Role of Macrophage Colony Stimulating Factor in Drug Resistance

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

By

CHOCKALINGAM S

Department of Biotechnology
Indian Institute of Technology Guwahati
Assam, India

December 2013

DECLARATION

I, hereby, declare that the matter embodied in this thesis entitled **"Role of Macrophage Colony Stimulating Factor in Drug Resistance"** is the result of investigations carried out by me under the supervision of Prof. Siddhartha Sankar Ghosh, Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India for the award of degree of Doctor of Philosophy. This work has not been submitted elsewhere for any degree, diploma, associateship or membership etc. of any institute or university.

IIT Guwahati **Chockalingam S**

December, 2013 Roll No. 08610608

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

This is to certify that the thesis entitled ***"Role of Macrophage Colony Stimulating Factor in Drug Resistance"*** being submitted to the **Indian Institute of Technology Guwahati** by **CHOCKALINGAM S** for the award of the degree of **Doctor of Philosophy** is a bonafide record of research work carried out by him under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India. The contents of this thesis have not been submitted to any other University or Institute for the award of any degree.

December, 2013

Prof. Siddhartha Sankar Ghosh

(Supervisor)

Acknowledgement

It gives me immense pleasure and satisfaction to thank and acknowledge all the people who have played a role in helping me to complete my Ph.D. Writing this section is the most challenging part of writing my thesis and perhaps, unintentionally I may face the risk of missing certain names. However, I am putting in my best efforts to show my appreciation to all the people and my sincere apologies to those, whose names are not mentioned here.

First of all, I would like to express my deepest sense of gratitude to my thesis supervisor Prof. Siddhartha Sankar Ghosh for giving me an opportunity to work in his lab and for providing me with the state-of-the-art facilities to carry out my research. I am very much indebted to him for giving me a scientific environment full of freedom, faith and support. It is because of him that I had started my sailing and exploration through this fascinating field of cancer biology. Thank you very much sir.

Next, I would sincerely like to acknowledge the members of my doctoral committee, Prof. Pranab Goswami, Dr. Biplab Bose and Dr. Subrata K. Majumder for evaluating my work and giving critical comments from time to time which ultimately shaped the progress of my work. I would specially like to thank Dr. Biplab Bose for involving me in numerous interesting discussions through which I have learnt a lot.

I am very much thankful to Department of Biotechnology, Govt. of India for providing me full travel grant support to attend World Cancer Congress in Montréal, Canada. It gave me the chance to interact with eminent scientists from around the world and gain deeper insight into the field of oncology. I also owe my gratitude to IIT Guwahati for giving me financial assistance to attend the 3rd International Conference on Stem Cells and Cancer (ICSCC-2012): Proliferation, Differentiation, and Apoptosis in New Delhi, India.

I acknowledge all our former and present Heads of the Department, faculty members and all staff members of the Department of Biotechnology, IIT Guwahati for providing me

with the necessary facilities during my Ph.D. tenure. I would also like to thank Centre for Nanotechnology, IIT Guwahati for providing me instrumentation facilities.

My interest in research started during my experience working in IISc, Bangalore and I would like to show my utmost gratitude to Dr. Dipshikha Chakravortty for giving me her constant support and encouragement. Words are insufficient to thank Dr. Arvindhan and Dr. Sandhya. They gave me the confidence and most importantly the first guidance as how to do research. The concepts and the research culture which they inculcated in me are largely responsible for making me a good researcher. I am indebted to my school teacher Mr. Gomathi Vinayagam and my UG teacher Dr. Chittibabu for giving me very good guidance and support, not just in academics but also in life.

My sincere thanks to Dr. Gopinath, my senior in IIT Guwahati. I cannot expect a better senior than him. He was in our lab just for two months after I joined. But till this day, he has helped me in every possible way. Thank you anna. I would also like to thank my other seniors Dr. Pallab, Dr. Vinod who were more of friends than seniors.

I was fortunate to have a collaborative work with Dr. Shilpa. Besides being one of my best friends, it was she who taught me the little practical knowledge which I have in the field of nanobiotechnology.

I sincerely appreciate the help of all my labmates and juniors- Kohila, Nidhi, Subhamoy, Dr. Amit, Amaresh, Upashi, Archita, Swati, Shubaash, Sharmila, Deepshika, Asif, Neha, Bandhan and Deepanjalee. I specially thank Archita and Sharmila for giving me suggestions and comments when I was writing my papers or thesis.

Next, I take this opportunity to acknowledge my batch mates and other colleagues- Mitun, Dr. Atul, Manab, Ashok, Dr. Asim, Pojul, Thyahoo, Sandipan, Shekhar, Himangshu, Anil, Santhosh, Lingaraj, Ritesh and numerous others. I had a wonderful time in hostel and core-III tea shop, thanks to my hostel mates and colleagues from other departments. Those 'useless' talks and 'chit-chats' actually acted as the stress busters on

numerous occasions. I thank Mr. Krishna and other mess workers in Umiam hostel for keeping food for me even when I came late to mess at times.

I am blessed to have friends like Arvind, Ark, Vini, Neha, Sankarnarayanan and Vignesh. They have readily helped me whenever I approached them. I am also lucky to get the sisterly love of Sandhya and Deepika. I have understood through them that a sibling relationship can be shared even without necessarily having a blood relation.

Finally, my parents and brother deserve a special mention here. I would not have reached the position that I am in now, if not their love, care and encouragement. Even in the most difficult situations, they have always supported me to choose the career I wished. Whatever I have achieved today is only because of them.

In the last five years, I have realized that Ph.D. is not only about doing research. It is also a training to face failures in life. And I am always indebted to the supreme power, the God Almighty for giving me the strength and mental toughness to face failures and success alike.

Chockalingam S

CONTENTS

ABSTRACT

Cancer cells acquiring resistance to chemotherapeutic drugs remain the most serious obstacle to the development of an efficient anti-cancer therapy. Overexpression of ABC transporter genes is associated with active expulsion of chemotherapeutic drugs from cancer cells. Many tumor cells overexpress Macrophage Colony Stimulating Factor (MCSF), which acts as the chemo-attractant, infiltrating the tumor with circulating monocytes and promoting metastasis of tumor. Moreover, tumors with augmented expression of MCSF show poor prognosis with treatment.

This thesis aims to explore the properties of Macrophage Colony Stimulating Factor when combined with the conventional anti-cancer drug 5-fluorouracil (5-FU) on a human glioblastoma cell line, U87MG. **Introductory section** of this thesis describes the current strategies employed in anti-cancer therapies and the genes responsible for drug resistance. An extensive review on molecular signaling mechanisms of MCSF is described in **Review of literature**. This section also provides deeper insight into the role played by MCSF in supporting tumor growth. **In Materials and Methods,** information about the chemicals and reagents used along with the methodologies employed in the work are discussed in detail. The fourth section of the thesis, **Results and Discussion** started with cloning of MCSF from mammalian cells followed by generation of a stable cell line of U87MG constitutively expressing MCSF, named as U87-MCSF cells. Upon 5-FU treatment, the proliferation of U87-MCSF was significantly affected as determined by cell cycle analysis and cyclin expression studies. The retardation in the cell proliferation did not lead to apoptosis; rather resulted in a change in morphology. Spindle shaped mesenchymal type cell populations and an

upregulation in markers of epithelial-mesenchymal transition (EMT) were noted in U87-MCSF cells upon 5-FU treatment. EMT in U87-MCSF cells culminated in the appearance of cancer stem cells with an increase in expression of ABC transporter genes, which accounted for the drug resistive properties of U87-MCSF cells. Finally, **Conclusion and Future Prospects section** summarizes the essential findings and the significance of the work. The current experimental evidence demonstrates the role of MCSF in increasing the drug resistance of U87MG cells through formation of cancer stem cells. Future anti-cancer therapeutic strategies aimed at targeting MCSF and cancer stem cells are briefly discussed.

ABBREVIATIONS

5-FU	-5-fluorouracil
ABC	-ATP-binding cassette
ANOVA	-Analysis of variance
AO	-Acridine orange
BAD	-Bcl-2-associated death promoter
BK channels	-Big potassium channels
CFDA-SE	-Carboxyfluoresceindiacetate, succinimidyl ester
CSC	-Cancer stem cells
CSF	-Colony stimulating factor
CSF-1R	-Colony stimulating factor-1 receptor
DAG	-Diacylglycerol
DAPI	-4', 6-diamidino-2-phenylindole
DMEM	-Dulbecco's modified eagle's medium
EMT	-Epithelial-mesenchymal transition
EtBr	-Ethidium bromide
FGF	-Fibroblast growth factor
FITC	-Fluorescein isothiocyanate
G-CSF	-Granulocyte colony stimulating factor
GFP	-Green fluorescent protein
GM-CSF	-Granulocyte-macrophage colony stimulating factor
HIFs	-Hypoxia-inducible factor
IFN	-Interferon
IL	-Interleukin
IP3	-Inositol 1, 3, 4 P3
JC-1	-5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide

LPS	-Lipopolysaccharide
MCSF	-Macrophage colony stimulating factor
MDR	-Multidrug resistance
MMP	-Mitochondrial membrane potential
PAGE	-Polyacrylamide gel electrophoresis
PBS	-Phosphate buffered saline
PCR	-Polymerase chain reaction
PE	-Phycoerythrin
PI	-Propidium iodide
PI3K	-Phosphatidylinositide 3-kinase
PIP2	-Phosphatidylinositol 4, 5-bisphosphate
PIP3	-Phosphatidylinositol (3, 4, 5)-triphosphate
PKC	-Protein kinase C
PMA	-Phorbol myristate acetate
PMSF	-Phenylmethanesulfonyl fluoride
ROS	-Reactive oxygen species
SDS	-Sodium dodecyl sulfate
TAM	-Tumor associated macrophage
TDF	-Tumor derived factor
TGF	-Transforming growth factor
TNF	-Tumor necrosis factor
uPA	-Urokinase plasminogen activator
VEGF	-Endothelial growth factor
XTT	-2, 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt
EDTA	-Ethylenediaminetetraacetic acid
TE	-Tris-EDTA
TAE	-Tris-acetate-EDTA
TSS	-Transformation and storage solution

LIST OF TABLES

LIST OF FIGURES

SECTION 1

INTRODUCTION

Section 1

INTRODUCTION

Over the last six decades, we have witnessed significant strides over advancements in the field of medical research. Severe and the most crippling diseases of the past like plague and smallpox, are no more a threat to humanity now. Such a vast array of vaccines, drugs and bio-medical technologies have been developed that it is perhaps now possible to live with an artificial heart for as long as two years. However, be it the lifestyle changes or nature's way of response, a spectrum of new diseases is adding to the teething troubles of already existing ones. Mutated, resistant and highly evolved strains of bacteria, viruses and other pathogens are wreaking global havoc within a short time. Sadly, the dazzling pace of development in medical science is not able to cope efficiently with the challenges these little bugs offer. Our use, rather, abuse of antibiotics is hardly a deterrent for these microbes.

Nevertheless, it is not always the microbes challenging our big human brain. Complications arising out of malfunctions in cells, organs and systems of human body are not uncommon. One such condition is cancer, which continues to be a global burden affecting both developed and developing countries. Although treatment strategies in the last decade have scaled new heights of accomplishments in combating this deadly disease, cancer still remains a mystery in many ways. The most common methods of anti-cancer treatments that are in use today are

- Chemotherapy
- Radiotherapy
- Surgery

More often than not, a combination of all these three therapies is administered for treating cancer patients. However, complete removal of cancer by modern anti-cancer treatments are rare with the treatment benefits often limited only to a gain in survival time of the patient by few additional years. The major hindrance in success of these therapeutic approaches is the relapse of the disease with time. A small number of tumor cells within cancer develop resistance and evade drug toxicity by a mechanism still largely unknown. This minority of cells left behind by current treatment methods including chemotherapy are responsible for the recurrence of cancer and further, metastasis. Hence, the challenge of future anti-cancer therapies lies in developing strategies to overcome the drug resistivity of tumor cells.

1.1. Concerns associated with present anti-cancer therapies

Cancer cells can develop resistance to drugs through different mechanisms such as active efflux of the drug and reducing the effective concentration of it in the cell, by modifying the target of the drug by mutation, through overexpression of the target protein or by simply inactivating the drug itself. Expulsion of the drug through molecular pumps located at the cell surface is the primary mechanism of acquiring multidrug resistance (MDR) in many types of cancer. Transporter proteins belonging to two distinct classes help in acquiring MDR: one, the members of ATP-Binding Cassette (ABC) transporter superfamily and the other, solute carrier transporter superfamily (Liu, 2009). While the overexpression of former group of proteins actively expels the drugs from the cell, the

downregulation of the latter can reduce the uptake of anti-cancer drugs by the cells.

There are 48 proteins belonging to ABC transporter superfamily found in humans. Among them, only three have been shown to be frequently associated with MDR both *in vitro* and *in vivo*. They are P-glycoprotein (ABCB1 or MDR1), MDR associated protein (ABCC1 or MRP1) and breast cancer resistance protein (ABCG2 or BCRP) (Stavrovskaya and Stromskaya, 2008). All these three membrane transporter proteins have broad substrate specificity and can expel a variety of chemotherapeutic drugs used in cancer treatment.

An exciting outcome of stem cell research is the finding that many of the normal stem cells have high expression of ABC transporter proteins. The principal necessity for increased expression of ABC proteins in stem cells is the need to protect them from environmental toxins and drugs (Schinkel et al., 1994). This raises an intriguing possibility of normal stem cells in tissues being transformed into cancerous cells and retaining their stemness properties with constitutive expression of MDR1 and other associated ABC transporter proteins. Such a pool of quiescent cancer stem cells within cancer will be a tumor reservoir for the relapse of the disease at a later stage.

Currently existing methods of anti-cancer treatments focus largely on the rapidly proliferating bulk populations of cells within cancer. This majority of tumor cells is sensitive to drugs, thereby making the tumor shrink in size with treatment. However, as and when the treatment ceases, there appears to be a minor population of cells left un-killed with an indefinite proliferating potential and an ability to self-renew, giving rise to any type of cell within the overall tumor population. This small subset of tumor cells within bulk populations of cancer is known as tumor initiating cells or cancer stem cells (CSCs). These tumor cells are

highly resistant to chemotherapeutic drugs and have high expression of MDR associated proteins.

CSCs resemble normal stem cells in many ways including the expression of various anti-apoptotic genes (Opferman et al., 2005; Madjd et al., 2009) and ABC transporter genes (Zhou et al., 2001; Liu, G. et al., 2006; Dean, 2009). They are quiescent, with a slow rate of proliferation (Moore and Lyle, 2011) and can exhibit symmetric as well as asymmetric cell division (Clarke et al., 2006). These overlapping properties between normal stem cells and cancer stem cells make it a daunting task to develop a targeted therapeutic regimen for cancer stem cells. Any treatment method developed to target cancer stem cells also carries with it the risk of destroying normal stem cells. Our current understanding of cancer stem cell biology is limited and hence, further insights into the differences in the physiological and immunological aspects of cancer stem cells are required to identify drug targets that are unique to cancer stem cells.

1.2. Immune system and Cancer

The immune system is a complex and sophisticated machinery designed to distinguish between 'self', 'non-self' materials, and safe guard the body against invasion by foreign pathogens, in addition to the routine clearance of aged and damaged cells. It is therefore surprising to see the magnitude of cancer development under the vigil of immune system. While cells of the innate immune system promote tumor progression by angiogenesis, tissue remodeling, release of growth factors and pro-tumoral cytokines, cells of adaptive immune system help in inhibition of tumor growth through efficient antigen presentation and cytotoxic T-cell activity (de Visser et al., 2006). In the tumor, the primary antigen presenting dendritic cells are differentiated poorly along with accumulation of immature myeloid cells, making the adaptive immune system relatively poor in defending

against tumor development. Further, microenvironment of the tumor is reported to have heavy infiltration of cells of innate immune system, especially macrophages, and these tumor-associated macrophages (TAM) are known to enhance tumor growth and metastasis (Condeelis and Pollard, 2006; Sica et al., 2008).

1.3. Macrophage Colony Stimulating Factor

A number of cytokines and growth factors including MCSF, IL-4, IL-10, IL-13 etc., play an important part in recruiting monocytes and macrophages into the tumor. Among them, the role of Macrophage Colony Stimulating Factor (MCSF) is well pronounced. MCSF is a growth factor responsible for survival, proliferation and differentiation of cells of haematopoietic lineages. Tumor cells have increased expression of MCSF, which acts as a chemo-attractant for monocytes and macrophages. Moreover, tumors that express MCSF have been reported to show poor prognosis with treatment. While the mechanism of MCSF promoting tumor development through macrophages is well deciphered, the reason behind their association with dismal response to treatment is not known. The main question that the current research work intends to address is whether MCSF plays any role in increasing the drug resistance of tumor cells.

This thesis aims to explore the properties of Macrophage Colony Stimulating Factor (MCSF) when combined with the conventional anti-cancer drug 5-fluorouracil (5-FU) on a human glioblastoma cell line, U87MG. We attempt to look into the details and decipher the molecular events associated with poor prognosis in this tumor, which overexpresses MCSF.

1.4. Objectives

- To clone Macrophage Colony Stimulating Factor (MCSF) into a mammalian expression vector.
- To generate a stable cell line of U87MG, glioblastoma cells expressing MCSF.
- To explore the effect of anticancer drug, 5-fluorouracil (5-FU) on MCSF expressing U87MG cells.
- To investigate the molecular events unfolding in U87-MCSF cells upon 5-FU treatment.

1.5. Salient features of the present work

MCSF, as one of the primary cytokines attracting monocytes into the tumor, plays a vital role in supporting tumor growth. Further, MCSF acts in synergy with macrophages in increasing the concentrations of angiogenic growth factors like VEGF. Although there are some contradictory reports on the role played by MCSF in tumors, many of the pro-tumoral pathways of MCSF have already been deciphered. However, there is an intriguing puzzle that needs be solved. The reason behind the poor prognosis in many tumors which overexpress MCSF has to be found. Solving this mystery needs further research and understanding of the molecular events taking place upon treatment with drugs. This thesis attempts to gain deeper insights into the molecular events which take place in MCSF expressing U87MG, human glioblastoma cell line upon treatment with 5-FU. The work started with establishing U87-MCSF, a stable cell line having constitutive expression of MCSF. Then, detailed studies were carried out in U87MG and U87-MCSF cells after treatment with 5-FU. Our results demonstrated that MCSF expression resulted in retardation of cell proliferation. Further, U87-MCSF cells

underwent epithelial-mesenchymal transition, which ultimately culminated in the augmentation of the cancer stem cell population and increase in the resistance of U87-MCSF cells to 5-FU treatment.

SECTION 2

REVIEW OF LITERATURE

Section 2

REVIEW OF LITERATURE

2.1. Growth factors

Growth factors occupy a central stage in controlling cellular growth, proliferation and differentiation. Growth factors exert their functional properties in the target cells by binding to their specific receptors, which then trigger key downstream signaling pathways. Advancements in the field of genetic engineering and recombinant DNA technology have facilitated the cloning and purification of individual recombinant growth factors to study their functions *in vitro* and *in vivo*. Depending on their type, growth factors can mediate autocrine, paracrine or endocrine signaling pathways. Growth factors can be subdivided into different families each having a group of related proteins with specific functions. One such group of growth factors controlling the growth, proliferation and differentiation of hematopoietic cells is known as hematopoietic growth factors. They are also called colony stimulating factors, as they stimulate the proliferation of hematopoietic cells in the form of colonies in semisolid culture media.

2.2. Colony Stimulating Factors

In early 1960s and 1970s, an unknown cell product was found to be responsible for the stimulation of colony formation in specific populations of granulocyte and macrophage cultures (Metcalf, 2008). It was soon named as colony-stimulating factor (CSF) owing to its function. CSFs are secreted glycoproteins, responsible for

the growth, proliferation and differentiation of cells of hematopoietic lineages. The initial expectation of a single CSF mediating the proliferation of all hematopoietic cells was thwarted with the discovery and purification of macrophage colony stimulating factor (MCSF or CSF-1) that stimulated only macrophages (Stanley et al., 1975; Stanley and Heard, 1977), granulocyte-macrophage colony stimulating factor (GM-CSF or CSF-2) that stimulated granulocytes, macrophages and mixed colonies depending on the concentration used (Burgess et al., 1977), granulocyte colony stimulating factor (G-CSF or CSF-3) that stimulated only granulocytes (Nicola et al., 1983) and a multi-CSF (commonly termed as interleukin 3 or IL3) that stimulated a wide variety of hematopoietic cell types (Ihle et al., 1982b; Ihle et al., 1982a). CSFs are primarily glycoproteins with their molecular weight ranging from 18 to 70 kDa with a half-life of 1-6 hrs. The amount of production and secretion of CSFs is very low under normal conditions but is increased drastically in response to infection by microorganisms, endotoxin or any other foreign cells (Metcalf, 2010). Although each CSF is capable of acting independently through their unique membrane receptors located in the target cells, their proliferative potential is enhanced in the presence of other CSFs (Metcalf, 1990).

2.3. Macrophage Colony Stimulating Factor (MCSF or CSF-1)

Macrophage colony stimulating factor is the primary growth factor regulating the growth, proliferation and differentiation of cells of hematopoietic lineages including monoblasts, promonocytes, monocytes, macrophages and osteoclasts (Stanley et al., 1978; Stanley et al., 1997; Yeung and Stanley, 2003). MCSF is encoded by a unique gene; however, through alternative mRNA splicing and differential post-translational modification, three different forms of MCSF, such as a secreted glycoprotein, a secreted proteoglycan and a short membrane bound isoform are found (Stanley et al., 1997). MCSF is secreted by various types of cells

like monocytes, fibroblasts, osteoblasts, stromal cells, endothelial cells and tumor cells. All the biological effects of MCSF are mediated through a type III receptor tyrosine kinase (Hamilton, 1997). MCSF requires the synergistic action of IL-1 and IL-3 during differentiation of early myeloid bone marrow cells (Bartelmez et al., 1989). However, at a later stage, MCSF can be a self-regulator in controlling the proliferation and differentiation of cells of the mononuclear phagocytic system.

In humans, the MCSF gene is located at 1p21 – p13 and in mice, it is located at chromosome 3, (51 cM) (Douglass et al., 2008). This single gene produces a primary mRNA, from which five mRNA species of varying length arises through alternative splicing (Cerretti et al., 1988; Shadle et al., 1989) (Figure 2.1). The mRNA transcripts of length 1.6 kb and 3.1 kb give rise to a shorter, membrane bound MCSF, while the transcripts of length 2.6, 3.7 and 4 kb are processed to form secreted MCSF. Various proteases including a signal peptidase, MCSF β-convertase, and MCSF α-convertase are involved in the processing of nascent protein into different forms of mature MCSF protein. The MCSF protein, in general consists of five sections: an N-terminal signal peptide composed of 32 amino acids, a receptor binding domain of 149 amino acids, a spacer region with varying length, a transmembrane domain with 24 amino acids and a cytoplasmic tail composed of 35 amino acids (Douglass et al., 2008).

MCSF is processed to form a disulfide-linked homodimer (Wang et al., 1993) which can either stay attached to the membrane or proteolytically processed to form the secreted forms of MCSF. The secreted MCSF can either be a proteoglycan or a glycoprotein. All isoforms of MCSF are biologically active and can stimulate cell proliferation on target cells (Stein et al., 1990; Wang et al., 1993).

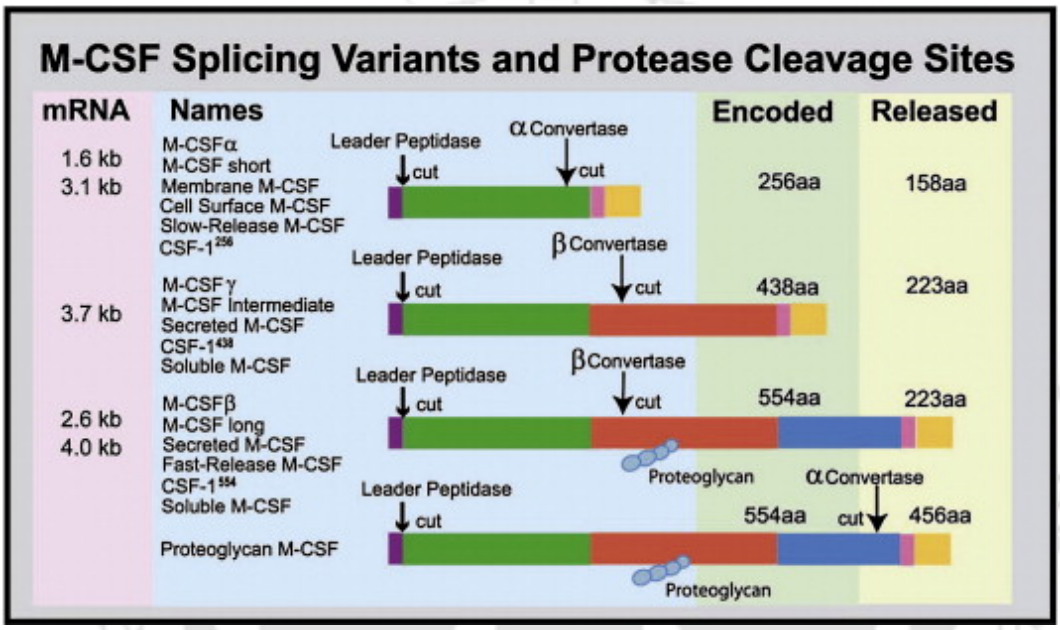

Figure 2.1. Splicing variants of Macrophage Colony Stimulating Factor and location of specific proteolytic cleavage sites. Purple color denotes the leader sequence, green-MCSF core region, red-the spacer region, pink-transmembrane region, orange-intracellular region. Reproduced with permission from reference (Douglass et al., 2008). Copyright @ 2008 Elsevier.

2.4. MCSF receptor and signaling cascades

All the biological effects of MCSF are mediated through CSF-1R, a receptor belonging to type III receptor tyrosine kinase family. It is encoded by the proto-oncogene *c-fms* (Hamilton, 1997). CSF-1R belongs to a family of growth factor receptors which also includes stem cell factor receptor (SCF), α and β receptors for platelet derived growth factor (PDGF-R) and Flt3/flk2 proteins (Roussel, 1994; Bourette and Rohrschneider, 2000). This family of receptors share similar structural features and possess an extracellular N-terminal ligand binding domain, a hydrophobic transmembrane domain and the cytoplasmic tyrosine kinase domain (Bourette and Rohrschneider, 2000). Binding of MCSF to CSF-1R initiates the signaling cascades, the first step of which is the dimerization of the receptor. It is followed by trans-phosphorylation of various tyrosine residues and binding of signaling proteins having SH2 domains to the phosphorylated sites of the receptor.

Although there are twenty tyrosine residues found in the cytoplasmic kinase domain of CSF-1R, phosphorylation of four are shown to mediate the major downstream signaling cascades (Hamilton, 1997; Bourette and Rohrschneider, 2000). In murine CSF-1R, these tyrosine phosphorylation sites are Y697, Y706, Y721 and Y807, while in human CSF-1R, the sites are Y699, Y708, Y723 and Y809. All of these auto-phosphorylation sites are located in the kinase domain of CSF-1R (Hamilton, 1997). The major signaling pathways activated by binding of MCSF to CSF-1R are shown in figure 2.2.

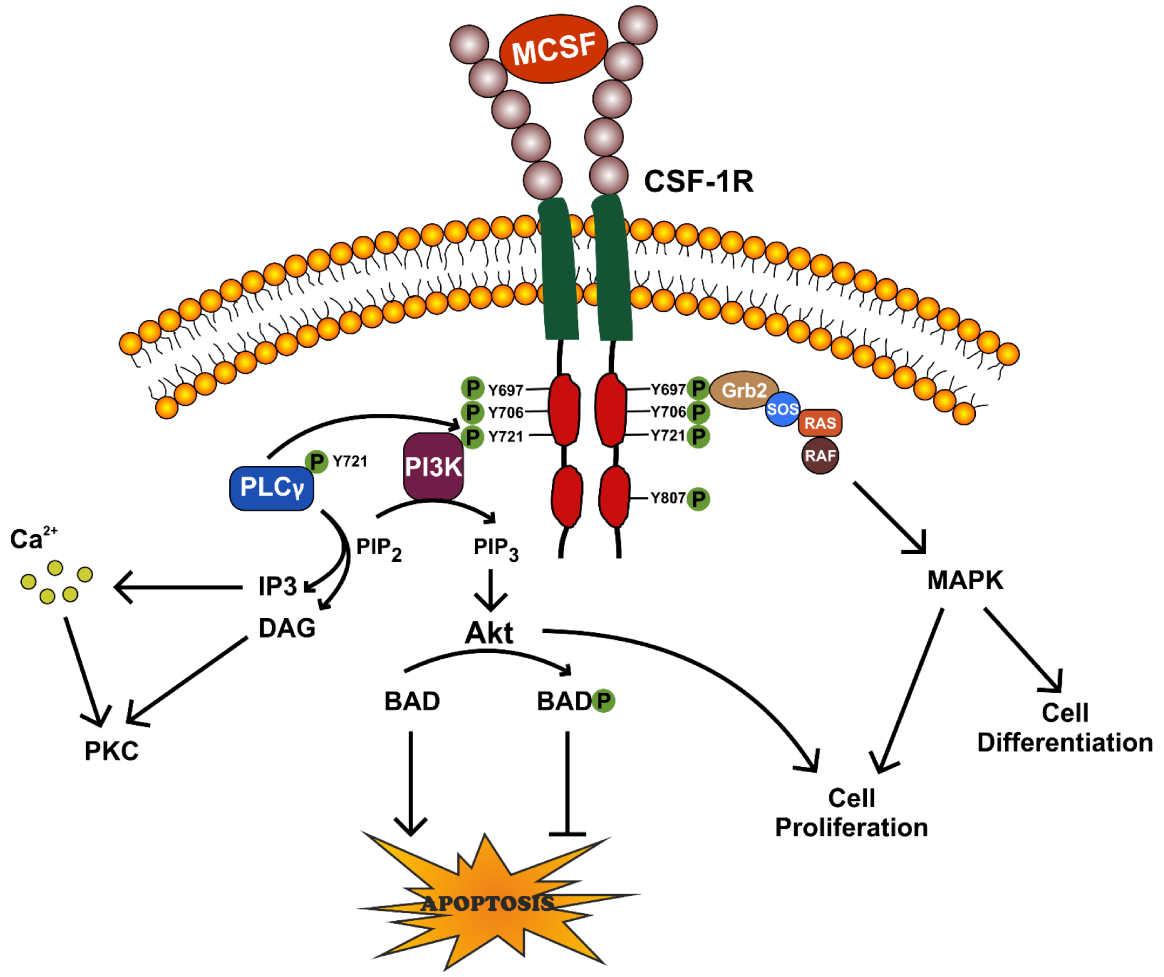

Figure 2.2. MCSF signaling pathways in murine CSF-1R. Binding of MCSF to CSF-1R results in dimerization of the receptor and phosphorylation of tyrosine residues at various positions in the cytoplasmic kinase domain of CSF-1R. While phosphorylation of Y697 activates MAPK signaling pathway, phosphorylation of tyrosine residue at position 721 can initiate PI3K or PLCγ signaling pathways. Picture adapted based on references (Hamilton, 1997; Bourette and Rohrschneider, 2000).

Phosphorylation of the tyrosine residue at position 697 (Y697) in CSF-1R binds Grb2 (van der Geer and Hunter, 1993) and activates the MAP kinase pathway through SOS, Ras and Raf (Bourette and Rohrschneider, 2000), which can eventually lead to cell proliferation or cell differentiation. Phosphorylated Y721 is shown to bind with PI3K (Reedijk et al., 1992) and further activate the PKB/Akt mediated signaling pathways. Phosphatidylinositide 3-kinases (PI3K) phosphorylates phosphatidylinositol 4, 5-bisphosphate (PIP_2) to phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3). PIP_3 then activates Akt, which further initiates the signaling pathways involved in cell proliferation. Activated Akt also plays an important role in the suppression of apoptosis through phosphorylation of Bcl-2-associated death promoter (BAD).

Besides PI3K mediated signaling, phosphorylated Y721 also recruits PLCγ2 (Bourette et al., 1997) which cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2), into two products namely, diacylglycerol (DAG) and inositol 1, 3, 4 P_3 (IP3). IP3 increases the concentration of Ca^{2+} in the cytoplasm by binding with IP3 receptors on the smooth endoplasmic reticulum and releasing Ca^{2+} through calcium channels. Increasing concentrations of Ca^{2+} and DAG trigger the signaling pathways mediated through protein kinase C (PKC). Activation of PI3K or PLCγ2 signaling cascades thus requires binding to the same phosphorylated Y721. Moreover, both PI3K and PLCγ2 share a common substrate, PIP_2. This represents that a fine balance in the activation of these two distinct signaling pathways plays a decisive role in determining the outcome of cell differentiation. Although a protein binding to the phosphorylated Y706 is yet to be determined (Bourette and Rohrschneider, 2000), STAT1 activation in CSF-1 stimulated cells is shown to require phosphorylation of Y706 (Novak et al., 1996). Phosphorylation of the tyrosine residue, Y807 situated in the main kinase domain of CSF-1R does not involve directly in any of the signaling pathways, but is instrumental in bringing

about a conformational change that can relay all the proliferation and differentiation signals.

2.5. Physiological functions of MCSF

Hematopoietic stem cells located in adult bone marrow, spleen and fetal liver continuously give rise to lymphoid and myeloid progenitor cells. These progenitor cells further proliferate and differentiate to give rise to a variety of cells belonging to their respective lineages. One of the lineages of myeloid progenitor cells yield monoblasts, promonocytes, monocytes and macrophages. Another class of specialized cells, the osteoclasts, also develops from monocyte/macrophages cell lineage. MCSF is the primary regulator of cells of mononuclear phagocytic system. All these cells express CSF-1R, the receptor for MCSF. All actions of MCSF in these target cells are by signaling through CSF-1R.

Under normal conditions, there is an abundance of MCSF *in vivo*, with the three different isoforms of MCSF playing distinct yet overlapping roles. Most of the tissues and organs in the human body have resident macrophages. Monocytes and macrophage development in most of the tissues require MCSF as a crucial factor. Although langerhans cells, bone marrow monocytes and macrophages present in lymph nodes and thymus can develop independent of MCSF, tissue specific macrophages present in other tissues like muscles, tendons, synovial membranes etc., require MCSF for their development and maturation (Cecchini et al., 1994). Further, MCSF stimulates macrophage activation in response to infection with various pathogenic organisms. In addition to macrophage development, MCSF stimulates the production of several other cytokines including IL-1α, IL-1β, IL-6 and GM-CSF in murine peritoneal cells (Evans et al., 1995). The regulation of monokine production by MCSF is dose-dependent and also time-dependent. Human monocytes when incubated with MCSF secrete

interferon (IFN), tumor necrosis factor (TNF) and also exhibit increased myeloid colony stimulating activity upon stimulation with other inducing agents such as LPS and PMA (Warren and Ralph, 1986). However, the response of MCSF activated monocytes is not uniform and it depends on the type of secondary priming signals. Generally MCSF activated monocytes produce pro-inflammatory cytokines when challenged with LPS. But when the activating stimulus is bacterial CpG-containing DNA, the response is anti-inflammatory and when the stimulus is lipopeptide, no response is observed (Sweet and Hume, 2003).

MCSF plays a crucial role in the regulation of bone physiology. Bone is a dynamic structure which undergoes continuous remodeling involving formation and resorption. Osteoblasts, which are cells of mesenchymal/stromal origin, regulate the formation of bones while osteoclasts, the cells differentiated from mononuclear phagocytic precursors, control bone resorption. For normal and healthy bone structure, a fine balance between osteoblasts and osteoclasts is maintained. MCSF is required for the formation of osteoclasts. Mouse having defective MCSF production were reported to be small, toothless, suffering from low body weight, severe abnormalities in skeletal structure and deficiency in macrophages (Ryan et al., 2001). In 1990, Wiktor-Jedrzejczak et al., reported that a null mutation in MCSF gene in *op*/*op* mouse led to a severe deficiency of osteoclasts and subsequent development of osteopetrosis. This defect was partially rectified when CSF-1 producing L929 cells were implanted in *op*/*op* mouse (Wiktor-Jedrzejczak et al., 1990). However, external administration of MCSF was not enough to overcome all the defects seen in *op*/*op* mouse.

Outside the hematopoietic system, MCSF has an important role in the development and regulation of placenta, mammary gland and brain. Almost all functions of MCSF are deciphered by using a mouse model where a homozygous null mutation in the coding region of MCSF gene was generated by a single

basepair insertion (*op*/*op* mouse). This *op*/*op* mice completely lacking MCSF production, have severe malfunctioning of central nervous system including visual and auditory defects and this abnormal shortcomings in functioning of brain can be rescued by daily postnatal administration of MCSF (Michaelson et al., 1996). *Op*/*op* mice also develop an aberrant mammary gland because of the failure to form lactating ducts during pregnancy (Pollard and Hennighausen, 1994). Further, MCSF was also reported to be a crucial factor necessary for normal fertility (Pollard et al., 1991).

2.6. MCSF and cancer

In order to sustain incessant proliferation, many tumors of non-hematologic origin secrete different types of endocrine and other growth factors. MCSF is produced by different types of cells including tumor cells. MCSF is overexpressed especially in breast cancers (Ramakrishnan et al., 1989; Kacinski et al., 1991) and tumors of female reproductive tract like ovarian (Ramakrishnan et al., 1989; Kacinski et al., 1990) and endometrial cancers (Kacinski, 1995). High MCSF levels are also seen in patients with colorectal cancer (Mroczko et al., 2007), pancreatic cancer (Groblewska et al., 2007), prostate cancer as well as head and neck cancer (McDermott et al., 2002). The circulating level of MCSF is often used as a method of estimating prognosis of patients. Overexpression of MCSF and its receptor, CSF-1R in tumors have been associated with poor prognosis (Chambers et al., 1997).

MCSF is known to infiltrate sites of injury and inflammation with mononuclear phagocytes. Excess amount of MCSF secreted by tumors acts as a chemo-attractant, infiltrating tumors with cells of mononuclear phagocytic system. In addition to MCSF, tumor cells also produce other chemokines and growth factors, like CCL3, CCL4, CCL5, CXCL12, transforming growth factor-α (TGF-α),

transforming growth factor-β (TGF-β), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (Sica et al., 2006; Richards et al., 2013). These tumor derived factors (TDFs) are responsible for the bulk mobilization of cells of immune system into tumor. The immune infiltrate in tumor is comprised of polymorphonuclear granulocytes, monocytes, immature dendritic cells and various types of T lymphocytes. The presence of NK cells in tumor is very rare. The accumulation of immune cells in tumor has given rise to conflicting reports with some reporting better prognosis and others, providing evidences for tumor growth and metastasis.

MCSF has been specifically implicated in the process of metastasis in breast cancer though the incidence and the initial growth of tumor is not affected in the absence of MCSF. Homozygous null mutation of MCSF gene in mice shows a depleted macrophage population in breast cancer, resulting in reduced malignancy and metastasis (Lin et al., 2001; Lewis and Pollard, 2006). Restoring the local concentrations of MCSF in this mouse model of mammary tumor by transgenic expression of MCSF gene resulted in the promotion of tumor development. The pro-tumoral actions of MCSF are exerted mainly through macrophages. In general, monocytes can get differentiated locally to form either M1 (classical) or M2 (alternate) polarized macrophages, depending on the stimulus and the type of cytokines present in the tumor (Mantovani et al., 2002; Sica et al., 2006; Qian and Pollard, 2010). The monocytes that infiltrate into tumors, differentiate predominantly into M2 polarized macrophages. They are also known as tumor associated macrophages (TAM). The M2 polarized macrophages are characterised by elevated production of interleukin-10 (IL-10), IL-1 receptor antagonist (IL-1ra) and low interleukin-12 (IL-12). IL-10 and other immunosuppressive cytokines secreted by TAMs inhibit the development of tumor specific T-cell immunity. Further, heavy infiltration of monocytes and

macrophages into tumor also facilitate angiogenesis that caters to the continuous supply of nutrients necessary for tumor growth and progression. Tumor cells and the surrounding stromal cells secrete large quantities of angiogenic factors which enhance the vascularisation of tumor. The excess vascularisation also provides an entry point for tumor dissemination into blood vessels moving to distant organs, thereby promoting metastasis.

The presence of monocytes and macrophages in tumor promotes angiogenesis by increasing the level of secretion of various growth factors and chemokines including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF), thymidine phosphorylase (TP) and urokinase plasminogen activator (uPA). Tumor associated macrophages (TAMs) often accumulate in the low oxygen and avascular regions within the tumor (Murdoch et al., 2004; Coffelt et al., 2009). This hypoxia condition of TAMs upregulates the expression of Hypoxia-Inducible Factors (HIFs), which in turn drives the transcription of various mitogenic growth factors including VEGF (Lewis and Murdoch, 2005). VEGF is a well-known angiogenic growth factor which increases the proliferation of endothelial cells and stimulates the formation of blood vessels. Large quantities of VEGF are secreted by the tumor cells as well as by TAMs (Harmey et al., 1998; Schoppmann et al., 2002; Mantovani et al., 2006). Tumor growth and invasion resulting from the elevated production of VEGF and its receptors have been documented in many tumors including breast cancer (Bachelder et al., 2002), prostate cancer (Soker et al., 2001), glioblastoma (Plate et al., 1994), ovarian cancer (Boocock et al., 1995), colon cancer (Takahashi et al., 1995) and liver cancer (Yoshiji et al., 1999). MCSF, apart from attracting monocytes into the tumor also acts as a transcriptional regulator of VEGF production in monocytes (Eubank et al., 2003; Curry et al., 2008). Further, the importance of MCSF in the degradation of extracellular matrix through production of urokinase,

and augmentation of invasive properties is noted in ovarian cancer cells (Chambers et al., 1995).

Nevertheless, MCSF has a potential role in eliciting anti-tumor response as well. Addition of purified MCSF to the human ovarian cancer cells has been documented to induce concentration dependent growth inhibition *in vitro* (Kawakami et al., 2000). Rat T9 glioma cells transfected with the gene corresponding to membrane isoform of MCSF (mMCSF) is killed by macrophages in a concentration dependent fashion (Jadus et al., 1996). This direct killing of mMCSF expressing tumor cells by macrophage *in vitro* occurs through phagocytosis (Jadus et al., 1998; Jadus et al., 2003). When injected into an immunodeficient mice, the site of mMCSF transduced human glioblastoma cells is reported to be heavily infiltrated by macrophages within 4 hours and these infiltrating macrophages are seen killing the tumor cells by paraptosis (Jadus et al., 2003). When monocytes or macrophages come into contact with tumor cells expressing mMCSF, they release reactive oxygen species (ROS). ROS then activates big potassium channels (BK channels) which in turn initiate paraptosis, a process where excessive swelling and vacuolization leads to cell death (Hoa et al., 2007). Glioblastoma cells transduced with mMCSF gene also conferred protective immunity in rat when challenged with glioblastoma cells, suggesting the potential application of these mMCSF expressing tumor cells as tumor vaccines (Jadus et al., 2003). The overall role played by MCSF within the tumor microenvironment is depicted in Figure 2.3.

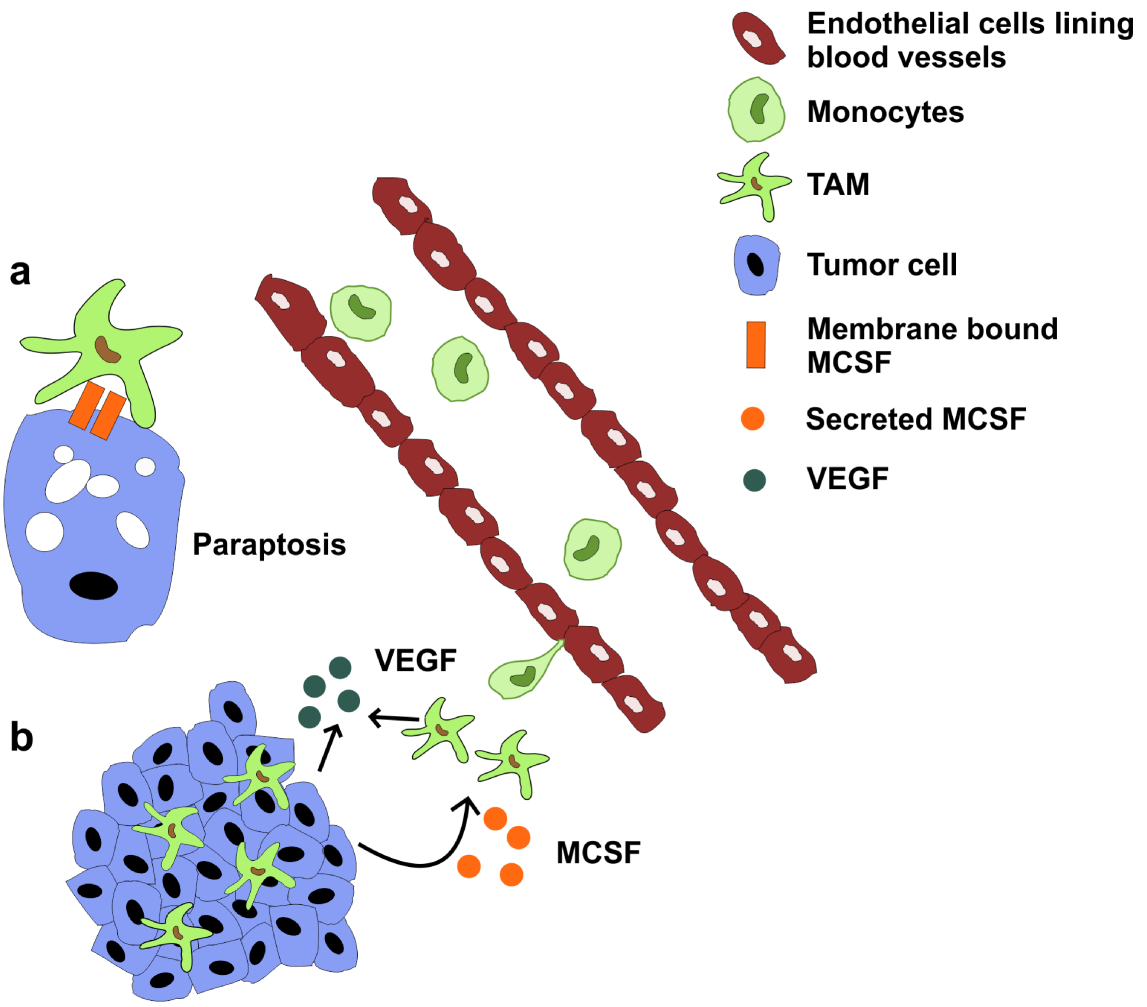

Figure 2.3. Scheme for the role of MCSF in cancer. (a) The anti-tumor properties are elicited when TAMs comes into contact with tumor cells expressing mMCSF. (b) Pro-tumor role of MCSF where TAMs increases angiogenesis and growth of tumor.

2.7. Cancer stem cells

One of the characteristic features of tumor is the presence of heterogeneous population of cells within it. The heterogeneity can be envisaged in both phenotypic as well as genotypic variations in the cells comprising tumor. A small percentage of cells within tumor has been seen to have remarkably high pleiotropic properties. They are biologically distinct and resistant to commonly used anti-cancer therapies. This minor population of cells also has the capacity to initiate tumor when injected into a mice. Owing to this property, these cells are named as cancer initiating cells. As the functional properties of cancer initiating cells are exhibited by undergoing self-renewal and differentiation just like normal stem cells, they are also called as cancer stem cells (CSC).

The biology of cancer stem cells is a union between stem cell biology and oncology. CSCs share many of the properties of normal stem cells including self-renewal, the ability to proliferate indefinitely and the pleiotropic property of giving rise to cells of multiple lineages (Reya et al., 2001; Jordan et al., 2006; Ailles and Weissman, 2007). This leads to a puzzling question that whether organ stem cells acquire the tumorogenic phenotypes and become CSCs or is it the tumor which develops the stem cell like properties through genetic and epigenetic changes. Although it is more likely that the normal stem cells get mutated to become CSCs, more evidences are accumulating to show the possibility of committed progenitor cells acquiring the mutations required for the self-renewal capacity and becoming CSCs (Huntly et al., 2004; Krivtsov et al., 2006). It is also possible that tumor cells undergo de-differentiation process by cellular fusion with stem cells (Bjerkvig et al., 2005). Incidence of organ stem cells from transplanted kidney migrating and fusing with skin epithelium to undergo neoplastic transformation is also reported (Aractingi et al., 2005).

The CSCs in tumor populations are isolated based on the expression of certain cell surface markers (Bjerkvig et al., 2005; Jordan et al., 2006). CSCs have been identified in many types of cancer including blood, brain, colon, breast and pancreas. The commonly expressed stem cell markers for identification of CSCs in various types of tumor are given in table 2.1

Table 2.1. Commonly identified CSC markers in various types of tumors. Table Courtesy: (Ailles and Weissman, 2007). Copyright @ 2007 Elsevier.

Tumor type	CSC phenotype
Breast	CD44+ CD24−/low
CNS	CD133+
Multiple myeloma	CD138−
Melanoma	CD20+
Prostate	CD44+ α2β1+ CD133+
HNSCC	CD44+
Colon	CD133+
Colon	CD44+ EpCam+ CD166+
Pancreatic	CD44+ EpCam+ CD24+

CSCs form a small minority of cells within the bulk tumor. Although the remaining major population of cells in tumor possess the ability to proliferate extensively, their life-span is limited and they are destined to die. Current anti-cancer therapies actively target this bulk component of tumor. However, the silent

persistence of resistant and self-renewing CSCs leads to relapse of the disease with the cessation of therapy.

2.8. Implications of CSCs in drug resistance

The functional properties of normal stem cells play an important role in the maintenance of adult tissues over the lifetime of an individual. The decision of a stem cell to self-renew and differentiate is commanded by tightly controlled regulatory mechanisms. Any breakdown in the regulatory machinery of self-renewal and proliferation can result in a mayhem and that happens precisely in CSCs (Reya et al., 2001). Apart from having a deregulated self-renewal and proliferation, CSCs are quiescent, possess highly efficient DNA repair mechanism and they rarely undergo apoptosis. However, the most important gain of tumor from the presence of CSCs is the development of drug resistance. Normal stem cells express high levels of drug effluxing proteins like ABCB1 and ABCG2 (Bunting, 2002). The ABC transporter family of proteins exerts a protective effect against environmental toxins in normal stem cells which helps the host to avoid accidental damage and retain the pool of stem cells necessary for tissue replenishment. But in tumor, this property of CSCs results in undesirable outcomes. Overexpression of ABC transporter proteins confers resistance to common anti-cancer drugs like cisplatin (Shafee et al., 2008), doxorubicin (Tada et al., 2002), 5-fluorouracil (Oguri et al., 2007), mitoxanthrone and topotecan (Scheffer et al., 2000). CSC mediated drug resistance has been observed in breast cancer (Shafee et al., 2008), pancreatic cancer (Hermann et al., 2007), glioblastoma (Bao et al., 2006) and colorectal cancer (Dylla et al., 2008).

The current knowledge about the biology of CSCs is limited. Further, the presence of CSCs is yet to be proved beyond doubt in many types of tumor. Although many efficient therapies have been developed against the bulk tumor,

drugs targeting CSCs is yet to gain any significant success. Drugs developed against CSCs should spare normal stem cells from being killed. In order to achieve that, existence of any hierarchy in the pathways concerning the development of differentiated tumor cells from CSCs should be investigated. This could also delineate normal stem cell pathways from CSC pathways. Another challenge lies in overcoming the intrinsic drug resistance of these CSCs. Navel drugs and small molecule inhibitors against the drug efflux proteins should be developed. For achieving all these stated objectives, a combinatorial approach targeting the bulk tumor, CSCs and ABC transporter proteins has to be established. Such a therapeutic strategy may have the potential to stop cancer from recurring, thereby leading to a complete cure.

SECTION 3

MATERIALS AND METHODS

Section 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and Reagents

Common chemicals including Sodium dodecyl sulphate (SDS), Sodium hydroxide, Acetic acid, Potassium acetate, Ammonium acetate, Ethylenediaminetetraacetic acid (EDTA), Ampicillin, Kanamycin, Isopropyl alcohol, Hydrochloric acid, Ethyl alcohol, Methyl alcohol were procured from SRL, India or Merck, India.

Molecular biology and cell culture grade chemicals, such as Dimethyl sulfoxide, Glycerol, Phenol, Chloroform, Glucose, G418, Agarose, Dulbecco's Modified Eagle Medium (DMEM), Sodium bicarbonate, Penicillin-Streptomycin antibiotic solution, Trypsin-EDTA solution, Triton X-100, 5-fluorouracil were purchased from Sigma Aldrich, USA.

Fetal Bovine Serum (FBS) was purchased from PAA Laboratories. PCR components including Taq polymerase and dNTP, DNA markers, such as 1kb DNA ladder and 100bp DNA ladder were obtained from Bioline, Spinchrom Life Sciences, India. GenElute Plasmid DNA isolation (Miniprep) kit, GenElute Total RNA extraction (Miniprep) kit and GenElute Gel extraction kit were purchased from Sigma Aldrich, USA.

Luria Bertoni Broth and Agar powder were procured from Himedia, India. cDNA synthesis kit was from Thermo Scientific, Restriction enzymes and T4 DNA

ligase were acquired from New England Biolabs. pGEMT-easy vector was purchased from Promega, USA.

3.1.2. Plastic and glass wares

All the glass wares used were obtained from Borosil International, India. PCR tubes was from Greiner, micro tips, centrifuge tubes, bacterial culture petridishes and other common plastic wares were procured from Tarsons Products Pvt. Ltd., India. Plastic wares used for mammalian cell culture works were purchased from Greiner bio-one, Corning and BD Falcon, BD Biosciences, India.

3.1.3. List of Bacterial strains

Strain	Genotype
1. *Escherichia coli* DH5α	F– Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *recA*1 *endA*1 *hsdR*17 (r_K^-, m_K^+) *deo*R *thi*-1 *pho*A *sup*E44 λ^- *gyr*A96 *rel*A1

3.1.4. Mammalian cell lines

U87MG (Human Glioblastoma), HeLa (Human Cervix adenocarcinoma) and ACHN (Human Renal carcinoma**)** cells were procured from National Centre for Cell Science (NCCS), Pune, India.

3.1.5. Media, solutions and buffers

3.1.5.1. Luria Bertani broth media for culture of bacterial cells

25 g of Luria Bertani broth powder was dissolved in 1 litre of double distilled water. The media was aliquoted into small volumes in test tubes or conical flasks and sterilised by autoclaving at 121 °C, 15 psi for 20 minutes.

3.1.5.2. LB Agar media for culture of bacterial cells

LB agar media were prepared by using 1.5 % Agar in Luria Bertani broth and sterilised by autoclaving at 121 °C, 15 psi for 20 minutes. LB agar plates were made by pouring 20 ml of LB agar media along with desired antibiotics into petridishes.

3.1.5.3. Dulbecco's Modified Eagles Media (DMEM) for mammalian cell culture

DMEM media was prepared by adding 13.47 g of DMEM powder having high glucose content, 3.75 g of Sodium bicarbonate, and Penicillin (50U/ml)-Streptomycin (50mg/ml) solution in 1 litre of double distilled water. For routine culture of mammalian cells, DMEM media was supplemented with 10 % of Fetal Bovine Serum (FBS).

3.1.5.4. Antibiotics

Table 3.1. List of antibiotics used for culturing bacterial cells

	Final concentration (μg/ml)	Manufacturer
1. Ampicillin	100	Himedia, India.
2. Kanamycin	50	Himedia, India.

Table 3.2. List of antibiotics used for mammalian cell culture

	Final concentration (μg/ml)	Manufacturer
1. G418	600 (Initial selection) 400 (Regular maintenance)	Sigma Aldrich, USA

3.1.5.5. 0.5 M EDTA solution

Disodium EDTA (Ethylenediaminetetraacetic acid) salt (186.12 g) was dissolved in 0.8 litre of double distilled water. To facilitate the dissolving of EDTA salt, NaOH pellets were added (approximately 20 g of NaOH pellets for 1 litre of EDTA solution). The pH was adjusted to 8.0 and the solution volume was made up to 1 litre. The solution was sterilised by autoclaving.

3.1.5.6. Solutions used in plasmid isolation

Solution I: 50 mM glucose, 25 mM Tris-cl (pH-8.0) and 10 mM EDTA (pH-8.0). The solution was autoclaved at 121 °C, 15 psi for 15 minutes and stored at 4 °C.

Solution II (prepared freshly): 0.2 N NaOH, 1 % SDS (w/v).

Solution III: 5 M potassium acetate-60 ml, glacial acetic acid-11.5 ml and water 28.5 ml. The solution was stored at 4 °C.

3.1.5.7. Tris-EDTA (TE) buffer

This buffer contained 10 mM Tris-Cl (pH 7.4) and 1 mM EDTA (pH 8.0). It was sterilized by autoclaving at 121 °C, 15 psi for 20 minutes and stored at 4 °C.

3.1.5.8. Tris-acetate-EDTA (TAE) buffer

Working solution of TAE buffer contained 40 mM Tris-acetate and 1 mM EDTA (pH 8.0). It was prepared as 50 X concentrated stock solution (242 g of Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA (pH-8.0)) and used at 1X concentration.

3.1.5.9. Transformation and storage solution (TSS) buffer

TSS buffer contained 85 % (v/v) LB media, 10 % (w/v) polyethylene glycol (PEG, MW 8000), 5 % (v/v) DMSO and 50 mM $MgCl_2$.

3.1.5.10. 6X DNA Loading dye

The DNA loading dye is prepared by adding 0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol in water.

3.1.5.11. Phosphate buffer saline (PBS)

Phosphate buffer saline contained 140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM di-sodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate. The pH of the buffer was adjusted to 7.4.

3.1.5.12. Propidium iodide staining solution

The propidium iodide staining solution contained 50 μg/ml PI, 0.1 mg/ml RNase A and 0.05 % triton X-100 in PBS.

3.1.5.13. SDS-Polyacrylamide gel electrophoresis

Table 3.3. Solutions and buffers used in SDS-PAGE

30 % acrylamide-bisacrylamide solution	29.2 g Acrylamide and 0.8 g of bis-acrylamide was dissolved in 100 ml of water.
1.5 M Tris.Hcl, pH 8.8	18.18 g of Tris base was dissolved in 80 ml of water. The pH was adjusted to 8.8 with 2 N HCl and the volume was made up to 100 ml.
1 M Tris.Hcl, pH 6.8	12.1 g of Tris base was dissolved in 80 ml of water. The pH was adjusted to 6.8 with 2 N HCl and the volume was made up to 100 ml.

1x Tris-glycine electrophoresis buffer	25 mM Tris, 250 mM glycine, 0.1 % (w/v) SDS.
1x SDS-gel loading buffer	50 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 1 % β-mercaptoethanol, 0.1 % bromophenol blue, 10 % (v/v) glycerol

3.1.5.14. Western blotting

Table 3.4. Solutions and buffers used in western blotting

PBST solution	PBS containing 0.1 % Tween-20.
Transfer buffer (Towbin buffer)	25 mM Tris, 192 mM glycine and 20 % methanol.
Blocking solution	5 % BSA in PBST
Ponceau solution	0.1 % ponceau solution in 5 % acetic acid

3.1.5.15. Primers

Table 3.5. List of primers used

Gene	**Primer sequence**
β-actin	Forward: 5`-CTGTCTGGCGGCACCACCAT-3` Reverse: 5`-GCAACTAAGTCATAGTCCGC-3`
MCSF	Forward: 5`-GGAATTCCCATGACCGCGCCGGGCGCC-3` Reverse: 5`-AAGGGCCCCACTGGCAGTTCCAC-3`
CSF-1R	Forward: 5`- CTGGAGCTCCGGGACCTGCT-3`

	Reverse: 5`- CCACTTCACAGGCAGGCGGG-3`
GFAP	Forward: 5`- CGGCTCGATCAACTCA-3` Reverse: 5′-CTCCTCCAGCGACTCAAT-3′
hTERT	Forward: 5′-GTACATGCGACAGTTC-3′ Reverse: 5′-TTCTACAGGGAAGTTCAC-3′
CyclinD1	Forward: 5`-CGCCCCACCCCTCCAG-3` Reverse: 5`-CGCCCAGACCCTCAGACT-3`
CyclinE	Forward: 5`-CCACACCTGACAAAGAAGATGATGAC-3` Reverse: 5`-GAGCCTCTGGATGGTGCAATAAT-3`
CyclinA2	Forward: 5`-ACGGCGCTCCAAGAGGACCA-3` Reverse: 5`-AGCCAGGGCATCTTCACGCT-3`
CyclinB1	Forward: 5`-TCTGGATAATGGTGAATGGACA-3` Reverse: 5`-CGATGTGGCATACTTGTTCTTG-3`
CyclinB2	Forward: 5`-AAAGTTGGCTCCAAAGGGTCCTT-3` Reverse: 5`-GAAACTGGCTGAACCTGTAAAAAT-3
mdm2	Forward: 5′-CAGCAGGAATCATCGGACTCA-3′ Reverse: 5′-CCTTATTACACACAGAGCCAGGC-3′
ABCB1	Forward: 5`-CCCATCATTGCAATAGCAGG-3` Reverse: 5` -TGTTCAAACTTCTGCTCCTGA-3`
ABCG1	Forward: 5′- CAGGAAGATTAGACACTGTGG -3′ Reverse: 5′- GAAAGGGGAATGGAGAGAAGA-3′
ABCG2	Forward: 5′- CCGCGACAGTTTCCAATGACCT -3′ Reverse: 5′- GCCGAAGAGCTGCTGAGAACTGTA -3′

CD44	Forward: 5'-AGAAGGTGTGGGCAGAAGAA-3' Reverse: 5'-AAATGCACCATTTCCTGAGA-3'
CD24	Forward: 5` CCCACGCAGATTTATTCCAG 3` Reverse: 5` GACTTCCAGACGCCATTTG 3`
Notch-1	Forward: 5′- TCCACCAGTTTGAATGGTCA -3′ Reverse: 5′- AGCTCATCATCTGGGACAGG -3′
E-cadherin	Forward: 5` TGCCCAGAAAATGAAAAAGG 3` Reverse: 5` GTGTATGTGGCAATGCGTTC 3`
N-cadherin	Forward: 5` ACAGTGGCCACCTACAAAGG 3` Reverse: 5` CCGAGATGGGGTTGATAATG 3`
Fibronectin	Forward: 5` CAGTGGGAGACCTCGAGAAG 3` Reverse: 5` TCCCTCGGAACATCAGAAAC 3`
Vimentin	Forward: 5` GAGAACTTTGCCGTTGAAGC 3` Reverse: 5` GCTTCCTGTAGGTGGCAATC 3`
Caspase-3	Forward: 5`-TTTGTTTGTGTGCTTCTGAGCC-3` Reverse: 5`-ATTCTGTTGCCACCTTTCGG-3`
Bax	Forward: 5`-AAGCTGAGCGAGTGTCTCAAGCGC-3` Reverse: 5`-TCCCGCCACAAAGATGGTCACG-3`
Bcl-xL	Forward: 5`-ATGGCAGCAGTAAAGCAAGCGC-3` Reverse: 5`-TTCTCCTGGTGGCAATGGCG-3`
RalBP1	Forward: 5`-ACTGTGCAGATCAGCAATCG-3` Reverse: 5`-CCTGATCTCCTCCTTGATGC-3`

3.2. Methods

3.2.1. Culture of bacterial strains

Bacterial cultures were grown in Luria Bertani Broth at 37 °C under shaking conditions (180 rpm). Stocks of bacterial strains were prepared in 20 % glycerol solution and stored at -80 °C.

3.2.2. Isolation of plasmid DNA from bacterial cells

The rapid alkaline lysis method of plasmid isolation (Birnboim and Doly, 1979) was followed with minor modifications. Bacterial pellet from 3ml of overnight grown bacterial cultures was resuspended in 100 µl of ice-cold solution I by vortexing. After 5 min incubation at room temperature, 200 µl of freshly prepared solution II was added and the contents were mixed, by gently inverting the tube several times. This was followed by the addition of 150 µl ice-cold solution III and gentle mixing. The tube was incubated on ice for 5 min and centrifuged at 12000 rpm for 15 min at 4°C. The clear supernatant was transferred into a fresh tube and the sample was treated for 30 min with DNase free RNase at a final concentration of 20 µg/ml at 55°C. The sample was extracted with an equal volume of phenol: chloroform mixture. The aqueous phase was carefully transferred into another tube and the plasmid DNA was precipitated with two volumes of cold ethanol in ice for 30 min. The plasmid DNA was pelleted by centrifugation at maximum speed for 15 min. A clear translucent pellet obtained was washed with 70 % ethanol solution, vacuum dried and dissolved in appropriate volume of nuclease free water.

3.2.3. Quantification of DNA and RNA

DNA and RNA samples diluted in water were quantified using a spectrophotometer. Absorbance was measured at 260 nm and 280 nm. The optical

density (O.D.) of 1 at 260 nm was considered to denote the presence of 50 μg/ml of DNA and 40 μg/ml of RNA. The actual concentration of DNA and RNA present in the samples were calculated using their respective dilution factors.

3.2.4. Agarose gel electrophoresis

The nucleic acid samples were mixed with the appropriate volumes of 6X DNA loading dye and subjected to electrophoresis through 0.6 %-1 % agarose gel in 1X TAE buffer. The gel was stained in ethidium bromide staining solution containing 0.5 μg/ml of ethidium bromide for 30 min at room temperature. The stained gel was washed briefly in distilled water and the bands were visualized by fluorescence under UV-light. When required, the results were documented using a gel documentation system (Molecular imager ChemiDoc XRS+ image system, Bio-Rad).

3.2.5. Restriction enzyme digestion of DNA

Plasmid DNA (0.5-1 μg) was used for restriction enzyme digestion analysis. 2-4 units of the restriction enzyme along with their appropriate 10X buffers supplied by the manufacturers were used in a total reaction volume of 20 μl. 1 % concentration of BSA was added whenever specified by the manufacturer. The digestion was allowed to proceed for 6 h at the temperature recommended by the manufacturer. The DNA fragments after restriction digestion were visualized by ethidium bromide staining following electrophoresis on 0.6-1 % agarose gels. Commercially available DNA ladders and the corresponding undigested plasmid DNA were run along with the digestion samples to compare with and to estimate the sizes of the restriction fragments. The digested samples were stored at -20 °C for future use.

3.2.6. Elution of DNA from agarose gel

Digested DNA products and PCR amplified DNA fragments were eluted from agarose gel by using GenElute Gel Extraction Kit (Sigma Aldrich, USA). The band of interest from agarose gel was sliced out and solubilized with 3 gel volumes of gel solubilization solution at 50-60 °C for 10 minutes with periodic vortexing. 1 gel volume of 100 % isopropanol was added to the solubilized gel solution and mixed until it became homogenous. The binding column supplied with the kit was prepared according to the manufacturer's instructions. The gel-isopropanol mixture was loaded into the binding column and centrifuged at maximum speed for 1 min. The binding column was washed with the wash solution and finally eluted with elution buffer or nuclease free water. The eluted DNA was preserved at -20 °C for further use.

3.2.7. Ligation of DNA

Typically, 100-200 ng of DNA was used in each ligation reaction. The molar ratio of vector to insert was maintained between 1:3 and 1:6. The ligation reaction was usually set to 15 µl total reaction volume with appropriate 10 x ligation buffer (provided by the manufacturer) and 2 units of T4 DNA ligase, at 16 °C for 12-16 h.

3.2.8. Preparation of DH5α competent cells

A single colony from a plate of DH5α culture was picked and grown overnight in a test tube having 3 ml LB media. The overnight culture was subcultured in 50 ml of fresh LB broth at a ratio of 1:500 and grown at 37 °C till mid logarithmic phase. Cells were chilled on ice for 15 minutes and pelleted at 5000 rpm at 4 °C for 10 minutes. The supernatant was discarded and the cells were suspended in 1/10th volume of Transformation and Storage Solution (TSS) (Chung et al., 1989). The cells were kept on ice for 20 minutes. The prepared competent cells were aliquoted

into small volumes (100-200 µl) in pre-chilled micro centrifuge tubes and stored at -80 °C.

3.2.9. Transformation of DNA into DH5α competent cells

100 µl of DH5α competent cells were mixed with 0.1-0.5 µg of DNA and incubated for 30 minutes on ice. Cells were given a heat shock for 90 seconds at 42°C. The cultures were rapidly chilled on ice for 1-2 minutes, mixed with 0.9 ml of LB and incubated at 37°C for 45-60 minutes. Transformed cultures were plated on selective media at various dilutions.

3.2.10. Polymerase chain reaction

Polymerase chain reaction was carried out in flat capped PCR tubes (Greiner bio-one). The reaction was carried out for each sample with 25-50 ng DNA in 20 µl reaction volume. 1-5 units of Taq DNA polymerase was used along with the supplied reaction buffer. Each reaction mixture contained 1.5 mM $MgCl_2$, 500 µM dNTP mix and 20 picomoles each of forward and reverse primers. The polymerisation was performed in a thermal cycler (Palm-Cycler, Genetix Biotech Asia Pvt. Ltd).

3.2.11. Maintenance of mammalian cells

Mammalian cell lines were routinely maintained in DMEM having 10 % serum and penicillin (50 U/ml)-streptomycin (50 mg/ml) antibiotic solution at 5 % CO_2 in a humidified incubator at 37 °C. The media was changed at regular intervals of three days. Confluent cells were subcultured by washing with sterile PBS and adding 0.2 to 1 ml of trypsin-EDTA. After cells were detached at 37 °C, the trypsin-EDTA solution was removed and the cells were suspended in complete DMEM media. A fraction of the uniform cell suspension was transferred to a fresh

culture flask having complete media and the cells were kept at 37 °C with 5 % CO_2.

3.2.12. Isolation of RNA from mammalian cells

Total RNA from mammalian cells was isolated by using GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, USA). Cells grown as monolayers in culture flasks were lysed directly by using lysis solution containing 2-mercaptoethanol. The cellular debris and sheared DNA were removed by passing the lysate through a GenElute Filtration Column and centrifuging at 12,000 x g for 2 min. Equal volume of 70 % ethanol solution was added to the filtered lysate and mixed thoroughly. The lysate/ethanol mixture was then loaded into a GenElute Binding Column and centrifuged at 12,000 x g for 15 seconds. The column was washed first with wash solution 1 and then subsequently washed twice with ethanol diluted wash solution 2. The binding column was centrifuged for an additional 1 min to remove any residual wash solution 2. The binding column was finally transferred to a fresh centrifuge tube and the RNA was eluted in elution buffer or nuclease-free water.

3.2.13. Synthesis of cDNA by reverse transcription

The synthesis of cDNA from total RNA was performed by using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Around 1 μg of total RNA was used for each sample. For a 20 μl reaction mixture, the following protocol was followed.

The sample mixture was prepared as follows:

Template RNA	-1 μg

Random hexamer	-1 µl
Nuclease-free water	-to 12µl

The components are mixed gently, centrifuges briefly and incubated at 65 °C for 5 min. The tubes were immediately chilled on ice. To the denatured RNA sample, the following components were added.

5x reaction buffer	-4 µl
RiboLock RNase inhibitor (20 U/µl)	-1 µl
10 mM dNTP mix	-2 µl
RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µl)	-1 µl

The components were mixed gently and centrifuged briefly. The samples were incubated for 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating the samples at 70 °C for 5 min. The cDNA synthesised was stored at -20 °C until used for further downstream PCR amplifications.

3.2.14. Transfection of mammalian cells

Mammalian cell lines were transfected by using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. DNA and lipofectamine were diluted in serum free media at appropriate ratios recommended by the manufacturer. After 5 minutes of incubation, the diluted DNA and lipofactamine were mixed and kept for 20 min at room temperature. The lipofectamine-DNA complex formed was added to mammalian cells and the cells were kept at 37 °C for tranfection. After 6 hours incubation, the media containing lipofectamine-DNA complex was removed and fresh, complete DMEM was added. Stable clones of the

transfected cells were selected by adding G418 antibiotic after 72 hours of transfection. After initial selection of the stable cells in media containing 600 μg/ml G418, the cells were maintained in complete DMEM in presence of 400 μg/ml G418.

3.2.15. Protein estimation

Cells grown to 70-80 % confluency were lysed by RIPA buffer (Sigma Aldrich, USA) containing 1mM PMSF (Sigma Aldrich, USA). Total protein content in the cell lysates were quantified by Lowry's method of protein estimation using BSA as standards. Briefly, 100 μl of protein sample was mixed with 250 μl of freshly prepared complex forming reagent (2 % Na_2CO_3 in 0.1 N NaOH, 2 % $CuSO_4.5H_2O$, 2 % potassium sodium tartrate in the ratio of 100:1:1). The mixture was incubated at 37 °C for 10 min and then 25 μl of freshly diluted Folin reagent (Folin reagent diluted with distilled water in 1:1 ratio) was added. The mixture was vortexed and kept at room temperature for 30 min in dark. 200 μl reaction mixture of each sample was then transferred to a 96 well plate and the absorbance was measured at 660 nm.

3.2.16. SDS-PAGE analysis and Western blotting

SDS-PAGE was done loading equal amount of proteins in each well. The samples were blotted onto PVDF membrane and detected using antibodies for β-actin (BD Transduction Laboratories) and MCSF (Sigma Aldrich, USA). The antibody against β-actin was used at a concentration of 0.05 μg/ml and antibody for MCSF was used at a concentration of 0.1 μg/ml. Blots were developed using chemiluminescent peroxidase substrate-1 kit (Sigma Aldrich, USA) and imaged using gel documentation system (Molecular imager ChemiDoc XRS+ image system, Bio-Rad).

3.2.17. Cell viability assay

Cell viability was measured by adopting the method reported by Roehm et al., (Roehm et al., 1991), using In Vitro Toxicology assay kit, XTT based (Sigma Aldrich, USA). Cells were seeded in 96 well microplates at a density of $1.5x10^3$ cells per well and allowed to grow overnight. 5-FU at various concentrations were added to the cells and kept at CO_2 incubator. XTT (2, 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) assay was performed at different time intervals using manufacturer's protocol. The soluble orange formazan product was measured by a multiplate reader (Tecan, Infinite M200) at 450 nm and the background was measured at 690 nm. Cell viability (%) of treated cells was calculated relative to untreated cells, taken as 100 % viable.

% cell viability= [($A_{450} - A_{690}$) in treated sample/ ($A_{450} - A_{690}$) in control sample] × 100.

3.2.18. Acridine orange-Ethidium bromide dual staining

Dual staining of cells with acridine orange-ethidium bromide dyes to detect apoptosis was done according to the previously reported method (Sharma et al., 2013). Cells were grown to 70-80 % confluency in 6-well plates and treated with desired concentrations of 5-FU. The culture media was removed after treatment and the cells were washed twice with PBS. Fresh complete media containing 2 μg/ml acridine orange and 6 μg/ml ethidium bromide was added to each well and cells were stained for 5 minutes in the dark. The cells were then washed twice with PBS and visualized under a fluorescence microscope (Nikon ECLIPSE, T*i*-U, Japan) with an excitation filter of 480/15 nm(for acridine orange) and 540/25 nm (for ethidium bromide).

3.2.19. CalceinAM-DAPI dual staining

Simultaneous staining of cells with calceinAM and DAPI (4', 6-diamidino-2-phenylindole) was done to detect the presence of viable and apoptotic cells respectively (Onishi et al., 2006). Following treatment for 120 h, cells were washed thoroughly with PBS and then 2.5 μM calceinAM in PBS was added. After 15 minutes of staining at room temperature in dark, the cell plate was washed twice with media containing serum and finally 300nM DAPI was added in complete media. The DAPI staining solution was removed after 3 min of incubation in dark and the cells were washed thrice with PBS. Samples were viewed under a fluorescence microscope (Nikon ECLIPSE T*i*-U, Japan) with an excitation filter of 480/15 nm(for calceinAM) and 360/20 nm (for DAPI).

3.2.20. Methylene blue staining

Methylene blue staining to study morphological features was performed following the method reported by Das et al., (Das et al., 2008) with minor modifications. Cells seeded in six well plates were treated with various concentrations of 5-FU for the desired time intervals. After treatment, the plate was washed twice with ice-cold PBS and cells were briefly fixed with 50% ice-cold ethanol. 0.2% (W/V) methylene blue staining solution was added to the plate and staining was carried out for 30 seconds. The solution was aspirated and the cells were washed thrice with ice-cold water. Samples were air dried and visualised under a light microscope to observe for any morphological changes associated with possible cell differentiation.

3.2.21. Actin cytoskeleton staining

Cells seeded in six well plates were treated with 5-FU. At the end of treatment period, cells were washed with PBS and fixed with 3.7 % paraformaldehyde

solution containing 0.1 % Triton X-100 at 37°C for 30 min. After washing with PBS for three times, cells were blocked with 1 % BSA blocking solution for 30 min. Then, cells were incubated with anti β-actin primary antibody (BD Transduction Laboratories) and subsequently with FITC tagged secondary antibody (BD Transduction Laboratories). After staining with FITC tagged secondary antibody, cells were washed thrice and incubated with media containing 300nM DAPI for 3 min. Cells were washed thoroughly, finally resuspended in PBS and visualised under fluorescence microscope (Nikon ECLIPSE Ti-U, Japan) with an excitation filter of 480/15 nm(for FITC) and 360/20 nm (for DAPI).

3.2.22. Trypan blue dye exclusion assay

Cell viability calculations by using the dye trypan blue was done according to the method described by Das et al., (Das et al., 2008) with slight modifications. Cells in six well plate were treated with 5-FU for 120 h. After treatment, cells were harvested, mixed with equal volume of 0.4 % trypan blue (Invitrogen) and loaded over a counting chamber. The healthy and viable cells had intact membrane and hence, they excluded the dye. The compromised cells took up the dye and were counted as dead. The percentage (%) of cell viability was calculated by using Countess-automated cell counter (Invitrogen).

3.2.23. CFSE cell proliferation assay

Cells were adjusted to a density of $1x10^6$ cells/ml in PBS containing 0.1% BSA. About 10µl of 0.5mM CFDA-SE (carboxyfluoresceindiacetate, succinimidyl ester) (Sigma Aldrich, USA) was added to make final working concentration of 5µM. Staining was carried out at 37°C for 10 min and then 5 volumes of ice-cold media was added to the cells to quench any free dye. Cells were pelleted by centrifugation and washed twice with fresh media. Stained cells were analysed

immediately by a flow cytometer (FacsCalibur, BD Biosciences, NJ) for 0 h readings, and a batch of same cells were seeded in six well plates for subsequent desired time periods. The data acquired were analysed in Cell Quest Pro software. The doubling time was calculated according to the formula $T_d=T/\log_2 (F_0/F_T)$, where F_0 is the geometric mean fluorescence intensity at 0 h and F_T is the geometric mean fluorescence intensity at T h (Das et al., 2012). In our study, T was chosen as 72 h.

3.2.24. Cell cycle analysis

The method reported by Sanpui et al., (Sanpui et al., 2011) was followed for cell cycle analysis. Briefly, cells were seeded in six well plates at density of $5x10^4$ cells per well. After overnight attachment, various concentrations of 5-FU was added. At the end of the treatment period, cells were trypsinised, washed with PBS and fixed with 70% alcohol solution for 15 min in ice. The fixed cells were collected by centrifugation and stained with propidium iodide (PI) staining solution at 37°C for 30 min in dark. At least 10000 events per sample were acquired by a flow cytometer (FacsCalibur, BD Biosciences, NJ) and the percentage of cells distributed in various phases of the cell cycle was calculated using ModFit LT software.

3.2.25. Determination of Mitochondrial Membrane Potential (MMP)

Changes in mitochondrial membrane potential (MMP) was investigated by using the method developed by Cossarizza et al.,(Cossarizza et al., 1993) with minor modifications. Briefly, $5x10^4$ cells per well were plated in six well plate. Desired concentrations of 5-FU were added to cells and after treatment, cells were trypsinized, centrifuged and resuspended in complete medium containing 10 μg/ml JC-1 (Sigma Aldrich, USA). The cell suspension was kept in dark for 10 min at room temperature. After incubation, the cells were washed twice with PBS,

resuspended in 500 µl PBS and analysed with a flow cytometer (FacsCalibur, BD Biosciences, NJ). A minimum of 10000 events were acquired for each sample and the data was analyzed with the CellQuest Pro software.

3.2.26. MCSF Localization study

Briefly, cells were washed with PBS and fixed with 3.7 % paraformaldehyde solution (with or without 0.1 % Triton X-100) at 37°C for 30 min. After washing with PBS for three times, cells were blocked with 1 % BSA blocking solution for 30 min. Then, cells were incubated with anti-MCSF (Sigma) primary antibody and subsequently with FITC tagged secondary antibody (BD Transduction Laboratories). After staining with FITC tagged secondary antibody, cells were incubated with media containing 300nM DAPI for 3 min, finally washed and visualised under fluorescence microscope (Nikon ECLIPSE Ti-U, Japan) with an excitation filter of 480/15 nm(for FITC) and 360/20 nm (for DAPI).

3.2.27. Real time PCR analysis

Quantitative real time PCR was performed using SYBR Green as reporter dye (Power SYBR Green PCR master mix, Applied Biosystem) and 7500 Real time PCR system (Applied Biosystem). Raw data was analysed and efficiency of each reaction was calculated by LinRegPCR software. β-actin was used as the endogenous control and the fold change in expression of genes were calculated by ΔΔCt method.

3.2.28. CD24/CD44 analysis by flow cytometry

Flow cytometry identification of surface markers was done following the method used by Al-Hajj et al., (Al-Hajj et al., 2003) with minor modifications. Cells after treatment were dissociated into single suspension cells by trypsinization and

washed twice with PBS. All cells were incubated with 10% human serum for 20 minutes on ice to block Fc receptors. Then the antibodies, FITC Mouse Anti-Human CD24 and PE Mouse Anti-Human CD44 (both from BD Pharmingen) were added and incubated for 20 minutes on ice in the dark. The cells were washed twice with PBS, finally suspended in PBS and analysed by a flow cytometer (FacsCalibur, BD Biosciences, NJ).

3.2.29. Statistical analysis

The values for all experiments were expressed as mean ± s.e.m of three or more individual experiments. The data were analysed by Student's t test or by ANOVA whichever applicable using GraphPad Prism 5.01. Statistically significant values are denoted by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$)

SECTION 4

RESULTS AND DISCUSSION

Section 4

RESULTS AND DISCUSSION

4.1. Cloning of MCSF gene and generation of stable cell lines

Human renal cell carcinomas were previously reported to have significant expression of macrophage colony stimulating factor (Gerharz et al., 2001; Komohara et al., 2011). In 1987, Sakai et al., purified MCSF from urine and sequenced its N-terminal region up to position 44 (Sakai et al., 1987). In our studies, renal cell adenocarcinoma cell line ACHN was chosen as the source of MCSF. MCSF was initially cloned into pGEMT-easy vector and then subsequently into pEGFP-N1 mammalian expression vector. A stable cell line of U87MG, human glioblastoma cells expressing MCSF (U87-MCSF) was successively generated. The cloning strategy employed is depicted in Figure 4.1.

Initially, total RNA was isolated from the ACHN cells and cDNA was synthesised by using cDNA synthesis kit (Fermentas). The gene corresponding to the membrane bound isoform of MCSF was amplified by polymerase chain reaction and the 0.771 kb PCR product was first cloned into the pGEMT-easy vector (Promega). The clone pGEMT-MCSF was confirmed by restriction digestion analysis with EcoRI, BamHI and XmnI which gave bands of 3 and 0.781 kb, 3.786 kb, 2.232 and 1.554 kb, respectively (Figure 4.2). The clone was also confirmed by DNA sequencing (Figure 4.3). The 0.771 kb MCSF gene cloned into pGEMT-easy vector was then PCR amplified by primers having EcoRI and ApaI as flanking restriction sites and the product was cloned into pre-digested EcoRI and ApaI sites

of the mammalian expression vector pEGFP-N1. The clones were confirmed by restriction digestion analysis with multiple combinations of restriction enzymes: digestion with EcoRI and ApaI generated bands of 4.676 and 0.783 kb, digestion with XmnI and NheI revealed bands of 4.930 and 0.541 kb and digestion with XmnI which yielded a single band of 5.471 kb (Figure 4.4). The recombinant plasmid, pEGFP-N1-MCSF was then transfected into the glioblastoma cell line, U87MG using lipofectamine 2000 (Invitrogen). After 72 h of transfection, U87MG cells stably expressing MCSF gene (U87-MCSF) were selected in culture media by adding the antibiotic, G418 at the concentration of 600 μg/ml. After initial selection for three weeks in media containing 600 μg/ml G418, cells were routinely maintained in culture media having 400 μg/ml G418. A mixed population of clones of U87-MCSF was selected in order to exclude the potential role of any other compensatory process arising out of transfection in a single clonal population.

The expression of transgene MCSF in U87-MCSF cells was confirmed by semi-quantitative RT-PCR analysis (Figure 4.5). The expression of MCSF protein was analysed by western blotting with anti-MCSF antibody. A band corresponding to molecular weight of 28 kD in U87-MCSF protein sample confirmed the overexpression of membrane bound isoform of MCSF protein in U87-MCSF cells (Figure 4.6). A mock transfected cell line was generated by transfecting U87MG cells with empty vector backbone of pEGFP-N1. The resultant U87-GFP cells were also selected with G418 and the expression of GFP was examined by fluorescence microscopy. As shown in Figure 4.7, bright green fluorescence was seen in U87-GFP cells. Next, the cytotoxicity of 5-fluorouracil was evaluated on all three cell lines of U87MG, U87-MCSG and U87-GFP.

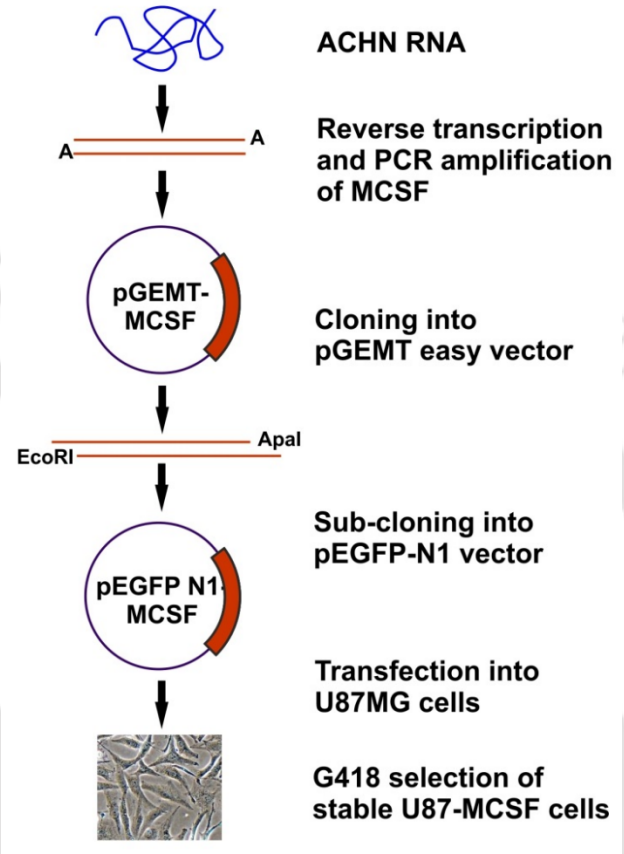

Figure 4.1. Strategy for cloning and generation of U87-MCSF cells. The membrane bound isoform of MCSF, amplified using cDNA of ACHN cells was first cloned into pGEMT-easy vector and subsequently into the mammalian expression vector pEGFP-N1. The pEGFP-N1-MCSF was transfected into U87MG cells by lipofectamine and U87-MCSF cells were selected in cell culture media having G418.

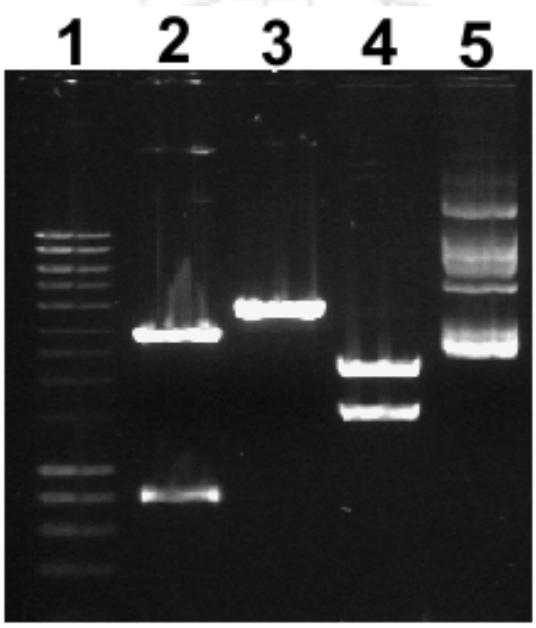

Figure 4.2. Confirmation of pGEMT-MCSF clone by restriction digestion analysis. Lane 1: 1 kb DNA ladder; Lane 2: pGEMT-MCSF digested with EcoRI (3 and 0.781 kb); Lane 3: pGEMT-MCSF digested with BamHI (3.786 kb); Lane 4: pGEMT-MCSF digested with XmnI (2.232 and 1.554 kb); Lane 5: uncut plasmid.

CTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGC
GGGAATTCGATTCACCATGGGTATGACCGCGCCGGGCGCCGCCGGGCGCTGCCCT
CCCACGACATGGCTGGGCTCCCTGCTGTTGTTGGTCTGTCTCCTGGCGAGCAGGA
GTACCACCGAGGAGGTGTCGGAGTACTGTAGCCACATGATTGGGAGTGGACACCT
GCAGTCTCTGCAGCGGCTGATTGACAGTCAGATGGAGACCTCGTGCCAAATTACAT
TTGAGTTTGTAGACCAGGAACAGTTGAAAGATCCAGTGTGCTACCTTAAGAAGGCA
TTTCTCCTGGTACAAGACATAATGGAGGACACCATGCGCTTCAGAGATAACACCCC
CAATGCTATCGCCATTGTGCAGCTGCAGGAACTCTCTTTGAGGCTGAAGAGCTGCT
TCACCAAGGATTATGAAGAGCATGACAAGGCCTGCGTCCGAACTTTCTATGAGACA
CCTCTCCAGTTGCTGGAGAAGGTCAAGAATGTCTTTAATGAAACAAAGAATCTCCT
TGACAAGGACTGGAATATTTTCAGCAAGAACTGCAACAACAGCTTTGCTGAATGCT
CCAGCCAAGGCCATGAGAGGCAGTCCGAGGGATCCTCCAGCCCGCAGCTCCAGG
AGTCTGTCTTCCACCTGCTGGTGCCCAGTGTCATCCTGGTCTTGCTGGCCGTCGGA
GGCCTCTTGTTCTACAGGTGGAGGCGGAGGAGCCATCAAGAGCCTCAGAGAGCGG
ATTCTCCCTTGGAGCAACCAGAGGGCAGCCCCCTGACTCAGGATGACAGACAGGT
GGAACTGCCAGTGTAGCTAGCATAATCACTAGTGAATTCGCGGCCGCCTGCAGGT
CGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGT
GTCACCTAAA

Figure 4.3. Annotation of the sequence of pGEMT-MCSF. The sequence of the clone pGEMT-MCSF was verified by DNA sequencing. The blue region indicates the nucleotide sequence of the cloned MCSF gene.

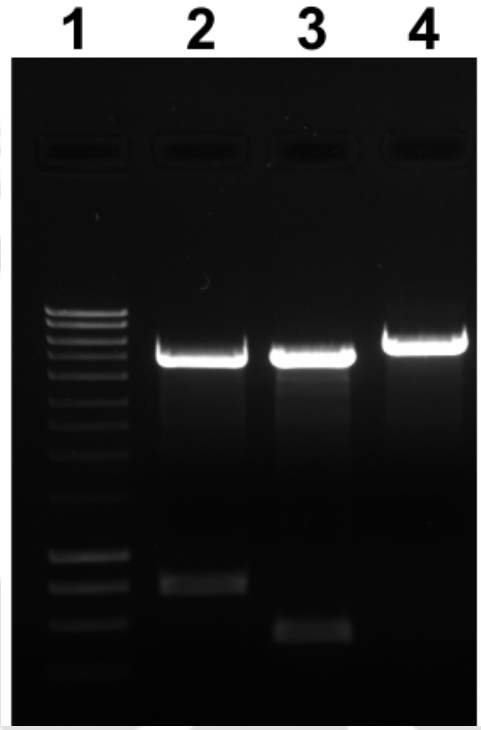

Figure 4.4. Confirmation of clones of pEGFP N1-MCSF by restriction digestion analysis. Lane 1: 1 kb DNA ladder; Lane 2: pEGFP N1-MCSF digested with EcoRI and ApaI (4.676 and 0.783 kb); Lane 3: pEGFP N1-MCSF digested with XmnI and NheI (4.930 and 0.541 kb); Lane 4: pEGFP N1-MCSF digested with XmnI (5.471 kb).

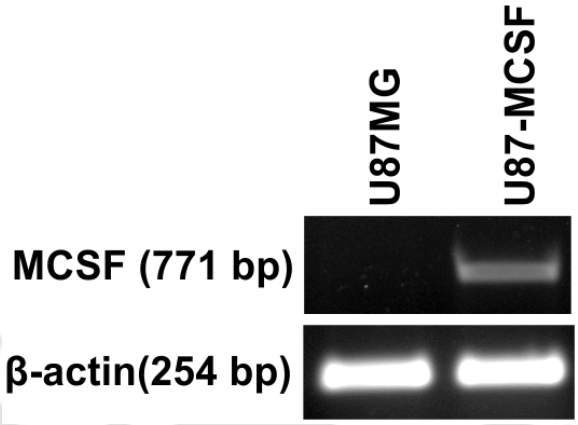

Figure 4.5. Semi-quantitative RT-PCR analysis to study the overexpression of MCSF gene in U87-MCSF cells. β-actin was used as the internal control.

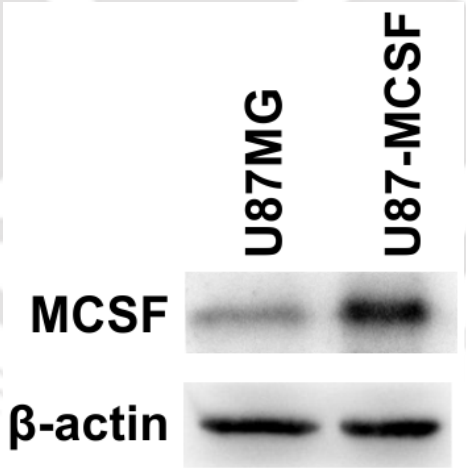

Figure 4.6. Western blotting analysis to confirm the overexpression of MCSF in U87-MCSF cells. A 28 kD band confirmed the overexpression of MCSF in U87-MCSF cells. β-actin was used as the loading control.

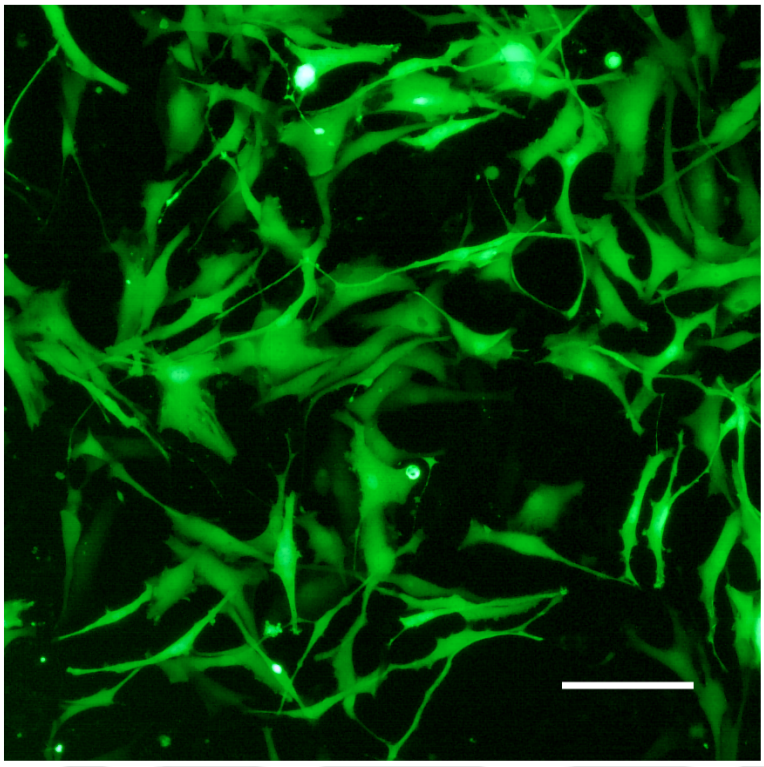

Figure 4.7. Generation of U87-GFP cells. U87MG cells were transfected with pEGFP N1 vector and the transfected cells were selected with 400 μg/ml G418. Bright green fluorescence was seen in the transfected cells when visualised under a fluorescence microscope. Scale bar: 100μm.

4.2. Effect of 5-FU on U87MG, U87-MCSF and U87-GFP cells

5-FU is one of the most widely used anti-cancer drugs used in the treatment of solid tumors. 5-FU mediates its cytotoxic effects by inhibiting thymidylate synthase enzyme and blocking DNA synthesis (Longley et al., 2003). We assessed the effect of 5-FU on U87MG and U87-MCSF cells by XTT assay. Cells were treated with various concentrations of 5-FU ranging from 5-100 μM, for 48 h and 72 h. We did not observe any difference in cell proliferation or viability between U87MG and U87-MCSF cells after 48 h of 5-FU treatment (Figure 4.8). However, the proliferation of U87-MCSF cells was found to be significantly reduced when treated with 5-FU of above 10 μM for 72 h as evident from the reduced absorbance values seen in treated U87-MCSF cells (Figure 4.9). Similar reduction in cell proliferation with 5-FU treatment was not observed in U87-GFP cell line, where the proliferation was almost similar to that of the treated U87MG cells. This retardation of cell proliferation observed with 5-FU treatment did not occur when U87-MCSF cells were treated with cisplatin (Figure 4.10).

We did not see any cell death by visual observation even after treatment with higher concentrations of 5-FU for a prolonged period of time. Acridine orange-ethidium bromide dual staining of treated U87MG and U87-MCSF cells did not show the presence of any apoptotic cell population (Figure 4.11 and 4.12). Morphological changes for characteristic apoptosis were also not observed after 5-FU treatment. Additionally, we performed JC-1 staining to investigate changes in mitochondrial membrane potential after treatment of cells with 5-FU for 120 h. As shown in figure 4.13, JC-1 staining revealed the absence of apoptosis in 5-FU treated U87MG or U87-MCSF cells. Furthermore, quantitative calculation using trypan blue dye exclusion assay revealed intact cell membrane with healthy cell populations after 120 h of 5-FU treatment (Figure 4.14).

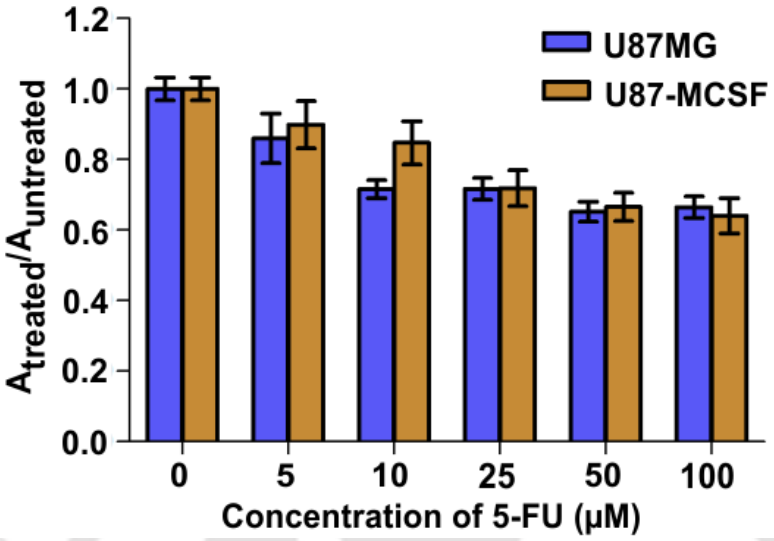

Figure 4.8. Effect of 5-FU on U87MG and U87-MCSF after 48 h of treatment.

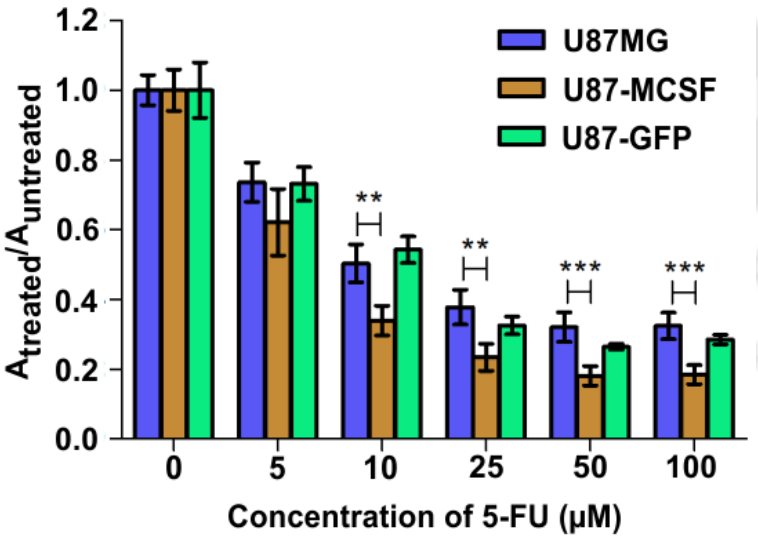

Figure 4.9. Effect of 5-FU on U87MG and U87-MCSF after 72 h of treatment. Treated U87-MCSF cells recorded less absorbance values than treated U87MG and treated U87-GFP cells. Statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

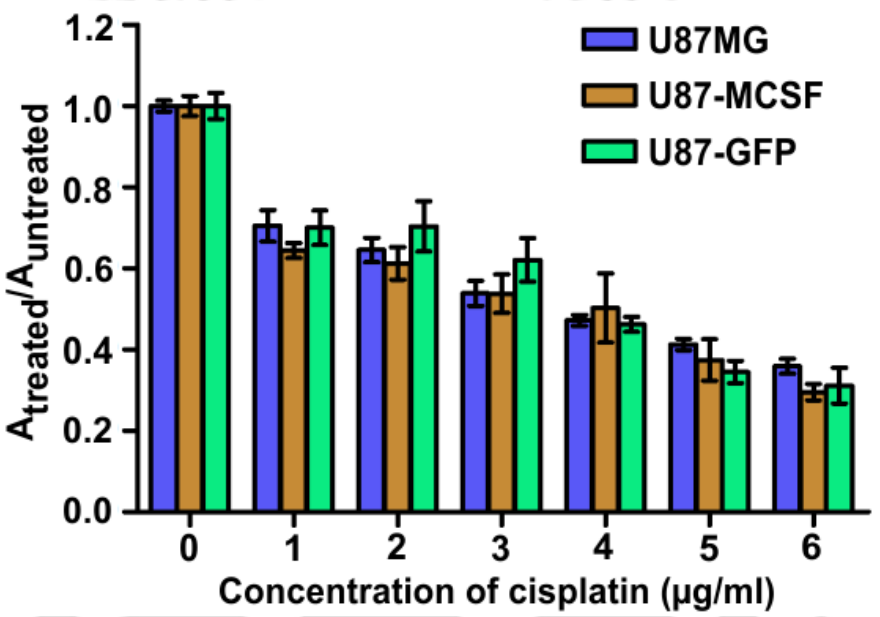

Figure 4.10. Studies on U87MG, U87-MCSF and U87-GFP cells after 48 h treatment with cisplatin. No difference in cytotoxicity was observed between treated samples of U87MG, U87-MCSF and U87-GFP cells.

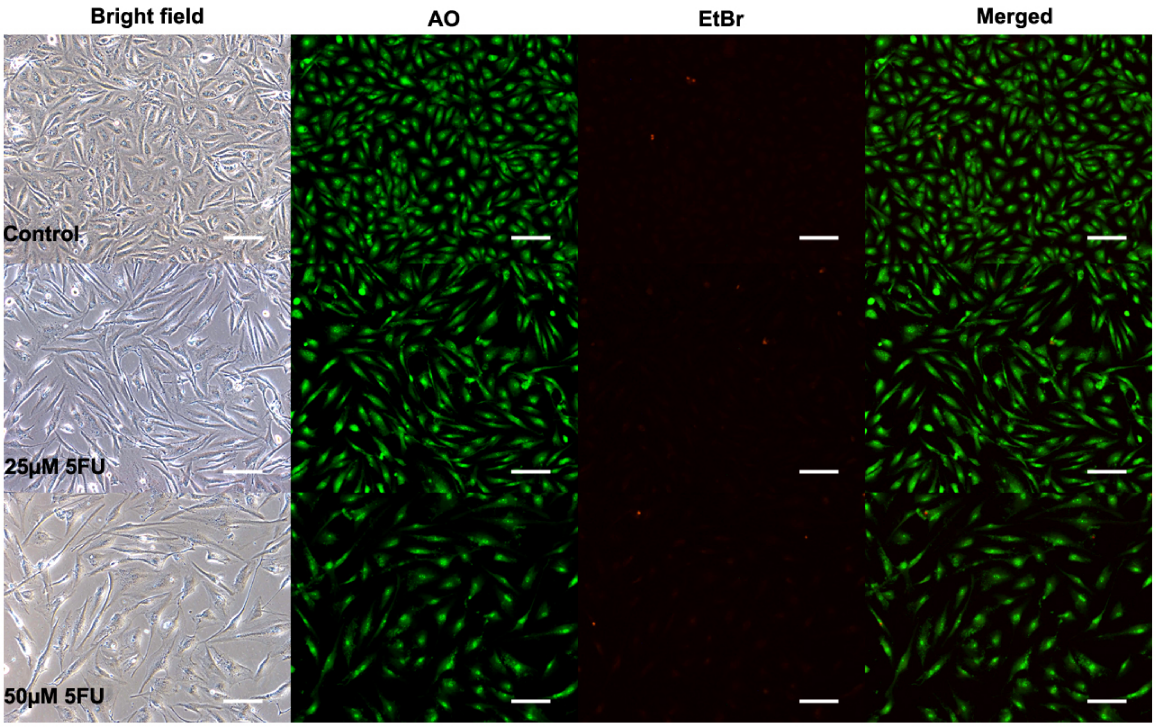

Figure 4.11. Acridine orange-ethidium bromide dual staining of U87MG cells. U87MG cells were treated with 5-FU for 72 h and stained with AO/EB dual staining solution. Cells were visualised under a fluorescence microscope. The pictures did not show any indication of apoptotic nuclei in treated cells. Scale bar: 100 μm.

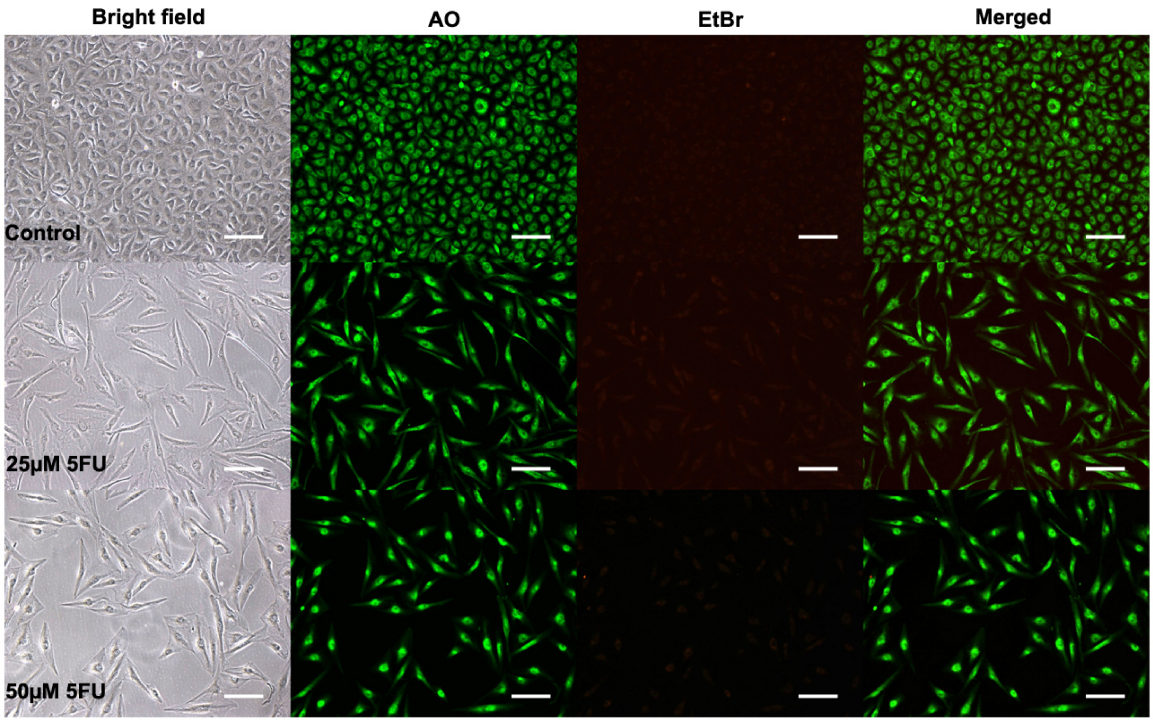

Figure 4.12. Acridine orange-ethidium bromide dual staining of U87-MCSF cells. U87-MCSF cells were treated with 5-FU for 72 h and stained with AO/EB dual staining solution. Cells were visualised under a fluorescence microscope. No apoptotic nuclei were seen in treated cells. Scale bar: 100 μm.

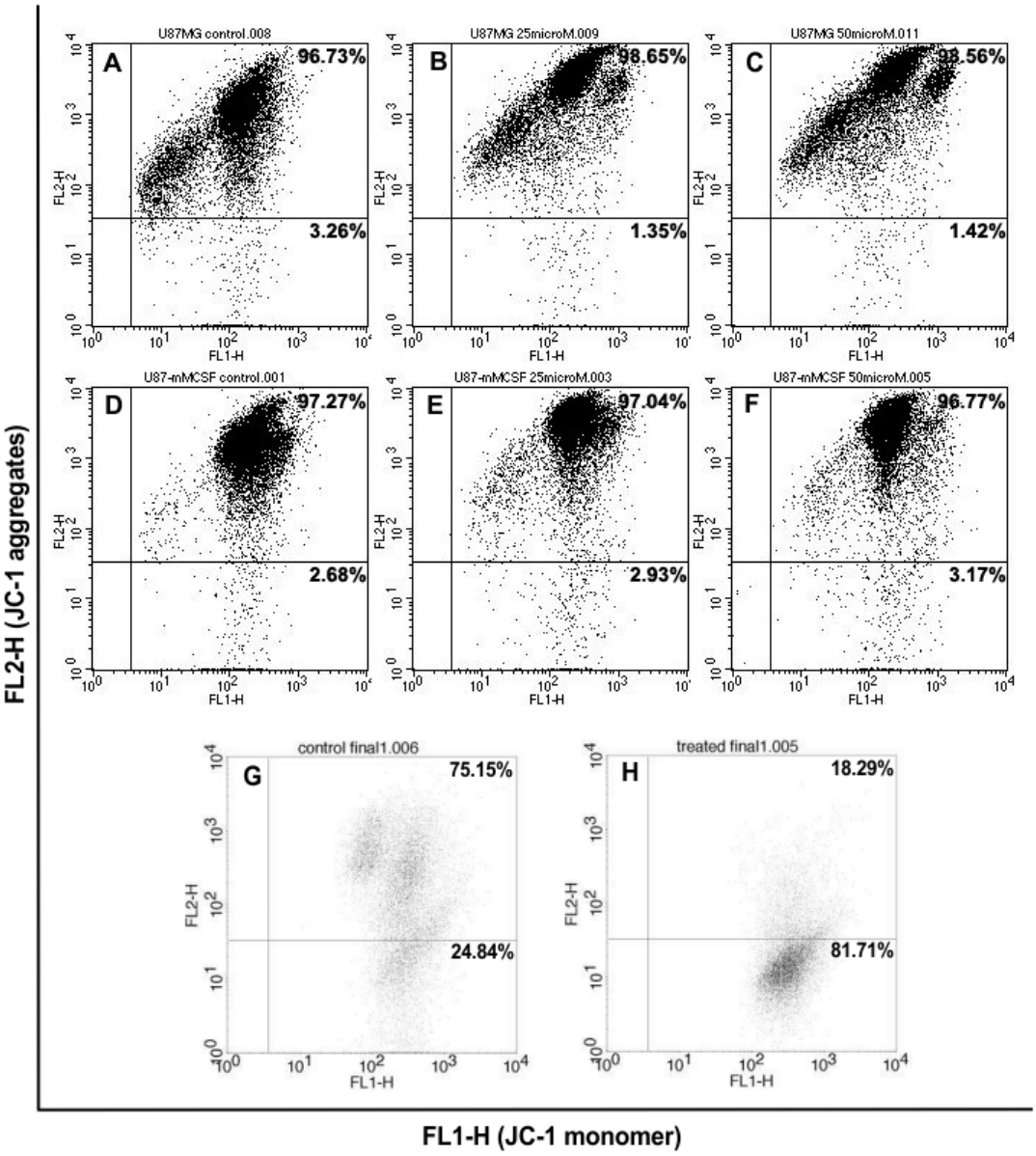

Figure 4.13. JC-1 analysis of treated U87MG and U87-MCSF cells. (A-C) U87MG. (D-F) U87-MCSF. The results revealed that MCSF expression failed to induce apoptosis after treatment with 25 μM 5-FU (B, E) and 50μM 5-FU (C, F) for five days. As U87MG cells were resistant to 5-FU and cisplatin, Hela cells were used as appropriate controls-(G) untreated HeLa cells and (H) HeLa cells treated with cisplatin.

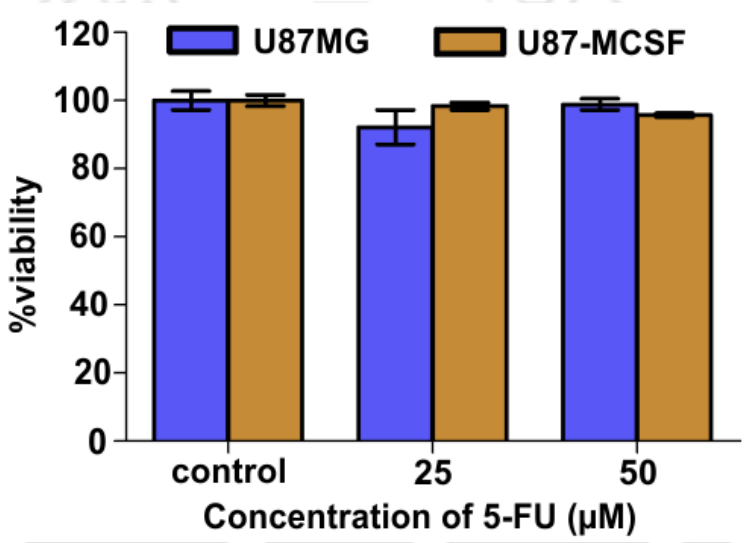

Figure 4.14. Trypan blue assay for U87MG and U87-MCSF cells. Cells were treated with 5-FU for 120 h and the viability of treated cells was investigated by trypan blue dye exclusion assay. Treated samples of both U87MG and U87-MCSF cells were healthy and viable.

We investigated the expression of some of the pro-apoptotic and anti-apoptotic genes like caspase-3, Bax and Bcl-xL. As shown in Figure 4.15, the expression of pro-apoptotic gene, Bax was increased in treated samples of both U87MG (1.90 fold) and U87-MCSF cells (1.29 fold). The expression of anti-apoptotic gene, Bcl-xL was also increased in treated U87MG and treated U87-MCSF cells. However, the increase in Bcl-xL expression was 2.11 fold in treated U87-MCSF cells as compared to the 1.25 fold increase in treated U87MG cells. Caspase-3 expression remained unchanged in treated samples of U87MG and U87-MCSF cells.

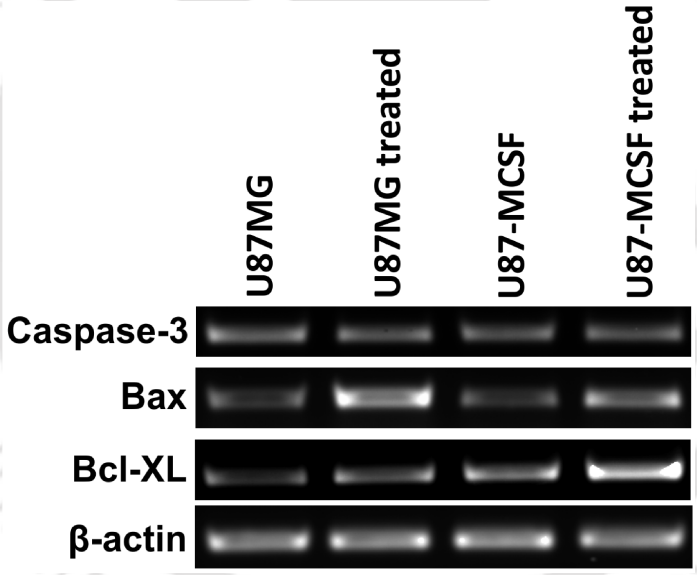

Figure 4.15. Semi-quantitative RT-PCR analysis of pro and anti-apoptotic genes. U87MG and U87-MCSF cells were treated with 5-FU for 72 h and the expression of Caspase-3, Bax and Bcl-xL was investigated. Upregulation in the expression of pro-apoptotic gene, Bax is counter-balanced by the upregulation in the expression of anti-apoptotic Bcl-xL in both treated U87MG and treated U87-MCSF cells. Expression of β-actin was used as the control. Fold changes in gene expression was quantified by densitometry analysis using ImageJ software.

A balance in the expression of pro and anti-apoptotic genes determine the fate of the cells in entering apoptotic pathway (Finkel, 2001). Analysis of expression of pro and anti-apoptotic genes revealed that upregulation in the expression of pro-apoptotic gene, Bax is counter-balanced by the upregulation in the expression of anti-apoptotic Bcl-xL in both treated U87MG and treated U87-MCSF cells. However, treated U87-MCSF cells had lesser expression of pro-apoptotic gene Bax and higher expression of anti-apoptotic Bcl-xL as compared to treated U87MG cells. Our results showed that although treated samples of both U87MG and U87-MCSF cells did not undergo apoptosis, 5-FU treated U87-MCSF cells had more resistance to apoptosis than 5-FU treated U87MG cells.

Two possible reasons could account for the decreased absorbance values seen by XTT assay in 5-FU treated cells. One, the cytotoxicity of 5-FU may trigger cell death, thereby decreasing the absorbance and the other, only the cell proliferation is retarded without affecting the viability of cells. Visual observation and the results from JC-1 staining and trypan blue dye exclusion assay negated the presence of apoptosis or any other form of cell death. Hence, the only other reason that is possible for reduced absorbance values is retardation in cell proliferation. In order to validate this, the cell cycle of treated samples of U87MG, U87-MCSF and U87-GFP cells were examined next by flow cytometry.

4.3. Cell proliferation studies for U87MG and U87-MCSF cells

As MCSF is a growth factor, mere expression of it alone can change the growth pattern of U87-MCSF cells. Hence, before cell cycle analysis of treated samples, the rate of proliferation of untreated U87MG and U87-MCSF cells has to be investigated. Untreated U87MG and U87-MCSF cells were stained with carboxyfluoresceindiacetate, succinimidyl ester (CFSE), analysed by a flow cytometer and the doubling time of both cells were calculated. As shown in Figure

4.16, the expression of MCSF did not change the growth rate of U87-MCSF cells. The doubling time of U87MG and U87-MCSF cells was found to be 12 h.

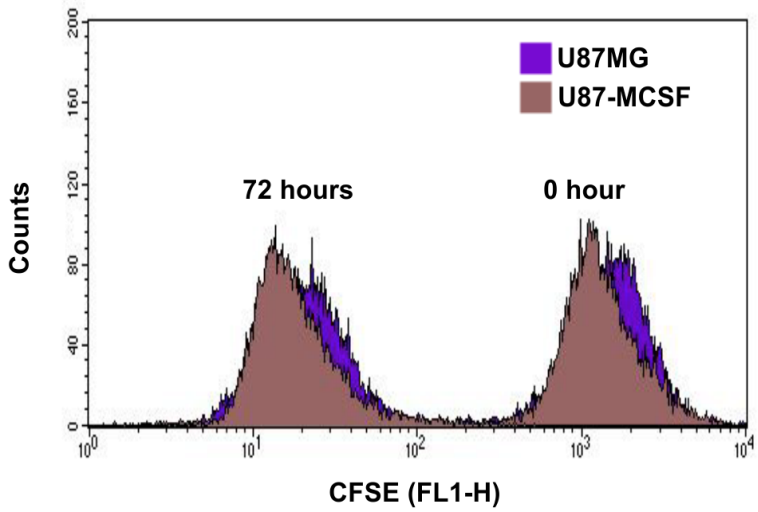

Figure 4.16. CFSE cell proliferation assay. The results showed that the rate of proliferation of untreated U87MG and U87-MCSF cells remained unaltered.

The pattern of proliferation of U87MG and U87-MCSF cells was also compared by examining the distribution of cells in various phases of cell cycle by flow cytometry analysis of propidium iodide stained cells. Initially, both U87MG and U87-MCSF cells were synchronised in G0/G1 phase by serum starvation for 48 h (Abal et al., 2004). FACS analysis for measurement of DNA content of cells at this stage (0 h) showed that the relative percentage of cells in G0/G1 phase was 96 ± 2%. Serum containing media was added to release the cells from G0/G1 arrest, and the cell cycle pattern of U87MG and U87-MCSF cells was carefully observed at regular time intervals (Figure 4.17).

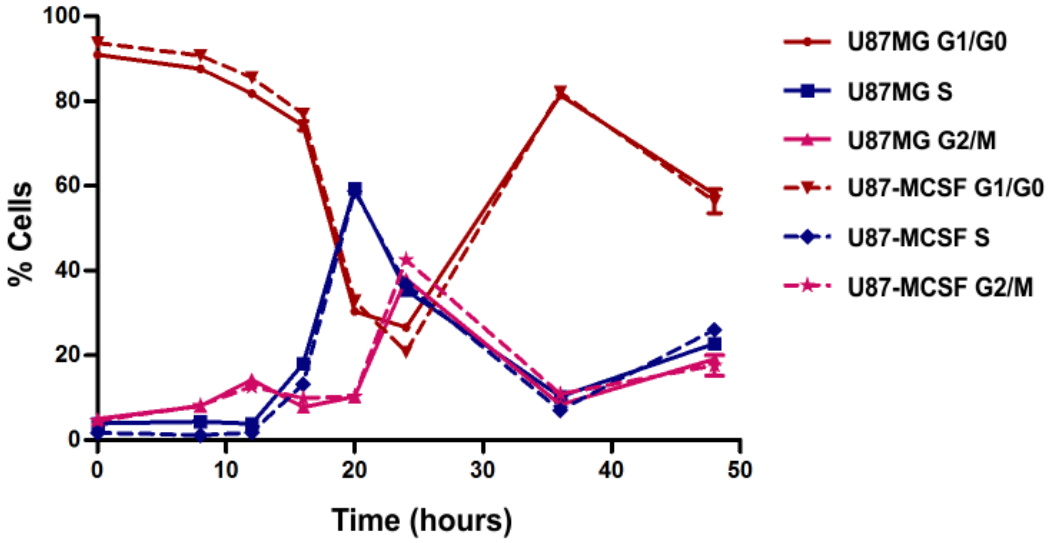

Figure 4.17. Comparison of cell cycle between U87MG and U87-MCSF cells. No difference in pattern of cell cycle between U87MG and U87-MCSF cells was observed.

The data obtained by cell cycle analysis clearly showed that there was no difference in the rate of progression of cell cycle between U87MG and U87-MCSF cells, which correlated with the results obtained in CFSE cell proliferation assay. This finding is in contrast to the previous reports, where addition of MCSF to the human ovarian cancer cells was reported to induce G0/G1 arrest *in vitro* and thereby, significantly inhibited cell growth (Kawakami et al., 2000).

4.4. Effect of 5-FU on cell cycle of U87-MCSF

Absence of apoptosis suggested that cell cycle retardation might be the possible reason for reduced cell growth measured by XTT assay. Therefore, the cell cycle of U87MG and U87-MCSF upon 5-FU treatment was studied by propidium iodide staining using flow cytometer. Cells were first synchronised in G0/G1 phase by serum starvation for 48 h before treatment. Then fresh media containing serum was added and the cells were treated with 25 μM 5-FU for 24 h and their distribution pattern in various phases of cell cycle was analysed by FacsCalibur. The treatment period was chosen as 24 h based on the data obtained by comparing the cell cycle of U87MG and U87-MCSF cells. 5-FU treatment is known to arrest cells in G1/S phase (Liu, H. C. et al., 2006). At 24 h post starvation period, the percentage of cells in G0/G1 phase was less (Figure 4.17) and hence, any blocking of cells in G0/G1 phase by 5-FU treatment will be distinctly seen after 24 h treatment.

The cell cycle data acquired by using CellQuest Pro software was analysed by ModFit LT software (Figure 4.18). The results were plotted and the statistical analysis was done for treated U87MG and treated U87-MCSF cells (Figure 4.19). Cell cycle analysis of treated U87-GFP cells was also performed and results were compared with treated U87MG cells (Figure 4.20).

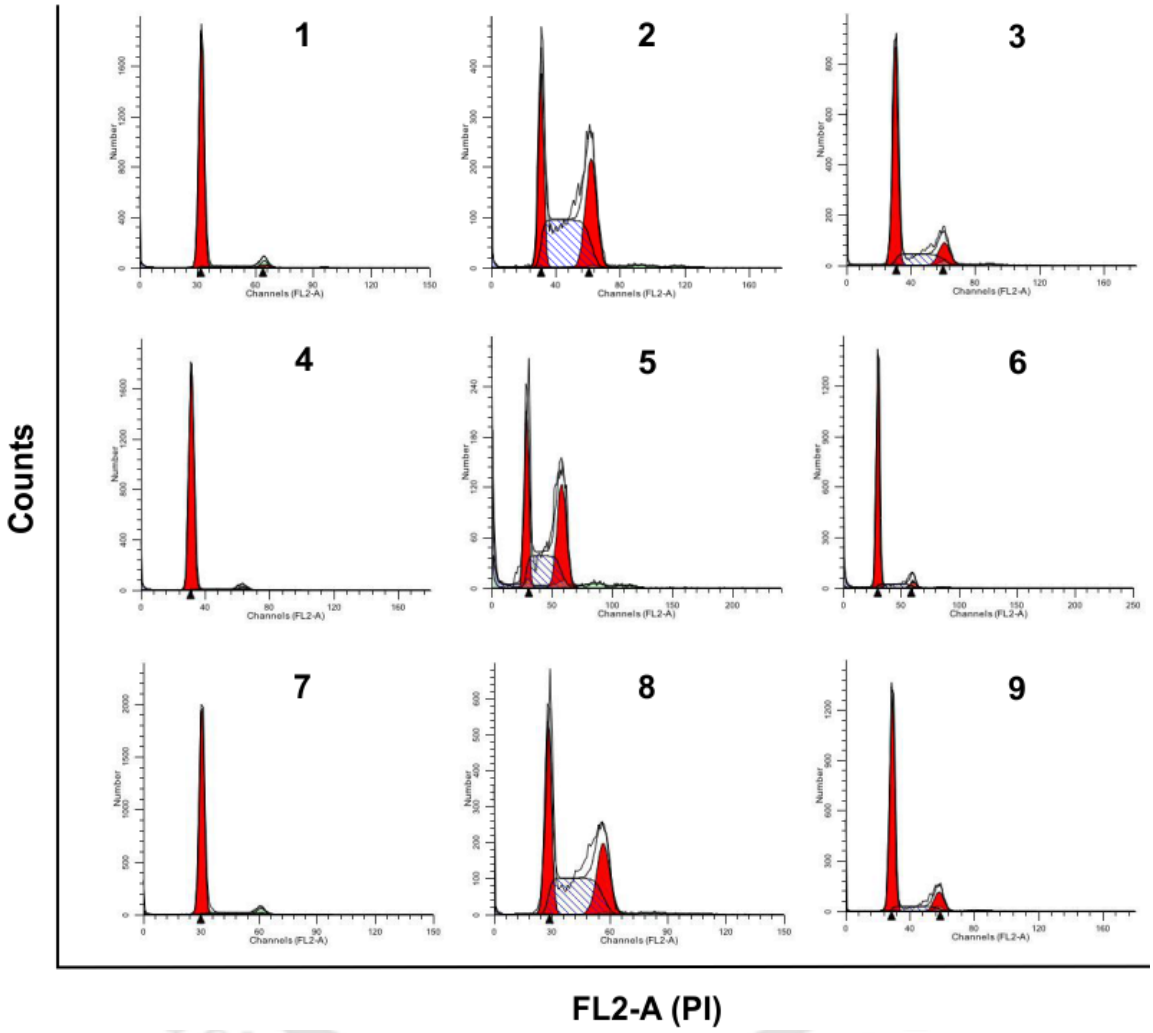

Figure 4.18. Histogram plots for cell cycle analysis of treated U87MG, U87-MCSF and U87-GFP cells. Cells were treated with 25μM 5-FU for 24 h and cell cycle was analysed by flow cytometer. Data was acquired by CellQuest Pro software and analysed by ModFit LT software. (1-3) U87MG cells; (4-6) U87-MCSF cells; (7-9) U87-GFP cells. (1,4,7) G0/G1 synchronised cells; (2,5,8) 24 h post synchronisation, untreated; (3,6,9) 24 h post synchronisation, 5-FU treated.

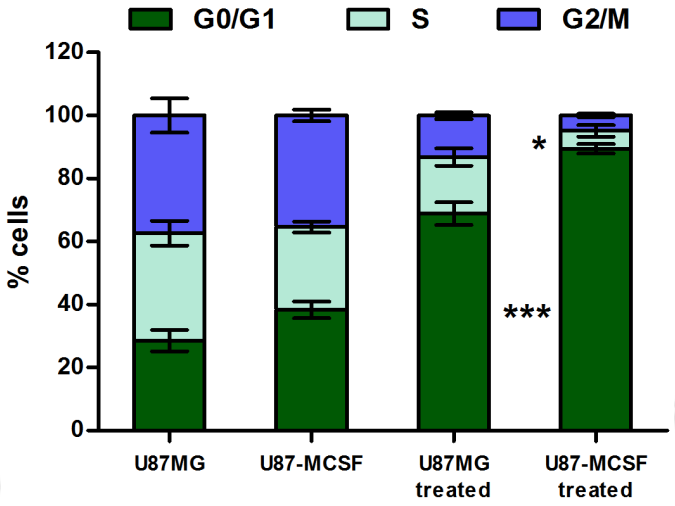

Figure 4.19. Cell cycle analysis of U87MG and U87-MCSF cells after treatment with 5-FU for 24 h. Majority of cells were accumulated in G0/G1 phase in treated population of U87-MCSF cells. Statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

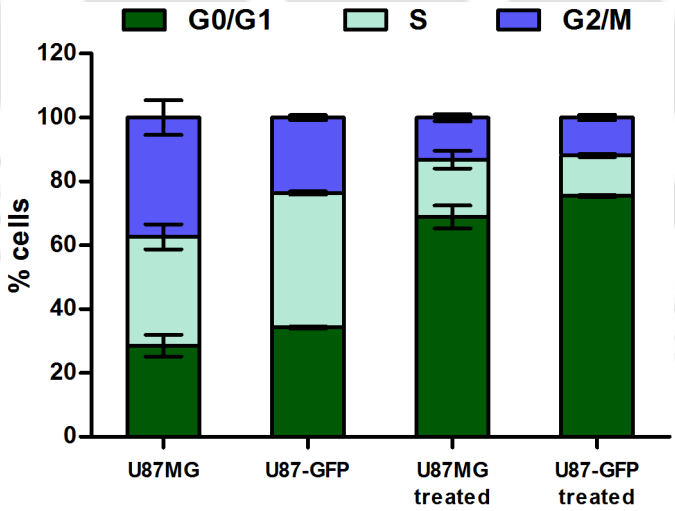

Figure 4.20. Cell cycle analysis of treated U87MG and U87-GFP cells. No difference in proportion of cells accumulated in G0/G1 phase was noted between treated U87MG and treated U87-GFP cells.

The data obtained showed significantly higher percentage (90 %) of U87-MCSF cells in G0/G1 phase than that of U87MG (69 %) and U87-GFP (75 %) cells after 5-FU treatment. This was accompanied by a corresponding decrease in proportion of cells in S and G2/M phases. These results indicated that MCSF expression augmented the accumulation of cells in G0/G1 phase upon treatment with 5-FU. However, this accumulation of cells in G0/G1 phase did not affect their viability and the cells did not enter into apoptosis. As the cell cycle of U87-MCSF cells was more affected by 5-FU treatment than treated U87MG or treated U87-GFP cells, we investigated the expression profiles of cyclins in our next experiments.

4.5. Effect of 5-FU on expression of cyclins

The expression levels of various cyclins were analysed by quantitative real time PCR. Specific cyclins are expressed transiently during the progression of cell cycle and are turned off at the end of individual phase. Before treatment, the cells were synchronised in G0/G1 phase by serum starvation for 48 h. Based on the results obtained in Figure 4.17, cyclins involved in G1 phase and G1-S transition phase were quantified after 18 h of 5-FU treatment, and the cyclins involved in late S phase and G2/M phase were quantified after 24 h of 5-FU treatment. A significant drop in the level of expression of cyclin E and a slight decrease in expression of cyclin A2 in 5-FU treated U87-MCSF cells were observed in comparison with 5-FU treated U87MG cells (Figure 4.21). We also measured the expression of p21, the cyclin-dependent kinase inhibitor. p21 expression was increased in the treated samples of both U87MG and U87-MCSF cells.

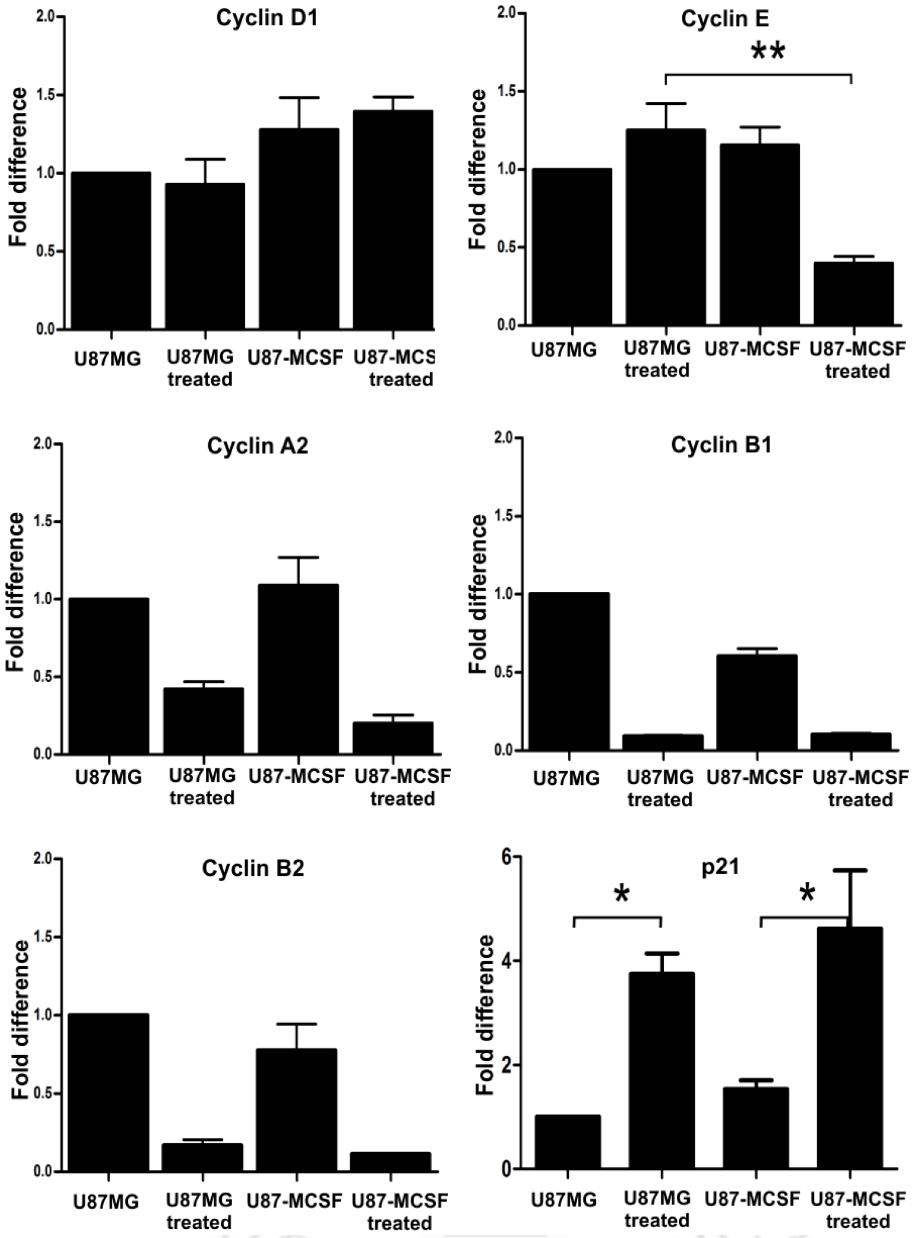

Figure 4.21. Real time PCR analysis to study the expression levels of various cyclins and p21. A significant decrease in the expression of cyclin E and a slight decrease in the expression of cyclin A2 was observed in treated U87-MCSF cells. The expression of p21, the cyclin dependent kinase inhibitor was increased in treated samples of both U87MG and U87-MCSF cells. Statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Results from Figure 4.21 revealed that Cyclin D1 expression remained unaltered between treated U87MG and treated U87-MCSF cells. After 18 h of 5-FU treatment, cyclin E expression was significantly decreased in treated U87-MCSF cells but not in treated U87MG cells (Figure 4.21). However, after 24 h of 5-FU treatment, down regulation in cyclin E expression was seen in treated samples of both U87MG and U87-MCSF cells (Figure 4.22). This denoted that the response of U87-MCSF cells to 5-FU treatment was faster than U87MG cells. A slight decrease in expression of cyclin A2 was also observed in 5-FU treated U87-MCSF cells in comparison to 5-FU treated U87MG cells after 24 h of 5-FU treatment (Figure 4.21). It is to be mentioned here that Cyclin E is involved in the G1 to S phase transition and cyclin A2 is expressed throughout the S and G2 phase. Hence, any decrease in expression of cyclin E and cyclin A2 denote the decrease in the transition of cells from G1 to S phase.

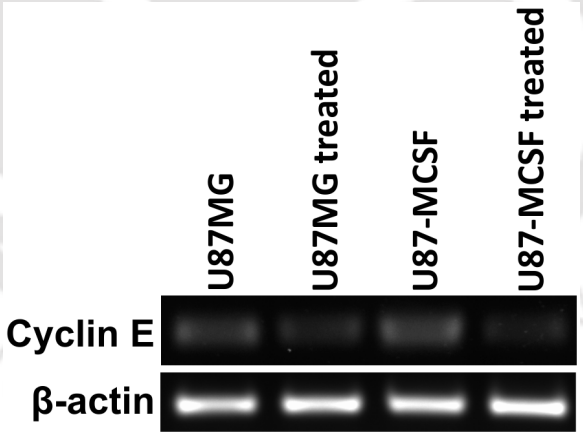

Figure 4.22. RT-PCR analysis of expression of cyclin E after 24 h of 5-FU treatment. The results showed decrease in expression of cyclin E in treated samples of both U87MG and U87-MCSF cells. β-actin expression was used as the control.

The expression of cyclin B1 and cyclin B2 was less in treated samples of both U87MG and U87-MCSF cells, correlating with the lesser number of cells progressing to the G2/M phase (Figure 4.21). There was an increase in the expression of p21, the cyclin-dependent kinase inhibitor, in the treated samples of both U87MG and U87-MCSF cells substantiating the increased G1 accumulation of cells after 5-FU treatment. Thus, differential expression of cyclins and the cdk inhibitor, p21 at various stages of cell cycle corroborated the cell growth retardation observed in 5-FU treated U87-MCSF cells.

4.6. Change in cell morphology on treatment with 5-FU

Cell growth retardation was observed in U87-MCSF cells after 5-FU treatment. Although apoptosis was absent, we observed morphological changes of U87-MCSF cells even when treated with low dose (25 μM) of 5-FU for 72 h. Methylene blue staining of U87MG and U87-MCSF cells showed the appearance of elongated, thin and spindle shaped morphology consistent with a mesenchymal appearance in U87-MCSF cells with 25 μM 5-FU treatment, but not in U87MG cells under the similar conditions (Figure 4.23). Treatment of U87MG and U87-MCSF cells with 50 μM 5-FU conferred elongated morphology in both cells.

Further, cytoskeleton staining of untreated and treated samples of U87MG and U87-MCSF cells was done using anti β-actin antibody (Figure 4.24). The results obtained reinforced the morphological changes observed in methylene blue staining. Elongated and mesenchymal cells were observed in 25 μM 5-FU treated U87-MCSF cells but not in 25 μM 5-FU treated U87MG cells after 72 h treatment. But, elongated cells were seen in both U87MG and U87-MCSF cells when treated with 50 μM 5-FU for 72 h.

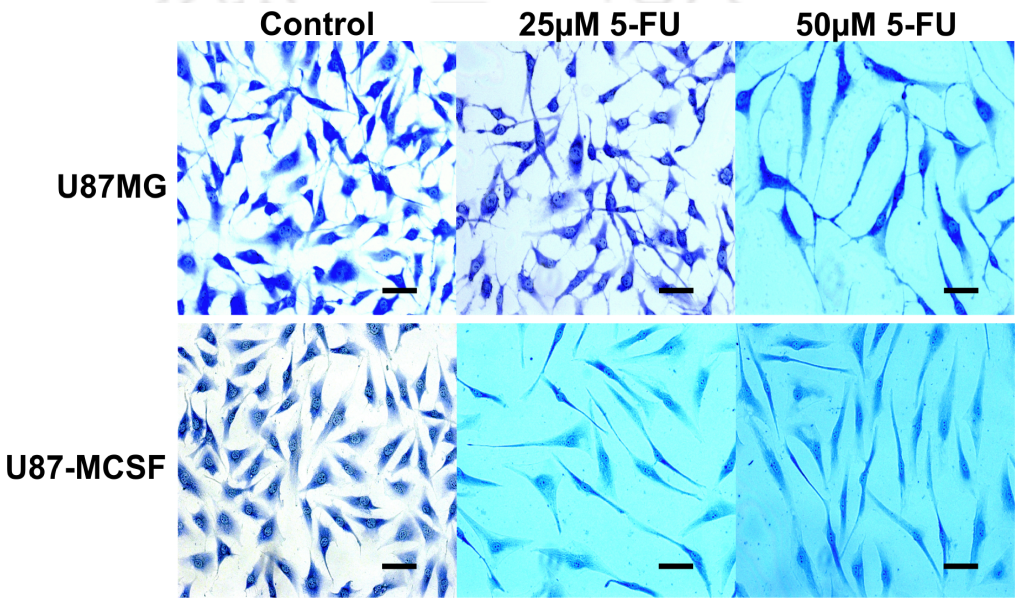

Figure 4.23. Methylene blue staining after 5-FU treatment for 72 h. Elongated and spindle shaped cells was seen in U87-MCSF cells treated with 25 μM 5-FU but not in treated U87MG cells. However, 50 μM 5-FU treatment conferred elongated morphology in treated samples of both U87MG and U87-MCSF cells. Scale bar: 50μm.

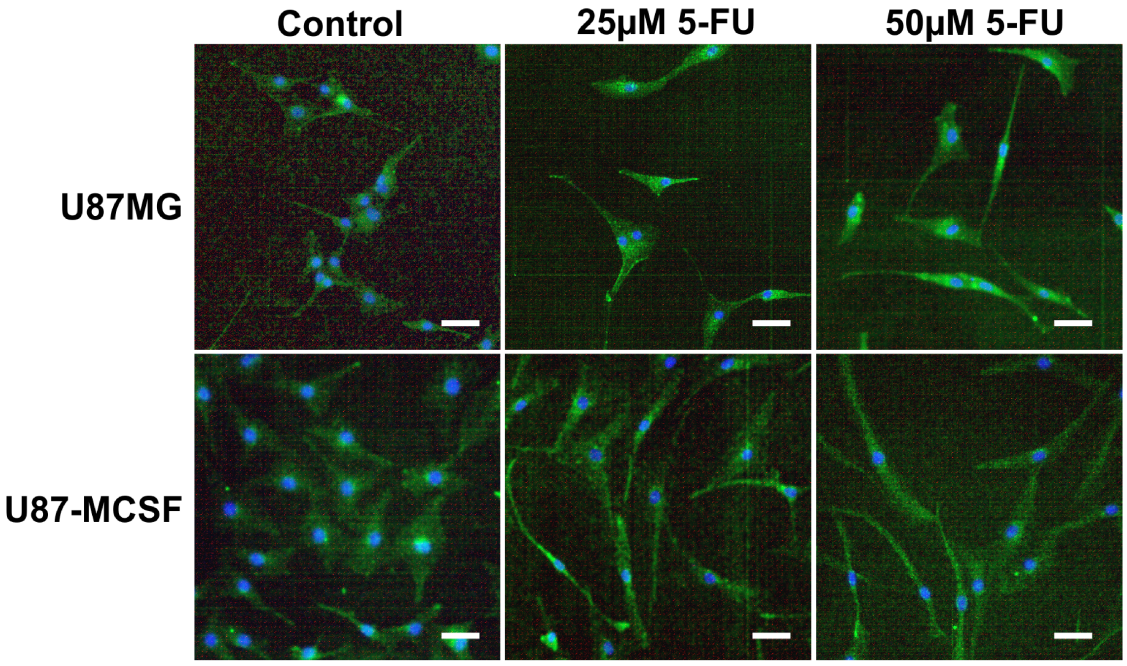

Figure 4.24. Actin cytoskeleton staining of cells using anti β-actin antibody. The morphological pictures showed the presence of elongated and mesenchymal cells in U87-MCSF cells treated with 25 μM 5-FU but not in U87MG cells treated with 25 μM 5-FU. Upon 50 μM 5-FU treatment, elongated cells were seen in both treated U87MG and treated U87-MCSF cells. Scale bar: 50 μm.

Additionally, microscopic examination of treated cells was carried out after dual staining with DAPI and CalceinAM. Cells were treated with 25 and 50 μM of 5-FU for 120 h and upon DAPI staining, cells showed intact nuclei. CalceinAM staining under same conditions, showed live cells with elongated morphologies (Figure 4.25), which was similar to the observation in methylene blue staining. This result indicated that upon prolonged treatment of cells with 5-FU for 120 h, both U87MG and U87-MCSF cells acquire mesenchymal morphology.

The morphological changes seen in the treated cells of U87MG and U87-MCSF prompted us to check the expression of GFAP and hTERT. Expression of telomerase is an important biochemical marker for assessment of cancerous cells. Germline tissues and cancerous cells have higher level of telomerase activity (Kim et al., 1994). Hence downregulation of telomerase is a very important indicator in successful cancer therapy. Increase in expression of GFAP and decrease in expression of hTERT were reported to be associated with astrocytic differentiation of glial cells (Das et al., 2008; Das et al., 2009). In order to evaluate these morphological features to examine the possibility of differentiation, the expression of classical biochemical marker for astrocytic differentiation namely, the glial fibrillary acidic protein (GFAP) and human telomerase reverse transcriptase (hTERT) catalytic subunit was analysed by semi-quantitative RT-PCR (Figure 4.26). The expression pattern of GFAP was unchanged in treated U87-MCSF cells which showed the absence of astrocytic differentiation. However, the expression of hTERT was significantly reduced in both U87MG and U87-MCSF cells after 5-FU treatment, justifying the suppression of cell proliferation.

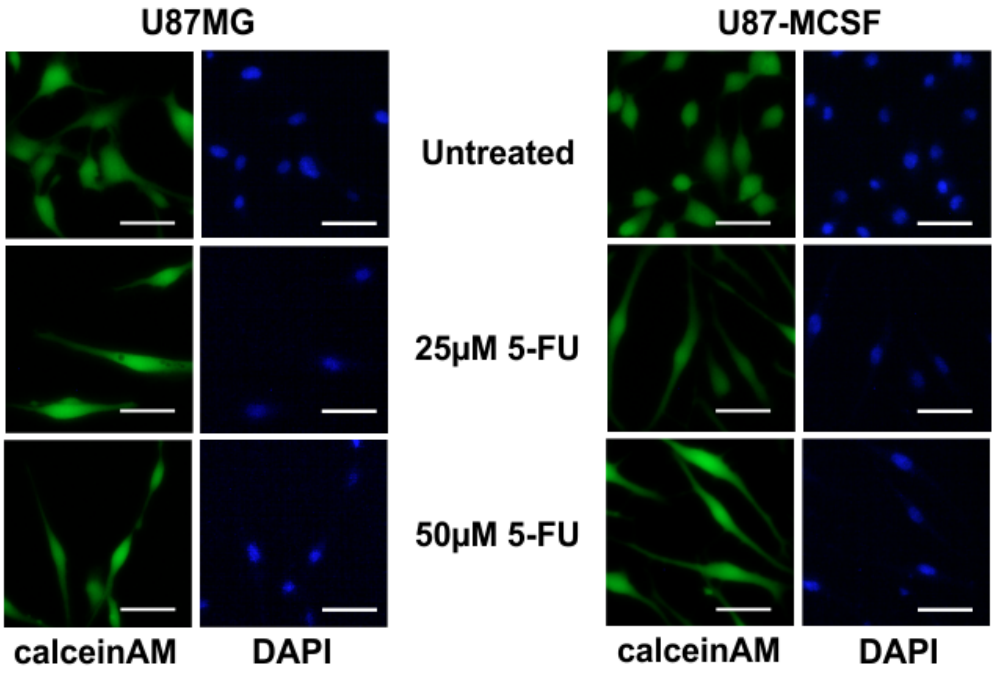

Figure 4.25. Microscopic examination of treated cells by DAPI/CalceinAM staining. Cells treated with 5-FU for 120 h were stained with DAPI/CalceinAM and observed under a fluorescence microscope. The results revealed the presence of elongated cells with intact nuclei in treated samples of both U87MG and U87-MCSF cells. Scale bar: 50μm.

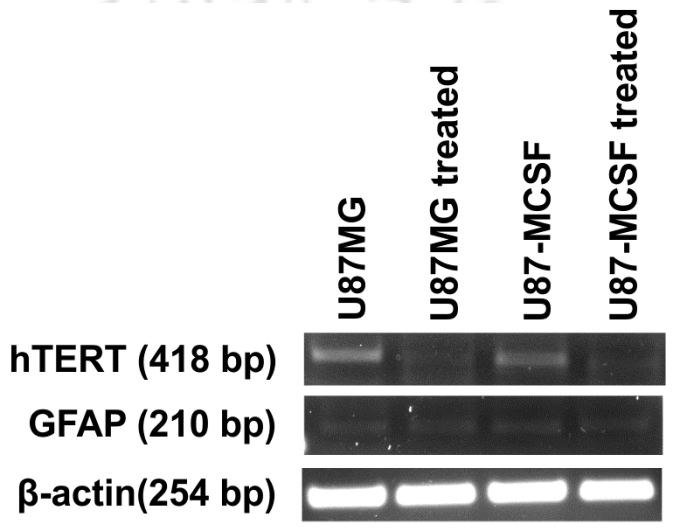

Figure 4.26. Semi quantitative RT-PCR analysis of expression of hTERT and GFAP genes. No change in expression of GFAP was observed between untreated and treated samples of U87MG and U87-MCSF cells. There was a down-regulation in the expression of hTERT in treated samples of both U87MG and U87-MCSF as compared to their corresponding untreated samples. Expression of β-actin was same across all the samples analysed.

4.7. Epithelial-mesenchymal transition (EMT) of U87-MCSF cells

The morphology of untreated U87MG and U87-MCSF cells was examined carefully with an inverted microscope. Spindle shaped cells with more mesenchymal-like appearance were observed in untreated U87-MCSF cells (Figure 4.27). The spindle shaped and elongated morphology was more prominent after treatment with 5-FU as seen by methylene blue staining (Figure 4.23) and actin cytoskeleton staining (Figure 4.24) in treated U87-MCSF cells. These morphological features indicated the possible occurrence of epithelial-mesenchymal transition in U87-MCSF cells. To investigate this, expression of epithelial cell marker E-cadherin and mesenchymal cell markers vimentin, N-cadherin, fibronectin was analysed by semi-quantitative RT-PCR.

EMT is a conserved transdifferentiation cellular process conferring the features of mesenchymal cells over the basal epithelial cells, increasing their invasive properties and metastasis (Singh and Settleman, 2010; Sethi and Kang, 2011). Cells which have undergone EMT are resistant to apoptosis (Kalluri and Weinberg, 2009) and have increased expression of mesenchymal markers such as N-cadherin, vimentin, fibronectin etc., (Lee et al., 2006). In our study, semi-quantitative RT-PCR analyses of epithelial and mesenchymal markers were performed. MCSF expression in U87MG cells resulted in the appearance of spindle shaped, more mesenchymal type cells indicating the occurrence of EMT. A corresponding upregulation in expression of mesenchymal markers like N-cadherin and vimentin (1.98 and 2.18 fold respectively) was also noted in U87-MCSF cells (Figure 4.28). E-cadherin expression was found to be absent in all the samples analysed (data not shown) which correlated with the previously reported data showing lack of E-cadherin expression in glioblastoma (Bellail et al., 2004).

With 5-FU treatment, the expression of the mesenchymal marker, N-cadherin was further increased in treated U87-MCSF cells by 3.32 fold while the

expression of vimentin remained unchanged and similar to untreated U87-MCSF cells (Figure 4.28). In treated U87MG cells, only a marginal increase in the expression of N-cadherin and vimentin (1.26 and 1.41 fold respectively) was noted. The expression level of fibronectin was unaltered in treated samples of both cell types. 5-FU treatment induced mesenchymal properties in both U87MG and U87-MCSF cells as seen in Figure 4.23 and Figure 4.24. However, the concentration of 5-FU required to induce these changes varied between U87MG and U87-MCSF cells. While elongated and spindle shaped mesenchymal cells were seen in treated U87-MCSF when treated with 25 μM 5-FU for 72 h, mesenchymal morphology can be seen in treated U87MG cells only when treated with 50 μM 5-FU for 72 h. But when treatment with 25 μM 5-FU was prolonged for 120 h, elongated mesenchymal type cells were seen in treated samples of both U87MG and U87-MCSF cells (Figure 4.25).

We also investigated the expression of Notch-1, one of the inducers of EMT. As seen in Figure 4.28, Notch-1 expression was upregulated in untreated U87-MCSF cells. Quantitative expression analysis by real time PCR analysis showed that notch-1 expression was increased by around 4-fold in untreated U87-MCSF cells when compared with untreated U87MG cells (Figure 4.29). The role of Notch-1 signalling in inducing EMT had been reported previously in pancreatic cancer cells (Bao et al., 2011). Numerous reports have shown the association between EMT and the progression of cancer (Berx et al., 2007; Burk et al., 2008; Bao et al., 2011). Invasion of tumor was decreased when Notch-1 expression was downregulated by using siRNA (Wang et al., 2006). Consistent with these reports, in our study we found that an increase in the expression of Notch-1 was associated with increase in expression of mesenchymal markers in U87-MCSF cells indicating the occurrence of EMT.

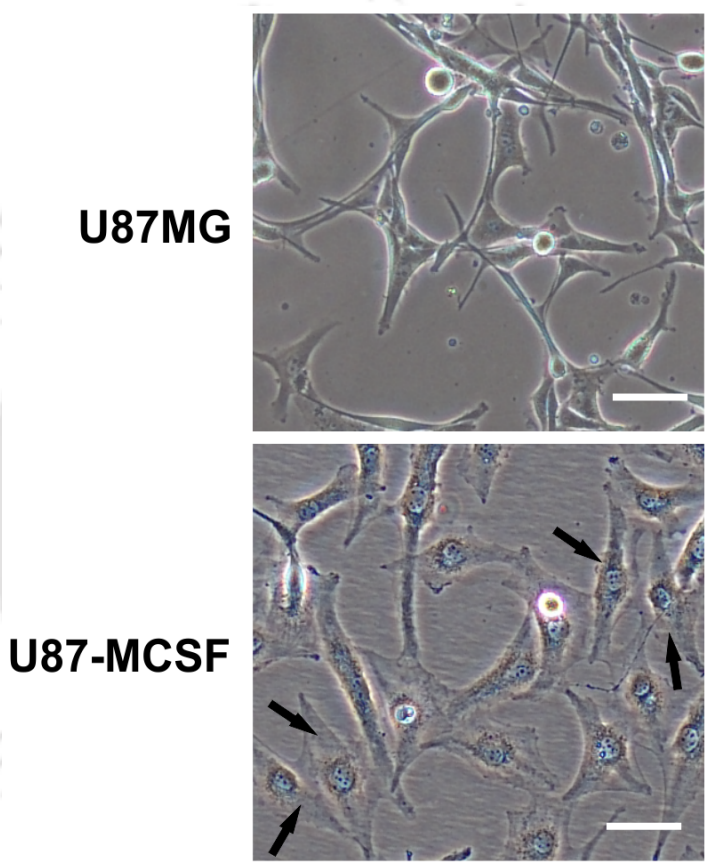

Figure 4.27. Morphology of untreated U87MG and U87-MCSF cells. U87-MCSF cells showed spindle shaped, mesenchymal like cells (indicated by arrows). Scale bar: 50 μm.

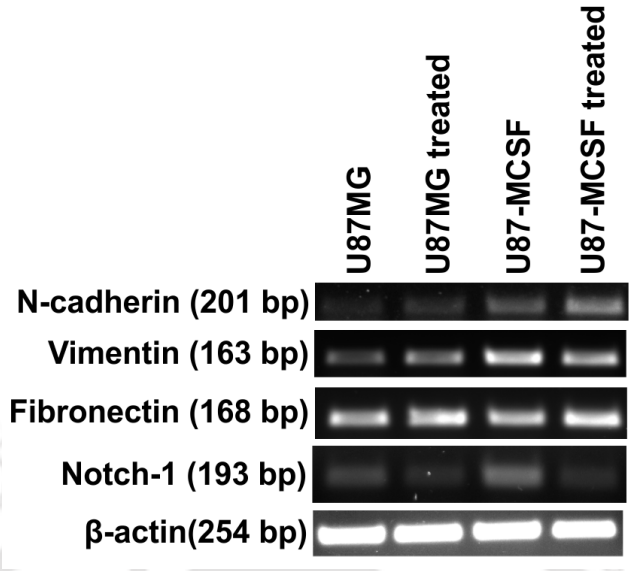

Figure 4.28. Semi quantitative RT-PCR analysis for expression of EMT markers. Upregulation in the expression of mesenchymal markers, N-cadherin and Vimentin was observed in untreated and treated U87-MCSF cells. The expression of Notch-1 was also increased in U87-MCSF cells. Beta actin was used as the control.

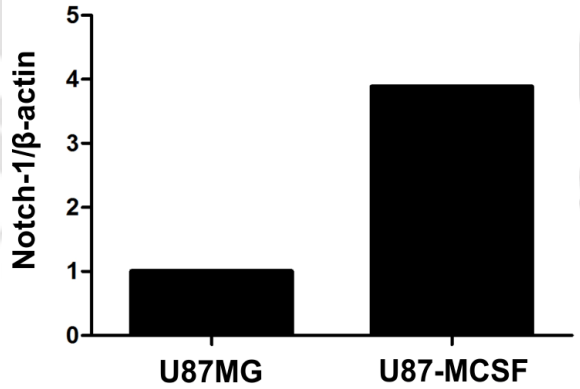

Figure 4.29. Real time PCR analysis of Notch-1 expression. U87-MCSF cells showed elevated expression of Notch-1 gene as compared to U87MG cells.

4.8. Expression of CSF-1R and Localization of MCSF

All the downstream signalling of MCSF is mediated through the receptor, CSF-1R. We checked the expression of CSF-1R in U87MG and U87-MCSF cells. As shown in Figure 4.30, CSF-1R expression was reduced in U87-MCSF cells as compared to U87MG cells. This result was not surprising and it was in agreement with the previous report (Yue et al., 1993), which showed down regulation of mRNA of CSF-1R by MCSF in primary macrophages. However, this result raises queries about the location of MCSF within U87-MCSF cells. Therefore, we investigated the localization of MCSF using anti-MCSF antibody and subsequent staining with a FITC tagged secondary antibody.

Microscopic examination of U87-MCSF cells was done by fixing the cells with 3.7 % paraformaldehyde solution with or without Triton X-100. After fixing, the cells were incubated with primary antibody and subsequently, with the corresponding FITC tagged secondary antibody. Triton X-100 was used as a membrane perforating agent in the fixing solution (Lleo et al., 2009). If MCSF is present on the membrane of U87-MCSF cells, both samples, irrespective of being fixed with or without the membrane perforating agent Triton X-100 will show FITC stained fluorescence. However, if it is located inside the cytoplasm, only the cells fixed in the presence of Triton X-100 will give fluorescence. In our study, we observed very little fluorescence in the absence of Triton X-100. On contrary bright green fluorescence was seen in samples fixed with 3.7 % paraformaldehyde solution containing Triton X-100 (Figure 4.31).

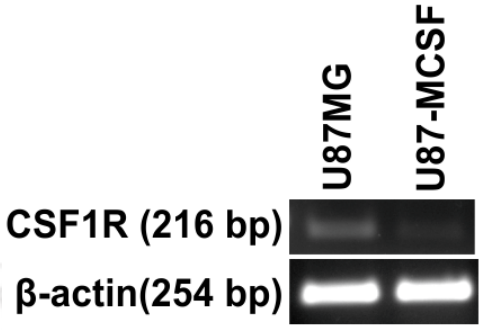

Figure 4.30. RT-PCR analysis of CSF-1R. CSF-1R expression was down-regulated in untreated U87-MCSF cells. Beta actin expression was taken as the internal control.

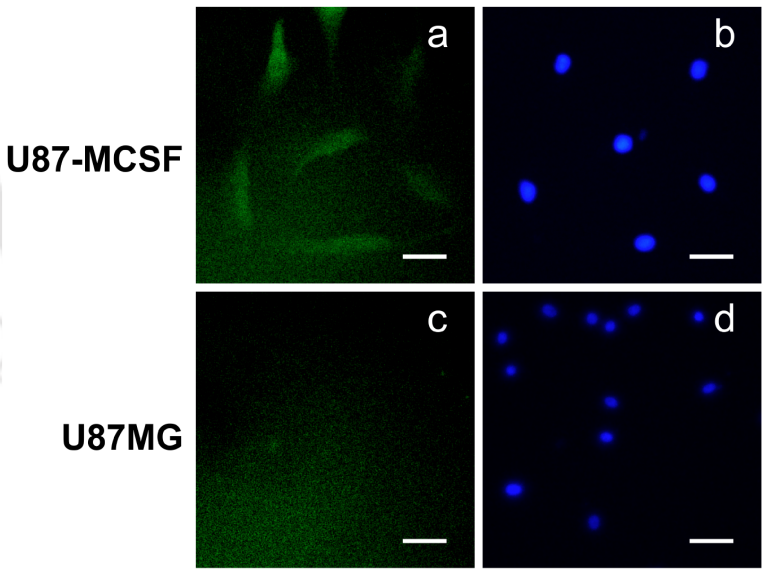

Figure 4.31. Examination of localization of MCSF in U87-MCSF cells. Microscopic studies using anti-MCSF antibody revealed the cytoplasmic location of MCSF. Triton X-100 was used as membrane perforating agent in fixing solution. DAPI stained nuclei of the cells were also shown. Scale bar: 50 μm.

These results revealed that MCSF is located predominantly in the cytoplasm and not at the cell membrane of U87-MCSF cells. Moreover, the expression of the receptor for MCSF, CSF-1R was down regulated in U87-MCSF cells. The known receptor for MCSF, the CSF-1R is present at the plasma membrane. But recent evidence showed the presence of functional receptor for MCSF at the nuclear envelope of various cancer cells and macrophages (Zwaenepoel et al., 2012). This explains that the observed effects of MCSF in U87-MCSF cells upon 5-FU treatment could be mediated through the receptor located at the nuclear envelope.

4.9. Appearance of cancer stem cells in treated cell population

Many of the recent reports indicated that EMT can trigger the acquisition of stem-like properties in the tumor cells (Mani et al., 2008; Polyak and Weinberg, 2009; Singh and Settleman, 2010). A sub population of cells within several tumors may exhibit stem-like properties or tend to convert into stem cells. The cancer stem cells (CSC) have extremely slow rate of proliferation, increased expression of multidrug resistance genes and are considered to be the primary reason for the relapse of the tumor after treatment (Dembinski and Krauss, 2009; Roesch et al., 2010; Moore and Lyle, 2011).

The reduced proliferation, change in cell morphology, indications for the presence of EMT and the increased resistance to apoptosis suggested a possible induction of cancer stem cells in U87-MCSF cells upon treatment with 5-FU. We analysed the expression of markers for cancer stem cells, CD24 and CD44 by flow cytometry and real time PCR. Flow cytometry analysis showed that the expression of CD24 was increased by 6.93% and 33.37% in U87MG cells and U87-MCSF cells, respectively, when treated with 25μM 5-FU (Figure 4.32). No increase in CD44 expression was noted in treated samples of both U87MG and U87-MCSF cells.

Quantitative expression studies by real time PCR was also performed. The results revealed the increase in CD24 expression by around 4 fold in treated U87-MCSF cells, whereas only 2 fold increase in CD24 expression was seen in treated U87MG cells (Figure 4.33). CD44 expression remained unchanged in treated cells of both U87MG and U87-MCSF.

Previous reports showed that CSCs isolated from breast cancer were predominantly $CD44^{high}/CD24^{low}$ cells (Mani et al., 2008). In contrast to this, breast cancer cells with $CD44^{low}/CD24^{high}$ phenotype was also reported to have poor prognosis with treatment (Ahmed et al., 2012). Additionally, $CD44^{high}/CD24^{high}$ CSCs were found in gastric, pancreatic, ovarian and colorectal cancers (Huang et al., 2008; Dembinski and Krauss, 2009; Zhang et al., 2011; Jaggupilli and Elkord, 2012). The expression of CD133 was considered to be indicative of CSCs in glioblastoma (Singh et al., 2003; Zeppernick et al., 2008). However, there are evidences for the presence of CD133 negative CSCs in glioblastoma (Wang et al., 2008; Brescia et al., 2012). In our study, we did not find any significant expression of CD133 in U87MG and U87-MCSF cells, both before and after 5-FU treatment. Recently, Deng et al. reported overexpression of CD24 in higher grade gliomas and the patients with CD24 positive tumors had poor prognostic outcome after surgery (Deng et al., 2012). The definition of what exactly constitutes cancer stem cells remains unclear and there are no universally accepted cancer stem cell markers for different types of cancers (Jaggupilli and Elkord, 2012). Here, in our study, we noted an increase in $CD24^{high}/CD44^{low}$ population of cells in both treated U87MG and U87-MCSF cells. However, treated U87-MCSF cells had 33.37% of $CD24^{high}/CD44^{low}$ cells as compared to 6.93% in treated U87MG cells. In addition, real time PCR analysis revealed that CD44 expression was uniform in 5-FU treated U87MG and U87-MCSF cells, but the CD24 expression was considerably higher in

treated U87-MCSF cells. This increased expression of CD24 surface marker indicated the exacerbated cancer stem cell properties of treated U87-MCSF cells.

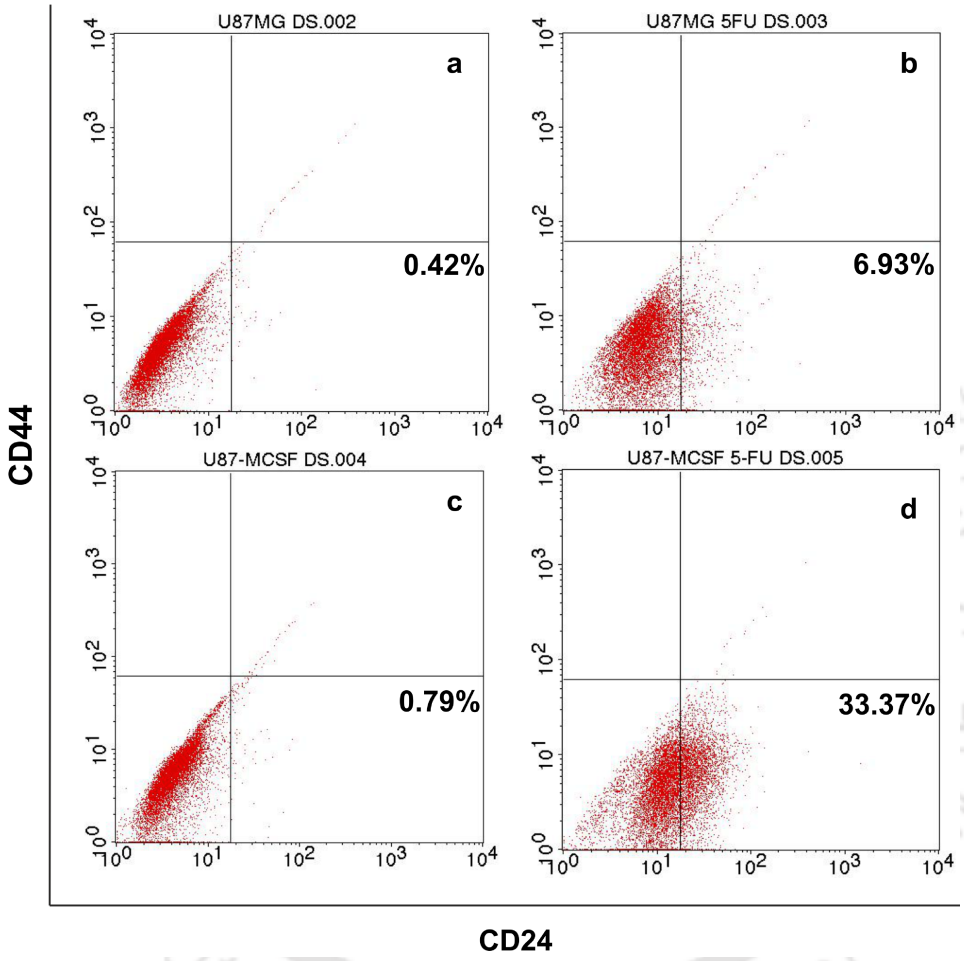

Figure 4.32. Flow cytometry analysis for the expression of CD24 and CD44. Cells were treated with 25 μM 5-FU for 120 h and stained with FITC tagged mouse Anti-Human CD24 and PE tagged mouse Anti-Human CD44. Samples were acquired and analysed with CellQuest Pro software.

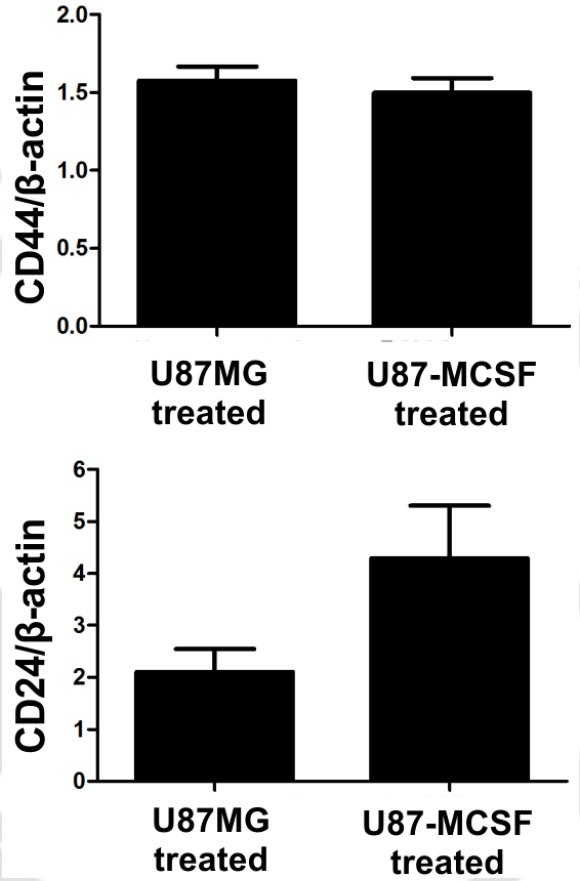

Figure 4.33. Real time PCR analysis of expression of CD44 and CD24 genes. The data was plotted as the ratio of gene expression in treated sample to untreated sample for the respective cells.

4.10. Upregulation of ABC transporter genes in treated U87-MCSF cells

Tumor cells are known to acquire resistance to chemotherapeutic drugs through expression of various multidrug resistance genes and cancer stem cells can effectively increase the drug resistance through upregulation of drug efflux transporter genes (Dean et al., 2005; Vinogradov and Wei, 2012). CSCs isolated by forming spheroid cultures of glioblastoma tumor cells were previously shown to have high expression of ABCB1 gene and were found to be significantly resistant to chemotherapeutic agents (Nakai et al., 2009). The ABC transporter genes have been associated with the expulsion of drugs in many types of cancers. The presence of cancer stem cells in treated samples of both U87MG and U87-MCSF cells prompted us to analyse the expression of ABC transporter genes-ABCB1, ABCG1, ABCG2 and the oncogene, mdm2.

Real time PCR analysis showed that the expression of ABCG1 and ABCB1 was increased in both treated U87MG and treated U87-MCSF cells (Figure 4.34). However, the expression of ABCB1 or multi-drug resistant 1 (MDR1) gene was increased 10.2 fold in treated U87-MCSF cells as compared to the 2 fold increase in treated U87MG cells. Similarly, the expression of mdm2 oncogene was also increased 6 fold in treated U87-MCSF cells in comparison to the 3.3 fold increase found in the treated U87MG cells. Upregulation in ABCB1 and ABCG1 genes explained mechanism of drug resistance in treated U87MG cells. However, the elevated increase in the expression of ABCB1 and mdm2 genes in treated U87-MCSF cells as compared to the treated U87MG cells further intensified the drug resistive properties of the glioblastoma cells expressing MCSF.

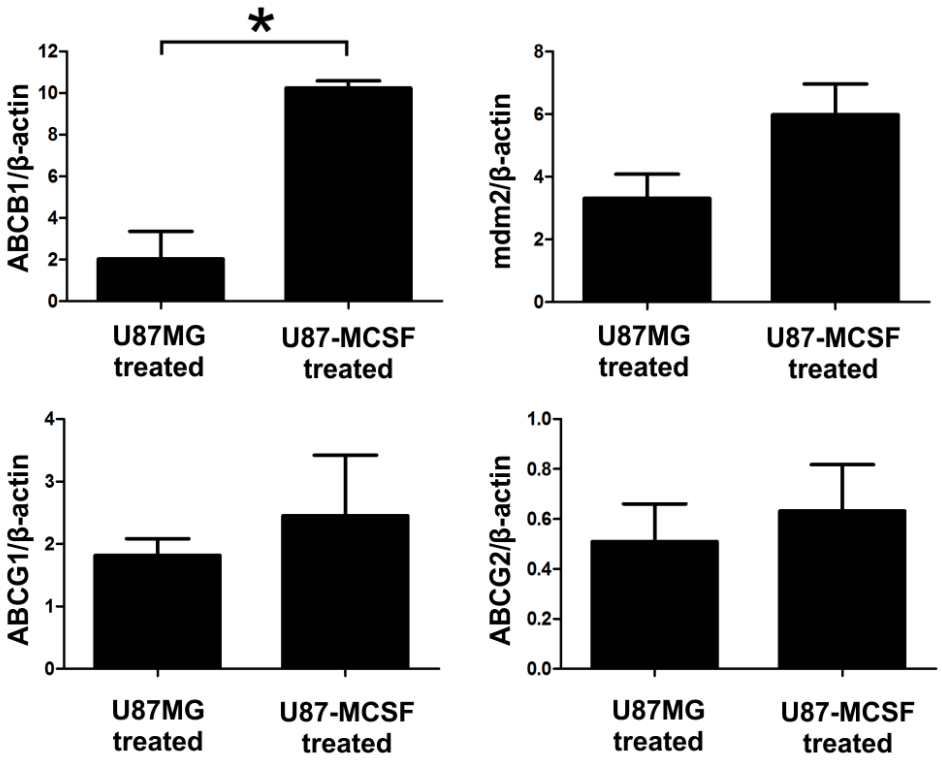

Figure 4.34. Real time PCR analysis of expression of ABCB1, mdm2, ABCG1 and ABCG2 genes. The data was plotted as the ratio of gene expression in treated sample to untreated sample for the respective cells. Statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

We also examined the expression level of RALBP1 gene, a non-ABC transporter associated with MDR (multidrug resistance). RALBP1 is a ubiquitous protein present in humans and is one of the non-ABC transporter proteins implicated in resistance to many chemotherapeutic drugs (Awasthi et al., 2005; Drake et al., 2007). In our study, although the expression of RALBP1 was slightly increased in untreated U87-MCSF cells (1.29 fold) as compared to untreated U87MG cells, no upregulation in RALBP1 expression was observed in 5-FU treated samples of both U87MG and U87-MCSF cells with respect to their corresponding untreated samples (Figure 4.35).

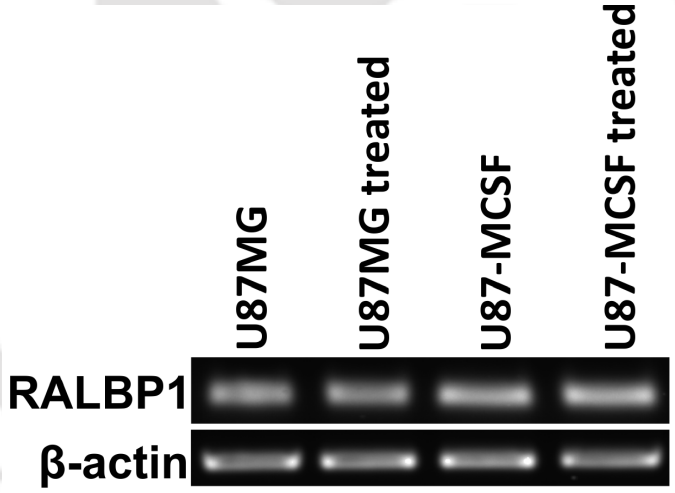

Figure 4.35. Semi-quantitative RT-PCR analysis of expression of RALBP1. A slight increase in expression of RALBP1 was observed in untreated U87-MCSF cells. However, no increase in RALBP1 expression was found after 5-FU treatment. Fold changes in RALBP1 expression was quantified by densitometry analysis using ImageJ software.

Glioblastoma remains one of the most prevalent malignant tumors with poor therapeutic response (Sharma et al., 2013). Expression of various multi-drug resistant proteins has mainly contributed to the ability of glioma cells to develop chemoresistant phenotype (Bredel and Zentner, 2002; Nakai et al., 2009). In our study, U87-MCSF cells exhibited retarded cell growth than U87MG cells after 5-FU administration as shown by XTT assay. However, this reduced cell growth did not lead to any significant apoptosis even after prolonged treatment with 5-FU. On contrary, a significant retardation in the proliferation of treated U87-MCSF cells was observed by cell cycle analysis.

We observed the morphological changes resembling EMT in majority of U87-MCSF cells. However, EMT in untreated U87-MCSF cells did not lead to the formation of $CD24^{high}/CD44^{low}$ CSCs as seen in Figure 4.32. Only after treatment with 5-FU, we noted an increase in $CD24^{high}/CD44^{low}$ CSCs in U87-MCSF cells. Further, the percentage of $CD24^{high}/CD44^{low}$ CSCs in treated U87-MCSF cells were 33.37 and in treated U87MG cells, the percentage of $CD24^{high}/CD44^{low}$ cells were only 6.93. Ideally, the remaining cells should be susceptible to 5-FU treatment and undergo apoptosis. But we did not observe any cell death even after prolonged treatment with 5-FU. This observation hints that there exists another line of defence to 5-FU apart from CSC mediated drug resistance. EMT and its regulators were shown to directly impart resistance to drugs (Arumugam et al., 2009; McConkey et al., 2009). Hence, it is possible that EMT in U87-MCSF cells increases the resistance to 5-FU through other pathways independent of CSC formation.

Further, results from XTT assay (Figure 4.9) and cell cycle analysis (Figure 4.18 and Figure 4.19) showed that the rate of proliferation was impaired in a vast majority of 5-FU treated U87MG and U87-MCSF cells. This suggests for the presence of slow proliferating and quiescent cells in treated U87MG and treated U87-MCSF cells. This quiescent nature of certain tumor cells was previously

reported to be the source of continuous tumor growth (Roesch et al., 2010). This quiescent cells were also reported to have morphological changes corresponding to EMT (Dembinski and Krauss, 2009) and they were resistant to chemotherapy (Moore and Lyle, 2011). This quiescent property is also shared by CSCs (Jordan et al., 2006; Visvader and Lindeman, 2008). In our study, occurrence of EMT and increase in drug resistance were seen in majority of U87-MCSF cells. However, the percentage of $CD24^{high}/CD44^{low}$ CSCs was found to be only 6.93 and 33.37 in treated U87MG and treated U87-MCSF cells respectively. So, it can be assumed that the quiescent nature of treated cells may not always culminate in the formation of CSCs. It may also be possible that treated cells do have more CSCs, expressing surface markers other than CD24 and CD44.

Taken together, our results demonstrated that U87MG cells developed resistive phenotype when subjected to 5-FU treatment. This drug resistance was further enhanced when MCSF gene was expressed in U87MG cells. MCSF is known to be a pro-tumoral cytokine and it is a major chemoattractant for monocytes and macrophages in tumor cells (Dorsch et al., 1993; Green et al., 2009). The tumor associated macrophages (TAMs) gets preferentially polarized to mount distinct M2 functions, thereby promoting angiogenesis and metastasis of tumor cells (Sica et al., 2008). The elevated expression of MCSF along with its receptor CSF-1R in breast, uterine and ovarian cancer was associated with tumor progression (Smith et al., 1995; Kacinski, 1997). Further, high level of MCSF in several tumors had been strongly associated with poor prognosis (Chambers et al., 1997; Mroczko et al., 2007). Our results provide evidence for MCSF increasing the resistance of tumor cells to 5-FU, by upregulating the expression of MDR associated genes. To the best of our knowledge, this is the first report implicating the cancer stem cells as a major nodal point for the MCSF associated drug resistance in a tumor cell type. This information would be useful for developing

therapeutic strategies against drug resistant cancers. The pivotal genes involved in resistance towards 5-FU treatment and the emergence of stem-like properties in treated U87-MCSF cells are depicted in Figure 4.36.

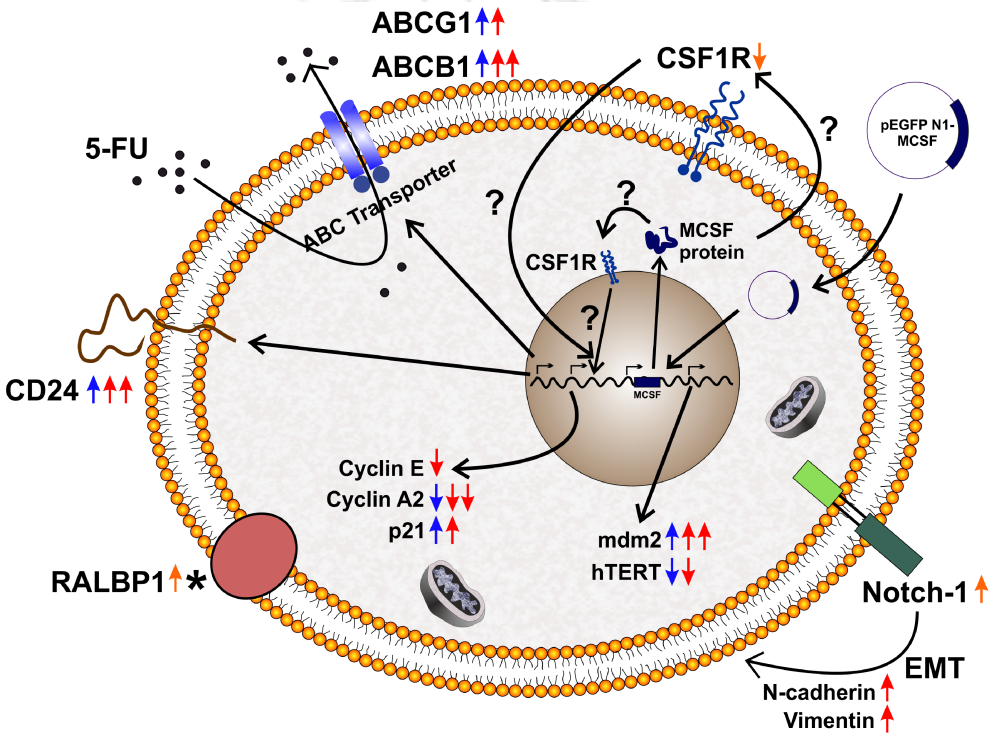

Figure 4.36. Schematic representation for the mechanism of 5-FU resistance in U87MG and U87-MCSF cells. The scheme depicts the upregulation and downregulation of various genes involved in 5-FU resistance and CSC formation. (Orange coloured arrows indicated untreated U87-MCSF cells; Blue coloured arrows indicated 5-FU treated U87MG cells; Red coloured arrows indicated 5-FU treated U87-MCSF cells; * indicated that RALBP1 expression remained unchanged in treated samples with respect to their corresponding untreated samples).

SECTION 5

CONCLUSION AND FUTURE PROSPECTS

Section 5

CONCLUSION AND FUTURE PROSPECTS

Macrophage Colony Stimulating Factor (MCSF) is traditionally classified as a pro-tumor cytokine, owing to its role in tumor development and metastasis. However, recent reports demonstrated the potential anti-tumor properties of membrane bound isoform of MCSF. These conflicting findings kindled our interest to gain deeper insights into the role played by MCSF in cancer. The present thesis work focuses mainly on exploring the properties of MCSF on a human glioblastoma cell line and understanding potential therapeutic outcome in MCSF expressing glioblastoma upon treatment with 5-fluorouracil.

Treatment with 5-FU resulted in retardation of cell proliferation in both U87MG and U87-MCSF cells. However, the expression of MCSF augmented the effect by accumulating over 90% of cells in G0/G1 phase as compared to the 69% in 5-FU treated U87MG cells. There was a substantial decrease in the expression of G1-S phase transition cyclins- cyclin E and cyclin A2 in 5-FU treated U87-MCSF cells, which reinforced the cell growth retardation observed in cell cycle analysis. Prolonged treatment with 5-FU did not exhibit apoptosis, but ensued epithelial-mesenchymal transition in 5-FU treated U87-MCSF cells. Notch-1, the key mediator of EMT was upregulated in U87-MCSF cells and there was an increase in expression of mesenchymal markers, N-cadherin and vimentin in 5-FU treated U87-MCSF cells. The entire sequence of events unfolding in U87MG and U87-MCSF cells upon treatment with 5-FU is summarized in the figure 5.1.

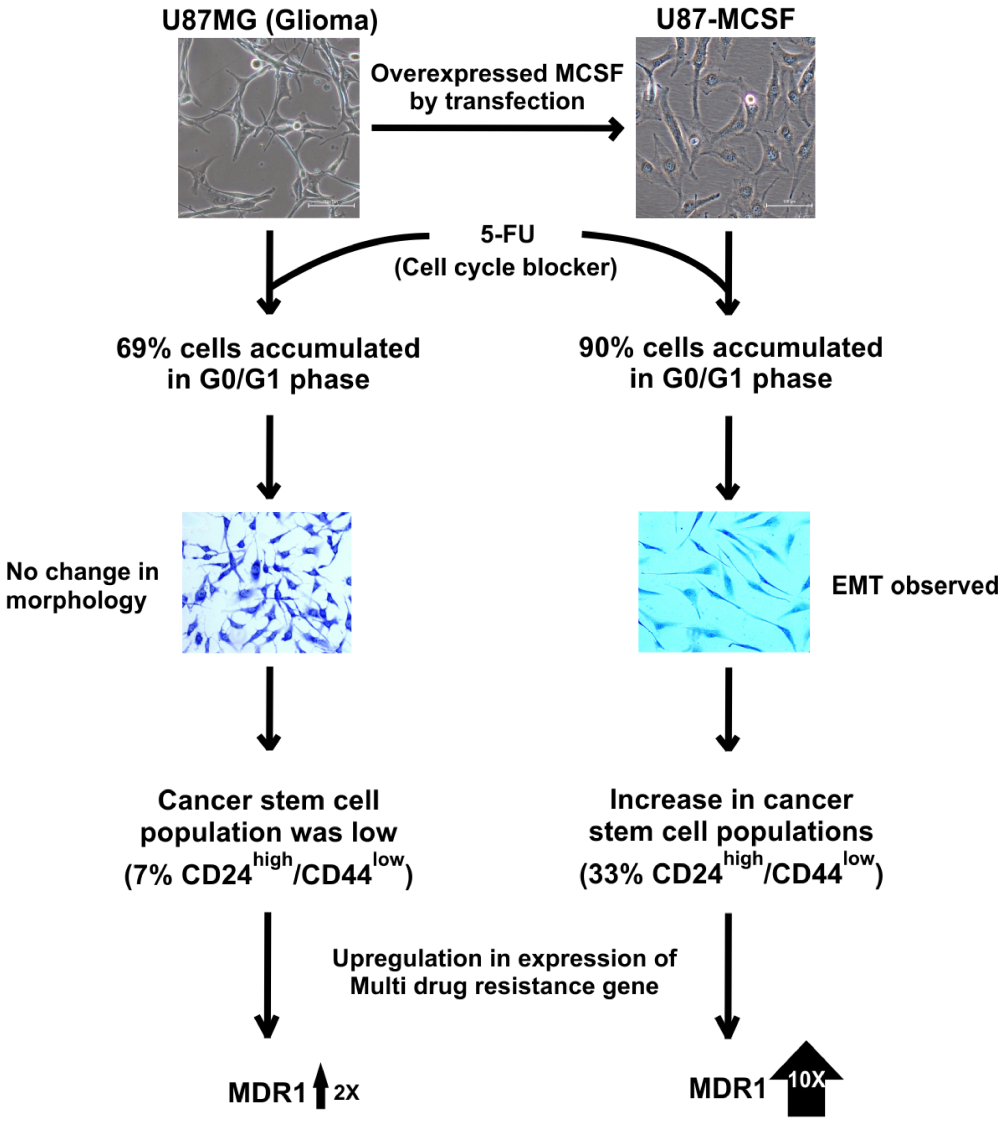

Figure 5.1. Summary of events in U87MG and U87-MCSF cells upon 5-FU treatment.

While U87MG cells acquired 6.93% $CD24^{high}/CD44^{low}$ cancer stem cells (CSC) after treatment with 5-FU, the MCSF expression and subsequent occurrence of EMT, resulted in an increase in the proportion of $CD24^{high}/CD44^{low}$ cancer stem cells to 33.37% in 5-FU treated U87-MCSF cells. This increase in CSCs in 5-FU

treated U87-MCSF cells also accounted for the 10-fold upregulation in expression of MDR1 gene as compared to the 2-fold upregulation in 5-FU treated U87MG cells. Increase in CSCs and upregulation in MDR1 ultimately conferred the drug resistive properties in U87-MCSF cells upon treatment with 5-FU.

The pro-tumor activities of MCSF are mainly associated to the context of MCSF infiltrating tumor with monocytes and macrophages. While numerous studies have reported the dismal response of MCSF expressing tumor cells to chemotherapy, the reason behind this poor response remains unknown. The notable outcome of this research work is the finding that MCSF expression contribute to the drug resistive phenotype in U87MG cells through formation of $CD24^{high}/CD44^{low}$ cancer stem cells upon treatment with 5-FU.

Taking a step further, in future, the major molecular signaling pathways involved in formation of cancer stem cells in 5-FU treated glioblastoma cells will be studied. Current therapeutic strategies, however successful in eradicating majority of drug sensitive cancer cells, are largely ineffective in killing the minor population of drug resistive cancer stem cells. These cancer stem cells should be the focus in all future anti-cancer therapeutic endeavors. Understanding the signaling pathways for cancer stem cell formation in presence of MCSF will give ample scope for development of therapeutic strategies aimed at targeting relevant signaling molecules. Silencing the expression of key genes involved in drug resistance by RNA interference or by developing small molecule inhibitors can serve as a significant step towards a highly efficient anti-cancer therapy.

REFERENCES

Abal, M., Bras-Goncalves, R., Judde, J. G., Fsihi, H., De Cremoux, P., Louvard, D., Magdelenat, H., Robine, S. and Poupon, M. F. (2004). Enhanced sensitivity to irinotecan by Cdk1 inhibition in the p53-deficient HT29 human colon cancer cell line. *Oncogene* **23**, 1737-1744.

Ahmed, M. A., Aleskandarany, M. A., Rakha, E. A., Moustafa, R. Z., Benhasouna, A., Nolan, C., Green, A. R., Ilyas, M. and Ellis, I. O. (2012). A CD44(-)/CD24(+) phenotype is a poor prognostic marker in early invasive breast cancer. *Breast Cancer Res Treat* **133**, 979-995.

Ailles, L. E. and Weissman, I. L. (2007). Cancer stem cells in solid tumors. *Curr Opin Biotechnol* **18**, 460-466.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* **100**, 3983-3988.

Aractingi, S., Kanitakis, J., Euvrard, S., Le Danff, C., Peguillet, I., Khosrotehrani, K., Lantz, O. and Carosella, E. D. (2005). Skin carcinoma arising from donor cells in a kidney transplant recipient. *Cancer Res* **65**, 1755-1760.

Arumugam, T., Ramachandran, V., Fournier, K. F., Wang, H., Marquis, L., Abbruzzese, J. L., Gallick, G. E., Logsdon, C. D., McConkey, D. J. and Choi, W. (2009). Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. *Cancer Res* **69**, 5820-5828.

Awasthi, S., Hallene, K. L., Fazio, V., Singhal, S. S., Cucullo, L., Awasthi, Y. C., Dini, G. and Janigro, D. (2005). RLIP76, a non-ABC transporter, and drug resistance in epilepsy. *BMC Neurosci* **6**, 61.

Bachelder, R. E., Wendt, M. A. and Mercurio, A. M. (2002). Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res* **62**, 7203-7206.

Bao, B., Wang, Z., Ali, S., Kong, D., Li, Y., Ahmad, A., Banerjee, S., Azmi, A. S., Miele, L. and Sarkar, F. H. (2011). Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells. *Cancer Lett* **307**, 26-36.

Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D. and Rich, J. N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756-760.

Bartelmez, S. H., Bradley, T. R., Bertoncello, I., Mochizuki, D. Y., Tushinski, R. J., Stanley, E. R., Hapel, A. J., Young, I. G., Kriegler, A. B. and Hodgson, G. S. (1989). Interleukin 1 plus interleukin 3 plus colony-stimulating factor 1 are essential for clonal proliferation of primitive myeloid bone marrow cells. *Exp Hematol* **17**, 240-245.

Bellail, A. C., Hunter, S. B., Brat, D. J., Tan, C. and Van Meir, E. G. (2004). Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. *Int J Biochem Cell Biol* **36**, 1046-1069.

Berx, G., Raspe, E., Christofori, G., Thiery, J. P. and Sleeman, J. P. (2007). Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer. *Clin Exp Metastasis* **24**, 587-597.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513-1523.

Bjerkvig, R., Tysnes, B. B., Aboody, K. S., Najbauer, J. and Terzis, A. J. (2005). Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer* **5**, 899-904.

Boocock, C. A., Charnock-Jones, D. S., Sharkey, A. M., McLaren, J., Barker, P. J., Wright, K. A., Twentyman, P. R. and Smith, S. K. (1995). Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst* **87**, 506-516.

Bourette, R. P. and Rohrschneider, L. R. (2000). Early events in M-CSF receptor signaling. *Growth Factors* **17**, 155-166.

Bourette, R. P., Myles, G. M., Choi, J. L. and Rohrschneider, L. R. (1997). Sequential activation of phoshatidylinositol 3-kinase and phospholipase C-gamma2 by the M-CSF receptor is necessary for differentiation signaling. *EMBO J* **16**, 5880-5893.

Bredel, M. and Zentner, J. (2002). Brain-tumour drug resistance: the bare essentials. *The Lancet Oncology* **3**, 397-406.

Brescia, P., Richichi, C. and Pelicci, G. (2012). Current Strategies for Identification of Glioma Stem Cells: Adequate or Unsatisfactory? *J Oncol* **2012**, 10.

Bunting, K. D. (2002). ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* **20**, 11-20.

Burgess, A. W., Camakaris, J. and Metcalf, D. (1977). Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. *J Biol Chem* **252**, 1998-2003.

Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S. and Brabletz, T. (2008). A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep* **9**, 582-589.

Cecchini, M. G., Dominguez, M. G., Mocci, S., Wetterwald, A., Felix, R., Fleisch, H., Chisholm, O., Hofstetter, W., Pollard, J. W. and Stanley, E. R. (1994). Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* **120**, 1357-1372.

Cerretti, D. P., Wignall, J., Anderson, D., Tushinski, R. J., Gallis, B. M., Stya, M., Gillis, S., Urdal, D. L. and Cosman, D. (1988). Human macrophage-colony stimulating factor: alternative RNA and protein processing from a single gene. *Mol Immunol* **25**, 761-770.

Chambers, S. K., Wang, Y., Gertz, R. E. and Kacinski, B. M. (1995). Macrophage colony-stimulating factor mediates invasion of ovarian cancer cells through urokinase. *Cancer Res* **55**, 1578-1585.

Chambers, S. K., Kacinski, B. M., Ivins, C. M. and Carcangiu, M. L. (1997). Overexpression of epithelial macrophage colony-stimulating factor (CSF-1) and CSF-1 receptor: a poor prognostic factor in epithelial ovarian cancer, contrasted with a protective effect of stromal CSF-1. *Clin Cancer Res* **3**, 999-1007.

Chung, C. T., Niemela, S. L. and Miller, R. H. (1989). One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci U S A* **86**, 2172-2175.

Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H., Jones, D. L., Visvader, J., Weissman, I. L. and Wahl, G. M. (2006). Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* **66**, 9339-9344.

Coffelt, S. B., Hughes, R. and Lewis, C. E. (2009). Tumor-associated macrophages: effectors of angiogenesis and tumor progression. *Biochim Biophys Acta* **1796**, 11-18.

Condeelis, J. and Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263-266.

Cossarizza, A., Baccaranicontri, M., Kalashnikova, G. and Franceschi, C. (1993). A New Method for the Cytofluorometric Analysis of Mitochondrial Membrane Potential Using the J-Aggregate Forming Lipophilic Cation 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine Iodide (JC-1). *Biochem Biophys Res Commun* **197**, 40-45.

Curry, J. M., Eubank, T. D., Roberts, R. D., Wang, Y., Pore, N., Maity, A. and Marsh, C. B. (2008). M-CSF signals through the MAPK/ERK pathway via Sp1 to induce VEGF production and induces angiogenesis in vivo. *PLoS One* **3**, e3405.

Das, A., Banik, N. L. and Ray, S. K. (2008). N-(4-Hydroxyphenyl) retinamide induced both differentiation and apoptosis in human glioblastoma T98G and U87MG cells. *Brain Res* **1227**, 207-215.

Das, A., Banik, N. L. and Ray, S. K. (2009). Molecular mechanisms of the combination of retinoid and interferon-gamma for inducing differentiation and increasing apoptosis in human glioblastoma T98G and U87MG cells. *Neurochem Res* **34**, 87-101.

Das, A. B., Loying, P. and Bose, B. (2012). Human recombinant Cripto-1 increases doubling time and reduces proliferation of HeLa cells independent of pro-proliferation pathways. *Cancer Lett* **318**, 189-198.

de Visser, K. E., Eichten, A. and Coussens, L. M. (2006). Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* **6**, 24-37.

Dean, M. (2009). ABC transporters, drug resistance, and cancer stem cells. *J Mammary Gland Biol Neoplasia* **14**, 3-9.

Dean, M., Fojo, T. and Bates, S. (2005). Tumour stem cells and drug resistance. *Nat Rev Cancer* **5**, 275-284.

Dembinski, J. L. and Krauss, S. (2009). Characterization and functional analysis of a slow cycling stem cell-like subpopulation in pancreas adenocarcinoma. *Clin Exp Metastasis* **26**, 611-623.

Deng, J., Gao, G., Wang, L., Wang, T., Yu, J. and Zhao, Z. (2012). CD24 Expression as a Marker for Predicting Clinical Outcome in Human Gliomas. *Journal of Biomedicine and Biotechnology* **2012**.

Dorsch, M., Hock, H., Kunzendorf, U., Diamantstein, T. and Blankenstein, T. (1993). Macrophage colony-stimulating factor gene transfer into tumor cells induces macrophage infiltration but not tumor suppression. *Eur J Immunol* **23**, 186-190.

Douglass, T. G., Driggers, L., Zhang, J. G., Hoa, N., Delgado, C., Williams, C. C., Dan, Q., Sanchez, R., Jeffes, E. W., Wepsic, H. T. et al. (2008). Macrophage colony stimulating factor: not just for macrophages anymore! A gateway into complex biologies. *Int Immunopharmacol* **8**, 1354-1376.

Drake, K. J., Singhal, J., Yadav, S., Nadkar, A., Pungaliya, C., Singhal, S. S. and Awasthi, S. (2007). RALBP1/RLIP76 mediates multidrug resistance. *Int J Oncol* **30**, 139-144.

Dylla, S. J., Beviglia, L., Park, I. K., Chartier, C., Raval, J., Ngan, L., Pickell, K., Aguilar, J., Lazetic, S., Smith-Berdan, S. et al. (2008). Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One* **3**, e2428.

Eubank, T. D., Galloway, M., Montague, C. M., Waldman, W. J. and Marsh, C. B. (2003). M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. *J Immunol* **171**, 2637-2643.

Evans, R., Kamdar, S. J., Fuller, J. A. and Krupke, D. M. (1995). The potential role of the macrophage colony-stimulating factor, CSF-1, in inflammatory responses: characterization of macrophage cytokine gene expression. *J Leukoc Biol* **58**, 99-107.

Finkel, E. (2001). The mitochondrion: is it central to apoptosis? *Science* **292**, 624-626.

Gerharz, C. D., Reinecke, P., Schneider, E. M., Schmitz, M. and Gabbert, H. E. (2001). Secretion of GM-CSF and M-CSF by human renal cell carcinomas of different histologic types. *Urology* **58**, 821-827.

Green, C. E., Liu, T., Montel, V., Hsiao, G., Lester, R. D., Subramaniam, S., Gonias, S. L. and Klemke, R. L. (2009). Chemoattractant signaling between tumor

cells and macrophages regulates cancer cell migration, metastasis and neovascularization. *PLoS One* **4**, e6713.

Groblewska, M., Mroczko, B., Wereszczynska-Siemiatkowska, U., Mysliwiec, P., Kedra, B. and Szmitkowski, M. (2007). Serum levels of granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) in pancreatic cancer patients. *Clin Chem Lab Med* **45**, 30-34.

Hamilton, J. A. (1997). CSF-1 signal transduction. *J Leukoc Biol* **62**, 145-155.

Harmey, J. H., Dimitriadis, E., Kay, E., Redmond, H. P. and Bouchier-Hayes, D. (1998). Regulation of macrophage production of vascular endothelial growth factor (VEGF) by hypoxia and transforming growth factor beta-1. *Ann Surg Oncol* **5**, 271-278.

Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M., Bruns, C. J. and Heeschen, C. (2007). Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* **1**, 313-323.

Hoa, N. T., Zhang, J. G., Delgado, C. L., Myers, M. P., Callahan, L. L., Vandeusen, G., Schiltz, P. M., Wepsic, H. T. and Jadus, M. R. (2007). Human monocytes kill M-CSF-expressing glioma cells by BK channel activation. *Lab Invest* **87**, 115-129.

Huang, P., Wang, C. Y., Gou, S. M., Wu, H. S., Liu, T. and Xiong, J. X. (2008). Isolation and biological analysis of tumor stem cells from pancreatic adenocarcinoma. *World J Gastroenterol* **14**, 3903-3907.

Huntly, B. J., Shigematsu, H., Deguchi, K., Lee, B. H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I. R. et al. (2004). MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell* **6**, 587-596.

Ihle, J. N., Keller, J., Henderson, L., Klein, F. and Palaszynski, E. (1982a). Procedures for the purification of interleukin 3 to homogeneity. *J Immunol* **129**, 2431-2436.

Ihle, J. N., Keller, J., Greenberger, J. S., Henderson, L., Yetter, R. A. and Morse, H. C., 3rd. (1982b). Phenotypic characteristics of cell lines requiring interleukin 3 for growth. *J Immunol* **129**, 1377-1383.

Jadus, M. R., Irwin, M. C., Irwin, M. R., Horansky, R. D., Sekhon, S., Pepper, K. A., Kohn, D. B. and Wepsic, H. T. (1996). Macrophages can recognize and kill tumor cells bearing the membrane isoform of macrophage colony-stimulating factor. *Blood* **87**, 5232-5241.

Jadus, M. R., Williams, C. C., Avina, M. D., Ly, M., Kim, S., Liu, Y., Narasaki, R., Lowell, C. A. and Wepsic, H. T. (1998). Macrophages kill T9 glioma tumor cells bearing the membrane isoform of macrophage colony stimulating factor through a phagocytosis-dependent pathway. *J Immunol* **160**, 361-368.

Jadus, M. R., Chen, Y., Boldaji, M. T., Delgado, C., Sanchez, R., Douglass, T., Al-Atar, U., Schulz, W., Lloyd, C. and Wepsic, H. T. (2003). Human U251MG glioma cells expressing the membrane form of macrophage colony-stimulating factor (mM-CSF) are killed by human monocytes in vitro and are rejected within immunodeficient mice via paraptosis that is associated with increased expression of three different heat shock proteins. *Cancer Gene Ther* **10**, 411-420.

Jaggupilli, A. and Elkord, E. (2012). Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity. *Clin Dev Immunol* **2012**, 708036.

Jordan, C. T., Guzman, M. L. and Noble, M. (2006). Cancer stem cells. *N Engl J Med* **355**, 1253-1261.

Kacinski, B. M. (1995). CSF-1 and its receptor in ovarian, endometrial and breast cancer. *Ann Med* **27**, 79-85.

Kacinski, B. M. (1997). CSF-1 and its receptor in breast carcinomas and neoplasms of the female reproductive tract. *Mol Reprod Dev* **46**, 71-74.

Kacinski, B. M., Carter, D., Mittal, K., Yee, L. D., Scata, K. A., Donofrio, L., Chambers, S. K., Wang, K. I., Yang-Feng, T., Rohrschneider, L. R. et al. (1990). Ovarian adenocarcinomas express fms-complementary transcripts and fms antigen, often with coexpression of CSF-1. *Am J Pathol* **137**, 135-147.

Kacinski, B. M., Scata, K. A., Carter, D., Yee, L. D., Sapi, E., King, B. L., Chambers, S. K., Jones, M. A., Pirro, M. H., Stanley, E. R. et al. (1991). FMS (CSF-1 receptor) and CSF-1 transcripts and protein are expressed by human breast carcinomas in vivo and in vitro. *Oncogene* **6**, 941-952.

Kalluri, R. and Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest* **119**, 1420-1428.

Kawakami, Y., Nagai, N., Ohama, K., Zeki, K., Yoshida, Y., Kuroda, E. and Yamashita, U. (2000). Macrophage-colony stimulating factor inhibits the growth of human ovarian cancer cells in vitro. *Eur J Cancer* **36**, 1991-1997.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L. and Shay, J. W. (1994). Specific

association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011-2015.

Komohara, Y., Hasita, H., Ohnishi, K., Fujiwara, Y., Suzu, S., Eto, M. and Takeya, M. (2011). Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma. *Cancer Sci* **102**, 1424-1431.

Krivtsov, A. V., Twomey, D., Feng, Z., Stubbs, M. C., Wang, Y., Faber, J., Levine, J. E., Wang, J., Hahn, W. C., Gilliland, D. G. et al. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature* **442**, 818-822.

Lee, J. M., Dedhar, S., Kalluri, R. and Thompson, E. W. (2006). The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* **172**, 973-981.

Lewis, C. and Murdoch, C. (2005). Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. *Am J Pathol* **167**, 627-635.

Lewis, C. E. and Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66**, 605-612.

Lin, E. Y., Nguyen, A. V., Russell, R. G. and Pollard, J. W. (2001). Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* **193**, 727-740.

Liu, F. S. (2009). Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review. *Taiwan J Obstet Gynecol* **48**, 239-244.

Liu, G., Yuan, X., Zeng, Z., Tunici, P., Ng, H., Abdulkadir, I. R., Lu, L., Irvin, D., Black, K. L. and Yu, J. S. (2006). Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* **5**, 67.

Liu, H. C., Chen, G. G., Vlantis, A. C., Leung, B. C., Tong, M. C. and van Hasselt, C. A. (2006). 5-fluorouracil mediates apoptosis and G1/S arrest in laryngeal squamous cell carcinoma via a p53-independent pathway. *Cancer J* **12**, 482-493.

Lleo, A., Selmi, C., Invernizzi, P., Podda, M., Coppel, R. L., Mackay, I. R., Gores, G. J., Ansari, A. A., Van de Water, J. and Gershwin, M. E. (2009). Apotopes and the biliary specificity of primary biliary cirrhosis. *Hepatology* **49**, 871-879.

Longley, D. B., Harkin, D. P. and Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* **3**, 330-338.

Madjd, Z., Mehrjerdi, A. Z., Sharifi, A. M., Molanaei, S., Shahzadi, S. Z. and Asadi-Lari, M. (2009). CD44+ cancer cells express higher levels of the anti-apoptotic protein Bcl-2 in breast tumours. *Cancer Immun* **9**, 4.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M. et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704-715.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P. and Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* **23**, 549-555.

Mantovani, A., Schioppa, T., Porta, C., Allavena, P. and Sica, A. (2006). Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev* **25**, 315-322.

McConkey, D. J., Choi, W., Marquis, L., Martin, F., Williams, M. B., Shah, J., Svatek, R., Das, A., Adam, L., Kamat, A. et al. (2009). Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer. *Cancer Metastasis Rev* **28**, 335-344.

McDermott, R. S., Deneux, L., Mosseri, V., Vedrenne, J., Clough, K., Fourquet, A., Rodriguez, J., Cosset, J. M., Sastre, X., Beuzeboc, P. et al. (2002). Circulating macrophage colony stimulating factor as a marker of tumour progression. *Eur Cytokine Netw* **13**, 121-127.

Metcalf, D. (1990). The colony stimulating factors. Discovery, development, and clinical applications. *Cancer* **65**, 2185-2195.

Metcalf, D. (2008). Hematopoietic cytokines. *Blood* **111**, 485-491.

Metcalf, D. (2010). The colony-stimulating factors and cancer. *Nat Rev Cancer* **10**, 425-434.

Michaelson, M. D., Bieri, P. L., Mehler, M. F., Xu, H., Arezzo, J. C., Pollard, J. W. and Kessler, J. A. (1996). CSF-1 deficiency in mice results in abnormal brain development. *Development* **122**, 2661-2672.

Moore, N. and Lyle, S. (2011). Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. *J Oncol* **2011**.

Mroczko, B., Groblewska, M., Wereszczynska-Siemiatkowska, U., Okulczyk, B., Kedra, B., Laszewicz, W., Dabrowski, A. and Szmitkowski, M. (2007). Serum

macrophage-colony stimulating factor levels in colorectal cancer patients correlate with lymph node metastasis and poor prognosis. *Clin Chim Acta* **380**, 208-212.

Murdoch, C., Giannoudis, A. and Lewis, C. E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* **104**, 2224-2234.

Nakai, E., Park, K., Yawata, T., Chihara, T., Kumazawa, A., Nakabayashi, H. and Shimizu, K. (2009). Enhanced MDR1 expression and chemoresistance of cancer stem cells derived from glioblastoma. *Cancer Invest* **27**, 901-908.

Nicola, N. A., Metcalf, D., Matsumoto, M. and Johnson, G. R. (1983). Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. *J Biol Chem* **258**, 9017-9023.

Novak, U., Nice, E., Hamilton, J. A. and Paradiso, L. (1996). Requirement for Y706 of the murine (or Y708 of the human) CSF-1 receptor for STAT1 activation in response to CSF-1. *Oncogene* **13**, 2607-2613.

Oguri, T., Bessho, Y., Achiwa, H., Ozasa, H., Maeno, K., Maeda, H., Sato, S. and Ueda, R. (2007). MRP8/ABCC11 directly confers resistance to 5-fluorouracil. *Mol Cancer Ther* **6**, 122-127.

Onishi, H., Jimi, S., Tsugu, H., Lee, S. and Fukushima, T. (2006). Mechanisms of small globular protein-induced plasma membrane permeability and cytotoxicity in U87-MG human malignant glioblastoma cells. *Anticancer Res* **26**, 4033-4041.

Opferman, J. T., Iwasaki, H., Ong, C. C., Suh, H., Mizuno, S., Akashi, K. and Korsmeyer, S. J. (2005). Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* **307**, 1101-1104.

Plate, K. H., Breier, G., Weich, H. A., Mennel, H. D. and Risau, W. (1994). Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. *Int J Cancer* **59**, 520-529.

Pollard, J. W. and Hennighausen, L. (1994). Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc Natl Acad Sci U S A* **91**, 9312-9316.

Pollard, J. W., Hunt, J. S., Wiktor-Jedrzejczak, W. and Stanley, E. R. (1991). A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. *Dev Biol* **148**, 273-283.

Polyak, K. and Weinberg, R. A. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* **9**, 265-273.

Qian, B. Z. and Pollard, J. W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* **141**, 39-51.

Ramakrishnan, S., Xu, F. J., Brandt, S. J., Niedel, J. E., Bast, R. C., Jr. and Brown, E. L. (1989). Constitutive production of macrophage colony-stimulating factor by human ovarian and breast cancer cell lines. *J Clin Invest* **83**, 921-926.

Reedijk, M., Liu, X., van der Geer, P., Letwin, K., Waterfield, M. D., Hunter, T. and Pawson, T. (1992). Tyr721 regulates specific binding of the CSF-1 receptor kinase insert to PI 3'-kinase SH2 domains: a model for SH2-mediated receptor-target interactions. *EMBO J* **11**, 1365-1372.

Reya, T., Morrison, S. J., Clarke, M. F. and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111.

Richards, D. M., Hettinger, J. and Feuerer, M. (2013). Monocytes and macrophages in cancer: development and functions. *Cancer Microenviron* **6**, 179-191.

Roehm, N. W., Rodgers, G. H., Hatfield, S. M. and Glasebrook, A. L. (1991). An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J Immunol Methods* **142**, 257-265.

Roesch, A., Fukunaga-Kalabis, M., Schmidt, E. C., Zabierowski, S. E., Brafford, P. A., Vultur, A., Basu, D., Gimotty, P., Vogt, T. and Herlyn, M. (2010). A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* **141**, 583-594.

Roussel, M. F. (1994). Signal transduction by the macrophage-colony-stimulating factor receptor (CSF-1R). *J Cell Sci Suppl* **18**, 105-108.

Ryan, G. R., Dai, X. M., Dominguez, M. G., Tong, W., Chuan, F., Chisholm, O., Russell, R. G., Pollard, J. W. and Stanley, E. R. (2001). Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1(op)/Csf1(op)) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood* **98**, 74-84.

Sakai, N., Umeda, T., Suzuki, H., Ishimatsu, Y. and Shikita, M. (1987). Macrophage colony-stimulating factor purified from normal human urine. Amino-terminal sequence and amino acid composition. *FEBS Lett* **222**, 341-344.

Sanpui, P., Chattopadhyay, A. and Ghosh, S. S. (2011). Induction of apoptosis in cancer cells at low silver nanoparticle concentrations using chitosan nanocarrier. *ACS Appl Mater Interfaces* **3**, 218-228.

Scheffer, G. L., Maliepaard, M., Pijnenborg, A. C., van Gastelen, M. A., de Jong, M. C., Schroeijers, A. B., van der Kolk, D. M., Allen, J. D., Ross, D. D., van der Valk, P. et al. (2000). Breast cancer resistance protein is localized at the plasma membrane in mitoxantrone- and topotecan-resistant cell lines. *Cancer Res* **60**, 2589-2593.

Schinkel, A. H., Smit, J. J., van Tellingen, O., Beijnen, J. H., Wagenaar, E., van Deemter, L., Mol, C. A., van der Valk, M. A., Robanus-Maandag, E. C., te Riele, H. P. et al. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491-502.

Schoppmann, S. F., Birner, P., Stockl, J., Kalt, R., Ullrich, R., Caucig, C., Kriehuber, E., Nagy, K., Alitalo, K. and Kerjaschki, D. (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol* **161**, 947-956.

Sethi, N. and Kang, Y. (2011). Notch signalling in cancer progression and bone metastasis. *Br J Cancer* **105**, 1805-1810.

Shadle, P. J., Aldwin, L., Nitecki, D. E. and Koths, K. (1989). Human macrophage colony-stimulating factor heterogeneity results from alternative mRNA splicing, differential glycosylation, and proteolytic processing. *J Cell Biochem* **40**, 91-107.

Shafee, N., Smith, C. R., Wei, S., Kim, Y., Mills, G. B., Hortobagyi, G. N., Stanbridge, E. J. and Lee, E. Y. (2008). Cancer stem cells contribute to cisplatin

resistance in Brca1/p53-mediated mouse mammary tumors. *Cancer Res* **68**, 3243-3250.

Sharma, S., Chockalingam, S., Sanpui, P., Chattopadhyay, A. and Ghosh, S. S. (2013). Silver Nanoparticles Impregnated Alginate-Chitosan-Blended Nanocarrier Induces Apoptosis in Human Glioblastoma Cells. *Adv Healthcare Mater*.

Sica, A., Schioppa, T., Mantovani, A. and Allavena, P. (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* **42**, 717-727.

Sica, A., Larghi, P., Mancino, A., Rubino, L., Porta, C., Totaro, M. G., Rimoldi, M., Biswas, S. K., Allavena, P. and Mantovani, A. (2008). Macrophage polarization in tumour progression. *Semin Cancer Biol* **18**, 349-355.

Singh, A. and Settleman, J. (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **29**, 4741-4751.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J. and Dirks, P. B. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Research* **63**, 5821-5828.

Smith, H. O., Anderson, P. S., Kuo, D. Y., Goldberg, G. L., DeVictoria, C. L., Boocock, C. A., Jones, J. G., Runowicz, C. D., Stanley, E. R. and Pollard, J. W. (1995). The role of colony-stimulating factor 1 and its receptor in the etiopathogenesis of endometrial adenocarcinoma. *Clin Cancer Res* **1**, 313-325.

Soker, S., Kaefer, M., Johnson, M., Klagsbrun, M., Atala, A. and Freeman, M. R. (2001). Vascular endothelial growth factor-mediated autocrine stimulation of

prostate tumor cells coincides with progression to a malignant phenotype. *Am J Pathol* **159**, 651-659.

Stanley, E. R. and Heard, P. M. (1977). Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *J Biol Chem* **252**, 4305-4312.

Stanley, E. R., Chen, D. M. and Lin, H. S. (1978). Induction of macrophage production and proliferation by a purified colony stimulating factor. *Nature* **274**, 168-170.

Stanley, E. R., Hansen, G., Woodcock, J. and Metcalf, D. (1975). Colony stimulating factor and the regulation of granulopoiesis and macrophage production. *Fed Proc* **34**, 2272-2278.

Stanley, E. R., Berg, K. L., Einstein, D. B., Lee, P. S., Pixley, F. J., Wang, Y. and Yeung, Y. G. (1997). Biology and action of colony--stimulating factor-1. *Mol Reprod Dev* **46**, 4-10.

Stavrovskaya, A. A. and Stromskaya, T. P. (2008). Transport proteins of the ABC family and multidrug resistance of tumor cells. *Biochemistry (Mosc)* **73**, 592-604.

Stein, J., Borzillo, G. V. and Rettenmier, C. W. (1990). Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. *Blood* **76**, 1308-1314.

Sweet, M. J. and Hume, D. A. (2003). CSF-1 as a regulator of macrophage activation and immune responses. *Arch Immunol Ther Exp (Warsz)* **51**, 169-177.

Tada, Y., Wada, M., Migita, T., Nagayama, J., Hinoshita, E., Mochida, Y., Maehara, Y., Tsuneyoshi, M., Kuwano, M. and Naito, S. (2002). Increased

expression of multidrug resistance-associated proteins in bladder cancer during clinical course and drug resistance to doxorubicin. *Int J Cancer* **98**, 630-635.

Takahashi, Y., Kitadai, Y., Bucana, C. D., Cleary, K. R. and Ellis, L. M. (1995). Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* **55**, 3964-3968.

van der Geer, P. and Hunter, T. (1993). Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. *EMBO J* **12**, 5161-5172.

Vinogradov, S. and Wei, X. (2012). Cancer stem cells and drug resistance: the potential of nanomedicine. *Nanomedicine (Lond)* **7**, 597-615.

Visvader, J. E. and Lindeman, G. J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* **8**, 755-768.

Wang, J., Sakariassen, P. Ø., Tsinkalovsky, O., Immervoll, H., Bøe, S. O., Svendsen, A., Prestegarden, L., Røsland, G., Thorsen, F., Stuhr, L. et al. (2008). CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *International Journal of Cancer* **122**, 761-768.

Wang, Z., Banerjee, S., Li, Y., Rahman, K. M., Zhang, Y. and Sarkar, F. H. (2006). Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. *Cancer Res* **66**, 2778-2784.

Wang, Z. E., Myles, G. M., Brandt, C. S., Lioubin, M. N. and Rohrschneider, L. (1993). Identification of the ligand-binding regions in the macrophage colony-stimulating factor receptor extracellular domain. *Mol Cell Biol* **13**, 5348-5359.

Warren, M. K. and Ralph, P. (1986). Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity. *J Immunol* **137**, 2281-2285.

Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A. W., Jr., Ahmed-Ansari, A., Sell, K. W., Pollard, J. W. and Stanley, E. R. (1990). Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A* **87**, 4828-4832.

Yeung, Y. G. and Stanley, E. R. (2003). Proteomic approaches to the analysis of early events in colony-stimulating factor-1 signal transduction. *Mol Cell Proteomics* **2**, 1143-1155.

Yoshiji, H., Kuriyama, S., Hicklin, D. J., Huber, J., Yoshii, J., Miyamoto, Y., Kawata, M., Ikenaka, Y., Nakatani, T., Tsujinoue, H. et al. (1999). KDR/Flk-1 is a major regulator of vascular endothelial growth factor-induced tumor development and angiogenesis in murine hepatocellular carcinoma cells. *Hepatology* **30**, 1179-1186.

Yue, X., Favot, P., Dunn, T. L., Cassady, A. I. and Hume, D. A. (1993). Expression of mRNA encoding the macrophage colony-stimulating factor receptor (c-fms) is controlled by a constitutive promoter and tissue-specific transcription elongation. *Mol Cell Biol* **13**, 3191-3201.

Zeppernick, F., Ahmadi, R., Campos, B., Dictus, C., Helmke, B. M., Becker, N., Lichter, P., Unterberg, A., Radlwimmer, B. and Herold-Mende, C. C. (2008). Stem

cell marker CD133 affects clinical outcome in glioma patients. *Clinical Cancer Research* **14**, 123-129.

Zhang, C., Li, C., He, F., Cai, Y. and Yang, H. (2011). Identification of CD44+CD24+ gastric cancer stem cells. *J Cancer Res Clin Oncol* **137**, 1679-1686.

Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H. et al. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* **7**, 1028-1034.

Zwaenepoel, O., Tzenaki, N., Vergetaki, A., Makrigiannakis, A., Vanhaesebroeck, B. and Papakonstanti, E. A. (2012). Functional CSF-1 receptors are located at the nuclear envelope and activated via the p110delta isoform of PI 3-kinase. *FASEB J* **26**, 691-706.

PUBLICATIONS AND PRESENTATIONS

Journal Publications

1. **S. Chockalingam** and Siddhartha Sankar Ghosh. *Amelioration of Cancer Stem Cells in Macrophage Colony Stimulating Factor-Expressing Human Glioblastoma upon 5-Fluorouracil Therapy*. **Accepted in PLoS One.** PONE-D-13-38673R1 10.1371/journal.pone.0083877.

2. Shilpa Sharma, **S. Chockalingam**, Pallab Sanpui, Arun Chattopadhyay and Siddhartha Sankar Ghosh. *Silver Nanoparticles impregnated Alginate-Chitosan blended Nanocarrier Induces Apoptosis in Human Glioblastoma Cells.* **Advanced Healthcare Materials**. 2013 Jul 15. doi: 10.1002/adhm.201300090.

3. Debashree Saikia, Naba K. Bordoloi, Pronobesh Chattopadhyay, **S. Chockalingam**, Siddhartha S. Ghosh, Ashis K. Mukherjee. *Differential mode of attack on membrane phospholipids by an acidic phospholipase A_2 (RVVA-PLA_2 - I) from Daboia russelli venom*. **Biochim Biophys Acta**. 2012 Dec; 1818(12):3149-57.

4. Navjot Kaur, Nitin Choudhary, Rajendra N. Goyal, S. Viladkar, I. Matai, P. Gopinath, **S. Chockalingam**, Davinder Kaur. *Magnetron sputtered Cu3N/NiTiCu shape memory thin film heterostructures for MEMS applications.* **J Nanopart Res**. 2013; 15:1468.

Manuscripts under Preparation

1. **S. Chockalingam** and Siddhartha Sankar Ghosh. *MCSF and Cancer: A Review. (Manuscript under preparation).*

Conference Presentations

1. **S. Chockalingam** and Siddhartha Sankar Ghosh. *Macrophage colony stimulating factor (CSF-1) expression potentiates 5-fluorouracil sensitization of drug resistant cancer cells.* **2012 UICC-World Cancer Congress**, Montreal, Quebec, Canada. ***(e-Poster presentation).***

2. **S. Chockalingam** and Siddhartha Sankar Ghosh. *Overexpression of MCSF increases the sensitivity of human glioblastoma cells to 5-fluorouracil.* **3rd International Conference on Stem Cells and Cancer (ICSCC-2012): Proliferation, Differentiation, and Apoptosis**, New Delhi, India. ***(Oral presentation).***

3. Shilpa Sharma, **S. Chockalingam**, Arun Chattopadhyay and Siddhartha Sankar Ghosh. *Induction of Apoptosis in human glioblastoma cancer cells at low silver nanoparticle concentration using Alginate-Chitosan blended nanocarrier*. **Frontiers in Chemical Sciences (FICS) – 2012**, Indian Institute of Technology Guwahati, India. ***(Poster presentation).***

Awards and Recognitions

1. Recipient, **Full travel grant award** from Department of Biotechnology, New Delhi to attend World Cancer Congress 2012 in Montreal, Quebec, Canada.

2. Recipient, **Best Oral presentation award**, 3rd International Conference on Stem Cells and Cancer (ICSCC-2012): Proliferation, Differentiation, and Apoptosis in New Delhi, India.

PERMISSIONS

This section contains the copies of original *permissions obtained for reproducing Figures and Tables* in this thesis from other sources, as listed below:

Figure No.	Page No.	Source
2.1	16	Douglass, T. G., Driggers, L., Zhang, J. G., Hoa, N., Delgado, C., Williams, C. C., Dan, Q., Sanchez, R., Jeffes, E. W., Wepsic, H. T. et al. (2008). Macrophage colony stimulating factor: not just for macrophages anymore! A gateway into complex biologies. Int Immunopharmacol 8, 1354-1376.

Table No.	Page No.	Source
2.1.	28	Ailles, L. E. and Weissman, I. L. (2007). Cancer stem cells in solid tumors. Curr Opin Biotechnol 18, 460-466.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 26, 2013

This is a License Agreement between Chockalingam S ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Chockalingam S
Customer address	Department of Biotechnology Guwahati, Assam 781039
License number	3276440309096
License date	Nov 26, 2013
Licensed content publisher	Elsevier
Licensed content publication	International Immunopharmacology
Licensed content title	Macrophage colony stimulating factor: Not just for macrophages anymore! A gateway into complex biologies
Licensed content author	Thomas G. Douglass,Lara Driggers,Jian Gang Zhang,Neil Hoa,Christina Delgado,Christopher C. Williams,Qinhong Dan,Ramon Sanchez,Edward W.B. Jeffes,H. Terry Wepsic,Michael P. Myers,Kirston Koths,Martin R. Jadus
Licensed content date	October 2008
Licensed content volume number	8
Licensed content issue number	10
Number of pages	23
Start Page	1354
End Page	1376
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No

TH-1247_08610608

Will you be translating?	No
Title of your thesis/dissertation	Role of Macrophage Colony Stimulating Factor in Drug Resistance
Expected completion date	Dec 2013
Estimated size (number of pages)	175
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

TH-1247_08610608

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable

to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx . As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

TH-1247_08610608

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK501168004.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

TH-1247_08610608

TH-1247_08610608

ELSEVIER LICENSE
TERMS AND CONDITIONS

Nov 26, 2013

This is a License Agreement between Chockalingam S ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Chockalingam S
Customer address	Department of Biotechnology Guwahati, Assam 781039
License number	3276440682100
License date	Nov 26, 2013
Licensed content publisher	Elsevier
Licensed content publication	Current Opinion in Biotechnology
Licensed content title	Cancer stem cells in solid tumors
Licensed content author	Laurie E Ailles,Irving L Weissman
Licensed content date	October 2007
Licensed content volume number	18
Licensed content issue number	5
Number of pages	7
Start Page	460
End Page	466
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No

TH-1247_08610608

Title of your thesis/dissertation	Role of Macrophage Colony Stimulating Factor in Drug Resistance
Expected completion date	Dec 2013
Estimated size (number of pages)	175
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage

incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx . As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

TH-1247_08610608

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK501168009.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

TH-1247_08610608