Chapter 4

Discussion
4.1 Cross-regulation of Stat3 activation and Wnt/β-catenin pathway

Oncogenic role of Stat3 is a much established fact and warrants no further discussion (Bowman et al. 2000; Bromberg et al. 1999; Bromberg 2002). Activation of this protein is mainly by phosphorylation at its Tyr-705 residue whereby it dimerizes, enters the nucleus and mediates its role as a transcription factor (Bromberg 2002). β-catenin, a key protein required during early embryogenesis and development, is critical transcription factor of several oncogenic players like Cyclin D1 and c-Myc.

Stat3 and β-catenin are crucial oncoproteins which are overexpressed in most human cancers as evident from existing literature and also what we found in cell lines of various cancers. In addition to their individual function, a cross-regulatory nature of regulation exists between these two crucial molecules of cell signaling, enumerated in some cancers and stem cell studies (Hao et al. 2006). Stat3 activation has been associated with β-catenin nuclear accumulation and activation linked to poor patient survival in colorectal cancer and breast cancer (Kawada et al. 2006; Armanious et al. 2010). We obtained a similar result in vitro in glioma and PCa cell lines where Stat3 activation by EGF treatment or knockdown using antisense RNAs resulted in concomitant overexpression or decreased accumulation of β-catenin, mainly in the nucleus. Interestingly, the two proteins do not interact physically even if they do modulate the function of each other. Additionally β-catenin promoter contains Stat3 binding site (Armanious et al. 2010) which adds a new dimension of understanding in the regulation of β-catenin by Stat3.

Conversely, β-catenin was also able to activate Stat3 and induce expression of its downstream target genes. β-catenin is reported to be overexpressed in anaplastic large cell lymphoma and could induce Stat3 activation (Anand, Lai and Gelebart 2011). β-catenin was also found to crosstalk with the EGFR pathway as knock down of β-catenin in human glioma cells could decrease expression of molecules downstream in the EGFR pathway, including Stat3 (Yue et al. 2010). In our experiments, activation of β-catenin in PCa cell lines could result in increased tyrosine phosphorylated forms of Stat3 in the nucleus which is required for its role as transcription factor. Transient overexpression of β-catenin in the cell lines also had a similar effect on Stat3 activity and expression of downstream target genes like bcl-xl, MCL1. β-catenin is known to be a co-transcription factor for Stat3 as Stat3 promoter has putative TCF/LEF binding sites,
established in esophageal squamous cell carcinoma (Yan et al. 2008) and can thus result in increased transcription of Stat3 molecules. This heightened accumulation of Stat3 molecules in the cytoplasm may ultimately lead to its increased phosphorylation and entry into the nucleus. Increased number of Stat3 molecules in the cytoplasm may also result in more number of unphosphorylated Stat3 entering the nucleus. This would induce the expression of a new set of Stat3 target genes which require Stat3-NFκB as the transcription factor; study in this realm of Stat3 is still at its nascent stage, a lot more research has to be done to study its implication in cancer progression.

![Figure 4.1: Model showing Stat3 and β-catenin cross-regulation.](image)

The model depicted here shows a part of the crosstalk between Stat3 activation and Wnt/β-catenin pathway which function in many parts with EGF as a mediator. EGFR is a target of the co-transcription factor β-catenin which in turn regulates Stat3 phosphorylation and activity (Guturi et al. 2012). These two proteins also regulate each other’s expression as each promoter contains binding site of the other.

The feedback loop established in this study resulting in increased activity of one protein upon stimulation of the other is only reflective of the cross-regulatory nature of signaling mechanisms inside the cell. Cancer has a widespread prevalence and incurable nature as it becomes impossible to target a single pathway responsible for tumorigenesis. Identification of novel cross-regulations and crosstalks enhancing tumor progression thus holds the key to multi-targeted combinatorial approach. The identification and elucidation of crosstalk between EGFR/Stat3 and Wnt/β-catenin signaling is thus a determined step towards this combinatorial therapeutic approach.
4.2 Regulation of Stat3 activation by CK2

Regulation of Stat3 phosphorylation by CK2 is another branch of cross-talk between the Wnt/β-catenin and Stat3 signaling. Novel regulatory mechanisms in Stat3 Ser-727 phosphorylation may lead to better understanding of the nature of tumor progression. Surprisingly, the effect of the serine/threonine kinase CK2, which could already activate the JAK-Stat signaling pathway via interaction with JAK2, was found to be negative in pStat3^S727^ regulation.

![Figure 4.2: Model depicting regulation of Stat3 phosphorylation by CK2.](image)

Figure 4.2: Model depicting regulation of Stat3 phosphorylation by CK2. Stat3 is regulated by CK2 via both its tyrosine phosphorylation and serine phosphorylation. Both regulation works concurrently to promote oncogenesis. PI3K-Akt signaling is another major oncogenic pathway in tumor cells which works independently of the CK2 signaling axis to regulate Stat3 activation.

Stat3 Ser-727 phosphorylation has been reported to be effected by various kinases like ERK, H7 sensitive kinase, protein kinase Cδ, nemo-like kinase, cyclin-dependent kinase 5, mammalian target of rapamycin kinase, death-associated protein kinase 3 and mitogen- and stress activated kinase 1 depending on the cytokine stimulation and the type of cell used for the experiment (Chung et al. 1997; Decker and Kovarik 2000; Jain et al. 1999; Yokogami et al. 2000; Wakahara et al. 2012). On evaluating the role of pStat3^S727^, it was found that it could either positively affect the Stat3-mediated gene activation (Aznar et al. 2001; Wen, Zhong and Darnell 1995) or
could also repress the activity of Stat3 (Chung et al. 1997; Ghosh et al. 2005; Wakahara et al. 2012). Another report also suggests that there may be no effect on Stat3 DNA binding activity as a result of Stat3 Ser-727 phosphorylation (Wen and Darnell 1997). This contradictory role of Stat3 Ser-727 phosphorylation may be due to the different stimulus used or the difference in cellular environment which leads to altered post-translational modifications of proteins thus affecting their function. In certain tumors, where Stat3 was implicated for oncogenesis, activation of Stat3 was found to be a result of phosphorylation at both Tyr-705 and Ser-727 residues (Bowman et al. 2000; Kim et al. 2007; Hazan-Halevy et al. 2010; Lee et al. 2009). Although no report till date details the role of pStat3S727 in glioma progression, elevated levels of this phosphoprotein has been reported in GBM patient samples but not in primary GBM cell lines (Brantley et al. 2008). Contrary to this finding of Brantley et al, we found that pStat3S727 was negligibly elevated in glioma when compared to normal tissue and was either cytoplasmic or poorly expressed in the nucleus of tumor samples. Expression of pStat3S727 was also reduced in glioma cell lines when compared to normal rat astrocytes suggesting a deleterious effect of this form of Stat3 on glioma progression. Regulation of this post-translational modification of Stat3, therefore, is vital in glioma pathogenesis.

Novel regulatory mechanisms of Stat3 Ser-727 phosphorylation may lead to better understanding of the nature of tumor progression. In our study, we checked the effect of a serine/threonine kinase CK2 in pStat3S727 regulation; CK2α is known to activate the JAK-Stat signaling pathway via interaction with JAK2 (Zheng et al. 2011) which mediates phosphorylation of Stat3 at Tyr-705 residue. Supporting the report by Dixit et al (Dixit et al. 2012), we found heightened CK2α expression in glioma, which was prominently nuclear in tumor cells, substantiating its pro-oncogenic role and also exhibiting a converse relation to pStat3S727 levels. As expected, upon overexpression, CK2α could induce Stat3 transcriptional activity as a result of reduced pStat3S727 levels with concomitant increase in pStat3Y705 levels. This effect was observed both by promoter analysis studies and quantitative PCR where increased transcriptional activity of Stat3 upon reduced phosphorylation at its Ser-727 residue was reflected in the increased expression of its target genes like bcl-xl, SOCS3, MCL1 and Cyclin D1. The negative relationship between pStat3S727 and pStat3Y705 has been discussed in previous reports where enhancement in pStat3S727 levels were associated with decreased pStat3 Y705.
levels and reduced transcriptional activity (Andersson et al. 2007; Shi et al. 2006). Wakahara et al. has proposed a mechanism for this negative regulation which works largely through a protein tyrosine phosphatase, TC45 (Wakahara et al. 2012). That the reduced Ser-727 phosphorylation level of Stat3 was responsible for its enhanced transcriptional activity in our findings provide an insight as to why the pStat3S727 level is not enhanced in glioma when compared to normal tissues.

We found increased Ser-727 phosphorylation of Stat3 upon Akt activation but reduced Stat3 Tyr-705 phosphorylation thereby affecting its transcriptional activity which was concomitant to the report by Ghosh et al. (Ghosh et al. 2005). According to Ghosh et al., Stat3 DNA-binding activity was affected by phosphorylation at multiple serine residues in its TAD domain and not dependent on the Ser-727 phosphorylation. Sun et al. also describes that rather than the Ser-727, the LPMSP motif around the Ser-727 of Stat3 was important to recruit p300 to the DNA bound Stat3 (Sun et al. 2006). These two reports suggest involvement of the entire Stat3 serine motif in its transcriptional activity and effect due to Ser-727 may only be a small fraction of the overall effect. In our report, we establish that a fully activated Akt, with phosphorylation at Thr-308 and Ser-473 is required for it to act as a kinase for Stat3 whereas the Ser-129, which is the only Ser residue to be phosphorylated by CK2, is not absolutely essential for Stat3 phosphorylation. CK2, therefore, must act through some other factor to mediate its negative regulation of Stat3 Ser-727 phosphorylation.

PP2A forms stable complexes with various protein kinase molecules, thereby establishing itself to be a major regulator in cellular signaling by reversible protein phosphorylation. Though PP2A has been suggested to be a tumor suppressor (Westermanck and Hahn 2008), caution must be exercised before referring to the different PP2A complexes in generic terms. Reports exist where PP2A tumor suppressive activity has been inhibited for promotion of cellular transformation (Neviani et al. 2005; Juntila et al. 2007; Mumby 2007). Indeed, in our study, we suggest PP2A to have an indirect tumor promoting function by dephosphorylating Stat3 Ser-727 which in turn increases the transcriptional activity of Stat3. Thus, in glioma, CK2 may play its pro-oncogenic role by associating with PP2A, activating it and resulting in altered activity of Stat3.
Targeting Stat3 activity has been the method of choice for therapies against cancer (Zhao, Jiang and Gao 2011; Madoux et al. 2010; Walker, Xiang and Frank 2013) and our study provides a novel axis for exploration in this direction.

4.3 Stat3 activation by reduced serine phosphorylation promotes oncogenesis

Enhanced activation of Stat3 is a major cause for glioma progression. In addition to reports in published literature, widespread data from public repositories also confirm this notion. Patient sample data sets from databases like Oncomine or Rembrandt for Stat3 mRNA or patient survival plots validate the designation of Stat3 as a crucial oncoprotein.

Rat C6 glioma cell lines have been used widely for generating both xenograft and orthotopic brain tumor models which helps in understanding the molecular pathophysiology of tumors. Implantation of C6 cells into rat brain tissue forms an orthotopic tumor model which closely resembles in vivo tumor growth. Advantage of this model over simplified models is that the inflammatory and vascular mechanisms are activated inside the rat brain. However, as these models are complex, it is difficult to identify the individual processes involved in sustained tumor growth, angiogenesis and invasion. This problem can be rectified to an extent by using in vitro models such as cell lines to study the effect of growth factors, extracellular matrix components, proteases and adhesion molecules.

C6 cells stably expressing a mutant form of Stat3, Stat3 S727A and its corresponding control cells were used as the cell line models for our in vitro and in vivo work. From our in vitro data, it was evident that as Stat3 target genes are involved mainly in cell survival (BCL2 family genes) and cell cycle (Cyclin D1), the stable cell line overexpressing the Stat3 S727A mutant had a greater survival and proliferative capacity. These cells were also more invasive and expressed increased amount of the angiogenic protein VEGF, MMP9 or MMP2 distinguishing them as precursors to cancer progression. Indeed, these cells could form larger tumors when injected in vivo to form xenograft models. Orthotopic models generated using the same mutant cells had similar
fate and analysis of the molecular pathology confirmed that Stat3 serine phosphorylation hindered tumor growth.

Thus, Stat3 activation by reduced Ser-727 phosphorylation confers multiple advantages on the glioma cells that are essential for successful malignancy. Understanding the role of serine phosphorylation of Stat3 for its activation in the context of cancer provides newer avenues of research in the field of regulation of Stat3 activity and search for better therapeutic options.

4.4 Future Perspectives

Stat3 is an interesting molecule in the sense that it is an essential protein for cell survival and proliferation but deregulation in its expression or overactivation promotes a cell to become cancerous. Detailed analysis and understanding of the molecular mechanisms to regulate such an important molecule thus becomes very crucial. The numerous signaling networks that requires active participation of Stat3 for their functioning also needs to be studied in detail. Though a wealth of literature already exist in the field of Stat3 research, newer information are emerging everyday to implicate Stat3 in one way or the other in various aspects of cell physiology.

Some of the novel aspects of Stat3 which are gaining current interest are:

a) **Stat3 in immune responses:** Recent studies have suggested Stat3 to have function in both innate and adaptive immunity. Loss of Stat3 in immune cells caused them to have severe inflammatory responses when afflicted by pathogens. Inflammation is one of the key enabling characteristics in tumor progression. Inflammatory cells provide various components to the tumor microenvironment which promotes tumorigenesis. As reducing Stat3 activity is a major therapeutic option for cancer treatment, the exact role of Stat3 in immune responses needs to be elaborately studied. Loss of Stat3, which in one hand can promote cancer cell apoptosis, on the other hand may induce inflammatory responses in pro-cancerous cells. Thus the balance between the two factors has to be rigorously determined to provide effective cancer therapy using targeted Stat3 inhibition.

b) **Targeting Stat3 in cancer stem cells:** Among the various components of the tumor microenvironment, the most serious factor which evades therapy is the cancer stem
cells. These stem cells are now proved to be the cells which drive tumor formation. Stat3 has been implicated in regulating cancer stem cell particularly in glioblastoma multiforme. When Stat3 is inhibited, cancer stem cells in glioblastomas lose their stem-cell characteristics permanently, suggesting that Stat3 regulates growth and self-renewal of stem cells within glioblastomas. Thus this area of research would provide novel ways of treating cancer.

c) **Regulation of Stat3 using microRNA:** In addition to inventing various inhibitors for Stat3, which includes synthetic compounds like small molecule inhibitors, peptides and molecules from natural compounds, newer ways of identifying Stat3 inhibitors from the cell itself are now being encouraged. Frontrunner among these is the microRNAs which may provide safer mode of inhibition of activity of a molecule with less adverse side effects than chemical compounds. miR124 has been identified to have a effect on Stat3 inhibition in some cell types. But the major challenge in this field of research is to determine specific target for the microRNA which would target Stat3 specifically, otherwise deleterious effect on the cell may take place.

d) **Role of unphosphorylated Stat3:** With the recent discovery that Stat3 may act as a transcription factor despite being unphosphorylated opens up newer challenges in its regulation. As unphosphorylated Stat3 acts conjugated to another well known transcription factor NFκB, the signaling cascades initiated by these two major proteins have to be studied minutely. The study of the role they may play in other disease progression in general and cancer progression in particular would prove to be particularly interesting as they have also been implicated in stem cell regulation.