CHARACTERIZATION OF CANCER STEM CELLS IN CHORDOMA

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“this thesis is dedicated to
my family and my best friend…”

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ABSTRACT

Chordoma is the most malignant type of bone tumors. Studies had revealed that chordoma was originated from the remnants of notochord. It is a very rare type of tumor with little tendency to metastasize to nearby tissues, however recurrence rate is quiet high. Although it is a very slow growing tumor, death age is around sixty and survival is between 5 to 10 years. Due to the rare cases of chordoma, studies performed are very limited and especially difficult in determining molecular markers in the diagnosis of chordoma. Since chordoma has got similar characteristics such as self-renewal, and pluripotency, with those of stem cells, they might contain a subgroup of cells called cancer stem cells. In order to detect the presence of cancer stem cells in chordoma, several methods can be used such as differentiation therapy, scratch, and proliferation assays.

Differentiation therapy is based on arresting cells at an immature state and differentiating them into a more committed stage and eventually leading to death. As differentiating agents, retinoic acid and osteogenic medium are used in this study to detect the variation in the expression of genes, which are known to play important roles in maintenance and development of cancer stem cells. Addition to this therapy, scratch assay and proliferation assays were performed in order to see the effects of differentiation on the migration and the proliferation of cells, respectively.

Using the above methods, an alteration in the expression of genes involved in cancer stem cell has been found. For instance, differentiation by retinoic acid and osteogenic medium had caused a slower migration and proliferation rate when compared to those of untreated ones. As a result, the detection of cancer stem cells in chordoma might become a very useful tool in targeting this tumor and providing an alternative treatment method to chordoma.

ÖZET

Kordoma, kemik tümörleri arasında en kötü huylu tümördür. Çalışmalar, kordomanın notokordal kalıntılardan köken aldığını göstermiştir. Çok nadir görülmekte olan kordoma vakalarında, düşük oranlarda da olsa yakın doku metastazı görülebilmektedir ve vakalarda tekrarlama oranı oldukça yüksektir. Çok yavaş seyreden bir tümör olmakla birlikte, ölüm yaşı ortalama 60'lı yaşlardır ve yaşam süresi 5 ile 10 yıl arasında değişmektedir. Kordoma vakalarının seyrek görülmesinden dolayı, yapılan çalışmalar çok sınırlı olup özellikle kordomanın moleküler teşhisi için kullanılacak olan moleküler işaretçileri belirlemek zordur. Bir kısım kordoma hücrelerinin, kök hücrelerde görülen kendini yenileme ve pluripotensi gibi özellikleri olmasından dolayı, kordomanın kanser kök hücre alt grubuna sahip olabileceği düşünülmektedir. Kordomalarda kanser kök hücre varlığını tespit etmek için, farklılaştırma tedavisi, çizme ve çoğaltma testleri gibi farklı metotlar uygulanabilir.

Farklılaştırma tedavisi, hücreleri olgunlaşmamış bir safhada yakalayıp durdurma ve onları farklılaştırarak daha olgun bir safhaya zorlamak ve sonuçta da ölüme götürme esasına dayanır. Bu çalışmada kanser kök hücrelerde etkin rol alan genlerin tespiti için farklılaştırma ajanları olarak retinoik asit ve osteojenik besi yerleri kullanılmıştır. Bu tedaviye ek olarak farklılaştırmanın hücre göçü ve çoğalmasında etkisini görmek için, çizme ve çoğalma metotları da uygulanmıştır.

Bu çalışmada farklılaştırma tedavisi, scratch (çizme) ve çoğaltma testleri gibi metotlar uygulanarak kordomalarda kanser kök hücreler etkinliği olan genlerin ifadelerinin değiştiği bulunmuştur. Örneğin retinoik asit ve osteojenik besiyerleriyle farklılaştırma, hücrelerin farklılaştırılmayanlara kıyasla daha yavaş göç etmesine ve çoğalmasına sebep olmuştur. Sonuç olarak kordomada kanser kök hücre genlerini tespit etmek, bu tümörü hedefleme de yardımcı bir araç olarak kullanılabilir ve kordoma tedavisine de alternatif bir yöntem sağlayabilir.

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LIST OF SYMBOLS / ABBREVIATIONS

p	Short arm of a chromosome
q	Long arm of a chromosome

AFM	Atomic force microscopy
BCE	Before the Common Era
EMA	Epithelial Membrane Antigen
p-AKT	Phosphorylated Akt
p-TSC2	Phosphorylated tuberous sclerosis 2
p-mTOR	Phosphorylated Mammalian Target of Rapamycin
P-70S6K	Phosphorylated Ribosomal Protein S6 kinase beta-1
p-RPS6	Phosphorylated Plastid ribosomal protein S6
elF-4E	The translation initiation factor4E
EGFR	Epidermal Growth Factor Receptor
CD	Cluster of differentiation
GFAP	Glial fibrillary acidic protein
CEA	Carcino Embryionic Antigen
Sox2	Sex determining region Y-box 2
Oct4	Octamer-4
Klf4	Kruppel-like family of transcription factor4
APL	Acute promyelocytic leukemia
ATRA	All-trans retinoic acid
SSEA1	Stage-specific embryonic antigen 1
IMDM	Iscove's Modified Dulbecco's Medium
NP	Nucleus Pulposus
CH	Chordoma
RA	Retinoic Acid

1. INTRODUCTION

Cancer is the second leading cause of death according to the statistics done in USA [1]. It is a neoplasm, which has the ability to proliferate uncontrollably and generally migrates to the other tissues and organs of the body and causes destruction. The battle against cancer has been going on for hundreds of years showing the first case of cancerous cells in mummies as early as 3000 BCE in Egypt. As the years go by many physicians, chemists, and even philosophers had shown interest in this disease and tried different ways to treat it. Intensive research has been undertaken up to date and now in 21st century scientists know more about cancer and the mechanisms that lead to cancer. With increasing knowledge new treatment methods are also found each passing day, however cancer maintains its unbeatable power and continues to be one of the deathly diseases of the century.

When a change in the genetic material, called mutation, an abnormality, occurs in the genetic material which codes for oncogenes, tumor suppressors, and stability genes then tumor formation is expected to arise. There are many reasons for these abnormalities to occur, such as exposure to carcinogens namely radiation, chemicals, or infectious agents. As a result cells lose their control over division and therefore proliferate immensely [2]. Depending on the division and recurrence rate tumors are classified as benign and malignant. Benign tumors have a non-invasive nature and hence give a better prognosis; however malignant tumors are more aggressive and cause a worse prognosis.

In our study we had focused on a rare type of bone tumor called chordoma. Bone tumors among all the other cancers are very rare with an estimated death rate of 0.2 per cent (1470 out of 562,340 for both sexes) according to American Cancer Society statistical database posted in 2009 [3]. Depending on the location, bone tumors are classified as primary, residing in the bone and secondary bone tumors, which are located elsewhere and metastasized from another organ or tissue in the body [4]. Chordoma is a rare primary bone tumor which is malignant and locally metastatic, however gets confused with benign tumors because of its slow growing nature.

It is known that chordoma has its origin from the remnants of notochord, which is the name for spine in the embryonic stage during the mesodermal development [5]. Because of high incidence of local recurrence, invasiveness, tendency to metastasize to other organs, and resistance to chemotherapy and radiotherapy, we suspect that cancer stem cells might be involved in chordoma and aim to determine the presence of cancer stem cells by different techniques such as scratch assay, proliferation assay, and differentiation therapy.

2. THEORETICAL BACKGROUND

2.1. CHORDOMA

Chordoma is one of the rarest bone tumors of all times occurring in average age of sixty except for those of skull base origins, which lead to death in early ages. It is also considered to be the second most malignant tumors of spine [6]. Chordoma was first described by Virchow in 1856 as a small tumor of clivus. Microscopically he observed these tumor cells containing bubble-like vacuoles which were called physaliphorous (Figure 2.1) structures [7]. Chordomas are rare primary bone neoplasms, which are accepted as being originated from the remnants of notochord based on histological and immunohistochemical similarities. Incidences of chordomas range between 1 per cent to 4 per cent among all bone tumors and annually 0.08 case per 100,000 population are diagnosed with more common occurrence in men (1.0) than women (0.6), in patients younger than age 40 [8]. Almefty *et al.* had proposed that, between 1990-2006, patients were treated against chordoma were grouped by their age and among them out of 67 patients, ages between 0 -17 were 16 per cent, 18-40 were 34 per cent, >40 were 49 per cent [9-15].

As Sundaresan *et al*. described evidently, "Grossly, chordomas are lobulated, gray, partially translucent, glistening, cystic, or solid masses that resemble cartilage tumors or occasionally a mucin-producing carcinoma. The consistency varies from firm and focally ossified or calcified tissue to extremely soft, myxoid, gelatinous, or even semifluid material… This tumor may show a wide range in its histologic appearance and pattern. In addition to the areas showing physaliphorous cells, an occasional tumor may show a typical spindle cell sarcoma arrangement or a round cell pattern, whereas others may show an epithelial arrangement"[16].

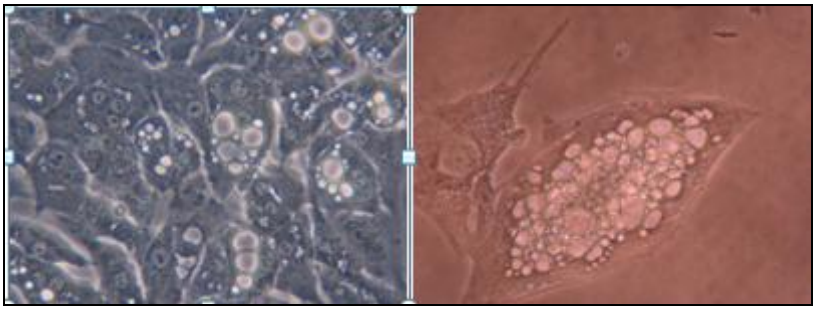

Figure 2. 1. Physaliphorous structures of chordoma tumor cells

Chordomas are traditionally considered as slow growing and locally invasive neoplasms with little tendency to metastasize and have high potential of recurrence rate because of the unsuccessful negative surgical margins [17;18]. Metastasis usually occurs at the late stage of the disease and the incidence of metastasis to places such as lung, bone, liver, and soft tissue has been reported so far [19]. The obscurity of chordoma lays beneath its aggressive behavior especially occurrence in the younger ages [20]. Frequently these tumors are found at the axial skeleton and more prone to occur at spheno-occipital and around the sacrum. Depending on the location on the spine 50 per cent of chordomas in adult occur in the sacrococcygeal (in close relationship with sacrum and the tailbone) region, 35per cent cranium (at the base of skull), and 15per cent along the spine [21]. Cranial and cervical chordomas can spread locally however metastasizing chordomas are rare.

Chordomas are considered to be low grade tumors because they are slow growing and tend to relapse and metastasize locally which means they only grow into the nearby tissues [22]. Depending on the various locations there are three types of chordoma tumors, namely the sacrum, intracranially at the clivus, and along the spinal axis and histopathologically chordomas are divided into three groups, conventional, chondroid, and dedifferentiated [5]. Conventional chordomas almost contain no cartilegeous structures however, in some incidences they may contain areas of cartilage and hence get confused with chondrosarcoma, which is another type of low grade primary bone tumor. They differ from the chondrosarcomas based on the expression of epithelial markers such as epithelial membrane antigen (EMA) and cytokeratins, specifically cytokeratin 19 [23]. One other

technique aims to distinguish chordoma from other tumors such as adenocarcinoma and cartilage like tumors is immunoperoxidase staining [8].

Common genetic changes that occur in chordoma are loss of 1p36, 3p, and 12p and gain of 1q, 7q, and 9q. It also has been shown that chordoma tumors are found to be associated with the locus 7q33 [24]. Further studies showed that there are duplications in the region of 6q27 and suggesting that the inheritance of chordoma can be resulted from these duplications [24]. However, sporadic chordomas are considered to be caused by loss of heterozygosity existed on the region of 1p36 [5;9].

Because of its rare nature, there has not been a human cell line model to work on until 2001 when Silke Bruderlein and her coworkers raised the first human chordoma cell line, U-CHI. The tumor tissue from which the cell line was generated was a recurrence of a sacrococcygeal chordoma [21].

2.2. TREATMENT OF CHORDOMA

So far there are only limited treatment methods that are applied to cure chordoma. One of them and the most important treatment is radical surgical resection [10]. In chordoma treatment maximum resection called *en bloc* method, is highly demanded since any remaining residual tumor after the surgery. For these reasons surgeons must be able to excise the tumor and scrape an extra healthy layer of the tissue so that no residue will be left behind. As this method has its limitations radiotherapy and/or proton therapy following the surgery is also applied [25]. Radiotherapy and proton beam therapies help to prevent chordoma cells from growing aggressively and leads to a better prognosis. Surgeons also need to be very careful in choosing the proper approach during surgery and consider all the parameters such as location, size extension, patient's distinguishing anatomy, and technical requirements. Experience is the most important factor that needs to be taken into consideration. If the tumor is very close to the parts of the brain that possess vital importance for the patient's life it might cause a life-threatening danger, therefore experience is highly required in these types of surgery. In some cases where surgery is not a solution, radiotherapy alone is considered to be the second choice [26]. Although chordoma cells are known to have chemoresistance, recently a drug called imatinib

mesylate has been used in treating chordoma patients. Imatinib mesylate has an anti-tumorigenic role in advanced chordoma cases [27]. Another recommended therapy is carbon ion radiotherapy which is effective and has minimum side effects for those skull based chordomas [28].

2.3. ORIGIN OF CHORDOMA

Up to date there has not been an exact mechanism revealed for the initiation and progression of chordoma, however recent studies put so much effort in order to explain some possible mechanisms for this disease. As recently reported, chordoma tumors are originated from the remnants of notochord. All chordates possess this structure at the embryonic stage of their lives [29]. Some chordates continue to have this structure throughout their life time, however most vertebrates replace the notochord with vertebral column, also known as the spine or the backbone, in which intervertebral discs, sacrum and thirty-three vertebrae were separated by intervertebral discs [30]. Each disc consists of three parts; a nucleus pulposus (Figure 2.2) the gelatinous central structure which gives the disc its flexible feature, annulus fibrosis which surrounds this inner part, and cartileganeous end plates [31].

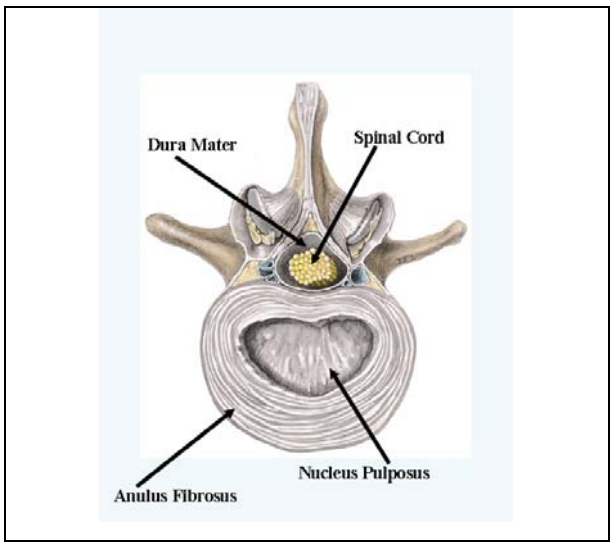

Figure 2. 2. Intervertebral disc containing nucleus pulposus and annulus fibrosis [82]

A recent data by Choi *et al*. (2008) suggested that in a mouse model, embryonic notochord gives rise to all cell types that are found in the nucleus pulposi part of intervertebral discs. In the same study it was shown that notochordal cells had begun to aggregate in the developing embryos and localized in positions where the intervertebral discs resided at the day of 12.5. Three days later notochordal cells had started to aggregate where the nucleus pulposi were forming and after one day almost all the cells had formed in the nucleus pulposi, however some of them had been detected in between intervertebral discs indicating that the completion of nucleus pulposi formation was not always the case. According to this study it was suggested that notochordal cells between discs had transformed and became responsible for the initiation of chordoma tumor [32].

2.4. A POSSIBLE MECHANISM OF HOW CHORDOMA OCCURS

According to the study done recently, through immunohistochemistry and western blot studies potential molecules in signaling pathway have been analyzed and found out that 65 per cent of chordomas were positive for p-AKT, p-TSC2, p-mTOR, p-70S6K, p-RPS6, p-4E-BP-I, and elF-4E and may be responsive to mTOR, inhibitors [33;34]. EGFR signaling pathways are also found to be activated in chordomas and may provide a potential therapeutic target in the treatment of chordoma [35].

2.5. CHORDOMA MARKERS

For the diagnosis of chordoma, not only morphological features but also immunostaining is being used. Epithelial markers such as cytokeratin 19, and epithelial membrane antigen (EMA) as well as cell adhesion markers such *e-cad*, *CD24*, and *CD44* are used to detect chordoma. However, all of these genes are not specific to chordomas therefore cannot be considered as specific markers to be able to detect chordomas [36]. An immunohistochemistry study in 2008 showed that *brachyury* gene is expressed in conventional chordoma cases with a specificity of 90per cent, whereas other genes such as, *CD24*, *GFAP*, and *CEA* showed less specificity [37-40]. Therefore *brachyury* gene became irrevocable in detecting chordoma.

2.6. POTENTIAL TO DIFFERENTIATE INTO MESENCHYMAL LINEAGES BY *"BRACHYURY"*

Brachyury is one of the members of T box family, which is an important protein responsible for the formation of short tail (*T* for the tail) in mice has been found to be expressed in all chordomas [41]. It is known to play an important role in the differentiation of notochord and mesoderm formation during notochordal development in mice. Brachyury is a transcription factor, which is found in the nucleus as an early mesenchymal marker, albeit showing no longer expression after the late differentiation of mesoderm. It has a potential to induce the differentiation of mesenchymal originated cells into other mesenchymal lineages such as bone, chondrocyte, and cartilage. In a study, mice lacking brachyury gene had died because of insufficient mesoderm formation leading to the absence of notochord at ten day of gestation [42]. For instance in one study, forced expression of brachyury led to the differentiation of mesenchymal cells into chondrogenic cell lineage. This finding indicates that brachyury gene has a potential of inducing differentiation [43].

2.7. CANCER STEM CELL CHARACTERIZATION IN CHORDOMAS

Self-renewal and differentiation are very important properties that a cell can possess especially for the tissues where major losses and repairs occur. The basic mechanism of self-renewal is that stem cells divide either symmetrically where two alike daughter cells are produced or asymmetrically with one daughter stem cell and one non-stem cell [44]. Differentiation is a process where a cell differentiates into other lineages. These two distinguished features make a normal cell a stem cell. The idea of the existence of cancer stem cells arises from the similar properties with those of stem cells. Tumors are formed as a mixture of heterogeneous cell groups in which some cells possess similar properties with those of stem cells such as the ability for self-renewal and differentiation into multiple lineages [45-47]. In addition to this, due to the self-renewal capability cancer stem cells maintain longer lifespan [48].

Due to the differentiation potential in cancer stem cells, transcription factors such as *sox2*, *oct4*, and *nanog* are found to play important roles in the fate of cancer [49].

Especially *sox2* and *oct4* form a heterodimer to regulate nanog, which altogether play a role in the differentiation [50]. A recent study supports the idea of co-expression between oct4, sox2, and nanog hence we might expect to see the joint expression of these genes in chordoma as well. Additional to these particular genes *klf4* and *c-myc* are found to be involved in cell proliferation during carcinogenesis [13].

The phenomenon of cancer stem cell has become especially important due to the fact that cancer stem cells show more resistant to chemotherapeutics or other anti-cancer related treatments than normal cancer cells [51]. Liu *et al.* (2009) proposed that in glioblastoma, CD133+ cancer stem cells showed more resistance to chemotherapy compared to the negative ones. They have concluded that it was important to target this small subset of cells to increase the rate of survival in glioblastoma [52].

Cancer stem cells are known for having a role in metastasis as well [53]. Metastasis through epithelial mesenchymal-transition was first described by Jean Paul Thiery [54]. Various studies also confirmed the presence of cancer stem cells in metastatic tumors. For instance, cancer stem cells from bone marrow of breast cancer patients showed the phenotype as cancer stem cells isolated from breast tissue [55].

Due to the similar characteristics that cancer stem cells and chordoma cells share such as self-renewal, proliferation, and differentiation potential, it is suspected that chordoma tumors might be arising from cancer stem cells.

2.8. DIFFERENTIATION THERAPY BY RETINOIC ACID

For decades retinoids have been used in differentiation therapy due to their role in growth and differentiation [56]. In differentiation therapy, the aim is to be able to arrest cancer stem cell proliferation, and to differentiate them into a more committed stage in which they no longer have the ability to differentiate. In normal stem cell differentiation mechanism, cells divide asymmetrically retaining one daughter cell and a progenitor cell, which will differentiate into a committed cell. On the other hand a similar situation occurs during cancer stem cell differentiation process except that transit amplifying progeny cell does not divide asymmetrically instead divides symmetrically or a mixture of symmetrical

and asymmetrical divisions take place. Therefore no maturation occurs and cells won't go into senescence. The main idea for differentiation therapy is to target this mechanism and reverse it so that cancer stem cells may undergo commitment and eventually die [57].

For the first time differentiation therapy was applied to acute promyelocytic leukemia (APL) patients as a part of the treatment. As a differentiation agent, all trans retinoic acid (ATRA), a derivative of vitamin A, was used in these patients and had shown a major efficacy by inducing remission in leukemia [58]. In 1982, a group of scientist from Canada revealed that when exposed to retinoic acid, embrional carcinoma cell line F9 differentiated into various cell types including neurons (when treated with high concentrations of retinoic acid, $> 10^{-6}$ M), glial astrocytes, and fibroblast like cells [59]. They had identified the neurons by their morphology with branched interconnected network appearance under the light microscope and scanning electron microscope. They had also applied immunostaining with antitubulin and showed the neuronal filaments in their cytoplasm and also tetanus toxin receptor on their surfaces was specific to neurons. In 1990, all *trans* retinoic acid was used in the treatment of acute promyelocytic leukemia as an alternative therapy. It also helped to eliminate problems such as disseminated intravascular coagulation [60]. Differentiation therapy was also used in the treatment of thyroid cancers and represented an alternative therapeutic approach as well [61].

Studies indicate that the expression of various genes is involved in pluripotency, an ability of embryonic cells to differentiate into all kinds of cells, during differentiation process. The expression level of pluripotency markers such as *c-myc*, *oct3/4*, *klf4*, and *sox2* has been found to be downregulated upon retinoic acid treatment in previous studies [62]. In one study Cha et al. showed that during retinoic acid differentiation, oct3/4 whose promoter region is primary unmethylated undergoes a methylation process leading to gene repression [63]. According to Schaniel et al. loss of *nanog* and *oct3/4* is required for the differentiation of embryonic stem cells. It is highly expected to see alterations in the expression of these genes in cancer as well.

Due to its embryonic origin, in chordoma tumor we expect to see the expression of embryonic stem cell markers specifically *ssea1*, *oct4*, *nanog*, and *sox2*, which are also involved in the self-renewal and differentiation of cancer stem cells [64]. If chordoma

tumor is consisting of cancer stem cells, then this can be detected through induction of differentiation. Once cells are differentiated, genes involved in self renewal, differentiation, proliferation, and metastasis should vary in terms of expression level or even become turned off. We sought to determine whether chordoma cells consist of a group of these so called stem like cells using this differentiation method.

3. MATERIALS

3.1. CELL CULTURE SUPPLEMENTS AND MOLECULAR MATERIALS

All the media including RPMI and IMDM, which were used in the cell culture experiments were purchased from GIBCO in USA. The sera and the antibacterial and antifungal supplements which were added into the media were purchased also from GIBCO in USA. In order to passage the cells Tyrpsin EDTA enzyme was used and purchased from GIBCO in USA. In the differentiation experiment, retinoic acid used for the neural differentiation was bought from SIGMA in USA and STEMPRO Osteogenesis Differentiation Kit for the osteogenic differentiation was used from GIBCO in USA. In order to measure the alkaline phosphatase activity as an indicator for the osteogenic differentiation, alkaline phosphatase detection solution was purchased from RANDOX laboratories ltd from United Kingdom. In the same experiment, 0.2 per cent Triton X-100 which was added into the lysis buffer and bought from SIGMA in USA. In proliferation as well as the differentiation detections, viable and proliferated cells were quantified via MTS method by using One Solution Reagent from Promega in USA.

In RNA isolation, total RNA was extracted by using Trizol reagent which was purchased from GIBCO in USA. Total RNA was used in cDNA synthesis by using Transcriptor Reverse Transcriptase kit from ROCHE in Germany. To detect the mesenchymal stem cell characteristic of chordoma cells, cells were labeled with the FITC labeled primary and secondary antibodies which were purchased from Santa Cruz Biotechnology in USA. In gene expression experiments, polymerase chain reaction mix buffer was purchased from Fermentas in Canada.

4. METHODS

All samples were obtained in accordance with approved ethical standards of the responsible committee of Yeditepe University Hospital. Chordoma cell line U-CHI was generated by Silke Bruederlein at University of Ulm, Germany, and we obtained it from Chordoma Foundation (Durham NC, USA). We also gathered total of ten chordoma tumor specimens and ten healthy nucleus pulposi materials from Neurosurgery Department at Yeditepe University, Istanbul. As described by Bruederlein, U-CHI cells were grown on gelatinized tissue culture flasks and cultured with Iscove's Modified Dulbecco's Media (IMDM)/RPMI with a ratio of (4:1) (GIBCO, USA) containing 10 per cent Fetal Bovine Serum and 1 per cent Penicillin Streptomycin Antifungal (100 μg/ml streptomycin and 10,000 units/ml penicillin) solutions. Cells were cultured in a 5 per cent CO_2 incubator with temperature set in 37 °C. Culturing media were changed twice a week until the confluency has reached to 60-70 per cent.

Primary cell cultures for nucleus pulposi tissue specimens were also generated by treating the tissues with collagenase mix (type I, II, and IV) (GIBCO, USA) for overnight in a 37° C incubator and following day removing the collagenase mix and resuspending the degraded tissues in Dulbecco's Modified Eagle Medium and letting cells to adhere to the bottom of tissue flasks and grow. Culturing media were changed twice a week and when cells have reached to the confluency of 60-70 per cent some of the cells were collected for RNA isolation and the rest were left to continue to grow.

4.1. OSTEOGENIC DIFFERENTIATION AND RETINOIC ACID INDUCTION

Through differentiation process we aimed to see if the chordoma cells have the potential to differentiate into other cell lineages such as osteogenic or neuronal. First of all chordoma cells grown on gelatinized tissue flasks were tyrpsinized with 0.5 per cent Tyrpsin- EDTA (GIBCO, USA) and seeded into two gelatinized T-25 tissue flasks and two 35 mm petri dishes at $6x10^5$ cell density using Osteogenesis Differentiation Kit (GIBCO, USA), STEMPRO Osteogenesis Differentiation Medium containing no

antibacterial/antifungal additive and serum was added into one of the flasks and petri dishes according to the manufacturer's protocol for fourteen days. At the end of this period cells inside the petri dish were collected for RNA isolation and the ones in the flask had continued to grow with the same treatment.

The other group of chordoma cells, which were seeded onto one T-25 flask and one 35 mm petri dish were treated with 10μM retinoic acid (Sigma, USA) in growth medium (IMDM:RPMI mix). Medium containing 10μM retinoic acid was changed two to three times a week for up to four weeks. By the third week of treatment most of the chordoma cells had died in the flasks and the petri dishes, however the surviving cells were collected for RNA isolation and these cells were called primary cells. In the same flask a group of cells had been observed to form a colony and continue to grow. This subgroup of newly formed cells were transferred into a new flask by tyrpsinization and continued to grow with retinoic acid. On the other hand, the live cells that resided inside the petri dish after treatment of retinoic acid had been collected for RNA extraction for further analysis.

4.2. ALKALINE PHOSPHATASE ACTIVITY ASSAY

The principle of Alkaline Phosphatase Activity Assay is based on the ability of alkaline phosphatase enzyme, which is mainly found in the liver and bone in human, to hydrolyze the phosphate esters in an alkaline environment, resulting in the formation of an organic radical and inorganic phosphate. The formation of yellow color indicates the hydrolysis of p-nitrophenyl phosphate into phosphate and p-nitrophenol molecules by ALP enzyme. Elevated ALP indicates that there could be active bone deposition taking place as ALP is a byproduct of the osteoblast activity.

Chordoma cells treated with osteogenic differentiation medium were analyzed via alkaline phosphatase activity assay to determine the amount of osteogenic differentiation. First, media were removed and treated and untreated cells were washed for three times with Phosphate Buffer Saline (PBS) solution. The cells were then tyrpsinized with 0.5 per cent Tyrpsin- EDTA (GIBCO, USA) and centrifuged at 1500 rpm for 5 minutes. The pellets in triplicates were lysed in 500 μl lysis buffer containing 0.2 per cent Triton X-100 (Sigma, USA). Protein lysates were vigorously vortexed for half an hour. Lysates (25 μl)

were then mixed with 75 µl ALP detection solution (Randox Laboratories, Ltd, UK). With the help of a 96-well microplate reader, each cell with triplicates was measured at 405 nm absorbance for every five minutes up to two hours. Standard deviations were calculated.

4.3. RNA ISOLATION AND cDNA SYNTHESIS

At the end of osteogenic and retinoic acid induction period cells were collected via tyrpsinization and total RNA was extracted by using Trizol reagent (GIBCO, USA).

4.3.1. RNA Isolation from Chordoma Tissues, U-CHI cells, Cell Lines Generated from the Nucleus Pulposi Tissues

Cells were collected by tyrpsinization and homogenization with Trizol reagent (1 ml of the reagent per 5-10 x 10^6) by pipetting rapidly. Homogenized cells were incubated at room temperature for 5 minutes for the complete dissociation. After the dissociation, 200 µl chloroform per 1 ml Trizol was added and continued to pipette rapidly for another 5 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4º C. The pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. After the centrifugation three separate phases were observed. The cleared phase which contains RNA had been transferred into a new tube. 500 µl of isopropyl alcohol per 1 ml of Trizol was added into the tubes containing the cleared homogenate. Samples were incubated for 10 minutes at room temperature and centrifuged at 12,000 x g for 10 minutes at 4º C. This step precipitates the RNA and usually makes it visible.

Resultant supernatant was discarded and the pellet was washed by adding 1 ml 75 per cent ethanol mixing by vortex and centrifuged at 7,500 x g for 5 minutes at 4º C. In the final step the RNA pellet was left to air dry until the ethanol disappeared entirely. The RNA pellet was redissolved in molecular grade nuclease free water.

In the mean time nucleus pulposi derived primary cell cultures were also tyrpsinized and used for total RNA isolation with the same procedure mentioned above. RNA amounts for each cell line were measured by spectophotometry.

4.3.2. cDNA Synthesis

cDNA synthesis was accomplished by Anchored-oligo(dT) Primer (Roche, Germany) method using 1 µg of total RNA. All the reagents were thawed and centrifuged before use. They were left on ice during the synthesis process. In an eppendorf tube, total RNA, anchored-oligo(dT) primer, and PCR grade water were mixed with appropriate amounts (Table 4.1). Primer-template mixture was denatured by heating the tube for 10 minutes at 65ºC in order to denature the RNA secondary structures. The tube was cooled on ice immediately.

Table 4.1. Reagents and proper amounts for cDNA synthesis

Reagent	Volume (µl)	Final Concentration
Total RNA	Variable	1 µg
Anchored-oligo(dT) Primer	1	2.5 mM
PCR Grade Water	Variable	To make total volume upto 11.4 µl
Transcriptor RT Reaction buffer, 5x	4	1x(8mM $MgCl_{2)}$
Protector RNase Inhibitor (40 U/µl)	0.5	20 U
dNTP Mix 10mM	2	1mM each
DTT	1	5 mM
Transcriptor Reverse Transcriptase	1.1	10 U

4.3.3. Addition of the Remaining Components

The remaining reagents were added into the tube containing primer-template mix with the appropriate amounts shown in Table 4.1. The final mixture was placed on a thermal block and heated between 45ºC to 55ºC for 30 minutes.

4.3.4. Inactivation of Transcriptor High Fidelity Reverse Transcriptase

The total mix of reagents including Transcriptor High Fidelity Reverse Transcriptase was inactivated by heating at 85ºC for 5 minutes. After the incubation, the cDNA mix was stored at -20 ºC for further gene expression analyses.

4.4. SCRATCH ASSAY

In order to measure the cell migration *in vitro* a method called scratch assay has been developed. It is a very easy procedure and costs less. To perceive the effect of differentiation on migration of chordoma cells this method was performed. Cells treated with STEMPRO Osteogenesis Differentiation Medium (GIBCO, USA), 10 µM retinoic acid, and untreated negative control cells were seeded into three 35 mm petri dishes (25,000 per dish). According to the protocol cell monolayers were scraped in a straight line with a pipette tip [65]. For twenty one days, observations were made via an inverted microscope (Leica, GA) and images were saved.

4.5. PROLIFERATION ASSAY

To see the effects of differentiation on proliferation of chordoma cells treated with osteogenic differentiation medium as well as retinoic acid. Cells were seeded and grown onto 12-well plates. Treated cells and untreated control cells ($15x\ 10^3$ per well) in triplicate were used for this experiment. Proliferation assay was done according to the manufacturer's protocol for cells treated with osteogenic medium and analyzed on the days of 7 and 14 and for those treated with retinoic acid at the end of 21 day. Viable cells were quantified via MTS method by using One Solution Reagent (Promega, USA) on a Elisa microplate reader (ELx800, BIOTEK, USA). Standard deviations were calculated.

4.6. GENE EXPRESSION PROFILES FOR STEM CELLS

In this experiment chordoma tissues from ten patients, nucleus pulposi tissues, and the chordoma cell line U-CHI were analyzed to detect the presence of stem cells. According to the protocols that were described before, cDNAs synthesized from the total

RNAs isolated from all the tissues and the cell line were used as templates in a conventional Polymerase Chain Reaction (PCR) with proper primers (Table 4.2). PCR was performed by using 3 µl cDNA, and 15 cycles of 95 °C for 30 s, 68 °C with an increment of temperature by 1° C for 45 s, 72° C for 45 s, 30 cycles for 95° C for 30 s, 52° C for 45 s, and 72° C for 45 s. The products were then resolved by gel electrophoresis on a 2per cent agarose gel and visualized with ethidium bromide using a benchtop UV transilluminator (AVEGENE, Taiwan).

Table 4.2. Primers

Gene	Primers	PCR Product (bp)	Reference
Brachyury	F: 5’ TGAGACCCAGTTCATAGCGG 3’ R: 5’ TGCTGGTTCCAGGAAGAAGC 3’	194	Custom made
c-myc	F: 5' CCTTGCAGCTGCTTAGACGC 3' R: 5' TCTGCTGCTGCTGCTGGTAG 3'	176	Custom made
Klf4	F: 5’- ATTAATGAGGCAGCCACCTG -3’ R: 5’- GGTCTCTCTCCGAGGTAGGG -3	292	[66]
Oct4	F: 5’ CTTGAATCCCGAATGGAAAGGG 3’ R: 5’ CCTTCCCAAATAGAACCCCCA 3’	206	[67]
Sox2	F: 5’- CCCCCGGCGGCAATAGCA -3’ R: 5’- TCGGCGCCGGGGAGATACAT -	448	[68]
nanog	F: 5’ CCTCCAGCAGATGCAAGAAC 3’ R: 5’ CCAGGTCTGAGTGTTCCAGG 3	346	Custom made
Smad2	F: 5' CCCAGCAGGAATTGAGCCAC 3' R: 5' GTGAGGGCTGTGATGCATGG 3	272	Custom made
Nestin	F: 5’ CCTGGAGCAGGAGAAACAGG 3’ R: 5’ GGAGCAAAGATCCAAGACGC 3	248	Custom made
CD24	F: 5’ GCCAGTCTCTTCGTGGTCTC 3’ R: 5’ CCTGTTTTTCCTTGCCACAT 3’	142	[69]

4.7. FLOW CYTOMETRY FOR THE DETERMINATION OF MESENCHYMAL STEM CELLS

In order to confirm the mesenchymal origin of chordoma cells, U-CHI cells were labeled with various stem cells markers including neural marker, CD133. Primary antibodies FITC labeled, CD90, CD73, CD105, CD166, CD34, CD45, CD133, CD29, CD14, and CD44 (Santa Cruz Biotechnology, USA) were added into the tubes (100,000 cells per tube) at a concentration of 200 μg/ml. Cells were incubated for one hour at 4° C and washed with PBS once and finally stored in a mixture of PBS and paraformaldehyde in cold chain. For antibodies CD73 and CD133, a secondary anti-mouse antibody was used and incubated for another hour at 4° C. When the incubation time was over, cells were transferred into the flow cytometry polypropylene tubes for the analysis which was performed with FACSCalibur (BD FACSCalibur, USA).

5. RESULTS AND DISCUSSION

5.1. OSTEOGENIC DIFFERENTIATION MEASURED BY ALP ASSAY

To analyze the osteogenic differentiation potential of chordoma cells, ALP Assay was performed. After induction, ALP activity in cells was measured on the seventh and fourteenth days and compared to that of the cells which were not treated with the differentiation medium. According to the results, measurements for both the seventh and fourteenth days of treatment, cells induced with differentiation medium possessed more than 3 folds higher alkaline phosphatase levels compared to the untreated ones (Table 5.1). As mentioned before, chordoma cells may differentiate into other mesenchymal originated lineages when induced with osteogenic/chondrogenic differentiation agents [70]. In previous studies it was shown that when induced with osteogenic differentiation medium human mesenchymal stem cells would differentiate into the osteogenic lineage supporting the cancer stem cell existence [71]. In our study differentiation into the osteogenic lineage was expected and not surprisingly ALP levels were found to be increased compared to the untreated ones.

Table 5.1. ALP activity of U-CHI cells treated with osteogenic differentiation medium vs. untreated controls (d: day, h: hour, m: minute)

Cells	7d/30m	7d/1h	7/2h	14d/30m	14d/1h	14d/2h
U-CHI treated	0.231 ± 0.003	0.322 ± 0.005	0.496 ± 0.007	0.295 ± 0.01	0.473 ± 0.02	0.863 ± 0.04
U-CHI untreated	0.127 ± 0.003	0.138 ± 0.005	0.158 ± 0.009	0.149 ± 0.05	0.172 ± 0.09	0.216 ± 0.22

5.2. SCRATCH ASSAY

Many recent studies had demonstrated that malignant tumor cells share the same characteristics like self renewal, extensive proliferation, and differentiation with that of stem cells. In previous studies, in order to demonstrate the migration and further metastasis property of chordoma caused by the presence of cancer stem cells, scratch assay have been employed [72]. According to the observations starting from day 1, chordoma cells treated with retinoic acid had died in higher amounts than the cells treated with osteogenic differentiating medium. Parallel to these results, cells with retinoic acid had a slower migration rate than those with osteogenic medium. On the other hand untreated control cells had covered the scratched area in a much faster rate. This evidence indicates that differentiation therapy may bring a disadvantage to the cancerous cells causing them to grow and migrate in a much slower rate leading to decrease metastasis rate of chordoma (Figure 5.1). We suspect that cancer stem cells involved in metastasis of various tumors, may be silenced during the differentiating procedure.

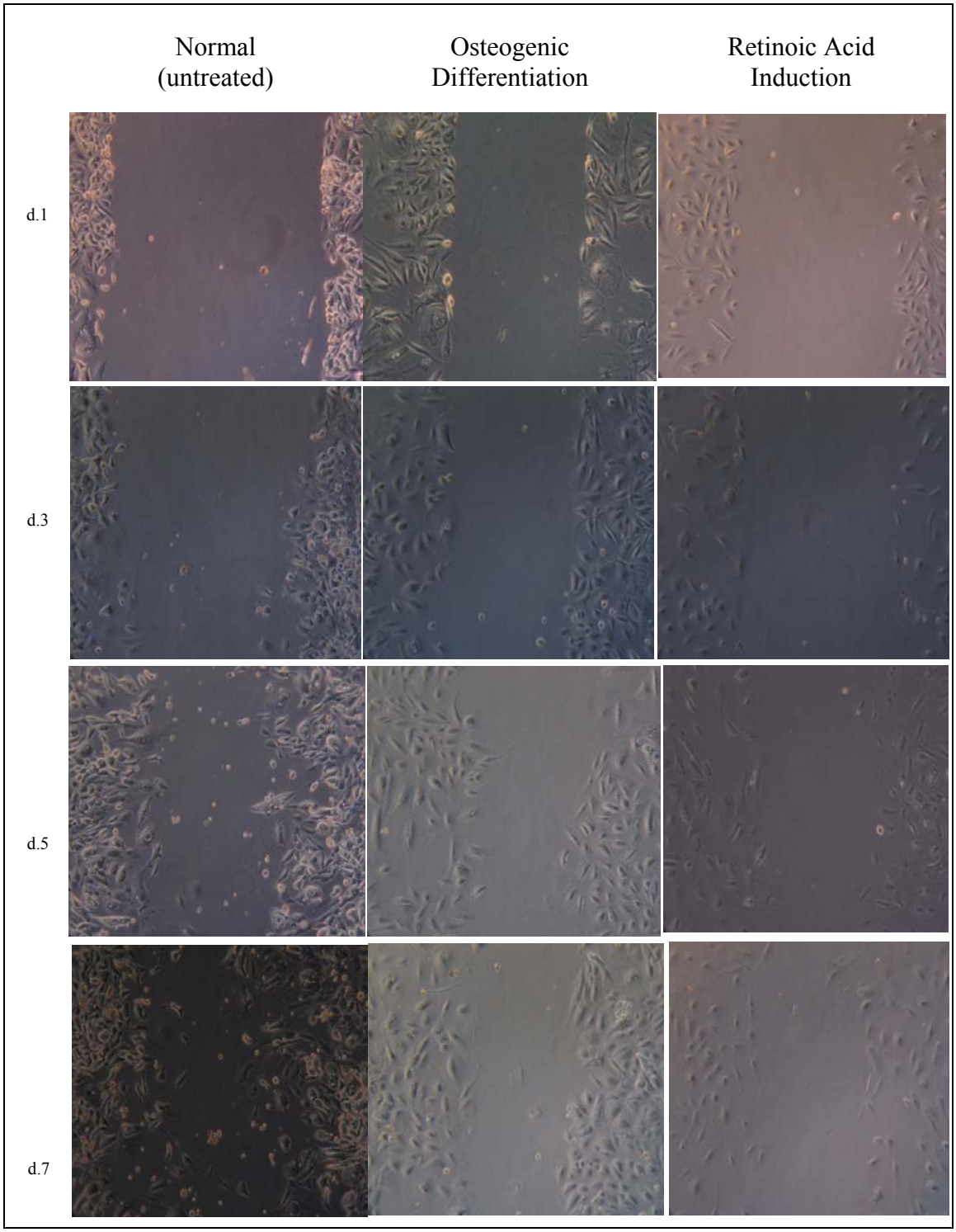

Figure 5.1. Scratch assay observations for U-CHI cells treated with RA and osteogenic medium and untreated ones beginning from day 1 to day 21 (10 x obj)

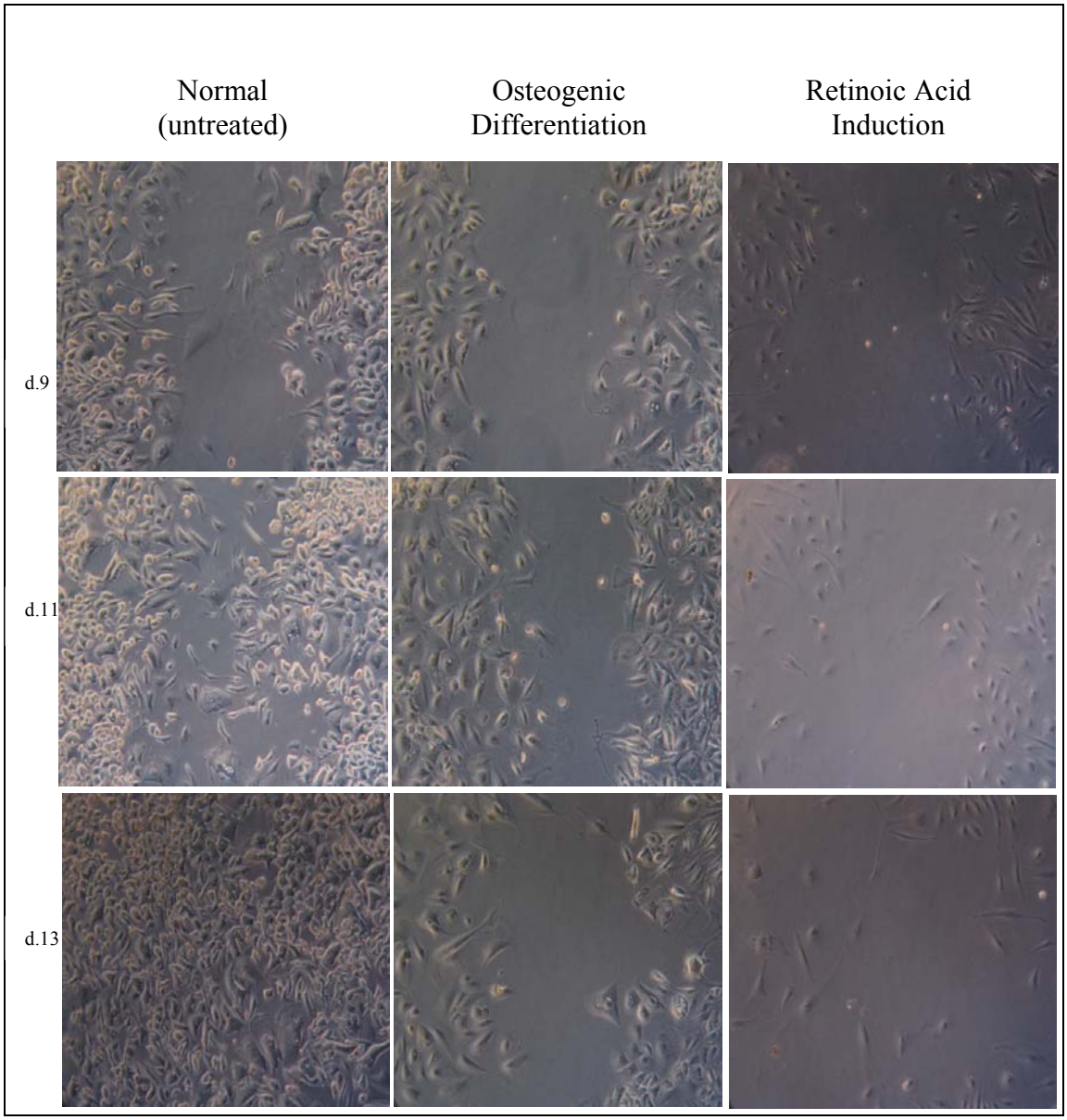

Figure 5.2. Scratch assay observations for U-CHI cells treated with RA and osteogenic medium and untreated ones beginning from day 1 to day 21 (10 x obj) (continue)

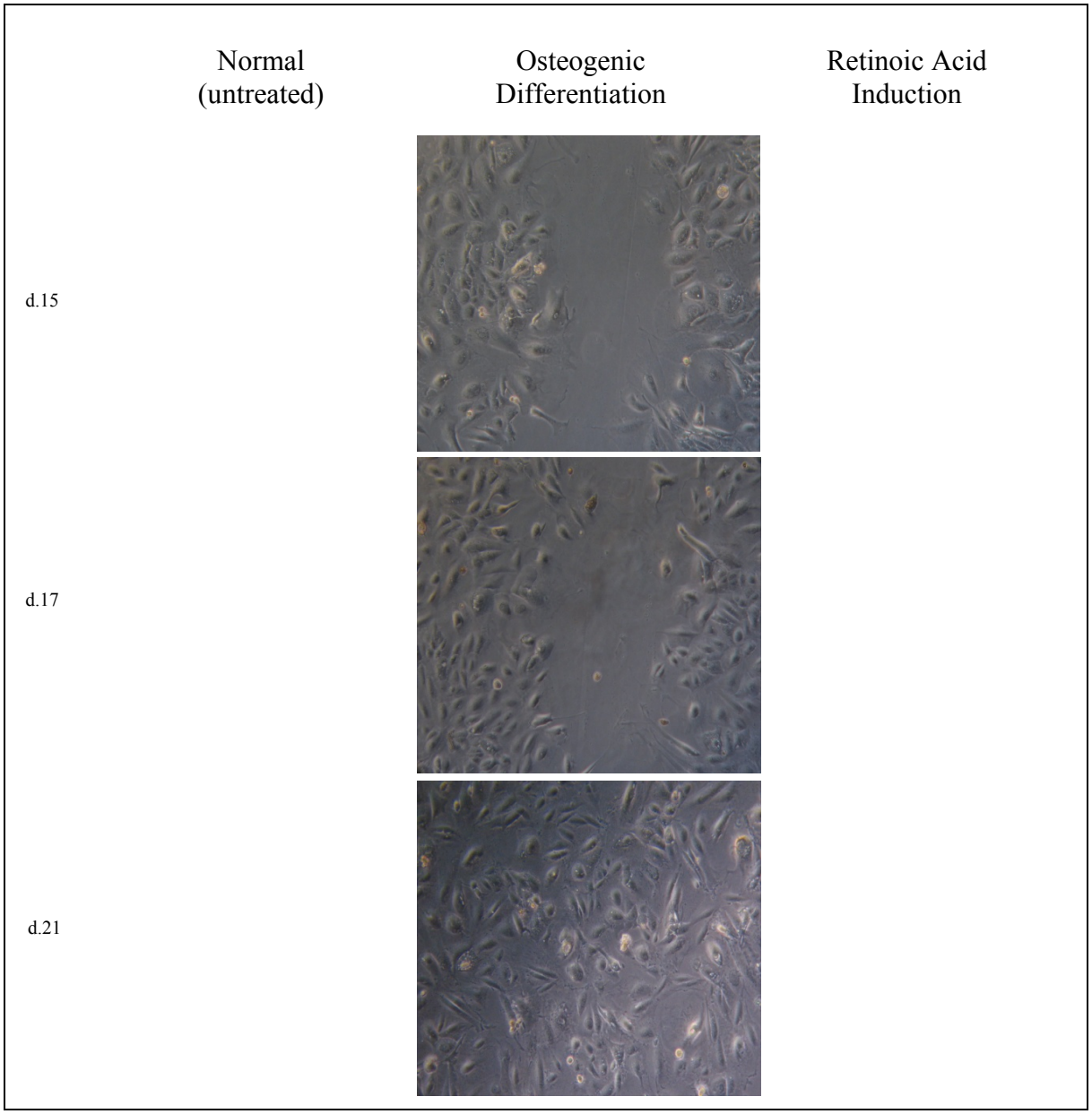

Figure 5.3. Scratch assay observations for U-CHI cells treated with RA and osteogenic medium and untreated ones beginning from day 1 to day 21 (10 x obj) (continue)

5.3. EFFECT OF DIFFERENTIATION THERAPY ON CHORDOMA PROLIFERATION

Cancer stem cells had become a major challenge against the treatment of cancer. Studies indicate that limitless proliferation capacity which cancer stem cells possess makes this challenge unbearable. We wanted to elucidate whether differentiating with retinoic acid or osteogenic medium had an effect on proliferation rate of chordoma cells using MTS assay. The results showed that cells treated with retinoic acid for fourteen days had dramatically decreased the proliferation rate down to 49 per cent whereas osteogenic differentiation medium had a much lower, almost no, effect on proliferation (Figure 5.2). During retinoic acid induction, cells might undergo apoptosis by TRAIL (Tumor necrosis (TNF)-related apoptosis-inducing ligand), which is a member of the TNF family of cytokines that promotes apoptosis [73]. It was reported that retinoids induce TRAIL-mediated death signaling in several cancers. In another study it was found out that TRAIL induced apoptosis in chordoma cells [74].

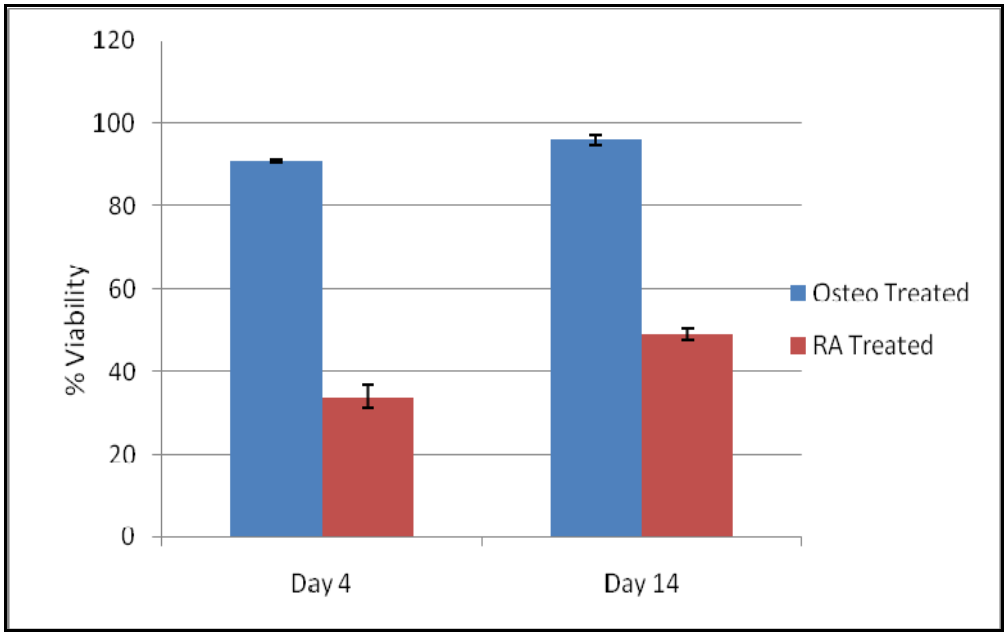

Figure 5.4. Effect of differentiation on the proliferation of U-CHI cells treated with retinoic acid and osteogenic differentiation medium.

5.4. GENE EXPRESSION PROFILE

Molecular approach is another way to detect the presence of cancer stem cells. Genes which were found to be highly and commonly expressed in cancer stem cell detection studies were analyzed in this study. Conventional polymerase chain reaction (PCR) was used to detect the presence of cancer stem cells. The first and foremost gene to be detected was *brachyury* in chordoma cells as well as in tissue samples. Once the characterization of chordoma tumor was established, the next analyses were performed to determine the presence of cancer stem cells in chordoma tumors. According to previous studies genes like *oct4*, *sox2*, *c-myc*, *klf4*, *smad2*, *CD90*, *nanog* and *nestin* were commonly found to be expressed in cancer stem cells [75]. Based on these findings we performed similar experiments with the addition of more related genes possibly involved in chordomas. We are also suspecting that some of these genes might be responsible for the metastasis while others for the recurrence of chordoma.

Gene expression profile performed through conventional PCR on RNA templates extracted from chordoma cells induced with retinoic acid for three weeks showed that cancer stem cell markers namely c-myc, klf4, smad2, CD90, brachyury, and oct4 were inactive (Figure 5.3).

Our data was consistent with previous studies. For instance, a recent study showed that retinoids had an opposing effect on c-myc levels because c-myc prevented cell cycle arrest in response to the growth inhibitory signals, differentiation stimuli, or mitogen withdrawal [76]. Activation of c-myc, a transcription factor, which is a member of myc family, was also demonstrated to inhibit differentiation; therefore, ectopic differentiation stimuli may have downregulating effects over c-myc. On the other hand, c-myc expression was observed after the induction of osteogenic differentiation medium confirming the previous studies that indicated the involvement of c-myc in osteogenic differentiation [77].

Oct4, *sox2*, and *nanog* are other transcription factors known to maintain the pluripotency of embryonic stem cells. Corresponding with the previous studies, *oct4* was not expressed in U-CHI cells treated with RA initially. Recent data demonstrated that *oct4* and *sox2* coregulated the maintenance of self renewal and pluripotency under

differentiation inducing culture conditions [78]. *Nanog* expression, on the other hand, was expressed weakly during RA treatment. Besides, *sox2* was expressed during and after differentiation [79]. Our data also showed that *oct4* expression is very weak in U-CHI cells treated with the osteogenic differentiation medium whereas *sox2* was expressed in a much stronger level.

In parallel with the transcription factors mentioned above *klf4* whose role in differentiation, proliferation, and survival has been studied extensively, was also found to be repressed in U-CHI cells when induced with retinoic acid for the first three weeks. Depending on the type of protein that is interacting with, klf4 has a transcription activation (i.e. in oncogenesis) as well as suppression in cancer [80]. For instance, in one study *klf4* is found to be required for the reprogramming of pluripotent stem cells [81].

Just like the other pluripotency markers, nestin was found to be expressed very weakly in chordoma cells initially treated with retinoic acid. Nestin, a neural precursor marker is an intermediate filament found in the nerve cells was also analyzed to detect the differentiation into a neural lineage by the induction of retinoic acid in chordoma cells. This would indicate that during differentiation process nestin expression was not needed.

Tissue samples obtained from the Neurosurgery Department in Yeditepe Hospital were also consistent in gene expression with that of untreated U-CHI cells which express all the cancer stem cell markers (Table 5.2) including chordoma gene markers such as brachyury, EMA, and cytokeratin 19 indicating that chordoma cells might consist of cancer stem cells.

After three weeks of induction by retinoic acid, a colony of surviving cells had been transferred into a new flask and allowed to grow. When cells had reached to confluency, they were collected for RNA isolation and further expression analyses. According to the gene expression studies we have found that surviving and proliferating after the transfer, U-CHI cells regained their cancer stem cell expression potential, which may be caused by the insensitivity to retinoic acid induction. In this transferred and grown subculture the cancer stem cell markers were seen to be re-expressed (Table 5.3). We might conclude that

these surviving cells must have been escaped from the commitment and further apoptosis induced by retinoic acid.

Table 5.2. Gene expression profile for chordoma tissue samples (CH1-10) and U-CHI cells. CH is short for chordoma. (+) means positive expression whereas (-) means for no expression

chordoma tissues	CH1	CH2	CH3	CH4	CH5	CH6	CH7	CH8	CH9	CH10	U-CHI	positive/ negative
Smad2	+	+	+	+	+	+	-	+	-	+	-	8/3
oct4	+	+	+	+	+	+	+	-	-	+	+	9/2
sox2	+	+	+	+	+	+	+	+	+	+	+	11/0
nanog	+	+	+	+	+	+	-	+	+	+	+	10/1
c-myc	+	+	+	+	+	+	+	-	+	+	+	10/1
klf4	+	+	+	+	+	+	-	+	+	+	+	10/1
CD44	+	+	+	+	+	+	+	+	-	-	+	9/2
CD24	+	+	+	+	+	+	+	+	+	+	+	11/0
CD90	+	-	-	+	+	+	-	-	+	+	+	7/4
GAPDH	+	+	+	+	+	+	+	+	+	+	+	11/0
SSEA-1	+	+	+	+	+	-	-	-	-	-	+	6/5

Next step was to compare the gene expression profile of U-CHI cells treated with retinoic acid and untreated U-CHI cells with those of nucleus pulposi cells. The main reason was to be able to compare chordoma cells with healthy nucleus pulposi cells so that we can determine the specificity of cancer stem cell markers. If there was a change in the expression of genes specific to differentiation and proliferation, then we could conclude that chordoma tumor might consist of cancer stem cells.

According to our results very few nucleus pulposi cells expressed the genes such as *oct4*, *sox2*, and *nanog*. This was expected because proliferating and differentiating cells should be expressing these specific genes rather than healthy nucleus pulposi cells (Table 5.4).

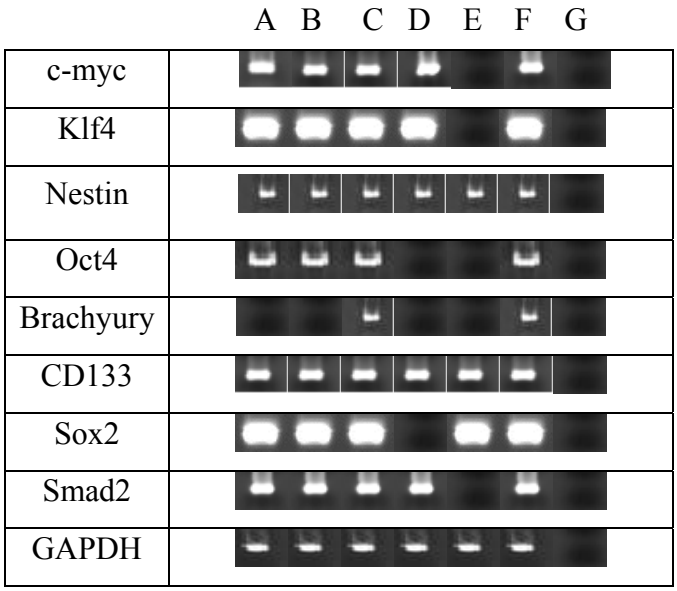

Figure 5.5. Gene expression analysis of U-CHI cells treated with different differentiating agents for the stem cell markers. A: U-CHI w/ 10μM RA(subculture of the primary culture treated with 10μM RA for three weeks), B: U-CHI w/ 50μM RA(subculture of the primary culture treated with 50μM RA for three week, C: U-CH1 w/ osteogenic medium, D: NP (nucleus pulposus), E: U-CHI w/ 10μM RA(primary culture), F: U-CHI (negative control) G: Negative Control

Table 5.3. Gene expression profile for subculture of U-CHI cells treated with 10 μM and 50 μM Retinoic Acid, primary U-CHI cells treated with 10 μM Retinoic Acid, U-CHI cells treated with osteogenic differentiation medium, Nucleus Pulposus (NP) cells, and U-CHI Negative Control (NC)

Genes	sub-RA-10μM	sub-RA-50μM	U-CHI osteo	NP	prim-RA-U-CHI	U-CHI NC
Brachyury	-	-	+	-	-	+
CD133	+	+	+	+	+	+
c-myc	+	+	+	+	-	+
Nestin	+	+	+	+	+	+
oct4	+	+	+	-	-	+
klf4	+	+	+	+	-	+
sox2	+	+	+	-	+	+
GAPDH	+	+	+	+	+	+

Table 5.4. Gene expression profile for nucleus pulposus cells from 1 to 9

Nucleus Pulposus	np1	np2	np3	np4	np5	np6	np7	np8	np9	positive/negative
Smad2	+	+	-	+	+	-	+	+	+	7/2
oct4	+	-	-	-	+	-	-	-	+	3/6
sox2	-	+	-	+	+	+	-	-	-	4/5
nanog	+	+	-	+	-	-	-	-	+	4/5
c-myc	+	-	+	+	+	+	-	+	+	7/2
klf4	+	+	+	+	+	+	+	+	+	9/0
CD44	+	-	+	-	+	-	-	-	+	4/5
CD24	+	+	+	+	-	+	+	+	+	8/1
CD90	+	+	-	+	+	+	-	-	-	5/4
GAPDH	+	+	+	+	+	+	+	+	+	9/0
SSEA-1	-	-	-	-	-	+	-	-	-	1/8

5.5. MESENCHYMAL ORIGIN OF CHORDOMA CELLS BY FLOW CYTOMETRY ANALYSIS

Figure 5.4 Flow cytometry analysis for mesenchymal stem markers in U-CHI cells Chordoma cells are known to be originated from the remnants of notochord during mesoderm formation. We aimed to determine the mesenchymal origin of chordoma cells by staining the cells with the antibodies used for the presence of stem cell markers [Figure 5.4]. Hematopoietic stem cell markers CD34 and CD45 were observed in very low levels in chordoma cells (only CD45 was illustrated in the figure). A neural antibody CD133, one of the most well known neural stem cell markers, was also found to be expressed in very low levels of chordoma cells. Altogether these findings show that chordoma cells express mesenchymal stem cell markers.

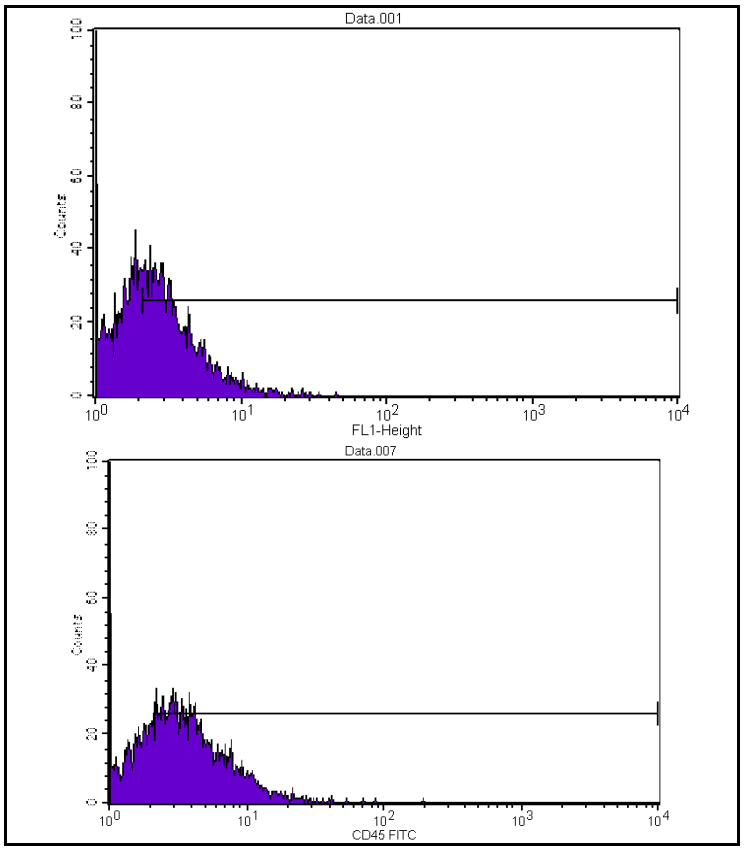

Figure 5.4 Flow cytometry analysis for mesenchymal stem markers in U-CHI cells

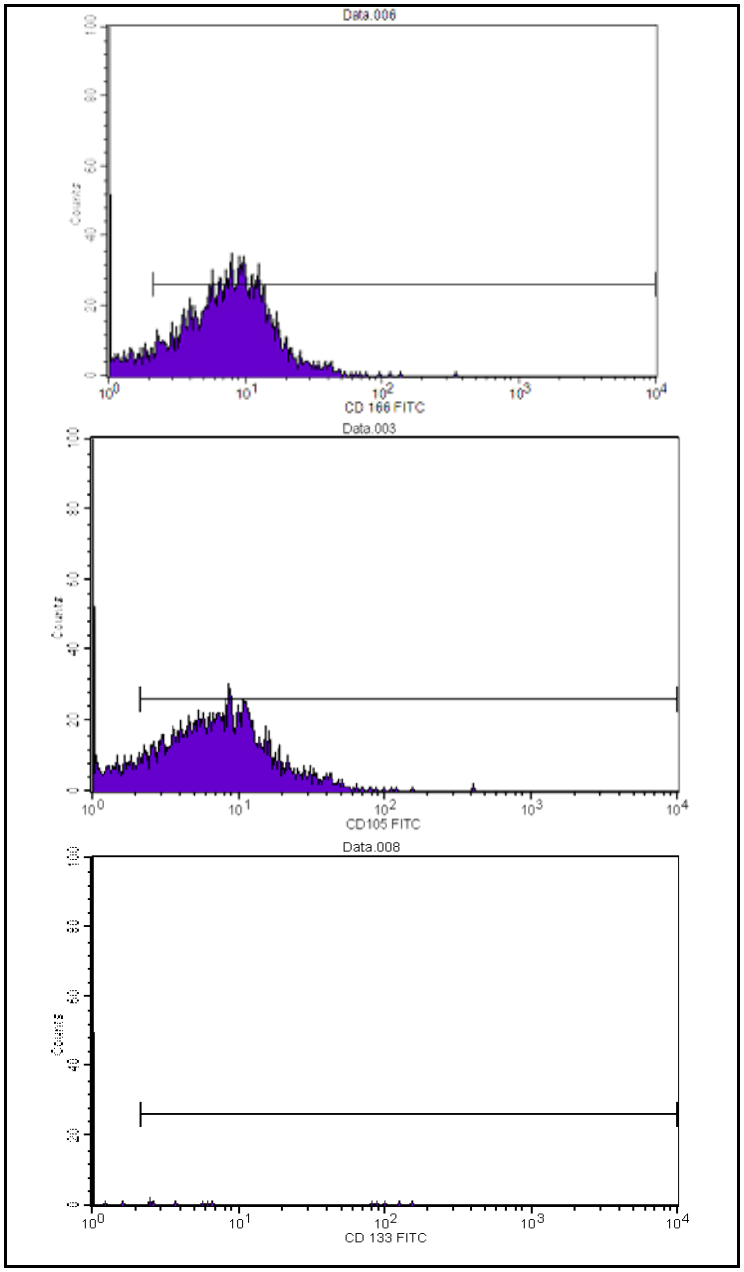

Figure 5.4 Flow cytometry analysis for mesenchymal stem markers in U-CHI cells (continue)

6. CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION

Chordoma, the second most malignant of bone tumors existing, is originated from the remnants of the notochord. Brachyury, a member of T-box gene family, was found to be expressed in chordomas and became the most important protein marker in distinguishing chordomas from a very similar bone tumor type called chondrosarcoma. Brachyury has an important role in the differentiation of notochord and mesoderm formation during notochordal development in mice.

Based on its slow growing yet recurring and metastasizing features, chordoma cells are considered to contain a group of cells similar to cancer stem cells. Cancer stem cells are a group of cells, which are capable of self renewing, differentiation and migration in cancer. They are known to play a role in initiation, progression, and chemoresistance in many cancers. Although chordoma is considered to be a low grade malignant tumor, it metastasizes to nearby tissues and show resistance to chemotherapy and radiotherapy.

There are several ways to determine the presence of cancer stem cells. In this study chordoma cell line U-CHI and tumor samples were analyzed to determine the presence of cancer stem cells by flow cytometry using stem cell surface antibodies. It was found out that chordoma cells do contain a group of cells that express stem cell markers on their surfaces.

Another method, a well known cancer treatment method in addition to chemotherapy namely differentiation therapy can be carried out by the application of differentiation agents on chordoma cells. As a differentiating agent, 10 μM retinoic acid was applied to chordoma cells for three weeks. To see the proliferation and migration rate of treated cells and compare them to those that are untreated, other methods such as scratch assay and proliferation assays were performed. The results depict that chordoma cells that lost their cancer stem cell characteristics through differentiation therapy had also demonstrated a lower migration and proliferation rate as opposed to the untreated ones. This indicates that

chordoma cells might consist of a group of cells with high metastatic and proliferation potential.

This is the first study regarding the existence of cancer stem cells in chordoma. Findings in this study may provide new insights to prospective upcomings related to molecular based chordoma studies. New methods based on these findings may contribute to the new developments towards the treatment of chordoma.

Targeted therapy against a particular tumor type is very crucial in the treatment of cancer. Therefore the first thing that should be done is to detect the cancer promoting genes and target them. This way, better and more efficient targeted therapies might be achieved to increase one's survival. Cancer stem cell detection is a very promising approach in the treatment of cancers including chordoma.

6.2. RECOMMENDATION

For the further studies cancer stem cells in chordoma, may be separated using appropriate stem cell surface markers by magnetic cell separation or flow cytometry. These isolated cells later on can be used in several studies such as dosage-dependent chemotherapy trials, migration assays, gene expression analyses and so on. In addition immunocytochemistry and western blot analyses might reveal more about the protein levels of cancer stem cell markers.

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