Also, GR has been shown to stimulate BRG1 chromatin remodeling in in-vitro assays using Hela nuclear extracts, ATP, purified GR and MMTV-LTR DNA reconstituted into a polynucleosomal array (Fletcher et al., 2000). Using in-vitro reconstituted nucleosomes, it was demonstrated that GR-mediated stimulation of nucleosome disruption by Swi/Snf depended on the presence of GRE; and GR is able to target Swi/Snf complex to the nucleosome (Ostlund Farrants et al., 1997). Furthermore, remodeling proteins including the Swi/Snf factors that interact with GR can directly alter structure and flexibility of chromatin to promote transcription (Fryer and Archer 1998; Muchardt and Yaniv 1993; Yoshinaga et al., 1992).

The role of histone modification in modulation of transcription by GR is not only limited to the status of acetylation of histone H3 and H4, but phosphorylation of linker
histone H1 has been implicated also. The importance of histone H1 in transcriptional regulation is supported by a number of studies (Bouvet et al., 1994; Cheung et al., 2002; Laybourn and Kadonaga 1991). As shown on the MMTV promoter, a loss of promoter-associated linker histone H1 occurred during GR-mediated chromatin remodeling (Bresnick et al., 1992). Prolonged GC treatment results in H1 dephosphorylation and inactivation of chromatin-assembled MMTV promoter but not transiently transfected promoters despite the presence of GR and required transcription factors in the nucleus (Lee and Archer 1998). However, this H1 dephosphorylation associated inactivation of the chromatin-assembled MMTV promoter upon prolong GC exposure can be reversed by removing the hormone for 24 h. Transcription factor NF-1 loading and rephosphorylation of histone H1 accompany this reactivation (Lee and Archer 1998).

The transcription factor NF-1 also has been shown to have a critical role in GR-mediated chromatin remodeling of the MMTV promoter (Archer et al., 1991; Cordingley et al., 1987; Prado et al., 2002). Chromatin remodeling of the MMTV promoter requires the binding of NF-1. Neither BRG1 nor GR is able to associate with the promoter if it is absent (Hebbar and Archer 2003).

Despite that much has been discussed about the mechanisms by which GR modulates transcription, GR induced chromatin remodeling upon ligand binding is still not fully understood. According to a model describing GR mediated chromatin remodeling of the MMTV promoter (Deroo and Archer 2001b), upon binding to ligand, the GR becomes associated with the BRG-1 complex. The GR-remodeling factors complex transiently associates with the GREs, which leads to transient displacement of histone H1. The remodeling of DNA then ensues as indicated by an increase in DNA
sensitivity to restriction enzymes, nuclease and chemical agents; allowing transcription
factors such as TBP and NF-1 to bind to DNA and initiation of transcription takes place.

The Human GR Gene and Protein (Figure 4A&B, Figure 5)

In every species examined to date, only one GR gene has been identified. The
human GR gene (hGR) is located on chromosome 5 q11-q16 (Hollenberg et al., 1985).
The gene is comprised of nine exons (Encio and Detera-Wadleigh 1991), with protein
coding regions containing exons 2-9 (Encio and Detera-Wadleigh 1991; Oakley et al.,
1996). The promoter region of the hGR contains binding sites for various transcription
factors including Sp1, AP-2, YY 1, NF-κB and even is responsive to GCs
(glucocorticoids) (Breslin et al., 2001; Nobukuni et al., 1995; Webster et al., 2001). The
hGR gene is driven by multiple promoters 1A, 1B (Breslin et al., 2001), and 1C
(Nobukuni et al., 1995; Zong et al., 1990). They are so named for being located upstream
of corresponding exons 1A, 1B and 1C; in which promoter 1A is GC responsive, both
positively and negatively depending on the cell line tested (Breslin et al., 2001). No
obvious TATA or CAAT elements were found in these promoters (Breslin and Vedeckis
1998; Encio and Detera-Wadleigh 1991). The promoter usage of the hGR gene is cell
type specific (Breslin et al., 2001). The existence of multiple binding sites for various
transcription factors allows one to predict that GR expression levels depend on the
transcription factors profile of specific cell types or tissues. In fact, the strength of
promoter 1B, thus the expression of the hGR, requires the Sp1 sites in Hela cells while
YY1 sites are required in HepG2 and Jurkat cells (Nunez and Vedeckis 2002). This
would provide the explanation for differential modulation of GR expression in response
Figure 5: human GR proteins

A-F: individual domains
GRα and GRβ: share the same first 727 residues. Residues 728-777 are unique for GRα and residues 728-742 unique for GRβ
GRα and GRβ: each has two iso-forms A & B resulting from differential translation initiation start sites
to treatment with GCs (also see Regulation of GR actions through regulation of GR levels in the cell).

The human GR exists in multiple iso-forms, among which hGR\(\alpha\) and hGR\(\beta\) are the most known (Hollenberg et al., 1985; Nunez and Vedeckis 2002; Weinberger et al., 1985a). The generation of multiple iso-forms is thought to be due to alternative splicing. Exons 1-8 are common for both mRNAs of the hGR\(\alpha\) and \(\beta\), while mRNA of the hGR\(\alpha\) contains an additional exon 9\(\alpha\) and mRNA of the hGR\(\beta\) contains exon 9\(\beta\) (Oakley et al., 1996) (Figure 4B). A third hGR mRNA has been identified that is more abundant than the other two mRNAs and is thought to be translated into hGR\(\alpha\). This mRNA is peculiar in that besides exons 1-8, it contains the entire exon 9 including exon 9\(\alpha\), the J region and exon 9\(\beta\) (Oakley et al., 1996). In addition, two other iso-forms hGR-P/hGR\(\delta\) and hGR\(\gamma\) exist as a result of two different alternative splicing events. The truncated 676 residue hGR\(\delta\) results from exon 8 being replaced by intron G (Moalli et al., 1993); the 452 residue hGR\(\gamma\) from an insertion of an additional arginine coding codon GTA between exons 3 and 4 (Rivers et al., 1999). Recently, it has been shown that alternative translation initiation results in the generation of two different iso-forms (A and B) of hGR (Yudt and Cidlowski 2001). Thus there exist hGR\(\alpha\) A and B and hGR\(\beta\) A and B.

The hGR is comprised of three distinct domains and a hinge region: the N-terminal domain consisting of residues 1-420; a central DNA-binding domain (DBD) of residues 428-488, and a C-terminal ligand-binding domain (LBD) of residues 527-777 for hGR\(\alpha\) and residues 527-742 for hGR\(\beta\) (Bamberger et al., 1996; Giguere et al., 1986; Mangelsdorf et al., 1995; Weinberger et al., 1985b). Residues 527-727 are common for
the LBDs of both hGRα and β. In hGRα, residues 727-777 are from exon 9α and in hGRβ, residues 727-742 from exon 9β (Figure 5).

In most human tissues examined, hGRα has been found to be in much greater abundance than hGRβ (Bamberger et al., 1995; Dahia et al., 1997; Hecht et al., 1997; Oakley et al., 1996; Pujols et al., 2002). In mice the iso-form GRβ has not been found to exist (Otto et al., 1997). In human neutrophils, the generation of hGRβ has been shown to be mediated by serine-arginine rich protein p30 (Xu et al., 2003). While hGRα is fully functional, hGRβ is not able to bind ligand. It is not capable of transcriptional activity as tested on a consensus GRE, an AP-1 and an NF-κB promoter driven system (Bamberger et al., 1997; de Lange et al., 1997). It is, however, capable of binding the consensus GRE with greater affinity than hGRα regardless of whether GCs are added or not (Oakley et al., 1999a). Furthermore, in several in vitro systems, co-transfection of hGRα with hGRβ resulted in inhibition of Dex-induced MMTV promoter transcriptional activity (Bamberger et al., 1995; Oakley et al., 1996, 1999a). Therefore, it is proposed that hGRβ may form heterodimer with hGRα in living cells that would have much lower transcriptional activity than that of hGRα homodimer (Bamberger et al., 1995; de Castro et al., 1996; Oakley et al., 1996). Indeed, the heterodimer hGRα-hGRβ has recently been demonstrated to exist in vitro and in human neutrophils (Strickland et al., 2001).

However, the theory that hGRβ acts as a dominant negative of hGRα is still a subject of ongoing debate. Some investigators attributed the inhibiting effect of hGRβ on hGRα to non-specific squelching of transcriptional co-activators because when the total amount of transfected plasmids was kept constant, transfection with increasing amounts of hGRβ did not influence hGRα mediated MMTV promoter transcription activity (Hecht et al.,
Works from other investigators also suggested that hGRβ did not act as an inhibitor for hGRα (Brogan et al., 1999; de Lange et al., 1999). Advocates for the inhibitory effects of hGRβ on hGRα have found that the 15 unique C-terminal residues in hGRβ are responsible for the mechanism for the dominant-negative activity of hGRβ (Oakley et al., 1999a). Insertions of hGRβ specific amino acids into hGRα result in a dominant-negative form of the receptor. In addition to demonstrating that nuclear localization and heterodimerization to be critical for the dominant negative activity of hGRβ, mutagenesis analyses have mapped this function to two amino acids 733-734 within the unique hGRβ region (Yudt et al., 2003). Regardless, elevated tissue levels of hGRβ have been found to associate with steroid resistant asthma (Hamid et al., 1999; Leung et al., 1997; Sousa et al., 2000) and colitis ulcerosa (Honda et al., 2000), suggesting a role for hGRβ in tissue insensitivity to GCs.

**Post-translational Modification of GR**

Glucocorticoid receptor is a phosphoprotein. The otherwise constitutive phosphorylation of GR is enhanced after ligand addition and occurs on serine and threonine residues (Bodwell et al., 1998; Weigel 1996). Five phosphorylation sites, all serines and located in the τ1, have been identified in hGR (positions 113, 141, 203, 211, 226) (Almlof et al., 1995). Mutations of these sites did not affect the trans-activation activity of the τ1 as tested in yeast (Almlof et al., 1995); and mutation of all putative phosphorylation sites in hGRα did not affect the transactivation properties of the receptor on the MMTV promoter (Almlof et al., 1997). Some studies, however, have shown that the mutations of phosphorylation sites of the mouse GR modulates GR-mediated transcription both positively and negatively relative to the activity of the wild type
Phosphorylation of GR Ser 226 by JNK (c-Jun N-terminal kinase) has been shown to result in enhanced GR nuclear export which may contribute to termination of GR-mediated transcription (Itoh et al., 2002).

Besides modification by phosphorylation, GR has been found to be sumoylated in a ligand enhanced manner (Tian et al., 2002). Small ubiquitin related modifier-1 (SUMO-1, also known as phosphoinositidase c1, sentrin, …) has been found to be attached to many transcription factors such as p53, c-Jun, c-Myb and AR as a result of a post-translational modification event (Bies et al., 2002; Muller et al., 2000). Sumoylation of the hGR in the N-terminal domain and LBD was found to regulate its transcriptional activity in a promoter context dependent manner (Tian et al., 2002).

Regulation of GR Actions Through Regulation of GR Levels in the Cell

The biologic activities mediated by GR also can be controlled by regulation of GR and co-activator levels. Oligonucleotide microarrays experiments on leukemic cells have shown that GCs up-regulate both hGR-α and hGR-β and SRC-1 (Yoshida et al., 2002). The up-regulation of GR and SRC-1 by GCs suggests a feed-forward auto-regulatory mechanism that exists to control the biologic actions of GCs. The up-regulation of GR after GCs administration has been reported to occur mostly in lymphocytes (Antakly et al., 1989; Ashraf et al., 1991; Eisen et al., 1988; Gomi et al., 1990). However, some other studies have demonstrated that liganded GR underwent down-regulation instead (Bellingham et al., 1992; Burnstein et al., 1990, 1991; Cidlowski and Cidlowski 1981; Hoeck et al., 1989; McIntyre and Samuels 1985; Silva et al., 1994). This occurs both at the mRNA level (Dong et al., 1988; Okret et al., 1986) as well as at the protein level (Dong et al., 1988; Hoeck et al., 1989). Upon GCs treatment, GR mRNA
levels were shown to be down-regulated by 50%-80% in many cell lines examined (Burnstein et al., 1994; Dong et al., 1988; Okret et al., 1986). Since the sensitivity of the cell to GCs has been shown to be dependent on the amount of GR in the cell (Bellingham et al., 1992; Cidlowski and Cidlowski 1981; Silva et al., 1994), the above seemingly contradictory data could support the hypothesis that GCs are capable of bringing about differential effects mediated through regulation of GR levels on specific cell types and tissues in their appropriate physiologic and/or pathologic states.

In addition to treatment with GCs, treatment of MCF-7 cells with Estradiol (E2) led to increased degradation of GR protein that involves ERα mediated up-regulation of Mdm2 (murine double minute 2, an E3 ubiquitin ligase shown to target the GR for degradation), resulting in decrease GR mediated transcriptional activities (Kinyamu and Archer 2003). The activity of GR is also subject to regulation by ubiquitin-proteosome pathway mediated degradation, which permits ligand-dependent degradation of the receptor in some but not all cell types (Wallace and Cidlowski 2001; Wang et al., 2002).

Cross-talk Between GR and Cellular Signaling Pathways

Not all GR-mediated biologic effects are elicited by its interaction with DNA (Reichardt et al., 1998). In fact many of the glucocorticoid effects are mediated by GR interactions with other proteins or cellular signaling pathways. The GR has been found to be involved in cross-talk with other signaling pathways such as Ras/Raf/MAPK (Croxtall et al., 2000; Widen et al., 2000). There was a report that GR was involved in trans-repression of AP-1 by interfering with JNK mediated c-Jun activation by blocking TNFα induced phospho-activation of JNK (Gonzalez et al., 2000); in trans-repression of NF-κB by interference with activation of NF-κB by the catalytic subunit of protein kinase A.
(PKA) probably via its direct binding to GR (Doucas et al., 2000). Also, GR mediated transcriptional activities have been shown to be inhibited by activation of ERK (extracellular signal-regulated kinase), a member of the MAPK family (Jamieson and Yamamoto 2000), and by JNK (Rogatsky et al., 1998). Also, GR has been shown to participate in GCs induced activation of phosphotidyl-inositol 3 kinase (PI3 K)/Akt pathway. Corticosteroids can activate PI3 K and Akt in a ligand dependent manner and this activation is blocked by co-treatment with RU-486 (a GR antagonist) and a PI3 K inhibitor but not by transcription inhibitors (Hafezi-Moghadam et al., 2002; Limbourg et al., 2002). The involvement of GR was demonstrated in COS7 cell (lacking endogeneous GR) where a GR agonist (Dex) was able to activate PI3 K/Akt only after transfection of GR or A458T (a dimerization defective GR mutant) (Limbourg et al., 2002). This indicates that the activation of PI3 K/Akt is independent of DNA binding. On the contrary, GR activity was found to be enhanced by cAMP of PKA pathway (Medh et al., 1998).

**Functional Interactions of GR with Other Transcription Factors**

The GR also has been shown to regulate transcription via interaction with various transcriptional factors:

**AP-1 and NF-κB**

The GR can trans-repress target genes through AP-1 (Gottlicher et al., 1998; Jonat et al., 1990; Karin et al., 1997; Lucibello et al., 1990; Resche-Rigon and Gronemeyer 1998; Schule et al., 1990; Tuckermann et al., 1999; Vayssiere et al., 1997; Yang-Yen et al., 1990). This can occur through blocking e-jun activation by JNK (Gonzalez et al., 2000); direct protein-protein interaction (Schule et al., 1990; Tuckermann et al., 1999)
between the AP-1 complex and GR in which the DBD (Tao et al., 2001) particularly its
1st zinc finger (Heck et al., 1994) is required. Direct interaction between GR and AP-1
can lead to recruitment of GRIP-1, an otherwise co-activator for GR in the context of the
consensus GRE, that confers trans-repressive action in this case (Rogatsky et al., 2001,
2002). Sine GR and AP-1 transcriptional activities involve recruitment of the same co-
activators, GR trans-repression of AP-1 has been proposed to be due to their competition
for the limited supply of co-activators (Kamei et al., 1996; Lee et al., 1998). However,
this view is challenged by evidence that GR repression of AP-1 is independent of CBP
levels in the cell (De Bosscher et al., 2001).

The GR also can trans-repress target genes through NF-κB (Caldenhoven et al.,
1995; Cato and Wade 1996; De Bosscher et al., 2000; Gottlicher et al., 1998; McKay and
Cidlowski 1999; Resche-Rigon and Gronemeyer 1998; Scheinman et al., 1995b; Wissink
et al., 1997). Nuclear factor κB (NF-κB) is a group of transcription factors consisting of
homo- or hetero-dimers of subunits including p65 (RelA), c-Rel, RelB, p105/p50 (NF-
κB1) and p100/p52 (NF-κB2) (Baeuerle and Baltimore 1996). The subunits are
characterized by a conserved stretch of 300 amino acids called the rel homology domain
(Ghosh et al., 1998). The classic and most abundant NF-κB is a hetero-dimer of p65/p50.
NF-κB proteins are bound by inhibitory proteins (IκB proteins) including IκBα (Thanos
and Maniatis 1995), IκBβ (Thompson et al., 1995), and IκBe (Whiteside et al., 1997).
Normally, the NF-κB/IκB complex is predominantly cytoplasmic due to the presence of a
nuclear export sequence (NES) in IκBα despite there is a constant nucleo-cytoplasmic
shuttling of the complex in the cell (Schmid et al., 2000). Activation of NF-κB involves
activation of IκB kinases including IKKα and IKKβ which in turn phosphorylate IκB
proteins at the N-terminal serines (Mercurio et al., 1997; Zandi et al., 1997).

Phosphorylated IκB proteins then are targeted for degradation through the ubiquitin-proteasome pathways (Coux and Goldberg 1998; Yaron et al., 1998), releasing NF-κB with an unmasked nuclear localization sequence (NLS) that mediates translocation of NF-κB to the nucleus and activation of transcription. The NF-κB/IκB complex is the preferred substrate for IκB kinases rather than IκB alone (Nelson et al., 2002), allowing efficient activation of NF-κB. Decreased NF-κB-mediated transcription is thought to occur through newly synthesized IκB entering the nucleus and binding to NF-κB, leading to nuclear export of the NF-κB/IκB complex to the cytoplasm (Arenzana-Seisdedos et al., 1995; Rodriguez et al., 1999). However, inhibition of NF-κB does not require its cytoplasmic re-localization because IκB can inhibit NF-κB in the nucleus (Nelson et al., 2002; Rodriguez et al., 1999). So the regulation of NF-κB activation actually is more complex than the known model about the interplays between IκB kinases, IκB proteins and NF-κB/IκB complex.

Various mechanisms have been described by which GR can trans-repress NF-κB. These include direct interaction between GR and p65 (subunit of the classic p50/p65 heterodimer NF-κB) (Caldenhoven et al., 1995; De Bosscher et al., 1997; Ray and Prefontaine 1994; Scheinman et al., 1995b; Unlap and Jope 1997) in which case the DBD of GR is involved (Liden et al., 1997; Wissink et al., 1997) probably providing a unique interaction surface (Tao et al., 2001); GR acting both as a direct inhibitor of CBP-associated HAT activity and as recruiter of HDAC2 to the p65-CBP HAT complex (Ito et al., 2000); PKA-dependent cross-repression of NF-κB by GR involving p65 subunit of NF-κB competing against GR for association with PKA (Doucas et al., 2000); induction
of IkBα by GCs (Auphan et al., 1995; Deroo and Archer 2001a; Scheinman et al., 1995a); GR and NF-κB competition for co-activators including p300/CBP which are present in limited amounts in the cell (Sheppard et al., 1998). However, there have been reports demonstrating that the trans-repression of NF-κB by GR can occur in the absence of IkBα induction (Heck et al., 1997; Unlap and Jope 1997) or even with over-expression of the co-activators p300 and SRC-1 (De Bosscher et al., 2000). In one study, over-expression of CBP increased GR antagonism by NF-κB (McKay and Cidlowski 2000), whereas in another it decreased this effect (Sheppard et al., 1998). In Dex treated Hela cells, the rate of p65 nuclear export is increased without concomitant induction of IkBα (Nelson et al., 2003). Furthermore, GR trans-repression of NF-κB has been shown due to its interference with the interaction between p65 and the basal transcription machinery irrespective of the cell’s co-activators levels (De Bosscher et al., 2000). Direct interaction between GR and NF-κB can lead to recruitment of GRIP-1, an otherwise co-activator for GR in the context of the consensus GRE, that exhibits trans-repressive effect on NF-κB (Rogatsky et al., 2002). Recently, a novel protein named glucocorticoid-Induced Leucine Zipper (GILZ) that is up-regulated by Dex and can directly interaction with p65 and p52 subunits was shown to inhibit NF-κB DNA binding and nuclear translocation, thus inhibiting its transcriptional activity (Ayroldi et al., 2001; D'Adamio et al., 1997).

Others

Glucocorticoid receptor also can interact with other transcription factors such as STAT3, STAT5 and GATA-1 as part of the diverse mechanisms by which GCs elicit their biologic effects (Chang et al., 1993; Stocklin et al., 1996; Zhang et al., 1997). STAT3 and GR have been shown to serve as co-activators for one another (Zhang et al.,
Immuno-precipitation experiments have shown that GR and STAT5 proteins interact physically (Cella et al., 1998; Stocklin et al., 1996) leading to potentiation of STAT5 and repression of GR transcriptional activities, respectively. Also, on the β-casein gene promoter, GR acts as a co-activator for STAT5 (Stoecklin et al., 1997). On the contrary, STAT5 has been shown to suppress GR transcription activity on the MMTV promoter (Biola et al., 2001; Stocklin et al., 1996). Glucocorticoid receptor tethering to GATA-1 leads to suppression of this transcription factor activity (Chang et al., 1993). Some nuclear proteins can interact and sequester GR in the nuclear matrix and negatively regulate GR transcription activity as demonstrated in the case of heterogenous nuclear ribonucleoprotein U (hnRNP U/SAF-A) (Eggert et al., 2001; Tang et al., 1998).

**Glucocorticoid Resistance and GR**

Resistance to glucocorticoids has been seen in a number of diseases (Bray et al., 1999; Hurley et al., 1991; Sher et al., 1994). Glucocorticoid resistance has been attributed to the following:

i) Down regulation of GR: this stems from the observations that GR down regulation associates with reduced GCs mediated activities (Bellingham et al., 1992; Cidlowski and Cidlowski 1981; Silva et al., 1994), and has been implicated in some pathological condition (Bray et al., 1999).

ii) The role of hGRβ (also see The human GR gene and protein). Several studies have suggested that glucocorticoid resistance is associated with increased expression of hGRβ in a variety of pathological conditions (Christodouloupolous et al., 2000; Hamid et al., 1999; Hamilos et al., 2001; Honda et al., 2000; Leung et al., 1997; Shahidi et al., 1999; Sousa et al., 2000; Strickland et al., 2001). Human neutrophils are exceptionally
resistant to GCs and the generation of hGRβ has been shown to be readily generated by serine-arginine rich protein p30c SRp30c (one of several highly conserved serine-arginine rich [SR] proteins involving in both constitutive and alternative splicing in eukaryotic cells). Knocking down SRp30c by antisense oligonucleotide inhibits expression of hGRβ while antisense oligonucleotide targeted to non-specific proteins does not yield the same effect (Xu et al., 2003).

iii) Trans-repression of GRα by NF-κB: several studies have shown that NF-κB can repress hGRα transcriptional activity (Caldenhoven et al., 1995; McKay and Cidlowski 1998; Ray and Prefontaine 1994; Scheinman et al., 1995b). Because NF-κB is expressed ubiquitously in a variety of tissues, its repressive effects on hGRα may be an important basis for glucocorticoid resistance.

**Histone Deacetylases and Their Inhibitors**

Mammalian HDACs (hHDACs) have been grouped into three classes (Kelly et al., 2002; Marks et al., 2001). Class I HDACs including HDAC1-3, HDAC8, HDAC11 are homologous to the yeast HDAC Rpd3. Class II HDACs including HDAC4-7, HDAC9 and HDAC10 are homologous to the yeast HDAC Hda1. The third class of HDACs consists of homologues of yeast Sir2 and requires NAD$^+$ for activity. The crystallographic structure of the catalytic core of HDACs has been resolved (Finnin et al., 1999). The HDACs share a homologous deacetylase core region consisting of approximately 390 residues. Residues that form the active site are highly conserved across all known HDACs. The HDACs were found to be associated with co-repressors (Hassig et al., 1997, 1998; Nomura et al., 1999; Zhang et al., 1999). Particularly, the HDAC1 exists in a large multiprotein complex, named HDAC1/mSin3A complex that
contains N-CoR, SMRT and others (Nomura et al., 1999); the HDAC2 also exists in the NURD complex that contains N-CoR and others (Zhang et al., 1999).

Known compounds that inhibit HDAC activity (HDAC inhibitors) have been grouped into four classes: i) the short chain fatty acid including the butyrates (Carducci et al., 2001; Gore and Carducci 2000; Newmark et al., 1994), valproic acid (Phiel et al., 2001) ii) the bezamides including CI-994 (Prakash et al., 2001), MS-27-275 (Lee et al., 2001; Saito et al., 1999; Weidle and Grossmann 2000), iii) the hydroxamic acids including suberoylanilide hydroxamic acid (SAHA) (Richon et al., 1998), trichostatin A (TSA) (Tsuji et al., 1976; Yoshida et al., 1990), oxamflatin (Kim et al., 1999), and iv) the cyclic peptide such as depsipeptide, also known as FR 901228 or FK228 (Nakajima et al. 1998; Weidle and Grossmann 2000), apicidin (Darkin-Rattray et al., 1996) and trapoxin A (Kijima et al., 1993). Inhibition of HDACs by HDAC inhibitors such as hydroxamic acids can lead to accumulation of acetylated histones as assessed with acetylated histones specific antibodies (Richon et al., 1998). The effects elicited by HDAC inhibitors include induction of cell growth inhibition, cell differentiation and apoptotic cell death in a variety of transformed cells; and some of them also have been shown to inhibit the growth of tumor xenografts in animal models with little or no toxicity (Cohen et al., 1999; Glick et al., 1999; Kim et al., 1999; Marks et al., 2001; Qiu et al., 1999; Saito et al., 1999; Yoshida et al., 1990). The effects elicited by HDAC inhibitors such as trichostatin A and SAHA are gene selective. Only about 2% of expressed genes are influenced by treatment of SAHA or TSA (Marks et al., 2000; Van Lint et al., 1996).

The mechanisms by which various HDAC inhibitors inhibit HDACs are not clear. However, radiograph crystallographic studies reveal that the histone deacetylase
inhibitors, SAHA and TSA, fit into the catalytic site of histone deacetylase, which forms a tubular pocket with a zinc atom at its base, and the hydroxamic acid moiety of the compounds binds to the zinc atom (Finnin et al., 1999).
Enhancement of Folate Receptor $\alpha$ Expression in Tumor Cells Through the Glucocorticoid Receptor: A Means to Improved Tumor Targeting$^{@,*}$

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$^{@}$ The findings in this report are covered by pending patents.

$^*$ Supported in part by NIH grants CA 80183 and CA 103964 (to M.R.). T.T was supported by NIH Institutional Pre-Doctoral NRSA grant CA 79450.

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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. In HeLa cells, the endogenous FR-α, its mRNA and its promoter activity were coordinately upregulated approximately 7-fold by the glucocorticoid receptor (GR) agonist, dexamethasone (Dex), progressively, between 24h and 96h of treatment. The Dex-induced FR-α had the ability to bind folate and was anchored to the plasma membrane by glycosyl-phosphatidylinositol (GPI). Optimal FR-α promoter activation by Dex occurred at < 50 nM, was inhibited by the GR antagonist, RU486, and was enhanced by co-activators, supporting GR mediation of the Dex effect. In contrast to a control glucocorticoid response element (GRE) driven promoter, the delayed response of the FR-α promoter was obtained even when Dex was withdrawn after an initial 6h of treatment, suggesting an indirect regulation of FR-α by Dex/GR. The action of Dex required the combination of a non-sequence specific G/C-rich Sp1 binding element and the initiator region in the proximal FR-α P4 promoter, without associated changes in major Sp family proteins suggesting selectivity of the Dex effect for the initiator context of the FR-α or other TATA-less promoters. Histone deacetylase (HDAC) inhibitors (valproic acid or trichostatin A), potentiated Dex induction of FR-α at both early (0-24h) and late (after 24h) stages of Dex action. Dex treatment, with or without HDAC inhibition, increased FR-α gene expression in FR-α-positive cell lines but did not cause de novo FR-α expression in the receptor-negative cells. Dex treatment also enhanced FR-α expression in vivo in murine HeLa cell tumor xenografts. 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.
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 (1-10); (ii) FR-α expression in proliferating normal tissues (2;5;7;10-13) 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); (iii) other FR isoforms are either expressed in a non-functional manner in mature hematopoietic cells (FR-β) (14;15) or poorly expressed and constitutively secreted (FR-γ/γ′) (16;17) and (iv) FR-α quantitatively recycles between the cell surface and intracellular compartments (18), effectively internalizing receptor-bound folate/antifolate compounds and folate conjugates (19;20). Various FR-α-targeted therapeutics and imaging agents have shown promise in pre-clinical models and in early clinical trials (19;21-25). 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 (20;26-48). 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 (49-64). 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 (65-68). Soluble FR-α is not detected in normal sera and therefore, the protein shed into the circulation is a potential serum marker for FR-α-positive tumors (69).

Even though major subtypes of malignant tissues show consistent patterns of FR-α expression it is clear, from quantitative flow cytometry (70) and in situ hybridization (71) analyses of ovarian tumors, that there is a considerable variability in the expression levels of the receptor, among tumors obtained from different patients, as well as heterogeneity in expression within the same tumor. The variability in the receptor expression in ovarian tumors occurs over a range of almost two orders of magnitude. The successful experimental FR-targeted therapies in animal models have used xenografts of human tumor cells (eg., 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.
We have previously reported that FR genes are non-classical targets of regulation by nuclear receptors. Thus, FR-α is negatively regulated by the estrogen receptor (72) and FR-β is positively regulated by retinoids (73;74). Here we report that the FR-α gene is indirectly and positively regulated at the transcriptional 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. These considerations provided the impetus for the present study of the nature of GR regulation of FR-α in vitro and in vivo.

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 (75;76). The FR-α gene contains two promoters, named P1 and P4, located upstream of exons 1 and 4, respectively. Products from both promoters encode identical proteins, but P1 promoter-driven transcripts are heterogeneous in the 5'UTR due to alternate splicing of exon 3 and the presence of multiple initiation sites (77). 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 (76). In the less well characterized P1 promoter, optimal activity has been attributed to the AATAATT sequence spanning a putative NP3/4 binding element located in exon 1 (78). However, the P4 promoter activity appears to be predominant in malignant cells (72) and further, P1 promoter-
driven transcripts appear to be translated several-fold less efficiently than the P4 promoter-driven transcript (77).

The present study further identifies the proximal P4 promoter elements, in particular the G/C-rich and initiator regions, as the ultimate site of Dex action in the FR-α gene. Furthermore, there appears to be no sequence specificity within the G/C-rich region or any associated changes in basal promoter activation through Sp family proteins. This finding suggests selectivity of this manner of Dex action for TATA-less promoters with preferred initiator sequences. Even though this regulation appears to be indirect and is likely to be a downstream event in glucocorticoid action, the studies show that it is associated with the hallmarks of transcriptional regulation by nuclear receptors in terms of co-activator dependence and potentiation by HDAC inhibitors. The findings are discussed in the context of the clinical utility of FR-α modulation through GR.
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 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) and valproic acid (VPA) from Sigma. Affinity-purified rabbit anti-human Sp1, anti-human Sp3, and anti-human Sp4 antibodies were purchased from Santa Cruz Biotechnology. Mouse anti-\( \alpha \) 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-\( \alpha \)-promoter construct (-3394 nt to +33 nt, relative to the transcription initiation site at +1 nt) in the PGL3 basic plasmid. The FR-\( \alpha \)(-3394nt)-(+33nt)/SV40(GC)\(_6\) and the FR-\( \alpha \)(-3394nt)-(+33nt)/SV40(INR) constructs were described elsewhere (72). The FR-\( \alpha \)(-3394nt)-(+33nt)/SV40(GC)\(_6\) construct was generated using the upstream primer 5'-GTCAGCATATGTAGTCCCGCCC-3' containing a synthetic restriction site (\( NdeI \)) and the downstream primer 5'-AAACTTAAGCAGCGATGGGGC-3' containing a synthetic restriction site (\( AflIII \))
corresponding to regions in the SV40 early promoter of the pGL3-control plasmid (Promega, catalog number E1741), the FR-α (-3394nt)-(+33nt)/SV40(INR) construct was generated using the upstream primer 5'-ATTCTCCGCGGCATCGCTGAC-3' containing a synthetic restriction site (SacII) corresponding to a region in the SV40 early promoter and the downstream primer 5'-CACTGCATACGACGATTCTGTG-3' corresponding to a region in the luciferase gene of the pGL3-control plasmid. The downstream restriction site (NarI) used in the subcloning occurred naturally in the plasmid. The recombinant plasmids were amplified in XL1Blue and purified using the Qiagen plasmid kit (Qiagen). The entire cloned sequence was verified by sequencing.

The expression plasmids for hSRC-1, hSRC-2, hpCAF, the corresponding vector plasmid pCR 3.1 and the GRE$_{2e1b}$ 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 kindly 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% CO$_2$ 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.

**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 KH₂PO₄, 2.7 mM KCl, 10 mM Na₂HPO₄, 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 (AAGTGCCGAGTGGGAGCT) and reverse primer (CATTGCACAGAACAGTGGGTG), and 0.5 µl of the Taqman probe (6FAM-CCTGCCAACCTTTCCATTCTACTTCCCCC-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.

**Binding assay for 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, 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 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.

**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 in PBS containing 1% Triton-X100 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 (79), 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 mixed with an appropriate volume of 5 x sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% 2-mercaptoethanol; and 0.001 25% bromophenol blue). The samples were resolved on 8% 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 then was similarly re-probed with a primary mouse anti-α tubulin antibody and secondary goat-anti-mouse
IgG conjugated to HRP and also subjected to Coumasie blue staining to ensure uniform sample loading.

**Murine tumor xenograft model:** Fox chase out-bred SCID female mice (29-35 days old) purchased from Charles River Laboratories were maintained under controlled conditions and fed with folate-free rodent chow ad libidum during the course of experiment. After a period of acclimation, the mice were injected with $5 \times 10^6$ Hela cells sub-cutaneously into the interscapular region. Dexamethasone pellets (0.001 mg/pellet for 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 tumor tissue collected, snap-frozen in liquid nitrogen and stored at $-80^\circ$C. The frozen tumor tissue was ground using mortar and pestle, lysed in PBS containing 1% Triton-X100 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 an approx. 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. 6B, 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 by phosphatidylinositol-specific phospholipase C (PI-PLC), which is used as a diagnostic test for the GPI plasma membrane anchor (Fig. 2A). 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. 2B). 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. 3A). RU-486 similarly inhibited Dex activation of the control GRE₂E₁b promoter-luciferase reporter construct in transfected HeLa cells (fig. 3B). The GRE₂E₁b 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 GRE₂E₁b 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:** In HeLa cells transfected with FR-α promoter-luciferase, although appreciable induction of the promoter activity and endogenous FR-α occurred only after 48h of Dex treatment, withdrawal of Dex at 48h or 72h did not appreciably decrease the FR-α promoter activity compared to the value after a 96h treatment (Fig.4A). In contrast, under similar conditions, Dex activation of the GRE₂E₁b promoter began within 3h of Dex treatment; however, the activity of the GRE₂E₁b promoter declined dramatically at 96h after
beginning Dex treatment, if Dex was withdrawn at 48h or at 72h (Fig.4A). In fact, an initial 6h treatment with Dex was adequate to produce an appreciable induction of FR-α promoter activity measured at the end of 96h, even though the induction was less than that obtained by longer treatments (Fig 4B). In contrast, withdrawal of Dex after 6h of treatment did not result in a significant activation of the GREe1b promoter, when measured at the end of 96h. These results indicate that the Dex enhancement of the FR-α promoter is likely mediated indirectly through the action(s) of an upstream target(s) of Dex and that the mode of action of Dex on the FR-α promoter is likely fundamentally different from its activation of a GRE-driven promoter.

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 FR-α promoter activity as well as its activation by Dex was measured in HeLa cells co-transfected with the expression plasmids for the individual co-activators and FR-α promoter-luciferase or the control GREe1b promoter-luciferase (Table1). Compared to the GREe1b promoter, each of the co-activators considerably enhanced the basal FR-α promoter activity. However, each of the co-activators further potentiated Dex induction of the FR-α promoter to levels comparable to that for the GREe1b promoter, which is a classical GR target 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 +33 fragments of the promoter, retained the Dex responsiveness of the full length construct in transfected HeLa cells (Fig. 5A). The time course of the Dex response was also unaltered for the truncated promoter fragments (Fig. 5A). 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.5B.

To determine whether another Sp1-dependent TATA-less 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 TATA-less and 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. 5C). 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 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. 5C). 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. 5C). 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. 6A). 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 under conditions in which endogenous FR-α was up-regulated (fig. 6B). These results exclude changes in the relative 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 (HDAC) 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 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. 7A) and TSA (fig.7B) 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 (fig. 7, A and B). VPA also potentiated Dex induction of the endogenous FR-α in HeLa cells, in a dose-dependent manner (fig.7C). 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. 7D). Under the conditions of the above treatments, the HeLa cells were viable and growing (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 (Table 2). As seen in Table 2, in human hematopoietic cells, fibroblasts and epithelial cell lines that were FR-α-negative, there was no detectable increase in the receptor mRNA expression upon Dex/TSA treatments;
however, in JAR, Ishikawa and SKOV-3 cells, that are FR-α-positive, Dex, alone or in combination with TSA, increased FR-α expression.

**Up-regulation of FR-α by Dex in murine tumor xenografts *in vivo*:** A murine tumor xenograft model was used to test whether the *in vitro* observations of FR-α induction by Dex could extend to the regulation of the FR-α gene in the physiologic milieu. Two groups of three fox chase out-bred SCID female mice were implanted subcutaneously at a single site with HeLa cells (5 x 10⁶ cells per mouse) grown in culture. When the tumors had grown to approximately 0.5 cm diameter, low dose slow release Dex pellets or placebo pellets were implanted subcutaneously to achieve a circulating concentration of 0.24 μM Dex, 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). As seen in fig.8, Dex treatment caused a uniform and substantial increase in FR-α protein in the tumors as seen in by western blot analysis of the tumor cell lysates.
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 (72-74) 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 derepress FR-α transcription (72). 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 upregulate its expression (73;74). 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, at levels of 2000-30,000 binding sites per cell (80). Further, the many clinical applications of Dex and the observation that variations in endogenous cortisol levels do not impact the physiological effects of Dex (81) also indicate that gene activation by endogenous glucocorticoids is suboptimal.
The results of this study clearly demonstrate that Dex upregulates 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 are 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 releasable tumor marker i.e., its ability to bind ligand and its anchoring to the membrane by GPI. A substantial induction of FR-α by Dex was also observed in a murine tumor xenograft model, confirming the relevance of this regulation to a 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 to help to understand, anticipate and address associated problems. FR-α upregulation in response to Dex treatment was considerably delayed (> 24h) and the delayed response (albeit lesser in magnitude) also occurred by as little as 6h of Dex 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 upregulation. 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 such as direct recruitment of components of MAP kinase (82) and PI3 kinase (83) pathways. This is evident from the GR co-activator dependence and potentiation of Dex induction of FR-α by HDAC inhibition in the early (within 24h) phase 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 (84-86). All of the GR co-activators that were shown 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 (87). Nuclear receptor corepressors generate a transcriptionally repressed state by recruiting class II HDACs (88-92). Therefore it was logical to test the effect of HDAC inhibitors on Dex induction of FR-α. The short chain fatty acid, VPA (an antineoplastic and antiseizure drug), and the hydroxamic acid, TSA (a well characterized laboratory reagent), are both inhibitors of class I and II HDACs (84;93). VPA and TSA both potentiated Dex induction of FR-α at the transcriptional level, in their pharmacologically effective milimolar and nanomolar concentration ranges, respectively (84;93). Even though one may expect HDAC inhibitors to have global effects on gene expression and to have toxic effects, 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 (84). The profound effects of HDAC
inhibitors on Dex induction of FR-α may be used to advantage in the receptor targeted
therapies 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 (93) and that act on HDACs functionally associated with nuclear receptors
are currently available (84;94-96).

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 region. Devoid of other regulatory elements, this portion of the FR-α
promoter region represents the essential elements of a basal TATA-less promoter. To
determine the minimal requirements for the delayed promoter activation by Dex, the
basal TATA-less promoter of the SV40 gene, which is also dependent on its G/C-rich
elements, was tested. The G/C-rich region of the SV40 promoter contains a cluster of six
Sp1 elements. The SV40 basal promoter did show a delayed response to Dex, similar to
the basal P4 promoter. However, chimeric promoters in which the G/C-rich and initiator
regions of the two promoters were interchanged, revealed that the combination of the
G/C-rich region of the SV40 promoter and the initiator region of the P4 promoter resulted
in increased Dex response. This result implies that a G/C-rich region is essential for the
Dex response, the magnitude of which also depends 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 (97), 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-α promoter in a similar manner and further, Dex did not alter their expression levels. It thus appears that the ultimate action of Dex in relation to FR-α regulation involves modulation of some components 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 are specific to the basal promoter context (98).

The FR-α gene thus belongs to a class of indirect target genes of Dex/GR that lack a glucocorticoid response element and are characterized by TATA-less promoters typically dependent upon Sp proteins for basal promoter activity. Based on the foregoing studies, it is reasonable to anticipate that only a few days of treatment of an individual bearing an FR-α-positive tumor with the combination of innocuous doses of a GR agonist such as Dex and a HDAC inhibitor will optimize FR-α expression in FR-α-positive tumors and greatly improve the outcome of FR-targeted diagnostics and therapies. Similar principles of inducing or even down-regulating 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.
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ovarian carcinoma-associated antigen folate receptor alpha (FRalpha) induces


FIGURE LEGENDS

Figure 1: Effect of Dex on FR-α gene expression and promoter activity. 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 duplicate 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). B: HeLa cells grown in 6-well plates were transfected with the full length FR-α promoter-luciferase reporter plasmid (500ng) 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).

Figure 2: Membrane anchoring and ligand binding properties of Dex induced FR. A: Hela cells in duplicate 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. B: 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 3: 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 GRE\textsubscript{2}e1b promoter-luciferase plasmid (B), and grown for a further 96h 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 4: Time course and reversibility of Dex induction of promoter activity.
A: Hela cells were transfected with either FR-α promoter-luciferase plasmid (filled bars) or the control GRE\textsubscript{2}e1b promoter-luciferase plasmid (open bars), and grown further for the indicated periods either in the absence or in the presence of Dex (1\textmu M) followed by harvesting for measurement of luciferase activity in the cell lysates (top panel). As indicated (top panel), in some cases, Dex was removed by replenishing the media without Dex after either 48h or 72h, but the cells were harvested for luciferase assay at the end of 96h. In the bottom panel, cells were grown in parallel for 96h either in the presence of vehicle alone or with the initiation of Dex (1\textmu M) treatment at the indicated times before harvesting the cells for western blot analysis using rabbit anti-FR antibody. B and C: At 24h following transfection of Hela cells with the FR-α promoter-luciferase plasmid (B) or the GRE\textsubscript{2}e1b promoter-luciferase plasmid (C), Dex (1\textmu M) was introduced in the media; after a further 6h, Dex was removed by replenishing fresh media or alternately, Dex was retained in the media. The cells were harvested for luciferase assay at 6h or 96h after the time at which Dex was introduced.
Figure 5: 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). The cells were then treated with Dex (1µM) and harvested at the indicated times for luciferase assay. Promoter activity is plotted as fold increase over that in cells that were not treated with Dex. 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 FR-α or SV40 promoter-luciferase constructs containing the indicated promoter fragments of FR-α, SV40 or chimeric FR-α/SV40 promoter chimeras. The cells were then treated with Dex (1µM) and harvested at the indicated times for luciferase assay. Promoter activity is plotted as fold increase over that in cells that were not treated with Dex. (top panel). The bottom panel shows a schematic of the promoter constructs that were used. The dark shaded bars represent regions of the SV40 promoter.

In chimeric constructs, the light portions were from FR-α promoter, the dark portion from SV40 promoter in the pGL3-control vector (Promega). Numeric nt in bold and italic were referenced from Promega data sheet accompanied the pGL3-control vector with 1 nt being the start of the multiple cloning site in this vector. The construct ((-3394)-(+33))/SV40(INR) is a chimaera in which the Inr of the SV40 early promoter is substituted for Inr of P4. The construct ((-3394)-(+33))/SV40(GC6) is also a chimera in which the cluster of 6 Sp1 elements of the SV40 early promoter is substituted for Sp1 sites of P4 promoter.
Figure 6: 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 FRα promoter-luciferase (500ng) 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-FRα antibody or with an antibody to tubulin (bottom panels). The nuclear extracts were probed by western blot using antibodies specific for Sp1, Sp3 or Sp4.

Figure 7: 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 antibody to tubulin. D: Hela cells were transfected with 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 8: Effect of Dex on FR-α expression in vivo in murine tumor xenografts.** Two groups of 3 mice bearing subcutaneous HeLa cell tumors 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.
Figure 1A

Fold increase in mRNA

<table>
<thead>
<tr>
<th>Time</th>
<th>Vehicle</th>
<th>Dex (100nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96h</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>12h</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>24h</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>48h</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>72h</td>
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<td>5</td>
</tr>
<tr>
<td>96h</td>
<td>1</td>
<td>6</td>
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</tbody>
</table>

Fold in molecular size

<table>
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<tr>
<th>Time</th>
<th>Vehicle</th>
<th>Dex (100nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96h</td>
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<td>2</td>
</tr>
<tr>
<td>12h</td>
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<td>5</td>
</tr>
<tr>
<td>96h</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 1B
Figure 2A
Figure 2B

Cell surface bound PLF (Relative fluorescence)

Vehicle

Dex (50nM)
Figure 3A
Figure 3B
Figure 4A

![Graph showing increase in promoter activity](image)

**Legend:**
- **FRα promoter**
- **GREα1b promoter**

<table>
<thead>
<tr>
<th>Time of harvest (h)</th>
<th>96</th>
<th>96</th>
<th>96</th>
<th>96</th>
<th>96</th>
<th>96</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of treatment (h)</td>
<td>Vehicle</td>
<td>0</td>
<td>6</td>
<td>24</td>
<td>48</td>
<td>72</td>
<td>96</td>
</tr>
</tbody>
</table>

**Graph Details:**
- **x-axis:** Time of treatment (h) and Time of harvest (h)
- **y-axis:** Increase in promoter activity (percent of maximum)
- **Bars:** Represent the increase in activity at different time points.
Figure 4B

FR-α Promoter-luc

<table>
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<th>Time of treatment (h)</th>
<th>6</th>
<th>96</th>
<th>6</th>
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</thead>
<tbody>
<tr>
<td>Dex (1 μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Promoter activity RLU ($\times 10^6$)

Time of harvest (h) 6 96 96

Time of treatment (h) 6 96 6
Figure 4C

GRE2e1b Promoter-luc

<table>
<thead>
<tr>
<th>Promoter activity RLU (x10^3)</th>
<th>Vehicle</th>
<th>Dex (1µM)</th>
</tr>
</thead>
</table>

Time of harvest (h) 6 96 96
Time of treatment (h) 6 96 6
**Figure 5**

### A

![Graph showing fold increase in promoter activity over time](image)

- Fold increase in promoter activity from 6h to 96h.
- Data points for time points 6h, 24h, 48h, 72h, and 96h.

### B

**P4 promoter**

- **-272**
  - `tgaaacattgaataaggcatccttgacatggcaaatctctgctattctgtttgcacccaatactccttgaa`
  - AP-1 like: `atgaggtggtggt`
  - Sp1: `cagggaggggtggt`

- **-116**
  - `ttatatcacatcctgtgacaccaccttggaagaaggca`
  - Sp1: `atgaggtggtggt`
  - Sp1: `cagggaggggtggt`

**Sp1**

- `gagggcagggggtggt`

**+1 Inr**

- `gtccagggcccaacccctcc`

**agctcttttctcagg**

116
Figure 5C

Fold increase in promoter activity

-3394 (+33)  SV40  (-3394) (+33) /SV40(INR)  (-3394) (+33) /SV40(GC6)

6h  24h  48h  72h

Fold increase in promoter activity

+33  -18
-28
-147
-394
-272
-394
-272
-394
-394

144

72
Figure 6A
Figure 6B
Figure 7A
Figure 7B

![Figure 7B](image-url)

**Figure 7B**

- **X-axis**: TSA (ng/ml)
- **Y-axis**: Increase in promoter activity (fold difference)

Legend:
- Dex (0mM) (open bars)
- Dex (100mM) (solid bars)
Figure 7C
Figure 7D

<table>
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<tr>
<th>Duration of VPA treatment (h)</th>
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<th>0-24</th>
<th>24-72</th>
<th>0-72</th>
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<tbody>
<tr>
<td>Time of harvest (h)</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
</tbody>
</table>
Figure 8

Mouse 1 2 3 1 2 3

Dex Placebo
Table 1

Effect of co-activators on FR-α promoter and GRE$_2$e1b promoter

<table>
<thead>
<tr>
<th>Coregulators</th>
<th>FR-α promoter</th>
<th>GRE$_2$e1b promoter</th>
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<tbody>
<tr>
<td></td>
<td>No Treatment</td>
<td>Dex (1μM)</td>
</tr>
<tr>
<td>Vector</td>
<td>1.0 +/- 0.1</td>
<td>5.0 +/- 0.7</td>
</tr>
<tr>
<td>SRC-1</td>
<td>34.0 +/- 3.8</td>
<td>118.0 +/- 6.9</td>
</tr>
<tr>
<td>SRC-2</td>
<td>38.0 +/- 3.2</td>
<td>111.0 +/- 7.2</td>
</tr>
<tr>
<td>pCAF</td>
<td>12.0 +/- 0.5</td>
<td>48.0 +/- 4.3</td>
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Table 2:

Effect of Dex on various cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cell types</th>
<th>FRα expression</th>
<th>Dex (0nM)</th>
<th>Dex (100nM)</th>
<th>TSA (25ng/ml)</th>
<th>Dex (100nM)+ TSA (25ng/ml)</th>
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<td>KG-1</td>
<td>Acute myelogeneous leukemia</td>
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<tr>
<td>Kcl-22</td>
<td>Myeloblastic leukemia</td>
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<td>…</td>
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<td>K-562</td>
<td>Erythroleukemia</td>
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<td>Ishikawa</td>
<td>Uterine adenocarcinoma</td>
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<td>+</td>
</tr>
<tr>
<td>Skov-3</td>
<td>Ovarian carcinoma</td>
<td>positive</td>
<td>…</td>
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</table>

♠ FR-α mRNA levels in FR-α-negative cell lines tested were at least 1000-fold less than those in Hela cells as assessed by real time RT-PCR and not detectable on Western blot.

…       There was no increase in FR-α mRNA as assessed by real time RT-PCR
+       There was an increase in FR-α mRNA as assessed by real time RT-PCR
Regulation of the Folate Receptor α Promoter Activity by the Progesterone Receptor Isoforms a and b @*  

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@ The material in this manuscript is the subject of pending patent applications by the Medical College of Ohio.  

* Supported in part by NIH grants CA 80183 and CA 103964 (to M.R.). T.T was supported by NIH Institutional Pre-Doctoral NRSA grant CA 79450.  

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ABSTRACT

The folate receptor (FR) type α gene, which is devoid of the classical hormone response elements, is regulated at the transcriptional level, directly and negatively by the estrogen receptor (ER) and indirectly but positively by the glucocorticoid receptor (GR). Based on the general physiologic antagonism between ER and the progesterone receptor (PR) and the functional similarities between PR and GR, it was of interest to explore the ability of PR to regulate transcription by the FR-α promoter. In HeLa cells, both PRα and PRβ upregulated the FR-α promoter activity up to 10-fold in a ligand (promegestone, R5020)-dependent manner. The promoter activation occurred progressively, between 12h and 72h of treatment and was optimal at < 5 nM R5020. The delayed response of the FR-α promoter was obtained even when R5020 was withdrawn after an initial 6h of treatment. The action of R5020 only required the core promoter elements of the proximal P4 promoter of the FR-α gene that includes G/C-rich Sp1 binding elements and the initiator and flanking sequences. The results suggest that the FR-α promoter is not a direct target of PR and that it is regulated by the product(s) of an upstream target gene(s) of PR. The nature of (indirect) activation of the FR-α promoter by PR was distinct from its (direct) activation of a classical PRE/GRE driven promoter in that (i) both PRα and PRβ activated the FR-α promoter to a comparable degree; (ii) PRα and PRβ were not mutually inhibitory; (iii) the R5020 concentration required for optimal activation of the FR-α promoter was greater by at least an order of magnitude and (iv) the PR antagonist, RU486,
acted as an agonist of PRb. These unique regulatory features may reflect the nature of PR regulation of its upstream target gene(s). In T47D cells, in which the FR-α promoter was unresponsive to R5020, either expression of the co-activator, SRC1, or treatment with the histone deacetylase inhibitor trichostatin A (TSA) enabled R5020 induction of the promoter activity. The above results support the potential utility of PR agonists in upregulating FR-α to improve FR-dependent diagnostics and therapies.
INTRODUCTION

The expression of the glycosyl-phosphatidylinositol (GPI)-anchored folate receptor (FR) in proliferating normal tissues is restricted to the luminal surface of certain epithelial cells where it is inaccessible to the circulation. On the other hand, the receptor is consistently 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 (1-10). FR-α expressed in these tumors is accessible via the tumor vasculature. FR-α quantitatively recycles between the cell surface and intracellular compartments (11), effectively internalizing receptor-bound folate/antifolate compounds and folate conjugates (12;13). Other FR isoforms are either expressed in a non-functional manner in mature hematopoietic cells (FR-β) (14;15) or poorly expressed and constitutively secreted (FR-γ/γ′) (16;17). Consequently, FR-α is a model tumor marker and target for tumor specific delivery of a wide variety of pharmacological and immunological experimental therapies. Diverse strategies for therapeutic and diagnostic (imaging) targeting of FR-α-targeted have shown promise in pre-clinical models and in early clinical trials (12;18-22). Approaches utilizing folate conjugation include radiopharmaceutical and cytotoxic conjugates such as prodrugs, prodrug activating enzymes, nanoparticles and liposomal drugs (23-36). Potent novel antifolates have been developed that are selectively transported into FR-α-rich cells (13;37). Immunological therapies have also been developed to target FR-α and 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 (38-53). Membrane bound FR-α is the precursor for a soluble folate binding protein released into the circulation by the combined action of a membrane associated protease and GPI-specific phospholipase (54-57). The soluble protein is a potential serum marker for FR-α-positive tumors (58).

Quantitative flow cytometry (59) and in situ hybridization (10) analyses of ovarian tumors have shown that there is a considerable variability in the expression levels of the receptor among tumors obtained from different patients as well as heterogeneity in expression within the same tumor. The variability in the receptor expression in ovarian tumors occurs over a range of almost two orders of magnitude. The successful experimental FR-targeted therapies in animal models have used xenografts of human tumor cells (eg., KB cells) that express the receptor uniformly and at levels closer to the high end of this range, underscoring a major potential limitation of the usefulness of the receptor as a tumor marker/target in human cancer. We have previously addressed this problem through the discovery that FR genes are non-classical target genes for regulation by nuclear receptor ligands. Thus, FR-α is regulated negatively by the estrogen receptor (60), and positively by glucocorticoid receptor and FR-β is positively regulated by retinoids (61;62).

The potential to up-regulate FR-α in gynecological tumors through additional members of the steroid receptors superfamily, may be expected to overcome limitations due to the
variability in the presence, functionality and level of expression of each individual steroid receptor among FR-α+ tumors. Therefore, we explored the possibility that the FR-α gene is regulated by progesterone. Our expectation that progesterone may positively regulate the FR-α gene at the transcriptional level was based on the observations that progesterone and estrogen generally produce opposing physiologic effects and that the progesterone receptor (PR) shares functional similarities with GR. Progesterone and glucocorticoid receptors bind with high affinity and high specificity to the same cognate DNA sequence (the PRE/GRE) (63-67) and subsequently recruit a similar set of co-activators (68;69).

Further FR-α is required in placenta where it is expressed at relatively high levels (70) and even though estrogen levels are higher in pregnancy, the ratio of progesterone to estrogen is also elevated.

The physiologic actions of progesterone are principally mediated by the progesterone receptor (PR). PR has two subtypes (PRa/PRb). PRa is identical to PRb except for the lack of a 164 residue amino-terminal peptide (71). Transcriptional activation through ligand-induced binding to the progesterone/glucocorticoid response element (PRE or GRE) through PRb is much greater than that through PRa. Furthermore, PRE/GRE-dependent transactivation by PRb is inhibited by PRa (72). PRa and PRb are variably expressed in approximately a third and two-thirds of ovarian cancers, respectively (73). PRa is the predominant subtype in breast cancer (74) whereas both PRa and PRb are expressed at variable levels but with equal frequency (one-third) in endometrial cancers (75).
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 (76;77). The FR-α gene contains two promoters, named P1 and P4, located upstream of exons 1 and 4 respectively. Products from both promoters encode identical proteins, but P1 promoter-driven transcripts are heterogeneous in the 5'UTR due to alternate splicing of exon 3 and the presence of multiple initiation sites (78). All seven FR-α cDNAs that were isolated from different carcinoma cell lines have identical ORF and 3’ UTR sequences but differ in their 5’ UTR (79-82). The basal TATA-less P4 promoter activity is primarily directed by a cluster of three GC-rich sequences that are non-canonical Sp1 binding sites, each of which contributes to promoter activity (77). Additional interactions between Sp1 and the initiator region sequence (5’-CATTCCTT-3’) also contribute to the initiation of transcription which starts at –45nt and nt –44nt (76). In the less well characterized P1 promoter, optimal activity has been attributed to the AATAATT sequence spanning +27nt to +33nt of a putative NP3/4 binding element located in exon 1 (83). The P4 promoter activity appears to be predominant in malignant cells (10) and further, P1 promoter-driven transcripts appear to be translated several-fold less efficiently than the P4 promoter-driven transcript (78).

Here we report that the PR agonist, progestone (R5020) regulates the FR-α promoter positively in a manner that is potentiated by inhibiting histone deacetylase (HDAC). Similar to the regulation of the FR-α promoter by dexamethasone, the effect of R5020 was delayed and occurred at a later time point after exposure of cells to the PR agonist.
The proximal P4 promoter region, that lacks steroid hormone response elements and only includes core promoter elements, was mapped as the ultimate target site of PR action in the FR-α promoter, similar to that for GR. It appears therefore that the FR-α promoter is the downstream target of the product(s) of a direct GR target gene(s). In striking contrast to direct activation of a PRE/GRE-dependent promoter, both PRa and PRb activated the FR-α promoter with comparable efficiency. In further contrast, the R5020 concentration required for optimal FR-α promoter activation was at least an order of magnitude greater and the PR antagonist, RU486 acted as an agonist of FR-α promoter activation by PRb. These results may reflect unique characteristics of the regulation of the upstream target gene(s) by PR. They also support the potential utility of PR agonists in enhancing FR-α expression in malignant tissues.
MATERIALS AND METHODS

**Chemicals and Reagents:** DMEM 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 from Roche Diagnostics, luciferase assay reagents from Promega, Progestosterone (R5020) from NEN Life Science Products, dexamethasone (Dex) and Trichostatin A (TSA) from Sigma. Vent DNA polymerase was purchased from New England Biolabs and custom oligonucleotide primers from Life Technologies, Inc.

**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). The recombinant plasmids were amplified in XL1Blue and purified using the Qiagen plasmid kit (Qiagen). The entire cloned sequence was verified by sequencing.

The expression plasmids for hPR and the GRE2e1b promoter-luciferase plasmid were provided by Dr. Brian Rowan (Medical College of Ohio, Ohio).

**Cell Culture and Transfection:** All of the cell lines were purchased from American Type Culture Collection (Rockville, MD). 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 Hela cells were routinely cultured in DMEM. T47D cells were grown
in RPMI-1640. For treatment with various agents (Dex 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.

**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.
RESULTS

PR Ligand and Receptor Dose Response of the FR-α Promoter and Promoter-Specificity: Fig. 1A shows that in HeLa cells transiently transfected with PR, the potent PR agonist R5020 (promegestone) increased FR-α promoter-luciferase reporter activity in a dose-dependent manner, with the maximal effects at 10-50nM for PRa and PRb; the maximal activation produced by PRa was greater than that produced by PRb. In contrast, in a control promoter (GRE\textsubscript{2e1b}) containing classical hormone (progesterone/glucocorticoid) response elements (GRE), the activity of PRb was much greater (nearly two orders of magnitude) than that of PRa and the maximal activation occurred at ~ 1nM (Fig. 1B). The relative PR dose response (in terms of ng PR plasmid DNA/10\textsuperscript{6} cells transfected) was in the range of 10ng-200ng for both PRa and PRb for the FR-α promoter (Fig. 2A) whereas maximum activation, produced by PRb on the GRE\textsubscript{2e1b}-promoter was at 100ng plasmid (Fig. 2B). The PR antagonist, RU486, inhibited transactivation of the GRE\textsubscript{2e1b} promoter by both PRa and PRb in the presence of R5020 (Fig. 3) RU486 also inhibited the action of PRa/R5020 on the FR-α promoter but not that of PRb/R5020; on the other hand, PRb/RU486 effectively activated the FR-α promoter (Fig. 3). The above results demonstrate specific ligand and receptor-mediated activation of the FR-α promoter by PR and show that it differs from a classical PR target promoter in terms of ligand and receptor subtype specificities and the much higher progestin dose required for substantial activation of the FR-α promoter.
Combined Effect of PRa and PRb: PRa and PRb are frequently co-expressed in vivo. When co-expressed in HeLa cells at sub-optimal levels, PRa and PRb produced additive enhancement of the FR-α promoter activity (in the presence of 50nM R5020) but at optimal levels, their combined degree of activation corresponded to that of PRa (the higher value) (Fig. 4A). In contrast, in the GRE$_2$e$_1$b promoter, PRa strikingly inhibited the activation by PRb (Fig. 4B); this inhibition is presumably due to effective competition for GRE by PRa, which as seen above is much less transcriptionally active than PRb. These results suggest mechanistic differences in PR action on FR-α vs. the GRE-driven promoter. They also suggest that PRa and PRb will work together to modulate FR-α in tumor tissues in which they are co-expressed.

Time Course and Reversibility of PR Effects: For the time course experiments in Fig. 6, R5020 (50nM) was introduced 48 h after transfection of HeLa cells with PR to ensure that PR levels did not change appreciably during the time course (western blot data for PR, not shown). In Fig. 5A, activation of the FR-α promoter began at 12 h-24 h with both PRa and PRb. In contrast, for the GRE$_2$e$_1$b-promoter, activation was observed at $\leq$ 3 h with both PRa and PRb (Fig. 5B). To understand this apparent delayed response of the FR-α promoter, an experiment was designed in which the transfected cells were treated with R5020 for only 6 h and then either immediately harvested or washed free of the agonist and incubated for a further 66 h before harvesting (Fig. 5C). The absence of a significant amount of residual R5020 after the wash was ensured in a separate experiment in which R5020-treated and washed cells were transfected with a ligand-sensitive promoter and transactivation measured (data not shown). As seen in Fig. 5C, even
though, as expected from the previous time course, there was no promoter activation at the time R5020 was withdrawn (i.e., 1-fold activation), a substantial amount of promoter activation occurred during a later period. This result strongly suggests that the FR-α gene may not be a direct target for PR action and that the promoter may be modulated by the product(s) of a specific upstream gene(s) target of PR.

Co-activator Limitation and Potentiation by TSA of Endogenous PR action in T47D Cells: T47D breast carcinoma cells express endogenous PR. Treatment of these cells with R5020 (50nM) did not activate transfected FR-α promoter-luciferase (i.e., 1-fold increase) (Fig. 6). However, co-transfection with expression plasmid for SRC1, a NR co-activator, resulted in activation of the FR-α promoter by R5020 (Fig. 6). Figure 6 also shows that activation of the FR-α promoter in T47D cells by R5020 was greatly potentiated by treatment of the cells with the HDAC inhibitor, TSA (25ng/ml). TSA and/or R5020 did not detectably alter PR expression under these conditions (western blot data not shown). These results illustrate that in certain cells at least one of the reasons for the inability of PR to mediate enhancement of FR-α promoter activity appears to be a limitation in co-activator availability and that in these cells a HDAC inhibitor (TSA) can substantially potentiate the activity of PR.

Identification of the Target Site of R5020 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 +33
fragments of the promoter, retained the R5020 responsiveness of the full length construct in transfected HeLa cells (Figure 7).
DISCUSSION

The results of this study clearly demonstrate positive regulation of the FR-α promoter by progestin, in a PR-dependent manner. It is also clear that FR-α promoter elements that respond to this regulation exist within the core promoter region, which is devoid of hormone response elements. This observation, together with the observed delayed response of the promoter even to a brief exposure of cells to progestin strongly support the view that similar to its regulation by Dex/GR, the regulation of the FR-α promoter by progestin/PR is indirect, i.e., the FR-α promoter is the downstream consequence of the action of progestin/PR on some more immediate target(s). Such an indirect regulation of the FR-α promoter was not a surprise in view of a similar regulation by Dex, but a number of aspects of this regulation are interesting because they presumably reflect regulatory features of the putative upstream target gene(s) for the action of progestin/PR that impact FR-α promoter activity. These regulatory features were in striking contrast to the classical progestin/PR regulation of a PRE/GRE-driven promoter and include (i) a comparable degree of activation by both PRa and PRb either individually or in combination; (ii) an optimal concentration of progestin that is about an order of magnitude greater than that for the activation of a PRE/GRE-driven promoter and (iii) the agonist action of the classical PR antagonist, RU486, for the action of PRb. The nature of regulation of this upstream target of progestin action is likely to be transcriptional because of its potentiation by an HDAC inhibitor (TSA) as well as a nuclear receptor co-activator SRC1. It thus appears that in several respects, the mechanism of action of progestin on the putative upstream target gene(s) is non-classical. It appears likely that the ultimate product of this regulation is an unidentified transcription factor that either
forms a component of or sequesters components from the core transcription initiation complex of the proximal FR-α P4 promoter. Such a supposition is reasonable in light of recent findings that showed that even though PRa is transcriptionally weak in transient transfection experiments in most cell lines, PRa and PRb regulated unique subsets of genes in T47D breast cancer cell line in response to progesterone (84). Unlike trans-activation through the classical mechanism in which PRa is a weak trans-activator, PR trans-activation through non-classical mechanism can be effectively mediated by PRa. For example, the regulation of fibronectin promoter (85) or IGFBP-1 (Insulin-like growth factor binding protein-1) (86), whose core promoters did not contain canonical GRE/PRE elements, could be elicited by both PRa and PRb in which PRa was a stronger trans-activator. Like these examples, the effect of PR/progestin on FR-α possibly represents an aspect of gene regulation by PR/progestin through non-classical mechanisms.

The non-classical features of FR-α promoter regulation by progestin favor the potential utility of PR agonists to induce FR-α in PR+ tumors. One reason for this is that PRa may be expected to mediate FR-α induction by PR agonists rather than inhibit the action of PRb as observed in the regulation of classical PR target genes. Further, the results of our studies are consistent with the view that endogenous levels of progesterone may be inadequate to support optimal expression of FR-α. This is because, the FR-α, gene, which is a non-classical target of PR, required a higher progestin dose for optimal activation, compared with a classical direct target of PR. Moreover, ovarian and endometrial cancers are more common among older (peri- and post-menopausal) women in whom the progesterone status is relatively low. The high expression of FR-α in
placenta and fetal tissues is presumably facilitated by the high progesterone levels and the high progesterone/estrogen ratios during pregnancy. Regardless of endogenous hormone levels, HDAC inhibitors may be expected to significantly impact FR-\(\alpha\) expression.

As seen in the case of T47D cells, in which the FR-\(\alpha\) promoter was unresponsive unless the cells were either treated with TSA or made to express SRC1, HDAC inhibitors may be able to overcome limitations of co-regulator expression in FR-\(\alpha^{+/PR^+}\) tumors that are unresponsive to PR induction of FR-\(\alpha\). Thus the PR-mediated regulation of the FR-\(\alpha\) promoter activity expands the repertoire of nuclear receptor ligands that may be useful to modulate FR in tumors. In contrast to our previous studies of FR regulation by retinoids, glucocorticoid and antiestrogens, we have thus far been unable to demonstrate upregulation of endogenous FR-\(\alpha\) by endogenous PR. This is because of an apparent scarcity of FR-\(\alpha^{+/PR^+}\) tumor cell lines, despite the frequent co-expression of these two proteins in primary tumors. Efforts are underway to procure primary cells and to generate recombinant cell lines in which to test endogenous FR regulation by PR agonists.


based therapeutic nanodevice targeted to tumor cells through the folate receptor. 


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    receptor isoform hPR-A is a stronger transactivator than hPR-B for the expression
    of IGFBP-1 (insulin-like growth factor binding protein-1) in human endometrial
FIGURE LEGENDS

Figure 1: R5020 up-regulated FR-α promoter in a dose dependent manner; PRb but not PRa activated GRE/PRE promoter. Hela cells were grown in phenol red free DMEM supplemented with 5% charcoal stripped serum in 6-well plates to 80% confluence, transfected with 500 ng FR-α promoter-luc (A) or GRE2e1b promoter-luc (B) and with 50 ng of PRa (open) or PRb (filled), treated with increasing concentrations of R5020 as indicated for 72 h then harvested for luciferase assay.

Figure 2: Both PR iso-forms activated FR-α promoter in a dose dependent manner; only PRb but not PRa activated PRE promoter. Hela cells were grown as in Figure 1 and transfected with 500ng of FR-α promoter-luc (A) or GRE2e1b promoter-luc (B), and with increasing amounts of PRa (gray) or PRb (black) expression vectors, treated with and without 50nM R5020 for 72 h then harvested for luciferase assay, data expressed relative to untreated controls.

Figure 3: RU486 antagonized the PRa mediated R5020 effect on both FR-α and GRE/PRE promoters whereas it antagonized the PRb mediated R5020 effect only on GRE/PRE promoter but not FR-α promoter. Hela cells were grown and transfected with various combinations of PR and with FR-α promoter-luc or with GRE2e1b promoter-luc as indicated, treated with or without 10 nM R5020 or various combinations of 10 nM R5020 and 10nM RU-486 as specified for 72h then harvested for luciferase assay, data expressed relative to untreated controls.
**Figure 4: PRa did antagonize PRb on the PRE promoter but not on the FRα promoter.** Hela cells were grown as in Figure 1 and transfected with 500ng of FR-α promoter-luc (A) or 500ng of GRE₂e₁b promoter-luc (B) and with various amounts of PRa (open) or PRb (filled) or a combination of the two, treated with and without 50nM R5020 for 72h, data expressed relative to untreated controls.

**Figure 5: A. Activation of FR-α promoter by R5020 through PR was a delayed effect.** Hela cells were grown as in Figure 1 and transfected with 500ng of FR-α promoter-luc and with 50ng of PRa (open) or PRb(filled) for 48h to make sure PR expression reached maximum, then treated with and without 50nM R5020 and collected at increasing time intervals, data expressed relative to untreated controls. **B. Activation of GRE₂e₁b promoter-luc by R5020 through PR.** Hela cells were grown as in Figure 1 and transfected with 500ng of GRE₂e₁b promoter-luc and with 50ng of PRa (open) or PRb(filled) for 48h to make sure PR expression reached maximum, then treated with and without 50nM R5020 and collected at increasing time intervals, data expressed relative to untreated controls. **C. Activation of FR-α promoter was not reversed after complete withdrawal of brief R5020 treatment.** Hela cells were grown as in Figure 1 and transfected with 500ng of FR-α promoter-luc and with 50ng of PRa (open) or PRb(filled) for 24h, treated with and without 50nM R5020 for 6h then either harvested (6h) or washed twice and medium replenished and harvested later (72h), data expressed relative to untreated controls.
Figure 6: Trichostatin A and SRC-1 synergized with R5020 to up-regulate FRα promoter in non-responsive T47D cells. T47D cells were maintained as in Figure 1, transfected with 500ng FR-α promoter-luc and with 500ng of SRC-1 expression vector or the same amount of pCR3.1 control vector, treated with or without 50 nM R5020 and with or without 25ng/ml TSA for 72 h as indicated, harvested for luciferase assay, data expressed relative to untreated pCR3.1 control.

Figure 7: The FRα P4 promoter is the site of both PRα and PRb actions. Hela cells were maintained as in Figure 1, transfected with various constructs and with 50ng of PRα or PRb as indicated, treated with or without 50nM R5020 for 72h. Data was expressed relative to untreated controls. The full-length FR-α promoter spans from (-3394 to (+33), the FR-α P4 promoter from (-272) to (+33). The construct spanning (-116)-(+33) has deletion of the most upstream Sp1 element relative to the transcription initiation site (+1).
Figure 1B

![Bar graph showing the activity of the GRE2e1b promoter in response to R5020 concentrations.

- Y-axis: GRE2e1b promoter activity (RLU × 10^6)
- X-axis: R5020 concentration (nM)

- Open bars represent PRa activity.
- Solid bars represent PRb activity.

The graph shows an increase in promoter activity with increasing R5020 concentration for both PRa and PRb. The error bars indicate variability among replicates.](image-url)
Figure 2A

Fold Increase in FR-α promoter activity

ng of transfected PR expression vector/10^6 cells
Figure 2B

Fold increase (10^2) in GRE_2e1b promoter activity

ng of transfected PR expression vector/10^6 cells

PRa

PRb
Figure 3

Promoter activities RLU (10^6)

- FR-alpha-Luc + PRa
- FR-alpha-Luc + PRb
- GRE-Luc + PRa
- GRE-Luc + PRb

50nM R5020
50nM RU486
Figure 4A

Fold increase in FR-α promoter activity

PRα (ng plasmid) 0 50 0 50 200 0 200
PRβ (ng plasmid) 0 0 50 50 0 200 200
Figure 4B

Fold increase ($10^2$) in GRE 2'1b promoter activity

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Figure 5A

Fold increase in FR-α promoter activity

- PRA
- PRB

Time (h) after R5020 addition

0 3 6 12 24 36
Figure 5B

Fold increase ($10^3$) in GRE$_{e1b}$ promoter activity

Time (h) after R5020 addition

- PR$_a$
- PR$_b$
Fold increase in FR-α promoter activity

Time of harvest (h)

6 72 6 72

PRa PRb
Figure 6

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Figure 7

P4 promoter

-272

tgaacaccttgataaggccattgtggacctatgccaactctctgcattttgctatttgcccaatccatccttgaaattatctcctgtgaccacctgagaagga 

AP-1 like

Sp1

-116

Sp1

\[ \text{atgaggctcaagc} \]

\[ \text{cagggaggggtggt} \]

\[ \text{cttatctctactttcacttccttttcttggatctgggaaaactgagggagatgggggcagggccttctatctgccccaggctccgtcaca} \]

\[ \text{gagggcctgcccttccttccca} \]

\[ \text{ggcccccaccccctc} \]

\[ \text{ctggtgctcactgaacacagcagctttctctttagg} \]
SUMMARY

Our work has laid the basis for using steroids such as Dexamethasone and R5020 alone or in combination with an HDAC inhibitors to up-regulate FR-α for the purpose of improving efficacy of FR-α mediated tumor targeting. Also, since FR-α over-expression is specific for cancer tissues, its up-regulation induced by the above steroids can be used for specific screening of some insidiously progressive cancers such as ovarian cancer that have been proven not to have any available reliable marker for use in the clinic. In addition, the study of the mechanism of Dex-induced up-regulation of FR-α uncovers another aspect of the function of the Inr in transcription control. It appears that the ultimate action of Dex in relation to FR-α regulation involves modulation of some component of the transcription initiation complex, whose exact composition is dictated by both the G/C-rich and initiator regions.
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Appendix

It is known that Dex through GR can repress NF-κB through various mechanisms such as induction of IκB expression and competition for co-activator. Therefore, I decided to explore the possibility of Dex activation of FRα through NF-κB. As shown in the data, p65 (a subunit of NF-κB) represses the FRα promoter in a dose dependent manner (Figure A). Conversely, IκB super-repressor, a specific inhibitor of NF-κB activates the FRα promoter (Figure B). Treatment of Dex diminishes the availability of p65 in the nucleus of Hela cells (Figure C), possibly linking the activating effect of Dex with the repressive effect of p65 on FRα. Consistent with this view, TNFα (an activator of p65 nuclear translocation) decreases FRα expression (Figure D&E). On the other hand, aspirin (one of its established effects is to suppress p65 activation) increases FRα expression (Figure E). However, the decrease in nuclear p65 occurs in < 24h (the earliest time point for this decrease has not been determined) whereas as presented in the main body in this thesis, Dex induction of FR-α promoter activity occurs at 48h suggesting a possible indirect effect. Based on these preliminary observations, it appears possible that the Dex action on the FRα gene is mediated by NF-κB. These correlative data will need to be examined further by more vigorous mechanistic studies to address the pleiotropic effects of TNFα and aspirin on cell physiology and homeostasis. Besides the possibility of using aspirin to induce FRα, these findings may provide a link between FRα regulation and cell signaling since the activation of NF-κB is elicited through various cell signal pathways. Secondly, they might also provide a link between the FRα expression
and NF-κB related cancer behaviors since NF-κB has been shown to be anti-apoptotic and pro-metastatic, all of which are characteristics of malignancy. This warrants further investigations into the relationship between NF-κB and FRα expression since the underlying mechanisms are surely complex and may shed light on why FRα expression is up-regulated in some but not in other cancers.
Figure legends for appendix

A: Hela cells were transfected with 500 ng FRα-promoter-luc alone or with 50 ng pCMV4 vector or with increasing amounts of pCMV4-p65 expression vector for 72 h then harvested for luciferase assay.

B: Hela cells were transfected with 500 ng FRα-promoter-luc alone or with increasing amounts of pCMV4-IκB super-repressor expression vector, incubated for 72 h in DMEM supplemented with 5% charcoal stripped serum then harvested for luciferase assay.

C: Hela cells were maintained in in DMEM supplemented with 5% charcoal stripped serum, treated with 1 μM of Dex for increasing durations so that they all seeded and harvested at the same time (cells treated for 24 h were first plated in appropriate medium at the same time with other cells, grown for 72h then treated for 24h to harvested at the same time as cell treated for 96h), nuclear extracts prepared for Western blot, the cytoplasmic portion tested for confirmation of up-regulation of FRα in response to Dex.

D. Hela cells were treated with increasing concentrations of TNFα for 72h then harvested for Western blot.

E. Hela cells were grown in 6 well plates treated with or without 10ng/ml TNFα for 72h then subjected to tritiated-FA binding assay.
F. Hela cells were treated with increasing concentrations of aspirin for 72h then harvested for Western blot.
FR-α promoter activity is inhibited by p65

A
IkB super-repressor increases FR-α promoter activity
Time of treatment (1μM Dex) 0 24 96 (h)

C

p65 →

Time of treatment (1μM Dex) 0 24 96 (h)
<table>
<thead>
<tr>
<th>Alc</th>
<th>0</th>
<th>0.0615</th>
<th>0.125</th>
<th>0.250</th>
<th>0.500</th>
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[Image of a gel with a protein band labeled FRα]
Abstract

Folate receptor (FR) is a GPI-anchored cell surface protein. Three iso-forms of FR have been identified and characterized, FRα, FRβ and FRγ/FRγ’. Folate receptor is capable of transporting folate/antifolate compounds and folate conjugates into the cell functions in transport of folates into the cell. Folate receptor α is found on certain normal epithelial cells and highly expressed in various cancer tissues including non-mucinous ovarian cancers, uterine endometrial endometrioid adenocarcinoma, ependynomas, menigioma and some colon and breast cancers. In normal tissues, FRα is restricted on the apical (luminal) aspect of the polarized epithelial cells, thus rendering it inaccessible through the circulation. However, it is accessible through the circulation in malignant tissues owing to the hypervascularization seen in these tissues. Folate compounds or conjugates can be effectively taken up into the cell by means of FRα due to its ability to shuttle back and forth between the cell membrane and endosomal compartment.

Folate receptor mediated targeting has been shown to be highly specific and effective and dependent on the density of FR on the cell membrane. However, the expression of FR in tumors is highly variable among tumors of certain type and heterogeneous within a tumor. Therefore, an optimal expression of FR in malignant tissues will help optimize the effectiveness of FR-mediated targeting.

We have found that both in cell culture and in tumor xenograft mouse model, Dexamethasone (Dex) can up-regulate the FRα gene in Hela cells and this is significantly enhanced when either valproic acid (VPA) or trichostatin A (TSA), both HDAC inhibitors, is used in conjunction with Dex. In addition, in Hela cells co-transfected with
a FRα promoter luciferase construct and an expression vector for either PRa or PRb, the progestin R5020 can up-regulate the FRα promoter up to ten-fold. Furthermore, in non-responsive cells such as T47D, co-treatment of R5020 with TSA can up-regulate the expression of the FRα promoter. Our findings establish the usefulness of employing Dex, R5020 and HDAC inhibitors to up-regulate FRα in order to improve FRα-mediated targeting. We report mechanistic aspects of the regulation of FRα by progestins and glucocorticoids that are relevant for its utility as a tumor target.