Novel functions of folate receptor alpha in CNS development and diseases

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Introduction

Folate receptor gene family consists of four members in humans, namely Folr1, Folr2 and Folr3, respectively localized to chromosome 11q 13.3-q14.1 encoding the proteins FRα, FRβ and FRγ [1]. The fourth Folr4 gene localized to 11q 14 encodes FRδ [2]. FRα, FRβ and FRγ are extracellular receptors attached by a glycosylphosphatidylinositol (GPI) anchor. In contrast, FRγ exist as a soluble receptor, the Folr4 gene localized to 11q 14 encodes FRδ [1]. The fourth Folr4 gene localized to 11q 14 encodes FRδ [2]. FRα, FRβ and FRγ are extracellular receptors attached by a glycosylphosphatidylinositol (GPI) anchor. In contrast, FRγ exist as a soluble receptor, the Folr4 gene localized to 11q 14 encodes FRδ [1]. The fourth Folr4 gene localized to 11q 14 encodes FRδ [2]. FRα, FRβ and FRγ are extracellular receptors attached by a glycosylphosphatidylinositol (GPI) anchor. In contrast, FRγ exist as a soluble receptor.

Folate receptors (FRα, FRβ and FRγ) are cysteine-rich cell-surface glycoproteins that bind folate with high affinity to mediate cellular uptake of folate. FRα expression is restricted to few epithelial tissues, whereas the remaining isoforms have primarily been found to be expressed in myeloid tissues [1]. Although expressed at very low levels in most tissues, folate receptors, especially FRα, are expressed at high levels in numerous cancers to meet the high folate demand of rapidly dividing cells under low folate conditions [1,3-5]. This dependency has been therapeutically and diagnostically exploited by administration of anti-FRα antibodies, high-affinity anti-folates [6,7], folate-based imaging agents and folate-conjugated drugs and toxins [8-10]. Although folate is required for rapidly dividing cancer cells, the role of FRα behaving like a transcription factor and activating oncogenic genes point out to the fact that FRα have other undiscovered functions [11] which aid tumorogenesis.

The role of FRα in neural tube defects has been very well documented [12]. Inactivation of the murine folate binding protein-1 (Folbp1) in nullizygous embryos (Folbp1/-/-) show significant malformations of the neural tube, craniofacial abnormalities, and conotruncus, and invariably die in utero by gestational day (E10) [13]. On the contrary Folbp2/-/- embryos developed normally [14] suggesting that it is not just the folate delivery into the cytoplasm by folate binding proteins that is critical, but additional properties of folate receptor alpha must also be looked into.

Autoantibodies against folate receptor alpha were identified as the cause of the infantile-onset cerebral folate deficiency (CFD) syndrome [15,16] and autism spectrum disorder [17]. Additionally, mutations in FRα have been reported to cause CFD [18] as well as cerebral folate transport defect –a neurological disorder associated with disturbed myelin metabolism [19].

In general, folate receptors are believed to mediate the uptake of folates and anti-folates by receptor mediated endocytosis [20,21], primarily because of the initial finding by Kamen et al., [22] which suggested that FRs traffic between an acid resistant (interior) and acid labile (exterior) state [22]. Endocytosis of FRα is assisted by low-density lipoprotein (LDL) receptor-related protein 2 (LRP2), a multifunctional cell-surface receptor expressed in the embryonic neuroepithelium [23] as well as by protein kinase Ca [24].

A more useful role of folate receptor alpha was recognized in its having a high affinity for folic acid and the circulating form of folate, (6S) N5-methyltetrahydrofolate (KD<10⁻⁹ M). The glycosylphosphatidylinositol (GPI) membrane anchored FRs can mediate internalization of receptor bound (anti)folate compounds and folate conjugates [25-27]. In most normal tissues, FRα is absent, non-functional, or expressed on luminal surfaces
that are inaccessible through the bloodstream [28]. Whereas in pathological tissues including malignant cells and activated macrophages FRα is overexpressed [25-36]. This makes FRα as an excellent route for the selective delivery of a broad range of experimental pharmacological agents to these tissues.

In this review we will comprehensively cover different functions of FRα in central nervous system development, and diseases such as neural tube defects, cerebral folate deficiency, autism, and cancer treatment strategies.

### Review

**Folate receptor alpha in folate/anti-folate transport**

Folate or anti folate transport inside the cell via FRα mediated endosomal transport is very well documented [21]. Elnakat et al., [24] showed that Protein Kinase Ca (PKCa) substrate, annexin II, is required for FR internalization. When activated PKCa is recruited to FR-rich membrane caveolar microdomains, it inhibits FRα internalization. Bandara et al., [37] demonstrated that FRα occupancy has no impact on the rate of FRα internalization in association with RACK1. Additionally they showed that multivalent FA conjugates that bind and crosslink FRα at the cell surface internalize at the same rate as monovalent folate conjugates. These FA conjugates have no impact on FRα clustering. These data suggested that FRα endocytosis occur at a constitutive rate, regardless of FRα occupancy or cross-linking due to multivalent ligand binding.

A recent study by Kur et al., [23] showed that low-density lipoprotein (LDL) receptor-related protein 2 (LRP2), mediates folate uptake in the developing neuroepithelium. LRP2-deficient neuroepithelial cells are unable to mediate the uptake of folate bound to soluble folate receptor 1 (sFOLR1). Moreover, the folic-acid dependent gene Axl3 is significantly downregulated in Lrp2 mutants, clearly suggesting that LRP2 is essential for cellular folate uptake in the developing neural tube. Figure 1 shows the summary of the FRα receptor internalization via endocytic pathway: GPI-anchored FRα bind to folic acid and the uptake of the complex is mediated through endocytic mechanisms [38]. High-efficiency internalization of GPI–FRα requires its interaction with a co-receptor LRP2, spanning the plasma membrane. Once FRα is internalized in the endosome, the endosome becomes increasingly acidic [39] and fuses with a lysosome [40]. In the lysosomal FA is released [21] and lysosomal GPI specific phospholipase D [41] cleaves off the GPI anchor on FRα, which is then set free.

There is also a different pathway for folate delivery especially in brain parenchyma. Grapp et al., [42] very elegantly demonstrated that choroid plexus via transcytosis and exosome shuttling deliver folate in the brain parenchyma. According to them, 5-methyl tetra hydro folate (5-MTHF)-FRα complex is internalized by receptor-mediated endocytosis, translocated into GPI-anchored protein-enriched early endosomal compartments (GEECs) and further transferred to multi-vesicular bodies (MVBs). MVBs are late endosomal compartments localized in the endocytic route. The intra-luminal vesicles (ILVs) of MVBs containing FRα are generated by inward budding of the limiting membrane. These ILVs are released as exosomes into the cerebrospinal fluid (CSF) after fusion of the MVB with the apical cell membrane. FRα-containing exosomes circulate in the CSF, cross the ependymal cell layer and are distributed in the brain parenchyma. FRα-positive exosomes might initially be taken up by astrocytes and from these further delivered to neurons.

Thus FRα is not only important for high affinity folate uptake via receptor mediated endocytosis, but also to activate genes when it behaves as a transcription factor. Its recent role is to transport FA in brain parenchyma via transcytosis and exosome shuttling (Figure 2).

**Folate receptor alpha as a transcription factor**

Free FRα translocates into the nucleus where it binds to cis-regulatory elements of target genes and directly activates transcription [11] of Hes1 and Fgfr4. This novel role of FRα as a transcription factor is very significant because it provides insight into developmental mechanisms associated with FA responsiveness. It also provides an exciting new avenue to explore for treatment of diseases associated with FA deficiency, FRα misregulation and cancers which express FRα as a biomarker.

**Folate receptor alpha in neural crest cell migration and neural tube defects**

FRα plays a key role in the development of embryo [12,13]. Nullizygous FRα embryos (Folbp1−/−) have significant malformations of the neural tube, craniofacies, and conotruncus, and die in utero by gestational day (E10). The affected genes in these embryos belong to the category of transcription factors, G-proteins, growth factors, methyltransferases, and those related to cell proliferation. In nullizygote embryos which showed open cranial neural tube defects, there was downregulation of Pax-3 and En-2 in the impaired midbrain, along with an observed upregulation of the ventralizing marker Shh in the expanded floor plate. Additionally, the nullizygotes also exhibit craniofacial abnormalities, such as cleft lip and palate, suggesting that FRα affects neural crest cell migration. This hypothesis was later confirmed by a brief and critical interruption of FRα expression by siRNA during embryo development which caused a failure of neural crest cell migration into pharyngeal arches resulting in abnormal development of pharyngeal arch artery and heart [43].

The above observations strongly suggest that disruption FRα expression causes neural crest cell migration and associated craniofacial anomalies and abnormal heart development, in addition to cranial neural tube defect. Accumulating evidences suggest that disruption of FRα function also can lead to neural tube defects. Fumonisin, a common mycotoxin contaminant of maize causes neural tube and craniofacial defects in mouse embryos in culture [44]. Fumonisins inhibit ceramide synthase, causing accumulation of bioactive intermediates of sphingolipid metabolism (sphinganine and...
other sphingoid bases and derivatives) as well as depletion of complex sphingolipids. This interferes with the function of human folate receptor alpha.

A small nucleotide polymorphism (SNP) screen across the three folate receptor genes (FOLR1, FOLR2, FOLR3) and the reduced folate carrier gene (SLC19A1) in a large population sample consisting of approximately 60% Hispanics of Mexican descent showed a statistically significant association to meningomyelocele (MM) in the patient population that was tested [45].

**Folate receptor alpha in cerebral folate deficiency syndrome and autism spectrum disorders**

Cerebral folate deficiency (CFD) can be defined as any neurological syndrome associated with low cerebrospinal fluid (CSF) 5-methyltetrahydrofolate (SMTHF), the active folate metabolite, in the presence of normal folate metabolism outside the nervous system. CFD is associated with low levels of 5-methyltetrahydrofolate in the cerebrospinal fluid (CSF) with normal folate levels in the plasma and red blood cells. CFD could result from either disturbed folate transport or from increased folate turnover within the central nervous system (CNS) [46]. The onset of symptoms caused by the deficiency of folates in the brain is at around 4 to 6 months.
of age, followed by delay in development, with deceleration of head growth, hypotonia, and ataxia. About one-third of children show dyskinesias (choreo-atetothetosis, hemiballismus), spasticity, speech difficulties, and epilepsy. The CFD can occur because of mutations in FRα or because of FRα autoantibody; both contribute to low levels of folate in the brain.

**FRα mutations**

Mutations resulting in the loss of intact FRα lead to congenital CFD causing a severe and complex neurologic disease [18, 47]. Steinfeld et al., [19] identified an inherited brain-specific folate transport defect that is caused by mutations in the folate receptor 1 (FOLR1) gene coding for folate receptor alpha (FRα). Three patients carrying FOLR1 mutations developed progressive movement disturbance, psychomotor decline, and epilepsy and showed severely reduced folate concentrations in the cerebrospinal fluid (CSF). Brain magnetic resonance imaging (MRI) in these patients demonstrated profound hypomyelination suggestive of disturbed myelin metabolism owing to mutations in FOLR1 (FRα protein). Grapp et al., [18] 2012 showed that the FOLR1 mutants’ p.C65W, p.C105R, p.C169Y and p.N222S were mistargeted to intracellular compartments and partially co-localized with the endoplasmic reticulum marker protein disulphide isomerase. This apparent mistargeting of FRα to other intracellular compartments but not to plasma membrane makes it not available to extracellular folate for active and high affinity uptake within the cell.

**FRα autoantibodies**

In human brain, preferentially expression of FRα in the choroid plexus, indicate that the major supply route for brain 5-MTHF occurs via the blood–CSF barrier [19,46,48]. FRα autoantibody can lead to autism spectrum disorders [17]. The low level of 5-methyltetrahydrofolate in the CSF can result from decreased transport across the blood-brain barrier, because of the blocking of folate transport into the CSF by the binding of FRα autoantibodies to FRα in the choroid plexus [49,50]. Perhaps one of the best reviews written which describes CFD syndromes attributed to FRα autoimmunity according to age is by Ramaekers et al., [52,53]. From prenatal conditions to adulthood and beyond, FRα and folate levels is critical to proper central nervous system functioning.

**Folate receptor alpha autoantibody in diagnostic utility**

Prevalence of FRα autoantibodies (AuAbs) are seen in various conditions such as NTD, mothers with a history of neural tube defect pregnancy, CFD, children with cerebral folate deficiency syndrome [49-53]; LFA, children with low-functioning autism [52]; ASD, children with autism spectrum disorder [16,17,52]; RS, children with Rett syndrome [50,54]. The discovery of FRα AuAbs that block the uptake of folate offers one of the many mechanisms explaining the response to folate in these disorders. The association of FRα AuAbs with pregnancy-related complications, CFD syndrome, and autism spectrum disorders and response to folate therapy suggests the involvement of these AuAbs in the disruption of brain development and function via folate pathways. All subjects with FRα AuAbs autoimmune condition had IgG antibodies, with IgG1 as the predominant isotype. Mothers with NTD pregnancy (40% IgG) and ASD subjects (14% IgG) also contained IgG2; CFD (21% IgG) and ASD (7% IgG) subjects also had IgG3 isotype. Although the occurrence of IgG4 is rare, 79% of the CFD subjects and 14% of the ASD subjects had this isotype. Thus it appears that the predominant antibodies in women with NTD pregnancy belong to the IgG1 and IgG2 isotype and in CFD children, the IgG1 and IgG4 isotype.

**Folate receptor alpha in cancer and its use in targeting cancer by immunotherapeutics and nanotherapeutics**

Folate is a basic component of cell metabolism and DNA synthesis and repair. Rapidly dividing cancer cells have an increased requirement for folate to maintain DNA synthesis. This prompted use of anti-folates in cancer chemotherapy. FRα levels are high in specific malignant tumors of epithelial origin compared to normal cells [3,20]. A recent study by Boshnjaku et al., [11] 2012 showed that FRα transcriptionally regulates several Pax3 downstream target genes such as Hes1 (a stem cell maintenance gene) and Fgfr4, suggesting FRα might confer a growth advantage to the tumor by generating transcriptionally regulatory signals. Cell culture studies show that expression of FOLR1 which codes for FRα is regulated by extracellular folate depletion, increased homocysteine accumulation [55], and steroid hormone concentrations [56]. It is quite possible that FRα in tumors decreases in vivo in individuals who are folate sufficient. It is also equally plausible that the tumor’s machinery sustains FRα levels to meet the increased folate demands of the tumor [1].

Owing to its high affinity binding property (Kd ~100 pM) and high substrate specificity FRα has been exploited for its therapeutic and diagnostic potential. In a series of experiments, Leamon and Low [57] showed that covalent conjugation of folic acid with horseradish peroxidase, IgG, serum albumin and ribonuclease, resulted in the intracellular delivery of these molecules via FRα. Low group pioneered the use of vitamin folic acid to target PET agents, γ-emitters, MRI contrast agents and fluorescent dyes to FR+ cancers for the purpose of diagnosing and imaging malignant masses with improved specificity and sensitivity [58]. In patients with ovarian cancer, intraoperative tumor-specific fluorescence imaging with a FRα–targeted fluorescent agent (generated by Low lab) showcased the potential applications in patients with ovarian cancer for improved intraoperative staging and more radical cyto-reductive surgery [59].

In subsequent elegant experiments, Low and colleagues [60] constructed a reduced and alkylated form of folic acid, N⁵, N¹⁰-dimethyl tetrahydrofolate (DMTHF) that exhibits selectivity for FRα. DMTHF–⁹⁹mTc was injected into mice bearing FRα–expressing tumor xenografts and imaged by γ-scintigraphy.
The selectivity for FRα over FRβ in vivo was examined by γ-scintigraphic images of animal models of various inflammatory diseases and they concluded that targeting ligand DMTHF enables selective noninvasive imaging and therapy of tumor tissues in the presence of inflammation.

Folate receptor α has been used for active targeting of cancer nanotherapeutics [61]. Recently folate-bovine serum albumin (BSA)-cis-aconitic anhydride-doxorubicin pro-drug was used for tumor target drug delivery by Du et al., [62]. They observed that the folate-bovine serum albumin (BSA)-cis-aconitic anhydride-doxorubicin prodruig, selectively targeted tumor cells and tissues with associated reduction in non-specific toxicity to the normal cells. The therapeutic efficacy of the pro-drug for FRα positive tumors was higher than that of non-conjugated doxorubicin.

Folate receptor alpha (FRα) is a unique tumor-associated antigen (TAA) with many characteristics that make it an attractive target for immunotherapy in cancer [63]. FRα is largely shielded from the immune system in normal tissue but is exposed in cancer cells. It is functionally active in cancer pathogenesis; and it is immunogenic. A variety of different immunotherapeutic methods targeting FRα are being explored to treat cancer. Passive immunotherapy includes (i) monoclonal antibodies; (ii) antibodies to deliver treatments and (iii) modified T cell therapy. Active immunotherapy has focused on using FRα to increase the immunogenicity of cancer or to generate active FRα-directed immunity through a range of vaccination techniques. For TAA to be an effective target, (i) the TAA antigen must have relative specificity, over-expression or hyper-activity in a target cancer type: (ii) TAA antigen displaying cancer cells must be visible to the immune system to prevent autoimmune toxicity; (iii) TAA antigen must also contain epitopes that are conserved and immunogenic [63].

Conclusions and future perspectives

It is quite evident that FRα has different fates in and out of the cell. A summary of the different fates of FRα is shown in Figure 3. FRα binds to FA and undergoes endocytosis. FA is released and the FRα is set free to act like a transcription factor, or is recycled. Another route that has been recently described is the translocation of FRα+FA into GPI-anchored protein-enriched early endosomal compartments (GEECs) which is further transferred to multi-vesicular bodies (MVBs). MVBs are late endosomal compartments localized in the endocytic route. The intra-luminal vesicles (ILVs) of MVBs containing FRα are generated by inward budding of the limiting membrane. These ILVs are released as exosomes into the cerebrospinal fluid (CSF) after fusion of the MVB with the apical cell membrane. FRα-containing exosomes circulate in the CSF, cross the ependymal cell layer and are distributed in the brain parenchyma. FRα-positive exosomes might initially be taken up by astrocytes and from these further delivered to neurons.

Mutations in FRα protein or autoantibodies against FRα, impairs proper high affinity folate transport inside the choroid plexus cell, causing CFD. Folinic acid or 5-MTHF supplementation is suggested for treatment of CFD. Cellular metabolism of 5-MTHF depends on the route of folate entry into the cell. 5-MTHF taken up via a non-FRα –mediated process is rapidly metabolized to folylpolyglutamates, whereas 5-MTHF that accumulates via FRα remains non-metabolized, supporting the hypothesis that FRα may be part of a pathway for transcellular movement of the vitamin. Additional function of FRα as a potential transcriptional regulator of genes underscores the importance of FRα as not just a high affinity folate carrier but as a regulator of genes involved in autism spectrum disorder and cerebral folate deficiency.

FRα, with high tumor specificity and overexpression in a broad range of cancers, has attracted considerable attention as a target for these various immunotherapeutic and FA-conjugated nano-therapeutic modalities. Novel methods and efforts to stimulate active immunity against FRα-expressing cancer include the use of folate-localized molecules to enhance cancer immunogenicity, genetically modified autologous T cells, and techniques to raise FRα-specific immunity via viral vector, as well as multiple vaccine strategies to include modified whole tumor cells, DNA, dendritic cell and peptide vaccines [63]. Active immunotherapy, with the potential to not only attack tumors but also to generate long-lasting protection has the potential to add a new important therapeutic approach to the already multimodal treatment of cancer.

Next major advances will see the active use of FRα dependent exosome-mediated folate or folate-drug conjugates delivery into the brain parenchyma as a mode of cerebral drug targeting, which has been prevented because of the impenetrable blood brain barrier. Selective targeting of FRα-expressing exosomes to the brain parenchyma not only substantiate the biological significance of this transport shuttle but also opens up new avenues for therapeutic approaches. By designing their protein expression, exosomes may serve as organ-specific delivery vehicle for therapeutic agents. Targeted manipulation of the
choroid plexus or direct application of FRα-positive exosome-like vesicles into the CSF may be a novel strategy to deliver biological active substances into the brain [42].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

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|------------------------|-----|-----|----|
| Research concept and design | -- | -- | -- |
| Collection and/or assembly of data | -- | -- | -- |
| Data analysis and interpretation | -- | -- | -- |
| Writing the article | ✓ | -- | -- |
| Critical revision of the article | -- | ✓ | ✓ |
| Final approval of article | ✓ | ✓ | ✓ |
| Statistical analysis | -- | -- | -- |

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