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Production of IVF transgene-expressing bovine embryos using a novel strategy based on cell cycle inhibitors

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Abstract

The objective was to evaluate the effects of cell cycle inhibitors (6-dimethylaminopurine [DMAP], and dehydroleukodine [DhL]) on transgene expression efficiency and on mosaic expression patterns of IVF bovine zygotes cytoplasmically injected with oolema vesicles coincubated with transgene. The DNA damage induced by the transgene or cell cycle inhibitors was measured by detection of phosphorylated histone H2AX foci presence (marker of DNA double-stranded breaks). Cloning of *egfp* blastomeres was included to determine continuity of expression after additional rounds of cellular division. The pCX-EGFP [enhanced green fluorescent protein gene (EGFP) under the chimeric cytomegalovirus IE-chicken-β-actin enhancer promoter control] gene plasmid (50 ng/μL) was injected alone (linear or circular exogenous DNA, *leDNA* and *ceDNA*, respectively) or associated with ooplasmic vesicles (*leDNA-v* or *ceDNA-v*). The effects of 2 mM DMAP or 1 μM DhL for 6 h (from 15 to 21 h post IVF) was evaluated for groups injected with vesicles. The DMAP increased ($P < 0.05$) *egfp* homogenous expression relative to transgene alone (21%, 18%, and 11% for *leDNA-v* + DMAP, *leDNA-v*, and *leDNA*, respectively) and also increased ($P < 0.05$) the phosphorylated histone H2AX foci area. Expression of *egfp* was higher ($P < 0.05$) for linear than for circular pCX-EGFP, and *egfp* blastocyst rates were higher ($P < 0.05$) for groups injected with linear transgene coincubated with vesicles than for linear transgene alone (95%, 77%, 84%, and 52% for *leDNA-v* + DMAP, *leDNA-v* + DhL, *leDNA-v*, and *leDNA*, respectively). Moreover, DMAP tended to improve *egfp* blastocysts rates for both circular and linear transgenes. Based on fluorescent in situ hybridization (FISH) analysis, there was evidence of integration in *egfp* embryos. Finally, clones derived from *leDNA-v* + DMAP had the highest *egfp* expression rates (96%, 65%, and 65% for *leDNA-v* + DMAP, *leDNA-v*, and *leDNA*, respectively). Transgenesis by cytoplasmic injection of *leDNA-v* + DMAP is a promising alternative for transgenic animal production.

Keywords: 6-Dimethylaminopurine; Dehydroleukodine; Transgenesis; Phosphorylated; Histone H2AX; Cattle

1. Introduction

Several techniques are currently used to produce transgenic mammalian embryos, including pronuclear microinjection [1], SCNT [2], sperm mediated gene transfer [3], and intracytoplasmic sperm injection (ICSI)-mediated gene transfer [4]. However, mechanisms involved are not yet fully understood. The first critical step for stable transgenesis is introduction of exogenous DNA into the host genome. After transgene incorporation into a pronucleus, exogenous DNA (eDNA) can be integrated into the host genome mainly as result of the activity of DNA repair machinery associated with replication [5,6]. It has been suggested that during pronuclear microinjection, sponta-

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neous chromosomal ruptures, exacerbated by the micromanipulation procedure, become eDNA integration sites by ligation reaction [7]. In addition, using sperm-mediated transgenesis, the interaction between transgene and the spermatozoon is responsible for an increase in paternal chromosome ruptures, which are proportional to transgenesis efficiency [8]. All of these observations indicate that chromosomal ruptures and integration rates are closely related.

The early integration of the eDNA into the host genome is a critical step for homogeneous transgenic embryo production. The chemical agents 6-dimethylaminopurine (DMAP) and dehydroleukodine (DhL) are used for parthenogenic activation, SCNT, and ICSI [9–14]. In addition, effects of DMAP on the inhibition of DNA synthesis [15] and on cell synchronization [16] are also known.

Dehydroleukodine was originally discovered as an agent capable of arresting the cell cycle of smooth muscle vascular cells at the G2 stage [17]. Treatment with these agents during the first pronuclear phase of zygotes injected with transgene could arrest the cell cycle and provoke DNA ruptures, increasing the activity of the repairing mechanisms associated with replication. Consequently, they could improve incorporation of transgenes into the bovine genome.

A group of cellular proteins are responsible for arresting the cell cycle and for activating DNA repair pathways in response to DNA double-stranded breaks (DSBs) or other DNA damage. The phosphorylation of histone H2AX (γH2AX) is a marker of DNA DSBs; [18] it plays a key role in DNA damage checkpoint activation by recruiting numerous repairing proteins to the vicinity of DNA lesions [19,20]. To date, γH2AX has apparently not been measured in bovine zygotes exposed to exogenous DNA and to various agents during the first embryonic S phase.

Our group recently developed a new technique for transgene-expressing embryo production, involving generation of small oolema vesicles by oocyte microsurgery, their short coincubation with eDNA, and finally their injection into the cytoplasm of presumptive zygotes generated by IVF [21]. This method was efficient for bovine and ovine transgene-expressing embryo production; however, as was the case for pronuclear microinjection and ICSI-mediated gene transfer, mosaic expression was observed [1,4].

In the present study we exploited these observations as opportunities to design strategies which could be applied to improve transgenesis efficiency and to reduce mosaic expression patterns of bovine transgene-expressing embryos. The effects of two cell cycle synchronizers (DMAP and DhL) incubated with zygotes on the expression of eDNA and on the induction of DNA breaks (measured by immunocytochemistry against γH2AX), were evaluated. Cytoplasmic injection of plasmid alone or coincubated with vesicles (both linear and circular eDNA) were also tested. The integration status of the transgene was assessed by fluorescent in situ hybridization (FISH) analyses. Finally, cloning of transgene expressing blastomeres was included to evaluate homogenous transgene expression after additional rounds of cellular division.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1: effect of cell cycle inhibitors (DMAP and DhL) during first pronuclear phase on IVF zygotes development

The IVF-derived presumptive zygotes were incubated in DMAP or DhL for 9 h (from 15 to 24 h post IVF) or for 6 h (15 to 21 h post IVF). First cellular division and development to blastocysts were evaluated.

2.1.2. Experiment 2: effect of cell cycle inhibitors (DMAP and DhL) on transgene expression of bovine embryos produced by vesicle-mediated transgenesis

Exogenous DNA alone (eDNA alone) or coincubated with vesicles (eDNA-v) was injected into bovine presumptive zygotes immediately after IVF. A group of IVF presumptive zygotes injected with vesicles + eDNA was incubated in DMAP (eDNA-v + DMAP) or DhL (eDNA-v + DhL) for 6 h (from 15 to 21 h post IVF). All of these treatments were repeated for circular (*ceDNA*) and linear (*leDNA*) structures of the transgene. Transgene expression was evaluated daily.

2.1.3. Experiment 3: FISH analysis of bovine embryos produced by vesicle and by transgene alone-mediated transgenesis

Bovine *egfp*-expressing blastocysts produced by IVF, followed by injection with linear transgene alone (*leDNA* alone) or coincubated with vesicles (*leDNA-v*), were subjected to FISH analysis to evaluate transgene integration status.