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Review

## Cloning in cattle: from embryo splitting to somatic nuclear transfer

Yvan Heyman<sup>a\*</sup>, Xavier Vignon<sup>b</sup>, Patrick Chesné<sup>a</sup>,  
Daniel Le Bourhis<sup>c</sup>, Jacques Marchal<sup>d</sup>, Jean-Paul Renard<sup>a</sup>

<sup>a</sup> Unité de biologie du développement, Inra, 78352 Jouy-en-Josas cedex, France

<sup>b</sup> Unité de biologie cellulaire et microscopie électronique, Inra, 78352 Jouy-en-Josas cedex, France

<sup>c</sup> Services techniques, UNCEIA, 13, rue Jouët, 94703 Maisons-Alfort, France

<sup>d</sup> Unité expérimentale de Bressonvilliers, Inra, 91630 Leudeville, France

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**Abstract** — The ability to obtain genetically identical offspring in cattle (clones) is useful for research and for potential applications to breeding schemes. Experimental possibilities for generating such animals have evolved considerably in the last two decades. Embryo splitting has become a relatively simple technique but is limited to twinning. Embryonic nuclear transfer has improved and is associated with sexing to generate sets of clones despite a great variability of results between parent embryos. The factors of progress are reviewed here. Recently, somatic cells used as a source of nuclei in bovine nuclear transfer has been demonstrated. Here we present the results of the developmental potential of nuclei from skin and muscle cells. © Inra/Elsevier, Paris.

**bovine / embryo cloning / somatic cloning / nuclear transfer**

**Résumé** — **Clonage chez les bovins : de la bissection d'embryons jusqu'au transfert de noyaux somatiques.** L'obtention de bovins génétiquement identiques (clones) est utile pour la recherche et les applications potentielles en sélection. Les possibilités expérimentales de produire de tels animaux ont considérablement évolué depuis 10 ans. La bissection d'embryons est devenue une technique relativement simple mais elle est limitée à l'obtention de jumeaux. Le clonage embryonnaire a progressé et peut être associé au sexage pour faire naître des clones de veaux, malgré une grande variabilité selon les embryons donneurs. Les facteurs de progrès sont analysés dans cette revue. Récemment l'aptitude de noyaux somatiques à être reprogrammés après transfert nucléaire a été démontrée et nous présentons les premiers résultats obtenus à partir de cellules de peau ou de muscle. © Inra Elsevier, Paris.

**bovin / clonage embryonnaire / clonage somatique / transfert de noyaux**

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\* Correspondence and reprints  
E-mail: heyman@biotec.jouy.inra.fr

## 1. INTRODUCTION

The possibility of generating genetically identical offspring in cattle has evolved considerably during the last two decades from the first embryo splitting experiments that provided twin calves, up to the very recent results obtained after the 'Dolly breakthrough' achieved with somatic cloning. This opens up new fields of applications for genetically identical animals primarily for medical use when associated with transgenesis. Somatic nuclear transfer using modified cell lines could become an efficient way to generate transgenic animals for medical protein production that would benefit the society. Bovine clones are also interesting as animal models for research and in the future, they could be of potential use to improve genetic selection. The aim of this article is to review the progress and evolution of the methods which are both the results of basic studies on nucleocytoplasmic interactions and technical improvements.

## 2. TWINNING BY BLASTOMERE SEPARATION

Under normal reproductive conditions, the birth of identical twin offspring accounts for less than 0.2 % of all births in cattle [20]. In 1979, the pioneer work of Willadsen [54] in sheep demonstrated the possibility of obtaining artificially produced monozygotic sheep twins developed from microsurgically separated blastomeres from two-cell embryos. This first success in sheep has initiated many experiments in cattle in order to achieve relatively simple methods of generating monozygotic twin calves. Blastomere separation from four and eight cell stage embryos into two groups of two and four blastomeres, respectively, showed that such 'half embryos' appeared to possess normal viability when embedded in agarships before an initial transfer to ligated oviducts of intermediate recipient ewes, followed by definitive transfer to the cow

uterus [55]. To avoid this delicate step of intermediate recipients, several groups have attempted to separate bovine embryos at later stages of development from day 6 compacted morula to expanded day 8 blastocysts [27, 36] using splitting with a microblade. At these development stages the two half embryos can be transferred directly and non-surgically to the uterus of the recipient heifers. The splitting is surprisingly efficient. A retrospective literature analysis indicates that, provided grade 1 embryos are used, more than 90 % of the embryos result in pairs of half embryos that rapidly form a mini blastocyst in culture. When such blastocysts are transferred by pairs to the same recipient, the twinning rate is around 40 % of the established pregnancies. When used with normal embryo transfer procedures, this technique provides a method for routine production of identical twin calves for experimental purposes. It can also improve the efficiency of embryo transfers in breeding schemes when limited numbers of highly valuable embryos are available after superovulation since splitting can nearly double the number of calves from embryo transfer. In a former study [53], 150 calves were produced from 143 embryos split at the compacted morula or blastocyst stage (105 %). Recently at Inra (France), 18 sets of twin calves and 16 singles were obtained in one experiment of simple splitting of 60 expanded day 8 blastocysts (87 %, Chesné, unpublished data). All the twins are currently involved in a research programme concerning the clearance of pharmaceutical compounds. One of the twins is kept as the control animal, thus the variability of the results can be analysed with a more powerful test which allows reduction of the overall number of animals needed in the experiment.

The potential of embryo splitting is limited, however, and does not provide more than two identical animals. A previous attempt to divide morula or blastocysts into quarter embryos failed to produce quadruplets and a maximum of two calves were

obtained by quartering a bovine morula [52]. Another approach using blastomere separation and culture of isolated blastomeres exceptionally produced one set of triplets from 8 cell stage embryos [55]. One set of quadruplets was reported in Canada after culture and transfer of blastomeres from a four cell stage embryo [21]; however, this result could not be reproduced (Betteridge, pers. comm.). Due to the very early loss of cellular totipotency in mammalian embryos, the only way to increase the number of identical offspring was to use nuclear totipotency after nuclear transfer to recipient cytoplasms.

### **3. EMBRYO CLONING BY NUCLEAR TRANSFER**

Much progress has been achieved since the first report in 1987 of two calves born from nuclear transfer of blastomeres of eight to 16 cell stage bovine embryos into enucleated oocytes [38]. Nuclear totipotency has been assessed for different developmental stages of donor embryos. Nuclei from a late compact morula stage can support the development of nuclear transfer embryos into blastocysts at a rate similar (about 30 %) to that of nuclei from earlier stage morula [16, 56]. Then the more advanced morula stage embryos, used as donor embryos, result in a higher mean number of cloned blastocysts per parent embryo. After the first differentiation into blastocysts, however, embryonic nuclei have limited potential for nuclear transfer as inner cell mass (ICM) cells for bovine cloning result in a low rate (3–7 %) of development to blastocysts [9, 23].

#### **3.1. Oocyte cytoplasm improvement**

One of the principal improvements in embryo cloning by nuclear transfer concerns the recipient cytoplasm with the use of in vitro matured oocytes in cattle. The influ-

ence of the recipient oocyte cell cycle stage on deoxyribonucleic acid (DNA) synthesis, nuclear envelope breakdown and chromatin behaviour has been intensively studied [2, 5], indicating that DNA synthesis is regulated differently if the recipient cytoplasm is in the metaphase II (MII) or S phase at the time of fusion. Consequently, the decay of the maturation promoting factor (MPF) activity in recipient cytoplasm was found to be favourable for nuclear remodelling of most of the embryonic donor nuclei which are in a high interphasic proportion in a given parent embryo. In the rabbit model, studies conducted on the behaviour of nuclei transferred to different cytoplasms [1] clearly showed that cytoplasms of MII oocytes close to interphase, with a low MPF activity, are able to remodel the blastomere nucleus even though the nuclear envelope is not broken down. Furthermore, NT embryos develop better into blastocysts when premature chromosome condensation is avoided. For bovine oocytes several pre-activation treatments have been developed to provide cytoplasms with a low level of MPF at the time of fusion. After enucleation of the oocyte at the MII stage, a further period of a 12 h in vitro culture followed by cooling [7] allows the cytoplasm to be in a pre-activated stage in which MPF activity has decreased (as revealed by the H1 kinase assay) but the MAPKinase remains elevated [13]. Currently for bovine nuclear transfer, the recipient oocyte matured in vitro is enucleated and generally submitted to an activation treatment before fusion with the blastomere. Oocyte activation may be induced by electric stimulus [24], or chemical treatments such as ethanol/cycloheximide [39] or ionophore followed by 6-dimethylaminopurine (6-DMAP) incubation [32]. The use of a preactivated recipient cytoplasm at the time of nucleus introduction resulted in improved development of the nuclear transfer embryos into blastocysts in vitro [11, 25, 44].

### **3.2. Development stage of donor embryos**

In the donor embryo produced either in vivo or in vitro, the early embryonic process is characterized by an asynchronous cleavage which increases progressively among blastomeres throughout preimplantation development. Thus, within the same parent embryo at the morula stage, nuclei can be at different cell cycle stages at the time of reconstitution and fusion with the recipient oocyte cytoplasm. This could partly explain the great variations observed in the in vitro development of series of nuclear transfer embryos depending on the parent morula used. In bovine nuclear transfer, attempts to synchronize blastomere nuclei in the same cell cycle stage to better fit the recipient oocyte cytoplasm have not yet proved efficient. The use of nocodazole [48] to block bovine blastomere nuclei in the metaphase stage allows most of the cells to enter G1 after the release of the drug. This treatment, however, remains toxic and further development after nuclear transfer is impaired (pers. obs.). Similarly, an inhibitor of MPF (6-DMAP) can be used to synchronize nuclear donor cells at late G2 phase [40], but again, further development is reduced by this agent compared to controls. G1 nuclei should develop better when transferred into MII recipient oocytes than other cycle stages, as demonstrated in the rabbit species [10]. In bovine embryos used as donors, most blastomeres (about 80 %) are in the S phase at any specific time of development, so in practice, preactivated oocytes are routinely used as host cytoplasts so that their low MPF activity is beneficial to the nuclear membrane maintenance, protecting the donor chromatin from harmful effects caused by cell cycle asynchrony [5].

### **3.3. Evaluation and improvement of in vitro culture methods**

The progress in in vitro culture methods for in vitro fertilized (IVF) embryos during

the last few years has also benefited the development of nuclear transfer embryos which are successfully developed in vitro up to the blastocyst stage in different culture systems (SOF, CR1 medium) or co-culture systems with Vero cells [34]. Bovine nuclear transfer blastocysts developed entirely in vitro have been assessed in our laboratory by nuclei counting and morphological evaluation using the electron microscopy. These blastocysts did not differ from control IVF embryos in terms of developmental kinetics, cell number or degree of differentiation [18]. However, further in vivo development of NT blastocysts after transfer to recipient uteri is still limited and somewhat lower than that reported for IVF embryos [15]. Increased embryonic or foetal mortality has been reported previously [4, 17]. A survey of at least 200 transfers of cloned embryos in the experimental farm of our institute indicates a decrease of the confirmed pregnancy rate from 49.5 % by day 35 of pregnancy to a calving rate of 31.4 %. Eighty percent in the calves born survived 2 weeks after calving [19]. It is also noteworthy that some neonatal anomalies such as the 'big calf syndrome' were observed [14] with different incidences depending on the laboratories and culture conditions used [26].

### **3.4. Multiple generational embryo cloning**

One way to increase the number of blastocysts derived from one parent embryo is to recycle first-generation nuclear transfer embryos as nuclear donors for a second- or third-generation nuclear transfer. Several reports indicate that the in vitro development rates are not decreased after recycling first-generation nuclear transfer embryos [43]. Furthermore, cloned blastocysts have been produced even after six consecutive nuclear transfers. Data on viability of such embryos and on their development into live calves are limited, however. The birth of calves has been reported for second- and

third-generation NT embryos [4, 12, 30], but pregnancy rates seem to be dramatically reduced for third-generation NT embryos [43] and to our knowledge, no term calves have yet been reported for bovine embryos cloned past the fourth generation. In 1996, Takano et al. [47] reported that NT blastocysts of the fifth generation were able to initiate pregnancy when transferred to recipients but resulted in abortion. It was also suggested that the incidence of chromosomal abnormalities may have increased with repeated nuclear transfer and extension of the culture period.

Re-cloning experiments, however, indicate a high variability between different parent embryos to support development by cloning; a maximum of 43 cloned blastocysts were produced from one morula while other donor morula yielded only five blastocysts after repeated nuclear transfer [47]. Recent experiments in Australia [31, 37] combined multi-generational nuclear transfer and freezing by vitrification according to the open pulled straws (OPS) method [49] and reported the production of more than 50 identical embryo clones in three rounds of nuclear transfer. Nevertheless, to date, a limited number of calves have been born after transfer of vitrified multi-generational cloned embryos.

## **4. PROGRESS TOWARDS SOMATIC CLONING**

### **4.1. The use of cultured embryonic cells**

The number of available nuclei from a morula stage embryo is limited and this situation has generated research to investigate the nuclear totipotency of cultured embryonic cells in order to multiply the source of nuclei. Ever since it was demonstrated in sheep [42] and cattle [9, 22, 23] that nuclei isolated from inner cell mass of blastocysts were able to develop after nuclear transfer, several groups have attempted to culture

bovine ICM in order to establish stem cells. As yet, there has been no success in obtaining bovine ES cell lines, i.e. embryonic cells that are able to multiply in vitro without differentiation and while maintaining their totipotency. In 1993, Sims and First [41] established cell lines from immunosurgically isolated ICM of three blastocysts and cultured them as a low cell density suspension to prevent aggregation and differentiation for 6 to 100 days before nuclear transfer. Some cell lines had multiplied to more than 2 000 cells and after nuclear transfer yielded 0 to 40 % blastocysts according to the cell line. Four calves were born after transfer of such blastocysts derived from cultured IMCs. Another group [45, 46] established that nuclear transfer embryos derived from bovine pluripotent stem cells could establish pregnancies and foetal development up to 55 days at which time the pregnancies were lost. Placental deficiencies were observed in these initiated pregnancies, indicating that improper reprogramming of certain developmental genes may occur and limit the potential of generating offspring from such pluripotent embryonic cell lines [45].

### **4.2. Attempts with gonial cells**

Another possibility was investigated [33] using germ cells as a source of nuclei. Such cells are known to be pluripotent at least in the mouse. Gonial cells are diploid and can be isolated in large numbers from bovine foetal gonads [28]. The pluripotency of bovine gonial cells has been tested through nuclear transfer by three separate laboratories, leading to the temporary conclusion that germ cells can direct in vitro development up to blastocysts in some cases (2–4 %), but no live offspring could be obtained after transfer to recipient heifers. Only two cases of initiated pregnancy were reported [29, 35] but both were lost by days 35 to 40 and full nuclear totipotency of bovine diploid germ cells remains to be established.

### 4.3. First results with differentiated cells

A report in sheep on the birth of three viable offspring derived from nuclei of foetal fibroblasts [6] has generated new insights into cloning research. In that experiment, the foetal cells were obtained from a mixture of tissues of eviscerated young foetuses and it could not be excluded that part of the cell population in the primary culture was not somatic. In cattle we have explored the potential for the reprogramming of nuclei from cultured cells obtained from very specific somatic tissues, to avoid any contamination with embryonic or germinal cells. Two types of explants were used: muscle and skin which were collected from day 50 to 80 foetuses or from newborn calves. Skin and muscle biopsies were cultured over six to 12 passages and characterized immunohistochemically before nuclear transfer. The markers used were antibodies to cytoplasmic or nuclear intermediate filaments, such as lamina A/C, cytokeratin or vimentin, and clearly indicated that at the time of nuclear transfer, the cultured bovine cells grown from explants of muscle and skin were in a differentiated state and largely fibroblastic [50]. The potential for development of embryos reconstructed with nuclei of muscle or skin origin was assessed in vitro by their development into blastocysts. The blastocyst rate ranged from 2 to 14 % according to the cell line used but, globally, the skin cell used as a source of nuclei yielded significantly more blastocysts than muscle cells (8.7 versus 4.1 %, respectively). Data are still scarce concerning in vivo development of somatic cloning in cattle throughout the world. In our laboratory, several pregnancies were established and three live calves were born and were apparently normal. Four other calves are expected, which are derived from both kinds of somatic cells, demonstrating full-term reprogramming of clearly differentiated cells in cattle [51]. A limited number of other calves have been born in the United States

[8] and New Zealand (Wells, pers. comm.) which are derived from cells of transgenic foetuses. Cibelli et al. [8] reported three healthy identical transgenic calves that were generated through nuclear transfer from a foetal fibroblast cell line that had been genetically modified by transfection with a marker construct of a  $\beta$ -galactosidase-neomycin resistance fusion gene. From 276 nuclear transfer embryos made from fibroblasts of a neomycin resistant colony, 33 blastocysts were obtained and resulted in three live offspring. Contrary to the report on sheep [6] which claimed that cell quiescence is a prerequisite for somatic nuclear transfer, we and others [8, 51] have shown in cattle that full-term development can also be achieved from nuclei taken from non-confluent cultured cells, without serum starvation that induces G0 exit. In our experiments, we compared the in vitro development of somatic nuclear transfer embryos made from quiescent (serum deprived) donor cells to cycling (not deprived) donor cells from skin or muscle biopsies. In both cases the blastocyst rates were similar and pregnancies were established, indicating that complete reprogramming can be achieved in the nucleus of cycling and actively dividing somatic cells.

## 5. CONCLUSIONS AND PERSPECTIVES

Somatic cloning introduces the possibility to combine more intensively nuclear transfer and transgenesis in large domestic species such as cattle. In the past years, transgenic livestock has been produced by microinjections of DNA into the pronuclei of fertilized oocytes. However, only a small proportion of animals integrated the transgene DNA into their genome, thus making this approach rather expensive and of low efficiency. During in vitro culture of somatic cells over several passages it is possible to stably transfect the cells with a construct containing, for example, a human gene

linked to a promoter known to provide a high expression in the mammary gland. Then, a co-transfection of cells in culture with a selectable marker makes it possible to isolate the transgenic cell clone. These selected cells which are all transgenic, can be used as a source of nuclei for somatic nuclear transfer to generate more efficiently transgenic embryos and offspring. In the future, precise genetic modifications will be achieved by site-specific recombination in the somatic cells before nuclear transfer. This will broaden the scope of applications of transgenic cattle and introduce new opportunities for genetic studies in order to define the role of any gene product and the mechanisms that regulate gene expression.

Much progress has been made since the first generation of identical twin calves by blastomere separation to the very recent birth of calves derived from somatic cloning. Research efforts have to be focused on basic studies to understand the reprogramming of somatic nuclei for improved efficiency, but also on the follow-up of pregnancies and calves derived from somatic nuclear transfer. Some developmental problems found in non-surviving calves could be attributed to abnormal placentation and these pathology risks must be carefully investigated in order to develop cloning techniques that are safe and that generate normal and viable offspring.

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