# NOVEL APPROACHES TO THE ISOLATION OF FARM ANIMAL EMBRYONIC STEM CELLS

**by**

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# TABLE OF CONTENTS

# ABSTRACT

The establishment of stable immortal ES cell lines using embryos as a source of isolation in domesticated farm animals, in particular for pigs, which are closer to humans than other ungulates, has not been reported; hence this information could contribute to the improvement of regenerative medicine in humans, biotechnology and agriculture. Therefore, the discovery of effective protocols to derive and maintain ES cells and the induction of purified somatic cells from ES cells in pigs is of importance.

The objectives of this study were to produce pES-like cells and direct differentiation of the ES-like cells obtained by improving the culture conditions. *In vivo*-derived porcine blastocysts at day 6-8 were classified into two groups distinguished by the exhibition of ICMs and epiblasts of the embryos. In each group, intact blastocysts and isolated ICMs or epiblasts were designed to culture in either KO4bh or DM40bh medium on mitotically inactivated MEFs under the humidified air of 5%$CO_2$ at 39°C until the primary outgrowth of ES-like cells was observed. Two morphologically distinct pES-like cells, pESA-like and pESB-like cells were isolated from the epiblasts, whereas no cell lines were generated from ICMs. pESA-like cells were observed as individual small round cells containing one or multiple nucleoli along with a high ratio of nucleus to cytoplasm, while pESB-like cells formed dome-like colonies. The pESA-like cells were stained both negative and positive with the alkaline phosphatase enzyme, while pESB-like cells were all stained positive. With immunofluorescence staining of OCT-4 and nanog, the nuclei of pESB-like cells appeared not to be stained positive with these two antibodies, while the designed self-renewing genes such as OCT-4, nanog, SOX-2, REX-1 and DPPA-3 were detectable as similar to mES cells. Regarding the pluripotent abilities of pESB-like cells, they could be induced to form neuronal-like, neuronal supporting-like, smooth muscle-like and hepatic-like cells in a variety of desirable differentiation media under the feeder-free culture system. The cytoplasmic contents of certain induced mature cells were stained positive with nestin, α-smooth muscle actin and α-fetoprotein in association with the expression of differentiated genes specific to each germ layer such as nestin, α-smooth muscle actin, smooth muscle myosin heavy chain, α-cardiac actin, transthyretin, α-fetoprotein, albumin and HGF1β. In conclusion, pESB-like cells obtained in this study may possibly have the potential to be authentic ES cells isolated from early epiblast origin as mES cells.

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Kwanta Thansa

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| α | Alpha |
| A | Adenine; Adenosine |
| aa | Amino acid |
| AFP | α-fetoprotein |
| AKT | Protein Kinase B (PKB) |
| ALK4/7 | Activin-like kinase receptor type 4 and 7 |
| AP | Alkaline phosphatase |
| ATP | Adenosine triphosphate |
| ß | Beta |
| BECM-3 | Beltsville embryo culture medium-3 |
| bFGF | Basic fibroblast growth factor |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| ß-ME | Beta mercaptoethanol |
| BMP-4 | Bone morphogenetic protein-4 |
| cAMP | Cyclic adenosine 3-,5-monophosphate |
| C | Cytidine; Cytosine |
| ºC | Degrees Celcius |
| $CaCl_2$ | Calcium dichloride |
| $Ca^{2+}$ | Calcium ion |

| | |
|---|---|
| cDNA | Complementary DNA |
| cm | Centimetre ($10^{-2}$ m) |
| $cm^2$ | Square Centimetre |
| c-Myc | Cellular myelocytomatosis oncogene |
| CDX-2 | Caudal type homeobox transcription factor-2 |
| $CO_2$ | Carbon dioxide |
| COCs | Cumulus oocyte complexes |
| CS | Calf serum |
| CTP | Cytidine triphosphate |
| Cy3 | Cyanine3 |
| DAPI | 4,6-diamidino-2-phenylindole |
| DC | Direct current |
| D ∞ G | Donkey anti-goat |
| D ∞ R | Donkey anti-rabbit |
| DEPC | Diethylpyrocarbonate |
| DIA | Differentiation inhibitory activity |
| DMEM | Dulbecco's modified eagle's medium |
| DMEM/F-12 | Dulbecco's modified eagle's medium:Nutrient mixture F-12 media |
| DMSO | Dimethyl sulfoxide |
| DPBS | Dulbecco's phosphate buffered saline |
| DPPA-3 | Developmental pluripotency associated-3 |
| DNA | Deoxyribonucleic acid |

| | |
|---|---|
| cDNA | Complementary DNA |
| DNase | Deoxyribonuclease |
| dNTP | Deoxyribonucleotide triphosphate |
| $dH_2O$ | Deionised or distilled water |
| Dsh | Dishevelled protein |
| EB | Embryoid body |
| ED | Embryonic disc |
| EDTA | Ethylenediamintetraacetic acid |
| EGF | Epidermal growth factor |
| ES | Embryonic stem |
| ERK | Extracellular signal-regulated protein kinases |
| ERK-1 | Extracellular signal-regulated protein kinases-1 |
| ERK-2 | Extracellular signal-regulated protein kinases-2 |
| FCS | Foetal calf serum |
| FGF | Fibroblast growth factor |
| FGF-2 | Fibroblast growth factor type 2 |
| FITC | Fluorescein isothiocyanate |
| FRV | Fast red violet LB base |
| FSH | Follicle stimulating hormone |
| g | Gram |
| gp-130 | Glycoprotein-130 |
| GAB1 | GRB2 associated binder protein-1 |

| | |
|---|---|
| GDFs | Growth differentiation factors |
| GFAP | Glial fibrillary acidic protein |
| GRB2 | Growth factor receptor bound protein-2 |
| GSK3-β | Glycogen synthase kinase3-β |
| G | Guanine; Guanosine |
| G ∞ M | Goat anti-mouse |
| GTP | Guanosine triphosphate |
| h | Hour |
| hES | Human embryonic stem/pluripotent |
| hLIF | Human leukemia inhibitory factor |
| HCl | Hydrochloric acid |
| HGF | Hepatocyte growth factor |
| HMG | High-mobility-group |
| HNF-1β | Hepatocyte nuclear factor 1 homeobox B |
| ICM | Inner cell mass |
| Ids | Inhibitor of differentiation proteins |
| IgG | Immunoglobulin G |
| IGF | Insulin-like growth factor binding protein |
| IL-6 | Interleukin-6 |
| iPS | Induced pluripotent stem |
| ITS | Insulin, transferrin and selenium |
| IVF | *In vitro* fertilization |

| | |
|---|---|
| IVM | *In vitro* maturation |
| JAK | Janus tyrosine kinase |
| kb | Kilobase |
| kDa | KiloDalton |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Potassium dihydrogen orthophosphate |
| KLF-4 | Kruppel-like factor 4 |
| KO-DMEM | Knockout DMEM |
| KSR | Knockout serum replacement |
| L | Litre |
| $lb/in^2$ | Pound per square inch |
| LH | Luteinizing hormone |
| LIF | Leukemia inhibitory factor |
| LIFR | Leukemia inhibitory factor receptor |
| LIF-gp130 | Leukemia inhibitory factor-glycoprotein130 |
| LIN28 | LIN-28 homolog (C. elegans) |
| LEF | Lymphoid enhancer factor |
| MAPK | Mitogen-activated protein kinases |
| $MgCl_2$ | Magnesium chloride |
| $Mg^{2+}$ | Magnesium ion |
| $MgSO_4$ | Magnesium sulfate |
| m | Metre |

| | |
|---|---|
| M | Molar |
| µg | Microgram ($10^{-6}$ g) |
| µl | Microlitre ($10^{-6}$ L) |
| µm | Micrometre ($10^{-6}$ m) |
| mg | Milli-gram ($10^{-3}$ g) |
| ml | Milli-litre ($10^{-3}$ L) |
| mm | Milli-metre ($10^{-3}$ m) |
| mM | Milli-molar ($10^{-3}$ M) |
| min | Minute |
| mEBs | Mouse embryoid bodies |
| mES | Mouse embryonic stem |
| MEFs | Murine embryonic fibroblasts |
| MEM NEAA | Minimum essential medium non essential aa |
| mEpiSC | Mouse epiblast stem |
| mLIF | Mouse leukemia inhibitory factor |
| mOsm | Mili-osmole |
| M-cadherin | Myotubule cadherin |
| Myf5 | Myogenic factor 5 |
| Myf6 | Myogenic factor 6 |
| MyoD | Myogenic differentiation |
| ng | Nanogram ($10^{-9}$ g) |
| $N_2$ | Dinitrogen |

| | |
|---|---|
| NaCl | Sodium chloride |
| $Na_2HPO_4$ | Disodium hydrogenphosphate |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium hydroxide |
| NCAM | Neural cell adhesion molecule homolog |
| NCSU-23 | North Carolina State University-23 medium |
| NOG | Noggin |
| NT | Nuclear transfer |
| Oct-3A/4 | Octamer-3A/4 |
| OCT-4 | Octamer-binding transcription factor-4 |
| $O_2$ | Oxygen |
| O4 | Oligodendrocyte marker O4 |
| Oligo dT | Thymidine oligodeoxynucleotide |
| OSM | Oncostatin M |
| PA | Parthenogenetic activation |
| PAX-6 | Paired box gene 6 |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PEDF | Pigment epithelium derived factor |
| PEF | Porcine embryonic fibroblast |
| pES | Porcine embryonic stem |

| | |
|---|---|
| PFA | Paraformaldehyde |
| PI3K | Phosphoinositide-3-kinase |
| PTB | Phosphotyrosine-binding |
| PTEN | Phosphatase and tensin homolog |
| P/S | Penicillin: Steptomycin |
| PUEC | Porcine uterine epithelial cells |
| PVA | Polyvinyl alcohol |
| PVA-TL-HEPES | Hepes-buffered Tyrode medium containing 0.1% PVA |
| PVP | Polyvinylpyrrolidone |
| PZM3 | Porcine zygote medium-3 |
| R ∞ M | Rabbit anti-mouse |
| RER | Rough endoplasmic reticulum |
| REX-1 | RNA exonuclease 1 homolog |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| RT | Reverse transcriptase |
| RT-PCR | Reverse transcriptase PCR |
| SER | Smooth endoplasmic reticulum |
| SH-2 | Src-homology-2 |
| SHP-2 | SH-2 domain-containing protein tyrosine phosphatase-2 |
| SMADs | Mothers against decapentaplegic protein |

| | |
|---|---|
| SMAD1/5/8 | Mothers against decapentaplegic protein type 1, 5 and 8 |
| SMAD2/3 | Mothers against decapentaplegic protein type 2 and 3 |
| SMAD4 | Mothers against decapentaplegic protein type 4 |
| SOX-1 | SRY-related HMG box-containing gene 1 |
| SOX-2 | SRY-related HMG box-containing gene 2 |
| SOX-17 | SRY-related HMG box-containing gene 17 |
| SRY | Sex determining region Y |
| STAT-3 | Signal transducer and activator of transcription-3 |
| STO | SIM (Sandoz inbred mouse) embryo-derived thioguanine-and ouabain-resistant fibroblast cell line |
| T | Thymine; Thymidine |
| TAE | Tris acetate EDTA buffer |
| TBS | Tris-EDTA buffer solution |
| TCF | T-cell factor |
| TCM199 | Tissue culture medium 199 |
| TDGF-1 | Teratocarcinoma-derived growth factor 1 |
| TE | Tris EDTA buffer |
| TGF-β | Transforming growth factor β |
| TH | Tyrosine hydroxylase |
| TIGR | The Institute for Genome Research |
| TTF-1 | Thyroid transcriptional factor-1 |
| TTP | Thymidine triphosphate |

| | |
|---|---|
| Tuj1 | Neuronal specific class III β-Tubulin |
| u | Unit |
| U | Uracil; Uridine |
| UTP | Uridine triphosphate |
| UV | Ultraviolet |
| VE | Visceral endoderm |
| V/mm | Volts per milimetre |
| Wnt | Wingless |
| x | Time |
| ZFP-42 | Zinc finger protein-42 |
| ZP | Zona pellucida |

# CHAPTER 1

## LITERATURE REVIEW: EMBRYONIC STEM CELLS

### 1.1 INTRODUCTION

Nowadays, stem cell research attracts more public and scientific attention than any other topic in developmental biology. An understanding of the specific properties of stem cells may generate deep insights and crucial impacts in cell biology and also provide great hope for the treatment of a variety of degenerative diseases such as Parkinson's, diabetes mellitus and leukaemia. Moreover, isolation of embryonic stem cells in domestics species would benefit agriculture production systems (Prelle *et al*., 2002; Melton and Cowen, 2006; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006; Prentice, 2006; Brevini *et al*., 2007a; Brevini *et al*., 2007b; Keefer *et al*., 2007; Renard *et al*., 2007; Vackova *et al*., 2007; Talbot and Blomberg, 2008).

In order to understand the concepts of stem cells and how they function, it is necessary to have some general knowledge of the properties of stem cells. Basically, stem cells can be isolated from all stages of the developmental embryo, foetus and adult and all share the common important characteristics of self-renewal, clonality and potency. Self-renewal is characterised by two patterns of mitotic cell division; firstly the stem cell may divide symmetrically to give rise to two identical immortal stem cells. Alternatively, asymmetric division may occur resulting in a daughter cell which subsequently differentiates and a stem cell which remains undifferentiated. In this model the uniparental stem cell usually retains the unreplicated copy of the nuclear genome. Second, clonality is a capacity of single cells to create more pluripotent stem cells to increase their indefinite numbers and, finally, potency represents an ability to elicit an expected result, which is identified its abilities of stem cells as unipotency, multipotency and pluripotency (Watt and Hogan, 2000; Melton and Cowen, 2006).

The number of embryonic stem (ES) cell research studied in human cell therapy increase rapidly when compare to foetal and adult stem cells, primarily because of its pluripotency, an ability of cells to differentiate to become certain types of cells in three germ layers, e.g. ectoderm, mesoderm and endoderm, and secondly the limitations of foetal stem cells; for example, finding the places to get foetuses from which to derive this type of stem cells and ethical problems perceived in ES cells. Regarding adult stem cells, isolation and maintenance of undifferentiated adult stem cells in culture system are difficult and they have a more definite expansion and restricted potency than do ES cells (Nardi, 2005; Pouton and Allsopp, 2005; Rossant, 2007). However, the research limitations in human ES cells, such as ethical issues, political concerns and long-term transplantations, has lead scientists to find an alternative method called induced pluripotent stem cells (iPS cells) by reprogramming somatic cells into an embryonic stem cell like state using certain stem cell-associated genes inserted into the required adult cells (Takahashi and Yamanaka, 2006; Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007; Yu *et al*., 2007; Kim *et al*., 2008b; Nakagawa *et al*., 2008). This artificial induced ES-like cell state technique seems to have the potential to replace ES cells originally derived from embryos, but a great deal of proof is still required that certain characteristics of iPS cells are equivalent to those of natural ones. Moreover, mutagenesis may possibly occur by using retroviral transfection as a method to insert the required genes to reprogramme the adult cells (Kim *et al*., 2008b). Although in the future, some studies might be able to reprogramme somatic cells without using viruses, it must be borne in mind that an induced pluripotent ES-like state may not reveal itself authentically in reality, leading to some occurrences of unknown disadvantages which must be investigated over a long period of time. Finally, different types of mature cells of individuals may need different compositions of genes to dedifferentiate them due to having variations in sensitivity or possibly varying in levels of endogenous gene expressions of each tissue in response to certain factors, as neural stem cells need only two defined factors to induce pluripotent stem cells (Kim *et al*., 2008b), while fibroblasts need three or four factors (Takahashi and Yamanaka, 2006; Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007; Yu *et al*., 2007; Nakagawa *et al*., 2008). Furthermore, it cannot be denied that tissues or organs induced from iPS cells *in*

*vitro* will either work effectively as naturally pluripotent ES cells initiated from embryos or not, when those iPS cells are permitted for use in live animals serving as specific medical models. Therefore, one of the best alternate ways to avoid the difficulties of human rights and political policies in conducting ES research created from embryos is to handle animals as models of medical diseases (Wolf *et al*., 2000; Prelle *et al*., 2002; Li *et al*., 2004a; Li *et al*., 2004b; Wang *et al*., 2005b; Vackova and Madrova, 2006; Taupin, 2006; Brevini *et al*., 2007a; Keefer *et al*., 2007; Vackova *et al*., 2007).

## 1.2 EMBRYONIC STEM CELL DERIVATIONS IN MAMMALS

Since the first successful isolation and characterisation of ES cells has been reported from mouse blastocysts (Evans and Kaufman, 1981), intensive attempts to derive pluripotent cell lines from other mammalian species have been made in order to discover the most suitable animal models specific to each medical disease. A variety of mammals have been used to establish pluripotent ES cell colonies, as shown in **Table 1.1**.

Unfortunately, to date, the establishment of stable ES cells has only been reported in mice, non-human primates and human (**Table 1.1**), and not in other mammals (Prelle *et al*., 2002; Keefer *et al*., 2007; Lovell-Badge, 2007; Rossant, 2007; Talbot *et al*., 2008). This is because the pluripotent cells obtained from those certain species reach all of the definitions of ES cells, which can be determined using 1) *in vitro* technique examinations such as staining and investigation of self-renewing and differentiation gene expressions, embryoid body (EB) formation and determination of morphology of undifferentiated and differentiated cells, and 2) *in vivo* evaluation such as derivation of teratomas in mice and production of species specific chimeras with germline transmission, which it is the best key to evaluate stable ES cell line establishment. However, creation of chimeras of primate ES cells especially for human is absolutely untestable due to restriction of ethical concerns (Thomson *et al*., 1995; Thomson *et al*., 1996; Thomson *et al*., 1998; Smith, 2001; Vackova *et al*., 2007).

*Table 1.1 Establishment of embryonic stem cells in mammals.*

This table shows some previous studies of embryonic stem cell establishment across mammalian species.

| **Species** | **References** |
|---|---|
| Cattle | Saito *et al*., 1992; First *et al*., 1994; Talbot *et al*., 1995; Van Stekelenburg-Hamers *et al*., 1995; Stice *et al*., 1996; Cibelli *et al*., 1998a; Cibelli *et al*., 1998b; Iwasaki *et al*., 2000; Mitalipova *et al*., 2001; Talbot *et al*., 2002; Saito *et al*., 2003; Vejlsted *et al*., 2005; Wang *et al*., 2005b; Yadav *et al*, 2005; Lazzari *et al*., 2006; Roach *et al*., 2006; Talbot *et al*., 2007b; Verma *et al*., 2007; Munoz *et al*., 2008 |
| Dog | Hatoya *et al*., 2006; Hayes *et al*., 2008 |
| Goat | Meinecke-Tillmann and Meinecke, 1996 |
| Horse | Saito *et al*., 2002; Li *et al*., 2006 |
| Hamster | Doetschman *et al*., 1988 |
| Human | Thomson *et al*., 1998; Richards *et al*., 2002; Cheng *et al*., 2003; Strelchenko *et al*., 2004; Lee *et al*., 2005; Xu *et al*., 2005a; Xu *et al*., 2005b; Klimanskaya *et al*., 2006; Ludwig *et al*., 2006; Strelchenko and Verlinsky, 2006; Agarwal *et al*., 2008; Bigdeli *et al*., 2008; Meng *et al*., 2008 |
| Mink | Sukoyan *et al*., 1992; Sukoyan *et al*., 1993 |
| Mouse | Evans and Kaufman, 1981; Martin, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Brook and Gardner, 1997; Chung *et al*., 2006; Lee *et al*., 2006b; Brons *et al*., 2007; Okita *et al*., 2007; Shinmen *et al*., 2007; Brons *et al*., 2007; Tesar *et al*., 2007; Wakayama *et al*., 2007a; Wakayama *et al*., 2007b; Huang *et al*., 2008 |
| Non-human primate | Thomson *et al*., 1995; Thomson *et al*., 1996; Suemori *et al*., 2001; Cibelli *et al*., 2002; Torii *et al*., 2002; Kuo *et al*., 2003; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006; Yamashita *et al*., 2006; Byrne *et al*., 2007 |
| Pig | Piedrahita *et al*., 1988; Evans *et al*., 1990; Piedrahita *et al*., 1990a; Piedrahita *et al* 1990b; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Wianny *et al*., 1997; Chen *et al*., 1999; Miyoshi *et al*., 2000; Talbot and Garrett, 2001; Talbot *et al*., 2002; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Ock *et al*., 2005; Shiue *et al*., 2006; Vackova and Madrova, 2006; Brevini *et al*., 2007a; Brevini *et al*., 2007b; Kim *et al*., 2007; Talbot *et al*., 2007a; Xu *et al*., 2007; Blomberg *et al*., 2008b |
| Rabbit | Graves and Moreadith, 1993; Fang *et al*., 2006 |
| Rat | Iannaccone *et al*., 1994; Brons *et al*., 2007 |
| Sheep | Piedrahita *et al*., 1990b; Talbot *et al*., 1993a; Meinecke-Tillmann and Meinecke, 1996; Wells *et al*., 1997; Iwasaki *et al*., 2000; Zhu *et al*., 2007 |

By sharing more phylogenic characteristics, physiological responses in immunological activities, enzymatic reactions and metabolism, life span, and body size with humans than other mammals, except for non-human primates, pigs are the most closet animal models to humans (Suemori *et al*., 2001; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006). In addition, ethical and political problems dealing with researching on ES cells as a tool of medical illnesses in pigs are less serious than those for non-human and human primates (Thomson *et al*., 1995; Thomson *et al*., 1996; Thomson *et al*., 1998; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006). Therefore, pigs have a great potential to be a very good medical model of human diseases and may contribute to testing suitable strategies for regenerative medical treatments before any innovative therapeutic applications reproduced from those pluripotent cells are applied to man (Prelle *et al*., 2002; Gjorret and Maddox-Hyttel, 2005; Wang *et al*., 2005b; Brevini *et al*., 2007a; Brevini *et al*., 2007b; Keefer *et al*., 2007) as well as benefiting their own agricultural development and biotechnology. For these reasons, pigs were designed as the model for deriving pluripotent ES cells in this present study.

### 1.2.1 Sources of embryos used to establish embryonic stem cell lines in mammals

According to two breakthrough papers proposing that establishment of mouse embryonic stem (mES), and mouse epiblast stem (mEpiSC) and human embryonic stem (hES) cells obtained should originate from different stages of epiblasts, early and late origin (see **Section 3.1**), respectively (Brons *et al*., 2007; Tesar *et al*., 2007), it could be inferred that immortal stem cells in any mammalian species might share the similar pattern of biological mechanisms. For example, intracellular signalling transduction pathways, at the same stage, but different in terms of genetic properties such as epigenetic and sequences of genes required, are related to their hierarchy to sustain a state of self-renewal and differentiation in their own species. For instance, a phenomenon of late development that is found only in the mouse (Kawase *et al*., 1994; Brook and Gardner, 1997) and most ungulates (Evans *et al*., 1990), and not in primates, theoretically increases the numbers of ICMs and primes the proliferation of ES cells via LIF-JAK-STAT3 pathway, as described elsewhere in this review, by hypersensitisation of LIF receptors during a delayed implantation before a

supplement of LIF to the culture medium results in the improvement of the efficiency of mES cell lines (Kawase *et al*., 1994; Brook and Gardner, 1997; Nichols *et al*., 2001; Buehr and Smith, 2003). Although human do not have a delayed phenomenon, the fact that they have a higher number of inner cell mass than mice and ungulates presumably increases the possibility of hES cell line derivation (Liao, 2005). Based on this idea, it is of interest to try to pinpoint the successful and unsuccessful establishment of ES cell lines previously studied in mammals.

#### *1.2.1.1 Techniques used to reproduce pre-implantation embryos*

To date it is known that there are five sources producing embryos to establish ES cell lines. First, regarding embryos from pregnant animals at the required stage of gestation, it is strongly believed that such embryos are the best for deriving ES cells in terms of having more numbers and quality of inner cell mass (ICM) and epiblasts than any other sources resulting in a higher chance of pluripotent ES cell derivation (Bavister, 2004). These *in vivo* embryos have been used in rodents, domestic ungulates and non-human primates, as summarised in **Table 1.2**. Second, pre-implantation embryos produced by the *in vitro fertilization* (*IVF*) technique have been manipulated in cattle, human, non-human primates, pigs, rabbits and sheep (**Table 1.2**). Although this technique has been widely used to reproduce embryos, it raises concerns about polyploidy and polyspermy (Li *et al*., 2003b) producing abnormal embryos resulting in abnormality and low rate of immortal ES cell establishment. Third, parthenogenetic embryos have been reported in cattle, mice, non-human primates, pigs and rabbit (**Table 1.2**). However, due to a lot of high incidences of abnormality in polyploidy, regulation of insulin growth factor and apoptotic rate (Newman-Smith and Werb, 1995; De La Fuente and King, 1998; Hao *et al*., 2004) generated from this tool, it is preferable to avoid establishing ES cells from these embryos, although they are claimed to be an alternative way to overcome the ethical and political issues of using fertilised conceptuses to derive pluripotent ES cells. Fourth, nuclear transfer (NT), a time-consuming, complicated and expensive source of embryos, has been performed to produce pre-implantation embryos for deriving immortal ES cells in cattle, mice, non-human primates, pigs and rabbits (**Table 1.2**). Certainly, these embryos are not as frequently used as *in vivo* and *IVF* derived-

embryo techniques because of the limitations listed above and also because they provide a lower rate and lower grade of blastocysts than the other two methods (Byrne *et al*., 2007; Wakayama, 2007). Finally, embryos may be obtained through the intracytoplasmic sperm injection (ICSI) method, an operative technique that principally involves in injecting a selective sperm into the cytoplasm of matured oocytes in order to increase the chance of fertilization, have been used to derive ES cells in mice and non-human primates (**Table 1.2**). Again, it is not well-generated source for the production of pre-implantation embryos from which to derive ES cells. Nevertheless, the achievement of the production of pluripotent ES cells from a single blastomere of embryos will definitely be of benefit in a clinical preimplantation genetic diagnosis, in which a technique is usually used to monitor pregnant women who fulfil some of the criteria indicating the possibilities of genetic disorders for their babies, in order to study and understand some of the mechanisms of genetic diseases if the isolated single blastomeres from inheritable genetic disorders could generate pluripotent ES cells.

#### *1.2.1.2 Different stages of early embryonic development*

Not only ICMs and epiblasts, but also blastomeres of pre-implantation embryos have been used to generate pluripotent embryonic stem cells in mammalian species.

##### ***1.2.1.2.1*** *Blastomeres of embryos*

A number of blastomeres obtained from different stages of embryos have been manipulated to create ES cells in a variety of mammals. However, isolated blastomeres has been successfully generating immortal pluripotent ES cells only in mice (Chung *et al*., 2006; Wakayama *et al*., 2007a) and human (Strelchenko *et al*., 2004; Klimanskaya *et al*., 2006; Strelchenko and Verlinsky, 2006) as shown in **Table 1.3**. Several attempts to establish ES cells from blastomeres in other mammalian species have also been summarised in **Table 1.3**. It is, of course, well-known that unsuccessful ES cell line establishment may result from the disturbances of certain procedures and chemical substances occurring during the protocols of the isolation of blastomeres and the processes of deriving immortal

pluripotent ES cell lines; therefore, it is not a standard way of generating stable ES cells.

#### *1.2.1.2.2 Blastocyst stage*

ICMs and epiblasts of blastocysts are well-known sources for producing mammalian ES cells, and a number of groups have carried out the isolation of ICMs in rodents, domesticated ungulates, non-human and human primates (**Table 1.3**). Regarding epiblasts deriving pluripotent ES cell lines, they have been revealed in cattle, mice, pigs, rats and sheep (**Table 1.3**). However, these two sources of isolated cells show no significant difference in derivation of certain stable ES cell lines; as previously reported, epiblasts seems to provide a better rate of immortal ES cells than do ICMs (Brook, 2006).

Taking into consideration all the results explained above, *in vivo* blastocysts were selected to generate the pluripotent ES cell lines in this present study.

### 1.2.2 Techniques used to isolate ICMs and epiblasts of blastocysts

To date, there are only five methods used to establish immortal stem cells, which are the following.

#### *1.2.2.1 Intact blastocyst*

The easiest, and theoretically the safest, way to derive pluripotent ES cell lines is to use a pipette to plate a blastocyst that is surrounded by zona pellucida or without zona pellucida in designed culture system, and then wait for the time of embryo attachment on feeders or surface of tissue culture dish, and the establishment of primary ES colony outgrowth. This method may give a good result of as far as the derivation of ES cells is concerned, but some undesirable cells such as trophectoderm, epithelial-like cells, primitive endoderm and so forth have been revealed (Talbot *et al*., 1995; Wang *et al*., 2005b; Roach *et al*., 2006; Talbot *et al*., 2007a; Talbot *et al*., 2007b) which lead to the transference of some pluripotent ES cells obtained into new culture dishes. However, this technique is still popular for the establishment of ES cells in rodents, ungulates and non-human primates (**Table 1.4**).

*Table 1.2 Sources of embryos used for the establishment of embryonic stem cells in mammals.*

This table shows five common methods used to isolate immortal embryonic stem cells across mammalian species.

| **Source of embryos** | **Species** | **References** |
|---|---|---|
| *In vivo* | Cattle | Talbot *et al*., 1995; Talbot *et al*., 2007b |
| | Dog | Hatoya *et al*., 2006; Hayes *et al*., 2008 |
| | Goat | Meinecke-Tillmann and Meinecke, 1996 |
| | Horse | Saito *et al*., 2002; Li *et al*., 2006 |
| | Mink | Sukoyan *et al*., 1992; Sukoyan *et al*., 1993 |
| | Mouse | Evans and Kaufman, 1981; Piedrahita *et al*., 1990b; Brook and Gardner, 1997; Lee *et al*., 2006b; Brons *et al*., 2007; Tesar *et al*., 2007; Huang *et al*., 2008 |
| | Non-human primate | Thomson *et al*., 1995; Thomson *et al*., 1996; Mitalipov *et al*., 2003a |
| | Pig | Evans *et al*., 1990; Piedrahita *et al*., 1990a; Piedrahita *et al* 1990b; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Gerfen and Wheeler, 1995; Wianny *et al*., 1997; Chen *et al*., 1999; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Ock *et al*., 2005; Shiue *et al*., 2006; Talbot *et al*., 2007a; Vackova *et al*., 2007; Blomberg *et al*., 2008b |
| | Rabbit | Graves and Moreadith, 1993; Fang *et al*., 2006 |
| | Rat | Brons *et al*., 2007 |
| | Sheep | Piedrahita *et al*., 1990b; Talbot *et al*., 1993a; Meinecke-Tillmann and Meinecke, 1996; Wells *et al*., 1997 |
| *In Vitro* Fertilisation | Cattle | Van Stekelenburg-Hamers *et al*, 1995; Stice *et al*., 1996; Cibelli *et al*., 1998a; Mitalipova *et al*., 2001; Vejlsted *et al*., 2005; Wang *et al*., 2005b; Talbot *et al*., 2007b; Verma *et al*., 2007; Munoz *et al*., 2008 |
| | Human | Thomson *et al*., 1998; Lee *et al*., 2005; Klimanskaya *et al*., 2006 |
| | Non-human primate | Suemori *et al*., 2001; Byrne *et al*., 2007 |
| | Pig | Miyoshi *et al*., 2000; Li *et al*., 2003a; Ock *et al*., 2005; Kim *et al*., 2007 |
| | Rabbit | Fang *et al*., 2006 |
| | Sheep | Iwasaki *et al*., 2000; Zhu *et al*., 2007 |
| Parthenogenetic activation | Cattle | Wang *et al*., 2005b; Lazzari *et al*., 2006; Talbot *et al*., 2007b |
| | Mouse | Allen *et al*., 1994 |
| | Non-human primate | Cibelli *et al*., 2002 |
| | Pig | Brevini *et al*., 2005; Ock *et al*., 2005; Brevini *et al*., 2007a; Brevini *et al*., 2007b; Kim *et al*., 2007; Xu *et al*., 2007 |
| | Rabbit | Fang *et al*., 2006 |
| Nuclear transfer | Cattle | Cibelli *et al*., 1998a; Cibelli *et al*., 1998b; Wang *et al*., 2005b; Lazzari *et al*., 2006; Talbot *et al*., 2007b |
| | Mouse | Wakayama, 2007; Wakayama *et al*., 2007b |
| | Non-human primate | Mitalipov *et al*., 2003a; Byrne *et al*., 2007 |
| | Pig | Chen *et al*., 1999; Kim *et al*., 2007 |
| | Rabbit | Fang *et al*., 2006 |
| Intracytoplasmic sperm injection | Mouse | Shinmen *et al*., 2007; Wakayama *et al*., 2007b |
| | Non-human primate | Suemori *et al*., 2001; Torii *et al*., 2002; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006 |

*Table 1.3 Embryonic stages used for the establishment of embryonic stem cells in mammals.*

This table summarises embryonic stages used for the derivation of pluripotent embryonic stem cells across mammalian species.

| **Embryonic stage** | **Species** | **References** |
|---|---|---|
| Single blastomere | Mouse | Chung *et al*., 2006; Wakayama *et al*., 2007a |
| | Human | Klimanskaya *et al*., 2006 |
| 4-8 cells | Pig | Li *et al*., 2003a; Li *et al*., 2004a |
| Morula | Cattle | First *et al*., 1994; Verma *et al*., 2007 |
| | Dog | Hatoya *et al*., 2006 |
| | Human | Strelchenko *et al*., 2004; Strelchenko and Verlinsky, 2006 |
| | Mink | Sukoyan *et al*., 1993 |
| | Pig | Chen *et al*., 1999; Li *et al*., 2003a; Li *et al*., 2004a |
| Inner cell mass | Cattle | First *et al*., 1994; Talbot *et al*., 1995; Wang *et al*., 2005b; Lazzari *et al*., 2006; Verma *et al*., 2007 |
| | Dog | Hatoya *et al*., 2006; Hayes *et al*., 2008 |
| | Goat | Meinecke-Tillmann and Meinecke, 1996; Zhu *et al*., 2007 |
| | Horse | Saito *et al*., 2002; Li *et al*., 2006 |
| | Human | Thomson *et al*., 1998; Richards *et al*., 2002; Lee *et al*., 2005 |
| | Mink | Sukoyan *et al*., 1992; Sukoyan *et al*., 1993 |
| | Mouse | Piedrahita *et al*., 1990b; Lee *et al*., 2006; Brons *et al*., 2007; Tesar *et al*., 2007 |
| | Non-human primate | Thomson *et al*., 1995; Thomson *et al*., 1996; Cibelli *et al*., 2002; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006; Byrne *et al*., 2007 |
| | Pig | Piedrahita *et al*., 1990b; Wianny *et al*., 1997; Talbot and Garrett, 2001; Li *et al*., 2003a; Li *et al*., 2004b; Brevini *et al*., 2005; Ock *et al*., 2005; Shiue *et al*., 2006; Vackova and Madrova, 2006; Kim *et al*., 2007; Xu *et al*., 2007; Blomberg *et al*., 2008b |
| | Rabbit | Fang *et al*., 2006 |
| | Rat | Iannaccone *et al*., 1994 |
| | Sheep | Piedrahita *et al*., 1990b; Meinecke-Tillmann and Meinecke, 1996; Wells *et al*., 1997; Iwasaki *et al*., 2000 |
| Epiblast | Cattle | Talbot *et al*., 1995; Talbot *et al*., 2002 |
| | Mouse | Brook and Gardner, 1997; Brook, 2006; Brons *et al*., 2007 |
| | Pig | Talbot *et al*., 1993a; Talbot *et al*., 1995; Wianny *et al*., 1997; Talbot and Garrett, 2001; Talbot *et al*., 2002; Talbot *et al*., 2007a; Blomberg *et al*., 2008b |
| | Rat | Brons *et al*., 2007 |
| | Sheep | Talbot *et al*., 1993a |

*Table 1.4 Methods used to isolate inner cell masses and epiblasts of embryos for establishment of embryonic stem cells in mammals.*

This table describes five techniques manipulated to isolate ICMs and epiblasts for derivation of embryonic stem cells across mammalian species.

| **Isolation technique** | **Species** | **References** |
|---|---|---|
| Intact | Cattle | Talbot *et al*., 1995; Vejlsted *et al*., 2005; Roach *et al*., 2006; Talbot *et al*., 2007b; Verma *et al*., 2007 |
| | Dog | Hayes *et al*., 2008 |
| | Goat | Meinecke-Tillmann and Meinecke, 1996; Zhu *et al*., 2007 |
| | Horse | Saito *et al*., 2002 |
| | Mink | Sukoyan *et al*., 1992; Sukoyan *et al*., 1993 |
| | Mouse | Piedrahita *et al*., 1990b; Allen *et al*., 1994; Brook and Gardner, 1997; Huang *et al*., 2008 |
| | Non-human primate | Byrne *et al*., 2007 |
| | Pig | Piedrahita *et al*., 1988; Evans *et al*., 1990; Piedrahita *et al*., 1990b; Gerfen and Wheeler, 1995; Chen *et al*., 1999; Miyoshi *et al*., 2000; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Shiue *et al*., 2006; Vackova and Madrova, 2006; Brevini *et al*., 2007b; Kim *et al*., 2007; Xu *et al*., 2007 |
| | Rabbit | Graves and Moreadith, 1993 |
| | Rat | Iannaccone *et al*., 1994 |
| | Sheep | Piedrahita *et al*., 1990b; Meinecke-Tillmann and Meinecke, 1996 |
| Mechanical | Cattle | Cibelli *et al*., 1998a; Iwasaki *et al*., 2000; Mitalipova *et al*., 2001; Saito *et al*., 2003; Wang *et al*., 2005b; Roach *et al*., 2006; Lazzari *et al*., 2006; Verma *et al*., 2007 |
| | Dog | Hatoya *et al*., 2006; Hayes *et al*., 2008 |
| | Goat | Meinecke-Tillmann and Meinecke, 1996 |
| | Horse | Saito *et al*., 2002 |
| | Mouse | Brook and Gardner, 1997; Lee *et al*., 2006; Brons *et al*., 2007; Tesar *et al*., 2007 |
| | Non-human primate | Byrne *et al*., 2007 |
| | Pig | Wianny *et al*., 1997; Li *et al*., 2004a; Vackova and Madrova, 2006 |
| | Rabbit | Fang *et al*., 2006 |
| | Rat | Brons *et al*., 2007 |
| | Sheep | Piedrahita *et al*., 1990b; Meinecke-Tillmann and Meinecke, 1996; Iwasaki *et al*., 2000 |
| Immunosurgery | Cattle | Talbot *et al*., 1995; Van Stekelenburg-Hamers *et al*., 1995; Lazzari *et al*., 2006; Munoz *et al*., 2008 |
| | Horse | Li *et al*., 2006 |
| | Human | Thomson *et al*., 1998; Richards *et al*., 2002; Lee *et al*., 2005 |
| | Mink | Sukoyan *et al*., 1992; Sukoyan *et al*., 1993 |
| | Mouse | Brons *et al*., 2007 |
| | Non-human primate | Thomson *et al*., 1995; Thomson *et al*., 1996; Cibelli *et al*., 2002; Mitalipov *et al*., 2006; Byrne *et al*., 2007 |
| | Pig | Piedrahita *et al*., 1990b; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Wianny *et al*., 1997; Chen *et al*., 1999; Talbot *et al*., 2001; Li *et al*., 2003a; Li *et al*., 2004b; Brevini *et al*., 2005; Ock *et al*., 2005; Shiue *et al*., 2006; Brevini *et al*., 2007b; Kim *et al*., 2007; Blomberg *et al*., 2008b |
| | Sheep | Talbot *et al*., 1993a |
| Enzymatic digestion | Cattle | Yadav *et al*, 2005; Verma *et al*., 2007 |
| | Dog | Hatoya *et al*., 2006 |
| | Pig | Li *et al*., 2003a; Li *et al*., 2004b; Shiue *et al*., 2006 |
| Laser | Mouse | Takeuchi *et al*., 2005; Tanaka *et al*., 2006; Cortes *et al*., 2008 |

### *1.2.2.2 Mechanical technique*

The mechanical technique, the most harmless and also the most time-consuming method used to isolate ICMs or epiblasts of embryos when compared to the other techniques, apart from intact embryos generating ES cells, has been used with rodents, domestic ungulates and non-human primates (**Table 1.4**). This application is usually used a couple of fine needle connected with 1-5 ml syringe to isolate ICMs and epiblasts. Under a light microscope, one of the needles is used to fix a blastocyst on surface of culture dish, while the other is used to cut around the area of required cells by avoiding trophectoderm and primitive endoderm as much as possible. In fact, some pluripotent ES cells are still contaminated with those two types of differentiated cells (Wianny *et al*., 1997; Wang *et al*., 2005b; Roach *et al*., 2006).

### *1.2.2.3 Immunosurgery*

Immunosurgery is a well-known and established technique to isolate ICMs of embryos since the first pluripotent ES cell line has been created in mice (Evans and Kaufman, 1981). This method, a selective killing of trophectoderm cells, requires two types of extra-cellular signals to activate the intracellular response in a trophectoderm cell. First, a species-specific antiserum is used to pre-incubate with blastocysts for binding to the outer layer of trophectoderm cells. Then, a complement is applied to the blastocysts to activate intracellular response via the binding of complement-binding co-receptor complex to cross-linkage with antigen receptor. Therefore, trophectoderm cells are loosened or damaged, and then these cells are mechanically pipetted until isolated ICMs are obtained (Solter and Knowles, 1975). Isolation of ICM using immunosugery application has been applied to derive ES cell lines in a wide range of mammals (**Table 1.4**). However, certain other cells such as trophectoderm, primitive endoderm and epithelial cells, found growing besides pluripotent ES cells, immortal ES cells have to be removed and transferred to new culture plates (Sukoyan *et al*., 1993; Talbot *et al*., 1995; Thomson *et al*., 1995; Thomson *et al*., 1996; Wianny *et al*., 1997).

#### *1.2.2.4 Enzymatic digestion*

Another method used to isolate ICMs of blastocysts is enzymatic digestion, trypsin, the serine protease, one of enzymatic hydrolysis widely used in cell culture, is the first enzyme applied to separate ICMs from trophectoderm in cattle, dogs and pigs (**Table 1.4**). First of all, if zona pellucida of blastocyst is still present, pronase enzyme will be treated to remove zona pellucida. Zona-free blastocyst is then transferred to a 20-50 µl drop of 0.25% trypsin-EDTA for 3-5 minutes. When trophectoderm cells start dispersing, blastocyst is moved to another drop of culture medium supplemented with serum in order to stop the enzymatic reaction. Thereafter, ICMs are separated from trophectoderm cells using two sets of fine needle connected with syringe as mentioned in **Section 1.2.2.2**, and a pulled mouth micropipette is finally used to wash ICMs with culture medium until trophectoderm cells are clearly removed from ICMs resulting from the high pressure produced inside the micropipette during washing. According to previous reports, it was found that primary ES cell colonies could not be identified easily and trophectoderm colonies were still established near pluripotent ES cells, suggesting that lysis of trophectoderm cells was not completed and that the reaction of the enzyme might interfere with the derivation of ES cells (Shiue *et al*., 2006).

#### *1.2.2.5 Laser application*

To avoid exposure to allogenic antibodies and complement such as using immunosurgery or other chemical substances as well as to reduce time of ICMs exposure to any technical processes and substances, laser technology has been developed and used in the dissection of ICMs of blastocysts (Takeuchi *et al*., 2005; Tanaka *et al*., 2006; Cortes *et al*., 2008). Basically, two capillary micropipettes, the holding and injecting pipettes, are used to isolate ICMs of blastocyst. Under a microscope, one side of blastocyst is held by the holding, and the other side of blastocyst revealing ICMs is slightly attached to the injecting pipette. The pipettes and ICMs are needed to be aligned before laser is applied to cut around the area of ICMs, and then micropipettes are manipulated to pull ICMs apart from the trophectoderm cells. After that, a pulled mouth micropipette is used to transfer ICMs to designed culture system. Recently, it was found that the establishment of mES cell lines plated on mouse embryonic

feeders using laser-assisted ICM excision and intact blastocysts was 30% and 20.5%, respectively. In addition, the cell line produced from laser dissection revealed similar growth patterns determined by morphological appearance and markers to ES cell lines originating from whole blastocysts (Takeuchi *et al*., 2005). Therefore, this technique should be another alternative efficient method to derive ES cells in other mammals, but it is costly and it requires skillful scientists to handle this innovative application.

### 1.2.3 Factors affecting mechanisms of self-renewal and differentiation of embryonic stem cells in mammals

Like other kinds of cells in our body, immortal ES cells have two major factors manipulating their functional responses, intrinsic genetic controls and extrinsic factors such as ontological factors stimulating pluripotent ES cells via specific transmembrane receptor proteins or nuclear receptors. As regards the intrinsic factor affecting self-renewal and differentiation, immortal pluripotent ES cells might carry both inherited undifferentiating genes such as Octamer-binding transcription factor-4 (OCT-4) (Rosner *et al*., 1990; Scholer *et al*., 1990; Nichols *et al*., 1998; van Eijk *et al*., 1999; Kirchhof *et al*., 2000; Niwa *et al*., 2000; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2003b; Brevini *et al*., 2005; Lee *et al*., 2006a; He *et al*., 2006b; Vejlsted *et al*., 2006b); Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 2 (SOX-2) (Wegner, 1999); and Nanog (Cavaleri and Scholer, 2003; Chambers *et al*., 2003; Mitsui *et al*., 2003; Pan and Pei, 2003; Oh *et al*., 2005; He *et al*., 2006b; Brevini *et al*., 2007b; Brons *et al*., 2007; Tesar *et al*., 2007), as well as inheritable differentiating genes such as nestin, SOX-1, paired box gene 6 (PAX-6) and β-III tubulin revealed in neuroectoderm (Kuo *et al*., 2003; Scardigli *et al*., 2003; Brons *et al*., 2007); brachyury, vimentin, α-actin and α-cardiac myosin found in mesoderm cells (Piedrahita *et al*., 1990b; Do and Scholer, 2005; Brons *et al*., 2007), and; finally α-fetoprotein (AFP), SOX-17 and cytokeratin exhibited in endoderm (Piedrahita *et al*., 1990b; Wianny *et al*., 1997; Do and Scholer, 2005; Brons *et al*., 2007), as shown in **Figure 1.1**. The extrinsic factors influencing the pluripotency of ES cells, e.g. surrounding environments, ontology and dedifferentiating factors, will be discussed in greater detail later.

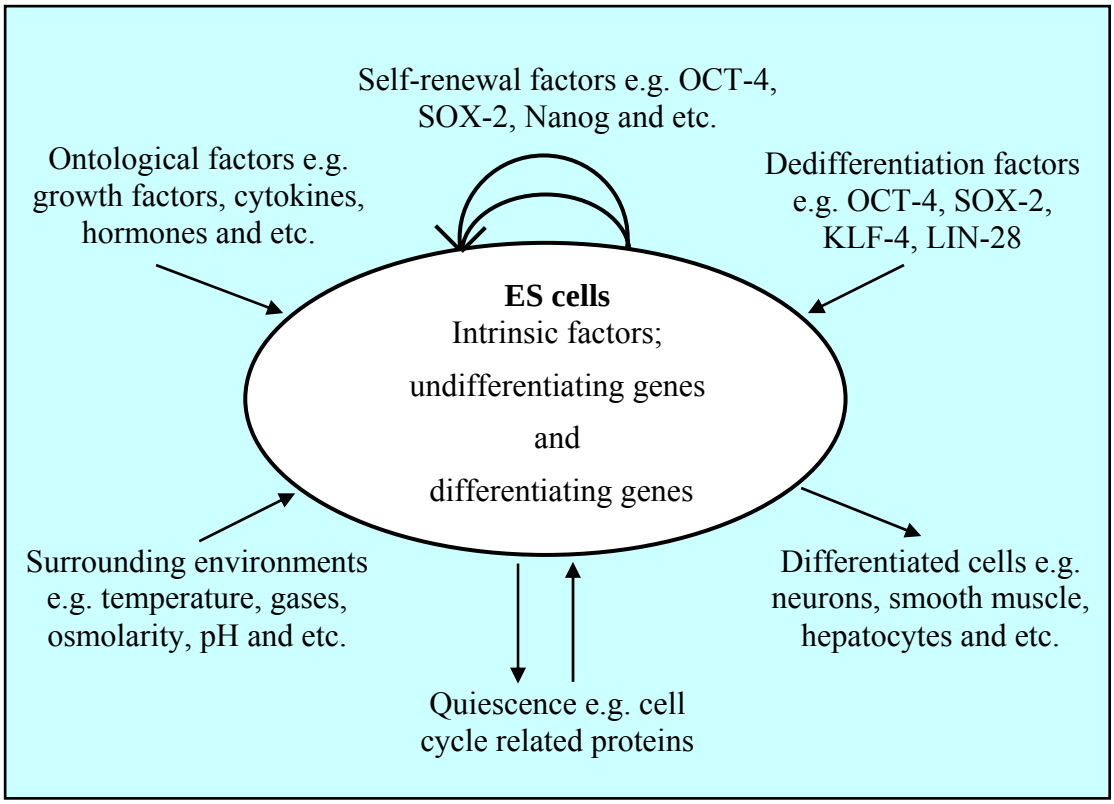



*Figure 1.1 Conceptual framework model of intrinsic and extrinsic factors affecting embryonic stem cells related to self-renewal and differentiation mechanisms, and cell cycle in mammals.*

Hypothetically, intrinsic genetic control of pluripotent ES cells, inheritable undifferentiating genes such as OCT-4, SOX-2 and Nanog (Rosner *et al*., 1990; Scholer *et al*., 1990; Nichols *et al*., 1998; Wegner, 1999; Chambers *et al*., 2003; Mitalipov *et al*., 2003a; Mitsui *et al*., 2003), and differentiating genes of three embryonic germ layers such as nestin, SOX-1, PAX-6 and β-III tubulin revealed in neuroectoderm (Kuo *et al*., 2003; Scardigli *et al*., 2003; Brons *et al*., 2007), brachyury, vimentin, α-actin and α-cardiac myosin expressed in mesoderm (Piedrahita *et al*., 1990b; Do and Scholer, 2005; Brons *et al*., 2007), and AFP, SOX-17 and cytokeratin exhibited in endoderm (Piedrahita *et al*., 1990b; Wianny *et al*., 1997; Do and Scholer, 2005; Brons *et al*., 2007) in a cooperation with extrinsic factors such as ontological signals and surrounding environments (Smith *et al*., 1988; Niwa *et al*., 1998; Thomson *et al*., 1998; Burdon *et al*., 1999a; Burdon *et al*., 1999b; Nichols *et al*., 2001; Ying *et al*., 2003a; Boyer *et al*., 2005; Cartwright *et al*., 2005; James *et al*., 2005; Kang *et al*., 2005; Li *et al*., 2005; Vallier *et al*., 2005; Wang *et al*., 2005a; Xu *et al*., 2005a; Hamazaki *et al*., 2006; Loh *et al*., 2006; Takahashi and Yamanaka, 2006; Brons *et al*., 2007; Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007; Yu *et al*., 2007; Chen *et al*., 2008; Jaenisch and Young, 2008; Kim *et al*., 2008a; Kim *et al*., 2008b; Nakagawa *et al*., 2008; Saha *et al*., 2008) play a combinative role in the regulation of self-renewal and differentiation in mammalian immortal ES cells. Abbreviaitons: AFP, α-fetoprotein; KLF-4, Kruppel-like factor 4; LIN-28, LIN-28 homolog (C. elegans); OCT-4, Octamer-binding transcription factor-4; PAX-6, paired box gene 6; SOX-1, Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 1; SOX-2, Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 2; SOX-17, Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 17.

#### *1.2.3.1 Factors regulating self-renewal and differentiation mechanisms of embryonic stem cells in mammals*

Many recent studies on mES cells and hES cells represented as early and late epiblast embryonic stem cell origin, respectively, have shown that these two different stages of ES cells have their own extracellular signals controlling their mechanisms of self-renew and differentiation, although they share the same key transcriptional factors sustaining pluripotent ES cell state regulated by three signalling transduction pathways, such as receptor tyrosine kinases (RTK), transforming growth factor-β (TGF-β) and wingless (Wnt). Otherwise, those transcriptional factors may be activated directly from some lipid soluble factors that can directly bind to the certain nuclear receptors regulating self-renewal and differentiation states. Moreover, it is generally accepted that most extracellular ligands can activate at least one intracellular signalling pathway when those external molecules bind to their certain transmembrane protein receptors, subsequently in additive, synergic or inhibiting effects of the cell responsiveness governed by a cascade of cell-cell communication. Thus, only well-focused intracellular signalling pathways triggered by some well-known factors regulating immortal ES cells are discussed in this review.

##### *1.2.3.1.1 Extracellular signalling molecules*

**1.2.3.1.1.1** Protein tyrosine kinase receptors

The response of this pathway is activated when external ligands bind to the extracellular domain of the transmembrane receptor protein and results in receptor dimerisation and an elevation in the activation of the tyrosine receptor kinase itself, leading to a provision of docking sites for Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains of a variety of signalling molecules (reviewed by Anneren, 2008). To date, there are two well-known key extrinsic factors activating via tyrosine kinase receptor pathway to control immortal ES stem cells, as described below.

Leukemia inhibitory factors (LIF)

It is well-known that LIF, a member of the interleukin-6 (IL-6) family of cytokines, is secreted by a variety of cell types such as fibroblasts, lymphocytes and liver cells (also known as differentiation inhibitory activity; DIA). Its

molecular weight ranges between 20-67 kDa and this gene is highly conserved in mammals (Smith and Hooper, 1987; Gearing *et al*., 1988; Smith *et al*., 1988; Williams *et al*., 1988; Stahl *et al*., 1990; Willson *et al*., 1992). To maintain pluripotency of ES cells, LIF activates its physiological functions when it binds to transmembrane Leukemia inhibitory factor receptor (LIFR) and consequently in heterodimerisation with another transmembrane protein, glycoprotein-130 (gp-130) (Nichols *et al*., 2001) via at least two different downstream pathways such as Janus tyrosine kinase (JAK)-Signal transducer and activator of transcription-3 (STAT3) and Ras-Extracellular signal-regulated protein kinases1 and 2 (ERK1/2), as shown in **Figure 1.2-1.3**.

In mES cells, LIF can maintain the pluripotent state via JAK-STAT3 pathway resulting from a recruitment of phosphorylated JAK by the intracellular domains of the LIF-gp130 heterodimer (Yoshida *et al*., 1994; Nichols *et al*., 2001), as depicted in **Figure 1.2**, where it acts as docking sites for proteins containing SH2 domains, of which it maintains transcription factor STAT3. Phosphoryated STAT3 by JAK is then dimerised and translocates to the nucleus to induce transcription of self-renewal genes such as OCT-4 (Niwa *et al*., 1998; Burdon *et al*., 1999a; Matsuda *et al*., 1999; Niwa *et al*., 2000), cellular myelocytomatosis oncogene (c-Myc) (Cartwright *et al*., 2005) and Kruppel-like factor-4 (KLF-4) (Li *et al*., 2005), thereby sustaining ES cell self-renewal state. In contrast, stimulation of LIF via Ras-ERK1/2 pathway in mES cells promotes differentiation by the activation of intracellular domains of LIF-gp130 heterodimer through SH2 domain-containing protein tyrosine phosphatase-2 (SHP2), where it performs as an adapter protein, collaborating with growth factor receptor bound protein-2 (GRB2) and consequently stimulating the activities of Ras, mitogen-activated protein kinase (MAPK), ERK and ERK1/2, respectively (Burdon *et al*., 1999b), as seen in **Figure 1.2**. In addition, it shows that activation of LIF-Ras-ERK1/2 signalling pathway can also suppress Nanog (Hamazaki *et al*., 2006), while the inhibition of this pathway supports self-renewal in terms of energising STAT3 responsiveness (Burdon *et al*., 1999a).

However, LIF does not play a role in ES cell self-renewal in the late epiblast origin (**Figure 1.3**). This is because with or without hLIF supplement in culture

medium consisting of serum in a feeder-dependent culture system, pluripotent ES cells can be derived in human (Thomson *et al*., 1998). It has been revealed that activation of LIF-gp130-JAK-STAT3 signalling pathway results in pluripotent cell differentiation in humans by down-regulating pluripotent transcription markers such as OCT-4 and Nanog (Daheron *et al*., 2004; Humphrey *et al*., 2004). Moreover, no lines of mEpiSC cells have been reported when mLIF is added to the culture medium (Brons *et al*., 2007).

Regarding the pluripotency of ES cells, LIF can generally induce self-renewal ES cells to become neurons, myoblasts, fibroblasts and so forth when it is added to the culture medium containing with serum (Talbot *et al*., 1993b; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005), as it has been well documented that LIF plays various biological roles in regulation of physiological systems such as neurotransmitters, myoblast proliferation, bone metabolism and etc. (reviewed by Hilton and Gough, 1991).

Fibroblast growth factor (FGF)

The FGFs consist of a huge family of polypeptides of at least 23 isoforms, also known as heparin-binding growth factors. The range of molecular mass of FGF members is 17-38 kDa and they are also highly conserved across vertebrates. FGFs are expressed in various types of cells and many of them are secreted and stored in the extracellular matrix (reviewed by Hossner, 2005). The functions of FGFs in regulation of immortal ES cells occur when their ligands bind to fibroblast growth factor receptors (FGFR) in plasma membrane, thereby providing a various alternative splices resulting in activations of at least two different intracellular signalling pathways such as Ras-ERK1/2 and Phosphoinositide-3-kinase (PI3K)-AKT, as depicted in **Figure 1.2-1.3**. These two intracellular signalling pathways share the same downstream activation until GRB-2 is formed. Consequently, GRB-2 affects Ras-ERK1/2 pathway as described above and then GRB-2 may bind to GRB2 associated binder protein-1 (GAB-1) as a complex to provoke the actions of PI3K and AKT (also known as Protein Kinase B; PKB), respectively, in FGF-PI3K-AKT signalling pathway.

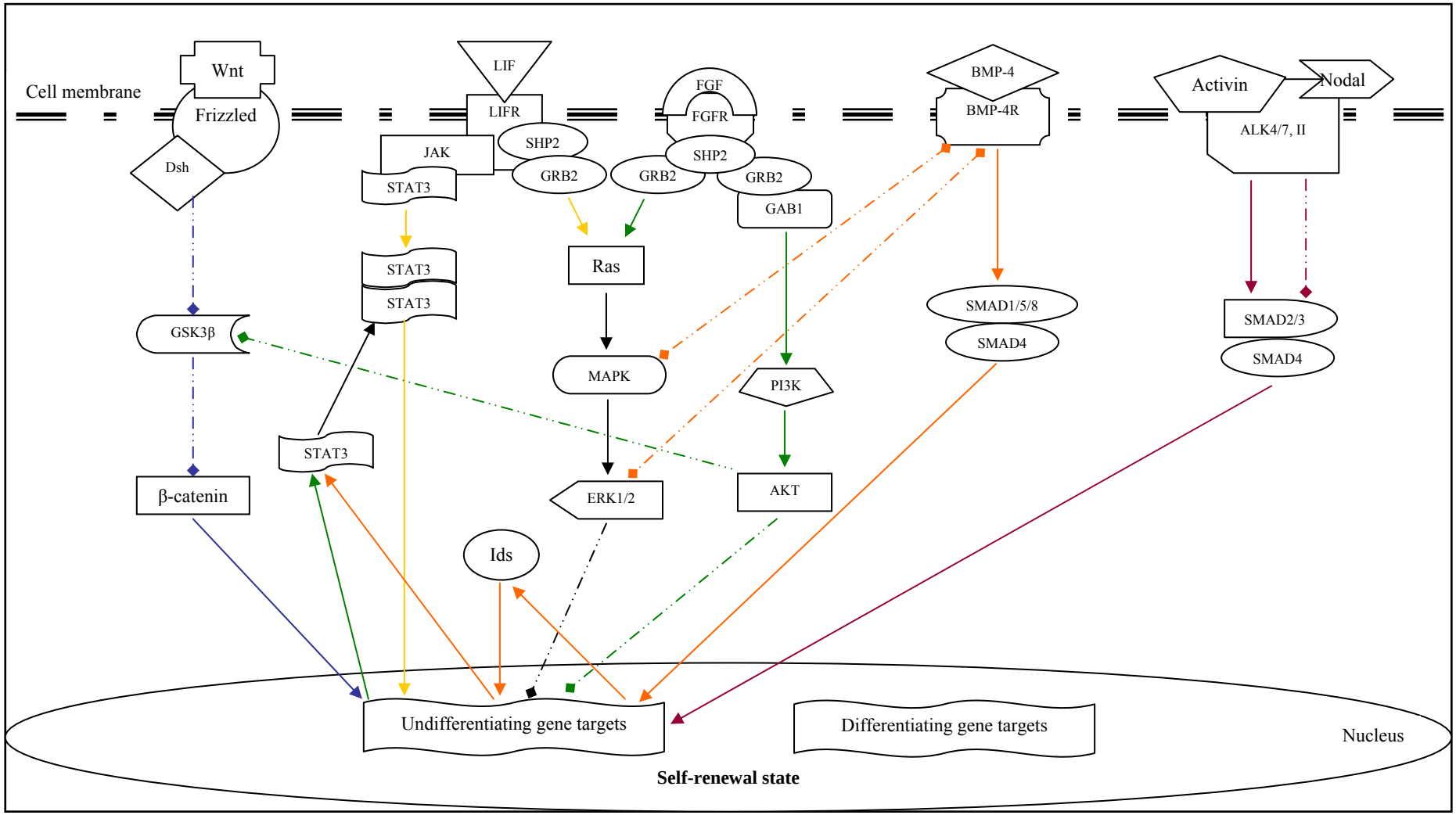

Cell membrane
Wnt
Frizzled
Dsh
LIF
LIFR
JAK
STAT3
SHP2
GRB2
FGF
FGFR
SHP2
GRB2
GRB2
GAB1
BMP-4
BMP-4R
Activin
Nodal
ALK4/7, II
STAT3
STAT3
Ras
GSK3β
MAPK
PI3K
SMAD1/5/8
SMAD4
SMAD2/3
SMAD4
STAT3
β-catenin
ERK1/2
AKT
Ids
Undifferentiating gene targets
Differentiating gene targets
Nucleus
Self-renewal state

**Figure 1.2**

*Figure 1.2 A diagram of possible cell-cell communication relates to intrinsic genetic control and extracellular signals to maintain pluripotency in early epiblast ES cell origin proposed by a model of mES cells.*

LIF activates its actions when forming a complex with LIFR-gp130 (Yoshida *et al*., 1994; Nichols *et al*., 2001) and then this complex sends its signal via LIF-JAK-STAT3 and Ras-ERK1/2 pathways. Activation of JAK-STAT3 signals results in phosphorylation of STAT3 and afterwards phosphorylated STAT3 translocates to the nucleus for induction of transcriptional factors e.g. OCT-4 (Niwa *et al*., 1998; Burdon *et al*., 1999a; Matsuda *et al*., 1999; Niwa *et al*., 2000), c-Myc (Cartwright *et al*., 2005) and KLF-4 (Li *et al*., 2005) leading to a sustenance of ES cells. On the other hand, stimulation of LIF via Ras-ERK1/2 pathway promotes differentiation due to suppressing pluripotent marker Nanog (Hamazaki *et al*., 2006) by the activation of LIF-gp130 heterodimer through a complex of SHP2-GRB2 resulting in stimulation of Ras, MAPK, ERK and ERK1/2, respectively (Burdon *et al*., 1999b), while alleviation of this intracellular signalling pathway supports pluripotent state by activating STAT3 response (Burdon *et al*., 1999a). Binding FGF to transmembrane FGFR gives a number of alternative splices in order to stimulate its functions through Ras-ERK1/2 and PI3K-AKT cascade. Activation of FGF-Ras-ERK1/2 pathway promotes differentiation of pluripotent mES cells by suppressing some pluripotent factors (Burdon *et al*., 1999b; Kunath *et al*., 2007). On the contrary, FGF supports mES cell proliferation by triggering PI3K-AKT, of which a pathway that FGF-FGFR complex stimulating SHP2, GRB2 and GAB1 to activate PI3K and AKT productions, respectively, for an inhibition of GSK3-β activity following by an accumulation of β-catenin and STAT3 in cytoplasm, respectively (Sharma *et al*., 2002). It is also believed that STAT3 produced will serve as a product for LIF-JAK-STAT3 signal to mediate its effect for sustenance of mES cells (Burdon *et al*., 2002; Hao *et al*., 2006; Ogawa *et al*., 2006; Singla *et al*., 2006). BMP-4 binding receptor complex send its signal via intracellular SMAD1/5/8 coupling SMAD4 to elevate protein Ids for collaboration with transcriptional factor STAT3 triggered by LIF under serum-free culture condition (Ying *et al*., 2003a) to support pluripotency in mES cells. In addition, it possibly suppresses neuronal differentiation through inhibition of MAPK and ERK stimulated by intracellular Ras-ERK1/2 pathway. Moreover, administration of MAPK and ERK inhibitors can mimic the effect of BMP to promote pluripotency in immortal mES cells (Finley *et al*., 1999; Ying *et al*., 2003a; Qi *et al*., 2004). Wnt-Dsh-β-catenin maintains pluripotency of mES cells via an activation of Wnt-Frizzled complex resulting in stimulating Dsh proteins to repress GSK3-β activity leading to accumulation of intracellular β-catenin. Thereafter, β-catenin passes through nucleus and collaborates with TCF/LEF to sustain pluripotent markers e.g. OCT-4, Nanog and REX-1/ZFP-42 (Aberle *et al*., 1997; Barker and Clevers, 2000; Bienz and Clevers, 2000; Bienz and Clevers, 2003; Sato *et al*., 2004; Reya and Clevers, 2005; Miyabayashi *et al*., 2007; Takao *et al*., 2007). Wnt also inhibits neuroectoderm formation by suppressing BMP-4 expression in mice (Haegele *et al*., 2003). Moreover, inhibition of GSK3-β activity via PI3K-AKT pathway can increase β-catenin level leading to sustenance of pluripotency in mice (Sharma *et al*., 2002; Naito *et al*., 2005; Tian *et al*., 2005). On one hand, signal from SMAD2/3, an essential pathway in sustaining pluripotent hES cells, is not required in mES cells *in vitro* because with or with out this cascade pluripotent markers are still revealed (James *et al*., 2005). Abbreviations: AKT, Protein Kinase B (PKB); ALK4/7, Activin-like kinase receptor type 4 and 7; bFGF or FGF-2, Basic fibroblast growth factor; BMP-4, Bone morphogenetic protein 4; c-Myc, Cellular myelocytomatosis oncogene; Dsh, Dishevelled protein; ES, Embryonic stem; ERK, Extracellular signal-regulated protein kinases; ERK-1, Extracellular signal-regulated protein kinases-1; ERK-2, Extracellular signal-regulated protein kinases-2; FGF-2, Fibroblast growth factor type 2; gp-130, Glycoprotein-130; GAB1, GRB2 associated binder protein-1; GRB2, Growth factor receptor bound protein-2; GSK3-β, Glycogen synthase kinase3-β; hES, Human embryonic stem; Ids, Inhibitor of differentiation proteins; JAK, Janus tyrosine kinase; KLF-4, Kruppel-like factor 4; LEF, Lymphoid enhancer factor; LIF, Leukemia inhibitory factor; LIFR, Leukemia inhibitory factor receptor; LIF-gp130, Leukemia inhibitory factor-glycoprotein130 protein complex; MAPK, Mitogen-activated protein kinases; mES, Mouse embryonic stem; OCT-4, Octamer-binding transcription factor-4; PI3K, Phosphoinositide-3-kinase; REX-1, RNA exonuclease 1 homolog; SH-2, Src-homology-2, SHP-2, SH-2 domain-containing protein tyrosine phosphatase-2; SMAD1/5/8, Mothers against decapentaplegic protein type 1, 5 and 8; SMAD2/3, Mothers against decapentaplegic protein type 2 and 3; SMAD4, Mothers against decapentaplegic protein type 4; STAT-3, Signal transducer and activator of transcription-3; TCF, T-cell factor; ZFP-42, Zinc finger protein-42. ⟶ = activating signal, —··—◆ = inhibiting signal.

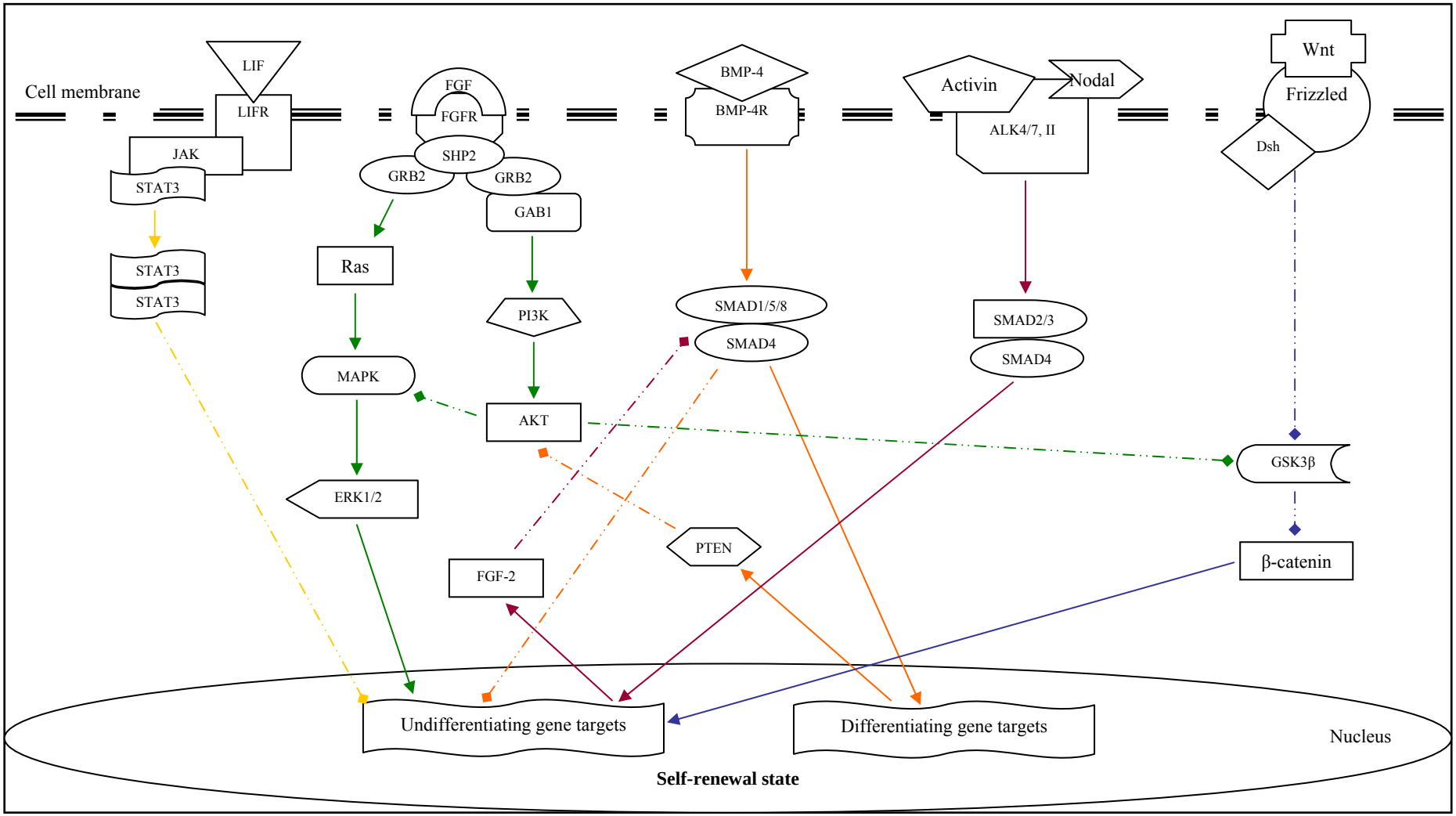

Cell membrane
LIF
LIFR
JAK
STAT3
STAT3
STAT3
FGF
FGFR
SHP2
GRB2
GRB2
GAB1
Ras
PI3K
MAPK
AKT
ERK1/2
FGF-2
BMP-4
BMP-4R
SMAD1/5/8
SMAD4
PTEN
Activin
Nodal
ALK4/7, II
SMAD2/3
SMAD4
Wnt
Frizzled
Dsh
GSK3β
β-catenin
Undifferentiating gene targets
Differentiating gene targets
Nucleus
Self-renewal state

**Figure 1.3**

*Figure 1.3 A diagram of possible cell-cell communication relates to intrinsic genetic control and extracellular signals to maintain pluripotency in late epiblast ES cell origin proposed by models of hES and mEpiSC cells.*

It is well-evident that LIF is not necessary in controlling self-renew in hES cells as it shows that addition of hLIF in culture medium or not, immortal hES cells can be obtained (Thomson *et al*., 1998). Also, stimulation of LIF-gp130-JAK-STAT3 pathway promotes pluripotent hES cells through a down-regulation of transcriptional factors e.g. OCT-4 and Nanog (Daheron *et al*., 2004; Humphrey *et al*., 2004). Moreover, no lines of mEpiSC cells established when mLIF is supplemented in the culture system (Brons *et al*., 2007). FGFs play a major role in supporting hES cells by mediating FGF-Ras-ERK1/2 signalling pathway (James *et al*., 2005; Kang *et al*., 2005; Vallier *et al*., 2005; Wang *et al*., 2005a; Xu *et al*., 2005a; Levenstein *et al*., 2006; Greber *et al*., 2007). In addition, differentiation of hES cells could be suppressed by a reduction in BMP-4-SMAD1/5/8 signal via a stimulation of FGF-PI3K-AKT signalling pathway (Kim *et al*., 2005) resulting in a decrease in a tumour suppressor protein PTEN, of which suppressing PI3K-AKT pathway, subsequently in exhibiting PI3K-AKT and Ras-ERK effects (Stambolic *et al*., 1999; Waite and Eng, 2003a; Waite and Eng, 2003b). As well as LIF does not take a task in supporting hES and mEpiSC cells (Daheron *et al*., 2004; Humphrey *et al*., 2004; Brons *et al*., 2007), triggering BMP-4 signalling through SMAD1/5/8 cascade could not only maintain human pluripotent ES and mEpiSC cells, but also promotes differentiation by down-regulating such OCT-4 and Nanog, instead (Xu *et al*., 2002; Pera *et al*., 2004; Beattie *et al*., 2005; Brons *et al*., 2007; Tesar *et al*., 2007). A combined binding between activin or nodal and activin receptors, ALK4/7 and ALK type II, stimulates a complex of SMAD2/3 and SMAD4 to elevate undifferentiation marker and supporting pluripotent hES cells, respectively. Also, this cascade can suppress a formation of neurons by inactivation of BMP-4 signals (Hashimoto *et al*., 1990; Amit *et al*., 2004; Besser, 2004; Vallier *et al*., 2004; Beattie *et al*., 2005; James *et al*., 2005; Vallier *et al*., 2005; Xiao *et al*., 2006; Saha *et al*., 2008). Activin possibly supports hES cells by stimulating bFGF expression and consequently inhibiting BMP-4-SMAD1/5/8 signalling pathway (Xiao *et al*., 2006). Moreover, mEpiSC cells requires activin and nodal to derive and maintain their property (Brons *et al*., 2007). Wnt-Dsh-β-catenin maintains pluripotency of hES cells when Wnt-Frizzled complex activates Dsh, a protein of which suppressing an action of GSK3-β, following by an increase in β-catenin level and sustenance of transcriptional pluripotent markers e.g. OCT-4, Nanog and REX-1/ZFP-42 (Aberle *et al*., 1997; Barker and Clevers, 2000; Bienz and Clevers, 2000; Bienz and Clevers, 2003; Sato *et al*., 2004; Reya and Clevers, 2005; Miyabayashi *et al*., 2007; Takao *et al*., 2007). Additionally, inhibition of GSK3-β activity via PI3K-AKT pathway can increase β-catenin level leading to sustenance of pluripotency (Sharma *et al*., 2002; Naito *et al*., 2005; Tian *et al*., 2005). Abbreviations: AKT, Protein Kinase B (PKB); ALK4/7, Activin-like kinase receptor type 4 and 7; bFGF or FGF-2, Basic fibroblast growth factor; BMP-4, Bone morphogenetic protein 4; Dsh, Dishevelled protein; ES, Embryonic stem; ERK, Extracellular signal-regulated protein kinases; ERK-1, Extracellular signal-regulated protein kinases-1; ERK-2, Extracellular signal-regulated protein kinases-2; FGF-2, Fibroblast growth factor type 2; gp-130, Glycoprotein-130; GAB1, GRB2 associated binder protein-1; GRB2, Growth factor receptor bound protein-2; GSK3-β, Glycogen synthase kinase3-β; hES, Human embryonic stem; hLIF, Human leukemia inhibitory factor; JAK, Janus tyrosine kinase; LIF, Leukemia inhibitory factor; LIFR, Leukemia inhibitory factor receptor; LIF-gp130, Leukemia inhibitory factor-glycoprotein130 protein complex; MAPK, Mitogen-activated protein kinases; mEpiSC, Mouse epiblast stem; mLIF, Mouse leukemia inhibitory factor; Oct-3/4, Octamer-binding transcription factor-4; PI3K, Phosphoinositide-3-kinase; PTEN, Phosphatase and tensin homolog; REX-1, RNA exonuclease 1 homolog; SH-2, Src-homology-2; SHP-2, SH-2 domain-containing protein tyrosine phosphatase-2; SMAD1/5/8, Mothers against decapentaplegic protein type 1, 5 and 8; SMAD2/3, Mothers against decapentaplegic protein type 2 and 3; SMAD4, Mothers against decapentaplegic protein type 4; STAT-3, Signal transducer and activator of transcription-3; ZFP-42, Zinc finger protein-42. ⟶ = activating signal, —··—◆ = inhibiting signal

FGFs play an important role in supporting pluripotent ES cell state via activation of FGF-Ras-ERK1/2 pathway (Kang *et al*., 2005) in hES cells (James *et al*., 2005; Vallier *et al*., 2005; Wang *et al*., 2005a; Xu *et al*., 2005a; Levenstein *et al*., 2006; Greber *et al*., 2007) (**Figure 1.3**), but this signalling pathway promotes mES cell differentiation by down-regulating some pluripotent genes (Burdon *et al*., 1999b; Kunath *et al*., 2007). However, some reports recently suggest that FGF can sustain proliferation of mES cells via activation of intracellular PI3K-AKT signalling pathway resulting from Glycogen synthase kinase3-β (GSK3-β) activity is inhibited and following by the accumulation of β-catenin (Sharma *et al*., 2002), of which believed increase in the level of STAT3 and mediating the action of LIF via LIF-JAK-STAT3 pathway (Burdon *et al*., 2002; Hao *et al*., 2006; Ogawa *et al*., 2006; Singla *et al*., 2006) (**Figure 1.2**). Moreover, stimulation of FGF-PI3K-AKT signalling pathway inhibits differentiation in hES cells by suppressing BMP-4-SMAD1/5/8 intracellular signalling cascade, as described elsewhere in this review (Kim *et al*., 2005), leading to a reduction of a phosphatase and tensin homolog protein (PTEN), a tumour suppressor protein suppressing PI3K-AKT pathway, resulting in activation of PI3K-AKT and Ras-ERK effects (Stambolic *et al*., 1999; Waite and Eng, 2003a; Waite and Eng, 2003b) (**Figure 1.3**).

There is no doubt that FGFs could promote immortal ES cells to become neuroectoderm cells, myoblasts, fibroblasts and other differentiated cells when a medium consisting of serum and FGFs is provided to the culture system (Talbot *et al*., 1993b; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b). Moreover, it is well-known that FGFs elicit a wide range of physiological responses, especially in the musculoskeletal system (reviewed by Hossner, 2005).

**1.2.3.1.1.2** Transforming growth factor-β (TGF-β)

The TGF-β superfamily was discovered about 25 years ago, it can be classified into two major groups. The first consists of Bone morphogenetic protein 4 (BMP-4) and growth differentiation factors (GDFs) and the other comprises of TGF-β, activin and nodal. These conserved growth factors play a major role in developmental biology and also have a wide range of physiological responsiveness (reviewed by Stewart *et al*., 2006; Puceat, 2007). Only three

members of this superfamily, BMP-4, activin and nodal, which play a critical role in controlling pluripotent ES cells through two main intracellular signalling pathways, SMADs (also known as mothers against decapentaplegic protein) and Ras-ERK1/2, are of concern in this study.

Bone morphogenetic protein 4 (BMP-4)

It has been reported that BMP-4 signalling works when it forms a complex with its BMP receptors leading to a stimulation of SMAD1/5/8 to couple with SMAD4 and then the binding complex translocates to the nucleus in order to activate its function by an increase in inhibitor of differentiation proteins (Ids) resulting in sustenance of self-renewal in mES cells; it is believed that Ids co-operate with STAT3 triggered by LIF under the serum-free culture condition (Ying *et al*., 2003a), as shown in **Figure 1.2**. In addition, BMP-4 possibly suppresses neuroectoderm differentiation via inhibition of Mitogen-activated protein kinases (MAPK) and ERK expressed in Ras-ERK1/2 pathway, it increases the chance to derive mesoderm instead. Moreover, administration of MAPK and ERK inhibitors can mimic the effect of BMP signalling to support self-renewal in mES cells (Finley *et al*., 1999; Ying *et al*., 2003a; Qi *et al*., 2004). As LIF does not affect sustenance of hES and mEpiSC cells previously mentioned above (Daheron *et al*., 2004; Humphrey *et al*., 2004; Brons *et al*., 2007), there is little doubt that activation of BMP-4 through SMAD1/5/8 pathway cannot maintain self-renewal state of human pluripotent ES and mEpiSC cells; this signal induces differentiation by down-regulation of transcriptional factors, OCT-4 and Nanog, instead (Xu *et al*., 2002; Pera *et al*., 2004; Beattie *et al*., 2005; Brons *et al*., 2007; Tesar *et al*., 2007), as shown in **Figure 1.3**.

Activin and nodal

Intracellular responses of activin and nodal begin when those extrinsic factors bind to their own receptor complexes consisting of activin receptor type I (activin-like kinases4/7; ALK4/7) and II and then its complexes send their signals through SMAD2/3 coupling SMAD4 pathway, leading to the expression of self-renewal markers subsequently in maintenance of human pluripotent ES cells, as well as the inhibition of neuroectoderm formation via suppressing BMP-4 signalling (Hashimoto *et al*., 1990; Amit *et al*., 2004; Besser, 2004; Vallier *et*

*al*., 2004; Beattie *et al*., 2005; James *et al*., 2005; Vallier *et al*., 2005; Xiao *et al*., 2006; Saha *et al*., 2008), as demonstrated in **Figure 1.3**. Furthermore, it suggests that activin can synergise its action to support human pluripotency by stimulating FGF-2 (also known as basic FGF; bFGF) expression in hES cells, playing a role in the suppression of BMP-4 signalling cascade (Xiao *et al*., 2006). Moreover, it has recently been shown that mEpiSC cells require an addition of activin and nodal in their medium in order to establish and support their identity. However, the level of activin and nodal supplemented in the medium is crucial, as high concentration of activin and nodal promotes differentiation (Brons *et al*., 2007; Takenaga *et al*., 2007; Tesar *et al*., 2007). Conversely, although the SMAD2/3 signalling pathway is inhibited in mES cells, their transcriptional pluripotent markers are still expressed, as depicted in **Figure 1.2**, implying that the intracellular SMAD2/3 pathway is not essential in mES cells *in vitro* (James *et al*., 2005). However, this signalling pathway is important during embryogenesis in mice resulting from a loss of SMAD2/3 signal reducing epiblast numbers and OCT-4 content suggesting a low efficiency in derivation and sustaining immortal ES cells (Gendall *et al*., 1997). Furthermore, the administration of activin in association with BMP-4 can induce mesoderm differentiation in mES cells (Johansson and Wiles, 1995).

**1.2.3.1.1.3** Wingless (Wnt)

It is well-known that Wnt glycoproteins play widespread roles in cell proliferation, tissue differentiation and the formation of organs (Cadigan and Nusse, 1997). Wnt-Dishevelled (Dsh)-β-catenin is the most classical cascade that has been explained to maintain pluripotency triggered by Wnt proteins. After Wnt forms a complex with its Frizzled receptor, Dsh proteins are activated, thereafter suppressing the action of GSK3-β, an enzyme to degrade the production of intracellular β-catenin, leading to accumulation of β-catenin in cytoplasm. Afterwards, β-catenin translocates to the nucleus and co-activates with T-cell factor (TCF, otherwise known as lymphoid enhancer factor; LEF) to maintain self-renewal in both mES and hES cells by sustenance of pluripotent markers such as OCT-4, Nanog and RNA exonuclease 1 homolog (REX-1, also known as Zinc finger protein-42; ZFP-42) (Aberle *et al*., 1997; Barker and Clevers, 2000; Bienz and Clevers, 2000; Bienz and Clevers, 2003; Sato *et al*.,

2004; Reya and Clevers, 2005; Miyabayashi *et al*., 2007; Takao *et al*., 2007), as shown in **Figure 1.2-1.3**. Wnt signalling also inhibits neural differentiation by down-regulating BMP-4 expression in mice (Haegele *et al*., 2003). Moreover, recent studies suggest that inhibition of GSK3-β activity via the PI3K-Akt pathway can provide β-catenin accumulation resulting in maintenance of pluripotency (Sharma *et al*., 2002; Naito *et al*., 2005; Tian *et al*., 2005). On the other hand, activation of Wnt-β-catenin cascade also regulates differentiation of three embryonic germ layers (Muroyama *et al*., 2004; Otero *et al*., 2004; Sumi *et al*., 2008).

#### ***1.2.3.1.2*** *Transcriptional factors*

The network of transcriptional genes regulating the homeostasis of pluripotent ES cells investigated thus far is highly complex (Chen *et al*., 2008; Jaenisch and Young, 2008; Kim *et al*., 2008a). Therefore, only a small well-known set of core pluripotent factors, OCT-4, SOX-2 and Nanog (Orkin, 2005; Jaenisch and Young, 2008) is of interest for discussion in this study.

It has been well established that OCT-4, a Pit-Oct-Unc (POU)-domain transcriptional factor, SOX-2, a member of the SRY-related transcription family, and Nanog, an NK-2 family homeobox transcriptional factor (also known as Thyroid transcription factor 1; TTF-1) are essential factors in regulation of embryonic development and identity of pluripotent ES cells (Nichols *et al*., 1998; Chambers *et al*., 2003; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2003b; Mitsui *et al*., 2003; Boiani and Scholer, 2005; Cavaleri and Scholer, 2006; Fujimoto *et al*., 2006; Ivanova *et al*., 2006; Niwa, 2006; Niwa, 2007). These three transcriptional factors are expressed in ICMs, epiblasts and immortal ES cells (Nichols *et al*., 1998; Wegner, 1999; Chambers *et al*., 2003; Mitsui *et al*., 2003; Niwa, 2006; Brons *et al*., 2007; Niwa, 2007; Tesar *et al*., 2007). Knock out of these genes results in a failure of generation of ICMs, but it forms trophectoderm and primitive endoderm instead. Additionally, it causes loss of pluripotency in ES cells (Nichols *et al*., 1998; Niwa *et al*., 2000; Chambers *et al*., 2003; Mitsui *et al*., 2003; Ivanova *et al*., 2006; Niwa *et al*., 2006).

Moreover, the binding of OCT-4, SOX-2 and Nanog to their own promoters or the promoters of the genes encoding the other two transcriptional factors could maintain pluripotency of immortal ES cells by affecting two sets of target genes. The first one is actively expressed and the other, which is revealed during differentiation, remains quiescent in pluripotent ES cells (Boyer *et al*., 2005; Loh *et al*., 2006; Chen *et al*., 2008; Kim *et al*., 2008a). Recently, it has been shown that OCT-4 forms a heterodimer complex with the HMG-box transcriptional factor SOX-2 and can maintain self-renewal in ES cells by regulating the levels of OCT-4 and Nanog (Yuan *et al*., 1995; Kuroda *et al*., 2005; Okumura-Nakanishi, 2005; Rodda *et al*., 2005; Masui *et al*., 2007).

Thus, the complex regulation formed by OCT-4, SOX-2 and Nanog proposed above may help to explain why overexpression of various combinations of these transcription factors could reprogramme somatic cells (Takahashi and Yamanaka, 2006; Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007; Yu *et al*., 2007; Kim *et al*., 2008b; Nakagawa *et al*., 2008). Those combined exogenous transcriptional factors might possibly directly stimulate the endogenous OCT-4, SOX-2 and Nanog into control their own pluripotency (Jaenisch and Young, 2008), or suppress the set of nuclear differentiation genes. Furthermore, the difference in the number of combined transcription factors used to reprogramme adult cells possibly results from various levels of endogenous gene expressions in different types of somatic cells. Thus, it may have a proper range of required transcriptional factor concentrations to induce pluripotent cells from mature cells when those levels of endogenous transcriptional genes are calibrated as a ratio responsive to certain exogenous transcription factors.

In summary, the achievement of maintaining self-renewal state in pluripotent ES cells depends on a balance between proliferation, inhibition of differentiation state and prevention of apoptosis or senescence during cell division controlled by intrinsic and extrinsic factors, which are believed to play their precise roles through cross-talk interactions between cellular signalling pathways.

### *1.2.3.2 Culture conditions*

The most important classical parameter, the key to establish any type of pluripotent ES cell lines is a suitable culture condition, which is an issue that needs be considerate critically. This is because although the cells isolated from any kind of pre-implantation embryo or technique are not absolutely ideal for deriving ES cells, if the culture condition suits them perfectly, they could adapt themselves, as other types of fundamental cells behave to survive in harsh situations whenever they are weak until they become healthy enough to perform their own functions effectively.

Two well-known culture conditions used to produce ES cells are feeder and non feeder culture systems, which share some similar aspects in their surrounding environments, such as temperature, gases, pH and etc., but different in that of known and unknown factors affecting pluripotent ES cells given to the culture system by manmade such as synthetic chemical substances as well as by natural factors such as those produced by feeder fibroblasts and ES cells themselves.

#### ***1.2.3.2.1*** *Feeder culture system*

It is understandable why feeder culture system has been playing an outstanding role in the derivation of ES cells to date. This may be because a number of undiscovered required factors are still unclear (Amano *et al*., 2006). To avoid the lack of some invaluable known and unknown factors in the culture medium that feeder layers produce to support ES cells, a great number of research groups have selected mouse embryonic fibroblasts (MEFs) or STO (SIM (Sandoz inbred mouse) embryo-derived thioguanine-and ouabain-resistant fibroblast cell line)) as their source of feeder layers to generate ES cells across mammalian species (**Table 1.5**). However, some groups reported that they used species-specific feeders, which are believed to be ideal sources to produce some useful factors to derive pluripotent ES cells and avoid cross contamination between species (**Table 1.5**) cultured in a variety of basic culture media such as DMEM, KO-DMEM and DMEM/F-12 along with the addition of various concentrations of factors related to the regulation mechanisms of self-renewal state of ES cells, e.g. 5-20% foetal calf serum (FCS), 10-20% knock out serum replacement (KSR), 4-100 ng/ml bFGF, 10-40 ng/ml mLIF and 10-40 ng/ml hLIF (**Table**

**1.6**) in a variety of culture environments such as a humidified atmosphere of 5% $CO_2$ in air at 37°C (Evans and Kaufman, 1981; Sukoyan *et al*., 1992; Sukoyan *et al*., 1993; Richards *et al*., 2002; Cheng *et al*., 2003; Lee *et al*., 2006b; Yamashita *et al*., 2006; Okita *et al*., 2007), at 38.5°C (Talbot *et al*., 1995; Meinecke-Tillmann and Meinecke, 1996; Mitalipova *et al*., 2001; Saito *et al*., 2002; Saito *et al*., 2003; Vackova and Madrova, 2006; Zhu *et al*., 2007; Xu *et al*., 2007), or a humidified atmosphere of 3% $CO_2$, 5% $O_2$ and 92% $N_2$ at 37°C (Byrne *et al*., 2007).

As previously described, some stable lines of ES cells can be established in feeder-dependent culture system and they can be passaged for a long period of time, but some cannot even though their protocols have been slightly modified (Evans and Kaufman, 1981; Thomson *et al*., 1995, Thomson *et al*., 1996; Thomson *et al*., 1998; Brons *et al*., 2007; Tesar *et al*., 2007). This means that all parameters involved in the derivation of pluripotent ES cells are still not suitable, even when cultured with feeders. This raises the question as to what the differences between the successful and unsuccessful derivation of embryonic stem cells are and why these differences exist. First of all, it should be noted that immortal ES cells derived from different origins needs different factors to sustain their own self-renewing state, but different mammalian species do not affect the derivation of pluripotent ES cell protocols (Brons *et al*., 2007; Tesar *et al*., 2007). Second, it should be borne in mind whether all required factors supplemented in the culture system are made at the right time and doses to generate ES cells. Therefore, it is worth analysing all critical parameters of culture conditions based on their effects on the mechanisms of self-renewal and differentiation, basic knowledge of cell biology and pharmacology, in order to select suitable factors to culture immortal ES cells.

*Table 1.5 Feeder culture system used for the establishment of embryonic stem cells in mammals.*

This table summarises types of feeder layers used for derivation of embryonic stem cells in mammalian species.

| **Type of feeders** | **Species** | **References** |
|---|---|---|
| Mouse feeders | Cattle | Saito *et al*., 1992; Van Stekelenburg-Hamers *et al*., 1995; Stice *et al*., 1996; Cibelli *et al*., 1998a; Cibelli *et al*., 1998b; Mitalipova *et al*., 2001; Talbot *et al*., 1995; Saito *et al*., 2003; Vejlsted *et al*., 2005; Wang *et al*., 2005b; Lazzari *et al*., 2006; Roach *et al*., 2006; Talbot *et al*., 2007b |
| | Dog | Hatoya *et al*., 2006; Hayes *et al*., 2008 |
| | Goat | Meinecke-Tillmann and Meinecke, 1996 |
| | Hamster | Doetschman *et al*., 1988 |
| | Horse | Saito *et al*., 2002; Li *et al*., 2006 |
| | Human | Thomson *et al*., 1998; Richards *et al*., 2002; Cheng *et al*., 2003; Klimanskaya *et al*., 2006; Agarwal *et al*., 2008; Bigdeli *et al*., 2008 |
| | Mink | Sukoyan *et al*., 1992; Sukoyan *et al*., 1993 |
| | Mouse | Evans and Kaufman, 1981; Martin, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Brook and Gardner, 1997; Chung *et al*., 2006; Lee *et al*., 2006b; Okita *et al*., 2007; Tesar *et al*., 2007; Wernig *et al*., 2007; Huang *et al*., 2008 |
| | Non-human primate | Thomson *et al*., 1995; Thomson *et al*., 1996; Kuo *et al*., 2003; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006; Yamashita *et al*., 2006; Byrne *et al*., 2007 |
| | Pig | Piedrahita *et al*., 1988; Evans *et al*., 1990; Piedrahita *et al*., 1990a; Piedrahita *et al* 1990b; Talbot *et al*., 1993a; Chen *et al*., 1999; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Ock *et al*., 2005; Shiue *et al*., 2006; Vackova and Madrova, 2006; Brevini *et al*., 2007a; Brevini *et al*., 2007b; Kim *et al*., 2007; Xu *et al*., 2007; Blomberg *et al*., 2008b |
| | Rabbit | Graves and Moreadith, 1993; Fang *et al*., 2006 |
| | Rat | Iannaccone *et al*., 1994 |
| | Sheep | Piedrahita *et al*., 1990b; Talbot *et al*., 1993a; Meinecke-Tillmann and Meinecke, 1996; Wells *et al*., 1997; Zhu *et al*., 2007 |
| Species-specific feeders | Cattle | First *et al*., 1994; Van Stekelenburgh-Hamers *et al*., 1995; Verma *et al*., 2007; Munoz *et al*., 2008 |
| | Goat | Meinecke-Tillmann and Meinecke, 1996 |
| | Horse | Li *et al*., 2006 |
| | Human | Richards *et al*., 2002; Cheng *et al*., 2003; Lee *et al*., 2005; Bigdeli *et al*., 2008; Meng *et al*., 2008 |
| | Mouse | Evans and Kaufman, 1981; Martin, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Brook and Gardner, 1997; Chung *et al*., 2006; Lee *et al*., 2006b; Okita *et al*., 2007; Tesar *et al*., 2007; Wernig *et al*., 2007; Huang *et al*., 2008 |
| | Mink | Sukoyan *et al*., 1992; Sukoyan *et al*., 1993 |
| | Pig | Piedrahita *et al*., 1990b; Li *et al*., 2004b; Kim *et al*., 2007 |
| | Rat | Iannaccone *et al*., 1994 |
| | Sheep | Piedrahita *et al*., 1990b; Meinecke-Tillmann and Meinecke, 1996; Zhu *et al*., 2007 |

*Table 1.6 Culture conditions used for the establishment of embryonic stem cells in mammals.*

This table summarises culture conditions used to derive embryonic stem cells in mammalian species. Abbreviations: bFGF, Basic fibroblast growth factor; DMEM, Dulbecco's modified eagle's medium; DMEM/F-12, Dulbecco's modified eagle's medium:Nutrient mixture F-12 media; FCS, Foetal calf serum; KO-DMEM, Knockout Dulbecco's modified eagle's medium; KSR, Knockout serum replacement; hLIF, human leukemia inhibitory factor and mLIF, mouse leukemia inhibitory factor. Colours of letter: red = addition of bFGF, green = addition of mLIF, blue = addition of hLIF and pink = addition of bFGF and LIF in culture medium.

| **Basic medium** | **Serum culture system** | **Ontological factors added** | **Species** | **References** |
|---|---|---|---|---|
| DMEM | FCS | No or others | Cattle | Van Stekelenburg-Hamers *et al*., 1995; Munoz *et al*., 2008 |
| | | | Mouse | Kawase *et al*., 1994; Lee *et al*., 2006b |
| | | | Non-human primate | Kuo *et al*., 2003; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006 |
| | | | Pig | Piedrahita *et al*., 1988; Evans *et al*., 1990; Shiue *et al*., 2006 |
| | | bFGF or LIF | Cattle | Talbot *et al*., 2007b; Verma *et al*., 2007 |
| | | | Mouse | Evans and Kaufman, 1981; Brook and Gardner, 1997; Okita *et al*., 2007 |
| | | | Non-human primate | Thomson *et al*., 1995; Thomson *et al*., 1996 |
| | | | Pig | Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Ock *et al*., 2005; Kim *et al*., 2007; Xu *et al*., 2007 |
| | | | Rabbit | Graves and Moreadith., 1993 |
| | | | Sheep | Zhu *et al*., 2007 |
| | KSR | bFGF | Pig | Xu *et al*., 2007 |
| KO-DMEM | FCS | No or others | Human | Richards *et al*., 2002 |
| | | | Mouse | Lee *et al*., 2006b |
| | | bFGF or LIF | Cattle | Wang *et al*., 2005b; Roach *et al*., 2006 |
| | | | Mouse | Tesar *et al*., 2007; Huang *et al*., 2008 |
| | KSR | bFGF or LIF | Human | Cheng *et al*., 2003 |
| | | | Mouse | Tesar *et al*., 2007 |
| | | | Pig | Vackova and Madrova, 2006 |
| DMEM/F-12 | FCS | No or others | Horse | Li *et al*., 2006 |
| | | | Mouse | Allen *et al*., 1994 |
| | | | Non-human primate | Byrne *et al*., 2007 |
| | | bFGF or LIF | Dog | Hatoya *et al*., 2006; Hayes *et al*., 2008 |
| | | | Sheep | Wells *et al*., 1997 |
| | KSR | No or others | Non-human primate | Yamashita *et al*., 2006 |
| | | bFGF or LIF | Human | Lee *et al*., 2005; Tesar *et al*., 2007; Agarwal *et al*., 2008 |
| | | | Rabbit | Fang *et al*., 2006 |

In the first place, it is quite important to bare in mind that any types of feeder layers supportive culture ES cells are one type of differentiated cells, not only producing undifferentiated factors, but also certainly secreting some kinds of differentiated factors to the culture system, which disturbs the self-renewal state of ES cells, such as by transforming growth factor-β (TGF-β), bovine serum albumin (BSA), insulin-like growth factor (IGF), pigment epithelium derived factor (PEDF) and etc. (Lim and Bodnar, 2002; Nagano *et al*., 2005; Levenstein *et al*., 2006; Buhr *et al*., 2007; Chin *et al*., 2007; Prowse *et al*., 2007). Therefore, the most important thing to be focused on is the numbers of feeders managed in the experiments, as the ratio between undifferentiated factors and differentiated factors that feeder cells release into the culture medium must play a master role in the derivation of ES cells. By focusing on the density of feeder cells placing on the culture dishes, it has been shown that most of them have been plating in the range between 0.5-4.0 x $10^5$ cells/$cm^2$ in mice (Kawase *et al*., 1994; Brook and Gardner, 1997; Brook, 2006), goats (Meinecke-Tillmann and Meinecke, 1996), sheep (Meinecke-Tillmann and Meinecke, 1996), horses (Saito *et al*., 2002), human (Richards *et al*., 2002; Lee *et al*., 2005), pigs (Li *et al*., 2004a; Kim *et al*., 2007; Blomberg *et al*., 2008b), cattle (Vejlsted *et al*., 2005; Roach *et al*., 2006; Talbot *et al*., 2007b; Munoz *et al*., 2008), monkeys (Yamashita *et al*., 2006) and dogs (Hatoya *et al*., 2006) to set up pluripotent ES cell lines, which results in obtaining some stable ES cell lines from different origins of epiblasts ES cells in mice, rats and primates. Moreover, the greater the density feeder cells applied to culture ES cells, the larger and flatter individual undifferentiated cells become. Thus, it is clear that the numbers of MEFs applied to culture or maintaining ES cells in previous studies were not suitable, and were too high to derive stable pluripotent ES cell lines using those factors supplemented in the culture system. Taken altogether, it is possible that the proper ratio of pluripotent factors such as hLIF and BMP-4 to activin, nodal and bFGF previously stated above in each culture system might be the key role in driving cells to be early epiblast ES cells like mES or late epiblast pluripotent ES cells as mEpiSC and hES cells when embryos at the early stage were used to establish ES cells. Otherwise, an improper ratio between undifferentiated and differentiated levels in any culture systems induces differentiated cells instead of

the pluripotent ES ones. Therefore, another question has been raised concerning the density of feeder layers to be placed on the culture dishes.

Based on basic knowledge of drug development, the dosage of any substance treated to any kinds of rodents should be higher than that for humans, mainly due to rodents' high metabolism. However, as the mouse is not a well-known model used to test drug toxicity like the rat and dog, it is rather difficult to estimate how much higher a metabolism the mouse has than humans, and other mammals. Although the rat's metabolic process is quite close to the mouse (Andress, 1992; Cook, 1992), no stable rat ES cells have been established in feeder culture system to date. By virtue of the reasons explained above, to derive ES cells, it was decided that the density of MEFs in this study was to be lower than any other works, correlating as far as possible to reaching their confluence in the tissue culture dish in case of feeders producing paracrine factors affecting immortal ES cell establishment.

However, another problem to be kept in mind is whether supplements of other exogenous factors need to be administered to the feeder dependent culture system in this present study, and if so, what kinds of factors and concentrations should be used. To begin by considering some factors secreting from feeders and playing a role in self-renewal, LIF and bFGF are of interest to add in the culture system in terms of increasing the chance of generating ES cells in this present study. As regards LIF, it is important to consider feeder cells secreting LIF and, of course, the properties of LIF itself. As mentioned above, mouse feeders have been manipulated to generate ES cells since 1981 and it is obvious that they produce mLIF to bind with mLIF receptors in order to activate self-renewal mechanisms in mice (Smith *et al*., 1988). Also, mLIF can bind to hLIF receptors because the sequences of mLIF and hLIF have just six defined residues differences, which are $Asp^{57}$, $Ser^{107}$, $His^{112}$, $Ser^{113}$, $Val^{155}$ and $Lys^{158}$. When these six amino acids, usually contained in most basic media, which vary in their concentrations of non-essential amino acids and certain other factors, are substituted into the mLIF framework, mLIF could activate hLIF receptors (Layton *et al*., 1994b) resulting in derivation of hES cells (Layton *et al*., 1994b; Thomson *et al*., 1998; Meng *et al*., 2008). Recently, human foreskin fibroblasts,

which certainly secrete hLIF into the culture system, were used to derive and sustain mES cells without supplement of mLIF. Conversely, mES cells generated from MEFs need an extra 10 ng/ml mLIF to maintain their self-renewal state (Meng *et al*., 2008). This is possibly due to hLIF having a 100- to 500-fold higher primary affinity binding than the low affinity of mLIF receptors and a lower kinetic dissociation rate than mLIF to stimulate LIF-JAK-STAT3 pathway (Layton *et al*., 1994a; Layton *et al*., 1994c). Overall, this indicates that hLIF has a higher potency in the maintenance of the self-renew state via the LIF pathway than does mLIF. Therefore, to achieve derivation of pluripotent ES cells, exogenous hLIF has to be added to the culture medium to support the low level of mLIF produced from mouse feeders in this present study. In the case of bFGF, as mentioned above, the addition of bFGF supports mEpiSC (Brons *et al*., 2007) and hES cells dose-dependently (Xu *et al*., 2005a; Xu *et al*., 2005b) in a feeder-free culture system. Therefore, it is of interest to examine the effect of supplementing optimized concentrations of bFGF used in other species in the feeder culture system in this present study.

Another two common main factors, FCS and KSR, previously added into culture medium to derive pluripotent ES cells in a feeder culture system in other works have to be carefully considered. It has been well-documented that to avoid some differentiation factors affecting self-renewal of ES cells such as platelet-derived growth factor (PDGF), IGF, FGF, hydrocortisone and so forth contained in serum (Freshney, 2005), KSR, of which the composition is modified, containing amino acids, vitamins, transferrin and insulin or their substitutes, trace elements, precursors of some collagens, albumin and some other factors that the providers of which have not been reported, in order to avoid the unwanted effects of other factors as the sera do, has been focused on to compliment the culture medium to derive ES cells instead (Cheng *et al*., 2003; Brevini *et al*., 2005; Lee *et al*., 2005; Wang *et al*., 2005b; Lee *et al*., 2006b; Vackova and Madrova, 2006; Yamashita *et al*., 2006; Brevini *et al*., 2007a; Brevini *et al*., 2007b; Xu *et al*., 2007; Wakayama *et al*., 2007a). Hypothetically, the lower the density of feeder layers uses to culture ES cells, the less necessity of KSR supplements to the culture medium to derive pluripotent ES cells. Although, FCS contains some differentiating factors interrupting self-renewal state of ES cells, it could

facilitate the attachment of cells onto the surface of culture dishes. Therefore, the decision was made to add FCS into the culture medium to derive ES cells in a feeder culture system in this present study.

Furthermore, it is necessary to bear in mind that a variety of basic media used to culture ES cells mainly contain different concentrations of essential nutrients and chemical substances that cells need (Freshney, 2005). Therefore, any medium used should not greatly influence the mechanisms of ES cell self-renewal more than the factors supplemented in the culture system, as stated above. However, basic media used in this present study were selected based on other previous works in mice and pigs. Also, to reduce as much as possible factors that may differentiate pluripotent ES cells, in this present study, unless otherwise stated, no tissue culture dishes used were coated with any substance. This is because intact or isolated porcine ICMs and epiblast cells are normal cells, so they ought to be able to attach to the surface of culture containers themselves in the proper culture system. They should not need any substances such as gelatin, fibronectin, matrix gel, collagen type I, laminin and etc. previously reported (Richards *et al*., 2002; Ludwig *et al*., 2006; Roach *et al*., 2006), which possibly play a role as one of the factors differentiating pluripotent ES cells, to coat the culture plate in order to help them sit on the surface of culture dish. Moreover, as it has been reported, mouse and primate ES cells are clearly obtained under various temperature conditions; therefore temperature used to incubate pluripotent ES cells may not be a key parameter affecting pluripotency of immortal ES cells. However, the higher the temperature used to keep the ES cells warm, the better the derivation and sustenance of immortal ES cells should be. During lab work, if the door is opened, the temperature of the incubator might fall and possibly affect the ES cells unintentionally, therefore, in this present study the incubator was set to be a humidified atmosphere of 5%$CO_2$ in air at 39°C.

Finally, the suitable surrounding environments to culture pluripotent ES cells, such as pH and osmolarity of the culture medium, which depend on the basic components of culture medium, supplemented factors and different percentages

of gases provided in the incubator, are also important and they are still under investigation by many research groups (Ludwig *et al*., 2006).

#### ***1.2.3.2.2*** *Feeder-free culture system*

In general, all other factors affecting the generation of pluripotent ES cells in feeder-independent culture system are the same as mentioned in feeder-dependent culture system, but to be successful in derivation of immortal ES cell lines obtained from non-feeder culture system all required factors controlling self-renewal of ES cells have to be completely investigated and added to the culture medium at the right time and concentrations to create ES cells as shown that supplement of BMP-4 and mLIF could establish and maintain mES cells (Ying *et al*., 2003a), but activin and nodal are needed to add into the culture medium for derivation and sustenance of mEpiSC and hES cells in feeder-free culture system (James *et al*., 2005; Camus *et al*., 2006; Brons *et al*., 2007). This indicates that those factors hypothetically benefit in generating and support other mammalian immortal ES cells in feeder-free culture system and again certain time and doses of crucial factors added to the culture system have to be considered in different species.

## 1.3 PLURIPOTENT ABILITY OF EMBRYONIC STEM CELLS

To indicate the definite establishment of new ES cell lines, not only is the evaluation of the self-renewal capability of ES cells needed, but their pluripotency must also be closely examined. It is well-known that two types of ES cell differentiation methods, *in vitro* and *in vivo* differentiations are mainly used to test the pluripotency of ES cells.

### 1.3.1 *In vitro* differentiation

Not only are derivation and maintenance of a high quality of pluripotent ES cells critical to induce successfully differentiation of all *in vitro* differentiation, but the process of direct differentiation of pluripotent ES cells to become required cell types in order to benefit the study of mechanisms of cells and then applying that knowledge to curing human diseases is also important.

Although a wide variety of alternative acceptable differentiation protocols have been reproduced mostly from using the mouse as a model (Tarasov *et al*., 2002; Tarasova *et al*., 2006; Wiles and Proetzel, 2006), there is much that remains unknown and unclear in the derivation of the specific tissues effectively and efficiently even in mice and it is of course essential to discover these things in order to fill the gaps in the knowledge required to provide better treatment of human diseases in the future, as well as for the benefits of their own species in agriculture and biotechnology.

#### *1.3.1.1 Embryoid body formation*

Embryoid body (EB) is the aggregates of pluripotent ES cells, which is the intermediate step of ES cells differentiate to be other types of cells in three embryonic germ layers, such as ectoderm, mesoderm and endoderm. These aggregates are initially formed as a ball and then become more and more complex until the hollow, cystic embryoid body, is obtained. Thereafter, internal structures are organized in those larger cystic embryoid bodies by the specific factors affecting differentiation of cells. To form embryoid bodies of ES cells, only two well-established techniques, hanging drop and suspension culture, have been used (**Figure 1.4**). Basically, hanging drop is a method that requires the dissociation of pluripotent ES cells digested by enzymatic or non-enzymatic solutions to single cells before those dissociated single cells are placed into the drop of 10-50 µl differentiation medium prepared on top of non-adherent culture dishes, such as bacteriological dishes, which are used to protect ES cells or the aggregate cells from attaching onto the surface of the culture containers, and any kind of sterile solutions such as PBS or differentiation medium is provided in the bottom of the dish in order to prevent drying of the hanging drops containing ES cells or aggregated cells. The advantage of this method is that the certain numbers of each embryoid body could be controlled at the beginning of the culture and this technique also prevents the fusion between EBs, as it gives only one embryoid body in each drop. Yet, mass production of EBs from this application should be concerned due to a limitation of drops prepared on top of the bacteriological dishes. Regarding the suspension culture technique, a simple key to forming embryoid bodies effectively is to plate a high density of dissociated single cells of pluripotent ES cells in the differentiation medium

contained in non-coated dishes or flasks (Pera *et al*., 2003; Pera *et al*., 2006). This method provides a lot of EBs, but numbers of fusion between EBs as big balls floating around the culture medium are usually observed. Therefore, to derive required differentiated cells from EBs based on these two techniques depends on the objectives of each experiment. Differentiation media used to generate EBs from these two methods have to be changed half every other day. To evaluate the formation of embryoid bodies, morphology and expression of differentiated genes of three germ layers mentioned above must be undertaken.

#### *1.3.1.2 Derivation of three germ layer cell types*

Two major *in vitro* techniques are applied to create cells of three embryonic germ layers for evaluation of the pluripotent ability of ES cells explained in this review.

##### ***1.3.1.2.1*** *Spontaneous differentiation*

This method of differentiation is for culturing a high density of pluripotent ES cells in a feeder-dependent culture system, the medium of which is changed half every other day. Certainly, any kind of cells in three embryonic germ layers, such as ectoderm (Evans *et al*., 1990; Talbot *et al*., 1993b; Wheeler, 1994; Gerfen and Wheeler, 1995; Chen *et al*., 1999; Talbot *et al*., 2002; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Blomberg *et al*., 2008b), mesoderm (Evans *et al*., 1990; Talbot *et al*., 1993b; Anderson *et al*., 1994; Wheeler, 1994; Gerfen and Wheeler, 1995; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b), and endoderm (Evans *et al*., 1990; Talbot *et al*., 1993b; Talbot *et al*., 1995; Moore and Piedrahita, 1996; Talbot *et al*. 1996; Chen *et al*., 1999; Talbot *et al*., 2005; Talbot *et al*., 2007a; Talbot *et al*., 2007b) could be obtained in this differentiation approach. Therefore, sometimes it is difficult to judge which type of germ layers those differentiated cells belong to in order to confirm by the staining of specific markers or analysis of gene expressions. Undoubtedly, the more experience in histological examination scientists have, the easier to identify which type of germ cells that ES cells differentiate to be should be.

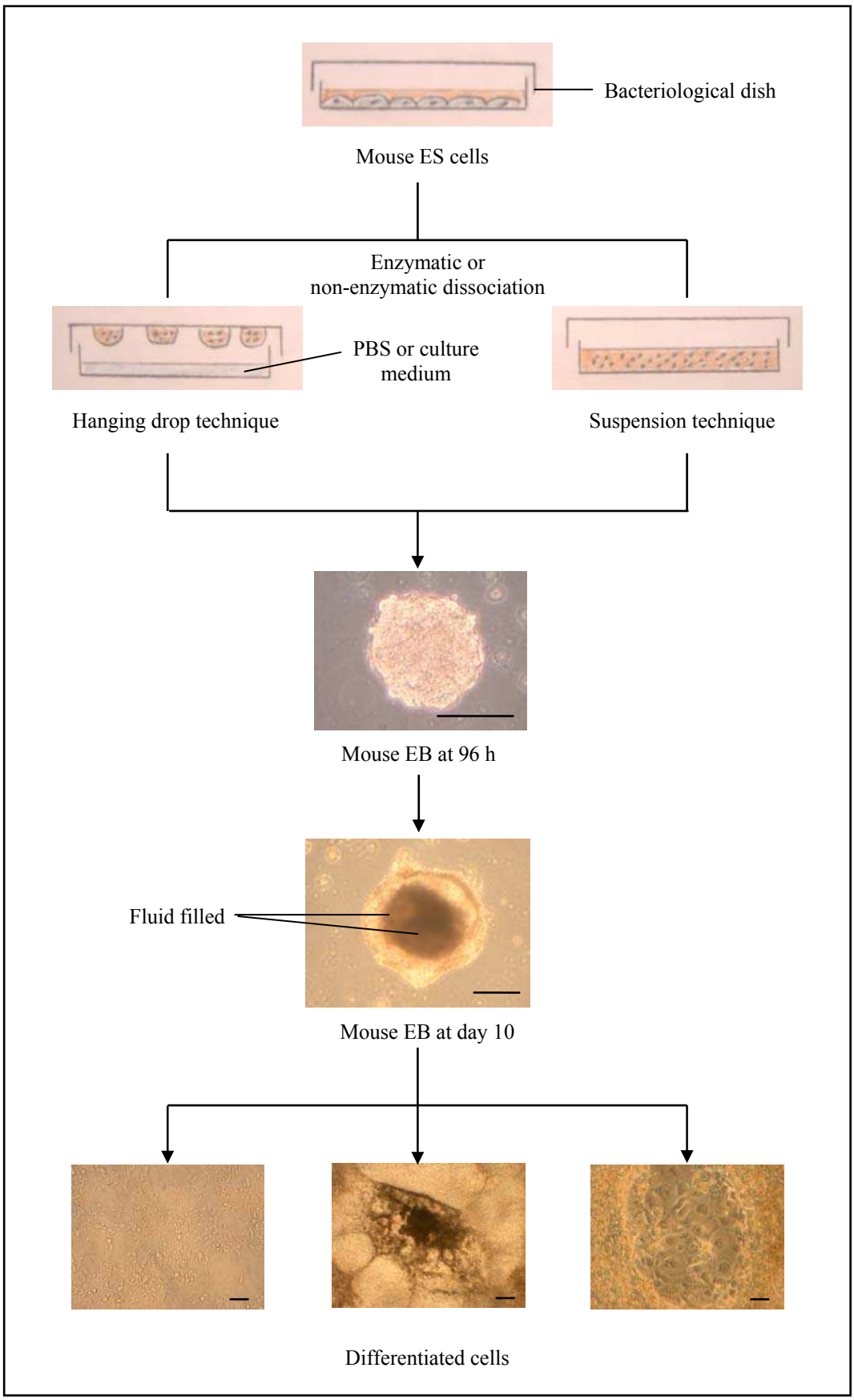

Bacteriological dish
Mouse ES cells
Enzymatic or
non-enzymatic dissociation
PBS or culture
medium
Hanging drop technique
Suspension technique
Mouse EB at 96 h
Fluid filled
Mouse EB at day 10
Differentiated cells

**Figure 1.4**

*Figure 1.4 A diagram of techniques used to form embryoid bodies in mammals.*

Two well-known methods used to produce embryoid bodies in mammalian species, which are hanging drop and suspension, are shown in this figure. Embryoid bodies obtained from these two applications could be used to derive any other types of differentiated cells in the body. Yet, to derive EBs and thereafter differentiated cells from these two techniques depends on the purposes of researchers. Abbreviations: EB, Embryoid body; ES, Embryonic stem and PBS, Phosphate buffer saline. Scale bar = 50 μm.

#### *1.3.1.2.2 Directed differentiation*

The induction of ES cell differentiation is the best method used to conduct specific cell types of three embryonic germ layer cells, although to date most improvement techniques have been carried out in mice (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Tarasov *et al*., 2002; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Yin *et al*., 2007). This is because a supplement of certain critical factors into the culture medium at the right doses and time to produce specific types of cells is necessary, otherwise unpurified cells will be found in the culture systems (Morishita *et al*., 2007). So far, two sources of cells for directed differentiation are used to evaluate the potency of ES cells in a conditioned medium prepared in the absence of feeders for a period of time; these are EBs (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Yin *et al*., 2007) and original pluripotent ES cells themselves (Yao *et al*., 2006).

### 1.3.2 *In vivo* differentiation

Not only has the evaluation of pluripotency of embryonic stem cells been conducted *in vitro*, but it has also been carried out *in vivo* in animals to cover all of the abilities these ES cells have.

#### *1.3.2.1 Teratoma*

Basically, pluripotent ES cells are introduced to the immunosuppressive mice and thereafter formation of teratoma-producing cells of three germ layers is determined. Many attempts to create tumors injected with immortal ES or ES-like cells derived from most ungulates (Piedrahita *et al*., 1990a; Piedrahita *et al*., 1990b; Anderson *et al*., 1996). This might be because the pluripotent ES cells claimed from those previous studies are not real ES cells, as most scientists believe that true pluripotent ES lines have not yet been established in domestic farm animals (Prelle *et al*., 2002; Rossant, 2007; Keefer *et al*., 2007; Talbot and Blomberg, 2008). Certainly, a great many researchers have been successful in generating teratomas from pluripotent ES cells originated from mice, non-human and human primates (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al*., 1995; Thomson *et al*., 1996; Thomson *et al*., 1998; Chung *et al*., 2006; Takahashi and Yamanaka, 2006; Yu *et al*., 2007; Brons *et al*., 2007; Tesar *et al*., 2007).

#### *1.3.2.2 Chimera*

There are a variety of different stages of recipient embryos injected with ES or ES-like cells in terms of the production of chimeras such as 4 to 8-cell stage embryos (Kawase *et al*., 1994; Golueke *et al*., 1998; Chung *et al*., 2006; Brons *et al*., 2007; Huang *et al*., 2008), morula (Onishi, 1994; Cibelli *et al*., 1998b; Brons *et al*., 2007) and blastocysts (Kawase *et al*., 1994; Chen *et al*., 1999; Shiue *et al*., 2006; Brons *et al*., 2007; Wakayama *et al*., 2007a). However, only ES cells derived from mice have a capacity to reproduce chimeras with germline transmission, of which it is the key marker indicating the establishment of stable pluripotent ES cell lines (Evans and Kaufman, 1981; Bradley *et al*., 1992; Kawase *et al*., 1994; Buehr and Smith, 2003; Chung *et al*., 2006; Wakayama *et al*., 2007a).

## 1.4 OBJECTIVES OF THIS STUDY

The objectives of this study are to establish stable porcine ES lines and to induce required adult cells from pluripotent ES cells obtained by improving the culture conditions based on the mouse ES and differentiation standard protocols, respectively, in consideration with the fundamental biology of the pig and new drug discovery. One of hypotheses proposed is whether mammals share the similar fundamental ES biology at the same stage due to having highly conserved ontological factors and intracellular signalling pathways, but different in the sense of genetic sequences and evolution, as the experiments will be performed in **Chapter 3** and **Chapter 4**. Another assumption is that induction of required differentiated cells of ES cells originating from the equivalent stage among mammalian species has the similar pattern of differentiation, but different in terms of cellular maturation timing, which is specific to their own species, as the experimentations will be done in **Chapter 5**.

# CHAPTER 2

## GENERAL MATERIALS AND METHODS

### 2.1 PORCINE EMBRYO COLLECTION

A cross of landrace x large white x duroc (PIC Camborough 12) pregnant sows were provided from J.C. Lister Farms Ltd., North Yorkshire, UK. Gilts between 230-250 days of age were artificially inseminated with pooled semen from PIC (Pig Improvement Company, UK), they were then transferred to the School of Biosciences, Sutton Bonington Campus, The University of Nottingham after 6-8 days of insemination. Day 0 was referred as a day of insemination. The sows were slaughtered and the uteri were removed and transferred to the lab. The uterine horns were flushed twice with 20 ml sterile phosphate buffer saline solution (PBS: Sigma P4417) supplemented with 1% BSA (Sigma A3311) and 1% penicillin streptomycin (P/S: Sigma P0781) each time. The PBS solution was calibrated in a humidified atmosphere of 5%$CO_2$ in air at 39°C for at least 2 hours before use. Thereafter, the aggregates in the PBS solution were collected and spread on a 92 x 10 mm Petri-dish (Nunclon, Roskilde, Denmark). Porcine embryos were selected from the solution under the microscope connected with the heated stage at 39°C, they were then transferred into one well of 4-well dish (Nunclon, Roskilde, Denmark) containing 500 µl of pre-calibrated BECM-3 (**Appendix A**) supplemented with 3% BSA or 10%FCS (Gibco 10106-169) under a humidified atmosphere of 5% $CO_2$ in air at 39°C before those porcine blastocysts were divided into two groups determined by their source of ES cell productions, inner cell mass and epiblasts (**Fig. 2.1**).

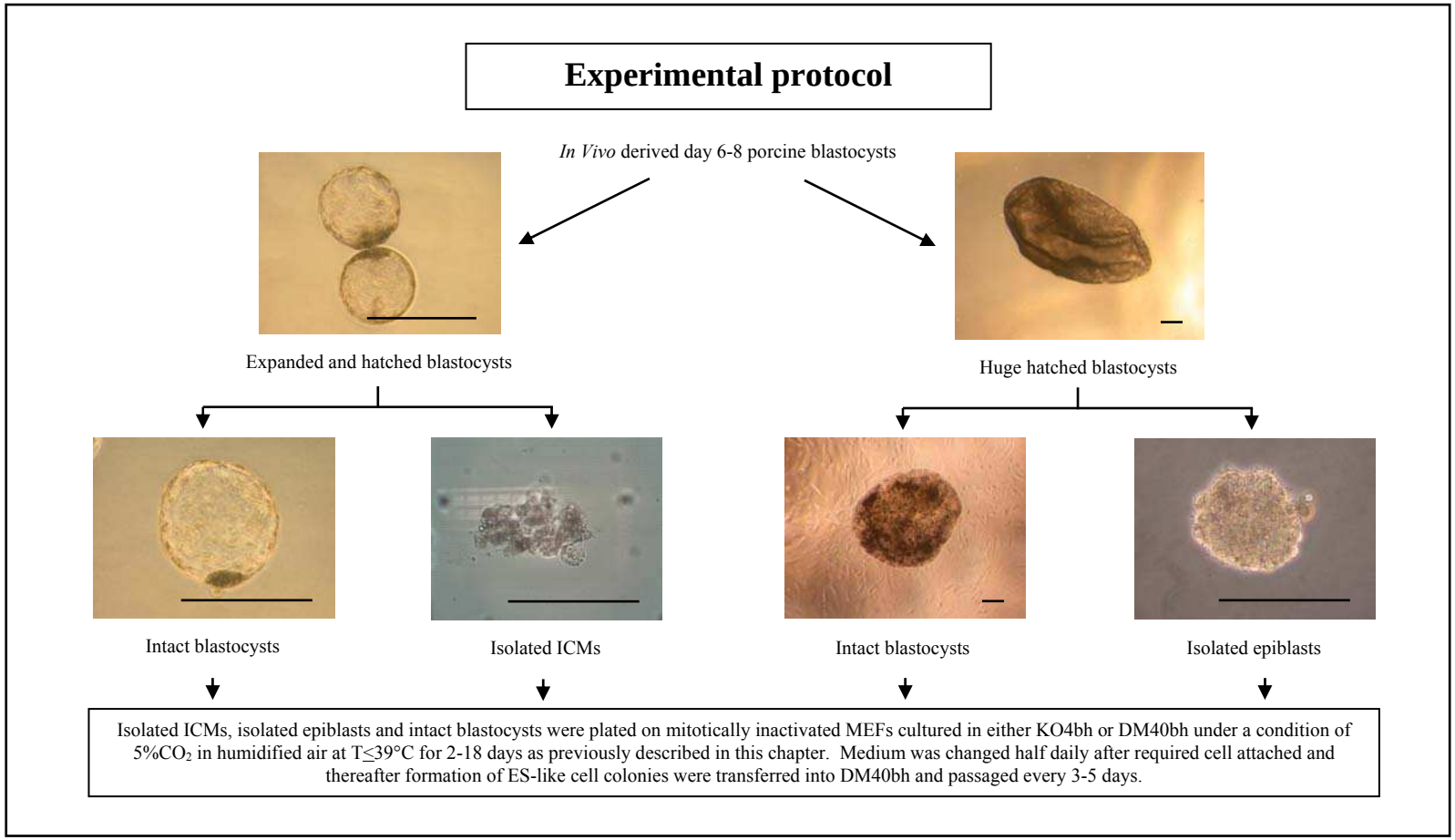

Experimental protocol
*In Vivo* derived day 6-8 porcine blastocysts
Expanded and hatched blastocysts
Huge hatched blastocysts
Intact blastocysts
Isolated ICMs
Intact blastocysts
Isolated epiblasts
Isolated ICMs, isolated epiblasts and intact blastocysts were plated on mitotically inactivated MEFs cultured in either KO4bh or DM40bh under a condition of 5%$CO_2$ in humidified air at T≤39°C for 2-18 days as previously described in this chapter. Medium was changed half daily after required cell attached and thereafter formation of ES-like cell colonies were transferred into DM40bh and passaged every 3-5 days.

**Figure 2.1**

*Figure 2.1 A diagram of experimental design to derive porcine ES-like cells isolated from pregnant pigs at day 6-8 of gestation.*

Pregnant PIC Camborough 12 sows which ranged in age between 230-250 days were purchased from J.C. Lister Farms Ltd., North Yorkshire, UK were moved to the slaughterhouse after insemination for 6-8 days. Day 0 was referred as a day of insemination. The uteri from those sacrificed pigs were removed and transferred to the lab. Porcine embryos were flushed with sterile PBS added with 1% BSA and 1% P/S, they were then selected and incubated in BECM-3 (**Appendix A**) supplemented with 3% BSA or 10%FCS in a humidified atmosphere of 5% $CO_2$ in air at 39°C before they were classified in two groups by identification of their reproducing sources of immortal ES cells. Thereafter, those two groups were once again divided into two groups, intact blastocysts and either mechanical surgery of isolated ICMs or epiblast cells. After that, those required cells were placed on top of inactivated murine feeders cultured in either KO4bh or DM40bh under a humidified atmosphere of 5% $CO_2$ in air at 39°C as mentioned in **Section 2.4**. Monitoring of cell growth was performed daily and medium was changed half everyday until the primary outgrowth of pES-like cells was revealed within 2-18 days. Then, those pES-like cells were mechanically cut into 4 pieces before transferring to new prepared culture medium DM40bh incubated in murine feeder-dependent system every 3-5 days, or evaluating their self-renewal and pluripotency as detailed in **Chapter 4-5** of this thesis. Scale bar: isolated ICMs and epiblasts = 50 μm and the rest of samples = 200 μm.

## 2.2 CULTURE OF MOUSE EMBRYONIC FIBROBLASTS

Primary mouse embryonic fibroblasts were isolated from 13.5 days old Swiss out-bred murine foetuses. In brief, pregnant animals were sacrificed and transferred to the laminar flow hood (BioMat$^2$, Medical Air Technology, Ltd.). The abdomens were cleaned with 70% alcohol (Fisher Scientific Code:E/0650DF/P17), the skin was cut and then torn to open the abdomen, the peritoneum was then cut, the uterine horns were exposed, removed and immersed fully but briefly in 70% ethanol. The uterine horns were then placed into sterile PBS in a 92 x 10 mm Petri-dish. The fetuses were covered, decapitated and the visceral organs of each foetus were removed as quickly as possible. The decapitated fetuses were moved to a fresh Petri-dish containing sterile PBS supplemented with 1% P/S and then washed until clean. Foetuses obtained from the same pregnant mouse were pooled together in 5 ml of 1x Trypsin/EDTA (Sigma T3924) in a bacterial Petri-dish. They were then chopped into small pieces using fine scissors and scalpel blades and then transferred into a 50 ml conical tube (Fisherbrand Cat.no.0553913). More trypsin was added up to a volume of 2 ml per foetus and the mixture was incubated under an atmosphere of 5%$CO_2$ in air at 39˚C for 5 minutes. The small pieces of foetuses were then completely repeatedly mashed into a thick soup using 2 ml syringe and followed by an addition of 3-5 ml complete medium consisting of 10% FCS (Gibco 10106-169) in DMEM (Sigma D6429) per foetus in a 15 ml conical tube to stop enzymatic reaction from Trypsin. The tissue suspension was then centrifuged at 1250 rpm for 10 minutes, the supernatant was discarded and the pellet was resuspended in 1 ml of complete medium. Cells were placed into T175 flask (Nunclon, Roskilde, Denmark) containing 50 ml complete medium at the density of 2 foetuses per flask in the incubator supplied with humidity under the condition of 5%$CO_2$ in air at 39°C. The growth of cells was monitored everyday and medium was changed every two days. When the cells become confluent, which is normally about 80-90% of the surface of the container, they were washed once with warm sterile 10 ml of PBS to remove serum, which is a factor interrupting the reaction of enzyme, supplemented in complete medium and incubated with 1x Trypsin-EDTA solution for 5 min at 39°C. The detached cells were aspirated into a 50 ml conical tube and diluted with 10 ml complete medium. The cell suspension was centrifuged at 1250 rpm

for 10 minutes. The pellets of cells were resuspended in 50 ml fresh medium for further culture or preserved at passage 0-2 in aliquots of 1-2 x $10^6$ cells/ml per vial. Briefly, harvested cells were collected in 1 ml of freezing medium, consisting of 10% Dimethyl Sulfoxide (DMSO: Sigma D5879) and 90%FCS (Gibco Cat.no. 10106-169), per cryotube (Nunclon, Roskilde, Denmark), which the freezing medium was prepared in the cryotubes at least 20 minutes at 4ºC or on ice to reduce the toxicity of DMSO to the cells (Lenka, 2006). Then, the tubes were wrapped in polyvinyl bubbles and stored at -80ºC for 24 h, the vials were then transferred to liquid $N_2$ and stored until required.

## 2.3 PREPARATION OF MOUSE EMBRYONIC FIBROBLASTS FOR DERIVATION OF PORCINE ES-LIKE CELLS

A cryotube containing MEFs was removed from the tank of liquid $N_2$, sprayed with 70% alcohol, the lid of the vial was loosened and incubated in a water bath (Grant, Grant Instruments (Cambridge) Ltd., UK) at 39ºC until the sample was almost completely thawed. The suspension of cells was then transferred into a 15 ml conical tube, and 10 ml of calibrated complete medium 39ºC was slowly added. The suspension was gently mixed and then centrifuged at 1250 rpm for 10 minutes (Biofuge Primo, Heraeus, Kendro Laboratory Products., Ltd., Germany). The supernatant was discarded and the pellet of cells was resuspended in 1 ml of complete medium and then transferred into a T75 flask (Nunclon, Roskilde, Denmark) containing 20 ml of complete medium and incubated in a humidified atmosphere of 5% $CO_2$ in air at 39ºC. Cultures were observed daily and medium was changed every other day until cells reached 80-90% confluence. The confluent cells of MEFs were washed once with 10 ml sterile PBS, and then 2 ml of 1x Trypsin-EDTA was placed into the flask followed by incubation at 39˚C for 5 minutes or until cells were detached from the surface of the container. To stop the enzymatic reaction of Trypsin, 10 ml of complete medium, which was pre-warmed at 39˚C for at least 1 h, was added, the suspension of cells were mixed gently and transferred to a fresh 15 ml conical tube. The cell suspension was centrifuged at 1250 rpm for 10 minutes, after that the supernatant was removed and the pellet of cells was resuspended again in 1 ml of complete medium, which was incubated at 39˚C for at least 1 h, before cells were counted, transferred into the new containers and cultured in a

humidified atmosphere of 5%$CO_2$ in air at 39˚C (Hera Cell 240, Heraeus, Kendro Laboratory Products., Ltd., Germany). Cells were observed everyday and the medium was changed every other day until confluence was monitored. The maximum passage of MEFs used to derive and maintain ES-like cells in this study was the sixth. This is because the most efficient passage of MEFs used to derive and maintain ES cells in other mammals is between passage 6 or 7 (Lenka, 2006).

### 2.3.1 Preparation of mitotically inactivated mouse embryonic feeder layers using Mitomycin C

The medium of the flasks containing approximately 80-90% confluent cultures of MEFs was discarded and replaced with 10 ml complete medium containing 10 μg/ml Mitomycin C (Sigma M4287). The cells were then incubated with Mitomycin C at 39˚C for 2 hours and 30 minutes in 5% $CO_2$. The medium was then removed and the monolayers were washed twice with 10 ml sterile PBS. Cells were harvested by trypsinisation as previously described and finally they were plated directly onto the surfaces of culture dishes at the density of 2-2.5 x $10^4$ cells per $cm^2$ in a humidified atmosphere of 5%$CO_2$ in air at 39˚C until needed.

### 2.3.2 Preparation of mouse embryonic feeders before seeding porcine intact blastocysts or isolated ICMs and epiblasts into the culture system

Prior to use as feeder layers, mitotically inactivated MEFs were washed twice with fresh complete medium and once with relevant culture medium used to produce pluripotent ES cells incubated in the condition of 5%$CO_2$ in humidified air at 39˚C. Crucially, floating cells of MEFs, which were occurred in this study due to placing them on non-coated tissue culture containers, had to be removed as much as possible because they are source of bacterial contamination and these dead cells release a lot of intrinsic substances such as enzymes or other chemical substances to disrupt the establishment of porcine ES-like cells.

## 2.4 MEDIUM FOR PORCINE ES-LIKE CELL ISOLATION AND CULTURE

Intact porcine blastocysts, isolated inner cell masses and isolated epiblasts were cultured in 500 µl of either KO4bh [Knockout-Dulbecco's modified eagle's medium (KO-DMEM: Gibco Cat.no. 10829), 15%FCS (Hyclone CIB perbio), 1x P/S (Sigma P0781), 1x minimum essential medium non-essential amino acid (MEM NEAA: Sigma M7145), 2 mM L-glutamine (Sigma G7513), and 100 µM ß-mercaptoethanol (ß–ME) consisting of 7 µl of ß–ME (100x) (Sigma M7522) diluted in 10 ml PBS (Sigma P4417) supplemented with 4 ng/ml bFGF (Sigma F0291) and 10 ng/ml hLIF (Chemicon Inc. LIF1010)] or DM40bh [DMEM (Sigma D6429), 16%FCS (Hyclone CIB perbio), nucleosides [0.03 mM adenosine (Sigma A4036), 0.03 mM guanosine (Sigma G6264), 0.03 mM cytidine (Sigma C4654), 0.03 mM uridine (Sigma U3003) and 0.01 mM thymidine (Sigma T1895)], 1x P/S, 1x MEM NEAA and 100 µM ß–ME supplemented with 40 ng/ml bFGF and 10 ng/ml hLIF] on mitotically inactivated MEFs at 39°C in 5%$CO_2$. Cells were observed under the microscope (Leica DMIL, Resolution Microscope Service, Ltd.). Medium was first changed half when those required porcine cells mentioned above attached on top of the feeders, and then it was changed half daily until the primary colonies of ES-like cells were revealed within 2-18 days. Porcine embryonic stem cell (pES)-like cells were mechanically passaged every 3-5 days and placed into fresh DM40bh on mouse feeder layers.

## 2.5 ISOLATION OF ICMs OR EPIBLASTS FROM PORCINE EMBRYOS

Under microscopic analysis, porcine blastocysts obtained from the pregnant pigs at day 6-8 were assigned into two groups; group 1 was for porcine blastocysts that exhibited ICMs and group 2 was for those that already developed epiblasts. Each group was divided into two sub-groups, intact blastocysts and mechanically isolated inner cell mass or epiblasts, based on the techniques used to handle the cells before they were given to culture in feeder culture system as shown in **Figure 2.1**. Importantly, to isolate epiblasts of porcine blastocysts, the dark field of microscope was used in stead of the bright one in order to easily spot the epiblastic area, which was whitish. All processes dealing with living cells were

managed on the heating stage (Lincam M60, Lincam Scientific Instruments, UK) connected to the power supply (Leica CLS150X, ISS Ark and General, Ltd.) and observed under the microscope (Leica DMIL, Resolution Microscope Service, Ltd.) in laminar flow hood (BioMat$^2$, Medical Air Technology, Ltd.).

### 2.5.1. Intact embryos

Each intact pig blastocyst was directly transferred into assigned culture medium using a defined 10 µl Pipetman (Gilson, France).

### 2.5.2 Mechanical technique

ICMs or epiblasts of porcine embryos were mechanically dissected using yellow 30G x ½ inch needle (BD Microlance Ref.no.304000) with 1ml insulin syringe (Sabre Regd.) on heated stage at 39°C and thereafter they were placed into the designed medium and then cultured in a humidified atmosphere of 5%$CO_2$ in air at 39°C.

## 2.6 IN VITRO PRODUCTION OF PARTHENOGENETIC PORCINE EMBRYOS

Parthenogenetic porcine embryos were provided by Dr. Amarnath Dasari. Briefly, gilt ovaries collected from local slaughterhouse were transferred to the laboratory in warm PBS within 3-4 h of their collection. Antral follicles greater than 2 mm were aspirated using a hypodermic syringe fitted with an 18G needle. The follicular fluid was pooled and collected into 50 ml conical tubes and allowed to settle for 10 minutes at 39°C. The supernatant was removed and the sediment was washed 3 times with Hepes-buffered Tyrode medium containing 0.1% polyvinyl alcohol (PVA-TL-HEPES) (**Appendix A)** allowing the contents to settle down for 10 minutes between the washes. Good quality cumulus oocyte complexes (COCs), oocytes surrounded by at least three layers of cumulus cells, were selected microscopically for maturation. Selected COCs were washed with handling medium, consisting of Tissue culture medium 199 (TCM199: Gibco cat.no.41150), 10% FCS, 0.57 mM L-Cystein (Sigma C7352), and 1x P/S, and matured in *in vitro* maturation (IVM) medium, composing of handling medium supplemented with 0.5 µg/ml follicle stimulating hormone (FSH: Vetrepharm, Ireland), 0.5 µg/ml luteinizing hormone (LH: Vetrepharm, Ireland) and 10 ng/ml

epidermal growth factor (EGF: Sigma E4127) for the first 22 h under a humidified air of 5%$CO_2$ at 39°C and then COCs were moved to new batch of in vitro maturation medium, but with out hormones and incubation continued for a further 22 h. After 44 h of IVM, COCs were briefly exposed to 300 IU/ml hyaluronidase (Sigma H3506) and were denuded of cumulus cells by gentle pipetting. Oocytes with evenly granulated cytoplasm with first polar body were selected for parthenogenic activation. Selected oocytes were briefly exposed to activation medium consisting of 0.28 mM Mannitol (Sigma M4152), 50 µM calcium dichloride ($CaCl_2$: Sigma C3881), 100 µM magnesium sulfate ($MgSO_4$: Sigma M1880) and 0.1% BSA (Sigma A3311). Then the oocytes were lined up between the two wire electrodes of fusion chamber (Eppendrof, USA) overlaid with activation medium. Two direct current (DC) pulses of 120V/mm for 60 µseconds duration each were applied with fusion machine (Eppendrof, USA) to activate the oocytes (Day 1). The activated oocytes were then cultured in porcine zygote medium-3 (PZM3) (**Appendix A**) supplemented with 0.4% BSA (Sigma A3311) and 5 µg/ml cytochalasin B (Sigma C6762) for 4 h. After 4 hours, the activated oocytes were washed in PZM3 containing 0.4% BSA and then cultured in the same medium for 7 days at 39˚C in a humidified atmosphere of 5%$O_2$, 5%$CO_2$ and 90%$N_2$. On day 7, blastocysts were harvested to extract total RNA using Absolutely RNA® nano prep kit as described in **Section 2.10.1.1**.

## 2.7 CULTURE OF MOUSE EMBRYONIC STEM CELLS

The mouse embryonic stem cell line, CGR8, originally derived from a 3.5 day pre-implantation embryo of 129 mice was provided by European Collection of Cell Culture, UK, was cultured in feeder-free condition at a density of 2-4 x $10^4$ cells/$cm^2$ in KO-DMEM (Gibco Cat.no. 10829), 10%FCS (Hyclone CIB perbio), 1x P/S, 1x MEM NEAA, 2 mM L-glutamine, 100µM ß–ME supplemented with 10 ng/ml mLIF (Chemicon Inc. LIF2010) at 39°C in a humidified atmosphere of 5%$CO_2$ in air for 2-3 passages before they were wash twice with sterile PBS and disaggregated with 1x Trypsin-EDTA at 39°C for 5 minutes before washing once again with PBS and then those cells were used to extract total RNA using RNeasy Mini kit as described in **Section 2.10.1.2**.

## 2.8 DIFFERENTIATION PROTOCOLS USED TO PRODUCE CELLS OF THREE GERM LAYERS

### 2.8.1 Derivation of neuroectodermal-like and myocyte-like cells from porcine ES-like cells obtained in this study

Porcine ES-like cells obtained from this study were cultured directly in non-coated 4-well tissue culture dish containing 500 µl of either DMh [DMEM, 16%FCS, nucleosides (0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine), 1x P/S, 1x MEM NEAA and 100 µM ß–ME supplemented with 10 ng/ml hLIF], DM4bh [DMEM, 16%FCS, nucleosides (0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine), 1x P/S, 1x MEM NEAA and 100 µM ß–ME supplemented with 4 ng/ml bFGF and 10 ng/ml hLIF], DM40bh [DMEM, 16%FCS, nucleosides (0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine), 1x P/S, 1x MEM NEAA and 100 µM ß–ME supplemented with 40 ng/ml bFGF and 10 ng/ml hLIF], or KOh [KO-DMEM, 15%FCS, 1x P/S, 1x MEM NEAA, 2mM L-glutamine and 100 µM ß–ME supplemented with 10 ng/ml hLIF] in a humidified atmosphere of 5%$CO_2$ in air at 39°C. Cells were observed daily and medium was changed half every other day until neuroectodermal-like and myocyte-like cells were obtained within 1-2 weeks and 2-4 weeks, respectively.

### 2.8.2 Derivation of hepatocyte-like cells from porcine ES-like cells obtained in this study

The protocol applied to derive porcine hepatocyte-like cells from porcine ES-like cells generated in this study was modified from some previous reports of murine protocols (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Yin *et al*., 2007). In brief, porcine ES-like colonies were cultured directly in the chamber slides (Nunclon, Roskilde, Denmark) containing 500 µl of either 10KOm [KO-DMEM, 10%FCS, 1x P/S, 1x MEM NEAA, 2mM L-glutamine and 100 µM ß–ME supplemented with 10 ng/ml mLIF], 10KOh [KO-DMEM, 10%FCS, 1x P/S, 1x MEM NEAA, 2mM L-glutamine and 100 µM ß–ME supplemented with 10 ng/ml hLIF], or 20KOh [KO-DMEM, 20%FCS, 1x P/S, 1x MEM NEAA, 2mM

L-glutamine and 100 µM ß–ME supplemented with 10 ng/ml hLIF], as referred to as Hep10KOm, Hep10KOh and Hep20KOh, respectively, and cultured in a humidified atmosphere of 5%$CO_2$ in air at 39°C for 3 days. 10 ng/ml bFGF was then added to all cultures for 2 days followed by an addition of 20 ng/ml recombinant mouse hepatocyte growth factor (HGF: R&D System Cat.no.2207-HG) for a further 2 days. Then the combination of 20 ng/ml HGF, 10 ng/ml recombinant mouse Oncostatin M (OSM: R&D System Cat.no.495-MO), 100 nM dexamethasone (Sigma D4902) and 5 µg/ml ITS (based on transferrin, Sigma I3146) was added until hepatocyte-like cells were formed in the culture system. Those cells were observed daily and medium was changed half every other day.

## 2.9 METHODS USED TO STAIN PORCINE ES-LIKE AND DIFFERENTIATED CELLS DERIVED IN THIS STUDY

### 2.9.1 Alkaline phosphatase staining

The method was modified from Sigma-Aldrich procedure number 86 (2003). Medium was removed from a required chamber slide used to culture porcine ES-like and differentiated cells. Cells were washed twice with 1x Tris-EDTA buffer solution (TBS) (Bio-Rad Laboratories Cat.no.170-6435). Samples were then fixed with MeOH:Formaldehyde (9:1) for 30 seconds and thereafter, samples were rinsed with deionised water ($dH_2O$) for 45 seconds. After $dH_2O$ removal, 200 µl alkaline-dye mixture was added to each chamber slide [(50 µl of 0.1 mol/L sodium nitrite (Sigma 914) and 50 µl of 5 mg/ml FRV alkaline solution (Sigma 862) were mixed gently and left for 2 minutes. Then, this solution was added into 2.25 ml $dH_2O$ and mixed gently. Thereafter, 50 µl of 4 mg/ml Naphthol AS-BI phosphate (Sigma 861) was blended with the solution prepared above)] and incubated at room temperature in the dark for 15 minutes. After removal of alkaline dye, samples were washed once again with 1x TBS, and then samples were observed under a light microscopy and results were recorded. AP positive cells stained red, while negative cells were colourless or brownish.

### 2.9.2 Immunofluorescence staining

#### *2.9.2.1 Double staining of Oct-4 and Nanog*

The culture medium was aspirated from the chamber slide and then washed quickly with 500 µl PBS-PVA to remove culture medium before cells were fixed with 4% PFA (Sigma P6148) for 20 minutes at room temperature. They were then washed twice with 500 µl rinse buffer [1% BSA (Sigma A4503), 0.05% Na Azide (Sigma S2002) in PBS (Sigma P4417) and 0.1% Tween 20 (Sigma P1379)] for 5 minutes each time. After that, samples were permeabilised with 500 µl 0.5% TritonX-100 (Sigma T8532), which was dissolved in warm PBS-PVA (0.2 mg PVA (Sigma P8136)/1 ml PBS), for 20 minutes at room temperature and then they were washed twice with rinse buffer for 5 minutes each time again. 500 µl Blocking solution (5% BSA and 0.05% Na Azide in PBS) was applied to the cells for 1 h at room temperature. Then, 150 µl primary antibody, Oct-3A/4 (N-19) goat immunoglobulin G (IgG) (Santacruz SC8628; 1:100) and rabbit anti-human Nanog IgG (Peprotech 500-P236; 1:100) was diluted with blocking solution and incubated with the samples overnight at 4˚C. The day after, samples were washed with rinse buffer, 10 minutes each, for three times and then diluted secondary antibody with blocking solution, donkey anti goat (D ∞ G) IgG Texas Red (Jackson ImmunoResearch Laboratories 705-075-147; 1:400) for Oct-3A/4 and donkey anti rabbit (D ∞ R) IgG FITC (Jackson ImmunoResearch Laboratories 711-097-003; 1:20) for Nanog, was added and incubated with the specimens for 1-2 hours in the dark at room temperature. Samples were again washed with rinse buffer three times, 10 minutes each. Finally, cells were incubated with DAPI (Vectashield H1200, Vector Laboratories, Inc. Burlingarne, CA) for another 10 minutes before they were covered with cover slides sealed with nail polish and thereafter observed under the epifluorescence microscope (Leica DMR, Heidelberg, Germany). Images were captured and stored using a digital camera (Hammamatsu, ORCA-er, Japan) and image analysis, and quantification were performed using Simple PCI software (Compix Imaging System, USA).

#### *2.9.2.2 Staining of nestin, alpha-smooth muscle actin and alpha-fetoprotein*

All procedures for the immunofluorescence staining of differentiated cells were as described for staining of OCT-4 and Nanog of porcine ES-like cells, except the primary and second antibodies were different. Primary antibodies, rabbit anti nestin IgG (Chemicon AB5922; 1:200 or Abcam AB5968; 1:100), mouse anti-human actin IgG (Chemicon CBL171; 1:100) and mouse anti-human alpha-fetoprotein IgG (Chemicon 2004189; 1:50) were used to stain neuronal-like cells representative of ectodermal cells, smooth muscle-like cells representative of mesodermal cells and hepatocyte-like cells representative of endodermal cells, respectively. For secondary antibodies, D ∞ R IgG FITC (Jackson ImmunoResearch Laboratories 711-097-003; 1:40) was used for nestin, rabbit anti mouse (R ∞ M) IgG FITC (DAKO; 1:40) for alpha-smooth muscle actin and goat anti-mouse (G ∞ M) IgG Cy3 (Jackson ImmunoResearch Laboratories 115-165-174; 1:50) for alpha-fetoprotein. Finally, all samples were observed using the same microscope and image analysis system as previously described.

## 2.10 GENE EXPRESSION ANALYSIS OF PORCINE ES-LIKE AND DIFFERENTIATED CELLS DERIVED FROM THIS STUDY

### 2.10.1 Extraction of total RNA contents

#### *2.10.1.1 Extraction from porcine blastocysts, porcine ES-like and differentiated cells obtained from this study*

The most important factor during extractraction of total RNA is to avoid denaturation of RNA and premature cDNA synthesis, therefore, all procedures while dealing with RNA were performed on ice at all times. Total RNA of each sample was extracted according to the protocol of Absolutely RNA® nano prep kit (Stratagene Cat.no.400753, UK). Briefly, all samples were mechanically selected from the culture dishes and then they were washed with PBS to remove any serum, cooled on ice and homogenised by pipetting and vortexing (Clifton cyclone, Electro Ltd., UK) with the well-mixed of 100 µl of lysis buffer added with 0.7 µl of β-ME contained in 250 µl microfuge tube. Thereafter, 100 µl of 70% ethanol stored at room temperature was added to the lysate of cells and

mixed by vortexing for 5 seconds. The mixture was transferred to an RNA-binding nano-spin cup sitting within a 2 ml collection tube and then centrifuged in a microcentrifuge (Biofuge Pico, Heraeus, Kendro Laboratory Products., Ltd., Germany) at 13,000 rpm for 1 min. The filtrate was discarded and the spin cup was held in the same collection tube. DNA was removed from the samples by the addition of 300 µl of 1x Low-Salt Wash Buffer provided with the kit and spinning in a microcentrifuge at 13,000 rpm for 1 min. The filtrate was again discarded and the spin cup was re-seated again in the same collecting tube and spun in the same centrifuge at 13,000 rpm for 2 min to allow drying of the matrix. After that, 15 µl DNase solution, a combination of 2.5 µl of reconstituted RNase-Free DNase I and 12.5 µl of DNase Digestion Buffer, was added directly onto the fiber matrix and incubated at 37°C for 15 minutes. Then, 300 µl of 1x High-Salt Wash Buffer was applied to the sample and centrifuged in the microcentrifuge at 13,000 rpm for 1 min, the filtrate was removed and the spin cup retained in the same collection tube. The same procedure of washing the samples was repeated twice more using 300 µl of 1x Low-Salt Wash Buffer instead of 1x High-Salt one. Afterward, the fiber matrix was dried by centrifugation at 13,000 rpm for 3 minutes, and the spin cup was then transferred into a new collection tube. Finally, 10 µl of Elution Buffer was added directly onto the fiber matrix of the spin cup, incubated for 2 minutes at room temperature and consequently centrifuged at 13,000 rpm for 5 minutes, the eluate containing purified total RNA was recovered and stored in 250 µl-microfuge tubes (Sarstedt Aktlengesellschaft & Co., Germany) at -80°C (Hera Freeze, Heraeus, Kendro Laboratory Products, Ltd., Germany) until required.

#### *2.10.1.2 Extraction from mouse embryonic stem cells, murine and porcine neurons, hearts and liver cells in this study*

All organs were homogenised using Polytron®PT4000 (Kinematica AG, Switzerland) prior to extraction of total RNA using an RNeasy Mini kit (Qiagen Cat.no.74104, UK). Briefly, homogenised tissues and mES cells were adjusted to a volume of 100 µl with RNase-free water and then 350 µl Buffer RLT was added and mixed gently. 250 µl absolute ethanol (Fisher Scientific Code:E/0650DF/P17) was then added to samples and mixed well by pipetting, thereafter, all of the solution was placed into an RNeasy Mini Spin column

sitting in a 2 ml collection tube. The lid was closed on top of the spin column and centrifuged for 15 seconds at 13,000 rpm. Solution in the collection tube was discarded and 350 µl Buffer RW1 was added to wash the spin column, the lid was closed gently and centrifuged for another 15 seconds at 13,000 rpm. The flow-through was removed and a mixture of 10 µl DNase I stock solution diluted in 70 µl Buffer RDD was applied directly onto the spin column membrane and incubated at 37°C for 15 minutes in a hot box (Heraeus, Kendro Laboratory Products, Ltd., Germany). 350 µl Buffer RW1 was then added to the spin column, the lid was closed gently and the column centrifuged for 15 seconds at 13,000 rpm. The filtrate was removed and 500 µl Buffer RPE was added to wash the spin column, again the lid was closed gently and centrifuged for 15 seconds at 13,000 rpm. The flow-through was discarded and 500 µl Buffer RPE was added to the spin column, the lid was closed gently and the column centrifuged for 2 minutes at 13,000 rpm. The collection tube and filtrate were then disposed and spin column was transferred to the new 1.5 ml collection tube and centrifuged again for 1 min at 13,000 rpm. Thereafter, the collection tube and filtrate were again discarded and the spin column was transferred to the new 1.5 ml collection tube. 30-60 µl Elution Buffer (Stratagene, UK) was pipetted directly onto the spin column membrane. The lid was then closed gently and centrifuged for 1 min at 13,000 rpm. Diluted purified total RNA was stored at -80°C until required.

### 2.10.2 RT-PCR

**Reverse Transcriptase step**

First-stranded cDNA of purified total RNA obtained from this study was transcribed using Sensiscript® Reverse Transcription kit (Qiagen Cat.no.205211, UK), total RNA was determined using a NanoDrop™ ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific) less than 50 ng/sample and using Omniscript® Reverse Transcription kit (Qiagen Cat.no.205111, UK) in case of total RNA yielded more than 50 ng up to 100 µg/sample. According to the manufacturers protocol, the solution of required total RNA, anchored Oligo-dT primer (Thermo Scientific ABgene AB-1247), 10x Buffer RT (Qiagen, UK), dNTP mix (Qiagen, UK), RNasIn® Plus RNase inhibitor (Promega N2611), Sensiscript RT or Omniscript RT and RNase-free water (Qiagen, UK) was

thawed on ice. Firstly, a total volume of 12 µl diluted RNA composing of 1-11 µl RNA and 1-11 µl RNase-free water was denatured at 65°C for 5 minutes (Eppendorf AG, Hamburg, Germany) before the RT reactions were set up. The variation of volume of RNA template and RNase-free water depended on the concentration of total RNA, which ranged between 20-200 ng/sample measured using NanoDrop™ ND-1000 UV-Vis spectrophotometer, and the conditions of reverse transcriptase enzyme used in the RT reactions. The reactions of RT master mix were prepared as listed in **Table 2.1**. In case of no addition of reverse transcriptase enzyme in the RT reactions, 1 µl RNase free water was added to the solution to make up the total volume of no reverse transcriptase group to be 20 µl in total. Thereafter, the RT reactions were incubated at 37°C for 60 minutes (Eppendorf AG, Hamburg, Germany) and then they were used immediately for PCR or stored at -20°C (Iced Diamond) until needed.

*Table 2.1 Compositions of RT reactions (20µl/reaction) set up to produce the first-stranded cDNA from total RNA of required samples in this present study.*

This table summarises the constituents of each RT reaction which required template RNA was denatured first in RNase free water in the total volume of 12 µl under 65°C for 5 minutes and thereafter denatured RNA was mixed well with 8 µl RT master mix composing of 2 µl each of 10x Buffer RT, dNTP and anchored Oligo-dT primer, 0.25 µl RNase inhibitor, 0 or 1 µl Reverse Transcriptase, and 0.75 or 1.75 µl RNase-free water before the blended RT solution was incubated at 37°C for 60 minutes in order to generate the first-stranded cDNA from the required samples.

| Components | Volume | Final concentration |
|---|---|---|
| **Denatured RNA solution** | | |
| RNase free water | 1-11 µl | - |
| Template RNA | 1-11 µl | 20-200 ng/sample |
| **Total** | **12.0 µl** | |
| **RT master mix** | | |
| 10x Buffer RT | 2.0 µl | 1x |
| dNTP mix (5mM) | 2.0 µl | 0.5 mM |
| Anchored Oligo-dT primer (10 µM) | 2.0 µl | 1.0 µM |
| RNase inhibitor (40 units/µl) | 0.25 µl | 10 units |
| Sensiscript or Omniscript Reverse Transcriptase | 0 or 1.0 µl | - |
| RNase-free water | 0.75 or 1.75 µl | - |
| Denature of RNA template | 12.0 µl | |
| **Total volume** | **20.0 µl** | |

**Polymerase Chain Reaction (PCR)**

All required primers were reconstituted to 100 x $10^{-9}$/µl with Tris EDTA buffer (TE buffer) (a mixture of 500 µl 1M Tris HCl and 100 µl 0.5M EDTA in 50 ml Diethyl pyrocarbonate (DEPC) treated water). 5 µl each of forward and reverse primers of genes of interest were diluted to 1 in 10 with molecular grade water (Sigma W4502). The PCR reactions were set up to a total volume of 25 µl/reaction, composed of 12.5 µl BioMix™ Red (Bioline BIO-25005), 0-12.5 µl molecular grade water, 0-5 µl of required first strand cDNA template, no RT template and diluted forward and reverse primers (1:10). Thereafter, the PCR reactions were performed using a PCR Thermal Cycler (Eppendorf AG, Hamburg, Germany). For pig samples, the amplification of cDNA was done by denaturation at 95°C for 4 minutes followed by amplification of 40-55 cycles of denaturing at 95°C for 30 seconds, annealing at 50.6-59.2°C for 20 seconds, extension at 68°C for 45 seconds and one cycle of final extension at 72°C for 2 minutes, while the amplification of cDNA of mouse specimens was performed by denaturation at 95°C for 4 minutes followed by amplification of 40 cycles of denaturing at 95°C for 30 seconds, annealing at 59.2°C for 30 seconds, extension at 68°C for 1 minute and one cycle of final extension at 72°C for 2 minutes Then, PCR products were collected and analysed by agarose gel electrophoresis.

**Agarose Gel Electrophoresis**

A 1.2% agarose gel was prepared consisting of 80 ml 1x Tris acetate EDTA buffer (TAE buffer) and 0.96 g Low EEO agarose (Biogene Cat.no.300-300). Agarose powder was dissolved completely in the buffer by boiling in the microwave (Belling M384 TCGR, UK) for 90 seconds, 8 µl of Ethidium Bromide (Fisher Scientific E/P800/03) was then added and mixed well with the solution. The agarose was then poured into the prepared electrophoresis tray prepared and left at room temperature. Once the agarose gel had set, the comb and tape were removed and the tray containing the agarose gel placed into the Gel Electrophoresis chamber (BioRad Mini-Sub® Cell GT, UK). The chamber was filled with 1x TAE buffer until it covered the agarose gel. 10 µl of 100 bp DNA ladder (Promega G2101) or PCR products were pipetted into individual wells. The lid was placed on the chamber connected to power supply (Sigma

Techware PS250-2) and the gel run between 70-90 volts for 45 minutes or until the markers moved to the right place. The gel was then removed from the tray and placed on the UV eliminator (GelDoc-IT™ Imaging System, USA) to detect the bands of required genes by fluorescence of Ethidium Bromide. The photos were captured using a Cohu-camera and results were printed using a Sony Digital Graphic Printer UP-D897.

### 2.10.3 Primers

The primers used in these present studies were shown in **Table 2.2-2.5** and they were designed using the modification between Primer 3 Input version 4 (http://frodo.wi.mit.edu/) and Oligo calculator programmes (http://www.pitt.edu/~rsup/OligoCalc.html) based on porcine-specific sequences reported from GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or The Institute for Genome Research (TIGR, Rockville, MD; http://www.tigr.org/tdb/tgi/ssgi) or from previous reports (Johansson and Wiles, 1995; Mitsui *et al*., 2003; Do and Scholer, 2005, Lee *et al*., 2006a; Talbot *et al*., 2007a). All required gene sequences of mice and pigs picked up by the designed primers were confirmed for their species-specific using BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) prior to ordering from Invitrogen life technologies.

*Table 2.2 Primers and PCR conditions used to examine genes of interest in porcine ES-like cells obtained in this present study.*

This table provides the accession numbers, sequences of primers, product length and annealing temperature of each gene examined in porcine ES-like cells by RT-PCR technique. TIGR accession numbers were initiated by TC and all other accession numbers were reported from GenBank. Abbreviations: A, Adenine; bp, Base pair; β-actin, Beta actin; C, Cytosine; CDX-2, Caudal type homeobox transcription factor-2; DPPA-3, Developmental pluripotency associated-3; F, Forward primer; G, Guanine; OCT-4, Octamer-binding transcription factor-4; R, Reverse primer; REX-1, RNA exonuclease 1 homolog; SOX-2, Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 2; T, Thymine and Tm, Melting temperature.

| **Gene** | **Accession no.** | **Primers** | **Product (bp)** | **Annealing Tm (°C)** |
|---|---|---|---|---|
| β-actin | DQ845171 | F: 5'-CTCTTCCAGCCCTCCTTC-3'<br>R: 5'-ATCTCCTTCTGCATCCTGTC-3' | 170 | 59.2 |
| OCT-4 | NM_001113060 | F: 5'-GAGGAGTCCCAGGACATCAA-3'<br>R: 5'-CTCTCCAGGTTGCCTCTCAC-3' | 341 | 59.2 |
| Nanog | DQ447201 | F: 5'-TTCCTTCCTCCATGGATCTG-3'<br>R: 5'-ATCTGCTGGAGGCTGAGGTA-3' | 214 | 50.6 |
| SOX-2 | DQ159208 | F: 5'- AACCAAGACGCTCATGAAGAA-3'<br>R: 5'- ATGTAGGTCTGCGAGCTGGT-3' | 306 | 58.7 |
| CDX-2 | AM778830 | F: 5'-CCTGTGCGAGTGGATGCG-3'<br>R: 5'-TCGCTCTGCGGTTCTGAAACC-3' | 227 | 52.5 |
| REX-1 | TC272492 | F: 5'- TTTCTGAGTACGTGCCAGGCAA-3'<br>R: 5'-GAACGGAGAGACGCTTTCTCAGAG-3 | 200 | 59.2 |
| DPPA-3 | AJ655743 | F: 5′-AGGATGACGGTGATGACAGT-3′<br>R: 5′-TCATCTTCTAGACTTGGGGC-3′ | 333 | 59.2 |

*Table 2.3 Primers and PCR conditions used to examine genes of interest in induced differentiated cells obtained from porcine ES-like cells and other porcine tissues in this present study.*

This table reports the accession numbers, sequences of primers, product length and annealing temperature of each differentiated gene examined in induced differentiated cells obtained from porcine ES-like cells by RT-PCR technique. TIGR accession numbers were initiated by TC and all other accession numbers were reported from GenBank. Abbreviations: α, Alpha; A, Adenine; α-SM actin, Alpha-smooth muscle actin; bp, Base pair; C, Cytosine; F, Forward primer; G, Guanine; HNF-1β, Hepatocyte nuclear factor 1 homeobox B; R, Reverse primer; SM myosin, Smooth muscle myosin; T, Thymine and Tm, Melting temperature.

| **Gene** | **Accession no.** | **Primers** | **Product (bp)** | **Annealing Tm (°C)** |
|---|---|---|---|---|
| Nestin | TC265906 | F: 5'-GCCACAGCTTAGAGGTCAA-3' | 494 | 59.2 |
| | | R: 5'-CAGAGATTGCAATGTCTGCAG-3' | | |
| α-SM actin | DQ400922 | F: 5'-AATGGCTCTGGGCTCTGTAA-3' | 219 | 59.2 |
| | | R: 5'-CTTTTCCATGTCGTCCCAGT-3' | | |
| SM myosin | AU058791 | F: 5'-GGCAACAGAGCGTAGCGCA-3' | 243 | 59.2 |
| | | R: 5'-ACCTCCTTCAGCTTCTTGTCC-3' | | |
| α-cardiac actin | TC249159 | F: 5'-CTTGAGTCGGACGGAGAAAG-3' | 376 | 59.2 |
| | | R: 5'-GGACTGGGCTGCCTACATTA-3' | | |
| Transthyretin | X87846 | F: 5'-ATGGTCAAAGTCCTGGATGCT-3' | 316 | 59.2 |
| | | R: 5'-TGGAGTAAGAGTAGGGGCTAA-3' | | |
| α-fetoprotein | NM_214317 | F: 5'-ACACAAAGAAAGCCCCTCAG-3' | 185 | 59.2 |
| | | R: 5'-ACCAGGGTTTATGGGCATCT-3' | | |
| Albumin | NM_001005208 | F: 5'-TGTTGCTGATGAGTCAGCTGA-3' | 222 | 59.2 |
| | | R: 5'-TGGAAGTCAGCGCATAAAGCA-3' | | |
| HNF-1β | TC296208 | F: 5'-GTTGCAGAAAAGCGCAGTGA-3' | 251 | 59.2 |
| | | R: 5'-CGTCGAAGGAGCACAAGGTA-3' | | |

*Table 2.4 Primers and PCR conditions used to examine genes of interest in mouse embryonic stem cells cultured in this present study.*

This table demonstrates the accession numbers, sequences of primers, product length and annealing temperature of genes investigated in mES cells by RT-PCR technique. All accession numbers were available from GenBank. Abbreviations: A, Adenine; bp, Base pair; β-actin, Beta actin; C, Cytosine; DPPA-3, Developmental pluripotency associated-3; F, Forward primer; G, Guanine; OCT-4, Octamer-binding transcription factor-4; R, Reverse primer; REX-1, RNA exonuclease 1 homolog; SOX-2, Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 2; T, Thymine and Tm, Melting temperature.

| **Gene** | **Accession no.** | **Primers** | **Product (bp)** | **Annealing Tm (°C)** |
|---|---|---|---|---|
| β-actin | NM_007393 | F: 5'-CGTGCGTGACATCAAAGAGAAGC-3'<br>R: 5'-ATCTGCTGGAAGGTGGACAGTGAG-3' | 441 | 59.2 |
| OCT-4 | X52437 | F: 5'-GAACAGTTTGCCAAGCTGCTG-3'<br>R: 5'-CCGGTTACAGAACCATACTCG-3' | 405 | 59.2 |
| Nanog | AY278951 | F: 5'-AGGGTCTGCTACTGAGATGCTCTG-3'<br>R: 5'-CAACCACTGGTTTTTCTGCCACCG-3' | 364 | 59.2 |
| SOX-2 | NM_011443 | F: 5'-ATGGGCTCTGTGGTCAAGTC-3'<br>R: 5'-CCCTCCCAATTCCCTTGTAT-3' | 300 | 59.2 |
| REX-1 | NM_009556 | F: 5'-AAAGTGAGATTAGCCCCGAG-3'<br>R: 5'-TCCCCATCCCCTTCAATAGCA-3' | 944 | 59.2 |
| DPPA-3 | NM_139218 | F: 5'-ACAGACTGACTGCTAATTGGG-3'<br>R: 5'-TGGGCCTCACAGCTTGAG-3' | 739 | 59.2 |

*Table 2.5 Primers and PCR conditions used to examine genes of interest in mouse embryoid bodies and other mouse tissues in the present study.*

This table shows the accession numbers, sequences of primers, product length and annealing temperature of genes examined in induced differentiated cells obtained from porcine ES-like cells by RT-PCR technique. All accession numbers were reported from GenBank. Abbreviations: α, Alpha; A, Adenine; α-SM actin, Alpha-smooth muscle actin; bp, Base pair; C, Cytosine; F, Forward primer; G, Guanine; HNF-1β, Hepatocyte nuclear factor 1 homeobox B; R, Reverse primer; SM myosin, Smooth muscle myosin; T, Thymine and Tm, Melting temperature.

| **Gene** | **Accession no.** | **Primers** | **Product (bp)** | **Annealing Tm (°C)** |
|---|---|---|---|---|
| Nestin | NM_016701 | F: 5'-GGGCAGAGAAGACAGTGAGG-3'<br>R: 5'-CATCCTGGACCTTGACACCT-3' | 494 | 59.2 |
| α-SM actin | X13297 | F: 5'-CTGACAGAGGCACCACTGAA-3'<br>R: 5'-ATCTCACGCTCGGCAGTAGT-3' | 311 | 59.2 |
| SM myosin | D85924 | F: 5'-TGACTTCCATGAGCAGATCG-3'<br>R: 5'-TTCAGCACTGTCACCTCCTG-3' | 345 | 59.2 |
| α-cardiac actin | NM_009608 | F: 5'-TGCCAACAATGTCCTATCTG-3'<br>R: 5'-GAAGCACAAACAAACTGCAC-3' | 379 | 59.2 |
| Transthyretin | NM_013697 | F: 5'-GCTTCCCTTCGACTCTTCCT-3'<br>R: 5'-CAGAGTCGTTGGCTGTGAAA-3' | 358 | 59.2 |
| α-fetoprotein | NM_007423 | F: 5'-CTCAGCGAGGAGAAATGGTC-3'<br>R: 5'-CCGAGAAATCTGCAGTGACA-3' | 340 | 59.2 |
| Albumin | NM_009654 | F: 5'-GTCTTAGTGAGGTGGAGCAT-3'<br>R: 5'- ACTACAGCACTTGGTAACAT -3' | 569 | 59.2 |
| HNF-1β | AB008176 | F: 5'-AGCCCACCAACAAGAAGATG-3'<br>R: 5'-GTTGTAGCGCACTCCTGACA-3' | 386 | 59.2 |

# CHAPTER 3

# ESTABLISHMENT OF PORCINE EMBRYONIC STEM CELL-LIKE CELLS

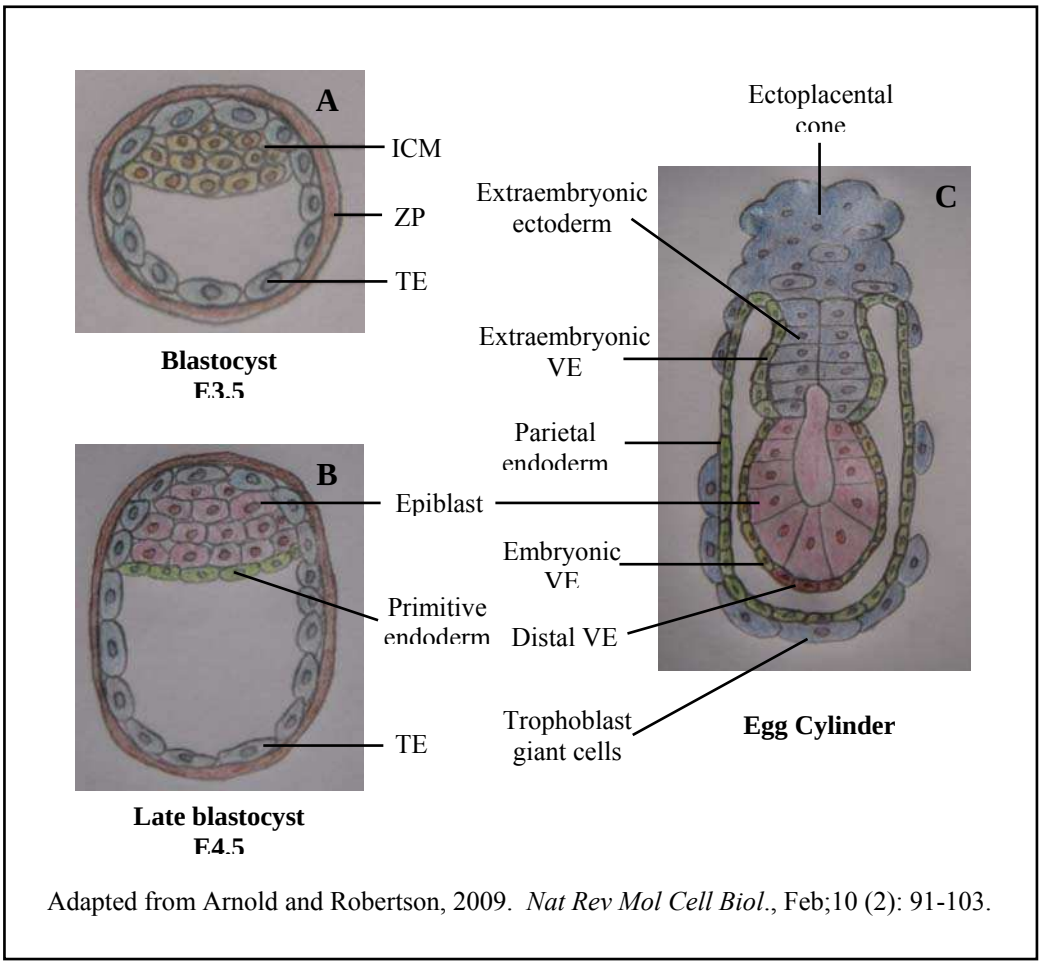
## 3.1 INTRODUCTION

As previously discussed in **Section 1.2**, three critical factors are thought to play a crucial role in the generation of stable pluripotent ES cell lines in most mammals. Firstly, the quality of the embryos used, secondly the techniques used to isolate the required cells from the embryos, and thirdly the culture used to derive ES cells. Therefore, to success in generation of porcine ES-like cells originating from the early epiblast ES cell origin as mES cells is to use the equivalent conditions manipulated to obtain mES cells in consideration with the basic knowledge of porcine biology and new drug development as much as possible, as the details will be discussed later.

### 3.1.1 Sources of embryos used to establish porcine ES cells

As stated in **Section 1.2.1**, porcine pre-implantation blastocysts used to generate ES cells in this study were obtained from pregnant pigs due to their superior quality as assessed by higher numbers of ICMs and epiblasts, which are the cells required for isolation of ES cells, when compared to other sources, thereby increasing the chance to get pluripotent ES cell lines (Bavister, 2004). However, not only the quality and number of embryos themselves are of importance to create stable ES cell lines, but also the embryonic stages may play a critical role in resolving this situation resulting from the differences in embryonic development between mice and pigs in terms of the mechanisms controlling gastrulation and their general appearances before implantation (Blomberg *et al*., 2008a; Arnold and Robertson, 2009). Generally, *in vivo* murine pre-implantation blastocysts, which they are identified by their morphology and consist of two distinct lineages, trophectoderm and ICMs, are fomed around day 3.5 (**Figure 3.1A**). Thereafter the ICMs of expanded blastocysts will give rise to epiblasts and primitive endoderm (also known as hypoblast cells) no later than day 4.5 (**Figure 3.1B**), whose epiblasts usually contribute to early epiblast ES cells (Brook and Gardner, 1997; Tesar *et al*., 2007; Arnold and Robertson,

2009). The formation of epiblast and primitive endoderm lineages is regulated by Nanog and GATA-binding factor 6 (GATA-6), respectively. After implantation, a cavity is formed in the central part of the epiblasts and the embryo elongates along the proximal-distal axis to create the late stage of murine conceptus called the egg cylinder, which is composed of ectoplacental cone, extraembryonic ectoderm, trophoblast giant cells, visceral endoderm (VE) and epiblasts. This development is controlled by a complex of networks of the TGF-β family such as nodal and BMP, Wnt and FGF families (Brook and Gardner, 1997; Brons *et al*., 2007; Tesar *et al*., 2007; Arnold and Robertson, 2009), as seen in **Figure 3.1C**. This late stage of epiblasts can still be used for derivation of pluripotent ES cells from late epiblast origin (Brons *et al*., 2007; Tesar *et al*., 2007). In contrast to the mouse, the *in vivo* derived porcine blastocysts develop distinct ICMs on day 5 or 6 (**Figure 3.2A**), thereafter the spherical conceptus will hatch from ZP. Undefined epiblast formation starts on late day 6 or 7 (Murray *et al*., 1971; Hunter 1974; Anderson 1978; Geisert *et al*., 1982a; Geisert *et al*., 1982b; Rodriguez-Martinez and Persson, 1993; Flechon *et al*., 2004) before ICMs develop to be hypoblastic cells by days 8 and 9 (Flechon *et al*., 2004). Porcine embryos naturally remain detached from the uteri for trophoblastic elongation and increase in their diameter, and then pass through the spheroid (**Figure 3.2A**), ovoid (**Figure 3.2B**), tubular (**Figure 3.2D**), filamentous (**Figure 3.2E**), and primitive streak stages (**Figure 3.2F**), respectively, coincident with the whitish embryonic disc, a source of late epiblast stage reproducing pluripotent ES cells, has been developed from early epiblasts in order to await for the time of implantation around day 16 or 17, which is controlled by some certain ontological factors, (Anderson 1978; Mattson *et al*., 1990; Vejlsted *et al*., 2006a; Brevini *et al*., 2007b). Importantly, vimentin, one of the key markers of mesoderm differentiation, has been detected since day 9 of *in vivo* derived porcine blastocysts (Prelle *et al*., 2001). In this sense, it suggests that *in vivo* porcine pre-implantation blastocysts between days 6 and 8, which are theoretically equivalent to murine embryonic stages for deriving pluripotent ES cells, may be the most suitable stage to derive early epiblast ES cells. As seen in **Table 3.1**, many research groups have been using this stage of porcine embryos to establish ES cells (Vackova & Madrova, 2006; Blomberg *et al*., 2008b).




Adapted from Arnold and Robertson, 2009. *Nat Rev Mol Cell Biol*., Feb;10 (2): 91-103.

*Figure 3.1 Characteristics of murine embryos at different stages during embryonic development.*

After fertilization, the mouse embryo will develop from a single cell until the blastocyst stage, which consists of inner cell mass and outermost trophectoderm cells around embryonic day 3.5 (E3.5) (**Fig. 3.1A**). A few hours later ICMs will be programmed to give rise to primitive endoderm and epiblast lineages and these two types of cells are founded around E4.5 (**Fig. 3.1B**) before implantation begins. After implantation, later stages of murine embryos will form an egg cylinder, which consists of ectoplacental cone, extraembryonic ectoderm, trophoblast giant cells, visceral endoderm (VE) and epiblasts (Brook and Gardner, 1997; Brons *et al*., 2007; Tesar *et al*., 2007; Arnold and Robertson, 2009), as shown in **Fig. 3.1C**. Abbreviations: ICM, Inner cell mass; TE, Trophectoderm; VE, Visceral endoderm and ZP, Zona pellucida.

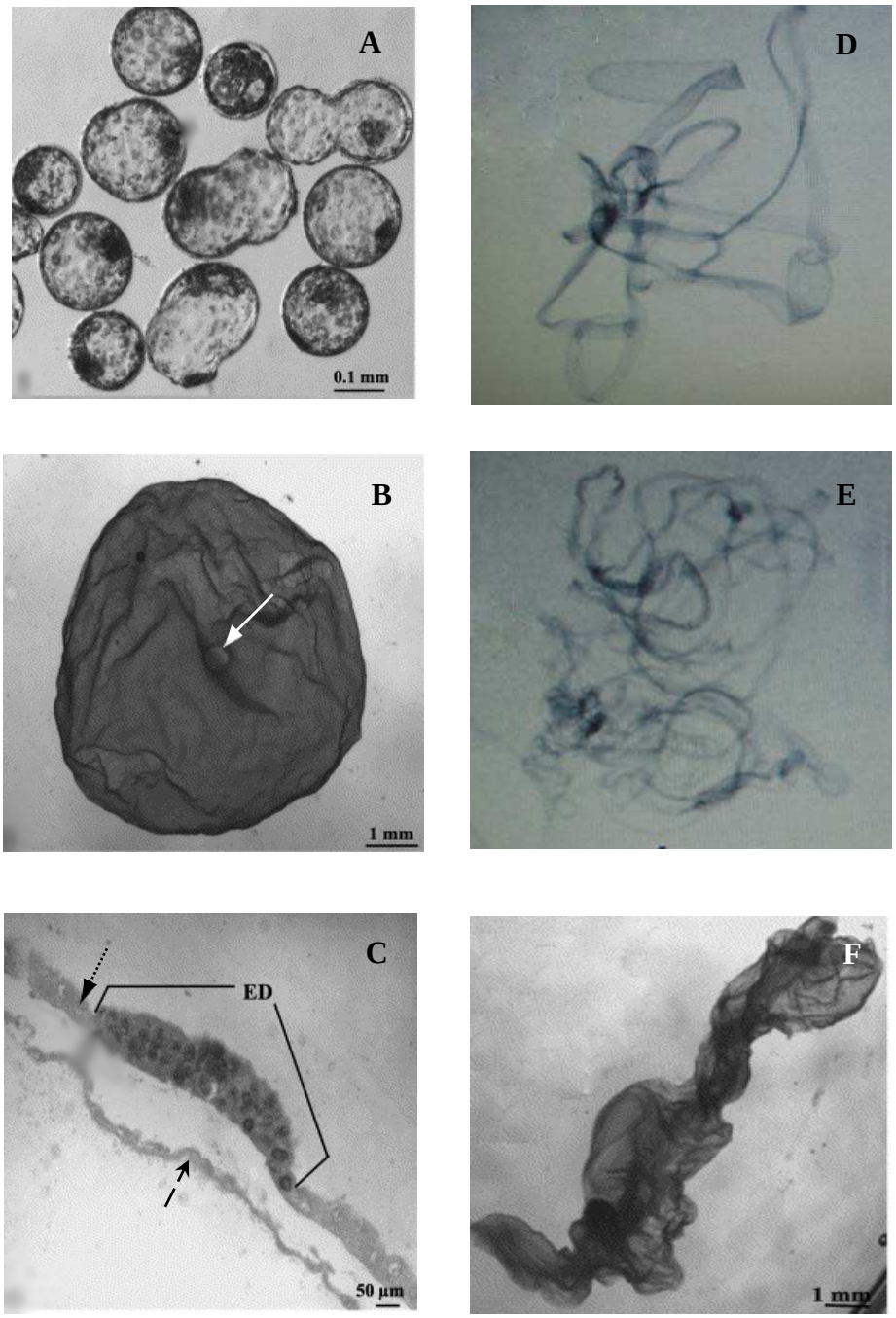



**Fig. 3.2A-C** and **3.2F** were adapted from Vejlsted *et al*, 2006a. *Theriogenology,* 65**,** 153-165.

**Fig. 3.2D-E** were modified from Mattson *et al*., 1990. *Biol. Reprod.*, 42, 195-205.

**Figure 3.2**

*Figure 3.2 Characteristics of porcine embryos at various stages during embryonic development.*

Fertilised porcine embryos develop from a single cell through to the blastocyst stage similarly to the pre-implantation mouse embryos, but porcine embryos will take more time for elongation and increase in their size than does the mouse in correlation with forming embryonic disc during development. *In vivo* porcine blastocysts develop around day 5 or 6, thereafter the spherical conceptus will start hatching from zona pellucida (**Fig. 3.2A**). The embryos then expand and develop themselves and pass through ovoid (**Fig. 3.2B**), tubular (**Fig. 3.2D**), filamentous (**Fig. 3.2E**) and primitive steak periods (**Fig. 3.2F**), respectively, before they begin attaching to the uteri. During all of these stages of porcine embryonic development, epiblasts established in the ovoid blastocysts will give rise to the embryonic disc (ED) (**Fig. 3.2B**) that lies nearby a lineage of trophoblast cells over the hypoblastic area around day 8 or 9 (**Fig. 3.2C**). The primitive streak stage of porcine conceptus will implant to the uteri around day 16 or 17 of pregnancy under the regulation of ontological factors (Murray *et al*., 1971; Hunter 1974; Anderson 1978; Geisert *et al*., 1982a; Geisert *et al*., 1982b; Mattson *et al*., 1990; Rodriguez-Martinez and Persson, 1993; Flechon *et al*., 2004; Vejlsted *et al*., 2006a; Brevini *et al*., 2007b; Blomberg *et al*., 2008a). Abbreviations: ED, Embryonic disc and ICMs, Inner cell masses. ⟶ = embryonic disc, ⋯▶ = trophectoderm, ⟶ = hypoblasts

*Table 3.1 Summary of selected papers describing establishment of porcine ES cells that provides some ideas to create culture conditions used to generate porcine ES-like cells in this present study.*

| Embryonic sources | Isolation methods | Feeders | Culture medium | Maximum Passage number | Undifferentiation evaluation | Differentiation evaluation | References |
|---|---|---|---|---|---|---|---|
| In vivo B d7-9 | Intact or isolated ED | STO | DMEM,10%CS, 5%FCS, 0.1mM β-ME, antibiotics | >50 | Morphology, Vimentin: negative | Morphology, EB, muscle cells, fibroblasts, neurons, endoderm cells | Evans *et al*., 1990 |
| In vivo B d7-8 | Intact or immunosurgery | STO, PEF | DMEM,10%CS, 10%FCS, 0.1mM β-ME | 32 | Morphology, vimentin & cytokeratin18: negative | EB, vimentin, cytokeratin18, no tumors, no chimeras | Piedrahita *et al*., 1990b |
| In vivo B d7-8 | Three step isolation | STO | DMEM with 10%FCS, 0.1mM β-ME, nucleosides*, 1mM L-glutamine or DMEM:M199, 5%CS, 15%FCS in 5%$CO_2$ at 37°C | 80 | Morphology , AP | Muscle cells, neurons, fibroblasts, endoderm cells and adipocytes | Talbot *et al*., 1993b |
| In vivo B d7-9 | Intact or enzyme digestion | MEF | DMEM, 16%FCS, 0.1mM MEM, P/S, 0.1mM β-ME, nucleosides**, 20ng/ml bFGF, 40ng/ml hLIF | 9 | Morphology , AP | EB, spontaneous differentiation e.g. neurons, smooth muscle cells, epithelial cells, trophoblasts | Li *et al*., 2003a |
| IVF | Intact | MEF | DMEM, 16%FCS, 0.1mM MEM, P/S, 0.1mM β-ME, nucleosides**, 20ng/ml bFGF, 40ng/ml hLIF | 4 | Morphology , AP | EB, spontaneous differentiation e.g. neurons, fibroblasts | Li *et al*., 2004a |
| In vivo B d7-9 | Enzyme digestion or immunosurgery | MEF, PEF, STO | DMEM, 16%FCS, 0.1mM MEM, P/S, 0.1mM β-ME, nucleosides**, 20ng/ml bFGF, 40ng/ml hLIF | 9 | Morphology, AP | Spontaneous differentiation e.g. neurons, fibroblasts, smooth muscle cells, epithelial cells | Li *et al*., 2004b |
| PA | Immunosurgery | STO | DMEM:F10, 10%KSR, 5%FCS, 10 ng/ml mLIF | Not shown | Morphology, Oct-4, Nanog, interferon-τ, α-amilase, BMP-4, neurofilaments | Oct-4, Nanog, interferon-τ, α-amilase, BMP-4, neurofilaments, EB, spontaneous differentiation | Brevini *et al*., 2005 |
| In vivo B d6-7 | Microsurgery | MEF | KO-DMEM, 20%KSR, 10ng/ml hLIF, 5ng/ml bFGF in 5%$CO_2$ at 38.5°C | 19 | Morphology, Oct-4, Nanog | Nestin, AFP, stat III, leptin, osteonectin, osteocalcin | Vackova & Madrova, 2006 |
| PA, IVF, NT | Intact or immunosurgery | STO, MEF, PUC, gelatin | DMEM or DMEM:Ham's F10 or DMEM:NCSU-23 with 15%FCS, 2mM L-glutamine, 1%MEM, 1%P/S, 0.1mM β-ME, 10ng/ml hLIF in 5%$CO_2$ at 37°C | 5 | Morphology, AP | EB, spontaneous differentiation | Kim *et al*., 2007 |
| In vivo B d8 | Three step isolation | STO | DMEM with 10%FCS, 0.1mM β-ME, P/S, L-glutamine:M199 with 10%FCS, P/S, L-glutamine in 5%$CO_2$ at 38°C | Not shown | Morphology, Oct-4, Nanog, SOX-2, BMP-4, NOG, LIFR, REX-1, TDGF-1, CDX-2, NCAM | Neurons, trophectoderm, endoderm cells, Oct-4, Nanog, SOX-2, BMP-4, NOG, LIFR, REX-1, TDGF-1, CDX-2, NCAM | Blomberg *et al*., 2008b |

**Table 3.1** summarises factors, which have been suggested to affect the derivation of porcine ES-like cells such as source of embryos, embryonic stage, technique used to isolate required cells, culture conditions, maximum cell passage and parameters used to evaluate self-renewal and pluripotent abilities previously reported and modified to generate pES-like cells in this study. By consideration of the maximum passage number of porcine ES-like cells established together with the evaluation of self-renew and differentiation, it is believed that only porcine ES-like cells obtained from six research groups (Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Vackova & Madrova, 2006; Kim *et al*., 2007) show the reasonable results that their culture conditions could be considered to modify for derivation of porcine ES-like cells in this study. All components that are considered to add into the culture medium in this study are explained in **Section 3.1.3** and they are labelled in three colours. Red is labelled for FCS and KSR, blue is labelled for nucleosides and green is labelled for ontological factors such as bFGF and LIF. *0.01mM guanosine, 0.03mM adenosine, 0.01mM cytidine, 0.01mM uridine and 0.01mM thymidine, **0.03mM guanosine, 0.03mM adenosine, 0.03mM cytidine, 0.03mM uridine and 0.01mM thymidine. Abbreviations: AFP, Alpha fetoprotein; AP, Alkaline phosphatase; B, Blastocysts; bFGF, Basic fibroblast growth factor; β-ME, β-mercaptoethanol; BMP-4, Bone morphogenetic protein 4; CDX-2, Caudal type homeobox transcription factor 2; CS, Calf serum; DMEM, Dulbecco's modified eagle's medium; EB, Embryoid body; ED, Embryonic disc; hLIF, Human leukemia inhibiting factor; IVF, *In vitro* fertilization; LIFR, Leukemia inhibitory factor receptor; MEF, Mouse embryonic fibroblasts; MEM, Non essential amino acids; NOG, Noggin; NCAM, Neural cell adhesion molecule homolog; NCSU-23, North Carolina State University-23 medium; NT, Nuclear transfer; OCT-4, Octamer-binding transcription factor-4; PA, Parthenogenetic activation; P/S, Penicillin/streptomycin; PEF, Porcine embryonic fibroblasts; PUEC, Porcine uterine epithelial cells; REX-1, RNA exonuclease 1 homolog; SOX-2, Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 2; STO, SIM (Sandoz inbred mouse) embryo-derived thioguanine-and ouabain-resistant fibroblast cell line and TDGF-1, Teratocarcinoma-derived growth factor 1.

### 3.1.2 Technique used to isolate ICMs and epiblasts of porcine blastocysts

Following reviews of the literature **Section 1.2.2**, it was decided to isolate the ICMs or epiblasts of *in vivo* porcine pre-implantation embryos by using the most harmless and common methods, either the blastocyst was cultured intact or mechanical dissection techniques were used, to derive immortal porcine ES cell colonies in this study. This method benefits in an avoidance of interactions between the required cells and artificial chemical substances. Even though these two techniques have been reported to produce of some unwanted differentiated cells nearby outgrowths of self-renewal colonies of immortal ES cells (Anderson *et al*., 1994; Talbot *et al*., 1995; Wianny *et al*., 1997; Li *et al*., 2004a; Talbot *et al*., 2007a; Talbot and Blomberg, 2008), they are still popular techniques which have been reported to establish pluripotent ES cell lines in pigs (Piedrahita *et al*., 1988; Evans *et al*., 1990; Piedrahita *et al*., 1990b; Gerfen and Wheeler, 1995; Wianny *et al*., 1997; Chen *et al*., 1999; Miyoshi *et al*., 2000; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Shiue *et al*., 2006; Vackova and Madrova, 2006; Brevini *et al*., 2007b; Kim *et al*., 2007; Xu *et al*., 2007) as seen in **Table 3.1**.

### 3.1.3 Culture conditions used to establish porcine ES cells

To succeed in establishment of porcine ES cells in this study, determination of the optimal culture system is a challenge. By consideration of all known and unknown factors affecting pluripotent ES cells self-renewal and differentiation in mammals proposed in **Section 1.2.3**, it is clear that a feeder culture system is still the most effective procedure to establish immortal ES cells. Although, species-specific feeders may be the best choice to yield ES cell lines, to begin with mouse embryonic feeder fibroblasts are more characterised. This is because a number of supportive reports have shown that MEFs have a potential to produce and sustain stable pluripotent ES cells not only in mice (Evans and Kaufman, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Brook and Gardner, 1997; Wakayama *et al*., 2005; Chung *et al*., 2006; Lee *et al*., 2006b; Shinmen *et al*., 2007; Tesar *et al*., 2007; Wakayama *et al*., 2007a; Wakayama *et al*., 2007b), but also in non-human primate (Thomson *et al*., 1995; Thomson *et al*., 1996; Kuo *et al*., 2003; Mitalipov *et al*., 2003a; Mitalipov *et al*., 2006; Yamashita *et al*., 2006; Byrne *et al*., 2007) and human (Thomson *et al*., 1998; Cheng *et al*., 2003; Agarwal *et al*., 2008; Bigdeli *et al*., 2008). Moreover, it is

well-documented that MEFs could support ICMs to produce a higher percentage of primary ES-like cell colony formation and maintain longer passage numbers than STO and PEFs in pigs resulting from their high number of microtubules and distribution thereby secreting a high ratio of cytokines involving in self-renewal state to the culture medium (Li *et al*., 2004b). Therefore, a number of pES-like cells have been established using MEFs as feeders (Piedrahita *et al*., 1990a; Piedrahita *et al*., 1990b; Gerfen and Wheeler, 1995; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Vackova & Madrova, 2006; Kim *et al*., 2007; Rasmussen *et al*., 2009; Son *et al*., 2009).

However, other factors associated with the derivation of ES cells in feeder-dependent culture system, which cannot be ignored, include the optimal culture conditions such as medium, synthetic growth factors and the culture environments. As discussed in **Section 1.2.3**, the density of MEFs in this study was designed to be lower than other reports in order to aware of metabolic rate differences among mammalian species, and of course, reduce the levels of some obscure factors affecting self-renewal state produced by feeder fibroblast cells. Moreover, to increase some possibilities to establish procine ES-like cells in this study, addition of some extrinsic factors in which they benefit proliferation of ES cells as previously mentioned such as FCS, bFGF and hLIF into the culture medium is quite essential in this present study.

To determine suitable concentrations of those ontological factors to supplement into the culture medium to derive porcine pluripotent ES cells in this study, all factors affecting the establishment of porcine ES-like cells previously published were considered. The criteria used to select certain components to add in culture medium were based on the results of reports of undifferentiated morphology resembling mES cells, culture medium, maximum passage number, and evaluation methods fulfilling the definition of immortal ES cells as much as possible.

To begin with the morphology of undifferentiated immortal mES cells, it is well-established that mES cells have a distinct domed-like colony consisting of small round cells with a high ratio of nucleus-cytoplasm, each mES cell contains one

or two nucleoli as depicted in **Figure 3.3A** (Evans and Kaufman, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Brook and Gardner, 1997; Tesar *et al*., 2007). By consideration of the previous reports of the characteristics of porcine ES-like cells, it shows that some groups have obtained similar morphology of porcine ES-like cells to the mouse, which porcine ES-like cells have a distinct colony composed of a number of small round cells with a high nucleo-cytoplasmic ratio containing at least one nucleolus (**Figure 3.3B**), by using a variety of medium combinations (Piedrahita *et al*., 1990b; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Anderson *et al*., 1994; Wheeler, 1994; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Vackova & Madrova, 2006; Kim *et al*., 2007) as shown some in **Table 3.1**. On the other hand, some research groups have reported that defined colonies of their porcine ES-like cells are flatter and bigger than those previous studies as shown in **Figure 3.3C** (Evans *et al*., 1990; Strojek *et al*., 1990; Gerfen and Wheeler, 1995; Wianny *et al*., 1997; Chen *et al*., 1999; Son *et al*., 2009).

As mentioned above that this study is focused on isolating porcine pluripotent ES cells with characteristics similar to mES cells. Therefore the culture conditions used to derive porcine ES-like cells with a similar appearance of immortal ES cells in mice have been firstly considered. As shown in **Table 3.1**, many previous research groups have generated pES-like cells which resemble the morphology of mES cells, however these cells could not create teratomas or chimeras, which are important in *in vivo* pluripotent evaluation of self-renewing ES cells (Piedrahita *et al*., 1990b; Talbot *et al*., 1993b; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Vackova & Madrova, 2006; Kim *et al*., 2007). Some reports demonstrated that the pES-like cells could be maintained over 30 passages by supplementing FCS in their culture medium in mouse feeder culture system (Piedrahita *et al*., 1990b; Talbot *et al*., 1993b). Although, some research groups (Anderson *et al*., 1994; Wheeler, 1994; Chen *et al*., 1999) have proposed that pES-like cells produced from their experiments are capable to create coat colour chimeras, those pigs show just a few of coated-hair colour areas and also there are no negative controls done such as injection, combining of somatic cells or different levels of multipotent stem cells or progenitors, which these quality controls could be applied to use with formation

of teratomas, so that percentage of chimerism production from those cells reproduced would be considered to compare with the ones created from ES-like cells injected to the host embryos alone. Also, the possibility of cell fusion between ES-like cells and embryonic stage host embryos used for chimera production needs to be considered, especially in cases of a low percentage of chimera contribution. Henceforth, no ideal percentage production of chimeras and teratomas has been considered and reported even in mice (Bradley 1984; Piedrahita *et al*., 1990b; Anderson *et al*., 1994; Wheeler, 1994; Notarianni *et al*., 1997; Chen *et al*., 1999; Keefer *et al*., 2007; Tesar *et al*., 2007; Talbot and Blomberg, 2008). On one hand, there is an attempt to avoid adding FCS, which it is well-known in containing some undefined factors affecting pluripotency of ES cells, to culture medium by adding KSR instead (Vackova & Madrova, 2006). This group also shows that their porcine ES-like cells reach a higher passage number than some other research groups applying FCS to the culture medium when a standard density of mouse feeders is applied to the experiments (Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Kim *et al*., 2007). Additionally, that group also administers 10ng/ml hLIF and 5ng/ml bFGF, which are well-known factors influencing the derivation and maintenance of a pluripotent ES cell state in early (Evans and Kaufman, 1981; Martin 1981; Smith *et al*., 1988; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Yoshida *et al*., 1994; Brook and Gardner, 1997; Niwa *et al*., 1998; Burdon *et al*., 1999a; Matsuda *et al*., 1999; Niwa *et al*., 2000; Nichols *et al*., 2001; Cartwright *et al*., 2005; Li *et al*., 2005; Chung *et al*., 2006;b Lee *et al*., 2006b; Shinmen *et al*., 2007; Tesar *et al*., 2007) and late epiblast ES cell origins (Cheng *et al*., 2003; James *et al*., 2005; Kang *et al*., 2005; Vallier *et al*., 2005; Wang *et al*., 2005a; Xu *et al*., 2005a; Levenstein *et al*., 2006; Brons *et al*., 2007; Greber *et al*., 2007; Tesar *et al*., 2007; Agarwal *et al*., 2008), respectively, in their feeder-dependent culture system in a humidified atmosphere of 5%$CO_2$ in air at 38.5°C possibly in order to elevate some beneficial factors to enhance the chance of immortal ES cell establishment. In this sense, it means that using a very low density of mouse feeders in this study, a lot of unwanted side effects which may have originated from feeders would be alleviated as compared to other previous experimental groups. Therefore, FCS was supplemented to the culture system instead of KSR in this study. However, the concentration that should be used is of importance to

be carefully concerned. Henceforth, some successful generation of pluripotent ES cells in pigs with a reasonable maximum passage obtained by applying FCS to their culture system have been counted to use as a parameter to make a decision in designing dosage of FCS undertaken in this study. Administration of 16%FCS, 10 ng/ml hLIF, 20 ng/ml bFGF and nucleosides has been reported that they are capable to reproduce pluripotent ES cells in pigs nearly 10 passages (Li *et al*., 2003a; Li *et al*., 2004b), which their maximum passages are closed to the group reporting that addition of a compostion of 20%KSR, 10ng/ml hLIF and 5ng/ml bFGF could sustain their self-renewal ES cells for 19 passage (Vackova & Madrova, 2006) as detailed in **Table 3.1**. Although, adding FCS hypothetically benefits in derivation of porcine ES cells in this study, the concentration of hLIF and bFGF decided to supplement in this mouse feeder culture system is needed to be highly aware. Therefore, it was decided to undertake a combined dosage of 10 ng/ml hLIF and 4 or 40 ng/ml bFGF because those concentrations of LIF and bFGF have been demonstrated to support isolation and maintenance in ES cells isolated from early (Evans and Kaufman, 1981; Martin 1981; Smith *et al*., 1988; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Yoshida *et al*., 1994; Brook and Gardner, 1997; Niwa *et al*., 1998; Burdon *et al*., 1999a; Matsuda *et al*., 1999; Niwa *et al*., 2000; Nichols *et al*., 2001; Cartwright *et al*., 2005; Li *et al*., 2005; Chung *et al*., 2006; Shinmen *et al*., 2007; Tesar *et al*., 2007) and late epiblasts (Cheng *et al*., 2003; James *et al*., 2005; Kang *et al*., 2005; Vallier *et al*., 2005; Wang *et al*., 2005a; Xu *et al*., 2005a; Levenstein *et al*., 2006; Brons *et al*., 2007; Greber *et al*., 2007; Tesar *et al*., 2007; Agarwal *et al*., 2008).

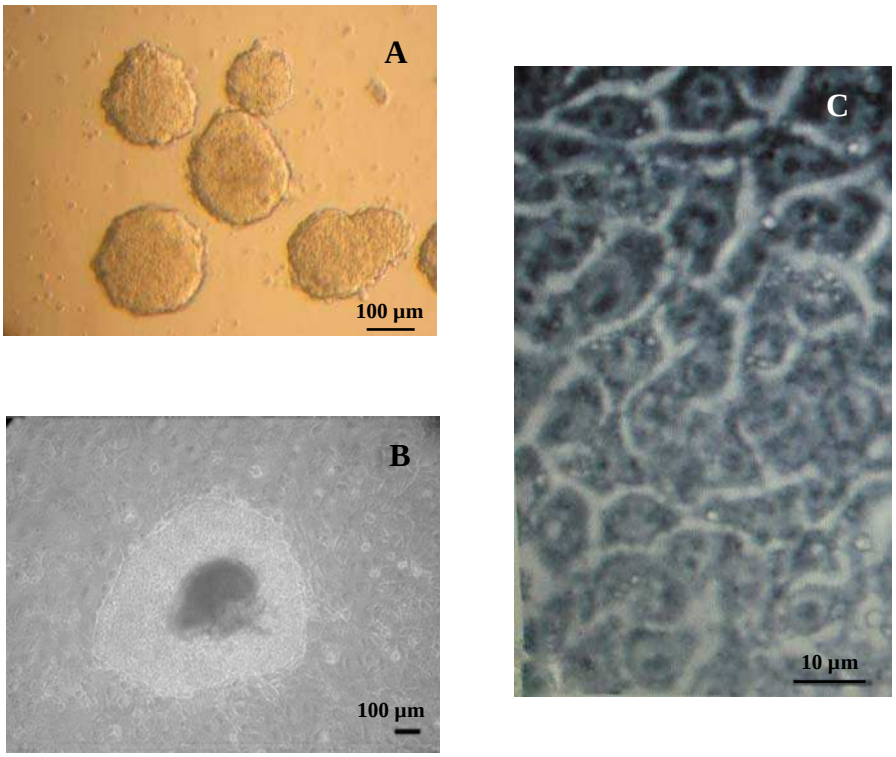



**Fig. 3.3B** was adapted from Kim *et al*., 2007. *Zygote,* 15**,** 55-63.

**Fig. 3.3C** was modified from Wianny *et al*., 1997. *Biol. Reprod.,* 57**,** 756-764.

*Figure 3.3 Morphology of murine and porcine embryonic stem cells.*

**Fig. 3.3A**: murine pluripotent ES cells, CGR8, which were cultured in non-feeder culture system as detailed in **Section 2.7**, showed small round shape with a high ratio of nucleus to cytoplasm. Those immortal ES cells gathered together and formed a domed-like colony as previously reported (Evans and Kaufman, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Brook and Gardner, 1997). In the case of porcine pluripotent ES cells, they have been found either as small round cells (Piedrahita *et al*., 1990b; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Wheeler, 1994; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Vackova & Madrova, 2006; Kim *et al*., 2007) as shown in **Fig. 3.3B**, or large flat cells (Evans *et al*., 1990; Strojek *et al*., 1990; Gerfen and Wheeler, 1995; Wianny *et al*., 1997; Chen *et al*., 1999) containing a high ratio of nucleocytoplasmic content with one or two nucleoli in a defined colony as depicted in **Fig. 3.3C**.

Furthermore, it is well-documented that most basic culture media have provided a variety concentration of nutrients and chemical constituents affecting pH and osmolarity of culture system, therefore, two basic culture media, KO-DMEM and DMEM, were designed to use in this study. Essentially, nucleosides managed to yield porcine ES-like cells prior described (Talbot *et al*., 1993b; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b) potentially influencing on pH and osmolarity of culture conditions, and some intracellular signalling pathways in either pluripotent ES cells or feeder fibroblasts in order to support establishment of self-renewing ES cells were also recruited to supplement into the culture medium in this study. This is because nucleosides, which are composed of some base purines and pyrimidines, and ribose or deoxyribose sugar, are served as subunits of nucleic acid, RNA and DNA when they are phosphorylated by some specific kinases. In addition, they could provide energy for cells in the forms of ATP, GTP, and so on. Also, they would be able to join with other chemical groups to form coenzymes such as coenzyme A, which is essential to metabolism of cells. Moreover, they are served as second messengers of the cells such as cyclic AMP, cyclic GTP and so forth to activate or inactivate cellular responses later on (Alberts *et al*., 2002).

Considering all these factors, *in vivo* derived hatched porcine blastocysts between days 6-8 post-fertilisation were used as starting material. ICMs and epiblasts isolated mechanically, or intact embryos were directly placed onto inactivated mouse feeder fibroblasts, and cultured in either KO4bh or DM40bh for the first series experiments as detailed in **Section 2.4**, or two further media, KO40bh and DM4bh for the second series in a humidified atmosphere of 5%$CO_2$ in air at 39°C.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Porcine embryo collection

As described previously in **Section 2.1-2.5**, briefly, the first group of pregnant PIC Camborough 12 sows provided from J.C. Lister Farms Ltd., North Yorkshire, UK obtained during 31 January-8 June 2007 and the second group received a year later on 10 September 2008 were transferred to the slaughterhouse 6-8 days after insemination. The uteri from the sacrificed pigs were removed and transferred to the lab. Porcine blastocysts were flushed from the uteri with sterile PBS supplemented with 1% BSA and 1% P/S, thereafter embryos were collected and placed into pre-calibrated BECM-3 supplemented with 3% BSA or 10% FCS in a humidified atmosphere of 5%$CO_2$ in air at 39°C. They were then assigned into two groups by determination of their sources of pluripotent ES cell production, ICMs and epiblasts, and they were subsequently divided into two groups, intact blastocysts and either isolated ICMs or epiblasts before those required cells were directly placed into the designed culture medium in mouse feeder culture system in a humidified atmosphere of 5%$CO_2$ in air at 39°C as described in **Figure 2.1**.

### 3.2.2 Cell isolation techniques and feeder-dependent culture system used to derive porcine ES-like cells in this study

Intact porcine blastocysts, isolated ICMs and epiblast cells cultured in the designated medium, KO4bh or DM40bh in the first series and KO40bh or DM4bh in the second series of experiments, were examined and their outgrowth were recorded daily. A 50% media change was carried out everyday, since those cells required were attached on top of the inactivated mitotically mouse feeder layers, until pES-like cells were observed in the culture dishes. Thereafter, those pES-like cells were mechanically passaged every 3-5 days by cutting into 4 pieces before some of those pieces were either placed onto the fresh inactivated MEFs cultured in DM40bh as shown in **Section 2.4**, or used for evaluation of their self-renewal and pluripotent abilities as described in **Chapter 4-5**. The reasons for selecting two novel culture media in the second series of experiments was to determine whether high concentration of bFGF or osmolarity affected the establishment of pES-like cells obtained in the first series of experiments

because the osmolarity of media composed of KO-DMEM and DMEM supplemented with exogenous nucleosides measured between 270-280 and 330-340 mOsm/kg$H_2O$, respectively, before they were applied to culture with the required cells. The inactivated MEFs originally used to culture the required producing ES-like cells of the first and second lots of *in vivo* derived porcine blastocysts in this study were from passage 2 and 4, respectively.

## 3.3 RESULTS AND DISCUSSION

### Derivation of porcine ES-like cells and their general appearance

As shown in **Table 3.2**, it was found that no intact porcine blastocysts attached to MEFs in group 1, however, isolated ICMs of this group could attach on top of the feeders between days 2 and 3 of culture. Nevertheless, no porcine ES-like cells obtained from this group, which it is contrast to previous reports showing that ICMs of *in vivo*, *in vitro* fertilised, parthenogenetic and somatic cell nuclear transfer derived porcine blastocysts isolated by different methods are able to establish immortal ES cells in mouse feeder culture system (Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Kim *et al*., 2007; Vackova and Madrova, 2007; Rasmussen *et al*., 2009; Son *et al*., 2009). Nevertheless, it is not a surprise that there were no porcine blastocysts in group 1 giving any pluripotent ES cells in this study and the isolated ones also tended to attach to the mouse feeders slower than those cells in group 2 (**Table 3.2**). This might be because zona pellucida of *in vivo* derived porcine blastocysts in group 1 was not removed when those embryos were plated in designed culture media KO4bh and DM40bh because it was an aim not to disturb them as much as possible. Therefore, those embryos lost a lot of their energy for overcoming the hatching process due to the high resistance of the zona pellucida (Gonzales and Bavister, 1995; Checiu and Checiu, 1996; Cheon et al., 1999; Montag et al., 2000; Freistedt et al., 2001). It means that removal of zona pellucida of conceptus possibly increases the chance of attachment of the embryo to the mouse feeder fibroblasts or establishment of ES cells by saving their energy for those tasks otherwise it will loose during the unsuccessful hatching process (Jelinkova et al., 2003; Kim *et al*., 2007). Yet, some naturally hatched porcine blastocysts in this group still could not attach on the mouse feeders, they were formed themselves as a huge ball containing a lot

of trophectodermal cells, which contain many lipid droplets (Talbot *et al*., 1993a; Talbot *et al*., 1993b; Talbot *et al*., 2001), floating around in the culture medium until they were discarded on day 7 of culture. As regards to the mechanical isolated ICMs in group 1, they seemed to attach on top of mouse feeders much later than the isolated epiblasts in group 2 possibly because those dissected ICMs were mixed with some trophectoderm cells, so that they were usually floating around together in the culture medium for a few days before attaching to the mouse feeder fibroblasts while those isolated epiblasts slowly sink on top of the feeder layers within a day. Moreover, isolated ICMs were easily dispersed and damaged during manual excision (Anderson *et al*., 1994) meanwhile dissected epiblasts were held tightly together as it has previously been described that the porcine epiblasts have developed strongly complex and tight junctions (Talbot *et al*., 2001) and it was easier to separate epiblasts from trophectoderm than the ICMs by mechanical surgery because the size of embryos establishing epiblastic cells was bigger than the ICMs ones, that is another possible reason why isolated ICMs used in this study did not produce any pluripotent ES-like cells possibly due to unskillful dissection. Furthermore, other previous studies (Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Kim *et al*., 2007; Vackova and Madrova, 2007) have used a higher density of MEFs than in this present study so that higher production of some chemical substances reproduced by mouse feeders (Lim and Bodnar, 2002; Nagano *et al*., 2005; Amano *et al*., 2006; Levenstein *et al*., 2006; Buhr *et al*., 2007; Chin *et al*., 2007; Prowse *et al*., 2007) might help ICMs to attach on top of the feeder fibroblasts quicker than this study thereby generating immortal ES-like cells.

*Table 3.2 Summary of porcine embryonic stem cell-like cell lines isolated from intact blastocysts, isolated ICMs and epiblasts of in vivo derived pig embryos at day 6-8 of gestation cultured in mouse feeder culture system during 2 May-August 2007.*

| Group | Source of cells | Attached to feeder cells | Isolation medium | Culture medium | Number of blastocysts | Lines obtained |
|---|---|---|---|---|---|---|
| 1 | Intact blastocysts | No | KO4bh | DM40bh | 4 | - |
| | | No | DM40bh | DM40bh | 8 | - |
| | Isolated ICMs | Day 2-3 | KO4bh | DM40bh | 4 | - |
| | | Day 2-3 | DM40bh | DM40bh | 8 | - |
| 2 | Intact blastocysts | Day 1 | KO4bh | DM40bh | 10 | 2 (20%) |
| | | Day 1 | DM40bh | DM40bh | 4 | 2 (50%) |
| | Isolated epiblasts | Day 1 | KO4bh | DM40bh | 8 | - |
| | | Day 1 | DM40bh | DM40bh | 8 | 4 (50%) |

This table summarises the results of pES-like cell line establishment. It was found that intact porcine blastocysts in group 1 did not attach to inactivated mouse feeders in any designed medium used to culture them resulting in no pES-like cell lines obtained. Although, isolated ICMs in the same group attached on top of mouse feeder layers during day 2-3 in both original culture medium KO4bh and DM40bh, however pluripotent ES-like cells were isolated. In case of group 2 which the epiblasts were the source of pES-like cell production, it was revealed that hatched blastocysts and isolated epiblasts of this group could attach on mouse feeder cells within 24 hours in both original culture medium. 2 from 10 and 2 from 4 of hatched porcine blastocyts cultured in KO4bh and DM40bh were capable to produce pES-like cells, respectively. For isolated epiblasts of this group, it was shown that 4 primary outgrowth of pES-like cells were observed only cells cultured in DM40bh in mouse feeder culture system. Notably, KO4bh was made of KO-DMEM, 15%FCS, 1x P/S, 1x MEM NEAA, 2 mM L-glutamine, 100 µM ß–ME, 4 ng/ml bFGF and 10 ng/ml hLIF, and DM40bh contained DMEM, 16%FCS, nucleosides [0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine], 1x P/S, 1x MEM NEAA, 100 µM ß–ME, 40 ng/ml bFGF and 10 ng/ml hLIF. Abbreviations: bFGF, Basic fibroblast growth factor; β-ME, β-mercaptoethanol; DMEM, Dulbecco's modified eagle's medium; FCS, Fetal calf serum; hLIF, Human leukemia inhibiting factor; KO-DMEM, Knockout-DMEM; MEM NEAA, Minimum essential medium non essential amino acids and P/S, Penicillin/streptomycin.

Likewise, intact blastocysts, which they already revealed epiblast cells as a source of immortal ES cell production, and isolated epiblasts of group 2 were all attached on mouse feeder fibroblasts within 24 hours after they were managed to the culture system (Talbot *et al*., 1993a; Talbot *et al*., 1993b; Talbot *et al*., 2001). Moreover, it was shown that 20% of porcine ES-like cells were produced from intact blastocysts originally cultured in KO4bh (2/10), 50% of porcine ES-like cells were generated from intact blastocysts (2/4) and isolated epiblasts (4/8) originally cultured in DM40bh.

According to the morphological characteristics of porcine ES-like cells obtained in this present study, two types of pES-like cells were observed in the culture system between days 2 and 18. The first one increased their ES-like cell numbers by spreading an individual small round ES-like cell with a high ratio of nucleus to cytoplasm with one or up to multiple nucleoli referred as pESA-like cells (**Figure 3.4A-B**). The other formed a small round cell gathering together in a distinct domed-like colony with a high nucleocytoplasmic ratio with one or up to multiple nucleoli called pESB-like cells (**Figure 3.4C-D**) as found in mouse ES cells (Evans and Kaufman, 1981; Martin 1981; Piedrahita *et al*., 1990b; Brook and Gardner, 1997; Chung *et al*., 2006; Lee *et al*., 2006b; Shinmen *et al*., 2007; Tesar *et al*., 2007; Huang *et al*., 2008). Although several lines of pES-like cells appeared in the culture conditions of this study at the same time, only one line of pESA-like cells and two lines of pESB–like cells were able to be maintain to evaluate the characteristics of stable immortal ES cells. Notably, two lines of pESA-like cells were only reproduced from hatched blastocysts forming distinct epiblasts and they were more difficult to handle than pESB–like cells in terms of maintaining their self-renewal state and passaging. Interestingly, the primary outgrowth of the two pESA–like cell lines reproduced in this study was not contaminated with any other types of cells when they first appeared on top of the mouse feeder fibroblasts. They just increased their number and changed their shapes if they could not maintain their self-renewal ground state on top of the feeders. Hatched blastocysts acting as a source for generating pluripotent cells was manually excised into 4 pieces when they were passaged to the fresh inactivated mouse feeders every 7-10 days until the pieces

of the embryos had gone. Unluckily, it must be the wrong decision to cut the source producing pluripotent ES cells of pESA-like cells while they were changed into the fresh culture system, thereby losing a great chance to study more on this rare ES-like cell line. On the contrary, the primary outgrowth of each pESB–like cell line was mixed with other types of cells such as epithelial like cells similarly to some prior works (Talbot *et al*., 1995; Wianny *et al*., 1997; Li *et al*., 2004a; Talbot *et al*., 2007a; Talbot and Blomberg, 2008) before those differentiated cells were mechanically removed while those cells were passaged to the new inactivated MEFs every 3-5 days. Interestingly, the embryonic stem cell knife used to passage the pESB-like cells mechanically could pass through the colonies of pESB-like cells easily without doing any huge damage to the cells resulting from those pluripotent ES-like cells adhered loosely together similar to the result of mES cells mechanically passaged in this study. Moreover, pESB-like cells generally started differentiating spontaneously at the edge of the colony to become larger and flatter cells with finally undefined colony while those colonies were completely differentiated, this is similar to mES cells, but in contrast to primate ES and domesticated ungulate ES-like cells, whose spontaneous differentiation tends to appear in the central part of the well-defined colony (Talbot *et al*., 1993a; Talbot *et al*., 1993b; Talbot *et al*., 1995; Thomson *et al*., 1995; Thomson *et al*., 1998; Keefer *et al*., 2007; Talbot and Blomberg, 2008). Remarkably, 2 lines of pESB-like cells handled to evaluate their self-renewing and pluripotent abilities both originated from KO4bh randomly selected by their good morphology of small round cells forming as a distinct domed-like colony. The maximum passage number of MPM8 and MPM28 was 17 and 15, respectively. Their self-renewal state could be maintained in the designed culture conditions approximately 2 months and a half.

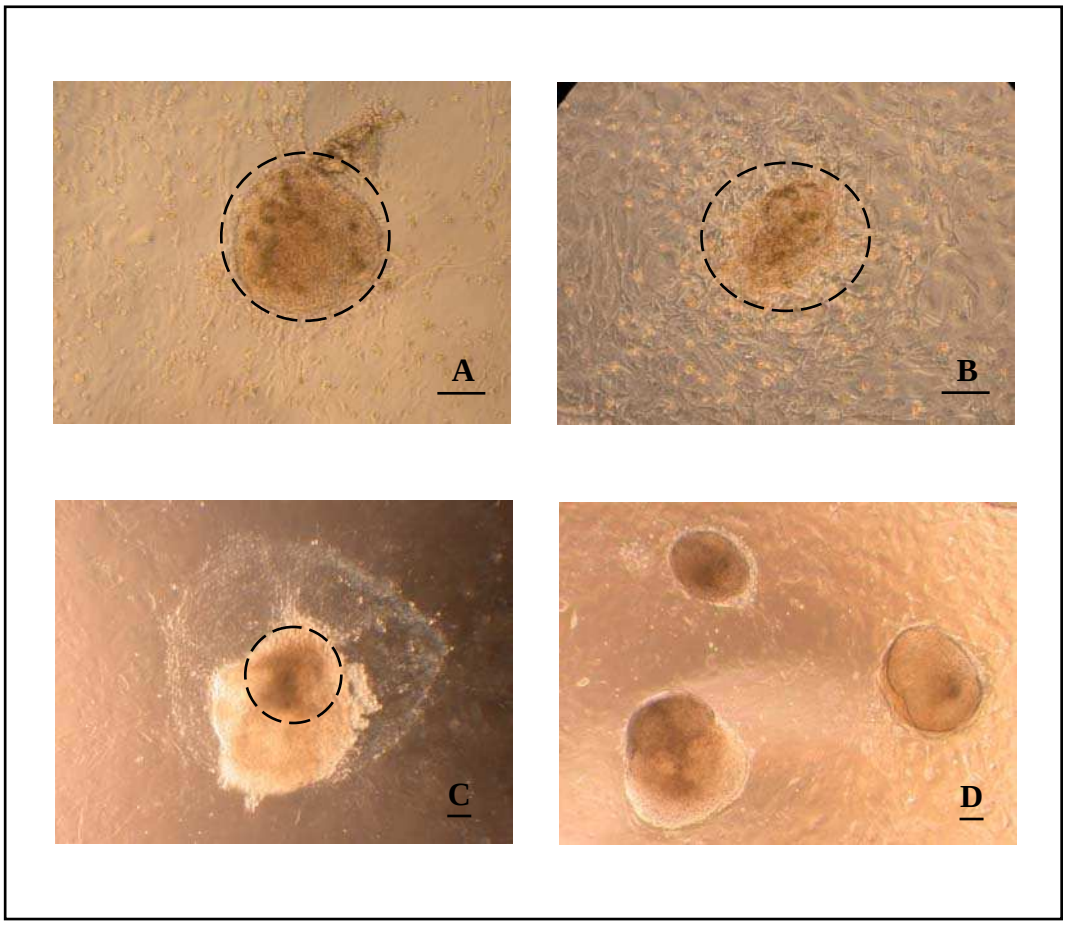



*Figure 3.4 Isolation and outgrowth of porcine ES-like cells cultured in mouse feeder culture system in this study.*

**Fig. 3.4A**: morphology of primary outgrowth of spreading individual small round porcine ES-like cell, pESA-like cells at day 7 of culture, **Fig. 3.4B**: morphology of pESA-like cells at passage 6, **Fig. 3.4C**: morphology of primary outgrowth containing a number of small round porcine ES-like cells in a distinct colony, pESB-like cells at day 5 of culture, **Fig. 3.4D**: morphology of pESB-like cells at passage 5. Scale bar = 50 μm.

◌ = Hatched blastocysts producing pluripotent pES-like cells

Consideration the results of these studies it appears that both KO4bh and DM40bh were suitable media to derive porcine ES-like cells from blastocysts that already revealed epiblasts. DM40bh seemed to have an improved ability to derive pluripotent ES-like cell lines in both intact blastocysts and mechanically isolated epiblast cells of group 2 than KO4bh. One explanation of this may be that DM40bh contained nucleosides that are subunits of nucleic acids, also nucleosides could generate energy for cells, interact with other chemical components to form coenzymes, and act as second messengers in some intracellular transduction pathways in order to affect cellular responsiveness later on (Alberts *et al*., 2002). Hence, supplementation of nucleosides in the medium might be one of key factors to successfully isolate and maintain porcine ES-like cells in this study. DM40bh also had higher concentration of bFGF, previously it was reported that adding high concentrations (100 ng/ml) of this growth factor alone could maintain human ES cells in feeder-free culture system (Xu *et al*., 2005b). Nevertheless, it cannot be concluded that using a high concentration of bFGF is essential to derive or maintain porcine ES-like cells in non-feeder culture system because pES-like cells obtained from this study were cultured on mouse feeder fibroblasts, which are a well-known source producing factors affecting self-renewal and pluripotency of immortal ES cells (Evans and Kaufman, 1981; Martin 1981; Smith *et al*., 1988; Lim and Bodnar, 2002; Nagano *et al*., 2005; Amano *et al*., 2006; Levenstein *et al*., 2006; Buhr *et al*., 2007; Chin *et al*., 2007; Prowse *et al*., 2007). Moreover, bFGF is a well-known factor utilised during development to induce cellular differentiation of three embryonic germ layers (Austin and Burgess, 1991; Austin *et al*., 1992; Kinoshita *et al*., 1995; Rao and Kohtz, 1995; Milasincic *et al*., 1996; Okabe *et al*., 1996; Brustle *et al*., 1999; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Kim *et al*., 2003; Ying *et al*., 2003b; Ying and Smith, 2003; Schmandt *et al*., 2006; Yin *et al*., 2007; Roa *et al*., 2009). However, it is not only the percentage of derivation of pES-like cells isolated here, but also the good quality of pES-like cells is an important consideration. The two lines of pESB-like cells which were maintained to examine their undifferentiated and differentiated characteristics were both produced from KO4bh, not DM40bh, therefore, it may be possible that the osmolarity or the different levels of nutrients and chemical substances of the basic culture medium affects the mechanism of immortal ES-like cell

production in pigs. This is because the osmolarity of KO4bh ranged between 270-280 mOsm/kg$H_2O$, the range of optimal osmolarity for culturing mammalian cells is 260-320 mOsm/kg$H_2O$ (Freshney, 2005), while the osmolarity of DMEM containing nucleosides was 330-340 mOsm/kg$H_2O$. However, most cultured mammalian cells have a variety of osmotic pressure tolerance (Waymouth, 1970), therefore, it cannot be concluded that the osmolarity of DMEM is not suitable to derive pES-like cells as previously mentioned that the most suitable culture conditions for pluripotent ES cells such as pH and osmolarity are still being determined (Ludwig *et al*., 2006) and surely that DM40bh was a culture medium used to maintain pESB-like cells after primary outgrowth of pESB-like cells derived from KO4bh were observed in the culture system because it seemed to be that those outgrowth stopped growing for a few days, this is why they were transferred to culture in DM40bh instead. For the different concentrations of nutrients and chemical constituents provided by basic culture medium, KO-DMEM and DMEM, hypothetically, they should be enough for cells to consume each day because the culture medium was changed half daily. Thus, a variation of nutrients and ions should not have a huge effect on derivation of pES-like cells in this present study. Regarding to the pH of culture medium, it would be possible that a combination of gases and temperature given to grow ES cells in this study was suitable for calibrating the effective pH of culture medium to the immortal porcine ES-like cells when they were kept culturing in the incubator.

Since the number of samples was low, it was difficult to determine whether the different culture conditions used in this primary study were suitable to establish porcine ES cells and whether they were reproducible. Therefore, two further pregnant pigs were ordered to confirm the methods used to produce pES-like cells as previously explained. In this experiment, two more culture media, KO40bh and DM4bh, were designed to use for deriving immortal ES-like cells based on previous data which showed that addition of 4 ng/ml bFGF was able to generate pES-like cells. Additionally, if an outgrowth of pES-like cells was observed in the culture system, it was an aim to passage these pluripotent ES-like cells into fresh medium adding higher concentration of bFGF in the same

basic culture medium, KO-DMEM or DMEM adding extra nucleosides, in order to determine whether the higher dose of bFGF was controlling cellular proliferation of pES-like cells, rather than the effect of different osmolarity of the basic culture medium. As shown in **Table 3.3**, even hatched blastocysts attached on the mouse feeders within one day, but no pES-like cells were observed in any medium. This may be due to obtaining earlier stage of porcine hatched blastocysts to generate pluripotent ES cells because the second series of these two pregnant pigs gave only 3 distinct embryos exhibiting epiblast cells, while the epiblastic area of other *in vivo* derived porcine blastocysts could not be identified so that these porcine blastocysts were directly placed into the culture medium because it was quite difficult to mechanically dissect undefined epiblasts from those embryos and there were a few embryos with distinct epiblastic area. Moreover, the size and total number of cells of those *in vivo* derived porcine blastocysts determined by microscopic examination was smaller and less than the ones which successfully gave rise to ES-like cells previously stated in this study. This observation is related to the explanations of previous reports explaining that a huge variation of embryonic stages of *in vivo* derived porcine blastocyst collection has been observed when uteri of pregnant pigs at the same age of gestation period have been flushed. It indicates that even using the same batch or the same day of *in vivo* derived blastocysts in pigs to generate pluripotent ES cells does not guarantee the successful establishment of pES-like cell lines resulting from an individual response of each conceptus during development to the maternal environment (Talbot *et al*., 2001; Vejlsted *et al*., 2006a; Brevini *et al*., 2007b). Undoubtedly, each embryo might develop a wide range and levels of receptors and intracellular signalling molecules to interact with other external stimuli as seen that *in vivo* derived porcine blastocysts at day 6-7 have a weak signal of gp130 and express receptors of leukemia inhibiting factor, while the isolated epiblasts of *in vivo* derived porcine hatched blastocysts at day 10-11 exhibit receptors of LIF and FGF (Hall *et al*., 2009). This means that those porcine blastocysts which successfully generated pES-like cells in this study had possibly already developed their own receptors and were waiting for stimulation from LIF and bFGF, which are well-known factors regulating self-renewal and differentiation of early (Evans and Kaufman, 1981; Martin 1981; Smith *et al*., 1988; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*.,

1994; Yoshida *et al*., 1994; Brook and Gardner, 1997; Niwa *et al*., 1998; Burdon *et al*., 1999a; Matsuda *et al*., 1999; Niwa *et al*., 2000; Nichols *et al*., 2001; Cartwright *et al*., 2005; Li *et al*., 2005; Chung *et al*., 2006;b Lee *et al*., 2006b; Shinmen *et al*., 2007; Tesar *et al*., 2007) and late epiblast ES cell origins (Cheng *et al*., 2003; James *et al*., 2005; Kang *et al*., 2005; Vallier *et al*., 2005; Wang *et al*., 2005a; Xu *et al*., 2005a; Levenstein *et al*., 2006; Brons *et al*., 2007; Greber *et al*., 2007; Tesar *et al*., 2007; Agarwal *et al*., 2008), respectively, produced by mouse feeder fibroblasts (Evans and Kaufman, 1981; Martin 1981; Smith *et al*., 1988; Lim and Bodnar, 2002; Nagano *et al*., 2005; Amano *et al*., 2006; Levenstein *et al*., 2006; Buhr *et al*., 2007; Chin *et al*., 2007; Prowse *et al*., 2007) or supplemented in the culture medium. Anyway, not only are these factors needed in the culture conditions, but also the suitable levels of those factors controlling self-renew and differentiation are important in establishment of stable porcine pluripotent ES cells as described elsewhere in **Section 1.2.3**. Furthermore, another factor needed to be considered in the failure to derive porcine pluripotent ES-like cell lines in the second series of experiments in this study was the use of different passage of mouse feeder layers to establish the primary outgrowth of pES-like cells, which was the use inactivated MEFs at passage 2 and 4 for the first and second series of experiments, respectively. Possibly, different passages of MEFs secreted different amount of cytokines which were beneficial to derive and maintain immortal ES-like cells in the culture conditions (Smith *et al*., 1998; Li *et al*., 2003a; Li *et al*., 2004b).

Taken together, these observations suggest that different origins of epiblast embryonic stem cells need different factors to generate and maintain their self-renewal state.

*Table 3.3 Attempts to derive porcine embryonic stem cell-like cell lines from intact in vivo pig blastocysts at day 8 of gestation theoretically revealing epiblast cells as a source of isolating embryonic stem cells, which were cultured in mouse feeder culture system during 10-30 September 2008.*

| Source of cells | Attached to feeder cells | Original culture medium | Number of blastocysts | Lines obtained |
|---|---|---|---|---|
| Intact blastocysts | Day 1 | KO4bh | 4 | - |
| | Day 1 | KO40bh | 3 | - |
| | Day 1 | DM4bh | 5 | - |
| | Day 1 | DM40bh | 2 | - |

This table demonstrates the attempts to confirm the methods used to derive pES-like cells previously performed during 2 May-August 2007, it was shown that even those *in vivo* derived porcine hatched blastocysts at day 8, which theoretically exhibit epiblast cells as a production source of immortal ES cells, attached on top of mitotically inactivated mouse feeders in all designed culture medium such as KO4bh, KO40bh, DM4bh and DM40bh within 1 day, but no pluripotent outgrowth were reproduced. Possibly the embryonic stage of porcine embryos obtained in this experiment was earlier than the ones previously used successfully to establish pES-like cells as stated in **Table 3.2** as shown that only 3 hatched porcine blastocysts in this experiment had distinct area of epiblasts. Notably, KO4bh contained KO-DMEM, 15%FCS , 1x P/S, 1x MEM NEAA, 2 mM L-glutamine, 100 µM ß–ME, 4 ng/ml bFGF and 10 ng/ml hLIF, KO40bh was composed of KO-DMEM, 15%FCS , 1x P/S, 1x MEM NEAA, 2 mM L-glutamine, 100 µM ß–ME, 40 ng/ml bFGF and 10 ng/ml hLIF, DM4bh consisted of DMEM, 16%FCS, nucleosides [0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine], 1x P/S, 1x MEM NEAA, 100 µM ß–ME, 4 ng/ml bFGF and 10 ng/ml hLIF, and DM40bh was made of DMEM, 16%FCS, nucleosides [0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine], 1x P/S, 1x MEM NEAA, 100 µM ß–ME, 40 ng/ml bFGF and 10 ng/ml hLIF. Abbreviations: bFGF, Basic fibroblast growth factor; β-ME, β-mercaptoethanol; DMEM, Dulbecco's modified eagle's medium; FCS, Fetal calf serum; hLIF, Human leukemia inhibiting factor; KO-DMEM, Knockout-DMEM; MEM NEAA, Minimum essential medium non essential amino acids and P/S, Penicillin/streptomycin.

# CHAPTER 4

# *IN VITRO* SELF-RENEWAL EVALUATION METHODS USED TO CONFIRM PORCINE EMBRYONIC STEM CELL-LIKE CELLS

## 4.1 INTRODUCTION

To confirm that pES-like cells isolated in this study have the potential to become stable ES cell lines, their self-renewal and pluripotent abilities in which covering all the definitions of ES cells both *in vitro* and *in vivo* evaluations previously described in **Chapter 1** have to be tested. However, it was not possible to examine their *in vivo* pluripotency, such as making teratomas and chimeras, in this present study due to the time and expenses required. Thus, *in vitro* evaluation was the only remaining choice for the investigation of the self-renew and pluripotency of the pES-like cells obtained in this study. Along with the morphological characterisation of undifferentiated (**Chapter 4)** and induced differentiated cells further presented in **Chapter 5**, staining and determination of specific self-renewing (**Chapter 4)** and differentiating markers (**Chapter 5)** were also performed to test the *in vitro* abilities of pES-like cells in this study.

To begin with the evaluation of self-renewing characteristics, it is generally accepted that staining of alkaline phosphatase is the principal method used to test the outgrowth of immortal ES and ES-like cells across the mammalian species (Pease *et al*., 1990; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Iannaccone *et al*., 1994; Talbot *et al*., 1995; Thomson *et al*., 1995; Moore and Piedrahita, 1996; Thomson *et al*., 1996; Thomson *et al*., 1998; Chen *et al*., 1999; Suemori *et al*., 2001; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Wang *et al*., 2005b; Yadav *et al*, 2005; Dattena *et al*., 2006; Fang *et al*., 2006; Hatoya *et al*., 2006; Shiue *et al*., 2006; Takahashi and Yamanaka, 2006; Brevini *et al*., 2007b; Brons *et al*., 2007; Kim *et al*., 2007; Verma *et al*., 2007; Zhu *et al*., 2007; Hayes *et al*., 2008; O'Conner *et al*., 2008; Son *et al*., 2009) because this enzymatic activity disappears during immortal ES and ES-like cell differentiation (Pease *et al*., 1990; Talbot *et al*., 1993a) with the exception of the mouse and rat EpiSC cells

stained negative with AP enzyme (Brons *et al*., 2007; Tesar *et al*., 2007). Likewise, some well-known specific self-renewal markers such as OCT-4 and Nanog are basically undertaken to stain with immortal ES and ES-like cells among mammals in association with the determination of their gene productions (Nichols *et al*., 1998; Chambers *et al*., 2003; Mitalipov *et al*., 2003a; Mitsui *et al*., 2003; Brevini *et al*., 2005; Wang *et al*., 2005b; Yadav *et al*, 2005; Fang *et al*., 2006; Hatoya *et al*., 2006; Takahashi and Yamanaka, 2006; Vackova and Madrova, 2006; Brons *et al*., 2007; Byrne *et al*., 2007; Chambers *et al*., 2007; Tesar *et al*., 2007; Verma *et al*., 2007; Blomberg *et al*., 2008b; Buehr *et al*., 2008; Hall, 2008; Hayes *et al*., 2008; O'Conner *et al*., 2008; Rasmussen *et al*., 2009; Son *et al*., 2009). Therefore, immunofluorescence staining of OCT-4 and Nanog proteins was investigated in this study in correlation with the examination of a pile set of acceptable self-renewing genes such as OCT-4, Nanog, SOX-2, REX-1 and developmental pluripotency associated-3 (DPPA-3, also known as Stella) (**Section 1.2.3**). In addition to identify in which epiblast ES cell origin pESB-like cells obtained in this study belonged to, REX-1 and CDX-2 were used as pivotal reference genes because REX-1, one of specific markers for germ cells, is revealed only in the murine pluripotent ES cells previously stated as early epiblast ES cell origin, not in hES and mEpiSC cells, which are known as late epiblast ES cells. In consideration of a specific marker for trophoblast lineage CDX-2, it is well-established that mES cells do not express this gene; hence they could not create trophectoderm, where CDX-2 is mainly found (Niwa *et al*., 2005; Strumpf *et al*., 2005; Deb *et al*., 2006; Brons *et al*., 2007; Tesar *et al*., 2007) unless certain trophoblastic transcriptional regulators such as CDX-2 or knock down of some undifferentiated genes such as OCT-4 and Nanog are introduced to mouse pluripotent ES cells (Niwa *et al*., 2000; Velkey and O'Shea, 2003; Hay *et al*., 2004; Strumpf *et al*., 2005; Hough *et al*., 2006; Tolkunova *et al*., 2006). In the meantime, hES cells could spontaneously differentiate to be trophectoderm cells (Thomson *et al*., 1998) and definitely either supplement trophoblastic inducing factors such as BMP-4 in the culture medium without the presence of bFGF, or silencing some self-renewing genes, as previously claimed in the mouse, could conduct trophoblastic differentiation in the human ES cells (Xu *et al*., 2002; Hay *et al*., 2004; Hyslop *et al*., 2005; Xu *et al*., 2005b; Schulz *et al*., 2008).

## 4.2 MATERIALS AND METHODS

### 4.2.1 Alkaline phosphatase staining of porcine ES-like cells

As explained in **Section 2.9.1**, AP staining was applied to stain the porcine ES-like cells potentially defined as stable porcine ES cell lines, such as MPM3, MPM8 and MPM28, since their primary colony outgrowths had been observed in this study. Results were recorded every time and photos were taken every 5 passages.

### 4.2.2 Double immunofluorescence staining of OCT-4 and Nanog with pESB-like cells

MPM8, MPM28 and mES cells were stained with OCT-4 and Nanog antibodies using the double immunofluorescence staining technique as described in **Section 2.9.2.1**. Results were recorded and photos were captured each time.

### 4.2.3 Examination of self-renewing genes in pESB-like cells by RT-PCR technique

To identify exactly which origin of epiblast ES cells porcine ES-like cells isolated in this study belong to, gene expression of mES cells referenced as a positive control of early epiblast ES cells was managed to compare to the results of porcine ES-like cells in this study. Another positive control used in this study was porcine blastocysts due to their outstanding expression of all required pluripotent gene markers (Lee *et al*., 2006a). The mouse blastocysts could not be collected from the pregnant mice (**Appendix E**). Moreover, MEFs were used as one of negative controls of pluripotent genes, and their results were managed to compare with the self-renewing gene expression of pES-like cells because those pES-like cells were cultured in mouse feeder system. In addition, PEFs were another negative control used in this study in order to confirm that differentiated pig cells themselves did not reveal any acceptable pluripotent genes. No addition of reverse transcriptase enzyme in RT reactions of each sample was manipulated as a negative control of the sample. β-actin, a housekeeping gene, was first used to test cDNA of each sample before those samples were used to investigate the required self-renewal genes and certainly it was reserved as one of positive controls of each sample. Self-renewal genes such as OCT-4, Nanog,

SOX-2, REX-1, DPPA-3 and CDX-2 were used to test with pESB-like cells, MPM8 and MPM28, obtained in this study.

Regarding the total RNA extraction, RNA of required samples were extracted following the description in **Section 2.10.1**, and examination of gene expression using RT-PCR technique was done following the methods in **Section 2.10.2**.

## 4.3 RESULTS AND DISCUSSION

### *In Vitro* undifferentiated characteristics of porcine ES-like cells in this study

To evaluate *in vitro* self-renewal characteristics of pES-like cells isolated in this study, staining of those pluripotent ES cells with specific markers such as AP, OCT-4 and Nanog, and examination of designed self-renewing genes was undertaken. However, it was unfortunate that only morphology and alkaline phosphatase staining results were successfully made to evaluate self-renewal characteristics of pESA-like cells generated in this study as they were easily differentiated to be other types of cells and it was extremely difficult to identify in which germ layer those somatic cells belonged when the cells were cultured on mouse feeder layers due to an individual disperse style of pESA-like cells. Therefore, most data of undifferentiated properties of pES-like cells reported in this study were generously provided by pESB-like cells, MPM8 and MPM28 at different passages. To begin with the evaluation of AP staining, it was found that AP stained positive with all huge hatched blastocysts producing pESA-like cells. AP even showed either negative or positive staining with the individual small round pESA-like cell as shown in **Figure 4.1A** and **Figure 4.1B**, respectively. On one hand, self-renewal pESB–like colonies of MPM8 and MPM28 were one hundred percent dyed positive with AP (**Figure 4.1C-D**) throughout this study up to the maximum passage of 17 and 15, respectively, before some of those cells were kept frozen in liquid nitrogen for further study. The positive AP staining results of pES-like cells obtained in this study are related to some previous works claiming that their porcine ES-like cells, which usually form a monolayer-like area containing either small round cells or large flat cells with a high ratio of nucleus to cytoplasm, were stained positive with AP enzyme (Talbot *et al*., 1993a; Talbot *et al*., 1993b; Moore and Piedrahita, 1996;

Chen *et al*., 1999; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2007b; Kim *et al*., 2007). These results indicate that pES-like cells obtained in this study have one of basic undifferentiated characteristics of true immortal ES cells. This conclusion led to another step, which was the further testing of pES-like cells with some more specific self-renewal proteins, OCT-4 and Nanog. As regards to the double immunofluorescence staining of OCT-4 and Nanog markers, it was found that PEFs, one type of differentiated cells served as a negative control of pig tissues, and MEFs, another somatic cells type referenced as feeder fibroblasts supporting pES-like cell derivation and a negative control of mouse cells, did not express OCT-4 and Nanog proteins, which was anticipated to be due to their cellular maturity and their not being types of somatic cells, such as trophectoderm, neurons and etc. that potentially produce some certain acceptable self-renewal proteins (Hart *et al*., 2004; Kirchhof *et al*., 2000; Blomberg *et al*., 2008b), as demonstrated in **Figure 4.2B-C and 4.2E-F**, respectively. Moreover, it was shown that OCT-4 and Nanog proteins had not been revealed in any passages of pESB–like colonies, MPM8 and MPM28, throughout this study as sampled in **Figure 4.2E-F**. It may be that the compact domed-like colony of porcine ES-like cells were not spread out individually or their nuclei had not been attached on the glass slide before staining so that the complex or gap junction formation of ES or ES-like cells still tightly remained (Talbot *et al*., 2001; Todorova *et al*., 2008) thereby decreasing the chance of desirable antibodies to stain with the nucleus of their cells, while previous studies normally spread only nuclei of their ES cells or ES-like cells on the slide first when wishing to proceed with OCT-4 and Nanog staining (Mermoud *et al*., 1999; Chambers *et al*., 2007; Silva & Smith, 2008). Even pESB-like cells were not stained positive with OCT-4 and Nanog antibodies as described above, but their abilities could still be proved by investigating some more well-known undifferentiated genes and their capability of differentiation in order to ensure whether pES-like cells obtained in this study had the potential to be stable ES cell lines or not.

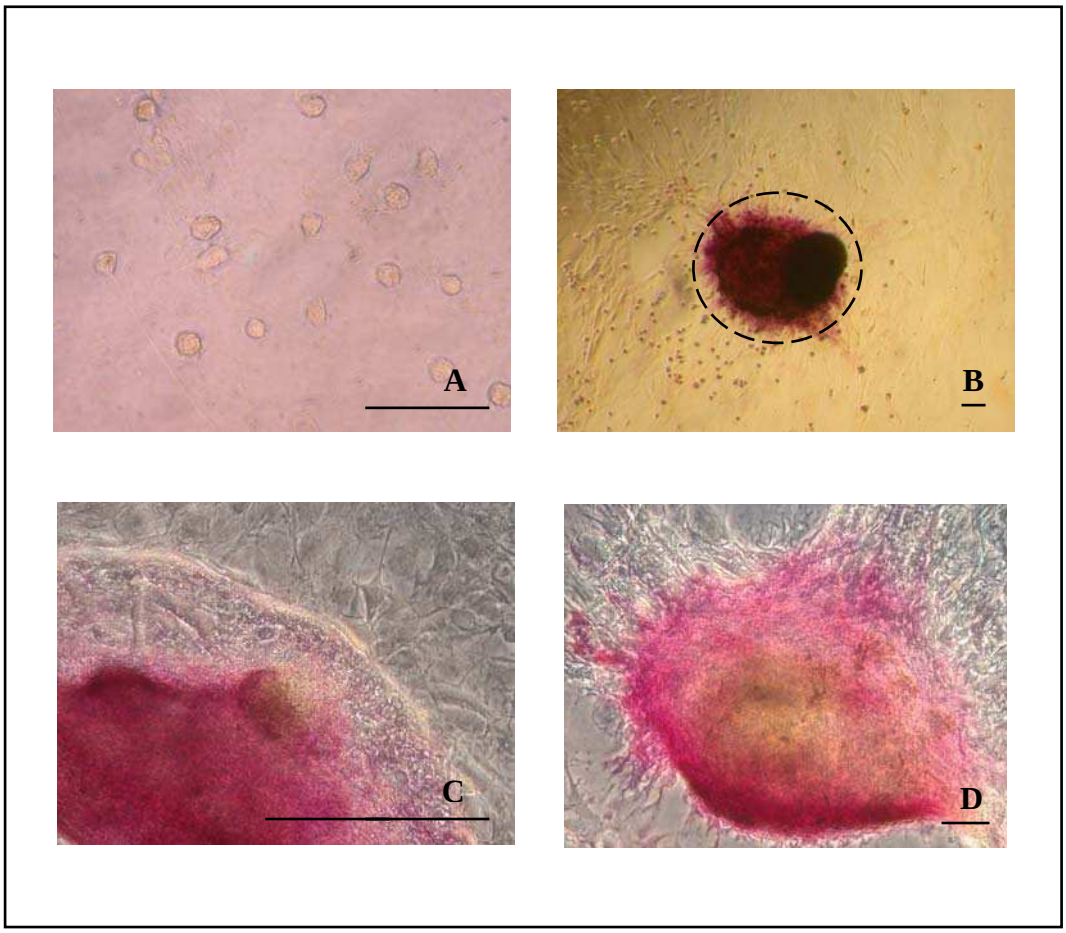



*Figure 4.1 Evaluation of in vitro self-renewal state of pES-like cells cultured in mouse feeder culture system in this study.*

**Fig. 4.1A-B**: AP stained positive with all hatched blastocysts generating pESA-like cells, either pESA-like cells themselves were stained negative (**Fig. 4.1A**) or positive (**Fig. 4.1B**) with their primary outgrowth, and **Fig. 4.1C-D**: AP revealed positive with those pESB-like cells at passage 5 and 8, respectively. Scale bar = 50 µm. A photograph of **Fig. 4.1B** was provided by Mrs. Patricia Fisher, a senior experimental officer, Stem Cell Laboratory, Division of Animal Sciences, School of Biosciences, the University of Nottingham, UK. Staining colour results of AP: pink = positive and grey or brown = negative. Abbreviation: AP, Alkaline phosphatase.

= Hatched blastocysts producing pluripotent pES-like cells

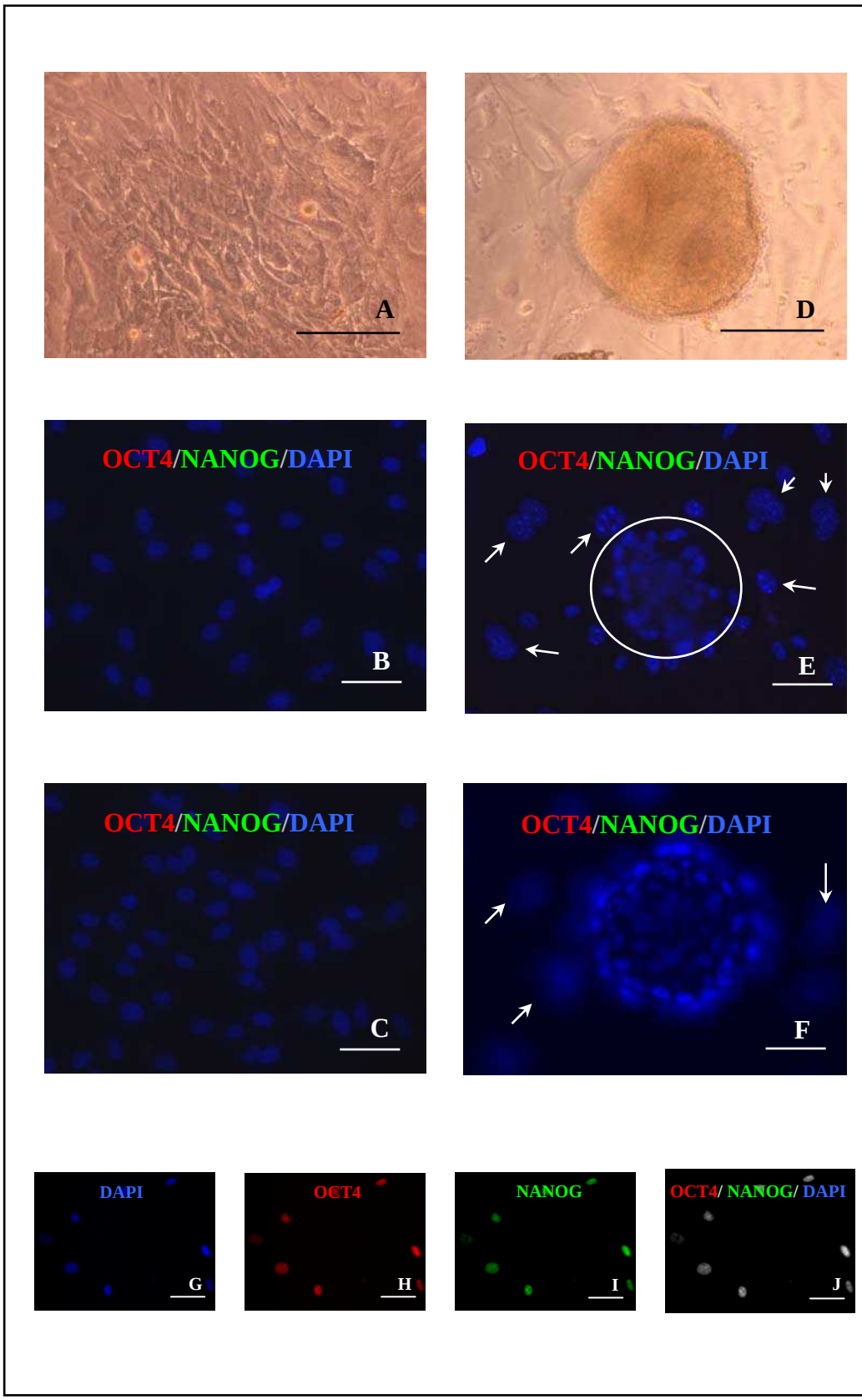

A
D
OCT4/NANOG/DAPI
B
OCT4/NANOG/DAPI
E
OCT4/NANOG/DAPI
C
OCT4/NANOG/DAPI
F
DAPI
G
OCT4
H
NANOG
I
OCT4/ NANOG/ DAPI
J

**Figure 4.2**

*Figure 4.2 Immunofluorescence staining of pESB-like cells MPM8 at passage 15 cultured on mouse feeder fibroblasts with OCT-4 and Nanog antibodies.*

**Fig. 4.2A**: morphology of PEFs at passage 4, **Fig. 4.2B**: negative control of double staining of OCT-4 and Nanog of PEFs at passage 4 was shown negative, **Fig. 4.2C**: double staining of OCT-4 and Nanog of PEFs at passage 4 revealed that PEFs did not generate OCT-4 and Nanog proteins, **Fig. 4.2D**: morphology of pESB-like cells cultured in mouse feeder culture system, **Fig 4.2E**: negative control of OCT-4 and Nanog staining of MPM28 and MEFs showed that these cells were stained negative, **Fig. 4.2F**: double immunofluorescene staining of OCT-4 and Nanog of MPM28 demonstrated that MPM28 cultured on mouse feeders at this passage did not produce OCT-4 and Nanog proteins, and **Fig. 4.2G-J**: double staining of OCT-4 and Nanog of mES cells at passage 16; **Fig. 4.2G** showed staining of DAPI with mES cells, **Fig. 4.2H** revealed that OCT-4 was stained positive with the nucleus of each mES cell, **Fig. 4.2I** demonstrated that Nanog was also stained positive with the nucleus of each mES cell, and **Fig. 4.2J** showed the overall colour of OCT-4, Nanog and DAPI staining with the nucleus. Staining colours of positive results: blue with DAPI, red with OCT-4 and green with Nanog. Scale bar = 50 μm. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; OCT-4, Octamer-binding transcription factor-4; mES; Mouse embryonic stem and PEFs, Porcine embryonic fibroblasts. → = MEFs, and ◯ = area of pESB-like colony

Thus, considering the investigation of some designed self-renewing genes of pESB-like cells shown in **Figure 4.3** was an important issue. This figure revealed that some specific outstanding undifferentiated genes such as OCT-4, Nanog, SOX-2 and REX-1 were determined in mES cells maintained in non-feeder culture system in this study, similar to other reports (Okamoto *et al*., 1990; Rosner *et al*., 1990; Scholer *et al*., 1990; Chambers *et al*., 2003; Mitsui *et al*., 2003; Okumura-Nakanishi *et al*, 2005; Takahashi and Yamanaka, 2006; Chambers *et al*., 2007; Masui *et al*., 2007; Tesar *et al*., 2007; Ying *et al*., 2008). Moreover, those four genes were also detected in both lines of MPM8 and MPM28 generated in this present study until passage 15 and 8, respectively, which the results are inconsistent with previous works (Brevini *et al*., 2005; Blomberg *et al*., 2008b; Hall, 2008; Rasmussen *et al*., 2009). However, the self-renewing genes examined are reported as positive or negative depending on the passage of their ES-like cells. Previous data states that OCT-4 disappeared from their porcine parthenogenetic embryos deriving pluripotent ES-like cells since passage 5, but Nanog remained (Brevini *et al*., 2005). The contrasting results may possibly be attributed to different sources reproducing porcine embryos being used to establish ES-like cells and the more advanced in cellular differentiation relating to the size and shape of pES-like cells being obtained. In the first place, pESB-like cells yielded in this study were derived from *in vivo* porcine blastocysts, which are the best choice among embryos producing pluripotent ES cells resulting in a higher percentage of ES cell production being found, especially when natural ones are compared to parthenogenetic embryos (Bavister, 2004). This is because embryos derived from parthenogenetic activation have very high rate of abnormalities in polyploidy, apoptotic index and controlling functions of insulin growth factor (Newman-Smith and Werb, 1995; De La Fuente and King, 1998; Hao *et al*., 2004), so they behave in a more erratic way than *in vivo* ones. Secondly, the bigger and flatter the pluripotent ES cells become, the more advanced in differentiation they would be, as it was noticed that pES-like cells generated in this study were smaller than those reported in some prior works (Evans *et al*., 1990; Strojek *et al*., 1990; Gerfen and Wheeler, 1995; Wianny *et al*., 1997; Chen *et al*., 1999) in correspondence with the general appearance of adult somatic cells such as PEFs and MEFs that are larger and flatter than immortal ES and ES-like cells as depicted in **Figure**

**4.1-4.2**. In Addition, one type, pESB-like cells, kept forming a compact dome-like colony similar to the mouse pluripotent ES cells (Evans and Kaufman, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994; Kawase *et al*., 1994; Brook and Gardner, 1997; Tesar *et al*., 2007), while other studies demonstrated only a flattened shape of their pES-like cells (Evans *et al*., 1990; Piedrahita *et al*., 1990b; Strojek *et al*., 1990; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Anderson *et al*., 1994; Wheeler, 1994; Gerfen and Wheeler, 1995; Wianny *et al*., 1997; Chen *et al*., 1999; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Vackova & Madrova, 2006; Kim *et al*., 2007). Hence, it is possible that the porcine ES-like cells previously reported have more advanced differentiation in terms of their morphology and inherited intrinsic factors, as described in **Section 1.2.3**, than pES-like cells established in this study so that the initial expanding in size and flattened colony of pES-like cells probably could be used as basic monitoring differentiated cells. This presumably results from the complex or gap junctions or the communication between cells via those junctional areas having been disturbed by other chemical substances, thereby losing the cellular attachment of one to another or disconnecting signals among cells, respectively. This is because when pESB-like cells cannot confine themselves in a three dimensional ball-like colony, their own cells begin expanding in size, starting at the rim of the distinct colony, with the consequence of the occurrence of complete differentiation of other types of cells observed in the culture medium. That is the reason why the initiation of cell expansion and flattened-like colony are proposed as being among the differentiation markers in this study. According to the expression of Nanog in pES-like cells reported by Brevini group (2005), even Nanog is detectable in their porcine ES-like cells, but it does not mean that those ES-like cells could be clearly claimed as true pluripotent ES cell lines as certain differentiated cells such as neurons are able to produce Nanog, and another self-renewal factor, SOX-2, but the levels of certain self-renewing genes are quite different among cell types (Talbot *et al*., 2002; Blomberg *et al*., 2008b; Kim *et al*., 2008b). Another group reported that the primary colony outgrowth of their pluripotent ES-like cells generated from *in vivo* derived porcine blastocysts at day 5 of gestation by using intact blastocysts or immunosurgery procedure to isolate ICMs could exhibit OCT-4, Nanog and SOX-2, but the passages were generally either negative or positive with those

self-renewing genes particularly (Rasmussen *et al*., 2009), these data exactly indicate precisely that the most important thing in derivation of mammalian ES and ES-like cells is suitable culture conditions, not the source of embryos or technique used to isolate the source of ES or ES-like cell production, as previously described in **Section 1.2.3**. The result is that the undifferentiated markers could hypothetically be determined infinitely. However, to have all the best parameters to derive and maintain pluripotent ES cells would be excellent in terms of enhancing the percentage of ES cell line production. Besides the investigation of OCT-4, Nanog and SOX-2 expression stated above, determination of a pile set of other well-known self-renew genes is also needed to evaluate ES cell characteristics in order to ensure that pluripotent ES-like cells obtained in this study are potentially or truly stable ES cell lines. To investigate the expression of DPPA-3, one of specific genes found in germ cell lineage, this gene was examined in MPM8 at passage 15, whereas MPM28 at passage 8 did not express it. Moreover, neither pESB-like cell lines exhibited a trophectoderm specific gene CDX-2, corresponding to previously reports that neither mES cells express this trophectoderm specific marker (Niwa *et al*., 2005; Deb *et al*., 2006; Brons *et al*., 2007; Tesar *et al*., 2007). Therefore, it may be that pESB-like cells obtained in this study originated from early epiblast embryonic stem cell origin the same as mES cells. However, it was an interesting finding that SOX-2, one of the core transcriptional factors controlling pluripotent ES self-renewal state, was also detected in both MEFs and PEFs as previous works have shown that undifferentiated markers are not only revealed in undifferentiated cells, but also in other differentiated ones. For instance, REX-1 is detected in primitive endoderm and lung (Talbot *et al*., 2007a; Blomberg *et al*., 2008b); Nanog could be determined in neurons, brain, lung, liver and etc. (Hart *et al*., 2004; Blomberg *et al*., 2008b); DPPA-3 has been found in heart, lung, liver, kidney, spleen, germ cells and TE (Lee *et al*., 2006a; Brons *et al*., 2007; Tesar *et al*., 2007); SOX-2 is detectable in skeletal myocytes, astrocytes, brain and lung (Talbot *et al*., 2002; Blomberg *et al*., 2008b; Kim *et al*., 2008b) and OCT-4 could be examined in trophectoderm cells (Kirchhof *et al*., 2000; Kuijk *et al*., 2008). Therefore, by taking into consideration the most important characteristics of stem cells self-renewal, which consist of an asymmetric mitotically cell divisional style that gives one daughter cell with the ability of differentiation, with the other acting as

the unlimited parental stem cells, and a symmetrical division that provides only immortal stem cells (Watt and Hogan, 2000; Melton and Cowen, 2006), it is possible that all acceptable self-renew markers currently used to evaluate embryonic stem cells originate from differentiated cells that function by regulating the homeostasis between self-renewing and differentiation statuses. In any case, it is not normal to find SOX-2 in PEFs and MEFs because they are not differentiated cell types hypothetically reproducing this type of undifferentiated gene. Therefore, this means that these porcine and mouse feeder fibroblasts are presumably contaminated with certain visceral differentiated cells, which SOX-2 has been reported (Talbot *et al*., 2002; Blomberg *et al*., 2008b; Kim *et al*., 2008b) during primary culture of porcine and murine feeder fibroblasts. Or, another explanation is that the PCR primers were not sufficiently specific to SOX-2 resulting in amplification of other homologous transcripts. These are why SOX-2 had been investigated in those two differentiated feeder layers.

Altogether, these results indicate that pES-like cells generated in this study have a basic capability of some *in vitro* self-renewal characteristics.

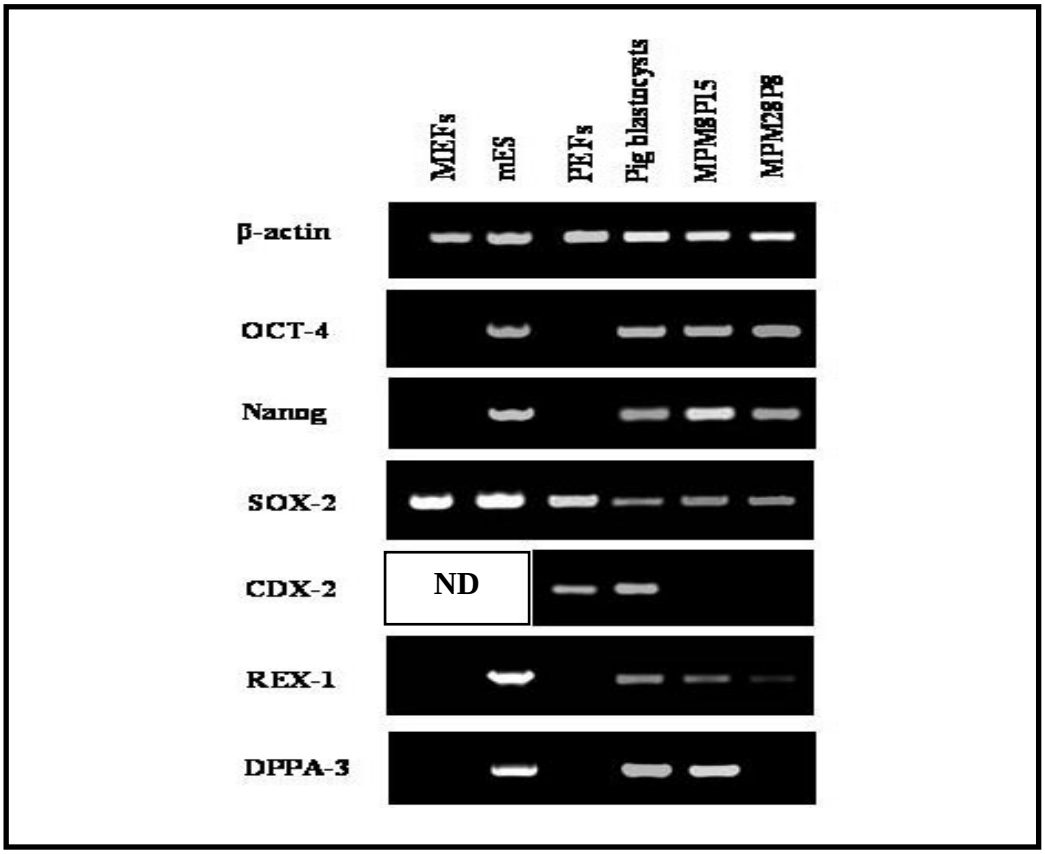



*Figure 4.3 Determination of self-renewal gene expression in pESB-like cells, MPM8 at passage 15 and MPM28 at passage 8 by using RT-PCR technique.*

β-actin was used as house-keeping genes of mouse and porcine cells. A set of mouse self-renewing genes such as OCT-4, Nanog, SOX-2, REX-1 and DPPA-3 obtained from mES cells were represented as a control of early epiblast ES cells when they were compared to the results of those genes produced from MPM8 and MPM28. Parthenogenetic porcine blastocysts were used as a positive control of those self-renewing genes stated above. It was shown that OCT-4, Nanog, SOX-2 and REX-1 were found in mES cells, as previously reported (Okamoto *et al*., 1990; Rosner *et al*., 1990; Scholer *et al*., 1990; Chambers *et al*., 2003; Mitsui *et al*., 2003; Okumura-Nakanishi *et al*, 2005; Takahashi and Yamanaka, 2006; Chambers *et al*., 2007; Masui *et al*., 2007; Tesar *et al*., 2007; Ying *et al*., 2008). In addition, the four self-renewing genes were also provided by both pESB-like cell lines, which relates to some previous works (Brevini *et al*., 2005; Blomberg *et al*., 2008b; Hall, 2008; Rasmussen *et al*., 2009). Moreover, DPPA-3, one of self-renewing gene found in germ cells, was determined in mES cells and MPM8P15, while it was not detected in MPM28P8. Furthermore, the two lines of pESB-like cells did not yield a specific trophectoderm lineage CDX-2. To sum up, these data presumably indicate that pESB-like cells originate from early epiblast ES cell origin, which is similar to mES cells. Abbreviations: CDX-2, Caudal type homeobox transcription factor 2; DPPA-3, Developmental pluripotency associated-3; MEFs, Mouse embryonic fibroblasts; mES, mouse embryonic stem; MPM8P15, pESB-like cells named MPM8 at passage 15; MPM28P8, pESB-like cells named MPM28 at passage 8; ND, Not detected; OCT-4, Octamer-binding transcription factor-4; PEFs, Porcine embryonic fibroblasts; REX-1, RNA exonuclease 1 homolog and SOX-2, Sex determining region Y (SRY)-related high-mobility-group (HMG) box-containing gene 2.

# CHAPTER 5

# *IN VITRO* PLURIPOTENT EVALUATION METHODS USED TO CONFIRM PORCINE EMBRYONIC STEM CELL-LIKE CELLS

## 5.1 INTRODUCTION

To determine the pluripotent ability of immortal ES and ES-like cells among mammals, embryoid body formation is normally undertaken, and then it is followed by either examination of self-renewing and/or differentiating genes, or generation of required differentiated cells using EBs as a source, producing differentiated cells under a variety of culture conditions in order to confirm that those ES and ES-like cells established are able to differentiate to be any types of cells in the body (**Table 5.1**).

As pESB-like cells isolated in this study could not form EBs or be passaged using trypsin digestion (**Appendix D**) possibly due to the formation of complex junctions among those ES-like cells as proposed in the epiblasts (Talbot *et al*., 2001; Talbot and Blomberg, 2008), induction of representative cells of three embryonic germ layers therefore proceeded directly from their own pluripotent ES colonies. One research group states that hES colony could be used as a source of differentiated cell production (Yao *et al*., 2006) in association with the preliminary results of inductive differentiation of mES cells demonstrated in **Appendix C**, indicating that both mEBs and mES cells themselves could serve as the original sources to conduct required differentiated cells in other parts of the mouse body.

*Table 5.1 In vitro pluripotent evaluation used to determine embryonic stem cells in mammals.*

This table demonstrates the best-known means of embryoid body formation used to examine *in vitro* pluripotency of mammalian embryonic stem cells.

| ***In vitro* pluripotent evaluation** | **Specific determination** | **References** |
|---|---|---|
| Embryoid body formation | No | Piedrahita *et al*., 1990a; Piedrahita *et al* 1990b; Chen *et al*., 1999; Li *et al*., 2003a; Pera *et al*., 2003; Li *et al*., 2004; Pera *et al*., 2006; Kim *et al*., 2007 |
| | Examination of self-renewing and/or differentiated genes | Okabe *et al*., 1996; Hamazaki *et al*., 2001; Tropepe *et al*., 2001; Chinzei *et al*., 2002; Brevini *et al*., 2005; Blyszczuk *et al*., 2006; Schmandt *et al*., 2006; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Yin *et al*., 2007; Hayes *et al*., 2008; O'Conner *et al*., 2008 |
| | Derivation of three embryonic germ layers | Wiles and Keller, 1991; Okabe *et al*., 1996; Thomson *et al*., 1996; Wiles and Johannsson, 1999; Hamazaki *et al*., 2001; Tropepe *et al*., 2001; Chinzei *et al*., 2002; Tarasov *et al*., 2002; Kim *et al*., 2003; Ying *et al*., 2003b; Ying and Smith, 2003; Brevini *et al*., 2005; Wang *et al*., 2005b; Blyszczuk *et al*., 2006; Dattena *et al*., 2006; Fang *et al*., 2006; Hatoya *et al*., 2006; Schmandt *et al*., 2006; Shiue *et al*., 2006; Takahashi and Yamanaka, 2006; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Byrne *et al*., 2007; Verma *et al*., 2007; Yin *et al*., 2007; Hayes *et al*., 2008; O'Conner *et al*., 2008; Son *et al*., 2009 |

In order to achieve conduction of required differentiated cells representing each embryonic germ layer originating from pESB-like cells obtained in this study, the previous hypothesis raised as to whether mammals share the similar fundamental ES biology at the same stage (Brons *et al*., 2007; Tesar *et al*., 2007) due to having highly conserved ontological factors and intracellular signaling pathways, but different in the sense of genetic sequences and evolution. This is described in **Section 1.2.3** is drawing an attention to another assumption, which is that inducing differentiation of required somatic cell types of immortal ES cells originating from the equivalent stage among mammals has the same pattern of differentiation protocols, but is distinct in terms of cellular maturation time related to their own species. Therefore, the culture conditions designed to conduct differentiated cells from pESB-like cells generated in this study are modified based on the mouse differentiation protocols, as pESB-like cells derived in this study probably have a similar origin to mES cells, as reported in **Section 3.3**, so that applying murine differentiation methods along with consideration of the basic knowledge of fundamental biology of pigs and the discovery of a new drug to direct differentiated cells from pESB-like cells could hypothetically reproduce all desired representative adult cells that belong to each embryonic germ layer in this study.

Generally, induction of required differentiated cells in any germ layers could be classified into two groups, deriving and sustaining certain mature cells in serum and serum-free culture medium in the feeder-independent system. It is well-established that supplement of 5-20% FCS could drive mES cells to differentiate to become any types of cells in three germ layers (Wiles and Keller, 1991; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Kim *et al*., 2003; Blyszczuk *et al*., 2006; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Yin *et al*., 2007). At the beginning of development, it is not essential to add any other growth factors into the culture medium containing serum, as FCS has the greatest affect on differentiation (Wiles and Keller, 1991; Wiles and Proetzel, 2006), possibly due to its known and unknown constituents (Freshney, 2005). However, since the progenitors are founded, addition of exogenous specific ontological factors into the culture medium is crucial to maintain their own mature differentiated cells (Wiles and Keller, 1991; Wiles and Proetzel, 2006). Consequently, these data

give the scientists a way to find out exactly which factors influence each stage of differentiation in each type of cells by using serum-free culture conditions (Okabe *et al*., 1996; Wiles and Johannsson, 1999; Tropepe *et al*., 2001; Kim *et al*., 2003; Ying *et al*., 2003b; Ying and Smith, 2003; Schmandt *et al*., 2006; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Rao *et al*., 2009). Undoubtedly, most research groups are currently still continuing to adapt their own protocols to derive and maintain their required differentiated cells, thereby probably creating suitable protocols that are useful for human regenerative medicine and agriculture (Prelle *et al*., 2002; Hamazaki and Terada, 2003; Wiles and Proetzel, 2006; Keefer *et al*., 2007). Thus, FCS was designed to add in the culture medium to induce differentiated cell types of three germ layers from pESB-like cells established in this study in correlation with supplementing certain additional ontological factors to the culture medium based on the prior studies, concluding that once the precursors of required differentiated cells have been revealed, certain specific ontological factors must be administered into the culture medium to support derivation of those progenitors and adult cells (Wiles and Keller, 1991; Wiles and Proetzel, 2006). Henceforth, it is necessary to take into careful consideration certain factors and their concentration in facilitating the production of desire differentiated cells.

To achieve generation of differentiated cells of three germ layers, certain well-documented pleiotropic factors affecting somatic cells of all germ layers are in the first group to be considered and supplemented into the differentiation medium in this study. As previously mentioned in **Section 1.2.3**, the outstanding key factors maintaining self-renewal state of immortal ES cells in both early and late epiblast ES cell origins could also regulate the differentiation depending on the balance between the mechanisms of self-renewing and differentiation states; thus, it was decided to utilize the same factors successfully yielding pES-like cells in this study, which are LIF and bFGF to induce required differentiated cells. It is well-documented that addition of LIF ranging between 0.2-1,000 units/ml to the serum-free culture medium in non-feeder culture system can produce and sustain neural stem cells, one of the well-known somatic cell types that belong to ectoderm layer, originating from both pluripotent ES cells and neural multipotent stem cell, thereby generating adult neurons and neuronal

supporting cells under the complex controls among certain ontological factors such as bFGF and TGF-β in mice (Lendahl *et al*., 1990; Tropepe *et al*., 2001; Kim *et al*., 2003; Ying *et al*., 2003b; Ying and Smith, 2003; Schmandt *et al*., 2006; Cohen and Fields, 2008; Rao *et al*., 2009). Moreover, the same range of LIF concentration also plays a critical role in development of some differentiated cells in the mesoderm layer such as muscles, by stimulating myoblast proliferation leading to the formation of mature muscle tissues by interacting with some other growth factors, e.g. bFGF and IGF, in the serum-dependent culture system in the mouse (Austin and Burgess, 1991; Austin *et al*., 1992; Bower *et al*., 1995; White *et al*., 2001). In case of inductive differentiation by bFGF, it is well-known that a complement of 10-100 ng/ml bFGF into serum-independent culture medium of a non-feeder culture system could promote murine neuronal stem cell precursors initiated from both immortal ES and neural multipotent stem cells in correspondence with other pleiotropic factors such as LIF and PDGF, resulting in the development of adult neurons and neuronal supporting cells (Okabe *et al*., 1996; Brustle *et al*., 1999; Kim *et al*., 2003; Ying *et al*., 2003b; Ying and Smith, 2003; Schmandt *et al*., 2006; Roa *et al*., 2009). Likewise, the addition of bFGF in between the dosage range managed to induce neuronal stem cells in the serum-dependent condition could activate mouse myoblastic growth, and afterwards formation of muscle tissue has been revealed via the complex regulations among intracellular signalling pathways as previously reported in LIF (Austin and Burgess, 1991; Austin *et al*., 1992; Kinoshita *et al*., 1995; Rao and Kohtz, 1995; Milasincic *et al*., 1996). Hence, a combined concentration of LIF and bFGF to supplement the culture medium for producing certain somatic cells that come from ectoderm and mesoderm layers in this study was the same as the ones managed to derive pES-like cells in this study, as the levels of those two factors were still in the range previously used by other research groups, i.e. 1,000 units/ml LIF and a variable of 4 and 40 ng/ml bFGF.

Regarding the direction of representative cell types of endoderm layer, hepatocytes are the best candidate for the cell type to be generated in this study because of their unique morphology is easily determined under the microscope. Furthermore, a number of research groups have successfully generated this type

of cell from the mouse pluripotent ES cells, albeit that it is quite difficult to reproduce and maintain hepatic cells in *in vitro* because of the specific ontological factors that are required to be added at the different time points of hepatic development by imitating each step of *in vivo* murine hepatocyte formation (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Blyszczuk *et al*., 2006; Yin *et al*., 2007). Therefore, the designed protocols of directing hepatic differentiation of pESB-like cells produced in this study were modified based on certain studies (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Yin *et al*., 2007), but were different in terms of the percentage of FCS managed to complement into the culture medium, and the concentration and type of LIF used for priming hepatic differentiation, as detailed in **Section 2.8.2**, despite all other processes being the same. Briefly, 10 ng/ml bFGF was applied to the culture medium for 2 days after 3 days of initiation treatment. Then, 20 ng/ml HGF was added to the culture medium for two days for the induction of the mid stage and finally the combination of 20 ng/ml HGF, 10 ng/ml OSM, 100 nM dexamethasone and 5 μg/ml ITS (based on transferrin, Sigma I3146) was supplied to the culture medium to induce the late stage of hepatic differentiation until hepatocyte-like cells were observed under the microscope.

In summary, the cells anticipated to be shown in this study by using the required differentiation protocols described above were neurons, neuronal supporting cells, myocytes and hepatocytes. Hence, it is of considerable importance to be clear as to how to classify those certain differentiated cells by understanding their distinctive characteristics, and of course how those differentiated cells behave during development in order to make sure that pESB-like cells obtained in this study have an ability to change themselves to become any other type of cell in the body.

Before beginning to discuss the outstanding morphology of each designed differentiated cells, it is necessary to give a general idea of the initiation of differentiation. During embryonic development in mammals, immortal ES cells principally give rise to be multipotent stem cells before each lineage of

progenitors is developed and yields a specific mature cell line that functions in the certain organs of three germ layers. Therefore, neurons and neuronal supporting cells, the building blocks of nervous system that represent the ectoderm layer cell types (**Figure 5.1A**), are derived from the multipotent neuronal stem cells called neuroblasts. In the vertebral central nervous system, neuroblasts usually change to be common progenitors known as neuroglioblasts that provide the lineage of both progenitors for neurons and glial cells and consequently the mature cells of neurons and glial cells are established. By microscopic analysis, a neuronal cell principally consists of a cell body containing a large spherical or ovoid nucleus in the centre, many processes of dendrites and a single axon that varies in length **(Figure 5.1)**. Neuronal cells are identified and named based on the numbers of processes extending from their cell body, such as unipolar, bipolar and multipolar (**Figure 5.1C**). They communicate with each other by sending electrical or chemical signals in the synaptic area and afterwards cellular responsiveness occurs. Several neurons usually form a chainlike network for sending impulses from one part of the system to another. Cluster groups of neuronal cell bodies that aggregate in the central nervous system and peripheral nervous system are recognized as nuclei and ganglia, respectively. For neuronal supporting cells in the nervous system, they have been named differently according to which nervous system they are located in. In the central nervous system, they are called neuroglia or glia, for example, oligodendrocytes, astrocytes and microglias, while they are known as Schwann and satellite cells in the peripheral nervous system (**Figure 5.1A-B**). The supporting cells basically surround the neurons acting as protective walls of neuronal cells, and also perform as the metabolic exchange pathways between vascular system and neurons of nervous system. Each neuronal supporting cell has a cell body with the nucleus in the centre and several processes that vary in length dealing with their functions (Ross and Pawlina, 2006; Squire *et al*., 2008). To confirm the establishment of the nervous system, nestin is the best-known marker usually used for investigation, as neural multipotent stem cells, neurons and neuronal supporting glial cells express this protein (Lendahl *et al*., 1990; Okabe *et al*., 1996; Kim *et al*., 2003; Ying *et al*., 2003b; Schmandt *et al*., 2006; Brevini *et al*., 2008; Cohen and Fields, 2008; Squire *et al*., 2008; Rao *et al*., 2009) (**Table 5.2**). Hence, nestin was on the list to determine if adult neuronal-

like and neuronal supporting-like cells were observed in the culture medium in this present study.

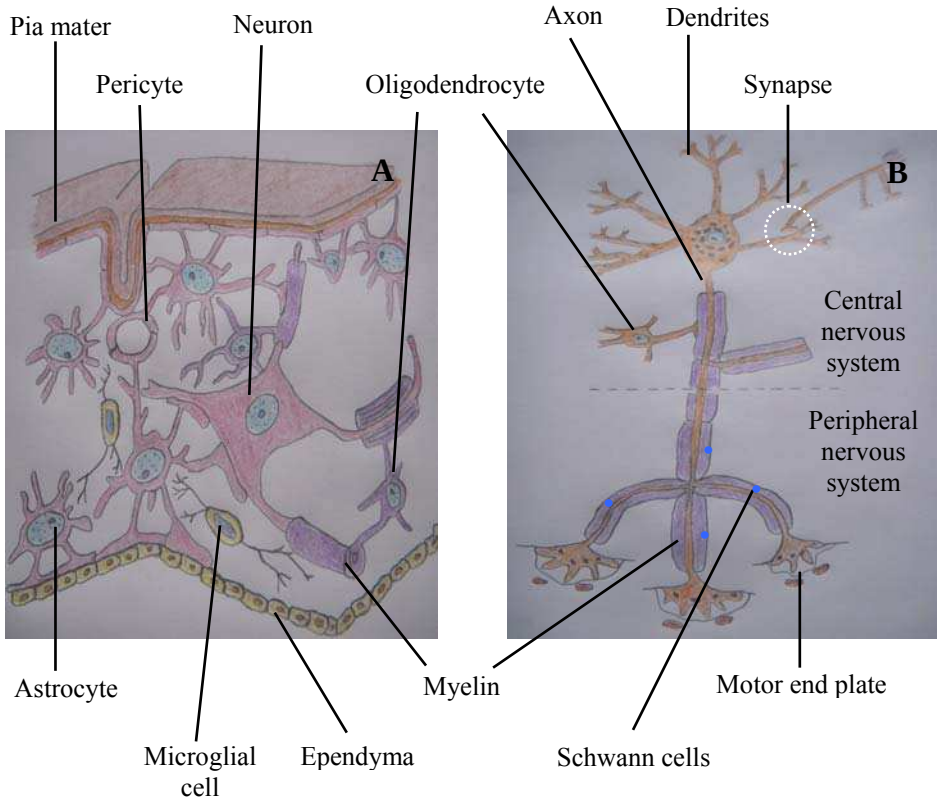
According to the development of mesoderm layer, myoblasts are multipotent stem cells providing three categories of myocytes such as cardiac, skeletal and smooth muscles (**Figure 5.2**). Their outstanding morphology and physiological functions are quite useful in terms of rough identification of these three differentiated cell types under the microscope. To start with their structure, the cardiac muscle tissue consists of the interlacing bundles of cardiomyocytes that are striated due to the formation of narrow dark and light bands composed of actin and myosin filaments that extend from end to end of each other in a parallel arrangement, which myosin molecules are organized as a helical parallel-antiparallel design resulting in a bare zone being formed in the centre of the filaments, as shown in skeletal muscle (**Figure 5.2A-B**), while the dark and light bands are not observed in smooth muscle cells because of myosin molecules forming as a side polar non-helical parallel-antiparallel arrangement that provides no bare zone in the middle of the filaments (Ross and Pawlina, 2006). Regarding their size, cardiac myocytes seem to be shorter and narrower than the skeletal muscle and they are more rectangular than the smooth muscle cells, which generally form a spindle shape (**Figure 5.2**). Another factor used to distinguish these three muscles is the location of their nucleus, as each cardiomyocyte and smooth muscle cell have only single nucleus in the centre of their own myocyte, while a skeletal muscle cell carries multiple nuclei on its periphery (**Figure 5.2**) (Ross and Pawlina, 2006). Regarding their muscular functions, only cardiac myocytes have been recorded as the obvious involuntary rhythmic beating cells in *in vitro* culture system (Tarasov *et al*., 2002; Tarasova *et al*., 2006). Furthermore, it is also important to determine a few specific markers of the certain adult muscle cells to identify the existence of those myocytes (**Table 5.2**). For instance, α-smooth muscle actin and smooth muscle myosin heavy chain should be investigated for the exhibition of smooth muscle cells (Tarasova *et al*., 2006; Kolodziejska *et al*., 2008; Sinha *et al*., 2009), and α-cardiac actin for cardiomyocytes (Talbot *et al*., 2007a; Sinha *et al*., 2009). Thus, morphological analysis of myocyte-like cells was determined in parallel with the

expression of some specific genes and proteins in order to be confident that differentiated cells derived from pESB-like cells in this present study originated from mesoderm lineage.

As far as hepatic development is concerned, it is well-known that hepatoblasts are the multipotent stem cells reproducing mature hepatocytes. The hepatocytes are described as large and flat cells that have a variety of shapes such as cuboid, spheroid and polygonal (Ross and Pawlina, 2006). Normally, mature mammalian hepatocytes are binucleate or have multiple nuclei that may be observed under the microscope, and their large nuclei are basically located in the central part of cytoplasm. The dimensions of each hepatocyte could be between 20-30 μm in each side, and they are surrounded by the hepatic sinusoids that are composed of endothelial and Kuffer cells, and bile canaliculi (**Figure 5.3**). The hepatic sinusoids play a crucial role in exchanges of gases and nutrients between hepatocytes and vascular system in the liver, while bile canaliculi are pathways that pass bile products secreted from hepatocytes to the duodenum. The Ito cell established in between the hepatocytes contains cytoplasmic vacuoles carrying vitamin A and also produces collagen fiber into the space of Disse. Numerous mitochondria, rough endoplasmic reticulum and lipid droplets are also found in the cytoplasm of hepatocytes (Ross and Pawlina, 2006). Not only is it necessary to determine morphological analysis of hepatocytes, but the investigation of well-known specific genes of these adult cells such as transthryretin, α-fetoprotein, albumin and HNF-1β must also be undertaken in order to indicate exactly the generation of hepatocytes (Talbot *et al*., 1996; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Meng *et al*., 2005; Blyszczuk *et al*., 2006; Talbot *et al*., 2007a; Yin *et al*., 2007; Agarwal *et al*., 2008; Ma *et al*., 2008) (**Table 5.2**). Therefore, hepatocyte-like cells derived from pESB-like cells in this study were first specified by morphology and were then subjected to test the expression of those genes specific to hepatic lineage.

Finally, one more parameter which it is important to validate along with the evaluation of undifferentiated and differentiated abilities of immortal ES and ES-like cells, is the monitoring of the chromosome characteristics when those

pluripotent ES and ES-like cells have been continuously passaged for a long period of time by using a karyotyping method so that cellular abnormality would be noticed and recorded before the cells used in ways that are crucial and risk human lives (Thomson *et al*., 1996; Thomson *et al*., 1998; Chen *et al*., 1999; Mitalipova *et al*., 2001; Suemori *et al*., 2001; Cibelli *et al*., 2002; Wang *et al*., 2005b; Dattena *et al*., 2006; Fang *et al*., 2006; Shiue *et al*., 2006; Takahashi and Yamanaka, 2006; Byrne *et al*., 2007; Talbot *et al*., 2007a; Verma *et al*., 2007; Hayes *et al*., 2008; O'Conner *et al*., 2008). A karyotyping procedure was carried out to observe the abnormal cellular division of pES-like cells in this study.



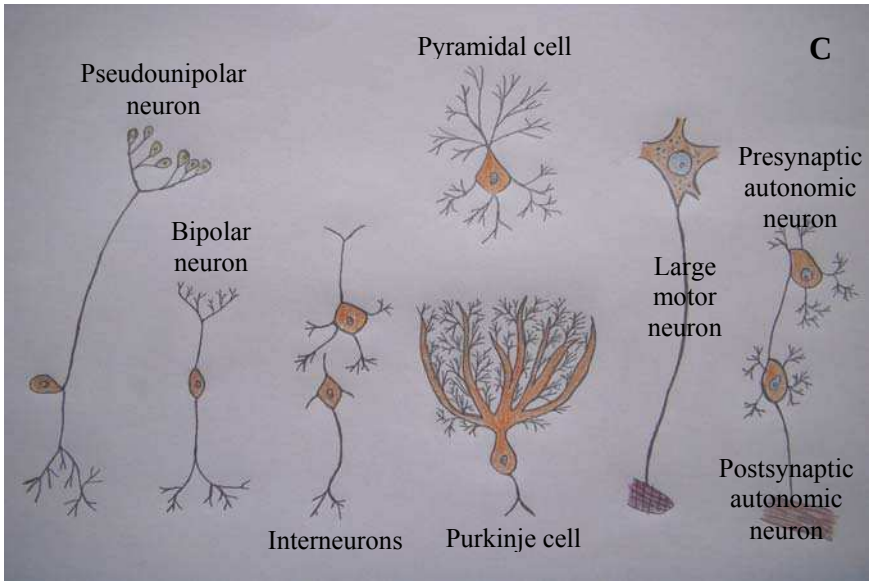




**Fig. 5.1A-C** (adapted from Ross and Pawlina, 2006). Nerve tissue. In *Histology: A text and atlas*, p. 338, p. 320, and p. 321, respectively.

*Figure 5.1 Characteristics of neuronal and neuronal supporting cells in mammalian nervous system.*

During nervous system development, neuroblasts are the multipotent stem cells producing the progenitors of neurons and neuronal supporting cells, thereby establishing of adult neurons and neuronal supporting cells, respectively. As seen from **Fig. 5.1A**, nervous system basically consists of neurons and neuronal supporting cells such as oligodendrocytes, astrocytes and microglias. Principally, neuron is composed of a large nucleus contained in the cytoplasm of its cell. Most neuronal cells have only one axon that is fundamentally the longest process of the cell, which passes the impulses away from the cell body to the synaptic area where neurons communicate to other cells. Each neuron normally has many dendrites the shorter processes to deliver the signals from the peripheral part of the cell to its cell body. Neuronal cells are identified and named based on the numbers of processes that extends from their own cell body such as unipolar, bipolar and multipolar (**Fig. 5.1B-C**). According to the neuronal supporting cells, they have been named based on which part of nervous system they are located in. For example, they are known as neuroglia or glia if they are established in the central nervous system, otherwise they would be recognised as Schwann and satellite cells in the peripheral nervous system (**Fig. 5.1A-B**). The neuronal supporting cells act as the protective wall of neurons and also operate as the metabolic pathway for exchanging the basic nutrients and gases between neurons and neurovascular system so that neurons are capable of functioning effectively. Likewise, each neuronal supporting cell has a cell body with the nucleus contained in the central part and it also has several processes that vary in length used to communicate with other cells (Ross and Pawlina, 2006; Squire *et al*., 2008). Finally, specific gene expression of these neuronal cell types such as nestin is highly suggested to be identified in correlation with the morphological analysis in order to make sure that derived differentiated cells belong to the ectoderm lineage (Okabe *et al*., 1996; Kim *et al*., 2003; Ying *et al*., 2003b; Ross and Pawlina, 2006; Schmandt *et al*., 2006; Brevini *et al*., 2008; Cohen and Fields, 2008; Squire *et al*., 2008; Rao *et al*., 2009).

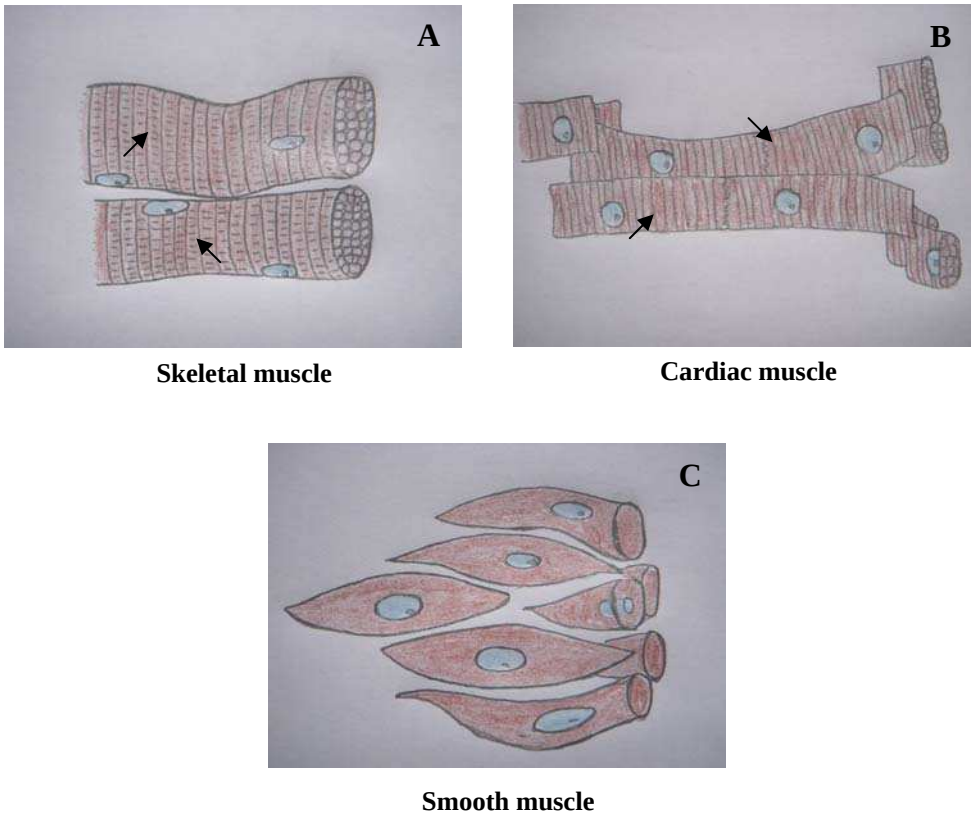
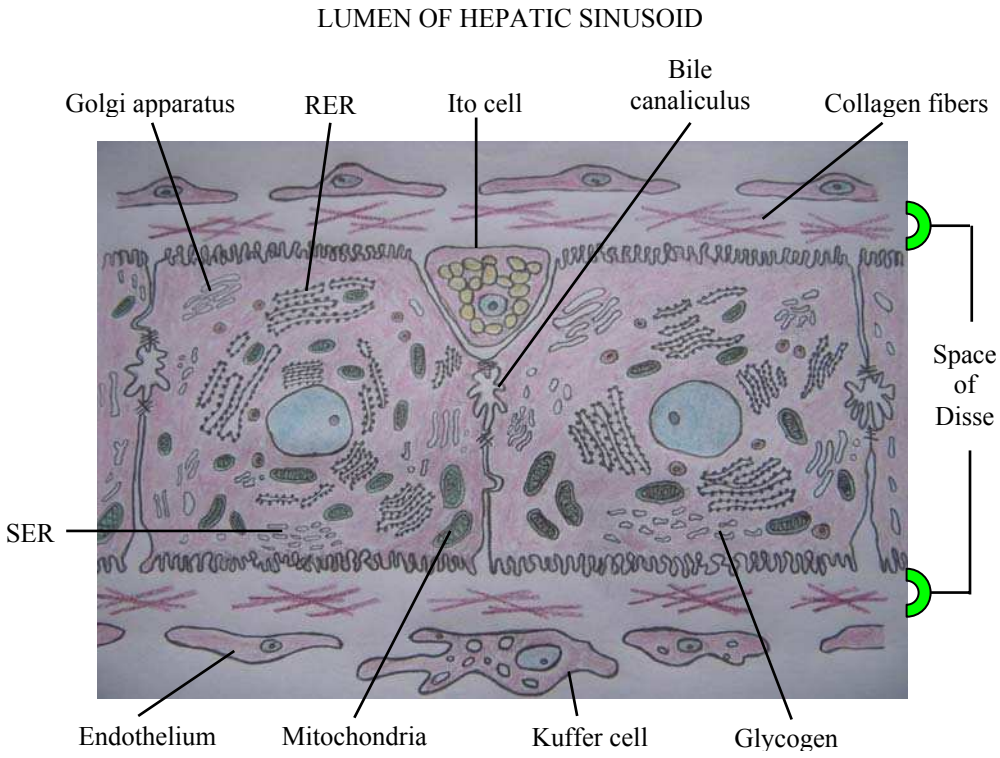



**Fig. 5.2A-C** (adapted from Ross and Pawlina, 2006). Muscle tissue. In *Histology: A text and atlas*, p. 306.

*Figure 5.2 Characteristics of mammalian muscle tissue.*

During development of muscle tissue, myoblasts are multipotent stem cells that give rise to a lineage of three categories of myocytes, which are skeletal, cardiac and smooth muscle cells (**Fig. 5.2**). By microscopic analysis, the outstanding morphology and physiological functions are the basic indicators to determine these three types of muscles separately in this study. Fundamentally, skeletal and cardiac muscles are striated cells composed of dark and light bands (**Fig. 5.2A-B**), whereas smooth muscle cells do not have the dark and light bands as shown in **Fig. 5.2C** (Ross and Pawlina, 2006). As regards their size, cardiac cells seem to be shorter and narrower than the skeletal muscle, and their shape is noticed to be more rectangular than smooth muscle cells, which normally has been seen as fusiform (**Fig. 5.2**). In addition to the nucleus of these muscles, it is well-documented that cardiomyocyte (**Fig. 5.2B**) and smooth muscle cell (**Fig. 5.2C**) have only one nucleus in the central part of their cells, while a skeletomyocyte carries the multiple nuclei on its periphery (**Fig. 5.2A**) (Ross and Pawlina, 2006). To consider their function roughly, only cardiac myocytes have been well-recorded as involuntary beating cells in *in vitro* culture system (Tarasov *et al*., 2002; Tarasova *et al*., 2006). Moreover, some specific markers such as α-smooth muscle actin, smooth muscle myosin heavy chain and α-cardiac actin (Tarasova *et al*., 2006; Talbot *et al*., 2007a; Kolodziejska *et al*., 2008) need to be examined along with the morphological analysis in order to confirm the derivation of mature myocytes. ⟶ = stratiated cells




**A**dapted from Ross and Pawlina, 2006. Digestive system III: Liver, gall bladder, and pancreas. In *Histology: A text and atlas*, p. 587.

*Figure 5.3 Characteristics of mammalian hepatocytes.*

During hepatic development, hepatoblasts are the mutipotent stem cells producing the lineage of hepatocytes. Generally, the mammalian hepatocyte has large polygonal shape containing at least one nucleus in the central part of the cell. As seen from the photo, normally two sides of each hepatocyte are surrounded by the hepatic sinusoids that consist of endothelial and Kuffer cells and also play an important role in hepatic metabolic exchanges, whereas the other two sides communicate with the bile canaliculi in order to pass the bile secreted from itself to the duodenum. Moreover, numbers of Vitamin A are found in the Ito cell, which is a source producing collagen fiber into the space of Disse (Ross and Pawlina, 2006). To prove the derivation of hepatocytes in mammals, some specific proteins and genes such as transthryretin, α-fetoprotein, albumin and HNF-1β have been continuously investigated along with morphological analysis (Talbot *et al*., 1996; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Meng *et al*., 2005; Blyszczuk *et al*., 2006; Talbot *et al*., 2007a; Yin *et al*., 2007; Agarwal *et al*., 2008; Ma *et al*., 2008). Abbreviation: HNF-1β, Hepatocyte nuclear factor 1 homeobox B; RER, Rough endoplasmic reticulum and SER, Smooth endoplasmic reticulum.

*Table 5.2 Morphology and specific gene expressions of some selected differentiated cells.*

This table summarises the outstanding morphology and expression of some genes specific to required differentiated cells. Abbreviation: AFP, α-fetoprotein; GFAP, Glial fibrillary acidic protein; HNF-1β, Hepatocyte nuclear factor 1 homeobox B; M-cadherin, Myotubule cadherin; Myf5, Myogenic factor 5; Myf6, Myogenic factor 6; MyoD, Myogenic differentiation; O4, Oligodendrocyte marker O4; PAX-6, Paired box gene 6; TH, Tyrosine hydroxylase and Tuj1, Neuronal specific class III β-Tubulin.

| Germ layer | Morphology | Gene expression | References |
|---|---|---|---|
| **Ectoderm:** Neuron | A cell body with several short process containing a large nucleus in the cytoplasm along with a long single axon | nestin, Tuj1, PAX-6 and TH | Okabe *et al*., 1996; Kim *et al*., 2003; Ying *et al*., 2003b; Ross and Pawlina, 2006; Schmandt *et al*., 2006; Brevini *et al*., 2008; Cohen and Fields, 2008; Squire *et al*., 2008; Rao *et al*., 2009 |
| Glia | A cell body with several process that vary in length containing a large nucleus in the cytoplasm | nestin, GFAP and O4 | Brustle *et al*., 1999; Ying *et al*., 2003b; Ross and Pawlina, 2006; Schmandt *et al*., 2006; Cohen and Fields, 2008; Squire *et al*., 2008; Rao *et al*., 2009 |
| **Mesoderm:** Cardiac muscle | Short and narrow striated cells with a single nucleus in the centre of the cell | α-cardiac actin, brachyury and troponin I | Ross and Pawlina, 2006; Talbot *et al*., 2007a; Sinha *et al*., 2009 |
| Skeletal muscle | Large and elongate striated cells with multiple nuclei on the periphery | Myf5, Myf6, myogenin, MyoD and M-cadherin | Ross and Pawlina, 2006; Tarasova *et al*., 2006 |
| Smooth muscle | Non-striated cells with fusiform shape containing a single nucleus in the centre of the cell | α-smooth muscle actin and smooth muscle myosin heavy chain | Ross and Pawlina, 2006; Tarasova *et al*., 2006; Kolodziejska *et al*., 2008; Sinha *et al*., 2009 |
| **Endoderm:** Hepatocyte | Large polygonal shape with at least one nucleus in the centre of the cell | transthryretin, AFP, albumin and HNF-1β | Talbot *et al*., 1996; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Meng *et al*., 2005; Blyszczuk *et al*., 2006; Ross and Pawlina, 2006; Talbot *et al*., 2007a; Yin *et al*., 2007; Agarwal *et al*., 2008; Ma *et al*., 2008 |

## 5.2 MATERIALS AND METHODS

Each differentiated protocol was performed at least 5 replicates, except for two groups of inducing hepatocyte-like cells due to there being no possibility of maintaining the living differentiated cells in feeder-free culture conditions for more than 10 days.

### 5.2.1 Directed differentiation of neuroectodermal and myoblast-like cells derived from pESB-like cells in this study

Both fresh and re-cultured pESB-like cells, MPM8 and MPM28, were induced to differentiate to neuroectodermal and myoblast-like cells every 2-3 passages, as described in **Section 2.8.1**. Results were recorded daily and photos were captured every 5-7 days.

### 5.2.2 Directed differentiation of hepatocyte-like cells derived from pESB-like cells in this study

MPM8 colonies frozen in the liquid nitrogen were re-cultured and used for conducting differentiation, as described in **Section 2.8.2**. Results were recorded every day and photos were taken every 5-7 days.

### 5.2.3 Immunofluorescence staining of specific proteins with differentiated cells derived from pESB-like cells in this study

Induced differentiated cells from MPM8 and MPM28 colonies at different passages cultured in a variety of designed non-feeder culture medium (**Section 2.8**) were randomly selected to stain with the specific marker proteins to three embryonic germ layers using immunofluorescence staining, as stated in **Section 2.9.2.2**. Results were recorded and photos were taken every time.

### 5.2.4 Examination of differentiated genes in induced differentiated cells obtained from pESB-like cells by RT-PCR technique

Induce differentiated cells obtained from MPM8 colonies at different passages cultured in a variety of designed non-feeder culture media (**Section 2.8**) were use to examine with a pile set of differentiated genes specific to each required representative germ layer cell type, as explained in **Section 5.1**. Briefly, nestin was used to determine cells specific to the ectoderm layer, α-smooth muscle

actin, smooth muscle myosin, and α-cardiac actin were used for cells that belong to mesoderm lineage. Finally, a set of genes, e.g. transthyretin, α-fetoprotein, albumin and HGF1-β, were used for a candidate cell type of endoderm layer hepatocytes. As with the determination of self-renewal gene expression in pESB-like cells stated above, investigated genes revealed in designed mouse cells were considered to be a set of gene references of early epiblast ES cells, which benefit the comparison to gene expression of differentiated cells reproduced from induced pESB-like cells in this study. Neurons, hearts and liver cells collected from adult pregnant murine strain 129/Sv and porcine fetuses obtained from the slaughterhouse were used as a positive control of ectoderm, mesoderm, and endoderm layer, respectively. MEFs and PEFs were used as differentiated gene references for their own group and their results were managed to compare to each other in order to have some rough ideas of genes they produced which were likely to benefit the establishment of pES-like cells, as those pES-like cells obtained in this study were cultured on mouse feeders. No addition of reverse transcriptase enzyme in RT reactions of each sample was manipulated as a negative control of the sample. β-actin, a housekeeping gene, was first managed to test the cDNA of each sample before the expression of required differentiated genes of the sample were determined and it also served as the internal control of each sample. Finally, mEBs that were formed to prove the pluripotency of immortal ES cells and the induced neuronal-like, smooth muscle-like and hepatocyte-like cells derived from pESB-like cells in this study were studied to examine all required differentiated genes.

As regards total RNA extraction, the RNA of required samples was extracted following the description in **Section 2.10.1** and examination of gene expression using RT-PCR technique was carried out according to the processes outlined in **Section 2.10.2**. Only neuronal-like and neuronal supporting-like cells induced in DMh, smooth muscle-like cells generated from DM40bh and hepatocyte-like cells conducted in Hep20KOh in the non-feeder culture system were managed to investigate the differentiated gene expression in this study.

### 5.2.5 Karyotype analysis

The confluent myoblast-like cells derived from MPM8 colonies at passage 11 and 14 cultured in the differentiation medium DMh in a non-feeder culture system were blocked in a metaphase II stage using 0.1 µg/ml colcemid (Sigma) for 1-2 hours. Then, they were trypsinised and resuspended in hypotonic 75mM KCl solution and thereafter those cells were left at RT for 10 minutes before they were transferred to the conical tube and centrifuged at 1,000 rpm for 5 minutes. KCl solution was aspirated, leaving approximately 200 µl behind with cell pellet and then methanol:acetic acid (3:1) was added and incubated for 15 minutes at room temperature before the solution was transferred to the conical tube and centrifuged at 1,000 rpm for 5 minutes. Fixation of the cells with methanol:acetic acid (3:1) had to be repeated twice more before those arrested cells were dropped onto the slides that were cleaned by using 70% ethanol and kept cold at -20°C for 3-4 drops/slide, and subsequently the slides were left to dry in a hot box. After this, the slides were placed in a glass jar containing Wright-Giemsa:Gurr Buffer (1:10) for 15-20 minutes before they were rinsed with distilled water and then allowed to air dry before they being observed under the microscope.

## 5.3 RESULTS AND DISCUSSION

### *In Vitro* pluripotent ability of porcine ES-like cells

Not only do the self-renew characteristics of any mammalian pluripotent ES and ES-like cells have to be determined, but also an ability to differentiate to any type of cell in three germ layers must be established to confirm that those new cell lines established are authentic ES cells. Therefore, pESB-like cells, MPM8 at different passages, were used to induce desirable differentiated cells that belong to all three embryonic germ layers by culturing in a variety of designed non-feeder culture medium, as previously described. Before presenting an explanation of the directing differentiation results, brief outlines must be given in order to facilitate understanding of all the processes. First, cellular changes of inductive adult cells cultured in different feeder-independent culture medium designed to generate a specific type of cells in each germ layer were described, then those cells were judged by observation with morphological analysis as to which type of germ layer they should belong to. After that, a specific antibody of each germ layer was subjected to testing with them by using the immunofluorescence staining method. Finally, all the derived differentiated cells were managed to determine their differentiated gene expression and the results obtained were consequently used to compare with the mouse data concurrently operational in this study. Beginning with the conducting ectoderm layer cell types, it was shown that a many undefined cells had begun to produce at the rim of the colony of MPM8 at passage 10 that were cultured in feeder-free culture medium KOh within 24 hours (**Figure 5.4A**). Then, a number of neuronal-like cells, each cell consisting of a cell body that extended one long process to communicate with other cells in a synaptic-like area, were firstly noticed growing on a layer of neuronal supporting-like cells on day 3 (**Figure 5.4B**) and those gathering neuronal-like cells obviously formed a nuclei-like or ganglionic-like area on day 26 (**Figure 5.4D**). Moreover, another feeder-dependent culture medium, DMh, used to conduct differentiation of MPM8 at passage 8 was capable of yielding a number of neuronal-like and neuronal supporting-like cells (**Figure 5.5A-C**) as KOh did. The neuronal-like cells cultured in DMh were definitely defined on day 11 expanding on top of neuronal supporting-like cells and they were primarily noticed to produce a nuclei-like or

ganglion-like area on the same day (**Figure 5.5C**). Subsequently, clear formation of the nuclei-like or ganglionic-like area was established approximately two weeks later (photos were not taken). When the neuronal-like and neuronal supporting-like cells reproduced from MPM8 cultured in non-feeder culture media, KOh and DMh, were managed to test with a specific antibody of neuroectoderm cell types, nestin, using the immunofluorescence staining method, it was found that a cytoplasmic area of neuronal-like cells were stained positive with nestin (**Figure 5.6D** and **5.6F**), whereas the PEFs ones (**Figure 5.6B**) and their own negative control (**Figure 5.6A, 5.6C** and **5.6E**) were not stained positive with this marker indicating that PEFs do not generate nestin protein as they should, as these types of differentiated cells originated from mesoderm lineage (Ross and Pawlina, 2006). Therefore, this means that neuronal-like cells reproduced from MPM8 colonies cultured in non-feeder culture media, KOh and DMh, in this present study presumably have a potential to be mature neurons resulting from their morphology and positive staining results with nestin marker are consistent with previous records (Lendahl *et al*., 1990; Okabe *et al*., 1996; Kim *et al*., 2003; Ying *et al*., 2003b; Schmandt *et al*., 2006; Brevini *et al*., 2008; Cohen and Fields, 2008; Squire *et al*., 2008; Rao *et al*., 2009). However, the result of differentiated gene expression of these induced neuronal-like cells that could assist in determining the neuronal-like cell derivation exactly would be tested and described along with the rest of somatic cells yielded in this study after the other two inductive differentiated cell types belonging to the mesoderm and endoderm layers were completely explained.

According to the conduction of mesoderm cells, it was demonstrated that a number of unclassified induced cells started reproducing at the edge of the colonies of MPM8 at passage 8 cultured in non-feeder culture medium such as DMh, DM4bh and DM40bh within one night as depicted in **Figure 5.5D**, **Figure 5.7A** and **Figure 5.7D**, respectively. Thereafter, smooth muscle-like cells, which were distinguishably determined by their parallel arrangement among fibers that extended from each end to each end of cells leading to a long spindle-shaped formation (**Figure 5.5F**, **5.7C** and **5.7F**) along with a single nucleus contained in the central part of the cell (**Figure 5.8D**, **5.8F** and **5.8H**) without

any observation of rhythmic beating in the culture system as similar as the characteristics of smooth muscle tissue previously explained (Ross and Pawlina, 2006), were definitely identified during days 11-15 when those differentiated cells were cultured in the certain non-feeder culture medium, DMh, DM4bh and DM40bh as shown in **Figure 5.5F**, **5.7C** and **5.7F**, respectively. Moreover, those smooth muscle-like cells obtained from MPM8 cultivated in feeder-independent culture media, DMh, DM4bh and DM40h, were all subjected to examine with a specific antibody of smooth muscle cells, α-smooth muscle actin, by using immunofluorescence staining technique in order to confirm that those induced differentiated cells originated from mesoderm layer. It was clearly determined that each cytoplasm of smooth muscle-like cells derived from non-feeder culture medium, DMh, DM4bh and DM40bh, was stained positive with α-smooth muscle actin, as revealed in **Figure 5.8D**, **5.8F** and **5.8H**, respectively, while the results of their own negative control were shown negative in **Figure 5.8C, 5.8E** and **5.8G**, respectively. In addition, the cytoplasmic contents of PEFs were stained positive with this specific marker specific to mesoderm layer (**Figure 5.8B**), as was to be expected because fibroblasts are a type of cell that belong to mesoderm lineage (Ross and Pawlina, 2006). Thus, smooth muscle-like cells induced from MPM8 that were cultured in the designed non-feeder culture media, DMh, DM4bh and DM40bh, in this study probably have a capability to be adult smooth muscle tissue, as their properties such as general appearances and positive staining with α-smooth muscle actin can be associated with some previous works (Ross and Pawlina, 2006; Tarasova *et al*., 2006; Kolodziejska *et al*., 2008; Sinha *et al*., 2009).

By the inductive differentiation of MPM8 in non-feeder culture media, DMh and KOh, it was shown that differentiated cell types originally come from the ectoderm layer, such as neuronal-like and neuronal supportive-like cells, and mesoderm lineage e.g. smooth muscle-like cells could be generated in these two culture media. So, these data mean that the differentiation culture media used to induce ectoderm and mesoderm cell types in this study are still not perfectly designed to differentiate only one type of cells that originated from only one germ layer. These records hypothetically result from the reasons that the

multipotent ES cells established from MPM8 colonies cultured in those two certain media without any supports of feeders either could be the neuronal multipotent ES cell progenitors that basically give rise to the precursors of mature neurons and neuronal supporting cell lineages and consequently adult cells have been founded, or the myoblastic multipotent stem cells themselves that principally have an ability to generate all three types of adult muscle tissues. However, addition of FCS to the culture medium is capable of stimulating the neuronal progenitors to create mature smooth muscle cells instead of neuronal cell types by activating the mechanisms of G-protein-coupled receptors, as reported in bFGF (Morishita *et al*., 2007). Moreover, two types of ectoderm cells observed in non-feeder culture media, DMh and KOh, in this study may result from known and unknown constituents contained in the FCS driving immortal ES cells to change to any types of cells (Wiles and Keller, 1991; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Kim *et al*., 2003; Freshney, 2005; Blyszczuk *et al*., 2006; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Yin *et al*., 2007), thereby yielding more than one type of cell in the same culture condition, as seen in this study. Regarding directing ectoderm and mesoderm differentiation of MPM8 colonies cultivated in the other two non-feeder culture media DM4bh and DM40bh, smooth muscle-like cells were the only type of cells observed in those two culture media. These findings mean that bFGF probably plays an important role in activation of adult smooth muscle-like cell formation in a dose-dependent manner because of its functions as a key factor stimulating the differentiation of the certain progenitors via the cascades of G-protein-coupled receptors (Morishita *et al*., 2007).

Regarding the induction of hepatocyte-like cells, a representative somatic cell type of endoderm layer in this study, it was recorded that three types of differentiated cells were observed in the designed hepatic differentiation media, Hep10KOm, Hep10KOh and Hep20KOh, in non-feeder culture system. When MPM8 at passage 8 were cultivated in Hep10KOm and Hep10KOh, neuronal–like and neuronal supporting-like cells served as somatic cell types of ectoderm layer, and smooth muscle-like cells represented as an adult cell type of mesoderm lineage were all clearly monitored in the medium stated above on day

7 (**Figure 5.9A-B**) and 9 (**Figure 5.9C-D**) of the culture period, respectively. These records correspond with reports from some research groups that the addition of LIF, bFGF or HGF can induce the proliferation of neuroblasts and myoblasts, thereby forming adult neurons (Lendahl *et al*., 1990; Ray & Gage, 1994; Okabe *et al*., 1996; Brustle *et al*., 1999; Maina & Klein, 1999; Santa-Olalla & Covarrubias, 1999; Tropepe *et al*., 2001; Kim *et al*., 2003; Ying *et al*., 2003b; Ying and Smith, 2003; Bauer & Patterson, 2006; He *et al*., 2006a; Schmandt *et al*., 2006; Martin-Ibanez *et al*., 2007; Xiao *et al*., 2007; Cohen & Fields, 2008; Nicoleau *et al*., 2009; Rao *et al*., 2009) and myocytes (Austin and Burgess, 1991; Austin *et al*., 1992; Bower *et al*., 1995; Kinoshita *et al*., 1995; Rao and Kohtz, 1995; Milasincic *et al*., 1996; White *et al*., 2001), respectively. Notably, those certain types of differentiated cells generated above began to die when they were cultured in those two designed media, Hep10KOm and Hep10KOh, more than 10 days. This means that those two hepatic differentiation protocols are not suitable for maintaining the two types of cells revealed in this present study. On the contrary, MPM8 at the same passage cultured in feeder-free medium Hep20KOh yielded a number of unidentified differentiated cells rising at the border of the colonies within a day (**Figure 5.9E**). Thereafter, those unclassified somatic cells incubated in the certain culture system became larger and flatter than the ones reproduced on day 1 depending on the time passing, relating to the occurrence of variable shapes such as cuboid, spheroid and polygons, each side of which measured between 20-50 μm and at least one large nucleus was contained in the central part of the cytoplasm (**Figure 5.9F-H**), similar to the morphology of hepatic cells, as stated previously (Meng *et al*., 2005; Ross and Pawlina, 2006; del-Castillo *et al*., 2008). Some binucleated cells were first noticed and determined as hepatocyte-like cells when the differentiated cells were cultured in the medium for 10 days (**Figure 5.9F**) and the binucleated hepatocyte-like cells gradually increased their numbers depending on the time of the culture period that passed (**Figure 5.9F-H**). However, some differentiated cells started dying and floating in the culture dishes when they were incubated in the designed medium for more than a month, similar to the data of inductive neuronal-like and neuronal supporting-like cells recorded in this study. Furthermore, those hepatocyte-like cells derived from MPM8 incubated in feeder-free culture medium Hep20KOh were determined

with a specific antibody of hepatic cells, α-fetoprotein, using immunofluorescence staining technique to ensure that those induced hepatocyte-like cells potentially belonged to the endoderm lineage. It was clearly demonstrated that a cytoplasmic area of each hepatocyte-like cell was shown positive with α-fetoprotein and an individual polygonal cell exhibited at least one large nucleus or multiple (up to four) nuclei in the central part of its cytoplasm (**Figure 5.10D-F**) in association with certain experiments (Talbot *et al*., 1996; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Meng *et al*., 2005; Blyszczuk *et al*., 2006; Talbot *et al*., 2007a; Yin *et al*., 2007; Agarwal *et al*., 2008; Ma *et al*., 2008), while the PEFs ones (**Figure 5.10B**) and the results of their own negative control (**Figure 5.10A** and **5.10C**) were not stained positive with this specific marker as they should have been, as PEFs belong to the mesoderm layer (Ross and Pawlina, 2006). Hence, hepatic-like cells induced from MPM8 cultivated in the designed feeder-independent culture medium Hep20KOh reported in this present study could possibly be claimed to be mature hepatocytes. However, the incubation period of differentiation protocol used to direct differentiation is still not suitable for the pig species. It is clearly demonstrated in **Section 5.1** that the protocol of hepatic induction at each stage including the levels of ontological factors added in this study are based on the development of *in vivo* hepatic organ in the mouse (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Yin *et al*., 2007), not the pigs themselves. Therefore, the right dosage of those factors supplemented and the exposure period of each designed culture medium at each step of hepatic development should be considered again, although Hep20KOh medium was successfully used to establish some mature appearances of porcine hepatic cells. Importantly, to improve the differentiation protocols of required somatic cells induced from ES and ES-like cells in the pigs, it is necessary to focus on each *in vivo* developmental stage of the desirable adult cells in the porcine organs.

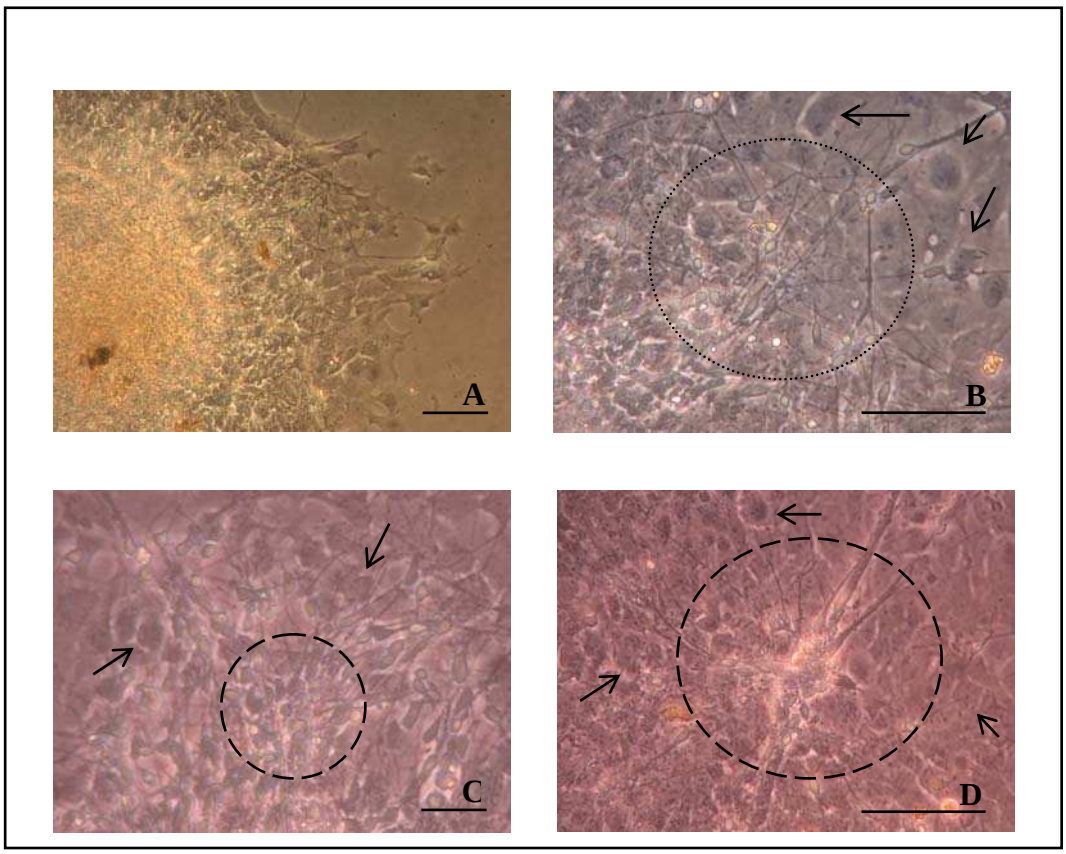



***Figure 5.4 Differentiated cells obtained from pESB-like cells MPM8 at passage 10 induced in non-feeder culture medium, KOh.***

**Fig. 5.4A**: a number of unidentified differentiated cells were revealed at the edge of induced MPM8 colony on day 1, **Fig. 5.4B**: neuronal-like cells were obviously observed on the supporting neuronal-like cells on day 3, **Fig. 5.4C**: a lot of neuronal-like and supporting neuronal-like cells were reproduced from pESB-like cells on day 7, neuronal-like cells forming a nuclei-like or ganglionic-like area was also observed on the same day, and **Fig 5.4D**: outstanding nuclei or ganglion-like area created by neuronal-like cells was demonstrated on day 26. Scale bar = 50 µm. The composition of culture medium KOh used for differentiation of neuronal-like and smooth muscle-like cells was described in **Section 2.8.1**.

→ = area of neuronal-like cells

= neuronal supporting-like cells

= neuronal-like cells forming nuclei-like or ganglionic-like area

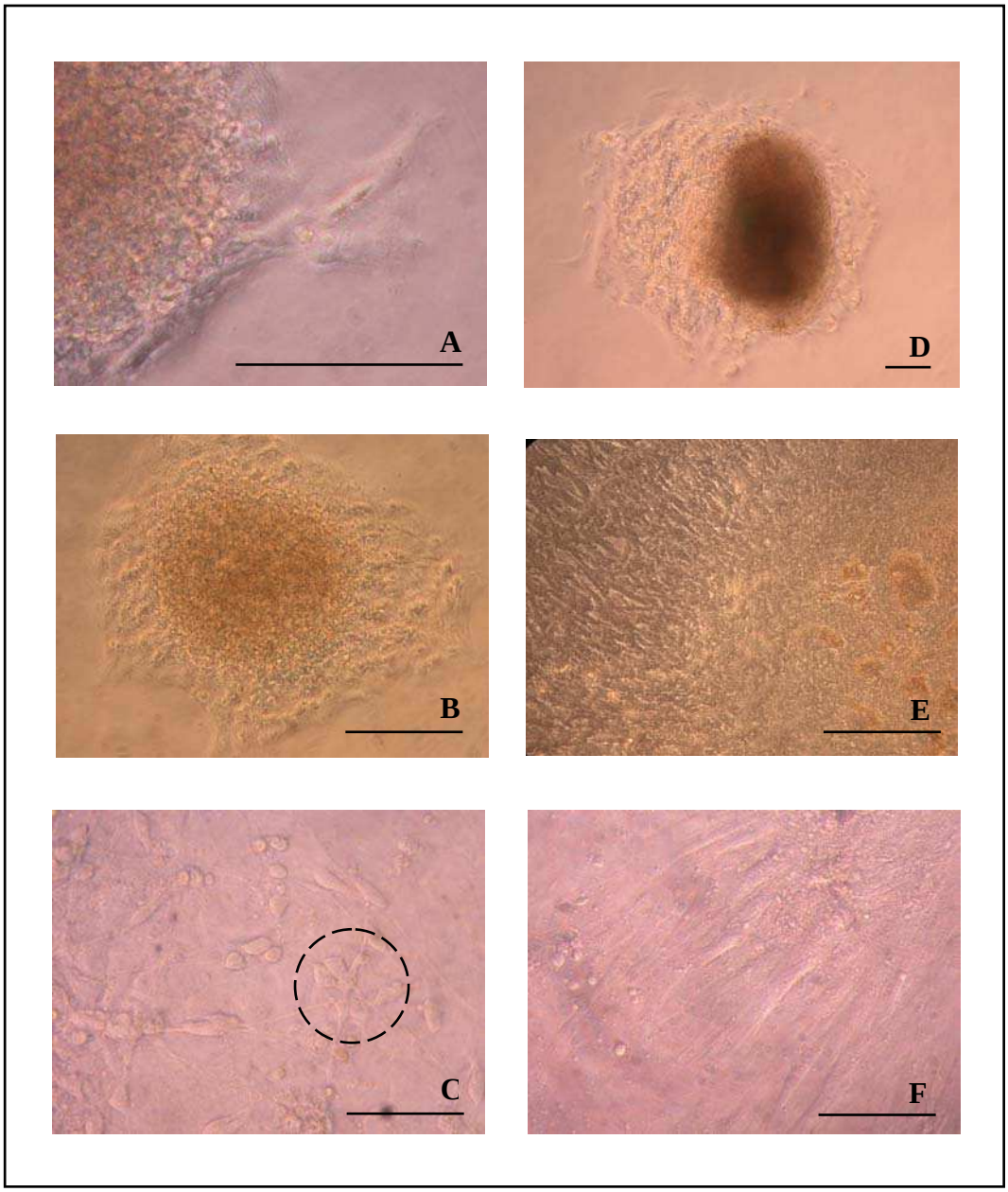



***Figure 5.5 Differentiated cells obtained from pESB-like cells MPM8 at passage 8 induced in non-feeder culture medium, DMh.***

**Fig. 5.5A-B**: some unclassified cells were found at the edge of the colony of induced MPM8 on day 1 and 2, respectively, **Fig. 5.5C**: neuronal-like cells were clearly identified on day 11, **Fig. 5.5D-E**: a number of differentiated cells were produced from pESB-like colony on day 1 and 3, respectively, and **Fig 5.5F**: smooth-muscle like cells were revealed in the culture medium on day 11. Scale bar = 50 µm. The composition of culture medium DMh used for differentiation of neuronal-like and smooth muscle-like cells was described in **Section 2.8.1**.

◌ = forming nuclei-like or ganglionic-like area

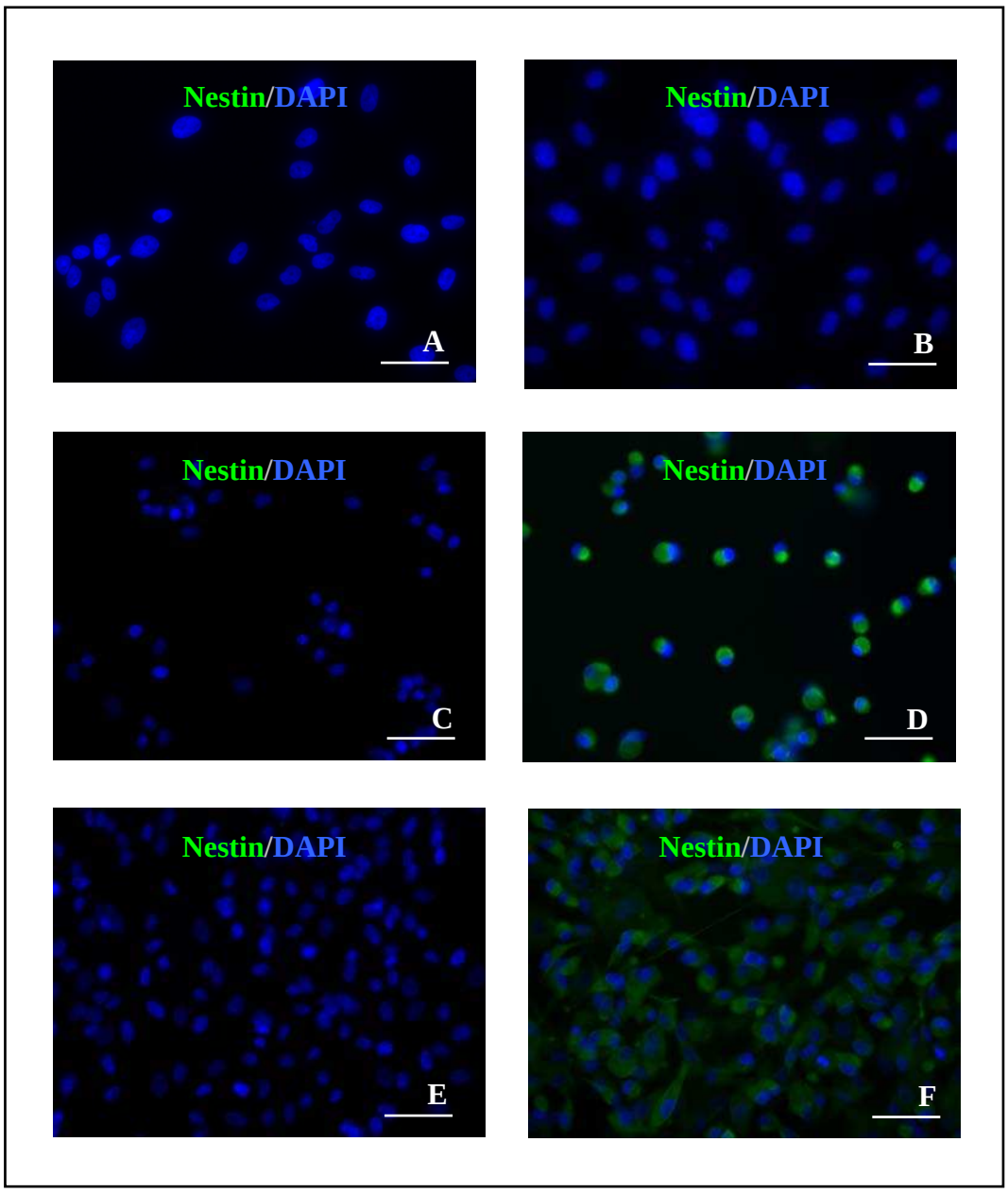



*Figure 5.6 Immunofluorescence staining of neuronal-like cells induced from pESB-like cells MPM8 at different passages with nestin antibody.*

**Fig. 5.6A**, **5.6C** and **5.6E**: negative control of nestin staining, which nestin antibody was not added into the blocking solution, PEFs at passage 4 (**Fig. 5.6A**), neuronal-like cells obtained from MPM8 at passage 12 induced in non-feeder culture medium DMh (**Fig. 5.6C**) and neuronal-like cells obtained from MPM8 at passage 14 induced in non-feeder culture medium KOh (**Fig. 5.6E**) showed negative results of nestin staining. **Fig. 5.6B**, **5.6D** and **5.6F**: experimental treatments that nestin antibody was added into the blocking solution, PEFs did not produce nestin protein (**Fig. 5.6B)**, but neuronal-like cells obtained from pESB-like cells cultured in non-feeder culture medium DMh (**Fig. 5.6D**) and KOh (**Fig. 5.6F**) generated nestin protein. Staining colours of positive results: blue with DAPI and green with nestin. Scale bar = 50 µm. The composition of each culture medium used for differentiation of neuronal-like cells was described in **Section 2.8.1**. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole and PEFs, Porcine embryonic fibroblasts.

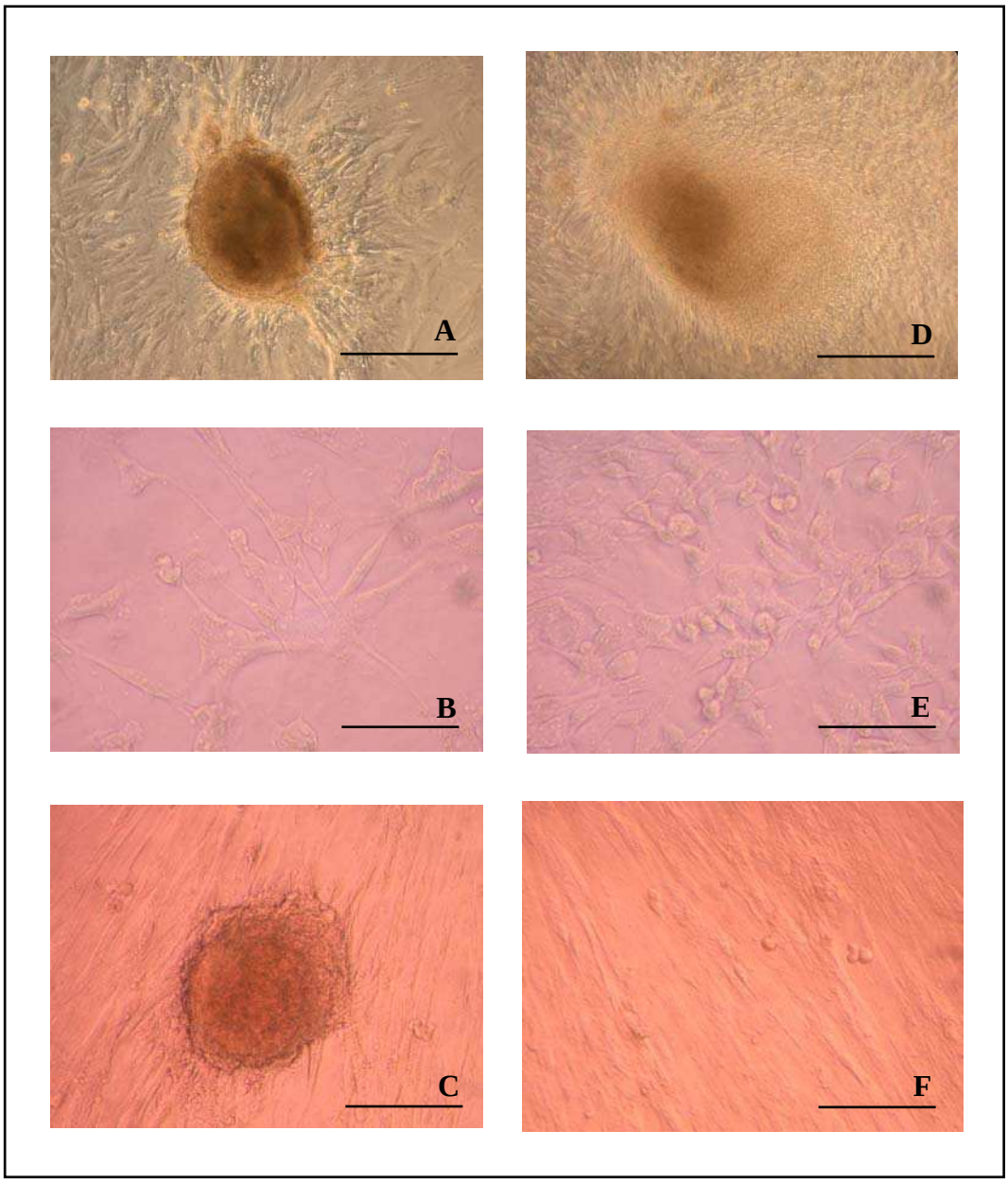



*Figure 5.7* ***Differentiated cells*** *obtained from pESB-like cells MPM8 at passage 8 induced in non-feeder culture media, DM4bh (Fig. 5.7A-C) and DM40bh (Fig. 5.7D-F).*

**Fig. 5.7A-B**: undetermined cells were monitored at the edge of colony of induced certain pESB-like cells on day 1 and 8, respectively, **Fig. 5.7C**: smooth muscle-like cells were found on day 15, **Fig. 5.7D-E**: a lot of differentiated cells were generated from directed differentiation of pESB-like cells on day 1 and 8, respectively, and **Fig. 5.7F**: smooth muscle-like cells were observed in the culture medium on day 15. Scale bar = 50 µm. The composition of each culture medium used for differentiation of smooth muscle-like cells was described in **Section 2.8.1**.

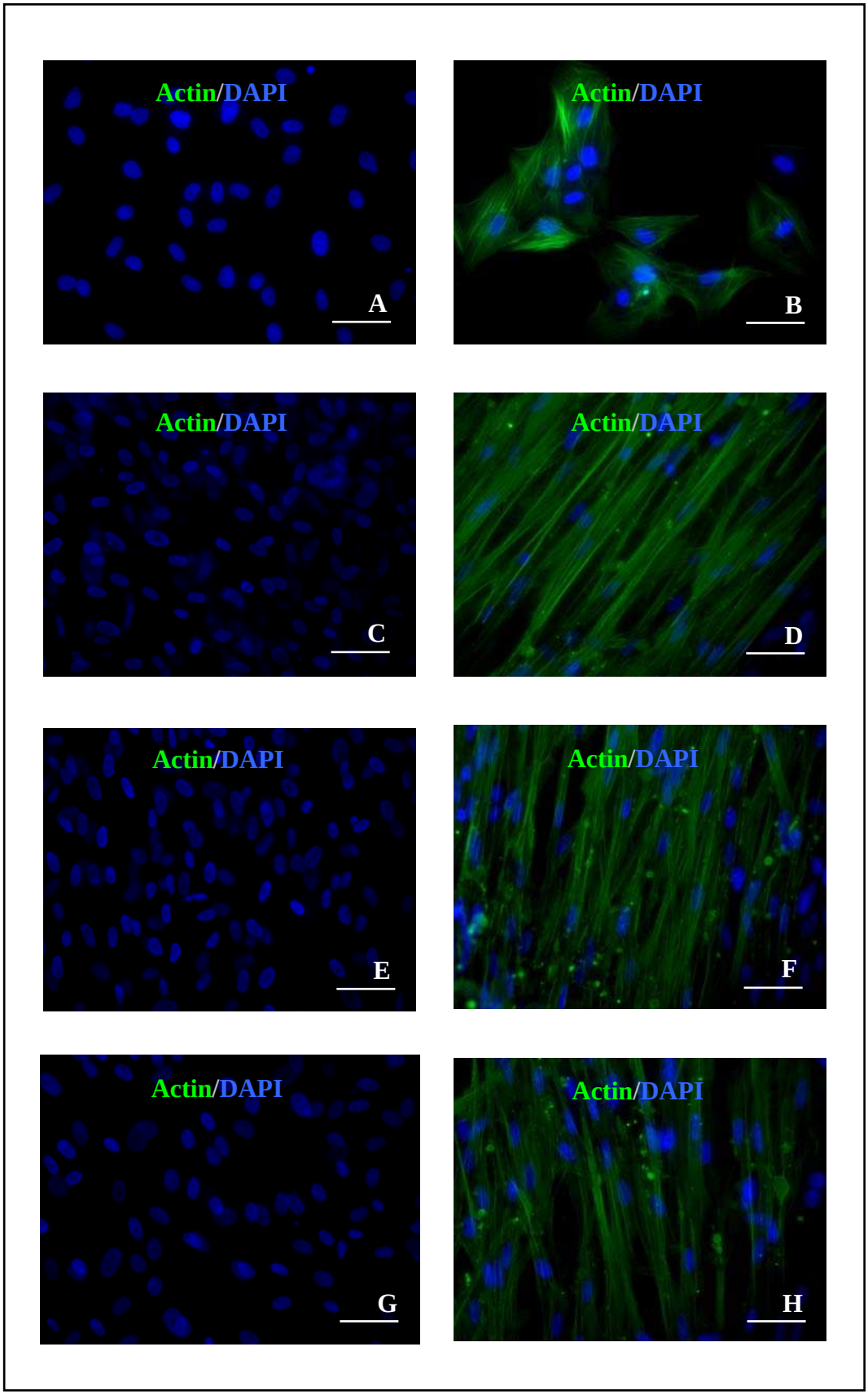

Actin/DAPI
A
Actin/DAPI
B
Actin/DAPI
C
Actin/DAPI
D
Actin/DAPI
E
Actin/DAPI
F
Actin/DAPI
G
Actin/DAPI
H

**Figure 5.8**

*Figure 5.8 Immunofluorescence staining of smooth muscle-like cells obtained from pESB-like cells MPM8 at passage 10 with α-smooth muscle actin antibody.*

**Fig. 5.8A**, **5.8C**, **5.8E** and **5.8G**: negative control of α-smooth muscle actin staining, which α-smooth muscle actin antibody was not added into the blocking solution, PEFs at passage 4 (**Fig. 5.8A**), and smooth muscle-like cells obtained from MPM8 at passage 10 induced in non-feeder culture medium DMh (**Fig. 5.8C**), DM4bh (**Fig. 5.8E**) and DM40bh (**Fig. 5.8G**) showed negative results of α-smooth muscle actin staining. **Fig. 5.8B**, **5.8D**, **5.8F** and **5.8H**: experimental treatments that α-smooth muscle actin antibody was added into the blocking solution, PEFs (**Fig. 5.8B**) and smooth muscle-like cells obtained from pESB-like cells cultured in non-feeder culture medium DMh (**Fig. 5.8D**), DM4bh (**Fig. 5.8F**) and DM40bh (**Fig. 5.8H**) expressed α-smooth muscle actin protein. Staining colours of positive results: blue with DAPI and green with α-smooth muscle actin. Scale bar = 50 μm. The composition of each culture medium used for differentiation of smooth muscle-like cells was described in **Section 2.8.1**. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole and PEFs, Porcine embryonic fibroblasts.

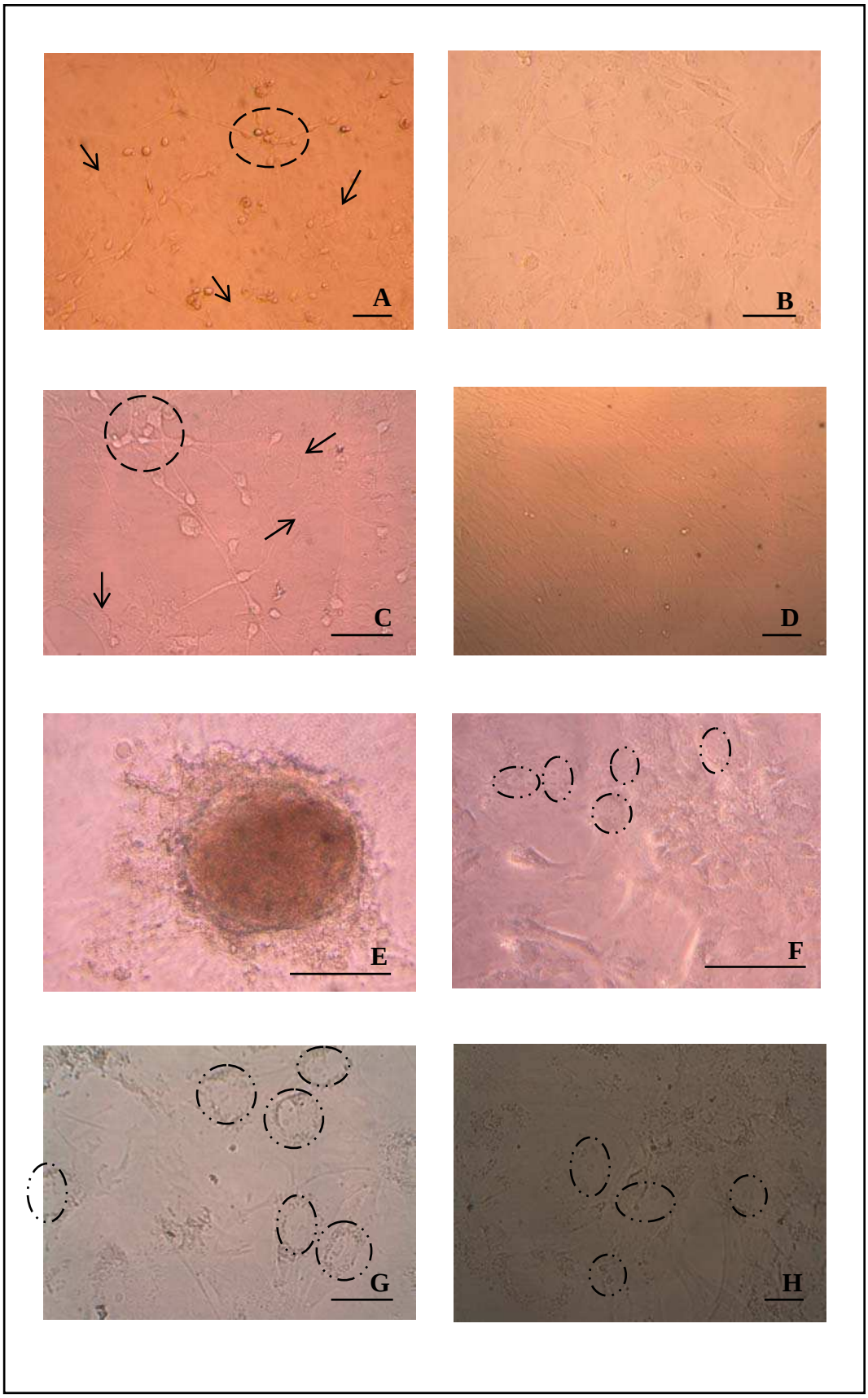



**Figure 5.9**

*Figure 5.9* ***Differentiated cells*** *obtained from pESB-like cells MPM8 at passage 8 induced in non-feeder culture media, Hep10KOm (Fig. 5.9A-B), Hep10KOh (Fig. 5.9C-D), and Hep20KOh (Fig. 5.9E-H).*

**Fig. 5.9A** and **5.9C**: neuronal and neuronal supporting-like cells were clearly formed in non-feeder culture medium Hep10KOm on day 7 and Hep10KOh on day 9 of directed differentiation of MPM8, respectively, **Fig. 5.9B and 5.9D**: smooth muscle-like cells were produced in feeder-free culture media, Hep10KOm on day 7 and Hep10KOh on day 9, respectively, and **Fig. 5.9E-H**: development of hepatocyte-like cells induced from MPM8 colony cultured in non-feeder culture medium Hep20KOh was photographed on day 1, 10, 15 and 23, respectively. The more time those differentiated cells spent in the culture medium, the larger and flatter their cells became. Mainly, one or two nuclei was found in each hepatocyte-like cell obtained in this study, but multiple nuclei up to four could be observed. Scale bar = 50 μm. Notably, those differentiated cells reproduced from Hep10KOm and Hep10KOh started dying after they were cultured in the medium for 10-14 days. Therefore, it was decided to terminate them around that time without staining with any specific antibodies or investigating their gene of expression. The composition of each culture medium used for differentiation of hepatocyte-like cells was described in **Section 2.8.2**

→ = neuronal supporting-like cells

= neuronal-like cells forming nuclei-like or ganglionic-like area

= binucleated hepatocyte-like cells

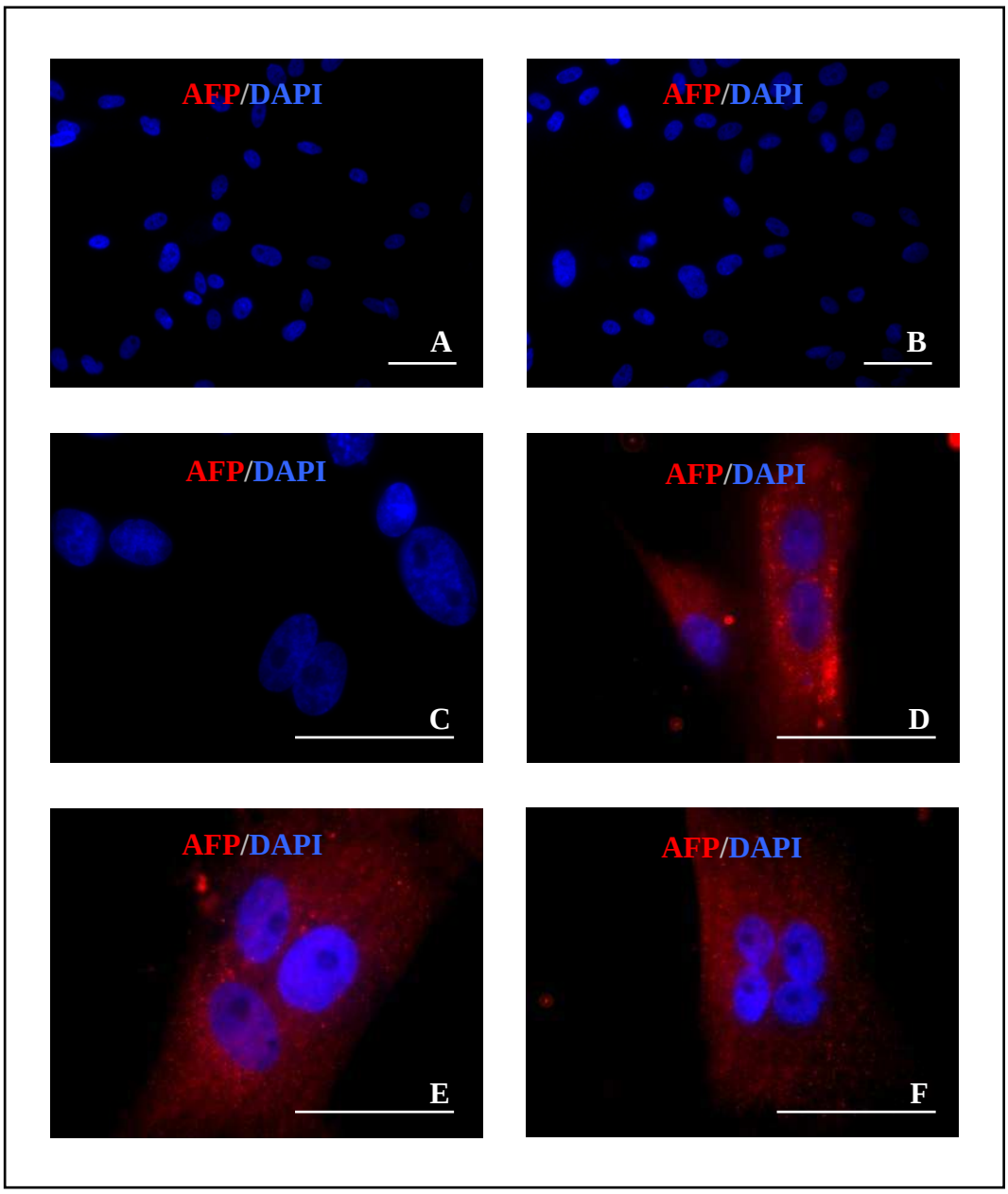



*Figure 5.10 Immunofluorescence staining of hepatocyte-like cells induced from pESB-like cells MPM8 at passage 10 with α-fetoprotein antibody.*

**Fig. 5.10A** and **5.10C**: negative control of AFP staining, which AFP antibody was not added into the blocking solution, PEFs at passage 4 (**Fig. 5.10A**) and hepatocyte-like cells derived from directed differentiation of MPM8 at passage 10 cultured in feeder-free culture medium Hep20KOh for 26 days (**Fig. 5.10C**) showed negative results of AFP staining. **Fig. 5.10B** and **Fig. 5.10D-F**: experimental treatments that AFP antibody was added into the blocking solution, PEFs did not generate AFP protein (**Fig. 5.10B**), but hepatocyte-like cells obtained containing at least one big nucleus or up to 4 nuclei per each cell (**Fig. 5.10D-F**) were stained positive with AFP antibody. Staining colours of positive results: blue with DAPI and red with AFP. Scale bar = 50 µm. The composition of culture medium Hep20KOh used for induced differentiation of hepatocyte-like cells was described in **Section 2.8.2**. Abbreviations: AFP, α-fetoprotein; DAPI, 4,6-diamidino-2-phenylindole and PEFs, Porcine embryonic fibroblasts.

Through consideration of the expression of specific differentiated genes depicted in **Figure 5.11**, it was found that the required differentiated genes tested in this study were either negative or positive depending on each sample. Beginning with the differentiated gene investigation of mouse samples, MEFs provided not only α-smooth muscle actin and smooth muscle myosin heavy chain, two acceptable differentiated genes specific to mesoderm cells, as anticipated (Ross and Pawlina, 2006), but they also produced HNF1β, one of outstanding genes of hepatocyte lineage, which they should not do, as to culture primary MEFs or other feeder fibroblasts the internal organs have to be completely removed from the fetuses before the blended soup of fetuses is placed into the prepared tissue containers for culturing in the incubator (Freshney, 2005). Therefore, MEFs may possibly have been contaminated with a number of hepatic cells during their primary culture procedures. In the case of mouse adult neurons, nestin, one of specific ectoderm layer markers, and α-smooth muscle actin were detectable in this sample, similar to previous works reporting that they had found nestin (Lendahl *et al*., 1990; Ross and Pawlina, 2006; Cohen and Fields, 2008; Squire *et al*., 2008) and α-smooth muscle actin in this type of mature cells (Ross and Pawlina, 2006; Squire *et al*., 2008). As previously documented, α-smooth muscle actin and smooth muscle myosin heavy chain, specific mesoderm layer markers, could not only be detected in mesoderm tissue derivatives, but were also found in the other two germ layers because the mesoderm cells actually can give rise to be the epithelium, mesothelium and endothelium of almost visceral organs of three embryonic germ layers (Ross and Pawlina, 2006), so that these are the reasons why α-smooth muscle actin and smooth muscle myosin heavy chain could be determined in a variety of cell types in this study, such as neurons, as described above or other tissues, as shown below. In addition, a set of genes specific to endoderm cells such as transthyretin, AFP and albumin were also investigated in the murine brain tissue, the results of which results are incompatible with others (Ross and Pawlina, 2006; Squire *et al*., 2008). It may be because those tissues were contaminated with some endoderm cells or the certain equipment, e.g. homogeniser, scissors and forcept used to collect the mouse tissues or extract RNA contents at the same time, although in each step of those processes great care was taken with sterile and well-performed technique. For the cardiac cells of adult mice, not only did they generated α-smooth muscle

actin, smooth muscle myosin heavy chain and α-cardiac actin, one of cardiac differentiated gene marker, as they should, but the murine cardiomyocytes also reproduced transthyretin, AFP and albumin, which is in contrast to the other data (Ross and Pawlina, 2006). This is probably due to the same reasons previously stated in the mouse brain tissue, i.e. that the tissues were contaminated with some endoderm cells or equipment, e.g. homogeniser, scissors and forcept, used to obtain the mouse tissues or extract RNA masses at the same time. As regards the mouse liver cells, it was found that they yielded α-smooth muscle actin, transthyretin, AFP, albumin and HNF1β, and these positive results are related to other studies (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Blyszczuk *et al*., 2006; Ross and Pawlina, 2006; Yin *et al*., 2007), except that AFP was not found in the adult livers of the mouse performed in those experiments. This is because AFP is a well-known marker expressed only in endoderm cells and fetal hepatocytes, and this gene will be inactive after birth. Thus, it is difficult to explain why AFP could be detected in the adult livers of the mouse in this study. One supposition is that AFP could not be absolutely detected in the mature liver organs as the hepatic cells are considered to be one of the organs capable of regeneration when damaged by toxic substances, diseases or surgery (Ross and Pawlina, 2006). In this sense, the hepatic tissues should hypothetically store sources that could act as hepatoblasts, or hepatoblasts themselves in some parts of the organ so that AFP genes could be identified in this type of cells. Finally, pooled 96 hours old mouse embryoid bodies were found producing all designed differentiated genes, except that albumin was not detected in this stage of murine embryoid bodies, as demonstrated in some documents reporting that albumin could be examined since day 12 of embryoid body formation (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Hamazaki and Terada, 2003; Yin *et al*., 2007). Regarding differentiated gene investigation in the pig samples, it was recorded that PEFs exhibited not only α-smooth muscle actin and smooth muscle myosin heavy chain, as anticipated (Ross and Pawlina, 2006), but α-cardiac actin was also detected in these feeders, which hypothetically should not be the case, resulting from PEFs being contaminated with some heart cells during the culture processes of primary porcine feeder fibroblasts. For pig foetal brain, it was found that not only did nestin, α-smooth muscle actin and smooth muscle myosin heavy chain reproduced, as they are

supposed to do, but that they also exhibited α-cardiac actin, transthyretin, albumin and HNF1β as they should not do, according to the results related to other records (Ross and Pawlina, 2006; Squire *et al*., 2008). This presumably caused by brain tissue of the pigs being contaminated with some cardiac and endoderm cells, or cross contamination between equipments used to receive the pig feotal tissues or extract RNA contents. For the cardiac sample of the pigs, it was shown that they yielded not only α-smooth muscle actin, smooth muscle myosin heavy chain and α-cardiac actin, but that they also produced nestin, albumin and HNF1β, results which are not associable with other work (Ross and Pawlina, 2006), probably due to contamination with some neuronal and endoderm cells, or equipments used to obtain the tissues or extract the RNA, as previously described in other tested tissues in both mice and pigs. As regards the porcine foetal hepatic cells, α-smooth muscle actin, smooth muscle myosin heavy chain, transthyretin, AFP, albumin and HNF1β were detectable in this type of cells, and these results are correlated to others (Ross and Pawlina, 2006). While nestin and α-cardiac actin should not have been observed in this tissue, this was possibly caused by this hepatic tissue being contaminated with some neuronal and cardiac cells, or cross-contaminated through the equipments administered to collect the tissues or extract the RNA as described previously.

In terms of genes expression in the differentiated cells derived from MPM8 cultured in a variety of designed non-feeder culture media, it was found that neuronal-like cells generated nestin, α-smooth muscle actin, smooth muscle myosin heavy chain, α-cardiac actin and transthyretin, while smooth muscle-like cells exhibited nestin, α-smooth muscle actin, smooth muscle myosin heavy chain, α-cardiac actin, transthyretin, albumin and HGF1-β, and hepatocyte-like cells showed the expression of nestin, α-smooth muscle actin, smooth muscle myosin heavy chain, albumin and HGF1-β. These results imply that those induced differentiated cells obtained in this study are not sufficiently well-developed to be the type of mature somatic cells required. That is the reason why they could express other genes that are not specific to their own expected germ layer.

Taking all results together, this means that even those three types of somatic cells produced by MPM8 colonies cultured in different kinds of non-feeder culture media have definitely shown the mature appearances of their own cell type, but the intrinsic factors they carrying are still not completely programmed to be specific to germ layer of each. These data possibly result firstly from the culture medium and induction period used to cultivate those differentiated cells still not being perfectly suitable for reproducing those certain induced cells. It was previously shown and described that two types of differentiated cells belonging to ectoderm and mesoderm lineages were observed in DMh and that two types of differentiated cells of the same germ layer were found in DMh and KOh. Secondly, those pESB-like colonies probably started to produce all receptors of three germ layers when they were cultured in the designed differentiated culture media as the aggregated embryoid bodies wait for the stimulation of ontological factors and thereafter enter to the germ layer, as those factors force them to do, as explained in **Section 1.2.3**. Thirdly, pESB-like cells presumably have their own sensitivity in response to those exogenous factors added in the culture medium, thereby providing a different time period of differentiated cell formation in association with variable exhibitions of specific markers of their own mature differentiated cells, as it was clearly seen that hepatic-like cells produced from pESB-like colonies cultured in Hep20KOh medium for 19-26 days were stained positive with AFP antibody using the immunofluorescence staining method, whereas the hepatocyte-like cells cultivated in the same hepatic differentiation medium for 13 days did not express the AFP gene by RT-PCR technique, as they should. Finally, it is possible that the levels of all required self-renewing or differentiated genes are naturally expressed quite low or that the starting point of RNA contents of samples are still insufficient for application to investigate all those designed genes using RT-PCR technique. However, these results indicate that pESB-like cells derived in this present study theoretically have an ability to differentiate to become any type of cell in the porcine body.

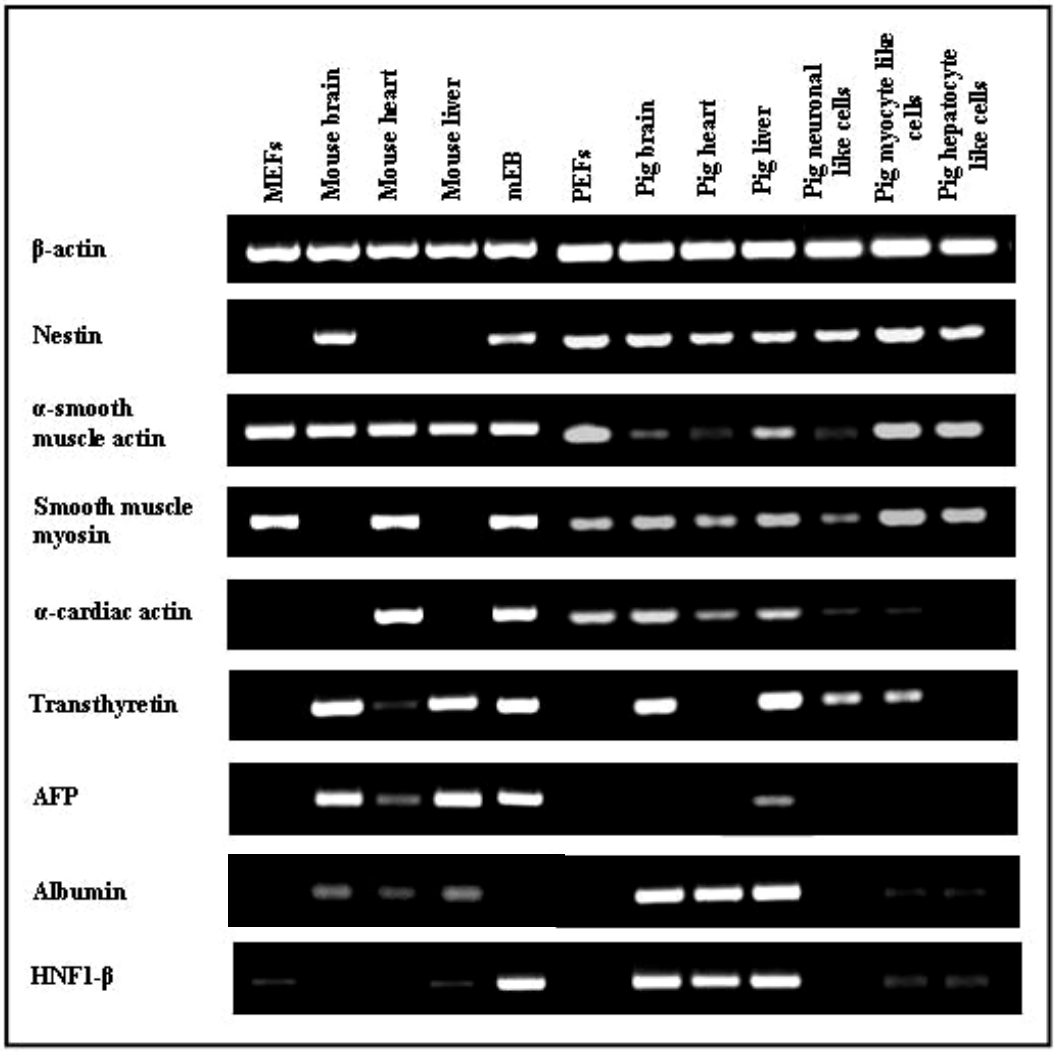



***Figure 5.11 Determination of differentiated gene expression in neuronal-like, smooth muscle-like and hepatocyte-like cells induced from pESB-like cells cultured in different culture media in this study by using RT-PCR technique.***

β-actin served as house-keeping genes of murine and porcine samples. A set of desirable differentiated genes investigated in mouse specimens were used as differentiated gene references of early epiblast ES cells when they were compared to the ones produced from pig samples. Gene expressions obtained from murine and porcine brains, hearts and livers were used as positive controls of required differentiated genes. It was found that mEBs were shown expressing all desirable differentiated genes, except for albumin, which are relevant to previous works claiming that albumin could be investigated since day 12 of embryoid body formation (Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Yin *et al*., 2007). In addition, neuronal-like cells obtained from pESB-like cells were found producing nestin, α-smooth muscle actin, smooth muscle myosin heavy chain, α-cardiac actin and transthyretin, while smooth muscle-like cells generated nestin, α-smooth muscle actin, smooth muscle myosin heavy chain, α-cardiac actin, transthyretin, albumin and HGF1-β, and hepatocyte-like cells yielded nestin, α-smooth muscle actin, smooth muscle myosin heavy chain, albumin and HGF1-β. These results state that differentiated cells induced from pESB-like cells in this study are not well-developed to behave as their adult cell type required. Abbreviations: AFP, α-fetoprotein; HNF1β, Hepatocyte nuclear factor 1 homeobox B; MEFs, Mouse embryonic fibroblasts; mEBs, mouse embryoid bodies and PEFs, Porcine embryonic fibroblasts.

Finally, the last technique administered to validate the normal cell lines, karyotyping, was undertaken in this study. As clearly noticeable before, the maintenance of self-renewing state of pESB-like cells was not easy and pluripotent pES-like cells obtained in this study could not be trypsinised, so that induced smooth muscle-like cells cultured in the certain non-feeder culture conditions were used to examine the chromosomal characteristics of MPM8 cell line instead. It was demonstrated that induced smooth muscle-like cells obtained from MPM8 colonies cultured in feeder-free culture medium DMh at passage 11 and 14 for approximately 2 weeks together with sustaining these type of cells in the same medium without the addition of hLIF for a few days revealed a normal set of diploid metaphase II chromosomes as depicted in **Figure 5.12A** and **Figure 5.13A**, respectively. Nevertheless, the sex of MPM8 could not be determined because these chromosomes were not stained with the special technique producing the G- or R-banding patterns of the chromosomes so that the homologous chromosomes could not be arranged in line precisely (Gustavsson, 1988). Yet, an attempt to provide the roughly idea about the sex of MPM8 cell line was made as demonstrated in **Figure 5.12B** and **Figure 5.13B**. Therefore, MPM8 possibly was a normal female cell line.

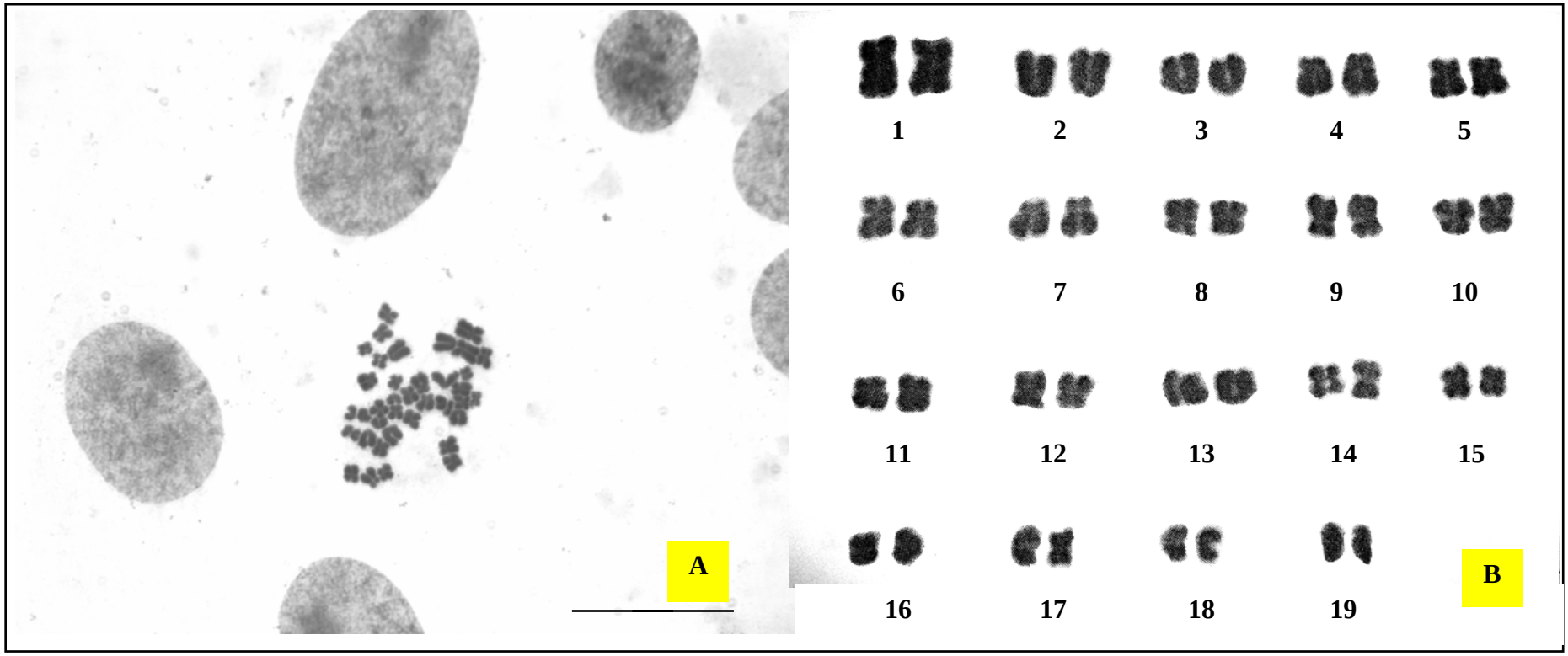



*Figure 5.12 Metaphase chromosomes of smooth muscle-like cells induced from pESB-like cells MPM8 at passage 11 grown in differentiation medium DMh.*

**Fig. 5.12A**: normal diploid karyotype, scale bar = 25 µm, and **Fig. 5.12B**: arrangement of normal diploid 38 chromosomes.

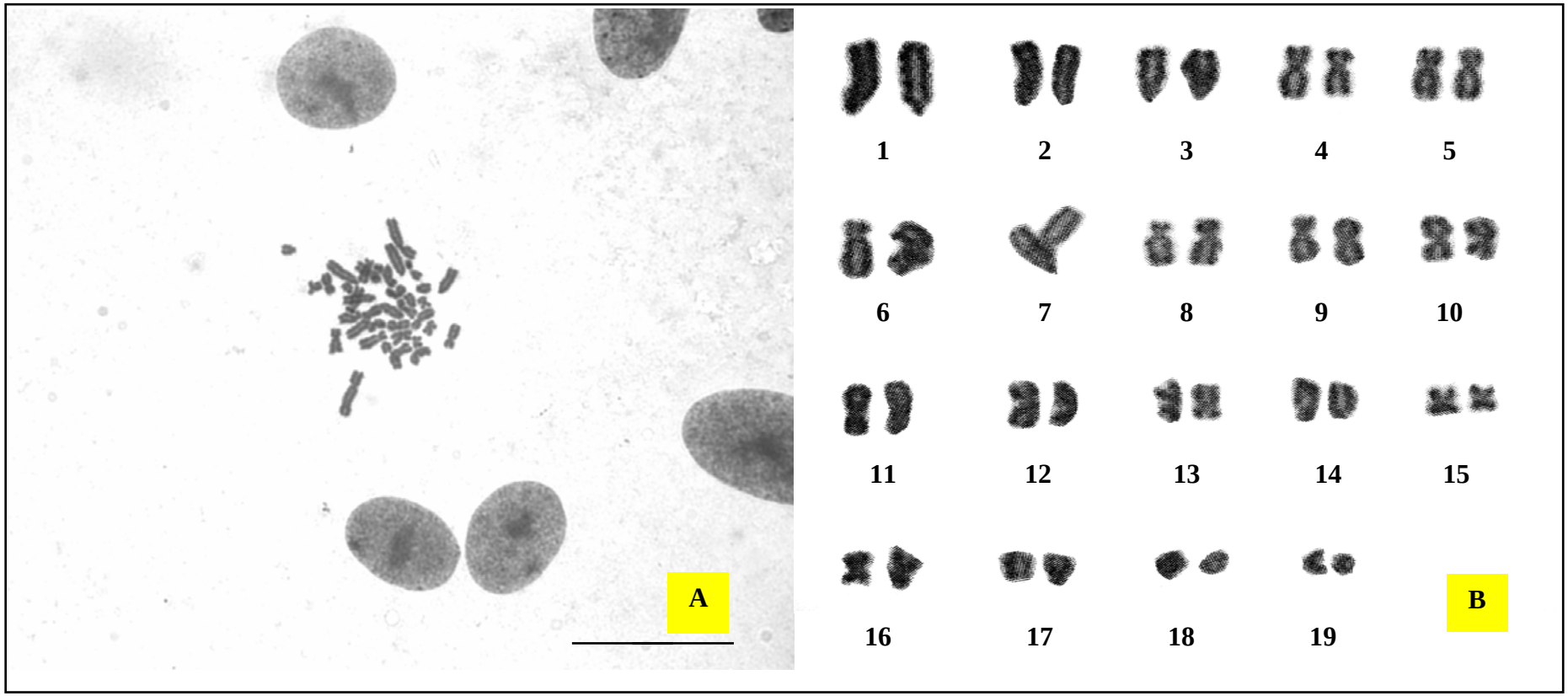



*Figure 5.13 Metaphase chromosomes of smooth muscle-like cells induced from pESB-like cells MPM8 at passage 14 grown in differentiation medium DMh.*

**Fig. 5.13A**: normal diploid karyotype, scale bar = 25 µm, and **Fig. 5.13B**: arrangement of normal 38 chromosomes.

To sum up of all results obtained from **Chapter 3, Chapter 4** and **Chapter 5**, pESB-like cells obtained in this study hypothetically have a potential to be true embryonic stem cells.

In conclusions, firstly, the factors supplemented in the culture medium used in this study may be suitable to derive and maintain porcine ES lines in feeder culture system in terms of interaction with the appropriate stage of embryos that all required receptors and intracellular signalling pathways having already been developed, and suitable environments such as nutrients, chemical constituents, pH and osmolarity to the cells. Secondly, different stages of porcine embryos need different culture media to produce and sustain their own ES cells. Thirdly, each pESB-like cell line has its own pattern of gene expressions. Fourthly, acceptable self-renewing gene markers in current use hypothetically originate from differentiated cells. Fifthly, pESB-like cells obtained from this present study have a potential to be stable ES cells originating from early epiblast stage, as demonstrated in mouse pluripotent ES cells. Sixthly, not all ES-like cell lines are able to create chimeras with germ line transmission. Seventhly, pluripotent ES cells naturally established probably have more than one style reproducing immortal ES cells, as two patterns of pES-like cell production were seen in this study. Eighthly, the culture media designed to induce differentiation of pESB-like cells in this study are capable of generating certain types of somatic cells that belong to all three embryonic germ layers, even the evidence for hepatocyte differentiation was less clear. Ninthly, the colonies of pluripotent ES cells themselves could be used as a source of differentiated cell production, as shown in embryoid bodies. Tenthly, the maturity of general appearance of differentiated cells induced from pES-like cells does not guarantee the complete programme of the intrinsic one. Finally, mammals probably share similar pattern of fundamental biology of ES cells in terms of self-renew and differentiation, as it can be seen that using basic knowledge of derivation and sustenance of undifferentiated and differentiated cells in the mouse in association with fundamental biology of the pigs and new drug discovery could generate numbers of self-renewing ES-like and induced somatic cells that belong to all three embryonic germ layers in the pigs in this present study.

# CHAPTER 6

# GENERAL DISCUSSION

## 6.1 INTRODUCTION

According to the objectives of this study, which were to establish pluripotent ES cell lines, and to induce required mature cells from the ES cells obtained by improving the culture conditions based on basic knowledge of the mouse, together with fundamental porcine biology and new drug development, it is found that the hypotheses raised (**Section 1.4**) are achieved, as pluripotent pES-like cells are derived (**Section 3.3**), and they have some self-renewing (**Section 4.3**) and pluripotent (**Section 5.3**) characteristics as similar to mES cells, which were determined by their cellular morphology, immunofluorescence staining of specific proteins and gene expression of required self-renewal and differentiation markers using RT-PCR technique.

As seen previously in **Chapter 1** and **Section 3.1**, establishment of stable domesticated ungulate embryonic stem cell lines, especially for pigs, has been frequently performed; unfortunately, there is still no evidence proving that any farm animal ES-like cells isolated from any other groups have been set up properly (Piedrahita *et al*., 1988; Prelle *et al*., 2002; Keefer *et al*., 2007; Son *et al*., 2009). This is because, firstly, those pluripotent ES-like cells do not have all the characteristics conforming to the definitions of ES self-renewal. Those scientists could not manage to handle those derived ES-like cells growing in their own designed culture system, as the mouse and their appearances of ES-like cells are varied among groups, as stated elsewhere. Secondly, the ability of those ES-like cells to form other cell types in the body is poorly demonstrated; no data have been clearly proposed to support the properties of their pluripotent ES-like cells, neither in the expression of all the acceptable self-renewal nor differentiated markers required in both *in vitro* and *in vivo*, especially for the production of chimeras with germ line transmission, which is the most important key to distinguishing between early and late origins of epiblast embryonic stem cells.

## 6.2 CHALLENGES AND PROBLEMS IN DERIVATION AND MAINTENANCE OF EMBRYONIC STEM CELLS AND INDUCTION OF REQUIRED DIFFERENTIATED CELLS IN FARM ANIMALS

As previously described in **Chapter 1**, **Chapter 3**, **Chapter 4** and **Chapter 5**, using the basic information of establishing and maintaining self-renewing ES and differentiated cells in the mouse in correlation with the fundamental biology of pigs and drug discovery could provide undifferentiated pES-like cells and induce required differentiated cells from those immortal pESB-like cells in this present study, so it could be concluded that mammals possibly share the similar pattern of fundamental ES biology in the aspects of regulating self-renewal and differentiation mechanisms. Therefore, other ungulate ES or ES-like cells could be established by imitating the ideas of derivation of pES-like cells in this study based on the fundamental biological studies that are related to the desirable domesticated farm animal species as far as possible.

As regards the successful derivation of pES-like cells in this study showing that two styles of epiblast producing pES-like cells are observed and one type of them, pESB-like cells, has almost all the capabilities of mouse ES cells in terms of their undifferentiation and pluripotency *in vitro* (**Chapter 3**, **Chapter 4** and **Chapter 5**), this indicates that pESB-like cells generated in this study presumably have a potential to be true porcine ES cells if they could further produce teratomas in the immunosuppressive mice and chimeras after these pluripotent ES-like cells are introduced to the designed porcine embryonic stage, even if they could not reveal germ cells. However, they are still claimed to be stable porcine ES cells originating from late epiblasts, not the early ones that are capable of creating chimeras with germ line lineage. Undoubtedly, to succeed in derivation of stable farm animal ES cell line productions, the factors probably playing the critical roles in derivation and maintenance of pES-like cells established in this study are a focus for discussion. As previously explained in **Section 1.2.3**, **Section 3.1** and **Section 3.3**, the quality of embryos, isolation techniques and culture conditions are the most important parameters used to derive embryonic stem cells in any kinds of mammals, so these are the reasons

why some prior studies would not be able to get a good quality of ES-like cells from their own experiments to cover all *in vitro* and *in vivo* evaluation of pluripotent ES cells in most domesticated farm animal species. Even pESB-like cells reproduced in this study have almost all the *in vitro* characteristics of ES cells, but the percentage of establishment and numbers of starting *in vivo* derived porcine embryos used is still very low and surely there are no guarantees that the same properties of intrinsic factors containing in each embryo suitable to generate ES cell lines could be obtained from the same pregnant pigs slaughtered at the same time, so that reproducibly of these pESB-like cells is still unclear. Nevertheless, the most essential issue is to administer the right culture conditions to the right embryonic stages in order to achieve an increase in the rate of ES and ES-like cell line generation. Hence, the first task that needs to be completed is to have the database of intrinsic characteristics of developing ungulate embryos at each stage along with the data of their general appearances for further making a decision as to the use of the culture conditions imitating the exhibition of their developed receptors and intracellular signalling cascades that are important in controlling the balance between self-renewing and differentiation states as much as possible. Moreover, the suitable levels or ratio of the factors supplemented into the culture medium must be considered, as explained in **Section 1.2.3**, the achievement in producing and sustaining the self-renew of immortal ES cells relies on a homeostatic balances between the regulation of the intrinsic and extrinsic factors hypothetically playing their precise roles in self-renew and differentiation via the cross-talk communications between the intracellular signalling pathways. However, there is still some doubt as to the extent to which the concentration of each key factor is required to be added in the culture medium, which is equal to the natural ones, so more undiscovered knowledge of ES cells will be revealed and further applied to obtain many more benefits for human and animals themselves in some aspects of medicines and agricultures. As reported in this study, two patterns of ES-like cell production are established, each pESB-like cell lines has its own gene expression and not all the ES-like cells could be used to generate chimeras with germ line transmission. Thus, these obvious recorded evidences prove a general idea that the ES cells naturally reproduced in living creatures possibly have many unknown events to be investigated and considered. Furthermore, suitable surroundings provided to

yield ES cells, such as nutrients, chemical substances, pH, osmolarity and etc. are also in the process of being determined by many research groups in order to prolong the *in vitro* self-renewal state of ES cells for their studies (Prelle *et al*., 2002; Ludwig *et al*., 2006; Keefer *et al*., 2007; Vackova *et al*., 2007). Taken together, if these aspects are clearly explained, they will bring the great hope for the successful cultivation of the farm animal ES cells, including those of pigs, in a feeder-free culture system, which will greatly facilitate the handling of ES cells. It is also much more practical to use non-feeder culture system than the feeder ones in terms of avoiding other sources of contamination and differentiation induced by certain known and unknown factors produced by fibroblasts (Smith, 1988; Lim and Bodnar, 2002; Nagano *et al*., 2005; Amano *et al*., 2006; Levenstein *et al*., 2006; Buhr *et al*., 2007; Chin *et al*., 2007; Prowse *et al*., 2007).

According to the isolation techniques used to isolate the sources of ES cell production, ICMs and epiblast cells, to establish ES cell lines, it cannot be identified exactly which technique is the best among these methods, as described in **Section 1.2.2**. This is because even those isolated ICMs or epiblasts are damaged during the processes of dissection, but could positively grow if the culture media used to culture them are most suitable for them. Otherwise, those isolated cells would die when they are cultivated in improper culture conditions. Moreover, if the differentiated cells have been found near the outgrowth colonies of ES or ES-like cells resulting from contamination of some somatic cells with the source reproducing ES cells having occurred during one of the isolation methods being applied, the unwanted cells will be removed absolutely when the time to passage the ES or ES-like colonies comes. These are the reasons why any technique could be managed to separate the required cells as they all have their own advantages and disadvantages. As for the techniques managed to passage pES-like cells derived in this study, mechanical method is clearly the most suitable application for passaging pES-like cells in this study otherwise they would die by trypsin digestion (**Appendix D**), which is inconsistent with one prior report showing that trypsin, the basic enzyme used to passage mouse ES cells (Evans and Kaufman, 1981; Piedrahita *et al*., 1990b; Allen *et al*., 1994;

Kawase *et al*., 1994; Brook and Gardner, 1997; Wakayama *et al*., 2005; Chung *et al*., 2006; Lee *et al*., 2006b; Tesar *et al*., 2007; Wakayama *et al*., 2007a; Wakayama *et al*., 2007b), is capable of passaging their pES-like cells (Brevini *et al*., 2005). Nevertheless, some other enzymes have been considered to dissociate ES cells in other species such as rat ES (Buehr *et al*., 2008; Ueda *et al*., 2008) and human ES cells (Richards *et al*., 2002; Ludwig *et al*., 2006; Tesar *et al*., 2007; Bigdeli *et al*., 2008; O'Conner *et al*., 2008;) instead of trypsin due to a very low re-plating efficiently and high possibility of chromosome abnormalities (Brevini *et al*., 2007a). For example, collagenase (Ueda *et al*., 2008) and accutase (Buehr *et al*., 2008) have been used to passage rat pluripotent ES cells, and collagenase (Tesar *et al*., 2007; Bigdeli *et al*., 2008; O'Conner *et al*., 2008) and dispase (Richards *et al*., 2002; Ludwig *et al*., 2006; Tesar *et al*., 2007) have been applied to passage the human ones. These records state that each type of mammalian species has its own properties of juctional formation between cells that react differently to the chemical substances, and even in the same species, the ES or ES-like cells reproduced from different research groups seem to have a variable responsiveness to the enzymes used to separate them. Hence, to discover the appropriate enzyme to use for passaging porcine pluripotent ES or ES-like cells in order to elevate the mass production in the future is another interesting aspect.

The results of designed culture conditions undertaken to yield pES-like cells in this study (**Section 2.4** and **Section 3.3**) imply that there should be a proper ratio of the outstanding factors regulating both early and late epiblast ES cell derivation, and surely driving differentiation when the ratio is changed as prior described in **Section 1.2.3**. In addition, it is presumably important that suitable dosages of LIF and bFGF appear in the culture medium to yield and maintain immortal ES cells. It is seen that LIF, the most best-known factor controlling early epiblast ES cell origin, and bFGF, one of the well-known growth factors playing a role in self-renewal state of the late origin of the epiblasts, supplemented into the feeder-dependent culture system consisting of a very low density of mouse feeders could establish pES-like cells that have almost the *in vitro* characteristics of standard ES cells in this present study, while the other

could not reproduce the good quality of ES-like cells that fulfill all the conditions of *in vitro* and *in vivo* properties by using the normal density of mouse feeders together with supplementing certain factors related to the sustenance of self-renewing mechanisms in their own culture system (Evans *et al*., 1990; Piedrahita *et al*., 1990a; Piedrahita *et al*., 1990b; Strojek *et al*., 1990; Talbot *et al*., 1993a; Talbot *et al*., 1993b; Anderson *et al*., 1994; Wheeler, 1994; Gerfen and Wheeler, 1995; Wianny *et al*., 1997; Chen *et al*., 1999; Li *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Brevini *et al*., 2005; Vackova & Madrova, 2006; Kim *et al*., 2007; Rasmussen *et al*., 2009; Son *et al*., 2009). These data indicate that the level of LIF composed of exogenous hLIF addition and mLIF provided by the mouse feeders, and the total amount of bFGF concentration made up by external administration and mouse feeder fibroblasts are in the range naturally producing ES cell lines when the porcine embryos developing epiblasts and all required intrinsic factors were used in this study. Thus, these results give more information to discover the most suitable culture environments for establishing the pluripotent ES and ES-like cells of the pigs and other farm animals in non-feeder culture system. In addition, deriving ES and ES-like cells from the inner cell masses of the porcine blastocysts is another challenge which requires further investigation.

However, neither the culture media successfully used to derive pES-like cells, the derivatives of those two culture media, or the mouse ES medium could be applied to support self-renewal state of pES-like cells obtained in this study when those pES-like cells were cultivated in a feeder-free culture system, thereby generating differentiated cells, which are very important in the conduction of required differentiated cells instead, together with the previous explanation stated in **Section 5.1** that FCS influences the cellular differentiation most (Wiles and Keller, 1991; Wiles and Proetzel, 2006), presumably because of its defined and undefined compositions (Freshney, 2005). Moreover, the concentration of 5-20% FCS basically drives the pluripotent ES cells to differentiate to other types of adult cells in the body when those ES cells are cultured in non-feeder culture conditions (Wiles and Keller, 1991; Hamazaki *et al*., 2001; Chinzei *et al*., 2002; Kim *et al*., 2003; Blyszczuk *et al*., 2006;

Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Yin *et al*., 2007). This implies that a variable percentage of KSR should be used instead of FCS, or either a reduction or elevation of the combination among dosages of KSR, FCS and other factors such as LIF and bFGF must be managed to derive the immortal ES or ES-like cells in feeder-independent culture system of the pigs and thereafter modification of the protocols could be applied to use with other farm animals. This is because most of the trial culture media handled to maintain self-renewal state of pESB-like cells in feeder-free culture system in this study consisting of 10-20% FCS and some ontological factors such as LIF and bFGF are not capable of supporting the undifferentiated state of the pESB-like cells. However, only the standard mouse ES medium used to support the ES cells in feeder-free culture system (**Appendix C**) has not been tested to sustain self-renewal state of fresh pESB-like cells obtained in this experiment, it was administered to examine the undifferentiated state maintenance only with the pESB-like cells that had been frozen in the liquid nitrogen by using slow freezing technique, in which 10% DMSO and 90% FCS had been made up and used as a freezing medium for the pESB-like cells. Therefore, it cannot be concluded that the mouse ES medium is not able to keep the self-renewing state of unfrozen pESB-like cells incubated in non-feeder culture system in this study because the properties of pESB-like cells hypothetically changed when they were put in the freezing medium resulting from the toxic effects of DMSO and high concentration of FCS. The effects of slow freezing processes could themselves positively prime the differentiation stage for pESB-like cells as shown that mouse ES cells have a poor re-culture quality (Kashuba Benson *et al*., 2008). Hence, finding the proper combination of freezing medium or other freezing techniques could help maintaining the intrinsic characteristics of ES cells, thereby decreasing the percentage of differentiation and cellular death. In addition, the standard mouse ES medium is highly recommended for examining the self-renewal supporting effect with the freshly-derived pESB-like cells again.

Recently, improving derivation and maintenance of mES cells in the artificial culture environments under a regulating condition based on well-known basic knowledge of self-renew and differentiation mechanisms previously described in **Section 1.2.3** has been proposed (Ying *et al*., 2008) and thereafter this

information could be used to establish the authentic rat ES cells in the same year (Buehr *et al*., 2008; Li *et al*., 2008). The scientists have announced that administration of some inhibitors that eliminate the inductive differentiation signals from ERK1/2 pathway controlled by FGF and GSK3β pathway without the supportive effects of STAT3 affected by LIF could derive and maintain the self-renew state of ES cells in those two species of the rodents. Thus, these findings could be theoretically applied to establish pluripotent ES cells in feeder-independent culture system of other mammalian species. However, one thing that must be kept in mind is that there should be many more classical intracellular signalling molecules controlling the self-renew and differentiation mechanisms that no one knows about. In addition, although those inhibitors are able to reproduce the stable ES cell lines in the rodents, this does not mean that their ES cells have the same quality and properties as the natural ones, as clearly seen in **Section 3.3** and **Section 4.3**.

As stated elsewhere in **Section 1.3** that induction of the effective certain types of cells from mammalian ES cells is another important step providing the great hope for human cell therapies, drug development and agriculture; therefore neuronal-like, neuronal supporting-like, smooth muscle-like and hepatocyte-like cells obtained from pESB-like cells in this study need to be further tested for their *in vitro* and *in vivo* functions. It is seen that somatic cells obtained in this study lack the functional analysis results, so some functional abilities of each cell type should be examined. For example, the action potential properties, ability of production of myelin sheath, capability of contraction, and secretion of some specific chemical substances such as albumin and urea should be determined in neuronal-like, neuronal supporting-like, smooth muscle-like and hepatic-like cells derived in this study, respectively, as documented in some prior reports (Chinzei *et al*., 2002; Blyszczuk *et al*., 2006; Schmandt *et al*., 2006; Yin *et al*., 2007; Agarwal *et al*., 2008; Sinha *et al*., 2009) in order to ensure that these certain differentiated cells are capable of performing their own functions effectively and efficiently; thereafter their results could be modified for manipulation with other mammalian species. Additionally, investigation of the most suitable differentiation protocol of each differentiated cell type must be

further studied in the pigs, as reported in **Section 5.3**, not only one type of differentiated cells was observed, but those somatic cells that belong to two germ layers were also established in the same culture medium such as DMh. Moreover, the maturity of general appearances of differentiated cells does not guarantee the complete programme of the intrinsic properties. This idea comes from the results received from the morphological analysis and gene expression not being ideally matched, except for the hepatic-like cells that seem to exhibit only the differentiated genes specific to their own germ layer cell type. Firstly, these records theoretically result from the factors such as FCS, LIF and bFGF, of which each has some capabilities to force undifferentiated cells to change to be any type of cell in the body (Austin and Burgess, 1991; White *et al*., 2001; Chinzei *et al*., 2002; Ying and Smith, 2003; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Yin *et al*., 2007), being composed in the designed culture media in this study. Nevertheless, the level of each combined ontological factors is the most important parameter to direct the required adult cells, as seen in some prior experiments showing that they all have received only adult skeletal muscle tissue, not the smooth muscle cells (Austin and Burgess, 1991; Austin *et al*., 1992; Bower *et al*., 1995; Kinoshita *et al*., 1995; Rao and Kohtz, 1995; Milasincic *et al*., 1996; White *et al*., 2001). It means that the combination of FCS, higher concentration of hLIF compared to some prior works (Austin and Burgess, 1991; Austin *et al*., 1992; White *et al*., 2001), and low to medium dosages of bFGF supplemented to the culture medium in this study plays a critical role via their certain intracellular signalling pathways in driving the myoblastic multiple stem cells to create smooth muscle cells instead of skeletal muscle. Hence, to achieve establishment of the desirable differentiated cells, suitable dosages and incubation period of differentiation protocol managed to direct differentiation must be considerate in association with its own species, as detailed in **Section 5.1** and **Section 5.3**. In addition, the timing of each gene expression of each induced somatic cell type along with cellular changes must be monitored to understand the development of each stage of differentiated cells as it has been definitely seen that no results of these aspects have been investigated in this study due to running out of good quality of undifferentiated pESB-like cells that could be used in directing differentiation. Secondly, pESB-like cells may possibly have the same internal properties as embryoid bodies in terms of

starting to yield all kinds of three germ layer lineage receptors awaiting for the activation of external factors when they were cultivated in the designed differentiation culture conditions in consequence with generating the certain types of somatic cells regulated by the effects of those factors. Thirdly, pESB-like cell presumably has its own sensitivity of responsiveness to the factors added in the culture medium, thereby giving the different formation time period of differentiated cells. Although the same differentiation processes were applied to induce the certain types of cells, is variety of specific differentiated gene expression of adult differentiated cells was exhibited in this study, similar to the results of undifferentiated characteristics of pES-like cells obtained in this study. Finally, it is possible that the concentration of each desirable differentiated gene is naturally reproduced so little or the RNA content of each specimen extracted is insufficient to manage to examine those required genes using the RT-PCR technique, as happened in the investigation of undifferentiated genes of pESB-like cells previously reported in **Section 4.3**.

Hence, the best way of yielding the undifferentiated and differentiated cells in farm animals is to use the defined serum-free medium in order to avoid the unwanted side effects originated from FCS, so there is still a long way to reach this point as it is seen that the designed serum-independent differentiation protocols providing required differentiated cells even in the mouse are still currently being investigated and improved (Okabe *et al*., 1996; Wiles and Johannsson, 1999; Tropepe *et al*., 2001; Hamazaki and Terada, 2003; Kim *et al*., 2003; Ying *et al*., 2003b; Ying and Smith, 2003; Schmandt *et al*., 2006; Tarasova *et al*., 2006; Wiles and Proetzel, 2006; Rao *et al*., 2009).

## 6.3 FUTURE ASPECTS OF DOMESTICATED FARM ANIMAL EMBRYONIC STEM CELLS USED AS MEDICAL TOOLS OF HUMAN DISEASES

Although embryonic stem cells are now considered to be one of the most powerful tools in drug development and human cell therapies, but it cannot be denied that great concerns of political policies and ethical issues dealing with living creatures, especially for human, lead scientists to find out other ways to study and cure human diseases rather than terminating embryos and to conquer immunoreactive rejection, so that reprogramming of somatic cells into pluripotent ES cell-like state, one of the most burning issues claimed to be the best technique used to replace embryos producing ES cells, has been proposed and is currently being studied (Yamanaka, 2008). To establish iPS cells by this technique, either the introduction of egg extract contents (Briggs and King, 1952), embryonic stem cells (Tada *et al*., 2001; Cowan *et al*., 2005; Yu *et al*., 2006) or certain outstanding self-renewing factors to the required differentiated cells (Takahashi and Yamanaka, 2006; Maherali *et al*., 2007; Okita *et al*., 2007; Wernig *et al*., 2007; Yu *et al*., 2007; Kim *et al*., 2008b; Nakagawa *et al*., 2008; Kaji *et al*., 2009; Woltjen *et al*., 2009) could successfully reprogramme adult cells to behave as pluripotent ES cells. However, induced ES-like cell state technique has still a long way to go before replacing fertilised embryos reproducing immortal ES cells resulting from there is still a lack of the complete stories confirming that the properties of iPS cells are equal to the authentic pluripotent ES cells and surely the incidence of unwanted effects such as tumors has presumably occurred through the use of any type of transfection as a tool to insert the required self-renewal genes to reprogramme the adult somatic cells (Kim *et al*., 2008b) stands as low as the natural ES cells. Hence, the best way is to study the iPS cells in parallel with the embryos deriving ES cells in order to compare the occurrence of advantages and disadvantages between them and another alternative way to avoid the controversial difficulties related to human rights and political issues concerning the performance of human ES studies generated from embryos is to use animal pluripotent ES cells as models of human diseases such as neurodegenerative diseases, muscle injuries, hormonal imbalances and etc. by applying the modification of validatory developed

knowledge that fulfills the gap between the species and human beings. The reasons stated above are leading the way for farm animals, especially for pigs, to be the first in line to be focused on as the most suitable animal model to study human illnesses, as their physiological responses, life span, body size and phylogenic tree are closer to human than those of other mammals, except for non-human primates. Additionally, the critical topics dealing with ethics are less serious than those for non-human primates (Thomson *et al*., 1995; Thomson *et al*., 1996; Thomson *et al*., 1998; Wolf *et al*., 2000; Suemori *et al*., 2001; Prelle *et al*., 2002; Mitalipov *et al*., 2003a; Li *et al*., 2004a; Li *et al*., 2004b; Gjorret and Maddox-Hyttel, 2005; Wang *et al*., 2005b; Mitalipov *et al*., 2006; Vackova and Madrova, 2006; Taupin, 2006; Brevini *et al*., 2007a; Brevini *et al*., 2007b; Keefer *et al*., 2007; Vackova *et al*., 2007; Hall, 2008). Thus, to develop the conceptual frameworks of derivation of immortal ES cell lines and induction of purified mature differentiated cells from the pluripotent ES cells in pigs or other domesticated ungulates not only benefits in the regenerative medicine and drug discovery, but also agriculture. Thus far, the data in the generation and sustenance of stable farm animal embryonic stem cells and their induced differentiated cells are still poorly understood, as detailed in **Chapter 3**, **Chapter 4** and **Chapter 5**. Moreover, only a few groups have been trying to develop some differentiation protocols by using their ES-like cells reproduced in some farm animal species such as bovine (Lazzari *et al*., 2006) and porcine (Talbot *et al*., 1996; Brevini *et al*., 2008), probably resulting from most procedures and chemical substances dealing with generation of good quality of embryos from such *in vivo* and *in vitro fertilization* techniques to be the source of producing ES cells that could be further used for inducing required differentiated cells being complicated and expensive, respectively. Hence, a great deal of effort and expense are also required in this research area in order to provide the alternative methods for human cell therapies, biotechnology and so forth.

In final conclusions, as described in **Section 3.3, Section 4.3** and **Section 5.3**. It is possible that, in the first place, some factors added into the culture media used in this study may be suitable to establish and sustain pES cell lines in feeder

culture system in the sense of interaction with the right embryonic stage under suitable environments. In the second place, different stages of porcine embryos need different culture media to derive and maintain their own pluripotent ES cells. In the third place, each pESB-like cell line has its own gene expression pattern. In the fourth place, acceptable undifferentiating gene markers currently used presumably originate from differentiated cells. In the fifth place, pESB-like cells obtained from this present study are highly potential to be stable pluripotent ES cells originating from early epiblast ES cell origin as mES cells. In the sixth place, not all ES-like cell lines are capable of generating chimeras with germ line transmission, as seen that MPM28P8 did not express DPPA-3. In the seventh place, immortal ES cells naturally derived theoretically have more than one pattern of ES cell production, as two patterns yielding pES-like cell were observed in this present study. In the eighth place, differentiation culture media used to induce pESB-like cells obtained in this study are able to produce some required adult cells belonging to all three embryonic germ cell lineages, even the evidence for hepatic differentiation was less clear. In the ninth place, colonies of ES cells themselves could serve as a source producing differentiated cells, as demonstrated in embryoid bodies. In the tenth place, the maturity of general appearance of differentiated cells obtained from pES-like cells induced in different culture media could not guarantee the complete programme of the intrinsic characteristics. Finally, mammals possibly share similar pattern of fundamental ES cell biology in the sense of self-renewal and differentiation states, as it is found that using basic knowledge of derivation and maintenance of self-renewing and differentiated cells in the mouse in consideration with fundamental porcine biology and new drug discovery could produce numbers of immortal pES-like and induced porcine adult cells belonging to all three embryonic germ cell lineages in this study.

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# APPENDIX A:

## MEDIA AND STOCK SOLUTIONS

## I Media and stock solutions for porcine embryo collection

### 1.1 PBS for flushing of porcine embryos

1 tablet of PBS (Sigma P4417) was dissolved in 200 ml of $dH_2O$, and then a combination of 1 mg/ml BSA (Sigma A3311) and 1x P/S (Sigma P0781), composed of 10,000 units/ml penicillin and 10 mg/ml streptomycin, was added to the solution and mixed gently using a magnetic stirrer until the solution was clear. Thereafter, the medium was filter sterilised using a 0.22 µm Millipore filter and then stored at 4ºC for up to 2 weeks.

### 1.2 BECM-3

All components used to make up this medium are listed in the table. Medium was adjusted to pH 7.25 using 1 M HCl (Sigma S2770) and osmolarity was between 270-300 $mOsm/kgH_2O$, then filtered sterilised using a 0.22 µm Millipore filter and stored at 4ºC for up to 1 month. Medium was supplemented with 3 mg/ml BSA (Sigma A3311) or 10%FCS (Gibco 10106-169) and then incubated under a humidified atmosphere of 5%$CO_2$ in air at 39ºC before use.

**BECM-3:**

| **Component** | **Company** | **Concentration** | **Weight or volume/ 100 ml medium** |
|---|---|---|---|
| NaCl | Sigma S5886 | 94.59 mM | 552.80 mg |
| KCl | Sigma P5405 | 6.00 mM | 44.70 mg |
| $MgSO_4$ | Sigma M2643 | 1.19 mM | 29.30 mg |
| $CaCl_2.H_2O$ | Sigma C3881 | 1.71 mM | 25.10 mg |
| $NaHCO_3$ | Sigma S5761 | 25.07 mM | 210.60 mg |
| Phenol red | Sigma P5530 | 5mg/ ml | 20 µl |
| D-glucose | Sigma G6152 | 5.56 mM | 100 mg |
| Sodium pyruvate | Sigma P5280 | 0.33 mM | 3.60 mg |
| L-glutamine | Sigma G1517 or Sigma G7513 (200mM) | 1.00 mM | 14.60 mg 50 µl |
| Sodium lactate (60%solution) | Sigma L7900 | 23 mM | 383 µl |
| MEM NEAA (100x) | Sigma M7145 | 1x | 1 ml |
| β-ME (50x) | Sigma B6766 | 1x | 2 ml |
| P/S (100x) | Sigma P0781 | 1x | 1 ml |
| $dH_2O$ | - | - | Up to 100 ml |

## II Media and stock solutions for culture of MEFs

### 2.1 Complete medium

| Component | Company | Concentration | Volume/ 100 ml medium |
|---|---|---|---|
| DMEM | Sigma D6429 | 90% | 90 ml |
| FCS | Gibco 10106-169 | 10% | 10 ml |
| MEM NEAA (100x) | Sigma M7145 | 1x | 1 ml |
| P/S (100x) | Sigma P0781 | 1x | 1 ml |

This medium was prepared and stored at 4ºC for up to one month.

### 2.2 Preparation of Mitomycin C

2 mg mitomycin C (Sigma M4287) was dissolved and mixed well in 200 ml complete medium. Thereafter, the solution was aliquoted into 10 ml volumes and stored at -20°C. Importantly, all waste solutions containing mitomycin C need to be collected in plastic bottles containing blue roll paper and Virgon and disposed of via clinical waste.

## III Media and stock solutions used to culture porcine ES-like cells and induced differentiated porcine ES-like cells

Two basic stock media, 16DM and 15KO, were prepared before the addition of the required supplements. Any medium coding with DM in this study was based on 16DM, while media coding with KO were based on 15KO unless otherwise stated.

### 3.1 16DM

| Component | Company | Concentration | Weight or volume/ 100 ml medium |
|---|---|---|---|
| DMEM | Sigma D6429 | 84% | 84 ml |
| FCS | Hyclone CIB perbio | 16% | 16 ml |
| Adenosine | Sigma A4036 | 0.03 mM | 0.80 mg |
| Thymidine | Sigma T1895 | 0.01 mM | 0.24 mg |
| Cytidine | Sigma C4654 | 0.03 mM | 0.73 mg |
| Guanosine | Sigma G6264 | 0.03 mM | 0.85 mg |
| Uridine | Sigma U3003 | 0.03 mM | 0.73 mg |
| MEM NEAA (100x) | Sigma M7145 | 1x | 1 ml |
| 7µl of BME (100x) diluted in 10ml of PBS | Sigma M7522<br>Sigma P4417 | - | 1 ml |
| P/S (100x) | Sigma P0781 | 1x | 1 ml |

### 3.2 15KO

| Component | Company | Concentration | Volume/ 100 ml medium |
|---|---|---|---|
| KO-DMEM | Invitrogen 10829-018 | 85% | 85 ml |
| FCS | Hyclone CIB perbio | 15% | 15 ml |
| MEM NEAA (100x) | Sigma M7145 | 1x | 1 ml |
| 7µl of BME (100x) diluted in 10ml of PBS | Sigma M7522<br>Sigma P4417 | - | 1 ml |
| P/S (100x) | Sigma P0781 | 1x | 1 ml |
| L-glutamine (200mM) | Sigma G7513 | 2 mM | 1 ml |

These two stock media were filter sterilised using a 0.22 μm Millipore filter and then stored at 4°C for up to 2 weeks.

## IV Media and stock solutions used to culture parthenogenetic porcine embryos

### 4.1 PVA-TL-HEPES

| Component | Company | Concentration | Weight or volume/ 1000 ml medium |
|---|---|---|---|
| NaCl | Sigma S5886 | 114.00 mM | 6.6633 g |
| KCl | Sigma P5405 | 3.20 mM | 0.2386 g |
| $NaHCO_3$ | Sigma S5761 | 2.00 mM | 0.1680 g |
| $NaH_2PO_4.H_2O$ | Fisions S/3760/60 | 0.34 mM | 0.0530 g |
| $MgCl_2.6H_2O$ | Sigma M2393 | 0.50 mM | 0.1017 g |
| Sodium lactate (60%solution) | Sigma L7900 | 10.00 mM | 1.868 ml |
| $CaCl_2.H_2O$ | Sigma C3881 | 2.00 mM | 0.2940 g |
| HEPES | Sigma H6147 | 10.00 mM | 2.3830 g |
| Sodium pyruvate | Sigma P5280 | 0.19 mM | 0.0220 g |
| Gentamycin | Sigma G3632 | 25 μg/ml | 0.0250 g |
| Penicillin G | Sigma P7794 | 100 IU/ml | 0.0650 g |
| PVA | Sigma P8136 | - | 0.1000g |
| MiliQ water | - | - | Up to 1000 ml |

All chemicals were dissolved together by using a magnetic stirrer at a low speed, the pH was adjusted to be 7.4 and the osmolarity was between 280-290 mOsm/kg$H_2O$. The solution was filtered through a 0.22 μm Millipore filter and then stored at 4ºC for up to 2 weeks. Medium was equilibrated in a humidified atmosphere of 5%$CO_2$ in air at 39ºC for at least 2 hours before use.

### 4.2 *In Vitro* Maturation medium (IVM medium)

**Handling medium:**

| Component | Company | Concentration | Weight or volume/ 20 ml medium |
|---|---|---|---|
| TCM 199 | Gibco cat.no.41150 | - | 20 ml |
| Cysteine | Sigma C7352 | 0.57 mM | 1.38 mg |
| PVA | Sigma P8136 | - | 20 mg |
| P/S (100x) | Sigma P0781 | 1x | 1 ml |
| L-glutamine (200mM) | Sigma G7513 | 2 mM | 20 µl |

The solution was filtered through a 0.22 µm Millipore filter and then stored at 4ºC for up to 2 weeks. Before use the solution was equilibrated in a humidified atmosphere of 5%$CO_2$ in air at 39ºC for at least 2 hours.

**FSH stock:**

400 mg Follitropin®-V (Vetrepharm, Ireland) was dissolved in 80 ml normal saline to make a stock solution of 5 mg/ml. The solution was then dispensed into 20 µl aliquots and stored at -20ºC. Working concentration of FSH was 5 µg/ml prepared from a dilution of FSH stock solution in IVM medium.

**LH stock:**

25 mg Lutropin-V (Vetrepharm, Ireland) was mixed gently with 5 ml normal saline to make up the stock solution of 5 mg/ml. The solution was then dispensed in 20 µl aliquots and stored at -20ºC. LH working concentration, 5 µg/ml, was obtained from a mixture of FSH stock solution diluted in IVM medium.

**IVM medium:**

| Component | Company | Concentration | Volume/ 2 ml medium |
|---|---|---|---|
| Handling medium | - | - | 2 ml |
| FSH stock (5 mg/ml) | Vetrepharm, Ireland | 5 µg/ml | 2 µl |
| LH stock (5 mg/ml) | Vetrepharm, Ireland | 5 µg/ml | 2 µl |
| EGF stock (10 µg/ml) | Sigma E4127 | 10 ng/ml | 2 µl |

All components of IVM medium were mixed gently prior to calibration in a humidified atmosphere of 5%$CO_2$ in air at 39ºC for at least 2 hours before use.

**4.3 Porcine Zygote Medium (PZM3)**

**PZM stock:**

| Component | Company | Concentration | Weight or volume/ 100 ml medium |
|---|---|---|---|
| NaCl | Sigma S5886 | 1.08 M | 6.3127 g |
| KCl | Sigma P5405 | 100.03 mM | 0.7457 g |
| $KH_2PO_4$ | Sigma P5655 | 3.57 mM | 0.0486 g |
| $MgSO_4.H_2O$ | Sigma M1880 | 4.02 mM | 0.0991 g |
| Water for embryo transfer | Sigma W1503 | - | Up to 100 ml |

All substances listed above were mixed gently and kept at 4°C until required.

**$NaHCO_3$ stock:**

1.5013 g $NaHCO_3$ (Sigma S5761) was homogeneously dissolved in 125 ml embryo transfer water and the solution was stored at 4°C until needed. Its final concentration was made up to be 142.98 mM.

**Gentamicin stock:**

50 mg Gentamicin sulfate salt (Sigma G3632) was dissolved in 1 ml normal saline and then stored at 4°C. A working concentration of 50µg/ml Gentamicin was used in maturation medium.

**PZM3:**

| **Component** | **Company** | **Concentration** | **Weight or volume/ 100 ml medium** |
|---|---|---|---|
| PZM stock | - | - | 10 ml |
| $NaHCO_3$ stock | - | - | 25 ml |
| Na Pyruvate | Sigma P5280 | 0.20 mM | 0.0022 g |
| Ca.lactate.$5H_2O$ | Sigma C8356 | 2.03 Mm | 0.0627 g |
| L-glutamine | Sigma G1517 | 1 mM | 0.0146 g |
| Hypotaurine | Sigma H1384 | 4.99 mM | 0.0544 g |
| BME (50x) | Sigma B6766 | 1x | 2 ml |
| MEM NEAA (100x) | Sigma M7145 | 1x | 1 ml |
| Gentamicin stock | - | 50 µg/ml | 100 µl |
| Water for embryo transfer | Sigma W1503 | - | Up to 100 ml |

All components were added and mixed using a magnetic stirrer at a low speed, the pH was adjusted to 7.3 and the osmolarity was between 280-290 mOsm/kg$H_2O$. The solution was sterilised using a 0.22 µm Millipore filter and then stored at 4ºC for up to 2 weeks. 10 ml PZM3 medium supplemented with 4 mg/ml BSA was filtered and equilibrated in a humidified atmosphere of 5%$CO_2$ in air at 39ºC for at least 2 hours before use.

### 4.4 Hyaluronidase stock (300 IU/ml)

100 mg Hyaluronidase (Sigma H3506)

1.1 g Polyvinylpyrrolidone (PVP; Sigma P5288)

110 ml $Ca^{2+}$-, $Mg^{2+}$-free Dulbecco's phosphate-buffered saline (DPBS; Sigma C8537)

All chemicals were gently dissolved and then the stock solution of 300 IU/ml Hyaluronidase was dispensed in 1 ml aliquots and stored at -20°C.

### 4.5 Cytochalasin B stock

10 mg Cytochalasin B (Sigma C6762)

1 ml Dimethyl Sulfoxide (DMSO; Sigma D5879)

Stock solution of cytochalasin B was placed in 10 µl aliquots and stored at -20°C. It was diluted with required medium to a final concentration of 5µg/ml before use.

## V Stock solutions for immunofluorescence staining

### 5.1 Rinse buffer

| **Component** | **Company** | **Concentration** | **Weight or volume / 100 ml medium** |
|---|---|---|---|
| BSA | Sigma A4503 | 1% | 1 g |
| Sodium azide | Sigma S2002 | 0.05% | 0.05 g |
| TWEEN-20 | Sigma P1379 | 0.10% | 0.10 ml |
| PBS | Sigma P4417 | - | ½ tablet |
| $dH_2O$ | - | - | Up to 100 ml |

### 5.2 Blocking solution

| **Component** | **Company** | **Concentration** | **Weight or volume / 100 ml medium** |
|---|---|---|---|
| BSA | Sigma A4503 | 5% | 5 g |
| Sodium azide | Sigma S2002 | 0.05% | 0.05 g |
| PBS | Sigma P4417 | - | ½ tablet |
| $dH_2O$ | - | - | Up to 100 ml |

Well-mixed rinse buffer and blocking solution were stored at 4 °C until required.

## VI Stock solutions prepared for examination of gene expressions using RT-PCR technique

### 6.1 DEPC treated water

2 ml Diethyl pyrocarbonate (DEPC; Sigma D5758)

998 ml Ultra pure water

A bottle that was pre-baked at 15 lb/in$^2$ for 20 minutes was used to mix 2 ml DEPC and 998 ml ultra pure water vigorously and then the solution was left overnight in a fume hood with a slightly opened lid. Thereafter, the solution was autoclaved at 15 lb/in$^2$ for 20 minutes to destroy DEPC by hydrolysis.

### 6.2 Tris EDTA buffer (TE buffer)

500 µl 1 M Tris HCl

100 µl 0.5 M Ethylenediamintetraacetic acid (EDTA)

49.4 ml DEPC treated water

500 µl 1 M Tris HCl and 100 µl 0.5 M EDTA were well-mixed in a 2 ml microcentrifuge tube, then transferred and diluted with DEPC treated water up to 50 ml. The solution was mixed gently and aliquoted into 5 ml volumes and stored at -20°C. It is important to bear in mind not to introduce any RNase contamination to the solution at any steps as this solution is used to reconstitute stock primers.

### 6.3 0.5 M EDTA

18.61 g Disodium EDTA.2$H_2O$

2 g NaOH

80 ml Ultra pure water

80 µl DEPC (Sigma D5758)

18.6 g disodium EDTA.2$H_2O$ and 2 g NaOH were added to 80 ml ultra pure water in a bottle that was prior baked at 15 lb/in$^2$ for 20 minutes. The solution was well mixed using a magnetic stirrer at a low speed. Then 80 µl DEPC was added to the solution in a fume hood and left overnight, and it was autoclaved at 15 lb/in$^2$ for 20 minutes to destroy DEPC by hydrolysis.

### 6.4 1 M Tris HCl

| | |
|---|---|
| 12.11 g | Tris base |
| 4.2 ml | HCl |
| up to 100 ml | DEPC treated water |

12.11 g Tris was dissolved in 80 ml DEPC treated water in a bottle that was pre-baked at 15 lb/in$^2$ for 20 minutes. 4.2 ml HCl was then added and mixed gently in a fume hood. DEPC treated water was added to a volume of 100 ml and then it was left overnight before autoclaving at 15 lb/in$^2$ for 20 minutes. It shuld be noted that solutions containing Tris should not be treated directly with DEPC as Tris inactivates DEPC.

### 6.5 50x Tris acetate EDTA buffer (TAE buffer)

| | |
|---|---|
| 242 g | Tris base |
| 57.1 ml | Glacial acetic acid |
| 100 ml | 0.5 M EDTA, pH 8.0 |

The components were well-mixed and the pH of the solution was adjusted to 7.2 with acetic acid. Then, water was added to make up the final volume of this solution to 1 L. To make up 10 litres of 1x TAE buffer for preparing and running agarose gel by the electrophoresis method, 200 ml of 50x TAE buffer was added with water up to 10 litres and then left on the bench at room temperature.

# ANTIBODIES

Primary and secondary antibodies were used with the immunofluorescence method in this study.

| **Markers** | **Primary antibody** | **Secondary antibody** | **Colour expression** |
|---|---|---|---|
| Oct-3/4 | Oct-3A/4 (N-19) goat IgG (Santacruz SC8628; 1:100) | D ∞ G IgG Texas Red (Jackson Immuno-Research Laboratories 705-075-147; 1:400) | Red |
| Nanog | Anti-Human Nanog IgG (Peprotech 500-P236; 1:100) | D ∞ R IgG FITC (Jackson Immuno-Research Laboratories 711-097-003; 1:20) | Green |
| Nestin | Rabbit anti-nestin IgG (Chemicon AB5922; 1:200 or Abcam AB5968; 1:100) | D ∞ R IgG FITC (Jackson Immuno-Research Laboratories 711-097-003; 1:40) | Green |
| α-smooth muscle actin | Mouse anti-human actin IgG (Chemicon CBL171; 1:100) | R ∞ M IgG FITC (DAKO; 1:40) | Green |
| AFP | Mouse anti-human alpha-fetoprotein IgG (Chemicon 2004189; 1:50) | G ∞ M IgG Cy3 (Jackson ImmunoResearch Laboratories 115-165-174; 1:50) | Red |
| DAPI (Vectashield H1200) | - | - | Blue |

# APPENDIX C:

## PRELIMINARY DATA OF INDUCED DIFFERENTIATED CELLS IN MOUSE

### AIM

The objective of these preliminary experiments was to testify whether mouse pluripotent ES cells themselves, not only the embryoid bodies, could be used as a source to conduct mouse immortal ES cells to be required specific types of cells as claimed in human ES cells (Yao *et al*., 2006). This was in order to gain ideas concerning the modification of induced differentiated cell protocols for application to the pESB-like cells obtained in this study resulting from no embryoid bodies being formed from porcine ES-like cells generated in this study.

### METHOD

The mouse ES cell line, CGR8, was cultured, as mentioned in **Section 2.7**, in a 4 well-dish at the density of 2-4 x $10^4$ cells/$cm^2$ in 10KOm [KO-DMEM, 10%FCS, 1x P/S, 1x MEM NEAA, 2 mM L-glutamine, and 100 μM ß–ME supplemented with 10 ng/ml mLIF], referred as mouse ES medium, in a humidified atmosphere of 5%$CO_2$ in air at 39°C for a few passages before they were induced into EBs or directly conducted into other types of cells by differently designed media and protocols. Each differentiated protocol had been carried out for at least 3 replicates before the results were concluded and the protocols were modified to apply to induced differentiation in pigs.

To form embryoid bodies, confluent mES cells were trypsinised for 5 minutes in a humidified atmosphere of 5%$CO_2$ in air at 39°C before mouse ES medium without addition of mLIF, called mouse differentiation medium, were applied to stop enzymatic reaction and then centrifuged at 1250 rpm for 10 minutes. A

high density of mES cells at 1-2 x $10^5$ cells/ml were suspended in mouse differentiation medium and cultured for 96 hours. The medium was changed half every other day, before the EBs were collected and subjected to induction into specific types of cells.

To conduct the differentiated cells represented as candidate cells of each germ layer, morphology of the cells observed by microscope was the only roughly key point used to identify the differentiated cells in these preliminary experiments. Therefore, those factors complimented in the culture medium were assumed to directed differentiation of the specific types of cells in which they were to be classified according to the embryonic germ layers they belonged to by determining their outstanding morphological appearance. Hence, it was expected that neuronal and neuronal supporting-like cells would be formed as representative cells for ectoderm, myocyte-like cells such as cardiomyocyte, skeletal and smooth muscle-like cells for mesoderm, and finally hepatocyte-like cells for endoderm induced by the designed culture conditions.

Induction of neuroectodermal cells was performed in 10KO40bm [KO-DMEM, 10%FCS, 1x P/S, 1x MEM NEAA, 2 mM L-glutamine, and 100 µM ß–ME supplemented with 40 ng/ml bFGF (Sigma F0291) and 10 ng/ml mLIF] and beating-like cells were cultured in 10KOm and 20KOm [KO-DMEM, 20%FCS, 1x P/S, 1x MEM NEAA, 2 mM L-glutamine, and 100 µM ß–ME added with 10 ng/ml mLIF] in a humidified atmosphere of 5%$CO_2$ in air at 39°C in non-feeder culture system. Medium was changed half every other day until those differentiated cells were observed under a light microscope. In case of directed differentiation of murine hepatocyte-like cells initiated from mouse EBs (mEBs) and mouse ES cells themselves, either mouse ES medium or 20KOm was used to culture those cells for 3 days in a humidified atmosphere of 5%$CO_2$ in air at 39°C, then addition of 10 ng/ml bFGF and 20 ng/ml HGF (R&D System Cat.no.2207-HG) in the mouse differentiation medium was supplied for 2 days each, respectively. Consequently, the combination of 20 ng/ml HGF, 10 ng/ml OSM (R&D System Cat.no.495-MO), 100 nM dexamethasone (Sigma D4902) and 5 µg/ml ITS (based on transferrin, Sigma I3146) were administered to the

mouse differentiation medium until the hepatocyte-like cells were observed in the culture system and the medium was changed half every other day. However, a decision was made to stop adding other growth factors in the group beginning with addition of 20KOm because neuronal-like cells and other undefined cells were revealed within 3 days.

## RESULTS

Induced differentiated cells of three embryonic germ layers were successfully performed in both mouse EBs, and ES cells served as sources of mouse pluripotent ES cells producing required differentiated cells. To start with directed differentiation of ectoderm cells, it was shown that 96 hour-old mouse EBs (**Fig. C1A**) could be changed to be neuronal-like cells forming nuclei or ganglion-like area in non-feeder culture medium, 10KO40bm, when they were cultured for 14 days (**Fig. C1B**). In the case of 20KOm medium conducting mouse EBs, neuronal-like cells forming nuclei or ganglionic-like areas and neuronal supporting-like cells were found at a certain period of time as 10KO40bm, as depicted in **Fig. C1C-D**, respectively. As regards to induced mES cells themselves into ectodermal cells, it was demonstrated that 2 day-old mouse pluripotent ES cells (**Fig. C2A**) shaped themselves like neuronal-like cells while they were cultured in feeder-free culture medium, 10KO40bm, for 14 days (**Fig. C2B**). In terms of directed mesoderm and endoderm cells, it was shown that 10KOm and 20 KOm were capable of generating beating-like cells originating from mEBs within 17 days of the experiment, as revealed in **Fig. C1E and C1F**, respectively. Moreover, the medium used to conduct mouse hepatocytes primarily from mEBs was also able to induce the beating-like cells between day 9-11 (photo not shown, only in video file) and thereafter binucleated hepatocyte-like cells were observed from day 14 in the different area of the culture chambers (**Fig. C1G**). In contrast, 20KOm was unable to induce beating-like cells originally from mES cells, but it provided undefined differentiated cells when those pluripotent ES cells were maintained in the medium for 7 days (**Fig. C2D**). Furthermore, the designed inducible mouse hepatocyte medium motivated some mES cells to differentiate into beating-like cells between days 7-9 (photo not shown, only in video file) and afterwards

mature binucleated hepatocyte-like cells occurred at day 21 (**Fig. C2C**). Notably, hepatocyte-like cells obtained from both mEBs and mES cells were mainly binucleated; one or up to 5 nuclei with different shapes of cells were possibly observed under the microscope when they were further cultured in the medium.

To sum up, these data indicate that the combination of the culture medium desgined above is able to induce representative cells of all three embryonic germ layers originally from both mEBs and mES cells in a certain period of time. Although the designed protocols are still not suitable to provide only the purified specific types of cells, but they are still good enough to apply to differentiate pESB-like cells.

## CONCLUSION

Mouse ES cells themselves could be used as an originated source to induce three embryonic germ layer cell types, as previously reported in human by certain culture conditions being applied to the cells, as explained above, so that pESB-like cells derived in this study could hypothetically be conducted into other types of cells in the body by applying the modified differentiated protocols suitable to the pig based on basic knowledge of drug development and fundamental biology of the pig.

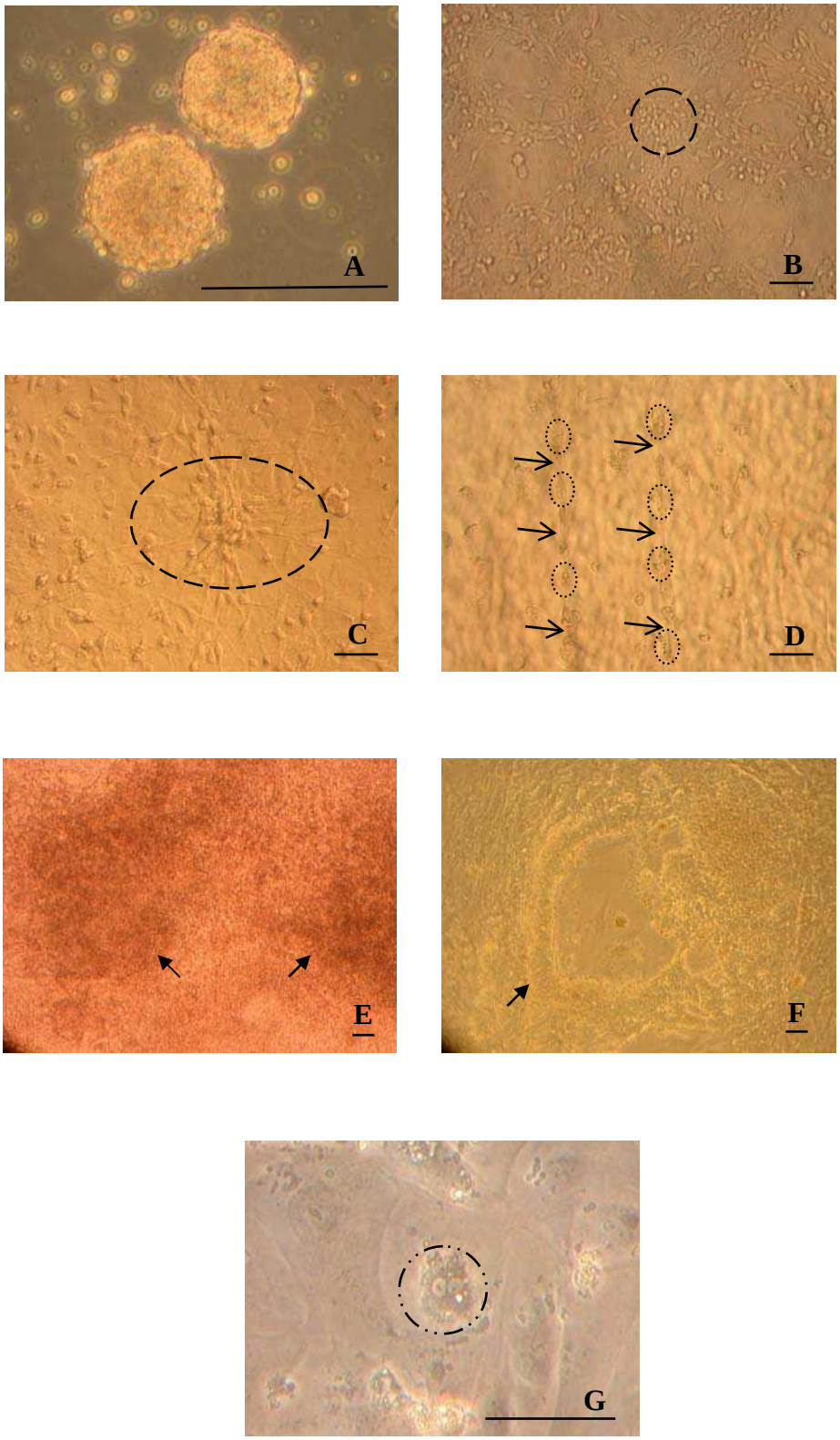


Figure C1

*Figure C1 Three embryonic germ layer cell types induced from murine embryoid bodies obtained at 96 hours in different feeder-free culture conditions.* **Fig. C1A**: murine embryoid bodies cultured for 96 hours by suspension technique in mouse differentiation medium, **Fig. C1B**: derived neuronal-like cells cultured in 10KO40bm for 14 days, **Fig. C1C-D**: generated neuronal and neuronal supporting-like cells cultured in 20KOm for 14 days, Fig. **C1E-F**: induced beating-like cells cultured in 10KOm and 20KOm for 17 days, respectively. **Fig. C1G**: conducted hepatocyte-like cells cultured in induced mouse hepatocyte medium Hep10KOm for 14 days. Scale bar = 50 μm

= neuronal-like cells forming nuclei-like or ganglionic-like area,

= migrating neuronal-like cells

= radial process of neuronal supporting-like cells

= area of beating cells

= binucleated hepatocyte-like cells

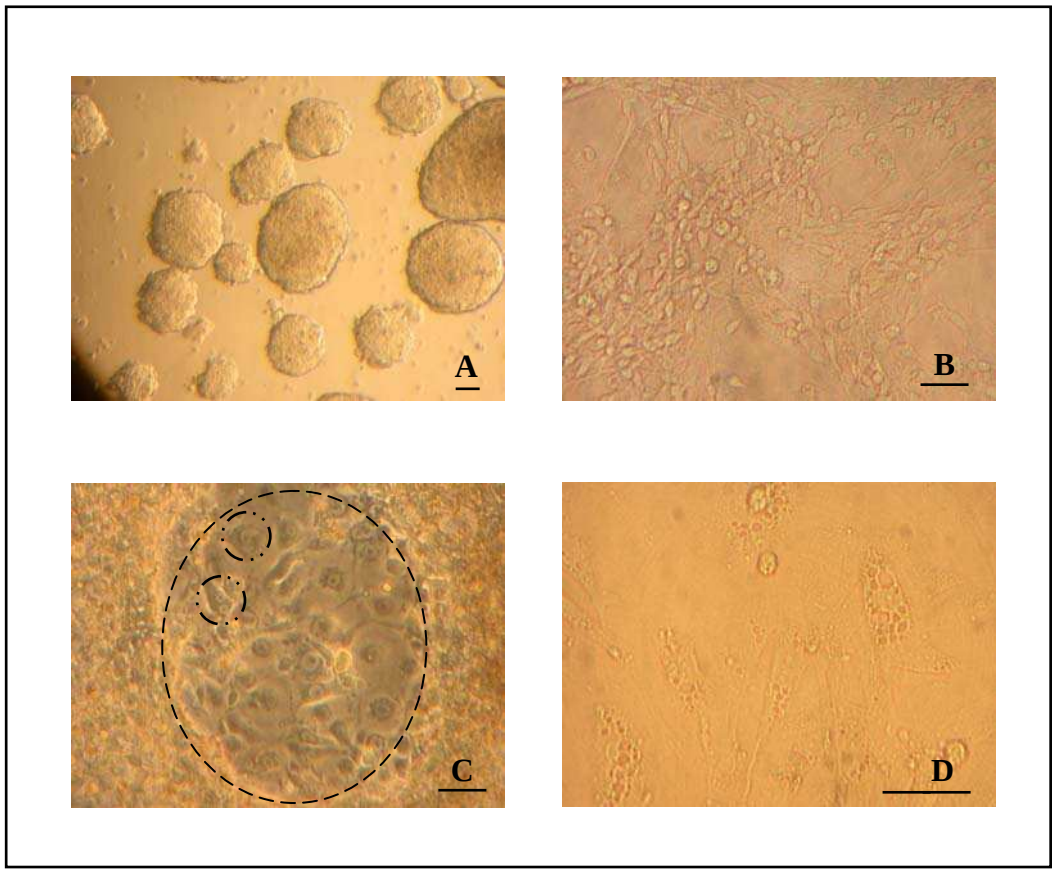


*Figure C2 Three embryonic germ layer cell types induced from originated murine embryonic stem cells themselves in different feeder-free culture conditions.* **Fig. C2A**: murine pluripotent ES cells cultured in mouse ES cell medium, **Fig. C2B**: derived neuronal-like cells cultured in 10KO40bm for 14 days, **Fig.C2C**: obtained hepatocyte-like cells cultured in induced murine hepatocyte medium Hep10KOm for 21 days, and **Fig.C2D**: reproduced unclassified cells cultured in 20KOm for 7 days. Scale bar = 50 μm.

= area of hepatocyte-like cells

= binucleated hepatocyte-like cells

# APPENDIX D:

## PRELIMINARY DATA OF TRYPSIN DISSOCIATION OF PORCINE ES-LIKE CELLS

### AIM

The objective of these preliminary experiments was to examine whether pESB-like cells could be dissociated into single cells by using enzymes in order to further manage them for other purposes such as passaging, and making EBs.

### METHOD

pESB-like cells were cultured as described in **Section 2.4**. A colony of pESB-like cells containing 2-4 x $10^3$ cells were placed into 20 µl of 1x Trypsin-EDTA for 5 minutes in a humidified atmosphere of 5%$CO_2$ in air at 39°C. Pipetting was performed to dissociate the colonies for a minute on a heated stage and then the solution containing pESB-like cells was either added into a fresh prepared 500 µl DM40bh for further culturing, or washed with 500 µl DM40bh for 2-3 minutes in order to stop the enzymatic reaction before the pESB-like cells were transferred to a hanging drop of 50 µl DM40bh without the addition of bFGF and hLIF for making EBs. Cells were observed and results were recorded daily.

### RESULTS

By microscopic analysis, 1x Trypsin-EDTA could not dissociate pESB-like cells when incubated and then pipetted on a heated stage. The colonies of pESB-like cells were held tightly after the enzyme was used to digest them. A day after, these pESB-like cells stopped growing and floated around in the culture medium of the ones for which trypsin was used for passaging. The result of these colonies of pESB-like cells stopping growing was related to the result of formation of EBs (photos were not taken). Those colonies were all discarded within 2-3 days.

### CONCLUSION

Trypsin could not be used to dissociate the colonies of pESB-like cells obtained in this study.

# APPENDIX E:

## MOUSE BLASTOCYST COLLECTION

### AIM

The objective of this experiment was to collect *in vivo* derived murine blastocysts at day 3.5 for investigation of gene expression.

### METHOD

Three pregnant 129/sv mice at day 3 of gestation purchased from the Harlan Company, UK, were transferred to the School of Biosciences, Sutton Bonington Campus, the University of Nottingham. The day of mating was referred to as Day 0. The mice were sacrificed and uteri were removed and transferred to lab by Dr. Jie Zhu. The uterine horns were then flushed and mouse blastocysts were collected under the microscope.

### RESULTS

By microscopic analysis, no blastocysts were determined among the embryos obtained from one pregnant mouse, while the other two mice were not pregnant. All data were confirmed by Dr. Jie Zhu. Moreover, there are no guarantees for pregnant mice under the age of 14 days of gestation ordered from Harlan. Furthermore, it is well-known that CDX-2, one of the most important genes designed to identify the origin of pESB-like cells in this study, can be detected in the trophectoderm of mouse blastocysts (Niwa *et al*., 2005; Strumpf *et al*., 2005; Deb *et al*., 2006; Brons *et al*., 2007; Tesar *et al*., 2007). Therefore, it was decided not to order any more pregnant mice for blastocyst collection in order to discover the condition of CDX-2 gene in this study.