Reproductive BioMedicine Online (2013) xxx, xxx–xxx

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A rapidly evolving revolution in stem cell biology and medicine

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Alan Trounson, PhD, FRCOG, FANZCOG and President of the California Institute for Regenerative Medicine (CIRM) is responsible for the management of the US$3 billion fund for stem cell research in California. Under his leadership, CIRM has constructed 12 new Californian Stem Cell Research Institutes, raising more than US$800 million in donor contributions. He has developed training programmes for new scientists entering stem cell science for a large number of MD–PhD and PhD researchers and university, college and high school students. He has overseen an extraordinary development of stem cell research which has led to more than 1000 peer-reviewed publications (24% in high impact factor journals) in the last 4 years. He has globalized the stem cell research programme and has led the translation of basic science discovery into translation and clinical trials.

Abstract The developments arising from human IVF are remarkable. Embryos were studied for developmental patterns that have consequences for viability and fertility. Growing human blastocysts *in vitro* allowed further exploration of the differentiation of primitive embryonic cells, leading to the discovery of human embryonic stem cells (ESC). The availability of perhaps unlimited numbers of human ESC could inform the study of differentiation and also provide cells for therapies in human regenerative medicine. The developments in cell biology have been impressive, including the discovery of induced pluripotent stem cells – adult cells transduced by specific transcription factors to behave like human ESC. Key regulators of development such as activators or inhibitors of lineage progression have also been explored, particularly the fibroblast growth factor, Wnt and transforming growth factor β signalling pathways and miRNA. Such regulators can be utilized in algorithms to predict how cells differentiate *in vitro*. Using multistep differentiation protocols, many different cell types can be formed and matured into functionally effective cells, some of which are already in translational research for clinical applications. Possible future developments include destruction of cancer stem cells, reversal of type I diabetes, restoration of vision, repair of motor function, cure for HIV/AIDS and heart muscle regeneration.

KEYWORDS: blastocysts, differentiation, embryonic stem cells, gametes, germ stem cells, human embryos, key regulators of differentiation, pluripotent stem cells, somatic cell nuclear transfer, translation to the clinic

Introduction

Beginning in the late 1960s and early 1970s, human preimplantation embryos were first made by IVF techniques by the British cell biologist Robert Edwards and his colleagues (Edwards et al., 1970; Steptoe et al., 1971). Independently Carl Wood and his colleagues in Australia began research into human IVF in 1970 and they published on the first IVF pregnancy that lasted a very short time *in vivo* (De Kretzer et al., 1973). Work continued in both groups studying human IVF using fertility drugs and laparoscopic procedures to recover multiple mature oocytes for fertilization and transfer to infertile patients with some encouraging results (Steptoe and Edwards, 1976). Edwards and Steptoe in 1978

1472-6483/$ - see front matter
http://dx.doi.org/10.1016/j.rbmo.2013.07.005

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(Edwards et al., 1980; Steptoe et al., 1980) demonstrated that the mature oocyte recovered in the natural ovulatory cycle could be fertilized and that viable embryos developed using IVF could be returned to infertile patients for delivery of healthy babies. These developments were the basis for a well-deserved Nobel prize in Physiology or Medicine for Robert Edwards in 2010. The Australian group was the first to confirm independently that successful IVF could be performed in the natural ovulatory cycle of infertile women (Lopata et al., 1980). However, the method required careful tracking of the surge in the concentration of the preovulatory LH in the blood or urine from predicted days of probable ovulation. There was no control of the timing of ovulation (which could therefore occur at any time of the day or night) and there was usually only a single follicle with a maturing oocyte present. The low efficiency of single oocyte recovery and the inconvenience of untimed laparoscopic procedures made this natural cycle method difficult to sustain.

Using clomiphene for mild ovarian stimulation and the administration of human chorionic gonadotrophin to induce preovulatory oocyte maturation instead of the natural LH surge, Trounson et al. (1981) demonstrated for the first time multiple births and pregnancies using IVF. This was an effective way to recover multiple mature oocytes on a preplanned programme of laparoscopic surgery for infertile women. This method of superovulation, which evolved through clomiphene, clomiphene + human menopausal gonadotrophin to human menopausal gonadotrophin alone or FSH, became the basic procedure used for clinical IVF studies from then on. This method resulted in multiple embryos developing from a single cycle of superovulation and required the introduction of embryo freezing to preserve patients' embryos for future transfer if necessary (Trounson and Mohr, 1983; Zeilmaker et al., 1984). The fertility drug-based ovulatory control of IVF enabled the method to improve and increase its efficiency and enabled human embryos to be produced for all stages of preimplantation development (Trounson et al., 1982) as the source for the development of human embryonic stem cells (ESC) (Reubinoff et al., 2000; Thomson et al., 1998). The recovery of multiple oocytes enabled oocyte and embryo donation (Lutjen et al., 1984; Trounson et al., 1983) and the development of embryo biopsy techniques for preimplantation genetic diagnosis of inherited genetic disease (Handyside et al., 1990; Verlinsky et al., 1990). These developments form a continuum on the timeline of developments that have evolved from IVF and continue to develop into the future (**Figure 1**).

Eggs, embryos and human ESC

The ability to cryopreserve human embryos that were donated for stem cell research, as excess to the patient's own needs, provided a source of human ESC for studying: the developmental processes during differentiation; the biology of stem cells and their developmental potential; possible cell products for therapeutic purposes in regenerative medicine; cells for tissue engineering; a source of cells for discovery of small molecules and biologics as new drugs for regenerative medicine; the understanding of disease pathology and cancer (cancer stem cells); and the manipulation of cell phenotype using transcription factors, small RNA and other key signalling molecules. Thus, this approach is likely to underpin a whole new revolution in cell biology and medicine.

What was the impetus for exploring human ESC? Firstly, the ability to develop human embryos *in vitro* to the blastocyst stage with properly formed inner cell mass (ICM) and trophectoderm (Mohr and Trounson, 1982), suggested that methods similar to those used in mouse embryology could be used to develop pluripotential stem cells from isolated

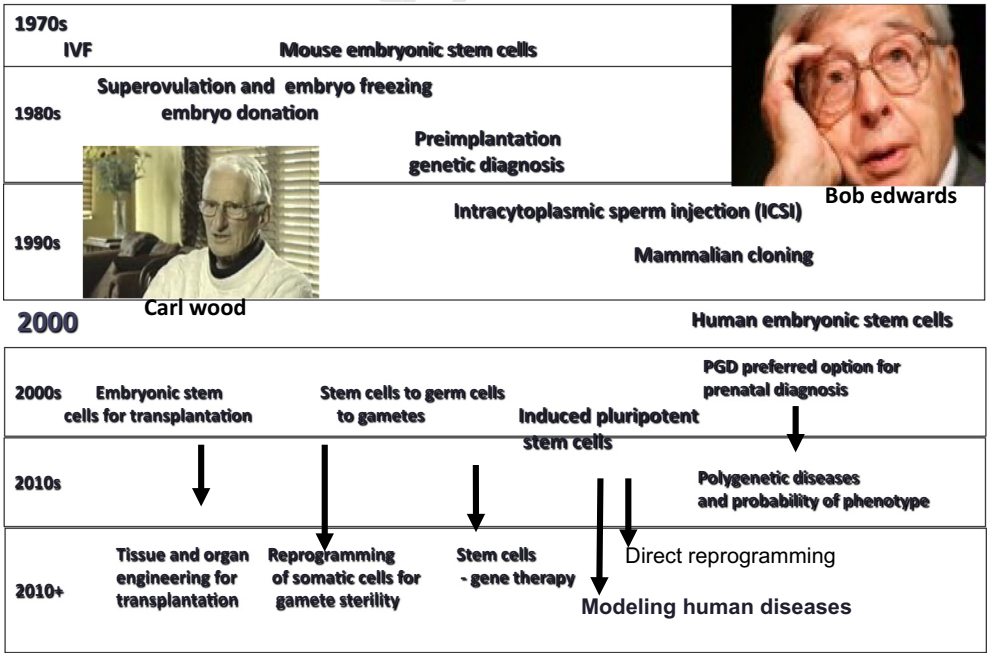

Figure 1 Timeline of human reproductive/stem cell discovery.

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ICM (Evans and Kaufman, 1981). This was in fact shown to be the case in independent studies by Thomson et al. (1998) and Reubinoff et al. (2000). Furthermore, it was not possible to grow human embryos normally beyond the hatched blastocyst stage *in vitro*, which prevented studies of the differentiation of the primitive undifferentiated embryonic cells into the germinal lineages. It was important to understand early development in the human if birth defects and early cancer formation were to be understood. There was also a possibility that human ESC would be an immortal source of cells for therapeutics. While there had been little work on the differentiation of mouse ESC, they had been used extensively for functional genomics. The capacity to genetically manipulate human ESC was also considered critical for understanding human developmental biology and to treat monogenic inheritable disease.

In order to develop human ESC, human blastocysts in excess of those needed by patients were required for isolation of ICM on fibroblast feeder layers. The ability to efficiently develop blastocysts *in vitro* and grow human embryonic cells needed to be established (Bongso et al., 1994; Trounson, 1994, 2001, 2002). Given these developments, the production of pluripotent ESC became reasonably efficient by the early 2000s. Presently there are large numbers of euploid human ESC lines available for research and these are likely to be sufficient for present research interests (Lomax and Trounson, 2013). The development of preimplantation genetic diagnosis also enabled the production of a wide range of ESC lines that have the genetic mutations responsible for monogenic diseases (Pickering et al., 2005). Genetically affected embryos are discarded and are readily donated for stem cell research. Interestingly, some two-thirds of embryos determined to be aneuploid, or mosaic for aneuploidy, were able to develop to euploid ESC (Biancotti et al., 2010; Verlinsky et al., 2009). This finding may suggest either that the fluorescent in-situ hybridization (FISH) assay was erroneous at chromosomal diagnosis and/or that there is strong selection for survival of only euploid cells, against aneuploid cells, in ESC being established *in vitro*. Biancotti et al. (2012) showed there is a clear bias against the formation of human ESC from embryos with autosomal monosomies, and in addition few trisomies survived as human ESC.

In an interesting analysis of the use of ESC lines for research up until 2008–2009, Löser et al. (2010) showed that 1071 human ESC lines were derived by 87 institutions in 24 countries, although data on their full characterization was limited. Two of these cell lines accounted for 70.5% of all world publications on human ESC research. While this may in part be due to the strength of research in the USA, there is clearly a preference to working with cell lines that are well known and well represented in the research community. In some countries, there is a preference for those derived nationally. While it is expected that human ESC derived by clinical good medical practice may be preferred for clinical cell therapeutic studies, there is rather little need at present to accelerate the expansion of the number of human ESC lines currently available for research (Lomax and Trounson, 2013). The most interest for new human ESC lines is presently from embryos with diagnosed genetic diseases and specially manipulated human ESC lines that have fluorescent reporter genes integrated into genes of interest for studies on differentiation (e.g. Goulburn et al., 2011).

Very large numbers of human ESC can be produced for large-scale research studies and clinical trials using an automated manufacturing system (Chen et al., 2012). The system involves suspension cultures of human ESC as aggregates for more than 20 passages, expanding cell numbers more than 10^{13}-fold with unaltered differentiation capacity and ploidy. This suspension expansion method has been used to establish master cell banks of human ESC lines for clinical studies.

Differentiating human ESC

Differentiation of the primitive pluripotent stem cells occurs through a process of epigenetic regulation governed by gene networks that programme cells to adopt the required phenotype (Bruce, 2013; Torres-Padilla, 2013). A hypothetical approach to understanding the nature of cell differentiation, stability of phenotype and integration in development was conceived by Conrad Hal Waddington (Waddington, 1957), who proposed an undulating epigenetic landscape governed by gene networks regulating valleys of stable cell states and ridges that impede cell transition from one state to another. It is possible to mathematically map predictions of cell response as in Waddington's epigenetic landscape. Models may predict pathways of differentiation and states of pause for renewal. These pathways and states are typically governed by the activity of gene pathways operative during the progressive cell differentiation and development (Bhattacharya et al., 2011). This model provides an informative and instructive perspective on what primary factors drive differentiation and where this may cease (become subject to other factors for complete maturation). The epigenetic landscape may facilitate the prediction of regenerative processes that otherwise falter because of an over-simplification of approach, for example the robust and sustained axonal regrowth induced by blocking dual gene pathways by the combined action of PTEN and SOCS3, that separately have brief and minor effects (Sun et al., 2011). It is necessary to further inform Waddington's epigenetic landscape by defining the influence of all the relevant gene networks in development and differentiation (including those that cause pathological effects), thereby enabling predictions of genes and their quantitative influence on spontaneous and directed differentiation pathways (the gullies of Waddington's landscape), stable states (progenitor cell types) and states of stochastic impedance (raised landscape in Waddington's model) that govern the flow and direction of cell differentiation. Gene mutation can define developmental abnormalities that appear when induced pluripotent stem cells (iPSC) from patients with diseases are differentiated *in vitro* ('disease in a dish' models, Trounson et al., 2012b) and this approach can further inform the source of pathologies by showing gain of function and loss of function phenotypes with such iPSC. Multiple genetic and micro-environmental (dimensional, matrix, secretory, plasticity, movement) determinants exist that need eventually to be accounted for in cell-differentiating algorithms.

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Progressively, researchers are generating the data to inform the directed differentiation of ESC. Tissues of many cell types may be obtained from human ESC (Metallo et al., 2008). The variety of cell types is impressive and many have shown function *in vitro* and when transplanted into animal models. These include pancreatic β-islet cells (Schulz et al., 2012), retinal epithelium (Hu et al., 2012), cardiomyocytes (Ardehali et al., 2013) and dopaminergic neurons (Kriks et al., 2011). Among those master regulators for differentiation and development, the Wnt pathways are possibly the most potent (Nusse, 2012; Willett and Nusse, 2012). Wnt proteins are also needed for renewal and the prevention of spontaneous differentiation of mouse ESC to epiblast stem cells (Berge et al., 2011) and for many other cellular responses necessary for development. The present perspective on Wnt signalling in development needs to be considered through 'multiple, often simultaneous inputs at the level of both Wnt-receptor binding and the downstream intracellular responses' (Amerongen and Nusse, 2009). Their role in tissue growth and expansion in development and specific differentiation processes makes Wnt signalling a very attractive target for small molecules in regenerative medicine. Both the Wnt/β-catenin canonical (nuclear β-catenin active) and non-canonical pathways control differentiation to ectoderm, mesendoderm, endoderm and mesoderm derivatives (see Davis and Nieden, 2008).

Other key signalling pathways that are critical in differentiation include the fibroblast growth factor (FGF) pathway. FGF signalling is involved in many of the early lineage specification decisions (Villegas et al., 2010). Members of the transforming growth factor β (TGFβ) signalling pathway are needed together with Wnt signalling for self-renewal of pluripotent stem cells and their early differentiation (Payne et al., 2011). In order to derive pancreatic β-islet cells for treating type I diabetes, it has been shown that these signalling pathways need to be inhibited in a stepwise manner to enable efficient production of the islet cell progenitors of the definitive endoderm lineage (see Baetge, 2008). This demonstration involved removal of insulin and basic FGF to inhibit phosphatidylinositol 3-kinase and simultaneously activating Activin/Nodal (TGFβ signalling) to produce definitive endoderm (chemokine receptor 4 expression) followed by elimination of TGFβ signalling to induce foregut patterning (FOXA2 expression) by induction of FGF signalling through FGFR2IIIb. Retinoic acid signalling is used to specify the pancreatic lineage (expression of a large number of transcription factors including PDX1, NKX6.1, PTF1a, PROX1, HNF6, HLXB9 and SOX9). Mature islet cells are formed *in vivo*. Similar differentiation protocols have evolved for ectodermal lineages (neurons and glia; Selvarai et al., 2012) and mesoderm (e.g. cardiac cell types; Doss et al., 2012; Gessert and Kuhl, 2010).

In the primate (monkey), chimeras cannot be made by injection of dispersed ESC into the blastocyst, but intact ICM are able to form fetuses and live-born young when inserted into the blastocoelic cavity (Tachibana et al., 2012). Primate ESC appear to more resemble mouse epiblast stem cells than mouse ESC, which do readily form chimeras after blastocyst injection. The difference between mouse and primate ESC is important because of the differences in the requirements for key regulating molecules, such as leukaemia inhibiting factor in the mouse. Furthermore, there are key signalling molecule differences between human ICM and human ESC (Reijo Pera et al., 2009). Notably FGF signalling is not required for human ICM to form the hypoblast (Roode et al., 2012) nor for the segregation of epiblast and hypoblast lineages (Kuijk et al., 2012), in contrast to the mouse. It is possible that new human ESC lines could be derived without FGF and these pluripotent stem cell lines may behave differently from those derived by the conventional methods. However, they still may not behave as do mouse ESC in their renewal and differentiation, given the differences between mouse and primate ICM.

Research has also identified microRNA (miRNA) as effective regulators of renewal and differentiation of pluripotent stem cells (Gangaraju and Lin, 2009; Hinton et al., 2012). This approach provides another dimension to gene control of development and these data should be included in the algorithms for directing lineage differentiation *in vitro*. The hierarchy of dominance in the key factors regulating the fate of stem cells remains to be determined but the power of multiple active and then redundant regulators may be required for achieving the subtlety of variation in progenitor and end-differentiated cell types observed *in vivo*.

Pluripotent stem cells and reproduction

Somatic cell nuclear transfer (SCNT) was proposed as a way of taking cells from the adult and converting them to genomically histocompatible ESC that could be directed to germ stem cells and potential spermatozoa and oocytes for sterile patients (**Figure 2**). While SCNT as a source of ESC can be performed in mice and in non-human primates, it has failed in the human to date (Grieshammer et al., 2011). The only progress more recently is the development of triploid human ESC by SCNT where the recipient oocyte nucleus is left intact after nuclear transfer of a diploid adult cell nucleus (**Figure 2**; Noggle et al., 2011). This observation suggests that the oocyte nucleus contains reprogramming factors necessary for human embryo viability. Clearly this is different from mice and farm animals (cattle, sheep and pigs).

The possibility that ESC could be used for the production of viable gametes has been an intriguing hypothesis that has been considered for some time (**Figure 2**). The discovery of iPSC in mice (Takahashi and Yamanaka, 2006) using adult cells reprogrammed by transcription factor transduction raised the possibility of iPSC-derived histocompatible gametogenesis. This possibility has been recently achieved in the production of spermatozoa and oocytes in mice (Hayashi et al., 2011, 2012). Given that iPSC can be produced in the human (Takahashi et al., 2007), the work of Hayashi and colleagues may prove to be the next major development in human reproductive medicine. This technology offers a solution for human sterility, failed IVF and a way to generate human gametes, embryos and ESC for research without the ethical issues of human gamete and embryo donation (**Figure 3**).

Understanding the need for stepwise guidance of pluripotent stem cells into germ stem cells and then the need for ovarian somatic cell contributions to mature the oocyte germ cells to viable oocytes in ovarian follicles is another

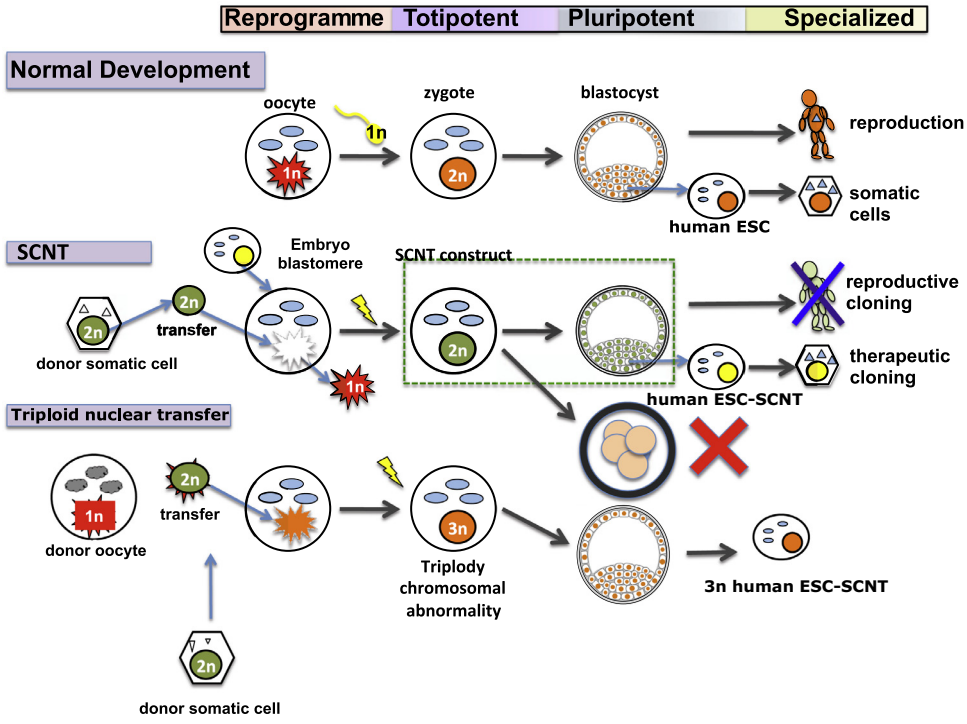

Figure 2 Somatic cell reprogramming in the human (modified from Grieshammer et al., 2011). Human oocytes are able to reprogramme spermatozoa (1n) after fertilization and somatic nuclei (2n) after somatic cell nuclear transfer (SCNT). Some limited development has been reported of SCNT embryos *in vitro* to early cleavage stages and occasionally blastocysts but not human ESC. However, if the oocyte nucleus is retained, embryos develop to triploid (3n) blastocysts relatively efficiently and form 3N human ESC (Noggle et al., 2011). These are not cloned cells because they include the genomic contributions of the donor and recipient nuclei.

very good example of the need to mimic the developmental pathways for functional tissues (**Figure 4**). In the case of Hayashi et al. (2012), normal embryonic development, birth of live young and reproduction of mice produced from the original female iPSC was demonstrated.

Translation of ESC discoveries

Some of the recent developments in translation of HECS and other cell types to clinical medicine have been reviewed and summarized recently (Trounson, 2012, in press; Trounson and DeWitt, 2012; Trounson et al., 2011). ESC-derived retinal pigmented epithelium (RPE) has entered clinical trials for the treatment of dry or atrophic macular degeneration (AMD), which is the commonest cause of blindness (80–90% of age-related macular degeneration). Present studies in the clinic involve injection of RPE into the subretinal space. However, approaches that involve RPE as monolayers on micro-thin scaffolds are evolving rapidly to clinical trial. These approaches will address both dry AMD and wet or exudative AMD and it is anticipated they will be more effective than the use of cell suspensions. The other human ESC application moving rapidly to the clinic is the ViaCyte pancreatic β-Islet cell programme that involves differentiation of human ESC to islet progenitors (Schulz et al., 2012) and their maturation in thin capsules inserted subcutaneously. These protected islet cells are not destroyed by the host immune system and are able to effectively control diabetes in rodent models of type I diabetes. The whole field is moving rapidly forward to attempt an effective therapy for this challenging disease (Lysy et al., 2012).

The translation of iPSC-derived cell therapies is lagging behind human ESC studies. There are some concerns about the genetic stability of, and epigenetic differences to, ESC derived from human embryos. However, the ability to derive apparently normal offspring from iPSC-derived germ cells in mice is strong support for their continued evaluation as a source of genetically compatible tissues in human medicine. Studies on the use of iPSC-derived skin cells that have had a collagen VII mutation corrected in patients with the very severe condition of epidermolysis bullosa are likely to appear in clinical trials within the next 3 years. Also studies on iPSC-derived retinal epithelial cells are likely to begin within the next 2 years.

In the mean time, research involving the genetic manipulation of adult blood (haematopoietic) stem cells (HSC) is also entering early clinical trials. Targeting disruption of the HIV blood cell gene encoding the HIV co-receptor CCR5 using zinc-finger nuclease transduction (Li et al., 2013) or down-regulation of CCR5 and CXCR4 (HIV R region-long terminal repeat) products using shRNA transcripts in HSC (Ringpis et al., 2012) can provide protection against HIV fusion and entry into susceptible blood cells (particularly T cells). These approaches offer a potential cure for HIV/AIDS as demonstrated by a bone marrow transplant from a homozygous mutated CCR5 donor to a patient who had AIDS-related cancer (Allers et al., 2011). Clinical trials are about to begin using these approaches to determine their effectiveness and safety for the cure of AIDS. If these ex-vivo manipulations of

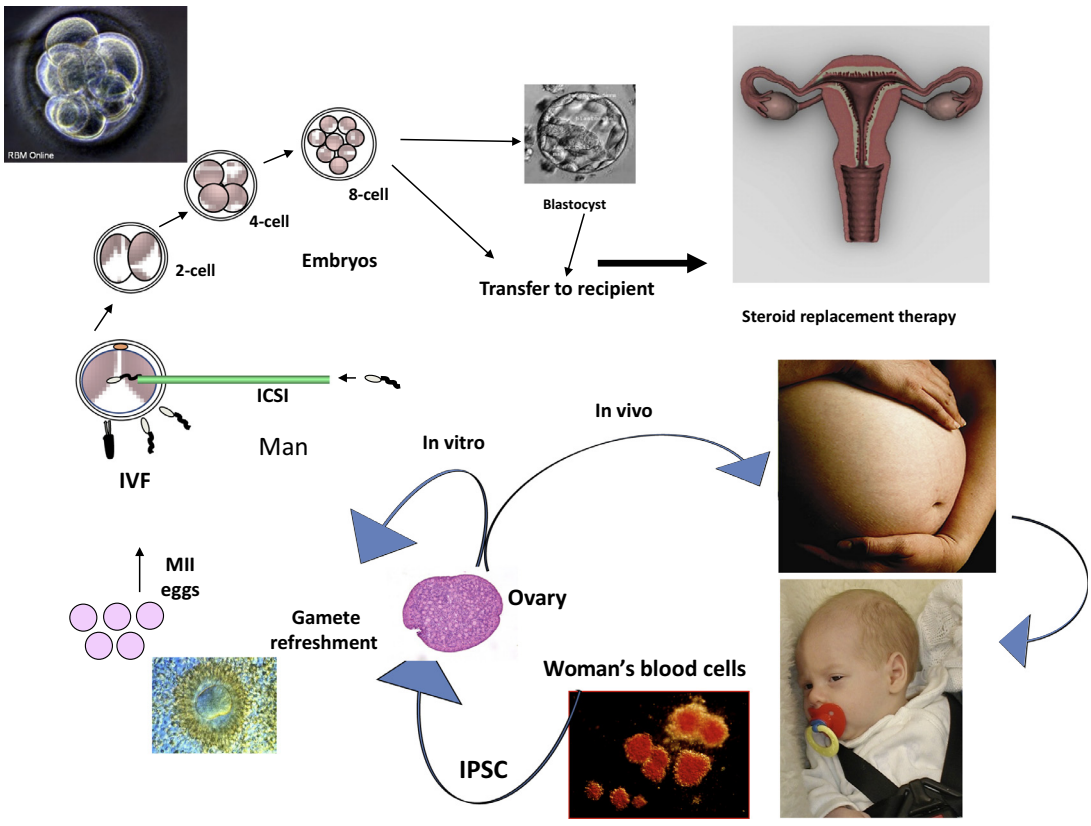

Figure 3 The potential utilization of iPSC for treating human sterility. Germ stem cell refreshment of the ovary from patient's blood cells transduced to induced pluripotent stem cells (iPSC) could in the future enable IVF procedures to generate embryos *in vitro* for otherwise sterile patients or the germ stem cell refreshment may be effective enough to allow conception *in vivo* for these patients.

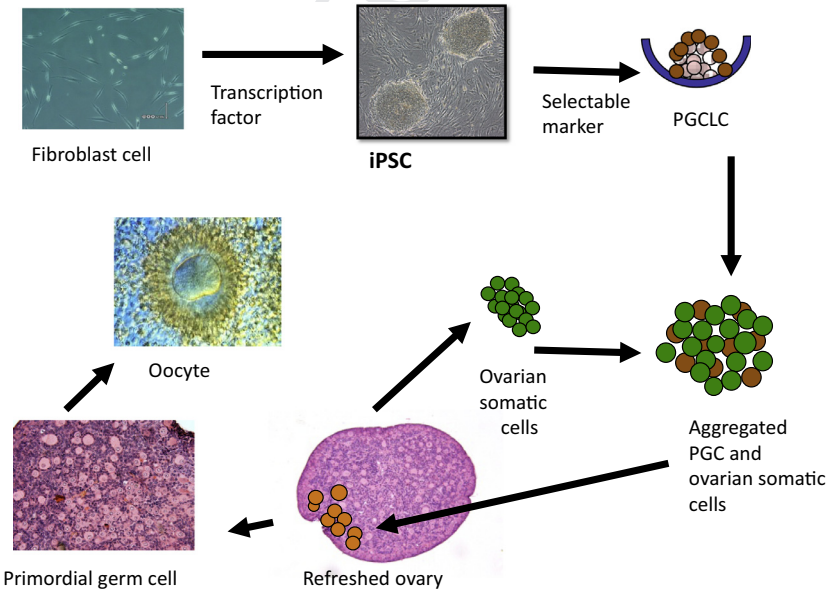

Figure 4 Producing viable oocytes from adult somatic cells in the mouse (Hayashi et al., 2012). The method used by Hayashi et al. (2012) in mice involved the production of iPSC from skin fibroblasts and their directed differentiation and selection of primordial germ cell-like cells (PGCLC) using germ cell markers. These PGCLC are aggregated with fetal ovarian somatic cells (devoid of gem cells) and transferred to a recipient ovary to produce new follicles of the original skin donor genome. This may be developed in the human to enable production of viable oocytes from patient's own somatic cells (fibroblasts or blood).

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the CCR5 gene are successful then the use of this approach for gene transduction *in vivo* should be explored in the future as a possible way to eradicate HIV/AIDS from susceptible populations. One generation of mutated CCR5 genes in the renewable long-term engrafting HSC could see the end of this terrible disease.

Other approaches for the cure of genetic diseases of the blood system are also entering the clinic, by utilizing gene-targeting technologies of HSC for the correction of mutations that include thalassaemia and sickle cell disease. Patients with children with such diseases may often seek preimplantation genetic diagnosis to avoid birth of additional children with these serious diseases. Hope for cures will be important to these families.

Conclusions

The remarkable number of studies that are now progressing through translation towards regulatory approval in California (Trounson, in press; Trounson et al., 2012a) and elsewhere gives a very strong impetus to a field that is moving quickly to establish a new paradigm for cell therapies based on the discoveries involving pluripotent stem cells. This revolution of cell-based therapies in medicine is likely to have impacts across the whole community. Like IVF, it is expected to demonstrate the good sense of exploring the nature and potential of the human ESC. The pioneers of human embryology would be pleased that their work has made such significant progress in so many fields of medicine. However, it is all yet to be verified and confirmed that cell therapies are the next major contribution to regenerative medicine.

Uncited reference

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Please cite this article in press as: Trounson, A A rapidly evolving revolution in stem cell biology and medicine. Reproductive BioMedicine Online (2013), http://dx.doi.org/10.1016/j.rbmo.2013.07.005

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Declaration: The author reports no financial or commercial conflicts of interest.

Received 14 February 2013; refereed 29 April 2013; accepted 8 July 2013.