Article

Efficient blastomere biopsy for mouse embryo splitting for future applications in human assisted reproduction

Dr Panayiotis Zavos received his Bachelor's (Biology), Master's (Biology Physiology) and Education Specialist degrees from Emporia State University, Kansas, USA. His PhD in Reproductive Physiology was awarded at the University of Minnesota. He is a Professor Emeritus of Reproductive Physiology–Andrology at the University of Kentucky, Director and Chief Andrologist of the Andrology Institute of America, Associate Director of the Kentucky Centre for Reproductive Medicine and President and CEO of ZDL, Inc., USA. Dr Zavos has published over 400 peer-reviewed publications. His research interests are in reproductive cloning, andrology, male reproductive physiology and infertility, and ART. He is a past Board Member of the China Academy of Science, receiving its first ever Honorary Professorship awarded to an American.

Dr Panayiotis Zavos

K Illmensee[1,2], K Kaskar[2,3], PM Zavos[1,2,3,4]
[1]Reprogen Ltd, Limassol 3106, Cyprus; [2]Andrology Institute of America, PO Box 23777, Lexington, Kentucky 40523, USA; [3]Kentucky Centre for Reproductive Medicine, Lexington, Kentucky 40523, USA
[4]Correspondence: Tel. +1 859 2786806; Fax: +1 859 2786906; e-mail: zavos@zavos.org

Abstract

The objective of the current study was to establish a safe, efficient biopsy procedure for embryo splitting using the mouse model for future applications in human assisted reproduction. From mouse embryos at the 2-, 4-, 6- and 8-cell stage, half the number of blastomeres were microsurgically biopsied and transferred into empty mouse zonae pellucidae. Twin embryonic development was monitored during in-vitro culture. Blastocyst developmental rate using 2-, 4-, 6-, and 8-cell splitting was 74.4, 75.0, 66.7 and 38.4 respectively, with corresponding hatching rates of 94.9, 97.5, 92.7 and 83.8%. Blastocysts from 2-, 4-, and 6-cell splitting resulted in elevated hatching rates compared with non-operated blastocysts (87.5%), due to the Tyrode-assisted hatching effect. Blastocyst morphology was superior from 2- and 4-cell splitting when compared with 6- and 8-cell splitting. Furthermore, outgrowth of twin blastocysts from 2- and 4-cell splitting showed well-developed colonies with trophoblast cells and clusters of ICM cells, whereas those obtained from 6- and 8-cell splitting frequently formed small-sized colonies. Due to the high twinning success rate obtained under the experimental conditions employed in this study, it appears that with further modifications and proper safeguards, such embryo splitting efforts could have potential applications in humans.

Keywords: *blastomere biopsy, culture in vitro, embryo splitting, mouse preimplantation, Tyrode-assisted hatching*

Introduction

Mammalian embryo splitting and isolation of blastomeres for the creation of twins or multiples have seen a long-standing history and have advanced over recent decades to a variety of applications in veterinary and human medicine.

In pioneering studies on mouse embryos, Tarkowski (1959a,b) examined the developmental potential of single blastomeres from 2-cell stage embryos and reported on their totipotency giving rise to adult mice. In a similar study, separated 2-cell blastomeres with this potential were able to promote full-term development resulting in twin mice (Mullen *et al.*, 1970). In chimaeric mouse experiments, by aggregating single blastomeres from the 4-cell stage with genetically different carrier embryos, Kelly showed that following transfer of these embryo aggregates into surrogate females, live-born mice could be obtained that, in a few instances, appeared to originate exclusively from isolated 4-cell blastomeres (Kelly 1975, 1977). Following another experimental approach, Tarkowski and co-workers (2001) investigated the developmental potential of single diploid (2n) blastomeres isolated from 4-cell mouse embryos and aggregated with non-viable tetraploid (4n) carrier embryos. Following transfer of these 2n/4n chimaeric embryos to surrogate females, young mice were successfully reared by foster mothers and showed a 2n karyotype together with specific genetic markers, thus demonstrating their origin from single blastomeres of 4-cell stage embryos.

Over the past decades, a variety of techniques for embryo splitting have been reported with varying degrees of success

for twinning. Mechanical division of preimplantation mouse embryos often led to cellular damage thereby reducing drastically the viability of split embryos (Agrawal and Polge, 1989). When bisecting mouse morulae to create twin embryos for transfer into surrogate female mice, 25% developed to term (Nagashima *et al*., 1984). Bovine embryos when quartered at the morula stage, and transferred *in utero* developed to term, resulting in multiple calves (Voelkel *et al*., 1985). From bisected bovine blastocysts transferred *in utero*, twin calves developed to term (Ozil, 1983). Similarly, bisected porcine morulae and blastocysts were able to promote full-term development, giving rise to twin piglets (Nagashima *et al*., 1989; Reichelt and Niemann, 1994). In goats, monozygotic twins have been obtained from bisected half embryos following transfer *in utero* (Tsunoda *et al*., 1984).

By developing more refined methods for blastomere biopsy from preimplantation embryos, further progress was reported on embryo splitting and the developmental potential of blastomeres isolated from various preimplantation stages. In sheep, by splitting early embryos via blastomere biopsy and transferring them *in utero*, 36% of split embryos developed to term (Willadsen, 1980). By applying similar blastomere biopsy to cattle embryos at the 8-cell stage to create twin embryos, 18% gave rise to viable calves (Willadsen and Polge, 1981). In the mouse, half-embryos derived from 8-cell stage biopsies, when transferred into surrogate females, gave very poor results with regards to embryonic development *in utero* (Rossant, 1976). On the other hand, split embryos derived from the 2-cell stage developed to term at a survival rate of 65% (Tsunoda and McLaren, 1983). Papaioannou and co-workers (1989) have shown that half-mouse embryos derived from 2-cell splitting gave rise to live offspring similar in size to control mice.

In the rat, embryo splitting at the 2-cell stage resulted in nine pairs of identical twins that following transfer into surrogate females resulted in 38% success rate of normal fetal development (Matsumoto *et al*., 1989). Concerning embryo splitting in the horse, 44 twin embryos created from blastomere biopsy at the 2-cell and 8-cell stages has resulted in two monozygotic pairs of healthy offspring (Allen and Pashen, 1984). In cattle, Canadian researchers reported on successful embryo splitting at the 4-cell stage (Johnson *et al*., 1995). From two embryos split into four pairs that were transferred into four heifers, four identical calves were delivered by elective Caesarean section at term pregnancy. Also in cattle, Japanese researchers showed for the first time that frozen–thawed twin embryos after time-separated transfer *in utero* gave rise to monozygotic calves of different ages (Seike *et al*., 1991).

When embryo splitting was applied to non-human primates, surprisingly low twinning results were reported for rhesus monkeys. Embryo splitting at the 8-cell stage into quadruplets, each containing two cells, has resulted in only 12% blastocyst formation (Chan *et al*., 2000). 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 (Chan *et al*., 2000). Similarly, embryo twinning in rhesus monkeys had been attempted by blastomere separation at the 2-cell and 4-cell stage (Mitalipov *et al*., 2002). Transfer *in utero* of 44 embryos (22 pairs of twin embryos) led to seven chemical pregnancies, including two twin pregnancies. However, none resulted in term birth of monozygotic twins.

Human embryo splitting has so far been reported only on genetically abnormal embryos (Hall *et al*., 1993). Polyploid embryos derived from IVF cycles destined to be discarded and donated for research were split at the 2- to 8-cell stage, coated with an artificial zona pellucida (1% sodium alginate) and cultured up to the morula stage. Using this technique, Hall and co-workers created 48 twin embryos from 17 abnormal polyploid embryos, some of which developed to the 32-cell stage. However, none of these non-viable split embryos developed beyond this stage, and they were discarded (Fackelmann, 1994). In a commentary referring to these experiments and to embryo splitting in general, Jones, Edwards and Seidel (1994) acknowledged the merits of these attempts for future application in reproductive medicine.

Human embryo splitting can be envisaged for patients enrolled in IVF programmes, and could serve the needs of infertile couples by increasing the number of embryos available for immediate or subsequent transfer *in utero*. Before considering possible applications of human embryo splitting in assisted reproductive technology, the aim of the present study was to develop and establish the relevant techniques for safe, efficient blastomere biopsy for embryo splitting on mouse embryos at various stages of preimplantation. It was also aimed to investigate and determine the effect of blastomere biopsy *per se* on the developmental potential of split embryos under in-vitro culture conditions.

Materials and methods

Mouse embryos

Commercially available frozen 2-cell stage mouse embryos commonly used for quality control testing were obtained (Embryotech Laboratories, Wilmington, MA, USA). Those embryos were derived from matings between mouse strains B6C3F-1 × B6D2F-1. The mouse embryos were thawed according to the manufacturer's guidelines, washed carefully and cultured in IVC-One medium (Invitro Care, San Diego, CA, USA) with 10% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) at 5%CO_2 and 37°C.

Microsurgical biopsy

Mouse embryos at the 2-cell, 4-cell, 6-cell, and 8-cell stages were placed in microdrops in embryo biopsy medium (Irvine Scientific) covered with equilibrated mineral oil (Sigma-Aldrich, St Louis, MO, USA).To further facilitate the separation of the mouse blastomeres, 5 µg/ml cytochalasin B (Sigma-Aldrich) was added to the biopsy medium commonly used in human blastomere biopsy procedures employed during preimplantation genetic diagnosis (PGD). The authors do not recommend that cytochalasin B should be used in human clinical embryology. Mouse embryos were attached to a holding pipette of 95 µm outside diameter (OD) (Conception Technologies, San Diego, CA, USA). With an assisted hatching pipette of 14 µm OD containing acidified Tyrode solution (Irvine Scientific), an opening of 30–50 µm in the zona pellucida was prepared by applying this solution locally to the zona pellucida. With a blunt biopsy pipette of 35 µm OD, half the blastomeres were removed

from 2-, 4-, 6- and 8-cell donor embryos (**Figure 1a**,**c**,**e**,**g**) and microinjected into empty recipient mouse zonae pellucidae (**Figure 1b**,**d**,**f**,**h**). The empty mouse zonae pellucidae were created by removing and discarding the blastomeres from the mouse embryos.

Culture *in vitro*

Donor and recipient embryos were removed from the embryo biopsy medium, washed carefully and cultured individually in microdrops of IVC-One medium supplemented with 10% SSS, covered with equilibrated mineral oil at 5% CO_2 and 37°C. Development of donor and recipient embryos was observed through the preimplantation period every 12 h up to the blastocyst stage, including hatching ability and outgrowth of twin blastocysts and was photographically documented. Non-operated (non-manipulated) 2-cell mouse embryos were cultured in-vitro in microdrops of IVC-One medium supplemented with 10% SSS and covered with equilibrated mineral oil at 5%CO_2 and 37°C; these served as controls. Development of these non-operated control embryos was monitored up to the blastocyst stage.

Results

Embryo splitting was achieved on a total of 198 mouse embryos from 2-, 4-, 6- and 8-cell stages, of which 191 started to cleave (96.5%). After embryo splitting, the next cleavage division was delayed for about 4 h when compared with non-operated controls, most likely due to the biopsy procedure. During cleavage, twin embryos appeared smaller in size when compared with normal control embryos.

From 39 embryos that were split at the 2-cell stage, 58 twin embryos developed into blastocysts (74.4%), from which 55 hatched (70.5%). From 52 embryos that were split from the 4-cell stage, 78 twin embryos progressed to blastocysts (75.0%) and 76 hatched (73.1%). From 51 embryos that were split at the 6-cell stage, 68 twin embryos advanced to the blastocyst stage (66.7%) and 63 hatched (61.8%). From 56 embryos split at the 8-cell stage, 43 twin embryos reached the blastocyst stage (38.4%) and 36 hatched (32.1%). In comparison, of the 70 non-operated control 2-cell embryos, 64 embryos reached the blastocyst stage (91.4%) and 56 hatched (80.0%; **Table 1**, **Figure 2**).

In general, the success rate for development of blastocysts derived from embryo splitting was most efficiently elevated for the 2-cell and 4-cell stages, decreased at the 6-cell stage and further regressed at the 8-cell stage (**Table 1**). The graphic representation of results on mouse embryo twinning shows that 2-cell and 4-cell splitting appears to be superior to 6-cell and 8-cell splitting with regard to blastocyst development (**Figure 3**).

Besides the quantitative differences in successful twinning between these four embryonic stages analysed, twin blastocysts derived from 2-cell and 4-cell embryo splitting showed superior morphological quality, with a regularly developed trophoblast and a cluster of inner cell mass (ICM) cells very similar to normal control blastocysts. The nature of early blastomere differentiation in human 4-cell embryos, and its consequences for embryonic development, have been reported, showing that precursor cells of inner cell mass, germline, and trophectoderm may be formed as early as the 4-cell stage (Hansis and Edwards, 2002; Edwards and Hansis, 2005). In contrast, twin blastocysts derived from 6-cell and 8-cell embryo splitting showed inferior morphological quality in terms of trophoblast development and ICM formation. Furthermore, twin blastocysts derived from 8-cell embryo splitting showed frequently abnormal cavitation in conjunction with reduced size and limited formation of ICM cells.

Concerning the developmental capacity of twin embryos, the success rate for blastocyst formation of donor embryos was consistently and slightly superior to the one obtained for recipient embryos (**Table 1**). A second biopsy was therefore performed on donor embryos following the first biopsy for creating recipient embryos. In this second biopsy, the remaining blastomeres were removed from donor embryos and reinjected into their empty zonae pellucidae, thereby carrying out the same microsurgical procedure as for the recipient embryos. This control biopsy was aimed to determine the effect of biopsy on embryonic developmental potential (**Table 2**). The results of these biopsies on donor embryos showed that the success rate for blastocyst formation of control-biopsied donor embryos was quite similar and comparable with that obtained for recipient embryos, although slightly inferior to that for donor embryos (**Table 1**). This implies that blastomere biopsy for embryo splitting had only negligible effects on developmental potential of twin embryos.

The success rate for hatching ability of twin blastocysts derived from 2-cell, 4-cell and 6-cell splitting was very similar, and ranged between 91 and 97% (**Table 3**). The elevated hatching rate of these twin blastocysts was superior to the non-operated control embryos of 87.5%, with the exception of a hatching rate of 83–85% obtained for 8-cell twin blastocysts (**Figure 4**). This high hatching success was facilitated by the opening in the zona pellucida created by the Tyrode-assisted hatching, a prerequisite for blastomere biopsy.

Hatched twin blastocysts frequently attached to the culture dish and showed cellular outgrowths after 2 days post-hatching, similar to hatched blastocysts from non-operated controls. At 3–4 days post-hatching, large trophoblast cells proliferated and spread out at the periphery, whereas ICM cells remained as a distinct cluster in the central part of such embryo colonies (**Figure 5**). Outgrowth was most substantially observed from twin blastocysts derived from 2-cell and 4-cell splitting, was less efficient from twin blastocysts derived from 6-cell splitting, and even less for those derived from 8-cell splitting. Moreover, outgrowth from 8-cell twin blastocysts frequently lacked an ICM cluster, when compared with control non-operated hatched blastocysts (**Figure 2B**).

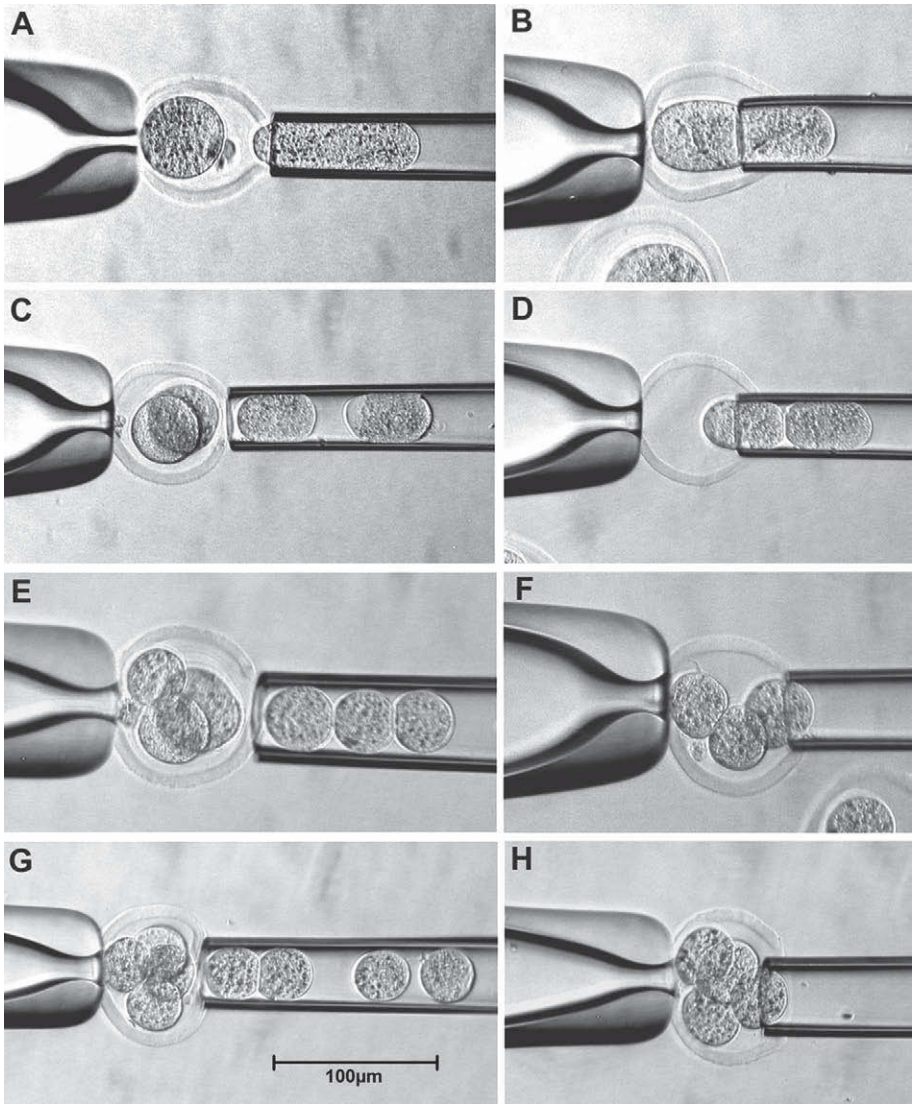

Figure 1. Blastomere biopsy of 2-, 4-, 6- and 8-cells mouse donor embryos (**A**, **C**, **E**, **G**) and reinjection into empty recipient zonae pellucidae (**B**, **D**, **F**, **H**).

Table 1. Embryo splitting at various stages of mouse preimplantation development.

Embryo stage	*Embryos split* (n)	*Blastocysts developed (%)*		*Blastocysts hatched (%)*	
		Donor	*Recipient*	*Donor*	*Recipient*
2-cell	39	76.8 (30/39)	70.0 (28/39)	71.8 (28/39)	69.3 (27/39)
4-cell	52	76.9 (40/52)	73.0 (38/52)	75.0 (39/52)	71.2 (37/52)
6-cell	51	68.6 (35/51)	64.6 (33/51)	62.7 (32/51)	60.8 (31/51)
8-cell	56	39.3 (23/56)	35.7 (20/56)	33.9 (19/56)	30.4 (17/56)
Controls[a]	70	91.5 (64/70)		80.0 (56/70)	

[a]Non-operated 2-cell embryos.

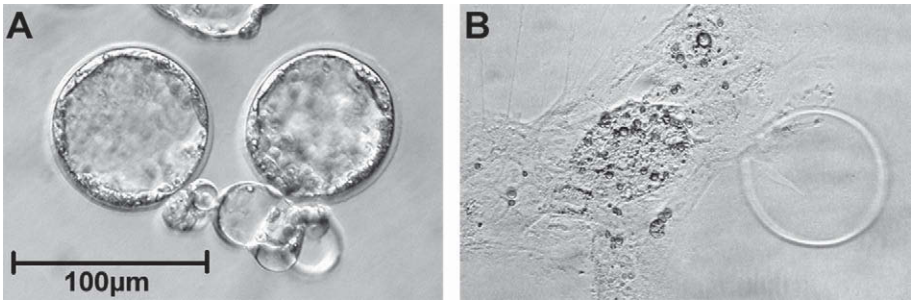

Figure 2. Hatched blastocyst (**A**) and outgrowth (**B**) derived from control non-operated 2-cell mouse embryos.

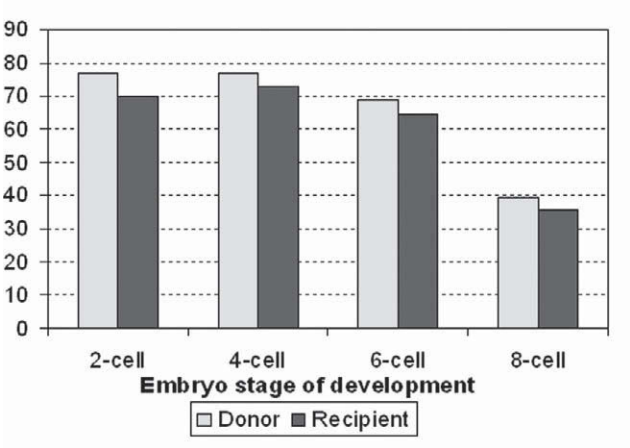

Figure 3. Blastocyst developmental rate for mouse embryo splitting at various stages of preimplantation.

Table 2. Effect of blastomere biopsy and reinjection into empty zona pellucida on embryonic developmental potential.

Embryo stage	*Blastomeres reinjected*	*Embryos operated*	*Blastocysts developed (%)*	*Blastocysts hatched (%)*
2-cell	1	20	75.0 (15/20)	70.0 (14/20)
4-cell	2	25	72.0 (18/25)	72.0 (18/25)
6-cell	3	34	64.7 (22/34)	61.7 (21/34)
8-cell	4	26	38.4 (10/26)	30.7 (8/26)

Table 3. Hatching ability of twinned blastocysts derived from embryo splitting at various stages of preimplantation.

Embryo stage	*Blastocysts hatched from blastocysts developed (%)*	
	Donor	*Recipient*
2-cell	93.3 (28/30)	96.4 (27/28)
4-cell	97.5 (39/40)	97.3 (37/38)
6-cell	91.4 (32/35)	93.9 (31/33)
8-cell	82.6 (19/23)	85.0 (17/20)
Controls[a]	87.5 (56/64)	

[a]Non-operated 2-cell embryos.

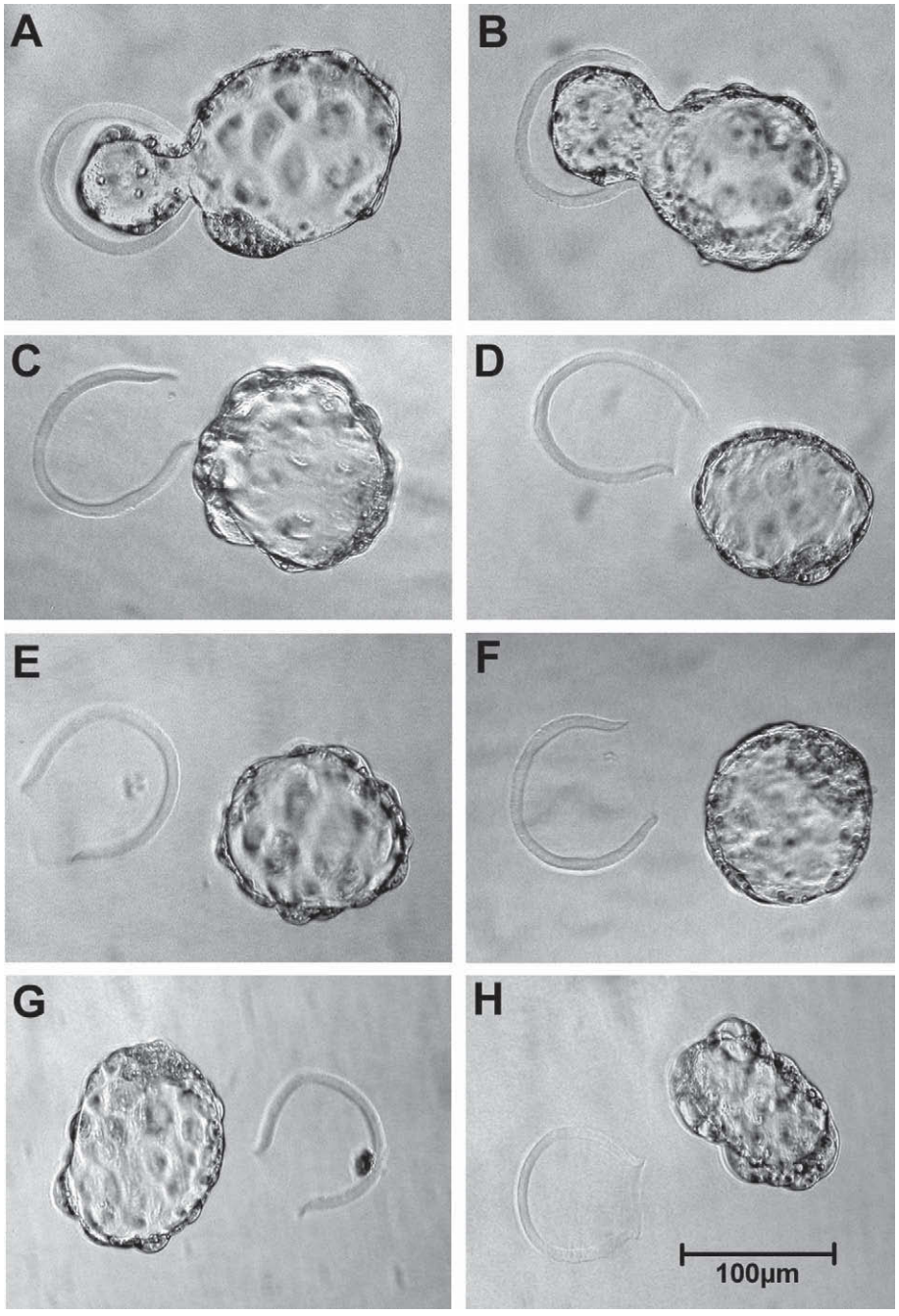

Figure 4. Blastocysts derived from mouse embryo splitting at 2-cell (**A** and **B**), 4-cell (**C** and **D**), 6-cell (**E** and **F**) and 8-cell stage (**G** and **H**). Donor blastocysts on the left and recipient blastocysts on the right.

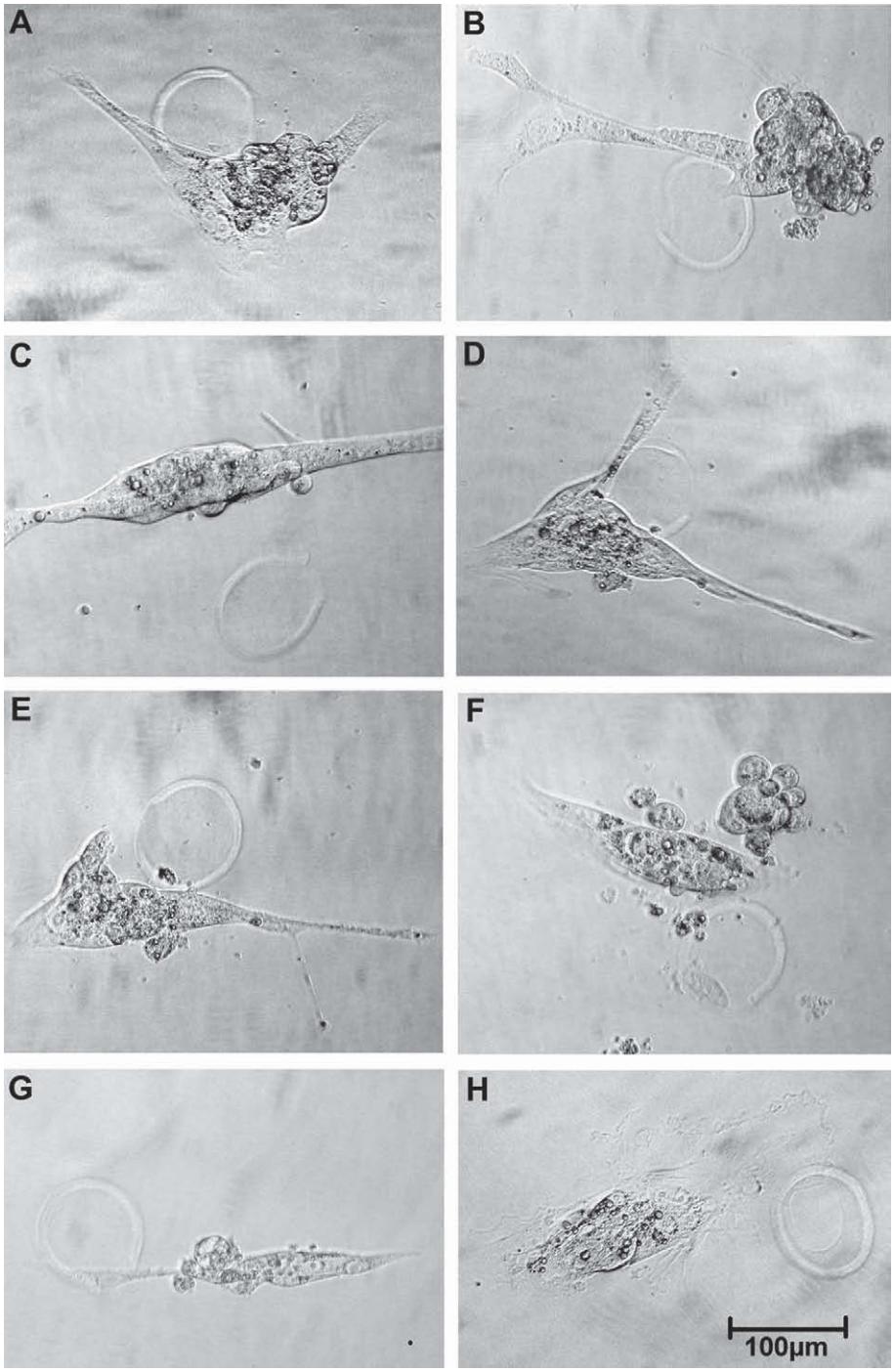

Figure 5. Outgrowths of hatched blastocysts derived from mouse embryo splitting at 2-cell (**A** and **B**), 4-cell (**C** and **D**), 6-cell (**E** and **F**) and 8-cell stage (**G** and **H**). Donor outgrowths on the left and recipient outgrowths on the right

Discussion

Various new technologies have been developed and proposed for the treatment of infertility such as somatic cell nuclear transfer, haploidization of somatic cells and creating of artificial gametes using totipotent embryonic stem cells (Nagy and Chang, 2005). In several mammalian species including non-human primates, embryo splitting has been successfully carried out using a variety of different techniques to create twin embryos. Mechanical bisection of early embryos has turned out to be rather harmful and reduces significantly the viability of split embryos. More efficiently, the successful creation of twins and multiplies via blastomere biopsy from early embryos has opened new possibilities for application in veterinary and human medicine.

Further progress in refining the biopsy procedures has led to improved success rates for split embryo survival and live-born offspring of monozygotic twins. Most of the basic research on embryo splitting has been carried out in the mouse. In different studies, the developmental capacities of blastomeres from various preimplantation stages have been investigated by blastomere separation and transfer into empty zona pellucida recipients to create twins or quadruplets. Surprisingly, there are no documented reports showing a systematic and comparative analysis of mouse embryo splitting at 2-, 4-, 6- and 8-cell stages as presented in this study. In addition, the potential of these four types of blastomeres for development and hatching of twin blastocysts has been examined.

It has been found that there is a high success rate for obtaining twin blastocysts from 2-cell and 4-cell split embryos (about 75%). 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%). In comparison, non-operated control 2-cell embryos showed a blastocyst development rate of 91.4%. The morphological quality of twin blastocysts derived from 2-cell and 4-cell embryo splitting was clearly superior to those obtained from 6-cell and, in particular, from 8-cell embryo splitting. Such regression in morphogenetic potential has been reported in mouse embryos derived from 8-cell blastomeres (Tsunoda and McLaren, 1983). Frequently, blastocysts originating from 8-cell split embryos were smaller in size and were composed of fewer cells forming trophoblast and ICM. When isolated blastomeres from 8-cell mouse embryos were investigated for their developmental capacities, Rossant (1976) discovered that embryos developing from these isolated blastomeres after their transfer into surrogate females were capable of inducing decidual formation and only occasionally developed into embryos with abnormal morphology. These findings have been interpreted that abnormal blastocysts with reduced cell number formed from these isolated blastomeres were responsible for abnormal embryonic development *in utero*. This phenomenon has been discussed in the context of programmed morphogenetic processes that continue with embryo compaction following the 16-cell stage (Ziomek *et al.*, 1982). Split 8-cell embryos are therefore not capable of dividing frequently enough before compaction to reach the cell number required for regular blastocyst formation and cavitation.

Hatching success rate for twin blastocysts was generally high and independent of the embryo stage used for twinning. This was certainly due to the assisted hatching effect that resulted from the Tyrode-prepared opening in the zona pellucida required for blastomere biopsy. Assisted hatching has been successfully applied in human assisted reproduction programmes to enhance pregnancy outcome in certain clinical indications such as repeated implantation failure, frozen–thawed embryo transfers and advanced maternal age (Germond *et al.*, 2004; Primi *et al.*, 2004; Petersen *et al.*, 2005).

Hatched blastocysts attached to the culture dish and showed cellular outgrowth after 2 days post-hatching. Best results concerning trophoblast outgrowth and distinct cluster of ICM cells were obtained from 2-cell and 4-cell twin blastocysts. This has important implications for future attempts to establish embryonic stem (ES) cells from ICM cells of twin blastocysts. As has been shown previously for mouse and human embryos, ICM cells derived from blastocysts can be utilized to establish ES cells under in-vitro culture conditions (Evans and Kaufmann, 1981; Thompson *et al.*, 1998).

From comparative studies on mouse embryo twinning, it is concluded that for future applications of human embryo splitting in assisted reproduction, the 2-cell and 4-cell stage twinning seems to be the most promising approach. In sheep (Willadsen, 1980), mouse (Tsunoda and McLaren, 1983), horse (Allen and Pashen, 1984) and cattle (Johnson *et al.*, 1995), blastomeres biopsied from 2-cell and 4-cell embryos have shown to be totipotent and can give rise to healthy offspring, including twins and multiples. The biopsy procedure as described in this study can, in principle, be applied to many species, with adequate modifications appropriate to the species involved. One example would be the exclusion of cytochalasin B from the protocol when applying this procedure to human embryos.

About a decade ago, the merits of embryo splitting had already been acknowledged as a valuable future application in reproductive medicine (Jones *et al.*, 1994). Embryo splitting in assisted reproduction 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 intracytoplasmic sperm injection. 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. In poor responders, there may be inherent genetic abnormalities, such as heteroploidy, that may be carried over into the twin embryos. For these patients, it is recommended that the embryos be screened via routine preimplantation genetic diagnosis (PGD) to eliminate any abnormal embryos from the pool of embryos available for transfer. PGD has been shown to be safe and efficient and transferring normal screened embryos yields higher take-home baby rates (Kuliev and Verlinksy, 2004; Gianaroli *et al.*, 2005). Furthermore, embryos that show binucleated and/or multinucleated blastomeres need to be evaluated prior to embryo transfer and which may impact on pregnancy potential (Meriano *et al.*, 2004). Since identical twins are often born independently of assisted reproduction 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 byproduct from 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 problem in 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 (ASRM), it has been stated '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' (2004). According to these recommendations, the splitting biopsy procedure as developed and described in this study did not reveal adverse effects on the developmental capacity of mouse twin embryos, and therefore it is these authors opinion that with further modifications and proper safeguards employed, such embryo splitting efforts could have potential applications in humans.

Acknowledgements

The authors wish to thank Embryotech Laboratories (Wilmington, MA, USA) for donating 2-cell mouse embryos used in this study.

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Received 15 July 2005; refereed 25 July 2005; accepted 29 September 2005.