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Role of folate receptor genes in reproduction and related cancers

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1. ABSTRACT

The expression patterns of folate receptor (FR) isoforms, α and β, in normal and malignant male and female reproductive tissues is described. The significance of the receptor in reproductive and developmental physiology is discussed. The potential value of the receptor expressed in malignant tissues including ovarian and endometrial cancers as a diagnostic marker and a therapeutic target is reviewed. Finally, the various transcriptional and post-transcriptional mechanisms that govern the tissue/tumor-specificity of the receptor and its regulation by folate and steroid receptor ligands are described; the potential value of this knowledge in developing better methods for the early detection and treatment of certain cancers is discussed.

2. INTRODUCTION

In its reduced states the B vitamin, folate, is an essential co-enzyme in the biosynthesis of purines and thymidine that are in turn required for DNA synthesis. In humans, there are three functionally relevant isoforms of the high affinity (Kd $<10^{-9}$ M) receptor for folate (1;2), termed folate receptor (FR) types α (3;4), β (5) and γ/γ′ (6;7). The FR isoforms are N-glycosylated polypeptides containing 220 to 236 amino acids and sharing amino acid sequence identity of 70-%-80% (3-7). FR-α and FR-β, which are attached to the cell surface by a glycosyl phosphatidylinositol (GPI) anchor (4;8-10), recycle between extracellular and endocytic compartments and are capable of transporting folate into the cell (11). The circulating form of folate is (6S) 5-methyltetrahydrofolate,

for which FR-α has a 50-fold greater affinity than FR-β (12). FR-γ and its truncated mutant variant, FR-γ′ lack a GPI anchor and are constitutively secreted (7). The three FR isoforms are encoded by distinct genes that are regulated by a variety of transcriptional and post-transcriptional mechanisms resulting in rather narrow tissue specificities (13). FR-α is expressed in a variety of epithelial tissues including those of the female reproductive tract, placenta, breast, kidney proximal tubules, choroid plexus, lung and salivary glands. FR-β is expressed at relatively high levels in placenta (5), mature neutrophils (14) and activated monocytes and macrophages (15). The secreted FR-γ only appears to be expressed by hematopoietic tissues, in particular, lymphoid cells (7). Paradoxically, with the exception of placenta, the receptor expressed in epithelial tissues cannot come in contact with circulating folate owing to its localization on the apical (luminal) surface. It is equally paradoxical that FR-β that is expressed in mature neutrophils is unable to bind folate, likely due to an as yet undetermined post-translational modification (16). Consequently, a physiologic role for membrane anchored FR in folate transport is clear in only certain instances; they include renal folate reabsorption (17;18), transplacental folate transport (19) and embryonic nerve tube development (20). Most tissues lack a folate receptor and their need for folate uptake is largely met by a low affinity/high capacity anion exchange protein called the reduced folate carrier (RFC) (21). The RFC is a structurally and functionally distinct and ubiquitous transmembrane protein that belongs to the glucose transporter superfamily (22). In addition to FR-γ, soluble forms of FR may also be derived by the action of proteases or phospholipase on membrane anchored FRs (23-25). The physiologic function of soluble FRs is not well understood, although there is some evidence that soluble FR-α present in milk facilitates intestinal folate absorption (26).

The narrow tissue specificity of membrane anchored FR combined with its inaccessibility/non-functionality in normal tissues offers an obvious advantage in its utility as a target for selective drug delivery and imaging in FR-positive malignant tissues. Since a large portion of the membrane anchored FR is shed into the circulation in a soluble form, the protein is also a potential serum marker for those tumors. Indeed in recent years, a large body of work has rendered FR-α targeting as a paradigm for tumor selective delivery of a broad range of experimental therapeutic agents (27-34). The research in this area has predominantly focused on malignancies of the female reproductive tract, although other FR-positive cancers have also been investigated.

An aspect of FR expression that is currently under investigation because of its significance in both developmental physiology and in cancer therapy is the regulation of its expression by tissue specific mechanisms, by folate and by steroid hormones (13). A number of the transcriptional and post-transcriptional mechanisms that govern FR expression also represent novel mechanisms of gene regulation. The following sections will review our current understanding of the physiologic role of FR with a particular emphasis on reproductive tissues and fetal development as well as the potential importance of FR in the detection and treatment of cancers originating from reproductive tissues. Accordingly, this review will focus on FR-α and FR-β expressed in the relevant normal and malignant tissues, including the mechanisms by which their expression is regulated. FR-γ/γ′ will not be discussed further.

3. EXPRESSION OF FR IN NORMAL REPRODUCTIVE TISSUES

The earliest immunocytochemical studies that attempted a detailed investigation of the tissue distribution of FR-α in normal and malignant tissues of the female genital tract used the monoclonal antibodies MOv18 and MOv19 (35-40). The antibodies, generated against a membrane preparation from a human ovarian tumor, recognized different epitopes on the same 38 kDa GPI anchored protein (gp38) subsequently identified as FR-α (41;42). The published immunocytochemical studies are conflicting in some respects, likely owing to methodological limitations (37). The technical problems associated with these antibodies include their non-reactivity with paraffin-embedded sections, the variability in their reactivities in different frozen section preparations, and their possible binding to target proteins other than FR. The use of a quantitative *in situ* hybridization method to examine the expression of FR-α mRNA circumvented these problems (43). This method also overcame the subjective nature of positive antibody reactivity that further failed to take into account variability in cell density within the tissue sections (43). The studies also indicated that the relative levels of FR mRNA in normal and malignant tissues generally reflect relative FR-α protein levels (44). The consensus that emerges from the various published studies (36-40;45;46) of FR-α expression in the female reproductive tract is that the receptor is expressed in the epithelial cells of the fallopian tube, the surface epithelium of the uterus and endocervix, the germinal epithelium of the ovary and the glandular epithelium of the cervix. Other female reproductive tissues that express FR-α include the trophoblasts of the placenta and the acinar cells of the breast (36;37;39;47). Placenta expresses FR-β in addition to FR-α (5). FR-β detected in other normal tissues is likely derived from infiltrating hematopoietic cells (neutrophils, monocytes and activated macrophage) (15;44).

Immunocytochemical studies using MOv19 localized FR-α in the normal male reproductive tract mostly in the epididymal epithelium and in the basal cells of the vas deferens epithelium (39). Superficial epithelial cells of the vas deferens did not show any reactivity to the antibody (39). A high affinity folate binding protein has also been identified in semen (48) and in testicular (49) and prostate tissues (50).

4. ROLE OF FR DURING PREGNANCY AND LACTATION

The best known physiologic functions of FR are related to its ability to transport folate or to promote folate

absorption, although alternate functions in regulating cell growth have been also suggested (51-53). As discussed below, in reproduction and development, detailed physiologic studies combined with FR gene knockout and knockdown studies have clearly delineated the physiologic significance of FR in folate uptake. As noted above the physiologic function of FR expressed on the luminal surface of epithelial cells of the normal genital tract has remained elusive.

4.1. Trans-placental folate uptake

Folate is essential for normal fetal development and deficiency in this vitamin has been shown to result in neural tube defects (54;55). The fetal folate level is determined by the level of folate in the maternal circulation; nevertheless it is higher than that of the mother (2). Significantly more folate bound to a folate binding protein (FBP) was measured in serum samples from the neonates' umbilical cords than from their maternal serum (56). Based on this finding, it was hypothesized that a folate binding factor mediated acquisition of folate by the fetus (56). Subsequently, a study by Henderson and colleagues using an *ex vivo* human placental cotyledon perfusion model elucidated the role of human placental FR in the delivery of folate to the fetus (19). That study showed that placental FR attached to the microvilli facing the maternal circulation is needed for sequestering 5-methyltetrahydrofolate, the circulating form of folate, thus trapping it until it is released into the intervillous blood (19). This generated a folate concentration gradient which led to passive diffusion of folate into the fetal circulation (19). In other studies, a Mg^{2+}-dependent metalloprotease which converts hydrophobic placental FRs into hydrophilic forms was purified from placenta (23;25;57). The physiologic significance of this protease is not clear although its action may be expected to interfere with the amount of FR available for ligand binding on the surface of trophoblasts (25).

4.2. Embryonal development

Further support for the importance of FR during development was obtained from transgenic mouse models with targeted inactivation of FBP1 (or Folbp1) and FBP2 (or Folbp2), which are the two murine homologs of the human FR-α and FR-β respectively (20;58;59). Folbp1 was shown to be expressed in the central nervous system of mouse embryos, at relatively high levels in the yolk sac at embryonic day (E)8 and E(9), and in placental tissue (59;60). The high expression of Folbp1 in the yolk sac was suggested to play a role in folate transport to the embryo at the stage of neural tube closure until the formation of the chorioallantoic connection (60). In a cross between $Folbp1^{+/-}$ mice, the embryos homozygous for the deletion in Folbp1 ($Folbp1^{-/-}$) died in utero by E(10) exhibiting failure of neural tube closure (20). In contrast $Folbp1^{+/-}$ embryos showed normal development despite the fact that $Folbp1^{+/-}$ dams had approximately 30% lower circulating folate compared to wild-type mice (20). Daily administration of a high oral dose of folinic acid (25mg/kg) to $Folbp1^{+/-}$ dams starting two weeks prior to their mating with $Folbp1^{+/-}$ males, resulted in rescue of the normal phenotype in a significant number of the $Folbp1^{-/-}$ embryos until they were sacrificed for genotyping on E(18) (20). Moreover, when a lower dose of folinic acid of 6.25mg/kg was administered daily to $Folbp1^{+/-}$ dams starting one week before mating with $Folbp1^{+/-}$ males, $Folbp1^{-/-}$ embryos developed neural and craniofacial abnormalities such as unilateral or bilateral cleft lip, or cleft palates by E(13.5) (61). Interestingly, it appears that deletion of the Folbp1 gene also alters the expression of a number of other genes including those involved in normal development (61;62).

In another study three different antisense oligonucleotides were microinjected into the amniotic sac of mouse embryos to disrupt Folbp1 expression (63). These sequences targeted the 5′ and 3′ coding regions, and the 5′ untranslated region in *Folbp1* (63). Microinjection of relatively higher concentrations of the antisense oligonucleotides resulted in a higher percentage of embryos with open neural tubes than those observed in the untreated control group or in mice microinjected with relatively lower concentrations of the oligonucleotides (63). In order to test whether exogenous folate could compensate for the effects of decreased Folbp1 expression, embryos were coinjected with the antisense sequences for the coding regions of *Folbp1*, 5-methyltetrahydrofolate, or a combination of both (63). Embryos receiving the combination treatment had a normally developing heart and a significant decrease in the occurrence of open neural tubes as compared to control embryos treated with the antisense sequence alone.

Recently, it was shown that circulating anti-FR antibodies in the serum of pregnant rats could bind to FR expressed on embryonic and extraembryonic tissues (64). This binding was hypothesized to block the folate uptake pathway via FR and cause a decrease in the amount of folate supplied to the embryos thus leading to abnormal development or death of the embryos (64). The intraperitoneal injection of different doses of FR antiserum to pregnant rats on E(8) caused resorption of all the embryos within 48 hours for the two highest doses (0.5 and 0.4 ml/rat) and within 7 days for the lower dose (0.3 ml/rat) (64). Moreover, subcutaneous injection of a pharmacologic dose (12mg/kg) of folinic acid in a three-dose regimen for two days starting on the day of the injection with 0.3ml of the antiserum prevented the resorption of the embryos (64). The protective effect of folinic acid was suggested to result from the uptake of folate in embryonic cells via other mechanisms such as RFC and/or passive diffusion when FR, the preferred uptake route, was absent or non-functional (64). These findings led to the investigation of whether autoantibodies against FR were present in the serum of women with a history of a pregnancy complicated by neural tube defects. (65). Indeed in a preliminary pilot study, 75% of the women (9/12 subjects) tested who had been or were pregnant with a fetus with neural tube defects had anti-FR antibodies in their serum whereas none were detectable in the serum of 90 % of the control subjects (65).

Mouse embryos homozygous for the deletion in Folbp2 ($Folbp2^{-/-}$) developed normally into fertile adults (20). They had comparable serum folate levels as the wild type mice when fed a normal diet (20). Folbp2 nullizygous

mice were further tested for any increased susceptibility to *in utero* exposure to teratogenic compounds such as sodium arsenate and valproic acid (VPA) (66;67). Administration of two intraperitoneal injections of arsenate (40 mg/kg) on E(7.5) and E(8.5) to Folbp2$^{+/-}$ and Folbp2$^{-/-}$ pregnant mice resulted in a significant increase in embryonic lethality and exencephaly compared to control embryos injected with vehicle alone (67). Furthermore, Folbp2$^{-/-}$ but not Folbp2$^{+/-}$ pregnant mice fed a folate-deficient diet had a further increase in the number of embryos with exencephaly. Based on the analysis of the arsenicals excreted in the urine, the higher susceptibility of Folbp2$^{-/-}$ mice to arsenate was not likely due to differences in metabolizing sodium arsenate but depended on proper folate transport mechanisms (67). No definitive proof exists for an increased susceptibility of Folbp2$^{-/-}$ mice to in utero exposure to VPA (66).

Taken together, the data on the consequences of inactivation of the *Folbp1* gene or protein in rodents stress the importance of Folbp1 in delivering necessary amounts of folate to the embryo. They also show that adequate supplementation with dietary folate given from conception and beyond neurulation will have a protective role against developmental abnormalities resulting from the absence of functional Folbp1 (20;63;68). The role of Folbp2 appears to be more subtle (20). As discussed above, emerging evidence also supports the relevance of these findings to the human population. No association was found between human FR gene polymorphisms and defective nerve tube development (69-72) but future studies may be expected to provide further insights into the exact nature of FR-related defects in developmental abnormalities in humans.

4.3. Soluble FR in milk

Folate binding proteins have been purified from cow, goat, and human milk principally by affinity chromatography using folate-Sepharose (73-76). Like other biological fluids including urine (77), cord blood (56), cerebrospinal (78) and amniotic fluid (79), milk contains significant levels of a high-affinity soluble folate binding protein apparently derived from a membrane-associated precursor (FR) (73).

A number of studies in rodents and goats have demonstrated the importance of the soluble form of FR in milk in increasing the bioavailability of folate to the newborn (80-84). The studies showed that the acidic pH of the stomach causes dissociation of folate from the soluble FR (81;82). Once in the intestine, folate reassociates with the soluble FR (81;82). Folate bound to FR is absorbed in the small intestine more slowly and by a mechanism that is distinct from that for the absorption of free folate (81;84). However, the total folate absorbed is not influenced by its binding to FR (84). On the other hand, urinary excretion of folate absorbed by this mechanism is lower and the folate is more bioavailable (80). In addition, it has also been shown that folate bound to soluble FR is protected against uptake by intestinal flora such as *Streptococcus faecalis* R and *Lactobacillus casei* that would decrease the bioavailability of folate to the newborn (80).

5. EXPRESSION OF FR IN MALIGNANT TISSUES OF THE REPRODUCTIVE SYSTEM

The expression of FR-α in a number of cancers of reproductive tissues has been examined by immunocytochemistry (36;38;40;46), flow cytometry (85), quantitative *in situ* hybridization (43) and microarray analyses (86). Variable patterns of FR-α expression were observed in relation to transformation of normal epithelial cells of the female genital tract into malignant cells (43). Differentiation of the pluripotent germinal epithelium into benign serous or benign mucinous lesions resulted in down-regulation of FR-α. Down-regulation of FR-α also occurred in malignant transformation of the germinal epithelium into mucinous cystadenocarcinoma or clear cell carcinoma. In contrast, high FR-α expression was observed in specimens from endometrioid adenocarcinomas of the ovary and serous cystadenocarcinomas. Tumors from patients with stage IV ovarian carcinomas expressed significantly higher levels of FR-α than tumors from patients with stage I (85). Endometrioid adenocarcinomas showed relatively high FR-α expression whereas uterine epithelial malignancies of small cell, clear cell and squamous cell differentiation did not have significant FR-α expression. Both ovarian and uterine carcinomas displayed heterogeneity in FR-α expression linked to apparent heterogeneity in relative differentiation. Poorly differentiated areas in the tumors expressed higher levels of FR-α than well-differentiated foci in the same sections. In contrast to *de novo* expression of FR-α in adenocarcinomas of the uterine endometrium, transformation of the glandular epithelium of the cervix into adenocarcinoma was associated with down-regulation of FR-α. Squamous cell carcinomas of the cervix did not appear to express significant amounts of FR mRNA (43) but it appears that the FR-α protein may be induced in these tumors by a translational mechanism related to folate status (87). FR-α was also expressed in ~ 20% of breast carcinomas (46). FR-α was expressed in testicular choriocarcinoma but not in seminomas (44;49). The β isoform of FR was co-expressed with FR-α in some malignant tissues (44).

The FR-α expressed in malignant tissues retains its cell surface distribution and its ability to bind folate (44). As discussed below, this allows for the utilization of the tumor cell FR as a means to selectively target various therapeutic and imaging agents.

6. CLINICAL UTILITY OF FR IN MALIGNANT TISSUES OF THE REPRODUCTIVE SYSTEM

6.1. FR as a target for tumor-selective drug delivery

FR-α targeted drug delivery is currently a paradigm in the development of innovative drug targeting strategies for the following reasons: (i) FR-α is expressed in a number of malignancies; (ii) FR-α expression in proliferating normal tissues 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 (28); (iii) other FR isoforms are either expressed in a non-functional manner in mature hematopoietic cells (FR-β) (16) or poorly expressed and constitutively secreted (FR-γ/γ′) (6;7) and (iv) FR-α quantitatively recycles between the cell surface and intracellular compartments (11), effectively internalizing receptor-bound folate/antifolate compounds and folate conjugates (88;89). A large body of literature has revealed considerable promise for FR-α as a therapeutic target in FR-α-rich tumors (reviewed in (27-34)), largely in ovarian cancer. Recent findings indicate that the FR-targeted agents may also target FR type β that is expressed in activated macrophages present in the tumors (90). It has been suggested that this simultaneous targeting of macrophages may be useful in checking tumor growth by preventing the production of tumor-promoting molecules by these cells (90).

A broad range of experimental strategies for targeting FR have been explored. In pre-clinical and clinical studies, FR has shown considerable promise as a potential means of delivering a wide variety of novel therapeutic agents, including genes, to solid tumors and leukemic cells and as a tumor and serum marker (29;32;88;91-93). The novel therapeutic approaches include the use of folate or antibody conjugates of cytotoxics (94-96), and radiopharmaceuticals (97-102), folate-coated liposomes containing antisense oligonucleotides, genes or cytotoxics (16;103-109), folate conjugates of pro-drugs (110) or a pro-drug activating enzyme (111) or folate-linked nanoparticle carriers for therapeutic drugs and genes (112-115). In cells expressing high levels of FR, the receptor also offers the preferred uptake route for novel antifolate drugs, which target glycineamide ribonucleotide formyltransferase and thymidylate synthase (116); antifolate drugs that are selectively transported by FR have recently been developed (89;117). A variety of immunological therapies have also been developed (118). Bifunctional antibodies, which bind FR and T-cell antigens, have induced a profound immune response against tumors in xenogenic animal models (119;120) and in patients with advanced ovarian cancer (121-124). Similarly, a chimeric molecule consisting of interleukin 2 and a single-chain Fv of an antibody against FR effectively inhibited tumor growth in vivo (125). FR may also be used to produce DNA and polypeptide vaccines against tumor cells (126-131). Recently, strong in vivo antitumor responses were obtained using dual-specific T-cells that could be expanded with an allo-antigen (132) and by targeting immunogenic haptens to FR (133).

6.2. FR as a target for tumor imaging agents

A distinctive advantage that FR offers as a target for whole body imaging of tumors is its ability to bind to an innocuous small molecule (folic acid), which is amenable to simple chemical conjugation with other molecules without a decrease in its binding affinity. Therefore, imaging FR-α+ tumors with folic acid-conjugates would circumvent a number of limitations associated with immunoscintigraphy using antibody probes such a immunogenicity, slower clearance from the circulation and the need to use multiple steps in the procedure (29;134-137).

Low-molecular weight folate-chelate conjugates radiolabeled with ^{111}-Indium (^{111}In) or ^{99m}Technetium (^{99m}Tc) have been or are being tested in clinical trials for imaging pelvic and abdominal masses in women (29;138). Phase I and II clinical studies using intravenously injected ^{111}In- diethylenetriamine penta-acetic acid (DTPA)-folate show that it is well tolerated by patients and that it has a good accuracy in distinguishing malignant from benign abdominal masses 4h post-injection (138). Nevertheless, assessment of patients with recurrent ovarian and endometrial carcinomas appears to be more challenging (138). Consistent with the biodistribution in mice studies, uptake of ^{111}In-(DTPA)-folate occurred in the kidneys of all patients probably due to FR-α in proximal kidney tubules which mediate the absorption of folate from urine (17;18;138-140). ^{99m}Tc-(DTPA)-folate conjugates like ^{111}In-(DTPA)-folate are mostly eliminated by the kidneys (101;139). However unlike ^{111}In's 68h long half-life, ^{99m}Tc has a shorter half-life of 6h (29). This relatively short half-life and the rapid clearance of ^{99m}Tc-folate conjugates from circulation are main health-related reasons that render them more attractive radioimaging agents than their predecessors (29;139;141;142). Based on preclinical experiments, ^{99m}Tc–based folate conjugates appear to be promising for *in vivo* tumor imaging in ovarian cancer patients and clinical trials are currently in progress to determine the efficacy of some of these imaging agents (29;101;143-145).

Radiolabeled bioactive peptides are beginning to replace monoclonal antibodies in tumor imaging mostly because they may be designed to have better pharmacokinetic characteristics and to be excreted via the kidney (146). Combining labeled folic acid with labeled peptides that target other tumor markers may be expected to improve the efficacy of tumor imaging.

6.3. FR as a serum marker for early detection of cancer

As previously mentioned, normal human serum contains virtually no detectable soluble FR. The inability to detect high affinity folate binding proteins in normal serum may be explained by the fact that FR-α is normally expressed on luminal surfaces that are not in contact with the bloodstream and that FR-β expressed on the surface of mature neutrophils is non-functional (16). In contrast, when FR-α-positive epithelial cells become malignant, the cells lose their polarity and the cell surface is in contact with the bloodstream within a vascularized tumor mass. Since soluble FR-α is low or undetected in normal human sera, the protein shed into the circulation is a potential serum marker for FR-α-positive tumors. Using a radioimmunoassay that had a rather poor sensitivity owing in part to the modest affinity of the monoclonal antibodies used, approximately a third of serum samples from ovarian cancer patients tested positive for FR-α (40). Recent findings in a mouse HeLa tumor cell xenograft model showed that treatment with an innocuous dose of dexamethasone (Dex), a glucocorticoid receptor agonist, upregulated the expression of FR-α in tumor cells and also led to a significant increase in the serum FR compared to

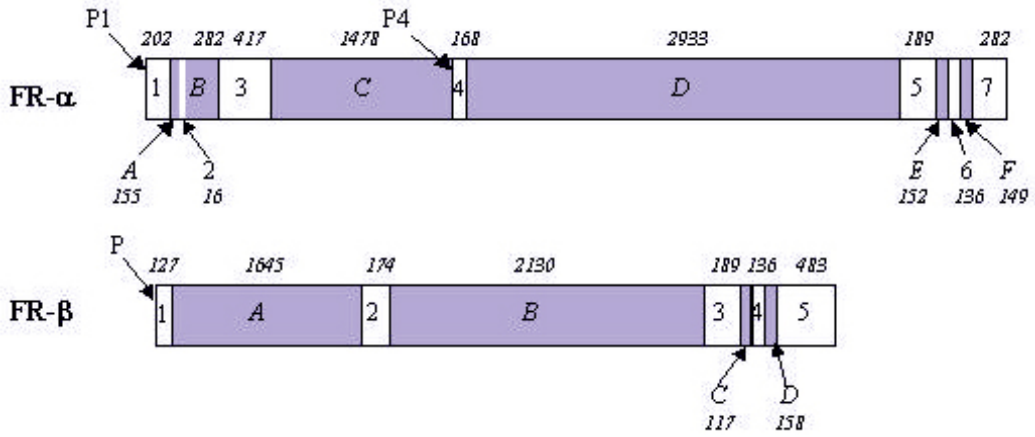

Figure 1. Organization of the human FR-α and FR-β genes. The two FR-α promoters (P1 and P4) and the single promoter (P) of FR-β gene are indicated. The numbers and italicized letters indicate exons and introns, respectively. The italicized numbers indicate the number of nucleotide base pairs in the corresponding exon or intron.

the placebo group (147). Moreover, serum FR levels were not significantly affected by administration of Dex to normal mice (147). Thus Dex treatment could be used to render FR-α-positive tumors more easily detectable from a serum assay (147).

Any progress in the early detection of ovarian cancer can result in major improvements in morbidity and mortality since early stage ovarian cancers have a better prognosis than stage III and IV cancers (148;149). Despite the advent of transcriptomics and proteomics approaches no potential serum marker for ovarian cancer, including CA-125, has been found that is expressed at high enough levels for early detection or that may be measured by a superior assay. Based on recent studies (40;147), the soluble form of FR-α is a candidate protein that begs development at this time as a serum marker for epithelial ovarian and endometrioid cancers.

7. CONTROL OF FR EXPRESSION

As noted in previous sections, each FR isoform has a narrow and distinct tissue specificity. In addition, several studies have established that FR-α expression is regulated by extracellular folate (150-153). More recently, FRs have been found to be regulated in a ligand-dependent manner by nuclear receptors; specifically, FR-β expressed in leukemic cells is regulated by retinoic acid receptors (154;155) and FR-α is regulated by steroid receptors (147;156). FR gene regulation occurs by a variety of mechanisms at both transcriptional and post-transcriptional levels (157-160). Studying FR gene regulation will obviously lead to a better understanding folate physiology. It is also clear that regulatory studies of FR genes will enable the development of means to optimize their expression selectively in the receptor-rich tumors to improve FR-targeted diagnostics and therapeutics. The following sections will discuss the molecular mechanisms of gene regulation with emphasis on FR-α, the relevant FR isoform in gynecological tissues.

7.1. Tissue-specific regulation

The FR-α gene has 7 exons, 6 introns and two TATA-less promoters called P1 and P4 located upstream of exons 1 and 4, respectively (Figure 1) (157). Whereas the P1 promoter generates multiple alternatively spliced transcripts only differing in the 5′ untranslated region (UTR), the P4 promoter generates a single mRNA species (158). Because P1 and P4 promoter-driven mRNA transcripts have the same open reading frame and 3′ UTR, they encode identical FR-α proteins (157;158). To date, the tissue-specific expression of FR-α seems to be governed by both transcriptional and post-transcriptional mechanisms. These include differential promoter usage (157), alternative splicing (157), differential hnRNA stability (159) and differential mRNA translation (158).

The basal FR-α P4 promoter activity is primarily directed by a cluster of three non-canonical Sp1 binding sites (161). The P1 promoter lacks a functional Sp1 element and its optimal activity has been attributed to a putative NP3/4 binding element (162). Whereas the transcription of the FR-α gene in normal human tissues from lung, placenta, salivary gland, uterus, breast and stomach appear to be primarily driven by P4, FR-α transcripts from kidney, testis, and brain originate from P1 (157;158). There was no significant difference in the stability of the FR-α mRNA whether the transcription was governed by P1 or P4 (158). The FR-α mRNA transcripts in malignant cells appear to be primarily transcribed from the P4 promoter (159).

At a post-transcriptional level, a discrete 60-base mRNA element within the open reading frame of FR-α confers differential instability to the FR-α hnRNA in the nucleus in a tissue-specific manner (159). This results in the selective degradation in the nuclear compartment of the FR-α transcripts in FR-α-negative cells but not in FR-α-positive cells. Insertion of the 60-base mRNA element upstream of heterologous gene promoters conferred the expression pattern of the FR-α gene (159). It has also been noted that the P4 promoter-driven transcript is translated

several fold more efficiently than P1-driven transcripts (158).

In contrast to FR-α, the FR-β gene contains only a single promoter (Figure 1) (163;164). The basal promoter activity of FR-β is governed by a transcriptional complex, which contains a non-canonical Sp1 element, ets binding sites, and two AP-1 like repressor elements (154;164). The different tissue-specificities of FR-α vs. FR-β may be attributed in large part to the differences in their promoter organization.

7.2. Regulation by folate

The expression of FR-α in a number of cell lines has been shown to be regulated by changes in the intracellular folate pool caused by changes in the extracellular folate concentration (151;152). *In vitro*, an inverse relationship exists between the level of folate in the tissue culture medium and FR-α expression by the cells (150-152;165-168).

Folate dependent regulation of FR-α expression in cervical carcinoma cells was attributed to the interaction of an mRNA binding transfactor called heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), with an 18-base *cis*-element in the 5′ UTR of the FR-α transcript (169;170). When these cells were grown in low-folate media, FR-α expression was up-regulated without any significant change in the expression of hnRNP E1 (168). The increase in FR-α expression was attributed to the accumulation of homocysteine in these cells which led to an increase in the interaction of hnRNP E1 with the 18 base *cis*-element of FR-α (168). Interestingly, *in vivo*, a number of tissues of gestational day 17 fetuses from pregnant dams fed a low folate diet (400 nmol folate/kg chow) up-regulated both hnRNP E1 and FR-α expression compared to dams fed a higher folate diet (1200 nmol folate/kg chow) (171).

Folate-dependent regulation of FR has also been found to occur by alteration of FR mRNA levels (150). Results of RNA gel shift assays are suggestive that binding of cytosolic proteins to *cis*-elements in the 5′ and 3′ regions of FR-α mRNA decreases the half-life of the FR-α transcripts in KB cells grown in folate-replete medium vs. folate-depleted medium (172). In cultured Chinese hamster lung fibroblasts, severe folate restriction has also been shown to result in FR gene amplification (153). In some malignant cells whose principal folate and antifolate drug uptake pathway is not FR, transport-defective resistance to certain antifolate drugs such as methotrexate (21) may be accompanied by upregulation of FR, which is a poor transporter of methotrexate, to ensure adequate supply of folate to the cell.

As seen above, there is clear evidence in the literature for a profound influence of folate and antifolates on FR expression both *in vitro* and *in vivo*. In addition to the potential physiologic significance of this regulation under conditions of limiting dietary folate, regulation of FR expression in response to folates or a combination of folates and antifolates is a potentially important consideration in developing novel FR-mediated therapies.

7.3. Regulation by steroid receptors

FR genes are regulated by nuclear receptors, even though their promoters do not contain the classical hormone response elements. Hormonal regulation of FR may have evolved due to the need for overproduction of FRs in certain tissues for the purpose of folate uptake during specific developmental stages. A detailed understanding of the molecular mechanisms involved in the regulation of FR-α expression is potentially useful for the development of selective steroid receptor modulators or combination drug therapies that could be used in the treatment of FR-α-positive cancers. Quantitative analysis by flow cytometry (85) and quantitative *in situ* hybridization (43) have established that despite consistent patterns of FR-α expression in normal and malignant female reproductive tissues, the actual level of expression of the receptor varies not only among tumors from different patients but also within the same tumor; this variability occurs in a range of up to two orders of magnitude. Therefore, up-regulating the expression of FR-α may be expected to result in more efficient tumor targeting. The following section will discuss the regulation of the FR-α gene by steroid receptor ligands.

7.3.1. Estrogen receptor

The estrogen receptor (ER) is expressed in about two thirds of epithelial ovarian (173), endometrial (174), and breast cancers (175). In breast cancer, a negative correlation has been observed between ER and FR-α expression (176). It has more recently been demonstrated that ER represses both the FR-α promoter and the endogenous FR-α gene expression (156). Whereas treatment with estrogen increased this repression further, treatment with the antiestrogens tamoxifen or ICI 182, 780 caused derepression (156). Functional mapping experiments narrowed the ER-responsive element in the FR-α promoter to the basal P4 promoter. Further mutational analysis identified the Sp1 element in the P4 promoter as the site of ER action. Both tamoxifen and ICI 182,780 prevented this interaction, offering a mechanistic explanation for the antagonistic effects of the ligands on ER repression of the FR-α gene (156). The unique manner in which ER associates with the FR-α promoter may enable it to recruit transcriptional corepressors rather than coactivators. Indeed, coactivators of the major SRC family (SRC-1, SRC-1E, TIF-2, and RAC3) as well as Creb binding protein did not appreciably alter estrogen/ER-mediated repression of the FR-α promoter, but the corepressor SMRT promoted ER repression. In summary, recruitment of one or more corepressors by ER to the FR-α gene results in repression of the promoter activity whereas derepression by antiestrogens is achieved by passive dissociation of ER (156). These findings predict that treatment of many ER+/FR-α+ tumors with antiestrogens should result in the up-regulation of FR by these cells. More importantly, since antiestrogens did not induce FR-α expression in a variety of ER+/ FRα- cell lines, they will not alter the tissue specificity of FR-α expression (156). Taken together, these results establish an experimental groundwork for optimal exploitation of FR-α modulation with ER ligands for imaging and treatment in major types of gynecological cancers.

7.3.2. Glucocorticoid receptor

The glucocorticoid receptor (GR) is almost ubiquitously expressed at levels of 2000-30,000 binding sites per cell (177). The GR agonist, dexamethasone (Dex), activates the FR-α promoter by an indirect mechanism (147). The action of Dex/GR was found to be mediated through the Sp1 element in the P4 promoter as well as the initiator, including its flanking region (147). This effect was delayed and it was shown that the immediate target of Dex action in FR-α upregulation was a gene(s) other than the FR-α gene (147). Presumably, such a gene product (s) in turn acts on the FR-α promoter to mediate an indirect upregulation by Dex. Consistent with a transcriptional regulation by Dex/GR, the nuclear receptor co-activators, SRC-1, SRC-2 and pCAF promoted the Dex effect. In addition, histone deacetylase inhibitors greatly potentiated Dex induction of the FR-α promoter and the endogenous FR-α in HeLa cells, in a dose dependent manner (147). The induction of FR-α by Dex occurred selectively in FR-α-positive cells and did not result in a global increase in FR-α gene expression i.e., in *de novo* expression of the receptor in FR-α-negative cells. Moreover, since the subcellular localization and function of FR-α was unaltered by Dex treatment, the receptor may be expected to retain its ability to bind FR-α-targeted agents. Thus, similar to antiestrogens, GR agonists could serve as innocuous agents that may be used to optimize the expression of tumor FR-α to obtain more effective diagnostic and therapeutic targeting of the tumor.

8. CONCLUSIONS AND PERSPECTIVES

In the absence of supplementation with supraphysiologic amounts of folate, the membrane bound and soluble forms of the folate receptor play a critical role in embryonal development and in ensuring the bioavailability of folate through milk. The role of the folate receptor expressed in reproductive tissues other than placenta and breast remains enigmatic, considering the fact that in those tissues, the receptor is not exposed to folate available through extracellular fluids. Nevertheless, the same considerations render the receptor a virtually ideal tumor target in malignancies originating from those tissues. The expression of FR genes is regulated both transcriptionally and post-transcriptionally, in a manner dependent not only on cell type but also on folate and nuclear receptor ligands including steroid receptor agonists and antagonists. Many of these mechanisms are novel or non-classical and offer means for improving the utility of FR in cancer diagnosis and therapy. There is much to be understood about the physiologic significance of FR expression and regulation in normal reproductive tissues even as new findings are applied in translational research for the detection and treatment of related cancers.

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