Characterization of Cancer Stem Cells in Retinoblastoma

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

MURALI MOHAN SAGAR BALLA

Under the Supervision of

Prof. Geeta K Vemuganti

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2011

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN)

CERTIFICATE

This is to certify that the thesis entitled **"Characterization of Cancer Stem Cells in Retinoblastoma"** which is submitted by **Murali Mohan Sagar Balla**, ID No. **2006PHXF025**, for award of Ph.D. degree of the institute, embodies original work done by him under my supervision.

Date: 12th Sept 2011

Geeta

Supervisor

Prof. GEETA. K. VEMUGANTI

Dean, School of Medical Sciences,

Hyderabad Central University,

Hyderabad - 500 046.

Dedicated to My Family and

Supervisor

Acknowledgements

I am grateful to my advisor ***Dr. Geeta K Vemuganti*** for providing me a healthy educative working environment. Under her guidance it was a great opportunity to learn both from professional and personal front, which inspired me at every step of my Ph.D. tenure. I am indebted to adviser for bringing a significant difference in myself.

I take this opportunity to thank my co-supervisor ***Dr. Chitra Kannabiran*** for giving valuable guidance whenever it was needed.

I am thankful to ***Dr. Santosh G Honavar, Ramesh Murthy, Anirban*** and ***Javed*** for clinically evaluating all the study patients and sending me the fresh samples. Without their support, it would not have been possible to pursue my study.

I am thankful to ***Prof. Paturu Kondiah*** for taking care and allowing me to work with him for my Micro Array experiments at Indian Institute of Science, Bangalore. I also thank ***Imran Khan, Ravi Kiran Reddy*** and ***Neeraj*** for assisting me in doing my experiments without any problem.

I am thankful to ***Prof. D. Balasubramanian*** for his efforts in registering me at BITS Pilani, arranging classes at BLSO Kismatpur, so as to avail semester fee waiver and letting us chat with some of the eminent scientists including nobel laureates. I also thank him for initially funding my project, when I was not having project. It is with this help I started my work, and the results of which helped me to propose a project to ICMR.

I am grateful to ***Dr. G.N. Rao*** for making a great place to work at L V Prasad Eye Institute. I am honored to be a part of LVPEI.

I appreciate the support from the research and consultancy division of ***BITS Pilani*** for taking care of my PhD related matters. It was very kind of ***Prof. B.N Jain, Prof. Ravi Prakash and Prof. A.K Das*** for helping me and clarifying my doubts whenever I was in need. I would also like to thank ***Mr. Dinesh*** and ***Mr. Sharad Srivastava*** for taking care of my Ph.D. related matters.

I would like to sincerely acknowledge ***Dr. Nishant*** and ***Dr. Yashoda*** for giving valuable suggestions in trouble shooting of certain difficult experiments in my work.

I am especially thankful to Purush (Pu), Afia (Moti), Hardeep (Jassi), Saritha (Katta), Shubha, Rachna (Rac) and Surya (Su) for having most wonderful and memorable time with them all through my PhD tenure.

I am fortunate to have a junior like Rohini (Ro), who is always enthusiastic in doing work and helping especially in the last set of experiments.

I am grateful to Mangalangi (Manku baby), Savitri (Vadi Vedi), Srujana (Szu Szu), Sonika (souuunikaaaaa), Nishika (Nishku) and Rachana (Rac) for tolerating my bantering all the time in student's cabin.

I am expressing my appreciation to Sridhar, Naidu, Arsha and Elizabeth of pathology lab, who aided me in getting samples, tissue sections and pathology reports.

I am grateful to Saritha (Kat), Ganeshwar (Ganni Boyle) and Subash (Buddy) for providing me the full text research articles for my independent studies and thesis.

I am thankful to my colleagues Purush, Afia, Hardeep, Surya, Saritha, Pulla Rao, Kiranpreet (Moti), Venu, Kalyan, Joveeta, Aparna, Anees, Rachana, Shuba, Savitri, Soumya, Neeraja (Rooni), Mounika, Nageswara Rao, Subash, Ganeshwar, Rajeswari, Sreelatha, Maithili,

Rohini, Mangalangi, Srujana, Nishika, Vasundara, Rajya Lakshmi and Indushree (Ms.CS) for maintaining a friendly environment and helping me in the time of need.

I am grateful to BD biosciences team, who initially taught me to learn operation of difficult instrument like FACS.

LVPEI is great as the people who work for it are sincere and dedicated to their work. I would like to thank Bhanu and Venkat of the department of library, housekeeping, stores, purchase, maintenance (specifically Ravi and co), biomedical, ISD, communications, graphics, reception, cafeteria, and security for their help at the time of need.

I am highly grateful and thankful to my ***Parents, Sister's family*** and ***my wife Sunanda*** for their continuous support and allowing me to pursue my PhD in spite of severe societal obligations.

I am thankful to all other faculty members for their valuable suggestions and encouragement during my stay at LVPEI.

Finally I am thankful to the study patients for providing their valuable samples and I sincerely acknowledge the financial support from HERF and CSIR for my fellowship.

Balla Murali Mohan Sagar

Abstract

Objectives:

The purpose of this study was to evaluate the presence of stem cells in retinoblastoma tumor using the cumulative data on cancer stem cells in all tumors especially the neuro ectodermal stem cell markers and attempt to correlate it with the well established histological prognostic factors. In view of the extensive necrosis (due to the rapidly dividing tumor that outgrows its blood supply), this study attempted to evaluate for CSC in primary tumors and Y 79 retinoblastoma cell lines, using flow cytometry, multiple parameter approach (size and phenotype, cell cycle analysis) and validating it by gene expression (RT PCR, microarray), colony assay and drug induced cell death.

Methods:

After Institutional Review Board (IRB) approval, tumor tissue (n=7) was harvested from unfixed eye specimens with Rb. The isolated cells were evaluated for stem/differentiated cell markers (CD44, ABCG2, CXCR4, CD133, and CD90), size and cell cycle status. The presumptive CSC (FSC^{low}/SSC^{low}), $CD44^{+}$ $CD133^{-}$ cells were sorted and evaluated for primitive neural stem cell markers (human Syntaxin1A, PROX1, CD133 and NSE), and correlated with histologic risk factors with the tumor cells. In the second objective, these properties were evaluated and validated in Y79 cell line using cell cycle analysis, drug resistance, gene expression using microarray, clone forming ability and regenerating the same phenotype as original from the stem cell clone. Finally gene expression was evaluated in eleven tumors by hybridizing to expression arrays. Results were validated by real time PCR and Immunohistochemistry (IHC).

Results:

Results of the first objective showed two different sub-populations of cells in seven Rb cases. The small cells, assigned FSC^{low}/SSC^{low} (Forward scatter low/Side scatter low, ranging from 1.7% to 17.7%) were characterized as positive for CD44 and negative for CD133, CXCR4 and CD90. The large cells were designated as FSC^{hi}/SSC^{low} (ranging from 2.7% to 35.1%) and characterized as positive for all the analyzed markers. RT-PCR analysis revealed that sorted cells of FSC^{low}/SSC^{low} sub-population expressed retinal progenitor cell markers PROX1 and Syntaxin1A.

In the second objective, similar to the human Rb tumors, Y79 cell line also expressed putative stem cell markers (Oct4, Nanog, ABCB1 and Bmi-1) and differentiated retinal cell markers (CD90 and CD133), except for the expression of CD44. The possible reason for absence of this marker in Y79 cells might be contributed by high serum culture conditions. Unlike in Rb tumors, cells were exposed to high hyaluronic acid containing vitreous fluid, hence this might have caused over expression of hyaluronate receptor CD44 in these tumors. The $CD133^-$ cells, which are similar to the phenotype of FSC^{low}/SSC^{low} in human Rb tumors, expressed PROX1, Oct4, Nanog ABCB1 and Bmi-1. Microarray results showed that embryonic and neural stem cell markers like HOXB2, HOXA9, Sall1, ABCB1, ABCB5, Lefty and Mushashi2 markers were expressed more in $CD133^-$ cells. Moreover 83.3±4.1% of the $CD133^-$ cells were shown to be in G_0/G_1 phase and the 81.1±10.6% of $CD133^+$ cells in $S/G_2/M$ phase. $CD133^-$ cells showed high clone forming ability compared to positive cells. These results demonstrated that $CD133^-$ phenotype is the cancer stem cells in Y79 cells. Finally, microarray results of human Rb tumors showed that, in 11 Rb tumors evaluated, 5593 genes were up-regulated and 4864 genes were down-regulated ($p \leq 0.05$ and fold

change ≥ 1.5 folds). Analysis shows significant up-regulation of neural stem cell signaling HMGA2 and its downstream regulator LIN-28b, which is involved in self-renewal ability of neural stem cells. IRS1 and CXCL14 showed significant difference in expression between tumors with and without histopathological risk factors (HRF's).

Conclusions:

In summary, CSC in retinoblastoma, could be ascertained on cytometric analysis by two distinct features, the FSC^{low}/SSC^{low} , stem cell ($CD44^{+}$) and retinal progenitor markers (PROX1 and Syntaxin 1A) combined with relatively lower percentage of differentiated markers and G_0/G_1 state. In contrast the differentiated cells were FSC^{hi}/SSC^{lo} with higher percentage of differentiated markers (CD90 and CD133).

In addition to the similar observation in Y 79 cell line (expect for CD44 expression), further evidence of expression of primitive stem cell markers like (Oct4, Nanog, Bmi-1 and PROX1), more clone- forming ability, and resistance to drug induced cell death was observed. The gene expression suggested high neural stem cell signaling with marginal up regulation of notch signaling and down regulation of Wnt, SHH. Clinico pathologic and expression profile correlation indicate that ACVR1C, IRS1 and CXCL14 could possibly serve as novel prognostic markers for tumors with and without risk factors. Additional studies are required to evaluate functional importance of these markers in Rb tumors.

Contents

List of Tables

Appendix Tables

List of Figures

List of Abbreviations and Symbols

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
bp	Base pair
cDNA	Complimentary DNA
cRNA	Complimentary RNA
dNTP	Deoxynucleotide Triphosphate
mg	Milligram
ml	Milliliter
mM	Millimolar
ng	Nanogram
nm	Nanometer
^0C	Degree Centigrade
OD	Right Eye
OS	Left Eye
OU	Both Eyes
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rpm	Rotations Per Minute
µg	Microgram
µl	Microliter
µM	Micromolar
GKV	Geeta K Vemuganti

min	Minutes
Ig	Immunoglobulin
FITC	Fluroscein Isothiocyanate
PE	Phycoerythrin
APC	Allophycocyanin
PI	Propidium Iodide
FACS	Florescent Activated Cell Sorter
SSC	Side Scatter
FSC	Forward Scatter
Rb	Retinoblastoma
RNase	Ribonuclease
M	Molar
sec	Seconds
hrs	Hours
pmol	Pico Mole
cy3	Cyanine 3
cy5	Cyanine 5
PMID	Pubmed ID
Ct	Threshold Cycle
ΔCt	Difference in Threshold Cycle

Chapter-1
Introduction and Literature Review

1 Introduction to stem cells

The discovery of stem cells was made, while dealing with another serious medical problem during World War II i.e Radiation. People were exposed to lethal doses of radiation and to treat these patients, transplants from the spleen and bone marrow were found to be effective in rescuing these victims. Later stem cell transplantations were pioneered using bone marrow derived cells by E. Donnall Thomas et al., and his work helped in reducing the likelihood of developing a life threatening complication called Graft Versus Host Disease (Thomas et al. 1957). It was then, the first quantitative descriptions of the self-renewing cells were characterized as hematopoietic stem cells (HSCs) (Becker et al. 1963). Since then HSCs in humans were further characterized and cultured (Morstyn et al. 1980; Sutherland et al. 1989; Sutherland et al. 1990).

During the 1980's, two groups independently isolated embryonic stem cells (ESCs) from mouse models, one being Martin Evans et al., and the other Gail R Martin et al. They have demonstrated that embryos can be cultured *in vitro* and ESC's can be derived from these embryos (Evans et al. 1981; Martin 1981). After many advances in techniques of culturing human cells in the laboratory, it was in the year 1998, cells from spare embryos were successfully removed and grown *in vitro* (Thomson et al. 1998). Since this discovery, many studies have emerged that suggested the pluripotency of ESCs and have shown that they are capable of differentiating into any of the specialized cell in the body (Shamblott et al. 1998; Reubinoff et al. 2000). Evidences from research on hematopoietic and ESCs gave a direction for determining the cells having stem cell characteristics in various other tissues.

This quest led to the evidence of the adult stem cell plasticity, challenging the long-held belief that adult mammalian stem cells are lineage restricted (Ferrari et al. 1998). These

plasticity studies have shown that a small number of cells have the capability to repair damaged tissues and these cells are called as '*stem cells*'.

1.1 Definition

With reference to the current knowledge and new theories of stem cells being postulated, it is quite a difficult task to define stem cells. A lot of definitions have been given by several groups, but Potten et al., have given the most elaborate definition of stem cells, in the context of gastrointestinal stem cells (Potten et al. 1990).

A stem cell can be defined as a cell which can divide (self replicating) for indefinite periods, often throughout the life span of an organism and under right conditions and signals would give rise (differentiate) to several specialized cells that make up the organism (http://stemcells.nih.gov/info/scireport/chapter1.asp. ; Lanza 2006)

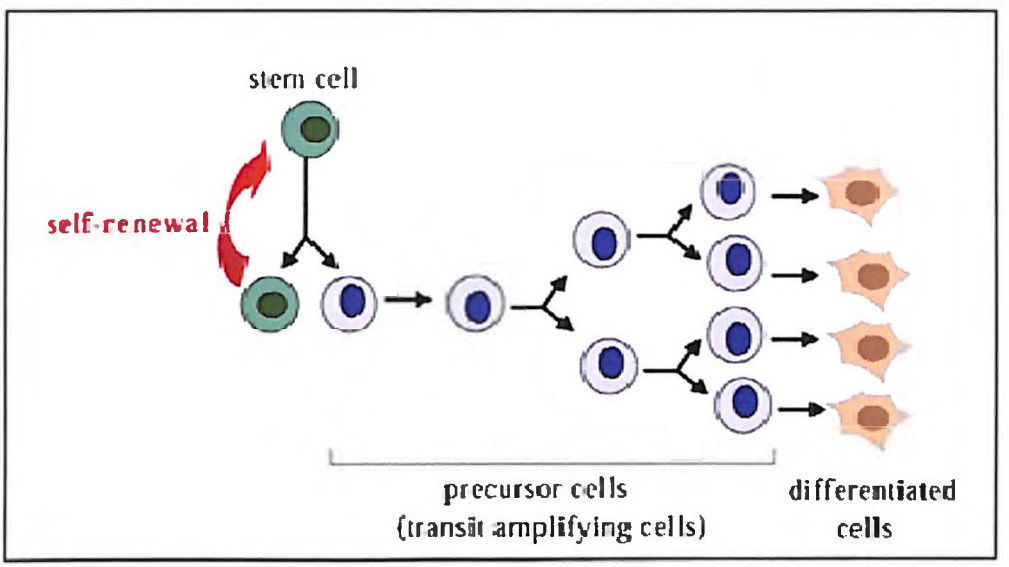


Figure 1.1: Division of a stem cell resulting in either daughter stem cell (Self-renewal) or precursor cell leading to form differentiated cells (Raff 2003)

1.2 Characteristics of stem cells

Stem cells differ from other cells in having three general properties: They are capable of dividing and self renewing for long periods, they are unspecialized in function, and they have capability to form specialized cell types.

These characteristics are briefly explained below:

1. Self-renewal and proliferation: Stem cells replicate many times i.e. a starting population of stem cells that can proliferate for many months in *in vitro* (approximately about 160 population doublings) and yield millions of cells without oncogenic transformation. These cells are said to be capable of long-term self-renewal. Most of the somatic cells, when cultured *in vitro* display a finite number of population doublings (i.e. less than 80) before they becomes senescent.

2. Stem cells are unspecialized: One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. For example, a stem cell cannot work with its neighbors to pump blood through the body (like a heart muscle cell), and it can't carry oxygen molecules through the bloodstream (like a red blood cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle, blood, or nerve cells.

3. Differentiation: When unspecialized stem cells give rise to specialized cells, the process is called differentiation. While differentiating, the cell usually goes through several stages, becoming more specialized at each step. Scientists are just beginning to understand the signaling inside and outside of the cells that trigger the differentiation process. The internal signals are controlled by cellular genes, which are interspersed across long strands of DNA, and carry coded instructions for all cellular structures and functions. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment. The interaction of signals during differentiation causes the cell's DNA to acquire epigenetic marks that

restrict DNA expression in the cell and can be passed on through cell division (http://stemcells.nih.gov/info/scireport/chapter1.asp. ; Lanza 2006).

1.3 Classification:

Stem cells can be classified into four types on basis of their origin: Embryonic stem cells (ESCs), Fetal Stem Cells (FSCs), Umbilical Cord blood Stem Cells (UCSCs) and Adult Stem Cells (ASCs)

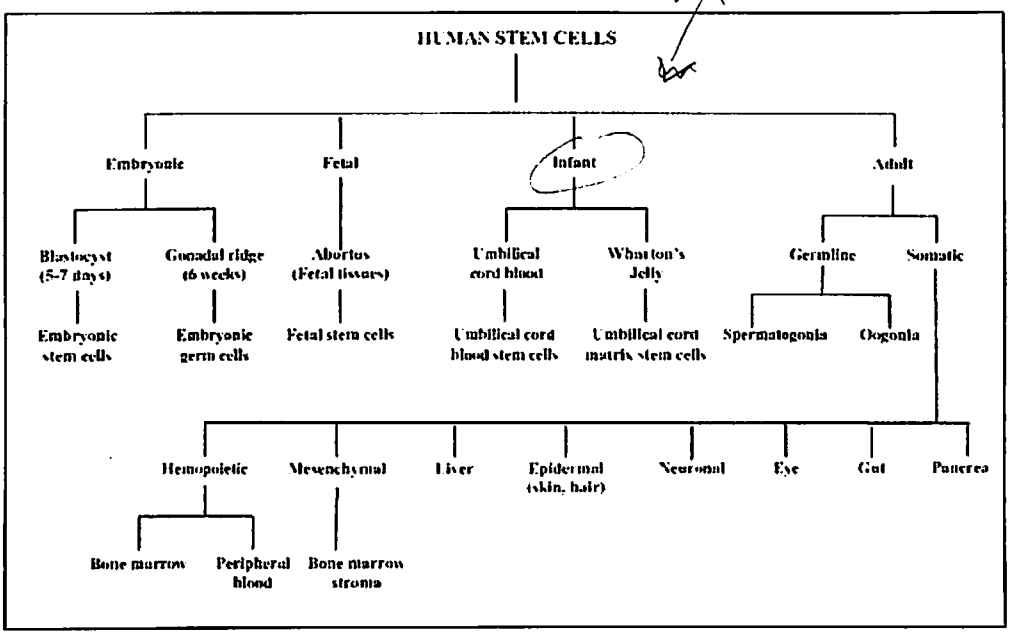


Figure 1.2: A detailed classification of human stem cells (www.worldscibooks.com/etextbook/5729/5729_chap1.pdf).

Stem cells can further be classified into 4 different types of cells based on their potentiality; it has been observed that the capability of stem cells in differentiating into various cell types' decrease as the organism develops.

the immense interest that has been generated in stem cell technology has intensified with the reports of the isolation of primate and human ESCs using surplus embryos produced by *in vitro* fertilization (IVF) and donated for research purpose (Thomson et al. 1998; Reubinoff et al. 2000).

ES cells are isolated from the inner cell mass (ICM) of the blastocyst stage embryo and, if maintained in optimal conditions, will continue to grow indefinitely in an undifferentiated diploid state.

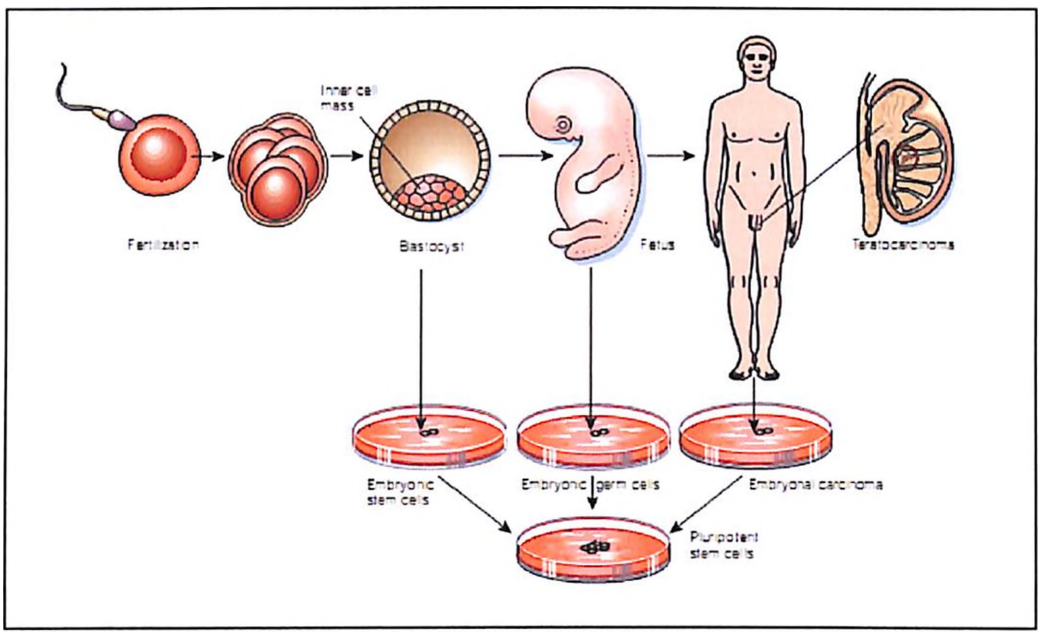


Figure 1.3: ESCs are derived from the inner cell mass of the pre-implantation embryo. Embryonic germ cells (EGCs) are derived from primordial germ cells (PGCs) isolated from embryonic gonad. Embryonal carcinoma cells (ECCs) are derived from testicular tumors in adult. All of the three pluripotent stem cells are derived by culture on layers of mitotically inactive fibroblasts, termed feeder layers (Donovan et al. 2001).

Characteristics of ESCs: Embryonic stem cells have characteristic properties in respect to their isolation, surfaces markers, culture properties etc which gives them a hallmark identity. These features are listed as follows.

i. They are derived from the inner cell mass/epiblast of the blastocyst and are capable of undergoing an unlimited number of symmetrical divisions without differentiating (long-term self-renewal).

ii. Exhibit and maintain a stable, diploid, normal complement of chromosomes (karyotype).

iii. Pluripotent ESCs can give rise to differentiated cell types that are derived from all three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm).

iv. ESCs are clonogenic, that is, a single ES cell can give rise to a colony of genetically identical cells, or clones, which have the same properties as the original cell.

v. Expresses the transcription factor Oct-4, which then activates or inhibits a group of target genes and maintains ES cells in a proliferative and non-differentiating state.

vi. Lacks the G_1 checkpoint in the cell cycle. ES cells spend most of their time in the S phase of the cell cycle, during which they synthesize DNA. Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication.

vii. Do not show X inactivation. In every somatic cell of a female mammal, one of the two X chromosomes becomes permanently inactivated. X inactivation does not occur in undifferentiated ES cells (Donovan et al. 2001).

1.3.2 Fetal stem cells

Fetal stem cells are primitive cell types found in the fetal tissue. Neural crest stem cells, fetal hematopoietic stem cells and pancreatic islet progenitors have been isolated from abortuses (Beattie et al. 1997). Fetal neural stem cells found in the fetal brain were shown to differentiate into both neurons and glial cells (Brustle et al. 1998; Villa et al. 2000).

Characteristics of ESCs: Embryonic stem cells have characteristic properties in respect to their isolation, surfaces markers, culture properties etc which gives them a hallmark identity. These features are listed as follows.

i. They are derived from the inner cell mass/epiblast of the blastocyst and are capable of undergoing an unlimited number of symmetrical divisions without differentiating (long-term self-renewal).

ii. Exhibit and maintain a stable, diploid, normal complement of chromosomes (karyotype).

iii. Pluripotent ESCs can give rise to differentiated cell types that are derived from all three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm).

iv. ESCs are clonogenic, that is, a single ES cell can give rise to a colony of genetically identical cells, or clones, which have the same properties as the original cell.

v. Expresses the transcription factor Oct-4, which then activates or inhibits a group of target genes and maintains ES cells in a proliferative and non-differentiating state.

vi. Lacks the G_1 checkpoint in the cell cycle. ES cells spend most of their time in the S phase of the cell cycle, during which they synthesize DNA. Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication.

vii. Do not show X inactivation. In every somatic cell of a female mammal, one of the two X chromosomes becomes permanently inactivated. X inactivation does not occur in undifferentiated ES cells (Donovan et al. 2001).

1.3.2 Fetal stem cells

Fetal stem cells are primitive cell types found in the fetal tissue. Neural crest stem cells, fetal hematopoietic stem cells and pancreatic islet progenitors have been isolated from abortuses (Beattie et al. 1997). Fetal neural stem cells found in the fetal brain were shown to differentiate into both neurons and glial cells (Brustle et al. 1998; Villa et al. 2000).

Fetal blood, placenta and umbilical cord are rich sources of fetal hematopoietic stem cells (HSCs).

1.3.3 Umblical cord blood stem cells (UCB)

Umblical cord blood is a rich source of circulating stem cells. The UCB cells are distinct in terms of size of the colonies formed, presence of different growth factors *in vitro* and the cells has long telomeres, when compared with those of bone marrow and adult peripheral blood. Cord blood stem cells are hypoimmunogenic compared with bone marrow, possibly due to the presence of high levels of interleukin-10 produced by the cells and or decreased expression of beta-2 microglobulin. Cord blood stem cells have been shown to be multipotent by being able to differentiate into neurons and liver cells (Mitchell et al. 2003; Rogers 2004).

1.3.4 Adult stem cells

i. HSCs: Bone marrow contains two types of stem cells one being hematopoietic in origin the other mesenchymal. The HSCs were originated early in embryogenesis from mesoderm and becomes deposited in very specific hematopoietic sites in the embryo. These sites include the bone marrow, liver, and yolksac. Bone marrow stem cells may be more plastic and multipotent than expected. These cells were shown to be differentiated into many cell types both *in vitro* and *in vivo*.

ii. Mesenchymal stem cells (MSCs): MSCs are found in the adult bone marrow stroma. Marrow stromal tissue is made up of different population of cells, which include reticular, osteogenic, smooth muscle, endothelial cells, adipocytes and macrophages (Bianco P 1998). In response to injury, stem cells found in the stromal tissue divide and produce the cells necessary for repair (Owen 1988). Apart from bone marrow stroma, these cells can also be derived from adipose tissue, skin and periosteum. These cells are multipotent and have capability to form cartilage, bone, muscle, tendon, ligament and fat (Caplan 1994).

iii. Gut stem cells: Epithelial cells of gastrointestinal gut, undergoes continuous and rapid renewal throughout lifespan. Differentiation programs exist in specific regions of the tract. The renewal of the epithelial cells is sustained with the presence of multipotent stem cells located in the crypts of Lieberhahn. The epithelial cells differentiate as they migrate from a crypt up an adjacent villus and leave the intestine once they reach the villus tip (Alison et al. 2002).

iv. Liver stem cells: Liver is a highly regenerative organ of the human body and it was said that surgical removal of at least 75% of the liver can be regenerated back in 2-3 weeks. This is in contrast to most other organs in the body like kidney or pancreas. Recent evidence suggests that different cell types and mechanisms are responsible for organ regeneration (Alison et al. 2004).

v. Bone and cartilage stem cells: Like in other tissues bone itself has its own stem cells as well as committed osteoprogenitor cells (Nuttall et al. 1998; Gronthos et al. 1999). In-vivo, articular cartilage has a very limited capacity to repair. In the presence of injury to cartilage, stem cells do participate in the repair process, but the numbers are small and the regulatory factors are limited (Metsaranta et al. 1996; Nakajima et al. 1998).

vi. Epidermal stem cells: The epidermis contains stem cells at the base of the hair follicle and their self-renewing properties allow for the re-growth of hair and skin cells. New keratinocytes are produced continuously during adult life to replace the dying cells from the outer skin layers and hairs. Stem cells initially differentiate to form transient amplifying cells which give rise to the more differentiated cell types keratinocytes and sebocytes (Blanpain et al. 2004).

vii. Neural stem cells: It has been shown that a neurogenic cell turn over occurs in some limited areas like the subventricular zone (SVZ) of the forebrain and the dental gyrus of the hippocampus of the central nervous system (CNS) (Reynolds et al. 1992; Lois et al. 1993;

Luskin 1993; Seaberg et al. 2002). In *in vitro* conditions NSCs have the capability to generate neurons, astrocytes and oligodendrocytes (Bottai et al. 2003).

viii. Pancreatic stem cells: It was shown that the endocrine cells of rat pancreatic islets of Langerhans, including insulin-producing beta cells, divide for every 40-50 days by proliferation and differentiation of new islet cells (neogenesis) from progenitor epithelial cells located in the pancreatic ducts (Zulewski et al. 2001). The same authors showed that rat and human pancreatic islets contained an undifferentiated nestin marker. These nestin positive cells were distinct from ductal epithelium and have extended proliferative capacity *in vitro*, could be cloned repeatedly and appeared to be multipotential. They were able to differentiate *in vitro* into cells that expressed liver and exocrine pancreatic markers and aid in neogenesis of islet endocrine cells.

ix. Eye stem cells: Stem cells have been identified in the adult mouse eye. These cells can be located at different regions of the eye like Limbus (corneoscleral junction), the conjunctive, and cilliary margins.

a) Limbal Stem Cells: The stem cells of cornea are located at the limbus (corneo sclera junction). Limbal location of these cells was first proposed by Davanger and Evensen in 1971 and later it was confirmed by the findings of Schermer et al., and Cotsarelis et al.(Davanger et al. 1971; Cotsarelis et al. 1989). In the event of corneal injury, cells from the limbus migrate towards the wounded cornea and become transiently amplifying cells (TAC) with lesser stemness and contribute to the corneal wound healing.

b) Stem cells of conjunctiva: It is the transparent tissue that covers the outer surface of the eye. The conjunctiva is nourished by tiny blood vessels and also involved in moistening of the eye. The conjunctiva, like the corneal surface undergoes constant renewal. Although the conjunctival stem cell hypothesis is well accepted, the

precise location of these stem cells within the conjunctiva is still under debate (Seigel from http://www.isscr.org/public/eye.htm.).

c) The Ciliary Margin: The ciliary body includes the group of muscles that allow the eye lens to contract and also produces that clear fluid. There are primitive cells located in the cilliary margin which have capability to form clones *in vitro* and can be differentiated to retinal specific cell types, including rod photoreceptors, bipolar neurons and muller glia. The adult retinal stem cells are localized to the margins of pigmentary cilliary epithelium and not to the central and peripheral retinal pigmented epithelium (Seigel from http://www.isscr.org/public/eye.htm. ; Tropepe et al. 2000).

1.4 Homeostasis and repair:

The regulation of stem cell division must balance the regenerative needs and the potential for cellular overgrowth, transformation and cancer. The process of self-renewal requires the coordination of cell cycle progression and cell-fate choices (i.e. commitment versus self-renewal). Any effort to understand the homeostasis of stem cells should include the understanding of the relationship between these processes.

1.4.1 Cell-cycle regulation in ESCs:

Classically, cell cycle is the process of events that takes place in a cell leading to its division and duplication. This process can be divided into two phases: interphase and M phase.

Interphase is in turn divided into G_1 phase, S phase and G_2 phase. M Phase is divided into mitosis and cytokinesis. Cells that have temporarily or reversibly stopped dividing are said to have entered a quiescence called G_0 phase.

Progression through the cell cycle is highly regulated, particularly transition from G_1 to S phase and from G_2 to M phase. These two check points assess cell intrinsic signals and are governed by the cyclin dependent kinases CDK_2 and CDK_1 respectively. A combination of intrinsic and extrinsic signals regulates the passage from early to late G_1 phase in most cells. This point is termed as restriction (R) point. R point represents the 'point of no return' for the cell, after which the cell has committed to enter the cell cycle and mitogenic stimuli are no longer required.

Late G_1 phase is characterized by mitogen-independent activation of cyclin E-CDK_2 complex activity and concomitant hyperphosphorylation and inactivation of the retinoblastoma tumor suppressor protein (RB). The transition from early to late G_1 is mainly regulated by the D-type cyclins and their enzymatic counterparts, CDK_4 and CDK_6. Cyclin D-CDK_4 and cyclin D-CDK_6 complexes function by enzymatic and non-enzymatic mechanisms to partially inactivate RB and activate expression of cyclin E. Upon reaching a threshold level of cyclin E-CDK_2 activity, RB becomes fully inactivated by hyperphosphorylation and cyclin D-CDK_4 and cyclin D-CDK_6 is no longer required for the G_1/S transition to occur (Orford et al. 2008).

Two families of cyclin-dependent kinase inhibitors (CDKIs) regulate the transition through G_1 phase. In particular, members of the Ink_4 family are direct inhibitors of the early G_1 cyclin D-CDK_4 and cyclin D-CDK_6 complexes, and members of the CIP/KIP family ($p21^{CIP}$, $p27^{KIP1}$, $p57^{KIP2}$) are direct inhibitors of the late G_1 cyclin E-CDK_2 complexes. These inhibitors generally slow down or prevent the transition through the cell cycle by blocking these activities. Paradoxically, p27 can actually enable the formation of cyclin D-CDK_4 complexes, presumably by stimulating the cyclin D-mediated transition to late G_1 while, at the same time, preventing the transition across the R point by inhibiting cyclin E-CDK_2 activity (Orford et al. 2008).

Murine ES cell growth *in vitro* is characterized by extraordinarily rapid proliferation and a short cell cycle (11-16 hours). Short cell cycle is due to reduction in the duration of G_1 phase, whereas cyclin E-CDK_2 is periodic in somatic cells, peaking at the G_1 to S transition and this effectively omits the early G_1 phase by bypassing the R point. Furthermore, mESCs express low levels of the D-type cyclins and have almost undetectable levels of CDK_4 associated kinase acivity. Throughout the cell cycle of mES cells, RB is hyperphosphorylated and therefore remains inactive. This is another reason why these cells lack R point (Lanza 2006; Orford et al. 2008).

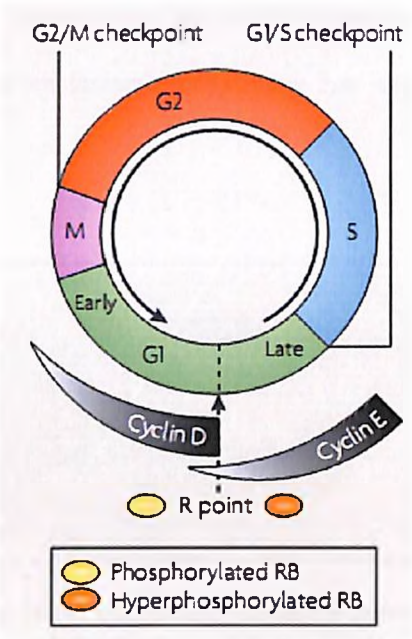


Figure 1.4: Cell cycle in mammalian cells (Orford et al. 2008)

1.4.2 Cell-cycle regulation in adult stem cells:

In contrast to ESCs, a hallmark feature of adult stem cells is their relative proliferative quiescence. HSCs which are the most studied adult stem cell population in both humans and mice, are largely in the G_0 or G_1 phase of the cell cycle and, of these cells, the large

Murine ES cell growth *in vitro* is characterized by extraordinarily rapid proliferation and a short cell cycle (11-16 hours). Short cell cycle is due to reduction in the duration of G_1 phase, whereas cyclin E-CDK_2 is periodic in somatic cells, peaking at the G_1 to S transition and this effectively omits the early G_1 phase by bypassing the R point. Furthermore, mESCs express low levels of the D-type cyclins and have almost undetectable levels of CDK_4 associated kinase acivity. Throughout the cell cycle of mES cells, RB is hyperphosphorylated and therefore remains inactive. This is another reason why these cells lack R point (Lanza 2006; Orford et al. 2008).

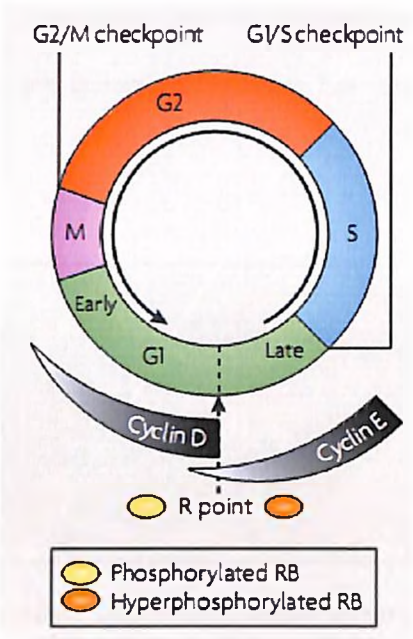


Figure 1.4: Cell cycle in mammalian cells (Orford et al. 2008)

1.4.2 Cell-cycle regulation in adult stem cells:

In contrast to ESCs, a hallmark feature of adult stem cells is their relative proliferative quiescence. HSCs which are the most studied adult stem cell population in both humans and mice, are largely in the G_0 or G_1 phase of the cell cycle and, of these cells, the large

majority have exited the cell cycle completely. Approximately 75% of the most primitive LT-HSCs are resting in G_0. It is widely accepted that the quiescent state is a functionally important characteristic of adult stem cells. Quiescent state of adult stem cells is mostly related with p21, growth factor inhibitor 1 (GFI1), PTEN homologue and Forkhead box proteins. It was demonstrated in knockout models of the above proteins resulted in increased proliferation and eventual exhaustion of HSCs (Lanza 2006; Orford et al. 2008).

1.4.3 Stem cell Niche:

Virtually every tissue of adult organism maintains a population of stem cells and are regulated and supported by the specialized microenvironment, referred to as stem cell 'niche'. The important niche components include both cellular (Cell-cell, cell-matrix contacts) and non-cellular (growth factors) components that interact actively with each other. (Fig.1.5)

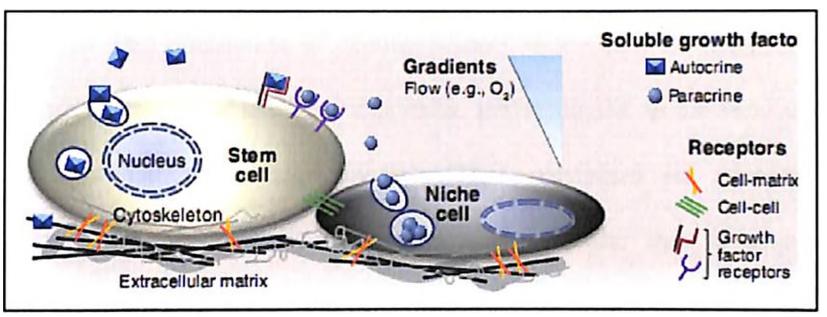


Figure 1.5: Stem cell niche: Soluble and matrix-bound factors combine with cell-cell contact, cell-matrix adhesion, and gradients to direct cell fate (Discher et al. 2009).

Adult stem cell niches are more complex due to the presence of complex interactions between niche cells and extra cellular components that are involved in the regulation and maintenance of the tissue specific stem cells. In mammals the well characterized microenvironments are Neural, Epithelial and Hematopoietic. These niches are briefly described below:

i. Neural stem cell (NSC) niche: In the mammalian brain, there are two specialized areas, where stem cells reside, one in the sub ventricular zone (SVZ) and second in the sub granular zone (SGZ). The best characterized is the SVZ niche. The stem cells isolated from SVZ have the capacity to differentiate into both neurons and astrocytes *in vitro* and the cells are regulated by both contact dependent factors and diffusible signals (Reynolds et al. 1992).

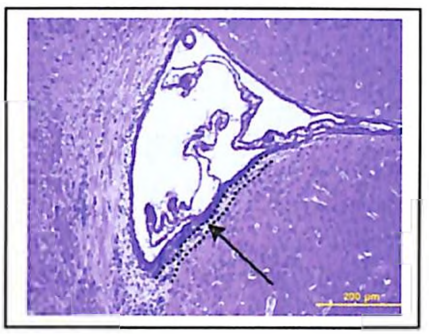


Figure 1.6: Portion of the SVZ surrounding the lateral ventricles, which shows the region where stem cells reside by the dotted line (Walker et al. 2009).

Physical contact- The orientation of mitotic spindle determines the type of division in NSCs. Vertical orientation of the mitotic spindle, perpendicular to the ventricular surface pushes cells towards symmetrical division. These divisions are necessary for the embryonic development of the brain in size. The protein that is associated with symmetrical type of division is ASPM (Fish et al. 2006). In order to generate asymmetric divisions of NSCs, Notch1 signaling plays an important role and is exhibited by the differentiating daughter cell (Chenn et al. 1995). *Numb* gene, which is the inhibitor of Notch signaling, also plays an important role in generating asymmetric divisions. Cells with symmetric *Numb* distribution gave rise to identical daughter cells, whereas cells expressing asymmetric distribution of *Numb* resulted in two different daughter cells (Lindsell et al. 1996). Expression of *Numb* gene is important for the repair and regeneration of SVZ niche and the loss of expression results in the neuroblast death (Shen

et al. 2002). Cell-cell contact between stem cells is achieved through adherins and tight junctions, which aid in the establishment of apical-basal polarity (Zhadanov et al. 1999). These studies show that cell-cell contact is important in keeping NSCs close to the niche where they can interact.

ECM, which is underlying the basal surface has also been shown to be involved in the regulation of NSCs. ECM helps in concentrating the growth factors in the niche like N-sulphate heparin sulphate proteoglycans (HSPG) binds to FGF-2 (Kerever et al. 2007). It was also shown that integrin $\alpha_6\beta_1$ increased NSC adherence to the endothelial cells and regulated the proliferation (Shen et al. 2008).

Diffusible factors: In the NSCs niche bone morphogenic proteins (BMPs) are important for the fate decisions. Noggin gene inhibits the BMPs and favours the differentiation of neurons over glial cells in SVZ (Lim et al. 2000). Epidermal growth factor (EGF) and Fibroblast growth factor (FGF-2) are the important growth factors for the maintenance and proliferation of the neural niche. EGF induces NSCs to differentiate into glial cells *in vivo* (Gregg et al. 2003). FGF2 regulates the rate of proliferation and neurogenesis in the organisms (Jin et al. 2003; Shetty et al. 2005). These above studies suggest that the stem cell niche possesses plasticity and can respond to signaling cues to initiate regeneration and differentiation.

ii. Epidermal stem cell niche: The epidermis has two important stem cell niches which aid in maintaining and regulating the stem cells responsible for the epidermis (Fig 1.7) and for the hair follicle. Stem cells of the hair follicle reside in the bulge region and stem cells of the skin reside in the basal layer of the epidermis (Potten 1981; Rochat et al. 1994).

Physical contact- Intergrin β_1 maintains the contact with the basement membrane and aid in maintaining the self-renewal capacity of the epidermal stem cell. The daughter cells resulting from the division of stem cells, which rapidly proliferate before differentiation

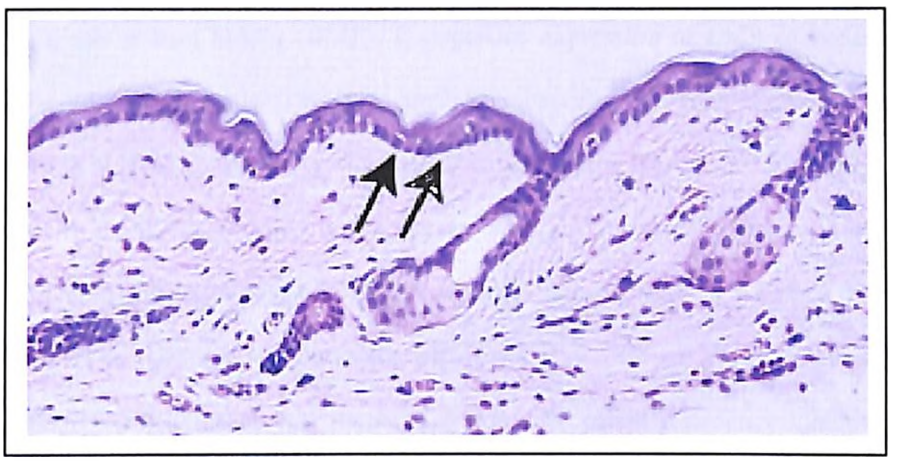


Figure 1.7:The epidermal stem cells reside in the basal layer of the skin (arrows) (Walker et al. 2009)

are called as transit-amplifying cells (TACs). These TACs express high levels of Ki67 and low levels of Intergrin β_1 (Jones et al. 1993; Jensen et al. 1999). Above studies show the importance of the niche, and its role in deciding the maintenance of self renewal property or leading to a differentiated pathway. In the hair follicle two different cell populations maintain the stemness and multipotency. One of the population shows BrdU label retaining capacity and the other cells express high levels of integrin α_6. Both these populations showed properties of stem cells like they were quiescent *in vivo*, clonogenic and engrafted in nude mice, forming patches of hair-producing epidermis (Blanpain et al. 2004).

Diffusible factors: The signaling pathways that are actively involved in the maintenance of skin and hair are Wnt and BMP signaling. Loss of β-catenin transcription factor in the skin and hair during embryogenesis causes loss of hair follicles and postnatal results in hair loss (Huelsken et al. 2001). Recent studies have shown that Wnt signaling is implicated in

the regulation of the epidermal stem cell in response to injury. In the absence of this signaling regeneration does not takes place (Ito et al. 2007).

Another important signaling pathway in the hair regeneration cycle is the bone morphogenic protein family (BMP). Endogenous expression of BMP prevents the cells entering into a new cycle. This was proved by blocking the BMP signaling by adding antagonist Noggin in keratinocytes, which subsequently increased hair follicle cycling (Plikus et al. 2008). Further investigation into this signaling pathway will help in understanding the niche organizational structure and the way in which it manages to maintain communication with the stem cell.

iii. Haematopoietic stem cell niche: Two very important microenvironments that are associated with HSCs are osteoblastic and vascular niches (Yin et al. 2006; Kiel et al. 2008). The primary constituent of the osteoblastic niche is, osteoblast (OB). The markers that OBs were designated are N-cadherin $^{+}$ and CD45^{-}. Increased Jagged 1 gene expression by OBs showed a subsequent increase in the number of HSCs (Calvi et al. 2003). Recent studies have shown that *in vitro* cultures of endothelium with HSCs demonstrated the ability of these primary vascular cells to support and maintain stem cells. This work was corroborated by the localization of HSCs marked by signaling lymphocyte activating molecules (SLAM family molecules) adjacent to the endothelium *in vivo* (Li et al. 2004; Kiel et al. 2005).

2 Carcinogenesis:

2.1 Introduction

In the western world cancer is the second most reason for causing death and current estimation project that one in every three people will develop cancer and one in five will die from the disease. Generally, the cancer cells are the cells which are altered and have escaped from the normal growth-regulating mechanisms to form mass of cells.

2.2 Origin and terminology

In most tissues and organs of a mature adult, a balance is usually maintained between cell renewal and cell death. The various types of differentiated cells in an organ have a particular life span, after which the cells undergo apoptosis leading to cell death. Newer cells are generated by the proliferation and differentiation of various types of stem cells. Under normal situations, the production of new cells is regulated so that the number of any particular type of cell remains constant; however very rarely certain cells arise that no longer respond to normal growth-control mechanisms. These cells give rise to a clone of cells that can self-renew and expand, producing mass of heterogeneous cells, which result in a 'tumor' or 'neoplasm'. The nomenclature of tumors is, however based on the parenchymal component.

i. Benign tumor: A tumor that is not capable of indefinite growth and does not invade the healthy surrounding tissue is Benign tumor. In general, these are designated by attaching the suffix "-oma" to the cell of origin eg: Tumors of mesenchymal cells fall under this category and a tumor of fibroblastic cell is called as fibroma. A cartilaginous tumor is a chondroma and a tumor of the osteoblasts is an osteoma. In contrast, nomenclature of benign epithelial tumors is different as some tumors were classified based on their cell of origin and some tumors based on microscopic characters eg: adenomas, papillomas, polyps and cystadenomas.

ii. Malignant tumor: A tumor that continues to grow and becomes progressively invasive is malignant tumor. The term 'cancer' refers specifically to a malignant tumor. In addition to uncontrolled growth, malignant tumors exhibit metastasis; in this process, small clones of cancerous cells dissociate from the main tumor, and invade other tissues through blood or lymphatic vessels. This way the primary tumor causes secondary tumor and spreads to the whole body.

Malignant tumors are classified based upon the embryonic origin of the tissue. Around 80% of the tumors are carcinomas i.e tumors that arise from endodermal or ectodermal tissues like skin or the epithelial lining of the internal organs and glands. The majority of cancers that arise from colon, breast, prostate and lung are carcinomas. Leukemias and lymphomas are malignant tumors of hematopoietic cells of the bone marrow. The incidence of this tumor is about 9% in the United States alone (Richard A. Goldsby 2000). Lymphomas tend to grow as tumor masses and leukemia's proliferate as single cells. Sarcomas are less frequent and are mesodermal in origin. These are generally derived from connective tissues like bone, fat and cartilage (Richard A. Goldsby 2000).

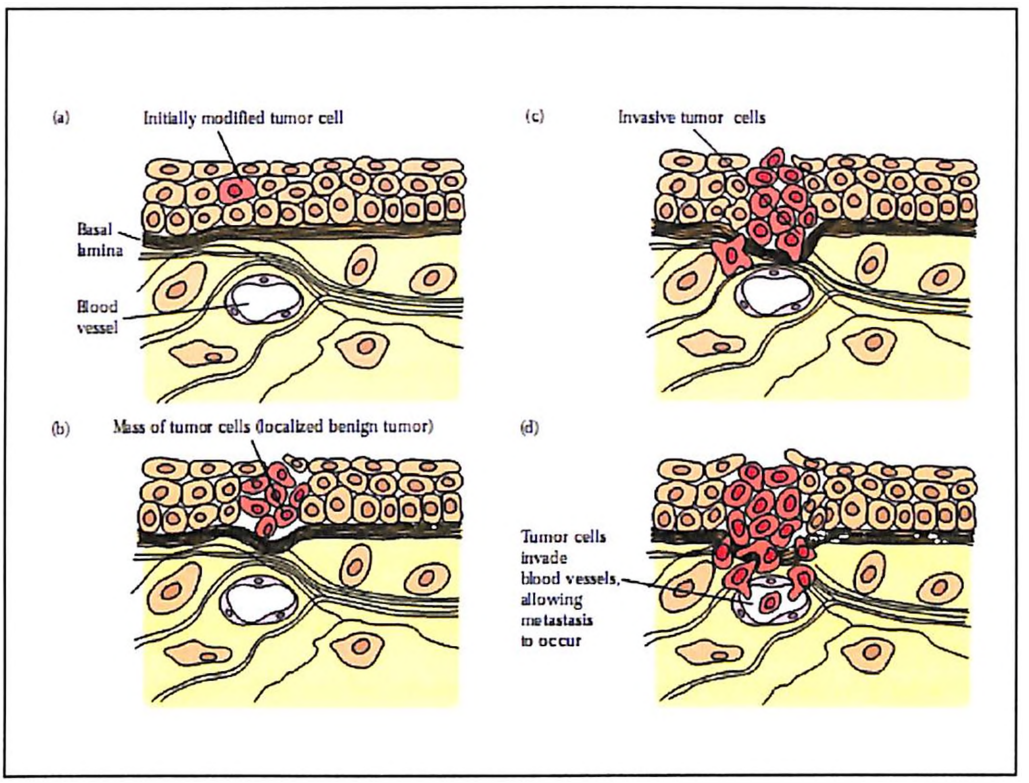


Figure 1.8: (a) A single modified cell developed altered growth properties at a tissue site. (b) Altered cell, producing a mass of localized tumor cells or benign tumor. (c) The cancer cells become progressively more invasive, invading the basal lamina, the tumor at this stage is said to be malignant. (d) Clusters of cells from malignant tumor dislodge and are carried to different organs by blood or lymphatic vessels (Richard A. Goldsby 2000).

2.3 Malignant transformation of cells

Transformation is the term used to describe the alteration of normal cultured cells when exposed to various carcinogens (DNA-alkylating agents), irradiation (U.V light, gamma irradiation) and certain viruses that result in cells with changed morphology and growth characteristics. This process leads to tumor formation when they are injected into animals. Such cells are said to have undergone malignant transformation.

Multiple steps involved in invasion and metastasis:

Invasion and metastasis of a carcinoma can be described by the following sequence of steps:

1. While the carcinoma proliferates and extends laterally, the tumor cells become less adherent to each other and to adjacent normal epithelial cells.
2. The underlying stroma becomes activated and inflammation may occur. The basement membrane which separates the epithelium from the underlying mesenchymal connective tissue is partially or completely destroyed.
3. The tumor continues its growth into the connective tissue. This is one of the more variable steps. Some carcinomas continue to grow as solid, coherent masses compressing the neighboring connective tissue or develop processes that spread into it, breaking up the extracellular matrix (ECM). From other carcinomas, small groups of cells or single cell splits off and migrate into the underlying tissues.
4. The invasion of stroma by tumor cells is accompanied by the altered gene activity in stromal cells, with some changes promoting and others inhibiting invasion.
5. A critical step in invasion is reached when the growing tumor cells encounter blood or lymph vessels and invade them. This process can occur either by a tumor mass growing through the vessel wall into the lumen or by single tumor cells squeezing through the vessel lining and is called as intravassation step. By this

process, tumor cells gain access to the circulation via lymphogenic or hematogenic routes.

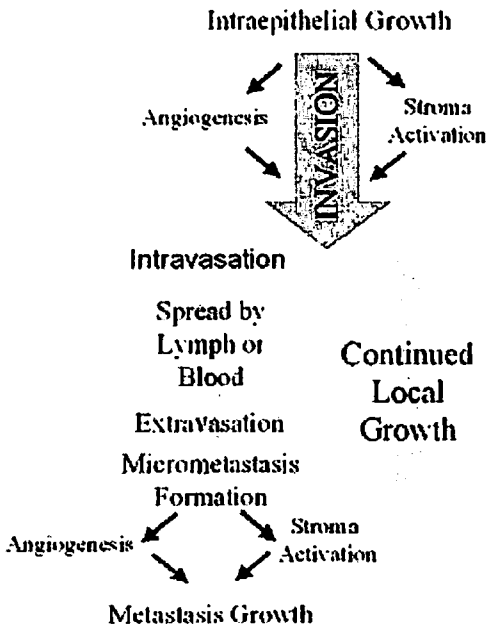


Figure 1.9: Steps of invasion and metastasis (Schulz 2005)

6. When tumor cells have entered into lymph vessels, they are transported to the filtering system of the local lymph nodes, where some may survive and start lymph node metastasis. Cells from this metastasis may eventually penetrate towards the main lymph vessels and enter the blood.
7. Tumor cells having entered into blood vessels can theoretically spread to any part of the body.
8. To form metastasis, tumor cells must exit from the circulation by extravassation. Most often, this appears to take place in organs with microcapillary systems, such as liver, lung, kidney and bone.

9. In the new tissue microenvironment, carcinoma cells have to reattach to the matrix, survive, sometimes for extended periods, and eventually start to expand into micro metastasis.
10. The final step in metastasis is the expansion of micro metastasis to actively growing tumors. This requires establishment of a sufficient nutrient supply and interaction with a different type of stroma (Schulz 2005).

2.4 Oncogenes and cancer induction

Induction of transformation can be either by physical, chemical and biological carcinogens or endogenous processes which are listed in Table below.

Table 1.2: Types of carcinogens

Type of Carcinogen	Examples
Chemical carcinogens	Nickel, cadmium, arsenic, nitrosamines, trichloro ethylene, arylamines, benzopyrene, aflatoxins, reactive oxygen species
Physical carcinogens	UV irradiation (Specifically UVB), ionizing radiation
Biological carcinogens	Human papilloma virus, Epstein Barr virus, Hepatitis virus B, *Helicobacter pylori*, *Schistosoma mansoni*

Physical Carcinogens: Any energy rich radiation can acts as a carcinogen, depending on dose and absorption. Visible light is not usually carcinogenic, unless it is absorbed by 'photosensitizing agents' which generate reactive oxygen species. UVB is an important carcinogen for skin, and its effect is augmented by UVA. γ- Radiation from natural, industrial and iatrogenic sources can penetrate into and through the body. Carcinogenicity depends upon the dose of γ- radiation. It damages the DNA and cells by direct absorption but also indirectly by generating reactive oxygen species. Damage and carcinogenicity by γ- radiation therefore depend on the concentration of reactive oxygen species and also on

the repair capacity. Radioactive α, β- radiation is most dangerous when nuclides are incorporated into the cells e.g. cesium, uranium and plutonium (Schulz 2005).

Eg: xeroderma pigmentosum, a rare disorder which is caused by a defect in the gene that encodes a DNA-repair enzyme called UV-specific endonuclease, individuals with this disease are unable to repair UV-induced mutations and consequently develop skin cancers (Goldsby 2000).

Chemical carcinogens: These carcinogens come from different sources and comprise very different chemicals. Inorganic compounds like nickel, cadmium, or arsenic are encountered at the workplace or are present as contaminants in water. Organic compounds acting as carcinogens can be aliphatic, like nitrosamines, which occur in smoked and pickled foods, or trichloro-ethylene, which is used for cleaning. Nitrosamines are thought to contribute to stomach cancer, in particular aromatic compounds like benzopyrenes and arylamines are generated from natural sources by burning and are among the many carcinogens in tobacco smoke. Arylamines are thought to cause bladder cancer. Natural compounds produced by plants and molds can be highly carcinogenic for eg: Aflatoxin B1 (Schulz 2005).

Biological carcinogens:

A number of DNA and RNA viruses (like SV40 and polyoma) have also been shown to induce malignant transformation. The genomes of both viruses integrate randomly into the host DNA and causes transformation. One of the best-studied RNA virus is Rous sarcoma virus. This virus carries an oncogene called v-src (v-onc), which encodes protein kinase that catalyzes the addition of phosphate to tyrosine residues on proteins. This was proved by cloning and transfecting this oncogene into normal cells which resulted in malignant transformation (Goldsby 2000).

Howard Temin hypothesized that oncogenes might not be unique to the transforming viruses but might also be found in the normal cells and he called these genes as proto-oncogenes, or cellular oncogenes (c-onc). J. M. Bishop et. al, identified a DNA sequence in normal chicken cells that is homologous to v-src from Rous sarcoma virus. This cellular oncogene was named as c-src. Since then many cellular oncogenes have been identified. Comparisions of cellular and viral oncogenes reveal that they are highly conserved during evolution. Most cellular oncogenes possess both exons and introns, but their viral counterpart contains only introns. It has now become evident that most of the oncogenes are derived from cellular genes which encode various growth controlling proteins.

The proteins encoded by a particular oncogene and its corresponding proto-oncogene have almost similar functions.

Oncogenes and tumor suppressor genes have been shown to play an important role in the homeostasis process, by regulating the cell cycle of these cells. These genes can be classified into three categories (Table 1.3).

i. Genes involved in induction of cellular proliferation: Some proto-oncogenes and oncogenes encode the proteins that induce cellular proliferation. These proteins function either as growth factors or growth factor receptors (eg: sis- encodes a form of platelet-derived growth factor and fms, erbB, neu- encode growth factor receptors). Some oncogenes in this category encode proteins that function in signal-transduction pathways or a transcription factors. The src and abl oncogenes encode tyrosine kinases, and the ras oncogene encodes a GTP- binding protein. The products of these genes act as signal transducers. The myc, jun and fos oncogenes may result in unregulated proliferation (Goldsby 2000).

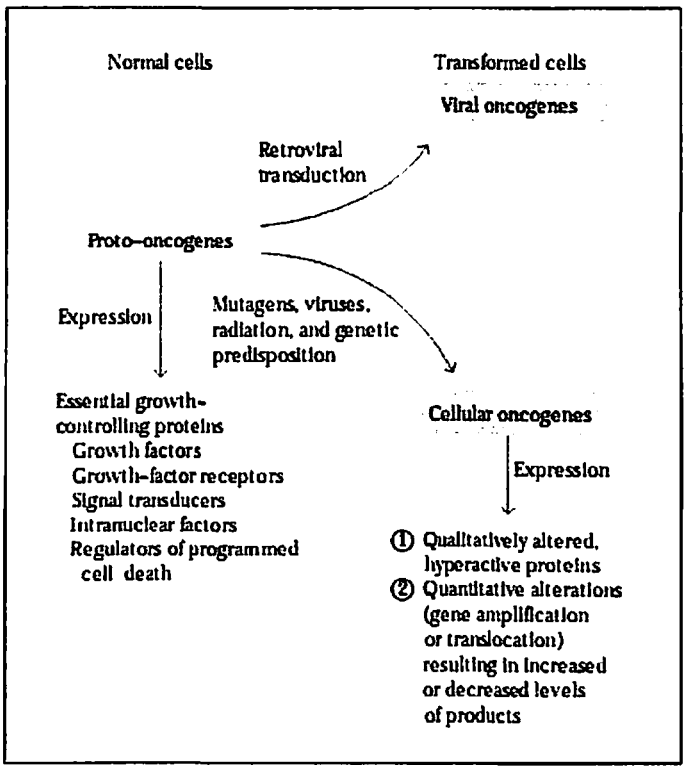


Figure 1.10: Conversion of proto-oncogenes into oncogenes can involve mutation, resulting in production of qualitatively different gene products or DNA amplification or translocation, resulting in increased or decreased expression of products (Goldsby 2000).

Table 1.3: Functional classification of cancer associated genes (Richard A. Goldsby 2000)

Type/Name	Name of the gene product
Category-I: Genes that induce cellular proliferation	
Growth factors- sis	A form of platelet-derived growth factor (PDGF)
Growth factor receptors	
Fms	Receptor for colony stimulating factor 1 (CSF-1)
erbB	Receptor for epidermal growth factor (EGF)
neu	Protein (HER2) related to EGF receptor

erbA	Receptor for thyroid harmone
Signal Transducers	
src	Tyrosine kinase
abl	Tyrosine kinase
Ha-ras	GTP-binding protein with GTPase activity
N-ras	GTP-binding protein with GTPase activity
K-ras	GTP-binding protein with GTPase activity
Transcription factors	
Jun	Component of transcription factor AP1
Fos	Component of transcription factor AP1
myc	DNA-binding protein
Category-II: Tumor suppressor genes	
Rb	Suppressor of retinoblastoma
P53	Nuclear phosphoprotein that inhibits formation of small cell lung cancer and colon cancers
DCC	Suppressor of colon cancer
APC	Suppressor of adenomatous polyposis
NF1	Suppressor of neurofibromatosis
WT1	Suppressor of Wilm's tumor
Category-III: Genes that regulate programmed cell death	
bcl-2	Suppressor of apoptosis

ii. Genes involved in inhibition of cellular proliferation: This category involves tumor suppressor genes or anti-oncogenes. These genes encode proteins that inhibit excessive

cell proliferation. Inactivation of these genes results in uncontrolled proliferation. Genes of this category are *Rb, p53, DCC, APC, NF1* and *WT1* (Table 1.3).

iii. Genes involved in regulation of programmed cell death:

This category of genes regulates apoptosis and these genes encode proteins that either block or induce the process. The oncogene in this category include bcl-2, an anti apoptotic gene. This gene plays an important role in regulating cell survival during hematopoiesis and in the survival of selected B cells and T cells during maturation. Epstein-Barr virus contains a gene that has sequence homology to bcl-2 and may function in a similar manner to suppress apoptosis (Goldsby 2000).

3 Cancer stem cells (CSCs):

3.1 Stem cells and cancer

Initial studies have given strong evidence that stem cells exist in the hematopoietic system and these studies have shown a way to the prospective isolation of various tissue specific stem and progenitor cells. The identification and characterization of their properties was helpful in designing regenerative cell therapies for various diseases. The most important characteristic of stem cells is self-renewal ability and the same property was found in cancer cells, it has given a thought to the interesting concept that the tumors may often originate from the transformation of normal stem cells or similar signaling pathways which regulate stem cells self renewal may be involved in cancer cells and/ or cancer cells may contain small subpopulation of cells called cancer stem cells with indefinite capability for self renewal that drive the formation of tumor (Fig 1.11).

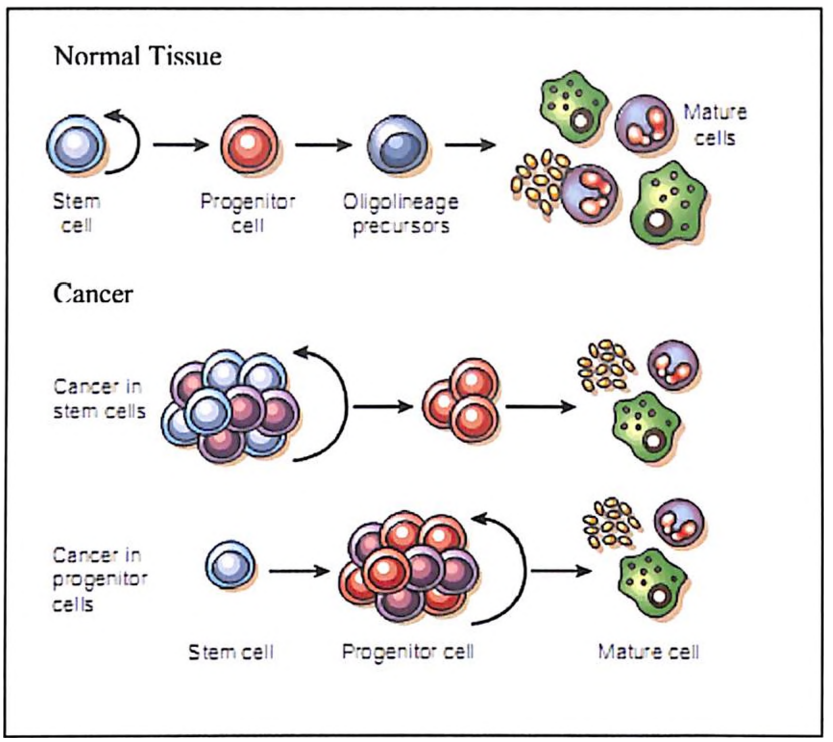


Figure 1.11: Comparision of self-renewal during hematopoietic stem cell development and leukaemic transformation. Because of their high levels of self renewal, stem cells are particularly good targets of transformation (Reya et al. 2001).

CSCs express similar markers and signaling pathways as that of stem cells (Sox2, Nanog , oct4, Wnt, Notch, Sonic Hedgehog, BMI-1 and EZH2 etc.), (Reya et al. 2001; Kolligs et al. 2002; Rask et al. 2003; Duncan et al. 2005; Hopfer et al. 2005; Katano 2005; Sanchez et al. 2005; Wilson et al. 2006) as shown in Fig 1.11.

Besides these properties they have elongated telomeres, increased telomerase activity, express ABC transporters which confer resistance to many chemotherapy drugs, capable to grow without growth factors by secreting cytokines of their own and finally augment

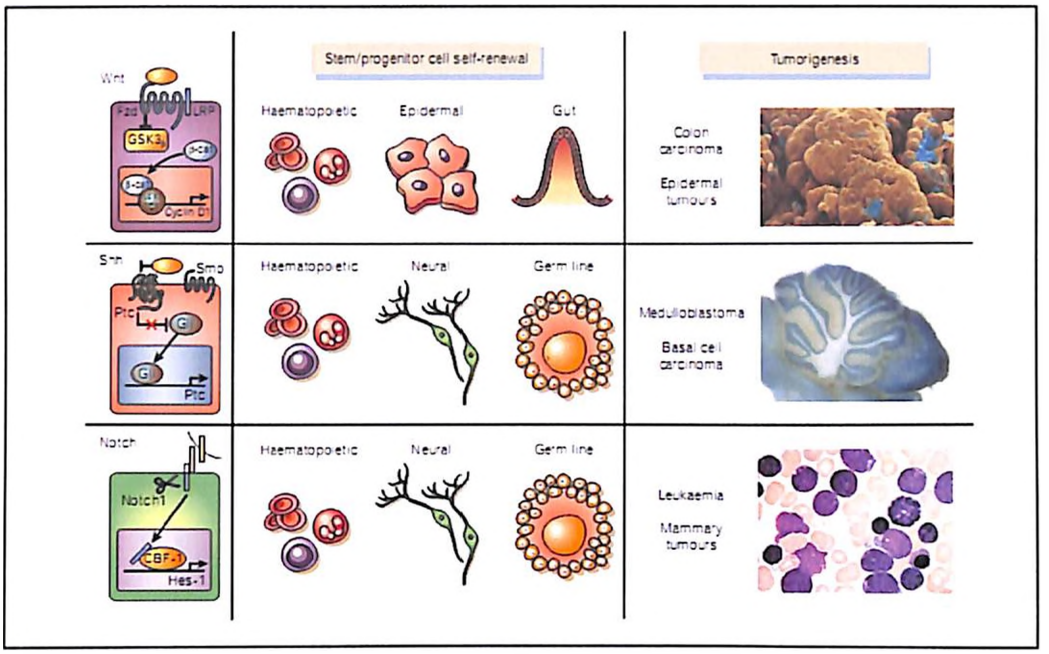


Figure 1.12: Signaling pathways that regulate self-renewal mechanisms during normal stem cell development and during transformation (Reya et al. 2001).

neoangiogenesis by secreting angiogenic factors and finally expressing receptors and adhesion molecules associated with metastasis CXCR4, LIF-R, c-met, c-kit etc (Kolligs et al. 2002; Zagzag et al. 2005; Peeters et al. 2006; Bapat 2009).

3.2 Concept of carcinogenesis based on CSCs:

The formation of tumor mass is a complex process that is influenced by multiple factors such as doubling time of tumor cells, angiogenesis and host responses elicited by tumor cells or their products. It is evident that by the time a solid tumor is detected, it has already completed a major portion of its life cycle as measured by cell doublings (thirty population doublings which is equal to 10^9 cells and weighs 1g) and it takes another ten population doublings to reach the maximum size (Cotran 1994).

The cell cycle of tumors, like that of all other cells, has the same five phases observed in normal cells (G_0, G_1, S, G_2, and M). As the cell population expands in the tumors, progressively higher percentage of tumor cells leave the proliferative or replicative pool

by reversion to G_0 phase or the cells divide very slowly i.e. about 5% of cells and the remaining differentiate and perish (Cotran 1994).

The growth fraction i.e. S, G_2 and M phase fraction of tumor cells has a profound effect on their susceptibility to cancer chemotherapy. Most anticancer agents act on cells that are actively synthesizing DNA, and the tumor that contains 5% of cells that reenter into G_0 phase or are slowly growing, will relatively be refractory to such treatment (Cotran 1994) as shown in Fig 1.13.

The possible reason for recurrence of the tumors even after chemotherapy is the expression of drug excluding channel called ATP binding cassette G2 gene (ABCG2) in certain primitive cells (Marx 2003; Seigel et al. 2005). These cells have been

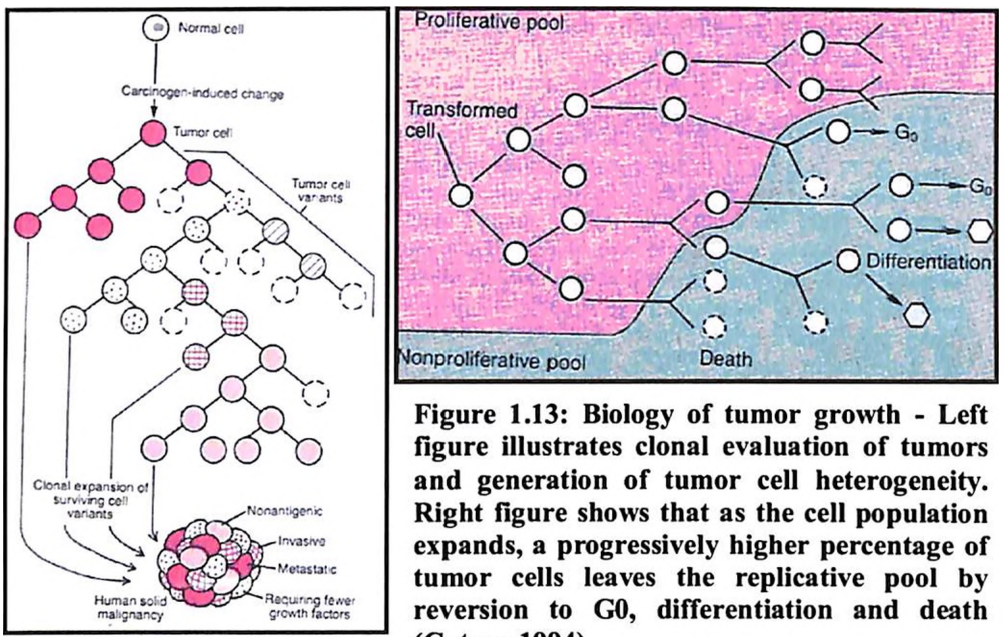


Figure 1.13: Biology of tumor growth - Left figure illustrates clonal evaluation of tumors and generation of tumor cell heterogeneity. Right figure shows that as the cell population expands, a progressively higher percentage of tumor cells leaves the replicative pool by reversion to G0, differentiation and death (Cotran 1994).

characterized by Hoechst 33342 dye exclusion and confer resistance to atleast 20 different chemotherapeutic agents. The side population has been identified as a group of cells able to exclude the Hoechst 33342, a characteristic abolished with 50μM verapamil treatment. This dye excluding "side population" (SP) phenotype has been used in a variety of tissues

to sort out presumptive stem cells, including stem cells from hematopoietic populations, bone marrow, skeletal muscle, mammary gland, lung, and developing retina (Zhou et al. 2001; Scharenberg et al. 2002; Welm et al. 2002; Bhattacharya et al. 2003; Summer et al. 2003). The SP phenotype, coupled with expression of stem cell markers is considered as hallmark of both stem cells and cancer like stem cells. The existing therapies for treating cancer are likely to be improved by characterization of cancer stem cells, as there is a hope that these will aid in targeting the de regulated self-renewal (Al-Hajj et al. 2004).

It was well understood that tumor arises in proliferating competent adult stem cells, which has undergone mutations and survived the natural elimination process of such a mutant cell. This mutant cell, with repeated divisions gives rise to a tumor, and produces a clonal population of cells that becomes clinically detectable after nearly 30 tumor population doublings (Cotran 1994). There could be additional mutations in these tumor cells, contributing to poor prognostic features, which is evident in the mutation screening studies of RB1 gene in Indian patients at our institute (Kiran et al. 2003). Some of the cells in the tumors are in the proliferative compartment of the tumor, called the "S" phase fraction of the tumor cells, while others are either dead or differentiated (Cotran 1994).

One of the main problems in cancer biology is identification of the cell type capable of sustaining the growth of the neoplasm clone. In recent years, the concept of Cancer stem cells evolved, suggesting that each tumor may have a population of cells that possess the capacity to proliferate extensively, resist chemotherapy or radiotherapy and survive to form new tumors in the body (Vercauteren et al. 2001; Al-Hajj et al. 2003; Kiran et al. 2003; Marx 2003; Pardal et al. 2003; Singh et al. 2003; Al-Hajj et al. 2004; Al-Hajj et al. 2004; Singh et al. 2004; Ponti et al. 2005; Burkert et al. 2006; Mohan et al. 2006; Yilmaz et al. 2006).

3.2.1 Concept and Model of Cancer Stem Cells

The hypothesis of cancer "stem cells" first originated from blood related cancers, in which it was shown that certain sub-population was responsible for tumor formation and this subpopulation have the characters of normal stem cells like slow dividing, chemo resistant and are capable of tumor progression. The fundamental property of both CSC and normal stem cells is their capacity for self-renewal. In addition, both stem cells and cancer cells have the ability to migrate and differentiate. Several signaling pathways implicated in stem cell self-renewal and maintenance of multipotentiality has also been associated with carcinogenesis in the hematopoietic system, colon, breast etc and these include the Notch, Wnt, Sonic hedgehog (SHH) pathways (Allenspach et al. 2002; Pasca di Magliano et al. 2003).

Emerging evidence reveals from leukemia cases and solid tumors show that there could be significant similarities with adult stem cells (for e.g. Wnt signaling, Sonic hedgehog, Notch signaling etc.), but the difference is that they have chromosomal abnormality, which was shown by karyotype analysis (Vercauteren et al. 2001; Camassei et al. 2003; Galli et al. 2004; Ponti et al. 2005; Yilmaz et al. 2006). The possible existence of cancer stem cells was first shown in acute myeloid leukemia and these are called as leukemia initiating cells ($CD133^+$, $CD34^+/CD38^-$) because when these cells transplanted into the non-obese diabetic/severe compromised immunodeficient mice (NOD/SCID mice) they form the secondary tumors (Lapidot et al. 1994; Bonnet et al. 1997; Singh et al. 2003). As $CD34^+/CD38^-$ is the marker for normal hematopoietic stem cells, which indicate that, the initially transformed cell was a primitive stem cell that took the wrong developmental pathway, presumably depending upon the mutation that occurred (Lapidot et al. 1994; Bonnet et al. 1997; Vercauteren et al. 2001). It has been shown that Phosphotensin homologue (PTEN) dependence distinguishes hematopoietic stem cells from leukemia-initiating cells and has shown that this property can be exploited in targeting only

leukemia initiating cells but not the HSC's (Yilmaz et al. 2006). Recently in ISSCR 2006 meeting it has been shown that bronchio alveolar stem cells were observed in established tumors suggesting that they may play a role in later stages of tumorigenesis in addition to a role in tumor initiation (Kruger. 2006). In another study prospective identification of tumorigenic cells in breast cancer was shown to express $ESA^{+}CD44^{+}CD24^{-/low}$ $lineage^{-}$ in eight of nine patients (Al-Hajj et al. 2003; Ponti et al. 2005). In pediatric brain tumors, tumor derived progenitors form neurospheres that can be passaged at clonal density and are able to self-renew. These cells expressed many genes characteristic of neural and other stem cells including $CD133^{+}$, nestin, Sox_2, musashi-1, bmi-1, maternal embryonic leucine zipper kinase, Oct_4 etc (Singh et al. 2003; Galli et al. 2004).

Two hypothesis were proposed to describe the tumor formation based upon the continuing mutagenesis and abberant differentiation of cancer cells:

i. Stochastic model: This model postulates that each subpopulation of cells with in the tumor has an equal but very low tumorogenic potential. Example of this model is colonic cancer which harbours mutated stem cells (Nowell 1976; Kruh 2003; Bapat 2009) as shown in Fig 1.12 a

ii. Hierarchial model: This model postulates that each subpopulation in the tumor don't have equal tumorogenic potential, only limited population of cells have clonogenic potential (i.e. CSC's) and the remaining part of the tumor are the descendants of these cancer stem cells.

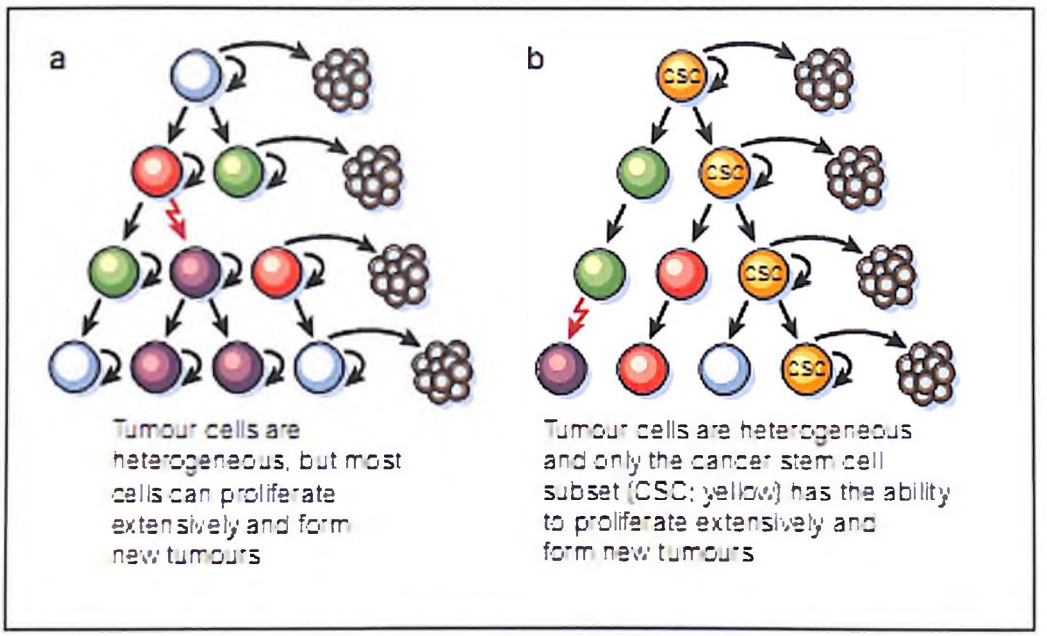


Figure 1.14: Two general hypothesis for heterogeneity in solid cancer cells (a) Stochastic model (b) Hierarchical model (Reya et al. 2001).

These descendents do not have self-renewal capacity and they are differentiated. This model explains why most tumors are heterogenous despite their clonal origin. Ex: leukaemias, brain tumors, breast, pancreatic tumors etc as shown in Fig 1.14 b. (Lapidot et al. 1994; Bapat 2009).

3.3 CSC plasticity and microenvironment:

The self renewal property and plasticity gained by CSCs is maintained because of complex microenvironment that these cells reside in. This microenvironment includes intrinsic factors (which may involve changes in DNA sequences or gene silencing through epigenetic effects or altered chromatin structure) and extrinsic factors (like paracrine factors derived from the tumor microenvironment).

i. Intrinsic factors: Deregulated gene expression of tissue specific stem cells promoting proliferation and/ or aberrant differentiation is the important characteristic of tumor (Knudson 1971; Caussinus et al. 2007). Uncontrolled expansion of cancer cells would not takes place by itself, as it requires genetic mutations on both the alleles to drive them

toward transformation (Gostjeva et al. 2005). This can be achieved either by oncogene activation or by inactivation of tumor suppressor genes. Further divisions accumulate more mutations leading to the clonal amplification and disease progression. Several members of the polycomb proteins (PcG) have been identified as important regulators of self renewal and also reported to be altered in various cancers (Liu et al. 2006)

ii. Extrinsic factors: The stem cell microenvironment is nothing but the stem cells surrounded by other differentiated cells within a tissue at defined locations including the factors that were released by these cells. These heterologous cell types influence their fate by having cell to cell and cell to matrix contacts, thereby regulating the balance between quiescence and activation, thus supporting the ongoing tissue repair and/or renewal. A number of mutagenic agents have been shown to confer a risk of cancer, which disturbs the microenvironment and tissue homeostasis. These disturbances may remain in a state of frequent activation by intercellular communications through ECM proteins or through autocrine or paracrine factors produced by niche cells. Such continuous alterations further create changes in gene expression and protein profiles that drive these stem cells toward differentiation, cell death and transformation pathways. Another important characteristic of the tumor niche is the active growth of new vessels and stromal cells into tumor that enhances tumor survival under adverse micro environmental conditions (Ganss 2006). The best example of normal niche may evolve to become proto-oncogenic is exemplified in lung cancer as shown in Fig 1.15 (Reynolds et al. 2000; Giangreco et al. 2002; Minna et al. 2003; Hong et al. 2004; Giangreco et al. 2007).

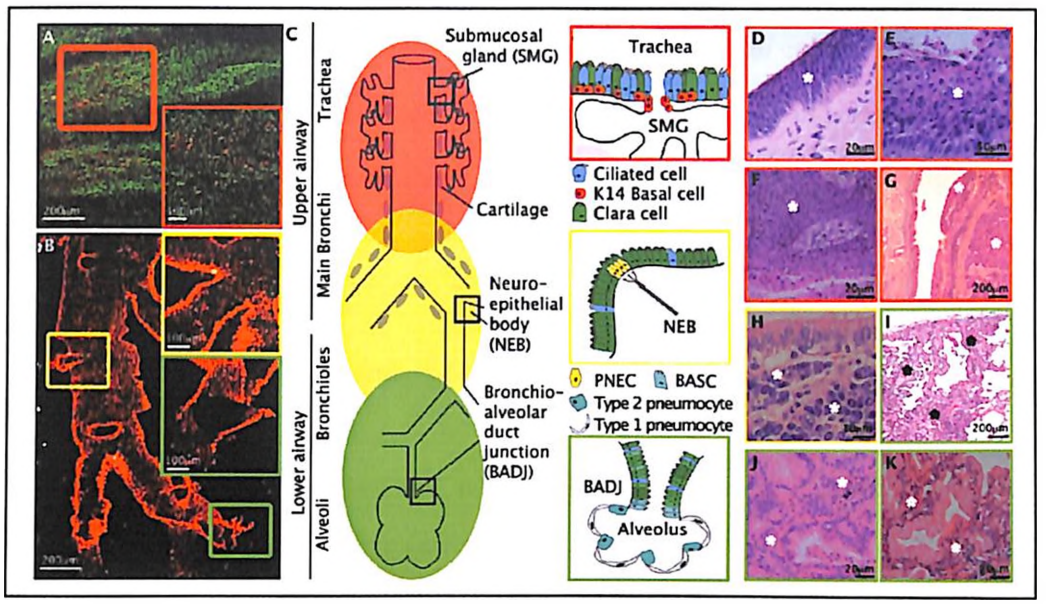


Figure 1.15: Lung stem cell microenvironments and associated human carcinomas. (A) Upper airways likely contain submucosal gland and intercartilagenous stem cell niches (red) defined by keratin 14 (K14) expressed in basal cells adjacent to ciliated cells expressing acetylated tubulin (green) ((C)-red). In humans basal cells may generate squamous cel carcinomas via transitional stages, including metaplasia (D), moderate dysplasia (E), carcinoma insitu (F), and invasive carcinoma (G). (C)-highlighted in yellow are midlevel, bronchiolar airway-mediated stem cell populations localized with calcitonin gene related peptide (CGRP) expressing neuroepithelial bodies (NEBs) adjacent to pulmonary neuroendocrine cells. These are shown in (B) stained with clara cell secreatory protein (CCSP) (red) and CGRP (green) and drawn schematically in (C) highlighted in yellow. Within the NEBs of humas, neuroendocrine cells themselves likely generate small cell carcinomas (H). CCSP expressing bronchioalveolar stem cells (BASCs) within green highlighted terminal airways (B-red portions) and BASCs drawn in C function as distal airway stem cells and in humans may generate atypical adenomatous hyperplasia (I), adenocarcinomas (J), and bronchioalveolar cell carcinomas (K). Abnormal human lung tissues are indicated by asterisks (D-K) (Giangreco et al. 2007).

Oncogenic niches have also been described in the field of hematopoietic stem cells. The retinoblastoma (RB) gene identified as a tumor suppressor, has been shown to regulate HSCs normal hematopoiesis. Loss of RB expression in the niche leads to degradation of osteoblastic niche and consequent disturbance in HSC niche resulting in a myeloproliferative disease (MPS) (Walkley et al. 2007).

Another important common characteristic of tumor and tissue stem cells is having similar signaling pathways the normally control the cell fate during early embryogenesis. Such pathways include components of the Wnt, Notch, Hedgehog and bone morphogenetic proteins (Rask et al. 2003; Ying et al. 2003; Doucas et al. 2005; Katano 2005; Sanchez et al. 2005; Wilson et al. 2006).

4 Characterization and isolation of CSCs

4.1 Phenotypic characteristics:

Most of the cancers are now believed to be heterogeneous containing mixture of self re-newing stem cells, transiently amplifying progenitors and proliferative cells that can undergo differentiation resulting in various types of cells in whole tumor (Jordan et al. 2006; Krivtsov et al. 2006). All these factors make the identification and isolation of the initial cancer forming cell within tumors a challenging problem in cancer biology.

Characterization of cells in the field of stem cell biology has elucidated in detail in hematopoietic system based upon the cluster of differentiation (CD) cell surface marker expression. Firstly leukemia initiating cells were identified in acute myeloid leukemia (AML) using limiting dilution assays. This study has demonstrated that in AML malignant cells were probably derived from primitive bone marrow cells, which express a similar surface marker expression ($CD34^+/CD38^-$) (Lapidot et al. 1994; Bonnet et al. 1997; Cobaleda et al. 2000).

Other than hematopoietic system, presence of stem cells in solid tumors was shown in breast, brain, prostrate lung, pancreas, liver, colorectal tumors and listed in table 1.4 (Al-Hajj et al. 2003; Singh et al. 2004; Bapat et al. 2005; Li et al. 2007; O'Brien et al. 2007; Ricci-Vitiani et al. 2007).

Table 1.4: Surface markers identified in various tumors

Cancer tissue/cell lines	Markers/ stem cell like properties characterized	References
Acute myeloid leukaemia	$CD34^+/CD38^-$, $CD90^-$, KIT^- Tumorigenic *in vivo*	(Lapidot et al. 1994; Bonnet et al. 1997; Cobaleda et al. 2000)
Multiple myeloma	$CD138^-$, Tumorigenic *in vivo*	(Matsui et al. 2004)
Neural Medulloblastoma, astrocytoma, glioblastoma, ependymoma	$CD133^+$/$nestin^+$, Neurosphere forming capability, Tumorigenic *in vivo*	(Singh et al. 2004)
Prostrate	$CD133^+$, $CD44^+$, $\alpha_2\beta_1$ integrin high, spheres formed, SMO^+, Tumorigenic *in vivo*	(Collins et al. 2005)
Breast	$CD44^+/CD24^-$, Oct4, Mammospheres formation observed, Tumorigenic *in vivo*	(Al-Hajj et al. 2003; Ponti et al. 2005)
Hepatocellular	$CD133^+$	(Suetsugu et al. 2006)
Lung	$Sca1^+$, $CD45^-$, $CD31^-$, $CD34^+$	(Kim et al. 2005)

Pancreas	$CD44^+/CD24^+/ESA^+$, $CD133^+/ABCG_2$, Tumorigenic *in vivo*	(Li et al. 2007)
Colorectal	$CD133^+$, Colon spheres, Tumorigenic *in vivo*	(O'Brien et al. 2007; Ricci-Vitiani et al. 2007)
Melanoma	$CD20^+$, Spheroid formation is observed	(Fang et al. 2005)
Ovarian cancer	$CD44^+$, Oct3/4, Nanog, EGFR, Vimentin/Snail, Tumorigenic *in vivo*	(Hopfer et al. 2005)
Head and neck cancer	$CD44^+$, Tumorigenic *in vivo*	(Prince et al. 2007)
Retinoblastoma	$ABCG_2$, MCM_2, $CD44^+$, $CD133^-$,$CD90^-$, Oct4, Nanog	(Krishnakumar et al. 2004; Seigel et al. 2005; Mohan et al. 2006; Seigel et al. 2007; Balla et al. 2009)

4.2 Clonogenic assays in cancer cells:

These assays were first carried out by Puck et al. (Puck et al. 1956) and the first attempt of agar assay on primary tumor cells were done with mouse myeloma (Ogawa et al. 1973; Park et al. 1980). After that human myeloma and other tumors were also cultured

(Hamburger et al. 1977; Courtenay et al. 1978). Later, studies described a stem cell model of human tumor growth in these clonal assays (Salmon et al. 1978). Eventually these clonogenic assays were used for clinical drug testing and pre clinical drug screening (Hamburger et al. 1977; Courtenay et al. 1978; Park et al. 1980). This assay gives the number of clone forming cells in certain number of tumor cells, thereby giving evidence of cancer stem-like cells in the heterogeneous population of tumors.

4.3 Side population cells /ALDH assay:

Side population (SP) is a method of characterizing cell populations which are highly enriched in primitive and undifferentiated cells (Decraene et al. 2005; Larderet et al. 2006). The isolation and characterization of cells by this method was initially described by Goodell et al (Goodell et al. 1996). It was shown that when whole bone marrow cells were stained using Hoechst 33342, they discovered that cells which display fluorescence simultaneously at two emission wavelengths (blue 450 nm and red 675 nm) shows a small, yet distinct non-stained cell population that expresses stem cell markers ($Sca1^{+}$ $Lin^{-/low}$) (Challen et al. 2006). The degree of efflux is correlated with the stemness state. The cells exhibiting the highest efflux are the most primitive and the cells that take up the dye are more differentiated (Challen et al. 2006). Hoechst 33342 binds to the A-T rich regions of the minor DNA groove, and the intensity of its fluorescence is a measure of DNA content, chromatin structure and cell cycle (Challen et al. 2006). It has been proposed that the exclusion of Hoechst 33342 by SP cells is an active process involving ATP-binding cassette (ABC) transporter transmembrane proteins (Goodell et al. 1996). To prove this hypothesis, the ATP-binding cassette inhibitor verapamil (Ca^{+2} Channel blocker) was used to demonstrate that Hoechst 33342 exclusion by SP cells decreases, when the ATP-binding cassette transmembrane protein is blocked. Moreover, the Multi drug resistant 1 (MDR1) is expressed by 65% of bone marrow cells and SP cells represent 0.1% of these cells (Goodell et al. 1996). Thus, it can't be taken as a single marker to identify and isolate

SP cells. Then it was demonstrated that the breast cancer resistance protein is a marker of the SP phenotype in breast tissues; in addition to this integrin β_3 expression is also correlated with the properties of quiescent stem cells possessing in SP phenotype (Umemoto et al. 2006).

In addition to their identification in a broad spectrum of normal cells, SPs have also been identified in cancer cell lines and tumors for ex: neuroblastoma, melanoma, retinoblastoma, ovarian, hepatocellular carcinoma, and glioma cell lines. (Hirschmann-Jax et al. 2004; Kondo et al. 2004; Seigel et al. 2005; Chiba et al. 2006; Grichnik et al. 2006; Szotek et al. 2006).

4.4 Telomerase activity:

It was shown by many studies that normal human cells progressively lose telomeric DNA with passage in culture, whereas telomere length is stable in neoplastic cells (Harley et al. 1994). These telomeres are specialized nucleotide sequences composed of large concatamers of the guanine-rich sequences 5'-TTAGGG-3'. These sequences serve to protect the chromosomal damage, thus increasing the genetic stability (Greider et al. 1996). Telomeres are maintained by telomerase, a multi subunit enzyme comprised of an RNA component, hTR_2, which provides the template for the synthesis of telomeric repeats and a protein reverse transcriptase component, hTERT, which catalyzes the synthesis reaction (Nakamura et al. 1998). Human TERT activity is absent in human normal cells but highly present in the majority of tumors (Kim et al. 1994). It was also shown that the expression of hTERT in many mortal cells permits immortalization and inhibition of this enzyme in various tumor cell lines lead to the apoptotic cell death (Bodnar et al. 1998; Hahn et al. 1999; Zhang et al. 1999). Co-expression of telomerase and oncogenes converts normal cells into tumor cells (Elenbaas et al. 2001), these evidences made telomerase enzyme a novel target for cancer therapy.

4.5 Tumor forming ability in animals:

In order to determine whether tumors grow by a stem cell concept, primary human or mouse tumors should be fractionated and sorted for cells expressing stem cell markers in heterogeneous tumor tissue. These sorted cell populations will then be injected into the specified area depending upon the tumor type in NOD/SCID mice. After 2-4 weeks, if a particular fraction of the primary tumor can produce the original tumor, then the tumor needs to be isolated and serially transplanted to see the tumor forming ability of the cells which confirms the stem cell concept in tumors (Bonnet et al. 1997; Collins et al. 2005; O'Brien et al. 2007).

4.6 Gene expression in tumors:

A looming concept of cancer stem cells indicate that the presence of a rare cell similar to the population of stem cells, is required for tumor progression and metastasis (Nowell 1976; Lapidot et al. 1994; Al-Hajj et al. 2003; Matsui et al. 2004; Bapat et al. 2005; Collins et al. 2005; Fang et al. 2005; Kim et al. 2005; Ponti et al. 2005; Lee et al. 2006; Mohan et al. 2006; Li et al. 2007; Prince et al. 2007; Ricci-Vitiani et al. 2007; Balla et al. 2009; Kusumbe et al. 2009).

It was shown that among the tumor, there is heterogeneous mixture of cell populations, thus it is not appropriate to look for gene expression in whole tumor samples, as it leads to high variability and misrepresentation of data from sample to sample. Hence, it will be informative to look the gene expression in the subpopulations of cells (Balla et al. 2009).

It was shown in human breast carcinoma that as few as 200 cells expressing $CD44^{+}CD24^{-/low}lin^{-}$ markers, when injected were capable of generating tumors in NOD/SCID mice, whereas the remaining population was not tumorigenic. Furthermore, in this $CD44^{+}CD24^{-/low}lin^{-}$ cell population, there was increased expression of Hh pathway components $PTCH_1$ (1.7 fold), Gli_1 (30 fold), Gli_2 (6 fold) and also poly comb gene Bmi-1 (5 fold) compared

with the cell population isolated from the same tumor which lacked these cancer stem cell markers (Liu et al. 2006).

Previous studies have reported an increased expression of Bmi-1 in other tumors like human non-small-cell lung cancer, human prostate cancer, human medulloblastomas, human breast cancers and established breast cancer cell lines (Vonlanthen et al. 2001; Dimri et al. 2002; Raaphorst et al. 2003) .

In prostrate cancers it was shown that patients with higher levels of Bmi-1 and Ezh_2 expression manifested clinically aggressive disease characters and is inclined to develop disease recurrence after radical prostatectomy (Berezovska et al. 2006).

Gene expression signature studies determined in the TRAMP transgenic mouse model of prostate cancer indicated the elevated levels of Bmi-1 mRNA expression was observed in late stage invasive primary tumors and multiple distant metastatic lesions. This increased Bmi-1 expression implies de regulation of self renewal pathways in these cells resulting in aggressive tumors (Glinsky et al. 2005).

Recent studies regarding gene expression signatures of the tumor cells in primitive state like Bmi-1 (poly comb genes), sonic hedgehog (SHH) might be useful in determining the outcome of the cancer therapy (Glinsky et al. 2005; Liu et al. 2006; Glinsky 2008).

4.7 Modules of gene expression:

In all tumors, de regulation of important proliferation and survival pathways are common while some pathways may be specific to certain tumors depending upon the origin of the tumors. As mentioned below there are very few studies trying to understand, which pathways are similar and which are specific. Addressing this issue may have important therapeutic implications. The genes which are general and specific category are arranged into modules as it was shown that modules related to neural processes are repressed in a subset of brain tumors, an intermediate filament module is induced in squamous cell lung carcinomas and reduced in lung adenocarcinomas (Segal et al. 2004).

Similarly, invasive hepatocellular carcinoma (HCC) is characterized by induction of cell cycle modules and repression of modules related to metabolism, detoxification, extra cellular matrix proteins and their signaling. Estrogen receptor positive breast tumors are characterized by regression of modules containing keratins and other intermediate filaments.

Steroid catabolism module is repressed in a subset of HCC and hepatic cell lines. The ESC like gene module is significantly activated in various human cancers when compared to the corresponding normal tissues. In contrast, adult tissue stem module is activated in various normal tissues compared to the tumor tissues. Thus, these findings implicate that various human cancers show an ESC like expression pattern (Segal et al. 2004).

It was shown that the primary breast tumors with the ESC activated signature were significantly associated with poorly differentiated tumor ($p<10^{-16}$) consistent with cancers expressing a stem cell like phenotype. In addition, those tumors with activated ESC signature were more likely to progress to metastasis and death ($p<10^{-4}$ and $p<10^{-6}$) (Wong et al. 2008).

ESC signature observed in 71 patients with stage I and II lung adenocarcinomas have shown that the tumors with ESC activated signature were associated with significantly higher risk of death compared to tumors with the ESC repressed signature ($p<0.03$). These results indicate that the degree of ESC module activation is associated with poor differentiation and increased risk of metastasis and mortality in multiple tumor types (Wong et al. 2008).

In adult epithelia the activation of c-Myc induces the reactivation of the ESC like module. It was shown in *in vitro* studies that c-Myc activated tumors demonstrated lost expression of differentiated epidermal markers including transglutaminase 1 and keratin 10, showing

that c-Myc is involved in inhibiting differentiation of the keratinocyte derived carcinoma (Wong et al. 2008).

The tumors with c-Myc activation retained keratins 5 and 14, which are expressed by the epidermal stem cells, demonstrating that they still express the undifferentiated markers of the keratinocyte lineage. c-Myc activated tumors showed the expression of keratin 8, which is a marker of simple epithelia suggesting that c-Myc induces de-differentiation and it was also shown that c-Myc is sufficient to induce increase in the cancer stem cell population (Wong et al. 2008).

4.8 Epigenetics:

Recent studies suggest that cancer pathogenesis is strongly related with aberrant losses and gains of DNA methylation and aberrant modifications of histones (Jones et al. 2002; Feinberg et al. 2004). As typical differentiation process is regulated by epigenetic modifications, it is possible that these modifications also contribute to the abberant differentiation present in tumors (Feinberg et al. 2006). Even though genetic studies have shown to be involved in neoplasia, these studies probably insufficient to explain the origin of the tumors. Report on glioblastoma showed that there are phenotypic and molecular similarities between normal stem cells and cancer stem like cells (Lee et al. 2006). Even though, they have similarities in the self-renewal properties of normal stem cells, they do not have control over their division. This suggests that both genetic and some non genetic events (epigenetic) are involved in proliferation (Feinberg et al. 2006).

Insight about epigenetic events associated with tumor stem cells will result in deep understanding of carcinogenesis in general and also aids in designing new therapeutic targets. Recent evidence showed that in advanced stage ovarian cancers DNA methylation and aberrant patterns of histone modifications was reported (Wei et al. 2002; Shi et al. 2003). Most of the chromatin modifications in cancer are related to the repression of gene expression and they silence tumor suppressor genes such as $RASSF_1A$, $p16INK_4$ and

$hMLH_1$ (Jones et al. 1999; Balch et al. 2005). Most of the epigenetic modifications occur early in cancer formation. It was also reported that epigenetic gate keeper genes such as SFRP (secreated frizzled receptor protein) was involved in Wnt signaling and aid in cell proliferation (Das et al. 2004; Baylin et al. 2006; Jones et al. 2007). Frequently recognized DNA methylations in adult tumors have shown the bivalent pattern of both transcriptionally activating (H3K4me3) and repressing (H3K27me3) chromatin modifications in embryonic stem cells were involved (Bernstein et al. 2006; Gan et al. 2007; Mikkelsen et al. 2007; Ohm et al. 2007). In general, promoter-associated CG rich regions are unmethylated (Gardiner-Garden et al. 1987), but in cancers it was shown that these islands were aberrantly methylated and these regions represent potential therapeutic targets (Jones et al. 2002).

5 Treatment implications: Understanding the mechanisms of resistance

Illustration of the CSC hypothesis in diverse solid tumors has provided new insights into new generation therapies for cancer. The main obstacle towards effective treatment remains the failure of current therapy in eradicating all tumor cells to prevent disease recurrence, thereby affecting long-term survival. Traditionally, drug therapies have been developed based on the ability of these agents to cause tumor regression in animal models. Since it has now been shown that a majority of the cancer cells within the tumor are non-tumorigenic, therapies directed against these cells would initially cause tumor regression, followed by recurrence as a consequence of persisting CSCs. Thus, the goal of cancer therapy should be to generate drugs that target CSCs. However, such therapeutic strategies against CSC fractions require elucidation of key mechanisms by which these cells resists existing therapies.

$hMLH_1$ (Jones et al. 1999; Balch et al. 2005). Most of the epigenetic modifications occur early in cancer formation. It was also reported that epigenetic gate keeper genes such as SFRP (secreated frizzled receptor protein) was involved in Wnt signaling and aid in cell proliferation (Das et al. 2004; Baylin et al. 2006; Jones et al. 2007). Frequently recognized DNA methylations in adult tumors have shown the bivalent pattern of both transcriptionally activating (H3K4me3) and repressing (H3K27me3) chromatin modifications in embryonic stem cells were involved (Bernstein et al. 2006; Gan et al. 2007; Mikkelsen et al. 2007; Ohm et al. 2007). In general, promoter-associated CG rich regions are unmethylated (Gardiner-Garden et al. 1987), but in cancers it was shown that these islands were aberrantly methylated and these regions represent potential therapeutic targets (Jones et al. 2002).

5 Treatment implications: Understanding the mechanisms of resistance

Illustration of the CSC hypothesis in diverse solid tumors has provided new insights into new generation therapies for cancer. The main obstacle towards effective treatment remains the failure of current therapy in eradicating all tumor cells to prevent disease recurrence, thereby affecting long-term survival. Traditionally, drug therapies have been developed based on the ability of these agents to cause tumor regression in animal models. Since it has now been shown that a majority of the cancer cells within the tumor are non-tumorigenic, therapies directed against these cells would initially cause tumor regression, followed by recurrence as a consequence of persisting CSCs. Thus, the goal of cancer therapy should be to generate drugs that target CSCs. However, such therapeutic strategies against CSC fractions require elucidation of key mechanisms by which these cells resists existing therapies.

$hMLH_1$ (Jones et al. 1999; Balch et al. 2005). Most of the epigenetic modifications occur early in cancer formation. It was also reported that epigenetic gate keeper genes such as SFRP (secreated frizzled receptor protein) was involved in Wnt signaling and aid in cell proliferation (Das et al. 2004; Baylin et al. 2006; Jones et al. 2007). Frequently recognized DNA methylations in adult tumors have shown the bivalent pattern of both transcriptionally activating (H3K4me3) and repressing (H3K27me3) chromatin modifications in embryonic stem cells were involved (Bernstein et al. 2006; Gan et al. 2007; Mikkelsen et al. 2007; Ohm et al. 2007). In general, promoter-associated CG rich regions are unmethylated (Gardiner-Garden et al. 1987), but in cancers it was shown that these islands were aberrantly methylated and these regions represent potential therapeutic targets (Jones et al. 2002).

5 Treatment implications: Understanding the mechanisms of resistance

Illustration of the CSC hypothesis in diverse solid tumors has provided new insights into new generation therapies for cancer. The main obstacle towards effective treatment remains the failure of current therapy in eradicating all tumor cells to prevent disease recurrence, thereby affecting long-term survival. Traditionally, drug therapies have been developed based on the ability of these agents to cause tumor regression in animal models. Since it has now been shown that a majority of the cancer cells within the tumor are non-tumorigenic, therapies directed against these cells would initially cause tumor regression, followed by recurrence as a consequence of persisting CSCs. Thus, the goal of cancer therapy should be to generate drugs that target CSCs. However, such therapeutic strategies against CSC fractions require elucidation of key mechanisms by which these cells resists existing therapies.

$hMLH_1$ (Jones et al. 1999; Balch et al. 2005). Most of the epigenetic modifications occur early in cancer formation. It was also reported that epigenetic gate keeper genes such as SFRP (secreated frizzled receptor protein) was involved in Wnt signaling and aid in cell proliferation (Das et al. 2004; Baylin et al. 2006; Jones et al. 2007). Frequently recognized DNA methylations in adult tumors have shown the bivalent pattern of both transcriptionally activating (H3K4me3) and repressing (H3K27me3) chromatin modifications in embryonic stem cells were involved (Bernstein et al. 2006; Gan et al. 2007; Mikkelsen et al. 2007; Ohm et al. 2007). In general, promoter-associated CG rich regions are unmethylated (Gardiner-Garden et al. 1987), but in cancers it was shown that these islands were aberrantly methylated and these regions represent potential therapeutic targets (Jones et al. 2002).

5 Treatment implications: Understanding the mechanisms of resistance

Illustration of the CSC hypothesis in diverse solid tumors has provided new insights into new generation therapies for cancer. The main obstacle towards effective treatment remains the failure of current therapy in eradicating all tumor cells to prevent disease recurrence, thereby affecting long-term survival. Traditionally, drug therapies have been developed based on the ability of these agents to cause tumor regression in animal models. Since it has now been shown that a majority of the cancer cells within the tumor are non-tumorigenic, therapies directed against these cells would initially cause tumor regression, followed by recurrence as a consequence of persisting CSCs. Thus, the goal of cancer therapy should be to generate drugs that target CSCs. However, such therapeutic strategies against CSC fractions require elucidation of key mechanisms by which these cells resists existing therapies.

5.1 Quiescence

In view of the fact that majority of cancer therapies are designed against rapidly proliferating cells, quiescent populations would automatically be shielded from therapeutic attack. Thereby quiescence, a defining trait for stem cells has been speculated extensively as mechanism of therapeutic resistance since the origin of CSC paradigm. However, experimental proof for the same was lacking until recently, when through long-term label retention potential, we demonstrated CSCs to remain quiescent within tumor niche. Long-term label retention that exploits relatively quiescent nature of stem cells is a classical approach for identification of tissue specific stem cells (Blanpain et al. 2004; Tumbar et al. 2004; Yue et al. 2005). Using the membrane labeling vital dyes PKH26/67, Kusumbe et al. identified CSCs in experimentally generated ovarian tumors as marked, *in vivo* residing label retaining cells (LRCs) within the tumor niche. Therapeutic refractoriness demonstrated by the long-term label retaining CSCs coupled with the potential to regenerate the tumors post therapeutic regression substantiated their role in tumor dormancy (Kusumbe et al. 2009). In corroboration with our results, a recent study has demonstrated the label retention potential of CSCs from breast cancer (Pece et al. 2010). These initial studies exploiting label retention for claiming CSC quiescence provide robust models for further investigations to identify mechanisms involved in maintenance of quiescent state.

5.2 Niche

Successful *insitu* identification of CSCs as LRCs prompted us to identify LRCs within the cell lines. However, in contrast to developing tumors, cell lines seemed to be devoid of LRCs (Kusumbe et al. 2009). Such failure to identify quiescent cells in culture suggests that analogous to the NSCs, maintenance of CSCs apart from intrinsic factors also substantially depends on external signals. These external signals collectively make up the

microenvironment or niche (classically defined as an interactive structural unit, organized to facilitate cell-fate decisions in a proper spatiotemporal manner) that has been implicated to play a crucial role in maintenance of the CSC compartment (Moore et al. 2006). Evidence for this notion came from a recent study that suggests involvement of vascular niches in maintenance of brain CSC compartment (Calabrese et al. 2007). Specifically, the study illustrates vascular niches within the brain tumors are aberrant and drives CSC self-renewal and proliferation that is in contrast to the niches found in the normal tissue that usually control the stem cell function. Microenvironment mediated drug resistance envisaged and studied since a long time is a form of *de novo* resistance that protects the tumor cells from the effects of therapies (Meads et al. 2009). Above preliminary investigations in field imply that even CSCs may depend on their microenvironment to resist the therapeutic regimes.

If indeed vascular niches are required for self-renewal and proliferation of CSCs as discussed above then development of such niches within tumor tissue would not only contribute tumor vasculature but also ensure long-term maintenance of CSCs in these protective niches thereby augmenting disease progression. Support for this notion comes from our finding that CSCs demonstrate an active recruitment of primitive endothelial stem cells that are capable of establishing an entire tumor endothelial hierarchy. Further the recruited endothelial stem cells also demonstrate an intimate physical association with CSCs; thereby claiming the instructive role of niche in CSC maintenance (Kusumbe et al. 2009). These recruited endothelial stem cells initiated and established the entire endothelial hierarchy thus contributing to tumor vasculature which augmented tumor growth and progression. These findings also support the previous studies proposing that the reciprocal interaction between the tumor cells and microenvironment controls the switch between proliferation and quiescence (White et al. 2004; Aguirre-Ghiso 2007).

5.3 Stress induced CSC enrichment

Above evidences suggest that oncogenic signaling might not always be dominant and that other programs (such as stem cell quiescence and microenvironment/niche restrictions) might overcome oncogenic signals, thereby governing CSC function and behavior. Application of treatment regime has been known to generate stress within the local tumor environment that leads to activation of stress pathways in CSCs enabling their persistence and accumulation (White et al. 2004). One such example is stress induced up-regulation of transcription factors Snail and Slug that not only aids cell survival but also leads to acquisition of chemoresistance and radioresistance (Kurrey et al. 2009). The fact that CSCs specifically exploit such pathways is affirmed by frequent documentation of their enrichment under various stress conditions including chemotherapy, radiotherapy, hypoxia, serum depletion etc. Further stress induced disease progression that mainly involves metastasis is also a frequent observation in cancer biology. To metastasize, cancer cells must detach from neighboring epithelial cells and adopt a mesenchymal phenotype i.e. cells must undergo epithelial to mesenchymal transition (EMT). Research in the field has revealed a number of pleiotropically acting transcription factors including Snail, Slug and Twist that play critical roles in EMTs not only during embryogenesis but also during tumorigenesis. Recent investigations illustrates that these transcription factors on upregulation under stress, besides orchestrating EMT and aiding therapeutic resistance also mediate acquisition of stem cell characteristics (Mani et al. 2008; Kurrey et al. 2009). Thus, orchestration of transcription machinery leading to cooperative modulation of gene expression seems to be an important mechanism for achieving therapeutic resistance through enrichment of CSC under stress.

5.4 Genetic instability

Cancer is classically recognized as a disease of clonal evolution. Aneuploid cells, due to their genetic instability, possess adaptive growth advantages and hence are thought to be

crucial determinants of caner recalcitrance (Sieber et al. 2003; Weaver et al. 2007). Consistent with this, we identified that apart from CSCs that contribute to tumor dormancy, aneuploid population too constitute major determinants of cancer dormancy. Aneuploid cells that are proliferation arrested / quiescent under steady state undergo selective pressure / stress acquired proliferation potency induced by chemotherapeutic exposure. These findings suggest that the existing pool of CSCs constantly generates a highly aneuploid progeny that stays proliferation arrested under the no / minimal stress conditions hence constituting just a dormant subset within the tumors. However on exposure to a stress condition or selective pressure (e.g. drug shock) these cells are recruited into cell cycle. Persistence of such genetically unstable dormant aneuploid cells packaged with remarkable adaptive and selective capacities has profound clinical implications for neoplastic progression and cancer therapy. Such stress induced acquisition of proliferation potency along with additional adaptive capacities inherent to these cells would lead to an emergence of a new CSC pool. The new CSC pool may dominate and take over the existing CSC pool or both of them may be retained concurrently thereby accounting for the CSC heterogeneity that has been documented (Visvader et al. 2008). This genetic instability leading to CSC evolution further explains the inefficacy in obtaining complete eradication of these cells during the treatment regimes.

6 Introduction to retinoblastoma (RB)

6.1 Introduction and epidemiology of RB

The distribution of retinoblastoma is worldwide, affecting all racial groups without sex predilection. Retinoblastoma is a rare malignant tumor with a prevalence of about one in 23000 live births. The incidence of retinoblastoma appears to be constant among the various populations of the world, suggesting that environmental influences play little role in its etiology

Knudson formulated his two hit hypothesis that the development of any retinoblastoma requires two complementary tumor inducing events to convert a normal retinal cell into a neoplastic cell. In cases of heritable retinoblastoma, the first mutation is in the germ cell and therefore every cell in the patient has the first hit. The second hit occurs in the somatic cell that will give rise to the retinoblastoma. In non-heritable retinoblastoma, both hits must occur in the somatic cell, which explains why patients with non-heritable retinoblastoma are usually older than those with the heritable type. An inherited autosomal dominant type constitutes 30 to 40% of cases, and non-heritable sporadic form, which constitutes the remaining 60 to 70% (Jones et al. 1999).

Patients with chromosomal deletion of retinoblastoma show a measurable defect in one of the long arms of chromosome 13, involving the 14 band. These children also have various somatic and mental developmental abnormalities, unlike children with other forms of retinoblastoma.

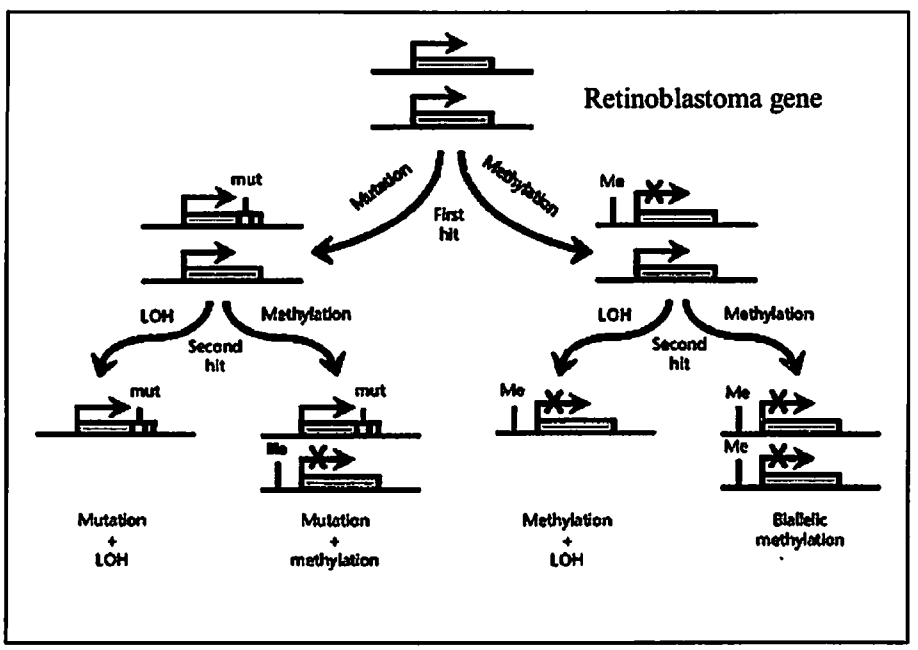


Figure 1.16: Knudson's two hit hypothesis (Jones et al. 1999).

It appears that a single locus exists for all the forms of retinoblastoma in the region 13q 14. The heritable retinoblastomas, including those associated with the chromosomal deletion and sporadic cases, show virtually identical histopathologic changes; however in the chromosomal deletion cases, the enucleated globes harboring the tumor may reveal proliferation of pars plana ciliaris epithelium. There are mutations reported in RB1 gene in Indian patients, almost all mutations produced nonsense codons or frame shifts. Recurrent mutations were predominantly present at CpG sites (Ata-ur-Rasheed et al. 2002; Kiran et al. 2003).

In 1809, Wardrop established retinoblastoma as an entity and advocated enucleation as treatment. Virchow considered the tumor to be a glioma of retina, whereas Flexner, in 1881, proposed the term neuroepithelioma of the retina based on the characteristic rosettes seen on histologic evaluation. Although many names have been proposed for the retinal tumor, Verhoeff suggested "retinoblastoma" in 1922, this name was adopted by the American ophthalmologic society and recently by the world health organization.

The rosettes described and illustrated by Flexner in 1881 and by Wintersteiner in 1897 were given their names, and the presence of such rosettes in a tumor was considered to be the highest degree of differentiation. In 1969 Tso et al. described further differentiated tumor cells that were cytologically benign appearing and showed clear photoreceptor differentiation. These cells were called "fleurettes," and were seen as a small component within a tumor otherwise showing typical histologic features of retinoblastoma. In 1983, Margo and coworkers described tumors that were entirely benign appearing with numerous fleurettes. Based on the histologic features and on follow up examinations conducted from 3 to 16 years after the diagnosis, they concluded that these tumors were a benign variant of retinoblastoma, and named them retinocytomas. In 1982, based on clinical observations, Gallie et.al, introduced the term retinoma for the benign

counterpart of retinoblastoma. It is possible that retinomas and retinocytomas are identical tumors. Although clinically and histologically retinocytoma and retinomas are distinct from retinoblastomas, the benign retinal tumor appears to carry the same genetic implications as retinoblastoma.

6.2 Genetics of RB

Retinoblastoma is a childhood retina related cancer that arises due to the inactivation of both alleles of RB gene, which results in the defective formation of pRB protein (Tumor suppressor) affecting cell cycle and leading to uncontrolled cell proliferation (*RB* Tumorigenesis) (William 1996). When these tumors are present in one eye, it is referred to as unilateral retinoblastoma, and when it occurs in both eyes it is referred to as bilateral retinoblastoma. Normally this tumor occurs in 1 out of every 15000 to 20000 live births (Ralph 1999). The tumor is caused in patients either with (familial form) or without family history of tumor (sporadic form). Knudsons proposed the hypothesis based upon the occurrence of retinoblastoma tumor, in the early 1970's and identification of retinoblastoma gene in 1987 confirmed Knudsons hypothesis (Kiran et al. 2003).

Knudson performed a statistical analysis on cases of retinoblastoma, which occurred both as bilateral and unilateral disease. The bilateral form is hereditary while the unilateral form is generally non-hereditary (Ata-ur-Rasheed et al. 2002). In the hereditary form, the predisposition to tumor formation is inherited from a parent who is a carrier of one mutant allele of the RB1 gene. The presence of one copy of the mutant gene through germ line transmission predisposes the child to lose second copy at a rate 1000 times more likely than a spontaneous mutation. This form is to be multifocal since one copy of mutant RB1 is present in all cells and mutation of the 2nd allele could occur in several retinal cells. Lack of RB1 in non-retinal cells can also predispose patients to second malignant neoplasm like osteo sarcomas. Unilateral retinoblastoma involves somatic mutation/loss of both copies of the RB1 gene in the developing retina and is generally unifocal (Ralph

1999). Nowadays this tumor is considered highly curable, with an overall 3-year survival rate of over 90%.

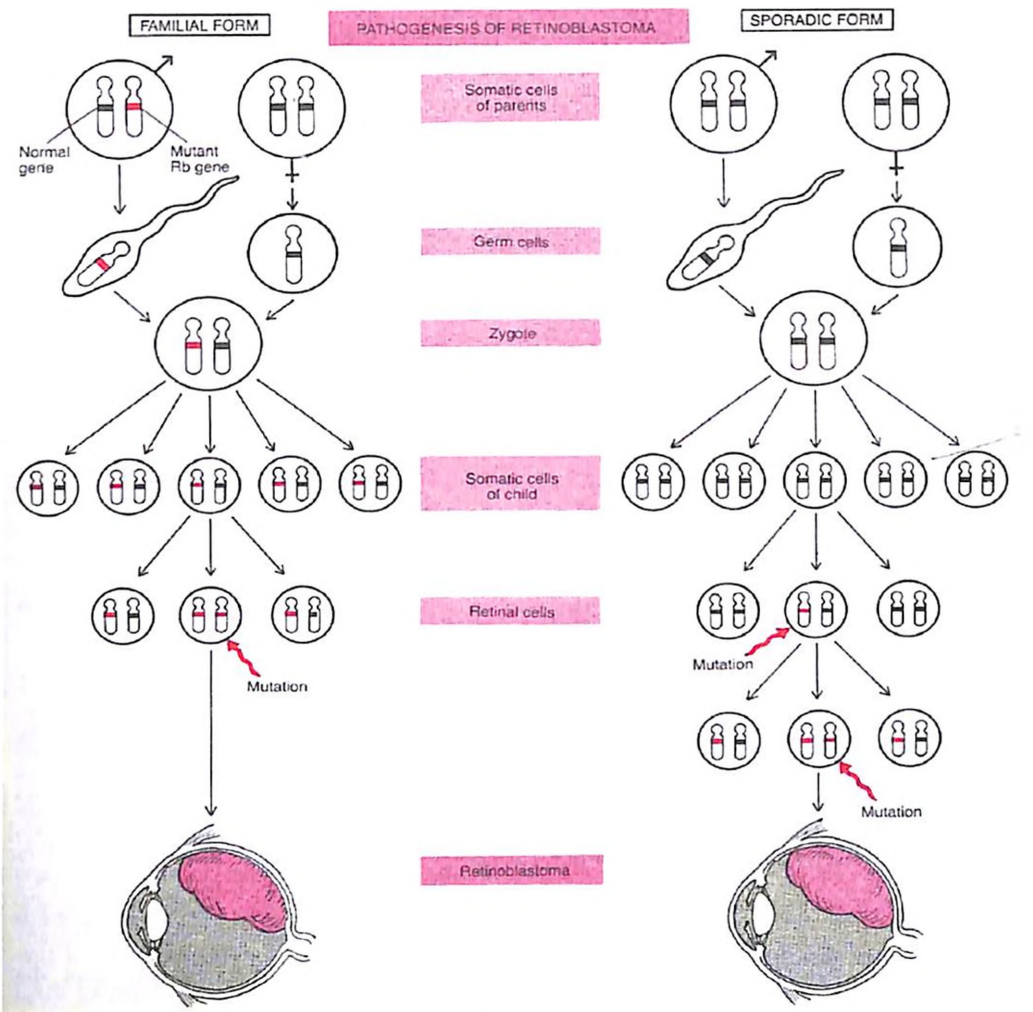


Figure 1.17: Pathogenesis of retinoblastoma – Two mutations at the Rb locus on chromosome 13q14 lead to neoplastic proliferation of the retinal cells. In the familial form, all somatic cells inherit one mutant Rb gene from a carrier parent. The second mutation affects the Rb locus in one of the retinal cells after birth. In the sporadic form, on the other hand, both mutations at the Rb locus are acquired by the retinal cells after birth (Cotran 1994).

However untreated, Rb is invariably fatal. Prior to the 1990's, the standard treatment approach to unilateral Rb was enucleation and in the bilateral cases the treatment typically involved enucleation of the worst eye and external beam radiation of the other eye (Ralph 1999). Chemotherapy is normally used for high risk (Choroidal invasion, extra ocular extension) and metastatic Rb.

Primary chemo reduction is used for intra ocular retinoblastoma and systemic chemotherapy is used following enucleation in patients with optic nerve, deep choroidal invasion, orbital extension and metastatic disease (Ralph 1999). It was observed in most cases that there was initially good response for chemotherapy, but eventually, it recurs and the possible reason for the recurrence or chemo resistance was hypothesized as the expression of P-glycoprotein protein or cancer stem cells expressing MDR phenotype (Dean et al. 2005). In contrast to the above report it was shown that retinoblastoma tumor express P-glycoprotein and lung resistance protein, but interestingly it was shown that none of the above proteins predicted the response to chemotherapy (Krishnakumar et al. 2004).

6.3 Animal models of RB:

Development of a mouse model for retinoblastoma posed a great challenge over a period since the discovery of the gene in 1986. Rb null mice die embryonically at E13.5 and Rb heterozygous mice do not develop retinoblastoma (Clarke et al. 1992). Later mouse models of retinoblastoma were developed with the expression of viral oncoproteins, SV40 large T antigen (Balch et al. 2005), human papilloma virus E7 and adenovirus E1A that bind and inactivate RB. These proteins not only bind RB but also RB family proteins- p107, p130 in addition to several other cellular proteins. Therefore, the transformation observed in these mouse models is not just due to Rb inactivation but also because of other proteins and may not reflect events in human Rb (Jones et al. 1997; Duensing et al. 2003). When vector carrying SV40 large T antigen (T_{ag}) was used to induce retinoblastoma in

mice, the vector was integrated into a site that induced retinal expression of T_{ag} and the mice developed retinoblastoma-like tumors. The LHß-T_{ag} induced tumors when analyzed by immunohistochemistry, had characteristics of amacrine/horizontal cells and occasionally Muller glial cells (Mills et al. 1999). Expression of T_{ag} and E_7 transgene under inter-photoreceptor retinoid-binding protein (IRBP) promoter induced tumorogenesis and apoptosis (al-Ubaidi et al. 1992; Howes et al. 1994). Both Tag and E7 bind RB family proteins but Tag also targets p53. Expression of E7 in a p53-null background was accompanied by tumor formation (Howes et al. 1994). So it appears that when p53 pathway is intact, apoptosis is the natural response of photoreceptor transition cells while RB family proteins and viral protein targets are inactivated. In contrast, the p53 pathway is intact in human retinoblastoma (Elison et al. 2006) and models of retinoblastoma using viral oncoproteins that targets RB family proteins along with p53 are not the best models of human retinoblastoma to study the cell of origin.

The tumor cells of these chimeric models expressed markers of amacrine and horizontal neurons (Robanus-Maandag et al. 1998). They also contained some Muller glia. The earliest defect in RB/p107 deficient retinal cells was not examined in these chimeric models and so it was not possible to detect whether it was amacrine transition cell or a progenitor cell of amacrine fate. Development of chimeric models is difficult and they also had a major disadvantage of not being heritable. Blastocyst injections are required each time to generate chimeric models. The knockout ES cells that were used for injection into the blastocysts had no marker gene and it was impossible to examine the effect of Rb or Rb/p107 loss on different retinal cell types.

6.4 Debate on cell of origin:

In the early days, Flexner and Wintersteiner hypothesized that photoreceptors might give rise to retinal tumors. This assumption was based on the morphology of rosettes observed in tumor samples (Cotran 1994). Early diagnosis of this tumor and availability of human

samples is very rare, investigators have concentrated in creating animal models to study this disease. In the 1960's, intraocular injection of adenovirus 12 produced retinal tumors in young rats, mice and baboons. These studies revealed that viral oncoproteins blocked Rb and its family members p107, p130 and many other proteins (Ogawa et al. 1966; Ogawa et al. 1969; Mukai et al. 1973). Later heritable retinal tumor mouse models were generated (LH-beta TAG mice) by expression of a viral oncogene simian virus 40T-antigen. The tumors that were resulted by this method were comparable to human tumors in histological, ultra structural and immunohistochemical characteristics (Windle et al. 1990).

Development of conditional knockout mouse (Feinberg et al. 2004; 2006) models gave more insight into the retinoblastoma cell-of-origin. The conditional knock-out models that were developed to mimic human retinoblastoma used Cre recombinase in the developing retina to excise Rb exons flanked by lox P sites. When Cre is cloned under a specific promoter, tissue-specific expression can be obtained depending on the promoter and thus the loss of Rb_1 could be achieved conditionally in the specific cell types of retina and not in all other tissues. Pax_6 is expressed only at E9.5 in the peripheral retina, and when the Cre expression is under the enhancer of Pax_6 gene, floxed alleles of Rb are deleted at E9.5 prior to differentiation so that all RPC, transition cells and differentiating cells lack the target gene. When Rb was inactivated at E9.5 using a Pax_6 transgene in the peripheral retinal progenitors in a $p107^{-/-}$ background, 68% of animals developed retinoblastoma. This finding provided evidence for p107 as a tumor suppressor in Rb^{-} deficient mouse retina (Chen et al. 2004). Double mutants lacking p107 or p130 and Rb mosaics were generated; no double mutants of $p107^{-/-}$ and Rb mosaics survived. They died prior to weaning age. In contrast, $p130^{-/-}$ plus Rb mosaics were viable and they developed retinoblastomas similar to humans in histology (MacPherson et al. 2004). Chx_{10}-Cre

transgene inactivates Rb in a mosaic fashion in retinal progenitors starting at E11 and mice developed tumors in $p107^{-/-}$ background (Zhang et al. 2004).

The inner nuclear layer consists of horizontal, amacrine and bipolar cells and all the conditional knockout models suggested that it is the transition cells that form the inner nuclear layer that could be the cell of origin in retinoblastoma and in particular amacrine cells. When Rb was inactivated in $p130^{-/-};p107^{+/-}$ background there was an increased proliferation of a cell type characteristic of horizontal cells and located at the outer edge of the inner nuclear layer. These cells expressed the markers of horizontal cells (Lim_1, calbindin and PROX1) and also formed synaptic processes and neurites that are characteristic of horizontal cells by P30. By 8-14 weeks of age these mice developed aggressive metastatic tumors in which proliferating cells showed the characteristics of horizontal interneurons (Ajioka et al. 2007). This study argued for the horizontal cell as the cell of origin in retinoblastoma.

A very recent study in the field of cell of origin in retinoblastoma was elegantly designed to distinguish between normal cells and tumor cell that lack both the alleles of *RB1* in human tumors. The normal cells express RB and can be distinguished from tumor cells. Using in situ hybridization, tumor cells with homozygous *RB1* deletions were selected. Co-labeling with cell specific markers showed positivity for markers of cone photoreceptors. Markers include retinoic acid-activated nuclear receptor RXR-gamma, homeodomain protein CRX, cone-specific thyroid hormone-activated nuclear receptor, THRß2, and two cone opsins (M/L). (Xu et al. 2009). This study suggests cone photoreceptors as the possible cell-of-origin. If this were true, since cones are dense in the foveal region it would be expected that more tumors should be at the fovea. When tumors were observed in newborn children with initial stages of Rb they first appear at the posterior pole (in 85% of the cases) (Abramson et al. 2002) and not at the fovea (Xu et al.

transgene inactivates Rb in a mosaic fashion in retinal progenitors starting at E11 and mice developed tumors in $p107^{-/-}$ background (Zhang et al. 2004).

The inner nuclear layer consists of horizontal, amacrine and bipolar cells and all the conditional knockout models suggested that it is the transition cells that form the inner nuclear layer that could be the cell of origin in retinoblastoma and in particular amacrine cells. When Rb was inactivated in $p130^{-/-};p107^{+/-}$ background there was an increased proliferation of a cell type characteristic of horizontal cells and located at the outer edge of the inner nuclear layer. These cells expressed the markers of horizontal cells (Lim_1, calbindin and PROX1) and also formed synaptic processes and neurites that are characteristic of horizontal cells by P30. By 8-14 weeks of age these mice developed aggressive metastatic tumors in which proliferating cells showed the characteristics of horizontal interneurons (Ajioka et al. 2007). This study argued for the horizontal cell as the cell of origin in retinoblastoma.

A very recent study in the field of cell of origin in retinoblastoma was elegantly designed to distinguish between normal cells and tumor cell that lack both the alleles of *RB1* in human tumors. The normal cells express RB and can be distinguished from tumor cells. Using in situ hybridization, tumor cells with homozygous *RB1* deletions were selected. Co-labeling with cell specific markers showed positivity for markers of cone photoreceptors. Markers include retinoic acid-activated nuclear receptor RXR-gamma, homeodomain protein CRX, cone-specific thyroid hormone-activated nuclear receptor, THRß2, and two cone opsins (M/L). (Xu et al. 2009). This study suggests cone photoreceptors as the possible cell-of-origin. If this were true, since cones are dense in the foveal region it would be expected that more tumors should be at the fovea. When tumors were observed in newborn children with initial stages of Rb they first appear at the posterior pole (in 85% of the cases) (Abramson et al. 2002) and not at the fovea (Xu et al.

2009). Until the response of retinal cells to the early loss of RB1 are clearly understood, the retinoblastoma cell-of-origin remains debatable.

It was also reported that p53 pathway was inactivated in retinoblastoma because of over expression of MDMX gene in this tumor and if this MDMX gene is targeted using nutlin-b and it resulted in the death of tumor cells (Laurie et al. 2006).

In addition to the mouse models of retinoblastoma, there are two very well characterized human retinoblastoma cell lines (Y79 and WERI-RB27) obtained from the patients diagnosed for retinoblastoma (Reid et al. 1974; Sery et al. 1990). These cell lines provide close models to the human cases for experimentation. Main difference between human and mouse models of Rb is that mouse Rb requires inactivation of RB and its family members but it is not the case for humans where RB inactivation is sufficient to produce tumors (Pacal et al. 2006).

6.5 Morphological studies and histopathological risk factors

Gross and Macroscopic examination

The gross features of intraocular retinoblastoma depend on the growth pattern of the tumor. Five growth patterns are recognized in retinoblastomas, which explain certain clinical variations as well as differences in intraocular and extraocular spread.

1. Endophytic retinoblastoma grows from the inner surface of the retina into the vitreous. Thus, on opthalmoscopic examination, the tumor is viewed directly. Retinal vessels are typically lost from view as they enter the tumor. As endophytic tumors grow large and become friable, tumor cells tend to be shed from the tumor into the vitreous, there they grow into separate tiny spheroidal masses.

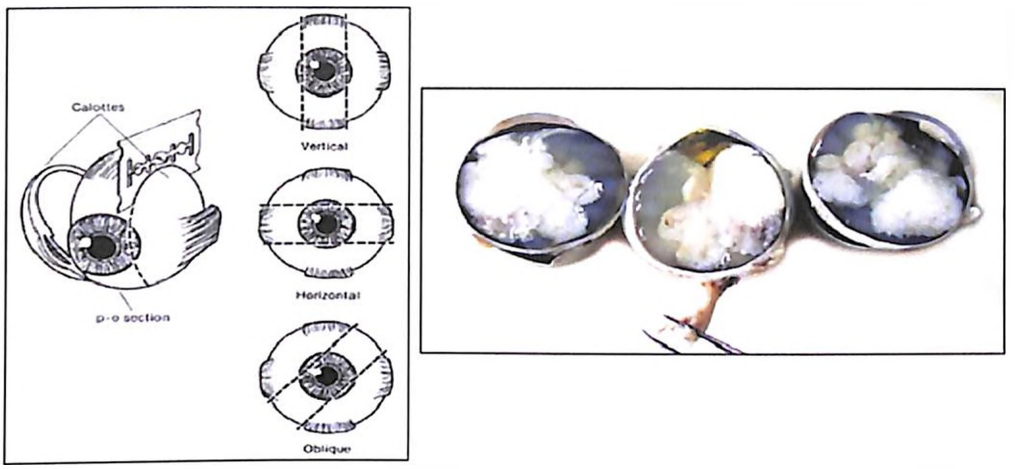


Figure 1.18: Showing the grossing of the retinoblastoma eye, normally done in Ocular pathology laboratory of LVPEI

That appears as fluff balls or cotton balls. The spheroidal masses of tumor can mimic inflammatory conditions such as, mycotic or nematoidal endophthalmitis, tumor cells in the vitreous will seed onto the inner surface of the retina, where they may invade into the retina, making it very difficult to distinguish between seeding and multicentricity histologically. Multicentric retinoblastoma should be distinguished from retinal seeding because the presence of multiple tumors indicates a germinal mutation, this distinction is impossible once extensive vitreous seeding has occurred. If one can discern that the tumor lies mainly on the inner surface of the retina rather than within it, or if one can also see tumor cell cluster within the vitreous, such observations would suggest that retina seeding has occurred.

Tumor cells in the vitreous may also spread into the posterior chamber and then into the anterior chamber by aqueous flow. Secondary deposits on the lens, zonular fibers, cilliary epithelium, iris, corneal endothelium and trabecular meshwork may be observed, and tumor cells may follow the aqueous outflow pathways out of the eye. In such cases, the anterior segment changes may be misinterpreted clinically as those of granulomatous iridocyclitis.

2. Exophytic retinoblastomas grow from the outer retinal surface toward the choroid, producing first an elevation, and then a detachment, of the retina. On opthalmoscopic examination, the tumor is viewed through the retina, and the retinal vessels course over the tumor. As the tumor grows larger, it may give rise to total retinal detachment, and tumor cells may escape into the sub retinal exudate. Secondary implants may then develop on the outer retinal surface, where they can invade into the retina or onto the inner surface of the RPE. The implants may then replace the pigment epithelium and eventually infiltrate through Bruch's membrane into the choroid. From the choroid, tumor cells may escape along cilliary vessels and nerves into the orbit and conjunctiva. From the orbit and conjunctiva, they can gain access to blood vessels and lymphatics and metastasize.

3. Mixed endophytic – Exophytic tumors are probably commoner than either purely endophytic or Exophytic retinoblastomas, especially among the larger tumors. The combined features of both endophytic and Exophytic growth characterize these tumors.

4. Diffuse infiltrating retinoblastomas are the least common and often are the most difficult to clinically diagnose. These tumors grow diffusely within the retina without greatly thickening it. Tumor cells are discharged into the vitreous, often with seeding of the anterior chamber, thereby producing a pseudohypopyon. Because of the absence of a mass, this type of retinoblastoma masquerades as retinitis, vitritis, or toxocara endophthalmitis. With anterior chamber involvement, hyper acute iritis with hypopyon, juvenile xanthogranuloma, or tuberculosis may be suspected.

Complete spontaneous regression is believed to occur more frequently in retinoblastoma than in any other malignant neoplasm. Typically, a severe inflammatory reaction occurs, followed phthisis bulbi. The mechanisms by which regression occurs are unknown. In several cases from the registry of ophthalmic pathology, bilateral retinoblastomas have been observed to have undergone total necrosis and phthisis bulbi on one side, while on

the other, a viable tumor massively filled the eye and invaded the orbit. Such cases would seem to exclude the possibility of a systemic mechanism for tumor necrosis. Occlusion of the central retinal artery has been observed in eyes with necrotic retinoblastomas, but whether this phenomenon occurs before or after the tumor becomes necrotic cannot be established (William 1996).

6.5.1 External examination

External examination of the globe will usually reveal a white retrolental tumor through a transparent cornea, but it would be uncommon for transillumination to allow precise localization of a tumor. The choice of plane of section is often empirical, but when a large tumor is present, several blocks should be taken from the calottes for histological examination. It is uncommon to find transcleral tumor spread in a retinoblastoma; if present, irradiation of the orbit would be required (Lee 1992).

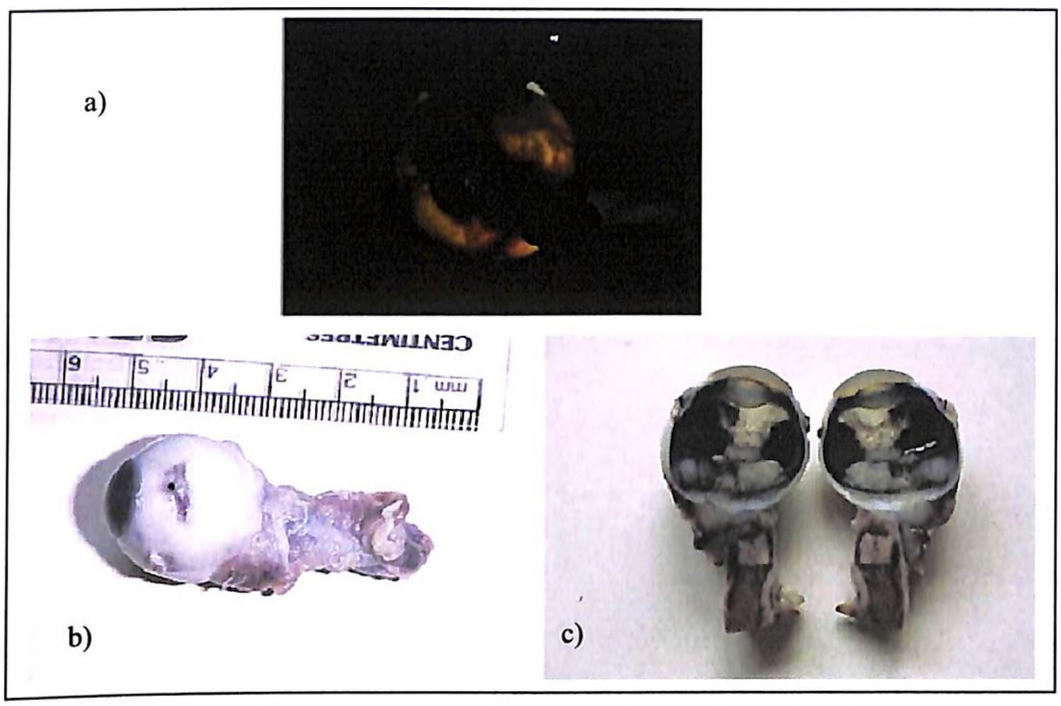


Figure 1.19: a) Transillumination of the retinoblastoma eye performed at LVPEI b) Enucleated Eye ball c) Grossed section of eyeball.

6.5.2 Tumor appearances

The macroscopic features of the cut surface of a retinoblastoma vary according to the size of the tumor and depend on the fact that there is limited blood supply. It is sometimes surprising to see how large a bulk of tumor tissue can be maintained by the central retinal artery. Endophytic tumors (i.e. those growing into the vitreous from a retina in situ) are pinkish white, smooth surfaced and homogeneous. Larger tumors exhibit darker grey areas of necrosis, brown foci of hemorrhage and firm white granules or flecks of dystrophic calcification. Intra retinal spread may be extensive and disintegration of the poorly cohesive tumor leads to deposits or seedlings in the vitreous.

Further growth of the tumor fills the interior of the globe and necrosis becomes more extensive.

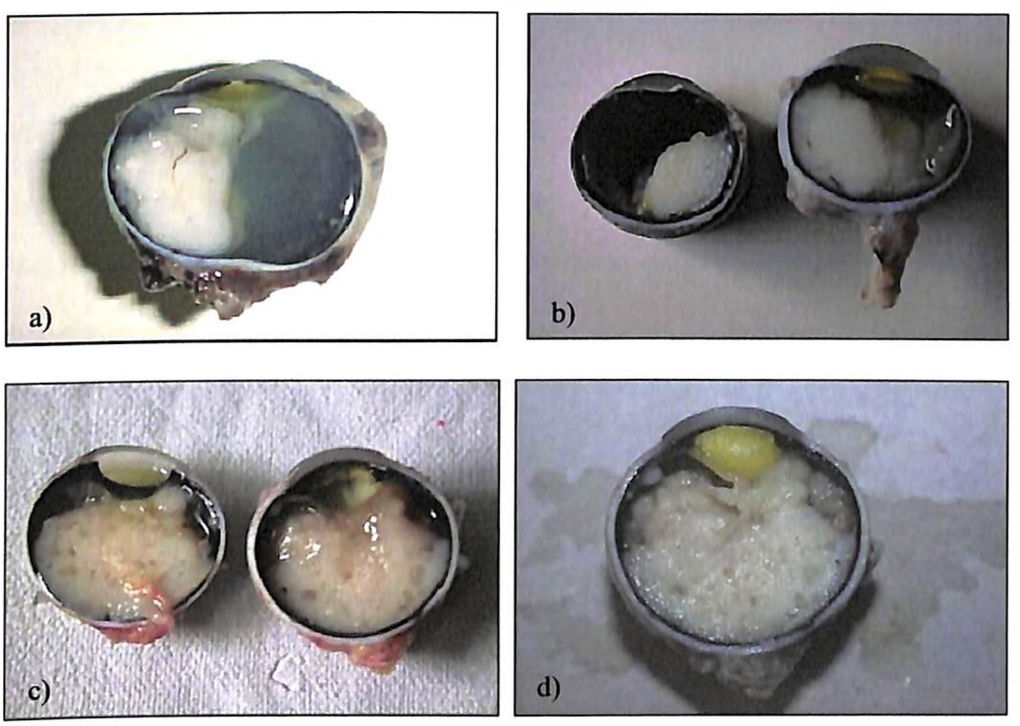


Figure 1.20: (a,b) Tumor appearance in the eyeball at LVPEI. (c,d) Microwave fixation of the RB tumor at LVPEI.

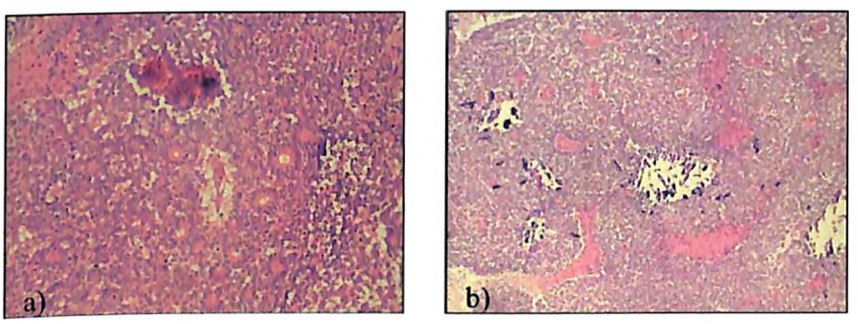
In Exophytic retinoblastomas, the tumor proliferates in the sub retinal space rather than the vitreous and a secondary exudate contributes to the detachment. The subretinal space also contains tumor seedlings. A layer of viable tumor tissue is frequently present on the inner surface of the choroid from which nutrition is derived. Macroscopic identification of choroidal invasion should be sought in multiple blocks.

Advanced tumor proliferation is associated with extensive necrosis leaving a honeycomb pattern of surviving cells around blood vessels. Spontaneous necrosis of the major part of the tumor produces milky white mass, which resembles a purulent exudate. In the rare, diffuse form of retinoblastoma, which carries a poor prognosis, there is widespread nodular thickening of the retina. Extra ocular spread will only be seen when medical aid has not been sought by the parents or is not available (Lee 1992).

6.6 Microscopic features:

6.6.1 Poorly differentiated tumors:

In the majority of specimens, the tumor is formed by uniform small round cells with a high mitotic rate and a tendency to individual cell necrosis. The nucleus is round or oval and the nuclear chromatin is finely granular. Nucleoli are inconspicuous and the cytoplasm and cell membranes are poorly defined.



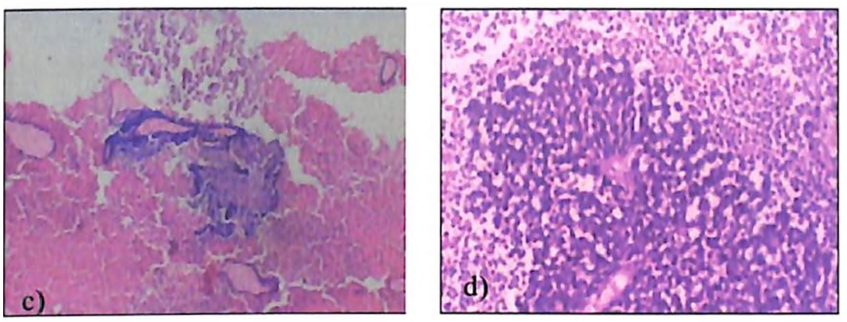


Figure 1.21: a) Retinoblastoma with perithelial arrangement, rosettes and necrotic areas b) Calcification is recognized as basophilic fragmented granules c) In necrotic retinoblastomas the dissolution of nuclei leads to the precipitation of DNA and accumulation of DNA lakes d) Perithelial arrangement of tumor around the blood vessel and above which is the necrotic cells present (All images are 100x magnified)

The tumor cells stain positively with anti S100, anti NSE and anti GFAP (glial fibrillary acidic protein) (Molnar et al. 1984). The dependence on blood supply for survival is manifest as rings (200-300 μm diameter) of viable cells around widely spaced blood vessels.

A large part of the tumor is often occupied by necrotic eosinophilic amorphous material. If the tissue block is not exposed to decalcifying fluids prior to processing, the necrotic tissue is in part, basophilic and granular due to dystrophic calcification (demonstrable by von kossa and alzarin red stains). On microtomy of this material, the calcified foci become fragmented and notch the microtome blade with subsequent scratching and tearing of the sections. Widespread necrosis of the tumor cells also leads to precipitation of DNA in the walls of blood vessels and as pools in the tissue spaces. DNA is also basophilic and stains positively with the feulgen stain: confirmation is obtained by pretreatment with DNA-ase. Multinucleate cells are a rarity in retinoblastomas but have been documented.

6.6.2 Differentiation within retinoblastoma

The cell of origin in retinoblastoma is a totipotent germinal retinoblast. Evidence has accumulated to show that in some tumors there is neuroglial differentiation and that in

many tumors groups of cells can adopt some of the morphological characteristics of photoreceptor cells. The specific entities are:

1. "Fleurettes": circular or oval groups of cells in which cytoplasmic processes (resembling a fleur-de-lys) have the ultra structural characteristics of inner segments of photoreceptors. These structures are most commonly found in irradiated tumors.

2. Flexner – Wintersteiner rosettes: a circle of cells is limited internally by a continuous membrane and cells are united by adherent junctions. The lumen of the rosette contains acid mucopolysaccharides.

3. Homer – Wright rosettes: A multilayered circle of nuclei surrounds eosinophilic fibrillary material.

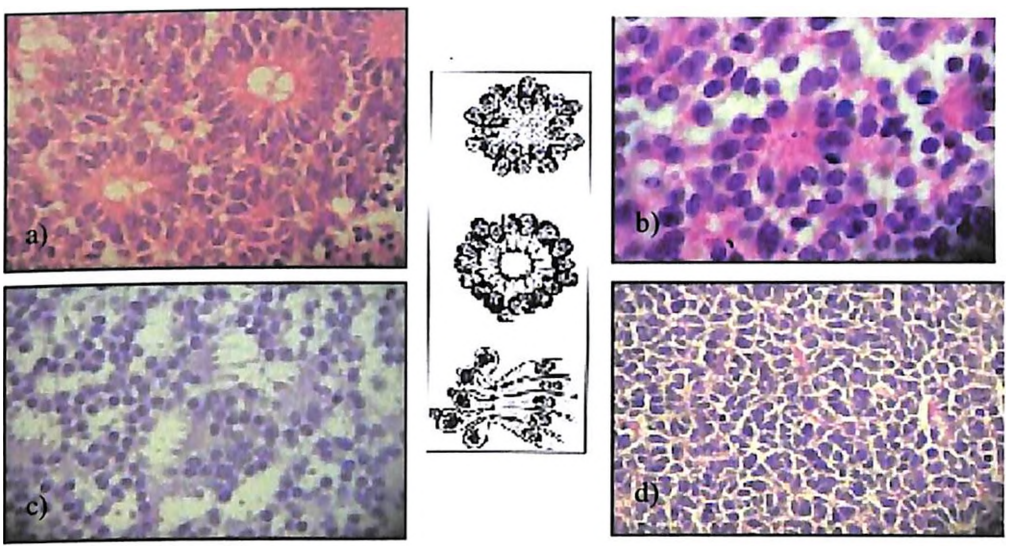


Figure 1.22: Various types of differentiation pictures of retinoblastoma. Flexner and Wintersteiner rosettes and fleurettes (All images are 200x magnified)

Glial cell differentiation is rare but has been of interest in recent literature. The response to ischaemia and the release of vasoformative factors is also of interest and occasionally endothelial budding is present within the tumor blood vessels.

6.6.3 Extra ocular spread and metastasis:

Most retinoblastomas exhibit relentlessly progressive, rapidly invasive growth. If left untreated, they usually fill the eye and completely destroy the internal architecture of the globe. The commonest method of spread is by invasion through the optic disk into the optic nerve.

Optic nerve involvement was classified according to the degree of invasion (Magramm et al. 1989):

a) **Grade I** is superficial invasion of the optic nerve head only

b) **Grade II** is involvement up to and including the lamina cribrosa

c) **Grade III** is involvement beyond the lamina cribrosa, and

d) **Grade IV** is involvement up to and including the surgical margin

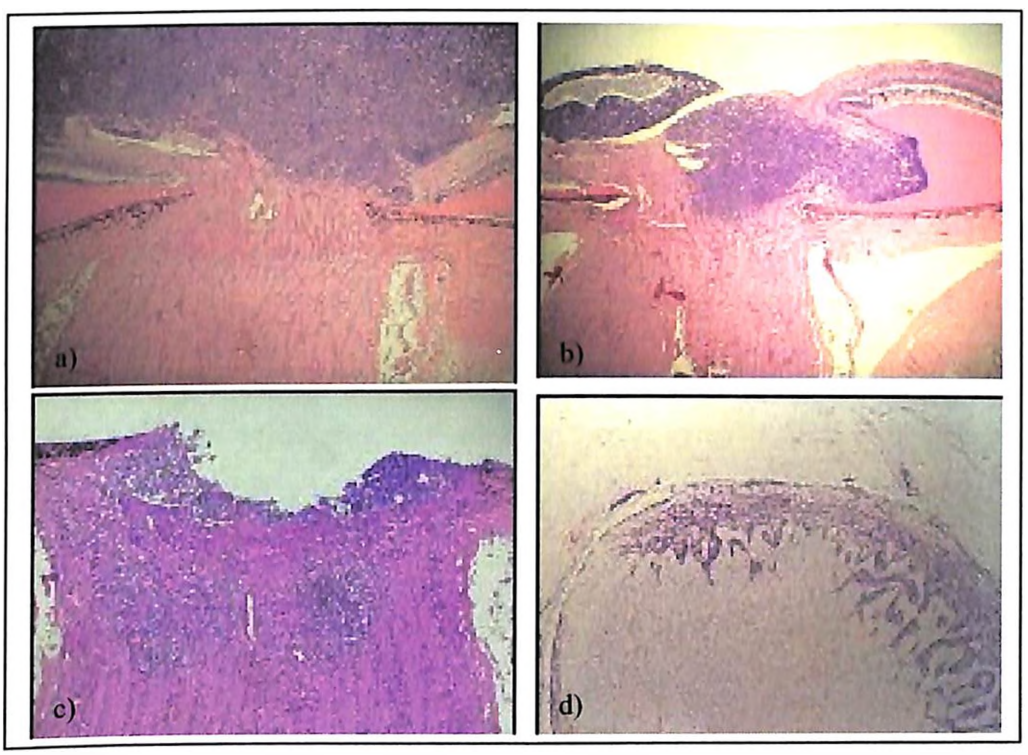


Figure 1.23: Various stages of optic nerve involvement (All images are 40x magnified)

Once into the optic nerve, the tumor may spread directly along the nerve fiber bundles back toward the optic chiasm, or it may infiltrate through the pia into the sub arachnoid space. From the subarachnoid space, tumor cells may be carried via the circulating cerebrospinal fluid to the brain and spinal cord. The choroidal invasion was morphologically divided into four stages:

Stage 1: only retinal pigment epithelium is involved, the Bruch's membrane was intact (1.24 a).

Stage 2: the Bruch's membrane was destroyed and the choroidal capillaries was not infiltrated (1.24 b).

Stage 3: choroial capillary and middle blood vessel layer in small limits were infiltrated.

Stage 4: invasion involved in choroid in great limits and involvement of sclera existed simultaneously (Yang et al. 1999) (1.24 c).

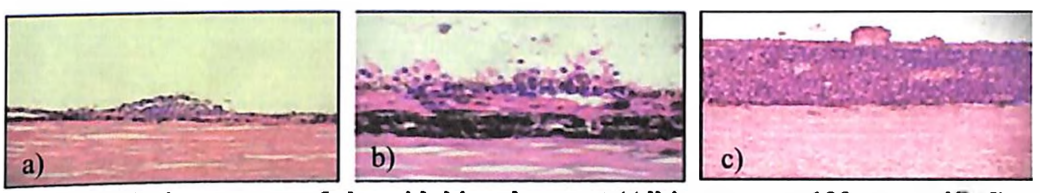


Fig 1.24: Various stages of choroidal involvement (All images are 100x magnified)

Once the tumor has invaded the choroid, it may then spread into the orbit via the scleral canals or by massively replacing the sclera. Extraocular invasion dramatically increases the chances of hematogenous dissemination and permits access conjuctival lymphatics and metastasis to regional lymphnodes.

6.6.4 Retinocytoma:

It is a rare, form of retinal tumor and like retinoblastoma it arises from mutation in RB1 gene. The term retinocytoma was first given by Margo to the group of tumors lacking any malignant features (Margo et al. 1983). Margo described 6 retinal tumors that were treated primarily with enucleation but these tumors showed none of the histologic features or

intraocular growth characteristics of malignant neoplasms. In all of these tumors, no immature neuroblastic elements were found. Similarly, Gallie et.al. have shown 34 retinal tumors exhibiting no clinical malignant features. Most of these patients had either family history or atypical retinoblastoma in the contra lateral eye (Gallie et al. 1982). Retinocytomas rarely undergo malignant transformation after a period of dormancy, although malignant transformation has been reported (Eagle et al. 1989; Singh et al. 2000).

Microscopic effects of Irradiation:

Successful destruction of the tumor is seen as residual fragments of amorphous calcified debris in a loose glial supporting framework. Successful treatment followed by recurrence will be manifest as typical well-differentiated retinoblastoma with fleurettes and Flexner – Wintersteiner rosettes adjacent to loosely spaced glial tissue. In quiescent static tumors, evenly spaced uniform cells with clear cytoplasm replace the retinal tissue.

6.7 Hazards in the histological assessment of retinoblastoma

Some difficulties are encountered in the histological assessment of retinoblastoma:

1. When the tumor cells penetrate the choroid, physical compression causes them to resemble lymphocytes. Subsequent chemotherapy may depend on accurate identification of choroidal invasion.

2. When the tumor destroys axons in the optic nerve, reactionary proliferation of astrocytes may give a misleading impression as to the extent of tumor cell invasion.

3. Necrosis and karyolysis of tumor nuclear protein in the anterior chamber can, in an aqueous sample, mimic bacterial events in giemsa and gram stained smears (Lee 1992).

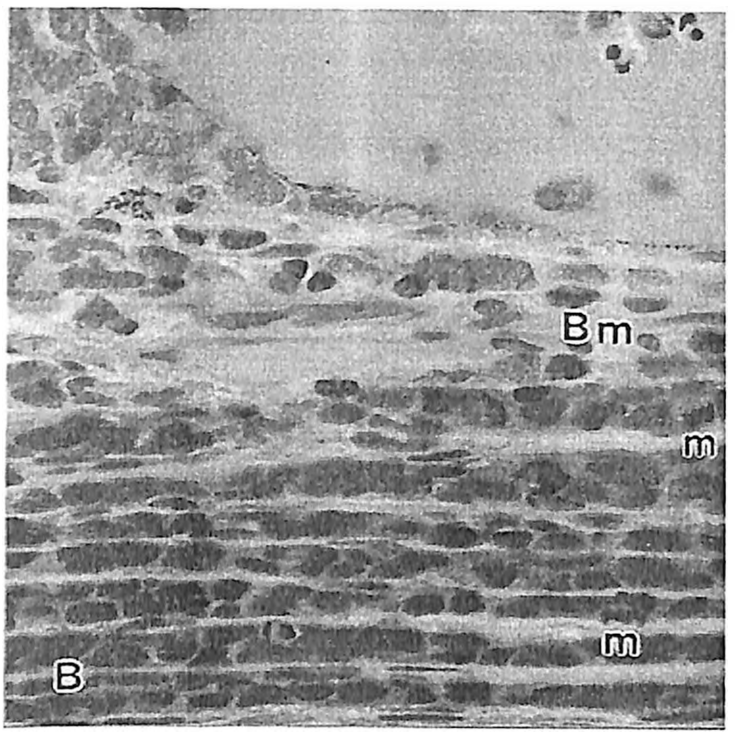


Figure 1.25: Undifferentiated tumor cells have invaded the choroid and parts of the choriocapillaries under bruch's membrane (Bm), but deeper in the choroid compression leads to a resemblance to lymphocytes. Note the mitotic figures (m). (430x)

6.8 Prognostic indications

The prognostic criteria in terms of metastatic risk, used in the past were tumor size, degree of differentiation, choroidal invasion, optic nerve invasion etc,. are even more relevant now and delayed enucleation (after initial diagnosis) carry correspondingly greater mortality. The significance of small foci of choroidal invasion in the risk of metastatic spread remains uncertain; large and obvious choroidal tumor masses will indicate the need for systemic chemotherapy in some centers.

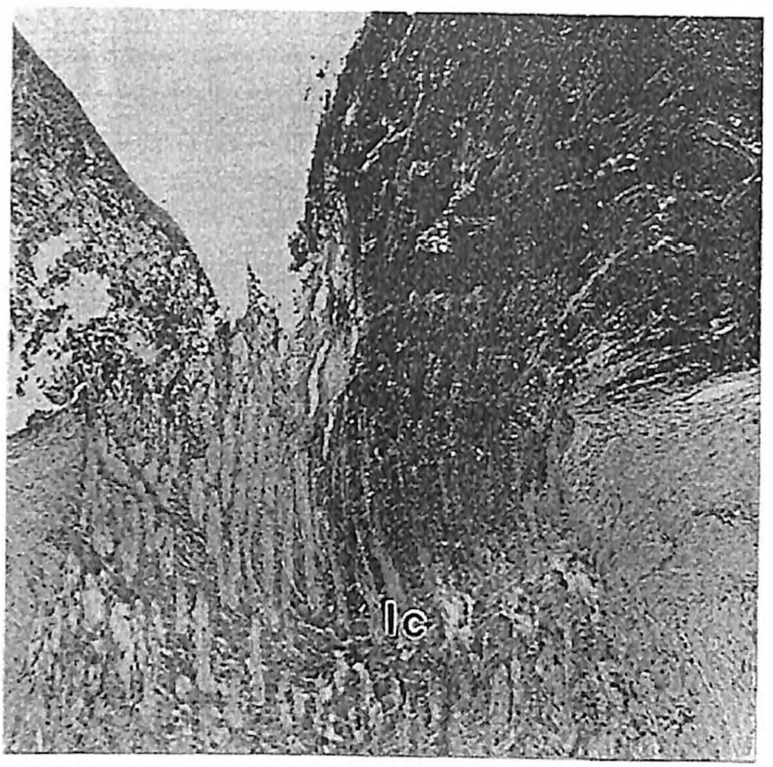


Figure 1.26: Tumor cell infiltration into the lamina cribrosa (lc) of the optic nerve has a significant effect on prognosis. [5]

6.9 Treatment of RB:

The management of retinoblastoma is complex, and the best treatment for each patient must be determined on an individual basis. The method of treatment should depend on the size and extent of the tumor. The age of the patient is also an important consideration because younger patients are likelier to have a germinal mutation and develop additional retinoblastomas.

Six types of standard treatment are used and is mentioned in table 1.5:

Table 1.5: Standard treatment procedures for retinoblastoma

SNo.	Type of treatment	Procedure details
1	Enucleation	Surgery to remove the eye and part of the optic nerve. It is commonly done when the tumor is large and no chance that vision can be saved.
2	Radiation therapy	Uses high energy X-Rays, depends upon the type and stage of cancer to be treated.
3	Cryotherapy	Uses an instrument that freezes and destroys abnormal tissue such as carcinoma in situ. Used when there is small primary or residual tumor after radiotherapy
4	Photocoagulation	Uses laser light to destroy blood vessels causing the tumor cells to die. This can be used to treat small tumors
5	Thermotherapy	Uses heat to destroy cancer cells. This can be given by using laser beam aimed through the dilated pupil or onto the outside of the eyeball or by ultrasound, microwaves or infrared radiation.
6	Chemotherapy	Uses drugs to stop the growth of cancer cells either by killing the cells or by stopping the cells from dividing.

6.10 Chemo resistance in RB

The clinical profile of patients seen at LVPEI is different from the previous studies available, representing more advanced disease than the series published from the West. At presentation, proptosis was seen in 53% of the eyes, and optic nerve extension or extra ocular extension in 41% of the eyes. In our series, viable tumor cells persisted in 70% of the eyes after primary chemotherapy, including 4/8 (50%) of the clinically phthisical eyes.

Previous studies have also reported persistence of viable tumor cells after primary chemotherapy in 50-80% of the eyes. The majority of the eyes evaluated by us showed undifferentiated tumors with high mitotic figure counts (83%). Only two eyes showed well-differentiated retinocytoma like areas. Previous studies have shown the association of such retinocytoma like appearance in tumors, which showed a fish-flesh pattern of regression (Thomas 2005; Magramm et al. 1989; Yang et al. 1999; Folberg et al. 2003). However, our patients had more advanced disease at presentation, and the criteria for judging the response to therapy were different, as were the indications for enucleation. In this study we had concluded that the eyes with advanced retinoblastoma, while showing good clinical response to chemotherapy, may still harbor viable cells; such eyes need to be evaluated carefully for residual risk factors, and management with appropriate adjuvant therapy may improve the outcome of these patients.

6.11 Evidences of stem cells in retinoblastoma:

The study on retinoblastoma has changed fundamentally the understanding of tumor biology, primarily genetics, mode of inheritance and the means to survival. Previous studies have shown the expression of drug resistance markers like multi drug resistant p-glycoprotein and lung resistance protein has provided the evidence of stem cells in RB (Krishnakumar et al. 2004)

Previously expression of ABCG2 a cell surface marker has been used to characterize stem cells and have shown that cells expressing this marker are present with in a pool of Hoechst 33342 low cells and it was shown for the first time in hematopoietic populations, and latter in skeletal muscle, mammary gland, lung and developing retina etc. (Zhou et al. 2001; Scharenberg et al. 2002; Welm et al. 2002; Bhattacharya et al. 2003; Summer et al. 2003).

Recent studies have shown the presence of cells expressing ABCG2 positive and hoechst33342 low cells in mouse RB cells and human RB cell lines and it generally ranges from 0.1% to 0.4% of the total population (Seigel et al. 2005). In addition to these markers Seigel et.al have detected immunoreactivity to other markers like $ALDH_1$, oct3/4, Nanog, MCM_2 and sca-1 was detected in mouse models (Seigel et al. 2005).

Expression of MCM_2 (mini chromosome maintenance gene) have reported in large number of retrospective samples in which Mohan et al. have shown MCM_2 and ABCG2 in more than 50% of RB tumor samples examined (Mohan et al. 2006). These expressions of markers have correlated with highly invasive tumors. Recently it was shown in human tumors that retinoblastoma cell MDM_2 expression was regulated by the cone-specefic RXR gamma transcription factor, human RXR gamma consensus binding site and cone specific thyroid harmone receptor-$beta_2$. It was also shown that CRX^+ cells are Rb^- and was the neoplastic component, results of this study provided support for a cone precursor cell of origin in retinoblastoma (Xu et al. 2009).

7 Hypothesis and aims of the study

7.1 Hypothesis

The proposed study is addressed at the identification and functional characterization of cancer stem cells present in retinoblastoma. Till date there are some evidences of stem cells in retinoblastoma cell lines and retrospective Rb cases, that they express ABCG2, $ALDH_1$, MCM_2 markers and cells with low uptake of Hoechst 33342 dye (Seigel et al. 2005; Mohan et al. 2006; Seigel et al. 2007), but studies on primary tumors are lacking. Functional characterization of these tumor stem cells, with convincing evidence from fresh & retrospective tumor samples, correlating with clinical outcome is lacking. In this study we propose to look for the presence of putative stem cell markers in primary retinoblastoma tumors. We will compare the gene expression profiles of RB tumor cells and clone derived cells (if possible to establish from fresh tumor) or from Y79

retinoblastoma cell line. Cells will be characterized using primitive markers (ABCG2, CD44, Hoechst 33342 dye, cell cycle analysis) and differentiation markers (CD133, CD90, CXCR4, Neuron specific enolase etc.) using fluorescent activated cell sorter, RT-PCR, and micro array.

7.2 Objectives of the study

1. To evaluate the presence of stem cell and retinal progenitor cell markers in tumor cells of retinoblastoma.

2. To characterize stem cell functional properties of different subpopulations in Y79 cell line.

3. To evaluate stem cell self renewal signaling pathways by gene expression studies.

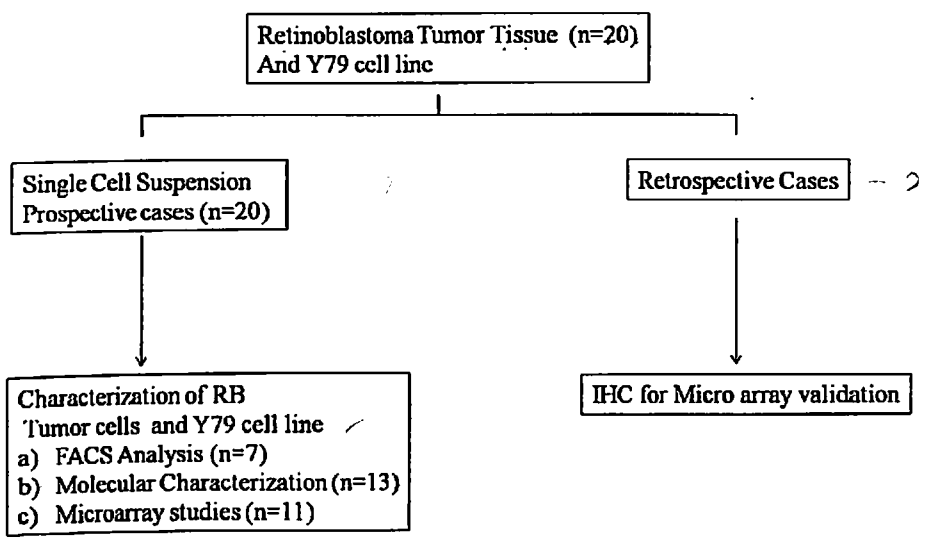
Chapter-2
Materials and Methods

8 Materials and Methods

8. 1 Tissue collection:

In this study, tumor samples of patients were obtained from the enucleated eyeballs diagnosed for retinoblastoma. Samples were collected with the approval of the institutional review board at LVPEI and in accordance with the Helsinki Declaration. Fresh unfixed eyeballs of patients diagnosed as Retinoblastoma, with and without any prior treatment, were included in the study. The enucleated eye balls were collected as per children's oncology group (COG) guidelines. After harvesting cells from tumor tissue, the eyeballs were fixed in formalin for further pathological evaluation. Harvested cells were eliminated for dead cells using 7-AminoActinomycin D by flow cytometry and RNA was isolated from sorted populations for further characterization. The histopathological slides were reviewed by an experienced ocular pathologist (GKV), specifically for features of differentiation, histological risk factors such as involvement of optic nerve, choroid, anterior segment, and other associated features. Cases with high risk factors were further evaluated for systemic spread by bone marrow and cerebro-spinal fluid (CSF) examination. The clinical profile of the patients was obtained from their medical records.

Figure 2.1: Schematic representation of the study design



8. 2 Cell Culture: Y79 cells (Obtained from Caucasian female Rb patient) were cultured in RPMI-1640 (Sigma) with 10% FCS (Hyclone), 0.37% sodium bicarbonate, 0.058% l-glutamine, 10mM HEPES and 1% pencillin-streptomycin (Gibco). Cells were incubated at 37^0C in 5% CO_2 and medium was changed every 3 days. Cells were passaged after 80% confluence and cultured in same conditions.

8. 3 Flow cytometry analysis and sorting:

Procedure:

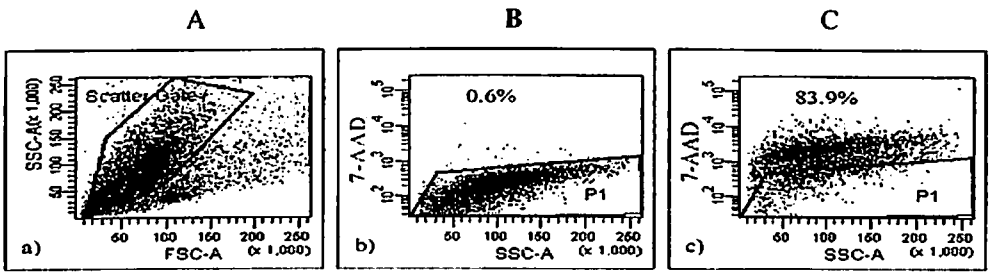
1. The cells were washed thrice with PBS containing 0.1% sodium azide.
2. One million cells were stained by incubating with 50 µl of diluted primary or direct conjugated antibodies at 4^0C for 45min (different antibodies as listed in Appendixtable-2).
3. The cells were washed thrice with PBS containing 0.1% sodium azide.
4. For unconjugated primary mouse IgG antibodies, cells were incubated with secondary anti-mouse IgG FITC, for 45min.
5. Stained cells were analyzed by flow cytometry. Isotype controls for the corresponding antibodies were used.
6. 7-AminoActinomycin D (50µg/ml) (BD Biosciences, California) staining was done to eliminate dead cells from analysis.
7. Cells were analyzed with FACS Diva software on a customized BD-FACS Aria (BD Biosciences, CA) using 488 nm blue laser (FITC, PE, 7-AAD) and 633nm red laser (APC).
8. Drop delay was calculated using BD^{TM} Accudrop beads (BD Biosciences, CA).
9. The cells of case 6 and were sorted based on the expression of CD44 and CXCR4 markers. Y79 cells were sorted based on the expression of CD133 marker.

(List of antibodies is given in Appendix-I)

8. 4 Elimination of doublets and dead cells from final analysis:

On an average, $12.3x10^6$ cells were obtained from tumor samples. This included many dead cells (ranging from 50.1±2.9% to 93.1±0.7%), which were characterized by their small size. high granularity and positivity for 7-AAD dye. These were eliminated from the final analysis by appropriate gating (Fig 2.2 A, B, and C). A fraction of cells ranging from 6.8±0.7 to 49.8±2.9% was selected from the scatter gate; these are shown in doublet discrimination plots (SSC-w vs SSC-h and FSC-w vs FSC-h). Subsequently the samples

Figure 2.2: Elimination of dead cells from final analysis



were evaluated for dead cells and marker expression in viable cells (viability ranged from 57.3±1.4% to 98.5±0.2%). Percentages of scatter gate, non-scatter gate, FSC gate, viable and dead cells across all samples were given in Table 2.1.

Case No.	Cells in Scatter gate %	Cells in non Scatter gate %	Cells after doublets discrimination FSC Gate %	% Viable cells in FSC gate	% dead cells in FSC gate
Case 1	6.8±0.7	93.1±0.7	6.3±0.6	67.1±12	32.1±10.8
Case 2	19±0.2	80.9±0.2	13.6±0.2	65.4±3.4	34.6±3.4
Case 3	26.9±1.1	73±1.1	23.6±1.2	61.7±6.7	38.3±6.7
Case 4	7.4±1.2	92.6±1.2	6.6±0.9	57.3±1.4	42.7±1.4
Case 5	35.1±3.6	64.8±4.6	34.3±3.6	98.5±0.2	1.5±0.2
Case 6	30±4.2	69.6±3.9	29.4±4.1	84.6±11.7	15.3±11.7

Case 7	49.8±2.9	50.1±2.9	48.7±2.9	96.3±0.9	3.6±0.9

Table 2.1: Shows the percentage of cells which are in scatter gate, non scatter gate, FSC gate, dead and viable cells. Results of multiple experiments were reported as means ± standard deviation

8. 5 Isolation of total RNA from tissue and sorted samples:

The isolation of total RNA from Rb tissue and sorted cells involved the following steps:

1. Homogenization, 2. Phase Separation, 3. RNA precipitation, 4. RNA wash 5. Re-suspension of the RNA pellet.

1. Homogenization

Cells were dislodged from 40-50 mg of tissue using 1 ml TRIzol (0.2ml for sorted cells) and vigorously passed several times through 1ml pipette for homogenization. After homogenization selected samples were stored at -80^{0}C.

2. Phase Separation

Homogenized samples were incubated for 5 mins at 15-30^{0}C. 0.2 ml of chloroform was mixed for every 1 ml TRIzol reagent used. Tubes were shaken vigorously for 15-20 seconds, incubated at 15-30^{0}C for 2-3mins and centrifuged at 12,000g for 15 mins at 2-8^{0}C. After centrifugation the sample will separate into lower red phenol-chloroform phase, an interphase, and an upper aqueous phase.

3. RNA Precipitation

The aqueous phase containing (organic phase - for DNA, Protein isolation) RNA was transferred to a fresh tube. RNA was then precipitated by isopropyl alcohol (0.5 ml/1ml TRIzol) and incubated at 15-30^{0}C for 10 mins. The sample was then centrifuged at 12,000g for 10mins at 2-8^{0}C. The RNA precipitate, often invisible forms a gel like pellet on bottom/side of the tube.

4. RNA Wash:

The supernatant was decanted and RNA pellet was washed in 75% ethanol (1ml/1ml of TRIzol). The sample was mixed by vortexing and centrifuged at 8000x*g* for 8 mins at 2-8^{0}C.

5. Re suspension of the RNA

RNA pellet was then air dried and dissolved in RNase-free water and stored at -70^{0}C.

8. 6 Quality and Quantification of RNA

Nanodrop 1000 Spectrophotometer:

Good quality RNA will have an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 of 1.8 or greater. This is because nucleic acid is detected at 260nm, whereas protein, salt and solvents are detected at 280 and 230 nm respectively. A high OD of 260/280 and OD of 260/230 ratios therefore indicates that RNA is devoid of any these contaminants. The isolated RNA (and DNA) was quantified by nanodrop spectrophotometer by adding 1 µl of sample to the NanoDrop 1000 spectrophotometer and measuring the OD of 260/280 and 230/260 ratio.

8. 7 Purification of RNA:

R Neasy mini spin column kit

A maximum of 100 µg of RNA can be cleaned up in this protocol. This amount corresponds to the RNA binding capacity of the RNeasy column.

Procedure:

1. The sample was made up to 100 µl with RNase free water to which 350 µl of buffer RLT was added.
2. 250µl Alcohol (96-100%) was added to the diluted RNA and mixed well by pipetting.

3. 700 µl of the sample was transferred to an RNeasy mini spin column, which was placed in a 2ml collection tube. The lid was closed gently and centrifuged for 15s at 8,000x*g*. After centrifugation, the flow through was discarded.

4. 500 µl of buffer RPE was added to the RNeasy spin column. This was centrifuged for 15s at 8,000x*g*.

5. 500 µl of RPE was added again to the RNeasy spin column and centrifuged for 2 min at 8,000x*g*.

6. After centrifugation the RNeasy spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1 min.

7. Again the RNeasy spin column was placed in a new 1.5 ml collection tube to which 30 µl of RNase free water was directly added. The lid was gently closed and centrifuged for 1 min at 8,000x*g*.

9 Quality and Quantity of RNA after Column Purification

After mini column purification of RNA, the quality and quantity was checked by Nanodrop 1000 spectrophotometer (Appendix-4) and also by electrophoresing on a denatured agarose gel (0.8%).

Agarose gel electrophoresis:

Gel Preparation:

1. Required amount (0.8%) of agarose was added to RNAase free water.
2. Agarose was boiled in microwave and the flask was swirled to ensure even mixing.
3. Melted agarose was cooled to a tolerable temperature (~55°C).
4. Eight ml of 10X MOPS buffer and 14.4 ml of formaldehyde was added to it.
5. The melted gel was poured in the casting apparatus with an inserted comb.
6. The gel was allowed to stand till it solidified.
7. The comb was removed gently from the gel plate after solidification.

Sample preparation:

RNA (500ng) products were mixed with 2 µl of RNA loading Dye (Bromophenol blue 0.025%), 10 µl of formamide, 4 µl of formaldehyde and 2 µl of 10X MOPS.

1. It was incubated at 65^0 C for 15 min.

Gel loading and running

1. The gel plate was placed in the electrophoresis tank
2. 1X MOPS buffer was poured to cover the wells
3. The samples were loaded along with standards - 250ng and 500ng of RNA. The gel was run in 1x MOPS buffer at 100V.
4. The gel was run for approximately half an hour at a voltage supply of 10V/cm till bromophenol was migrated to atleast half the distance of the gel
5. The gel was removed from the tank and was placed on UV transilluminator (UV tec) and the amplification was documented in a gel doc system.
6. After running, the amount of RNA was quantitated by comparing with known concentrations of 250ng and 500ng of RNA.

10 cDNA and cRNA Preparation

1. 500 ng of total RNA was added to 1.5 ml micro centrifuge tube.
2. 0.48 µl of T7 promoter primer was then added to control sample and tumor samples.

Table 2.2: Ingredients for cDNA preparation

Sample	T7 promoter primer (µl)	RNA (500ng) (in µl)	Nuclease free water (in µl)	Total volume (in µl)
Retina control	0.48	1.8	2.32	4.6
Case 1	0.48	1.4	2.72	4.6
Case 2	0.48	2.6	1.52	4.6

Case 3	0.48	1.0	3.12	4.6
Case 4	0.48	2.4	1.72	4.6
Case 5	0.48	1.35	2.77	4.6
Case 6	0.48	1.0	3.12	4.6
Case 7	0.48	2.3	1.82	4.6
Case 8	0.48	1.0	3.12	4.6
Case 9	0.48	0.8	3.22	4.6
Case 10	0.48	1.2	2.92	4.6
Case 11	0.48	0.8	3.22	4.6
$CD133^-$	0.48	2.0	2.12	4.6
$CD133^+$	0.48	3.6	0.52	4.6

3. Nuclease free water was used to bring total reaction volume to 4.6 µl for control and samples.

4. The primer and the template were denatured by incubating the reaction at 65^0 C in a circulating water bath for 10 min.

5. Thereafter the samples were placed on ice for 5 min.

6. Prior to use the components were mixed gently and added in the order mentioned below and placed on ice (Table 2.3).

Table 2.3: cDNA master mix

Component	Volume per Reaction for control (in µl)	Volume per Reaction for tumor samples (in µl)
5X First Strand Buffer	1.6	1.6

0.1M DTT	0.8	0.8
10mM dNTP mix	0.4	0.4
MMLV-RT	0.4	0.4
RNase Out (RNA Inhibitor)	0.2	0.2
Total volume	3.4	3.4

7. Each sample was spun briefly to bring down the contents from the walls of the tube and lid.

8. 3.4 µl of cDNA master mix was added to the control and each tumor samples respectively

9. Samples were incubated at 40^0C in a circulating water bath for 2 hrs.

10. Samples were then placed at 65^0C circulating water bath and incubated for 15 min

11. Samples were then placed on ice for 5 min and spun to bring down the contents along the wall and lid.

12. Just prior to use, the components were mixed gently and added in the following order (Table 2.4).

Table 2.4: Transcription Master Mix

Component	Volume per reaction For control (in µl)	Volume per reaction For samples diluted by 2.5 times (in µl)
Nuclease free water	6.12	6.12
4X transcription buffer	8	8
0.1 M DTT	2.4	2.4
NTP mix	3.2	3.2

50%PEG	2.56	2.56
Rnase out	0.2	0.2
Inorganic pyrophosphatase	0.24	0.24
T7 RNA polymerase	0.32	0.32
Cyanine-3-CTP or cyanine 5-CTP	0.96	0.96
Total volume	24	24

1. 24 µl of transcription master mix was added to control and each tumor sample respectively

2. Samples were incubated in a circulating water bath at 40^0 C for 2 hrs.

10.1 Purification of the Cy3 and Cy5 labeled/amplified RNA

1. Nuclease free water was added to the cRNA sample to make up the total volume to 100µl.

2. 350 µl of Buffer RLT was added and mixed well by pipetting

3. 250 µl of ethanol (96% to 100% purity) was added and mixed well by pipetting

4. 700 µl of the cRNA sample was transferred to an Rneasy mini column in a 2 ml collection tube. The samples were then centrifuged at 13,000rpm at 4^0 C for 30 seconds. After centrifugation the flow-through was discarded.

5. The Rneasy column was transferred to a new collection tube and 500µl of buffer RPE (containing ethanol) was added to the column. The samples were then centrifuged at 13000 rpm, 4^0C for 30 sec.

6. 500 µl buffer RPE was added to the column. The samples were centrifuged at 13,000 rpm, 4^0 C for 60 sec.

7. The cleaned cRNA sample was eluted by transferring the Rneasy mini column to a new 1.5 ml collection tube. 30 µl of Rnase free water was directly added on to the membrane and centrifuged at 4^0C for 30 seconds at 13,000 rpm

8. The flow-through containing cRNA was placed on ice.

10.2 Quality and Quantification of cRNA

Labelled cRNA was quantified by using Nandodrop 1000 UV-VIS spectrophotometer. The specific activity of cRNA was calculated as follows

Specific activity = Conc. Of Cy3 or Cy5 / Concentration of cRNA *1000 = pmol cy3 per µg of cRNA.

Quality of cRNA was checked by running the sample (200ng each) in 0.6% agarose gel.

Hybridization

10.3 Preparation of 10X blocking agent

1. 500 µl of nuclease free water was added to a vial containing lyophilized 10X blocking agent supplied with agilent gene expression hybridization kit. Mixed by vortexing.

2. Any material adhering to the walls and cap of the tube was brought down by centrifuging for 5 to 10 sec.

10.4 Preparation of hybridization samples

1. Water bath was set at 60^0 C

2. For each microarray, the components were added as indicated in the table 2.5

3. Samples were incubate at 60^0 C for exactly 30 min to fragment RNA

4. 55 µl of 2x hybridization buffer HI-RPM was added to makeup total volume to 110 µl.

5. Samples were mixed by centrifugation and used immediately.

Table 2.5: Ingredients for hybridization

Sample	Cy3 825ng in µl	Cy5 825ng in µl	Blocking agent in µl	Nuclease free water in µl	25X fragmentati-on buffer in µl	Total in µl
Retina control	6.2	0	6	16.6	1.2	30
Case 1	0	5.4	6	17.4	1.2	30
Case 2	0	7.4	6	15.4	1.2	30
Case 3	0	4.8	6	18.0	1.2	30
Case 4	0	7.1	6	15.7	1.2	30
Case 5	0	5.3	6	17.5	1.2	30
Case 6	0	4.9	6	17.9	1.2	30
Case 7	0	6.85	6	15.95	1.2	30
Case 8	0	1.61	6	21.19	1.2	30
Case 9	0	1.46	6	21.34	1.2	30
Case 10	0	6.69	6	16.11	1.2	30
Case 11	0	1.95	6	20.85	1.2	30
$CD133^-$	0	1.1	6	21.7	1.2	30
$CD133^+$	1.4	0	6	21.4	1.2	30

10.5 Preparation of hybridization assembly

1. A clean gasket slide was loaded into the agilent SureHyb chamber base with the label facing up

2. 100 µl of hybridization sample was slowly dispersed on to the gasket well in a drag and dispense manner, being sure not to touch the gasket walls.
3. The array "active side' down onto the SureHyb gasket slide, so that the "Agilent"-labeled barcode is facing down and the numeric barcode is facing up.
4. The SureHyb chamber cover was placed onto the sandwitched slides and slide the clamp assembly onto both pieces.
5. The clamp was hang-tightened on the chamber
6. The assembled slide chamber was placed in rotisserie in a hybridization oven set to 65^0 C at 10rpm for 17hrs.

10.6 Microarray Wash

1. The slide was placed onto a dish containing gene expression wash buffer 1 and rotated for 1 min.
2. The slide rack was transferred to a slide containing gene expression wash buffer 2, which is pre warmed at 37^0 C overnight.
3. The slide rack was removed slowly as to minimize droplets on the slides.
4. The slides were scanned to minimize the impact of environmental oxidants on signal intensities.

10.7 Microarray image and data analysis

Microarray image analysis was done using Feature extraction version 9.5.3.1 (Agilent Technologies) and data analysis was done using Gene Spring version 10 (Agilent Technologies). The background corrected intensity values were used for analysis. Normalization was done using LOWESS algorithm. Similar expressed genes were filtered on the basis of standard deviation between two biological replicates with the cutoff of less than one. Fold changes were calculated and genes more than 1.5 fold expression were

selected. Array image analysis was done using Agilent's Feature Extraction Software. Filtering and compilation of data have been done using Microsoft Excel. Spots of compromised quality and with low intensity were eliminated from the analysis, background subtracted signal intensities are normalized using LOWESS method (Gene spring version 10). T-test was performed in order to identify differentially regulated genes in retinoblastoma samples in comparison to normal and Cy5:Cy3 ratios are established. The genes showed consistent regulation in at least 75% of the samples and showed fold change greater than +1.5 fold and less than -1.5 fold were considered for functional enrichment analysis and further for validation using real-time PCR.

10.8 Enrichment analysis of biological functional groups and pathways

We studied the significance of differentially regulated genes on the biological process using DAVID bioinformatics resources (PMID: 12734009). In order to eliminate general biological process we choose the gene ontology level 5 and also picked those biological processes that are highly enriched and showed p-value less than or equal to 0.05.

11 Validation of Microarray using Real-time-PCR

To confirm the gene expression profile determined by microarray, a number of selected genes listed in Appendix table-3 were subjected to Real time PCR analysis, using total RNAs derived from the two retina controls and thirteen tumor samples. GAPDH was used as an internal control (Barber et al. 2005).

A 2 µg quantity of RNA was reverse transcribed using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA, http://www.appliedbiosystems.com), and 1/100th of the reaction was used per 20µl PCR reaction. PCR reactions were performed with DyNAZYME master mix (Finnzymes Oy, Espoo, Finland, http://www.finnzymes.com). The PCR products were resolved on a 2% agarose gel containing ethidium bromide. Real-Time PCR quantitation was performed in an ABI prism 7900 HT sequence detection

system and analysed with SDS 2.1 software (Applied Biosystems). The reactions were identical to those described above, except that DyNAMO^{TM}SYBERgreen 2X mix (Finnzymes) was used in place of DyNAZYME MIX. The sequences of primers are shown in table 2.6. Amplification of GAPDH was performed for each cDNA (in triplicate) for normalization of RNA content. Threshold cycle number (Ct) of amplification in each sample was determined by ABI Prism Sequence Detection System software (Applied Biosystems). Relative mRNA abundance was calculated as the average for Ct for amplification of a gene-specific cDNA minus the average Ct for GAPDH and fold change over control has been calculated as follows

$\Delta ct = ct_{gene} - ct_{GAPDH}$

$\Delta\Delta ct = \Delta ct$ (one cell type) $- \Delta ct$ (another cell type)

Fold Change $= 2^{-\Delta\Delta ct}$

Three individual gene-specific values thus calculated were averaged to mean ± standard deviation and fold change was expressed as log 2 ratios.

12 Reverse transcriptase-polymerase chain reaction:

Sorted cells (Q4 and Q2 quadrant) were counted on haemocytometer. Total RNA was isolated from equal number of cells using TRIzolTM reagent (Invitrogen Life Technologies, Inc. California). RNA was reverse transcribed using reagents from the first strand cDNA synthesis kit (Invitrogen Life Technologies, Inc. California). GAPDH was used as an internal control and reaction mixture without template as a negative control. Product size and primers for amplification of Oct4, Nanog, Bmi-1, Pax6, human Syntaxin1A, PROX1, CD133, NSE and GAPDH are given in Table 2.6. The PCR conditions for human Syntaxin1A, PROX1, CD133 and NSE were: 1 cycle of 3 min at 94^{0}C; 32 cycles of 1 min at 94 ^{0}C, 30 sec at 57 ^{0}C and 30 sec at 72 ^{0}C; and 1 cycle of 7 min at 72 ^{0}C. GAPDH was amplified under similar conditions for 25 cycles.

Sr.No	Primer name	Forward Primer (primer length)	Reverse Primer (primer length)
1.	CD133	5'-CCTCTGGTGGGGTATTTCTT-3'	5'-AGGTGCTGTTCATGTTCTCC-3
2.	PAX6	5' TTCAGCACCAGTGTCTACCA 3'	5' TAGGTGTTTGTGAGGGCTGT 3'
3.	Oct4	5'ATGCATTCAAACTGAGGT GCCTGC3'	5'CCACCCTTTGTGTTCCCAAT TCCT3'
4.	PROX 1	5' CAAGTTGTGGACACTGTGGT 3'	5' GCAGACTGGTCAGAGGAGTT 3'
5.	Syntaxin 1a	5'CTGCAGTCAGTCCCTCAAG 3'	5' CTGCCGAATACTGCATCTG 3'
6.	GAPDH	5'CCAGGTGGTCTCCTCTGAC TTC3'	5'GTGGTCGTTGAGGGCAATG 3'
7.	Nanog	5'CAACCAGACCCAGAACAT CC3'	5'TTCCAAAGCAGCCTCCAAG 3'
8.	Bmi-1	5'GCTTCAAGATGGCCGCTTG 3'	5'TTCTCGTTGTTCGATGCATT TC3'
9.	NSE	5'CATCGACAAGGCTGGCTA CACG3'	5'GACAGTTGCAGGCCTTTTC TTC3'

Table 2.6: Primer sequences

13 Cell cycle analysis of RB sub-populations:

Sorted $CD133^+$, $CD133^-$ and unsorted total cell populations of one of the RB sample were pelleted by centrifugation and resuspended in a solution containing 4mM sodium citrate (pH 7.6) and 50 µg/ml propidium iodide. After incubation on ice for 30mins, cell populations were treated with 0.25 mg/ml RNase A for 45mins at 37^0C to remove double-stranded RNA. Cells were finally analyzed by flow cytometry at an excitation wavelength of 488nm.

14 Clonal assays:

Single cell suspensions were obtained by passing the cells many times through pipette. Cell clumps were centrifuged at 1200 rpm for 1 min to precipitate the cell clumps. The single cells in the supernatant were harvested. The procedure was repeated until enough cells were obtained. The viable cells were counted in a haemocytometer by trypan blue dye exclusion procedure and serial dilution was made to get final cell number of 1000 cells/ml. 300µl of this cell suspension was made up to 30.0 ml with RPMI containing 10% FCS. Hundred cells of CD133 positive and negative population of Y79 cell line were sorted directly into 24 well plate (Becton Dickinson, USA) using BD FACS Aria automatic cell deposition unit and maintained as mentioned above.

The cells were incubated at 37^0C in a humidified atmosphere of 5% CO_2 in air for 14 days. After 14 days incubation colonies containing more than 50 cells were counted manually under an inverted microscope (Olympus, Tokyo, Japan). The experiment was repeated thrice to get statistically significant data. One of the CD133$^-$ clone was expanded and further characterized for CD133 at passage number 2, 3, 4 and 5.

15 p53 expression analysis:

One million cells were counted and plated in normal growth medium. Once confluency was achieved cells were washed with plain media and then maintained in 10µM Nutlin-3 or DMSO for 24 hrs. Cells were then processed for flow cytometric staining of the p53 to see the expression.

16 Immunohistochemistry:

Procedure:

1. Selection and sectioning of tissue: Sections are cut in 3 µm, use a clean water bath with distilled water, and the sections dry upright in order to facilitate adhesion between the

section and the slide. To facilitate the adhesion the slides are coated with sialne which enhances the adhesion of sections on slides.

2. Deparffinization and dehydration of sections: Sections are deparaffinized by keeping in hot air oven at 100°C for half-an-hour.

3. Wash with xylene for 10' for 2 times and dehydrated in graded alcohol.

4. Antigen retrieval: Antigen Retrieval (AR) is a high-temperature heating method to recover the antigenicity of tissue sections that had been masked by formalin fixation. Microwave Oven, Pressure Cooker and Steamer are the most commonly used heating methods. Enzyme digestion by proteases has also been used to retrieve antigen. In present method of Immunohistochemistry, antigen retrieval performed using microwave method in which the sections are heated at boiling temperature in microwave for 15' in citrate buffer of pH-6.

5. Blocking of non-specific staining: Blocking of endogenous peroxidase is done using 3% H2O2 in distilled water incubated for 10 min at room temperature.

6. Incubating with primary antibody: After washing with PBS the primary antibody mouse anti-human p53 is added which is pre diluted and section is incubated for 1 hour at room temperature. Washings were done to remove unbound 1^0 antibody.

7. Incubation with secondary antibody: Slides are exposed to the anti- mouse IgG secondary antibody-HRP (dilution 1:200) and incubated for 30 min at room temperature.

8. Addition of Avidin biotin complex: After PBS wash ABC complex (Avidin biotin complex) which is HRP (Horseradish peroxide) is added and incubated for 30 min or by using Biogenix enhancer kit.

9. Addition of substrate Diamino benzidine (DAB): The peroxidase reaction is obtained by incubating the slides with 0.005% hydrogen peroxide and 0.02% of diaminobenzidine tetrachloride for 15'.

10. After DAB reaction the section is stained with Harris haematoxylin for 1 min and mounted with DPX.

(List of antibodies is given in Appendix-2)

Chapter-3
Phenotypic Evaluation of Tumor Cells

3.1 Introduction

Rb tumors are typically comprised of morphologically small round cells that express a variety of undifferentiated stem cell and differentiated retinal lineage markers (Krishnakumar et al. 2004; Seigel et al. 2005; Mohan et al. 2006; Seigel et al. 2007; Zhong et al. 2007). These studies have demonstrated that tumor cells expressing Oct4, Nanog, ABCG2, ALDH1, MCM2 and cells which uptake low Hoechst 33342 dye are present in human retinoblastoma. Recent evidence suggests the presence of cancer stem cells in various malignancies such as acute leukemia, neural, breast and ovarian tumors (Pardal et al. 2003; Singh et al. 2003; Al-Hajj et al. 2004; Ponti et al. 2005; Szotek et al. 2006; Yilmaz et al. 2006). The marker expression profile that is commonly expressed in leukemia include $CD133^{+}$ $CD34^{+}/CD38^{-}$, $CXCR_4^{+}$ and in breast tumors $ESA^{+}CD44^{+}CD24^{-/low}$ lin^{-} markers (Lapidot et al. 1994; Al-Hajj et al. 2003; Ponti et al. 2005). In pediatric brain tumors, tumor-derived progenitors, when passaged at clonal density form neurospheres. These neurospheres express many genes characteristic of neural and other stem cell markers including CD133, nestin, SOX2, musashi-1, Bmi-1, Oct4 etc (Singh et al. 2003; Singh et al. 2004).

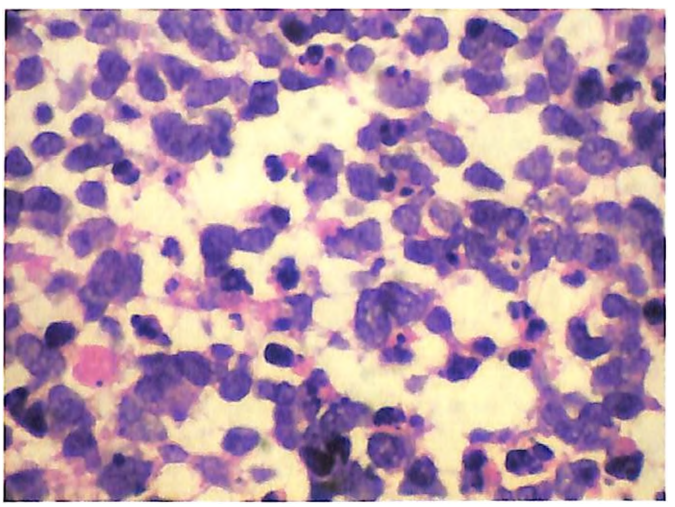
In Rb tumors study of the basic morphology and phenotype has only yielded a limited amount of knowledge regarding the clinical behavior of the tumor; in addition there is lack of information about the culture conditions and functional assays of these tumor cells. Determination of key subpopulations in the tumor will give insight into the mechanism of tumorigenesis. The majority of the tumor cells in Rb are dead, necrotic or apoptotic. Analyzing the expression of markers in the heterogeneous tumor constituting many dead and necrotic cells is inappropriate. To overcome this anomaly, we attempted to show the expression of neuro ectodermal stem and differentiated cell markers (CD44,

$ABCG_2$, CD133, CD90, and $CXCR_4$) in the freshly isolated tumor cells by flow cytometry.

3.2.1 Results

A. I) Clinical profile: The clinical features of the seven cases are summarized in Table 3.1. The mean age of patients included in this study was 4.7±2.2 yrs (1.5 yrs to 7 yrs) comprising of three females and four male patients. All the tumors were sporadic cases; with right eye involvement in three and left eye in four cases. Four cases presented with a history of white reflex/ leucokoria, two with history of pain and redness, and one case with progressive visual loss. Six of the seven patients had an advanced form of retinoblastoma; with five cases having ICIR (International Classification of Intraocular Retinoblastoma) group E and one ICIR group D tumor.

Figure 3.1: Anaplastic features in case 6



II) Histologic examination: The summary of clinical and histological findings is provided in Tables 3.1 and 3.2. Except for case 4, which was a well differentiated tumor, the other cases were poorly differentiated and case 6 manifested features of anaplasia (Figure 3.1). High risk factors of massive choroid involvement were noted in case 1, while cases 3, 4 and 7 showed minimum choroid involvement. Tumor in optic nerve (up

to cribrosa) was seen in case 3 and 6. The resected margin of the optic nerve was however free in all the cases.

B. Clinical outcome: On histo pathological examination Case 1 and 7 had high risk factors for choroid and anterior segment involvement. Both patients were advised to undergo bone marrow/cerebrospinal fluid (BM/CSF) evaluation. Report for tumor cells in BM/CSF was negative. During the last follow up, four cycles for case 1 and 2 cycles for case 7 with a 3-drug regimen of vincristine, etoposide and carboplatin had been administered. The remaining 5 patients were doing well at the last follow up.

C. Phenotypic analysis of tumor cells using flow cytometry: (Figure 3.1; rows A to G)

a) Expression of putative stem cell (ABCG2, CD44 and CXCR4) and differentiated retinal cell markers (CD90 and CD133):

ABCG2: ABCG2 was positive in five out of seven tumors and its expression ranged from 0.08% to 1.2% of the selected population. In case 4, a well-differentiated tumor, it constituted 0.08% of all cells. Among the poorly differentiated tumors, there was no significant difference observed in the percentage of these cells irrespective of massive choroid or lamina cribrosa involvement.

CD90: All the tumors had a high and variable expression of CD90 ranging from 45.4 ± 9.6% to 94.8 ± 0.5%. CD90 expression was high in case 3 which exhibited involvement of anterior layers of cribrosa (94.8 ± 0.5%). There was no disparity in its expression in well differentiated or poorly differentiated tumors.

Table 3.1: Clinical and histo pathological features in Rb tumors:

Case No.	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Age	7 yrs	7 yrs	4 yrs	1 1/2 yrs	4 yrs	3 yrs	7 yrs
Sex	F	F	F	M	M	M	M
Laterality	U	U	U	U	U	U	U
Eye	OD	OS	OS	OD	OS	OD	OS
Heredity	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic	Sporadic
Duration of symptoms	10 days	10 days	3 months	9 months	3 days	2 months	10 days
Clinical features	H/O pain redness and watering	white reflex	White reflex	Leukokoria	H/O pain redness and watering	Progressive loss of vision	H/O pain redness and watering
Retinoblastoma group (ICIR)	Group E	Group D	Group E	Group E	Group E	Group E	-
Histopathological risk factors:							
Differentiation	Poorly differentiated	Poorly differentiated	Poorly differentiated	Well Differentiated with Flexner wintersteiner and Holmer Wright rosettes	Poorly differentiated	Poorly differentiated	Poorly differentiated
Choroid involvement	Massive choroid involvement	–	Minimum choroid involvement is present	Minimum choroid involvement is present	–	–	Minimum choroid involvement is present
Anterior segment	+	–	–	–	–	–	-
Optic Nerve	–	–	anterior layers of the cribrosa involved	–	Pre-laminar involvement of cribrosa	–	-
Others	Angle and Iris deposits	Focal areas of sub RPE deposits seen	NVI	NVI	–	–	-

Bone Marrow/CSF involvement.	negative	ND	ND	ND	ND	ND	negative
Duration of follow up	Received adjuvant chemotherapy 8 weeks	-	1 week	1month	-	-	-
Outcome	On adjuvant chemotherapy, 4 cycles were completed. Two more to be given. Patient is doing well	Doing well	Doing well	Doing well	Doing well	Doing well	On adjuvant chemotherapy, 2 cycles were completed. Patient is doing well.

Foot notes: Yrs =Years; F = Female; M = Male; U = Unilateral; OD = Right eye; OS = Left eye; ND = Not done; (+) = Positive; (-) = Negative; RPE = Retinal pigment epithelium; CSF = Cerebrospinal fluid; NVI = Neovascularization of Iris.

CD133: CD133 showed variable expression in all the tumors ranging from 13.0% to 91.1% with no specific relationship with differentiation or involvement of optic nerve or choroid.

CD44: Expression of CD44 ranged from 5.9 ± 1.9% to 98.1 ± 0.2% with the lowest expression in poorly differentiated tumor case 5, and highest in case 3 and 6, which exhibited a marked involvement of anterior layers of lamina cribrosa.

CXCR4: Expression of CXCR4 was variable in all tumors, ranging from 3.6 ± 0.8% to 92.4 ± 4.2% of all cells. The lowest expression was observed in case 5 and highest in the tumor with cribrosa involvement.

Table 3.2: Marker expression in Rb patients

Case No.	Age/Sex	Clinicopathological Features and high risk factors	% of $ABCG_2$ expression	% of CD90 expression	% of CD133 expression	% of $CXCR_4$ expression	% of CD44 expression
Case 1	7yrs/F	OD, PD Choroid and anterior segment involved. Iris and sub RPE deposits noted	1.14%	69.3± 1.6%	73.30%	64.5 ± 8.5%	86.6 ± 1.7%
Case 2	7yrs/F	OS, PD Focal areas of sub RPE deposits seen	0.44%	46.8 ± 3.1%	91.10%	82.1 ± 12.5%	83.9 ± 8.4%
Case 3	4yrs/F	OS, PD Anterior layers of cribrosa involved. Neovascularisation of iris with ectopion of uvea, with sub RPE deposits seen	1.2%	94.8 ± 0.5%	90.80%	92.4 ± 4.2%	97.8 ± 1.6%
Case 4	1.5yrs/M	OD, WD sub retinal tumor deposits noted	0.08%	85.70%	87.10%	46.3 ± 15.6%	36.5 ± 8.6%

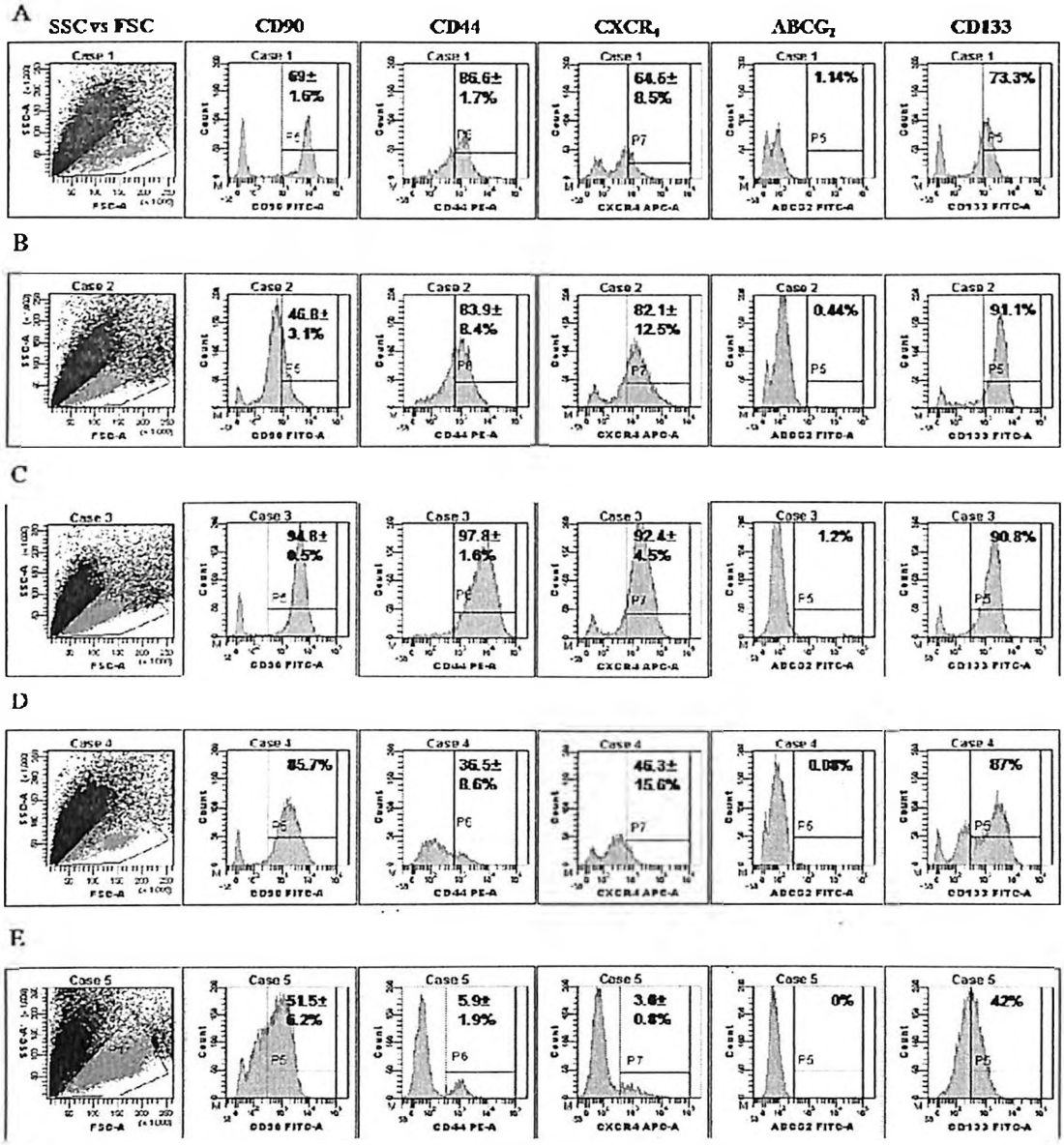
Case 5	4yrs/M	OS, PD	0%	51.5±6.25%	42%	3.6±0.8%	5.9±1.9 %
Case 6	3yrs/M	OD, PD Anterior layers of cribrosa involved	0.2%	46.4±9.4%	25.5%	18±4.4%	98.1±0. 2%
Case 7	7yrs/M	OS. PD Superficial choroidal involvement is seen	0%	45.4±9.6%	13.0%	42.6±4.2%	88.1±0. 5%

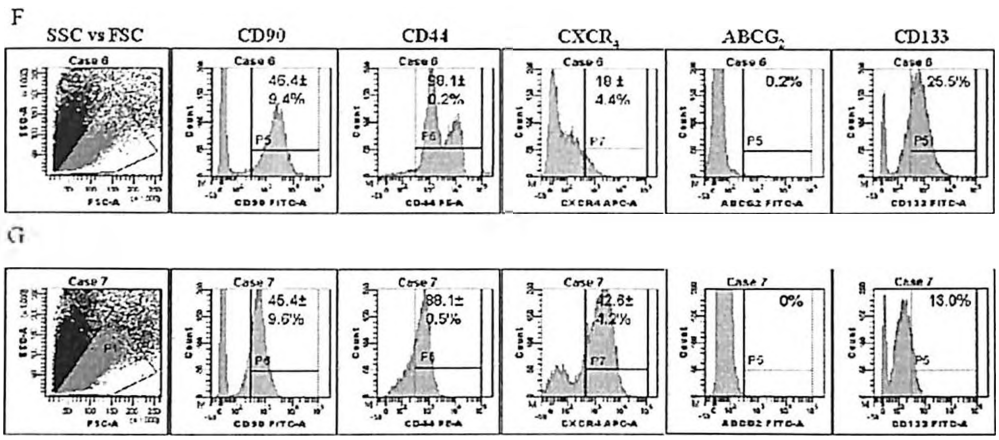
Foot Notes: Yrs = Years; F = Female; M = Male; OD = Right eye; OS = Left eye; RPE = Retinal pigment epithelium; PD = Poorly differentiated; WD = Well differentiated.

The percentage of expression of markers along with histological risk factors for different tumors is shown in Table 3.2 and 3.3.

b) Combination of markers expressed in retinoblastoma: The percentage of cells co-expressing ABCG2, CD44 and CXCR4 ranged from 0% to 1.08%. Cells expressing CD133, CD44 and CXCR4 ranged from 0.14% to 93%. Those expressing CD90, CD44 and CXCR4 ranged from 0.07% to 84.7%. Other combination of markers had variable expression, as shown in Table 3.3.

Figure 3.2: Expression of markers in Rb tumors





D. Sub-populations of cells in retinoblastoma: We observed two different sub-populations present in the retinoblastoma that varied in surface marker expression and scatter properties. The data of all the 7 cases is provided in Table 3.3, 3.4 and 3.5. Two sub-populations include FSC^{low}/SSC^{low}, FSC^{hi}/SSC^{low} and are termed as P2 and P3 respectively (Figure 3.4, Panel B). P2 population (ranging from 1.7% to 17.7%) exhibited more cells positive for CD44 (34.6±3.5% to 91±2.2% of selected population), and negative for CD133, CXCR4 and CD90 markers (Q4 quadrant, Fig 3.3, A-G). In P2 population, cells which were positive for CD44 and CXCR4 co-expressed CD133 and CD90 (Q_2 quadrant, Fig 3.3 and 3.4 C, D, E).

Table 3.3: Multiple markers expressed in Rb tumors

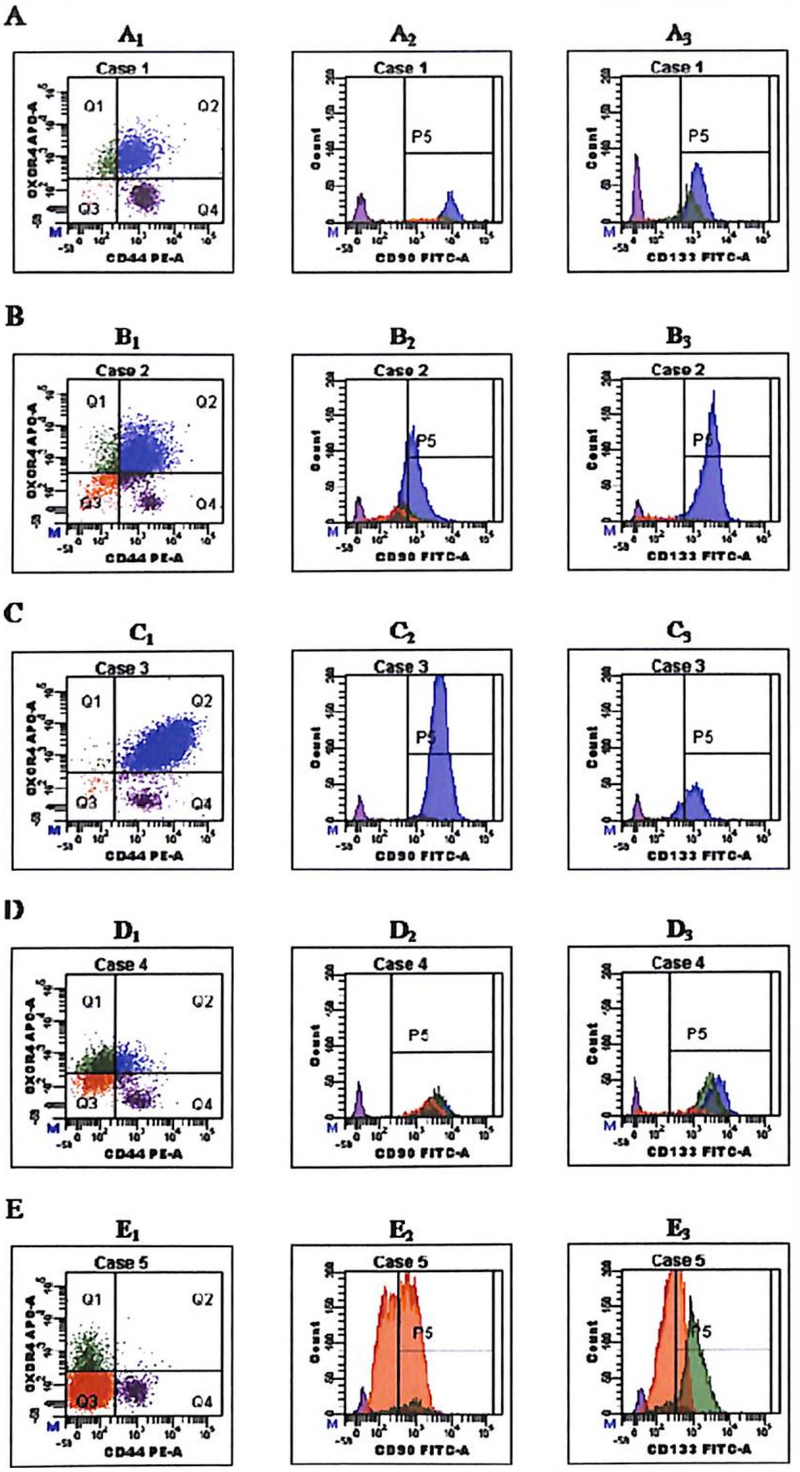
Combination of Markers	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
CD44+CXCR$_4$+	55.5±3.7	75.8±14.9	92.2±1.6	7.3±5.4	0.15±0.05	20.2±6.5	35.9±2.9
ABCG$_2$+CD44+	1.032	0.435	0.8	0.028	0	0.2	0
CD133+ CD44+	51.9	81.9	95.3	20.13	6.5	34.9	21.3
CD90+ CD44+	62.8	47.6	91.9	15.9	0.9	33.3	34.7
ABCG$_2$+ CXCR$_4$+	0.32	0.435	0.79	0.028	0	0	0
CD133+ CXCR$_4$+	71.9	81.8	95.6	60.084	8.8	11.7	18.3
CD90+ CXCR$_4$+	49.2	48	91.24	28.95	3.1	0.7	31.5
CD44+ ABCG$_2$-	81.9	80.3	98.15	19.13	5.7	95.7	88.3
CD44+ CD133-	25.2	11.1	4.31	10.81	5.4	64.3	65.5
CD44+ CD90-	29.8	34.7	4.8	12.28	5.1	60.7	53.1
ABCG$_2$+ CD44-	0.14	0.013	0	0.055	0	0	0
CD133+ CD44-	19.8	0.311	0.015	62.36	47.5	0.4	0.1
CD90+ CD44-	6.9	3.03	1.34	71.57	51.5	3.1	2.4
ABCG$_2$+ CXCR$_4$-	0.85	0.03	0.154	0	0	0.2	0
CD133+ CXCR$_4$-	0.26	0.73	0.093	20.95	41.9	24.6	3.3
CD90+ CXCR$_4$-	21.4	4.05	2.17	58.37	53.1	36.4	10.6
ABCG$_2$- CXCR$_4$+	65.6	85.8	91.8	29.3	2.3	7	34
CD133- CXCR$_4$+	3.06	3.37	1.45	0.42	1.3	1.1	20.8
CD90- CXCR$_4$+	0.1	23.92	0.78	0.12	0.7	0	8.9
ABCG$_2$+CD44+CXCR$_4$+	0.6	0.35	1.08	0.002	0	0.1	0
CD133+CD44+CXCR$_4$+	39.63	88.35	93	7.34	0.14	12.5	21.9
CD90+CD44+CXCR$_4$+	30.78	37.3	84.7	6.4	0.07	17.9	29.9

Table 3.4: Marker expression in FSC^{low}/SSC^{low}

FSC^{low}/SSC^{low} population			
Case No. and Selected scatter gate cells (%)	$CD44^+$ $CXCR4^+$ cells (%)	$CD44^-$ $CXCR4^+$ and $CD90^+$ cells (%)	$CD44^+$ $CXCR4^+$ and $CD133^+$ cells (%)
Case 1 (1.7)	17.9±3	17.5	17.1
Case 2 (4.6)	30.7±11.1	29.6	30.5
Case 3 (4.6)	60.7±6.2	58.57	58.2
Case 4 (2.5)	4.7±5.3	2.8	4.7
Case 5(2.1)	0.9±0.9	14.3	79.2
Case 6(12)	2.85±1.6	40	89.8
Case 7(17.7)	14.7±4.2	84.2	73.9
Case No. and Selected scatter gate cells (%)	$CD44^+$ and $CXCR4^-$ cells (%)	$CD44^+$ and $CXCR4^-$ cells which are $CD90^+$ cells (%)	$CD44^+$ and $CXCR4^-$ cells which are $CD133^+$ cells (%)
Case 1 (1.7)	54.2±15.6	3.7	0
Case 2 (4.6)	39.9±7.3	4.5	1.8
Case 3 (4.6)	34.6±3.5	4.1	0.3
Case 4 (2.5)	66.5±12.9	2.5	1.8
Case 5(2.1)	51.5±1.8	0.9	1.2
Case 6(12)	91±2.2	2.5	0.5
Case 7(17.7)	84.6±6.4	5.2	3.8

Table 3.5: Marker expression in FSC^{hi}/SSC^{low}

FSC^{hi}/SSC^{low} population			
Case No. and Selected scatter gate cells (%)	$CD44^+$ $CXCR4^+$ cells (%)	$CD44^+$ $CXCR4^+$ and $CD90^+$ cells (%)	$CD44^+$ $CXCR4^+$ and $CD133^+$ cells (%)
Case 1 (2.7)	92.7±3.4	91.8	91.5
Case 2 (12.2)	95.4±5.5	93.5	94.9
Case 3 (16.9)	99±0.5	98.8	98.7
Case 4 (3.7)	52.5±16.1	52.5	52.5
Case 5(32.2)	0.16±0.1	66.7	99.6
Case 6(15.9)	38.6±17.1	96.9	88.2
Case 7(35.1)	51.7±6.5	81.7	57.5
Case No. and Selected scatter gate cells (%)	$CD44^+$ and $CXCR4^-$ cells (%)	$CD44^+$ and $CXCR4^-$ cells which are $CD90^+$ cells (%)	$CD44^+$ and $CXCR4^-$ cells which are $CD133^+$ cells (%)
Case 1 (2.7)	4.5±3.1	2.56	0.21
Case 2 (12.2)	1.8±2	0.99	0.2
Case 3 (16.9)	0.6±0.4	2.9	0.81
Case 4 (3.7)	4.8±3.8	4.3	1.4
Case 5(32.2)	2.7±0.11	33.6	32.5
Case 6(15.9)	70.8±22.4	61	35
Case 7(35.1)	37.6±6.6	27.3	6.5


A
A1
Case 1
Q1
Q2
Q3
Q4
CD44 PE-A
A2
Case 1
P5
CD90 FITC-A
A3
Case 1
P5
CD133 FITC-A
B
B1
Case 2
Q1
Q2
Q3
Q4
CD44 PE-A
B2
Case 2
P5
CD90 FITC-A
B3
Case 2
P5
CD133 FITC-A
C
C1
Case 3
Q1
Q2
Q3
Q4
CD44 PE-A
C2
Case 3
P5
CD90 FITC-A
C3
Case 3
P5
CD133 FITC-A
D
D1
Case 4
Q1
Q2
Q3
Q4
CD44 PE-A
D2
Case 4
P5
CD90 FITC-A
D3
Case 4
P5
CD133 FITC-A
E
E1
Case 5
Q1
Q2
Q3
Q4
CD44 PE-A
E2
Case 5
P5
CD90 FITC-A
E3
Case 5
P5
CD133 FITC-A

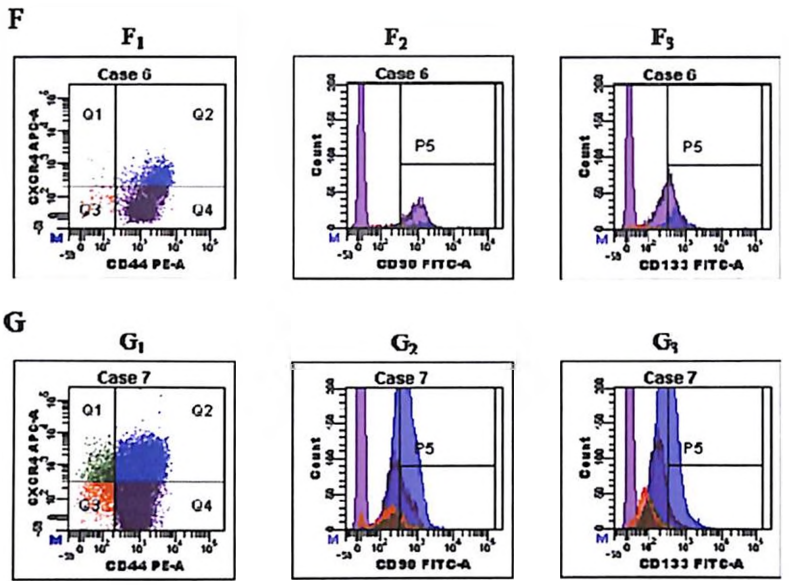


Figure 3.3: Combination of markers expressed in Rb tumors- Rows A to G represent the expression profile of a combination of markers (CXCR4 + CD44 + CD90 and CXCR4 + CD44 + CD133) expressed in cases 1 to 7. *Panels A_1 to G_1,* shows the population of cells expressing CXCR4 alone (Q1), CD44 alone (Q4), CD44 and CXCR4 (Q2) and negative for both the markers (Q3). *Panels A_2 to G_2,* shows the populations from various quadrants of panel A_1 to G_1 which show expression of CD90. *Panel A_3 to G_3,* shows the populations from various quadrants of panel A_1 to G_1 which show expression of CD133

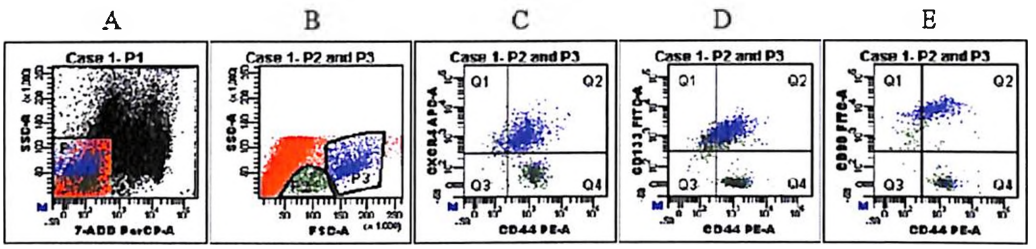


Figure 3.4: Representation of different populations on FSC vs SSC- A- elimination of dead cells B- P2 gate cells (Majority of them are $CD44^+$ $CXCR4^-$ $CD133^-$ $CD90^-$), P3 gate cells (Majority of them are $CD44^+$ $CXCR4^+$ $CD133^+$ $CD90^+$), C,D,E- Dot plots showing expression of CD44, CXCR4, CD133 and CD90 markers

P2 cells were present in low percentage (Table 3.4). P3 population showed (2.7% to 35.1% of total cells) higher percentage of cells expressing the combination of CD44, CD133, CXCR4 and CD90 markers when compared to CD44 alone (0.16±0.1% to

99±0.5%) (Table 3.4&3.5). Since a consistency was observed across 7 samples, the cells of case 6 were sorted for RTPCR analysis.

E. Gene Expression Profile of FSC^{low}/SSC^{low} (P2) Vs FSC^{hi}/SSC^{low} (P3) sub-population:

The two sub-populations (P2 and P3) were sorted based on the expression of CD44 and CXCR4 with a sorting efficiency of 97% and 61% respectively (Figure 3.5 A, B, C). The gene expression profile of the P2 population showed a strong expression of retinal progenitor cell markers PROX1 and Syntaxin1A. In contrast, the P3 population showed low expression of retinal progenitor cell markers (Fig 3.5 D, Q4, Q2 quadrant). Differentiated cell markers CD133 and NSE were expressed in both the groups.

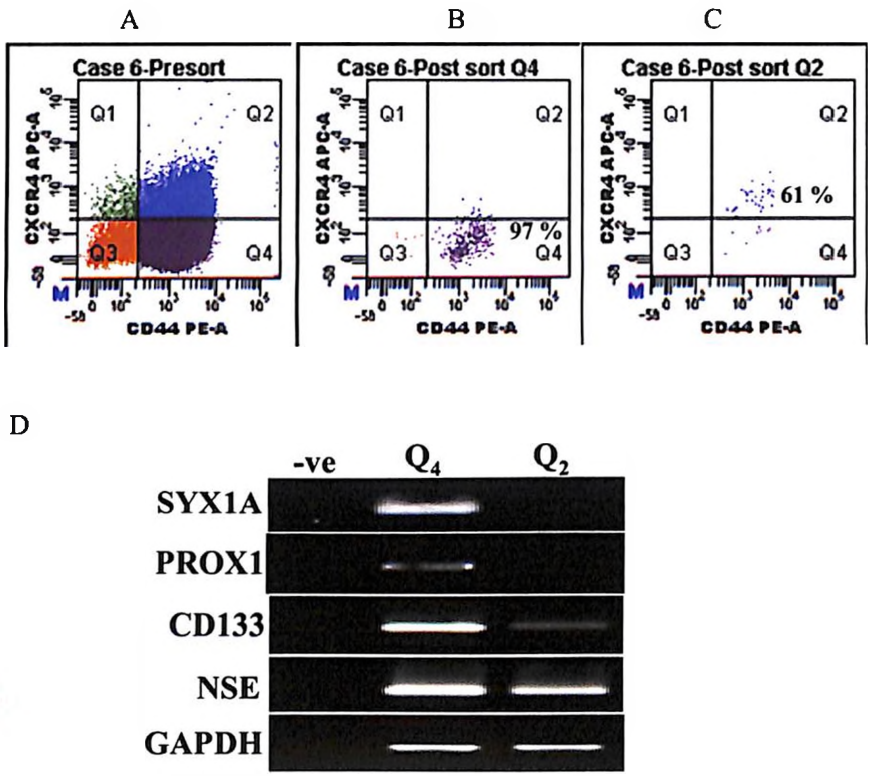


Figure: 3.5: RT-PCR of genes expressed in sorted population of cells- A) Dot plot representation of CD44 and CXCR4 expression B) Post sort purity of $CD44^{+}$ cells

C) Post sort purity of $CD44^{+}CXCR4^{+}$ cells

3.3 Discussion:

Our observation regarding the expression of ABCG2, CD133, and CD90 are in accordance with earlier studies (Seigel et al. 2005; Mohan et al. 2006). We identified two sub-populations in retinoblastoma based on surface marker expression and scatter properties. This prompted us to hypothesize that the cells in P2 population (mean 6.4% of total cells) which were positive for CD44 and negative for other markers are primitive, when compared with the P3 group (mean 16.9% of total cells). We corroborated our hypothesis by analyzing for the expression of retinal progenitor cell markers (PROX1 and Syntaxin1A) in the sorted population using RT-PCR. Results have shown strong expression of retinal progenitor cell markers in the P2 population when compared with P3 group (Figure 3.5D). In addition, the expression of differentiated cell markers of retina CD90 (retinal ganglion cell marker), CD133 (Photo receptor cell marker (Maw et al. 2000)) was more in the P3 group.

The other studies also showed the existence of cells with stem cell markers like multi drug resistance (MDR) related P-glycoprotein (P-gp) and lung resistance protein , provided further insight of stem cells in Rb (Krishnakumar et al. 2004). Immunoreactivity to P-gp was frequently observed in well-differentiated retinoblastoma, especially those treated by chemotherapy before enucleation (Krishnakumar et al. 2004; Filho et al. 2005). In a retrospective study of 20 non-invasive Rb tumors, ABCG2 expression was in 12 and MCM2 in 14. Furthermore in 19 invasive Rb tumors ABCG2 was positive in 15 tumors and MCM2 in 16 of the tumors (Mohan et al. 2006). Previous reports have demonstrated that stem cells expressing Oct4, Nanog, ABCG2, ALDH1, MCM2 and low Hoechst 33342 are present in human retinoblastoma. However these studies were based on fixed tissues and Rb cell lines (Seigel et al. 2005; Mohan et al. 2006; Seigel et al. 2007).

This is the first study to document the percentage of cells expressing CD44 and CXCR4 in primary retinoblastoma using flow cytometry. Previous studies have shown CD44 to be the hallmark of cancer stem cells in leukemia, pancreatic and breast cancers (Reber et al. 1990; Gunthert et al. 1991; Hofmann et al. 1991; Rudy et al. 1993; Seiter et al. 1993; Penno et al. 1994). Nevertheless, its expression is repressed in certain tumors of the neuroectoderm, like neuroblastoma, small cell lung carcinoma and gastrinoma (Shtivelman et al. 1991; Penno et al. 1994).

The over expression of CD44 (hyaluronate receptor) in retinoblastoma may be attributed to the micro environment of vitreous fluid as it contains hyaluronic acid. Although our sample size is limited, we did note that the proportion of CD44 positive cells was high in case 6, which is primitive and poorly differentiated. It is difficult to correlate the histo pathological risk factors with markers, since there is variability of the expression from sample to sample.

Chapter-4

Functional Characterization of Cancer Stem Cells in Y79 Cell line

Introduction:

Although the hypothesis of "cancer stem cells" originated in 1930's, it was only demonstrated in the past decade due to technological advancements. It was first documented in acute myeloid leukemia that only certain population, when transplanted would form and represent the original phenotype of the tumor and not all the tumor cells had the same ability (Lapidot et al. 1994; Bonnet et al. 1997). Latter this concept was established not only in other tumors like breast cancer, human brain tumors but also in well established, long standing cell lines (Lapidot et al. 1994; Bonnet et al. 1997; Al-Hajj et al. 2003; Singh et al. 2003; Kondo et al. 2004). Kondo et al. has shown that a few cancer cell lines (C6 glioma cell line, MCF7 breast cancer cell line, B104 neuroblastoma cell line and HeLa adenocarcinoma cell line) contain side population cells (SP). These sorted SP population, displayed the ability to form the non SP phenotype, and metastatic tumors in nude mice. These results have suggested that many of the cell lines might contain their own cancer stem cells, which are responsible for malignancy (Kondo et al. 2004).

It was also demonstrated that these cancer stem cells express high levels of ATP binding cassette (ABC) transporters contributing to multi drug resistance (MDR). The development of MDR phenotype was one of the major causes of resistance to cancer chemotherapeutic drugs, which is ultimately responsible for disease relapse (Choi 2005; Dean et al. 2005; de Jonge-Peeters et al. 2007). As these cells must be eliminated, there is immediate necessary for methods to identify and isolate cancer stem cells in every tumor in order to prevent the relapse of the disease.

In Rb tumors, stem cell markers like Nanog, Oct4, ABCG2, ALDH1 and MCM2 were reported, however the functional characterization of these cells was not adequate

(Krishnakumar et al. 2004; Seigel et al. 2005; Mohan et al. 2006; Seigel et al. 2007; Zho al. 2007). In our earlier work, using a two parameter analysis (size and phenotype) observed two populations $FSC^{low}SSC^{low}$: $CD44^{+}CD133^{-}CD90^{-}$ and $FSC^{hi}SS($ $CD44^{+}CD133^{+}CD90^{+}$ (Balla et al. 2009). $FSC^{low}SSC^{low}$ cells expressed primitive stem markers, lacking the expression of differentiation markers, while the reverse was obser for $FSC^{hi}SSC^{low}$ population. Further *in vitro* functional characterization of cancer stem c in human tumor samples was not possible due to lack of culture conditions and also limited availability of primary tumors without chemo-treatment. Hence the present study w primarily aimed to evaluate the phenotypical and *in vitro* functional characterization of the stem-like cells in the well established Y79 Rb cell line.

Results:

I) Phenotypic characterization of Y79 Cells:

Similar to the human Rb tumors, Y79 cell line also expressed putative stem cell markers (Oct4, Nanog ABCB1 and Bmi-1) and differentiated retinal cell markers (CD90 and CD133). Flow cytometric evaluation of markers was shown in Figure 4.1. In contrast to human Rb tumor cells, CD44 marker was not expressed in Y79 cells and the percentage positivity was 0.1±0.1% (Figure 4.1 f). The expression of CD133, CD90 and CXCR4 (Figure 4.1 e, g, h: 90.3±3.4%, e: 79.7±1.3% and h: 14.4±0.5% respectively) were comparable to the expression in human Rb tumors (Table 3.2 in chapter 3).

CD44 (+) in Rb
(-) in Y79
CD133, CD90, CXCR (+) in both

Figure 4.1:

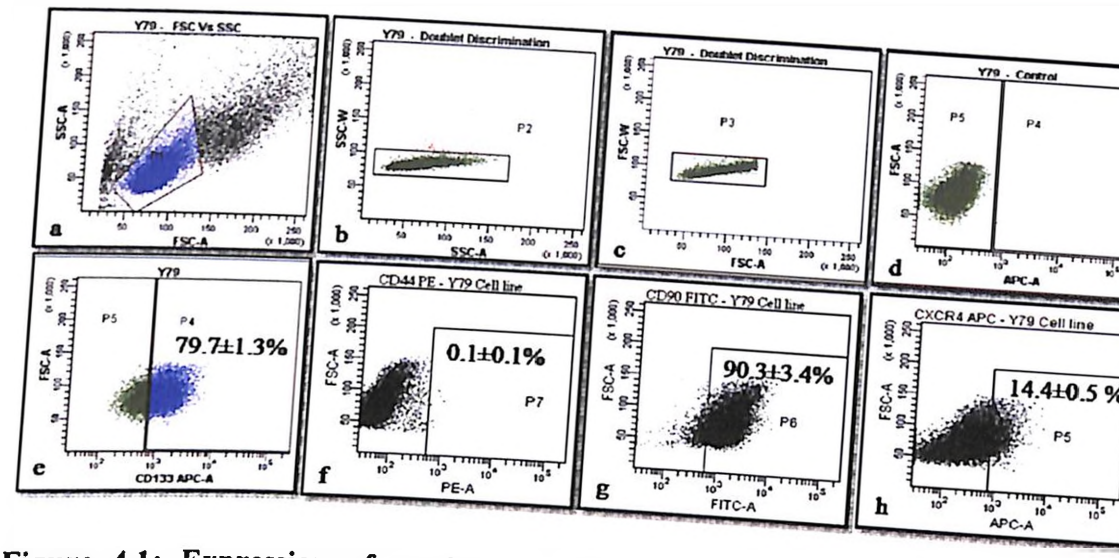


Figure 4.1: Expression of markers in Y79 cells – a) FSC vs SSC b&c) Dou discrimination plots d) unstained control e) expression of CD133 APC f) CD44 P CD90 FITC and h) CXCR4 APC

As the expression levels of these markers was similar to that of human Rb tumors, for furt experiments CD133 was considered as a marker for sorting, microarray, cell cycle analys RT-PCR and clonal assay experiments. Based upon the expression of CD133 marker, bo $CD133^+$ (98.1% purity) and $CD133^-$ cells (95.7% purity) were sorted (Figure 4.2 a, b) a then evaluated for $ABCG_2$, Bmi-1, Nanog, Pax6, Oct4 and PROX1 (stem cell and retin progenitor cell marker) and CD133 (photo receptor cell lineage marker). In negativ population PROX1, Oct4, Bmi-1 and Nanog are highly expressed and CD133 is absen Figure 4.2 e. In addition the stem cell markers like Nanog and Pax6 is 8.57 and 2.73 folds up regulated in CD133 negative cells compared to positive population as shown in Figure 4.2 c, d.

CD133 for sorting, Microarray, cell cycle & RT

CD133⊖ -> Nanog, Oct4, Bmi-1, Pax6 1 poten

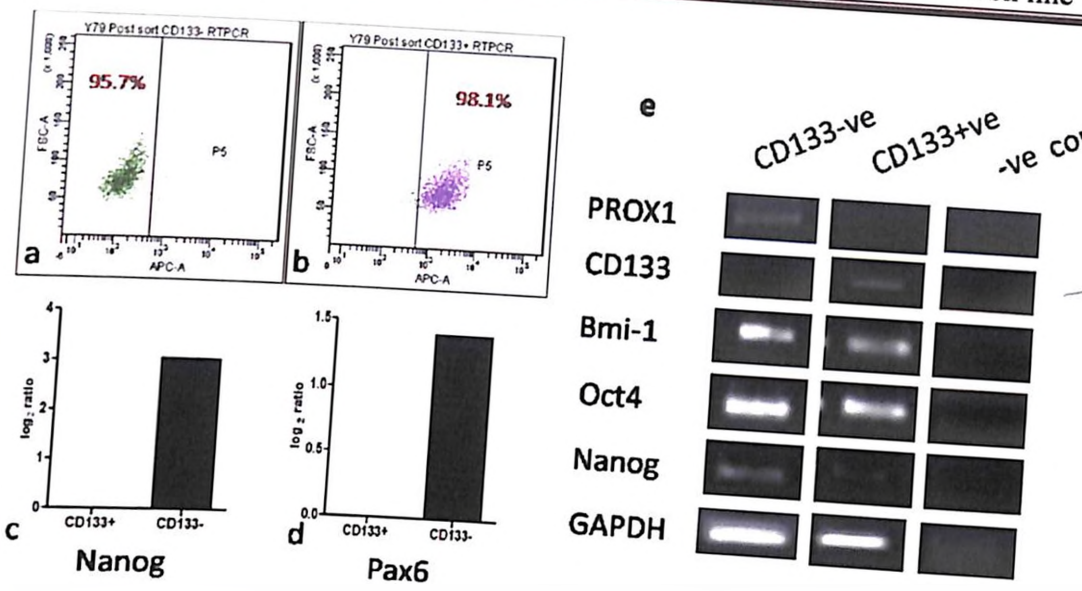


Figure 4.2: Marker expression in CD133⁻ and CD133⁺ sorted populations – a) Post s purity of CD133⁻ cells is 95.7% b) Post sort purity of CD133⁺ cells is 98.1% c&d) R time PCR data of Nanog and Pax$_6$ expression e) RT-PCR expression of GAPI CD133, PROX$_1$, Oct4, Nanog and Bmi-1 expression in CD133 negative and posit sorted populations

III) Gene expression profile of putative stem cells in Y79 cells:

In the Y79 cell lines sorted by CD133, there was up-regulation of 2945 genes (≥1.5 fold) and dow regulation of 4531 genes (≤ 1.5 fold) in CD133⁻ cells. We identified Purine metabolism pathwa (p=0.009), TGF-beta signaling pathway (p=0.009), p53 signaling pathway (p=0.017), Jak-St signaling pathway (p=0.047), cytokine-cytokine receptor interaction pathway (p=0.034) an oxidative phosphorylation pathway (p=0.012) that are highly deregulated in CD133⁻ cells, whe compared to CD133⁺ population. List of top 25 up regulated and down regulated genes are listed i Table 4.1& 4.2.

Table 4.1: List of top 30 genes that were up regulated in CD133⁻ cells

Gene Name	PValueLogRatio	Mean log2 fold change (upregulation)
AF334588	1.25E-16	4.041897

KLF17	3.17E-17	4.037766
A_24_P649507	1.12E-18	3.992264
AK125176	5.17E-16	3.863978
PDE4DIP	1.05E-10	3.443785
U22172	5.80E-16	3.371156
VNN3	4.99E-08	3.134369
ZDHHC15	4.15E-15	3.10141
A_24_P817490	8.75E-17	3.094543
A_23_P63447	8.78E-06	3.034319
C1orf131	3.60E-16	3.004795
PTGS2	2.08E-09	2.986919
LOC154761	6.41E-10	2.874717
NP083564	2.20E-08	2.833521
GIMAP1	2.47E-06	2.786683
PPP1R14C	2.21E-08	2.736343
MECOM	3.30E-07	2.656235
CD69	1.23E-05	2.593014
A_24_P925901	4.37E-07	2.589578
MORN5	1.41E-05	2.525711
ENST00000442408	9.55E-07	2.521708
RNF175	2.12E-12	2.477196
AF090887	6.31E-08	2.473979

SYT4	3.03E-07	2.436962
STK32B	1.69E-05	2.389932
KIAA1377	3.73E-03	2.384472
ENST00000390632	1.32E-04	2.355071
HOXA11	4.57E-07	2.319228
ATF7IP2	2.59E-05	2.313631

Table 4.2: List of top 30 genes that were down regulated in CD133$^-$ cells

Gene Name	PValueLogRatio	Mean log2 fold change (down regulation)
A_24_P938577	2.31E-04	-2.61517
A_24_P911519	4.63E-05	-2.62426
C12orf12	3.55E-14	-2.62559
A_24_P942870	1.29E-02	-2.68738
TSHR	4.27E-02	-2.68954
A_24_P800363	2.28E-01	-2.74867
CDH6	5.85E-02	-2.74995
A_24_P376029	1.00E+00	-2.77371
GFM1	6.18E-02	-2.79011
A_24_P642426	5.80E-02	-2.80546
A_32_P849727	1.00E+00	-2.80911
DLX2	2.77E-16	-2.8165
A_24_P931377	1.56E-18	-2.8605

RORI	1.17E-06	-2.86722
TFEC	3.05E-02	-2.87202
MITF	4.87E-02	-2.87412
ENST00000328752	2.47E-06	-2.91985
A_24_P923789	2.10E-02	-2.92733
KNG1	1.33E-04	-2.96233
SCN9A	1.00E+00	-2.99605
ENST00000436580	1.00E+00	-3.11519
LMOD3	1.00E+00	-3.13762
A_24_P923439	9.65E-11	-3.14399
ENST00000481704	4.02E-06	-3.15966
FGF5	3.19E-01	-3.25497
A_32_P53093	3.03E-01	-3.45255
CCDC68	2.63E-20	-3.50641
ENST00000354854	2.72E-06	-3.516
CXorf57	6.69E-03	-3.5772
AK000119	9.79E-04	-3.67492

Micro array results were confirmed by real time PCR for Nanog and Pax6. Reverse transcriptase PCR was done for CD133, PROX1, ABCG2, Oct4, Bmi-1 and Nanog genes to see the differential expression in both the genes (Figure 4.2 c, d, and e).

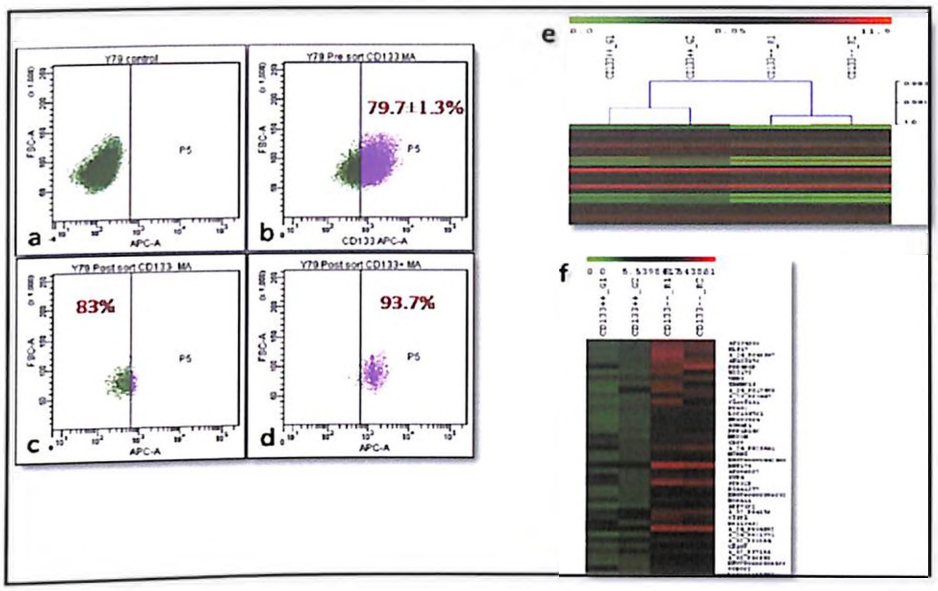


Figure 4.3: Dot plots of a) control Y79 cells b) cells expressing CD133 marker (79.7±1.3%) c&d) Post sort purity of CD133$^-$ (83%) and CD133$^+$ (93.7%) e) Hierarchial clustering of CD133 negative and positive populations f) Heat map generated for the genes in CD133 negative population de regulated by 2 fold change compared to CD133$^+$ cells.

The other important stem cell genes that were significantly up regulated in CD133$^-$ cells was listed in Table 4.3. The embryonic stem cell genes up regulated were HoxB2, HoxA9, Sall1 and Lefty. The neural stem cell genes up regulated consisted of ABCB1, ABCB5, Mushashi 2 and Bmi-1.

Table 4.3: Embryonic and Neural stem cell markers up regulated in CD133$^-$ cells

S.No	Genes highly expressed in CD133 negative cells	Fold expression
1	HOXB2	2.4
2	HOXA9	2.3
3	Sall1	2.2
4	ABCB1	2.2
5	ABCB5	2.1

6	**Lefty**	**2.0**
7	**Mushashi 2**	**1.6**
8	**Bmi-1**	**1.6**

II) Cell Cycle Analysis of Sorted Populations:

Cell sorting was done based on CD133 marker expression. The unsorted Y79 cells present in G0/G1 phase was shown to be 47.7% and in S/G2/M phase were 51.9%. Important observation made in this assay was that the sorted cells of $CD133^+$ population in S/G2/M phase was about 81.1±4.1% and the sorted cells of $CD133^-$ population in G0/G1 phase about 83.3±4.1% of the cell cycle as shown in Figure 4.4 a, b, c, d, e and f.

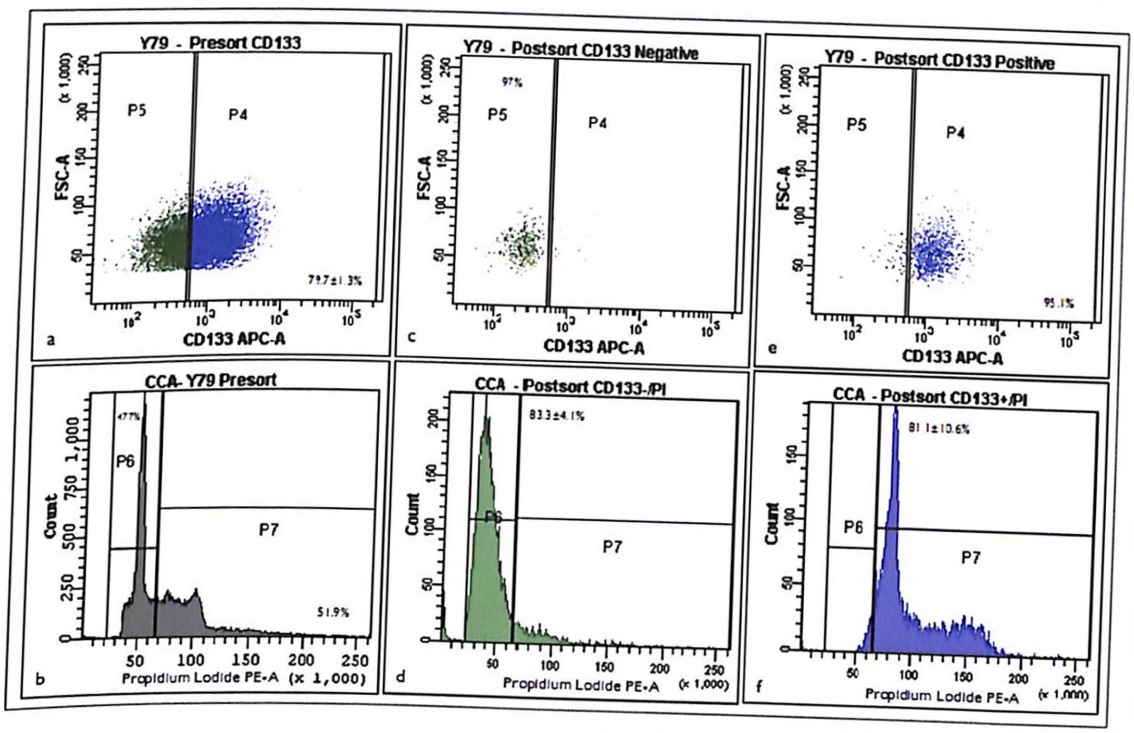


Figure 4.4: Cell cycle analysis of unsorted and sorted populations of Y79 cells – a) CD133 expression in Y79 cells was 79.7±1.3% b) CCA of Y79 cells c) Purity of sorted population of $CD133^-$ cells was 97% d) $CD133^-$ cells in G_0/G_1 phase e) Purity of sorted population of $CD133^-$ cells was 95.1% f) $CD133^+$ cells in $S/G_2/M$ phase.

III) Clone forming ability of sorted populations of Y79 cells:

Clone forming ability of $CD133^-$ cells was 23±4.3% and in $CD133^+$ cells the ability was 9.3±0.5%.

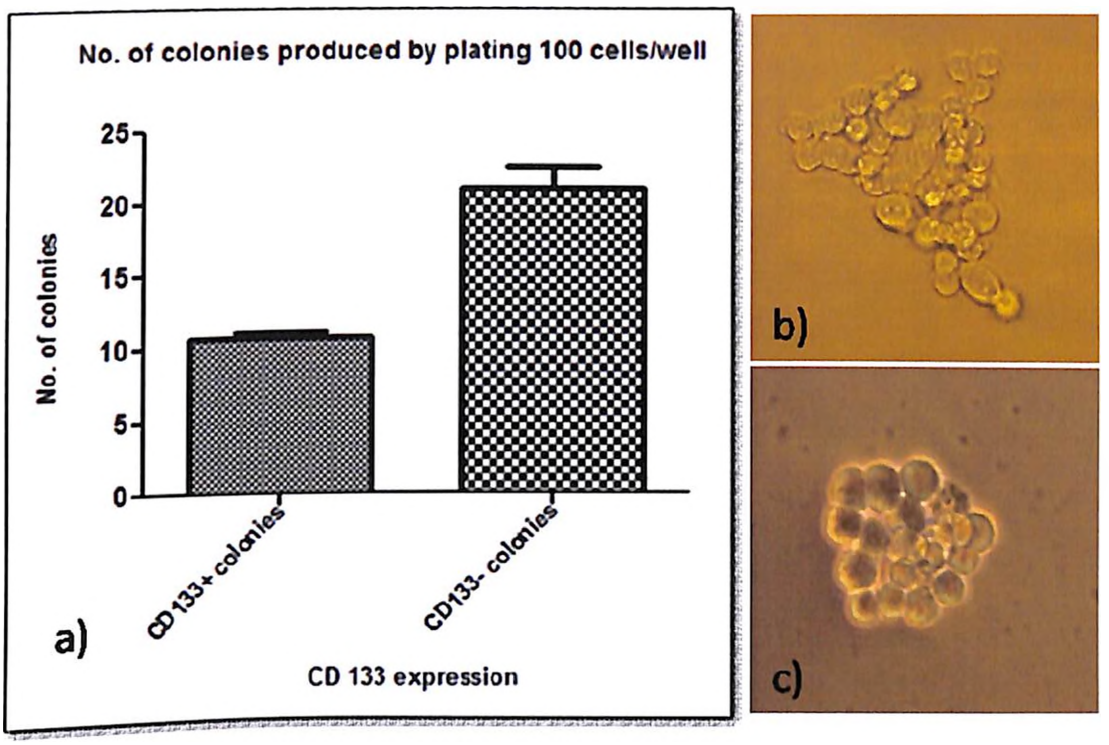


Figure 4.5 a) shows the graphical representation of the clones plated per 100 cells of CD133 negative and positive populations b) Clone of $CD133^+$ cell c) Clone of $CD133^-$ cell – Magnification (200x)

IV) Expansion of CD133 Negative clones:

One of the clones generated from single cell of CD133 negative cell was expanded to confluence and then characterized for the expression of CD133 marker in passage 2, 3, 4 and 5. The expression of CD133 was very minimal in passage 2, 3 and the expression ranged

from 12.4±0.55 to 14.4±1.1%. Its expression increased in passage 4, 5 and it ranged from 34.5±0.1 to 38.8±1.3% as shown in Figure 4.6.

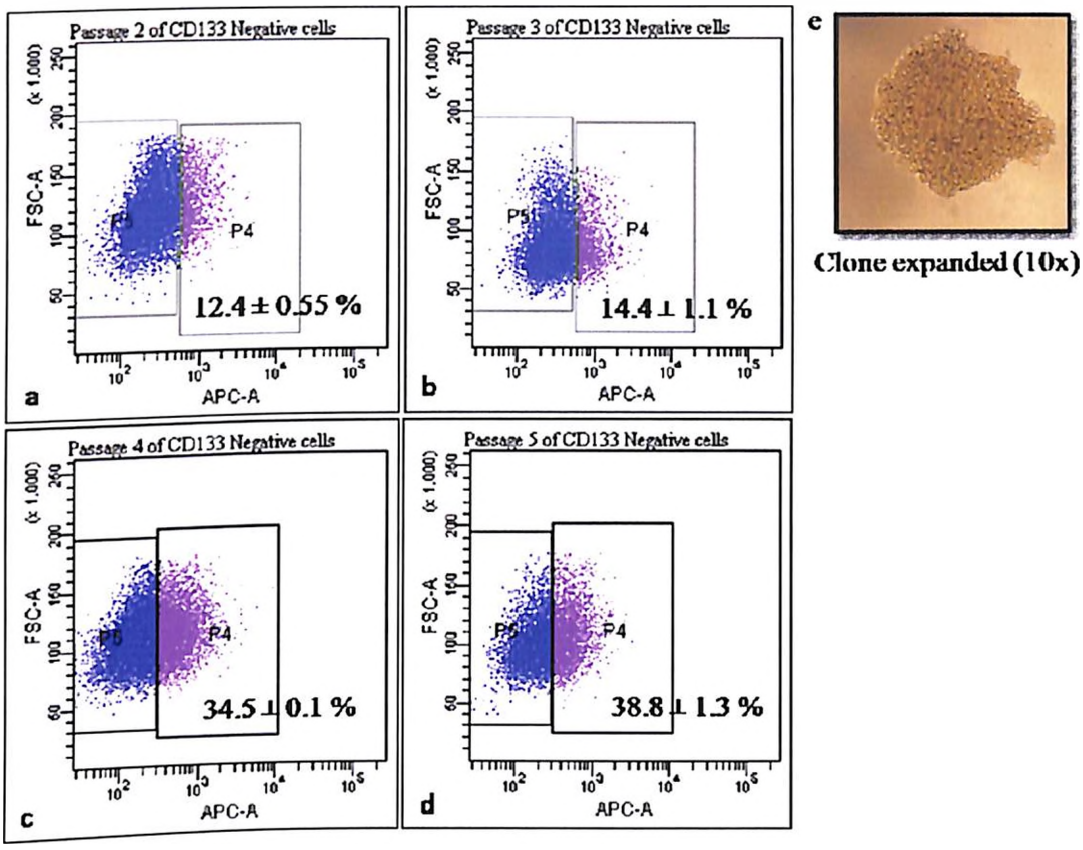


Figure 4.6: Characterization of expanded single cell clone of $CD133^-$ population – a) CD133 expression in cells of passage 2 b) passage 3 c) passage 4 d) passage 5 and e) expanded clone at 40x magnification

V) Effect of Nutlin-3 drug on the p53 expression:

Unsorted Y79 cells on exposure to the drug Nutlin-3 showed 55.2±12.4% cells positive for p53 marker and interestingly all these cells were present at $FSC^{hi}SSC^{hi}$ region. The p53 negative cells were seen in $FSC^{low}SSC^{low}$ region, suggesting that the putative stem cell population is resistant to drug induced over expression of p53 (Figure 4.7).

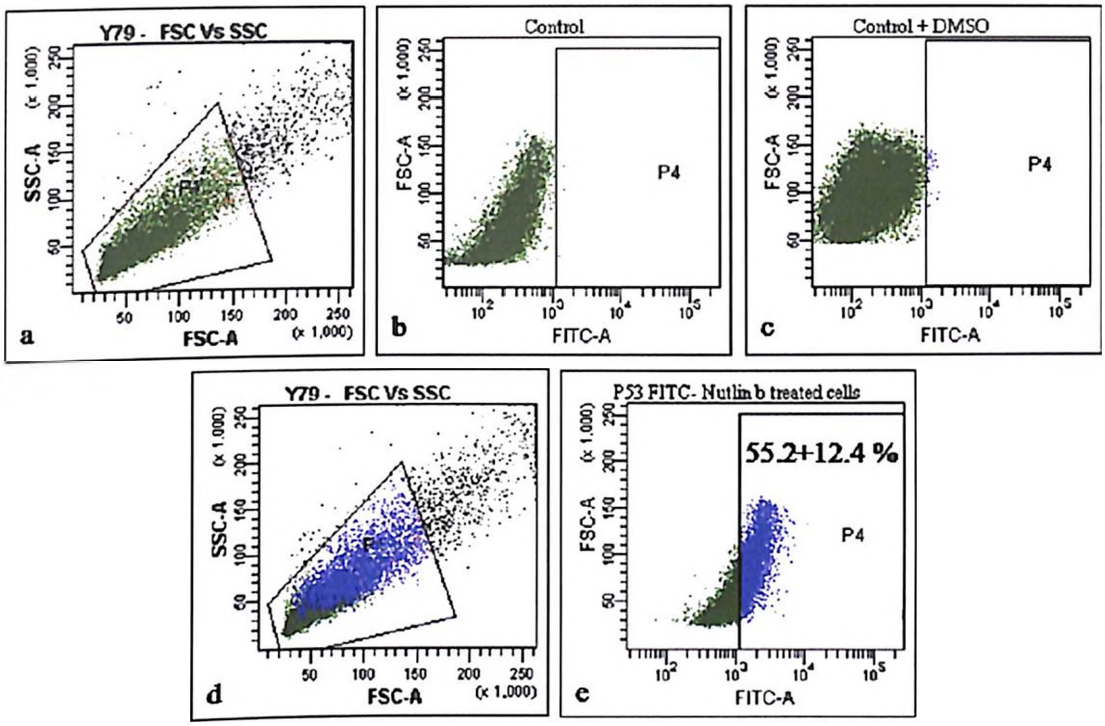


Figure 4.7: Effect of Nutlin-3 drug on the expression of p53 in Y79 cells: a) Dotplot showing the FSC Vs SSC scatter b) Control without drug c) DMSO treated cells d) FSC Vs SSC showing p53 positive (blue region) and negative cells (green region) e) Y79 cells showing p53 expression (55.2±12.4%) on treatment with Nutlin-3 drug

Discussion: Rb, being a small round cell tumor, shows extensive areas of ischemic necrosis as the rapidly dividing tumor cells outgrow their own. Our FACS data also supported the same- the dead cells were more and constituted in the range of 50.1±2.9% to 93.1±0.7% of all cells (Inomata et al. 1994; Balla et al. 2009). In view of this limitation as well as limited availability of samples from metastatic or recurrent tumors, the evaluation of CSC in tumor specimens becomes a limitation. After identification of putative cancer stem-like cells in the freshly isolated tumor cells, this study focused on identifying the CSCs in Y79 cell lines and also expanded the panel of tests that could validate the CSC properties (Balla et al. 2009).

This study confirmed our hypothesis as well as previous reports that CSCs are present in Y79 cell line. This also fulfills the size phenotype criteria laid by us in our earlier work. However we observed one difference as the cell lines were CD44 negative, hence this could not be considered for isolating the CSC population in cell lines. Instead, we used CD 133 negative cells as representative of, small cells with $CD44^+$ $CD133^-$ in primary tumor cells. The possible reason for down regulation of this marker in Y79 cells might be contributed for the high serum culture conditions. It is possible that due to constant exposure to high hyaluronic acid containing vitreous fluid, these are seen in primary tumor cells but not in culture conditions where they are exposed to serum and not hyaluronic acid, hence over expression of hyaluronate receptor CD44 in these tumors (Balla et al. 2009). Similar to the observations made in Rb cells and other tumors, the Y79 cells show a distribution in all phases of cell cycle. As the tumor grows in size, small percentage of cells leaves the proliferative pool by getting back to G_0/G_1 phase and these cells divide very slowly (Cotran 1994). Remaining cells which are larger fraction of tumor cells eventually differentiate and perish. The cells which are susceptible for chemotherapy (like vincristine, etoposide and carboplatin) are the cells which are in $S/G_2/M$ phase and the cells which become resistance would be in G_0/G_1 phase (Cotran 1994). As evident from literature review now, the stem cells are not in the actively proliferation cell pool but rather in quiescent state. This was shown in ovarian cancer, that stem cell activity is enriched in the quiescent fraction of a tumor that shows the capability to revert to a state of self-renewal and regeneration (Kusumbe et al. 2009). Our study also supports the same. $CD133^-$ cells were in G_0/G_1 phase implicating that these are the cells which are slow cycling and quiescent. They are small,

with less granularity and express stem cell markers like Bmi-1, Oct4, Nanog, Pax6 and PROX1 suggesting that these cells might have cancer stem cell properties.

One of the strategies of promoting cell death was through drug induced over expression of p53 that pushes the cells into apoptosis or cell death. In Rb cells, Laurie et al., shown the down regulation of the MDM2 expression by using Nutlin-3 drug and there by elevating the expression p53 this resulted in the apoptosis of these tumor cells (Laurie et al. 2006). In our study, when Y79 cells were challenged using Nutlin-3 drug and looked for the expression of p53, majority of the cells positive for p53 were having large size and high granularity and the cells negative for p53 are showing lesser size and granularity. As it was shown that majority of CD133 negative cells falls in the region of $FSC^{low}SCC^{low}$ region, these cells might be the cells resistant to Nutin-3 drug.

Another feature of CSC was formation of clones, Siegel et al. has shown the evidence for clone forming ability of Y79 cells, but this study didn't addressed, which population has more ability to form clones (Seigel et al. 2007). Furthermore our results have shown that negative cells showed higher clone forming ability compared to positive cells. One of the $CD133^-$ clone, that was expanded to passage 5 showed that from passage 2 to passage 5, the number of cells positive for CD133 also increased, suggesting that these negative cells regenerate the original phenotype of tumor cells. In another study by Zong et al. which showed that $CD133^+$ cells, when cultured in neurosphere assay medium have shown the sphere forming ability (Zhong et al. 2007). Our results have shown that, even though CD133 positive cells forms clones, which is in agreement with the study of Zong et al. negative cells have higher clone forming ability and when expanded these cells they again formed positive population, showing that the $CD133^-$ cells are cancer initiating cells in Y79 cell line.

Chapter-5
Microarray of Human Rb Tumors

Introduction

Rb is a childhood malignant tumor, which generally develops within 5 years of age. This tumor is the result of mutations in *RB1*, a tumor suppressor gene and it may be manifested in genetic or sporadic forms. *RB1* gene, functions in various mechanisms like anti apoptosis, differentiation, cell cycle regulation, DNA repair and DNA replication (Burkhart et al. 2008).

The differentiation or grade of human tumors is assessed routinely in the histopathology laboratories, with poorly differentiated tumors generally having the worst prognosis. However this grading is based upon histology criteria and the molecular pathways controlling the tumor behavior is poorly understood. The introduction of high-throughput analyses using oligonucleotide arrays has given the ability to assess RNA levels in tumor samples and correlate the signaling pathways that are deregulated in particular type of tumors. Previously, Glinksky and colleagues identified a set of genes called 11-gene signature that were deregulated and has the ability to differentiate tumors that have good or poor prognosis (Glinsky et al. 2005). When this signature had applied on various human tumors it showed high predictive value than other potential sets like 14 gene signature, which did not demonstrate high predictive value (Glinsky et al. 2005). Moreover, evidences from other tumors showed that the cells in tumor have elevated stem cell pathway regulators and as a result may exhibit the resistance to chemotherapy (Kolligs et al. 2002; Dean et al. 2005; Hopfer et al. 2005; Katano 2005; Liu et al. 2006; Rossi et al. 2006). A broad description of stem cell associated regulatory pathways in Rb tumors is lacking and hence, evaluation of various signaling pathways specific for stem cells in Rb tumor may aid in identifying novel biomarkers for better prognosis of the tumor.

The present study has evaluated the expression of various stem cell markers and signaling pathways in Rb tumors as compared to normal retinas. Specifically, we looked at HMGA2 gene and it's signaling in these tumors. In addition, we looked for novel biomarkers expressed in different types of Rb tumors. The expression of these genes was correlated with histo pathological features of the tumors.

Results:

Clinical features: The clinical features of 26 Rb cases are summarized in Appendix Table-1. The mean age of patients was 2.6 yrs in which sixteen patients were males and eleven were females. Out of twenty six cases, there were four eyeballs from bilateral cases and remaining from unilateral. None of the case had BM/CSF involvement. Adjuvant chemotherapy was given to seven cases after enucleation and in other two cases enucleating was done post chemotherapy (Evaluated for IHC). Eleven tumors were categorized as International Classification of Intraocular Retinoblastoma (ICIR) group D and five tumors as ICIR group E.

Histopathology examination:

Sixteen tumors were well & moderately differentiated, while nine were poorly differentiated. Retinocytoma like areas were present in three cases. Histopathologic high risk factors were noted in 12/23 cases, which include 3/11 cases with optic nerve, lamina cribrosa or beyond, 4/11 pre laminar cribrosa, one case each had full thickness choroid, with optic nerve and anterior layers of cribrosa involvement. Two had lamina cribrosa with full thickness choroid involvement and one had post cribrosa involvement. None of the cases showed BM/CSF involvement.

Gene expression in retinoblastoma tumors:

There was up-regulation of 5593genes (≥1.5 fold) and down-regulation of 4864genes (≤ 1.5 fold) in comparison to control retina. A comprehensive analysis of the micro array data revealed genes belonging to cell cycle, DNA repair, amino acyl t-RNA biosynthesis were deregulated. In neuronal tissues like neuron apoptosis, vaculogenesis, somatigenesis, neural crest cell development and differentiation, gene expression was negatively regulated. This study also identified VEGF signaling pathway (p=0.0019), TGF-beta signaling pathway (p=0.009), p53 signaling pathway (p=0.017), Insulin signaling pathway (p=0.032), Chemokine signaling pathway (p=0.011) and Wnt signaling pathway (p=0.05) that are highly deregulated in retinoblastoma tumors. List of top 25 up regulated and down regulated genes in Table 5.1&5.2

Table 5.1: List of top 25 up regulated genes sorted by fold change

Gene symbol	Accession number	Adj p value	average-log2-Fold upregulated
HMGA2	NM_003483	5.83E-10	5.4
HS3ST4	NM_006040	4.41E-09	4.2
KCNC2	NM_139136	4.15E-05	4.1
RNF175	AK091509	1.61E-07	3.7
ENST00000343505	ENST00000343505	2.38E-10	3.6
NPTX1	NM_002522	3.59E-09	3.5
PXDN	AF200348	8.27E-07	3.5
STK32B	NM_018401	7.64E-08	3.5

CD24	L33930	6.19E-05	3.4
DKFZp667G2110	NM_153605	2.68E-07	3.4
CNTNAP4	AK054786	6.61E-05	3.3
CDK6	NM_001259	1.28E-05	3.3
PCDHB10	NM_018930	1.72E-06	3.2
ZNF533	NM_152520	1.44E-06	3.1
CYP20A1	NM_177538	1.14E-07	3.1
MTX3	AK091375	4.72E-07	3.1
OR5T2	AK098491	1.31E-06	3.1
GRIK2	ENST00000333309	9.69E-07	3.1
MFAP4	NM_002404	2.75E-09	3.0
SLC27A6	NM_001017372	2.36E-05	3.0
CCNJL	NM_024565	5.61E-05	3.0
KIAA1509	NM_001080414	2.40E-05	3.0
TTN	NM_133378	8.25E-05	3.0
SEMA5A	ENST00000382496	4.49E-05	3.0
DOCK3	NM_004947	1.09E-05	2.9

Microarray results were confirmed by real time PCR for MycN, HMGA2, Lin 28b and ACVR1C in 13 RB tumors compared to n=2 control retina tissues (Fig. 5.2 a, b, c, d). The mRNA expression levels of MYC N, HMGA2 and Lin28-b was statistically significant and the p values of the genes were 0.001, 0.0012 and 0.0048 respectively. The p value for ACVR1C is statistically significant (p=0.015) by micro array analysis (Table 5.4), which

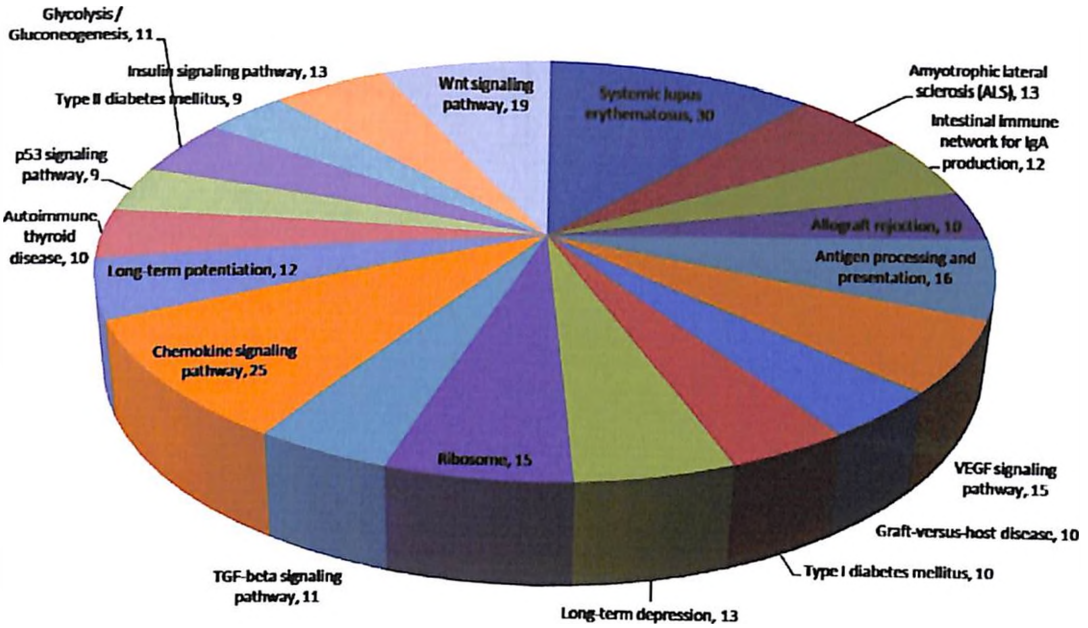
was further validated by using IHC and real time PCR analysis as shown in figure 5.2&5.3. The expression in WD tumors, especially in areas of rosettes is very less compared to PD areas. In retinocytoma areas, the expression is very less/almost absent as shown in figure 5.3-D, F, G, J and L.

Table 5.2: List of top 25 down regulated genes sorted by fold change

Gene symbol	Accession number	Adj p value	Average-log2-Fold down regulation
GALP	NM_033106	3.27E-07	5.4
SAG	NM_000541	1.19E-09	5.0
TTR	NM_000371	2.43E-08	4.4
HBB	NM_000518	1.60E-07	4.2
PROCR	NM_006404	4.50E-10	4.2
TEX12	NM_031275	6.17E-05	4.1
ATXN3L	ENST00000380622	1.92E-04	4.1
NOX4	NM_016931	1.13E-06	4.0
C1orf168	NM_001004303	7.88E-05	3.9
MYCNOS	BC002892	1.06E-04	3.9

EFTUD1	NM_024580	2.55E-04	3.8
BTN3A1	NM_007048	7.52E-05	3.8
OSTbeta	NM_178859	3.99E-04	3.8
CKM	NM_001824	3.02E-07	3.7
HIST1H1A	NM_005325	1.97E-04	3.7
PIK3R5	NM_014308	2.92E-04	3.7
ZCCHC13	NM_203303	4.81E-05	3.7
PPP3CC	NM_005605	1.25E-07	3.7
TMPRSS3	NM_032401	9.73E-05	3.7
KRT12	NM_000223	9.46E-07	3.7
FGA	NM_021871	4.35E-05	3.6
CGB1	NM_033377	4.49E-04	3.5
EGR3	NM_004430	1.21E-05	3.4
WNT2B	NM_004185	5.42E-05	3.4
SLC45A4	BC033223	5.70E-04	3.4

Figure 5.1: Pie diagram representing cumulative data of genes involved in specific pathways in Rb tumors



HMGA2 Signaling: Microarray results showed that the fold expression of HMGA2 is higher in PD compared to MD tumors (Mean expression in five each of MD, PD were 45.66 and 52.4 respectively). Real-time PCR validation showed that mRNA expression levels of HMGA2 and LIN-28B in 13 tumor samples were significantly up regulated and p-values were 0.0012 and 0.0048 respectively. Our results suggest that HMGA2 signaling is highly elevated in human Rb tumors.

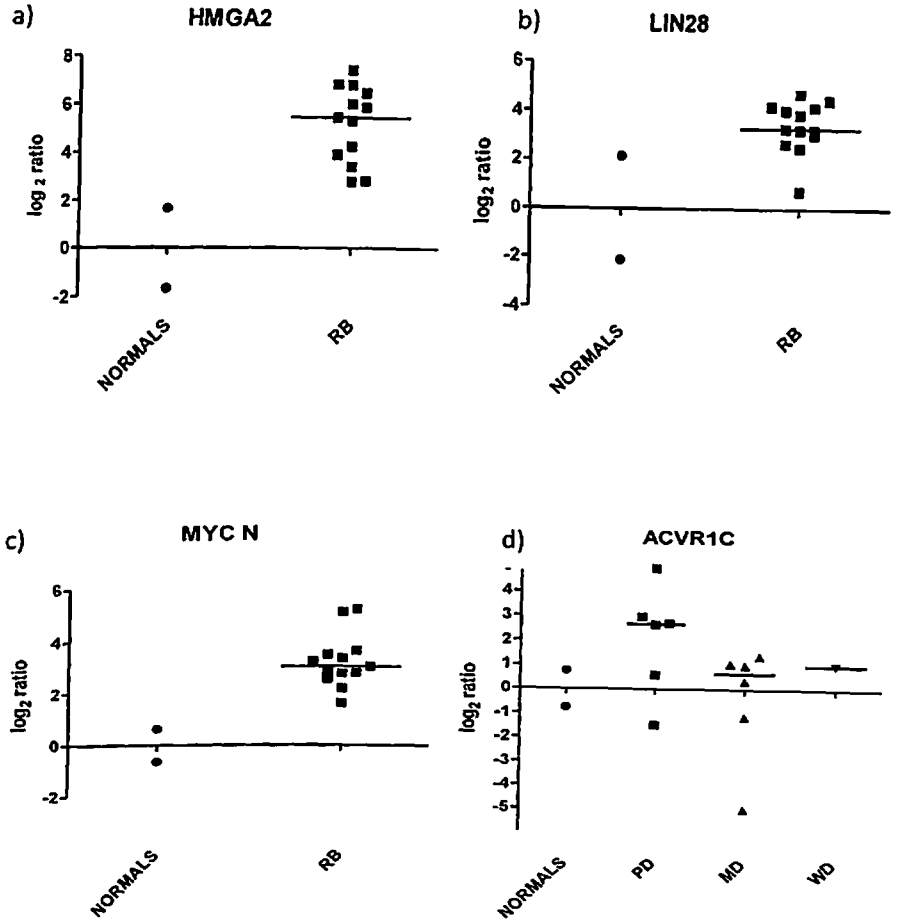


Figure 5.2: Real time expression data of genes a) HMGA2 b) Lin-28b c)MYC N and d) ACVR1C in Rb and control retina samples (Bar indicated median value for all the samples)

Regulators of various other signaling pathways that were deregulated i.e. ≥ or ≤1.5 fold change was listed in Table 5.3.

Table 5.3: Various signaling pathways deregulated in Rb tumors

SNO	Signaling Pathways	Up regulated Genes ≥1.5 folds In at least 5 cases	Down regulated Genes ≤ 1.5 folds In at least 5 cases
1	ERK-MAPK pathway	SRF, SAPS3 and CREB1	PTPRU and MAPK1
2	AKT Signaling Pathway	TP53, RPS6KB1, PDCD4, MYT1, MAP3K8, CHUK, BCL2L11 and BCL2	PIK3R5, PFKFB2, NOS3, MDM2, FOXO1, CDKN1B, CCND1 and BAD
3	IGF Signaling pathway	PTPN11 and IRS1	PIK3R5, MAPK1, IGF1 and HRAS
4	Integrin signaling pathway	TSC1, PTK2, MAPK8, ITPR3, ARHGAP10 and APAF1	RHO, PRKCA, PIK3R5, MAPK1, MAP2K2, HRAS, EIF4E and BAD
5	TGF-β signaling pathway	SMAD7, SMAD4, SMAD3, SMAD2, RPS6KB1, MAPK8, MAPK14, MAP3K4, MAP2K6, MAP2K4 and LIMK1	SMAD6, SHC1, PRKCA, PIK3R5, MLC1, MAPK1, HRAS, DIAPH3, CFL1 and CDC42
6	Jak-Stat pathway	SUMO1, SRC, MCL1 and EGFR	STAT3, SOCS3, PIK3R5, MAPK1 and HRAS
7	NOTCH Pathway	NOTCH1, EP300, RBPJ and MAML	LFNG
8	WNT pathway	TGFβ1, F2R, CREBBP and APC	DVL1 and BMP1
9	SHH pathway	SMO and GLI2	PRKACA
10	Oct4-Sox2	NANOG, JARID2, C3orf63 and BRCA1	FOXD3, FOXA2 and FOXA1
11	BMP signaling pathway	SMURF2, SMURF1,SMAD1, MAPK14, MAP2K6, LIMK1 and CREBBP	SMAD6 and CFL1
12	HMGA2 signaling pathway	HMGA2 and CDK6	RB1, CDKN2A, CDKN2D, CDKN1B and CCND1

The genes that were having differential expression in five PD cases and 6 MD/WD tumors i.e. ≥ or ≤ 2 fold change were listed in Table 5.4

ACVR1C nodal signaling regulator is highly up regulated in PD compared to MD/WD and retinocytoma tumors as shown in Figures 5.2 & 5.3 and Table 5.4. In addition to this pro-apoptotic regulator BAD is significantly down regulated in MD/WD compared to PD tumors ($p=0.015$). Other gene which is down regulated in MD/WD tumors and unregulated in PD tumors was GNB2 and the $p=0.015$ value was shown to be statistically significant.

RASA1 was up regulated in 50% of the MD/WD cases, while it is unregulated in all of the PD tumors. RRM2 was up regulated in 60% of PD tumors and unregulated in 83% of MD/WD cases. SHH was down regulated in 60% of the PD tumors and unregulated in 83% of WD/MD tumors. SMAD3 was highly up regulated in 83.7% of MD/WD tumors and unregulated in 60% of PD tumors. IRS1 gene was shown to be highly up regulated in tumors without HRF's compared to cases with HRF's ($p=0.015$). CXCL14 was down regulated in 80% of cases with HRF's and unregulated in 83.3% of cases without HRF's. LTBP1 was highly up regulated 66.7% of cases without HRF's and unregulated in 80% of cases with HRF's. ZFYVE16 was highly up regulated in 66.7% of cases without HRF's. MAPK8 and ZMIZ1 genes were highly up regulated in 83.4% of cases without HRF's and unregulated in 60% of cases with HRF. The genes that were having differential expression in five tumors with HRF's and six tumors without HRF i.e. $\geq$ or ≤ 2 fold change were listed in Table 5.5.

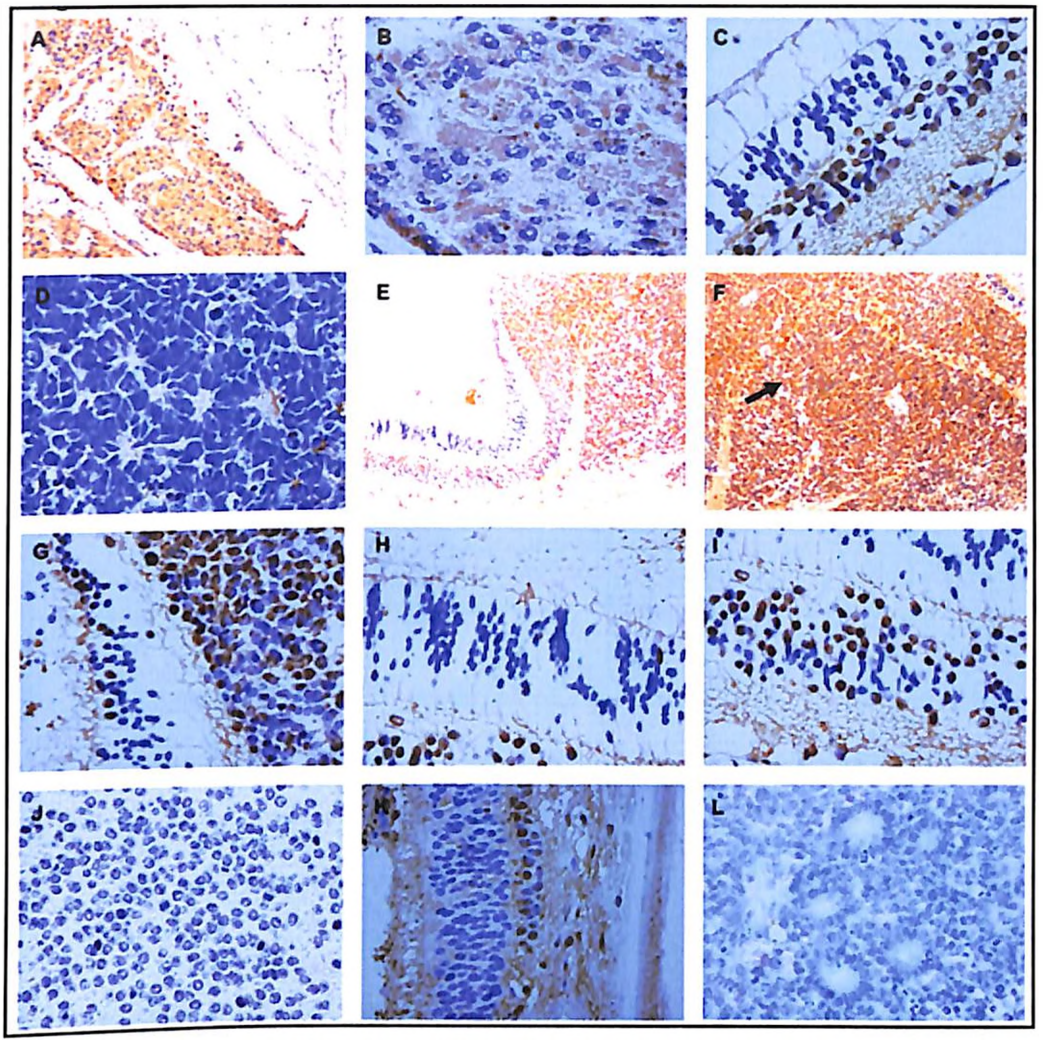


Figure 5.3: IHC pictures of ACVR1C expression in A, B: Pictures of Hepato cellular carcinoma, used as a positive control for ACVR1C marker. C: Normal retina (internal control) and D: Differentiated areas of MD tumor, where there is less expression of ACVR1C. E, F, G, H, I: Retina and tumor of PD Rb tumor, higher expression of ACVR1C in PD tumors. J: Retinocytoma area of Rb tumor. Negative expression of ACVR1C is clearly evident. K, L: Retina of WD tumor. Rosettes were shown to be negative for ACVR1C. A, E, F: Magnification 100x. B, C, D, G, H, I, J, K, L: Magnification 400x

Table 5.4: Significant difference in gene expression between cases of MD/WD Vs PD

S. No	Genes	MD/WD (Fold Change >2.0/<-2.0)	PD (Fold Change >2.0/<-2.0)	p value
1	ACVR1C Homo sapiens activin A receptor, type IC (ACVR1C), mRNA [NM_145259]	0/6	4/5	0.015
2	BAD Homo sapiens BCL2-antagonist of cell death (BAD), transcript variant 1, mRNA [NM_004322]	1/6	5/5	0.015
3	GNB2 Homo sapiens guanine nucleotide binding protein (G protein), beta polypeptide 2 (GNB2), mRNA [NM_005273]	5/6	0/5	0.015
4	RASA1 Homo sapiens RAS p21 protein activator (GTPase activating protein) 1 (RASA1), transcript variant 1, mRNA [NM_002890]	3/6	0/5	0.18
5	RRM2 Homo sapiens ribonucleotide	1/6	3/5	0.24

	reductase M2 polypeptide (RRM2), mRNA [NM_001034]			
6	SHH Homo sapiens sonic hedgehog homolog (Drosophila) (SHH), mRNA [NM_000193]	1/6	3/5	0.24
7	SMAD3 Homo sapiens SMAD family member 3 (SMAD3), mRNA [NM_005902]	5/6	2/5	0.24

Table 5: Significant difference in gene expression between cases with and without HRF's

S. No	Genes	HRF (Fold Change >2.0/<-2.0)	No HRF(Fold Change >2.0/<-2.0	p value
1	IRS1 Homo sapiens insulin receptor substrate 1 (IRS1), mRNA [NM_005544]	0/5	5/6	0.015
2	CXCL14 Homo sapiens chemokine (C-X-C motif) ligand 14 (CXCL14), mRNA [NM_004887]	4/5	1/6	0.08

3	LTBP1 Homo sapiens latent transforming growth factor beta binding protein 1 (LTBP1), transcript variant 1, mRNA [NM_206943]	1/5	4/6	0.24
4	ZFYVE16 Homo sapiens zinc finger, FYVE domain containing 16 (ZFYVE16), mRNA [NM_014733]	2/5	4/6	0.24
5	MAPK8 Mitogen-activated protein kinase 8 (EC 2.7.11.24) (Stress-activated protein kinase JNK1) (c-Jun N-terminal kinase 1) (JNK-46). [Source:Uniprot/SWISSPROT;Acc:P45983] [ENST00000374189]	2/5	5/6	0.24
6	ZMIZ1 Homo sapiens zinc finger, MIZ-type containing 1 (ZMIZ1), mRNA [NM_020338]	2/5	5/6	0.24

Discussion

Rb is the most common intraocular childhood tumor arising from mutations in the both alleles of *RB1* gene. Few studies have demonstrated the presence of markers expressed by slow cycling self-renewal stem like cells in Rb tumors (Kyritsis et al. 1984; Krishnakumar et

al. 2004; Seigel et al. 2005; Mohan et al. 2006; Seigel et al. 2007; Zhong et al. 2007; Balla et al. 2009). As there is limited amount of literature regarding the gene expression profile in these tumors, the present study aimed at analyzing stem cell pathway gene expression profiles in these tumors. The results of our study show that HMGA2, which is a neural stem cell signaling regulator, is highly up regulated. HMGA2 gene and its downstream regulator LIN 28b was highly up regulated in Rb tumor samples in comparison to normal retina. Micro array results have shown that there is an average of 48.6 fold up regulation of HMGA2 gene. Whereas the downstream regulators of HMGA2 signaling like CDKN2A and LIN-28b were significantly deregulated. The expression of CDKN2A was unregulated and LIN-28b gene is up regulated by 2.1 folds. These results suggest the hypothesis that neural stem cell self-renewal signaling is highly up regulated in Rb tumors.

In humans, HMGA2 is strongly expressed in early stages of development and its expression is restricted to particular cell types. In adults, the function of HMGA2 gene is replaced by Bmi-1 in certain tissues (Molofsky et al. 2005; He et al. 2009). Recent studies have shown that HMGA2 gene is involved in self-renewal of fetal neural stem cells and retinal stem/progenitor cells in mouse models (Lord-Grignon et al. 2006; Nishino et al. 2008). It was shown that HMGA2 deficient mice have defects in self-renewal ability of fetal stem cells. This study has suggested that HMGA2 loss do not result in overall decrease in cellular proliferation but specifically affects stem cell self-renewal (Nishino et al. 2008). Expression of HMG family of genes was correlated in various other tumors like neuroblastomas, breast, small cell lung carcinoma and human prolactinomas (Fedele et al. 2002; Finelli et al. 2002; Hisaoka et al. 2002; Hunter et al. 2002; Van Dorpe et al. 2002, Quade et al. 2003; Cerignoli et al. 2004). HMGA2 derepression was observed in retinoblastoma tumors and was shown to

be expressed in high risk metastatic tumors (Mu et al. 2010; Chau et al. 2003; Venkatesan et al. 2009). Moreover, it was shown that micro RNA's like let-7b was down regulated in 39% of Rb tumors (Mukai et al. 1973). This study provides evidence of up regulation of LIN 28b (avg. 2.1 fold) which indirectly suggests that let-7b is not activated and hence could result in increased self renewal signaling in these tumors. Targeting let-7b micro RNA might reduce this signaling and proves to be a therapeutic target in these tumors.

This data also suggests that nodal signaling is highly deregulated as higher ACVR1C expression is observed in PD tumors, the downstream regulators like Smad2/3 was unregulated in 60% of PD tumors. BAD, a pro-apoptotic gene is highly down regulated in MD/WD tumors suggesting that apoptosis is minimal in these subtypes (MD/WD).

The expression of IRS1 was highly up regulated in Rb tumors and this gene was shown to be involved in regulating the self renewal capability of murine embryonic stem cells (Rubin et al. 2007). This gene interestingly showed significantly up regulated ($p=0.015$) in tumors without HRF's compared to the tumors with HRF's. Other gene CXCL14 was shown to be highly down regulated in 80% of tumors with HRF's and unregulated in tumors without HRF's and it was shown that in tongue carcinoma cells over expression of this gene reduced the rate of tumor formation and size of tumor xenografts (Sato et al. 2010).

Micro-array results also revealed that other stem cell self-renewal regulators like Notch pathway for example Notch1, EP300, RBPJ and MAML were marginally up regulated and the negative regulators of this signaling like NUMB and HDCA2 were unregulated. Wnt signaling pathway regulators were down regulated in our data set which is in agreement with the study of Hackam AS et.al. (Silva et al. 2010). This data suggests that stem cell signaling

like HMGA2 and Notch were shown to be activated in these tumors and further supports the hypothesis of presence of cancer stem cells.

This study also evaluated the data set for retinal specific genes that were differentially regulated. The micro-array data was submitted to ret chip database and results show that ten genes related to retina that were highly up regulated are NPTX1, MYO10, GALNT13, PFTK1, CNTN1, PCSK2, PLAGL1, SSTR2, MBP and FZD8 respectively. The genes that were highly down regulated are STAT4, HES1, CLDN5, PAX6, FBOX2, IRX5, GDNF, ROM1, PDE6A and ARR3.

Previous two gene expression studies have shown contradicting results regarding the deregulation of PI3K/AKT/mTOR (insulin pathway) pathway (Ganguly et al. 2010; Chakraborty et al. 2007). Chakraborty et.al has shown that there is up-regulation of this signaling, which is marginally up regulated in our data set. In contrast, this signaling was not regulated in study conducted by Ganguly et.al (Ganguly et al. 2010). In the present study, the primary regulators of this pathway like TP53, RPS6KB1, PIK3R5, ACACA, PCK2, IRS1, PRKX, CBLB, TSC1, MAPK8, CRK, AKT3 and SHC4 were up regulated.

The other reported genes in retinoblastoma tumors like MDM2, MYC-N, CRX and RXRG that were shown to be up regulated in human Rb tumors (Ajioka et al. 2007; Xu et al. 2009). In this study MYC-N is highly up regulated in all tumors, MDM2 and RXRG is marginally up regulated in 4 & 5 cases respectively and unregulated in other tumors. Both CRX, THR beta were down regulated in six cases that were observed. LHX1 (LIM homeobox gene, horizontal cell marker), which was shown to be present in Rb cell of origin (horizontal cells)

in mouse models of retinoblastoma (Laurie et al. 2006), this study showed that this gene is down regulated by 2.5 fold in human Rb samples.

Unlike other tumors where, there is vast amount of gene expression data available in databases, but in Rb, limited data is available. For the same reason in one report they have compared the expression of genes in tumors against adult retina (Chakraborty et al. 2007). In other report they have compared against normal appearing retina in the retinoblastoma eye (Ganguly et al. 2010). However keeping in view that there is non-availability of human infant/juvenile retina, this study attempted to evaluate gene expression profiles of human Rb tumors compared to the retina collected from non tumor calotte of eyeball diagnosed for Rb.

Conclusions

In conclusion the present study has shown that in Rb, a malignant round cell tumor, there is presence of two distinct sub-populations of cells. Among these sub- populations, the P2 cells manifest as primitive when compared with the P3 group. P2 group of cells shows the expression of primitive stem cell, retinal progenitor markers (PROX1 and Syntaxin1A). In addition, we have shown that P3 group shows higher percentage of differentiated cell markers when compared with P2 population. Further studies involving sorting of distinct cell populations and analyzing the functional capability of these cells by clonal and transplantation assays are warranted. Further understanding of the basic biology of these cells would require analysis of differential gene expression and karyotype which is paramount to target these cells for effective therapy.

In support of the evidence in primary tumor cells where $CD44^{+}CD133^{-}$ cells showing the markers for progenitor cell markers PROX1 and Syntaxin 1a, Y79 cell line showed similar marker expression as that of primary tumor cells except for CD44 expression. Primitive markers like Oct4, Nanog, Bmi-1 and PROX1 were highly expressed in $CD133^{-}$ population. Cell cycle analysis also showed that majority of $CD133^{-}$ cells are in G0/G1 phase. In addition to these properties $CD133^{-}$ population have also shown high clone- forming ability and have capability to regenerate the tumor phenotype, which further substantiates our hypothesis.

In Rb tumors, neural stem cell signaling HMGA2 is highly up regulated. Notch signaling is marginally up regulated and other stem cell self renewal pathways like Wnt, SHH were

down regulated. Nodal signaling is also deregulated in these tumors. Along with ACVR1C used for distinguishing the subtypes, the genes IRS1 and CXCL14 can be used as novel prognostic markers for tumors with and without risk factors. Additional studies are required to evaluate functional importance of these markers in Rb tumors.

Specific Contributions of the Study

1. This is the first study in Rb to show two distinct sub-populations expressing variability in stem cell and retinal progenitor markers using freshly isolated tumor cells. Among these populations, the FSC^{low}/SSC^{low} sub-population appeared to be more primitive, since they expressed stem cell (CD44) and retinal progenitor markers (PROX1 and Syntaxin 1A) combined with relatively lower percentage of differentiated markers. Moreover FSC^{hi}/SSC^{low} sub-population showed higher percentage of differentiated markers (CD90 and CD133).

2. This is the first study that has attempted to evaluate the cancer stem cells in Y79 cell line drawing similarities with primary tumors. Cells which are negative for CD133 also showed primitive markers like Oct4, Nanog, Bmi-1 and PROX1. Cell cycle analysis also showed that majority of $CD133^-$ cells are in G_0/G_1 phase. In addition to these properties $CD133^-$ population have also shown high clone forming ability and have capability to regenerate the tumor phenotype, which further substantiates cancer stem cell hypothesis.

3. This study highlights significant up regulation of neural stem cell signaling with marginal up regulation of Notch signaling, down regulation of other stem cell self renewal pathways like Wnt and SHH. Nodal signaling is also deregulated in these tumors.

4. This is the first study to suggest a possible application of ACVR1C to segregate the different subtypes of Rb tumor and that, genes IRS1 and CXCL14 can be used as novel prognostic markers for tumors with and without risk factors.

Future Scope of the Work

1. The bimodal selection of CSC (FSC^{low}/SSC^{low}, $CD44^{+}CD133^{-}$) in Rb could be further validated on larger sample size with survival pattern.
2. Further validation of CSC could be done by xeno-transplantation and chemo/radio resistance. The characterized CSC phenotype i.e. $CD133^{-}$ cells can be further confirmed by serial transplantation assays in animal models. Drugs targeting this phenotype can be screened for possible therapeutic efficacy in patients.
3. Exploring the option of targeting Lin28b as a viable therapeutic target to block HMGA2 signaling in human Rb tumors.
4. Makers that have shown significant differential expression in different subtypes of tumors and tumors with and without HRFs can further be validated in large number of samples for possible use of these markers as novel prognostic markers (optional).

Limitations of the Study

1. Though the number of enucleated eyes with Rb included in this study were n=14 only n=7 number could fulfill the inclusion criteria for flow cytometry and gene expression studies. Further validation of results on larger samples would aid in getting results with statistical significance.

2. Primary cultures of Rb tumor cells could not be established and standardized from the experiments planned in this study as most of the primary cultures were established from highly metastatic samples and it is very rare to get such samples at our center. To compensate for it, *in vitro* functional characterization of cancer stem cells was carried out in Y79 Rb cell lines. Similar characterization in tumor cells would aid in better understanding of the tumor regulation.

3. In situ localization of some of important list of markers could not be done to validate the results obtained in FACS and microarray studies. *In vivo* characterization of these cells would confirm the phenotype as cancer stem cells and further drug therapies can be designed to target these cells.

4. Lack of adequate number of controls for micro array studies – this is an inherent problem, as there is no normal retina available from the age matched controls for the study purpose.

References

Abramson, D. H., T. T. Du, et al. (2002). "(Neonatal) retinoblastoma in the first month of life." Arch Ophthalmol **120**(6): 738-42.

Aguirre-Ghiso, J. A. (2007). "Models, mechanisms and clinical evidence for cancer dormancy." Nat Rev Cancer 7(11): 834-46.

Ajioka, I., R. A. Martins, et al. (2007). "Differentiated horizontal interneurons clonally expand to form metastatic retinoblastoma in mice." Cell **131**(2): 378-90.

Al-Hajj, M., M. W. Becker, et al. (2004). "Therapeutic implications of cancer stem cells." Curr Opin Genet Dev **14**(1): 43-7.

Al-Hajj, M. and M. F. Clarke (2004). "Self-renewal and solid tumor stem cells." Oncogene **23**(43): 7274-82.

Al-Hajj, M., M. S. Wicha, et al. (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A **100**(7): 3983-8.

Al-Ubaidi, M. R., R. L. Font, et al. (1992). "Bilateral retinal and brain tumors in transgenic mice expressing simian virus 40 large T antigen under control of the human interphotoreceptor retinoid-binding protein promoter." J Cell Biol **119**(6): 1681-7.

Alison, M. R., R. Poulsom, et al. (2002). "An introduction to stem cells." J Pathol **197**(4): 419-23.

Alison, M. R., P. Vig, et al. (2004). "Hepatic stem cells: from inside and outside the liver?" Cell Prolif **37**(1): 1-21.

Allenspach, E. J., I. Maillard, et al. (2002). "Notch signaling in cancer." Cancer Biol Ther **1**(5): 466-76.

Ata-ur-Rasheed, M., G. Vemuganti, et al. (2002). "Mutational analysis of the RB1 gene in Indian patients with retinoblastoma." Ophthalmic Genet **23**(2): 121-8.

Balch, C., J. S. Montgomery, et al. (2005). "New anti-cancer strategies: epigenetic therapies and biomarkers." Front Biosci **10**: 1897-931.

Balla, M. M., G. K. Vemuganti, et al. (2009). "Phenotypic characterization of retinoblastoma for the presence of putative cancer stem-like cell markers by flow cytometry." Invest Ophthalmol Vis Sci **50**(4): 1506-14.

Bapat, S., Collins, A., Dean, M., Nephew, K. and Rasheed, S. (2008) Cancer Stem Cells: Similarities and Variations in the Theme of Normal Stem Cells, in Cancer Stem Cells: Identification and Targets (ed S. Bapat), John Wiley & Sons, Inc., Hoboken, NJ, USA. doi: 10.1002/9780470391594.ch1

Bapat, S. A., A. M. Mali, et al. (2005). "Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer." Cancer Res **65**(8): 3025-9.

Barber, R. D., D. W. Harmer, et al. (2005). "GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues." Physiol Genomics 21(3): 389-95.

Baylin, S. B. and J. E. Ohm (2006). "Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction?" Nat Rev Cancer **6**(2): 107-16.

Beattie, G. M., T. Otonkoski, et al. (1997). "Functional beta-cell mass after transplantation of human fetal pancreatic cells: differentiation or proliferation?" Diabetes **46**(2): 244-8.

Becker, A. J., C. E. Mc, et al. (1963). "Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells." Nature **197**: 452-4.

Berezovska, O. P.. A. B. Glinskii, et al. (2006). "Essential role for activation of the Polycomb group (PcG) protein chromatin silencing pathway in metastatic prostate cancer." Cell Cycle 5(16): 1886-901.

Bernstein, B. E., T. S. Mikkelsen, et al. (2006). "A bivalent chromatin structure marks key developmental genes in embryonic stem cells." Cell **125**(2): 315-26.

Bhattacharya, S., J. D. Jackson, et al. (2003). "Direct identification and enrichment of retinal stem cells/progenitors by Hoechst dye efflux assay." Invest Ophthalmol Vis Sci **44**(6): 2764-73.

Bianco P, Riminucci M.(1998). The bone marrow stroma invivo: Ontogeny, structure, cellular composition and changes in disease. In Marrow Stromal Cell Culture. Hand books in Practical Animal Cell Biology, ed. J. N. Beresford and M. E. Owen, p.1025. Cambridge, UK: Cambridge University Press.

Blanpain, C., W. E. Lowry, et al. (2004). "Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche." Cell **118**(5): 635-48.

Bodnar, A. G., M. Ouellette, et al. (1998). "Extension of life-span by introduction of telomerase into normal human cells." Science **279**(5349): 349-52.

Bonnet, D. and J. E. Dick (1997). "Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell." Nat Med **3**(7): 730-7.

Bottai, D., R. Fiocco, et al. (2003). "Neural stem cells in the adult nervous system." J Hematother Stem Cell Res **12**(6): 655-70.

Brustle, O., K. Choudhary, et al. (1998). "Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats." Nat Biotechnol **16**(11): 1040-4.

Burkert, J., N. A. Wright, et al. (2006). "Stem cells and cancer: an intimate relationship." J Pathol **209**(3): 287-97.

Calabrese, C., H. Poppleton, et al. (2007). "A perivascular niche for brain tumor stem cells." Cancer Cell **11**(1): 69-82.

Calvi, L. M., G. B. Adams, et al. (2003). "Osteoblastic cells regulate the haematopoietic stem cell niche." Nature **425**(6960): 841-6.

Camassei, F. D., R. Cozza, et al. (2003). "Expression of the lipogenic enzyme fatty acid synthase (FAS) in retinoblastoma and its correlation with tumor aggressiveness." Invest Ophthalmol Vis Sci **44**(6): 2399-403.

Caplan, A. I. (1994). "The mesengenic process." Clin Plast Surg **21**(3): 429-35.

Caussinus, E. and F. Hirth (2007). "Asymmetric stem cell division in development and cancer." Prog Mol Subcell Biol **45**: 205-25.

Challen, G. A. and M. H. Little (2006). "A side order of stem cells: the SP phenotype." Stem Cells **24**(1): 3-12.

Chen, D., I. Livne-bar, et al. (2004). "Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma." Cancer Cell **5**(6): 539-51.

Chenn, A. and S. K. McConnell (1995). "Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis." Cell **82**(4): 631-41.

Chiba, T., K. Kita, et al. (2006). "Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties." Hepatology **44**(1): 240-51.

Clarke, A. R., E. R. Maandag, et al. (1992). "Requirement for a functional Rb-1 gene in murine development." Nature **359**(6393): 328-30.

Cobaleda, C., N. Gutierrez-Cianca, et al. (2000). "A primitive hematopoietic cell is the target for the leukemic transformation in human philadelphia-positive acute lymphoblastic leukemia." Blood 95(3): 1007-13.

Collins, A. T., P. A. Berry, et al. (2005). "Prospective identification of tumorigenic prostate cancer stem cells." Cancer Res 65(23): 10946-51.

Cotran, K., Robbins (1994). Pathologic basis of disease, W.B. Saunders company.

Cotsarelis, G., S. Z. Cheng, et al. (1989). "Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells." Cell 57(2): 201-9.

Cotran K. R., Ed. (1994). Pathologic basis of disease. Robbins Pathologic basis of disease. 5th Ed. USA, W.B. Saunders & Co, 81.

Courtenay, V. D. and J. Mills (1978). "An in vitro colony assay for human tumours grown in immune-suppressed mice and treated in vivo with cytotoxic agents." Br J Cancer 37(2): 261-8.

Courtenay, V. D., P. J. Selby, et al. (1978). "Growth of human tumour cell colonies from biopsies using two soft-agar techniques." Br J Cancer 38(1): 77-81.

Das, P. M. and R. Singal (2004). "DNA methylation and cancer." J Clin Oncol 22(22): 4632-42.

Davanger, M. and A. Evensen (1971). "Role of the pericorneal papillary structure in renewal of corneal epithelium." Nature 229(5286): 560-1.

Dean, M., T. Fojo, et al. (2005). "Tumour stem cells and drug resistance." Nat Rev Cancer 5(4): 275-84.

Decraene, C., R. Benchaouir, et al. (2005). "Global transcriptional characterization of SP and MP cells from the myogenic C2C12 cell line: effect of FGF6." Physiol Genomics 23(2): 132-49.

Dimri, G. P., J. L. Martinez, et al. (2002). "The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells." Cancer Res 62(16): 4736-45.

Discher, D. E., D. J. Mooney, et al. (2009). "Growth factors, matrices, and forces combine and control stem cells." Science 324(5935): 1673-7.

Donovan, P. J. and J. Gearhart (2001). "The end of the beginning for pluripotent stem cells." Nature 414(6859): 92-7.

Doucas, H., G. Garcea, et al. (2005). "Changes in the Wnt signalling pathway in gastrointestinal cancers and their prognostic significance." Eur J Cancer 41(3): 365-79.

Duensing, S. and K. Munger (2003). "Human papillomavirus type 16 E7 oncoprotein can induce abnormal centrosome duplication through a mechanism independent of inactivation of retinoblastoma protein family members." J Virol 77(22): 12331-5.

Duncan, A. W., F. M. Rattis, et al. (2005). "Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance." Nat Immunol 6(3): 314-22.

Eagle, R. C., Jr., J. A. Shields, et al. (1989). "Malignant transformation of spontaneously regressed retinoblastoma, retinoma/retinocytoma variant." Ophthalmology 96(9): 1389-95.

Elenbaas, B., L. Spirio, et al. (2001). "Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells." Genes Dev 15(1): 50-65.

Elison, J. R., D. Cobrinik, et al. (2006). "Small molecule inhibition of HDM2 leads to p53-mediated cell death in retinoblastoma cells." Arch Ophthalmol 124(9): 1269-75.

Evans, M. J. (1972). "The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratoma cells." J Embryol Exp Morphol **28**(1): 163-76.

Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." Nature **292**(5819): 154-6.

Fang, D., T. K. Nguyen, et al. (2005). "A tumorigenic subpopulation with stem cell properties in melanomas." Cancer Res **65**(20): 9328-37.

Feinberg, A. P., R. Ohlsson, et al. (2006). "The epigenetic progenitor origin of human cancer." Nat Rev Genet **7**(1): 21-33.

Feinberg, A. P. and B. Tycko (2004). "The history of cancer epigenetics." Nat Rev Cancer **4**(2): 143-53.

Ferrari, G., G. Cusella-De Angelis, et al. (1998). "Muscle regeneration by bone marrow-derived myogenic progenitors." Science **279**(5356): 1528-30.

Filho, J. P., Z. M. Correa, et al. (2005). "Histopathological features and P-glycoprotein expression in retinoblastoma." Invest Ophthalmol Vis Sci **46**(10): 3478-83.

Fish, J. L., Y. Kosodo, et al. (2006). "Aspm specifically maintains symmetric proliferative divisions of neuroepithelial cells." Proc Natl Acad Sci U S A **103**(27): 10438-43.

Folberg, R., D. Salomao, et al. (2003). "Recommendations for the reporting of tissues removed as part of the surgical treatment of common malignancies of the eye and its adnexa. The Association of Directors of Anatomic and Surgical Pathology." Hum Pathol **34**(2): 114-8.

Galli, R., E. Binda, et al. (2004). "Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma." Cancer Res **64**(19): 7011-21.

Gallie, B. L., R. M. Ellsworth, et al. (1982). "Retinoma: spontaneous regression of retinoblastoma or benign manifestation of the mutation?" Br J Cancer **45**(4): 513-21.

Gan, Q., T. Yoshida, et al. (2007). "Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells." Stem Cells **25**(1): 2-9.

Ganguly, A. and C. L. Shields (2010) "Differential gene expression profile of retinoblastoma compared to normal retina." Mol Vis **16**: 1292-303.

Ganss, R. (2006). "Tumor stroma fosters neovascularization by recruitment of progenitor cells into the tumor bed." J Cell Mol Med **10**(4): 857-65.

Gardiner-Garden, M. and M. Frommer (1987). "CpG islands in vertebrate genomes." J Mol Biol **196**(2): 261-82.

Giangreco, A., K. R. Groot, et al. (2007). "Lung cancer and lung stem cells: strange bedfellows?" Am J Respir Crit Care Med **175**(6): 547-53.

Giangreco, A., S. D. Reynolds, et al. (2002). "Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction." Am J Pathol **161**(1): 173-82.

Glinsky, G. V. (2008). ""Stemness" genomics law governs clinical behavior of human cancer: implications for decision making in disease management." J Clin Oncol **26**(17): 2846-53.

Glinsky, G. V., O. Berezovska, et al. (2005). "Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer." J Clin Invest **115**(6): 1503-21.

Goodell, M. A., K. Brose, et al. (1996). "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo." J Exp Med **183**(4): 1797-806.

Gostjeva, E. V. and W. G. Thilly (2005). "Stem cell stages and the origins of colon cancer: a multidisciplinary perspective." Stem Cell Rev **1**(3): 243-51.

Gregg, C. and S. Weiss (2003). "Generation of functional radial glial cells by embryonic and adult forebrain neural stem cells." J Neurosci **23**(37): 11587-601.

Greider, C. W. and E. H. Blackburn (1996). "Telomeres, telomerase and cancer." Sci Am **274**(2): 92-7.

Grichnik, J. M., J. A. Burch, et al. (2006). "Melanoma, a tumor based on a mutant stem cell?" J Invest Dermatol **126**(1): 142-53.

Gronthos, S., A. C. Zannettino, et al. (1999). "Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells." J Bone Miner Res **14**(1): 47-56.

Gunthert, U., M. Hofmann, et al. (1991). "A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells." Cell **65**(1): 13-24.

Hahn, W. C., S. A. Stewart, et al. (1999). "Inhibition of telomerase limits the growth of human cancer cells." Nat Med **5**(10): 1164-70.

Hamburger, A. W. and S. E. Salmon (1977). "Primary bioassay of human tumor stem cells." Science **197**(4302): 461-3.

Harley, C. B., N. W. Kim, et al. (1994). "Telomerase, cell immortality, and cancer." Cold Spring Harb Symp Quant Biol **59**: 307-15.

Hirschmann-Jax, C., A. E. Foster, et al. (2004). "A distinct "side population" of cells with high drug efflux capacity in human tumor cells." Proc Natl Acad Sci U S A **101**(39): 14228-33.

Hofmann, M., W. Rudy, et al. (1991). "CD44 splice variants confer metastatic behavior in rats: homologous sequences are expressed in human tumor cell lines." Cancer Res **51**(19): 5292-7.

Hong, K. U., S. D. Reynolds, et al. (2004). "Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium." Am J Pathol **164**(2): 577-88.

Hopfer, O., D. Zwahlen, et al. (2005). "The Notch pathway in ovarian carcinomas and adenomas." Br J Cancer **93**(6): 709-18.

Howes, K. A., J. G. Lasudry, et al. (1994). "Photoreceptor cell tumors in transgenic mice." Invest Ophthalmol Vis Sci **35**(2): 342-51.

Howes, K. A., N. Ransom, et al. (1994). "Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53." Genes Dev **8**(11): 1300-10.

http://stemcells.nih.gov/info/scireport/chapter1.asp.

Huelsken, J., R. Vogel, et al. (2001). "beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin." Cell **105**(4): 533-45.

Inomata, M., A. Kaneko, et al. (1994). "Culture of retinoblastoma cells from clinical specimens: growth-promoting effect of 2-mercaptoethanol." J Cancer Res Clin Oncol **120**(3): 149-55.

Ito, M., Z. Yang, et al. (2007). "Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding." Nature **447**(7142): 316-20.

Jensen, U. B., S. Lowell, et al. (1999). "The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis." Development **126**(11): 2409-18.

Jin, K., Y. Sun, et al. (2003). "Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice." Aging Cell 2(3): 175-83.

Jones, D. L., R. M. Alani, et al. (1997). "The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2." Genes Dev 11(16): 2101-11.

Jones, P. A. and S. B. Baylin (2002). "The fundamental role of epigenetic events in cancer." Nat Rev Genet 3(6): 415-28.

Jones, P. A. and S. B. Baylin (2007). "The epigenomics of cancer." Cell 128(4): 683-92.

Jones, P. A. and P. W. Laird (1999). "Cancer epigenetics comes of age." Nat Genet 21(2): 163-7.

Jones, P. H. and F. M. Watt (1993). "Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression." Cell 73(4): 713-24.

Jordan, C. T., M. L. Guzman, et al. (2006). "Cancer stem cells." N Engl J Med 355(12): 1253-61.

Kahan, B. W. and B. Ephrussi (1970). "Developmental potentialities of clonal in vitro cultures of mouse testicular teratoma." J Natl Cancer Inst 44(5): 1015-36.

Katano, M. (2005). "Hedgehog signaling pathway as a therapeutic target in breast cancer." Cancer Lett 227(2): 99-104.

Kerever, A., J. Schnack, et al. (2007). "Novel extracellular matrix structures in the neural stem cell niche capture the neurogenic factor fibroblast growth factor 2 from the extracellular milieu." Stem Cells 25(9): 2146-57.

Kiel, M. J. and S. J. Morrison (2008). "Uncertainty in the niches that maintain haematopoietic stem cells." Nat Rev Immunol 8(4): 290-301.

Kiel, M. J., O. H. Yilmaz, et al. (2005). "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells." Cell 121(7): 1109-21.

Kim, C. F., E. L. Jackson, et al. (2005). "Identification of bronchioalveolar stem cells in normal lung and lung cancer." Cell 121(6): 823-35.

Kim, N. W., M. A. Piatyszek, et al. (1994). "Specific association of human telomerase activity with immortal cells and cancer." Science 266(5193): 2011-5.

Kiran, V. S., C. Kannabiran, et al. (2003). "Mutational screening of the RB1 gene in Indian patients with retinoblastoma reveals eight novel and several recurrent mutations." Hum Mutat 22(4): 339.

Kleinsmith, L. J. and G. B. Pierce, Jr. (1964). "Multipotentiality of Single Embryonal Carcinoma Cells." Cancer Res 24: 1544-51.

Knudson, A. G., Jr. (1971). "Mutation and cancer: statistical study of retinoblastoma." Proc Natl Acad Sci U S A 68(4): 820-3.

Kolligs, F. T., G. Bommer, et al. (2002). "Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis." Digestion 66(3): 131-44.

Kondo, T., T. Setoguchi, et al. (2004). "Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line." Proc Natl Acad Sci U S A 101(3): 781-6.

Krishnakumar, S., K. Mallikarjuna, et al. (2004). "Multidrug resistant proteins: P-glycoprotein and lung resistance protein expression in retinoblastoma." Br J Ophthalmol 88(12): 1521-6.

Krivtsov, A. V., D. Twomey, et al. (2006). "Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9." Nature **442**(7104): 818-22.

Kruger., G. M., Carla F., (2006). "Bronchioalveolar stem cells and chemotherapy resistance in lung cancer". Proceedings of the 4th ISSCR annual meeting. Metro Toronto Convention Center, Toronto, Ontario, Canada.

Kruh, G. D. (2003). "Introduction to resistance to anticancer agents." Oncogene **22**(47): 7262-4.

Kurrey, N. K., S. P. Jalgaonkar, et al. (2009). "Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells." Stem Cells **27**(9): 2059-68.

Kusumbe, A. P. and S. A. Bapat (2009). "Cancer stem cells and aneuploid populations within developing tumors are the major determinants of tumor dormancy." Cancer Res **69**(24): 9245-53.

Kusumbe, A. P., A. M. Mali, et al. (2009). "CD133-expressing stem cells associated with ovarian metastases establish an endothelial hierarchy and contribute to tumor vasculature." Stem Cells **27**(3): 498-508.

Lanza. R., Ed. (2006). Essentials of stem cell biology, Elsevier Academic Press.

Lapidot, T., C. Sirard, et al. (1994). "A cell initiating human acute myeloid leukaemia after transplantation into SCID mice." Nature **367**(6464): 645-8.

Larderet, G., N. O. Fortunel, et al. (2006). "Human side population keratinocytes exhibit long-term proliferative potential and a specific gene expression profile and can form a pluristratified epidermis." Stem Cells **24**(4): 965-74.

Laurie, N. A., S. L. Donovan, et al. (2006). "Inactivation of the p53 pathway in retinoblastoma." Nature **444**(7115): 61-6.

Lee, J., S. Kotliarova, et al. (2006). "Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines." Cancer Cell 9(5): 391-403.

Lee W.R., Intraocular Tumors. A Text and Atlas. WB Saunders; 1992:117-136.

Li, C., D. G. Heidt, et al. (2007). "Identification of pancreatic cancer stem cells." Cancer Res 67(3): 1030-7.

Li, W., S. A. Johnson, et al. (2004). "Hematopoietic stem cell repopulating ability can be maintained in vitro by some primary endothelial cells." Exp Hematol 32(12): 1226-37.

Lim, D. A., A. D. Tramontin, et al. (2000). "Noggin antagonizes BMP signaling to create a niche for adult neurogenesis." Neuron 28(3): 713-26.

Lindsell, C. E., J. Boulter, et al. (1996). "Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development." Mol Cell Neurosci 8(1): 14-27.

Liu, S., G. Dontu, et al. (2006). "Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells." Cancer Res 66(12): 6063-71.

Lois, C. and A. Alvarez-Buylla (1993). "Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia." Proc Natl Acad Sci U S A 90(5): 2074-7.

Luskin, M. B. (1993). "Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone." Neuron 11(1): 173-89.

MacPherson, D., J. Sage, et al. (2004). "Cell type-specific effects of Rb deletion in the murine retina." Genes Dev 18(14): 1681-94.

Magramm, I., D. H. Abramson, et al. (1989). "Optic nerve involvement in retinoblastoma." Ophthalmology 96(2): 217-22.

Mani, S. A., W. Guo, et al. (2008). "The epithelial-mesenchymal transition generates cells with properties of stem cells." Cell **133**(4): 704-15.

Margo, C., A. Hidayat, et al. (1983). "Retinocytoma. A benign variant of retinoblastoma." Arch Ophthalmol **101**(10): 1519-31.

Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." Proc Natl Acad Sci U S A **78**(12): 7634-8.

Marx, J. (2003). "Cancer research. Mutant stem cells may seed cancer." Science **301**(5638): 1308-10.

Matsui, W., C. A. Huff, et al. (2004). "Characterization of clonogenic multiple myeloma cells." Blood **103**(6): 2332-6.

Maw, M. A., D. Corbeil, et al. (2000). "A frameshift mutation in prominin (mouse)-like 1 causes human retinal degeneration." Hum Mol Genet **9**(1): 27-34.

Meads, M. B., R. A. Gatenby, et al. (2009). "Environment-mediated drug resistance: a major contributor to minimal residual disease." Nat Rev Cancer **9**(9): 665-74.

Metsaranta, M., U. M. Kujala, et al. (1996). "Evidence for insufficient chondrocytic differentiation during repair of full-thickness defects of articular cartilage." Matrix Biol **15**(1): 39-47.

Mikkelsen, T. S., M. Ku, et al. (2007). "Genome-wide maps of chromatin state in pluripotent and lineage-committed cells." Nature **448**(7153): 553-60.

Mills, M. D., J. J. Windle, et al. (1999). "Retinoblastoma in transgenic mice: models of hereditary retinoblastoma." Surv Ophthalmol **43**(6): 508-18.

Minna, J. D., J. M. Kurie, et al. (2003). "A big step in the study of small cell lung cancer." Cancer Cell **4**(3): 163-6.

Mitchell, K. E., M. L. Weiss, et al. (2003). "Matrix cells from Wharton's jelly form neurons and glia." Stem Cells **21**(1): 50-60.

Mohan, A., M. Kandalam, et al. (2006). "Stem cell markers: ABCG2 and MCM2 expression in retinoblastoma." Br J Ophthalmol **90**(7): 889-93.

Molnar, M. L., K. Stefansson, et al. (1984). "Immunohistochemistry of retinoblastomas in humans." Am J Ophthalmol **97**(3): 301-7.

Moore, K. A. and I. R. Lemischka (2006). "Stem cells and their niches." Science **311**(5769): 1880-5.

Morstyn, G., N. A. Nicola, et al. (1980). "Purification of hemopoietic progenitor cells from human marrow using a fucose-binding lectin and cell sorting." Blood **56**(5): 798-805.

Mu, G., H. Liu, et al. (2010). "Correlation of overexpression of HMGA1 and HMGA2 with poor tumor differentiation, invasion, and proliferation associated with let-7 down-regulation in retinoblastomas." Hum Pathol **41**(4): 493-502

Mukai, N. and S. Kobayashi (1973). "Human adenovirus-induced medulloepitheliomatous neoplasms in Sprague-Dawley rats." Am J Pathol **73**(3): 671-90.

Nakajima, H., T. Goto, et al. (1998). "Characterization of the cells in the repair tissue of full-thickness articular cartilage defects." Histochem Cell Biol **109**(4): 331-8.

Nakamura, T. M. and T. R. Cech (1998). "Reversing time: origin of telomerase." Cell **92**(5): 587-90.

Nowell, P. C. (1976). "The clonal evolution of tumor cell populations." Science **194**(4260): 23-8.

Nuttall, M. E., A. J. Patton, et al. (1998). "Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteopenic disorders." J Bone Miner Res **13**(3): 371-82.

O'Brien, C. A., A. Pollett, et al. (2007). "A human colon cancer cell capable of initiating tumour growth in immunodeficient mice." Nature **445**(7123): 106-10.

Ogawa, K., K. Hamaya, et al. (1969). "Tumor induction by adenovirus type 12 and its target cells in the central nervous system." Gann **60**(4): 383-92.

Ogawa, K., A. Tsutsumi, et al. (1966). "Histogenesis of malignant neoplasm induced by adenovirus type 12." Gann **57**(1): 43-52.

Ogawa, M., D. E. Bergsagel, et al. (1973). "Chemotherapy of mouse myeloma: quantitative cell cultures predictive of response in vivo." Blood **41**(1): 7-15.

Ohm, J. E., K. M. McGarvey, et al. (2007). "A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing." Nat Genet **39**(2): 237-42.

Orford, K. W. and D. T. Scadden (2008). "Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation." Nat Rev Genet **9**(2): 115-28.

Owen, M. (1988). "Marrow stromal stem cells." J Cell Sci Suppl **10**: 63-76.

Pacal, M. and R. Bremner (2006). "Insights from animal models on the origins and progression of retinoblastoma." Curr Mol Med **6**(7): 759-81.

Pardal, R., M. F. Clarke, et al. (2003). "Applying the principles of stem-cell biology to cancer." Nat Rev Cancer **3**(12): 895-902.

Park, C. H., M. Amare, et al. (1980). "Prediction of chemotherapy response in human leukemia using an in vitro chemotherapy sensitivity test on the leukemic colony-forming cells." Blood 55(4): 595-601.

Pasca di Magliano, M. and M. Hebrok (2003). "Hedgehog signalling in cancer formation and maintenance." Nat Rev Cancer 3(12): 903-11.

Pece, S., D. Tosoni, et al. (2010) "Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content." Cell 140(1): 62-73.

Peeters, S. D., D. M. van der Kolk, et al. (2006). "Selective expression of cholesterol metabolism genes in normal CD34+CD38- cells with a heterogeneous expression pattern in AML cells." Exp Hematol 34(5): 622-30.

Penno, M. B., J. T. August, et al. (1994). "Expression of CD44 in human lung tumors." Cancer Res 54(5): 1381-7.

Plikus, M. V., J. A. Mayer, et al. (2008). "Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration." Nature 451(7176): 340-4.

Ponti, D., A. Costa, et al. (2005). "Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties." Cancer Res 65(13): 5506-11.

Potten, C. S. (1981). "Cell replacement in epidermis (keratopoiesis) via discrete units of proliferation." Int Rev Cytol 69: 271-318.

Potten, C. S. and M. Loeffler (1990). "Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt." Development 110(4): 1001-20.

Prince, M. E., R. Sivanandan, et al. (2007). "Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma." Proc Natl Acad Sci U S A 104(3): 973-8.

Puck, T. T. and P. I. Marcus (1956). "Action of x-rays on mammalian cells." J Exp Med **103**(5): 653-66.

Puck, T. T., P. I. Marcus, et al. (1956). "Clonal growth of mammalian cells in vitro; growth characteristics of colonies from single HeLa cells with and without a feeder layer." J Exp Med **103**(2): 273-83.

Raaphorst, F. M., C. J. Meijer, et al. (2003). "Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene." Neoplasia **5**(6): 481-8.

Raff, M. (2003). "Adult stem cell plasticity: fact or artifact?" Annu Rev Cell Dev Biol **19**: 1-22.

Ralph C.E. (1999). Eye Pathology. An Atlas and Basic text. Retinoblastoma and other pediatric intraocular tumors. WB Saunders Co, 1-306.

Rask, K., A. Nilsson, et al. (2003). "Wnt-signalling pathway in ovarian epithelial tumours: increased expression of beta-catenin and GSK3beta." Br J Cancer **89**(7): 1298-304.

Reber, S., S. Matzku, et al. (1990). "Retardation of metastatic tumor growth after immunization with metastasis-specific monoclonal antibodies." Int J Cancer 46(5): 919-27.

Reid, T. W., D. M. Albert, et al. (1974). "Characteristics of an established cell line of retinoblastoma." J Natl Cancer Inst **53**(2): 347-60.

Reubinoff, B. E., M. F. Pera, et al. (2000). "Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro." Nat Biotechnol **18**(4): 399-404.

Reya, T., S. J. Morrison, et al. (2001). "Stem cells, cancer, and cancer stem cells." Nature **414**(6859): 105-11.

Reynolds, B. A. and S. Weiss (1992). "Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system." Science **255**(5052): 1707-10.

Reynolds, S. D., A. Giangreco, et al. (2000). "Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration." Am J Pathol **156**(1): 269-78.

Ricci-Vitiani, L., D. G. Lombardi, et al. (2007). "Identification and expansion of human colon-cancer-initiating cells." Nature **445**(7123): 111-5.

Richard A. Goldsby, T. J. K., Barbara A. Osborne (2000). Immunology, W.h. Freeman & Company.

Robanus-Maandag, E., M. Dekker, et al. (1998). "p107 is a suppressor of retinoblastoma development in pRb-deficient mice." Genes Dev **12**(11): 1599-609.

Rochat, A., K. Kobayashi, et al. (1994). "Location of stem cells of human hair follicles by clonal analysis." Cell **76**(6): 1063-73.

Rogers I, Casper RF. (2004). Umbilical cord blood stem cells. Best Pract Res Clin Obstet Gynaecol. **18**(6):893-908.

Rosenthal, M. D., R. M. Wishnow, et al. (1970). "In vitro growth and differetiation of clonal populations of multipotential mouse clls derived from a transplantable testicular teratocarcinoma." J Natl Cancer Inst **44**(5): 1001-14.

Rudy, W., M. Hofmann, et al. (1993). "The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: each one individually suffices to confer metastatic behavior." Cancer Res 53(6): 1262-8.

Salmon, S. E., A. W. Hamburger, et al. (1978). "Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs." N Engl J Med **298**(24): 1321-7.

Sanchez, P., V. Clement, et al. (2005). "Therapeutic targeting of the Hedgehog-GLI pathway in prostate cancer." Cancer Res 65(8): 2990-2.

Sato, K., S. Ozawa, et al. (2010). "Expression of tumour-suppressing chemokine BRAK/CXCL14 reduces cell migration rate of HSC-3 tongue carcinoma cells and stimulates attachment to collagen and formation of elongated focal adhesions in vitro." Cell Biol Int 34(5): 513-22.

Scharenberg, C. W., M. A. Harkey, et al. (2002). "The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors." Blood 99(2): 507-12.

Schulz, W. A. (2005). Molecular biology of human cancers. Springer, 1st Ed. 1-504.

Seaberg, R. M. and D. van der Kooy (2002). "Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors." J Neurosci 22(5): 1784-93.

Segal, E., N. Friedman, et al. (2004). "A module map showing conditional activity of expression modules in cancer." Nat Genet 36(10): 1090-8.

Seigel, G. from http://www.isscr.org/public/eye.htm.

Seigel, G. M., L. M: Campbell, et al. (2005). "Cancer stem cell characteristics in retinoblastoma." Mol Vis 11: 729-37.

Seigel, G. M., A. S. Hackam, et al. (2007). "Human embryonic and neuronal stem cell markers in retinoblastoma." Mol Vis 13: 823-32.

Seiter, S., R. Arch, et al. (1993). "Prevention of tumor metastasis formation by anti-variant CD44." J Exp Med 177(2): 443-55.

Sery, T. W., E. Y. Lee, et al. (1990). "Characteristics of two new retinoblastoma cell lines: WERI-Rb24 and WERI-Rb27." J Pediatr Ophthalmol Strabismus 27(4): 212-7.

Shamblott, M. J., J. Axelman, et al. (1998). "Derivation of pluripotent stem cells from cultured human primordial germ cells." Proc Natl Acad Sci U S A **95**(23): 13726-31.

Shen, Q., Y. Wang, et al. (2008). "Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions." Cell Stem Cell **3**(3): 289-300.

Shen, Q., W. Zhong, et al. (2002). "Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts." Development **129**(20): 4843-53.

Shetty, A. K., B. Hattiangady, et al. (2005). "Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes." Glia **51**(3): 173-86.

Shi, H., S. H. Wei, et al. (2003). "Triple analysis of the cancer epigenome: an integrated microarray system for assessing gene expression, DNA methylation, and histone acetylation." Cancer Res **63**(9): 2164-71.

Shtivelman, E. and J. M. Bishop (1991). "Expression of CD44 is repressed in neuroblastoma cells." Mol Cell Biol **11**(11): 5446-53.

Sieber, O. M., K. Heinimann, et al. (2003). "Genomic instability--the engine of tumorigenesis?" Nat Rev Cancer **3**(9): 701-8.

Silva, A. K., H. Yi, et al. (2010). "Lithium chloride regulates the proliferation of stem-like cells in retinoblastoma cell lines: a potential role for the canonical Wnt signaling pathway." Mol Vis **16**: 36-45.

Singh, A. D., C. M. Santos, et al. (2000). "Observations on 17 patients with retinocytoma." Arch Ophthalmol **118**(2): 199-205.

Singh, S. K., I. D. Clarke, et al. (2003). "Identification of a cancer stem cell in human brain tumors." Cancer Res **63**(18): 5821-8.

Singh, S. K., C. Hawkins, et al. (2004). "Identification of human brain tumour initiating cells." Nature **432**(7015): 396-401.

Stevens, L. C. (1967). "Origin of testicular teratomas from primordial germ cells in mice." J Natl Cancer Inst **38**(4): 549-52.

Suetsugu, A., M. Nagaki, et al. (2006). "Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells." Biochem Biophys Res Commun **351**(4): 820-4.

Summer, R., D. N. Kotton, et al. (2003). "Side population cells and Bcrp1 expression in lung." Am J Physiol Lung Cell Mol Physiol **285**(1): L97-104.

Sutherland, H. J., C. J. Eaves, et al. (1989). "Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro." Blood 74(5): 1563-70.

Sutherland, H. J., P. M. Lansdorp, et al. (1990). "Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers." Proc Natl Acad Sci U S A **87**(9): 3584-8.

Szotek, P. P., R. Pieretti-Vanmarcke, et al. (2006). "Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness." Proc Natl Acad Sci U S A **103**(30): 11154-9.

Thomas E.O., David. R. H., Stephen J.R. (2005). Retinoblastoma. Retina, 4rth Ed. 1-3014.

Thomas, E. D., H. L. Lochte, Jr., et al. (1957). "Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy." N Engl J Med **257**(11): 491-6.

Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." Science **282**(5391): 1145-7.

Tropepe, V., B. L. Coles, et al. (2000). "Retinal stem cells in the adult mammalian eye." Science **287**(5460): 2032-6.

Tumbar, T., G. Guasch, et al. (2004). "Defining the epithelial stem cell niche in skin." Science **303**(5656): 359-63.

Umemoto, T., M. Yamato, et al. (2006). "Expression of Integrin beta3 is correlated to the properties of quiescent hemopoietic stem cells possessing the side population phenotype." J Immunol **177**(11): 7733-9.

Vercauteren, S. M. and H. J. Sutherland (2001). "CD133 (AC133) expression on AML cells and progenitors." Cytotherapy **3**(6): 449-59.

Villa, A., E. Y. Snyder, et al. (2000). "Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS." Exp Neurol **161**(1): 67-84.

Visvader, J. E. and G. J. Lindeman (2008). "Cancer stem cells in solid tumours: accumulating evidence and unresolved questions." Nat Rev Cancer **8**(10): 755-68.

Vonlanthen, S., J. Heighway, et al. (2001). "The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression." Br J Cancer **84**(10): 1372-6.

Walker, M. R., K. K. Patel, et al. (2009). "The stem cell niche." J Pathol **217**(2): 169-80.

Walkley, C. R., G. H. Olsen, et al. (2007). "A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency." Cell **129**(6): 1097-110.

Walkley, C. R., J. M. Shea, et al. (2007). "Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment." Cell **129**(6): 1081-95.

Weaver, B. A. and D. W. Cleveland (2007). "Aneuploidy: instigator and inhibitor of tumorigenesis." Cancer Res **67**(21): 10103-5.

Wei, S. H., C. M. Chen, et al. (2002). "Methylation microarray analysis of late-stage ovarian carcinomas distinguishes progression-free survival in patients and identifies candidate epigenetic markers." Clin Cancer Res **8**(7): 2246-52.

Welm, B. E., S. B. Tepera, et al. (2002). "Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population." Dev Biol **245**(1): 42-56.

White, D. E., N. A. Kurpios, et al. (2004). "Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction." Cancer Cell **6**(2): 159-70.

William H.S. (1996) Retinoblastomas. 4^{nh} edition, Ophthalmic Pathology. WB Saunders & Co, 1-3044.

Wilson, A. and F. Radtke (2006). "Multiple functions of Notch signaling in self-renewing organs and cancer." FEBS Lett **580**(12): 2860-8.

Windle, J. J., D. M. Albert, et al. (1990). "Retinoblastoma in transgenic mice." Nature **343**(6259): 665-9.

Wong, D. J., H. Liu, et al. (2008). "Module map of stem cell genes guides creation of epithelial cancer stem cells." Cell Stem Cell **2**(4): 333-44.

Xu, X. L., Y. Fang, et al. (2009). "Retinoblastoma has properties of a cone precursor tumor and depends upon cone-specific MDM2 signaling." Cell **137**(6): 1018-31.

Yang, H., H. Schilling, et al. (1999). "Morphologic classification of choroidal invasion of retinoblastoma." J Tongji Med Univ **19**(2): 149-54.

Yilmaz, O. H., R. Valdez, et al. (2006). "Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells." Nature **441**(7092): 475-82.

Yin, T. and L. Li (2006). "The stem cell niches in bone." J Clin Invest **116**(5): 1195-201.

Ying, Q. L., J. Nichols, et al. (2003). "BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3." Cell **115**(3): 281-92.

Yue, Z., T. X. Jiang, et al. (2005). "Mapping stem cell activities in the feather follicle." Nature **438**(7070): 1026-9.

Zagzag, D., B. Krishnamachary, et al. (2005). "Stromal cell-derived factor-1alpha and CXCR4 expression in hemangioblastoma and clear cell-renal cell carcinoma: von Hippel-Lindau loss-of-function induces expression of a ligand and its receptor." Cancer Res **65**(14): 6178-88.

Zhadanov, A. B., D. W. Provance, Jr., et al. (1999). "Absence of the tight junctional protein AF-6 disrupts epithelial cell-cell junctions and cell polarity during mouse development." Curr Biol **9**(16): 880-8.

Zhang, J., J. Gray, et al. (2004). "Rb regulates proliferation and rod photoreceptor development in the mouse retina." Nat Genet **36**(4): 351-60.

Zhang, X., V. Mar, et al. (1999). "Telomere shortening and apoptosis in telomerase-inhibited human tumor cells." Genes Dev **13**(18): 2388-99.

Zhong, X., Y. Li, et al. (2007). "Identification of tumorigenic retinal stem-like cells in human solid retinoblastomas." Int J Cancer **121**(10): 2125-31.

Zhou, S., J. D. Schuetz, 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**(9): 1028-34.

Zulewski, H., E. J. Abraham, et al. (2001). "Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes." Diabetes **50**(3): 521-33.

Appendix Table-1: Clinical and histo pathological information of Rb cases used for microarray work

Sl. No	Case No	Age/Sex	Laterality/Eye	Hereditary	Duration of symptoms	Grade of tumor	HRF	BM/CSF involvement	Outcome	Technique performed
1	Case 1	4/F	UL/OD	No	2 mo	D	MD, AC, Sub-RPE tumor deposits, anterior layers of cribrosa involved	Nil	Enucleation done	Microarray, Real time PCR and IHC
2	Case 2	6mon/M	UL/OS	No	2 mo	D	WD, AC, anterior layers of cribrosa involved	Nil	Enucleation done	Microarray, Real time PCR
3	Case 3	2/M	UL/OD	No	7 days	D	MD, Retinocytoma like areas, no HRF	Nil	Enucleation done	Microarray, Real time PCR and IHC
4	Case 4	5/F	UL/OD	No	3 mo	D	PD, No HRF	Nil	Enucleation done	Microarray, Real time PCR and IHC
5	Case 5	1/F	UL/OS	No	9 mo	D	MD, pre laminar cribrosa involve ment	Nil	Enucleation done	Real time PCR and IHC
6	Case 6	3/M	UL/OD	No	2 mo	D	PD, No HRF	Nil	Enucleation done, adjuvant chemotherapy	Microarray, Real time PCR and
7	Case 7	2/F	UL/OD	No	10 days	D	MD, sub-retinal tumor deposits	Nil	Enucleation done	Microarray, Real time PCR and IHC
8	Case 8	4/M	UL/OS	No	15 days	D	PD, Laminar cribrosa, ON involvement	Nil	Enucleation done, adjuvant chemotherapy	Microarray, Real time PCR
9	Case 9	4/F	UL/OS	No	6 mo	E	PD, full thickness choroidal involvement	Nil	Enucleation done, adjuvant chemotherapy	Microarray and Real time PCR

10	Case 10	6/M	UL/OS	No	1 mo	E	PD, iris and angle seedlings, neovascularisation of iris, angle closure	Nil	Enucleation done, adjuvant chemotherapy	Microarray and Real time PCR
11	Case 11	1/F	UL/OD	No	13 days	E	MD, multiple sub-RPE deposits	Nil	Enucleation done	Microarray and Real time PCR
12	Case 12	1.3/F	UL/OS	No	2 mo	-	MD, Post cribrosa involvement	Nil	Enucleation done	Real time PCR
13	Case 13	1/F	UL/OS	No	-	-	MD	Nil	Enucleation done	Real time PCR
14	Case 14	4/M	UL/OD	No	-	-	PD	Nil	Enucleation done	Real time PCR
15	Case 15	5/M	UL/OD	No	5 mo	Vb	PD, ON, Laminar cribrosa, focal area of choriocapillaries involved	Nil	Enucleation done	IHC
16	Case 16	4/M	BL/OU	Yes	15 days	Vb	PD, No HRF	Nil	Enucleation done	IHC
17	Case 17	5 mon/M	BL/OU	Yes	4 mo	Va	WD, Full choroidal involvement, laminar cribrosa, Post cribrosa involvement, neovascularization of iris, anterior and posterior synechiae	Nil	Enucleation done, adjuvant chemotherapy	IHC
18	Case 18	3/M	UL/OD	No	2 mo	Vb	MD, Laminar cribrosa involved, full thickness choroid involvement	Nil	Enucleation done, adjuvant chemotherapy	IHC
19	Case 19	1/F	UL/OS	No	2 mo		MD, Laminar cribrosa involved, neovascularization of iris	Nil	Enucleation done, adjuvant chemotherapy	IHC

20	Case 20	1/M	UL/OS	No	3 mo		WD, involvement of choriocapillaries and sub-RPE region, No HRF	Nil	Enucleation done	IHC
21	Case 21	1yr 5 mon/M	UL/OD	No	6 mo	Vb, D	WD, No HRF	Nil	Enucleation done	IHC
22	Case 22	16 mon/ F	UL/OS	No	3 mo	D	WD, Involvement of choriocapillaries, No HRF	Nil	Enucleation done	IHC
23	Case 23	18 mon/M	UL/OD	No	15 days	Va	PD, ON, Anterior layers of Laminar Cribrosa	Nil	Enucleation done	IHC
24	Case 24	6 mon/M	BL/OU	Yes	2 weeks	OD-A. OS-E	WD, involvement of superficial choroid, choriocapillaries, No HRF	Nil	Enucleation done	IHC
25	Case 25	6/M	UL/OD	No	16 days, white patch at the iris since birth	E	WD, Retinocytoma like areas, No HRF	Nil	Enucleation done post chemotherapy	IHC
26	Case 26	2/F	BL/OU	Yes	1-2 mo	OD-D. OS-E	Retinocytoma like areas	Nil	Enucleation done post chemotherapy	IHC

Appendix-2: List of antibodies

S.No	Primary Antibody	Standardized Dilution	Secondary Antibody	Standardized Dilution	Intended Use
1	Anti Human CD133(Milteyini Biotech, Germany)	50µg/ml at a dilution of 1:10	FITC conjugated anti mouse IgG1 (eBiosciences, California)	0.5mg/ml at a dilution of 1:100	Flow Cytometry
2	FcR blocking reagent (Milteyini Biotech, Germany)	10 µl/million cells	NA	NA	Flow Cytometry
3	Anti Human CD44-PE (eBiosciences, California)	0.2mg/ml at a dilution of 1:100	NA	NA	Flow Cytometry
4	Anti Human CXCR4-APC (eBiosciences, California)	0.5mg/ml at a dilution of 1:100	NA	NA	Flow Cytometry
5	Anti Human CD90-FITC (eBiosciences, California)	0.5mg/ml at a dilution of 1:100	NA	NA	Flow Cytometry
6	Mouse IgG2a APC Isotype control (eBiosciences, California)	50 µg/0.5ml at a dilution of 1:100	NA	NA	Flow Cytometry
7	Rat IgG2b PE Isotype control (eBiosciences, California)	0.2mg/ml at a dilution of 1:100	NA	NA	Flow Cytometry
8	Mouse IgG1 FITC Isotype control (eBiosciences, California)	0.2mg/ml at a dilution of 1:100	NA	NA	Flow Cytometry
9	mouse anti human ABCG2 (Abcam, UK)	250µl culture supernatant at a dilution of 1:50	FITC conjugated anti mouse IgG1 (eBiosciences, California)	0.5mg/ml at a dilution of 1:100	Flow Cytometry
10	Anti Human CD133- APC	50µg/ml at a dilution of	NA	NA	Flow Cytometry

	(Milteyini Biotech, Germany)	1:10			
10	Mouse anti human p53 (Dako, Denmark)	1mg/ml at a dilution of 1:200			IHC
11	Rabbit anti human ACVR1C (Abcam, UK)	1mg/ml at a dilution of 1:50			IHC

Appendix Table-3:

S No.	Gene	Forward primer	Reverse primer
1	GAPDH	ccaggtggtctcctctgacttc	gtggtcgttgagggcaatg
2	MYCN	tgatgaagatgatgaagaggaagatg	cagtgatggtgaatgtggtgac
3	HMGA2	aggcagacctaggaaatggc	gatccaactgctgctgaggt
4	Lin 28b	cctgtttaggaagtgaaagaagac	cacttctttggctgaggaggtag
5	Syntaxin1A	ctgcagtcagtccctcaag	ctgccgaatactgcatctg
6	PROX1	caagttgtggacactgtggt	gcagactggtcagaggagtt
7	CD133	cctctggtggggtatttctt	aggtgctgttcatgttctcc
8	NSE	catcgacaaggctggctacacg	gacagttgcaggccttttcttc
9	GAPDH	gccaaggtcatccatgacaac	gtccaccaccctgttgctgta

Appendix Table-4: Concentration of RNA samples used for Microarray experiments

S No.	Samples	Concentration (ng/µl)
1	Retina Control	256.8
2	Case 1	349.7
3	Case 2	191.8
4	Case 3	487.2
5	Case 4	206.2
6	Case 5	370.1
7	Case 6	487.8
8	Case 7	219.4
9	Case 8	501.2
10	Case 9	541.3
11	Case 10	416.6
12	Case 11	542
13	$CD133^-$	250
14	$CD133^+$	138.8

Publications

- **Murali MS Balla[1]**, Imran Khan, Ravi Kiran Reddy Kalathur, Rohini Nair, Chitra Kannabiran, Santosh G Honavar, Kondiah P, Geeta K Vemuganti. "Fetal neural stem cell signaling in human retinoblastoma tumors" (Manuscript in submission)

- **Murali MS Balla[1]**, Imran Khan, Ravi Kiran Reddy Kalathur, Rohini Nair, Chitra Kannabiran, Santosh G Honavar, Kondiah P, Geeta K Vemuganti."CD133 negative cells of Y79 cell line shows the properties of cancer stem cells" (Manuscript in preparation)

- Shubha Tiwari, Javed Ali, **Murali MS Balla[2]**, Milind N Naik, Santosh G Honavar, Vijay Anand P Reddy, Geeta K Vemuganti. "Establishing human lacrimal gland cultures with secretory function" *Plos One.* 2011; (Manuscript in consideration)

- **Murali Mohan Sagar Balla[1]**, Geeta K Vemuganti, Chitra Kannabiran, Santosh G Honavar, Ramesh Murthy. "Phenotypic characterization of retinoblastoma for the presence of putative cancer stem cell markers by flow cytometry" *Invest Ophthalmol Vis Sci.* **2009**;50:1506-14.

- **Murali Mohan Sagar Balla[1]**, Satyanarayana Rentala, P.N.V. Gopal, Shalini Sharma, Asok Mukhopadhyay. "Fibronectin and laminin enhance engraftibility of cultured hematopoietic stem cells" *Biochem Biophys Res Commun.* **2006** Dec 1;350(4):1000-5.

- **Murali Mohan Sagar Balla[1]**, Rentala S, Khurana S, Mukhopadhyay A. "MDR1 gene expression enhances long-term engraftibility of cultured bone marrow cells" *Biochem Biophys Res Commun.* **2005** Sep 30;335(3):957-64.

Book Chapters

- **Murali Mohan Sagar Balla[1]**, Geeta K Vemuganti, Anjali P Kusumbe and Sharmila Bapat. "Cancer stem cells" **Springer Link, London 2011. (Title of the book: "Regenerative Medicine" Editor: Steinhoff, Gustav,** 1st edition **(ISBN: 978-90-481-9074-4))**

- **Murali Mohan Sagar Balla**[1], Geeta K Vemuganti, Shubha Tiwari " Limbal stem cells and corneal regeneration" **John Wiley & Sons, Inc. 2010 (Title of the book: "Applications of flow cytometry in stem cell research and tissue regeneration" Editors: Awtar Krishan, Krishna Murthy H, Satish Totey (ISBN: 978-0-470-54398-6))**

Presentations

- Participated for a poster on "Evaluation of Cancer Stem Cells in Human Y79 Retinoblastoma Cell Line" in the Indian Eye Research Group Annual Conference, July **2011**, LVPEI, Hyderabad.
- Participated for a poster on "Evaluation of Human Lacrimal Gland Cultures for Secreetory Function and Putative Stem Cells" in the Indian Eye Research Group Annual Conference, July **2011**, LVPEI, Hyderabad.
- Presented an oral presentation on "Characterization of Cancer Stem Cells in Y79 cell line" at the Indian Eye Research Group Annual Conference, July **2010**, LVPEI, India.
- Presented a poster on "Characterization of Cancer Stem Cells in Y79 cell line" in ARVO Annual meeting, May **2010** Fort Lauderdale, USA.
- Presented an oral talk on "Phenotypic Characterization of Cancer Stem-like Cells in Retinoblastoma" in the Asia ARVO meeting, January **2009**, Hyderabad, India.
- Appeared for an oral talk on "Phenotypic Characterization of Cancer Stem-like Cells in Retinoblastoma" in the Indian Eye Research Group Annual Conference, July **2008**. Aravind Eye Care Systems, Madurai.
- Presented poster in the 76th annual meeting of Society for Biological Chemists India (SBCI), held at Sri Venkateswara University, November **2007**, Thirupati, Andhra Pradesh, India.
- Participated for a poster in the Indian Eye Research Group Annual Conference, July **2006**. LVPEI Hyderabad.
- Appeared for an oral talk on "Micellar Electrokinetic Capillary Chromatography" at Andhra University.

CURRICULUM VITAE

Murali Mohan Sagar Balla
Plot#59, Sarada Nagar, Phase-I, Road#6,
Near Loyola Model High School,
Vanasthalipuram, Hyderabad- 070, A.P, INDIA
Home (091) 40- 24111212; Mobile: 9493186301
E-mail: *bsagar327@gmail.com*

Academic Qualifications

Present educational position (2006-present)	:	Graduate student (Ph.D.) at L.V.Prasad Eye Institute (Working as CSIR Senior Resesarch Fellow)
M. Tech. (Biotechnology) (2002-2004)	:	Jawaharlal Nehru Technological University, Hyderabad (First class with distinction)
B. Pharmacy. (Pharmacy) (1997-2001)	:	Osmania University (First class)

Research Interests

I am interested in studying the strategies/mechanisms (involving both biological and biomolecular engineering concepts) by which the microenvironment is involved in maintaining the stem/cancer stem cells (SC/CSC) in a given tissue or tumor.

Tissue homeostasis and regeneration is highly orchestrated process, which is critically dependent on the microenvironment and the number of stem cells in the tissue. It was shown that if this highly regulated process is de regulated, leads to the abnormal division of cells thus forming tumor.
Despite of the extensive research conducted in the field of SC/CSC biology, yet difficult problems need to overcome in order to expand SCs *in-vitro* or target CSCs *in-vivo*. One of the important challenges is controlling stem cell behavior *in-vitro*. In order to accomplish this challenge, studying microenvironment would answer many questions that would help in understanding the behavior of SC/CSC in a normal/cancerous tissue.

I would like to address questions like how to develop artificial *in-vitro* niche for maintaining stem cells? Using these niches how to delineate the mechanisms of regulation? How to expand these meager stem cells into more numbers for application in cell therapy? To observe whether such mechanisms are deregulated in tumor tissues? and finally how to target these individual diseased cells to address chemo resistance?

This research would aid in understanding the dynamics of stem cell behavior in both normal and diseased conditions.

Fellowships Received

- Awarded a pre-doctoral travel award of **US$ 1100** by the Association for Research in Vision and Ophthalmology to attend the ARVO2010 Annual meeting at Fort Lauderdale, USA.
- Awarded the Junior Research Fellowship (2006-2008) from Hyderabad Eye Research Foundation and Senior Research Fellowship (2008-2011) from the Council of Scientific and Industrial Research (CSIR)
- Awarded teaching assistantship for coordinating and conducting science practical classes at Bausch & Lomb school of optometry, Hyderabad (2006-2010)
- Successfully cleared JNTU Entrance exam in the year 2002 (7^{th} rank in India) for studying M.Tech (biotechnology)

Research Experience

- Presently working on **"Microarray analysis of $CD133^-$ and $CD133^+$ sorted populations"** in the laboratory of Dr.Geeta K Vemuganti at the L.V.Prasad Eye Institute, Hyderabad, India
- Carried out microarray studies of human Rb tumors under the guidance of Prof. Kondiah at **Indian Institute of Science,** Bangalore.
- Designed the cGMP facility and established the tissue culture laboratory. Worked in a cGMP facility for **"*Exvivo* expansion of mesenchymal stem cells"**. Maintaned and characterized Harvard university human embryonic stem cell lines **(HUES-5, HUES-6 and HUES-7)** at **Stempeutics pvt.Ltd. Bangalore, India** (2005-2006)
- **Characterized and expanded murine bone marrow stem cells** (hematopoietic stem cells and mesenchymal stem cells) in *in-vitro* by using flow cytometry, ICC, RT-PCR, Colony assays, clonal assays, Marrow repopulation assays at **National Institute of Immunology. NewDelhi, India** (2003-2004). (Part of M.Tech Degree)
- Prepared periodontal films of tetracycline hydrochloride by Solvent evaporation technique using ethyl cellulose, hydroxypopylmethylcellulose 4000cps, polyethylene glycol 4000 and Eudragit rs100 as polymers. **"Evaluation of periodontal films for antimicrobial activity"**. (Part of my Bachelor degree)

Technical Skills

Cell Culture: Culturing primary mouse, human stem and tumor samples, adherent and suspendend mammalian cell lines, soft agar assays, clonal assays, single cell assays, immunocytochemistry, immunohistochemistry, flourescent microscopy, MTT asays, colony forming unit assays, long term cultute initiating cell assays, methyl cellulose colony assays, drug assays.

Flow Cytometry: Involved in developing Flow cytometry lab at L.V. Prasad Eye Institute. Handled and maintained **BD FACS Aria I** instrument for 4 years. Experienced in analyzing, sorting (single cells, two populations and 4 populations) using BD FACS Aria instrument.

Molecular Biology: RNA isolation from tissues and sorted cells, PCR, RT-PCR, Real time PCR, Primer designing, preparing FISH probes etc.

Microarray: Preparation of cRNA probes, Hybridization of probes, Analysis of data using Gene Spring version 10, normalizing the data, calculating fold change based on ratio of Cy5/Cy3 intensities, Pathway analysis using KEGG pathway database, evaluation of differentially regulated genes using DAVID bioinformatics resources.

Bioinformatics: DNA and protein sequence alignment using BLAST and ClustalW software, Using STRING database for analyzing predicted protein-protein interactions

Animal Experiments: Bone marrow (HSC's and MSC's) stem cell isolation, retro-orbital blood collection, Marrow repopulation assays, intravenous injections in mice etc.

Summary of Ph.D. work

My PhD project involves the characterization of cancer stem cells in Y79 retinoblastoma tumor and identification of different populations and novel prognostic markers in human retinoblastoma tumors. The project can be broadly divided into three parts.

(A) I have characterized two different sub-populations in human Rb tumor samples. Among these sub- populations, the FSC^{lo}/SSC^{lo} cells manifest as primitive when compared with the FSC^{hi}/SSC^{lo} group. Since FSC^{lo}/SSC^{lo} group showed the expression of primitive stem cell CD44, retinal progenitor markers (PROX1 and Syntaxin1A). In addition I have shown that FSC^{hi}/SSC^{lo} group shows higher percentage of differentiated cell markers (CD133&CD90) when compared with FSC^{lo}/SSC^{lo} population.

(B) In the second part, I have shown that in support of the evidence in primary tumor cells where $CD44^{+}CD133^{-}$ cells showing the markers for progenitor cell markers PROX1 and Syntaxin 1a, Y79 cell line also showed similar marker expression as that of primary tumor cells except for CD44 expression. Primitive markers like Oct4, Nanog, Bmi-1 and PROX1 were highly expressed in $CD133^{-}$ population. Cell cycle analysis also showed that majority of $CD133^{-}$ cells are in G_0/G_1 phase. In addition to these properties $CD133^{-}$ population have also shown high clone- forming ability and have capability to regenerate the tumor phenotype, which further substantiates our hypothesis.

(C) In the third part, I analyzed that in human Rb tumors; neural stem cell signaling (HMGA2) is highly up regulated. Notch signaling is marginally up regulated and other stem cell self renewal pathways like Wnt, SHH were down regulated. Nodal signaling is also deregulated in these tumors. Along with ACVR1C used for distinguishing the subtypes, the genes IRS1 and CXCL14 can be used as novel prognostic markers for tumors with and without risk factors. Additional studies are required to evaluate functional importance of these markers in Rb tumors.

Summary of work at Stempeutics Pvt. Ltd

(A) Establishment of Stromal Cultures: Isolated bone marrow mononuclear cells from minimal volumes of unstimulated, discarded diagnostic marrow taps using simple density gradient centrifugation and established stromal cultures by virtue of their affinity to culture flask. Phenotypic cells were analyzed and characterized stromal cells for mesenchymal specific marker expression like CD29, CD71, CD90, CD105, and CD106 by flow cytometry and ICC.

(B) Single cell Assays were evaluated for the clonal properties of stromal cells. Differentiation was induced to push mesenchymal stem cells into osteocytic, adipocytic and chondrocytic lineages. Characterized BMSCs differentiated into osteo, adipo and chondrocytic lineages by simple histological stains -von kossa, oil red 'O' and alizarin red.

Summary of M.Tech thesis work

(A) Stem cell isolation: Treated mice with 5-flouro uracil (5-FU) through tail vein injection and after 3rd & 5th day, mice were sacrificed for isolation of 5-FU resistant hematopoietic stem cells (HSCs).

(B) Phenotypical and functional characterization: Evaluated stem cell properties by phenotypical (FACS, giemsa staining, hoechst33342 staining, MTT assay) & functional characterization (invitro assays). Expanded Sca-1 enriched HSCs in presence of various cytokine & hormonal combinations for 5 & 10 day period. Harvested expanded cells and characterized for primitive and differentiated cell markers.

(C) Transplantation Experiments: Characterized cells, then transplanted into lethally irradiated mice (analyzed marker expression by collecting peripheral blood by retro orbital collection) to clarify the marrow repopulating ability. Evaluated effect of laminin and fibronectin individually on the stem ness of 5-FU resistant HSCs.

Selected Presentations

- Participated for a poster on "Evaluation of Cancer Stem Cells in Human Y79 Retinoblastoma Cell Line" in the Indian Eye Research Group Annual Conference, July **2011**, LVPEI, Hyderabad.
- Participated for a poster on "Evaluation of Human Lacrimal Gland Cultures for Secreetory Function and Putative Stem Cells" in the Indian Eye Research Group Annual Conference, July **2011**, LVPEI, Hyderabad.
- Presented an oral presentation on "Characterization of Cancer Stem Cells in Y79 cell line" at the Indian Eye Research Group Annual Conference, July **2010**, LVPEI, India.
- Presented a poster on "Characterization of Cancer Stem Cells in Y79 cell line" in ARVO Annual meeting, May **2010** Fort Lauderdale, USA.
- Presented an oral talk on "Phenotypic Characterization of Cancer Stem-like Cells in Retinoblastoma" in the Asia ARVO meeting, January **2009**, Hyderabad, India.
- Appeared for an oral talk on "Phenotypic Characterization of Cancer Stem-like Cells in Retinoblastoma" in the Indian Eye Research Group Annual Conference, July **2008**. Aravind Eye Care Systems, Madurai.
- Presented poster in the 76th annual meeting of Society for Biological Chemists India (SBCI), held at Sri Venkateswara University, November **2007**, Thirupati, Andhra Pradesh, India.
- Participated for a poster in the Indian Eye Research Group Annual Conference, July **2006**. LVPEI Hyderabad.
- Appeared for an oral talk on "Micellar Electrokinetic Capillary Chromatography" at Andhra University.

Workshops/International Conferences attended

1. ARVO Annual meeting, May **2010** Fort Lauderdale, USA.
2. Attended Asia ARVO meeting, January **2009**, Hyderabad, India.
3. Flow cytometry workshop on "Handling, Maintenance and Analysis of data on BD FACS Aria I" held at Rajiv Ghandhi Center for Biotechnology, Trivandrum, Kerala, 2007.

Other academic activities

I have trained summer interns and short-term workers from Massachusetts Institute of Technology and Birla Institute of Technology and Science. I was involved in training flow cytometry to these students for their projects at LVPEI.

Membership of professional societies

Association for Research in Vision and Ophthalmology
Indian Society of Biological Chemists
The Cytometry Society
Indian Eye Research Group
Asian Association for Research in Vision and Ophthalmology

Publications

- **Murali MS Balla[1]**, Geeta K Vemuganti, Chitra Kannabiran, Santosh G Honavar, Ramesh Murthy. "Phenotypic characterization of retinoblastoma for the presence of putative cancer stem cell markers by flow cytometry" *Invest Ophthalmol Vis Sci.* **2009**;50:1506-14. (Impact factor# 3.766)

- **Murali MS Balla[1]**, Satyanarayana Rentala, P.N.V. Gopal, Shalini Sharma, Asok Mukhopadhyay. "Fibronectin and laminin enhance engraftibility of cultured hematopoietic stem cells" *Biochem Biophys Res Commun.* **2006** Dec 1;350(4):1000-5. (Impact factor# 2.548)

- **Murali MS Balla[1]**, Rentala S, Khurana S, Mukhopadhyay A. "MDR1 gene expression enhances long-term engraftibility of cultured bone marrow cells" *Biochem Biophys Res Commun.* **2005** Sep 30;335(3):957-64. (Impact factor# 2.548)

- **Murali MS Balla[1]**, Imran Khan, Ravi Kiran Reddy Kalathur, Rohini Nair, Chitra Kannabiran, Santosh G Honavar, Kondiah P, Geeta K Vemuganti. "Fetal neural stem cell signaling in human retinoblastoma tumors" *Mol Vision.* 2011; (Manuscript in submission)

- **Murali MS Balla[1]**, Imran Khan, Ravi Kiran Reddy Kalathur, Rohini Nair, Chitra Kannabiran, Santosh G Honavar, Kondiah P, Geeta K Vemuganti."CD133 negative cells of Y79 cell line shows the properties of cancer stem cells" *Invest Ophthalmol Vis Sci.* 2011; (Manuscript in Preparation)

- Shubha Tiwari, Javed Ali, **Murali MS Balla[2]**, Milind N Naik, Santosh G Honavar, Vijay Anand P Reddy, Geeta K Vemuganti. "Establishing human lacrimal gland cultures with secretory function" *Plos One.* 2011; (Manuscript in revision)

Book Chapters

- **Murali MS Balla[1]**, Geeta K Vemuganti, Anjali P Kusumbe and Sharmila Bapat. "Cancer stem cells" ***Springer Link, London 2011.*** (Title of the book: "Regenerative Medicine" Editor: Steinhoff, Gustav, 1st edition (ISBN: 978-90-481-9074-4))

- **Murali MS Balla**[1], Geeta K Vemuganti, Shubha Tiwari " Limbal stem cells and corneal regeneration" ***John Wiley & Sons, Inc. 2010*** (Title of the book: "Applications of flow cytometry in stem cell research and tissue regeneration" Editors: Awtar Krishan, Krishna Murthy H, Satish Totey (ISBN: 978-0-470-54398-6))

References

1. Prof. Geeta K Vemuganti, (MD, DNB, FAMS, FICP)
Dean, School of Medical Sciences
University of Hyderabad, Hyderabad 500046
Phone: 91-40-23013279/23014781
Email: deanmd@uohyd.ernet.in
gkvemuganti@yahoo.com
gkvemuganti@gmail.com

2. Prof. D. Balasubramanian,
Director, Research, L. V. Prasad
Eye Institute, Banjara Hills, Hyderabad- 500 034, India
Tel: +91-40-23543652/30612501
E-mail: dbala@lvpei.org

3. Dr. Asok Mukhopadhyay,
Staff Scientist-VI, Stem cell biology lab,
National Institute of Immunology,
Aruna Asaf Ali Marg, New Delhi.
Tel: 91-11-26703781
E-mail: ashok@nii.res.in

Brief Biography of the Supervisor

Dr Geeta K Vemuganti is a physician- pathologist by training and has contributed extensively to the field of ophthalmic pathology and translational research. In order to combat blindness due to severe ocular surface disease patients, post chemical burn, she developed a simple, cost-effective, feeder cell free method of culturing the limbal stem cells from patient's own limbal tissues through explant culture, using human amniotic membrane as a substrate. This membrane could be transplanted onto more than 700 patients so as to restore the ocular surface and thereby visual recovery. The highlights of this technique was it was simple, cost-effective, feeder cell free, xeno free and submerged technique of generating a sheet of corneal epithelium within 10-14 days of culture. For patients with more severe disease, she designed a novel method of co-culturing two types of ocular surface epithelium (peripheral conjunctiva and central limbal epithelium) on a single membrane using a self-designed ring barrier. This was useful in reconstructing the ocular surface in a single step surgery using one membrane in 50 patients, who otherwise were incurably blind. To obviate the need for immunosuppression and allogenic limbal transplantation in severe bilateral disease with no other treatment option, she developed an alternate source of epithelium using oral mucosa. The pilot study using oral epithelium showed a success with 35% of patients. Within the same culture system, she reported a novel finding of the presence of stromal cells which show a striking resemblance to mesenchymal cells derived from bone marrow. Based on the phenotype, in-situ localization and gene expression studies, she proposed that these cells could be acting as intrinsic feeder cells in the explant culture system, which possibly could be limbal niche cell. She has also explored the option of using collagen scaffolds for growing limbal cells as an alternative to human amniotic membrane. Her current approach is to establish

the mesenchymal stem cells of autologus human bone marrow, provide proof of plasticity, hypoimmunogenicty and to provide proof of concept in animal model through homing and integration of the transplanted cells.

Her combined knowledge and expertise of pathology and stem cell biology has helped her to bridge the gap between clinical and basic sciences. Using this knowledge she reported the presence of putative cancer stem cell in Retinoblastoma, the most common intraocular tumor using flow cytometric analysis. This work throws new light on cancer stem cells especially on the hierarchy of stem cells within the tumor population.

The nominee has established and nurtured the field of Ophthalmic Pathology in India. For the last 12 years and more, the nominee has single handedly evaluated more than 10,000 diagnostic ocular specimens, the world's largest archival base, thus contributing to patient care and management. Based on her evaluation of more than 3000 corneal specimens, she has postulated pathobiology of various forms of corneal infections and reported the largest series of microsporial keratitis, a new and emerging form of corneal infection contributing to blindness. Her study on fungal keratitis has shed new light on the pathobiology of the disease: that it could progress in two phases- an agent dependant inflammatory phase and an agent- independent, host-dependant inflammation in late phases. She has documented for the first time that the keratocyte loss in corneal infections is by the process of apoptosis. She has contributed immensely to the understanding of genotype and phenotype correlation in various forms of inherited diseases of the eye like Retinoblastoma- the most common malignant intraocular tumor in children. She negated the role of Human Papilloma Virus (HPV) in genesis and prognosis of Ocular Surface Squamous Cell Neoplasia (OSSN) in south India, and found out some epigenetic changes and high expression of cell cycle markers which could be

useful as prognostic indicators. Her work on genotype and phenotype correlation of different types of corneal dystrophies is significant.

She established the Indian Association of Ophthalmic Pathologists, and subsequently facilitated its affiliation to the International Society of Ophthalmic Pathology. She is a mentor of many ophthalmology and pathology residents and fellows and a Phd guide to research scholars. Her contributions to Translational research and Ophthalmic Pathology has won her several grants, awards and honors at both national and international level, even breaching the border conflicts with Pakistan. She has also contributed to various ethics committees, Institute Review Boards, Biosafety committees and was invited to formulate guidelines for stem cell therapy in India. She has more than 160 publications to her credit, nearly 100 invited talks at international meetings and grants worth 3 million USD.