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Role of STAT3 signaling pathway in oncogenesis and cancer therapy

Úloha signální dráhy STAT3 v nádorovém bujení a terapii rakoviny

Bachelor Thesis

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Dedication:

Chtěla bych touto cestou poděkovat svému školiteli, MUDr. Zdeňkovi Hodnému, CSc., za trpělivou a ochotnou pomoc při vedení mé bakalářské práce. Zároveň také děkuji celému laboratornímu týmu za vlídné přijetí do jejich kolektivu. A na závěr, ale ne naposledy, bych chtěla poděkovat svým rodičům a nejbližším přátelům, kteří mě podporovali a stáli při mě NEJEN po celou dobu psaní této bakalářské práce a doufám, že tomu tak bude i nadále. Nebýt mých rodičů, nebyla bych dnes tam, kde právě jsem.

Statement:

I declare I am the only author of this Thesis. I have submitted references of all sources I have used for creation of this Thesis. This Thesis nor any significant part of it were used for obtaining any other academic degree.

In Prague, 14th of May

Abstract

STAT3 (Signal Transducer and Activator of Transcription 3) is considered to be one of the possible targets of cancer treatment. The ability of STAT3 constitutive activation to form tumors is a foundation of such theories. Additionally, constitutively activated STAT3 is present in many types of cancer with high occurrence, such as breast and prostate carcinoma. This protein is required in normal body cells as well. STAT3 is a transcription factor targeting many genes that are essential for the cell. STAT3 is activated by phosphorylation of its tyrosine residue and homodimerization. Proteins transcribed with help of STAT3 function in cell cycle progression, cell growth, replication, negative regulation of apoptosis, and other roles, typical for cancer. Moreover, STAT3 is modulating mitochondrial function and maintaining ROS production in mitochondria, but in form of transcriptionally inactive monomers. The purpose of this Thesis is to review known data about STAT3 in oncogenesis and by that, to show STAT3 has great potential to become the target of cancer treatment. This Thesis contains a short overview of known STAT3 inhibitors as well.

Key words:

Signal Transducer and Activator of Transcription 3 (STAT3), JAK/STAT3 pathway, constitutive activation, cancer, tumor, inhibitor, mitochondria, apoptosis

Abstrakt

Velká pozornost je v dnešní době upírána na STAT3 (Signální transduktor a aktivátor transkripce 3) jako na možný cíl terapie rakoviny. Je dokázáno, že STAT3 je konstitutivně aktivován u mnoha typů rakoviny, jako je rakovina prsu či prostaty, a samotná tato konstitutivní aktivace stačí k rozvinutí nádorového bujení. Tento protein funguje jako transkripční faktor. Je aktivován fosforylací tyrosinu a následnou homodimerizací. Role STAT3 je nezastupitelná i v nenádorových buňkách; je transkripčním faktorem genů, jejichž proteinové přepisy se podílí na progresi buněčného cyklu, růstu a replikaci buňky, rovnováze mezi apoptotickými a anti-apoptotickými proteiny a dalších funkcích. Poměrně nově objevenou rolí STAT3 je modulace chodu mitochondrií a ochrana buňky před tvorbou kyslíkových radikálů. Tato bakalářská práce má za cíl shrnout podporující údaje, že STAT3 je perspektivním cílem nádorové terapie, a to zaměřením se na funkci tohoto proteinu v aspektech typických pro nádorové buňky. V této bakalářské práci je obsažena i kapitola věnovaná pokroku, kterého bylo dosaženo ve snaze vytvořit ideální STAT3 inhibitor.

Klíčová slova:

Signální transduktor a aktivátor transkripce 3 (STAT3), signální dráha JAK/STAT3, konstitutivní aktivace, rakovina, tumor, inhibitor, mitochondrie, apoptóza

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Introduction

Cancer belongs to the most frequent causes of death and as such, principles of the transformation from normal cell to malignant one are studied with utmost efforts. Researchers are trying to determine how cancer occurs for decades and I believe we reached quite high level of understanding this disease. However, logically, with more data known, whole issue is becoming more and more complex. Many scientists study specific types of cancer, their characteristic signs and then come up with new treatment strategies for them. I see a problem in this approach because I think we should focus more on general characteristics of cancer to find solution working for at least most common types, in need of only minor modifications.

These days, such hopes may be directed towards STAT3 protein which has its role in fundamental functions of the cell. STAT3 is constitutively activated in most tumors and it is very important that tumors with high occurrence are among them, for example tumors of lungs (Yeh, H. H. et al., 2006), breast (Garcia, R. et al., 1997), prostate (Mora, L. B. et al., 2002), and myeloid leukemia (Steensma, D. P. et al., 2006) (as indicated in Surveillance Research done by American Cancer Society, Inc in year 2012).

As a general outline of this Thesis, I utilize main hallmarks of cancer as defined by D. Hanahan and R. A. Weinberg (Hanahan, D., Weinberg, R. A., 2000), (Hanahan, D., Weinberg, R. A., 2011) to demonstrate functional contribution of this potent oncogene to characteristic features probably every cancer cell has, with focus on tumors of breast and myeloid leukemia. Moreover, this Thesis involves chapter dedicated to the known inhibitors of STAT3 that can be used for therapy treatments or for development of new effective drugs.

1 STAT family (Signal transducers and activators of transcription)

Existence of STAT proteins was discovered during studies of interferon receptor signaling (Larner, A. C. et al., 1984). To this date, we know seven ‘standard’ STAT family members in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5 and STAT6; moreover, some of them have various forms emerging from alternative splicing (Azam, M. et al., 1995), (Nielsen, M. et al., 1997).

They are important participants in vital processes of body, like cell proliferation, differentiation, anti-inflammatory responses (Matsukawa, A. et al., 2003), apoptosis, and cancer formation (as mentioned below).

STATs are very uniform in their structure and their DNA-binding domains recognize very similar sequences, a consensus with two adenine bases on 5´ terminus, two thymine bases on 3´ terminus, and there are four to six various bases between them (5´AAXXXX(X)(X)TT3´) (Seidel, H. M. et al., 1995). Despite that, every STAT has different function and regulates different set of genes. For example, STAT3 is vital in embryo and no other STAT can compensate for its absence (Takeda, K. et al., 1997). They also may act antagonistically towards each other. STAT1 is a pro-apoptotic factor (Janjua, S. et al., 2002) while STAT3 is generally counteracting apoptosis (Aoki, Y. et al., 2003).

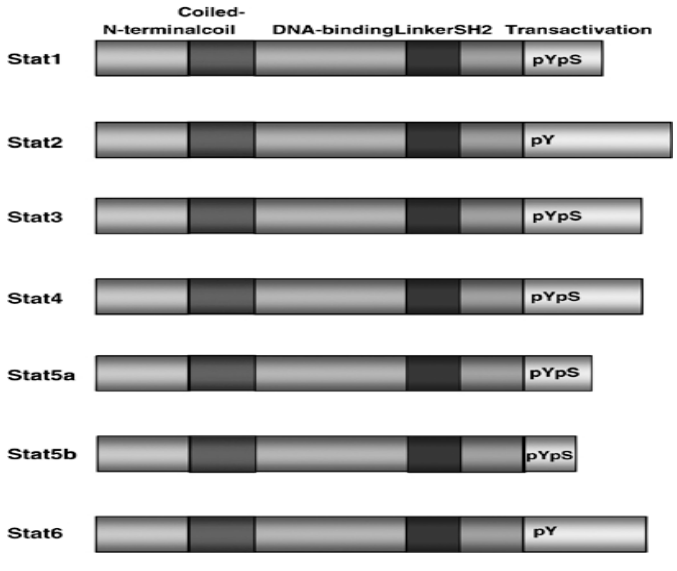

Figure 1. Organization of domains in STAT proteins. There is N-terminal domain significant for STAT3-STAT3 interaction, coiled-coil which is a structural motif used often in transcription factors, specific DNA-binding domain, and SH2 (Src homology 2) domain that binds phosphorylated tyrosine residue and leads to dimerization and thus activation. C-terminal transactivation domain is where activating tyrosine and serine residues are located (Baker, S. J. et al., 2007).

2 STAT3

STAT3 was first noticed as a transcription factor of acute phase genes in hepatocytes and thus was named Acute Phase Response Factor (Wegenka, U. M. et al., 1993). Later on, STAT3 was associated with several more functions; some of them will be described later. Additionally, it was found STAT3 is essential for survival of mammals. Embryos lacking STAT3 are just not viable. It has been speculated embryonic lethality of STAT3 knock-out mice is caused by starvation because results showed that mRNA coding STAT3 is expressed in both maternal tissue and embryonic visceral endoderm of STAT3 wild-type individuals, which is in charge of nourishment transport to the embryo (Takeda, K . et al., 1997) .In mature tissues, for example in epidermis, STAT3 affects keratinocyte migration and through that wound healing. It also has a role in second and later hair cycle (Sano, S. et al., 1999). Its other functions in different tissues can be seen in Figure 2.

Target cells	Phenotypes	Affected functions
Keratinocytes	Impaired 2nd hair cycle, keratinocyte migration, wound repair	Migration
Thymic epithelium	Age-dependent thymic hypoplasia, hypersensitivity to stress	Survival
T lymphocytes	Impaired IL-6–dependent survival, impaired IL-2Rα expression	Survival /proliferation
Monocytes/ Neutrophils	Enhanced inflammatory response, chronic colitis and Th1 differentiation	–
Mammary epithelium	Delayed mammary involution	Apoptosis
Liver	Impaired acute-phase response	Gene expression
Sensory neurons	Enhanced neuronal apoptosis	Survival
Motoneurons	Impaired survival after nerve damage	Survival (cytokine-stimulated)

Figure 2. STAT3 functions in specific tissues and effects of STAT3 missing in them (Levy, D. E., Lee, C., 2002).

STAT3 has wide sphere of competence; it affects activities in one cell that are counteractive to the ones in other cells, also mediated by STAT3. It can induce both inhibition

of myoblasts differentiation (Kataoka, Y. et al., 2003) and cardiomyocytes differentiation (Snyder, M. et al., 2010).

Very discussed topic is also a role of STAT3 in cancer. Constant presence of activated STAT3 induces oncogenic transformation and tumorigenesis (Bromberg, J. F. et al., 1999). Many researchers are interested in mechanisms of this phenomenon in hope to find a treatment to cancer.

STAT3 protein was found even in mitochondria (Wegrzyn, et al. et al., 2009), therefore it is supposed that it has to be transported there. STAT3 functions in protection against apoptosis and free radical production (Negoro et al., 2001; Oshima et al., 2005)(Oshima, Y. et al., 2005) so its location in mitochondria is not surprising.

Proteins having their expression regulated by STAT3 are Survivin, Mcl-1, HSP27, Adrenomedullin, and Bcl-xL, Bcl-2, c-Fos, MEK5, and c-Myc, VEGF, COX-2, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-10 and several others (Hsieh, F. C. et al., 2005) (Yuan, G. et al., 2008) (Senft, C. et al., 2010).

2.1 Activation of STAT3

STAT3 is activated by various stimuli, mostly by cytokines and many growth factors, for example IL-6 (interleukin 6), EGF (epithelial growth factor) (Zhong, Z. et al., 1994), IGF (insulin-like growth factor) (Zong, C.S. et al., 2000) and so on. The process can operate through membrane receptors with kinase activity or non-receptor tyrosine kinases, such as Src (Sarcoma kinase) or Abl (Abelson leukemia protein). JAKs, Janus kinases, is family of receptor tyrosine kinases. In mammals, they comprise of Jak1, Jak2, Jak3 and Tyk2.

2.1.1 Canonical pathway

STAT3 was discovered as Acute Phase Response Factor activated via gp130, common transmembrane subunit of IL-6 receptor family (Wegenka, et al. et al., 1993). Since then, many kinases with the ability to activate STAT3 were identified but for this historical reason, I chose to describe STAT3 phosphorylation via IL-6 receptor as representative of IL-6 family.

The receptor contains gp130 subunit and other subunit fundamental for recognition of specific cytokine. In IL-6 receptor, it is gp80 (glycoprotein 80) (Kurotani, R. et al., 2001). It has been proved that homodimerization of two gp130 subunits in case of IL-6 receptor (Murakami, M. et al., 1993) or heterodimerization of gp130 with gp130-like subunit in the other receptors (Davis, S. et al, 1993) follows stimulation of the receptor. gp130 subunits

autophosphorylate ots own tyrosine residues, and that forms docking site for proteins with SH2 domain, for example STAT3. Tyrosine residues with the highest affinity toward STAT3 are Y905 and Y915, then Y767 and the one with weakest affinity toward STAT3 is Y814. Y759 functions as negative regulation site and attract SHP2 and SOCS3.(Lehmann, U. et al., 2006). Then gp130-associated tyrosine kinase, one member of JAK, is phosphorylated and phosphorylates STAT3 on Y705a hen STAT3 is phosphorylated, connection between kinase and protein is dissolved. Two STATs (complextcan bonsist of different tTAT members) (Schulze, H. et al, 1999) reciprocally hold on phosphorylated tyrosine residue of the other one by SH2 domain.

Nuclear import signal for STAT3 is in its coiled-coil domain and thus the transfer does not depend on phosphorylation or dimerization of STAT3 (Liu, L. et al., 2005). The transfer occurs continuously and happens via MgcRacGAP being NLS-containing (nuclear localization signal) nuclear chaperone of STAT3. MgcRacGAP is a protein bound to GTPases; it makes hydrolysis of their GTP faster. MgcRacGAP binds STAT3 and via its nuclear localization signal importin-α and importin-β. GTP-bound Rac1 is bound to this complex as well. In the nucleus; MgcRacGAP catalyzes GTP hydrolysis od GTP-bound Rac1 which causes dissasembly of importins and the interaction of STAT3 to MgcRacGAP (Kawashima, T. et al., 2009).

Effectivity of STAT3 can be increased by phosphorylation of serine residue within transactivation domain. (Wen, Z. et al., 1995) When S727 is phosphorylated, trans-activation domain of STAT associates with transcriptional co-activators, for example with NcoA/SRC1a. Transcriptional co-activators have histone acetyltransferase activity regulating the expression of genes via decompressing DNA (Giraud et al, 2002). Acetylation of STAT3 lysine residue K685 plays a role in STAT3 activation as well, it is critical for dimerization of STAT3 (Yuan, Z. L. et al., 2005). Under normal circumstances, STATs signalization is quite rapid and transient, lasting from half an hour to several hours (Yang, J. et al., 2005).

2.1.2 Non-canonical pathway

Non-canonical pathway is another way STAT3 can affect expression of certain genes. It has been determined that activated dimers of STAT3 regulate beside expression of specific genes also expression of STAT3 gene itself (Narimatsu, M. et al, 2001). After fast response to stimuli via activated dimers of STAT3, which happens from half an hour to 4 hours with maximal peak between 4 and 8 hours, other part of reaction occurs. Amount of unphospohorylated STAT3 greatly increases and reaches its maximum level 16 to 32 hours

after stimuli, usually around 24 times (Yang, J. et al., 2005). Unphosphorylated STAT3 probably locates to the nucleus because there are genes in the cell responding to the unpohosphorylated STAT3 as to their transcription factor, for example cdc2 and cyclin B1 mras (Yang, J. et al., 2005). Plausible idea is also that it is interacting with other transcription factors, such as Smad-1 (Nakashima, K. et al, 1999), in form of heterodimers (Yang, J. et al., 2005).

Of course, unphosphorylated STAT3 can be found in cell at all times, just not in such quantity; its level is stable. But until there is an appropriate stimuli, it cannot regulate transcription of genes requiring its dimer form (Yang, J. et al., 2005).

2.2 Inhibition of STAT3

Like nearly every pathway, also STAT3 has negative regulation of its signaling in addition to positive one. For STAT3, there are at least 3 mechanisms.

The first one attenuates signalization through dephosphorylation of gp130, tyrosine kinases or STAT3 itself. The responsible tyrosine phosphatases are for example SHP1, SHP2 and TC-PTP with its nuclear form TC45 (Kim, D. J. et al., 2010).

Function counteracting to STAT3 has also PIAS3 (Protein Inhibitor of Activated STAT3) which belongs to the PIAS family. There are 5 members of PIAS family and they generally inhibit function of STATs. PIAS3 makes complex with STAT3 specifically, probably dephosphorylates Y705 residue within STAT3 and interfere with STAT3 DNA-binding domain. Both its nuclear location and binding to STAT3 is dependent on phosphorylation of STAT3 Y705 (Dabir, S. et al., 2009). PIAS1 inhibits STAT1-STAT3 dimers via their DNA-binding activity (Diao, Y. et al., 2009).SOCSs (Suppressors Of Cytokine Signaling) are a third way of attenuating STAT3 signalization. SOCS-1 attaches itself to JAK and inhibits its activity; SOCS-3 binds to the gp130 with the same purpose (Diao, Y. et al., 2009). GRIM-19 protein is also negatively regulating STAT3 signaling by prohibiting STAT3 translocation to the nucleus (Lufei, C. C. et al., 2003).

3 The role of STAT3 in cancer

In this part of Thesis, I am going to analyze whether STAT3 plays a significant role in oncogenesis and whether is as a promising target in the treatment of cancer.

D. Hanahan and R. A. Weinberg proposed a very plausible generalization that says most, if not all, types of cancer share some basic features (hallmarks). Here, I want to summarize the functional contribution of STAT3 to the main hallmarks of cancer.

"We suggest that the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. " (Hanahan, D., Weinberg, R. A., 2000)

These hallmarks of cancer cell were extended in their following article. *"Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list - reprogramming of energy metabolism and evading immune destruction."* (Hanahan, D., Weinberg, R. A. et al., 2011)

In following pages, I am going to concentrate on STAT3 role in these listed features to show STAT3 is very important strong oncogene and thus may be used as a target in cancer treatments with high efficiency.

3.1 STAT3 constitutive activation

STAT3 was found constitutively activated in many types of cancer, for example in breast carcinoma (Garcia, R. et al., 1997), and myeloid leukemia, both chronic and acute (Steensma, D. P. et al., 2006) (Coppo, P. et al, 2006). STAT3 constitutive activation can be reached in many ways and probably not all of them were found yet.

A possible cause of such activation can be, for example via ligand-independent constitutively active gp130 mutant. Several variants of this protein, along with the normal one, were discovered in inflammatory hepatocellular adenomas. Majority of altered gp130 receptors had small in-frame deletions within the cytokine binding site. These receptors can undergo dimerization resulting in STAT3 activation independently on ligand stimulation. They can form complexes with non-mutated gp130 as well. (Sommer, J. et al., 2012)

STAT3 can be constitutively activated through constant stimulation of IL-6 receptor as well. Monocytes within liver cancer creates ideal tumor microenvironment via consistent

production of IL-6. Hepatocellular carcinoma (HCC) cells are constantly stimulated by IL-6 and that way STAT3 is constitutively activated. HCC cells become addicted to IL-6/STAT3 pathway. (Wu, W. Y. et al., 2011) The cause for IL-6 dependency is, perhaps, irreversible metabolism switch of the cell to aerobic glycolysis. Without STAT3, the cell cannot produce required enzymes and becomes apoptotic. (Demaria, M. et al., 2010)

In chronic myeloid leukemia cells, phosphorylation of STAT3 via Jak2 and Bcr-Abl was observed. One of the hypotheses is that Bcr-Abl constitutively but weakly phosphorylates Tyr705 through JAK. Coppo et al. suggested Tyr705 phosphorylation can be greatly increased by receptor inducement of JAK activity, for example by G-CSF receptor stimulation. It was showed by Nakamura et al. that MEKK1 is required pathway as well. (Nakamura et al., 2005) Neverthless, the possibility of other kinases taking part in STAT3 phosphorylation is not ruled out. Activated STAT3 is up-regulating expression of STAT3 which cause positive loop regulation; constitutive phosphorylation of Tyr705 residue keeps the level of phosphorylated Tyr705 stable and thus maintains constitutive expression of this protein (Coppo, P. et al, 2006).

Additionally, it has been reported MgcRacGAP is needed for activation of STAT3 by IL-6 dependent pathway. STAT3 and Rac1/Rac2 binds to MgcRacGAP, even more so under IL-6 stimulation. Overexpression of MgcRacGAP makes cells hypersensitive to IL-6 stimulation and thus is also increasing transcription of genes regulated by STAT3. Yukio Tonozuka et al. propose a hypothesis MgcRacGAP may act as an effector in Rac-STAT3. B its function is still unclear and even Rac-STAT3 signalization is not completely understood (Tonozuka, Y. et al., 2004). While there are doubts about the way Rho GTPases interact with STAT3, it was proved RhoA, Rac1 and Cdc42 are able to activate STAT3 (Debidda, M. et al., 2005).

Based on diversity of presented data, the manner in which STAT3 is constitutively activated depends on many factors and it is a great example of how adaptive tumor cells are.

3.2 Unceased proliferation and unlimited replication

Unceased proliferation is probably the most characteristic feature of cancer. Hayflick and Moorhead stated there is a limit of dividing one cell can sustain. This limit is around 50 population doublings and it is called Hayflick limit (Hayflick, L. Moorhead, P. S., 1961). This regulation of replication may be caused by shortening of telomeres. Blackburn, Greider and Szostak, winners of Nobel Prize in Physiology or Medicine in year 2009, suggested telomeres shorten with each replication and there is a minimal limit how telomeres have to be long to

survive. Unlimited replication is crucial for chronic proliferation. Enzyme telomerase is increasing number of these terminal sequences and maintaining to keep cell able of proliferation.

In the cell, there is complex of cyclin E and Cdk2 (cyclin dependent kinase 2) that is responsible for transition from G1 to S phase of cell cycle. This complex is inhibited by $p27^{Kip1}$or $p21^{Cip1}$ (Perez-Rodger, I. et al., 1999). Complex consisting of cyclin D (either cyclin D1 or D2) and of Cdk4 (cyclin dependent kinase 4) has higher affinity towards both inhibitors, i.e. $p27^{Kip1}$and $p21^{Cip1}$, compared to cyclin E/Cdk2 complex so it bounds them. That way, complex of cyclin E and CDK2 is left active and can induce replication (Perez-Rodger, I. et al., 1999). Targets of c-Myc, transcription factor and downstream effector of STAT3 (Hsieh, F. C. et al., 2005), are genes coding cycline D1, cycline D2, cycline E, and Cdk4 (Hermeking, H. et al., 2000) (Steiner, P. et al., 1995). In turn, cyclin D1 is negatively affecting STAT3 DNA-binding activity (Bienvenu, F. et al., 2001). Moreover, c-Myc is able to negatively regulate cell proliferation through increase in expression of p53. p53 upregulates transcription of $p21^{Cip1}$ thus inhibits cell cycle progression. (Wagner, A. J. et al., 1994)

c-Myc is transcription factor of hEST2 as well. hEST2 is telomerase subunit essentially needed for the telomerase activity. Experiments with overexpression of hEST2 resulted in the increase of total abundance of telomeric sequences and elongation of their length. On the other hand, when both hEST2 and c-Myc are present, telomeric abundance and length show improvement but not as strong as when hEST2 was provided without c-Myc. These results show c-Myc is not increasing but maintaining the length of telomeres. Also it was reported activated telomerase without c-Myc is not enough for cell to bypass replicative senescence (Wang, J. et al, 1998).

STAT3 is responsible for progression of M phase as well. Survivin, anti-apoptotic protein, binds to the centromere. When centromere starts to divide, survivin relocates to the spindle midzone and then to microtubule midbodies. In both cases, survivin is onto microtubules. However, it localization is not dependent on them. Survivin deficient cells do not form mitotic spindle or midbodies. These cells display morphologically irregular nuclei of very large size or bi- and multinucleated cells. Network consisting of microtubules was impaired too (Uren, A. G. et al, 2000). Uren et al. reviewed that Survivin is partaking in segregation of chromosomes and cytokinesis, along with INCENP and Aurora1. Considering that homologues of these proteins can be found even in yeast, this mechanism seems to be ancient (reviewed in Uren, A. G. et al., 2000).

3.3 Disregulation of carcinoma cell growth

In order to divide, the cell must to grow into sufficient size. There are several ways to obtain increased growth: via insensitivity to inhibitors of growth factors, hypersensitivity to growth factors or independence on them. STAT3 is participating in growth mediation through both up-regulation of growth signals and down-regulation of anti-growth signals.

IF4E is limiting part of the complex initiating protein translation. eIF4E binds to the capped mRNAs and regulates their translation (reviewed in Dong, K. et al., 2008). After eIF4E inhibition, breast carcinoma cells displayed suppressed cell growth and cell cycle arrest in G0/G1 phase. Cell cycle arrest was caused by descent in cyclin D1 expression. These cells were treated with cisplatin and after repression of growth they displayed enhanced sensitivity towards chemotoxines. (Dong, K. et al., 2008).

p53, tumor-suppressor, plays a role in growth deregulation as well. It was proven that p53 negatively affects cell growth. p53 deficient cell had significantly increased growth compared to the cell line with wild-type p53 (Ito, D. et al., 1999) Ito et al. think it is plausible p53 down-regulates the response to EFG and IL-1 without decreasing expression of EGF and IL-1 receptors (Ito, D. et al., 1999). p53 is expressed at low level in many breast carcinoma cells and while these cells carry alteration of p53 gene (Miller, L.D. et al., 2005), they are usually in late phase of tumorgenesis when mutation of p53 occurs. (Baker, S.J. et al., 1990) It was discovered promoter of p53 contains three STAT3-binding sequences and inhibition of p53 correlates with STAT3 activation. The principle of STAT3 attenuating expression of p53 is not known yet, but at least important part of it functions through transactivating function of STAT3. (Niu, G. et al., 2005).

3.4 Angiogenesis

Angiogenesis is growth of new blood vessels. New carcinoma cells do not have any source of nutrients so growth of tumor in size is limited without new blood vessels. And they can also migrate and reach long distances through them.

The main inducer od angiogenesis is VEGF whhich sufficient for angiogenesis to occur (Phillips, G. D. et al, 1994). Endothelial cells rapidly divide so they can form new blood vessels and important factor in this event is cell migration. (principle is same as described below in the next section) (reviewed in Terman, B.I. and Stoletov, K. V., 2001)

STAT3 initiates angiogenesis through both VEGF (Niu, G. L. et al. 2002) and Hif-1α (Niu, G. et al, 2009), a subunit of Hif-1, because STAT3 is binding to the promoter of both of

them as their transcription activator. Together with STAT3, Hif-1 is needed for optimal expression of VEGF. However, a principle of this regulation is not known yet (Xu, Q. et al, 2005). Hif-1α is also regulated post-transcriptionally via oxygen-dependent hydroxylation that ends up in degradation when level of O2 is normal (Lando, D. et al., 2002). In tumor cell, hypoxia and hyperactivated Src and PI3K are increasing mRNA stability which leads to up-regulation of Hif-1α and thus VEGF expression.

3.5 Migration

Migration of the cell is essential for metastasis - spreading of the carcinoma. Metastasis is complex phenomenon including angiogenesis for spreading via blood vessel system, insensitivity to the apoptotic stimuli or anti-apoptotic defense once the cell is detached from its supposed location and of course migration. The cell is attached to the substrate through several ways; for example via integrins, cadherins, selectins etc. Integrin is a cytoplasmic receptor attaching itself to the extracellular matrix. It consists of two subunits, α and β. There is many different versions of these subunits and their composition specialize them

STAT3 is important factor in regulation of transcription of matrix metalloproteinases MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, and possibly of some other MMPs through binding to theirs promoters (Yuan, G. et al., 2008) (Senft, C. et al., 2010). MMPs are extracellular proteinases; each one binds different participate in migration and metastasis of tumor cells (Sehgal, G. et al., 1998). For example in keratinocyte migration, α2β1 integrin binds collagen type I strongly. There is also MMP-1, forming a complex with α2β1 integrin and collagen, and MMP-1 specifically breaks collagen helix. The cell detaches itself from substrate and migrates. When α2β1integrin recognizes collagen type I, cell attaches itself again and whole circle is repeated (Dumin, J. A. et al., 2001). The adhesion of the cell is monitored through integrin signaling. If β1 integrin is bound to fibronectin, PKC is activated and via PKC, c-Src induces transcription of c-Myc. One of the possibilities is that c-Src activates STAT3 and thus c-Myc will be transcribed. This way, proliferation and apoptosis of the cell is affected when the cell is not attached to the substrate (reviewed in Benaud, M. B., Dickson, R. B., 2001).

3.6 Aerobic glycolysis - Warburg effect

Warburg effect is an irreversible metabolic switch in cancer cells toward aerobic glycolysis.

Hif-1α is a transcription factor of many glycolytic genes. It is encoding for example glucose transporter 3 (Liu, Y. et al., 2009), phosphoglycerate kinase 1, and pyruvate kinase M2 (Kress, S. et al., 1998). At the same time, overexpressed STAT3 reduces activity of complex I and II. In this case, STAT3 has to be phosphorylated on S727 residue and not on Y705. That is leading to assumption STAT3 is interacting with some proteins instead of binding to DNA (Wegrzyn et al. et al., 2009).

As it was stated above, Hif-1 expression is regulated through several factors, STAT3 and the level of oxygen in cell are among them. STAT3 is up-regulating transcription of Hif-1α and its RNA is more stable during hypoxia. Even at normal oxygen level, Hif-1 is up-regulated during STAT3 constitutive activation, however, not as strongly as during hypoxia. This enhancement is sufficient for metabolic switch toward aerobic glycolysis (Demaria, M. et al., 2010). Marco Demaria thinks glycolytic cells are dependent on STAT3 because when activated STAT3 is not present in cell in higher levels, metabolism goes back to normal and apoptosis occurs (Demaria, M. et al., 2010). As it was established above, there is STAT3 in mitochondria that does not function as a transcription factor but it probably affects subunits of electron transport chain (ETC) via modulation of protein kinases activity. (Boengler, K. et al., 2011) The translocation of STAT3 to the nucleus occurs probably via Tom20-dependent pathway (Boengler, K. et al., 2011). STAT3 is uncoupling subunits of complex I (NADH dehydrogenase) which attenuates flow of electrons and ETC effectivity. This would protect cell if ischemia was to occur. Production of ROS would decline and that means ROS cannot oxidize cardiolipin in significant numbers. Oxidation of cardiolipin would mean releasing cytochrom c from inner membrane of mitochondria and its leaving mitochondria through mitochondrial permeability transition pore. MPTP opening is activated by high concentration of calcium and ROS (Boengler, K. et al., 2010). STAT3 protected cells show decrease in these levels and also other study claim STAT3 inhibits opening of MPTP.

3.7 Insensitivity to pro-apoptotic stimuli

Apoptosis is a type of programmed cell death. It is an important function of multicellular organism, usually an animal one. This type of cell death does not induce immune reaction. When cells are not needed or they are damaged, body disposes of them without any problem. The dying cell undergoes characteristic events such as leakage of chromosome c from mitochondria, degradation of DNA and cytoskeleton as well, disorganization of cytoplasmic membrane and fragmentation of the nucleus and whole cell altogether into

apoptotic bodies. It is natural for body cells to eliminate themselves by apoptosis when they do not conform to norm. Thus cancer cells have to find a way to bypass this trait.

STAT3 plays an important role in apoptosis; it prevents it through up-regulating transcription of anti-apoptotic proteins Mcl-1, Bcl-2, Bcl-xL, and survivin, and through down-regulating expression of anti-apoptotic protein p53. STAT3 participates in the DNA repairs as well.

Both Mcl-1and Bcl-x_L bind Bak, a pro-apoptotic protein of the same family (Bcl-2 family), and thus keep it inactivated. Bak binds preferentially to Mcl-1 which suggests Mcl-1 is primary inhibitor of Bak. Rate of Mcl-1 and Bcl-xL bound to Bak depends on amount of functional Mcl-1. (Willis, S. N. et al., 2010) This way of regulating apoptosis is called 'intrinsic'; a stimulus inducing apoptosis comes from the inside of the cell. When cell detects any inconsistency of itself such as DNA damage, it activates pro-apoptotic BH3-only members of Bcl-2 family. These BH3-only proteins replace Bak in the Bak/Mcl-1 or Bak/Bcl-xL and by that, Bak is released. Moreover, Noxa, another proapoptotic mediator, binds specifically to Mcl-1 and supports its proteasome-dependent degradation (Willis, S. N. et al., 2010). NBK/BIK, on the other hand, binds both Mcl-1 and Bcl-Xl with the same result. (Shimazu, T. et al., 2007). Some of BH3-only proteins activate other pro-apoptotic proteins universally and some do not. Once Bax is free, it clusters and forms pores in cellular organelles by oligomerizing in them (reviewed in Danial, N. and Korsmeyer, S. J., 2004). Cytochrome c and other apoptotic factors like SMAC leak through these pores and trigger apoptosis. Bax is considered a functional equivalent of Bak (Lindsten, T. et al. 2000).

STAT3 is also protecting the cell through modulation of DNA damage response pathway. Cells deficient in STAT3 show aggravation of DNA repair, which leads to apoptosis. (Barry, S. P. et al., 2010). Moreover, STAT3 is also down-regulating p53 expression. It has been shown p53 can activate Bax when there is no other BH3-only protein that available. Bax and p53 make a complex together and then permeabilize outer mitochondrial membrane (Chipuk, J. E. et al., 2004).

There is another anti-apoptotic protein regulated by STAT3 – survivin. This protein does not appear in mature cells often but its occurrence in fetal and cancer cells is common (Reed, J. C., 2001 – Ambrosini C. et al, 1997). For example in primary effusion lymphoma cells, STAT3 inhibition caused apoptosis. It was, however, unusual that STAT3 inhibition did not caused down-regulation of Mcl-1, Bcl-x_L and Bcl-2; a decline of survivin was the reason for induction of apoptosis (Aoki, Y. et al., 2003). O´Connor reported that survivin, when

phosphorylated on Thr34, binds and thus inhibits caspase-9. Activation of caspase-9 by cytochrome c would lead to activation of caspase-3 and apoptosis. It is important to note survivin is a part of the intrinsic pathway along with Bcl-2 family (O´Connor, D. S., 2000).

On top of it all, there is MPTP (mitochondrial permeability transition pore) which opening is activated by high concentration of calcium and ROS. Opening of MPTP causes apoptosis (Boengler, K. et al., 2010). STAT3 is supposed to inhibit opening of MPTP.

From these data, it is clear STAT3 inhibits apoptosis mainly via intrinsic pathway. That means it counteracts induction of apoptosis based on detected abnormalities in the cell. The cell is still sensitive toward extracellular apoptosis-inducing stimuli.

4 Inhibitors of STAT3

As it was covered in previous chapters of this Thesis, STAT3 is a good target in cancer treatment. STAT3 appears in every hallmark that is characteristic for cancer cells, thus all of them will be targeted at the same time. It is essential for treatment purposes that some carcinoma cells are addicted to STAT3 (Demaria, 2010) and that high levels of activated STAT3 are needed for cancer cell survival due to its anti-apoptotic function. Other advantages to the other treatments are prevention of metastasis and the most importantly, its universal use to many types of cancer due to its presence in many various frequently occurring tumors.

There are four possible domains which STAT3 can be targeted at. One of them is SH2 domain of STAT3. It is essential for STAT3 activation through gp130-associated kinase, such as JAK. After stimulation of receptor and its autophosphorylation, docking site for STAT3 in form of phosphotyrosine arises. In order to be phosphorylated by gp130-associated kinase, STAT3 has to be bound to this site which is not possible without functional SH2 domain. At the same time, inhibition of SH2 domain disables STAT3 to form dimers. Taken together, SH2 domain inhibition means for the cell attenuation of cytokine-induced STAT3 phosphorylation which is independent on type of cytokine (assuming its receptor contains gp130). Other type of inhibition involves SH2 domain as well. STAT3 dimers are formed reciprocally through binding between SH2 domains and phosphotyrosines. If the inhibitor contains SH2 domain, phophotyrosine of STAT3 will be bound to it and STAT3 cannot form dimers and thus be activated. The third way to attenuate activity of STAT3 is to inhibit its translocation to the nucleus. If the presence of STAT3 in the nucleus is to be inhibited, STAT3 cannot function as a transcription factor and soon, even expression of STAT3 will be reduced. As it was mentioned above, it is highly probable STAT3 can translocate to the nucleus independently on its phosphorylation and dimerization. The last way of STAT3 inhibition is via its DNA-binding domain. STAT3 perform in mitochondria as well but I have not noted any efforts of inhibition of this STAT3 function.

There are various types of known STAT3 inhibitors. They can be in form of peptides or peptidomimetic compounds, non-peptidic small molecules, and metal-based inhibitors. Usually, researchers put their efforts in inhibition of SH2 domain.

C. Gomez et al. designed a small peptidomimetic inhibitor of STAT3 with incorporated 7-membered Freidinger lactam structure. Results showed Lys591, Arg609, Ser611, and Ser613 residues within STAT3 are important for its affinity to phosphotyrosine (in these circumstances belonging to the STAT3 inhibitor) so this is the sequence of STAT3

researchers are concentrating on in their efforts to target it. According to data, presence of Leu and especially Gln residues within STAT3 inhibitor is significant as well. Their amide side chains form hydrogen bonds with backbone carbonyls of STAT3 Ser, resp. Glu residue. Gln residue in STAT3 inhibitor may cause its higher affinity to STAT3 due to its role in specific gp130 recognition by STAT3 (Gomez, C. et al., 2009).

Scientists are trying to come up with new solution to SH2 domain targeting because inhibitors containing phosphotyrosine have two serious disadvantages. Their membrane permeability is poor due to their negative charge and they are sensitive to hydrolysis by phosphatases present in the cell. For example, P. K. Mandal with his colleagues designed a bis-POM phosphonodiester building block that can be attached to peptides and peptidomimetics. After attachment, two POM groups within inhibitors cause its higher permeability. For SH2 binding, there is aryl difluoromethylphosphonate that emerges after POM groups cleavage by carboxyesterases of the cell. The aryl difluoromethylphosphonate is strong enough to separate already formed dimers (Mandal, P. K. et al., 2009).

Chen with his team chose a different approach. They designed STAT3 inhibitor and then they tested the use of various modifications to it. The resulting compound with highest STAT3 inhibition efficiency had incorporated positively charged nitrogen into the place of carbon atom. That resulted in shift of overall charge towards positive charge and compound became more permeable; however, its ability to bind STAT3 was slightly reduced, compared to the compound with a carbon in its place. Treatment of breast carcinoma cells with positively charged nitrogen containing compound led to their apoptosis which leads to conclusion this compound is effective (Chen, J. et al., 2010).

However, peptides and peptidomimic molecules are more sensitive to degradation and denaturation. They can also cause an immunity response of organism. For this reasons, new non-peptidic inhibitors are developed. Scientists were either inspired by already existing inhibitors or they were discovered by both in-silico and in-vitro screening. For example, Song et al. discovered STA-21 during a structure-based virtual database screening of approximately 429,000 different compounds. STA-21 was the most fitting inhibitor of STAT3 SH2 domain (Song, H. et al., 2004). A structure of STA-21 was used by B. Fuh et al. in the search for an improved model of STA-21. LLL-3 displayed the closest resemblance to STA-21. After a treatment with this inhibitor, cancer cells showed significant decrease in viability and at the same time, treated animals survived two-times longer (Fuh, B et al., 2009). There are also metal-based inhibitors. Another way of STAT3 inhibition through SH2-pTyr bond was presented by Drewry et al. They focused on the other part of the bond, phosphotyrosine, and

designed STAT3 inhibitor mimicking SH2 domain. These bis-dipicolylamine copper complexes disrupt STAT3 homodimers quite selectively (Drewry, J. A. et al., 2010).

To present other types of inhibition as well, Duan discovered SD-1029, a compound able to attenuate both STAT3 activation and its translocation to the nucleus. Inhibition of STAT3 activation is via inhibition of JAKs activity (Duan, Z. et al., 2006). Differently, Nagel-Wolfrum et al. found several inhibitors in form of peptide aptamers and among them, there were some interacting with DNA-binding site of STAT3. Use of one of them induced apoptosis in 50% of the cells.

It is important to note there is already ongoing clinical trial of STAT3 inhibitor for solid tumors. This study started in 2009 and there are no available up-dates of progress yet. Patients can include a special diet in their treatment. Natural compounds inhibiting STAT3 activation are for example catechins contained in green tea extract (Leong, H. et al., 2009), curcumin (Bharti, A. C. et al., 2003), etc. It is vitally important in cancer treatment consisting of STAT3 inhibition to prescribe right doses of the STAT3 inhibitor as STAT3 is needed for the function of normal cells.

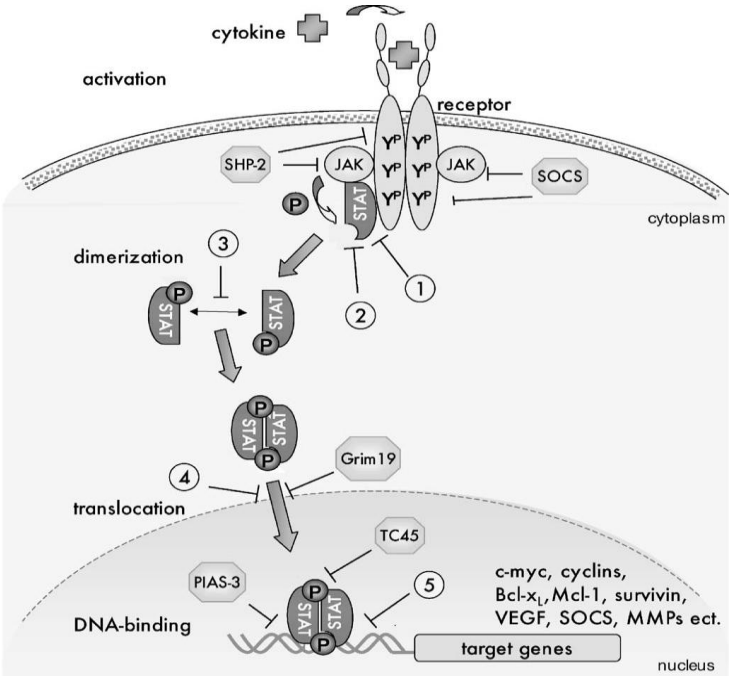

Figure 4. Mechanism of STAT3 canonical activation and possible ways of its attenuation. Ligand in form of cytokine binds to its receptor. That causes dimerization and phophorylation of gp130 receptor. It results in Jak kinase and thus STAT3 phosphorylation. STAT3 dimer translocates to the nucleus and acts as transcription factor there. This pathway can be inhibited at these sites: 1.) binding of STAT3 to the gp130 receptor, 2.) STAT3 phosphorylation, 3.) STAT3 dimerization, 4.) STAT3 trans-location to the nucleus and 5.) DNA-binding of STAT3.*(Sylvane Desrivières at al., 2006)*

5 Conclusions

Since STAT3 was discovered, scientific world is realizing more and more how important this protein actually is regarding cancer and especially cancer treatment. However, aside thinking about STAT3 as the partial effector in forming of many types of cancer, we ought to acknowledge the phenomenon that STAT3 surely is. For that, it is essential to understand what cancer is.

Vincent presents his idea: *"Each trait (of cancer[1]) can be categorized under one of three main ideas:* ***phylogenation*** *(cancer is not only type of animal, but one entirely different from its originating host);* ***re-primitivization*** *(the path from archaic unicellularity to multicellularity is bi-directional); and* ***adaptive resilience*** *(cellular survival sometimes must be decoupled from identity, through genomic alteration). These organizing principles service a single higher goal: "any-cost cellular survialism"* (Vincent: M., 2011)

But which one is true? Is cancer a type of parasite waiting for the right conditions to attack its host? Or does time-shift occur in our cells? And if it is, is this time-shift going backward to random re-primitivization or toward adaptive resilience? And are these two theories compatible or contradictory?

Following part of this chapter is going to be resume of my theories based on reading previously mentined review (Vincent: M., 2011) and recapitulation of already presented data. Constitutively active STAT3 is able to transform human body cells into cells displaying features typical for Protozoa . One conclusion, however quite unprobable, could be that primitive historical cells had had STAT3 activated and when they were becoming specialized and more sophisticated, they 'enslaved' STAT3. Its activity had to be suppressed in order to transform group of independent cells into an organism. It is easier to fulfill their common goal - to pass down their genetic material to the next generations - when united. It may seem as some cells are waiting for the opportunity to start a 'revolution'. When it happens, they stop contributing to their organism and orient on their own needs. Or, perhaps, this transformation is random and STAT3 functions as a possible 'time-machine' with an ability to transform cells into the form th were in a distant history.

Other, more likely, explanation is that cancer is an ancient survival program. When the organism does not have any other option left because all of tumor suppressive mechanisms failed then individual cells need to take care of their own fate. The cell re-acquires its

[1] Noted by the autor of this Thesis

autonomy in exchange for its identity. When circumstances are not benefiting the cell, identity is not such an important concept. The cell, as it is mentioned below, increases its proliferation, mutation rate and survival which results in many cell phenotypes. This way, the cell protects itself in means of biological fitness; at least some of these phenotypes can continue grow and proliferate in new environment. Carcinoma cells are more oriented toward 'any-cost cellular survialism' so they claim higher amount of nutrition in order to keep fast proliferation.

In many cell types, STAT3 has an essential role in this survival program. In regular body cells, STAT3 is a protector against too high production of ROS. It mildly decreases activity of electron transport chain complexes I and II. STAT3 is maintaining specific level of ROS, since it is essential for the cell to keep ROS homeostasis. STAT3 has also a role in DNA damage repair. Its presence is required for sufficient repair.

In the contrast, when the cell is deficient in STAT3 or STAT3 is constitutively active, level of ROS increases. It is clear why that happens in STAT3 deficient cells but the same question regarding cancer cells with constitutive STAT3 activity helps to understand the principle of tumorigenesis.

When STAT3 is activated, it enhances expression of itself through its positive-loop regulation; STAT3 targets its own promoter. More STAT3 can locate into the mitochondria where it strongly attenuates activity of complex I and II within electron transport chain. It has been proved potent down-regulation of ECT complexes I and II in aerobic environment causes elevation of ROS (Li, N. et al., 2002). Not oriented flow of electrons in the presence of oxygen and absence of protons is the reason for it; they attack oxygen molecules and that results in ROS production. STAT3, at the same time, protects the cell against apoptosis that would usually follow ROS homeostasis disruption. It enhances repair of DNA damage; up-regulates transcription of survivin and some anti-apoptotic members of Bcl-2 family, down-regulates expression of p53; and inhibits MPTP opening and thus release of cytochrome b. High concentration of ROS can alter cancer cell DNA and make mutations which is the whole purpose of ROS elevation in these circumstances (various phenotypes). However, STAT3 protects DNA against breakes. (Barry, S. P. et al., 2010)

Parallel to this, switch to aerobic glycolysis and angiogenesis through Hif-1 occurs. Aerobic glycolysis is great opportunity for the cancer cell. Mitochondria no longer functions as a distributor of ATP and the cell has to find new one. For this reason, there is Hif-1which is a transcription factor of enzymes needed for aerobic glycolysis. When source of glucose is unlimited or at least very high, ATP is produced more quickly. This way, cancer cell can

sustain its unceasing growth and proliferation. In order to achieve good nutrient saturation, Hif-1 induces angiogenesis as well. Constitutively activated STAT3 is sufficient for inhibition of apoptosis thus tumor will very fast out-grow the location where it is supplied with nutrients and oxygen from already existing blood vessels. There is a time frame when cancer cell reach hypoxia which will cause higher Hif-1 activity. When Hif-1 is more active, angiogenesis is promoted more and cancer cells will be provided with needed nutrients sooner. Other positive fact for cancer cells is that forming new blood vessels helps cancer to migrate and spread to the whole body.

STAT3 also plays a role in others, for cancer cell essential, functions. It enhances growth of the cell through both inducing c-Myc and causes cell insensivity to factors counteracting cell growth. Also STAT3 has a role in immortality of cancer cells, their fast proliferation, and ability to migrate.

Every version of the reason and purpose of cancer brings new approach to cancer treatment. Considering cancer a parasite would mean use of drugs specific for different species but this theory seems very unlikely. If a cancer is an atavism, the treatment would consist of targeting the trigger. Methods of current cancer treatment, such as radiotherapy and chemotherapy, are in that case not much useful. Before cells started to form communities of sorts, they had to be accustomed to UV-light and chemotoxines; they were always present in the environment. However, some cancer cells will, with the highest probability, respond to the treatment and the rest of them will be weakened by aggravation of the bodily condition. They depend on their organism with maintainance of homeostasis and income of nutritions. But this brings harm to the organism as well. The third outlook is the most probable theory. If cancer is an ancient survival mechanism, then carcinoma cells are protected against impact of such drastic theraphy better than our non-transformed cells. There is possibility this treatment will result in much more aggressive response because more cells will be in need of survival program. Cellular damage by UV-light and chemotoxines were probably the reason such protective mechanism had to evolve. That means therapies based on them are highly unsuitable.

Their compensation could be found in various specific inhibitors of oncogenes. As it was presented here for several times, STAT3 is definitely one of them. Great progress in designing and discovering these inhibitors was reached during last decade. Based on that, I presume in 20 years from now, cancer will be cured more effectively without such side effects that are occurring now.

I believe I roughly outlined the role of STAT3 in tumorigenesis and cancer therapy which was an assignment of my Thesis.

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