How identical would cloned children be? An understanding essential to the ethical debate

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The ban on human cloning in many countries worldwide is founded on an assumption that cloned children will be identical to each other and to their nuclear donor. This paper explores the scientific basis for this assumption, considering both the principles and practice of cloning in animals and comparing genetic and epigenetic variation in potential human clones with that in monozygotic twins.

Key words: cloning/epigenesis/ethics/human/monozygotic twins

Introduction

The prospect of cloned children, especially in large identical groups, raises widespread alarm. In virtually every country that has passed legislation on assisted human conception, a clause has been inserted to ban the application of cloning to humans on pain of severe penalties. Concern about cloning arose after the initial studies in amphibians, and re-emerged when human eggs were first fertilized in vitro. There were abounding fears about self-cloning, identical football teams, and fetuses cloned to produce spare organs for the nuclear donor. Considered an affront to human dignity and individuality, cloning was bracketed with other non-permitted activities including the creation of animal/human hybrids, genetic modifications of embryos and, strange to say, the cryostorage of embryos. Alarm was sustained by films such as ‘Boys from Brazil’.

Underlying these concerns is the fundamental assumption that cloned children will be identical to their nuclear donor or their nuclear siblings. In this commentary, we consider the scientific basis for this assumption. Clones, defined as ‘a group of living organisms sharing the same nuclear gene set’ (opinion of the group of advisors on the Ethical Implications of Biotechnology to the European Commission, unpublished) are essentially monozygotic twins formed by biological manipulation. Two experimental methods have been used in animals to produce cloned embryos: embryo splitting and nuclear transfer. While embryo splitting gives rise to monozygotic twins at a high efficiency, up to 30% in cattle (Williams et al., 1984) and sheep (Chesné et al., 1987), and may be considered as an acceptable application to humans in the next decade, we confine our discussion to cloning by nuclear transfer since it is this process which has generated such adverse reactions. We outline details of the cloning process, and explore the effects of uterine and environmental factors on biological variability in cloned animals and monozygotic human twins.

Principles of cloning embryos

Studies on nuclear cloning in mammals followed initial work in amphibians by Briggs and King (1952) and Gurdon (1964). The method involves the fusion of a nuclear karyoplast taken from cells in various tissues with an enucleated metaphase II oocyte or an enucleated pronucleate egg (Baranska and Koprowski, 1970; Lin et al., 1973; Bromhall, 1975; Tarkowski and Balakier, 1980; Illemensee and Hoppe, 1981; McGrath and Solter, 1986; Willadsen, 1986; Tsunoda, 1987; Marx, 1988; Szollosi et al., 1988). Metaphase oocytes are enucleated by excising their spindles.

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and chromosomes, while pronuclei are excised from fertilized eggs. DNA dyes applied to excised ooplasm are used to verify the full removal of all chromosomal material (Bondioli, 1993). Up to one-quarter of the ooplasm can be excised during this process in sheep (Smith and Wilmot, 1989). The donor karyoplast, containing the nucleus with some cytoplasm, is then placed in the perivitelline space, adjacent to the oocyte membrane, and fusion and activation are induced by electrical, viral or chemical stimulation (Stice and Robl, 1988; Cheong et al., 1993; Prather, 1996). Cytochalasin B and other microtubule blockers are necessary to control cytoskeletal movements and allow enucleation (Stice and Robl, 1988; Smith and Wilmot, 1989; Cheong et al., 1993; Kwon and Kono, 1996; Prather, 1996).

Ooplasmic factors reprogramme the nucleus, conferring on it an ability to regulate embryonic growth to full term. Understanding of the key processes is still incomplete, but underlying mechanisms are being clarified. Blastomere karyoplasts are widely used, and others are taken from fetuses or adults. Nuclei in 2- and 4-cell embryos are totipotent, capable of supporting full-term development and this characteristic may require the expression of particular genes capable of supporting full-term development and this characteristic may require the expression of particular genes. Nuclei in 2- and 4-cell embryos are totipotent, and other microtubule blockers are necessary to control cytoskeletal movements and allow enucleation (Stice and Robl, 1988; Smith and Wilmot, 1989; Cheong et al., 1993; Kwon and Kono, 1996; Prather, 1996).

Status of the recipient oocyte

The status of the recipient oocyte is an important factor in cloning. Ooplasm changes its properties during maturation and fertilization as the cell is released from metaphase II arrest and activated at fertilization by a series of calcium discharges (Swann and Ozil, 1994) possibly involving cortical rotation (Edwards and Beard, 1997). Maternal RNA transcripts then control development and this characteristic may require the expression of particular genes including non-specific alkaline phosphatase, Tnap, transcription factors, Oct3/4, proto-oncogene, c-kit, and the DNA methyltransferase, Mt (Urven et al., 1993). In most mammals, blastomere nuclei are widely used, and others are taken from fetuses or adults. Nuclei in 2- and 4-cell embryos are totipotent, capable of supporting full-term development and this characteristic may require the expression of particular genes including non-specific alkaline phosphatase, Tnap, transcription factors, Oct3/4, proto-oncogene, c-kit, and the DNA methyltransferase, Mt (Urven et al., 1993). In most mammals, blastomere nuclei become committed by the 8–16-cell stage or even earlier so that later stages are no longer totipotent, and their nuclei may have to reprogrammed during cloning.

Histone H1 kinase activity has been used in reconstructed bovine embryos to measure MPF concentrations which decline rapidly after activation, e.g. down to 30% after 1 h and 20% after 2 h (Campbell et al., 1993) although reactivation may occur after a single activation pulse (Collas et al., 1993). Since MPF is associated with the spindle-chromosome complex (Fulka et al., 1996; Sagata, 1996) it may be depleted by the process of enucleation prior to activation. Several authors stress the importance of MPF in nuclear envelope breakdown, premature chromosome condensation (PCC) and reorganization of the cytoskeleton (Campbell et al., 1993; Prather, 1996), while Leno et al. (1992) suggest that nuclear membrane breakdown may occur with G1 donor nuclei but not with those in G2. Dissolution of the donor nuclear envelope presumably proceeds in a manner resembling disassembly of the sperm membrane during normal fertilization (Poccia and Collas, 1997). MPF may also play a beneficial role in remodelling G2-phase donor nuclei (Fulka et al., 1996).

Thus cloning may best be achieved with aged MPF-deficient oocytes rather than fresh oocytes, and with enucleated fertilized or activated eggs rather than with enucleate metaphase II oocytes since MPF decays during such a delay (Prather et al., 1987; Campbell et al., 1993, 1994; Prather, 1996). Some investigators prefer to clone activated oocytes some hours later, e.g. during their S phase, when MPF concentrations have subsided (Campbell et al., 1993; Kwon and Kono, 1996). In bovine embryos, the combination of ageing, cooling and enucleation results in low MPF activity but high MAP kinase activity (Gall et al., 1996). Matching cell cycles in donor nuclei and recipient enucleated oocytes may be advantageous, particularly
when MPF concentrations are high, to yield good rates of growth to full term (Gurdon, 1964; Chastant et al., 1996).

Transferred nuclei must interact with a sequence of cytoplasmic activators and repressors which control normal zygotic gene transcription (Majumder et al., 1993; Yang et al., 1995; Davis et al., 1996). Metabolic variations such as methylation of insulin-like growth factor (IGF) II receptor could occur in cloning as in parthenogenetic mouse embryos stimulated by electroactivation (Kono et al., 1996). The whole procedure must involve stress, and possibly the production of heat shock proteins (Kurtz et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986) in far greater amounts than under normal conditions of culture (Gardner and Leese, 1990; Conaghan et al., 1986). Stress invokes numerous effects, such as changes in protein synthesis in embryos growing in vitro (Reik et al., 1993) as well as apparent long-lasting effects such as large birthweights in cattle and sheep (Walker et al., 1992; Behboodi et al., 1995). Such large birthweights are more common after cloning (Keefer et al., 1994; Renard, 1998).

Cloning of isolated plant stem cells also involves stress responses to growth factor starvation, these responses involving somatic meioses, homeotic transformation and somatic embryogenesis in response to this form of starvation (Nuti Ronchi, 1995). Isolated plant protoplasts display somatic meiosis when fused after removal of their chitinous cell membranes, and have been used to establish wide and variable crosses between species (Lowe et al., 1996; Parkonny et al., 1997). All these observations illustrate the profound effect of environmental factors on early development and the importance of culture techniques and conditions on the outcome of cloning.

**Status of the donor karyoplast**

Initial studies on cloning in mammals utilized nuclei from blastomeres of preimplantation embryos, and shifted later to nuclei from fetal, and adult tissues. The blastomeres are in their formative periods of cellular differentiation and sex determination (Antczak and Van Blerkom, 1997; Edwards and Beard, 1997), yet they have the advantage of containing nuclei capable of reverting easily to an oocyte-like condition. Adult nuclei presumably need to reverse changes imposed by ≥30 cell cycles, i.e. the number of cycles estimated to occur in the transition from single cell to a 1 × 10⁹ cell adult, so it is hardly surprising that cloning is more successful with younger nuclei. Two-cell nuclei are more effective than 8-cell nuclei in mice (Cheong et al., 1993), nuclei from rabbit inner cell mass give better development than granulosa cell nuclei (Collas and Barnes, 1994), and embryonic rather than adult cell nuclei give higher success rates in sheep and cattle (Keefer et al., 1994; Wilmut et al., 1997). The birth of Dolly (Wilmut et al., 1997), whose cloned status is now fully confirmed by DNA microsatellite analysis (Ashworth et al., 1998) and DNA fingerprinting (Singer et al., 1998) and supported by recent work in mice (Wakayama et al., 1998) decisively widens the scope of cloning, to the use of fully differentiated adult nuclei.

Successive nuclear modifications during growth to adulthood must presumably be reversed in cloning. These include DNA methylation and imprinting (Monk et al., 1987), X inactivation, histone acetylation (Turner, 1991), and modifications in other nuclear proteins (Pinto-Correia et al., 1995), enzyme activation (Worrad et al., 1995), and telomere shortening (Harley et al., 1990). Telomeres, centromeres and chromosome-specific subsatellite regions display their own typical arrangements in interphase somatic nuclei, e.g. centromeric DNA can be held on the nuclear envelope, dispersed throughout the nucleus or concentrated at one pole particularly in S and G₂ phases, although there are wide species differences (Vourc’h et al., 1993). These nuclear modifications may have to be reversed in cloning. Genetic modifications arising in adult nuclei during growth could include somatic mutagenesis (presumably at a rate of 1 in 10⁹ base pairs per nucleus), mitotic errors, deletions and disomy. These factors will be present in cloned nuclei and will influence embryonic growth. Reversal of these modifications, together with epigenetic effects of cloning, could cause wide differences from the original genotype of the nuclear donor.

The process of nuclear de-differentiation and remodelling is not understood although several nuclear markers have been studied in order to identify changes occurring in transferred nuclei. Nuclear lamins A and C, polymerize during early cleavage stages in mice, pigs and cattle, and during nuclear swelling in cloned embryos (Prather et al., 1989; Kubiak et al., 1991). The appearance of phosphorylated polypeptides perhaps involved in DNA repair, replication, recombination and transcription, is another indicator. They normally appear 2 h after fertilization in rabbit eggs during sperm head decondensation, and are first formed in clones at 10 h post-fusion (Pinto-Correia et al., 1995). Blastocoel formation can be delayed in rabbit clones (Stace and Robl, 1988). Nucleolar reprogramming is delayed in some cloned 32-cell rabbit embryos, since fibrillarin forms in only a proportion of cloned blastomeres, in comparison with all cells of normal 32-cell embryos. Fibrillarin associates with nucleolar small ribonuclear RNA and marks the onset of nucleolar transcription (Pinto-Correia et al., 1995), and its delayed synthesis in clones may be the
consequence of defects arising in nucleolus organizer chromatin, which is heterochromatic. Changes in nucleolar ultrastructure and a large increase in free ribosomes suggested that reconstituted rabbit embryos at the 32-cell stage had disrupted ribosomal transport mechanisms (Kanka et al., 1996). In contrast, some cell surface antigens are expressed simultaneously in fertilized 32-cell embryos and clones (Van Stekelenburg-Hamers et al., 1994).

The de-differentiation of somatic cells in vitro may help to understand cloning, even though it is induced via the cell surface rather than by cytoplasmic messengers. Both systems involve gross changes in gene expression. Modifications to tissue culture substrates has been shown to alter phenotypic expression in a variety of systems. Chick retinal pigmented epithelial cells (PECs) de-differentiate, proliferate and re-differentiate into lens cells as they activate α-, β- and γ-crystallin genes when placed in media containing phenylthiourea and hyaluronidase, whereas collagen substrates inhibit this transition. The expression of certain PEC-specific genes such as MMP115 and pP344 is repressed as the cells de-differentiate and re-activated as they revert to PECs (Agata et al., 1993; Mazaki et al., 1996).

Culture media containing glucocorticoids help to up-regulate expression of the breast cancer genes, BRCA1 and BRCA2 in mammary epithelium during proliferation and differentiation, but not when deprived of serum (Rajan et al., 1996).

Wide morphological changes can be induced in vitro. When primary explants of bovine mammary epithelial cells are cultured on fibronectin or collagen, rather than laminin, an epithelial pavement can form over 4 weeks, with tight junctions, desmosomes, apical villi and a high transepithelial electric resistance (Delabarre, 1997). When placed on plastic or connective tissue (Matrigel) substrates, fetal enterocytes de-differentiate, whereas they remain fully differentiated, displaying typical enzymes in their brush border membranes, if cultured on laminin or co-cultured with fibroblasts (Sanderson et al., 1996).

Cartilage cells de-differentiate when cultured to high cell densities with bone morphogenic protein on various matrices and can be switched into specific cell types for use in reconstructive surgery. Human cells are being placed on biodegradable mesh scaffolds with specific substrates in order to construct replaceable organs such as skin, breast, nipples, ears and cornea in work approaching clinical trials (Vacanti, 1988; Sittinger, 1995; Cao, et al., 1997). With both nuclear de-differentiation and cloning, further experimentation is required to understand the signals and responses involved in these processes.

**Synchronization of donor and recipient cell cycles**

Most forms of cloning have utilized enucleated metaphase II oocytes. Results with cloning blastomere nuclei vary according to the cell cycle stage in the donor and recipient. Some investigators use donor nuclei in G1/S (Cheong et al., 1993; Pinto-Correia et al., 1995); others prefer G0 nuclei from confluent cell cultures in ‘starvation culture media’ (Wilmut et al., 1997). Metaphase nuclei are used rarely but produce normal embryos and offspring in mice (Kwon and Kono, 1996). Nuclei from mouse 2–8-cell embryos in G1, S and G2 phase produce blastocysts at a rate of 78, 0 and 21% respectively, and G1 nuclei yield 18–29% young per transferred embryo (Cheong et al., 1993). Reservations are expressed about these results since the G1 phase is very short or absent in blastomere nuclei (Pinto-Correia et al., 1995; Kwon and Kono, 1996), and it is notable that Kwon and Kono (1996) could not repeat the work of Cheong et al. (1993). Cloning G0 nuclei was successful with Dolly following a G0 induced by cell starvation in culture. Usually, G0 occurs during or at the end of G1, and the activation of the nucleus during cloning would resemble the natural progression from G1 to early S phase of the mitotic cycle or gamete fusion to post-S phase in a fertilization cycle (Figure 1a,b). G0 can also arise at the S–G2 transition in some cell types, e.g. Drosophila nurse cells (Su et al., 1988). It is not clear which of these was the G0 phase used by Wilmut et al. (1997).

Synchronization of the cell cycle phases in the donor nucleus and recipient oocyte may be very important for the success of cloning. When MPF concentrations are high in fresh enucleated oocytes, PCC and nuclear envelope breakdown occur allowing reinitiation of DNA synthesis (Barnes et al., 1993; Campbell et al., 1993). When donor nuclei are in G2 phase, DNA replication must be completed (centromeric DNA synthesis) prior to PCC. Aneuploidy will result from complete re-replication of DNA in a single nucleus, although the 4S status and normal cleavage will follow extrusion of a polar body (Figure 1c).

PCC was first described in HeLa cells fused at different stages of the mitotic cell cycle (Johnson and Rao, 1970). The fate of chromosomes following PCC depends on the cell cycle stage of the karyoplast. Early S nuclei can form rare double spindles, sticky chromosomes, micronuclei and chromosomal mosaicism. With S phase nuclei, chromosome pulverization may occur as was observed in the original studies on HeLa cells (Johnson and Rao, 1970). Late S nuclei can produce irregular metaphase plates, and half or disorganized spindles, chromatin patches and
Figure 1. Models of the normal mitotic cell cycle, events at fertilization and cloning in relation to DNA content and chromosome number, as well as chromatid and centromere structure. S values indicate DNA levels which double in the S phase as chromatids replicate. Maternal (M) and paternal (P) chromatids are indicated. (a) DNA content during the normal mitotic cell cycle. The extended period of DNA synthesis in heterochromatic, including centromeric regions, is indicated. Cell division at metaphase restores 2S in daughter cells. (b) Modifications of the cell cycle and DNA content during fertilization. Metaphase II oocytes and first polar bodies are 2S. At fertilization, the oocyte and second polar body become 1S, and the spermatozoon introduces 1S. Fertilized eggs thus become 2S immediately after fertilization in their G1 phase, and 4S after their S phase. The first cleavage division produces 2S in each daughter blastomere. (c) Cloning nuclei in late S and G2 phase. Successive illustrations show the consequences of fusing donor karyoplasts with recipient enucleated oocytes in meiosis metaphase II. A single chromosome pair is shown. Nuclei in late S are approaching 4S and are replicating chromatids, while those in G2 have completed this process. Neither stage has replicated centromeres since heterochromatic regions replicate late in the cell cycle. Nuclei presumably undergo premature chromosome condensation (PCC) and replicate centromeres before the onset of spindle formation. A complete DNA synthesis in the pronucleus involving chromatids and centromeres would establish 8S. If a polar body and a pronucleus are formed, they will each be 4S and the eggs would be ready for normal cleavage. If polar body expulsion is prevented, the egg forms one or two pronuclei, with a total of 8S, and this could be incompatible with normal development. Species differences appear to result in polar bodies being expelled in cloned mouse eggs but not in those of farm animals and rabbits.
larger-sized chromosomes. Nevertheless, the overall impression is of similar events in all of these stages, so that many cloned embryos inherit normal chromosome counts and morphology and cleave normally (Collas et al., 1992a,b).

The situation in cloning is entirely different if donor nuclei are fused several hours after oocyte enucleation and activation, or with enucleated pronucleate eggs. Many recipient eggs will then be in S, a perfect match for donor nuclei also in their S phase. Campbell et al. (1993, 1994) stress that PCC does not occur at all under these conditions, and that oocytes activated some hours before fusion are ‘universal cytoplast recipients’. Almost all of their nuclei were in S, and therefore synchronous with the recipient egg. Fusion several hours after activation is also effective in cattle, and produces few chromosomal anomalies in the embryos (Barnes et al., 1993). Mouse pronuclei in S phase have been recloned into recipient enucleated pronucleate eggs which were also in or close to S phase, and have yielded very high rates of normal growth among the recloned offspring (Figure 2) (Kwon and Kono, 1996). Cell cycle problems could still arise with G2 and M donor nuclei fused with enucleated recipient eggs in S phase. They could be forced back into an abnormal S phase involving centromeric regions or even whole chromosomes. Wilmut et al. (1997) evidently abandoned the universal recipient system when cloning Dolly, and instead returned to enucleated metaphase II oocytes and G0 donor nuclei.

Many unknowns about cloning remain to be solved. Cell cycle phases stated by many investigators may not be formally correct, since G1 is brief or non-existent in blastomere nuclei. G1 phase must be induced artificially by arresting blastomeres at metaphase, e.g. using nocodazole. The embryos which are to produce the donor nuclei are then transferred to medium without nocodazole and containing hydroxyurea, and checked hourly for cleavage; immediately after cleavage, the temperature is lowered on the assumption that blastomeres now in G1 or at the G1/S boundary will remain arrested there (Campbell et al., 1993). Whether this procedure produces the desired result is debatable, since the S phase can begin before mitosis is completed in some types of cell, or nuclei may enter the S phase during the delay in extracting and fusing them.

**Cytogenetic modifications and abnormal development after nuclear fusion**

**Nuclear and chromosomal situations**

Major developmental, chromosomal and centromeric epigenetic modifications arise in cloning, particularly when recipient oocytes and donor nuclei differ in their cell cycle stages. Abnormalities, such as polyploidy (Campbell et al., 1993) and disomies as well as imprinting defects may arise and this could account for some of the high levels of embryonic and fetal loss.

The production of either two ‘pronuclei’, a pronucleus and one or two polar bodies, or a single (diploid?) pronucleus after nuclear transfer could be influenced by the use of nocodazole or cytochalasin which impair tubulin function and polar body extrusion (Kwon and Kono, 1996). Species variations seemingly arise, since polar bodies are apparently not regular features of cloning in rabbits (Stice and Robl, 1988; Collas et al., 1992), sheep (Robl et al., 1987; Collas et al., 1992) and cattle (Prather...
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Figure 3. Segregation of chromatids in a chromosome pair as premature chromosome condensation (PCC) and metaphase arise during cloning. Maternal (M) and paternal (P) chromosomes are shown individually, with their chromatids.

et al., 1987; Bondioli et al., 1990; Campbell et al., 1993, 1994). Polar body extrusion apparently leaves some eggs with insufficient chromosomes for a normal first cleavage division (Cheong et al., 1993). The observation that cloned embryos extrude polar bodies, is astonishing and perhaps a further sign that oocyte microtubules control donor chromosomes and impose a late form of meiosis. These successive changes presumably utilize ooplasmic factors and chromosome-associated centromeres, centrioles, and lamins. Normally, the sperm centriole is involved in spindle formation in the embryo, and it seems that the cloned nucleus can assume this function. Nucleoli may have to adapt to a different function during cloning, initially being inactivated and then producing compounds such as nucleolin and fibrillarin (Baran et al., 1995). Chromosomal damage, as yet undefined, may arise during PCC (Collas et al., 1992; Campbell et al., 1994; Kwono et al., 1996; Prather, 1996). Chromosome association or pairing, the formation of 0, 1 or 2 polar bodies (Kwon and Kono, 1996), unipolar and uneven sized spindles (Collas et al., 1992) and other disorders may distort normal chromatid separation to the poles after PCC. Meiotic-like phenomena similar to PCC, but not chiasmata, occur in plant cells cloned from individual stem cells (Nuti Ronchi, 1995). Anomalous segregation could cause disomy and other wide genetic variations in the cloned nuclei (Figure 3). Disomy, which is of immense clinical significance for various cancers, involves two simultaneous events: loss of one parental chromosome set, and a (simultaneous?) doubling of the other. Perhaps unusual forms of centromere synthesis in somatic cells, just as in PCC, are responsible for these complex chromosomal events.

**Centromeric DNA synthesis**

A poorly understood event of centromeric DNA synthesis has been observed in cloned rabbit embryos just before PCC (Pinto-Correia et al., 1995). Two distinct phases of DNA synthesis thus occur in cloned metaphase II recipients. One phase occurs before or during PCC when oocytes are ‘still at transient spindle stage’ and is restricted to centromeric regions. The second phase occurs after PCC in pronuclei and involves a complete DNA synthesis including previously-replicated centromeric regions. Pinto-Correia et al. (1995) suggest that this could lead to developmental defects, although many of their embryos developed normally to blastocysts. Although steps are often taken to synchronize donor nuclei in the early S phase, the DNA synthesis that began in cells of the original donor embryo was probably being completed just after fusion occurred, since centromeric and other heterochromatic regions are known to prolong DNA synthesis until the M phase (Wolfe, 1981). This situation also explains the low-level synthesis of DNA until PCC (reported by Collas et al., 1992a) and which was possibly due to the synthesis of heterochromatic centromeric DNA late in the cell cycle of the donor nucleus. Other forms of heterochromatin could also replicate very late in the cell cycle. With blastocyst nuclei, for example, one X chromosome is inactivated in female embryos by actions of the gene Xist, and this heterochromatic chromosome contains less acetylated isoforms of histone H4 and replicates late in the S phase (Panning and Jaenisch, 1998). The brief period between fusion and PCC would thus be best explained as a completion of late-replicating heterochromatin in the S phase. A subsequent and later synthesis of all of the genomic DNA synthesis could then result in 4 S polyploidy in the absence of polar body formation.

Aberrant centromere synthesis at these stages could duplicate centromeres on single chromatids (Figure 4). Unusual forms of centromere replication occur in mitotic cells. Neocentromeres can form in latent DNA regions devoid of the typical α satellite repeats and other sequences in normal centromeres, and are apparently induced by epigenetic factors (du Sart et al., 1997; Murphy and Karpen, 1998; Wiens and Sorger, 1998). Centromeres in higher eukaryotes, including mammals, consist of an essential DNA core flanked by highly repeated nucleotide sequences (Murphy and Karpen, 1995). Centromere-associated proteins in an anaphase-promoting complex control anaphase separation by proteolysis (Jorgensen et al., 1998). Dicentric human chromosomes, as found in Roberstonian translocations, seemingly function by

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<th>Chromosome structure</th>
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<td>MM  PP</td>
<td>Donor nucleus</td>
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<td>MMPP</td>
<td>Chromosomes at PCC</td>
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<td>MM  PP</td>
<td>Combinations in the egg and polar body, or in the two promi nuclei after polar body expulsion is suppressed</td>
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Figure 4. Aberrant centromere synthesis. If premature chromosome condensation (PCC) and incomplete (centromeric) DNA synthesis occur (Pinto-Correia et al., 1995), chromatids would become dicentric. This could result in chaos, interferring with spindle formation and function, perhaps inducing conditions such as short spindles producing anomalous divisions or even unipolar spindles leading to chromosome doubling to 2S. Anomalies in spindle and chromosome structure have been reported in rabbits although they seem to be more severe with late S clones (Collas et al., 1992), and it is even possible that nuclei are forced backwards to G1 and mitosis by maturation promoting factor (MPF). If complete DNA synthesis occurs following centromeric synthesis as found by Pinto-Correia et al. (1995), this would effectively convert the nucleus to 4S DNA. Since G1 is very short in blastomere nuclei, these comments will apply only to nuclei from fetal or adult tissues.

Unipronucleate human eggs, possibly precursors of androgenetic moles, apparently undergo two distinct pronuclear phases, and perhaps two cycles of DNA synthesis (Figure 5) (Edwards et al., 1990). Their single pronucleus initially decondenses in preparation for syngamy, but is replaced by another distinct nucleus which forms and persists for some hours before the onset of a normal syngamy and first cleavage. This evidence almost suggests that hydatidiform moles are clones of the fertilizing spermatozoon! Other unusual forms of pronuclear reprogramming include the discovery of a single pronucleus in human eggs after IVF. The pronucleus was diploid 18,18,XY in four and 18,18,XX in two eggs, indicating that they contained maternal and paternal components (Levron et al., 1995). Synchronous nucleoplasts and unipronucleate eggs can be fused to produce diploid human blastocysts (Azambuja et al., 1996). There is no knowledge about DNA synthesis in these studies.

Unipronucleate human eggs injected with spermatids during ICSI (Tesarik and Mendoza, 1996). Among 28 eggs, 10 formed two pronuclei which apparently fused by 16 h into a single...
‘syngamy nucleus’ with many nucleoli (Figure 5). Poor activation and embryogenesis as well as the death of human embryos in utero after spermatid injections resembles some aspects of cloning (Tesarik et al., 1998). Spermatid nuclei must de-differentiate and recondense, replace protamines by histones, and adapt to the cell cycle of the recipient oocyte (Fishel et al., 1996). Such reprogramming events may have similarities to cloning.

**Epigenetic regulators in cloning**

The phenomenon of epigenesis, i.e. heritable changes in gene function which cannot be explained by changes in DNA sequence (Nan et al., 1998) is clearly of prime importance in cloning. DNA methylation does not affect the base pair sequence but is associated with gene silencing and is now known to operate on a wide variety of genes including developmental genes, specific allelic-inhibition sites, transgenes and the inactive X chromosome in female mammals. Gene silencing at these sites suppresses transcription, and imposes position effects leading to variegated patterns of gene expression. Fetal overgrowth in the human Beckwith–Wiedeman syndrome could involve imprinting defects in IGFII (Wutz et al., 1998); similar systems could produce large calf syndrome during bovine IVF and cloning.

Methylated sequences are not rare; they may comprise the 35% of human DNA classes associated with transposons and retroviruses in parasite DNA sequences with high copy numbers (Bestor, 1998). Methylation and imprinting may ensure that a stabilized chromatin structure sustains transcriptional repression (Wolffe, 1998), a role that may be more important than the regulation of mammalian differentiation. Some genes function better in heterochromatic sites, and perhaps in centromeric sites, than in euchromatin. An onset of methylation at implantation is associated with X inactivation in female mammalian embryos, although the process may be activated by the gene Xist and then sustained by methylation (Jaenisch et al., 1998). Once established, this form of inactivation could be maintained, self-perpetuating systems active over many cell cycles, such as late DNA replication and modifications in histone assembly which maintain dosage compensation of X-linked genes (Panning and Jaenisch, 1998). Likewise, allele-specific repression may be invoked by initial silencing of DNA elements and then sustained by methylation (Brenton et al., 1998). All cells in the trophectoderm display X inactivation, compared with 15% in the epiblast (Jaenisch et al., 1998).

It is clear that such epigenetic modifications occurring during cloning could play a decisive role in embryogenesis. Even a seemingly simple operation such as transferring mouse pronuclei into a different ooplasm can impair embryonic growth (Latham and Solter, 1991), while exchanging pronuclei, or male and female pronuclei, evokes transcriptional repression, fetal growth retardation and abnormal methylation of urinary proteins in offspring even though they are diploid with paternal and maternal genomes (Norris et al., 1990; Reik et al., 1993). The focus on this area will be enhanced by the recent demonstration that these epigenetic modifications can be passed to >50% of the next generation by transmission through the male germline in mice (Roemer et al., 1997). Indeed, appropriate chromosomal modifications establishing active or repressed states in gene function may be built into chromatin as a memory of the developmental decisions taken by individual cells, and genomic or gametic imprinting may be a significant form of epigenetic inheritance (Surani, 1998). It is highly probable that similar mechanisms occur during cloning, or have to be reversed after nuclear fusion, with considerable effects on the resulting form of development in cloned embryos.

**Practical aspects of cloning**

**Growth of cloned embryos**

Whatever its complexities, cloning is being commercially applied in livestock production, with hundreds of calves born (Bondioli, 1993) and used to increase efficiency in research studies, e.g. in pigs (Lamberson, 1994). The first transgenic cloned lamb, Polly, carried a human gene for a therapeutic protein (Nature, 1997) which takes cloning into human medicine. Any clinical applications in human embryology are precluded as long as the reasons for poor success rates, incomplete development and high pre- and perinatal losses in animals, particularly with late embryonic, fetal, or adult nuclei, are not fully understood.

The success of embryonic development after cloning in cattle has been notably poor when compared with embryos established by IVF. Approximately 60% of those cloned from blastomeres establish pregnancies, compared with a pregnancy rate of 78% with IVF (Renard, 1998). These proportions reduce to 22 and 33% respectively, at birth. Bovine blastomere nuclei produce more blastocysts (30%) than do germ cell or fibroblast nuclei (2 and 6% respectively), and this is typical of the poorer results with highly differentiated nuclei. Large calf syndrome is more common after cloning than after IVF. Bovine embryos cloned from embryonic nuclei produce similar numbers of embryos in comparison with IVF, but these clones produce more heterogeneous RNA, many of their cells die and embryonic mortality is high, implying that reprogramming is
Incomplete (Heyman et al., 1995; Lavoir et al., 1997). In contrast, comparisons of 30 datasets obtained worldwide for several breeds of cattle showed higher rates of increased birthweight, dystocia and perinatal death in offspring produced by in-vitro maturation and culture techniques than by nuclear transfer (Kruip and den Daas, 1997).

Bovine inner cell mass nuclei have been used moderately successfully in cloning. One study yielded 34 blastocysts transferred to 27 cows, resulting in 13 pregnancies and four normal births (Sims and First, 1994). Another study on 948 nuclear transfers produced 5% blastocysts, and six pregnancies from 26 transferred embryos. Two pregnancies aborted after 60 days and two live and two stillborn calves were delivered. A stillborn male and a liveborn female had limb deformities, and all four died within 15 days of birth. The average birthweight of cloned calves was significantly greater than that of normal dairy calves (Keef er et al., 1994). Varying nuclear/cytoplasmic ratios and cytoplasmic vesicles characterize bovine clones established from embryonic cell lines. Better results followed the use of small, rather than large, cells but all embryos died by 60 days post-cloning (Stice et al., 1996). Bovine cells derived from fetal male gonads supported limited development to blastocyst (4%), although none survived beyond 40 days after transfer of three blastocysts and one morula (Delhaise et al., 1995).

Sheep blastomere nuclei allowed development to blastocyst (35%) and established pregnancies in four out of 22 (18%) recipients (Smith and Wilmut, 1989). Embryos cloned with nuclei from cultured cell lines failed to develop normally, and fewer than 17% developed to blastocysts. Of eight lambs born from 34 blastocysts transferred to seven recipients, three died pre-birth, two died within minutes of birth and another at 10 days (Campbell et al., 1996). The birth of Dolly was accompanied by the death of 62% (12/21) of the fetuses, far more than an estimated 6% after natural mating (Wilmut et al., 1997). Two fetuses had abnormal liver development, and losses at birth reached 12.5% (one lamb in eight), a ratio similar to the 8% loss in commercial flocks (Nash et al., 1996).

Recloning of cloned embryos has been achieved in cattle and mice. In cloning steps carried out over four successive generations in cattle, the number of cloned embryos rose from one to 54 identical morulae (Stice and Keefer, 1993). Fusion rates declined with later generations of clones, and developmental rates were highest with first and third generation embryos. Several first, second and third generation calves were born, but pregnancies were lost in all generations as calving rates declined from 10 to 2–3% between the first and third generations (Stice and Keefer, 1993). Recloning was also achieved using nuclei from 32-cell cow embryos (Ectors et al., 1995). Electrofusion was followed by co-culture on feeder cells until day 6 when the embryos were recloned and five pregnancies arose after transfer.

Excellent success rates have been obtained with recloning in mice. Each metaphase donor nucleus taken from 4-cell embryos following fusion using Sendai virus and then electrical activation was successfully cloned (Kwon and Kono, 1996). The resulting embryos were cultured in cytochalasin to prevent polar body expulsion and their two pronuclei were transferred singly into enucleated fertilized oocytes. Over 80% of the recloned embryos developed into blastocysts, including three sets of identical septuplets derived from a single original embryo (Figure 2). An astonishing figure of 57% of embryos transferred to 11 recipients developed into offspring (Kwon and Kono, 1996). One set of identical sextuplets and one set of identical quadruplets were obtained among 25 live offspring which were normal and fertile.

These recent studies in mice provide the brightest outlook yet for the future applications of cloning. It is not known what differences between species may arise and the range has now extended to the rhesus monkey (Meng et al., 1997). This stresses yet again that further studies are needed in all species to resolve problems in the cloning process including the high fetal and perinatal mortality and the many abnormal forms. Even as this article was being completed, outlooks on the success of cloning using nuclei from adults has expanded to mice (Wakayama et al., 1998) and to 28 cattle cloned from adult oviductal nuclei (Y.Tsunoda, personal communication). The pace of work is obviously quickening.

Variations between cloned offspring

Information on the variability of cloned offspring, a most important outcome, is inadequate to draw any definite conclusions. Patterns of growth, malformation and death imply that clones are highly variable and that long-acting genetic, developmental and epigenetic effects cause catastrophic forms of embryonic, fetal and post-natal development.

The highly unusual and stressful events involved in the cloning process could impose enormous effects on the normality of embryogenesis. Epigenesis is widely studied in relation to methylation, position effects, switches in identifiable gene activity, gene silencing and imprinting. It is less well studied in relation to inherited variation and variance analysis, although Wolffe (1998) sums up the whole matter: ‘Gene regulation is dependent not only on DNA sequence per se but also on the appropriate compartmentalization of the DNA sequence. The compart-
mentalization concept emphasizes the general, rather than the local, control of gene activity. Epigenesis has been a major matter for embryologists, who wish to know if its expression is regular, invariant and decisive, and how its effects compare with gene mutation and recombination as a source of variation (Waddington, 1956).

Sporadic information on phenotypic variation in clones is available from several studies. Cattle cloned from the same nuclear donor vary in size and in their colour markings (Renard, 1998; Stice and Robl, 1988). Birthweights ranged from 70 to 86 lb in four calves cloned from the same donor (Sims and First, 1994) and multiple generation recloning produced eight calves in the first generation and one each in the second and third generations, with high variations in birthweights. Two calves had high birthweights of 160 and 162 lb respectively, against a mean of 84 ± 10 lb in eight others (Stice and Keefer, 1993). In contrast, responses to parasites and leukocytic reactions to immunological challenge probably varied less in clones than in normal siblings (Renard, 1998).

Models have been constructed to estimate the degree of variation expected in cloned pigs. Three traits, litter size, days to slaughter and backfat thickness, represent wide ranges of heritabilities with well-defined causes of phenotypic variation. Data from four large trials were used to partition phenotypic variation into additive and dominant genetic, environmental and remaining non-additive genetic and common environmental variance. Animals were compared by range of performances comprising 95% of the group. Littersize was predicted to range from 5.4 to 14.6 offspring per litter for clones compared with 5.2 to 14.8 for full sibs and 5.0 to 15.0 for unrelated animals. Litter size has a low heritability which accounts for these low inter-group differences. Backfat thickness, with the highest heritability, was estimated to range from 12.6 to 17.4 mm for clones compared with 11.8 to 18.2 mm for full sibs and 11.0 to 19.0 mm for unrelated individuals (Lamberson, 1994). This author comments that the range of performance is still wide even in clones.

Cloned animals could reduce the number of animals needed in various trials. The degree of reduction depends on trait heritability. Numbers required were estimated to be reduced by 65 and 37% using clones or full sibs for days to slaughter, whereas savings were only 12 and 5% using clones or full sibs for litter size in pigs (Lamberson, 1994). In cattle, 30 clones would be expected to provide the same information as 53 unrelated animals for highly heritable characteristics \( h^2 = 0.5 \), where variability between treatments is similar to variance within treatments. Differences were few for lowly heritable traits \( h^2 = 0.1 \).

Additive genetic variability using clones was found to be four times less than with unrelated animals (Colleau et al., 1998). The value of clones over full sibs was much less, although clones were far less variable than random animals. Wide differences still remained among clones, perhaps greater than those between monozygotic twins as suggested by Lamberson’s (1994) study in which intraclass correlations among clones were predicted as 0.15, 0.66 and 0.64 for litter size, days to slaughter and backfat thickness in pigs. These values are generally lower than for production traits in monozygotic twin dairy cattle, which range from 0.83 for linear body measurements to 0.98 for butterfat production.

### Monozygotic human twins: nature’s clones

**Variations in embryological origins and development of monozygotic twins**

The frequency of human monozygotic twinning was stable at four per 1000 pregnancies before ovarian stimulation and IVF were introduced in the 1970s. Dizygotic twinning is inherited and has a frequency of 4–16 per 1000 pregnancies, depending on race and age (Derom et al., 1993; Tong et al., 1997). Monozygotic twins may separate at the 2-cell stage or at a later preimplantation stage (Corner, 1955; Boyd and Hamilton, 1970). Occasionally, two human embryos form within one zona pellucida, or a blastocyst within a human blastocyst (Hardy et al., 1996). Twinning in early cleavage stages may be a consequence of totipotency in mammalian blastomeres (Johnson et al., 1995). 2-cell splitting could produce dichorial and mono- or dichorionic placenta (Corner, 1955).

Monozygotic twins might also separate as the diffuse human inner cell mass divides. Rare human blastocystos possess two separate inner cell masses within one trophoectoderm, but they might fuse as observed in mouse blastocysts with an extra transferred inner cell mass producing chimaeric offspring rather than twins (Gardner, 1975). Inner cell mass duplication could result in monochorionic, diamniotic placenta (Corner, 1955), and two-thirds of monozygotic twins are monochorionic (Machin, 1996).

The higher rate of human dizygotic twinning since ovarian stimulation and IVF were introduced stems from multiple ovulations and embryo transfers. The 2–3-fold increase in monozygotic twins could be due to anomalies in hatching human blastocysts (Edwards et al., 1986; Derom et al., 1993; Wenstrom et al., 1993). This is a consequence of ovarian stimulation rather than IVF, as it occurs in similar frequencies with both situations (Derom et al., 1993). A hardened zona pellucida after hyperstimulation...
could impair hatching (Edwards and Brody, 1995), trapping and splitting half-hatched blastocysts with inner cell mass and trophectoderm in each half (Hardy et al., 1996). Resulting monozygotic twins could be highly variable, inheriting different amounts of inner cell mass and trophectoderm, and grow quite separately or in various membranal relationships. Since many of them could be overlooked or misclassified as dizygotic from their membranes, identifying DNA tests are mandatory. Splitting at the head process stage, as in some armadillos (Bulmer, 1970), could produce unequal or joined twins. A monozygotic twin pair at an estimated post-ovulatory age of 17 days had a chorionic sac containing twins, each with a yolk sac (Heuser, 1954).

These forms of monozygotic twinning do not differ greatly from cloning nuclei of preimplantation embryos and may display similar developmental sequences. It is well known that discordances can arise within monozygotic twin pairs, involving genetic diseases, intrauterine development, birth weights, congenital anomalies and behaviour (for reviews, see Hall, 1996; Machin, 1996). Machin (1996) writes ‘While it is true that most monozygotic twins are phenotypically very similar, there are significant numbers of monozygotic pairs who are neither genotypically nor phenotypically ‘identical’. Hall (1996) comments ‘there are clearly major exceptions to the assumption that monozygotic twin pairs are genetically identical.’ If cell allocations to twins differ during early embryogenesis, discordances could arise through somatic non-disjunction, skewed or differing forms of X inactivation, or a non-random inactivation or allocation of inner cells to one twin (Machin, 1996). Chromosomal mosaicism or aneuploidy, resulting from non-disjunction at the first cleavage division or later explains why numerous twin sets differentially inherit various monosomies and trisomies, and even mosaics (Machin, 1996). One twin in a pair might have to wait for the formation of a correct number of cells in its tissues, and sharing one placenta can be dangerous for a disadvantaged twin. A mother’s lifestyle and placental history could emphasize such differences or invoke new ones. These events could impose effects persisting throughout life (Machin, 1996), thus questioning beliefs that monozygotic twins are genetically identical in all tissues, and explain why neurological, immunological and structural aspects of twin development have a genetic and a non-genetic component (Hall, 1996).

Twinning is nevertheless less stressful compared with cloning. A need for de-differentiation is minimal, and stress should be slight during 2-cell separation, and not great even during incomplete hatching. Problems unique to monozygotic twins involve inequalities in dividing tissues during separation, and competition within the uterus. While these causes of discordance are absent in cloning, it is possible that variations between uterine environments will impose wider variations on singleton clones.

Genotypic and phenotypic variation among monozygotic twin pairs

Monozygotic twins are invaluable for analyses of human genetic and environmental variation, and as a model for inheritance in clones. Most twins share sources of post-natal variation since they are nurtured together. Others, separated at birth, develop in separate environments until reunited many years later, and provide invaluable data on environmental variation distinct from genetic effects.

Twin sets had been separated at an average age of 0.5 years among 146 twins reared apart (Bouchard et al., 1990a). Their numerous analyses have permitted heritability estimates of numerous human traits to be calculated, and only a sample can be considered here. Even though separate, genetic factors such as the choice of identical environments by separated twins could cause them to grow similarly, and such effects of sharing might be mistakenly classified as genetic. Similar factors may emerge among twins reared together (Bouchard et al., 1990a). This characteristic is termed genetic-environmental covariance, and could involve learning through experience. The Minnesota studies assume that any resemblances between twins reared apart are genetic, that assortive mating does not occur, genetic effects are additive (i.e. with no dominance or epistasis), and that genetic and environmental effects operate differently, i.e. without genotype/environment interactions.

The heritability ($h^2$) of psychological and behavioural traits between separated twins was 0.70, i.e. the genetic component was 70% of the total. This estimate did not include extreme cases since very few twins had been reared in great poverty (Bouchard et al., 1990a). Other multiple measures of personality, temperament, interests and social attitudes were similar whether twins were raised together or apart. The authors concluded that although personality and social attitudes such as traditionalism and religiosity could be influenced by parenting and environment, this does not happen in most families in Western societies (Wilson, 1983).

Two mental ability batteries, Hawaii and Comprehensive Ability, were used to measure verbal, spatial, perceptual speed and accuracy and memory domains among 146 twins reared apart (Bouchard et al., 1990b). Twin sets had been separated at an average age of 0.5 years.
and reunited at 32.6 years, and the chance of misclassifying monozygosity was <0.001. Intraclass correlations of several characteristics were 0.45 and 0.48 respectively for the two batteries of tests on monozygotic twins and 0.34 and 0.35 respectively for dizygotic twins. A biometric model indicated an average heritability of 0.50. Most sub-tests on the Hawaii Battery fitted the model, with spatial ability having the largest variability and visual memory the least. High heritabilities ($h^2 \geq 0.48$) were associated with card rotations, identical pictures, pedigrees, mental rotations and hidden patterns and lowest heritability with things, subtraction and multiplication, lines and dots and cubes. With the Comprehensive Ability Battery, most variables fitted the model, verbal ability had the highest genetic component, and genetic components were significant in characteristics other than word fluency, memory span and flexibility of closure. Variables with high $h^2 (>0.60)$ included verbal, spelling, word fluency, number space, and those with low $h^2 (<0.49)$ included memory span, flexibility of closure, speed of closure, perceptual speed, and meaningful and associative memory. Many variables with lowest heritabilities displayed non-significant or borderline $P$ values in tests for no genetic effects (Bouchard et al., 1990b). Comparisons between reared-apart and reared-together twin pairs for verbal, spatial, perceptual speed and accuracy, and memory tests highlighted shared environment as having an important role in determining some special mental abilities, but a minor importance in personality and cardiovascular domains among twin sets reared apart.

Genetic and environmental influences on motor learning measured in monozygotic twins reared apart (Fox et al., 1996), based on a rotary pursuit task, were fitted to a combined genetic and environmental model. A high heritability in performance increased with practice, and the rate of learning was also largely inherited. Acquisition of motor skills was strongly influenced by practice with feedback, although non-additive genetic effects affected the results. Practice thus decreased environmental variation dependent on previous learning and increased a role for inheritance of motor performance far more in monozygotic than in dizygotic twin pairs. This observation on reunited twins could be very relevant to clones gestated separately and then brought together.

As in animal cloning, the variation in human clones would largely depend on the heritability of each particular trait. Low heritability traits would be little affected by cloning. Much data is available concerning genetic inheritance in man. A few examples (see Table I), illustrate the wide differences in heritabilities resulting from a range of conditions from single point mutations and structural chromosomal anomalies to multifactorial inheritance and predominantly environmental factors. It is therefore essential to consider each feature individually. Facial features, for example, which play such an important role in social recognition, could be a trait of some interest with respect to cloning. The major components of the face are derived from cells migrating from the neural crest of the 2–3-week fetus and anomalies arise from external environmental factors such as alcohol, intra-uterine trauma and thalidimide, single gene mutations such as Treacher–Collins mandibulofacial dysostosis and multifactorial inheritance including cleft lip (with or without cleft palate) as shown in Table I (Kaye, 1981). Thus for a number of different traits it would be possible to predict the variation expected between clones as between monozygotic twins and, therefore, society should analyse and clarify the nature of its abhorence for the concept of cloning in humans.

### Table I. Wide differences in heritability ($h^2$) estimates for some chosen human conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>$h^2$</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendicitis</td>
<td>0.56</td>
<td>Polygenic or multifactorial model based on 80 families with histopathological acute appendicitis and matched surgical controls</td>
<td>Basta et al. (1990)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>0.3–0.4</td>
<td>Analysis of 45 monozygotic twins and 77 dizygotic twins</td>
<td>Holm et al. (1980)</td>
</tr>
<tr>
<td>Cleft lip</td>
<td>0.77–0.97</td>
<td>Comparison of Danish and Japanese populations</td>
<td>Chung et al. (1986)</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>0.91</td>
<td>Prominent history in two large families</td>
<td>Rotter et al. (1982)</td>
</tr>
<tr>
<td>Electroencephalogram</td>
<td>0.74 dominance</td>
<td>Average heritabilities of $\delta$, $\theta$, $\alpha$ and $\beta$ frequencies (76, 89, 89, 86% respectively)</td>
<td>Van Beijsterveldt et al. (1996)</td>
</tr>
</tbody>
</table>

Examples of discordances within monozygotic twin sets

Unusual differences and unusual similarities among twin sets often indicate de novo embryological events or rare genetic differences. Discordance for juvenile chronic myelogenous leukemia (JCML) in a twin set confirmed by DNA analyses arose through their joint inheritance of a de-novo deletion in the pericentric region of chromosome 7, a known cause of this disorder (Najfield et al., 1997). The more severely affected twin could have inherited more cells carrying a mutation arising before uneven splitting of the original embryo. Shared environments and common genetic factors of monozygotic twin pairs could explain similarities in within-pair spontaneous mutation frequency of hypoxanthine–guanine phosphoribosyltransferase (hprt) in circulating T-lymphocytes, when compared with the wider variations between pairs (Curry et al., 1997).

Environmental influences may evoke discordances. One twin in a monozygotic set checked by DNA analyses had hand abnormalities and hearing loss in oculo-oto-radial syndrome whereas the other had strabismus only, perhaps as a consequence of differing epigenetic or environmental factors (Elcioglu and Berry, 1997). Discordancies for isolated congenital complete heart block within two twin sets checked by DNA analyses, arose soon after birth in one twin and at 3 years in the other, despite their identical genetics and common environmental exposure to anti-Ro antibody (Cooley et al., 1997). Leptin concentrations were almost 4-fold higher in the obese twin among 23 discordant monozygotic twin pairs assessed for fasting concentrations of plasma leptin and distribution of visceral and subcutaneous fat (Ronnemaa et al., 1997). Intrapair differences correlated with corresponding variations in the percentage of body fat in women (r = 0.73, P = 0.003) but not in men, and with differences in visceral fat area in men and women, and had arisen independently of genetic background (Ronnemaa et al., 1997). Among 53 monozygotic twin pairs reared apart, with a mean body mass index (BMI) of 24.2 ± 4.7, heritability was 0.79 for all twins, 0.63 for Finnish twins in the sample, 0.73 for Japanese twins and 0.85 for ‘archival’ twins (Allison et al., 1996). One-half or more of the variance in heritability not accounted for by covariates was genetic. In two of 14 monozygotic twins sets discordant for endometriosis when adult, one twin reached stage II–IV of the disease whereas the other was unaffected (Hadfield et al., 1997); secondary effects such as uneven splitting could explain the discordance.

Discordances for characters displaying lateral asymmetry include handedness, which is well known to diverge in monozygotic pairs. More left than right handedness among 1616 monozygotic twins was independent of chorion type and zygosity (Derom et al., 1996). Families likely to have monozygotic twins display a tendency not to use the right hand. Machin (1996) considers that an abnormal blastogenesis could explain discordances for lateral body wall defects and other conditions.

Some genetic differences could arise between monozygotic twin pairs as in the X-linked condition, Aicardi syndrome, a condition involving agenesis of the corpus callosum, seizures and anomalies of the chorioretina. Slightly more methylated sequences flanking the Dxs225 locus in the afflicted twin of a discordant dichorionic pair may have arisen through a post-zygotic mutation and unequal distribution of mutated cells, skewed X inactivation, germ cell mosaicism or other X-linked disorders (Costa et al., 1997). Post-zygotic de-novo mutations within two monozygotic twin pairs may have caused discordancies for tuberous sclerosis and a form of muscular dystrophy (Brilliant et al., 1990; Tawil et al., 1993). One non-afflicted monozygotic twin in a 47 year old pair did not carry a familial BRCA1 mutation nor suffer from repeated cancers as did all other family members (Diez et al., 1997). The discordance could not be traced to age of menarche, contraceptive use, pregnancy, hormone therapies, smoking or drinking, environment, exercise, habits or diet. Discordance for the Schimmelpenning–Feuersstein–Mims syndrome (SFM) was attributed to a post-zygotic lethal mutation in one monozygotic twin who suffered from childhood epilepsy and developmental and mental retardation (Schworm et al., 1996).

Genetic differences involved higher concentrations of two cell recognition molecules (CRMs) and neural-cell adhesion molecule (N-CAM) and lower values of antigen L1 in cerebrospinal fluid in affected twins within monozygotic sets discordant for schizophrenia (Poltorak et al., 1997). N-CAM and L1 in cell membranes regulate cell/environment contacts essential for neural development and synapsis formation. Genomic mutations or imprinting might cause discordances preceding the onset of disease, or be associated with its pathological course.

A ‘non-shared environment’ can involve wide differences between children within a family as in members of the same pair among 93 monozygotic sets aged 10–18 years, chosen in order to relate the effects of shared and non-shared influences on children of the same genotype (Pike et al., 1996). Parental negativity and the twins’ anti-social behaviour were related perhaps by differences in parental treatment, although effects of twins behaviour on parents, or other factors, could not be excluded. Carried out on middle-class children, this study requires confirmation
by similar work on other classes, or on extrafamilial influences such as peers. A delay in talking and problems with relationships outside those with their twin characterize the difficulty of some monozygotic pairs in establishing their identity (Bryan, 1998). Length of gestation influences tempramental discordancy within discordant monozygotic pairs developing to full-term, but not those born pre-term (Riese, 1996). Pre-term twins also grow more alike in emotionality, and full-term discordant twins soon overcome adverse uterine influences on early behaviour.

According to Machin (1996), such considerable physical, emotional and psychological divergencies among monozygotic twin pairs imply that ‘identical’ should be replaced by ‘monozygotic’. Hall (1996) concurs, suggesting that many twin discordances arise through varying intrauterine environments, different cell allocations to each twin, different placental vascular supply and stochastic developmental differences. She also infers that some discordances may be actually cause twinning. The twinning process must occur independently of specific embryological characteristics if monozygotic sets are to be acceptable models of cloning. In other words, discordance within monozygotic pairs must be a consequence and not a cause of twinning. Although still theoretical, the formation of two or more cell clones in an inner cell mass could stimulate twinning or variations in the formation and vascularization of the placenta.

**How closely would cloned children resemble their parents and each other?**

This fundamental question can be answered only by evidence from human cloning. Animal studies provide some clues, since all eutherian mammals may share the same form of developmental genetic regulation (Edwards and Beard, 1997). Species differences in implantation, in organogenesis, and in numbers of gestated fetuses complicate comparisons with humans. Clones could vary if derived from nuclei of different tissues of the donor, with varying post-fertilization ages or differing numbers of previous cell divisions. At present, high frequencies of anomalies and intrauterine or post-natal deaths characterize animal clones produced from fetal or adult nuclei, unlike those derived from blastomere nuclei. Variations in growth may also be imposed by incomplete activation of cloned nuclei, differing components in recipient ooplasm, and genetic differences in maternal transcripts and mitochondria. Outcomes not unlike those of cloning characterize other micromanipulations on mammalian eggs, implying a common etiology of disorders due to stress or other underlying factors.

Human clones from the same donor are likely to vary considerably. They will have no ‘mother’ for gestation, and will grow in surrogates, as already happens for the gestation of IVF fetuses for commissioning couples, undoubtedly offering highly diverse uterine environments. Physiological variations within the uterus, caused by smoking, drinking, drugs and infections, will distort normal growth patterns in fetuses as reported in mothers taking cocaine (Rizk et al., 1997). The site of implantation, degree of vascularity and presence of structural or other disorders will also dictate the birth characteristics of clones, including variations in their multicigenic characteristics. Divergences in jawbone structure among newborn mice were initially ascribed to mutations induced by embryo cryopreservation, but are now suspected to be experimental or statistical artefacts (Testart, 1998). Disorders imposed on human embryos during uterine or post-partum life can affect the child and adult for years to come. Nor can there be much doubt that uterine and social environments for cloned children will differ from those experienced by their nuclear donor.

Monozygotic twins identify some of the variations likely to arise in clones. They are suitable models since most originate at the 1–64-cell stages (1–6 cleavages), which are the embryonic stages widely used for cloning. Embryo splitting might expose twin embryos to similar forms of stress as in cloning. Discordancies in genetic, developmental and psychological characteristics within monozygotic pairs must be caused by epigenesis or environment. Studies on monozygotic twins reared apart have proved extremely valuable, since their post-natal lives are not shared, so their degrees of environmental variation arising after birth can be calculated. Discordances within monozygotic pairs can lessen among those reunited at a later age, and among those who are discordant at birth becoming more concordant post-natally.

All this evidence suggests that clones will be at least as divergent as monozygotic twins, who are accepted without question in society. The one medical group to propose a reduction in monozygotic twinning by banning fertility drugs (Derom et al., 1993), did so on obstetric rather than psychological or behavioural grounds. Most twins live happily within a family circle, and many consider themselves highly fortunate in having such a close friend throughout life. Many dizygotic twins feel the same. Personality and other difficulties seem to stem partially from their environment within the family (Bryan, 1998), but none are of such an order as to question their existence or happiness.

Could clones be accepted in society, just like twins? Widely-quoted objectors stress that human personality must
be inherited absolutely freely and randomly, since it is a fundamental right never to be jeopardized by the whim of its parents. Yet society already imposes restrictions to prevent consanguinity and by restricting the number of births from sperm donors. Parents already choose particular characteristics in their children. Female fetuses are aborted or newborn girls killed in some societies for various complex reasons about the value of boys. Embryos are selected, and fetuses aborted, because they inherit a particular genetic trait. Indeed, science itself is demeaned by a familiar process, starting with the identification of a new disease gene, followed by the consequential abortion of fetuses carrying it. A well-known sperm bank (apparently in California!) stores spermatozoa from Nobel laureates, suggesting that both donors and recipients of such gametes approve this attempt to ‘improve’ the human race. What, then, is so special about cloning? An American lawyer, Robertson (1997), stresses that cloning is less intrusive than fetal gene therapy and that cloned children will be loved for themselves, have all rights of existing persons, be no more the property of the commissioning couple than any other child and will be unrecognizable from other children. Desires to clone emerge in forces as diverse as love for a recently-dead child or lust of power-hungry despoths to ensure their succession. The first example is a product of love, the second of power. Should a sterile couple be denied the right to clone a donor of their choice because of society’s fears and therefore be compelled to accept donor embryos unknown and genetically unrelated to either of them? Cloning could be desirable or objectionable, and perhaps more desirable if the children display differences wider than monozygotic twins.

Cloning from embryonic nuclei does not hold the same moral fears as adult cloning. It involves neither ‘carbon copies’ nor the many anomalies associated with fetal or adult nuclei. Cloning monozygotic twins from blastomeres provides an origin very similar to the origins of most naturally-occurring monozygotic twins. Viewed from this perspective, blastomere cloning offers a procedure to increase numbers of available embryos in infertility treatments without imposing a drastic departure from natural embryological events. Although limited to the use of inbred strains, possibly restricting epigenetic variation, a successful model already exists in mice (Kwon and Kono, 1996). Re-cloning existing blastomere clones, also highly successful in mice, is more problematic. It opens dangers of storing many cloned embryos while a few are transferred to test their social and personal attributes. Unwelcome aspects of cloning, such as this form of progeny testing, could be controlled by law, just as the number of offspring permitted from a semen sample is restricted legally.

Is the inheritance of a highly variable genetic trait due to gene recombination and mutation an inalienable and essential right that is threatened by cloning? Monozygotic twins do not think so. We have been taught throughout our lives that man (like animals and plants) is a product of a highly variable genetic inheritance, modified in an ever-changing environment. This has been true in the past, but need not be so in the future. Mankind’s gene pool and its variability has been based on family structures and infant survival rates, processes frozen by the spread of contraception, medical care and infertility treatments. New forms of variation could become essential one far-off day. The significance of variation as an essential evolutionary human characteristic has stimulated debate among geneticists, behaviourists and sociobiologists. We initially concurred with, but now question, a recent judgement about the importance of modern society from Bouchard et al., (1990a): ‘A human species whose members did not vary genetically with respect to significant cognitive and motivational attributes, and who were uniformly average by current standards, would have created a very different society to the one we know. Modern society not only augments the influence of genotype on behavioural variability…but permits this variability to reciprocally contribute to the rapid pace of cultural change’. Is this statement correct, and does it apply to societies with a high level of modern contraception? How significant is epigenesis to our quantitative inheritance and variation? Dolly seems to be healthy and happy, even if her birth was at the expense of an enormous mortality rate. Polly and recently-born calves carry genes inserted for human needs. Genes could be inserted to improve the life of the recipient, e.g. for a long life or to introduce new forms of variation. Such procedures could prove of enormous potential significance and value to future human generations, and cannot be dismissed too easily. Furthermore, fears of cloning for spare-parts surgery for donors could be unfounded, outdated by the clinical application of cell de-differentiation in vitro and reconstructive surgery. Within a few years, let us hope that desired cell lines will be available for self-grafting by de-differentiating cells taken from our own tissues.

Conclusions

Society always reacts unfavourably to new concepts, especially in reproduction. Fears of the unknown raised about contraception, IVF, embryo cryopreservation, surrogacy, embryo donation, genetic engineering and other matters have proved to be largely unfounded, and without earth-shattering consequences. Indeed, most of them have already increased the overall sum of human happiness and are now practised
worldwide. Sadly, recent consultative documents from various European authorities revert to an earlier and more restrictive age (Shenfield, 1998). Moral and philosophical strictures against cloning are raised again, as a threat to uniqueness and ‘autonomy and dignity’ of each human being which creates an ‘intolerable lowering of a person to the status of an object’ and introduces ‘new kinds of slavery’ by ‘the creation of human varieties’ (Boué, 1998). The same thing was said about contraception, abortion and IVF. What scientific rationale lies behind such statements? Hottois (1998), a philosopher himself, seems to be highly unimpressed on philosophical grounds by these arguments. Parents often interfere with the fundamental interests of their children. Their self-interest can often outweigh those of the child during adoption, genetic diagnosis of embryos and fetuses, gamete donation and surrogacy. Only one progressive European organization, the European Society of Human Reproduction and Embryology, (ESHRE), dissented from general criticisms of cloning as it appealed to members to delay attempting it for 5 years until its ethical and biological implications were clearer (ESHRE, 1998). We agree with this stance, and with a US approach requesting a 5 year ban and a re-evaluation at the end of this time (National Bioethics Advisory Commission, 1997). They wrote that ‘More speculative psychological harms to the child, and effects on the moral, religious and cultural values of society may be enough to justify continued prohibitions in the future, but more time is needed for discussion and evaluation of these concerns’.

We suggest that human cloning is premature, until animal studies reveal its potential and disadvantages, and until variance among clones is assessed in detail. Before cloning is accepted, it would be more important to know much more about epigenesis as a source of human variation. A delay of 5 years should be sufficient for some decisions on cloning, e.g. whether to duplicate 2- or 4-cell embryos to produce monozygotic twins for sterile couples with only a single embryo in vitro. We find no scientific reasons for an outright ban, nor any rational basis for some of the objections. We welcome an open approach to clarify potential benefits to particular individuals and couples, and look forward to understanding the long-term effects of cloning.

References


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How identical would cloned children be?  


