Label Free Quantitative Proteomics Approach Unravels the Pleotropy of Buffalo Leukemia Inhibitory Factor (BuLIF) in COS-1 cells

Sudarshan Kumar*, Syed Azmal Ali, and Ashok Kumar Mohanty

Proteomics and Cell Biology Lab, Animal Biotechnology Center, India
National Dairy Research Institute Karnal, Haryana, India

Key words: Leukemia Inhibitory Factor, Cytokine, Pathways, Mass Spectrometer

Leukemia Inhibitory Factor (LIF) may be a pleotropic molecule which performs diverse functions during a context dependent manner. Bovine LIF (BuLIF) is an important media component in in-vitro somatic cell culture and also considered essential within the early stages of pregnancy. However, the exact molecular mechanism behind the diverse actions of this molecule is unknown except the stat3 mediated canonical pathways in stem cell pluripotency. One of the "cells that are self-replicating, are derived from human embryos or human fetal tissue, and are known to become cells and tissues of the three primary germ layers. Although human pluripotent stem cells could also be derived from embryos or fetal tissue, such stem cells aren't themselves embryos." (From the National Institutes of Health Guidelines for Research Using Human Pluripotent Stem Cells.)

"Self-replicating" means the cell can divide and to make cells indistinguishable from it. The three primary germ layers (called the ectoderm, mesoderm, and endoderm) are the first layers of cells within the embryo from which all tissues and organs develop. Human pluripotent stem cells also are referred to as human embryonic stem cells.

We amplified BuLIF from cumulus oophorus cells of cow ocytes by RT-PCR. The nucleotide sequence was determined by bidirectional sequencing and submitted to NCBI database as GenBank (accession number HQ616665). A multiple sequence alignment from different species revealed its high similarity with sheep (98.77%) and cattle (96.62%) in comparison to pig (86.77%), dog (88.15%), and human (87.38%). The signal peptide was of 22 amino acids which was highly conserved in all organisms, except for goat where at the third position valine was replaced with aspartic acid (V–D). Further amino acid changes in position 124–132 were seen, and it was clearly remarkable that the C-terminal end of the sequences is fully conserved among the species.We produced a stably transfected COS-1_BuLIF cell line which expressed high amount of LIF in media. The integration of BuLIF into genome was confirmed by PCR followed by sequencing.

We found that LIF induces dome like structure formation which is indicative of BuLIF action via stat3 pathway (https://doi.org/10.1159/000465507). Further, pure rBuLIF was purified from this cell line which was found to be 58.99 kDa and 48.9 kDa protein with and without glycosylation respectively which was confirmed by western blot and nLC-MS/MS. The time lapse and concentration-dependent assay of purified LIF showed maximum inhibition at 72 hours and half-maximal effective concentration (EC50) to be 0.0555 ng/mL, corresponding to a specific activity of >1.6×107 units/mg and identified IC50 value for migrating cells to be 77.8ng/ml. The biological activity of pure rBuLIF was tested using multiple assays like BrdU, MTT, migration, Caspase 3/7, western and RT-qPCR which indicated that it's growth inhibitory in nature and it doesn't activate apoptosis. To further, elucidate the molecular mechanism behind its growth inhibitory action we used high-resolution LC-MS/MS-based LFQ approach to identify the DEPs (Differentially Expressed Proteins) and deep bioinformatics analysis on Cytoscape platform for determination of non-canonical pathways. The MS/MS data recognized 2083 proteins which consequently, illustrated the LIF-mediated cascade for the activation of MEK/ERK, Ras, mTOR, Hippo, and RAP1 pathways in addition to three well know PIP3, STAT3, and MAPK pathways.

Thus, we conclude that rBuLIF is growth inhibitory in nature in fibroblast cells (COS-1) and this action is mediated via the regulation of multiple signalling pathways additionally to 3 canonical pathways in a highly context-dependent manner. In a further study to investigate the power of BuLIF in the maintenance of pluripotency of bovine stem cells, we used glycosylated BuLIF which was expressed in COS-1 cells. The purified rBuLIF was used in three diferent concentrations on bovine stem cells culture made from inner cell mass obtained from embyos produced through in vitro fertilization (IVF). The final concentration of purified protein was adjusted to 10µg/µl. Bovine embryonic stem cell were cultured in the presence of purified rBuLIF and observed for 6 days. The cells maintained their colonies properly with characteristic morphological features of bovine stem cells. We conclude that the purified rBuLIF fused with GFP from stably transfected COS-1 cells can be used for application in the culture of bovine stem cells. However, more trials with stem cells are needed to confirm the comparative

efficacy of rBuLIF. The comparative study is important in relation to the use of human and murine LIF for the culture of bovine stem cells. There are differences in the sequence of BuLIF in comparison to human and murine LIF. These differences are reflected in the differential pattern of glycolsylation (glycosylation is a chemical reaction where a carbohydrate (a glycosyl donor) is attached to the hydroxyl or other functional group of a glycosyl acceptor. In biochemistry, glycosylation refers to the process wherein a carbohydrate (referred to as glycan) and other organic molecules are combined through the aid of certain enzymes) and phosphorylation(the process of phosphorylating a compound either by reaction with phosphate or by transfer of phosphate from another organic phosphate especially : the enzymatic conversion of carbohydrates into their phosphoric esters in metabolic processes). Since, LIF is a signaling molecule, such postranslational modifications are expected to create difference in the way it triggeres signaling in various tissues. We further, investigated (unpublished data) rBuLIF for potential sumoylation. The degree of sonmoylation determines the biological half-life of the molecule. Sicne, LIF is regarded as pleotropic molecule it is not surprising that different pattern of post translational modifications will exert different functions in variuos tissues like blood cells, mammary epithelial cells and stem cells.

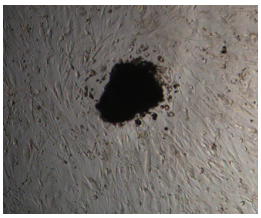

Fig A: Individual Embryonic stem cell colony on feeder layer with human LIF (control)

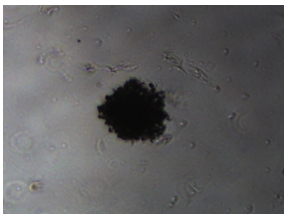

Fig B: Individual Embryonic stem cell colony on feeder layer with recombinant BuLIF at day 3

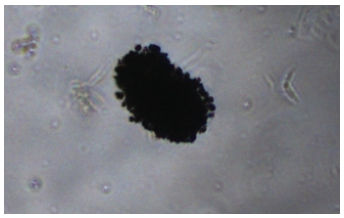

Fig C: Individual Embryonic stem cell colony on feeder layer with recombinant BuLIF at day 6