Milk-derived epithelial cells area a potential source of donor genome for the production of buffalo (*Bubalus Bubalis*) cloned embryos

In most somatic cell nuclear transfer (SCNT) experiments, traditional skin-derived fibroblast cells are commonly used as the donor genome. In addition, alternative sources of donor genome include those derived from hair follicles[1,2], colostrum/milk[3,4], blood[5] and semen[6]. Milk is easily obtained from live animals without any biopsies and can be a safe mode of somatic cell source for those animals with high risk of bacterial infection. Therefore, milk-derived somatic cells may be an important source donor genome for the production of cloned embryos and for endangered species conservation programmes as part of genome resource banking. Recently, Golla *et al.*[4] reported the production of cloned embryos using milk-derived somatic cells in buffalo. However, they were unable to culture epithelial-type somatic cells due to non-optimized culture conditions for such cell types and the study was limited to cloned embryo yield only. In the present study, we have not only successfully optimized the culture conditions for milk-derived epithelial cells, but have also studied the developmental competence, quality, freezing potential and mRNA abundance of *in vitro*-produced cloned embryos from these cells compared to traditional skin-derived fibroblast cells from the same donor animal.

In vitro culture of somatic cells, oocytes and embryos was done at 38.5°C in a CO_2 incubator (5% CO_2 in air). Animal experiments were conducted after following the guidelines laid down by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and Institute Animal Ethics Committee (IAEC).

Isolation and culture of somatic cells of freshly collected milk and ear-skin tissue from the same animal were carried out as described by Golla *et al.*[4], except that collagen IV-coated dish was used for optimum proliferation. Characterization of culture cells was carried out as described earlier on the basis of expression of cytoskeleton markers such as cytokeratin, keratin, vimentin and tubulin[6]. Also, population doubling time and effect of attachment factors such as collagen IV and laminin on proliferation rate were estimated according to the method described by Selokar *et al.*[6].

Somatic cells were synchronized in G1/G0 stage by contact inhibition, and preparation of recipient oocytes (maturation, cumulus/zona removal and manual enucleation), fusion, activation and culture of fused embryos were performed as described previously[7]. For examining the quality of embryos, total cell number and level of apoptosis in day-8 blastocysts were determined using TUNEL staining and freezing potential by open pull straw (OPS) vitrification as described earlier[8].

In case of gene expression analysis, the relative mRNA abundance of *HDAC1*, *DNMT1*, *DNMT3a*, *P53* and *CASPASE3* (Supplementary Information Table S1; see online) was determined in blastocysts by qPCR as described earlier using β-actin as the housekeeping gene[6]. For comparison, the average expression level of each gene from skin-derived embryo group was set as 1 and three separate experiments were performed with three replicates for each gene.

Differences among groups were analysed using Student's *t*-test of the proportional data of cell proliferation, cleavage rate, blastocyst rate, total cell number, apoptotic index, re-expansion rate and gene expression analysis. The percentage values were arc-sine transformed before analysis, and differences were considered significant at $P < 0.05$.

Milk can be non-invasively obtained from the animals without any biopsies, which invariably lead to high risk of

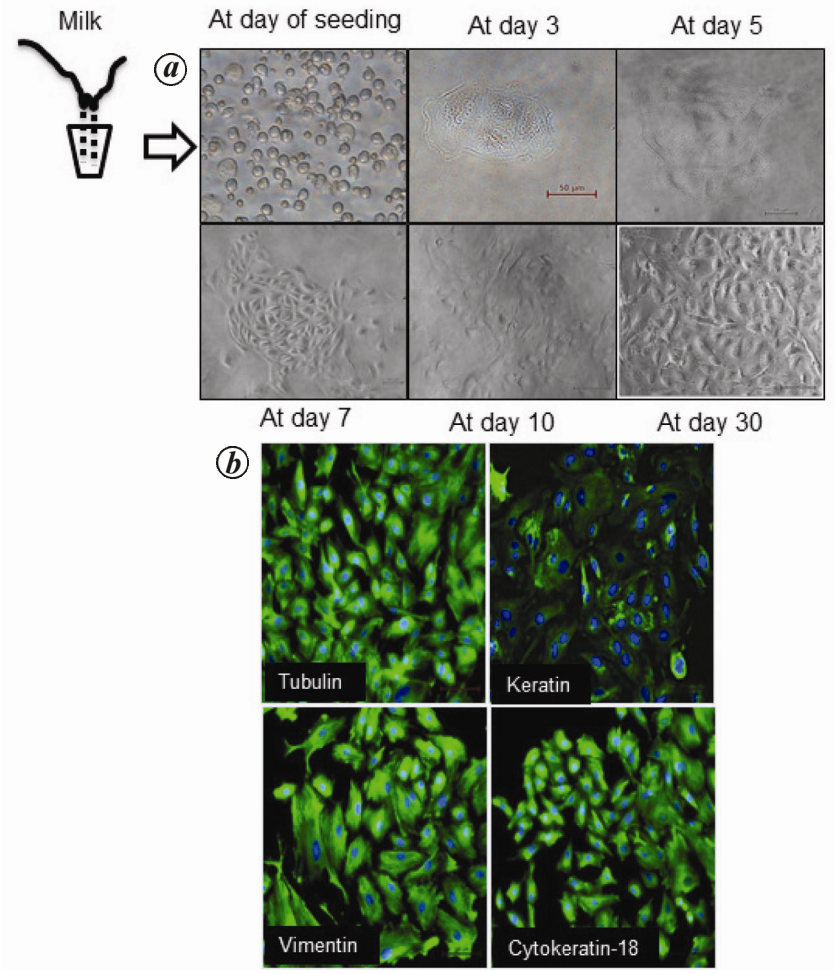

Figure 1. ***a***, Morphological appearance of milk-derived somatic cells during different days of culture. ***b***, Expression of cell-specific markers for characterization.

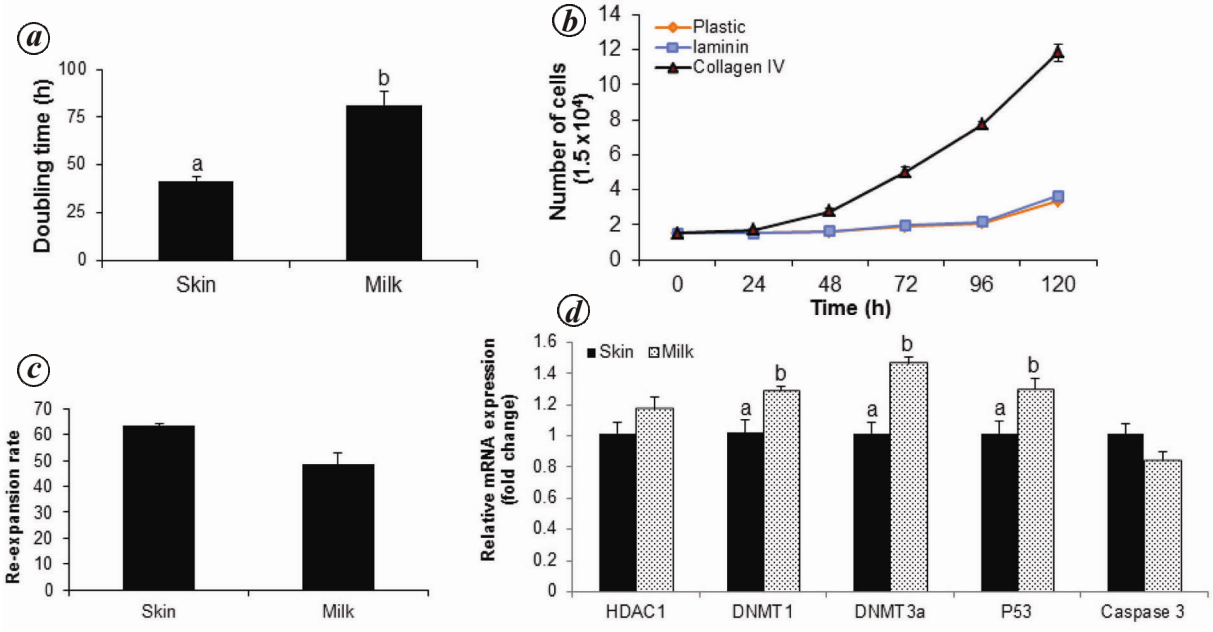

Figure 2. Population doubling time (***a***), effect of attachment factors on proliferation rate (***b***), freezing potential of cloned embryos (***c***) and relative mRNA abundance of genes (***d***) in cloned embryos produced from skin or milk-derived somatic cells. Values with different superscripts differ significantly ($P < 0.05$).

Table 1. Developmental competence and quality of cloned embryos produced using donor cells isolated from skin and milk

Cell type	Reconstructed embryos	Cleaved *n* (%)	Blastocyst *n* (%)	Total cell number	Apoptotic index
Skin	174	159 (92.0 ± 2.6)	96 (54.6 ± 4.2)	514.1 ± 63.5	4.23 ± 0.86[a]
Milk	147	141 (96.2 ± 1.4)	70 (48.98 ± 6.8)	460.1 ± 79.3	8.02 ± 1.29[b]

Data from eight trials. Values with different superscripts within the same column differ significantly ($P < 0.05$).

bacterial infection. In the present study, we successfully cultured somatic cells from milk and cloned blastocyst stage embryos were produced from these cells. We found that cultured cells were of epithelial type and their proliferation rate was slower than that of skin-derived fibroblast cells. Extracellular matrix supports such as bovine collagen IV and laminin enhanced proliferation rate. We have also demonstrated that cloned embryo production rate and freezing potential are similar to those of traditional skin-derived somatic cells. However, gene expression pattern was different.

In the present study, cells cultured from freshly collected milk were classic cobblestone morphology (Figure 1 *a*), and expressed cytokeratin-18, keratin and vimentin (Figure 1 *b*), which is contradictory to a previous report[4], which stated that milk-derived cells expressed only vimentin, confirming that culture cells were of an epithelial origin type. This might have happened due to the use of collagen IV-coated dishes in the present study, which provide proper growth matrix for epithelial cells. Highly specific culture conditions have been described for seminal plasma-derived epithelial cells[6] and incorporating these conditions to culture cells isolated from milk improves their proliferation rates. Epithelial cells require specific culture conditions for optimum proliferation. We found that milk-derived cells were proliferating slower compared to skin-derived cells (Figure 2 *a*), and their proliferation rate was significantly improved in the order of bovine collagen IV > laminin > uncoated surface (controls) at 72, 96 and 120 h of culture (Figure 2 *b*). Similarly, proliferation of epithelial cells cultured from fresh semen obtained from buffalo was improved when cells were cultured on collagen IV-coated surface than that uncoated plastic surface[6]. These results suggest that proliferation of milk-derived epithelial cells shows improvement on using collagen IV-coated dishes.

The blastocyst rate and total cell number were not significantly ($P > 0.05$) different between cloned embryos produced from skin- and milk-derived cells of the same animal (Table 1), which contradicts a previous report stating that the developmental competence of cloned embryos produced from milk-derived cells was poor in comparison to skin-derived somatic cells[4]. This might be due to the presence of high concentrations of antibiotics and inappropriate culture conditions for somatic cells. In addition, re-expansion rate after OPS vitrification–warming was similar between both types of embryo, indicating that cloned blastocysts produced from milk-derived cells had a similar ability to withstand the stress of vitrification–warming (Figure

2 *c*). These results confirm that milk-derived epithelial cells are equally competent as traditional skin-derived fibroblast cells as donor genome for production of cloned buffalo embryos. However, the capability of these embryos to result in pregnancy and live birth of normal offspring needs to be examined further.

Apoptosis and DNA hypermethylation are important aberrations occurring in cloned embryos during chromatin remodelling, resulting in developmental abnormalities and embryonic death. In the present study, we found that apoptotic index was higher in blastocysts produced from milk-derived epithelial cells than of skin-derived cells (Table 1). In case of gene expression pattern, relative mRNA abundance of *DNMT1*, *DNMT3a* and *P53* was found to be significantly ($P < 0.05$) higher in epithelial cell-derived cloned blastocysts than fibroblast cell-derived blastocysts. However, no change was found in case of *HDAC1* and *CASPASE 3* among these blastocyst types. Similar observation was found in our previous study stating that mRNA abundance of *DNMT1* and *P53* apoptotic genes was higher in cloned blastocysts produced from epithelial cells of seminal plasma than fibroblast cells of skin[6]. These results indicate that the apoptotic status and pattern of expression of important genes may vary among embryos of different cell origin, even though they may be from the same animal.

In conclusion, we have demonstrated that milk-derived epithelial cells are equally competent as traditional skin-derived fibroblast cells as a donor genome for the production of cloned buffalo embryos. Further studies need to be carried out with focus on determination of live offspring rate following transfer of cloned embryos produced from milk-derived epithelial cells to recipients for comparing their overall cloning efficiency.

Declaration of interest. The authors declare that there is no conflict of interest which could be perceived as prejudicing the impartiality of the research reported.

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