CHAPTER – 3

Materials and Methods
3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 PLASTIC WARE AND GLASS WARE

All the glasswares used in the present investigation were made of high-grade pyrex glass. The glassware, wherever used, were thoroughly cleaned, rinsed with Ultrapure water and then heat sterilized at 250°C for 4 h. Pasteur pipettes were from Labco, Ambala, India. Disposable 35 mm x 10 mm cell culture Petri dishes, 15 and 50 ml Falcon tubes, 100 mm x 100 mm square Petri dishes with 13 mm grid were purchased from Nunc Roskilde, Denmark. Disposable plastic syringes were non-toxic and non-pyrogenic from Sigma Aldrich Chemicals (Norm-Ject, Henke-Sass Wolf GmbH, Germany). Disposable 19-guage hypodermic needles were from Dispovan (Hindustan syringes and medical devices Ltd. Faridabad, India) whereas the 0.22 and 0.45 µm filters were from Millipore Corporation, Bedford, MA, USA. Autoclavable disposable tips for micropipettes were obtained from Labware, USA. French straws (0.25 ml) were from IMV, L’Aigle, France.

3.1.2 CHEMICALS, CELL CULTURE MEDIA AND SUPPLEMENTS

The culture media used in the present study, which included tissue culture medium-199 (M-199), Dulbecco’s modified eagle’s medium (DMEM) Dulbecco’s phosphate buffered saline (DPBS) and the additives which included bovine serum albumin (BSA), porcine follicle stimulating hormone (FSHp) and antibiotics (gentamicin, penicillin and streptomycin were purchased from Sigma Chemical Co., St. Louis, MO, USA. All the cell culture media were in the form of ready-to-use liquid media. Mineral oil, enzymes (Hyaluronidase, Pronase E, Trypsin-EDTA), Phytohemagglutinin (PHA-L) and other chemicals used were also from Sigma Chemical Co., unless otherwise indicated. Most of the chemicals used were embryo culture tested or of cell culture grade. Fetal bovine serum (FBS) was from Hyclone (Logan, Utah, US). Knock-out DMEM and Knock-out serum replacer were purchased from Gibco, NY, USA.

For the characterization of ES cells by examining the expression of pluripotency markers, which included surface-antigens and/or intracellular proteins, the
primary antibodies were purchased from Chemicon International, Inc., Temecula, CA, USA. Secondary antibodies like goat anti-rabbit IgG-FITC conjugate was purchased from Chemicon International, Inc., Temecula, CA, USA, whereas goat anti-rat IgM-FITC conjugate was purchased from Pierce Biotechnology Inc., Rockford, IL, USA. Antibody details are provided in annexure.

3.1.3 EQUIPMENTS

3.1.3.1 MICROSCOPE

i) Zoom stereomicroscope: Low magnification zoom stereomicroscopes (NIKON, Japan, Model SMZ-2T) were used for searching the aspirated oocytes, evaluating the topography and quality of the oocytes, cell cultures and tissue explants.

ii) Inverted microscope: An inverted microscope (NIKON, Japan, Model TMD) was used for the examination of cell cultures, monitoring the cell health, morphological characteristics and growth of the cultured cells. The microscope with the light source at the top and a long working distance condenser allowed cells in culture dishes or flasks to be viewed and photographed whenever needed. The microscope was equipped with an incubator attachment, so that optimum temperature conditions could be maintained during working. The inverted microscope was also equipped with UV fluorescence and differential interface contrast (DIC) attachment, which helped in micromanipulation of the oocytes and capturing the images of the cell cultures and in vitro produced embryos. The microscopes were equipped with programmable still photography and video recording facilities.

iii) Compound microscope: A compound microscope (NIKON, Model MICROPHOT-FXA) with a movable slide holding stage and photography facilities was used to count cells in the culture, differentiate between viable and non-viable cells, examining the cell cultures for morphological evaluations and capturing the images of karyotypes of the cells at different passages.

3.1.3.2 CO₂ INCUBATOR
A Thermo Forma Scientific (Marietta, Ohio, USA, Model 3131) make CO₂ incubator, with facility to maintain humidified environment, adjustable incubation temperature and CO₂ concentrations was used for the culture of somatic cells, oocytes and embryos.

### 3.1.3.3 LAMINAR FLOW CABINET

Experiments including cleaning, processing and culturing of oocytes and somatic cells, in vitro produced embryos and cloning procedures were carried out in Laminar flow cabinet (CLEANAIR Laminar Flow Systems, India), which served the purpose of minimizing the incidences of microbial contamination and ensuring the safety of the operator. UV irradiation and thorough cleaning of working places with ethanol (70% v/v) was used to maintain hygienic and sterile environment throughout the experiments.

### 3.1.3.4 CENTRIFUGE

Refrigerated Centrifuge (Sigma 3K30, Germany) with facilities to adjust centrifugation speed, time and temperature was used for centrifugation of the tissue explants, washing of the cells etc. as and when needed

### 3.1.3.5 VORTEX SHAKER

Vortex shaker was used for the dissociation of cells after trypsinizing the tissue pieces for establishing primary cell cultures of various somatic cells. Rapid vortexing was also required for dissociating the cumulus cells for denuding the *in vitro* matured oocytes etc.

### 3.1.3.6 THERMAL CYCLER

A thermal cycler (My Cycler, BIO-RAD, Hercules, CA, USA) was used for synthesizing cDNA from mRNA of buffalo embryos, putative ES cells or differentiated cells through reverse transcription in the presence of reverse transcriptase enzyme for amplification of genes of interest with gene specific primers and heat stable taq polymerase. It gave $2^n$ number of DNA strands, where $n$=number of cycles.

### 3.1.3.7 ELECTROPHORESIS UNIT AND GEL DOCUMENTATION
Agarose gel electrophoresis was performed for the resolution of PCR products. The electrophoretic unit (Bio-Rad, Hercules, CA, USA) included the buffer chamber, safety lid with cables, UV transparent tray, casting gates, comb set and power supply. For analyzing the PCR products and for capturing the images, gel documentation system (Molecular Imager, Gel Doc™ XR Imaging System, BioRad) was used.

3.1.4 BIOLOGICALS

Ovaries, oocytes and fetus: The ovaries served as the source of immature oocytes, follicular fluid and cumulus cells during the present investigation. The buffalo ovaries and fetus were obtained from a nearby abattoir (New Delhi).

3.2 METHODS

3.2.1 PREPARATION OF DIFFERENT MEDIA

For details regarding the composition of various media used in the present study, please see ANNEXURE-I.

3.2.2 COLLECTION OF BUFFALO FOLLICULAR FLUID

For the collection of buffalo follicular fluid (buFF), buffalo ovaries were obtained from abattoir and transported to the laboratory in sterile isotonic saline at 4°C within 4-5 h of collection. Follicular fluid was aspirated from all visible surface follicles with a 19-gauge needle. The cellular debris was removed from the pooled aspirate by centrifugation at 10000 rpm for 15 min at 4°C. The buffalo follicular fluid (buFF) was sterilized by filtration through 0.22 µm filters and stored in aliquots in sterile culture tubes at -20°C. The same pool of buFF was used throughout the study.

3.2.3 IN VITRO PRODUCTION OF PARTHENOGENETIC BUFFALO EMBRYOS

3.2.3.1 COLLECTION AND GRADING OF OOCYTES

Buffalo ovaries were collected from Delhi slaughterhouse immediately after slaughter. After removing the extra tissues, these were washed thrice with warm isotonic saline (32-37°C) containing 400 I.U./ml penicillin and 500 µg/ml streptomycin. The washed ovaries were then put in a thermos flask containing
warm saline and antibiotics. The collected ovaries were transported to the laboratory within 4-5 h of slaughter. In the laboratory, the ovaries were rinsed twice with warm saline containing antibiotics. Oocytes were collected by aspiration of surface follicles (2-8 mm diameter) with a 19-gauge needle attached to a 10 ml syringe containing the aspiration medium which consisted of TCM-199 + 0.6% bovine serum albumin (BSA) + 50 µg/ml gentamycin sulfate. The contents of the syringe, which included the aspirated oocytes, follicular fluid, granulosa cells and other debris, were poured in 100 mm x 15 mm square Petri dishes with 13 mm grid (Searching dishes). The oocytes were searched under a zoom stereo microscope (Olympus, SZ40 or SZX7, Japan) at around 20x magnification. The oocytes were then shifted to 35 mm x 10 mm cell culture Petri dishes containing the washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 µg/ml gentamycin sulfate). The collected oocytes were graded on the basis of their morphology as described below:

**Usable quality (A+B grade):** Compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having ≥2 layers of cumulus cells, and with homogenous, evenly granular ooplasm.

**Unusable quality (C+D grade):** Oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm.

Oocytes of both qualities were separately used for in vitro maturation.

### 3.2.3.2 IN VITRO MATURATION OF OOCYTES

The method used for IVM was the one followed routinely in the laboratory. Briefly, the oocytes of both quality were washed 4-6 times with the washing medium, then twice with the IVM medium (TCM-199 + 10% FBS + 5 µg/ml pFSH + 0.81 mM sodium pyruvate + 5% buFF). For IVM, groups of 15-20 COCs were placed in 100 µl droplets of the IVM medium, overlaid with sterile mineral oil in 35 mm Petri dishes, and cultured for 24 h in a humidified CO₂ incubator (5% CO₂ in air) at 38.5°C.

### 3.2.3.3 ACTIVATION OF IVM OOCYTES
For production of embryos, both usable (A+B) and unusable (C+D) grade oocytes were activated parthenogenetically. The three artificial activation reagents (i) 7% ethanol (ii) calcium ionophore (CaI) and (iii) electrical pulse and three types of culture medium (i) mCR2 (ii) mSOF and (iii) RVCL were used, which were compared in this study. Briefly, oocytes were denuded of cumulus cells by incubation in 0.2% hyaluronidase in Dulbecco’s phosphate buffered saline for 2 min, after 24 h of IVM. The denuded oocytes with a prominent polar body were parthenogenetically activated by exposure to following treatments (i) 7% ethanol for 7 min (ii) 5 μM calcium ionophore A23187 (CaI) for 5 min, (iii) electrical pulse (double D.C. pulse of 1.2 kV/cm or 2.1 kV/cm or 3.3 kV/cm for 20 ls each) using BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA) followed by incubation with 2 mM 6-dimethyl aminopurine in respective culture medium for 3.5 h in a CO₂ incubator (5% CO₂ in air, 90-95% relative humidity) at 38.5°C and then subjected to IVC for 8 days in the following
medias (i) mCR2 (ii) mSOF and (iii) RVCL.

3.2.3.4 IN VITRO CULTURE

After the end of oocytes incubation with DMAP, the activated oocytes were separately washed several times with mCR2, mSOF and RVCL media respectively. After this, the embryos were shifted to the IVC medium (mCR2aa + 0.6% BSA + 10% FBS, mSOF with 1% FAF-BSA and RVCL supplemented with 1% FAF-BSA.) and cultured in 100 µl droplets of these medium on original beds of granulosa cells for up to 9 days post activation in a humidified CO₂ incubator (5% CO₂ in air) at 38.5°C. The medium was replaced with 50% of fresh IVC medium every 48 h.

3.2.3.5 ASSESSMENT OF BLASTOCYST QUALITY

For examining of the health of the embryos, the total cell number of trophectoderm (TE) and inner cell mass (ICM) of day 7 blastocysts was determined by differential staining as described by Thouas et al., (2001). With little modifications, briefly the blastocysts were washed with DPBS for 10 to 15 sec. and transferred into 500µL of solution I (5µg/mL Hoechst 33342) and incubated for 40 min. at 37°C. Then blastocysts were washed with DPBS and immediately transferred into 500µL of solution II (0.04% Triton X 100) for 1 min. after that blastocysts were washed again with DPBS, followed by incubation in 25µg/mL Propidium Iodide (PI) for 40 sec. The number of nuclei was counted
using an inverted microscope (Nikon Diaphot, Japan) fitted with an UV lamp and excitation filters (Excitation wavelength: 330–380 nm; barrier filter: 420 nm). Inner cell mass cells stained blue while trophectodermal cells stained red colour.

3.2.4 PRODUCTION OF BUFFALO PARTHENOGENETICALLY DERIVED EMBRYONIC STEM CELLS

3.2.4.1 PREPARATION OF BUFFALO FEEDER LAYERS

Buffalo fetus (around 60 days old) obtained from slaughtered animals were separated from uteri and were washed twice with antibiotic fortified saline. The surface of fetus was made aseptic by dipping in 70% ethanol after which the fetus was washed several times with saline. Skin biopsies were taken and washed 4-6 times with DPBS containing 10% FBS and 50 µg/ml gentamicin sulphate. These were then cut with the help of a pair of scissors into small pieces (approximately 1 mm³), again washed 3-4 times with DPBS + 10% FBS and 50 µg/ml gentamicin sulphate and then finally with the cell culture medium (DMEM supplemented with 20 % FBS and 50 µg/ml gentamicin sulphate). The tissue pieces were transferred to tissue culture flasks or cell culture dishes and were cultured in the culture medium in a CO₂ incubator (5% CO₂ in air) at 37°C. The explants were removed after proliferation and establishment of fibroblasts, which usually took 6-8 days. Monolayer fibroblasts were allowed to grow till confluence. Confluent monolayers were split and subcultured for cell multiplication after treatment with 0.25% trypsin-EDTA. Cells from initial passages were cryopreserved in small aliquots for future use. For the preparation of feeder layer, buffalo embryonic fibroblasts were inactivated by treatment with 10 µg/ml mitomycin-C for 3 h after which the monolayer was trypsinized and the cells were washed 3-4 times with DPBS + 10% FBS + 50 µg/ml gentamicin sulphate and then finally with DMEM + 10% FBS + 50 µg/ml gentamicin sulphate. The feeder layer was prepared by seeding these harvested cells in 4 well plates/ tissue culture dishes one day prior to culture of putative buES cells.

3.2.4.2 CRYOPRESERVATION OF BUFFALO FEEDER LAYER CELLS

To reduce variability, the passage number of feeder cells was kept constant. At initial passages, when the monolayers reached a confluence of 70-80%, the culture medium was removed, the cells were rinsed with Ca²⁺ Mg²⁺-free DPBS,
after these were detached and made to a single cell suspension by trypsin-EDTA
treatment for 3-5 min at 37°C. An equal volume of FBS containing medium was
added to stop the remaining action of trypsin. The cells were counted with a
haemocytometer and were transferred to the freezing medium (culture medium +
10% DMSO) at a concentration of 10^6 cells /ml. The cell suspension was
transferred to cryovials, which were stored at -80°C overnight and then directly
transferred to liquid nitrogen. The frozen aliquots were thawed by placing the
cryovials in a 37°C water bath for 1 min followed by transfer of cells to a tube
containing the culture medium. The cells were washed twice by centrifugation for
5 min at 1000 rpm. The cells were then plated in tissue culture flasks, grown to
form a monolayer of 60-70% confluences, and were treated with mitomycin-C for
feeder layer preparation.

3.2.4.3 ISOLATION OF INNER CELL MASS AND THEIR MAINTENANCE IN
CULTURE

The hatched and expanded blastocysts obtained on days 8 or 9 post activation
were used for the isolation of inner cell mass (ICM) cells, which were plated on
feeder layers for the derivation of buffalo ES cells. Following culture, the ICM
cells were found to proliferate leading to formation of a morphologically distinct
colony. This was separated from the trophectodermal cells and was then seeded
on a fresh feeder layer to obtain a distinct colony hereafter referred to as ‘primary
colony’ of buES cells. For expanded blastocysts, zona pellucida (ZP) was
removed by treatment with 0.1% pronase. The zona-free blastocysts were treated
with 0.25% trypsin-EDTA and observed under a zoom stereomicroscope until the
trophectodermal cells became loose. These were then dispersed to release the
ICM. From hatched blastocysts the ICM were isolated mechanically with the help
of fine needle it was separated from the trophectoderm by cutting then it was
cultured on feeder layers and were allowed to attach and proliferate.

The isolated ICM cells were washed and seeded on feeder layer. The cells were
cultured in 200 µl of ES culture medium in 4-well plates (Nunc, Roskilde,
Denmark) in 5% CO₂ at 38.5 °C and examined at 12h intervals. The ES culture
medium was replaced every day. The ES culture medium used was Knock out
DMEM supplemented with 20% Knock out serum replacer, 2 mM L-glutamine,
1000 units/ml recombinant murine leukemia inhibitory factor (rmLIF), 1X
nonessential amino acids and 50 µg/ml gentamycin sulphate. Subculture was performed by mechanically cutting out the colonies and dividing them into pieces with the aid of two fine glass needles under a zoom stereomicroscope. The observations recorded included the primary colony formation rate, primary colony formation time, the maximum number of passages for which the ES cells survived and the colony morphology.

3.2.4.4 VITRIFICATION OF PUTATIVE BUFFALO EMBRYONIC STEM CELLS

The putative buPES cells at different passages were vitrified in clumps of about 150-200 cells in French mini straws (250 µl; IMV, L'Aigle, Cedex, France). Two vitrification solutions (VS), both based on holding medium (HM) consisting of DMEM supplemented with 20% FBS were used. The first vitrification solution (VS1) included 10% dimethyl sulphoxide (DMSO) and 10% ethylene glycol (EG). The second vitrification solution (VS2) included 20% DMSO, 20% EG and 0.5 M sucrose. The cell clumps were incubated in VS1 for 1 min, were transferred to VS2 in which they were incubation for 25 sec. The clumps were loaded in the straws in such a way that the first part of the straw contained 30 µl VS2, a small air bubble, VS2 having the putative buES cell clumps, an air bubble, and VS2. Just after sealing the open ends of the straws with polyvinyl alcohol powder, these were immediately plunged into liquid nitrogen (LN₂).

For thawing, the straws were taken out of LN₂ and plunged into water at 37°C. After holding the straws for 30 sec, the straws were wiped with 70% ethanol and the sealed ends were cut off to allow the cell clumps to enter the HM supplemented with 0.2 M sucrose for 1 min then clumps were transferred in 0.1 M sucrose solution for 5 min. The clumps were further incubated twice (5 min each) in HM before being seeded on mitomycin C-treated feeder layer.

3.2.5 CHARACTERIZATION OF PARTHENOGENETICALLY DERIVED BUFFALO EMBRYONIC STEM CELLS

The characterization of the stem cells was carried out at different passages by the following methods.

3.2.5.1 ALKALINE PHOSPHATASE STAINING

For alkaline phosphatase staining, the medium was removed from the buPGES cell cultures, which were then washed twice with DPBS. The cells were fixed in
citrate-acetone-formaldehyde fixative solution for 1 min. After fixation, the cells were washed 3 times with deionized water for 1 min and incubated for 15 min at room temperature with the alkaline dye. The cells were rinsed again 2-3 times with deionized water and counter stained with neutral red stain for 1-2 min. Finally, colonies were washed several times to remove the extra neutral red stain and the response of the cells to alkaline phosphatase staining was observed under a microscope. The cells that stained red were considered to be the putative buPGES cells.

3.2.5.2 IMMUNOFLUORESCENCE STAINING OF BUFFALO PGES CELLS

The expression of surface markers like SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, were examined by carrying out immunofluorescence staining of colonies of ES cells at various passages. All the following steps were carried out at room temperature. The ES cell colonies were fixed in 4% paraformaldehyde in DPBS for 30 min, washed 3 times with DPBS and then permeabilized by treatment with 0.1% Triton X-100 in DPBS for 30 min. After thorough washing with DPBS, the ES cell colonies were incubated with the blocking solution (4% normal goat serum) for 30 min, and then with the primary antibody at a dilution of 1:10 to 1:20 for 1 h. In the respective controls, the addition of the primary antibody was omitted. After washing 3 times with DPBS, the ES cell colonies were incubated with the appropriate FITC-labeled secondary antibody (goat anti-rat IgM or goat anti rabbit IgG or anti-mouse IgG or IgM, diluted 1:100 to 1:200) for 2 h. The ES cell colonies were then examined under a fluorescence microscope (Diaphot, Nikon, Tokyo, Japan).

3.2.5.3 GENE EXPRESSION ANALYSIS IN EMBRYOS AND EMBRYONIC STEM CELLS

(i) PREPARATION OF RNASE FREE PLASTICWARE

RNase free plasticware were prepared by soaking the plastic ware like microtips, tip boxes, eppendorf tubes etc, in 0.1% diethylprocarbazine (DEPC) at room temperature overnight. The DEPC solution was then drained off and the treated material was dried in a hot air oven at 50°C. For removing the remaining traces of DEPC which is a potent inhibitor of RT reaction, the dried plastic ware were
wrapped in aluminium foil and were autoclaved twice at 15 lbs pressure for 45 min. DEPC treated water was prepared by adding 1 ml of DEPC to 1 liter of MilliQ water and shaking vigorously for half an hour.

**(ii) PRIMER DESIGNING**

To detect the expression of pluripotency genes in buffalo COCs/embryos and buPGES cells the specific primers are essential for amplification of target gene of interest. PCR primers were designed based on nucleotide sequences available or were based on sequence homology analysis (using ClustalW and Bioedit software) between murine, human, sheep, goat and bovine sequences obtained from NCBI (www.ncbi.nlm.nih.gov) and ENSEMBL genome browser, for the respective genes. Primers were designed and verified using the web-based software PRIMER-3 (www-genome.wi.mit.edu/cgi-bin/prime/primer3-www.cgi).

**(iii) RNA EXTRACTION**

Prior to using the laboratory surfaces e.g., work table, bench tops, laminar hoods centrifuge and electrophoresis tanks, the surfaces were made RNase - free by wiping it with RNase inhibitor solution, RNase Zap (Ambion, USA).

For total RNA isolation from oocytes, cumulus cells, embryo of different stages and embryonic stem cells of different passage the TRIZOL method was used. TRIZOL Reagent is a widely used ready-to-use reagent for the isolation of total RNA from a variety of cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation. In the present study, TRI™ Reagent (Sigma) was used for RNA isolation according to the manufacturer’s instructions, with some modifications. Briefly, the oocytes/embryos (10 of each stages) and cells (5 clumps of cells) were washed several time with PBS to completely remove the culture medium then these were collected in DEPC treated tubes with little amount of PBS and added 200µl of TRI reagent and vortex 2-3 min. and incubate at room temperature for 5 min. then added 40µl of chloroform mix gently and incubate for 10 min. at room temperature, then centrifuge at 12000rpm for 10 min. the aqueous phase were collected in another tube and precipitated with isopropanol alcohol. The RNA pellet were dried and dissolved in DEPC treated water. The
quality and quantity of RNA were measured by using Nano Quant (Tecan, Germany).

(iv) cDNA Synthesis

The cDNA were synthesized using Omniscript cDNA synthesis kit (Qigene, Germany). For cDNA synthesis, 70 ng of RNA was taken from oocytes/embryos and ES cells in 200 µl DEPC- treated tube and 2 µl dNTP mix (10mM), 2 µl oligo dT (50µM), 2 µl RT buffer, 1 µl RNase inhibitor, 1 µl RT enzyme and nuclease free water was added to make volume up to 20 µl. The mixture was mixed, centrifuged and incubate at 37°C for 1 hrs. Then 75°C for 5 min. To nullify the genomic DNA presence in the RNA sample a negative RT control reaction was always set. In the negative RT control all components were same as that of normal RT reaction except that of MMLV Reverse Transcriptase. After completion of the reverse transcription, cDNA was stored in -20 °C until use in PCR. To confirm the cDNA synthesis, PCR was done with internal control. In present study the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal control.

(v) AMPLIFICATION OF PLURIPOTENCY GENES IN EMBRYOS AND EMBRYONIC STEM CELLS

Gene expression analysis of transcription based pluripotent markers like REX-1, OCT-4, NANOG, SOX-2, NUCLEOSTEMIN, FOXD-3, TELOMERASE, STAT-3 and c-MYC genes was performed in different embryonic developmental stages (immature oocytes, mature oocytes, 2-cell, 4-cell, 8-16-cell, morula blastocyst), trophectoderm cells (TE), ES cells, embryoid body (EB) cells, fetal and adult fibroblast cells. The PCR cycle included denaturation for 3 min at 94°C, followed by repeated cycles of 94°C for 30 sec, annealing at the temperature as indicated in Table 3.1 for 30 sec, and extension at 72°C for 30 sec followed by a final extension at 72°C for 5 min. The PCR primers and the reaction conditions used are as described in Table 3.1. A –ve PCR without template was also set up. GAPDH was amplified at each stage as a house keeping marker gene.

<p>| Table 3.1: List of primers used for characterization of embryos and embryonic stem cells |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Cycles/Temp</th>
<th>Size (bp)</th>
<th>Accession#</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT-4</td>
<td>F- 5'- GATATACCCAGCGGATGTG-3'&lt;br&gt;R- 5'- TCGATACTCGTCGCCGTTTCT -3'</td>
<td>33/58</td>
<td>232</td>
<td>EU926737</td>
</tr>
<tr>
<td>NANOG</td>
<td>F- 5'-'GGGAAAGGTAAAGTGATCACA-3'&lt;br&gt;R- 5'-AGCCTCCCTATCCAGAAA-3'</td>
<td>35/57</td>
<td>211</td>
<td>DQ487022</td>
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<tr>
<td>REX-1</td>
<td>F- 5'- CAGCTGCCATAGGCTCTACC-3'&lt;br&gt;R- 5'-GTCTGCCTGACAAAGGTGT-3'</td>
<td>35/55</td>
<td>239</td>
<td>XM_001255545</td>
</tr>
<tr>
<td>FOXD-3</td>
<td>F- 5'- CTATTCGTATGCAGCTCCTACC-3'&lt;br&gt;R- 5'-GGGTCCAGCAGGTCCAGT-3'</td>
<td>35/58</td>
<td>228</td>
<td>ENSBTAG00000008501</td>
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<tr>
<td>NUCLEOS TEMIN</td>
<td>F- 5'-AGAATCGGATGAGCTGTTGG-3'&lt;br&gt;R- 5'-ATCAGCATTGCCAGAGGT-3'</td>
<td>35/60</td>
<td>400</td>
<td>NM_001034307.1</td>
</tr>
<tr>
<td>STAT-3</td>
<td>F- 5'- GCGAAGAATCAAGCAGTTCC-3'&lt;br&gt;R- 5'-CCAGGGCAGTAAGCATCTGT-3'</td>
<td>35/60</td>
<td>383</td>
<td>DQ487026</td>
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<tr>
<td>SOX-2</td>
<td>F- 5'- TCCACATTGCAGATCGCAGA-3'&lt;br&gt;R- 5'-CATGAGCGTCTTGGTTCC-3'</td>
<td>35/50</td>
<td>162</td>
<td>DQ487021</td>
</tr>
<tr>
<td>TELOMER ASE</td>
<td>F- 5'- GACTTCCAGCGGTACCTGAG-3'&lt;br&gt;R- 5'-GATCCCCCTGACACTGAGATGT-3'</td>
<td>35/58</td>
<td>192</td>
<td>NM_001046242.1</td>
</tr>
<tr>
<td>c-MYC</td>
<td>F- 5'- GGAAGAATTCGAGCTGCTG-3'&lt;br&gt;R- 5'- CGCCTCTTGTCTAGTCCCTCTC-3'</td>
<td>35/60</td>
<td>922</td>
<td>Q2HJ27</td>
</tr>
</tbody>
</table>

**3.2.5.4 KARYOTYPING**

The chromosomal integrity of the putative buPGES cells at different passage and after freezing thawing were analyzed by performing karyotyping. The cells were subjected to chromosomal analysis as described by Dyban (1983) with slight modifications in the procedure. The colonies of putative buPES cells were collected and incubated in DMEM supplemented with 0.1 µg/ml colcemid for 2-3 h at 37°C. The colonies were then trypsinized, and the activity of trypsin was neutralized by addition of FBS containing medium. The cells were centrifuged at 1000 rpm for 10 min and the cell pellet obtained was mixed thoroughly in 2 ml of hypotonic fluid (0.56% KCl) and incubated at 37°C for 8 min. The cell suspension was centrifuged and after removal of the supernatant, the pellet was suspended...
in 2 ml of chilled fixative solution (glacial acetic: methanol, 1:3) for 30 min. The suspension was centrifuged again after which the pellet was resuspended in 5 ml of chilled fixative for another 10 min. This step was repeated once more. The supernatant was removed leaving about ½ ml of fluid at the bottom of the tube. Then 2 to 3 drops of the fluid were dropped from a height of about 24-30 inches on ice cold glass slides. The slides were allowed to dry. The dried material was then stained by immersion in freshly prepared 2% Giemsa stain for 7-10 min. The glass slides were then rinsed with deionized water until the excess stain was removed. The slides were dried and were observed oil immersion (1000X) using a compound microscope (Nikon, Microphot, FXA, Japan).

3.2.6 EMBRYOID BODY FORMATION

When ES cells are cultured in suspension without the support of LIF, FGF-2 and feeder layer, they form 3-dimensional aggregates called ‘embryoid bodies’ (EBs). For the preparation of EBs, ES cell colonies were removed from the feeder layers and were disaggregated into small clumps using two fine needles. The clumps were cultured for two days in hanging drops (20 µl of ES cell culture medium without LIF and FGF-2) in which the cells were dispersed and suspended from the lid of a Petri dish. Third day onwards, the EBs were transferred to bacteriological dishes for further culture. Compact or cystic EBs was formed within 3-7 days.

3.2.6.1 SPONTENEOUS DIFFERENTIATION

For spontaneous differentiation these EBs were placed on tissue culture dishes for adhesion and growth. The cells were fed every alternate day by tilting the plate, allowing them to settle, and carefully replacing the medium. The EBs were differentiated spontaneous into several types of cells, like neuron, epithelial, mussels and fibroblast cells depending on the duration of culture. The EBs and epithelial cells was characterized by the lineage-specific gene expression by RT-PCR.

3.2.6.2 CHARACTERIZATION OF EBs

For detection of lineage specific genes and differentiated cells specific markers the reverse transcriptase polymerase chain reaction (RT-PCR) were used using “Cells-to-cDNA kit-II” (Ambion, Austin, TX, USA) as per manufacturer’s
instructions. Briefly, the cells were washed with ice cold PBS after which 20-50 µl of cold cell lysis buffer was added and the reaction mixture was incubated in a thermal cycler (Bio-Rad, USA) at 75°C for 10 min. The cell lysate was treated with DNase-I at 37°C for 30 min to degrade the genomic DNA and was then heated to 75°C for 5 min to inactivate DNase-I. The cell lysate (10 µl) was used for making cDNA using random primers. After completion of synthesis of cDNA the PCR was run with lineage specific primers sets. The PCR reaction included 2µl cDNA, 5µl PCR buffer, 10mM dNTPs, 10pM gene specific primers and 0.2 units of Taq polymerase (Promega, Madison WI, USA) total reaction volume was 25µl. cycle included denaturation for 5 min at 94°C, followed by repeated cycles of 94°C for 30 sec, annealing at the temperature as indicated in (Table 3.2) for 30 sec, and extension at 72°C for 35 sec followed by a final extension at 72°C for 10 min. A –ve RT reaction (i.e., parallel RT reaction but without MMLV enzyme) was set up with every batch of cDNA preparation to check genomic DNA contamination. A –ve PCR without template was also set up. GAPDH was amplified at each stage as a house keeping marker gene. The amplified products were resolved in 2% agarose gel electrophoresis (Bio-Rad, USA) with 100bp marker and the image of gel was capture by gel documentation system (Bio-Rad, USA).

**Table 3.2: List of primers used to characterization of EBs and differentiated cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer F</th>
<th>Primer R</th>
<th>Cycles/Temp</th>
<th>Size (bp)</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF-4</td>
<td>5'-ACATCCCAGCCTTCTGTGAG-3'</td>
<td>5'-TCGTCACACACTGAGCTCTTG-3'</td>
<td>35/58</td>
<td>238</td>
<td>AF250028</td>
</tr>
<tr>
<td>GATA-4</td>
<td>5'-CGGAAAGAGGGGATTCAAAC-3'</td>
<td>5'-ATCTCCTCACTGCTGCTGGT-3'</td>
<td>35/58</td>
<td>152</td>
<td>DQ487031</td>
</tr>
<tr>
<td>AFP</td>
<td>5'-TCTTCCCCATGTTCTTCAGG-3'</td>
<td>5'-TTTCACGGCAAATTTCTTCC-3'</td>
<td>35/55</td>
<td>205</td>
<td>NM_001034</td>
</tr>
<tr>
<td>ASA</td>
<td>5'-GGCATCATACCAACTGGGA-3'</td>
<td>5'-TTGCCGATGGTGACCTG-3'</td>
<td>35/55</td>
<td>536</td>
<td>Sritanauadomch et al., 2007</td>
</tr>
</tbody>
</table>
### 3.2.6.3 DIRECTED DIFFERENTIATION

In directed differentiation, the EBs produced by the hanging drop method were placed in suspension for 3 additional days and were then transferred to gelatin-coated dishes containing stimulating agent. After 2 days, the EBs had adhered and had started to grow. The stimulating agents examined in the present study included retinoic acid evaluated at DMEM supplemented with 10% FBS and $10^{-6}$ M all *trans* retinoic acid $10^{-6}$ M for directing the differentiation to neuron cell-like cells. The neurons cells were characterized by RT-PCR with mRNA expression of *NF-68*.

### 3.2.7 EXPERIMENTAL DESIGN

#### 3.2.7.1 To compare different methods for production of parthenogenetic blastocysts and isolation culturing of the embryonic stem cells.

In this experiment the in vitro matured oocytes of both usable (A+B grade) and unusable (C+D grade) were subjected to parthenogenetically activation as follows:

1. 7% ethanol + DMAP, cultured in mCR2, mSOF, RVCL
2. Calcium Inophore + DMAP, cultured in mCR2, mSOF, RVCL
3. Electric Pulse + DMAP, cultured in mCR2, mSOF, RVCL

After cultured on 8-9 days the ICM were isolated from expanded and hatched blastocyst by mechanically isolation method.
3.2.7.2 To study the expression of pluripotent genes

In this experiment the panel of pluripotency genes was analyzed in immature, mature oocytes, 2-cell stage, 4-cell stage, 8-16-cell stage, morula and blastocyst. The expression of pluripotency gene was also analyzed in embryonic stem cells of buffalo generated by parthenotes.

3.2.7.3 To study the in vitro differentiation of buPGES cells

In this study when buPES cells were cultured without LIF and FGF they form embryoid body. The presence of three germ layers in embryoid body they were characterized by RT-PCR using lineage specific markers (see table). When these EBs were cultured long time in tissue culture dish they formed different types of cells like neuron, epithelial, mussels and fibroblast cells. Some of these cells were characterized by RT-PCR using specific primers.

3.2.8 STATISTICAL ANALYSIS

Data were analyzed using SYSTAT 7.0 (SPSS Inc. USA) after arcsin transformation. Differences between mean percentages were analysed by one way ANOVA followed by Fisher’s LSD test for pair wise comparison of means. The determination of total cell number of hatched blastocysts on day 8 and expanded blastocysts on day 9 post insemination were analyzed by unpaired t-test.