Theory and praxis of cloning via nuclear transfer: ‘Never say never’ or ‘mission impossible’?

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Introduction

The announced death of Dolly the sheep has brought back the attention of the specialists as well as of the laymen to the problems of the transfer of somatic nuclei into enucleated oocytes, also known as reproductive cloning (NAS, 2002).

In biology, even after the sequencing of hundreds of genomes, including our own, empiricism overwhelms theory, dogmas prevail on principles. This has been remarked with unchallengeable reliability by F. Crick, Ph. D. in physics and Nobel laureate in medicine as co-discoverer of the DNA double helix (Crick, 1988). Perhaps that is why biologists often wriggle out of challenging problems stating ‘Never say never!’. Like James Bond. Or the Raelians. That may also be why the hoaxes about Clonaid’s as well as others’ cloned babies have not been flatly ignored, as probably it should have been done, especially by scientists. Had they elected to do so, they would have enacted the best defence not of the alleged clones, since they do not exist, and anyhow they would be a matter to be left to the law. Had they done so, they would have afforded a due protection to science, the major victim of the cloning paranoia culminated at the end of 2002 with the unfounded announcement of baby Eve, the clone which once more shook the world (after that of 1997 (Wilmut and Griffin, 1997)).

In science not just everything does happen: what violates scientific laws does not. This is true in physics (take the perpetual motion), in chemistry (the transmutation of metals in gold): is this true also in biology?

Adult human cloning may well be the biological equivalent of the perpetual motion, or the philosopher’s stone. Indeed we have heard human cloning frequently announced by thoroughly unpredict-

able and often unreliable quarters. Admittedly cloning could be attempted by almost anybody, even if, compared to announcements, the experiments would be more demanding, in terms of equipment and competence. Finally its ethical implications have been debated *ad nauseam*. Rarely as in the case of cloning, visibility has paved the way to credibility.

Here we shall argue that the creation of even one identical copy of an adult human being is impossible to achieve, is a ‘never!’ issue. And this is based on both empirical and theoretical reasons.

In the last seven years scientific journals of high standard have been enthusiastically promoting animal cloning: it was even elected as the scientific breakthrough of 1997 (Science, 1997). But the quality of most of the published papers has constantly been embarrassingly poor. The same journals which in the past have vehemently exalted it, recently have changed position, and furthermore have found themselves involved in a row of scandals (e. g., the Bell Laboratories mishap). The flaws of the extant reviewing and publishing systems are hardly deniable, but they have been superciliously refused (Anonymous, 2002). In this field there are corrections, additions, retractions, responses to challenges (Sgaramella and Zinder, 1998) signed by a minority of the authors of the challenged paper (Campbell et al., 1998), ‘sworn’ supporting statements meant to accredit witnesses and controls otherwise inconclusive (Solter, 1998; Ashworth et al., 1998; Signer et al., 1998; Shiels et al. 1999; Evans et al., 1999), by far more than in any other field of science. This denounces an underlying weakness of the ‘art’.

In an area where outstanding biomedical developments are being unyieldingly heralded, it is important to assess if there is any substance in the purported achievements beyond the chauvinistic support by

some scientific journals and the sensationalism of most mass media. We ought to understand whether the transfer of a somatic nucleus in an oocyte may be equated to the transfer of naked DNA in bacterial transformation, where the uptake by the host organism of the relevant sequences (indeed in a vast excess of copies over the recipient genome) elicits the appearance of the relevant phenotype; and, as a consequence, whether adult reproductive cloning is possible through nuclear transfer into oocytes. Last but not least whether therapeutic cloning aimed at the production of organs, is practicable (NAS, 2002).

Too many purported achievements and inflated projections keep being disavowed by a steadily increasing body of empirical evidence. It may be time to assess the value of underlying theories, if any. Here I am proposing that cloning via nuclear transfer into enucleated oocytes is at odds with at least two general principles that are gaining momentum in genetics.

Zygote-soma genomic discontinuities

Genomic changes, scheduled or not, reversible or not, steadily accompany the genome of the fertilized egg (the zygote) through differentiation, disease, senescence. In the somatic cells of adults, the genomic DNA base sequences are not photocopies of those of the originating zygote, and differ from each others.

DNA-gene differences

The preservation of the same base sequence in specific positions of the genome may not mean the preservation of the same function. If this is true it follows that not every coding DNA is a gene. Genes are portions of DNA that are either expressed or silenced through interactions with several and diverse cell components, often different from one type of cell to another, from one growth stage to another, from one sex to the other. Accordingly a stretch of DNA acts as a gene in one kind of cell (say, liver) and not in another (kidney); in one life phase (lactation) and not in another (pregnancy). Genes are specific DNA sequences that work through association with specific ligands. They are complexes made up mostly by nucleic acids and proteins. The resulting interactions elicit a reproducible modulation of gene expression, and perhaps are just too complex to be harmlessly severed at cloners' command.

The remodelling of a somatic genome backward to the zygotic status, or sideways to a different cell lineage, as demanded by either goal of cloning, may thus be exceedingly difficult. Two non-exclusive reasons may be invoked. First, the somatic genomes may be discontinuous versus the zygotic one: so the specific base sequence of a gene may be different in different cells. Second, the oocyte cytoplasm may be inadequate for the effective remodelling of the somatic chromosomes: so the activation of potential genes is impaired. In most protocols, cloning via transfer of a somatic nucleus into an oocyte requires that this is previously stripped of its own nucleus: enucleation can be traumatic per se, and also for what it removes. A critical portion of the material (cytoplasm) surrounding the nucleus may be accidentally taken away with it. This may compromise the correct remodelling and the ensuing proper functioning of the transferred nucleus.

In this regard, it is of interest to note that also the other cloning method, the splitting of multicellular embryos in sub-embryonic particles each made up of several blastomeres, is distressingly inefficient. In the early phases of natural development, as the fertilized oocyte steadily divides into a multicellular embryo, all the daughter cells contain a semi-conservative copy of the zygotic DNA, originally contributed half by the maternal and half by the paternal germ cells. Compared to the zygote, the DNA of each embryo cell is similar in quantity, most probably but not necessarily, identical in quality (see point 1, above). Problems may also arise from the cytoplasm component of the early embryo cells, the blastomeres. This may be explained as follows. The zygote has an exceptionally large amount of cytoplasm: it is the *viaticum* endowed by the mother to the oocyte, by far the largest of the mammalian body. The paternal contribution, the sperm, is negligible in term of quantity of cytoplasm, but certainly not in term of importance of the nucleus and its genomic content, fully comparable to the maternal counterpart. The development of the zygote initiates after fertilisation: its single cell divides into two 'halves' (identical in size but not quite so as to cytoplasm content). Concomitantly, the fully duplicated and properly reassorted genomes segregate in each daughter cell as allegedly faithful copies of the zygotic genome. In due time the daughter cells will enact a similar process, and so on: the approximately five-six cycles of cell divisions will form the hundred plus cells constituting the blastocyst. This represents the first instance of cellular differentiation: trophectoderm at the outside, inner cell mass internally. The

embryo proper will develop from the latter. In the successive fifty or so division cycles several thousand billions cells will appear: they are us, with our two hundred plus different kinds of cells. Throughout the early embryonic phase, smaller and smaller parts of the oocyte cytoplasm end up in each blastomere: eventually these will contain less than one hundredth of the original zygotic cytoplasm endowment, given the absence of any synthesis and/or uptake during embryogenesis. Conversely the DNA content will remain constant in each new cell thanks to its replication at each cell division. Only at the end of the embryonic stage the cells will start incorporating food, and thus replenish their endowment of cytoplasm so that they can divide without halving it, but differentiating it from tissue to tissue, and possibly growing the oocyte. In the whole process the genomes steadily replicate and thus accumulate discontinuities, as from point 1 above.

During embryogenesis, the interactions between cytoplasm and DNA, the positionally and compositionally unequal allocations of the former and the discontinuities of the latter, will cause different interactions to occur between the partners, diverse genes to be switched on and off. This in turn contributes to determine the formation of the different cell lineages, the various tissues and the organs of our body.

In each cell a complicated choreography is staged between a changing DNA and the interacting cellular compounds. This is now better known as 'epigenetics'. It affects inter- and intra-generational discontinuities and sanctions the differences between any given DNA portion and its corresponding, eventual 'gene' in the various cell lineages.

Introduced by Waddington (1940), epigenetics has just been revisited by Lederberg (2001), who stated that 'epigenetic information regulates the manifestation of nucleic acids' potentialities in development'. In so doing it must steadily erode and may eventually erase the original zygotic totipotency from mature body cells. The cloners' dream to rescue from them the plan for the A-to-Z creation of an organism, may be unattainable.

Totipotency is a quantitative trait, borne by 'nucleic, epinucleic and extranucleic' structures (Lederberg, 2001). Perhaps it is fully present only in the zygote, and fades away with different rates in different cells at different stages. In adults there are some (stem) cells competent for tissue regeneration; just a few are so potent as to reprogram an embryo; even less retain enough of it to develop a fetus; very rare

are those eventually capable of leading to adults, especially among primates. This explains why animal cloning is occasionally possible, but constantly inefficient. If and when it works, clones are few, sick, different from each other and from the donor. The recent suppression of Dolly has highlighted the issue of the poor health of most clones.

The present cloning crisis asks for an urgent re-examination of epigenetics, because of its steady removal of the original zygotic totipotency from the somatic cells as they are shaped in the course of development.

The potency of any cell is a peculiar quantitative trait, perhaps fully present as totipotency only in the zygote and then fading away in the progeny cells, with different rates in different tissues, as the organism ages, only to come back regenerated through sexual reproduction. This may explain how we succeed occasionally at cloning animals, only to discover that the laboriously produced clones are a flop. That is so because in adults there may be just a few cells which retain genetic information adequate to program back an embryo; those which retain enough information to develop a pseudo-zygote into a fetus, are probably less; an even fewer are those capable of leading the development of an organism all the way to adulthood. Some of the body cells, such as lymphocytes, are scheduled to contain genomic rearrangements that are part of an ontogenic programme (Panelli et al., in preparation). They may appear also in the genome of most cells of the resulting clone(s), if any, variably affecting their viability (Wakayama and Yanagimachi, 2001). This suggests that totipotency does not require a donor nucleus be identical to the original zygotic one: for sure, the closer they are, the better it is. If the somatic genomes have diverged, some clones may still issue from their transfer: they are likely to be rare, dysfunctional and different from the donor. The complexity of the phenomena concurring to the success of cloning is demonstrated by the reports on its independence on whether the transferred nucleus came from an embryo, a fetus or a grown-up (Wilmut et al., 1997): but who on earth would be interested in cloning an embryo or a fetus? Does all this mean 'never!' for clones and for stem cells?

Probably no. But certainly it means that reproductive cloning is impracticable with animals. Given the current yields, the popular slogan 'a flock of clones' coined to celebrate the appearance of one individual sheep genetically identical to the donor of the some 434 udder cells (Wilmut et al., 1997), may have to be

replaced by another dictum: 'e pluribus unum', even more popular, since it appears in most USA bills and coins.

Also with humans the issue of no-better-qualified clones via nuclear transfer is technically possible. Conversely the production of adult individuals genetically identical to a pre-existing adult is hard. In humans, the identity between donor and clone should extend beyond mere physical traits. If so, then one has to add the irretrievable contributions of nurture, that is to say of biography, affecting the biology itself of the individual and probably dwarfing the contributions brought about by nature. If cloning a human adult means creating an identical copy of the donor of a somatic cell, then cloning can be safely defined a 'mission impossible' for everybody. Apart from the Raelians, of course.

Science may accept vetoes (if posed by science itself), but must treasure exceptions. It is well-known that plants can be cloned successfully from an adult cell. Explanations for this are tentative: among them the lower number of tissues, close to one tenth of those of mammals, and the peculiarities of soma-germ alternation in plants *versus* animals.

Along this line it is also well-known that animals produce offspring which resemble very closely their parents: how is this, in view of the above? In nature animals reproduce sexually and species do change, albeit very slowly. It is indeed thanks to sex that newborns receive from their parents the most protected and integer (and thus most functional) genomes they could possibly start with, in spite of the discontinuities undergone by both parental genomes. This beneficial legacy occurs because parents patch up the variously battered portions of their genomes through recombination. The resulting genomes are packaged in germ cells, and the coupling of two such cells of different genders produces an offspring. Its quality depends on that of the two nuclei contributed by the merging germ cells, thus on their genomes. Sex is a great evolutionary success not so much because it helps biodiversity, or provides fun: these features enjoy wide circulation, but are just by-products. The prior function of sex is seeing that the new generations receive the best repaired genomes from both parents (Michod, 1995). Still the rate of success of sexual reproduction is low: about 20% of the naturally conceived zygotes make it to birth. In cloning this protection is lost, and thus the rate of success is much lower (less than 1% of the attempted couplings of somatic nucleus to enucleated oocyte (NAS, 2002)).

Finally, a few words are due on the most recent development of nuclear transfer. Since it is now being eagerly scrutinized also as a means to produce embryonic stem cells, we should try to understand whether the caveats plaguing reproductive cloning may also affect its 'therapeutic' version. Here the remodelling of the somatic genome would be no longer all the way back to square zero, to the zygotic status, as in reproductive cloning, but rather sideways, to a different somatic lineage. Compared to starting a new embryo, conceptually this is slightly less heretic and technically slightly less demanding. But morally it is not going unnoticed. Indeed it is the target of the analysis by the most supercilious ostracism of some bioethicists.

The point is that also this cloning is mediated by the transfer of a somatic nucleus into an enucleated oocyte and thus it is impregnated with the ambiguous fascination of a reproductive act. Therapeutic cloning is being characterized by a goal (an organ), different from that of reproductive cloning (an organism), but this is seen as almost irrelevant. The stigmas affecting the latter are heavy even if it were unable to recreate a functional pseudo-zygote or if its efficiency were close to zero. Paradoxical but not entirely trivial would be a position maintaining that if the 'never!' clause were applicable to reproductive cloning (it cannot produce a living individual), then its therapeutic version would be freed of most of the rigid ethical constraints now hindering its acceptance. Experimentation could be facilitated and may help to check whether clonal embryo stem cells really match the optimism now surrounding them (a critical analysis of the problem is presented in the accompanying paper by Borge and Evers).

Still, given the importance attributed to embryo stem cell research, one wonders whether in these crucial experimental stages one could use other stem cells donors, possibly better than cloned embryos. This point is of a particular importance since in reproductive cloning the correct functioning of the whole process is demonstrated by an unequivocal signal: the healthy survival of the clone. If that is sub-optimal, the clone is counterselected and doomed to illness or death. Conversely in the therapeutic version, some cells of the cloned embryo, as issued from nuclear transfer, are amplified in vitro and then injected by the millions in the patient, with both the legitimate expectation of no antigenic reaction and the appraisable hope to fix a disease. Also in view of the practical and conceptual problems (not entirely unpredictable since the early times) now plaguing genetic therapy

(Fisher et al., 2002; Kaiser, 2003) (where 'safe' genes are borne by 'disarmed' vectors, but more often than desirable cause problems, tumours and death), it is mandatory to check preliminarily whether stem cells, derived from embryos cloned via nuclear transfer, at least do not come with predictable and assessable dangers, stemming from genomic discontinuities.

At this stage it is unavoidable to bring in another serious problem: the fate of the spare IVF embryos. It is known that the fertility clinics around the world host millions of them, either leftovers from successful interventions or of substandard quality. One way out is to forget those already there, and either create only those which can be reasonably implanted (generally three), or go on with the excess of in vitro fertilizations, and spare embryos. The point is that there are spare embryos, now: they are all bound either to be left in the cold or to be destroyed by the fire. This contrasts vehemently against a different option: those embryos may help mankind by helping biomedical research. If they were considered abortions, they could be entrusted by informed parents to internationally recognized health organizations. These could act as centres for the distribution of stem cells and for the promotion of a global effort to understand better the therapeutic potentials and the bioethical qualms of regenerative medicine.

Conclusion

De-differentiation and trans-differentiation are complex and poorly understood cellular phenomena. Their control in a purposeful way is not an easy task. It is proposed here that there may be obstacles to the important scientific, biomedical and biotechnological goals linked to them. These may be both technical and theoretical, stemming from discontinuities between the zygotic and the somatic genomes, and the subtleties of the regulatory interactions involved in gene expression. Hundred years ago Haeckel proposed a 'biogenetic law', stating that ontogeny recapitulates phylogeny (Haeckel, 1900). Even if it was subsequently shown to be of limited value, it may be appropriate to rescue it from the dust and reconsider it for some suggestive aspects. Thus it may have some merit in the current appraisal of the commonalities between evolution and development (Wilkins, 2001). In these days where manipulations are the rule, nobody would reasonably work for reversing evolution. It may be equally difficult, if not plainly

impossible, to obtain the reversal of the developmental program of a fully differentiated cell and reproduce an identical copy of the adult human individual who initially donated the genome, especially if he/she were endowed of outstanding multifactorial features.

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