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REVIEW

Genetics, epigenetics and gene silencing in differentiating mammalian embryos[1]

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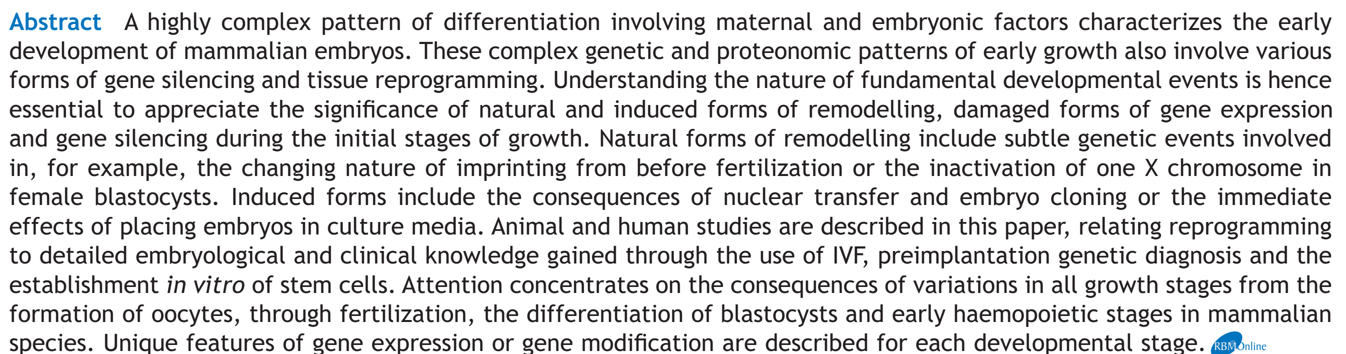

Abstract A highly complex pattern of differentiation involving maternal and embryonic factors characterizes the early development of mammalian embryos. These complex genetic and proteonomic patterns of early growth also involve various forms of gene silencing and tissue reprogramming. Understanding the nature of fundamental developmental events is hence essential to appreciate the significance of natural and induced forms of remodelling, damaged forms of gene expression and gene silencing during the initial stages of growth. Natural forms of remodelling include subtle genetic events involved in, for example, the changing nature of imprinting from before fertilization or the inactivation of one X chromosome in female blastocysts. Induced forms include the consequences of nuclear transfer and embryo cloning or the immediate effects of placing embryos in culture media. Animal and human studies are described in this paper, relating reprogramming to detailed embryological and clinical knowledge gained through the use of IVF, preimplantation genetic diagnosis and the establishment *in vitro* of stem cells. Attention concentrates on the consequences of variations in all growth stages from the formation of oocytes, through fertilization, the differentiation of blastocysts and early haemopoietic stages in mammalian species. Unique features of gene expression or gene modification are described for each developmental stage. RBMOnline

KEYWORDS: cloning, early embryogenesis, early haemopoietic stages, gene silencing, mammalian embryos, tissue reprogramming

Introduction

Oocytes and embryos are sensitive to various factors capable of modifying their differentiation. Some of these factors are natural, others are experimental. An early example emerged ca. 50 years ago when Conrad Waddington, then my Professor of Genetics in Edinburgh, raised the incubation temperature of *Drosophila* eggs. He obtained offspring with two legs instead of eyes, having apparently reinstated a silent gene that bred true thereafter. He coined the term epigenetics to cover genetic events determined by factors outside the genome, and named the re-awoken gene *aristopedia*. He pondered whether a gene silent for centuries had been reactivated or a position effect related to this particular gene had been induced. Since those early days, various forms of remodelling have been widely assessed in searches for epigenetic systems and also for recent moves towards human reproductive and therapeutic cloning.

Reprogramming cells and embryos is hence a major feature of the present manuscript although relevant data are still limited. Detailed knowledge is essential to understand the basis of the proteonomic and genetic regulation of numerous tissues forming in early post-implantation embryos from oocyte to blastocyst, and is presented here in review form. The differentiation of the haemopoietic system is also discussed in this review as an example of an early-forming tissue in post-blastocyst stages. The intention of this paper is to clarify this mass of knowledge.

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Analyses on the preimplantation development of human and mouse embryos

Epigenetic and remodelling events may involve obscure proteins or carbohydrates that have not yet been traced since the necessary analytical methods are still imprecise. Others may involve large and even massive and simultaneous alterations in numerous developmental systems, such as when epigenetic changes affect single genes or enzymes and so distort normal gene control from the earliest stages of oocyte maturation and fertilization. Natural forms of reprogramming include the formation of sex-linked imprints identified in preimplantation mouse embryos, which are modified during cleavage and the formation of the blastocyst.

The ovarian oocyte, fertilization and the early embryo

Extensive studies have clarified the formation and growth of ovarian follicles. Early follicles form in the ovaries of early fetuses in mice and humans and grow under the control of various factors, including the gonadotrophins. Belief over many years suggested that the oocytes and follicles formed late in gestation or soon after birth in some mammalian species. Those formed in the fetus enter a prolonged dictyate phase as they form a germinal vesicle, which is, in essence, an arrested diplotene capable of persisting for 50 years or longer in some human oocytes until the onset of ovulation and fertilization. Their prolonged existence may result in many human embryos possessing unbalanced chromosome complements, multiple nuclei, cytoplasmic fragments and other errors in early development. Such errors could arise through genetic or epigenetic errors at meiotic checkpoints, through distortions in a 'production line' of oocytes, or when ovarian oocytes suffer long exposures to epigenetic agents. Similar embryonic disasters are apparently rarer in mouse oocytes, perhaps due to their much shorter life span. This view of oogenesis was queried when numerous oocytes originating in adult life were discovered. They arose from dedifferentiating bone marrow cells that migrated to the ovaries, to add a further aspect of ovarian function (Johnson *et al.*, 2004, 2005).

Over the last decade, new approaches in proteonomics and gene transcription have revolutionized the study of embryogenesis. Earlier methods of measuring proteins or carbohydrates in various cells or tissues by means of gels or columns were reinforced by the introduction of 2-dimensional protein assays and by the introduction of fluorescent in-situ hybridization and the use of fluorescent G protein markers. Van Blerkom *et al.* (1976) introduced such assays to study the changing distributions of hundreds of genes at successive developmental stages in preimplantation mouse embryos, as analyses of preimplantation embryos at the molecular level revealed global expression patterns of RNAs (Piko and Clegg, 1982) and proteins (Van Blerkom *et al.*, 1976). Antczak and Van Blerkom (1997, 1999) also produced classic examples of proteonomics in relation to polarities in mouse and human embryos as described below.

A total of 14,000 known oocyte proteins were classified into distinct families. Moving to specific sites in ooplasm, their variations in culture included surface markers, folding and development to new shapes, juxtaposing groups, protein/ligand coupling, conserved residues, and various mutants (Thornton, 2001). Variations affected substrate specificity and catalytic residues, and frequent mutational changes involve Arg and Gly substitutions. Toxic compounds can modify each of these characteristics, and inherited variations may vary between tissues or individuals to result in modified protein structures regulating developmental or disease genes.

Earlier means of studying gene expression in early mammalian embryos measured the functions of individual genes, analysed crossover locations of related genes, studied homologues with other species or analysed mutant forms of the genes under study. Gene homologies also yielded valuable information from analyses on *Caenorhabditis elegans*, *Xenopus* and *Drosophila* (Edwards, 2005a). Today, gene analyses have been strengthened by the acquisition of microarrays and the utilization of RNAi. Thousands of genes can be grouped into various classes or allocated to specific developmental systems such as successive waves of genetic activity during preimplantation stages (Ko *et al.*, 2005). Hamatani *et al.* (2003) assessed four transcription waves: in 2–4-cell stages (zygotic genome activation) perhaps determined by a maternal clock, 8-cell stages (mid-preimplantation development), and finally in morulae and blastocysts. Each wave was soon largely down-regulated. Earlier knowledge was confirmed such as the metabolic switch from pyruvate to glucose (Leese, 1995). Differentiating mouse embryos displaying successive waves of gene activity typify the situation in cleaving embryos. Epigenetic alterations in transcription waves may be induced by nuclear transfer (NT), since genetic effects have been identified by Boiani *et al.* (2002) and Bortvin *et al.* (2003), as described below.

Analyses using RNAi are also invaluable adjuncts to the study of gene expression and function. Originally described by Fire et al. (1998), RNAi are significant controllers of gene function during development and are also invaluable for gene knock-out. Groups of genes controlling polar body extrusion, pronuclear growth and cell division were identified in *C. elegans* embryos between the 1–4-cell stages when hundreds of genes active in specific developmental functions

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were identified and classified (Sonnischen et al., 2005). Many of the identified genes had human homologues, so these data may be relevant to human embryos. This information was applied to form molecular machines coordinating the topology of this integrated network, and it identified similarities in transcription profiling in different tissues (Gunsalus et al., 2005). One model identified high densities of genes with specific functions such as ribosomes, mitochondrial systems and anaphase-promoting complexes. A second model measured genes participating in systems such as RNA/protein metabolism, while a third model on cell polarity identified all the Par proteins and a mammalian homologue of Cdc37 shuttling in and out of nuclei as polarity was established.

Embryonic polarities and cleavage planes in early mammalian differentiation

The study of polarities in mouse and human embryos is currently attracting considerable attention. Body axes are clearly essential as a basis for controlled gene action and tissue differentiation. Induced genetic defects could be disastrous for the embryo and offspring, as witnessed in some anomalies of the left/right (L/R) axis in humans. The existence of polar axes was initially based on morphological evidence of germinal vesicles sited in polar positions and the extrusion of the first polar body at the animal pole. Among many attempts at clarifying the existence of embryonic axes, studies using radiolabelled proteins and RNA were inconclusive in identifying gradients in their distribution in mouse oocytes (Edwards and Sirlin, 1956). Evidence then emerged of the existence of an animal/vegetal axis as mouse and human oocytes initiated their formation and differentiation (Antczak and Van Blerkom, 1997; Edwards and Beard, 1997). Important roles were suggested for granulosa cells, especially those surrounding the animal pole, which seemingly inserted proteins such as leptin and STAT3 into cortical ooplasm at this pole in the formative stages of oocyte growth. Once established, these and other proteins retained their polarized positions throughout oogenesis, fertilization and early embryogenesis, and were finally confined to trophectoderm and absent from inner cell mass (ICM). Similar distributions to trophectoderm only were also detected for *bax, Bclx, TGF-2 VEGF, c-kit and c-erbB (EGF-R*) (Antczak and Van Blerkom, 1999).

The gene *staufen* has a significant role in establishing polarities in oocytes, and BLAST searches and cDNA expressed sequence tags have confirmed homologies among humans, mice, rat and *C. elegans* and its role in relation to microtubules and endoplasmic reticulum in several species. A second dissimilar *staufen* gene has been identified in mouse and human embryos (Saunders *et al.*, 2000). Other well-known examples of the significance of closely related gene systems involved the six *par* genes originally identified in *C. elegans* but now found in several species including humans (Guo and Kemphues, 1995, 1996). Divided into two groups: *par-1* and *par-4* in group 1 are primarily cytoplasmic whereas *par-2, 3, 5, 6* in group 2 affects spindle orientation. Weak cross-reactions occur between groups. Polarities in kinases and *par* genes in somatic and germline cells in *Drosophila*, *C. elegans* and *Xenopus* proved to be relevant to mammals, for example in relation to the PAR-6/PAR-3/PKC-3 complex, which is also widely conserved (Hung and Kemphues, 1999). MARKs (MAP/microtubule affinity-regulating kinase) are human homologues of *par-1* and phosphorylate a repeated motif in the microtubule binding domains of Tau, MAP2 and MAP4 (Drewes *et al.*, 1997). Their influence on microtubule binding and depolymerization resembles the regulation of microtubule dynamics in *Drosophila*.

Observations on developing *Drosophila* oocytes by Cáceres and Nilson (2005) clarified and confirmed the studies of Antczak and Van Blerkom (1997) on the formation of polarities in mouse and human oocytes. Previous authors had revealed the asymmetric distributions of *gurken* mRNA and protein in flies and amphibians. These genes were essential in defining the anteroposterior and dorsoventral axes of the embryo, and Cáceres and Nilson (2005) showed how nurse cells, and not the oocyte, inserted gurken protein into ooplasm at the correct polarized point in the *Drosophila* oocyte.

Another approach to studying mammalian embryos involves the formation and function of various axes, although there is no direct information on their genetic control. Their expression in oocytes and early embryos could dictate the physical characteristics of oocytes and early embryos, and the shape and size of blastomeres in cleaving embryos. Such forms of prepatterning in 1-cell embryos, in the sense that embryonic structure was informative, were measured by Gardner (2001). When bilateral symmetries of embryo cells were measured the A/P axis was found to be orthogonal to the plane of the first cleavage division and to the embryonic/abembryonic polarity in blastocysts. Some investigators dismiss such 'pre-programming' events, proposing instead that polarizing systems are established post-fertilization. Consequently, fierce debates emerged regarding the induction of changed polarities at sperm entry. This topic is clearly an essential aspect of embryonic patterning. Thus, Zernicke-Goetz (2003), Gardner and Davis (2003) and Gardner (2006) insisted on the significance of pre-programming and the plane of the first cleavage division, whereas Hiragi and Solter (2004, 2005) suggest that polar axes are determined by the topology of the two pronuclei and the shape of the zona pellucida. A third approach suggests that human ooplasm rotates after ovulation, enabling it

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arrange itself with a polarity synchronous with the point of sperm entry and driven by the sperm aster (Edwards and Beard, 1997). This situation remains to be resolved.

The fundamental gene regulating axis formation in various species is *Cdc42*. It produces a GTPase expressed under tight spatial and temporal control, and responsive to internal and external stimuli. Regulated by downstream effectors and diverse regulators, guanine nucleotide exchange factors are stimulatory while guanine nucleotide dissociation inhibitors are inhibitory (Etienne-Manneville, 2004). Fundamentally a molecular switch, *Cdc42* regulates microtubules and responds to internal and external agents via integrins and cadherins. As yet undetected, it must be involved in the earliest stages of oogenesis and embryogenesis in mammals, although it has not been detected so far.

Emerging conflicts on the degree of differentiation in mammalian blastomeres during cleavage stages in mammalian embryos are again a matter of great significance in embryogenesis. Opinions expressed by many embryologists state that blastomeres in cleaving embryos are genetically similar and undifferentiated. This concept has now been questioned in favour of the importance of combined effects of embryonic polarities and cleavage planes in determining the fate of individual blastomeres by the 4-cell stage.

Polarized locations of proteins and mRNAs in mammalian embryos became highly significant for early embryogenesis. These factors are distributed into 4-cell blastomeres by maternally controlled cleavage planes during the first two cleavage divisions. A meridional first cleavage division is followed in 2-cell stages by a second meridional division in one blastomere and an equatorial division in the other (Gulyas, 1975). This results in 4-cell embryos possessing two blastomeres with complete A/V axes, which are possibly the precursors of ICM. The third blastomere possesses animal ooplasm only and is apparently the trophectoderm stem cell; it was later discovered to produce *HCG-β*. The fourth and distant blastomeres inherit vegetal ooplasm and might be germline precursor. Therefore, there was no clear knowledge on the transmission of germplasm in early mammalian embryos as compared with other Orders. In many 4-cell embryos, this form of cleavage also resulted in three blastomeres being linked to the polar body while the fourth lay distant, was sited at the base of the other blastomeres, and may have been germline as just discussed (Edwards and Beard, 1997, 1999; Edwards and Hansis, 2005; Hansis, this Symposium). This matter will be discussed in greater detail below.

Correlations between cleavage planes and polarities were assessed by gene markers including the transgene *CAG-CAT-2* (Fujimori *et al.*, 2003). Independent markers identified each individual 2-cell blastomere and revealed their derivatives were mixed randomly by day 8.5 post-coitum indicating they were equal, had mixed at random and differentiated together. In contrast, marking individual 4-cell blastomeres revealed three distinct categories of development. One revealed random mixing, resembling the situation with 2-cell blastomeres. The second category migrated to extra-embryonic tissues including trophoblastic giant cells and extraplacental cone, and not to the embryos proper. The third category was identified in the embryo proper and in its extra-embryonic mesoderm. Similar variations were identified in the fates of individually marked 2- and 4-cell embryos. This evidence must have been determined by forms of ordered cell growth, as reported by several other workers.

As this paper was being written, Piotrowska-Nitsche *et al.* (2006) reported that injecting markers into single blastomeres enabled their fate in blastocysts to be determined (**Figure 1**). Embryos where the first-dividing 2-cell blastomere divided meridionally and the second divided transversely or obliquely produced

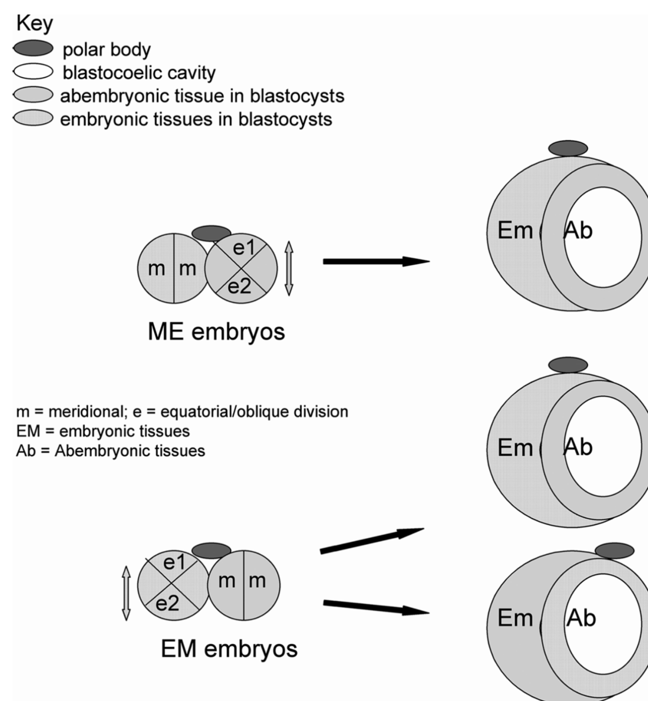

Fig. 1. Analyses of the fate of cytoplasm of 4-cell mouse embryos in blastocyst tissues. The relationships between the patterns of cleavage of 2-cell blastomeres is demonstrated, with some cleaving meridionally initially, then equatorially and obliquely, which led to the early-cleaving blastomere at the 4-cell stage colonizing embryonic tissue and later-cleaving blastomeres colonizing extra-embryonic tissues. In contrast, some 4-cell embryos display an initial equatorial/oblique first division, followed by a meridional second division to produce 4-cell embryos. The first-dividing 4-cell blastomere with this pattern of formation can contribute to the embryonic regions of the blastocyst but also to the abembryonic regions. These two classes of embryos, as indicated in the figure, amounted to >80% of the blastocysts examined. Based on a figure from Piotroska-Nitsche *et al.* (2006).

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fast-growing tissues colonizing embryonic regions of the blastocyst. This combination of cleavage divisions arose in a greater proportion of 4-cell embryos. In contrast, the later-dividing blastomeres produced daughter cells, which congregated in abembryonic tissues. If the first-dividing blastomeres displayed an oblique or transverse cleavage, some descendants colonized abembryonic tissues, although the other blastomeres were still able to colonize embryonic tissues in blastocysts. This evidence thus agrees with that of Fujimori et al. (2003) and the earlier models of mammalian development. These data each confirm that cleavage planes are highly significant and that errors in their patterns may not necessarily lead to lethal developments in the embryo.

Among other examples of polarity detected in mouse morulae and blastocysts, *Par 3* induced trophectoderm cells to polarize at right angles to the blastocoelic cavity. Consequently, some inner daughter cells projected towards the blastocoel and became incorporated in ICM while the remaining outer cells remain trophectodermal. Hence, ICM cells consist of two fractions, initially from inner cells in the early embryo and later from outer cells derived from trophectoderm (Handyside and Johnson, 1978). This polarizing system fails to operate in $Par3^{-/-}$ mice, which inherit a reduced ICM (Plusa *et al.*, 2005). The two ICM layers may have differing functions in development, and their isolation may help to clarify the origin and functions of embryonic stem (ES) cells.

Homologies in various species also arise in relation to the cadherin–catenin system. Cell polarity depends on cell adhesion, the cytoskeleton and signalling involving atypical protein kinase C (Izumi *et al.*, 1998). Catenins are essential for adhesion between cells, cell shape and morphogenetic movements, and form complexes with cadherins. Mediated by the E-cadherins and extracellular matrices, *ASIP* and PKCλ are essential to assemble the actin cytoskeleton and signalling networks in epithelial cells (reviewed by Eaton and Simons, 1995). Trophectoderm itself is a modified epithelium with outward-directed polarized apical domains permitting fluid transport to the blastocoelic cavity at ca. 3 days post-fertilization (Fleming and Johnson, 1988). Complexes of maternal E-cadherin and α- and β-catenin in mouse oocytes and cleaving embryos associate with cytoplasmic polyadenylation elements in cleaving embryos, and may interact with EGF-R, a mitogen located in basal regions of epithelial cells, which concentrates and polarizes at outer cell surfaces in 8-cell stages and at apical cell surfaces in morulae (Wiley *et al.*, 1992; Ohsugi *et al.*, 1996). These interactions persist into trophectoderm, to regulate compaction and cell polarization (Fleming *et al.*, 1991; Eaton and Symons, 1995; Ohsugi *et al.*, 1996). The role of cadherins in establishing polarities will be discussed below.

Vinot *et al.* (2004) recently described another polarized situation in murine oocytes whereby two active *PAR6* genes were polarized on the metaphase-1 spindle. Surprisingly, these authors failed to quote earlier and relevant works of Antczak and van Blerkom (1997) and Edwards and Beard (1997).

Imprinting syndromes

Another major area of embryonic development that is full of gene silencing and other major epigenetic effects concerns the imposition of imprints on gametes and preimplantation embryos. These were first identified some 20 years ago, and have since attracted immense attention (see Surani *et al.*, 1990; Reik and Walter, 2001). They involve major genetic changes leading to gene activation or silencing and to the reprogramming of embryo cells. Imprints can be characteristic of particular alleles in males and females, characterize male and female gametes, and emerge in cells specializing also germline or stem cells. Specific genes expressed in embryos may hence be derived from one maternal or paternal allele or both. Peg3, for example, is expressed paternally in transgenic mice, and is regulated by elements lying some distance away on the genome, which interact with a responder sequence in the gene (Szeto *et al.*, 2004). Closely timed and regulated, the actions of such genes can be reversed, e.g. in germline precursors. Correct imprinting is essential since lethality or abnormality can arise through errors or mutations in mouse and possibly human embryos involving allele-specific imprints leading to the human Prader–Willi syndrome. A human global maternal-effect mutation has recently been identified, which disrupts all normal maternal imprints that assume a paternal pattern (Judson *et al.*, 2002). Afflicted embryos resemble the androgenetic complete hydatidiform mole.

Grafting nuclei or pronuclei into oocytes was among the early findings that led to studies on gene methylation and imprinting (Reik *et al.*, 1993). The biochemical systems involved included gene imprinting, DNA methylation, epigenesis, the suppression of ectopic genes, and the induction of differential forms of gene expression. These are each potential regulators of early forms of embryo differentiation. Genomic modifications involve parental imprinting and X inactivation. Anomalies emerging after pronuclei were exchanged between mouse eggs included a repression of transcription and the re-methylation or demethylation of individual genes. Retarded fetal growth was seemingly due to epigenetic disorders similar to those described above. Similar observations were reported in lambs and calves (Mayne and McEvoy, 1993). Parthenotes seldom grow to later embryonic stages for similar reasons, until adult parthenogenetic

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Table 1 Stages of methylation and imprinting in oocytes and embryos in late oogenesis and immediately post-fertilization in mice (modified from Arney *et al.*, 2002).

Stage of development	Time pre- or post-fertilization (h)	Form of methylation and HP1b recruitment	
		Maternal	Paternal
Oocyte at MII	0	Methylated histone H3	–
Sperm entry	1–6	HP1b recruited + de-novo methylation	DNA methylation
Pronuclear	6–8	–	HP1b recruited
Pronuclear	12	–	Histone methylated

mice were obtained by grafting pronuclei into oocytes and modifying the expression of H19 and Igf2 (Kono *et al.*, 2004). Other examples of the significance of epigenetic mechanisms involve hsp90 acting as a 'capacitor' of the evolution of morphological systems (Sollars *et al.*, 2003), and the role of chromatin imbalance apparently affecting the expression of *Krüppel*, which is normally active in the eyes of *Drosophila melanogaster*. Chromatin remodelling and other strong reinforcing mechanisms can also influence the silencing of active genes and reprogramming of silent genes.

In mammalian eggs, epigenetic characteristics typify features of the two parental genomes as imprints are imposed on gametogenic cells. Differing patterns of methylation patterns are then imposed on sperm heads and on maternal and paternal pronuclei at fertilization. Some timing patterns vary in maternal and paternal pronuclei as they are imposed initially in the latter and then in the former. Hence, methylation influences paternal genomes in early pronuclear stages as compared with newly ovulated or fertilized eggs in the maternal genome at a time when sporadic defects are common (Buiting *et al.*, 1998; El-Maarri *et al.*, 2001; Arney *et al.*, 2002; Surani, 2002) (**Table 1**). Extraneous factors can impair these developmental systems as in sheep embryos grown *in vitro* after fertilization *in vivo* where the imprinting control of maternally derived Igf2r may be hypomethylated (Young *et al.*, 2001). In mice, substances stored in ooplasm bind differentially to parental genomes, e.g. the heterochromatin-associated protein HP1b interacts with histone H3, itself methylated at lysine 9 (Bannister *et al.*, 2001). High concentrations of lysine 9-methylated histone H3 on oocyte chromosomes persist in unfertilized eggs and in maternal pronuclei. These events are reinforced by the methylation of stored HP1 b in ooplasm and by its exclusive and preferential binding to this pronucleus. Initial binding at the centromeres spreads to entire chromosomes between 1 and 5 h post-fertilization.

Biochemical aspects of imprinting have been closely investigated. Histones coat the paternal genome, which binds neither HP1b nor mtH3. Epigenetic asymmetry between the parental genomes in mouse oocytes may involve interactions between HP1b, lysine 9-methylated histone H3 and the maternal genome whereas the paternal binding of HP1b occurs in pronuclear stages (Arney *et al.*, 2002). This lack of paternal binding of HP1b in later pronuclear stages complies with its earlier preferential binding there immediately after fertilization (Santos *et al.*, 2001; Arney *et al.*, 2002). Protected from this methylation, it may link with histone methylation organized by HP1 proteins associated with DNA methyltransferase activity (Bachman *et al.*, 2001). This sequence of events could explain why methylated histones and HP1 proteins help to initiate maternal imprints in the oocyte, and why most DNA methylation associates with imprinted genes arising from maternal sources (Reik and Walter, 2001). In mammalian germ cells, zygotes and early embryos, epigenetic reprogramming of the genome regulates gene functions at critical developmental stages (Hajkova *et al.*, 2002). Such forms of de-novo methylation in migrating primordial germ cells lead to their erasure over a 1-day period as they enter the genital ridge. Germ cells of both sexes thus acquire similar epigenetic fates, which are then lost as these cells differentiate into male and female cells, each of which acquires specific imprints as the gametes develop. Genome-wide specific gene loci are methylated, although DNA methylation might be aberrant. Demethylation in the genome invokes epigenetic reprogramming in early embryos and primordial germ cells, in the form of many single-copy sequences acting actively and passively. Imprinted gene methylation itself is not affected in embryos; in contrast, single copied and imprinted sequences are demethylated in primordial germ cells (Lane *et al.*, 2003). Demethylation affected Line 1 elements but not IAP (intracisternal A-particle) elements. This procedure may prevent IAP retransposition, which is a cause of mutations, while also aiding the transgenerational inheritance of IAP epigenetic states, which may sustain heritable epimutations in neighbouring genes.

Interference with the timing or expression of methylation patterns results in anomalies of development in mammals. Various factors could be causative, leading to overgrowth in sheep fetuses after culture *in vitro* due to hypomethylation of the

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control element of maternally imprinted Igf2r, or changed expression of imprinted H19, Grb7, Grb10 and Igf2r, and low body weight, in mouse embryos cultured *in vitro* (Khosla *et al.*, 2001). Such long-expressed concern about risks of human imprinting associated with intracytoplasmic sperm injection (ICSI) was partially confirmed when two ICSI-derived children were recently diagnosed as having Angelman's syndrome (Cox *et al.*, 2002), a neurogenic disorder involving severe mental retardation, delayed motor development, poor balance, failure to speak and a happy disposition. Loss of functions in maternal alleles possibly arose through disomy or defective imprinting. The condition was not found in the fetal stage, nor was it expressed in the parents. One 3-year-old girl showed delay in speaking, had poor balance, had an overall a happy personality and showed a developmental age of 18 months. She displayed macrosaemia, obesity and microbrachycephaly and a flexed arm. A strong unmethylated band and a faint methylated band were found on her chromosome 15, and also characterized exon 1 of the *SNPRN* gene. Normally, embryos have a methylated maternal band and unmethylated paternal band. A second child had similar developmental anomalies, her chromosome 15 having an unmethylated band on chromosome 15 identified via an SNPRN probe (Cox *et al.*, 2002). Originally thought to be a consequence of ICSI, no such child had deleted 15q at birth, and the fact that chromosome 15 is methylated after ovulation and fertilization indicates a post zygotic epigenetic effect. Later studies identified other very rare children carrying various imprinting syndromes, indicating that this situation must be closely monitored to discover if it is aggravated by the use of assisted reproduction.

From a practical perspective, the large calf syndrome was first identified by culturing bovine embryos in media containing fetal calf serum, similar effects emerged in mouse embryos and fetuses cultured in M16 medium containing calf serum. Many embryos died by day 14 as the activity of the imprinted genes *H19* and *IGF2* declined, an upstream control gene of *H19* and a non-imprinted growth factor, receptor-binding protein Grb7, were hypermethylated, and the maternally expressed growth-suppressor *Grb10* displayed a heightened expression (Khosla *et al.*, 2001). In embryos, numbers of cytoplasmic lipid droplets increased and immature mitochondria may have reduced oxygen levels. Avoiding fetal calf serum improved embryonic growth and reduced risks of large calf syndrome, and scanning electrochemical microscopy has been used to measure oxygen consumption non-invasively and detected viable embryos (Khosla *et al.*, 2001). So far, there have been no indications of similar effects on human embryos growing *in vitro*. Imprinting defects in IGFII have also been proposed as arising in the large calf syndrome, as a result of cloning or as a defect arising in the human Beckwith–Wiedemann syndrome (Wutz *et al.*, 1998). Details on the methods used by investigators studying conception in cattle have been described by Hoshi (2003).

Many lessons can be learnt from the study of imprinting. Slight changes in methylation can lead to considerable effects on embryos. Clinical risks remain low at present, since these forms of epigenetic damage are very rare in human populations. Close attention will be paid to ICSI, which interferes with normal embryogenesis at the very stage when major epigenetic changes are occurring, although controlled trials have so far noted no greater risks than after IVF and possibly after natural conception.

Genes involved in specific blastomeres and ICM cells during early development

Over many years, individual blastomeres in cleaving embryos, and even the component cells of the ICM, were thought to be identical and totipotent. It is now known that gene action varies between individual blastomeres in 4-cell stages. Among the many identified genes controlling early development, *Oct3/4* is a major regulator, expressed in oocytes, cleavage stages and blastomeres from the initial stages of development. It is then restricted to inner cells as blastocysts differentiate and finally confined to germline in both sexes. Its quantitative expression determines the precise differentiation, dedifferentiation or self-renewal of embryonic cells. A master-regulator of pluripotency and resembling a morphogen (Niwa *et al.*, 2000), its knock-out impairs the growth of ICM. As the blastocyst differentiates, the lineage-related differentiation of trophectoderm is regulated by genes such as *Caudal-related homeobox 2 gene* (*Cdx2*), which acts in concert with *Oct3/4* and invokes loss-of-potency in trophectodermal cells (**Table 2**) (Niwa *et al.*, 2000, 2005; Tolkunova *et al.*, 2006). Trophoblast fails to form in knock-out *Cdx2*$^-$

Table 2 Gene expression in human trophectoderm (Edwards and Hansis, 2005).

Stage	Genes expressed
A. Early cleavage, mostly maternal	*Leptin*, *STAT3*, *β-HCG*, *β-LH*, *sHLA-G*, *TGFβ2*, timers, polarities[a]
B. Blastocysts and implantation	*LIF-R*, *H19*, *cyclin D1*, *integrin-β1*, *FGFr1–4*, *ATP synthetase U6*, *aldose reductase*, *PBK1*, *bFGF*, *MAPK*, *placenta lactogen*, *ID2*, *MASU2*, *STRA13*, *TCF5*

[a] Deb et al. (2006) state that *Cdx2* is also specifically expressed in mouse trophectoderm.

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blastocysts, which develop abnormally and generate ES cells with full pluripotency; this approach has been claimed as an ethical advance in avoiding the use of blastocysts to make ES cells (Meissner and Jaenisch, 2006). In fact, it actually raises major ethical queries about the establishment and use of such debilitated 'blastocysts'.

Moderation of promoter activity could explain varying levels of activity in *Oct3/4* and *Cdx2*. Their reciprocal inhibition may be an initial step in mammalian differentiation, although curiously *Cdx2* becomes redundant for trophectodermal differentiation when *Oct3/4* is repressed. Genes such as *Eomeso* overlap its functions, indicating that *Oct4, Cdx3, Eomeso* and *Elf5* are each essential for normal trophectodermal development (Rossant, 2001). Perhaps *Cdx2* defines the separation of polar and mural trophectoderm, *Eomes* acts as ICM separates from trophectoderm in morulae, and *Elf5* separates extra-embryonic ectoderm and ectoplacental cone in implanted embryos (Niwa *et al.*, 2005). This model also indicates that extra-embryonic ectoderm instructs patterning in epiblast.

Further evidence has recently revealed how *Cdx2* mRNA segregated near vegetal ooplasm at metaphase-1 in mice and was then restricted mostly to a single and later-dividing 2-cell blastomere (**Table 2**) (Deb *et al.*, 2006). It was then expressed in two blastomeres in 4-cell stages, being cytoplasmic in one and nuclear in the other in many embryos. In blastocysts, its presence was identified in trophectoderm and in the outer layers of ICM, which may be trophectodermal in origin. Queries about the work of Deb *et al.* (2006) imply that evidence is conflicting and difficult to repeat. If this evidence can be confirmed, it adds further support to opinions expressed by Edwards and Beard (1997, 1999), Antczak and Van Blerkom (1997) and Hansis and Edwards (2003). Decisive evidence requires the discovery that all four trophectodermal markers, namely leptin, STAT3 and HCGβ proteins, and mRNA for *Cdx2* are produced exclusively by one blastomere. Valuable genes to this end have been described above, and another could be *Gata6*, a transcription factor involved in the differentiation of primitive endoderm. It is expressed at day 3.5 in the early blastocyst, again its location indicates a separation of cell types in cleaving embryos at the 16–32 cell stage (Rossant *et al.*, 1997, 2003).

Unexpected support of gene expression in single blastomeres and ICM cells emerged from studies on the global amplification of mRNAs and quantitative high-density oligonucleotide microarray analyses. The method was based on a small number of PCR cycles followed by the linear amplification of disaggregated inner cell mass cells (Kurimoto *et al.*, 2006). For the first time, definite but different distributions of gene markers at the single-cell level were detected among a group of apparently homogeneous cells. Two groups of cells were revealed in ICM cells from day 3 blastocysts. Active genes in one group were typical of early epiblast, while those in the second group resembled primitive endoderm. One day later, the embryos possessed two populations, which were clearly separated by their individual genetic activities. Numerous genes characteristic of either epiblast or primitive endoderm were identified and classified. Kurimoto *et al.* (2006) were able to identify *Oct4* and *Cdx2* in single cells as the two groups were separated. One cluster of nine cells expressed the epiblast genes *nanog* and *Fgf4*, in numbers as high as 1000 per cell, while the other cluster consisted of 11 cells expressing genes typical of primitive endoderm including *Gata4*, *Gata6* and *cubulin*, in copy numbers reaching a few hundred per cell. Cells in cluster 1 also expressed several genes known to be transcription factors (*Sox2, c-Myc, Kf2, SpiC*), signal transduction factors, and apoptosis genes. Cells in cluster 2 expressed specific genes of various classes of gene, including different transcription factors (*Sox17, Runx1*), which were associated with specific cell-surface receptors, a basement membrane component and DNA methyl transferases. By day 4, ICM cells expressing *Gata4* and *Gata6* formed 57% of the disaggregated cells and expressed other markers of primitive endoderm. Some genes including *Hhex and Hnfa* had previously been expressed in epiblast and primitive endoderm at day 3.5 but were restricted to the latter tissue only by day 4.5. Similar findings were made for the genes active in epiblast. Kurimoto *et al.* (2006) conclude that morphologically indistinguishable ICM cells are following separate fates by day 3.5, and are clearly differentiating by day 4.5. They intend to trace back to earlier stages. Based on the results of previous studies, they will find trophoblastic markers and genes responsible for allocating cells to specific lineages. A similar approach to profiling single cells and involving real-time PCR-based 220-plex miRNA expression profiling to analyse microRNA expression was recently reported by Tang et al. (2006).

Trophectoderm is polarized in later embryonic stages as forecast by Edwards and Beard (1997) and Antczak and Van Blerkom (1997). A local group of its constituent cells possessed specific functions in 'hatching', i.e. as the embryo divested itself of its surrounding zona pellucida. This is achieved as blastocysts undergo several contractions and then 'hatch' from their zona pellucida through the action of 'plump' cells located at the vegetal pole in trophectoderm (Sathananthan *et al.*, 2003). An enzyme produced from these cells dissolves adjacent regions of the zona pellucida and permits the embryo to escape.

Further evidence of the autonomy of single cells or localized tissues emerged from analyses on the

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asymmetric pattern of cells in primitive endoderm in blastocysts. Roles of the gene *Lefty* were assessed in relation to the formation of the left—right axis in mouse embryos (Takaoka *et al.*, 2006). Initially expressed randomly among ICM cells, it became regionalized and tilted to one side of the ICM in blastocysts as implantation approaches, and regulated the migration of dorsal visceral endoderm to the future anterior side. It is an antagonist of *Nodal*, which is expressed symmetrically in ICM. Takaoka *et al.* (2006) conclude that this form of asymmetry reveals the origin of the anterior/posterior (A/P) body axis at the peri-implantation stage. Children and adults with disorders in L/R polarity offer clear insights into damage caused by these mutations. This axis is involved in major anomalies in human embryos including primary ciliary dyskinesis, which involves four homozygous and six heterozygous mutants of the DNAH5 protein as identified by sequence analysis (Olbrich *et al.*, 2002). The L/R axis is established in mutant mice by breaking asymmetry, establishing midline and Nodal signalling in mesoderm of the left lateral plate (Purandare *et al.*, 2002). Disorders in these syndromes include situs inversus, among other defects in heart, neural tubes and other organs, leading to the early death of afflicted embryos. Phenotypes also include recurrent infections due to weak ciliary action in clearing mucus in patients with various disorders including Kartagener's syndrome with a randomized L/R symmetry and immobile cilia syndrome and infertility in men. A randomized L/R asymmetry could also involve secretions from mutant forms that are non-functional. Recurrent infections due to the weak ciliary clearance of mucus may be a cause of syndromes involving immotile spermatozoa in men. The randomization of their left—right (L/R) asymmetry also led to one-half of afflicted offspring inheriting situs inversus (reversed organs). Similar anomalies associated with other polar axes have not been detected as yet.

The early cleavage stages described in this section have an immense significance for the differentiation of individual blastomeres in early embryo. The distribution of ooplasm is a fundamental matter in embryogenesis, and much more study is needed to confirm the nature of disorders in embryonic and fetal polarities and the fates of individual blastomeres in embryogenesis.

Does germline form in cleaving embryos in mammals?

The concept that a single blastomere in 4-cell embryos is the germline stem cell (Edwards and Beard, 1997, 1999; Edwards and Hansis, 2005) has been strongly criticized and germline is proposed instead to differentiate from epiblast. It is essential to decide if this new concept on germline inheritance is legitimate. Germline is desperately important. Induced mutations or other forms of damage to it during cleavage stages would be transmitted to the fetus. Experience with Angelman's and Beckwith—Wiedemann syndromes indicates that epigenetic factors may be active in germline. It is interesting to note that the absence of germline factors typical of other Orders (**Table 2A**) has led to concepts that germline reforms at post-blastocyst stages in mammals. This viewpoint was criticized by Edwards and Beard (1999) as being a most uncertain system, since germline precursors would be exposed to the massive genetic changes occurring in blastocysts including X inactivation, expansion of triplet repeats and massive waves causing alterations in gene activity.

A closer analysis of these reports from Saito *et al.* (2002) and Ohinata *et al.* (2005) also indicates that the 'primordial germ cells' identified by these authors had not in fact formed from epiblast and primitive streak as claimed. This can be done by measuring the timings of these successive stages in the embryo. Assuming a 20—24 h cell cycle, cells in epiblast would require ca. two divisions to reach primitive streak (**Table 2B**). Timings between the 4-cell stage and posterior epiblast would require 4—5 cleavage division at 24 h apart. This seems to be a reasonable estimate. The alternative concept thus suggests that germline was inherited through one 4-cell trophectodermal blastomere and then sustained at later stages of development by *Blimp, Stella* and *fragilis*. This proposal obviously has to be verified.

This section has related modern knowledge on polarities and related examples of gene expression in preimplantation embryos. It reveals the delicate state of successive developmental stages, and the considerable changes that could be affected by localized epigenetic interference. The brief mention of germline inheritance via 4-cell stages also stresses the potential epigenetic risks to mammalian fetuses.

X inactivation: the Lyon hypothesis

An outstanding example of constant reprogramming and gene silencing is found in the inactivation of many genes on one X chromosome in female mouse embryos. Discovered by Mary Lyon in 1961, the hypothesis was named after her. Earlier forms of the Lyon Hypothesis proposed one X chromosome was inactivated to induce equivalent dosages of maternal and paternal genes at implantation (Lyon, 1961). Paternal genes in female embryos were active only briefly in blastocysts, perhaps due to the methylation of cystine residues in regulatory regions and gene promoter sites on the X chromosome, and of CpG clusters (Wolf and Migeon, 1985). Variations on this model arose in particular tissues, e.g. in one-half

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of brain cells, and not at all in chorionic villi. In mice, this form of inactivation began in the paternal X chromosome in trophectoderm, then in inner cell mass and finally in embryonic ectoderm. Maternal and paternal X chromosomes were inactivated at random inn cells contributing to the embryo proper. Findings were overall similar in human embryos, especially in neural tissues, while CpG repeats arose in cleaving embryos and blastocysts (Hinds et al., 1993).

Modifications in the Lyon Hypothesis arose in the 1990s. Various genes on 'inert' X chromosomes remained active, some of them being located adjacent to the pairing region with the Y chromosome (Brown *et al.*, 1991). This situation meant that maternal and paternal regions remained active at particular sites along the X chromosomes. The gene *xist* (X_i-*specific transcripts*) was expressed from the inactive X, was female specific and located near the centre of X inactivation. These remarkable situations may explain some characteristics typical of XO patients. It is also noteworthy that other genes are methylated in the ICM in mouse embryos but not in trophectoderm or its descendants (Kratzer *et al.*, 1983; Singer-Sam *et al.*, 1992; Edwards and Brody, 1995).

Concepts have changed, including suggestions by Huynh and Lee (2003) that X inactivation has a much earlier onset, for example at meiosis in paternal germline, prior to fertilization or during the first transcription wave in 2-cell embryos prior to *Xist* taking over. This gene produces a *cis*-acting RNA, which recruits silencing complexes to the inactive X chromosome to maintain inactive states in related genes. Stably repressing *Xist* requires either DNA methylation, which occurs without DNA replication or the synthesis of RNA and protein, and precedes reprogramming and *Oct4* transcription in *Xenopus* oocytes by affecting its promoter (Simonsson and Gurdon, 2004), or the roles of *Dnmt3a* and *Dnmt3b* in mediating the expression of the Xist promoter (Sado et al., 2004). This process of X-inactivation silences the Xp (paternal) or Xm (maternal) chromosome at random except for extra-embryonic trophectoderm and primitive endoderm where Xp is inactivated, and may vary along the X chromosome (Mak et al., 2002). Maternal but not paternal PgK expression in cleavage stages implied that X^m and not X^p were active; X^m was expressed in trophoblastic stem cells along most of its length after implantation. Extensive silencing along Xp is related to a switch in Xist chromatin from an early to a later form.

Further developments in the new century led to even further refinements of the Lyon Hypothesis. Plasticity in gene silencing in mice as assessed by Huynh and Lee (2003) using a *Cot-1* probe detected no transcription in 1-cell stages, weak signals in 2-cell stages and rising levels in later stages except where *Xist* was active. 'Holes' were detected in chromosomal gene expression, and variations also arose among heterochromatic markers such as *eed* (*embryonic ectoderm development*) and *enx1* (*enhancer of zeste*). Maternal and paternal genes were expressed unequally. Repression was highest near the X-inactivation site, and some genes including *Chic1*, *Xnp* and *Pgk* retained maternal expression. Gradients in gene silencing along chromosomes in morulae were identified among maternal transcripts for *BZm*, *Yy1* and other genes. Shifts in *Xist* expression from early to later forms occurred at implantation as heterochromatic factors such as *Eed* and *Enx1* were recruited (Chadwick and Willard, 2003). One-fifth of blastomeres now expressed low levels of *Xist* or none at all, and many imprints were erased in epiblast. *Eed* and *EZh2* are members of the *Polycomb* group and associate with the inactive X chromosome in trophoblast stem cells. This association is stable, and may be a mechanism maintaining X inactivation in these cells (Mak et al., 2002). After X inactivation, *Eed* maintains gene silencing or repression, and it also acts on imprinted loci in autosomes. Null mutants of either gene suppress development post-gastrulation as trophoblast is repressed and X inactivation fails to respond to changes in methylation status. Embryonic lineages are also regulated and timed as *Xist* recruits the *Eed/EZh2* complex as a template while awaiting permanent forms of silencing.

Imprinting at day 7.5 post-coitum, for example, affected four paternally inherited and six maternally inherited genes among others that were seemingly unaffected (Ferguson-Smith and Reik, 2003). Many genes escape *Eed*, such as maternally expressed *Igf2r* and others locating on paternally inherited chromosomes, which are regulated by paternally expressed anti-sense RNA. *Eed* also regulates parent-of-origin silencing via a modified form of imprinting. Some genes may escape this type of control as methylation is induced by an alternative system of modifying histones. Apparently, *Eed* does not regulate global imprinting and is involved locally in a subset of imprinted genes including Angelman's, Beckwith–Wiedemann and Prader–Willi syndromes (Mager *et al.*, 2003). ES trophectodermal cells also undergo X inactivation during differentiation, with a shift from reversible to irreversible inactivation corresponding with a leaky silencing of in embryos before but not after implantation (Wutz and Jaenisch, 2000). A weak expression of *Xist* may lead to leaky forms of silencing in the paternal X chromosome (X^p).

Mice made deficient for methyltransferase, an active component in methylation, were not prevented from the normal regulation of Xist and X inactivation (Sado *et al.*, 2004). This evidence is held to cast doubts on their causative role of methylation. Either other systems can regulate inactivation, or the methyltransferase gene may have to be expressed

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at specific stages of preimplantation development as discovered by Wutz and Jaenisch (2000) for ES cells. The possibility of changes in the expression of X inactivation led Sandovici *et al.* (2004) to perform a longitudinal study on X inactivation in normal women. No differences were discovered in the activation ratio in most women, except for women aged 60 and over. This led these authors to suggest that discontinuous or catastrophic factors may be responsible as they call for further research.

These successive developments spell out a remarkable series of studies on X activation and inactivation. It is an amazing model of reprogramming, active in every embryo from its earliest developmental stages. Immense amounts of information clearly remain to be clarified and applied to knowledge on reprogramming systems.

Epigenetic modifications in preimplantation NT embryos

Grafting somatic cell nuclei into oocytes during cloning offers an immense scope for imposing chosen characteristics on somatic and stem cells. Yet it also evokes major embryonic disasters when used to produce NT embryos in some mammalian species but not in others. Wilmut *et al.* (1997) discovered that NT for cloning was possible at the risk of damaged embryos and offspring in various species, and even Dolly expressed epigenetic changes. The principles underlying SCNT are shown in **Figure 2**.

Cloning laboratory and farm animals

Nuclear transfer using somatic cell donors (SCNT) may nevertheless have important roles in various aspects of reproductive and stem cell medicine. Reproductive cloning involves producing newborn children cloned from a donor nucleus. Therapeutic cloning is designed to produce stem cell lines identical with the nuclear donor, in case they are needed by the donor for transplantation in later life. Infertility cloning involves preparing gametes for infertile mean and women lacking their own gametes (Lacham-Kaplan *et al.*, 2001; Nagy, 2004). Each of these approaches utilizes essentially similar techniques that are based on initial studies in mice (Tarkowski and Balakier, 1980), which are being applied in humans (Palermo *et al.*, 2002). When these workers transferred nuclei of somatic cells into ooplasm of non-activated eggs, chromosomal condensation was premature and various structures formed in recipient oocytes, some resembling a spindle and a metaphase plate. Later studies have, overall, confirmed these results, with modified methylation in oocytes, further changes in genomic methylation, histone modifications, X chromosome inactivation, and non-coding RNA interference as closely linked genes displayed varying responses to methylation (Tesarik *et al.*, 2001; Nagy, 2004; Takeuchi and Palermo, 2004; Verdel *et al.*, 2004).

Long experience with cloning in farm and laboratory animals revealed significant effects of the cell cycle stage in achieving successful cloning, as variable responses in cloned oocytes modified normal development in NT embryos, as reviewed by Edwards and Beard (1998).

NT may be improved by selecting the optimal stage of the cell cycle in donor nuclei. Some investigators preferred using donor nuclei in the G_1/S phase whereas others used G_0 nuclei as with the birth of Dolly (Cheong *et al.*, 1993; Wilmut *et al.*, 1997). Yet the G_1 phase is short or absent in some early post-implantation stages (Pinto-Correia *et al.*, 1995; Kwon and Kono, 1996). Likewise, Galat *et al.* (2005) induced human cumulus cell nuclei to undergo haploidization by grafting them to enucleated human oocytes in

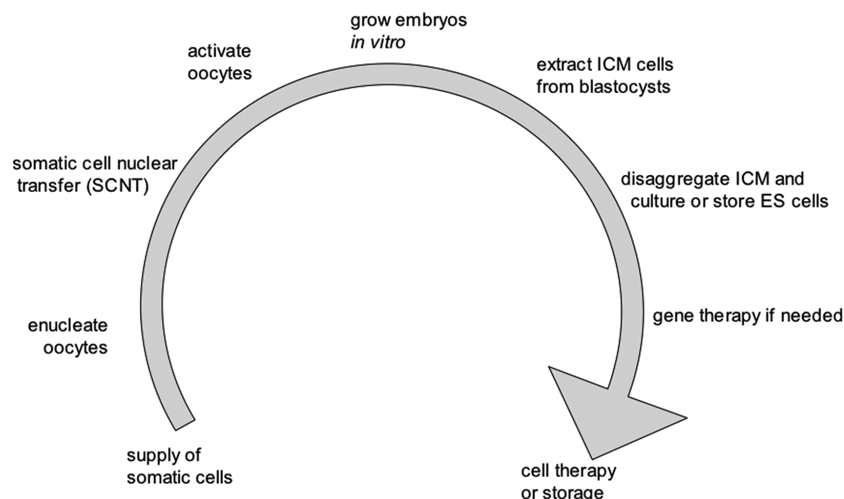

Fig. 2. A general model showing the successive steps involved in somatic cell nuclear transfer (SCNT). Gene therapy may be used when the method becomes more successful with human embryos to prepare special cell lines that can be used therapeutically. ES = embryonic stem; ICM = inner cell mass.

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Table 3 Chromosomal aneuploidies after haploidization involving various periods between nuclear transfer (NT) and oocyte activation to permit nuclear remodelling (Galat *et al.*, 2005).

Group	Interval NT to activation	Details of NT oocytes (%)				
		Normal haploid	1 error	2 errors	3 errors	Total NT
1	4–7	2 (11.1)	9 (50)	7 (38.9)	0	18
2	8–14	2 (18.2)	1 (9.0)	4 (36.4)	4 (36.4)	11
3	15–21	0	4 (33.3)	4 (33.3)	4 (33.3)	12
All groups	4–21	4 (9.8)	14 (34.1)	15 (36.6)	8 (19.5)	41

metaphase 2 and then using electroactivation to induce the differentiation of the grafted oocytes at various intervals thereafter. The majority of oocytes were activated and displayed similar pronuclei derived from the donor nucleus and differing from normally fertilized eggs in their close location in ooplasm. Only 4/10 of the resulting embryos were diploid (**Table 3**).

Culture conditions, the 'freshness' of the recipient oocyte, the status of the cell cycle in donor nuclei and other factors influence the success of NT. Some earlier workers preferred to utilize ageing oocytes deficient in maturation promoting factor (MPF). Yet MPF is linked to the actions of histone H1 kinase activity, i.e. mos protein, on the meiotic spindle during polar body extrusion, and to the breakdown of the nuclear envelope among other effects (Collas *et al.*, 1993; Sagata, 1997). Some investigators even recommended the use of aged MPF-deficient oocytes in NT (Prather *et al.*, 1987; Campbell *et al.*, 1993). Attitudes have changed concerning human NT, since recent workers stress the significance of fresh oocytes to obtain the best results as summarized by Stojkovic *et al.* (2005). This team recommended using oocytes by 1 h post-collection, removing the oocyte nucleus and spindle before metaphase II arrest, avoiding human oocytes matured *in vitro*, performing NT using specific approaches to spindle removal, avoiding factors in medium and improving the means of micromanipulation to achieve NT.

Specific endogenous demethylases with the dinucleotide sequence CpG modify developmental patterns by modifying DNA methylation (Ramchandani *et al.*, 1999). Patterns of methylation and demethylation regulating gene expression may be reversed or modified by internal and extracellular signals or through simple changes in the local environment. High degrees of methylation do not necessarily harm gene expression, and variations might accrue in the degree of methylation among linked genes. Applying NT with embryonic stem cells and cumulus cell nuclei led to anomalies in gene expression in mouse embryos that persisted until birth without undue effects on the offspring (Humpherys *et al.*, 2001). This evidence implies that some epigenetic changes may be tolerated. NT may even be improved under abnormal forms of gene expression in donor nuclei from cumulus cells, and in mice cloned from embryonic stem cells. Some conditions of DNA methylation and histone modification seemingly invoked epigenetic changes and improved success with NT (Hochedlinger and Jaenisch, 2003).

The source of the nucleus can also influence success. For example, in clones derived from cumulus cells, three or more genes were largely silenced or expressed aberrantly, in contrast to the situation in clones derived from nuclei of primordial germ cells (Boiani *et al.*, 2002). A faulty expression of *Oct4* was also found among one-third or more of the genes related to *Oct4* expression when clones were derived from cumulus cell nuclei (Bortvin *et al.*, 2003). In contrast, the use of ES cell nuclei resulted in the normal expression of these genes and a related improvement in embryo development to full term. Differential afflictions emerged with each of these forms of cloning in relation to several genes related to *Oct4* that are incompletely activated after NT in mouse oocytes (Bortvin *et al.*, 2003). Controlled methylation of some genes or histones, or natural silencing with *Oct4* could well differ from silencing induced by NT and its complex situations.

Damage to embryos may be manifest some time after birth. For example, 12 NT male mice cloned from immature Sertoli cells grew at similar rates to controls (Ogunuki *et al.*, 2002). Cloned offspring at birth were seemingly normal since only two serum parameters were higher in the clones as compared with controls. Yet 10 died from severe pneumonia, liver necrosis, or leukaemias by 810 days, compared with three controls, possibly due to compromised antibody production as in cloned goats (Keefer *et al.*, 2001). Epigenetic effects were also induced in cloned embryos derived from NT in rabbits. They grew more slowly than controls, and only 15/54 embryos implanted and none survived when transferred to a host mother in synchrony with her uterine differentiation (Chesné *et al.* (2003). In contrast, waiting for 22 h before transfer produced 10/27 implants and six offspring that were fully fertile, a result similar to those in normal untreated controls. Rabbit NT had proved to be highly successful, provided epigenetic effects leading to placental insufficiency were avoided.

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Hiiragi and Solter (2005) recently summarized current findings on the success of NT and its problems. Overall success is low. Uncertainties as to whether reprogramming is essential were solved in studies comparing cloned nuclei from cumulus or zygote nuclei. Results were poor using nuclei from embryos in later developmental stages, which proved harder to reprogramme. Nuclei from naturally ovulated oocytes were superior to those obtained after induced ovulation. This evidence again revealed a need for a greater understanding of oocyte biology plus a need for technical improvements in NT.

Details of the responses of donated nuclei and recipient oocytes are being clarified. A study on fusing ES and somatic cells resulted in hybrid cells with 4n nuclei resembling that of ES cells (Cowan *et al.*, 2005). Somatic nuclei were reprogrammed to an embryonic state, indicating that ES cells can modify the transcriptional state of somatic cells. Cloning oocytes may be a different matter, although grafting nuclei from cells of adult mammalian donors into amphibian oocytes reprogrammed them to produce Oct-4, which is typical of stem cells (Byrne et al., 2003). Perhaps not surprisingly, factors such as using nuclei taken from cells in varying stages of their cell cycle can affect the success of cloning in some species. A somatic nucleus transferred into an oocyte may fail to act like a fertilizing spermatozoon. This tiny gamete possesses a very dominant anterior centriole forming a huge aster to re-establish oocyte polarities after fertilization, and this may not happen in its absence during cloning. Species variation may be significant. Mouse embryos, for example, do not have such dominant centrioles as other species. The significance of cell-cycle changes was stressed, as it affects successful cloning in some species.

Epigenetic disorders in human embryos during assisted reproduction

Human embryos also respond to agents capable of inducing epigenetic changes in the embryonic genome. They may be induced by procedures commonplace in assisted reproduction. Many investigators consider the 'simple' matter of placing human oocytes or embryos in culture media during IVF or cloning can raise very sensitive moments for induced epigenesis. Changes in gene expression can also be induced when uncultured human stem cells are placed *in vitro* (Boquest *et al.*, 2005).

Epigenetic disorders described in mice by Reik *et al.* (1993) have been found in rare in newborn children conceived by assisted reproduction. Primary defects apparently arise in ICM (Maher *et al.*, 2000). Occasional examples of imprinting syndromes such as Beckwith–Wiedemann syndrome and Angelman's syndrome have been identified as in several children who had an unmethylated band and a weak methylated band on chromosome 15 (Cox *et al.*, 2002). Methylated regions were also found in the chromosomal region 11p5.5 that interfered with paternal *IGF2* and maternal *H19*. NT must be improved before therapeutic cloning is possible and only after stem cell lines tolerant to a particular individual have been prepared. Greater success may be gained by NT into somatic cells. Recently, Strelchenko *et al.* (2006) reported that cybrids could be constructed by transferring nuclei into enucleated somatic cells; a study is discussed in detail in this Symposium by Strelchenko and Verlinsky.

Experience with large calf and other epigenetic events in animals have alerted clinicians to potential risks to IVF and ICSI children have been mooted, based on data emerging from studies on the elevated risks of children with imprinted defects such as Beckwith–Wiedemann and Angelman's syndromes. While rare, these syndromes are unpleasant and would be best avoided. Warnings have been received from studies on farm animals, and especially the large calf syndrome.

Human NT has been very unsuccessful to date, the three published papers on human cloning having drawn similar conclusion, as embryos failed to cleave and rarely reached the blastocyst stage (Zavos, 2003; Lavoir *et al.*, 2005; Stojkovic *et al.*, 2005). Potential causes have been discussed extensively, including the stage of the cell cycle at nuclear transfer. Disturbed polarities may be another factor leading to embryonic anomalies although unlikely to explain why reproductive cloning in humans is so fruitless. Reproductive cloning has been achieved in members of 11 mammalian species including laboratory and farm animals although success rates were extremely low since some of them required 1000 or more attempts. Perhaps the human embryo is especially sensitive to damage and destruction. Various forms of early death in routine IVF, for example, display high frequencies of fragmented embryos and delayed cleavages. The occurrence of such conditions seems similar in human oocytes conceived *in vivo* then flushed from the human female reproductive tract although data are scarce (**Table 4**).

Modern views on human epigenesis

Conrad Waddington, then Professor of Animal Genetics in Edinburgh University, coined the word 'epigenetics' in the 1940s, outlining this new concept as 'the interactions of genes with their environment, which bring the phenotype into being' (Waddington, 1943). In modern terms, it refers to all stimuli affecting the actions of genes without altering their DNA sequence.

The implication of his work bears heavily to-day. Epigenetic changes may explain many human characteristics. These may include differences in the growth of identical twins such as recorded in the Minnesota database, since twins are exposed

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Table 4 Growth of human embryos *in vivo* and *in vitro* (from Edwards and Brody, 1995). (**A**) Human embryos flushed from the uterus after natural conception. (**B**) Comparison of natural cycle IVF and stimulation with clomiphene or human menopausal gonadotrophin (HMG).

Origin	No. inseminations	Ova flushed from uterus	Stage of growth and condition of the embryos				
			1-cell	Fragments	2–18 cells	Morulae	Blastocysts
A							
UCLA[a]	84	35	6	2	17	2	8
Pavia	64	26	2	5	6	6	7
Total	148	61	8	7	23	8	15
B							
Natural cycle	88	37	4	6	9	7	11
Clomiphene	17	13	4	1	1	2	5
HMG	22	22	9	2	3	2	6
Total	127	724	17	9	13	11	22

[a] Data from Buster *et al.*, Sauer *et al.*, Formigli *et al.* (see Edwards and Brody, 1995).

to different stimuli as they grow. This has been confirmed in a recent study where identical twins between the ages of 3 and 74 were examined for gene differences (Fraga *et al.*, 2005). Epigenetic differences were rare in younger identical twins, but increased considerably by threefold or more in ageing pairs. Qiu (2006) summarizes the current situation by pointing out that cancers may arise as a consequence of epigenetic change and that recent problems with embryo stem cells *in vitro* were due to epigenetic changes involving the fusion of stem cells with others or with different cells, to produce tetraploid and other chromosomally imbalanced cells. Epigenetic codes are seemingly far more sensitive to outside influences than DNA sequences. It is also curious that the cells in the first-ever ES cell lines formed from rabbit ICM rarely displayed signs of fusion or tetraploidy (Cole *et al.*, 1966).

The Sanger Centre in Cambridge University, UK, is studying the epigenome to identify changes to the genome arising through the actions of external factors. In epigenesis, the DNA is altered for example by methylation, which involves adding methyl groups to it. Or the surrounding histone proteins and their packaging around DNA may undergo changes in their protruding tails under the influence the structure of chromatin. The European Human Epigenome Project Consortium, founded in 2000, is the most prominent centre at the present time (Qiu *et al.*, 2006). Debate has centred on whether to begin with studies searching for differences between eight and 10 tissues, including blood. Cells grown in a laboratory may offer another easiest approach since their genomes can be closely controlled. Large amounts of data may have to be stored and analysed to assess methylated sites in DNA, and the favourite species may be yeast, fruit fly and mice. These tasks will be very expensive.

A discussion in the ethics of reproductive cloning revealed differences of opinion on whether it should be made available even if proved to safe in later years. The relationship between this procedure and reproductive freedom was held to remain an open matter for further enquiry (Birnbacher, 2005; Strong, 2005).

Genetics of early post-implantation stages

Differentiation of embryonic and extra-embryonic tissues

Soon after the blastocyst stage, various tissues differentiate in mammalian embryos. Discussed briefly here, maternal effects are now minor as an avalanche of genes is activated in many differentiating tissues. Each stage is thus highly susceptible to epigenetic changes in gene expression, although there is insufficient data on how each tissue initiates its differentiation at this early stage of embryonic development.

Embryonic and extra-embryonic development

Numerous studies have assessed the initial formation of embryonic and extra-embryonic tissues in mice as they differentiate from ICM and trophoblast during days 4–5, a stage when studies on reprogramming embryonic cells are rare. Extra-embryonic ectoderm is significant for its role in embryo patterning (**Figure 3**). With ectoplacental cone, it differentiates from polar trophectoderm and signals to epiblast to ca. 40 founder germline cells via the synthesis of *Bmp4* and *Nodal*. Some investigators suggest these cells are germline precursors based on the expression of germline markers including *Trap*, *fragilis*, *Stella* and the recently discovered *Blimp1* as discussed above (**Table 5**) (Saitou *et al.*, 2002; Ohinata *et al.*, 2005). Also known as *Prdm1*, *Blimp1* has significant

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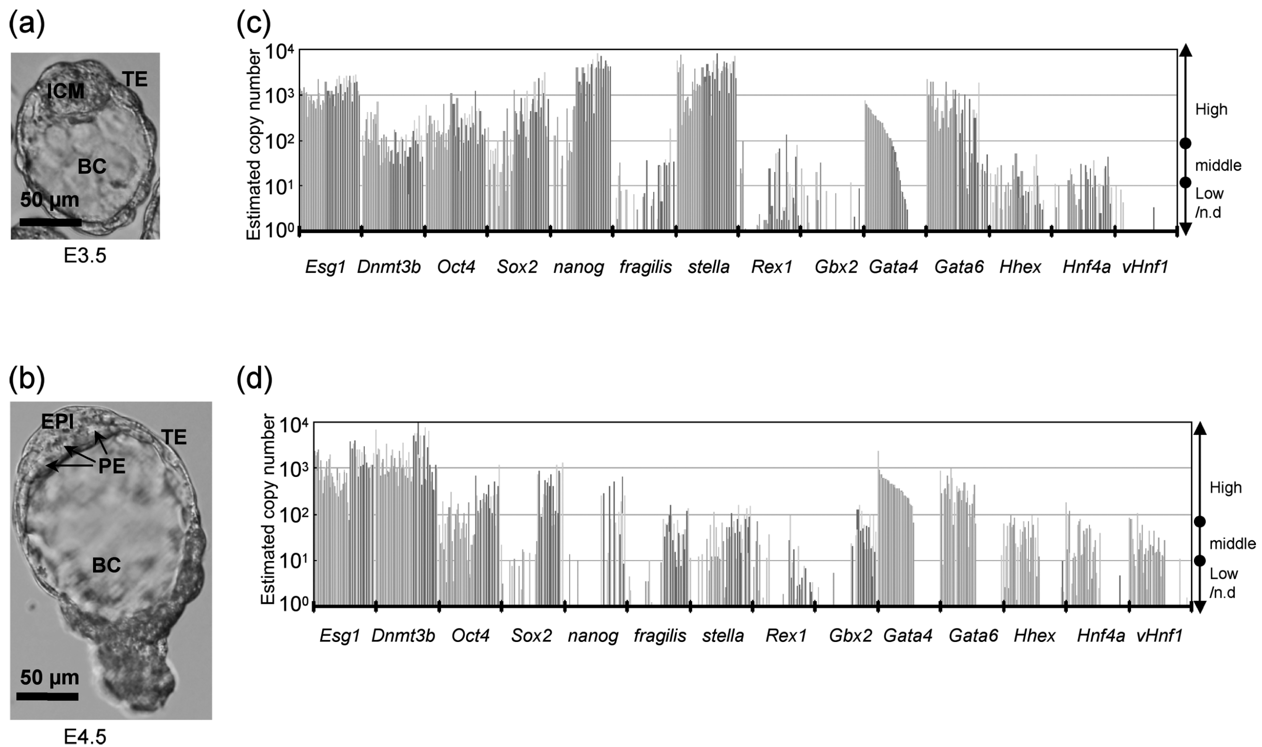

Fig. 3. Analysing single inner cell mass (ICM) cells from mouse day 3.5 blastocysts reveals two distinct cell populations in the hierarchical clustering of single ICM cells into two cell populations. Related gene expression was preserved between days 3.5 and 4.5. (**a**) and (**b**) represent data gained from blastocysts at days 3.5 and 4.5 and typical blastocysts for these stages are shown in this illustration. Levels of gene expression on day 3.5 (**c**) and 4.5 (**d**) are indicated. Expression levels of key genes related to primitive endoderm (PE) and to epiblast at day 3.5 (**c**) and day 4 (**d**) are shown. BC = blastocoelic cavity; TE = trophectoderm. This figure is adapted from Kurimoto et al. (2006), and is reprinted here by permission of Mitinori Saitou and Oxford University Press.

Table 5 Formation of mammalian germline.

A. Genes controlling germline in various orders

C. elegans	*Mex-3*, *Par-1*, *Par-2*
Drosophila	*Oskar*, *vasa*, *tudor*, *nanos*, *Capucchino*, *spire*, *staufen*, *Germ-cell less*, *Pgc*
Xenopus	*X-cat2*, *XVLGI*, *Xklpl*
Mouse	*Blimp1*(*Prdm1*), *stella*, *fragilis*

Blimp 1 is a transcriptional repressor involved in 20 clustered primordial germ cells from posterior epiblast; they do not repress homeobox genes as in other species (Ohinata *et al.*, 2005).

B. Germline cell numbers at successive stages in mouse embryos

Gene or marker	Tissue with germline cells	Estimated time of development (days)	Estimated numbers of germline cells
4-cell stage[a]	Blastomere	2.0	1
Blimp[b]	Posterior epiblast	4.5	20
Stella/*fragilis*[b]	Late primitive streak	5.5	43

[a] Data from Hansis and Edwards (2003).
[b] Data from Saitou *et al.*, (2002) and Ohinata *et al.* (2005).

inductive relationships with trophectoderm. It may help to initiate lineage-restricted primordial germ cell precursors in proximal posterior epiblast.

Genes determining the nature of extra-embryonic development in mice include *Dp1* is an essential gene sustaining the embryo, active in extra-embryonic tissues. Its inactivation leads to defects in this tissue including reduced numbers of trophoblastic giant cells and their endoduplication, fewer ectoplacental cone and chorionic cells and growth retardation (Kohn *et al.*, 2003). Murine *Elf5,* a transcription factor (*ESE2* in humans), sustains tissues derived from epiblast. Targeted mutants lack extra-embryonic ectoderm, form undersized fetuses and survive briefly and form

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Table 6 Changing ratios of haemopoietic stem cell progenitors in yolk sac or per embryo at gestation days 8–9 (Wong *et al.*, 1986).

	No. of progenitors per yolk sac or per embryo			
	Day 8		Day 9	
	Yolk sac	Embryo	Yolk sac	Embryo
Day 5 CFU-E	113 ± 26	0	1 ± 1	0
SBFU-E	32 ± 8	0	20	2 ± 1
LBFU-E	22 ± 6	0	62 ± 1	3 ± 2
E-mix	1 ± 1	0	32 ± 4	0
Non-E	10 ± 3	0	86 ± 2	1 ± 1

Data are means ± SEM. Labels: CFU-E: colony-forming units; SBFU-E: small burst CG+FU; LBFU-E: large burst CFU; E-mix: mixtures; Non-E: other colonies.

an ectoplacental cone lacking trophoblast stem cell (Donnison *et al.*, 2005).

E2f1 also acts at these stages, its knock-out leading to minor embryonic defects. Some of its mutants share the effects of retinoblastoma (*Rb*), the first known tumour suppressor gene. The retinoblastoma tumour suppressor gene (*pRB*) is involved in permitting entry to S phases in cell division. $Rb^{-/-}$ mice produce abnormal offspring, a situation long considered as due to the knock-out. Yet deeper investigations revealed that major anomalies and the death of fetuses *in utero* at day 16 was due to an excess of trophoblast cells, which suppressed the normal placental transfer by decreasing blood spaces and so reducing blood flow (Wu *et al.*, 2003). Neurological and other effects ascribed to $Rb^{-/-}$ has thus turned out to be epigenetic defects acting through placental insufficiency (Kohn *et al.*, 2003). They reveal that epigenesist in specific tissues may be induced by interference with closely related genetic systems in other tissues.

Origins of haemopoietic and mesenchymal stem cells

This section is based on a recent review (Edwards, 2005b). It describes the early and rapid differentiation of haemopoietic and mesenchymal systems (HSC and MSC) as they establish blood cells, vasculature and endothelial tissues. Stem cells may possess a chromatin structure that is wide open and maintains multipotential that is quenched as they enter a differentiation pathway. This was revealed using analyses of oligonucleotide arrays targeted for various haemopoietic systems (Akashi et al., 2003). DNA methylation and histone deacetylation can also invoke gene silencing and the expansion of cells with phenotypes resembling HSC. Exposing human marrow cells with proliferative and phenotypic characteristics to 5-aza 2′deoxycytidine and trichostatin A resulted in $CD34^+$ expanded cells identical to primitive HSC (Milhem et al., 2004). These cells were capable of engrafting immunodeficient mice without necessarily expressing $CD34^+$. Such evidence hints that regulators of epigenetic systems might help to determine the fate of primitive HSC *in vitro*.

HSC and MSC may differentiate from a single stem cell named the haemangioblast or the blast-forming cell (BL-CFC), which can be isolated from primitive streak (Choi *et al.*, 1998; Huber *et al.*, 2004). Their angioblast potential is queried by some investigators since these cells may also form vascular smooth muscle (Ema *et al.*, 2003). Green (2005) suggests that BL-CFC establish haemopoiesis in yolk sac and that HSC formed in para-aortic splanchnopleure, a tissue closely associated with endothelium. They may fail to differentiate as far as lymphoid or haemopoietic stem cells (HSC).

Many reports have proposed that bone marrow cells carry progenitors for stem cells capable of colonizing and resuscitating sick recipients. The multifactorial abilities of some HSC precursor cells, perhaps angioblasts, may render them capable of forming mesenchyme cells, endothelium and perhaps other forms in addition to HSC (e.g. Kabrun *et al.*, 1997). These HSC cells are *Flk-1*$^+$, which characterizes cells identified initially in yolk sac at day 8.5, increasing to day 10 and also expressed in fetal liver in fetuses. They are also found in cells developing from embryoid bodies, maximizing 4 days after their initial identification, producing cells with early haemopoietic potential and preceding cells with a transient wave of haemopoietic potential that express $CD34^+Sca\text{-}1^+AA4.1^+$.

Murine erythropoietic precursors *in vivo* emerge on day 7–8 in yolk sac and produce primitive nucleated erythroid cells followed by haemopoietic lineages in blood islands, which colonize liver (**Table 6**) (Wong *et al.*, 1986). Endothelial cells may arise from angioblasts, defined as isolated mesodermal cells expressing *TAL-1 and Flk-1* between days 6.5–9 (Drake and Fleming, 2000). They differ from yolk sac cells producing *TAL-1* but not *Flk-1*. In blood islands, haemopoietic cells express *TAL1 and* not *Flk-1*, while blood vessels form *TAL1*$^+$ *and Flk-1*$^+$ initially, succeeded by *PECAM*$^+$*CD34*$^+$*VE-cadherin*$^+$ and then by

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Table 7 Timing the expression of TAL-1, FLK-1, PECAM CD34, VE-cadherin and Tie 2 during intra-embryonic vasculogenesis (Drake and Fleming, 2000).

Protein	Embryos day 8.2–8.3 pc			Embryos day 8.5 pc		
	Endocardium	Dorsal aorta	Lateral vascular networks	Endocardium	Dorsal aorta	Lateral vascular networks
TAL1	+	+	–	–	+	+
Flk1	+	+	+	+	+	+
PECAM	+	+	–	+	+	–
CD34	–	+	–	+	+	–
VE-cadherin	–	+	–	+	+	–
Tie2	–	–	–	+	+	–

Table 8 Marker expression in successive stages of blood formation from embryonic stem (ES) cells (from Nishikawa *et al.*, 1998 and based on an illustration in Edwards, 2005b).

Type of cell	Characteristics
ES cell	E-cadherin^{+}Flk1^{-}PDGFRα^{-}
Proximal lateral mesoderm	Flk1^{+}VE-cadherin^{-}PDGFR$\alpha^{-\text{ or }+}$CD34^{-}CD45^{-}E-cadherin^{-}
Haemangioblast	Flk1^{+}VE-cadherin^{+}CD34^{+}CD31^{+}CD45^{-}Ter119^{-}
Haemopoietic progenitor	CD45^{+}c-Kit^{+}Ter119^{-}Flk1^{-}VE-cadherin^{-}
Erythroid cell	CD45^{+} or Ter119^{+}c-Kit^{-}
Other lineages	CD45^{+}Ter119^{-}

Haemangioblasts and committed angioblasts are difficult to discriminate with these markers.

Tie2^{+} (**Table 7**). *TAL1* is down regulated in endothelial cells of mature vessels. Haemopoiesis in mice may thus involve an initial primitive stage and then a definite stage associated with mesodermal cells expressing *Flk-1*$^{+}$*VE*$^{-}$*cadherin*$^{-}$ and endothelial cells expressing *Flk-1*$^{+}$*VE*$^{-}$*cadherin*$^{+}$. Vasculogenesis may be initiated in mesoderm by cells later producing endocardium (Drake and Fleming, 2000).

Later studies revealed that Flk-1^{+}CD41^{+}TER119^{+} proteins were expressed in haemopoietic subsets. Flk-1 marks early cells and possibly the earliest haemopoietic precursors (Ferkowitz et al., 2003). CD41 discriminates the onset of primitive and definitive erythroid progenitor cells, while TER119 regulates primitive erythroblasts and erythrocytes. Flk-1 receptor, characteristic of endothelial cells, is expressed by putative haemangioblasts characteristic of the earliest haemaopoietic cells responding to the ligand VEGF. This cell line develops through primitive erythroid lineages, and then disappears in later stages to mark the switch from embryonic to fetal haemopoiesis although endothelial cells may retain it in some haemopoietic tissues in adult life. Progressive lineage analysis has enabled plotting successive stages from ES cells to mature blood cells (**Table 8**) (Nishikawa et al., 1998). This table shows the position of haemangioblasts, again considered as founder cells of haemopoietic cells and mesenchymal cells. Some of them identified *in vitro* were able to respond via populations of cells expressing CD34.

Blood and endothelium were then discovered to be sustained by the murine gene *stem cell leukaemia* (*SCL*), and neither differentiates in mutant forms. *SCL* expression is limited, perhaps to the period when the *Tie2Cre* transgene becomes active. *SCL*$^{+}$/*Tal-1*$^{+}$ act in mesodermal cells, committing them to the initial phases of HSC formation, before V-cadherin is expressed, and as HSC development and endothelial fate are separated (Endoh *et al.*, 2002). Recently, BL-CFC were reported to arise from embryoid bodies at day 3, forming individual progenitors within 24 h of colony formation and becoming committed to form haemangioblasts involving *SCL*/*Tal-1* (D'Souza *et al.*, 2005). *SCL* is not required in later stages of HSC differentiation (D'Souza *et al.*, 2005; Schlaeger *et al.*, 2005), and haemopoietic cells do not form in *SCL*$^{-/-}$ mutants where BL-CFC produce vascular smooth muscle. *SCL* remains active since among its other functions, it is essential for the differentiation of primitive and definitive erythrocytes and megakaryocytes.

Other genes regulating HSC include *Notch* and *Wnt*. They stimulate self-renewal in murine HSC by inhibiting differentiation and inducing proliferation (Duncan *et al.*, 2005). *Notch* is a key inhibitory factor regulating early HSC differentiation, and when down-regulated accelerates their differentiation *in vitro*. Its signals also maintain *Wnt* support for undifferentiated HSC, but neither their survival nor their entry into the cell cycle. It acts via surface receptors reactive with ligands of various gene families, releasing a *Notch* fragment, which enters the nucleus to associate

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Table 9 Comparison of transcriptomic and proteonomic analyses of gene expression in LSK$^+$ and LSK$^-$ cells (Unwin *et al.*, 2006).

Protein (iTRAQ)	mRNA (Affymetric array)		
	Up	No change	Down
Up	35	27	1
No change	27	325	22
Down	0	47	27

Data presenting relative changes in mRNA concentrations for all proteins found to be differentially expressed by iTRAQ labelling in two closely related cell lines and for those displaying no differences between the two cell lines.

with the transcriptional repressor CBF-1. If expressed constitutively in HSC, immortalized murine cell lines capable of generating lymphoid and myeloid cells *in vitro* are generated in long-term cell grafts. These properties involve *Notch* expression in a variety of differentiating tissues.

Wnt is also active in a variety of developmental systems, exerting strong regulatory influences in colon, breast, prostate and skin via catenins. It also regulates the fate of embryonic, neural, epidermal and gut epithelial stem cells, and is a major player in several forms of cancer (Duncan *et al.*, 2005). *Kruppel-like factor* (*EKLF*) expressed in extra-embryonic mesoderm in yolk sac, especially in blood islands and then in liver, is regulated by the *BMP4/Smad* pathways. Related systems include the expression of *lac-2* in blood islands and fetal liver, and the endogenous expression of *EKLF*, which regulates haemopoiesis in specific cells from day 7.5 (Xue *et al.*, 2004).

Similarities and differences in protein and gene expression in two classes of primary haemopoietic stem cells were recently assessed by Unwin *et al.* (2006) (**Table 9**). Two-dimensional liquid chromatography and mass spectrometric assay with an isobaric covalent modification of peptides prior identified 948 proteins between long-term reconstituting haemopoietic stem cells expressing Lin$^-$Sca$^+$Kit$^+$;LSK$^+$ and non-long-term reconstituting progenitor cells expressing Lin$^-$Sca$^+$Kit$^-$;LSK$^-$. A total of 145 differences were detected in the proteomes, of which 54% were extra to those identified from transcriptome analyses. Proteonomics had detected differences between the two cell lines involving proteins controlling metabolism and oxidative protection related to hypoxia and implied that LSK$^+$ cells but not LSK$^-$ cells could survive in anaerobic environments.

Bone marrow stem cells

Mesenchymal stem cells form several lineages cells in bone marrow. They regulate haemopoietic cells there and modify their differentiation via the transcription factors Runx2 and PPARγ. These are driven by TAZ, a transcriptional co-activator that activates Runx2 and represses PPARγ to balance the production of osteoblasts and adipocytes (Hong *et al.*, 2005). Mesenchymal cells can be identified and separated from HSC using serum-free medium in suspension culture. Their initial exposure to stem cell factor (SCF) and interleukin 3 (IL-3) produces CD45$^+$ haemopoietic cells and CD45$^-$ mesenchymal cells (Baksh et al., 2005). CD45$^-$ cells respond to soluble factors from CD45$^+$ cells to induce the proliferation of CD45$^-$CD123$^+$CD117$^+$ cells (CD123 is the IL-3 receptor; CD117 is the SCF receptor). CD45$^-$CD123$^+$ cells are oestrogenic and produce 24% of the fibroblast colony-forming units (CFU-F) and 22% of the osteoblast colony-forming units (CFU-O). Knowledge of cytokine interrelationships between haemopoietic and mesenchymal cells should help to improve the simultaneous mass culture of these fundamental stem cells (Baksh et al., 2005).

Self-renewing angioblasts identified by Bailey *et al.* (2004) might be CD31 precursors for endothelial and haemopoietic cells, which display a common clonal origin after the transfer of a single cell. Marrow-derived HSC in adults could then serve as a reservoir for precursors of endothelial cells. Some multipotent cells including HSC persist in adults although they have early limits to their multipotency and do not undergo reprogramming to hepatocytes or endothelial cells according to Stadfield and Graf (2004). These investigators supported classic interpretations on developmental boundaries being established by the specific germ layers. They reported that HSC did not produce endothelial cells, a point queried by other investigators. Markers including CD34 specify cell lines with vascular endothelial cells capable of establishing short- or long-term recoveries after grafting (Osawa *et al.*, 1996). Hence, multipotential mesenchymal CD14$^-$CD34$^-$CD35$^-$ precursors of various adult progenitor cells (MAPS) might form chrondrocytic, osteogenic and adipocytic cell lines and other tissues, as single c-kit$^+$Thy$^-$1.1^{lo}Lin$^-$Sca$^-$ HSC engraft recipients except for two distinct lines that express neither Sca$^-$ or Sca$^+$ (Cao *et al.*, 2004).

Perhaps the capacity of certain stem cells for therapeutic reprogramming has been underestimated. The most extreme example concerns the formation of oocytes after the transfer of bone marrow cells into the ovaries of recipient mice (Hubner *et al.*, 2003; Johnson *et al.*, 2004, 2005). In some cases,

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certain genes including *ZP3, synaptonemal complex* (*Scp3*), *growth differentiation factor 9* (*Gdf9*) were not expressed in the newly produced oocytes although *Oct-3/4* and *stage-specific antigen* (*SSEA-1*) were expressed. In most cases, oocytes were normal morphologically and expressed many specific genes. Variants remain to be explained and might be determined via variations in the properties of donor bone marrow cells.

Reservations expressed about the morphology of the grafted cells seem to be unjustified since histological examination revealed the oocytes in grafted ovaries were normal. Curiously, an illustration by Pietro Motta on the normal growth of the human ovary had earlier revealed follicles surrounded by small cells that resembled embryo stem cells. This is the route that Johnson *et al*. (2004, 2005) identified as the colonizing pathway. Woodbury *et al*. (2002) reported that germline genes were among several somatic tissues expressed in adult bone marrow cells, and Weismann (2005) recently declared that HSC were perhaps the only stem cells capable of lifelong tissue regeneration.

Epigenetic changes in stem cells due to cell fusions

Many investigators now query the ultimate therapeutic potential of human stem cells since epigenetic variations arose so frequently *in vitro* in many published reports. These variations were identified on a massive scale a few years ago, and cast doubt on the value of many stem cell lines derived from haemopoietic, muscle and brain tissues. The nature of the initial epigenetic stimuli remained obscure, but later modifications involved fusions between stem cells and other cells, even ES cells, which had apparently switched them *in vitro* to an apparently unrelated type. This situation has clouded major aspects of stem cell therapies and possibly emerged as gene activity in transferred nuclei of stem cells was modified by somatic cell cytoplasm (Cowan *et al.*, 2005). Somatic cells thus differentiated as their own genes were transcribed in the hybrid cells, regulated by the totipotent nucleus, which possibly lead to modifications in DNA and chromatin. Hyperacetylation at H3 and H4, and the hypermethylation of lysine 4 in H3, is associated with reprogramming. Similar effects in the *Oct4* promoter region are associated with the activation of both somatic and reprogrammed somatic genes, although genes remain silent in both genomes in some promoter regions. This implies that reprogramming does not always lead to modified gene actions (Kimura *et al.*, 2004).

Much remains to be learnt about factors causing fusion (reviewed in Edwards, 2004). Mouse hepatocytes derived from bone marrow *in vitro* repopulated liver corrected symptoms of disease in mutants with fumarylacetoacetate hydrolase deficiency (Wang *et al.*, 2003). Serial transplants of these cells to later recipients revealed many repopulating hepatocytes had lost their homozygosity and were now heterozygous for alleles typical of bone marrow. Some cells were 80 XXXY (2n fused with 2n cells) and others were 120 XXXXYY (2n/4n), clear evidence of fusion. Another example concerned donor cells that were originally *Rag*$^{+/+}$ that became diluted with mutant cells and hybrids when transferred to non-irradiated *Rag1*$^{-/-}$ mice. Studies on the deletion of *Fanconi anaemia gene* (*Fancc*$^{-/-}$) and *Fah*$^{+/+}$ also revealed how donor cells had been transformed. Chromosome painting with FISH detected XY variants that must have arisen through fusion, forming cells now dominant in repopulated tissues. Fusion may have involved later rather than earlier stages of stem cell development, and could have been due to macrophages, and T or B cells. This work was helpful in confirming conclusions made by Lagasse *et al*. (2000) that fused cells may offer distinct therapeutic possibilities.

Takahashi and Yamanaka (2006) published a highly significant paper on inducing pluripotent stem cells from mouse embryonic and adult fibroblasts by means of defined genetic factors. Initially, they incorporated 24 genes specifically expressed in mouse embryo stem (mES) cells into adult fibroblasts, which induced the cells to form pluripotent stem cells with specific ES cell markers and expressed similar morphology and growth properties. They then discovered that four such factors, *Oct4*, *Sox*2, c-*Myc*, *Klf4,* were also effective, and unexpectedly that *Nanog* was dispensable. They designated their cells as iPS (induced pluripotent cells), which formed chimaeras in all tissues when placed into blastocoelic cavities of host blastocysts. Transplanting iPS cells into nude mice produced tumours, a characteristic also typical of mES cells. This work clearly opens new approaches to more simple means of generating pluripotent stem cells and suggests that current techniques of preparing ES cells may become obsolete.

Summary

This paper has described examples of reprogramming and gene modification in early mammalian embryos via methods as distant as nuclear transfers to oocytes or via innate factors such as X inactivation in female embryos. Epigenetic factors, and especially methylation, seem to be involved in distinct forms of reprogramming, especially at conception and following the transfer of somatic cell nuclei to oocytes and during X chromosome inactivation. Cell fusions *in vitro* also involve tissue stem cells and consequential effects on gene expression. Epigenetic effects on the establishment of normal embryonic

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polarities after NT could well be disastrous, and may be responsible for some of the fetal defects associated with disorders in establishing left/right polarity. Every scrap of knowledge is needed to master these techniques if therapeutic cloning is to be successfully applied in medicine or if cell remodelling is to be achieved. In a sense, discoveries outlined in this paper also make major contributions to understanding of normal development. For example, it is possible that various haemopoietic and mesenchymal tissues arise from single progenitors. The current hesitant phase in applying stem cell knowledge therapeutically will doubtless be terminated, to be replaced by the future application of individual blastomeres and the therapeutic potential of ES and other stem cells. One recent example in sheep illustrates the potential value of using ES cells committed to cardiomyogenic phenotype to a gain improvements in myocardial function (Ménard *et al.*, 2005). Fascinating studies clearly lie ahead in the search for new clinical applications, and lack of space and time has precluded detailed attention in this review on other developmental systems, notably in the clocks timing differentiation, the power of circadian rhythms and the roles of telomeres.

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