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# *The role of the Soluble Folate Receptor Alpha in cancer development*

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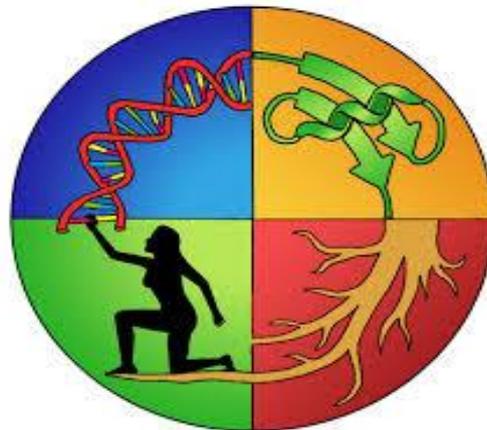
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# List of abbreviations

BSA: Bovine Serum Albumin

cFBS: Charcoal stripped fetal bovine serum

ddH$_2$O: Double distilled water

DTT: Dithiothreitol

FA: Folic Acid

FBS: Fetal Bovine Serum

FRα: Folate Receptor alpha

FRβ: Folate Receptor beta

FR: Folate Receptor delta

FRγ: Folate Receptor gamma

GPI: glycosylphosphinositol

IFNβ: Interferon beta

(s)IL-6R: (soluble) Interleukin 6 receptor

NC: Negative Control

NTD: Neural Tube Defects

ON: Overnight

PBS: Phosphate-buffered saline

PBS-T: Phosphate-buffered saline w. Tween 20

PCFT: Proton-Coupled Folate Transporter

PI-PLC:Phosphatidylinositol-Specific Phospholipase C

P/S: Penicillin/ Streptomycin

RFC: Reduced Folate Carrier

RT: Room temperature

sFRα: Soluble Folate-Receptor alpha

STAT: Signal transducer and activator of transcription

TBS: Tris-buffered saline

TBS-T: Tris-buffered saline w. Tween 20

# I. Abstract

Folates are essential vitamins for cell growth and replication which shows an important role of folates in development of embryos, deficits may result in congenital malfunctions. In order to avoid diseases such as neural tube defects (NTD) due to folate deficiency during pregnancy, different countries approved the folic acid supplementation and fortification of foods. As a result NTD development decreased, but adverse effects have been reported for several years. Some studies have shown that folic acid elevates the growth of pre-existing tumours when supplied. To understand folic acid uptake from cells and cancer development, we have been focused on one of the transport mechanisms, folate receptor alpha (FRα), is highly restricted to certain tissues and is upregulated in several cancers.

Previous experiments performed at *Department of Molecular Biology and Genetics (University of Aarhus)* have shown that folic acid activates STAT3 oncogene via the FRα. Furthermore FRα can be found bound to membrane by GPI-anchored or as a soluble protein (sFRα). This soluble form has been associated with cancer because of its high concentration in serum of patients with cancer and poor folates levels, whereas healthy individuals exhibit low sFRα concentrations and high folates levels. Results demonstrated that the soluble FRα is able to activate STAT3 gene via transsignaling independently of its membrane-bound counterpart. Earlier analysis were performed to investigate the effect of folic acid on tumour development and it was found that folic acid cause elevated growth of pre-existing tumours when folic acid doses are similar to those from fortified foods. The high FRα expression in breast tumours suggested that it contributes to tumour development. Consequently, larger tumours expressed higher levels of STAT3 protein.

The aim of this project was to study the sFRα expression and how is it involved in cancer development through the transsignaling process prior proposed. Before starting to investigate the soluble protein is necessary to study its predecessor FRα. We show here that FRα is expressed at higher levels in cancer cells and we expect to find this protein in its soluble form (sFRα). In addition, the PI-PLC, an activator of phospholipase C, is used in to demonstrate its ability to release FRα by cleavage of GPI-anchored proteins. Levels of the soluble FR were lower and precipitations were performed to detect proteins at higher concentrations. Additionally, to analyse protein concentrations in tumours, a group of mice fed a high or low folic acid diet were analysed. After find out which of them were PyMT mice (MT protein develops cancer) tumours from one of these mice together with serum from previously analysed mice were extracted. From tumours cell cultures were generated to study expression levels of FRα, and from serum sFRα levels were investigated. From tumours cultures no FRα was detected but the soluble protein was found in serum although it was not possible to measure the protein concentration.

The higher expression of FRα in cancer cells shows its involvement in disease development. Moreover, the soluble protein presence confirms its expression and even though concentration was not determined the preliminary data suggest that FRα levels increase when tumours are bigger and so the expression of FRα proteins is larger in these tumours than in small tumours from mice fed a low folic acid diet. Further studies will focused on determine sFRα levels and its effect on tumour development.

## II.    Introduction

Folates are B vitamins necessary for cell growth and metabolism. Many studies have shown the important role of these vitamins to prevent diseases such as folate deficiency syndrome, schizophrenia, autism (Mitchell ES, et al. 2014) or anemia in pregnant women (Gunaratna NS et al. 2015). These vitamins are used as supplements in food to fight against diseases such as mentioned, but it has been found a high concentration of folates activates the STAT3 oncogene and can develops in cancer (Hansen MF, et al. 2015). Because of that, folic acid and folate receptor alpha (the major folate transport) have become in the key of several studies involved in cancer.

## Folates

Folates are involved in essential one-carbon transfer reactions that are important in DNA synthesis and replication, cell division, and growth and survival, particularly for rapidly dividing cells.

Folates are water soluble $B_9$ vitamins found in food such as leafy vegetables, legumes and liver in their processed active forms such as dihydrofolates (DHF) and tetrahydrofolates (THF) whereas folic acid is a synthetic, oxidize folate not found in nature, used to fortified food and as supplements.
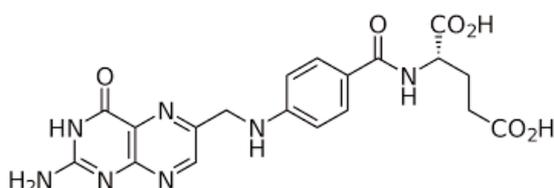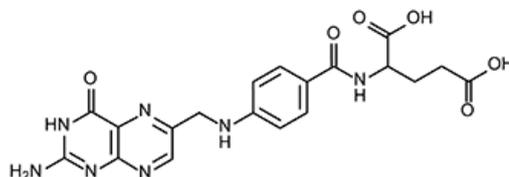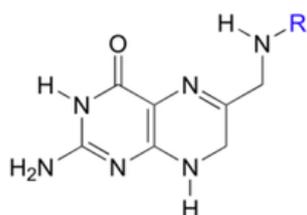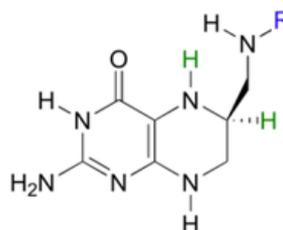


**Figure 2.1.; Chemical structures of folates**. Folate and folic acid differ in the protonation state of the glutamate moiety. Reduction of aromatic bonds produces the conversion of folate/folic acid to its reduced forms dihydrofolate (DHF) and tetrahydrofolate (THF).

These vitamins can arrive to cells by two different ways; the first one the anion channel called the Reduced Folate Carrier (RFC) with low affinity for folate (this is the mechanism used by most of

cells to get folate). The second one has a high affinity for folate and only cancer cells and those involved in embryonic development follow this via.

Recent studies have shown differences between both types of cells. Those cells which need low concentrations of folates take them up by the RFC, while cells that need high concentrations of folates take them up by the present folate receptors on their membranes.

## Folate deficiency

In the 1990s, the increase of digested amounts of folate revealed the reduction of occurrence of neural tube defects (NTD) by as much as 70%. Since this finding, the use of periconceptional folic acid supplementation and folic acid fortification of foods to prevent folate-deficiency emerged. (Andrew E et al. 1992). The neural tube is the embryo's precursor to the central nervous system and will later develop into the child's brainstem and spine Neural tube defects are birth defects of the brain, spine, or spinal cord. *Spina bifida* and *anencephaly* are the two most common neural tube defects. In *spina bifida* there is usually nerve damage that cause some paralysis of the legs, it is developed when the fetal spinal column does not close completely. When new-borns are affected by *anencephaly*, most of the brain and skull is not develop and they are usually either stillborn or die shortly after birth. The lower lesions observed with *spina bifida* cause a range of morbidities, including urinary and fecal incontinence and paralysis of the lower limbs.

In 1998, in the United States and Canada folic acid fortification became mandatory and around 20-50% reduction of NTD rates was observed (Honein et al. 2001). Furthermore, a reduction in the progression of atherosclerosis was reported. Since then, 82 countries worldwide have implemented mandatory fortification of food, but just two southeastern European countries (Kosovo and the Republic of Moldova) have implemented the mandatory fortification because of the risk of adverse effect.

It is well known that periconceptional folate supplementation reduces the risk of NTDs in developing embryos, but the mechanism by which they are decreased are poorly understood and it seems to be that folate deficiency is not the exclusively cause that develops in NTDs.

The folate deficiency could be associated to mutation in the FOLR gene that encodes for the FRα. The study performed by *Kelemen et al. (2014)* revealed that mutations in this gene are associated with reductions of folate concentrations in the cerebral folate deficiency (CFD), and FRα is the responsible to mediate folate transport across the blood-brain barrier.

## Folates and cancer

In the United States (1996) and Canada (1997) began the fortification of enriched uncooked cereal grains with folic acid to reduce the number of births complicated by neural tube defects (a deficiency of folic acid through gestation can affect the new born). Few years later the trend in colorectal cancer (CRC) started to increase in both countries, becoming in the two countries with higher incidence of CRC cases (Mason et al. 2007). Although, other studies revealed no correlation between folic acid treatment and cancer development, except for melanomas (Qin et

al. 2013). The study by *Song et al. 2000* revealed the key to explain these opposite effects. Mice fed with folic acid supplementation **before** establishment of malignant lesions prevent tumour formation, whereas folic acid supplementation **after** establishment of malignant lesions acted as a promoter of tumour expression.

An explanation of preventive tumour expression could be that folic acid increases the extent of DNA repair in healthy tissues, so this reparation in DNA prevent cancer. In contrast, when the malignant lesions are established the increase amount of folic acid interferes in DNA synthesis, DNA reparation is not allowed, wrong synthesis goes on and replication of this DNA develops on tumour progression.

## Folate transporters

Mammals cannot synthesize folates, are dependent from dietary sources and those proteins are absolutely necessary to synthesize DNA, RNA or repair them. Folates are polyglutamate forms of 5-methyl THF, hydrophilic molecules that circulates in serum as anions, because of that these proteins do not penetrate biological membranes by diffusion (Matherly LH, et al. 2003) The action of cells to uptake folates is needed and there are three different mechanisms that allow it: the Reduced Folate Carrier (RFC), the Proton-Coupled Folate Transporter (PCFT) and the Folate Receptors (FRs). (Table 1.1.)

| | RFC | PCFT | FRα | FRβ | FRγ | FRδ |
|---|---|---|---|---|---|---|
| **Membrane attachment** | Transmembrane domain | Transmembrane domain | GPI-anchor soluble | GPI-anchor | Soluble | GPI-anchor |
| **Distribution** | Ubiquitous | Restricted | Restricted (epithelial) | Restricted (non-epithelial) | Restricted (hematopoietic) | Restricted (hematopoietic) |
| **Affinity:** Reduced folate Folic acid | High: $K_M$ 1-10µM Low: $K_M$ 200-400 µM | High: $K_D$ 1-5µM High: $K_D$ 1-5 µM | High: $K_D$ 1-10nM Very high: $K_D$ <1nM | High:$K_D$1-10nM | Very high: $K_D$ 0.42nM | |
| **pH optimum** | Physiological | Acidic (pH 5.5) | Physiological | Physiological | Physiological | Physiological |
| **Mechanism for folate uptake** | Bidirectional anion antiporter | Proton symporter | Receptor mediated endocytosis | Receptor mediated endocytosis | | Receptor mediated endocytosis |

**Table 1.1.; Properties of folate transporters.** RFC: Reduced Folate Carrier, PCFT: Proton-Coupled Folate Transporter, FR: Folate receptor, $K_D$: Dissociation constant (folate binding), $K_M$: Michaelis constant (folate uptake). The table is adapted from (Hansen 2013).

### Widely expressed folate transporters: RFC and PCFT

Both mechanisms, RFC and PCFT are transmembrane proteins and high-affinity folate transporters widely expressed on cells, the difference between them remains in their active functions at different pH, whereas RFC is active at neutral pH the PCFT works at acidic pH (Matherly LH, et al. 2007).

The RFC has higher affinity for reduced forms of folate and has low affinity for its substrates, and thus the uptake of folate takes place when there is an excess of folates in serum (Hou Zhanjun et al. 2014), the same occurs if we focused on the PCFT protein.

A flow of positive charges is generated by the PCFT transporter, caused by the cotransport of protons in excess of folate negative charge. The transporter operates even when there is no transmembrane pH gradient; what is means that transport occurs also with the presence of the folate gradient and in such case when the membrane potential is increased, folate-induced currents are increased at pH 7.4. (Rongbao Zhao, et al. 2011).

It has been demonstrated the role that PCFT plays in intestinal folate absorption, mutations on this gene cause a loss-of function what affects to patients with hereditary folate malabsorption (HFM) (Qiu A, et al. 2006).

## Low expression of folate transporters: FRs
Folate receptors are glycosyl-phosphatidylinositol (GPI)-anchored proteins in cell membranes. Their expression is restricted to a limit cell lines, which include those cells involved in embryonic development and cancer. FRs folate-uptake is mediated by endocytosis and it occurs when the folate concentration in serum is low, becoming in high-affinity receptors. Because folate and folate-drug conjugates bind to FR with high affinity (Kd ~1 nM) and because FR rapidly recycles between the plasma membrane and the cell interior (Sabharanjak, et al. 2004), studies have focused on understanding folate receptors role in tumours development and design of therapeutic agents against those tumours (Yang J, et al.2007).

# The FRs family

In humans there are three genes; FOLR1, FOLR2 and FOLR3 that codify for the folate receptors FRα, FRβ and FRγ, respectively (Wibowo Ardian S., et al. 2013) and the putative FOLR4 gene encoding FRδ. The FOLR multigene family is localized to chromosome 11q13.3–q14.1. FRα, FRβ and FRδ are extracellular receptors anchored to the membranes by GPI. In contrast, FRγ is a soluble receptor and its function has not been widely studied.

The three extracellular FRs isoforms share highly conserved sequence (71-79%) in the open reading frame encoded by exons 4 through 7 in the 3′ region of the gene but differ in amino acid residues in the 5′ untranslated region (UTR) encoded by exons 1 through 4. Due to this difference tissue expression, function and biochemical properties can differ depending on the isoform (Kelemen LE 2006).

All of the FR isoforms bind folic acid (an unphysiologic form of the vitamin) with a high affinity ($K_D$<1 nM). FRs-α and –β bind reduced folate coenzyemes ($N^5$-methyltetrahydrofolate and $N^5$-formyltetrahydrofolate) with different ligand specificities, whereas FR-α binds the physiologic (6$S$)diastereoisomer of the circulating $N^5$-methyltetrahydrofolate as tightly as folic acid, In contrast FR-β displaysmore tan 50-fold lower affinity for the compound, these different specifities are

consequence of the amino acid sequence divergence involving Leu-49 in FR-β and Ala-49, Val-104 and Glu-166 in FR-α (Elnakat H, et al. 2004*).*

# Folate Receptor Alpha (FRα)

Many studies of cancer research area have been focused on understand FRα structure, how it works and its relation to cancer development because of its high expression in epithelial cancers, and highest expression in more aggressive cancers.

## FRα protein structure

The FRα protein is encoded by the FOLR gene and its sequenced is composed by 257 amino acids. The N-terminal signal peptide targets the protein to the endoplasmatic reticulum and the C-terminal signal peptide contains a Serine at position 234, which corresponds to the signal necessary for the attachment site for GPI-anchor proteins, an important site because FRα is a GPI (glycophosphatidylinositol) protein. The amino acid sequence contains three putative N-glycosilation signals and 16 Cysteine residues which form disulphide bridges (Figure 2.2). These N-glycosilations are important for correct folding of the protein, since it was found deletion of N-glycosilations decreased the level of FRα proteins expressed on cell surfaces. (Shen et al.1997). However, glycosilations are not involved in ligand binding affinity.
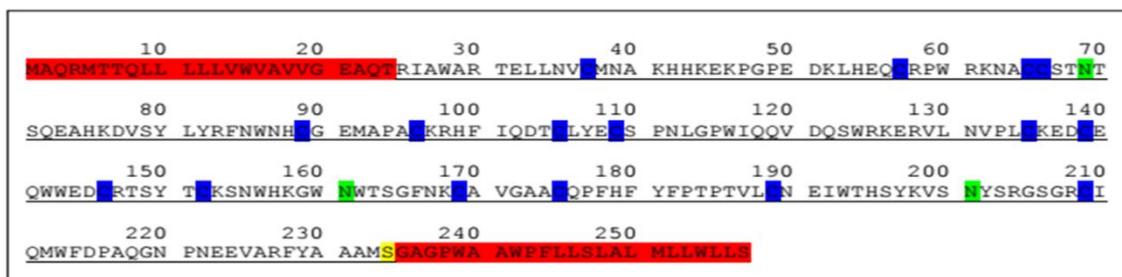


**Figure 2.2.; Amino acid sequence of the FRα protein.** The N-terminal and C-terminal signals are highlighted in red. The serine at 234 position, highlighted in yellow, is the attachment site for GPI-anchor. In green, three N-glycosilations important for the correct folding of the protein, and the cysteine residues in blue.

The FRα structure is globular and folic acid binds in a pocket deep within FRα. The N-glycosylations situated on Asparagine residues at positions 69, 161 and 201 are away from the binding site, what shows they are not important for folic acid binding. (Figure 2.3). Folic acid is stabilized in the binding pocket y both interactions: hydrogen bonds and hydrophobic interactions. The amino acids Aspartate 103, Serine 196, Histidine 157, Arginine 125 and Arginine 128 form hydrogen bonds to the positively charged pterin ring of folic acid. Some studies have revealed the importance of the Aspartate residue at 103 position, when Aspartate 103 was mutated to Alanine, the folic acid dissociation constant ($K_D$) increased approximately 12-fold whereas mutations of other residues only increased by 3 fold.
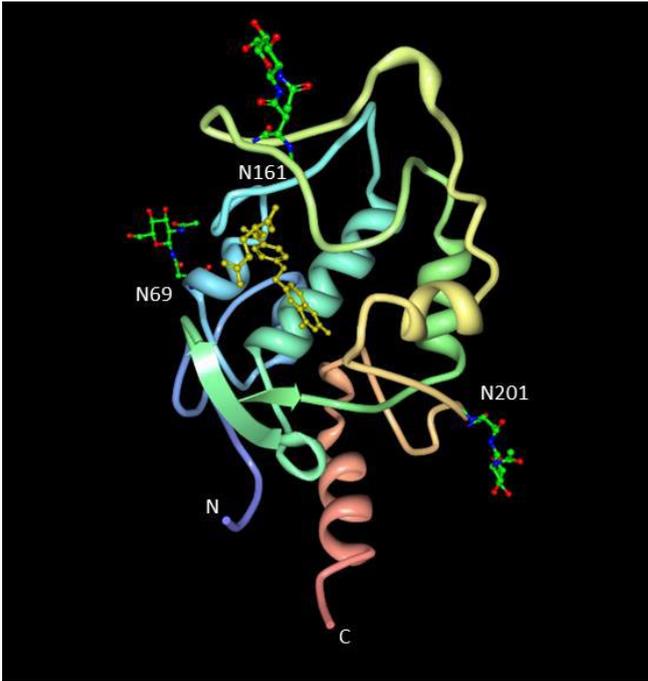
**Figure 2.3.; Structural model of FRα protein.** Folic acid is bound in a deep pocket in FRα. The N-glycosilations show they do not interfere with ligand binding and are marked by number, their position at sequence (N69, N161, and N201). The N-terminal (N) is marked with blue colour and the C-terminal (C) with red. The figure is from (Hansen 2013)

## FRα expression

Expression patterns between the different isoforms of this receptors differ due to the changes produced in their sequences, as is explained above. The high degree of sequence similarity is reflexed in some of the tissues and cell types where their expression is shared; placenta, hematopoietic tissues and cells.

FRα expression is restricted to certain tissues and cell types, and expression patterns differ for the different isoforms as well as for normal tissues.

FRα expression is restricted to certain epithelial tissues, such as kidney tubule and intestine where prevents folate loss from excretion, and placenta and choroid plexus, FRα mediates folate transport. FRβ is mainly expressed in hematopoietic tissues and placental cells as well as soluble FRγ is secreted from hematopoietic tissues. The FRδ is the most recently discovered isoform and is expressed exclusively in splenic lymphocytes, but it has not been found in malignant tissues or cell types. (Table 1.2.).

|  | FRα | FRβ | FRγ | FRδ |
|---|---|---|---|---|
| **Normal tissues and cell types** | Placenta (trophoblasts) Proximal Kidney tubules Bladder Testes Female genital tract (ovary, fallopian tubes, uterus, cervix) Choroid plexus Cerebrospinal fluid Salivary, bronchial and breast glands (*Acinar cells*) Colorectum Lung (type I and II pneumocytes) Human milk Serum Erythrocytes | Placenta Hematopoietic tissues* (spleen, thymus) Hematopoietic cells of myelomonocytic lineage* (neutrophil granulocytes*, activated macrophages*) Erythrocytes | Hematopoietic tissues (bone marrow, spleen, thymus) Neutrophil granulocytes | Regulatory T cells Splenic lymphocytes Mature lymphocytes |
| **Malignant tissues and cell types** | Pancreatic, renal lung, testicular, endometrial, uterine, cervical, **ovarian** and **breast carcinomas** Ependymal brain tumours | Leukemia (Leukemic granulocytes) Lymphoma Rheumatoidal arthritis (Synovial macrophages) | Leukemia | |

**Table 1.2. Tissue expression of FR isoforms.** FRα is widely expressed in normal tissues and cell types, followed by the FRβ isoform, whereas FRδ has restricted expression. Malignant tissues and cell types are clearly dominated by FRα expression (ovarian and breast carcinomas in bold text because of its high relation to this isoform). FRδ is has not been found in cancer. An asterisk (*) is referred to the inactive form. The table is modified from (Kelemen 2006), with the use of results from (Hoier-Madsen et al. 2008), (Henderson 1990), (Basal et al. 2009) and (Tian et al. 2012).

A high expression of FRβ and FRγ is only found in leukemia, lymphomas and macrophages. In contrast FRα is highly expressed in several malignant tissues and is believed that its expression is upregulated in these epithelial tissues to maintain the necessities required for folates in nucleic acid synthesis and cellular growth. (Leung et al. 2013).

## The Signal Transducer and Activator of Transcription 3 (STAT3)

STAT3 is a cytoplasmatic transcription factor that belongs to STATs family (Signal Transducer and Activator of Transcription), composed by seven different STAT proteins: STAT1, 2, 3, 4, 5a, 5b and 6. These proteins are activated by a large number of extracellular signalling polypeptides, such as cytokines and growth factor that interact with specific cell surfaces receptors

Members of STAT family share a high degree of homology and structurally and functionally conserved domains, Src (sarcoma receptor tyrosine kinase) homology 2 (SH2) domain.

STAT3 is responsible for the up-regulation of several genes involved in cancer development, by inducing genes in survival, proliferation, angiogenesis, metastasis and cell adhesion. Not only is

it necessary for tumour promoting effects, but also for maintaining a cancer promoting tumour microenvironment. This tumour microenvironment is created by cancer cells, which release cytokines such as IL-6 and IL-11 due to the action of STAT3 (Jarnicki A et al 2010).

STAT3 is a pro-oncogene constituvely active in several cancer types, for example leukemia, breast cancer, lung cancer, renal cell carcinoma and ovarian carcinoma (Yu H. et al 2009). In addition, another relation has been found to high STAT3 levels and poor survival prognosis in many cancers, for example renal, colorectal and ovarian carcinoma

The role of several signalling pathways is related to constitutive STAT3 activation or overexpression in cancer. The EGFR pathway is linked to overexpression or constitutive activation, in addition to overexpression of SFKs as well as constitutively active JAKs. Furthermore gp130 is constituvely active and it also cause an increased STAT3 activation.

One more reason to understand the relation between STAT3 and cancer development is this protein has a direct inhibitory effect on the transcription of p53, which allows apoptosis to those malignant cells and is considered the most important defense against cancer (Niu G. et al. 2005)

STAT3 is a contributor to the oncogenic transformation and is therefore an important target for cancer therapy.

It has been discovered STAT3 and STAT1 produce opposing effects in relation to cancer, activated STAT3 is oncogenic, whereas activated STAT1 acts as a tumour suppressor (Johnston PA. et al. 2011).

## The JAK-STAT Pathway

STAT proteins are 750 and 795 amino acids long (STATS 1, 3, 4, 5a and 5b) or ~850 amino acids longs (STATs2 and 6). The tyrosine residue located around position 700 has to be phosphorylated for STAT activation. Ligand-activated receptors that catalyse this phosphorylation include receptors with intrinsic tyrosine kinase activity such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), as well as receptors that lack intrinsic tyrosine kinase activity but to which Janus Kinases (JAKs) are noncovalently associated. Receptors to which JAKs are bound are known as cytokine receptors and their ligands include IFN-α, -β, and -γ; interleukins (IL) 2 to 7, 10 to 13, and 15; and erythropoietin, growth hormone, prolactin, thrombopoietin, and other polypeptides (James E, et al. 1997). When a cytokine binds to its cognate receptor the signaling through the JAK-STAT pathway starts and it means a tyrosine residue from a cytokine residue is phosphorylated, which deserves a docking site for STAT proteins. Latent cytoplasmatic STATs are recruited to the receptor and phosphorylated on a tyrosine residue, which trigger the activation of STATs and its dimerization via interactions between the SH2 domain of one monomer and the phosphotyrosine (Y705 in STAT3) on the other monomer. These STAT dimers translocate to the nucleus where they activate transcription of target genes (Kisseleva et al. 2002).
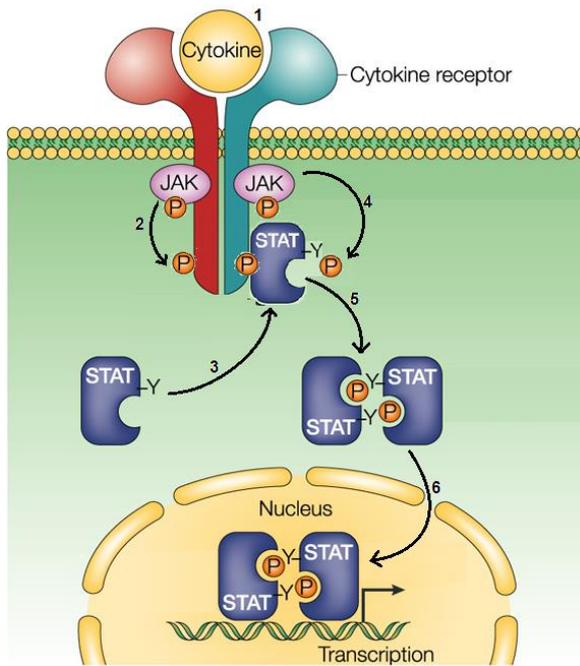
**Figure 2.4 The JAK-STAT pathway.** When no ligand is bound the cytokine receptor is associated with Janus Kinases (JAKS). (1) Upon cytokine binding, JAKs autophosphorylate each other (2) and the cytokine receptor. 3 The phosphorylated receptor generates a docking site and recruits STAT proteins (4) which are phosphorylated by the receptor associated JAKs on a specific tyrosine residue (Y). Due to the phosphorylation, STAT proteins form dimers (6) which translocate to the nucleus and induce transcription of STAT target genes. The figure is modified from (Shuai et al. 2003)

## The proposed FRα-STAT3 signaling pathway

Previous experiments performed in the laboratory where I have been working for five months have shown that binding folic or folinic acid to the FRα results in activation of STAT3 in a Janus Kinase (JAK) dependent manner.

In order to understand the process is necessary to make a brief introduction of the gp130 (glycoprotein 130). This gp130 is the universal signal-transducing receptor subunit for the IL-6-type cytokines, IL-6, IL-11, LIF (leukaemia inhibitory factor), OSM (oncostatin M), CNTF, cardiotropin-1 and cardiotrophin-like cytokine. IL-6 and IL-11 cytokines binds to their respectives α-receptors and results in homodimerization of gp130. Signal transduction through gp130 activates JAK/STAT and MAPK (mitogen-activated protein kinase) cascades. Gp130 receptor dimerization causes autophosphorylation of receptor-associated JAKs, and therefore the JAK-STAT pathway starts. The proposed FRα-STAT3 signaling pathway is shown and explained on Figure 2.5.
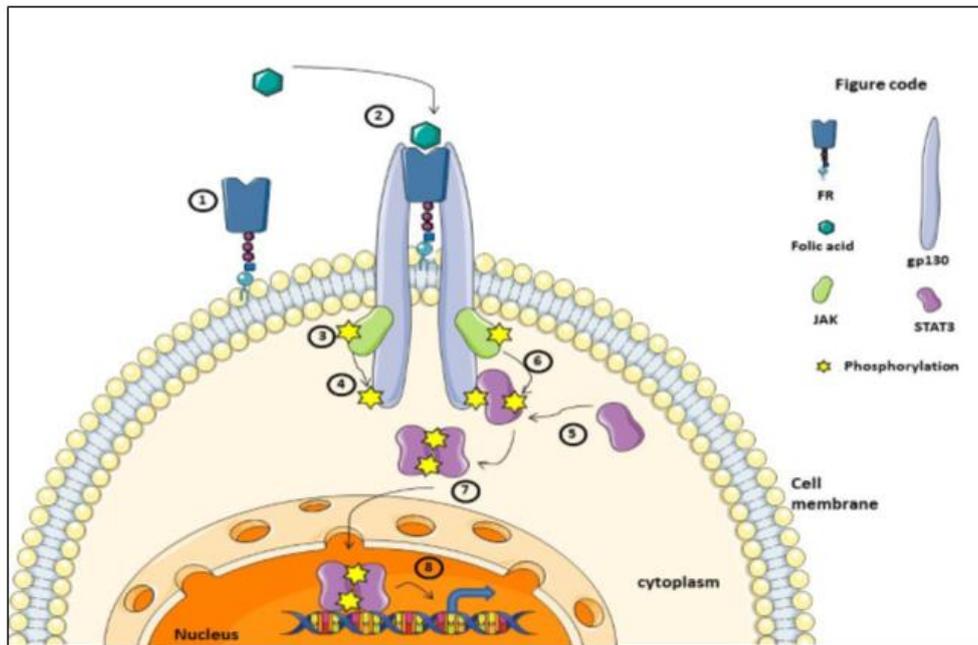
**Figure 2.5.; Folic acid induces STAT3 activation via FRα and gp130.** FRα is attached to the cell surface through a GPI anchor (1). The folic acid binding to FRα results in the association of gp130 and FRα (2). This association causes the homodimerization of gp130 and its autophosphorylation associated to Janus Kinases (JAK) (3). Activated JAKS are phosphorylated and at the same time, they phosphorylate gp130 (4) and creates a docking site for STAT3 proteins (5), which are consequently phosphorylated by the receptor associated JAKs (6).STAT3 proteins form homodimers and migrate to the nucleus (7), where they bind to promoter elements and initiate the transcription of several genes (8) involved in cancer development, survival, proliferation, angiogenesis, metastasis and cell adhesion.
The figure is from (Hansen 2013).

## Transsignaling process and receptors

Cytokine receptors are found either membrane-bound or soluble form which affinity to bound their ligands is similar. Transsignaling process occurs when a soluble receptor in a complex with its ligand activates receptor signalling without the intervention of the membrane-bound form. As a well-known and established example of receptor transsignaling the interleukin 6 receptor (IL-6R) has been described by several studies. In order to understand this process I have decided to make a brief from this receptor transsignaling (IL-6R) and its role in cancer.

The receptor binds interleukin 6 in the circulation system as a soluble receptor (sIL-6R) or on the cellular membrane (IL-6R). The receptor complex binds to the gp130 transmembrane receptor and activates the STAT3 pathway. In addition to the cellular gp130, there is the soluble form of gp130 (sgp130), and is described as an antagonist to IL-6.

The soluble IL-6R receptor together with IL-6 stimulates cells that only express gp130, the transsignaling process (Heike Knüpfer et al. 2010). The importance of this process remains in the fact that cells that were originally unresponsive to IL-6 (because of the lack of the membrane-bound IL-6R) become in responsive via the soluble complex. (Figure 2.6.).
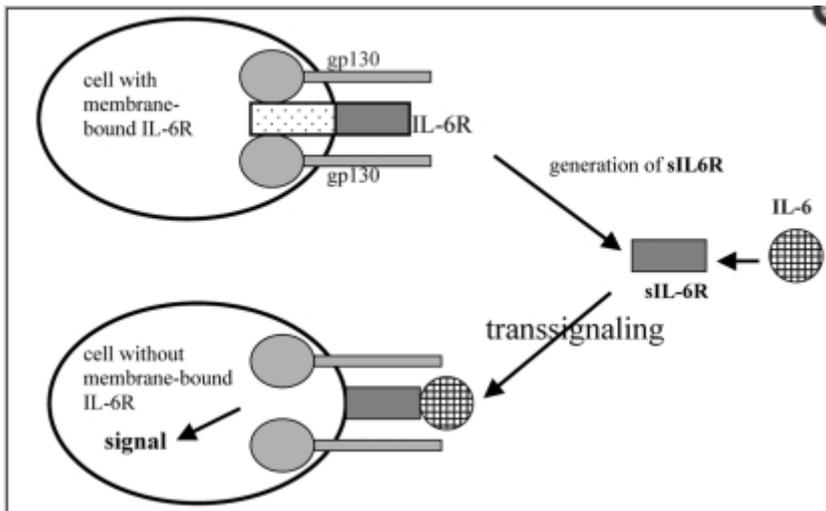
**Figure 2.6.; The transsignaling process via the sIL-6R.** The transsignaling process occurs when the soluble IL-6 receptor is generated due to the presence of the membrane-bound gp130. The figure is from (Heike Knüpfer 2010).

About 70% of the secreted IL-6 forms a complex with the sIL-6R in the blood and binds to membrane gp130. The other 30% has a transient existence in the blood, or binds to the membrane-bound receptor. Some studies have shown the importance of the sIL-6R in cancer, such as the study performed by Vermes et al. (2002), they found cell surface IL-6R expressed in osteoblasts, but this membrane-bound form was unable to transmit IL-6 induced signals until it is shed in its soluble form. Another finding from Modur et al. (1997), shows that the limiting factor is the sIL-6R, because of the fact that endothelial cell activation depends on sIL-6R concentration. The study of Becker et al. (2004), using a colon tumour mouse model shows that IL-6 signal transduction was mediated by the soluble rather than the membrane-bound IL-6R, indicating that tumour growth is controlled by IL-6 transsignaling via the soluble form of the IL-6R.

## Soluble folate receptor alpha (sFRα)

Previous studies found the soluble form of the folate receptor alpha (sFRα) in serum and human milk, which has the same affinity to bind folic acid as the membrane-bound receptor.

Lactating cells express high levels of membrane-bound FRα and are release into milk due to the cleavage performed by GPI-specific phospholipases. These soluble proteins form aggregates in micelles because of the presence of the hydrophobic GPI residue and micelles can be converted to soluble form (sFRα) by cleavage of the GPI-anchor. sFRα is secreted into milk and serve to concentrate folates in milk, protect folates from degradation and facilitate intestinal absorption in the neonate.

High levels of sFRα have been associated to pregnancy and some diseases such as folate deficiency and ovarian cancer. In contrast when healthy individuals serum levels of sFRα have been tested, the levels are almost undetectable. This is in correlation to folate levels in serum, when folate levels are high the concentration of sFRα decreased, whereas this concentration increased when folate levels are low (Kelemen 2006).

The conditions associated to undetectable levels of sFRα in healthy individuals were studied by Linda E. Kelemen (2014), and found that serum FRα levels are significantly higher in women than in men. Furthermore, this concentration is related to pregnancy and oral contraceptive use, which leads to think about the involvement of hormones to increase the presence of soluble folate binding proteins. Other factors that may influence serum levels include vitamin A (retinoic acid), age and body mass index, but no association with folate nutritional status have been found.

The sFRα could be a promising biomarker candidate in ovarian cancer. The soluble form has been detected in elevated levels in several studies, even in patients with early disease. Studies from Eati Basal et al. (2009), found that in early stages of disease the circulating levels of FRα similar to those from advanced stages, and Daniel J O'Shannessyfound et al. (2013) a correlation to both stage and grade of disease.

The biological functions of soluble FRs in serum are not uncovered, but suggested functions include protection against enzymatic degradation of folates, bacteriostatic effects, high expressions levels are correlated with tumor progression (Kelemen 2006).

## Folate receptors: Glycosylphosphatidylinisotol (GPI) –anchored proteins.

Folate binding proteins (FBP) are characterized by their high affinities for folic acid and reduced physiologic folates and antifolates. Folate receptors were originally known as FBP, which are bound to cell membranes by Glycosylphosphatidylinisotol (GPI) -anchored.

There are some degrading enzymes present in eukaryotes and prokaryotes that cleave GPI anchors: PI-PLC from bacteria and PI-PLD from mammals. These enzymes are used to determine the presence and the structures of GPI anchors to search for the intermediates in the process of GPI biosynthesis. GPI-PLC of *Bacillus thuringiensis* has been extensively used for study of GPI-anchored proteins than other enzymes. On the other hand, GPI-PLD is effective for hydrolysis of GPI anchors with an acylated inositol which is resistant to PI-PLCs and GPI-PLC (Ikezawa H 2002).

FRα membrane bound (mFRα) are release from cells due to the role of different metalloproteases and phospholipases C and D, which cleaves the GPI anchor. When FRα is released it becomes in soluble FRα (sFRα). Folate binding proteins exhibit a hydrophobic anchor to attach to the membrane and, several experiments performed, show that the use of a degrading enzymes activator increase the role of the enzyme, increasing the activity of phospholipase C, which deletes the hydrophobic anchor from the sequence. In result, the concentration of sFRα is higher.

## III.  Aim of the study

The aim of this project was to study the FRα protein and its association with cancer development. Previous studies shown that folic acid activates STAT3 oncogene via FRα, but soluble form of FRα has become in the new object of the study, because of its high expression in several cancers and high affinity to bind folic acid. The sFRα is directly involve in transsignaling process, is able to activate cell signaling independently of its membrane-bound counterpart and in this project the soluble protein has been studied in both, human and mouse cells. Human cell lines were used to identify those cells which express high FRα and sFRα. Mice were selected for the study to understand the effect of high and low folic acid diet in tumour expression and growth and at the same time, the expression of sFRα in tumours of different size.

# IV.    Materials and methods

## Charcoal stripped FBS (cFBS)

Fetal Bovine Serum was needed to grow cells. FBS was filtered to avoid the activation of compounds that could interfere in cellular activity. The filtration was performed incubating 50mL FBS with 1g dextran coated charcoal (Sigma) and incubated ON at 4ºC on a rotator. The following day charcoal was removed by centrifugation (2x15 minutes at 4000rpm) and serum was filtered using a 0.2μL syringe filter.

## Mammalian cell culture

Cells were cultured as monolayers in T75 flasks and grown in an incubator with 5% $CO_2$ at 37ºC. Cells were split up twice a week, washing cells in PBS and adding 2ml of Trypsin-EDTA, which acted for 3-5 minutes while cells were in the incubator. After the action of trypsin, a new T75 flask was prepared with 15ml of RPMI and 10ml of RPMI were added to the old one, 1.7 mL of cells were transfected to the new flask.

| Cell lines | Origin | FRα expression (Previous experiments) * |
|---|---|---|
| HeLa | Human epithelial carcinoma (Cervical cancer) | ↑↑ (High) |
| HEK 293 | Human embryonic Kidney | -    (No expression) |
| KB3 -1 | Human epithelial carcinoma (Cervical cancer) (HeLa derivative) | ↑↑↑↑ (Highest expression) |
| HeLa pcDNA3 | HeLa cells transfected with pcDNA3 plasmid as control | ↑ (Lower than HeLa) |
| HeLa – FRα | HeLa cells transfected with a plasmid that increases the expression of FRα | ↑↑↑ |
| HeLa pcDNA3 FRα – D103A | HeLa cells transfected with pcDNA3 encoding the FRα – D103A mutant | ↓ (Low) ? ** |

NOTES:

*Last column "FRα expression" shows results from previous experiments

**FRα expression has never been analysed in this mutant (D103A), we just supposed that the concentration will be lower.

All cell lines were grown in a RPMI 1640 media composed by + L-glutamine, +/- folic acid with 10% cFBs (50mL) and 1% P/S (5mL).

## STAT3 activation experiment

HeLa cells were seeded out in a 12 well plate (2mL/well) and they were growing until the following day. To continue the experiment, cells must arrived to a confluence of 50-75%. After check the confluence, cells were starved for 3 hours, cell medium was removed by jet suction and replaced by 1,5mL/well FFRPMI media (Free Folic acid medium),  was supplemented with 1%P/S and 10% cFBS. The rest of FFRPMI was in the incubator until FA solutions were prepared. To run the dose experiment FA solutions stock was prepared using different folic acids, the old one, the new one and folinic acid. The old and the new folic acid solutions were dissolved in 50μL 1M NaOH to a concentration of 50mg/mL and 450μL FFRPMI while folinic acid was dissolved in ddH$_2$O. As a positive control IFNβ was used.

In a new 12 well plate 500μL FFRPMI were added in each well together with the right concentration of FA solutions (250μg/mL or 500μg/mL).
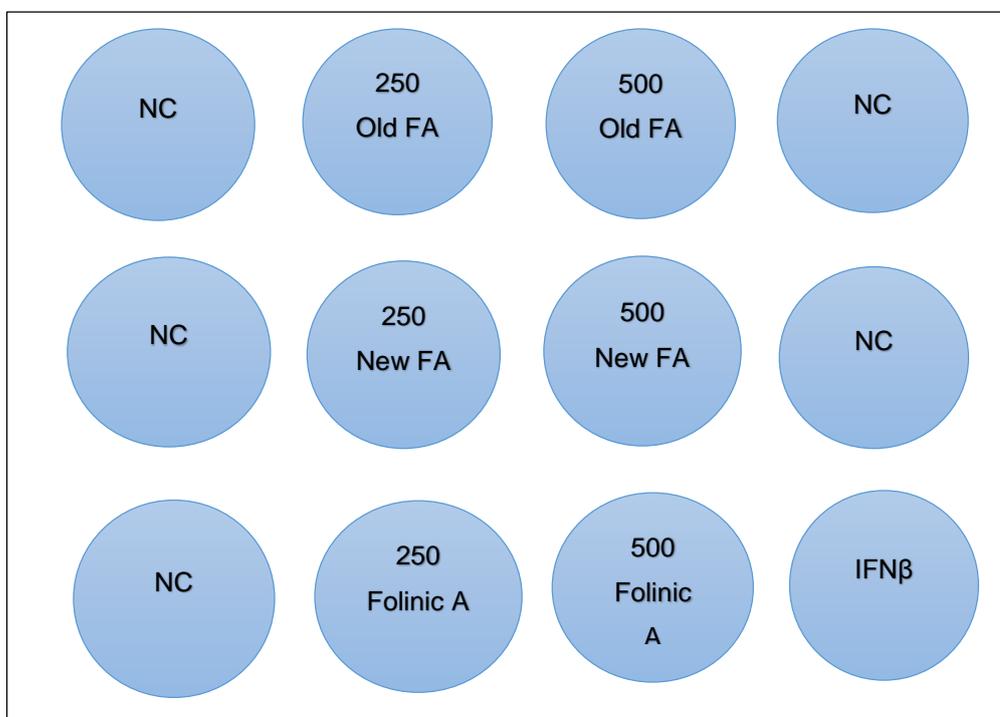


Starvation medium from cells was removed by jet suction and 500μL solutions prepared in the new plate were transferred. Activation of STAT3 stimulating cells was performed for 10-12min in the incubator. Medium from wells was removed and cells were clean in cold PBS. After wash, cell lysis was induced with 150μL PONG buffer for 10min and 0,5μL benzonase was added. Lysates were mix on a thermomixer and plate was cover by fix film and incubated ON at 4ºC. Lysates were collected and centrifuged at 12000rpm for 5 min, pellets were discarded and protein extracts were frozen at -80ºC or treated to be loaded in gels.

## Seed out the cells

When cells were splitted up the rest of them were counted using a hemocytometer, Neubauer Chamber. In order to know the quantity of cells and media to seed out the cells, cells from two different squares were counted, and the calculations done (Equation 4.1.):

Concentration of Cells/mL · x media volume (mL) = 100.000 cells/mL · ** 25/8mL media

Equation 4.1. Cells to seed out and media measures

**Cells were seeded out in a 12 well plate (media volume was 25mL) or 6 well plate (media volume was 8mL).

HeLa cells were used for STAT3 activation experiments and they were seeded out in a 12 well plate (2mL/well) while for FRα experiments; HeLa, HEK 293, KB, HeLa pcDNA3, HeLa FRα, HeLa pcDNA3 FRα-D103A 2mL/well to get more concentration).

## Expression of FRα in different cell lines

Cell cultures from HeLa, HEK 293, KB, HeLa pcDNA3, HeLa FRα, HeLa pcDNA3 FRα-D103A were grown in a 6-well plate in a volume of 2mL for 4 days. Confluence from each cell line was checked and media was collected in Falcon 2059 tubes and centrifuged at 4ºC for 10min and 4000rpm. Cells were washed in 1mL cold PBS and lysis was induced by 200µL Shriuangpong (PONG) buffer for 10 min at RT, before 1µL benzonase was added. Media samples and protein extracts were stored at -80˚C.

## Addition of PI-PLC in cell cultures

To increase the concentration of FRα in the media (sFRα) Phosphatidylinositol-Specific Phospholipase C (PI-PLC) was used. This enzyme is able to release folate-binding proteins (FBP) attach to membranes of some cells (Leamon CP et al. 1993) which were identified as FRα proteins.

Cell cultures from HeLa, HEK 293, KB, HeLa pcDNA3, HeLa FRα, HeLa pcDNA3 FRα-D103A were grown in a 6-well plate in a volume of 2mL for 4 days. The *Bacillus cereus* PI-PLC (*Molecular Probes*) was added to the growth medium in a concentration of 0.1U/mL and incubated for 30 min at 37ºC (Leamon CP et al. 1993). Media and protein extracts were prepared as described above.

## SDS-PAGE

This electrophoresis were used for samples from STAT3 activation experiment which were prepared by addition of 6X SDS sample buffer and incubated for 5 min at 95ºC to denature proteins, while samples from FRα project were prepared in 6X SDS sample buffer without DTT because the Mov18 antibody used for Western blots only recognized proteins in their native structures. Markers used in these gels were PageRuler™ Plus Prestained Protein Ladder #26619 and PageRuler™ Plus Prestained Protein Ladder #26616.Samples were run on 10% SDS gels in 5X PAGE buffer for 1h and 30min at 400mÅ and 100V.

## Western blot

Western blot technique was used to detect proteins of interest which were recognized by specific antibodies.

After SDS-PAGE electrophoresis, proteins were transferred to a PolyScreen® PVDF transfer membrane. First, the membrane was activated in 96% ethanol for 5min, the ethanol was removed with water and membrane was moistened with transfer buffer.

In order to run the transfection the assembly of the blotting sandwich was prepared using a blotting apparatus with the black side down, the different papers wet in transfer buffer, the gel with protein side up and the membrane. The blotting sandwich was introduced in the gel box with two ice pack and transfer buffer. Blot was run for 1h and 15min at 400mÅ and 200V.

When transfection was done the protein membrane was incubated in 20ml blocking buffer and shaken on a rotator at RT for 1h. Blocking buffer was composed by 1g of 5% skimmed milk and 20ml TBS-T or PBS-T (MPBS-T) in proteins which conserved their native structures. For STAT3 activation experiments membranes were washed 3x5min in TBS-T, put in sealed plastic bags and incubated ON at 4°C with 5ml primary antibodies, shaking. Primary antibodies used were prepared with 0,25g BSA, 5mL TBST-T and 1µl STAT3 or 2,5µl pSTAT 3 antibodies.

## Stripping Western Blot membranes

This method is used to repeat a western blot with the same membranes, the primary antibody is eliminated and some of the proteins too, thus membranes are stripped one time.

Membranes to be stripped are introduced into a glass holder, between two meshes, with 20mL stripping buffer. The glass holder is put into an incubator with rotator at 37ºC for 30min. Blocking buffer is prepared, 1g skimmed milk and 20mL 1X TBS-T. After 30min, stripping buffer is discarded and 20mL blocking buffer are added, incubation for 1h. Membranes are washed three times for 5min with TBS-T and western blot is performed.

## Acetone precipitation

This technique was used when western blots were run to observe soluble FRα in the medium but it was not found, no bands of 38kDa. A reason could be because of the low concentration of soluble FRα, so the acetone precipitation was done to concentrate this receptor. 1mL media samples from the 6 different cell lines were defrosted and 500µL of cold acetone were added. These mixtures were incubated for 10min at -20°C and centrifuged at 12000rpm for min at 4°C. The supernatant was removed and samples were dried for 15 min in opened eppendorf tubes.

Samples were prepared to be loaded and run in a SDS-PAGE; the pellets containing the protein of interest were resuspended in 10µL ddH$_2$O and 3µL sample buffer was added. Proteins from the gel were transferred to a membrane and Western Blot was run, incubating membranes in Mov18 as primary antibody and SAM as secondary antibody.

## Breeding and diet from cancerous mice

To understand if FRα has an important role in development of cancer, results from the experiments above performed by the used of human healthy cells were compared with results from experiments run with both healthy and cancerous mouse cells. Some mice followed a high folic acid diet and other mice a low folic acid diet.

## DNA extraction

A male mouse affected by breast cancer and a healthy female were crossed and the breeding was tested to understand which mice had the allele to produce cancer. A DNA extraction was performed, samples taken from mice were tail-tips and ear-clips, which were introduced in eppendorf tubes and store at 4ºC until the moment they were treated.

First, for the DNA extraction samples were incubated with 75µL lysis buffer at 95ºC for 1,5h. To improve the lysis we made sure tail-tips and ear-clips were in the buffer and not in the surface. Samples were cooled down at 4ºC in the fridge for 20min. After that, samples were neutralized when 75µL neutralization buffer were added to each tube and centrifuged at 14000rpm for 5min. Preparation was done and a PCR was run.

## PCR: cDNA synthesis

After DNA extraction a PCR was performed to find out which mice carried the PyMT gene, the allele which develops cancer.

From the samples centrifuged, 1µL from each one was introduced in PCR tubes and prepared for the reaction with 24µL Mastermix solution. PCR was run using the MFH PyMT program (see Appendix II.)

After running the PCR, 4,2 µL loading buffer were added to each sample and mix properly before loading the 2% agarose gel, 15 µL samples and 10µL 6X DNA loading dye.

## Cell cultures from tumours and seeding out

After results from PCR, mice which had developed cancer were detected and tumours were collected to generate cell cultures in T25 flasks. Tumour cells grew slowly and sometimes media from cultures (RPMI media) was just changed, when cell confluence was enough they were splitted.

To run my experiments I used four tumours, two from down the legs and two from the middle of the stomach and all of them were extracted from the same mice. Cells were seeded out in two 6 well plate (2mL/well), but their confluence and characteristics were checked before. After 5 days, the amount of cells was lower than expected and fibroblast cells dominated over epithelial cells, cells which express FRα. Media was collected and cells were lysed, as is explained above. Media samples and protein extracts were prepared and loaded in a SDS-PAGE gel and blotted using the FRα mouse antibody.

## Serum samples from PyMT mice

15µL of serum extracted from different mice, were taken and mix for 5min at 95ºC with 3µL 6X sample buffer without DTT. After the preparation, samples were store at 4ºC until the following day. A total of 38 samples were treated and loaded in a SDS-PAGE gel, plus the kidney sample used as a positive control that express the sFRα. When samples were run, a western blot was performed to recognize the sFRα protein by the FRα mouse antibody.

# V.    Results

## Folic acid induces STAT3 activation in HeLa cells

HeLa are FRα-positive cells that were stimulated with folic acid to induce STAT3 activation. Prior to stimulation, HeLa cells were starved of folic acid to reduce background of STAT3 activation. After 3-4 hours of starvation STAT3 was activated to pSTAT3 running a dose experiment and assayed by western blots.
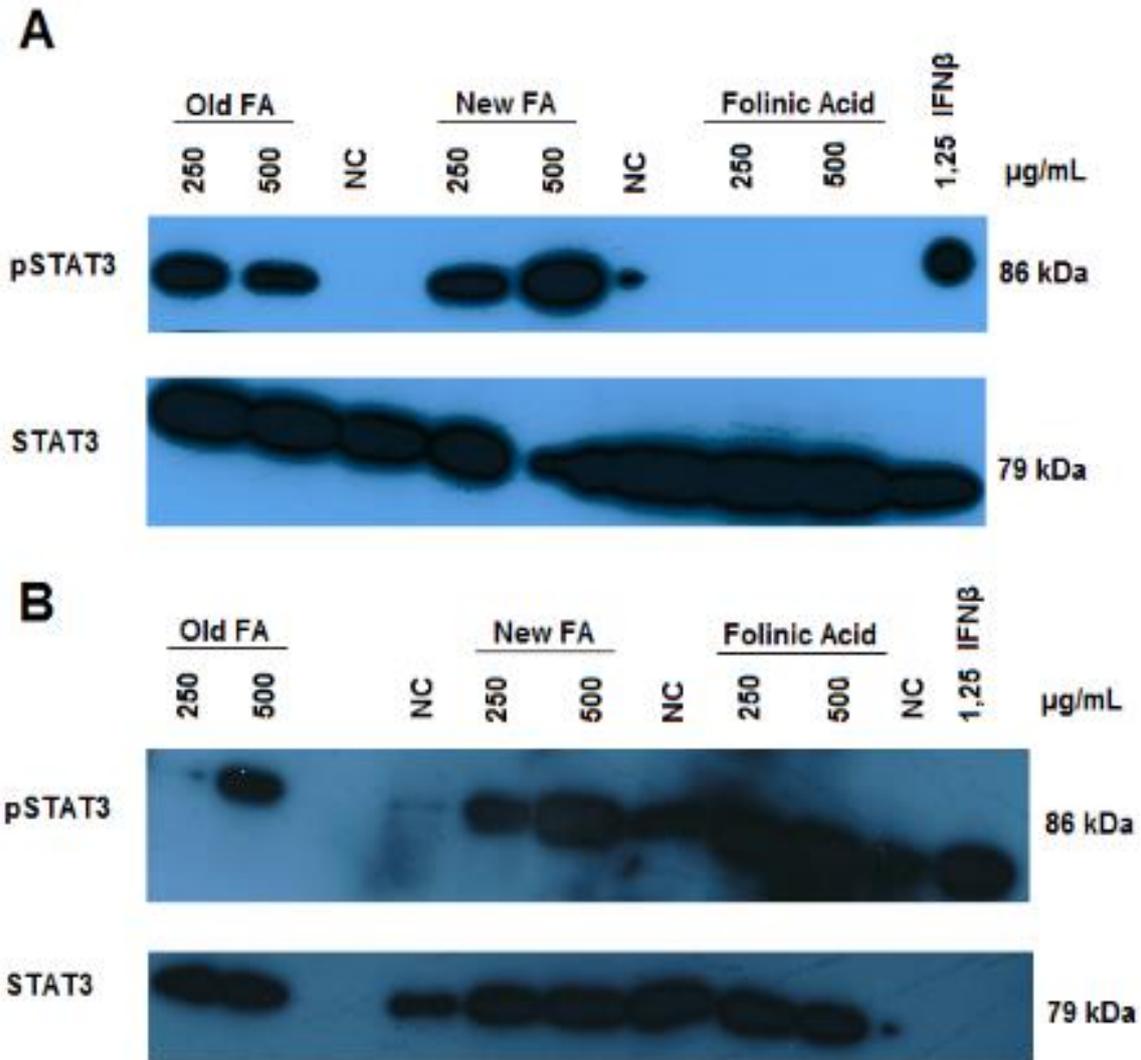


**Figure 5.1.; Folic acid dose experiment to activate STAT3.**
**A. STAT3 activation is dose dependent.** HeLa cells were starved for 3 hours prior to stimulation. Cells were stimulated with 250 and 500 µg/mL of folic acid. This dose experiment was performed with different types of folic acid, old and new from 2012 and 2015, respectively (see Appendix 2). After stimulation, cells were lysed with 150 µL PONG lysis buffer. The samples were blotted for the presence of STAT3 and pSTAT3, recognized as two bands of 79 and 86 kDa. **B. Stripping membranes.** The experiment was repeated, bands from pSTAT3 appeared in western blot, but no results were observed from STAT3. To get STAT3 results the pSTAT3 membrane was stripped and western blot performed with STAT3 antibodies.

The dose experiment shows that STAT3 is constituvely expressed, even though different type of folic acid and concentrations were used, the bands appear to be expressed at the same level (Figure 5.1.A).

STAT3 activation response to folic acid is detectable at doses of 250 and 500 μg/mL. When the old folic acid induces the activation it appears to reach a lower expression than when the new folic acid is used. Furthermore, when the concentration is higher the STAT3 activation is stronger, it is observed with thicker bands (Figure 5.1.B). In addition, folinic acid was tested in this experiment to find out if there were significant differences between this compound and folic acid.

We repeated this experiment several times to get better results but we thought that different factors are involved and consequently, the results are not what we expected. Some of these factors could be cell stress from medium changes, serum starvation and distortion in pH balance of the folic acid solutions. Probably the combination of these aspects would made STAT3 activation experiments tricky and very sensitive to minor variations, as you can see folinic acid did not induce the activation on Figure 5.1 A, but it did it on Figure 5.1. B. Negative controls (NC) solutions were free folic acid and pSTAT3 should not be expressed, but this expression is observed in Figure 5.1 A and B and as positive control interferon-beta (IFNβ) was used. Although higher concentrations of folic acid should increase the activation of STAT3, some of the factors mentioned were involved and 250μg/mL of folic acid reach more activation of STAT3 than 500μg/mL. In order to perform better experiments they were repeated and membranes stripped (results from strip membranes are represented in Figure 5.1 B). STAT3 membrane did not show bands, but pSTAT3 bands were clear so, pSTAT3 membrane was stripped and incubated with STAT3 antibodies to observe the STAT3 bands.

## FRα expression levels in six different human cell lines

### FRα membrane-bound

The expression levels of FRα were assayed by immunoblot with the Mov18 antibody in six cell lines used: HeLa, HEK 293, KB, and modified HeLa cells; HeLa pcDNA3, HeLa FRα, HeLa pcDNA3 FRα-D103A.

**A** Cell lysates (Mov 18)

HeLa
HEK 293
KB
HeLa pcDNA3
HeLa FRα
HeLa pcDNA3 FRα D103A

FRα                                                38 kDa

**B** Cell lysates (α -tubulin)

HeLa
HEK 293
KB
HeLa pcDNA3
HeLa FRα
HeLa pcDNA3 FRα D103A

α-tubulin                                          50 kDa
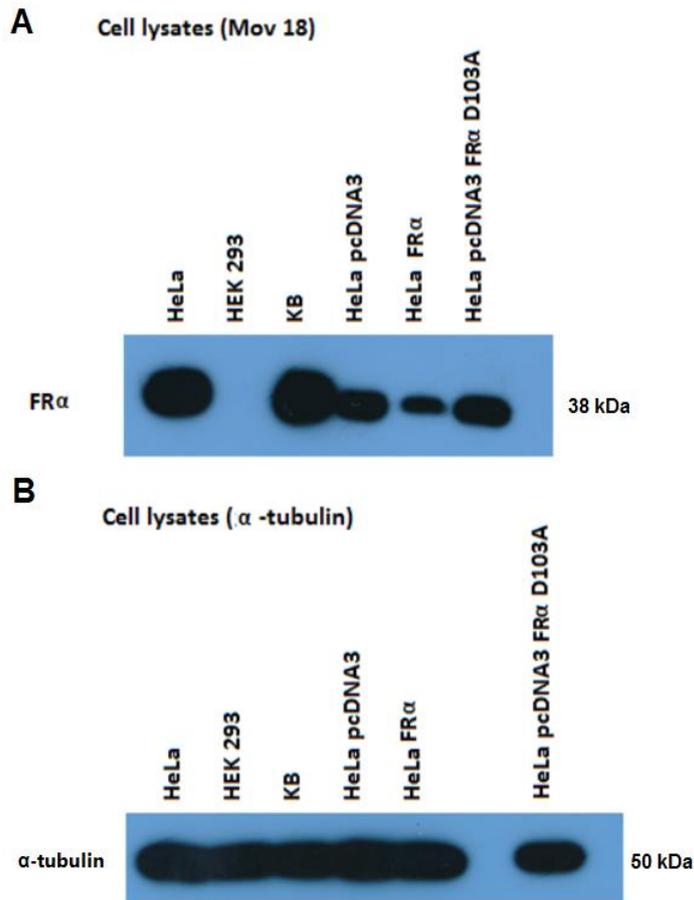
**Figure 5.2.; FRα expression in human cell lines.**
**A. FRα in the cell membrane.** Cells were seeded out in a 6-well plate. After 4 days cells were lysed with 120 PONG lysis buffer and samples were blotted. FRα was recognized by the Mov18 antibody as a single band of 38 kDa.
**B. α-tubulin expression in cells.** Protein extracts from the same lysates were blotted but looking for another protein, α-tubulin expressed in all cells, used as a quality control, and recognized by the α-tubulin antibody, detected as a band of 50 kDa.

As it was expected HEK 293 cells were FRα-negative, whereas HeLa and KB cells express FRα membrane-bound at high level (Figure 5.2 A). The significant FRα level in KB cells correlates with previous findings, as it is explained in Elwood PC et al. (1986) article.

In relation to modified HeLa cells the expression levels of FRα were different than expected. HeLa pcDNA3 cell line was used as a control and the amount of FRα is higher than in HeLa FRα, a plasmid to overexpress the protein was inserted into these cells thus, should be the cell line with higher expression, but is the one with the lowest (Figure 5.2 A).

Another modification was introduced in HeLa, an Aspartate at position 103 was mutated to Alanine and as other studies shown, the affinity to bind folic acid ($K_D$) increases, but I did not know if this mutation affects the FRα expression. FRα concentration in HeLa compares to its concentration in HeLa pcDNA3 D103A is higher, it means that the mutation causes a decrease of the FRα levels (Figure 5.2 A).

To clarify any doubts about wrong practise or steps during the experiment the same protein extracts were blotted to detect another protein recognized by the α-tubulin antibody. The α-tubulin is present in all cells, one of the major constituent of microtubules, and allows to work with samples using this protein as a quality control. In Figure 5.2 B the α-tubulin is present in all cell lines and the expression is equal.

## Soluble FRα (sFRα)

FRα expression was studied in its soluble form, from the same samples were FRα membrane-bound was assayed. Before cells were lysed medium was collected and prepared to be blotted and detect sFRα by the Mov18 antibody.
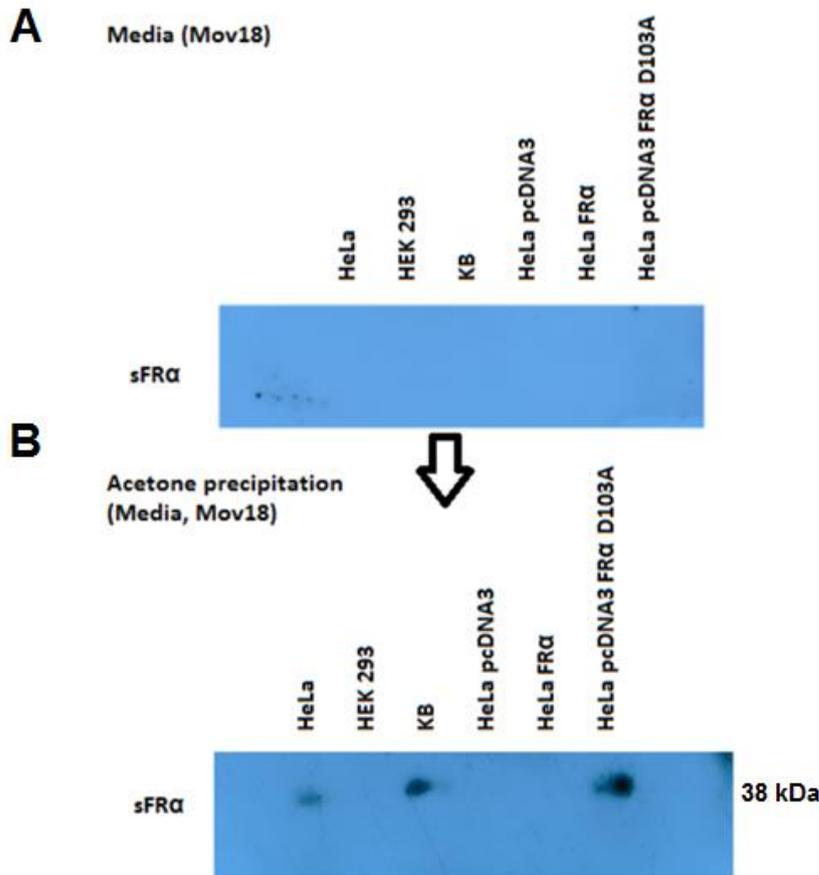


Figure 5.3.; sFRα expression (sFRα). A. Study of [sFRα] in medium. Media from the different cells lines was centrifuged and prepared with sample buffer without DTT to run a Western Blot. B. Acetone precipitation from media. SFRα concentration was low. In order to concentrate the proteins 1mL of media samples were precipitated with 500mL acetone, incubated for 10 min at -20ºC and centrifuged for 5 min at 1200rpm. Then supernatant was discarded and pellet dried. Pellet was dissolved in 7µL of water and 3µl sample buffer without DTT. Samples were blotted and single bands of 38 kDa were detected. Bands detected are not really clear.

When media from the same cell lines was blotted no proteins were observed (Figure 5.3 A). The cause was unknown, maybe there was some trouble with the experiment or the concentration of the soluble FRα was too low in medium to be detected. In order to get an explanation, we decided to perform an acetone precipitation of media samples and concentrate sFRα. When these precipitated samples were assayed by Western Blot with the Mov18 antibody, some bands appeared, but these were not the bands expected because of its bad visualization (Figure 5.3 B). As this experiment was performed several times, previous to this try, other protein extracts were tested together with media samples that were precipitated and blotted, and even though results

were not satisfactory they are included in this report them (Figure 5.4) to show the presence and concentration of FRα in media (sFRα).



**Acetone precipitation (2)**
**(Media, Mov18)**

Lanes: HeLa, HEK 293, KB, HeLa pcDNA3, HeLa FRα, HeLa pcDNA3 FRα D103A

sFRα    38 kDa
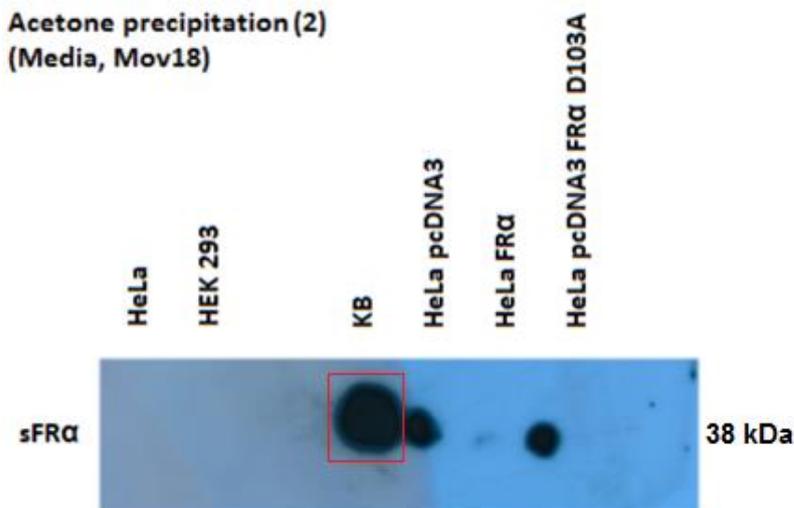
**Figure 5.4.; Acetone precipitation from previous media samples.** These results correspond to media samples from a previous experiment, the procedure followed was exactly the same. On the western some bands of 38 kDa were detected (sFRα).

Even though these results corresponds to different samples and are not what we supposed, they led us to think that sFRα is present in media at significant high levels in KB cells, as it was found in FRα membrane-bound, and no presence in HEK293. Something was wrong with the treatment of HeLa because no proteins appear, the same is observed in HeLa FRα.

Moreover, times this experiment was performed again when no proteins were found in acetone precipitated media from Figure 5.3 B, and in none of them proteins were observed. One reason to explain the absence of proteins could be the antibody used. It seems to be that results from several experiments indicated the Mov18 antibody stopped working and made difficult to work on later experiments. (See VI. Discussion).

## PI-PLC releases FRα from the cell membrane

FRα are proteins bound to the cell membrane by GPI-anchor, which is cleaved by some enzymes such as the phospholipase C, and release them. Consequently the sFRα concentration increases in medium. In this experiment cells were incubated for 30 min at 37ºC with an activator of phospholipase C, PI-PLC, to increment the amount of sFRα. Then two western blots were performed with media samples and protein extracts, looking for sFRα and FRα, respectively. Unfortunately there are not results to be shown, as it is mentioned above, the Mov18 antibody did not recognized the proteins because it probably stopped working. As it was expected no proteins were observed in protein extracts because all FRα proteins should had been released from cell membranes, but in media samples nothing appeared neither.

## Study of FRα and sFRα expression in mouse cancer models

### Mouse cancer models detection by PCR

Mice used for this new experiment were fed differently: high folic acid and low folic acid diets. DNA was extracted from tail-tips and ear-clips, after the preparation of these samples, the PCR was run to distinguish mice that express the MT protein, which cause cancer (PyMT mice), than those without its expression.

**A**

M 633 634 635 636 637 638 639 640 641 1Ha 1Va 1H1Va 1H 1V 1H1V PC NC M 633 634 635 636 637 638 639 640 641

**B**

M IV IV IV PC NC M

**C**

M 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 PC NC M

FEMALE MALE FEMALE MALE
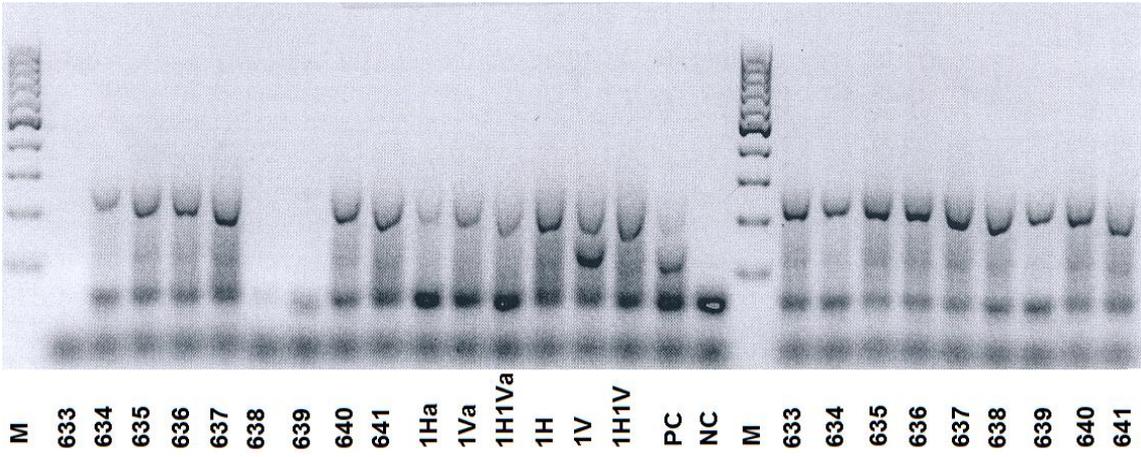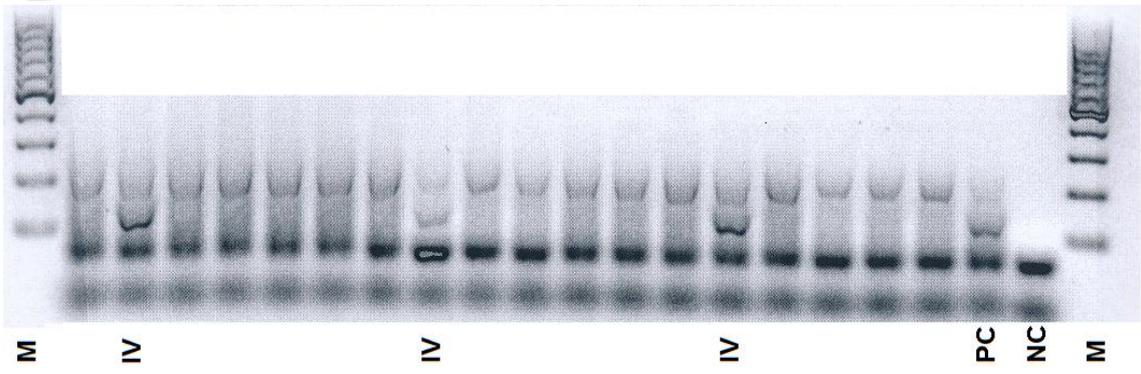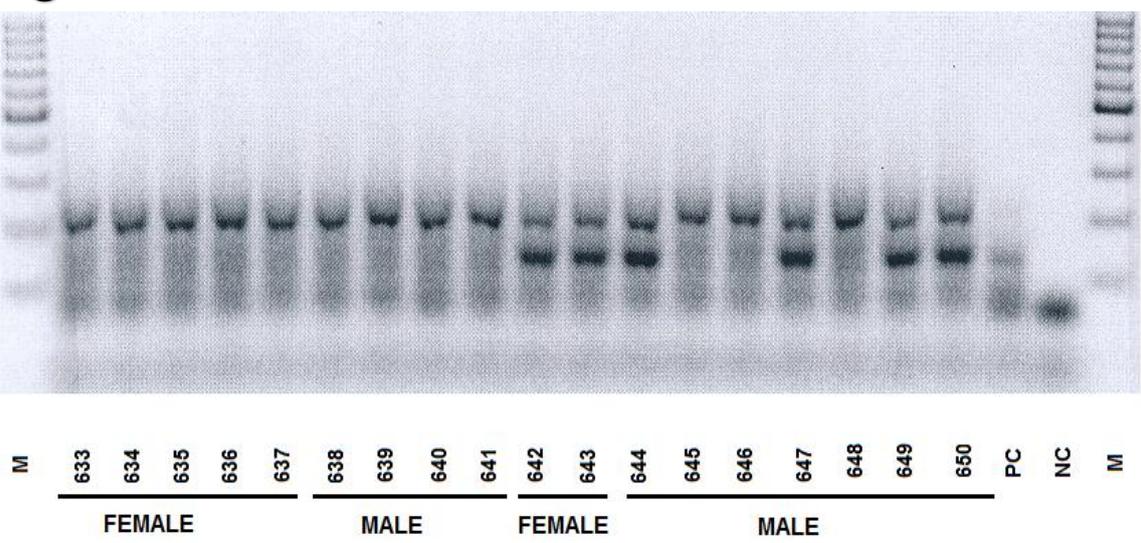
**Figure 5.5.; Detection of PyMT mice by PCR.** Tail-tips and ear-clips samples were incubated with 75µL lysis buffer at 95ºC for 1,5h to extract the DNA, cooled down at 4ºC for 20 min, neutralized with 75µL neutralization buffer and centrifuged at 14000rpm for 5min. 24µL Master mix were added to 1µL sample and PCR MHPyMT program was used in PCR machine (Appendix II). Finally 4.2µL 6X DNA loading dye were added to all samples and loaded on a 2% agarose gel, together with 10µL marker (M), positive control (PC) and negative control (NC). **A.** Samples from different mice, but just one shows a clear band that corresponds to the MT protein, IV sample shares this band with the positive control (PC). **B.** In order to ensure IV sample was positive to develop cancer, another PCR was run. This sample was loaded in several lanes and MT protein was detected in all of them. **C.** More samples from new mice were analysed, together with previous samples from the first picture (A). In this PCR is possible to ensure that mice from 633 to 641 were MT negative, while six new mice were MT positive. Two of these PyMT mice were female (642 and 643) and four were males (644, 647, 640 and 650).

The different PCRs allowed to detect PyMT mice that developed cancer, a total of six different mice. IV samples corresponds to big tumours from previous mice, IV a samples corresponds to mice without MT expression. IH and IH a samples corresponds to small tumours.

## FRα expression in tumours from PyMT mice

After finding which mice were MT positive (PyMT) their tumours were extracted and seed in T25 flasks. When tumour cells reached a high confluence, where seeded out in a 6 well plate and treated with or without PI-PLC after 5 days. As we did with previous experiments, cells were lysed and media collected. All samples were prepared to be blotted, using the FRα mouse antibody. The western blots below show the FRα expression in tumours experiment that was performed several times.
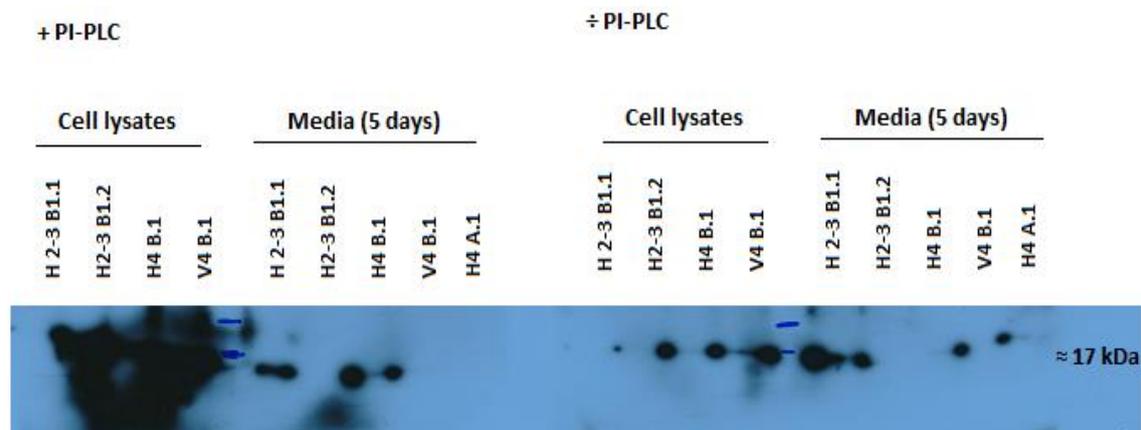


**Figure 5.6.; FRα expression in tumour cells.** Tumour cells were seeded in T25 flasks and seeded out in duplicates in 6 well plates (4mL). After 5 days, the fibroblast cells confluence was higher than the epithelial cells confluence. One of the duplicates were treated with 2µl/well PI-PLC for 30min at 37ºC. After this treatment, media was collected and centrifuged at 12000rpm for 5min and cells were clean with 1mL PBS, lysed with 200µL PONG buffer lysis and stored overnight at 4ºC. The numbers 2-3 and 4 denotes the position of the tumours: 2-3 in the middle of the stomach, 4 down from the legs, whereas the letters H and V express right and left, respectively.
Media samples and protein extracts were blotted using the FRα mouse antibody, but no bands were observed and we decided to reactivate the membranes with the same antibody. Some bands appeared after the reactivation, but their molecular weight was around 17 kDa and does not correspond to FRα mouse protein of 30 kDa.

These results were got when membranes were reactivated because no bands were visible after the western blot was performed. The bands observed in these membranes have a molecular weight of 17 kDa approximately, as we knew the murine FRα (around 30 kDa), these 17 kDa bands were dismissed.

Probably FRα bands were not detected because the level of this protein was too low. When tumour cells were seeded out in a 6 well plate and confluence was checked after 5 days, it was clear that fibroblast cells predominated in the culture, while epithelial cells were present in poor amounts. FRα protein is expressed in epithelial cells and no in fibroblasts, so this knowledge led us to think the quantity of FRα was low due to the minor concentration of epithelial cells.

## sFRα expression in serum from PyMT mice

Earlier experiments performed in our laboratory showed the effects and role of folic acid in cancer development. In one hand, a group of mice were fed a high doses of folic acid and develop cancer, big tumours were observed in most of these mice. On the other hand, a different group of mice followed a low folic acid diet and those mice that developed cancer expressed small tumours. Serum samples from all these mice were extracted and store at -80ºC for further experiments and studies.

In order to go on with this project about the sFRα the study went on working on serum samples from these mice. A total of 38 serum samples were analysed, 34 serum samples from the old mice and 4 from the new mice analysed by the PCR (IV, IVa, IH and IHa). This experiment was repeated several times, running several western blots using the FRα mouse antibody.
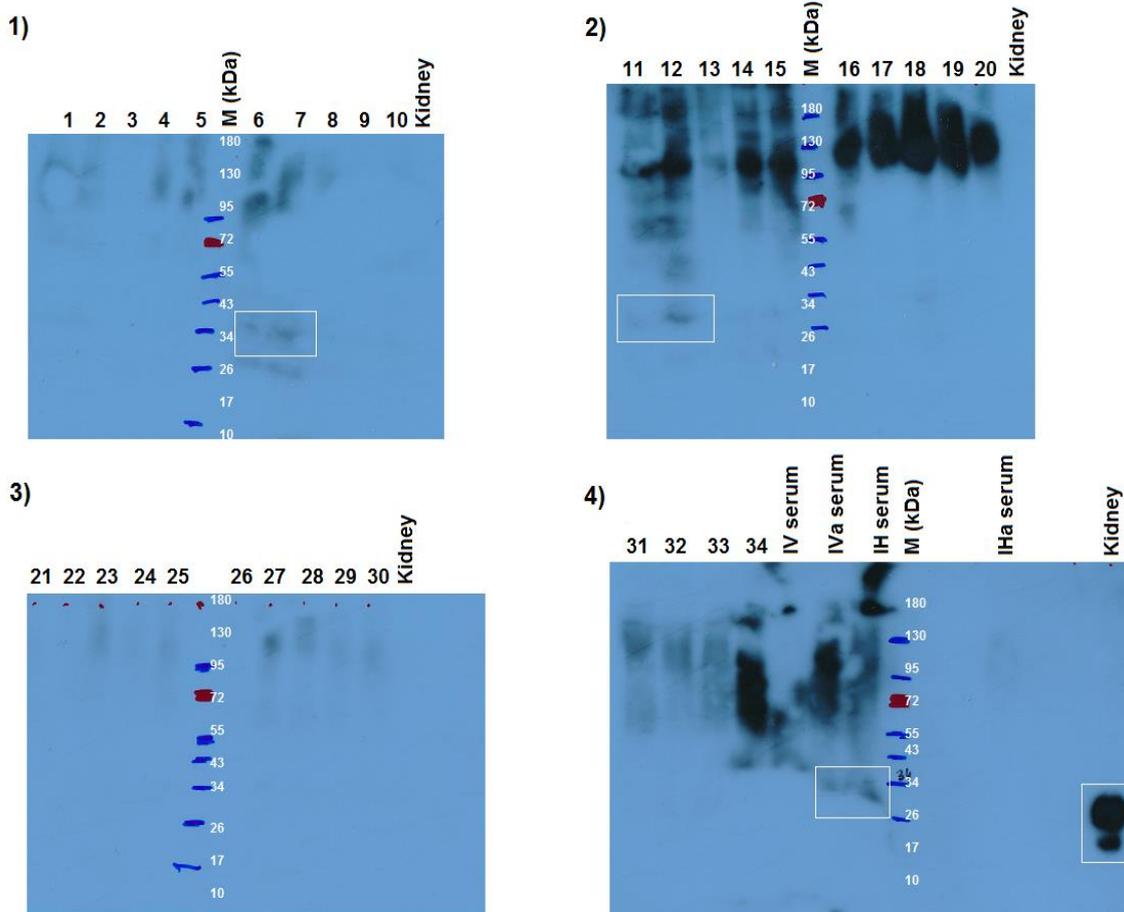
**Figure 5.7.; Serum samples from PyMT mice (A).** Serum samples were vortex before start the experiment. 15µL serum were taken, 3µL 6X sample buffer without DTT were added and mix for 5min at 95ºC. All samples were loaded in SDS-PAGE gels, 10 samples in each one plus the sample extracted from kidney that was used as positive control that express sFRα. Results from western blot show some bands at 34 kDa in 1,2 and 4 gels. The positive control (kidney) is visible just in the last western blot, a clear band at 34 kDa approximately, that corresponds to the sFRα. The rest of bands have a high molecular weight, from 180 to 55 kDa,that corresponds to proteins from hemolysis.

These results are different to what we supposed, the positive control should be expressed in all gels, instead is just expressed in the last one (4). Probably something was wrong with these gels. In gels 1, 2 and 4 is possible to observed some bands of 30kD (white box) that could be sFRα proteins. Furthermore, the clear bands observed have a high molecular weight that could correspond to protein from hemolysis, the lysis of hematopoietic cells.

As these results were confused we prepared again the samples and repeated the experiment (Figure 5.8.). Some problems were found because there was not enough volume of serum samples and some of them were over (27 and 28). About the kidney sample was completely over and it was not possible to use another sample as a positive control, so it made more difficult the analysis of results.
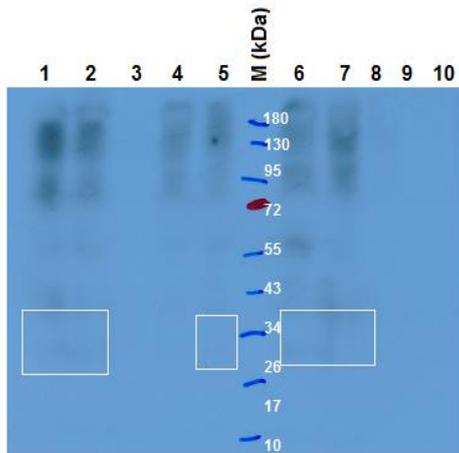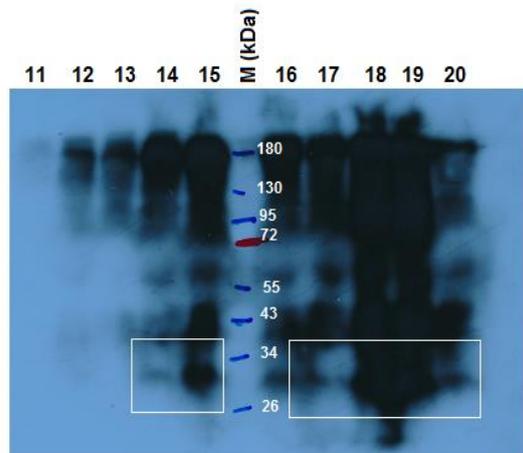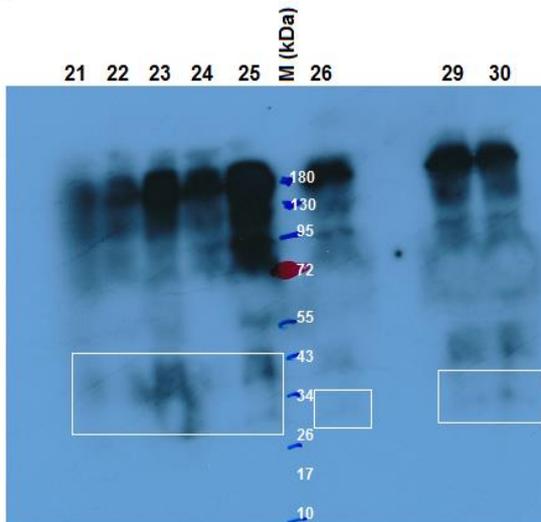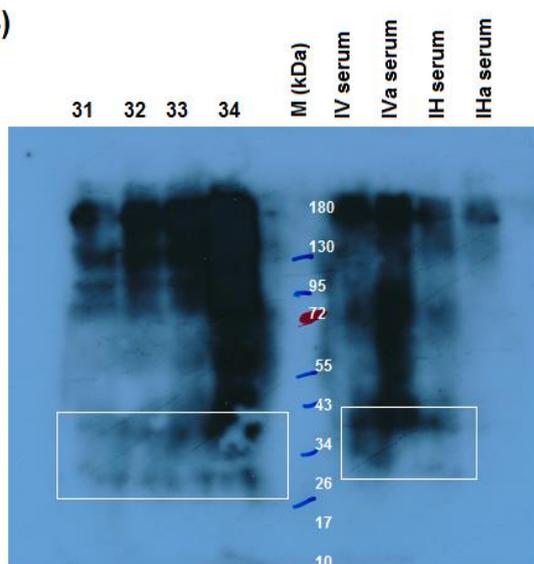
**Figure 5.8.; Serum samples from PyMT mice (B).** Samples were prepared as it is explained in Figure 5.7. Unfortunately, no positive control was loaded in these gels. In these four gels sFRα is recognized as bands of ~30 kDa (white boxes) of different intensity. Proteins that could be from hemolysis are still highly expressed in all samples, just in the first gel the intensity of these bands is lower.

The different amount of samples loaded could be an explanation of several intensities from sFRα bands, as it is mentioned above some serum samples were over and other were insufficient, less than 15μL. It supposes a difficulty, we could established the presence of sFRα in almost all serum samples, but its expression levels are not clear. The lack of a positive control makes even harder the interpretation of results because it is not possible compare the size from bands.

If we focus on the last four samples (IV, IVa, IH and IHa) is possible to observed bands of ~30 kDa in IV and IH samples, big and small tumours, respectively. In contrast, IHa sample does not express the protein, because is a sFRα negative serum sample.

# VI.    Discussion

The aim of this study was to understand the role of soluble folate receptor alpha (sFRα) in cancer development. It was found in previous studies that STAT3 oncogene is activated by folic acid via FRα, but new findings show the importance of the soluble form of this protein, sFRα. This soluble receptor is able to activate cell signaling independently of its membrane-bound counterpart, it is involved in transsignaling process and widely expressed in several cancers, and this is the reason why it has been studied in this project.

In order to clarify the role of folic acid in cancer, we performed the STAT3 activation experiment which is based on working with different concentration of folic acid to demonstrate its direct involvement. This dose experiment clearly shows the constituvely expression of STAT3 oncogene, but we want to show the folic acid effect at different concentration levels. It is clear, using a new folic acid the activation to pSTAT3 is higher than using the old one. As it has been mentioned, this experiment was repeated several times because results obtained did not correspond to explanations. In Figure 5.1.A is possible to observe a higher expression of pSTAT3 when 250 μg/mL folic acid were added to the medium than the activation obtained with 500μg/mL folic acid. To solve these problems we stripped some membranes and got better results shown in Figure 5.1.B, new folic acid and higher concentrations induce a stronger activation of STAT3. In contrast, the negative controls were expressed in this last experiment, something unexpected. All the problems mentioned are explained by different factors: cell stress from medium changes, serum starvation and distortion in pH balance of the folic acid solutions.

The widely expression of FRα in different types of cancer makes this protein the key of the study and to understand and confirm its high expression I performed several times an experiment with six different human cell lines. From these cell lines, just one does not express the protein, HEK293, and the rest express it at different levels. HeLa and KB cells express the FRα in high concentrations, while mutants from HeLa show levels unexpected. HeLa FRα should has more protein, overexpression, than HeLa pcDNA3, a control. Every time the experiment was repeated the FRα concentration in HeLa FRα was lower than in HeLa pcDNA3, so something was wrong in the cell line with overexpression of the protein. At this point, when it was sure all cell lines, except HEK293, express the protein bound to membrane at different levels, the objective was to study the concentration of its soluble form in the same cell lines. Data may suggest that sFRα concentration is really low in the media to be detected, so it is necessary to performed another step before it is recognized, an acetone precipitation. After concentrate the most amount of soluble protein it appears, strongly expressed in KB and it correlates to the results from its membrane-bound counterpart. Some problems with the Mov18 antibody made difficult to get the expected results, and some of the cell lines did not express the protein as they should, but it is possible to confirm the presence of the sFRα in HeLa, KB (higher expression), HeLa pcDNA3, HeLa FRα and HeLa pcDNA3 FRα D103A (this mutation does not change the concentration of the protein, just the affinity to bind folic acid).

In order to increase the concentration of sFRα in the medium, an activator of phospholipase C was added, PI-PLC. To demonstrate that phospholipase C cleaves the GPI-anchor between the protein and the cell membrane, same cell lines were incubated with PI-PLC. Western blots do not show bands from cell lysates, so no FRα proteins were bound to membranes, all of them were released. Therefore, the sFRα should be expressed at high levels. In contrast, when media was analysed and blotted no proteins were found, so we supposed it was because the Mov18 antibody did not work. At this time several colleagues of the laboratory were using the same antibody and did not get any results, which could be the reason to explain the absence of results.

Another part of this project is the study of FRα and sFRα in mice breast model cancer, which present the MT protein that develops cancer, PyMT mice. Earlier studies in our laboratory shown that folic acid has an effect in cancer development and the size of tumours depends on folic acid levels. The high murine FRα expression in PyMT breast tumours contributes to tumour development. Mice that followed the high folic acid diet generated big tumours, whereas tumours from mice with low folic acid diet were small. Moreover the larger tumours expressed higher levels of STAT3 protein. Once this study was performed, serum from these mice was stored at -80°C for further experiments related to sFRα, the experiments that were ran later. Before starting to explain the serum samples experiment and correlate the presence and concentration of sFRα with FRα levels, is important to interpret results obtain from a previous experiment.

A new group of mice, that followed a high or low folic acid diet, were analysed to find out which of them develop cancer. After DNA extraction, the PCR results reveal which mice were PyMT and tumours from one of them were extracted to generate a culture and study the FRα expression. Tumours were extracted from different parts: down from the legs and in the middle of the stomach. Unfortunately, western blot results show some bands with a molecular weight of 17 kDa approximately and they do not correspond to murine FRα, which has a molecular weight around 30 kDa. No FRα proteins were observed probably, because tumours overexpressed fibroblast cells and epithelial cells population was poorly expressed. Epithelial cells are the ones that express the protein and due to its low concentration no FRα bands were visible in western blot membranes.
This kind of tumour cell cultures were generated for the first time in our laboratory, maybe the conditions of growth were not suitable and fibroblast population predominated over epithelial cells. For further experiments new conditions will be settled.

Murine serum samples from old experiments ran at our laboratory were analysed looking for the expression of FRα soluble form considering that the same protein bound to cell membranes was found in these mice. Results achieved from the first experiment (Figure 5.7) do not correspond to sFRα protein because the size of bands are different from the size of the positive control (kidney). It is possible to observe only six bands that have the same size as the positive control, thus six serum samples that express the protein. Therefore, this experiment was repeated and new results were obtained (Figure 5.8), almost all samples express the protein but the intensity of bands is completely different. As there is no positive control loaded on these gels I cannot confirm the

bands around 30kDa are sFRα proteins. Assuming that bands which size is 30kDa approximately and compared to positive control band from Figure 5.7, I can explain a new problem detected. The different intensity from bands is due to diverse amount of serum loaded in gels. Some samples were over and other had less than 15μL (quantity loaded in each well), thus these results just allow to confirm the presence of sFRα in serum but it is not possible to understand its expression levels in serum, it is mean the concentration of this protein in serum is unknown.

Lack of samples made impossible to repeat the experiment again but the intention before starting this study was to compare the effect of folic acid in cancer development. In order to do this comparison, it is needed to observe results achieved from old experiments. These results show that mice following a high folic acid diet developed larger tumours than those that were fed a low folic acid diet. Furthermore, larger tumours expressed more FRα proteins, thus the objective was to investigate if levels of this protein are higher in serum too. As I have explained is not possible to do this comparison, because results are not quantitative and lack of serum samples together with lack of time made impossible to finish this study.

# VII. Conclusions

After all experiments ran in our laboratory, it is possible to draw some conclusions about FRα and cancer development. First of all, STAT3 experiments seems to explain the folic acid effect on cells, it means, high doses of folic acid (similar to levels from food fortification) increase STAT3 oncogene activation via FRα pathway. Moreover cancer cells overexpress FRα proteins, which can be found as GPI-anchored proteins bound to membranes or as a soluble proteins in medium which are found at lower concentration. The soluble form of this protein (sFRα) is being widely studied because of its ability to activate receptor transsignaling and therefore, to induce STAT3 gene transcription.

Preceding studies shown that a high folic acid diet increased tumour volume in PyMT mice, and these larger tumours expressed higher levels of STAT3 protein. Furthermore, it was found PyMT tumours have a high FRα expression and suggests that FRα may contribute to tumour development. In addition, PyMT serum has been analysed and it seems that sFRα is present, but at unknown concentrations. Further experiments will answer this question, is there higher sFRα levels at serum when tumours are larger?

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# Appendices

## Appendix I. Buffers and gels
### Buffers

| PAGE buffer | PBS (PBS-T) | TBS (TBS-T) | Wet transfer buffer |
|---|---|---|---|
| 25mM Tris base | 137mM NaCl | 150mM NaCl | 26mM Tris base |
| 0,25M glycine | 2,7mM KCl | 50mM Tris | 190mM glycine |
| 0,1% SDS | 10mM $Na_2HPO_4$ | pH 7,6 | 20% ethanol |
| | 1,8Mm $KH_2PO_4$ | (0,5% Tween-20) | |
| | (0,5% Tween-20) | | |

| 6X sample buffer | PBS (for cell culture) | Sriuranpong (PONG) lysis buffer |
|---|---|---|
| 350mM Tris-HCl (pH 6,8) | 150mM NaCl | 62,5mM Tris-HCl (pH 7,5) |
| 30% glycerol | 1,5mM $KH_2PO_4$ | 2% SDS |
| 10% DTT | 6,5mM $Na_2HPO_4$ | 10% glycerol |
| 10% SDS | pH 7,2 | 1µL/ml protease inhibitor (*Sigma*) |
| | | 1µL/ml phosphatase inhibitor (*Sigma*) |

| TBE buffer (10X) |
|---|
| 1,0M Tris |
| 0,9M Boric Acid |
| 0,01M EDTA |
| pH 8,4 |

### Gels

| 10% SDS-PAGE | |
|---|---|
| **Separation gel** | **Stacking gel** |
| 10% Protogel (acrylamide/bisacrylamide) | 5% Protogel (acrylamide/bisacrylamide) |
| 0,38M Tris pH8.8 | 0,2M pH 6.8 |
| 0,1% SDS | 0,1% SDS |
| 0.07% TEMED | 0.12% TEMED |
| 0.07% APS | 0.12% APS |
| | Methylene blue |

| 2% Agarose gel |
| --- |
| 4 spoons of agarose |
| 200mL 1X TBE |
| 10µL Gel Red |

## Appendix II . PCR:   Buffers, primers, PCR reaction mix and PCR program

### Buffers for PCR

| Lysis buffer | Neutralization buffer |
| --- | --- |
| 10mL ddH$_2$O | Stock: 1M Tris pH 5.0 |
| 250µL 1M NaOH | |
| 4µL 0.5M EDTA pH 8.0 | |

### Primers for PCR

| Primer name | Primer sequences |
| --- | --- |
| PyMT-3P | CGC CGC AGC GAG GAA CTG AGG AGA G |
| PyMT-4m | TCA GAA GAC TCG GCA GTC TTA GGC G |
| Plg-in2-3' | TGT GGG CTC TAA AGA TGG AAC TCC |
| Plg-ex2-5' | GAC AAG GGG ACT CGC TGG ATG GCT A |

### PCR MFH PyMT program and PCR Mastermix

| Step | Temperature (ºC) | Time | Comment |
| --- | --- | --- | --- |
| 1 | 94 | 45s | Initial denaturation |
| 2 | 58 | 45s | Denaturation |
| 3 | 72 | 45s | Annealing |
| 4 | Go to step 1-3, 35 times | | |
| 5 | 72 | 10min | Elongation |
| 6 | 4 | "forever" | Final elongation |

| PCR Mastermix | 1X |
| --- | --- |
| Sterile ddH$_2$O | 8.5µL |
| MgCl$_2$ (50Mm) | 2.5µL |
| 10X PCR buffer | 2.5µL |
| dNTP mix 100mM | 2µL |
| Primers x4 | 0.2µL (x4) |
| Taq pol. | 0.2µL |
| Betaine 4M | 6.25µL |
| DMSO 5% | 1.25µL |

## Appendix III. Antibodies for western blot

| Primary antibody | Bands detected | Origin | Dilution | Buffer for primary antibody | Wash buffer | Secondary antibody |
|---|---|---|---|---|---|---|
| STAT3 (*Cell Signaling*) | 79/86 kDa | Rabbit | 1:5000 | 5% BSA in TBS-T | TBS-T | Goat anti-Rabbit IgG/HRP (*GaR*) (*Dako*) 1:5000 |
| pSTAT3 (*Cell Signaling*) | 79/86 kDa | Rabbit | 1:2000 | 5% BSA in TBS-T | TBS-T | (GaR) (*Cell signaling*) 1:1000 |
| Mov18 (Anti-FRα) (*Enzo Life-Sciences*) | 38 kDa | Murine | 1:2000 | 5% skimmed milk powder in PBS-T (MPBS-T) | PBS-T | Sheep Anti-Mouse Antibody (SaM) 1:5000 (*Amersham Biosciences*) |
| α-tubulin (*Sigma*) | 50 kDa | Murine | 1:20000 | 5% MPBS-T | PBS-T | SaM 1:5000 |

## Appendix IV. Starvation medium (FFRPMI)

**Composition**

$$1{,}5mL\ RPMI \cdot 12\ wells = 18mL \approx 20mL\ RPMI$$

Supplemented with 10% cFBS

$$\frac{20}{100}mL\ RPMI \cdot \frac{100}{10}mL\ cFBS = 2mL\ cFBS$$

$$20mL\ RPMI + 2mL\ 10\%\ cFBS = 22mL\ FFRPMI$$

## Appendix V. Folic acid stock and calculations

| Folic Acid | Date | Company |
|---|---|---|
| **Old folic acid** | 25.05.12 | SIGMA-ADRICH |
| **New folic acid** | 19.01.15 | SIGMA-ADRICH |
| **\*Folinic acid calcium hydrate** | 10.01.15 | SIGMA-ADRICH |

Calculations

Preparation of FA solutions stock:

2,5 mg FA, dissolved in 1M NaOH to a concentration of 50mg/mL

Calculations to prepare FA solutions dissolved to a concentration of 250µg/mL and 500µg/mL:

$$Concentration_{old} \cdot Volume_{old} = Concentration_{new} \cdot Volume_{new}$$

   a.  *Concentration of 250µg/mL*

$2,5\dfrac{mg}{mL} \cdot x = 250\dfrac{\mu g}{mL} \cdot 500\mu L$

$x = 50\mu L \ FA \ solution$


   b.  *Concentration of 500µg/mL*

$2,5\dfrac{mg}{mL} \cdot x = 500\dfrac{\mu g}{mL} \cdot 500\mu L$

$x = 100\mu L$ FA solution


FA solutions were dissolved in 50µL NaOH * and 450µL FFRPMI.
*Folinic acid was dissolved in 50µL ddH$_2$O.