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Embryonic Stem Cells and the Capture of Pluripotency

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1. Introduction

Stem cells have the ability to produce daughter cells, which may either differentiate into specialized cell types or remain uncommitted over repeated mitotic divisions, thereby maintaining the stem cell population. As such, stem cells offer great promise for research and medicine. They have potential uses in cell-based therapies such as blood or organ replacement, genetic engineering, drug and toxic substance screening and for studies in the fields of reproductive development and cancer. A number of different types of stem cell have been described which possess varying degrees of developmental potential. These may either be naturally-occurring *in vivo* (adult, fetal or primordial germ cells) or *in vitro* derived stem cell lines (embryonal carcinomas, embryonic germ cells, embryonic stem cells, extra-embryonic stem cells, trophoblast stem cells, epiblast stem cells or induced pluripotent stem cells). Here, we concentrate on the most extensively-researched type, embryonic stem cells (ESC), and how they are derived in mice. In order to understand why mouse ESC are regarded as the 'gold standard' for pluripotency to which other stem cell types are compared, we shall begin by outlining the origin, developmental potential and logistical suitability of the various forms of stem cell described so far.

2. The spectrum of stem cell types

2.1 Adult stem cells

The organs and tissues of adults harbour stem cells, many of which are multipotent and are, therefore, able to follow multiple differentiation pathways within a particular primary germ layer lineage. Adult stem cells have been derived from tissues such as the brain, blood, muscle, skin, pancreas, liver and from bone marrow, which contains both haematopoietic and mesenchymal stem cells. These somatic stem cells typically reside in small numbers in specific niches within the tissue or organ to which they contribute. They have a limited capacity for proliferation and may lie quiescent over many years until stimulated to divide in order to replace or repair injured tissue. Autologous somatic stem cells offer the potential for patient-specific treatment without the concern of transplant rejection. However, adult stem cells are difficult to isolate and propagate in cell culture, with a limited lifespan. The differentiation repertoire of any particular type of adult stem cell is restricted to a relatively

narrow range of cell types, and most somatic stem cell research has centered on haematopoietic stem cells, isolated from bone marrow and blood.

2.2 Fetal stem cells

Further types of stem cell that arise at an early stage during the course of natural reproductive development, include fetal and umbilical stem cells. Whilst umbilical cord blood contains a population of multipotential haematopoietic stem cells (HSC), fetal stem cells are capable of differentiation towards a wider range of cell types. Cord blood-forming stem cells differ from adult haematopoietic stem cells in gene expression, developmental potential, self-renewal, and regulation (Kim et al., 2007). At three to four weeks post partum, fetal stem cells take on adult HSC properties and become quiescent. Fetal HSCs display greater regenerative capacity on adoptive transfer to irradiated hosts than do adult HSCs. Pluripotent fetal stem cells can be isolated from amniotic fluid and placenta, and their capacity to differentiate into a wider range of cell types may offer an alternative to bone marrow transplantation because they elicit a weaker immune response from the host.

2.3 Germline stem cells

Also present in the juvenile and adult are germline stem (GS) cells that reside within a specific stem cell niche and are consistently proliferating asymmetrically to produce the progenitor cells for sperm and egg. Their developmental potential is even more restrictive within the body. Embryonic germ (EG) cells, however, are mouse cell culture lines derived from isolates of primordial germ cells from the genital ridges of post-implantation conceptuses (day E8.5 – E12.5). These stem cell cultures are pluripotent, capable of generating tissues from each of the three primary germ-layers (ectoderm, endoderm and mesoderm). They are non-tumorigenic when transplanted to immunologically matched host but have a limited proliferative capacity *in vitro* (70 – 80 cell divisions). They have been reported to produce chimeras when introduced into a host embryo and permitted to continue development within the maternal environment. Although EG cells have been derived in a number of species (mouse, pig, cow, human and chicken) germline transmission to the offspring of chimeras has only been reported for the mouse (Laborsky et al., 1994; Stewart et al., 1994) and, on one occasion, in the chicken (Chang et al., 1997).

2.4 Embryonic stem cells

During mammalian pre-implantation development, the period extending from fertilization to uterine implantation, the mouse zygote, following the first four cleavage divisions, gives rise to a rounded mass of compacted cells called a morula. Over the course of the next few divisions, two morphologically-distinct tissues are generated which form a blastocyst. The blastocyst comprises a monolayer spheroid of outer trophectoderm surrounding an inner cell mass (ICM) which becomes asymmetrically located within the blastocoelic cavity. By injecting single cells from early (3.5d) and mature (4.5d) mouse ICM into genetically dissimilar host blastocysts and mapping their contributions to the post-gastrulation embryo, Papaioanou and Gardner demonstrated that the mature ICM consisted of two populations of cells, hypoblast (primitive endoderm) and epiblast (primitive ectoderm). These elegant experiments revealed that it was the epiblast tissue which contributed most to the embryo proper, with hypoblast and trophoblast restricted to extra-embryonic regions of the resulting chimeras (Papaioanou & Gardner, 1975).

ESC are an *in vitro* cultured cell line derived from the epiblast of the early embryo. They represent the earliest developmental stage from which stem cells have been obtained. Perhaps reflecting their more primitive origin, these small, highly proliferative adherent cells can generate cell and tissue types from all three primary germ layers (ectoderm, endoderm and mesoderm) of the embryo, both *in vitro* and when used to generate teratomas *in vivo*. Furthermore, when introduced into a host embryo and replaced in the maternal environment, murine ESC can populate the gonads of the resulting chimeric fetus and transmit its genome to subsequent offspring.

Since their derivation in mouse was first described in 1981 (Evans & Kaufman, 1981; Martin, 1981), ESC have engendered much excitement and expectation within the scientific community and much debate amongst the public. Their arrival opened up the possibility of a vehicle for genetic manipulation and delivery to a host, making cell replacement therapy a more achievable goal. As such, murine ESC could be used to produce novel models for human diseases. Mouse ESC can be grown and expanded through many generations in culture, their genomes can be readily altered, and they can be reproducibly differentiated along various developmental pathways *in vitro*. The capacity of ESC when placed *in vivo* to differentiate into every cell type or organ within the body also permitted cell lineage and genetic investigations via the use of gene knockout mice. Thus they provide valuable insights into the earliest stages of embryonic development.

Characteristic of ESC are their morphology, growth pattern and expression of a range of genes now known to be associated with pluripotency. Mouse ESC (mESC) are small cells with a high nucleus to cytoplasm ratio. On feeder cell layers they grow as distinct domed colonies with no discernable intercellular space between neighboring cells. They have a population doubling time of ~12 hours, with almost no gap phases (G1 and G2) in their cell cycle, such that individual colonies display discernable daily growth. If maintained properly, mESC colonies should not show high incidence of spontaneous differentiation between passages and should retain a normal karyotype. Though routinely grown on feeder layers of mitotically inactivated fibroblasts in the presence of fetal bovine serum (FBS) containing medium to which the growth factor, leukemia inhibitory factor (LIF) is often added, mouse ESC can also be cultured under feeder-free conditions. With feeder-free culture on gelatin-coated or specially-treated tissue culture plastic, mESC have a tendency to form monolayers of cells with prominent nucleoli. When grown as a cell suspension in the absence of LIF and feeders, either in hanging drops, on a surface which resists attachment or in a matrix such as methylcellulose; ESC will aggregate together to form rounded, partially-organized structures known as embryoid bodies (EB) (Doetschman et al., 1985; Kurosawa, 2007). The EB provides an environment for ESC to coordinate and differentiate in a fashion similar to embryogenesis. Initially consisting of an outer rind of endoderm surrounding a core of primitive ectoderm after only just a few days' culture, EB can be grown either clonally from a single cell, or formed from aggregates of up to a thousand or more cells. They provide a useful tool for studying progenitor cell interactions and a means of assessing gene targeted mutagenesis effects. With use of differing growth factors and attachment matrices, EB can be encouraged to differentiate to a wide variety of tissue types such as cardiomyocytes (Wobus et al., 1991), skeletal muscle (Miller-Hance et al., 1993) or neuronal (Bain et al., 1995), pancreatic (Skoudy, 2004) and haematopoeitic (Schmitt et al., 1991; Fairchild et al., 2000) cell types. The opinion voiced by many who work on ESCs, that they are like babies – expensive to look after and requiring constant attention – may be something of an exaggeration, but ESC nevertheless require daily changes of medium

("feeding") and passaging to freshly-prepared feeders on average every 3 days (Brook, 2006). A distinct advantage of ESC is that they can be stored frozen under liquid nitrogen for many years without loss of viability.

The pluripotent status of ESC is evident by their expression of a range of cell surface, protein and transcriptional markers. Stage specific embryonic antigen-1 (SSEA-1) is expressed on the cell surface of mouse ESC as well as murine early epiblast and EC cells, and it becomes down-regulated as these cell types differentiate. Interestingly, undifferentiated human ESCs don't express SSEA-1; instead SSEA-3 and SSEA-4 are indicative of pluripotency. Alkaline phosphatase activity also correlates with the undifferentiated state in these cell types, as it does in the mouse. Foremost amongst pluripotency markers are the transcription factors, Octamer binding protein 4 (Oct4), SRY-box containing gene 2 (Sox2) and Nanog which are required to maintain the pluipotent state. Other genes that are highly expressed in uncommitted ESC but which are down regulated at the transcriptional level upon differentiation, include Rex1, Dppa5, utf1, Rex2 and Rif1 (Surani et al., 2010). Early differentiation marker genes, such as Brachyury (mesoderm), nestin (neuroectoderm) and GATA 4 (endoderm) are absent from ESC until differentiation is induced by removal of LIF.

2.5 Other stem cell lines derived from the mouse conceptus

The mouse pre-implantation embryo can also give rise to other types of stem cell. Each of the three primary germ layers of the blastocyst (trophoblast, primitive endoderm and primitive ectoderm) can yield stem cells, though only the epiblast is capable of giving rise to pluripotent stem cells. Trophoblast stem cells (TS) can be derived from the mural trophectoderm layer of mouse blastocysts cultured in the presence of fibroblast growth factor (FGF)-4 and heparin on feeders, or in feeder conditioned medium (Tanaka et al., 1998). FGF-4 is produced by the ICM and binds to FGF-4 receptors on trophoblast cells. The transcription factors Cdx-2 and Eomesodermin (Eome) have been found necessary for maintenance of this cell type. Extra-embryonic endoderm (XEN) stem cells arise if no growth factors (such as LIF or FGF) are added to serum-containing culture medium on feeders (Kunath et al., 2005). These are characterized by expression of GATA-4 and GATA-6 transcription factors. Both TS and XEN cells are restricted to their parental lineages when used to make chimeras. It is not just the mouse blastocyst which can give ESC; lines have been derived from as early in development as day 2.5 (Tesar, 2005; Chung et al., 2006). These cells were found to be equivalent to ESCs derived from blastocyst and demonstrate that the window of development from which ESC can be derived extends from as early as the 4-cell stage until the late blastocyst. That the implantation period represents the closure of this window was revealed with the discovery by two independent investigations, of mouse epiblast stem cells (EpiSC) (Tesar et al., 2007; Brons et al., 2007). EpiSC are stable pluripotent cells derived from the post-implantation epiblast (E5.5-E7.75), requiring FGF-2 for self-renewal. Like ESC they can be expanded almost indefinitely in culture as undifferentiated cells yet still retain their pluripotency. In culture, they can be readily differentiated into a variety of tissue types, perhaps representing a more committed developmental stage than mESC.

EpiSC can be derived from a variety of mouse strains including those considered non-permissive for ESC derivation, such as (B6 x CBA)F_1 and NOD. However their generation from a later stage in embryonic development is reflected by their inability to populate ICM and give rise to chimeras when injected into host blastocysts. Mouse EpiSC are considered to more closely resemble human ESC than mouse ESC. Both human ESC and mouse EpiSC

grow as flat epithelial colonies and require FGF and Activin/Nodal signaling to maintain their pluripotency. EpiSC represent the stem cell type which is developmentally closest to gastrulation, and so should provide valuable insight into the events pertaining to somatic and germ cell lineage determination.

2.6 Induced pluripotent stem cells

August 2006 saw the publication of potentially one of the most important findings in the field of stem cell biology. Takahashi and Yamanaka revealed that forced expression of just four reprogramming factors could transform fully differentiated somatic (mouse skin fibroblast) cells into embryonic-like stem cells, which they termed induced pluripotent stem cells (iPSC) (Takahashi & Yamanaka, 2006). Although this first iPSC line failed to generate viable chimeras, subsequent iPSC lines produced by retroviral integration of the four classical reprogramming factors, Oct3/4, Sox2, Klf4 and c-Myc, gave chimeras with germ-line transmission (Okita et al., 2007) and live mice by means of the tetraploid complementation assay (Zhao et al., 2009). Importantly other groups were able to reproduce these findings and, where feasible, extend them to human tissues (Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Lowry et al., 2008). iPSCs could be readily differentiated into a wide variety of somatic tissue types. However, concerns arose that retroviral and lentiviral vectors used in reprogramming adult tissues would permanently integrate into the cells' genome and that the oncogenic nature of some of the reprogramming factors, especially c-Myc, were responsible for tumours found in chimeras made from iPSC. The rapidly-expanding field of iPSC research soon led to the development of alternative reprogramming strategies and refinements to existing protocols. It was found, for instance, that the number of reprogramming factors could be reduced from four and that others (Nanog, LIN28) could be substituted. Although retroviral vectors remain the most common and efficient method of reprogramming, the possibility that random integration of transgenes into the genome might lead to tumorigenesis, prompted the development of vector-free systems. These include non-integrating adenovirus-based vectors, plasmids, modified insect-specific baculovirus and 'gene-free' systems such as charged protein transfection (Cho et al., 2010) and small molecules, which mimic the activity of the reprogramming factors and promote cell survival. However, the initial enthusiasm that iPSCs represented 'an ethical version of ESC' that could be used for patient-specific treatment of disease or injury, has been tempered by the concerns that iPSC may have a greater propensity to form tumours than ESC (Gutierrez-Aranda et al., 2010). As iPSC came under increasing scrutiny it emerged that they retain an epigenetic memory of the cell type from which they were derived, and that they preferentially differentiated towards a specific lineage linked to their cell of origin. These transcriptional, epigenetic and differentiation differences linked to the cell of origin are seemingly, mostly but not entirely, erased with continuous passaging. Furthermore, evidence is emerging that iPSC are not as indistinguishable from ESC as first thought. Gene expression mapping techniques indicate that they have a novel gene expression profile that is different from that of ESC, but similar amongst iPSC lines (Chin et al., 2009). Additionally, there are more than 500 genes which are differentially expressed between low and high human iPSC passage number, with iPSC becoming more like human ESC at higher passage, though still distinct. It has become evident that the potential for regenerative medicine that has been opened up by the discovery of iPSC elevates the continued study of ESC to an even greater degree of importance.

3. A historical perspective on the derivation of ESC

3.1 Establishing cultures conditions

The advent of mouse ESC was preceded by pioneering research into the establishment of cell culture lines from testicular teratomas (Stevens, 1967) and teratocarcinomas (Kleinsmith & Pierce, 1964). Teratocarcinomas are a subset of germ cell tumours that contain a disorganized array of many somatic and extra-embryonic cells, together with foci of stem cells. Their isolation led to embryonal carcinoma (EC) cell lines becoming the first self-renewing pluripotent cell line to be characterized. EC cells are thought to arise from transformed germ cells in the testis or ovary, they are now regarded as the malignant counterpart of ESC. Classic experiments conducted by Kleinsmith and Pierce in which transplantation of a single EC cell to a new recipient mouse was sufficient to regenerate a new tumour, suggested the pluripotent nature of EC cells. Human EC cell lines were then later obtained from testicular tumours (Andrews et al., 1984). Although of immense value as a research tool, EC cells differ from ESC in several aspects. EC cells have a limited capacity for differentiation, with many lines becoming nullipotent at higher passage number. In addition they are often karyotypically abnormal, particularly the human EC lines. These disparities between ESC and EC may reflect differences in adaptation to culture. The development and refining of culture techniques required for EC isolation and expansion proved to be fundamental to the derivation of mouse ESC.

Studies by Edwards and Paul in Glasgow during the 1960s, into the derivation of cell lines from *in vitro* cultured rabbit pre-implantation embryos, helped pave the way for the discovery of ESC in mice. These early investigations by Edwards and Paul resulted in two long lasting cell lines being isolated from the ICMs of 6d rabbit blastocysts explanted onto a collagen-coated culture surface. The cell lines possessed good proliferation rates, and stability in their secretion of enzymes, morphology and chromosomal complement. One was a fibroblastic type cell line, the other epithelial. Although developmentally-earlier rabbit embryos failed to yield cell lines (Cole et al., 1965; Cole et al., 1966), these investigations focused interest on the mature ICM as a source of progenitor cells. They established techniques and procedures that were to be of fundamental value in revealing the role played by the epiblast in fetal development, such as micro-injection of embryo cells into a host blastocyst to generate chimeras. In 1975, Sherman performed similar experiments with the mouse. Pooled intact blastocysts outgrown *in vitro* gave four cell lines which could be maintained in culture for over a year. However they became chromosomally abnormal and were not able to generate tumours when injected into syngeneic hosts. Sherman had employed the same highly rich medium (NCTC-109) with which Cole and Paul (1965) had achieved limited success. Whilst Cole and Paul reported observing proliferation of 5 – 20% of blastocysts cultured *in vitro*, Sherman obtained 90% hatching and attachment of blastocysts in culture (Sherman, 1975). The improvement may have been attributed to differences between mouse strains (see below) or to the careful selection of the heat-inactivated Fetal Bovine Serum (FBS) used. Currently, the batch testing of FBS for ESC/embryo culture work is standard practice in every laboratory, since commercial supplies can vary extensively in their suitability. Heat-inactivation may have destroyed both contaminants, such as mycoplasma, and heat-labile growth factors that encourage differentiation, such as FGF. FBS likely consists of a milieu of growth factors and biomolecules which probably varies significantly from batch to batch. However bone morphogenetic proteins (BMPs) have now been identified as one such component that has

an important role in maintaining ESC in their undifferentiated state (Ying et al., 2003; Qi et al., 2004). BMP, in particular BMP4, acts via the SMAD pathway to induce Inhibition of Differentiation (ID) genes, which suppress neural differentiation of ESC in the mouse. Why then, with critical components (medium and FBS) for long-term blastocyst culture apparently in place, was the attainment of undifferentiated pluripotent, self-renewing cell lines still so elusive?

It is now understood that the BMP contained within the FBS acts in conjunction with another growth factor produced by fibroblast feeder cells, or which can be added as a medium supplement, to maintain self-renewal of mouse ESC. That the fibroblast feeder cell layer produced both an attachment surface and secreted factor(s) conducive to stem cell growth, was known from the studies on EC cell lines. This secreted factor has now been identified as Leukemia Inhibitory Factor (LIF) (Smith et al., 1988; Williams et al., 1988). LIF is an interleukin-6 (IL-6) family multifunctional cytokine that is essential for maintaining self-renewal of undifferentiated mouse ESC, but is not required for such in either the human or rat. Adding recombinant LIF to mouse ESC culture medium removes the need for feeder cells; though many laboratories now routinely culture ESC on feeders with LIF supplemented medium. LIF, a factor also secreted by trophoblast cells, binds to a cell surface complex composed of the LIF receptor LIFRβ, and the transmembrane glycoprotein, gp130. This subsequently actives the transcription factor STAT3 (Signal Transducers and Activators of Transcription-3) that is essential and sufficient for suppression of mESC differentiation (Smith et al., 1988; Niwa et al., 1998; Matsuda et al., 1999). Whilst BMP induces ID genes to block the neural differentiation pathway, LIF stimulates STAT3 to constrain BMP from inducing mesodermal and endodermal differentiation. Recently, a chemically defined medium supplement (Knockout Serum Replacement; KSR) has been formulated which directly substitutes for FBS in media and requires only LIF to be added in order to maintain mESC in the pluripotent condition, either on feeders or under feeder-free conditions. KSR has been identified in our laboratory as an essential component in the efficient derivation of mESC lines, previously regarded as non-permissive.

One further point of interest when considering reasons for Sherman's improved mouse embryo cell line generation, is that blastocysts were cultivated in groups, rather than being plated out individually. Four hundred blastocysts were pooled together for his initial successful experiment, and cohorts of 25 blastocysts for subsequent experiments. Mouse embryos cultured in micro-drops under oil, in the absence of feeders or LIF, often give better morphological development to blastocyst stage when a number of embryos are pooled together, than when cultured individually (T.D and P.F. unpublished observations). During natural pregnancies, LIF is produced maternally by the endometrium and has an important role in trophoblast giant cell differentiation, which is a vital early step in implantation and invasion of the uterus. Addition of LIF to culture medium has been shown to enhance *in vitro* pre-implantation embryo development in several different species, including mice (Kauma & Matt, 1995), cows (Maquant-Le et al., 1993) and sheep (Fry et al., 1992), though the situation is not clear in the human (Jurisicove et al., 1995; Chen et al., 1999). Human ESC in culture do not require LIF, instead FGF-2 must be added to maintain the undifferentiated state. Mouse (and human) pre-implantation embryos produce both LIF and LIR receptor (LIF-R) mRNA transcripts, and the proportion of embryos expressing transcripts increases at the morula and blastocyst stages (Chen et al., 1999). Pooling a number of embryos together may have the effect of enhancing their collective responsiveness to LIF, in a paracrine or autocrine manner, and may result in more robust epiblast development. This

conjecture is supported by a study in which mouse embryos, cultured from zygote to blastocyst in medium supplemented with or without LIF, were used for ESC derivation. LIF supplementation had beneficial effects, increasing blastocyst total cell number through increased proliferative activity, especially of the ICM, and enhanced derivation of ESC (Rungsiwiwut et al., 2008). That mESC are able to promote their own growth has been indicated by the description of a Stem cell Autocrine Factor (SAF) secreted by the ESC themselves which supports their clonal propagation in serum-free medium and which is augmented by up-regulated autocrine Nodal signaling during early colony formation (Ogawa et al., 2006). Finally, Sherman reported fibroblasts often dominated his cultures, and these would have been a source of secreted LIF.

3.2 Derivation of ESC

In 1981 two laboratories working independently, brought together the factors crucial for generation of undifferentiated proliferating mouse stem cells. These factors included:

- Culture conditions conducive to good embryo development
- Expansion of the epiblast/stem cell progenitor population
- Removal of the trophoblast
- Separation of the epiblast from the hypoblast
- Establishment of primary colonies in the presence of BMP4 and LIF
- Propagation of primary colonies in the presence of LIF

Evans and Kaufman explanted batches of six whole blastocysts onto a tissue culture plastic surface and allowed them to attach and outgrow *in vitro* for four days. Under these culture conditions, outgrown blastocysts have a 'fried egg' appearance. The trophectoderm firmly attaches and spreads out resembling the flattened egg-white, whilst the ICM elongates to form an egg-cylinder (the yolk). Endodermal cells tend to migrate away from the base of the largely ectodermal egg-cylinder structure. At this stage Evans and Kaufman picked off the epiblast, enzymatically dissociated it and dispersed the resulting cell suspension into plates containing a layer of STO fibroblast feeder cells that had previously been mitotically inactivated. The medium used was Dulbecco's modified minimal essential medium supplemented with 10% FBS and 10% newborn calf serum. Colonies of pluripotent epiblast cells became visible by five days and these could be picked and passaged to fresh feeder plates following trypinisation. In addition to employing conducive culture conditions in a protocol that successfully removes epiblast from the influences of trophectoderm, Evans and Kaufman attempted to maximize the number of stem cell progenitors in the embryo by using delayed-implanting blastocysts. Delay of implantation is a natural occurrence in many species and can be induced in mice either by lactation or by maternal ovariectomy during the early pre-implantation period. Delayed embryos develop normally *in vivo* until the expanded blastocyst stage, shedding their zona pellucida as usual but then become quiescent before giant cell transformation and fail to implant into the uterus. They reach a maximal total cell number of about 130 (half of them epiblast) and can be stimulated to exit diapause and resume normal development, by explanting to culture or by giving maternal hormone injections. By utilizing facultative delay, Evans and Kaufman hoped to maximize the number of epiblast cells available at the time of plating. Using blastocysts from 129SvE mouse strain, over a period, they achieved up to 30% derivation success, establishing some 15 novel ESC lines. The four lines from their initial experiment described above, were all karyotypically normal, with an even sex ratio. These cells were termed "EK" cells by Evans and Kaufman, but they were essentially the first true ESC derived.

Interestingly, the second laboratory to derive pluripotent cell lines at that time, used a slightly different approach. Martin (1981) did not use delayed embryos, nor did she outgrow the blastocyst in order to pick the epiblast. Instead she isolated ICMs by use of immunosurgery to destroy the outer trophectoderm layer. ICMs from about thirty (ICR X SWR/J)F_1 blastocysts produced by superovulation and cultured overnight prior to immunosurgery, were plated onto mitotically-inactivated STO feeders in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The DMEM was supplemented with conditioned medium from EC cells cultured on fibroblasts, which had then been dialyzed and lyophilized and was effectively concentrated five fold at working dilution. Four colonies emerged within a week, and these were dissociated and re-plated onto feeder cells in conditioned medium, giving rise to embryonic stem cell lines which could be passaged on feeders without conditioned medium after the fifth passage. Isolated ICMs similarly cultured but with no conditioned medium, failed to yield ESC. The concentrated conditioned medium produced from EC cells cultured on STO feeders, would have been a rich source of LIF (and BMP4) sufficient to inhibit differentiation of the epiblast and to promote self-renewal. EC conditioned medium may contain other ESC self-renewal factors that are independent of the LIF/Stat3 pathway (Kawazoe et al., 2009). The four lines isolated, which Martin termed 'embryonic stem cells', showed some karyotypic abnormalities but expressed SSEA-1 and like the cell lines of Evans and Kaufman, were shown to be pluripotent both *in vitro* and *in vivo*. Martin was also able to demonstrate that ESC could be derived from other strains eg (C3H x C57BL/6)F_1 mice, albeit with a similar low efficiency (12%). That feeder cells can produce enough LIF has been demonstrated by Stewart et al. who showed that LIF null fibroblasts could not support self-renewal of mESC (Stewart et al., 1992). Why then were Evans and Kaufman able to derive ESC lines on feeders without additional LIF when Martin had found it an absolute requirement?

3.3 Significance of mouse strain on permissiveness of ESC derivation

It may have been serendipitous that Kaufman & Evans chose the 129 strain of mouse from which to attempt to derive ESC, or the success of their experiments may have been related to the propensity of this strain to produce teratocarcinomas from which EC cells had previously been derived (Stevens, 1958). However, other strains of mouse have since proven to be much more refractory to the derivation of ESC. Lines have been obtained from inbred stocks of C57 and BALB/c, but most emanate from 129 strain, though, genetically, this is not the ideal strain and requires time-consuming and expensive backcross breeding programs to transfer any genetic modifications to a more useful background. Despite these drawbacks, 129 are often still favoured over the commonly used C57BL/6 mouse because it has better germline competence (Seong et al., 2004). Until recently, strains such as SVB, CBA and in particular, NOD have been regarded as non-permissive for the derivation of ESC. Even when the occasional lines could be generated from these strains, they often fared badly at producing chimeras and were not able to show germline transmission (Roach et al., 1995; Chen et al., 2005). The CBA/Ca strain was originally selected for its low incidence of mammary tumours, and it seems that genetic background has a profound effect on propensity to generate ESC lines. It is probably for this reason that Martin was not able to generate ESC lines from (ICR x SWR/J)F_1 and (C3H x C57BL/6)F_1 mice by culturing on feeders alone, whilst Evans and Kaufman succeeded using the 129 strain. When she added conditioned medium equivalent to a five fold concentration of LIF, ESC lines were obtained.

Stage	Authors	Technique	Attachment substrate	BMP4	LIF	Mouse strain	Efficiency of derivation
Expand Epiblast	MJE & MHK	Delayed-implantation (2.5d for 4-6 days)	In vivo	-	Endogenous, pooled embryos (x6)	MJE & MHK 129 SvE	4x colonies from 6-12x blastocysts. (30%)
	GM	Overnight culture (3.5d to 4.5d)	None	FBS	Endogenous, pooled embryos (x30)		
Remove trophoblast	MJE & MHK	TB attaches and outgrows.	TC plastic + trophectoderm	FBS	Endogenous, pooled embryos (x6)		
	GM	Immunosurgery	-	-	-		
Isolate Epiblast from Hypoblast	MJE & MHK	Whole blastocyst cultured (4 days). Epiblast picked.	TC plastic + trophectoderm	FBS(10%)			
	GM	Cultured whole ICMs(<7 days). Epiblast picked.	Feeders cells	FBS(10%)	Feeder cells +con. med.	GM ICR x SWR/J C3H x C57BL6	4/30(13%) 1/8(12%)
Establish Primary colonies	MJE & MHK	Dissociated picked epiblast	Feeder cells	FBS(10%) + NCS(10%)	Feeder cells		
	GM	Dissociated picked epiblast	Feeders cells	FBS(10%)	Feeder cells + con. med.		
Expand Primary colonies	MJE & MHK	Dissociated colonies and passaged	Feeder cells	FBS(10%) + NCS(10%)	Feeder cells		
	GM	Dissociated colonies and passaged	Feeders cells	FBS(10%)	Feeder cells + con. med.		

Table 1. Comparison of important stages in the initial description of mESC derivation.

At this time, Evans & Kaufman iterated a number of criteria for a cell line to be regarded as a true ESC line. These included:

- The ESC must remain undifferentiated through repeated passages in cell culture
- The cells must maintain their normal karyotype
- They should retain their pluripotential capacity to differentiate both *in vitro* and *in vivo*
- An additional demonstration of true ESC status which can be tested for in mouse but not human, is that ESC should be capable of giving rise to chimeras when introduced into a host embryo and transferred to the oviducts/uterus of a pseudopregnant recipient
- That chimeric offspring display the capacity for germ line transmission to their descendents is compelling evidence of stem cell status

4. Benchmark assays for true mouse ESC status

4.1 Germline transmission

Obtaining germline transmission (GLT) is an important test of ESC pluripotency status, since it proves that the stem cell is capable of generating functional germ cells as well as somatic cell types. It involves injection of a small number (10 – 15) of ESC into the blastocoelic cavity of a genetically dissimilar host blastocyst, usually differing in coat colour. Successfully injected ESC will become incorporated into the ICM of the blastocyst (Figure 1) and when the host embryo is transferred to the oviducts or uterus of a pseudopregnant recipient mouse, the ESC can contribute fully to the normal embryological development following implantation. Contribution to the chimera is always quite variable, even for injected ESC from the same line, and some lines routinely give more chimeric off-spring than others. If the ESC are included in the formation of the gonads, germline transmission may be observed.

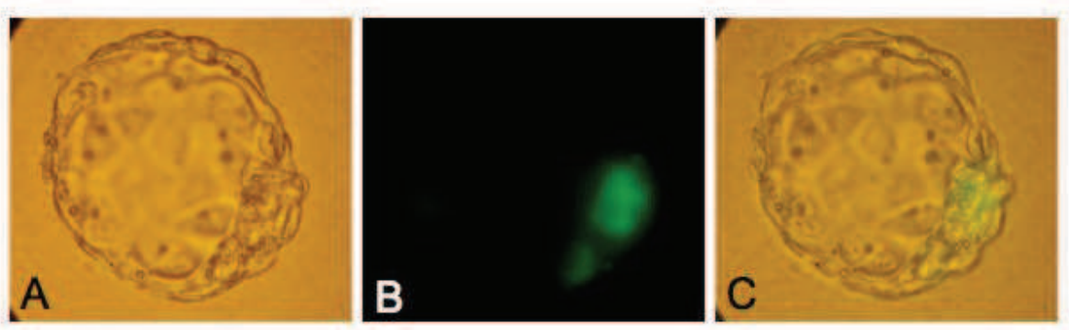

Fig. 1. Embryonic stem cells transgenic for GFP injected into GFP-negative blastocyst and cultured for 1 day become incorporated into host ICM. A) Bright-field phase contrast of an injected embryo. B) dark-field fluorescence showing the introduced ESC and
C) combination of low light plus fluorescence, revealing the location of the cells within the ICM.

4.1 Tetraploid complementation

Tetraploid complementation is a very stringent test of the ESC ability to form an entire embryo. In this assay, a tetraploid host embryo is formed by fusing together two cell blastomeres and culturing until morula formation. The resulting tetraploid morula cells are then dissociated and aggregated together with a similar number of ESCs. The cell aggregate re-organizes to form a blastocyst which can be transferred to a pseudopregnant recipient, where implantation will occur. Alternatively, the ESC may be injected into the blastocoelic cavity of a tetraploid blastocyst. The tetraploid host embryo is only able to contribute to extra-embryonic tissues, so that all embryonic lineages must be ESC derived. Live offspring with germ-line transmission represent powerful evidence of the ESC pluripotentiality. The strain-to-strain genetic differences inherent in mice that make ESC derivation more refractory in some strains are not fully understood, but may include chromosomal aberrations such as small changes in DNA methylation affecting gene expression. It has been recognized that, for blastocyst injection experiments, certain combinations of ESC and host embryos are preferable. 129 ESC are often injected into C57BL/6 hosts, whilst Balb/c blastocysts are often used as recipients for C57BL/6 ESC. However, for morula aggregation experiments using 129 ESC, CD1 or ICR are

the strains of choice. The reasons why some combinations work better than others may involve strain compatibility issues or relative rates of proliferation of ESC to ICM. Arai et al. have observed that NOD ESC, which typically had a very low GLT (1%), showed improved incorporation into the testes of chimeras when injected into NOD host blastocysts compared to genetically-dissimilar C57BL/6 recipient embryos (Arai et al., 2004). This may have been because their NOD ESC were significantly slower growing than the C57BL/6 ICM cells and so were out-competed at some stage in embryonic development.

4.2 Generation of chimeras by ESC injection

Strain variations are not the only factors affecting mouse chimera contribution, and subsequent germ-line transmission. Not least amongst these factors is the routine day-to-day culturing of stem cells prior to blastocyst injection. Mouse ESC are usually grown in humidified incubators at 37^{o}C under 5% CO_2. Chromosomal aberrations accumulate with time in culture, and each passage represents an opportunity for trauma. To minimize cumulative DNA damage, passage number for blastocyst injection should be kept low. Protocols for optimal culturing of stem cells vary from laboratory to laboratory, and different cell lines may have differing requirements. All protocols agree that to avoid increased spontaneous differentiation, ESC should not be allowed to approach confluency and that their medium should not be allowed to become exhausted before feeding (as evidenced by discoloration of the medium indicating a pH change due to metabolic by-products). In addition to the quality of reagents (FBS, LIF, medium, water source, etc.) and the requirement to use fresh feeders, as we shall discuss later, plating cell density is important. At passage, ESC should be dissociated to single cells/small clusters by gentle pipetting with a 1ml 'Gilson' tip or silliconised Pasteur pipette to avoid subsequent differentiation that would occur if larger clumps persisted. However ESC are gregarious and so should not be sub-divided too much on plating to fresh feeders (p2 – p4). For routine passaging every 2 to 3 days, depending on the rate of proliferation, the range 1:3 to 1:10 is typically observed when sub-dividing. The best way to determine when to sub-divide ESC, is to observe colony morphology by phase-contrast microscopy. The colonies should not coalesce, nor lose their smoothly domed appearance by the accumulation of small rounded cells at their surface. A typical figure for cell density in a flask ready for passaging is between 1-2 x 10^5 cells per cm^2. ESC intended for blastocyst injection to make chimeras should be split 1:1 to 1:3 the day before injection so that the cells are actively proliferating when harvested.

4.3 Homologous recombination in embryonic stem cells

Within three years of the initial description of ESC, Bradley et al. had demonstrated germ line transmission of the ESC genome from three lines, with a rate of chimera formation of more than 50% of live born pups. Seven of these were functional germ line transmitters (Bradley et al., 1984). A few years after that, germ line transmission of genetically altered mESC was achieved (Gossler et al., 1986; Robertson et al., 1986) and with the establishment of homologous recombination techniques (Smithies et al., 1985), gene targeting of specific alleles has since enabled the production of more than 6000 gene knockout mice (Koller et al., 1989; Thomas & Capecchi, 1990). These early endeavors have come to fruition with the setting up of the International Mouse Phenotyping Consortium (IMPC). This is an international co-operation that aims to generate germ line transmission of targeted knockout

mutations inESC for each of the 20,000 plus mouse genes on identical genetic backgrounds. The knockouts will be tested and phenotyped and a freely available database established which should provide an invaluable resource for mouse modeling of human diseases.

5. Advances in the derivation of ESC

5.1 Fibroblast feeder cells and conditioned medium

In addition to the huge influence that mouse strain has on the generation of ESC lines, other factors are also of importance. Foremost amongst these factors are the fibroblast feeder cells onto which the blastocyst/ICM is plated. A fibroblast feeder layer may not be an absolute requirement for the derivation of mESCs, but they have been found to improve growth and passaging of primary colonies, a critical stage in obtaining a cell line. Mouse primary embryonic fibroblast (MEF or PEF) feeder cells are best used at low passage (p2 – p4 for ESC derivation) and seeded the day before explanting the blastocyst. They are made mitotically inactive either by gamma irradiation or mitomycin C treatment so as not to overgrow the flask. The fibroblast seeding density should be between 5-10 x 10^4 cells per cm^2. The first ESC lines were isolated on STO fibroblasts (as described above), however mouse ESC can be derived and maintained on a range of fibroblasts. Rabbit spleen-derived fibroblasts (RSF) which express high levels of LIF and Wnt3A, have recently been shown to support mESC self-renewal in an exogenous LIF-free culture system. STO (fibroblasts from SIM mice that are thioguanine- and ouabain-resistant) are a continuous cell line utilized for EC culture. They have the advantage over MEF of not having a limited life span before they lose their proliferation enhancing abilities. However, STO have the disadvantage of requiring optimal culture conditions to prevent aberrant growth which may result in too much differentiating or too little proliferating growth factors. For some strains of mice, MEF have been shown to permit better ESC derivation than STO (Brook & Gardner, 1997). MEF are derived from E13.5 mouse fetus which has been decapitated and eviscerated prior to mincing and plating onto tissue culture plastic in medium containing 10% FBS. Fibroblasts may have their genomes transformed to include drug resistance genes which allow their survival along with ESC during selection experiments. Schoonjans et al. found that medium conditioned by rabbit fibroblasts transduced with the rabbit LIF gene permitted more efficient derivation and maintenance of mESC from ten inbred mouse strains, than adding recombinant mouse or rabbit LIF to unconditioned medium. In each case the ESC were derived on MEF (Schoonjans et al., 2003). Which factor(s) in the conditioned medium secreted by the rabbit fibroblasts were responsible are not yet known. However other IL-6 family cytokines are known to activate the gp130 signaling process that is triggered by LIF. These include related members ciliary neurotrophic factor and oncostatin M and the combination of interleukin-6 (IL-6) plus soluble interleukin-6 receptor (sIL-6R), which have been shown to support ESC derivation (Nichols et al., 1994).

5.2 Efficiency of mESC derivation

Schoonjans et al. (2003) achieved derivation rates of between 5% and 66% efficiency across the ten different inbred strains, including the CBA/CaOla mouse which was previously thought non-permissive. It is difficult to compare efficiency of derivation between publications because of inconsistencies in the criteria used to assess success rates. The efficiency rate described by Schoonjans et al. is based on number of cultured blastocysts that gave rise to ESC lines. Other reports are based on number of lines generated from pooled

blastocysts (McWhir et al., 1996) or from ICMs/epiblasts isolated after blastocyst culture period (Roach et al., 1995). Another inconsistency is that a number of separate clonal lines can be raised from primary colonies originating from the same embryo, perhaps from different epiblast progenitor cells, whereas on other occasions several primary colonies from a single embryo may be mixed and regarded as one polyclonal cell line (Robertson, 1987).

5.3 Genetic modification

A number of different strategies and refinements have been used to improve the efficiency of ESC derivation. The approach employed by WcWhir et al. (1996) was to derive ESC lines from hybrid embryos genetically modified for drug resistance linked to expression of a pluripotency marker gene. Non-permissive CBA strain mice were mated with the permissive C57BL/6J strain transgenic for a neomycin-resistance gene linked to Oct3/4 promoter, such that hybrid embryos possessing different levels of CBA genetic background were generated. Under G418 selection conditions, ESC lines could be generated from embryos containing as much as 87.5% CBA genetic background, although at a lower efficiency than embryos containing just 25% CBA (10.5% compared to 22.8%). The influence of strain difference on the capture of pluripotency was also highlighted by the work of Brook and Gardner who achieved very high success rates from (NOD x 129)F_1 x 129 hybrid embryos (88%) which could not be replicated with two strains of pure NOD genetic background (Brook et al., 2003).

5.4 Serum replacement

The defined serum-free substitute for FBS, KSR has been designed for use with an osmolarity optimized DMEM formulation, Knockout D-MEM. The KSR overcomes the problems of variability between FBS batches and is free from unknown differentiation-inducing factors. Using KSR as a direct substitute for FBS, Cheng et al. observed improved establishment of ESC lines from C57BL/6J blastocysts on feeders with LIF (10^3 U/ml) at 19% - 36% efficiency, with stable karyotype and GLT (Cheng et al., 2004). The authors observed that cells cultured in KSR medium are more readily dissociated following trypsinization, which is regarded as a critical step in early culture of ESC. Fibroblast feeder cells lay down a thicker basement matrix in KSR medium, which can often be removed intact during gentle trypsinization thereby removing more fibroblasts and enriching the remaining ESC population. ESC colonies adopt a more prominent domed morphology when KSR is substituted for FBS, which may provide a cell-to-cell contact environment more suitable to ESC proliferation (Figure 2).

Whilst KSR alleviates the uncertainties associated with FBS, it seems that a low concentration of serum may be beneficial. For an ESC line (MGZ5), normally resistant to clonal growth in medium supplemented with 10% KSR and LIF (10^3 U/ml), addition of a small amount (0.3%) of FBS to the KSR medium permitted ESC propagation from single cells in low-density culture (<100 cells/cm^2) (Ogawa et al., 2006). Bryja et al. described using KSR for generating mESC from C57BL/6J blastocysts, also in conjunction with feeders and LIF (10^3 U/ml), with a success rate of 50% – 75%. In these experiments KSR was alternated between pulses of FBS used following trypsinization steps to dissociate the ICM and primary colonies. These authors also reported that blastocysts explanted on feeders in KSR retained more ESC-like properties, such as expression of Oct-4 and decreased Erk kinase activation, than embryos similarly cultured in FBS (Bryja et al., 2006). KSR medium

has recently been shown to be more effective than serum-containing medium for obtaining iPSC from mouse embryonic and adult fibroblasts (Okada et al., 2010).

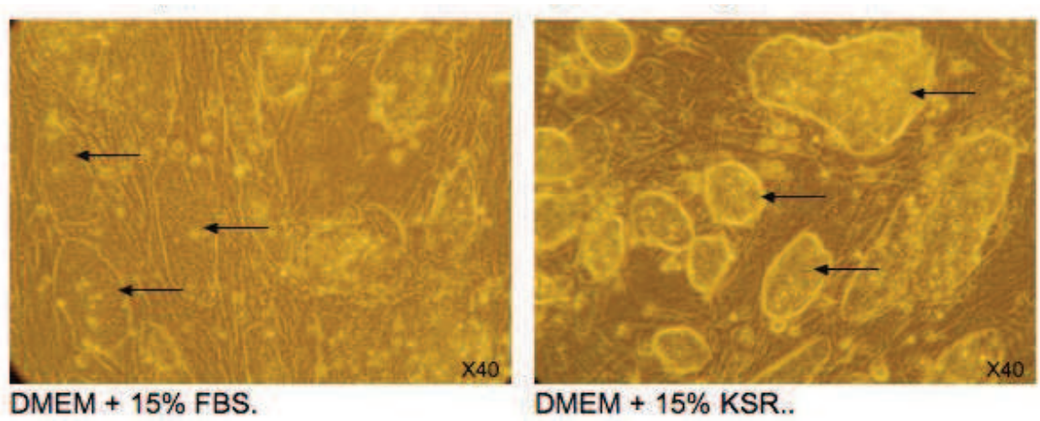

Fig. 2. Comparison of colony morphology between C57BL/6 ESC (EST29) cultured on feeders in either 15% FBS- or 15% KSR-containing medium with LIF. The arrows indicate individual ESC colonies which appear much flatter when FBS is used.

5.5 Growth factors

A number of external factors are known to contribute to stem cell self-renewal in the mouse. The LIF/gp130/JAK/Stat3, BMP4/Smad/Id and Wnt/GSK3β/β-catenin pathways combine to promote self-renewal without differentiation by activation of nuclear transcription factors such as Nanog, KLf4 and Oct3/4. Other growth factors such as SAF are also likely to be involved. However LIF plays a significant role in the induction of pluripotency. Insufficient activation of JAK/Stat3 restricts the acquisition of ESC status (Yang et al., 2010). Although it has become the standard to use LIF at 10^3 U/ml for both ESC maintenance and derivation, the range reported varies between 400 – 10^4 U/ml. A number of successful attempts of derivation from non-permissive strains have featured high levels of LIF (Baharvand et al., 2004; Yang et al., 2009). It is interesting to speculate, that, had Martin not used concentrated conditioned medium, she may not have been amongst the first to describe the derivation of ESC. Results from our own laboratory have indicated that by using elevated levels of LIF (5000 U/ml; ESGRO; Millipore UK) in conjunction with KSR medium, highly efficient rates of ESC derivation can be achieved from C57BL/6, Balb/k, CBA/Ca and even NOD mice (T.J. Davies & P.J. Fairchild, manuscript in preparation).

5.6 Small molecules

Small molecules which target specific signaling pathways have been shown to be useful chemical tools in manipulating cell developmental fate and function, by such means as replacing transcription factors. The POU transcription factor Oct-4 is a specific marker for pluripotent cells and is expressed by ICM and ESCs. Oct-4 expression by epiblast *in vivo* is lost just prior to differentiation following implantation and *in vitro* when explanted for ESC derivation. Hence it was considered that if Oct-4 could be maintained, then the number of ESC progenitor cells in the outgrowing epiblast might be increased leading to more efficient derivation. In 2003, Buehr and Smith reported that, by combining immunosurgical isolation of delayed-implanting ICM with culture in the presence of a small molecule that inhibits

Oct-4 loss, they could obtain CBA ESC at 25% efficiency (Buehr & Smith, 2003). The small molecule was PD98059, an inhibitor of the Erk activating enzyme MEK-1 that was known to be able to enhance self-renewal in established ESC lines. Cell based screening of synthetic chemical libraries revealed other candidate small molecule inhibitors that proved to be successful in mESC derivation from CBA and Balb/c strains (Lodge et al., 2005; Umehara, 2007). However these reagents, MEK-1 inhibitors PD98059 and U0126 and the GSK-3 inhibitor BIO, still required the presence of feeders and LIF. A small molecule, pluripotin (otherwise known as SC-1), was identified through feeder-, serum- and LIF-free screening which acted as a dual inhibitor of RasGAP and ERK1. Inhibition of RasGAP promotes ESC self-renewal by enhancing the phosphoinositide-3 kinase (PI3K) signaling pathway, whereas ERK1 inhibition blocks ESC differentiation. Pluripotin could be used to maintain undifferentiated ESC without LIF and when used in combination with LIF (2 x 10^3 U/ml), novel ESC lines were successfully derived from the normally refractory Balb/c (63%), CD-1 (80%) and NOD-scid (57%) strains (Yang et al., 2009). These derivations were conducted under an alternating FBS – KSR regime in the presence of 2x LIF (2 x 10^3 U/ml). However, the NOD-scid ESC required continuous presence of pluripotin and one out of the four lines displayed chromosomal abnormalities.

5.7 ESC from NOD mice

The Non-Obese Diabetic (NOD) strain is an important mouse model for human type 1 diabetes. More than 20 insulin dependent diabetes (Idd) loci are involved in the disease. It would be very desirable to have NOD ESC lines in which to perform gene knockout of potential Idd gene candidates or allele-shuffling strategies in which deletion of potential susceptibility genes could be followed by their replacement with identical genes from a diabetes-resistant strain. Unfortunately, the NOD mouse has proven to be the most refractory of all strains from which to produce ESC; and the pre-implantation NOD embryo is notoriously fragile *in vitro*. Until recently only one NOD ESC line had been generated but this had very low GLT (1%), was incompatible with feeder-free culture and prone to spontaneous differentiation on MEF (Nagafuchi et al., 1999). Interestingly, the line had been isolated using relatively high levels of LIF (10^4 U/ml) and required continuous culture in 3 x 10^3 U/ml LIF to prevent differentiation. The GLT of this line was later improved by batch testing of FBS and using NOD blastocysts as recipient embryos for injection of the NOD ESC (Arai et al., 2004). Even strategies which had proven successful in deriving ESCs from other non-permissive strains, such as CBA/Ca, were not able to succeed with NOD. Brook and Gardner had previously shown that by removing the inhibitory influence of the hypoblast (primitive endoderm) and trophoblast from 5 day mouse blastocysts by means of micro-dissection combined with brief enzymatic digestion; isolated epiblasts gave improved efficiency of ESC derivation when compared to whole embryos (Brook & Gardner, 1997). When epiblasts from implantation-delayed embryos were used, the efficiency of derivation of 129 ESC was increased even further, from 52% to 100%. Efficient rates of derivation using delayed isolated epiblasts cultured on MEF, were also demonstrated for other strains, including PO (56%) and CBA/Ca (21%). When LIF (10^3 U/ml) was also included 56% of the CBA/Ca embryos yielded ESC lines. Would these techniques, when applied to NOD embryos, result in novel ESC lines?

The pluripotency-suppressing nature of the NOD genome could not be overcome using these techniques (Brook et al., 2003). Brook et al. were also able to demonstrate that the ICR

mouse strain, from which the NOD strain was originally derived, was equally as refractory as the NOD. Researchers sought other avenues to explore and NOD EpiSC were produced, as described above (Brons et al., 2007). The generation of NOD EpiSC from 6.75d post-implantation epiblast required FGF-2 plus Activin A/Nodal signaling, rather than LIF/BMP4. However, these pluripotent cells, in common with other EpiSC, were not able to contribute to chimeras and were, therefore, unable to transmit their genome. Using two of the reprogramming factors reported by Takahashi and Yamanaka (2006) to be responsible for the production of iPSC, Hanna et al. infected NOD ICM with constitutive lentiviruses encoding Klf4 or c-Myc (Takahashi & Yamanaka, 2006; Hanna et al., 2009). Under routine conditions for mouse ESC derivation (DMEM + FBS + LIF on feeders), the ICM produced NOD ESC with a normal karyotype, which expressed pluripotency markers and gave chimeras with GLT. Unfortunately, ectopic expression of integrated c-Myc transgene lead to tumour formation in the chimeras. When small molecule inhibitors that are known to substitute for Klf4 and c-Myc in the generation of iPS cells, were applied to NOD ICM outgrowths then genetically unmodified NOD ESC were produced. The reagents used were Kenpaullone, a GSK3β and CDK1/ cyclin B inhibitor, the glycogen synthase kinase 3 inhibitor, CHIR99021, and the ERK-cascade inhibitor PD184352. Their use in various combinations generated NOD ESC which remained stable but which required the continuous presence of the inhibitors to prevent differentiation of the ESC. Applying the same principle of using a cocktails of either two or three small molecules ('2i' or '3i') to prevent differentiation and promote ESC expansion, Ying et al. applied a serum- and feeder-free system to generate ESC from both permissive 129 and non-permissive CBA and MF1 strains (Ying et al., 2008). The important question that arose from these studies was whether this approach would finally break the NOD barrier?

5.8 The ground state of pluripotency.

In 2009, Nichols et al. cultured NOD embryos from eight-cell to blastocyst in KSOM-2 medium in the presence of the mitogen-activated protein kinase kinase (MEK) inhibitor PD0325901(1μM) and the glucogen synthase kinase-3 (GSK3) inhibitor CHIR99021(3μM) – collectively called '2i'. The blastocysts were then expanded by culturing for a day in N2B27 medium with 2i and LIF, at an undisclosed concentration, prior to removing the zona pellucida and immunosurgery to destroy the trophoblast. Isolated ICM were then plated into gelatinized wells in N2B27 medium with 2i and LIF for 5-7 days. The ICM outgrowths were then trypsinized and replated under the same feeder-free conditions until primary colonies were given which could then be expanded to give NOD ESC lines. Thirty NOD embryos yielded sixteen ESC lines. Of the eleven lines tested, seven were karyotypically normal and four of these generated chimeras, three with GLT (Nichols et al., 2009). By removing exogenous signaling factors other than LIF and attachment on gelatin, coupled with inhibition of differentiation via MEK/ERK inhibition in combination with the promotion of proliferation and self-renewal via GSK3-inhibition, true NOD ESC were at last obtained. These cells were stable and pluripotent both *in vitro* and *in vivo*, and offspring of germ-line competent chimeras could be induced to develop diabetes. Furthermore, the ESC could be genetically modified and still retain the capacity for germ-line transmission, demonstrating that they represent a functional model for the human disease. The authors speculate that pluripotency is the basal state for epiblast/ESC and is self-maintaining in the absence of commitment cues. If perturbed by exposure to extrinsic factors, such FGF or

serum, differentiation occurs. Although LIF signaling is known to sustain self-renewal in mESC, it has been demonstrated not to be absolutely required for ESC generation under certain conditions (Ying et al., 2008). However its addition greatly improves efficiency. Activation of the Jak/Stat3 pathway (by LIF) is a limiting process in induction of pluripotency. The current picture is of a metastable pluripotent condition where the ESC exists in one of two states: as either in the naïve (ground state) or in the primed (committed) state (Nichols & Smith, 2009). The capture of pluripotency is, therefore, a balance between differentiation and self-renewal, increased LIF signaling tips the scales towards naïve ESC status.

5.9 Pluripotent stem cells from other species

Whilst ESC-like lines from some fifteen different species, including other mammals, chick and teleost fish, have been reported that meet some of the criteria as outlined by Evans and Kaufman and restated above, until recently only the mouse fulfilled all. In particular, no other species consistently gives as high levels of chimerism and none support germline transmission at high passage, as does the mouse. Nevertheless, the derivation, in 1995, of ES-like cells from the Rhesus monkey (Thomson et al., 1995) demonstrated that lines could indeed be made from primates. This raised the expectation that human ESC lines could also be made and with it the prospect of real advances in understanding the genetic and molecular processes of cell differentiation and proliferation that are central to conditions such as birth defects and cancer. In 1998 human ESC (Thomson et al., 1998) were isolated for the first time from surplus pre-implantation embryos produced *in vitro* by IVF and donated with consent. Traditionally, culture conditions for human ES have differed from those of mice; mice requiring LIF/BMP4 and/or feeders, whilst human require FGF-2/Nodal-Activin signaling. There are now many human ESC lines established. The advent of 2i/3i small molecule induction of pluripotency has opened up the door to production of ESC from species other than mouse (rat, vole, and rabbit). The first rat ESC derived using 3i culture conditions did not yield GLT (Buehr et al., 2008), however Li et al. derived rat ESC from explanted ICM in 2i culture conditions with an efficiency of 30-60%. Two of the nine rate ESC lines generated produced GLT (Li et al., 2008). Interestingly, none of the three rat iPSC lines so far described have been shown to be germline competent (Jacob et al., 2010). Although pluripotent stem cells have been derived from mouse, rat, monkey, pig and rabbit by reprogramming differentiated cells back to the ground state, only mouse and rat iPSC have shown GLT, although human iPSC can not be tested for obvious ethical reasons. The continued study of mouse ESC therefore remains the gold standard by which other pluripotent stem cell types should be measured.

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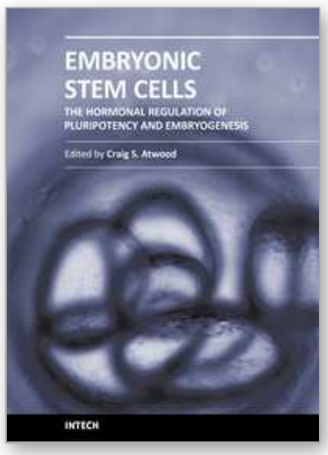

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