PRODUCTION AND PURIFICATION OF RECOMBINANT BUFFALO LEUKEMIA INHIBITORY FACTOR (rBuLIF) FROM STABLY TRANSFECTED COS-1 CELLS

A DISSERTATION

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by

Gurjeet Kaur

(Reg. no. 301301005)

Supervisor:

Dr. NIRANJAN DAS

Professor
Thapar University, Patiala

Co supervisor:

Dr. SUDARSHAN KUMAR

Scientist
NDRI, Karnal

DEPARTMENT OF BIOTECHNOLOGY
THAPAR UNIVERSITY, PATIALA (PUNJAB)-147004
JULY 2015

THAPAR UNIVERSITY

ndri

CERTIFICATE

This is to certify that the dissertation entitled **"Production and purification of recombinant buffalo Leukemia inhibitory factor (rBuLIF)"** submitted by **Gurjeet Kaur** in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

14-07-2015

Dr. Niranjan Das
Professor
Department of Biotechnology
Thapar University, Patiala

14.7.15

Dr. Sudarshan Kumar
Scientist, Animal Biotechnology Centre
National Dairy Research Institute, Karnal

Dr. Dinesh Goyal
Professor, Head of Department
Department of Biotechnology
Thapar University, Patiala

Dr. S.S. Bhatia
Dean, Academic Affairs
Thapar University, Patiala

CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this dissertation entitled **"PRODUCTION AND PURIFICATION OF RECOMBINANT BUFFALO LEUKEMIA INHIBITORY FACTOR (rBuLIF)"** in partial fulfilment of the requirements for the award of the degree of **Masters of Science in Biotechnology, Department of Biotechnology (DBT), Thapar University, Patiala,** is an authentic record of my work during the period of six months from January 2015 to July 2015, under the guidance of **Dr. Niranjan Das, Professor, Thapar University, Patiala.**

I have not submitted the embodied in this thesis for the award of any other degree or diploma.

Gurjeet Kaur.

Gurjeet Kaur
(Reg. No. 301301005)

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Gurjeet Kaur

301301005

Abbreviations

Aa	**:**	**Amino acid**
AHC	**:**	**Adrenal hypoplasia congenital**
BHK	**:**	**Baby Hamster Kidney**
BMP	**:**	**Bone morphology protein**
Bp	**:**	**Base pair**
BuLF	**:**	**Buffalo Leukemia inhibiting factor**
CD	**:**	**Complementary of Determination**
Cm	**:**	**Centimeter**
CnBr	**:**	**Cynogen bromide**
CREB	**:**	**cAMP response element-binding**
Da	**:**	**Dalton**
DAB	**:**	**3,3'-diaminobenzidine**
DEAE	**:**	**Diethylaminoethyl**
^{o}C	**:**	**Degree centigrade**
DEPC	**:**	**Diethylpyrocarbonate**
DIC	**:**	**Differential interference contrast microscopy**
DNA	**:**	**Deoxyribonucleic acid**

dNTPs	**:**	**deoxy Nucleotide Tri Phosphates**
DMEM	**:**	**Dulbecco's Modified Eagle's Medium**
DMSO	**:**	**Dimethyl sulphoxide**
DSS	**:**	**Dosage-sensitive sex reversal**
e.g.	**:**	**Example**
EB	**:**	**Embryoid body**
EDTA	**:**	**Ethylene diamine tetra acetate**
Eed	**:**	**Embryonic ectoderm development**
EpiSC	**:**	**Epiblast stem cells**
ERK	**:**	**Extracellular Regulated Kinase**
ESCs	**:**	**Embryonic Stem Cells**
FBS	**:**	**Fetal Bovine serum**
FGF	**:**	**Fibroblast growth factor**
Fig	**:**	**Figure**
GABP	**:**	**GA-binding protein**
GE	**:**	**Gel Electrophoresis**
GFP	**:**	**Green Fluorescent Protein**
GM-CSF	**:**	**Granulocyte-macrophage colony-stimulating**
Grb2	**:**	**factor Growth-factor-receptor-bound protein 2**

bLIF	**:**	**Bovine leukemia inhibitory factor**
hLIF	**:**	**Human leukemia inhibitory factor**
GSK	**:**	**Glycogen synthase kinase**
H	**:**	**Hour**
HEK	**:**	**Human Embryonic Kidney cells**
HiPSC	**:**	**Human induced pluripotent cell**
HIV	**:**	**Human immunodeficiency virus**
HPRT	**:**	**Hypoxanthine guanine Phosphoribosyl transferase**
HSV	**:**	**Herpes simplex virus**
IBS	**:**	**inclusion bodies**
ICM	**:**	**Inner Cell Mass**
Ig	**:**	**immuno globulin**
Inc.	**:**	**Inclusion**
IL	**:**	**Interleukin**
JAK	**:**	**Janus kinase**
Kb	**:**	**Kilo basepair**
KDa	**:**	**Kilodalton**
Klf	**:**	**KrOppel-like factor**
LB	**:**	**Luria Bertaini**
LIF	**:**	**Leukemia inhibitory factor**

LPS	**:**	**Lipo Poly Saccharides**
Ltd.	**:**	**Limited**
mAb	**:**	**Monoclonal Antibody**
MAPK	**:**	**Mitogen activated protein kinase**
MEF	**:**	**Mouse embryo fibroblasts**
MEK	**:**	**MAPK kinase**
MEM	**:**	**Minimal essential medium**
MEL	**:**	**Mouse Erythroleukaemia**
µg	**:**	**Microgram**
Mg	**:**	**Milligram**
ML	**:**	**Millilitre**
Mmol	**:**	**Millimolar**
Min	**:**	**Minute**
M	**:**	**Molar**
MS	**:**	**Multiple sclerosis**
N	**:**	**Normality**
NCBI	**:**	**National centre Bioinformatics**
NCCS	**:**	**Information National centre for cell science**
Ng	**:**	**Nanogram**
NIH	**:**	**National Institute of Health**
Ntd	**:**	**Nucleotide**
O.D	**:**	**Optical Density**

PAcGFP	**:**	**Pl-derived artificial chromosome**
PAGE	**:**	**Polyacrylamide Gel Electrophoresis**
PBS	**:**	**Phosphate Buffer solution**
PCR	**:**	**Polymerase Chain Reaction**
PI3K	**:**	**Phosphatidylinositol-3 phosphate kinase**
PKC	**:**	**Protein kinase C**
PLC	**:**	**Phospholipase C**
PTEN	**:**	**Phosphatase and tensin homolog**
PTMs	**:**	**Post-transcriptional modification**
PVDf	**:**	**Polyvinylidene difluoride**
Pvt.	**:**	**Private**
pUC	**:**	**plasmid of university of California**
rBuLIF	**:**	**recombinant buffalo Leukemia Inhibitory Factor**
Rpm	**:**	**Revolution per minute**
RSK	**:**	**Ribosomal S6 kinase**
SDS	**:**	**Sodium Dodecyl Sulfate**
SGK	**:**	**serum/glucocorticoid-regulated kinase**

SHP	**:**	**Small heterodimer partner**
SOB	**:**	**Super Optimal Broth**
SOCS	**:**	**Suppressor of cytokine signaling protiens**
SOS	**:**	**Son Of Sevenless**
SRL	**:**	**Sisco Research Laboratories**
STAT	**:**	**Signal transducers and activators of transcription**
SV	**:**	**Simian vacuolating virus 40**
TAE	**:**	**Tris base, acetic acid and EDTA.**
T-box	**:**	**TATA-box**
TBST	**:**	**Tris-Buffered Saline and Tween 20**
TE	**:**	**Tris EDTA**
TEMED	**:**	**Tetra methyl ethylene diamine**
TEV	**:**	**Tobacco Etch Virus**
TMD	**:**	**Transmembrane domain**
TNF	**:**	**Tumor Necrosis Factor**
TK	**:**	**Thymidine Kinase**
UV	**:**	**Ultra violet**
Vac	**:**	**Vacuum**
VIS	**:**	**Visible**

CONTENTS

ABSTRACT

Leukemia inhibitory factor (LIF) is a secreted glycoprotein that was initially identified through its ability to induce macrophage differentiation in the murine M1 myeloid leukemic cell line. LIF also maintain pluripotency in Embryonic Stem cells (ESCs). Mouse LIF (mLIF) is in use for buffalo stem cells production whose efficiency is unclaimed. We successfully cloned the buffalo LIF gene in pAcGFP-N1 vector which was subsequently transfected into eukaryotic cell line (COS-1). After several rounds of selection in the presence of G418 and single cell clonal expansion, a stably transfected cell line of COS-1_BuLIF was developed which expressed BuLIF_GFP at high level. The strong expression of BuLIF was observed at 70^{th} passage and cells could grow in the absence of selection pressure without losing GFP signal. PCR based identification to detect GFP and BuLIF gene in the COS-1_BuLIF genome further confirmed the genomic integration of BuLIF. Co-immunoprecipitation technique was employed for the purification of rBuLIF_GFP where Cynogen Bromide activated Sepharose was used as matrix to couple with anti GFP monoclonal antibody. Pure BuLIF_GFP protein was obtained from several T75 cm^2 confluent flasks. The SDS-PAGE revealed a single band of around 65-70kDa molecular weight protein which corresponds to the expectedmolecular weight of BuLIF_GFP fusion protein (22.9kDa+35.0 kDa=55.9kDa). LIF protein is highly glycosylated which increase the size of protein from 10-30kDa. The purified protein will further confirmed by Mass spectrometry based identification (Q-TOF) which revealed the identity of protein as BuLIF. Thus, this work demonstrates the successful purification of rBuLIF_GFP which may find its applications in bovine stem cell and related biological field.

INTRODUCTION

Leukemia Inhibitory factor (LIF) is pleiotropic molecule synthesized and secreted in various body tissues such as uterine gland cells, blastocyst, thymus and lung, cardiac muscle, kidney, neuronal tissue following injury, skin, oocyte. Its existence has been reported in many species such as human, porcine, ovine, rat, mink including cattle and buffalo. Some of the common biological functions regulated by LIF are like proliferation of cells, differentiation, calcium and bone metabolism, induction of acute phase proteins, cachexia in organism affected with neoplastic disorders etc. The first evidence of this molecule was found from the work of Metcalf and colleagues at the Walter and Eliza Hall Institute ofMedical Research in Melbourne, Australia in the year 1986 wherein, the LIF molecule was described as a factor that induced the differentiation of mouse myeloid leukemic M1 cells into macrophages. However, it plays many other functions like it regulates the growth and differentiation of embryonic stem cells, primordial germ cells, peripheral neurons, osteoblasts, adipocytes, and endothelial cells also. A number of reports came on characterization of LIF, its production and purification and about elucidation of its function and its application in various species (mouse, human, cattle, buffalo, porcine, ovine, rat, goat etc.).

One of the major applications of LIF is in the form of its use for the maintenance of pluripotency in mouse and human embryonic stem cell. An important role for LIF in implantation was shown on LIF knockout mice, when embryo implantation did not occur. Propagation of mouse embryonic stem is mediated via co-culture with a feeder layer of embryo fibroblasts or provision of a cytokine such as leukemia inhibitory factor (LIF) that acts through the LIF-R/gp130 complex. LIF-deficient fibroblasts are reported to be incapable of supporting embryonic stem cells self-renewal which indicates that LIF is essential for pluripotency of embryonic stem cell. LIF binds to the specific LIF receptor (LIFR-α) which forms a heterodimer with a specific subunit common to all members of that family of receptors, the GP130 signal transducing subunit. This leads to activation of the JAK/STAT and MAPK cascades.

LIF is normally expressed in the tropho-ectoderm of the developing embryo and endometrium particularly at the time of implantation with its receptor LIFR expressed throughout the inner cell mass. LIF maintains the pluripotency of ESCs through the production of Sox2 and Nanog via two different pathways JAK-STAT3 and MAPK

respectively. This property of LIF makes it a suitable media component to be used in *in-vitro* experiments of ESCs.

The first report on the production of embryonic stem cell in bovine came in 2007 and on buffalo embryonic stem cells in the year 2009. In all such studies media was supplemented with LIF from mouse or human source. Recombinant human LIF is added to bovine embryo culture media at a concentration of 100ng/ml**.** Buffalo ESCs have been derived from *in-vitro* produced embryos by using mouse LIF. It is understood that although seemingly similar pathways are operational in different species for the maintenance of pluripotency in embryonic stem cells, there are miniscule differences at molecular level in the specific signaling pathways. For example, the major cell signaling pathways that govern ESC self-renewal in mice, are the JAK-STAT, WNT, BMP4 and MEK-ERK, whereas in human the FGF and MEK-ERK paths are more critical. Because of this, although mouse LIF is effective in both the mouse and human embryonic stem cell pluripotency, human LIF cannot be used in mouse stem cells. It has been observed that bovine and buffalo LIF sequences (nucleotides) are closer to each other and are relatively different from mouse or human LIF. The nucleotide sequence of the human LIF gene indicates 78% sequence identity with murine LIF. *Bostaurus* LIF with 202 amino acids including 18 amino acids as signal peptide mapped to chromosome 17 shares 88.61% (nucleotides) and 89.11 % (amino acid) homology with human LIF. Post translational modifications (PTMs) like glycosylation and phosphorylation are different in buffalo LIF than that of mouse or human LIF as predicted from sequence analysis. Thus, use of mouse or human LIF for bovine embryonic stem cells is a compromised choice in the absence of a suitable bovine LIF.

When recombinant LIF is used, its host cell of expression becomes important. LIF is considered a heavily glycosylated protein. LIF expressed in bacterial host may not have the identical PTMs as that of native bovine LIF. Further, required refolding step limits the recovery pure protein. It is desirable that the recombinant LIF should be as close as native LIF. For this, eukaryotic systems like yeast, insect and mammalian cell lines have been used for the production of various proteins as well as human LIF. Bovine LIF was never produced in recombinant form and hence, its functional characterization and application in embryonic stem cells remained unfulfilled. It is envisaged that, bovine origin LIF will help overcoming shortcomings of current state of embryonic stem cell science. Production of buffalo ESCs will be easier and faster. Cost of production of buffalo ESCs may be much lower. It is critical to understand and characterize the molecule and its pathway involved in maintenance of pluripotency of buffalo ESCs.

2.1 Embryonic Stem Cells

Embryonic stem (ES) cells are pluripotent cells, ES cells are derived from the inner cell mass (ICM) of blastocyst-stage embryos and possess the capacity for long-term propagation and broad differentiation capability.Mouse embryonic stem cells are derived from the epiblast cells within the inner cell mass of a blastocyst (Brook and Gardner *et al.,* 1997; Martin *et al.,* 1981). The related property of totipotency is a characteristic restricted to the fertilized egg and early cleavage stage blastomeres, because they alone can give rise to all cells required for development (Lovell- Badge *et al.,* 2001). Following the reports of their isolation in mouse (Martin*et al.,* 1981) and human (Reubinoff*et al.,* 2000), attempts have been made to establish ES cell-like cell lines from various mammals like rat (Iannaccone*et al.,* 1994), rabbit (Graves and Moreadith*et al.,* 1993), hamster, mink (Saroyan *et al.,* 1992) and rhesus monkey (Thomson*et al.,* 1995). In farm animals, ES cell-like cells have been established in sheep,cattle (Strelchenko*et al.,* 1996). pig (Wheeler *et al.,* 1994), horse (Saito *et al.,* 2002) and Recently ES-like cells have been established from buffalo (Verma*et al.,* 2007). The IPS (induced pluripotent stem cells) cells generation from somatic cells with defined transcription factors in human (Takahashi *et al.,* 2007) and mouse from farm animal species also. Pluripotent cells from farm animals could provide a powerful tool for studies on early embryonic development, gene targeting, cloning, transgenesis and chimera formation

2.1.1 Pluripotency of Embryonic Stem (ES) Cells

Embryonic stem (ES) cells derived from pre-implantation embryos have the potential to differentiate into any cell type derived from the three germ layers of ectoderm (epidermal tissues and nerves), mesoderm (muscle, bone, blood), and endoderm (liver, pancreas, fgastrointestinal tract, lungs) and adult cells (Thomson *et al.,* 1998). The basis of pluripotentiality resides in conserved regulatory networks composed of numerous transcription factors and multiple signaling cascades. Together, these regulatory sequences maintain ES cells in aundifferentiated form and pluripotent (Yamanaka*et al.,* 2008).

2.1.2 Self-Renewal of ES Cells

The detail study of Austin Smith and colleagues (Smith *et al.,* 2001).revealed most critical pathway by which cytokine signaling acts to promote mES cell self-renewal They isolated a soluble glycoprotein that prevents stem cell differentiation and established that ES cell self-renewal is dependent on paracrine signals from feeder cells of mouse embryonic fibroblasts (MEF) on which mES cells were initially cultured (Hooper *et al.,* 1987). The factor required for self-renewal of mES was LIF (Williams *et al.,* 1988) that firstly was identified as the cytokine capable of inducing the differentiation of M1 myeloid leukemia cells into macrophages (Ichikawa *et al.,* 1970).

2.2 Leukemia Inhibitory Factor (LIF)

Leukemia inhibitory factor (LIF) was first identified by Metcalf and colleagues in 1986 at the Walter and Eliza Hall Institute of Medical Research in Melbourne, Australia. Leukemia inhibitory factor (LIF) is a secreted glycoprotein that was initially identified through its ability to induce macrophage differentiation in the murine myeloid leukemiccell line M1 (Hilton *et al.,* 1988).

2.2.1 LIF as an Extrinsic Signal

ES cells have been maintained in co-culture with mitotically fibroblasts in inactive(Martin *et al.,* 1981). If the medium is supplemented with leukemia inhibitory factor (LIF) then the co-culture system is unnecessary to maintain (Williams *et al.,* 1988). Moreover, LIF signaling is largelyresponsible for the pluripotency-promoting activity of the fibroblasts in these co-cultures (Chambers and Smith, 2004) In brief, the positive event occurring upon LIF stimulation of ES cells is the dimerization, tyrosine phosphorylation and translocation to the cell nucleus of the signal transducer and activator of transcription, STAT3 (Matsuda *et al.,* 1999). In the absence of LIF signaling, induced either by LIF withdrawal or by the expression of a dominant interfering form of STAT3, ES cells differentiate into a morphologically mixed cell population expressing genes characteristic of endoderm and mesoderm (Niwa*et al.,*1998; Niwa*et al.,* 2000).

2.2.2 Pleotropic Nature of LIF

Three laboratories simultaneously discovered and cloned the LIF cytokine through its pleiotropic biological activities on i) the proliferation of adult human T cells (HILDA)(Moreau *et al.,*1988), ii) the inhibition of leukemic cell differentiation(Gough *et al.,* 1988). iii) the maintenance of ES cells pluripotency (DIF)(Smith *et al.,* 1992) LIF was thus characterized as a pleiotropic cytokine with pro or anti-differentiation, pro or anti-survival effects depending upon cell maturity and cell types (Trouillas*et al.,* 2009).

2.2.3 LIF Production in different tissues

LIF mRNA is transcribed in multiple organs (Brown *et al.,* 1994) like most cytokines, LIF production is highly inducible with a wide range of inducing agents according to the cell type involved. In uterine tissue, LIF production by uterine gland cells changes abruptly prior to implantation (Song *et al.,* 2000). LIF production has also been reported in the blastocyst (Sun *et al.,* 1998), hypophysis (Chesnokova*et al.,* 2000), cardiac muscle (Ancey*et al.,* 2002), kidney (Morel *et al.,* 2000), thymus and lung (Fukada*et al.,* 1997), neuronal tissue following injury (Tofaris*et al.,* 2002), and in the skin (Bonifati*et al.,* 1998).

2.3 Molecular Biology of LIF and LIF receptor (LIFR)

2.3.1 Molecular biology of LIF

LIF was originally characterized and cloned as a differentiation factor for the murine leukemic M1 cell line.

2.3.1.1 LIF genes in the mammalian systems

To date, the murine (GenBank Accession: X06381, M63419 J05435, X12810 m60289, S73374)(Gearing *et al.,*1987; Hsu*et al.,* 1994; Stahl *et al.,*1994), human (GenBank Accession: M63420 J05436, X13967) (Moreau *et al.,*1988; Sutherland *et al.,*1989),ovine (Willson*et al.,*1992), porcine (Willson*et al.,*1992), bovine (GenBank Accession: D50337, U63311) (Kato *et al.,*1992), rat (Takahama*et al.,*1992), and mink (GenBank Accession: AF048827) (Song *et al.,*1998) genes for LIF have been cloned. Southern blot analysis with human and murine probes of the LIF coding region yield a unique hybridization pattern(Stahl *et al.,* 1990),indicating a single gene locus. The murine LIF gene is located on chromosome number 11A1 (Bottorff*et al.,* 1992), while the human LIF gene is located on chromosome number 22q12.1–12.2 (Sutherland *et al.,* 1989). In the murine and human genome, the LIF

gene is in close proximity to the OSM gene (Budarf*et al.,* 1989). The human and murine LIF gene consist of 3 exons and 2 introns (Stahl et *al.,* 1992). The length of the murine and human LIF gene is approximately 6.0 kb and 6.3 kb, respectively.

2.3.1.2 LIF expression

LIF gene expression can be induced by several pro-inflammatory agents, *e.g*., lipopolysaccharide (Brown *et al.*,1994; Wang *et al.*,1996),IL-1 (Aricie*t al.*,1995), IL-17 (Gollner*et al.,* 1999) or inhibited by anti-inflammatory agents, *e.g*., glucocorticoids (Grosset*et al.*,1992), IL-4 (Gollner*et al.,* 1999), and IL-13 (Auernhammer*et al.,* 1998), respectively.

2.3.1.3 Structural attributes of LIF

LIF is a long-chain four-a-helix bundle cytokine (Bazan*et al.,* 1991; Sprang *et al.,* 1993). The four-a-helix bundle cytokines are subdivided into short chain and long-chain cytokines, as their helices comprise approximately 15 or 25 residues, respectively (Walter *et al.,* 1997).

2.3.2 LIF Receptor—Gene Structure and Regulation

2.3.2.1 LIFR gene and structure

The human (GenBank Accession: X61615) (Gearing *et al.,* 1991), murine (GenBank Accession: D26177, S73496, S73495, S81861, X99778, X99779) (Owczarek*et al.,* 1996) and rat (GenBank Accession: D86345) (Aikawa*et al.,* 1997) gene for LIFR a have been cloned. The LIFR gene is located on human chromosome 5p12–13 and murine chromosome 15

2.3.2.2 Membrane-bound LIFR

Human LIFR (GenBank Accession: X61615), is an approximately110-kDa protein that is highly glycosylated to about 190 kDa at multiple potential N-linked glycosylation sites (Gearing *et al.,* 1991).

2.3.2.3 Soluble LIFR

Murine LIFR exists in both a membrane-bound and a soluble form, the latter lacking the transmembrane and cytoplasmic domains.

2.3.2.4 LIF binding and LIFR expression

Low- and high-affinity binding sites for LIF have been described in several cell types (Godard *et al.,* 1992). A low number of approximately 150–400 high-affinity binding sites are found on most cells responsive to LIF. Furthermore, approximately 1,000–6,000 low-affinity binding sites are present on many cell types. While LIFR a constitutes the low-affinity binding site, association of the LIF-LIFR complex with gp130 results in its conversion to a high-affinity binding site (Gearing *et al.,*1992). Several human cell lines exhibiting no detectable binding of non-glycosylated human LIF, revealed 3,000 to 40,000 binding sites for glycosylated human LIF, due to the Man-6-P/IGFII-R (Blanchard *et al.,* 1992. Binding of LIF to the Man-6-P/IGFII-R caused no downstream functional effects, but mediated a rapid internalization and degradation of LIF (Blanchard *et al.,* 1993).

2.4 LIF Signaling

2.4.1 General Principles of Cytokine Signaling

Leukemia inhibitory factor (LIF) belongs to the family of interleukin (IL)-6-type cytokines, which signal through the common receptor subunit gp130 in association with a ligand-specific receptor subunit. The binding of LIF to the LIFR induces its hetero-dimerization with gp130.The cytokine receptor superfamily comprises single transmembrane proteins that can homo or heterodimerize upon binding of one or more ligands.(David *et al.,*2005)When LIF binds to the LIF receptor, it triggers three signaling pathways: (i) the JAK (Janus kinase)/STAT3 (signal transducer and activator of transcription 3) pathway; (ii) the PI3K (phosphoinositide 3-kinase)/AKT pathway; and (iii) the SHP2 (SH2 (Src homology 2) domain-containing tyrosine phosphatase 2)/MAPK (mitogen-activated protein kinase) pathway.

2.4.1.1 LIF/JAK/STAT3 signaling pathway

The LIF/JAK/STAT3 pathway has been extensively studied (Murray *et al.,* 2007; Levy *et al.,* 2002; Heinrich *et al.,* 2002; Heinrich *et al.,* 1998), and its central role in self-renewal has been well established by the fact that activation of STAT3 is sufficient to maintain mESC self-renewal (Matsuda *et al.,* 1999; Murray *et al.,* 2007; Levy *et al.,* 2002). STAT3 is a ubiquitously expressed protein containing six domains: a dimerization domain, a coiled-coil domain, a DNA-binding domain, a linker domain, an SH2 domain and a transactivation domain (Schindler *et al.,* 2007; Leonard *et al.,* 1998). It is activated by JAKs.

JAKs contain seven domains called JH (JAK homology) domains 1–7, which are conserved among family members (Schindler *et al.,* 2007, Leonard et *al.,* 1998). This heterodimer activates the gp130-bound JAK through trans-phosphorylation within a single JAK or between two JAKs (Murphy *et al.,* 2010).

Phosphorylation of the tyrosine at position 1022 is critical for activation of JAK1. Among the four JAKs, JAK1 and JAK2 are the ones primarily involved in the LIF signaling pathway (Kisselev*aet al.,* 2002). Dimerized STAT3 is then imported into the nucleus via the binding of nuclear import proteins, importin-α3 and importin-α6, to the coiled-coil domain of STAT3 (Liu *et al.,* 2005; Reich *et al.,* 2006). STAT3 is constantly shuttled between the cytoplasm and nucleus independently of Tyr705 phosphorylation. Dimerized STAT3 finally binds to the consensus sequence TTCCSGGAA (S C or G) at the enhancer of its target genes to regulate their expression (Chen *et al.,* 2008; Leonard *et al.,* 2008). Although LIF is the only member of the IL-6 family routinely used to culture mESCs, other family members such as oncostatin M, ciliaryneurotrophic factor and cardiotrophin 1 can also maintain self-renewal of mESCs because of their shared signaling mechanisms that converge on STAT3 (Conover *et al.,* 1993).

2.4.1.2 LIF/PI3K/AKT signaling pathway

In a second LIF signaling pathway, JAKs activate PI3Ks, potentially through phosphorylation of the regulatory subunit p85 (Migone*et al.,* 1998), which then activates the AKT serine/ threonine kinases (Paling *et al.,* 2004).AKTs inhibit their major target protein GSK3β (glycogen synthase kinases 3β) by two independent mechanisms, resulting in an increase of the levels of Nanog and c-Myc, both of which are important for self-renewal of mESCs (Bechard*et al.,* 2009). GSK3β promotes ubiquitin dependent degradation of c-Myc by phosphorylation of Thr58 (Cartwright *et al.,* 2005). GSK3β also inhibits Nanog expression, although the exact mechanism remains unknown (Storm *et al.,* 2007). Although GSK3α and GSK3β are structurally and functionally distinct (Hoeflich*et al.,* 2000), they are functionally redundant for self-renewal and differentiation of mESCs (Doble*et al.,* 2007). Therefore, although previous studies have focused on GSK3β, GSK3α might function in a similar way in the LIF/PI3K/AKT signaling pathway. Another pluripotency gene Tbx3 (T-box3) is also up-regulated by AKTs, although the involvement of GSK3α/β remains unknown (Niwa*et al.,* 2009). The LIF/PI3K/AKT pathway also induces acetylation of lysine residues on STAT3 (Wang*et al.,* 2005). Acetylated STAT3 forms more stable dimers and

more actively transcribes target genes without phosphorylation of Tyr705 (Braunstein*et al.*, 2003).

In mESCs, the PI3K/AKT pathway can also be activated by FGF4 (Ying *et al.*,), insulin, IGF1 (insulin-like growth factor 1) (Alessi*et al.*, 1996; Rubin *et al.*, 2007) and the ESC-specific Ras-like protein Eras (Takahashi *et al.*, 2003, Takahashi *et al.*, 2007), and inhibited by the tumour suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Sun*et al.*, 1999). FGF4 functions as an autocrine stimulator for differentiation of mESCs by inducing several signalling pathways, including the PI3K/AKT pathway.

2.4.1.3 LIF/SHP2/MAPK pathway and other LIF signaling pathways

The third LIF signaling pathway is less well characterized than the preceding two. When combined with the available evidence for other IL-6 family members, the third pathway is thought to signal through SHP2/MAPK (Figure 1) (Schiemann*et al.*, 1997). Binding of LIF to the LIF receptor induces JAK-mediated tyrosine phosphorylation on gp130, which leads to the recruitment and subsequent phosphorylation of SHP2 by JAKs. SHP2 then interacts with the Grb2 (growth-factor-receptor-bound protein 2)–SOS (son of sevenless) complex to activate the MAPK pathway involving the Ras/RAF/MEK/ERK cascade. Unlike the two pathways mentioned above, this system induces differentiation of mESCs by down-regulating Tbx3 and Nanog. The Ras/RAF/MEK/ERK cascade is also activated by autocrine FGF4, inducing differentiation of mESCs as described above.

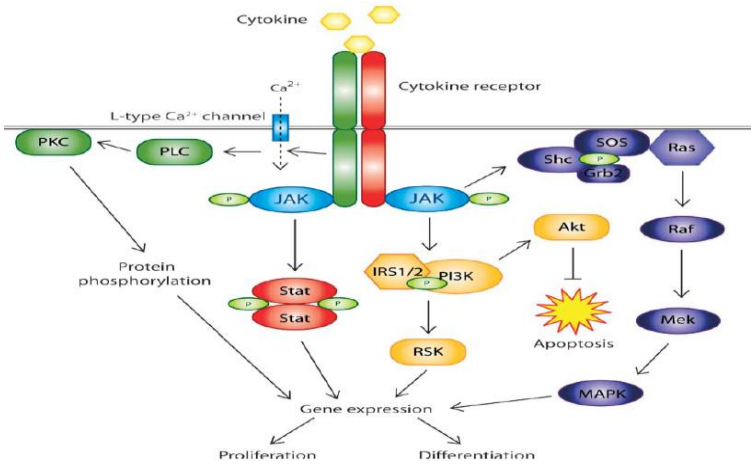


Fig.1: Different LIF (cytokine) induced signaling pathways in mouse Embryonic stem cells: IRS: Insulin receptor substrate; JAK: Janus kinase; MAPK: Mitogen-activated protein kinase; MEK: MAPK

kinase; PKC: Protein kinase C; RSK: Ribosomal S6 kinase; PI3K: Phosphatidylinositol-3 phosphate kinase; P: Phosphate group; PLC: Phospholipase C; SOS: Son of sevenless; STAT: Signal transducers and activators of transcription (Kristensen*et al.,* 2005).

2.5 Applications of LIF

Leukemia inhibitory factor (LIF) is a glycoprotein that has been increasingly recognized to possess a wide range of physiological activities. Some of the therapeutic applications LIF determined are as follows;

2.5.1 Therapeutic applications of LIF

i)A role for LIF in the host response against bacterial infection .The administration of LIF may confer beneficial effects and enhance host resistance against lethal endotoxemia. A single intravenous dose of recombinant human D factor completely protected C57/B16 mice from the lethal effect of Escherichia coli endotoxin (lipopolysaccharide [LPS]. When human D factor was combined with sub-protective doses of IL-I or TNF, there was dramatic synergistic protection against a subsequent lethal LPS challenge. This is so because IL-1 and TNF help in synthesis of LIF (Alexander *et al.,* 1992).

ii) The therapeutic efficacy of recombinant human leukemia inhibitory factor (LIF) was examined in a nonhuman primate model of radiation-induced marrow aplasia. Thrombocytopenia and neutropenia remain as dose-limiting consequences after high-dose irradiation or cytotoxic drug exposure. Cytokines such as recombinant human granulocyte and granulocyte-macrophage colony stimulating factor have been effective in reducing the duration of neutropenia after radiation- or drug-induced marrow aplasia in preclinical and clinical protocols (Farese*et al.,* 1994).

iii) The role of leukemia inhibitory factor in the establishment of pregnancy: Leukemia inhibitory factor (LIF) is required for blastocyst implantation in mice. Uterine expression of LIF and that of its receptors has been demonstrated in a number of mammalian species indicating that LIF may have widespread importance in the establishment of pregnancy. Recombinant human LIF (AM424) has been reported in clinical trials (Kurek*et al.,* 1998) making this a testable hypothesis.

iv) RecombinantmLIF induced an increase in blood platelet counts, an increase in megakaryocyte numbers in the spleen, elevated serum Ca levels, (Metcalf *et al.,*1990).

v) Therapies for multiple sclerosis (MS) reduce the relapse rate but are unable to stop neurological decline. Leukemia inhibitory factor (LIF) has the potential to act as a novel therapeutic in diseases with a neurodegenerative and inflammatory component, such as MS. (Slaets*et al.,* 2010)

2.5.2 Target Molecules of the LIF/STAT3 Pathway in ES Cells

Chip-on-chipand Chip-seq analyses have revealed that STAT3 binds to the regulatory regions of severalself-renewal genes in ES cells (Chen *et al.,* 2008; Kidder *et al.,* 2008).

2.5.2.1 Transcription factors

GA-binding protein (GABP) belongs to the transcription factor family and forms a hetero-tetramer consisting of two α-subunits and two beta-subunits (Rosmarin*et al.,* 2004). Thealpha-subunit (GABPα) mediates DNA binding, while the beta-subunit (GABPbeta) enhances thetranscriptional activity of thealpha-subunit.

Krüppel-like factor (Klf)-4 belongs to the KLF zinc finger protein family that shareshomology with the *Drosophila* Krüppel segmentation protein (Rowland and Peeper, 2006).Klf4 is expressed in a variety of tissues and plays an important role in many physiologicalprocesses, including cell proliferation and terminal differentiation. Klf4 can either activateor repress transcription, depending on the specific target gene, and it can function as anoncogene or a tumor suppressor gene in certain cellular contexts.

2.5.2.2 Transcriptional repressors

The orphan nuclear receptor Dax1 (DSS-AHC critical region on the X-chromosome gene 1) was originally identified as a gene responsible for thecongenital disease dosage-sensitive sex reversal (DSS) and for adrenal hypoplasia congenital (AHC); in humans, gene duplication causes male-to-female sex reversal, while mutations inDAX1 result in AHC (Niakan and McCabe, 2005). Dax1 is expressed in self-renewing ES cells under the regulation of STAT3 andOct3/4 (Clipsham*et al.,*2004; Sun *et al.,*2008), and its importance in ES cell self-renewal hasbeen suggested by both knockdown and knockout experiments (Niakan*et al.,*2006; Wang *et al.,*2006; Khalfallah*et al.,*2009).

2.5.2.4 Kinases

In addition to transcription-related genes, the LIF/STAT3 pathway regulates expression ofother classes of genes, for example, kinases. The *pim*genes encode

serine/threonine kinases, Pim-1, Pim-2 and Pim-3, regulate cell growth and apoptosis (Bachmann and Moroy,2005). The expression of Pim-1 and Pim-3 is regulated by STAT3 in ES cells (Aksoy*et al.,* 2007). Overexpression of Pim-1 and Pim-3 promotes self-renewal and knockdown ofthese genes increases the rate of differentiation and apoptosis (Aksoy*et al.*,2007), suggesting that these kinases have important roles in ES cell self-renewal. The Akt-related kinase serum/glucocorticoid-regulated kinase (SGK) can phosphorylateand inactivate GSK-3, a kinase that is known to regulate beta-catenin levels in ES cells (Tessier&Woodgett, 2006). SGK is downstream of both the STAT3 and PI3K pathways (Kobayashi& Cohen, 1999; Park *et al.*,1999; Bourillot*et al.*,2009).

2.6 Functions of LIF

LIF performs many functions and hence known as pleiotropic molecule. Some of the important functions LIF are mentioned below.

2.6.1 LIF as a Candidate Hematopoietic Regulator

Murine LIF is a glycoprotein with a 180-amino-acidsingle 4-alpha-helix polypeptide chain. LIF was purified frommedium conditioned by Krebs-II ascites tumor cells andthen cloned from a murine T-lymphocyte cDNA library asa factor able to induce macrophage maturation and terminate self-renewal of the undifferentiated and highly clonogenicmurine myeloid leukemia, M1 (Hilton*et al.*,1988). Combination ofthese actions suppressed the leukemic population, hence thename assigned. Cloning of the corresponding human LIFcDNA was performed using the murine cDNA as a probe(Gough *et al.*,1988). The name “leukemia inhibitory factor” has proven to bequite inappropriate for this highly poly-functional molecule but at least it has preserved LIF from the indignity of endingup merely with an anonymous IL- or CD-barcode number.LIF was presumed to be a factor playing some regulatoryrole in hematopoiesis and possibly having a special suppressiveaction on some myeloid leukemia.

2.6.2 Actions in Kidney

In collaboration with transforming growth factor-2,LIF produced by the ureteric buds induces clumps of culturedmesenchymal cells to differentiate into glomeruli andtubules (Barasch*et al.*,1999).

2.6.3 Neuronal Functions

There have been multiple reports of LIF action on neuronaltissue, which extend the original findings in LIF–/–mice: A) LIF enhances the survival of sensory and motorneurons (Murphy *et al.*,1991); B) LIF stimulates the formation of sensoryneurons from cultures of neural crest cells (Murphy *et al.*,1991); C) LIF with fibroblast growth factor (FGF) and epidermal growthfactor, can allow the protracted in vitro proliferation of multipotential human neural progenitor cells (Carpenter *et al.*,1999); D) LIF prevents oligodendrocyte death in animal models of multiplesclerosis(Butzkueven*et al.*,2002).

2.6.4 Endocrine Actions

LIF has been reported to have multiple effects onendocrine organs or their target tissues: A) LIF suppressesthe proliferation in vitro of normal human breast epithelialcells and breast cancer cells (Grant *et al.*,2001); B) LIF is a major regulatorof ACTH production in the pituitary, and its actions areblocked by SOCS-3 (Chesnokova*et al.,* 2002); C) Conversely, LIF inhibits theproduction of prolactin and growth hormone (Tomida*et al.*,2003

2.6.5 Actions in Bone

In vitro studies have extended the original observation of excess bone formation in mice with excess LIF levels:A) LIF increases calcium re-sorption from bone and increases osteoclast numbers (Reid *et al.*,1990) and B) conversely, LIF enhances bone formation by binding directly to osteoblasts and increasing osteoblast numbers (Dazai*et al.*,2000).

2.6.6 Actions in Muscle

LIF stimulate the proliferation of muscle satellite cells (Spangenburg EE, *et al.*,2002)and can ameliorate muscle fiber degeneration in vivo in mdx mice lacking dystrophin (Austin *et al.*,2000). LIF is a hypertrophic agent for cardiac muscle (Murata *et al.*,1999) and reduces apoptosis in such cells (Negoro*et al.*,2001).

2.6.7 Miscellaneous Effects

LIF has been reported to stimulate the proliferation ofneonatal mouse epidermal melanocytes (Hirobe*et al.*,2002) and keratinocytesfrom patients with amyotrophic lateral sclerosisand to enhance mast cell proliferation (Hu *et al.,* 2000). Whencombined with basic

FGF, LIF was noted to enhance theformation of capillary-like structures in cultures of anembryonic endothelial cell line (Paradis*et al.*,2000).

2.6.8 LIF Induces Apoptosis in Mammary Epithelial Cells

In this study the LIF is expressed at low but detectable levels in postpubertal, adult virgin, and mammary glands of pregnant mouse. However, LIF expression decreases after parturition to become almost undetectable and unmeasurable in lactating glands. LIF expression shows increase shortly after weaning that is maintained for 3 days. During this period, known as the first stage of mammary gland the lack of suckling promotes local factors that cause extensive epithelial cell death. Stat3 play major role in signaling the initiation of apoptosis, but the mechanism of its activation still unclear. LIF expression is induced by milk stasis. Implantation of pellets (LIF containing)in lactating glands results in increase in epithelium apoptosis. Moreover, this treatment also induces phosphorylation on Stat3. LIF regulated expression in mouse mammary gland may play important role during the first stage involution of mammary gland.It describes that LIF also induce mammary epithelium apoptosis partly because of Stat3 activation (Carolina Schere-Levy*et al.,* 2003)

2.7 Mouse and Human LIF

Mouse and Human LIF has been extensively used in embryonic stem cells culture media**.** Some of the features of mouse and human LIF are as follows.

2.7.1 Mouse LIF

Propagation of mouse ES cells is mediated via co-culture with a feeder layer of embryo fibroblasts or provision of a cytokine such as leukemia inhibitory factor (LIF) that acts through the LIF-R/gp130 complex (Yoshida *et al.,* 1994; Burdon *et al.,* 1999a). LIF-deficient fibroblasts are reported to be incapable of supporting ES cell self-renewal (Stewart*et al.,* 1992), indicating that supply of LIF is a key attribute of feeders. However, although no self-renewal factors have been identified other than gp130 cytokines, there is evidence for operation of a gp130-independent pathway that can maintain ES cell identity (Dani*et al.*, 1998).

In mouse embryonic stem cells (ESC) it blocks processes of spontaneous differentiation and formation of embryoid bodies (EB, analogs of early mammal embryos) maintaining thus the stem cells in pluripotent state *in vitro* (Gearing *et al.,* 1989; Raz*et al.,* 1999); (Nagy *et al.,* 1993). High dependence of mouse ESC lines from LIF is evidenced by

the fact that at a LIF-free culture medium the cells spontaneously differentiate into EB loosing thus their pluripotent properties. Main mechanisms of LIF effects, through cell membrane receptors, are comprehensively investigated (Haines *et al.,* 1999, 2000; Heinrich *et al.,* 2003; Gonzalez *et al.,* 2004; Giese *et al.,* 2005) and widely recognized; nevertheless dynamics of events at early stages of the protein interaction with ESC needs further clarifying.

Two major pathways have been characterized so far, leading to the identification of "master genes" critical for the maintenance of mouse ES cell pluripotency: the LIF/ STAT3 pathway, which synergizes with BMP2/4 and/or Wnt family members (as Wnt3a, Wnt5a and Wnt6, to maintain ES cell pluripotency alone (Boeuf *et al.,* 1997; Niwa*et al.,* 1998; Matsuda *et al.,* 1999), and the OCT4/SOX2 and NANOG pathways, the last one identified in cells in which the LIF pathway has been knocked down (Chambers*et al.,* 2003; Mitsui*et al.,* 2003). In mouse ES cells, LIF induces signaling pathways including JAK1/STAT3/MYC/CD9/SOCS3/PI3K and ERK/RSK/ CREB leading to activation of both anti- and pro-differentiate signals (Chapman *et al.,* 1999; Kritikou*et al.,* 2003; Boeuf*et al.,* 2003).

2.7.2 Human LIF

Human leukemia inhibitory factor (hLIF), also known as differentiation-stimulating factor (D factor) or melanoma-derived LPL inhibitor (MLPLI), is a cytokine that demonstrates multipleeffects on cells, disease (Hilton *et al.,* 1992; Chodorowska*et al.,* 2004). hLIF is absolutely required for maintaining the characters of stemness of embryonic stem cell (ESC) lines (Smith *et al.,* 1998; Williams *et al.,* 1998; Hirai *et al.,* 2011). LIF-deficient mice demonstrate difficulties in blastocyst implantation (Chen *et al.,*2000), which suggests thatadministration ofhLIF may aid the implantation rate of women displaying some forms of infertility (Aghajanova*et al.,* 2004). The efficient production of hLIF has been continuouslypursued because of its uses in biomedical research and clinical medicine. The expression of LIF in eukaryotic cells has been reported (Gough *et al.,* 1988; Hilton *et al.,* 1988), although the yields are low. *Escherichia coli* (*E. coli*) produces better expression, however the protein misfolds and aggregates to form inclusion bodies (IBs) in this expression host (Samal*et al.,* 1995). In general, the solubilization of IBs requires high concentrations of denaturants, such as urea orguanidine hydrochloride, and subsequent refolding via theremoval of the denaturants. In many cases the overall yield ofbiologically active protein from IBs is low (Fahnert*et al.,* 2004).

2.8 Effects of Human vs. Mouse Leukemia Inhibitory Factor on the In Vitro Development of Bovine Embryos

Leukemia inhibitory factor (LIF) is a cytokine that shows conflicting effects on in vitro produced (IVP) bovine embryos. Bovine LIF (bLIF) has been cloned and used in culture, but there is no commercially available bLIF (Rodrıguez *et al.*,2007). Thus, researchers use human LIF (hLIF) to supplement the culture medium for bovine embryos because of its greater sequence homology compared to murine LIF (mLIF). The human cytokine shares 89.1% sequence homology with bLIF and 76.8% sequence homology with mouse LIF (mLIF) .However, the use of hLIF has yielded controversial results as compared to bLIF (Rodrıguez *et al.*,2007).During the whole culture period, concentrations of recombinant 100 ng ml_1 bovine LIF (bLIF) or lower have been shown to improve bovine embryonic development (Yamanaka *et al.*,1990). These effects of bLIF are observed during the second half of the culture period (Yamanaka *et al.*,2001). Despite these findings, most studies assessing the effects of LIF in bovine embryo cultures have used human LIF (hLIF), since there is no commercially available bLIF. When added from the morula stage, hLIF has been reported to have no effect on subsequent development (Sirisathien*et al.*,2003; Funston *et al.*,1993). During the course of blastocyst formation, hLIF has been found to stimulate expansion and hatching (Marquant-Le Guienne*et al.*,1990; Sirisathien*et al.*,2003), but other authors report no comparable effect (Fukui *et al.*,1994). Recently, hLIF has been reported to have adverse effects on embryonic development kinetics, morphology, cell counts and expression of Oct4 and laminin, having no apparent influence on the subsequent formation of outgrowths (Vejlsted*et al.*,2005). In the present study, embryonic development was impaired by hLIF, while the number of ICM cells was reduced by mLIF, in disagreement with the effects described by Yamanaka *et al,* for bLIF(Yamanaka *et al.*,1999; Yamanaka *et al.*, 2001). While bLIF has been described to increase TE cell counts without affecting the ICM (Yamanaka *et al.*,1999; Yamanaka *et al.*, 2001), hLIF has been noted to increase (Sirisathien*et al.*,2003; Funston*et al.*,1993), decrease (Vejlsted*et al.*,2005) or have no effect on the ICM. In addition, mLIF was found here to have no effect on the number of TE cells yet to reduce the ICM. Thus, neither hLIF nor mLIF were able to mimic the effects reported for bLIF. The detrimental effects of hLIF during embryonic development we observed are consistent with the findings of a recent report by Vejlsted*et al*,(Vejlsted*et al.*,2005). These disparate effects exhibited by hLIF and mLIF during blastocyst formation provide additional evidence that these compounds should not be used to replace bLIF. Indeed, this has been

previously pointed out (Yamanaka *et al.*,1999; Yamanaka *et al.*,2001). In mice, reduced blastocyst development and cell numbers have been attributed totreatment with LIF antisense nucleotides (Cheng *et al.*, 2004). These effects and the existence of LIF mRNA in the TE and low affinity LIFR (a 190 kDa transmembrane protein) mRNA in the ICM but not in the TE (Nichols *et al.*, 1996), suggest that TE produced, endogenous LIF can bind to the ICM. In contrast, exogenous LIF does not bind to the ICM of whole mouse embryos (Fry *et al.*, 1992). This issue has not been addressed in cattle, but ES-like cells have been derived using hLIF(Saito *et al.*,2003) in the absence of exogenous LIF (Mitalipova*et al.*,2001), and the generation of cell colonies from blastomeres is not influenced by exogenous hLIF(Rexroad*et al.*,1993). Therefore, according to the results of the present and former reports, heterospecific LIF should not be used for experiments on bovine embryos and embryonic stem cells.

2.9 Expression and Purification of recombinant LIF protein

2.9.1 Expression of Recombinant LIF Protein

The process of introducing nucleic acids into eukaryotic cells by non-viral methods is defined as transfection. Using various chemical, lipid or physical methods, this gene transfer technology is a powerful tool to study gene function and protein expression in the context of a cell. Development of reporter gene systems and selection methods for stable maintenance and expression of transferred DNA have greatly expanded the applications for transfection. Assay-based reporter technology, together with the availability of transfection reagents, provides the foundation to study mammalian promoter and enhancer sequences, trans-acting proteins such as transcription factors, mRNA processing, protein-protein interactions, translation and recombination events (Groskreutz and Schenborn, 1997)

2.9.1.1 LIF Expression in Prokaryotic System

Methods for the production and purification of recombinant hLIF from Escherichia coli have previously been attempted and reported by many groups. Gearing *et al.*, made recombinant mLIF and rhLIF expressed as a glutathione- S-transferase based fusion construct (Gearing *et al.*, 1989). Tomala*et al.*, reported hLIF in a thioredoxin based fusion protein as soluble form with subsequent TEV protease cleavage and membrane chromatography (Tomala*et al.*, 2010). Imsoonthornruska*et al.*, reported a soluble thioredoxin-histidine LIF fusion protein using a single purification step (Imsoonthornruska*et al.*,2011).

Although both the murine (Ruan*et al.,* 2004; Dubendorff*et al.,* 1991) and human LIF have been earlier expressed in *E.coli,*yeast and COS cells (Nishi *et al.,* 2004), high level expression of human LIF (hLIF) in *E.coli* and its purification, and physicochemical characterization has also been done by Samrul*et al*.

However, in prokaryotic system the required refolding step limits the recovery rate. A novel strategy was designed by Lin J*et al,* in 2011 to produce a soluble recombinant human LIF (rhLIF) in the prokaryotic system in order to obtain higher yields of the bioactive protein with simpler steps. This optimal hLIF gene was cloned, and it successfully expressed the soluble recombinant protein in E. coli using the thioredoxin (Trx) protein as a fusion partner. A simple purification procedure is established to purify the recombinant fusion protein from the soluble supernatant of the lysed culture cells.

Leukemia inhibitor factor (LIF) is a three disulfide bridge-containing cytokine with numerous regulatory effects. The high level expression of a soluble recombinant human LIF (rhLIF) in Escherichia coli was done by codon-optimized ProfinityeXact™-tagged hLIFcDNA cloned into pET3b vector, and transformed into E. coli OrigamiB(DE3) harboring the bacterial thioredoxin co-expression vector. (Ruan*et al.,*2004) The recombinant protein was purified via a single chromatographic step using an affinity tag-based protein purification system that processed by cleavage with sodium fluoride, resulting in the complete proteolytic removal the N-terminal tag. Soluble rhLIFyield was estimated to be approximately 1 mg/g of wet weight cells, with above 98% purity.

2.9.1.2 LIF Expression in Eukaryotic System

The baculovirus expression vector system has been widely used to express a variety of heterologous genes in insect cells and caterpillars (Luckow*et al.,* 1988; Miller *et al.,* 1988). Insect cells provide a suitable environment for post-translational modifications and folding of the protein product such that the foreign proteins synthesized resemble their authentic counterparts in almost all respects (O'Reilly *et al.,*1992), and the insect larval system offers an exciting alternative because of lesser equipment requirements and simple operations involved in the mass-scale production (rearing and maintenance of larvae) compared to tissue-cultured cells.Mammalian expression systems comprise a plethora of different cell lines used for protein production. Most commonly, Chinese hamster ovary (CHO) cells (Page*et al.,* 1991; Konig*et al.,* 1989; Cosgrove *et al.,* 1995; Cockett*et al.,* 1999), baby hamster kidney (BHK) cells (Wirth *et al.,* 1989), human embryonic kidney (HEK) 293 cells

(Berg *et al.*, 1993), mouse L-cells (Misrahi*et al.*, 1994; Venkatgopal*et al.*, 1989), and myeloma cell lines like J558L (Trauneckeret *al.*, 1991; Lane *et al.*, 1993;) and Sp2/0 (Gillies*et al.*, 1992; Lo *et al.*, 1989) are employed as hosts for the establishment of stable transfectants.Less frequently the use of NIH 3T3 cells (Kane *et al.*, 1992), lymphoblastoid cell lines like Namalwa (Okamoto *et al.*, 1990) and RPMI 1788 (Lopez *et al.*, 1994), and murine erythroleukemia (MEL) cells (Needham *et al.*, 1992) has been described.

The expression of the cytokine human Leukemia Inhibitory proteins (hu-LIF) in five of the most commonly used systems, namely in CHO, Sp2/0, MEL, COS, and insect cells has been done by Sabine G *et al*.

In result, the stably transfected cell lines, CHO, Sp2/0, and MEL cells, gave rise to production of fully glycosylated hu-LIF at variable product titers; incompletely glycosylated, albeit biological active hu-LIF could be rapidly produced by transient expression in COS cells or by baculovirus-mediated infection of insect cells.

Table 1: Expression of Human LIF in different expression systems (Sabina *et al.*, 1996)

System	Cell growth	Mode of gene transfer	Gene amplification
CHO cells	Adherent/suspension	Stable transfection	Yes
Sp2/0 cells	Suspension	Stable transfection	Yes
MEL cells	Suspension	Stable transfection	Optional
COS cells	Adherent/microcarrier	Transient transfection	No
BEVS	Adherent/suspension	Infection	No

A fusion gene coding human granulocyte-macrophage colony stimulating factor (GM-CSF) and LIF (cDNAs was inserted into the transfer vector pSXIVVI+ X3 with the control of Syn and XIV promoters. The Sf9 cells (Spodopterafrugiperda) were co-transfected with the recombinant plasmid and TnNPV DNA (Trichoplusiani nuclear polyhedrosis virus DNA) (Zhang *et al.*, 1999). The fusion protein recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) could be synthesized in cells infected with recombinant virus at a level of about 23% of their total cellular protein. Activity analysis of the fusion protein in infected cells revealed that it exhibited the dual activities of GM-CSF and LIF (Zhang *et al.*, 1999). Western blot analysis of the expressed

fusion protein in infected larvae showed that the virus-mediated fusion protein, with a molecular weight of 35 kDa, is confirmed with immunoreactivity (Zhang *et al.,*1999).

LIF is highly glycosylated when it binds to receptor to activate the signal transduction. Therefore, expression of LIF through eukaryotic system is the best way to obtain the correct glycosylation. Human LIF cDNA with the sequence of signal peptide was cloned from adult blood cells by RT-PCR, and then sub-cloned into pcDNA3 for expression in HEK-293T cells. After transfection of the recombinant plasmid pcDNA3/LIF into HEK-293T cells, the conditioned medium containing the secreted LIF was obtained.

2.9.1.2 COS-1 Cell Line as Mammalian Host for Expression

The expression of heterologous proteins has been described for many unicellular organisms and cell lines from a variety of species. While recombinant proteinsexpressed in the cytoplasm of bacteria are often insoluble and therefore inactive, soluble, and bioactive proteins can frequently be obtained from eukaryotic cells. Furthermore, eukaryotic cells have the capacity to carry out posttranslational modifications, such as glycosylation, phosphorylation on tyrosine, serine, and threonine residues, or the addition of fatty acidchains. Mammalian expression systems comprise a plethora of different cell lines used for protein production.

Transient expression of genes in COS cells is a powerful and frequently used method for the rapid production of cell supernatants for structural and functional analyses of DNA and proteins as well as for rapid expression cloning (Dietsch*et al.,* 1993; Hollenbaugh*et al.,* 1992; Aruffo*et al.,* 1992).Aside from a few other lines also suitable for the transient expression of heterologous genes driven by SV40 large T-antigen expression (Boast *et al.,* 1983; Gerard *et al.,* 1985), the cells almost exclusively used are the COS cell lines developed in 1981 by Y.Gluzman (Gluzman*et al.,* 1981). Transformation of the African Green Monkey cell line CV-1 with an origin-defective SV40 genome gave rise to three cell lines (COS-1, -3, and -7), which constitutively express SV40 large T-antigen. Upon transfection with an expression plasmid containing a functional SV40 origin of replication, the interaction between the SV40 origin of DNA replication and SV40 large T-antigen leads to extrachromosomal replication of the expression plasmid to high copy numbers (Mellon *et al.,* 1981). Hence high transcription and translation of the gene of interest from a suitable eukaryotic promotor will lead to remarkable product titers (Edwards *et al.,* 1993; Trill *et al.,* 1995)

Plasmid replication in COS cells peaks at around 48 h post-transfection. Thereafter the cells begin to slowly shed the high amount of plasmid copies, accompanied by signs of cytopathic effect and subsequently cell death, presumably, because they cannot tolerate the presence of high levels of extra chromosomally replicating DNA (Gerard *et al.,* 1985). As a consequence, the system is not suitable for large scale production over a prolonged period of time. Yet, recombinant protein expression in COS cells reaches its maximum after 72 h post transfection, and continues, despite the above described slow deterioration of cells, over a period of approximately 5–10 days (Edwards *et al.,* 1993). This offers the possibility of using the COS system in an extended batch fashion. By subjecting a large number of cells to transfection (in the range of 108 cells per batch), the adherently growing cells can be seeded onto roller bottle surfaces or on micro-carrier beads for use in spinner culture; multiple harvests of spent culture medium can lead to the cumulative production of several milligrams of recombinant protein (Ridder*et al.,* 1995). The success of this system varies, largely depending on the nature of the individual protein to be expressed and its sensitivity toward proteolytic degradation, and a careful experimental design with respect to transfection method, ratio of plasmid to cell number, and media composition (Kluxen*et al.,* 1993). Transient expression by means of extra-chromosomal replication in COS cells is frequently used to check the functional integrity of genes/plasmid and to produce small quantity of cell supernatants containing the protein of interest (Edwards *et al.,* 1993; Trill *et al.,* 1995).Yet COS cell supernatants produced on a large scale can also serve as a source of pure recombinant protein.

2.9.2 Purification of recombinant LIF protein

Molecular clones encoding murine and human LIF have been isolated (Gearing *et al.,* 1987; Gough *et al.,* 1988; Moreau *et al.,* 1988) and the recombinant protein tested in animal model systems (Metcalf and Gearing, 1989; Metcalf *et al.,* 1990). LIF has been known under a variety of synonyms (Moreau *et al.,* 1988; Baumann and Wong, 1989; Lowe *et al.,* 1989; Mori *et al.,* 1989) and is naturally produced by a wide range of hematopoietic, mesenchymal and endodermic cell types as both a conventionally secreted form (Gearing *et al.,* 1987; Moreau *et al.,* 1988), and as a matrix-associated form (Rathjen*et al.,* 1990).

LIF and several other growth factors/cytokines are commercially available, but these recombinant proteins represent the major costs of the culture medium in PSC/ESC research. It would therefore be desirable to have sources of highly purified and biologically active cytokines, including LIF, in large quantities.

2.9.2.1 Purification of GFP fusion proteins

The green fluorescent protein (GFP), originally isolated from the bioluminescent jellyfish *Aequoreavictoria*, has become one of the most widely studied and exploited proteins in biochemistry and cell biology (Wiedenmann*et al.*,2006;Bjornberg*et al.*,2006). GFP is a 35 kDa protein, containing 238 amino acid residues, and is able to emit intense and stable fluorescence, without any cofactors, in many different organisms. GFP fluorescence is produced when energy is transferred from the Ca2+-activated photoproteinaequorin to GFP. It is highly stable and resistant to many biological denaturants, including most proteases, pH effects (Chalfie*et al.*,1998), temperature (Tm = 78°C), and chaotropic agent (8M urea)(Chalfie*et al.*,1998). Given the fact that many species, such as bacterial (Escherichia coli), fungi (Dictyostelium), plant (tobacco), and animal (including mammalian) cells, can express recombinant GFP (rGFP), it has been extensively used in a variety of assays. It is an ideal marker of gene expression, and has been widely used for tracking the localization of target proteins in intact cells, living or fixed tissues, and organisms, or the analysis of molecular interactions, among others (Meima*et al.*,2007; Peckham*et al.*,2006). With its continued use, several reports on GFP purification methods have emerged, including hydrophobic interaction, size-exclusion and ion-exchange chromatography, phase partitioning, organic solvent extraction, and salt and metal precipitation (Cabanne*et al.*,2004; Penna*et al.*,2004; Skosyrev*et al.*,2003). GFP, for purification of GFP and GFP fusion proteins.The binding of the monoclonal antibody to the GFP epitope allows isolation of GFP, as well as many active GFP-fused recombinant proteins, directly and easily from crude cellular sources, utilizing a mAb-coupled affinity column. (Zhang *et al.*,2008)

2.9.2.2 pAcGFP1-N1 as vector for expression in mammalian host

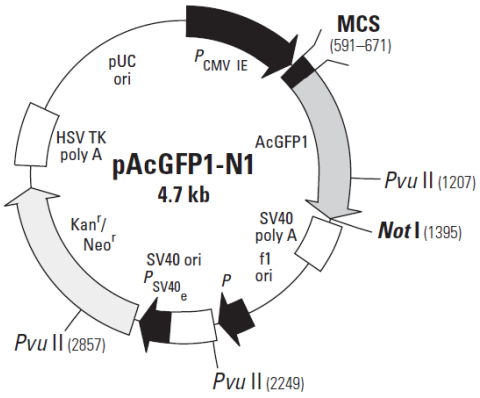


Fig. 2: Mammalian expression vector pAcGFP-N1 map

Description

PAcGFP1-N1 encodes a green fluorescent protein (GFP) from *Aequoreacoerulescens* (excitation maximum = 475 nm; emission maximum =505 nm).The coding sequence of the AcGFP1 genecontains silent base changes which corresponds to human codon usage preferences. The MCS in pAcGFP1-N1 is between the immediate early promoter of CMV (Pcmv) and the AcGFP1 coding sequences (Haas *et al.*,1996). Genes cloned into the MCS will be expressed as fusions to the N-terminus of pAcGFP1 if they are in the same reading frame as pAcGFP1 and there are no intervening stop codons. SV40 polyadenylation signals downstream of the pAcGFP1 gene direct proper processing of the 3' end of the pAcGFP1 mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin- resistance cassette (Neor),the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of the gene expresses kanamycin resistance in *E.coli.* The pAcGFP1-N1 backbone also provides also provides a pUC origin of replication for propagation in *E.coli* and an f1 origin for single-stranded DNA production (Gorman *et al.,* 1995).

2.9.2.3 Stable Transfection in Mammalian Host Cell

A very large number of techniques for the transfection of mammalian tissue culture cells have been described. The general requirements for a useful transfection reagent are that it is applied using simple, robust protocols to effect efficient transfection in a wide range of cell types and transfection formats, without excessive cytotoxicity. Techniques for DNA transfection of cultured cells hasprovided powerful methods for examining the function of various parts of complex mammalian genes. Various mammalian genes including thymidine kinase (Peruchoet *al.*,1980), hypoxanthine guanine phosphoribosyltransferase(HPRT) (Jolly *et al.*,1982), thymidylate synthase (Takeishi *et al.*,1984), transferrin receptor (Kuhn *et al.*,1984), DNA repair gene (Westerveld*et al.*,1984), and new oncogenes (Schechter*et al.*,1984; Shih *et al.*,1982) havebeen isolated by calcium phosphate-mediated transfection of cells which involve the use of either calcium phosphateor DEAE-dextran (or its analogs) as a carrier to deliver DNA into cells (Corsaro*et al.*,1982; Graham *et al.*,1973;McCutchan*et al.*,1962). Under these conditions, many common cultured mammalian celllines like NIH3T3, C127, CV1, BHK, CHO, and HeLa cell lines were transformed at efficiencies of 10 to 50%.

In some methods, osmotic shock or treatment with lysosomal inhibitors is used to enhance the transfection efficiencies (McCutchan*et al.*,1962).A method involving the use of high-voltage electric pulses tocreate pores in membranes has been devised for deliveringDNA into cells (Neumann *et al.*,1982).

2.10 Origin of the problem

Leukemia inhibitory factor (LIF), a pleiotropic cytokine, has been used mainly in the maintenance and survival of in vitro grown mouse embryonic stem cell pluripotency. In this current work, we had expressed the recombinant LIF in COS-1 cells and purified through affinity chromatography. To examined the effect of the LIF in embryonic stem (ES) cell proliferation to a differentiation cell fate. From literature survey and in vitro studies, leukemia inhibitory factor (LIF) is a glycoprotein thought to be involved in mouse hematopoiesis, neuropoiesis, and embryogenesis as recently determined. It is known to maintain embryonic stem cells (ES cells) in a pluripotent state in culture. ES cells are closely related to cells of the inner cell mass and of the embryonic ectoderm. These cells express high-affinity LIF receptor. The LIF gene is transcribed during mouse embryogenesis as early as the blastocyst stage. A single copy of the LIF gene has been shown to give two RNA molecules differing by their first coding exon. The corresponding proteins, M-LIF and D-LIF, present different signal peptides suggested to be functional. Consequently, the mature proteins D-LIF and M-LIF have exactly the same polypeptide sequence, but they show different specific localizations. D-LIF is thought to be secreted and diffusible and M-LIF remains associated with the extracellular matrix.

The mechanisms that could target those molecules remain unclear. As a first step toward further understanding the significance of LIF expression, we designed an experiment of gain of LIF function by targeted overexpression of LIF cDNA in COS-1 cell line. Keeping in view with the above, the following objectives were framed for this study.

2.11 Objectives

- Production of the recombinant Buffalo Leukemia Inhibitory Factor (rBuLIF) from stably transfected COS-1 cells

- Purification of LIF-GFP protein from stably transfected COS-1 cells

3.1 Chemicals

All chemicals used in this study were of analytical and cell culture grade. The chemicals were procured from Sigma-Aldrich Inc. (St. Louise, M.O., USA), Sisco Research Laboratories Pvt. Ltd., Mumbai, Bangalore Genei, Bangalore, Genetix Asia Pvt. Ltd., New Delhi, HiMedia Laboratories Pvt. Ltd., Mumbai; Pharmacia Fine Chemicals, Sweden; Thermo Scientific, Lithuania;Biochem, Life Sciences, New Delhi; Merck Specialities Pvt. Ltd., Mumbai; Thomas Baker (chemicals) Pvt. Ltd. Mumbai; Qualigens Fine Chemicals, Mumbai; LobaChemie, Mumbai; Technoconcept, New Delhi.

3.1.1 Chemicals

The experimental work was performed using chemicals from various companies based on the study requirements and purity level required are mention Table 3.1

Table 2. Chemicals required

Chemicals	Company Name
Agarose	Hi-media
Phenol:chloroform:isoamyl alcohol (25:24:1)	
Tris buffer	
Sodium dihydrogen Phosphate	Sisco Research Lab Pvt Ltd (Mumbai)
Glacial acetic acid	
Sodium bicarbonate	
Sodium acetate	
Sodium Chloride	
Glycine	Ficher Scientific(Mumbai)
Acrylamide	Sisco Research Lab Pvt Ltd(Mumbai)
TEMED	Stratagene, Santa Clara(USA)
DAB system	GeNei™, Mumbai, India
Nitrocellulose membrane	Sigma Aldrich
Unstained Protein Marker(10kDa-200kDa)	Ficher Scientific(Mumbai)

Page-Ruler prestained marker (10 kDa-170 kDa)	Sigma Aldrich
pH standard buffers(pH4.0, pH7.0, pH 10.0)	Amercho
Tween-20	Merck
Methanol	
HCL	
Mitomycin-C	
G418 reagent	Hyclone Laboratories. Inc.
Monoclonal Anti –GFP antibody produced in mouse CnBr-activated Sepharose 4B	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium (DMEM)	
Trypsin	
L-glutamine	
Fetal Bovine serum (FBS)	
Dimethyl sulphoxide (DMSO)	
Gentamicine	
DEPC	

3.1.2 **Instruments**

The experimental work was performed using the instruments mentioned in Table 3.2

Table 3. List of Instruments

Instrument	Company
Incubator shaker	Innova
Q-Sepharose Column	GE healthcare biosciences
SDS-PAGE unit	Biorad/GE healthcare lifescience
UV-VIS Spectrophotometer	Shimadzu
Vacuum Pump	Tarsons
Benchtop centrifuge	Hermle
PCR thermo cycler	Biorad
V.D.R.I rotor	Tanco
Rocking table	Lukham
Pippette	

SpeedVac concentrator	Thermo Scientific
Nanoquant	Tecan
Laminar air flow cabin	Toshibha(India)
Microscope	
Fluorescent microscope	Olympus
Neubers chamber	Celeromics
-20 freezer	Vestfrost
-80freezer	New Brunswick scientific

3.2 Plastic wares

The plasticwares were purchased from Tarsons Products Pvt. Ltd., Kolkata; Axygen, Inc., USA; Millipore Pvt. Ltd.;Costar, Corning, NY, USA.

The plastic wares used in research work viz, 75 cm^2 and 25 cm^2 Tissue culture flask for adherent type cell culture, 50 ml falcon tubes, 15 ml falcon tubes, 96 well plates , 24 well – plate and 6- well plate ,microblade , 2 ml and 1.5 ml eppendorf tubes, microtips

3.3 Microscopes

3.3.1 Inverted Microscope

An inverted microscope (Nikon, Japan, Model TMD) was used for the examination of health, morphological characteristics and growth of cultured cells. The microscope, with the light source at the top and a long working distance, allowed cells in cell culture dishes or flasks to be viewed and photographed whenever needed. The inverted microscope was also equipped with UV fluorescence and differential interference contrast (DIC) attachment, which helped in capturing the immune-fluorescent images of BuLIF-GFP transfected COS-1 cells. The microscope was equipped with programmable still photography and video recording facilities.

3.3.2 Compound Microscope

A compound microscope (Nikon, Model MICROPHOT-FXA) with a movable slide holding stage and photography facilities was used for counting the cells in culture, differentiating viable from non-viable cells, examining cell cultures for morphological of cells at different passages.

3.4 pAcGFP-N1 mammalian expression vector

The pAcGFP1-N1 is designed for mammalian expression systems which encodes a green fluorescent protein from *Aequoreacoerulescens*. This vector allows expression of a protein of interest as an N-terminal fusion to AcGFP1. As the unmodified vector will express fluorescent protein, the

fusion vector can also be used as a co-transfection marker. The fluorescent protein coding sequence in this construct has been human codon-optimized for efficient expression and enhanced brightness in mammalian cells. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen.

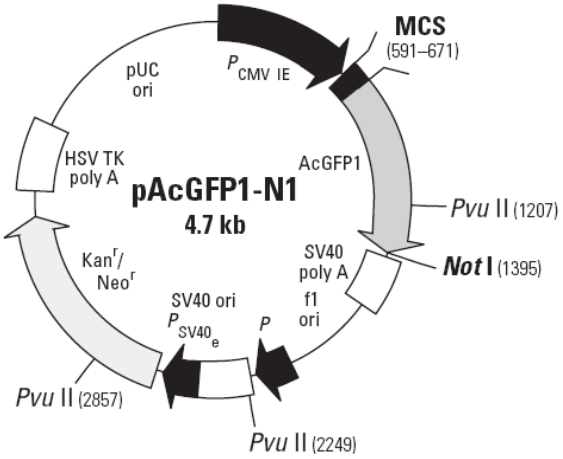


Figure 3: Mammalian expression vector pAcGFP-N1 map.

A neomycin-resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells selection using G418. A bacterial promoter upstream of the gene expresses kanamycin resistance in *E. coli*. The pAcGFP1-N1 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

3.5 Scale up of rBuLIF production in COS-1 cell line:

3.5.1 COS-1 and COS-LIF cell culture

COS-1 cells were obtained from the NCCS pune. Cells were cultured in growth medium containing DMEM supplemented with 20% FBS, 2mmol/L L-Glu and antibiotics (Penicillin 100 U/mL, Streptomycin 30 µg/mL). They were incubated at 37°C in humidified atmosphere containing 5% CO2. The monolayer became confluent 4-5 days after seeding $1x10^6$ cells/flasks ($25cm^2$ flasks), and the cells were subcultured at split *ratio* of 1:3 by trypsinization (0.5% trypsin and 0.05% EDTA). The medium was changed regularly. The cells used in this study were at passage 30^{th} onwards. The stably transfected COS_BuLIF was used in this study which was previously cloned and produced in the laboratory (Shikha et. al., 2013). In brief, the reagents and methodology used henceforth are mentioned below.

3.5.3 Media

i) Growth Medium

DMEM, pyruvate free	50mL
Fetal bovine serum	20%
Gentamicine	50µg/mL
Glutamine	2mM

Equilibrate with $NaHCO_3$ to pH 7.4 under 5% CO_2 and filter sterilized with 0.22 micron syringe PVDF filter.

ii) PBS (Phosphate buffer solution)

NaCl	**8.0 g/L**
KCl	**0.2 g/L**
Na_2HPO_4	**1.15 g/L**
KH_2PO_4	**0.2 g/L**

The above mentioned chemical components were dissolved in distilled water, adjusted the pH 7.4 with 1N NaOH and autoclaved at 120 lbs pressure for 15 min.

3.5.4 Microscopic examination

In order to ensure the health and confluence status of the cells repeated microscopic examinations were done. The morphology, surface area covered and other parameters were checked out. The experiments were conducted only after the culture were 95% to 100% confluent.

3.5.5 Trypsinization

After ensuring 95% to 100% confluency of the flask cells were made to detach with 0.02% trypsin EDTA (1 mL/flask), cold growth media with 10% serum was added to the same flask to stop trypsin action such that finally there was 1mL trypsin EDTA with detached cells along with 4 mL of media with FBS and antibiotics. These cells were transferred to 15mL falcon tube and subjected to

centrifugation at 1100 rpm for 5min. Supernatant was discarded and pellet was reconstituted in 1mL growth media.

3.5.6 Cell Counting

Diluted aliquot (10 times) was made from the above cell suspension for cell counting. 10 µL of the diluted aliquot was placed on neubauer chamber and observed under 20x. Cells in the large four quarter were counted and average of the 4 quarter was taken. The cell number per ml of the solution was calculated by the given formula:

cells/mL = average no of cells in four chambers $X10^4$ X dilution factor

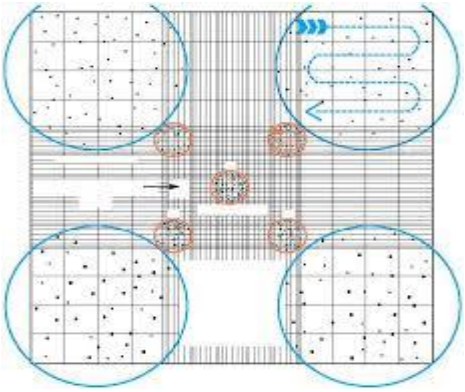
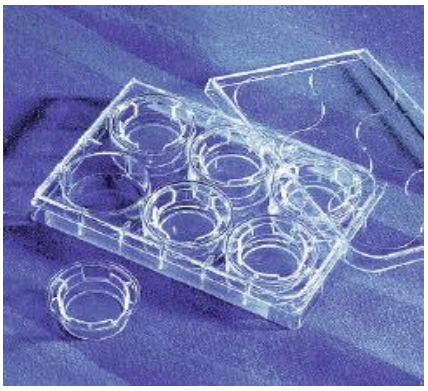


Fig 4: Cell counting using neubauer chamber

3.5.7 Seeding

According to the cell density of the culture suspension, 1mL of the cell suspension having 3 x 10^4 cells were added to the apical chamber of each well along with 1 mL of growth medium such that the total volume was 2 mL per well. Likewise 2 mL of the growth medium was added to the basal chamber. The plate was moved to & fro and left & right for the equal distribution of the cells while circular movements were avoided. The medium was changed every regularly till sub-culture. Regular microscopic examination was done to check for contamination. The plates were kept at 37ºC, 5% CO_2. Culture attained confluence within four to five days.



3.5.8 Procedure for Isolation of DNA from Cell Culture

From a T75 flask or equivalent standard trypsinization protocol was performed and pelleted the cells by centrifugation in a 15 mL tube. The supernatant was discarded and resuspended the pellet in 3 mL of TE and 100µL of 20% SDS. 20 µL of Proteinase K 20 mg/mL stock solution was added in the above solution. Mixed gently by inversion and incubated overnight at 55°C. Added 1 mL of saturated NaCl solution and then10 mL of 100% EtOH was added. Inverted to precipitate DNA. Incubated overnight on a slow rocker. With the help of pipette transfer the DNA to a 15 mL microfuge tube containing 5 mL of 70% EtOH. Incubated at room temperature overnight on a slow rocker. Spooled the DNA out of the tube and place in a new 1.5 mL centrifuge tube. Aspirated as much EtOH from the tube as possible with a pipette. Let the DNA air-dried. Resuspended the DNA in 100-300 µL of water. It may be necessary to let the mixture sit at room temperature for one or more hours to allow the DNA to fully resuspend. Determined the DNA concentration using nanoquant. In the unlikely event that the DNA solution was opaque in appearance and nanoquant 260/280 readings indicate the presence of proteins, the DNA was further isolated using phenol/chloroform/isoamyl alcohol protocol. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. Vigorously shaked the tube until an emulsion formed.Centrifuged at 1600xg for 3 minutes. Pipette off the aqueous phase and transfer to a new polypropylene 1.5 mL tube. Added an equal volume of chloroform/isoamyl alcohol (25:24).Vigorously shake the tube until an emulsion forms. Centrifuged at 1600xg for 3 minutes. Pipette off the aqueous phase and transferred to a new 1.5 mL tube.Extract the DNA by Ethanol precipitation. Added 2.5 volumes of 100% EtOH and 0.1 volume of 3M sodium acetate. Mixed by inversion and either spool out the DNA and centrifuged on high for 15 minutes to pellet the DNA. Resuspended in water. Determined the DNA concentration using a nanoquant. Stored at -20°C.

3.6 Designing of primer for PCR amplification

The BuLIF gene sequence of Bubalusbubaliswas retrieved from NCBI nucleotide data base (HQ616665). By analyzing the conserved regions of the aligned sequences of, Primers were designed and got custom synthesized from Sigma Pvt. Ltd., Bangalore.

Table 4. Primers for PCR amplification of LIF gene fragment

Forward primers: LifRT-F	**5'- CCATCACCCCGGTCAACGCT-3'**
Reverse primers: LifRT-R	**5'-AAAGAGGCTGTTGGCACTGCTGCTGT-3'.**

3.6.1 Primer dilution

The primer was provided in powdered form and the powder form of primer was re-constituted in suspension form by following method. 597µL nuclease free water was added in forward primer to make 100 µM of stock solution and likewise 545µL nuclease free water was added in reverse primer to make 100 µM stock solution under laminar air flow. These above informations was provided in data sheet.

Preparation of working solution: for working solution 10 µL of stock solution of forward primer was added in 90 µL to nuclease free water likewise 10 µLof stock solution of reverse primer was added in 90 µL to nuclease free water under laminar air flow.

3.6.2 POLYMERASE CHAIN REACTION

Procedure

- Initialization step: This step was consist of heating the reaction mixtureto a temperature of 95°C which was hold for 5 minutes
- Denaturation step: This step was the first regular cycling event and consist of heating the reaction at 94°C for 2 min. It causes melting of the DNA template by disrupting the hydrogen bonds.
- Annealing step**:** The reaction temperature was lowered to 55°C for 30 seconds to allow annealing of the primers to the single-stranded DNA template.
- Extension/elongation step: The temperature at this step depends basically on the DNA polymerase .This step was performed at 72°C for 45 seconds.
- Final elongation: This single step was occasionally performed at a temperature of 72°C for 7 minutes. Last PCR cycle to ensure that no single-stranded DNA are remain.

- Final hold:This step at 4°C for 1hr only for short-term storage of the reaction. A 50µLreaction mixture was prepared using the following components in the given proportion as mentioned in the below mentioned table.

Table 5. Composition for PCR reaction

Reagents	Volume
Master mix (2x)	31.5 µL
Forward primer (10nM)	2.0µL
Reverse primer (10nM)	2.0 µL
Nuclease free water	8.5µL
Template DNA	6.0µL
Total	50 µL

PCR samples were run in 1.2 % agarose gels and documented using Gel documentation system (GE, USA).

3.6.3 Agarose gel electrophoresis

Gel casting tray, running tray, buffer tank were washed with detergent, rinsed with water several times to remove traces of detergent then finally rinsed with distilled water, swabbed with 70% alcohol and dried. The cleaned unit was used immediately.

i) Preparation of agarose gel

For electrophoresis, 1.2% agarose gel was prepared in 1X TAE and used for electrophoresis. The contents were heated to allow solubilization of agarose. The flask was swirled from time to time till agarose was completely dissolved. After cooling the agarose solution around 50°C, ethidium bromide was added to a final concentration of 0.5 µg/mL. Gel running tray was placed in casting tray holder. Comb (5.2 cm long, and 4 mM width) was inserted in such a way that at least 0.5 mM (in length) of the comb remains above the running tray. The warm (~ 40°C) agarose solution was carefully poured in tray containing a comb and bubble formation was avoided to obtain gel thickness of about 3-5 mM. The gel was allowed to solidify resulting into a matrix. The gel solidified within 20-30 minutes of pouring and was ready to use. A little amount of buffer (1X TAE) was layered over the

solidified gel and then comb was gently removed. The running tray was placed at appropriate place in electrophoresis tank containing 1X TAE buffer to submerge the gel.

ii) Preparation of samples and electrophoresis

2 µL of loading dye was properly mixed with 10 µL of each amplified PCR products. Separate tips were used for pipetting and mixing of different samples. Entire volume of samples was dispensed carefully to the wells. A 100 bp DNA ladder (Fermentas, USA) was also run along with PCR products to confirm the desired product size. After loading the sample, electrophoresis was carried out at constant voltage (100 V) at room temperature for 20-30 min. 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). The gel was handled after wearing gloves and then photographed by Gel Doc imaging system (Bio-Rad, USA).

3.6.4 Gel extraction purification

The PCR products were observed under UV trans-illuminator and excised immediately. PCR products were purified from the gel as per manufacturer's protocol using the QIAquick gel extraction kit.

With the help of a clean, sharp scalpel, DNA fragment was excised from agarose gel with minimum extra agarose. The gel slice containing DNA was weighed in a colourless tube and 3 volumes of buffer QG was added to 1 volume of gel (100 mg ~ 100 µL) and incubated at 50°C for 10 min (or until the gel slice was completely dissolved) and mixed by vortexing the tube every 2-3 min during the incubation. After the gel slice dissolved completely, the mixture was checked for yellow colour (similar to buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.0, and mixed to change the colour to yellow. Then one gel volume of isopropanol was added to the sample and mixed. QIAquick spin column was placed in a 2 mL collection tube provided. To bind DNA, applied the sample to the QIAquick column, and centrifuged for 1 min. Then maximum volume of the column reservoir is 800 µL. For sample volumes of more than 800 µL, simply load and spin again. Flow-through discarded and place QIAquick column back in the same collection tube. After this, 0.75 mL PE buffer was added to QIAquick column and centrifuged for 1 min. Then flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 13,000 rpm. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µL of buffer EB (10 mMTris·Cl, pH 8.5) was added to the center of the QIAquick membrane and allowed to stand for 1 min, and then centrifuged for 1 min and sent to Sigma for Sequencing

Culture of recombinant *E. coli* cells was grown in 20 mL LB broth media with antibiotics for overnight at 37°C with constant shaking (225 rpm/min). The culture was centrifuged at 10,000 x g for 5 min after the overnight incubation. Then pellet was dissolved in 500 µL of chilled P1 buffer (with RNase) by vortexing and transferred in 2 mL microcentrifuge tube. 500 µL of P2 buffer was added slowly and mixed gently by inverting the tube 4-6 times. The tube was kept at room temperature for 3-4 min. After which 700 µL of P3 buffer was added drop wise and immediately mixed by inverting tube 4-6 times. The lysate was centrifuged at 10,000 x g for 10 min at room temperature. The supernatant was transferred in fresh QIAprep spin column tube and centrifuged at 10000 x g for 1 min. Then flow-through was discarded. QIAprep spin column was washed by adding 0.75 mL buffer PE and centrifuged at 10000 x g for 1 min and discarded the flow-through. Then again centrifuged at 10000 x g for 10 min to remove residual wash buffer. Then QIAprep column was placed in a clean 1.5 mL micro-centrifuge tube. 50 µL EB buffer (10 mMTrisHCl, pH 8.5) was added in center of each QIAprep spin column, kept 1 min at room temperature and centrifuged for 1 min to elute DNA,. Finally plasmid DNA was checked by 1% agarose gel electrophoresis in 1X TAE and concentration was determined by using a Nanoquant spectrophotometer (Tecan Sales, Austria GmbH).

3.7 Purification of Recombinant BuLIF Protein from Stably Transfected COS-1_BuLIF Cell Culture

3.7.1 Purification of recombinant BuLIF using CnBr-activated Sepharose 4B (GE-Pharmacia)

3.7.1.1 Materials

i) CnBr-activated Sepharose 4B (GE-Pharmacia)

ii) Coupling Buffer (pH 8.3) (500 mL)

0.1 M $NaHCO_3$ (pH8.3) [stock used: 1 M $NaHCO_3$]

0.5 M NaCl

Final volume adjusted with DEPC- treated H_2O, and stored at 4°C.

iii) Blocking Buffer (pH 8.0)

0.1 M Tris-HClpH8.0 [stock used: 1 M Tris-HClpH8.0]

Final volume adjusted with DEPC- treated H_2O, and stored at 4°C.

iv) Binding Buffer (pH 7.4)

1x PBS and stored at 4°C.

V) Elution Buffer (pH 4.5)

0.1 M Glycin-HCl (pH4.5) [stock used: 1 M Glycin-HCl pH4.5]

Final volume adjusted with DEPC- treated H_2O, and stored at 4°C.

vi) High pH Regeneration Buffer (pH 8.5)

0.1 M Tris-HCl (pH8.5) [stock used: 1 M Tris-HClpH8.5]

Final volume adjusted with DEPC- treated H_2O, and stored at 4°C.

vii) Low pH Regenration Buffer (pH 4.5)

0.1 M Na-acetate (pH4.5) [stock used: 1 M Na-acetatepH4.5]

Final volume adjusted with DEPC- treated H_2O, and stored at 4°C.

3.7.1.2 Method

Preparation of GFP affinity column using cyanogen bromide-activated Sepharose 4B

The method used was previously developed in the lab by (Shikha et al. 2013). Briefly, The CnBr-activated Sepharose 4B was freshly prepared prior to GFP mAb binding. 500 mg CnBr-activated Sepharose 4B was swelled in 15 mL Coupling Buffer for 5min (500 mg swells about 1.5 mL final volume). Final volume was washed with 15 mL ice-cold Coupling Buffer. Spin at 500 rpm briefly. Supernatant was removed. 2.5 mL of cold Coupling Buffer was added, and 100-500 ug of GFP mAb was added in 500 µL Coupling Buffer (Maximum GFP ab = 2-5 g/ 100 mg CnBr-activated Sepharose 4B). The Sepharose/GFP mAb mix was rotated using a rotating disc mixer at 4°C 2 h to overnight. After spinning, all of the supernatant was removed. The remaining active groups was blocked by 15 mL ice-cold Blocking Buffer (0.1 M Tris-HClpH8.0) at 4°C for 30 min. Final Sepharose/GFP mAb mix was rinsed with 15 mL ice-cold 1x PBS. The column was packed. 1 mL pre-cleared cell lysates was added with protease inhibitors and PMSF onto GFP mAb-coupled affinity chromatography column. The column was washed with 10 column volumes of ice-cold 1x PBS. GFP-tagged BuLIF protein was eluted with 1 mL ice-cold Elution Buffer. The elutes were immediately neutralized by adding 100 µL, 1 M Tris-HCl pH 9.0.The elutes were dialyzed in ice-cold, 0.1x PBS at 4°C for 1-2 h. The protein sample was concentrated by 4x vol. of acetone. The column was washed with high pH Regeneration buffer and then with low pH Regeneration buffer. Final column was resuspended in Binding buffer.

3.7.2 SDS-PAGE ANALYSIS

3.7.2.1 Reagents

Composition of stock solutions for use in SDS_PAGE

1. Acrylamide / bis Acrylamide (30% T, 2.67% C)

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

(Total volume)

gmbis-Acrylamide

Cross linking monomer concentration (% C) = ——————————————— X 100
rylamide + gm bis-Acrylamide)

Acrylamide 29.2 g / 100 ml.

N' N' bis-methylene – Acrylamide 0.8 g / 100 ml

Filtered and stored at 4 ^{0}C in the dark (30 days maximum)

2.1.5 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

3. 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

4. 10 % SDS	
4X sample buffer	
1 M Tris –HCl, pH 6.8	2.5 mL
SDS	0.8 g
2-Mercapto ethanol	1.0 mL
Glycerol	3 mL
Bromophenol Blue	2 mg
Total volume	10 mL
5X Running Buffer, pH 8.3	
Tris base	15g / L
Glycine	72 g / L

SDS	5 g / L

3. 12 % Separating Gel Preparation – 0.375 M Tris, pH 8.8	
Deionized water	3.35 mL
1.5 M Tris-HCl, pH 8.8	2.5 mL
10 % SDS	80 µL
Acrylamide / Bis (30% stock)	4.0 mL
10% Ammonium per sulfate	80 µL
TEMED	8µL
Total volume	10 mL

4. 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	80 µL
Acrylamide / Bis (30% stock)	0.665 mL
10% Ammonium per sulfate	25 µL
TEMED	5 µL

Total volume	5 mL

Running conditions

At constant current of 18 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 (30% Methanol and 10 % glacial Acetic acid).

Destaining

Destain with several changes of 30% methanol and 10% glacial acetic acid to remove background.

3.7.2.2 Method

Purified recombinant BuLIF protein was analyzed by standard SDS-PAGE method. PAGE is composed of polymerized acrylamide that is cross-linked by a bifunctional agent such as N, N'- methylene bis-acrylamide. Most Native-PAGE gels are prepared with 1:30 molar ratio of acrylamide to bis-acrylamide.

i) Casting of gel

A slab mini vertical gel electrophoresis apparatus (10 x 8 cm) from Bio-Rad was used. Resolving gel (15%) and stacking gel (5%) was prepared as given in Table 3.12. The glass plates were cleaned, well dried and arranged the assembly along with spacers. The assembly was fitted into the gel casting stand. The resolving gel solution was poured slowly into the gap between the glass plates and kept undisturbed for half an hour in gel casting apparatus. It was overlaid with distilled water saturated butanol for even surface of the resolving gel polymerization. The overlay was poured off and the top of the gel was washed with deionized water was added to remove any unpolymerized acrylamide after completion of polymerization. The stacking gel solution mixture was poured directly above the surface of the resolving gel. After polymerization the comb was removed and the gel was assembled into the electrophoresis running apparatus. Tris-Glycine running buffer (pH 8.3) was added to the upper and lower buffer reservoirs. Protein sample and the SDS page loading buffer were mixed well to prepare the sample. After loading the samples, the electrophoresis apparatus was connected to the power pack for electrophoresis.

ii) Electrophoresis of sample

The electrophoresis was carried out in Tris-Glycine buffer on a Vertical slab gel electrophoresis system (Bio-Rad, USA). The lysate was analyzed for expression of recombinant human lysozyme in SDS-PAGE (15% resolving and 5% stacking gel) at 50 V for 30 min. and 100 V for 60 min. About 15 µl of sample was loaded in each well. Standard protein molecular weight marker (Fermentas, USA) was also loaded in adjacent well to compare the molecular weight of cellular protein components. After the completion, the gel was removed from the plates and the gel was stained using Coomassie brilliant blue. The molecular size of the expressed protein was estimated by standard molecular weight markers.

3.8 In gel trypsin digestion for mass spectrophotometry

Reagents

40% Acetonitrile (CH_3CN)

1. Acetonitrile (CH_3CN)= 20 mL
2. LC-MS-Grade Water= 30mL
   Total Volume= 50 mL

40mM Ammonium bicarbonate (NH_4HCO_3) (m.w= 79.06)

1. Ammonium bicarbonate= 36.6mg
2. LC-MS-Grade Water= 10 mL
   Total Volume= 10 mL

5mM DTT in 40mM Ammonium bicarbonate (DTT m.w. = 154.25)

1. DTT = 3.83mg
2. 40mM Ammonium bicarbonate =5 mL
   Total Volume = 5 mL

20mMIodoacetamide (IAA) in 40mM Ammonium bicarbonate (IAA m.w. = 184.96)

1. Iodoacetamide =18.49 mg
2. 40mM Ammonium bicarbonate = 5.0 mL
3. Total Volume = 5.0 mL

5% Formic acid

1. Formic acid = 250 µL
2. LC-MS-Grade Water = 4.750 mL

   Total Volume = 5.0 mL

5% Formic acid in 40% ACN

1. Formic acid = 250 µL
2. 40% ACN = 4.750 mL

   Total Volume = 5.0 mL

50 mM Ammonium bicarbonate (NH_4HCO_3) (m.w= 79.06)

1. Ammonium bicarbonate = 19.76 mg
2. LC-MS-Grade Water = 5.0 mL

   Total Volume = 5.0 mL

3.8.1 Method

The COS and COS-LIF protein bands were cut from SDS-PAGE with fresh surgical blade and chopped into fine pieces and transferred to two different eppendrofs. Added 100 µL of 40mM ABC (Ammonium Bi-carboante) and 100 µL of 40% CAN (Acetonitrile) to every eppendrof. Wait until all the bands got destained completely. Spin briefly and removed the supernatant with micro tip. 100% ACN was added for dehydration of gel, gel pieces were shrinked and appeared opaque. Incubate for 10-15 min. at room temperature. In the next step, 100% ACN was removed and rehydration solution (5mM DTT in 40mM ABC) was added. Incubate the gel bands at 60^0C for 45 min. Cooled the tubes at room temperature for 10 min., Spin and discarded the supernatant with micro tip. Alkylation solution (20mM IAA in 40mM ABC) was added to both Eppendorf's and incubated for 10 min. at room temp. in dark. After 10 min. IAA was removed and added 100% ACN. Volume should be sufficient to cover gel pieces. Incubate the tubes for 15 min. at room temp. Removed the 100% ACN and added freshly prepared 50mM ABC buffer, containing 12.5 ng/µL of sequencing grade modified Trypsin. Added just enough Trypsin solution to cover the gel pieces (about 30 µL) incubated the tubes in ice for 45 min. to re-swell the gel pieces. In the last added enough 40mM ABC to cover the gel pieces. Incubated the tubes at 37^0C for overnight.

3.8.2Peptide Extraction

After overnight incubation, Cooled the tubes at room temp.and added 100 µL of 5% Formic acid to each tube and incubated for 10 min. at 37^0C. Spin the tubes briefly and transferred the supernatant into a fresh tube. For extraction, added 100 µL of extraction Buffer (5% formic acid in 40% ACN)

incubated for 10 min. spin the tubes and pool the Supernatant with the supernatant from earlier step. For final extraction added 100% ACN Repeat the above step. Dried the pooled supernatant by spinning tubes in speedvac, Stored at -20^{0}C

3.9 Western Blot Analysis of Recombinant Protein

3.9.1 Reagents

Transfer buffer

3.03 g Tris base and 14.4 g glycine are dissolved in 800 mL of distilled water. Then 200 mL of methanol was added before using.

TBS Buffer

20 mM Tris-HCl (pH 7.5)

150 mMNaCl

DAB system (Bangalore Genei, Bangalore, India)

TBST Buffer

TBS buffer with 0.05% Tween 20

3.9.2 Method

The gel was removed from the SDS – PAGE apparatus and washed in de-ionized water, equilibrated the gel in transfer buffer for 15 – 20 minutes. The Nitrocellulose membrane was first made wet in distilled water and then kept in transfer buffer for 15 minutes. Placed wet blotting paper on the negative terminal of the blotting cassette and then the gel. Over the gel, the nitrocellulose membrane was placed taking care to avoid trapping of air. This was then overlaid with blotting paper. The sand witched gel in the cassette was tightly closed and blotting was done in 1X transfer buffer at a constant current of 30-40 V overnight.

After overnight transfer, the membrane was removed and washed in distilled water, and then it was blocked with 5% BSA (in TBS) by incubating for overnight. The blocked membrane was rinsed in de-ionized water and then incubated for three hour in primary antibody (mAbH10), which was diluted 1: 1000 times in TBST containing 5% BSA. The contents were drained and washed with TBST buffer for 10 minutes, this was repeated three times. Then the membrane was incubated in secondary antibody solution (diluted to 1: 500 in TBST) for 1 hour, drained the contents and washed with TBST buffer for 10 minutes once and 5 minutes twice. Then 10 uL of H_2O_2 (30%) to 10 mL of 0.05% DAB (3, 3'-diaminobenzidine) in TBS was added and mixed well immediately and poured on to the membrane, incubated at room temperature with gentle shaking in the dark. The progress of the reaction was monitored carefully. When the bands were of desired intensity (2-5 min), washed the developed membrane briefly in water, and in TBS.

In recent years, the mammalian cell system has been extensively utilized for the production of biochemically complex proteins requiring post-translational modifications. In addition, baculovirus and yeast expression systems have been used extensively for the expression of recombinant proteins. Buffalo LIF is a glycosylated protein which is expressed in minute amount in specific tissue location. The requirement of biologically active buffalo LIF for its application in bovine stem cell made it imperative to develop an eukaryotic cell based expression system so that bovine LIF could be produced continuously as and when demanded.

The objective was to scale-up homogenous cell population of stably transfected mammalian cells with Buffalo LIF (BuLIF) to increase the production of recombinant BuLIF protein. The idea behind taking mammalian cell line (COS-1) as host to express BuLIF was to produce perfectly glycosylated LIF with exact amino acids composition as that of native BuLIF expressed in buffalo.

GFP as a fusion tag has widely been used to trace the expression of target protein (Kanda *et al.*,1998). Green fluorescent protein (GFP) of the jellyfish *Aequoreavictoria* retains its fluorescent properties when recombinant GFP proteins are expressed in eukaryotic cells (Chalfie*et al.*,1994).

4.1 Factors Influencing protein expression

With any transfection reagent or method, cell health, degree of confluence, number of passages, contamination, and DNA quality and quantity are important parameters that can greatly influence recombinant protein production efficiency.

A. Cell Health

Contaminated cells and media (e.g., contaminated with yeast or mycoplasma) should never be used for culturing. If cells have been compromised in any way, discard them and reseed from a frozen, uncontaminated stock. Make sure the medium is fresh if any components are unstable. Medium lacking necessary factors can harm cell growth. Be sure the 37°C incubator is supplied with CO2 at the correct percentage (usually 5–10%).

B. Confluency

As a general guideline, transfect cells at 40–80% confluency. Too few cells cause the culture to grow poorly without cell-to-cell contact. Too many cells results in contact inhibition. So before sub-culturing, flask should be 80-90% confluent.

C. Number of Passages

In addition, the number of passages for cells used in a variety of experiments was also consistent. Cell characteristics can change over time with number of passages. As the number of passages increases the rate of expression of recombinant protein also increase.

4.2 Optimization of Scale-up parameters

1. Optimization of Transfected cell density
2. Optimization of FBS concentration to be used
3. Determination of the minimum dose of G418 for COS-1 cells

1. Optimization of transfected cell density

To optimize concentration oftransfected cell density so that 70-80% confluency is achieved in 2-3 days, host cells were plated in individual wells of a 6-well plate, 12 well plate, 24- well plate and 96 well plate at varying densities (e.g., 5×10^4, 1×10^5, 2×10^5, 4×10^5) keeping all parameters constant. The optimized cell density for the culture of COS-1 cells in different culture configuration are shown in table 4.1

Table 4.1: Showing cell density after 30th passage and after 70th passage in different culture vessels

Cuure vessel	cell density after 30th passage	cell density after 70th passage
96-well plate	3×10^4	6×10^4
24-well plate	8×10^4	2.6×10^5
12-well plate	1.6×10^5	3×10^5
6-well plate	3×10^5	6×10^5

2. Optimization of FBS concentration

Fetal bovine Serum was used with DMEM medium. FBS is an important component for cell culture as it provides all essential required proteins. Firstly, DMEM was provided to cells with 5% concentration of FBS which caused high rate of cell death. It was seen that as the concentration of FBS continuously increased from 10% upto 20% the survival rate increased further increasing the concentration of FBS has no effect on stably transfected COS-1 cell line. For our later studies we used optimum concentration of 20% FBS.

3. Determination of the minimum dose of G418 for COS-1 cells

The vector pAcGFP_LIF contains neomycine resistance gene, the transfected cell can survive in the presence of G418 whereas, non-transfected cells cannot survive in G418. G418 (Geneticin) is an aminoglycoside antibiotic similar in structure to gentamicin. It is produced by *Micromonosporarhodorangea*.G418 is commonly used in laboratory research to select genetically engineered cells (Carpenter S *et al.*,2011) G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. A wide range of concentrations were tried according to the confluency, cell health and configuration of experiment. The duration of treatment was critically observed as at too low concentration of (200µg/mL) proper selection could not be achieved and at too high

concentration of (1200 µg/mL), the cells started dying with severe morphological aberration. Eventually, the final selection was started at 600µg/mL of G418 for continuous supplementation in media.

4.3 Selection of stably transfected COS-1 cells expressing rBuLIF

T25 flasks exhibiting very less fluorescence at 30th passage and were kept under serial selection with G418 (fig. 5). Following the addition of G418, a large number of cells lost adherence and started floating in to medium. The medium was decanted and the floating cells were washed off with continuous supplementation with fresh medium containing same dose of G418 (fig. 6). Since, cell density decreased drastically the adhered and surviving cells were harvested and reseeded into smaller wells (6 well or 12 well plates). Specific colonies of highly fluorescence were picked with trypsin digestion and vigorous pipetting and seeded into 24 well plate (fig. 7). After 90-95% confluency cells were sub-cultured into 6 well plates and following repeated retrograde and progressive sub-culturing most of the cells were exhibiting fluorescence (fig. 8) Prolongedupto 70th passageculture of COS-1_LIF cells which still exhibited fluorescence was indication that plasmid DNA (pAcGFP_BuLIF) had integrated into the genome of COS-1 cells.

Table 4.4: Showing % of transfected cells, cell density and passage number in different culture vessels with increasing G418 concentration

Culture Vessel	% of transfected cells (approx.)	Cell Density	Passage number	G418 selection reagent used
6 well plate	50%	6×10^5	30 – 33	500 µg/Ml
12 well plate	65%	3×10^5	33 – 45	600 µg/mL
24 well plate	75%	1.6×10^5	45 – 65	800 µg/mL
96 well plate	90%	4×10^4	65 – 70	1000 µg/mL

Fig 4.5: Showing propagation and expansion of single cell colony to bigger culture vessels

4.4DNA isolation and quantification for PCR

Genomic DNA was isolated from both COS-1 and COS-LIF cells by Phenol:Chloroform:Isoamyl alcohol method and further quantified by nanoquant system. At 260 nm nanoquant readings were 20.9 ng/uL for COS-1 cells and 29.5 ng/uL for COS-LIF. Isolated DNA was used for PCR for confirmation of rBuLIF gene. (Fig. 10A)

4.5 Confirmation of rBuLIFgene by PCR

PCR amplification revealed that specific sequence of recombinant buffalo LIF is transfected in COS-1 cells whereas, that sequence was absent in non- transfected COS-1 cells. BuLIF and GFP genes were amplified by RT-PCR specific primers. The specificity of amplification were checked by Agarose Gel Electrophorsis.COS-1_BuLIF and non-transfected bands are shown in Fig. 10B whereas COS-LIF (112 bp) and GFP (150 bp) bands are shown in Fig. 10C. These bands were digested by gel digestion kit and sent for sequencing.

Sequence of LIF gene

Forward Primer Seq.Start Codon
CTCTGGAGTGCAGCCCATA
1 ATGAAGGTCTTGGCGGCAGGAGTCGTGCCCCTGCTGCTGGTTCTCCACTGGAAACACGGG 60
61 GCCGGGAGCCCCCTTCCCATCACCCCGGTCAACGCTACCTGTGCCACCCGCCATCCCTGT 120
121 CCCAGCAACCTCATGAACCAGATCAGAAACCAGCTGGGACAACTCAACAGCAGTGCCAAC180
181 AGCCTCTTTATCCTCTATTACACGGCCCAGGGGGAGCCCTTCCCCAACAACCTGGACAAG240
241 CTGTGCAGCCCCAACGTGACTGACTTCCCGCCCTTCCACGCCAACGGCACGGAGAAGGCC 300
301 CGGCTGGTGGAGCTGTACCGCATCATAGCGTACCTGGGCGCCTCCCTGGGCAACATCACG 360
361 CGGGACCAGAAGGTCCTCAACCCCTACGCCCACGGCCTGCACAGCAAGCTGAACACCACG420
421 GCTGACGTCCTGCGGGGTCTTCTCAGCAACGTGCTCTGCCGCTTGTGCAGCAAGTACCAC480
481 GTGAGCCACGTGGACGTGACCTACGGCCCCGACACCTCGGGCAAGGACGTCTTCCAGAAG 540
541 AAGAAGCTGGGCTGTCAGCTCCTGGGGAAGTACAAGCAGGTCATCGCCGTGCTGGCCCAG 600
601 GCCTTCTAG
ACGGGAGGTCTTAGATAGTAGG Reverse Primer Seq. Stop Codon

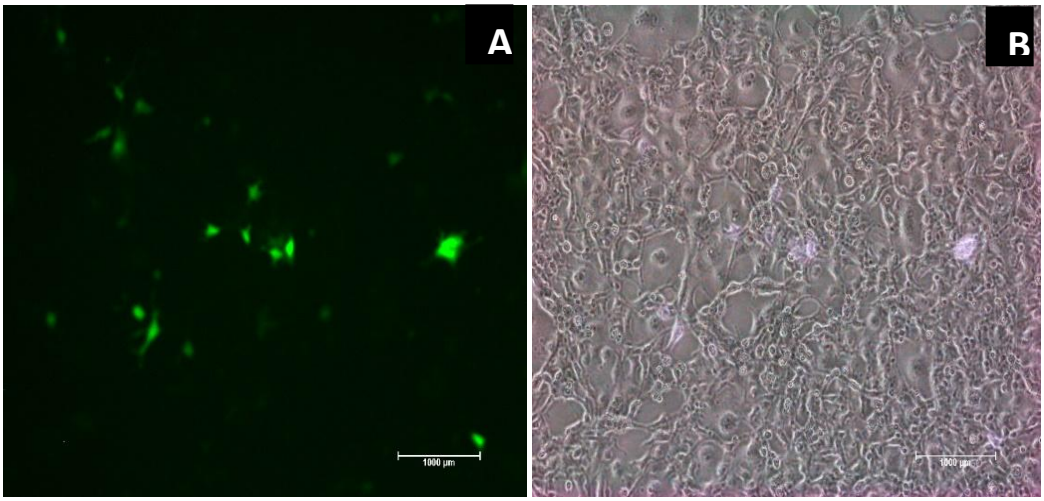


Fig. 5: Image of transfected COS-1 cells under blue light at 30th passage. A: Fluorescent image of COS-1 transfected with pAcGFP_LIF using lipofectamine(40X), B: white light image of COS-1 1 transfected with pAcGFP_LIF using lipofectamine(40X)(same focus).

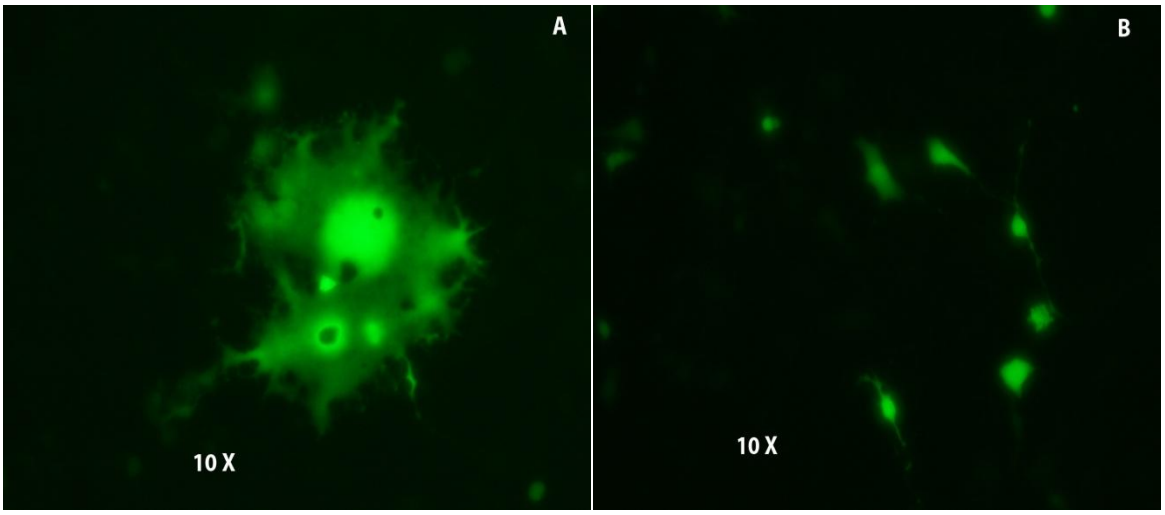


Fig. 6: Floating transfected cells with High Intensity that describes over expression of LIF-GFP Conjugated protein.

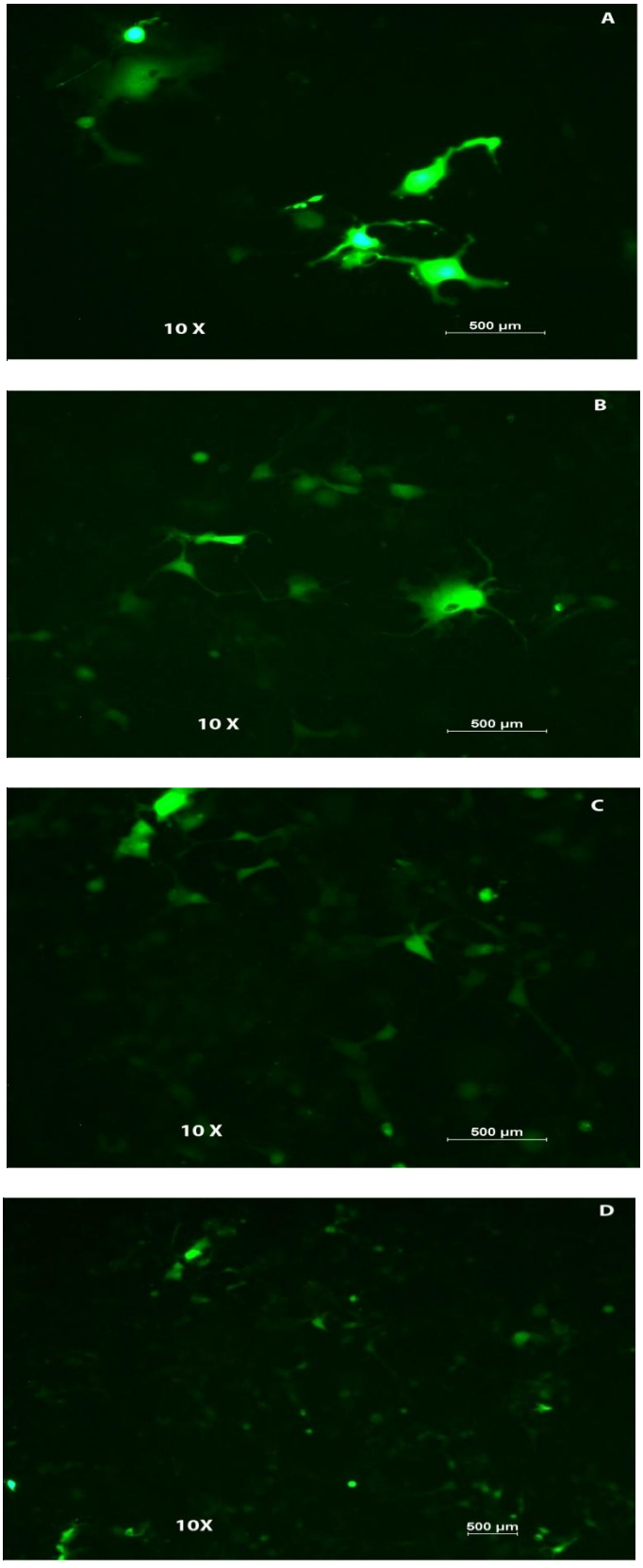


Fig. 7: Image of Retrograde Passaging of transfected COS-1 cells from 6 well plate to 96 well plate.

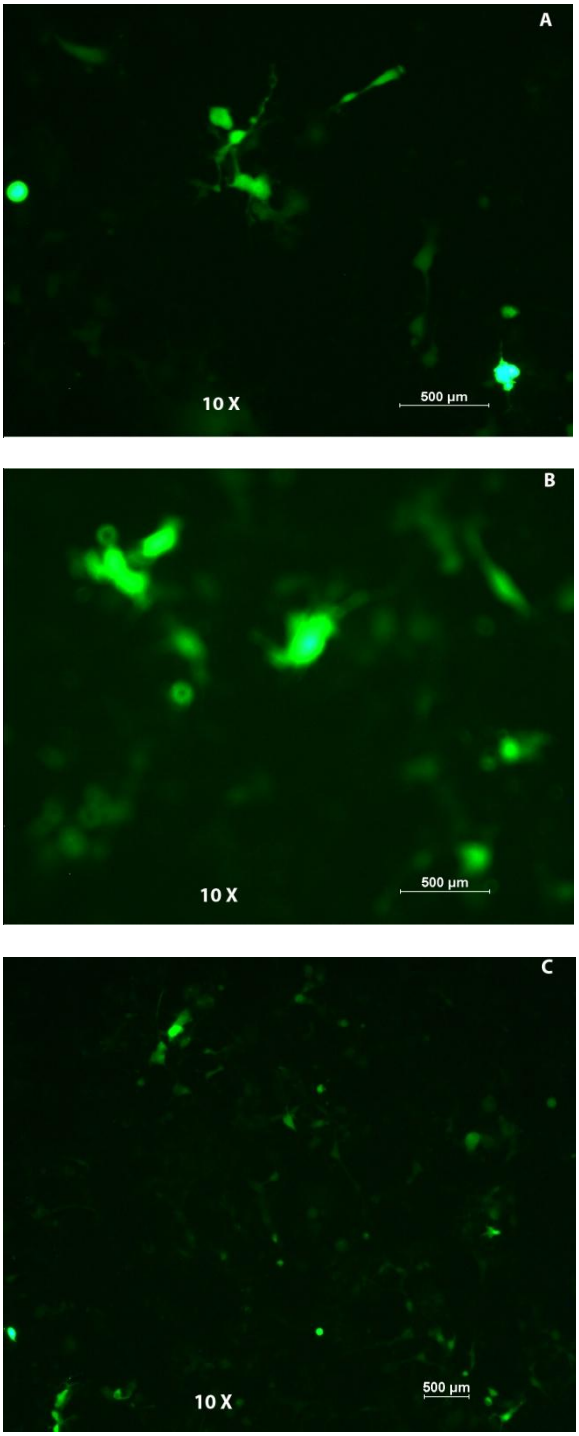


Fig. 8: Image of transfected COS-1 cells under blue light. **A:** Fluorescent image of transfected cells at 35th passage. **B:** Fluorescent image of transfected cells at 55th passage. **C:** Fluorescent image of transfected cells at 70th passage

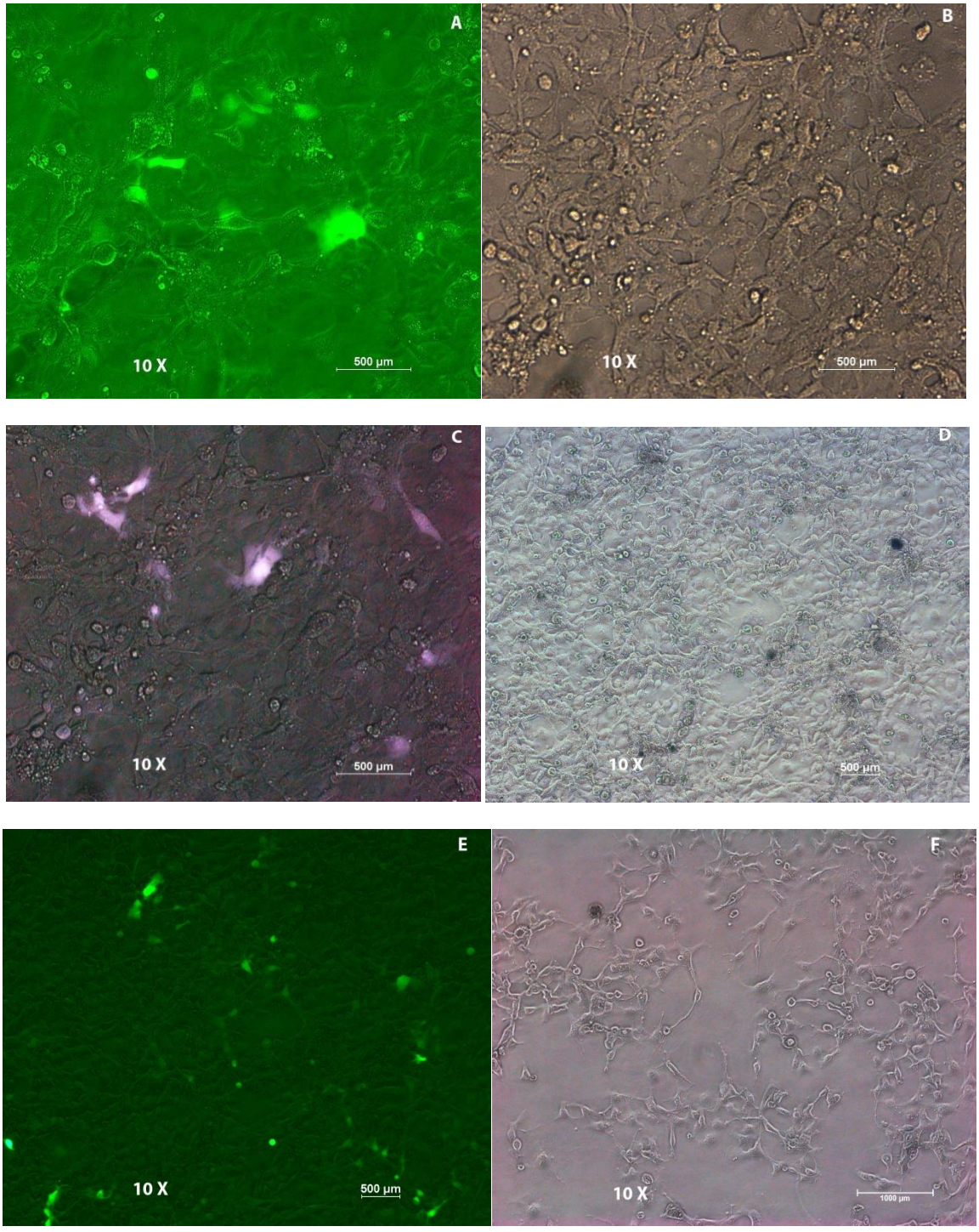


Fig. 9: Stably transfected COS-1 cells with G418 under Bright Field Microscope.

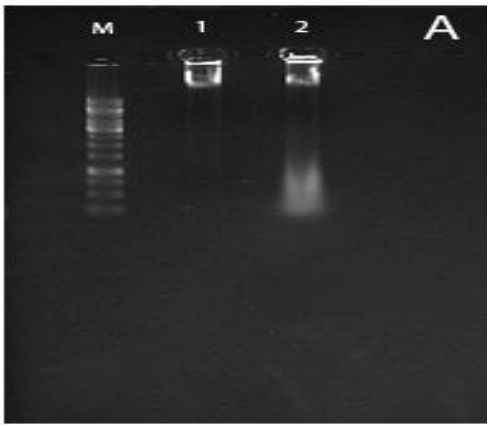


Fig. 10A: Image represents, M: DNA Marker, Lane-1: Isolated DNA from COS-LIF cell line, Lane-2: Isolated DNA from COS-1 cell line.

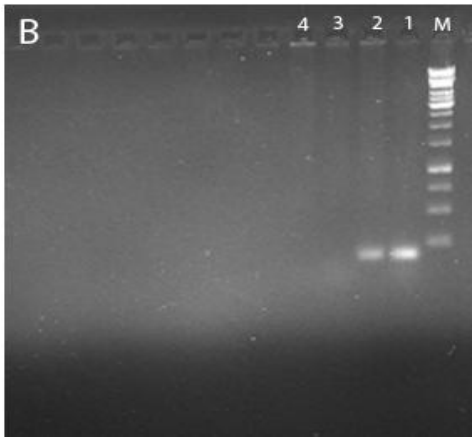


Fig. 10B: M: DNA marker (1Kbp-250bp), Lane-1: PCR amplified product from COS-LIF cells, Lane-2: NO PCR amplification occurred in COS cell

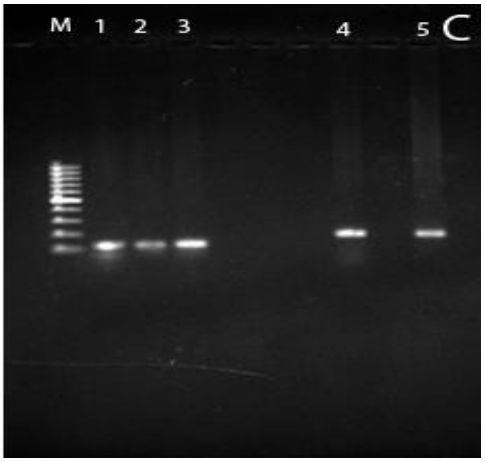


Fig. 10C: M: DNA marker (1 kb-100 bp), Lane-1 to 3 PCR amplification of LIF sequence by using RT-LIF primers (112 kb), Lane-4 and 5: PCR amplification of GFP by using RT-GFP primers (150 kb).

4.6 Purification of rBuLIF

The quantity of exogenous protein expressed in eukaryotic cell is comparatively less than those produced in bacteria or yeast. Usually, in bacterial system the yield varies and some reports finds up to 17-34 mg proteins from a 50mL cell culture. Use of CMV promoter is very popular to drive high level expression of heterologous protein. pAcGFP possesses CMV promoter upstream to the cloned BuLIF which is followed by the in frame expression of GFP. AntiGFP antibodies were used for trapping the GFP tagged BuLIF. Monoclonal antibodies from Sigma and Merck were used. A cynogen bromide activated sepharose 4B matrix was used to coupleAntiGFP antibodies and packed into column. This is a covalent interaction between matrix and immunoglobulins. A large number of cells are required to obtain enough purified protein. Two T75cm^2 flasks at their full confluency were harvested in RIPA and PMSF buffer. The COS-1 and COS-LIF samples were sonicated before use for purification (fig. 11). After sonication both samples were quantified by using Bradford quantification method, protein concentration of COS-LIF and COS-1 samples were 238.533µg/mL and 3028.33µg/mL respectively. The total protein yield 20µg/uLwas used for SDS-PAGE and Western-blot. Cynogen Bromide activated Sepharose 4B coupled with anti GFP antibodies resulted in production of approximately 2 mg total protein. This purified protein was checked on SDS-PAGE which showed one single band between 65-70 kDa, which is the combined molecular size of BuLIF_GFP fused protein (fig.12 and fig. 13). The purified protein was also checked by western blotting which resulted in intense staining at approx. 65-70 kDa molecular size. SDS-PAGE of unpurified cell lysate and western blot of purified protein is shown in fig (14 and 15) respectively.Thus it was confirmed that we had successfully purified BuLIF protein from the stably transfected COS-1-LIF cells. The SDS protein bands were cut and digested by In-gel trypsin digestion for Mass Spectrophotometry but unknown proteins were identified in trial. Furthermore, to confirm the above statement of hyper glycosylation we have to perform the peptide mass finger printing of purified protein in LC- MS/MS for accurate determination of molecular mass and glycosylation moiety. Western blot results conclude that identified SDS band was rBuLIF that can be used for application in the culture of Bovine stem cells.

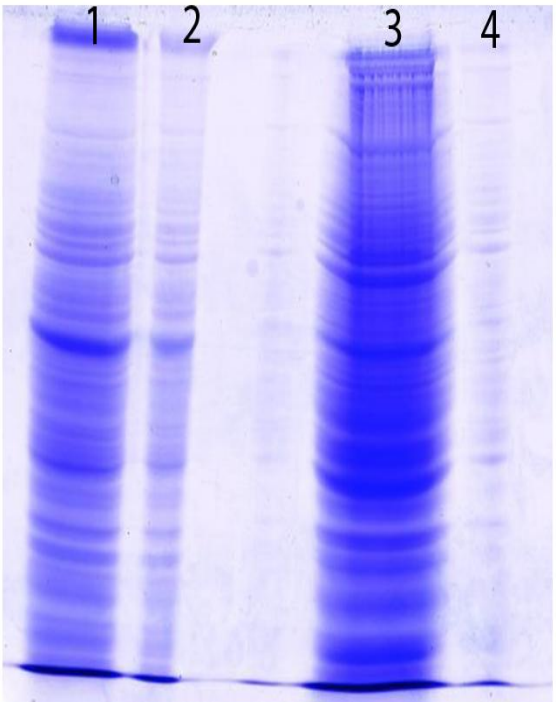


Fig. 11: SDS-PAGE images showing sonicated and non-sonicated cell lysates, Lane no.1 Cell lysate of transfected COS-1 cells after sonication and Lane no.2:Cell lysate of transfected COS-1 cells before sonication , Lane no.3: cell lysate of non-transfected COS-1 cells after sonication and, Lane no.4: cell lysate of non-transfected COS-1 cells before sonication.

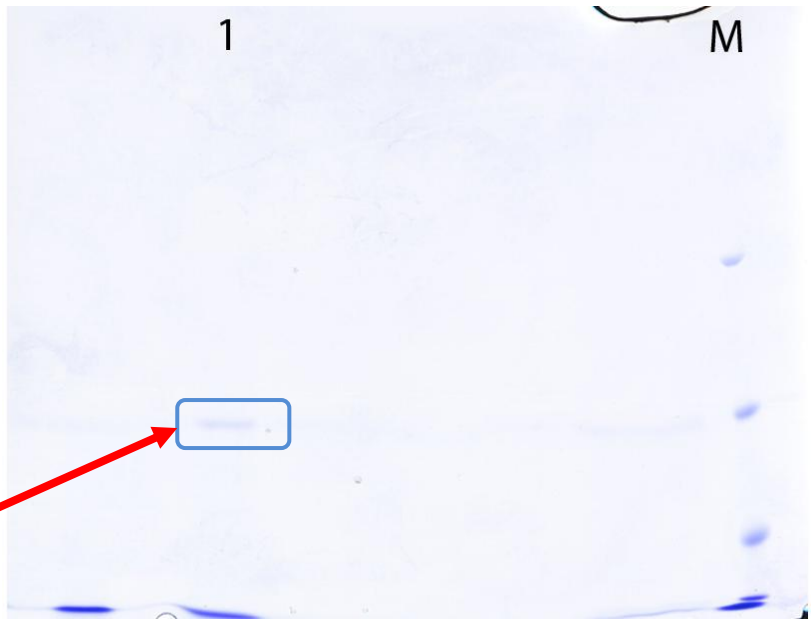


Fig. 11:SDS-PAGE of purified recombinant BuLIF-GFP protein with monoclonal Anti-GFP antibodies.

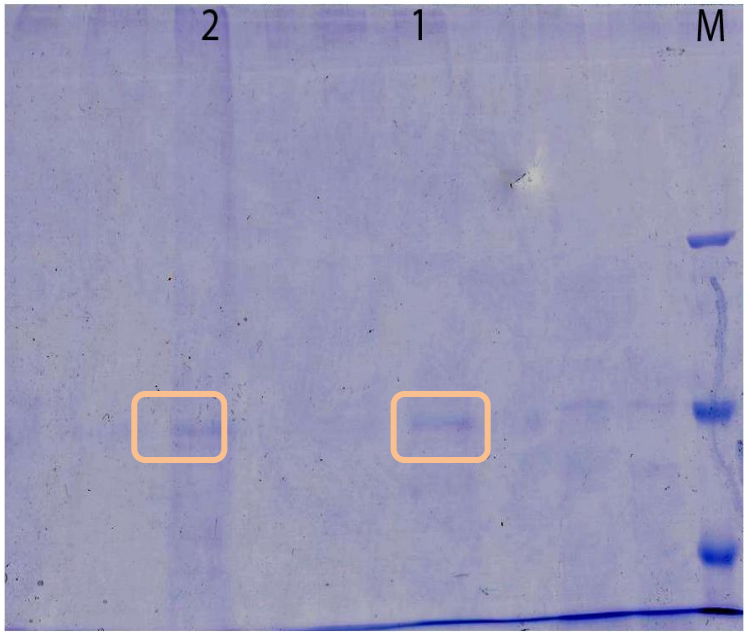


Fig. 12:SDS-PAGE of purified recombinant BuLIF-GFP protein with Polyclonal Anti-GFP antibodies.

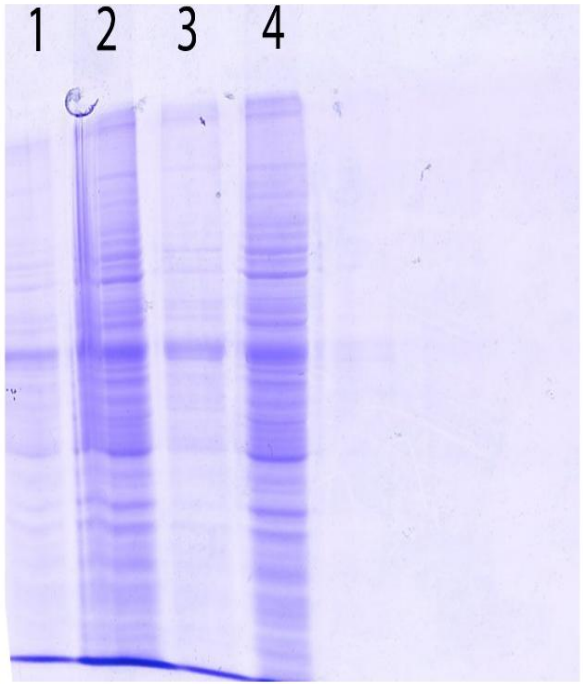


Fig. 13: SDS PAGE image showing Lane no.1 and 3: cell lysate of transfected COS-1 cells after purification procedure, Lane no.2 and 4: cell lysate of transfected COS-1 cells before purification procedure (duplicates).

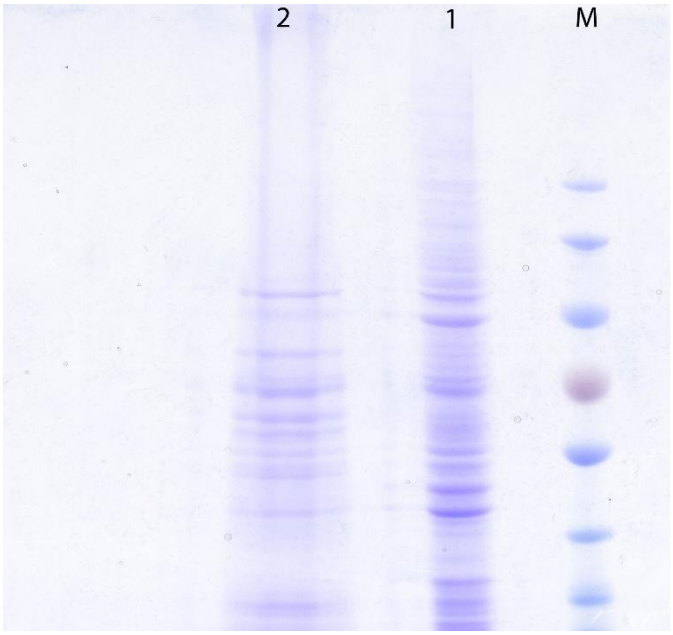


Fig. 14:SDS-PAGE image showing expression of target rBuLIF as fusion protein with GFP. Lane No M: Marker, Lane no.1: Cell lysate of COS-LIF, Lane no.2 Cell lysate of COS-1 cell line.

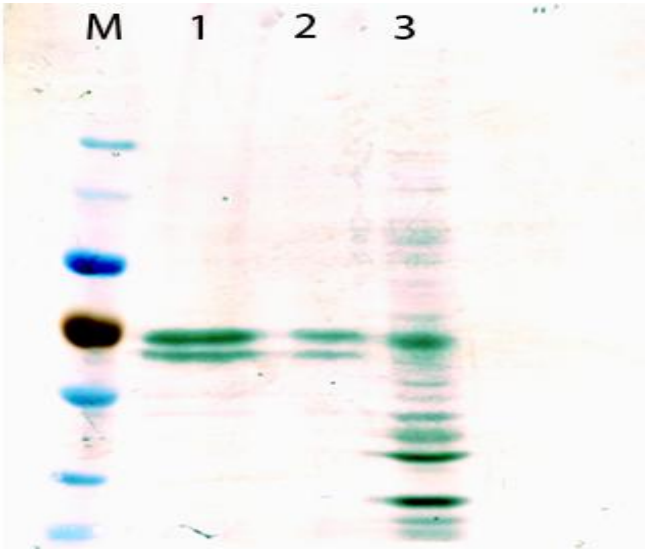


Fig. 15: Image of western-blot showing purified rBuLIF-GFP protein and cell lysate. Lane M: Protein Marker, Lane no.1 and 2 Cell lysate of Transfected COS-1 cells, Lane no.3: Non-Transfected COS-1 cell lysate.

CONCLUDING REMARKS

Leukemia Inhibitory factor (LIF) is pleiotropic molecule synthesized and secreted in various body tissues. Some of very important biological functions regulated by LIF are like proliferation, differentiation, and metabolism of cells. LIF is proved to maintain pluripotency in mouse and human embryonic stem cells. LIF is also considered as an important molecule for bovine stem cells. The present study was aimed to scale up of rBuLIF (recombinant Buffalo Leukemia Inhibitory Factor) production in COS-1 cell line and purification of recombinant BuLIF protein from COS-1 cell culture.

Our study constitute 2 objectives (i) Scale up of rBuLIF production in COS-1 cell line. (ii)Purification of recombinant BuLIF protein from COS-1 cell culture. To achieve the first objective, the stably transfected COS-1 cells were grown upto 70^{th} passages. Different conditions were optimized to achieve high rate of protein like FBS (Fetal Bovine Serum) concentration, G418 concentration etc. Different parameters like cell health, degree of confluency, number of passages, contamination. Transfection efficiency was found above 80% after 70^{th} passage with high cell density. Stably transfected cells were selected under 600 μg/mLconcentration of G418. FBS concentration was also optimised from 5%% to 20% for healthy growth of cells. The final FBS concentration 20% was used. Expression of BuLIF-GFP was observed under fluorescent microscopy and the expression of BuLIF protein was detected by PCR and Western-blot. The results confirmed the stably transfection of LIF gene in COS-1 cell line.

To achieve second objective, COS-1 culture was grown to purify the expressed recombinant BuLIF protein. Buffalo LIF-GFP fusion protein was purified from crude cellular sources. Crude cellular sources were lysed by RIPA with PMSF cell lysis buffer (400μL/T-75cm^2). Cell lysates were sonicated for complete extraction of proteins from cellular sources. Recombinant BuLIF protein was purified from lysed cell lysate utilizing a monoclonal Anti-GFP antibody produced in mouse coupled with CnBr-activated sephrose affinity column. SDS-PAGE and western-blot results revealed that purified protein is of approx. 60kDa to 70kDa size as different unknown hyper glycosylation occurs in rBuLIF. The SDS protein bands were cut and digested by In-gel trypsin digestion for Mass Spectrophotometry but unknown proteinsthe peptide mass finger printing of purified protein in LC- MS/MS for

accurate determination of molecular mass and glycosylation moiety. Western blot results conclude that identified SDS band was rBuLIF that can be used for application in the culture of Bovine stem cells.

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Aghajanova, L. 2004. Leukemia inhibitory factor and human embryo implantation. *Ann. N Y Acad. Sci*., 1034: 176-183. doi: 10.1196/annals.1335.020. PubMed: 15731310.

Aikawa, J., Ikeda-Naiki, S., Ohgane, J., Min, K.S., Imamura, T., Sasai, K., Shiota, K. and Ogawa, T.1997. Molecular cloning of rat leukemia inhibitory factor receptor alpha-chain gene and its expression during pregnancy. *Biochim. Biophys. Acta.*, 1353: 266–276.

Aksoy, I., Sakabedoyan, C., Bourillot, P.Y., Malashicheva, A.B., Mancip, J., Knoblauch, K., Afanassieff, M. and Savatier, P. 2007. Self-renewal of murine embryonic stem cells is supported by the serine/threonine kinases Pim-1 and Pim-3. *Stem Cells*, 25: 2996-3004.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B.A. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO. J.*, 15: 6541–6551. [PubMed: 8978681].

Ancey, C., Corbi, P., Froger, J. et al. 2002. Secretion of IL-6, IL-11 and LIF by human cardiomyocytes in primary culture. *Cytokine*, 18: 199-205.

Arici, A., Engin, O., Attar, E. and Olive, D.L.1995. Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. *J. Clin. Endocrinol. Metab.*, 80: 1908–1915.

Aruffo, A. and Seed, B. 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA*, 84: 8573–8577.

Auernhammer, C.J., Chesnokova, V. and Melmed, S. 1998. Leukemia inhibitory factor modulates interleukin-1b-induced activation of the hypothalamo-pituitary-adrenal axis. *Endocrinology*, 139: 2201–2208.

Austin, L., Bower, J.J., Bennett, T.M., et al. 2000. Leukemia inhibitory factor ameliorates muscle fiber degeneration in the mdx mouse. *Muscle Nerve*, 23: 1700-1705.

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jorgensen, H.F., John, R.M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M. and Fisher, A.G. 2006. Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.*, 8: 532-538.

Bachmann, M. and Möröy, T. 2005. The serine/threonine kinase Pim-1. *Int. J. Biochem. Cell Biol.*, 37: 726-730.

Barasch, J., Yang, J., Ware, C.B., et al. 1999. Mesenchymal to epithelial conversion in rat metanephros is induced by LIF. *Cell*, 99: 377-386.

Baumann, H. and Wong, G.G. 1989. Hepatocyte-stimulating factor III shares structural and functional identity with leukemia-inhibitory factor. *J. Immunol.*, 143: 1163.

Bazan, J.F.1991. Neuropoietic cytokines in the hematopoietic fold. *Neuron*, 7: 197–208.

Bechard, M. and Dalton, S. 2009. Subcellular localization of glycogen synthase kinase 3β controls embryonic stem cell self-renewal. *Mol. Cell Biol.*, 29: 2092–2104. [PubMed: 19223464].

Berg, D. T., McClure, D. B., and Grinnell, B. W. 1993. High-level expression of secreted proteins from cells adapted to serum-free suspension culture. *BioTechniques*, 14:972–978.

Bjornberg, O., Ostergaard, H. and Winther, J.R. 2006. Measuring intracellular redox conditions using GFP-based sensors. *Antioxid Redox Signal*, 8: 354–361.

Blasey, H. D. and Bernard, A. R. 1994. Transient expression COS cells on spinner scale, *in* ‘‘Animal Cell Technology: Products of Today, Prospects for Tomorrow’’ (Spier, R. E., Griffiths, J. B., Berthold, W., Eds.). *Butterworth and secretion of human Tissue Plasminogen Activator in recombi-Heinemann, Oxford*, 331–332.

Boast, S., La Mantia, G., Lania, L. and Blasi, F. 1983. Highefficiency of replication and expression of foreign genes in SV40-transformed human fibroblasts. *EMBO. J.*, 2: 2327–2331.

Boeuf, H., Hauss, C., Graeve, F.D., Baran, N. and Kedinger, C. 1997. Leukemia inhibitory factor-dependent transcriptional activation in embryonic stem cells. *J. Cell Biol.*,138(6): 1207-1217.

Bottorff, D., and Stone, J.C. 1992. The murine leukemia inhibition factor (LIF) is located on proximal chromosome 11, not chromosome 13. *Mamm Genome*, 3: 681–684.

Bourillot, P.Y., Aksoy, I ., Schreiber, V., Wianny, F., Shulz, H., Hummel, O., Hubner, N. and Savatier, P. 2009. Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog. *Stem Cells*, 27: 1760-1771.

Boussif, O. et al. 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. USA*, 92: 7297–301.

Braunstein, J., Brutsaert, S., Olson, R. and Schindler, C. 2003. STATs dimerize in the absence of phosphorylation. *J. Biol. Chem.*, 278: 34133–34140. [PubMed: 12832402].

Bromberg, J.F., Horvath, C.M., Besser, D., Lathem, W.W. and Darnell, J.E. Jr. 1998. Stat3 activation is required for cellular transformation by v-src. *Mol. Cell Biol.*, 18: 2553–8.

Brook, F., and Gardner, R. 1997. The origin and efficient derivation of embryonic stem cells in the mouse.*Proc Natl Acad Sci USA*, 94(11): 5709-12.

Brown, M.A., Metcalf. D. and Gough. N.M.1994. Leukemia inhibitory factor and interleukin 6 are expressed at very low levels in the normal adult mouse and are induced by inflammation. *Cytokine*, 6: 300–309.

Budarf, M., Emanuel, B.S., Mohandas, T., Goeddel, D.V., Lowe, D.G. 1989. Human differentiation-stimulating factor (leukemia inhibitory factor, human interleukin DA) gene maps distal to the Ewingsarcoma breakpoint on 22q. *Cytogenet Cell Genet*, 52: 19–22.

Burdon, T., Stracey, C., Chambers, I., Nichols, J. and Smith, A. 1999. Suppression of SHP-2 and ERK signaling promotes self-renewal of mouse embryonic stem cells. *Dev. Biol.*, 210: 30-43.

Butzkueven, H., Zhang, J.G., Soilu-Hanninen, M., et al. 2002. LIF receptor signaling limits immune-mediated demyelination by enhancing oligodendrocyte survival. *Nat. Med.*, 8: 613-619.

Capaccioli, S. et al. 1993. Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. *Biochem.Biophys. Res. Commun.*, 197: 818–25.

Carpenter, S., Wochal, P., Dunne, A., O'Neill, L.A., 2011. Toll-like receptor 3 (TLR3) signaling requires TLR4 Interactor with leucine-rich Repeats (TRIL).*J. Biol. Chem.*, 286: 38795 – 38804.

Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K. and Dalton, S. 2005. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development*, 132: 885–896. [PubMed: 15673569].

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. *Science*, 263 pp. 802–805.

Chambers, I. 2004. The molecular basis of pluripotency in mouseembryonic stem cells. *Cloning Stem cells*, 6(4): 386-391.

Chapman, R.S., Lourenco, P.C., Tonner, E., Flint, D.J., Selbert, S., Takeda, K., Akira, S., Clarke, A.R. and Watson, C.J. 1999. Suppression of epithelial apoptosisand delayed mammary gland involution in mice with aconditional knockout of Stat3. *Genes Dev.*, 13(19): 2604-2616.

Chen, J.R., Cheng, J.G., Shatzer, T., Sewell, L., Hernandez, L., et al. 2000. Leukemia inhibitory factor can substitute for estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology*, 141: 4365-4372. doi: 10.1210/en.141.12.4365. PubMed: 11108244.

Chen, L. and Khillan, J.S. 2010. A novel signaling by vitamin A/retinol promotes self -renewal of mouse embryonic stem cells by activating PI3K/Akt signaling pathway via insulin-like growth factor-1 receptor. *Stem Cells*, 28: 57–63. [PubMed: 19890980].

Dani, C., Chambers, I., Johnstone, S., Robertson, M., Ebrahimi, B., Saito, M., Taga, T., Li, M., Burdon, T., Nichols, J., and Smith, A. 1998. Paracrine induction of stem cell renewal by LIF-deficient cells: a new ES cell regulatory pathway. *Dev. Biol.*, 203: 149–162.

Dazai, S., Akita, S., Hirano, A., et al. 2000. Leukemia inhibitory factorenhances bone formation in calvarial bone defect. *J. Craniofac. Surg*., 11: 513-520.

Debs, R.J. et al. 1990. Regulation of gene expression in vivo by liposome-mediated delivery of a purified transcription factor. *J. Biol. Chem.*, 265: 10189–92.

Deng, X.F., Rokosh, D.G. and Simpson, P.C. 2000. Autonomous and growth factor-induced hypertrophy in cultured neonatal mouse cardiac myocytes. Comparison with rat. *Circ. Res.*, 87: 781-788.

Dietsch, M.T., Smith, V.F., Cosand, W.L., Damle, N.K., Ledbetter, J.A., Linsley, P.S., and Aruffo, A. 1993. Bispecific receptor globulins, novel tools for the study of cellular interactions. *J. Immunol. Methods*, 162: 123–132.

Doble, B.W., Patel, S., Wood, G.A., Kockeritz, L.K. and Woodgett, J.R. 2007. Functional redundancy of GSK-3αand GSK-3βin Wnt/β-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev. Cell.*, 12: 957–971. [PubMed: 17543867].

Dubendorff, J.W. and Studier, F.W. 1991. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *J. Mol. Biol.*, 219: 45–59.

Edwards, C. P. and Aruffo, A. 1993. Current applications of COS cell based transient expression systems. *Curr.Opinion Biotechnol.*, 4: 558–563.

Escary, J.L., Perreau, J., Dumenil, D., et al. 1993. Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature*, 363: 361-364.

Fahnert, B., Lilie, H. and Neubauer, P.2004. Inclusion bodies: formation and utilisation. *Adv. Biochem. Eng. Biotechnol.*, 89: 93-142. doi: 10.1007/ b93995. PubMed: 15217157.

Farese, A.M., Myers, L.A. and MacVittie, T.J. 1994. Therapeutic efficacy of recombinant human leukemia inhibitory factorin a primate model of radiation-induced marrow aplasia. *Blood*, Vol 84, No 11: pp 3675-3678.

Farhood, H. et al. 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta.*, 1235: 289–95.

Felgner, P.L. et al. 1995. Improved cationic lipid formulations for in vivo gene therapy. *Ann. NY Acad. Sci.*, 772: 126–39.

Fraley, R. et al. 1980. Introduction of liposome-encapsulated SV40 DNA into cells. *J. Biol. Chem.*, 255: 10431–5.

Fry, R.C. 1992. The effect of leukemia inhibitory factor (LIF) on embryogenesis. *Reprod. Fertil. Dev.*, 4: 449–58.

Fukada, K., Korsching, S. and Towle, M.F. 1997. Tissue-specific and ontogenetic regulation of LIF protein levels determined by quantitative enzyme immunoassay. *Growth Factors*, 14: 279-295.

Fukui, Y. and Matsuyama, K. 1994. Development of in vitro matured and fertilized bovine embryos cultured in media containing human leukemia inhibitory factor. *Theriogenology,* 42: 663–73.

Funston, R.N., Nauta, W.J. and Seidel Jr., G.E. 1997. Culture of bovine embryos in buffalo rat liver cell-conditioned media or with leukemia inhibitory factor. *J. Anim. Sci.*, 75: 1332–6.

Gao, X. and Huang, L. 1995. Cationic liposome-mediated gene transfer. *Gene Ther.,* 2:710–722.

Gerard, R. D. and Gluzman, Y. 1985. New host cell system, for regulated Simian Virus 40 DNA replication. *Mol. Cell. Biol.*, 601–621; 5: 3231–3240.

Giese, B., Roderburg, C., Sommerauer, M., et al. 2005. Demonization of the cytokine receptors gp130 and LIFR analyzed in single cells. *J. Cell Sci.*, Vol. 118: 5129-5140.

Gillies, S. D., Dorai, H., Wesolowski, J., Majeau, G., Young, D., Boyd, J., Gardner, J., and James, K. 1989. Expression of human anti-tetanus toxoid antibody in transfected murine myeloma cells. *BioTechnology,* 7: 799–804.

Gorman, C., *et al.* 1995. In DNA cloning: A practical appoarch. *Vol. II. Ed. D. M. Glover (IRL press, Oxford, UK),* pp. 143-190.

Gough, N.M., Gearing, D.P., King, J.A., Wilson, T.A., Hilton, D.J., Nicola, N.A. and Metcalf. D. 1988. *Proc. Natl. Acad. Sci.*, 85: 2623-2627.

Gough, N.M., Willson, T.A., Stahl, J. and Brown, M.A.1992. Molecular biology of the leukemia inhibitory factor gene. *Ciba Found Symp,* 167: 24–46.

Graham, F. L. and Van der Eb, A. J. 1973. Transformation of rat cells by DNA of human adenovirus 5. *Virology,* 52: 456-467.

Graham, F.L. and van der Eb, A.J. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*, 52: 456–67.

Grant, S.L., Douglas, A.M., Goss, G.A., et al. 2001. Oncostatin M and leukemia inhibitory factor regulate the growth of normal human breast epithelial cells. *Growth Factors*, 19: 153-162.

Graves, K.H. and Moreadith, R.W. 1993. Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. *Mol Reprod Dev.*, 36: 424–433.

Guo, G., Yang, J., Nichols, J., Hall, J.S., Eyres, I., Mansfield, W. and Smith, A. 2009. Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development*, 136: 1063-1069.

Haensler, J. and Szoka, F.C. 1993. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconj.Chem.*, 4: 372–9.

Haines, B.P., Voyle, R.B., Pelton, T.A., Forrest, R. and Rathjen, P.D. 1999. Complex conserved organization of the mammalian leukemia inhibitory factor gene: regulated expression of intracellular and extracellular cytokines. *J. Immunol*., 162: 4637–4646.

Hamazaki, T., Kehoe, S.M., Nakano, T. and Terada, N. 2006. The Grb2/Mek pathway represses Nanog in murine embryonic stem cells. *Mol. Cell Biol.*, 26:7539–7549. [PubMed: 16908534].

Harland, R. 2000. Neural induction. *Curr.Opin.Genet. Dev.*, 10: 357–362.

Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F. and Graeve, L. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.*, 334: 297–314. [PubMed: 9716487].

Hilton, D.J., Nicola, N.A. and Metcalf, D. 1991. Distribution and comparison of receptors for leukemia inhibitory factor on murine hemopoietic and hepatic cells. *J. Cell Physiol*, 146: 207-215.

Hirai, H., Karian, P. and Kikyo, N. 2011. Regulation of embryonic stem cell self-renewal and pluripotency by leukaemia inhibitory factor. *Biochem J.*, 438: 11-23. doi: 10.1042/BJ20102152. PubMed: 21793804.

Hirobe, T. 2002. Role of leukemia inhibitory factor in the regulation of the proliferation and differentiation of neonatal mouse epidermal melanocytes in culture. *J. Cell Physiol.*, 192: 315-326.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O. and Woodgett, J.R. 2000. Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation. *Nature*, 406: 86–90. [PubMed: 10894547].

Hsu, L.W. and Heath, J.K.1994. Identification of two elements involved in regulating expression of murine leukemia inhibitory factor gene. *Biochem J.*, 302: 103–110.

Hu, J., Ono, S., Katayama, H., et al. 2000. Leukemia inhibitory factor induces epidermal hyperplasia in patients with amyotrophic lateral sclerosis. *J. Invest Dermatol*., 115: 486-492.

Huang, B., Li, T., Wang, X.L., et al. 2010. Generation and characterization of embryonic stem-like cell lines derived from in vitro fertilization Buffalo (Bubalus bubalis) embryos. *Reprod. Domest. Anim.*, 45: 122–128.

Iannaccone, P.M., Taborn, G.U. and Garton, R.L. 1994, Pluripotent embryonic stem cells from the rat are capable of producing chimeras. *Develop. Bio.*, 163: 288–292.

Ivanova, N., Dobrin, R., Lu, R., Kotenko, I., Levorse, J., DeCoste, C., Schafer, X., Lun, Y. and Lemischka, I.R. 2006. Dissecting self-renewal in stem cells with RNA interference. *Nature*, 442: 533-538.

Jiang, J., Chan, Y.S., Loh, Y.H., Cai, J., Tong, G.Q., Lim, C.A., Robson, P., Zhong, S. and Ng, H.H. 2008. A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat. Cell Biol.*, 10: 353-360.

Jolly, D. J., Esty, A. C., Bernard, H. U. and Friedman, T. 1982. Isolation of a genomic clone partially encoding human hypoxanthine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA*, 79: 5038-5041.

Kabanov, A.V. and Kabanov, V.A. 1995. DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate Chem.*, 6: 7–20.

Kawai, S. and Nishizawa, M. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.*, 4: 1172–4.

Khalfallah, O., Rouleau, M., Barbry, P., Bardoni, B. and Lalli, E. 2009. Dax-1 knockdown in mouse embryonic stem cells induces loss of pluripotency and multilineage differentiation. *Stem Cells*, 27: 1529-1537.

Kidder, B.L., Yang, J. and Palmer, S. 2008. STAT3 and c-Myc genome-wide promoter occupancy in embryonic stem cells. *PLoS ONE*, 3: e3932.

Kinoshita, K.; Ura,H.; Akagi,T.; Usuda, M.; Koide, H. & Yokota, T. (2007) GABPαregulates Oct-3/4 expression in mouse embryonic stem cells.*Biochem.Biophys. Res. Commun.*, 353, 686-691.

Kisseleva, T., Bhattacharya, S., Braunstein, J. and Schindler, C.W. 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene*, 285: 1–24. [PubMed: 12039028].

Kluxen, F.-W., and Lu¨ bbert, H. 1993. Maximal expression recombinant cDNAs in COS cells for use in expression cloning. 208, 352–356.

Ko, S.Y., Kang, H.Y., Lee, H.S., Han, S.Y. and Hong, S.H. 2006. Identification of Jmjd1a as a STAT3 downstream gene in mES cells. *Cell Struct. Funct.*, 31: 53-62.

Ko¨nig, R., Ashwell, G. and Hanover, J. A. 1989. Overexpression and biosynthesis of CD4 on Chinese hamster ovary Coamplification using the multiple drug resistance gene. *Proc. Natl. Acad. Sci. USA*, 86: 9188–9192.

Kobayashi, T. and Cohen, P. 1999. Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochem. J.*, 339: 319–328.

Kouzarides, T. 2007. Chromatin modifications and their function. *Cell*, 128: 693-705.

Kristensen, D.M., Kalisz, M. and Nielsen, J.H. 2005. Cytokine signalling in embryonic stem cells. *APMIS*, 113: 756–72.

Kritikou, E.A., Sharkey, A., Abell, K., Came, P.J., Anderson, E., Clarkson, R.W. and Watson, C.J. 2003. A dual, non-redundant, role for LIF as a regulatorof development and STAT3-mediated cell death in mammarygland. *Development*, 130(15): 3459-3468.

Kubo, M., Hanada, T. and Yoshimura, A. 2003. Suppressors of cytokine signaling and immunity. *Nat. Immunol.*, 4: 1169-1176.

Kuhn, L. C., MacClelland, A. and Ruddle, F. 1984. Gene transfer, expression, and molecular cloning of the human transferring receptor gene. *Cell*, 37:95-103.

Kukowska-Latallo, J.F. et al. 1996. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc. Natl. Acad. Sci. USA*, 93: 4897–902.

Laine, J., Kunstle, G., Obata, T., Sha, M. and Noguchi, M. 2000. The proto-oncogene TCL1 is an Akt kinase coactivator. *Mol. Cell*, 6: 395-407.

Lamb, B.T. and Gearhart, J.D. 1995. YAC transgenics and the study of genetics and human disease. *Curr. Opin. Genet. Dev.*, 5: 342–8.

Lane, P., Brocker, T., Hubele, S., Padovan, E., Lanzavecchia, A., and McConnell, F. 1993. Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell dependent activation. *J. Exp. Med., 177:*1209–1213.

Leonard, W.J. and O'Shea, J.J. 1998. Jaks and STATs: biological implications. *Annu. Rev. Immunol.*, 16: 293–322. [PubMed: 9597132].

Levy, D.E. and Darnell, J.E. Jr. 2002. Stats: transcriptional control and biological impact. *Nat. Rev. Mol. Cell Biol.* 3: 651–662. [PubMed: 12209125].

Li, Y., McClintick, J., Zhong, L., Edenberg, H.J., Yoder, M.C. and Chan, R.J. 2005. Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. *Blood*, 105: 635-637.

Lindberg, I., Shaw, E., Finley, J., Leone, D. and Deiniger, P. 1991. Posttranslational modifications of Rat Proenkephalin overexpressed in Chinese hamster ovary cells. *Endocrinology,* 128:1849–1856.

Lo, K.-M., Lynch, C. A., and Gillies, S. D. 1992. The use of a wild-type dihydrofolate reductase-encoding cDNA as dominant selectable marker and induction of expression by methotrexate. *Gene,* 121: 365–369.

Loh, Y.H., Zhang, W., Chen, X., George, J. and Ng, H.H. 2007. Jmjd1a and Jmjd2c histone H3 Lys9 demethylases regulate self-renewal in embryonic stem cells. *Genes Dev.*, 21: 2545-2557.

Lopez, C., de Chesnay, A., Tournamille, C., Ben Ghanem, A., Prigent, S., Drouet, X., Lambin, P., and Cartron, J.P. 1994. Efficient production of biologically active human recombinant

proteins in human lymphoblastoid cells from integrative andepisomal expression vectors. *Gene*, 148: 285–291.

Lowe, D.G., Nunes, W., Bombara, M., McCabe, S., Ranges, G.E., Henzel, W., Tomida, M., Yamamoto-Yamaguchi, Y., Hozumi, M. and Goeddel, D.V. 1989. Genomic cloning and heterologous expression of human differentiation-stimulating factor. *DNA (New York)*, 8: 351.

Loyter, S. et al. 1982. Mechanisms of DNA uptake by mammalian cells: Fate of exogenously added DNA monitored by the use of fluorescent dyes. *Proc. Natl. Acad. Sci. USA*, 79: 422–6.

Luckow, V.A. and Summers, M.D. 1988. Trends in the development of baculovirus vectors. *Biotechnology*, 6: 45–55.

Malaval, L. and Aubin, J.E. 2001. Biphasic effects of leukemia inhibitory factor on osteoblastic differentiation. *J. Cell Biochem.*, (suppl 36): 63-70.

Malone, R.W. et al. 1989. Cationic liposome-mediated RNA transfection. *Proc. Natl. Acad. Sci. USA*, 86: 6077–81.

Marquant-Le, Guienne, B., Humblot, P., Guillon, N. and Thibier, M. 1999. Murine LIF improves the development of IVF cultured bovine morulae. *J. Reprod. Fertil.*, 12: 61 (abs).

Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A., Ko, M.S. and Niwa, H. 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat. Cell Biol.*, 9: 625-635.

Matoba, R., Niwa, H., Masui, S., Ohtsuka, S., Carter, M.G., Sharov, A.A. and Ko, M.S. 2006. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. *PLoS One*, 1: e26.

Mauduit, C., Goddard, I., Besset, V., et al. 2001. Leukemia inhibitory factor antagonizes gonadotropin induced-testosterone synthesis in cultured porcine leydig cells: sites of action. *Endocrinology*, 142: 2509-2520.

Mayer, P., Geissler, K., Ward, M. and Metcalf, D. 1993. Recombinant human leukemia inhibitory factor induces acute phase proteins and raises the blood platelet counts in nonhuman primates. *Blood*, Vol. 1, No 12 pp 3226-3233.

McCutchan, J. H. and Pagano J. S. 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer Inst.*, 41: 351-357.

McDonald, N.Q., Panayotatos, N. and Hendrickson, W.A. 1995. Crystal structure of dimeric human ciliary neurotrophic factor determined by MAD phasing. *EMBO. J.*, 14: 2689–2699.

Meima, M.E., Weening, K.E. and Schaap, P. 2007. Vectors for expression of proteins with single or combinatorial fluorescent protein and tandem affinity purification tags in Dictyostelium. *Protein Expression and Purification*, 53: 283–288.

Mellon, P., Parker, V., Gluzman, Y. and Maniatis, T. 1981. Identification of DNA sequences required for transcription of the human a-1-Globin gene in a new SV40 host-vector system. *Cell*, 27: 279–288.

Meshorer, E. and Misteli, T. 2006. Chromatin in pluripotent embryonic stem cells and differentiation. *Nat. Rev. Mol. Cell. Biol.*, 7: 540-546.

Metcalf, D., Nicola, N.A., Gearing, D.P. 1990. Effects of injected leukemia inhibitory factor (LIF) on hemopoietic and other tissues in mice. *Blood*, 765.

Migone, T.S., Rodig, S., Cacalano, N.A., Berg, M., Schreiber, R.D. and Leonard, W.J. 1998. Functional cooperation of the interleukin-2 receptor β chain and Jak1 in phosphatidylinositol 3-kinase recruitment and phosphorylation. *Mol. Cell Biol.*, 18: 6416–6422. [PubMed: 9774657].

Miller, L.K. 1988. Baculovirus as gene expression vectors. *Annu. Rev. Microbiol*, 42: 177–199.

Mitalipova, M., Beyhan, Z. and First, N.L. 2001. Pluripotency of bovine embryonic cell line derived from precompacting embryos. *Cloning*, 3: 59–67.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. 2003. The homeoprotein Nanogis required for maintenance of pluripotency in mouse epiblastand ES cells. *Cell*, 113(5): 631-642.

Moon, C., Yoo, J.Y., Matarazzo, V., et al. 2002. Leukemia inhibitory factor inhibits neuronal terminal differentiation through STAT3 activation. *Proc Natl Acad Sci USA*, 99: 9015-9020.

Moreau, J.F., Donaldson, D.D., Bennett, F., Witek-Giannotti, J., Clark, S.C. and Wong, G.G. 1988. Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells. *Nature*,336: 690–692.

Morel, D.S., Taupin, J.L., Potier, M. et al. 2000. Renal synthesis of leukaemia inhibitory factor (LIF), under normal and inflammatory conditions. *Cytokine*, 12: 265-271.

Murphy, J., Tannahill, G., Hilton, D. and Greenhalgh, C. 2010. The negative regulation of JAK/STAT signaling. *Handbook of Cell Signaling. Elsevier; San Diego,* p. 467-480.

Murray, P.J. 2007. The JAK/STAT signaling pathway: input and output integration. *J. Immunol.*, 178: 2623–2629. [PubMed: 17312100].

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. 1993. Derivation of completely cell culture-derived mice from early passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA*, Vol. 90, No 18: 8424-8428.

Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO. J.* , 1: 841–845.

Niakan, K.K., Davis, E.C., Clipsham, R.C., Jiang, M., Dehart, D.B., Sulik, K.K. and McCabe, E.R. 2006. Novel role of the orphan nuclear receptor Dax1 in embryogenesis, different from steroidgenesis. *Mol. Genet. Metab.*, 88: 261-271.

Nilsson, E.E., Kezele, P. and Skinner, M.K. 2002. Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Mol. Cell Endocrinol*., 188: 65-73.

Nishi, K., Ueno, M., Murakami, Y., Fukunaga, N., Akuta, T., Kadowaki, D., Watanabe, H., Suenaga, A., Maruyama, T. and Otagiri, M. 2009. A site-directed mutagenesis study of drug-binding selectivity in genetic variants of human alpha(1)-acid glycoprotein. *J. Pharm. Sci.*, 98: 4316–4326.

Niwa, H., Ogawa, K., Shimosato, D. and Adachi, K. 2009. A parallel circuit of LIF signaling pathways maintains pluripotency of mouse ES cells. *Nature*, 460: 118-122.

O'Reilly, D.R., Miller, L.K. and Luckow, V.A. 1992. Baculovirus Expression Vectors—A Laboratory Manual. *WH Freeman and Company, New York.*

O'Shea, J.J., Gadina, M. and Schreiber, R.D. 2002. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell*, 109: S121–S131. [PubMed: 11983158].

Ogawa, K., Nishinakamura, R., Iwamatsu, Y., Shimosato, D. and Niwa, H. 2006. Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells. *Biochem. Biophys. Res. Commun.*, 343: 159–166. [PubMed: 16530170]

Okamoto, M., Nakayama, C., Nakai, M., and Yanagi, H. 1990. Amplification and high-level expression of a cDNA for human granulocyte-macrophage colony stimulating factor in human lymphoblastoid Namalwa cells. *BioTechnology*, 8: 550–553.

Owczarek, C.M., Layton, M.J., Robb, L.G., Nicola, N.A and Begley, C.G. 1996. Molecular basis of the soluble and membrane-bound forms of the murine leukemia inhibitory factor receptor

a-chain. Expression in normal, gestating, and leukemia inhibitory factor nullizygous mice. *J. Biol. Chem.*, 271: 5495–5504.

Page, M. J. and Sydenham, M. A. 1991. High level expression of the humanized monoclonal antibody Campath-1H in Chinese Hamster Ovary Cells. *BioTechnology*, 9:64–68.

Paling, N.R., Wheadon, H., Bone, H.K. and Welham, M.J. 2004. Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. *J. Biol. Chem.*, 279: 48063–48070. [PubMed: 15328362].

Paradis, H. and Gendron, R.L. 2000. LIF transduces contradictory signals on capillary outgrowth through induction of stat3 and (P41/43)MAP kinase. *J. Cell Sci.*, 113: 4331-4339.

Park, J., Leong, M.L.L., Buse, P., Maiyar, A.C., Firestone, G.L. and Hemmings, B.A. 1999. Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinasestimulated signaling pathway. *EMBO. J.*, 18: 3024–3033.

Patterson, B.K., Behbahani, H., Kabat, W.J. et al. 2001. Leukemia inhibitory factor inhibits HIV-1 replication and is upregulated in placentae from nontransmitting women. *J. Clin. Invest*, 107: 287-294.

Peckham, G.D., Bugos, R.C., Su, W.W. 2006. Purification of GFP fusion proteins from transgenic plant cell cultures, *Protein Expression and Purification*, 49: 183–189.

Pekarsky, Y., Koval, A., Hallas, C., Bichi, R., Tresini, M., Malstrom, S., Russo, G., Tsichlis, P. and Croce, C.M. 2000. Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. *Proc. Natl. Acad. Sci. USA*, 97: 3028-3033.

Perucho, M., Hanahan, D. and Wigler, M. 1980. Genetic and physical linkage of exogenous sequences in transformed cells. *Cell*, 22: 309-317.

Piquet-Pellorce, C., Dorval-Coiffec, I., Pham, M.D., et al. 2000. Leukemia inhibitory factor expression and regulation within the testis. *Endocrinology*, 141: 1136-1141.

Rathjen, P.D., Toth, S., Willis, A., Heath, J.K. and Smith, A.G. 1990. Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. *Cell*, 62: 1105-1114.

Raz, R., Lee, C.-K., Cannizzaro, L.A., d'Eustachio, P., and Levy, D.E. 1999. Essential role of STAT3 for embryonic stem cell pluripotency. *Proc. Natl. Acad. Sci. USA*, Vol. 96, No 6: 2846-2851.

Reich, N.C. and Liu, L. 2006. Tracking STAT nuclear traffic. *Nat. Rev. Immunol.*, 6: 602–612. [PubMed: 16868551].

Reid, L.R., Lowe, C., Cornish, J., et al. 1990. Leukemia inhibitory factor: a novel bone-active cytokine. *Endocrinology*, 126: 1416-1420.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., et al. 2000. Embryonicstem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.*, 18: 399–404.

Rexroad Jr., C.E. and Powell, A.M. 1997. Culture of blastomeres from in vitromatured, fertilized, and cultured bovine embryos. *Mol. Reprod. Dev.*, 48: 238–45.

Ridder, R., Geisse, S., Kleuser, B., Kawalleck, P. and Gram,H. 1995. A COS-cell-based system for rapid production and quantification of scFv::IgCk antibody fragments. *Gene*, 166: 273–276.

Ristevski, S., O'Leary, D.A., Thornell, A.P., Owen, M.J., Kola, I. and Hertzog, P.J. 2004. The ETS transcription factor GABP□is essential for early embryogenesis. *Mol. Cell. Biol.*, 24: 5844-5849.

Ruzinova, M.B., and Benezra, R. 2003. Id proteins in development, cell cycle and cancer. *Trends Cell. Biol.*, 13: 410–418.

Saito, S., Ugai, H. and Sawai, K. 2002. Isolation of embryonic stem-like cells from equine blastocysts and their differentiation in vitro. *FEBS Lett.*, 531: 389–396.

Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I. and Weinberg, R. A. 1984. The neuoncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature (London)*, 312: 513-516.

Schindler, C., Levy, D.E. and Decker, T. 2007. JAK-STAT signaling: from interferons to cytokines. *J. Biol. Chem.*, 282: 20059–20063. [PubMed: 17502367].

Schorpp-Kistner, M., Wang, Z.-Q., Angel, P. and Wagner, E.F. 1999. JunB is essential for mammalian placentation. *EMBO. J.*, 18: 934 – 948.

Sessa, G. and Weissmann, G. 1968. Phospholipid spherules (liposomes) as a model for biological membranes. *J. Lipid Res.*, 9: 310–8.

Shaulian, E. and Karin, M. 2001. AP-1 in cell proliferation and survival. *Oncogene*, 20: 2390-2400.

Shih, C. and Weinberg, R. A. 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. *Cell*, 29: 161-169.

Simon, J.A. and Kingston, R.E. 2009. Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat. Rev. Mol. Cell Biol.*, 10: 697-708.

Sirisathien, S., Hernandez-Fonseca, H.J., Bosch, P., Hollet, B.R., Lott, J.D. and Brackett, B.G. 2003. Effect of leukemia inhibitory factor on bovine embryos produced in vitro under chemically defined conditions. *Theriogenology*, 59: 1751–63.

Skosyrev, V.S., Rudenko, N.V., Yakhnin, A.V., Zagranichny, V.E., Popova, L.I., Zakharov, M.V., Gorokhovatsky, A.Yu., Vinokurov, Leonid M. 2003. EGFP as a fusion partner for the expression and organic extraction of small polypeptides. *Protein Expression and Purification*, 27: 55–62.

Slaets, H., Hendriks, J.J.A., Stinissen,P., Kilpatrick, T.J. and Hellings, N. 2010. Therapeutic potential of LIF in multiple sclerosis: *Trends in Molecular Medicine*, Vol. 16, No. 11.

Smith, A.G., Nichols, J., Robertson, M. and Rathjen, P.D, 1992. Differentiation inhibiting activity (DIA/LIF) and mouse development. *Dev. Biol.*, 151: 339-351. doi: 10.1016/0012-1606(92)90174-F. PubMed: 1601171.

Song, J.H., Houde, A. and Murphy, B.D. 1998. Cloning of leukemia inhibitory factor (LIF) and its expression in the uterus during embryonic diapause and implantation in the mink (*Mustela vison*). *Mol. Reprod. Dev.*, 51: 13–21.

Spangenburg, E.E. and Booth, F.W. 2002. Multiple signaling pathways mediate LIF-induced skeletal muscle satellite cell proliferation. *Am J Physiol Cell Physiol*, 283: C204-C21.

Sprang, S. and Bazan, J.1993. Cytokine structural taxonomy and mechanisms of receptor engagement. *Curr. Opin. Struct. Biol.*, 3: 815–827.

Sutherland, G.R., Baker, E., Hyland, V.J., Callen, D.F., Stahl, J. and Gough, N.M. 1989. The gene for human leukemia inhibitory factor (LIF) maps to 22q12. *Leukemia*, 3: 9–13.

Takahashi, K., Mitsui, K., and Yamanaka, S. 2003. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. *Nature*, 423: 541–545.

Takao, Y., Yokota, T. and Koide, H. 2007. β-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. *Biochem. Biophys. Res. Commun.*, 353: 699–705. [PubMed: 17196549]

Takeishi, K., Ayusawa, D., Kaneda, S., Shimizu, K. and T. Seno. 1984. Molecular cloning of genomic DNA segments partially coding for human thymidylate synthase from the mouse cell transformant. *J. Biochem*, 95: 1477-1483.

Tessier, M. and Woodgett, J.R. 2006. Serum and Glucocorticoid-Regulated Protein Kinases: Variations on a Theme. *J. Cell. Biochem.*, 98: 1391–1407.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. 1995. Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. USA*, 92: 7844–7848.

Trill, J. J., Shatzman, A. R. and Ganguly, S. 1995. Production of monoclonal antibodies in COS and CHO cells. *Curr.Opinion Biotechnol.*, 6: 553–560.

Trouillas, M., Saucourt, C., Guillotin, B., Gauthereau, X., Ding, L., Buchholz, F., et al. 2009. Three LIF-dependent signatures and gene clusters with atypical expression profiles, identified by transcriptome studies in mouse ES cells and early derivatives. *BMC Genomics*, 10: 73.

Vejlsted, M., Avery, B., Gjorret, J.O. and Maddox-Hyttel, P. 2005. Effect of leukemia inhibitory factor (LIF) on in vitro produced bovine embryos and their outgrowth colonies. *Mol. Reprod. Dev.*, 70: 445–54.

Venkatgopal, T., Polte, T., Arthur, P., and Seidman, M. 1989. Mouse hybrid cell line that supports gene expression from a variety of promotors in amplifiable vectors. *In vitro Cell. Dev. Biol., 25:*1147–1154.

Verma, V., Gautam, S.K., Singh, B., et al. 2007. Isolation and characterization of embryonic stem cell-like cells from in vitroproduced buffalo (Bubalus bubalis) embryos. *Mol. Reprod. Dev.*, 74: 520–529.

Vogiagis, D. and Salamonsen, L.A. 1999. The role of leukaemia inhibitory factor in the establishment of pregnancy. *Journal of Endocrinology*, 160: 181–190.

Walter, M.R.1997. Structural biology of cytokines, their receptors, and signaling complexes: implications for the immune and neuroendocrine circuit. *Chem. Immunol.*, 69: 76–98.

Wang, R., Cherukuri, P. and Luo, J. 2005. Activation of Stat3 sequence-specific DNA binding and transcription by p300/CREB-binding protein-mediated acetylation. *J. Biol. Chem.*, 280: 11528–11534. [PubMed: 15649887].

Wang, Z., Ren, S.G. and Melmed, S.1996. Hypothalamic and pituitary leukemia inhibitory factor gene expression *in vivo*: a novel endotoxin-inducible neuro-endocrine interface. *Endocrinology*, 137: 2947–2953.

Waring, P., Wall, D., Dauer, R., et al. 1993. The effects of leukaemia inhibitory factor on platelet function. *Br J Haematol*, 83: 80-87.

Westerveld, A., Hoejimakers, H. J., Van Duin, M., deWit, J., Odik, H., Pastink, A., Wood, R. D. and Bootsma, D. 1984. Molecular cloning of a human DNA repair gene. *Nature (London)*, 310: 425-429.

Wheeler, M.B. 1994. Development and validation of swine embryonic stem cells: A review. *Reprod. Fertil. Develop.*, 6: 563–568.

Wiedenmann, J. and Nienhaus, G.U. 2006. Live-cell imaging with EosFP andother photoactivatable marker proteins of the GFP family, *Expert Review of Proteomics*, 3: 361–374.

Wilson, T. et al. 1979. The introduction of poliovirus RNA into cells via lipid vesicles (liposomes). *Cell*, 17: 77–84.

Wirth, M., Bode, J., Zettlmeissl, G. and Hauser, H. 1988. Isolation of overproducing recombinant mammalian cell lines by a 22and simple selection procedure. *Gene*, 73:419–426.

Yamanaka, M., Amano, T. and Kudo, T. 2001. Effect of the presence period of bovine leukemia inhibitory factor in culture medium on the development of in vitro fertilized bovine embryos. *Anim. Sci. J.*, 72: 285–90.

Zhang, R., Howard J.K, Gourley J. 2008. Purification of GFP fusion proteins with high purity and yield by monoclonal antibody-coupled affinity column chromatography. *Protein Expression & Purification*, 59: 138-143.