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(54) Title: NUCLEAR TRANSFER EMBRYO FORMATION METHOD

(57) Abstract: A nuclear transfer embryo is formed by destabilizing microtubules of an oocyte, whereby essentially all endogenous chromatin collects at a second polar body during meiosis of an oocyte. The oocyte is fused with the nucleus of a donor somatic cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from the oocyte, thereby forming the nuclear transfer embryo. In one embodiment, the nuclear transfer embryo is employed to impregnate an animal, such as a mammal. In another embodiment, the donor nucleus is transgenic.



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## NUCLEAR TRANSFER EMBRYO FORMATION METHOD

## RELATED APPLICATION

5           This application claims priority to and is a continuation-in-part of U.S. Application No. 10/208,653, filed July 29, 2002, the entire teachings of which are incorporated herein by reference.

## GOVERNMENT SUPPORT

10           This invention was supported, in whole or in part, by Grant No. 2001-35205-09966 from the United States Department of Agriculture. The Government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

15           Nuclear transfer methods have been developed and used successfully to produce cloned sheep, cattle, mice, goats and pigs. Two cell components are combined to produce a cloned embryo; the donor nuclear genome (karyoplast) that is the target for clonal replication, and the enucleated oocyte (cytoplast) whose cytoplasmic constituency is sufficiently competent to facilitate genome reprogramming and support embryonic development to term.

20           Mammalian oocyte cytoplasts have been prepared by physically removing nuclear chromatin by micromanipulation techniques in preparation to receive the donor genome. Enucleated oocytes arrested at metaphase of meiosis II (MII) are subsequently "reconstructed" by the addition of the donor karyoplast typically using either electrofusion or microinjection techniques. However, physical enucleation is generally technically demanding, time consuming,  
25 inherently invasive and clearly damaging to cytoplast spatial organization. Moreover, in certain instances, development of reconstructed embryos is inherently inefficient.

          One alternative strategy to physical enucleation has been to treat oocytes with agents that modify the processes of karyokinesis and cytokinesis and result in chemically enucleated oocytes at high rates (>85%). However, certain studies have reported that exposure of metaphase I and  
30 MII oocytes to etoposide, a topoisomerase II inhibitor, and cycloheximide yields enucleated cytoplasts with limited ability to support cleavage or blastocyst development, and term development of reconstructed embryos has not been reported.

Hence, a need exists for improved methods for developing nuclear transfer embryos.

#### SUMMARY OF THE INVENTION

The present invention relates to methods of forming a nuclear transfer embryo by  
5 destabilizing microtubules of an oocyte (e.g., mammalian (human or non-human)), whereby  
essentially all endogenous chromatin collects at (e.g. segregates into) a second polar body during  
meiosis of the oocyte. The methods also include combining the oocyte with at least the nucleus  
of a donor cell of the same species of the oocyte prior to cessation of extrusion of the second  
polar body from the oocyte, thereby forming a nuclear transfer embryo. Certain conditions and/or  
10 compounds destabilize the microtubules of the meiotic spindle of the oocyte. Examples of  
chemicals that destabilize the microtubules of the oocyte include demecolcine, paclitaxel,  
phalloidin, colchicine, and nocodazole. Such conditions include alterations (e.g., increasing or  
decreasing) in electromagnetic radiation (e.g., x-rays or heat), pH or osmolality.

In one embodiment, the present invention further includes activating the oocyte prior to  
15 exposing the oocyte to the chemical that induces enucleation. Stages of an activated oocyte  
include telophase II or anaphase II stage of meiosis. In another embodiment, the oocyte is in a  
resting of meiosis (e.g., metaphase II stage) during injection of the donor nucleus.

The present invention utilizes donor cells that are in various stages of mitotic cell cycle, and  
can include several types of cells. The present invention encompasses use of an active (e.g., G<sub>1</sub>,  
20 S or G<sub>2</sub>/M stage of a mitotic cell cycle) or inactive (e.g., G<sub>0</sub> stage of a mitotic cell cycle) donor  
cell. The donor cell can be an active or inactive fibroblast cell, epithelial cell, a somatic cell (e.g.,  
adult or embryonic). The donor cell can also be transgenic.

The present invention also encompasses methods for cloning a mammal by forming the  
nuclear transfer embryo, as described herein, and impregnating a mammal of the same species as  
25 the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for  
gestation of the cloned mammal; and gestating the embryo, thereby causing the embryo to  
develop into the cloned mammal.

The present invention further includes methods for producing a transgenic mammals, by  
forming a nuclear transfer embryo using a transgenic donor cell, as described herein, and  
30 impregnating a mammal of the same species as the nuclear transfer embryo with the nuclear

transfer embryo under conditions suitable for gestation of the transgenic mammal; and gestating the embryo, thereby causing the embryo to develop into the transgenic mammal.

Yet another embodiment of the invention includes methods for cloning a mammalian fetus, by forming a nuclear transfer embryo as described herein, and impregnating a mammal of  
5 the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the cloned mammalian fetus; and gestating the embryo, thereby causing the embryo to develop into the cloned mammalian fetus.

The present invention further pertains to methods of producing a protein of interest in an animal, by forming a nuclear transfer embryo as described herein, and impregnating a mammal of  
10 the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the cloned mammal; gestating the embryo, thereby causing the embryo to develop into the cloned mammal; and purifying the protein of interest (e.g., tissue, cells or a bodily secretion) from the cloned animal. Examples of sources from which proteins that can be purified from cloned animals include milk, blood, urine, hair, mammary gland,  
15 muscle, viscera (e.g., brain, heart, lung, kidney, pancreas, gall bladder, liver, stomach, eye, colon, small intestine, bladder, uterus and testes).

The present invention also relates to methods of producing a heterologous protein in a transgenic animal by forming a transgenic nuclear transfer embryo, as described herein, and impregnating a mammal of the same species as the nuclear transfer embryo with the nuclear  
20 transfer embryo under conditions suitable for gestation of a transgenic cloned mammal; gestating the embryo, thereby causing the embryo to develop into the transgenic cloned mammal; and purifying the protein of interest from the transgenic cloned animal. In one embodiment, the genetically engineered nucleus includes an operatively linked promoter (e.g., a host endogenous promoter, an exogenous promoter and a tissue-specific promoter (e.g., mammary-specific  
25 promoter, blood-specific promoter, muscle-specific promoter, neural-specific promoter, skin-specific promoter, hair-specific promoter and urinary-specific promoter)).

In another embodiment, the present invention relates to methods of forming a nuclear transfer embryo by performing the steps in the following order: combining an oocyte with at least the nucleus of a donor cell of the same species of the oocyte; activating the oocyte; and  
30 destabilizing microtubules of the oocyte, whereby essentially all endogenous chromatin collects

at a second polar body during meiosis of the oocyte, thereby forming a nuclear transfer embryo. The oocyte can be in a metaphase II stage of meiosis prior to activation, or in a telophase II or anaphase II stage of meiosis after activation.

The present invention also includes methods of forming a nuclear transfer embryo, by  
5 activating an oocyte; combining the activated oocyte with at least the nucleus of a donor cell of the same species of the oocyte in less than about 45 minutes (e.g., 40, 35, 30, 25, 20, 15, 10 or 5 minutes) after activation of the oocyte; and destabilizing microtubules of the activated oocyte, whereby essentially all endogenous chromatin collects at a second polar body during meiosis of the oocyte, thereby forming a nuclear transfer embryo.

10 The methods of the invention have advantages over methods of nuclear transfer that employ mechanical enucleation (e.g., enucleation using a micropipette to remove the nucleus) of the oocyte for several reasons. For example, using physical enucleation, the nucleus along with other important spindle associated elements are also removed. These spindle associated elements include, for example, centrosome factors ( $\alpha$ -tubulin, pericentrin), kinases (e.g., C-mos, MPF,  
15 PAPK) and competence factors (e.g., cdc25C, spindlin). Several of these elements are important in forming a nuclear transfer embryo that is developmentally competent (e.g., embryo's ability or likelihood to develop successfully *in vivo* following an embryo transfer and implantation to a recipient mammal). The method of the invention removes the endogenous nucleus from the oocyte, while allowing several of these spindle associated elements to remain in the oocyte,  
20 thereby potentially significantly improving yield of competent embryos.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing the process of induced enucleation and the  
25 introduction of the donor nucleus before extrusion of the second polar body containing essentially all endogenous chromatin of the oocyte ceases.

FIG. 2 is a schematic diagram showing the process of induced enucleation and the introduction of the donor nucleus before cessation of extrusion of the second polar body containing essentially all of the chromatin of a telophase II oocyte.

FIGS. 3A-3B are a series of histograms showing percent (%) of complete second PB extrusion at several times post-activation (45 min, 75 min, 105 min and 135 min) for untreated ethanol-activated oocytes (EtOH) or demecolcine (Deme)-treated activated oocytes at different times (0, 5, 10 and 15 min) post-activation. FIG. 3A shows the time course for complete second PB extrusion for B6D2F1 strain mouse oocytes, and FIG. 3B shows the time course for complete second PB extrusion for CF-1 strain mouse oocytes. Oocytes were analyzed by immunofluorescence at several time-points after activation to assess the extent and completeness of second PB extrusion. Different superscripts represent significant differences ( $P < 0.05$ ) between different treatments for each time-point and strain.

FIG. 4 is a series of histograms showing percent (%) of complete second PB extrusion at several times post-activation (45 min, 75 min, 105 min and 135 min) for various types of B6D2F1 and CF-1 strain oocytes (type C, type D, type E and type F) that were treated with demecolcine (Deme) at different times (0, 5, 10 and 15 min) post-activation.

FIGS. 5 A-5B are a set of histograms showing percent (%) of enucleation rates in ethanol-activated B6D2F1 and CF-1 mouse oocytes treated with demecolcine (Deme) at different times (0, 5, 10 and 15 min) post-activation (p.a.). FIG. 5A shows the percentage of enucleated oocytes of only those activated oocytes that completed second PB extrusion, and Figure 5B shows the total of activated oocytes. The value in each treatment represents the combined results for all four time-points examined (45, 75, 105 and 135 min p.a.). Different superscripts represent significant differences ( $P < 0.05$ ) between treatments for each strain of oocytes. In both Figures 5A and 5B, values for each treatment differ significantly between strains.

FIG. 6 is a graphical representation showing the rates of activation and the second PB extrusion in goat oocytes upon treatment with demecolcine.

FIG. 7 is a schematic showing induced enucleation and the introduction of the donor nucleus pre-activation and post activation.

FIG. 8 is a histogram showing the meiotic status of bovine oocytes fixed and stained at various timepoints (30 min. (n=63), 1h (n=63), 1.5h (n=67) and 17 h (n=51)) after activation.

FIG. 9 is a histogram showing the activation rates of control and demecolcine treated oocytes fixed at 5 hours (N=26-45/group) post activation.

FIG. 10 is a histogram showing induced enucleation rates of demecolcine treated oocytes fixed at five hours (black bars, n=26-45/group) and 17 hours (n=18-32/group) post-activation.

FIG. 11 is a histogram showing TCN, pyknic, interphase and mitotic nuclei in day 8 blastocysts obtained by chemical enucleation and partenogenetic activation followed or not by donor cell nuclear transfer.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to new methods of cloning animals by enucleating an oocyte by induced enucleation and, prior to the cessation of protrusion of a second polar body containing essentially all of endogenous chromatin of the oocyte (e.g., the completion of the enucleation), combining (e.g., injecting or fusing) at least the nucleus from a somatic donor cell (e.g., karyoplast) with the oocyte. The nuclear material from the somatic donor cell is combined with the oocyte (e.g., cytoplasm) before the protrusion of the second polar body containing essentially all of the endogenous chromatin ceases.

The present invention utilizes "induced enucleation" which refers to enucleation of the oocyte by disrupting the meiotic spindle apparatus through the destabilization (e.g., depolymerization) of the microtubules of the meiotic spindle. Destabilization of the microtubules prevents the chromatids from separating (e.g., prevents successful karyokinesis), and induces the oocyte genome (e.g., nuclear chromatin) to segregate unequally (e.g., skew) during meiotic maturation, whereby essentially all endogenous chromatin of the oocyte collects in the second polar body.

Induced enucleation can be accomplished, for example, by exposing an oocyte with compounds or conditions (e.g., at least one compound and/or condition) that destabilize the microtubules, as described above. Examples of compounds that destabilize the microtubules include, but are not limited to, demecolcine, Taxol® (e.g., paclitaxel), phalloidin, colchicine, and nocodazole. Methods of chemically inactivating the DNA are known to those of skill in the art. Completion of enucleation can be determined by visually inspecting oocyte having stained microfilaments e.g., with rhodamine phalloidin. In addition, exposure of oocytes to certain conditions (e.g. increased or decreased temperature, pH, osmolality) that induce destabilization of

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the microtubules. In particular, oocytes are exposed to temperatures, pH and/or osmolality that are above or below normal body conditions for that species.

Before the enucleation process is completed, the nucleus (e.g., genome) from the donor cell is introduced into the oocyte. Completion of enucleation is signified by the cessation of the  
5 second polar body extrusion (e.g., completion of cytokinesis or effective cessation of active, or observable, continuing extrusion of the second polar body). For example, a compound, such as demecolcine, which destabilizes microtubules can, depending upon the concentration employed, slow extrusion of a second polar body to effectively terminate continuing extrusion and, thereby, prevent completion of cytokinesis which, normally would mark the end point of second polar  
10 body formation. The second polar body (PB), in the case of induced enucleation, contains all of the endogenous nuclear chromatin (e.g., the nucleus) of the oocyte. The introduction of the donor nucleus can occur before, during or shortly after the enucleation process begins (e.g., after exposure to the compound or condition that induces enucleation), but before the cessation of the second PB extrusion containing the nucleus of the oocyte. Representative examples of complete  
15 second PB extrusion, induced chemically with demecolcine, are described in the Exemplification. Preferably, the second PB is fully extruded and contains the nuclear chromatid of the oocyte. However, in some instances the second PB is not fully extruded. Descriptions of this process in the Exemplification demonstrate that in some instances, although enucleation is completed and the second PB is formed, the second PB is not fully extruded in all cases. Regardless of the  
20 extent to which extrusion of the second PB actually occurs, the end point for the completion of the enucleation process is cessation of extrusion of a second PB containing essentially all endogenous nuclear chromatin of the oocyte. Additionally, induced enucleation was performed on mouse, goat and bovine embryos, and for each species demecolcine caused the enucleation of the oocyte to form a second PB containing essentially all of the endogenous chromatin of the  
25 oocyte. See Exemplification.

When using a compound that destabilizes the microtubules to enucleate an oocyte, the induced enucleation begins upon exposure of the compound and continues until the protrusion of a second polar body containing essentially all endogenous chromatin ceases, such as by completing formation of the second polar body. The length of time needed to complete  
30 enucleation depends on a variety of factors including the specific compound or condition used for

the enucleation, and the species or strain within a species of oocyte used. In general, induced enucleation for mammals using demecolcine is about 5 hours post-activation. For example, when using demecolcine to enucleate bovine oocytes, induced enucleation (greater than 80%) generally requires about 5 hours post-activation. Induced enucleation with demecolcine generally takes  
5 about 1.5 hours for murine oocytes (greater than 50%), 4-6 hours for pig oocytes, 3-5 hours for human oocytes, and 5 hours for goat oocytes (greater than 80%). One of skill in the art can readily determine the length of time required to complete induced enucleation.

Activated oocytes are those that are in a dividing stage of meiotic cell division, and can include any meiotic phase except metaphase II (e.g., metaphase I, anaphase I, telophase I, and  
10 preferably, anaphase II and telophase II) stage. In particular, activated oocytes refer to those metaphase II oocytes that have been stimulated to resume meiosis naturally (e.g., fertilization) or by artificial means (e.g., ethanol, ionomycin, electrical change, or chemical activation). An activated oocyte is also defined as one that has a protruding second polar body. Oocytes in metaphase II are considered to be in a resting state and are therefore arrested. The oocytes can be  
15 in the resting stage of metaphase II (arrested), and then activated, using methods described herein. The stage that the oocyte is in can be identified by visual inspection of the oocyte under a sufficient magnification. Methods for identifying the stage of meiotic cell division are known in the art.

In another embodiment, the oocyte can be quiescent, in metaphase II. In accordance with  
20 the present invention, the donor nucleus is introduced to the metaphase II oocyte and then activated. See Figure 1. Once the metaphase II oocyte and the donor nucleus are combined, the oocyte is activated and enucleation of the endogenous nucleus is induced, as described herein, to thereby form a nuclear transfer embryo. This nuclear transfer embryo is ready to be implanted into an animal that is of the same species as the embryo.

25 In one embodiment, the oocyte can be activated prior to exposure to a microtubule destabilizing compound. For example, an activated oocyte can be in the anaphase II or telophase II stage of meiotic cell division, and then exposed to the microtubule destabilizing compound. Before enucleation is complete, the donor nucleus is introduced to the active, enucleated oocyte. See Figure 2. This process forms a nuclear transfer embryo which is ready to be implanted into  
30 an animal that is of the same species as the embryo.

Oocytes are activated by, for example, increasing their exposure to calcium levels. Increasing levels of calcium, e.g., by between about 10% and about 60% above the baseline levels, induces activation or meiotic cell division of the oocyte. Baseline levels are those levels of calcium found in an inactive oocyte. Rising levels of calcium, coupled with decreasing levels of phosphorylation further facilitates and sustains activation of the oocyte. Several methods exist that allow for activation of the oocyte. In particular, a calcium ionophore (e.g., ionomycin) is an agent that increases the permeability of the oocyte's membrane and allows calcium to enter into the oocyte. As the free calcium concentration in the cell increases during exposure to the ionophore, the oocyte is activated following reduction in MPF (maturation promoting factor) activity. Such methods of activation are described in U.S. Patent No. 5,496,720. Ethanol has a similar affect. Prior to or following enucleation, an oocyte in metaphase II can be activated with ethanol according to the ethanol activation treatment as described in Presicce and Yang, *Mol. Reprod. Dev.*, 37:61-68 (1994); and Bordignon & Smith, *Mol. Reprod. Dev.*, 49:29-36 (1998). Exposure of calcium to the oocyte also occurs through electrical stimulation. The electrical stimulation allows increasing levels of calcium to enter the oocyte. At least one application or exposure to an activating agent can be used for activating the oocyte. Also, more than one application or exposures to activating agents can be used for activating an oocyte. The time frame for a second activation can be, for example, from about one minute after the first activation to cessation of extrusion of the second polar body. The activating agents for each activation can be different or the same. A second activation can be used to ensure complete activation of the oocyte. This second activation can be timed after a recovery is indicated from the first activation.

In certain embodiments, the zona pellucida, can be removed (e.g. mechanically) for optimizing the efficiency of the somatic cell cloning. The removal prevents the potential possibility of reintroducing the chromatin in the oocyte which can occur with an incompletely extruded second polar body.

Oocytes can be obtained from a donor animal during that animal's reproductive cycle. For example, oocytes can be aspirated from follicles of ovaries at given times during the reproductive cycle (exogenous hormone-stimulated or non-stimulated). Also at given times following ovulation, a significant percentage of the oocytes, for example, are in telophase. Additionally, oocytes can be obtained and then induced to mature *in vitro* to arrested metaphase II stage.

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Arrested metaphase II oocytes, produced *in vivo* or *in vitro*, can then be induced *in vitro* to enter telophase. Thus, oocytes in telophase can readily be obtained for use in the present invention. In particular, oocytes can be collected from a female animal following super ovulations. Oocytes can be recovered surgically by flushing the oocytes from the oviduct of a female donor. Methods of inducing super ovulations in, for example, goats and the collection of the oocytes are described  
5 herein.

As described above, the enucleated oocyte is combined with the nucleus of the donor cell. The donor cell can be active or inactive. An activated (e.g., non-quiescent) donor cell is a cell that is actively dividing (e.g., not in a resting stage,  $G_0$  of mitosis). In particular, an activated  
10 donor cell is one that is engaged in the mitotic cell cycle, such as  $G_1$  phase, S phase or  $G_2/M$  phase. The mitotic cell cycle has the following phases,  $G_1$ , S,  $G_2$  and M. The  $G_2/M$  phase refers to the transitional phase between the  $G_2$  phase and M phase. The commitment event in the cell cycle, called START (or restriction point), takes place during the  $G_1$  phase. "START" as used herein refers to late  $G_1$  stage of the cell cycle prior to the commitment of a cell proceeding  
15 through the cell cycle. The decision as to whether the cell will undergo another cell cycle is made at START. Once the cell has passed through START, it passes through the remainder of the  $G_1$  phase (i.e., the pre-DNA synthesis stage). The S phase is the DNA synthesis stage, which is followed by the  $G_2$  phase, the stage between synthesis and mitosis. Mitosis takes place during the M phase. If prior to START, the cell does not undergo another cell cycle, the cell becomes  
20 arrested. In addition, a cell can be induced to exit the cell cycle and become quiescent or inactive. A "quiescent" or "inactive" cell, is referred to as a cell in  $G_0$  phase.

In one embodiment, the donor cell and oocyte can be "synchronous" with respect to the cell cycle. In this case, synchronization refers to cells that are in the same stage of cell division (e.g., in an active state). The meiotic cell stage of the activated oocytes correlates to the mitotic  
25 stage of the cell cycle of the activated donor cell. For example, an oocyte in telophase II fused with the genome of a donor cell in the  $G_1$  stage before completion of the enucleation process provides a synchronization between the oocyte and the donor nuclei.

It is preferable that the donor cells also be in the same stage of cell division. Using donor cells at certain phases of the cell cycle, for example,  $G_1$  phase, allows for synchronization of the  
30 donor cells. One can synchronize the donor cells and put them in the same stage by depriving

(e.g., reducing) the donor cells of a sufficient amount of nutrients in the media that allows them to divide. Once the donor cells have stopped dividing, then the donor cells are exposed to media (serum) containing a sufficient amount of nutrients to allow them to begin dividing (e.g., mitosis). The donor cells begin mitosis substantially at the same time, and are therefore, synchronous. For example, the donor cells are deprived of a sufficient concentration of serum by placing the cells in 0.5% Fetal Bovine Serum (FBS) for about a week. Thereafter, the cells are placed in about 10% FBS and they will begin dividing at about the same time. They will enter the G<sub>1</sub> phase about the same time, and are therefore, ready for the cloning process.

Methods of determining which phase of the cell cycle a cell is in are known to those skilled in the art, for example, U.S. Patent No. 5,843,705 to DiTullio *et al.*, Campbell, K. H. S., *et al.*, *Embryo Transfer Newsletter*, vol. 14(1):12-16 (1996), Campbell, K. H. S., *et al.*, *Nature*, 380:64-66 (1996), Cibelli, J. B., *et al.*, *Science*, 280:1256-1258 (1998), Yong, Z. and L. Yuqiang, *Biol. of Reprod.*, 58:266-269 (1998) and Wilmut, I., *et al.*, *Nature*, 385:810-813 (1997). For example, as described below in the Examples, various markers are present at different stages of the cell cycle. Such markers can include cyclins D 1, 2, 3 and proliferating cell nuclear antigen (PCNA) for G<sub>1</sub>, and BrDu to detect DNA synthetic activity. In addition, cells can be induced to enter the G<sub>0</sub> stage by culturing the cells on a serum-deprived medium. Alternatively, cells in G<sub>0</sub> stage can be induced to enter into the cell cycle, that is, at G<sub>1</sub> stage by serum activation (e.g., exposing the cells to serum after the cells have been deprived of a certain amount of serum).

The donor cell can be any type of cell that contains a genome or genetic material (e.g., nucleic acid), such as a somatic cell, germ cell or a stem cell. The term "somatic cell" as used herein refers to a differentiated cell. The cell can be a somatic cell or a cell that is committed to a somatic cell lineage. Alternatively, any of the methods described herein can utilize a diploid stem cell that gives rise to a germ cell in order to supply the genome for producing a nuclear transfer embryo. The somatic cell can originate from an animal or from a cell and/or tissue culture system. If taken from an animal, the animal can be at any stage of development, for example, an embryo, a fetus or an adult. Additionally, the present invention can utilize embryonic somatic cells. Embryonic cells can include embryonic stem cells as well as embryonic cells committed to a somatic cell lineage. Such cells can be obtained from the endoderm, mesoderm or ectoderm of the embryo. Embryonic cells committed to a somatic cell lineage refer

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to cells isolated on or after approximately day ten of embryogenesis. However, cells can be obtained prior to day ten of embryogenesis. If a cell line is used as a source for a chromosomal genome, then primary cells are preferred. The term "primary cell line" as used herein includes primary cells as well as primary derived cell lines.

5           Suitable somatic cells include fibroblasts (for example, primary fibroblasts), epithelial cells, muscle cells, cumulous cells, neural cells, and mammary cells. Other suitable cells include hepatocytes and pancreatic islets.

          The genome of the somatic cell can be the naturally occurring genome, for example, for the production of cloned animals, or the genome can be genetically altered to comprise a  
10 transgenic sequence, for example, for the production of transgenic cloned animals, as further described herein.

          Somatic cells can be obtained by, for example, disassociation of tissue by mechanical (e.g., chopping, mincing) or enzymatic means (e.g., trypsinization) to obtain a cell suspension followed by culturing the cells until a confluent monolayer is obtained. The somatic cells can  
15 then be harvested and prepared for cryopreservation, or maintained as a stalk culture. The isolation of somatic cells, for example, fibroblasts, is described herein.

          The nucleus of the donor cell is introduced before or upon exposure to the chemical or condition used to induce enucleation, or during any time prior cessation of protrusion of the second polar body containing essentially all of the endogenous chromatin. The donor nucleus  
20 and the enucleating oocyte can be combined in variety of ways to form the nuclear transfer embryo. For example, genome of a donor cell can be injected into the activated oocyte by employing a microinjector (i.e., micropipette or needle). The nuclear genome of the donor cell, for example, a somatic cell, is extracted using a micropipette or needle. Once extracted, the donor's nuclear genome can then be placed into the activated oocyte by inserting the  
25 micropipette, or needle, into the oocyte and releasing the nuclear genome of the donor's cell. See, for example, McGrath, J. and D. Solter, *Science*, 226:1317-1319 (1984), the teachings of which are incorporated by reference in their entirety.

          Alternatively, the genome of a donor cell can be combined with an oocyte by fusion; e.g., electrofusion, viral fusion, liposomal fusion, biochemical reagent fusion (e.g., phytohemagglutinin  
30 (PHA) protein), or chemical fusion (e.g., polyethylene glycol (PEG) or ethanol). The nucleus of

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the donor cell can be deposited within the zona pelliduca which contains the oocyte. The steps of fusing the nucleus with the oocyte can then be performed by applying an electric field which will also result in a second activation of the oocyte. Anaphase II and/or Telophase II oocytes (e.g., oocyte having an extruding second polar body) used are already activated, hence any activation subsequent to or simultaneous with the introduction of genome from a somatic cell would be considered a second activation event. With respect to electrofusion, chambers, such as the BTX® 200 Embryomanipulation System for carrying out electrofusion are commercially available from for example BTX®, San Diego. The combination of the genome of the donor cell with the oocyte results in a nuclear transfer embryo.

10 A nuclear transfer embryo of the present invention is then transferred into a recipient animal female and allowed to develop or gestate into a cloned or transgenic animal. Conditions suitable for gestation are those conditions that allow for the embryo to develop and mature into a fetus, and eventually into a live animal. Such conditions are known in the art. For example, the nuclear transfer embryo can be transferred via the fimbria into the oviductal lumen of each recipient animal female. In addition, methods of transferring an embryo to a recipient known to those skilled in the art and are described in Ebert *et al*, *Bio/Technology*, 12:699 (1994). The nuclear transfer embryo can be maintained in a culture system until at least first cleavage (2-cell stage) up to the blastocyst stage, preferably the embryos are transferred at the 2-cell or 4-cell stage. Various culture media for embryo development are known to those skilled in the art. For example, the nuclear transfer embryo can be co-cultured with oviductal epithelial cell monolayer derived from the type of animal to be provided by the practitioner.

The present invention encompasses the cloning of a variety of animals. These animals include, for example, human or nonhuman mammals, (e.g., canines, felines, murine species (e.g., mice, rats), and ruminants (e.g., cows, sheep, goats, camels, pigs, oxen, horses, llamas)). In particular, goats of Swiss origin, for example, the Alpine, Saanen and Toggenburg bread goats can be used. The donor cell and the oocyte are preferably from the same species, and once combined, an animal of the same species is impregnated with embryo.

“Cloning an animal” refers to producing an animal that develops from an oocyte containing genetic information or the nucleic acid sequence of another animal, the animal being cloned. The cloned animal has substantially the same or identical genetic information as that of

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the animal being cloned. "Cloning" also refers to cloning a cell, which includes producing an oocyte containing genetic information or the nucleic acid sequence of another animal. The resulting oocyte having the donor genome is referred to herein as a "nuclear transfer embryo."

The present invention also relates to methods for generating transgenic animals. A transgenic animal is an animal that has been produced from a genome from a donor cell that has been genetically altered, for example, to produce a particular protein (a desired protein). Methods for introducing DNA constructs into the germ line of an animal to make a transgenic animal are known in the art. For example, one or several copies of the construct can be incorporated into the genome of a animal embryo by standard transgenic techniques.

Embryonal target cells at various developmental stages can be used to introduce transgenes. A transgene is a gene that produces the desired protein and is eventually incorporated into the genome of the activated oocyte. Different methods are used depending upon the stage of development of the embryonal target cell. The specific lines of any animal used to practice this invention are selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness. In addition, the haplotype is a significant factor.

Genetically engineered donor cells for use in the instant invention can be obtained from a cell line into which a nucleic acid of interest, for example, a nucleic acid which encodes a protein, has been introduced.

A construct can be introduced into a cell via conventional transformation or transfection techniques. As used herein, the terms "transfection" and "transformation" include a variety of techniques for introducing a transgenic sequence into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE dextrane-mediated transfection, lipofection, or electroporation. In addition, biological vectors, for example, viral vectors can be used as described below. Samples of methods for transforming or transfecting host cells can be found in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual In Second Edition*, Cold Spring Harbor Laboratory, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989). Two useful and practical approaches for introducing genetic material into a cell are electroporation and lipofection.

The DNA construct can be stably introduced into a donor cell line by electroporation using the following protocol: donor cells, for example, embryonic fibroblasts, are resuspended in

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phosphate buffer saline (PBS) at about  $4 \times 10^6$  cells per mL. Fifty micrograms of linearized DNA is added to the 0.5 mL cell suspension, and the suspension is placed in a 0.4 cm electrode gap cuvette. Electroporation is performed using a BioRad Gene Pulser (Bio Rad) electroporator with a 330 volt pulse at 25 mA, 1000 microFarad and infinite resistance. If the DNA construct contains a neomycin resistance gene for selection, neomycin resistant clones are selected following incubation where 350 mg/mL of G418 (GIBCO BRL) for fifteen days.

The DNA construct can be stably introduced into a donor somatic cell line by lipofection using a protocol such as the following: about  $2 \times 10^5$  cells are plated into a 3.5 cm well and transfected with 2 mg of linearized DNA using LipfectAMINE<sup>®</sup> (GIBCO BRL). Forty-eight hours after transfection, the cells are split 1: 1000 and 1: 5000 and if the DNA construct contains a neomycin resistance gene for selection, G418 is added to a final concentration of 0.35 mg/mL. Neomycin resistant clones are isolated and expanded for cyropreservation as well as nuclear transfer.

It is often desirable to express a protein, for example, a heterologous protein, in a specific tissue or fluid, for example, the milk of a transgenic animal. A heterologous protein is a protein that is not naturally made by the cloned species (e.g., a protein that is derived from a different species than the species being cloned). The heterologous protein can be recovered from the tissue or fluid in which it is expressed. For example, it is often desirable to express the heterologous protein in milk. Methods for producing a heterologous protein under the control of a milk-specific promoter is described below. In addition, other tissue-specific promoters, as well as, other regulatory elements, for example, signal sequences and sequences which enhance secretion of non-secreted proteins, are described below. The transgenic product (e.g., a heterologous protein) can be expressed, and therefore, recovered in various tissue, cells or bodily secretions of the transgenic animals. Examples of such tissue, cells or secretions are blood, urine, hair, skin, mammary gland, muscle, or viscera (or a tissue component thereof) including, but not limited to, brain, heart, lung, kidney, pancreas, gall bladder, liver, stomach, eye, colon, small intestine, bladder, uterus and testes. Recovery of a transgenic product from these tissues are well known to those skilled in the art, see, for example, Ausubel, F. M., *et al.*, (eds), *Current Protocols in Molecular Biology*, vol. 2, ch. 10 (1991).

Useful transcriptional promoters are those promoters that are preferentially activated in mammary epithelial cells, including promoters that control the genes encoding protein such as caseins,  $\beta$ -lactoglobulin (Clark *et al.*, *Bio/Technology*, 7:487-492 (1989)), whey acid protein (Gordon *et al.*, *Bio/Technology*, 5:1183-1187 (1987)), and lactalbumin (Soulier *et al.*, *Febs Letts.*, 5 297:13 (1992)). Casein promoters can be derived from the alpha, beta, gamma, or kappa casein genes of any animal species; a preferred promoter is derived from the goat  $\beta$ -casein gene (Ditullio, *Bio/Technology*, 10:74-77 (1992)). Milk specific protein promoter or the promoters that are specifically activated in mammary tissue can be derived from cDNA or genomic sequences.

10 DNA sequence information is available for the mammary gland's specific genes listed above, in at least one, and often in several organisms. See, for example, Richards *et al.*, *J. Biol. Chem.*, 256:526-532 (1981) ( $\alpha$ -Lactalbumin rat); Campbel *et al.*, *Nucleic Acids Res.*, 12:8685-8697 (1984) (rat WAP); Jones *et al.*, *J. Biol. Chem.*, 260:7042-7050 (1985) (rat  $\beta$ -Casein); Yu-Lee and Rosen, *J. Biol. Chem.*, 258:10794-10804 (1983) (rat  $\alpha$ -Casein); Hall, *Bio. Chem. J.*, 15 242:735-742 (1987); ( $\alpha$ -Lactalbumin human); Stewart, *Nucleic Acids Res.*, 12:389 (1984) (Bovine  $\alpha$  S1 and  $\kappa$ 1 Casein, cDNAs); Gorodetsky *et al.*, *Gene*, 66:87-96 (1988) (Bovine  $\beta$ -Casein); Alexander *et al.*, *Eur. J. Biochem.*, 178:395-401 (1988) (Bovine and  $\kappa$ -Casein); Brignon *et al.*, *Febs Let.*, 188:48-55 (1977) (Bovine  $\alpha$  S2 Casein); Gamieson *et al.*, *Gene*, 61:85-90 (1987); Ivanov *et al.*, *Biol. Chem. Hopp-Seylar*, 369:425-429 (1988); Alexander *et al.*, *Nucleic Acid Res.*, 17:6739 (1989) (Bovine  $\beta$ - Lactoglobulin); Vilotte *et al.*, *Biochimie*, 69:609-620 20 (1987) (Bovine  $\alpha$ - Lactalbumin).

The structure and function of the various milk protein genes are reviewed by Mercier & Vilotte, *J. Dairy Sci.*, 76:3079-3098 (1993). If additional flanking sequences are useful in optimizing expression of the heterologous protein, such sequences can be cloned using the 25 existing sequences as probes. Mammary gland specific regulatory sequences from different organisms can be obtained by screening libraries from such organisms using known cognate nucleotide sequences, or antibodies to cognate proteins as probes.

Useful signal sequences such as milk specific signal sequences or other signal sequences which result in the secretion of eukaryotic or prokaryotic proteins can be used. Preferably, the 30 signal sequence is selected from milk specific signal sequences, that is, it is from a gene which

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encodes a product secreted into milk. Most preferably, the milk specific signal sequence is related to the milk specific promoter used in the construct. The size of the signal sequence is not critical. All that is required is that the sequence be of a sufficient size to effect secretion of the desired recombinant protein, for example, in the mammary tissue. For example, signal sequences  
5 from genes coding for caseins, for example,  $\alpha$ ,  $\beta$ ,  $\gamma$  or  $\kappa$  caseins and the like can be used. A preferred signal sequence is the goat  $\beta$ -casein signal sequence. Signal sequences from other secreted proteins, for example, proteins secreted by kidney cells, pancreatic cells, or liver cells, can also be used. Preferably, the signal sequence results in the secretion of proteins into, for example, urine or blood.

10 A non-secreted protein can also be modified in such a manner that it is secreted such as by inclusion in the protein to be secreted all or part of the coding sequence of a protein which is normally secreted. Preferably, the entire sequence of the protein which is normally secreted is not included in the sequence of the protein but rather only a sufficient portion of the amino terminal end of the protein which is normally secreted to result in secretion of the protein. For  
15 example, a portion which is not normally secreted is fused (usually at its amino terminal end) to an amino terminal portion of the protein which is normally secreted.

In one aspect, the protein which is normally secreted is a protein which is normally secreted in milk. Such proteins include proteins secreted by mammary epithelial cells, milk proteins such as caseins,  $\beta$ -lactoglobulin, whey acid protein, and lactalbumin. Casein proteins  
20 including, alpha, beta, gamma or kappa casein genes of any mammalian species. The preferred protein is  $\beta$ -casein, for example, goat  $\beta$ -casein. Sequences which encode the secreted protein can be derived from either cDNA or genomic sequences. Preferably, they are of genomic origin, and include one or more introns.

Other tissue specific promoters which provide expression in a particular tissue can be  
25 used. Tissue specific promoters are promoters which are expressed more strongly in a particular tissue than in others. Tissue specific promoters are often expressed exclusively in the specific tissue.

Tissue specific promoters which can be used include: a neural-specific promoter, for example, nestin, Wnt-1, Pax-1, Engrailed-1, Engrailed-2, Sonic-hedgehog; a liver specific  
30 promoter, for example, albumin, alpha-1, antitrypsin; a muscle-specific promoter, for example,

myogenin, actin, MyoD, myosin; an oocyte specific promoter, for example, ZP1, ZP2, ZP3; a testis specific promoter, for example, protamine, fertilin, synaptonemal complex protein-1; a blood specific promoter, for example, globulin, GATA-1, porphobilinogen deaminase; a lung specific promoter, for example, surfactin protein C; a skin or wool specific promoter, for example, keratin, elastin; endothelium-specific promoter, for example, TIE-1, TIE-2; and a bone specific promoter, for example, BMP. In addition, general promoters can be used for expression in several tissues. Examples of general promoters, include  $\beta$ -actin, ROSA-21, PGK, FOS, c-myc, Jun-A, and Jun-B.

A cassette which encodes a heterologous protein can be assembled as a construct which includes a promoter for a specific tissue, for example, for mammary epithelial cells, a casein promoter. The construct can also include a 3' untranslated region downstream of the DNA sequence coding for the non-secreted proteins. Such regions can stabilize the RNA transcript of the expression system and thus increase the yield of desired protein from the expression system. Among the 3' untranslated regions useful in the constructs for use in the invention are sequences that provide a polyA signal. Such sequences can be derived, for example, from the SV40 small t antigen, the casein 3' untranslated region or other 3' untranslated sequences well known in the art. In one aspect, the 3' untranslated region is derived from a milk specific protein. The length of the 3' untranslated region is not critical but the stabilizing effect of its polyA transcript appears imported in stabilizing the RNA of the expression sequence.

Optionally, the construct can include a 5' untranslated region between the promoter and the DNA sequence encoding the signal sequence. Such untranslated regions can be from the same control region as that from which the promoter is taken or can be from a different gene, for example, they can be derived from other synthetic, semisynthetic or natural sources. Again, there specific length is not critical, however, they appear to be useful in improving the level of expression.

The construct can also include about 10%, 20%, 30% or more of the N-terminal coding region of a gene preferentially expressed in mammary epithelial cells. For example, the N-terminal coding region can correspond to the promoter used, for example, a goat  $\beta$ -casein N-terminal coding region.

The construct can be prepared using methods known to those skilled in the art. The construct can be prepared as part of a larger plasmid. Such preparation allows the cloning and selection of the correct constructions in an efficient manner. The construct can be located between convenient restrictions sites on the plasmid so that they can be easily isolated from the remaining plasmid sequences for incorporation into the desired animal.

Transgenic sequences encoding heterologous proteins can be introduced into the germ line of an animal or can be transfected into a cell line to provide a source of genetically engineered donor cells as described above. The protein can be a complex or multimeric protein, for example, a homo- or heteromultimeric proteins. The protein can be a protein which is processed by removing the N-terminus, C-terminus or internal fragments. Even complex proteins can be expressed in active form. Protein encoding sequences which can be introduced into the genome of an animal, for example, goats, include glycoproteins, neuropeptides, immunoglobulins, enzymes, peptides and hormones. The protein can be a naturally occurring protein or a recombinant protein for example, a fragment or fusion protein, (e.g., an immunoglobulin fusion protein or a mutien). The protein encoding nucleotide sequence can be human or non-human in origin. The heterologous protein can be a potential therapeutic or pharmaceutical agent such as, but not limited to, alpha-1 proteinase inhibitor, alpha-1 antitrypsin, alkaline phosphatase, angiogenin, antithrombin III, any of the blood clotting factors including Factor VIII, Factor IX, and Factor X chitinase, erythropoietin, extracellular superoxide dismutase, fibrinogen, glucocerebrosidas, glutamate decarboxylase, human growth factor, human serum albumin, immunoglobulin, insulin, myelin basic protein, proinsulin, prolactin, soluble CD 4 or a component or complex thereof, lactoferrin, lactoglobulin, lysozyme, lactalbumin, tissue plasminogen activator or a variant thereof. Immunoglobulin particularly preferred protein. Examples of immunoglobulins include IgA, IgG, IgE, IgM, chimeric antibodies, humanized antibodies, recombinant antibodies, single chain antibodies and anti-body protein fusions.

Nucleotide sequence information is available for several of the genes encoding the heterologous proteins listed above, in at least one, and often in several organisms. See, for example, Long *et al.*, *Biochem.*, 23(21):4828-4837 (1984) (Alpha-1 antitrypsin); Mitchell *et al.*, *Prot. Natl. Acad. Sci. USA*, 83:7182-7186 (1986) (Alkaline phosphatase); Schneider *et al.*, *Embo J.*, 7(13): 4151-4156 (1988) (Angiogenin); Bock *et al.*, *Biochem.*, 27 (16):6171-6178

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(1988) (Antithrombin); Olds *et al.*, *Br. J. Haematol.*, 78(3): 408-413 (1991) (Antithrombin III); Lyn *et al.*, *Proc. Natl. Acad. Sci. USA*, 82(22):7580-7584 (1985) (erythropoietin); U.S. Pat No. 5,614,184 (erythropoietin) Horowitz, *et al.*, *Genomics*, 4(1):87-96 (1989) (Glucocerebrosidase); Kelly *et al.*, *Ann. Hum. Genet.*, 56(3):255-265 (1992) (Glutamate decarboxylase); U.S. Pat No. 5,707,828 (human serum albumin); U.S. Pat. No. 5,652,352 (human serum albumin); Lawn *et al.*, *Nucleic Acid Res.*, 9(22):6103-6114 (1981) (human serum albumin); Kamholz *et al.*, *Prot. Matl. Acad. Sci. USA*, 83(13):4962-4966 (1986) (myelin basic protein); Hiraoka *et al.*, *Mol. Cell Endocrinol.*, 75(1):71-80 (1991) (prolactin); U.S. Pat. No. 5,571,896 (lactoferrin); Pennica *et al.*, *Nature*, 301(5897):214-221 (1983) (tissue plasminogen activator); Sarafanov *et al.*, *Mol. Biol.*, 29: 161-165 (1995).

A transgenic protein can be produced in the transgenic cloned animal at relatively high concentrations and in large volumes, for example in milk, providing continuous high level output of normally processed protein that is easily harvested from a renewable resource. There are several different methods known in the art for isolation of proteins for milk.

Milk proteins usually are isolated by a combination of processes. Raw milk first is fractionated to remove fats, for example by skimming, centrifugation, sedimentation, (H.E. Swaisgood, *Development in Dairy Chemistry, I: Chemistry of Milk Protein*, Applied Science Publishers, NY 1982), acid precipitation (U.S. Patent No. 4,644,056) or enzymatic coagulation with rennin or chymotrypsin (Swaisgood, *ibid.*). Next the major milk proteins can be fractionated into either a clear solution or a bulk precipitate from which this specific protein of interest can be readily purified.

French Patent No. 2487642 describes the isolation of milk proteins from skim milk or whey by performing ultra filtration in combination with exclusion chromatography or ion exchange chromatography. Whey is first produced by removing the casein by coagulation with rennet or lactic acid. U.S. Patent No. 4,485,040 describes the isolation of an  $\alpha$ -lactoglobulin-enriched product in the retentate from whey by two sequential ultra filtration steps. U.S. Patent No. 4,644,056 provides a method for purifying immunoglobulin from milk or colostrum by acid precipitation at pH 4.0-5.5, is sequential cross-flow filtration first on a membrane with 0.1-1.2 mm pore size to clarify the product pool and then on a membrane with a separation limit of 5-80 kD to concentrate it. Similarly, U.S. Patent No. 4,897,465 teaches the concentration and

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enrichment of a protein such as immunoglobulin from blood serum, egg yolks or whey by sequential ultra filtration on metallic oxide membranes with a pH shift. Filtration is carried out first at a pH below the isoelectric point (pI) of the selected protein to remove bulk contaminants from the protein retentate, in next adding pH above the pI of the selected protein to retain  
5 impurities and pass the selected protein to the permeate. A different filtration concentration method is taught by European Patent No. EP 467 482 B 1 in which defatted skim milk is reduced to pH 3-4, below the pI of the milk proteins, to solubilize both casein and whey proteins. Three successive rounds of ultra filtration are diafiltration and concentrate the proteins to form a retentate containing 15-20% solids of which 90% is protein. Alternatively, British Patent  
10 Application No. 2179947 discloses the isolation of lactoferrin from whey by ultra filtration to concentrate the sample, fall by weak cation exchange chromatography at approximately a neutral pH. No measure of purity is reported in PCT Publication No. WO 95/22258, a protein such as lactoferrin is recovered from milk that has been adjusted to high ionic strength by the addition of concentrated salt, followed by cation exchange chromatography.

15 In all these methods, milk or a fraction thereof is first treated to remove fats, lipids, and other particular matter that would foul filtration membranes or chromatography medium. The initial fractions thus produce can consist of casein, whey, or total milk protein, from which the protein of interest is then isolated.

PCT Patent Publication No. WO 94/19935 discloses a method of isolating a biologically  
20 active protein from whole milk by stabilizing the solubility of total milk proteins with a positively charged agent such as arginine, imidazole or Bis-Tris. This treatment forms a clarified solution from which the protein can be isolated for example by filtration through membranes that otherwise would become clogged by precipitated proteins.

Methods for isolating a soluble milk component such as a peptide in its biologically active  
25 form from whole milk or a milk fraction by tangential flow filtration are known. Unlike previous isolation methods, this eliminates the need for a first fractionation of whole milk to remove fat micelles, thereby simplifying the process in avoiding losses of recovery of bioactivity. This method can be used in combination with additional purification steps to further remove contaminants and purify the product (e.g., the protein of interest).

30

5 The following examples are intended to be illustrative and not limiting in any way.

#### EXEMPLIFICATION

EXAMPLE 1: IMPROVED METHOD OF CLONING THROUGH THE  
INTRODUCTION OF DONOR CELL NUCLEUS PRIOR TO  
10 COMPLETION OF ENUCLEATION PROCESS

Progress has continued with experiments designed to compare *in vitro* development of control embryos (parthenotes) with that of reconstructed NT embryos prepared by conventional NT, telophase NT, IE and a novel simultaneous IE-NT paradigm (SIE/NT; see Table 1 below). As in all previous experiments, abattoir-sourced oocytes were obtained having been submitted to  
15 26h of maturation in ACM media @5%CO<sub>2</sub> in air. Oocytes were denuded (enzyme/vortex) and activated (5μM ionomycin for 5 min, +/- CHX). See Example 2 for detail on methods. IE was performed by treatment of oocytes with demecolcine (0.4μg/ml) starting at 1.5h and ending 5h post-activation (p.a.) and nuclei were injected 1.5-3h p.a. In the case of SIE/NT, donor karyoplasts (fibroblasts) were injected immediately prior to or immediately after activation. *In*  
20 *vitro* embryo development was evaluated over a period of 7 days.

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SUMMARY of Bovine NT *in vitro* development

GROUP	Total oocytes	Cleavage	Morula	Blastocyst	Frag-mented
<i>Control Parthnotes Zona-intact</i>	67	58% (39/67)	13% (5/39)	21% (8/39)	28%
5 <i>Control Parthnotes Zona-free</i>	30	86% (26/30)	NA	8% (2/26)	41%
<i>Conventional MII Enucleation (CMII)</i>	23	35% (8/23)	17% (4/23)	9% (2/23)	22%
10 <i>Telophase Enucleation (TE)</i>	55	62% (34/55)	NA	12% (4/34)	26%
<i>Induced Enucleation (IE)</i>	144	49% (71/144)	17% (12/71)	16% (11/71)	20%
15 <i>Simultaneous IE-NT (SIE/NT)</i>	145	54% (79/145)	NA	25% (20/79)	18%

15 In summary, these data further confirm the comparative *in vitro* development potential of NT embryos produced by conventional, telophase and IE protocols. Moreover, the SIE/NT protocol appears to support the highest rate of blastocyst development when compared to the other methods. This observation is further supports the mechanism by which higher NT development rates can be obtained using the IE method. This is founded on the concept that IE

20 affords spindle-associated enabling factors to compartmentalize within the enucleating cytoplasm in a manner and time frame so as to enhance cytoplasmic-nuclear synchronization and chromatin remodeling.

25 EXAMPLE 2: DEMECOLCINE-INDUCED OOCYTE ENUCLEATION FOR SOMATIC CELL CLONING: COORDINATION BETWEEN CELL CYCLE EGRESS, KINETICS OF CORTICAL CYTOSKELETAL INTERACTIONS, AND SECOND POLAR BODY EXTRUSIONS

30 Studies were designed to further explore the use of pharmacological agents to create developmentally competent enucleated mouse oocytes for animal cloning by somatic cell nuclear transfer. Metaphase-II oocytes from CF-1 and B6D2F1 strains were activated with ethanol and

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subsequently exposed to demecolcine at various times post-activation. Chromosome segregation, spindle dynamics and polar body (PB) extrusion were monitored by fluorescence microscopy using DNA, microtubule and microfilament selective probes. Exposure to demecolcine did not affect rates of oocyte activation induced by ethanol but did disrupt the coordination of cytokinesis and karyokinesis, suppressing the extent and completion of spindle rotation and second PB  
5 extrusion in a strain-dependent manner. Moreover, strain and treatment specific variations in the rate of oocyte enucleation were also detected. In particular, CF-1 oocytes were more efficiently enucleated relative to B6D2F1 and demecolcine treatments initiated early after activation resulted in higher enucleation rates than when treatment was delayed. The observed strain differences are  
10 possibly due to a combination of factors such as the time course of meiotic cell cycle progression after ethanol-activation, the degree of spindle rotation and the extent of second PB extrusion. These results suggest that developmentally competent cytoplasts can be produced by timely exposure of activated oocytes to agents that disrupt spindle microtubules.

In the present study, the temporal consequences of demecolcine-induced enucleation with  
15 reference to the cytoskeletal remodeling that occurs during early phases of oocyte activation in CF-1 and B6D2F1 mouse strains was investigated. In particular, manifestations of demecolcine treatment on spindle rotation/anchoring dynamics, and second PB formation and extrusion were investigated.

## 20 MATERIALS AND METHODS

### *Collection of mature oocytes*

Hybrid B6D2F1 (C57BL/6 x DBA/2) and outbred CF-1 female mice, 8-12 weeks of age, were used as oocyte donors. Animal care and procedures were conducted according to protocols approved by the Tufts University Institutional Animal Care and Use Committee. Females were  
25 induced to superovulate by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Calbiochem) followed 48 h later by 5 IU of human chorionic gonadotropin (hCG, Calbiochem). MII oocytes were collected from oviducts 16-17 h after hCG administration in HEPES-buffered KSOM (H-KSOM, Specialty Media). Cumulus cells were dispersed by incubation in 150 units/ml of bovine testicular hyaluronidase (Sigma) in H-KSOM at 37°C for 5

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min. Cumulus-free oocytes were then washed three times in fresh H-KSOM and immediately activated.

*Oocyte activation, treatment and culture*

5 Oocytes were parthenogenetically activated by a 5-min exposure to freshly prepared 7% (v/v) ethanol in H-KSOM at 37°C and then washed twice in H-KSOM. Removal of oocytes from ethanol was considered as time zero post-activation (p.a.). To monitor meiotic progression, activated control oocytes (EtOH groups) were cultured for up to 2 h and 15 min and fixed at 30 min intervals, starting 45 min p.a. Culture of activated oocytes was at 37°C under 5% CO<sub>2</sub> in air  
10 in KSOM medium containing 1 mg/ml BSA and amino acids (16; Specialty Media).

Other activated oocytes were treated with the microtubule-destabilizing drug demecolcine (Sigma) at a concentration of 0.4 µg/ml in KSOM (Deme groups). These oocytes were cultured in the continued presence of demecolcine starting either immediately after activation (Deme 0 groups) or with a delay of 5, 10 or 15 min after their removal from ethanol (Deme 5, Deme 10  
15 and Deme 15 groups, respectively). Demecolcine-treated oocytes were fixed at 30 min intervals, from 45 min to 135 min p.a., identical to control oocytes. To determine oocyte meiotic status at the onset of demecolcine treatments, some control activated B6D2F1 and CF-1 oocytes were fixed at the same time drug exposure was initiated in the treatment groups: 0, 5, 10 and 15 min p.a.

20

*Fixation of oocytes and processing for immunofluorescence analysis*

At defined time-points after activation, control and demecolcine-treated oocytes were fixed and extracted for 30 min at 37°C in a microtubule stabilizing buffer containing 0.1 M PIPES, 5 mM MgCl<sub>2</sub>, 2.5 mM EGTA, 3.7% formaldehyde, 0.1% Triton X-100, 1 µM taxol,  
25 0.01% aprotinin, 1 mM dithiothreitol (DTT) and 50% deuterium oxide. Fixed oocytes were stored until processing at 4°C in a PBS blocking solution containing 1% BSA, 0.2% powdered milk, 2% normal goat serum, 0.1 M glycine, 0.2% sodium azide and 0.01% Triton X-100. Wickramasinghe D, *et al.*, *Dev Biol* 152:62-74 (1992).

A triple labeling protocol was used for the detection of microtubules, microfilaments and  
30 chromatin by fluorescence microscopy. Oocytes were first incubated for 1 h at 37°C in a mixture

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of mouse monoclonal anti  $\alpha$ -tubulin and anti  $\beta$ -tubulin antibodies (Sigma) at a 1:1000 final dilution. After several washes in 0.1% polyvinylpyrrolidone (PVP)/PBS at room temperature, oocytes were incubated at 37°C in PBS blocking solution for 30 min and then in a 1:150 dilution of a donkey antimouse fluorescein-conjugated IgG (Jackson ImmunoResearch) for 45 min at  
5 37°C. Oocytes were washed again several times in 0.1% PVP/PBS and incubated at 37°C for 30 min in 10 units/ml of Texas Red-conjugated phalloidin (Molecular Probes) to stain actin filaments. Finally, after extensive washing in 0.1% PVP/PBS, oocytes were incubated at room temperature for 15 min in 10  $\mu$ Lg/ml Hoechst 33258 (Molecular Probes) and mounted in 50% glycerol/PBS containing 25 mg/ml sodium azide.

10 Labeled oocytes were examined using a Zeiss IM-35 inverted epi-fluorescence microscope fitted with filters selective for Hoechst, fluorescein and Texas Red and a 50 W mercury lamp. Selected images were acquired using a Photometrics Cool Snap CCD camera (Roper Scientific Inc., Trenton, NJ) running on Metamorph software (version 5.0, Universal Imaging Corp., Downingtown, PA).

15

#### *Statistical analysis*

All demecolcine treatments were repeated at least three times and approximately 50 oocytes were examined per treatment at each defined time-point. Data were analyzed by  $\chi^2$  test or Fisher's exact test. A probability value of  $P < 0.05$  was considered to be statistically significant.

20

## RESULTS

To monitor the effects of demecolcine on the meiotic cell cycle progression after activation and determine its efficiency in inducing oocyte enucleation, ethanol-activated oocytes of the B6D2F1 and CF-1 mouse strains were cultured in the presence of drug and analyzed at  
25 selected timepoints for microtubule (MT), microfilaments and chromatin organization. Exposure to demecolcine was continuous for 30-135 min, from 0, 5, 10 or 15 min p.a. Control activated oocytes were cultured for the same period of time in the absence of demecolcine.

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*Ethanol activation rates are not affected by demecolcine treatment:*

The effect of demecolcine on the meiotic spindle was evident 15 min. after the onset of treatment, since spindles in treated oocytes were smaller and displayed a lower MT density compared to untreated control activated oocytes. Although MT density decreased with extended exposure to drug, spindle MTs did not disappear completely and even after 2 h. of treatment a few short MTs were detected in the majority of the oocytes.

At 45 min p.a., 98% and 92.5% of the control activated B6D2F1 and CF-1 oocytes, respectively, had resumed meiosis as evidenced by chromatid segregation, spindle elongation and the presence of a large, actin-rich cortical protrusion or, in a few cases, a completely extruded second PB. These oocytes were considered activated. Similar rates of activation at 45 min p.a. (80-98%; Table 2) were observed in all groups of demecolcine-treated oocytes according to the same criteria, except that spindle elongation failed to occur. Although the extent of chromosome separation was reduced, due to spindle disruption, two distinct clusters of chromosomes were clearly visible in these treated activated oocytes indicating that an effective anaphase had occurred. The chromosomes were subcortical to the oocyte cortex, and usually connected by a spindle remnant that resembled a midbody. As in the control group, a small fraction of oocytes had already extruded a second PB. Interestingly, a single group of chromosomes and no detectable MTs were present in treated oocytes that failed to activate.

TABLE 2. Activation rates of ethanol-activated oocytes (EtOH) and ethanol-activated oocytes treated with demecolcine (Deme) at different times (0-15 min) post-activation

		activated oocytes (n)				
5	Strain	Treatment	45 min*	75 min*	105 min*	135 min*
	B6D2F1	EtOH	98 (51)	98.1 (52)	100 (58)	100 (53)
		Deme 0	96 (50)	98 (50)	96 (50)	96 (50)
		Deme 5	98 (50)	94 (50)	100 (55)	94.5 (55)
10		Deme 10	94 (50)	98.1 (54)	100 (52)	100 (52)
		Deme 15	97.9 (47)	96(50)	94 (50)	98 (51)
	CF-1	EtOH	92.5 (67)	97 <sup>a</sup> (67)	97.3 <sup>a</sup> (74)	100 (48)
		Deme 0	92 (50)	98 <sup>a</sup> (50)	100 <sup>a</sup> (50)	95.9 (49)
		Deme 5	82 (50)	76 <sup>b</sup> (50)	84.3 <sup>b</sup> (51)	98 (51)
15		Deme 10	80 (50)	84 <sup>b</sup> (50)	96 <sup>a,b</sup> (50)	94.7 (57)
		Deme 15	94.2 (52)	100 <sup>a</sup> (53)	96.1 <sup>a,b</sup> (51)	95.9 (49)

\*Time post-activation

a,b Values with different superscripts within the same column and strain differ significantly (P<0.05)

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In the B6D2F1 strain, activation rates of demecolcine treated oocytes were equivalent to those of non-treated control oocytes at all time-points examined (Table 2). While rates of activation in some groups of CF-1 treated oocytes were lower than in the control group at 75 min and 105 min p.a., this effect was transitory and reversible since at 135 min after ethanol exposure activation rates were again equivalent among all groups. Therefore, normal rates of activation are obtained when activated oocytes are cultured in the continuous presence of demecolcine.

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#### *Effect of demecolcine on spindle rotation in activated oocytes:*

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As noted by others, two cortical protrusions formed adjacent to each spindle pole shortly after activation in non-treated control oocytes. One protrusion then regressed as the spindle rotated towards the remaining protrusion, and assumed an orientation perpendicular to the plasma membrane. Eventually this structure was constricted at the oolemma and gave rise to the second PB. Activated oocytes treated with demecolcine yielded two classes that displayed either a single (type A oocyte) or double (type B oocyte) cortical protrusions overlying the remnants of the spindle. The two sets of chromosomes were closer to each other in type A oocytes than in type B

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oocytes, suggesting that the formation of one or two protrusions was probably dependent on the extent of meiotic cell cycle progression before spindle disruption. Consistent with this idea, type A oocytes were more frequently observed when demecolcine treatment started immediately or 5 min after activation (Deme 0 and Deme 5 groups, respectively) whereas the incidence of type B oocytes predominated when treatment was delayed for 10 or 15 min (Deme 10 and Deme 15 groups, respectively).

Initiation of spindle rotation occurred in all groups of demecolcine-treated oocytes, except for the group Deme 0 in the CF-1 strain, but at lower rates than in control activated oocytes (Table 3). Although only a few short spindle MTs were present in treated oocytes, orientation of spindle remnants and the two chromosomal sets relative to the plasma membrane was used as an indicator of spindle rotation. CF-1 oocytes treated with demecolcine consistently exhibited a comparatively low percentage of activated oocytes undergoing a partial or complete spindle rotation at all p.a. time points examined. Demecolcine also impaired spindle rotation in B6D2F1 oocytes at 45 min and 75 min p.a. when compared to the controls, but the effect was less pronounced than in CF-1 oocytes. However, a dramatic decrease in the percentage of B6D2F1 treated oocytes showing spindle rotation occurred in all treatment groups at 105 min and 135 min p.a., suggesting that spindle rotation was reversed with prolonged drug exposure. By 135 min p.a. oocytes showing complete spindle rotation were observed in only 0-9.6% of the CF-1 and B6D2F1 treated oocytes, compared to 100% in both control groups; and the lack of differences between demecolcine treatments further attested to the effectiveness of demecolcine on spindle rotation. In all, control and treated oocytes showing a completely rotated spindle at 135 min p.a. extrusion of the second PB had occurred. Together, these results indicate that continued exposure to demecolcine after oocyte activation inhibits spindle rotation independent of the time of initiation of the treatment and the strain of oocyte, although the kinetics of this inhibition varies between strains.

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TABLE 3. Spindle rotation in ethanol-activated oocytes (EtOH) and ethanol-activated oocytes treated with demecolcine (Deme) at different times (0-15 min) post-activation

		activated oocytes with a partially or completely rotated spindle						
Strain	Treatment	45 min	75 min*	105 min*	135 min*			
10	B6D2F1	EtOH	64.0 <sup>a</sup> (50)	98.0 <sup>a</sup> (51)	100 <sup>a</sup> (58)	100 <sup>a</sup> (53)		
		Deme 0	37.5 <sup>b</sup> (48)	30.6 <sup>b</sup> (49)	0 <sup>b</sup> (48)	8.3 <sup>b</sup> (48)		
		Deme 5	26.5 <sup>b</sup> (49)	38.3 <sup>b</sup> (47)	3.6 <sup>b</sup> (55)	7.7 <sup>b</sup> (52)		
		Deme 10	29.8 <sup>b</sup> (47)	47.2 <sup>b</sup> (53)	7.7 <sup>b</sup> (52)	9.6 <sup>b</sup> (52)		
		Deme 15	30.4 <sup>b</sup> (46)	41.7 <sup>b</sup> (48)	34.0 <sup>c</sup> (47)	4.0 <sup>b</sup> (50)		
15	CF-1	EtOH	58.1 <sup>a</sup> (62)	84.6 <sup>b</sup> (65)	94.4 <sup>a</sup> (72)	100 <sup>a</sup> (48)		
		Deme 0	0 <sup>b</sup> (46)	0 <sup>b</sup> (49)	0 <sup>b</sup> (50)	0 <sup>b</sup> (47)		
		Deme 5	4.4 <sup>b</sup> (45)	2.6 <sup>b,c</sup> (38)	2.3 <sup>b</sup> (43)	4 <sup>b</sup> (50)		
		Deme 10	2.5 <sup>b</sup> (40)	2.4 <sup>b,c</sup> (42)	4.2 <sup>b</sup> (48)	1.8 <sup>b</sup> (54)		
		Deme 15	6.1 <sup>b</sup> (49)	13.2 <sup>c</sup> (53)	6.1 <sup>b</sup> (49)	2.1 <sup>b</sup> (47)		

\*Time post-activation

a-c Values with different superscripts within the same column and strain differ significantly (P&lt;0.05)

*Complete extrusion of the second polar body is inhibited in the presence of demecolcine:*

25 While the onset of second PB formation was evident in all treated activated oocytes, forming one or two cortical protrusions overlying chromosomes, completion of second PB extrusion was impaired in the presence of demecolcine. By 45 min p.a., a small and similar percentage of activated control and treated oocytes displayed a completely extruded second PB (Fig. 3A & 3B). Whereas the rates of PB extrusion in B6D2F1 and CF-1 control oocytes

30 increased progressively with time, reaching 100% at 135 min p. a., complete PB extrusion in demecolcine-treated oocytes from both strains was significantly decreased, with rates ranging from 23.1 % to 70.2% at the various time-points p.a. examined. Even though some differences were detected between treatments in both strains, a correlation between rates of second PB extrusion and the onset of demecolcine treatment could not be established. On the other hand,

35 comparison of second PB extrusion rates in demecolcine-treated oocytes from both strains revealed significant differences between groups Deme 10 at 75 min p.a., between all treatment groups at 105 min p.a. and between groups Deme 0, Deme 10 and Deme 15 at 135 min p.a. These

results suggest a strain-dependent effect of demecolcine on the suppression of second PB extrusion, being more pronounced in oocytes from the CF-1 strain.

In those demecolcine-treated oocytes that failed to extrude a second PB, cortical protrusions enlarged over time and, in some oocytes, showed signs of constriction at the oolemma. To determine whether PB extrusion was merely delayed in these oocytes, they were  
5 cultured for a longer period of time (4 h) before fixation and analysis. In most of the oocytes the cortical protrusion/s were reabsorbed while the formation of two pronuclei indicated that the cell cycle progressed to early interphase. As described *infra*, Figure 7C shows oocytes (Types A and B) that have been exposed to demecolcine, and as a result completion of cytokinesis of the  
10 second PB was prevented. Figure 7C also depicts oocytes (Type D, E and F) that have fully extruded second PB.

*Characteristic phenotypes are observed in activated oocytes treated with demecolcine that complete second polar body extrusion:*

15 All control activated oocytes extruded a second PB containing half of the chromosomal complement and displayed a midbody perpendicular to the plasma membrane. This phenotype was classified as type C and was also observed in a fraction of demecolcine-treated oocytes with completely extruded second PBs. However, midbodies in type C treated oocytes were narrower and shorter than in type C control activated oocytes, and were defined as midbody-like structures.  
20 Other treated oocytes that completed second B extrusion displayed characteristic phenotypes that were never detected in control activated oocytes. Type D oocytes deployed one set of chromosomes in the oocyte cytoplasm and one set inside the extruded second PB, connected by a midbody-like structure as in type C oocytes. However, the midbody-like structure was oriented in parallel to the plasma membrane, indicating that spindle rotation had not occurred. Moreover,  
25 a prominent protuberance adjacent to the second PB was present in type D oocytes, probably due to the subcortical position of the chromosomal complement in the oocyte in the absence of spindle rotation. Other demecolcine-treated oocytes displayed two (Type E) or one (Type F) completely extruded second PBs that contained all chromosomes. Therefore, type E and F represent totally enucleated oocytes. Rotation of the spindle had not occurred in these oocytes

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either, as was evidenced by the parallel orientation of the remaining spindle MTs and the observation of two sets of chromosomes inside the PB to the plasma membrane.

The frequency of each of these phenotypes varied according to the onset of the demecolcine treatment with regards to activation, the duration of the treatment, and the strain of the oocyte, further indicating variability in the responsiveness of demecolcine (Fig. 4). In B6D2F1 oocytes, type C was the most frequent at 45 min and 75 min p.a. in all treatments, except for Deme 0 group in which it was detected at similar rates as type D. Prolonged exposure, independent of when treatment was initiated, caused a shift to type D phenotype, as seen by the high percentage of type D oocytes at 135 min p.a. in all treatments. CF-1 oocytes exhibited a strikingly different response. For all treatments, type F was the main phenotype in those oocytes that completed second PB extrusion by 45 min p.a. An increase in the frequency of type D oocytes was observed over time, and at 135 min p.a. type D and type F oocytes appeared at a similar frequency, except for the group Deme 5 in which most of the oocytes were still of type F.

15 *Treatment of activated oocytes with demecolcine induces enucleation in a strain-dependent manner:*

According to the previous results, most CF-1 oocytes that completed extrusion of the second PB were enucleated as a result of demecolcine exposure, while the majority of B6D2F1 treated oocytes retained half of the chromosomal complement. This result is summarized in Figure 10, which shows the combined results for all time-points examined of the total oocytes enucleated as a result of the various demecolcine treatments applied. Enucleation rates ranging from 48.3% to 76.7% were obtained in those CF-1 oocytes that completed second PB extrusion, and from 1% to 17.3% in the B6D2F1 strain (FIG. 5A). In the four treatments applied, the rates of enucleation were significantly higher in CF-1 than in B6D2F1 oocytes, suggesting that the efficiency of demecolcine in inducing oocyte enucleation is strain-dependent. Moreover, enucleation efficiency was also dependent on the time treatment was initiated p.a., as indicated by the higher enucleation rates obtained in both strains of oocytes when demecolcine treatment was initiated soon after activation rather than later.

Because of the low rates of complete PB extrusion in demecolcine-treated oocytes, when the total activated oocytes are considered there is a dramatic decrease in the rates of enucleation

(FIG. 5B). Maximum enucleation rates of only 21% and 6.9% in CF-1 and in B6D2F1 oocytes, respectively, were obtained and again the enucleation efficiency in all four treatments was higher in CF-1 than in B6D2F1 oocytes. Although enucleation rates of the total activated oocytes were equivalent between treatments in CF-1 oocytes, some differences were detected between B6D2F1  
5 oocytes subjected to different treatments. These differences indicated, again, that exposure to demecolcine early after activation results in higher rates of enucleation than when the treatment is delayed.

*Meiotic cell cycle progression after activation is strain-dependent:*

10 In order to determine if the different efficiency of demecolcine to induce enucleation in CF-1 and B6D2F1 oocytes could be related to variations in the oocyte meiotic progression after activation, some control activated oocytes were fixed at the same time-points p.a. when the demecolcine treatments were initiated. As demecolcine effects on the meiotic spindle are not immediate, the meiotic progression at 45 min p.a. was also recorded. Although the time course  
15 of activation was similar in oocytes from the two strains, the rate of cell cycle progression after the activation stimulus was slightly different (Table 4). Release from MR arrest and entry into anaphase followed a similar progression in the two groups of oocytes after ethanol exposure, but the anaphase-telophase transition proceeded faster in CF-1 oocytes. Thus, 2.6% and 4.4% of activated CF-1 oocytes were at telophase II 10 min and 15 min p.a., respectively, while all  
20 activated B6D2F1 oocytes remained at anaphase II. By 45 min p.a. 87.1% of activated CF-1 oocytes had entered telophase II, a value significantly higher than the 60% observed for activated B6D2F1 oocytes.

TABLE 4. Time course of activation rates and meiotic status of B6D2F1 and CF-1 oocytes after ethanol activation

5	Strain	Time p.a.*	n	%activated	
				oocytes	Meiotic status**
10	B6D2F1	0 min	50	64.0	100% A
		5 min	49	57.1	100% A
		10 min	49	69.4	100% A
		15 min	52	84.6	100% A
		45 min	51	98.0	40% A, 60% T <sup>a</sup>
15	CF-1	0 min	50	60.0	100% A
		5 min	50	74.0	100% A
		10 min	50	78.0	97.4% A, 2.6% T
		15 min	50	90.0	95.6% A, 4.4% T
		45 min	67	92.5	12.9% A, 87.1 % T <sup>a</sup>

\*Removal from ethanol is considered as t=0 min post-activation (p.a.)

20 \*\*Only of activated oocytes. A: anaphase; T: telophase

<sup>a</sup> Values significantly different between the two strains (P<0.05)

## DISCUSSION

The microtubule-destabilizing drug, demecolcine, was used to induce enucleation of pre-activated mouse oocytes of the B6D2F1 strain as a means to prepare competent cytoplasts for nuclear transfer procedures. Additionally, the data described herein shows the relationship between oocyte cell cycle control and the cytoskeleton during exit from meiotic metaphase (M-phase).

Resumption of meiosis after fertilization or artificial activation of M II-arrested oocytes is characterized by chromosome segregation to the spindle poles, elongation and rotation of the meiotic spindle, and extrusion of a second PB containing half of the chromosomal complement of the oocyte. M-phase exit is triggered by the inactivation of maturation-promoting factor (MPF) and it is now well established that cyclin B degradation, and thus MPF inactivation, requires an intact spindle. Consistent with this, MII oocytes treated with demecolcine or nocodazole prior to *in vitro* fertilization or parthenogenetic activation remain arrested in M-phase, despite the occurrence of a normal pattern of calcium oscillations. The exact mechanism by which the meiotic spindle mediates the transition from meiotic M-phase to embryonic interphase remains unclear. In the data described herein, oocytes activated with ethanol prior to demecolcine treatment exhibited activation rates comparable to activated control oocytes never exposed to

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demecolcine. Ethanol exposure induces an immediate increase in intracellular calcium and rapid progression into anaphase, as evidenced by the rapidity of meiotic cell cycle resumption in control activated oocytes from the two strains analyzed in this work. Because a delay exists between the onset of demecolcine application and detectable signs of spindle MT disruption, the acute effects of ethanol on cell cycle resumption are not impeded. In fact, as the results in control activated oocytes show, most oocytes (>60%) exited M-phase and progressed to anaphase by the end of the 5 min ethanol exposure (0 min p.a.). Therefore, most of the oocytes were already at anaphase II or at the anaphase-telophase transition when the demecolcine treatment was applied. When demecolcine is applied after the activation stimulus, activation proceeds in the presence of the drug. However, at later stages there were clear consequences of demecolcine exposure that altered the relationship between karyokinesis and cytokinesis.

Also as shown herein, demecolcine binds tightly to tubulin dimers and prevents MT polymerization, resulting in the loss of dynamic spindle MTs in mitotic and meiotic cells. Immunofluorescence staining with antitubulin antibodies confirmed the time course and extent of spindle disruption by demecolcine and showed further that few short MTs remain in the majority of oocytes even after prolonged (2 h) drug exposure. The presence of these spindle remnants reflects differential stability of some MTs in the spindle, and likely correspond to interpolar MTs. Spindle disruption impaired the extent of chromatid segregation under these conditions. However, because oocytes were activated prior to demecolcine treatment, the observed variable degrees of chromosome segregation most likely result from the time of demecolcine administration, its uptake kinetics and variations in anaphase onset or duration. These results establish that cell cycle activation occurs prior to gross disruptions of spindle stability.

In early telophase, the meiotic spindle rotates from a parallel to a perpendicular orientation relative to the plasma membrane coincident with the initiation of second PB formation. Although the mechanism of spindle rotation is unclear, the presence of an actin-rich cortical domain overlying the spindle coupled with the inhibition of spindle rotation in both mouse and *Xenopus* oocytes treated with cytochalasin suggests that the interaction of spindle MTs with actin filaments of the cell cortex mediates spindle rotation and serves to coordinate karyokinesis and cytokinesis. Consistent with this, disruption of the spindle should also inhibit its rotation, as the results with demecolcine demonstrate. In fact, some demecolcine treated

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oocytes undergo some degree of spindle rotation, especially in the case of B6D2F1 eggs, but the process is completed in less than 10% of the oocytes. These observations suggest that spindle rotation is initiated before demecolcine induces depolymerization of the spindle MTs, which as a result perturbs interactions between MTs and cortical microfilaments and impairs further rotation of the spindle. Although suppression of spindle rotation occurred in both strains of oocytes examined, the percentage of oocytes with partially or completely rotated spindles at 45 and 75 min p.a. was higher in the B6D2F1 than in the CF-1 strain. This result indicates strain-dependent variation in the kinetics of inhibition of spindle rotation induced by demecolcine, that can not be related to interstrain differences in (a) the initiation and progression of spindle rotation and in (b) the rate of cell cycle progression (as detected in control activated oocytes from the two strains). Thus, these strain-dependent variations could be due to other factors associated with elongation and anchoring of the spindle such as centrosome positioning.

An additional effect of demecolcine was inhibition of second PB extrusion. The initial phase of PB formation, described as a "furrowing" of the plasma membrane in the region overlying the spindle, occurred in most treated oocytes, as evidenced by the formation of one or two actin-rich cortical protrusions. However, later "furrow constriction" and abscission was generally impaired in oocytes activated in the presence of demecolcine. Nocodazole or demecolcine treatment prior to furrowing and cleavage in sea urchin eggs has shown that MTs are required for furrow stimulation and the formation of the actomyosin contractile ring. But once furrowing has been stimulated, MTs are unnecessary. MTs are important for abscission, as depolymerization of the central spindle in late anaphase blocks the completion of cytokinesis. Several proteins necessary for cytokinesis have been localized to the central spindle and it has been suggested that MTs could serve as tracks along which these proteins and other components of the cell move into the cleavage furrow. Specifically, the presence of a functional midbody is required in mammalian cells to complete division. Formation of the midbody begins in anaphase, when MT bundles assemble in the central spindle, but functional midbody assembly also requires formation of new MTs nucleated by  $\gamma$  tubulin centers during telophase. In view of this, suppression of new MT polymerization would be expected in demecolcine-treated oocytes and could underlie the inhibition of second PB extrusion. Interestingly, midbody-like structures were detected in some of the treated oocytes that completed second PB extrusion, and specifically in

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all type C and type D oocytes. As MTs that form the central spindle and the midbody are extremely stable, it is possible that some MT bundles could assemble in these oocytes before extensive MT depolymerization, forming a midbody-like structure that persisted. However, detection of these midbody-like structures in oocytes with a completely extruded second PB  
5 argues against the need of newly nucleated MTs for the completion of cytokinesis, unless this is not required for PB abscission or a different mechanism was used in these oocytes to complete division. In fact, second PB extrusion in type E and type F oocytes was completed in the absence of a midbody or a midbody-like structure. Spindle rotation had not occurred in these oocytes and the spindle remnants together with all chromosomes were extruded inside the second PB, leaving  
10 an enucleated oocyte. Interestingly, chemically enucleated mouse oocytes produced by a combined treatment with etoposide and cycloheximide also extrude PBs containing all oocyte chromosomes without involvement of the spindle. Completion of cytokinesis in the absence of MTs has also been reported in other studies and a midbody-independent mechanism for cytokinesis has been proposed to exist in mammalian cells. Thus, it is also possible that in all or  
15 some of the demecolcine-treated oocytes that completed second PB extrusion this alternative mechanism was used due to the absence of a midbody or the presence of a non-functional midbody-like structure. The mechanism of PB extrusion and its dependence on midbody integrity will require further study.

Suppression of second PB extrusion in the presence of demecolcine was independent of  
20 the time of treatment but was dependent on the strain of the oocytes tested. In general, the incidence of second PB extrusion was lower in CF-1 than in B6D2F1 treated oocytes. Almost all B6D2F1 treated oocytes with an extruded second PB were of type C or type D and exhibited a midbodylike structure. On the other hand, as the results in control activated oocytes show, extrusion of the second PB seems to proceed somewhat faster in the B6D2F1 strain. Therefore,  
25 strain specific variations in the time course of midbody formation and second PB extrusion can explain the observed differences between CF-1 and B6D2F1 treated oocytes.

Timely perturbation in spindle function during second PB extrusion also resulted in oocyte enucleation. Inhibition of spindle rotation and the extent of chromosome migration in the presence of demecolcine probably contributed to the expulsion of the entire chromosome  
30 complement inside one, or occasionally two, second PBs. As the results described herein, the

onset of the demecolcine treatment in relation to activation is key to achieving enucleation. Application of demecolcine immediately or a few minutes after ethanol exposure results in higher enucleation rates than application of the drug 15 min after activation, and this finding suggests that the extent of chromatid segregation is a key determinant of enucleation. In addition, a strain effect was also observed for enucleation efficiency, but the reasons for this are unclear. If the proximity of the two groups of chromosomes were decisive for enucleation, slower progression into telophase after activation would favor enucleation. However, a faster anaphase/telophase transition was observed in control activated oocytes of the CF-1 strain, with higher rates of enucleation in all demecolcine treatments, than of the B6D2F1 strain, with lower rates of enucleation. Thus, other parameters account for the strain-dependent efficiency of enucleation.

The majority of CF-I oocytes treated with demecolcine that completed second PB extrusion were enucleated, and enucleation rates close to 80% were obtained. However, since many activated oocytes failed to complete second PB extrusion, the overall enucleation efficiency approximated 20%. Therefore, at least in the CF-1 strain, impairment of PB extrusion is a limitation to enucleation. Shorter treatments with demecolcine, that would allow MT regeneration by late telophase, can promote the completion of second PB extrusion. In fact, preliminary studies with oocytes exposed to demecolcine for only 15, 30 or 45 min resulted in slightly higher rates of second PB extrusion but the rates of oocyte enucleation were also reduced (unpublished results). As the effects of demecolcine on MT depolymerization and regeneration are not immediate with respect to time of application and removal, synchronization of treatment with oocyte cell cycle stage can be difficult to achieve. Possibly the use of other MT-disrupting drugs such as nocodazole, which have more rapid and reversible effects, provide better control over the integration of cytokinesis and karyokinesis.

Culture of activated mouse oocytes in the presence of demecolcine results in normal rates of oocyte activation and progressive cytoskeletal changes after activation. Disruption of spindle MTs by demecolcine impairs chromosome migration, suppresses spindle rotation, inhibits second PB extrusion, alters chromosome partitioning and thereby results in the generation of enucleated oocytes. Enucleation efficiency depends both on the onset of the demecolcine treatment in relation to oocyte activation and on the genetic background of the oocyte. This protocol can then be applied to prepare recipient cytoplasts in nuclear transfer procedures.

EXAMPLE 3:           ACTIVATED BOVINE CYTOPLASTS PRODUCED BY INDUCED  
                          ENUCLEATION SUPPORT DEVELOPMENT OF NUCLEAR  
                          TRANSFER EMBRYOS *IN VITRO*

Poor efficiency of somatic cell NT has been associated with the preparation of  
5 developmentally competent enucleated cytoplasts. Induced enucleation (IE) of mouse oocytes  
has been shown to support enhanced term development of cloned mice. This study characterized  
the kinetics and phenotypic progression of bovine oocytes subjected to IE, and evaluated their  
developmental competence to support NT embryo development *in vitro*. *In vitro* matured (26h)  
oocytes were denuded, activated (5pM ionomycin, 5 min, then 10 µg/mL cycloheximide, 5 h) and  
10 cultured for up to 5h post-activation (pa). Oocyte enucleation was induced by demecolcine (0.4  
µg/ml, DM) exposure at 30, 60, 90 and 120 min post activation for various time periods (1 to  
4.5h). Activation rates and meiotic progression of control and DM treated oocytes (n=31-49/gp)  
was evaluated at 5 hpa by immunofluorescence microscopy (microtubule - Mab-FITC,  
microfilament -Texas red-phalloidin and chromatin-H33258). DM treatment at 30 min pa  
15 resulted in low activation rates (10-16%) whereas DM exposure at 1, 1.5 or 2 hpa resulted in  
higher (79-100%) oocytes activation rates. Onset and duration of DM treatment significantly  
altered IE rates, which varied from 60-91% at 5hpa. Maximum rates of IE were obtained when  
oocytes were exposed to DM between 1.5 and 5hpa (91% IE at 5hpa). DM treatments elicited a  
range of distinct oocyte spindle, chromatin, microfilament and PB phenotypes. Development of  
20 reconstructed IE embryos was evaluated by culture *in vitro* for 7 days. Mechanically isolated  
adult fibroblast nuclei were injected into IE cytoplasts between 1.5-3hpa (n=106).  
Parthenogenetically activated and DM treated oocytes were cultured simultaneously for 7 days  
and served as controls. Control group cleavage and morula/blastocyst rates were 49% (23/47)  
and 30% (7/23) respectively, whereas IE group rates were 48% (51/106) and 27% (14/51)  
25 respectively. These results demonstrate that the IE method can be used to produce enucleated  
bovine cytoplasts and that IE cytoplasts are competent to support *in vitro* development. This  
technically simple approach provides a more efficient method to prepare competent cytoplasts for  
use in nuclear transfer procedures.

Another set of experiments used bovine oocytes that have been subjected to induced  
30 enucleation by demecolcine and control Telophase II bovine oocytes. In these experiments, the

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extrusion of the second PB in bovine oocytes had ceased. Prior to cessation of the formation of the second PB, the nucleus of a donor cell can be introduced into the oocyte to obtain a nuclear transfer embryo.

#### 5 EXAMPLE 4: INDUCED ENUCLEATION OF MOUSE AND GOAT OOCYTES: KINETIC AND PHENOTYPIC CHARACTERIZATIONS

In general, the rates of successful somatic cell cloning of animals are poor, in part due to low efficiency in the production of competent cytoplasts when prepared by mechanical  
10 enucleation of MII oocytes. A method to induce enucleation of activated oocytes has been developed that supports enhanced rates of development of cloned mice to term. This study was designed to characterize the kinetics and progressive phenotypes observed during induced enucleation (IE) of activated mouse and goat oocytes. *In vivo* ovulated mouse oocytes (B6D2F1, n=959; CF 1, n=999) and *in vitro* matured goat oocytes (n=163) were denuded, activated (7%  
15 ethanol) and incubated for up to 3.5 hr in KSOM. Enucleation was induced by continuous exposure to demecolcine (0.4 g/ml) commencing at 0-30 min post-activation. Non-demecolcine treated activated oocytes served as controls (n=50/rep). At selected time points oocytes (n=11.50) were fixed/extracted and processed for immunofluorescence microscopy to document activation and meiotic progression based on spindle (microtubules), chromatin (H33258) and  
20 polar body (PB, microfilaments) phenotypes. In mouse oocytes, activation rates were high and similar in both strains (89-100%). In contrast, the pace of meiotic progression and PB extrusion was strain dependent. Maximum IE rates (23-100%) were observed when oocytes were exposed to demecolcine immediately after activation, and the efficiency was also strain-dependent. IE rates diminished progressively when demecolcine treatment was delayed with respect to the time  
25 of activation. A range of distinct spindle, chromatin, PB and oocyte phenotypes were observed in response to demecolcine. In goat oocytes, activation was evident by anaphase onset at 30 min and activation rates of 69-94% were observed. When treated continuously with demecolcine from 30 min post-activation, 63% of activated oocytes displayed all nuclear chromatin within the extruding second PB. Unlike the mouse, spindle, chromatin, PB and oocyte phenotypes  
30 displayed little variation. These results demonstrate that the IE method can be successfully

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employed to produce enucleated mouse and goat cytoplasts and therefore has broad utility for somatic cell cloning in many animal species.

Also studied were goat oocytes subjected to induced enucleation by demecolcine, and control Telophase II bovine oocytes. In these experiments, the extrusion of the second PB in goat oocytes had ceased. Prior to cessation of the formation of the second PB, the nucleus of a donor cell can be introduced into the oocyte prior to obtain a nuclear transfer embryo, as described herein.

#### EXAMPLE 5: ACTIVATED BOVIN CYTOPLASTS PREPARED BY INDUCED 10 ENUCLEATION SUPPORT DEVELOPMENT OF NUCLEAR TRANSFER EMBRYOS *IN VITRO*

The kinetics and phenotypic progression of bovine oocytes subjected to demecolcine induced IE and the evaluation of their ability to support NT embryo development to the blastocyte stage *in vitro* was studied. Demecolcine treatments elicited a range of distinct changes involving oocyte spindle, chromatin, microfilament and polar body phenotypes as observed by immunofluorescence microscopy. Onset and duration of demecolcine treatment significantly altered the activation and IE rates, which varied from 10-100% and 60-91%, respectively, at 5h post-activation. Maximum rates of induced enucleation (IE) were obtained when oocytes were exposed to demecolcine between 1.5 and 5 hpa (91% IE at 5h post-activation). Development of reconstructed demecolcine treated embryos was evaluated by culture *in vitro*. After mechanical isolation, adult fibroblast nuclei were injected into IE cytoplasts following two different protocols. These results demonstrate that the IE method can be used to produce enucleated bovine cytoplasts and that IE cytoplasts are competent to support development at least to the blastocyst stage *in vitro*. This technically simple approach may provide a more efficient method to enhance the success rate of nuclear transfer procedures.

#### MATERIALS AND METHODS

##### *Oocyte recovery, maturation and activation*

Bovine cumulus-oocyte complexes (COCs) were recovered by follicular aspiration from slaughterhouse-derived ovaries and shipped to the laboratory in maturation medium at 38.5°C.

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After 26 h of in vitro maturation, cumulus cells were removed from COCs by a 10 min treatment with 1200 UI/ml of hyaluronidase (Sigma) followed by vortexing. Activation of denuded oocytes was achieved by treatment with 5: M ionomycin (Sigma) for 5 minutes at 37°C followed by a 5-h culture (38.5°C, 5%CO<sub>2</sub> in air) in maturation medium supplemented with 10 µg/ml cycloheximide (CHX, Sigma). The beginning of ionomycin treatment was considered as time zero post-activation. To monitor normal meiotic cell cycle progression under our experimental conditions, activated oocytes (control oocytes) were fixed at 30 min (n=63), 1h (n=63), 1.5h (n=58), 5h (n=67) and 17h (n=51) post-activation and processed for immunofluorescence microscopy, as described below.

10

#### *Demecolcine treatment of activated oocytes*

To define the optimal protocol for induced enucleation, activated oocytes (n=652) were submitted to several treatments (2 replicates each) with demecolcine (0.4 µg/ml, Sigma) starting at 30, 60, 90, and 120 min post-activation. Demecolcine was removed at 30 min intervals, from 60 min to 270 min (4.5h) post-activation. After demecolcine treatment each group of oocytes was washed 3 times in demecolcine free media and transferred to fresh culture media (Cooks, cleavage) until fixed. Half of the oocytes in each demecolcine-treated group was fixed after 5 hours of activation, while the other half was fixed at 17 hrs post-activation. Oocytes were fixed with 2.5 % paraformaldehyde supplemented with 10% triton X-100 (Sigma, 5 min at 37° C) followed by Microtubule Stabilization buffer-extraction fixative (MTSB-EX, 10% Triton-X) incubation for 25min at 37°C.

20

#### *Oocyte fixation and immunofluorescence processing*

Activated control and demecolcine-treated oocytes were fixed by incubation for 5 min at 37°C in 2.5% paraformaldehyde supplemented with 10% Triton X-100 (Sigma) followed by 25 min at 37°C in a Microtubule Stabilization Buffer-Extraction Fixative (MTSB-EX, 3) containing 10% Triton X. Unspecific binding sites of fixed oocytes were blocked by overnight treatment at 4°C with a blocking solution composed of 0.2% sodium azide, 1% BSA, 0.2% powdered milk, 2% normal goat serum 0.1M glycine and 0.01% Triton-X 100 in phosphate-buffered saline (PBS). Spindle structure was assessed after primary labeling of the oocytes with a mixture of

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mouse monoclonal anti  $\alpha$ -tubulin and anti  $\beta$ -tubulin antibodies (Sigma) at a 1:500 dilution for 8 h at 4°C. Secondary labeling was performed with a 1:100 dilution of donkey anti-mouse fluorescein-conjugated IgG (Jackson ImmunoResearch) for 3h at 37°C. Texas red-conjugated phalloidin (5 U/ml, 30 min at 37°C) was used for the visualization of cell surface actin filaments.

5 Oocytes were then stained with a DNA-specific fluorochrome dye (10 $\mu$ g/ml of Hoechst 33258, Sigma), mounted onto glass slides using a 50% glycerol/PBS mounting solution, and examined by epifluorescence microscopy.

#### *Scoring criteria*

10 Oocytes were considered to be activated when the second PB was present at 5 h post-activation or when at least one pronucleus could be observed at 17 h post-activation. The normal pattern of meiotic progression was determined in ionomycin and cycloheximide (CHX) activated oocytes (controls) subjected to the same fixation and labeling procedures. The meiotic status was scored as follows: "Immature (MI-TI)" when a large meiotic spindle was  
15 detected with chromosomes aligned between the two spindle poles (MI) or with two sets of chromosomes each migrating towards the opposite spindle poles (AI-TI) and no PBs could be observed; "MII stage", when chromosomes were located along the midplane between the two spindle poles and the first PB was present in the perivitelline space; "AII stage" when two sets of chromosome migrating towards opposite poles and, "TII stage", when two chromosome groups  
20 were intercalated by the characteristic remainder of telophase spindle and one or two PBs were present in the perivitelline space. The oocytes in "pronuclear stage" were scored according to the number of pronuclei (PN) and PBs detected.

Oocytes were categorized as enucleated when all the DNA content was located in the PBs, as determined by Hoechst staining, and PB extrusion seemed to be complete, as assessed by  
25 actin labeling.

#### *Nuclear Transfer and In vitro culture of reconstituted embryos*

Recipient cytoplasts for NT experiments were produced by demecolcine treatment between 1.5 and 5 h post-activation, as described previously. Embryos were reconstituted by  
30 direct microinjection of mechanically isolated proliferating fibroblast nuclei (line ACT 00448

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BFF056H, passage 6). Prior to NT, monolayers of fetal fibroblasts were trypsinized and resuspended in manipulation media. Cytoplasmic membranes and content were removed by repetitive pipetting of individual cells using a 12-14 mm diameter glass micropipette in 12% polyvinylpyrrolidone (PVP-360, Sigma) microdrops.

5 Intracytoplasmic NT was performed in the first group (Treatment 1, n=107), 5 to 10 minutes prior to the initiation of the activation-demecolcine treatment while in the second group (Treatment 2, n=106) the embryos were reconstructed between 1.5 and 2.5h post activation (FIG. 7). Micromanipulation of the second group was performed in the presence of CHX and demecolcine. All the oocytes were exposed to cytochalasin B (5 mg/ml) treatment for 20 min  
10 prior to micromanipulation. Activated oocytes treated with demecolcine but not subjected to micromanipulation served as the control group (IE oocytes, n=47).

Reconstituted and IE embryos were transferred to culture media (Cooks, Cleavage®) supplemented with 10% heat-inactivated fetal bovine serum (FBS) on mouse fibroblast feeder layers under oil. After 3 days in culture, cleaved embryos were transferred to blastocysts culture  
15 media (Cooks, Blast®) supplemented as described for cleavage. Cleavage rates were determined 2 days post activation and blastocyst rates were assessed at day 7 of *in vitro* culture.

#### *Karyotype analysis of reconstructed embryos*

Day 8 blastocysts obtained after NT (n=8 Treatment 1, n=6 Treatment 2), or IE (n=4)  
20 were incubated with 0.4 µg/ml demecolcine for 16 h at 38.5°C for the induction of mitotic arrest. Each embryo was then transferred to a 72 well dish containing 15:1 of 0.65% KCl solution and incubated for 15 min. Embryos were then placed separately on methanol chilled slides and fixed with 3 drops of chilled methanol-acetic acid mixture (3:1). Slides were air dried overnight and stained with 4 % Giemsa for 10 min, washed with tap water and kept at -20°C until analyzed. All  
25 analyzable cells were counted, categorized accordingly to morphology, and recorded. The total cell number (TCN) was determined by counting all the cells including pycnotic, interphase and mitotic cells with distinguishable chromosomes. A nucleus was considered pycnotic and thus inactive, when the chromatin was extremely condensed and the nuclear periphery was defined. All nuclei containing distinguishable chromosomes were categorized as mitotic and further

classified as near haploid, near diploid and near tetraploid when around 30, 60 or 120 chromosomes, respectively, were encountered in the spreads.

## RESULTS

### 5 *Meiotic progression of control activated oocytes*

The meiotic cell cycle progression of control parthenogenetically activated bovine oocytes matured *in vitro* for 26 h was examined at 5 intervals, from 30 min to 17 h post-activation. The group of oocytes fixed at 30 min post-activation provided information as to the resumption of meiosis I, as an indicator of nuclear maturation status at the time artificial activation (26 hrs post maturation). Those fixed at the last interval indicated whether the parthenogenetically activated oocytes were capable of progressing to pronuclear stage within 17 hrs post-activation. Figure 8 shows the meiotic status of activated oocytes observed at 30 min, 1 h, 1.5 h, 5 h and 17 h post-activation. At 30 min post-activation the meiotic stage of oocytes ranged from MI to early TII, with most of the oocytes in MII (71.4%). Progression to AII and then to TII was observed in 10 oocytes fixed at 1 and 1.5 h post-activation. At 5 h post-activation the majority of oocytes (80.6%) were in TII, and at least one PN could be observed in 80.7% of the activated oocytes at 15 oocytes fixed at 17 h post-activation. The rate of immature oocytes (MI-TI) ranged from 6.9 to 12.7% between groups.

### 20 *Meiotic progression and induced enucleation of demecolcine treated activated oocytes*

Next, the effect of demecolcine on the meiotic progression of bovine oocytes after activation was evaluated. Several treatments were performed to establish an efficient protocol for demecolcine-induced enucleation of oocytes as described in the Materials and Methods section above. The results obtained after demecolcine exposure are shown in FIG. 9 for oocytes fixed at 5 and 17h post-activation. When demecolcine treatment was initiated 30 min post-activation and lasted for 1 h, 60% of the oocytes resumed meiosis, a value significantly lower than the estimated 100% observed in the control group (FIG. 9). Moreover, demecolcine treatment of oocytes starting at 30 min post-activation and lasting for 2 or 4.5 h seemed to arrest meiotic progression as low rates of activation were observed in these oocytes (10.3-16%, FIG.9). The remaining

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groups presented activation rates ranging from 79 to 100% (Fig. 3), equivalent to the control group.

Maximal enucleation rates, determined as the number of activated oocytes that were enucleated at 5 h post-activation, were observed when oocyte exposure to demecolcine was initiated at 1.5 h post-activation and lasted for 3.5 h (91%, FIG. 10). Further culture of oocytes after the withdrawal of the drug induced a decrease in IE rates as seen when oocytes were fixed at 17h post-activation (45 to 65% decrease depending on treatment, FIG. 10). A positive correlation of enucleation rates was observed between the results obtained from oocytes fixed at 5 and 17 h post-activation. Thus, demecolcine treatment between 1.5 and 5h post-activation also resulted in maximal IE rates when oocytes were fixed at 17 h post-activation.

Immunofluorescence analysis of demecolcine treated oocytes revealed that the microtubule inhibitor was able to disrupt the meiotic spindle and to enable PB extrusion with all the oocyte's DNA content. A more intense background was also observed in demecolcine treated oocytes indicating depolymerization of tubulin, which could be seen dispersed in the cytoplasm. Several ooplasmic membrane and spindle phenotypes were observed in demecolcine treated oocytes. Multiple PB extrusion and a variety of different PB sizes were also observed in the oocytes treated with demecolcine.

At 5h post-activation demecolcine treated oocytes often showed an acentric localization of the chromosomal plate, which approached the oocytes plasma membrane. The region of the plasma membrane right above the chromosomes displayed, in most cases a "cauliflower" like protrusion, which was repeatedly localized adjacent to the PB. The reason behind this difference in the membrane conformation near the chromosomal plate remains unclear however, we believe it could represent the initiation site for the oocyte induced enucleation.

Abnormal spindle shapes, such as multipolar spindles or aster-like spindle were observed in a minority (less than 1%) of oocytes treated with the microtubule inhibitor within 5 h post-activation. The majority of oocytes however did not display a complete spindle like structure, instead tubulin could be seen dispersed in the cytoplasm and aggregated around the chromosomes, with the remainder of the spindle contained within the PBs.

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*In vitro development of reconstructed chemically enucleated embryos*

Also, the *in vitro* developmental competence of cytoplasts prepared by chemical enucleation was evaluated. Nuclear transfer was performed before (treatment 1) or during (treatment 2) oocytes exposure to demecolcine, thus confirmation of enucleation prior to nuclear transfer was not possible. However, in order to access enucleation of demecolcine treated recipient oocytes, 9 oocytes from treatment 1 and 10 oocytes from treatment 2 were randomly chosen after NT, fixed at 7h post-activation and labeled as described above. Control IE oocytes that did not receive donor nuclei were also fixed and monitored. Of these 20% (1/5) were completely enucleated whereas 60% (3/5) were in pronuclear stage, indicating incomplete chromosomal extrusion. The remaining oocyte did not undergo maturation. Complete enucleation and incorporation of donor nuclei took place in 22 % (2/9) and 50% (5/10) of treatment 1 and 2 micromanipulated oocytes. Incomplete enucleation was observed in 67% (6/9) and 40% (4/10) of treatment 1 and 2 oocytes, respectively. One oocyte of each group did not undergo activation. Intracytoplasmic injection of the donor nucleus prior to oocyte activation and treatment with demecolcine yielded 54% cleavage rates and 19% of blastocysts (Table 5). Lower cleavage and blastocysts rates (48 and 16%, respectively) were observed when donor cell nuclei were transferred into the recipient cytoplasts between 1.5 and 3h post-activation. Moreover similar *in vitro* development was observed in IE activated oocytes (Table 5). High fragmentation rates were observed in control IE embryos (35%) and in NTs produced by nuclear injection after activation (treatment 2, 27%). Whereas the embryos produced by NT before activation displayed lower fragmentation (19%), indicating a possible effect of timing of nuclear injection on the fate of early embryo development.

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Table 5. Embryo development after intracytoplasmic injection of donor bovine fetal fibroblast into recipient oocytes prepared by chemical enucleation. Donor nuclei were injected 5 to 10 min prior to activation (treatment 1) or 1.5 to 2 h post-activation (treatment 2). Control oocytes were parthenogenetically activated and enucleated by demecolcine treatment (IE). Chromosome make up of reconstructed (chemically enucleated) embryos.

Table 5

Nuclear transfer	Oocytes		Embryo development		
	Manipulated (n)	Cleaved % (n)	Morula % (n)	Blastocyst % (n)	Fragmented % (n)
Control (IE)	47	49 (23/47)	4 (1/23)	26 (6/23)	35 (8/23)
Treatment 1	107	54 (58/107)	5 (3/58)	19 (11/58)	19 (11/58)
Treatment 2	106	48 (51/106)	12 (6/51)	16 (8/51)	27 (14/51)

Nuclear transferred and IE control blastocysts were examined to enumerate the cells according to their ploidy. The ratio of cells in mitosis to total cell number (TCN) was not significantly different between the control (43.2%, n=3), treatment 1 (48.8%, n=8) or treatment 2 (36.3%, n=5) groups. Ninety percent of the analysed NT embryos showed a majority of diploid ( $\pm 60$ ) chromosomal makeup, indicating successful enucleation by the microtubule inhibitor. Heteroploidy was observed in 100% of the control embryos (n=4), 62.5% of treatment 1 derived embryos (n=5) and 16.7% of blastocyst in treatment 2 (n=6). Additionally, 37.5% and 66.7% of embryos produced by treatment 1 and 2, respectively, were diploid in all cells analyzed. One embryo derived from treatment 2 displayed a tetraploid chromosomal make up.

None of the embryos in treatment 2 had haploid (30 chromosomes), or nearly haploid ( $\pm 30$ ) cells, whereas 50% (4/8) of the blastocysts in treatment 1 were heteroploid showing a range from haploid to diploid cells

No major differences were noted in the mean chromosome make up between the treatment groups. All blastocysts in the control group presented an heteroploid nuclear composition.

FIG. 11 shows the mean distribution pattern with reference to the type of nuclei (pycnotic, interphase or nuclei with visible chromosomes) observed in day 8 blastocysts subjected to chemical enucleation and nuclear transfer (treatment 1 and 2) or chemical enucleation alone (controls). No significant difference was observed in any of these parameters between the  
5 different groups.

## DISCUSSION

Microtubules and microfilaments are major cytoskeletal components that modulate the movement of chromosomes during meiosis and cell division. A non-invasive method for the  
10 preparation of enucleated oocytes for the production of NT embryos, based on the chemical disruption of the oocyte's microtubules during the second meiotic division is described. Demecolcine is a specific microtubule inhibitor shown to be capable of inducing the expulsion of the entire chromosome complement of an oocyte within the PBs, resulting in enucleated cytoplasts. This technique causes minimal harm to recipient oocytes during enucleation, as  
15 opposed to the mechanical preparation of enucleated cytoplast in which 10 to 30% of the oocyte's content is removed. The oocyte's cytoplasm surrounding the nucleus is believed to contain important organelles and proteins which are essential for the maintenance of embryonic developmental competence.

Demecolcine is a reliable and potent reversible microtubule inhibitor, which enabled  
20 enucleation of up to 91% of the activated bovine oocytes. Exposure of bovine oocytes to this drug starting at 1 h post-activation or later did not seem to affect the meiotic progression since the majority of the oocytes (>79%) reached telophase II stage before 5 h post-activation, and pronuclear stage before 17 h post-activation. Interestingly a significant decline in enucleation rates was observed at 17 h after activation, i.e. after 12 h of culture in demecolcine-free media.  
25 This decrease could have been caused by the reintegration of chromosomes into the oocyte after incomplete extrusion of PBs or by re-fusion of PBs to enucleated oocytes. Similar results were reported by Elsheikh *et al.* (J. Vet Med Sci 59(2):107-13 (1997)) who noted 23 % of spontaneous PB re-fusion in chemically enucleated mouse oocytes. Moreover, karyotype analysis of parthenogenetically activated blastocysts treated with demecolcine showed a majority of  
30 embryos with diploid chromosomal make up confirming incomplete PB extrusion and thus

enucleation of treated oocytes. This difficulty could certainly be overcome by the removal of the second PB after 5 h of activation, when the chromosomal content of 91% of the treated oocytes was present in the PBs, minimizing the invasiveness of the current mechanical enucleation procedure and enabling the removal of minimal amounts of cytoplasm.

5 This study shows that the overall developmental performance of *in vitro* cultured NT embryos is not affected by the chemical treatment of the oocytes with demecolcine as evidenced by our *in vitro* developmental rates, in which 24-28% of artificially enucleated oocytes able to cleave and reach the blastocyst stage following activation and/or nuclear transfer.

10 Interestingly, lower rates of embryonic fragmentation were observed when nuclei were transferred into enucleated cytoplasts before activation (19 vs 27%). Similar observations were published by Wakayama and Yanagimachi (Reproduction, 122:49-60 (2001)) based on nuclear transfer of mouse oocytes before and after activation using mechanical enucleation.

15 As shown above induced enucleation can be accomplished in bovine oocytes using demecolcine. It is likely that many technical factors such as oocyte activation, chemical treatments and *in vitro* manipulation may affect development of cloned embryos. The low number of haploid nuclei may indicate a loss of developmentally incompetent nuclei (haploid) moreover this loss does not seem to significantly influence development of embryos to blastocyst stage.

#### 20 EXAMPLE 6: INDUCED ENUCLEATION FOR SCNT IN BOVINE WITHOUT MICROMANIPULATION

By producing competent enucleated cytoplasts without micromanipulation, IE has the potential for application to large scale production of viable cloned embryos for basic research and commercial animal production. Bovine oocytes were first activated (Act I, 10  $\mu$ M ionomycin in Tyrodes-Hepes, 5 min) 22-25 hr post IVM and placed into M199 containing 5% FBS, 10  $\mu$ g/ml cycloheximide (CHX), 2  $\mu$ g/ml cytochalasin D (CD), 0.4  $\mu$ g/ml demecolcine (DEME) for 20 min, followed by a second activation (Act II) and subsequent culture (M199-5% FBS, 10  $\mu$ g/ml CHX) for 20 min. ZPs were removed by acidified Tyrodes solution, washed and transferred into M199-5%FBS, 10  $\mu$ g/ml CHX, 0.4  $\mu$ g/ml DEME, 2% DMSO and 8.5 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . After 1

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hour, the extruding second polar body (PB2) containing all nuclear chromatin is evident and removed using a hand-held glass pipette (15-20  $\mu\text{m}$  I.D.) under a stereoscope. Enucleation was assured by Hoechst 33342 staining and UV light. IE cytoplasts were treated with PHA (200  $\mu\text{g/ml}$ ) to facilitate adhesion of a donor somatic cell (fibroblast, passage 3-4). Nuclear transfer (NT) embryos were reconstructed by electric fusion (150 V/mm, 10  $\mu\text{sec}$ , 1 pulse). NT embryos were packaged in artificial ZPs (0.4% alginate and 0.1%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) and cultured in BARC medium for 7 days. At 1.6 hr post Act I, 80% of oocytes respond to the double activation-IE protocol. Of the responding oocytes, an enucleation rate of 75-90% was achieved. PB removal without manipulation is rapid taking 2-5 sec to enucleate a single IE oocyte. Culture of IE NT embryos resulted in 6-23% development to the blastocyst stage, which was similar to development rates of parthenogenetic control embryos. The results of this study demonstrate that the modified IE protocol is an effective method to produce cloned, blastocyst-stage bovine embryos. This method represents a novel strategy for more efficient somatic cell cloning without the need for micromanipulations. Further investigation of the cellular mechanisms and comparison with conventional cloning methods to produce healthy offspring is warranted, and will reveal the utility of this novel method.

This method, by preparing activated zona-free cytoplasts by IE results in the extrusion of oocyte nuclearchromatin in PB2 and simple agglutinin-facilitated reconstruction, represents a novel strategy for more efficient somatic cell cloning without the need for micromanipulation. Further investigation of the cellular mechanisms involved, and validation of this approach by comparison with conventional cloning methods to produce healthy offspring is ongoing, and will reveal the utility of this novel methodology.

## MATERIALS AND METHODS

The modified IE method employs double activation (ionomycin), zona pellucida removal, agglutin-facilitated reconstruction and *in vitro* development using an artificial zona pellucida (ZP). Slaughterhouse-sourced bovine oocytes were first activated (Act I, 10 mM ionomycin in Tyrodes-Hepes, 5 min) 22-25 hour post IVM and placed into M199 containing 5% FBS, 10 mg/ml cycloheximide (CHX), 2 mg/ml cytochalasin D (CD), 0.4 mg/ml demecolcine (DEME)

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for 20 min, followed by a second activation (Act II, 10 mM ionomycin) and subsequent culture (M199-5% FBS, 10 mg/ml CHX) for 20 min. ZPs were removed (acidified Tyrodes solution, pH 1.79), washed and transferred into M199-5%FBS, 10 mg/ml CHX, 0.4 mg/ml DEME, 2% DMSO and 8.5 mM CaCl<sub>2</sub>. After 1 hour, the extruding second polar body (PB2) containing all nuclear chromatin is evident and removed using a hand-held glass pipette (15-20 mm I.D, 100 mm O.D.) under a stereoscope. Enucleation was assured by Hoechst 33342 staining and UV illumination. IE cytoplasts were immediately treated with PHA (200 mg/ml) to facilitate adhesion of a donor somatic cell (primary fibroblast, passage 3-4). Nuclear transfer (NT) embryos were reconstructed by electric fusion of couplet units (150 V/mm, 10 msec, 1 pulse) in fusion medium (0.253 M D-Sorbitol, 0.5mM MgAc, 0.05% PVA). NT embryos were packaged in artificial ZPs, which were comprised of 0.4% alginate and 0.1% CaCl<sub>2</sub>·2H<sub>2</sub>O, and NT embryos were cultured in BARC medium for 7 days.

## RESULTS

At 1.6 hr post Act I, 80% of oocytes respond to the double activation-IE protocol. Of the responding oocytes, an enucleation rate of 75-90% was achieved. PB removal without manipulation is rapid taking 2-5 sec to enucleate a single IE oocyte. The Culture of zone-free reconstructed IE NT embryos in artificial zona resulted in 6-23% development to the blastocyst stage, which was similar to development rates of parthenogenetic control embryos. shown (Table 6) demonstrate that double activation can significantly increase the rate of development to the blastocyst stage when compared with a single activation.

Groups	No. Replicates	No. Oocytes	Stages of Development to (%)		
			Cleavage (2-8)	16 cell to morula	Blastocyst
control ACT I	11	271	182(67) <sup>a</sup>	57(21) <sup>a</sup>	35(13) <sup>a</sup>
control ACT II	8	207	152(73) <sup>a</sup>	35(17) <sup>a</sup>	27(13) <sup>a</sup>
IE NT	8	238	152(64) <sup>b</sup>	37(16) <sup>a</sup>	36(15) <sup>a</sup>

Significant difference b vs. a, p<0.05

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The teachings of all the patents, patent applications and publications cited herein are incorporated by reference in their entirety. In particular, U.S. patent application No.: 09/432,906, filed November 2, 1999, entitled, "Methods for Cloning Animals," by Baguisi *et al.* is incorporated herein by reference in its entirety.

5           While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

## CLAIMS

What is claimed is:

1. A method of forming a nuclear transfer embryo, comprising the steps of:
  - a) destabilizing microtubules of an oocyte, whereby essentially all endogenous chromatin collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
2. The method of Claim 1, wherein the microtubules are destabilized by combining the oocytes with a chemical selected from the group consisting of demecolcine, paclitaxel, phalloidin, colchicine, and nocodazole.
3. The method of Claim 2, further includes activating the oocyte prior to exposing the oocyte to said chemical.
4. The method of Claim 1, where the microtubules are destabilized by exposing the microtubules to electromagnetic radiation.
5. The method of Claim 4, where the electromagnetic radiation is selected from the group consisting of x-rays and heat.
6. The method of Claim 1, wherein the microtubules are destabilized by exposure of the oocyte to a change in pH or osmolality.
7. The method of Claim 3, wherein the activated oocyte is in a telophase II or anaphase II stage of meiosis.
8. The method of Claim 1, wherein the oocyte is in a metaphase II stage of meiosis.

9. The method of Claim 8, further including the step of activating the oocyte.
10. The method of Claim 1, wherein the donor cell is an activated donor cell.
11. The method of Claim 10, wherein the activated donor cell is in the G<sub>1</sub> stage of a mitotic cell cycle.
12. The method of Claim 11, wherein the somatic activated donor cell is a fibroblast activated donor cell.
13. The method of Claim 12, wherein the somatic activated donor cell is an epithelial activated donor cell.
14. The method of Claim 1, wherein the donor cell is a somatic cell.
15. The method of Claim 14, wherein the donor cell is an adult somatic cell.
16. The method of Claim 14, wherein the donor cell is an embryonic somatic cell.
17. The method of Claim 1, wherein the oocyte is mammalian.
18. The method of Claim 17, wherein the mammal is non-human.
19. The method of Claim 18, wherein the oocyte and the donor cell are of the same species.
20. The method of Claim 1, wherein the donor cell is transgenic.
21. A method of cloning a mammal, comprising:
  - a) destabilizing microtubules of an oocyte, whereby essentially all endogenous

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- genetic material collects at a second polar body during meiosis of said oocyte; and
- b) combining the oocyte with at least the nucleus of a donor cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
  - c) impregnating a mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the cloned mammal; and
  - d) gestating the embryo, thereby causing the embryo to develop into the cloned mammal.
22. The method of Claim 10, wherein the compound that destabilizes the microtubules is selected from the group consisting of demecolcine, nocodazole, colchicine, phalloidin and paclitaxel.
23. A method of producing a transgenic mammal, comprising the steps of:
- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor cell of the same species of said oocyte prior to cessation of extrusion of the second polar body for said oocyte, thereby forming a nuclear transfer embryo.
  - c) impregnating a mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the transgenic mammal; and
  - d) gestating the embryo, thereby causing the embryo to develop into the transgenic mammal.
24. A method of cloning a mammalian fetus, comprising the steps of:

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- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor somatic cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
  - c) impregnating a mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the cloned mammalian fetus; and
  - d) gestating the embryo, thereby causing the embryo to develop into the cloned mammalian fetus.
25. A method of forming a non-human, mammalian nuclear transfer embryo, comprising the steps of:
- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor somatic cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
26. A method of cloning a non-human mammal, comprising the steps of:
- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor somatic cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo;
  - c) impregnating a non-human mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the cloned non-human mammal; and
  - d) gestating the embryo, thereby causing the embryo to develop into the cloned non-

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human mammal.

27. A method of producing a transgenic non-human mammal, comprising the steps of:
- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor somatic cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
  - c) impregnating a non-human mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the transgenic mammal; and
  - d) gestating the embryo, thereby causing the embryo to develop into the transgenic non-human mammal.
28. A method of cloning a non-human mammalian fetus, comprising the steps of:
- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor somatic cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
  - c) impregnating a non-human mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the cloned mammalian fetus; and
  - d) gestating the embryo, thereby causing the embryo to develop into the cloned non-human mammalian fetus.
29. A method of producing a protein of interest in an animal, comprising the steps of:
- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and

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- b) combining the oocyte with at least the nucleus of a donor cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
  - c) impregnating a mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of the cloned mammal;
  - d) gestating the embryo, thereby causing the embryo to develop into the cloned mammal; and
  - e) purifying the protein of interest from the cloned animal.
30. The method of Claim 29, wherein purification of the protein of interest is expressed in tissue, cells or a bodily secretion of the cloned animal.
31. The method of Claim 29, wherein the tissue, cells or bodily secretion is selected from the group consisting of: milk, blood, urine, hair, mammary gland, muscle, viscera.
32. The method of Claim 31, wherein said viscera is selected from the group consisting of: brain, heart, lung, kidney, pancreas, gall bladder, liver, stomach, eye, colon, small intestine, bladder, uterus and testes.
33. A method of producing a heterologous protein in a transgenic animal comprising the steps of:
- a) destabilizing microtubules of an oocyte, whereby essentially all endogenous genetic material collects at a second polar body during meiosis of said oocyte; and
  - b) combining the oocyte with at least the nucleus of a donor cell of the same species of said oocyte prior to cessation of extrusion of the second polar body from said oocyte, thereby forming a nuclear transfer embryo.
  - c) impregnating a mammal of the same species as the nuclear transfer embryo with the nuclear transfer embryo under conditions suitable for gestation of a transgenic

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- cloned mammal;
- d) gestating the embryo, thereby causing the embryo to develop into the transgenic cloned mammal; and
  - e) purifying the protein of interest from the transgenic cloned animal.
34. The method of Claim 33, wherein the genetically engineered nucleus includes an operatively linked promoter.
35. The method of Claim 34, wherein said promoter is selected from the group consisting of: a host endogenous promoter, an exogenous promoter and a tissue-specific promoter.
36. The method of Claim 35, wherein said tissue-specific promoter is selected from the group consisting of: mammary-specific promoter, blood-specific promoter, muscle-specific promoter, neural-specific promoter, skin-specific promoter, hair-specific promoter and urinary-specific promoter.
37. A method of forming a nuclear transfer embryo, comprising the steps in the following order:
- a) combining an oocyte with at least the nucleus of a donor cell of the same species of said oocyte;
  - b) activating the oocyte; and
  - c) destabilizing microtubules of the oocyte, whereby essentially all endogenous chromatin collects at a second polar body during meiosis of said oocyte, thereby forming a nuclear transfer embryo.
38. The method of Claim 37, wherein the oocyte is in a metaphase II stage of meiosis prior to activation.
39. The method of Claim 38, wherein the oocyte is in a telophase II or anaphase II stage of

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meiosis after activation.

40. A method of forming a nuclear transfer embryo, comprising the steps of:
- a) activating an oocyte;
  - b) combining the activated oocyte with at least the nucleus of a donor cell of the same species of said oocyte in less than about 45 minutes after activation of the oocyte; and
  - c) destabilizing microtubules of the activated oocyte, whereby essentially all endogenous chromatin collects at a second polar body during meiosis of said oocyte, thereby forming a nuclear transfer embryo.

# Simultaneous IE – NT Just Prior to Activation

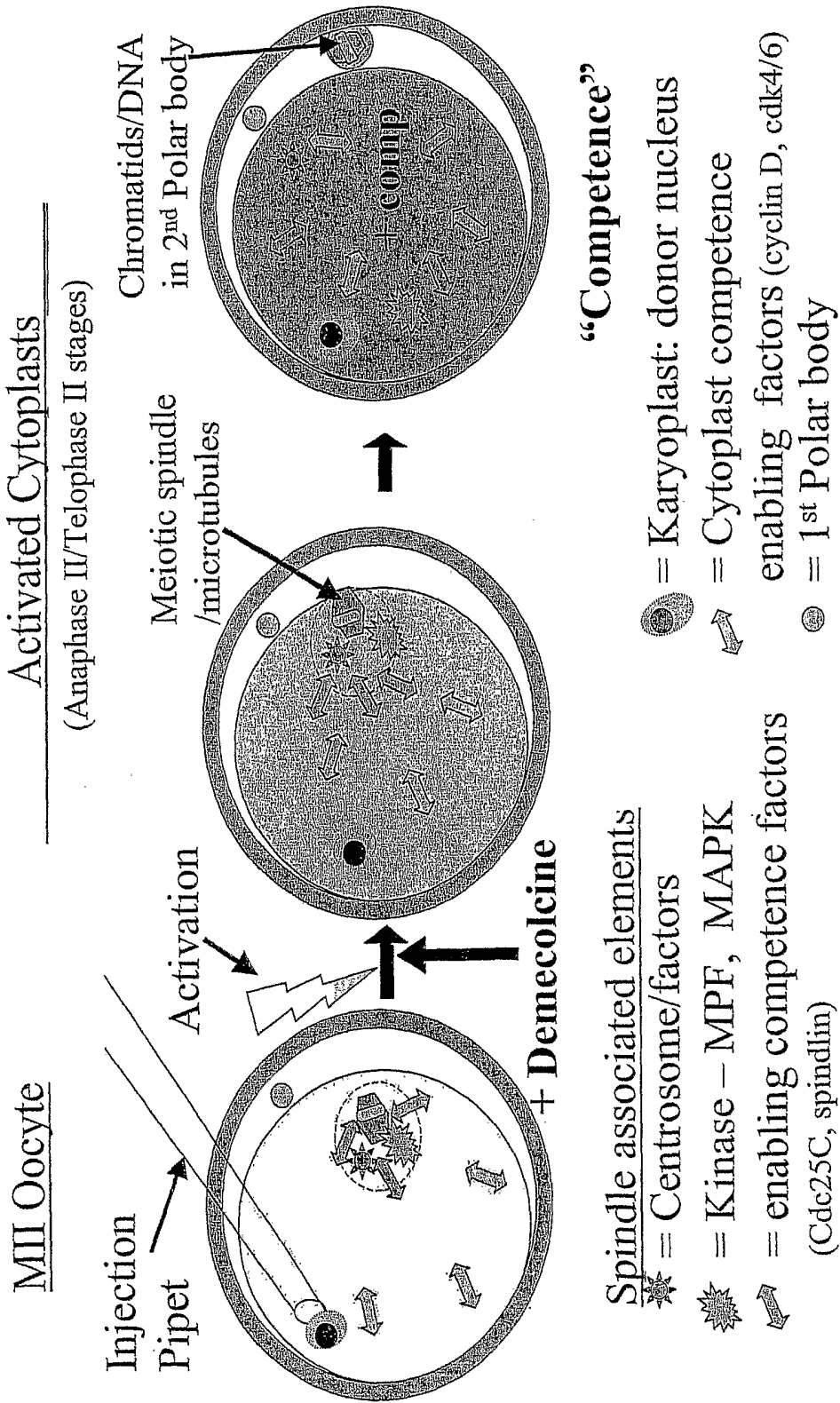


FIG. 1

# Simultaneous IE – NT Just After Activation

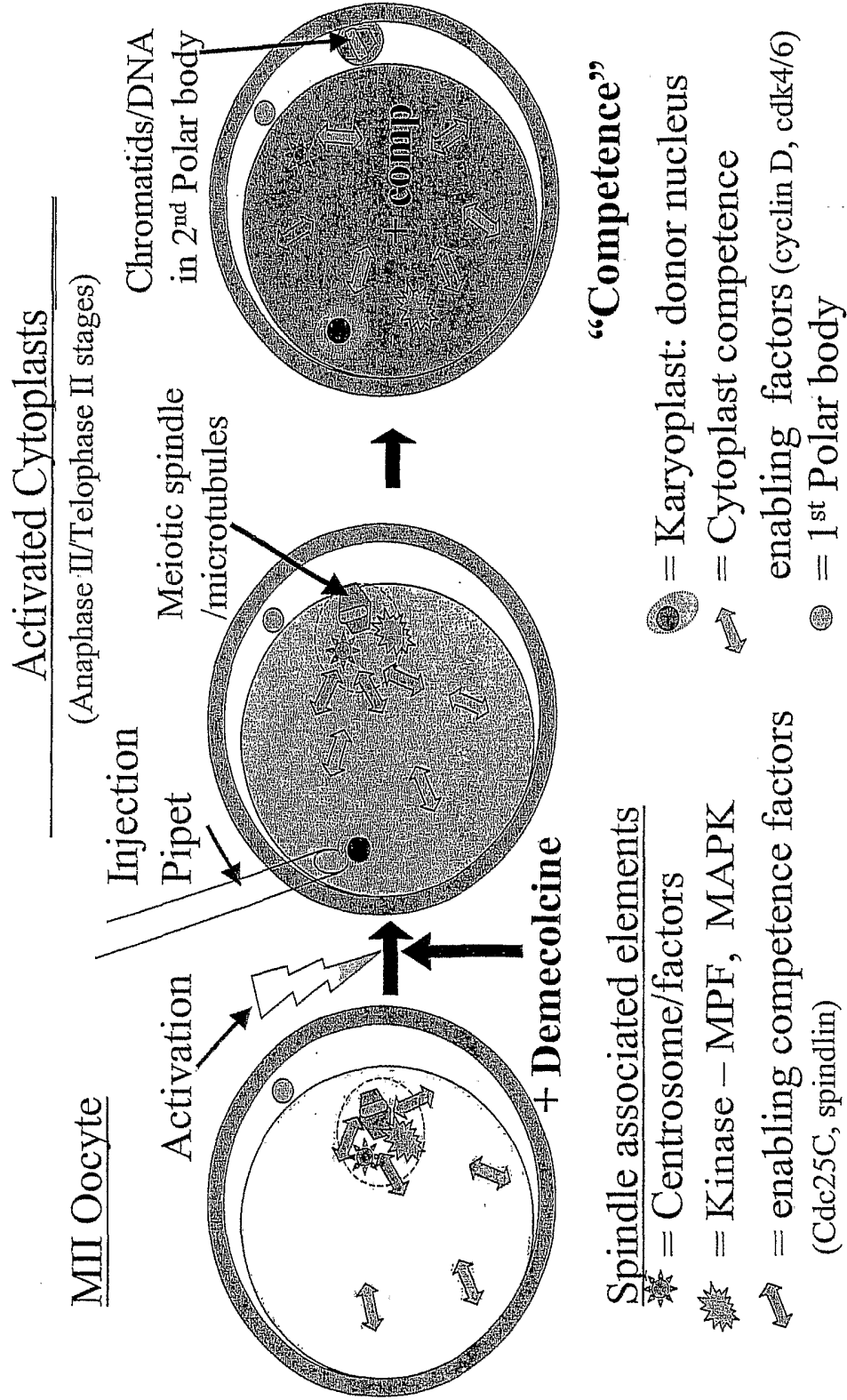


FIG. 2

FIG. 3A

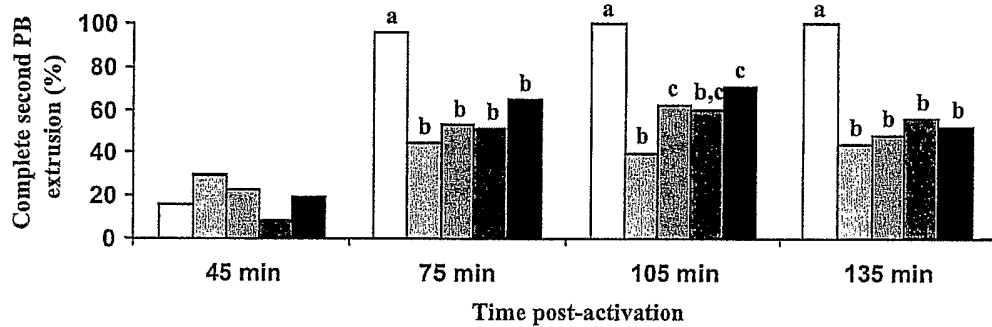
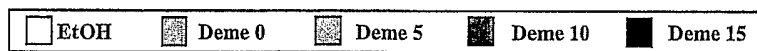
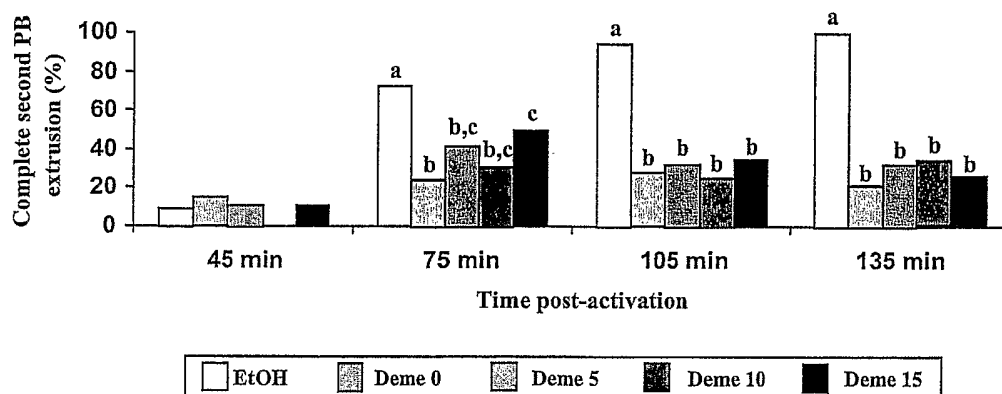


FIG. 3B





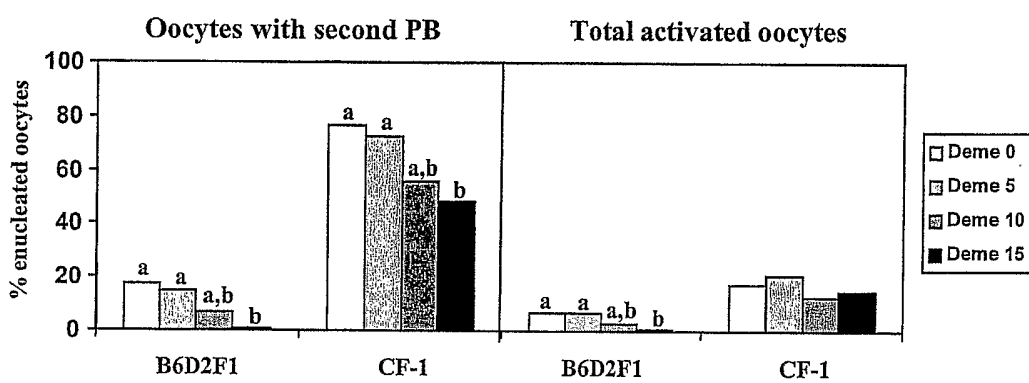


FIG. 5A

FIG. 5B

**RATES OF ACTIVATION, SECOND POLAR BODY EXTRUSION AND IE IN GOAT OOCYTES**

Treatment	% activated oocytes*	% IE (/activated)*	% IE (/extruded PB)*
EtOH (n=120)	93.3 (83.3-97.6)	-	-
Deme (n=105)	92.4 (88.9-100)	36.1 (16.7-52.5)	40.2 (17.6-53.5)

\*Average of results at 60min, 3h, 3.5h and 5h post-activation.

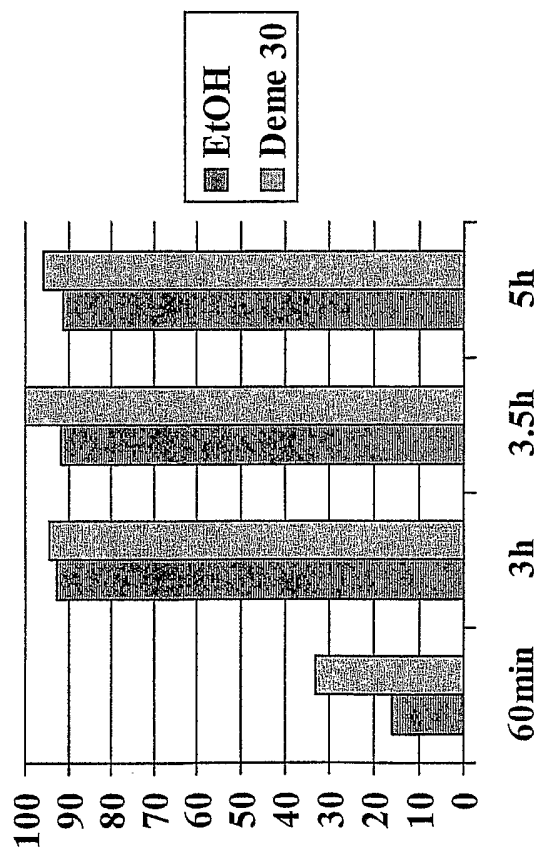


FIG. 6

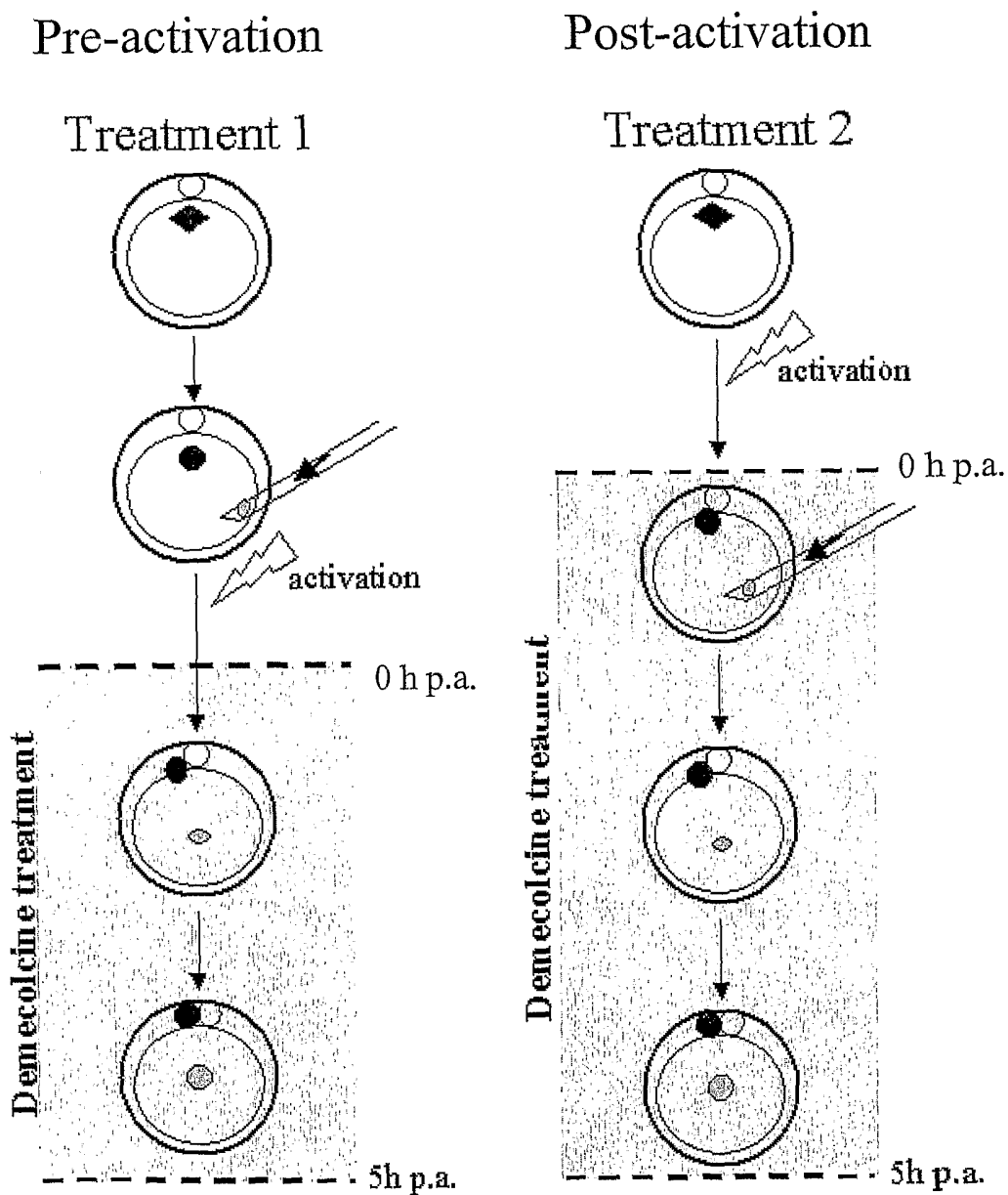


FIG. 7

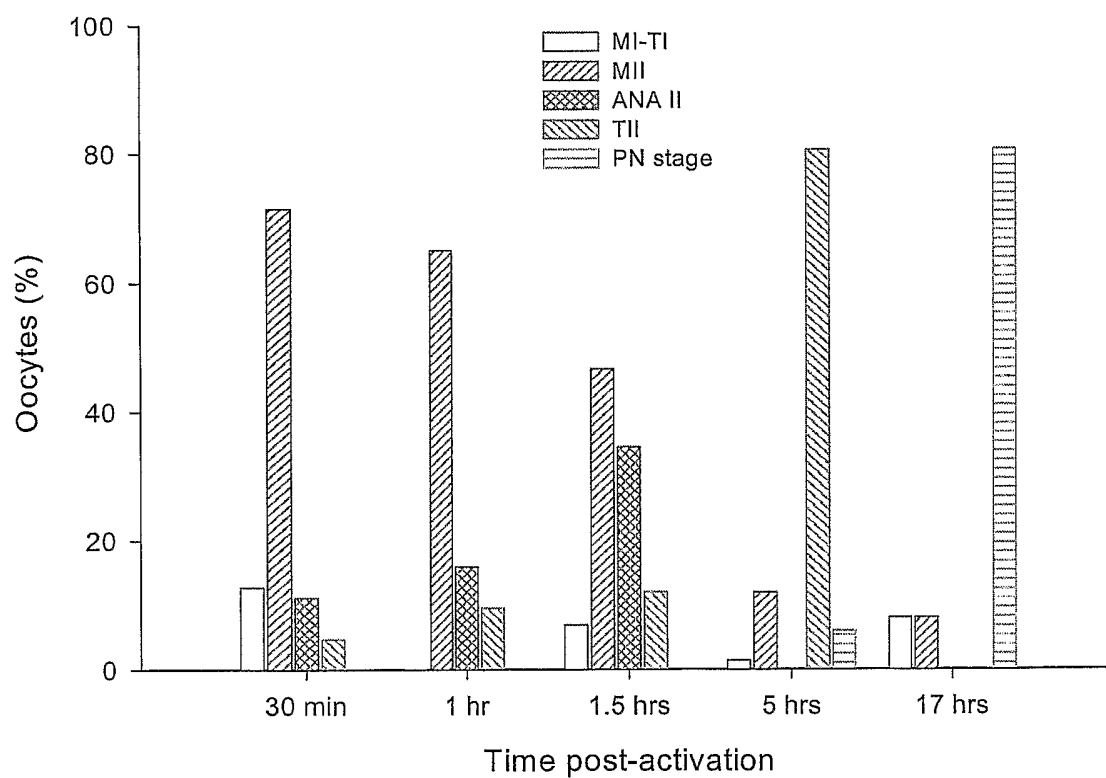


FIG. 8

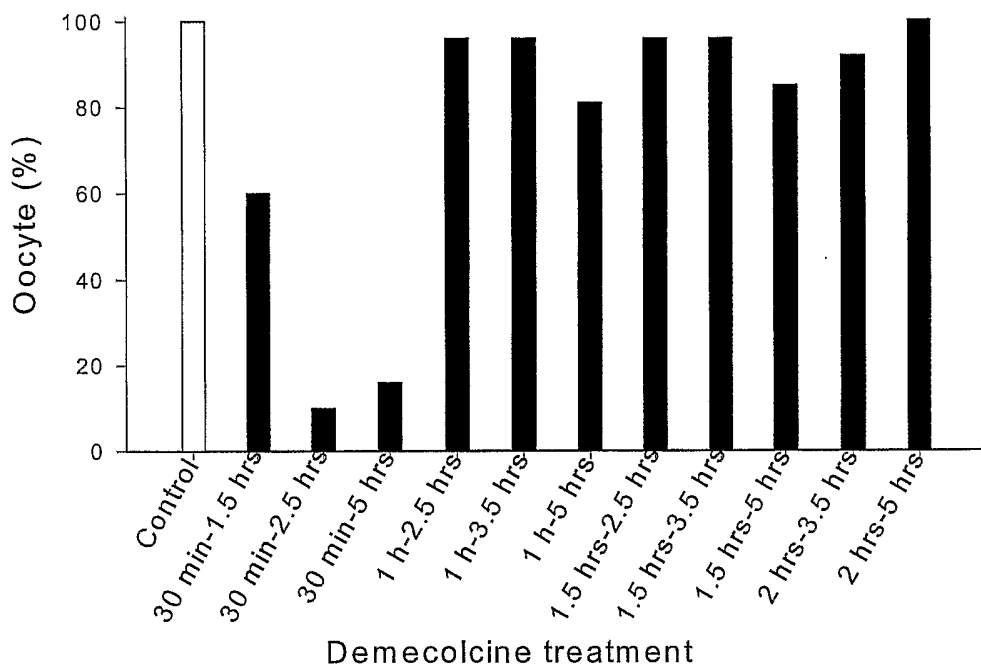


FIG. 9

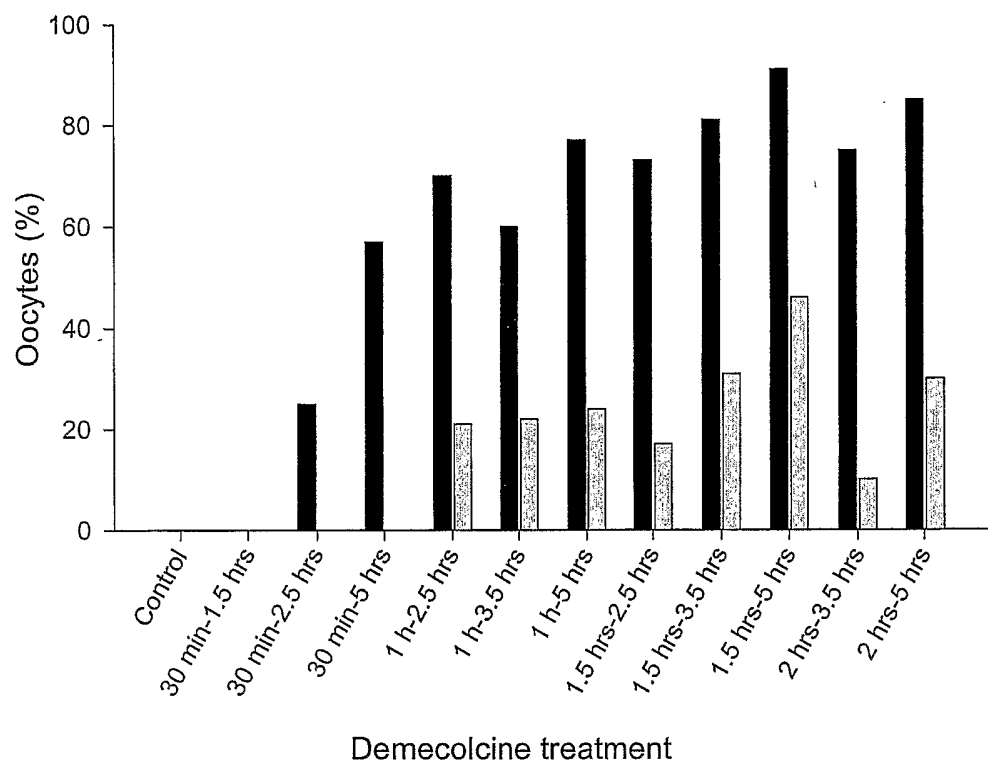


FIG. 10

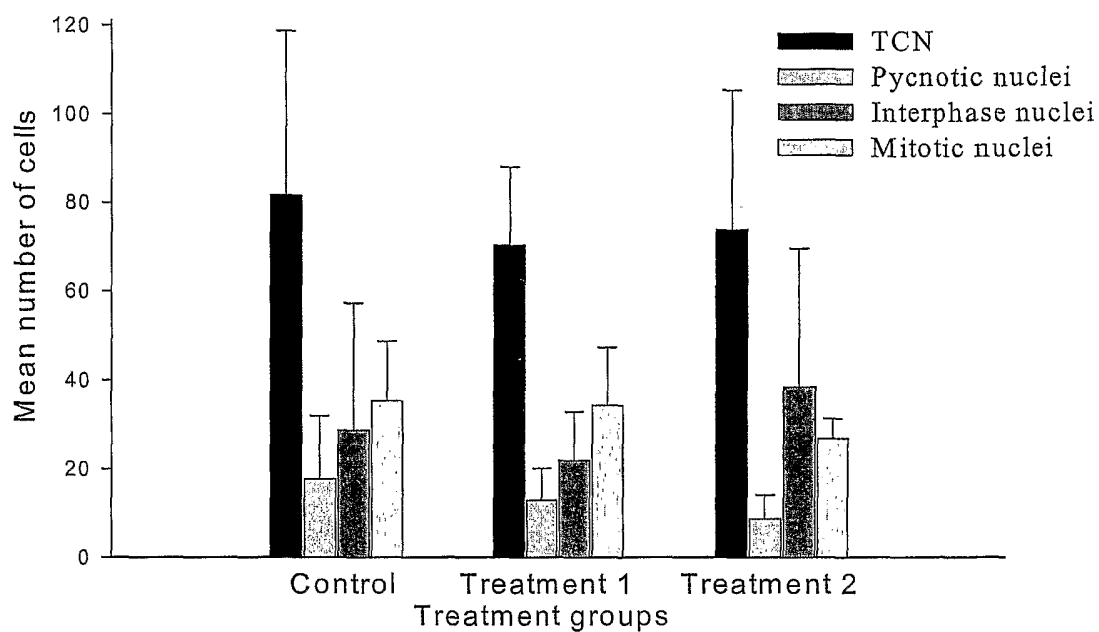


FIG. 11