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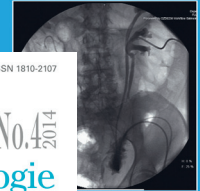**Journal für Reproduktions-medizin und Endokrinologie**
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Mammalian Cloning and its Discussion on Applications in Medicine

K. Illmensee

This review article summarizes the current state of the art concerning mammalian cloning and embryo biotechnology with its far-reaching consequences for multiple applications in reproductive and therapeutic medicine. It presents updated information on basic research to further the understanding of nuclear and cytoplasmic interactions required for genomic alterations from adult to embryo gene expression during cloning. Risk factors for reproductive cloning have been included for serious considerations. This article contains most recent developments in the field of interspecies cloning for embryonic stem (ES) cell research, human embryo cloning for reproductive purposes and for establishing patient-specific ES cells for future autologous transplantation. It also describes novel attempts for embryo splitting with its various implications for assisted reproduction, embryo cryopreservation, ES cell production, embryo sexing and preimplantation genetic diagnosis (PGD).

Predictive progress and prognostic views for mammalian cloning and patient benefits have been outlined for the near future. Furthermore, social and ethical issues concerning mammalian cloning have been discussed in reference to public opinion poll (APART) or ethics committee guidelines (ASRM).

Key words: somatic cell nuclear transfer (SCNT), reproductive, therapeutic and interspecies cloning, embryonic stem (ES) cells, embryo splitting, PCR and DNA analysis.

Säuger-Klonen – Diskussion hinsichtlich der medizinischen Applikation. Dieser Übersichtsartikel faßt den gegenwärtigen Stand der Erkenntnisse auf dem Gebiet von Säuger-Klonen und Embryo-Biotechnologie mit weitreichenden Konsequenzen für zahlreiche Anwendungsmöglichkeiten in der reproduktiven und therapeutischen Medizin zusammen. Er beinhaltet aktuelle Information zur weiteren Erkenntnis über Kern- und Zytoplasma-Wechselwirkungen, die für genomische Veränderungen von der adulten zur embryonalen Genexpression beim Klonen von fundamentaler Bedeutung sind. Risikofaktoren beim reproduktiven Klonen werden in Betracht gezogen. Dieser Artikel befaßt sich mit neuen Entwicklungen auf dem Gebiet von Interspecies-Klonen für embryonale (ES) Stammzellenforschung, von humanem Embryo-Klonen für die Reproduktion und zur Etablierung von patientenspezifischen ES-Zellen für zukünftige autologe Transplantation. Neue Ansätze für Embryo-Teilung mit verschiedenen Implikationen für die assistierte Reproduktion, Embryo-Kryokonservierung, ES-Zellproduktion, Embryo-Geschlechtsbestimmung und Präimplantations-Gendiagnostik (PGD) werden aktuell zusammengefaßt.

Prädiktiver Fortschritt und prognostischer Ausblick von Säuger-Klonen und Patientenbenefit werden für die nahe Zukunft aufgezeigt. Bezüglich sozialer und ethischer Überlegungen zum Säuger-Klonen sind öffentliche Umfragen (APART) und Ethik-Kommissions-Richtlinien (ASRM) mit einbezogen worden. **J Reproduktionsmed Endokrinol 2007; 4 (1): 6–16.**

Schlüsselwörter: somatischer Zellkern-Transfer (SCNT), reproduktives, therapeutisches und Interspecies-Klonen, embryonale (ES) Stammzellen, Embryoteilung, PCR- und DNA-Analyse

The term "clone" originates from the Greek expression for multiplication of genomically identical cells by non-sexual reproduction. In mammals, cloning requires oocytes, since so far, these germ cells represent a developmental stage to ensure the origin of mammalian life. The nuclear genome can be removed from these cells, thus being "enucleated" by microsurgical procedure. What remains is a "cytoplast" into which the nucleus from an embryonic or adult cell could be transferred via fusion and activation methods. Such cloning procedure via somatic cell nuclear transfer (SCNT) can be achieved several times by creating multiple, genomically identical siblings. SCNT-reconstructed oocytes with donor-cell genomes can be cultured in vitro for several days. The resulting cloned embryos can be transferred into the uterus of surrogate females giving birth to offspring which are genomically identical with the original donor-cell nuclei, but mainly are different concerning their mitochondrial (mt) DNA.

Reproductive Cloning in Animals

Reproductive cloning in mammals can be traced back to 1981 when cloning experiments were successfully performed in mice using embryonic cell nuclei from the inner cell mass (ICM) of blastocysts for their transplantation into enucleated egg cells [1]. At that time, this achievement sparked a worldwide dispute with arguments for and against cloning. In 1984, further experiments on cloning mice with embryonic cells as nuclear donors gave negative results. For this reason, the authors argued against the feasibility of cloning mammals via nuclear transplantation, concluded that "the cloning of mammals by simple nuclear transfer is biologically impossible" and hypothesized that "the inability of cell nuclei from these stages (i. e., preimplantation) to support development reflects rapid loss of totipotency" [2]. These negative findings influenced profoundly and stagnated temporarily further progress in the field of reproductive cloning. "Many people were deterred by this statement from pursuing mammalian cloning further" [3]. In the meantime and under different cloning conditions, Japanese researchers clearly documented that, indeed, ICM cell nuclei are suitable to promote full-term development of cloned mice [4]. In 1986, cloning technology was successfully established in farm animals and cloned sheep were created from embryonic cell nuclei transferred into enucleated oocytes [5]. During the next 10 years, cloning technology was further developed and extended to the pig [6], goat [7], bovine [8] and rhesus monkey [9], but always using embryonic cells as nuclear donors for cloning.

In 1997, for the first time, adult donor cells were employed for successful cloning of the sheep "Dolly" [10]. Subsequently in 1998, eight calves were cloned from adult cells of a single donor animal [11]. Analogously, cloned mice were created via SCNT using adult ovarian cumulus cells as nuclear donors [12]. In 2000, for the first time, cloned calves were produced from adult fibroblast cells after their long-term culture in vitro [13]. Several cloned

calves of female and male genotype were also generated from a variety of different adult cell types [14]. In addition, cloning technology has recently been extended and applied to domestic animals such as the cat [15] and dog [16]. Most importantly, reproductive cloning has been considered as a potential conservation strategy for maintaining endangered species and rescuing them from extinction [17].

Concerning primate cloning with adult somatic donor cells, and particularly relevant for the rhesus monkey model, American researchers recently reported the failure to obtain pregnancy from such primate-cloned embryos [18]. From their observations they concluded that "primate NT (nuclear transfer) appears to be challenged by stricter molecular requirements for mitotic spindle assembly than in other mammals" [18]. Since there is no further detailed information on plausible intrinsic biological or species-specific factors responsible for these negative findings on rhesus monkey cloning, reasonable conclusions should not be currently drawn and extrapolated to other primates.

The current success rates (between 2 to 20 %) for obtaining adult mammalian clones derived from SCNT with adult donor cells remain rather limited. Several still unknown cellular mechanisms that may contribute to such low success rates in reproductive cloning are under intense scientific analysis. Some of the key investigations focus on nucleocytoplasmic interactions and reprogramming of the transferred somatic cell nucleus by the cytoplasm of the recipient oocyte [19], chromatin remodelling of the somatic nucleus by oocyte factors [20], rebuilding of telomere length of somatic chromosomes [21] or cell-cycle coordination between nucleus and cytoplasm during the cloning procedure [22]. Cloning efficiency may be further improved by increasing biological uniformity between recipient oocytes and donor cells and by establishing selection techniques for functional reprogramming of the nuclear donor cell genome [23]. It was reported that following removal of different amounts of cytoplasm during enucleation of bovine oocytes, the volume of extracted ooplasm approximately equivalent to the volume of the somatic donor cells gave the best cloning results [24]. It was also shown that embryonic and somatic donor-cell nuclei used for SCNT in enucleated bovine oocytes require a different ooplasmic environment for organic reprogramming, most likely due to their different cell cycles and differentiation profiles [25]. Similarly, different treatments for oocyte activation seem to significantly influence the outcome of SCNT [25, 26].

Research has also focused on possible biological effects of mitochondrial contributions from both cell types (oocyte and donor cell) to cloned mammals. Since such reconstructed oocytes can transmit both oocyte-specific and donor cell-specific mtDNA to cloned offspring, transmission of two different populations of mtDNA could have biological consequences to offspring development and survival [27]. In sheep, mtDNA only from the recipient-oocyte type but not from the donor-cell type could be detected in cloned offspring, leading to mitochondrial homoplasmy [28]. In cattle, on the other hand, donor cell-specific mtDNA contributions between 0,4 to 4 % were found in healthy cloned calves, demonstrating that mitochondrial heteroplasmy is not necessarily preventing normal clonal development [29]. In a recent study using adult ovarian cumulus cells as nuclear donors for SCNT, some cloned healthy calves exhibited considerable mitochondrial heteroplasmy with 4 to 60 % mtDNA from the donor-cell type [30].

Risk Factors for Animal Cloning

In livestock, culture conditions have been shown to profoundly influence the development of normal (IVF) and cloned embryos during growth in vitro. After their intrauterine transfer, some of them developed into large-sized fetuses giving rise to large offspring (LO) [31, 32]. This LO syndrome [33] as well as other organic anomalies and postnatal malformations have turned out to be of major concern for farm animal IVF and cloning [34]. Scottish researchers reported that sheep fetuses with the LO syndrome, when compared with normal fetuses, expressed low levels of IGF2R, a multifunctional protein that is also involved in fetal organogenesis. This low protein expression is concomitantly associated with reduced methylation of the corresponding gene locus [35]. It has been argued that epigenetic changes in DNA methylation during embryogenesis could be responsible for the observed developmental defects. American researchers have recently shown that in artiodactyls (i. e. sheep, goat, cattle, pig) only the maternally inherited allele for the IGF2R gene locus is expressed, whereas in primates both paternal alleles remain active, most likely as a result of evolutionary processes [36]. Such species-specific differences in genomic imprinting may explain to some extent the observed differences in embryo culture between artiodactyls and primates. Whereas in farm animals a large proportion of in-vitro cultured embryos (irrespective of IVF- or clonally-derived) develop into abnormal fetuses and offspring, a significantly elevated percentage of malformations has not been observed in human assisted reproduction when compared to new-born from normal conception.

Several risk factors for reproductive cloning have been discussed and proposed for intensive investigation [37]. Not only epigenetic alterations in methylation of genes, but possibly also point mutations and changes in the structure of chromosomes can be envisaged as critical [38]. Unpredictable processes and multiple factors involved in DNA methylation, chromatin remodelling, genomic imprinting, telomere-size alteration of chromosomal ends and epigenetic inheritance profoundly influence nuclear reprogramming and the outcome of animal cloning [39]. In the sheep system, cloning research dealing with the investigation on chromosomal telomere size of different late-passage cultured cell lines has shown that re-established cell lines from cloned fetuses and sheep exposed the same telomere size and proliferative capacity as the original cell lines used for SCNT cloning. The authors therefore concluded that the proliferative lifespan is conserved after nuclear transfer and suggested that these are genetically determined properties inherent to the somatic cell genome [40]. In the cattle system, on the other hand, restoration of full lengths of chromosomal telomeres was documented in cloned calves derived from early-passage-cultured SCNT-donor cells [41]. This discrepancy may have its origin in the use of late-passage versus early-passage cell lines for SCNT in sheep and cattle, respectively. Japanese researchers reported that remarkable differences in telomere lengths can be observed among cloned cattle derived from different donor cell lines [42]. Further cloning experiments on this

subject of telomeric extension and restoration by exposing donor-cell nuclei to the telomerase pool of recipient oocytes are therefore desirable to advance our knowledge about aging and rejuvenating processes in cultured SCNT-donor cells and cloned animals.

As another issue of concern for reproductive cloning it has been pointed out that the short period of time for somatic donor-cell nuclei to be properly reprogrammed in cytoplasm of recipient oocytes may not be sufficient enough to initiate and promote normal embryogenesis. Cloning efficiency in goat [43] and cattle [44] can be improved by exposing donor nuclei from early cells (blastomeres) of a cloned embryo again to the cytoplasm of recipient oocytes during a second recloning step, thus prolongating the exposure time for genomic remodelling. By applying such serial (double) cloning techniques, Zhang and Li [43] reported the birth of 45 healthy goats originating from 141 double-cloned embryos and documented an impressive improvement via double cloning when compared to the still poor results obtained via conventional cloning.

Cloning in Reproductive Medicine

Whether complex and far-reaching embryobiotechnology (fig. 1) will be partially and successfully applied in human reproductive medicine depends on scientific progress and social acceptance [45]. In 2001, an opinion poll among medical practitioners and members of APART (international association of assisted reproductive technology centers) revealed that three quarters of them would be willing to provide human cloning for patients in clinically indicated cases [46]. Zavos and co-workers reported on a cloned human embryo for reproductive purposes via heterologous SCNT using an enucleated human oocyte that was fused with an adult ovarian granulosa cell from a donor patient [47]. This cloned embryo developed to the 4-cell stage and was subsequently transferred into the patient's uterus, but hCG levels showed a negative result for pregnancy [48].

Throughout the world, human reproductive cloning has again sparked a dispute for and against its application in medicine [49, 50]. Some crucial questions concerning reproductive cloning have already been answered in the animal model systems, but other imperfections linked to reproductive SCNT remain to be solved [51]. Some of the central questions focus on ethical issues and the impact on society and family. Reproductive cloning may be envisaged for infertile couples with a male partner totally devoid of any germ cells. For these couples, it could be desirable to conceive a child with the genes of at least one partner, if they have strict reservations about using donor gametes. In such cases, "reproductive SCNT would meet an infertile couple's desire to participate biologically in the development of a new human being" [52]. Other im-

portant questions relevant to reproductive SCNT are closely linked to yet unsolved imperfections and risk factors in cloning procedures. We also share proper respects to these biological and medical concerns. A currently negative position on possible applications of reproductive SCNT in humans is intimately and most likely connected to the limiting cloning results obtained in farm animals. Rhind and co-workers [53] proposed that further insights into the biological mechanisms and improvement of SCNT technologies are required as essential prerequisites so that, eventually, reproductive cloning can be safely applied in humans.

Recently, we transferred a cloned human embryo for an infertile couple [48]. The male patient was suffering from azoospermia and complete inability to produce sperm due to non-descended (cryptorchid) testicles. The couple was provided with full explanation of all the procedures to be performed and only after having obtained their consent and being approved by the Institutional Review Board (IRB) of REPROGEN Ltd., SCNT procedures were carried out. Skin tissue was harvested from the infertile husband and was allowed to grow in vitro to establish primary fibroblast cultures. Following hormonal hyperstimulation of the patient's wife and oocyte retrieval, three collected oocytes were used for SCNT and fused with the male patient's fibroblast cells. One resulting cloned embryo developed further in culture for 60 hours post-SCNT and reached the 4-cell stage. It was composed of four equally-sized blastomeres showing regular cytoplasmic morphology with very few tiny fragments at the site of microsurgical operation. This embryo was approximately 12 hrs behind the regular time schedule of embryos obtained from IVF or ICSI procedures. This may have resulted from initial adaptation of the somatic cell nucleus within the cytoplasm of such SCNT-reconstructed oocyte that can lead to delayed nuclear reprogramming during initial embryogenesis of cloned embryos [54]. Based upon its high morphological quality, the cloned embryo was subsequently transferred into the patient's uterus without applying any invasive testing. Moreover, the couple did not wish to pursue any invasive analyses that could damage the embryo because of their own religious beliefs. The patient's luteal phase was supported via progesterone injections for two weeks following embryo transfer, after which the blood β-hCG levels showed a negative pregnancy result [48]. Even though no pregnancy was established in this case, we showed and documented for the first time that human reproduction via

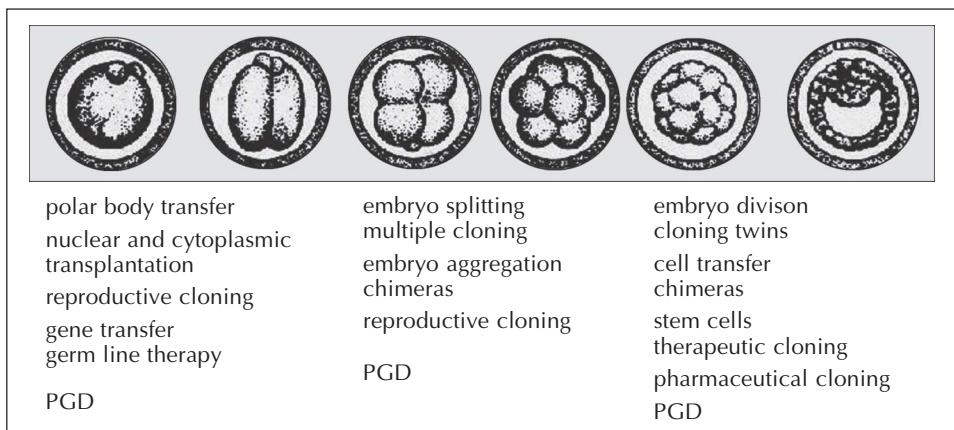| polar body transfer | embryo splitting multiple cloning | embryo divison cloning twins |
nuclear and cytoplasmic transplantation	embryo aggregation chimeras	cell transfer chimeras
reproductive cloning	reproductive cloning	stem cells therapeutic cloning
gene transfer germ line therapy		pharmaceutical cloning
PGD	PGD	PGD

Figure 1: Biotechnology on mammalian embryos at various stages of preimplantation development with multiple applications in reproductive and therapeutic medicine.

SCNT and transfer of human cloned embryos may eventually be possible in the future for patients who have no other alternative options for procreating their own offspring.

Interspecies Bioassay for Reproductive Embryocloning

For future applications in reproductive cloning, it will be very important to further advance in our understanding how to rejuvenate and reprogram human adult cells by modern bioengineering. In this context, we recently started a novel approach using adult human ovarian granulosa cells and skin fibroblasts for SCNT into enucleated bovine oocytes to test and extend the efficiency of interspecies-specific SCNT with the aim to establish a series of bioassays for different types of human nuclear donor cells. The bovine oocyte is an excellent and highly efficient model as it provides adequate parameters for SCNT [55]. Our recent results point out that SCNT procedures with enucleated bovine oocytes fused with adult human cells are feasible and can yield appreciable success rates of embryo development [56].

Based upon our experience with such interspecies bioassay, we tested fibroblast cells derived from skin biopsy of the male patient suffering from azoospermia and testicular cryptorchism to reveal their embryonic capacity for SCNT procedures [48]. Enucleated bovine oocytes were injected subzonally with single fibroblast cells from the male patient. Such bovine oocyte-fibroblast constructs were fused electrically and activated chemically and then cultured in vitro for several days. Interspecies-cloned embryos developed and reached various stages of preimplantation. These interspecies embryos were analysed individually for the presence of human genomic DNA, human and bovine mtDNA using species-specific microsatellite markers for genomic DNA and species-specific primers for mtDNA. The PCR data revealed that human genomic DNA was present together with human and bovine mtDNA in the interspecies embryos. Based upon these results we could demonstrate that adult fibroblast cells from the infertile male patient were capable of initiating and promoting embryonic development. These fibroblast cells were also successfully employed in SCNT using oocytes from the infertile patient's wife [48]. The interspecies bioassay will enable us to primarily examine, evaluate and explore the developmental potential of various human adult somatic cells in their usefulness as possible nuclear donor cells for reproductive and therapeutic embryo cloning.

Embryonic Stem Cells for Future Therapeutic Applications

A few years ago, American and Australian researchers managed to successfully culture embryonic cells from 5 to 6 day-old human preimplantation embryos and primordial germ cells from 5 to 9 week-old human embryos in suitable nutritional media [57, 58]. Embryonic stem (ES) cells and primordial germ (PG) cells remain diploid and maintain their embryonic and proliferative characteristics over many cell divisions in culture. ES cells are derived from the inner cell mass (ICM) of cultured human blastocysts produced by in-vitro fertilization for clinical purposes in assisted reproduction and donated by patients. Human ES cell lines can be established by selecting and expanding individual ICM colonies of uniform and undifferentiated morphology. Human ES cells express alkaline phosphatase and several cell surface markers including stage-specific antigens characteristic for embryonic and undifferentiated cells. In addition, human ES cells synthesize a high level of telomerase that is required for adding telomeric repeats to chromosomal ends and for maintaining the mitotic activity of cells. Somatic cells usually show a low level of telomerase, contain shortened telomeres after prolonged culture in vitro and finally terminate their proliferative division but can extend their lifespan when telomerase is added to aged cells [59].
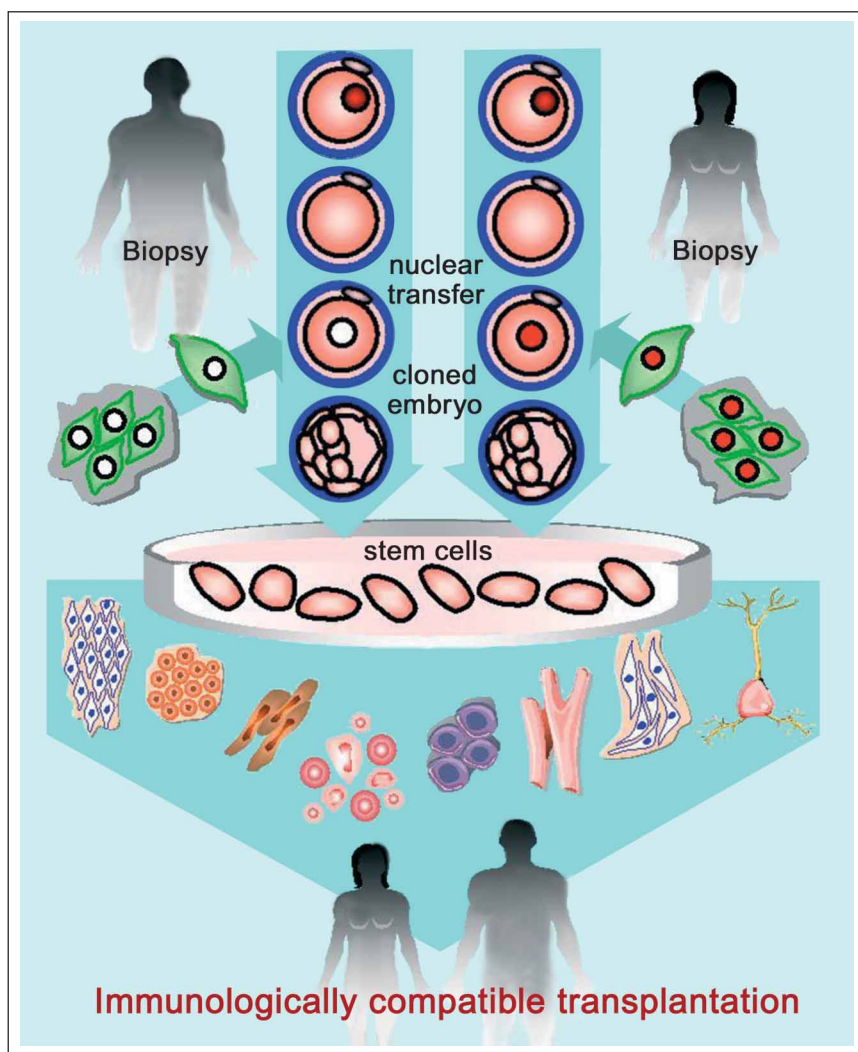

Figure 2: Somatic cell nuclear transfer (SCNT) for establishing embryonic stem (ES) cells in therapeutic medicine.

Although human ES cell lines were not established clonally from individual ICM cells but rather from morphologically homogeneous cell populations, they consistently expressed various biochemical markers unique to ES cells. When these cells started to pile up under crowded culture conditions, they spontaneously differentiated into morphologically different cell types. When human ES cells where transplanted to immune-suppressed SCID mice, they developed teratomas that contained a variety of differentiated tissues and clusters of undifferentiated stem cells, so-called embryonal carcinoma cells [60]. Since these cells can generate tumors it will be important to focus research on how to direct and channel these stem cells into benign precursor cells for various cell lineages. Cellular bioengineering on ES cells will tremendously help to provide particular precursor cells for specific organ therapy [61]. In the non-human primate system, ES cells from rhesus monkey blastocysts were successfully established in vitro and showed similar morphological, biochemical and developmental features as human ES cells [62]. It was proposed to utilize these monkey ES cells as a model system to investigate human differentiation and to develop therapeutic cloning strategies for human diseases.

Human embryo-derived ES cells may be employed for future therapeutic purposes to reconstitute or repair defective organ systems since they are capable of differentiating into a variety of tissues and possess a large regenerative potential [63]. First therapeutic applications concerning this subject have recently been attempted using human ES cells for treatment of patients with degenerative diseases [64, 65]. Human ES cell therapy can be made applicable to repair damaged heart tissue in patients with myocardial infarction and heart failure [66]. In the near future, patients suffering from Parkinson's disease [67] or diabetes [68] may also be treated successfully with human ES cells that have been channelled to synthesize organ-specific gene products such as dopamine or insulin, respectively. Therapeutic treatments using human ES cells with airway-epithelial developmental potential should also soon be envisaged for patients with pulmonary diseases including cystic fibrosis and bronchiolitis [69].

With respect to human therapeutic cloning by means of developing cloned embryos in assisted reproduction, a novel discipline of biotechnology dedicated to stem-cell therapy in regenerative medicine for a variety of human diseases will emerge for the benefit of patients' health. Our society will have to set up the proper scientific, legal and ethical rules concerning human cloning with its multiple applications and far-reaching consequences in medicine.

Embryo Cloning for Human Embryonic Stem Cells

Therapeutic cloning with the aid of assisted reproductive technologies (ART) for the extraction and expansion of human ES cells will open up novel and quite controversial applications for transplantation medicine [70]. ES cell research may be performed by utilizing donated pre-implantation embryos from IVF and ICSI programs or by creating cloned embryos via SCNT from patients' somatic cells into enucleated oocytes (fig. 2). The latter concept would offer a unique opportunity to establish patient-specific stem cells for regenerative therapy.

Cibelli and co-workers reported SCNT procedures in humans in the context of future therapeutic cloning [71]. Using skin fibroblasts and ovarian cumulus cells as nuclear donor types and human enucleated oocytes as SCNT recipients, they obtained two abnormal embryos that stopped development at the 4-cell and 6-cell stage, respectively. At this early embryonic stage, however, stem cells cannot yet be isolated under current biotechnology procedures. Embryos have to be capable of developing to the blastocyst stage, which is a necessary prerequisite for stem-cell isolation.

American researchers were successful in establishing non-human primate ES cells derived from parthenogenetically developing embryos [72, 73]. These undifferentiated stem cells can proliferate in vitro and are able to differentiate into a number of different cell types. Under in-vivo conditions by transplanting them into immunodeficient mice, these primate ES cells can also form a variety of different tissues, as judged from their specific morphological features and biochemical expression markers. For future medical applications in humans, the authors proposed that parthenogenetically de-
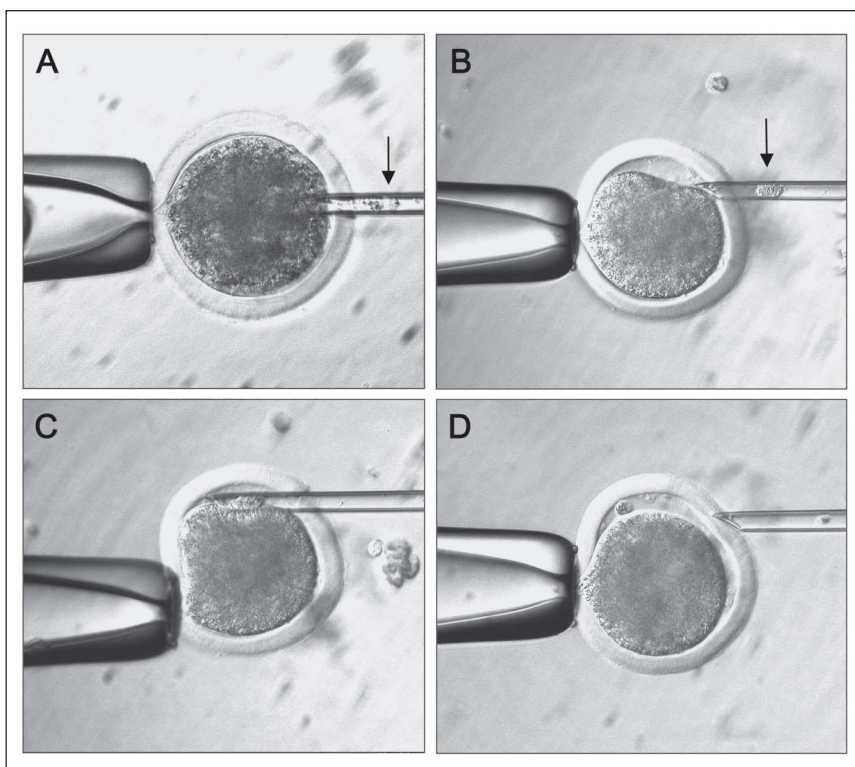
Figure 3: Interspecies SCNT. (**A**) Enucleation of bovine oocyte at metaphase II; (**B**) Selection of adult human donor cell; (**C**) Injection of adult human donor cell into the perivitelline space. (**D**) Human donor cell attached to the enucleated bovine oocyte.

rived human ES cells may be useful for female therapy with the advantage of bypassing the production of cloned embryos via SCNT procedures.

## Interspecies Embryo Cloning for Embryonic Stem Cells

Chinese researchers reported successful interspecies SCNT using enucleated rabbit oocytes fused with human adult fibroblast cells. From the developing cloned embryos they established in-vitro cultures of interspecies-derived ES cells. These interspecies ES cells could also develop into different tissue- and organ-specific cell clusters [74]. They also documented very clearly by DNA genotyping and cytogenetic karyotyping that the SCNT embryos and ES cells had originated from the genomic DNA of the human fibroblast donor cells. However, the authors did not present data on mitochondrial (mt)DNA heteroplasmy in the interspecies embryos originating conjointly from rabbit and human cytoplasm. The promising perspectives are that such interspecies embryos may serve as potential source for stem cell research. Future extensive investigations will be required to discover the embryonic capacity of human adult somatic cells in interspecies SCNT and their ability to create ES cells to be tested for their possible applications in therapeutic medicine.

Bovine oocytes were successfully employed in interspecies SCNT using adult fibroblast cells from pig, sheep, rat and rhesus monkey as nuclear donor types [75]. Development of cloned interspecies embryos was observed up to the blastocyst stage. No investigations at the molecular level have been reported to document the embryos' genomic origin from the particular donor cell types. American researchers reported interspecies SCNT using bovine oocytes and human lymphocytes or oral mucosal epithelial cells for cloning [76]. However, their data were presented
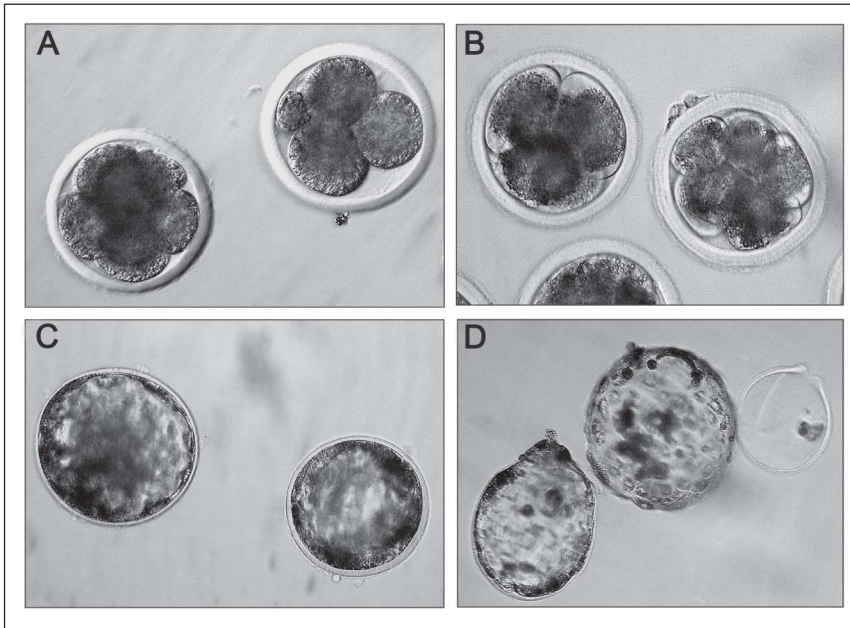
Figure 4: Interspecies-SCNT preimplantation embryos derived from human granulosa cells fused with enucleated bovine oocytes. Cleavage embryos (A) and blastocysts (C) derived from SCNT. Parthenogenetically developed cleavage embryos (B) and hatching blastocysts (D) as controls.
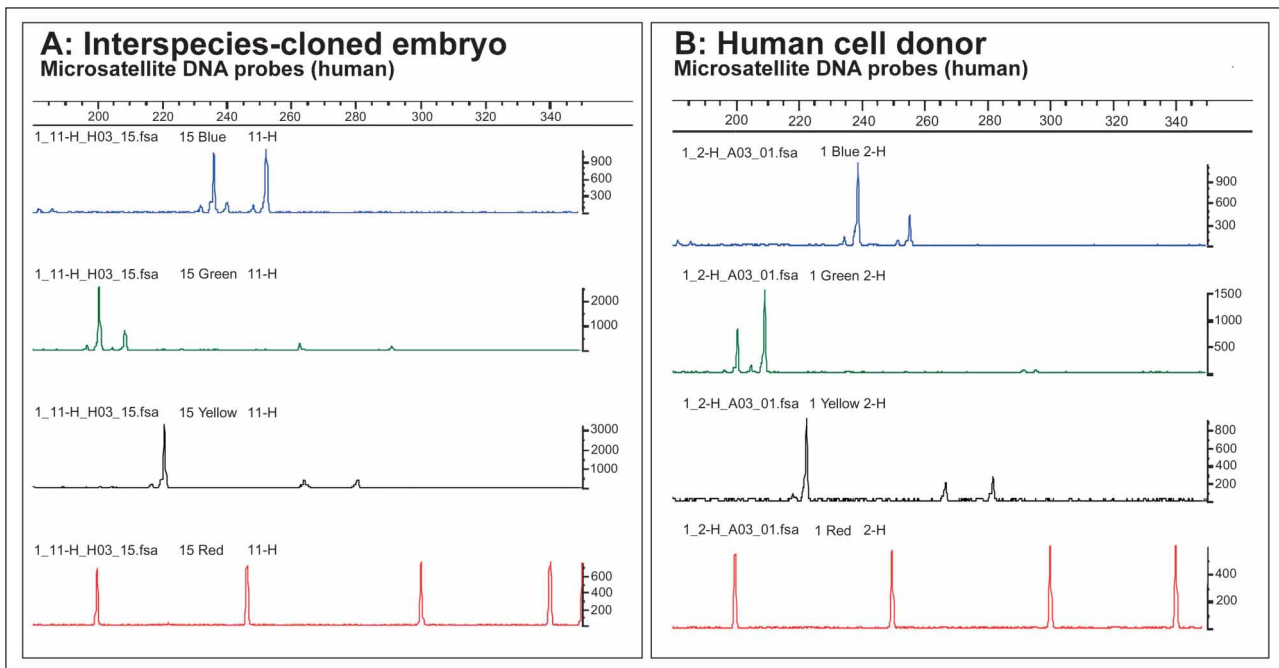
Figure 5: Human genomic DNA sequence analysis of interspecies-cloned embryo and human adult fibroblast donor cells used for SCNT. Identical DNA sequence profiles concerning peak positions are detectable for interspecies embryo (A) and human donor cells (B). Some variations in peak levels result from different sample analysis. Three chromosomal microsatellite probes were used for PCR amplification. FGA (chromosome 4q28), D21S11 (chromosome 21), D13S317 (chromosome 13q22–31). A standard marker (ABI Applied Biosystem ROX Reference Dye) served as internal control. For further details see [78].

briefly in a review on mammalian SCNT without providing specific information about fusion and activation of the bovine oocytes or characteristics of the human somatic donor cells, nor presenting any documentation of the obtained embryos to furnish proof for their origin from the donor cells' genome. In another more recent report, cytogenetic and molecular analyses of interspecies SCNT embryos derived from human cord fibroblasts fused with enucleated bovine oocytes clearly documented their genomic origin from the human donor cell nuclei [77].

In our interspecies SCNT studies [48, 56, 78], human ovarian granulosa cells obtained from IVF programs and fibroblast cells derived from skin biopsies were deliberately chosen as donor cells for fusion with enucleated bovine oocytes (fig. 3). The rationale for selecting these adult cell types was based on various cloning reports in different mammalian species that these cells seem to be currently the most efficient adult donor cells for SCNT procedures [14, 55, 79]. Our series of interspecies cloning with human granulosa cells and fibroblasts resulted in appreciable success rates of 31.3 % and 29.3 % of embryonic development up to the blastocyst stage (fig. 4).

In order to properly document that the human donor cell nuclei were capable of initiating and promoting embryonic development noted in our studies, the interspecies-cloned embryos were analysed at the molecular level by species-specific microsatellite DNA and mtDNA using PCR and DNA technology (these investigations were carried out at the Department of Molecular Biology, Veterinary Institute of the University of Göttingen, Germany). The detection of specific genomic microsatellites is a marker for the presence of genomic DNA of the corresponding species. Moreover, it also determines the specific genotype of the individual and therefore goes to prove the origin and stability of the donor cell nuclei in cloned embryos (fig. 5). The absence of PCR products originating from bovine genomic microsatellites in these interspecies embryos indicated that the bovine oocytes were efficiently depleted of their nuclear genome following microsurgical enucleation for SCNT. In these interspecies-cloned embryos, human mtDNA together with bovine mtDNA was detected which clearly proves that mitochondria from the fused human donor cells were carried over to the enucleated bovine oocytes. The resulting interspecies embryos can therefore be considered as heteroplasmic for mtDNA (fig. 6). In our bovine-human interspecies SCNT, human-specific mtDNA was still detectable up to the blastocyst stage, similar to what has been reported for interspecies blastocysts derived from rabbit-monkey SCNT [80]. Such variability in the persistence of donor cell-specific mtDNA in interspecies SCNT embryos needs further investigation. Viable presence and replicative ability of donor cell-specific mtDNA has been clearly documented in the bovine system by revealing a significant mitochondrial heteroplasmy in cloned adult cattle [29, 30]. Possible mixing and recombination of different mtDNA populations in heteroplasmic cloned animals have been discussed in the context of assisted reproduction and embryo biotechnology [81].

Figure 6: DNA sequence analysis of human and bovine mitochondrial (mt) DNA. From human donor cells and interspecies-cloned embryo (blastocyst). For detection of human and bovine mtDNA, species-specific primers were used that amplify parts of the mtD-loop region, respectively. The amplicons were sequenced using internal primers and products separated on an ABI3100. (A) Human mtD-loop region (position 16191–16239; GeneBank acc. no. AY275537) that was amplified from the human donor cells. (B) Human mtD-loop region obtained from interspecies-cloned embryo. Both mtDNA sequences show identical profiles. (C) Bovine mtDNA sequence from interspecies-cloned embryo obtained by using bovine mtDNA primers for PCR amplification of mtD-loop region (position 306 to 359; GeneBank acc. no. AF499248). For further details see [78].
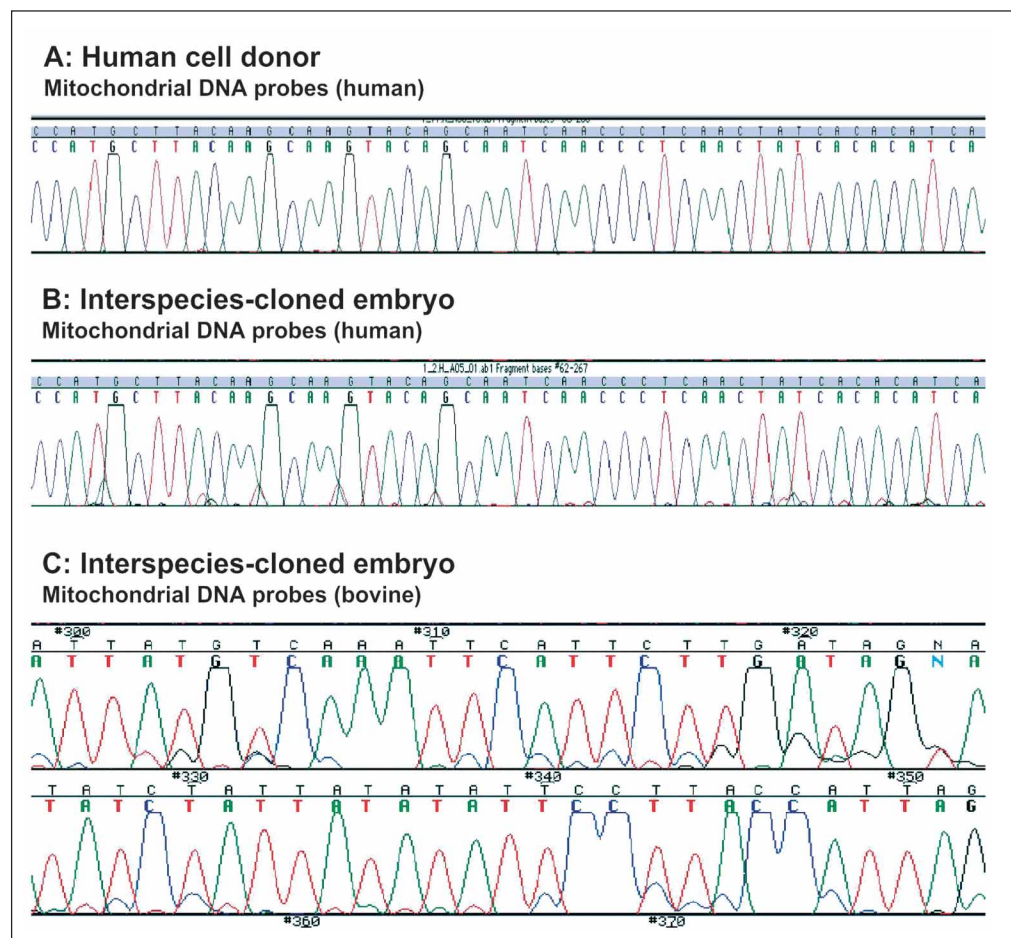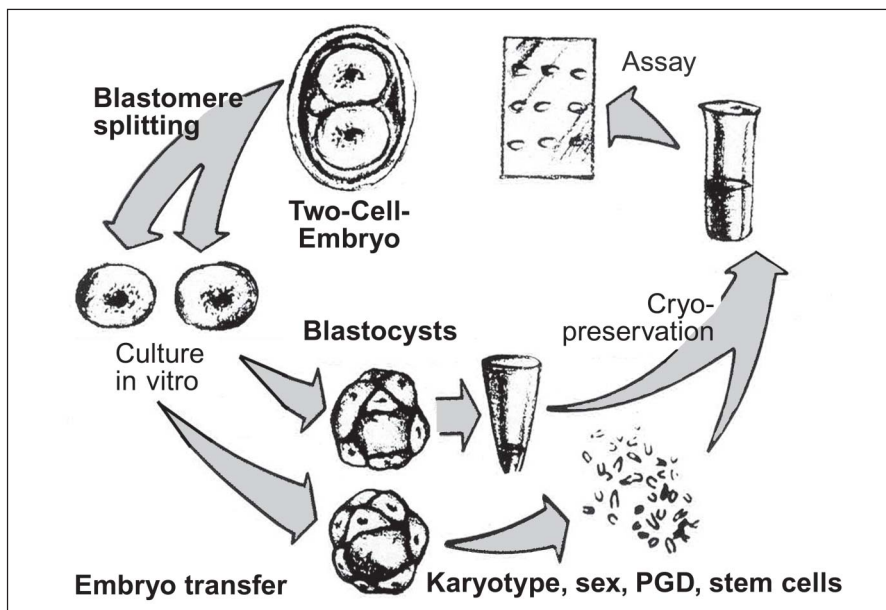
Cloning Via Embryo Splitting

Mammalian embryo splitting and isolation of blastomeres for the creation of genomically identical twins or multiples have seen a long-standing history and have advanced over the past decades to a variety of applications in veterinary and human medicine (fig. 7).

In sheep, by splitting early embryos via blastomere biopsy and transferring them in utero, 36 % of split embryos developed to term [82]. In cattle, Canadian researchers reported successful embryo splitting at the 4-cell stage [83]. From two embryos split into four pairs that were transferred into four heifers, four identical calves were delivered at term pregnancy. Also in cattle, Japanese researchers could show for the first time that frozen-thawed twin embryos after time-separated transfer in utero gave rise to monozygotic calves of different ages [84]. When embryo splitting was applied to nonhuman primates, surprisingly low twinning results were reported for rhesus monkey. Embryo splitting at the 8-cell stage into quadruplets, each containing two cells, resulted only in 1 % blastocyst formation [85]. From 13 transfers in utero with quadruplet-derived blastocysts, four pregnancies could be established from which one healthy female was born originating from a quarter of a biopsied 8-cell embryo. Similarly, embryo twinning in rhesus monkey was attempted by blastomere separation at the 2-cell and 4-cell stage [86]. Transfer in utero of 44 embryos (22 pairs of twin embryos) led to seven chemical pregnancies, including two twin pregnancies. However, none of them resulted in term birth of monozygotic twins.

Human embryo splitting so far has been reported only on genetically abnormal embryos [87]. Polyploid embryos derived from IVF cycles, destined to be discarded and donated for research, were split at the 2- to 8-cell stage. Of 48 twin embryos originating from 17 abnormal polyploid embryos, some developed to the 32-cell stage. However, none of these nonviable split embryos developed beyond this stage and were subsequently discarded. In a commentary referring to these experiments and to embryo splitting in general, Jones, Edwards and Seidel [88] acknowledged the merits of these attempts for future applications in reproductive medicine. Human embryo splitting can be envisaged for patients enrolled in IVF programs and could serve the needs of infertile couples by increasing the number of embryos available for immediate or subsequent transfer in utero and by offering preimplantation genetic diagnosis (PGD) or embryo sexing. In future applications, it may also be envisaged that early embryos, after splitting into twin embryos, will provide a unique opportunity to implant one embryo in utero and to cryopreserve the monozygotic twin embryo for future purposes (see fig. 7).

Before considering possible applications of human embryo splitting in ART, the aims of our current studies were to develop and establish the relevant techniques for safe, efficient blastomere biopsy for embryo splitting on mouse embryos at various stages of preimplantation (fig. 8). Also, we aimed to investigate and determine the effect of blastomere biopsy *per se* on the developmental potential of split embryos under in-vitro culture conditions up to the blastocyst stage [89]. We found that there is a high success rate (about 75 %) for obtaining twin blastocysts from 2-cell and 4-cell split em-

Figure 7: Splitting of early embryos (twinning) for various applications in reproductive and therapeutic medicine.
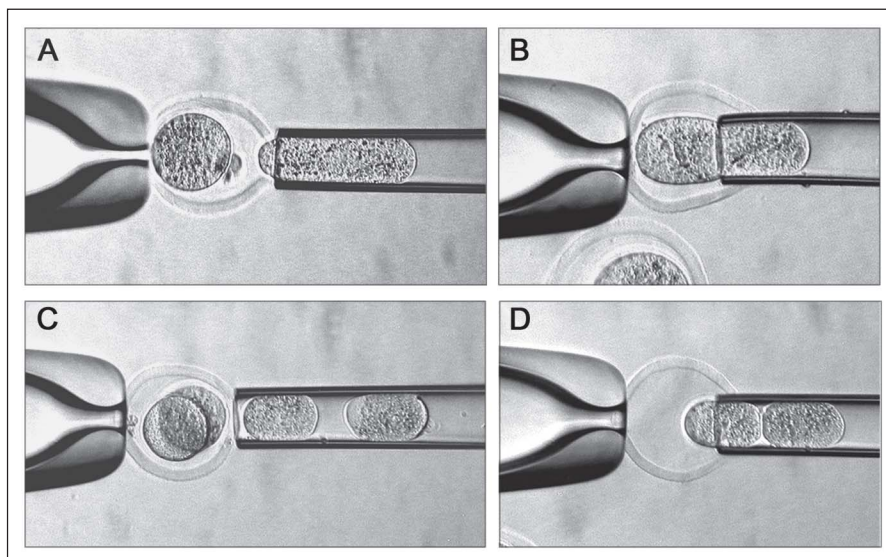
Figure 8: Blastomere biopsy from 2-cell and 4-cell mouse donor embryos (**A** and **C**) and blastomere reinjection into empty zona pellucida recipients (**B** and **D**).

bryos (fig. 9). Development of twin blastocysts derived from 6-cell split embryos was reduced (about 65 %) and was even less efficient for 8-cell embryo splitting (about 35 %). The morphological quality of twin blastocysts derived from 2-cell and 4-cell embryo splitting was clearly superior to the ones obtained from 6-cell and, in particular, from 8-cell embryo splitting. Such regression in morphogenetic potential has been reported on mouse embryos derived from 8-cell blastomeres [90].

From our comparative studies on mouse embryo splitting we conclude that for future applications of human embryo splitting in ART, 2-cell and 4-cell stage twinning seems to be the most promising approach [91]. In sheep [82], cattle [83], and mouse [90], blastomeres biopsied from 2-cell and 4-cell embryos have been shown to be totipotent and can give rise to healthy offspring, including twins and multiples.

More than a decade ago, the merits of embryo splitting were acknowledged as a valuable future application in reproductive medicine [88]. Embryo splitting in ART may be applicable and considered for those patients termed as "low responders" with only a few oocytes being usually recovered after hormonal stimulation and available for IVF or ICSI. Embryo splitting should increase the likelihood for obtaining a pregnancy since more embryos could be made available for transfer in utero. For couples with several embryos produced during one IVF cycle, embryo splitting may provide additional embryos for subsequent transfers without having to go through another retrieval cycle. Since identical twins are often born independently of ART and develop to perfectly normal human beings, the birth of twins as a result of embryo splitting should not be of major concern, neither ethically nor socially speaking. As a matter of fact, twinning happens unintentionally as a by-product of transferring multiple embryos in IVF cycles for infertile couples. As long as a couple is fully informed about the consequences of twinning outcome, there appears to be no concern about transferring two embryos in utero with the same genome with the hope of producing a pregnancy. In a report on embryo splitting as a modality for infertility treatment from the Ethics Committee of the American Society for Reproductive Medicine [92], it was stated that "concerns that embryo splitting could lead to more than one child born with identical genomes is a more realistic possibility if embryo splitting is clinically successful, but still is not a sufficient reason to discourage research in the technique" and „since embryo splitting has the potential to improve the efficacy of IVF treatments for infertility, research to investigate the technique is ethically acceptable" [92]. According to these recommendations, our splitting biopsy procedure as developed in the mouse model can be considered as a safe and efficient technique for future splitting of human embryos with its multiple applications in reproductive and therapeutic medicine.
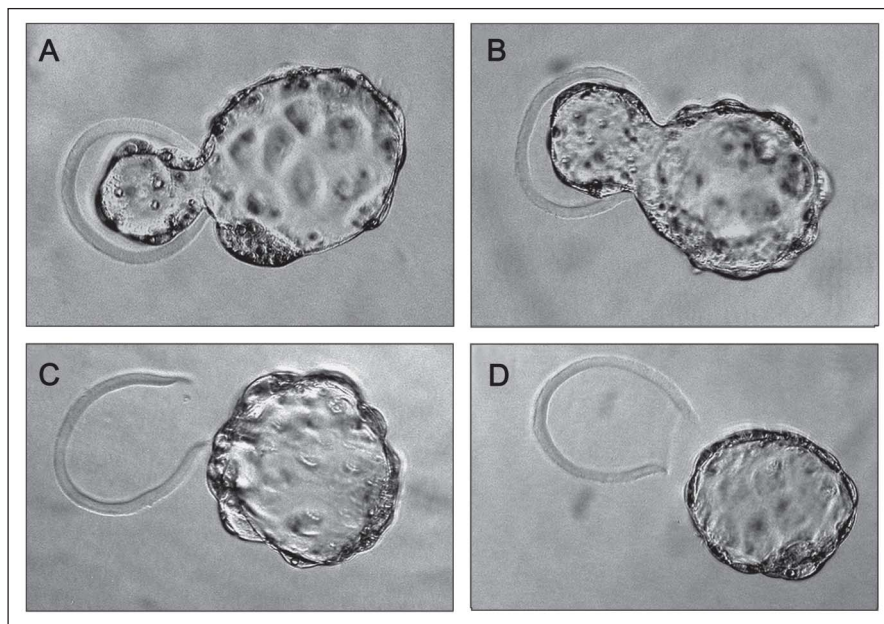
Figure 9: Hatching blastocysts derived from mouse embryo splitting at the 2-cell (**A** and **B**) and 4-cell (**C** and **D**) stage. Donor blastocysts on the left and recipient blastocysts on the right.

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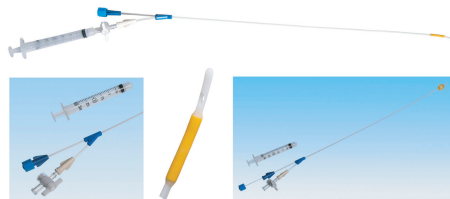
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