

**Development of transgenic goat embryos expressing human lactoferrin gene**



**THIS IS SUBMITTED TO THE  
ICAR- NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)  
KARNAL (HARYANA)  
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR THE AWARD OF THE DEGREE OF**

**DOCTOR OF PHILOSOPHY  
IN  
(ANIMAL BIOTECHNOLOGY)**

**By**

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KARNAL- 132001 (HARYANA), INDIA**

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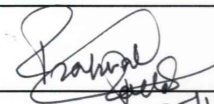
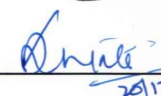


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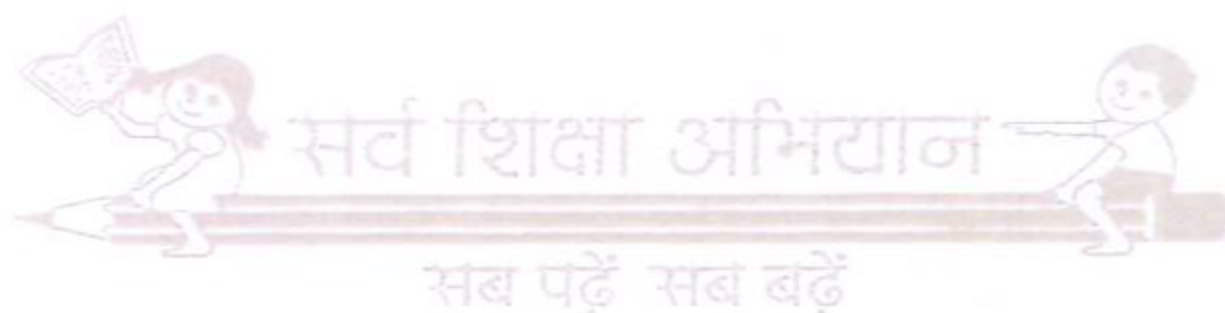
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## CERTIFICATE

This is to certify that the thesis entitled "**Development of transgenic goat embryos expressing human lactoferrin gene**" submitted by **Neha Saini** towards the partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Animal Biotechnology** of the **ICAR- National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 20.12.2016

  
M.S. Chauhan  
(MAJOR ADVISOR)





*Dedicated  
To  
My Dear Parents  
& Sisters  
For their  
Constant  
Encouragement,  
blessings & Love*

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---

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## **ABBREVIATIONS AND SYMBOLS**

$\alpha$	:	Alpha
$\beta$	:	Beta
APS	:	Ammonium Persulphate
bp	:	Base pairs
BSA	:	Bovine serum albumin
cDNA	:	Complementary DNA
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleoside triphosphates
EDTA	:	Ethylene diamine tetra acetic acid
EtBr	:	Ethidium Bromide
DMEM	:	Dulbecco's Modified Eagle Medium
h	:	Hour(s)
hLF	:	Human lactoferrin
IU	:	International unit
Kb	:	Kilo base pairs
KCl	:	Potassium chloride
kDa	:	Kilo Dalton
L	:	Litre
M	:	Molar
mg	:	Milligram
mg/ml	:	Milligram per millilitre
MgCl <sub>2</sub>	:	Magnesium chloride
min	:	Minute
ml	:	Millilitre
mm	:	Millimeter
mRNA	:	Messenger ribonucleic acid
ng	:	Nanograms
nm	:	Nanometer
°C	:	Degree centigrade

OD	:	Optical Density
PAGE	:	Poly Acrylamide Gel Electrophoresis
PBS	:	Phosphate buffer saline
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of hydrogen ion concentration
pmol	:	Pico Mole
rpm	:	Revolutions per minute
SDS	:	Sodium Dodecyl Sulphate
TAE	:	Tris Acetate EDTA
TEMED	:	NNNN – Tetra Methyl Ethylene Diamine
TRIS	:	Tris (hydroxymethyl) amino methane
V	:	Volt
µg :	:	Microgram
µl	:	Microlitre
µM	:	Micromole

## Development of transgenic goat embryos expressing human lactoferrin gene

### ABSTRACT

The purpose of the present study was to develop transgenic goat embryos for human lactoferrin gene, with the final aim of producing transgenic goats expressing human lactoferrin in milk. Therefore, the study was designed with the following objectives: i) To develop transgene constructs of human lactoferrin under different mammary gland-specific promoters. ii) To determine *in vitro* expression of the lactoferrin constructs in goat mammary epithelial cells. iii) To develop transgenic goat embryos containing human lactoferrin gene. We developed three transgenic constructs under mammary gland-specific promoter for human lactoferrin i.e. pBCL1, pBCL2 and p $\beta$ ChLF. For this, we amplified and got the construct elements sequenced which consist of 5' UTR of major milk proteins ( $\beta$ -casein: 3.5 and 6.5 kb;  $\alpha$ -casein: 2.97 and 5.27 kb;  $\beta$ -lactoglobulin: 2.1 and 2.7 kb), human lactoferrin gene and bGH 3'UTR. All 5' UTRs were assayed by luciferase activity. Both the fragments of  $\beta$ -casein had comparable activity. pBCL1 was designed under 6.5 kb  $\beta$ -casein promoter in which human lactoferrin was fused to GFP. pBCL2 was designed on the same lines under 3.5 kb  $\beta$ -casein promoter. To circumvent fusion protein and to detect transgene expression in all types of cells, p $\beta$ ChLF was designed under 3.5 kb  $\beta$ -casein promoter. This construct was transfected into mammary epithelial cells established and characterized in lab and found to grow best on ECM-coated surface. Flow cytometry results showed nucleofection as significantly more efficient than lipofection for transfection. Mammary epithelial cells were transfected and selected using G418. *In vitro* expression of human lactoferrin was checked by RT-PCR, Immunostaining, Western blotting and ELISA which demonstrated that hLF was being produced by GMECs and secreted in the medium at concentration 0.07- 0.11 ng/ml. Due to better reprogramming efficiency of fetal fibroblast cells, p $\beta$ ChLF was transfected by nucleofection into female GFFs established and characterized using RT-PCR, Immunostaining and karyotyping which were subsequently selected under G418 and showed the integration of transgene into their genome when subjected to PCR. These transgenic goat fetal fibroblast cells were used for HMC to produce transgenic goat embryos which were confirmed to contain human lactoferrin gene as verified by single embryo PCR. In conclusion, we were able to establish transgenic technology platform in farm animals for the first time in India.

## मनुष्य के लेक्टोफेरिन जीन को अभिव्यक्त करने वाले बकरी के ट्रांसजेनिक भ्रूणों का विकास

### सारांश

वर्तमान अध्ययन का उद्देश्य मनुष्य के लेक्टोफेरिन जीन को अभिव्यक्त करने वाले बकरी के ट्रांसजेनिक भ्रूणों का विकास करना था जिससे अंततः मनुष्य के लेक्टोफेरिन को अपने दूध में उत्पादित करने वाली ट्रांसजेनिक बकरी उत्पन्न की जा सके। अतः वर्तमान अध्ययन निम्न उद्देश्यों के साथ प्रस्तावित किया गया १) विभिन्न स्तन ग्रंथि के लिए विशिष्ट प्रमोटरों के नीचे मनुष्य के लेक्टोफेरिन से युक्त ट्रांसजीन कंस्ट्रक्ट का विकास करना. २) बकरी की मेमेरी एपिथेलियल कोशिकाओं में इन लेक्टोफेरिन कॉन्सट्रक्ट्स की इन विट्रो अभिव्यक्ति का निर्धारण करना ३) मनुष्य के लेक्टोफेरिन से युक्त बकरी के ट्रांसजेनिक भ्रूणों का उत्पादन करना। हमने अपने अध्ययन में मनुष्य के लेक्टोफेरिन के लिए स्तन ग्रंथि हेतु विशिष्ट निम्न तीन ट्रांसजेनिक कॉन्सट्रक्ट्स विकसित किये : pBCL1 , pBCL2 तथा pBChLF। इस उद्देश्य की पूर्ति के लिए हमने दूध के मुख्या प्रोटीनों के 5' UTR ( $\beta$ - केसिन : 3.5 तथा 6.5 Kb ,  $\alpha$ -केसिन: 2.97 तथा 5.27 Kb  $\beta$  लेक्टोग्लोब्यूलिन : 2.1 तथा 2.7 Kb), मनुष्य का लेक्टोफेरिन जीन तथा bGH 3' UTR को प्रवर्धित करके प्राप्त किया। सभी 5' UTR की लुसिफेरेस विधि द्वारा जाँच की गयी।  $\beta$ - केसिन के दोनों खंडों की सक्रियता तुलनात्मक पायी गयी। 6.5 Kb  $\beta$  केसिन प्रमोटर जिसमें मनुष्य के लेक्टोफेरिन को gfp के साथ जोड़ा गया हो, द्वारा pBCL1 संरचित किया गया। इसी क्रम में pBCL2 का निर्माण किया गया जिसमें 3.5 Kb  $\beta$  केसिन प्रमोटर उपयोग में लाया गया। संलयित प्रोटीन से बचने के लिए तथा सभी प्रकार की कोशिकाओं में ट्रांसजीन की अभिव्यक्ति ज्ञात करने के लिए 3.5 Kb  $\beta$  केसिन प्रमोटर के साथ pBChLF संरचित किया गया। इस कंस्ट्रक्ट को प्रयोगशाला में स्थापित तथा विशेषीकृत मेमेरी एपिथेलियल कोशिकाओं में ट्रांसफेक्ट करने के लिए उपयोग में लाया गया तथा इनकी वृद्धि ECM उपचारी सतह पर सर्वोत्तम पायी गई। फ्लोसायटोमेट्री के परिणामों ने यह दर्शाया कि नुक्लेओफेक्शन विधि ट्रांसफेक्शन हेतु लिपोफेक्शन कि तुलना में सार्थक रूप से प्रभावी पाई गयी। मेमेरी एपिथेलियल कोशिकाओं को ट्रांसफेक्ट करके G418 द्वारा चयनित किया गया. मनुष्य के लेक्टोफेरिन की इन विट्रो अभिव्यक्ति को RT-PCR, इम्म्युनोस्टेनिंग, वेस्टर्न ब्लॉटिंग तथा एलिस द्वारा ज्ञात किया गया जिसने दर्शाया कि GMECs कोशिकाओं ने hLF उत्पादित किया तथा 0.07-0.11 ng/ml की मात्रा में यह मीडिया में सावित हुआ। फीटल फाइब्रोब्लास्ट कोशिकाओं के बेहतर रिप्रोग्रामिंग क्षमता के कारण, pBChLF को नुक्लेओफेक्शन विधि द्वारा मादा GFFs में ट्रांसफेक्ट किया गया। ये कोशिकाएँ पूर्व में स्थापित तथा RT-PCR, इम्म्युनोस्टेनिंग तथा karyotyping द्वारा विशेषीकृत थीं तदुपरांत इन्हें G418 द्वारा चयनित किया गया एवं पीसीआर द्वारा ट्रांसजीन का जीनोम में एकीकरण ज्ञात किया गया। इन बकरी के ट्रांसजेनिक फीटल फाइब्रोब्लास्ट कोशिकाओं को HMC द्वारा बकरी के ट्रांसजेनिक भ्रूणों को उत्पन्न करने हेतु प्रयुक्त किया गया तथा एकक भ्रूण पीसीआर द्वारा इनमें मनुष्य के लेक्टोफेरिन का होना सत्यापित किया गया। निष्कर्ष के रूप में हिम भारत में सर्वप्रथम फार्म पशुओं में ट्रांसजेनिक तकनीक के लिए एक मंच स्थापित करने में सक्षम हो सके।

# CHAPTER – 1

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**Introduction**

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## **INTRODUCTION**

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Transgenic animals are the animals which have been genetically transformed by splicing and inserting foreign animal or human genes into their chromosomes. The inserted gene, when successful, enables an animal to make a certain pharmaceutical protein in its milk, urine, blood, sperm, eggs, or to grow rejection-resistant organs for transplant. This process of using transgenic animals to produce human drugs is known as animal pharming and is currently staking its claim in a lucrative world market. In 1998, less than one percent of the world supply of human therapeutic proteins came from production of recombinant proteins - proteins formed by laboratory manipulation of genes in plants, bacteria, or animals. That tiny percentage of overall production, however, was valued at almost \$12 billion, or 50 percent, of a \$24 billion global market for human protein. With greater integration of computers into laboratory functions, molecular biologists have drastically reduced the time needed to identify and isolate genes. As gene sequencing has become increasingly automated, each known sequence is recorded and stored in a data base. Animal producers are using this body of genetic information to produce transgenic animals. The automated gene sequencing and biological advantages of animals, when compared to more traditional methods of recombinant protein production, have combined to make pharming a preferred alternative to traditional methods of recombinant protein production that use laboratory cell cultures of transgenic bacteria, yeast or animal cells to produce proteins. Inherent disadvantages in traditional methods, when compared to using animals as bioreactors, include (1) constant monitoring and sampling of cell and bacterial cultures, (2) more costly expansion owing to purchase and maintenance of plant machinery (3) isolation and purification of proteins from microbial and cell cultures is more difficult than purifying proteins from an animal's milk or other bodily fluids. Overall, animal mammary gland provides a more cost-effective bioreactor, because the product is efficiently passed through the milk, with an average yield of 53 percent and with 99 percent purity.

The purifying process may become simpler if harvesting proteins from poultry eggs and urine becomes viable. Using animals as bioreactors is also cost-effective and advantageous because animals naturally carry the cellular mechanisms needed to produce complex proteins because the mechanisms required by genes to produce proteins are present in a living animal, but they may be difficult or impossible to replicate in a cell culture. Only a fraction of the raw material, capital equipment, and maintenance costs needed for traditional cell culturing are required with transgenic animals. Therefore, the unit cost per protein should be significantly less when animals are used as bioreactors to produce human proteins. Transgenic animals with alterations to the germ line are commonly produced through microinjection to produce changes in germ line which are heritable from generation to generation within the herd. But due to the expense and difficulty in producing a transgenic animal by this method, a second easier, cheaper and lesser technically demanding alternative known as reproductive cloning has been developed. Cloning is regarded as the method of choice to produce multiple transgenic animals without altering the animal's genes as traditional breeding would do. The industrialization of transgenic animals is continuously growing as the demand continues to grow for human protein and vaccines. The interest in transgenic proteins has leaped from more traditional therapeutic protein production to the production of food supplements and nutraceuticals to develop functional foods which were otherwise produced by addition of the fortifying supplements. Since transgenic technology provides the alternative of producing the fortified food directly from the source, without the need for the addition of the purified and tested supplement from the outside, it has garnered enough hopes for humanity to eat what we want or require rather than whatever worthy or unworthy is available e.g., vitamin A in Golden rice.

Transgenic technology in near future would enable us to enhance both the nutritive value as well as the taste of the food we eat. The technology gives us the hope of producing foods of medicinal value – something that serves both as food as well as medicine. In this direction, we planned this project to produce transgenic goats expressing human lactoferrin in milk.

Lactoferrin, formerly known as lactotransferrin, is a non-haem glycoprotein and a member of the transferrin family, thus belonging to those proteins capable of binding and transferring  $\text{Fe}^{3+}$  ions. It was first identified in bovine milk and then subsequently in human milk. It performs multiple biological roles, including inflammation and immune modulation, iron absorption in newborns, non-iron-dependent antimicrobial activity, regulation of the level of retinoblastoma protein, bone growth, protection against cancer development and metastasis, transactivation of the p53 tumor suppressor gene as well as in human and animal behavior modulation. Lactoferrin has been reported to benefit human and animal health when administered orally, due to its anti-microbial, anti-carcinogenic and anti-inflammatory activities. Bovine lactoferrin (bLF) is added as a supplement to several products, viz., infant formula, yogurt, specialized milk-based and other beverages, pet foods and cosmetics in Japan, Indonesia, South Korea and Spain. However, the activity of bovine lactoferrin has been found to be quite different in several aspects from its human counterpart, provoking the need in replacing its use with hLF in the products of human consumption. Human lactoferrin has been found to have larger nutritional and therapeutic applications. Therefore, the need for a recombinant source of hLF has increased and its production has been attempted using different sources viz., fungi, virus, cell culture, plants and animals. Among all the exogenous sources of hLF, transgenic animals provide an alternative approach for production of the protein in larger amounts and at relatively lesser costs. The mammary gland of dairy animals has been the organ of choice and milk the appropriate vector for successful expression of many recombinant drugs and proteins. In spite of the imperfectness of the mammary gland to accurately add some sugar residues, it continues to be the most desirable organ to which the exogenous gene expression is targeted, due to its potent biosynthetic machinery and the possibilities to amend the said incapacity to glycosylate appropriately all kinds of proteins. However, among all the transgenic mammals produced so far, goats have represented an excellent model of transgenesis in terms of such factors like market demand of protein, milk volume per lactation, gestation length and reproductive rate.

In India the transgenic animal production technology has been carried out in rodents and number of transgenic mice were produced (Singh et al., 2012). However, in large animals no work has been initiated so far. In this context, the present study has been planned to develop transgenic goats expressing human lactoferrin in milk with the following objectives:

1. To develop transgene construct(s) of human lactoferrin under mammary gland-specific promoter(s).
2. To determine *in vitro* expression of the lactoferrin constructs in goat mammary epithelial cells.
3. To develop transgenic goat embryos containing human lactoferrin gene.

# CHAPTER – 2

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## Review of Literature

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## **2. REVIEW OF LITERATURE**

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The objective of the present study was to develop transgenic goat embryos expressing human lactoferrin. This was to be achieved by isolation of human lactoferrin gene from human lymphocytes and preparing an expression construct(s) under goat mammary gland specific promoter(s) with subsequent transfection of the functional construct into goat mammary epithelial and/or fetal fibroblast cells so as to develop the transgenic cell lines expressing human lactoferrin. The stably transfected cell were to be used as donor cells for somatic cell nuclear transfer (Hand-guided cloning) so as to produce transgenic goat embryos, expressing human lactoferrin. The transgenic embryos so produced were to be transferred to suitable recipients for production of transgenic goats. The relevant literature regarding lactoferrin and transgenic expression systems is summarized in the current chapter under the appropriate headings.

### **2.1 Transgenic Technology**

Transgenic animals are the animals which have been genetically transformed by splicing and inserting foreign animal or human genes into their chromosomes. The inserted gene, when successful, enables an animal to make a certain pharmaceutical protein in its milk, urine, blood, sperm, eggs, or to grow rejection-resistant organs for transplant. This process of using transgenic animals to produce human drugs is known as animal pharming and is currently staking its claim in a lucrative world market. With the advent of transgenic technology to farm animals, it became possible to express recombinant proteins of high complexity in the body compartments and fluids of these animals. This was a tremendous achievement taking into consideration the high costs associated with conventional (cell based) production methods and the incapacity of lower organisms to adequately process complex proteins. Subsequently, the mammary gland was discovered to be the ideal organ and milk the appropriate medium for successful expression of many recombinant proteins, leading all the roads to milk. Henceforth, several exogenous proteins have been effectively produced in the

mammalian mammary gland, e.g., human  $\alpha$ 1-antitrypsin in milk of transgenic sheep (Wright *et al.*, 1991),  $\alpha$ -casein in rabbits, mice and goats (Lee and de Boer, 1994). The interest in transgenic proteins has leaped from more traditional therapeutic protein production to the production of food supplements and nutraceuticals to develop functional foods which were otherwise produced by addition of the fortifying supplements. Among the various proteins of interest produced through transgenic technology, lactoferrin lies at the top of the list.

## 2.2 Lactoferrin

Lactoferrin (LF) is a glycoprotein belonging to transferrin family. It was formerly known as lactotransferrin, owing to its capability of binding and transferring  $\text{Fe}^{3+}$  ions and its presence in milk (Metz-Boutique *et al.*, 1984). The family also includes transferrin, ovotransferrin and melanotransferrin. These proteins are able to bind, in reversible form, to two atoms of ferric iron in the presence of bicarbonate ions. The transferrin family proteins are widely distributed in vertebrates and invertebrates (Lambert *et al.*, 2005). Most of its members are presumed to have evolved from an ancient gene duplication event, which resulted in a single polypeptide, folded into two homologous lobes, of about 80,000 Da. LF was first isolated by Sorensen and Sorensen, who separated it as “red protein fraction” from cow’s milk in 1939 (Sorensen and Sorensen, 1939). It was subsequently determined to be the main iron binding protein in human milk and was further identified in secretions from exocrine glands and in specific granules of neutrophils (Groves, 1960; Iyer and Lonnerdal, 1993). Among all the members of its family, LF has attracted increasing scientific interest primarily due to its high concentration in human breast milk (Lonnerdal and Iyer, 1995). The molecule since then has been identified in a number of mammalian species like cow, pig, equine, buffalo, goat and mouse. A vast number of studies have been undertaken to pinpoint the biological role (s) of lactoferrin (Reiter, 1985; Sanchez *et al.*, 1992). In addition atleast four conferences have been devoted explicitly for unravelling the features of its structure and understand the structure-function relationships of this versatile and intriguing molecule (Shimazaki *et al.*, 1993; Hutchens *et al.*, 1994; Hutchens and Lonnerdal, 1997; Spik *et al.*, 1998).

### 2.3 Occurrence of lactoferrin

Lactoferrin is predominantly found in products of exocrine glands located in the gateways of digestive, respiratory and reproductive systems, suggesting a role in non-specific defence against invading pathogens. Thus, lactoferrin is confined not only to milk but also in tears, synovial fluids, saliva and seminal fluid. It is also present in blood where it is derived from neutrophils which degranulate and synthesize lactoferrin during inflammation (Levay and Viljoen, 1995). It has been reported that concentration of LF in milk changes during the mammary cycle (Schanbacher *et al.*, 1997). For example, in cow the concentrations of lactoferrin and its messenger RNA especially increase during development of the mammary gland, colostrum formation and involution of the gland, whereas during lactation the levels of lactoferrin decrease, as opposed to the increasing levels of casein. The latter suggests that casein has primarily a nutritional function for the calf, whereas lactoferrin may have growth factor-like activity and protective functions for the (non-) lactating gland and the critical phase for the new born, just after birth.

### 2.4 Sources of lactoferrin

Lactoferrin expression can first be detected in two- and four-cell embryos during embryonic development, then throughout the blastocyst stage up to implantation and until halfway through gestation. Later, it is found in neutrophils and epithelial cells of forming reproductive and digestive systems (Ward *et al.*, 2005). The predominant cell types involved in lactoferrin synthesis are of the myeloid series and secretory epithelia (Baynes and Bezwoda, 1994). In adults, higher levels of lactoferrin are present in milk and colostrum (Masson and Heremans, 1971; Brock, 1980). It is also found in most mucosal secretions such as uterine fluid, vaginal secretion, seminal fluid, saliva, bile, pancreatic juice, small intestine secretions, nasal secretion, and tears (Masson *et al.*, 1966; Baker *et al.*, 1994; Levay and Viljoen, 1995; Lonnerdal and Iyer, 1995; Kikuchi *et al.*, 2003; Baker and Baker, 2005). Its production has also been reported in human kidneys (Abrink *et al.*, 2000). It is expressed and secreted throughout the collecting tubules, while in the distal

part of the tubules it may be reabsorbed. These results show that the kidney produces lactoferrin in a highly ordered manner and that only a minor fraction of this protein is secreted in urine. Therefore, lactoferrin is thought to have important functions in both the immune defense of urinary tract and in general iron metabolism. Neutrophils are an important source of lactoferrin in adults. Indeed, most plasma lactoferrin originates from neutrophils (Iyer and Lonnerdal, 1993). Lactoferrin is predominantly stored in specific (secondary) granules (Baggiolini *et al.*, 1970). However, it can also be found in tertiary granules albeit in significantly lower concentrations (Saito *et al.*, 1993). Its presence has been shown in blood, plasma or serum in relatively low concentrations (Rumke *et al.*, 1971; Boxer *et al.*, 1982; Brown *et al.*, 1983). The concentration of LF in plasma may or may not correlate with the neutrophil count (Hansen *et al.*, 1975; Olofsson *et al.*, 1977; Baynes *et al.*, 1986). This depends on the extent of degranulation and perhaps on the contribution of other organs, such as bone marrow, endometrium (Masson *et al.*, 1968) and placenta (Niemela *et al.*, 1989). Lactoferrin plasma levels change during pregnancy as well as with menstrual cycle (Sykes *et al.*, 1982; Levay and Viljoen, 1995). Its concentration in blood increases during infection, inflammation (Birgens, 1985), excessive intake of iron or tumor growth (Levay and Viljoen, 1995).

## **2.5 Biosynthesis of lactoferrin**

Lactoferrin is synthesized by glandular epithelial cells and secreted into milk, tears, saliva, nasal fluids, pancreatic-, bronchial-, gastrointestinal- and reproductive tissue secretions (Ward and Conneely, 2005). The concentration of LF in milk varies considerably among species. Human milk has the highest LF concentration (1-6 mg/ml); mouse milk has moderate levels of LF (1-2 mg/ml) and milks from ruminants have relatively low levels of LF (0.01-0.1 mg/ml), while rabbit and rat milk contains virtually no LF (Nuijens *et al.*, 1996). The LF concentration in lacteal secretions varies also within the lactation phase. In human milk, the LF concentration can be as high as 10 mg/ml in colostrum declining to about 1-2 mg/ml in mature milk. The concentration of bovine LF (bLF) is about 1-2 mg/ml and 0.01-0.1 mg/ml in bovine colostrum

and mature milk, respectively (Nuijens *et al.*, 1996). Upon involution of the human and bovine mammary gland, the LF concentration increases to about 50 mg/ml and 20-100 mg/ml, respectively (Nuijens *et al.*, 1996). The concentration of LF in human saliva and tears is about 30 µg/ml and 2 mg/ml, respectively (Kijlstra *et al.*, 1983; Tanida *et al.*, 2003). LF is also released from the secondary granules of activated neutrophils. This process likely accounts for the presence of LF in normal blood plasma at a concentration of about 0.2 µg/ml (Nuijens *et al.*, 1996). In patients with sepsis, the levels of LF in plasma are increased to about 1 µg/ml (Nuijens *et al.*, 1992). The level of LF in synovial fluid of patients with non-inflammatory joint diseases is about 0.7 µg/ml, while in patients with inflammatory joint diseases the level of LF in synovial fluid is increased to about 4 µg/ml (Abbink *et al.*, 1991). LF is efficiently removed from circulation by liver. Studies in rats showed that about 95% of intravenously administered LF (0.25 mg/kg body weight) is cleared by liver within 5 minutes (Ziere *et al.*, 1992).

## **2.6 Regulation of lactoferrin synthesis**

The regulation of lactoferrin synthesis depends on the type of cells producing this protein. The amount of lactoferrin synthesized in mammary gland is controlled by prolactin (Green and Pastewka, 1978), whereas its production in reproductive tissues is determined by estrogens (Pentecost and Teng, 1987; Walmer *et al.*, 1992; Teng *et al.*, 2002). The synthesis of lactoferrin in endometrium is influenced by estrogens as well as epidermal growth factor (Nelson *et al.*, 1991).

## **2.7 Lactoferrin metabolism**

Lactoferrin is eliminated through receptor-mediated endocytosis of phagocytic cells (macrophages, monocytes, and other cells belonging to the reticuloendothelial system) with subsequent iron transfer to ferritin or through direct uptake by the liver. Endocytosis performed by Kupffer cells, liver endothelial cells and hepatocytes contributes to lactoferrin removal (Levay and Viljoen, 1995). Kidneys seem to be involved in the removal of lactoferrin from the circulation since lactoferrin and its fragments, mainly of maternal origin, have been found in the urine of breast-fed infants (Hutchens *et al.*, 1991).

## 2.8 Structure of lactoferrin

Lactoferrins are single chain polypeptides of about 80 000 Da containing 1±4 glycans, depending on the species (Spik *et al.*, 1994). The molecular structure and amino acid sequence of human lactoferrin were discovered in 1984. Lactoferrin was then classified as a member of the transferrin family, due to its 60% sequence identity with serum transferrin (Metz-Boutique *et al.*, 1984). Bovine and human lactoferrin consist of 689 and 691 amino acids, respectively. They share 69 % sequence identity (Pierce *et al.*, 1991). Three different isoforms of lactoferrin have been isolated. Lactoferrin- $\alpha$  is the iron binding form, but has no ribonuclease activity. On the other hand lactoferrin- $\beta$  and lactoferrin- $\gamma$  demonstrate ribonuclease activity but they are not able to bind iron (Furmanski *et al.*, 1989). The 3-D structures of bovine and human lactoferrin are very similar, but not identical.

## 2.9 Lactoferrin receptors

The biological properties of lactoferrin are mediated by specific receptors on surface of target cells. These receptors are typical for each cell type and can be found, for example, on mucosal epithelial cells, hepatocytes, monocytes, macrophages, polymorphonuclear leukocytes, lymphocytes, thrombocytes, fibroblasts, and on some bacteria such as *Staphylococcus aureus* or *Pseudomonas hydrophila* (Levay and Viljoen, 1995; Suzuki and Lonnerdal, 2002; Suzuki *et al.*, 2005). Some cells have also “main receptors”, which enable them to bind not only lactoferrin, but also transferrin or lactoferrins of other species (Kanyshkova *et al.*, 2001).

## 2.10 Iron binding properties of lactoferrin

The affinity of lactoferrin for iron is very high (about 260 times that of blood serum transferrin) with an affinity constant of about  $10^{20}$  (Baker *et al.*, 1994). The binding site appears to be optimized for the binding of ferric iron and (bi) carbonate with respect to size, charge and stereochemistry, as evidenced from a number of structural studies with different anions and cations, or using mutant recombinant lactoferrins (Harrington *et al.*, 1987; Baker *et al.*, 1994; Brodie *et al.*, 1994; Faber *et al.*, 1997).

## 2.11 Biological functions of lactoferrin

### i) Iron metabolism

Although the influence of lactoferrin on iron distribution in an organism is implied by its resemblance to transferrin, it has thus far not been unequivocally proven that lactoferrin plays an important role in iron transport. This may be due to the fact that lactoferrin plasma concentrations are very low under normal conditions. On the other hand, lactoferrin level increases when inflammation occurs. In such an environment iron exchange from transferrin is easier due to lower pH suggesting that lactoferrin may contribute to local iron accumulation at sites of inflammation (Brock, 2002). Lactoferrin has long been known to be responsible for hypoferraemia through binding free iron and shuttling it back to macrophages (Van Snick *et al.*, 1974). Lactoferrin from human milk seems to affect intestinal iron absorption in infants, but it depends on the organisms' need for iron. Specific receptors (SI-LfR), present on enterocytes, mediate binding of lactoferrin. After lactoferrin is bound to the enterocyte, 90% of it is degraded and Fe<sup>3+</sup> ions are released. The remaining intact 10% is transported through the cell membrane. A lack of intracellular iron may evoke increased expression of specific receptors on the surface of enterocytes and thereby elevated absorption of lactoferrin-bound iron (Suzuki *et al.*, 2005). Breast-fed infants have demonstrated better iron accessibility than babies on formula (Fairweather-Tait *et al.*, 1987). Counter to this, some research fails to identify a positive effect of lactoferrin on iron absorption in the intestines. Indeed, a possible suppressive effect of lactoferrin on absorption is described because higher iron absorption has been reported in infants fed lactoferrin-free human milk (Davidsson *et al.*, 1994). Even though lactoferrin does not play the most important role in iron metabolism, its capability of binding Fe<sup>3+</sup> ions has a significant influence on many of its other biological properties.

### ii) Antimicrobial activity

Lactoferrin is considered to be a part of the innate immune system. At the same time, lactoferrin also takes part in specific immune reactions, but in an

indirect way (Legrand *et al.*, 2005). Due to its strategic position on mucosal surface lactoferrin represents one of the first defense systems against microbial agents invading the organism, mostly *via* mucosal tissues. Lactoferrin affects the growth and proliferation of a variety of infectious agents including both Gram-positive and negative bacteria, viruses, protozoa, or fungi (Kirkpatrick *et al.*, 1971).

### **iii) Antibacterial activity**

The ability to bind free iron, which is one of the elements essential for growth of bacteria, is responsible for the bacteriostatic effect of lactoferrin (Arnold *et al.*, 1980). A lack of iron inhibits the growth of iron-dependent bacteria such as *E. coli* (Brock, 1980). In contrast, lactoferrin may serve as iron donor and in this manner support the growth of some bacteria with lower iron demands such as *Lactobacillus* sp. or *Bifidobacterium* sp., generally considered as beneficial (Petschow *et al.*, 1999; Sherman *et al.*, 2004).

Lactoferrin may contribute to defense against the invasion of facultative intracellular bacteria into cells by binding both target cell membrane glycoaminoglycans and bacterial invasins, which prevents pathogen adhesion to target cells. This ability was first reported against enteroinvasive *E. coli* HB 101 and later also against *Yersinia enterocolica*, *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, *Streptococcus pyogenes*, and *Staphylococcus aureus* (Valenti and Antonini, 2005).

### **iv) Antiviral activity**

Lactoferrin is capable of binding certain DNA and RNA viruses (Yi *et al.*, 1997). Nevertheless, its main contribution to antiviral defense consists in its binding to cell membrane glycosaminoglycans. In this manner lactoferrin prevents viruses from entering cells and infection is stopped at an early stage (Ward *et al.*, 2005). Such a mechanism has been demonstrated as being effective against *Herpes simplex* virus (Fujihara and Hayashi, 1995; Marchetti *et al.*, 1996), *Cytomegaloviruses* (Andersen *et al.*, 2001), and human immunodeficiency virus (Harmsen *et al.*, 1995), respectively.

**v) Antiparasitic activity**

Lactoferrin acts against parasites in various ways. For example, the infectivity of *Toxoplasma gondii* and *Eimeria stiedai* sporozoites is reduced after their incubation with lactoferricin. It is thought that lactoferricin breaches parasitic membrane integrity causing subsequent changes in interactions between the host and the parasite (Omata *et al.*, 2001). The competition for iron between the parasite and lactoferrin is basis of its antiparasitic activity against *Pneumocystis carinii* (Cirioni *et al.*, 2000). In contrast, some parasites such as *Tritrichomonas foetus* are able to use lactoferrin as a donor of ferric ions (Tachezy *et al.*, 1996).

**vi) Host defense**

Due to its iron binding properties and interactions with target cells and molecules, lactoferrin can both positively and negatively influence immune system cells and cells involved in inflammation reaction. In one way, lactoferrin may support proliferation, differentiation and activation of immune system cells and strengthen immune response. On the other hand, lactoferrin acts as an anti-inflammatory factor and prevent the development of inflammation and subsequent tissue damage caused by release of pro-inflammatory cytokines and reactive oxygen species (Legrand *et al.*, 2005).

**vii) Antitumorogenic**

The protective character of lactoferrin has on numerous occasions been demonstrated on chemically induced tumors in laboratory rodents. Lactoferrin has even been reported to inhibit the development of experimental metastases in mice (Bezault *et al.*, 1994; Wang *et al.*, 2000; Wolf *et al.*, 2003). Lactoferrin is able to halt the growth of human mammary gland carcinoma cells between the G1 and S stage. Such a negative effect on cell proliferation may be ascribed to altered expression or activity of regulatory proteins (Damiens *et al.*, 1999). Lactoferrin-mediated inhibition of tumor growth might be related to apoptosis of these cells induced by activation of the Fas signaling pathway. Nevertheless, the exact mechanism of this function has not been discovered so far (Fujita *et al.*, 2004).

## 2.12 Applications of lactoferrin

The diverse biological actions of LF may provide a basis for a large variety of potential nutraceutical as well as topical and systemic applications in human healthcare. The applications may include the prevention and treatment of local or systemic infections and (chronic) inflammations such as those occurring in patients with inflammatory bowel diseases, patients receiving high-dose chemotherapy and patients with allergic asthma. Furthermore, LF may be suitable for neutralization of heparin activity after its use as anticoagulant in surgery. Both hLF and bLF, obtained after fractionation of bovine milk whey, can be used in applications of LF in human health care. However, the use of bLF in human healthcare is limited to oral applications because of its immunogenicity. Furthermore, bLF may be inferior to hLF in applications where interactions with specific receptors are required. Recently, oral administration of lactoferrin has been reported to benefit human and animal health, due to these anti-microbial, anti-carcinogenic and anti-inflammatory activities (Wakabayashi *et al.*, 2006). These findings support the utility of lactoferrin as an ingredient in foods and special products (Tomita *et al.*, 2009). Large-scale manufacturing of bovine lactoferrin (bLF) was established more than 20 years ago, using bovine skim milk and whey as sources. Oleofina Company in Belgium and MILEI GmbH in Germany (using technology developed by Morinaga Milk Industry Co., Ltd.) pioneered industrial scale bLF production (Tomita *et al.*, 2009). Currently, bLF is added as a supplement to several products in Japan, including infant formula, yogurt, specialized milk-based and other beverages, nutritional supplements, pet foods, and cosmetics (Wakabayashi *et al.*, 2006). Similarly, infant formulas enriched with bLF are also available in other countries, including Indonesia, South Korea, and Spain. However, whether the activity of bovine lactoferrin is the same as that of human lactoferrin (hLF) for all proposed functions is unclear.

## 2.13 Recombinant human lactoferrin

Despite of their almost similar functions, there is considerable evidence that bLF is quite different in several aspects as compared to hLF and, mechanistically, it has been observed that bLF does not bind the same site on enterocytes in the infant small intestine (Kawakami and Lönnerdal, 1991). Bovine lactoferrin (bLF) is added as a supplement to several products, *viz.*, infant formula, yogurt, specialized milk-based and other beverages, pet foods and cosmetics in Japan, Indonesia, South Korea and Spain (Wakabayashi *et al.*, 2006). However, the activity of bovine lactoferrin has been found to be quite different in several aspects from its human counterpart, provoking the need in replacing its use with hLF in the products of human consumption (Conesa *et al.*, 2010) which has been found to have larger nutritional and therapeutic applications (Shu *et al.*, 2007). Consequently, there is significant interest in replacing the use of bLF with hLF in products for human consumption. However, obtaining hLF presents both technical and safety issues, the former constrained by supply and the latter by the risk of pathogen transmission. Numerous attempts have been made to produce recombinant human lactoferrin (rhLF) and its production has been attempted using different sources *viz.*, fungi (Liang and Richardson, 1993; Ward *et al.*, 1995), virus (Salmon *et al.*, 1997; Tutykhina *et al.*, 2009a), cell culture (Stowell *et al.*, 1991; Min *et al.*, 2006), plants (Lee *et al.*, 2002; Kamenarova *et al.*, 2007) and animals (Platenberg *et al.*, 1994; Yang *et al.*, 2008; Tutykhina *et al.*, 2009b). Among the recombinant lactoferrins, only a few systems can be expanded to industrial scale. These lactoferrins are rhLF from *Aspergillus awamori*, produced by Agennix (Houston, TX, USA), rhLF from rice, produced by Ventria Bioscience (Sacramento, CA, USA), and rhLF from transgenic cows, produced by Pharming (Leiden, The Netherlands).

### 2.13.1 Recombinant human lactoferrin from transgenic animals

Among all the exogenous sources of hLF, transgenic animals provide an alternative approach for production of the protein in larger amounts and at relatively lesser costs. Several heterologous proteins have been effectively

produced in the mammalian mammary gland. This production includes  $\beta$ -lactoglobulin in sheep and mice; whey acidic protein (WAP) in goats, mice and pigs;  $\alpha$ -casein in rabbits, mice and goats; and  $\alpha$ S1-casein in mice and rabbits (Lee and de Boer, 1994). The first transgenic produced rhLF was in mice with secretion into the milk. Transgenic mice were produced by using either hLF cDNA or genomic hLF sequences fused to regulatory elements of the bovine  $\alpha$ S1-casein gene. The concentrations of recombinant hLF in the milk ranged from less than 0.1  $\mu$ g/mL to 36  $\mu$ g/mL (Platenburg *et al.*, 1994). The murine transgenic expressed rhLF was immunologically indistinguishable from native hLF. In addition, purification of the recombinant protein by mono S cation-exchange and subsequent N-terminal protein sequencing revealed identical charge structure and amino acid sequences (Nuijens *et al.*, 1997). However, there were differences between this transgenic murine rhLF and native hLF. Murine rhLF was highly iron-saturated (approximately 90%) as compared to native hLF (3% iron-saturated) and there were also minor variations in the glycosylation pattern between the two lactoferrins (Nuijens *et al.*, 1997). The mammary gland of dairy cattle is considered ideal for largescale production of heterologous proteins due to the large capacity of protein synthesis, efficient secretion and low feed and housing costs of dairy animals relative to *in vitro* fermentation or tissue culture systems (Lee and de Boer, 1994). Consequently, in order to produce large-scale quantities of rhLF, the expression of rhLF in transgenic cows has been achieved (Van Berkel *et al.*, 2002) by Pharming, a Netherlands based company. The company focus is on development of rhLF for use as an ingredient in advanced nutritional products, however the product also has potential for pharmaceutical applications and it is found now in Phase II/III trials as a bionutrient. Transgenic cattle were obtained by microinjection and harbour the hLF gene under the regulatory control of the bovine  $\alpha$ S1-casein promoter, as was achieved for transgenic mice. The expression levels of rhLF in lactating dairy cows were in a range from 0.8 to 2.0 g/L and these levels remained consistent throughout the 280 days of the lactation period (Van Berkel *et al.*, 2002). Apart from differences in glycosylation, rhLF from transgenic cows and hLF have identical crystal structures (Thomassen *et al.*, 2005). Analysis of the

monosaccharide composition of the two lactoferrins indicated that rhLF contained relatively more mannose than native hLF. Furthermore, N-linked glycans of rhLF contain N-acetylgalactosamine next to galactose, and less sialic acid and fucose than the glycans of native hLF (Van Berkel *et al.*, 2002; Zhao *et al.*, 2006). The oral toxicity of recombinant human lactoferrin (rhLF) produced in the milk of transgenic cows was investigated in Wistar rats by daily administration *via* oral gavage for 13 consecutive weeks (Appel *et al.*, 2006). No treatment-related significant toxicological changes were observed. The few observations that may be related to the treatment (lower cholesterol in females administered a high rhLF dose, lower urinary pH in males and females given the high-dose rhLF, and slightly higher kidney weight in high-dose females) were not considered to be of toxicological significance. Based on the absence of toxicologically relevant changes related to treatment, the non-observed-adverse effect level (NOAEL) was considered to be at least 2000 mg/kg body weight/day (Appel *et al.*, 2006). The anti-bacterial activity of rhLF from cow's milk and native hLF was found to be similar in an experimental infection of mice with *Staphylococcus aureus* (Van Berkel *et al.*, 2002). However, in a study with an experimental *Escherichia coli* mastitis model using transgenic cows expressing rhLF in milk, it was shown that the high levels of rhLF (approximately 2.9 mg/mL) did not protect the animals from intramammary infection. Moreover, levels of rhLF in transgenic cows were constant throughout the experimental infection, whereas bovine lactoferrin levels were higher during mastitis (Hyvönen *et al.*, 2006a and b). This finding suggests that mammary gland expression of rhLF does not seem to be an effective strategy to enhance mastitis resistance in dairy cows. Recently, a new large-scale production of rhLF in cattle has been developed (Yang *et al.*, 2008). In this work, a 150 kb bacterial artificial chromosome (BAC) carrying the human lactoferrin gene and a marker gene was microinjected into bovine fetal fibroblast cells, followed by somatic cloning. For high-level and stable expression of rhLF in transgenic animals, this group had previously optimized the use of a construct carrying the entire hLF genomic sequence and obtained transgenic mice capable of producing rhLF at up to 8 g/L of milk (Liu *et al.*, 2004). Using this technique in cattle, two transgenic

cows were developed that secreted rhLF at 2.5 and 3.4 g/L. Moreover, this rhLF was found to have a similar pattern of glycosylation and proteolytic susceptibility as the natural hLF counterpart. Biochemical analysis revealed that the iron-binding and releasing properties were identical in both lactoferrins. Furthermore, the anti-microbial activity of this rhLF has been confirmed (Yang *et al.*, 2008). Apart from mice and cows, described above, other transgenic animals have been used for the production of rhLF at laboratory scale. These include rabbits, in which rhLF was produced using dimethylsulfoxide-sperm-mediated gene transfer (Li *et al.*, 2006), and goats, by using replication-defective adenoviral vectors (Han *et al.*, 2007) or by microinjecting sequence encoding hLF cDNA to the pronucleus (Zhang *et al.*, 2008). The expression levels of rhLF in goat milk were from 0.765 g/L (Zhang *et al.*, 2008) to 2.6 g/L (Han *et al.*, 2007).

#### **2.14 Mammary gland of transgenic animals as bioreactor**

The mammary glands are apocrine/ exocrine cutaneous glands of the tubulo-alveolar composition type and their secretory units consist of alveoli that lead to small excretory ducts. The groups of secretory units form lobules that are later grouped into larger structures called lobes, forming the mammary parenchyma. The alveoli are the fundamental secretory units of milk and are composed of epithelial cells capable of synthesizing fats, carbohydrates and proteins, expelling the product to inside the lumen of alveoli. The proteins secreted by the mammary gland are grouped into two classes, caseins and serum proteins. According to Park *et al.*,(2007), ruminants secrete mainly four caseins ( $\alpha$ s1,  $\alpha$ s2,  $\beta$  and  $\kappa$ ) and two serum proteins ( $\beta$ -lactoglobulin and  $\alpha$ -lactoalbumin). The genes that encode these proteins are in simple copies and are transcribed at high levels, specifically in the mammary gland during pregnancy and lactation (Clark, 1998). Therefore, pioneer works in 1980s explored the capacity of the production of recombinant proteins in milk of transgenic animals (Gordon *et al.*, 1987; Simmons *et al.*, 1987). Thus, promoters and regulatory regions of genes of specific milk proteins were utilized to direct the gene expression in the mammary gland (Maga and

Murray, 1995). The mammary gland can be currently considered the best bioreactor available (Cole, 1994), since milk represents a source of raw material, unprocessed, safe, abundant, renewable, easy to obtain and well accepted by the public. Besides, extensive studies have demonstrated the possibility of the production of a great variety of recombinant proteins in milk.

#### **2.14.1 Gene construction for expression in mammary gland**

Gene construction is a fundamental element in the production of transgenic animals and consists, generally, of a promotor region, a gene that encodes the protein of interest and other regulatory elements to optimize gene expression (Maga and Murray, 1995). The transgene or exogenous gene that codes for the protein of interest can be derived from another animal of the same species, different species or even of other kingdoms such as bacteria, yeasts or plants (Pesqueiro *et al.*, 2007). According to Keefer (2004), the selection of this gene should be based on social, scientific and economic factors. The expression of the transgene in mammary gland requires the use of promoters and regulatory regions of the genes of milk proteins, that is, sequences that direct the gene expression only for the mammary gland during lactation (Maga and Murray, 1995). In this manner, it is expected that the expression of the recombinant protein would be limited to the mammary gland to avoid the deleterious effects on the health of the animal. Promoters are sequences of a few kilobases located close to the 5' end of the transcribed region of the gene (Houdebine, 2003). In goats, various regulatory sequences of milk specific genes were already isolated and verified in the production of transgenic animals, such as a murine WAP (Ebert *et al.*, 1991), goat  $\beta$ -casein (Ko *et al.*, 2000; Parker *et al.*, 2004), bovine  $\beta$ -casein (Lee *et al.*, 1997; Huang *et al.*, 1998) and goat and bovine  $\alpha$ s1-casein (Freitas *et al.*, 2007). The expression levels of recombinant proteins vary from one bioreactor to another, which can be related to various factors, such as the expression vector; method utilized for the transfer of genetic material; number of copies and site of insertion of the transgene (Rosen *et al.*, 1996).

### 2.14.2 Post translational processing in mammary gland

The biological activity of many proteins is dependent on appropriate post-translational processing, including the removal of the signal peptide, formation of disulfide bonds, modifications of amino acids, proteolytic processing and addition of subunits. Bacteria possess limitations in their ability to carry out the post-translational modifications of proteins necessary for many functions, while some eukaryotic systems, such as yeasts, filamentous fungi and unicellular algae, do not have adequate post-translational processes, yielding recombinant products with little biological activity or immunogenicity (Dyck *et al.*, 2003). Cultured mammalian cells and transgenic animals are utilized for the production of recombinant proteins when complex post-translational modifications are necessary for bioactivity of the protein (Clark, 1998). One of the commonest and least well understood post-translational modifications of proteins is their glycosylation. Human glycoproteins are glycosylated with a bewilderingly heterogeneous array of complex N- and O-linked glycans, which are the product of the coordinated activity of enzymes resident in the endoplasmic reticulum and Golgi apparatus of the cell. Glycosylation of proteins is highly regulated and changes during differentiation, development, under different conditions and in disease. The glycosylation of recombinant proteins, especially those destined for potential administration to human subjects is of critical importance. Glycosylation profoundly affects biological activity, function, clearance from circulation, and crucially, antigenicity (Brooks, 2004). A DNA sequence provides all of the information necessary for a cell to produce a recombinant protein with the same amino acid sequence as the native protein but it does not dictate exactly how that protein will be glycosylated. Therefore, using a human DNA sequence does not guarantee that the molecule will be glycosylated as it is when synthesized by the human bodies. The peptide sequence may determine where glycosylation is added to the protein; but the mix of glycosyltransferases, cell within which the protein is expressed, and even the conditions under which those cells are cultured will determine what oligosaccharide structures are added to the molecule (Raju *et al.*, 2003; Betenbaugh *et al.*, 2004). Covalent binding between the sugars and the peptide chain is a central part of the structure of glycoproteins. The main

ones are: (1) N-glycosidic bonds, which occur between a residue of N-acetylglycosamine (GlcNac) and the amino acid asparagine (Asn) of the polypeptide chain; (2) O-glycosidic bonds, which occur between a residue of N-acetylgalactosamine (GalNac) and the OH group of serine (Ser) or threonine (Thr) of the polypeptide chain (Lubon *et al.*, 1996). Mammalian species are phylogenetically closest to human beings and can show common patterns of glycosylation. In general, the glycosylation found in human proteins secreted in the milk of transgenic animals has been similar to that of human plasma proteins (Meade *et al.*, 1999). Cole *et al.* (1994) found that antithrombin III (AT III) and LAtPA (the long-acting form of human tissue plasminogen activator) expressed in the goat milk had some N-acetylgalactosamine (GalNac) replacing the N- group of galactose on oligosaccharide complexes. Both the recombinant AT III and LAtPA were shown to be more fucosylated than their recombinant or plasma counterparts. Denman *et al.* (1991) observed that significantly lower levels of galactose, N-acetylglucosamine and sialic acid were present in LAtPA from transgenic goats, when compared to proteins produced by the murine cell line C127 and Chinese hamster ovary cells (CHO). Carboxylation is the introduction of a carboxyl group or carbon dioxide to form carboxylic acid (Bosze *et al.*, 2008). Coagulation cascade factors dependent on vitamin K and regulatory proteins require the conversion of glutamic acid residues (Glu) into gamma-carboxyglutamic acid (Gla). Gla residues bind to calcium which is an essential cofactor for its activity, thereby making it possible for the adhesion of coagulation factors to cell surface phospholipids, accelerating the coagulation process. Species-specific differences were observed in the ability of mammary epithelial cells with respect to the carboxylation of recombinant proteins, and these differences can reflect the enzyme levels and/or specificity of the substrate (Luboń *et al.*, 1996). Proteolytic processing is also a very important post-translational modification, where it is the first step of protein maturation in which the signal peptide is removed. The signal peptides are short sequences (generally 13-30 amino acids) at the N-terminal region of proteins, which direct the post-translational transport of new proteins (synthesized in the cytosol) to certain organelles which promote this processing (Bosze *et al.*, 2008).

## 2.15 Goat as transgenic animal

Human proteins have been utilized in medicine worldwide, and for some time, their supply was limited due to few sources for their extraction (Clark, 1998). With the advent of genetic engineering, this reality changed, because the genes of interest could be isolated, inserted into expression vectors and transferred to the cells or organisms that could be turned into producers of proteins on an industrial scale (Houdebine, 2003). The first attempts at the production of therapeutic proteins from the cloned genes were from microorganisms. However, some human proteins were synthesized only in small amounts, others became insoluble making their purification difficult. In addition, the products of gene expression can be immunogenic and can have little biological activity due to incorrect post-translational processing (Kues and Niemann, 2004). As an alternative, another expression system, such as animal cells, has been used, although it is a costly process and not very profitable. Since 1982, with the development of the first mouse expressing elevated levels of the human growth hormone (Palmiter *et al.*, 1982), it was found that animals could be utilized as bioreactors. Later, various transgenic animals were obtained in different species: rabbits, pigs and sheep (Hammer *et al.*, 1985), goats (Ebert *et al.*, 1991) and cattle (Krimpenfort *et al.*, 1991). According to Clark (1998), for the production of recombinant proteins, some aspects should be considered in the choice of the bioreactor species, such as the market demand, volume of milk produced per lactation and reproductive rate. Of all the transgenic mammalian bioreactors already produced, goats (*Capra hircus*) have represented an excellent model for transgenesis, since the production of founder animals and operating costs are significantly easier to manage compared to cattle. Goat also has lower age of sexual maturity and gestation period which further adds to its advantage when transgenic animals are desired to be produced in milk.

Species	Sexual maturity (month)	Gestation length (days)	Generation interval (month)	Potential milk volume (L)
Rabbit	3-4	30-32	6	1
Pig	5-8	114	12	>100
Sheep	3-6 (m), 5-10 (f)	150	18	>100
Goat	5-9 (m), 8-10 (f)	150	19	>100
Cattle	8-11	280	30	>10,000
Buffalo	18-24	310	32	>10,000

Abbreviations: m, male; f, female

(Wang *et al.*, 2013)

### 2.15.1 Approaches for transgenic goat production

#### i) Microinjection

Using this method, transgenic animals were obtained by direct microinjection of the DNA constructs into fertilized zygotes. The overall efficiency of such manipulations is poor, especially when compared to that obtained in mice, but is comparable to that achieved in sheep. In goat, around 1 % or less of the injected eggs gives birth to a transgenic kid as in sheep. In cow, this percentage is even lower, often below 0.1 % whereas in mice it is rather between 5 and 10 %. The levels of recombinant protein being secreted in the milk could reach several g/L, allowing purification processes to be applied and commercial uses, as mentioned above. However, despite the use of various expression cassettes, highly variable results were obtained between and within experiments, highlighting the site-integration dependency of the resulting transgene expression levels and the need for further improvements to increase the predictability of this outcome and consequently reduce the overall cost of such experiments. This latter point is not specific to the goat

species, but rather a general drawback of the current microinjection approach. As an alternative to the establishment of transgenic lines, *in vivo* direct transformation of mammary epithelial cells with stearylamine liposomes (Zhang *et al.*, 1997b) or replication-defective adenoviral vectors (Archer *et al.*, 1994; Sa´nchez *et al.*, 2004; Toledo *et al.*, 2006; Han *et al.*, 2007, 2009; Yang *et al.*, 2009) was investigated. Although again variable between experiments and manipulated goats, relatively high levels of recombinant proteins could be produced in the milk (up to several g/L), allowing its purification and biological characterization. It remains that such alternative strategies can only be used to assess the feasibility of the production of a recombinant protein over successive lactations but, as the transgene is not integrated into the germ line, it cannot be used for establishing a transgenic line. For the above-mentioned applications, a more goat specific major potential drawback could be the genetic heterogeneity of the goat population that could affect quantitatively and also qualitatively the produced recombinant protein. Alterations in the co- and post-translational modifications can indeed modify the properties of a biologically active protein, a situation poorly compatible with the principle of the comparability exercise required for biopharmaceuticals (Schiestl *et al.*, 2011). However, although differences were noticed between different goat transgenic lines expressing the same recombinant protein, transgenic animals derived from a same founder were found to express recombinant proteins with comparable glycosylation (Zhou *et al.*, 2005). The physiological impact of the production of the recombinant protein in the milk of lactating goat has been studied in details in some of these animals and/or in the young that suckle such modified milks. In some cases, a detrimental effect was reported on the lactation physiology. Expression levels above 1 g/L of human butyryl-cholinesterase negatively impacted the lactation performance of the transgenic goats, with decreased milk production, reduced milk fat and casein contents, alteration of milk composition through decreased expression of key enzymes and modification of epithelial tight junctions and increased amount of leucocytes in the milk independently of an infectious status (Baldassarre *et al.*, 2008a, b, 2009).

Some of these observations could be explained by detection in these mammary epithelial cells of an endoplasmic reticulum stress (Baldassarre *et al.*, 2004). Expression of human lysozyme resulted in a different outcome, with no impact on growth and reproductive traits (Jackson *et al.*, 2010), potential improvement of the health status of the lactating goats and their offspring through increased antimicrobial milk properties, modulation of intestinal micro-flora and improved gastrointestinal health (Maga *et al.*, 2006a, b; Brundige *et al.*, 2008; Cooper *et al.*, 2011). Overall, these observations suggests that the physiological impact of expressing recombinant proteins in milk is more related to the biological activity of these proteins than to its absolute level of secretion, if it remains in a reasonable range value. Biological activity of the expressed recombinant protein could also be used to modify milk nutritional value. This was the aim for targeting expression of stearoyl-CoA desaturase in the lactating mammary gland of goats (Reh *et al.*, 2004). It induces the production of less saturated fatty acids, a modification that could diminish the risk of human cardiovascular diseases. Several technical improvements have been tested to lower the cost of the microinjection technology in goats, such as the weight, length, position (right or left) of the ovaries, their genetic origin, the season, age of donors and recipients (Gootwine *et al.*, 1997, Islam *et al.*, 2007). These analyses highlighted potential significant differences on the number of collected oocytes when parameters such as the season, age and previous hormonal treatments were analyzed or when the right and left ovaries were compared. Other studies focused on alternative strategies to obtain fertilized zygotes and to increase transgenic rates. Oocytes collected by laparoscopic ovum pick-up techniques were successfully used to derive transgenic goats following *in vitro* maturation and fertilization (Wang *et al.*, 2002; Baldassarre *et al.*, 2003). Selection of the transgenic embryos by nested PCR was also applied to reduce the number of needed recipient goats (Huang *et al.*, 2001). Microinjection of RecA recombinase coated DNA increased the transgene integration rate (11 fold) and embryos survival (two fold) but led to a large proportion of mosaic transgenic founders (Maga *et al.*, 2003).

## ii) Sperm mediated gene transfer

Sperm-mediated gene transfer (SMGT) is an established procedure for transgenic production in several species and its potential use as a gene therapeutic approach is currently under consideration. SMGT is not yet applied in goats and recent experiments have assessed its feasibility. It was shown that although goat spermatozoa can uptake DNA, presence of the seminal fluid partially inhibits it, while alteration of the sperm membrane by various treatments increases it (Zhao *et al.*, 2012). Differences between breeds were also noticed for the sperm DNA-binding capacity (Zhao *et al.*, 2012). Incubation of sperm with goat DNA appears to alter its motility (Ma *et al.*, 2010), and expression of the transgene was only observed in embryos resulting from the use of live-immotile and dead sperm before ICSI (Shadanloo *et al.*, 2010). Altogether, these data suggest that SMGT is potentially applicable to goats using ICSI but genetic characterization of the resulting transgenic animals both in terms of transgene integrity and mosaicism are still lacking.

## iii) Goat stem cells

Although validated goat embryonic stem cells are still lacking, ES-like cells were recently isolated from inner cell-masses. These cells remained undifferentiated for several passages and possessed multiple differentiation potency (Tian *et al.*, 2006). Stem cells were also isolated from late gestating amniotic fluid of goat fetuses. These cells could be efficiently transfected and could differentiate into adipogenic cells (He *et al.*, 2011). The totipotency of these cells and their efficiency for both transformation by homologous recombination and NT remain to be evaluated. Transplantation of testis germ cells from a transgenic goat into testes of sexually immature wild-type recipient goats allowed transmission of the donor haplotype, suggesting an alternative procedure for the production of transgenic goats (Honaramooz *et al.*, 2003). Following this experiment, depletion of endogenous germ cells in male goats was achieved by fractionated irradiation of the testes of immature animals (Honaramooz *et al.*, 2005) and transplantation of AAV-mediated transduced germ cells into such syngeneic recipient testis was shown to allow the production of transgenic sperm successfully used for *in vitro* fertilization (Honaramooz *et al.*, 2008). The recent establishment of goat germline stem

cells from fetal testis and their ability to differentiate into germ cells is a step further towards the use of this alternative strategy for transgenic goat production. Their capacity to be genetically transformed *in vitro* prior to transplantation without alteration of their self-renewal and differentiation capabilities remains to be fully established (Hua *et al.*, 2011).

#### **iv) Nucleases**

The use of mega-nucleases, Zinc Finger Nuclease (ZFN) and TAL Effector Nucleases (TALEN) has recently emerged as a promising tool for targeted genetic manipulation of animals (Remy *et al.*, 2010). Successful gene inactivation or knock-in through direct cytoplasmic injection of ZFN-encoding RNA into fertilized eggs has been reported in rat (Geurts *et al.*, 2009; Mashimo *et al.*, 2010; Cui *et al.*, 2011) and mice (Carbery *et al.*, 2010; Cui *et al.*, 2011) and rabbit (Flisikowska *et al.*, 2011). However, none has yet been reported in larger mammals, including small ruminants. More precisely, targeting events could be obtained only in embryos that suffer from early developmental arrest. This observation could either suggest a toxic effect of the two used ZFN or a yet unexplained negative correlation between translation of the ZFN RNA and/or accessibility of the targeted DNA with the developmental capacity of the egg. The invalidation of the two targeted genes is not lethal in mice and although off-target cleavage of ZFN has been reported (Pattanayak *et al.*, 2011), the former hypothesis appears unlikely. The latter might be more relevant taking into account the known differences between ruminants and rodents in the timing of genome reactivation (Kopecny *et al.*, 1989a, b; Memili and First, 2000). Despite these difficulties, ZFN will probably be successfully used in goats to precisely manipulate their genome using *in vitro* manipulation of fetal fibroblasts or other cell types and NT as recently illustrated in pigs (Hauschild *et al.*, 2011) and cattle (Yu *et al.*, 2011). One potential current limitation is the absence of an available goat genome sequence. However, evolutionary closely related species have been sequenced, such as *Bos Taurus*, allowing precise amplification of the targeted locus using heterologous primers, a constantly increasing number of fragmented genomic goat sequences are available in the databases and the complete genome sequence should soon be released. Although less attractive than pigs in term of medical applications, such as animal models for human diseases and/or xeno-transplantations, and less advertised than cow or rabbits for the

production of pharmaceutical proteins in their milk, goat is currently a species with active research conducted for producing transgenic animals at lower cost and with precise genomic alterations. Promising new era involved isolation of germline stem cells and gene inactivation or replacement through the use of nucleases. Alongside large animals, goats might be the first species in which such combined transgenic approaches will soon become available.

#### **v) Nuclear transfer**

Transgenic goats producing malaria antigen were also obtained by nuclear transfer (NT) using fetal cell lines as donor cells (Behboodi *et al.*, 2005). Similarly, cloned embryos carrying human acid beta-glucosidase were produced (Zhang *et al.*, 2010). The overall efficiency of nuclear transfer in goat averages 2.6 % (total live kids/embryos transferred), making this technology an attractive alternative to microinjection. Most of the NT experiments use fetal fibroblastic cell lines as nuclei donor cells but other cell lines were also successfully tested such as skin cells (Behboodi *et al.*, 2004), cumulus cells (Lan *et al.*, 2006) or mammary gland epithelial cells. Fetal somatic cells derived from transgenic animals were similarly successfully employed to propagate transgenic lines (Baguisi *et al.*, 1999). Culture conditions favoring G0 stage of cell cycle for the donor cells, such as the use of serum-deprived medium, led to better outcomes (Zou *et al.*, 2002) whereas serial nuclear transplantation using 16- or 32-64-cell re-cloned embryos did not improve the overall developmental rate (Zhao *et al.*, 2007). The NT embryos could be produced at lower costs using oocytes derived from FSH-stimulated and from non stimulated abattoir-derived ovaries using various activating protocols (Reggio *et al.*, 2001; Lan *et al.*, 2006; Nasr-Esfahani *et al.*, 2011), without drastic differences in term of efficiency. NT approaches were also used to assess the role of genes or transcripts during embryonic development. Gene targeting in fetal fibroblast was achieved by homologous recombination at the  $\beta$ -casein and FoxL2 loci. However, the use of such genetically modified cells as donor cells for NT only resulted in pregnancies but not in the birth of live animals in these two experiments (Shen *et al.*, 2007). Successful gene inactivation by NT in goats has so far only been reported for the Prnp gene (Yu *et al.*, 2006; Zhu *et al.*, 2009). The production of Prnp-knockout goats, besides its intrinsic interest in potentially helping to assess the biological function of the PrP protein, could become a

public health issue as BSE was shown to infect goat under natural breeding conditions (Eloit *et al.*, 2005; Spiropoulos *et al.*, 2011). An alternative to gene inactivation by homologous recombination is gene knockdown by RNA interference. Lentiviral vectors were successfully used to deliver short-hairpin RNA expression cassettes targeting the prion protein mRNA in goat fibroblast cells that were subsequently used for nuclear transplantation. Brain tissue analyses of transgenic fetuses confirmed the knockdown of the targeted mRNA and of the encoded PrP protein, thus validating this approach as a potential alternative to gene knockout (Golding *et al.*, 2006). It remains that specific care should be taken using such procedures in goats to avoid potential trans-complementation with caprine arthritis encephalitis virus or CAEV (Perk, 1995).

### **2.16 Transgenic goat and human Lactoferrin**

Transgenic animals have been extensively used for producing human recombinant proteins in milk, by which it is possible to use transgenic cattle in the large-scale production of rhLTF (Van Berkel *et al.*, 2002). However, this transgenic system requires more time, and its inefficiency in large livestock makes it impossible for use in large scale of rhLTF production. Therefore, in an attempt to establish an efficient and economical way of producing rhLTF due to its nutritional and therapeutic applications, Han *et al.* (2007) directly infused replication-defective adenovirus encoding human lactoferrin cDNA into the mammary gland of goats via the teat canal and obtained a high level of expressed human lactoferrin up to 2 g/L in the milk of goats. The milk serum was collected from the infected mammary gland 48 h post-infection and subjected to a 10% SDS-PAGE and Western blotting. A ~80-kDa protein was visualized after viral vector infection. The rhLTF was purified and its expression in milk was determined. Furthermore, the amounts of hLTF detected in the milk were significantly higher than that of previous reported protein produced by other protein expression system (Mitra *et al.*, 1994; Legrand *et al.*, 1995, Salmon *et al.*, 1997, 1998; Liu *et al.*, 2005). These results showed that *in vivo* expression of heterologous genes is feasible by the direct intra-mammary infusion of adenoviral vectors, and that the expressed protein is secreted into milk of goats in the subsequent lactation.

Zhang *et al.* (2008) established transgenic goat model in order to produce human lactoferrin (hLf) in the mammary gland for large-scale application and research. Two transgenic male goats were generated by microinjecting expression cassette harboring hLf cDNA under the control of goat b-casein promoter with two insulators (to overcome the position effect) into the goat zygote. In the two lines, derived from the two founders, eight lactating female goats could secrete recombinant human lactoferrin (rhLf) at concentrations of up to 0.765 mg/ml as detected by ELISA. The method of purifying the rhLf from the milk was achieved using ion-exchange chromatography and resulted in 97% purity. Biochemical and physicochemical characteristics of rhLf were similar to native lactoferrin (nhLf); this included N-terminal sequence, isoelectric point, molecular mass, glycosylation, iron-binding/ releasing ability, thermal stability, and proteolysis. The rhLf showed broad spectrum antibacterial activity inhibiting the growth of several pathogenic bacterial strains. Their work proved the feasibility of the transgenic goat expressing rhLF.

In another study, Yu *et al.* (2012) reported dominant expression of recombinant human lactoferrin (rhLF) in transgenic cloned goats using a novel optimised construct made by fusing a 3.3 kb hLF minigene to the regulatory elements of the  $\beta$ -casein gene. The transgenic goat produced more than 30 mg/ml rhLF in its milk, and rhLF expression was stable during the entire lactation cycle. They analysed the rhLF that was isolated from goat milk and investigated its influence on tumour growth and metastasis in the mouse model and found that the rhLF demonstrated specific anti-tumour activity. They reported rhLF purification efficiency from whole goat milk was approximately 70%, and its purity is above 98%. Moreover, they found that Compared with natural hLF, the rhLF from transgenic goats has similar biological characteristics including molecular mass, N-terminal sequence, isoelectric point, immunoreactivity and digestive stability.

All these studies showed that the large-scale production of functional rhLF in transgenic goat milk could be an economical and promising source of human therapeutic use in the future. Therefore, in order to establish a basic platform for the transgenic technology to produce recombinant proteins through farm animals, we designed and performed the study as described in following chapters.

# CHAPTER – 3

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## Materials and Methods

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### 3. MATERIALS AND METHODS

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#### 3.1 MATERIALS

##### 3.1.1 Plasticware and Glassware

All the glassware used in the present study were made of high-grade pyrex glass. The glassware, wherever used, was thoroughly cleaned, rinsed with Ultrapure water and then heat sterilized at 250°C for 4 hrs. Pasteur pipettes were purchased from Labco, Ambala, India. Disposable 35 mm x 10 mm cell culture Petridishes, 15 ml and 50 ml Falcon tubes, 100 mm x 100 mm square petri dishes with 13 mm grid and capillary pipettes (10 µl, 20 µl capacity; Unopette®) were purchased from Becton, Dickinson and Co., Lincoln Park, NJ, USA or from Nunc, Roskilde, Denmark. Disposable plastic syringes, non-toxic and non-pyrogenic, were from Sigma-Aldrich, St. Louis, MO, USA. Disposable 18-gauge hypodermic needles were from Dispovan (Hindustan syringes and medical devices Ltd., India) whereas the 0.22 and 0.45 µm filters were from Millipore Corporation, MA, USA. Autoclavable disposable tips for micropipettes were obtained from ThermoFisher Scientific, MA, USA. French straws (0.25 ml) were from IMV, L'Aigle, France. Low profile 0.2 ml 8 tubes strips without cap and Optical flat 8-cap strips, for 0.2 ml PCR tubes used for real time PCR were purchased from Bio-Rad Laboratories, Hercules, CA, USA.

##### 3.1.2 Bacterial strains, plasmid vectors, cell lines and enzymes

*Escherichia coli* strain *XL-1 Blue MRF* (Stratagene, CA, USA) was used as transformation and propagation host for cloning of the goat promoters, 3' UTR of bovine growth hormone and human lactoferrin gene as well as the final gene constructs. The expression vectors pAcGFP1-1 and pAcGFP1-C1 were procured from Clontech, USA. Restriction enzymes used (Sall, MluI, SacII and XhoI) were purchased from ThermoFisher Scientific, MA, USA.

##### 3.1.3 Chemicals, Cell Culture Media and Supplements

The culture media such as tissue culture medium-199 (TCM-199), Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS) and the additives like bovine serum albumin (BSA), porcine follicle

stimulating hormone (pFSH) and antibiotics (Gentamicin sulfate, penicillin and streptomycin), mineral oil, enzymes (Hyaluronidase, Pronase, Trypsin-EDTA), phytohemagglutinin (PHA), were purchased from Sigma Chemical Co., MO, USA; unless otherwise stated. Most of the chemicals used in the present study were embryo culture tested or of cell culture grade. Fetal bovine serum (FBS) was from Hyclone (Logan, Utah, US). Transfection reagent for lipofection used, Fugene HD, was from Promega, Wisconsin, USA as was the Bright-Glo Luciferase Assay system. For nucleofection, P3 Primary Cell 4D-Nucleofector™ X Kit was procured from Lonza, Germany. InsTA clone PCR cloning kit was procured from ThermoFisher Scientific, MA, USA as were various components used for SDS-PAGE and western blotting like protein extraction reagents, Protease inhibitors, pre-stained protein ladder, CL-XPosure Film and chemiluminiscent substrate solutions. Immobilion PVDF membrane was purchased from EMD Millipore, MA, USA. Expression vectors pAcGFP1-1, pAcGFP1-C1 were procured from CloneTech, Takara Bio, USA. For cell characterization, the antibodies were purchased from Merck Millipore (Bioscience), Darmstadt, Germany and Santa Cruz Biotechnology Inc., Texas, USA.

Genomic DNA was extracted using Genomic DNA Purification kit (ThermoFisher Scientific, MA, USA). Total RNA extraction was performed using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) while cDNA was synthesized using SUPERSCRIPT III 1<sup>ST</sup> strand RT-PCR Kit from Invitrogen, CA, USA. DreamTaq DNA polymerase and Long PCR Enzyme Mix were used for RT-PCR which were procured from ThermoFisher Scientific, MA, USA. Purification of PCR products from agarose gel was performed by using GeneJET Gel Extraction Kit (ThermoFisher Scientific, MA, USA).

DNA ladders were also purchased from (ThermoFisher Scientific, MA, USA). Plasmid isolation was done using Plasmid Miniprep Kit (ThermoFisher Scientific, MA, USA). All gene constructs were sent for custom sequencing to Merck Specialities Pvt Ltd (Bangalore). ELISA for human lactoferrin was performed using Human Lactoferrin ELISA kit procured from AssayPro, MO, USA. Various primers (primers were designed using the internet-based software PRIMER-3 <http://www-genome.wi.mit.edu/cgi-bin/prime/primer3->

[www.cgi](http://www.cgi) or DNASTAR and analysed by OlidodT Analyzer), were purchased from Sigma-Aldrich, St. Louis, MO, USA.

### 3.1.4 Equipments

#### i) Microscopes

**a) Zoom stereomicroscope:** Low magnification zoom stereomicroscopes (NIKON, Japan, Model SMZ-2T) were used for searching the aspirated oocytes, evaluating their topography and quality, as well as cell cultures and tissue explants.

**b) Inverted microscope:** An inverted microscope (NIKON, Japan, Model TMD) was used for the examination of cell cultures, monitoring the cell health, morphological characteristics and growth of cultured cells. The microscope with the light source at top and a long working distance condenser allowed cells in culture dishes or flasks to be viewed and photographed whenever needed. The inverted microscope was also equipped with UV fluorescence and differential interface contrast (DIC) attachment, which helped in micromanipulation of the oocytes and capturing the images of the cell cultures and *in vitro* produced embryos. The microscopes were equipped with programmable still photography and video recording facilities.

#### ii) CO<sub>2</sub> incubators

CO<sub>2</sub> incubators from Thermo Forma Scientific (Marietta, Ohio, USA, Model 3131), with facility to maintain humidified environment, adjustable incubation temperature and CO<sub>2</sub> concentrations, were used for the culture of oocytes, embryos, somatic cells and embryonic stem cells.

#### iii) Biosafety and Laminar Flow Cabinet

Experiments including cleaning, processing and culturing of oocytes and somatic cells, *in vitro* produced embryos and cloning procedures were carried out in Class II Biosafety cabinet (ESCO, Portland, OR, USA) and Laminar flow cabinet (CLEANAIR Laminar Flow Systems, India), which served the purpose of minimizing the incidences of microbial contamination and ensuring the safety of the operator. UV irradiation and thorough cleaning of working places with ethanol (70% v/v) was used to maintain hygienic and sterile environment throughout the experiments.

**iv) Refrigerated Centrifuge**

Refrigerated centrifuge (Sigma, Model: 3K-30, Germany) with facilities to adjust centrifugation speed, time and temperature was used for centrifugation of the oocytes, embryos and various types of cells.

**v) Vortex Shaker**

Vortex shaker was used for the dissociation of cells after trypsinizing the tissue pieces for establishing primary cell cultures of various somatic cells. Rapid vortexing was also required for dissociating the cumulus cells for denuding the *in vitro* matured oocytes, etc.

**vi) Transfection equipment**

Amaxa Nucleofector (Lonza, Cologne, Germany) was used for generating pulse for nucleofection of different cell types.

**vii) Electrofusion Equipment**

Electrofusion machine (BTX ELECTRO CELL MANIPULATOR 200, San Diego, CA, USA), with facilities for manual as well as automatic controlled electrical inputs and outputs was used for generating the electrical pulses i.e. alternating current (AC) and direct current (DC) pulses for dielectrophoresis and membrane fusion, respectively. Electrofusion also served to activate the fused triplets through electro-activation.

**viii) Thermal cyclers**

Thermal cyclers used in the study were purchased from *Bio-Rad*, Hercules, CA, USA (1000 Touch Thermal Cycler; CT008917) and Applied Biosystems (Veriti, 96 Well TC).

**ix) Electrophoresis unit and gel documentation**

Agarose gel electrophoresis was performed for the resolution of PCR products. The electrophoresis unit from *BIO-RAD* Hercules, CA, USA included the buffer chamber, safety lid with cables, UV transparent tray, casting gates, comb set and power supply. For analyzing the PCR products and for capturing the images, gel documentation system (Molecular Imager, Gel Doc™ XR Imaging System, *Bio-Rad*, Hercules, CA, USA ) was used.

**x) Real Time PCR**

Real time thermal cycler (Bio-Rad, Model: CFX96 System, Hercules CA, USA) was used for quantification/ relative abundance of the genes in different types of cells like oocytes, embryos, ES cells and differentiated stem cells.

**xi) SDS-PAGE Unit and Semi dry transfer machine**

Polyacrylamide gel electrophoresis of the proteins was performed on Mini-PROTEAN Tetra cell unit from Bio-Rad (Hercules CA, USA) that runs both handcast and Precast gels. It includes a casting stand and glass plates with permanently bonded gel spacers that simplify hand casting and eliminate leaking. The cell can run one to four gels, and the mini tank is compatible with other Bio-Rad electrode modules for tank blotting, 2-D electrophoresis, and electroelution. The semi dry transfer apparatus (E5100) used for electroelution of the protein bands from PVDF membrane was also from (ThermoFisher Scientific, MA, USA).

**xii) Flow Cytometer**

Cell lab Quanta SC MPL (BR-10257A) used in the study was from Beckman Coulter, Inc. Fullerton, CA 92835. All the accessories and chemicals used for the machine operation like cleaning solution kits, sheath fluid, isodiluents, flowcheck fluorospheres, sample cups, filters and shutdown solution were also purchased from the same vendor.

The other equipments used in the study were; Analytical balance (Shimadzu Corp., Kyoto, Japan), Nano Quant reader (Model; Infinite M200, Tecan, Austria, GmbH), Deep Freeze (Vestfrost, India), Microwave oven (Kenstar, India), Micropipettes (Nichiryo, Japan & ThermoFisher Scientific, MA, USA), Water Purification System (Millipore, Massachusetts, USA), Dry Bath (Model-EL-01, Taiwan), Hot Air Oven (Labco, Ambala, India), and Digital pH meter (Eutech Instruments, Singapore) etc.

**3.1.5 Biologicals**

**3.1.5.1 Ovaries and oocytes**

The ovaries served as the source of immature oocytes during the present investigation. The goat ovaries were obtained from a nearby abattoir (New

Delhi) and were transported to the laboratory within 3-4 h in antibiotic (Gentamicin and PenStrep) fortified normal saline solution.

### **3.1.5.2 Tissues for Somatic Cell Cultures**

For establishment of fetal fibroblast cultures, ear biopsies from the goat fetus obtained from slaughter house were used. Goat mammary gland was also obtained from Delhi slaughterhouse to establish goat mammary epithelial cells.

## **3.2 METHODS**

### **3.2.1 Preparation of different media**

The detailed composition, preparation, handling and storage conditions of different media used in the study are dealt with in ANNEXURE.

### **3.2.2 Primer designing for RT-PCR and qPCR**

PCR primers were designed based on published nucleotide sequences when and where available or were based on sequence homology analysis (using DNASTAR and/ BioEdit software) between murine, human, porcine and bovine sequences obtained from NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and ENSEMBL genome browser, for the respective construct elements. Primers were designed and verified using the web-based software DNASTAR and Primer3 and analyzed in OligoAnalyzer for T<sub>m</sub>, hairpin, and homo- as well as hetero-dimer formation. qPCR primers were designed across exon-exon junctions in order to eliminate contaminating genomic DNA amplification.

### **3.2.3 Amplification of milk protein promoter regions**

#### **3.2.3.1 DNA extraction from goat uterine tissue**

Genomic DNA was isolated from goat uterine tissue obtained from slaughterhouse using Genomic DNA Purification kit (Thermo Fisher Scientific, MA, USA) as per the following manufacturer's instructions.

- 25-30 mg of mammalian tissue was pulverized in liquid nitrogen with mortar and pestle. Powder was placed in a 1.5 ml microcentrifuge tube and resuspended in 200µl of TE buffer.
- Mixed 200µl of sample with 400 µl of lysis solution and incubated at 65°C for 5 min.

- Immediately, added 600  $\mu$ l of chloroform, gently emulsified by inversion (3-5 times) and centrifuged the sample at 10,000 rpm for 2 min.
- Prepared precipitation solution by mixing 720  $\mu$ l of sterile deionized water with 80  $\mu$ l of supplied 10X concentrated Precipitation Solution.
- Transferred the upper aqueous phase containing DNA to a new tube and added 800  $\mu$ l of freshly prepared precipitation solution, mixed gently by several inversions at room temperature for 1-2 min and centrifuged at 10,000 rpm ( $\sim$ 9400 x g) for 2 min.
- Removed supernatant completely and dissolved DNA pellet in 100 $\mu$ l of NaCl solution by gentle vortexing.
- Added 300  $\mu$ l of cold ethanol, let the DNA precipitate (10 min at -20°C) and spinned down (10,000 rpm ( $\sim$ 9400 x g), 3-4 min).
- Removed the ethanol. Washed the pellet once with 70% cold ethanol and dissolved DNA in 100  $\mu$ l of sterile deionized water by gentle vortexing.

### **3.2.3.2 Qualitative and quantitative assessment of DNA**

Isolated DNA was analyzed by spectrophotometry. DNA concentration was determined by recording the absorbance at 260 nm (A<sub>260</sub>) using a Nanoquant spectrophotometer (Tecan, Austria, GmbH). The purity of the DNA was determined from the A<sub>260</sub>/A<sub>280</sub> ratio. The quality of the isolated DNA was also evaluated by (0.9% agarose) gel electrophoresis using 2  $\mu$ l of isolated DNA. The type of band pattern indicated the quality of the DNA isolated.

### **3.2.3.3 PCR amplification of $\beta$ -Casein promoter regions**

Two fragments of  $\beta$ -casein promoter region of sizes 3.5 kb and 6.5 kb were amplified from goat genomic DNA using specific primers designed from the sequence available at NCBI, GenBank (Table 3.1) using Long PCR Enzyme Mix (ThermoFisher Scientific, MA, USA) on a Mastercycler Gradient (Bio-Rad, USA). Gradient PCR was performed to optimize the annealing temperature. Among the various temperatures tried, the optimum amplification was achieved at the temperature as mentioned in Table 3.1,

and subsequent PCR was performed at those temperatures. PCR was carried out in a 25  $\mu$ l reaction containing 200ng of isolated DNA, 20 pm of each primer, 200  $\mu$ M of dNTPs, and 1.25 U of Enzyme Mix. The PCR was performed as follows: an initial denaturation cycle at 95°C for 3 min; followed by 35 cycles at 95°C for 30 seconds; Annealing Temperature (Table 3.1) for 30 sec; 68°C for 3.5 min or 7 min; with one final extension step at 68°C for 15 min. The PCR products were analyzed by 1% agarose gel electrophoresis along with DNA molecular weight marker.

**Table 3.1: Primers for amplification of  $\beta$ -casein promoter region**

Primer sequence	Product size (kb)	Annealing temp (°C)	Accession number
BC3.5F: TACGTGGACCATGAACTTC BC3.5R: TCCTAGAAATCTGCATGATTAG	3.5	55.7	AF409096.1
BC6.5F: TTTCACATTTATTATCTGATTACAAGCAGGA BC6.5R: AAGGATGAGGACCTTCAAGGCTCTC	6.5	55	

### 3.2.4 Agarose gel electrophoresis

#### 3.2.4.1 Preparation of agarose gel

For electrophoresis, 1% agarose gel was prepared and used. 40 ml of 1% agarose gel was prepared by placing 0.4 g agarose and 40 ml 1X TAE in 100 ml conical flask. The contents were heated to allow solubilization of agarose. It was allowed to cool slightly (~50°C) and ethidium bromide (0.5 $\mu$ g/ml) was added to it. The warm (~ 50°C) agarose solution (40 ml) was carefully poured in running tray, containing a comb, avoiding bubble formation to obtain gel thickness of about 3-5 mm.

#### 3.2.4.2 Preparation of samples and their loading

10  $\mu$ l of each sample was added to the 2  $\mu$ l of loading dye and mixed well. For different samples, separate tips were used for pipetting and mixing. All the samples were mixed with loading dye before loading of samples to the wells. Entire volume of samples was dispensed carefully to the wells. A 100 bp DNA

ladder (ThermoFisher Scientific, USA) was also run along with PCR products to confirm the desired product size.

#### **3.2.4.3 Electrophoresis run**

After loading the sample, the system was connected with an electric power supply unit (Bio-Rad, USA). Electrophoresis was carried out at constant voltage (100V) at room temperature. The progress of electrophoresis was judged by visualizing the migration of dyes present in loading buffer (Bromophenol blue dye appearing dark blue moves faster and Xylene Cyanol appearing light blue moves slower). After 20-30 min, electric supply was switched off. The visualization of DNA was done under UV trans-illuminator (Pharmacia, USA). Before placing gel on illuminator, the surface of illuminator was cleaned with distilled water and gel was placed carefully, not allowing entrapment of air bubbles between gel and surface. The gel was handled after wearing gloves and then photographed by Gel Doc imaging system (Bio-Rad, Hercules, CA, USA) for record.

#### **3.2.5 Gel extraction and purification**

- The PCR products were observed under UV transilluminator and excised immediately. PCR products were purified from the gel as per manufacturer's protocol using the GeneJET Gel Extraction Kit (ThermoFisher Scientific, MA, USA).
- Gel volume was minimized by cutting gel as close to DNA as possible. Gel slice was placed into a pre-weighed 1.5ml tube.
- 1:1 volume of Binding Buffer was added to the gel slice and incubated gel mixture at 50-60°C until the gel slice was completely dissolved.
- 800 µl of solubilized gel solution was transferred to the GeneJET purification column, centrifuged for 1 min and placed the column back into same collection tube after discarding the flow through.

- 700  $\mu$ l of Wash Buffer (diluted with ethanol) was added to the GeneJET purification column, centrifuged for 1 min and placed the column back into same collection tube after discarding the flow through.
- Empty GeneJET purification column was centrifuged for additional 1 min to completely remove the residual wash buffer.
- 30  $\mu$ l of Elution Buffer was added to the center of the purification column membrane after transferring GeneJET purification column into a clean 1.5 ml microcentrifuge tube and centrifuged for 1 min.

### **3.2.6 Cloning and sequencing of $\beta$ -casein promoter region**

#### **3.2.6.1 Preparation of *E. coli* XL1 competent cells by $\text{CaCl}_2$ treatment method**

- The frozen bacterial culture was activated by culturing on SOB agar plates (without antibiotics) at 37°C overnight. Then, well isolated colonies were transferred to 1 ml SOB with  $\text{MgSO}_4$  (20 mM).
- Colonies were dispensed by vortexing at moderate speed and then diluted the culture in 100 ml SOB with  $\text{MgSO}_4$  in 1 L flask. Culture was grown for about three hours until the O.D. at 600nm reached 0.3-0.4.
- The culture was transferred into 50 ml falcon tube and kept on ice for 10 min. Cells were recovered by centrifugation at 3000x g for 15 min at 4°C. Media was decanted from the pellet. Tube was inverted for 1 min to remove all traces of media.
- The pellet was resuspended by gentle vortexing in approximately 20 ml of ice cold 0.1M  $\text{CaCl}_2$ . The cells were allowed to cool on ice for 30 min followed by centrifugation at 3000x g at 4°C. All media was removed by inverting the tube for 1 min.
- The pellet was resuspended in 10 ml 0.1M  $\text{CaCl}_2$  containing 15% glycerol. The mixture was allowed to cool on ice for 60 min. Aliquots of 200  $\mu$ l cell suspension were made in microcentrifuge tubes and stored at - 80°C.

### 3.2.6.2 Ligation with cloning vector

The purified product was ligated into a TA cloning vector (InsTAclone PCR Cloning Kit, ThermoFisher Scientific, MA, USA) The InsTAclone PCR Cloning Kit takes advantage of the terminal transferase activity of Taq DNA polymerase and other non-proofreading thermostable DNA polymerases. Such enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favors direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Such overhangs at the vector cloning site not only facilitate cloning, but also prevent the recircularization of the vector. As a result, more than 90% of recombinant clones contain the vector with an insert. Recombinant clones are selected based on blue/white screening. TA cloning vector pTZ57R/T is ready to use for efficient ligation with PCR products providing high cloning yields and low background. The following reaction was set up for cloning.

Component	Quantity ( $\mu$ l)
5X Ligation buffer	6 $\mu$ l
PCR product (purified)	3 $\mu$ l
Vector pTZ57R/T (0.17 pmol ends)	3 $\mu$ l
water, nuclease-free	17 $\mu$ l
T4 DNA ligase	1 $\mu$ l
<b>Total volume</b>	<b>30 <math>\mu</math>l</b>

All components were mixed in one tube and vortexed briefly and centrifuged for 3-5 s. Then ligation mixture was incubated overnight at 4°C.

### 3.2.6.3 Bacterial transformation

- Competent cells and ligation product were kept in ice for 10 min. The ligation mixture (30  $\mu$ l) was added to 200  $\mu$ l of competent cells and kept in ice for 30 min.

- Heat shock was given at 42°C for 90 sec and immediately chilled on ice for 1min. 300µl of SOC medium was added to the mixture and incubated at 37°C for 1 hr in a shaker incubator.
- The transformed cells were spread on LB agar plates containing 100 µg/ml antibiotic ampicillin + 0.8 mg/ml X-Gal + 0.1mM IPTG solution.
- Plates were incubated overnight at 37°C and observed for growth of recombinant colonies. DNA was transformed into *E. coli XL1-Blue MRF'* host for propagation.

#### **3.2.6.4 Screening of recombinant vector by using colony PCR**

After overnight incubation blue and white bacterial colonies had developed on the agar plate. The white colonies, taken as recombinants, were selected for primary amplification by streaking them on fresh LB agar plate fortified with ampicillin. The plates were incubated overnight at 37°C. The colonies which developed were picked with an autoclaved toothpick, individually and swirled into 10 µl of nuclease free water in a microcentrifuge tube. The mixture was heated at 95°C for 3 min. The sample was spun at 10,000x g for 2 min. About 2 µl of the supernatant was taken as template in a 25 µl standard PCR reaction. The PCR was performed with primer set specific for β-casein promoter regions as per Table 3.1. PCR product was verified in 1% agarose gel along with 5 kb DNA ladder.

#### **3.2.6.5 Positive clone propagation and recombinant plasmid isolation**

2-3 positive clones were added into separate tubes containing ampicillin fortified LB broth (20ml) and incubated overnight in shaking incubator (37°C and 200 rpm) till the bacterial growth reached mid log phase. At this time, the bacteria were subjected to plasmid isolation in order to obtain the recombinant vector. Recombinant plasmids were isolated by GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, MA, USA) according to following protocol:

- The pellet, obtained by centrifuging the broth at 4000 rpm for 5min, was resuspended in 500 µl of chilled Resuspension solution (with RNase) by vortexing and transferred in 2 ml microcentrifuge tube.
- 500 µl of Lysis solution was added and mixed thoroughly by inverting the tube 4–6 times until the solution became viscous and slightly clear.
- 350 µl of Neutralization solution was added and mixed immediately by inverting tube 4-6 times. The lysate was centrifuged at 13,000 x g for 5 min at room temperature.
- The supernatant was transferred to fresh GeneJET spin column tube and centrifuged at 13,000x g for 1 min. Then flow-through was discarded.
- GeneJET spin column was washed by adding 500 µl Wash Solution (diluted with ethanol) and centrifuged at 13,000x g for 1 min and flow through was discarded. Then again centrifuged at 13,000 x g for 1 min to remove residual wash buffer.
- GeneJET spin column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Elution Buffer was added to center of each spin column membrane, incubated for 2 min at room temperature and centrifuged for 1 min.
- Plasmid concentration was measured using a Nanoquant spectrophotometer (Tecan Sales Austria GmbH) and stored at -20°C.

#### **3.2.6.6 Insert verification by Plasmid PCR**

PCR was carried out in a 25 µl reaction containing 100ng of isolated plasmid DNA, 10 pm of each primer, 200 µM of dNTPs, and 1U of *Taq* DNA polymerase. PCR conditions were - initial denaturation cycle at 95°C - 3 min; followed by 35 cycles at 95°C -30 seconds; Annealing temperature - 30 sec; 68°C -3.5 min or 7 min; with one final extension step at 68°C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis along with DNA molecular weight marker.

### 3.2.6.7 Plasmid sequencing and sequence analysis

After confirmation of the recombinant clones for insert sequence based on PCR amplification, clones were sent for custom sequencing to Merck Specialities Pvt Ltd (Bangalore). The sequence obtained was verified by BLAST and Pairwise alignment in DNASTAR. The sequence was annotated and submitted to GenBank, NCBI.

### 3.2.7 PCR amplification, cloning and sequencing of $\alpha$ -casein promoter regions

Two fragments of  $\alpha$ -casein promoter region of sizes 2.97 kb and 5.27 kb were amplified from goat genomic DNA using specific primers designed from the sequence available at NCBI, GenBank (Table 3.2) using Long Range Enzyme Mix, cloned into cloning vector, sequenced. The sequence obtained was verified by BLAST and Pairwise alignment in DNASTAR. The sequence was annotated and submitted to GenBank, NCBI. All the steps were carried out as described for  $\beta$ -Casein promoter regions above in sections 3.2.3.3 to 3.2.6.7.

**Table 3.2: Primers for amplification of  $\alpha$ -casein promoter region**

Primer sequence ( $\alpha$ -Casein)	Product size (kb)	Annealing temp ( $^{\circ}$ C)	Accession number
AC2.97F: CACAGCATACTGCCCATCACTT	2.97	59.5	AJ504710.2
AC2.97R: GTCTTTATCCTTTAGCCTGC			
AC5.2F: CTGTCATACA ACTGTGAATACAC	5.27	57	
AC5.2R: TCATTGAGGACTTCCTGTAG			

### 3.2.8 PCR amplification, cloning and sequencing of $\beta$ -lactoglobulin promoter regions

Two fragments of  $\beta$ -lactoglobulin promoter region of sizes 2.7 kb and 2.1 kb were amplified from goat genomic DNA using specific primers designed from the sequence available at NCBI, GenBank (Table 3.3) using Long Range Enzyme Mix, cloned into cloning vector, sequenced. The sequence obtained was verified by BLAST and Pairwise alignment in DNASTAR. The sequence was annotated and submitted to GenBank, NCBI. All the steps

were carried out as described for  $\beta$ -Casein promoter regions above in sections 3.2.3.3 to 3.2.6.7.

**Table 3.3: Primers for amplification of BLG promoter region**

Primer sequence (BLG)	Product size (kb)	Annealing temp (°C)	Accession number
BLG2.7F: GAAGGGCATGGTTTATGGTCA	2.7	56.9	Z33881.1
BLG2.7R: ACACGTGGCAACATTTGGATCTT			
BLG2.1F: GCCAGAGGTGCTTTATTTTC	2.1	60	
BLG2.1R: GTCGTGCTTCTGAGCTCTG			

### 3.2.9 Human Lactoferrin gene Amplification, Cloning and Sequencing

#### 3.2.9.1 RNA extraction from human blood

Human blood was collected in a sterile vacutainer coated with EDTA. 3 ml of blood was then layered on 3 ml of Histopaque 1077 (Sigma) and centrifuged at 400g for 30 mins. Buffy coat was then transferred to microcentrifuge tubes and washed with DPBS 2-3 times. Total RNA was extracted using TRIZOL as follows:

- 1 ml of TRI Reagent was added to the buffy coat. The tube was vortexed for 5 min to lyse the cells. The tube was kept at room temperature for 5 min.
- 0.2 ml of chloroform per ml of TRI reagent was added and shaken vigorously for 15 seconds and then kept at room temperature for 5 min, followed by centrifugation at 12000 x g for 15 min at 4°C.
- The upper colorless aqueous phase containing RNA was transferred carefully to fresh microcentrifuge tube.
- The total RNA was precipitated by adding isopropanol (0.5 ml/1.0 ml of TRI reagent).

- The resulting mixture was kept at room temperature for 10 min and centrifuged at 12000 x g for 15 min at 4°C.
- The supernatant was discarded and RNA pellet obtained was washed with 1.0 ml of 75 percent ethanol per ml of TRI reagent and then centrifuged at 7500xg for 5 min at 4°C. Finally, the RNA pellet was briefly air-dried and resuspended in appropriate volume of DEPC water.
- The resultant RNA was further treated with DNase to rule out the possibility of genomic DNA contamination. For this 1µl of DNase along with 1µl of its buffer was added and incubated at 37°C for 30 min. Added 1µl of EDTA and incubated at 65°C for 20 min. Then prepared RNA was subjected for its qualitative and quantitative analysis or was stored at -80°C till further use.

### **3.2.9.2 RNA quantitation and purity**

Total RNA was quantified by taking 2 µl RNA stock and absorbance was measured at 260nm and 280nm with a UV spectrophotometer (NanoQuant TECAN, Switzerland). Total RNA content of sample was determined using the following equation:

$$\text{Total RNA} = \{(A_{260} \times 40 \times 200)/1000\} \mu\text{g}/\mu\text{l}$$

In this equation,  $A_{260}$  is the absorbance value at 260 nm wavelength, 40 is constant for RNA quantification and 200 is the dilution factor. The purity of RNA preparation was determined by finding the  $A_{260}/A_{280}$  ratio which was found to be in the range of 1.8- 2.0.

### **3.2.9.3 Preparation of complementary DNA (cDNA)**

The 'Superscript III first strand synthesis Kit' (Invitrogen, USA) was used for cDNA synthesis from the RNA isolated from human blood buffy coat following the manufacturer's instructions. The reverse transcriptase (RT) reaction was set on ice. A negative RT control reaction was always set to nullify the genomic DNA presence in the prepared RNA sample. In the negative RT

control all components were same as that of normal RT reaction except that of Superscript III RT. After reverse transcription, PCR was done for the amplification of desired gene cDNA in which content of minus RT reaction product was used as template (RT –ve control) and also for all PCR reactions a PCR –ve control was used as quality control. The RT reaction is as follows: 10 µl of RT reaction was prepared by adding following components and named as tube-I

Components	Quantity (µl)
RNA	2.00
Oligo (dT)	1.00
10 mM dNTPs	1.00
DEPC- treated water	6.00
<b>Total Volume</b>	<b>10.00</b>

Kept the mixture at 65°C for 5 min then brought out on ice and prepared another cocktail and named as tube-II as per the following composition.

Components	Quantity (µl)
10x RT buffer	2.00
25 mM MgCl <sub>2</sub>	4.00
0.1M DTT	2.00
RNaseOUT	1.00
Superscript III RT	1.00
<b>Total Volume</b>	<b>10.00</b>

The contents of both the tubes-I and tube-II were mixed.

**Cycling conditions:** The reaction tube was kept in a thermal cycler programmed at 50°C for 50 min and at 85°C for 5 min followed by adding 1µl of RNase H and incubated at 37°C for 20 min. The prepared cDNA was stored at -20°C till the further use.

### 3.2.9.4 PCR amplification of human Lactoferrin gene

Full length human Lactoferrin gene, measuring 2259 bps was amplified using gene specific primers designed using DNASTAR and Primer3 and analyzed for various parameters in OligoAnalyzer, from the sequence available at NCBI (GenBank Accession No. AY165046.1) (Table 3.4). The optimized annealing temperature was achieved via gradient PCR and 60°C was chosen to be the optimal annealing temperature. hLF gene was amplified using Long PCR Enzyme mix (ThermoFisher Scientific, MA, USA) on a Mastercycler Gradient (Bio-Rad, USA). PCR was carried out in a 25 µl reaction containing 100ng of isolated cDNA, 20 pm of each primer, 200 µM of dNTPs, and 1.25 U of Enzyme Mix. The PCR was performed as follows: an initial denaturation cycle at 95°C for 3 min; followed by 35 cycles at 95°C for 30 seconds; 60°C for 30 sec; 68°C for 150 seconds; with one final extension step at 68°C for 15 min. The PCR products were analyzed by 1% agarose gel electrophoresis along with DNA molecular weight marker.

**Table 3.4: Primers used for amplification of human Lactoferrin gene**

Primer sequence	Product size	Accession no.
LF1F: CAGACCGCAGACATGAAACT		
LF1R: GCAATCCCCACCTTCAGCAG	2259	AY165046.1

The amplified product was cloned into cloning vector and sequenced. The sequence obtained was verified by BLAST and Pairwise alignment in DNASTAR. The sequence was annotated and submitted to GenBank, NCBI. All the steps were carried out as described for  $\beta$ -Casein promoter regions above in sections 3.2.3.3 to 3.2.6.7.

### 3.2.10 Amplification, Cloning and Sequencing of 3' UTR of bovine growth hormone

3' UTR of bovine growth hormone gene of size amplified from goat genomic DNA using specific primers designed from the sequence available at NCBI, GenBank (Table 3.5) using Long PCR Enzyme Mix, cloned into cloning vector, sequenced. The sequence obtained was verified by BLAST and

Pairwise alignment in DNASTAR. The sequence was annotated and submitted to GenBank, NCBI. All the steps were carried out as described for  $\beta$ -Casein promoter regions above in sections 3.2.3.3 to 3.2.6.7.

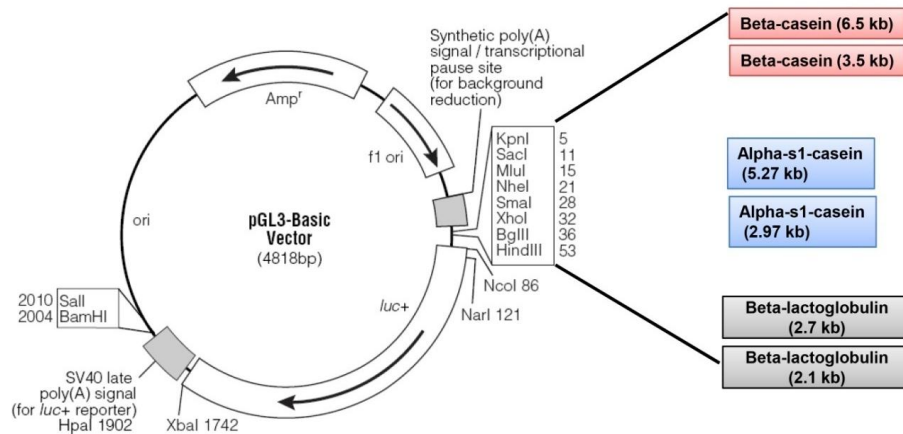
**Table 3.5: Primers used for amplification of 3' UTR of bovine growth hormone gene**

Primer sequence	Annealing Temp (°C)	Product size	Accession no.
BGHF- CTCGACTGTGCCTTCTA	58	250 bp	AJ000484.1
BGHR- TCTTTCCGCCTCAGAA			

### 3.2.11 Luciferase Assay

The luciferase reporter assay is commonly used as a tool to study gene expression at the transcriptional level. It is widely used because it is convenient, relatively inexpensive, and gives quantitative measurements instantaneously. We performed Luciferase Assay in order to find the promoter region with the highest activity. Each of the promoter fragment (2 each of  $\beta$ -casein,  $\alpha$ -casein and  $\beta$ -lactoglobulin) was amplified using the primers with the restriction enzymes Sall and XhoI and each of the product was gel purified. The vector as well as were digested with restriction enzymes as per the manufacturer's protocol. The pGL3 basic expression vector (Promega, USA) and each of the purified product of the promoters were digested with Fast Digest MluI and XhoI (ThermoFisher Scientific, MA, USA) restriction enzyme by incubating at 37°C for 5 min. Digested products were purified by gel extraction purification method using GeneJET gel extraction kit (ThermoFisher Scientific, MA, USA). Purified vector and promoter fragment was ligated together by T4 DNA ligase enzyme (ThermoFisher Scientific, MA, USA) by incubating at 4°C for 16 hrs. Ligated product was cloned into competent cells and recombinant plasmids were isolated by GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, MA, USA). After the quantification of the concentration of plasmids, equal copy number of each of the plasmids was transfected into goat mammary epithelial cells in a 24-well plate in duplicates at 70-80% confluency using Fugene HD (Promega, USA). After 48 hrs of

transfection, reconstituted Bright-Glo substrate (Promega, USA) with Bright-Glo buffer (Promega, USA) and added volume of reagent equal to that of the culture medium in each well, and mixed. Waited for 5 mins to allow complete cell lysis and measured in a Luminometer.



**Schematic representation of Luciferase Assay gene constructs where each of the 5'UTR was cloned into MCS of pGL3 basic vector.**

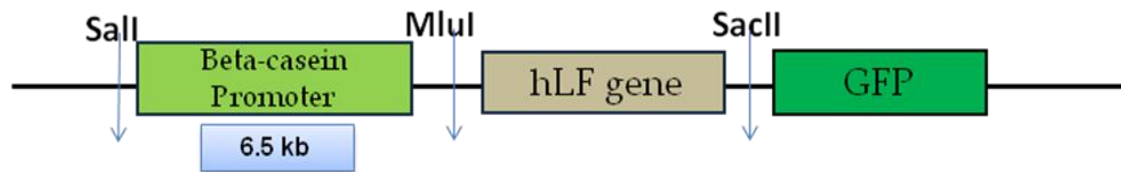
### 3.2.12 Preparation of gene constructs for expression of human lactoferrin gene

The promoterless vector pAcGFP1-1 was used as a backbone for preparation of gene constructs. The restriction sites at the end of each fragment were chosen in such a way that each restriction enzyme would cut the final transgene construct only once and restriction site for each enzyme was absent in any of the fragments. Each of the element of transgene construct was amplified using specific primers containing appropriate restriction site. Three mammary gland specific constructs were prepared for expression of human lactoferrin in milk of transgenic goat: pBCL1, pBCL2 and p $\beta$ ChLF.

#### 3.2.12.1 Construction of pBCL1 expression vector

Promoterless pAcGFP1-1 expression vector was digested by incubating with conventional Sall and SacII for 2 hrs at 37°C.  $\beta$ -casein 6.5 kb fragment was

digested with Sall and Mlul restriction enzyme by incubating at 37°C for 5 min. Human lactoferrin was digested with Mlul and SacII for 2 hrs at 37°C. All three purified fragments were ligated together in the ratio 1:1:1 by T4 DNA ligase enzyme by incubating at 4°C for 16 hrs. Ligated product was cloned into competent cells and recombinant plasmid pBCL1 was isolated using GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, MA, USA).



The final construct so obtained was verified by Double restriction digestion carried out by digesting with Sall and SacII for 2 hrs at 37°C and running the digested product on 1% agarose gel. The construct was also subjected to Triple restriction digestion carried out by digesting with conventional restriction enzymes Sall, Mlul and SacII for 2 hrs at 37°C and running the digested product on 1% agarose gel.

### 3.2.12.2 Construction of pBCL2 expression vector

Promoterless pAcGFP1-1 expression vector was digested by incubating with conventional Sall and SacII for 2 hrs at 37°C.  $\beta$ -casein 6.5 kb fragment was digested with Sall and Mlul restriction enzyme by incubating at 37°C for 5 min. Human lactoferrin was digested with Mlul and SacII for 2 hrs at 37°C. All three purified fragments were ligated together in the ratio 1:1:1 by T4 DNA ligase enzyme by incubating at 4°C for 16 hrs. Ligated product was cloned into competent cells and recombinant plasmid pBCL2 was isolated using GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, MA, USA).

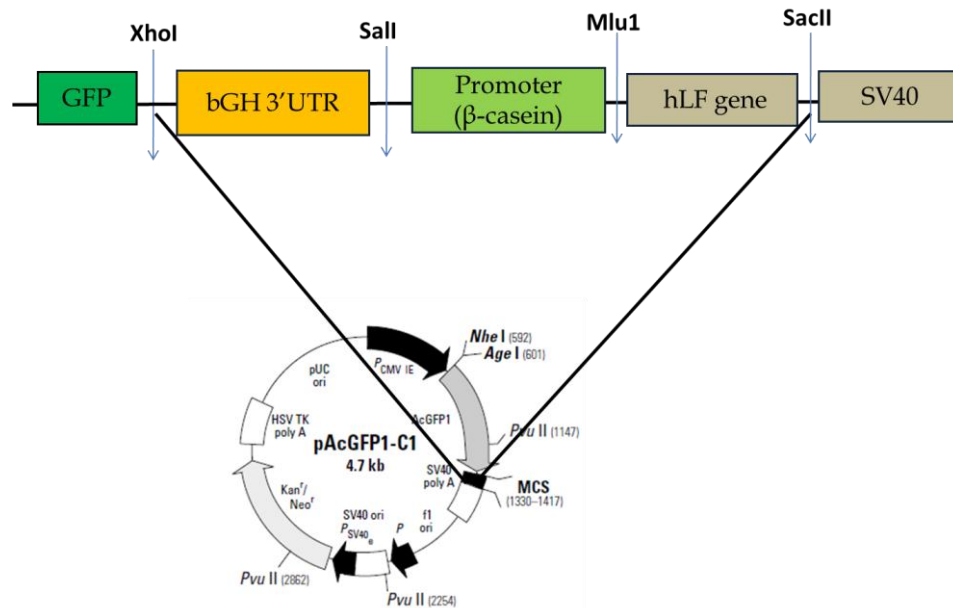


The final construct so obtained was verified by Double restriction digestion carried out by digesting with Sall and SacII for 2 hrs at 37°C and running the digested product on 1% agarose gel. The construct was also subjected to Triple restriction digestion carried out by digesting with conventional restriction enzymes Sall, MluI and SacII for 2 hrs at 37°C and running the digested product on 1% agarose gel.

### **3.2.12.3 Construction of p $\beta$ ChLF expression vector**

Both the above constructs would express GFP only in mammary epithelial cells and not in other somatic cell types. Therefore, the construct was redesigned as follows:

- Because the 5' UTR was to be ligated in frame with the GFP and contained exon1, therefore, to eliminate the chances of it getting transcribed and translated, 3' UTR of bGH was ligated before as shown above. bGH 3'UTR and plasmid vector pAcGFP1-C1 were digested using XhoI and Sall for 5 min at 37°C. Digested product was purified by gel extraction purification method (ThermoFisher Scientific, MA, USA). Purified vector and bGH 3'UTR were ligated together by T4 DNA ligase enzyme by incubating at 4°C for 2 hrs. Ligated product was cloned into competent cells and recombinant plasmid bGH3'UTR +pAcGFP1-C1 was isolated by GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, MA, USA).
- Due to size constraint, we took 3.5 kb  $\beta$ -casein promoter.  $\beta$ -casein promoter + human lactoferrin was released from pBCL2 expression vector using Sall and SacII by incubating at 37°C for 2 hrs. and ligated using T4 DNA ligase into the plasmid bGH3'UTR +pAcGFP1-C1 which was also digested with Sall and SacII restriction enzymes. Ligated product was cloned into competent cells and recombinant plasmid p $\beta$ ChLF was isolated by GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, MA, USA).



### 3.2.12.4 Cloning and sequencing of final gene construct

All gene constructs were sent for custom sequencing to Merck Specialities Pvt Ltd (Bangalore). Inserts were sequenced from both the ends. Afterwards the sequenced DNA was recognized by alignment with available sequence using CLUSTAL W software.

### 3.2.13 Establishment of goat mammary epithelial cells (GMEC)

#### 3.2.13.1 Collection of mammary tissue

Mammary tissue was obtained at Delhi slaughter house from a lactating goat. Immediately after slaughter, the tissue was transported to the laboratory in DPBS containing 50 IU/ml penicillin, and 50 µg/ml streptomycin.

#### 3.2.13.2 Primary culture of goat mammary epithelial cells (GMEC)

Two strategies will be followed for isolation of mammary epithelial cells: Digestion method and explant culture.

**Digestion method:** Goat's mammary parenchymal tissue was finely minced with scissors and digested for 4-6 h in DMEM/F12 (1:1) supplemented with collagenase (0.4%), Hyaluronidase (1%) and DNase (0.03%) in a shaker

incubator at 37° C at 200 rpm. The tissue suspension was then passed through a 80-µm- pore size filter. Cell suspension was centrifuged at 1,000 rpm for 10 min to obtain pellet containing mammary epithelial cells. The pellet was resuspended in Ca<sup>2+</sup> and Mg<sup>2+</sup>-free DPBS and washed for 3-5 times. Finally the cells were resuspended in DMEM/F12 supplemented with 10% FBS and growth factors including EGF (10ng/ml), Insulin (10µg/ml), Hydrocortisone (1µg/ml ) and Prolactin (4µg/ml) and seeded in T25 and cultured at 37°C in a 5% CO<sub>2</sub> incubator.

***Explant culture:*** The internal mammary tissue of goat was cut into 1 mm<sup>3</sup> and placed in T25 flasks. After the explants got tightly attached, 3 ml of DMEM/F12 supplemented with 10% FBS and growth factors including EGF (10ng/ml), Insulin (10µg/ml), Hydrocortisone (1µg/ml ) and Prolactin (4µg/ml) was added and cultured at 37° C, 5% CO<sub>2</sub> in air.

### **3.2.13.3 Enrichment of epithelial cells**

Whereas digestion method resulted in a relatively pure epithelial-like cell population, approximately 10% of the cells showed fibroblastic morphology. To isolate to homogeneity, we enriched the epithelial population by selective detachment and selective attachment method. We carried out selective detachment by adding 500 µl 0.25% trypsin-EDTA (GIBCO) to GMECs. The dish was incubated at 37° C for 2.5, 5, and 10 min. At each time point, the supernatant was removed and fresh trypsin-EDTA was added. After 2-3 min trypsinization, fibroblast cells got detached and were removed by washing with DPBS. 5 and 10 min trypsinized cells were washed with DPBS and the cell pellet was resuspended in medium and plated in 35-mm dishes. For selective attachment, the cells were transferred from one flask into another alongwith supernatant at interval of 2h and 4 h due to the fact that fibroblast cells get attached to substrate within 3-4 h of subculture while mammary epithelial cells take longer time.

### **3.2.14 Characterization of goat mammary epithelial cells (GMEC)**

#### **3.2.14.1 Immunofluorescence staining**

The GMEC cultures, so obtained were characterized for various epithelial cell-specific (cytokeratin 8, cytokeratin 18, E-cadherin and  $\beta$ -Tubulin) as well as non-specific markers (Vimentin, smooth muscle  $\alpha$ -actin) by immunocytochemistry. Briefly, epithelial cells at passage 2 were seeded into 24-well plate at 60-70% seeding density. After 16h of incubation at 37°C in 5% CO<sub>2</sub> incubator, the growth media was removed from the wells, followed by two washes with DPBS. The cells were fixed in 4% paraformaldehyde for 30min at room temperature, washed twice with DPBS and treated with 0.05% Triton-X-100 in DPBS for 15 min. This was followed by two DPBS washes and incubation with blocking buffer (5% BSA + 0.01% Triton-X-100 in DPBS) for 1h at room temperature. After this primary antibodies against cytokeratin 8, cytokeratin 18, E-cadherin,  $\beta$ -Tubulin, Vimentin and Smooth muscle  $\alpha$ -actin (all from Santa Cruz Biotechnology Inc. Texas, USA) diluted 1:300 in blocking buffer were added to the wells, followed by overnight incubation at 4°C. After three washes with DPBS, appropriate PE/ FITC-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Texas, USA), diluted 1:500 in DPBS, were added. This was followed by 1h incubation in dark and three more washes with DPBS. Hoechst 33342 nuclear stain (10 $\mu$ g/ ml) was finally added, followed by incubation in dark for 10-15 min. The cells were then washed thrice in DPBS and visualized under green/ blue filter of fluorescence microscope for immunodetection of the marker proteins.

#### **3.2.14.2 Reverse transcription PCR (RT- PCR)**

##### **3.2.14.2.1 RNA extraction from GMECs:**

Cells at Passage 2 were washed twice with DPBS and trypsinised for 5 min. Culture media was added and cell suspension was centrifuged at 1000 rpm for 5 min. Cell pellet was washed with DPBS 2-3 times. Total RNA was extracted using TRIZOL as follows:

- 1 ml of TRI Reagent was added to the cell pellet. The tube was vortexed for 5 min to lyse the cells. The tube was kept at room temperature for 5 min.

- 0.2 ml of chloroform per ml of TRI reagent was added and shaken vigorously for 15 seconds and then kept at room temperature for 5 min, followed by centrifugation at 12000 x g for 15 min at 4°C.
- The upper colorless aqueous phase containing RNA was transferred carefully to fresh microcentrifuge tube.
- The total RNA was precipitated by adding isopropanol (0.5 ml/1.0 ml of TRI reagent).
- The resulting mixture was kept at room temperature for 10 min and centrifuged at 12000 x g for 15 min at 4°C.
- The supernatant was discarded and RNA pellet obtained was washed with 1.0 ml of 75 percent ethanol per ml of TRI reagent and then centrifuged at 7500xg for 5 min at 4°C. Finally, the RNA pellet was briefly air-dried and resuspended in appropriate volume of DEPC water.
- The resultant RNA was further treated with DNase to rule out the possibility of genomic DNA contamination. For this 1µl of DNase along with 1µl of its buffer was added and incubated at 37°C for 30 min. Added 1µl of EDTA and incubated at 65°C for 20 min. Then prepared RNA was subjected for its qualitative and quantitative analysis or was stored at -80°C till further

#### **3.2.14.2.2 RNA quantitation and purity:**

Total RNA was quantified by taking 2 µl RNA stock and absorbance was measured at 260nm and 280nm with a UV spectrophotometer (NanoQuant TECAN, Switzerland). Total RNA content of sample was determined using the following equation:

$$\text{Total RNA} = \{(A_{260} \times 40 \times 200)/1000\} \mu\text{g}/\mu\text{l}$$

In this equation,  $A_{260}$  is the absorbance value at 260 nm wavelength, 40 is constant for RNA quantification and 200 is the dilution factor. The purity of RNA preparation was determined by finding the  $A_{260}/A_{280}$  ratio which was found to be in the range of 1.8- 2.0.

### 3.2.14.2.3 Preparation of complementary DNA (cDNA)

The ‘Superscript III first strand synthesis Kit’ (Invitrogen, USA) was used for cDNA synthesis from the RNA isolated from goat mammary epithelial cells following the manufacturer’s instructions. The reverse transcriptase (RT) reaction was set on ice. A negative RT control reaction was always set to nullify the genomic DNA presence in the prepared RNA sample. In the negative RT control all components were same as that of normal RT reaction except that of Superscript III RT. After reverse transcription, PCR was done for the amplification of desired gene cDNA in which content of minus RT reaction product was used as template (RT –ve control) and also for all PCR reactions a PCR –ve control was used as quality control. The RT reaction is as follows:

10 µl of RT reaction was prepared by adding following components and named as tube-I

Components	Quantity (µl)
RNA	2.00
Oligo (dT)	1.00
10 mM dNTPs	1.00
DEPC- treated water	6.00
<b>Total Volume</b>	<b>10.00</b>

Kept the mixture at 65°C for 5 min then brought out on ice and prepared another cocktail and named as tube-II as per the following composition.

Components	Quantity (µl)
10x RT buffer	2.00
25 mM MgCl <sub>2</sub>	4.00
0.1M DTT	2.00
RNaseOUT	1.00
Superscript III RT	1.00
<b>Total Volume</b>	<b>10.00</b>

The contents of both the tubes-I and tube-II were mixed.

**Cycling conditions:** The reaction tube was kept in a thermal cycler programmed at 50°C for 50 min and at 85°C for 5 min followed by adding 1µl of RNase H and incubated at 37°C for 20 min. The prepared cDNA was stored at -20°C till the further use.

#### **3.2.14.2.4 PCR amplification of mammary epithelial cells specific gene**

PCR primers were designed based on published nucleotide sequences when and where available or were based on sequence homology analysis (using BioEdit software) between murine, human, porcine and bovine sequences obtained from NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and ENSEMBL genome browser, for the respective genes. Primers were designed and verified using the web-based software PRIMER-3/Perl primer. Mammary epithelial cells specific primers are shown in Table 3.6 respectively.

PCR reactions were set up in a final volume of 25 µl having 10X PCR buffer, 10 mM dNTPs, 1.0 U of Dream Taq DNA polymerase and 10µM each of forward and reverse primers. The PCR cycling conditions for various genes were 94°C for 3 min, followed by a cycling program of 94°C for 30 sec, primer specific annealing temperature (mentioned in Table 3.1) for 30 sec and 72°C for 30 sec for 33 cycles. The reactions were ended with a final extension at 72°C for 10 min. RT negative and PCR negative controls were set for all the PCR reactions to negate the DNA contamination in cDNA templates and for ascertaining the authenticity of PCR amplifications. PCR products were visualized on 2% agarose gel in 1X TAE buffer containing 0.5 µg/ml of ethidium bromide and migrated at 100 V for 20 min.

**Table 3.6: Primers for mammary epithelial cells characterization**

Gene	Sequence	Annealing temp (°C)	Base pairs	Accession No.
<i>β-CASEIN</i>	F- CCAGGATAAAATCCACCCCT R- AGGAAGGTGCAGCTTTTCAA	60	274	DQ317447.1
<i>BLG</i>	F- CTTGTGCTGGACACCGACTA R- CACACAGCTGGTCTCCCTG	60	132	DQ785797.1
<i>BTN1A1</i>	F-TGTGTTGCTGCTGATAGAGTGTTAG R-CCTCCAAGTTCCTTTATGGGATTTTC	52	305	Z93323
<i>ACACA</i>	F-GGGAAGGAAGAAGGACTTGG R- TTTCGACCTGGATGGTTCTC	60	169	NM_174224.2
<i>CK18</i>	F-CAGAGTCAAGTATGAGACAGAG R-TGTAGACCCTTTACTTCTCC	60	182	NM_001192095.1
<i>CK 8</i>	F- CTTCAAGACCAAGTATGAGGA R - TGCCTGTAGAAGTTGATCTC	60	133	NM_001033610.1
<i>GAPDH</i>	F-ATCACCCCTGCTTCTACTGG R-AGGAGACAACCTGGTCCTCA	60	235	GU324291.1

### 3.2.15 Karyotyping and sex determination of goat mammary epithelial cells

Karyotyping analysis was performed on goat mammary epithelial cells. The cells were subjected to chromosomal analysis according to the method cited by Dyban (1983) with slight modifications. Briefly, the cells were incubated in DMEM supplemented with 0.1 mg/ml colcemid for 4 hr at 38.5°C. The cells were then washed, trypsinized and resuspended in hypotonic solution (0.9% sodium citrate) for 4hrs at 37°C. They were washed and then fixed in chilled fixative (3:1 methanol/glacial acetic acid) for 30 min at room temperature and centrifuged at 200g for 8 min. The pellets were resuspended in 5 ml of ice-chilled fixative for another 10 min and then centrifuged again. The metaphase spreads were prepared by dropping the cells onto ice cold glass slides. Chromosomes were stained with 2% Giemsa for 5-6 min and observed under oil immersion (1,000x) using a compound microscope (Nikon, Japan).

The sex of the mammary epithelial cells was confirmed by PCR amplification of *SRY* and *PLP* genes using genomic DNA isolated from the respective gonads as the template. Genomic DNA isolation was performed using Genomic DNA Purification kit (ThermoFisher Scientific, MA, USA) as described in section 3.2.3.1, following the manufacturers' protocol. The extracted DNA was used as the template for thermocycler reactions, set up in a final volume of 25  $\mu$ l having 10X PCR buffer, 10 mM dNTPs, 1.0 U of DreamTaq DNA polymerase, 10 pmol/ $\mu$ l each of forward and reverse primers (Table 3.7) and 2 $\mu$ l (500 ng) of the genomic DNA. The thermal cycling conditions were as follows: 94°C for 3 min, followed by a cycling program of 94°C for 1 min, annealing temperature of 58°C for 30 s and 72°C for 30 s, repeated for 36 cycles and followed by final extension at 72°C for 10 min. The primer sequences, annealing temperatures and the amplified product lengths are given in Table 3.10. The PCR products were visualized on 2% agarose gel in 1X TAE buffer containing 0.5  $\mu$ g/ml of ethidium bromide, after electrophoresis at 100 V for 20 min.

**Table 3.7: Primers for sex determination of mammary epithelial cells**

Gene	Sequence	Annealing temp (°C)	Base pairs	Accession No.
<i>SRY</i>	F-GAAGAAAGCAGAGCCTTAATATCC R-GAACATAGCAGAAGCATAACGAC	58	127	AF148462.1
<i>PLP</i>	F-AGAGTACCTTTCCCTTTCCCTTCC R-CTTCCTTCCTTAGCCCAGTC	58	97	AJ009913.1

### 3.2.16 Growth of goat mammary epithelial cells on different matrices

Three attachment factors i.e., gelatin, collagen matrix and MaxGel™ECM were compared for the culture of goat mammary epithelial cells. Collagen matrix and MaxGel™ ECM were prepared as per the manufacturer's (Sigma) protocol. Briefly, a mixture of 25  $\mu$ l rat tail collagen Type-I/ml of DMEM or 10  $\mu$ l of MaxGel ECM/ml of DMEM or 0.1% gelatin was pipetted out in 24-well plates (400  $\mu$ l/well), which were incubated for 2 h at 37°C in a CO<sub>2</sub> incubator. The medium was removed after 2 h, and fresh culture medium was added before

culture of goat mammary epithelial cells at 4000 cells/well. To compare the efficacy of different attachment factors, the cells were cultured in 24-well dish coated with gelatin or collagen or MaxGel ECM or with no attachment factor (controls) for 216 h. The cells were harvested every 24 h for determination of cell number using hemocytometer.

### **3.2.17 Determination of relative cell viability of goat mammary epithelial cells on different matrices**

For determining the metabolic activity by MTT assay, the cells grown on different matrices at density of ~5000cells per well of a 96-well plate were incubated with 5 mg/ml MTT at 37°C for 2 h after which DMSO (diluted 1:1 with the culture medium) was added and the reactants were mixed until the formazan crystals were completely dissolved. The optical density of dissolved formazan was measured at 570 nm using a Nanoquant infinite M200 ELISA reader. All the analyses were performed in at least three independent replicate cultures. Absorbance ratio of coated to non-coated control cells was calculated and presented as relative cell viability.

### **3.2.18 Optimization of the transfection of goat mammary epithelial cells**

pβChLF expression vector was transfected into GMECs at passage 3 by lipofection using FuGENE® HD Transfection reagent (Promega, USA) according to the manufacturer's recommendation. Briefly, one day before transfection, cells were seeded into 24 well plate  $0.5-2 \times 10^5$  cells in 500 µl of growth medium without antibiotics so that cells are 80-90% confluent at the time of transfection. Plasmid DNA and FuGENE HD Transfection reagent were diluted in 129 µl Opti-MEM® I Reduced Serum Medium without serum, mixed gently and incubated for 15 min at room temperature. Total 125 µl of each complexes was added to each respective well containing cells and medium and mixed gently by rocking the plate back and forth. The cells were then incubated at 37°C in a CO<sub>2</sub> incubator for 24 hours prior to test for transgene expression. Medium was changed after 4 hours.

DNA ( $\mu\text{g}$ ): FuGENE® HD Transfection ( $\mu\text{l}$ ) ratios 3:1 to transfect cells in different tissue culture formats, the ratio of FuGENE® HD Transfection and nucleic acid used varied, as shown in the Table 3.8.

**Table 3.8: Amounts of FuGENE® HD Transfection and nucleic acid in proportion to the relative surface area.**

Culture vessel	Volume of plating medium	Volume of dilution medium	DNA	Fugene
96-well	100 $\mu\text{l}$	5.2 $\mu\text{l}$	0.11 $\mu\text{g}$	0.3 $\mu\text{l}$
24-well	500 $\mu\text{l}$	26 $\mu\text{l}$	0.55 $\mu\text{g}$	1.7 $\mu\text{l}$

For nucleofection, P3 Primary Cell 4D-Nucleofector™ X Kit for epithelial cell was used according to manufacturer's instructions. Cells were cultured in T25 flask till they reached 70-90% confluency. Removed media from the cultured cells and washed cells once with DPBS. Trypsinised and neutralized the cells with culture medium. Counted an aliquot of cells and took 1 million cells in microcentrifuge tube and centrifuged at 90Xg for 5 min at RT. Resuspended cell pellet in RT 4D-Nucleofector solution (premixed 82 $\mu\text{l}$  Nucleofector solution+ 18 $\mu\text{l}$  Supplement). Added 6  $\mu\text{g}$  of p $\beta$ ChLF expression vector and transferred the mastermix into nucleocuvette supplied with the kit. Gently tapped the Nucleocuvette to make sure the sample covered the bottom of the cuvette. Placed Nucleocuvette with closed lid into the retainer of 4D-Nucleofector X Unit. Started Nucleofection Process by pressing the "Start" on the display of the 4D-Nucleofector Core Unit. After run completion, carefully removed the Nucleocuvette Vessel from the retainer. Incubated Nucleocuvette for 10 minutes at room temperature. Resuspended cells with pre-warmed medium and plated into 6-well plates.

After 48 hrs of lipofection and nucleofection, transfected cells were trypsinized and made into single cell suspension by repeated pipetting and filtering through 60  $\mu\text{m}$  filter and subjected to flow cytometry analysis the parameters for which were set by using non-transfected cells as control to define the measurement and gating parameters.

### **3.2.19 Optimization of working selection concentration of Geneticin by Titration**

The first critical step for stable cell line generation is determining the optimal antibiotic concentration for selecting stable cell colonies; the optimal concentration is cell type dependent. A kill curve is a dose-response experiment where the cells are subjected to increasing amounts of antibiotic to determine the minimum antibiotic concentration needed to kill all the cells over the course of one week. We performed kill curve assay for Geneticin (SIGMA, MO, USA). GMECs were plated in 0.5 ml complete growth medium per well in a 24-well tissue culture plate one day prior to introducing antibiotic selection. Added increasing amounts of G418 to duplicate wells of cells plated in complete media including a no-antibiotic control from 0, 200, 300, 400, 600, 800, 1000 and 1200 $\mu$ g/ml concentration. Replaced media containing selection antibiotic every 2-3 days for up to a week and examined the culture every day for signs of visual toxicity.

### **3.2.20 Transfection of goat mammary epithelial cells with p $\beta$ ChLF expression vector by Nucleofection**

After optimizing the method of transfection and dose for G418, GMECs were nucleofected using P3 Primary Cell 4D-Nucleofector<sup>®</sup> X (Lonza, Switzerland) according to the manufacturer's recommendations using Program EL110 as described in section 3.2.17. After 48 hrs of nucleofection, GFP expression was checked under inverted UV microscope. Cells were then trypsinised and plated in 35 mm dishes in the complete medium (DMEM/F12+ Antibiotics+ 10%FBS+ GFs) containing Geneticin@500 $\mu$ g/ ml. The cells were subjected to selection for 3-4 weeks continuously to obtain stably transfected cells.

### **3.2.21 Analysis of hLF production in mammary epithelial cells**

#### **3.2.21.1 Reverse transcriptase-polymerase chain reaction analysis**

Total RNA was extracted from transfected mammary epithelial cells at passage 3, 5 and 8, cDNA was prepared and PCR amplification for human lactoferrin was done as described in section 3.2.13.2 using primers shown in Table 3.9

**Table 3.9: Primers used for amplification of human Lactoferrin gene in transfected GMECS**

Primer sequence	Product size	Accession no.
LF1F: CAGACCGCAGACATGAAACT		
LF1R: GCAATCCCCACCTTCAGCAG	2259	AY165046.1

### 3.2.21.2 Immunocytochemical analysis

Staining for the presence of human lactoferrin protein in mammary epithelial cells was carried out using the Rabbit anti-human Lactoferrin primary antibody (Millipore) @ 1:300 dilution and PE-conjugated polyclonal anti-Rabbit IgG (SantaCruz) secondary antibody @ 1:500 dilution, by seeding 60,000 cells/well in 4-well flat-bottom culture plates as described in 3.2.13.1 section. After staining, the cells were observed for GFP under blue filter, human lactoferrin under green filter and Hoechst under UV filter.

### 3.2.21.3 Western blot analysis

Western blotting was done to detect the expression of human lactoferrin in cell lysates as well as secreted medium. Transfected cells from 1 T25 flask were harvested using Trypsin, washed briefly with DPBS and lysed on ice by NE-PER Nuclear and cytoplasmic extraction reagent (ThermoFisher Scientific, MA, USA), according to manufacturer's instructions and stored at -80 °C until use. Culture media of transfected GMEC cells was collected after 24 hour cultured in serum free media. Secretory proteins were concentrated from medium using Amicon Ultra Centrifugal Filters with 10 kDa cut off. For SDS-PAGE, the extracted proteins were mixed with sample loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% 2-Mercaptoethanol and 0.002% bromophenol blue) containing protease cocktail inhibitor i.e. HALT(tm) Protease Inhibitor Cocktail (100X). Contents were properly mixed by vortexing and then boiled for 5 min at 95°C. Proteins were loaded finally in 10% SDS polyacrylamide resolving gel overlaid by 4% stacking gel. The gel was loaded onto the running apparatus and run initially at 50 V for 30 min and

then at 100 V till the dye front reached the end of the gel. The gel was removed from the SDS-PAGE apparatus, washed in deionized water and equilibrated in transfer buffer for 15-20 min. The Hybond-LFP (Low fluorescent PVDF membrane) of suitable size was cut, and prepared by immersing in methanol (Cat no# 132977; SRL Pvt. Ltd) for 30 sec, then in ultrapure water for 30 sec and finally in cold transfer buffer for at least 5-10 min. Wet Hybond blotting papers (pre-wetted in transfer buffer) were placed on the anodic side of the semi dry transfer apparatus (E5100) followed by the prepared membrane, the gel and on the top again by pre-wet Hybond blotting papers. Trapped air between blotting papers, gel and membrane was removed by slightly pressing over the assembly with a pressure roller. The sandwiched gel in the cassette was tightly closed and blotting was done in 1X transfer buffer at a constant current of 80 mA for 90 min. After transfer, the membrane was removed and washed in distilled water, and then blocked with 5% BSA (in TBST) by incubating for 2 h at room temperature. The blocked membrane was rinsed in deionized water and then incubated for 2 h at RT or overnight at 4°C, with shaking, in primary antibody (specific to human lactoferrin), diluted (1:1000) in TBST containing 5% BSA. The contents were drained and washed thrice with TBST buffer for 10 min. Thereafter, membrane was incubated in appropriate HRP-conjugated secondary antibody solution ( $1\mu\text{g}\mu\text{l}^{-1}$ ) (diluted 1: 3000 in TBST) for 1 h at room temperature with constant shaking. Contents were then drained, rinsed thrice with TBST buffer and washed four times, 10 min each, with shaking at room temperature. Membrane was rinsed thrice with TBS (TBST but without Tween 20). The freshly prepared substrate solution DAB-  $\text{H}_2\text{O}_2$  was added and the membrane was visualized for color development in 10-30 min. After the optimum color was developed, the PVDF membrane was washed with excess water to stop the reaction.

#### **3.2.21.4 Enzyme-linked immunosorbent assay (ELISA)**

Before human lactoferrin content and secretion analyses,  $2 \times 10^6$  cells were seeded per well of six-well plates and allowed to attach overnight. After this,

the cells were rinsed three times with PBS (preheated to 37°C) to remove hLF secreted overnight. To assess hLF secretion, cells were then exposed to a serum-free medium containing 0.1% BSA for 24 h of incubation. After incubation, the medium was collected for enzyme linked immunosorbent assay (ELISA). ELISA was done using Human Lactoferrin ELISA kit (Cat No. EL2011-1; AssayPro, MO, USA) following manufacturer's instructions as described below.

- Prepared all the reagents, working standards, and samples as instructed.
- Added 50µl of human lactoferrin standard and sample per well. Covered wells with a sealing tape and incubated for 2 hrs.
- Washed 5 times with 200µl Wash buffer manually. Inverted the plate each time and decanted the contents.
- Added 50µl of biotinylated human lactoferrin antibody to each well and incubated for 1 hr. Washed the microplate as above.
- Added 50µl of Streptavidin-Peroxidase conjugate per well and incubated for 30 mins. Washed the microplate as above.
- Added 50µl of chromogen substrate per well and incubated for about 15 minutes till the optimal blue colour density developed.
- Added 50µl of Stop solution to each well. Colour changed from blue to yellow.
- Read the absorbance on an ELISA reader at wavelength of 450nm immediately.
- Calculated the mean value of duplicate readings for each standard and sample and analysed data accordingly.

### **3.2.22 Establishment of goat fetal fibroblast cells**

Goat fetus obtained, as gravid uterus, from a slaughtered animal was separated from the uterus and washed twice with antibiotic (Gentamicin and

Penstrep) fortified saline. The surface of fetus was disinfected by dipping in 70% ethanol followed by several washings with antibiotic fortified (Gentamicin and Penstrep) normal saline. Ear skin biopsies were washed 4-6 times with DPBS containing 10% FBS and 50 µg/ml gentamicin sulphate. The biopsies were then cut with the help of a pair of scissors into small pieces (~1 mm<sup>3</sup>) which were then again washed 3-4 times with DPBS + 10% FBS and 50 µgml<sup>-1</sup> gentamicin sulphate and then finally with the cell culture medium (DMEM supplemented with 2.0 mM L-Glutamine, 20% FBS and 50 µgml<sup>-1</sup> gentamicin sulphate). The explants were transferred into T25 tissue culture flasks and allowed to attach to the substrate, in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> in air) at 37°C. Fibroblast culture medium was then slowly added after 3-4 h, taking care not to detach the explants. The flasks were incubated undisturbed for 2-3 days or until cells started migrating out from the adhering explants. The media was replaced with the fresh medium every 3 days until 70-80% confluence was achieved.

#### **3.2.22.1 Sub-culturing of cells**

When a monolayer (60-70% confluent) was formed, the cells were ready for subculture. The media was decanted and cells were washed with Ca<sup>++</sup> and Mg<sup>++</sup> free DPBS (DPBS<sup>-</sup>) for 5 min with vigorous shaking so as to remove the explants. The monolayer was then disaggregated by addition of Trypsin-EDTA (0.25%) and incubated at 37°C for 2 minutes to ensure fractional disaggregation only of the fibroblasts while other cells, especially epithelial cells, remained attached to the culture flask. The passaged or sub-cultured cells were observed for *in vitro* growth for about 12 h. The media was decanted to remove the dead cells (unattached) and anchored cells were allowed to grow further after addition of fresh medium.

#### **3.2.22.2 Cryopreservation of fetal fibroblast cells**

Maintaining large number of cell culture flasks is skill oriented, expensive and a risky practice. Once the fibroblast cultures were characterized, the representative cell culture stocks were preserved by cryopreservation. The cells were allowed to grow *in vitro* to exponential phase of growth. Cell culture was checked for healthy growth, freedom from contamination and presence of

specific characteristics (morphology) for skin fibroblast cells. The cells were harvested by trypsinization of the monolayer and preparing cell suspensions at a final concentration of  $10^6$ - $10^8$  cells  $\text{ml}^{-1}$  in the holding medium (10% FBS in DMEM). Freezing medium (15% Glycerol + 30% FBS + DMEM) was prepared and sterilized through 0.22 $\mu\text{m}$  syringe filter. Care was taken that glycerol used was not more than one year old, because it becomes toxic after prolonged storage. After centrifugation, the cells were washed once with freezing medium. Cell pellet was suspended in 1 ml of freezing medium per T25 flask and transferred to a Cryovial (Corning, NY, USA). Cryovials were subjected to slow freezing in Cryo freezing container (Mr. Frosty, Nalgene) at  $-80^\circ\text{C}$  for 2-3 hours to achieve a slow and constant cooling rate equivalent to  $-1^\circ\text{C}$  per minute, and then quickly plunged into liquid nitrogen.

When required for further culture, the cryovials were taken out from liquid nitrogen, thawed in warm water at  $37^\circ\text{C}$  with vigorous intermittent shaking and then transferred into the complete medium, previously equilibrated in  $\text{CO}_2$  incubator for 15 to 20 mins. The survival of the cells was evaluated by thawing after 7 days and culturing the frozen thawed cells in DMEM supplemented with 10% FBS. Quantitative survivability of the cells was determined by staining the frozen thawed cells with Trypan blue (0.4%) whereas the qualitative survivability was evaluated by allowing the frozen thawed cells to grow *in vitro*.

### **3.2.23 Characterization of goat fetal fibroblast cells (GFFs)**

#### **3.2.23.1 Immunofluorescence staining**

The fetal fibroblast cultures, so obtained were characterized for various fibroblast specific ( $\beta$ -Tubulin, Vimentin) as well as non-specific markers (Keratin, CK18) by immunocytochemistry as described in section 3.2.13.1

#### **3.2.23.2 Reverse Transcription PCR**

RNA was extracted from GFFs using TRIZOL, cDNA was prepared and PCR amplification of fibroblast specific (positive markers; *FSP-1*, *Desmin*, *Vimentin*, *Tubulin*) and epithelial specific (negative markers; *CK8*, *CK18*) genes using specific primers (Table 3.10) was done as described for GMECS in section 3.2.13.2

**Table 3.10: Primers for fibroblast cells characterization.**

Gene	Sequence	Annealing temp (°C)	Product size	Accession ID
<i>DESMIN</i>	F- AGCGGAGAACAATTTGGCTG R- ACATGTCCATCTCCACCTGG	52	188 bp	XM_005676941.1
<i>VIMENTIN</i>	F- ACCCCTCCTTTATCAGCACC R- CTCTCGGCTTCCTCTCTCTG	52	146 bp	XM_005688054.1
<i>TUBULIN</i>	F- GAGGGAAATCGTGACATCC R- TAGTACACGGAGATGCGGTC	52	150 bp	XM_005696628.1
<i>CK8</i>	F- AAGCTGGTGTCTGAGTCCTC R- GGTGGAGGACAGGGGTAAAA	56	150 bp	XM_005679931.1
<i>FSP-1</i>	F- ACCTCCACAAGTACTCGGG R- CCACCTCGTTGTCCTTGTTG	52	169 bp	XM_005677502.1
<i>CK18</i>	F- GGAGCTGGACAAGTACTGGT R- ACTTCCCTCAGGCTGTTCTC	53	186 bp	XM_005680092.1
<i>β-ACTIN</i>	F-TCACGAAACTACCTTCAATTC R-TCTTGATCTTCATTGTGCTGG	60	164 bp	NM_001290932.1

### 3.2.23.3 Chromosomal analysis and sex determination

Chromosomal analysis was performed on goat fetal fibroblast cells as described in section 3.2.14 except that the hypotonic solution used was 0.56% KCl. Sex of the fetal fibroblast cells was also determined as described in section 3.2.14

### 3.2.24 Development of transgenic goat fetal fibroblast cells

The characterized goat fetal fibroblast cells were transfected with the expression vector pβChLF by both lipofection and nucleofection and analyzed by Flow cytometry for the efficiency of transfection as described in section 3.2.17. Optimization of Geneticin dose was also done by Kill Curve Assay as described already in section 3.2.18. Finally, goat fetal fibroblast cells were transfected by Nucleofection using the program EN150 and subjected to

selection using G418 for 8 weeks in order to obtain pure population of transfected goat fetal fibroblast cells at passage 8.

### **3.2.25 Detection of transgene integration into the transfected goat fetal fibroblast cells by PCR**

The molecular detection of hLF-transgenic donor cells was conducted by a PCR. Genomic DNA from transfected fetal fibroblast cells at passage 8 was extracted, and polymerase chain reaction (PCR) amplification was performed using one set of primers that amplified 122 bp of Lactoferrin. The PCR primer sequences were LF forward 5' CCTGGTGCTGAAAGGAGAAG 3' and LF reverse 5' GGTCAGTCTTTGTTGGGAT 3'. The PCR amplification conditions were 94°C for 3 minutes, then 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 minutes 30 seconds, 72°C for 10 minutes, and 4°C for 1 hour. A total of 35 cycles were performed. After amplification, products were analyzed on a 2% agarose gel stained with ethidium bromide.

### **3.2.26 Production of transgenic goat embryos by Hand-made cloning**

#### **3.2.26.1 Collection and classification of oocytes**

Goat ovaries were obtained from Delhi slaughterhouse. These were washed 3-4 times with antibiotics-fortified (400 I.U/ml penicillin and 500 µg/ml streptomycin) isotonic warm saline (32-37°C) and were then put in a thermos flask containing warm saline. The ovaries were transported to the laboratory within 6 h of slaughter. In the laboratory, the ovaries were rinsed twice with antibiotic fortified warm saline and were then trimmed to remove the extra tissue after which these were washed several times with antibiotic fortified warm saline. Oocytes were collected by puncturing the ovaries with a 18 gauge needle in a dish containing the aspiration medium (TCM-199 + 2 mM L-Glutamine + 0.3% BSA + 50 µg/ml gentamicin sulfate). The contents of the syringe, which included the aspirated oocytes, follicular fluid, granulosa cells and other debris, were poured in 100 mm x 100 mm Petri dishes with 13 mm grid (searching dishes). The oocytes were searched under a zoom stereomicroscope at around 20X magnification and were shifted to 35 mm Petri dishes containing the washing medium (TCM-199 + 2 mM L-Glutamine +

10% FBS + 0.81 mM sodium pyruvate + 50 µg/ml gentamicin sulfate). The aspirated oocytes were graded according to the following criteria already in use in the laboratory.

**Usable quality:** Compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having  $\geq 2$  layers of cumulus cells, and with homogenous, evenly granular ooplasm.

**Unusable quality:** Oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm.

Oocytes of only usable quality were used for *in vitro* maturation.

### 3.2.26.2 *In vitro* maturation of oocytes

The usable quality oocytes were washed 4-6 times with the washing medium and then twice with the IVM medium (TCM-199 + 10% FBS + 5 µg/ml FSH-p + 1 µg/mL estradiol-17 $\beta$  + 0.81 mM sodium pyruvate + 10ng/ml EGF+ 50 µM cysteamine + 1X ITS + 50 µg/mL gentamicin sulfate). For IVM, groups of 15-20 COCs were placed in 70 µL droplets of the IVM medium, overlaid with sterile mineral oil in 35 mm Petri dishes and cultured for 27 h in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub> in air) at 38.5°C.

### 3.2.26.3 Processing of IVM oocytes for preparation of cytoplasts

The recipient cytoplasts were prepared as follows. Various steps involved in the preparation of enucleated cytoplasts are elaborated below.

#### 3.2.26.3.1 Denuding the oocytes

COCs with expanded cumulus were transferred to 1.5 ml microcentrifuge tube containing 500 µl hyaluronidase (0.5 mg/ml) in T2 (where T denotes TCM-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 µg/ml gentamicin and the following number denotes percentage of FBS) and incubated for 5 min at 38.5°C. It was followed by gentle pipetting using 1 ml microtip. The contents of the tube were transferred to a 35 mm dish containing T2 and completely denuded oocytes with evenly granular

cytoplasm were selected and washed twice with fresh T2 for removal of residual cumulus cells.

### **3.2.26.3.2 Removal of zona pellucida**

The denuded oocytes were transferred to a 35 mm dish containing pronase (2.0 mg/ml in T10) and incubated for 5-6 min at 38.5°C with a periodic gentle rotation of the dish. Oocytes with completely digested zona pellucida were transferred to a dish containing T20 so as to stop the activity of the enzyme. Oocytes with undigested or partly digested zona were discarded. Zona-free oocytes were washed twice with T20 and were incubated in the same medium at 38.5°C for 30 min or until a prominent protrusion cone was easily visible under the zoom stereomicroscope.

### **3.2.26.3.3 Protrusion cone-guided bisection**

Protrusion cone-bearing oocytes were transferred (5-8 each time) to 35 mm dish containing 4 ml T20 and 2.5 µg/ml cytochalasin B and were manually bisected using microblade in such a way that the smaller half bore the protrusion cone and the larger half was enucleated. The bisected half-oocytes without protrusion cone were transferred to T20 and incubated for 10-15 min at 38.5°C to enable them to regain a spherical shape. The demioocytes were confirmed to be enucleated by Hoechst 33342 staining.

### **3.2.26.4 Preparation of donor cells for nuclear transfer**

Different donor cell types i.e., mammary epithelial cells, non-transfected fetal fibroblast cells, transgenic fetal fibroblast were processed for their use as nucleus donors. Each type of the donor cells were allowed to grow further for 3 days in order to achieve over-confluence. The culture medium was removed by aspiration and the cells were overlaid with Ca<sup>2+</sup> and Mg<sup>2+</sup>-free DPBS for 5 min. After removing DPBS, the cells were subjected to trypsinization using Trypsin-EDTA solution. The cells were then harvested in T20 medium and centrifuged to get a loose cell pellet. The pellet was resuspended in T20 and mixed by pipetting to get a single cell suspension in 1.5 ml microcentrifuge tube and was held at room temperature. The cells were now ready for use as nucleus donors.

### **3.2.26.5 Pairing and electrofusion of donor cells and cytoplasts**

The enucleated demi-oocytes were immersed in phytohemagglutinin (0.5 mg/ml in T2) for 3-4 seconds and transferred to T2 containing donor cells at a low density. Each demi-oocyte was then allowed to attach to a single, rounded, medium sized cell by gently rolling the demi-oocyte over it. The couplets (demi-oocyte-donor cell pairs) were transferred to fusion medium for equilibration. Electrofusion of the triplets (demi-oocyte-somatic cell-demi-oocyte) was then carried out by the following method.

### **3.2.26.6 Single step fusion method**

The couplets and the remaining demi-oocytes were transferred to northern and southern parts, respectively, of the fusion chamber (BTX microslide 0.5 mm gap, model 450; BTX, San Diego, CA, USA) containing the fusion medium. A single step fusion protocol was followed wherein a demi-oocyte and a couplet were picked using a fine pulled capillary pipettes (Unopette® Becton Dickinson, NJ, USA) having an inner diameter of 70-80 µm. Initially, the couplet was expelled and aligned with an A.C. pulse (4 Volts) using BTX Electrocell Manipulator 200 (BTX), so that the somatic cell faced the negative electrode. Immediately after alignment, another demi-oocyte was introduced into the fusion chamber close to the somatic cell. As soon as the somatic cell was sandwiched between the demi-oocytes, single D.C. pulse, was applied. The triplets were then incubated in T20 (for rounding up and subsequent reprogramming) for 6 h at 38.5°C.

### **3.2.26.7 Activation of reconstructed oocytes**

The reconstructed oocytes were then activated (~33h after the start of maturation) by incubating in T20 containing 5 µM calcimycin A23187 for 5 min at 38.5°C. The oocytes were washed thrice with T20 and were then incubated individually in 5 µl droplets of respective culture medium containing 2 mM 6-dimethylaminopurine (6-DMAP) and covered with mineral oil in CO<sub>2</sub> incubator, at 38.5°C for 4 h. Reconstructed embryos were removed from 6-DMAP and washed five times with the embryo culture medium.

### **3.2.26.8 *In vitro* culture of embryos**

After 4 h of activation, the reconstructed embryos were cultured in 4- well dishes. In this system, 400  $\mu$ L of Research Vitro Cleave medium (K-RVCL-50, Cook®, Queensland, Australia) was added to each well of a 4-well dish overlaid with 400  $\mu$ L mineral oil. Reconstructed embryos were gently placed (10-15 per well) along the periphery of the well at a distance from each other so as to avoid aggregation. The dish was handled carefully so as not to displace the embryos and incubated undisturbed at 38.5°C in CO<sub>2</sub> incubator for 7 days.

### **3.2.27 Single Embryo PCR**

Some of the embryos produced using HMC were randomly picked, one in each 0.2 ml tube in 2-3  $\mu$ l of water and immediately frozen. On thawing, the embryos were lysed by heating the tubes at 95 °C and immediately chilled on ice. Mastermix for PCR amplification of human Lactoferrin was added and PCR was carried out as described in section 3.2.24 above.

### **3.2.28 Statistical Analysis**

Analysis was performed using SPSS Statistics 17.0 software by students 't' test to compare the means of different groups. The percentage values were analyzed after arcsine transformation prior to analysis. The differences were considered to be statistically significant at  $P < 0.05$ . Data are presented as mean  $\pm$  SEM.

# **CHAPTER – 4**

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## **Results and Discussion**

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## **4. RESULTS AND DISCUSSION**

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The present study entitled, “**Development of transgenic goat embryos expressing human lactoferrin gene,**” precisely aimed to establish the transgenesis protocol for expression of the protein in goat milk. For this purpose, the study was carried out in three phases; i) designing and construction of mammary gland-specific expression vectors to carry the gene for our protein of interest i.e. human lactoferrin, ii) validating the expression of transgenic construct in goat mammary epithelial cell line taken as model against goat mammary gland. The transfected cell lines were established and characterized for the expressed protein. In the last and third phase of the study, transgenic fetal fibroblast cell line was established to be finally used as donor cells in hand-guided cloning so as to produce transgenic cloned embryos. In this chapter, all the results and corresponding discussions are presented under the appropriate sections and sub-sections.

### **Development of goat mammary gland-specific expression vectors for human lactoferrin**

The expression vectors for human lactoferrin were designed under various mammary gland specific promoters, so as to achieve the targeted expression of the desired protein in milk. The vector elements included: i) promoter regions of major milk proteins, ii) human lactoferrin cDNA and iii) termination sequences from 3'UTR of bovine growth hormone and  $\beta$ -casein gene. Overall experimental design to produce transgenic goat embryos expressing human lactoferrin gene is shown in Figure 1.

#### **4.1. Cloning and sequencing of milk protein promoter regions**

We selected promoter regions of  $\alpha$ -Casein,  $\beta$ -Casein and  $\beta$ -Lactoglobulin proteins in order to drive the expression of human lactoferrin in milk. Promoter regions of different milk proteins were amplified as there were no consistent reports available in literature about the efficiency of different promoter elements. In order to further ensure the promoter activity, we selected two different fragments from each promoter as indicated in the table 4.1.

**Table 4.1: Different promoters amplified from goat genomic DNA**

β- casein	6.5 kb
	3.5 kb
α- casein	5.27 kb
	2.97 kb
β- lactoglobulin	2.7 kb
	2.1 kb

#### 4.1.1 Amplification and sequencing of β-Casein promoter regions

The genomic DNA was isolated from goat uterine tissue and promoter regions of β-Casein protein were amplified using the specific primers. The primers for amplification of two promoter region fragments (3.5 and 6.5 kb) were designed and standardized for specific amplification by gradient PCR (Figure 2 and 3). Bulk PCR for the fragments was performed at the optimized temperature (55°C for 6.5 kb and 55.7°C for 3.5 kb) and the specific amplicons were gel eluted. The eluate was checked for purity on Nanodrop and only those eluates having 260/280 ratio equal to 1.8 were used in subsequent studies. The eluates were ligated with the TA cloning vector (pTZ57R/T). This was followed by primary amplification in *E.coli* strain *XL1 Blue*. The recombinant bacteria (white colonies) were selectively picked, checked for the presence of promoter region element by colony PCR (Figure 2 and 3). The positive clones were selectively amplified and propagated. Plasmid isolation was performed in the positive clones and the isolated plasmids were further verified by plasmid PCR (Figure 2 and 3). The verified positive plasmids were sent for sequencing in order to confirm the desired promoter fragments. Atleast three sequencing reactions were performed for each plasmid and sequence analysis was performed. The sequence has been submitted to the nucleotide database of National Center for Biological Information (NCBI)/ GenBank with the accession numbers (**KF992213** and **KF992210**) (Annexure IV and V). The beta-casein 5' UTR has been used for expression of various genes viz. human lactoferrin (Yu *et al.*, 2004; Zhao *et*

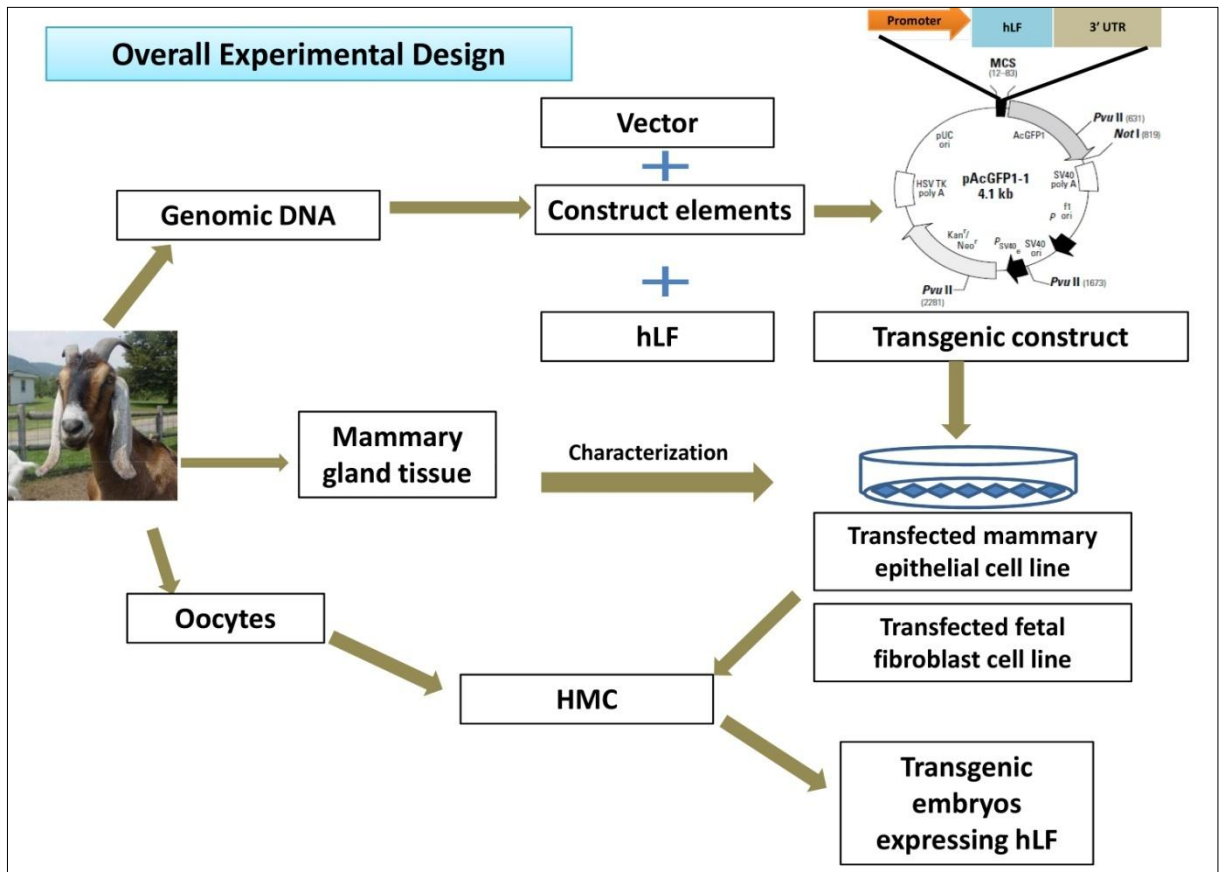


Figure 1: Overall experimental design to produce transgenic goat embryos expressing human lactoferrin gene

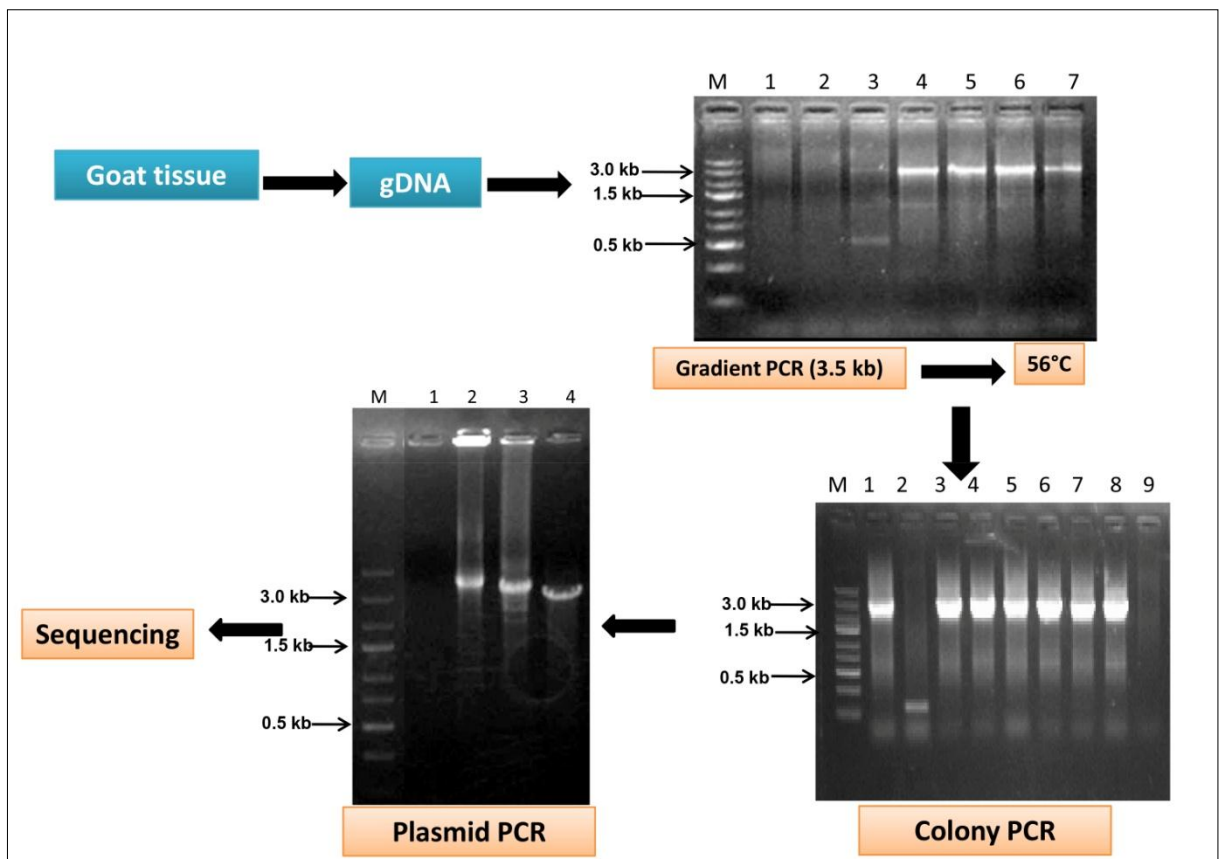


Figure 2: Amplification and cloning of  $\beta$ -Casein 5' UTR (3.5 kb) in XL1 strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

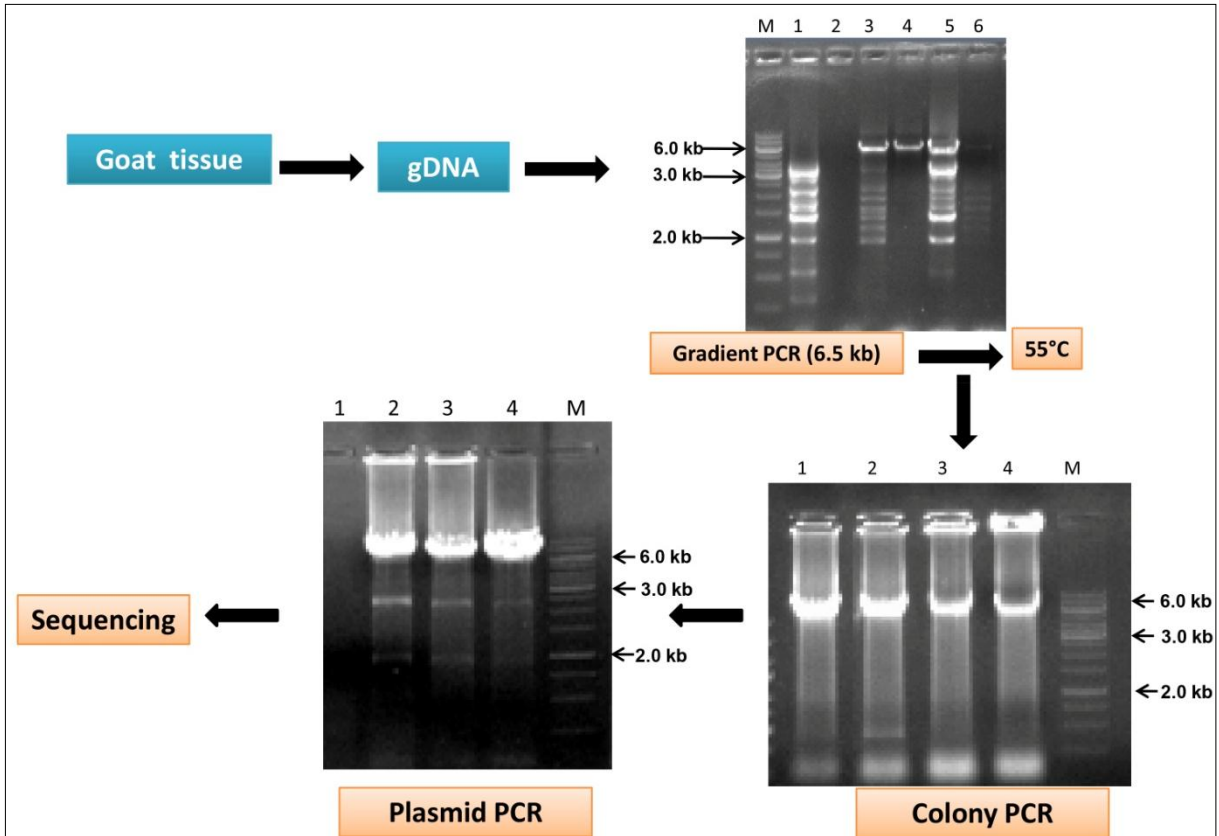


Figure 3: Amplification and cloning of  $\beta$ -Casein 5' UTR (6.5 kb) in *XL1* strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

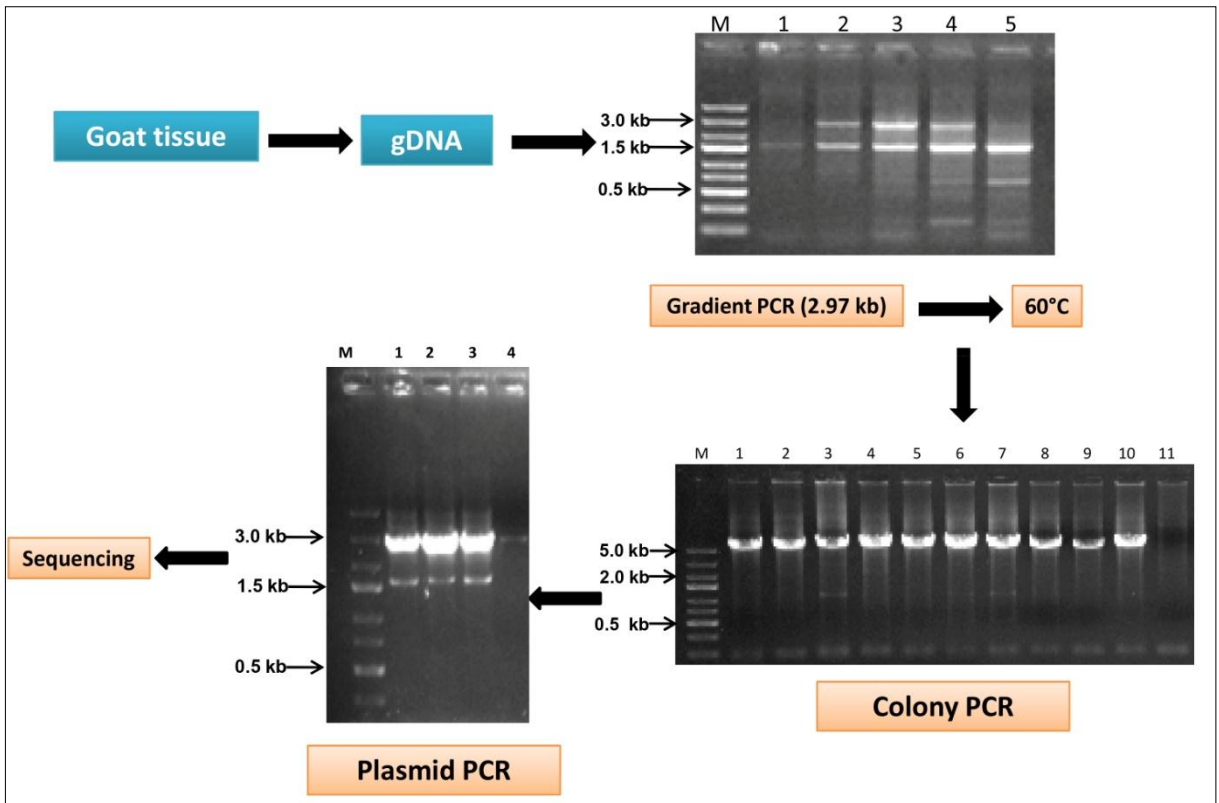


Figure 4: Amplification and cloning of  $\alpha$ -Casein 5' UTR (2.97 kb) in *XL1* strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

*al.*, 2009; Meng *et al.*, 2011), human clotting factor IX (hFIX) (Yekta *et al.*, 2013), Hirudin (Yen *et al.*, 2008), human  $\alpha$ -fetoprotein (Parker *et al.*, 2004), proinsulin (Qian *et al.*, 2014), human butyrylcholinesterase (Huang *et al.*, 2007)

#### **4.1.2 Amplification and sequencing of $\alpha$ -Casein promoter regions**

The genomic DNA was isolated from goat uterine tissue and promoter regions of  $\alpha$ -casein protein were amplified using the Long PCR Enzyme Mix (Thermo Scientific). The primers for amplification of two promoter region fragments (2.97 and 5.27 kb) were designed and standardized for specific amplification by gradient PCR (Figure 4 and 5). Bulk PCR for the fragments was performed at the optimized PCR (57°C for 5.27kb and 59.5°C for 2.97 kb) and the specific amplicons were gel eluted. The eluate was checked for purity on Nanodrop and only those eluates having 260/280 ratio equal to 1.8 were used in subsequent studies. The eluates were cloned in TA cloning vector (pTZ57R/T). This was followed by primary amplification in *E.coli* strain XL1Blue. The recombinant bacteria (white colonies) were selectively picked, checked for the promoter region element by colony PCR (Figure 4 and 5). The positive clones were selectively amplified and propagated. Plasmid isolation was performed in the positive clones and the isolated plasmids were further verified by plasmid PCR (Figure 4 and 5). The verified positive plasmids were sent for sequencing in order to confirm the desired promoter fragments. At least three sequencing reactions were performed for each plasmid and sequence analysis was performed. The plasmids with correct sequencing for the desired fragments were identified and their clones further propagated. The sequence has been submitted to the nucleotide database of National Center for Biological Information (NCBI)/ GenBank with the accession numbers (**KF992212** and **KF992209**) (Annexure II and III).

#### **4.1.3 Amplification and sequencing of $\beta$ -lactoglobulin promoter regions**

Two fragments of goat  $\beta$ -Lactoglobulin promoter (2.7kb and 2.1 kb) were amplified using Long PCR Enzyme Mix (Thermo Scientific) taking goat genomic DNA isolated from uterine tissue as the template employing specific

primers. The PCR products were analyzed by 1% agarose gel electrophoresis along with DNA molecular weight marker (Figures 6 and 7). These fragments were then cloned into TA cloning vector and sent for sequencing. The sequences were submitted to the nucleotide database of National Center for Biological Information (NCBI)/ GenBank with the accession numbers (**KF992214** and **KF992211**) (Annexure VI and VII).

After the sequences were obtained, all the promoters were checked for their activity by PROMOTER PREDICTION 2.0 software. All the six promoters were found to have activity at accepted levels.

#### **4.2 Promoter assay by luciferase assay**

The efficiency of different promoter fragments was verified by luciferase assay, in which the promoter fragment was cloned upstream to luciferase gene and assayed for luciferase activity after transfection into mammary epithelial cells (Figure 8). We observed that all the 6 promoter elements (2 each from  $\alpha$ -Casein,  $\beta$ -Casein and  $\beta$ -Lactoglobulin) lead to expression of luciferase when cloned upstream to this protein in the expression vector (pGL3 basic). By luciferase assay, we observed that 6.5 kb fragment of  $\beta$ -Casein had highest luciferase activity, indicating that this promoter fragment was most active and would lead to maximum expression of human lactoferrin in mammary epithelial cell population as well as in the transgenic goats so produced, in comparison to other promoter fragments (Figure 9). Additionally, 3.5 kb fragment of  $\beta$ -Casein had activity comparable to that of 6.5kb fragment. Based on the result of the luciferase assay, out of the 6 different promoters, we used 6.5 kb and 3.5 kb fragment of  $\beta$ -Casein promoter region for final transgenic expression vector construction to be used as transgenic donor cells.

#### **4.3 Amplification and sequencing of human lactoferrin gene**

The lactoferrin gene was amplified from cDNA prepared from total RNA isolated from human blood lymphocytes, using gene specific primers. The primers were first optimized in gradient PCR (Figure 10) and finally the optimized PCR conditions were applied for bulk PCR for amplification of the

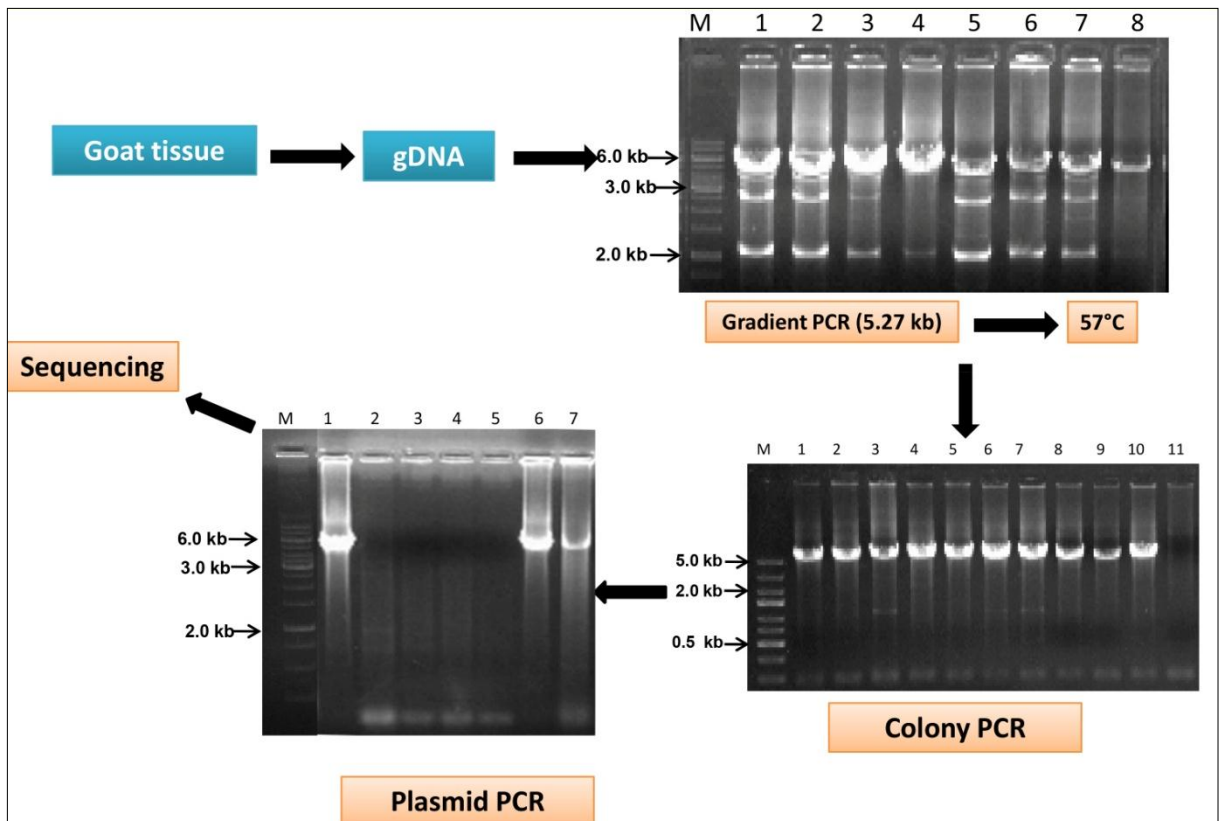


Figure 5: Amplification and cloning of  $\alpha$ -Casein 5' UTR (5.27 kb) in *XL1* strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

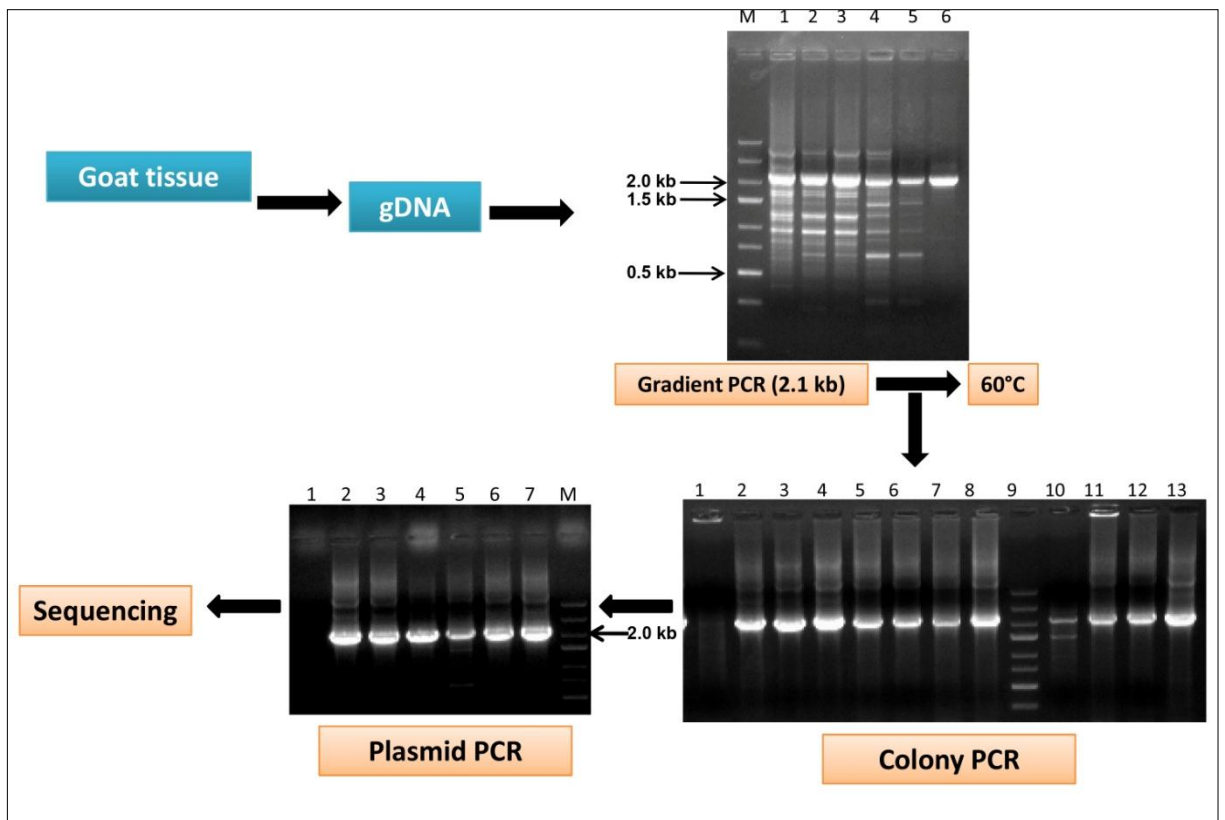


Figure 6: Amplification and cloning of BLG 5' UTR (2.1 kb) in *XL1* strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

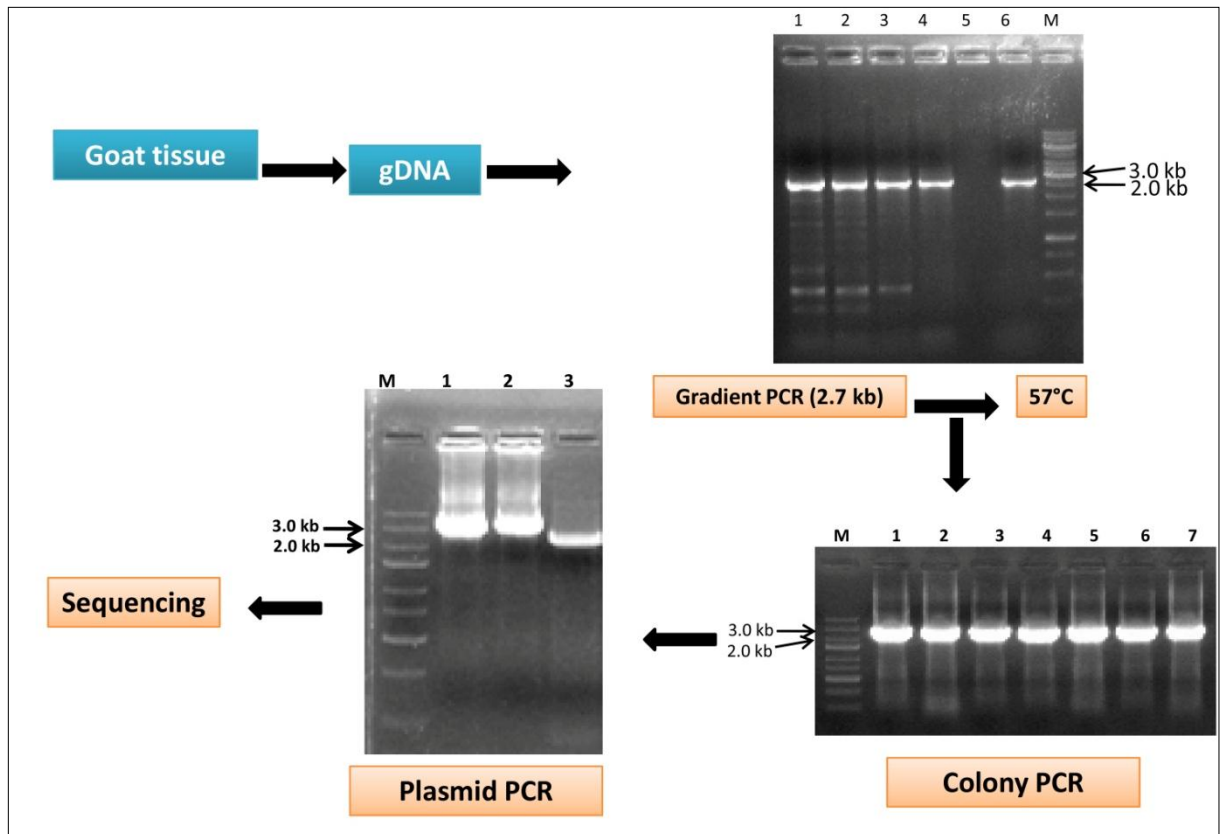


Figure 7: Amplification and cloning of BLG 5' UTR (2.7 kb) in XL1 strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

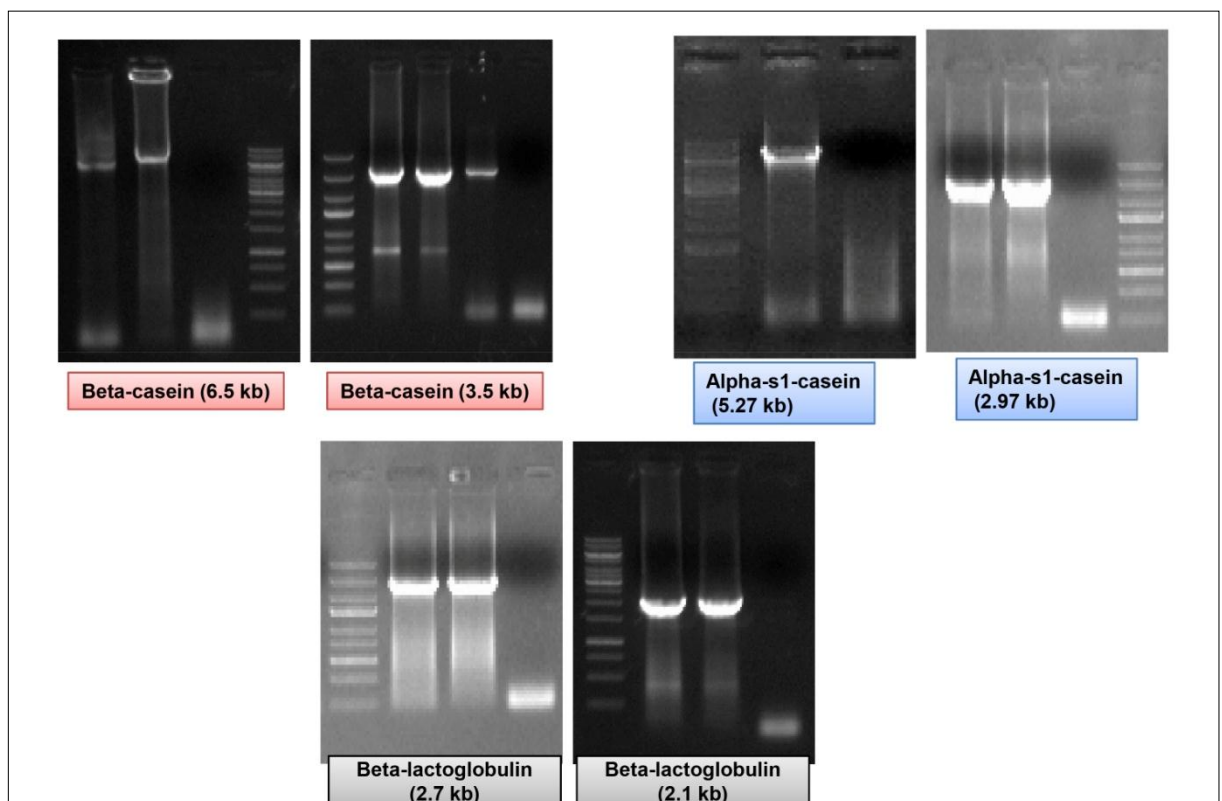


Figure 8: Amplification of various promoters cloned into pGL3 basic vector by Plasmid PCR for Luciferase Assay

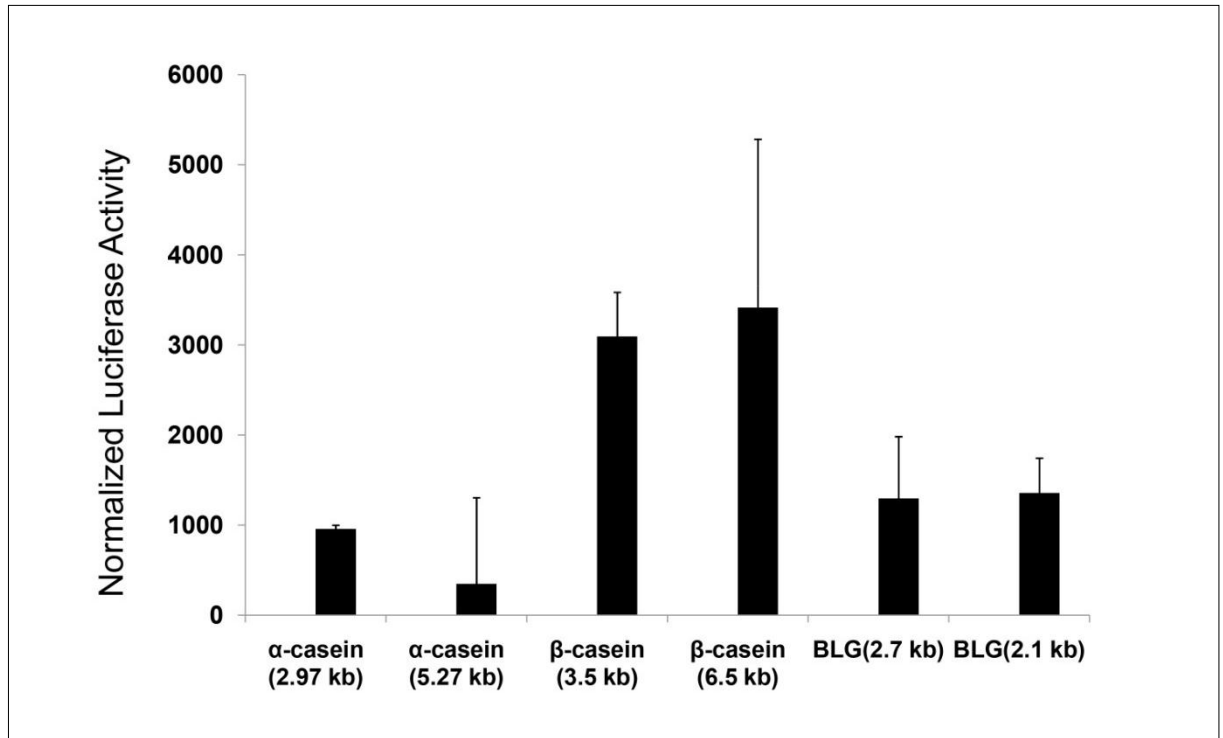


Figure 9: Luciferase Assay of various promoters cloned into pGL3 basic vector

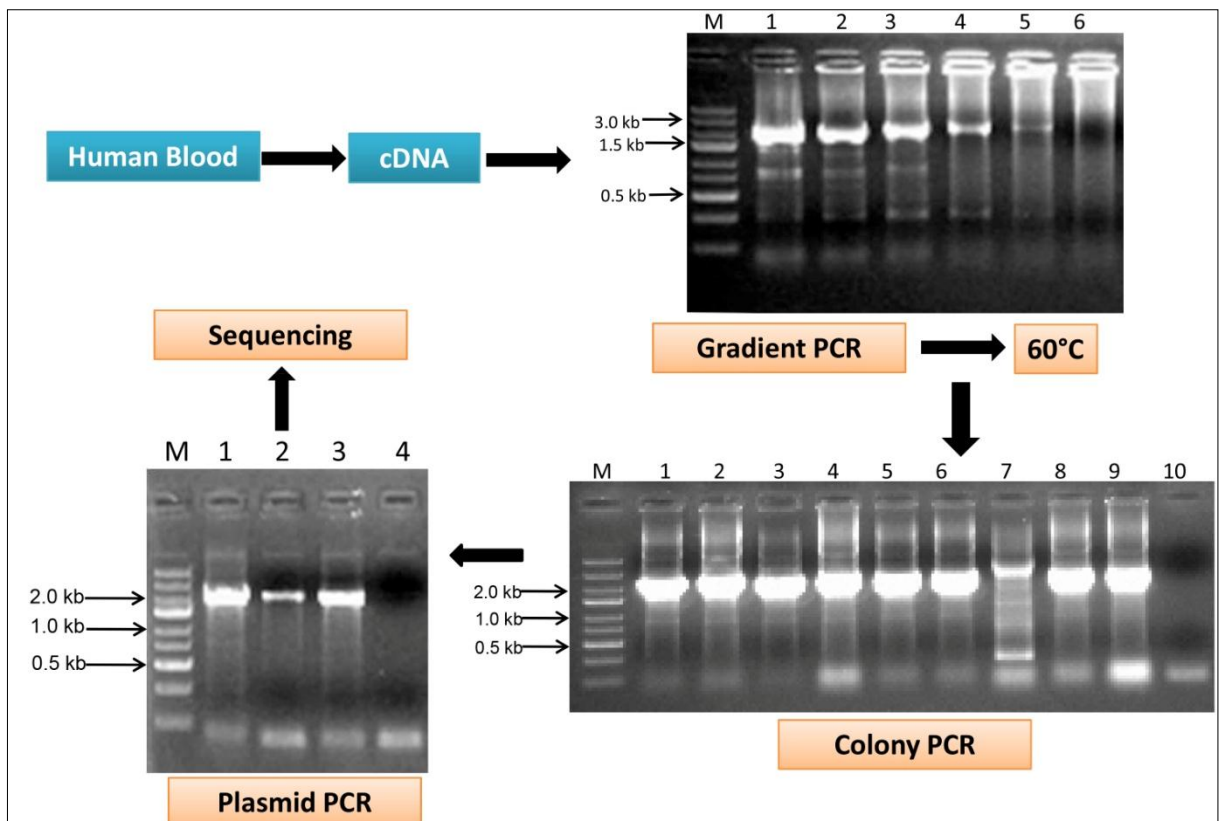


Figure 10: Amplification and cloning of human Lactoferrin CDS (2.2 kb) in XL1 strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

gene. The PCR was analyzed by 1% gel electrophoresis for the specific product formation. A 2.2 kb human lactoferrin gene was thus amplified. The gel eluted amplicon was checked for purity on a Nanodrop and only those samples with 260/280 ratio equal to 1.8 were used for cloning into the cloning vector (pTZ57R/T). *E. coli* strain XL1-Blue was transformed with the vector and plated on LB agar containing Ampicillin+ X-gal. The positive colonies were checked for the gene fragment by colony PCR (Figure 10) and further propagated for plasmid isolation. Plasmids were isolated from the positive clones and the gene fragment was further verified by plasmid PCR (Figure 10). The verified plasmids were sequenced for the desired gene and after sequence analysis, presence of lactoferrin cDNA was ensured. The sequence was submitted to the nucleotide database of the National Center for Biological Information (NCBI)/ GenBank (Figure 11) with the accession number **KF992208** (Annexure VIII).

#### **4.4 Amplification and sequencing of 3'UTR region**

3'UTR fragment of bovine growth hormone was amplified from genomic DNA isolated from cow blood. The primers as usual were standardized by gradient PCR (Figure 12) and finally bulk amplified in optimized PCR. The bulk amplified product was gel eluted and checked for purity on a Nanodrop at 260/280 absorbance ratio. The eluate was used for further studies only when the ratio equaled to 1.8. The 747 bp amplicon was cloned into TA cloning vector (pTZ57R/T) and transfected into XL1 Blue strain of *E. coli* for primary amplification. The positive clones were verified by colony PCR (Figure 12) and used for secondary amplification to isolate the plasmids. The plasmids were further verified for the desired product by plasmid PCR (Figure 12) and finally sequenced for the desired product. The sequence was submitted to the nucleotide database of the National Center for Biological Information (NCBI)/ GenBank with the accession number **KF992215** (Annexure IX). Computational analysis of the sequence showed 95 and 97% homology with bovine, and sheep, respectively.

The sequences of all the vector elements (promoter regions, human lactoferrin and 3'UTR) were submitted to Genbank and accession numbers obtained are summarized in Table 4.2.

**Table 4.2: Accession numbers for construct elements obtained from NCBI.**

<b>Element</b>	<b>Name</b>	<b>Size (kb)</b>	<b>Accession No.</b>
Promoter	Goat $\alpha$ -s1-casein	5.27	KF992212
Promoter	Goat $\alpha$ -s1-casein	2.97	KF992209
Promoter	Goat $\beta$ -casein	6.5	KF992213
Promoter	Goat $\beta$ -casein	3.5	KF992210
Promoter	Goat $\beta$ -lactoglobulin	2.7	KF992214
Promoter	Goat $\beta$ -lactoglobulin	2.1	KF992211
Gene	Human lactoferrin	2.2	KF992208
3' UTR	Bovine Growth Hormone	0.747	KF992215

#### **4.5 Construction of mammary gland-specific expression vector for human lactoferrin gene expression**

Since two fragments of  $\beta$ -casein promoter (6.5 kb and 3.5 kb) had comparable activity as evidenced by Luciferase assay, we decided to prepare transgenic constructs for human lactoferrin under both fragments and thus, two mammary gland-specific expression vectors were prepared for expression of human lactoferrin gene having final size as 12.8 kb (6.5 kb  $\beta$ -casein promoter+ 2.2 kb lactoferrin cDNA + 4.1 kb vector) and 9.8 kb (3.5 kb  $\beta$ -casein promoter+ 2.2 kb lactoferrin cDNA + 4.1 kb vector). Preparation of second construct was undertaken as it would provide the ease of transfecting the smaller construct into the cells due to the fact that smaller DNA molecules have greater transfection efficiency.

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ATGAAACTTGTCTTCCTCGTCTGCTGTTCTCGGGGCCCTCGGACTGTGTCTGGCTGGCCGTAGGAGAAGGAGTGT
CAGTGGTGCGCCGTATCCCAACCCGAGGCCACAAAATGCTTCCAATGGCAAAGGAATATGAGAAAAGTGCCTGGCCC
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CCTTGATGGTGGTTTCATATACGAGGCAGGCCTGGCCCCCTACAACTGCGACCTGTAGCGCGGAAGTCTACGGGA
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GACCCGAGATCTAATCTCTGTGCTCTGTGATTGGCGACGAGCAGGGTGAGAATAAGTGCCTGCCCAACAGCAACGA
GAGATACTACGGCTACTGTTGGGCTTCCGGTGCCTGGCTGAGAATGCTGGAGACGTTGCATTTGTAAAGATGTCAC
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CCTCGATGGCAAACGGAAGCCTGTGACTGAGGCTAGAAGCTGCCATCTTGCATGGCCCCGAATCATGCCGTGGTGTG
TCGGATGGATAAGGTGGAACGCCTGAAACAGGTGCTGCTCCACCAACAGGCTAAATTTGGGAGAAATGGATCTGACT
GCCCGGACAAGTTTTGCTTATTCCAGTCTGAAACCAAAAACCTTCTGTTCAATGACAACACTGAGTGTCTGGCCAGACT
CCATGGCAAAAACAACATATGAAAAATATTTGGACCACAGTATGTCGCAGGCATTACTAATCTGAAAAAGTGCTCAAC
CTCCCCCTCCTGGAAGCCTGTGAATTCCTCAGGAAGTAA

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Figure 11: Sequence of complete CDS of human Lactoferrin gene as submitted to GenBank (KF992208).

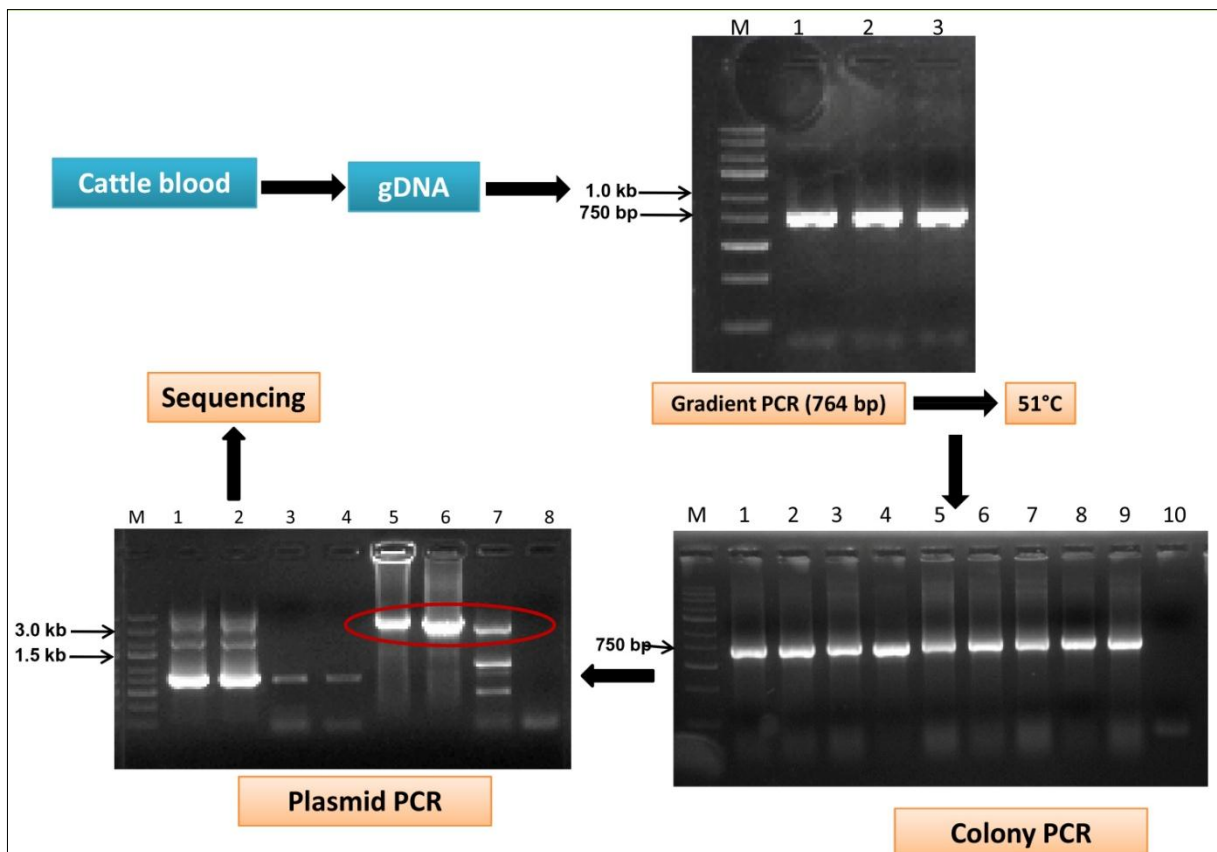


Figure 12: Amplification and cloning of 3'UTR of bovine Growth Hormone gene in *XL1* strain of *E. coli*, positive clone selection by colony PCR, insert confirmation by plasmid PCR and sequencing of the insert.

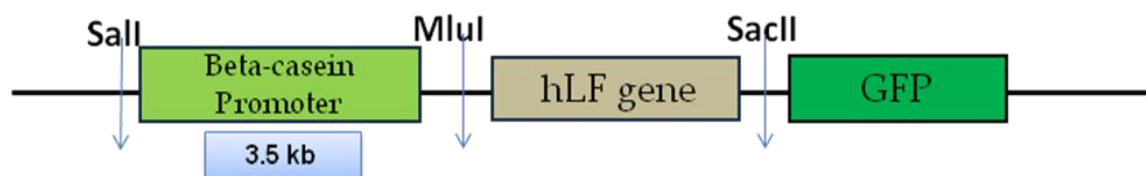
#### 4.5.1 pBCL1 expression vector

The promoterless vector pAcGFP1-1 was used as a backbone for preparation of gene constructs. The restriction sites at the end of each fragment were chosen in such a way that each restriction enzyme would cut the final transgene construct only once and restriction site for each enzyme was absent in any of the fragments. To prepare the construct for human lactoferrin under 6.5 kb  $\beta$ -casein promoter, the three elements of the construct i.e. promoter, human lactoferrin and vector backbone were digested by restriction digestion and ligated by T4 ligase in 1:1:1 ratio as per the following scheme. All the elements of this transgenic construct were confirmed by restriction digestion and PCR (Figure 13, 14, 15).



#### 4.5.2 pBCL2 expression vector

The transgene construct for human lactoferrin under 3.5 kb  $\beta$ -casein promoter was constructed by ligating the promoter element having restriction sites as Sall and Mlul, human lactoferrin having Mlul and SacII sites and expression vector backbone cut by Sall and SacII restriction endonucleases in 1:1:1 ratio with T4 DNA ligase as following.



All the elements of this transgene construct were found to be in correct order and orientation as verified by PCR and restriction enzyme digestion (Figure 16, 17, 18).

### 4.5.3 p $\beta$ ChLF expression vector

Both the expression vectors developed under  $\beta$ -casein promoter would lead to the production of human lactoferrin fused to GFP and led to very limited expression of GFP which would be out of the detection limits after few passages. Moreover, these expression vectors would drive the expression of human lactoferrin and thus, GFP (under mammary gland-specific promoters) only in mammary epithelial cells and not in fibroblast cells. Therefore, it was decided to rearrange the construct elements in order to express GFP under CMV promoter and human lactoferrin under the mammary gland-specific promoter. Therefore, the construct was re-designed as shown in Figure 19.

For the preparation of this transgenic construct, pAcGFP1-C1 was used as backbone in which GFP is under CMV promoter. Due to size constraint, we took 3.5 kb  $\beta$ -casein promoter. Because the 5' UTR was to be ligated in frame with the GFP and contained exon1, therefore, to eliminate the chances of it getting transcribed and translated, 3' UTR of bGH was ligated before as shown above. First, the 250 bp bGH flanked by XhoI and Sall sites was ligated into pAcGFP1-C1. After this,  $\beta$ -casein promoter+human lactoferrin was released from pBCL2 expression vector using Sall and SacII and ligated into the vector. All the inserts were confirmed by various combinations of a.) primers for PCR amplification (Figure 20) as indicated in Table 4.3 and b.) restriction enzymes for digestion (Figures 21 and 22).

**Table 4.3: Various primer combinations to confirm the sequence of construct elements.**

Lane	Fragments	Size
1.	bGH 3'UTR+ $\beta$ -casein promoter	3.75 kb
2.	$\beta$ -casein promoter + hLF	5.7 kb
3.	bGH 3'UTR+ $\beta$ -casein promoter+ hLF	5.95 kb
4.	hLF+SV40	2.4 kb

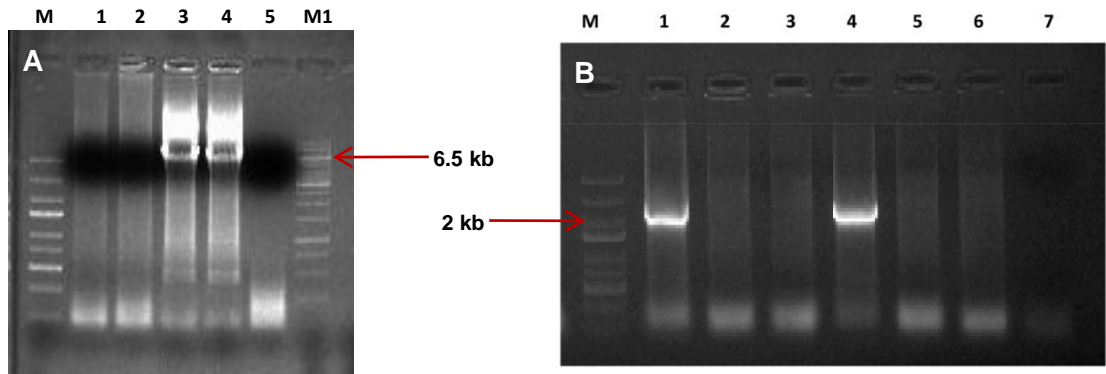


Figure 13: Amplification of  $\beta$ -casein promoter (6.5 kb) from bacterial clones transformed with pAcGFP1-1+ $\beta$ 6.5+ hLF (pBCL1) construct by Colony PCR (A); Amplification hLF from bacterial clones transformed with pBCL1 construct by Colony PCR (B) (M: 5kb ladder; M1: 1kb ladder)

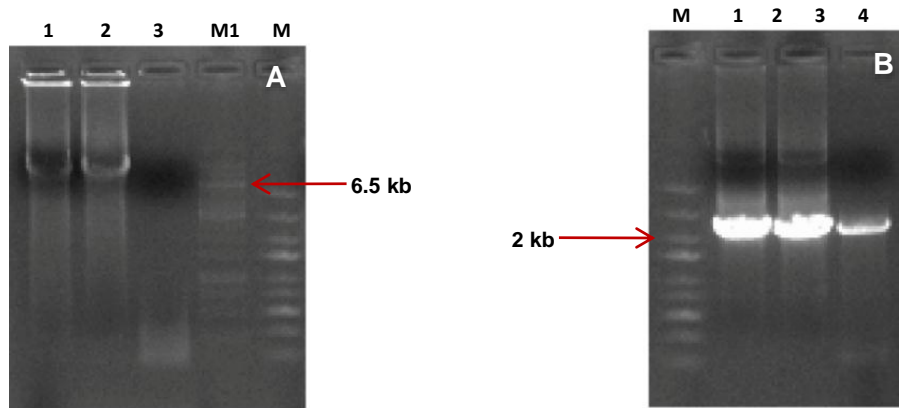


Figure 14: Amplification of  $\beta$ -casein promoter (6.5 kb) from plasmids isolated from positive clones transformed with pBCL1 construct (A); Amplification of hLF from plasmids isolated from positive clones transformed with pBCL1 construct (B) (M: 5kb ladder; M1: 1kb ladder)

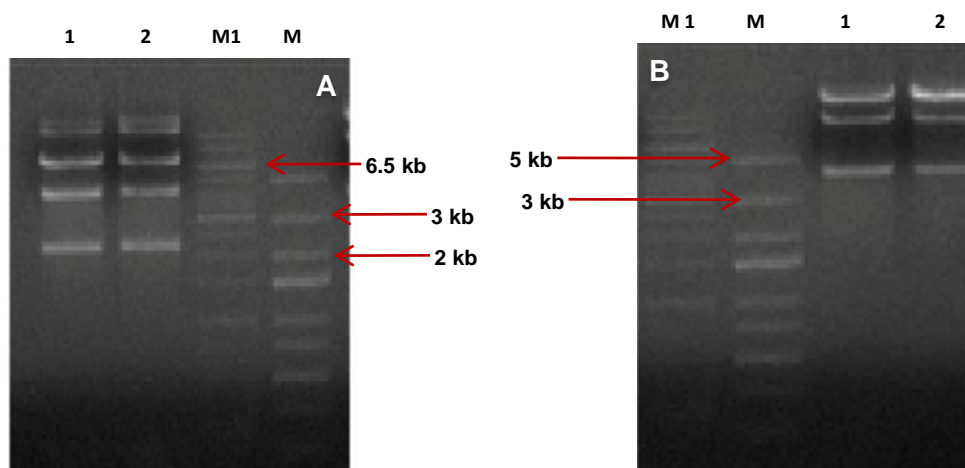


Figure 15: Triple Restriction enzyme digestion of pBCL1 construct using Sall, MluI and SacII yielding 6.5 kb  $\beta$ -casein promoter, 4.1 kb vector, 2.2 kb hLF (A); Double Restriction enzyme digestion of pBCL1 construct using Sall and SacII yielding 8.7 kb  $\beta$ -casein promoter+ hLF, 4.1 kb vector (B) (M: 5kb ladder; M1: 1kb ladder)

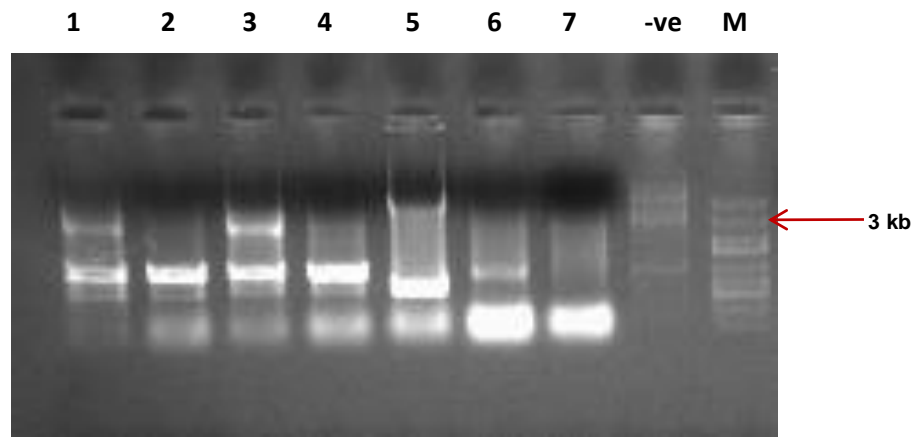


Figure 16: Amplification of  $\beta$ -casein promoter (3.5 kb) from bacterial clones transformed with pAcGFP1-1+ $\beta$ 3.5+ hLF (pBCL2) construct by Colony PCR (M: 5kb ladder)

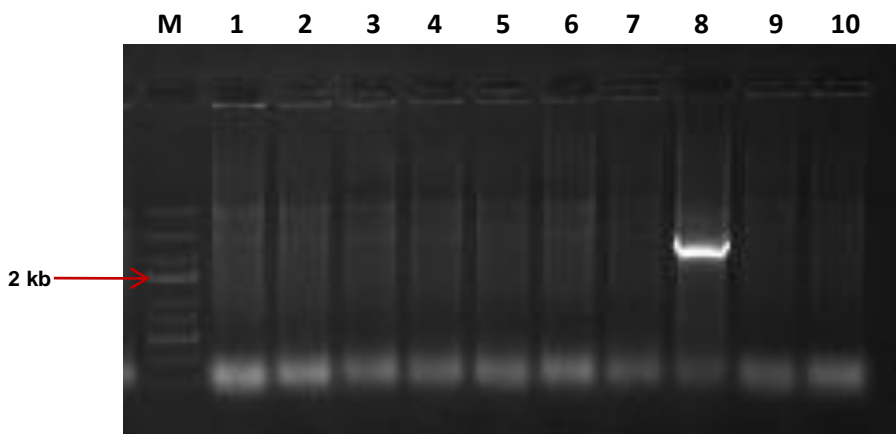


Figure 17: Amplification hLF from bacterial clones transformed with pAcGFP1-1 + $\beta$ 3.5+ hLF (pBCL2) construct by Colony PCR (M: 5kb ladder)

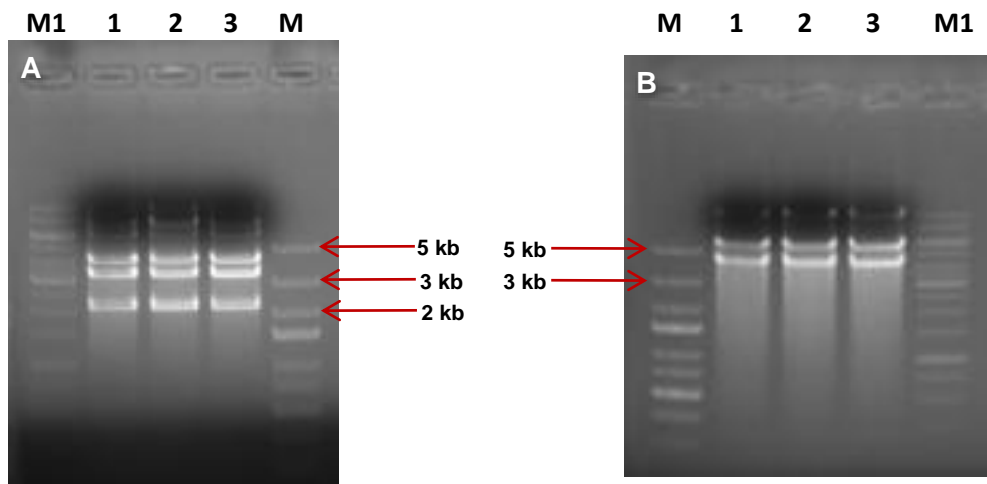


Figure 18: Triple Restriction enzyme digestion of pBCL2 construct using Sall, MluI and SacII yielding 3.5 kb  $\beta$ -casein promoter, 4.1 kb vector, 2.2 kb hLF (A); Double Restriction enzyme digestion of pBCL2 construct using Sall and SacII yielding 5.7 kb  $\beta$ -casein promoter+ hLF, 4.1 kb vector (B) (M: 5kb ladder; M1: 1kb ladder)

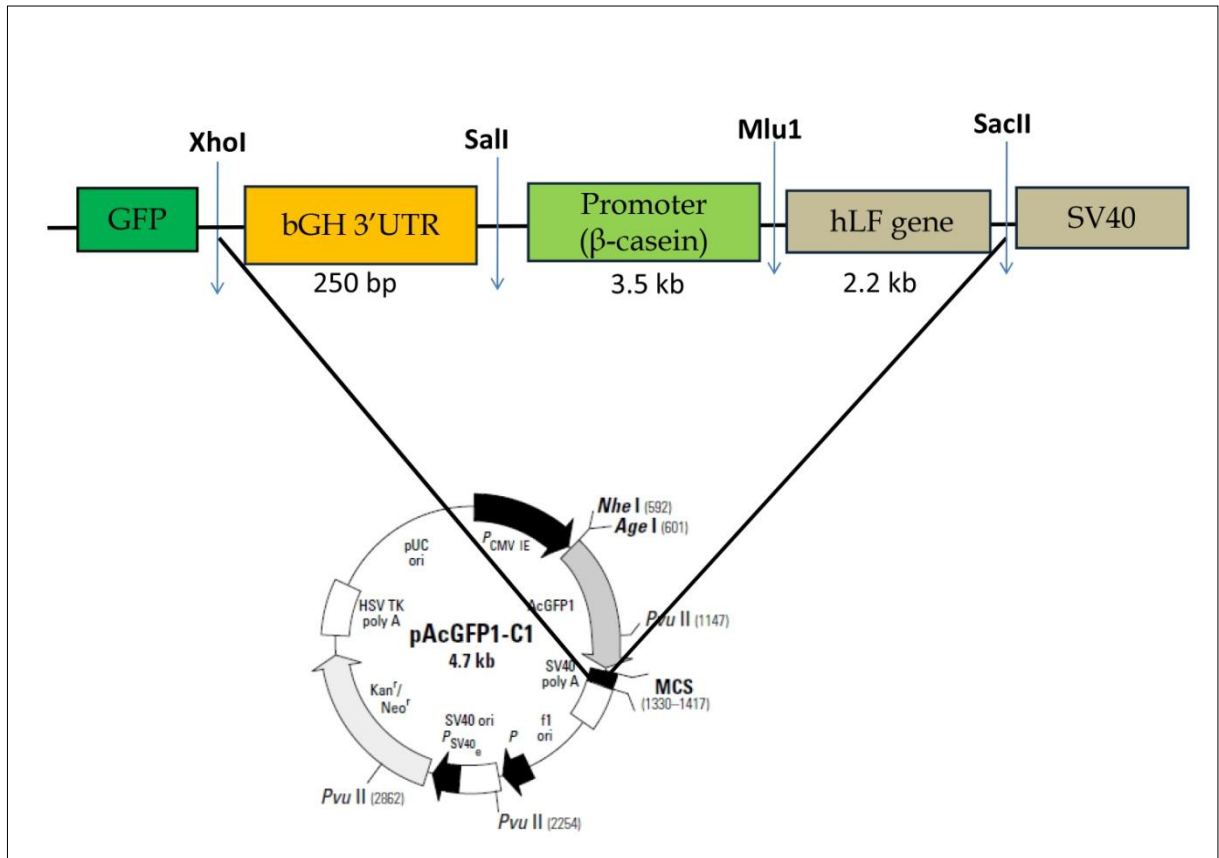


Figure 19: Diagrammatic representation of pβChLF expression vector

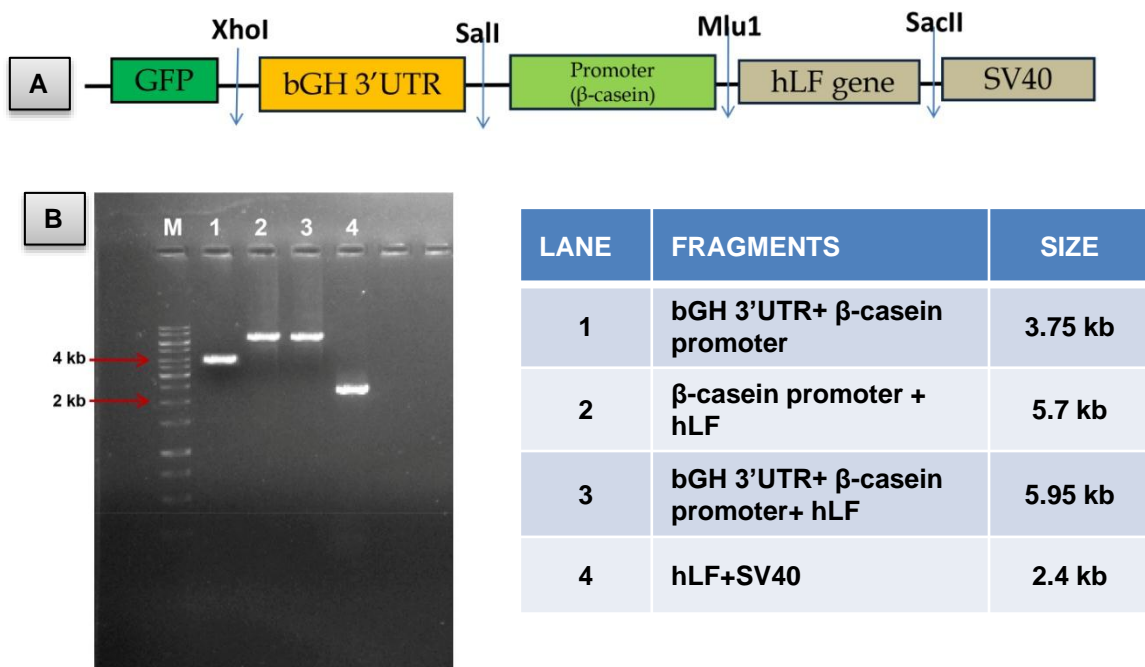
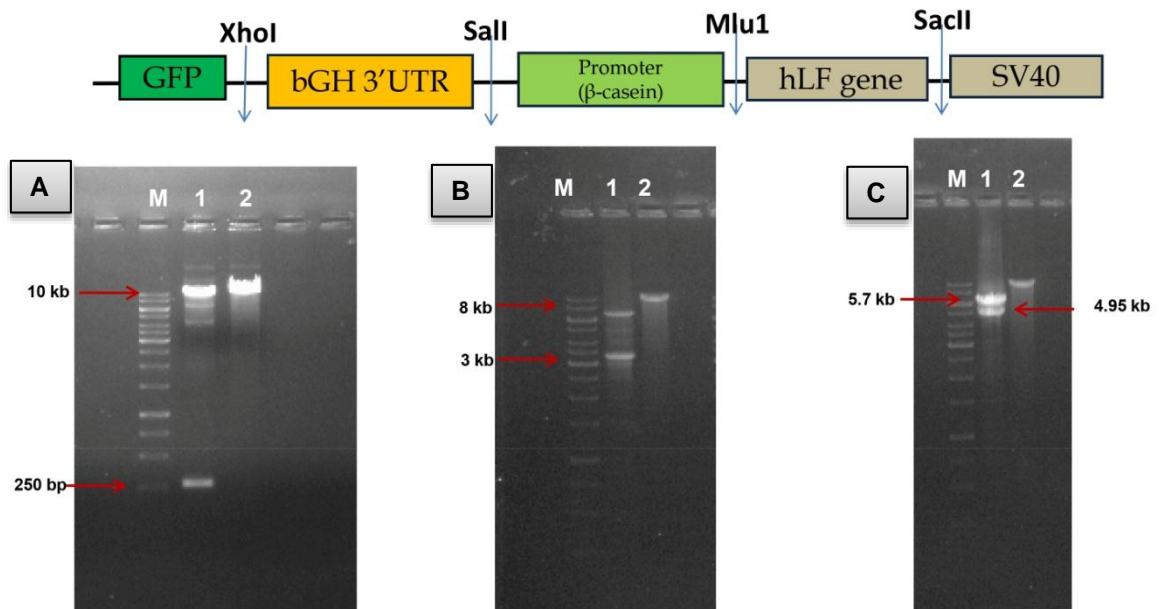
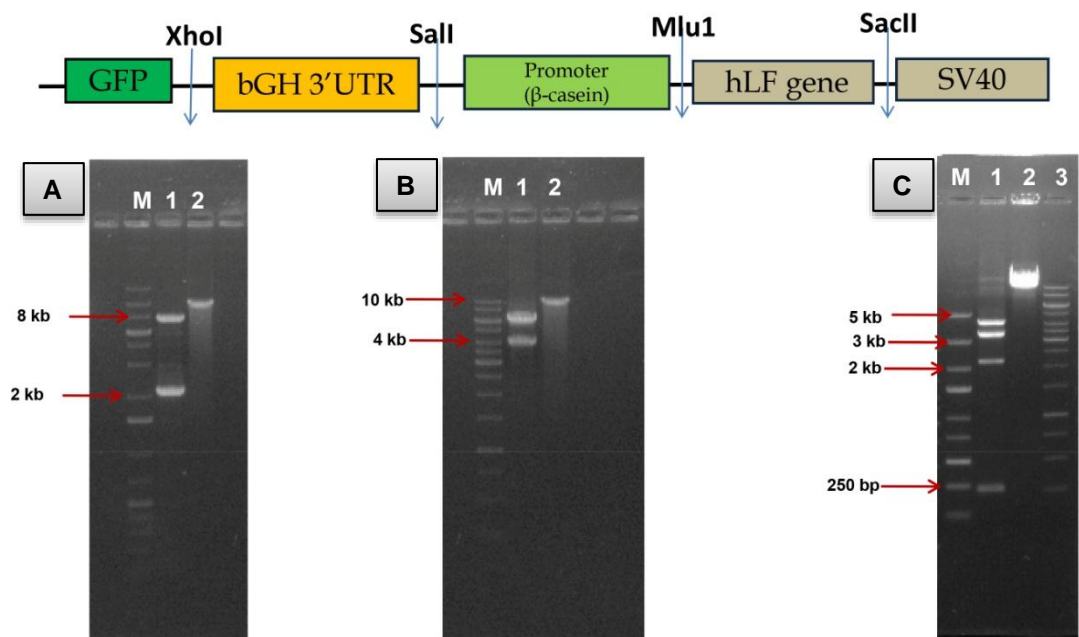


Figure 20: pβChLF gene construct : (A) Linearized pβChLF vector representing positions of all inserted fragments. (B) PCR amplification of all inserted fragments in pβChLF by different combination of primers as described in table followed (M: 1kb ladder)



**Figure 21: Restriction digestion of pβChLF gene construct :** Double Restriction enzyme digestion of pBCL1 construct (A) using XhoI, Sall yielding 250 bp bGH 3'UTR and 10.4 kb fragment (3.5 kb β-casein promoter, 4.7 kb vector, 2.2 kb hLF) (B) using XhoI and MluI yielding 250 bp bGH 3'UTR+ 3.5 kb β-casein promoter (3.75 kb) and 6.9 kb fragment (4.7 kb vector+2.2 kb hLF) (C) using Sall and SacII yielding 2.2 kb hLF+ 3.5 kb β-casein promoter (5.7 kb) and 4.95kb fragment (4.7 kb vector+250 bp bGH 3'UTR) (M: 1kb ladder)



**Figure 22: Restriction digestion of pβChLF gene construct :** Double Restriction enzyme digestion of pBCL1 construct (A) using MluI and SacII yielding 2.2 kb hLF and 8.45 kb fragment (3.5 kb β-casein promoter, 4.7 kb vector, 250 bp bGH 3'UTR) (B) using XhoI and SacII yielding 250 bp bGH 3'UTR+3.5 kb β-casein promoter+ 2.2 kb hLF (5.95 kb) and 4.7 kb vector (C) using XhoI, Sall, MluI and SacII yielding 250 bp bGH 3'UTR, 3.5 kb β-casein promoter, 4.7 kb vector, 2.2 kb hLF (M: 1kb ladder)

This vector construct was used for transfection of the mammary epithelial cell line in order to validate the expression of human lactoferrin and subsequently into goat fetal fibroblast cells so as to develop the transgenic cell lines for human lactoferrin which were used as donor cells in hand-guided cloning for development of transgenic goat embryos, expressing human lactoferrin gene.

The key step in the development of mammary gland-specific expression vectors is to select the appropriate regulatory elements. These regulatory elements should enable high and specific expression of exogenous gene in the mammary gland of transgenic animal with subsequent secretion of the desired protein in milk. The most effective regulatory elements would naturally be the promoter regions of proteins which are expressed in milk at appreciable quantities. Since caseins ( $\alpha$  and  $\beta$ ) and  $\beta$ -lactoglobulin are the main milk proteins, we used the promoter regions of these proteins in our expression vectors so as to achieve the mammary gland specific expression of human lactoferrin which was cloned directly underneath these promoter elements. The 5' regulatory regions of these genes have been widely used to regulate exogenous gene expression, specifically in mammary epithelial cells (VanKuik-Romeijn *et al.*, 2000; Wright *et al.*, 1991). These regulatory regions most often contained intron sequences and several studies have confirmed that these introns, especially the first intron, play an important role in foreign gene transcription and correct splicing (Palmiter *et al.*, 1982 and 1992). To ensure their activity in the final vector construct, we ensured their activity through PROMOTER 2.0 software. We further performed luciferase assay to ensure their activity as well as efficiency and observed that 3.5 kb and 6.5 kb  $\beta$ -casein promoter fragments had the highest and comparable efficiency and were the most powerful promoters in our study group. Further, due to shorter size of 3.5 kb fragment, we used this promoter region in our final recombinant expression vector (p $\beta$ ChLF) which was to be transfected into the goat mammary epithelial cells and goat fetal fibroblast cell line for production of transgenic cell lines. The amplification of these fragments was performed by specific primers carrying the appropriate RE sites for the later ligation with expression vector and hLF gene fragment. To construct the expression

vector, hLF gene fragment containing the compatible RE sites was ligated with the 5' flanking site of the promoter region already cloned into the expression vector. This resulted to development of an expression vector for hLF under the mammary gland specific regulatory elements. 3' UTR region of bovine growth hormone was used as it contains signals for mRNA termination and polyadenylation, thereby, leading to correct transcription termination and stable mRNA production of GFP gene.

The most common design for tissue specific vectors consists of a promoter region fused to gene of choice (genomic or cDNA sequence) and flanked at the 3' end by 3' untranslated DNA which contains signals for mRNA termination and polyadenylation. The gene construction is a fundamental element in the production of transgenic animals and consists generally of a promoter region, a gene that encodes the protein of interest and other regulatory elements to optimize gene expression (Maga and Murray, 1995). Promoters are sequences of few kilobases located close to the 5' end of the transcribed region of the gene (Houdebine, 2003). The selection of promoter element depends on the tissue or organ where the transgene is desired to be produced. Thus, in our case where human lactoferrin is desired to be produced in goat milk, we used mammary gland-specific promoters like  $\alpha$ - and  $\beta$ - caseins and  $\beta$ -lactoglobulin which function to produce milk proteins viz., casein and lactoglobulin in the milk. These promoters would direct the expression of the desired protein only in mammary gland during lactation. Thus, it could be expected that the expression of the transgene would be limited to the mammary gland to avoid the deleterious effects on the health of the animal.

In goats, various regulatory sequences of milk specific genes have already been isolated and verified in the production of transgenic animals. The most common of these sequences include murine WAP (Ebert *et al.*, 1991; Baldassarre *et al.*, 2004), goat  $\beta$ -casein (Ko *et al.*, 2000; Parker *et al.*, 2004), bovine  $\beta$ -casein (Lee *et al.*, 1997; Huang *et al.*, 1998) and goat and bovine  $\alpha$ -s1-casein (Freitas *et al.*, 2007). The transgene that codes for the protein of interest could be derived from another animal of the same species, different

species or even from other kingdoms like bacteria, yeast or plants (Pesqueiro *et al.*, 2007). The expression levels of recombinant protein vary from one bioreactor to another, which involves factors like expression vector, method utilized for transfer of genetic material, number of copies and site for insertion of the transgene (Rosen *et al.*, 1996). To date recombinant human lactoferrin has been expressed in several types of transgenic animals including mice, rabbit, cow and goat (Shi *et al.*, 2009; Li *et al.*, 2006; Yang *et al.*, 2008; Zhang *et al.*, 2008). The expression level of the desired protein varied dramatically in different transgenic animals, thereby making the analysis complex.

However, regardless of the mammary specific promoters or integration of the transgenic constructs, it has been found that the constructs which comprised of hLFcDNA always expressed at a relatively lower level (<1mg/ml). In contrast, rhLF is highly expressed by vectors containing the hLF genomic sequence. But the sequence is difficult to manipulate due to its large size (Yang *et al.*, 2008; Shi *et al.*, 2009). It is reported that gene introns might contain enhancer elements where the transcription factors can bind or essential elements needed for the stability of the transcribed DNA. Since, our main objective was to develop transgenic protocols in goat for the first time in the country, we used hLFcDNA and not the genomic DNA, as our main concern was the development of the protocols and not the expression level of the desired protein over which we would shift the attention later on.

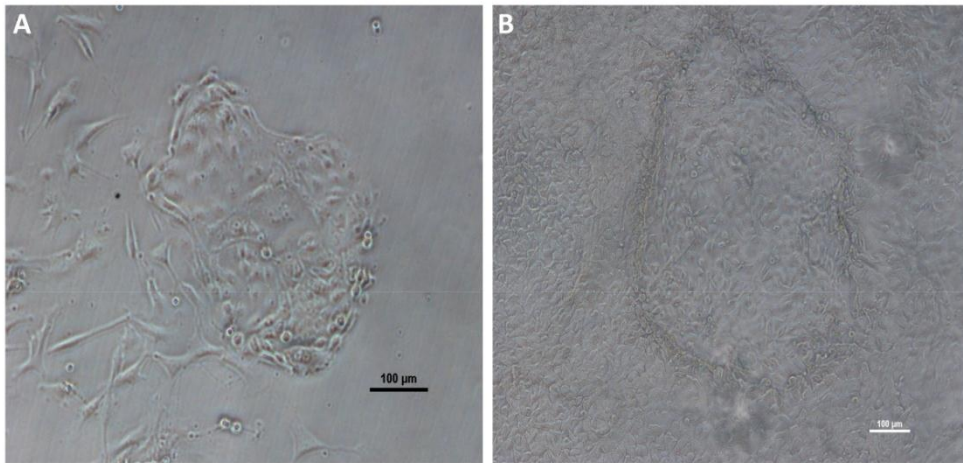
#### **4.6 Establishment of goat mammary epithelial cells (GMEC)**

The aim of the present study was to establish a platform where human lactoferrin would be secreted into the milk of the goat. This necessitates the verification of the *in vitro* expression of the transgene and proper screening of true transgenic cell lines to avoid the wastage of time, energy and resources at the end of the day. In true sense, transgene developed should be checked by transduction in mammary gland (Duchler *et al.*, 1998) but that would involve related ethical issues and long time period besides, the systemic effects of the procedure on the animals. A mammary epithelial cell line retaining mammary gland-specific functions can reasonably be used as a

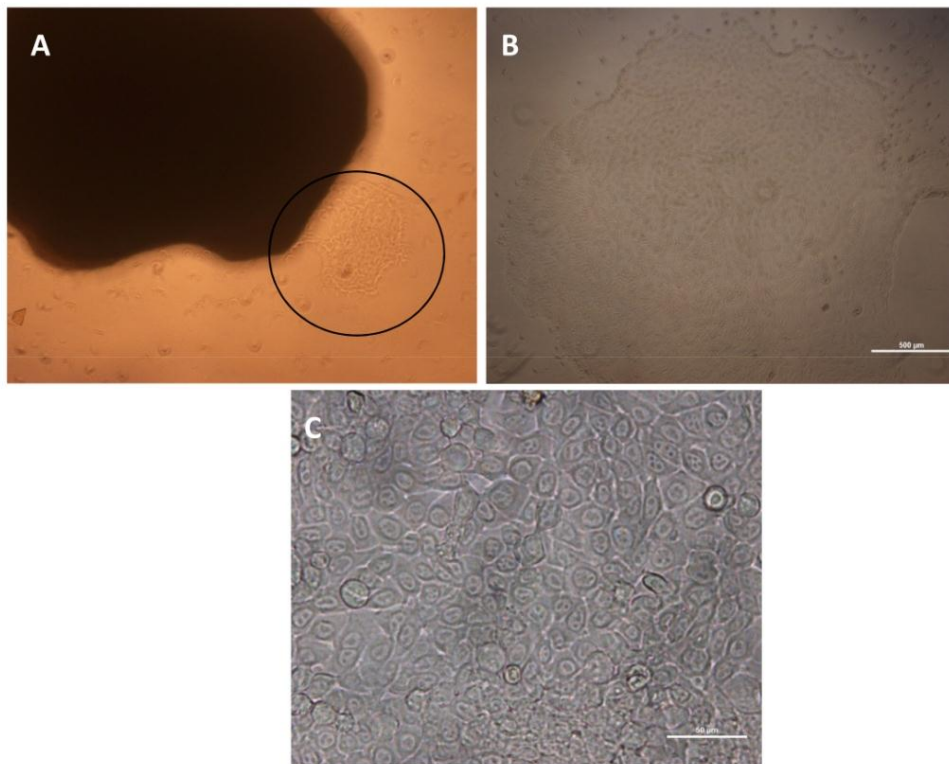
model to study the development, differentiation, and involution of mammary glands as well as an expression/ selection system for producing protein pharmaceuticals (Link *et al.*, 2006). Therefore, we developed goat mammary epithelial cell lines from lactating goat mammary epithelial tissue by both explant culture (Figure 23) as well as by digestion method (Figure 24). We were successful in developing the pure mammary epithelial cell lines by both the methods, though the cell lines derived by digestion method achieved faster confluence in earlier passages as compared to those derived by explant culture. The digestion method derived cell lines were also more homogenous and highly pure for mammary epithelial cells as compared to those derived by explants method. However, explants cultures were much easier and less time consuming than the digestion method. Explant culture also demanded no enzymatic treatments and were, thus, economical. One of the main disadvantages was that most of the explants failed to adhere. Keeping in view the obvious advantages, we preferred digestion method in our study for establishing the cell lines as used by many workers previously (Pantschenko *et al.*, 2000; Sun *et al.*, 2006; Tong *et al.*, 2012).

#### **4.7 Morphology of GMEC cells**

The primary cultures obtained contained a heterogenous population of cells which included fibroblast-like spindle shaped cells as well as setellate, polygonal and cuboidal cells (mammary epithelial cells). Morphology of the mixture of mammary epithelial cells and fibroblast cells is shown as Figure 23. These primary cultures were further enriched by differential digestion (selective detachment and attachment method) with Trypsin-EDTA in order to easily remove the fibroblast cell subpopulation as epithelial cells were found to be more resistant than fibroblast cells to treatment with trypsin and EDTA when the cells were detached from their substratum. By applying this strategy between subcultures 2 to 5, we were able to establish cultures containing only mammary epithelial cells. These cultures were positive for mammary epithelial cell markers and had growth kinetics and morphological features typical of goat mammary epithelial cell cultures (described later). The continuous



**Figure 23: Goat Mammary Epithelial Cell Colony from Digestion method on (A) Day 7 (10X) (B) Day 11 (10X)**

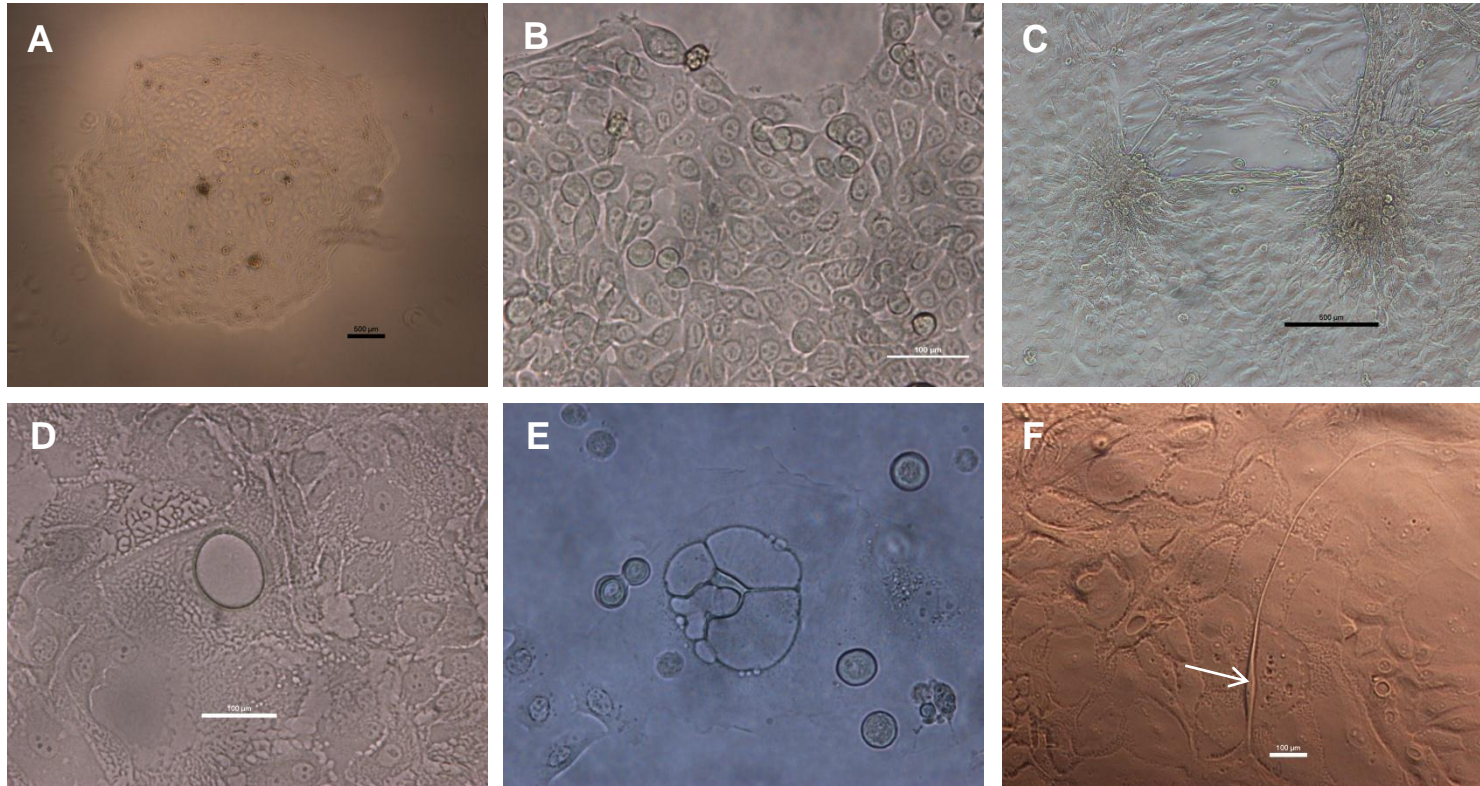


**Figure 24: Goat Mammary Epithelial Cell Colony from Explant on Day 8 (4X) (A), Day 11 (4X) (B), Day 11 (40X) (C)**

propagation (for more than 12 passages) and differential trypsinization of these cells on plastic substratum resulted in the selection of homogenous population of polygonal and cuboidal cells which formed monolayer island aggregates (Figure 25) with no evidence of contaminating stromal or myo-epithelial cells. When grown to confluency, the cells formed a monolayer and aggregated with the characteristic cobble-stone morphology of epithelial cells (Figure 25) in which cell nucleus and nucleoli were clearly visible. The post-confluent cultures (3-4 days after confluency) formed dome-shaped structures (Figure 25) initially where as lumen-like structures were observed later (Figure 25). Also, we could observe mammosphere-like (Figure 25) and duct-like structure (Figure 25). These cells could grow upto 25 passages without changes in growth properties or viability. GMECS were cryopreserved and maintained their growth characteristics without evidence of senescence after reviving them even after 24 months.

The purpose of establishment of mammary epithelial cell line in our study was to study the *in vitro* expression of the transgene so developed. We established mammary epithelial cell lines from a lactating gland tissue, considering the fact that the lactating caprine mammary gland retains its potential for proliferation (Knight and Peaker, 1984), in contrast to bovine mammary gland in which an intervening dry period is essential for glandular redevelopment between the successive lactations (Oliver and Sordillo, 1989). The isolated cells from mammary gland tissue were cultured and propagated in goat mammary epithelial cell culture medium, containing well described mammogenic factors like insulin and hydrocortisone. We used the established cell lines for limited number of passages (20-25) such that they more closely maintained the tissue-specific characteristics. The digestion method for obtaining mammary epithelial cell lines has been reported to lead to morphologically homogenous cells with fewer fibroblast and myoepithelial-like cells, as compared to explants culture or mincing of the tissue or only 1h digestion protocol (Pantschenko *et al.*, 2000).

During early passages we enriched the proportion of cells with epithelial morphology to purity based on their relative resistance to detachment by trypsinization and their ability to more rapidly attach to plastic substratum than cells with a setellatemyo-epithelial-like appearance. The cells which were to be the progenitors of the mammary epithelial cell line maintained a consistent proliferative capacity in medium with mammogenic factors (insulin and hydrocortisone) and did not show any evidence of senescence during successive subcultures. This demonstrates that we indeed cultured a population of epithelial cells which retained their proliferative capacity. Since, these cells are derived from a lactating rather than a developing caprine mammary gland, they are more likely to be responsive to the induction of lactation-specific protein expression. This ascribes to active promoters for milk protein like  $\alpha$ - and  $\beta$ -caseins and  $\beta$ -lactoglobulin. Thus, if such cell lines are transfected with expression vectors designed to express human lactoferrin under such promoters, the probability of achieving the expression of the desired proteins is much higher in comparison to other cell and promoter types. This can be related to report by Tong *et al.* (2012) who studied the lactating function of GMECs established from a lactating goat (DGMECs). They showed that DGMECs could secrete lactose and  $\beta$ -casein. Lactating function of the cells had no obvious change after 48 h treated by insulin, while prolactin could obviously raise the secretion of milk proteins and lactose. It has been reported that caprine mammary epithelial cells grow on plastic surfaces with epitheloid morphology and at low density form cobblestone monolayer aggregates (Pantschenko *et al.*, 2000), as observed in our cultures. The interesting observation was that despite through single cell suspension preparation prior to passage, the cells upon plating, predominantly formed island aggregates of 3-4 cells rather than attach in a random single cell pattern. Such morphological patterns were also observed by Pantschenko *et al.* (2000) in their studies. We also observed that the post-subcultured isolated cells and cell islands form pseudopodial extensions between neighbouring cells, suggestive of the production of some autocrine chemotactine factor production which facilitates the formation of cell groups.



**Figure 25: Morphology of GMEC cells: (A) Monolayer islands formed when grown at low density (40x); (B) Cobblestone morphology showing distinct round cell nucleus and nucleoli (200x); (C) Dome-shaped structures formed by confluent monolayer of GMEC (100x); (D) Lumen like structures (100x); (E) Mammosphere (100x); (F) Duct-like structure of GMECs (100x)**

With increasing density, the cells form lumen-like structures with elongated cells within the luminal area and elongated cells forming the boundaries where island groups converge. We also observed that the majority of the cells contained large and small vacuoles. Tong *et al.* (2012) established lactating dairy goat mammary epithelial cell line from goats at 35 d of lactation and reported similar results as ours.

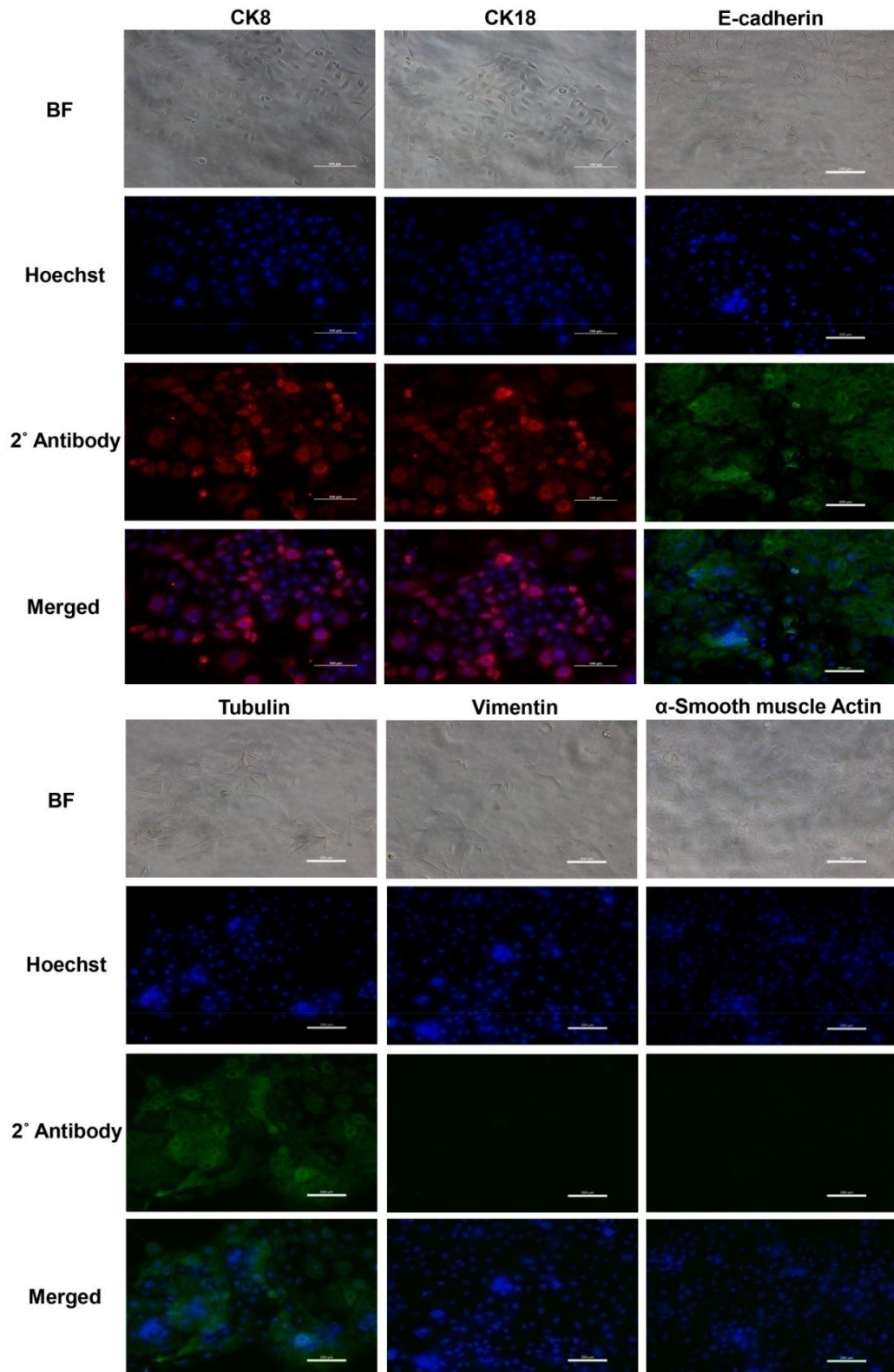
#### **4.8 Characterization of the goat mammary epithelial cell line by Immunofluorescence staining and RT-PCR**

The primary cell lines were characterized by Immunofluorescence staining between passages 5 and 20 for various mammary epithelial cell markers like Cytokeratin-8, Cytokeratin-18, E-cadherin and Tubulin. The cell lines were also stained for myo-epithelial cell marker,  $\alpha$ -smooth muscle Actin, and Vimentin, which is specific for stromal cells like fibroblast cells in order to ensure their absence in the cell population. We observed expression of such mammary epithelial cell specific proteins like Cytokeratin-8, Cytokeratin-18, E-cadherin and Tubulin in our established cultures in almost 100% of the cell population, while as very few or none of the cells stained positive for  $\alpha$ -smooth muscle Actin and Vimentin, reflecting to the absence of non-mesenchymal cells, like myoepithelial cell population as well as fibroblast cell population respectively, in our culture (Figure 26).

Cytoskeleton expression is important in identifying epithelial morphology, we investigated the cytoskeleton protein expression specific for epithelial lineage by immunocytochemistry. Cytokeratins are intermediate filaments of epithelial cells and are important in defining the cell phenotype (Bartek *et al.*, 1985). It has been reported that cytoskeleton filaments appear as interconnected bundles in the cytoplasm. The cytoskeleton network is denser around the nucleus, cytoplasmic vesicles and in the periphery of the cell where the filaments run parallel to the cell surface, which after several subcultures may reduce to the area surrounding the nucleus (Cifrain *et al.*, 1994). Cytokeratin 18 is normally associated with simple epithelium and all luminal epithelial cells of human mammary gland. Positive reaction of GMECs with anti-cytokeratin

18 antibody indicated their luminal epithelial lineage. It has been reported earlier (Pantschenko *et al.*, 2000) that cytokeratin expression is consistent with an epithelial cell line wherein the caprine mammary epithelial cell line was identified as more than 99% epithelial by a positive staining pattern with cytokeratin antibodies. We did not detect  $\alpha$ -smooth muscle antibody in GMECs, which confirmed the absence of myoepithelial cells. Our observation in GMECs is in line with the reported expression of cytokeratin 18 in goat (Pantschenko *et al.*, 2000), bovine (Schmid *et al.*, 1983) and human luminal MECs (Taylor-Papadimitriou *et al.*, 1989). Cadherin/ Cadherin-1 (E-CAD) is a transmembrane cell adhesion protein. It forms homophilic clusters between epithelial cells and is a critical component of adherens junctions. These junctions are critical for the maintenance of the epithelial barrier and epithelial tissue homeostasis. *In vivo*, a particularly important role for E-cadherin in epithelial biogenesis can be deduced from knockout experiments ablating E-cadherin in the skin, which disturbs the functional assembly of TJs (Tunggal *et al.*, 2005). Thus, expression of E-cadherin confirms the epithelial nature of GME cells. In contrast, we observed very low level staining of GMECs with vimentin. Though we did not observe vimentin expression in most of our analyzed cultures but vimentin staining has been reported to vary with culture conditions. This observation was similar to earlier report in caprine (Pantschenko *et al.*, 2000) and bovine (Hu *et al.*, 2009) where the vimentin staining was predominantly perinuclear with filament degradation.

Establishing optimal culture conditions to allow for protein synthesis in mammary epithelial cells is important, as it would more closely mimic the *in vivo* system. Upon RT-PCR analysis, we observed expression of mammary epithelial cell specific genes of milk proteins like  $\beta$ -Casein,  $\beta$ -lactoglobulin, *BTN1A1*, *ACACA* and cytoskeleton related genes *viz.* *Cytokeratin 8* and *Cytokeratin 18*, while no such bands were observed in fibroblast cell specific genes i.e. *Vimentin* and *FSP* as well as in RT-PCR negative reactions (Figure 27). This confirmed the ability of the isolated cells to synthesize mammary gland specific proteins. mRNA expression of milk proteins specific (*CSN2*, *BLG*, *BTN1A1*, *ACACA*) and mammary epithelial cells specific



**Figure 26: Immunocytochemistry for characterization of mammary epithelial cells. BF- Bright field images; Hoechst- Hoechst nuclear staining; 2° Ab– PE/ FITC- conjugated 2° Ab; Merged- merged images of 2° Ab and Hoechst (Magnification- 200X).**

(Cytokeratin-18, Cytokeratin-8) genes was observed in GMEC cells cultured in collagen coated dish with growth medium containing insulin, hydrocortisone, EGF and prolactin.

Previously some of these genes expression have been reported in bovine (Beswick and Kennelly, 1998; German *et al.*, 2002; Hu *et al.*, 2009; Zhao *et al.*, 2010; Monzani *et al.*, 2011) and pig (Kumura *et al.*, 2001; Sun *et al.*, 2005) mammary epithelial cells. Epithelial cell differentiation is characterized by expression of milk proteins, such as  $\beta$ -casein and whey acidic protein, production of milk fats rich in triglycerides, sources of energy and essential fatty acids (Aoki, 2006). Acetyl-CoA carboxylase (ACACA) enzyme is active in the lactating mammary gland and its activity level is affected by dietary and hormonal states of the animal (Abu-Elheiga *et al.*, 2000; Mao *et al.*, 2001). Butyrophilin (BTN1A1), a major milk-fat-globule transmembrane glycoprotein, is also a mammary gland specific protein in milk-fat secretion expressed on the apical surface of the mammary epithelial cells in the final stage of pregnancy and during lactation (Mather and Jack, 1993; Banghart *et al.*, 1998; Szyda and Komisarek, 2007). The expression of milk proteins like,  $\beta$ -Casein and  $\beta$ -lactoglobulin, as examined by RT-PCR by our cell lines reflects to the process of lactogenesis occurring within them, the primary purpose for which mammary epithelial cell lines are mostly established. These results likely reflect to the retaining of biological function in the mammary epithelial cells in our culture conditions, which may be representative of a semi-differentiated proliferating cell type found within the lactating gland.

#### **4.9 Chromosomal analysis of the goat mammary epithelial cell lines and sex determination by PCR**

Since the mammary gland to establish mammary epithelial culture was obtained from slaughterhouse, the confirmation of the species becomes very important. Moreover, the sex of the cells was determined using karyotyping and PCR. Upon karyotyping all the cell lines revealed a stable and normal female karyotype (58+XX) throughout the culture period (Figure 28) which is consistent with the normal diploid chromosome number for caprine species

(Mensher *et al.*, 1989). This also suggests that the cell lines maintain a stable and normal DNA content in the culture conditions and thus, could be presumed to be non-transformed cell lines. For sex determination of the cells using PCR, genomic DNA was extracted from GMECs and subjected to amplification using *SRY* and *PLP* genes (Figure 29). Specific amplification of only *PLP* gene which is present on X chromosome and non-amplification of *SRY* gene (present on Y chromosome) indicated that the cells obtained were of female origin.

#### **4.10 Comparison of growth kinetics of mammary epithelial cells on different matrices**

To determine the best niche for growth of mammary epithelial cells so that they secrete larger quantities of proteins, we further cultured the single cell suspension obtained after 3h digestion on different substrate types: i) bare (non-coated) plastic surface; ii) Extracellular cell matrix (ECM) coated surface; iii) Collagen coated surface; and iv) Gelatin coated surface. The cells, initially seeded at 4000 cells per well of a 24-well plate, were cultured for a period of 9 days, the time till they were near-confluent and cell count was taken on a daily basis using hemocytometer. We observed that cultures seeded on ECM-coated surface had fastest doubling time and thus, achieved confluence faster than collagen- and Gelatin-coated surfaces. The highest doubling time and hence, the slowest growth rate was observed on non-coated plastic surfaces, indicating the need of ECM coat for better and happy growth (Figure 30).

#### **4.11 Determination of relative cell viability of goat mammary epithelial cells on different matrices**

The relative cell viability of mammary epithelial cells was examined by determination of their metabolic activity by MTT assay. It was seen that the relative cell viability of mammary epithelial cells was significantly higher when cultured on ECM-coated surface as compared to gelatin- coated, collagen-coated and uncoated surfaces (Figure 31).

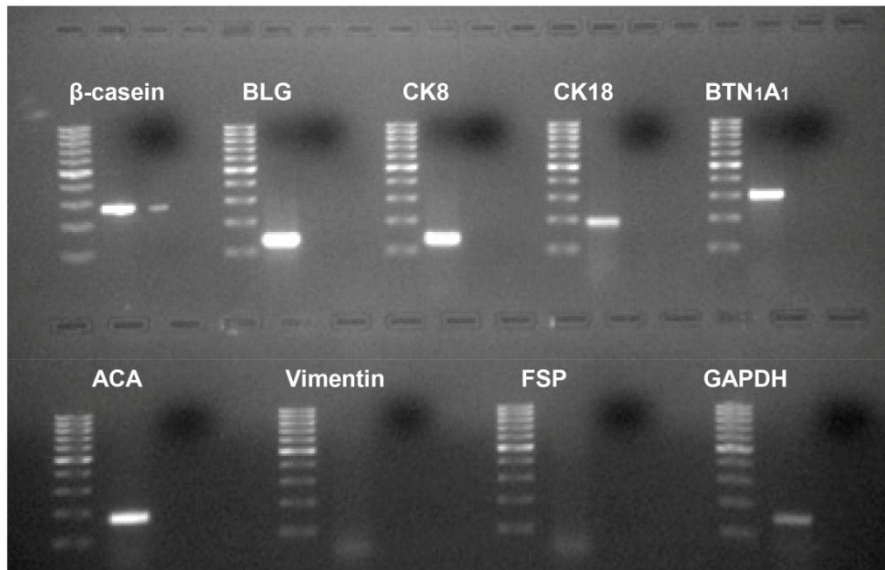


Figure 27: Characterization of goat mammary epithelial cells by RT-PCR

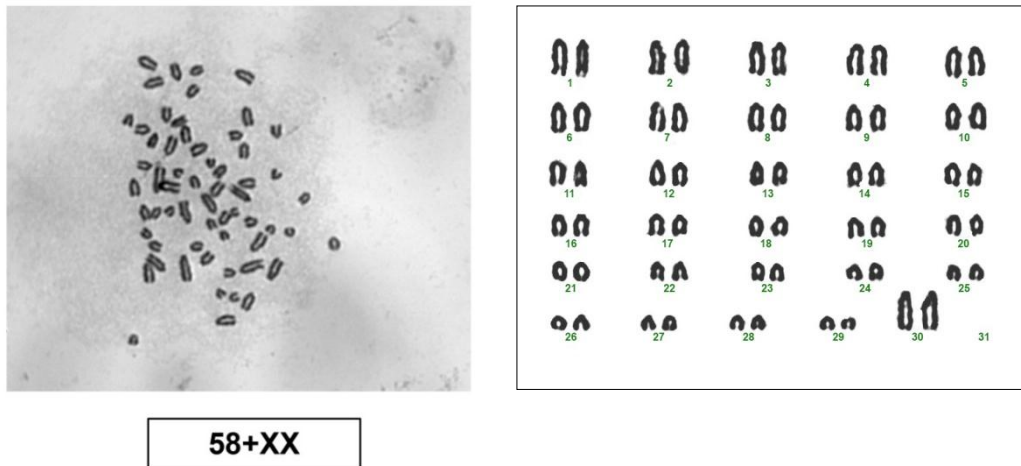


Figure 28: Chromosomal analysis of goat mammary epithelial cells depicting 58 + XX karyotype

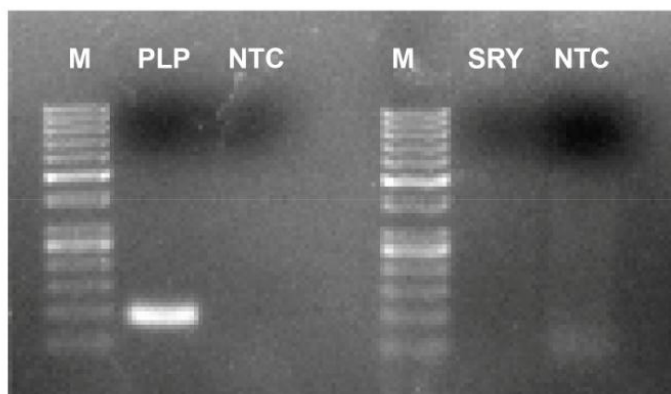


Figure 29: Sex confirmation of goat mammary epithelial cells by PCR by *SRY* and *PLP* gene amplification from genomic DNA Panel 1 - *PLP* gene amplification; Panel 2 - *SRY* gene amplification (M: 50bp DNA ladder).

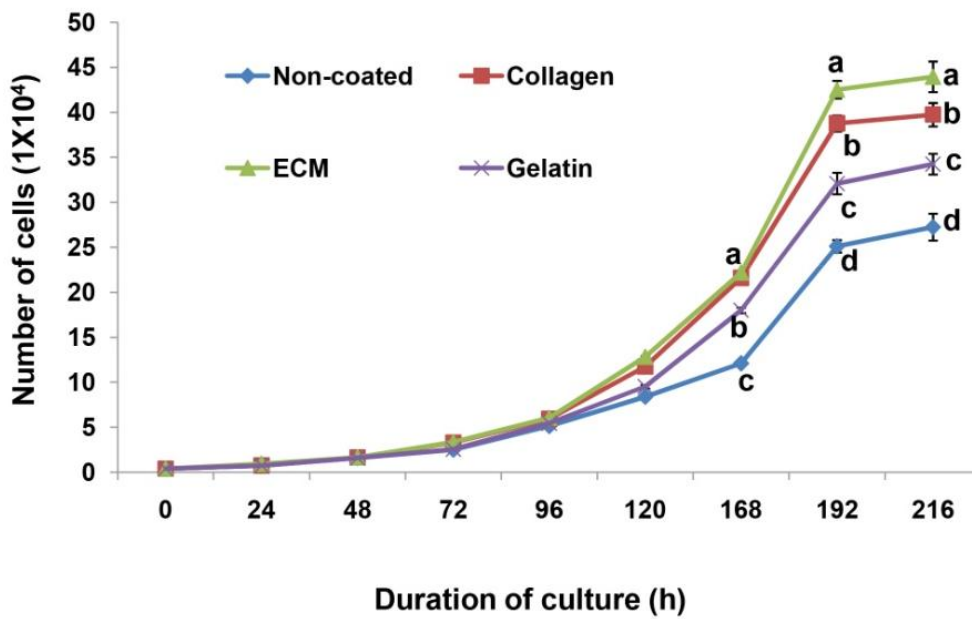


Figure 30: Proliferation rate of goat mammary epithelial cells on different matrices. Points with different superscripts differ significantly (P<0.05)

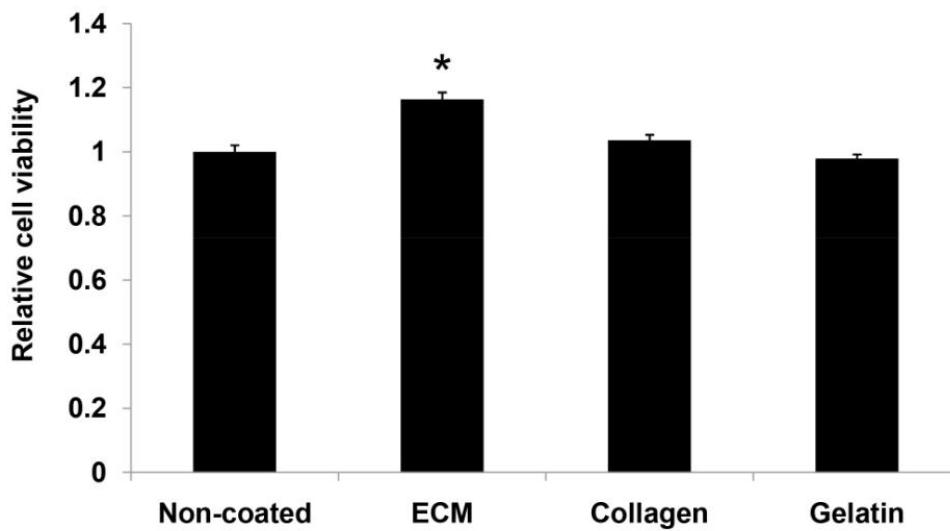


Figure 31: Relative cell viability of goat mammary epithelial cells on different matrices as determined by MTT assay. Bars with different superscripts differ significantly (P<0.05).

The influence of tissue microenvironment on cellular function and development is well recognized. Mammary epithelial cells have been shown to synthesize and secrete basement matrix components when cultured on plastic substratum. These components include laminin (Streuli and Bissell, 1990), collagen type IV, fibronectin and sulfated glycosaminoglycans (Parry *et al.*, 1985). It has also been proposed that the optimal expression of lactation specific proteins requires not only basement membrane but also stromal matrix which supports differentiation by organizing the basement membrane. This facilitates the interaction between extracellular matrix and the cell's integrin receptors (Howlett and Bissell, 1990) and allows for change in cell shape for enhanced cell-to-cell interaction. The coating of plastic surface with collagen and/or other matrix proteins thus, provides a basement membrane somewhat more similar to *in vivo* mammary gland microenvironment for cell-to-cell interaction as compared to the bare plastic surface, thereby leading to better growth and milk protein expression, as observed in our study.

#### **4.12 *In vitro* expression of the transgene construct in goat mammary epithelial cells**

##### **4.12.1 Optimization of the transfection of goat mammary epithelial cells**

The characterized goat mammary epithelial cell lines were transfected with the expression vector p $\beta$ ChLF by both lipofection and nucleofection (Figure 32) and analyzed by Flow cytometry for the efficiency of transfection. For this, the cells of the same passage were seeded for nucleofection as well as lipofection. After 48 hrs of culture, for nucleofection the cells were trypsinised and subjected to nucleofection using Nucleofection kit (Lonza, Germany) as per the manufacturer's instructions. The nucleofection pulse named EL110 worked the best for transfection into GMECs (known after standardization). For lipofection the cells were transfected using Fugene HD (Promega, USA) as per the protocol described. After 48 hrs of nucleofection and lipofection, the cells were trypsinised and prepared as single cell suspension and subjected to flow cytometry, the parameters for which were set by using non-transfected cells as control to define the measurement and gating parameters (Figure 33). The FACS results show that for mammary epithelial cells, **nucleofection** worked better as can be inferred from Table 4.4

**Table 4.4: Transfection efficiency of different methods in GMECs.**

Method of transfection	Transfection efficiency (%)
Lipofection	12.2 ± 1.6 <sup>a</sup>
Nucleofection	23.0 ± 1.2 <sup>b</sup>

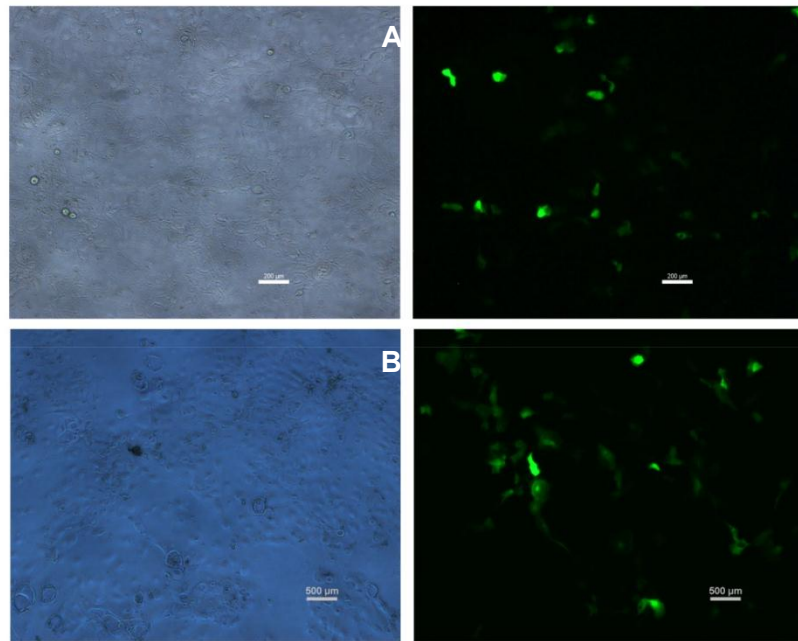
Values are Mean ± SEM.

Values with different superscript within the same column differ significantly ( $P < 0.05$ ).

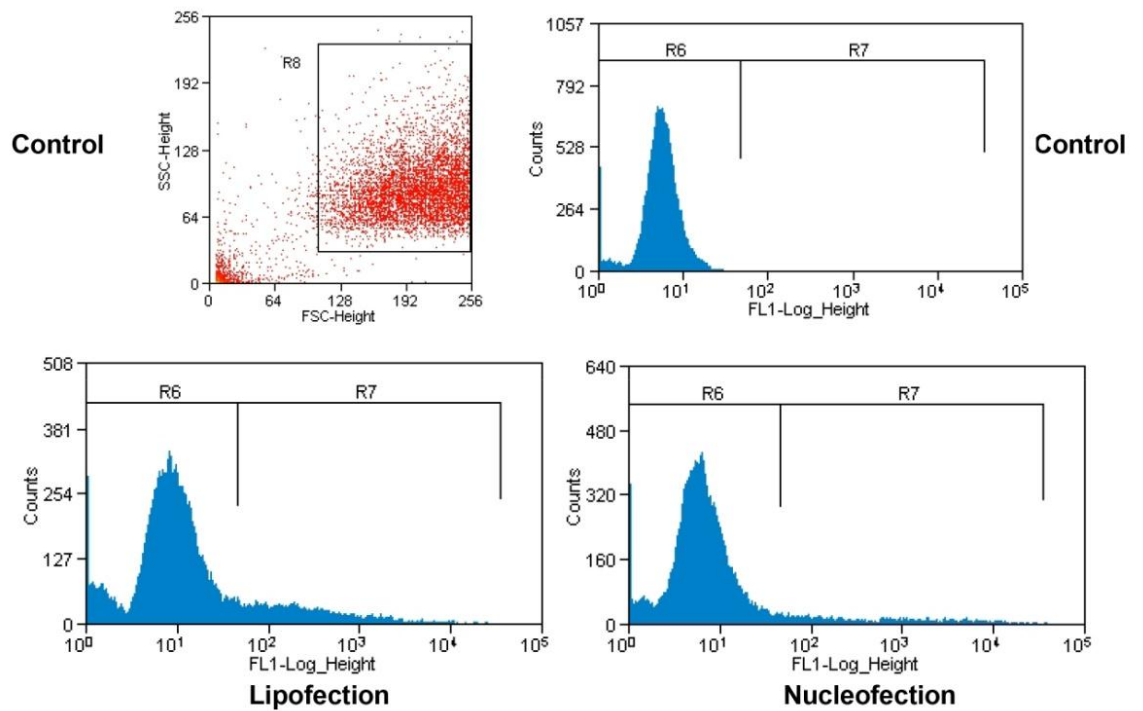
Efficient gene transfer into primary cells has been a challenge for quite some time. Available viral methods are complex and labor intensive, whereas non-viral techniques usually fail to transfect primary cells efficiently. Nucleofection has been reported by several authors as a valuable technique to efficiently transfer the gene constructs into the cells. Gresch *et al.* (2004) demonstrated that the Nucleofector technology represents a system that allows efficient and rapid transfection of a broad range of primary cells from different species in a reproducible manner besides hard-to-transfect cell lines. Distler *et al.* (2005) found Nucleofection as a highly efficient transfection method for primary human keratinocyte. Kurosawa *et al.* (2012) suggested that nucleofection based non-viral gene transfer is useful method for systematic generation of human gene-knockout cell lines.

#### 4.12.2 Optimization of working selection concentration of geneticin

The selectable drug chosen for selection of stably transfected cells depends on the selectable marker present in the expression vector. In our case, pAcGFP1-C1 was used as backbone vector which contained the Neomycin resistant gene to create selection pressure for mammalian cells. Therefore, the transfected cells were selected using Geneticin for stable transfection. For using any selectable marker, the optimum dose must be determined. The optimization of dose of geneticin was done by trypsinizing the mammary epithelial cells and plating in the growth medium containing different doses of Geneticin along with negative control with the omission of G418. The cells were observed daily and the minimum dose which killed maximum non-transfected cells was 500 µg/ml



**Figure 32: Expression of GFP in goat mammary epithelial cells after transfection via Lipofection (A: Bright field image; A': under blue filter) and Nucleofection (B: Bright field image; B': under blue filter) (Magnification: 100X)**



**Figure 33: Comparison of transfection efficiency of lipofection and nucleofection for goat mammary epithelial cells by Flow Cytometry**

(Figure 34). Various authors have used Geneticin concentration from 400 to 600 µg/ml (Liu *et al.*, 2013; Elenbaas *et al.*, 2001).

#### **4.12.3 GFP expression in transfected goat mammary epithelial cells**

Goat mammary epithelial cells were transfected with expression vector pβChLF by nucleofection using P3 Primary Cell 4D-Nucleofector® X (Lonza, Switzerland) according to the manufacturer's recommendations. After 48 hrs of nucleofection, expression of GFP in the cells was monitored under an inverted UV microscope (Nikon Ti-Eclipse, Tokyo, Japan). After 48 hrs of nucleofection, the cells were trypsinised and seeded in the growth medium containing growth factors alongwith 500 µg/ml of Geneticin. This was repeated until a pure population of transfected GMECs was obtained at Passage 8 (Figure 35). The GFP expressing mammary epithelial have been established in porcine (Zheng *et al.*, 2006, 2010), goat (Zheng *et al.*, 2010) for the transfection efficiency detection and transgene expression in mammary epithelial cells.

#### **4.13 Analysis of human lactoferrin production in mammary epithelial cells**

##### **4.13.1 Reverse transcriptase-polymerase chain reaction analysis**

Total RNA was extracted from transfected mammary epithelial cells at different passages (P3, P5 and P8) and cDNA was prepared using RevertAid Kit (Thermo Scientific, USA). Prepared cDNA was subjected to RT-PCR using primers specific for human lactoferrin. It was found that human lactoferrin mRNA was present at all the passages but the expression was found to be decreasing with increasing passage number (Figure 37). This might be due to the fact that transgene was transiently expressed in cytoplasm and nucleus but did not get integrated into the genome.

##### **4.13.2 Immunocytochemical analysis**

Transfection of expression vector into the goat mammary epithelial cells resulted into the translation of hLFcDNA and thus, production of human lactoferrin. This was evident as the Immunofluorescence staining of

transfected GMECs showed positive signals against both Anti-GFP and Anti-human lactoferrin antibodies (Figure 36) while no signal was observed in non-transfected cells. Antibody used against human lactoferrin (Millipore, USA) did not have species cross reactivity against goat and thus, it was made sure that the expression detected was of human lactoferrin and not goat lactoferrin. This staining showed that the human lactoferrin cDNA of the recombinant expression vector was getting transcribed into mRNA and then translated into the protein which again confirms we were heading onto the correct path leading to transgenesis in goats.

#### **4.13.3 Western blot analysis of recombinant human lactoferrin secreted by transfected GMEC cells**

In order to make sure that recombinant human lactoferrin so produced will be secreted into the milk; we did western blotting to confirm the presence of human lactoferrin in the secreted form in the culture medium. Cells were cultured for 24 hrs in medium without FBS. The proteins were concentrated using Amicon Ultra Centrifugal Filters with 10 kDa cut off. The proteins were then fractionated on SDS-PAGE and blotted onto a PVDF membrane and subjected to anti-human lactoferrin polyclonal antibody. 80 kDa lactoferrin was found to be present in cell extract of transfected GMECs as well as secreted in the medium supplemented with prolactin which works as an inducer for  $\beta$ -casein promoter (Figure 38). In contrast, control GMEC represented no human lactoferrin protein band.

#### **4.13.4 Enzyme linked immunosorbent assay (ELISA)**

Transfected mammary epithelial cells were grown in 35 mm dishes. Cells were washed three times with serum-free medium and incubated in 2 ml of serum-free medium for 24 hrs. After 24 hrs, conditioned media was collected, and human lactoferrin levels were measured using a human lactoferrin ELISA kit (animal serumfree) that detects human lactoferrin with no cross reactivity to goat or bovine. Conditioned medium from transfected cells were assayed for

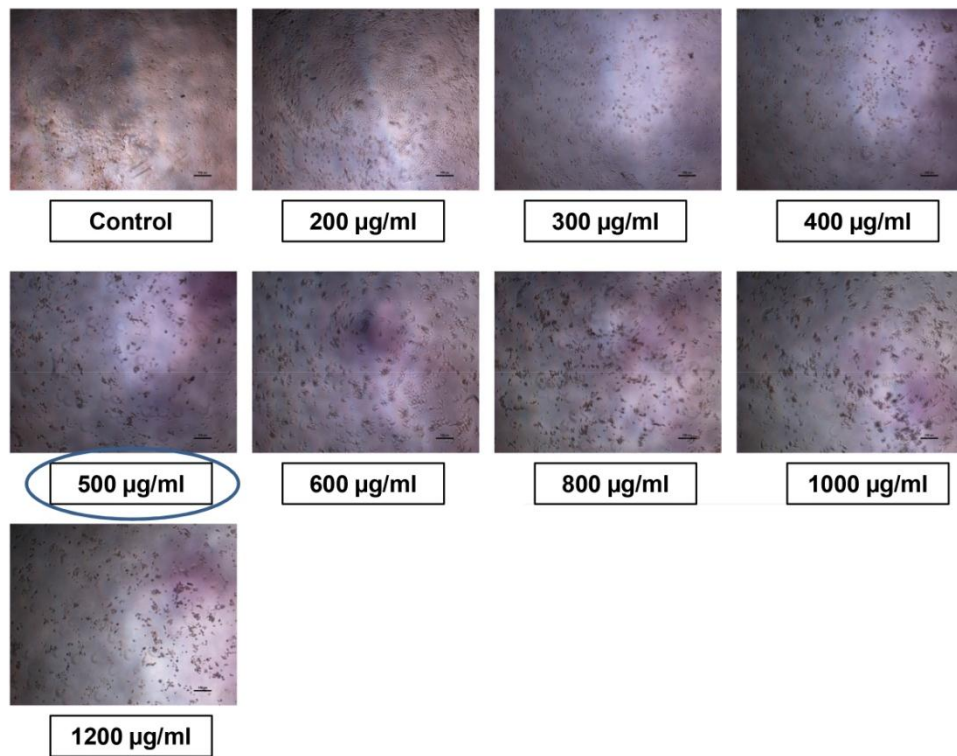


Figure 34: Optimization of G418 selection drug dose for goat mammary epithelial cells by titration.

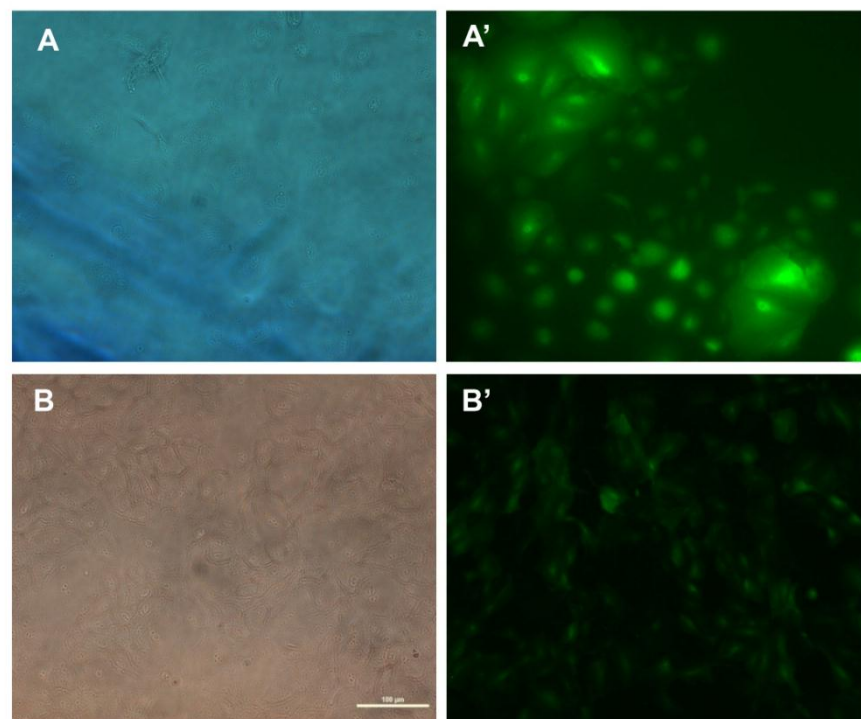
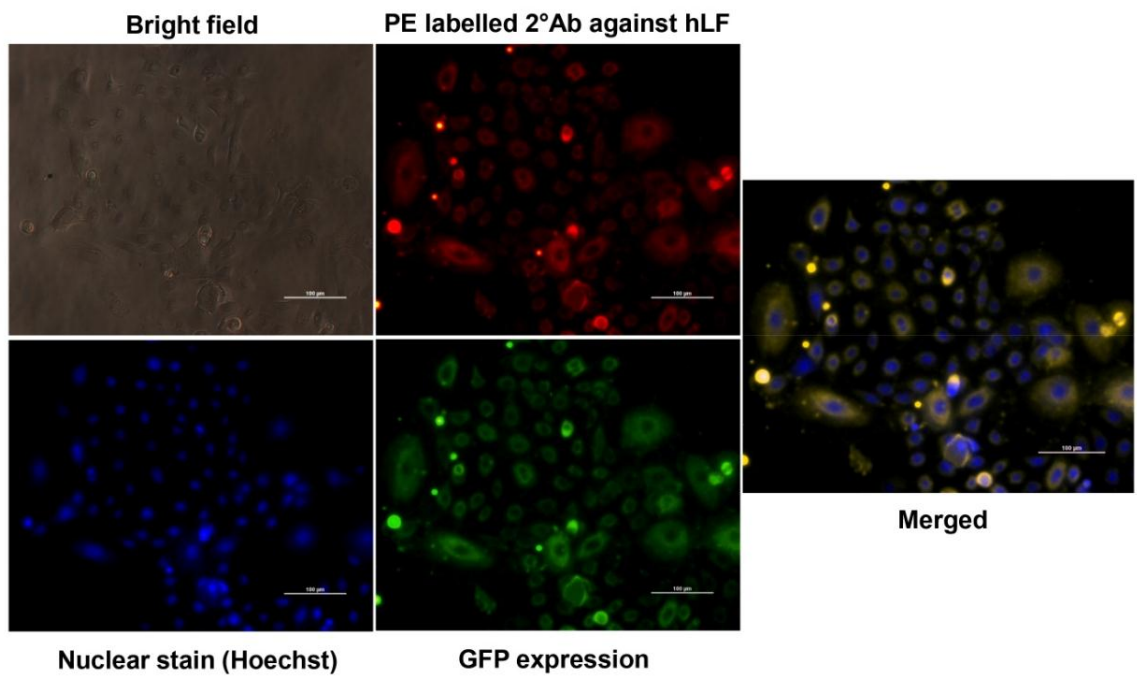
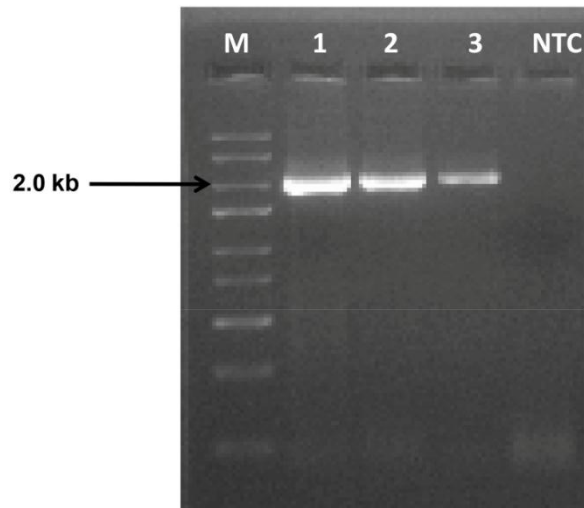


Figure 35: Transfection of pβChLF expression vector into goat mammary epithelial cells by Nucleofection; A, A': GMEC colony at Passage 6 after G418 selection (200X); B,B': Transfected GMEC population at Passage 8 (100X)



**Figure 36:** Immunocytochemical analysis for human Lactoferrin protein expression in transfected GMECs, 2 days after transfection. (Magnification- 100X, Scale bar- 100µm).



**Figure 37:** RT-PCR for human Lactoferrin expression in transfected GMECs at Passage3 (Lane 1) , Passage5 (Lane 2) and Passage 8 (Lane 3)

human lactoferrin and was found to contain 0.07- 0.11 ng/ml human lactoferrin (Figure 39).

All the experiments carried out indicated that 80 kDa human lactoferrin was being produced in a secretory form by goat mammary epithelial cells. To the best of our knowledge, this is probably the first report in which *in vitro* expression of human lactoferrin has been analyzed in goat mammary epithelial cells after transfecting with non-viral method of transfection. Earlier, Han *et al.* (2007) demonstrated that the direct transduction of goat mammary epithelial cells with adenoviral vector containing human lactoferrin resulted in transient expression of recombinant human lactoferrin at high concentration of 2 g/l. Also, expression of human lactoferrin was analyzed in bovine mammary epithelial cells by Shu *et al.* (2007). Many authors have analyzed the expression of human lactoferrin in the milk of transgenic goats (Zhang *et al.*, 2008; Yu *et al.*, 2012), mice (Nuijens *et al.*, 1997; Ward *et al.*, 1997; Kim *et al.*, 1999; Shi *et al.*, 2009). Transgenic lactoferrin (rhLF) was first produced in mice, with secretion into the milk, by using either hLF cDNA or genomic hLF sequences fused to regulatory elements of the bovine  $\alpha$ S1-casein gene. The concentrations of recombinant hLF in the milk ranged from 0.1  $\mu$ g/mL to 36  $\mu$ g/mL (Platenburg *et al.*, 1994).

Thus, we were able to prepare a transgenic construct for human lactoferrin under mammary gland-specific promoter which was verified for *in vitro* expression in goat mammary epithelial cells. As stated above, most of the work has been done on the milk secreted by transgenic animals which is a later stage. Had there been something wrong in the design of the construct, it would have come into notice at a very later stage or it would not have come into notice at all. In our approach, the transgene expression was first verified and then it was used to produce transgenic animal which ensured that our transgene construct was designed in appropriate manner and was enabling mammary epithelial cells to secrete human lactoferrin.

#### **4.14 Establishment of transgenic goat fetal fibroblast cell line.**

After confirming *in vitro* expression of human lactoferrin in mammary epithelial cells, due to following reasons, we established transgenic goat fetal fibroblast cell line: 1.) mammary epithelial cells are hard-to-transfect cells and thus, the expression of human lactoferrin was only transient 2.) the nuclear reprogramming efficiency of fetal fibroblast cells is better than mammary epithelial cells (discussed later).

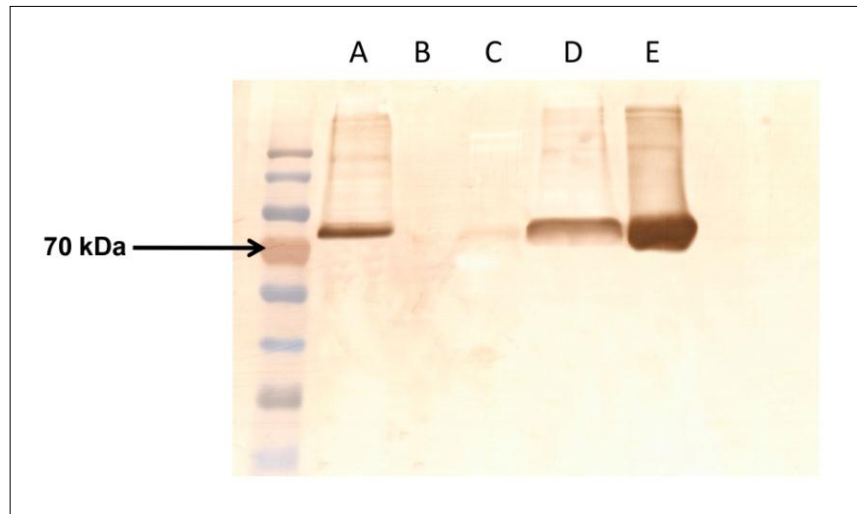
##### **4.14.1 Establishment of goat fetal fibroblast cells**

The fetal ear skin explants attached to culture flask surface within 3-4 h. Fibroblast cells started migrating from the explants after 2-3 day incubation interval. The migrating cells started proliferating by cell division and within 7-10 days occupied almost whole available surface of the T25 culture flask (Figure 40). The explants were removed and the primary cultures were subcultured in a split ratio of 1:4. Also, the cells at early passages, notably at passage 2, were cryopreserved in order to ensure a uniform and homogenous cell population, as and when required.

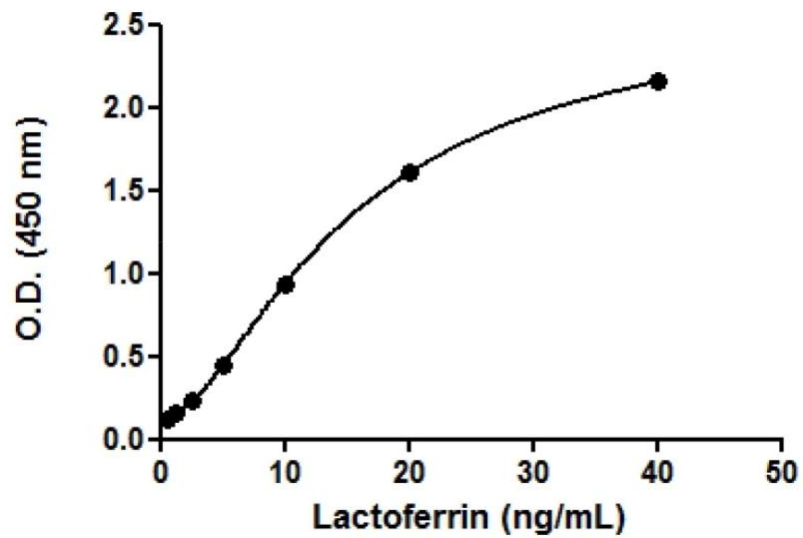
##### **4.14.2 Characterization of goat fetal fibroblast cells by immunostaining and RT-PCR**

Somatic cells isolated from ear explant were characterized by examining their morphology and expression of cytoskeletal markers by immunofluorescence staining. The ear skin-derived cells were found to express vimentin and Tubulin but not keratin and cytokeratin-18 indicating that these cells were of fibroblast nature (Figure 41). This is in agreement with many studies carried out earlier in our laboratory using buffalo ear skin-derived cells as donor cells in which these were found to be of fibroblast nature (Golla *et al.*, 2012; Selokar *et al.*, 2012).

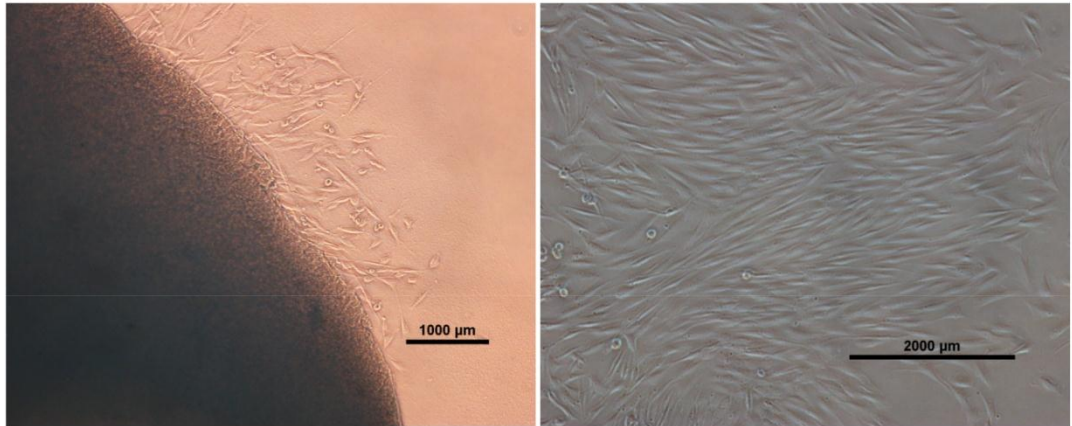
Further, the characterization was done by RT-PCR for markers like *FSP-1*, *DES-2*, *Vimentin*, *Tubulin*, *CK8* and *CK18*. The specific amplification of *FSP-*



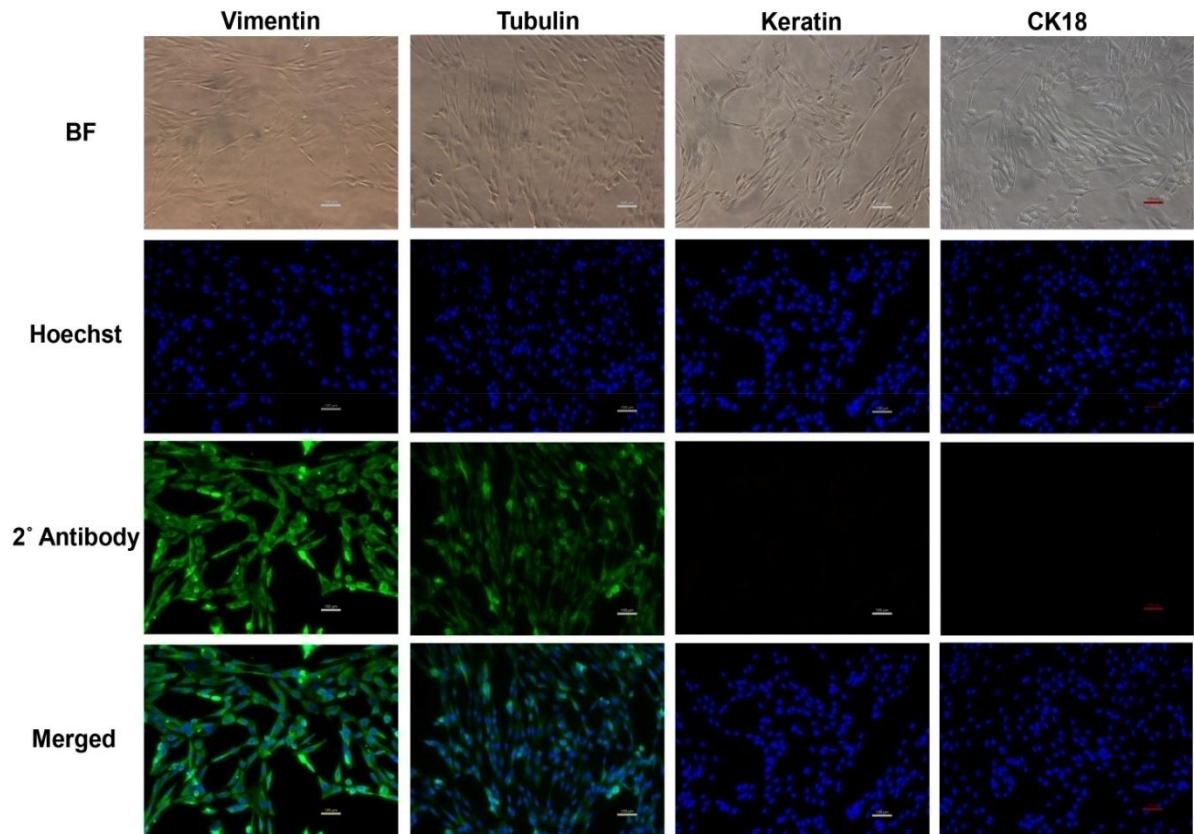
**Figure 38:** Western blot showing (A) presence of human lactoferrin in cell lysate of transfected GMECs; (B) absence of human lactoferrin in cell extract of non-transfected GMECs; (C) human lactoferrin secreted in medium supplemented with Growth factors; (D) Human lactoferrin from blood as positive control



**Figure 39:** Analysis of human lactoferrin protein expression in transfected GMECs. Total human lactoferrin protein in the conditioned media of transfected cells as determined by ELISA was found to be 0.07- 0.11 ng/ml



**Figure 40: Establishment of goat fetal fibroblast primary culture: Outgrowth of goat fetal fibroblast cells from ear skin tissue explant on Day 4 (100X) (A); Confluent monolayer of fibroblast cells (100X) (B)**



**Figure 41: Immunocytochemistry for characterization of goat fetal fibroblast cells. BF- Bright field images; Hoechst- Hoechst nuclear staining; 2° Ab-FITC- conjugated 2° Ab; Merged- merged images of 2° Ab and Hoechst (Magnification- 200X).**

1, *Desmin*, *Vimentin* and *Tubulin* and the absence of bands for PCR product corresponding to *CK 8* and *CK18* confirmed the fibroblast nature of the cells (Figure 42). FSP-1 is a fibroblast specific marker which was identified and characterized by Strutz *et al.* (1995). Through subtractive and differential hybridization for transcript comparison between murine fibroblasts and isogenic epithelium, they observed that the promoter/ enhancer region driving this gene is active in fibroblasts but not in epithelium, mesangial cells or embryonic endoderm. During development, FSP1 is first detected by *in situ* hybridization after day 8.5 as a post-gastrulation event, and is associated with cells of mesenchymal origin or of fibroblastic phenotype and thus, it can be used as a marker for identifying fibroblast cells. Desmin is another intermediate-filament associated protein specific for fibroblast cells (Lazarides and Balzer, 1978; Schmid *et al.*, 1982). Thus, through Immunofluorescence staining and RT-PCR, it was confirmed that the cells obtained were of fibroblastic nature.

#### **4.14.3 Chromosomal analysis of the goat fetal fibroblast cells and sex determination by PCR**

Upon karyotyping all the cells revealed a stable and normal female karyotype (58 +XX) throughout the culture period (Figure 43) which is consistent with the normal diploid chromosome number for caprine species (Mensher *et al.*, 1989). For sex determination of the cells using PCR, genomic DNA was extracted from GMECs and subjected to amplification using *SRY* and *PLP* genes (Figure 44). Specific amplification of only *PLP* gene (present on X chromosome) and non-amplification of *SRY* gene (present on Y chromosome) indicated that the cells obtained were of female origin. Sex determination of the cells is a crucial factor since the sex of the offspring depends on the sex of the donor cell. Thus, in our case, the human lactoferrin is intended to be produced in the milk of the transgenic, therefore, it was a mandatory condition that the sex of the donor cell must be female.

#### 4.14.4 Development of transgenic goat fetal fibroblast cells

The characterized goat fetal fibroblast cells were transfected with the expression vector  $\beta$ ChLF by both lipofection and nucleofection (Figure 45) and analyzed by Flow cytometry for the efficiency of transfection. For this, the cells of the same passage were seeded for nucleofection as well as lipofection. After 48 hrs of culture, for nucleofection, the cells were trypsinised and subjected to nucleofection using Nucleofection kit (Lonza, Germany) as per the manufacturer's instructions. The nucleofection pulse named EN150 worked the best for transfection into GFFs (known after standardization). For lipofection the cells were transfected using Fugene HD (Promega, USA) as per the protocol described. After 48 hrs of nucleofection and lipofection, the cells were trypsinised and prepared as single cell suspension and subjected to flow cytometry, the parameters for which were set by using non-transfected cells as control to define the measurement and gating parameters (Figure 46). The flow cytometry results show that for fetal fibroblast cells also, **nucleofection** worked better as can be inferred from Table 4.5. As observed from above, EN150 protocol of Nucleofection worked the best for fetal fibroblast cells. The only drawback of the nucleofection is the high mortality associated with it but in this case, it was observed that 90% of the cells remained viable out of which more than 50% of the cells were found to be transfected. The low efficiency of transfection is directly related to the size of the transgene construct. Therefore, in our case, it could be assumed that due to very large size of transgene construct, the efficiency could reach only upto 50%.

**Table 4.5: Transfection efficiency of different methods in goat fetal fibroblast cells.**

Method of transfection	Transfection efficiency (%)
Lipofection	18.5 $\pm$ 2.5 <sup>a</sup>
Nucleofection	52.7 $\pm$ 1.9 <sup>b</sup>

Values are Mean  $\pm$  SEM.

Values with different superscript within the same column differ significantly ( $P < 0.05$ ).

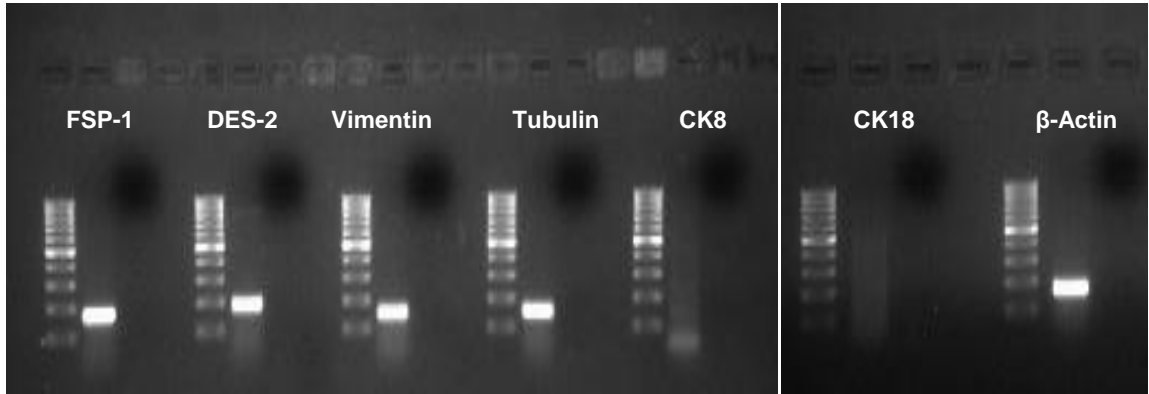


Figure 42: Characterization of goat fetal fibroblast cells by RT-PCR for genes specific to fibroblast and epithelial cells.

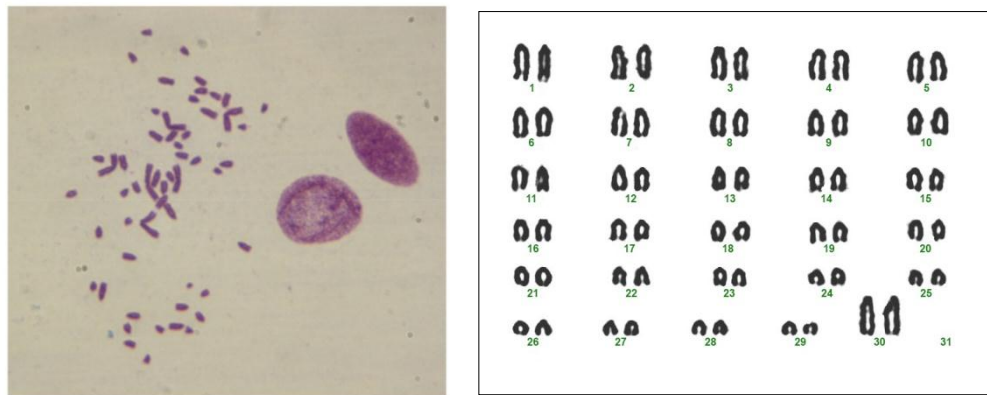


Figure 43: Chromosomal analysis of goat fetal fibroblast cells depicting 58 + XX karyotype

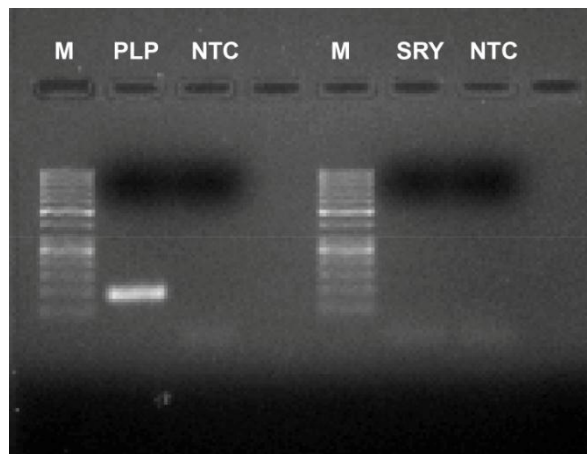
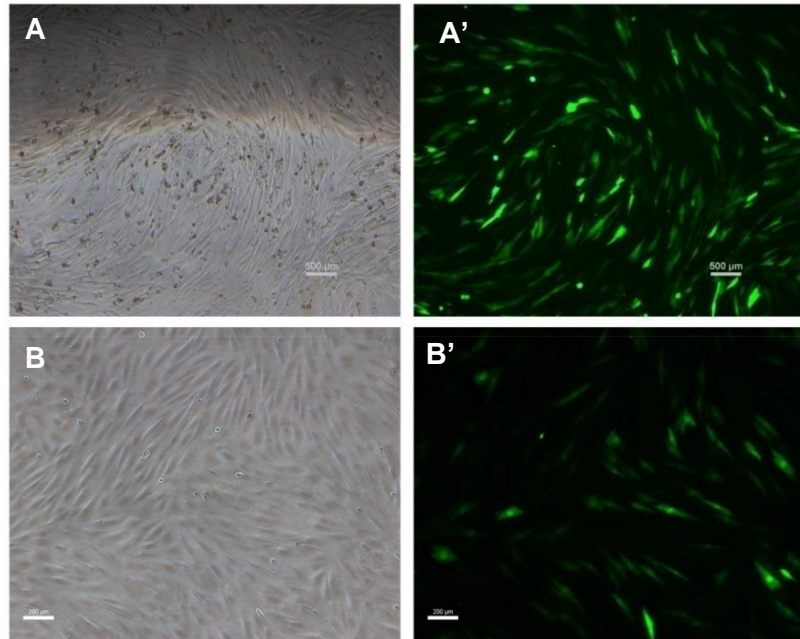
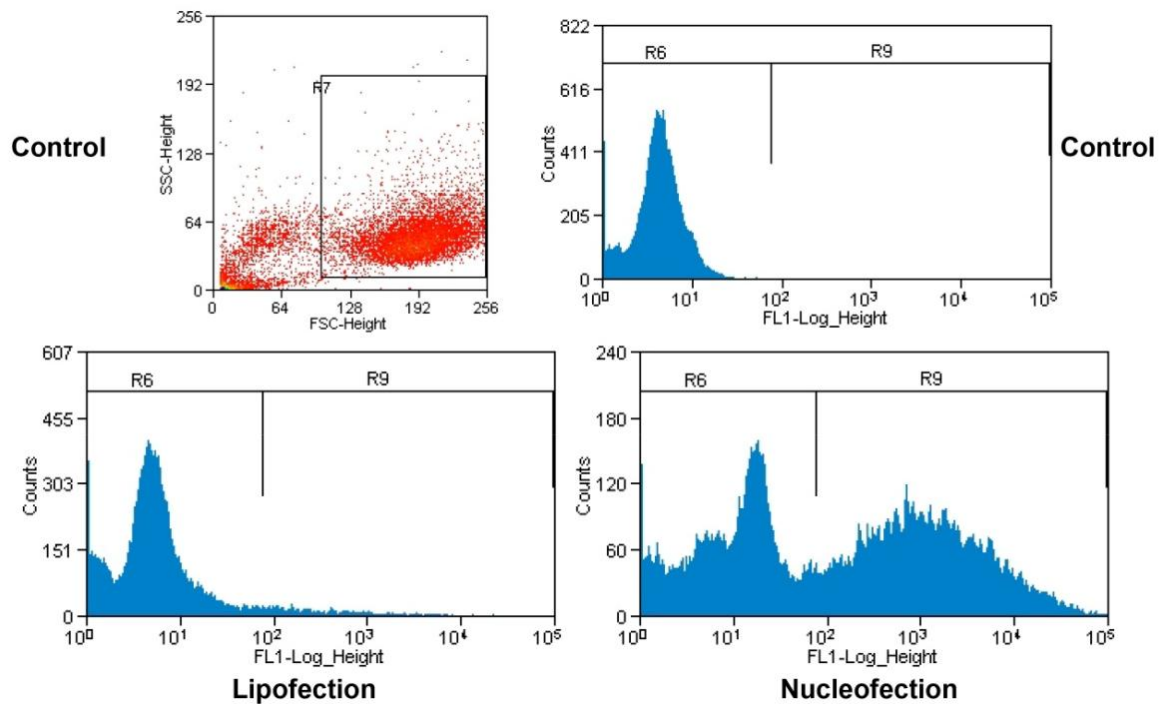


Figure 44: Sex confirmation of goat fetal fibroblast cells by PCR by *SRY* and *PLP* gene amplification from genomic DNA. Panel 1 - *PLP* gene amplification; Panel 2 - *SRY* gene amplification (M: 50 bp DNA ladder).



**Figure 45: Expression of GFP in goat fetal fibroblast cells after transfection via Lipofection (A: Bright field image; A': under blue filter) and Nucleofection (B: Bright field image; B': under blue filter) (Magnification: 100X)**



**Figure 46: Comparison of transfection efficiency of lipofection and nucleofection for goat fetal fibroblast cells by Flow Cytometry**

The transfected cells were trypsinised after 48 hrs of transfection and plated in the medium containing selection drug Geneticin@ 600 µg/ml. The dose of the G418 was optimized for goat fetal fibroblast cells as described for mammary epithelial cells in section 4.5.2. The cells were subpassaged at 70-80% confluency and kept under selection pressure of G418 for 8 weeks in order to obtain pure population of transfected goat fetal fibroblast cells at passage 8 (Figure 47).

#### **4.14.5 Detection of transgene integration into the transfected goat fetal fibroblast cells by PCR**

To confirm the integrity of the expression vector into the genome of transfected cells, RT-PCR for the human lactoferrin was performed. We observed specific bands in genomic DNA of transfected cells, while no such bands were observed in negative and non-transfected controls (Figure 48). This integration gave an assurance that the embryos developed after HMC would be transgenic embryos containing human lactoferrin gene.

Zhang *et al.* (2010) developed a mammary-specific expression vector for hGCcase and transfected into HC-11 mammary epithelial cells for bioactivity analysis *in vitro*; mRNA transcripts and hGCcase protein were correctly expressed in transfected HC-11 cells and subsequently introduced this gene construct into fetal fibroblasts (from dairy goats) to prepare competent transgenic donor cells. Similarly in our study, after developing the transgene construct and verification of *in vitro* functioning in goat mammary epithelial cells, the construct for human lactoferrin was transfected into the goat fetal fibroblast cell line and thus, transgenic cell line was established.

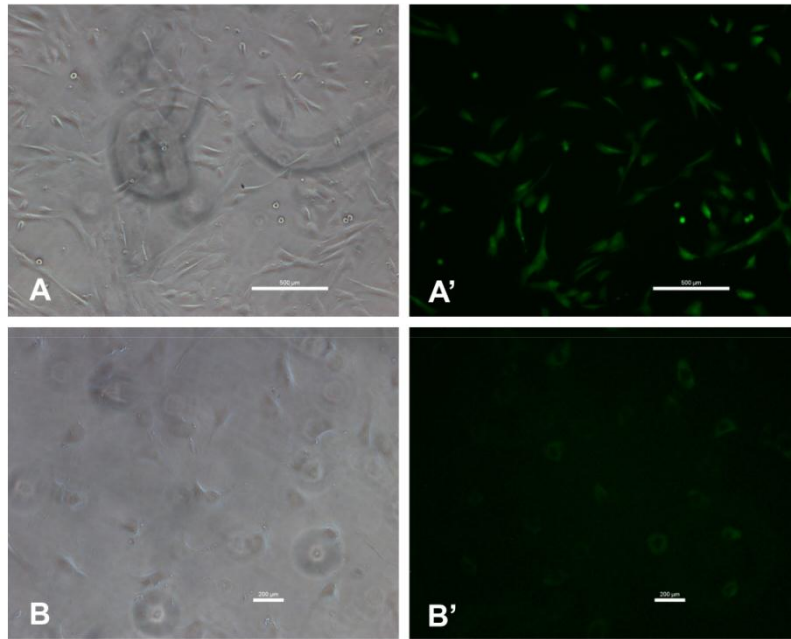
#### **4.15 To develop transgenic goat embryos containing human lactoferrin gene**

After confirming the proper functioning of transgene construct developed *in vitro* in goat mammary epithelial cells, to achieve the target of large scale

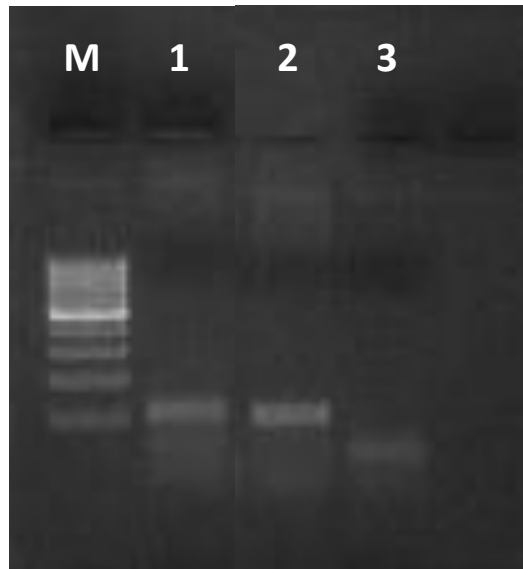
production of human lactoferrin, a method would have to be chosen wherein the protein will be secreted in large quantities. As compared to microbial and animal cell bioreactors, production of proteins of pharmaceutical importance in the milk of transgenic animals has gained popularity amongst scientists all over the world as it circumvents the improper post-translational modifications of proteins in microbial system as well as high cost and smaller quantities of proteins produced in animal cell culture system. Transgenic goats have been produced to date by pronuclear microinjection and somatic cell nuclear transfer (SCNT). In Brazil, the first goats were born from the microinjected embryos (Freitas *et al.*, 2003) and, later the birth of the first transgenic goat in Latin America was achieved (Freitas *et al.*, 2007). While successful, the traditional gene transfer by pronuclear injection had limited efficiency (Ebert and Schindler, 1993) and frequently generated mosaic founder animals (Wilkie *et al.*, 1986; Burdon and Wall, 1992; Whitelaw *et al.*, 1993). The use of recombinant somatic cell lines for NT allows the introduction of transgenes by traditional transfection methods, increases the efficiency of transgenic animal production to 100%, and overcomes the problem of founder mosaicism. Transgenic goats have been produced by SCNT by Behboodi *et al.* (2005), Zhang *et al.* (2010), Yu *et al.* (2012) and Yekta *et al.* (2013). Therefore, we decided to produce transgenic cloned goat embryos using the modified SCNT i.e. Hand made cloning which does not require use of sophisticated micromanipulators and the skilled person (Figure 49).

#### **4.15.1 Production of transgenic goat embryos by Hand-made cloning using different type of donor cells**

The type of donor cell used is one of the important factors affecting the efficiency of cloning. We used the goat mammary epithelial cells and goat fetal fibroblast cells as donor cells for hand made cloning. The developmental competence was compared by recording the cleavage and different stages that the cloned embryos developed to at Day 8 (Day 0 = day of IVC).



**Figure 47: Pure population of transfected goat fetal fibroblast cells after G418 selection at Passage 5 (A,A'; magnification-100X) and Passage 8 (B,B'; magnification-200X)**



**Figure 48: PCR amplification of human lactoferrin demonstrating integration of transgenic construct in genomic DNA of transfected fetal fibroblast cells (Lane 1: P8 Transgenic fetal fibroblast cell line; Lane 2: Positive control; Lane 3: Negative control)**

## Production of transgenic goat embryos by HMC

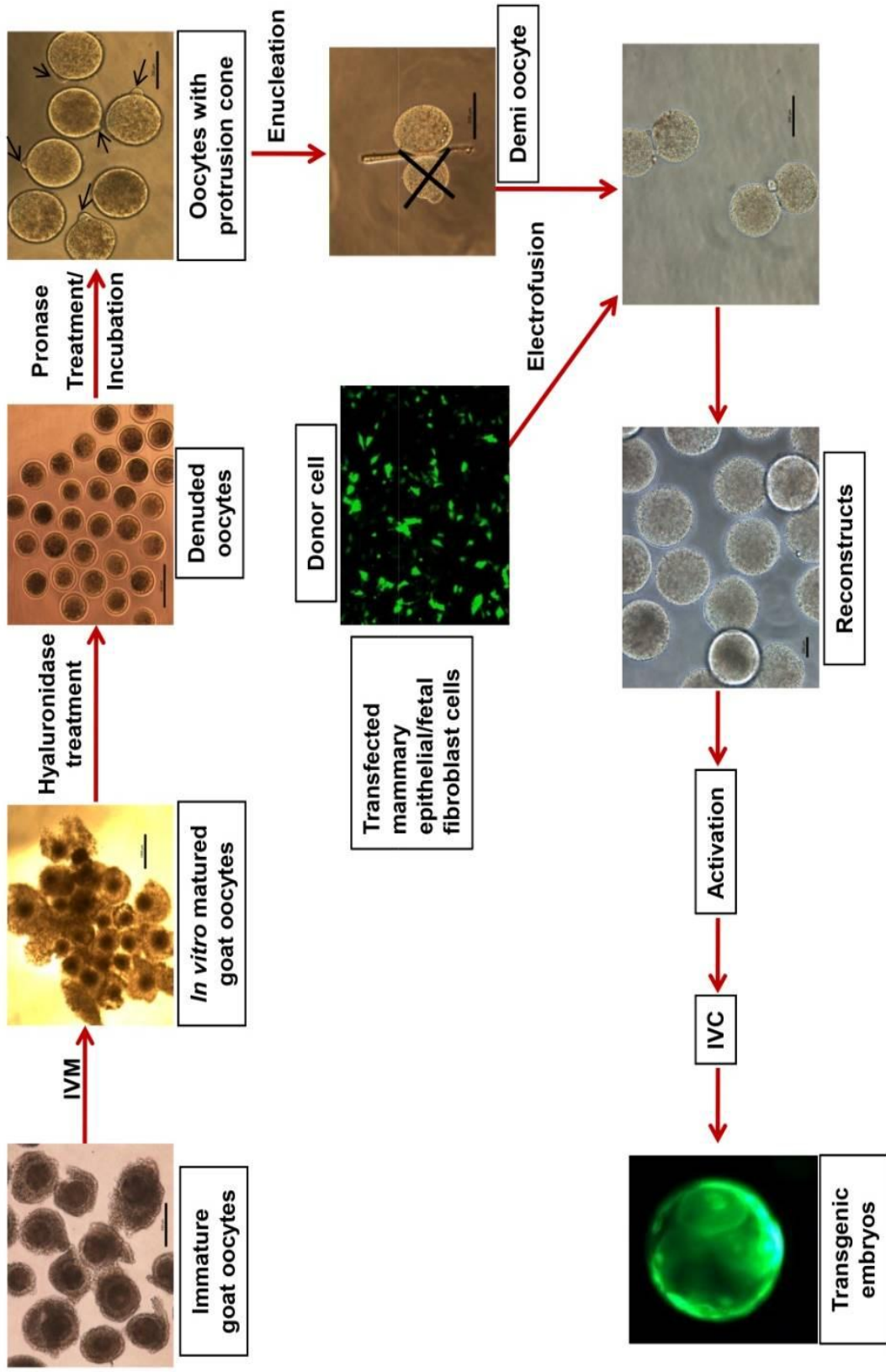


Figure 49: Pictorial representation of the technique of Hand-made cloning

**Table 4.6: Developmental competence of hand-made cloned goat embryos produced from different types of donor cells.**

Donor cell type	Reconstructed embryos	Cleaved embryos n (%)	4-cell stage embryos n (%)	8- to 16-cell stage embryos n (%)	Morulae n (%)
Fetal Fibroblast	191	167 (90.3±2.04)	136 (73.28±6.03)	100 (53.67±3.65)	38 (20.51±0.67) <sup>a</sup>
Mammary Epithelial	152	130 (89.5±2.93)	103 (70.97±3.24)	73 (50.56±3.59)	22 (15.34±1.65) <sup>b</sup>

Values are Mean±SEM.

Values with different superscript within the same column differ significantly ( $P < 0.05$ ).

A comparison of the developmental competence of different groups of cloned embryos produced using different types of donor cells at same passage (P4) and same cell cycle stage (G0/ G1) revealed that among the cloned embryos produced using fetal fibroblast cells and mammary epithelial cells, the cleavage rate (90.3±2.04% and 89.5±2.93%, respectively), the percentage of reconstructs that developed to 4-cell stage (73.28±6.03% and 70.97±3.24%, respectively) and 8-to 16-cell stage (53.67±3.65% and 50.56±3.59%, respectively) was not significantly different (Table 4.6). However, the percentage of reconstructs that developed to morulae was significantly lower ( $P < 0.05$ ) for cloned embryos produced using mammary epithelial cells (15.34±1.65%) than that for embryos produced from fetal skin fibroblast cells (20.51±0.67%). Various types of somatic cells have been used for production of cloned goats *viz.* caprine mammary gland epithelial cells (Yuan *et al.*, 2009), fetal fibroblast cells and granulosa cells (Keefer *et al.*, 2002). In almost all of the species in which transgenic clones have been produced, fetal fibroblast cells have been used as sources of donor nuclei in the production of transgenic animals because they grow rapidly and have the potential for multiple cell divisions before undergoing senescence in culture (McCreath *et al.*, 2000; Kuhholer *et al.*, 2001). The effect of differentiation status of donor cell on cloning efficiency is well documented. Briggs and King, (1952) reported a gradual decline in cloning efficiency with an increase in donor cell differentiation state. Blastomeres or embryo derived cells were observed to

provide better cloning efficiency as compared to somatic cells isolated from adult individuals. Kato *et al.* (1998) noted that clones derived from adult cells frequently aborted in the later stages of pregnancy, and calves developing to term showed a higher number of abnormalities than did those derived from newborn or fetal cells. Forsberg *et al.*, (2002) reported that, embryos cloned from fetal cells produced higher pregnancy and calving rates than those from adult cells. All these studies support our findings and we could conclude, in this study, that goat fetal fibroblast cells had better reprogramming efficiency as compared to goat mammary epithelial cells as evidenced from the results shown in Table 4.6 and hence, would serve as better donor cells for transgenic goat embryo production.

#### 4.15.2 Production of transgenic goat embryos expressing human lactoferrin gene by Hand-made cloning

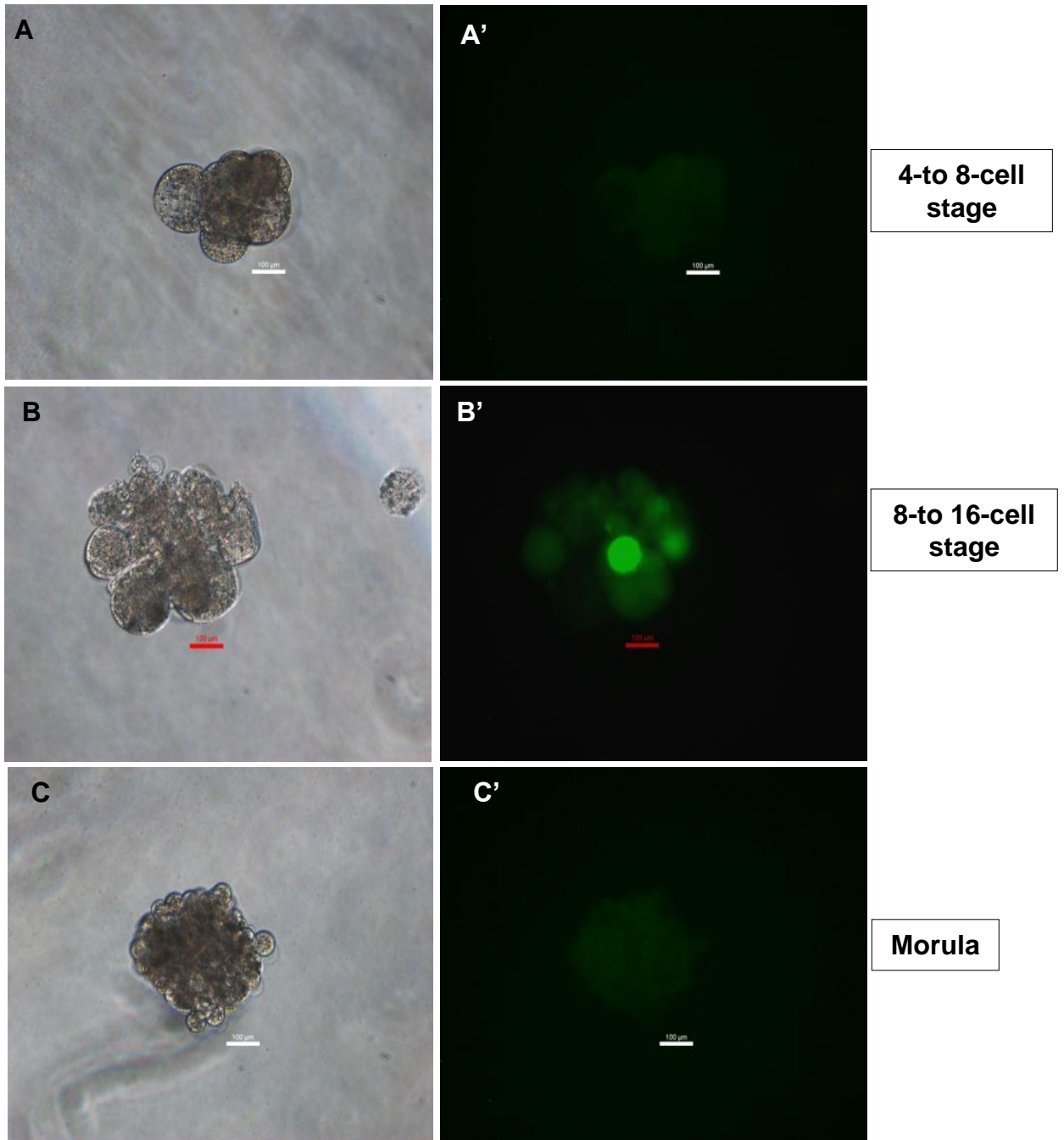
After finding the better prospects of using goat fetal fibroblast cells as donor cells for hand-made cloning, we used transgenic goat fetal fibroblast cell line in which the transgene construct integration was confirmed by PCR as described in section 4.14.5 for producing the cloned transgenic goat embryos, expressing human lactoferrin. The number of reconstructs developed, cleavage rate, number of 2-cell, 4-cell, 8-16-cell, morulae and blastocyst stage embryos developed in hand-guided cloning from using fetal fibroblast both control and transfected (Figure 50), is given in Table 4.7.

**Table 4.7: Developmental competence of hand-made cloned goat embryos produced from different types of donor cells.**

Donor cell type	Reconstructed embryos	Cleaved embryos n (%)	4-cell stage embryos n (%)	8- to 16-cell stage embryos n (%)	Morulae n (%)
Fetal Fibroblast	326	277 (87.78±2.71)	237 (74.54±3.67)	204 (63.88±3.06)	67 (21.03±0.55) <sup>a</sup>
Transgenic fetal fibroblast	253	207 (85.43±1.27)	174 (71.75±1.85)	146 (60.42±1.29)	39 (16.07±0.96) <sup>b</sup>

Values are Mean ± SEM.

Values with different superscript within the same column differ significantly ( $P < 0.05$ ).



**Figure 50:** Transgenic cloned goat embryos containing human lactoferrin gene expressing GFP under Blue light (A'-C') and under bright light (A-C) (200x).

It was found that despite the changes accompanying transfection and integration of the foreign DNA, the cleavage rate ( $87.78\pm 2.71\%$  and  $85.43\pm 1.27\%$ , respectively), the percentage of reconstructs that developed to 4-cell stage ( $74.54\pm 3.67\%$  and  $71.75\pm 1.85\%$ , respectively) and 8-to 16-cell stage ( $63.88\pm 3.06\%$  and  $60.42\pm 1.29\%$ , respectively) was not significantly different (Table 4.7). However, the percentage of reconstructs that developed to morulae was significantly lower ( $P < 0.05$ ) for cloned embryos produced using transgenic fetal fibroblast cells ( $16.07\pm 0.96\%$ ) than that for embryos produced from control cells i.e. non-transfected fetal fibroblast cells ( $21.03\pm 0.55\%$ ). An interesting observation while screening of GFP expressing embryos was that in all the embryos, GFP was expressed (Figure 50) and thus, visible only at 8-to16 cell stage and not at 2-cell or 4-cell stage. The appropriate reason for this comes from MZT i.e. Maternal-zygotic transition. This also indicates that the transgene had become the part of the genome and is getting transcribed and translated when the genome of the zygote becomes active.

Transgenic animal mammary gland bioreactors are desired for the production of recombinant protein for medical purposes (Wall *et al.*, 1996; Wright *et al.*, 1991; Krimpenfort *et al.*, 1991). Somatic cell nuclear transfer (SCNT) is an efficient method for producing recombinant therapeutic proteins in the milk of transgenic animals. Because fetal fibroblast cells grow rapidly and have more proliferative ability, they have been commonly used as sources of donor nuclei to produce transgenic animals (Schnieke *et al.*, 1997; McCreath *et al.*, 2000; Cibelli *et al.*, 1998; Lai *et al.*, 2002). We found a significant difference in the cloning efficiency in different cell lines when used for nuclear transfer. In a previous study, the development rate of bovine nuclear transfer embryos for non-transfected and transfected donor cells was similar (Arat *et al.*, 2002) because transfection of donor cells did not affect the development of nuclear transfer embryos (Lee *et al.*, 2005). In contrast, the blastocyst rate of reconstructed embryos from transgenic cells was significantly lower than that from normal cells (Gui *et al.*, 2012; Xiong *et al.*, 2011). One of the main advantages for using nuclear transfer is that it allows the sex of transgenic animals to be predetermined and thus offers a further two-fold increase in

efficiency relative to pronuclear microinjection when the sex of the transgenic founder animal is critical. If, for example, the primary interest is the expression of human proteins in milk, the founder generation can be all female. Goats with different random integrations of the transgene can be produced by nuclear transfer from independent cell clones and the milk analyzed. After a suitable clone has been identified, the corresponding stock of cells can be used to generate an "instant flock" by further nuclear transfer. Such a flock could be superior to those produced by conventional breeding as a source of proteins for human therapy because genetic identity would contribute to the consistency of the desired product.

Studies have been done to compare the developmental competence of cloned embryos produced using non-transfected and transfected cells as donor cells. Arat *et al.* (2002) examined the effect of genetic manipulation of donor granulosa cells on developmental rates and cell number of bovine NT embryos. Cells transfected with a plasmid containing the enhanced green fluorescence protein (EGFP) gene, and non-transfected cells were used for cloning between passage 10 and 15 as serum-starved and serum-fed donor cells. There were no significant differences ( $P > 0.1$ ) in cleavage rates or development to the blastocyst stage for NT embryos from transfected (60.4 and 13.5%, respectively) or non-transfected (61.9 and 14.1%, respectively). In pigs, Jura *et al.* (2013) showed that the developmental competence of embryos (morula and blastocyst stage) produced from zygotes collected from transgenic sows was decreased compared to that obtained from non-transgenic ones. Guojie *et al.* (2009) showed that the developmental rate of non-transgenic bovine SCNT blastocysts was higher than that of transgenic SCNT blastocysts. This is in agreement with the result that *in vitro* development of bovine transgenic SCNT embryos is negatively influenced by transfection of the human ProU gene into donor fibroblasts (Bhuiyan *et al.*, 2004). Similarly, *in vitro* development to the blastocyst stage of NT embryos was lower for the transfection versus non-transfection donors with EGFP gene in porcine and bovine embryos (Park *et al.*, 2001; Arat *et al.*, 2002). This finding indicated that extended culture associated with transfection and selection procedures may induce changes

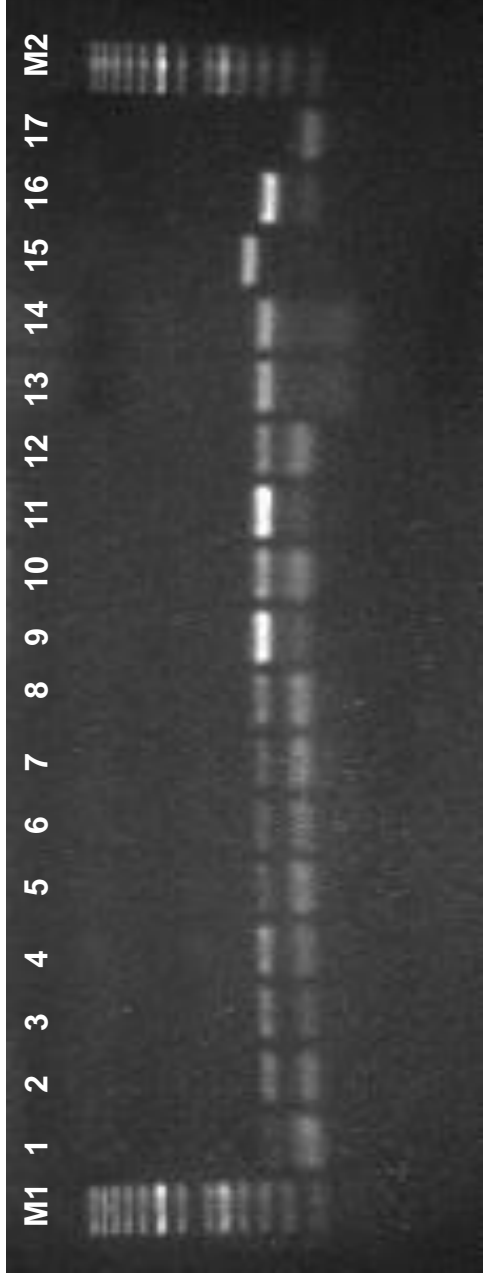
in donor cells which markedly decrease the efficiency of NT and lead to sub-populations of cells accumulating genetic and/or epigenetic defects, with potential deleterious effects on development following NT (Zakhartchenko *et al.*, 2001). This happens probably due to the fact that non-site directed make up of the gene construct causes random integration of a transgene. The random integration in the immediate vicinity or inside the sequence of the gene essential for the development can lead to its dysfunction. Lower developmental competence of hand-made cloned embryos obtained with transgenic cells, as donor cells in our case, also is in agreement with the above findings and can be attributed to random gene integration.

#### **4.16 Single embryo PCR**

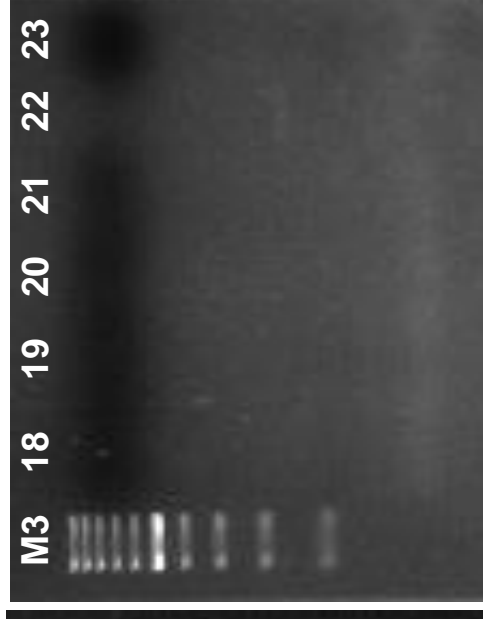
All the embryos produced using transgenic fetal fibroblast cells as donor cells appeared to be green (due to GFP expression) under blue light. Some of the transgenic embryos (14) and non-transgenic embryos produced using non-transfected fetal fibroblast cells as donor (6; taken as negative control) were selected randomly and checked for human lactoferrin DNA by PCR for amplification of lactoferrin gene (Figure 51) using the technique of single embryo PCR which was used for the first time in our lab. For this, single embryo was lysed using water and then subjected to PCR amplification using primers specific for lactoferrin gene. All the transgenic embryos showed the amplification of human lactoferrin gene while non-transgenic embryos showed no amplification. This clearly confirmed the presence and integration of the transgene into the genome of the transgenic cells and hence embryos, which guarantees the production of 100% transgenic founder upon the birth of live kid. In order to produce transgenic goat, embryo transfer was performed on synchronized recipient goats by laparoscopy. For this purpose, 30 goats were transferred with the morula stage transgenic embryos but no pregnancy was detected after 3 months. The reason for this may be attributed to the seasonality of the goat breeding besides the method being surgical in nature or the implanted embryos might be absorbed or not competent enough to establish pregnancy.

Through this study, we can conclude that, for the first time in India, we were able to establish a basic platform for the transgenic technology to produce recombinant proteins through farm animals, and more study is required to to established this technology, in India.

**Transgenic goat embryos**



**Non-transgenic goat embryos**



- M1, M2:** 50 bp ladder
- Lanes 1-14:** Transgenic cloned goat embryos with hLF gene integrated into genome
- Lane 15:** Positive control ( $\beta$ -Actin)
- Lane 16:** Positive control (hLF amplified from human blood)
- Lane 17:** Negative control (NTC)
- M3:** 100 bp ladder
- Lanes 18-23:** Cloned goat embryos (Control)

**Figure 51: Single Embryo PCR for amplification of human lactoferrin gene in individual embryo**



**Surgical embryo transfer in Goats**

# CHAPTER – 5

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**Summary and Conclusions**

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## **5. SUMMARY AND CONCLUSIONS**

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The purpose of the present study was to develop transgenic goat embryos for human lactoferrin gene, with the final aim of producing transgenic goats expressing human lactoferrin in milk. We were to develop and establish the transgenesis protocol for mammary gland specific expression. The study was completed in five phases; viz., i) development of transgenic construct for human lactoferrin under mammary gland-specific promoter; ii) development and characterization of goat mammary epithelial cell line iii) transfection of the expression vector into mammary epithelial cell lines to validate the functioning of the expression vector; iv) transfection of expression vector into goat fetal fibroblast cells and checking for integration; v) transgenic goat embryo production via hand-made cloning.

The expression vectors were designed so as to achieve the targeted expression of the desired protein in milk. The vector construct included the promoter regions of milk proteins, human lactoferrin cDNA and bGH termination sequence. The promoter regions were the genomic DNA fragments from promoter regions of  $\alpha$ -Casein,  $\beta$ -Casein and  $\beta$ -Lactoglobulin proteins in order to drive the expression of human lactoferrin in milk. The promoter regions were cloned and sequenced for ascertaining the specific amplification. The sequences were submitted to GenBank and corresponding accession numbers were obtained (Annexure II to VII). We checked each promoter fragment for its activity by luciferase assay after transfection into mammary epithelial cells. The highest activity was observed for the 6.5 Kb fragment of  $\beta$ -Casein which had comparable activity to 3.5 Kb fragment of  $\beta$ -casein. So the expression vector with this promoter fragment was used in the final expression vector to be transfected into the mammary epithelial cell line. The human lactoferrin cDNA was amplified, cloned and sequenced. The final verified sequence was submitted to GenBank and corresponding accession number obtained (Annexure VIII). Besides, 3' UTR of bovine growth hormone was also amplified and sequence was submitted to GenBank (Annexure IX). Lactoferrin cDNA was ligated into the expression vector under the promoter region of 6.5 and 3.5 Kb fragment of  $\beta$ -casein and in fusion with eGFP, so as to achieve GFP-fused lactoferrin for easy observation of the lactoferrin

expression. Later on, the construct was redesigned and final construct named p $\beta$ ChLF was developed in which GFP was under CMV promoter and hLF under mammary gland specific promoter. Goat mammary epithelial cell lines were developed from the lactating goat mammary gland tissue by both explants culture and digestion method. The cells were cultured and propagated in goat mammary epithelial cell culture medium, containing well described mammogenic factors like insulin and hydrocortisone. We observed that the cell lines derived by digestion method were better in terms of growth kinetics and contaminating other cell type sub-population. The digestion method derived cell lines were more homogenous and highly pure for mammary epithelial cells as compared to those derived by explants method. The primary cultures obtained contained a heterogenous population of cells which included fibroblast-like spindle shaped cells as well as setellate, polygonal and cuboidal cells (mammary epithelial cells). They were thus enriched for mammary epithelial cell population by differential digestion (selective detachment and attachment method) with Trypsin-EDTA in order to easily remove the fibroblast cell subpopulation which got detached faster than the epithelial cell population. By applying this strategy between subcultures 2 to 5, we were able to establish cultures containing more than 90% mammary epithelial cells. These cultures were positive for mammary epithelial cell markers and had growth kinetics and morphological features typical of goat mammary epithelial cell cultures. The continuous propagation (for more than 12 passages) and differential trypsinization of these cells on plastic substratum resulted in the selection of homogenous population of polygonal and cuboidal cells which formed monolayer island aggregates with no evidence of contaminating stromal or myo-epithelial cells. The confluent or near-confluent cultures consisted of large and small cells which were often vacuolated and organized into lumen-like structures. The post-confluent cultures (3-4 days after confluency) formed dome-shaped structures initially where as lumen-like structures were observed later. The primary cell lines were characterized by Immunocytochemistry between passages 5 and 20 for various mammary epithelial cell markers like Cytokeratin 8, Cytokeratin 18, E-cadherin and Tubulin. The cell lines were also stained for myo-epithelial cell marker,  $\alpha$ -smooth muscle Actin, and fibroblast marker, Vimentin, in order to ensure their absence in the cell population. We observed expression of such mammary

epithelial cell specific proteins like Cytokeratin8, Cytokeratin 18, E-cadherin and Tubulin and in our established cultures in almost 100% of the cell population, while as very few or none of the cells stained positive for  $\alpha$ -Actin and Vimentin, reflecting to the absence of non-mesenchymal cells, like myoepithelial cell population as well as fibroblast cell population, in our culture. Upon RT-PCR analysis, we observed expression of mammary epithelial cell specific genes like  $\beta$ -Casein,  $\beta$ -lactoglobulin, ACACA, *BTN1A1* Cytokeratin 8 and Cytokeratin 18, while no such bands were observed in fibroblast cells as well as in RT-PCR negative and blank control reactions. Karyotype examination was also performed throughout the culture period so as to ensure a stable and normal karyotype (58+XX) and sex was determined to be female by PCR amplification of *PLP* and *SRY* genes. The cell growth kinetics was also examined on bare-, extra-cellular cell matrix coated-, collagen surface-coated and gelatin-coated surfaces. We observed that cultures seeded on ECM-coated surface had fastest doubling time and thus achieved confluence faster than collagen- and gelatin-coated surfaces. The highest doubling time and hence, the slowest growth rate was observed on non-coated plastic surfaces, indicating the need of ECM coat for better cell growth. Similar results were obtained by MTT assay implying probably to the need of coating the plastic substrate in order to achieve lactogenesis in the cell population under *in vitro* conditions. Thus, it appears that ECM coated dishes should be ideally used for goat mammary epithelial cell cultures when expression of milk proteins is to be desired in the cell cultures. The expression vector was then transfected into the mammary epithelial cells by both nucleofection and lipofection and nucleofection was found to be more efficient method to transfect the robust epithelial cells as revealed by Flow cytometry analysis. The transfected cells were selected on Geneticin, after optimization of G418 dose by titration. rhLF production by mammary epithelial cells was confirmed by RT-PCR, immunocytochemistry and western blotting (in cell lysate as well as conditioned medium). The amount of human lactoferrin secreted was found to be 0.07-0.11 ng/ml as determined by ELISA. In the initial phase, hand-made cloning was done by using mammary epithelial and fetal fibroblast cells as donor cells and it was found that fetal fibroblast cells had a better reprogramming capacity. After the validation of the working of transgene construct, cells were transfected into goat fetal fibroblast cells which

were established as primary cultures from fetal ear skin. Fetal fibroblast cells were characterized by Immunocytochemistry, and RT-PCR. By karyotyping, it was ensured that the cells were of female goat which was cross-determined by PCR also. Fetal fibroblast cells were transfected with both lipofection and nucleofection and subjected to flow cytometry which revealed nucleofection to be better method as in case of mammary epithelial cells. After optimization of transfection method and G418 dose, fetal fibroblast cells were transfected with p $\beta$ ChLF and subjected to G418 selection for 8 weeks. The integration of expression vector into the transgenic fetal fibroblast cells was confirmed by PCR. We found positive expression of human lactoferrin in the transgenic cell lines selected for 8 passages on Geneticin while non-transgenic (control) cell lines showed no band. These results indicate that transgenic cell lines were stable for lactoferrin expression and the transgene was stably intergared within their genome. Thus, they could be used a donor cells for production of transgenic goat embryos expressing human lactoferrin gene. Hand-guided cloning was performed using these stably transfected cells as donor cells. Control (non-transgenic) goat fetal fibroblast cells were also used as donor cells for the purpose. The embryos produced using transgenic fetal fibroblast cells expressed GFP. Some of the transgenic embryos were selected randomly and checked for PCR for amplification of lactoferrin gene by single embryo PCR. The embryo transfer was performed on synchronized recipient goats by laproscopy. 30 goats were transferred with the morula stage transgenic embryos but no pregnancy was detected after 3 months.

In conclusion, we report the development of cloned-transgenic caprine embryos developed from transgenic goat fetal fibroblast cells. The strategies and protocols for expression vector construction, development of transgenic cell lines, and transgenic embryo production have been standardized and could be optimized for production of transgenic goat for any other pharmaceutical, nutritional or hormonal product by using the mammary gland as the bioreactor.

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# Annexure

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**ANNEXURE I****PREPARATION OF REAGENTS****For RNA isolation****Ethanol 70 %**

Ethanol 99.9 %	:	70 ml
Distilled water	:	30 ml

**TE buffer**

Tris	(1M, pH 8.0)	:	1 ml
EDTA	(0.5M, pH 8.0)	:	200 $\mu$ l

Make volume 100 ml and then autoclave.

**RNase (10 mg/ml)**

RNase	:	10 mg
Distilled water	:	1 ml

Heated in boiling water-bath for 15 min. Stored at – 20°C

**Electrophoresis Agarose 1.0 %**

Agarose	:	0.40 gm
TAE (1x)	:	40 ml

**Ethidium bromide**

Dissolve 10mg of Ethidium bromide in 1ml of distilled water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved; wrap the container with aluminum foil and store at 4°C

**Preparation of 6x gel loading dye (10 ml)**

6X dye	:	1ml
Glycerol	:	3ml
Autoclaved water	:	6ml

**TAE buffer (50x)**

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml

EDTA (0.05M, pH 8.0) : 100 ml

Double distilled water to make volume 1000, filter it and then autoclave

**Antibiotics:**

**Ampicillin**

Ampicillin : 50mg

Distilled water : 1ml

Sterilize by filtration and store in aliquots at  $-20^{\circ}\text{C}$

**Tetracyclin**

Tetracyclin : 50mg

Distilled water : 1ml

Sterilize by filtration and store in aliquots at  $-20^{\circ}\text{C}$

**Transformation**

**SOB (Salt optimizing buffer) broth**

Tryptone : 2g

Yeast extract : 0.5g

NaCl : 0.05g

250Mm KCl : 1 ml

2M MgCl<sub>2</sub> : 0.5ml

Total volume was made to 100 ml with distilled water and autoclaved before use.

**SOB media:** SOB media was prepared by dissolving 2g agar per 100ml SOB broth and autoclaved.

**SOB plates:** Autoclaved SOB media was allowed to cool to  $50^{\circ}\text{C}$ . Approx. 15 ml media was poured in petridishes (8ml) and it was allowed to harden for 20 min at room temp. These plates were either used immediately or stored at  $4^{\circ}\text{C}$  for further use.

**SOC solution:** 600 $\mu\text{l}$  SOC broth was mixed with 200 $\mu\text{l}$  of 1 M glucose (1 M glucose was prepared by dissolving 18g glucose in 90 ml double distilled water and sterilized by passing through 0.45 micron filter).

**LB agar plates**

Tryptone	:	1g
Yeast extracts	:	0.5g
NaCl	:	1g
Agar	:	2g

**LB broth**

Tryptone	:	1g
Yeast extracts	:	0.5g
NaCl	:	1g

**i. SDS-PAGE reagents****Acrylamide / bis Acrylamide (30% T, 2.67% C)**

$$\text{Total monomer concentration (\%T)} = \frac{(\text{gm Acrylamide} + \text{gm bis-Acrylamide})}{(\text{Total volume})} \times 100$$

$$\text{Crosslinking monomer concentration (\% C)} = \frac{(\text{gm bis-Acrylamide})}{(\text{Acrylamide} + \text{gm bis-Acrylamide})} \times 100$$

Acrylamide	29.2 g / 100 ml
N' N' bis-methylene – Acrylamide	0.8 g / 100 ml
Filtered and stored at 4 °C in the dark (30 days maximum)	

**1.5 M Tris-HCl , pH 8.8**

18.15 g Tris base / 100 ml. pH is adjusted with 6 N HCl and final volume was made to 100 ml with deionized water

**0.5 M Tris-HCl, pH 6.8**

6 g Tris base / 100 ml. pH is adjusted with 6 N HCl and final volume was made to 100 ml with deionized water

**10 % SDS**

**5X Running Buffer, pH 8.3**

Tris base	15g / lit.
Glycine	72 g / lit.
SDS	5 g / lit.

**4X sample buffer**

1 M Tris –HCl, pH 6.8	2.5 ml
SDS	0.8 g
2-Mercaptoethanol	1 ml
Glycerol	3 ml
Bromophenol Blue	2 mg
Total volume	10 ml

**10 % Separating Gel Preparation (0.375 M Tris, pH 8.8)**

Deionized water	3.1 ml
1.5 M Tris-HCl, pH 8.8	2.0 ml
10 % SDS	80 µl
Acrylamide / Bis (30% stock)	2.6 ml
10% Ammonium per sulfate (Freshly prepared)	80 µl
TEMED	3.2 µl
<b>Total volume</b>	<b>8.0 ml</b>

**4 % Stacking Gel Preparation (0.125 M Tris, pH 6.8)**

Deionized water	3.05 ml
1.5 M Tris-HCl, pH 8.8	1.25 ml
10 % SDS	50 µl
Acrylamide / Bis (30% stock)	0.665 ml
10% Ammonium per sulfate (Freshly prepared)	25 µl
TEMED	5 µl
<b>Total volume</b>	<b>5 ml</b>

**Running conditions**

At constant ampere of 16 mA till the dye reaches separating gel, then increased to 20 mA.

**Coomassie Blue staining**

Stain ½ hr with 0.1% Coomassie blue R-250 in fixative (40% Methanol and 10 % glacial Acetic acid).

**Destaining**

Destain with several changes of 40% methanol and 10% glacial acetic acid to remove background.

**ii. Western blot analysis reagents****Transfer buffer**

3.03 g Tris base, 50 mg SDS and 14.4 g glycine are dissolved in 800 ml of distilled water. Then 200 ml of methanol was added before using.

**TBST Buffer**

Tris Base	12g
Nacl	18.8g
Tween 20	0.1% (Add at last)
10% Ammonium per sulfate	25 µl
ddH <sub>2</sub> O	Up to 1L
Adjust pH to 7.5 with HCl.	

**TBS Buffer** : TBST buffer without 0.1% Tween 20

**Blocking** : TBST + 5% BSA

**ix) Antigen Masking reagent for IHC**

0.25% Trypsin-EDTA	:	2ml
CaCl <sub>2</sub>	:	0.01g
ddH <sub>2</sub> O	:	Upto 10ml

Adjust pH to 7.8 with 1N NaOH.

**x) Fixation Buffer**

**Formaldehyde (37-40%) : 100ml**

NaH <sub>2</sub> PO <sub>4</sub>	:	4g
Na <sub>2</sub> HPO <sub>4</sub>	:	6.5 g
ddH <sub>2</sub> O	:	900ml

## Solutions for oocyte collection and maturation

### **Normal saline containing antibiotics**

<b>Composition</b>	<b>Volume (1000ml)</b>
Sodium chloride	- 9.0 gm
Penicillin G	- 0.06 gm
Distilled water	- 1000 ml

### **Aspiration medium (for about 200-250 ovaries)**

<b>Composition</b>	<b>Volume (100 ml)</b>
TCM-199 (Hepes modified)	- 50 ml
BSA	- 0.15 gm
Gentamicin	- 50µg/ml
L-glutamine	-As recommended by the manufacturer

### **Washing medium**

<b>Composition</b>	<b>Volume (40 ml)</b>
TCM-199 (Hepes modified)	- 36 ml
FBS	- 4 ml
Sodium pyruvate	- 0.0036 gm
Gentamicin	- 50µg/ml
L-glutamine	- Recommended by manufacturer

### **Maturation medium**

<b>Composition</b>	<b>Volume (10 ml)</b>
Washing medium	- 10 ml
Porcine FSH	- 5 µg/ml
Follicular Fluid	- 500µl
Estradiol 17-β	- 1µg/ml
EGF	- 10ng/ml
Cysteamine	- 50 µM

## Handmade Cloning Media

### T0

<b>Composition</b>	-	<b>Volume (10 ml)</b>
HEPES (H-7006),	-	2.5 mM
NaHCO <sub>3</sub> (S-4019),	-	5 mM
Sodium pyruvate (P-3662)	-	0.2mM
Gentamycine sulfate (G-1264)	-	50 µg/mL
Adjusted to pH 7.4, 280 mOsm.		

### T2 & T20

T0 supplemented with 2 and 20% FBS, respectively.

**Hyaluronidase** - 0.5 mg/mL solution in T2.  
(Store in 500µL aliquots in 2 mL Eppendorf tubes)

**Protease** - 2 mg/mL solution in T10  
(Sore in 500 µL Centrifuge before use)

**Cytochalasin B** - 5 mg/mL stock dissolved in DMSO (Store in 2µL aliquots)

### Fusion medium

<b>Composition</b>	-	<b>Volume (10 ml)</b>
D-mannitol (M-9546)	-	0.3 M
MgSO <sub>4</sub> (M-2393)	-	0.1 mM
CaCl <sub>2</sub> (C-7902)	-	0.05 mM
Polyvinylalcohol (P-8136)	-	1 mg/mL
(Store in 5 mL aliquots)		

**Phytohemagglutinin** - 5 mg/mL stock solution in T0  
(Store in 50µL aliquots)

**Calcium ionophore II** - 1 mg/mL stock in DMSO  
(Keep in a small dark bottle with tightly closed cap)

**Embryo culture Media****Research Vitro Cleave**

RVCL	-	4 ml
BSA (FAF)	-	40 mg

**Cell Culture Media****Somatic Cell Culture Media**

<b>Composition</b>	-	<b>Volume (10 ml)</b>
DMEM	-	9 mL
Gentamycin	-	50 µg/ml
L-Glutamine	-	2.0 mM
Fetal Bovine Serum	-	1 mL

During the preparation of above all media, all the components were mixed well and incubated in a CO<sub>2</sub> incubator at 38.5°C, 5% CO<sub>2</sub> for 2 h for stabilization of pH and temperature before further use. The media were filtered through 0.22 µm filter just before use.

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## ANNEXURE-II

**Goat alpha casein promoter region (2.97 kb)****Accession number: KF992209**

LOCUS seq2 2958 bp DNA linear MAM 19-DEC-2013  
 DEFINITION Alpha casein (CSN1)DNA.  
 ACCESSION seq2  
 VERSION  
 KEYWORDS  
 SOURCE Capra hircus (goat)  
 ORGANISM Capra hircus  
     Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
     Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;  
     Pecora; Bovidae; Caprinae; Capra.  
 REFERENCE 1 (bases 1 to 2958)  
 AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
     Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Cloning and sequence characterization of mammary gland specific  
     promoters  
 JOURNAL Unpublished  
 REFERENCE 2 (bases 1 to 2958)  
 AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
     Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Direct Submission  
 JOURNAL Submitted (19-DEC-2013) Animal Biotechnology Center, National Dairy  
     Research Institute, NDRI Karnal, Karnal, Haryana 132001, India  
 COMMENT Bankit Comment: ALT EMAIL:syedalhyderi14@gmail.com.  
     Bankit Comment: TOTAL # OF SEQS:1.

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Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

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## ANNEXURE-III

**Goat alpha casein promoter region (5.27 kb)****Accession number: KF992212**

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 VERSION  
 KEYWORDS .  
 SOURCE Capra hircus (goat)  
 ORGANISM Capra hircus  
     Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
     Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;  
     Pecora; Bovidae; Caprinae; Capra.  
 REFERENCE 1 (bases 1 to 5272)  
 AUTHORS **Saini,N.**, Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Cloning and sequence characterization of goat mammary gland  
 specific promoters  
 JOURNAL Unpublished  
 REFERENCE 2 (bases 1 to 5272)  
 AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Direct Submission  
 JOURNAL Submitted (19-DEC-2013) Animal Biotechnology Center, National Dairy  
 Research Institute, NDRI Karnal, Karnal, Haryana 132001, India  
 COMMENT Bankit Comment: ALT EMAIL:syedalhyderi14@gmail.com.  
 Bankit Comment: TOTAL # OF SEQS:1.

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

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**ANNEXURE-IV****Goat alpha casein promoter region (3.5 kb)****Accession number: KF992210**

LOCUS seq3 3385 bp DNA linear MAM 19-DEC-2013  
 DEFINITION Beta casein (CSN2) DNA.  
 ACCESSION seq3  
 VERSION  
 KEYWORDS  
 SOURCE Capra hircus (goat)  
 ORGANISM Capra hircus  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
 Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;  
 Pecora; Bovidae; Caprinae; Capra.  
 REFERENCE 1 (bases 1 to 3385)  
 AUTHORS **Saini,N.**, Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Cloning and sequence characterization of goat mammary gland  
 specific promoters  
 JOURNAL Unpublished  
 REFERENCE 2 (bases 1 to 3385)  
 AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S., Singla,S.K.,  
 Palta,P. and Chauhan,M.S.  
 TITLE Direct Submission  
 JOURNAL Submitted (19-DEC-2013) Animal Biotechnology Center, National Dairy  
 Research Institute, NDRI Karnal, Karnal, Haryana 132001, India  
 COMMENT Bankit Comment: ALT EMAIL:syedalyhyderi14@gmail.com.  
 Bankit Comment: TOTAL # OF SEQS:1.

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

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**ANNEXURE-V****Goat alpha casein promoter region (6.5 kb)****Accession number: KF992213**

LOCUS seq 6530 bp DNA linear MAM 19-DEC-2013  
 DEFINITION 1 Beta casein (CSN2) DNA.  
 ACCESSION seq  
 VERSION  
 KEYWORDS .  
 SOURCE Capra hircus (goat)  
 ORGANISM Capra hircus  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
 Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;  
 Pecora; Bovidae; Caprinae; Capra.  
 REFERENCE 1 (bases 1 to 6530)  
 AUTHORS **Saini,N.**, Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Cloning and sequence characterization of goat mammary gland  
 specific promoters  
 JOURNAL Unpublished  
 REFERENCE 2 (bases 1 to 6530)  
 AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Direct Submission  
 JOURNAL Submitted (19-DEC-2013) Animal Biotechnology Center, National Dairy  
 Research Institute, NDRI Karnal, Karnal, Haryana 132001, India  
 COMMENT Bankit Comment: ALT EMAIL:syedalhyderi14@gmail.com.  
 Bankit Comment: TOTAL # OF SEQS:1.  
  
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 5581 ccaaggagca taaaattggg gactgggcaa gagaaactga caccctggtt aattaccaag  
 5641 agataagtac acagttctat gtagagaaaa taagcatagt gtatgatctc taaaattatg  
 5701 tgagacaaag gagagatgac attaggcatg tggggatgaa gactgagtag agaagaaca  
 5761 atctaatcag tccaagaaaa catctcgatc agtggacaaa atagaagaaa tgctaaaatg  
 5821 aaacagaagt ctactggaa ataaaagata tgcataagac aaaaattcat gaaaatcact  
 5881 tagtttagca gagaaaagat aaaaataaag tatgacctc ttcatataca ttgttgatt  
 5941 atatgcacct caataaaaat gagtctcaa cagaaatgaa acattaatat ttgttact  
 6001 gctctaatcc cagaatctaa gcgatatctg gcaataaaaa taataaatat atattttta  
 6061 ataatgaat caaccacta attttctgt aaatatctgt aacttctct ctgtcttcc  
 6121 aaaaacactc ataagtactg tgaatgagat gaaaaagagt gaagtaggat ataggctgtt  
 6181 agcagaaaac atctgaatgg ctggcagtga aacattaact tgaatgtaa gattaatgag  
 6241 taatagtaaa ttttaacctt ggccatagta taaaatgttc attaatatt ttctagaata  
 6301 cagggtttt tgttttgcc atgaggttg caggatctg gttccctgac caggatcaa  
 6361 acctgcactc cctggaagc atggagtctt ggacattgt attatacaca tcttggtc  
 6421 cttttaaagg gaagtaatt tacttaata agaaaataga ttgacaagta atacgctgtt  
 6481 tctcatctt cccattcaca ggaatcgaga gcatgaaggt cctcatcctt

//

## ANNEXURE-VI

## Goat alpha casein promoter region (2.7 kb)

Accession no: KF992214

LOCUS seq 2674 bp DNA linear MAM 20-DEC-2013  
 DEFINITION 1 Betalactoglobulin (BLG) DNA.  
 ACCESSION seq  
 VERSION  
 KEYWORDS .  
 SOURCE Capra hircus (goat)  
 ORGANISM Capra hircus  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
 Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;  
 Pecora; Bovidae; Caprinae; Capra.  
 REFERENCE 1 (bases 1 to 2674)  
 AUTHORS **Saini,N.**, Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Clonng and sequence characterization of goat mammary gland  
 specific promoters  
 JOURNAL Unpublished  
 REFERENCE 2 (bases 1 to 2674)  
 AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Direct Submission  
 JOURNAL Submitted (20-DEC-2013) Animal Biotechnology Center, National Dairy  
 Research Institute, NDRI Karnal, Karnal, Haryana 132001, India  
 COMMENT Bankit Comment: ALT EMAIL:syedalhyderi14@gmail.com.  
 Bankit Comment: TOTAL # OF SEQS:1.  
 ##Assembly-Data-START##  
 Sequencing Technology :: Sanger dideoxy sequencing  
 ##Assembly-Data-END##  
 FEATURES Location/Qualifiers  
 source 1..2674  
 /organism="Capra hircus"  
 /mol\_type="genomic DNA"  
 /db\_xref="taxon:9925"  
 /tissue\_type="Mamamry gland"  
 /country="India"  
 /collection\_date="21-Oct-2012"  
 gene <1..2674  
 /gene="Beta lactoglobulin"  
 promoter <1..2049  
 /gene="Beta lactoglobulin"  
 TATA\_signal 2013..2025  
 /gene="Beta lactoglobulin"  
 gene <2050..2185  
 /gene="Betalactoglobulin"  
 exon <2050..2185  
 /gene="Betalactoglobulin"  
 sig\_peptide <2090..2143  
 /gene="Betalactoglobulin"

intron 2186..2674  
 /gene="Beta lactoglobulin"  
 BASE COUNT 510 a 872 c 745 g 547 t  
 ORIGIN  
 1 gaagggcatg gtttatggc acggttcaca ggaacttggg agaccctgca gtcagacgt  
 61 cccgagggtg gtggcaccca gatttctaa gctcgtggg gaacggggcg cttgtttc  
 121 cctggctgac ctccctccg cctgcatcac ccagttctga gagcagagcg gtgctgggg  
 181 gcacagcctc tcgcatctga tgccggtgc caaacacccc gtgctgggtg tcggggggct  
 241 acctatgggg aagggtcct cactgcaggg tgccctccgt cccctctgag atcagaagt  
 301 ccagtcggga catgaacag gccaagctcc ctccagaggc tccagggagg gatccttgc  
 361 ccccgcgcc gcctccagct cctggtgcc caccctgag cctgatctg tagacgcctc  
 421 agtctagtct ctgctccgt gttcacatgc ctctccca tgtccctcc atgtccctg  
 481 tttctcac aaggacacc gacagtagat tagcccctgt tccagcctca cctgaacagc  
 541 tcacatctgt aaagacctag attccaaca agattccaac ctgaagtcc tggtgatg  
 601 gaggttggg gcaacatct tcaacccat cacagctgc agttcatcac aaaacatgga  
 661 acctggggtt tatcataaaa cctaggttct tcatgaaca ctgagcttcg aggcttgg  
 721 caagaattaa aggtgcta acagatcaag gcaaggactg aagctggcca agcctactc  
 781 ttccatcaca ggaaagggg atctggggg ggctgggggt ctgctcccgt gagtgagctc  
 841 tttctgta cagtaccaa cagtctcc gggaaggaaa ccagaggcca gagagcaagc  
 901 cagagctagt ttaggagacc cctgaacct caaccaagat gctgaccagg ccagcgggcc  
 961 ccctgaaaag accctacagt tcagggggga agaggggctg acccgccagg tcctgctat  
 1021 caggagacat ccccgctac aggagattcc cccacctgc tcccgttccg ctacccaat  
 1081 acgccaccc caccctgtg atgagtgtt tagtactta gaatgtcaac tgaaggctt  
 1141 tgcacctct ttgccagagg cacaaggcac ccacagcccg ctgggtacca acgccatg  
 1201 ggattcagc aggaggctg tctgcacc tcctgctcg ggccccttct gtactagca  
 1261 acacaccag caccagcatt cccactgctc ctgaggtctg caggcagctc gctgagcct  
 1321 gagcgtgtg gaggaagtg tctgggaga cttaaagt gggaggtgg aggtgggag  
 1381 ttggccctg tggcctgcc catcccgct gcctgcatgg agcccagtg ctgctagcc  
 1441 gtgccccgc cgcagggtca ggtcacttc cgtcctggg ggttattatg accgttga  
 1501 tttcattgc cattttgt acctaactg ggcagcaggt gttgcagag ccctcgata  
 1561 cgaccagtc cccctcga gctccactg aacccctgt caccctgcc ccagcctgca  
 1621 gaggatggg tactgcaga gatccctca ccaaggcca cggtcacatg gttggagga  
 1681 gctggtgcc aaggcagagg ccacctcca ggacacacct gtcccagtg ctggcttga  
 1741 cctgccctg tctaagagg tgacccgga agtgttctg gcactggcag ccagcctgac  
 1801 ccagagtcca gacaccacc tggccccca ctctgggt ctaccaggaa cgtctaggc  
 1861 ccagagggg acttctgtg tggccccga tggagaagg cctctattg tcctctaga  
 1921 ggaagccacc ccggggccc ggatgagcc aagtaggatt ccgggaacct cgtggctggg  
 1981 ggccggccc gggctggctg gctggcacgc ctctgtata aggcccag cccgctgt  
 2041 cagccctcca ctccctgag agctcagaag cagacccca gctgcagcca tgaagtgcct  
 2101 cctgctgc ctggcctg cctcgcctg tggcatccag gccatcatg tcaccagac  
 2161 catgaaaggc ctggacatcc agaaggttc agggggccg ggtgggtgt aggttcagg  
 2221 gcggcaggg gagctggcc tcagagacca agagaggctg tgacgtcgg ttcgcatcag  
 2281 tcagctagg ccgctgaca aatccccgc tgggcagct tggaccagg gttcactgcc  
 2341 ttgcattctg gaggctgaa gcccaagatc caggtgtcgg ccaggctggc ttctctgcg  
 2401 gccgcttc gcggagcaga cggcgtct ctccatgtcc tctcgagcc ctgattct  
 2461 ctctgtga gcccagc cctgctgaa acacgctgc ctgcacagcc tcacagacc  
 2521 tttctatct cftaaaggc cgtgtctca gagtcatgt tgaagtct ggggtagt  
 2581 gggacacagt tcagcccta aaagagtct tctgccctc aaatttccc cacctccagc  
 2641 catgtctcc caagatcaa atgtgccac gtgt

//

## ANNEXURE-VII

**Goat alpha casein promoter region (2.1 kb)****Accession number: KF992211**

LOCUS Seq4 2169 bp DNA linear MAM 19-DEC-2013

DEFINITION Betalactoglobulin (BLG) DNA.

ACCESSION Seq4

VERSION

KEYWORDS .

SOURCE Capra hircus (goat)

ORGANISM Capra hircus

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;

Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;

Pecora; Bovidae; Caprinae; Capra.

REFERENCE 1 (bases 1 to 2169)

AUTHORS **Saini,N.**TITLE CLONING AND SEQUENCE CHARACTERIZATION OF GOAT MAMMARY GLAND  
SPECIFIC PROMOTERS

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 2169)

AUTHORS Saini,N.

TITLE Direct Submission

JOURNAL Submitted (19-DEC-2013) Animal Biotechnology Center, National Dairy  
Research Institute, NDRI Karnal, Karnal, Haryana 132001, India

COMMENT Bankit Comment: ALT EMAIL:syedalhyderi14@gmail.com.

Bankit Comment: TOTAL # OF SEQS:1.

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..2169

/organism="Capra hircus"

/mol\_type="genomic DNA"

/host="Capra hircus"

/db\_xref="taxon:9925"

/tissue\_type="Mammary gland"

/country="India"

gene &lt;1..2169

/gene="Beta lactoglobulin"

promoter &lt;1..2150

/gene="Beta lactoglobulin"

TATA\_signal &lt;2106..2118

/gene="Beta lactoglobulin"

exon 2151..2169

/gene="Beta lactoglobulin"

BASE COUNT 424 a 699 c 595 g 451 t

ORIGIN

1 gccagaggtg cttatttc gtctctct ctggatgta ttcttgaa gctgaagatt  
 61 cctggaagtt atgaatactt cgcctgaag ggcattggtt atggcacgg ttcacaggaa  
 121 cttgggagac cctgcagctc agacgtccc aggttggtg caccagatt tctaagctc  
 181 gctggggaac ggggcgctg ttctccctg actgacctc ctccgcctg catcaccag  
 241 ttctgagagc agagcggtc tggggggcac agcctctgc atctgacgc ggtgtccaaa  
 301 ccaccctgc tgggttcgg ggggtacct atgggaagg gctctcact gcagggtgc  
 361 ctccgtccc tctgagatca gaagtcac tccggacatc gaacaggcca agctccctc  
 421 agaggctcca gggagggatc ctgcccccc gccgccct ccagctctg gtgccgacc  
 481 ctgagcctg atctttaga cgctcagtc tagtcttgc ctccgttgc acatgcctc  
 541 tccccatgc cctccatgt ccccgtttc ttcacaagg acaccggaca gtgattagc  
 601 cctgttcca gctcacctg aacagctcac atctgtaaag acctagattc caaacaagat  
 661 tccaacctga agttcctggt ggatgtgagt tctgggcaa catcctcaa cccatcaca  
 721 gcttgacgtt catcacaata catggaacct ggggtttatc ataaacctt ggttctcat  
 781 gaaacactga gcttcgaggc ttgtgcaag aattaaagg gtaatacag atcaaggcaa  
 841 ggactgaagc tggccaagcc tactcttcc atcacaggaa aggggggtct gggggcggct  
 901 ggggtctgc tccctgagt gagctcttt ctgctacagt caccaacagt ctctccgga  
 961 aggaaaccag aggccagaga gcaagccaga gctagttag gagaccctg aacctccaac  
 1021 caagatgctg accaggccag cgggcccct gaaagacc tacagttcag ggggaagag  
 1081 gggctgacc gccaggtccc tgctatcagg agacatccc gctatcagga gattcccca  
 1141 cctgtctcc gtcccctac cccaatacgc ccaccacc cctgtgatga gtggttagt  
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 1261 agcccgtgg gtaccagcg ccatgtgat tcagccagga ggcctgtct gcaccctcc  
 1321 tgctgggccc ccttctgtac tcagcaacac acccagcacc agcattcca ctgctctga  
 1381 ggtctgcagg cagctcgtg tagcctgagc ggtgtggagg gaagtgtct gggagacta  
 1441 aaatgtgga ggtgggagg gggaggtgg aggtgggccc ctgtggcct gccatccc  
 1501 tgtcctgca tggagccca gtgctgcta gccgtgccc cgccgaggg tcaggtcact  
 1561 tcccgtct ggggttatt atgaccgtg tcatttcat tgccatttt gctaccctaa  
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 1681 ctgaaccctg tgcaccctt gcccagcct gcagaggatg gggctactgc agagatccct  
 1741 tcaccaagg ccacggtcac atggttga ggagctggtg ccaaggcag aggccacgt  
 1801 ctaggacaca cctgtccca gtgctggctc tgacctgcc ttgttaaga ggctgacccc  
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 1921 ccacttctg ggtctaccag gaaccgtcta ggcccagagg gggacttct gcttgcccc  
 1981 ggatggaaga aggcctcta ttgctctg agaggaagcc accccggggc cgggggatga  
 2041 gccaaagtagg attccggaa cctcgtggt gggggcccgg cccgggctg ctggctgga  
 2101 gcctctctg ataaggccc gagcccactg tctagccct ccactcctg cagagctcag  
 2161 aagcacgac

//

## ANNEXURE-VIII

**Human Lactoferrin (2.2 kb)**  
**Accession number: KF992208**

LOCUS Seq1 2259 bp ss-RNA linear PRI 19-DEC-2013

DEFINITION Lactotransferrin (LTF)mRNA, Complete CDS.

ACCESSION Seq1

VERSION

KEYWORDS .

SOURCE Homo sapiens (human)

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;

Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;

Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 2259)

AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S., Singla,S.K. and Chauhan,M.S.

TITLE Sequencing, cloning and characterization of human lactoferrin gene

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 2259)

AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S., Singla,S.K. and Chauhan,M.S.

TITLE Direct Submission

JOURNAL Submitted (18-DEC-2013) Animal Biotechnology Center, National Dairy Research Institute, NDRI Karnal, Karnal, Haryana 132001, India

COMMENT Bankit Comment: ALT EMAIL:syedalhyderi14@gmail.com.

Bankit Comment: TOTAL # OF SEQS:1.

##Assembly-Data-START##

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..2259

/organism="Homo sapiens"

/mol\_type="transcribed RNA"

/isolation\_source="Human Blood"

/db\_xref="taxon:9606"

/plasmid="pTZ57R/T"

/collection\_date="10-Aug-2012"

gene <13..2148

/gene="Lactoferrin"

CDS <13..2148

/gene="Lactoferrin"

/note="Lactoferrin is a 80kDa non haem binding

glycoprotein of transferrin family"

/codon\_start=1

/product="LACTOTRANSFERRIN/ Lactoferrin"

/translation="MKLVFLVLLFLGALGLCLAGRRRRSVQWCTVSQPEATKCFQWQR

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PPEPIEAAVARFFSASCVPGADKGFNLCRLCAGTGENKCAFSSQEPYFSYSGAFKC

LRDGAGDVAFIRESTVFEDLSDEAERDEYELLCPDNTRKPVDFKDKCHLARVP SHAVV

ARSVNGKEDAIWNLLRQAQEKFGKDKSPKFQLFGSPSGQKDLLFKDSAIGFSRVPPRI  
 DSGLYLGSYFTAQNLKSEEEVAARRARVVWCAVGEQELRKCQNSGLSEGSVTCS  
 SASTTEDCIALVLKGEADAMSLDGGYVYTAGKCGLPVLAENYKSQQSSDPDPCVDR  
 PVEGYLAVAVVRRSDTSLTWNSVKGKKSCHTAVDRTAGWNIPMGLLFNQTSCKFDEY  
 FSQSCAPGSDPRSNLCALCIGDEQGENKCVPSNERYYYGYTGAFRCLAENAGDVAFVK  
 DVTVLQNTDGNNDAWAKDLKLADFULLCLDGKRKPVTEARSCHLAMAPNHAVVSRMD  
 KVERLKQVLLHQAKFGRNGSDCPDKFLFQSETKNLLFNDNTECLARLHGKTTYEY  
 LGPQYVAGITNLKCKSTSPLEACEFLRK"

BASE COUNT 537 a 574 c 652 g 496 t  
 ORIGIN

1 cagaccgcag acatgaaact tgtctcctc gtctctgtgt tctcggggc cctcggactg  
 61 tgtctggctg gccgtaggag aaggagtgtt cagtgtgca ccgatccca acccgaggcc  
 121 acaaaatgct tccaatggca aaggaatag agaagagtgc gtggccctcc tgtcagctgc  
 181 ataaagagag actccccat ccagtgatc caggccattg cggaaaacag ggccgatgtc  
 241 gtgacccttg atggtggtt catatacgag gcaggcctgg ccccctaca actcgcacct  
 301 gtacggcgg aagtctacgg gaccgaaga cagccacgaa ctactatta tgccgtggct  
 361 gtgtggaaga agggcggcag cttcagctg aacgaactgc aaggctgaa gtctgccac  
 421 acaggccttc gcaggaccgc tggatggaat gtcctatag ggacactcg tccattctg  
 481 aattggacgg gtccacctga gccattgag gcagctgtg ccaggttct ctacgccagc  
 541 tgtttcccg gtgcagataa aggacagtc cccaacctgt gtcgctgtg tgcggggaca  
 601 ggggaaaaca aatgtgcctt ctctcccag gaacctact tcagctact tggcgcctc  
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 841 gcacgaagtg tgaatggcaa ggaggatgcc atctggaatc ttcccgcca ggcacaggaa  
 901 aagttggaaggacaagtc accgaaattc cagctcttg gctcccctag tggcagaaa  
 961 gatctgtgt tcaaggactc tgccattggg tttcagagg tgcctccgag gatagattc  
 1021 gggctgtacc ttgctccg ctacttact gccatccaga acttgaggaa aagtgaggag  
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 1201 acagaggact gcctgcctt ggtgctgaaa ggagaagctg atgcatgag ttggatgga  
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 1681 gagaatctg gagacgtgc atttgaaa gatgtcactg tctgcagaa cactgatgga  
 1741 aataacaatg acgcatggc taaggattg aagctggcag acttgcgct gctgtcctc  
 1801 gatggcaaac ggaagcctgt gactgaggct agaagctgcc atctgcat ggccccgat  
 1861 catgccgtg tgtctcggat ggataagggt gaacgcctga aacaggtgt gctccacaa  
 1921 caggctaaat ttggagaaa tggatctgac tgccggaca agtttctt attccagtct  
 1981 gaaacaaaa acctctgtt caatgacaac actgagtgc tggccagact ccatggcaaa  
 2041 acaacatag aaaaatattt gggaccacag tatgtcag gcattactaa tctgaaaaag  
 2101 tgctcaacct cccccctct ggaagcctgt gaattcctca ggaagtaaaa ccgaagaaga  
 2161 tggcccagct cccaagaaa gcctcagcca ttcactgcc ccagctctc tcccagggtg  
 2221 tgtggggcc ttgctcccc tgctgaaggt ggggattgc

//

## ANNEXURE-IX

## Goat alpha casein promoter region (0.7 kb)

Accession no: KF992215

LOCUS seq 763 bp DNA linear MAM 20-DEC-2013  
 DEFINITION 1 Bovine Growth Hormone (BGH) 3'UTR DNA.  
 ACCESSION seq  
 VERSION  
 KEYWORDS .  
 SOURCE Bos indicus  
 ORGANISM Bos indicus  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
 Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla; Ruminantia;  
 Pecora; Bovidae; Bovinae; Bos.

REFERENCE 1 (bases 1 to 763)  
 AUTHORS **Saini,N.**, Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Amplification and characterization of trangene construct elements  
 JOURNAL Unpublished

REFERENCE 2 (bases 1 to 763)  
 AUTHORS Saini,N., Shah,S.M., Waiz,S.A., Kaushik,R., Singh,M.K., Manik,R.S.,  
 Singla,S.K., Palta,P. and Chauhan,M.S.  
 TITLE Direct Submission  
 JOURNAL Submitted (20-DEC-2013) Animal Biotechnology Center, National Dairy  
 Research Institute, NDRI Karnal, Karnal, Haryana 132001, India

COMMENT Bankit Comment: ALT EMAIL:syedalhyderi14@gmail.com.  
 Bankit Comment: Vecscreen Comment:Submitter says Vecscreen match is  
 due to: This is the sequence of bovine growth hormone 3' end (Poly  
 A) Since this is used as Poy A signal in most vectors, so this  
 might be the reason for the matches with cloning vector.  
 Bankit Comment: TOTAL # OF SEQS:1.

##Assembly-Data-START##  
 Sequencing Technology :: Sanger dideoxy sequencing  
 ##Assembly-Data-END##

FEATURES Location/Qualifiers  
 source 1..763  
 /organism="Bos indicus"  
 /mol\_type="genomic DNA"  
 /host="Bos indicus"  
 /db\_xref="taxon:9915"  
 /tissue\_type="Blood"  
 /country="India"  
 gene <1..763  
 /gene="Bovine growth hormone"  
 3'UTR <1..763  
 /gene="Bovine growth hormone"

BASE COUNT 150 a 223 c 220 g 170 t

ORIGIN

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61 agtccagcct tgaccaggg gaaaccttt cccctttga aacctcttc ctgcccttc  
121 tccaagcctg taggggaggg tggaaaatgg agcgggcagg agggagctgc tcctgagggc  
181 ccttcggcct ctctgtctct ccctccctg gcaggagctg gaagatggca cccccgggc  
241 tggcagatc ctcaagcaga cctatgacaa atttgacaca aacatgcgca gtgacgacgc  
301 gctgctcaag aactacggtc tgctctcctg ctccggaag gacctgcata agacggagac  
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