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Techniques and Conditions for Embryonic Germ Cell Derivation and Culture

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1. Introduction

Embryonic germ cells (EGCs) are pluripotent stem cells derived from primordial germ cells (PGCs), which are unipotent cells that *in vivo* give rise to the gametes (McLaren, 2003).
Specification of PGCs takes place in the proximal posterior part of the epiblast shortly before gastrulation, when the epiblast is about to give rise to the three germ layers ectoderm, mesoderm, and endoderm. PGCs can be identified by expression of tissue non-specific alkaline phosphatase (TNAP) activity, various surface antigens stage specific embryonic antigen (SSEA1,3,4), mouse vasa homolog (Mvh) and intracellular proteins (Stella, Fragilis, Oct-4, Nanog and Blimp1 among others). From the proximal epiblast, PGCs migrate along the extraembryonic mesoderm at the base of the allantois, and then move into the epithelium of the hindgut. Later, PGCs start to move through the dorsal mesentery reaching the aorta-gonad-mesonephros (AGM) region and finish their migration at the developing genital ridges. When in gonads, PGC proliferate by mitosis until males enter in mitotic quiescence and in females enter into meiosis (De Felici, 2009).
Under special conditions, PGCs become pluripotent stem cells. In vivo PGCs can generate embryonal carcinoma cells (ECC), the pluripotent stem cells of testicular tumors (Stevens, 1967; Oosterhuis& Looijenga, 2005), while in vitro they can generate EGCs (Matsui et al., 1992; Resnick et al., 1992; Surani, 2007; De Felici et al., 2009; De Miguel et al., 2010). PGCs could be isolated and cultured as such during short periods (up to 10 days) maintaining their phenotype, until they undergo apoptosis. When exposed to a specific mixture of growth factors, PGCs generate EGC colonies. EGCs could be an important source of cells for germ cell or stem cell therapy and a valuable model for understanding development processes involved in reprogramming such as the acquisition of pluripotency.
EGCs were first derived in mice (Matsui et al., 1992; Resnick et al., 1992), and afterwards in a wide variety of mammals like cow (Cherny et al., 1994), goat (Jia et al., 2008), pig (Shim et al., 1997), and sheep (Ledda et al., 2010) among others. Importantly, in 1998 Shamblott et al. derived the first human EGC line, providing a potential source of pluripotent stem cells for therapy.
In this chapter, differences and similarities of EGC derivation and culture of different species are discussed, including species in which long term EGC lines derivation has not yet been achieved.

2. Mouse embryonic germ cells

PGCs in mouse arise at the epiblast adjacent to the extra-embryonic ectoderm around 6.5 days post coitum (dpc) (Lawson et al., 1999). At this stage mouse PGCs constitute a small cluster of TNAP positive cells which also express the POU domain transcription factor Oct-4, both of which are pluripotent markers of the inner cell mass (ICM) of the blastocyst and embryonic stem cells (ES). At 8-8.5 dpc, PGCs migrate and are found in the hindgut endoderm and at the base of the allantois. From there they migrate along the hindgut mesentery and begin to colonize the developing genital ridges at 10.5 dpc. At 13.5 dpc, an established population of 25,000-35,000 PGCs in the gonad will form the future gametes (Tam & Snow, 1981; Ginsburg et al., 1990).

PGCs from embryonic days 8.5 to 12.5 dpc are prevalently used for EGC derivation. They express the pluripotent markers Oct-3/4, Sox-2 and Nanog. However, in culture, PGCs proliferate for 7-10 days and then disappear either because they differentiate or die (De Miguel & Donovan, 2003). *In vitro* culture of mouse PGCs has identified many growth factors that affect their survival and/or proliferation. When PGCs in culture are exposed to leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), stem cell factor (SCF) and serum (Matsui et al., 1992; Resnick et al., 1992; Durcova-Hills et al., 2001) they become immortalized. In addition, unipotent PGCs turn into pluripotent cells called embryonic germ cells through a molecular reprogramming event. These cells are similar to embryonic stem (ES) cells derived from the early embryo or induced pluripotent stem cells (iPS) generated from differentiated tissue in terms of cell morphology and gene expression profile (Sharova et al., 2007; Durcova-Hills et al., 2008). Mouse EG cells also share many features that define pluripotency including the ability to differentiate into a variety of cell types *in vitro*, contribution to germline-competent chimaeras when introduced into blastocysts and the capacity to form spontaneous teratomas (Matsui et al., 1992; Labosky et al., 1994; Stewart et al., 1994).

However, EG cells differ from iPS and ES cells in some properties. EG cells have a different epigenetic state demonstrated by the erasure of genomic imprints and chromatin remodeling from certain imprinted genes like Igf2, Igf2rr, Dlk1 and H19, a process that occurs after specification of PGCs (Lee et al., 2002). Therefore, the pluripotency state from EG cells is somewhat distinct from the naïve pluripotency state of mouse ES cells (Gillich & Hayashi, 2011).

Although the findings in this respect are increasing, the molecular and epigenetic mechanisms that control the reprogramming conversion from PGCs into pluripotent EGCs are not fully understood. However, the signaling pathways and genes that control several processes like proliferation and specification of PGCs and the epigenetic restriction that separate PGCs from pluripotency have been identified. Several lines of evidence highlight key molecules involved in the pluripotency acquirement of PGCs. It has been proposed that downregulation of Prdm1/Blimp1 is a prerequisite for EG cell derivation (Durcova-Hills et al., 2008) and PGC-specific inactivation of the tumor suppressor PTEN enhances both EG cell production and testicular teratoma formation (Kimura et al., 2003). Also, an enforced activation of Akt, one of the major downstream effectors of PI3K, augments the efficiency of EG cell establishment and enables derivation of EG cells from late PGCs, such as from 14.5 dpc embryos (Kimura et al., 2008; Gillich & Hayashi, 2011).

2.1 Isolation and culture of mouse embryonic germ cells

Several protocols have been described for culturing mouse PGCs, their posterior conversion into EG cells as well as derivation of mouse EG cell lines. This part describes the basic

protocol developed by the Donovan´s lab (Resnick et al., 1992; De Miguel & Donovan, 2003) for the derivation and establishment of mouse embryonic germ cells lines from mouse PGCs isolated from both 8.5 and 10.5 dpc embryos and some differences with protocols described by Matsui and Durcova-Hills & Surani (Matsui et al., 1992; Durcova–Hills & Surani, 2008).

2.1.1 Buffers and solutions

PBS: Phosphate buffered saline, without Ca^{+2} and Mg^{+2}, pH 7.0 (Gibco/Invitrogen).

Trypsin/EDTA: HBSS containing 0.05% trypsin and 0.53 mM EDTA (Sigma).

Fast Red/Naphtol phosphate solution (Alkaline phosphatase staining solution): a 1 mg/ml solution of Fast red TR salt (Sigma, stored at -20ºC) is made up in dH_2O. 40 µl/ml Naphtol AS-MX phosphate is then added (Sigma, stored at 4ºC). Used immediately.

2.1.2 Culture media

Basic culture medium for PGCs: DMEM (Dulbecco's modified Eagle's medium) high glucose, supplemented with 15% Fetal Bovine Serum (Gibco/Invitrogen) (we recommend to test different batches of Fetal Bovine Serum before purchasing since serum batch used for culture may be especially critical for the growth of PGCs and derivation of EG cells), 2 mM glutamine (Gibco/Invitrogen), 5 U/ml penicillin–streptomycin (Invitrogen), and 1 mM Na^+ pyruvate (Sigma).

Growth factors for EG cell derivation and culture: mSCF (R&D Systems) 10 ng/ml, mLIF (Millipore) 1000 U/ml, hbFGF (Gibco/Invitrogen) 1 ng/ml and Forskolin (Sigma) 100mM.

Basic culture medium for STO: (Sandoz Thioguanine- and Ouabain-resistant cell line, a transformed mouse embryonic fibroblast line): DMEM high glucose, supplemented with 10% Fetal Bovine Serum (Gibco/Invitrogen), 2 mM glutamine (Gibco/Invitrogen), 5 U/ml penicillin–streptomycin (Gibco/Invitrogen), 1 mM Na^+ pyruvate (Sigma). Protocol down below can be used with primary MEFs instead of STO cell line; in that case add 0.1mM non-essential amino acids (Gibco/ Invitrogen) to the medium.

Freezing medium: 9 ml of basic culture medium for PGCs (see above) plus 1 ml of dimethyl sulfoxide (DMSO). Mix thoroughly and keep on ice.

2.1.3 Fixatives

4% Paraformaldehyde: 4g paraformaldehyde in 100 ml PBS. To dissolve the paraformaldehyde, PBS is preheated at 90°C, and NaOH is added drop-wise until the solution turns clear. Solution is cooled down before use.

acid–ethanol: 1 ml acetic acid and 19 ml ethanol precooled at -20ºC.

2.1.4 PGCs Isolation and culture

8.5 days post coitum Embryo Dissection: C57BL/6J inbred mouse line is used to collect tissues containing PGCs (available from Jackson Labs). 8.5 day pregnant females are sacrificed by cervical dislocation. The day on which a vaginal plug is found is designated 0.5 dpc (coitus is assumed to take place at midnight). The abdomen is dissected using scissors and the uteri is removed. Using forceps, each implantation site is separated by cutting the uterus between them, very near to each embryo to allow the deciduum to project. The dissected implantation sites are placed in a Petri dish with ice cold PBS. The decidua is cut across with a pair of fine forceps (Dumont #55). Pressure is applied with fine forceps to the other

side and base of the decidua to pop the embryo out of the slit in the deciduum. The extraembryonic membranes are dissected away and the posterior third of the embryo is removed, including the caudal end of the primitive streak and allantois. 50-100 PGCs are localized at the junction of the primitive streak and the base of allantois at this embryonic stage. The embryo fragments are collected in PBS and kept on ice until trypsinization.

10.5 days post coitum Embryo Dissection: At this stage most PGCs are in the developing genital ridges and the wall of the hindgut of the embryo. The embryos are removed from the implantation site and dissected out from the uterus and extraembryonic tissues. The embryo is cut in half, below the forelimbs. The caudal half of the embryo is kept and the anterior portion is discarded. A cut down is made at the midline of the embryo. The genital ridges lay on the dorsal body wall either side of the hindgut mesentery. The skin is opened out on either side of genital ridges and the forceps lifted upwards and towards the tail of the embryo. The two genital ridges, the dorsal aorta that is between them and hindgut are close to the dorsal body wall. The genital ridges are separated from the anterior end and pulled out and replaced in the PBS to squeeze the blood out from the aorta to reduce the number of contaminating cells. The genital ridge/aorta/dorsal mesentery PGC-containing portions are collected. The estimated number of PGCs per embryo is $1x10^3$ at 10.5 dpc.

12.5 days post coitum Embryo Dissection: The dissection of 12.5 dpc embryos is similar to that for the 10.5 dpc embryos. All the PGCs are in the genital ridge. At this stage in embryogenesis it is preferable to dissect the genital ridges away from the adhering tissue with fine forceps. The expected increase in embryo size is associated with a higher yield of PGCs isolation. The estimated number of PGCs per embryo is $10x10^3$ PGCs at 12.5 dpc. PGCs from older embryos than 10.5 dpc are not routinely isolated in our labs because the efficiency of EG cells derivation in culture is lower due to reduced cell proliferation in PGCs of male embryos and the entry into meiosis of PGCs in female embryos.

Enzymatic treatment: The PBS is eliminated and trypsin/EDTA is added to the tube. The embryo fragments are incubated in the solution for 8 min at 37°C in a water bath and the trypsin/EDTA solution is removed as much as possible being careful not to aspirate the tissue fragments. An appropriate volume of PGC basic culture medium is added to break up the tissue fragments by pipetting slowly up and down with a micropipette tip to obtain a single cell suspension. The cells are diluted into the required volume of basic culture medium for PGCs supplemented with growth factors.

PGC Culture: PGCs are cultured using a transformed mouse embryonic fibroblast line as feeder layer, the Sandoz Thioguanine- and Ouabain-resistant cell line (STO). STO cells are commercially available from the American Type Culture Collection (CRL-1503™). PGCs are plated onto confluent mitotically inactivated STO feeder cells and cultured in incubator at 37°C in an atmosphere of 95% air and 5% CO_2. Although soluble form of recombinant SCF is further added to the basic culture medium, both the transmembrane and the soluble form of SCF produced by STO cells is required for enhancing proliferation and long-term survival of cultured PGCs (Resnick et al., 1992). Notably, Sl^4m220 feeder cell line is also used by other investigators to culture PGCs and derive EG cell lines. Sl4m220 cells are derived from a homozygous null (*Sl/Sl*) murine embryo which contains a deletion of the gene coding SCF (*Sl* gene) and have been stably transfected with the membrane bound murine form of SCF (Matsui et al., 1991; Toksoz et al., 1992).

STO cells are maintained in basic culture medium for STO cells. For convenience these feeder cells should be prepared the day before the dissection. The STO cells are plated at a density of $10x10^4$ cells/cm^2 onto 0.1% w/v pregelatinized coated culture dishes. Next

morning, STO cells are γ-irradiated with a dose of 50 Gy (5000 rads) to induce cell cycle arrest. After irradiation, the STO cells culture medium is removed and PGC basic culture medium supplemented with growth factors is added (STO cells can also be mitotically inactivated by treatment with mitomycin C (Sigma) at a concentration of 10µg/ml for 4 hours at 37°C). PGCs are plated on top of the STO feeder layer. The final dilution volume of basic culture PGC medium is calculated to plate approximately two 8.5-dpc embryos or 0.5 of one 11.5-dpc embryo per well of a 24-well culture plate. The number of PGCs isolated is estimated from the known numbers of PGCs present in each embryonic stage of the embryo. The medium is replaced every day by gentle aspiration of 2/3 of medium and fresh medium supplemented with growth factors is added.

2.1.5 Identification of PGCs and EG cells

Alkaline phosphatase staining: PGCs and EGCs colonies are distinguished from accompanying somatic and feeder cells by several techniques but the simplest method is alkaline phosphatase staining (Ginsburg et al., 1990). Note that this marker is not unique to germ cells. In mouse embryos, alkaline phosphatase activity is present also in the developing skeletal system (Kaufman, 1992), the developing gut (Merchant-Larios et al., 1985) and the neural tube (Kwong & Tam, 1984). Also, note that this marker stains dead cells, to stain live and dead cells use SSEA1 (see below).

The cultures are washed twice in PBS without Ca^{+2} and Mg^{+2}, and then fixed in 4% paraformaldehyde in PBS (see above) for 15 min at room temperature (RT). The cultures are washed three times in PBS, once in distilled water, and then are incubated in the dark in freshly made alkaline phosphatase staining solution for 20- 30 min at room temperature. After staining, cultures are washed in distilled water; the color reaction will stain PGCs red. The cells must be counted or photographed within a few days of staining, otherwise the cell morphology will deteriorate (Fig. 1).

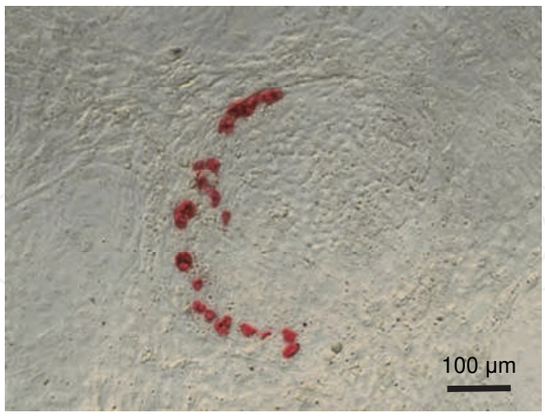


Fig. 1. Mouse primordial germ cells (8.5 dpc) cultured on STO feeder layer and stained for alkaline phosphatase activity in red.

Another way to distinguish between PGCs and EG cells is by their morphological characteristics and growth properties; PGCs grow as single cells, are mortal and will survive for only about 7-10 days in culture, whereas EG cells are immortal, form colonies and can be maintained indefinitely.

Immunofluorescence staining for SSEA-1 or Oct-3/4: PGCs and EG cells can be identified in culture using monoclonal antibodies that recognize pluripotency markers, such as SSEA-1 (anti-SSEA-1 monoclonal antibody can be obtained from the Developmental Studies Hybridoma Bank (http:// www.uiowa.edu/dshbwww/info.html) or commercially (R&D systems) (Donovan et al., 1986; Fenderson et al., 2006) and Oct-3/4 (BD Transduction Laboratories). Here we present a standard protocol for IF staining for SSEA-1 or Oct-3/4 on PGCs and EG cells (Durcova-Hills & Surani, 2008).

PGCs or EGCs are cultured on the appropriate feeder cells over 0.1% gelatin pre-coated microscope glass slides (Fisher) on 24-well culture plates. Cells are washed in PBS and the fixative solution is added for 15 minutes at RT. After that the fixative solution is removed and cells are washed with PBS. Cells are permeabilized and blocked with PBS solution containing 0.1% (w/v) Triton X-100 and bovine serum albumin (BSA; Sigma) at a concentration 1% (w/v) in PBS and incubated for 20 minutes at RT. Mouse anti SSEA-1 (1:200) or mouse anti Oct-3/4 (1:250) antibody diluted in antibody dilution buffer (PBS with 0.1% (w/v) BSA) are added to the fixed cells and incubated overnight at 4ºC in a humidified chamber. Then samples are washed and the appropriate secondary antibody (anti-mouse IgM- FITC (1:100; Sigma) for SSEA-1 or Anti-mouse IgG-Alexa (red, 1:500; Molecular Probes) for Oct-3/4) diluted in antibody dilution buffer is added and incubated in a humidified dark chamber for 60 min at RT. Afterwards cells are washed with PBS and nuclei are counterstained with DAPI solution (Sigma) for 10 min at RT in a humidified chamber in the dark. The DAPI solution is aspirated and a drop of fluorescence mounting medium (Vectashield; Vector) is placed on an immunohistochemistry glass slide. The microscope glass slide with stained cells is placed onto the drop of mounting medium and any excess is removed with paper tissue. The samples should be examined under a fluorescence microscope with appropriate filters as soon as possible as the signal diminishes over time (Fig 2). Slides should be stored at 4ºC (short term storage) or in a freezer at -20ºC (for few days) in the dark.

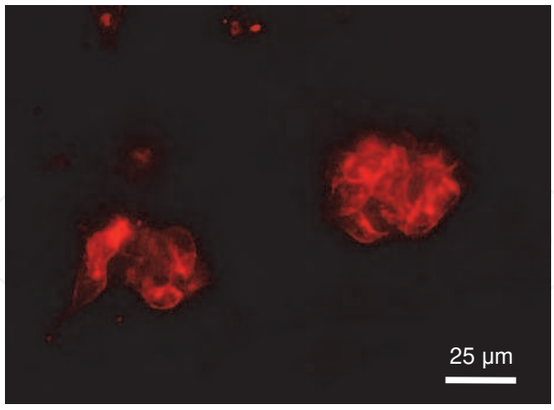


Fig. 2. Characterization of pluripotent EG cells. Detection of cell surface pluripotent marker SSEA-1 performed by immunofluorescence staining (red) in mouse EG cells.

2.1.6 Derivation of EG cells

For EG derivation, PGCs 7- 9 days are cultured in basic culture medium supplemented with leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and stem cell factor (SCF). The pharmacological agent forskolin is a cAMP agonist, and one of the most potent

PGC mitogens (De Felici et al., 1993). It can also be added to PGC culture to increase derivation efficiency, however is not required for mouse EG derivation. By 7–10 days of primary culture, large colonies of EG cells resembling ES cell colonies should be growing on the STO feeder layer. If the generation of a EG cell line is desired, colonies of EG cells can then be subcultured by trypsinization onto fresh, mitotically inactive primary mouse embryonic fibroblasts (MEFs) or STO. The CF-1 mouse strain feeder layer MEFs isolated from 13.5 dpc embryos (also available at Millipore, PMEF-CFL) is used to subculture EG cells since in our hands EG cells grow better on this cell type. However, feeder layer Sl4-m220 or STO have also been used to derive EG cell lines (Matsui et al., 1992). MEF feeder cells are plated the day prior to the EG colonies passage at a density 10 x 10^4 cells/cm^2 and irradiated (50 Gy) on the morning of use. EG tightly packed dome-shaped colonies can be visualized by fixing the cultures and staining for alkaline phosphatase or by observing living cultures under a microscope equipped with phase contrast or Hoffman modulation contrast optics.

To subculture EG cell colonies, primary cultures are washed twice with PBS and trypsin/EDTA solution is added, then the culture is incubated at 37ºC in a humidified CO_2 incubator for 5–10 min. The trypsin/EDTA solution is removed and PGC basic culture medium added to neutralize the action of the trypsin. The solution is pipetted up and down to obtain a single cell suspension. Importantly, the extent of cell disruption of EGC clumps must be controlled to obtain 2 to 5-cell clumps as large clumps of EG colonies will differentiate if passaged. The cell suspension is transferred to a centrifuge tube and centrifuged 5 min at 400 *g* at RT, and then the pellet is resuspended in fresh basic culture medium supplemented with LIF. The cell suspension is plated onto mitotically inactivated MEF containing wells. To generate a EG cell line, cells can be grown in the same way as mouse ES cell lines and cultured on medium with only LIF, since the previously mentioned factors are no longer necessary. The medium is replaced every 24 hr by removing as much of the existing medium as possible and fresh medium supplemented with LIF. At early stages of EG cell derivation it may be difficult to see the small numbers of EG cells present. After 3–5 days the EG cell colonies should start growing and must be visible on the culture dish (Figs. 3&4).

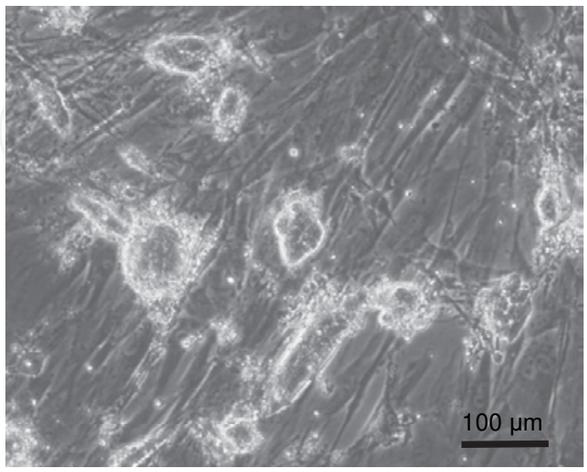


Fig. 3. Phase contrast image of mouse EGC colonies of a mouse EGC cell line cultured on MEFS.

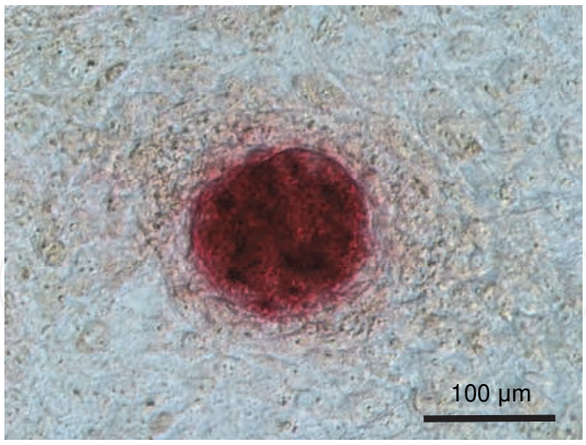


Fig. 4. Alkaline phosphatase staining of a mouse EGC colony.

Recently mouse EG cells have been derived from PGCs efficiently in similar conditions as those used for ES cell derivation from the ICM. By addition of SCF, bFGF, LIF and FBS to the primary culture of murine PGCs on Sl⁴m220 feeders, an efficiency of EGC derivation of 1-2% has been reported (Leitch et al., 2010; Gillich & Hayashi, 2011). The addition of two inhibitors (MEK and GSK3 inhibitors) and LIF after two days of previous culture conditions yielded an efficiency of 2-10% (Leitch et al., 2010; Gillich & Hayashi, 2011).

2.1.7 Colony selection and Passaging EGC colonies

Single colonies of EG cells can be selected and expanded. Mitotically inactivated MEF feeder cells must be prepared 24 h before and irradiated on the morning of use. The medium from a culture dish of EG cells is removed and washed with PBS. Using an inverted or dissecting microscope, individual colonies are picked with a pipettor and transferd to a centrifuge conical tube containing a trypsin/EDTA solution. As many colonies as possible should be picked in a short period of time (so as to avoid over trypsinization of the colonies because it can affect the viability of cells) and the suspension placed into the incubator at 37ºC for 5 to 10 min. The cell clumps are mixed by pipetting up and down until a single-cell suspension is obtained. To neutralize the effect of trypsin, complete medium is added and centrifuged for 5 min at 400 *g* at RT. The pellet is resuspended in complete medium and placed onto mitotically inactivated MEF feeder cells plate containing EGC complete medium with LIF. The medium with LIF factor should be replaced daily until the cells have expanded sufficiently that they can be passaged onto a larger culture dish plate.

EG colonies also can be picked using a pulled-glass pipette instead of using a pipettor to disaggregate cells in a trypsin-EDTA solution. Also picked EGCs colonies can be trypsinized in a microdrop under mineral oil before transferred onto feeder layer cells (Matsui et al., 1992; Durcova-Hills & Surani, 2008).

Once a EG cell line is derived as explained in the above paragraph, growing colonies can be picked and expanded on gelatin-coated plates without feeder layers albeit at a lower efficiency compared to the traditional method (Leitch et al., 2010).

3. Other mammalian EG cells

3.1. Rat embryonic germ cells

The first derivation of EG cells from rat has been recently reported (Leitch et al., 2010). Leitch and collaborators have efficiently derived and propagated rat EG cell lines with using culture conditions previously used for clonogenic expansion of rat pluripotent embryonic stem cells (Li et al., 2008). EG cell line generation was obtained adding a MEK inhibitor and a GSK3 inhibitor in combination with LIF without SCF or bFGF to the culture of PGCs at the first medium change. Rat EG cells express pluripotency markers similar to mouse EG cells (Nanog, Oct3/4, Sox2, AP, Klf4 and Rex1) and the EG cell lines derived were competent for multilineage colonization of chimaeras and embryoid body formation giving rise to cells of the three germ layers thus showing the ability to reprogram rat PGCs to the pluripotent state.

Rat EG cells were derived from rat embryos from embryonic day 10, equivalent to 8.5 dpc in the mouse. The protocol used for derivation of rat EG cell lines follows the same schedule as described above for mice (Leitch et al., 2010) but on day 3 of derivation (first medium change), the cultures were washed with PBS and the medium was replaced with serum-free N2B27 medium supplemented with 1μM PD0325901 MEK inhibitor, and 3 μM CHIR99021 GSK3 inhibitor and LIF. Cultures were then maintained continuously in these conditions. Picked colonies were expanded on gelatin-coated plates with neither feeders nor conditional medium. After colony picking, cells were expanded by dissociation with trypsin and replating every 2-4 days.

3.2 Buffalo embryonic germ cells

Recently, embryonic germ cell like-cells from 30-90 dpc fetuses of the Chinese buffalo have been derived. These EG-like cells were cultured for more than two weeks on buffalo embryonic fibroblast feeder cells in DMEM containing 20% FBS media and supplemented with LIF, bFGF and SCF. During isolation of EG-like cells, the mechanical method used for disaggregating cells was better than the trypsin digestion, enabling cells to reach a higher passage in culture. Buffalo EG-like cells grew in large densely packed colonies resembling mouse ES or EG cells colonies and were characterized by their expression of the pluripotency markers AP, SSEA-1, SSEA-3, SSEA-4 and Oct-4. EG-like cells were capable to differentiate into the three germ layers in vitro, although chimaera formation was not determined (Huang et al., 2007).

3.3 Pig EG cells

Pig EG cells have been derived from porcine primordial germ cells isolated from the gonadal ridges of fetuses on days 24-28 of gestation (approximate to human Carnegie Stages 18-20) (Piedrahita et al., 1998). Several breeds have been used: Hampshire X Yorkshire (Shim et al., 1997), Duroc and German Landrace (Mueller et al., 1999), and the Chinese mini pig (Tsung et al., 2003). Genital ridges are dissected from the embryos, washed with PBS and then underwent different enzymatic dissociation treatments depending on the author: 0.02% EDTA (Sigma) for 20 min at RT (Shim et al., 1997; Mueller et al., 1999), 0.25% trypsin + 0.02% EDTA or 1mg/ml dispase (Sigma) in PBS for 5 min or up to 15 min at 37ºC (Tsung et al., 2003). After incubation, PGCs are mechanically dissociated by gentle disruption of the tissues using fine forceps and then centrifuged at 800 x g for 5 min. Enzymatic treatment could be replaced by more roughly mechanically dissociation passing the disrupted tissues

several times through a 20-gauge needle, then centrifuged for 3-5 min at 250 g to settle tissue fragments, and supernatant containing mostly single cells is collected and centrifuged at 1000 g for 5 min (Piedrahita et al., 1998). Colonies are disaggregated with 0.25% trypsin-EDTA for 10-15 min and passaged to fresh feeder layers at 4- to 10-day intervals (Shim et al., 1997; Piedrahita et al., 1998).

Similar to other species, feeder cells expressing membrane-bound SCF are required for survival and establishment of porcine EG cells (Lee & Piedrahita, 2000). STO cells, mitotically inactivated with 10 µg/ml mitomycin C (Sigma) for 2 h, are plated at a density of ~1.5-3 x 10^5 cells/cm^2 (Shim et al., 1997; Piedrahita et al., 1998).

The growth medium used to derive and maintain porcine EGCs is DMEM or DMEM:Ham´s F10 (1:1) (Piedrahita et al., 1998) containing 15% FBS, 1 mM L-glutamine, 0.1 mM or 0.01 mM nonessential amino acids (Gibco) (Piedrahita et al., 1998), 0.1 mM 2-mercaptoethanol (10 µM by Shim et al., 1997), penicillin (100 U/ml), and streptomycin (0.5 mg/ml) (Shim et al., 1997). FBS can be replaced with knockout serum replacement (KSR) (Petkov & Anderson, 2008) and 0.1 M Na^+ pyruvate (Gibco) has also added (Mueller et al., 1999). Cultures are maintained at 37-39°C in 5% CO_2, 95% air, and medium is changed every day. Growth factor supplementation is not necessary, but is common to use SCF (40 ng/ml), hbFGF (20-25 ng/ml), and LIF (10-20 ng/ml) (Piedrahita et al., 1998; Tsung et al., 2003). Lee & Piedrahita (2000) reported that even if it is not required for EGCs culture, supplementation with all three growth factors increases eight times the number of EGC colonies TNAP positive. In addition to AP, pig EGCs are Oct4, SSEA-1 and SSEA-4 and TRA-1-81 positive (Petkov & Anderson, 2008).

3.4 Goat EG cells

First attempts to generate EGCs from goat resulted in lines that survived briefly and after 3-4 passages differentiated (Kuhholzer et al., 2000; Lee & Piedrahita, 2000). However, the most successful attempt achieved to subculture goat EGC cells over 12 passages before spontaneous differentiation. Moreover, two chimeras out of 29 injected blastocysts were obtained: one aberrant chimera, showing cells representing the three germ layers and one viable chimera, showing chimerism in skin and blood. However, germ line transmission of the chimerism could not be confirmed (Jia et al., 2008).

Goat primordial germ cells were isolated from the gonadal ridges of fetuses from slaughterhouse. The fetuses age varied between groups, from 25 (Lee et al., 2000) to 32 dpc (Kuhholzer et al., 2000) or 28-42 dpc (Jia et al., 2008).

Tissues were washed with PBS with 0.02% EDTA, mechanically disaggregated and then incubated in 0.25% type IV collagenase (Sigma) for 30 min. To remove most somatic cells, cell suspension was filtered through a 100 mesh sterile gauze. After washing again in PBS and centrifugation at 1000 g for 5 min, cells were cultured on a goat embryonic fibroblast (GEF) feeder layer mitotically inactivated with mitomycin C at 100% density (Jia et al., 2008). First attempts used STO cells as feeders and different media, such as DMEM: Ham's F10 medium (1:1), but colonies differentiated earlier in culture (Kuhholzer et al., 2000; Lee et al., 2000).

The growth medium used was DMEM supplemented with 15% KSR (Gibco), 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1 mM Na^+ pyruvate (Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma), 10 ng/ml recombinant human bFGF (Sigma), 10 ng/ml SCF and 1000 IU/ml recombinant murine LIF (Chemicon) at 37 °C in a humidified atmosphere with 5% CO_2 (Jia et al., 2008).

The EGC cells were subsequently passaged to a fresh MEF feeder layer in the same medium by picking up colonies after 10 to 12 days (Jia et al., 2008). Long term culture of EGCs was difficult because of spontaneous differentiation into epithelial-, neuronal-, and fibroblast-like cells after few passages (Kuhholzer et al., 2000; Lee et al., 2000). However, goat EGCs were positive for AP, SSEA-1, Nanog and c-kit, as their mouse counterparts (Jia et al., 2008).

4. Human EG cells

To date, six laboratories have reported successful derivation of human EGC lines (Shamblott et al., 1998; Turnpenny et al., 2003; Liu et al., 2004; Park et al., 2004; He et al., 2007; Hua et al., 2009). This very limited number of groups is due to the difficulties of acquiring human PGCs (therapeutic termination of pregnancy and ethical approval is needed), and the complexity of the derivation and culture of EGCs (Turnpenny et al., 2006; Kerr et al., 2006; Perret et al., 2008).

4.1 Growth medium

The methods used for human EGCs derivation are based in part in those described previously for mouse. The growth medium used to derive and maintain human EGCs is Dulbecco's modified Eagle's medium, DMEM-199 or KO-DMEM (Invitrogen) supplemented with 15% FBS or 20% Knockout serum (HyClone/Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 2mM glutamine (Invitrogen), 1mM Na^+ pyruvate (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen). For human EGC derivation, 1000 U/ml human recombinant LIF (hLIF, Chemicon), 1–2 ng/ml human recombinant bFGF (Genzyme/R&D Systems), and 10 μM forskolin (Sigma) is added (Shamblott et al., 1998; Kerr et al., 2006). bFGF doses vary between groups, from 1 ng/ml (Shamblott et al., 1998) to 10 ng/ml (Hua et al., 2009). In contrast to mouse EGC derivation, supplementation with soluble SCF is not necessary for human EGCs. In agreement with mouse, transmembrane SCF expression of the feeder layer promoted human PGC growth (Kerr et al., 2006).

It is also possible to derive and maintain human EGCs without serum using 15-20% KSR (Invitrogen) (Turnpenny et al., 2003; Pan et al., 2005; Hua et al., 2009; Hiller et al., 2011). Whereas serum provides essential nutrients also provides some factors that promote differentiation of EGCs and make the analysis of the experiments difficult. Thus, it is reported that the derivation efficiency increases using KSR instead of FBS (Hua et al., 2009).

Recently it has been reported that the addition of 5-20 ng/ml recombinant BMP4 (R&D Systems) increases, in a dose-responsive manner, the efficiency of EGC derivation and maintenance. The results revealed an increase in EGC derivation of 5-fold with 20 ng/ml BMP4 and, after 3 weeks the survival efficiency increased by 50-fold in the presence of BMP4 (Hiller et al., 2011). Survival appeared to be in part related to the ability of BMP4 to inhibit spontaneous differentiation in these cultures, an inherent problem with long-term maintenance of human EGC cultures.

4.2 Feeder layer

Human EGCs have been mainly derived using the transformed mouse embryonic fibroblast line STO (Swelstad & Kerr, 2010). Although it is a clonal cell line, there are several phenotypic variations in STO cells from different isolates or even depending on the time in culture that affects the human EGC derivation (Kerr et al., 2006). Some groups have also

successfully utilized primary MEFs (CF1) as feeder layers for EGC derivation (Liu et al., 2004; Hua et al., 2009) and even human embryonic fibroblast-like cells derived from gonadal ridges and dorsal mesenteries (see below, He et al., 2007).

The feeder layer can be mitotically inactivated with mitomycin C or by irradiation, either before or after plating. The most convenient method is to irradiate the STO cells after plating, although a large γ radiation unit is needed (Kerr et al., 2006). STO cells are cultured in the EGC growth medium without growth factor and are passaged for short periods, being disaggregated with 0.05% trypsin–EDTA. STO cells are plated at 5 x 10^4 cells per well in 96-well plates previously coated with 0.1% gelatin for 30 min. It is also possible to culture the STO cells in other plate types, maintaining a similar cell density of 1.5 x 10^5 cells/cm^2.

Feeder cells can be irradiated before plating, which has the advantage of the need of a smaller γ radiation unit, but the disadvantage of lower control of feeder cell density. Feeder cells are disaggregated as described, resuspended in growth medium and then exposed to 50 Gy of γ radiation or X rays. Finally, cells are cultured into gelatinized culture plates at ~1.5 x 10^5 cells/cm^2 and allowed to adhere overnight. Alternatively, feeder layers can be mitotically inactivated with 10 µg/ml mitomycin C (Sigma) (Park et al., 2004; Liu et al., 2004; Hua et al., 2009).

When MEFs are used instead of STO cells, these have to be seeded at 7.5 ×10^4 cells/cm^2, half the density of STO cells (Pan et al., 2005; Hua et al., 2009). There is not comparison reported between human EGCs derivation on MEFs versus STO cells or between irradiation against mitomycin C mitotic inactivation.

Co-culture of human EGCs with mouse feeder cells entails clinical restrictions due to the possible contamination by xenogenic proteins or pathogens. As a result, a new method has been developed for hEGCs culture using human embryonic fibroblast-like cells derived from gonadal ridges and dorsal mesenteries obtained from 5-10 week human embryos. The tissues are mechanical and enzymatically (0.25% trypsin 10 min) dissociated and then cultured in high glucose (HG)-DMEM, 10% FBS (Gibco), 1 mM Na^+ pyruvate, 100 U/ml penicillin, and 50 µg/ml streptomycin. These cells are plated at 3 x 10^5 cells in a 6-well plate (~3.2 x 10^4 cells/cm^2), and after 24 h treated with 12.5 mg/l of mitomycin C (Roche) (He et al., 2007).

4.3 PGC isolation

To derive human EGCs, PGCs are isolated from the fetal gonad between 5 and 11 weeks of gestation (Carnegie stage 15 onwards) at termination of pregnancy by using a drug protocol or surgically and with local research ethics committee approval with written informed consents (Kerr et al., 2006). Hua et al. (2009) used a wider margin with fetuses aged 4-13 weeks, but is not clear that EGC derivation is possible with PGCs of those younger or older ages since the authors did not specify the age of the PGCs that actually originated EGC colonies. Human EGC derivation is more difficult to standardize compared to mouse EGCs in part because of the age and genetic heterogeneity among the embryonic sources. The collection of mesenteries besides the gonadal ridges also varies between different groups. Most of them but two groups included mesenteries (Pan et al., 2005; Turnpenny et al., 2006). Here we are going to describe the detailed protocol reported by Kerr et al. (2006) pointing out the differences with other laboratories.

Dissected tissues are collected in 1 ml of ice cold growth medium, then soaked in Ca^{+2} and Mg^{+2} free Dulbecco's phosphate buffered saline (DPBS) for 5 min and then enzymatically disaggregated with 100µl of trypsin–EDTA solution (Invitrogen). The concentration of

trypsin and EDTA varies depending on the developmental stages, using 0.05% trypsin–0.5 mM EDTA solution at earlier stages, and 0.25% trypsin–0.5 mM EDTA at later stages.
Following that, the tissue is mechanically dissociated, using fine forceps and iris scissors, for 5–10 min at RT, and then placed at 37ºC for 5–10 min. Growth medium is added to the tube to stop the digestion and finally the cell suspension is pipetted 30–50 strokes with a 200 µl Pipetman (Gilson) (Kerr et al., 2006).
Slight variations in these protocols are used by other groups: A previous 0.01% EDTA treatment for 10 minutes (Turnpenny et al., 2003), different enzymatic dissociation treatments between using only trypsin (without EDTA) (Liu et al., 2004), collagenase IV + DNase I (Turnpenny et al., 2003; Park et al., 2004), and even without mechanically dissociation and just incubation with 0.125% trypsin and 0.02% EDTA for 10–20 min or with 0.125% collagenase for 20–40 min at 37°C (Hua et al., 2009). No comparisons between different PGC isolation protocols have been reported, so it is unknown which one is the more efficient.
The cell suspension is plated on the previously prepared feeder layer, culturing each gonad on 4–10 wells of a 96 well plate, keeping in mind that derivation efficiency could be affected by the plating density (Kerr et al., 2006).
To get rid of accompanying somatic cells, PGCs could be sorted before plating (Kerr et al., 2008; Hiller et al., 2011). PGCs are isolated using magnetic cell sorting technology and an indirect labeling of cells with magnetically tagged goat anti-mouse IgM antibodies towards a mouse-anti-SSEA1 antibody (Miltenyi Biotech). After tissue dissociation, cells are incubated with SSEA1 antibody (1:5 dilution) for 15 min on ice, then secondary antibody is applied at 1:100 dilution for another 30 min on ice and sorted on magnetic columns. Culturing a pure population of PGCs allows ruling out possible effects of the somatic cells of the gonads in EGC derivation. When PGCs are counted after sorting, approximately 50 PGCs are seeded in each well of a 96-well plate (Hiller et al., 2011).
The plate is incubated at 37ºC in 5% (or 8% (Shamblott et al., 1998) CO_2 with 95% humidity for 7 days. Growth medium is changed every day, removing 90% of the old medium (Kerr et al., 2006).

4.4 Passage of EGC cultures

No EGC colonies are normally seen in most human EGC cultures during the first 7 days and only solitary PGCs could be observed when staining for TNAP activity. Passages are performed after 7 days, subculturing the PGCs onto fresh feeder cells. Compared to other pluripotent stem cells, hEGCs are also challenging to maintain due to the difficulty in disaggregating colonies, so the passage is a critical step in the EGC culture (Kerr et al., 2006).
Medium has to be removed and the wells washed with Ca^{+2} and Mg^{+2} free DPBS. To disaggregate the cells, 40 µl of freshly thawed trypsin-EDTA solution (0.05%-0.25% trypsin and 0.5 mM EDTA) is added to each well and incubated for 5 min at 37ºC. Instead of trypsin, 0.1% type IV collagenase could be used (He et al., 2007). Then a Pipetman and 200 µl tip is used to scrape the bottom of the wells and gently pipetting the culture 20–30 times. To stop digestion fresh growth medium is added and pipetting another 10–30 times. Cell suspension is placed into twice the number of former wells on feeder cells (Kerr et al., 2006). Subsequent passages are repeated every 7 days and after 2-3 weeks EGC colonies can be seen in some of the wells (Kerr et al., 2006).
Although initially 50% of the wells on average produce EGC colonies (Kerr et al., 2006), after 2 to 3 weeks, large and recognizable EGC colonies are seen only at approximately 10 to 20% of the wells (Swelstad & Kerr, 2010) (Figs. 5-7).

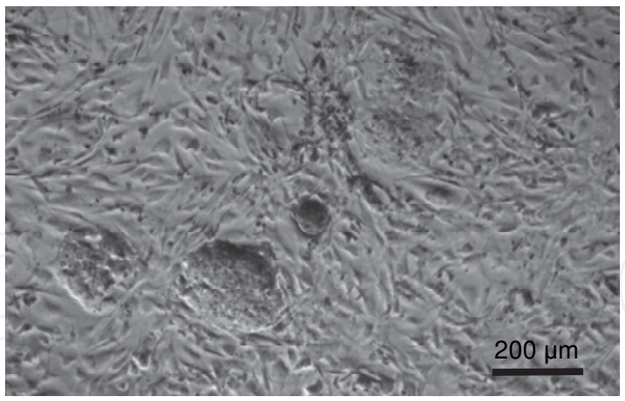


Fig. 5. Phase contrast image of human EGC colonies after derivation on STO feeder cells.

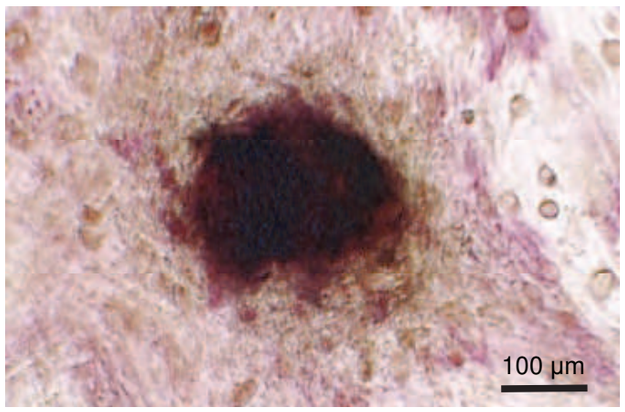


Fig. 6. Human EGC colony stained for alkaline phosphatase activity in red.

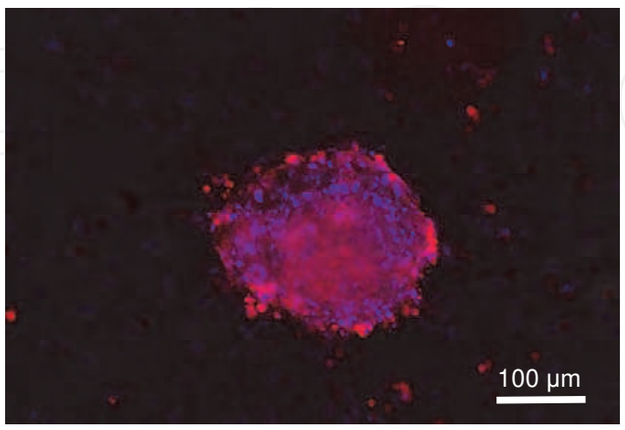


Fig. 7. Characterization of pluripotent human EG cells. Indirect immunofluorescent detection of pluripotent marker, Tra-1-81 (red). Dapi (blue) stains nuclei.

A relationship between the conversion efficiency and the embryonic stage or sex of the embryo has been reported in both male and female gonads to be associated with an increase in Oct-4 expressing PGCs which peak around 10 weeks of age for both sexes (Kerr et al., 2008a,b).

5. Attempts in challenging species

Finally, several attempts to derive EG cells from species are ongoing, and the challenges of EG derivation of these particular species are examined.

5.1 Rabbit EG cells

No stable EGCs lines have been derived yet from rabbit primordial germ cells. Kakegawa et al. (2008) reported the obtaining of EGC-like colonies that can form embryonic bodies (EBs) and differentiate in several cell types in vitro but did not develop teratomas after 20 days of injection of 3 x 10^6 cells. In addition, EGC-like colonies were lost in culture after 4 passages. These cells expressed Oct3/4, Sox2, and SSEA-1 and were AP positive as their mouse counterparts.

Rabbit PGCs were isolated from New Zealand White rabbit's embryos of 9.5-11.5 days of gestation, which resemble 8.5-11.5 dpc mouse embryos. Genital ridges were dissected and the dorsal mesentery was removed. Tissues were then washed with PBS and incubated in 0.04% trypsin, 0.25% EDTA (Sigma) for 5 minutes at RT. After the enzymatic digestion, PGCs were dissociated using a glass capillary tube and seeded on mitotically inactivated MEF feeder layer. Culture medium was composed of knockout-DMEM (Invitrogen) with 20 % KRS (Invitrogen), L-glutamine, Na^+ pyruvate, MEM nonessential amino acids and 2-mercaptoethanol. No growth factor addition was necessary for EG-like colonies formation, but the number of colonies increased with the addition of LIF (Chemicon), bFGF (Upstate), and forskolin (Sigma) at different concentrations. The greatest improvement was seen with 1000 IU/ml LIF, 20 ng/ml bFGF and 20 μM forskolin. For passaging, cultures were dissociated with 0.04% trypsin, 0.25% EDTA, and centrifuged onto fresh feeder cells at 4- to 7-day intervals (Kakegawa et al., 2008).

5.2 Sheep EG cells

Sheep EGC colonies have been derived and maintained over 14 passages. Their pluripotency has been proved by in vitro differentiation into a range of tissue types and by embryonic body formation. However, attempts to produce teratoma formation or chimaeric generation were attempted but without success (Ledda et al., 2010). Sheep EG expressed AP, Oct3/4, Sox2, Nanog and SSEA-1 (Ledda et al., 2010).

Primordial germ cells from sheep were isolated from fetuses of 20–28 days (similar to human Carnegie stages 13-19) following protocols similar to those previously described for mouse. PGCs were cultured on mitotically inactivated MEF feeder layers at near 100% of density on DMEM supplemented with 20% serum, L-glutamine, Na^+ pyruvate, MEM nonessential aminoacids, 2-mercaptoethanol and 1000 IU/ml LIF. Supplementation with 10-30 ng/ml bFGF and forskolin improved in vitro proliferation and long term survival of PGCs (Ledda et al., 2010).

5.3 Cow EG cells

Bovine EG-like cells were derived from 29-35 days of gestation PGCs. Genital ridges from the fetuses obtained from slaughterhouses were dissected without mesonephros. Tissues

were enzymatically disrupted with trypsin and EDTA and then seeded on a feeder layer of neomycin-resistant LIF-producing STO cells which express recombinant human LIF (McMahon & Bradley, 1990), or on bovine primary embryonic fibroblasts. Passages were performed picking the colonies from the feeder layers and subculturing (Cherny et al., 1994). As with sheep and rabbit EGCs, bovine EG-like cells also demonstrated the ability to generate embryoid bodies and differentiate to various cell types. Bovine EGCs were demonstrated positive for Oct-4, and Hes-1. Pluripotency in vivo was not demonstrated, as no teratomas or chimaeras were developed and EGCs only could be maintained in culture for up to 10 passages (7 weeks) (Cherny et al., 1994).

5.4 Non-human primate EG cells

Non-human primates pose an interesting alternative source for studying the highmarks of EGC derivation relevant to human biology and preclinical applications. While mouse has been the most extended animal model for EGCs research, there remain significant differences between mouse and human EGCs in terms of growth characteristics, marker expression, signaling patterns and imprinting status. Furthermore, human EGC derivation is limited to a diverse genetic background and human embryo donations are anonymous it is not possible to analyze epigenetic patterns relating them to parental lines. Thus, it seems reasonable to suggest that questions regarding the process of reprogramming and differentiation via genomic imprints could potentially be more easily and appropriately addressed using non-human primate EGCs.

To date, there are no reports on the isolation of stable long term EGC lines from non-human primate PGCs, but short term EGCs have been developed from the groups of Schatten and Simerly (Simerly et al., 2010).

Baboon embryos were collected between days 28-35 post coitum (similar to human Carnegie Stages 12-16), dissecting the lower half of the embryo and excising the genital ridge, dorsal mesentery and part of the gut. Tissues were dissociated with 0.05% trypsin treatment and cells cultured on irradiated STO feeder cells in 96-well plates at a density of ~$10x10^4$ cells/cm^2.

The growth medium used was DMEM high glucose with 15% FBS (Hyclone, Fisher), 2mM glutamine, 5 U/ml penicillin-streptomycin and 1mM Na^+ pyruvate, supplemented with various growth factor combinations, similar to both mouse and human protocols. These included different concentrations of hbFGF, 20 μM retinoic acid, 1000 U/ml hLIF, 10 ng/ml hSCF (Cell Signaling), 100 μM forskolin (Sigma), 20 ng/ml stromal cell-derived factors α & β (α,β-SDF), and 50 ng/ml BMP-4. BMP-4 was previously used to improve human EGC culture (Hiller et al., 2011) and retinoic acid has been shown to replace bFGF for mEGC derivation (Koshimizu et al., 1996). Three different protocols have been tried: 10 ng/ml hbFGF, LIF, hSCF and forskolin; 20 ng/ml hbFGF, LIF, hSCF, α,β-SDF and BMP-4; and retinoic acid, LIF, hSCF, α,β-SDF and BMP-4. Medium was removed and replenished with fresh growth medium daily and passaging onto fresh STO feeders was performed weekly. EGC colonies were established using all three protocols, but after 2-3 passages start to differentiate.

6. Conclusions

In summary, EGC lines have been derived from a variety of mammals including human and are being attempted in many others. Comparison of differences in methodology used as well as in pluripotency markers, achieved time of culturing and functional pluripotency demonstration, are summarized in Tables 1 and 2.

	Age (dpc)	Feeders	Medium	GFs	Passages	Pluripotency
Mouse (Matsui et al., 1992; De Miguel & Donovan 2003)	8.5-12.5	STO MEFs (P1 onwards)	DMEM + 15% FBS	LIF, bFGF, SCF, Forskolin	> 1 year	Chimaeras teratomas
Rat (Leitch et al., 2010)	10	Sl⁴-m220 MEFs (P1)	N2B27 serum free	LIF, MSKi, GSK3i	35	Chimaeras
Buffalo (Huang et al., 2007)	30-90	BuffaloEFs	DMEM + 20% FBS	LIF, bFGF, SCF	8	In vitro diff
Pig (Piedrahita et al., 1998)	24-28	STO	DMEM + 15% FBS	LIF, bFGF, SCF	> 10 months	Chimaeras
Goat (Jia et al., 2008)	25-42	GoatEFs MEFs	DMEM + 15% KSR	LIF, bFGF, SCF	12	Chimaeras
Human (Shamblott et al. 1998; Kerr et al., 2006)	5-11 week	STO	DMEM + 15% FBS	LIF, bFGF	>20	In vitro diff
Rabbit (Kakegawa et al., 2008)	9.5-11.5	MEFs	KO-DMEM + 20% KSR	LIF, bFGF, Forskolin	4	In vitro diff EBs
Sheep (Ledda et al., 2010)	20-28	MEFs	DMEM + 20% serum	LIF; bFGF + Forskolin	14	In vitro diff EBs
Cow (Cherny et al., 1994)	29-35	SNL BovineEF			>10	In vitro diff EBs
Baboon (Simerly et al., 2010)	28-35	STO	DMEM + 15% FBS	LIF, bFGF, SCF	3	ND

Table 1. Comparison of different methods of EGC lines derivation from different species. GF: growth factors. EBs: in vitro embryoid bodies. dpc: days post coitum. FBS: fetal bovine serum. KSR: knockout serum replacement. MEFs: mouse embryo fibroblasts. EFs: embryonic fibroblasts. KO: knockout. SNL: STO Neomycin-resistant LIF-producing cells. ND: not determined.

	Mouse	Rat	Buffalo	Pig	Goat	Human	Rabbit	Sheep	Cow	Baboon
TNAP	+ CC RT (De Miguel & Donovan, 2003)	+ CC (Leitch et al., 2010)	+ CC (Huang et al., 2007)	+ CC (Petkov & Anderson, 2008)	+ CC (Jia et al., 2008)	+ CC (Shamblott et al., 1998)	+ CC (Kakegawa et al., 2008)	+ CC (Ledda et al., 2010)	+ CC (Cherny et al., 1994)	**ND**
Oct4	+ IF; RT (Durcova-Hills et al., 2008)	+ IF; RT (Leitch et al., 2010)	+ IF RT (Huang et al., 2007)	+ ICC (Petkov & Anderson, 2008)	**ND**	+ ICC (Liu et al., 2004)	+ ICC (Kakegawa et al., 2008)	+ RT (Ledda et al., 2010)	+ RT (Cherny et al., 1994)	**ND**
Sox2	+ IF; RT (Durcova-Hills et al., 2008)	+ IF; RT (Leitch et al., 2010)	+ RT (Huang et al., 2007)	**ND**	**ND**	- RT (Perrett, et al., 2008)	+ ICC (Kakegawa et al., 2008)	+ RT (Ledda et al., 2010)	**ND**	**ND**
Nanog	+ RT (Durcova-Hills et al., 2008)	+ IF; RT (Leitch et al., 2010)	+ RT (Huang et al., 2007)	**ND**	+ RT (Jia et al., 2008)	+ RT (Turpenny et al., 2006)	**ND**	+ RT (Ledda et al., 2010)	**ND**	**ND**
cMyc	+ IF; RT (Durcova-Hills et al., 2008)	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
Klf4	+ IF; RT (Durcova-Hills et al., 2008)	+ RT (Leitch et al., 2010)	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
Rex1	+ RT (Durcova-Hills et al., 2008)	+ RT (Leitch et al., 2010)	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
SSEA1	+ IF (Durcova-Hills et al., 2008)	**ND**	+ IF (Huang et al., 2007)	+ ICC (Petkov & Anderson, 2008)	+ ICC (Jia et al., 2008)	+ ICC (Shamblott et al., 1998)	+ ICC (Kakegawa et al., 2008)	+ ICC (Ledda et al., 2010)	**ND**	**ND**
SSEA3	- µarry (Sharova et al., 2007)	**ND**	+ IF (Huang et al., 2007)	+ IF (Petkov & Anderson, 2008)	**ND**	+ ICC (Shamblott et al., 1998)	**ND**	**ND**	**ND**	**ND**

	Mouse	Rat	Buffalo	Pig	Goat	Human	Rabbit	Sheep	Cow	Baboon
SSEA4	**-** µarry (Sharova et al., 2007)	**ND**	**+** IF (Huang et al., 2007)	**+** ICC (Petkov & Anderson, 2008)	**ND**	**+** ICC (Shamblott et al., 1998)	**ND**	**ND**	**ND**	**ND**
Mvh	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**-** RT (Kakegawa et al., 2008)	**ND**	**ND**	**+** IF (Simerly et al., 2010)
TRA1-81	**-** µarry (Sharova et al., 2007)	**ND**	**ND**	**+** ICC (Petkov & Anderson, 2008)	**ND**	**+** ICC (Shamblott et al., 1998)	**ND**	**ND**	**ND**	**ND**
TRA1-60	**-** µarry (Sharova et al., 2007)	**ND**	**ND**	**ND**	**ND**	**+** ICC (Shamblott et al., 1998)	**ND**	**ND**	**ND**	**ND**
TERT	**ND**	**ND**	**ND**	**ND**	**ND**	**+** ICC (Turpenny et al., 2003)	**ND**	**ND**	**ND**	**ND**
Others	**Stella+** IF; RT (Durcova-Hills et al., 2008)	**ND**	**gp130+** RT (Huang et al., 2007)	**ND**	**ckit+** ICC (Jia et al., 2008)	**ckit+** IF (Hua et. al., 2009) **EMA1+** ICC (Turpenny et al., 2003)	**ND**	**ND**	**Oct6+** RT **Hes1+** RT (Cherny et al., 1994)	**ND**

Table 2. Comparison of differences in expression of markers in EGCs of different species. CC: Cytochemistry. IF: immunofluorescence. ICC: immunocytochemistry. RT: RT-PCR. µarry: microarray. ND: Not determined.

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8. References

Cherny, RA., Stokes, TM., Merei, J., Lom, L., Brandon, MR. & Williams RL. (1994). Strategies for the isolation and characterization of bovine embryonic stem cells.*Reproduction, Fertility and Development* Vol.6(No.5): 569-75.

De Felici, M. (2009). Primordial germ cell biology at the beginning of the XXI century, *International Journal of Developmental Biology* Vol.53(No.7): 891-4.

De Felici, M., Dolci, S. & Pesce, M. (1993). Proliferation of mouse primordial germ cells in vitro: a key role for cAMP, *Developmental Biology* Vol.157(No.1): 277-80.

De Felici, M., Farini, D. & Dolci, S. (2009). In or out stemness: comparing growth factor signalling in mouse embryonic stem cells and primordial germ cells, *Current Stem Cell Research&Therapy* Vol.4(No.2): 87-97.

De Miguel, MP. & Donovan, PJ. (2003). Isolation and culture of embryonic germ cells. *Methods in Enzymology* Vol.365: 353-63.

De Miguel, MP., Fuentes-Julian, S. & Alcaina, Y. (2010). Pluripotent stem cells: Origin, maintenance and induction, *Stem Cells Reviews and Reports* Vol.6: 633-49.

Donovan, PJ., Stott, D., Cairns, LA., Heasman, J. & Wylie, CC. (1986). Migratory and postmigratory mouse primordial germ cells behave differently in culture, *Cell* Vol.44: 831–8.

Durcova-Hills, G., Ainscough, J. & McLaren, A. (2001). Pluripotential stem cells derived from migrating primordial germ cells, *Differentiation* Vol.68(No.4-5): 220-6.

Durcova-Hills, G., Tang, F., Doody, G., Tooze, R. &Surani, MA. (2008). Reprogramming primordial germ cells into pluripotent stem cells, *PLoS One* Vol.3(No.10): e3531.

Durcova-Hills, G. & Surani, A. (2008). Reprogramming primordial germ cells (PGC) to embryonic germ (EG) cells, *Current Protocols in Stem Cell Biology* Vol.1:5:1A.3.1-1A.3.20.

Fenderson, BA., De Miguel, MP., Pyle, AD. & Donovan, PJ. (2006). Staining embryonic stem cells using monoclonal antibodies to stage-specific embryonic antigens, *Methods in Molecular Biology* Vol.325: 207-24.

Gillich, A. & Hayashi, K. (2011). Switching stem cell state through programmed germ cell reprogramming. *Differentiation*. [Epub ahead of print].

Ginsburg, M., Snow, MH. & McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* Vol.110(No.2): 521-8.

He, J., Wang, Y. & Li, YL. (2007). Fibroblast-like cells derived from the gonadal ridges and dorsal mesenteries of human embryos as feeder cells for the culture of human embryonic germ cells, *Journal of Biomedical Science* Vol.14(No.5): 617-28.

Hiller, M., Liu, C., Blumenthal, PD., Gearhart, JD. & Kerr, CL. (2011). Bone morphogenetic protein 4 mediates human embryonic germ cell derivation, *Stem Cells and Development* Vol.20(No.2): 351-61.

Hua, J., Yu, H., Liu, S., Dou, Z., Sun, Y., Jing, X., Yang, C., Lei, A., Wang, H. & Gao, Z. (2009). Derivation and characterization of human embryonic germ cells: serum-free culture and differentiation potential, *Reproductive Biomedicine Online* Vol.19(No.2): 238-49.

Huang, B., Xie, TS., Shi, DS., Li, T., Wang, XL., Mo, Y., Wang, ZQ. & Li, MM. (2007). Isolation and characterization of EG-like cells from Chinese swamp buffalo (Bubalus bubalis), *Cell Biology International* Vol.31(No.10): 1079-88.

Jia, W., Yang, W., Lei, A., Gao, Z., Yang, C., Hua, J., Huang, W., Ma, X., Wang, H. & Dou, Z. (2008). A caprine chimera produced by injection of embryonic germ cells into a blastocyst, *Theriogenology* Vol.69(No.3): 340-8.

Kakegawa, R., Teramura, T., Takehara, T., Anzai, M., Mitani, T., Matsumoto, K., Saeki, K., Sagawa, N., Fukuda, K. & Hosoi, Y. (2008). Isolation and culture of rabbit primordial germ cells, *Journal of Reproduction and Development* Vol.54(No.5): 352-7.

Kaufman, MH. (1992). *The Atlas of Mouse Development*, Academic Press, San Diego.

Kemper, CH. & Peters, PW. (1987). Migration and proliferation of primordial germ cells in the rat. *Teratology* Vol.36(No.1): 117-24.

Kerr, CL., Shamblott, MJ. & Gearhart, JD. (2006). Pluripotent stem cells from germ cells, *Methods in Enzymology* Vol.419: 400-26.

Kerr, CL., Hill, CM., Blumenthal, PD. &Gearhart, JD. (2008). Expression of pluripotent stem cell markers in the human fetal ovary, *Human Reproduntion* Vol.23(No.3): 589-99

Kerr, CL., Hill, CM., Blumenthal, PD. & Gearhart, JD. (2008). Expression of pluripotent stem cell markers in the human fetal testis, *Stem Cells* Vol.26(No.2): 412-21.

Kimura, T., Suzuki, A., Fujita, Y., Yomogida, K., Lomeli, H., Asada, N., Ikeuchi, M., Nagy, A., Mak, T. W. & Nakano, T. (2003). Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production, *Development* Vol.130: 1691-700.

Kimura, T., Tomooka, M., Yamano, N., Murayama, K., Matoba, S., Umehara, H., Kanai, Y. & Nakano, T. (2008). AKT signaling promotes derivation of embryonic germ cells from primordial germ cells. *Development* Vol.135(No.5): 869-79.

Koshimizu, U., Taga, T., Watanabe, M., Saito, M., Shirayoshi, Y., Kishimoto, T. & Nakatsuji, N. (1996). Functional requirement of gp130-mediated signaling for growth and survival of mouse primordial germ cells in vitro and derivation of embryonic germ (EG) cells. *Development* Vol.122(No.4): 1235-42.

Kühholzer, B., Baguisi, A. & Overström, EW. (2000). Long-term culture and characterization of goat primordial germ cells, *Theriogenology* Vol.53(No.5): 1071-9.

Kwong, WH. & Tam, PPL. (1984). The pattern of alkaline phosphatase activity in the developing mouse spinal cord, *Journal of Embryology & Experimental Morphology* Vol.82: 241-51

Labosky, PA., Barlow, DP. & Hogan, BL. (1994). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines, *Development* Vol.120(No.11): 3197-204.

Labosky, PA.& Hogan, BL. (2008). Mouse primordial germ cells: isolation and in vitro culture *Methods in Molecular Biology* Vol.461: 187-99.

Lawson, KA., Dunn, NR., Roelen, BA., Zeinstra, LM., Davis, AM., Wright, CV., Korving, JP. & Hogan, BL. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo, *Genes & Development* Vol.13(No.4): 424-36.

Ledda, S., Bogliolo, L., Bebbere, D., Ariu, F. & Pirino, S. (2010). Characterization, isolation and culture of primordial germ cells in domestic animals: recent progress and insights from the ovine species. *Theriogenology* Vol.74(No.4): 534-43.

Lee, CK., Moore, K., Scales, N., Westhusin, M., Newton, G., Im, KS. & Piedrahita, JA. (2000). Isolation and genetic transformation of primordial germ cell (PGC)-derived cells from cattle, goats, rabbits and rats, *Asian - Australasian Journal of Animal Sciences* Vol.13: 587-94.

Lee, CK. & Piedrahita, JA. (2000). Effects of growth factors and feeder cells on porcine primordial germ cells in vitro. *Cloning* Vol.2(No.4): 197-205.

Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A. & Ishino, F. (2002). Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells, *Development* Vol.129(No.8): 1807-17.

Leitch, HG., Blair, K., Mansfield, W., Ayetey, H., Humphreys, P., Nichols, J., Surani, MA. & Smith, A. (2010). Embryonic germ cells from mice and rats exhibit properties consistent with a generic pluripotent ground state. *Development* Vol.137(No.14): 2279-87.

Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, RE., Schulze, EN., Song, H., Hsieh, CL., Pera, MF. & Ying, QL. (2008). Germline competent embryonic stem cells derived from rat blastocysts, *Cell* Vol.135(No.7): 1299-310.

Liu, S., Liu, H., Pan, Y., Tang, S., Xiong, J., Hui, N., Wang, S., Qi, Z. & Li, L. (2004). Human embryonic germ cells isolation from early stages of post-implantation embryos, *Cell and Tissue Research* Vol.318(No.3): 525-31.

Matsui, Y., Toksoz, D., Nishikawa, S., Nishikawa, S., Williams, D., Zsebo, K. & Hogan, BL. (1991). Effect of Steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture, *Nature* Vol.353: 750-2.

Matsui, Y., Zsebo, K. & Hogan, BL. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture, *Cell* Vol.70(No.5): 841-7.

McLaren, A. (2003). Primordial germ cells in the mouse, *Developmental Biology* Vol.262: 1-15.

McMahon, AP. & Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain, *Cell* Vol.62(No.6): 1073-85.

Merchant-Larios, H., Mendlovic, F. & Alvarez-Buyulla, A. (1985). Characterisation of alkaline phosphatase from primordial germ cells and ontogenesis of this enzyme in the mouse, *Differentiation* Vol.29: 145-51.

Mueller, S., Prelle, K., Rieger, N., Petznek, H., Lassnig, C., Luksch, U., Aigner, B., Baetscher, M., Wolf, E., Mueller, M. & Brem, G. (1999). Chimeric pigs following blastocyst injection of transgenic porcine primordial germ cells, *Molecular Reproduction and Development* Vol.54(No.3): 244-54.

Oosterhuis, JW. & Looijenga, LH. (2005). Testicular germ-cell tumours in a broader perspective, *Nature Reviews Cancer* Vol.5(No.3): 210-22.

Pan, Y., Chen, X., Wang, S., Yang, S., Bai, X., Chi, X., Li, K., Liu, B. & Li, L. (2005). In vitro neuronal differentiation of cultured human embryonic germ cells, *Biochemical and Biophysical Research Communications* Vol.327(No.2): 548-56.

Park, JH., Kim, SJ., Lee, JB., Song, JM., Kim, CG., Roh, S. & Yoon, HS. (2004). Establishment of a human embryonic germ cell line and comparison with mouse and human embryonic stem cells, *Molecules and Cells* Vol.17(No.2): 309-15.

Perrett, R M., Turnpenny, L., Eckert, JJ., O'Shea, M., Sonne, SB., Cameron, IT., Wilson, DI., Meyts, ER. & Hanley, NA. (2008). The Early Human Germ Cell Lineage Does Not Express SOX2 During In Vivo Development or upon In Vitro Culture, Biology of Reproduction Vol.78 (No.5): 852-8.

Pesce, M., De Felici, M. (1995). Purification of Mouse Primordial Germ Cells by MiniMACS Magnetic Separation System, *Developmental Biology* Vol.170(No.2): 722-5.

Petkov, SG. & Anderson, GB. (2008). Culture of porcine embryonic germ cells in serum-supplemented and serum-free conditions: the effects of serum and growth factors on primary and long-term culture, *Cloning Stem Cells* Vol.10(No.2): 263-76.

Piedrahita, JA., Moore, K., Oetama, B., Lee, CK., Scales, N., Ramsoondar, J., Bazer, FW. & Ott, T. (1998). Generation of transgenic porcine chimeras using primordial germ cell-derived colonies, *Biology of Reproduction* Vol.58(No.5): 1321-9.

Resnick, JL., Bixler, LS., Cheng, L. & Donovan, PJ. (1992). Long-term proliferation of mouse primordial germ cells in culture, *Nature* Vol.359(No.6395): 550-1.

Shamblott, MJ., Axelman, J., Wang, S., Bugg, EM., Littlefield, JW., Donovan, PJ., Blumenthal, PD., Huggins, GR. & Gearhart, JD. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells, *Proceedings of the National Academy of Sciences* Vol.95(No.23): 13726-31.

Sharova, LV., Sharov, AA., Piao, Y., Shaik, N., Sullivan, T., Stewart, CL., Hogan, BL. & Ko, MS. (2007). Global gene expression profiling reveals similarities and differences among mouse pluripotent stem cells of different origins and strains, *Developmental Biology* Vol.307(No.2): 446-59.

Shim, H. & Anderson, GB. (1998). In vitro survival and proliferation of porcine primordial germ cells, *Theriogenology* Vol.49(No.3): 521-8.

Shim, H., Gutiérrez-Adán, A., Chen, LR., BonDurant, RH., Behboodi, E. & Anderson, GB. (1997). Isolation of pluripotent stem cells from cultured porcine primordial germ cells, *Biology of Reproduction* Vol.57(No.5): 1089-95.

Simerly, C., Castro, C., Gori, M., Turpin, J., Champagne, J., Grund, K., Bauer, C., De Miguel, MP., Donovan, P. & Schatten, G. (2010). Towards Deriving Baboon Pluripotent Embryonic Germ Cells. *8thAnnual Meeting of the International Society for Stem Cell Research*. San Francisco, CA., mes-2010.

Stevens, LC. (1967). Origin of testicular teratomas from primordialgerm cells in mice, *Journal of the National Cancer Institute* Vol.38(No.4): 549-52.

Stewart, CL., Gadi, I. & Bhatt, H. (1994). Stem cells from primordial germ cells can reenter the germ line, *Developmental Biology* Vol.161(No.2): 626-8.

Surani, MA. (2007).Germ cells: the eternal link between generations,*Comptes rendus biologies* Vol.330(No.6-7): 474-8.

Swelstad, BB. & Kerr, L. (2010). Current protocols in the generation of pluripotent stem cells: theoretical, methodological and clinical considerations, *Stem Cells and Cloning: Advances and Applications* Vol. 3: 13–27.

Tam, PP., Snow, MH. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos, *Journal of Embryology & Experimental Morphology* Vol.64: 133-47.

Toksoz, D., Zsebo, KM., Smith, KA., Hu, S., Brankow, D., Suggs, SV., Martin, FH. & Williams, DA. (1992). Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor, *Proceedings of the National Academy of Sciences* Vol.89: 7350–4.

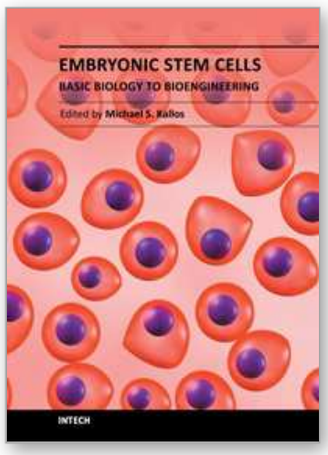
Tsung, HC., Du, ZW., Rui, R., Li, XL., Bao, LP., Wu, J., Bao, SM. & Yao Z. (2003). The culture and establishment of embryonic germ (EG) cell lines from Chinese mini swine, *Cell Research* Vol.13(No.3): 195-202.

Turnpenny, L., Brickwood, S., Spalluto, CM., Piper, K., Cameron, IT., Wilson, DI. & Hanley, NA. (2003). Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells, *Stem Cells* Vol.21(No.5): 598-609.

Turnpenny, L., Spalluto, CM., Perrett, RM., O'Shea, M., Hanley, KP., Cameron, IT., Wilson, DI. & Hanley, NA. (2006). Evaluating human embryonic germ cells: concord and conflict as pluripotent stem cells, *Stem Cells* Vol.24(No.2): 212-20.

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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