Review

Changing genetic world of IVF, stem cells and PGD. A. Early methods in research

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Abstract

Genetics proved essential to introduce IVF, preimplantation diagnosis (PGD) and embryo stem cells in the 1960s. Its small input in early years was confined to aspects such as timing follicle growth and ovulation. Modest understanding in the mid- to late 1980s, mostly on studies in mice, involved the actions of single genes and the balance between maternal and zygotic transcripts in preimplantation stages. Human IVF began after human oocytes were matured *in vitro*, and their meiotic chromosomes analysed. Their fertilization *in vitro* led to PGD and embryo stem cells. Unlike mouse embryos, most human embryos failed to implant, so the best had to be selected to improve IVF pregnancy rates. Initially, faster-growing embryos proved superior. Later, patterns of polarized nucleoli in pronuclei, the degree of blastomere fragmentation and growth of embryos *in vitro* to blastocysts provided excellent markers. Single cells could be isolated from embryos using micromanipulation. Stem cells from inner cell mass, a branch of IVF, differentiated into immortal stem cell lines *in vitro* if disaggregated. They formed virtually all body tissues in blastocysts cultured intact or when injected singly into recipient blastocysts. Later, the genetic controls of ES cell differentiation were assessed, together with factors switching them along specific differentiation pathways. Marker genes identified ES cells differentiating into various tissues.

Keywords: *human and mouse preimplantation embryos, introduction of IVF, preimplantation genetic diagnosis, stem cells*

Introduction

This review describes the increasing significance of various branches of genetics in widening the opportunities for research into human and mouse embryos and the introduction of novel and astonishing opportunities for improving clinical care. The opening sections of this review briefly describe earlier studies forming the basis for more detailed genetic analyses into IVF, preimplantation genetic diagnosis (PGD) and embryo stem cells (ES). Modern research emerged in the form of primary studies on the endocrine control of ovulation, fertilization and embryo culture *in vitro*, a wider understanding of developmental genetics, and studies on the early stages of differentiation including the formation of trophectoderm and early stages of haematopoiesis. Developmental variations among individual blastomeres of 4-cell embryos were discovered to arise from a series of meridional divisions and a transverse cleavage division involving spindle rotation in one 2-cell blastomere (e.g. Gulyas, 1975). Clinical opportunities emerged in mid-century from this advancing knowledge in mid-century with improved forms of patient care for infertile couples, couples wishing to clear their family of their inherited genetic disease using preimplantation genetic diagnosis, and the immense potential of organ repair achievable by ES cells. Specific items included highly effective means of ovulation induction, fertilization and embryo culture *in vitro* to blastocysts, the properties of rabbit embryo stem cells (ES cells) to undergo constant replication or to differentiate and colonize various organ systems.

These items promised a clinical revolution in the 1960s. Human eggs maturing in their ovarian follicles could be aspirated, fertilized *in vitro* and grown *in vitro* to day 9 blastocysts. Embryos were replaced *in vitro* to alleviate infertility, and PGD expanded to identify chromosomal and gene defects in

preimplantation embryos and enable those free of these disorders to be replaced. In the late twentieth century, major advances in genetics led to greater knowledge of genetically-regulated developmental systems, microarray analyses, and the use of RNAi to identify genes regulating specific organelles in various developmental systems in preimplantation embryos. The final part of the review considers the significance of these recent advances on knowledge of the gene control of development, the significance of inductive systems and the allocation of stem cells to specific tissues including trophectoderm, haemopoietic and mesenchyme stem cells. Successive sections then describe the introduction of human IVF, PGD, ES cells, the significance of polarized systems, gene expression in embryos and stem cells, and the origins and properties of inner cell mass (ICM) and stem cells including haemopoietic systems. Detailed reviews of the early history of these topics have been presented by Edwards and Brody (1995).

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Human IVF and infertility

Endocrinology of ovarian stimulation, ovulation and oocyte aspiration

Leading endocrinologists in the early twentieth century, including Zondek and Ascheim (1927), clarified the nature of the hypothalamo–pituitary–ovarian axis. The significance of pituitary and hypothalamic hormones in inducing ovulation in immature and adult animals clarified the successive phases of the oestrous cycle, as in mice where females had to be selected as being in oestrus as identified by daily vaginal smears. Methods differed in rabbits which were known to be in constant oestrus and their ovulation was induced by coitus. Artificial insemination introduced in species from mouse to cattle enabled more detailed studies to be carried out on the embryology of laboratory and farm animals.

The introduction of commercial preparations of pregnant mares' serum (PMS; Gestyl, Organon) and human chorionic gonadotrophin (HCG; Pregnyl, Organon) opened sources of FSH and LH respectively. Applied to induce follicle growth and ovulation in immature animals of various species, as in mice (e.g. Runner and Palm, 1953), mature females were believed to be refractory to such treatments. Large-scale studies using Gestyl and Pregnyl were then applied to adult mice to produce numerous ovarian follicles, and to induce oestrus, ovulation and mating. Multiovulation produced more than 30 fetuses in many treated mouse females, as it became clear that the numbers of ovulated oocytes could not be predicted or controlled by varying the dose of PMS (Fowler and Edwards, 1957). Oocyte maturation and ovulation were controlled by unidentified timing genes which imposed exact timings on the stages of meiosis until metaphase II in mature oocytes. These timings were typical for each species, a valuable aspect since these techniques are now standard in laboratories and clinics world wide. Human studies began using human pituitary extracts as a source of FSH, and HCG as a source of LH (Gemzell and Johansson, 1971). Human urinary menopausal gonadotrophins (HMG) then replaced pituitary extracts to stimulate follicle growth and ovulation in amenorrhoeic women (Lunenfeld and Insler, 1990). The high frequency of multiple births including octoplets revealed that the number of large follicles increased slowly with higher doses of HMG, as in mice (**Table 1**). Many patients switched to clomiphene which had been introduced as a mild and effective stimulant (Greenblatt *et al*., 1961).

Human IVF offered new benefits to infertile couples and its introduction accelerated research on human conception. Cyclic women were now treated with HMG and HCG (Steptoe and Edwards, 1970). Their oocytes matured *in vivo* over a 37-h period and could be aspirated from their follicles using laparoscopy or ultrasound. One unexpected consequence of treating patients with active reproductive cycles emerged as conflicts between natural and artificial control of the cycle led to complex endocrine situations controlling the reproductive organs. Short luteal phases, a typical feature, led to premature menstruation, prevented embryo implantation in many patients and produced short-lived biochemical pregnancies in others. Luteal phase support with progesterone or progestagens was essential. The addition of clomiphene to treatments with HMG/HCG improved the luteal phase and achieved a fully active corpus luteum (Edwards, 1980; Steptoe *et al*., 1980).

Modern genetic technologies have now transformed the induction of ovulation in infertile patients. Recombinant gonadotrophins, GnRH agonists and antagonists, now enable tight control to be imposed on the reproductive cycle and offer more specific controls over ovarian stimulation for patients with various disorders (Olivennes *et al*., 2003). Differing ratios of agonists, antagonists, rFSH and rLH and their timings have been widely tested in numerous stimulation protocols. It is uncertain if these new technologies offer higher pregnancy rates than obtained with urinary products, although they clearly offer more controllable systems and opportunities for future advances. Even today, luteal phase weakness still characterize cycles involving HMG and HCG.

A totally different approach may soon offer an alternative to ovarian stimulation. Attention had been drawn in the 1930s to an alternative approach to obtaining mature metaphase-II oocytes ready for fertilization. Led by Pincus and Enzmann (1935), immature rabbit oocytes matured *in vitro* when liberated from their follicles into culture media. They required 12 h to mature to metaphase II and emit their first polar body. Pincus and Saunders (1939) then attempted to mature human oocytes *in vitro*, again requiring 12 h to mature. This was an error which probably misled later investigators who failed to fertilize human eggs *in vitro* (e.g. Menkin and Rock, 1948). Pincus and Saunders had failed to characterize the characteriztic stages of diakinesis and metaphase I, and later detailed trials revealed human oocytes passing through diakinesis at 24 h and maturing fully to metaphase II and an emitted polar body at 37 h (Edwards, 1965). Oocytes of cows, sheep, pig, rhesus monkeys and others matured according to their own specific genetic timetable (**Table 2**). The vast majority of human oocytes matured successfully, and in unison, to offer a source of fertilizable human oocytes independent of hormonal stimulation. After many years of research, the necessary endocrine conditions in the ovary, and the conditions of oocyte culture have now improved to produce human embryos capable of development to full term. Birth rates with this technique, exceeding 20% per embryo, now promise

Table 1. Mean numbers of Graafian follicles in the human ovary after priming with gonadotrophins (Edwards, 1980).

Dose of HMG (IU)	*Mean number of follicles*	*Mean number of large follicles*
300–375	9.2	2.2
675	8.8	2.6
900	10.6	3.6
975–1125	11.4	5.6
1500–2600	–	6.3

Table 2. Maturation of mouse, pig and human oocytes *in vitro* (h) (Edwards, 1980). Timings *in vitro* for each species accord closely with those occurring *in vivo*.

	Mouse	*Pig*	*Human*
Germinal vesicle	0–2	0–20	0–24
Diakinesis	2–4	–	25–28
Metaphase I	3–8	20–35	26–32
Metaphase II	10–12	35–43	~36

to equal those gained with endocrine stimulation (Chian, 2004; Mikkelsen, 2005). It will be interesting to follow developments in this field, especially the risks of epigenetic changes induced by culture media. The addition of fetal calf serum induces the large calf serum in cows although this feature has not been reported in humans.

Fertilization and embryo cleavage *in vitro*

Heape (1890) is credited with the first transfer of a rabbit blastocyst grown *in vitro* and their development to full-term offspring. Investigators included Lewis and Wright (1935) and Pincus and Saunders (1939), who developed in-vitro techniques for embryo culture in the early to mid-twentieth century. Simple embryo culture media were designed by Earl and by Eagle. More complex media designed to support embryos over several days *in vitro* were designed by Richard Ham, Charity Weymouth and John Biggers and medium 199 also became widely used (Paul, 1965). Methods of tissue culture also improved in parallel. Culture media were designed with controlled pH and osmotic pressures when equilibrated against gas phases of 5% CO_2 and 95% air. The introduction of plastic culture vessels replaced the need for the endless rinsing of glass vessels. Culturing animal and human cell lines was improved by the introduction of CO_2 incubators, warm stages to prevent temperature loss, the use of feeder layers, holding microdrops of media beneath liquid paraffin, the use of antibiotics and strict training in handling embryos *in vitro* (Paul, 1965).

Until the 1970s, most observations on human embryos relied on flushing them from the oviduct or uterine cavity (e.g. Croxatto *et al*., 1972). This decisive maturation of human oocytes *in vitro* opened the way to modern IVF as they were fertilized *in vitro* (Edwards *et al*., 1969). All stages of sperm entry were recorded in human oocytes matured and inseminated *in vitro*, together with the formation and expansion of pronuclei and their distinct nucleoli. Since rabbit oocytes matured and fertilized *in vitro* failed to implant (Chang, 1955), it was decided to recover oocytes matured *in vivo* in patients who were stimulated by HMG and HCG. Previous experience in mice provided a model. Patients attending the Oldham and General Hospital were offered ovarian stimulation to induce the growth and enlargement of follicles, followed by laparoscopy at ca. 36 h later to aspirate ovulatory oocytes just before ovulation was expected (Steptoe and Edwards, 1970). Modest doses of gonadotrophins induced the growth and maturation of several follicles.

Simple culture media for oocyte recovery and insemination *in vitro* were based on Earle's and other simple saline media. Later stages of growth were sustained mostly by Ham's F10 and F12, and also by Weymouth's medium and medium 199, each reinforced with pyruvate if necessary, plus antibiotics and small amounts of freshly-prepared serum taken from each patient. Fertilization and cleavage were achieved *in vitro*. Embryonic growth was classified according to cleavage times and morphology, revealing many substandard embryos with uneven sized or fragmenting blastomeres, or failing to form blastocysts (Gardner *et al*., 1998). Some embryos were diploid, and a few were heteroploid (Edwards, 1980). Approximately 50% developed to morulae or blastocysts which expanded by day 5 and trebly enlarged by day 9 (Steptoe *et al*., 1971; Edwards and Surani, 1978). Their respective inner cell masses or embryonic discs promised numerous stem cells. Single blastocysts were transferred to their mothers' uteri from 1971, but few of them implanted.

Low implantation rates were related to short luteal phases in the patients (Edwards, 1980). Luteal phase support with progesterone or a progestagen was clearly essential to sustain a secretory endometrium, so progestagen support was given throughout the luteal phase and beyond the expected time of implantation. The world's first biochemical pregnancies were identified, and the first clinical IVF pregnancy arising stimulation with HMG and HCG was established. It proved to be an ectopic and had to be removed in the early weeks of pregnancy. Clomiphene stimulation was introduced, since it established a very normal luteal phase, and it was combined successfully with HMG. Natural cycle IVF, based on assays of urinary LH and steroids, led to four clinical pregnancies. Louise Brown was born in 1978 after natural cycle IVF and the transfer of an 8-cell embryo (Steptoe and Edwards, 1976). Two other children were also delivered, one very premature.

Later advances utilized ultrasound to score numbers and diameters of growing follicles in stimulated ovaries and to aspirate their pre-ovulatory oocytes (Wikland and Hamberger, 1984). It largely replaced laparoscopy. ICSI was introduced to replace IVF in cases of need (Palermo *et al*., 1992). Its advantages included the injection of a single spermatozoon, which produced high rates of fertilization and offered new

advances in the treatment of severely oligozoospermic men. It also helped PGD by avoiding the contamination of excised tissues by accidentally aspirating spermatozoa attached to the zona pellucida. This was especially significant when using sensitive genetic assays using the polymerase chain reaction (PCR.). Wider experience with IVF confirmed a very low incidence of implantation at 20% per embryo, a situation contrasting with animals where implantation rates were 80% per embryo or even more in some species (Edwards, 1980). It was essential to improve these results of human IVF by selecting the best embryos for transfer, and several different approaches were found to improve implantation rates.

Embryo selection via cleavage rates, morphology or chromosomal constitution

Bourn Hall opened in 1980 as the world's first dedicated IVF clinic. Reintroducing natural cycle IVF led to birth rates of 20% per treatment cycle, but persisting weak implantation and pregnancy rates were not due entirely to luteal phase weakness, since combining gonadotrophins with clomiphene for ovarian stimulation did not improve implantation rates per embryo (Edwards and Steptoe, 1983). Many similar failures also occurred *in vivo*, as shown by epidemiological evidence that natural pregnancy rates were also below 20% per embryo. Low IVF and natural implantation rates were seemingly due to inherited defects in oocyte growth and maturation. Mankind was seemingly in an unusual evolutionary situation where numbers of fertile oocytes were restricted to 20% of the total eggs ovulated. Causes were obscure, even though occasional embryos had been identified as having chromosomal anomalies (Edwards, 1980). Their frequency was not the apparent cause of implantation failure, although recent evidence suggests otherwise.

Similar findings by Trounson *et al*. (1981) led them to transfer two or three embryos after stimulation with clomiphene, and improved pregnancy rates then came at the high cost of numerous multiple births. A better alternative was to select the best embryos for transfer, and this was achieved initially by timing the onset of the first and second cleavage divisions (Edwards *et al*., 1984). Implantation rates as high as 42% were attained by embryos cleaving first at either division as compared with <12% in slow-cleavage embryos. The significance of timers, undoubtedly genetic, had again been encountered. Other indicators of quality included modest rates of blastomere fragmentation (Alikani *et al*., 1999; Antczak and Van Blerkom, 1999; Van Blerkom *et al*., 2001), good polarities in pronuclear nucleoli (Balaban *et al*., 2004), well-formed blastomeres, and healthy growth to blastocysts *in vitro* (Gardner *et al*., 1998). Clinics now chose their own markers to improve implantation rates to 30–40% with single embryo transfers (Vilska *et al*., 1999). Pregnancy rates per non-selected IVF embryo remained at ca. 20% or even 15% as calculated by the US Department of Health and Human Services. Other novel approaches also included scoring oocyte morphology, the presence of multiple nuclei in blastomeres, the use of testicular spermatozoa and assisted hatching by drilling holes in the zona pellucida (Boiso *et al*., 2002). Some of these methods were essential, e.g. testicular spermatozoa had to be combined with ICSI for men with severe non-obstructive azoospermia.

As clinics opened in Australia, France, Austria and Sweden, Bourn Hall was by far the largest and conceived 1000 babies by 1988. The first IVF babies born in Germany, USA, Greece, Austria, and Germany and elsewhere were conceived in Bourn Hall. A further 500 babies were conceived elsewhere by this date. No evidence emerged of seriously increased anomalies in IVF children as IVF exploded worldwide, even though some risks due to inherited imprinted genes gave some ground for concern. The worldwide number of IVF children at the latest estimate is 2.3 million (K Nygren, personal communication).

The successful introduction of IVF opened pathways to PGD, stem cells and other aspects of clinical care. These are illustrated in **Figure 1**.

Introduction of preimplantation genetic diagnosis

Early days

Preimplantation genetic diagnosis (PGD) was introduced in 1967 with the intention of combining it with IVF and embryo stem cells (Edwards and Gardner, 1967). This combined programme was meant to avert the birth of disabled babies, and repair individuals with damaged tissues by injecting stem cells. Its initial target was to introduce methods capable of identifying embryos free of gene or chromosomal defects. Initially, phase contrast microscopy was used in an attempt to identify the sex chromatin body in living cells of female rabbit blastocysts. This body is expressed in female rabbit blastocysts from 5 days after fertilization, but is not expressed in male embryos (Chang, 1950; Melander, 1962), so its presence would target female embryos (Edwards and Gardner, 1967). Lack of success with this approach led to a micromanipulative method involving the excision of small pieces of trophectoderm from rabbit blastocysts and staining them for chromatin and the sex chromatin body (Gardner and Edwards, 1968). Groups of ca. 10 cells were excised and stained within a very short time. They could be scored microscopically for sex chromatin very easily, and successfully identified female embryos (positive) and male embryos (negative). Embryos classified as male or female were then replaced in different recipients, and at full term, all offspring were correctly sexed. PGD had arrived!

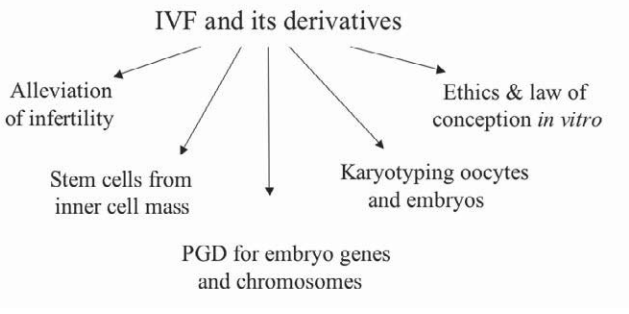

Figure 1. Derivatives of IVF.

A few spare human IVF blastocysts were also examined for sex chromatin and the Y body which characterizes males, again without any success. Searches began for Y-linked markers, produced a radiolabelled probe binding to sequences on the mouse Y chromosome. This was found to cross-react with the human Y, and it identified human Y-spermatozoa and nuclei in male embryos. This result permitted the first assessments of sex ratios in spermatozoa and preimplantation embryos (Jones *et al.*, 1987). Unfortunately, this approach demanded autoradiography which was too slow for clinical application. Attention also turned to the optimal method of excising cells from human embryos for genetic analysis. Small pieces of trophectoderm could be excised from human blastocysts, as in earlier studies on rabbits (Edwards and Hollands, 1988). Alternatively, a single cell was excised from human 4- or 8-cell embryos, again following earlier studies on animal embryos (Handyside *et al.*, 1990). After various trials, an 8-cell blastomere was assayed using PCR to identify embryos carrying cystic fibrosis. Replacing normal embryos resulted in the birth of the first unaffected child born after PGD and signified that human PGD was possible (Handyside *et al.*, 1990).

Modern PGD

Following this modest beginning, PGD was undertaken by several IVF clinics in the 1990s. Gene assays used the polymerase chain reaction (PCR), which had arrived at an optimal moment. It was used to score polar bodies or blastomeres excised from cleaving human embryos or blastocysts (Edwards, 1992; Verlinsky and Kuliev, 2003), while the success of the Human Genome Project now enabled hundreds of genes to be identified in the excised cells. FISH analyses of individual chromosomes also proved to be very effective, enabling deletions, translocations, polyploidy, aneuploidy and other karyotypic disorders to be identified in developing embryos. Today, PGD has virtually divided into these two differing topics dealing with genes or chromosomes respectively (Kuliev and Verlinsky, 2003).

PGD also opened studies on so-called 'designer babies' (Verlinsky *et al.*, 2000). This first case concerned a young child carrying the Fanconi anaemia gene, which is a severe inherited, life-threatening gene disorder. Wishing for a second child free of this disorder, the parents requested PGD to ensure their second child would be born free of this disease. Their embryos were accordingly typed for Fanconi anaemia, and those testing positive were withheld from their mothers. Those testing negative were free of disease, and stored for later transfers. Using the same excised tissue, embryos free of anaemia were then assessed for their HLA types. These were compared with those of the elder sick sibling suffering from Fanconia anaemia, to enable embryos with the closest match to be transferred to the mother. At full term, the baby was free of Fanconi anaemia, and its cord blood stem cells were isolated and grafted to its elder sibling with anaemia. Grafting unaffected cells enabled the child to make a rapid and complete recovery from its anaemia, as in irradiated and even in non-irradiated mice grafted with ES cells prepared from blastocysts (Hollands, 1988a,b). Many similar human cases have now confirmed this success (Rechitsky *et al.*, 2004), and similar treatments could be used to help parents, relations or even unrelated people suffering from Fanconi anaemia or other disease genes.

Unexpectedly, the use of PGD to identify and discard human embryos carrying particular mutants, e.g. for deafness, was not accepted by many deaf people. They preferred to have a child carrying their own affliction in order to establish good family relationships with its members sharing the same disorder. Deaf embryos were indeed transferred in some clinics, and those free of the deleterious gene were discarded at the request of the deaf parents. A debate at a recent meeting on the Ethics, Law and Moral Philosophy of Reproductive Biomedicine enabled embryologists to defend their stance on ethical issues such as this (Verlinsky, 2005).

FISH detects many anomalous embryo karyotypes

Improvements in applying FISH technology to single excised cells were also greatly improved. It was applied to classify

Table 3. Results of PGD for aneuploidy in embryos (from Munné *et al.*, 2003). Maternal age and anomalies in embryos. Values in parentheses are percentages.

	Maternal age (years)			*Total*
	<34.9	*35–39*	*>40*	
Diploid	243	301	211	755
Haploid/polyploidy	64 (12)	81 (11)	55 (9)	200
Aneuploid	59 (14)	109 (13)	151 (24)	319
Mosaic	184 (34)	203 (28)	169 (28)	556
Others	17	38	18	73
Total	567	732	604	1903

numerous anomalous karyotypes in many human embryos (**Table 3**) (Harper *et al*., 1995; Munné *et al*., 1995, 2003; Delhanty *et al*., 1997). FISH enabled normal embryos, i.e. those with no detected anomalies, to be identified and transplanted to their mothers as chromosomally-abnormal embryos were discarded (Munné *et al*., 2003; Kuliev and Verlinsky, 2003). Pregnancy rates were greatly improved when heteroploid embryos were excluded from transfer after PGD (**Table 4**). Abortion rates were also reduced in patients with chromosomal anomalies, repeated IVF failure and repeated abortion (Munné *et al*., 2003; Gianaroli *et al*., 2004).

FISH is indeed so successful that some commentators consider that screening embryonic karyotypes should be compulsory for every IVF clinic. This will add further costs to the treatment, plus extra training for specialized personnel. These extra costs for karyotyping are surely offset by attaining high rates of implantation and low abortion rates in patients rather than simply continuing with embryo selection on the basis of various characteristics in the embryos. The best approach to this problem is to identify those testicular or ovarian characteristics leading to meiotic and mitotic errors. The meiotic checkpoints governing such errors in chromosomal segregation may soon be identified, although whether they can be mended is a more difficult problem (Rienzi *et al*., 2005). Meiotic check points concerned with chromosomal attachment to the spindle, and especially the M-phase checkpoint, are the obvious targets to overcome heteroploidies involving single chromosomes. The meiotic spindle can also be assessed microscopically *in vitro*, and its position accords with the polar body in most oocytes, although its exact location has been a matter of debate. Its position and structure may be influenced by manipulating oocytes *in vitro*, including cryopreservation, and a deeper understanding these systems should help to avoid the the various forms of chromosomal imbalance in so many human embryos.

PGD has now spread world-wide. It hs helped to reduce the numbers of transferred embryos, even to single embryo transfers. It is also helping to avert the births of many children carrying adverse genes. This is exemplified by the situation in Cyprus, where the government has encouraged couples at risk of β-thalassaemia to undergo PGD, and had averted many of these problematic births. PGD is also essential for the preparation of embryo stem cells, to avert the use of those carrying chromosomal or genetic defects.

Introduction of embryo stem cells

The opening studies

The sequence of historical events described above also paved the way to preparing embryo stem cells derived from rabbit blastocysts (Edwards, 1964; Cole *et al*., 1965, 1966). Grown in media including Weymouth's, 199, Hams F10 and others with added serum, their properties were assessed in detail. Collagen-coated surfaces were widely used, the gas phase was controlled and occasionally a mesodermalizing inducer was added to the cultures. Feeder cell layers and conditioned media were widely tested. Two distinct approaches when preparing stem cells produced widely differing results. The first involved excising inner cell mass from rabbit and mouse blastocysts, and culturing them either intact in culture or after their disaggregation into their constitutent cells. In either case, cells from ICM divided endlessly through 200 or more generations. They could be cryopreserved, and resumed their divisions when thawed (Cole *et al*., 1965, 1966). These cells remained faithful in maintaining

Table 4. Results of PGD for aneuploidy in embryos (from Munné *et al*., 2003). Implantation rates among various classes of patients after PGD for aneuploidy. NS = not significant

Patient class	*No. patients*	*No. embryos transferred*	*Implantation rate*	P-*value*
All cases				
Controls	138	508	10.6	
PGD	138	272	17.6	<0.05
<2 previous failed cycles				
Controls	84	300	10.0	
PGD	84	174	19.5	0.01
2 or more previously failed cycles				
Controls	54	209	12.5	
PGD	54	98	14.3	NS
>8 zygotes				
Controls	81	311	9.3	
PGD	81	196	16.3	0.025

their enzymic profiles, their diploid chromosomal contents and morphology, and in the retention of their immortality while awaiting a differentiation stimulus. Details of the numbers of stem cell lines established *in vitro* are shown in **Table 5**. Slightly differing outcomes obtained with mouse stem cells isolated from inner cell masses included the formation of large round masses of cells *in vitro* (Cole and Paul, 1965). These were later named embryoid bodies (Evans and Kaufman, 1981).

The second approach involved the in-vitro culture of intact blastocysts on collagen surfaces. Results were totally different. Outgrowths of trophectoderm formed a thin pavement, and migratory ICM cells then migrated over it. ICM derivatives possessed an enormous pluripotency *in vitro*, as they differentiated into blood islands, muscle, neurons, phagocytes and many other body tissues. It seemed they were capable of developing into every tissue in the body (Cole *et al*., 1965, 1966). This pluripotency in rabbit stem cells was mirrored in mice when single ICM cells were injected into the blastocoel cavity of recipient blastocysts. In the resulting chimaeras, virtually every tissue except trophectoderm became a mosaic of host and donor cells (Gardner, 1968). Backcrosses between these chimaeras and mice of the donor strain produced spermatozoa and oocytes derived from the donated cells. They were obviously fully pluripotent and capable of producing gonads and gametes in addition to other tissues. In-vitro cultures or preparing chimaeras had thus revealed the astonishing pluripotential of stem cells *in vitro* and *in vivo*.

Similar investigations in mice involved the use of teratocarcinoma cells cultured on various substrates sometimes with feeder cells to prepare clonal cell lines (Pierce, 1967; Stevens, 1967). They also formed blastocyst injection chimaeras (Evans, 1972). As previously discovered in rabbits, blood islands and other tissue cells formed outgrowths of mouse ES cells in syngeneic mice or when cultured on allantoic membranes isolated from quails (Evans and Kaufman, 1981). Teratocarcinoma cells were more tumourigenic than ES cells.

Injecting single ES cells into blastocysts to form blastocyst injection chimaeras proved to be invaluable for several major aspects of embryo research. The potency of single blood, nerve and other forms of stem cells in chimaeras reflected their capacity to form numerous body tissues in the resulting chimaeras (Fleischman and Mintz, 1979, 1982; Risau *et al*., 1988). The exposure of ES cells to FGF encouraged them to undergo angiogenesis, IL3 stimulated the formation of macrophages and βFGF enriched neuroepithelial precursor cells (Risau *et al*., 1988). Donor cells also marked various forms of genetic change such as X-inactivation. Blastocyst injection chimaeras were also decisive in the introduction of homologous recombination by ensuring that donor genes had been inserted at the correct site in the genome of the donor cells (Smithies *et al*., 1985; Doetschman *et al*., 1987; Thomas and Capecchi, 1987; Williams *et al*., 1988).

Mouse embryo stem cells could also colonize tissues in adult animals. This was first achieved when disaggregates of mouse blastocysts grown for 3 days *in vitro* were grafted to X-irradiated recipients (**Figure 2**) (Hollands 1988a,b). Injected into recipients via their tail veins, the stem cell differentiated very quickly, becoming active in recipients within 4–5 days. They migrated to liver in a few days, and then moved to spleen and bone marrow. Controls died within a few days whereas injected recipients lived a full lifespan, sustained by donor cells which permanently colonized their bone marrow. Non-irradiated recipients could also be colonized but to a much lower degree, perhaps due to the absence of 'space' to colonize in bone marrow. Surprisingly, they did not reject the grafts. Genetic disease in recipients could also be overcome in many recipients by this treatment, and the colonizing cells did not induce cancers or other forms of damage.

As with IVF and PGD, the reported characteristics of ES cells led to severe criticisms about the methods and about the advantages to be gained. Hollands' work was criticized by Brent *et al*. (1989) and by Tomlinson (1989), who was a PhD student working with Alan Trounson, since they could not obtain similar outcomes. Hollands (1990) responded, defended his work, and has witnessed the enormous advances in stem cell biology over a decade or longer.

Early data on the genetic control of ES cell differentiation

Attention turned to the genetics of ES cells and their regulation *in vitro*. Media containing leukaemia inhibitory factor (LIF) were essential to prepare murine ES cell lines (Williams *et al*., 1984). LIF invoked proliferation among non-differentiating murine ES cells in the absence of feeder layers, and similar effects were noted with inerleukin-6 receptor (IL-6), interleukin-11, oncostatin M and ciliary neurotrophic factors (CNF). Later studies indicated that LIF was unnecessary when preparing human ES cells. Differentiation among murine ES cells was prevented by exposing them to polypeptide differentiation inhibitory ability (DIA) in contrast with human ES cells which had a different response (Gearing *et al*., 1987). Murine ES cells were also regulated *in vitro* by DIA, which induced the formation of macrophages. IL-7 induced B-cell lineages, erythroprotein induced myeloid and other lineages, IL-7 enhanced the formation of mast cells and neutophils, and FGF invoked angiogenesis.

Typical markers of static or differentiating ES cells included Oct-4, which regulated totipotency and held the cells in an undifferentiated state. SOX-2 had similar functions including its action via Fgf4, itself expressed in ICM, in establishing polar trophectoderm. Fgfr2 located in trophectoderm. Other factors driving ES cells to haemopoietic cell lines included retinoic acid, transferrin and IL-3. The ability of neuroepithelial precursors including glia and dopaminergic and serotoninergic neurons to be enriched in serum-free media containing βFGF was confirmed (Okabe *et al*., 1996). They expressed several specific genes including *Pax 2*, *Pax 4*, *Wnt1* and *En1* (Lee *et al*., 2000). Their output of dopamine was doubled when exposed to sonic hedgehog, (SHH), FGF8 and ascorbic acid. ES cells also differentiated into muscle cells, cardiomyocytes and various other tissues (Westfall *et al*., 1997).

Similar work on human ES cells to that just described became possible when day 5 and then day 9 blastocysts were grown *in vitro*, with distinct inner cell masses or embryonic discs respectively (Steptoe *et al*., 1971; Edwards and Surani, 1968). A first attempt at making human stem cells (Fishel *et al*., 1984) failed either because inner cell mass cells degenerated

Table 5. Characteristics of some cell lines established from inner cell mass/embryonic disc (Cole *et al*., 1965, 1966).

Name	*Origin*	*Time to establish*	*Characteristics*[a]	*Enzymes*	*Persistence*
RB1	Day 6, outgrowths of embryonic disc	Established at 6 weeks	Fibroblastic, spindle-shaped	High arginase	>11 months or 200 generations
RB2	Day 6		Fibroblastic diffuse		
RB3/3	Day 6		Epithelial	High alkaline phosphatase[b]	>9.5 months
RB3/4	Day 6		Fibroblastic, as RB1		

[a]RB1 and RB3/3 remained largely diploid.
[b]Alkaline phosphatase.

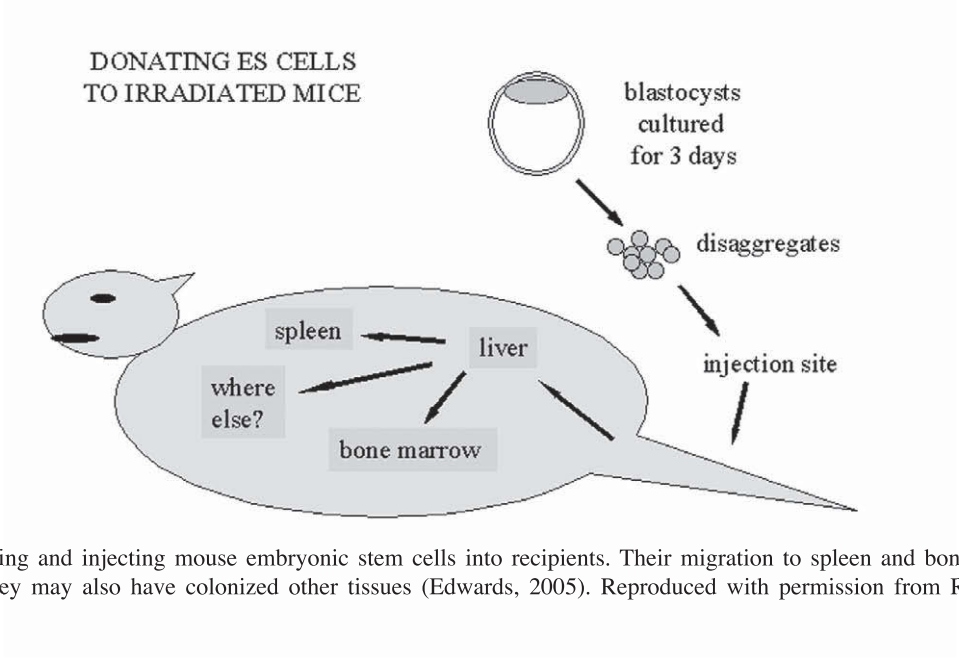

Figure 2. Preparing and injecting mouse embryonic stem cells into recipients. Their migration to spleen and bone marrow is indicated, and they may also have colonized other tissues (Edwards, 2005). Reproduced with permission from Reproductive Healthcare Ltd.

and did not grow over trophectodermal cells, or because the trophectodermal outgrowths were too weak. The first human ES cell lines were produced in 1997 using methods identical to those used in 1966 to prepare rabbit ES cell lines, and obtaining very similar results (Thomson *et al*., 1998). A summary of the avalanche of reports on human stem cells in the final years of the twentieth century is presented later in the second section of this extended review. Details on the differentiation of murine and human ES cells *in vitro* and their role in grafting to recipients were reviewed by Edwards (2004).

Summary

This review has described the early events resulting in human IVF, PGD and stem cells. Details of developments in endocrinology, ovulation induction, the culture of human embryos *in vitro* and problems associated with establishing the first human IVF pregnancies have been enunciated. The introduction of hormonal stimulation in animals and humans using gonadotrophin preparations have been described, followed by the recent introduction of recombinant gonadotrophins and GnRH agonists and antagonists. Problems with short luteal phases and biochemical pregnancies are described. Genetic approaches sustained many studies on the early stages of oocyte formation and maturation, and helped to assess the development of human embryos to blastocyst stages between day 5 and day 9. Clinical methods introduced for the introduction of human IVF and are still used today. Rates of implantation of embryos transferred to their mothers were very low, at ~20% per embryo. A similar situation *in vivo* implied that the embryonic errors were inherited and not due to the use of IVF. Currently, the best embryos are selected by means of early cleavage, well-formed polarities, few cytoplasmic fragments or rapid growth to blastocysts, These and other forms of selection identify embryos with implantation rates of 50% or more, while the remainder are largely ineffective. These studies opened wide clinical opportunities for the alleviation of infertility, the detection of anomalies in embryos and the value of stem cells in biomedicine. By today, more than 2 million IVF babies have been born.

The introduction of PGD has led to elegant methods to study mouse and human embryos, and to apply PGD and other genetic analyses to embryos. The introduction of FISH enabled the great majority of heteroploid embryos to be identified and discarded rather than replacing them in their mothers. Replacing embryos known to be diploid enhanced the rate of implantation, indicating that errors in the meiotic cycle may be the primary cause of embryonic death. PGD using PCR to detect gene anomalies in embryos has also expanded, and data from the Genome Project opens this method to the entire genome. Many gene defects in newborn children have been avoided by the application of this method. The introduction of 'designer babies' permitted the transfer of normal embryos with known HLA markers to their mother, so that cord blood collected at term could be grafted into an older sick sibling with Fanconi anaemia. This sibling recovered rapidly from its anaemia.

The first immortal and faithful ES cells were prepared from ICMs of rabbit blastocysts. They persisted unchanged through many generations *in vitro*. Culturing intact blastocysts led to outgrowths of trophectoderm and then ICM cells which differentiated into blood islands, muscle and many other tissues. Similar results accrued in mice when the grafting of single ICM cells to intact blastocysts produced chimaeras in which virtually all the recipients' tissues had been partly colonized by descendants of the grafted cells. Blastocyst chimaeras proved to be invaluable in many types of analysis, including the introduction of homologous recombination. The role of LIF in the maintenance of mouse ES cells *in vitro* is described, along with the actions of several other substrates driving them along differentiation pathways as they expressed Oct-4, Sox-2 and other genes. Human ES cells were introduced in 1984 and improved in1998 using methods identical to those used to prepare rabbit ES cells 35 years previously. All these studies have established a firm base for the therapeutic potential of ES cells.

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