

**INVESTIGATION OF PLURIPOTENT STEM CELLS
IN HUMAN DENTAL FOLLICLE**

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June 2008

**İNSAN DENTAL FOLİKÜLÜNDEKİ PLURİPOTENT
KÖK HÜCRELERİN ARAŞTIRILMASI**

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ABBREVIATIONS

HESC	: Human embryonic stem cells
FSCs	: Fetal stem cells
UCBSCs	: Umbilical cord blood stem cells
ICM	: Inner cell mass
IVF	: <i>in vitro</i> fertilization
FDA	: Food and Drug Administration
MEM	: Modified minimum essential medium
cGMP	: Current good manufacturing practice
GCTP	: Good tissue culture practice
EGCs	: Embryonic germ cells
PGCs	: Primordial germ cells
HAF	: Human amniotic fluid
HAFSCs	: Human amniotic fluid stem cells
BM	: Bone Marrow
UC	: Umbilical cord
SSC	: Somatic stem cell
HSCs	: Hematopoietic stem cells
MSCs	: Mesenchymal stem cells
MAPCs	: Multipotent adult progenitor cell
DPCs	: Dental pulp cells
DFCs	: Dental follicle cells
PDLCs	: Periodontal ligament cells
BMFs	: Buccal mucosa fibroblasts
BMPs	: Bone morphogenetic proteins
NS	: Nucleostemin
bFGF	: Basic fibroblast growth factor
ALP	: Alkaline phosphatase
NFM	: Neurofilament medium chain
DMEM	: Dulbecco's Modified Eagle Medium
PSA	: Penicillin/Streptomycin/ Amphotericin
NGF	: Nerve growth factor

PBS : Phosphate buffered saline
iPS : Induced pluripotent stem cells

INVESTIGATION OF PLURIPOTENT STEM CELLS IN HUMAN DENTAL FOLLICLE

SUMMARY

Stem cells hold promises for many people suffering from Parkinson, Alzheimer, Diabetes, spinal injuries and even cancer. It is quite important to find an ideal stem cell source to develop therapeutic applications for these diseases. Embryonic stem cells are classified as pluripotent stem cells since they have unique capacity to differentiate into various tissues and organs, offering replacement of damaged or non-functional tissues with new ones. Embryonic stem cells are characterized according to their morphology, surface markers, and expressions of developmentally regulated proteins such as, Oct-4, Sox-2, Klf-4...etc. As the isolation of embryonic stem cells requires killing of embryos, it gives rise to controversies all over the world. In addition to this, it is also difficult and expensive to maintain embryonic stem cells cultures as they require some special conditions.

On the other hand, stem cells derived from other sources such as umbilical cord blood, bone marrow, adipose tissue, dental pulp, etc., seem to be much more easily obtainable and ethically harmless. Adult stem cells are not pluripotent because they cannot give rise to all mature cells types coming from three different germ layers, ectoderm, mesoderm, and endoderm. They generally do differentiate into the cell type of the tissue where they reside. Recent studies showed that adult stem cells can differentiate into some other cells types if they are induced specifically, which is called as transdifferentiation or plasticity. For example, a Hematopoietic Stem Cell (derived from bone marrow) can give rise to a neuron or a hepatocyte instead of a leukocyte or erythrocyte. For this reason adult stem cells are also called as multipotent stem cells.

To achieve effective use of stem cells in clinics, it is necessary to find easily and less expensively obtainable and well characterized stem cell sources. So far, although bone marrow is used as the primary sources of adult stem cells for clinics, it brings about a surgical stress and contamination risk. Isolation of stem cells from other adult stem cell sources such as dental follicle and dental pulp as a by-product of routine treatment might be an advantageous approach over usage of bone marrow derived stem cells.

Stem cells isolated from human dental follicles (HDF) are being investigated to show their differentiation potential. They have relatively high potential of proliferation and can differentiate into osteocytes, adipocytes and chondrocytes. Most recent data show that human dental follicle cells (HDFCs) might transdifferentiate into neurons.

In this study, HDFCs from a 17 years old patient's wisdom tooth dental follicle, which was extracted because of some orthodontic reasons, were isolated. For the first time, it was tried to detect the expressions of some pluripotent stem cells markers such as Oct-4 and Nucleostemin (NS) in HDFCs with the aim of identifying pluripotent stem cells in dental follicle. Also, *in-vitro* neurogenic differentiation of HDFCs was investigated suggesting, for the first time, a retinoic acid mediated neurogenic differentiation model might work in HDFCs.

According to the results, HDFCs contained putative Oct-4 and NS expressing pluripotent stem cells in very rare number. In consistency with previous data, HDFCs can be differentiated into osteogenic and neurogenic cells. Based on this, it can be said that our retinoic acid mediated induction is a putative model of neurogenic differentiation for HDFCs.

As a conclusion, it can be suggested that HDFCs can be a valuable stem cell source for autologous transplantation which does not provoke immune rejection upon transplantation. Dental follicles might be collected after routine extractions of wisdom teeth and stored in liquid nitrogen as a readily available stem cell source for the future.

İNSAN DENTAL FOLİKÜLÜNDEKİ PLURİPOTENT KÖK HÜCRELERİN ARAŞTIRILMASI

ÖZET

Kök hücreler, Alzheimer, Parkinson, diyabet, omirilik yaralanması ve hatta kanser hastaları için bir ümit kaynağı olmaya devam etmektedir. Bu hastalıkların tedavisinde kullanılabilecek kök hücre tedavileri geliştirebilmek için en ideal özelliklere sahip kök hücrelerin elde edilmesi zorunludur. Embriyonik kök hücreler vücuttaki tüm hücre tiplerine farklılaşabildikleri için “pluripotent” olarak adlandırılırlar. Embriyonik kök hücreler yüksek farklılaşma potansiyellerinin yanı sıra yüzey antijenleri, morfolojileri ve gelişimsel olarak sentezledikleri Oct-4, Sox-2, Klf-4, vb, farklı proteinlerin varlığına bağlı olarak karakterize edilirler. Embriyonik kök hücre izolasyonu canlı embriyoların feda edimesini gerektirdiği için bu konu ile ilgili etik sorunlar dünyanın her yerinde tartışılmaktadır. Öte yandan, embriyonik kök hücrelerin kültürde çoğaltılması pahalı, zahmetli ve özel koşulların sağlanmasını gerektiren bir yöntemdir.

Kordon kanı, kemik iliği, yağ dokusu, diş pulpası vs, gibi kaynaklardan elde edilen kök hücreler, embriyonik kök hücrelere nisbeten etik açıdan daha az sorunlu olup, kolayca kültürde çoğaltılabilir ve genel olarak yetişkin kök hücreler olarak adlandırılırlar. Yetişkin kök hücreler pluripotent değildir. Çünkü ektoderm, mezoderm ve endoderm’den gelen tüm hücre tiplerine farklılaşma potansiyelleri yoktur. Yetişkin kök hücreler daha çok bulunduğu dokudaki hücrelere farklılaşırlar. Ancak yapılan son çalışmalar yetişkin kök hücrelerin çapraz farklılaşma (transdifferansiasyon) ya da plastisite gösterebildiklerini ortaya koymuştur. Örneğin bir kemik iliği kök hücresinin lökosit ve eritrosite dönüşmesi beklenirken, uygun ortamlar sağlandığında sinir ya da karaciğer hücresine dönüşebilmesi mümkündür. Bu sebepten, yetişkin kök hücreler için multipotent tanımı kullanılmaktadır.

Kök hücrelerin klinikte tedavi amacıyla başarılı bir şekilde kullanılabilmesi için kolay ve ucuz bir şekilde elde edilip tamamen karakterize edilmeleri gerekmektedir. Günümüzde her ne kadar kemik iliği klinikte kullanılan kök hücrelerin ana kaynağı olsa da, beraberinde cerrahi stres ve kontaminasyon riskini getirmektedir. Bu noktalar dikkate alındığı zaman, kök hücrelerin, diş folikülü ya da diş pulpasından rutin tedaviler sırasında açığa çıkan atık dokulardan elde edilmesi kemik iliğinden elde edilmesinden daha avantajlı görülmektedir.

Dental folikülden elde edilen kök hücrelerin karakterizasyonu ve farklılaşma potansiyeli ile ilgili çalışmalar devam etmektedir. Çalışmalar dental folikülden elde edilen hücrelerin, kemik, kıkırdak ve yağ dokusuna kolayca farklılaşabileceğini göstermiştir. Ayrıca, son zamanlarda bazı gruplar dental folikül kök hücrelerini sinir hücrelerine benzeyen hücrelere farklılaştırdıklarını açıklamışlardır.

Bu çalışmada 17 yaşında bir hastaya ait olan üçüncü molar diş folikülünden (yirmi yaş dişi) dental folikül hücreleri elde edilmiştir. Folikül, hastanın rutin devam eden tedavisinde hekimin gerekli görüp folikülü çıkarması sonucu elde edilmiştir. Bu çalışmada dental folikül hücrelerinin pluripotent hücre içerip içermediğini anlamak amacıyla, ilk defa üzerlerinde pluripotent kök hücre markeri olan Oct-4 ve NS (Nucleostemin) birlikte tespit edilmeye çalışılmıştır. Buna ek olarak dental folikül hücrelerinden ilk defa retinoik asit içeren bir yöntem kullanarak sinir hücrelerine benzeyen hücreler elde edilmesi amaçlanmıştır.

Çalışmanın sonunda, daha önce yapılan çalışmalar ile paralel olarak insan dental folikül hücrelerinden kemik hücresi ve sinir hücresi benzeri hücreler elde edilmiştir. Ayrıca çalışmada elde edilen sonuçlara göre, yirmi yaş dişi folikülünden elde edilen kök hücreler arasında çok az miktarda da olsa Oct-4 ekspresyonu yapan hücreler bulunmaktadır. Sinirsel farklılaşmada başarılı olunması retinoik asit aracılı bir sinirsel farklılaşmanın insan dental folikül hücrelerinde aktif olabileceğini göstermektedir.

Sonuç olarak yapılan çalışma, insan dental folikülü kök hücrelerinin kolay elde edilebilir ve kişiye özel otolog transplatasyon için çok uygun bir kaynak olabileceği tezini desteklemektedir. Öyleyse, rutin yirmi yaş dişi çekimlerinde atık olarak açığa çıkan foliküllerden kişiye ait kök hücrelerin izole edilip depolanması, kişinin ileride ihtiyaç duyabileceği kök hücre kaynağını oluşturabilme potansiyeli açısından oldukça önem kazanmaktadır.

1. INTRODUCTION

1.1 Stem Cells

The present excitement and controversies about stem cell research began with two breakthroughs: (i) successful cloning of “Dolly” by Ian Wilmut, Keith Campbell and coworkers in 1997; and (ii) the establishment of human embryonic stem cell (ESC) lines by the laboratory of James Thomson in 1998 (Ho A.D. et al.,2003). Today stem cell research gives hope that a lot of age related degenerative disorders such as heart disease, Parkinson’s disease, diabetes, and stroke could someday be cured by stem cell therapy.

Stem cells are unspecialized cells in the human body that are capable of becoming specialized cells, each with new specialized cell functions. The most well known example of stem cell is the bone marrow stem cells that are unspecialized and able to differentiate into blood cells, such as white and red blood cells. These new cell types have special functions, such as being able to produce antibodies, act as soldiers to combat infection and transport gases. Bone marrow transplantation is actually the transfer of these stem cells into donors having immune system problems. This “Stem cell” term is used as one cell type stems from the other. Basically, a stem cell remains uncommitted until it receives a signal to develop into a specialized cell. Apart from blood cells, stem cells have the remarkable properties of developing into a variety of cell types in the human body. They serve as a repair system by dividing without limitation to replenish other cells. When a stem cell divides, each new cell has the potential to either remain as a stem cell or become another cell type with new special functions, such as blood cells, brain cells, etc (Bongso and Lee, 2005).

During embryogenesis, one fertilized egg cell – the zygote – becomes two, and two become four. In these early stages, each cell is called totipotent – that is, a whole organism can be derived out of each of these cells. Within 5 to 7 days, blastocyst is formed by around 40 cells consist of the inner cell mass and surrounding cells

called trophoblasts. At this stage, each of these cells in the inner cell mass has the potential to give rise to all tissue types and organs including germ cells – that is, these cells are pluripotent (Figure 1.1). Ultimately, the cells forming the inner cell mass will give rise to the trillions of cells that constitute a human body, organized in 200 differentiated cell types. Many somatic, tissue-specific or adult stem cells are produced during fetal development. Such stem cells have more limited ability than the pluripotent embryonic stem cells (ESC) and they are called multipotent. These cells have the ability to give rise to multiple lineages of cells. These adult stem cells persist in the corresponding organs to varying degrees during a person's whole lifetime.

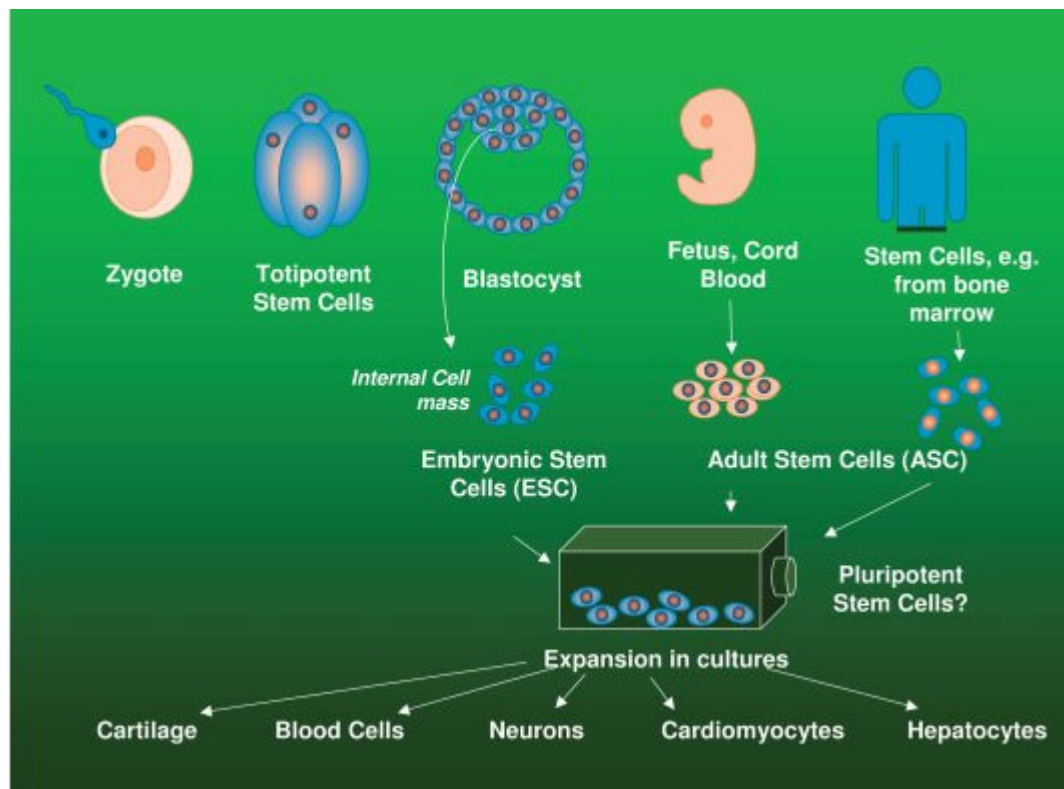


Figure 1.1: Sources of stem cells (Ho A.D. et al.,2003)

A stem cell can either remain a stem cell, that is called self-renewal, or follow a pathway resulting in differentiation. In differentiation pathway, daughter becomes a **precursor** cell which proliferates before it differentiates. **Progenitor** cell term is usually used instead of precursor term but some attribute progenitor cell to the cells with greater developmental potential than a precursor cell (Raff, 2003). Precursor cells within a tissue start to proliferate and finally differentiate upon an

injury, for example to restore damaged tissue. Although factors determining whether a stem cell remains a stem cell or undergoes differentiation are not clear, today we know that cell-fate determinants from the mother cell and environmental factors are playing important roles.

1.2 Classification and Sources of stem cells

Stem cells can be classified into four broad groups on the basis of their origin. These are **embryonic stem cells (ESCs)** from embryos; **fetal stem cells (FSCs)** from the fetus; **umbilical cord blood stem cells (UCBSCs)** from the umbilical cord; and **adult stem cells** from the adult (Bongso and Lee, 2005). Each of these can be grouped into subtypes (Figure 1.2).

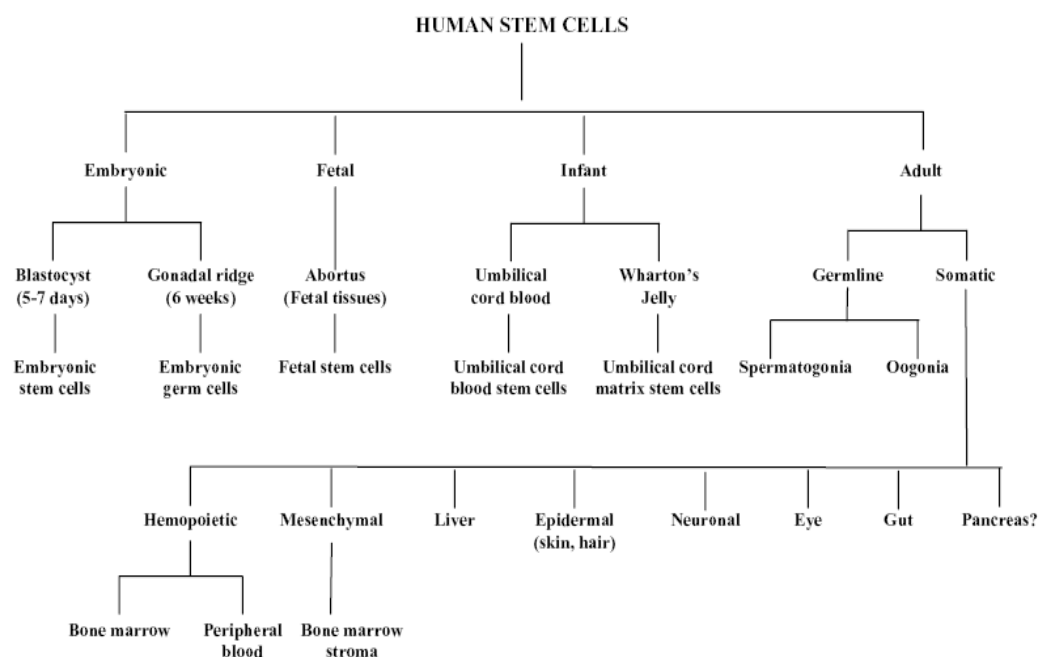


Figure 1.2: Types of stem cells (Bongso and Lee, 2005)

1.2.1. Embryonic Stem Cells (ESCs)

In mammals, zygote, 2-cell, 4-cell, 8-cell and morula stages contains totipotent cells that are able to form a complete organism. Pluripotent Human Embryonic Stem Cells (hESCs) are derived from inner cell mass (ICM) of the 5- to 6-day old human blastocyst. During embryonic development, the ICM develops into two distinct cell layers, the epiblast and hypoblast. The hypoblast forms the yolk sac which later becomes redundant in the human, and the epiblast differentiates into the three primordial germ layers: ectoderm, mesoderm and endoderm (Figure 1.3).

Human embryonic stem cells (hESCs) can be isolated from: i) surplus embryos left over after *in vitro* fertilization (IVF) treatment; ii) embryos created specifically for this purpose; and iii) embryos created by somatic cell nuclear transfer. The latter two approaches are heavily charged with emotional and ethical implications while the first is less controversial.

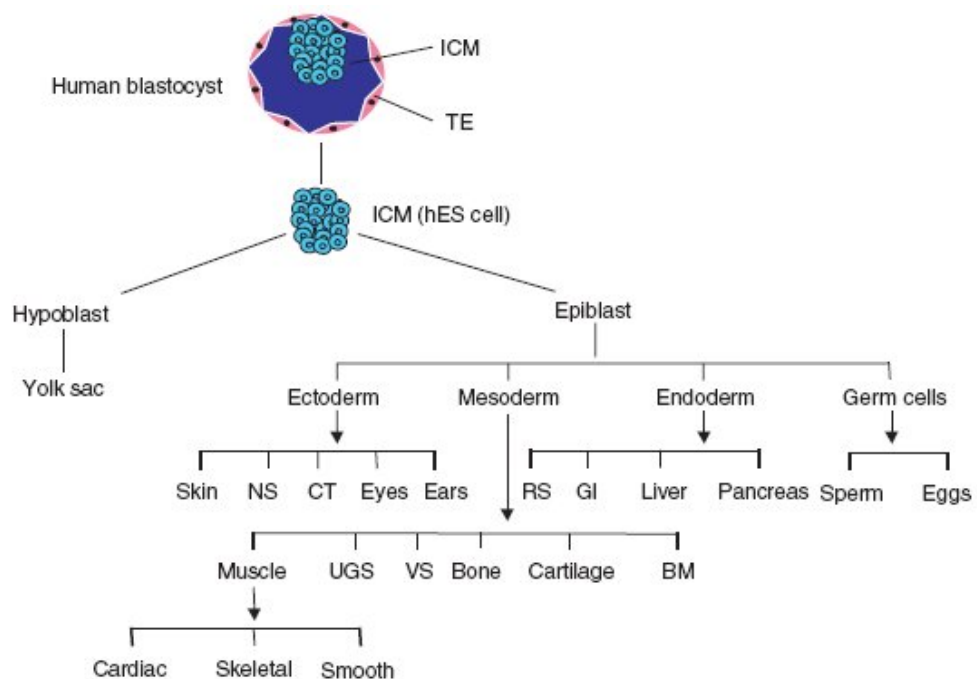


Figure 1.3: Cell lineages from the ICM of human blastocysts (Bongso et. al., 2005)

All of these approaches bring about some controversies and eventually affects the development of stem cell based therapies and its funding. The reason is that derivation of ESCs from embryos results in destruction of embryos. For many people of faith around the world, an embryo is fully a human being and therefore may not be killed (Suzanna et al., 2001). Although millions of people believe that hESCs research will improve the quality of their lives, the two major obstacles must be overcome, a) hESC culture technology *in vitro* must receive FDA (Food and Drug Administration) approval in USA, and b) enough hESC lines must be produced to generate the required number of cells for later clinical application. Proponents of embryonic stem cell usage admit that potential treatments include risk for both immune rejections by the patient and tumor formation (Jon S Odorico et. al., 2001). ESCs have unique properties that make them preferable among other stem cell sources (Table 1.1) but they are difficult and expensive to establish and maintain in culture. ESCs need a feeder cell layer to remain undifferentiated and continue its growth utilizing the secretions of feeder cells which are cytokines. Feeder cell layer also helps ESCs to attach to the cell culture flask. But, this brings about some risk of transmission of some infectious agents from feeder cells to ESCs. It was reported that embryonic stem cells are genomically unstable, exhibiting variable gene expression even in controlled conditions of the laboratory (Humpherys et. al., 2001)

Table 1.1: Unique Characteristics of hESCs (Bongso et. al., 2005)

Prolific growth, unlimited self renewal and abundant in numbers
Cell lines available and easy to scale up with bulk culture protocol
Pluripotent, versatile with wide plasticity
Maintains normal genetic make-up in low density cultures and on feeders
Telomerase levels high and consistent
No shortening of chromosome length with serial passaging
Late apoptosis
Customization of hESC lines possible via somatic cell nuclear transfer
Ethical issues limited to some countries and institutions
Can be used for (1) transplantation therapy (2) pharmaceutical screening (3) gamete and embryo production (4) studies on human development, congenital anomalies and infant cancers

Certain hESC lines (H7.S6 and H14.S9, University of Wisconsin) showed karyotypic changes involving the gain of chromosomes 17q and 12 with extended culture of 22 to 60 passages (2–6 months) (Draper et. al.2003). Interestingly, these aneuploid karyotypes were very similar to those seen with human embryonic carcinoma cells. Especially in feeder free culture conditions, stem cells might show adaptive genetic changes (Pera, 2004)

Another important issue is to keep differentiation conditions under control. ESCs can differentiate spontaneously. There are three approaches to achieve controlled differentiation: 1) Treating the hESCs with specific agents (growth factors, chemicals) to generate a specific differentiated cell type e.g. retinoic acid to produce neurons; 2) Growing hESCs in direct contact with companion fetal cells of the required cell type or a different cell type (co-culture) with the hope that the companion cell will release certain factors that will lead the hESCs to be converted to the desired cell type; 3) Transfecting specific gene constructs into hESCs and the gene then switching on in order to direct the hESCs to differentiate along a specific cell pathway, e.g. when the cardiomyosin gene inserted into mESCs and then switched on, it converts the mESCs into cardiomyocytes (Bongso et. al., 2005) .

In order to derive stable hESC lines, the gametes and ensuing embryos must be high quality and have no genetic and infectious diseases. Patients donating such material must be screened for HIV, hepatitis B, sex-linked and autosomal genetic diseases. hESCs must be grown under current good manufacturing practice (cGMP) and good tissue culture practice (GCTP) conditions. HLA typing for future tissue matching, tests for full characterization (using molecular genetic markers) and sustainability *in vitro* without karyotypic changes for prolonged passages should be performed.

Another embryo derived stem cell type is embryonic germ cells (EGCs). They are actually obtained by culturing Primordial germ cells (PGCs) *in vitro*. Primordial germ cells originate from proximal epiblast and they are the precursors of oogonia and prospermatogonia (Pera and Dottori, 2005). Primordial germ cells or diploid germ cell precursors transiently exist in the embryo before they closely associate with somatic cells of the gonads and then become committed as germ cells. Human embryonic germ cells (hEGCs) which are also stem cells are obtained from the primordial germ cells of the gonadal ridge of 5- to 9-week old fetuses (Figure 1.4).

hEGCs have been successfully isolated and characterized. These stem cells are pluripotent and are able to produce cells of all three germ layers, ectoderm endoderm and mesoderm.

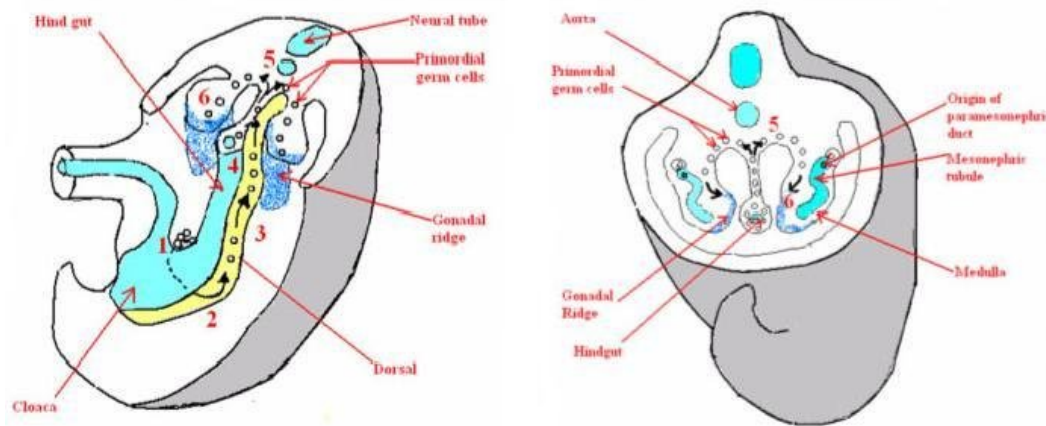


Figure 1.4: Primordial germs cells (URL-1)

1.2.2. Fetal Stem Cells

Fetal stem cells are primitive cell types found in the organs of fetuses. Neural crest stem cells, fetal hematopoietic stem cells and pancreatic islet progenitors have been isolated in abortuses. Human fetal pancreatic cells grow and differentiate after transplantation in nude mice (Beattie et. al.,1997). Fetal neural stem cells found in the fetal brain were shown to differentiate into both neurons and glial cells (Brustle et.al. 1998). However, isolation of pluripotent stem cells from fetuses increases people's skepticism about its ethical results. During isolation, abortuses is used and it is believed by some that this increases the rate of abortions indirectly. But some materials which do not require abortion such as fetal blood, placenta and umbilical cord can be used as rich sources of fetal hematopoietic stem cells.

Another ethically harmless fetal stem cell source seems to be amniotic fluid. Human Amniotic Fluid (HAF) obtained during the process of amniocentesis (Figure 1.5) contains a variety of stem cell originated from embryonic and extraembryonic tissues (Kim et.al. 2007). It was reported that HAF contains multipotent stem cells (Tsai et. al. 2004). In humans, the expansion potential of mesenchymal amniocytes exceeds that of bone-marrow-derived mesenchymal stem cells (Fauza, 2004). Human amniotic fluid epithelial cells are able to differentiate into neurons,

astrocytes and oligodendrocytes, therefore, Human amniotic fluid stem cells (HAFSCs) might be a possible candidate for transplantation therapy of neurodegenerative diseases (Kakishita et. al. 2003). Unlike their mesenchymal counterparts, cells that could be ESCs are very rare in the human amniotic fluid, representing 0-1% of the cells. This means ESCs cannot always be isolated from the amniotic fluid. In addition, these cells have been identified mostly through markers commonly present in ESCs, such as nuclear Oct-4, stem cell factor, vimentin, alkaline phosphatase, CD34, CD105, and c-Kit (Figure 1.6). These markers can be expressed alone or in various combinations in embryonic germ cells; embryonic fibroblasts; embryonal carcinoma cells; mesenchymal stem cells; hematopoietic stem cells; ectodermal, neural, and pancreatic progenitor cells; and fetal and adult nerve tissues (Fauza, 2004)

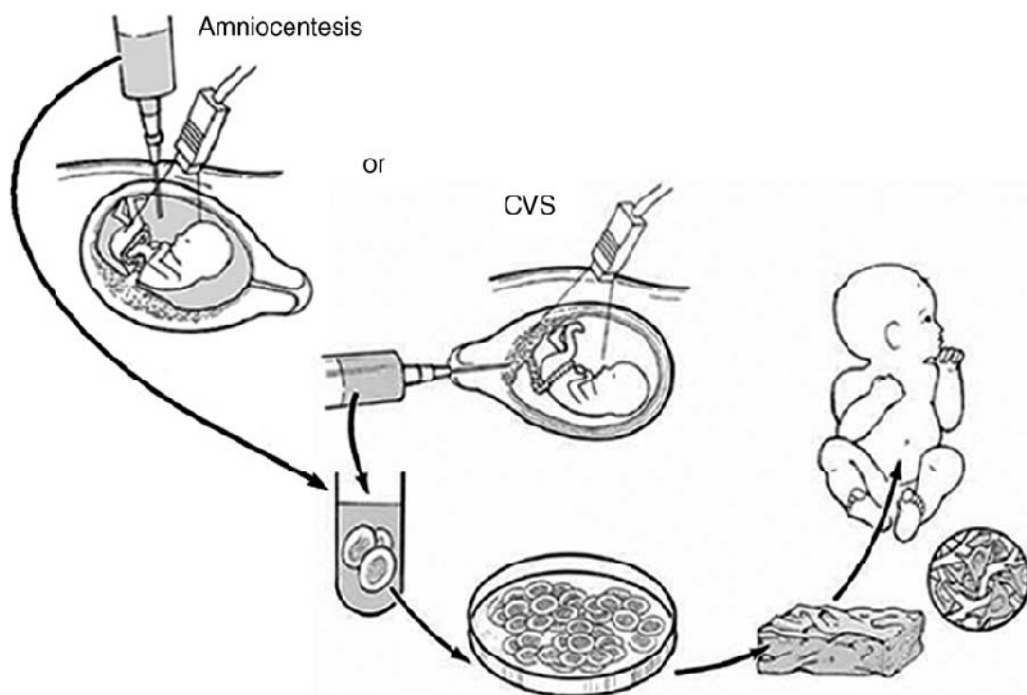


Figure 1.5: Amniocentesis (Fauza, 2004)

The Amniotic fluid mesenchymal stem cells (AFMSCs) can expand rapidly and they maintain their capacity to differentiate into multiple cell types *in vitro*. Aside from the common mesenchymal lineages (adipocytes and osteocytes), they also have been differentiated successfully into neuron-like cells (Figure 1.6). Most recently it was

reported that stem cell derived from amniotic fluid can differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages (De Coppi et. al. 2007). Same group also showed that when the cells were maintained for over 250 population doublings, they retained long telomeres and a normal karyotype.

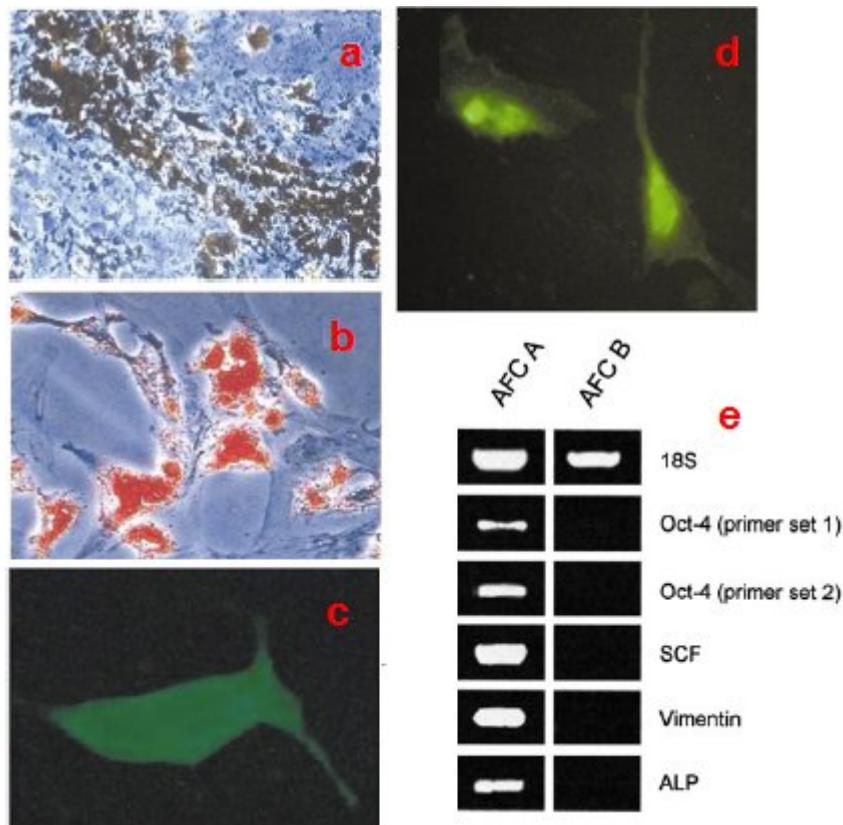


Figure 1.6: Multipotential stem cells derived from amniotic fluid: **a.** Osteogenic differentiation was shown by von Kossa staining; **b.** Adipogenic differentiation was shown by Oil Red O staining; **c.** Positive immunofluorescence stain of β -actin (Tuj-1); **d.** Immunocytochemical staining of Oct-4 positive cells in cultured AFMSCs; (Tsai, 2004) **e.** AFC A: Cells in Human amniotic fluid express Oct-4, Stem cell factor (SCF), vimentin, ALP mRNAs; AFC B: Representative sample of Oct-4 negative cells; 18S RNA is an internal standard expressed in both type of cells (Prusa et. al., 2003)

1.2.3. Umbilical Cord Stem Cells

Umbilical cord (UC) blood contains circulating stem cells and the cellular contents of umbilical cord blood appear to be quite distinct from those of bone marrow and adult

peripheral blood. It has been shown that, in contrast to its adult bone marrow (BM) counterpart, the stem cell compartment in CB (cord blood) is less mature. This has been documented extensively for the hematopoietic system, including a higher proliferative potential *in vitro* as well as *in vivo*, which is associated with an extended life span of the stem cells and longer telomeres (Szilvassy, 2001). The frequency of umbilical cord blood hematopoietic stem cells equals or exceeds that of bone marrow and they are known to produce large colonies *in vitro*, have different growth factor requirements, have long telomeres and can be expanded in long term culture. Cord blood shows decreased graft versus host reaction compared with bone marrow, possibly due to high interleukin-10 levels produced by the cells and/or decreased expression of the beta-2-microglobulin. Cord blood stem cells have been shown to differentiate into osteoblasts, chondrocytes, adipocytes, and neural progenitors as well as to differentiate *in vivo* into bone, cartilage, hematopoietic cells, neural, liver, and heart tissue (Figure 1.7) (Kögler, 2005). Since UC blood seem to contain mesodermal progenitors for MSCs, and are also an easily accessible source of cells, they should be considered as a potential candidate for supportive therapy (Kögler, 2005).

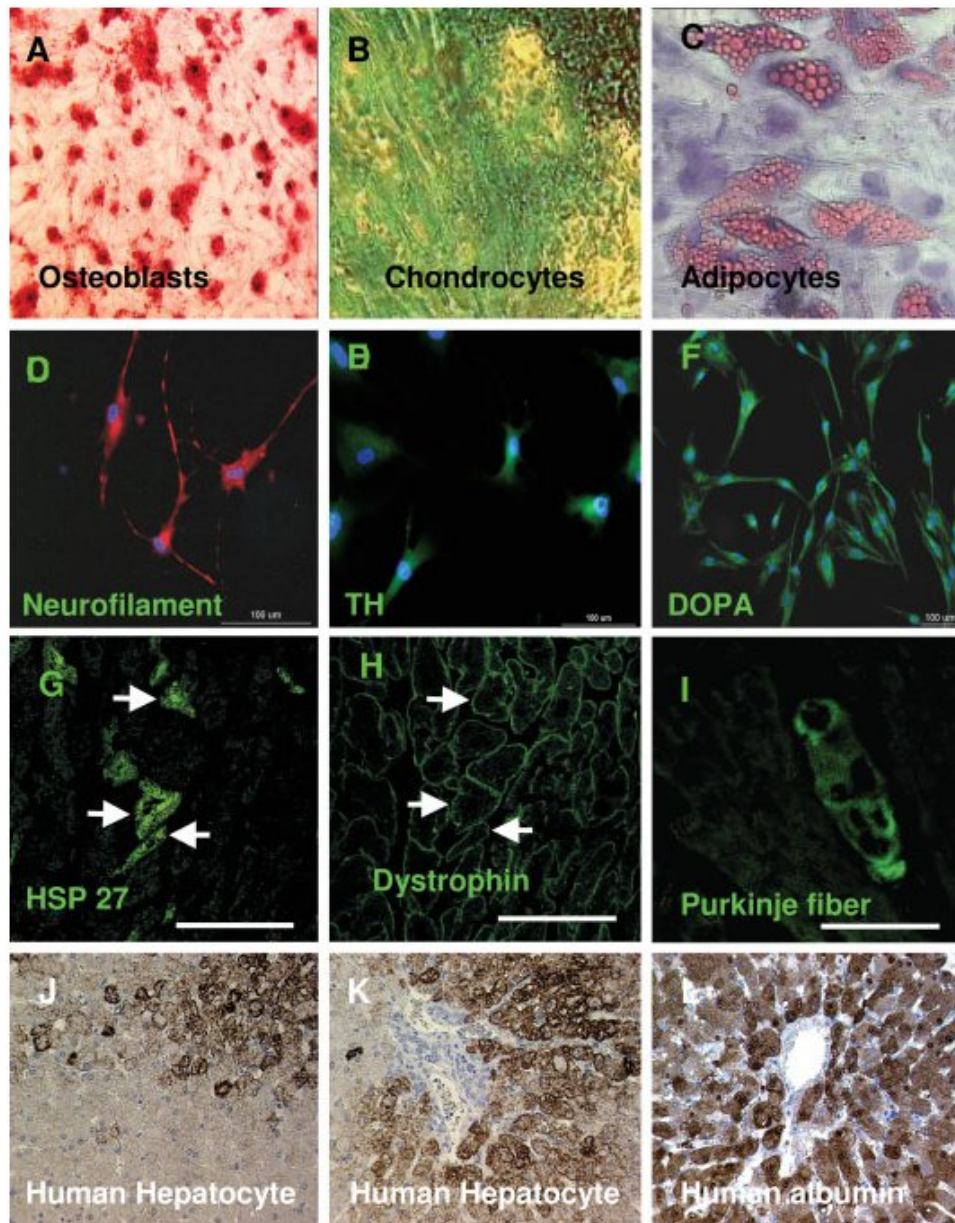


Figure 1.7. Multipotential stem cells derived from UC blood: A: Differentiation to osteoblasts is shown by Alizarin red staining to determine calcium deposition; B: Collagen type II staining to show chondrogenic differentiation; C: Oil red-O staining of the lipid vesicles showing adipogenesis; D–F: *In-vitro* differentiation of UC blood stem cells into neural cells: Differentiated cells showed positive immunoreactivity for the neuron-specific markers neurofilament (D), the enzyme tyrosine hydroxylase (E) and DOPA-decarboxylase(F); (G–I) *In-vivo* differentiation of UC blood stem cells to cardiomyocytes and Purkinje fibers: (G) and (H) are serial sections of the right ventricle, (I) A section of a Purkinje fiber. Scale bars: (A–E) = 50 mm; (F) = 100 mm; (J, K) *In-vivo* differentiation of USSC from CB into parenchymal liver cells; (L) Anti-human albumin staining of the liver.

1.2.4. Adult Stem Cells (ASCs)

Somatic stem cell (SSC) and ASC term are being used interchangeably to describe stem cells of adult origin. It was thought that adult mammalian stem cells were only present in organs such as blood, skin, gut and testis. Today, we know that if not all, adult organs contain stem cells. Such ASCs have been known to possess the ability to regenerate the corresponding tissue from which they are derived. Adult stem

cells usually form only 1-2% of the cell population, their main role is the replenishment of the tissue's cells in appropriate proportions and numbers in response to “wear and tear” loss or direct organ damage (Fang et. al., 2004). Adult stem and progenitor cells possess the capability of self-renewal and differentiation into at least one or more mature cell types. Most adult tissues contain multipotential stem cells, i.e. cells that are capable of lifelong renewal and that produce a limited range of differentiated cell lineages appropriate to their location (Levicar, 2007). Such properties make it possible to use adult stem cells to regenerate damaged or senescent cells throughout life span of the organism. Based on animal models, many studies have recently claimed that ASCs might exhibit developmental potentials comparable to those exhibited by ESCs through their having “plasticity potential” or “trans-differentiation” (Figure 1.8) (Wagers et. al., 2002). For example, unmanipulated bone marrow cells were found to participate in the muscle regeneration process when injected into skeletal muscle (Ferrari, et al. 1998).

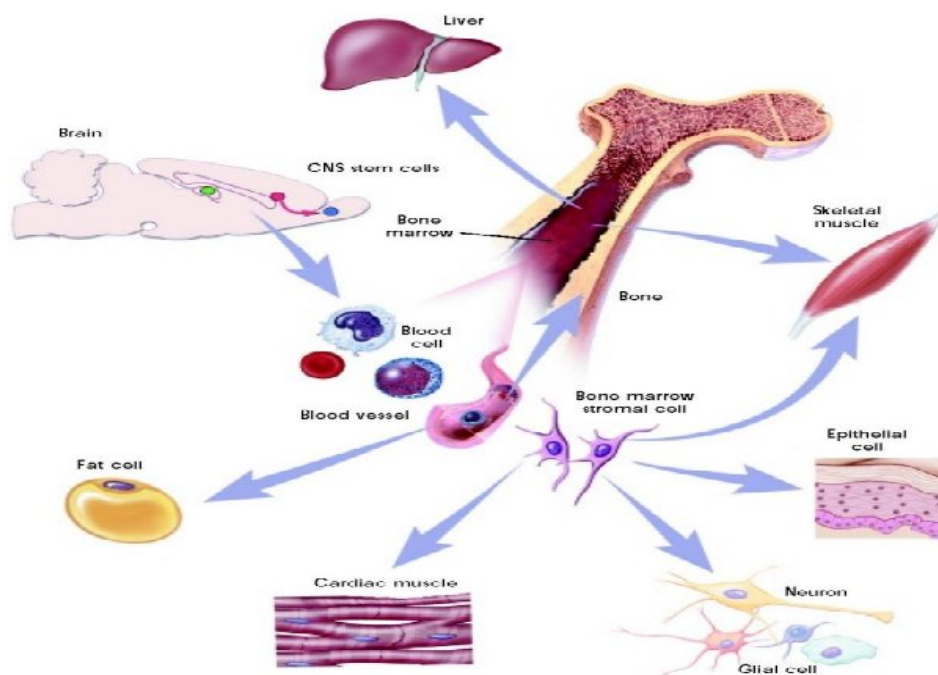


Figure 1.8: Plasticity of bone marrow derived ASCs (URL-2)

It was reported that bone marrow cells were able to differentiate into microglia, astroglia and neurons within the central nervous system (Eglitis and Mezey, 1997). Stem cells from the rat bone marrow have been shown to give rise to hepatocytes in recipients with artificially induced hepatic injury (Peterson et.al.1999).

1.2.4.1. Hematopoietic stem cells (HSCs)

HSCs are among the few stem cells to be isolated in adult humans. They reside in the bone marrow and under some conditions migrate to other tissues through the blood. HSCs are also normally found in the fetal liver and spleen and in umbilical cord and placenta blood. The ability of hematopoietic stem cells (HSCs) to self-renew continuously in the marrow and to differentiate into the full complement of cell types found in blood makes them as the premier adult stem cells (Figure 1.9)

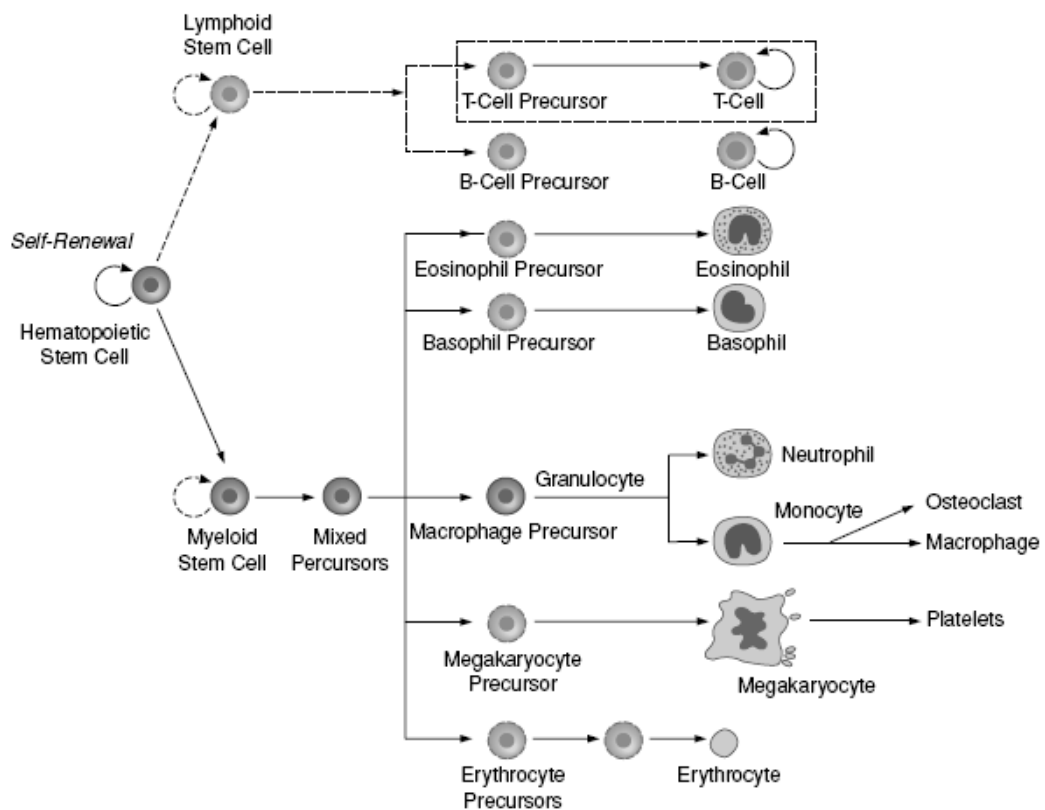


Figure 1.9: Blood cell differentiation from HSCs (NATIONAL ACADEMY PRESS 2001).

HSCs normally divide to generate either more HSCs (self-renewal) or progenitor cells, which are precursors to various blood cell types. HSCs are found mainly in

bone marrow, although T cells develop in thymus, and some other cell types develop from blood monocytes. Once HSCs partly differentiate into progenitor cells, further differentiation into one or a few types of blood cell is irreversible.

Recent studies showed that HSCs have the capacity for transdifferentiation. It was shown that HSCs can differentiate into liver cells (Lagasse et. al., 2000), cardiac and muscle tissue (Bittner et. al., 1999), and neuron-like cells (Brazelton et. al., 2000). These findings have raised expectations that HSCs will eventually be shown to be able to give rise to multiple cell types from all three germ layers.

On the other hand, there are some limitations in supplying HSCs for every patient. Number of HSCs in bone marrow is rare (one in every 10,000 bone marrow cells) and it is difficult to separate them from other components of the blood, thereby bone marrow stem cell transplants are generally impure. All cells of the body express on their surface a set of molecules called histocompatibility (i.e. tissue compatibility) antigens. If a patient receives a transplant of HSC cells from a donor that has histocompatibility antigens different from his own, the patient's body will recognize and react to the cells as foreign. To increase success of bone marrow transplantation, it is preferred that donors be a related sibling of the transplant recipient. In case their histocompatibility antigens do not match, the recipient's body will reject the material or can produce an immune reaction in which the T cells of the transplant attack the tissues of the recipient's body, leading to a potentially lethal condition known as graft versus host disease. However, autologous transplants, in which material from a person is implanted into the same person (for example, when a cancer patient deposits his own blood in advance of chemotherapy or irradiation) solve the problem of immune system rejection (National Academy Press, 2001). Purified and concentrated populations of autologous HSCs transplanted in breast cancer patients after chemotherapy have been shown to repopulate the blood more swiftly and with fewer complications (Negrin et al., 2000).

1.2.4.2. Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are found postnatally in the nonhematopoietic bone marrow stroma. Marrow stromal tissue is made up of a heterogeneous population of cells, which include reticular cells, adipocytes, osteogenic cells, smooth muscle cells, endothelial cells and macrophages (Bianco and Riminucci, 1998). Apart from bone marrow stroma, MSCs can also be derived from periosteum, fat and skin (Figure 1.10).

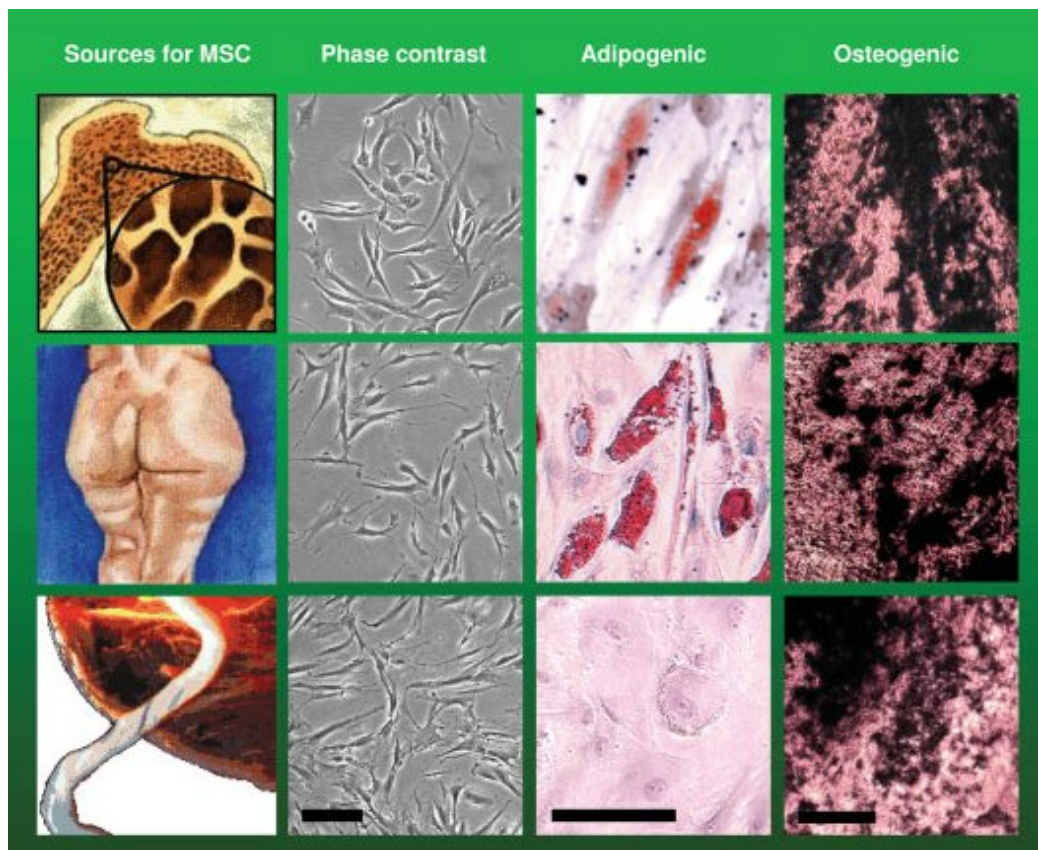


Figure 1.10: Mesenchymal stem cells (MSCs): MSCs can be isolated from various tissues including bone marrow, adipose tissue and umbilical cord blood. MSCs are plastic adherent with a spindle-shaped morphology. Adipogenic and osteogenic differentiation can be induced by appropriate culture conditions as examined by Oil Red-O staining or von Kossa staining. Scale bar: 100 μ m (Ho and Wagner, 2005).

MSCs are multipotent cells that are capable of differentiating into cartilage, bone, muscle, tendon, ligament and fat (Caplan, 1994). There is some recent evidence that there is a rare cell within MSC cultures that is pluripotent and can give rise not only to mesodermal but also to endodermal tissues (Jiang et. al., 2002). These cells are also Multipotent Adult Progenitor Cell (MAPCs). MSCs can be expanded *in vitro* without losing their “stemness” or self-renewal capacity and they do not need any feeder cell layer (Caplan, 2000). To reach desired goals in use of MSCs in clinics, many questions must be elucidated. Isolation, propagation and long term culture protocols must be universalized. Molecular characterization of MSCs has not still been performed completely. Although the reports demonstrated high differentiation capacity of these cells, exact pathways remains elusive.

1.3. Human craniofacial stem cells

Human craniofacial stem cells are recently discovered sources of mesenchymal stem cells. They represent great promise for autogenic or allogenic transplantation and tissue engineering. Prior to employing these cells in clinical applications, they must be thoroughly investigated and characterized. So far craniofacial stem cells were isolated from dental pulp cells (DPCs), dental follicle cells (DFCs), periodontal ligament cells (PDLCs), and buccal mucosa fibroblasts (BMFs) (Lindroos et. al., 2008).

The dental pulp contains connective tissue, mesenchymal stem cells, neural fibers, blood vessels, and lymphatics (Figure 1.11). Researchers have recognized that dental pulp contains *ex vivo* expandable cells called dental pulp cells. These cells express osteogenic markers, such as alkaline phosphatase, type I collagen, bone sialoprotein, osteocalcin, osteopontin, transforming growth factor β (TGF- β), and bone morphogenetic proteins (BMPs) (Chen et al., 2005). Human DPSCs were initially identified on the basis of their traits of forming single colonies in culture, self-renewal *in vivo*, and multidifferentiation *in vitro* (Gronthos et al., 2000).

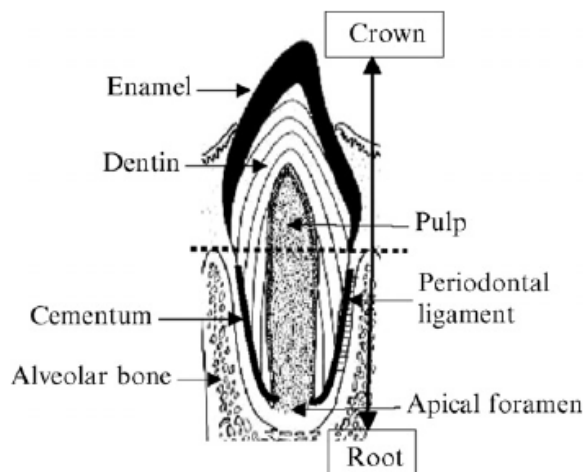


Figure 1.11: Diagram of a tooth structure (Liu et. al., 2005)

Most recently it has been shown that DPSCs express Oct-4 suggesting that there might be small population of pluripotent stem cells residing in dental pulp. (Huang et. al. 2008) (Figure 1.12)

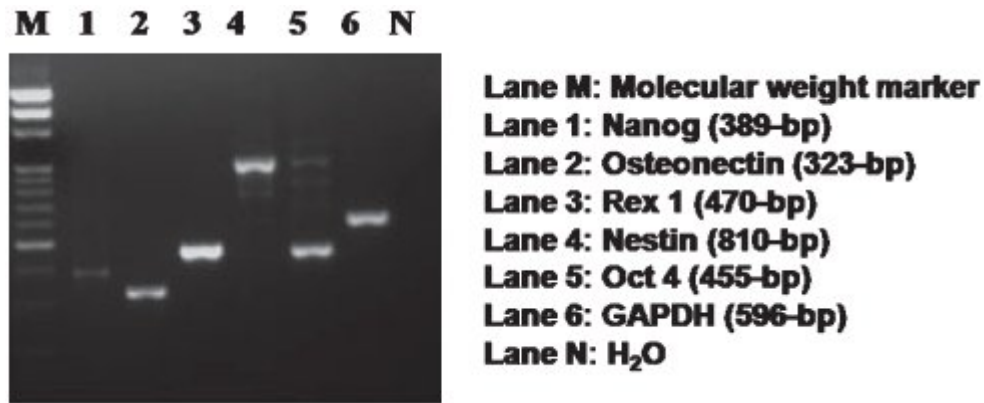


Figure 1.12: DPSCs express both stem cells and osteogenic markers (Huang et. al.2008)

There is also tissue engineering approaches in the maxillofacial region that attempt to isolate mesenchymal stem cells from the intraoral sites and utilize these cells for therapeutic purposes. It has been demonstrated that dental follicle cells remain stable in terms of transformation and do not undergo differentiation into osteoblast and osteocytes in culture (Yao et. al., 2004). The dental follicle surrounds developing tooth germ and is an ectomesenchymal derived connective tissue that contains progenitor cells for the development of the periodontium (Figure 1.13).

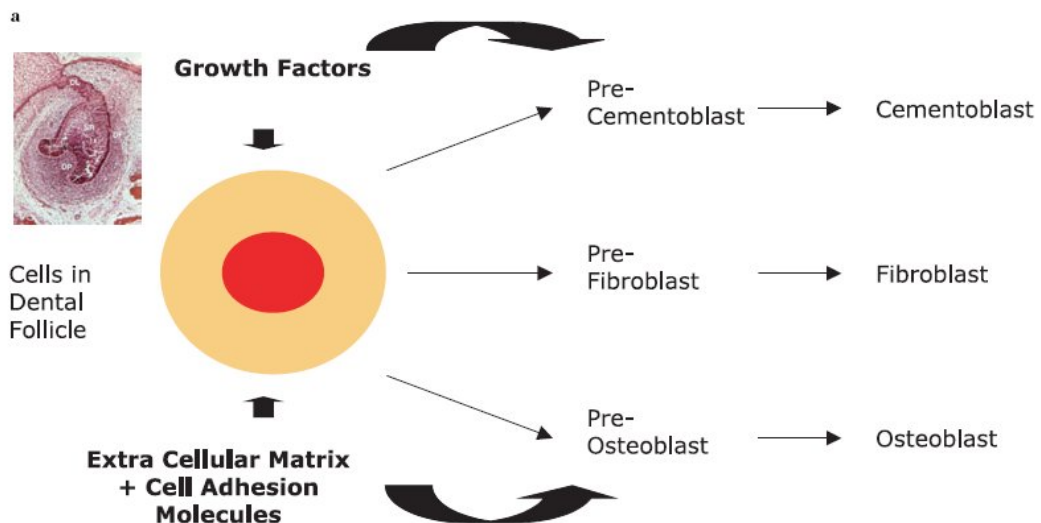


Figure 1.13: Dental follicle stem cells Dental follicle stem cells give rise to periodontium in the presence of growth factors, extra cellular amtrix and cell adhesion molecules (Bartold et. al.,2000).

Morsczeck et. al. (2005) isolated precursor cells from human dental follicle of wisdom teeth and suggested that these cells are able to differentiate into PDL-like structures. These periodontal ligament stem cells exhibited the capacity to generate clonogenic adherent cell colonies when plated under the same growth conditions as described for bone marrow stromal stem cells (Seo et. al, 2004). The identification of putative mesenchymal stem cell populations within the dental follicle has stimulated interest in the potential use of stem cell-based therapies to treat the damaged caused by trauma or various diseases.

1.4. Scope of the study

Although bone marrow is a potential cell source for the preparation of MSCs, an alternative source of adult stem cells that could be obtained in large quantities with minimum surgical stress would be advantageous. Recent evidences has suggested that adult stem cells are not exclusive to bone marrow, but are also present in a variety of tissues and organs such as liver, nerve, muscle, skin, synovium, cartilage and dental tissues. In this study, it was focused on extracted teeth as they are easily obtainable waste materials. However, extracted teeth are often decayed, i.e., already infected, and present the risk of contamination during cell preparation/culturing. Non-decayed third molars including impacted molars are sometimes extracted during orthodontic treatment. These third molars, especially impacted molars, might be good sources of cells for this purpose because impacted molars are generally thought to be free of infection. Impacted third molars from young adults (age about 10–16) generally contain immature tissue called tooth germ. The tooth germ is a budlike thickening of the dental lamina that is the primordium of a tooth. It consists of the dental papilla, the dental follicle, and the enamel organ (Ikeda et.al. 2006). Consequently, the dental follicles might be a target of interest as a good alternative source of adult stem cells. Although different investigations has been conducted on the biological potential of dental follicle cells showed that they are highly proliferative and have osteogenic adipogenic and chondrogenic differentiation, there is not enough data representing if dental follicles contains stem cells expressing pluripotent stem cells markers such as Oct-4 and Nucleostemin (NS). It is also little known about transdifferentiation capacity of dental follicle derived stem cells.

Oct-4 is a very important protein for maintenance of pluripotency of stem cells. It activates or represses patterns of gene expression that mediates cellular events during differentiation. Oct-4 is a key regulator of stem cell pluripotency and differentiation (Pan et.al. 2002). Investigation of adult tissues for the presence of Oct-4 expressing cells might be very important for finding new sources for stem cell-based therapies. NS is expressed by bone marrow derived stem cells. It has been shown that NS involves in regulation of telomerase activity thus, it controls proliferation of dividing cells (Zhu et. al., 2006). NS is stably expressed in proliferating cells. Expression of NS increased when the cells were induced to proliferate with FGF-2 (Kafienah, 2006). Therefore, NS expression might correlate with proliferation capacity of the cells. In order to prove the presence of MSCs in our cells, we also showed expression of ALP (Alkaline Phosphatase) and Vimentin as previously shown (Prusa et al., 2003 and Fauza, 2004).

In this study, in order to find out neurogenic differentiation capacity of HDFCs, firstly, it was shown that early neural markers Nestin and β 3-Tubulin are being expressed by HDFCs and secondly, some neuron-like cells were obtained by applying neural differentiation medium on HDFCs. Our data suggest for the first time that a retinoic acid mediated pathway might be triggered to induce neurogenic differentiation of HDFCs aiming to contribute to the literature by suggesting a potential molecular mechanism of neurogenic differentiation in HDFCs.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Dental Follicle Tissues:

Dental follicle tissue from a 17 years old patient was surgically removed with orthodontic reasons in Istanbul University School of Dental Medicine and was sent to our laboratory in Yeditepe University, Department of Genetic and Bioengineering.

2.1.2 Cell Lines

SH-SY5Y (Human metastatic neuroblastoma cell line) and HeLa (Human cervical cancer cell line) were kindly provided by Ömer Faruk Bayrak.

2.1.3 Tissue Culture

- Dulbecco's Modified Eagle Medium (DMEM) 1g/liter glucose (Sigma)
- α -modified minimum essential medium (MEM) (Sigma)
- Fetal Bovine Serum (FBS) (Gibco)
- L-Glutamine solution (Gibco)
- Penicilin/Streptomycin/ Amphotericin (PSA) solution (Biological Industries, Israel)
- 0,5% Trypsin-EDTA solution (Sigma)
- PBS (Gibco)
- T25 Tissue Culture Flasks (Nunc)
- T75 Tissue Culture Flasks (Nunc)
- T150 Tissue Culture Flasks (Nunc)

- 6-well culture plate (Nunc)
- Coverslip (Isolab)
- Steril scalpels
- Serological pipets, 5 ml, 10 ml, 25 ml (Isolab)
- Centrifuge tubes, 15 ml (Falcon)
- Cryovials, 2 ml (Falcon)

2.1.4 Reverse Transcriptase and PCR (RT-PCR) Analysis

- Rneasy RNA isolation kit (Qiagen)
- Sensiscript Reverse transcriptase (Qiagen)
- Go taq flexi DNA polymerase (Promega)
- Oligo-dT (Sigma)
- RNAase inhibitor (Fermentas)
- dNTP Mix (Biogen)
- PCR Tubes
- Agarose (Sigma)
- Loading Buffer, 6x (Sigma)
- DNA Ladder (Sigma)
- TAE Buffer (Biobasic)
- Ethidium Bromide (Sigma)

2.1.5 Immunocytochemistry Analysis

- Paraformaldehyde (Sigma)
- Anti- human Trk-a antibody, mouse origin (Santa Cruz)
- Anti human β 3-tubulin antibody , mouse origin (Santa Cruz)
- Anti human Osteocalcin antibody , mouse origin (Santa Cruz)
- Anti human NFM (Neurofilament Medium) , mouse origin (Santa Cruz)
- Anti human Oct-4 antibody , rabbit origin (Chemicon)
- Anti human NS antibody , rabbit origin (Chemicon)
- Triton X-100 (AppliChem)
- Goat polyclonal anti-rabbit IgG-Alexa 488 conjugate (Molecular Probes)
- Goat polyclonal anti-mouse IgG Alexa 488 conjugate (Molecular Probes)
- Goat polyclonal anti-rabbit IgG-Alexa 647 conjugate (Molecular Probes)

- Goat polyclonal anti-mouse IgG-Alexa 647 conjugate (Molecular Probes)
- Leica TCS SP2 SE Confocal Microscope
- TO-PRO3 dye (Invitrogen)
- Mounting Medium (Mowiol)

2.1.6 Differentiation assays

- RANDOX Alkaline Phosphatase detection reagent (Randox Laboratories Ltd.)
- Von Kossa Staining Kit (Bio-Optica)
- Dexamethasone (Sigma)
- β -Glycerophosphate (Sigma)
- Ascorbate (Sigma)
- Ripa Buffer (Pierce)
- Polystyrene 96-well microplate (Orange Scientific)
- Elisa Plate Reader (Bio-Tek)
- Retinoic Acid (Sigma)
- Basic fibroblast growth factor (bFGF) (Sigma)
- Nerve Growth Factor (NGF) (Sigma)
- β -mercaptoethanol (AppliChem)

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Culture of HDFCs

Dental follicle used in this study was obtained from 17 years old patient whose impacted third molar germ (wisdom tooth) was surgically removed as a prophylactic treatment due to orthodontic reasons. Written informed consent was obtained from the patient and his parents under approval of the local ethics committee of Istanbul University.

Dental follicle tissue was sliced into small pieces using sterile scalpels and plated on 6-well tissue culture plates in growth medium containing α -modified minimal essential medium (α -MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Biological Industries) and 1% PSA (penicillin, streptomycin and

amphotericin solution). The cells started to grow on the third day of incubation spreading from the tissue pieces and reached confluency in 4 days (Figure 3.1). The cells were detached from dishes by incubating in trypsin-EDTA solution for 3 minutes. After centrifugation at 1500 rpm for 5 minutes and resuspension in growth medium, cells were cultivated at 37 °C in a humidified atmosphere of 5% CO₂ in T-75 flasks. The medium was changed every day and cell cultures were passaged every other day.

2.2.1.2 Culture of SH-SY5Y Cells

SH-SY5Y cell line was used in neurogenic differentiation experiments due to its neuronal origin as previously reported. SH-SY5Y culture was maintained in DMEM containing 10% FBS, 2 mM L-Glutamine and 1% PSA solution. Cells were grown in T-75 flasks with 15 ml medium at 37°C in 5% CO₂ incubator until they reach to confluency. They were usually splitted every 2-3 days by tyripsinization.

2.2.2 Osteogenic Differentiation of HDFCs

HDFCs at passage 4 were used for osteogenic differentiation experiment. Cells were counted and cultivated in 6-well plates at 3000 cells/cm² in growth medium. After 2 days, medium was shifted to osteogenic medium (α-MEM supplemented with 10% FBS, 0.1 mmol/l dexamethason, 10 mmol/l β-glycerophosphate, 50 mmol/l ascorbate). Osteogenic medium was changed every other day. All solutions were freshly prepared immediately prior to use (Protocol described by Delo and De Coppi, 2006).

2.2.3 Neurogenenic Differentiation

HDFCs and SH-SY5Y cells were seeded at a concentration of 3000 cells/cm² in 6-well plates. At the frist step, cells were incubated in α-MEM supplemented with 20% FBS, 0.1M Retinoic acid, 5 ng/ml basic fibroblast growth factor (bFGF) for 24 hours. At the second step, medium was shifted into α-MEM without serum, supplemented with NGF (10ng/ml), and incubated 3 days by changing medium every day. All

solutions were freshly prepared immediately prior to use (Protocol described by Tatard et. al., 2007).

2.2.4 RT-PCR Analysis

Total RNAs were isolated from HDFCs at passage 4 and SH-SY5Y cells using RNeasy Mini Kit. First-strand cDNA synthesis was performed by using Sensiscript Reverse Transcriptase. Primer sets for each gene are listed in Table 2.1. PCR conditions were applied as reported in the literature Table 2.1. The amplified DNA fragments were visualized through 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed under UV light (Figure 3.2).

Table 2.1: Primers for PCR

Marker	Sequence	Length	Ref.
Oct-4	forward: 5'-cgaccatctgccgctttgag-3' reverse: 5'-ccccctgtccccattccta-3'	572bp	McLaughlin et al.,2006
NS	forward: 5'-ggagtcctggatttccttcc-3' reverse: 5'-gccctgaccactccagttta-3'	98bp	Kafienah et. al.,2006
B3-Tubulin	forward: 5'-ctcaggggcctttggacatc-3' reverse: 5'-caggcagtcgcagttttcac-3'	159bp	Xao et.al., 2001
Nestin	forward: 5'-ggagtcctggatttccttcc-3' reverse: 5'-gccctgaccactccagttta-3'	200bp	McLaughlin et al.,2006
B-actin	forward:5'-gacaggatgcagaaggagattact-3' reverse:5' tgatccacatctgctggaagg-3'	141bp	Kafienah et. al.,2006
Osteocalcin	forward:ggtgcaaagcccagcgactct-3' reverse; ggaagccaatgtgtccgcta-3'	190bp	Yoon et.al.2007
NFM	forward: 5' acg ctg gac tgc ctg ggc aa 3' revers: 5' gcg agc gcg ctg cgc ttg ta 3'	156bp	Estus et.al. 1997
hTERT	forward: 5'-cctctgtgctgggcctggacgata-3' revers: 3'-acggctggaggctgtcaaggtag-3'	282bp	Fujita et. al.,2005
ALP	forward: 5'-gacatcgctaccagctcat-3' reverse: 5'-tcacgttgttctgttcagc -3'	306bp	Tare et. al. 2005
Vimentin	forward: 5'-tgaggctgccaaaccggaaca-3' reverse: 5'-ttggccgcctgcaggatgag-3'	208bp	Kameda et. al. 2003

2.2.5 Immunocytochemistry Analysis

HDFCs and SH-SY5Y cells were seeded on glass coverslips at 3000 cell/cm² in 6-well plates, cultured for 2 days and fixed with 2% paraformaldehyde (Appendix 1, preparation of 2% paraformaldehyde). After rinsing with PBS, cells were permeabilized by incubating with 0.1 % Triton-X 100/PBS for 5 minutes. In order to avoid non-specific binding of antibodies, the cells were incubated with 2% FBS (diluted in PBS) for 20 min. Samples were incubated with primary antibodies overnight at 4°C. Each sample were washed twice for 5 minutes with PBS to remove unbound primary antibodies. After washing secondary antibodies were added and incubated for 1 hour. For Oct-4 and NS goat polyclonal anti-rabbit IgG-Alexa 488 conjugate was used. For NFM, β -3 Tubulin and Trk-A goat polyclonal anti- mouse IgG-Alexa 488 conjugate were used. TO-PRO3 dye (Invitrogen) was used to stain nuclei of the cells. Stained coverslips were mounted on clean glass slides and fixed with Mowiol mounting medium (Appendix 2). Prepared slides were observed under Confocal microscope (Leica TCS SP2 SE) immediately after preparation in order to prevent fading.

2.2.6 ALP Activity

ALP activities of HDFCs were determined using RANDOX Alkaline Phosphatase detection reagent 500 μ l of cold Ripa buffer was added on suspension of HDFCs (approximately 2.5×10^6 cells), pipetted well and centrifuged at 14,000 rpm for 15 minutes to pellet the cell debris. Supernatant containing proteins were carefully transferred into a new 1ml plastic tube. Fifty microliter of supernatant was mixed with 100 μ l of reagent in a 96-well plate and incubated at 37°C for one and a half hours. 50 μ l of enzyme free water was mixed with 100 μ l of reagent in order to use as a reference. Absorbance of the wells was measured at 405 nm by an ELISA (Enzyme-Linked ImmunoSorbent Assay) plate reader at 15 minutes intervals.

2.2.7 Von Kossa Staining

After 8 days incubation with Osteogenic medium in 6-well plates, the cells were fixed by incubating with 2% Paraformaldehyde at 4°C for 30 minutes. After fixation cells were rinsed with distilled water. Twenty drops Reagent A (Lithium carbonate saturated solution) was put on the cells and left to act for 10 minutes. Cells were rinsed with distilled water and 20 drops of reagent B (Silver nitrate solution) was put on the cells and left to act in dark for 1 hour. After incubation 20 drops of reagents C (Reducing Solution), D (Sodium sulphate solution) and E (Mayer's Carmalum) were put on the cells and left to act for 5 minutes respectively. After final rinsing 100% Ethanol was added and the samples were dehydrated. After drying the samples from the alcohol, the cells were observed under light microscope.

3. RESULTS

3.1 Expansion of HDFCs

Dental follicle cells grew on culture dishes and showed fibroblast like morphology (Figure 3.1). It was observed that actively dividing cells contain sphere shape nuclei. When the thesis had been written, the 35th passage cultures were completed in Yeditepe University Cell Culture Laboratories. Undifferentiated HDFCs expanded extensively without feeder cells and doubled approximately every 36 hours.

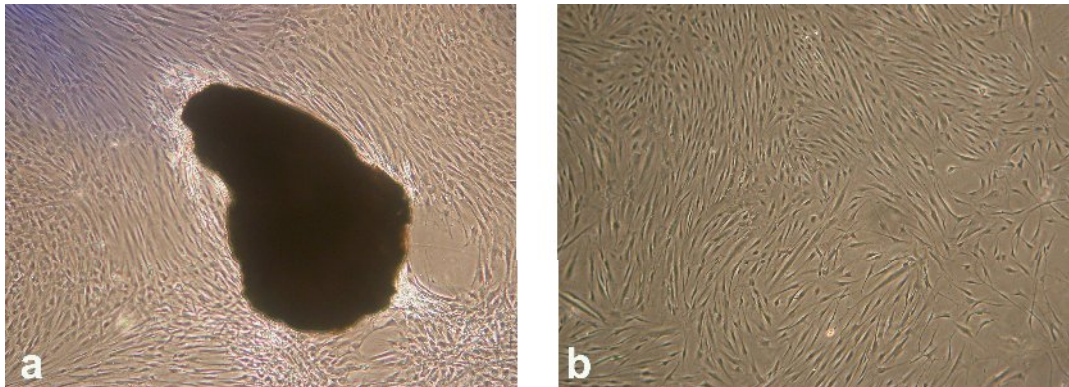


Figure 3.1: Growing DFCs, **(a)** Cells spreading from tissue pieces; **(b)** Confluency on day 4.

3.2 RT-PCR analysis

RT-PCR analysis showed that HDFCs express Oct-4 and NS, Nestin, β 3-tubulin, hTERT (Figure 3.2), ALP and Vimentin (Figure 3.3). Prior to differentiation Osteocalcin and NFM expressions were not detectable but after osteogenic and neurogenic differentiation both of these proteins were expressed (Figure 3.3).

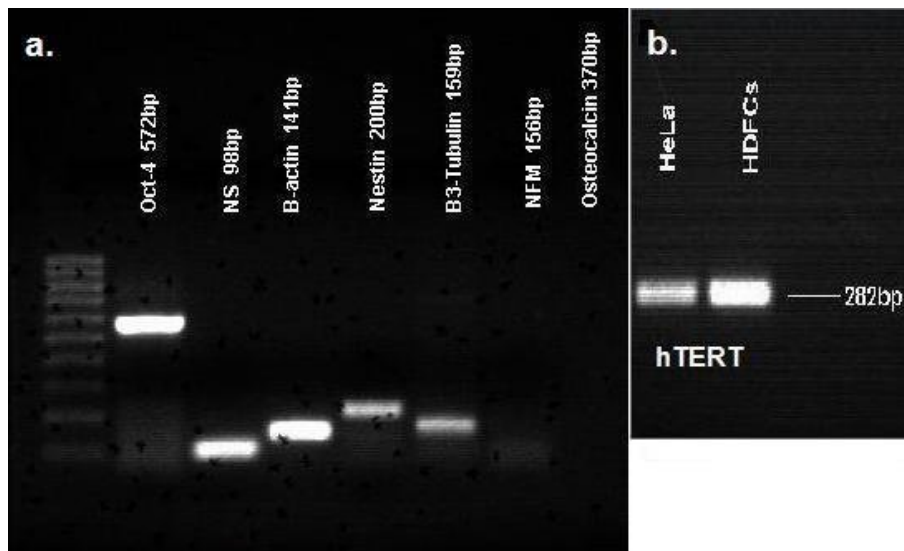


Figure 3.2: RT-PCR results before differentiation. (a) Oct-4, NS, β -actin, Nestin, β 3-Tubulin mRNAs were expressed; (b) Both HDFCs and HeLa cells expressed hTERT mRNA

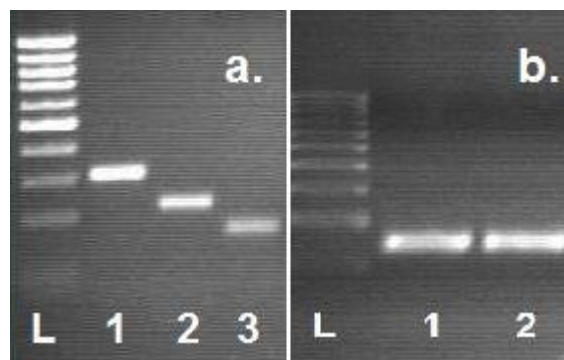


Figure 3.3: RT-PCR results after differentiation: **(a)** L:100 bp ladder, 1: ALP (306 bp), 2:Vimentin (208 bp), 3:Osteocalcin (190 bp); **(b)** L:100 bp ladder, 1: NFM expression in HDFCs , 2: NFM expression in SH-SY5Y (156 bp)

3.3 Immunocytochemistry analysis

In order to observe the localization of Oct-4, NS and β 3-tubulin immunocytochemistry experiment was carried out. Results demonstrated that Oct-4 and NS expressions localize in the to the nuclei of the cells (Figure 3.4). While around 0.1% of the cells expressed Oct-4 protein, 95-99% of the cells expressed NS protein. Localization of β 3-tubulin was shown in the cytoplasm of the cells.

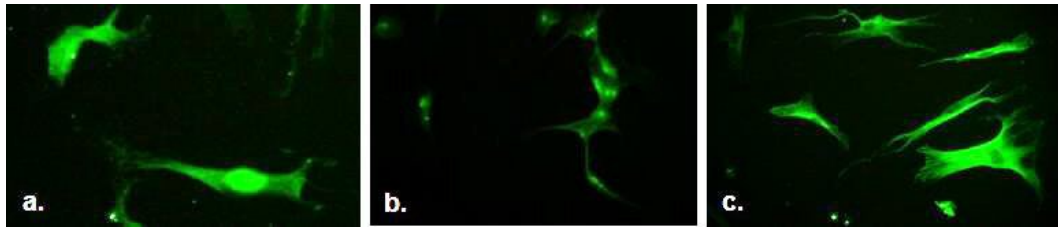


Figure 3.4: Expression of (a) Oct-4 (b) NS and (c) B-3 tubulin.

In order to demonstrate osteogenic differentiation, HDFCs were analyzed for Collagen type I expression. Results revealed that HDFCs expressed Collagen type I. (Figure 3.5)

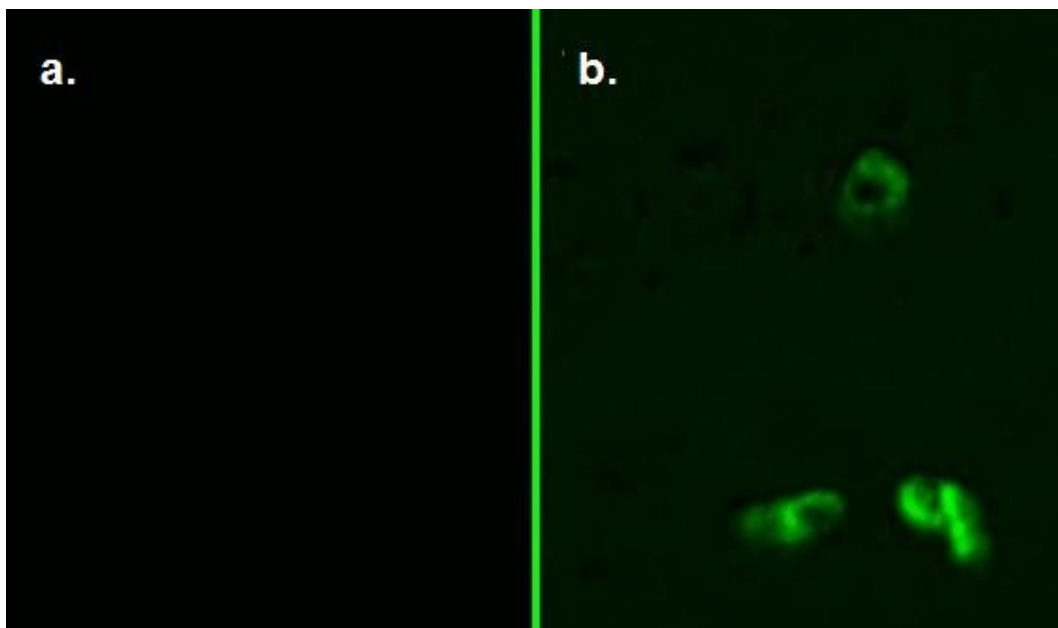
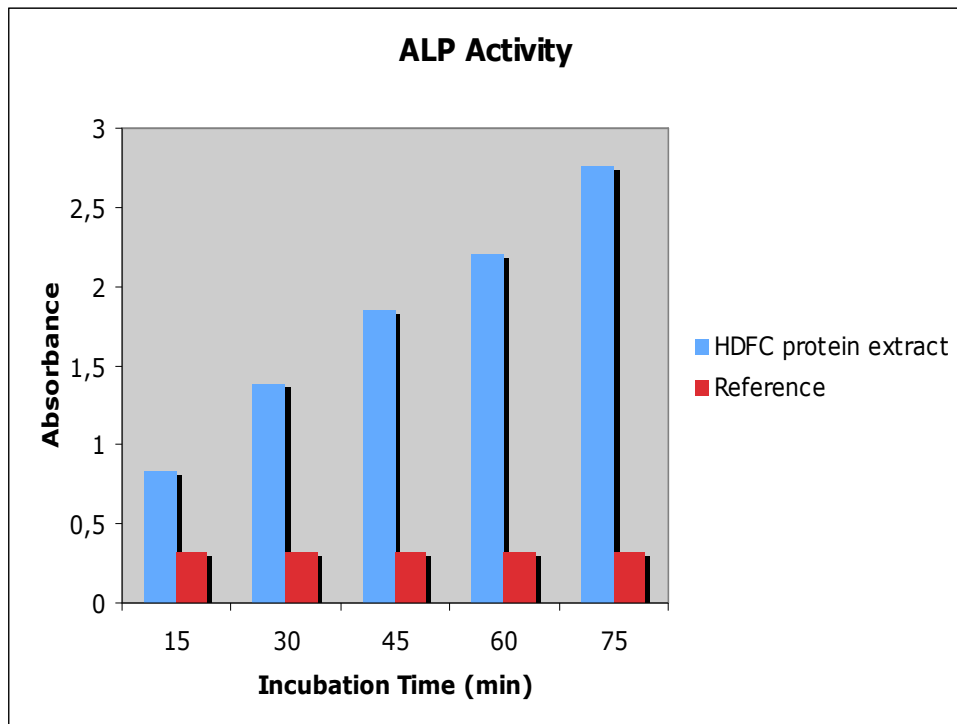


Figure 3.5. Collagen type I immunocytochemistry staining of HDFCs (a) before osteogenic differentiation, (b) after osteogenic differentiation.

3.4 ALP Activity

ALP activity of HDFCs was determined by measuring the absorbance with an ELISA plate reader (Figure 3.6).



Figure

3.6: ALP activity of HDFCs: ALP activity was detected in the protein extracts derived from HDFCs. Graph shows that while absorbance of wells including protein extract of HDFCs increases by time due to activity of ALP, absorbance in reference wells remains unchanged.

3.5 Von Kossa staining

After culturing HDFCs with osteogenic induction medium for 8 days, most of the cells showed the aggregates or nodules of calcium mineralization in the culture by von Kossa Staining. On the other hand HDFCs cultured in growth medium in this period did not show calcium mineralization (Figure 3.7).

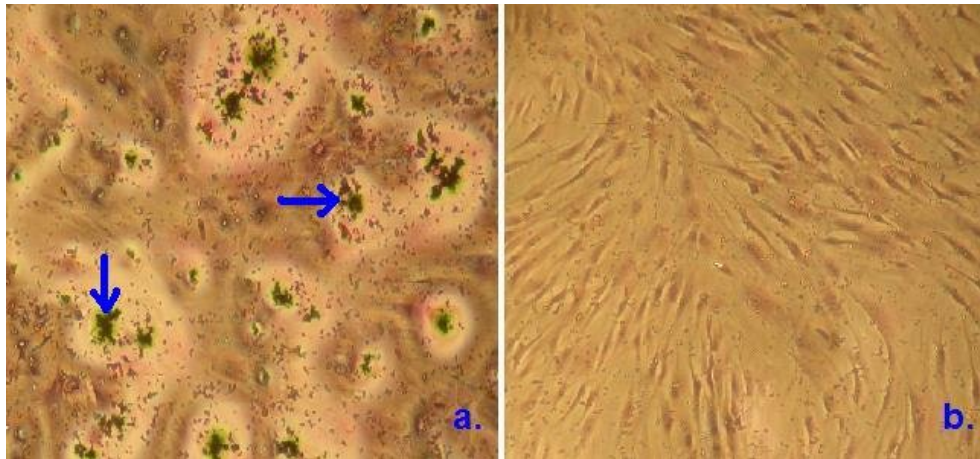


Figure 3.7 Osteogenic differentiation was demonstrated by von Kossa staining. **(a)** HDFCs cultured in osteogenic medium, **(b)** HDFCs cultured in growth medium. Blue arrows indicate aggregates of calcium mineralization.

3.6 Neurogenic differentiation

To induce neural differentiation, HDFCs and SH-SY5Y cells were cultured in neurogenic medium including retinoic acid and bFGF at the first step and NGF at the second step. During 3- day incubation, cells changed their morphology producing neurites (axon-like extensions) (Figure 3.8). Induced HDFCs exhibited positive immunocytochemistry staining for neuron specific proteins, Trk-A, β -3 Tubulin and NFM (Figure 3.9).

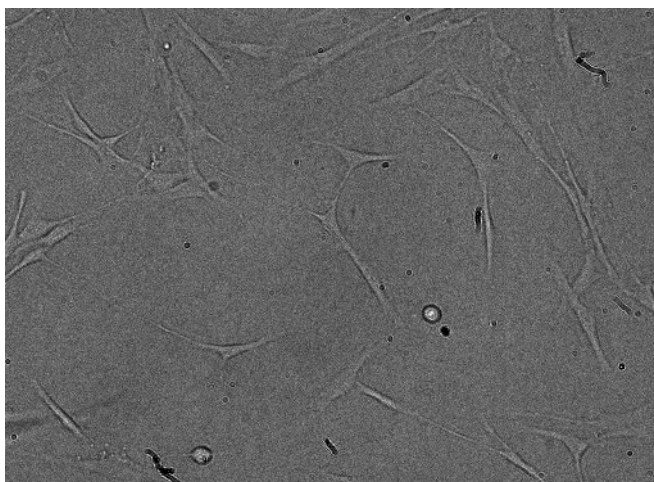


Figure 3.8. Neuron-like cells with long axon-like structure

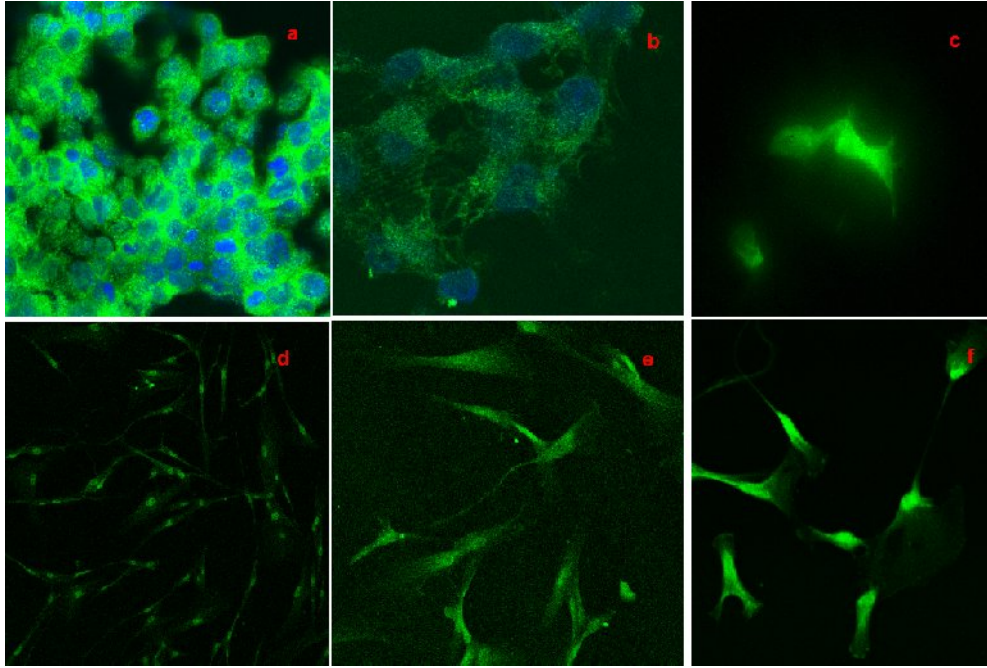


Figure 3.9: Immunocytochemistry staining of Neuron-like cell. Expression of neurogenic markers after differentiation. SH-SY5Y cells were positively stained with (a) Trk-A, (b) NFM, and (c) β -3 Tubulin; HDFCs were positively stained with (d) Trk-A (e) NFM, and (f) β -3 Tubulin

4. DISCUSSION

It has been reported that adult stem cells are not only found in bone marrow but also in a variety of tissues such as liver, nerve, muscle, skin, synovium, and cartilage (Spradling et. al. 2001; Gage, 2000). Bone marrow is one of the best potential sources of the mesenchymal stem cells (MSCs) and it is currently most preferred source to get large quantities of these stem cells. Despite this reputation, a surgical stress exists during isolation of stem cells from bone marrow. During the isolation of adult stem cells, reducing the surgical stress can be advantageous (Kose et. al.2003). Recent studies showed that extracted non-decayed impacted third molars, which are usually discarded, might be a very valuable source of these stem cells. Because dental follicles are being removed during standard prophylactic procedure and do not require additional surgical procedure. Utilization of such biological waste for the purpose of isolation of pluripotent stem cells present viable alternative to other sources. Isolation of MSCs-like stem cells from dental follicle of impacted third molars was successfully performed by different groups (Morsczech et. al. 2005). Stem cells isolated from the extracted third molars showed *in vitro/in vivo* osteogenic differentiation (Morsczech et. al. 2005). Investigation of pluripotency potential of dental follicle cells isolated from impacted third molars requires analysis of Oct-4 expression. NS expression indicates that HDFCs are actively dividing and proliferating cells.

In this research, HDFCs were successfully isolated from non-decayed impacted third molar of a 17 years old patient. There are several advantages of isolating HDFCs. First, it does not interfere with patient's orthodontic treatment. Secondly, it does not raise any ethical issues that are associated with human embryonic stem cell research. Thirdly, it is a potential source of MSCs which might be used in autologous transplantation without inducing tissue rejection.

Our results are consistent with the recent reports which demonstrated the presence of undifferentiated multipotent stem cells in dental follicles (Yao et. al. 2004). There

are also evidences that Oct-4 is expressed by cranio facial stem cells such as dental pulp, suggesting that there might be pluripotent stem cells in dental tissues (Huang, et. al. 2008). So far, there has been no report showing expression of Oct-4 and NS in dental follicle derived cells. To investigate if there are Oct-4 and NS expressing cells in HDFCs, we performed RT-PCR and immunocytochemistry analysis.

RT-PCR results showed that Oct-4 and NS mRNAs were expressed by HDFCs. Immunocytochemistry analysis also showed the existence and localizations of these two proteins in the nucleus. The number of Oct-4 expressing cell is very important in order to understand exact pluripotent stem cell capacity of HDFCs. During the analysis, the number of Oct-4 positive cells was less than 1% suggesting that it might be very difficult to detect and isolate Oct-4 expressing pluripotent stem cells from dental follicles. It has been reported that during the cultivation, spontaneously differentiating cells, by secreting some cytokines, cause the reduction of pluripotency of the stem cells in the population. pluripotent stem cells loose their pluripotency (Fauza, 2004). Therefore if the pluripotent cells are not isolated from the rest of the cells in very early passages, they might gain the characteristics of general population in the cell culture. Surface antigens can be utilized in this case. For example, c-kit (CD117, stem cell factor receptor) expressing cells were isolated from amniotic fluid samples, which contains 0-1% pluripotent stem cells (Fauza, 2004), and can cultivated in different culture conditions. These isolated pluripotent stem cells differentiated into all cell types coming from three embryonic germ layers (ectoderm, mesoderm and endoderm) (De Coppi et. al. 2007). Therefore, small number of Oct-4 expressing cells in HDFCs might be isolated by utilizing their surface antigens such as c-kit in magnetic separation systems. On the other hand, the majority of cells (90-95%) expressed NS proteins like stromal stem cells in human bone marrow (Kafienah, 2006). This means that although most of the cells are proliferative and actively dividing, majority of them are not pluripotent. Isolation of these rare pluripotent stem cells and their enrichment might provide researchers with an alternative source of pluripotent stem cells for tissue engineering and cell therapy applications.

There has been increased interest in studies of reprogramming the somatic cells to yield pluripotent stem cells received attention. By transfecting somatic cells with transcription factors, Oct-4, Sox2, cMyc, and Klf4, the mouse cells were

successfully reprogrammed producing induced pluripotent stem cells (iPS) without going through a nuclear transfer into the eggs (Takahashi et. al. 2006) (Figure 4.1)

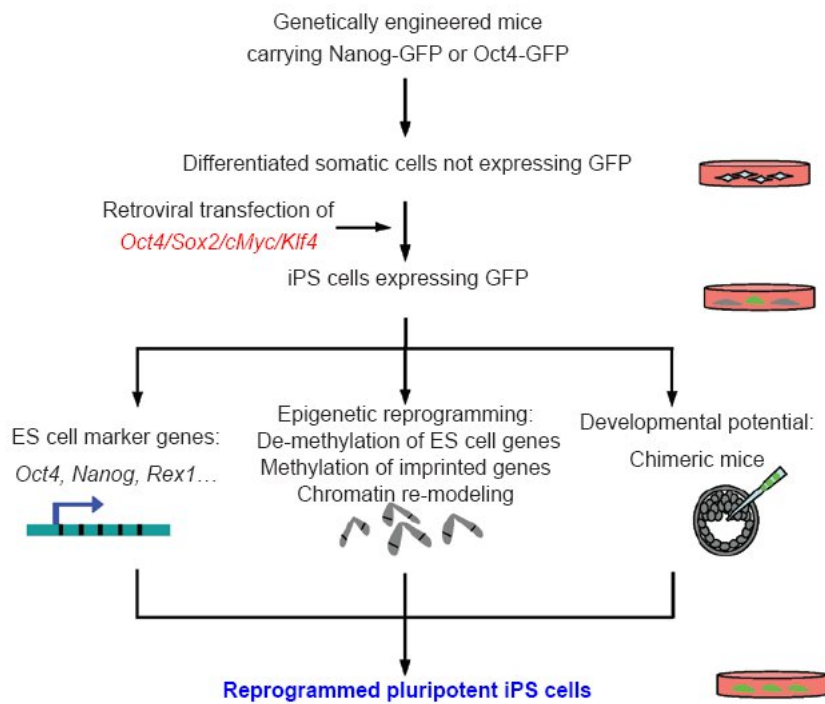


Figure 4.1: Generation of iPS cells

Application of this process in human therapy seems very exciting but there are still many questions to be answered. A major question is: Are these cells really exact counterparts of normal pluripotent stem cells? Usage of Oct-4 expressing pluripotent stem cells that already exist in adult tissues might be a safer strategy than generating iPS cells using extensive genetic manipulations.

Telomerase is required for the maintenance of stable telomere length in replicating cells (Greider et.al.1989). In this study, HDFCs showed telomerase activity suggesting that they protect their telomeres as they undergo cell division (Figure 3.2). ALP expression is also found in undifferentiated stem cells (Prusa et. al. 2003). During osteogenic differentiation, while ALP expression decreases, Osteocalcin expression elevates (Park et.al., 2007). HDFCs showed ALP activity (Figure3.6) but did not express osteocalcin before osteogenic induction. These data indicate that stem cells isolated from HDFCs are undifferentiated.

Previous studies showed that HDFCs have capacity to differentiate into osteocytes (Lindroos et. al., 2008). In this study it was also demonstrated HDFCs isolated from non decayed impacted third molar are able to differentiate into osteocytes. After osteogenic induction, HDFCs formed aggregates of calcium mineralization and expressed bone specific collagen type I (Figure 3.5 and Figure 3.7). This data proved that multipotent stem cells were successfully isolated from HDFCs.

Many studies investigated for a long time if adult stem cells can transdifferentiate, e.g. if a hematopoietic stem cell give rise to a muscle cell or a neuron instead of blastocytes or leukocytes. So far, there is promising evidence that some of the adult stem cells have transdifferentiation potential (Ho et al. 2005). Neurotrophic factors regulate survival, differentiation, growth and plasticity in the nervous system. Neurotrophic factors are expressed in developing dental tissues (Yamazaki et. al. 2007). Most recently, It has been reported that neuron like cells can be isolated from peridontium (Widera et. al. 2007). Also precursor cells derived from human dental follicle express neurofilament, map2 and β -3 tubulin expressions (Völlner et. al. 2007). Our results are consistent with previous data suggesting that HDFCs can differentiate into neurons. In this study a retinoic acid mediated neurogenic differentiation model were suggested for HDFCs. In this model, SH-SY5Y cells were used as positive control as they can show neurogenic differentiation in the presence of retinoic acid. Retinoic acid causes the cells to stop dividing and express Trk receptor which recognize NGF and Neurotrophin molecules (Kou et al. 2008). In this neurogenic differentiation experiments it was found that Trk-A and NFM proteins are expressed by both HDFCs and SH-SY5Y cells upon induction with Retinoic acid and NGF (Figure 3.9). After three days of incubation, NFM expressing cells were identified in both cell cultures by immunocytochemistry. RT-PCR data also showed that differentiated cells expressed NFM mRNA (Figure 3.3). Data shown in this experiment supports the evidences that HDF derived stem cells might be a readily accessible autologous stem cell source for therapeutic research in neuroscience.

5. CONCLUSION

In recent years, many technical and ethical problems in the clinical applications of ESC forced the scientists for searching new, novel and/or alternative adult stem sources and their characterization. Human dental follicle is one of this highly proliferative stem cell sources and It is easy obtain as a product of extracted teeth (Morsczeck et. al., 2005). Especially non-decayed impacted third molars contain stem cells without any infection. So far, the studies characterizing HDFCs have revealed that important marker proteins such as ALP, Vimentin, and Nestin are expressed by HDFCs. However, there was no study analyzing expression of Oct-4 and NS, which are markers of pluripotent stem cells, in HDFCs. By performing both RT-PCR experiment and immunocytochemistry it was proved that proved that these markers are being expressed together in a very small population of cells. As Oct-4 is a key regulator of differentiation, this small population of cells is of great significance. Isolation and enrichment of Oct-4 expressing pluripotent stem cells from HDFCs might provide us stem cells source having capacity to differentiate into all three embryonic germ layer cells. Full characterization of HDFCs will enable us an alternative source to bone marrow in autologous transplantations.

In consistent with the previous data suggesting neurogenic potential of HDFCs, it was shown in this study that HDFCs are expressing some early neural markers such as Nestin and β 3-Tubulin. In addition to this, it was also tested if a retinoic acid mediated neurogenesis might be triggered in HDFCs. Eventually, it was obtained that in the presence of retinoic acid and NGF, HDFCs gave rise to neuron-like cells expressing some mature neuron markers. Depending on these studies it can be hypothesized that HDFCs derived from human wisdom teeth represent a rational source for stem cells capable of differentiation into osteocytes and neurons.

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APPENDICES

Appendix 1. Preparation of 2% Paraformaldehyde

Preparation of a paraformaldehyde fixative solution

Reagent grade paraformaldehyde powder (Sigma). For 10ml of a 4% paraformaldehyde stock solution:

1. Weigh out 0.4 g of paraformaldehyde, transfer powder into 15 ml conical tube (Falcon).
2. Add 8 ml of ddH₂O
3. Add 5 µl of 1M NaOH
4. Heat to 70°C (cap on), mix frequently until complete solubilization (-10 min at 70°C; the solution should become clear)
5. Put on ice and allow the solution to cool down to room temperature
6. Adjust volume to 9 ml with ddH₂O
7. Add 1 ml of PBS 10X and mix.
8. Filter solution using a 0.2 µm 25 mm nylon syringe filter.
9. Prepare a 2% paraformaldehyde solution by diluting 4% paraformaldehyde solution in PBS.

Use the fixative at room temperature

Appendix 2. Preparation of Mowiol Mounting medium

Mowiol is a brand-name of polyvinyl alcohol solution. It hardens to match the refractive index of immersion oil, so it has gained a reputation for being a good mounting medium to use on fixed cells that are to be viewed with epifluorescence

or Confocal microscopes. Most users add an additional antifade reagent to retard photobleaching.

Mowiol 4-88 can be purchased from EMD Biosciences and Polysciences. There are many recipes for Mowiol mountants out there—the one below is from the Research Technologies Branch of the NIAID/NIH.

1. Put 6 g of glycerol in a plastic 50 mL centrifuge tube, along with a small stir bar.
2. Add 2.4 g Mowiol 4-88; stir to mix.
3. While stirring, add 6 mL distilled water, leave stirring at room temperature for 2 hours.
4. Add 12 mL 0.2 M Tris, pH 8.5. If desired, add sodium azide to a final concentration of 0.02%.
5. Incubate the tube in hot water (50-60°C) for 10 minutes to dissolve the Mowiol.
6. Add anti-fade reagent (optional).
7. Centrifuge at 5000 g for 15 minutes to remove any undissolved solids.
8. Store 1 mL aliquots in Eppendorf tubes at -20°C.
9. To use, warm to room temperature. Opened tubes can be stored at 4°C for approximately 1 month. Discard if you see crystalline material in the tube or on your slides.

You should only use about 15-20 µl of this for 22 x 22 mm coverslip, or 40-50 µl for a 22 x 50 mm coverslip. Leave your coverslipped slides in the dark overnight to harden before using an oil or water immersion microscope objective.

CURRICULUM VITAE

Mehmet Emir Yalvaç was born in Erzurum, in 1981. He entered Atatürk, Department of Biology Education in 2000. He graduated from this department as a top ranked student and was awarded with B.Sc. and M.Sc. without thesis. Since 2005, he has been employed in Yeditepe University, Department of Genetics and Bioengineering as a research assistant. In 2006, He started his Master of Science degree in Molecular Biology and Genetics Department at Istanbul Technical University. So far, he has seven abstracts published in national and international symposium books.