

## Investigating Differentiation Ability of Induced Pluripotent Stem cell (ips) & Stromal Endometrial (Ens) toward Pre\_Oligodendrocyte Cells, by the Means of Growth Factors in Vitro

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Oligodendrocytes are types of cells in central neural system (CNS). Their main function is protection of Axons and generation of Myelin sheath in CNS, this sheath insulates the Axons. Any disorder in the function of these cells leads to demyelination of neurons and causes neural disorders including multiple sclerosis (MS). Nowadays, cell therapy provides plenty of hope for cure of MS. So far it has used different sources such as stem cells or progenitor for cell therapy of neural system. But each of them had some limitations, for instance using neural stem cells requires certain amount of CNS tissue. Embryonic stem cells also introduced as another candidate for cell therapy but due to some moral problems, such as necessity to creating a Blastocyst, using these cells accompanied many limitations. In cell therapy, the most important factor is facility to acquiring stem cells. IPS cells are kinds of Induced Pluripotent Stem cells which directly created by transferring of 4 copying factors: oct<sub>4</sub>, sox<sub>2</sub>, klf<sub>2</sub> and c\_myc into the differentiated cells. IPS cells are like pluripotent embryonic stem cells although they don't require demolition of Blastocyte. Endometrial Stromal cells are kinds of mesenchyme or adult cells which have proven in human and mouse's womb and also access to them is easy. Both of these types of cells can be good candidates for cell therapy. In this research we use these two types of cells for differentiate to Oligodendrocytes and we are able to differentiate IPS cells which are from human's eye and also human Stromal Endometrial cells to pre\_Oligodendrocytes. Also we can compare their differentiation ability. These cells can be used for transplanting in MS patients.

**Keywords:** cell therapy, pre\_oligodendrocyte cells, Stem cells, IPS, stromal endometrial cells.

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MS disease is a neural chronic and inflammable disease which causes disorders in function of central neural system. In this disease, Oligodendrocyte cells which myelinate the nerve fibers in neural system affected with defection in their function. Today, the main reason of MS disease is unknown; therefore there is no affective remedy. Up to now drug therapy has been the

main remedy for this disease, but one of the main problems in cure of this disease is temporary remedy of the symptoms and only preventing the progress of the disease. One of the solutions for cure of MS disease is cell transplant and replacement of active Oligodendrocyte in the place of the defective one in the patients. The positive results of cell\_replacement remedies in transplant of immature neural cells in MS disease and in empirical animal models are reported (Uccelli *et al.* 2010). Limitation in access to the early Embryonic cells, in accompany culturing

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and screening of progenitor cells in adult's brain, which can differentiate neurons and Glia, resulting pay more attention to finding a better cell source, for vast function in cell therapy CNS. It has showed that different stem cells such as mesenchyme stem cell (MSC), Hematopoietic stem cell (HSC), adult's multipotent progenitor stem cell (MAPC), Umbilical Cord Blood Stem Cell (UCBSC), Embryonic Stem Cell (ESC) can differentiate to neuron cells (Daily *et al.* 2005), however these cells encounter to limitations because of unavailability and extract methods and in some cases low proliferation. Elder people's mesenchyme stem cells do not have a good function in culture because of the limit capacity (Chow *et al.* 2009). Therefore in this way our goal in the current study is consideration on differentiation ability of induced pluripotent stem cell (ips) and Stromal Endometrial (Ens) toward pre\_Oligodendrocyte cells, by the means of growth factors in vitro.

#### **Cure by replacing the cell**

Cell therapy means reversion of the missed functions of a cell by replacing a healthy cell in the place patient one. For doing this we need stem cells that in culture (in vitro) and in exposure of certain factors we can differentiate them to the special cells. In cell therapy there are two views: one of them is use adult stem cells from the same tissue, the other one is use Embryonic stem cells (ESC), but using of both of these views has special problems (Liao *et al.* 2009). In general a stem cell is a kind of cell that has the ability of self-renewal and also can produce other types of cells. Self\_renewal means proliferation and protection of genesis ability for differentiation to other types of cells. Stem cells are in different stages of genesis, so show different degrees of self-renewal and differentiation (Baharvand 1387). Stem cells have the potential for conveying to other types of the differentiated cells and act as a corrective system for the body, they can divide indefinitely until they can fill in the place of injured cells when the creature is still alive (Mattahai *et al.*, 2009). Stem cells have two important features which make discriminable them from other cells:

- 1) Ability to dividing and producing cells with the same features (self-renewal).
- 2) Creation of types of the differentiated cells (Colter *et al.* 2001).

Base on the differentiation ability and

plasticity of the stem cells, it can divide them to the following items:

#### **Totipotent stem cells**

The obtained cells from combination of ovum with spermatozoon and the derived cells after the first dividing of sperm, are fecundated and can make all of the cells such as person's cells and placental expulsion, like Blastomers of a two\_cell embryo that each cell of it can make a complete person.

#### **Pluripotent stem cells**

The stem cells are weaker than the totipotent stem cells and they can differentiate to the derived cells from every embryonic 3 layers and they have the ability to make the most or all of the person's cells, but they cannot make placental expulsion. The cells which gained from fetus gonads and they are called embryonic germ cells and the Embryonic Carcinoma cells: ECC are also pluripotent cells.

#### **Multipotent stem cells**

They create more limited types of cells (like stem cells that exist in adult tissues) these cells can differentiate to some of the cellular lineages which are related to a cellular level.

For instance: hematopoietic stem cell which differentiate to red blood cells, white blood cells and plaquettes (Katler *et al.* 2001, & Winston, 2001).

#### **Using the stem cells in cell therapy**

In cell therapy, one view is use embryonic stem cells; these cells have two clear characteristics: (Baharvandi, 1387):

- 1) Self\_renewal
- 2) These cells in vitro, in exposure of different factors can differentiate to all types of cell in body.

Therefore ICM cells and primordial germ cells are the best source for embryonic stem cells so embryonic stem cells are introduced as good candidates for cell therapy (Glaser *et al.*, 2004). However these cells have plenty of potential, but moral problems that are related to embryonic stem cells consider as a limitation (Chang *et al.*, 2007). So because of these moral problems, getting embryonic stem cells by Cloning therapy method creates limitations then scientists use the other way for obtaining these cells.

#### **Induced pluripotent stem cells (iPSC)**

Because of the Cloning problems, scientists were trying to activate the genes which

were in the early embryonic stages and express them. With progress in gene therapy methods, at first they suggested that the genes which express in the early stages of embryonic in Blastomers and make pluripotent feature in them, enter to a genome of a complete specialized cell by a series of special vectors until it show that whether these cells can reverse to pluripotent status or not. In 2006, Yamanaka and his colleagues analyze and compare the expression of genes in two fibroblastuses cell and embryonic stem cell of mouse, and they introduced 24 candidates of genes for transforming by vector, they consist of:

Ecat 1, Dpp5(Esg1), Fbx015, Nanog, Eras, Dnmt31, Ecat8, Gdf3, Sox15, Dppa 2, Fthl17, Sall4, Oct4, Sox2, Rex1, Utl1, Tcl 1, Dppa3, Klf4, b\_cat, cMyc, Stat3, Grb2.

Yamanaka and his colleagues could successfully enter these 24 factors into fibroblastus cells of mouse by retroviruses, after passing of two weeks they observed that these cells conveyed to pluripotent cells which they were similar to embryonic stem cells (ESC) in the way of morphology and growth features, and also they expressed the ES cells marker. These cells are called Induced Pluripotent stem cells or iPS cells. This group of researchers by decreasing these genes in the transfer process finally realized that 4 copying factors, include: oct4, c\_Myc, Klf2, sox2 are necessary for converting fibroblastus cells to IPSC. Then these researchers inject the iPS cells into a Blastocyst and they observed that these cells contribute in genesis of the fetus. By hypodermic injecting iPSC to the nude mice, it was seen that a cancerous gland in the existed tissues by each of 3 progenitive layers is created (figure 1\_1). This wonderful experiment without any moral problems and limitations in cellular source created a new point of view of reprogramming. Somatic cells and pluripotent stem cells in all of cellular populations in morphology, gene expression and functional activities are different, so it is wonderful that just expression of four or even expression of three genes result in this great structure change. It is obvious that all of these copying factors have specialized and important roles in reprogramming of destination of the differentiated cells (Takahashi *et al.* 2006). Ips cells similar to embryonic stem cells have all of the pluripotent features and ability

of self\_renewal and differentiate to different cellular levels stem cells. Using these cells are object point in this way that with availability of the patient's adult cells, by creating genetic changes in them can get stem cells and by differentiating of these cells we can use them for cell therapy for the same patient (Soldner *et al.* 2009), furthermore in joining of these cells will not exist some of the immune problems that are result of the incongruent of proteins on the cell surface and this is considered as a new source of the stem cells. These cells can create new hopes in the cell therapy (Ran *et al.*, 2009).

#### Adult stem cells

Adult stem cells are multipotent cells that have more limited differentiation ability than embryonic stem cells and they just can differentiate to more limited cell types. These cells are in many of the specialized tissues of the body such as: brain, marrow, liver, skin, alimentary canal, eye cornea and retina and even Destin pulp (Smith, 2001). In adults when there is injury or there isn't, these cells are active in a constant manner. Before this scientists thought the adult's stem cells, create just the cells of its own, but today they believe that these cells plasticity is very much and they can make another types of cells. For example adults' stem cells that are derived from marrow, in addition to making immune and blood cells, in trans differentiation way they can make another types of cells such as muscular cells, microglia and Astroglia in brain and liver hepatocytes (Herman *et al.*, 2004). These cells separated from different tissues and their differentiating potential may be reflector of their local place, they are lack of special features like ESCs, but in effect of good signals they can differentiate to specialized cells with a differentiated phenotype of early progenitor. Base on the observations it has suggested that maybe these cells can be usable in transplant medicine (Pittenger *et al.*, 1999). In general using adult stem cells in cell therapy with the same tissue encounters to limitation in two aspects:

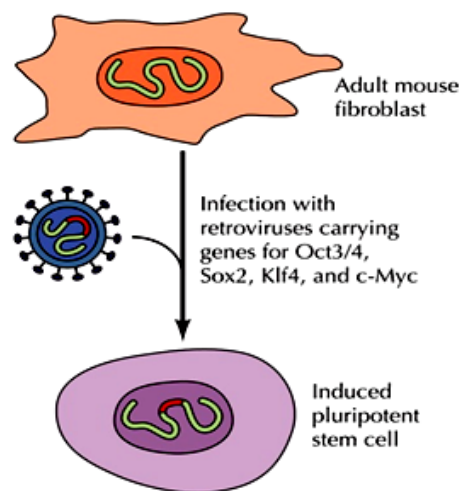
- 1) The source of these cells is limited.
- 2) These cells have less ability for proliferating in vitro (Liao *et al.*, 2008).

Recently some rare cells with the same activity of adult stem cells which attract scientists' attention for using in cell therapy has discovered in human's womb endometrium.

### Mesenchyme stem cells or endometrial stromal (EnSCs) or (eMSCs)

Human endometrium or mucous coat of womb has the prominent ability of restoration in woman's life, and it can go from thickness of 1\_0/5mm to 7\_5 mm after the menstruation (Mac *et al.* 1965). Endometrium has the cycling progress of proliferating, differentiation and finally stoppage of activity in menstrual cycle. The growth of functional endometr in proliferating phase happens in response to increasing of estrogen. Increasing of progesterone in secretory phase causes blockage of epithelial mitosis and start of differentiation of cells. In a situation which nidation of embryo does not happen, metrorrhagia happens and then a new cycle begins, the regeneration happens on the endometr surface during the metrorrhagia and it completes 24 hours after it (Frenzy *et al.* 1979). The continuous growth of endometr provides the possibility of pregnancy in people. Since endometrium is a kind of few tissues that exists in an adult which have defluxion and new growing cycle ,existence of stem cells in it and ability of angiogenesis was expressed as a hypothesis since previous years. Recently existence of stromal and epithelial stem cell populations is identified. The presence of stem cells in basal layer of endometrium was introduced since previous years (Report, 2004). Recently, mesenchyme stem cells by simultaneous expressing of two markers PDGFR\_â and CD<sub>146</sub>

has separated from human endometrium. These cells showed their ability in differentiating to Adipocyte, Myoblast, Chondrocyte, Osteoblast in appropriate differentiating places (Schap *et al.*, 2007) . The study showed that endometrial stem cells population contains mesenchyme stem cells that are similar to marrow and tooth pulp. Up to now different markers for identifying of endometrial stem cells are used. The oct<sub>4</sub> that is a basic factor for plasticity of stem cells has identified by Immunohistochemistry in human endometrium. The CD<sub>117</sub> which is stem cells marker expresses in separated stem cells from menstruation blood. The CD<sub>90</sub> is one of the phenotypic markers of mesenchemal stem cells which in accompanying by CD<sub>29</sub> and CD<sub>105</sub> are used for identifying mesenchyme stem cells from different tissues and endometrium (Tessie *et al* 2004). Also being enriched of endometrial stem cells from CD<sub>146</sub> shows it's similarity to tooth pulp and marrow stem cells. The endometrial stem cells have showed that they have the ability of angiogenesis. As well as, study on differentiation ability of these cells has showed these cells can differentiate to the cells of every three layers of Endoderm (Hepatocyte), Mesoderm (osteocyte, edipocyte, cardiomyocyte) and Ectoderm (nerve). They are appropriate to replacing with cell therapy. Likewise the researches indicate that these cells after 34 passages still they have a normal Karyotype, proliferation speed of



THE CELL 5e, Figure 17.27

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**Fig. 1.** Schematic figure of producing of Ips cells from mouse fibroblast cells, by transferring 4 factors c\_Myc, Klf2, oct4, sox2, by the means of retrovirus systems

these cells is higher than marrow stem cells (Schap *et al.*, 2005) and most prominent benefit of these cells in proportion to other stem cells is easy access to them.

### Pre Oligodendrocyte cells

Oligodendrocyte progenitors (Ops) after moving in Mammalia CNS put in range with fiber parts of the next white substance then change their forms to pre oligodendrocyte cells. Pre oligodendrocyte cells are oligodendrocyte progenitors which are multiprocess and preserve cell division features, have fewer movements and identify by Monoclonal antibody O4. After moving and missing the power of division they alter to immature Oligodendrocyte and finally they differentiate to adult Myelination Oligodendrocytes which express the MAG, MBP and also PLP (Bioman *et al.* 2005). The production of Oligodendrocyte progenitor cells (OPCs) by the neural stem cells (NSCs) of embryonic periods or Neuroepithelial cells, neonatal periods or neural progenitor and also adulthood periods (cell type B) is a result from complex interaction of induce

factors and copying organ in stem cells (Shear *et al.*, 2008).

### Research method

The methods in this research contain experiments for differentiation of two types of stem cells, HENS cells and IPS cells.

The culture of DMEM/F12, HIPS contains 10 % SR (Serum Replacement) and 10% FBS. This place contains pen/strep with final thickness 50µg/ml, unnecessary amino acids with final thickness 100µM and L Glutamine with final thickness 100mM and Beta mercaptoanol with final thickness 100µM, and 10ng/ml (Baharvand, 1387). The culture of Endometrial Stromal cells and MEF DMEM/F12 have 10 % FBS and pen/strep. The culture of IPS cells was changed every day and the culture of MEF and HENS every 2 or 3 days. For using MEF as feeder cells of IPS cells, at first it is necessary to stop their proliferation because of metabolic activities, and protect the IPS cells but do not divide. In current study it is done by using Mitomycin C. According to this purpose, 100ul of Mitomycin C (1mg/ml) was dissolve in 10ml

**Table 1.** The method of CDNA

The volume	Substance name	Line
1µm	Oligo Dt primer or Random Hexamer(50um)	1
1µm	Dntps Mixture	2
Total RNA:<5µg	Template RNA	3
Poly(A) <sup>+</sup> RNA:<1µg Up to 10µl	RNase_free water	4

A\_ The method of template RNA primer Mixture with Prime Script 1<sup>st</sup> strand CDNA synthesis Kit.

**Table 2.** The method of reaction with Prime Script 1ST strands CDNA synthesis Kit

The volume	Substance name	Line
The volume	Substance name	Line
10µl	Template RNA primer	1
4µl	5x primer script Buffer	2
0.5µl	Rnase inhibitor	3
1µl	Primer scribe RTase	4
Up to 20µl	RNase_free water	5

B\_ The method of reaction with Prime Script 1ST strand CDNA synthesis Kit.

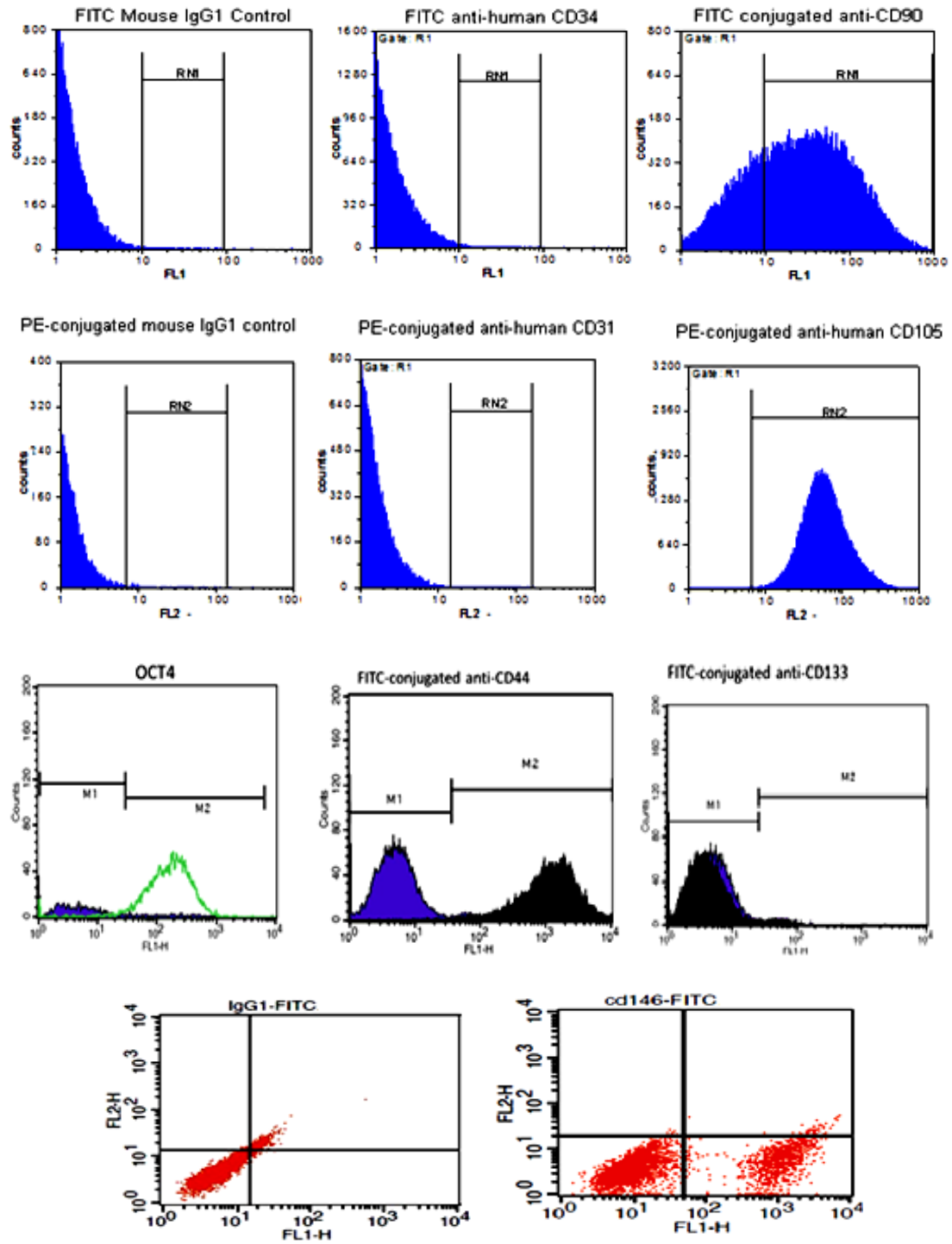


Fig. 2. Flow cytometric of expression of mesenchyme and non-mesenchyme in endometrial stem cells

culture that was without serum. The solution added to the flask contains MEF cells and we put the flask in incubator for 2\_3 hours, it leads to stopping of cell division. After 2 hours we discard the culture contains Mitomycin C and washed 3 times by PBS. After this phase we can cultivate the IPS cells on them.

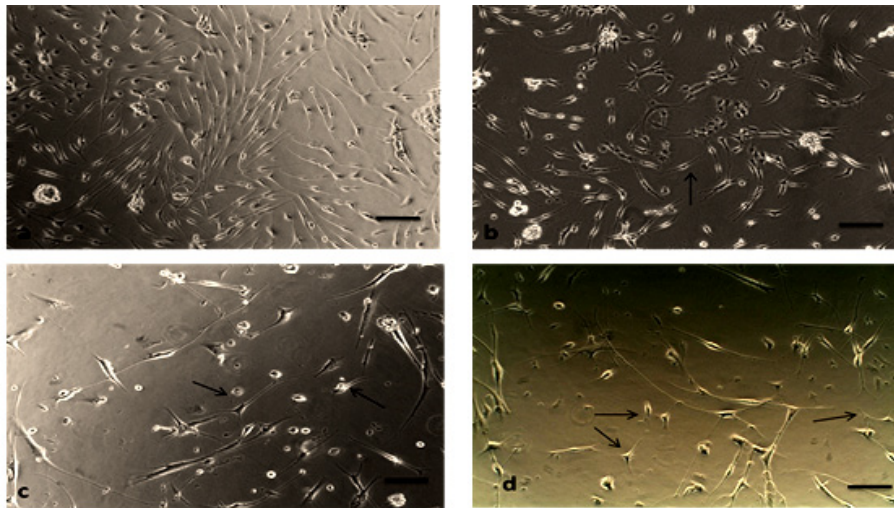
For each RNA sample, we made cDNA by profiting of Prime Script 1<sup>st</sup> strand CDNA synthesis kit (The used values were inserted in 6\_2

table). The reaction for the kit was 10 minutes in 30 degrees, then 30\_60 minutes in 42 degrees and finally 5 minutes in 95 degrees, respectively.

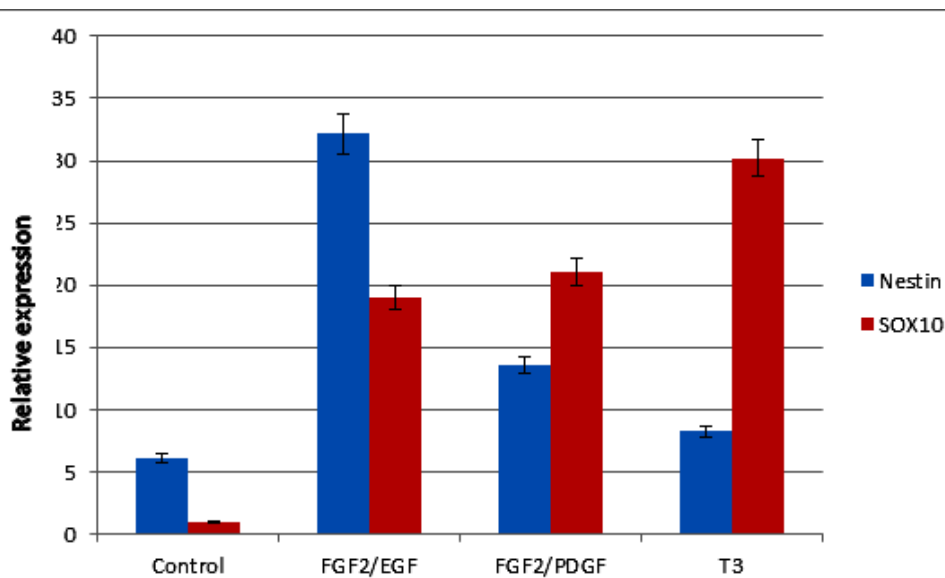
**RESULTS**

**The results of differentiation of hEnS cells**

The analyze of flow cytometric of construction in which Endometrial stem cells are positive for mesenchyme stem cells markers such



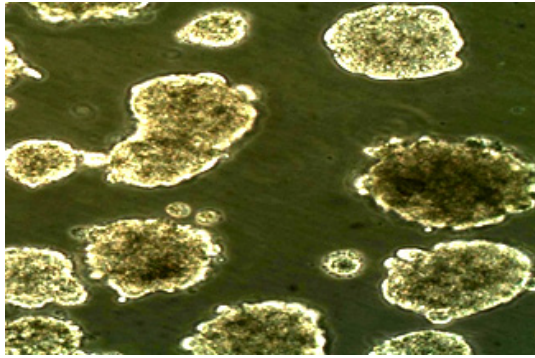
**Fig. 3.** The photomicrograph of differentiated stem cells to Oligodendrocyte in control environment (a) after adding FGF2/EGF, (b) after GF2/PDFG(c) after adding T3 (d) (Zoom ×100)



**Fig. 4.** The graph is formed by QRT\_PCR related to Oligodendrocyte \_ like cells of endometrial treatment cells in three phases with differentiation factors FGF2/EGF, FGF2/PDGF, and T3

as CD44,CD146,CD90,CD105 and pluripotent marker OCT4, but they are negative for Endometrial CD31 cells markers and Hemapoietic CD34 and CD133. The results are mentioned in Fig.\_2.

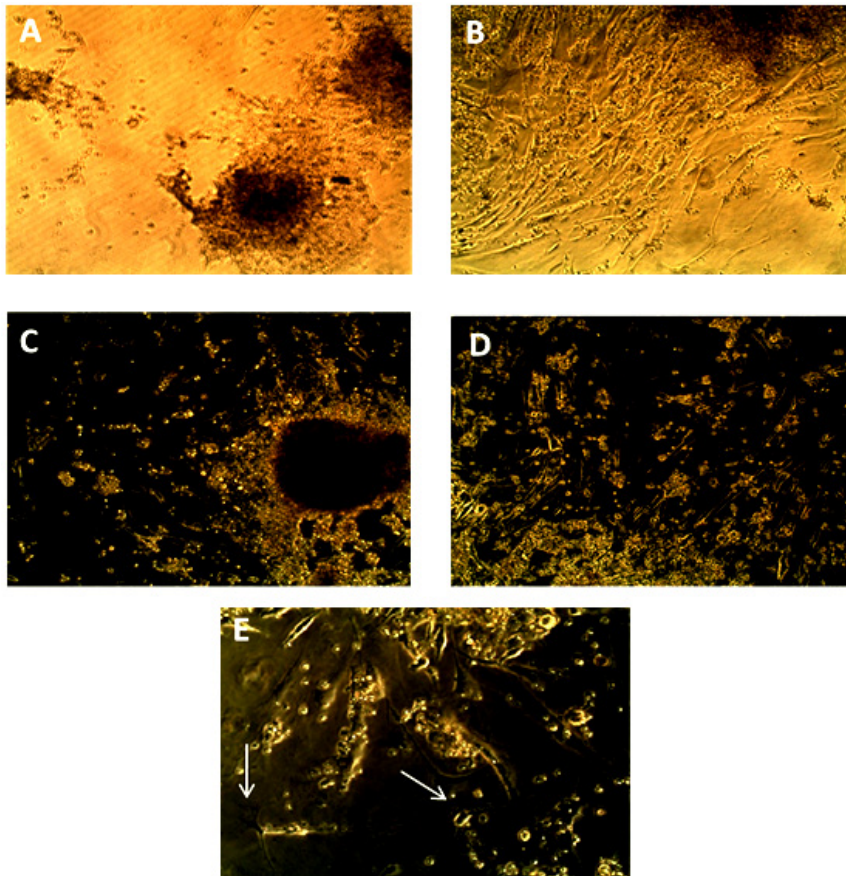
The results of differentiation to pre\_ Oligodendrocyts:



**Fig. 5.** Photomicrograph of embryoid bodies EB. (Zoom  $\times 100$ )

In current study, at the end of fourth, fifth and sixth phases differentiation of expression of markers in each phases are were considered by a qualitative technic Real time PCR.

It was expected to observing the progenitor neural cells markers (Nestin) in fourth phase and Oligodendrocyt marker SOX10 in fifth and sixth phases. In fourth, fifth, sixth phases the expression of specialized markers on the protein surface were considered by Immunocytochemistry method. The investigation of taken photos during the study indicated that after adding FGF2/EGF, morphology of treatment cells were starting to change step by step. The cell superfluities cleared and the cells began elongation. The longest state was seen after adding PDGF in treatment cells. The bipolar Oligodendrocyte\_ like cells began to be long, when the hormone T3 was added, some of them also appeared as multipolar. The control group cells were unchanged and these cells have kept the



**Fig. 6.** The photomicrograph of differentiated IPS cells to Oligodendrocyte in control environment (A) and after adding FGF2/EGF, (B) after FGF2/PDFG (C, D) and after adding T3, (E) (zoom $\times 100$ )



undifferentiated cells features (a lot of cytoplasm and superfluties). Accompanying Endometrial cells that differentiated to Oligodendrocyte\_like cells there were neural cells. Sometimes it is seen the scattered cells have contact by the means of the created superfluties in far distances, so that it is possible 4\_5 cells contact sequentially. The outcomes are mentioned in fig.3.

As well as the coloring by specialized neurons and Oligodendrocyte demonstrated these cells after treating with various growth factors, expressed neural marker Nestin and Oligodendrocyte A<sub>2</sub>B<sub>5</sub> and Olig2 markers.

#### Considering RT\_PCR

The PCR considerations are performed in three various stages: after adding FGF2/EGF and FGF2/PDGF and T3. The mRNAs consideration

results of neural markers and Oligodendrocyte include Nestin and sox10 indicated they were treated in cells with FGF2/EGF, Nestin was expressed in a high rate but in the next phases this rate reduces gradually. Sox10 that is an important copying factor for expressing Oligodendrocyte genes had a low expression rate but in progressing phases of differentiation, its expression rate raised. The outcomes are in fig.4.

#### The consequences from differentiation of hiPS cells to pre\_Oligodendrocyte cells

The hiPS cells are useful, pluripotent and feeder dependent cells which observe on feeder layer of MEF cells. At the end of the fifth, sixth and seventh phases, the expression of specialized markers were investigated on the surface of protein by Immunocytochemistry method.

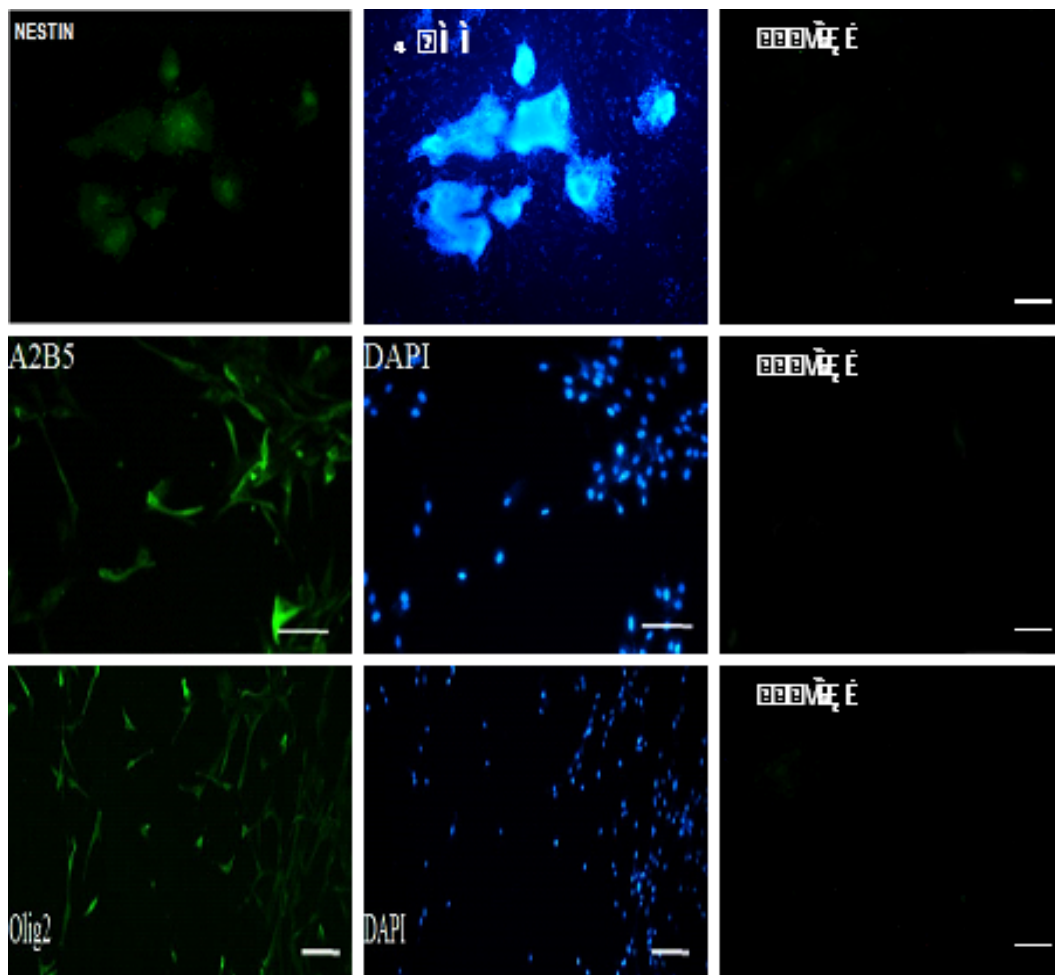


Fig. 7. Immunocytochemistry for progenitor neural markers (Nestin) and Oligodendrocyte(olig2) & (A2B5)

At the second differentiation phase, a stage was made for forming of embryoid bodies from hiPS cells in EB structures. The investigating of taken photos during the study indicated the IPS cells when lacks of BFGF were in Teflon dishes and in a place; they showed the ability of EB production. In the embryoid bodies, the desired features for differentiation of embryonic stem cells are spherical appearance and arranged margins, the EB structures had round and arranged margins (Fig.5).

After adding FGF2/EGF, the treatment EB morphologies began to change gradually. The EB cells started to separating, the superfluties cleared, and the cells began elongation. The longest state was seen after adding PDGF in treatment cells. The bipolar Oligodendrocyte\_like cells began to be long, when the hormone T3 was added, some of them also appeared as multipolar but the control group cells were unchanged. Accompanying IPS cells that differentiated to Oligodendrocyte\_like cells there were neural cells. Sometimes it is seen the scattered cells have contacts by the means of the created superfluties in far distances, so that it is possible 4\_5 cells contact sequentially. The outcomes are mentioned in fig.6.

The coloring by specialized markers of progenitor neural cells and Oligodendrocyte progenitors demonstrated these cells after treating with various growth factors, expressed progenitor neural marker Nestin and Oligodendrocyte A2B5 and Olig2 markers. The outcomes of Immunocytochemistry investigations are in fig.7.

In general in current study we used expressing investigation of genes in MRNA level and Real Time\_PCR method and neural markers of Immunocytochemistry and Oligodendrocyte for identifying the differentiation of neural progenitors and Oligodendrocyte. According to the performed investigations by the means of mentioned methods in these differentiated cells and comparing them to control group, we achieved this outcome that Endometrial stem cells have the ability of differentiating to Oligodendrocyte progenitors.

### CONCLUSION

The considering of the outcomes indicates that endometrial adult stem cells and induced pluripotent stem cells which are derived from

human's eye can differentiate to Oligodendrocyte progenitors when they put in the neural cultures accompanying growth factors. The hiPSC and hEnSC are appropriate replacements for restoring of neural system because they have important and potential advantages. The access to HENS cells is simpler and more available than other stem cells. Producing of IPS cells requires difficult stages like transferring of gen; unfortunately one of these genes is an oncogene that is called c\_Myc and cause cancer. Delivery method of gen is an important subject for producing of IPS cells. For being sure that pluripotent factor expressions are performed, the virus vectors should be used. It is necessary to silence the expression of transferred factors in following successful reprogramming. Since some of the required pluripotent factors are oncogics. The viruses that interpose the transferring of the gene are general methods for producing of IPS cells. Using them cause a problem because entrance of the viruses can break the genes in entrance place. In addition, the entrance of an active copying virus genome to adjacency of interior gene leads to activating of copying (and it is possible to silencing genes). This is a serious matter because the entrance activity has important results, for example retroviruses that interpose the entrance to Hematopoietic cells can increase the self\_renew in vitro which cause cancer in vivo. The EnS cells are available easily and do not need to gene transferring methods for production, they are not pluripotent like IPS cells and they have low differentiation ability. Although in cell therapy the main choice is access to these cells. One of the problems of EnS cells is their limited usage (only in women), this matter cause increase human need for using IPS cells in cell therapy.

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