# EMBRYONIC STEM CELLS DIFFERENTIATE INTO NEURAL-LIKE CELLS *IN VITRO* AND *IN VIVO* AFTER INTRACEREBRAL TRANSPLANTATION

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**Abstract**

**Background:** Pluripotent embryonic stem (ES) cells may provide a virtually unlimited donor source for transplantation, according to protocols that permit the *in vitro* generation of neural precursors from ES cells.

**Objectives:** To described conditions that induce differentiation of ES cells reliably and high efficiency into neural pathway.

**Materials & Methods:** Mouse ES cells obtained from cultured Blastocyst, were subcultured with addition of embryonic brain extract. The cells from these cultures as well as from cultured cells without addition of brain extract were transplanted into adult mice brain by injection using stereotaxic technique. Horse Raddish Peroxidase (HRP) was added to culture medium in the aim of labeling the transplanted stem cells, to enable the observation of transplanted location within the recipient brain.

**Results:** Results show that ES cells derived neural precursors have migrated for variable distances of different regions of the brain to replace damaged cells at the site of transplantation.

**Conclusion:** Thus, brain extract serves here as a source of neurotrophic factors for the induction of ES cells to neuronal pathway of differentiation, in which ES cell can serve as a valuable source of cell type-specific somatic precursors for neural transplantation. HRP histochemical marker was used for first time in culture medium for marking ES cells *in vitro* and *in vivo* to allow determination of the fate of the transplanted ES cells.

Key words: Embryonic stem (ES) cell/ neurogenesis/ neurotrophic factor / ES cell transplantation (cell transplant)

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## Introduction

Embryonic stem (ES) cells, derive from the inner cell mass (ICM) of blastocyst-stage embryos are pluripotent, undifferentiated, immortal cells, capable of differentiating into derivatives of all three embryonic germ layers[1,2]. ES cells can be maintained *in vitro* in the undifferentiated state for periods of several months without loss of their developmental capacity, by coculturing with mitotically inactivated feeder cells, such as embryonic fibroblast[3,4] or with the addition of a differentiation inhibiting activity, {Leukaemia inhibitory factor (LIF)}[5,6].

In the past fifteen years, pluripotential ES-cell lines have been used extensively as amodel system to study aspects of gene expression and early embryonic development[6-8]. But recently, these self-renewing pluripotent ES cells (after induced to differentiate *in vitro*) may considered the bases of new cell replacement therapies[9].

To initiate differentiation of ES cells into specialized population need to change the growth conditions of the ES cells, in specific ways, such as by adding growth factors to the culture medium Of ES cells aggregate and embryoid bodies[9], or changing the chemical composition of the surface on which ES cells are growing[10], or to introduce foreign genes into the cells via transfiecion or by using cloning technology[11-13] and this directed derivation of ES cells is then vital to the ultimate use of such cells in the development of new therapies (in cell transplantation)[14-17].

The present study deals with how to direct the differentiation of these ES cells *in vitro* into neural pathway to be used *in vivo*, by grafting and transplanting ES cells into the injured area of mouse brain tissue and by using Horse raddish peroxidase (HRP) as a histochemical marker[18] to follow the fate of these transplanted cells.

## Materials & Methods

### Culture conditions

ES cells derived from the ICM of blastocyst-stage mouse embryo (Albino mice of strain:*Blab/C*) (Hammash of Waheed, 2003 in preparation )[4] were used in this study.

ES cells were maintained under 5% $CO_2$, 37°C in minimum essential medium eagle modified (MEM) (Sigma) on mitotically inactive mouse

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embryonic fibroblast (feeder layer) supplemented with 20% new born bovine serum (NBBS) (Sigma).

To initiate differentiation, ES cells were dispersed with trypsin-EDTA 0.25% (w/v) powdered trypsin in 0.04% (w/v) Ethylene diamine tetra acetic acid (EDTA) in Calcium and Magnesium Free-Phasphate buffer saline (CMF-PBS) (PH 7.6) into single cell suspension, the later was cultivated without feeder layer or substrate attached layer, on non adhesive bacteriological petridish (to prevent attachment) with culture medium containing 20% NBBS, then incubated for few days, where they spontaneously form floating clusters of cells termed aggregates and simple embryoid bodies (EBs).

**Induction of differentiation**

As the aggregates and simple EBs were formed they were allowed to attach to the (0.01%) gelatin substrate coated tissue culture plate well and were left for 12 hr with MEM medium-plus 20% NBBS to form monolayer.

To initiate directed differentiation, the monolayer were plated in a defined medium (induction medium) that favor's the survival of ES cell-derived neural precursors and incubated for 10 days and this medium was changed every two days.

ES cells induction medium containing MEM medium plus 20% NBBS plus 25 µl/ml embryonic brain extract (prepared from 14-16 days mouse embryo following the general principles of embryo extract preparation as set by New[19] with some modification.

The directed differentiation pathway of these treated ES cells was evaluated after 10 days of incubation and as the cells form a monolayer.

**a-*In vitro* differentiation:**

Single cell suspension of these treated cells were prepared and passaged in the absence of feeder layer and cultured on gelatin substrate coated tissue culture plate and incubated for 2-5 days.

**b-*In vivo*: Preparation of marking ES cells for transplantation:-**

As a monolayer of directed and non directed (control group) ES cells was prepared, the medium was aspirated and change with MEM medium plus 20% NBBS plus 4% HRP-histochemical marker (Sigma), then incubated for 6 hours, washed for 5 times with CMF-PBS to remove the excess HRP. The cells dispersed into single cell suspension, centrifuged, then the cell number was determined at concentration of 20000 cells/ 10 µl of medium ready for transplantation.

**Animals and transplantation procedure**

Adult male mice (Albino mice: strain: *Balb/C*) were used as graft recipients. Mice were annaesthetized by inhalation of ether and placed in a stereotactic frame with tooth bar at +Z level, the rest of the body placed over thermoregulated operating table fixed at 37°C. Midline incision starting 5 mm anterior to orbital level extending back to the occipital region.

3mm X 3mm craniotomy was done just lateral to the midline and 1mm anterior to the lambda. The above flap is elevated, dura opened exposing by this the superior parietal area of the brain.

A needle was introduced for 2.5-3 mm into the brain in a fixed direction of stereotactic frame, in order to produce limited damaged in a specific location.The damaged may be considered severe when over manipulation was done by the needle.

Injection of ES cells (for both directed and non directed) was done as follow: 5 µl Hamilton's syringe loaded with the ES cells (two times) was fixed to X-Y-Z coordinate of the stereotactic frame. The tip of the needle advanced in brain tissue to adepth of 2.5-3 mm from the cortical surface. The coordinate of site of injection were L+Z, P-10, +2.3 mm and the tip of the needle was fixed at these coordinate for 3 minute befor injection for proper settlement in brain parenchyma.

ES cells were injected at rate of 1µl/4 minutes with a total volume of 10µl which contain 20000 cells, and the needle was kept in its position for additional 5 minute after the completion of injection.

The syringe withdrawn and the skin closed with interrupted skin suture and the animal was allowed to recover and returned to its cage alone to be re-explored after 17-20 days[10].

**Histochemical evaluation:-** These studies were carried out into the brain tissue in order to follow-up the fate of these transplanted marked ES cells and as follow:-

After 17-20 days from transplantation, the whole brain was obtained and HRP marker was detected histochemically following fixation of the frozen sections (4 µ thickness) with 4% formaline for 10 minutes and histochemical

visualization of the HRP is achieved by incubating the fixed tissue sections in a medium containing the substrate ($H_2O_2$) and a 3,3-diaminobenzidine tetrahydrochloride (DAB)[20].

## Results

*In vitro* and *in vivo* (in the transplantation study) we initiated and adopted this protocol which appeared more suitable to promote the differentiation of ES cells to the neuronal pathway, this protocol included three steps:

*First step*: ES cells were cultured in a non-adhesive dish, where they form floating clusters of cells, termed aggregates when these aggregate cultivated for 2-3 days in suspension they for simple EBs.

*Second step*: attached cultures were initiated by plating these aggregates with simple EBs onto gelatin coated dish to allow adherence.

*Third step*: these adhered (attached) cells was exposed to MEM medium plus serum plus embryonic brain extract, then cultivated in this condition without passage for 10 days.

The *in vitro* examination of this culture during this period, showed no differentiated ES cells as a neuronal-like cells in the monolayer of the culture.

**Differentiation of directed ES cells**

*In vitro* (after passaged) these cells were differentiated to neuronal-like cells as shown in (Figure 1).

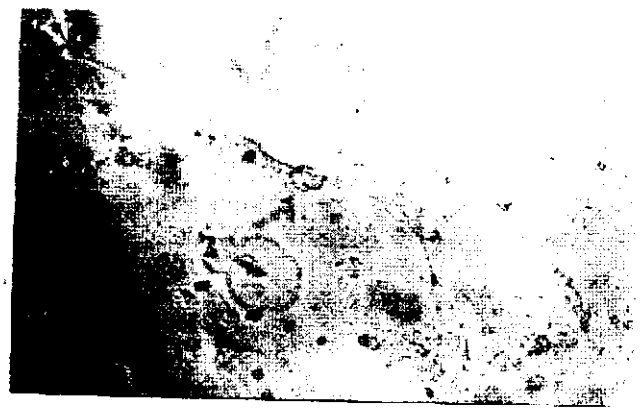

**Figure 1: In vitro differentiation of ES cells to neural-like cells (living materail) x 100.8**

The *in vivo*, grafting and transplanting of the ES cells into the adult mice brain, showed the following:

First: When there is severe damage in the brain tissue the transplanted cells settled in this damaged area then proliferate and differentiate enhancing the repair of damaged area.

Second: When there is no severe damaged carried in the brain tissue, some of the transplanted cells migrated away from the site of transplantation to different regions of the brain tissue, then proliferate and differentiate to cells of this brain tissue they migrated to it.

When these cells are transplanted via injection (without damage was carried, only the damaged of the site of injection) in the Hipocampus region (at the tip of dentate gyrus medial to the corn of Ammon layer (CA1), the transplanted cells migrated away from the site of injection to the gray matter of parietal lobule (sensory area) for distance of 1.5 mm surrounding the site of injection (Figures 2A & B) according to[21].

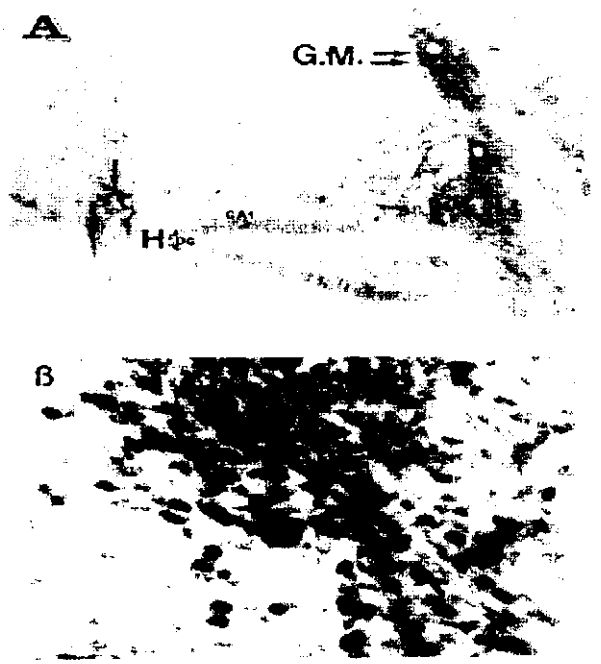


**Figure 2: Transplantation of the (treated and marked) ES cells in the mouse brain. A: shows the site of injection (↓) Hipocampus region (H) at the tip of the dentate gyrus (DG), medial to the corn of Ammon layer (CA1) and the site of the fate migrating these cells (→) the Gray matter of parietal lobule (sensory area (GM) x 60. B: The higher magification of the site of fate migrating ES cells (HRP) marked ES cells brown in color. X 600**

Cells transplanted into the damaged right superior parietal lobe showed the following possibites, as appeared in brain section:

A. Some of them are settled and diffused in damaged area (Figures 3A & B) and when the original brain cells and transplanted differentiated ES cells were examined under high magnification, showed similarity between

transplanted cells, and the host cells, in size and shape (Figures 4A & B).


**Figure 3: (A & B): Transplantation of the ES cells in the superior parietal lobe (Rt. Side) x 600.**


**Figure 4A & B: By using higher magnification in order to compare between the original cells at the site of injection (A) and transplantd (treated and marked) ES cells (B). These pictures indicate the similarity between these cells. X 1500**

B. Other transplanted cells migrated and were observed in the subcallosal area in the $CA_3$, Dentats Gyrus (DG), for distance 3-5 mm from the site of transplantation (Figure 5A) then proliferated and differentiated in this area (Figures 5B, C & D).


**Figure 5 A: Migration of the transplanted (treated and marked) ES cells from superior parietal lobe (Rt. Side) to the subcallosal area in the corn Ammon (CA3), dentate gyrus (DG). X60 B: High magnification of area (▼ ) from figure A. x 600 C: High magnification of area ( ➔) from figure A. x 300 D: More higher magnification of figure C. x600.**

C. Another group of transplanted cells migrated away from the site of transplantation and observed in the inferior parietal lobule of the right side for distance of 5.45 mm from the side of transplantation (Figure 6).

**Figure 6: Migration of the transplanted (treated and marked) ES cells from the superior parietal lobe (Rt. Side) to the inferior parietal lobule of the Rt. Side. X600**

D. Others migrated away from the superior parietal lobule to the inferior parietal lobule (or angular gyrus) of the left side for a distance of 6.5 mm from the site of transplantation (Figure 7A). They were setteld and has to proliferated and differentiated in to an isomorphous population of round to bipolar cells (Figure 7B).


**Figure 7 A: Migration of the transplanted (treated and marked) ES cells from the site of injection (superior parietal lobe) of the Rt. Side to the inferior parietal lobule (or angular gyrus) of the Lt. side and sattled, proliferate and differentiate in this area ( ↓ ). X150. B: shows an isomorphous population of round to bipolar (differentiate) transplanted ES cells. X 1500**

Third: When cells were transplanted in the right parietal area after sever damage most of ES cells were restricted and settled in the damaged area and proliferated and differentiated replacing the damaged brain tissue (Figure 8).

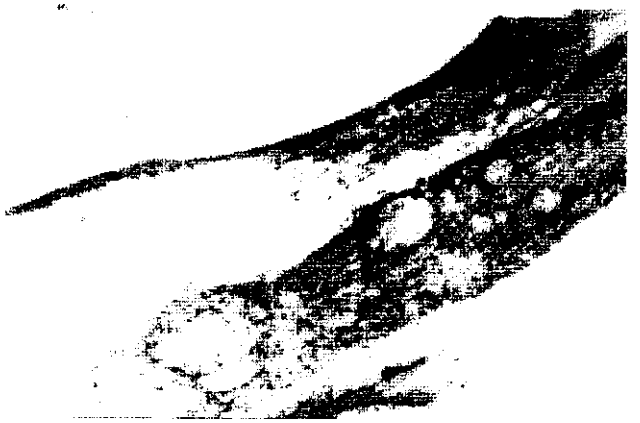

**Figure 8: Transplantation of (treated and marked) ES cells in the parietal area after severe damage was carried in this area, note the repaire and healing of damaged tissue were carried by ES cells, which appeared as brown stained layer. X300**

In all these three conditions of transplanted ES cells and after the end of the experiment, no signs of tumor growth or non-neural tissue in the transplanted recipients was noted.

Forth: Marked ES cells but not treated with embryonic brain extract were transplanted in the simillar location. They form a heterogeneous tissue inside the brain (Figure 9).

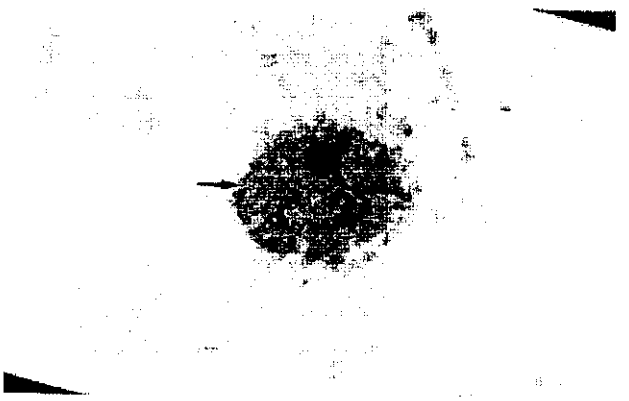

**Figure 9: Transplantation of marked ES cells (non-treated with brain extract) ( ▼ ) in the mouse brain tissue, the piocture indicates that these cells did not differentiate and they form a heterogenous tissue inside the brain. X250**

As described above these transplanted ES cells were tracked by prelabelling *in vitro* with histochemical marker HRP before transplantation and from the result of the present study, this marker can provide good information about events occuring within long period (for 17-20

days in this study) after transplantation. This marker appeared non deleterious or harm to the cells, because the cells survive and for long period after marking as shown in the result of transplantation.

## Discussion

In the present study, part of a long term goal to develop cell transplantation therapy for specific diseases (that results from the destruction and/or dysfunction of a limited number of cell types) using ES cells as a source of pluripotent cells.

The use of ES cells to generate replacement tissue for treating neurological diseases is a major focus of researches on spinal cord injury, stroke, multiple sclerosis, and Parkinson's diseases, at which the concept of replacing destroyed or dysfunctional cells in the brain or spinal cord seems plausable[22,23].

ES cells have the ability to choose between prolonged self renewal and differentiation. This fate choice is highly regulated by intrinsic signals and the external microenvironment, the elements of which are being rapidly elucidated[24]. These cells will need to be differentiated or otherwise modified before they can be used clinically.

ES cells are theoretically capable of differentiating into any cell type *in vitro*. The previous works have shown that they can be induced *in vitro* to become nural precorsers by using a number of protocols which depend on using compilation of several and different growth factors plus other compound and serum-free medium. *In vivo* neuronal histogenesis is regulated by neurotrophic factors, neuron-glia reactions, extracellular matrix molecules and sex steroid. Growth factors (such as fibroblast growth factors (FGF) have a broad survival and growth effect on cell. culture from different regions of the embryonic brain and large amount of these growth factors are present in all regions of fetal brain suggesting an important role for these growth factors in early neurogenesis[9,16,25,26].

Results of this study has shown that mouse ES cells, cultured with addition of embryonic brain extract can develop neuronal- like cells in the culture, as observed morphologically.

In this, we substituted, the specified growth factors by embryonic brain extract, as a crude source of neurotrophic factors for directing the differentiation of ES cells to obtain neural precursors *in vitro* and to be used for transplantation *in vivo*.

For induction of ES cells differentiation to the neuronal pathway, we used protocol given above which is different from other protocols[9,10,25,27], that the induction is carried to the ES cells monolayer.

To evaluate the lasting differentiation of ES cells grown under this protocol, *in vivo*, immortalized cells were implanted into the brain and where they seem to be migrate broadly,as if they differentiated and replaced the depleted cells. These cells induced to differentiate into a mixed population of cells enriched with neuronal precursors. These results are in agreement with a study[28] (using combination of growth factors with serum-free medium) and implanted in the spinal cord of myelin-deficient rats depleted of endogenous oligodondroglia and with Benninger *et al*[29] who used several different media to assess the influence of serum and growth factors on the differentiation pattern of the intracerebrally transplanted EB. It appeared that ES cells are capable of differentiating into circumscribed transplants of central nervous system (CNS) tissue containing neuron.

Previous transplant studies involving ES cells-derived neural cells generated without brain extract treatment or growth factors or retinoic acid treatment[9] were complicated by the formation of heterogenous tissue, while in this study, embryonic brain extract may play a role in directing the differentiation of ES cells to the neural pathway.

The ellucidation mechanism of migration of transplanted ES cells, need further study.

FGF have been shown to be involved in stem cell migration and lineage commitment and have been implicated in self-renewal[30,31].

Definitive identification will require phenotypic markers that discriminate between different cell types or different states of a common cells. Once a stem cell divided and the born cells migrate to specific regions, it matures further until it reaches a site when it stops and become fully differentiated into a functioning cell. From our and other studies, neural cells can be derived from more primitive cells, including ES cells, but the major obstacle of identifying and discovering markers that define a stem cell is that the most primitive cells are probably in a

quiescent state and do not express many unique antigens. Thus, as with other fields like hematopoisis, a combination of positive and negative markers will be required to define the primitive stem cell[32], that is why we thought the use of HRP as a useful marker[18], because there is a great need for a simple marker that could be applied on cells in tissue culture. ES cells appeared to have the ability to take up this large molecule marker into inside due to the active phagocytosis nature of these cells as shown by scanning electron microscope study[4]. This marker is a protein that could be kept inside the cells for long period usually as granules, although these cells proliferated and migrated for several millimeters distance from the site of injection, The labeled cells are in most cases completely distinguished from other cells in tissue.

## Conclusions

Using embryonic brain extract as a crude source of neurotrophic factors, for the first time, seems to direct the differentiation of these cells to neural precursors *in vitro* and vivo.

Pluripotent ES cells are of potential interest as a possible source for cell replacement therapies for the nervous system. These cells have been shown to proliferate, migrate and differentiate into neural cells after intracerebral transplantation by using mouse as an animal model of injury.

## References


1. Evans, M.J., and Kaufman, M.H.: Establishment in culture of pluripotential cells from mouse embryos. Nature, 1981; 292: 154-6.
2. Martin, G.R.: Isolation of a pluripotent cell line from early mouse embryos culture in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA, 1981; 78: 7634-8.
3. Wobus, A.M., Holzhausen, H., Jakel, P., and Schoneich, J.: Characterization of a pluripotent stem cell line derived from mouse embryo. Exptl Cell Res, 1984; 152: 212-9.
4. Hammash, M.H., and Waheed, I.N.: Isolation and Characterization of a pluripotent stem cells from mouse embryos-2003 (in press).
5. Smith, A.G., and Hooper, M.L.: Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol, 1987; 121: 1-9.
6. Williams, R.L., Hilton, D.J., Pease, Sh., Willson, T.A., Stewart, C.L., Gearing, D.P., et al.: Myeloid Leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature, 1988; 336: 684-7.
7. Bradley, A., Evans, M., Kaufman, M.H., and Robertson, E.: Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. Nature, 1984; 309: 255-6.
8. Schwartzberg, P.L., Goff, S.P., and Robertson, E.L.: Germ-line transmission of ac-able mutation produced by targeted gene disruption in embryonic stem cells. Science, 1989; 246: 799-803.
9. Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., et al.: Embryonic stem cell-derived glial precursors: A source of myelinating transplants. Science, 1999; 285: 754-6.
10. Liu, S.Q., Stewart, T.J., Howard, M.J., Chalkrabortty, S., Holekaamp, T.F., and McDonald, J.W.: Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci USA, 2000; 97: 6126-31.
11. Call, L.M., Moore, C.S., Stetten, G., and Gearhart, J.D.: Acre-lox recombination system for the targeted integration of circular yeast artificial chromosomes into embryonic stem cells. Hum Mol Genet, 2000; 9: 1745-51.
12. Wiles, M.V., Vaut, F., Otte, J., Funchtbauer, E.M., Ruiz, P., Fuchtbauer, A., et al.: Establishment of a gene-trop squence tag library to generat mutant mice from embryonic stem cells. Nat Genet, 2000; 24: 13-4.
13. Odorico, J.S., kaufman, D.S., and Thomson, J.A.: Multilineage differentiation from human embryonic stem cell lines. Stem cells, 2001; 19: 193-204.
14. Stocum, D.L.: Regenerative biology: A millenial revolution. Cell Dev Bio, 1999; 10: 433-40.
15. Rossant, J.: Stem cells from the mammalian blastocyst. Stem cells, 2001; 19: 477-82.
16. Shihabuddin, L.S., Ray, J., and Gage, F.: Stem cell technology for basic science and clinical applications. Arch Neurol, 1999; 56: 29-32.
17. Robey, P.G.: Stem cells near the century mark. J Clin Invest, 2000; 105: 1489-91.
18. Hammash, M.H., and Waheed, I.N.: Horse radish peroxides HRP: Biochemical marker for embryonic stem cells.2003 (in press).
19. New, D.A.T.: The culture of vertebrate embryos, Logos press, Academic press. Great Britain and London. 1966.
20. Pearse, A.G.E.: Histochemistry, Theoretical and Applied, 3rd edition. Edinburgh: Churchill Livingstone. 1972.
21. Paxinos, G., and Waston, C.: The rat brain in stereotaxic coordinates-2nd edithon. Academic press. 1985.
22. Gage, F.H., and Fisher, L.J.: Intracerebral grafting: a tool for neurobiologist. Neuron, 1991; 6: 1-12.
23. Fisher, L.J.: Neural precursor cells: applications for the study and repair of the centeral nervous system. Neurobiol Dis, 1997; 4: 1-22.
24. Watt, F.M., and Hogan, B.L.: Out of Eden: stem cells and their niches. Science, 2000; 287: 1427-30.
25. Bain, G., Kitchens, D., Yao, M., Huettner, J., and Gottleib, D.: Embryonic stem cells express neuronal properties *in vitro* Dev Biol, 1995; 168: 342-57.

26. Mckay, R.: Stem cells in the central nervous system. Science, 1997; 276: 66-71.
27. Bain, G., Ray, W.J., Yao, M., and Gottlieb, D.I.: From embryonal carcinoma cells to neurons: the P19 pathway. Bioessays, 1994; 16: 343-8.
28. McDonald, J.W., Liu, X.Z., Qu, Y., Liu, S., Mecay, S.K., Turetsky, D., et al.: Transplanted embryonic stem cells survives, differentiate and promote recovery in injured rat spinal cord. Nature Med, 1999; 5: 1410-12.
29. Benninger, Y., Marino, S., Hardegger, R., Weissmann, C., Aguzzi, A., and Brander, S.: Differentiation and Histological analysis of embryonic stem cell-derived neural transplants in mice. Brain Pathol, 2000; 10: 330-41.
30. Walsh, C., and Cepko, G.: Cell lineage and cell migration in the developing cortex. Experientia, 1990; 46: 940-7.
31. Schuldiner, M., Yanuka, O., Itskovitz, E.: Effects of eight growth factors on the differentiation of cell derived from human embryonic stem cells. Proc Natl Acad Sci USA, 2000; 97: 11307-12.
32. Gage, F.H.: Mammalin neural stem cells. Science, 1995; 287: 1433-56.