

DERIVATION OF ES CELLS FROM EARLY STAGE PREIMPLANTATION EMBRYOS AND CHARACTERISATION OF THEIR CARDIAC DIFFERENTIATION POTENTIAL IN MICE

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Most murine embryonic stem cell lines have been derived from the inner cell mass of blastocysts and extensively studied in different aspects including generation of organ specific cells. However, no detailed studies have been made on cardiac specific gene expression, immunocytochemical and electrophysiological characterisation of cardiomyocytes generated from early stage (preimplantation) embryo derived embryonic stem cells in mice. In the present study, new embryonic stem cell lines were derived from early stage preimplantation embryos in mice. *In vitro* differentiation of such cell lines readily generated cardiomyocytes, which expressed different cardiac specific genes in a temporally regulated manner as well as cardiac cells specific proteins. This is probably the first report, which showed the temporal pattern of cardiac specific genes as well as protein expression in cardiac cells generated from *in vitro* differentiation of preimplantation embryo derived ES cells.

Introduction

Since the discovery of the embryonic stem (ES) cells in mice in early eighties^[1,2], a number of stem cell lines have been derived in different species including human being. Providing specific culture conditions, ES cells can be maintained in undifferentiated state for a prolonged period of time.^[3,4,5,6] An important characteristic of ES cells is their ability to undergo a spontaneous differentiation *in vitro*^[3,7,8,9] forming a variability of progenitors and differentiated cells. The ability of ES cells to differentiate into any tissue represents an enormous therapeutic potential to treat different diseases. Further, the remarkable characteristic of ES cells to contribute germ line transmission has paved the way for generating transgenic animals more efficiently.^[10]

Apart from inner cell mass (ICM), ES cells have been derived from very early stage embryos in different species including human being.^[11,12,13,14,15,16] The analysis of these cells was mainly focused on different aspects of pluripotency. ES cell derived from early stage embryos may have added advantage than that of blastocyst derived ES cells not only in therapeutics, but also in animal reproduction. This is because the stage of embryonic development at which ES cells are derived seems crucial to their subsequent function in experimental models and cell therapy.^[14,17] For example, the preblastocyst period in murine development

constitutes a time when blastomeres are not yet committed to either ICM or the trophectoderm lineage.^[18] Therefore, it is assumed that ES cells derived from morula or even more earlier stage embryos could exhibit a broader differentiation potential than those of ICM or epiblast derived ES cells^[14]. This could primarily be the result of variation in epigenetic status of the ES cells as epigenetic memory of the embryo used to derive ES cells may influence the developmental potential of the ES cells obtained.^[14] The epigenetic changes would be more in blastocyst derived ES cells than morula derived ES cells as extended *in vitro* culture are known to cause methylation defects that can result in abnormal embryo development.^[19] At the morula stage, both parental genomes are hypo-methylated and genome wide de novo methylation occurs at the blastocyst stage preferentially in the ICM.^[20]

In the present study we report the derivation of new ES cell lines from early stage murine preimplantation embryos which readily exhibit cardiac differentiation potentiality.

Materials and Methods

Mice and embryos

I29/ SvPassico (I29S2/SvPassCrl) male and female mice

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(Charles River, Germany) were used in the present study. Mice were kept on a light dark cycle of 12:12 hr regime with dark period from 6.30PM to 6.30AM. Blastocyst for ES cell injections were obtained from BL6=C57BL6 mice. BL6/BalbC female were used as recipients for embryo transfer. All the embryos were flushed from the uterine horns with M2 medium (Sigma-Aldrich, Munich, Germany).

Superovulation and embryo collection

The adult 129/ SvPassico strain of mice were superovulated with injection of 5IU PMSG (Intervet) followed by 5IU human chorionic gonadotrophin (Intervet) after about 48 hrs of PMSG injection. Injected females were placed with 129/SvPassico male and mating was confirmed in the next morning by the presence of vaginal plug.

Culture of embryos and ES cell derivation

The culture of embryos and derivation of ES cell lines were done as described by Tesar.^[14] Briefly, morula stage embryos were isolated from 3.0 pc (post coital) mice. The zona pellucida of embryos was removed with brief exposure to Tyrodes saline acidified to pH 2.5.^[21] The zona pellucida-free embryos were placed on inactivated CF1 feeder (0.2×10^6 /well) in 6 well tissue culture plates. The culture medium used was DMEM (Invitrogen) with 15% fetal bovine serum (Sigma), 2mM L-glutamine (Invitrogen), 0.1mM 2-mercaptoethanol (Invitrogen), 1x non-essential amino acids (Invitrogen) and 1000 units/ml recombinant murine leukaemia inhibitory factor (ESGRO, Chemicon) in a humidified incubator at 5% CO₂ and 37°C. Penicillin-streptomycin (Invitrogen) was used only for culture of CF1 feeder and zona-free embryos but culture of established ES cell lines was always done in ES cell medium without antibiotics. Entire cell colonies formed by the individual embryos were cut manually by the tip of a needle and aspirated with a fine glass pipette. The individual cell clones derived from embryos were put in drop of PBS (phosphate buffered saline) for serial washing to remove medium with serum. The washed cell clones were put into droplets of 0.05% trypsin-EDTA (Invitrogen) for about five minutes at 37°C. The cell clumps were gently pipetted in and out through a fine glass tube and remaining large clumps were mechanically dissociated into small clumps by the tip of a needle. The dissociated cells as well as small cell clumps were put onto a fresh inactive CF1 feeder-layer in a 6-well culture dish in ES cell medium. After 4-6 days, cells were passaged again with trypsin-EDTA onto a fresh feeder layer. Resulting ES cell colonies were propagated 3 to 4 times depending on the number of cells before they were cryopreserved in liquid nitrogen. Cryopreservation of ES cells was done by suspending ES cells in freezing medium having 90% FBS and 10% dimethyl sulfoxide (DMSO, Sigma). The ES cells were kept in -80°C for 48 hrs and then transferred in liquid nitrogen vapour.

Immunocytochemistry

For immunostaining, the ES cells were cultured about 48 h or till small colonies of ES cells were formed on cover slips. The cells were washed with PBS twice and then fixed with absolute methanol for 15 minutes at room temperature. The

cells were then blocked with 5% bovine serum albumin (BSA) in PBS for one hour at room temperature. The cells were again washed with PBS for 30 minutes at 10 minutes interval. After washing, the cells were incubated at 4°C overnight with primary antibodies diluted in 1% BSA in PBS. After washing with PBS, the cells were again incubated with fluorescent secondary antibodies (Molecular probes, 1:1000) along with DAPI (1:2000) for 1 hr at room temperature. The cells were further washed with PBS for 30 minutes with changing PBS at 10 minutes interval and mounted with antifading agent (Pro Long Gold, Molecular probes). The primary antibodies used were: mouse monoclonal primary antibody against Oct-3/4 (Santa Cruz biotechnology, INC; 1:200), goat polyclonal Nanog (1:40) (Santa Cruz biotechnology, INC), mouse monoclonal SSEA-1 (MC-480, Developmental studies Hybridoma Bank; 1:400) and mouse monoclonal SSEA-3 (MC-631, Developmental studies Hybridoma Bank; 1:400). Alkaline phosphatase staining was done as described.^[22]

Flow cytometry

After about 48 hrs of culture, ES cells were trypsinised and a single cell suspension was prepared. Cell clumps were removed by passing through a cell sieve. The ES cells were immunostained with PE conjugated primary antibody SSEA-1 (Sigma). A parallel isotype control was also run. Acquisition of 20,000 cells was done with a FACScan (BD Biosciences, Heidelberg, Germany) and the data analysis was done with CellQuest software (Becton Dickinson, Heidelberg, Germany) for calculating SSEA-1 positive cells.

Sexing of ES cells

For sexing, genomic DNA was isolated from all the five cell lines. Sexing was done by PCR using Y-chromosome specific primer sequence (See table below).

Chromosome count

The number of chromosomes was studied in different cell lines by giemsa staining as per the protocol of Department of Genetics, School of Medicine, University of Pennsylvania, USA. In brief, ES cells after 2 days of passage were arrested in the metaphase by adding colcemid (0.02µg/ml at a final concentration, PPA laboratories, Pasching, Austria) to the culture medium for 1 hr. The cells were treated with trypsin-EDTA for 5 min at 37°C. After vigorous pipetting, the single cell suspension was centrifuged at 500g for 5 min. The pellet was exposed to hypotonic shock by 0.56% KCL solution for 10 minutes at room temperature. After centrifugation at 500g for 5 min, the hypotonic solution was removed and the pellet was loosened via gently flicking and fixed with methanol-acetic acid (3:1). After 5 min, the suspension was

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The list of primers used for amplification of each transcript were:

Name	Primer sequence	Product size
<i>A-MHC</i>	F----GATGGCACAGAAGATGCTGA R----CTGCCCTTGGTGACATACT	120 bp
<i>B-MHC</i>	F----CCCTCCTCACATCTTCTCCA R----GCGGCAATAACAGCAAATA	150 bp
<i>Gata-4</i>	F----TCAAACCAGAAAACGGAAGC R----GTGGCATTGCTGGAGTTACC	107 bp
<i>NKx2.5</i>	F----CCACTCTCTGCTACCCACCT R----CCAGGTTCCAGGATGTCTTTGA	107 bp
<i>CTnT</i>	F----GAGGAGGTGGTGGAGGAGTA R----GGCTTCTTCATCAGGACCAA	150 bp
<i>MLC2v</i>	F-----AAAGAGGCTCCAGGTCCAAT R-----TCAGCCTTCAGTGACCCTTT	140 bp
<i>Nanog</i>	F-----CAAACCAAAGGATGAAGTGC	111 bp
<i>Oct4</i>	F----AGGAAGCCGACAACAATGAG R---GAGCAGTGACGGGAACAGAG	429 bp
<i>Nestin</i>	F-----GGAAGAAGTTCCCAGGCTTC R-----ATTAGGCAAGGGGAAGAGA	139 bp
<i>AFP</i>	F----CCA GAA CCT GCC GAG AGT TGC R----GCC TTC AGG TTT GAC GCC ATT	552bp
<i>CdX2</i>	F-----GATACATCACCATCAGGAGGAAA R-----CAAGGAGGTCACAGGACTCAAG	236bp
<i>Tbrachyury</i>	F----CAT GTA CTC TTT CTT GCT GG R----GGT CTC GGG AAA GCA GTG GC	313 bp
<i>Y chromosome</i>	F-----GTA GGA AGA ATC TTT CTC ATG CTG G R-----TTT TTG AGT GCT GAT GGG TGA CGG	313 kb
<i>GAPDH</i>	F---GGTGCTGAGTATGTCGTGGA R---CGGAGATGATGACCCTTTTG	97 bp

centrifuged again for 5 min. Fixation was performed twice prior to spreading the cells on ice cold slides. The slides were air-dried, stained in freshly made Giemsa (Carl Roth, Germany) for 30-40 min and rinsed with tap water. Some slides were also stained with Hoechst dye for 10 min, washed with water and examined under a fluorescence microscope.

Production of chimeric mice

About 10-15 ES cells were introduced into the blastocoel cavity (BL6=C57Bl6) by microinjection using micromanipulator (Carl Zeiss). After microinjection, the blastocysts were cultured for 2-2.5 hrs in CO₂ incubator before being transferred into pseudopregnant (BL6/BalbC) foster mothers. Chimeric progeny were identified on the basis of their brown coat color.

In vitro differentiation of ES cells

The differentiation ability of the newly derived ES cells was examined by suspension^[23] as well as hanging drop culture^[24]. Briefly, for hanging drop culture, an ES cell suspension of 2.5 × 10⁴ cells/ml was prepared in differentiation medium (IMDM supplemented with 20% fetal calf serum, 1% non-essential amino acids, 2 mmol/l L-glutamine, and 100 μmol/l β-ME). Of this ES cell suspension, 20 μl was spotted on the inside of the upper lid of a 10 cm bacteriologic dish and then covered over its bottom dish

containing 5 ml phosphate-buffered saline. For mass culture, about one million ES cells were suspended in 10 ml of differentiation medium and agitated in bacteriological dishes. On day 2, the multicellular aggregates (EBs) that have formed were transferred to new dishes with 10 ml differentiation medium kept in culture up to day 19 with changing medium periodically. For transgenic cell lines (see below), medium was supplemented with 5μg/ml puromycin (Sigma-Aldrich) on day 8 or 9 of culture to select for cardiomyocytes. Medium was changed every 2-3 days. Selection was continued till day 20 of differentiation.

Generation of transgenic ES cell lines for cardiac lineage selection

For cardiac lineage selection, BHK-1 was used to generate transgenic ES cell lines. For this purpose, the αPIG vector described by Kolosov *et al.* 1998^[25] was used. The αPIG vector contains an expression cassette for puromycin-acetyltransferase under control of the cardiac specific alpha myosin heavy chain promoter. The coding sequence for enhanced green fluorescent protein (eGFP) is hooked up via an internal ribosome entry site to the puromycin-acetyltransferase sequence. The αPIG vector was linearised by SacI restriction enzyme. About 40 μg of this vector DNA was used for transfection. The transgenic cells were selected using geneticin (400μg/ml, Invitrogen). The transgenic clones were propagated at least 4 times before differentiating them into beating EBs. Twelve transgenic clones were differentiated *in vitro* and assayed for the formation of

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beating areas and eGFP expression. Clone five (CL-5) was selected for further characterisation of EBs and ES cell-derived cardiomyocytes.

Culture of cardiomyocytes and whole EB of transgenic BHK-1 for immunostaining

The beating EBs were gathered under a microscope and collected in a 15ml tube. The EBs were treated for 20-30 minutes at 37°C with 1ml of collagenase-B (1mg/ml, Roche Diagnostics GmBH, Germany) with intermittent pipetting. After incubation, the cell suspension was gently resuspended to achieve single cell suspension. Cells were washed with differentiation medium. The cell pellet was resuspended in differentiation medium and plated on fibronectin-coated cover slips. Plated cells were cultured for 3-4 days. Further, a collection of EBs having beating one were cultured on gelatin coated (0.1% in PBS) cover slip in differentiation medium for about 48 hrs for their attachment.

Immunostaining of cultured cardiomyocytes and EBs

The cultured cardiomyocytes and attached EBs were washed with PBS and fixed with 99% methanol for 15 minutes at room temperature. After fixation, cells were blocked with 5% BSA (Sigma) in PBS for 1 hr and washed for 30 minutes with PBS after changing PBS at 10 minutes interval. The cultured cardiomyocytes and EBs were incubated with primary antibodies singly or in combination viz. α -actinin, α -actinin+desmin, α -actinin+connexin-43, α -actinin+vimentin and α -actinin+cardiac troponin-I. After washing with PBS for 30 min at 10 min interval, cultured cardiac cells and EBs were incubated with fluorescent-labelled secondary antibodies (Molecular probes) for 1 hr. The cells and EBs were washed thoroughly with PBS and mounted with antifade reagent (Molecular probes). The fluorescence staining was observed using a Zeiss Axiovert 200 (Zeiss, Jena, Germany). The primary antibody used were anti mouse alpha-actinin (IgG1, 1:800, Sigma); anti mouse desmin (IgG1, 1:100, Neomakers, Fremont, CA), anti mouse vimentin (IgM, 1:200, Sigma-Aldrich), antimouse connexin-43 (IgM, 1:400, Sigma-Aldrich), antimouse monoclonal anti cardiac toponinI (IgG2b, 1:200, Chemicon).

RNA isolation from ES cells and differentiating EB during different days of in vitro culture.

For studying the expression of *oct-4* and *nanog*, total mRNA was isolated from about one million cells of each five ES cell clones. After about 48 hrs of culture, cells were trypsinized, spun down and used for isolation of total RNA. About 40-50 EBs were collected on different days of differentiation (described above) between day-0 (ES cells) and day-19 of culture. Prior to RNA isolation, EBs were dissociated into single cells using 0.05% trypsin. Total RNA was prepared using the pure link micro to midi Total RNA purification kit (Invitrogen).

Reverse transcription PCR (RT-PCR) analysis of marker gene expression in ES cells and differentiating EBs

Analysis of *Oct3/4*, *Nanog*, *nestin*, *AFP*, *cdX2*, *T-brachury* and different cardiac specific genes viz. α -MHC, β -MHC, *Gata-4*, *NKx2.5*, *CTnT*, *MLC2v* were performed by RT-PCR cDNA was synthesized from ~1 μ g of total RNA using superscript II reverse transcriptase (Invitrogen, Karlsruhe,

Germany). cDNA samples were subjected to PCR amplification with primers selective for different transcripts. The Jumpstart Red Taq ready master mix (Sigma) was used in the PCR. The PCR protocol was followed as: 94°C-2 min; 94°C-35s; 56-58°C for 45s; 72°C-1min 45s; step 2 to 4 were repeated 29 to 35 times; 72°C- 5 min; hold -4°C. The PCR products were size fractionated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Results and Discussion

Derivation of ES cells and their characterisation

Zona free embryos were plated on inactivated CF1 feeder (conditioned with ES cell medium) in 6-well culture dishes. The embryos were not disturbed until day 4 or 5 when they had formed colonies on the feeder layer (Fig.1A-G). Most of the embryos attached to the feeder within 24 hrs. Few embryos developed a blastocyst-like structure having a single layer of cells surrounding the embryos and a cell mass in one pole. These embryos also formed primary colonies. It took about 19 to 31 days to derive ES cell lines from morula stage embryos including 4 to 5 passages of the individual clones and cryopreservation of the cells (Table 1). Five independent cell lines (BHK-1, 2, 3, 4 and 5) were derived from culture of 22 morula stage embryos, which was about 22.7% of the total embryos cultured (Table 2). The newly derived ES cell colonies are shown in Figure 1H. The cell lines were passaged every 48h. All five ES cell lines were positive for alkaline phosphatase and stained positive for ES cell specific markers SSEA-1, SSEA-3, Nanog and Oct-3/4 (Fig. 2 A-E). These ES cells also expressed the transcription factors Nanog and Oct-3/4 (Fig. 2F). FACS analysis revealed about 94.0%, 94.2%, 91.4%, 88.7% and 67.7% SSEA-1 positive cells for BHK-1 to BHK-5, respectively.

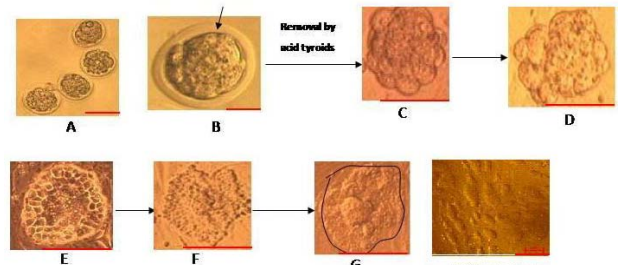


Fig. 1. Derivation of ES cells from early stage murine embryos (morula). Fig A to G indicates the sequential events during culture of murine early stage embryos. (A, B): Day-0 Morula (20x), (C): Morula after ZP removal, (D): Morula after Day-1 of culture, (E): Morula after Day-3 of culture, (F): Morula after Day-4 of culture, (G): Morula after Day-5 of culture (10x), (H): Established new cell colonies (4x).

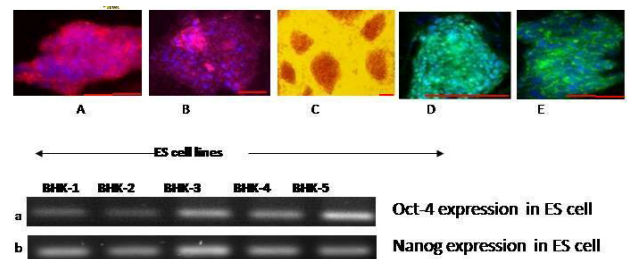


Fig. 2. Immunohistochemical (A-E) and transcriptional expression (F) of different stem cell specific markers in newly derived cell lines in mice. (A):SSEA-1 (40x), (B):SSEA-3 (20x), (C): Alkaline phosphatase (10x), (D): Oct-3/4 (63x), (E): Nanog (40x), (F): Expression of Oct4 (a), Nanog (b) in BHK-1.2.3.4.5. (c)-GAPDH.

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Table 1. Time taken for derivation of ES cells from morula of mice

ES cell name	Date of Embryo culture	1st passage (After)	2nd passage (After)	3rd passage (After)	4th passage (After)	5th passage (After)	6th passage (After)	Time taken for first freezing (From date of embryo culture)
BHK-1 (Morula)	D-0	D-7	D-10	D-15	D-17	D-19 (Frozen)		19 Days
BHK-2 (Morula)	D-0	D-3	D-10	D-15	D-19 (Frozen)			19 days
BHK-3 (Morula)	D-0	D-3	D-7	D-10	D-15	D-22 (Frozen)		22 Days
BHK-4 (Morula)	D-0	D-7	D-15	D-19	D-23	D-26	D-30 (Frozen)	30 Days
BHK-5 (Morula)	D-0	D-4	D-15	D-24	D-29	D-31 (Frozen)		31 days

Table 2. Table showing derivation of ES cell lines from early stage murine embryos

STRAIN OF MICE	NUMBER OF EMBRYOS CULTURED		NO OF ES CELL LINE DERIVATION FROM	
	Morula	Blastocyst	Morula	Blastocyst
Him-1	33	14	0	0
129 SV/Passico	22	0	5	---

Sexing, karyotyping and production of chimeric mice:

Out of five ES cell lines, 4 were male and one was female genotype (Fig.3A). Chromosome counting by giemsa analysis revealed that BHK-1, 2 and 3 contain 40 chromosomes within 90.90% (n=11), 65.51% (n=29) and 91% of the spreads. ES cell line BHK-4 was found to contain 38 chromosomes per cell in 87.5% of the chromosomal spreads (n=24) (Fig. 3B, Table 3). BHK-5 (n=38) showed abnormal

karyotype with more than 40 chromosomes per cell.

BHK-1 and 2 were injected into blastocysts and gave rise to chimeric mice. The BHK-1 was injected in 60 blastocysts and produced 4 chimeras whereas BHK-2 was injected into 44 blastocysts and produced 4 chimera (Fig. 3C, 3D).

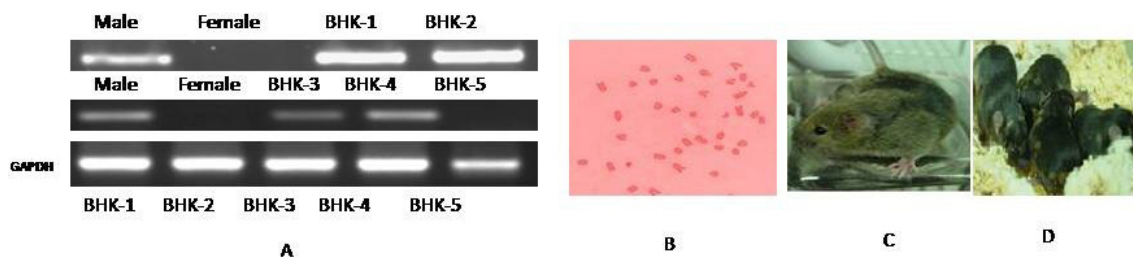


Fig. 3. Showing sexing (A), chromosomal spread (B) and generation of chimeric mouse (C-BHK-1, D-BHK-2) from new cell lines (See text for detail).

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Table 3. Chromosome numbers in newly derived ES cells.

Cell line		Chromosome numbers									
		38	39	40	41	42	43	56	77	76	80
BHK-1	P10			(90.10%)							9.9%
BHK-2	P28	10.34	6.89%	65.51%	13.79				12.5%		
BHK-3	P7			91%							
BHK-4	P7	87.5	6.25%								
BHK-5		Diploid and as well as abnormal chromosomal number(38-80)									

In vitro differentiation of ES cells and genomic expression

Since the methylation of such morula derived ES cells might be different than ICM derived ES cells, we tested the differentiation capacity. Cells of all five lines generated embryoid bodies when cultured in suspension or in hanging drops in differentiation. BHK-1 was tested for the expression of key markers of the three germ layers. The expression of ectodermal (*Nestin*), mesodermal (*T-brachyury*), endodermal (*AFP*, *cdx2*) and cardiac (*Nkx2.5*) cell specific genes during *in vitro* differentiation were tested for BHK-1(Fig. 4). The expression of *AFP* and *cdx2* were observed from day-7 and day-4 onwards. *T-brachyury* was expressed on day 4 and 5 only, while the expression of *Nkx2.5* was detected from day-5 onwards. *Nestin* was present at all days of differentiation.

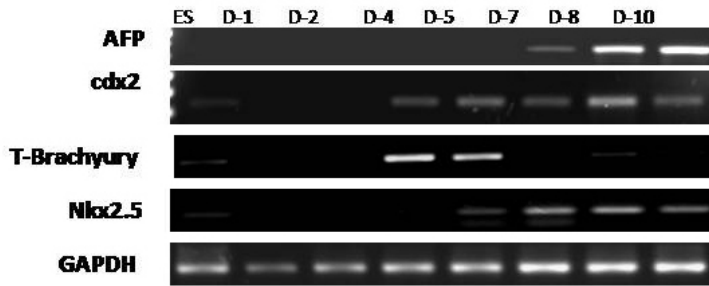


Fig. 4. Genomic expression during differentiation of ES cells derived from morula as a marker for multipotency in mice strain (BHK-1)

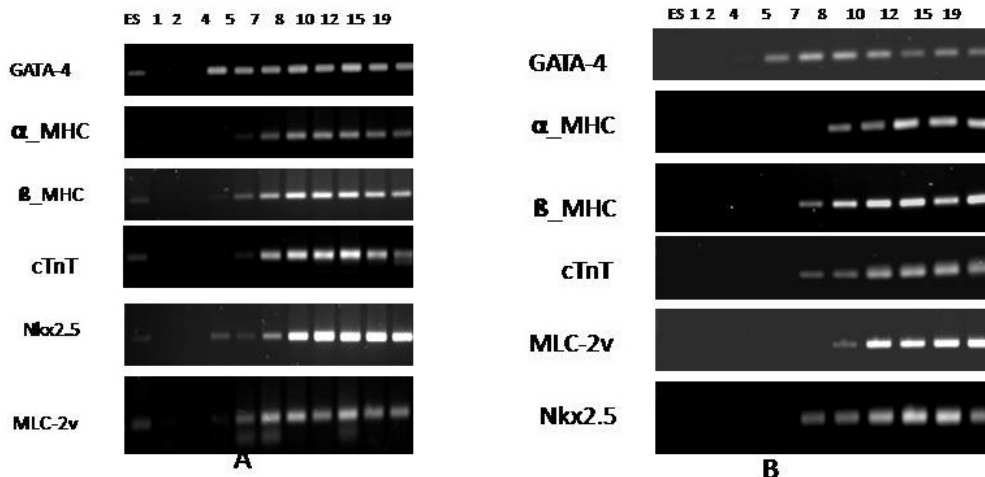


Fig. 5. Cardiac specific genomic expression during differentiation of ES cells derived from morula (A-BHK-1. B- BHK-2) in mice.

Expression of cardiac specific genes in differentiating ES cells

The expression of α -MHC, β -MHC, *cTnT*, *Nkx2.5*, *GATA-4* and *MLC2v* were analysed during the development of BHK-1 and BHK-2 EBs. In BHK-1 EBs, the expression of *GATA-4* and *Nkx2.5* started on day-4, β -MHC and *MLC2v* from day-5 onwards while the expression of α -MHC, *cTnT* was observed from day-7 onwards (Fig.5A). In BHK-2, the expression of *GATA-4* was observed from day-5 onwards, while α -MHC, β -MHC, *cTnT* were started from day-7 onwards. *MLC2v* expression was observed beginning on day-8 (Fig. 5B).

In vitro differentiation of transgenic ES cells and immunocytochemistry of cardiac specific marker proteins.

BHK-1 ES cell line was transfected with a cardiac lineage selection construct to express eGFP and puromycin-acetyltransferase under the control of the cardiac specific alpha myosin heavy chain promoter (see materials and methods for details). Twelve stable transfected clones were chosen randomly for *in vitro* differentiation. Four clones showed beating embryoid bodies and one clone showed maximum beating embryoid bodies with GFP expression (Fig. 6A,6B, 6C). In the other three clones, the GFP expression was detectable in a small number of EBs as well as in small areas of beating EBs. The clone exhibiting higher GFP expression in EBs during differentiation was used for immunostaining and electrophysiological studies. The

cultured cells (Fig.6D, 6E) of beating EBs expressed various cardiac specific marker and gap junctional proteins viz. α -actinin, desmin, connexin-43, vimentin and cardiac troponin (cTnI) and Titin (Fig.7). Similarly, the attached beating EBs also expressed i.e. α -actinin, cTnI, connexin-43 desmin and, vimentin (Fig.7).

SV129 strain of mice [26] while only 2.0 and 2.96% have been reported for C57BL/6 and BALB/c strain, respectively. [27] We could not derive ES cells from Him-OF1 (Table 2), neither from morula nor blastocyst (results not shown). The establishment of ES cells not only differs with culture condition [27] but also with strain of mice. [28,29] We observed a formation of primary colonies by morula of Him-OF1 strain mice but it was impossible to split the primary colonies with

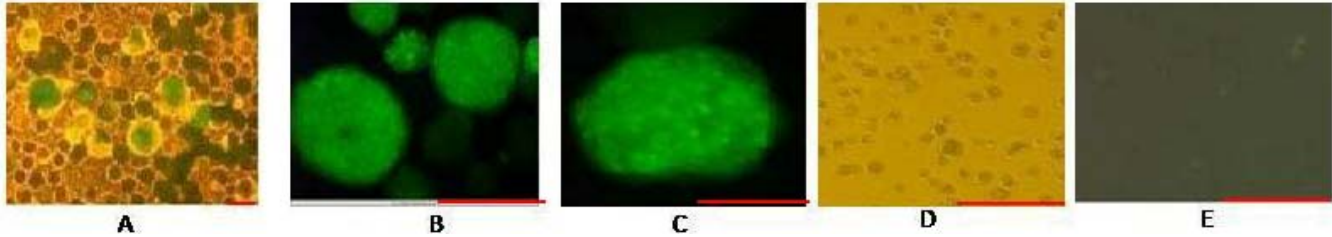
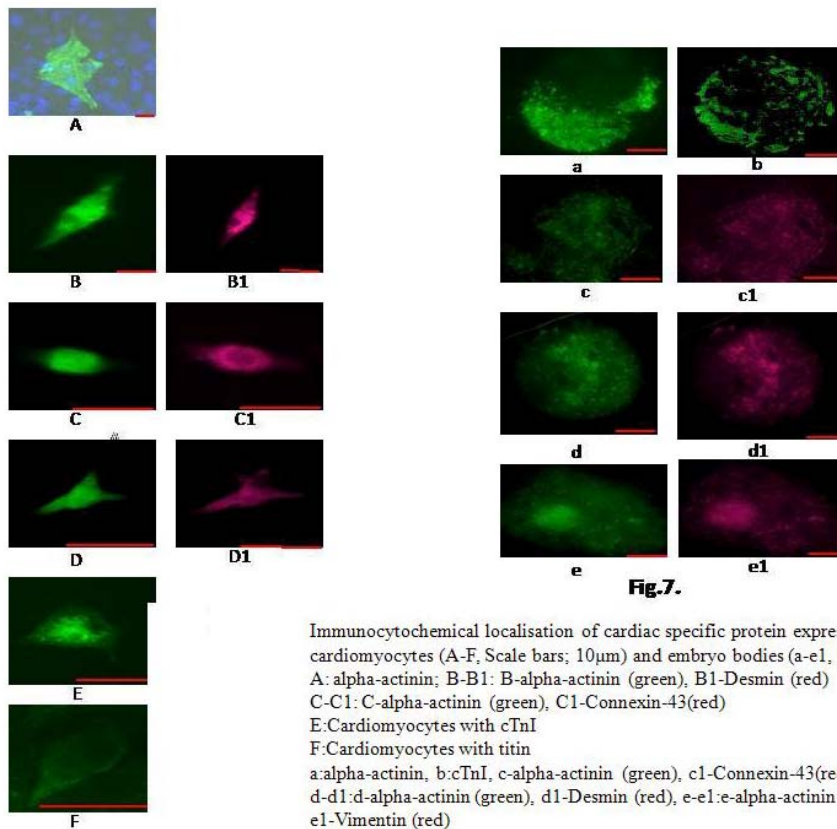


Fig. 6. GFP expression in beating EB under puromycin selection in transgenic cell line-1. (A): Day 14 of differentiation (4x), (B): Day-15 of differentiation (32X), (C): Day-24 of differentiation (32X), (D): Cardiac cells from puro selected beating EBs during culture (32X), (E): Cardiac cells showing GFP expression during culture (32X)

The present study was aimed at understanding the characteristics of ES cells derived from early stage preimplantation embryos in murine species. It took an average of 19 to 31 days to derive the ES cells after culture of morula stage embryos (Table 1). The percentage of ES cell derivation was about 23% of the total embryos cultured (Table 2). A frequency of 10-30% has been reported for

0.05-0.25% trypsin-EDTA, pronase (0.5%) or different types of collagenase because of the presence of fibrous matrix embedding the cells of primary culture. When some of the colonies were again plated after trypsinisation on CF1 feeder, the colonies they formed behaved similarly. We concluded that derivation of ES cell lines in Him-OF1 strain is quite difficult with the protocol followed here.



Immunocytochemical localisation of cardiac specific protein expression in cultured cardiomyocytes (A-F, Scale bars; 10 μ m) and embryo bodies (a-e1, Scale bars; 20 μ m).
 A: alpha-actinin; B-B1: B-alpha-actinin (green), B1-Desmin (red)
 C-C1: C-alpha-actinin (green), C1-Connexin-43(red)
 E:Cardiomyocytes with cTnI
 F:Cardiomyocytes with titin
 a:alpha-actinin, b:cTnI, c-alpha-actinin (green), c1-Connexin-43(red)
 d-d1:d-alpha-actinin (green), d1-Desmin (red), e-e1:e-alpha-actinin (green), e1-Vimentin (red)

Fig.7. Immunohistochemical localisation of cardiac specific sarcomeric protein expression in cultured cardiomyocytes (A-F) and Embryo bodies (a-e1).

(A): alpha-actinin, (B-B2): B-alpha-actinin (20x), B- Desmin (20x), (C-C1): C- alpha-actinin (40x), C1-Connexin-43(40x), (D-D1):D-alpha-actinin (40x), D1-Vimentin (40x); (E): Cardiomyocytes with cTnI (40x), (F):Cardiomyocytes with Titin (63x)

(a): alpha-actinin (10x), (b): cTnI(10x), (c-c1): c-alpha-actinin (10x), c1-Connexin-43(10x); (d-d1): d-alpha-actinin (10x), d1-Desmin (10x), (e-e1) : e-alpha-actinin (10x), e1-Vimentin(10x)

All the five ES cell lines derived from SV129 morulae showed positive immunostainings for SSEA-1, SSEA-3, Oct-4 and Nanog as well as alkaline phosphatase activity (Fig. 2). These results indicated that the newly derived independent cell lines satisfied some criteria for pluripotent cells which are exhibited by the ICM derived ES cell lines i.e. D3. The majority of our ES cell lines had a normal karyotype in more than 80% of the cells (Table 3). These data are well above the normal range (35%–73%) for normal ES and nuclear transfer-ES cell lines.^[30,31,32,33] ES cell lines having more than 40% of cells with a normal karyotype have been suggested to be efficient for germ line transmission due to successful segregation of chromosomes throughout meiosis^[12,31]

All the five cell lines formed embryoid bodies, which is a criterion of pluripotent ES cells derived from ICM. In the present study key markers were examined to confirm the ability of the morula derived ES cells to form different body cells- a pointer of pluripotency. During *in vitro* differentiation, the embryoid bodies of morula derived ES cells exhibited expression of genes specific for three germ layers i.e. ectoderm, endoderm and mesoderm (Fig. 4). Previous studies have also shown that differentiating embryoid bodies from ICM derived ES cells express different germ layer specific genes.^[34] However, these ES cells have less potentiality to contribute to trophoblastic cells which makes them pluripotent but not totipotent^[35]. Morula derived ES cells because of their more primitiveness can make trophoblastic cells more readily which has been partially proven by the result of cloning experiments where blastomeres from morula resulted more live births of cloned animals than ICM cells or cultured ICM cells.^[36,37]

The expressions of cardiac specific genes (Fig. 5A and 5B) were not quite similar as compared to ICM derived cells as reported earlier.^[38] Interestingly, the expression of Nkx2.5 were little delayed (Fig.5A,5B) in the morula derived ES cells.^[38] In the present study, the expression of GATA-4, B-MHC and MLC2v was earlier in morula derived embryonic stem cells than ICM derived ES cells. This may indicate that morula derived ES cells may be better in generation of cardiac cells than ICM derived ES cells. The ES cell derived cardiomyocytes express cardiac gene products in a developmentally controlled manner that recapitulate the development of cardiomyocytes from very early cardiac precursor cells (Nkx2.5, α -cardiac-actin and GATA-4) to terminally differentiated cells (α - and β -cardiac myosin heavy chain (MHC)^[39] (a-tropomyosin)^[40], myosin light chain 2v (MLC-2v)^[41], atrial natriuretic factor and type B natriuretic factor.^[42] These genes express phenotypes of atria (atrial natriuretic factor, α -heavy chain myosin, atrial light chain myosin) and ventricular cardiomyocytes (ventricular myosin light chain). The first gene to display enhanced expression has been observed as GATA-4^[43] a transcription factor known for its significant role in regulating different genes involved in the differentiation and development of the heart.^[44] As in early myocardial development, mRNAs encoding GATA-4 and Nkx2.5 transcription factors appear in differentiating EBs before mRNAs encoding myosin light chain (MLC-2v), alpha-myosin heavy chain (α -MHC), beta-myosin heavy chain (β -MHC) and cardiac troponin-T (cTnT).^[38] In the present study, almost similar temporal expression pattern were observed in both the cell tested (BHK-1 and BHK-2). This is probably the first report in expression profile of cardiac specific genes in morula derived ES cells in mice.

In conclusion, the ES cells derived from early stage murine embryos were able to differentiate easily towards cardiac lineages. This is probably the first report which showed the temporal pattern of cardiac specific genes as well as protein expression in cardiac cells derived ES cells developed from very early stage murine embryos.

ACKNOWLEDGEMENT:

We thank Department of Biotechnology, Government of India for providing financial support as fellowship to the first author for working in the Institute of Neurophysiology, University of Cologne, Germany.

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