PROJECT ON PROCESS OF CLONING APPLICATION

&

ETHICAL ISSUES OF COW CLONING

UNIVERSITY OF CALCUTTA

B.Sc Zoology (Honours) 6th Semester

Roll No- 183115-11-0174

Reg No- 115-1212-0699-18

INTRODUCTION

Nuclear transfer has been used to clone several mammalian species including sheep, cattle, goats, mice, and pigs . One of the primary goals of established cloning programs is to improve the efficiency of initiating and maintaining pregnancies and reduce the incidence of prenatal and postnatal abnormalities. Considerable effort is also directed toward identifying nuclear transfer donor cell types and donor cell culture conditions that support genetic modification of the cells prior to NT.

Several groups have expanded the use of nonembryonic cells in nuclear transfer to clone goats, mice, cattle, and pigs . Several cell types, including adult fibroblasts, fetal fibroblasts, and adult cumulous or granulosa cells have been used to produce these clones. Recent reports have also demonstrated the successful cloning of mice from ES cells . Mouse ES cells are attractive because certain strains of these cells are amenable to transgene integration by homologous recombination.

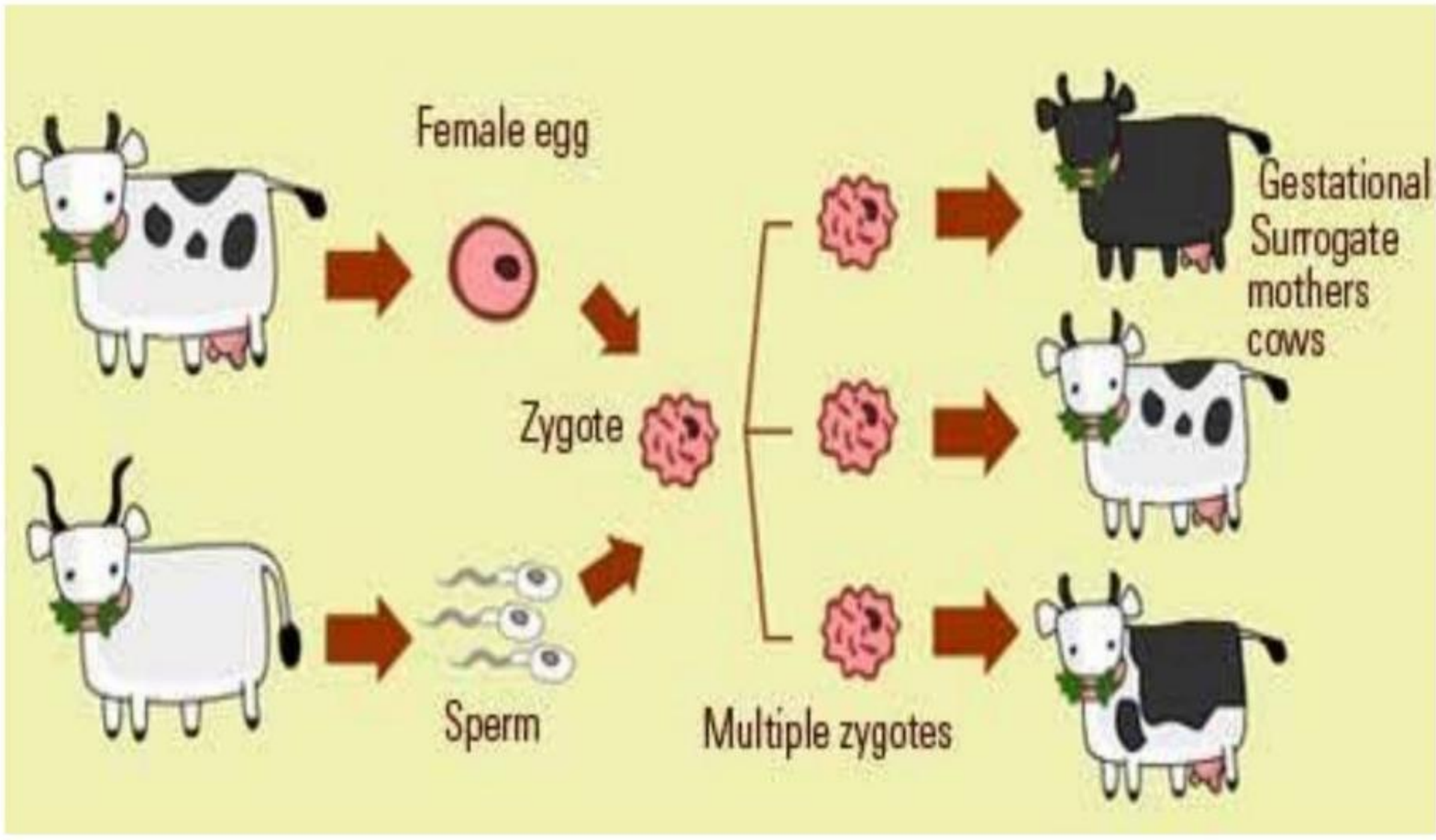
Plasmids, small bits of DNA in bacterial cells, are combined with the genes. Then, they are transferred into living bacteria. These bacteria are allowed to grow into colonies to be studied. When a colony of bacteria containing a gene of interest is located, the bacteria can be propagated to make millions of copies of the plasmids. Then, the plasmids can be extracted for gene modification and transformation. There are three different types of artificial cloning: gene cloning, reproductive cloning and therapeutic cloning. Gene cloning produces copies of genes or segments of DNA. Reproductive cloning produces copies of whole animals. Therapeutic cloning produces embryonic stem cells for experiments aimed at creating tissues to replace injured or diseased tissues.

The goal of the studies reported here was to compare the pregnancy initiation and maintenance rates of NT embryos produced from several bovine cell types and to determine which of these cell types produce healthy calves and have growth characteristics that would allow for genetic manipulation prior to NT.

What is cloning?

Cloning is a complex process that lets one exactly copy the genetic, or inherited, traits of an animal (the donor). Livestock species that scientists have successfully cloned are cattle, swine, sheep, and goats. Scientists have also cloned mice, rats, rabbits, cats, mules, horses and one dog. Chickens and other poultry have not been cloned.

Most people think of livestock breeding taking place through traditional mating, in which males and females physically get together to reproduce. In fact, this is not often the case. Traditional mating is not that efficient, if the goal is to produce as many offspring as possible. For example, a male has enough sperm to produce many more offspring than would be possible by traditional mating. Traditional mating also has certain risks: one or both of the animals may be injured in the process of mating. The female may be hurt by the male because he is often much larger, or an unwilling female may injure the male. There is also a risk of infection or transmission of venereal disease during traditional mating.



Why do we clone a cow ?

Beef cattle or other meat-producing animals such as swine need to have high fertility rates in order to replace animals that are sent to slaughter. Cloning allows farmers and breeders to clone those animals with high fertility rates so that they could bear offspring that would also tend to be very fertile. Some

cows produced much more milk than others. By cloning these cows, farmers could make milk more quickly and cheaply.

How to cloned a cow ?

The US is known to be one of a number of countries actively engaged in cloning bulls for breeding. The underlying idea is to use bulls with desired breeding characteristics for breeding even if, for example, the original bulls are too old to perform or already deceased. The process of cloning is the same as that developed for the cloned sheep, Dolly. In brief, the nucleus from a somatic cell is transferred to an oocyte. If the cells start to divide, as is the case with embryos, they are transferred to a surrogate cow. It takes several hundred attempts to produce one cloned animal that looks healthy, and might eventually be used for breeding purposes. Sperm from bulls is frozen and traded globally. Breeders in the EU use it for the artificial insemination of cows. As research shows, cows stemming from cloned bulls have already been registered by a professional UK breeding organisation.

Materials and Methods

Isolation and Culture of Genital Ridge Cells:

Genital ridges were aseptically removed from bovine fetuses of age 40–80 days. The genital ridges were minced with surgical blades in 1 ml of Tyrode Lactate Hepes medium (Biowhittaker, Inc., Walkersville, MD) containing protease from *Streptomyces griseus* (3 mg/ml) and incubated at 37°C for 45 min. The minced genital ridges were disaggregated by passing them through a 25-gauge needle several times. The disaggregated genital ridges were diluted with 10 ml of TL-Hepes medium and centrifuged at 300 × *g* for 10 min. A portion of the pellet corresponding to 50 000–100 000 cells was cultured on a mitotically inactivated mouse feeder cell layer in α-MEM containing 0.1 mM 2-mercaptoethanol, 4 mM L-glutamine, 100 ng/ml of recombinant human leukemia factor (rhLIF) (R&D System, Inc., Minneapolis, MN), 100 ng/ml bovine basic fibroblast growth factor (bFGF), and 10% fetal bovine serum . All cultured cells were kept in an atmosphere of humidified air/5% CO_2 at 37°C. After 5–7 days in culture, the rhLIF and bFGF concentrations were reduced to 40 ng/ml. After 9–12 days in culture, rhLIF and bFGF were removed from the medium. Upon reaching confluence, the cells were passaged using standard procedures .

In one experiment, primordial germ cells (PGCs) were isolated from the genital ridge (GR) digest based on their characteristic pseudopodia . Approximately 40%–60% of the cells in the digest displayed this morphology. The cells were drawn into a glass pipette (30-µm inner diameter) attached to a

micromanipulation station. Approximately 100 cells were cultured on a mouse feeder cell layer as described above, and another 100 cells were cultured in the same conditions except that neither rhLIF nor bFGF was added to the culture medium.

Isolation and Culture of Cells from Bovine Ear Tissue:

Small portions of the ear were aseptically removed and washed several times in phosphate buffered saline (PBS). The ear samples were minced with scalpel blades and then digested in 5 ml of a trypsin-EDTA phosphate-buffered saline solution for 45 min at 37°C. The digest was filtered through a 70-µm-mesh cell strainer and the effluent was centrifuged at 300 × *g* for 10 min. The pellet was resuspended and cultured in 35-mm culture dishes in α-MEM containing 0.1 mM 2-mercaptoethanol, 4 mM L-glutamine, and 10% fetal bovine serum. The cells were passaged upon confluence.

Feeder Cell Layer Preparation:

A feeder cell layer was prepared from mouse fetuses that were between 15 and 20 days gestation. The head, liver, heart, and alimentary tract were removed, and the remaining tissue was washed and incubated at 37°C in trypsin-EDTA phosphate-buffered saline solution. Cells dissociated from the tissue were cultured in supplemented α-MEM without rhLIF or bFGF but containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). The mouse feeder cells were cultured until confluent and then treated with mitomycin C (10 µg/ml; Calbiochem, La Jolla, CA) for 3 h to block mitosis. The mitotically inactivated feeder cells were cultured for 5–10 days before use.

Transfection Methods:

Multiple methods of transfection were used to optimize both for the type of cell used and the design of DNA construct. The methods used were electroporation and commercial reagents such as Lipofectamine (Gibco 8324SA), SuperFect (Qiagen, Valencia, CA; 301305), Effectene (Qiagen 301425), CLONfectin (Clontech, Palo Alto, CA; 8020-1), CalPhos (Clontech K2051-1), pGeneGrip (GTS, Inc., San Diego, CA; G10100K), LipoTAXI (Stratagene, La Jolla, CA; 204110), and TransIT (Mirus, Madison, WI; MIR2500). The manufacturers' recommended transfection protocols were typically used. Most of the DNA constructs contained the aminoglycoside phosphotransferase gene to confer resistance to the neomycin derivative G418 (Gibco 10131-035), and selection was maintained for 12–16 days (600 µM G418) with 0–1 passages.

Nuclear Transfer:

Oocytes aspirated from abattoir ovaries were matured overnight in maturation medium (medium 199; Biowhittaker) supplemented with luteinizing hormone (10 IU/ml; Sigma), estradiol (1 mg/ml; Sigma), and FBS (10%; Hyclone, Logan, UT) at 38.5°C in a humidified 5% CO_2 incubator. Typically, after 16–17 h in maturation medium, the cumulous cell layer had expanded and the first polar bodies had extruded in approximately 70% of the oocytes (referred to as young oocytes). The oocytes were stripped of cumulous cells by vortexing in 0.5 ml of TL-Hepes. The chromatin was stained with Hoechst 33342 (5 µg/ml; Sigma) in TL-Hepes solution supplemented with cytochalasin B (7 µg/ml; Sigma) for 15 min. Stained oocytes were enucleated in drops of TL-Hepes solution under mineral oil. Cells used in the NT procedure were prepared by removing a group of confluent cells from the culture dishes using a pipette tip. The isolated cells were incubated in a TL-Hepes solution containing 3 mg/ml *S. griseus* protease at 32°C for approximately 1 h. Once the cells were in a single cell suspension, they were washed with TL-Hepes and used for NT within 2–3 h. Single nuclear donor cells of optimal size (12–15 µm) were inserted into the perivitelline space of the enucleated oocyte. The cell and oocyte plasma membranes were fused by applying an electrical pulse of 90 V for 15 µsec in an isotonic sorbitol solution (0.25 M) containing calcium acetate (0.1 mM), magnesium acetate (0.5 mM), and fatty acid-free bovine serum albumin (BSA) (1 mg/ml; Sigma #A7030) (pH 7.2) at 30°C in a 500-µm chamber. Following 4 h of culture in CR1aa (CR2) medium containing 3 mg/ml BSA, the NT embryos were activated by a 4-min exposure to 5 µM ionomycin (Ca^{2+} salt) (Sigma) in hamster embryo culture medium (HECM) containing 1 mg/ml BSA, followed by a 5-min wash in HECM containing 30 mg/ml BSA. The activated embryos were then incubated in CR2 medium containing 1.9 mM 6-dimethylaminopurine (DMAP; Sigma) for 4 h followed by a wash in HECM and subsequently cultured in CR2 medium with BSA (3 mg/ml) at 38.5°C in a humidified 5% CO_2 incubator for 3 days. The embryos were transferred to CR2 medium containing 10% FBS and cultured for an additional 1–4 days.

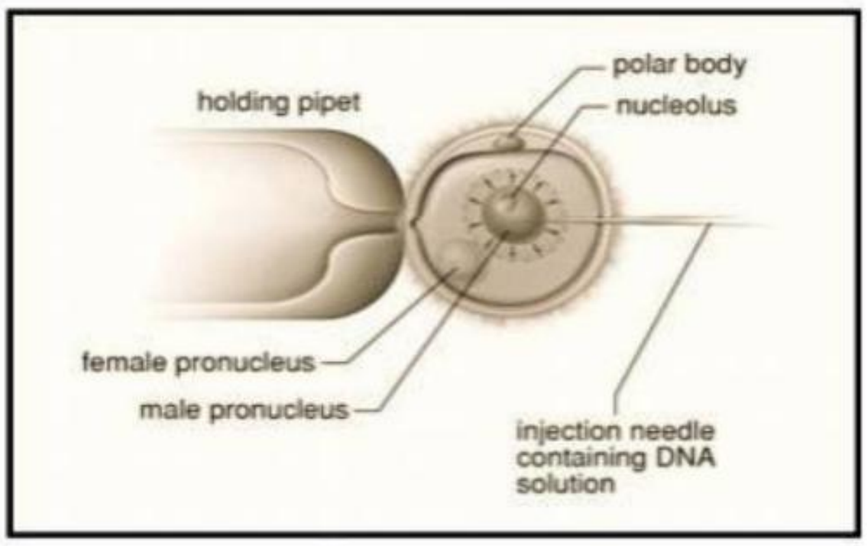
Second Nuclear Transfer (Recloning):

Morula stage NT embryos (generally after 4 days in culture) were disaggregated by treatment with cytochalasin B (7.5 µg/ml) in TL-Hepes for 20 min. Single blastomeres were placed into the perivitelline space of enucleated aged oocytes. Aged oocytes were produced by incubating matured young oocytes for an additional time in maturation medium (typically 40–44 h total). The blastomere was fused with the enucleated oocyte via electrofusion in a 500-µm chamber with an electrical pulse of 105 V for 15 µsec. Electrofusion of the aged oocytes with the nuclear donor blastomeres simultaneously activated the oocytes. After blastomere-oocyte fusion, the embryos from the second NT were cultured in CR2

medium supplemented with BSA (3 mg/ml) for 3 days. The embryos were cultured for an additional 4 days in CR2 medium containing 10% FBS. Good quality embryos as determined by morphology were nonsurgically transferred into recipients.

DNA microinjection:

Cloning is a complex process that lets one exactly copy the genetic, or inherited, traits of an animal (the donor). Livestock species that scientists have successfully cloned are cattle, swine, sheep, and goats. Scientists have also cloned mice, rats, rabbits, cats, mules, horses and one dog. Chickens and other poultry have not been cloned.



Most people think of livestock breeding taking place through traditional mating, in which males and females physically get together to reproduce. In fact, this is not often the case. Traditional mating is not that efficient, if the goal is to produce as many offspring as possible. For example, a male has enough sperm to produce many more offspring than would be possible by traditional mating. Traditional mating also has certain risks: one or both of the animals may be injured in the process of mating. The female may be hurt by the male because he is often much larger, or an unwilling female may injure the male. There is also a risk of infection or transmission of venereal disease during traditional matin

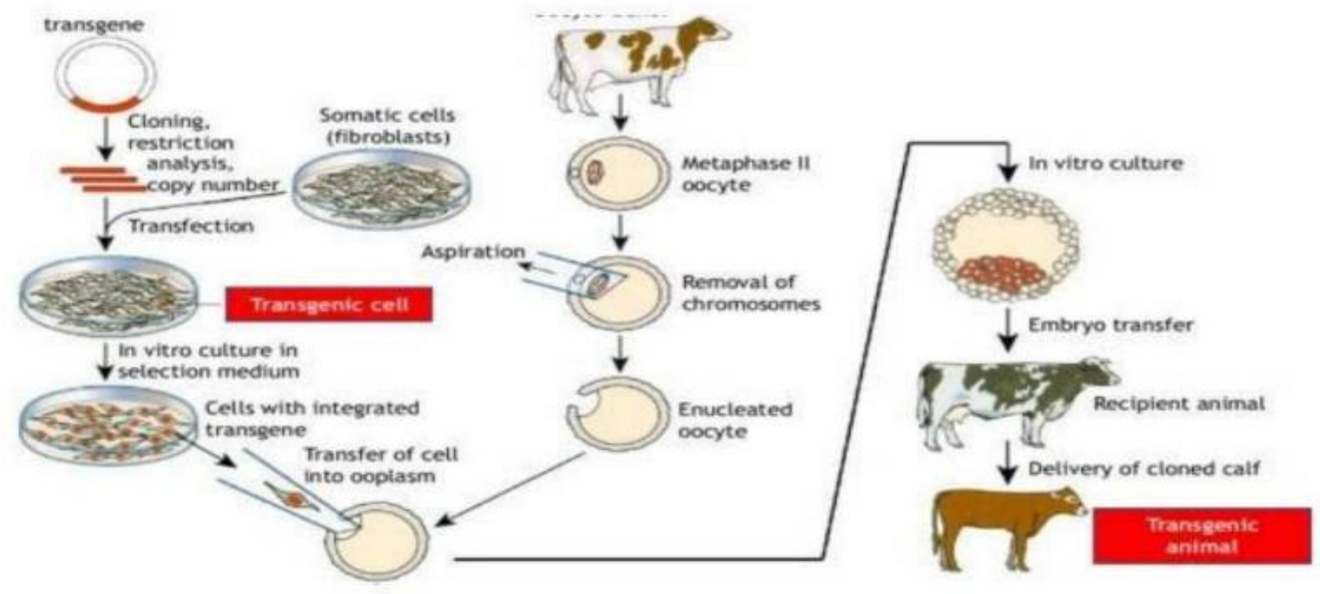


Fig: Cow cloning in a schematic way

Embryo Transfer:

Grade 1 or 2 blastocysts were used for transfer into recipients (1–3 embryos/recipient). Recipients were observed for natural estrus, and blastocysts were transferred into recipients whose predicted ovulation had occurred within 48 h of the time that the nuclear donor cells were fused into the enucleated oocytes. Transfers occurred 5–8 days postfusion.

Parentage Analysis:

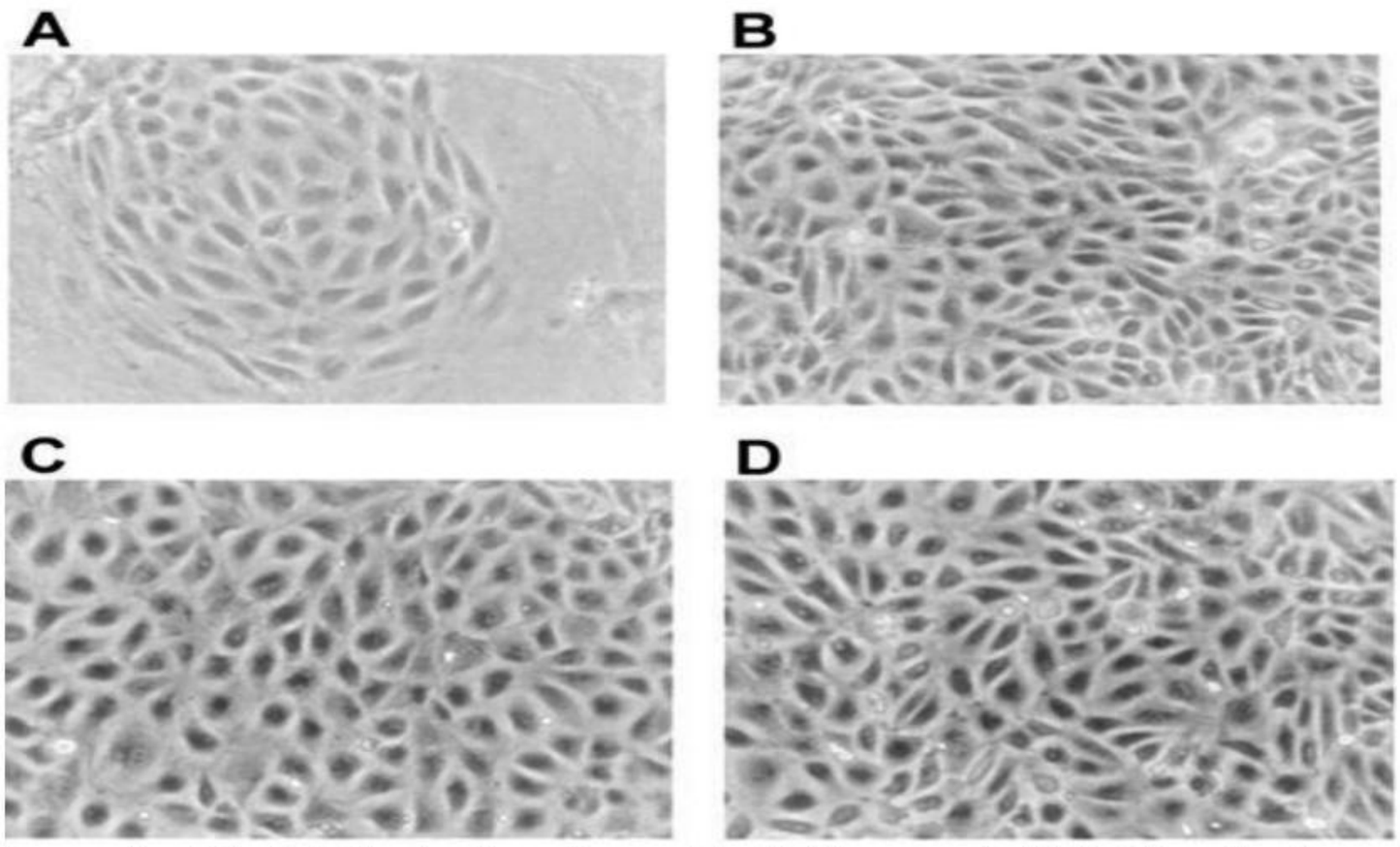
Parentage of the cloned calves was verified by comparing 20 DNA microsatellite markers from the donor cell line and the recipient with those from the cloned calf.

Fig: some cloned cow

Result

Cell Culture: Genital ridge digests from 40- to 80-day-old bovine fetuses formed small colonies of epithelial-like cells after 3–5 days when cultured on mitotically inactivated mouse feeder cells (Fig. 1A). The size and rate of appearance of these colonies were greater when the growth factors rhLIF and bFGF were included in the medium. Cell shape was irregular (10- to 25-µm horizontal dimensions), but typically the cells did not become elongated. After 7–12 days in culture, the cells became confluent, and thereafter proliferation appeared to slow based on the lack of cell multilayer formation (Fig. 1B). Proliferation rates increased after the first passage (data not shown). Cells cultured individually by diluting dissociated cells at first passage generally continued to divide and doubled up to 75 times after isolation . After 68 doublings, the cell line BF15c3 maintained a normal

karyotype Genital ridge digests contain several cell types in addition to the characteristic primordial germ cells (PGCs). We manually selected cells that matched the morphology of PGCs and cultured them on feeder cell layers with and without rhLIF and bFGF. Colonies that developed from the presumptive PGCs (Fig. 1C) were similar to those derived from complete GR digests, although their appearance was delayed (>14 days), particularly in the absence of rhLIF and bFGF.



Genital ridge and epithelial-like body cells used in NT (see text). **A**) A GR cell colony that formed on a feeder cell layer after 4 days. **B**) Confluent GR cells. **C**) Isolated PGCs after 33 days in culture. **D**) Epithelial-like cells from fetal body tissue after 29 days in culture

Cloning from Cloned Fetuses:

To determine if cells from cloned fetuses could themselves produce a second-generation cloned animal, GR cells from BF15 were used to make a 53-day cloned fetus that was collected from the recipient. Body (fibroblast-like) cells from this second fetus were used to make 28 blastocysts (from 151 NT embryos) that were transferred into 14 recipients. These recloned embryos initiated 9 pregnancies (64%), of which 4 went to term (29%) and produced 5 calves (including 1 set of twins).

Parentage Analysis of Cloned Cattle:

Microsatellite DNA markers on 20 chromosomes were used to analyze DNA extracted from recipient tissue, the nuclear donor cell line, and the calf. For each cloned calf, the recipient cow was excluded as a possible parent to the calf whereas the nuclear donor cell line could not be excluded as the genetic source for the calf. Parentage analysis of Gene's DNA is shown in . This figure shows that two

microsatellite markers from Gene match those from the donor GR cells and the placenta but not those from the recipient.

Application of cow cloning

Production of Pharmaceuticals:

The mammary glands of the dairy cattle can be used as a bioreactor for the production of pharmaceutical proteins and therapeutic agents. Many transgene constructs that have mammary gland-specific promoters and human gene sequences has been successfully introduced and expressed in the milk of transgenic sheep, goats, pigs, and rabbits. The advantage of the transgene-derived proteins is that these proteins are glycosylated and have other posttranslational modifications. The proteins secreted in the milk usually have biological activities similar to human proteins.

Strategy for expressing human genes in the milk of domestic animal:

To express the human hormone gene in cow, milk, the coding sequence of the human hormone gene is linked with the promoter of β-lactoglobulin gene, a gene is normally expressed in mammary glands cells. In addition a short signal sequence, necessary for protein secretion in the milk was also included in the transgene. These transgenes are incorporated in the sheep. In this way transgenic sheep producing human hormone in their milk can be created. The hormone can be purified from the milk and used to treat humans.Transgenic cattle have wide potential in production of pharmaceuticals and therapeutics.

High yielding varieties of cow can produce approximately 10,000 liters of milk annually. If amount of recombinant protein in the milk is 1 gram per liter of milk and it could be purified with 50% efficiency, 20 transgenic cows would yield about 100 kg of the recombinant protein per annum. This much yield suffices the annual global requirement for protein C, which is used for the prevention of blood clots. Similarly one transgenic cow would be sufficient for the production of the annual world supply of factor IX (plasma thromboplastin component), which is used by hemophiliacs to facilitate blood clotting. Transgenic goats and sheep can also be raised to produce pharmaceuticals in their milk.

Fig: Milk production

Recently U.S. Food and Drug Administration approved the human protein „antithrombin"produced in transgenic goat"s milk for the use in the individuals with a hereditary deficiency for this protein. Antithrombin is an anticlotting factor, prevents the excessive formation of blood clots, by inhibiting the activity of thrombin. Approximately 1 in 5,000 people is unable to produce this protein naturally. Therefore, they are at risk for heart attacks and strokes. Conventionally the antithrombin is extracted from the plasma of donated blood. This hashigher risk of contamination with pathogens. Also process of extraction is less efficient and more costly; the supply is also not sufficient to meet the needs of patients. The milk of transgenic goats is a significant source of human antithrombin, which yields 2 to 10 grams per liter of milk. It has been estimated that 75 transgenic goats are sufficient to meet the annual worldwide demand for antithrombin. Many other human therapeutic proteins such as antitrypsin, human clotting factors (factor IX for the treatment of hemophilia) and monoclonal antibodies have also been expressed in transgenic goats.

Ethical issues :

Health issue :

Of the live clones born, many experience compromised health status or early death. In one study of cloned pigs, researchers reported a 50% mortality rate forthe live offspring, with five out of 10 dying between three and 130 days of age from ailments including chronic diarrhoea, congestive heart failure, and decreased growth rate. In some studies, cloned mice experienced early deathdue to liver failure and lung problems. In others, they had a high tendency to develop morbid obesity.Of the live clones born, many experience compromised health status or earlydeath. In one study of cloned pigs, researchers reported a 50% mortality rate forthe live offspring, with five out of 10 dying between three and 130 days of age from ailments including chronic diarrhoea, congestive heart failure, and decreased growth rate. In some studies, cloned mice experienced early deathdue to liver failure and lung problems. In others, they had a high tendency to develop morbid obesity.

Risk Factors :

From the literature, the term “risk” is commonly used and has many different meanings. Risk is Defined as a “chance or possibility of danger, loss, injury, etc.” In relation to animal cloning, risk Can be defined as a possibility to cause potential effects to cloned animals, surrogate dams and the Environment. Risk assessment is the process of evaluating the possible risks arise due to animal Cloning process. The animals involved in animal cloning experiments are subjected to high risks adversely affecting .Them which includes abnormal placentation, pregnancy toxemia, and hydroallantois.

Philosophical Views :

Several debates have held over a period of time to discuss the validity of animal cloning. It was concluded to ban the use of products from cloned animals and to prevent the use of animal cloning. For example, the U.S. Humane society which is an animal protection organization has requested to ban on products coming from cloned animals and their offspring. The vice president of this Organization, Michael Appleby stated that the animal welfare problems that already exist will Increase with the new biotechnological practices e.g., disease vulnerability. He argued: “Already Animals are suffering from maladies at a rate unheard of before we applied biotechnology to the Barnyard. It would be disastrouslypremature to put this technology into commercial practice”

Religious views :

Many religions have various guidelines or principles on doing things in the right way, on how should we live. Various philosophers proposed many ethical traditions or principles which can be Classified into two categories 1) secular 2) spiritual. Some ethical or moral theories are included in the secular division or western division, whereas religion comes under the spiritual division. According to the Islamic religion research, one has to ultimately find the truth and to observe the signs of Allah's glory in order to understand Natural phenomena and the hidden story behind the universe. IN the early Christian era, St. Augustine a theologian and Neoplatonic philosopher Was highly influential. He mentioned that the percept "Thou shalt not kill" is not applicable to Animals, because as they do not object and are unlike humans. According to him, God created an Animal for the betterment of humans so people should also protect animals for their own uses.

CONCLUSIONS

Every year, around 30 to 40 tons of bull sperm from US enter the EU for the purpose of cattle breeding.

However, there are currently no requirements for registration or labelling of imports into the EU. Therefore, it is impossible to identify breeding material stemming from cloned bulls.

The European Parliament, as well as the German Bundestag and the German government all advocate a ban on cloned animals for food production in the EU. The reasons are mostly ethical, since cloning inherently means animal suffering due to disturbances in their gene regulation. Consequently, the EU Parliament is amongst those institutions demanding that transparency measures are established in order to register the clones, their offspring, relevant products and material. Without these measures, the animals and food derived thereof can enter the market unnoticed. As yet, there is no transparency in this sector, and no information is available to farmers, food producers or consumers.

REFERENCE

1. "Genetic Engineering". Genome.gov. Retrieved 16 March 2021.
2. EJForsberg · 2002https://www.google.com/url?sa=t&source=web&rct=j&url=https://academic.oup.com/biolreprod/article/67/1/327/2683927&ved=2ahUKEwiCmtbH-ZjyAhVCjuYKHVmhB6gQFnoECBcQAg&usg=AOvVaw0FV0f6tmE4c71DEQISw85v&cshid=1628134513071
3. https://www.google.com/url?sa=t&source=web&rct=j&url=https://www.sciencedirect.com/science/article/pii/S096098221000994 2&ved=2ahUKEwiCmtbH-ZjyAhVCjuYKHVmhB6gQFnoECA4QAg&usg=AOvVaw0n8epwTo0d-9Yv3x3o2Ncf&cshid=1628134513071
4. https://www.google.com/url?sa=t&source=web&rct=j&url=https://www.nature.com/articles/news011129-1&ved=2ahUKEwiCmtbH-ZjyAhVCjuYKHVmhB6gQFnoECBYQAg&usg=AOvVaw0rH6Hk92pcj39ZKt4mttP7&cshid=1628134513071
5. https://www.google.com/url?sa=t&source=web&rct=j&url=https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4517265/&ved=2ahUKEwiCmtbH-ZjyAhVCjuYKHVmhB6gQFnoECBwQAg&usg=AOvVaw3KCnI2iDiaTAGbSv32a2k1&cshid=1628134513071
6. .Barnes, F.L., Westhusin, M.E. and CR. Looney. 1990.https://www.google.com/url?sa=t&source=web&rct=j&url=https://edepot.wur.nl/202960&ved=2ahUKEwiCmtbH-ZjyAhVCjuYKHVmhB6gQFnoECAwQAg&usg=AOvVaw24i8kSzy8x3JG8fK9zVh2E&cshid=1628134513071
7. Vert M, Doi Y, Hellwich KH, Hess M, Hodge P, Kubisa P, Rinaudo M, Schué F (2012). "Terminology for biorelated polymers and applications (IUPAC Recommendations 2012)". Pure and Applied Chemistry. 84 (2): 377–410. doi:10.1351/PAC-REC-10-12-04. S2CID 98107080.
8. "How does GM differ from conventional plant breeding?". royalsociety.org. Retrieved 14 November 2017.
9. L Erwin E, Gendin S, Kleiman L (22 December 2015). Ethical Issues in Scientific Research: An Anthology. Routledge. p. 338. ISBN 978-1-134-81774-0.
10. Alexander DR (May 2003). "Uses and abuses of genetic engineering". Postgraduate Medical Journal. 79 (931): 249–51. doi:10.1136/pmj.79.931.249. PMC 1742694. PMID 12782769.
11. Nielsen J (1 July 2013). "Production of biopharmaceutical proteins by yeast: advances through metabolic engineering". Bioengineered. 4 (4): 207–11. doi:10.4161/bioe.22856. PMC 3728191. PMID 23147168.

ACKNOWLEDGEMENT

In the accomplishment of completion of my project on Process of Cloning, Application & Ethical issues of Cow Cloning, I would like to convey my special gratitude to Mrs. Subhadipa Majumdar and Mrs. Subhadra Roy of zoology department and as well as our principal Dr. Indranil Kar.Your valuable guidance and suggestions helped me in various phases of the completion of this project. I will always be thankful to you in this regard.