Forkhead box a2 (Foxa2) Gene differntiation of embryonic stem cells

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الملخص

Foxa2 gene differntiaion in neural stem cells exist in the mammalian developing and adult nervous system during the neural differentiation of the embryonic stem cells (ESCs). The potential of neural stem cells for the treatment of neurodegenerative diseases and brain injuries has substantially promoted research on neural stem cell self-renewal and differentiation.

About the history of SCs, because they are important for replacement therapy diseases.

Then, I will review literature, arguments that are dealing with, how the SCs differentiate into neural cells, and how could this process can be handled *in vitro*.

The unique capability of these cells to form various tissues under definite signals received from the body, it makes this cell an object of extensive research.

Subsequently, information has been compiled on the question how neural differentiation is controlled on the molecular level, and controlled *in vivo*.

Finally, ther is a major gene is investigated, which is involved in practical according to their expression patterns. This gene is forkhead box a2 (Foxa2).

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الكلمات المفتاحية: (Foxa2) forkhead box a2, (SCs) stem cells, (ESCs) embryonic stem cells.

History of stem cells

SCs have an interesting history that has been tainted with debate and controversy.

In the mid of 1800s, it has been interested in cell biology since the advent of microscopes. At the same time it was discovered that cells are basically the building blocks of life. And these cells had the ability to generate other cells that made them a key to understanding human development for a great focus of medical research. In

the early 1900s the first real SCs were discovered when it was discoverd that some cells generate blood cells. SCs are able to divide indefinitely, forming hundreds of copies of themselves, and to repair damaged body tissues. SCs are less likely than other foreign cells to be rejected by the immune system when they are implanted in the body. On the scientific front, it is clear that work on first embryonic stem cell (ESCs) cells has already generated new possibilities and stimulated development of new strategies for increasing our understanding of cell lineages and differentiation (Till and McCulloch, 1961). The first quantitative descriptions of the self-renewing activities of transplanted mouse bone marrow cells were documented by Canadian researchers (Ernest A. Till, 1963).

Other key events in SCs research include:

1978: Stem cells were discovered in human cord blood

1981: First *in vitro* SCs line developed from mice

1988: Embryonic SCs lines created from a hamster

1995: First embryonic SCs line derived from a primate

1997: Cloned lamb from SCs

1997: Leukemia origin found as hematopoietic stem cell, indicating possible proof of cancer SCs.

During the initial phase of regeneration it has been found that cells in the area of the injury can help repair defects and become SCs again. Known as dedifferentiation, the process produces cells that will later grow and dedifferentiate to form the new part or organ. The process is unique and is not observed in animals that lack regenerative powers, such as mammals. Scientists are looking for those genes that regulate regeneration and are investigating the possibility of transferring cells to mammals even to humans. Clearly, ESCs models are already providing opportunities for the establishment of limitless sources of specific cell populations (Turksen K, 2002).

Recently there has been lot of interest in the stem cell research finding a creative treatment for many diseases, including the cancers, spinal injuries, limb ischemia, myocardial infarction, and Parkinsonism and many more.The field requires dedicated team of basic researchers and clinicians to fully understand the cell physiology, modulators, their potentials and scope of applications more so in the diseases where presently there is no cure (Devendra K, 2008).

Characteristics that distinguish stem cells from other cells

1. Under certain conditions stem cells can be induced to become cells with special functions, such as cells of the heart muscle or insulin-producing cells of the pancreas
2. Stem cells give rise to specialized cells. When this occurs, the process is called differentiation. Signals from both inside and outside the cell may trigger this differentiation. External signals include signaling factors secreted by other cells, physical contact with other cells, and certain molecules called growth factors.
3. stem cells are responsible for replacing blood and tissues on a regular basis.
4. Embryonic germ cells are similar to embryonic stem cells except they are collected from the fetus later in development. The cells come from a region known as the gonadal ridge, which will later develop into the sex organs. Because the cells are farther along in the developmental process, they are slightly limited in their ability to give rise to organs of the body (Kelly E, 2007).

Classification of stem cells

Stem cells can be classified into three broad categories, based on their ability to differentiate. Totipotent stem cells are found only in early embryos. Each cell can form a complete organism. Pluripotent stem cells exist in the undifferentiated inner cell mass of the blastocyst and can form any of the over 200 different cell types found in the body. Multipotent stem cells are derived from fetal tissue, cord blood and adult stem cells. Although their ability to differentiate is more limited than pluripotent stem cells, they already have a track record of success in cell-based therapies. Here is a current list of the sources of stem cells: Embryonic stem cells are derived from the inner cell mass of the blastocyst seven to ten days after fertilization:

- Fetal stem cells are taken from the germ line tissues that make up the gonads of aborted fetuses.
- Cord stem cells - Umbilical cord blood contains stem cells similar to those found in bone marrow.
- Placenta derived stem cells - up to ten times as many stem cells can be harvested from a placenta as from cord blood.
- Adult stem cells - Many adult tissues contain stem cells that can be isolated.

ESCs, which are derived from the inner cell mass of mammalian blastocysts, have the ability to grow indefinitely while maintaining pluripotency and the ability to

differentiate into cells of all three germ layers. hESCs might be used to treat diseases, such as Parkinson's disease, spinal cord injury, and diabetes (Thomson et al., 1998).

Signaling in neurogenesis and genes controlling differentiation in the mouse

Both stem cells and neurons are not easy to maintain in culture, and it is hard to introduce , deoxyribonucleic acid (DNA) or, ribonucleic acid (RNA) molecules into them to target specific genes or pathways. Nonetheless, there is a successful in determining the expression of thousands of genes and comparing expression patterns between different cells or cells grown under different conditions. Such work has allowed to identify, for example, master regulators of stem-cell differentiation or neuronal survival. In addition, a wide variety of tools has been designed specifically for use in stem cells or neurons to control the expression of a gene of interest and study its function. One useful technique, Serial analysis of gene expression (SAGE) allows identifying all the genes involved in a particular process, such as the migration or the differentiation of stem cells. SAGE is an open platform for monitoring the expression patterns of thousands of transcripts in one sample and can lead to the discovery of novel genes. The technique relies on the generation of a library of short complementary DNA (cDNA) 'tags' each corresponding to a sequence near the 3 end of every transcript in a cell or tissue sample. The Genome Analysis System uses a tag amplification step on the surface of a glass flow cell. Methods such as SAGE and the ever-popular microarrays can be at the complement of transcripts isolated from a population of cells (Didier Trono, 2006).

Signaling that controls Foxa2 expression-importance of Foxa2in neurogenesis

The Foxa2 subfamily of winged helix/forkhead box (Fox) transcription factors has been the subject of genetic and biochemical studies for over 15 years. During this time its three members, Foxa1, Foxa2 and Foxa3, have been found to play important roles in multiple stages of mammalian life. Beginning with early development, continuing during organogenesis, and finally in metabolism and homeostasis in the adult. Foxa2 is required for the formation of the node and notochord, and in its absence severe defects in gastrulating, neural tube patterning, and gut morphogenesis result in embryonic lethality. Foxa1 and Foxa2 cooperate to establish competence in

foregut endoderm and are required for normal development of endoderm-derived organs such as the liver, pancreas, lungs, and prostate. In post-natal life, members of the Foxa family control glucose metabolism through the regulation of multiple target genes in the liver, pancreas, and adipose tissue. Insight into the unique molecular basis of Foxa function has been obtained from recent genetic and genomic data, which identify the Foxa proteins as 'pioneer factors' whose binding to promoters and enhancers enable chromatin access for other tissue-specific transcription factors (Friedman and Kaestner, 2006).

The role of transcription factors in regulating the development of midbrain dopaminergic (mDA) neurons is intensively studied owing to the involvement of these neurons in diverse neurological disorders. During specification, Foxa1 and Foxa2 regulate the extent of neurogenesis in mDA progenitors by regulating Neurog2 (Ngn2) expression. Interestingly, genetic evidence indicates that these functions require different gene dosages of Foxa1 and Foxa2 (Ferri et al, 2007).

Forkhead transcription factors are critical regulators of survival and longevity of the embryonic stem cells. The role of Fox transcription factor has been studied by knockout overxpression in the mouse. Foxa2 gene has the function to generate dopamine neurons during fetal development and from embryonic stem cells. Mice carrying only one copy of the foxa2 gene show abnormalities in motor behavior in old age and an associated progressive loss of dopamine neurons. Fox genes have evolved to acquire a specialized function in many key biological processes. Mutations in Fox genes have a profound effect on human health, disease-related phenotypes as varied as cancer, glaucoma and language disorders (Hannenhalli S and Kaestner KH, 2009).

Functions of Foxa2 can be summarized as follows:

-Foxa2 regulates a complex pulmonary program of epithelial cell maturation required for transition to air breathing at birth.

-Foxa2 regulates multiple pathways of insulin secretion.

-In preadipocytes Foxa-2 inhibits adipocyte differentiation by activating transcription of the Pref-1 gene.

- Foxa2 gene expression, it is controls in pancreatic beta-cells.

-Foxa2 plays an integral role in the formation of axial mesendoderm, which is required
to maintain the specification of the forebrain and the anterior definitive endoderm.

A novel role for Foxa2 has been reported in bile acid metabolism. The winged helix transcription factor Foxa2 is required to prevent intrahepatic cholestasis and liver injury in mice fed a colic acid enriched diet. Functional genomics were used to study how Foxa2 regulates its targets in a colic acid-dependent manner. Foxa2-deficient mice. This suggests that the deletion of Foxa2 in the hepatocyte affects the liver on a large scale. It was discovered distinct feed-forward regulatory loops controlling Foxa2-dependent targets in a colic acid dependent. it shows that Foxa2 interacts with different transcription factors to achieve gene expression responses appropriate for each physiologic state (Bochkis et al, 2009).

In the mammalian CNS an important contingent of dopaminergic neurons are localized in the substantial nigra and in the ventral segmental area of the ventral midbrain. They constitute an anatomically and functionally heterogeneous group of cells involved in a variety of regulatory mechanisms. mDA primary cultures represent a useful tool to study molecular mechanisms involved in their development and maintenance. Considerable information has been gathered on mDA neurons development and maturation *in vivo*, as well as on the molecular features of mDA primary cultures (Greco et al., 2009).

In vitro differentiation to neural cell types

The derivation of specific neuronal or glial cell types from ESCs invariably includes the production of neural stem cells. The basic mechanisms of neural induction during vertebrate embryogenesis are gave information to helpe formulate several protocols waich are used to generate NSCs from ESCs (Cai C aned Grabel L, 2007).

NSCs arise from embryonic ectoderm that forms neuroepithelial cells. The neuroepithelial cells generate radial glia that produce fetal and adult NSCs within the CNS. Adult NSC and restricted progenitors are found in the several regions of the CNS throughout life (Leslie P et al., 2008)

Mouse embryonic stem cells derivatives in EBs

During mouse embryogenesis, the primitive ectoderm of the epiblast forms three primary germ layers: the ectoderm, the mesoderm, and the definitive endoderm. These germ layers interact to form all tissues and organs of the developing embryo. Differentiation is induced by culturing ES cells as aggregates EBs in the absence of

the self-renewal signals provided by feeder layers or LIF, either in hanging drops, in liquid "mass culture", or in methylcellulose. (YingQLetal.,2003). Once differentiation has begun, cells representing primary germ layers spontaneously develop in vitro. Initially, an outer layer of endoderm-like cells forms within the EB, followed over a period of a few days by the development of an ectodermal and subsequent specification of mesodermal cells (fig. 1). Transfer of these EBs to tissue culture plates allows continued differentiation into a variety of specialized cell types including cardiac, smooth, and skeletal muscle as well as hematopoietic, pancreatic, hepatic, lipid, cartilage, or neuronal and glial cells (Rohwedel J et al., 1999).

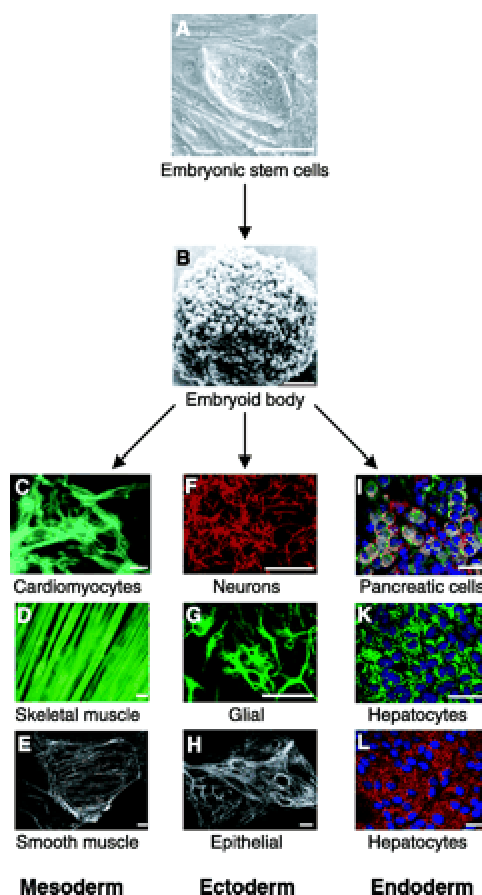


Fig. 1. In *vitro* differentiation of ES cells. Undifferentiated mouse ES cells, (*A*) develop in vitro via three-dimensional aggregates (embryoid body, (*B*) into differentiated cell types of all three primary germ layers, and (*C,F,I,D,G,K,E,H,L*) are cells differentiation into a variety of specialized cell types (Rohwedel J et al., 1999).

Human embryonic stem cells current technologies and applications

hESCs lines, which have recently been derived, may additionally serve as an unlimited source of cells for regenerative medicine. Before therapeutic applications

can be realized, important problems must be resolved. Ethical issues surround the derivation of hESCs from *in vitro* fertilized blastocysts. The number of human ES cell lines available for research also be insufficient to adequately determine their therapeutic potential. Recent molecular and cellular advances with mouse ES cells, however, portend the successful use of these cells in therapeutics. Mouse and human ES cells are respect to in vitro propagation and differentiation as well as their use in basic cell and developmental biology (Anna M et al., 2005).

The establishment of human ES cell lines from in vitro fertilized embryos (fig.2) and the demonstration of their developmental potential in vitro have widespread discussions concerning future applications of human ES cells in regenerative medicine. Primordial germ (PG) cells, which form normally within the developing genital ridges, represent a third embryonic cell type with pluripotent capabilities. Isolation and cultivation of mouse PG cells on feeder cells led to the establishment of mouse embryonic germ (EG) cell lines. In most respects, these cells are indistinguishable from blastocyst-derived ES cells and are characterized by high proliferative and differentiation capacities in vitro , and the presence of stem cell markers typical of other embryonic stem cell lines. These cell lines showed multilineage development in vitro but have a limited proliferation capacity, and currently can only be propagated as embryoid body (EB) derivatives (Shamblott MJ et al., 2001).

During cloning of Dolly sheep in 1997, a technique has been used called somatic cell nuclear transfer (SCNT) or, nuclear transfer (NT) provided a means of generating ES cells with defined genetic makeup. The advantage of using NT to derive hESCs is that the nuclear genomes of the resulting hESCs would be identical with those of the donors for the somatic cells. One obvious benefit is that this would avoid the problem of rejection if cells generated from the hESCs were to be transplanted into the donor (Alexander Capron et al, 2005).

More imminent, however, is the employment of stem cell technologies for drug discovery and development. Novel improved in vitro models based on physiologically relevant human cells will result in better precision and more cost-effective assays (Hyllner J et al, 2007). In this context, identifying soluble factors, in particular chemically synthesized small molecules, and signal cascades involved in specific differentiation processes. Defined tissue for specific cells types are crucial for

optimizing the generation of somatic cells in vitro for therapeutic approaches. However, experimental models are required allowing rapid and "easy-to-handle" parallel screening of chemical libraries to achieve this goa (Sachinids A et al, 2008).

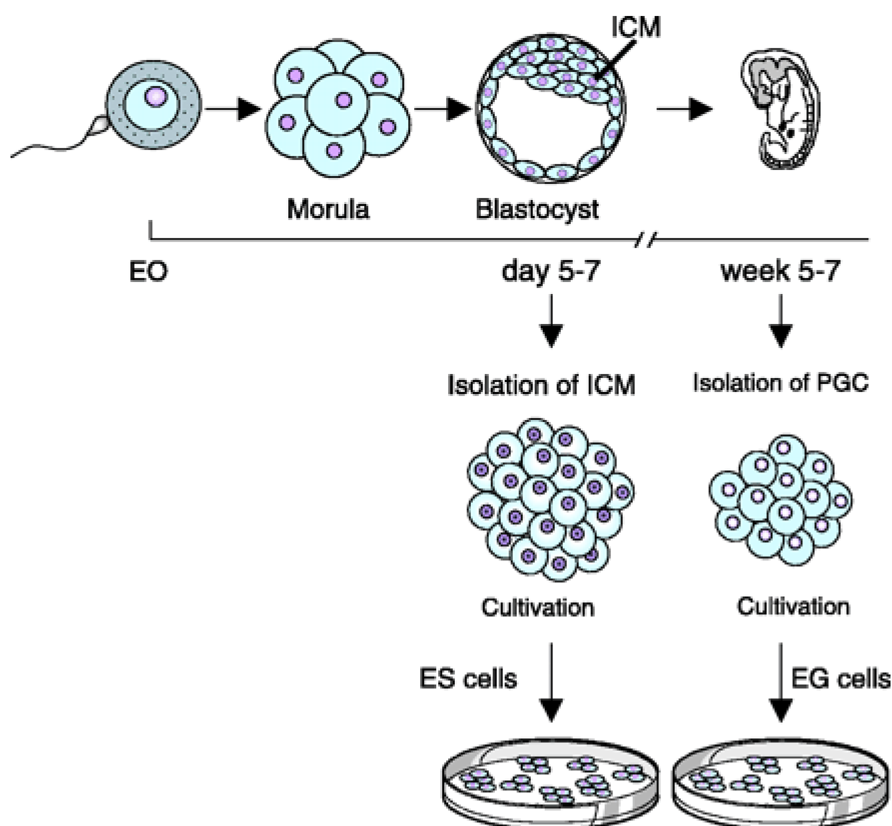


Fig.2. Human pluripotent ES and EGCs have been derived from in vitro cultured ICM

cells of blastocysts (after in vitro fertilization) and from primordial germ cells (PGC) isolated from aborted fetuses, respectively (Thomson JA et al., 1998).

MATERIALS AND METHODS

In the laboratory work we use modern methods in analyzing and investigated the expression of genes in the embryonic tissue by the way of the *situ*- hybridization.

Genes Investigated in the practical section

The three clones used in our study were isolated from different sources which are requested from the RZPD, and these clones are:

1- RZPDp471B0753D2 (TH)

The clones contain DNA from the following library:

Library No: 471
Creator: Minoru Ko
Source: embryo-derived stem cells
Vector: pSPORT1
Host: E.coli DH10B
Growth Conditions: Unconfirmed antibioiitics ampicillin

2-IRAVp968C04116D6,it (SOX2)

The clone contains DNA from the fo llowing library:

Library No: 968
Source: mammary gland,lung,liver,mouth,colon,kidney
Eye, thymus, uterus, olfactofy epithelium,
inner

Ear, embryo, limb, stem cell,embryonic stem cell, pancreas, Blastocyst,egg, bone,brain

Growth Conditions: temperature: 37ºC

Antibiotics : ampicillin

3-RZPDp981A0892D5, it is entry HNF3B(Foxa2)

The clone contains DNA from the following library:

Library No: 981

Creator: Thomas Schlueter, Minoru Ko

Source: muscle,bowel,mammary gland,liver,kidney,

Embryo,uterus,brain,embryonic stem cell, Gonad,spleen,testis,eye.lung,B-cell,inner ear,

Thymus,bone,bone marrow,testis,

Host: E.coli DH10B

Growth Conditions: unconfirmed

Antibiotics: ampicillin

Resources for coloned genes

For preparing the bacterial culture, LB media was prepared and bacterial colonies were propagated according to the colonies properties, transformed in the bacterial culture in to the LB medium, ampincilin, and plasmid preparation.

Plasmid preparation

The followed protocol was adopted by Birnboim Doley- protocol (Birnboim Doley) with some modifications. According to them it was essential the purification, and precipitation with Ammouniumacetate to get plasmid purification.

Linearization by suitable restriction enzymes

The plasmid was prepared from alkaline mini-preparation and linearized by the probe of end 5′ overhong and in vitro transcript to label diogoxygenin riboprobes using the promoters T3, T6 and sp6. Original vector survival from the library was known T7 or T3 promoter in order to allow riboprobes and then sub cloning in the blue script.

FOXa2

- vector name PME188-FL3
- promoter T3
- Restriction enzyme Xho I

- Sub cloning Foxa2 sequences in to pBs in order to generate riboprobes

The original cloning vector from the library does not contained RNA polymers promoter side, therefor we sub cloned the vector by cutting the original vector by the use of enzyme and cloning the dephosphorlation vector plasmid Blue script KS (pBSII KS).

Protocol for Dephosphorylation of DNA 5′-termini

Dephosphorylation of the vector was done by Calf-intestine alkaline phosphates (CIAP) and it was done by this way:

- Dissolve DNA in 10-40µl deionized water.
- Prepare reaction mixture by adding the following DNA solution 10-40 µl+10x reaction buffer 5µl+deionized water to 49 µl + CIAP (1u).Stop reaction by heating at 85ºC for 15 minutes.
- Purify vector by QIAquik extraction, test the Vector for complete dephsphorylation by ligation, then transformation in to bacteria.
- Count number of colonies in relation by used vector for relegation, transformation and vector known concentration by photometer(Gene Quant II).

Preparation of Foxa2 inserts

In order to generate plasmid which is suitable for RNA preparation in vitro production for diogoxygen in probes. The Foxa2 insert has to be sub cloning in to a vector for allowed produce stem cells to obtain portion region. This well done by restriction the original colonies isolated the fragments and coloning into special peaparation for pBSII KS vector. Then purification was treatment by QIAquik Gel Extraction Kit, according to the protocol.

Ligation protocol for plasmid cloning of DNA fragments with blunt ends

The ligation protocol for plasmid cloning of DNA feagnments was procceded according to the standard laboratory protocols.

In situ- hybridization, was performed by phenol /chloroform purification of the DNA and was subjected to RNA synthesis according to the Birnboim Doly- protocol.

Transformation of bacteria

This technique is to introduce a foreign plasmid into bacteria and to use those bacteria to amplify the plasmid in order to make large quantities of it. This is based on the natural function of a plasmid to transfer genetic information in to survival of the bacteria.

Method of preparation **:**

- Bring copmentent bacterial cells with ice and lived in ice from 10-15 minutes.

-put 25 µl of copmentent cells for control and 25 µl of copmentent cells + 10 ng of DNA.

-live the solutions 30 minutes in the ice.

-put at 42ºC for heating for 35 seconds.

-put in the ice for 1 minutes.

-take 100 µl LB in every tube, and take 2 plates LB agar with Ampcilin.

-pure 50 µl for every plate, make culture on the plates then put them at 37ºC over night, and next day see the growth of bacteria.

-Make the liquid culture from the plate to take one colone form transformation ligation reaction and prepare plasmid preparation(Alkaline lysis-mini preparation).

-Restrict the plasmid cloning (ligation) by ECOR I and Hind III.

- 14 µl water + 2 µl 10xbuffer red + 2 µl plasmid + 1 µl ECOR I + 1µl Hind III

-shake verey slowly, put it 1 hour in 37ºC and stop reaction in 70ºC for 10 mintues.Then run the gel and check it.

Preparation as a template for Sequencing

The template generation is to do probe generated of linerizated template for using 5′ overhong with the polymer with use Ruche libeling mixture.

Protocol:

-half volume of the volume of DNA solution(50 µl DNA + 10 µlNH4).

-2.5 times of the volume of DNA solution + NH4, if you have 60 µl, you need to put 150 µl 100% Ethanol and mix by hand.

-live it 2 minutes at room temperature, centrifuge for 10 minutes, remove away the liquid

-wash by 1ml of 70% ethanol. µl, remove the suprements,dry it about 5 minutes and dissolved the DNAin 30µl TE

- measure the DNA cocentation by photometer and QIA PrepSpin Miniprep Kit protocol using a microcntrifuge for purification.

In situ-hybridization

Situ –hybridization is done for this ways :

- Probes are prepared as Digoxigenin labeled RNA . The labeling mix as well as all antibodies are purchased from Boehringer
- All conditions and solutions should be totally ribonuclease (RNase) free.
    1. Linearize the plasmid and check the digest.
    2. Extract by Phenol.
    3. Extract twice with chloroform:isoamyl alcohol (24:1)
    4. Ethanol precipitate (1/2 vol 7.5 M NH4 OAc 2.5 vol 100% ethanol. Rinse with 125µl 75% ethanol. Let dry with caps open for 10 minutes).
    5. Resuspend in suitable volume of nuclease free water.
    6. Measure concentration.
    7. Set up transcription reaction.
        - 200ng DNA
        - 2 µl 10X transcription buffer (Stratagene)
        - 2 µl labeling mix
        - 1 µl RNAGUARD (Pharmacia)
        - 1 µl RNA polymerase (SP6, T3 or T7)
        - Add water to 20 µl
    8. Incubate for 2 hours at 37 °C.
    9. Run on 1% agarose gel (1.5µl probe 5µl of 1.2X running buffer.) for a short time. The RNA should appear as a single band with little degradation product and about 10 times more intense than the DNA band.
    10. Remove unincorporated free nucleotides with Quick-Spin Columns.

- Remove the caps (top first not to create air bubble trapped in the column) and spin for 5 min, at °C, 1800 rpm in the Sorval swing bucket centrifuge.
- Remove the elute and centrifuge 5 min again.
- Put the column in new tubes, add the transcription reaction onto them and spin 15 min.
- The volume of the final elute should be around 30-40µl .
- Run a gel (loading 1.5µl in 5µl loading buffer) to quantify the yield and to determine the amount to be used for the in situ.

Preparation of embryos

Dissect embryos (129V mice type) in cold phosphated-buffered saline (PBS) , and change solution often.

1. Punch a hole in brain cavities for embryos older than 9 dpc.
2. Transfer after dissecting a few embryos to a 5 ml screw cap flat bottomed glass vial containing 4% paraformaldehyde
3. When all the embryos of the same mother are dissected, renew the 4% paraformaldehyde and incubate at 4 °C for 4 hrs for 7.5d embryos or overnight for older embryos(9.5d),(or over day if dissection is done in the morning).
4. The next day, wash 2x with PBSw (PBSw=PBS with 0.1% Tween-20)
5. Dehydrate with methanol series (25%, 50%, 75%, and 100% in PBSw). Change 2x in 100% methanol.
6. Store the embryos at -20 °C (up to 2 months).

Situ hybridization protocol

Day 1

1. Dissolved 4% paraformaldehyde fresh in PBS. About 5 ml will be needed for each sample after proteinaseK treatment.
2. Preapartion and dissolves hybridization solution.
3. Rehydrate the embryos through 75, 50 and 25% methanol series in PBSw. Incubate each step for 5 min. on ice.
4. Wash the embryos 3 times for 5 min. with PBSw on ice. Staining for highly expressed gene requires less digestion, but for low expression genes longer digestion may help to get stronger staining. Make sure to thaw the proteinase

K stock completely and vortex to dissolve precipitate at the bottom of the tube. Use aliquots of the proteinase K stock 10mg/ml, do not thaw-freeze repeatedly.

5. Rinse them in PBSw.
6. Wash 2 times with PBSw for 5 min.
7. Refix in 5 ml of 4% paraformaldehyde-0.2% glutaraldehyde in PBSw for 15 min.
8. Rinse in PBSw.
9. Wash 3 times with PBSw for 5 min. each.
10. Wash in 1 ml of 50% PBSw:50% hybridization solution, followed by 100% hybridization solution for about 3 min. each standing.
11. Replace 900 µl of fresh hybridization mix in each Eppendot tubes.
12. Prehybridize samples for 3 hrs at 70ºC.
13. Heat 200 ng of the RNA probe in 100 µl of hybridization mix to 95 °C for 5 min.
14. Add the probe/hybridization mix to the embryos. The final probe concentration should be about 200ng/ml.
15. Hybridize overnight at 70 °C in a incubator.

DAY 2

1. Remove hybridization solution and add 800µl of prehybridization solution. Wash for 5 minutes at 70 °C.
2. Add 400µl of 2X SSC, ph 4.5 (without removing prehybridization solution.) C. Repeat the addition of the 2XSSC washes twice more.
3. Remove the mix and wash twice, 30 min each time, in 2XSSC pH7/0.1% CHAPS 70 °C.
4. Wash twice, 10 min each, in Maleic Acid Buffer (MAB; 100 mM maleic acid, 150 mM NaCl; pH 7.5) at room temperature. Wash twice, 30 min each time, in MAB at 70 °C.
5. Wash twice 10 min each in PBS at room temperature.
6. Wash 5 min in PBSw at room temperature.
7. Incubate the embryos in 1 ml antibody buffer for at least 2 hours at 4 °C with rocking.
8. BMblock-mouse antibody buffer 2.5ml needed for each sample:

- 10% Goat serum (heat inactivated 30 min at 56 °C)
- 1% boehringer blocking reagent in PBSw
- Heat the mixture at 65 °C until total dissolution, filter through 4.5 micron filters (several may be needed), then cool on ice.
- During the blocking step, preabsorbe the antibodies. The dilution for the Alkaline phosphatase conjugate (AP) is 1/10000 from a stock of 150 units/200 µl (Boehringer). Use this solution to replace the blocking solution.

9. Replace buffer with diluted antibody and incubate overnight at 4 °C .

DAY 3

1. Fast wash embryos with 0.1% bavine serum albumin(BSA) in PBSw.
2. Do another 5 washes with 5 ml 0.1%BSA in PBSw (fill to the top to minimize air bubble) rotating for 45 min. each.
3. Wash twice, 30 min. each in PBSw.
4. Take out staining solutions to warm in room temperature (RT).
5. Wash the embryos in AP1 buffer (100 mM Tris 9.5; 100 mM NaCl; 50 mM MgCl2) rocking for 10 min, two times each, at RT.
6. Replace with 1 ml BM purple and rock slowly in the37ºC . BM purple staining takes a few hours to several days, if necessary leave at 4 °C overnight or until background appears.
7. Stop staining reaction by washing in at least three changes of PBS.
8. After staining, dehydrate through methanol series (25%, 50%, 75%, and 2x 100%) and store in methanol at -20 °C.
9. Take pictures after placing back in methanol. BM purple becomes more blue and intense in methanol.

RESULTS AND DISCUSSION

In respect to this important of these gene, that already well done, discussed in the part of laboratory, and finally in the situ- hybridization was conformed.

Sub cloning of Foxa2 genes cDNA in to RNA in vitro transcription vectors

For the generation of the transcription factors, the probes (fig.3-A) were cloned according to the protocol with a number of promoters the probe generation was made by the cloning of the fragments in to blue script. For that it was vector which dephosphorylation by alkaline phosphates, dephosphorylation was successful, the linearization vector seen on the control gel have then insert and purified in order ligation and successful ligation is demonstrated in the Fig 4-B and C.

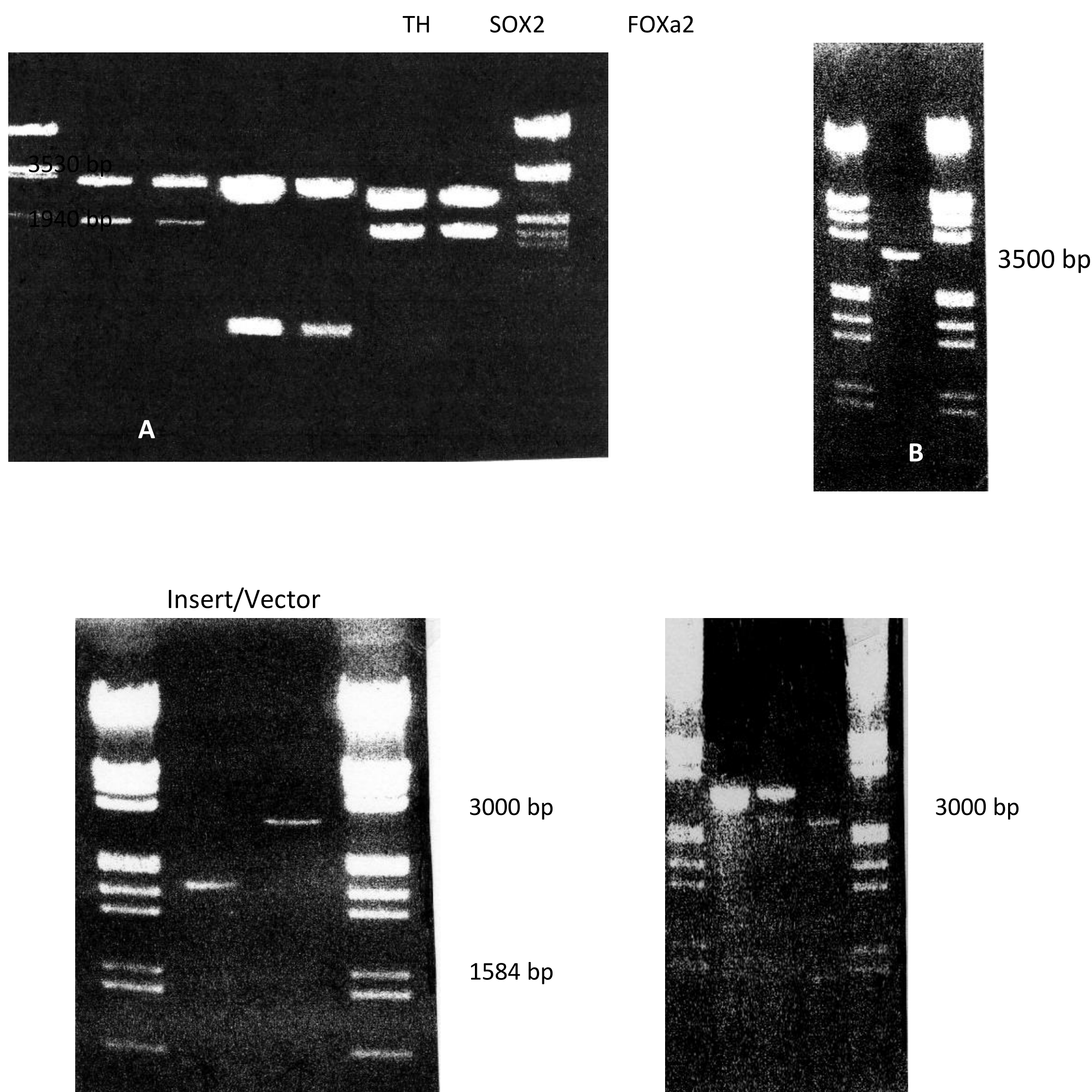


Fig 3. (A)-Restriction analysis for plasmid, (B)-Successful linearization of the vector (C)-Control gel demonstrated for successful ligation of vector to insert.

Results of the situ-hybridization in comparison to the literature

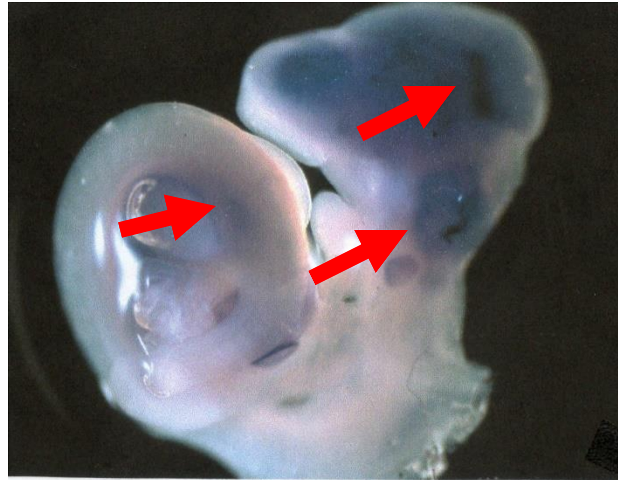
During the gene expression of the embryoid bodies in the mouse, it was different expression pattern in the embryoid bodies and have been shown in the mouse embryo for this ages D 8 and D 10. So, the experiment can manipulation in the mouse and expression during the embryoid development. The expression pattern with the relation to the results in summary reviews from the literature during the expression of the gene which is :

Foxa2 gene:

The investigated gene expression regions, are identical to those of the literature but there are existing differences, are shown in the fore brain, in the spinal cord and in the midbrain/hindbrain (Fig.4).

The forkhead transcription factor, foxa2, is specifically expressed in adult dopamine neurons and their precursors in the medial floor plate. Experiments show this gene, foxa2, is required to generate dopamine neurons during fetal development and from embryonic stem cells. Also show the abnormalities in motor behavior in old age and an associated progressive loss of dopamine neurons. Manipulating forkhead function may regulate both the birth of dopamine neurons and their spontaneous death, two major goals of regenerative medicine (Raja Kittappa et al, 2008).

In the mammalian central nervous system it is an important contingent of dopaminergic neurons are localized in the substantial nigra and in the ventral segmental area of the ventral midbrain. They constitute an an atomically and functionally heterogeneous group of cells involved in a variety of regulatory mechanisms, from locomotion to emotional/motivational behavior. mDA neuron primary cultures represent a useful tool to study molecular mechanisms involved in their development data (Dario Greco et al, 2009).



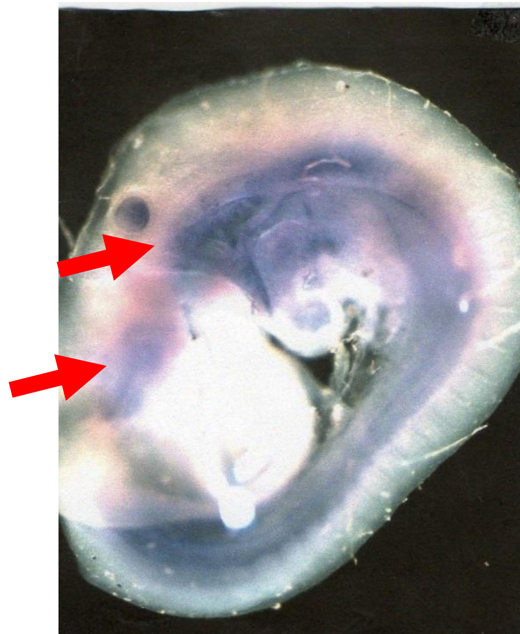


Fig.4 In situ-hybridization probes for Foxa2 antisense expression in mouse EBs , D8.and D10

4. SUMMARY

The study of neuronal differentiation of embryonic stem cells has raised major interest over recent years, because SCs directed to neural differentiation could be the source for a therapeutic in many human disorders. The interest has focus on SCs research in respect for neural differentiation to combined knowledge for medical material and the process for cell therapy. Gene expression regions, are identical to those of the literature but there are existing differences, are shown in the fore brain, in the spinal cord and in the midbrain/hindbrain.

Therefore, in order to understand the progress in the field of the SCs the current literature has been reviewed. The literature concerning the original SCs, the historical aspect of SCs research, and the understanding different diseases stability of cells to repair human diseases.

Then we investigated how neural differentiation occurs *in vivo* and how it is achieve perform *in vitro*. In addition we also made a special emphasis in this work, for genes which has been given to the molecular control of neural differentiation. In this respect there was also we make practical experiments for three of the most important genes have been investigated for the expression pattern in the mouse embryo.

It is still a new science hampered by lack of reliable experiences and thus suffering from numerous difficults. Before envisaging any therapeutic application of such approaches human has been solved brain disorder patients.

Several, still unsolved problems (i) to establish the most adequate in vivo and/or in vitro manipulations to obtain the appropriate cells for transplantation, (ii) to construct a rood map for clinical routes of focal and multifocal CNS disorders, (iii) to learn about the right timing for cell transplantation, and (iv) to exactly determine the appropriate number of cells for transplantation treatments.

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