Biotechnology and Genetic Engineering Reviews - Vol. 27, 285-304 (2010)

Stem cells: The therapeutic role in the treatment of diabetes mellitus

GARY G. ADAMS,[1/3*], LEE BUTTERY,[2], SNOW STOLNIK[2], GORDON MORRIS[3], STEPHEN HARDING[3] AND NAN WANG [1]

[1/3]University of Nottingham, Faculty of Medicine and Health Sciences, Insulin Diabetes Experimental Research Group, Clifton Boulevard, Nottingham NG7 2RD UK; [2]University of Nottingham, School of Pharmacy, Faculty of Science, University Park, Nottingham, NG7 2RD, UK; [3]University of Nottingham, National Centre for Macromolecular Hydrodynamics, School of Biosciences, University of Nottingham, Sutton Bonington, LE12 5RD, U.K.

Abstract

The unlimited proliferative ability and plasticity to generate other cell types ensures that stem cells represent a dynamic system apposite for the identification of new molecular targets and the production and development of novel drugs. These cell lines derived from embryos could be used as a model for the study of basic and applied aspects in medical therapeutics, environmental mutagenesis and disease management. As a consequence, these can be tested for safety or to predict or anticipate potential toxicity in humans. Human ES cell lines may, therefore, prove clinically relevant to the development of safer and more effective drugs for patients presenting with diabetes mellitus.

*To whom correspondence may be addressed (Gary.Adams@nottingham.ac.uk)

Abbreviations: EC: embryonic carcinoma cells; EG: embryonic germ cell; PGC: primordial germ cells; ES:Embryonic stem cells; MSC: mesenchymal stem cells; ICM: inner cell mass; LIF: Leukaemia inhibitory factor; IL-6: interleukin-6, IL-11: interleukin-11, OSM: oncostatin M; CNTF: ciliary neurotrophic factor; CT-1: cardiotrophin-1; STAT: signal transduction and activation of transcription; SSEA-1: specific cell surface antigens; BIO: 6-bromoindirubin-3'-oxime; GSK-3: glycogen synthase kinase-3; DM: diabetes mellitus

Introduction

Stem cells are cells with the capacity for unlimited or prolonged self-renewal that can produce at least one type of highly differentiated descendant (Watt and Hogan 2000). Under the right conditions, or given the right signals, stem cells can give rise (differentiate) to the many different cell types that make up the organism. Stem cells have the potential to develop into mature cells with characteristic shapes and specialized functions, such as heart cells, skin cells, or nerve cells.

The first entity of life, the fertilized egg, has the ability to generate an entire organism, a capacity, defined as totipotency, which is retained by early progeny of the zygote up to the eight-cell stage of the morula. Hence, totipotent stem cells have the ability to form any embryonic or extra-embryonic cell type, including germ cells. Subsequently, cell differentiation results in the formation of a blastocyst composed of an outer trophoblast and undifferentiated inner cells, which are no longer totipotent, but retain the ability to develop into all cell types of the embryo proper. These pluripotent stem cells are capable of differentiating into cells from each of the three embryonic germ layers. Multipotent stem cells, which are able to form multiple organ specific cell types, are progressively restricted in their potential to differentiate (Figure 1).

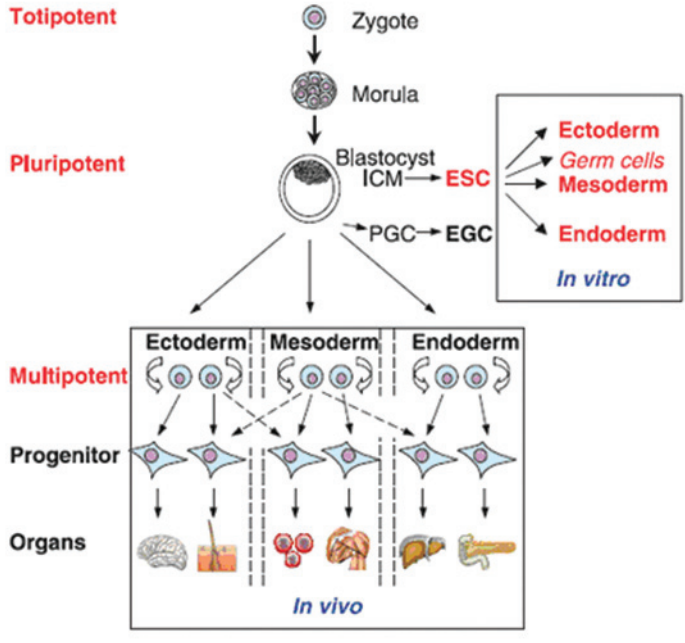

Figure 1. Stem cell and their hierarchy (Wobus, and Boheler 2005).

Stem cells research dates back to the early 1970s, when embryonic carcinoma (EC) cells, the stem cells of germ line tumours called teratocarcinomas (Smith, Heath *et al.* 1988), were established as cell lines (Jakob, Boon *et al.* 1973; Gearhart and Mintz 1974). Embryonic stem (ES) cells, and their tumorigenic counterparts, embryonal carcinoma (EC) cells, were among the first stem cells to be isolated and characterized extensively.

However, it has been suggested that the EC cells did not retain the pluripotent capacities of early embryonic cells and had undergone cellular changes during the transient tumorigenic state *in vivo* (Andrews 2002). In 1981, two groups succeeded in cultivating pluripotent cell lines from mouse blastocysts (Evans and Kaufman 1981; Martin 1981). These cells, originate from the inner cells mass of epiblast, termed as ES cells, can be maintained *in vitro* without any apparent loss of differentiation potential.

It has been confirmed that mouse ES cells showed their capacity to reproduce the various somatic cell types (Evans and Kaufman 1981; Wobus, Holzhausen *et al*. 1984; Doetschman, Eistetter *et al*. 1985), and moreover they were found to develop into cells of the germ line (Hubner, Fuhrmann *et al*. 2003; Geijsen, Horoschak *et al*. 2004). The other pluripotent embryonic cells type is primordial germ cells (PG cells), which form normally within the developing genital ridges. Isolation and cultivation of mouse PG cells on feeder cells led to the establishment of mouse embryonic germ (EG) cell lines (Resnick, Bixler *et al*. 1992; Labosky, Barlow *et al*. 1994; Stewart, Gadi *et al*. 1994). ES cells and EG cells differ in the conditions required for their isolation, culture, life span *in vitro*, and differentiation ability. ES cells can proliferate for as long as 300 population doublings and can be passaged for over a year in culture; EG cells can proliferate for only 80 population doublings (Thomson, Itskovitz-Eldor *et al*. 1998; Odorico, Kaufman *et al*. 2001). EG cells hold high ability to proliferate and differentiate *in vitro,* however, once transferred into blastocysts, EG cells retain the capacity to erase gene imprints. Human EG cell lines (Matsui, Zsebo *et al*. 1992) showed multilineage development *in vitro* but have a limited proliferation capacity and currently can only be propagated as embryoid body derivatives (Shamblott, Axelman *et al*. 2001). Over the past 4 decades, stem cells have been isolated from a wide range of sources, including mesenchymal stem cells (Prockop 1997) neural stem cells (Reynolds and Weiss 1992), and amniotic fluid stem cells (De Coppi, Bartsch *et al*. 2007) which were isolated from adult tissues (Oktem 2009).

Types of stem cells and their properties

Zygote and early cell division stages (blastomeres) to the ovular stage are defined as totipotent, because they can generate a complex organism. At the blastocyst stage, only the cells of the inner cell mass (ICM) retain the capacity to build up all three primary germ layers, the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGC), the founder cells of male and female gametes. In adult tissues, multipotent stem and progenitor cells exist in tissues and organs to replace lost or injured cells. At present, it is not known to what extent adult stem cells may also develop (transdifferentiate) into cells of other lineages or what factors could enhance their differentiation capability (dashed lines). Embryonic stem (ES) cells, derived from the ICM, have the developmental capacity to differentiate *in vitro* into cells of all somatic cell lineages as well as into male and female germ cells (Wobus and Boheler 2005).

Embryonic stem cell (ES cell)

The embryonic origin of mouse and human ES cells is the major reason that research in this field is a topic of great scientific interest and vigorous public debate, influenced

by both ethical and legal positions (Wobus and Boheler 2005; Bukovsky 2009). Undifferentiated ES cells share two unique properties, which are the unlimited self-renewal capacity and the ability to differentiate via precursor cells into terminally differentiated somatic cells.

ES cell is derived from the blastocyst stage of the embryo, a stage of embryonic development prior to implantation in the uterine wall. At this stage, the pre-implantation embryo is made up of 150 cells and consists of a sphere made up of an outer layer of cells (the trophectoderm), a fluid-filled cavity (the blastocoel), and a cluster of cells on the interior (the inner cell mass). Cells of the ICM are no longer totipotent but retain the ability to develop into all cell types of the embryo. Studies of ES cells derived from mouse blastocysts became possible 20 years ago with the discovery of techniques that allowed the cells to be grown in the laboratory. One of the current perceived advantages of using ESCs rather than adult stem cells is that ESCs have the ability to proliferate for long periods *in vitro* and can be directed to differentiate into a broad range of cell types.

Mouse ES cells

Mouse ES cell lines were first established in the early 1980s (Evans and Kaufman 1981; Axelrod 1984; Doetschman, Eistetter *et al.* 1985). The pre-implantation embryos (blastocysts) was isolated and cultivated on mouse embryonic fibroblasts followed by the expansion of primary ES cells outgrowths through careful trypsinization (Robertson 1987). The mouse ES cell lines displayed their unlimited *in vitro* proliferation ability (Smith 2001) and maintenance capacity to differentiate into all cell lineages (Ezhkova 2009; Teramura 2009).

The generation of mouse ES cell lines required the inactivated feeder layer cells; hence the property of feeder layer cells may be crucial to affect ES cells' proliferation and differentiation. It has been identified that Leukaemia inhibitory factor (LIF) is an important factor which responsible for controlling ES cells activities (Williams, Hilton *et al.* 1988). It has been demonstrated that cytokines, including LIF, IL-6, IL-11, OSM (oncostatin M), CNTF (ciliary neurotrophic factor), cardiotrophin-1 (CT-1), can be applied to maintain the pluripotency of mouse ES cells(Smith, Heath *et al.* 1988). They act via a membrane-bound gp130 signalling complex to regulate a variety of cell functions through signal transduction and activation of transcription (STAT) signalling (Burdon, Stracey *et al.* 1999). Quantitative measurements of ES cell phenotypic markers demonstrated a superior ability of LIF to maintain ES cell pluripotentiality at higher concentrations (>500 pM) which indicated a ligand /receptor signalling threshold model of ES cell fate modulation that requires appropriate types and levels of cytokine stimulation to maintain self-renewal (Viswanathan, Benatar *et al.* 2002).

It is now well established that undifferentiated mouse ES cells express specific cell surface antigens (SSEA-1) (Solter and Knowles 1978) and membrane-bound receptors (gp130) (Niwa, Burdon *et al.* 1998; Burdon, Chambers *et al.* 1999) and possess enzyme activities for alkaline phosphatase (ALP) (Wobus, Holzhausen *et al.* 1984) and telomerase (Prelle, Vassiliev *et al.* 1999; Armstrong, Lako *et al.* 2000). ES cells also contain the epiblast/germ cell-restricted transcription factor Oct-3/4 (Scholer, Hatzopoulos *et al.* 1989; Pesce, Anastassiadis *et al.* 1999). In ES cells, continuous Oct-3/4 function at appropriate levels is necessary to maintain pluripotency. A less than two-fold increase in expression causes differentiation into primitive endoderm and mesoderm, whereas loss of Oct-3/4 induces

the formation of trophectoderm concomitant with a loss of pluripotency (Niwa, Miyazaki *et al*. 2000).

Recently, two groups identified the homeodomain protein Nanog as another key regulator of pluripotentiality (Chambers, Colby *et al*. 2003; Mitsui, Tokuzawa *et al*. 2003). It has been demonstrated that the dosage of Nanog is a critical determinant of cytokine-independent colony formation, and forced expression of this protein confers constitutive self-renewal. Nanog may (Vallier 2009) therefore act to restrict the differentiation-inducing potential of Oct-3/4. Study also implicated that Wnt pathway activation by a specific pharmacological inhibitor (BIO; 6-bromoindirubin-3'-oxime) of glycogen synthase kinase-3 (GSK-3) maintains the undifferentiated phenotype in both mouse and human ES cells and sustains expression of the pluripotent stage (Sato, Meijer *et al*. 2004). Hence, the ES cell property of self-renewal, therefore, depends on a stoichiometric balance among various signalling molecules, and an imbalance in any one can cause ES cell identity to be lost (Figure 2).

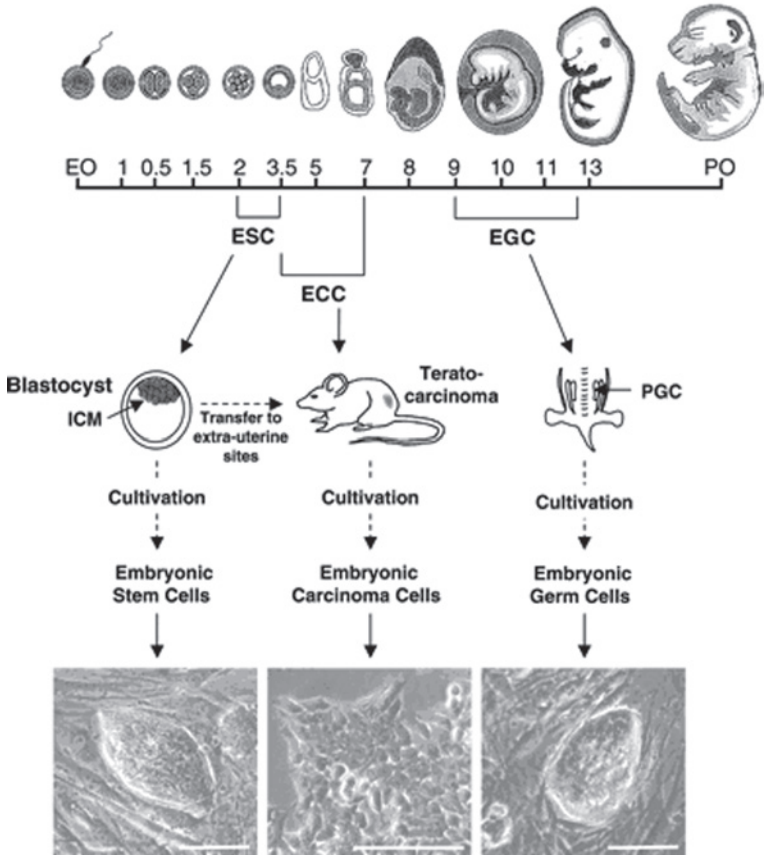

Figure 2. Developmental origin of pluripotent embryonic stem cell lines of the mouse. The scheme demonstrates the derivation of embryonic stem cells (ESC), embryonic carcinoma cells (ECC), and embryonic germ cells (EGC) from different embryonic stages of the mouse. ECC are derived from malignant teratocarcinomas that originate from embryos (blastocysts or egg cylinder stages) transplanted to extrauterine sites. EGC are cultured from primordial germ cells (PGC) isolated from the genital ridges between embryonic day *9* to *12.5*. Bar = 100 μm (Boheler, Czyz ***et al***. 2002).

Human ES cells

Pluripotent human embryonic stem (Doss MX) cell lines were successfully derived from the inner cell mass (ICM) of human blastocysts in the late 1990s. hES cells have similar characters with mES cells such as the Oct3/4 expression, telomerase activity, and the formation of teratomas containing derivatives of all three primary germ layers in immunodeficient mice (Thomson, Itskovitz-Eldor *et al*. 1998; Richards, Fong *et al*. 2002). Compared to mouse ES cells, hES cells present a longer average population doubling time (30–35 h vs. 12–15 h) (Amit, Carpenter *et al*. 2000). It has been demonstrated that LIF is insufficient to inhibit the differentiation of hES cells (Thomson, Itskovitz-Eldor *et al*. 1998; Reubinoff, Pera *et al*. 2000), which continue to be cultured routinely on feeder layers of MEFs or feeder cells from human tissues. LIF is unable to maintain the pluripotent state of hES cells (Daheron, Opitz *et al*. 2004), hence it has been demonstrated that the application of extracellular matrix-associated factors (such as Matrigel and laminin with MEF-conditioned media) need to be employed to improve the culture and maintenance of pluripotent hES cells (Xu, Inokuma *et al*. 2001; Kalkunte 2009).

Although the successful establishment of hES lines has been reported, these cells suffer from significant limitations including possible murine retrovirus infections (from the feeder cells) that have rendered them inappropriate for therapeutic applications in the previous studies (Wobus and Boheler 2005). By the end of 2001 about 70 hES lines had been established using feeder layers of mouse embryonic fibroblasts. As of December 2004, only 22 of the cell lines listed in the NIH register had been successfully propagated *in vitro* (Wobus and Boheler 2005). Importantly, hES cell lines have now been cultivated both on human feeder cells to avoid xenogenic contamination (Richards, Fong *et al*. 2002; Amit, Margulets *et al*. 2003; Amit and Itskovitz-Eldor 2006) and in the absence of feeder cells under serum-free conditions (Lee, Lee *et al*. 2005).

ES cells of other species

Pluripotent stem cell lines have been generated from other species, including chicken (Chang, Jeong *et al*. 1997), hamster (Doetschman, Williams *et al*. 1988), rabbit (Graves and Moreadith 1993) (Schoonjans, Albright *et al*. 1996), and rat (Brenin, Look *et al*. 1997; Vassilieva, Guan *et al*. 2000). Among all of these, the establishment of monkey ES cells represented special importance due to the possible application for human stem cell research, including Rhesus monkey (Lester LB 2004; Pau and Wolf 2004), common marmoset (Sasaki, Hanazawa *et al*. 2005) and cynomolgus monkey (Suemori, Tada *et al*. 2001). Monkey ES cells, characterized by typical markers of human ES (Oct-4, SSEA-4, TRA-1–60, TRA-1–81), have a high differentiation capacity *in vitro* (Thomson, Kalishman *et al*. 1995). These properties may qualify these cell lines as alternative and substitute model systems for hES cell lines. Moreover, after *in vivo* parthenogenetic development of *Macaca fascicularis* eggs to blastocyst-stage embryos, a pluripotent monkey stem cell line (Cyno-1) has been established that showed all the properties of hES cells, such as high telomerase and ALP activity; expression of Oct-3/4, SSEA-4, TRA 1–60, and TRA 1–81; and the ability to differentiate into various cell lineages (Vrana, Hipp *et al*. 2003).

Adult stem cell

It is already known that fetal organs as well as adult tissues, with high self-renewal capacity, contain regenerative stem cell populations : blood, skin and gut. Adult stem cells are also known as fetal/somatic stem cells or tissue-derived stem cells which include both multi-potentiality stem cells, such as adult mesenchymal stem cells (hMSC) (Meirelles 2009; Chugh 2009), and the uni-potentiality cell, such as epidermal stem cells. Adult stem cells are present in most tissues and are responsible for the replenishment of those tissues throughout life. Adult stem cells differ from embryonic stem cells not only in their different lineage potentials, but also in the mechanism by which they proliferate. Adult stem cells divide asymmetrically to maintain their number in the tissue, while at the same time giving rise to cells committed to becoming differentiated tissues and organs; however, embryonic stem cells normally divide symmetrically (Serakinci and Keith 2006).

Adult stem cells are rare but the list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas (2001) and their plasticity restricted. A number of recent investigations have suggested that they may be more plastic than previously thought. Haematopoietic stem cells (Ainsworth 2009; Parrish 2009) from the bone marrow had the capacity to develop into neural, myogenic and hepatic cell types and neuronal or muscle stem cells developed into the haematopoietic lineage (Bjornson, Rietze *et al*. 1999). Moreover only an estimated 1 in 10,000 to 15,000 cells in the bone marrow is a hematopoietic (blood forming) stem cell (HSC) (Weissman 2000).

Their primary functions are to maintain the steady state functioning of a cell (homeostasis), with limitations, to replace cells that die because of injury or disease (Holtzer 1978). These observations follow that somatic cells of the adult organism may yet have a high plasticity, and their developmental potential may not be restricted to one lineage, but could be determined by the tissue environment in the body (Watt and Hogan 2000). Furthermore, adult stem cells are dispersed in tissues throughout the mature animal and behave very differently, depending on their local environment.

Hence, adult stem cells offer new potential for the biomedical therapy, although the cells proliferation and differentiation mechanisms remains unclear. The limitation of adult stem cell include: 1) the proliferation and differentiation capacity may be lower than ES cells; 2) adult stem cells may contain DNA abnormalities caused by exposure to environmental factors, and stem cells of certain patients may contain genetic defects and therefore would be inappropriate for transplantation.

Stem cells: Therapeutic interventions in the treatment of Diabetes mellitus

Due to their unlimited unique self-renewal and potential development properties, ES cells can be derived into multi-lineage cells and used as alternative resource of transplantation therapy. To fulfill the therapeutic application, efficiency of directed differentiation into specific somatic cell populations need to be improved to satisfy clinic requirements. Stem cells represent the nature of embryonic development and tissue regeneration. The established permanent ES cell lines can be regarded as a ver-

satile biological model system that leads to major advances in cell and developmental biology and this includes the pancreas.

The mature pancreas plays a central role in glucose homeostasis and metabolism, has two morphologically and physiologically distinct components, the endocrine pancreas and the exocrine pancreas (White 1973). The exocrine pancreas comprises the bulk of the tissue and is made up of acinar cells that specialise in the secretion of digestive enzymes via the epithelial pancreatic ducts. The ductal system transports the digestive enzymes to the intestine where they ensure nutrient digestion and absorption. The endocrine part of pancreas is scattered irregularly throughout the exocrine pancreas, with the majority identified as small spheroid clusters of cells, called the islets of Langerhans (Soria, Roche *et al*. 2000). The islets are distributed throughout the whole pancreas and vary in size with a higher density in the tail of the organ. Apart from the typical islets, small endocrine clusters and single pancreatic endocrine cells can also be found occasionally in the pancreas. The islets are a heterogeneous population of 4 main types in the following proportions: 65-90% insulin-producing βcells, 15-20% glucagon-producing α-cells, 3-10% somatostatin-producing δ-cells and 1% pancreatic polypeptide producing PP-cells (Rahier 1988) and under normal physiological conditions glucose homeostasis and metabolism is maintained; in the patient presenting with DM, however, imbalance can lead to micro-and-macrovascular disorders.

During gastrulation, the epiblast, consisting of multi-potential cells, generates three embryo layers, including endoderm, mesoderm and ectoderm (Figure 3). The gastrointestinal organs, including the pancreas, are derived from the endodermal layer. The induction of the endoderm appears to be governed by nodal/transforming growth factor (TGF-β) signalling from the adjacent ectoderm and mesoderm within the primitive streak and the node (Wells and Melton 1999; Lewis and Tam 2006). After gastrulation, the endoderm is patterned in an anterior-posterior fashion in response to fibroblast growth factor (FGF-4) signals from adjacent mesoderm (Dessimoz, Opoka *et al*. 2006). It has long been recognized that mesenchymal-epithelial signalling is important during subsequent growth and differentiation of the pancreatic epithelium (Golosow and Grobstein 1962). Epidermal growth factor is another mesenchymal factor that has been shown to regulate proliferation of the developing pancreas both *in vitro* and *in vivo* (Miettinen, Huotari *et al*. 2000). Other mesenchymal factors, such as follistatin, regulate cell-type specific differentiation within the pancreatic epithelium. In the absence of mesenchyme, pancreatic epithelium gives rise to endocrine cells, but not exocrine cells (Gittes, Galante *et al*. 1996; Miralles, Czernichow *et al*. 1998). Notch signalling is another key regulator of pancreatic cell growth and differentiation. During the early stages following the formation of pancreatic primordial, Notch signalling is essential for the expansion of undifferentiated progenitor cells (Hart, Papadopoulou *et al*. 2003; Norgaard, Jensen *et al*. 2003). During subsequent stages, Notch is frequently used to control the sequential generation of different cell types from a common progenitor cell and consequently helps to specify endocrine cell differentiation (Esni, Ghosh *et al*. 2004). It has been reported recently that the Wnt signalling pathway is also crucial in pancreatic organogenesis (Murtaugh, Law *et al*. 2005) (Papadopoulou and Edlund 2005) and that activation of Wnt/β-catenin signalling at a later time point in pancreas development causes enhanced proliferation of acinar cells (Heiser, Lau *et al*. 2006).

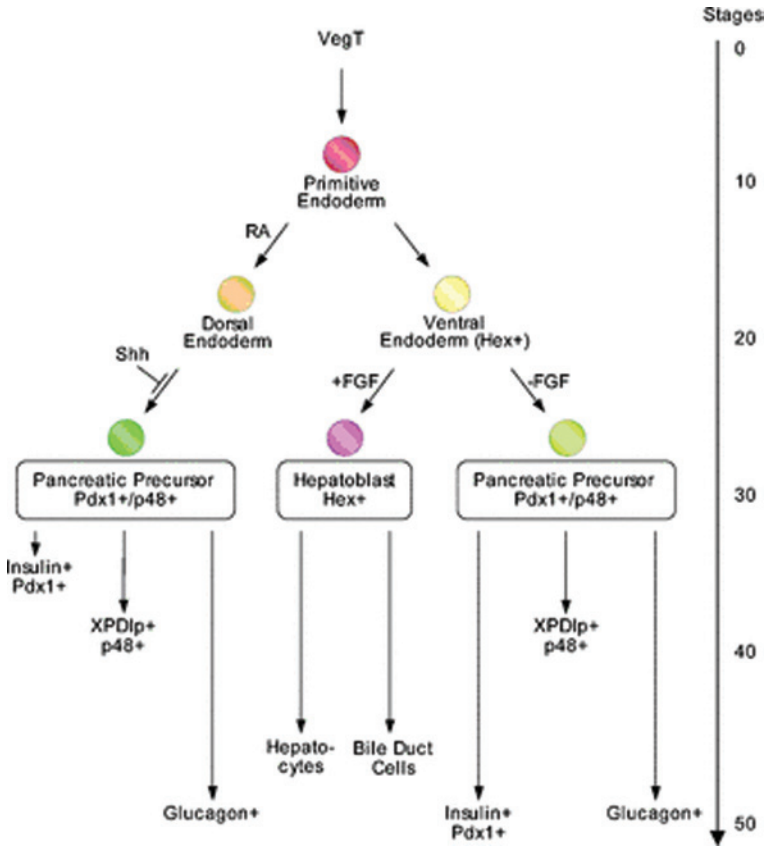

Figure 3. Forgotten and novel aspects in pancreas development (Pieler and Chen 2006).

Differentiation of ES cells is a heterogeneous process, and desired lineages can be enriched using various strategies (Zhao 2009), which includes the induction using chemokines, co-culture with differentiated cell types, and genetic manipulations on the ES cells. Differentiation is normally induced by culturing ES cells as aggregates (EBs) in the absence of the self-renewal signals provided by feeder layers or LIF, either in hanging drops (Yamada, Yoshikawa *et al*. 2002; Kurosawa 2007), in liquid "mass culture" (Doetschman, Eistetter *et al*. 1985), or in methylcellulose (Wiles and Keller 1991; Dang, Kyba *et al*. 2002). The adherent monolayer cultures in the absence of LIF (Ying, Stavridis *et al*. 2003) have also been used to differentiate mouse ES cells *in vitro*. Scalable production of ES-derived cells could furthermore be achieved through the use of stirred suspension bioreactors with encapsulation techniques (Dang and Zandstra 2005; Cameron, Hu *et al*. 2006).

During the differentiation process, ES cells spontaneously develop to form cells within all three germ layers (Qu 2008), endoderm, ectoderm and mesoderm (Figure 4).

Endodermal differentiation

In mammalian development, both the pancreas and liver originate from the definitive endoderm (Shiraki 2009). The pancreas develops from dorsal and ventral regions of

the foregut, and the liver from the foregut adjacent to the ventral pancreas compartment (Slack 1995). Pancreatic and hepatic cells are of special therapeutic interest for the treatment of hepatic failure (Gupta and Chowdhury 2002) and diabetes mellitus (Blyszczuk and Wobus 2006), and both pancreatic endocrine and hepatic cells could be developed successfully *in vitro* from ES cells.

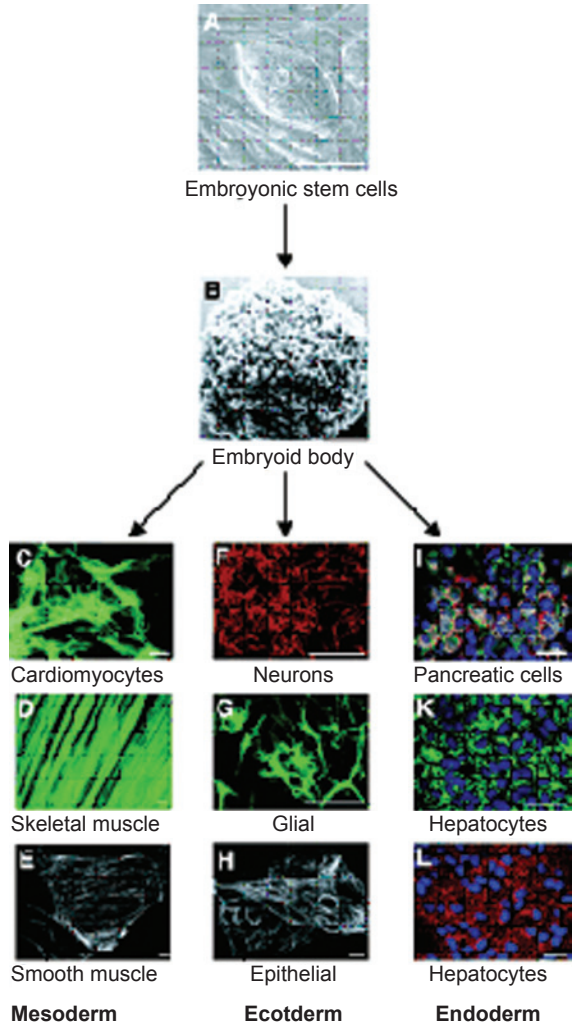

Figure 4. *In vitro* differentiation of ES cells. Undifferentiated mouse ES cells (***A***) develop *in vitro* via three-dimensional aggregates (embryoid body, ***B***) into differentiated cell types of all three primary germ layers. Shown are differentiated cell types labeled by tissue-specific antibodies (in parentheses). ***C***: cardiomyocytes (titin Z-band epitope). ***D***: skeletal muscle (titin Z-band epitope). ***E***: smooth muscle (smooth muscle α-actin). ***F***: neuronal (ßIII tubulin). ***G***: glial (glial fibrillary acidic protein, GFAP). ***H***: epithelial cells (cytokeratin 8). ***I***: pancreatic endocrine cells [insulin (red), C-peptide (Sato, Meijer ***et al.***), insulin and C-peptide colabeling (yellow)]. ***K*** and ***L***: hepatocytes (***K***, albumin; ***L***, α 1-antitrypsin). Bars = 0.5 µm (***H***), 20 µm (***I***), 25 µm (***C***, ***D***, ***E***), 30 µm (***K***, ***L***), 50 µm (***B***, ***G***), and 100 µm (***A***, ***F***). (Wobus and Boheler 2005)

The generation of ES derived insulin-producing cells holds great potential as an alternative treatment for T1DM. The first successful induction of pancreatic differ-

entiation from ES cells was obtained by stable transfection with a vector containing a neomycin-resistance gene under the control of the insulin promoter (Soria, Roche *et al*. 2000). In contrast, the spontaneous differentiation of mouse ES cells *in vitro* generated only a small fraction of insulin-producing cells (0.1%) (Shiroi, Yoshikawa *et al*. 2002). By modifying the differentiation protocols and using genetically modified mouse ES cells, the differentiation efficiency has been improved greatly (Lumelsky, Blondel *et al*. 2001; Blyszczuk P 2004; Miyazaki, Yamato *et al*. 2004). Although the modification of differentiation protocol allowed the generation of insulin-producing cells from mouse and human ES cells, further improvements are necessary to generate functional pancreatic islet like clusters.

Although ES cells could be used as an available source to obtain large amounts of transplantable cells as regenerative medicine as in the case of T1DMs, the major obstacle to the successful and safe clinical use of differentiated cells is the possibility of immune rejection and teratomas or teratocarcinoma formation in the recipients (Mimeault and Batra 2006; Trounson 2006).

More recently, Kroon *et al* showed that pancreatic endoderm derived from human embryonic stem (Doss MX) cells efficiently generates glucose-responsive endocrine cells after implantation into mice. Upon glucose stimulation of the implanted mice, human insulin and C-peptide are detected in sera at levels similar to those of mice transplanted with ~3,000 human islets. Moreover, the insulin-expressing cells generated after engraftment exhibit many properties of functional ß-cells, including expression of critical ß-cell transcription factors, appropriate processing of proinsulin and the presence of mature endocrine secretory granules. They also showed that implantation of hES cell–derived pancreatic endoderm protects against streptozotocin-induced hyperglycemia (Kroon 2008).

Encapsulation of embryonic stem cells

A way of ensuring that immune rejection does not occur is to establish an appropriate encapsulation method that releases insulin in a glucose-responsive manner and that the diffusivity of insulin being produced by the stem cells post-encapsulation can be assessed. In our systems, alginate was used in various concentrations: 0.5, 1.0 and 2% w/v in order to examine diffusion parameters. A key concern with the use of alginate is the long-term stability, and too low an alginate concentration may easily cause a collapse of the gel structure and cell release. 0.5% w/v alginate gel can only form relatively weak structure and cannot maintain cells long term in culture system and 2% w/v gels inhibit the influx of nutrients and the efflux of cellular wastes. As a result, only the 1% w/v alginate-based gel was used. An essential requirement for alginate micro-beads to serve as 3D environments for stem cells differentiation is the permeability towards diffusion of the growth factors and supplements required for cells' endocrinal differentiation into the micro-beads. Diffusion studies were conducted by monitoring penetration of fluorescently-labelled model molecules in a range of molecular weights (FITC of 327 Da, FITC-IgG of 150kDa and FITC-dextran of 500kDa) into alginate beads (Figure 5). We assessed the viability of encapsulated ES cells such that a scalable tissue culture environment using alginate encapsulation could be utilised to promote embryonic stem cell differentiation. The cells were released immediately post-encapsulation (within 12 hours) and cell viability analysis

carried out. 95.2% of the original 10.3 million confluent mouse ES cells harvested were live cells and following encapsulation and immediate disintegration of the beads, 9.6 million cells were collected (data not shown). There was no significant difference between cell viability before and after encapsulation (P=0.1205) and no indication that encapsulation significantly affects ES cell viability. This shows that in the initial stages, the cells may continue to follow a pre-programmed route until they become acclimatised to the local surrounding environment (Maguire 2006). Once acclimatised the viability begins to minimally diminish over time but this diminution is not statistically significant.

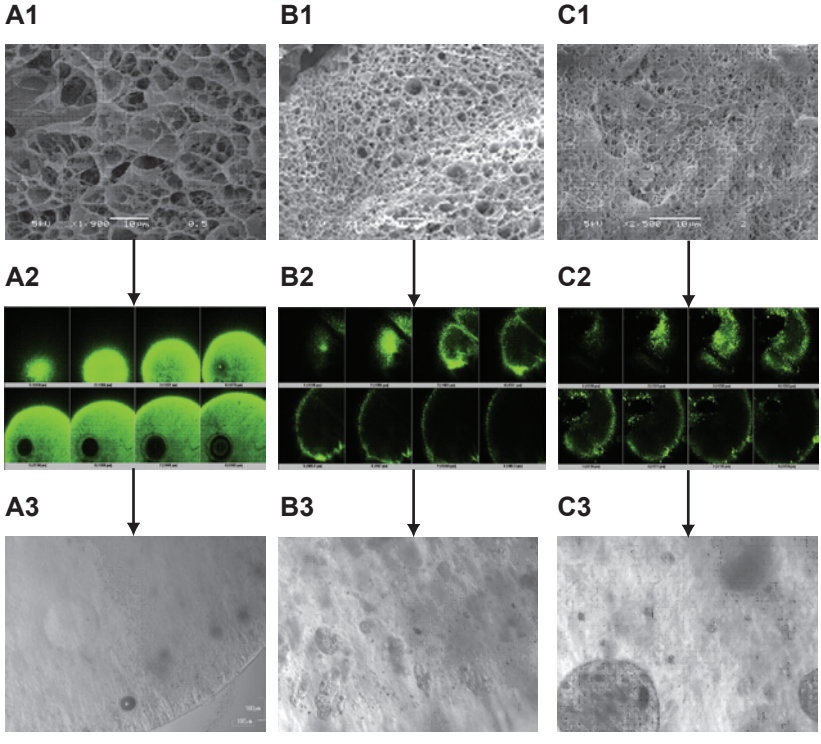

Figure 5. Cryo-SEM images of various concentrations alginates A1 (0.5% w/v), B1 (1% w/v), and C1 (2% w/v). Study of FITC conjugated Dextran (Mw 500KDa) diffusion into alginate micro-beads at 1 and 24 hours time points. A2) Confocal microscopy section images of 0.5% alginate micro-beads incubated with FITC- Dextran after 24 hours; B2) Confocal microscopy section images of 1% alginate micro-beads incubated with FITC- Dextran after 24 hours; C2) Confocal microscopy section images of 2% alginate micro-beads incubated with FITC- Dextran after 24 hours. Alginate beads with encapsulated stem cells A3) 0.5% alginate beads; B3) 1% alginate beads; C3) 2% alginate beads.

The use of alginate may promote entrapment either by cell–cell contact or decrease intercellular spacing, both of which could be conducive to intercellular signalling, which is mediated either by direct contact or soluble mediators. Any additional entrapment by alginate that followed this could further promote this interaction; the inclusion of hyaluronic acid as a constituent of the encapsulate does not, however, further significantly affect the viability of the cells.

These findings substantiate our method of encapsulation as opposed to the traditional formation strategies, for example those described by Cameron *et al*, where embryoid body formation is required (Hu 2003; Cameron 2006). These systems are probably used because they are known to generate three germ layers characteristic of embryo development, but EB formation is not an essential requirement in the production of insulin.

Conclusion

Due to their unique characters, the unlimited proliferative ability and plasticity to generate other cell types, stem cells represent a dynamic system suitable for the identification of new molecular targets and the development of novel drugs, which can be tested *in vitro* for safety or to predict or anticipate potential toxicity in humans (Ahuja, Vijayalakshmi *et al*. 2007). These cell lines derived from embryo could be used as a model for the study of basic and applied aspects in medical therapeutics, environmental mutagenesis and disease management. Moreover Human ES cell lines may, therefore, prove clinically relevant to the development of safer and more effective drugs for human diseases (Davila, Cezar *et al*. 2004) especially with those patients presenting with diabetes mellitus.

Acknowledgements

The authors would like to thank Pfizer Global and The University of Nottingham for funding.

References

Ahuja, Y. R., Vijayalakshmi, V. (2007). Stem cell test: A practical tool in toxicogenomics. *Toxicology* **231**(1), 1-10.

Ainsworth, C. D., Crowther, M.A., Treleaven, D., Evanovitch, D., Webert, K.E., Blajchman, M.A. (2009). Severe hemolytic anemia post-renal transplantation produced by donor anti-D passenger lymphocytes, case report and literature review. *Transfusion Medical Review* **23**(2), 155-9.

Amit, M., Carpenter, M. K. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Developmental Biology* **227**(2), 271-278.

Amit, M. and Itskovitz-Eldor, J. (2006). Maintenance of human embryonic stem cells in animal serum- and feeder layer-free culture conditions. *Methods Molecular Biology* **331**, 105-13.

Amit, M., Margulets, V. (2003). Human feeder layers for human embryonic stem cells. *Biological Reproduction* **68**(6), 2150-6.

Andrews, P. W. (2002). From teratocarcinomas to embryonic stem cells. *Philosophical Transactions of the Royal Society B* **357**(1420), 405-17.

Armstrong, L., Lako, M. (2000). mTert expression correlates with telomerase activity during the differentiation of murine embryonic stem cells. *Mechanisms of Development* **97**(1-2), 109-16.

Axelrod, H. R. (1984). Embryonic stem cell lines derived from blastocysts by a simplified technique. *Developmental Biology* **101**(1), 225-8.

Bjornson, C. R., Rietze, R. L. (1999). Turning brain into blood, a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* **283**(5401), 534-7.

Blyszczuk P, A. C., Rozzo A, Kania G, St-Onge L, Rupnik M, Wobus Am. (2004). Embryonic stem cells differentiate into insulin-producing cells without selection of nestin-expressing cells. *International Journal of Developmental Biology* **48**(10), 1095-104.

Blyszczuk, P. and. Wobus A. M (2006). In vitro differentiation of embryonic stem cells into the pancreatic lineage. *Methods Molecular Biology* **330**, 373-85.

Boheler, K. R., Czyz, J. (2002). Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circulation Research* **91**(3), 189-201.

Brenin, D., Look, J. (1997). Rat embryonic stem cells, a progress report. *Transplantion Proceedings* **29**(3), 1761-5.

Bukovsky, A., Caudle, M.R., Virant-Klun, I., Gupta, S.K., Dominguez, R., Svetlikova, M., XU, F. (2009). Immune physiology and oogenesis in fetal and adult humans, ovarian infertility, and totipotency of adult ovarian stem cells. *Birth Defects Research C Embryo Today* **87**(1), 64-89.

Burdon, T., Chambers, I. (1999). Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. *Cells Tissues Organs* **165**(3-4), 131-43.

Burdon, T., Stracey, C.(1999). Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Developmental Biology* **210**(1), 30-43.

Cameron, C. M., Hu, W. S.(2006). Improved development of human embryonic stem cell-derived embryoid bodies by stirred vessel cultivation. *Biotechnology Bioengineering* **94**(5), 938-48.

Cameron, C. M., Hu, W.S., Kaufman, D.S. (2006). Improved development of human embryonic stem cell-derived embryoid bodies by stirred vessel cultivation. *Biotechnology and Bioengineering* **94**(5), 938-948.

Chambers, I., Colby, D. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**(5), 643-55.

Chang, I. K., Jeong, D. K.(1997). Production of germline chimeric chickens by transfer of cultured primordial germ cells. Cell Biology International 21(8), 495-9.

Chugh, A. R., Zuba-Surma, E.K., Dawn, B. (2009). Bone marrow-derived mesenchymal stems cells and cardiac repair. *Minerva Cardioangiologica* **57**(2), 185-202.

Daheron, L., Opitz, S. L.(2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* **22**(5), 770-8.

Dang, S. M., Kyba, M. (2002). Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnology Bioengineering* **78**(4), 442-53.

Dang, S. M. and Zandstra P. W. (2005). Scalable production of embryonic stem cell-derived cells. *Methods Molecular Biology* **290**, 353-64.

Davila, J. C., Cezar, G. G. (2004). Use and application of stem cells in toxicology. *Toxicology Science* **79**(2), 214-23.

De Coppi, P., Bartsch, Jr., G. (2007). Isolation of amniotic stem cell lines with potential for therapy. *Nature Biotechnology* **25**(1), 100-6.

Dessimoz, J., Opoka, R. (2006). FGF signaling is necessary for establishing gut tube

domains along the anterior-posterior axis in vivo. *Mechanisms of Development* **123**(1), 42-55.

Doetschman, T., Williams, P. (1988). Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Developmental Biology* **127**(1), 224-7.

Doetschman, T. C., Eistetter, H. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines, formation of visceral yolk sac, blood islands and myocardium. *Journal of Embryological Experimental Morphology* **87**, 27-45.

Doss Mx, K. C., Gissel C, Hescheler J, Sachinidis A. (2004). Embryonic stem cells, a promising tool for cell replacement therapy. *Journal of Cell Molecular Medicine* **8**(4),465-73.

Esni, F., Ghosh, B. (2004). Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. *Development* **131**(17), 4213-4224.

Evans, M. J. and Kaufman M. H. (1981). Establishment in Culture of Pluripotential Cells from Mouse Embryos. *Nature* **292**(5819), 154-156.

Ezhkova, E., Pasolli, H.A., Parker, J.S., Stokes, N., Su, I.H., Hannon, G., Tarakhovsky, A., Fuchs, E. (2009). Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell* **136**(6), 1122-35.

Gearhart, J. D. and Mintz B. (1974). Contact-mediated myogenesis and increased acetylcholinesterase activity in primary cultures of mouse teratocarcinoma cells. *Proceedings National Academy of Sciences U S A* **71**(5), 1734-8.

Geijsen, N., Horoschak, M. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* **427**(6970), 148-54.

Gittes, G. K., Galante, P. E. (1996). Lineage-specific morphogenesis in the developing pancreas, Role of mesenchymal factors. *Development* **122**(2), 439-447.

Golosow, N. and Grobstein C. (1962). Epitheliomesenchymal Interaction in Pancreatic Morphogenesis. *Developmental Biology* **4**(2), 242-&.

Graves, K. H. and Moreadith R. W. (1993). Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. *Molecular Reproductive Development* **36**(4), 424-33.

Gupta, S. and Chowdhury J. R. (2002). Therapeutic potential of hepatocyte transplantation. *Seminars in Cell & Developmental Biology* **13**(6), 439-446.

Hart, A., Papadopoulou, S. (2003). Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. *Developmental Dynamics* **228**(2), 185-193.

Heiser, P. W., J. Lau, (2006). Stabilization of beta-catenin impacts pancreas growth. *Development* **133**(10), 2023-2032.

Holtzer, H. (1978). Cell lineages, stem cells and the quantal cell cycle concept. In, Stem cells and tissue homeostasis, Cambridge University Press.

Hu, A., Cai, J., Zheng, Q., He, X., Pan, Y., Li, L. (2003). Hepatic differentiation from embryonic stem cells in vitro. *Chinese Medicine Journal* (Engl) **116**(12), 1893-1897.

Hubner, K., Fuhrmann, G. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science* **300**(5623), 1251-1256.

Jakob, H., Boon, T.et al. (1973). [Teratocarcinoma of the mouse, isolation, culture and properties of pluripotential cells. *Annals of Microbiology* (Paris) **124**(3), 269-82.

Kalkunte, S. S., Mselle, T.F., Norris, W.E., Wira, C.R., Sentman, C.L., Sharma, S. (2009). Vascular endothelial growth factor C facilitates immune tolerance and

endovascular activity of human uterine NK cells at the maternal-fetal interface. Journal of *Immunology* **182**(7), 4085-92.

Kroon, E., Martinson, L.A., Kadoya, K., Bang, A.G., Kelly, O.G., Eliazer, S., Young, H., Richardson, M., Smart, N.G., and Cunningham J. (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells *in vivo. Nature Biotechnology* **26** 443-452

Kurosawa, H. (2007). Methods for inducing embryoid body formation, *in vitro* differentiation system of embryonic stem cells. *Journal of Bioscience and Bioengineering* **103**(5), 389-98.

Labosky, P. A., Barlow, D. P. (1994). Mouse Embryonic Germ (Eg) Cell-Lines - Transmission through the Germline and Differences in the Methylation Imprint of Insulin-Like Growth-Factor 2 Receptor (Igf2R) Gene Compared with Embryonic Stem (Es) Cell-Lines. *Development* **120**(11), 3197-3204.

Lee, J. B., Lee, J. E. (2005). Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. *Biological Reproduction* **72**(1), 42-9.

Lester Lb, K. H., Andrews L, Nauert B, Wolf Dp. (2004). Directed differentiation of rhesus monkey ES cells into pancreatic cell phenotypes. *Reproductive Biological Endocrinology*. **16**, 42.

Lewis, S. L. and Tam, P. P. (2006). Definitive endoderm of the mouse embryo, formation, cell fates, and morphogenetic function. *Developmental Dynamics* **235**(9), 2315-29.

Lumelsky, N., Blondel, O. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* **292**(5520), 1389-94.

Maguire, T., Novik, E., Schloss, R., Yarmush, M. (2006). Alginate-PLL microencapsulation, effect on the differentiation of embryonic stem cells into hepatocytes. *Biotechnology Bioengineering* **93**(3), 581-91.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings National Academy of Sciences U S A* **78**(12), 7634-8.

Matsui, Y., Zsebo, K. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**(5), 841-7.

Meirelles, L., Nardi, N.B. (2009). Methodology, biology and clinical applications of mesenchymal stem cells *Frontiers in Bioscience* **14**, 4281-98.

Miettinen, P. J., Huotari, M. A. (2000). Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. *Development* **127**(12), 2617-2627.

Mimeault, M. and Batra S. K. (2006). Concise review, recent advances on the significance of stem cells in tissue regeneration and cancer therapies. *Stem Cells* **24**(11), 2319-45.

Miralles, F., Czernichow, P. (1998). Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. *Development* **125**(6), 1017-1024.

Mitsui, K., Tokuzawa, Y. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**(5), 631-42.

Miyazaki, S., Yamato, E. (2004). Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells. *Diabetes*

53(4), 1030-7.

Murtaugh, L. C., Law, A. C. (2005). beta-Catenin is essential for pancreatic acinar but not islet development. *Development* **132**(21), 4663-4674.

Niwa, H., Burdon, T. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Development* **12**(13), 2048-60.

Niwa, H., Miyazaki, J. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics* **24**(4), 372-6.

Norgaard, G. A., Jensen, J. N. (2003). FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. *Developmental Biology* **264**(2), 323-338.

Odorico, J. S., Kaufman, D. S. (2001). Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* **19**(3), 193-204.

Oktem, O., Oktay, K. (2009). Current knowledge in the renewal capability of germ cells in the adult ovary. *Birth Defects Research C Embryo Today* **87**(1), 90-95.

Papadopoulou, S. and Edlund H. (2005). Attenuated Wnt signaling perturbs pancreatic growth but not pancreatic function. *Diabetes* **54**(10), 2844-2851.

Parrish, Y. K., Baez, I., Milford, T.A., Benitez, A., Galloway, N., Rogerio, J.W., Sahakian, E., Kagoda, M., Huang, G., Hao, Q.L., Sevilla, Y., Barsky, L.W., Zielinska, E., Price, M.A., Wall, N.R., Dovat, S., Payne, K.J. (2009). IL-7 Dependence in human B lymphopoiesis increases during progression of ontogeny from cord blood to bone marrow. *Journal of Immunology* **182**(7), 4255-66.

Pau, K. Y. And Wolf D. P. (2004). Derivation and characterization of monkey embryonic stem cells. *Reproductive Biological Endocrinology* **2**, 41.

Pesce, M., Anastassiadis, K. (1999). Oct-4, lessons of totipotency from embryonic stem cells. *Cells Tissues Organs* **165**(3-4), 144-52.

Pieler, T. and Chen Y. (2006). Forgotten and novel aspects in pancreas development. *Biol Cell* **98**(2), 79-88.

Prelle, K., Vassiliev, I. M. (1999). Establishment of pluripotent cell lines from vertebrate species--present status and future prospects. *Cells Tissues Organs* **165**(3-4), 220-36.

Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**(5309), 71-4.

Qu, X. B., Pan, J., Zhang, C., Huang, S.Y. (2008). Sox17 facilitates the differentiation of mouse embryonic stem cells into primitive and definitive endoderm in vitro. *Developmental Growth and Differentiation* **50**(7), 585-93.

Rahier, J. (1988). The diabetic pancreas, a pathologist's view. New York, Springer-Verlag.

Resnick, J. L., Bixler, L. S. (1992). Long-Term Proliferation of Mouse Primordial Germ-Cells in Culture. *Nature* **359**(6395), 550-551.

Reubinoff, B. E., Pera, M. F. (2000). Embryonic stem cell lines from human blastocysts, somatic differentiation in vitro. *Nature Biotechnology* **18**(4), 399-404.

Reynolds, B. A. and Weiss S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**(5052), 1707-10.

Richards, M., Fong, C. Y. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nature Biotechnology* **20**(9), 933-936.

Robertson, E. (1987). Embryo-derived stem cell lines. In, Teratocarcinoma and embryonic stem cells, a practical approach. Oxford, UK, IRL.

Sasaki, E., Hanazawa, K. (2005). Establishment of novel embryonic stem cell lines derived from the common marmoset (*Callithrix jacchus*). *Stem Cells* **23**(9), 1304-13.

Sato, N., Meijer, L. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature Medicine* **10**(1), 55-63.

Scholer, H. R., Hatzopoulos, A. K. (1989). A family of octamer-specific proteins present during mouse embryogenesis, evidence for germline-specific expression of an Oct factor. *Embo Journal* **8**(9), 2543-50.

Schoonjans, L., Albright, G. M. (1996). Pluripotential rabbit embryonic stem (ES) cells are capable of forming overt coat color chimeras following injection into blastocysts. *Molecular Reproductive Development* **45**(4), 439-43.

Serakinci, N. And Keith W. N. (2006). Therapeutic potential of adult stem cells. European *Journal of Cancer* **42**(9), 1243-6.

Shamblott, M. J., Axelman, J. (2001). Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively in vitro. *Proceedings of the National Academy of Sciences U S A* **98**(1), 113-118.

Shiraki, N., Higuchi, Y., Harada, S., Umeda, K., Isagawa, T., Aburatani, H., Kume, K., Kume, S. (2009). Differentiation and characterization of embryonic stem cells into three germ layers *Biochemical and Biophysical Research Communications* 278-283

Shiroi, A., Yoshikawa, M. (2002). Identification of insulin-producing cells derived from embryonic stem cells by zinc-chelating dithizone. *Stem Cells* **20**(4), 284-92.

Slack, J. M. W. (1995). Developmental Biology of the Pancreas. *Development* **121**(6), 1569-1580.

Smith, A. G. (2001). Embryo-derived stem cells, of mice and men. *Annual Review Cell Developmental Biology* **17**, 435-62.

Smith, A. G., Heath, J. K. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**(6200), 688-90.

Solter, D. and Knowles B. B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proceedings of the National Academy of Sciences, USA* **75**(11), 5565-9.

Soria, B., Roche, E. (2000). Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* **49**(2), 157-62.

Stewart, C. L., Gadi, I. (1994). Stem-Cells from Primordial Germ-Cells Can Reenter the Germ-Line. *Developmental Biology* **161**(2), 626-628.

Suemori, H., Tada, T. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Developmental Dynamics* **222**(2), 273-9.

Teramura, T., Onodera, Y., Murakami, H., Ito, S., Mihara, T., Takehara, T., Kato, H., Mitani, T., Anzai, M., Matsumoto, K., Saeki, K., Fukuda, K., Sagawa, N., Hosoi, Y. (2009). Mouse Androgenetic Embryonic Stem Cells Differentiated to Multiple

Cell Lineages in Three Embryonic Germ Layers *In Vitro*. *Journal of Reproductive Development*. **55**(3),283-92.

Thomson, J. A., Itskovitz-Eldor, J. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282(5391), 1145-1147.

Thomson, J. A., Kalishman, J. (1995). Isolation of a primate embryonic stem cell line. *Proceedings of the National Academy of Sciences, USA* **92**(17), 7844-8.

Trounson, A. (2006). The production and directed differentiation of human embryonic stem cells. *Endocrine Review* **27**(2), 208-19.

Vallier, L., Mendjan, S., Brown, S., Chng, Z., Teo, A., Smithers, L.E., Trotter, M.W., Cho, C.H., Martinez, A., Rugg-Gunn, P., Brons, G., Pedersen, R.A. (2009). Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. *Development* **136**(8), 1339-49.

Vassilieva, S., Guan, K. (2000). Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth. *Experimental Cell Research* **258**(2), 361-73.

Viswanathan, S., Benatar, T. (2002). Ligand/receptor signaling threshold (LIST) model accounts for gp130-mediated embryonic stem cell self-renewal responses to LIF and HIL-6. *Stem Cells* **20**(2), 119-38.

Vrana, K. E., Hipp, J. D. (2003). Nonhuman primate parthenogenetic stem cells. *Proceedings of the National Academy of Sciences, USA* **100** Suppl 1, 11911-6.

Watt, F. M. And Hogan B. L. (2000). Out of Eden, stem cells and their niches. *Science* **287**(5457), 1427-30.

Weissman, I. L. (2000). Stem cells, units of development, units of regeneration, and units in evolution. *Cell* **100**(1), 157-68.

Wells, J. M. And D. A. Melton (1999). Vertebrate endoderm development. *Annual Review Cell Developmental Biology* **15**, 393-410.

White, T. (1973). Surgical anatomy of the pancreas. Saint Louis, The CV Mosby.

Wiles, M. V. And Keller G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* **111**(2), 259-67.

Williams, R. L., Hilton, D. J. (1988). Myeloid-Leukemia Inhibitory Factor Maintains the Developmental Potential of Embryonic Stem-Cells. *Nature* **336**(6200), 684-687.

Wobus, A. M. And Boheler K. R. (2005). Embryonic stem cells, prospects for developmental biology and cell therapy. *Physiological Review* **85**(2), 635-78.

Wobus, A. M., Holzhausen, H. (1984). Characterization of a Pluripotent Stem-Cell Line Derived from a Mouse Embryo. *Experimental Cell Research* **152**(1), 212-219.

Xu, C., Inokuma, M. S. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnology* **19**(10), 971-4.

Yamada, T., Yoshikawa, M. (2002). In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. *Stem Cells* **20**(2), 146-54.

Ying, Q. L., Stavridis, M. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nature Biotechnology* **21**(2), 183-6.

Zhao, Y., Lin, B., Darflinger, R., Zhang, Y., Holterman, M.J., Skidgel, R.A. (2009). Human cord blood stem cell-modulated regulatory T lymphocytes reverse the autoimmune-caused type 1 diabetes in nonobese diabetic (NOD) mice. *PLoS ONE* **4**(1), e4226.