

# Retrotransposon expression as a defining event of genome reprogramming in fertilized and cloned bovine embryos

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## Abstract

Genome reprogramming is the ability of a nucleus to modify its epigenetic characteristics and gene expression pattern when placed in a new environment. Low efficiency of mammalian cloning is attributed to the incomplete and aberrant nature of genome reprogramming after somatic cell nuclear transfer (SCNT) in oocytes. To date, the aspects of genome reprogramming critical for full-term development after SCNT remain poorly understood. To identify the key elements of this process, changes in gene expression during maternal-to-embryonic transition in normal bovine embryos and changes in gene expression between donor cells and SCNT embryos were compared using a new cDNA array dedicated to embryonic genome transcriptional activation in the bovine. Three groups of transcripts were mostly affected during somatic reprogramming: endogenous terminal repeat (LTR) retrotransposons and mitochondrial transcripts were up-regulated, while genes encoding ribosomal proteins were downregulated. These unexpected data demonstrate specific categories of transcripts most sensitive to somatic reprogramming and likely affecting viability of SCNT embryos. Importantly, massive transcriptional activation of LTR retrotransposons resulted in similar levels of their transcripts in SCNT and fertilized embryos. Taken together, these results open a new avenue in the quest to understand nuclear reprogramming driven by oocyte cytoplasm.

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## Introduction

During normal preimplantation development in mammals, reprogramming is defined as extensive epigenetic modifications of differentiated gamete nuclei by the ooplasm that transforms them to a totipotent embryonic nucleus. In somatic cell nuclear transfer (SCNT) experiments, reprogramming refers to the transformation of somatic nuclei by the ooplasm to the state, which allows recapitulation of normal developmental process and gene expression programs (Yang *et al.* 2007, Niemann *et al.* 2008). These reprogramming events, dominated by the oocyte cytoplasm, affect fundamentally different genomes: while during fertilization, reprogramming occurs to two transcriptionally silent haploid gametic genomes 'designed' for this process by nature, after SCNT a transcriptionally active diploid somatic genome is reprogrammed. In both cases, the extent of ooplasm-driven reprogramming first manifests during transcriptional activation of the embryonic genome (EGA; Vassena *et al.* 2007). Indeed, during normal development EGA leads to a transition from development determined by maternal factors to the

'autonomous' embryonic control of development, which is mainly regulated at the transcriptional level.

Incomplete reprogramming of the somatic nucleus and misexpression of critical regulatory genes are considered the main reasons for developmental failures in clones (Bortvin *et al.* 2003, Gao *et al.* 2003, Santos *et al.* 2003). However, gene expression program of early mammalian embryos is quite flexible. For example, while *in vitro* culture (IVC) conditions greatly affect early transcriptomes of *in vitro*- versus *in vivo*-developed embryos, *in vitro* development remains compatible with full-term development (Wrenzycki *et al.* 2001, Corcoran *et al.* 2007). Consequently, a defining set of features manifesting the 'adequate' genome reprogramming remains a puzzle due to this gene expression plasticity.

To resolve this, we used a novel strategy to define the properties of the ooplasm reprogramming activity. First, two transitions in gene expression programs were analyzed and contrasted: the maternal-to-embryonic transition (MET) in normal embryos, and 'somatic' reprogramming of the SCNT donor nuclei before nuclear transfer (NT) and after EGA. Secondly, previous studies

demonstrated that EGA invokes expression of a very specific subset of genes and genomic loci (Evsikov *et al.* 2004, Peaston *et al.* 2004); therefore, we constructed an EGA-specific cDNA array that allowed focusing on the reprogramming of genes specifically expressed during this stage of development. In bovine embryos, EGA is 'progressive' in that MET extends over 3–4 cell cycles, and major wave of transcriptional activation occurs at the 8–16 cell stage (Camous *et al.* 1986), while in the mouse embryos, it spans over a single-cell cycle (Bensaude & Morange 1983). Moreover, the efficiency of NT experiments in bovine expressed as the percentage of NT blastocysts developing to term is about five times higher than in the mouse (Yang *et al.* 2007). For these reasons, bovine oocytes and early embryos were chosen as a superior model to conduct these studies.

## Results

To compare gene reprogramming in SCNT embryos (somatic reprogramming) with that occurring during MET in control embryos (MET reprogramming), SCNT embryos produced by oocyte *in vitro* maturation (IVM), followed by NT and IVC and control embryos produced by IVM, followed by IVF and IVC were used. This allowed alleviating the potential biases in the comparison of gene expression patterns due to IVC conditions. To factor in the epigenotype of a somatic nucleus as another variable, somatic reprogramming was analyzed on three types of SCNT embryos produced from different donor cell lines (OV0029, OV5538, and OV7711). All three cell lines provided high rates of embryonic development to the blastocyst stage after NT (32.8–42.2%). The corresponding SCNT embryo types were able to efficiently initiate pregnancy following their transfer at the blastocyst stage into foster recipients (D35 pregnancy rates ranging from 20.3 to 51.9%) and to produce healthy calves, although with different abilities (1.8, 7.8 and 12.7% for OV0029, OV7711, and OV5538 respectively over the period when SCNT embryos were produced for the molecular analyses). To account for the uniqueness of early embryonic transcriptome, we constructed and used an array specifically dedicated to early gene expression in the bovine.

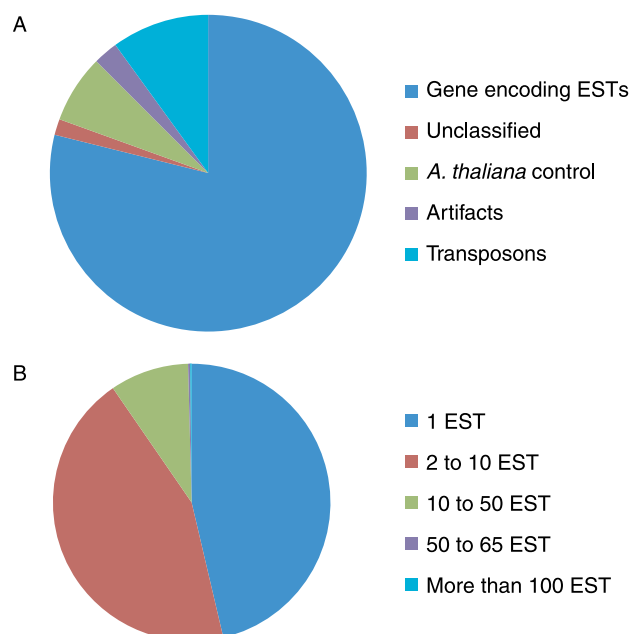
### Construction of a dedicated cDNA array

To obtain a cDNA library dedicated to early bovine embryonic transcription at EGA, we subtracted cDNAs from early post-MET embryos by cDNAs from late pre-MET embryos. We chose the morula (20–30 cell, grade 1 morulae) as the early post-MET stage because an increase in embryonic transcription is detected at this stage. The four-cell stage was chosen as the late pre-MET stage because it is the latest stage containing mainly residual maternal transcripts just before EGA

(Duranthon & Renard 2003). This work was done by suppressive subtractive hybridization (SSH). Analysis of the quality of this library demonstrated that even though it still contained maternally expressed transcripts, it was greatly enriched in embryo-specific transcripts and contained very rare embryonic transcripts (Bui *et al.* 2005). From this library we randomly isolated 6530 clones and spotted an array with the corresponding PCR inserts. This embryo-dedicated array was further characterized by sequencing the ESTs. Sequence analysis showed that among these 6530 ESTs, 5231 correspond to 1189 unique genes, 662 encode transposable elements (mostly long terminal repeat (LTR) retrotransposons), 108 were unidentified, 167 represent artifact 'chimeric' sequences, and 463 correspond to the *Arabidopsis thaliana* cDNA present in the starting material (see Bui *et al.* 2005, and Materials and Methods; Fig. 1). For 1189 unique genes on the array, the number of EST probes varies from one (for 550 genes) to more than one hundred (for two genes). This corresponds to our previous analysis of efficiency for normalization by SSH, when applied to embryonic amplified material (Bui *et al.* 2005).

### Somatic nuclei are extensively reprogrammed by NT as soon as the morula stage

Somatic nucleus reprogramming was assessed by comparison of gene expression among somatic cells and corresponding SCNT morulae using the dedicated bovine EGA-specific cDNA array. Hybridization signals



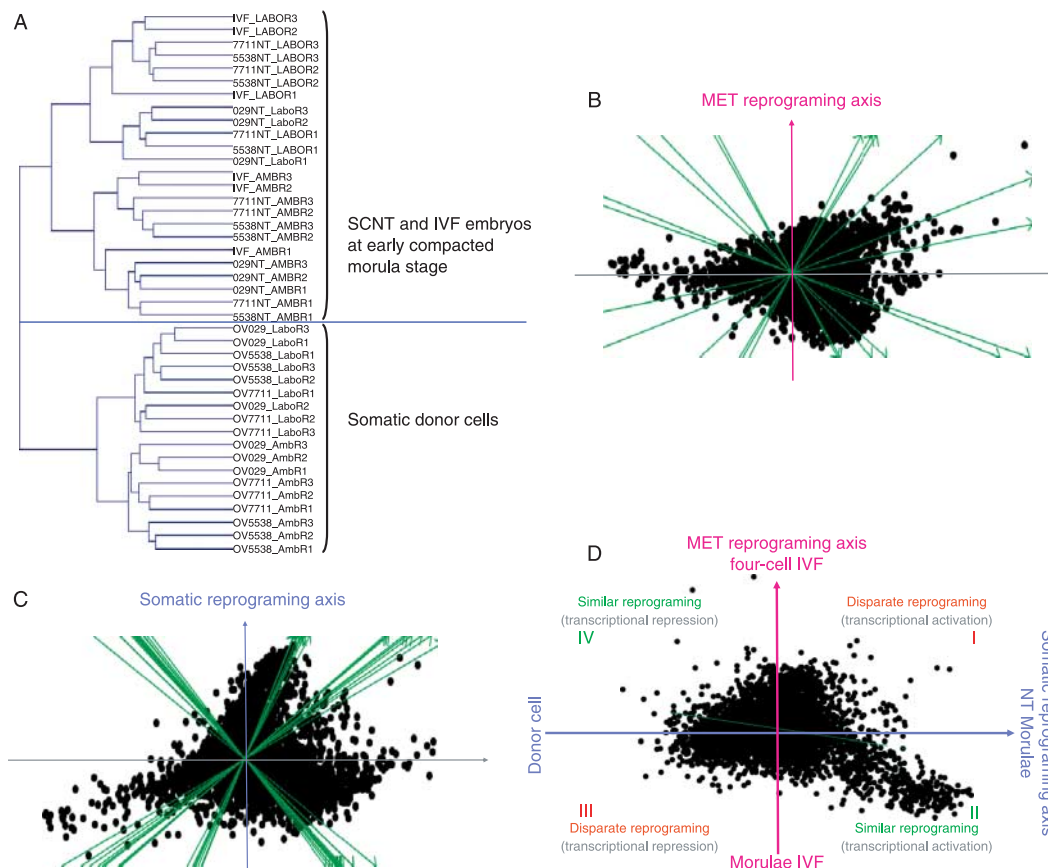
**Figure 1** Analysis of the EGA dedicated array. (A) Distribution of the 6523 arrayed ESTs among five broad categories. (B) Distribution of the array's ESTs among five groups according to their redundancy.

corresponding to the three different cell lines and the corresponding types of SCNT embryos were analyzed by ANOVA. Five variables were taken into account: array probe, epigenotype (i.e. donor cell line), nuclear state (i.e. somatic versus 'reprogrammed' SCNT embryonic), amplification method (see Materials and Methods), and experimental replication. We found that the 'probe-nuclear state' interaction was the most significant (Supplementary Table 1, which can be viewed online at [www.reproduction-online.org/supplemental/](http://www.reproduction-online.org/supplemental/)), and that the 'nuclear state' effect affected 4338 out of the 6523 probes (false discovery rate (FDR)=5%). These results highlight the dramatic extent of reprogramming after SCNT. Furthermore, hierarchical clustering of expression data obtained for SCNT and control embryos and somatic cells resulted in two major nodes – 'somatic' and 'embryonic' (Fig. 2A). Irrespective of the embryo types (control versus SCNT), their global gene expression

patterns were significantly different from those of donor cells. These data indicate that SCNT reprogramming instigates a gene expression program similar to control embryos by EGA at the morula stage.

### Dynamic comparison of somatic and MET reprogramming

To characterize the genome reprogramming in control bovine embryos (MET reprogramming), differential screening of EGA-specific array was performed using RNA of the four-cell-stage embryos (before MET) and early compacted morulae (after MET). Three independent replications and two different amplification methods of each RNA sample were analyzed by principal component analysis (PCA). This analysis evidenced the 'MET reprogramming axis' as the second most important axis displaying 13% of total variability. It separated the probes for genes overexpressed in embryos



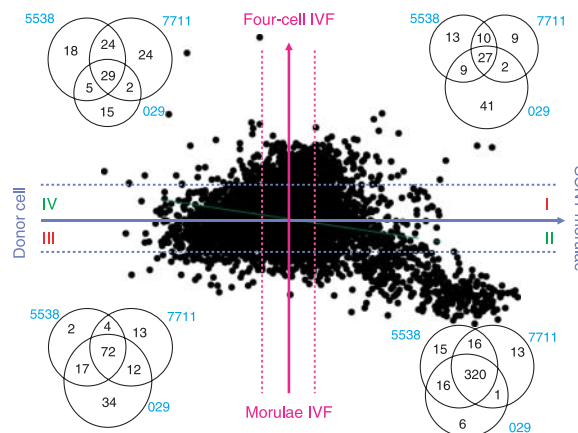
**Figure 2** Comparison of somatic and MET reprogramming. (A) Hierarchical cluster taking into account the three somatic donor cells (OV55838, OV7711, OV029), SCNT (5538NT, 7711NT, 029NT), and IVF (FIV) morula-stage embryos, three repetitions (R1, R2, and R3) and two amplification methods (Amb for Ambion and Labo for cDNA Laboratory protocol). The cluster of 6523 ESTs highlights extensive reprogramming of somatic donor nuclei by transfer to oocyte cytoplasm leading to an 'IVF-like' gene expression pattern. (B) PCA analysis of MET in control IVF embryos: differential screening between four-cell and morula stages. Horizontal axis corresponds to the analyzed genes. Vertical axis separates the ESTs overexpressed in morulae-stage embryos (negative coordinate) from those overexpressed in four-cell stage (positive coordinate). This axis is called MET reprogramming axis. (C) PCA analysis of somatic reprogramming: differential screening of three SCNT morulae and their corresponding donor cells. Horizontal axis corresponds to the analyzed genes. Vertical axis separates the ESTs overexpressed in SCNT morulae (positive coordinates) from those overexpressed in their donor cells (negative coordinates). This axis is called 'somatic reprogramming axis'. (D) Four distinct reprogramming behaviors evidenced by comparison of somatic and MET reprogramming.

at the morula stage (negative coordinates) from those overexpressed in embryos at the four-cell stage (positive coordinates; Fig. 2B and Supplementary Table 2, which can be viewed online at [www.reproduction-online.org/supplemental/](http://www.reproduction-online.org/supplemental/)). Similarly, genome reprogramming after SCNT was analyzed using RNA from three different cell lines and three different SCNT morulae types. PCA analysis of these data permitted separating the probes overexpressed in donor cells (negative coordinates) from those overexpressed in SCNT-embryos (positive coordinates) and to define the 'somatic reprogramming axis' displaying 16% of total variability (Fig. 2C and Supplementary Table 3, which can be viewed online at [www.reproduction-online.org/supplemental/](http://www.reproduction-online.org/supplemental/)). These two axes allow characterizing the behavior of any gene on the array simultaneously in either SCNT or MET transition.

The data for all 6523 array probes were plotted on the plane defined by the 'MET reprogramming' and 'somatic reprogramming' axes to compare the SCNT and MET reprogramming processes. This analysis exposed four distinct groups of genes with different 'reprogramming behaviors'. Specifically, all array probes in this plane belong to one of the four quadrants (Fig. 2D). The first and third quadrants contain probes with a disparate pattern of reprogramming during somatic and MET reprogramming. Namely, transcripts in first quadrant are up-regulated in SCNT morulae and four-cell-stage control embryos when compared with donor cells and control morulae respectively; the third quadrant contains probes for transcripts up-regulated in control morulae and donor cell lines, when compared with four-cell-stage control embryos and SCNT morulae respectively. The second and fourth quadrants contain probes with similar pattern of reprogramming over somatic and MET reprogramming. The second quadrant contains probes up-regulated in both SCNT and control morulae compared with donor cell lines and four-cell-stage embryos respectively, while the fourth quadrant contains probes up-regulated in four-cell-stage control embryos and somatic donor cells, compared with control and SCNT morulae.

### Correlations among reprogramming, gene expression, and gene function

To identify the transcripts that have similar reprogramming behaviors irrespective of the donor cell epigenotype, three independent PCAs were performed with the data on individual donor cell lines and corresponding SCNT embryos. Scatter plots of data points displayed very similar shapes (not shown). The probes whose absolute values along both axes were higher than one s.d. were identified for each donor cell line. Of these, reprogramming behavior for 27, 320, 72, and 29 probes in quadrants I–IV respectively were shared by all three SCNT embryo types and donor cells (Fig. 3). EST identities



**Figure 3** Identification of transcripts with similar reprogramming behavior irrespective of donor cell epigenotype. Number of probes displayed by each SCNT embryo type in each quadrant were reported in Venn diagrams: only probes whose absolute values along both axes were higher than one s.d. were taken into account. 27, 320, 72, and 29 probes in quadrants I–IV respectively were shared by all three SCNT embryo types.

corresponding to these probes provided further support for nonrandom distribution for specific classes of transcripts among four quadrants. Seven out of the 27 commonly expressed probes in quadrant I correspond to mitochondrial sequences. In quadrant II, 236 of the 320 commonly expressed probes represent LTR retrotransposons. Of 72 probes in quadrant III, 52 correspond to transcripts of 16 different ribosomal proteins (L3, L10a, L12, L14, L23, L30, L34, L35, L38, S15a, S19, S23, S25, S27, S29, and S16-2like respectively). No overt bias was found for the types of transcripts represented by 29 probes in quadrant IV. These data indicate that: a) somatic reprogramming results in an up-regulation of mitochondrial transcripts and a repression of genes encoding ribosomal proteins in SCNT embryos compared with donor cells, which is opposite to the behavior of these categories of genes during MET reprogramming and b) transcriptional activation of endogenous LTR retrotransposons is a major feature of both somatic and MET reprogramming.

### Retrotransposon activation during MET and somatic reprogramming

However, these results remained qualitative due to the unequal redundancy in the array (Fig. 1). Because one of the most striking results was the reprogramming of LTR transcripts, we focused on retrotransposon transcriptional activation within the context of both MET and somatic reprogramming. The 236 ESTs encoding LTR sequences present in quadrant 2 belong to three different types of retrotransposons, specifically identified in RepBase (Jurka *et al.* 2005) as ERV1-1\_BT, ERV1-2\_BT, and BTLTR1 (Table 1). We focused on ERV1\_1\_BT because it was the retrotransposon with the highest

**Table 1** Number of ESTs encoding for each retrotransposon sequence in quadrant II.

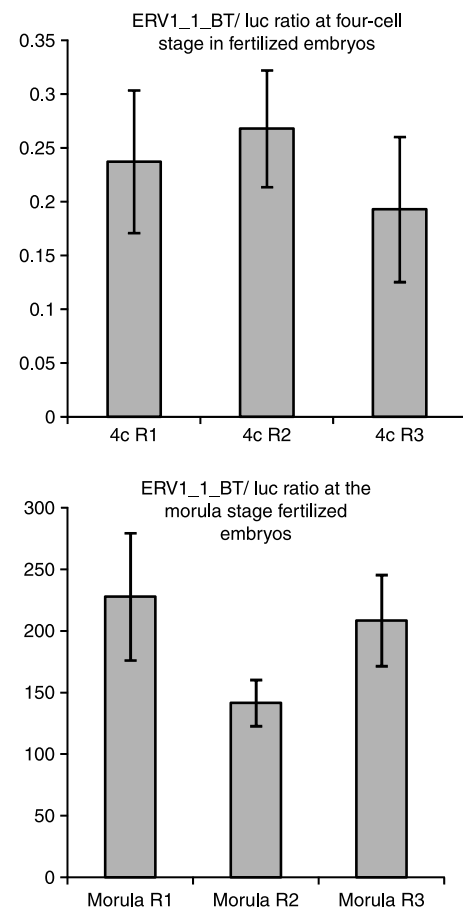
RepBase name (Jurka <i>et al.</i> 2005)	Estimated number of full-length elements in the genome	Total number of ESTs on the array	Number of ESTs in quadrant II (% of total)
BTLTR1	80	3	1 (33)
ERV1-1-LTR_BT	130	445	213 (47)
ERV1-2-LTR_BT	110	62	23 (37)

proportion of array's ESTs present in quadrant 2, irrespective of the epigenotype involved in somatic reprogramming. In order to quantify the extent of its transcriptional activation over both reprogramming processes, we performed quantitative RT-PCR experiments on the four-cell- and morula-stage control IVF embryos, as well as on the donor cells and the SCNT embryos. ERV1\_1\_BT transcripts were first quantified starting from batches of ten control four-cell or morula embryos. Three independent embryo pools were used for each category of embryos to provide independent replications, and each sample was analyzed in triplicate. By expressing quantification data as a ratio of ERV1\_1\_BT/luciferase reporter transcript, we observed a 1000-fold increase in the amount of ERV1\_1\_BT transcripts per embryo between the four-cell (pre-EGA) stage and the morula (post-EGA) stage (ratio values  $\sim 0.2$  and  $\sim 200$  respectively; Fig. 4). We then compared control IVF and 5538 SCNT morulae by performing three replications on three independent batches of embryos. The quantity of ERV1\_1\_BT transcripts expressed as ERV1\_1\_BT/luciferase ratios did not differ according to the type of embryos (Fig. 5), underscoring that cloned and fertilized morulae expressed ERV1\_1\_BT at the same level.

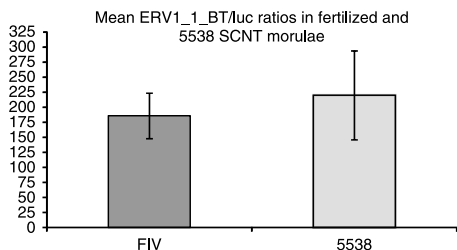
In order to quantify ERV1\_1\_BT transcripts in somatic donor cells, we used samples obtained by two-fold serial dilutions of cDNA synthesized from a cell suspension (from 30 000 to 468 cells). Such serial dilutions were performed thrice starting from three different culture dishes (Fig. 6). Linear regression analysis indicated that the mean number of ERV1\_1\_BT mRNA molecules per cell was about 100 ( $R^2 = 0.948$ ). Absolute quantification of ERV1\_1\_BT molecules in SCNT morulae was  $5.9 \pm 0.17 \times 10^6$  molecules. Since there are about 30 cells per morula, somatic reprogramming was accompanied by a 2000-fold increase in the number of ERV1\_1\_BT transcripts per transcribing nucleus. This somatic reprogramming resulted in the same amount of ERV1\_1\_BT transcript available in SCNT embryos as in fertilized ones at the morula stage. These data, thus faithfully recapitulated the results obtained by the comparison of MET and somatic reprogramming dynamics, and evidenced transcriptional activation of specific retrotransposon sequences as a major event delineating both MET and somatic reprogramming.

## Discussion

Studies of global changes in gene expression between SCNT and control embryos usually result in long lists of differentially expressed genes and are not successful in providing cues, or even reliable markers, for the process of ooplasm-driven genome reprogramming. Here, instead of direct comparison of SCNT to normal embryos, we sought to contrast the two types of genome reprogramming: the one that occurs to a somatic nucleus after SCNT and gametic reprogramming that occurs during normal MET. We analyzed and compared two shifts in gene expression patterns: the first one is MET itself, while the second is the transition from the somatic to embryonic pattern of gene expression. This bidirectional analysis allows delineating, with greater precision, the differences and similarities in the process of gametic versus somatic genome reprogramming, as reflected by the initial establishment of embryonic gene expression program at the morula stage. This analysis identifies genes with the greatest extent of expression changes during the SCNT and MET transitions, opposing them to



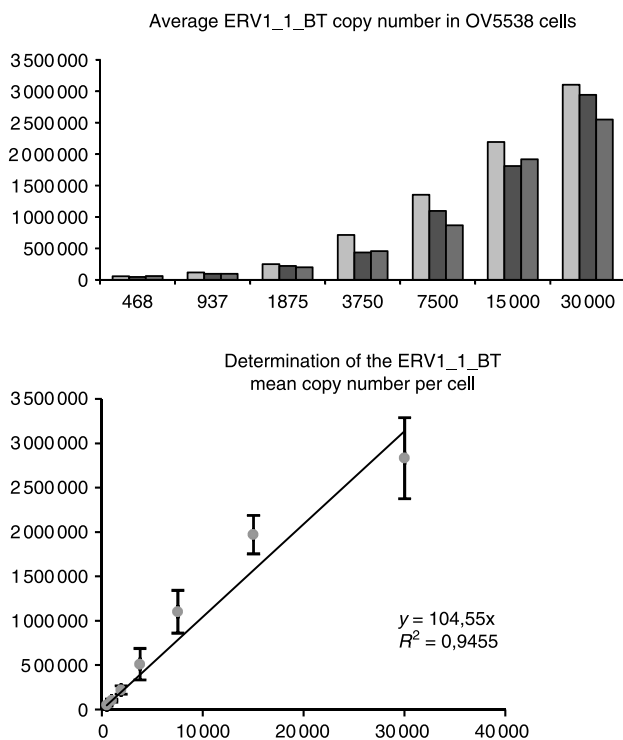
**Figure 4** ERV1\_1\_BT expression in four-cell and morula-fertilized embryos (quantitative RT-PCR results). At each stage, three batches (R1–R3) of IVM, IVF, IVC embryos were analyzed. Mean  $\pm$  s.d. for the ERV1\_1\_BT/luciferase reporter ratios were calculated for each batch of embryos.



**Figure 5** Comparison of ERV1\_1\_BT expression in fertilized and SCNT morulae. Three batches of fertilized and cloned morulae were analyzed. Mean  $\pm$  s.d. ERV1\_1\_BT/luciferase reporter ratios were calculated on the nine values obtained from three samples analyzed in triplicate.

those whose expression is unchanged. It focuses on new categories of genes that are the most sensitive to reprogramming processes and may thus be markers of their efficiency.

By comparing the SCNT embryo gene expression patterns with those of the nucleus donor cell lines and control embryos we established that gene expression patterns of SCNT embryos are different from the corresponding donor cells. Such differences were manifested just after the major EGA. Moreover, as soon as the early morula stage, gene expression pattern of SCNT embryos closely resemble that of control embryos. These results are in accord with the previous studies,



**Figure 6** ERV1\_1\_BT expression in somatic donor cells. (A) Quantification of ERV1\_1\_BT transcripts was performed on three independent serial two-fold dilutions of cDNA synthesized from cell suspensions. (B) Linear regression for the determination of a mean number of ERV1\_1\_BT copies per cell.

where efficient reprogramming after SCNT had been shown at the blastocyst stage (De Sousa *et al.* 1999, Smith *et al.* 2005). The data on earlier pre-blastocyst reprogramming events in SCNT embryos are scarce and deal with a handful of candidate genes and sometimes inconsistent results (Daniels *et al.* 2000, Winger *et al.* 2000, Park *et al.* 2003). Our results show, for the first time, that as early as the morula stage in the bovine, somatic nuclei have already undergone significant nuclear reprogramming that makes their gene expression program close to the embryonic one. This result differs from the analysis of mouse SCNT embryos. In mice, the pattern of gene expression in two-cell-stage SCNT embryos was reported to be grossly abnormal (Vassena *et al.* 2007). However, two important differences between these two mammalian systems may underlie these differences. First, in mouse embryos, the very early and abrupt EGA at the 2-cell stage narrows the window of opportunity for SCNT reprogramming comparing to the bovine, where the progressive nature of EGA provides a prolonged period (3–4 cell cycles) to ensure proper reprogramming. Secondly, the SCNT efficiency in mice is significantly lower (about five time less) than in bovine (Yang *et al.* 2007), and therefore ‘abnormality’ versus ‘normality’ of gene expression programs during EGA may be the ultimate determinants of the SCNT embryo viability.

Our analysis of genome reprogramming in the artificial epigenetic context of SCNT, when compared to the ‘normal’ context of fertilization (MET), produced four distinct categories of affected genes (Fig. 1D), which can be regrouped into either appropriate or disparate reprogramming.

Among the appropriately reprogrammed genes, those present in quadrant two (Fig. 2D) represent the genes that are typical for EGA, but are downregulated in somatic cells or pre-EGA embryos. Intriguingly, the major proportion of array probes in this group corresponds to the transcripts of LTR transposons. Unexpectedly, the LTR transposons identified here (Table 1) belong to the Class I LTR sequences, while the major LTR transposon expressed during EGA in the mouse, MuERV-L, is a member of Class III LTR sequences (Evsikov *et al.* 2004, Peaston *et al.* 2004). Our stringent analysis of current bovine genome assembly indicates that these retro-transposons are represented by ~100 copies per haploid genome (Table 1). Our result is the first experimental confirmation of robust LTR transposon up-regulation during EGA in a nonmurine embryo. This expression had not been evidenced by previous analyses of the massive changes in the embryonic transcriptome accompanying EGA in the bovine. This may be explained by the use of nondedicated arrays (Misirlioglu *et al.* 2006, Kues *et al.* 2008), by the size of the custom array, or the precise methods used in custom array construction (Vigneault *et al.* 2009). Moreover, our results show that the expression of LTR transposons is faithfully recapitulated in bovine

SCNT embryos, where their transcriptional activation results in a quantity of expressed transcripts that does not differ from fertilized embryos. Apparently, this is not the case in the mouse, where 2-cell stage SCNT embryos do not express the EGA-associated LTR transposon Mu-ERVL at the levels comparable with controls (Suzuki *et al.* 2006). Given the dramatic differences in developmental capacity between mouse and bovine SCNT embryos, we hypothesize that LTR transposon expression is an important marker of the viability in mammalian clones. Appropriate reprogramming of LTR elements in bovine clones shows that their expression is associated with the ooplasm-driven return to totipotency irrespective of the gametic or somatic origin of chromatin. Indeed, the link between retrotransposon expression and pluripotency had been suggested in mice, where expression of ETn LTR retrotransposons is limited to pluripotent cells in early embryogenesis (Brûlet *et al.* 1985). Overall, the conserved pattern of LTR transposon expression during EGA among mammals reinforces the interest about their functional role at this unique stage of development.

The fourth quadrant (Fig. 2D) contains probes for genes that have undergone transcriptional repression in both SCNT and control embryos and thus are also appropriately reprogrammed. Overall, this group is quite small and no functional bias was identified among these genes. However, our cDNA array used for these studies is specifically biased towards embryo-encoded transcripts (see Materials and Methods) and may be unsuitable to provide a faithful representation of genes that have this type of reprogramming behavior in SCNT or normal embryos.

Disparately reprogrammed genes distribute into two groups. The first is a group of genes that encode transcripts up-regulated during somatic reprogramming, while they are down regulated during MET reprogramming (Fig. 2D, quadrant 1). This disparate up-regulation is the result either of a transcriptional activation of somatic nucleus by the factors present in the ooplasm or of the persistence of a high level of maternal transcript, the stability of which may be greater in SCNT than in control morulae. Indeed, alterations of pre-EGA transcriptome in early SCNT embryos, and specifically the stabilization of certain maternal mRNAs, have been documented in the mouse (Vassena *et al.* 2007). Regulation of maternal mRNA stability and cytoplasmic polyadenylation was shown to require, sometimes, the expression of embryo-encoded transcripts across various species, such as zebrafish (Giraldez *et al.* 2006), African clawed frog (Sible *et al.* 1997, Bashirullah *et al.* 1999, Audic *et al.* 2001), and rabbit (our unpublished data). One of the molecular mechanisms for this regulation involves embryo-encoded micro-RNAs, which regulate a subset of maternal transcripts during zebrafish's early development (Giraldez *et al.* 2006). Therefore, it is possible that some regulatory RNAs expressed during EGA in normal

bovine embryos fail to be correctly reprogrammed after SCNT, resulting in the abnormal stabilization of maternal mRNAs in SCNT morulae. Concerning the mitochondrial genome and the transcripts that belong to this group, their disparate up-regulation during SCNT reprogramming, if linked to transcription of the somatically inherited nucleus, may again rely on two different mechanisms: either an increased number of mitochondria or an increased transcription of the mitochondrial genome. In normal embryos, mitochondria are maternally inherited organelles, and in the bovine, the levels of mitochondrial transcripts such as COX1 globally increase from the morula stage onward (May-Panloup *et al.* 2005), that is at or just after the stage we analyzed. On the other hand, in mouse SCNT embryos mitochondrial replication factors are over-expressed, most likely due to their expression in donor nuclei (Bowles *et al.* 2007). If the same is true for bovine, then up-regulation of mitochondrial transcripts during SCNT reprogramming is likely due to an increase in the number of mitochondria.

The third quadrant contains probes downregulated in SCNT morulae compared with the donor cells (Fig. 2D). Undoubtedly, these probes represent genes that have undergone transcriptional repression during somatic reprogramming (but transcriptional activation during MET reprogramming). Interestingly, most of these are the genes encoding ribosomal proteins. Translation increases dramatically during and after EGA, and protein synthesis is required for blastocyst formation (Kidder & McLachlin 1985). High increase in transcripts encoding ribosomal proteins has been reported during EGA of rabbit (Brunet-Simon *et al.* 2001) and mouse (Evsikov *et al.* 2004) embryos, and is confirmed in the bovine by our results. However, SCNT embryos are definitely viable, which indicates they possess functional protein synthesis machinery. We suggest that the reprogramming behavior of genes in the third quadrant may be explained by lower-than-somatic levels of expression of a gene during EGA. An alternative explanation is 'abusive' repression, or incomplete reactivation, during SCNT reprogramming.

According to our results, reprogramming of basic cell machinery, such as mitochondrial and ribosomal protein-encoding genes, undertakes different routes during somatic and MET reprogramming processes. This makes 'housekeeping genes' unexpected candidates for analysis of variations in reprogramming efficiency.

In conclusion, comparison of two transitions—MET and somatic reprogramming—made possible to analyze four different 'gene reprogramming behaviors' that are shared by three somatic donor cell lines, when exposed to the oocyte cytoplasm; at the same time, it delineated genes with similar and disparate reprogramming behaviors during MET versus SCNT. Faithful reprogramming of LTR retrotransposons and their activation during EGA in SCNT embryos suggests a functional, yet still elusive role for these transcripts in mammalian early development.

Their faithful reactivation in SCNT embryos provides an important and reliable marker of the ooplasm-driven somatic cell nuclear reprogramming.

## Materials and Methods

The experiment was performed in accordance with the International Guiding Principle for Biomedical Research involving animals as promulgated by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation. Researchers involved with direct work with the animals possessed an animal experimentation license by the French veterinary services.

### Oocyte and embryo recovery

IVF embryos were obtained by *in vitro* oocyte maturation, fertilization, and embryo culture as described (Parrish *et al.* 1986, Pavlok *et al.* 1989). Four-cell- and morulae-stage embryos were recovered at 41 and 120 h post-insemination respectively, from early two-cell-cleaved embryos picked at 32 h post-insemination.

### Somatic donor cell culture and NT

Primary cultures of bovine fibroblasts were derived from ear skin biopsies of three distinct Holstein heifers, two 12-month-old (OV029, OV5538) and one 18-month-old (OV7711). The cell types derived from these primary cultures were frozen-stored at passage 1, and used as sources of donor nuclei for NT between passages 3 to 13. Donor fibroblasts were grown to confluence in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies) at 38 °C with 5% CO<sub>2</sub> in air, and further cultured for an additional 2–5 days in DMEM containing either 0.5 or 10% FCS. The cells were collected by trypsinization for NT. For RNA extractions, the cell populations were prepared and collected the same way.

Recipient oocytes were *in vitro* matured as previously described by (Pavlok *et al.* 1989) and enucleated at 20–22 h post-maturation (hpm). SCNT embryos were reconstructed by electrofusion of enucleated oocytes with donor cells at 23–24 hpm (2.0 kV/cm 30 μs × 2 pulses). Reconstructed embryos were activated by incubation for 5 h after fusion, in 10 μg/ml cycloheximide and 5 μg/ml cytochalasin B in 199 medium (Sigma) with 10% FCS. They were then co-cultured under the same conditions as the IVF embryos (Parrish *et al.* 1986). Grade 1 (defined in Robertson & Nelson (1998)) morulae were selected at 120 h post-fusion and immediately dry-frozen for further molecular analysis.

### RNA extraction and amplification

Total RNAs were extracted from batches of embryos ( $n=30$  embryos) using the RNeasy Mini Kit (Qiagen). The purification procedure included a DNase I treatment (37 °C, 30 min).

Transcriptomic analyses starting from scarce biological materials require RNA amplification before labeling and hybridization. Two methods are available for such amplification:

antisense RNA *in vitro* transcription and PCR amplification of all cDNAs. Each of these methods may introduce bias into the amplified material. We have observed (data not shown) that the systematic use of both of these methods on each sample makes it possible to isolate the method effect in ANOVA analyses and the subsequent identification of a greater number of differentially expressed genes starting from the same number of scarce samples. For this reason our differential screenings are performed with two distinct targets for each sample corresponding to the two amplification procedures. We routinely use three repetitions of each biological sample.

Half of the extracted RNA was subjected to RNA amplification by *in vitro* transcription (Eberwine *et al.* 1992) using the MessageAmp aRNA Kit (Ambion) according to the manufacturer's instructions. One quarter of the sample was used for global RT-PCR amplification as described (Pacheco-Trigon *et al.* 2002). Briefly, the whole volume of RT-tailing product (10 μl) was subjected to two rounds of PCR amplification. PCRs were performed in a total volume of 50 μl using 15 units of Goldstar DNA polymerase (Eurogentec, Seraing, Belgium) for each round of the PCR cycles. Samples were incubated at 94 °C for 10 min before PCR. Eighteen and 15 cycles were performed for the first and the second PCR respectively. Cycle parameters were: 94 °C for 2 min, 63 °C for 50 s, and 72 °C for 6 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen).

### cDNA library and array construction

To obtain a cDNA library dedicated to early bovine embryonic transcription at MET; we subtracted cDNAs from early post-MET embryos by cDNAs from late pre-MET embryos. We chose the morula as the early post-MET stage since an increase in embryonic transcription is detected at this stage. The four-cell stage was chosen as the late pre-MET stage because it is the latest stage containing mainly residual maternal transcripts just before EGA (Duranthon & Renard 2003). This work was done by SSH with the PCR Select cDNA Subtraction Kit (Clontech) as described (Bui *et al.* 2005). Exogenous *A. thaliana* transcripts were added either to both the tracer and driver materials or only to the tracer material in order to analyze the subtraction and normalization efficiencies. Large-scale analysis of the library we obtained demonstrated that even though it still contained maternally expressed transcripts, it was greatly enriched in embryonic specific transcripts and contained even very rare embryonic transcripts (Bui *et al.* 2005). From this library, we randomly isolated 6523 clones.

cDNA inserts were amplified by PCR using M13 universal primers. Each reaction started from 2 μl bacterial culture, in 1 × PCR Buffer with 1 U Taq Polymerase (Qbiogen). The amplified products were run on a 2% agarose gel to verify the presence of the inserts. After purification and quantification, 6523 inserts were spotted onto nylon membranes (Hybond N+ Amersham) using the QBot robot (Genetix). DNA was denatured (NaOH 0.5 M, NaCl 1.5 M; 5 min), neutralized (Tris 0.5 M, NaCl 1.5 M; 5 min), and fixed to the membranes (2 h, 80 °C). This embryo-dedicated array was subjected to further differential screenings.



### Labeling and array hybridization

Two micrograms aRNA were reverse-transcribed and labeled with [ $\alpha$ - $^{33}$ P] dATP (Perkin Elmer) as described (Decraene *et al.* 1999). From global RT-PCR Amplification, 500 ng cDNA was labeled with [ $\alpha$ - $^{33}$ P] dATP (Perkin Elmer) using an Atlas SMART probe amplification kit (Clontech) according to the manufacturer's instructions. The labeled aRNA or cDNA targets were hybridized to macro-array replicates using ExpressHyb hybridization solution (Clontech) at 68 °C overnight. Washings were performed four times in  $2\times$  SSC, 1% SDS and once in  $0.1\times$  SSC, 0.5% SDS at 68 °C for 30 min each. Membranes were then exposed to phosphoscreen (Phosphorimager Amersham) for 5 days. The hybridization signals were quantified using the Imagen 3.1 software from BioDiscovery (Proteigene, Saint Marcel, France).

### Data analysis

Hybridization signals (Signal means) after quantification by Imagen were log transformed and normalized before data analysis.

Differential analysis was performed by Anova using GenAnova software (Didier *et al.* 2002). For each array probe, the equation used for ANOVA was the following:

$$Y_{ijkl} = \mu + E_i + S_j + M_k + R_l + \varepsilon_{ijkl}$$

where  $Y_{ijkl}$  is the array probe intensity and  $\mu$  is the mean of intensities of expression measured for the probe.  $E_i$ ,  $S_j$ ,  $M_k$ , and  $R_l$  are respectively, the effects of the analyzed epigenotype  $i$ , 'nuclear state' $j$  (the status of the nucleus: somatic versus reprogrammed after transfer into an oocyte), the amplification method  $k$ , and the biological repetition  $l$ .  $\varepsilon_{ijkl}$  is the residual error including all interactions between these factors.

The threshold of 5% for the FDR was chosen to select genes with significant expression changes. (The FDR is the expected proportion of erroneously rejected null hypotheses among the rejected ones).

**Hierarchical clustering:** to classify our different 'conditions', unsupervised hierarchical clustering was performed using free GenAnova software.

PCA was performed using GenAnova software (Didier *et al.* 2002).

### Analysis of array probes

The ESTs sequences representing array probes were initially filtered to eliminate the *A. thaliana* transcripts (internal control for cDNA library preparation; see Bui *et al.* 2005) and empty clones. The remaining 6152 ESTs were assembled in consensus sequences ('clusters') using CAP3 software (Huang & Madan 1999). Each sequence was assigned a unique identifying number, and analyzed for consistency of CAP3 assembly as described (Evsikov *et al.* 2004). The identities of these 2465 sequences were established by searching, using BLASTN (Altschul *et al.* 1990) and MEGABLAST (Zhang *et al.* 2000) programs, all mammalian mRNAs and *Bos taurus* ESTs available in GenBank. Genomic locations (*B. taurus* genome assembly Btau\_3.1, August 2006)

were established using the SSAHA and BLAST tools of ENSEMBL (Hubbard *et al.* 2002). Unambiguous genomic locations were determined for a large proportion of sequences (89.5%). Two thousand two hundred five of 2465 sequences are the transcripts of 1188 known or inferred *B. taurus* genes, i.e. genes curated by the National Center for Biotechnology Information Gene database (Maglott *et al.* 2005), or genes with unambiguous orthology to known *H. sapiens* and *Mus musculus* genes. Totally 155 sequences correspond to retrotransposable elements, and 105 sequences could not be classified. All 5921 ESTs that represent transcripts of known and novel *B. taurus* genes and retrotransposons have been submitted to GenBank EST database, dbEST (Boguski *et al.* 1993), under accession numbers GO349393–GO355313.

### Real-time RT-PCR

RNA were extracted from batches of ten embryos at the four-cell, or morula (20–30 cell) stage using the PicoPure RNA extraction kit (Arcturus, Sunnyvale, CA, USA). At the first step of extraction, 2.5  $\mu$ g carrier RNA (16S-28S carrier Roche Diagnostics) and 1 pg per embryo luciferase encoding reporter transcripts (Promega) were added to the extraction buffer. Carrier RNA recovery rate was estimated by OD measurement (embryonic RNA was considered as negligible compared with carrier RNA). This recovery rate was taken into account to normalize the samples and cDNA synthesis was performed starting from seven 'equivalent embryos'. cDNAs were synthesized from total RNA using the Superscript III enzyme (Invitrogen) and random primers hexamers (Roche Diagnostics) in 20  $\mu$ l final volume. RT reaction was diluted to 400  $\mu$ l in water. Quantitative PCR was performed on 0.5 equivalent embryos. A PCR mix was prepared as follows: 12.5  $\mu$ l of  $2\times$  SybrGreen Mastermix (Applied Biosystems, Courtaboeuf, France), 0.25  $\mu$ l Uracyl *N*-glycosylase (1 U/ $\mu$ l), 0.5  $\mu$ l each primer (10  $\mu$ M initial concentration), 1.25  $\mu$ l H<sub>2</sub>O, 10  $\mu$ l each diluted RT reaction. For each sample the PCR was carried out in triplicates and the mean  $C_t$  was determined. The thermal cyclic profile started with a 2 min step at 50 °C, followed by 10 min at 95 °C, and 45 cycles of 95 °C denaturation for 15 s, 60 °C annealing and extension for 60 s. The reactions were performed on an ABI Prism 7000 sequence detector (Applied Biosystems). Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified. A standard curve consisting of a tenfold dilution series of quantified amplicon was included in each run. Experiments were carried out thrice starting from distinct batches of embryos. Luciferase transcript was quantified according to the same procedure. Mean ratio values obtained for each batch of embryos were calculated with their s.d.s. Primers sequences were as follows: ERV1\_1\_BT P3\_F CTGCTTTACGGTTTCCATCC and ERV1\_1\_BT P3\_R ATCCTGCCACTTTCCTTCC for ERV1\_1\_BT; FLuc5: AGAGATACGCCCTGGTTCCT, and RLuc10: ATAAATAACGCGCC-CAACAC for luciferase.

**Real-time RT-PCR on cell samples.** Three independent quantifications were performed as follows. Four dishes were cultured concomitantly to confluence from a single-cell

suspension. One dish was used to count the cells, while cells from the three other dishes were recovered for RNA extraction using the Qiagen minikit, and cDNA synthesis. Two-fold serial dilutions of the cDNA were submitted to quantitative PCR. Each dilution was analyzed in triplicate experiments.

## Declaration of interest

There is no potential conflict of interest.

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