CHARACTERIZATION OF STEM LIKE CELLS IN A NEW ASTROBLASTOMA CELL LINE

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CHARACTERIZATION OF STEM-LIKE CELLS IN A NEW ASTROBLASTOMA CELL LINE

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ABSTRACT

CHARACTERIZATION OF STEM-LIKE CELLS IN A NEW ASTROBLASTOMA CELL LINE

Astroblastomas are uncommon neuroepithelial tumors of glial origin. It predominantly affects younger people mainly teenagers and children with a high female proportion. Only a single study so far reported that astroblastoma contains a high amount of neural stem like cells which had only partial proliferation capacity and differentiation.

In our study a patient derived astroblastoma cell line was generated and presence of cancer stem cells was investigated. Primary cell lines are powerful tools for clarifying the biological characteristics of a cancer and for developing effective treatment strategies. Characterization for astroblastoma and stem cell markers through flow cytometric and gene expression analyses, capacity to differentiate into other lineages and recapitulation of the original tumor has been studied through several methods.

The presence of stem cells in this heteregenous cell population might allow is to learn about the initiation, progression and chemoresistance of this specific astroblastoma cell line. This new cell line might be used as a model cell line for further studies that scientific people might have an interest in astroblastoma.

ÖZET

YENİ BİR ASTROBLASTOMA HÜCRE HATTINDA KÖK HÜCRE KARAKTERİZASYONU

Astroblastomlar glial kökenli yaygın olmayan nöroepitel tumörlerdir. Daha çok genç insanları ve çocukları etkilemekle birlikte kadınlarda görülme oranı daha yüksektir. Bugüne kadar sadece bir çalışmada astroblastomlarda yüksek oranda nöral kök hücre varlığı ve bu hücrelerin sadece kısmi oranda çoğaldıkları ve farklılaştıkları gösterilmiştir. Çalışmamızda astroblastomlu bir hastanın dokusundan primer hücre hattı geliştirilmiştir ve kök hücre varlığının tespiti araştırılmıştır. Primer hücre kültürleri kanserin biyolojik karakteristiğinin anlaşılmasında ve etkili tedavi stratejilerinin geliştirilmesinde çok güçlü bir araç olmaktadırlar. Astroblastomdaki kök hücre karakterizasyonu, flow sitometri ile kök hücre markerlerinin analizi, gen ekspresyonun analizi, diğer hücre soylarına dönüşebilmesi ve tümörün yeniden kendini oluşturması çalışmamızda pek çok yöntemle araştırılmıştır.

Astroblastom hücre hattındaki heterojen olan hücre populasyonlarındaki kök hücre varlığı, tümörün başlatılması, prognozu ve ilaça dirençliliğinin anlaşılmasına yardım olacaktır. Bu yeni hücre hattı devam eden çalışmalarda astroblastomla ilgilenen bilim insanlarına model olarak kullanılacaktır.

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ABB	Annexin Binding Buffer ABB
ABCG2	ATP-binding cassette sub-family G member 2
ACNU	4-Amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chlorethyl) 3-nitrosoharnstoff
Akt	Protein Kinase B
AML	Acute myeloid leukemia
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
ATRA	All-*trans*-Retinoic Acid
bFGF	Basic broblast growth factor
BTSC	Brain Tumor Stem Cells
CD	Clusters of differentiation
CDM	Chondrogenic Differentiation Medium
CGH	Comparative genomic hybridization
CSC	Cancer stem cell
CT	Computerized Tomography
DAPI	4',6-diamidino-2-phenylindole
dH2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DTAB	Dodecyltrimethylammonium bromide
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EtBr	Ethidium Bromide
ErbB2	Erythroblastic leukemia viral oncogene homolog
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast Growth Factor
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GS	Goat Serum
HIF	Hypoxia-inducible factor
HNSCC	Head and neck squamous cell carcinoma
ITS	Insulin-Transferrin-Selenium
KCI	Potassium chloride
LKB1	Liver kinase B1
MOP	Mitochondrial oxidative phosphorylation

MRI	Magnetic resonance imaging
MTS	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4 sulfo-phenyl)-2H-tetrazolium
MYC	C-Myc
MIC	Minimum inhibition concentration
NaCl	Sodium chloride
NF-κB	Nuclear Factor kappa B
NspI	NspI gene from Nostoc species
ODM	Osteogenic differentiation medium
P	Petite
PAF	Paraformaldehyde
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PI3K-Akt-mTOR	Phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin
PLL	Poly-L-Lysine
Prom1	Prominin 1
PS	Phosphatidylserine
PSA	Penicillin/Streptomycin/Amphotericin
PSA	Prostate specific antigen
Q	Queue (long in French)

Ras	Rat sarcoma
SKY	Spectral Karyotyping
STR	Short tandem repeat
TAE	Tris base, acetic acid and EDTA
TdT	Terminal deoxynucleotidyl transferase
TE	Tris Edta
TMZ	Temozolomide
TGF β3	Transforming Growth Factor β3
TP53	Tumor protein p53
TH	Tyrosine Hydroxylase

1. INTRODUCTION

1.1. CANCER

Cancer is the abnormal growth of cells in an organism. Being the second leading cause of death in USA, its origin goes back as early as 3000 BCE in Egypt. It is a neoplasm, which can uncontrollably proliferate and usually migrates by disseminating to the other tissues and organs of the body and causes destruction that can lead to organ failure and inevitably death. Intensive research and excessive budget has been spent on this deathly disease and as the years go by physicians, chemists, and even philosophers continue to show interest in finding different ways to treat it. With increasing knowledge, new treatment methods are being investigated, however cancer maintains its unbeatable power and continues to be one of the deathly diseases of the century.

Although the processes behind tumorigenesis are partially recognized, it is well known that the accumulation of mutations that occur in fundamental genes drive this process. After each mutation cells gain an advantage of outgrow over nearby cells which can be considered as Darwinian evolution model such that each mutation brings an adaptation potency to generate surviving cells against to overpowering the rules that regulate the normal cell growth hence namely clonal evolution [1]. It is very well known that cancer cells evades apoptosis, causes new blood vessels to form for nutrition supply, can replicate infinitely, and invade nearby tissues as well as migrate to distant tissues and organs through metastasis [2].

When Weinberg and Hanahan declared the hallmarks of cancer (Figure 1.1) a new era has begun in scientific community. According to authors, cancer promotes its proliferation without external signals. Normal cells need mitogenic growth factors to move from a dormant state into an active proliferative state. These factors are transmitted in a signaling way into the cells by transmembrane receptors, which bind unique groups of signaling molecules such as diffusing growth factors, extracellular matrix elements, and cell-to-cell contact molecules. They suggested without these signals normal cells will not be able to proliferate. A variety of oncogenes act by imitating these growth signals in normal cells. It

was demonstrated that when external mitogenic factors were introduced into normal cells in cell culture and proper conditions are maintained for cells' integrins, cells propagate as expected. This is exactly the opposite in cancer cells where appropriate propagation conditions are provided; cancer cells don't tend to proliferate like normal cells do. Various cancer cells acquire the capability to synthesize growth signals to which they are responsive and create an autocrine stimulation like proposed in human breast cancer cell lines which suggest that autocrine growth factors are secreted by the cancer cells and are essential for their growth *in vitro* [3].

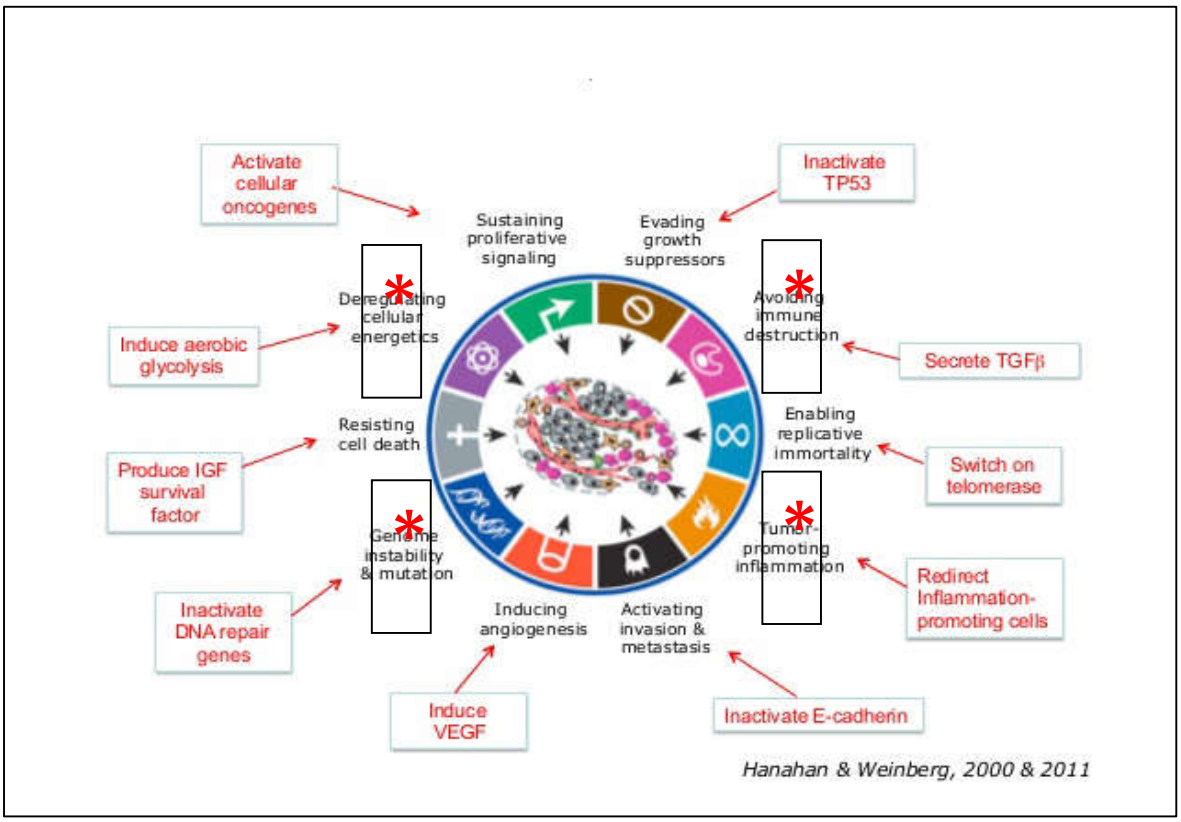


Figure 1.1 A diagram of hallmarks of cancer [4]

As a second hallmark cancer cells show resistance to the growth inhibitory signals via mutations of tumor suppressor genes like *TP53*. Predisposition to cancer is indicated by deletions or mutations in the *TP53* gene as observed in mice with homozygous p53 null mutation [5]. The disruption of the pRb signaling pathway governed by TGFb and other external factors, cause proliferation in certain types of human tumors [6]. Also, cancer cells can shut down the expression of integrins and other cell adhesion molecules, which

will send antigrowth factors, favoring their proliferation through pRb pathway [7]. The third hallmark of cancer is the evasion of apoptosis. The apoptotic mechanism can be divided into two classes of elements namely sensors and effectors. The sensors monitor the environment of inside and outside of the cell for conditions either normal or abnormal, which will decide whether a cell should survive or die. These sensors regulate the second element of apoptotic machinery, that function as effectors of apoptotic death [8]. Ultimately effectors of apoptosis including a group of intracellular proteases named caspases [9]. Two caspases 8 and caspace 9, are both activated by death receptors including Fas cell surface death receptor or by the cytochrome C which is released from mitochondria. These caspases promote the activation of a number of effector caspases that finish the death program, through destruction of structures inside the cells and organelles. In a study done bye Pitti et al., a mechanism for revoking the FAS death signal has been discovered in a high portion of lung and colon carcinoma cell lines [10]. It is highly expected that almost all cancer cells acquire alterations that enable evasion of apoptosis.

The first three principals of cancer should be enough for enabling the production of the massive cell populations that comprise macroscopic tumors. In 1997, in his early work Hayflick revealed that cells in culture have a limited replicative capacity [11]. When cell populations reach to a number of doublings, they stop proliferating a phenomenon called senescence. By disabling pRb and p53, one can establish a human fibroblast cells undergoing senescence, which will end up crisis (death). Most types of tumor cells propagated in cell culture seem to be immortalized, recommending that infinite replicative potential that was acquired *in vivo* as the tumor progress. Infinitely replication sustains the malignancy state of many tumors.

As the cancer cells get larger and bigger in size they require more oxygen and nutrients, which are supplied by the vasculature system. It is important for functioning and survival of cells within a tissue to exist within 100 μm of a blood vessel. It is also essential for the organogenesis is that this closeness maintains the growth of vessels and parenchyma. A process called angiogenesis, which is the formation of new blood vessels is tightly regulated even though transient. Due to its dependence on close capillaries, Because of this dependence on nearby capillaries, it would seem reasonable to believe the cells within a tissue might have an intrinsic capability to that proliferating cells within a tissue would have an intrinsic ability to inspire new blood vessel formation. Yet the findings suggest the

opposite, which is that cells in abnormal proliferative state don't have angiogenic ability limiting their capability for expansion. In order to progress to a bigger size, developing neoplasms must acquire angiogenic ability [12-14].

The last hallmark of cancer is the settlements of tumor cells as metastases, which are the reason of 90 per cent of human cancer deaths [15]. The ability for invasion and metastasis helps cancer cells to escape the primary tumor bulk and settle in a new territory in the organism in which nutrients and spaces are not limited. When cancer cells successfully invade the nearby tissue and metastasize to a new organ, it becomes difficult to tract down and control in place. Because of this acquired ability, cancer becomes malignant. A protein family namely cell–cell adhesion molecules (CAMs), which are the members of the immunoglobulin and calcium-dependent cadherin families, link cells to extracellular matrix components. Particularly, all of these "adherence" interactions transfer regulatory signals to the cells [16].

Alterations in expression of CAMs also play critical roles in invasion and metastasis [17]. The best example showing the switch in expression of N-CAM from a highly adherent phase to a poorly adherent phase as happened in neuroblastoma and small cell lung cancer in addition to reduction in overall expressions in pancreatic and colorectal cancers [18,19]. Experiments in transgenic mice support a functional role for the normal adhesive form of N-CAM in suppressing metastasis [20]. The other significant parameter of invasive and metastatic ability of cancer cells relates to extracellular proteases [21,22]. Upregulation of protease genes and downregulation of inhibitor genes plus the inactive form of zymogen proteases are turned into active enzymes, which lead to degradation of extracellular matrix and hence metastasis [23,24].

A decade later in 2011, the same group has added two more enabling characteristics and two hallmarks into the previous ones [4]. One of the enabling characteristics is the genome instability and mutations of tumor cells acquired during tumorigenesis. Acquisition of the multiple hallmarks mentioned above depends largely on a series of changes in the genomes of tumorigenic cells. This means that cells with distinct mutant genotypes have an advantage on the overgrowth and becoming dominant compared to the other cells in the environment. We have known that tumor progression is a multistep process where each subclone is promoted by a set mutations coded in their genomes. Inactivation of tumor suppressor genes can be inherent while they can also be acquired via epigenetic

mechanisms including DNA methylation and histone modifications. Advanced molecular genetic analysis of cancer cell genome has demonstrated function changing mutations genomic instability during the progression of tumor. A wide range of chromosomal abnormality analysis shows the gains and losses of genomic copy numbers in a variety of tumors, which revealed the evidence of loss of genomic integrity. Essentially relapse of specific genomic abnormalities like duplications and deletions at distinct sites of the genome implies that these distinct sites are more likely to have genes whose changes will favor neoplastic formation and progression [25]. Therefore, genome instability is noticeably an enabling characteristic that is related to the acquirement of hallmark capabilities.

The second enabling characteristic of tumors is tumor-promoting inflammation. Immunologist and pathologists are familiar with some tumors are being densely penetrated by cells of the innate and adaptive types of immune system which mimics inflammatory conditions occurring in non-neoplastic tissues. With the arrival of improved markers for the accurate identification of the certain cell types of the immune system, it is now obvious that almost every neoplastic tissue comprises immune cells ranging from minor infiltrations which can be only detectable with cell type specific antibodies to large inflammations that are deceptive even by standard immnunohistochemistry staining techniques [26,27].

Inflammation is even apparent in some cases of early stages of tumor progression. Also, inflammatory cells can release chemicals, especially reactive oxygen species, which have mutagenic effects on nearby cancer cells, leading their genetic makeup toward degrees of increased malignancy [28,29]. Overall, inflammation might be considered as an enabling characteristic for its roles on the achievement of hallmark capabilities.

As we know more about the characteristics of cancer in general, there are other hallmarks emerged in time. One of the emerging hallmarks of cancer is the reprogramming of the energy metabolism of cancer cells. Uncontrolled cell proliferation requires an adjustment of energy metabolism to provide fuel to the cell growth. Normal cells under aerobic conditions, convert glucose to first pyruvate via glycolysis in the cytoplasm, to carbon dioxide in the mitochondria. However when the conditions unfavor like occurrence of anaerobic conditions, glycolysis is preferred and little pyruvate is shipped to the mitochondria. This irregular characteristic of cancer cell energy metabolism was first

observed by Otto Warburg [30-32] who stated that no matter how much oxygen is available in the environment, cancer cells can reprogram their glucose uptake, and thus energy production, by restricting their energy metabolism largely to glycolysis, leading to aerobic glycolysis. Glycolytic energizing has been shown to be related to activated oncogenes like RAS and MYC, and mutant tumor suppressor genes including TP53 whose changes were found to be selectively beneficial presenting the hallmark abilities of infinite cell division, avoidance of cell cycle controls and reduction of apoptosis [33,34].

A second emerging hallmark evading immune destruction which still stays as an unresolved issue. It is the concept of immune system playing a role in fighting or destroying formation and progression of emerging neoplasias, late-stage tumors, and micrometastases. The role of imperfect immunological examining of tumors would appear to be proven by the noticeable increases of certain cancers in immunocompromised models. Recently a mounting body of evidence from genetically engineered mice as well as clinical trials suggests that the immune system works as a significant blockade to tumor development and progression. Deficiency of immune system in genetically modified mice has led the tumor development much faster than other mice with non-deficient immune system [35].

1.1.1. Cancer Initiation, Promotion, and Progression

When there is an abundance of food, oncogenes that drive certain pathways accommodate of carbon into molecules like proteins, nucleic acids, and lipids that are essential to metabolism. The driving force is to maintain cell survival and growth. In cancer, the pathways responsible for glucose and glutamine uptake and metabolism are regularly rearranged by mutations in genes such as *MYC*, *TP53,* the Ras-related oncogenes, and the LKB1-AMP kinase (AMPK) and PI3 kinase (PI3K) [4].

When the conditions are reversed like scarcity of nutrient, cancer cells are obliged to modify their metabolism and adapt to this new environment to optimize nutrient consumption. Cancer cells must be flexible both *in vitro* as well as *in vivo* to adapt the food availability. Certain mutations including KRAS cause colon cancer cells to survive under low glucose environment [36]. In cell culture environment cancer cells do reshape their metabolism balance the absence of glucose or glutamine by using one or the other to fulfill

the nutrient requirement [37-39]. For instance, when there is stably low oxygen in the environment cells survive through oxidative phosphorylation [40].

1.1.2. Oncogenes and Tumor Suppressors

There are three types of genes altered and are responsible for tumorigenesis including oncogenes, tumor-suppressor genes, and stability genes or caretakers. Oncogenes contribute cancer initiation when proto-oncogenes (genes that control division and growth) become mutated and active constantly thereby leading cells to grow out of control. Chromosomal translocations that lead to amplification of certain genes cause the activation of oncogenes. The most common genetic alterations occur in BRAF proto-oncogenes changing a valine to a glutamate located in the activation of a kinase domain [41].

Oppositely genetic alterations in tumor suppressor genes cause reduction in gene activity. For instance a mutation in TP53 protein contributes tumor formation by attenuating the apoptosis of genetically mutated cells [42]. Hence the accumulation of cells evaded from apoptosis leads uncontrolled cell growth.

The third class of genes responsible for cancer initiation is stability genes or caretakers. They form tumors more differently than oncogenes and tumor suppressors such that genes involved in mechanisms like mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) are responsible for repairing elusive mistakes created during regular DNA replication or exposition to several mutagens. Stability genes balances genetic changes to a minimum level so that if they are inactivated, mutations in other genes occur at a higher rate [43].

1.1.3. Cancer and Energy Metabolism

Cancer cells are grouped based on their growth rate as low, intermediate and fast. In *ex vivo* (animal studies), tumor degree is determined by its size, mitotic count, differentiation degree, and so on [44]. Based on their histological characteristics and clinical stage of tumor progression human tumors are categorized as slow growing or fast growing. Normal cells consume energy through mitochondrial oxidative phosphorylation (MOP). It

generates more adenosine triphosphate (ATP) when compared to glycolysis where produced only two ATP. The need for energy is very quick for cancer cells therefore cancer cells prefer aerobic glycolysis. However, one of the metabolic features of cancer cells is to avidly take up glucose for aerobic glycolysis. It was first defined by the German scientist Otto Warburg that this way of energy consumption in cancer cells is very inefficient and called as Warburg effect [30]. He thought that the reason behind the use of aerobic glycolysis was the defect function in MOP. Recent investigations deny this effect indicating that the MOPs do not exhibit in spontaneous tumors. Activated oncogenes, loss of tumor suppressors, mutations in mitochondria, and low levels of oxygen are general factors that cause tumor initiation [45]. Malignant tumors require mainly aerobic glycolysis, however MOPs are still used as energy route in some cancers [46].

During carcinogenesis metabolism of cancer cells are regulated in four waves as proposed by Smolkova et al [47]. According to him, transformation of oncogenes in cancer stem cells (CSC) is found to be the first wave of regulation. Low levels of oxygen, which ultimately induces hypoxia-inducible factor (HIF), AMP-activated protein kinase (AMPK), and NF-κB signaling is the second wave of regulation. The first and the second waves cause cells to use glycolysis as energy source and inhibits MOPs under the reprogramming of oncogenic and hypoxic controls as akin to the classic Warburg effect. Depletion of nutrition due to the high proliferation rate also induces glycolysis whereas MOPS are partially used. In this wave, through several pathways including LKB1-AMPK-p53, and/or the PI3K-Akt-mTOR genes have gone through reprogramming to adapt glycolysis. In the last wave, mitochondria are revived to cause MOPs to lead energy consumption, which contradicts Warburg effect generalizing the dysfunction of mitochondria in cancer cells.

1.2. CANCER STEM CELLS

1.2.1. The Cancer Stem Cell Hypothesis

Cellular bodies are comprised of stem cells that are organ-specific and undergo self-renewal and differentiation into multiple types of cells. These stem cells are separated from embryonic stem cells by their defined differentiation to the specific organ of interest. Cancer is comprised of heterogeneous cell populations (Figure 1.2) specifically a small group involved in self-renewal and drug resistance, that recapitulate the original tumor

[48-51]. Based on its similarity, this subgroup of cells is called cancer stem cells. The cancer stem cell phenomenon has two distinct but related components. The first component interests the origin of tumors in a cellular level, questioning of if tumors result from tissue stem cells. A second component of this hypothesis is that tumors are originated by cellular factors that show ‘‘stem cell properties.’’

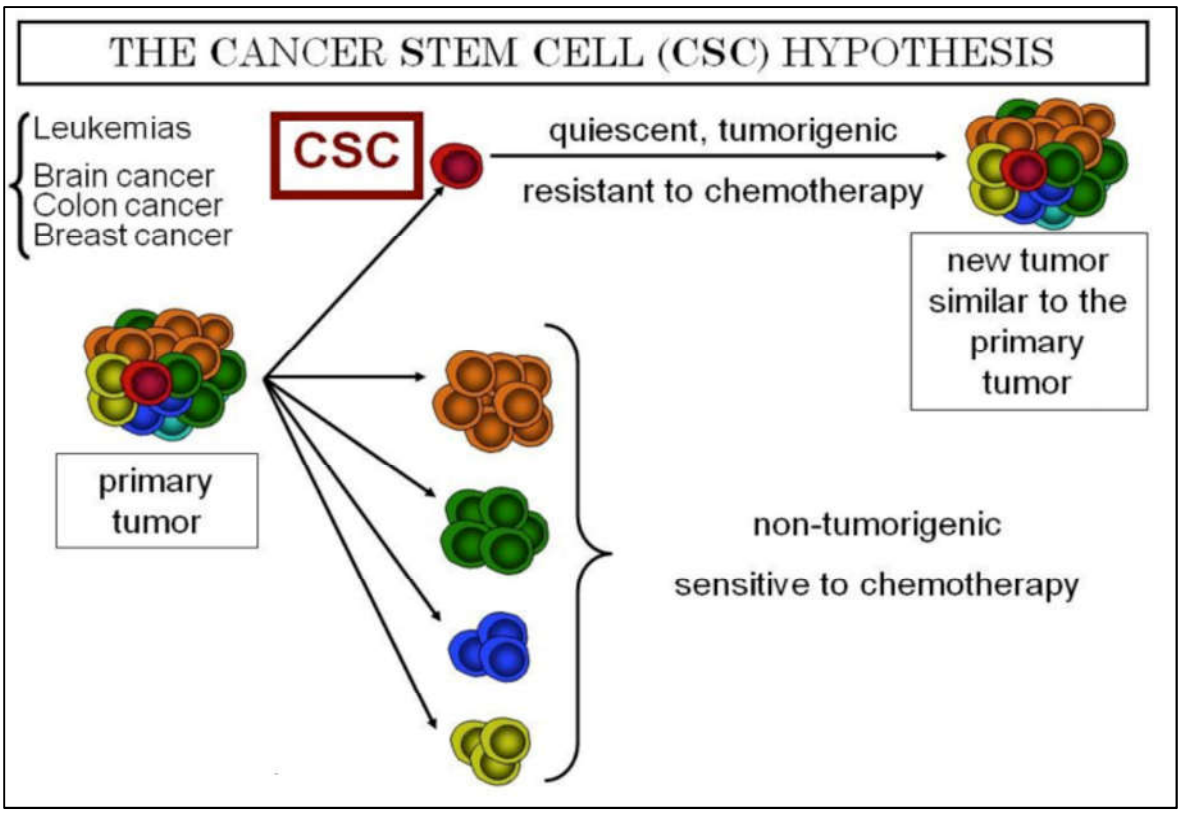


Figure 1.2. The cancer stem cell hypothesis[52]

This concept of cancers arising from a subset of population of cell that have stem cell properties was proposed 150 years ago [53]. It was suspected that tissue specific stem cells may be considered as the cell of origin of tumor [54]. A concept was proposed over 30 years ago by Pierce et al., that cancers are a stage of stem cells which underwent maturation arrest [55]. In addition, the concept of tumors containing populations of cells with stem cell features was brought by *in vitro* ‘‘clonogenic assays’’ that demonstrated subpopulations of tumor cells with higher proliferative capability as supported by colony formation in *in vitro* assays utilizing cells isolated from tumor samples [56]. One of the major drawback of these assays, however, was that they quantified *in vitro* proliferation rather than actual self-renewal. Also, it has been detected that the production of human

tumor xenografts in animal models needed a large number of cells and yet it was uncertain whether this was due to the incompetence of these cells in endorsing tumor growth or to the presence of rare populations within a tumor bulk that were uniquely tumorigenic.

Since its first description by Dick et. al, in acute myeloid leukemia in the late 1990s [57], it has been studied extensively in all of the solid and liquid tumor types. According to this hypothesis, tumor is composed of different groups of cells that create a heterogeneous structure in itself. Treatment with chemotherapy and/or radiotherapy causes tumor to shrink without eliminating it entirely. After a certain time, the tumor begins to grow even more aggressively and metastasize to other parts of the organism. A very small subgroup of cells is known to resist chemotherapy/radiotherapy through drug efflux mechanisms driven by ATP-binding cassette sub-family G member 2, ABCG2.[58,59]

1.2.2. Cancer Stem Cells in Solid Tumors

In a recent workshop Clarke et. al, stated that "cancer stem cells can only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor" [60]. In order to identify stem cell populations in a tumor, cells that are isolated from the patient are labeled with various antibodies against cell surface markers either singly or in combinations. One of the techniques generated to do this is termed fluorescence-activated cell sorting (FACS), which is based on separation of cells labelled with different cell surface marker against cells that are unlabeled [61]. These selected cells according to their specific surface markers are then used to initiate tumor in immunocompromised mice. The ability of these cells to recapitulate the original tumor defines whether these cells are cancer stem cells or not. To detect this several assays have been run through including immunostaining and flow cytometric analyses and clonal marking of the sequential tumors of the parental. Once all the assays prove the similarity of the cell populations to the cells composing the primary tumor then after the serial transplantation of these cells to the immunocompromised mice must reform the primary tumor.

At last, to show the multiple secondary tumors based on same clonal propagation, the capacity of these cancer stem cells to self-renew and raise the same tumor must be demonstrated. Therefore the original tumor can only be recapitulated after initiation and

serial transplantation in immunocompromised mice. It is simpler to show this in liquid tumors such as in acute myeloid leukemia. In the case of acute myeloid leukemia, cancer stem cells present a certain cell surface markers to isolate of distinct cells such as hematopoietic stem cells (HSCs) a phenotype with a phenotype CD34+CD38 [62] and CD90 [63]. These CD34+CD38 CD90 cells were only point one–one per cent of the total cells but had the ability to reform AML in NOD/SCID mice.

In contrast with liquid tumors, solid tumors require a more significant identification and characterization of CSCs due to the lack of knowledge on normal tissue hierarchy system. It is important to select the specific markers separating normal cells from the malignant ones to identify the original cells of the tumor. Unlike liquid tumors, solid tumors are not very easy to mimic as grafting of the cells into the origin of the tumor especially if orthotopically. Some of the organs including lung, colon, and bladder make even harder to inject the cells technically [64]. To overcome these problems several methods were developed such as subcutaneous injection or implantation under the kidney capsule, which still require confirmation that these xenografts resembles the histology of the patient tumors.

Among these solid tumors, breast cancer stem cells are the ones that was first described by Al-Hajj et al., [65]. He isolated and identified a subgroup of cells in a breast tumor tissue and demonstrated that these cells have the ability to reform the tumor unlike the remaining non-cancer stem cells did not reform the tumor even though injected at higher doses of amount. Based on their cell surface markers, the phenotype of this particular tumor type was indicated as Lineage-CD44+CD24 /low. They concluded that when as low as 200 cells of this phenotype were injected into immunocompromised mice, the cells initiate the tumor of the origin. After the first identification of solid tumor cancer stem cell phenotype, the others came immediately after. It was found that multiple myeloma consists of subgroup of cells that lack the expression CD138, which is a marker for plasma and epithelial cells [66]. Several other studies have shown the particular stem cell markers for other solid tumors including HNSCC [67], pancreatic cancer [68], and colon cancer [69,70].

1.2.3. Brain Tumor Stem Cells

Brain tumors were demonstrated to be one of the first solid tumors among others that possessed cellular hierarchy for tumor onset [71,72]. These subgroups of cells within the brain tumor cells show stem cell characteristics like resistance to conventional therapies [73]. They may also localize inside a vasculature place similar to normal neural stem cells [74-76]. Animal studies show that there is a similarity in the tumor initiation hierarchy of brain tumors between as to *in vitro* models, which highly indicates that the origin of the tumor is either progenitor cells or stem cells [77-79]. *In vitro* models improved from normal neural stem cells promise great opportunities for large drug or chemical screening for generating new molecular targets in drug discovery [80,81].

Stem cell approach has furthered the research in brain tumors while there is still a controversy about the exact distinctiveness of brain tumor initiating cells. Prior to findings in other solid tumors, human neural stem cells were found to express prominin one (prom1 or CD133) on their cell surfaces [82]. When as few as 100 CD133+ cells from human brain tumors including Glioblastoma Multiforme (GBM) and medulloblastomas were injected into immunocompromised mice, cells reformed the tumors. On the other hand CD133- cells did not constitute new tumors [83]. In a study done on metastatic colon cancer cells, CD133 expression was not found to be the initiator population. In fact, CD133+ and CD133- cells both formed spheres in vitro and accomplished lasting tumorigenesis in a NOD/SCID mouse model.

1.3. ASTROBLASTOMA

Astroblastomas are uncommon neuroepithelial glial tumors derived from the astroblasts that may invade the brain and spinal cord. It predominantly affects younger people mainly teenagers and children with a high female proportion. [84] In contrast to Fu et al. [85], recent study done by Ahmed et. al [86] shows that different age profile might have dissimilar prognosis resulted from genetic variations between young and elder people.

Since its discovery by Bailey and Cushing in 1924, rare cases have been reported that cause difficulty in terms of diagnosis and typing among other brain tumors [87]. The tumor was located in various parts of the brain as lobulated, cystic and solid masses however,

other locations such as cerebellum, brainstem, and ventricles (Figure 1.3) were also reported for the diagnosis of this tumor [88].

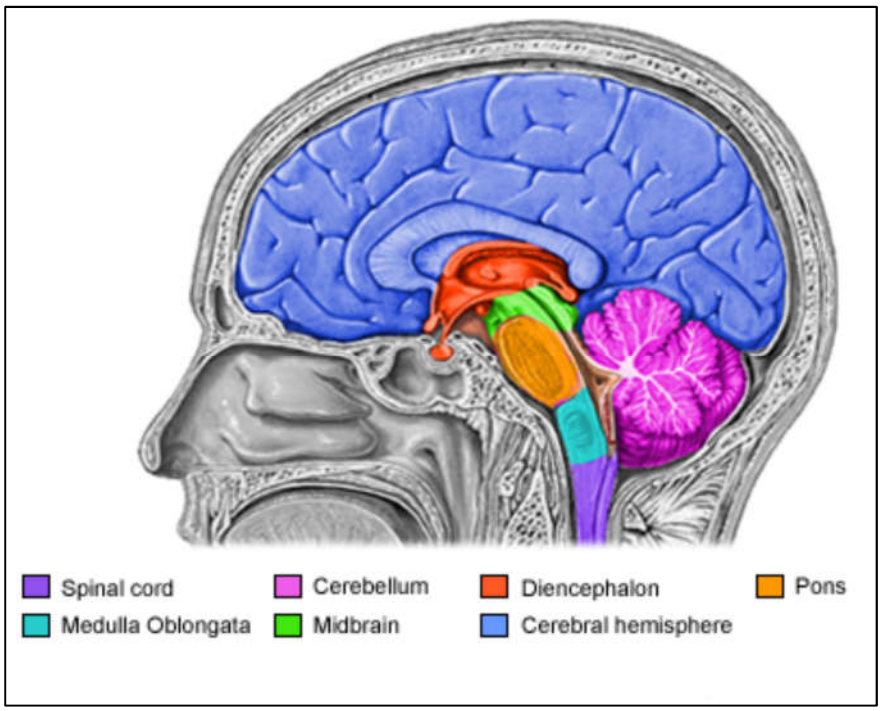


Figure 1.3. Parts of the brain

Only a single study so far reported that astroblastoma contains a high amount of neural stem like cells which had only partial proliferation capacity and differentiation [89]. In the same study authors declared that the functional role of this stem-ness in astroblastoma is unknown. This was the inspiration to us to investigate further the possible role of stem like cells in development, progression and chemotherapy resistance of astroblastoma.

1.3.1. Classification and Subtypes

Based on the recurrence rate astroblastoma might be divided into three subtypes namely, low-grade, well-differentiated, and high-grade, anaplastic astroblastoma [90]. Vivek et. al, reported a case of a low grade group without necrosis, filtration into brain parenchyma and high mitotic activity [91]. Poor prognosis is usually known with its high recurrence rate that requires more invasive surgeries. As opposed to poor prognosis, good prognosis is

known for patients with well-differentiated, low grade astroblastoma. These patients usually do not go through strict treatment regimens [92,93]. Low grade astroblastomas usually recover after the surgery and do not recur however there are some cases reported for the opposition [94,95]. Usually people who have been diagnosed with low grade astroblastoma do not tend to show the symptoms of the tumor after the surgery, however may suffer from the recurrence in one to two years. At this point the size of the tumor determines the follow up treatment [96,97], which most of the time do not require extra operation and other treatment methods remain sufficient.

As for the high grade astroblastomas, survival rate is quiet high although there are number of cases diagnosed with high grade and end up dying due to the recurrence. This type of astroblastoma tend to metastasize uncontrollably to other parts of the body [98]. Synergistic effect of chemotherapy and radiotherapy may only slow down the recurrence rate yet not kill the tumor entirely, although there are reports that show the survival benefit of chemotherapy after the surgery [99].

1.3.2. Epidemiology

Astroblastoma is a type of glial neoplasm seen infrequently with an incidence between 0.45 to 2.8 per cent of all brain gliomas [100]. The origin of this tumor is usually unknown with an indefinite prognosis. As opposed to older ages, younger people are being more affected. According to Kaplan-Meier analysis done by Sugruhe et. al, indicated that although astroblastoma is usually considered a pediatric brain tumor, the incidence of this tumor is bimodal with a noticeable five to ten and 21-30 are being more effected [84,96]. The inconsistency might be explained that parents of the younger children are more likely to complain their children's complaints than young adults do as they tend to ignore the symptoms like nausea and headache as insignificant illnesses.

In reported cases of astroblastoma the percentage of affected females show much higher incidence than male population with unknown reasons [98]. Sughrue et. al, shows that as opposed to the number of affected male against females is 30 per cent while female is 70 per cent. Some studies displays that there might be genetic predisposition of females to undergo astroblastoma than males yet there is not an explanation behind this [101,102]. Cancer is known to be caused by environmental effectors like viral infections, carcinogens,

and radiation as well as genetic changes and predispositions. Upto date there has not been a single case reported for astroblastoma to be caused by environmental factors.

1.3.3. Symptoms and Signs

Astroblastomas bear the prevalence of symptoms including headache, nausea, and damaged vision. One of the major symptoms of astroblastomas is intracranial pressure that leads to severe headaches. Both low and high grade astroblastoma patients suffer severe headaches with high grade going through more severely. These people usually end up staying home and minimize their routine work [103]. In a case report presented by Caroli et. al, a Computerized Tomography (CT) showed a large left temporal tumor with inhomogeneous enhancement and significant shift of the midline structures with scarce edema [104]. Other cases also report similar findings of a large fronto-parietal cystic mass [90] in CT findings, and other symptoms on examination, including imbalance and difficulty with walking are found. Symptoms also include vomiting, poor feeding, and [105], drowsiness, decreased sensation, and seizure [93,106,101,94]. Some high grade patients might even see hallucinations [107]

1.3.4. Clinical, Radiologic, and Histopathologic Features

In general, astroblastomas are observed as large and peripheral supratentorial tumors with bubbly appearance. Brat et. al, showed that out of 20 cases of astroblastomas, all were categorized histologically by astroblastic pseudorosettes, and most displayed noticeable perivascular hyalinization (glassy appearance), regional hyaline changes, and aggressive borders in regard to neighboring brain. As for the radiographic images, all of the lesions were contrast-enhancing and solid, frequently with a cystic element. Tumor cells were found to be highly immunoreactive S-100 positive as well as GFAP, and VIMENTIN.

As diagnostic markers, BETA III TUBULIN, GFAP, NESTIN, VIMENTIN, NEURO D, TYROSINE HYDROXYLASE are used to identify the astroblastoma cells. Beta III Tubulin is a microtubule element of the tubulin family and clinically when overexpressed it leads to aggressiveness and drug resistance of tumor. Glial fibrillariy acidic protein

(GFAP) is also an intermediate filament which is found in almost all types of cells in the central nervous system and is predicted to maintain the mechanical strength of astrocytes [108]. Nestin is a neural stem cell marker and also an intermediate filament type VI and a useful marker for exploratoring the infiltration of malignant cells into neighboring tissue [109-111]. Vimentin is found in mesenchymal cells and along with other tubulin and filament proteins comprise the entire cytoskeleton. Neuro D and TH are also found in certain parts of the brain [112]. Half of the cases were considered to be "well differentiated" and others as "malignant," due to the increased mitotic indices, vascularity growth, and necrosis [113].

1.3.5. Treatment and Prognosis

Complete surgical removal is the treatment of choice in astroblastomas [114], although high-grade tumors have a high recurrence rate. Previous studies show that surgical interventions have the greatest effect on handling these tumors [115,106,116]. Usually high-grade astroblastoma is treated with surgery and radiotherapy, it is difficult to predict the prognosis of the tumor even if the total resection is taken place [117]. Radical surgical resection is the treatment of choice for astroblastomas. Salvati et. al, claims that radiotherapy may assist in the treatment of high-grade tumors as opposed to chemotherapy whose role is still debating. As for high grade cases, they propose an aggressive treatment with temozolomide (TMZ), frequently used in Glioblastoma Multiforme, used parallel and subsequently to radiotherapy [118]. In a similar case, total resection and radiation therapy after the surgery would be efficient in high-grade astroblastomas [94].

Primary treatment in astroblastomas is total resection of the tumor. It is believed that the surgery would be sufficient to stop dividing the tumor cells turn them into the benign state. Until the second resection chemotherapy is not required. Then chemotherapy is used after the second round of surgery to kill the cells that were once at benign state after the first surgery [98]. We believe that after the first surgery, the cells that are left behind and believed to be benign might not be that benign as it is thought and may have the capacity to renew themselves and cause the tumor relapse. These cells might be stem-like cells which also are the source in many other solid and liquid cancers that lead cancer stem cells to evade chemotherapy and continue the grow and accumulate to reform the original tumor

and even metastasize to other parts of the body and resist to conventional drug therapies. Therefore, chemoresistance stay as a problematic method to continue after the surgery.

Hirano et. al, described a standard chemotherapy procedure start with two rounds of nimustine hydrochoride (4-Amino-2-methyl-5-pyrimidinyl)methyl]- 3-(2-chlorethyl)-3-nitrosoharnstoff), etoposide, vincristine, and interferon-beta [107]. The time required for drug administration is determined by whether there is a need for the second surgery. If after the second surgery tumor relapse occur, then other drugs including ifosfamide, cisplatin, and etoposide will be given to the patient in a six-round program and hope for the relapse would not occur again. At this stage chemotherapy would be the last hope, but still the recurrence might occur and also affects severely the patient's health state. Continuation of temozolomide intake orally would recommend to the patient [119,120,99].

1.3.6 Cytogenetics and CGH Array Profile

When Bailey and Cushing proposed described astroblasts in 1924, they depicted that the astroblasts are embryonic cells, which are fated to become astrocytes especially at a developmental stage between spongioblasts and astrocytes. They showed that astroblasts were clearly stained by Cajal's gold-sublimate method [121]. Although the exact cytogenesis profile for astroblastoma is unknown, a few cases in literature continue to enlighten characteristic of genetic background of astrocytoma.

Comparative genomic hybridization array (CGH array) is based detecting whether a DNA strand binds to its conjugated pair or not depends on the presence of chromosomal loss or gain. With this technology one can detect the chromosomal imbalances due to the subtle chromosome alterations. According to Brat et. al, the most prominent chromosomal changes detected by CGH were gains of chromosome arm 20q and chromosome 19 which occurred in two well differentiated and one malignant case of astroblastoma [113].

1.4. THE AIM OF THE STUDY

In this thesis, we explore a rare neoplasm that is composed of heterogeneous sub-populations of cells. Because these tumors are very scarce, it is not clearly known the

details of the behavior of the tumor and to cure them. Although gross resection of the tumor is highly recommended and applied for low-grade tumors, recurrent ones associated with bad prognosis require additional therapies such as radiotherapy and chemotherapy with a limited survival rate. Therefore, novel methodologies are needed to battle with this tumor. Due to the scarcity of this tumor there is inadequate models to learn about the pathways involved in the initiation and progression of the tumor and generate new technologies to cure it.

The presence of stem cells in cancer helped scientists to learn about the pathways associated with the nature of the tumor of any type in a better way. The progenitor and stem cells' occurrence were found to be the origin of the initiation, progression, and metastasis of various cancers. With this study we aim to learn the characteristics of this novel cell line and investigate the possible existence of stem like cells among these very heterogeneous cell populations, which may allow us to learn more about the characteristics of the tumor and provide a novel model for drug development and therapy.

2. MATERIALS AND METHODS

2.1. PATIENT HISTORY

A 24-year old male presented with headache and nausea. An MRI image showed contrast-enhancing lesion in the left frontal lobe that underwent “a gross total resection”. The pathologist noted it as a glial tumor that had some aspects of astroblastoma. Microscopic slides showed few mitotic figures although labeling index rather high and looked like an aggressive tumor. The patient did not re-checked into the hospital for any reason. Five years after the surgery patient died of an unknown reason due to the lack of information of patient prognosis after the surgery.

2.2. SPECIMEN COLLECTION AND MAINTENANCE OF A TUMOR CELL LINE

Tissue sample was obtained in accordance with approved ethical standards of the responsible committee of Yeditepe University Hospital. Tissue sample was lysed through mincing with a scalpel to one mm pieces and a let the cells to adhere to the bottom of tissue flasks and grow from there. Culturing media, Dulbecco’s Modified Eagle’s Medium, (DMEM, #41966-029, Invitrogen, Gibco, UK) supplemented with 10 per cent fetal bovine serum (FBS, #10500-064, Invitrogen, Gibco, UK) and 1 per cent Penicillin/Streptomycin/Amphotericin (PSA, Invitrogen, Gibco, UK) in a humidified chamber at 37 °C and 5 per cent CO_2, were changed twice a week and when cells have reached to the confluence of 60-70 per cent they were trypsinized. Briefly, cells were incubated with 0.25 per cent trypsin for 5 minutes in the humidified chamber at 37 °C and 5 per cent CO_2 until the cells detach. Detached cells were collected in a falcon tube and centrifuged at 500 g for 5 minutes. Supernatant was removed and cells were resuspended in culturing media. The first passage of the cells was frozen with culturing medium supplemented with five per cent dimethyl sulfoxide (DMSO) and 10 per cent FBS in a cryovial tube and stored in a liquid nitrogen tank as an original stock. The rest of the cells were left to continue to grow for further characterization assays. The name HERK is a

combination of the first/middle/last name of the patient of which for the confidentiality reason it won't be declared.

2.3. PROLIFERATION RATE ASSAY

The analysis of cell proliferation is significant to calculate the average proliferation rate of a cell population. Cells were seeded onto three 96-well plates (each plate for a day) as 1000 cells per well for six (replicates) wells and incubated at 37°C for three days. Next day the proliferation rate of one out of three plates was measured via a calorimetric assay namely 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK). The degradation of tetrazolium salt into formazan product at 490 nm gave an absorbance value for each well in the plate. An average of six wells was calculated and standard deviation was calculated based on it. A curve of three days was obtained and doubling time for this specific cell type was calculated accordingly.

2.4. SHORT TANDEM REPEAT (STR) ASSAY

Short tandem repeats (STR) found in the human genome are susceptible to copy number variation polymorphisms that can be amplified by PCR and analyzed by gel electrophoresis. Based on this one can quantify the size of the DNA fragment and detect the number of repeats because the both are proportional to each other in size. The number of repeats offers a genetic identification for each person. The cells in our study are unknown for its short tandem repeat. Short tandem repeat (STR) assay was accomplished according to the protocol described in AmpFlSTR® Identifiler® PCR Amplification (#4322288, Appliedbiosystems, USA). Briefly, AmpFlSTR® PCR Reaction Mix, was mixed with AmpFlSTR® Identifiler® Primer Set, AmpliTaq Gold® DNA Polymerase, and DNA sample with a concentration of one ng/ μL. after the PCR procedure sample was treated with formamide (#F9037, sigma-Aldrich, USA) before scanning through electrophoresis (#3100/3100-Avant, Appliedbiosystems, USA).

2.5. ARRAY COMPARATIVE GENOMIC HYBRIDIZATION (ARRAY CGH)

2.5.1. DNA Isolation

DTAB buffer (eight per cent DTAB, 100 mM Tris pH 8, 1.2 M NaCl, 50 mM EDTA) was added to HERK cells that were trypsinized and resuspended in 300 µL and incubated at 65 °C for five minutes. Onto this mixture 500 µL of chloroform was added and vigorously shaken for at least five minutes. The tubes were centrifuged at 13 000 rpm for five minutes and aqueous phase was transferred into a new tube followed by an addition of 100 µL of CTAB buffer (five per cent CTAB, 400 mM NaCl) and 900 µL of sterile deionized water (or nuclease free water). The mixture was gently mixed and incubated at -20 °C for 10 minutes. The mixture was then centrifuged at 10 000 rpm for five minutes and the supernatant was discarded. The pellet was resuspended in 200 µL of 1.2 M NaCl and 300 µL of cold 100 per cent EtOH. It was mixed gently and centrifuged at 10 000 rpm for five minutes and the supernatant was discarded. The pellet was washed with 700 µL 70 per cent EtOH and let the EtOH evaporate. The pellet was resuspended in either nuclease free water or TE buffer for the following experiments.

2.5.2. Dilution of DNA for CGH Array

Isolated DNA sample from the previous work was measured for the quantification as well as the purity. Depending on the required amount the dilution was made as final concentration of 50 ng/µL.

2.5.3. Restriction Digestion

All steps were done on ice. For one sample the following material was mixed and five µL DNA was added onto it.

Table 2.1. Restriction mixture

dH2O	11. 55 µL
NspI Buffer	2 µL
NspI Enzyme	1 µL
100X BSA	0.2 µL

Table 2.2. Restriction digestion thermal cycler program

37 °C	2 hours
65 °C	20 minutes
4 °C	5 minutes

Table 2.3. Ligation mixture

10X T4 DNA ligase buffer	2.5 µL
Adaptor, NSPI	0.75 µL
T4 Ligase Eznyme	2 µL

On top of this mixture 19.75 µL DNA sample digested with NspI enzyme was added. Ligation was performed by the following program as indicated in (Table 2.4).

Table 2.4. Ligation thermal cycler program

16 °C	3 hours
70 °C	20 minutes
4 °C	5 minutes

On top of 25 µL PCR product, 75 µL dH2O was added. For one sample the following four tubes of reaction mixture was prepared. This mixture was mixed with 10 µL ligation product (Table 2.5).

Table 2.5. PCR reaction mixture of ligation product

dH2O	50.3 µL
Titanium Taq Polymerase	10 µL
Betain	10 µL
dNTP Mix	3.5 µL
PCR Primer	4.2 µL
50X Enzyme	2 µL

Table 2.6. PCR program of ligation product

95 °C	1 minute	1 cycle
95 °C	30 seconds	30 cycles
60 °C	45 seconds	
68 °C	60 seconds	
68 °C	7 minutes	1 cycle
4 °C	5 minutes	1 cycle

2.5.4. Gel Electrophoresis for The PCR Products

The samples were run on two per cent agarose gel at 120 Voltage for 25 minutes and images were taken under UV light.

2.5.5. Purification of PCR Products

All the PCR products collected in a round bottom eppendorf tubes were mixed with purification beads and incubated at room temperature for 10 minutes. Then the samples were centrifuged at 13000 rpm for three minutes and the supernatant was discarded. One milliliter of washing buffer was added and vortexed for two minutes. They were at centrifuged at13000 rpm for three minutes and the supernatant was discarded. An eppendorf tube was placed in magnetic stand and washing buffer was removed from the pellet. Further drying step was done by letting the tube standing at room temperature for 10 minutes. Elution buffer (52 µL) was added onto the pellet and vortexed at maximum speed for 10 minutes. It was centrifuged at 13000 rpm for three minutes. An eppendorf tube was paced onto the magnetic stand and waited for 10 minutes. New sample (47 µL) was taken and transferred into a new tube.

2.5.6. Quantification of PCR Products

One microliter of PCR product was diluted with nine µL dH2O and measured by Nanodrop.

2.5.7. Fragmentation

All the steps are done on ice during the procedure. One microliter of fragmentation reagent was diluted in nine µL dH2O mixed with seven µL of dH2O, 11 µL 10X fragmentation buffer and two µL fragmentation enzyme. From this mixture five µL was transferred onto 45 µL of first PCR product and run through thermal cycler via following program indicated in the (Table 2.7).

Table 2.7. PCR program for fragmentation

37 °C	35 minutes
95 °C	15 minutes
4 °C	5 minutes

2.5.8. Gel Electrophoresis of Fragmentation Products

Two grams of agarose was weighed and mixed with 50 mL of Tris base, acetic acid and EDTA mixture (TAE) and boiled in microwave. When it is cooled down three µL of EtBr was added. The gel was poured onto a cassette with combs attached. When it is hardened the three µL from samples and one µL loading dye was mixed and added onto the gels and run through. The images were observed under the UV light.

2.5.9. Labeling Stage and Its Program

TdT and DNA labeling regent mixture was added onto the fragmented product and incubated at a thermal cycler with the following program as indicated in the (Table 2.8).

Table 2.8. Thermal cycler program for labeling

37 °C	4 hours
95 °C	15 minutes
4 °C	5 minutes

2.5.10. Hybridization Stage and Its Program

Hybridization oven must be turned on one hour before the hybridization. All the buffers must be left at the room temperature. A mixture of all the buffers as indicated in the Table must be prepared and added onto the sample and incubated in the thermal cycle with a program indicated in the (Table 2.9). Outcome product was loaded onto a chip.

Table 2.9. Hybridization mixture

Hybridization Buffer 1	82.5 µL
Hybridization Buffer 2	7.5 µL
Hybridization Buffer 3	3.5 µL
Hybridization Buffer 4	0.5 µL
Oligo Control Reagent	1 µL

Table 2.10. Hybridization program

95 °C	10 minutes
49 °C	5 minutes

2.5.11. Loading on to a Chip, Washing and Scanning Steps

Loaded chip was incubated at oven at 50 °C rotated at 60 rpm for 16 hours. After the incubation step the chip was washed with chip fluidics and placed onto the scanner and scanned.

2.6. INVASION ASSAY

Among other brain tumor cells HERK is a novel and an unknown kind for its invasiveness therefore it was compared with a Glioblastoma Multiforme (GBM) cell line, U-87 MG (ATCC® HTB-14). All cells were seeded as 200,000/well onto 8 µm porous chambers coated with growth factor reduced matrigel (#356230, Corning, USA), and a non-coated insert as a negative control. Cells were incubated for two days in a humidified chamber at 37 °C and 5 per cent CO_2. After incubation, media were removed from the chambers, and inner membrane of the insert was cleaned gently with a cotton swab to remove the non-invasive cells. Inserts were placed in new wells filled with Giemsa dye (provided in the kit) for ten minutes at room temperature. Excessive dye was removed and cleaned with dH2O for three times and let the chambers dry for a period of time before visualizing under a light microscopy. Cells stained with Giemsa were counted as invasive by Image J and compared with the control group. In addition each insert was transferred to an empty well, adding 200 µL of Extraction Solution per well and then incubated 10 minutes on an orbital shaker. Extracted solution (100 µL from each sample) was transferred into to a 96-well microtiter plate and the OD 560nm was in a plate reader and calculations were done.

2.7. MIGRATION ASSAY (WOUND HEALING)

The capacity of HERK cells for migration was measured and compared to U-87 MG. Wound healing assay was performed to measure the closure of a scratch and the migration rate of the cells according to the protocol described by Liang et. al [122]. Cells were seeded onto a well of 12-well plate as a confluent monolayer and incubated in a humidified chamber at 37 °C and 5 per cent CO_2 for one day. The next day a scratch with a p200 tip was created and media was renewed to remove the detached cells. Cells were incubated in a humidified chamber at 37 °C and 5 per cent CO_2 for two days and images were taken on each day under an inverted microscopy and closure was measured, quantified and comparisons between two cell lines were made.

2.8. IMMUNOCYTOCHEMISTRY

Astroblastoma cells are known to possess certain diagnostic markers including glial fibrillary acidic protein (GFAP), vimentin, nestin, neuro D, TH, and beta-III-tubulin. By immunocytochemistry these markers were detected on HERK cells. Briefly, cells were seeded onto poly-L-Lysine (PLL) coated microscope slides and incubated at a humidified chamber with 37 °C, five percent CO_2, and 18 per cent O_2. Adhered cells were washed with PBS three times and fixed with two per cent PFA for half an hour at 4° C. Cells were washed with PBS three times for five minutes on each wash. PBS was aspirated and permeabilized with 0.1 per cent Triton-X 100 for half an hour at room temperature. Cells were washed with PBS three times and blocked with 10 per cent Goat Serum (GS) containing PBS for one hour at room temperature. Primary antibodies diluted in three per cent Goat Serum containing PBS 4° C for overnight. Cells were washed with PBS three times and incubated with secondary antibody solution in the same buffer as used for the primary antibody. Phalloidin, which stains actin filaments in the cytoplasm of the cells, was added to the secondary antibody solution at a concentration of 1/1000 and cells were incubated with this mixture for one hour and 40 minutes at room temperature. After completion the cells were washed with PBS three times well before the induction of DAPI (five µg/mL) diluted in PBS for nuclear staining for 10 minutes. Cells were washed with PBS once and slides were removed from the wells and covered on another microscope slide reversely before viewing under confocal microscope. Slides were kept in dark. Images were taken at 20 X.

2.9. CHARACTERIZATION ASSAY BY FLOW CYTOMETRY

Through flow cytometric analysis stem cells markers were detected on antibody labeled HERK Cells. Cells were trypsinized and washed with Phosphate Buffered Saline (PBS) twice. Washed cells were fixed with two per cent Paraformaldehyde (PFA) for half an hour at 4° C and washed with PBS twice. Cells were permeabilized with 0.1 per cent Triton-X 100 for half an hour at room temperature. After completion cells were washed with PBS twice and incubated with selected antibodies, CD90 (#ab95700, ABCAM, USA), CD73 (#ab157335, ABCAM, USA), CD34 (#ab18227, ABCAM, USA), CD14 (#ab82434,

ABCAM, USA), CD105 (#ab53321, ABCAM, USA), CD31 (#ab27333, ABCAM, USA), CD44 (#ab58754, ABCAM, USA), CD29 (#ab27314, ABCAM, USA), CD45 (#ab134202, ABCAM, USA), and Integrin beta at a concentration of 10 µL/10^6 cells at 4° C for overnight. After labeling with antibodies, cells were washed with PBS and resuspended in 500 µL PBS to analyze through Flow Cytometry (FACS Calibur, BD, USA).

2.10. DIFFERENTIATION ASSAYS

Stem cells have differentiation potential to other lineages of interest when induced with various differentiating agents. Cells were plated onto six-well plates (4 X 10^4 cells/well) and cultured in osteogenic differentiation medium (ODM) consisting of DMEM (with high glucose), 10 per cent FBS, 50 µg/ml Ascorbic acid (#A92902, Sigma-Aldrich, USA), 10 mM β-glycerophosphate (#G9422, sigma-Aldrich, USA) and 10 nM Dexamethasone (#D1756, Sigma-Alrich, USA). The media were changed every other day for total of 21 days followed by washing in cold Phosphate Buffered Saline (PBS, # 20012068, Thermo-Fisher, USA), fixing with absolute EtOH and staining 40 mM Alizarin Red S (#33010, Sigma-Aldrich, USA), pH 4.1 [123] Calcium deposits were visualized under the light microscopy.

For chondrogenic differentiation, cells (1 X 10^5 cells/drop (50 µL)) were cultured as a drop in the middle of the well and incubated in Chondrogenic Differentiation Medium (CDM) consisting of DMEM (with high glucose), 100 µg/ml sodium pyruvate (#P2256, Sigma-Aldrich, USA), 10 ng/ml TGFβ3 (#SC83 P, Santa Cruz, USA), 100 nM dexamethasone, 25 µg/ml 2-phospho ascorbate (#49752 , Sigma-Aldrich, USA)[124]. The media were changed every other day for total of 21 days followed by washed, fixed in two per cent paraformaldehyde (PAF) (#P6148, Sigma-Aldrich, USA) for 20 min at 4° C, washing with PBS several times and staining with Alcian Blue (#A5268, Sigma-Aldrich, USA) in 3 per cent acetic acid (pH 2.5) for 30 min and observed at the light microscopy.

As for neural differentiation cells (3 X 10^4/well) were seeded onto 22x22 mm slides coated with one mg/mL Poly-D-Lysine (#27964-99-4, Sigma-Aldrich, USA) and placed in wells of six-well plate (#CLS3516, Corning, Sigma-Aldrich, USA). Cells were incubated in Differentiation Medium consisting of 1 µM all-*trans*-Retinoic Acid (ATRA) (#R2625, Sigma-Aldrich, USA) in DMEM (with high glucose and 10 per cent FBS and 1 per cent

PSA) [125]. Media were changed every other day for 21 days and terminated by washing with cold PBS, fixing in two per cent PAF for 20 minutes at 4° C, washing with PBS several times and stained with cresyl staining (#C5042, Sigma-Aldrich, USA) for 30 minutes. Active neurons were visualized under light microscopy.

2.11. GENE EXPRESSION ANALYSES

Differentiated HERK cells for all three lineages were collected for RNA isolation. RNAs were isolated according to protocol described in High Pure RNA Isolation Kit (#11828665001, Roche, USA). Briefly, cell pellet was treated with lysis buffer (supplemented in the kit) and lysed through several pipetting or vortexing for two minutes. Lysed cells were loaded onto blue spin columns provided in kit and centrifuged with bench top centrifuge at 9000 g for 30 seconds. Lysed product was mixed with 70 per cent EtOH, mixed well and loaded onto pink columns present in the kit and centrifuged for 30 seconds at the previous speed. The span down lysate was discarded and the collection tube was reattached to the column. The column was treated with DNAse mixed with DNAse I buffer supplemented in the kit and incubated at room temperature for 15 minutes. After the incubation step wash buffer 1 was added onto the lysate in the column and centrifuged for 30 seconds. Additional steps of washing were done for two times and finally elution buffer was added onto the column and centrifuged for 1 minute to elute RNA. Purity of RNAs were measured and the ratio for pure RNA A260/280 is ~2. Complementary DNAs were synthesized according to protocol described in QuantiTect Reverse Transcription Kit (#205310, Qiagen, USA) by using total one µg of total RNA in the reaction. Primers used in realtime PCR were described in (Table 2.1) and gene expression folds were calculated and normalized to house keeping gene, *BETA ACTIN*.

Table 2.11. Sequences of the primers

Primers	Sequences
GFAP	Hs00909233_m1
MGMT	Hs01037698_m1
IDH1	Hs01855675_s1
IDH2	Hs00158033_m1
NANOG	Hs04399610_g1
OCT3/4	Hs00999632_g1
C-MYC	Hs00153408_m1
BETA ACTIN	Hs01060665_g1
NESTIN	Hs04187831_g1
BETA ACTIN	5’ TTCTACAATGAGCTGCGTGTG 3’ 5’ GGGGTGTTGAAGGTCTCAAA 3’
COL1A1	5’ CCACGCATGAGCGGACCCTAA 3’ 5’ ATTGGTGGGATGTCTTCGTCTTGG 3’
ALP	5’ GGGTGGACTACCTCTTAGGTC 3’ 5’ ATGATGTCCGTGGTCAATCCTG 3’
RUNX2	5’ TAAGAAGAGCCAGGCAGGTGC 3’ 5’ AGGTACGTGTGGTAGTGAGTG 3’
SOX9	5’ GAACGCACATCAAGACGGAG 3’ 5’ TCTCGTTGATTTCGCTGCTC 3’

2.12. ALDEFLUOR ASSAY

The ALDEFLUOR™ assay (#01705, Stem Cell, USA) is used to isolate human stem/progenitor cells based on their aldehyde dehydrogenase (ALDH) activity. It has been reported that normal and cancer precursor cells including mesenchymal, hematopoietic,

and endothelial express high levels of ALDH, which is brighter and can be counted using regular flow cytometer or sorted through fluorescently activated cell sorting technology (FACS). Opposed to the conventional methods like immunostaining cells with certain antibodies against surface markers, isolation of stem and progenitor cells in a heterogeneous cell population is a good alternative in cancer stem cell research. In our study we aim to isolate the sub-population of stem/progenitor like cells via aldefluor assay.

According to the protocol cells were trypsinized and resuspended in one mL Aldefluor Assay Buffer supplied in the kit. In a separate tube, five µL of diethylaminobenzaldehyde (DEAB, provided in the kit) was pipetted. On top of one mL suspension of cells, five µL of Aldefluor Reagent was added and immediately half of the amount in the first tube (suspension of cell and aldefluor reagent) was added onto the second tube containing DEAB only. The tubes were incubated at 37 °C for half an hour. After the incubation, cells were centrifuged at 250 *g* for five minutes. Supernatant was discarded and pellets were resuspended in 500 µL aldefluor assay buffer. Flow cytometer was set with proper dot blots and cells were analyzed based on their expression of Aldehyde Fluor.

2.13. SPHERE FORMATION ASSAY

A single cell with a self-renewal capacity should reform a clone by consecutive divisions. To evaluate the self-renewal capacity of HERK cells sphere formation assay was done. Monolayer HERK cells grown in complete medium was switched to non-adherent conditions treated with serum-free medium containing two per cent B27, 20 ng/ml of basic broblast growth factor (bFGF, # F5392, Sigma-Aldrich, USA), 20 ng/ml of epidermal growth factor (EGF, # E9644, Sigma-Aldrich, USA), one per cent Insulin-Transferrin-Selenium (ITS, # I3146, Sigma-Aldrich, USA)), one per cent L-Glutamine, and one per cent PSA. Cells were incubated in 37 °C with five per cent CO_2 and 18 per cent O_2 replenishing sphere forming media every other day for the following two weeks until the spheres with a diameter between 100 and 200 µM are observed.

2.14. CHEMOTHERAPY INDUCTION (VIABILITY ASSAY)

As a healthy control, human astrocytes, kindly obtained from Dr. Huseyin Cimen, were seeded onto 96-well plates for the induction of drugs of interest namely Cisplatin, Velcade, Taxotere, Cytarabine and Fludarabine to determine the concentrations that does not have a lethal effect (IC_{50}). Treatment will be performed for four days and each day the percentage of viability was measured via a calorimetric assay. The absorbance of the formazan product at OD 490 nm was measured in a 96-well microtiter plate reader and percentage of viability was calculated. Based on the findings of proper doses, HERK cells were seeded onto 96-well plates and induced with the same drugs. Viability for each treatment was calculated against non-treated HERK cells.

2.14.1. Early Apoptosis Assays (Annexin V Binding Assay)

Translocation of membrane phosphatidylserine (PS) proteins from inside of the membrane is one of the earlier events of programmed cell death (apoptosis) to the surface of the cell membrane. A Ca2+-dependent phospholipid-binding protein namely Annexin V has high capacity to bind for PS. Annexin V FITC apoptosis detection kit on the BD Calibur system was then used for the detection of PS exposed on the membrane. Based on the previous chemotherapeutics analyses the selected drug, Velcade was introduced to the HERK cells and early apoptosis marker was analyzed through Annexin V labeling. Cells were treated with Velcade (five nM) for one day and followed with trypsinization and washing with PBS. Detached and washed cells then were resuspended in Annexin Binding Buffer (ABB) as mentioned in the protocol. Cells were incubated with five µL Annexin V and one µL Propidium Iodide (PI) as supplied in the kit for 15 minutes at room temperature followed by additional 500 µL ABB. Cells were then analyzed via BD Facs Calibur system and results were analyzed.

2.14.2. Cell Cycle Assay

The quantitation of DNA content through cell cycle analysis was performed by utilizing flow cytometry. The DNA of mammalian as well as other organisms' cells can be stained

via several DNA binding dyes. These dyes can bind proportionally to the amount of DNA found in the cell. According to this cells that are in the synthesis phase should have more DNA content than on the G1 phase. More cells should take up more dye and fluoresce more brightly. Therefore, the cells in G2 phase should bright as twice as cells in G1 phase.

When cells are treated with a chemical or a drug and die as a result, this might be because of termination of cell division leading to brightening less then the untreated control cells when a dye like PI given.

According to the protocol cells were trypsinized and washed with PBS, and fixed with 70 per cent EtOH for at least two hours at –20 °C. after fixation cells were incubated with a mixture comprising 40 µg/mL PI, 10 µg /mL RNase A, and 0.1 per cent Nonidet P40 for half an hour at 37 °C. Cells were then analyzed through Flow Cytometry (BD Facs Calibur).

2.15. STATISTICAL ANALYSIS

The data were statistically analyzed using one-way analysis of variance and student's t-test. The values of P 0.05 were considered statistically significant.

3. RESULTS

3.1. IMAGE OF THE PRIMARY CELL LINE, HERK

As seen in (Figure 3.1) HERK cells possess the typical appearance of neuron cells with an elongated star and spindle shape.

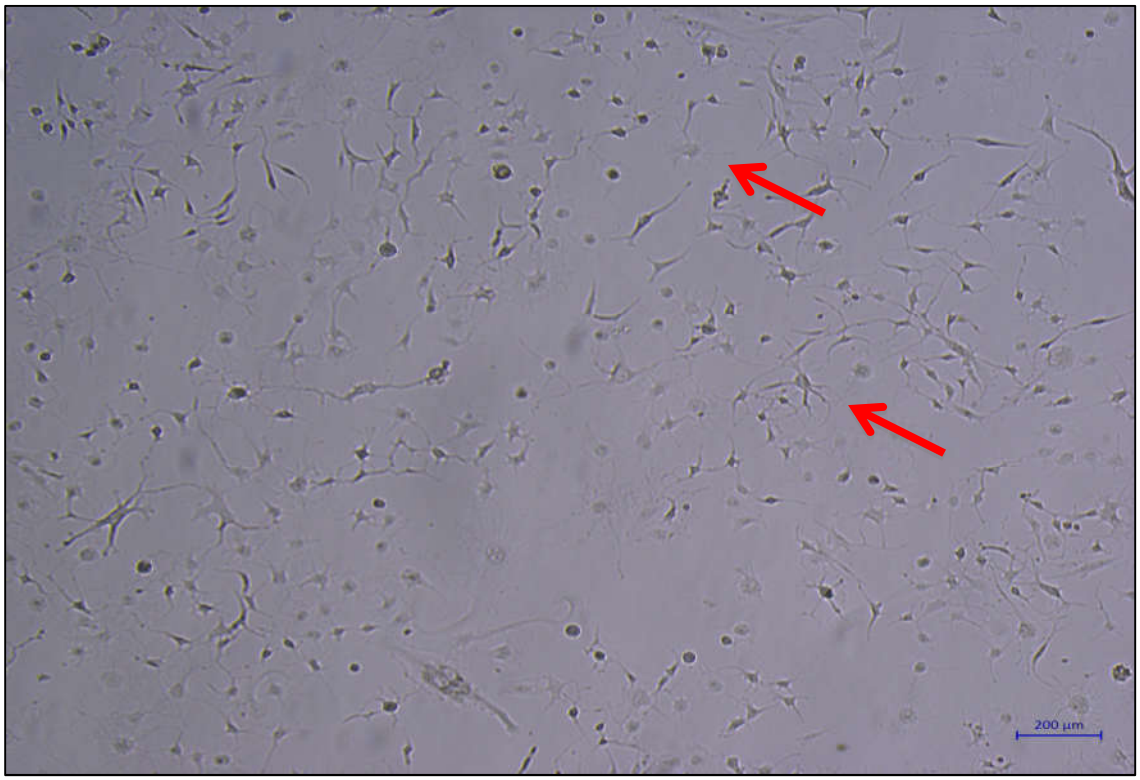


Figure 3.1. The star shaped astrobastoma cells pointed with red arrows showing typical appearance of neural cells

3.2. PROLIFERATION RATE DETERMINATION

Cells that were seeded as 1000 cells/well on a 96-well plate were incubated for three days and each day the number of cells were determined by the digestion of tetrazolium salts to colored end products namely formazans and the color indication show the increase in

viable cells. Based on this assay HERK cells showed doubling time of nearly 60 hours as in (Figure 3.2), which shows that the fold change of 2.5 is the fold in increase in numbers of cells in 72 hours.

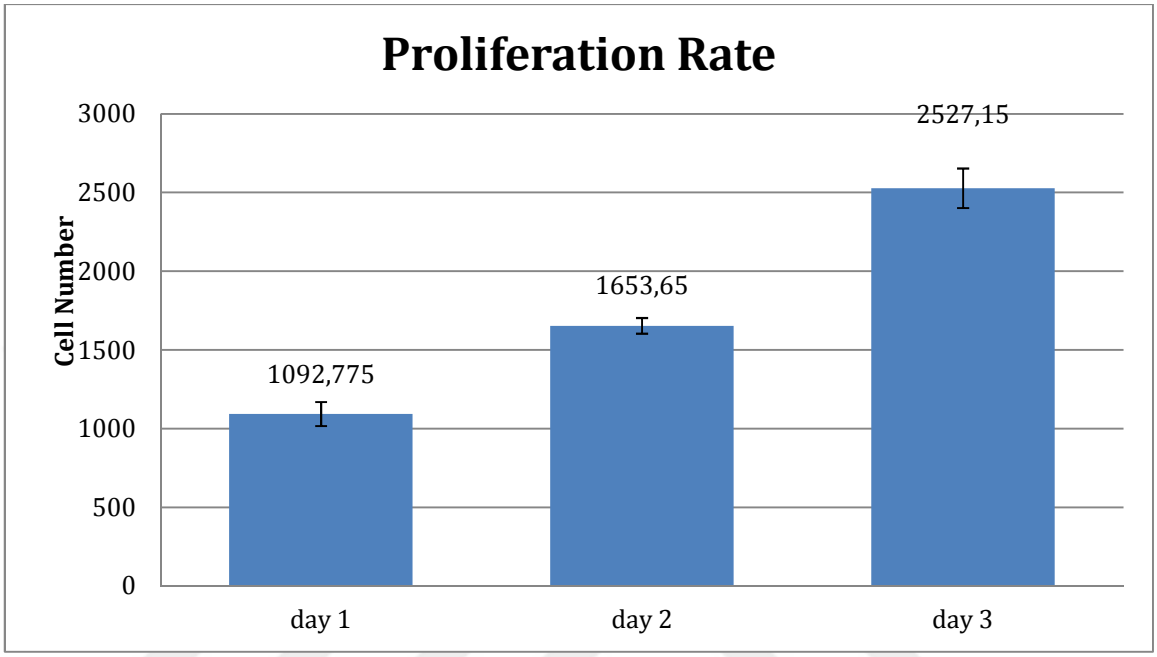


Figure 3.2. Doubling time of HERK cells

3.3. STR ANALYSIS

According to short tandem repeat analysis assay, the alleles are found designated loci as demonstrated in (Table 3.1). Due to the absence of patient's blood sample the confirmation of the STR analysis could not be done.

Table 3.1. STR analysis for HERK cells

Locus Designation	**Alleles included in AmpFlSTR® Identifiler® Allelic Ladder**	
D8S1179	10	
D21S11	31.2	32.2
D7S820	11	
CSF1PO	10	
D3S1358	18	
THO1	9	
D13S317	8	
D16S539	8	
D2S1338	20	26
D19S433	13	
VWA	18	
TPOX	9	10
D18S51	10	
AMEL	X	Y
D5S818	12	
FGA	24	

3.4. ARRAY CGH

According to CGH array, which shows the submicroscopic alterations in chromosomes, there are a number of gains in HERK cell line. Chromosomes number one, two, five, seven, nine, ten, 16, 19, 20, 21 and an extra Y chromosome appear to have many gains without any losses. In general, gains in all chromosomes possess the genes associated with neurological diseases including choreoathetosis/spasticity, spastic paraplegia, lateral sclerosis (rare neurological disease), anorexia nervosa, susceptibility retinitis pigmentosa, spinocerebellar ataxia, and retardation. Diseases associated with TSPAN2 (found duplicated in 1p13.3) include migraine with or without aura. In addition Alzheimer disease, nephropathy and susceptibility basal cell carcinoma related genes were found duplicated in this cell line. There is also duplication in *PLXNA2* gene, which has a role in axon guidance, invasion, and cell migration. Other chromosomal alterations include duplications in chromosome 5 that increases susceptibility to glioma highly compatible to this tumor type. In chromosome five there is also *TERT* gene that is highly expressed in cancers. Diseases like migraine, schizophrenia, glaucoma, and restless legs syndrome are also associated with the duplicated genes in this chromosome.

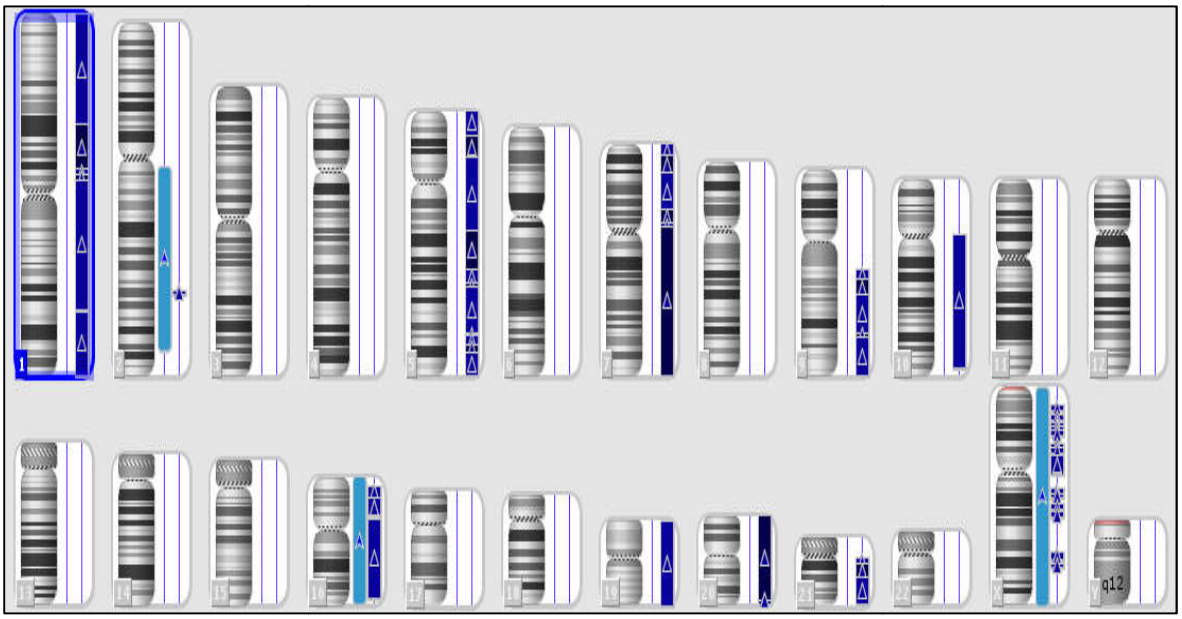


Figure 3.3. Array CGH, Karyoview of whole chromosomes

3.5. INVASION ASSAY

In reference to control group in which no matrigel coated inserts were used, 64.9 per cent (Figure 3.5) of HERK cells passed through the matrigel membrane. As a comparison 84.1 per cent of the U-87 MG cells passed through the membrane indicating that HERK cells might be almost as invasive as glioblastoma multiforme cells. This proves that the HERK is an aggressive type of brain tumor.

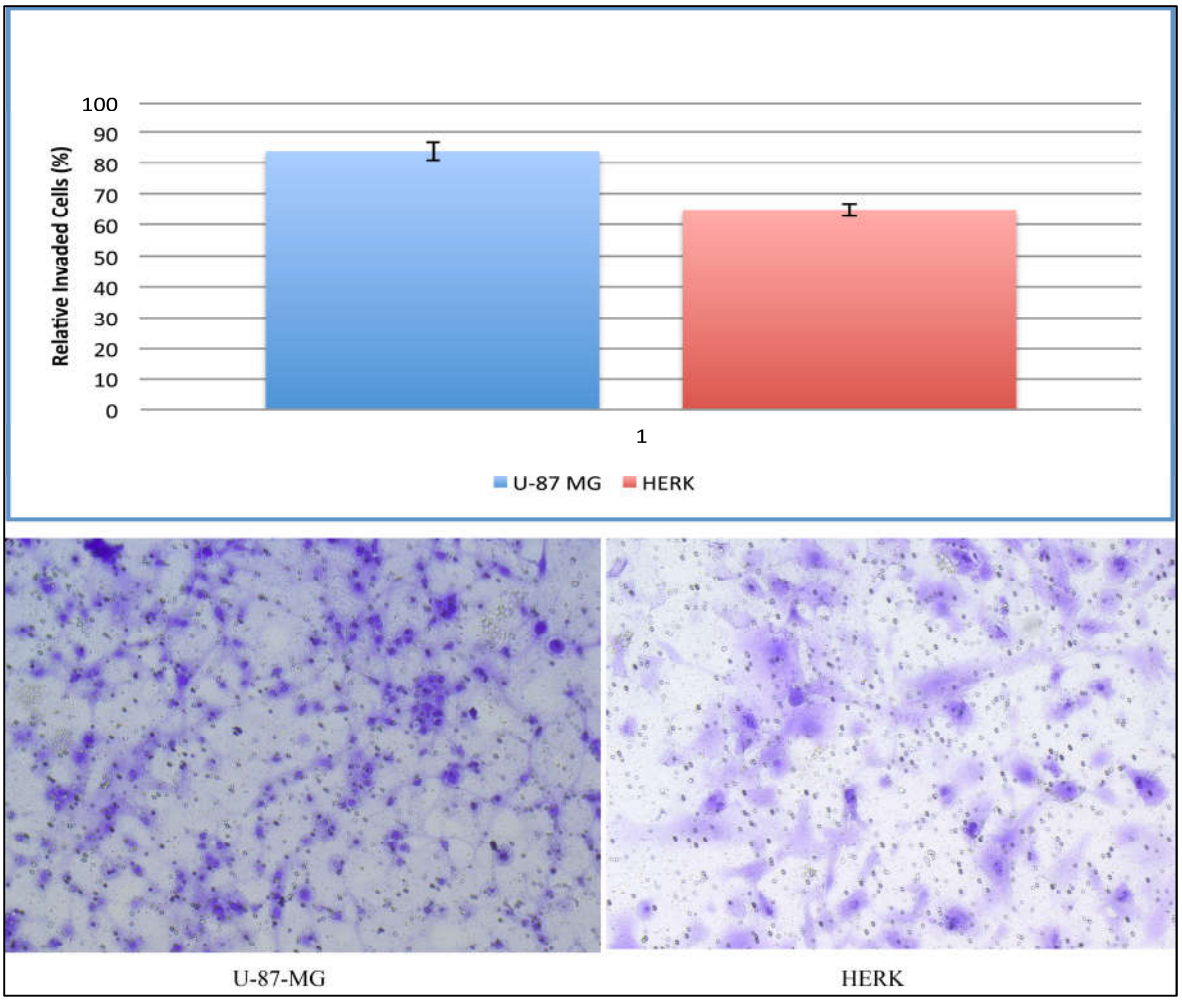


Figure 3.4. Comparison of invasioness between HERK cells and U-87 MG cells (numbers indicated in percentage)

3.6. MIGRATION ASSAY (WOUND HEALING)

Migratory ability of HERK cells was analyzed through wound healing assay. HERK cells closed the scratched area in 48 hours while U87 MG cells migrated at a faster rate closing the gap in 24 hours as shown in (Figure 3.5).

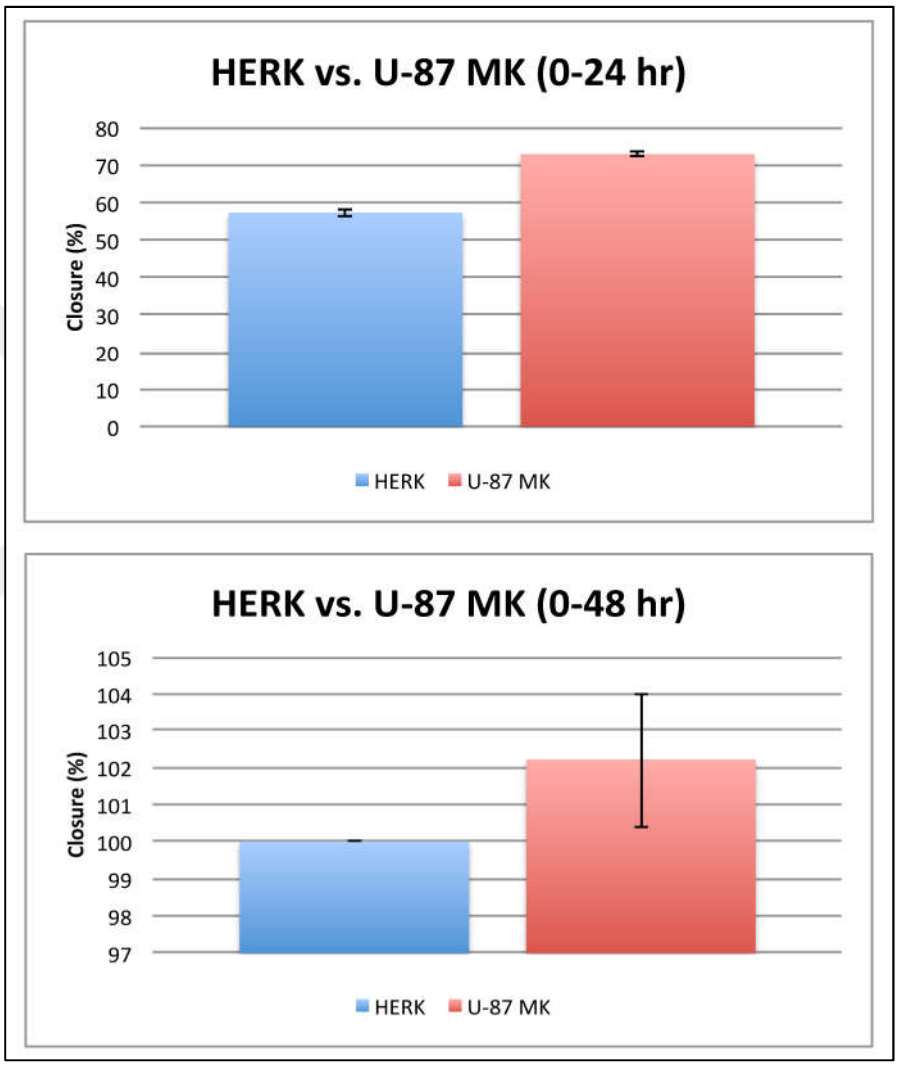
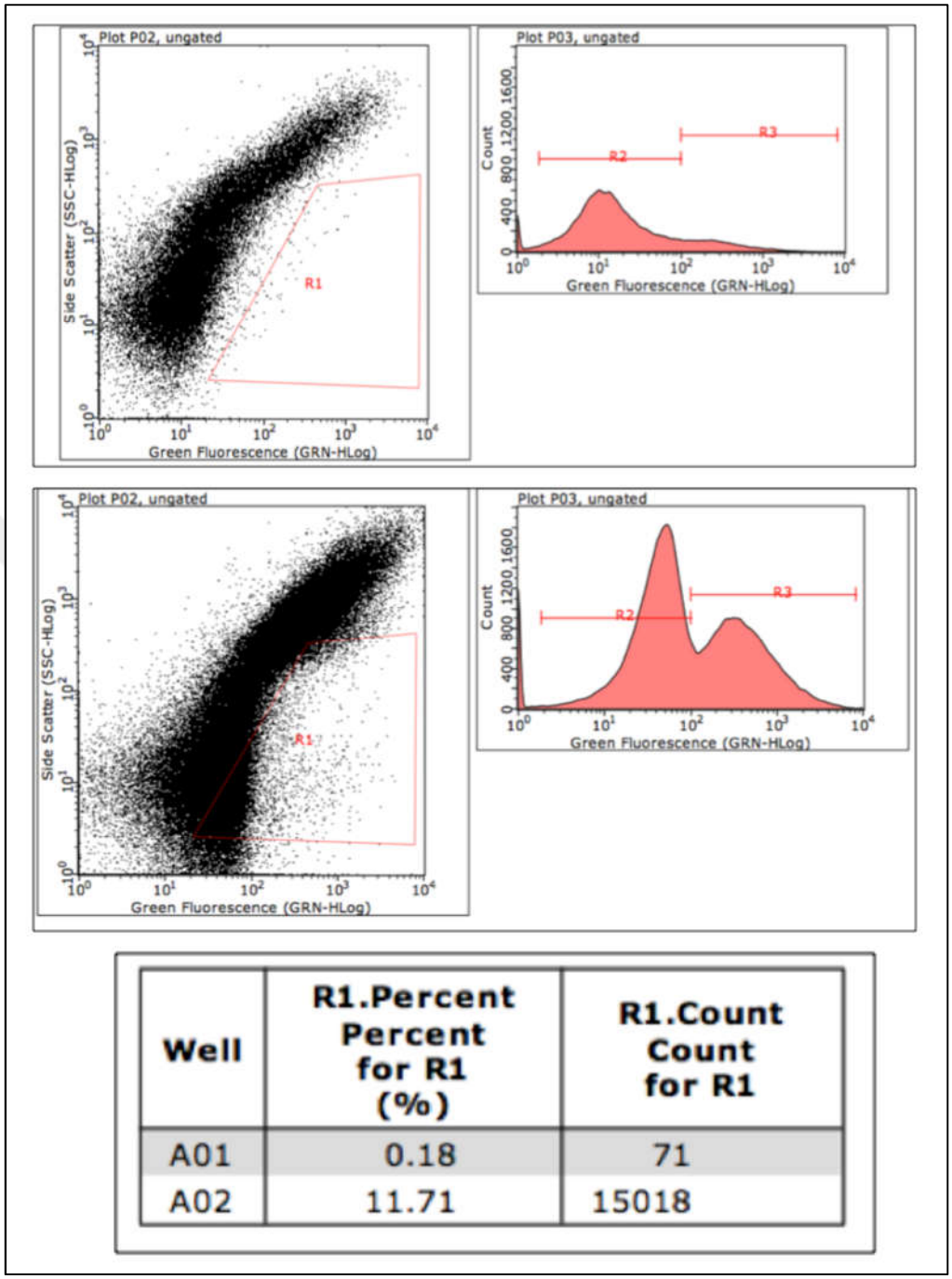


Figure 3.5. Migration ability of HERK cells compared to U-87 MK cells. Top is 0-24 hrs. Bottom is 0-48 hrs

3.7. ALDEFLUOR ASSAY

Aldehyde fluor assay was performed to separate stem like cells among other subpopulations of HERK. As shown while DEAB treated HERK cells are 0.18 per cent, there are 11.71 percent ALDH positive cells (Figure 3.6) in HERK cell line suggesting the presence of stem cells.



Well	R1.Percent Percent for R1 (%)	R1.Count Count for R1
A01	0.18	71
A02	11.71	15018

Figure 3.6. Aldehyde fluor negative cells in control group (top, DEAB treated) Aldehyde positive cells in tested cells (middle, treated with only Aldehyde Fluor Regent). Percentage distribution of aldehyde negative cells (A01) vs. positive cells (A02) ($P<0.05$)

3.8. STEM CELL CHARACTERIZATION BY SURFACE MARKERS

In order to determine the molecular signature of HERK cells and define subpopulations within, cells were labeled with stem cell surface markers and analyzed through flow cytometry. Based on the findings HERK cells appear to have mesenchymal stem cell characteristics with high percentage of CD90 (92.52 per cent) and CD73 (99.3 per cent). HERK cells also express highly of adhesion markers like CD44 (99.36 per cent), and a metastatic (diffusion) marker, CD29 (99.23 per cent) another name for beta integrin 1)). Other surface markers like CD34 and CD45, which are endothelial markers are found in low percentage as 8.58 per cent and 0.92 per cent, respectively.

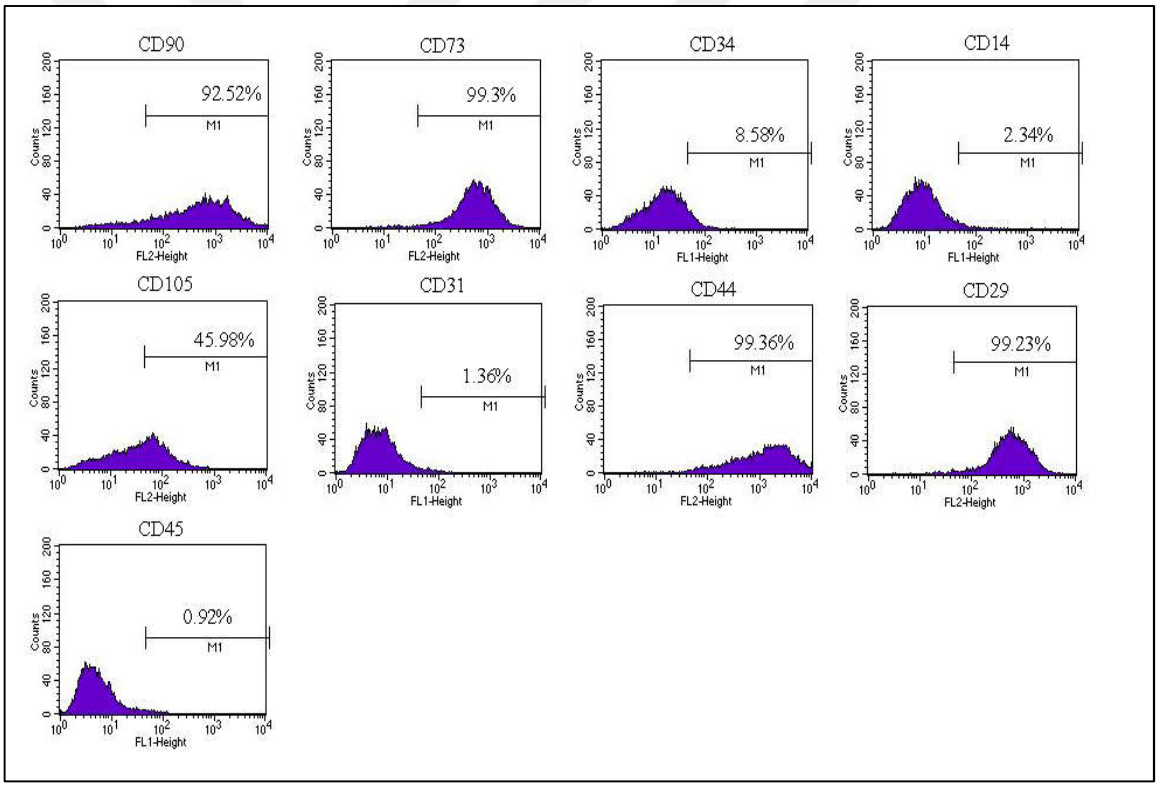


Figure 3.7. Stem cell characterization by mesenchymal stromal markers (CD90, CD105, CD29, CD44 positive, and CD45 negative)

3.9. CHARACTERIZATION OF ASTROBLASTOMA BY ICC

Astroblastoma is known to exhibit the certain diagnostic markers like Nestin, GFAP, Beta III Tubulin, Neuro D, TH, and Vimentin. For the confirmation of cells for astroblastoma origin, cells were stained with these markers via immunocytochemistry and images were taken under confocal microscopy. According to these images (Figures 3.8), cells express astroblastic characteristics positively except for the TH, which has a very low intensity of fluorescence.

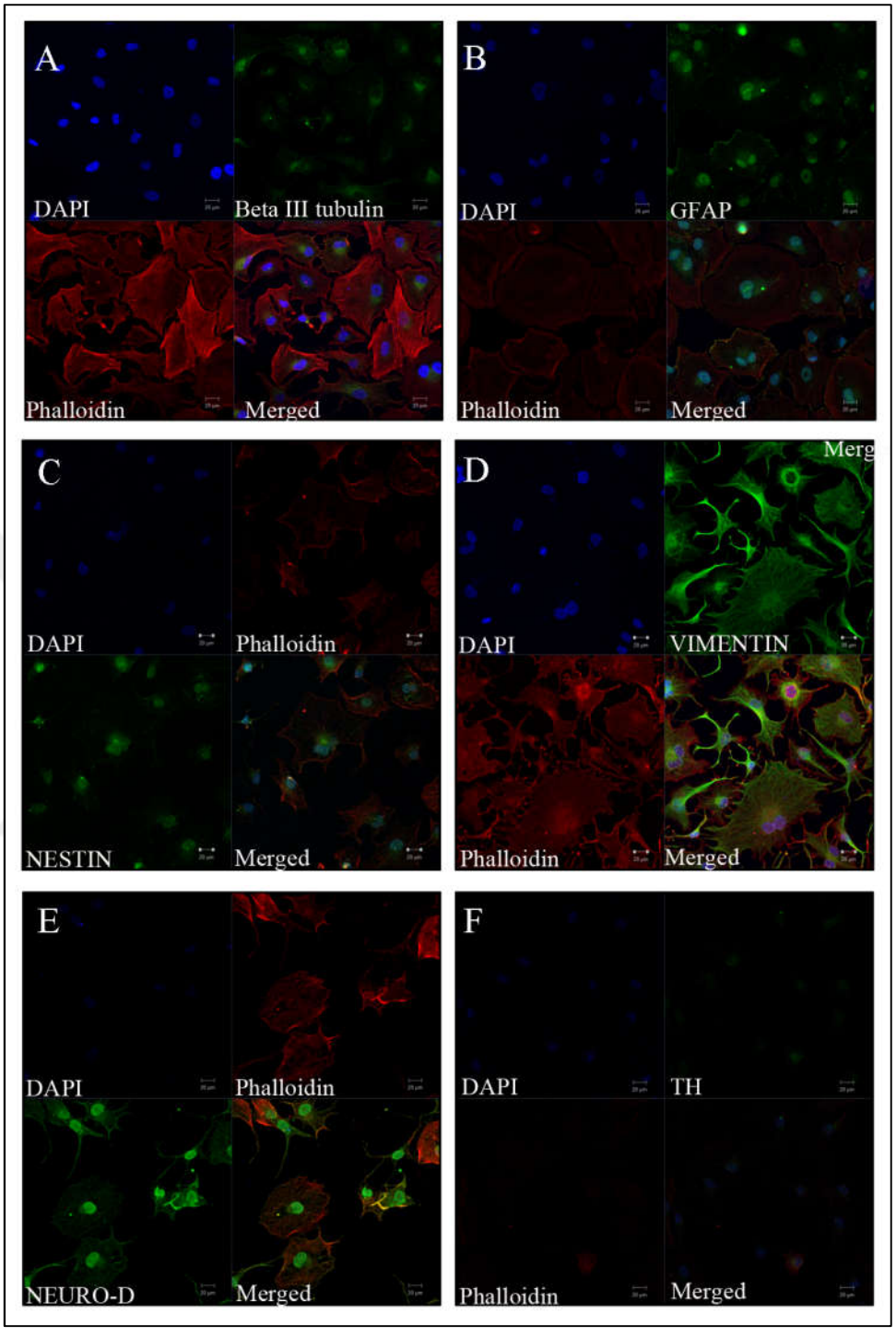


Figure 3.8. Immunocytochemistry images for A: Beta III tubulin, B: GFAP, C: Nestin, D: Vimentin, E: Neuro D, F: TH (obj, 20x)

3.10. SPHERE FORMING ASSAY

Stem like cells in a tumor tend to recapitulate the entire tumor under non-adherent sphere forming conditions. In our study HERK cells tend to form spheres as seen in (Figure 3.9). Each sphere has nearly more than 100 cells.

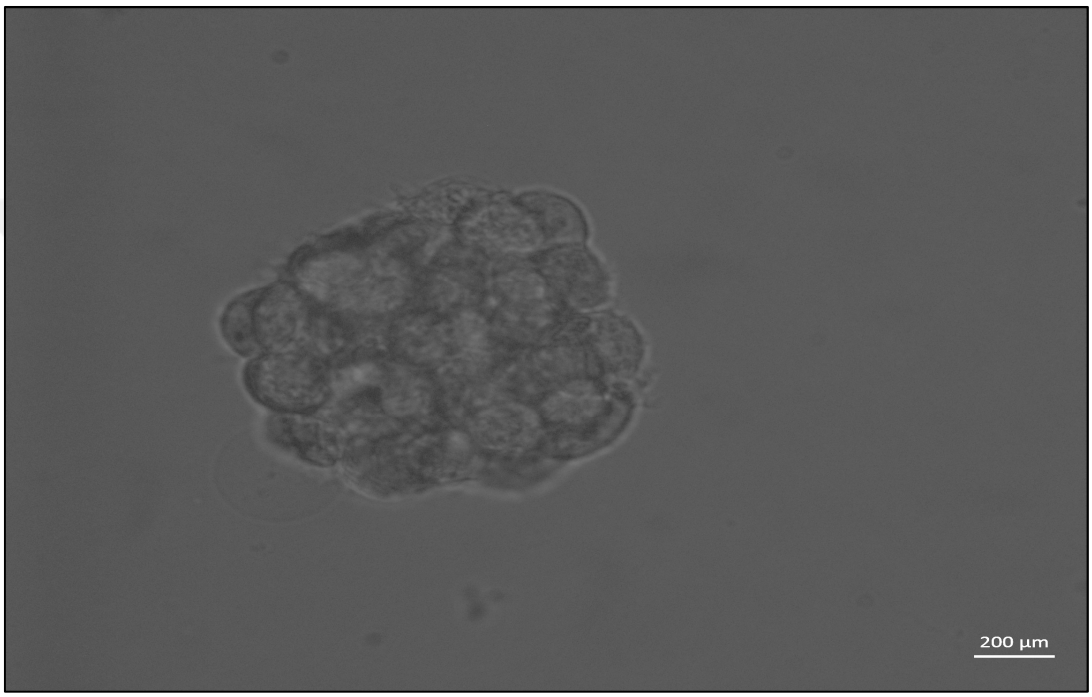


Figure 3.9. HERK cells form sphere under sphere forming conditions (20X obj)

3.11. DIFFERENTIATION ANALYSES

3.11.1. Osteogenic Differentiation

Alizarin Red dye can be used to identify calcium in cells. When cells treated with osteogenic differentiation media they were stained with Alizarin Red Dye more then the untreated control cells. Genetically cells were found to express some of the osteogenic markers such as *hCol1A1*, *hALP*, and *hRunx2* (Figure 3.10) higher when induced with an osteogenic differentiation media.

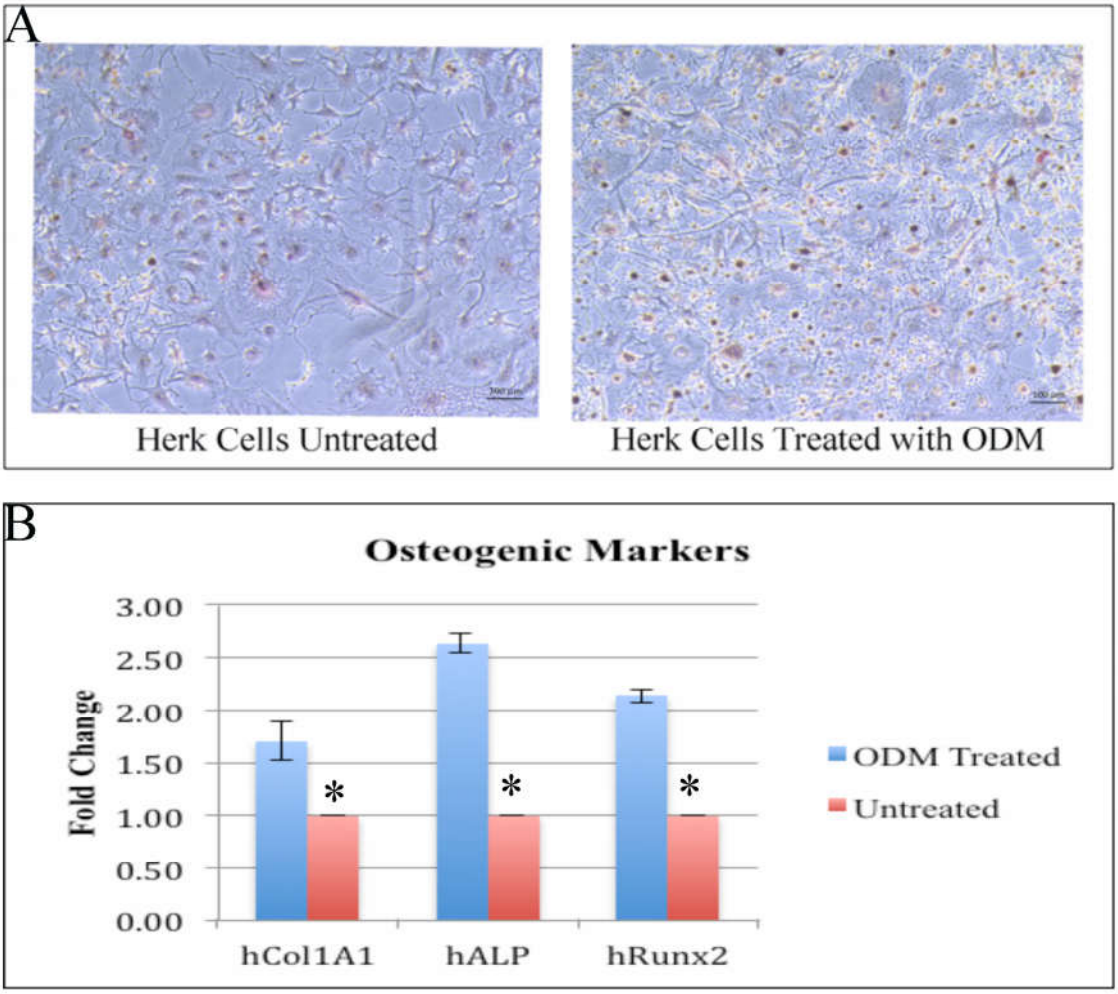


Figure 3.10. Osteogenic differentiation treatment. A: Alizarin red staining of HERK cells differentiated with osteogenic differentiation medium on top right (ODM), and untreated cells as negative control (top left, 10X obj), B: Gene expression analysis of osteogenic markers (increased levels of osteogenicity under ODM treatment)

3.11.2. Chondrogenic Differentiation

Alcian blue stains specifically acidic polysaccharides like glycosaminoglycans in cartilages of cells. One of the detection methods to show the increase in chondrogenicity is to dye glycosaminoglycans especially when induced with chondrogenic differentiation media. When HERK cells are induced with CDM they showed clearly the increased chondrogenicity. Gene expression analysis also indicates the increase in expression of selected chondrogenic markers including *Sox9*, *hCol2A*, and *CD44* (Figure 3.11).

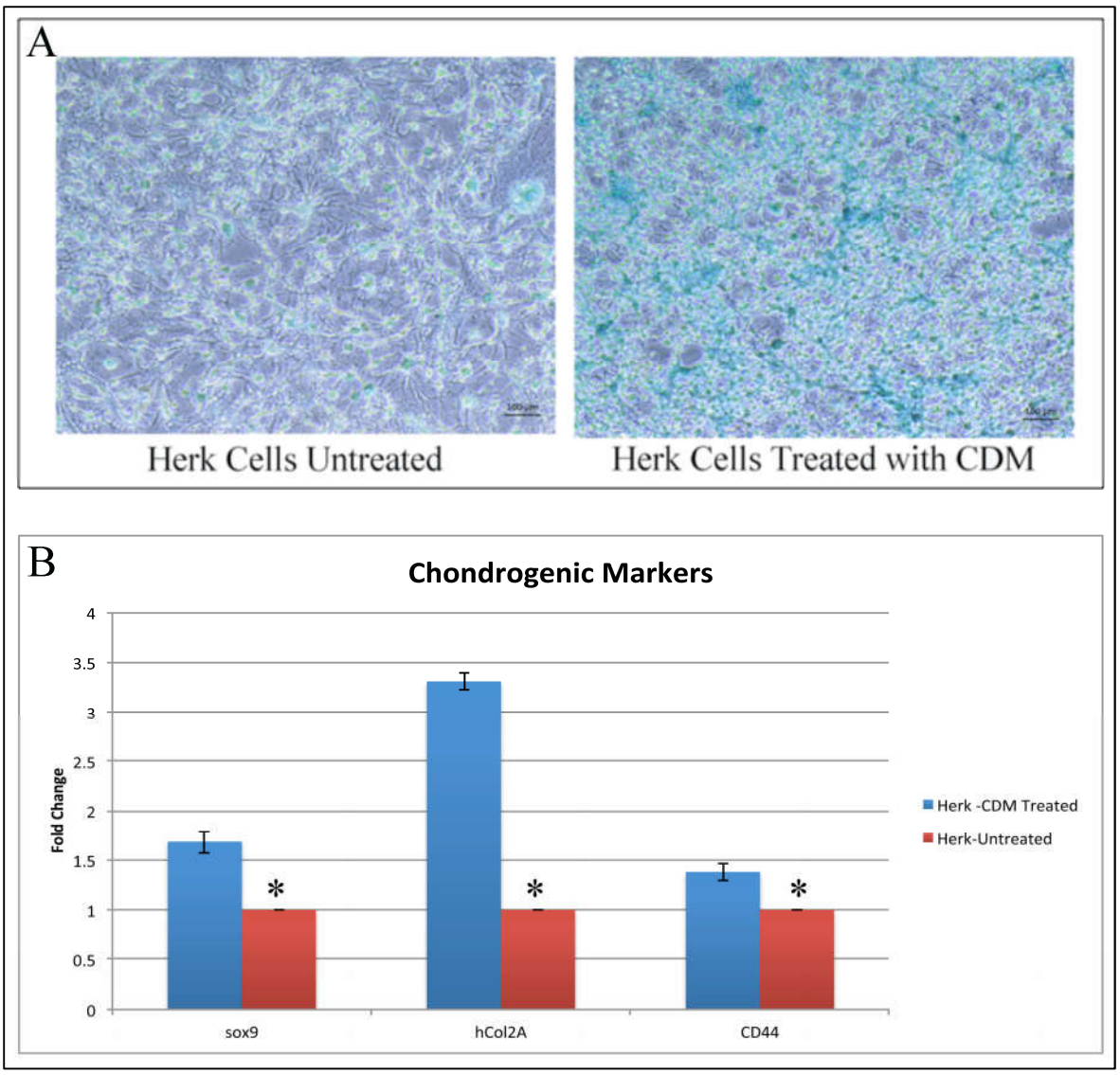


Figure 3.11. Chondrogenic differentiation treatment. A: Alcian blue staining of HERK cells differentiated with chondrogenic differentiation medium on right (CDM), and untreated cells as negative control (10X obj), B: Gene expression analysis of chondrogenic markers (increased levels of chondrogenicity under CDM treatment, (P<0.05))

3.11.3. Neurogenic Differentiation

HERK cells seemed to have a neural origin based on the tissue pathology and positivity for astroblastoma markers like, GFAP, Vimentin, Beta III tubulin, hence when induced with a neural differentiation media, it is not likely to see an increase in neurogenicity of the cells. This was shown when cresyl violet staining performed. According to the figure both

untreated and NDM treated cells have a similar staining profile. Cresyl violet acetate solution is used to dye endoplasmic reticulum in the cytoplasm of active neurons of the brain. It is difficult to measure the difference in dye intensity between two pictures (Figure 3.12). In addition to this, when the expression profiles of neural markers are compared there is not a significant change among most of the genes (Taqman Primer Probes were used) such as *GFAP* (Hs00909233_m1), *IDH1* (Hs01855675_s1), and *IDH2* (Hs00158033_m1). On the other hand stem cell markers like *Nanog* (Hs04399610_g1), *Oct3/4* (Hs00999632_g1), and *C-myc* (Hs00153408_m1) decreased significantly after the induction of neural differentiation marker, indicating that when cells become more differentiated and determined they have lost the stemness properties.

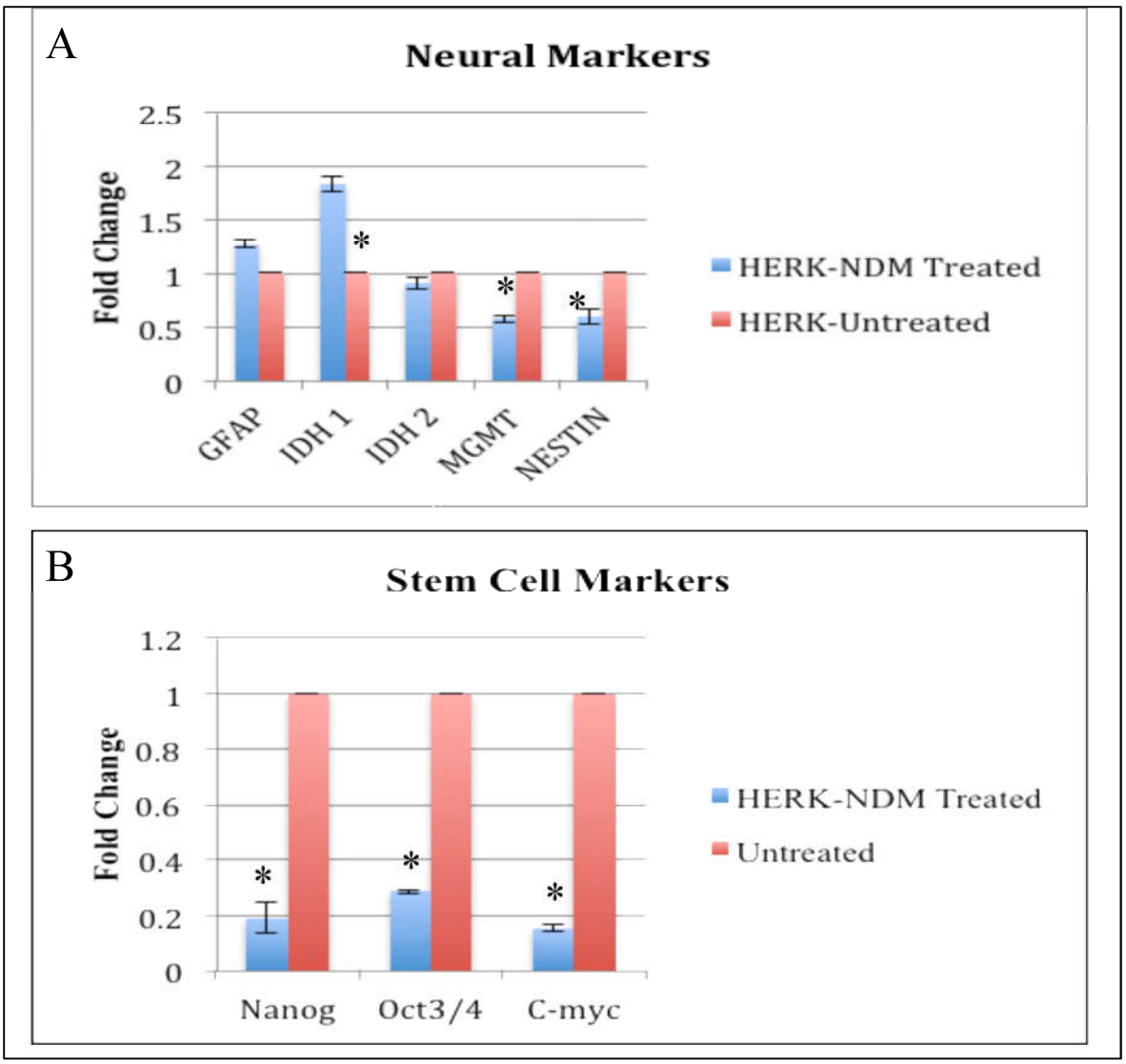


Figure 3.12. A: Gene expression analysis of altered levels of neurogenicity under CDM treatment, B: Gene expression analysis of decreased levels of stemness under NDM treatment (P<0.05)

3.12. CHEMOTHERAPY ASSAY

3.12.1. Dose Determination on Healthy Astrocytes

Healthy astrocytes were used as a reference to HERK cells in determination of the drug concentrations (IC_{50}) in drug assay trials. Drugs that decrease the viability over 70 per cent are chosen for drug trials. These drugs are namely Cisplatin (2.5 μM), Velcade (five nM),

Taxotere (five nM), Fludarabine (five µM), and Cytarabine (five µM) were not found lethal doses to healthy astrocytes as seen in (Figure 3.13)

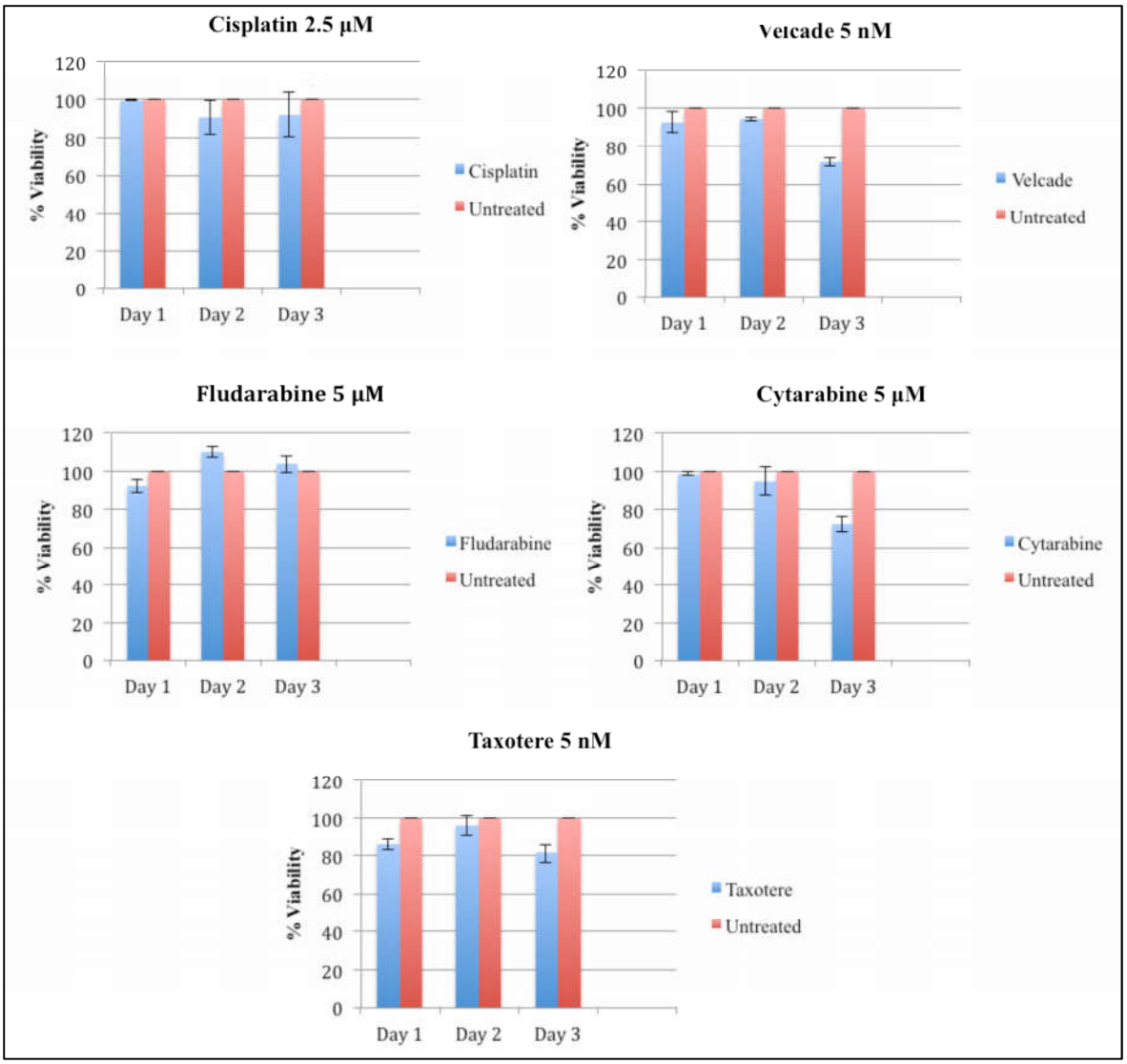


Figure 3.13. Determination of lethal and non-lethal dose on astrocytes when induced with Cisplatin (2.5 µM), Determination of lethal and non-lethal dose on astrocytes when induced with Velcade (5 nM), Taxotere (5 nM), Fludarabine (5 µM), with Cytarabine (5 µM)

3.12.2. Chemotherapy Trials on HERK cells

Based on previous findings on healthy astrocytes, the proper concentrations were determined to apply on HERK cells. After incubation for four days cells the viability of HERK cells went down to 57.25 per cent when treated with cisplatin, 8.38 per cent wit h velcade, 39.5 per cent with fludarabine, 62.6 per cent with taxotere, and 73.4 per cent with cyatarabine (Figure 3.14).

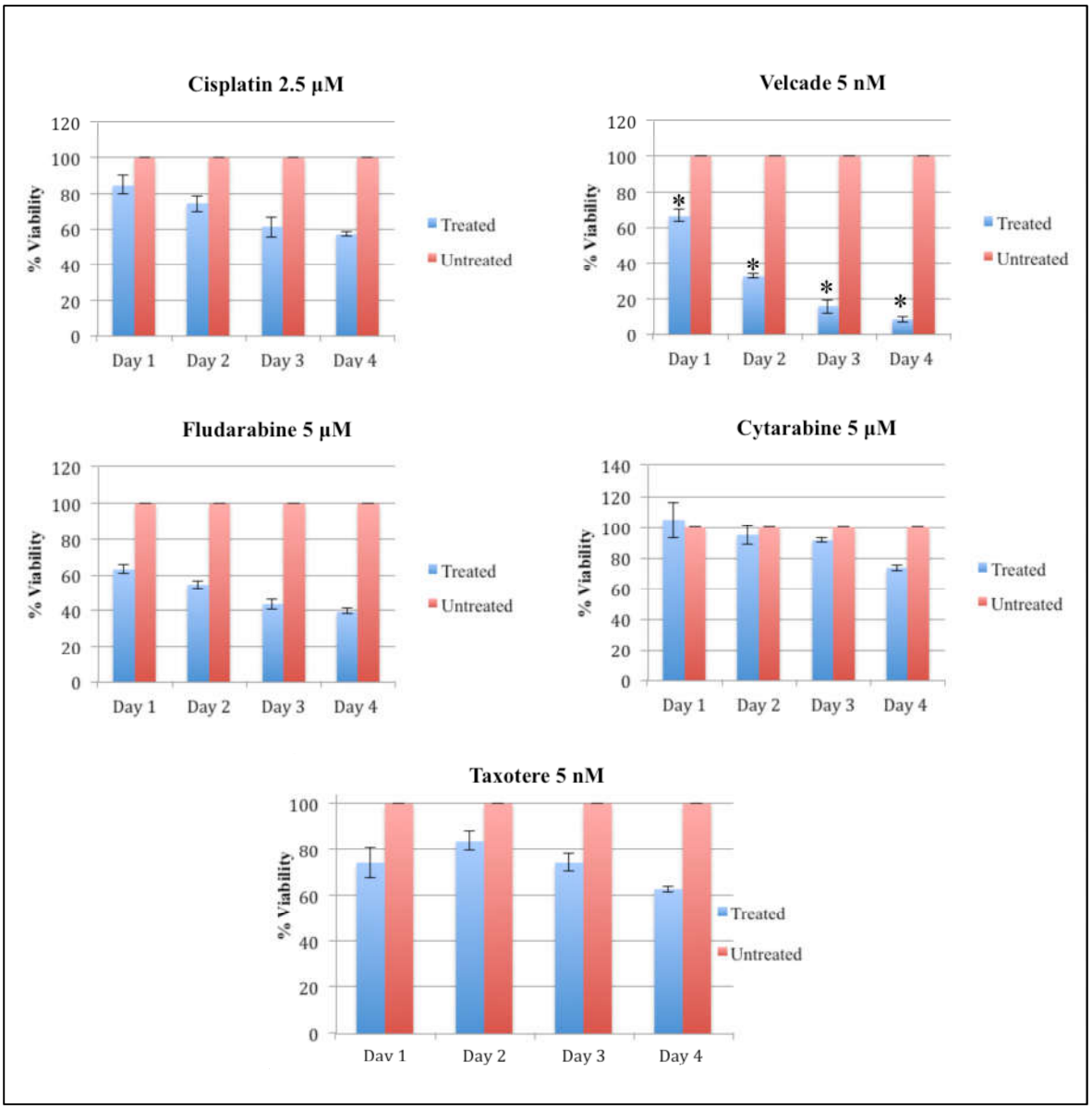


Figure 3.14. Determination toxicity on HERK cells when induced with Cisplatin (2.5 µM), Velcade (5 nM), Taxotere (5 n M), Fludarabine (5 µM), Cytarabine (5 µM) (P<0.05)

3.12.3. Early Apoptosis (Annexin V) Assay

One of the early markers of apoptosis was demonstrated via Annexin V assay. According to it, HERK cells went through en early apoptosis in after 24 hours of Velcade treatment. While 14 per cent untreated cells had early apoptosis, cells treated with Velcade was 38 per cent (Figure 3.15).

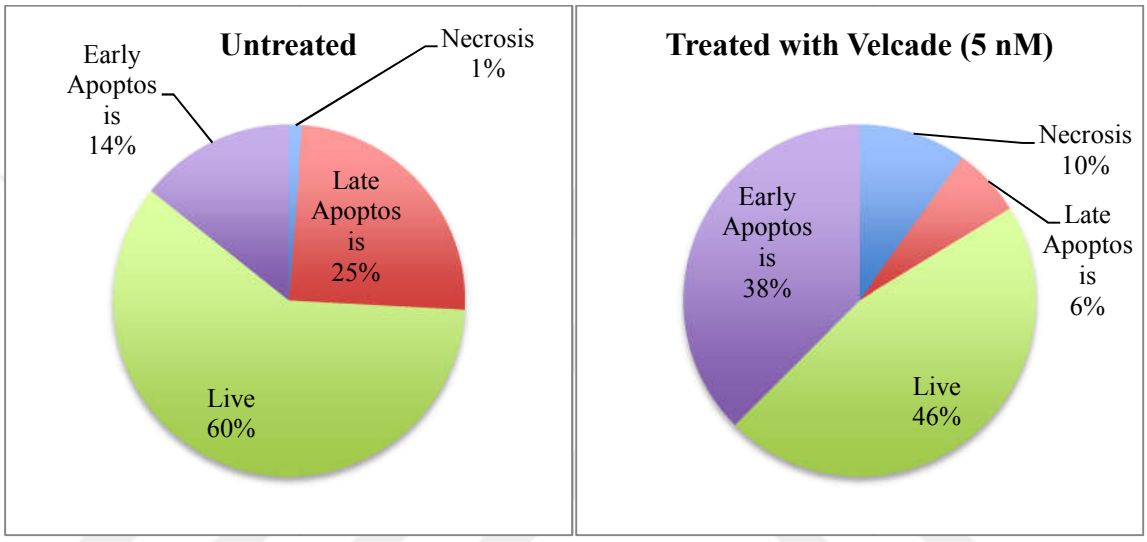


Figure 3.15. HERK cells go through early apoptosis in higher percentage when induced with Velcade (5 nM) for a day

3.12.4. Cell Cycle Analysis

The cell cycle assay is a powerful tool that indicates the G0/G1 phase versus S phase, and G2, in cells steps. Being treated with Velcade leads the decrease in the percentage of cells that are S (DNA Synthesis) phase of the cell cycle. Cells in G0/G1 phase did not seem to be affected by the treatment, however the number of cells that went to G2/M phase increased by 20 per cent (Figure 3.16).

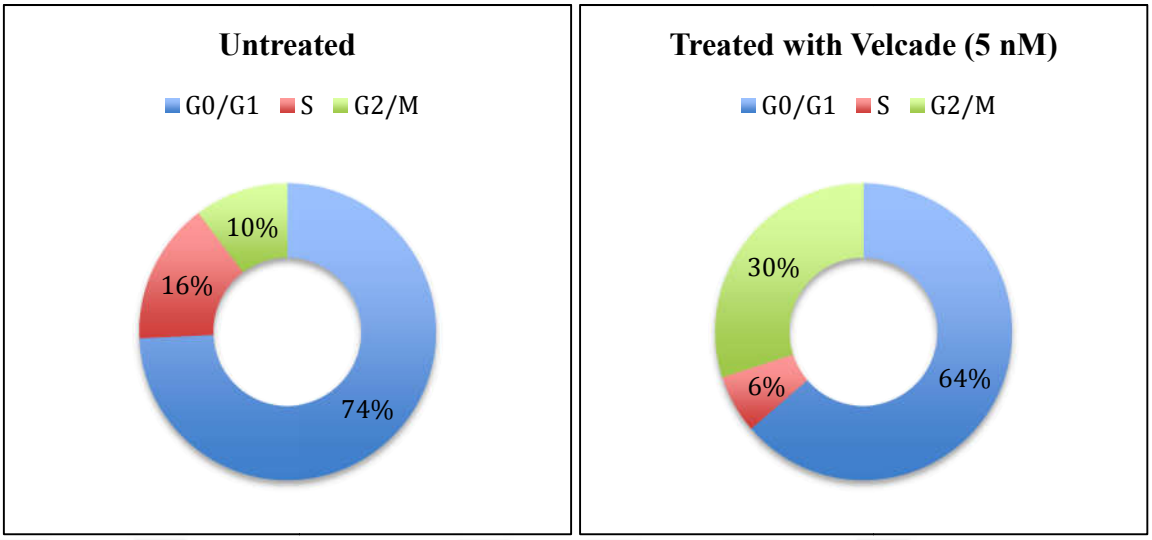


Figure 3.16. Cell cycle assay shows the arrest in HERK cells going through S phase when induced with Velcade (5 nM) for a day

4. DISCUSSION

Recent data in cancer research have suggested that tumor is a heterogeneous entity comprised of which especially a small group of cells having the properties of self-renewal and differentiation into other cells and still divide uncontrollably. Differentiation nature of these cells would help to understand the phenotypic dissimilarity of the tumors. In early 1990s a group of scientist proposed the cancer stem cell concept in leukemia and onward studies have grown like a snowball.

Among other solid tumors a subgroup of brain tumor-derived cells have several properties with the neural stem cells and named as Brain Tumor Stem Cells (BTSC) [126-128]. Two groups of scientists independently reported the existence of BTSC in heterogeneous tumors of neuro-epithelial tissue and later approved by others [129,130,72,131]. Cancer stem cells were identified based on the expressing the certain surface markers like CD133 [72] or forming neurospheres [127,131] in vitro conditions although CD133 is not considered as a cancer stem cell markers in several tumors as in our case.

In this study, we describe the generation of a novel human astroblastoma cell line (HERK) by using a surgical tissue derived from a patient who was diagnosed with astroblastoma. With knowing its characteristics, this cell line may allow us to study the molecular pathways of a rare type of a brain tumor, astroblastoma, and use it in biotechnological as well as biomedical purposes that use human rather than rodents or other animals. Successful establishment of primary human astroblastoma cells for in vitro studies is limited due to shortage of sufficient human material and the failure to passage these cells in culture environment for a long period of time.

Up to date there is only a single study suggesting a possible existence of a population of stem cells in a pediatric astroblastoma. The tissue specimen from an 11-year old was processed and cells were cultured in an established *in vitro* environment. Then, the cells were FACS sorted by surface markers, CD133, CD24, CD34, and CD45. CD133 positive cells versus negative others were chosen as the stem cell subgroup. As further studies they formed spheres in stem cells media and the cells proliferated but not for a persistent time of period and therefore, could not be used for further study. This still bears a problem since these cells do not exist anymore. The absence of these types of cells still stands as a big

gap in cancer stem like cells in astroblastoma. The role of cancer stem like cells in the initiation, progression, and chemoresistance of astroblastoma is yet unknown. Therefore more extensive studies are needed to be done to take this area into a higher level of research hence a better *in vitro* modeling is required.

Here we present a case diagnosed with astroblastoma with aggressive pathology. The patient had died for an unknown reason five years after being diagnosed. The patient may have gone through recurrence of the same tumor or a metastasis of the tumor into a new organ/tissue. The patient did not check in the same hospital as was diagnosed before that's why no further information has been gathered after the first surgery. The cell line was derived from his/her surgical material in regular tissue culturing methods and passaged over 40 times successfully with a same phenotypic appearance.

We characterized these cells as astroblastic via detection of diagnostic markers including Beta III Tubulin and GFAP, which was also confirmed with pathology results. First the doubling time of these cells were determined as approximately 60 hours which make this tumor is not very aggressive as other types including lung adenocarcinoma cell line, A549 owing nearly 22 hours of doubling time [132]. Yet, we cannot conclude this tumor as a benign one by doubling time since 60 hours is not very long time when compared to other slow growing but metastatic tumors [133]. The migration and invasion ability of HERK cells were compared to a glioblastoma cell line, U-87 MG, which is conferred for its high migratory and invasion capabilities through downregulation of NF-κB signaling pathway causing loss of endogenous interleukin 8 (IL-8) [134]. Accordingly, HERK cells are almost as invasive as U-87 MG cells suggesting the higher metastatic capacity for HERK cells.

Cytogenetic analysis of HERK using array CGH includes partial gains at chromosomes 1, 5, 7, 9, 10, 16, 19, 20, 21, and X. These minor chromosomal alterations are caused by duplications throughout the genome. Genes that may play roles in the formation of several diseases from choreoathetosis/spasticity, spastic paraplegia and lateral sclerosis (rare neurological disease) to retardation are found duplicated in HERK cells. Diseases associated with TSPAN2 (found duplicated in 1p13.3) include migraine with or without aura. In addition Alzheimer disease, nephropathy and susceptibility basal cell carcinoma related genes were duplicated in this cell line. *PLXNA2* gene has a role in axon guidance, invasion, and cell migration, therefore might be involved in astroblastoma formation or

progression. Other chromosomal alterations include duplications in chromosome 5 that increases susceptibility to glioma highly compatible to this tumor type. In chromosome five there is also duplication in *TERT* gene which is highly expressed in almost all cancers. Diseases like migraine, schizophrenia, glaucoma, and restless legs syndrome are also associated with the duplicated genes in this chromosome. There are four cases that have reported that astoblastomas have diploidy in karyotypes [135-138]. In addition to chromosomal gains and losses translocations are frequently found. Chromosome analysis by using spectral karyotyping (SKY) of an astroblastoma case done by [139] Jay, et al., discovered the loss of chromosomes 10, 21, and 22 unlike in our case indicating the variety in chromosomal mosaic in astroblastomas overall. Similar to our case, Brat et al., performed an array CGH [140] on seven astroblastoma cases, detecting genomic alterations that include gains of chromosome 20q (in well-differentiated and malignant astroblastomas) and 19. Overall previous findings support the karyotypic profile of astroblastomas with the one described in our case. In normal cells some of the signaling pathways like hedgehog, Wnt, TGF β, and notch [141-143] play a role in self renewal and when dysregulated these genes may contribute to cancer stem like cell phenotype. According to the CGH array these genes are found duplicated and therefore may cause the impairment of the regulation of self-renewal in astroblastoma. Cytogenetic analyses provide new target genes that may be associated with the initiation and progression of the disease and therefore will help us to understand the mechanisms behind astroblastoma.

Aldehyde dehydrogenase (ALDH) is an enzyme associated with the production of intracellular retinoic acid, which is involved in cellular differentiation during development [144,145]. The activity of ALDH is measured for identifying stem-like cells in tumor. In our case HERK cells possess ALDH positive cells indicating potential to have stem like cells. Further, it has been broadly used to isolate stem-like cells, either alone or in combination with other surface markers including CD44 and D133 in tumors [146-148]. HERK cells do not express CD133 (data not shown) whereas CD44 is expressed very highly. Therefore cells isolated the latter may help to understand the stem cell characteristics of HERK cells extensively.

Mechanisms that control cellular processes such as degradation of proteins involved in cellular division, growth and, death is the key regulator of cellular homeostasis [149-151]. Proteins like cyclins and cyclin dependent kinase inhibitors regulate the cell cycle [149].

When there is a problem in degradation of proteins then the regulatory proteins are accumulated in the cells leading an imbalance in cellular homeostasis affecting certain proteins involved in progression from the G1 to the S phase of the cell cycle [152,153]. Degradation of cellular proteins in eukaryotic cells is driven by the ubiquitin proteasome pathway (UPP), which comprises of a ubiquitin-conjugating system and the proteasomes [153-155]. UPP controls a number of proteins including cell-cycle regulatory proteins, oncogenes, tumor suppressors, proteins responsible form chromosome segregation and transcriptional activators as well as inhibitors [155-158]. Not only controlling these proteins but also removal of certain mutated, misfolded and degraded proteins is the ultimate function of UPP [149,156,158]. Inactivation of UPP leads to many diseases including numerous malignancies [149,155,156]. Proteasomal inhibitions may prevent cellular growth and division that leads to cells death. Since more than 80 per cent of cellular proteins are degraded by proteasomes its blockage would be unsuited in eukaryotic cells [153]. Opposing to this, clinical studies indicate that the growth of tumor cells can be arrested and apoptosis would occur when there is proteasomal degradation. Inhibition of proteasomes also increases the effect of chemotherapy by increase in sensitivity of tumor cells [159-161]. Hence, proteasomal inhibitors have become a valuable tool in developing new therapies against cancer cells. A proteasome inhibitory agent called Bortezomib (PS-341, Velcade), is the first proteasome inhibitor used in humans, and received US Food and Drug Administration (FDA) approval for multiple myeloma treatment [162-164]. In our case HERK cells treated with Velcade at a concentration of 5 nM went to early apoptosis by 2.7 fold as compared to untreated control. This indicates that velcade increases apoptosis in HERK cells. Velcade is well known for its induction of G2/M arrest in human colon cancers [165]. Increased number of HERK cells treated with velcade was found arrested in G2/M phase of the cell cycle suggesting the increase in apoptosis of damaged cells. This may suggest the selectivity of velcade in tumor subpopulations such as cancer stem like cells in this particular astroblastoma cell line.

5. CONCLUSION

In this study, we aimed to establish a novel astroblastoma cell line which may have stem cell characteristics. Stem cells in cancer have being long and extensively studied and yet more study is required to clarify the molecular pathways involved in initiation and/or progression stages of cancer.

Astroblastoma is a rare type of brain tumor that has a glial origin. Du to its scarcity *in vitro* models have not been established yet. We have a created a new cell line of astroblastoma origin, which possesses a subgroup of cells that may consists of stem like cells characteristics. The stem like cells in this novel cell line may be causing the metastatic and chemoresistance feature of the tumor. By targeting these cells, we may have less metastatic and more chemosensitive cells that may develop a basis to the generation of new therapies against astroblastoma. The cell line generated in our study may be used as a tool to proceed in this area of research but more studies are needed to be done for further confirmation of stem cell characteristics.

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