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METHODS AND PROTOCOLS

A combinatorial approach for robust transgene delivery and targeted expression in mammary gland for generating biotherapeutics in milk, bypassing germline gene integration

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Abstract

Protein expression in the milk of transgenic farmed animals offers a cost-effective system for producing therapeutics. However, transgenesis in farmed animals is not only cumbersome but also involves risk of potential hazard by germline gene integration, due to interruptions caused by the transgene in the native genome. Avoiding germline gene integration, we have delivered buffalo β-casein promoter-driven transgene construct entrapped in virosomes directly in the milk gland through intraductal perfusion delivery. Virosomes were generated from purified Sendai viral membrane, containing hemagglutinin-neuraminidase (HN) and fusion factor (F) proteins on surface (HNF-Virosomes) which initiate membrane fusion, devoid of any viral nucleic acids. Intraductal delivery of HNF-Virosomes predominantly transfected luminal epithelial cells lining the milk duct and buffalo β-casein promoter of the construct ensured mammary luminal epithelial cell specific expression of the transgene. Mammary epithelial cells expressed EGFP at lactation when *egfp* was used as a transgene. Similarly, human interferon-γ (hIFN-γ) was expressed in the mammary gland as well as in the milk when hIFN-γ was used as a transgene. This combinatorial approach of using Sendai viral membrane-derived virosomes for entrapment and delivery of the transgene and using buffalo β-casein promoter for mammary gland specific gene expression provided a better option for generating therapeutic proteins in milk, bypassing germline gene integration avoiding risks associated with animal bioreactor generated through germline gene integration.

Keywords Therapeutic protein · Animal bioreactor · β-Casein promoter · In vivo gene delivery · Mammary gland specific expression

Introduction

With increasing knowledge about various genes and functions of gene products (proteins), several clinical disorders have proven to be associated with deficiencies or malfunctioning of different proteins. About 45% of the human body consists of proteins which are essential for its repair, regulation, and protection. In various clinical conditions, exogenous replenishment of desired protein in the form of therapeutics is the choice of treatment (Morishita and Peppas 2006). It is essential to produce these proteins in bulk for remediation of diseases by appropriate replenishment. With rapid advancements in the field of diagnostics, our ability to know the deficiencies associated with diseases has increased remarkably, thereby increasing the need of using relevant therapeutic proteins for the treatment. There is a genuine need for developing methods for producing biopharmaceuticals in rapid and cost-effective

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manner. Both bacterial and mammalian systems are presently being used to produce such therapeutic proteins in vitro but cost of such therapeutics is exorbitantly high and beyond reach of masses (Fuh 2011). Protein expression in the milk of transgenic farmed animals offers a cost-effective system for producing large amounts of complex proteins with appropriate post-translational modifications (Meade et al. 2006; Freitas et al. 2014; Long 2014). Biopharmaceuticals which is produced in vitro required additional steps for introducing proper post-translational modifications such as glycosylation, γ-carboxylation, hydroxylation, etc., further escalating their cost (Pittius et al. 1988; Houdebine 2000). The large-scale production of biopharmaceuticals in the milk of transgenic animals is much more cost-effective and biologically efficient compared with in vitro cell culture-based systems (van Berkel et al. 2002). Biopharmaceuticals produced in animal milk are known to be modified post-translationally in a manner very similar to their human counterparts and have been demonstrated to be safe (Pittius et al. 1988; van Berkel et al. 2002).

Transgenesis in farmed animals is very tedious, time consuming, and costly procedure requiring maintenance of several females for collection of ova (Wall 2001). Efficiency of generating transgenic farmed animals is also poor (Rudolph 1999). Prolonged gestation time of farmed animals as well as major resources required for raising transgenic progeny until they start lactating (1–2 years of age) adds to the cost escalation (Wall et al. 1997; Rudolph 1999). Also, a global concern is raised about unintentional germline transmission of the integrated transgene by these transgenic animals in the normal herd leading to environmental risk (Muir and Howard 1999; Devlin et al. 2006; Houdebine 2014). Transgene integration at undesirable site in the genome can generate unforeseen hazards like increased susceptibility to certain unwanted disease, transcriptional suppression of some useful proteins, etc.

In this situation, use of mammary gland as a bioreactor without manipulating germinal cells may provide a better alternative. For this purpose, naked DNA delivery, receptor-mediated endocytosis, and viral approaches have been tried to directly transfect mammary epithelial cells in the past, with limited success (Sobolev et al. 1998; Russell et al. 2003; Kerr et al. 2009).

To this end, we have developed an efficient technique for generating therapeutic protein in the milk via direct transfection of transgene in epithelial cells of mammary gland using transgene entrapped virosomes. Virosomes were generated by reorganizing Sendai viral membranes containing hemagglutinin-neuraminidase (HN) and fusion factor (F) on surface (HNF-Virosomes) which initiate membrane fusion (Bagai et al. 1993). Such HNF-Virosomes (HNF-V) have been used in the past for gene delivery, in vitro (Loyter et al. 1983). It has also shown previously that virosome generated from Sendai virus containing only F receptor can be used for gene delivery in vitro or in vivo (Ramani et al. 1998). Use of HNF-V for in vivo gene delivery has never been attempted successfully as HNF-V interact and deliver the entrapped transgene non-specifically, in any cell types. Its robust fusion and delivery ability made it an attractive choice for gene delivery. We have used the construct having buffalo β-casein promoter (BuCSN2) isolated from buffalo genome for guiding transgene expression (Ganguli et al. 2015), which assured mammary luminal epithelial cells (LEC) specific expression of the transgene. This combination of HNF-V and BuCSN2 promoter was further assisted with specialized delivery method (mammary intraductal perfusion delivery) for increasing specific interaction of HNF-V with LEC restricting nonspecific interaction with other cell types of the mammary glands. This combinatorial approach led us to achieve higher level of transgene delivery followed by milk gland specific protein expression. Efficacy of this method in obtaining expression of transgene in LEC was tested in vitro using cell lines as well as in mice, in vivo, where virosomes were delivered directly into mammary glands. Transgene cannot be propagated by such animals reducing the potential risk posed by transgenic farmed animals generated by germline manipulation.

Materials and methods

Animal *Swiss Albino FBV/J mice*: FVB/J mice were bred and maintained at the Small Animal Facility of the National Institute of Immunology.

β-Casein-EGFP transgenic mice: Transgenic mice carrying *pBuCSN2-IRES2-EGFP* as transgene, generated previously (Ganguli et al. 2015) in FVB/J background were bred and maintained at the Small Animal Facility of the National Institute of Immunology.

Plasmid constructs: *pBuCSN2-IRES2-EGFP and pBuCSN2-hIFN-γ-IRES2-EGFP*: Both constructs were designed by us previously (Ganguli et al. 2015) for milk gland specific gene expression using buffalo β-casein promoter isolated and characterized by us (GenBank accession no. KF612339). Briefly, the buffalo β-casein gene promoter was amplified from the buffalo genome and cloned into pIRES2-EGFP vector between *Pst*I and *Xma*I restriction enzymes sites within the MCS to generate milk gland specific expression vector. In the same vector was used to clone the cDNA of hIFN-γ (procured from Open Biosystem, USA) in the *Sma*I site between pBuCSN2 and IRES (Fig. S1). In both constructs, expression of EGFP and hIFN-γ was driven by buffalo β-casein promoter.

Isolation and culture of mammary epithelial cells from mice: Primary mammary epithelial cells were cultured as per method described by Stingl et al. (2006). Dissociation solution was

prepared by 200 U/ml collagenase and 100 U/ml hyaluronidase in 20 ml of MEBM (Lonza, Switzerland) supplemented with 5% FBS. Minced mammary tissue was dissociated in dissociation solution for 2 h at 37 °C in a shaker incubator with 120 oscillation/min. After dissociation, cells were centrifuged at 350×*g* and cell pellet was washed with cold Hanks' Balanced Salt Solution (HBSS) supplemented with 2% FBS. Pellet was then resuspended with a 1:4 mixture of cold HBSS/ammonium chloride and centrifuged at 350×*g* for 5 min. The resultant pellet contained epithelial cell organoids as well as stromal cells and lymphocytes. One milliliter of pre-warmed (0.05%) Trypsin-EDTA was added to the partially dissociated tissue and mixed by pipetting followed by addition of 10 ml of cold HBSS supplemented with 2% FBS (HF) for 5 min and centrifuged at 350×*g* for 5 min. One hundred microliters of 1 mg/ml DNaseI was added and the sample was mixed by pipetting for 1 min with a P1000 micropipettor to further dissociate cell clumps. The cloudy cell suspension was then diluted with an additional 10 ml of cold HF and filtered through a 40-μm cell strainer into a fresh 50 ml centrifuge tube and centrifuged at 350×*g* for 5 min and the supernatant was discarded. The pellet was washed with cold HBSS twice. Mammary epithelial cells were seeded into tissue culture flasks at a density of $2–4 \times 10^3$ cells/cm^2 in complete MEBM medium supplemented with 10% FBS, 10 ng/ml EGF, 2.5 μg/ml ITS, 1 μg/ml hydrocortisone, 5 μg/ml prolactin, and 1% antibiotic antimitotic compounds. Contamination of fibroblast was removed as per method described by Pal and Grover (1983).

Preparation of transgene entrapped HNF-V: Sendai virus (Z strain, initially obtained from A. Loyter, Hebrew University, Jerusalem) was grown in the allantoic sac of 10–11 days old embryonated chicken eggs as per methods described by Bagai et al. (1993). Virosomes were prepared using viral membranes containing HN and F proteins (HNF-Virosomes) while plasmid DNA (transgene) was simultaneously added at the time of preparation for entrapment (Fig. S2). The amount of DNA (transgene) entrapped in the HNF-V was quantified by precipitating the entrapped DNA after dissolving the HNF-V membrane and was found to be within ~ 8 ng to ~ 12 ng/μg of total HNF-V protein. Total virosomal protein was estimated by Lowry's method. Heat treatment of HNF-V was carried out at 55 to 60 °C for 30 min. HNF-V doses (μg/μl) were prepared by diluting the virosomes stock as per required quantity in sterile MEBM for in vitro or in DPBS for in vivo treatment. Each dose was then passed through 26 gauge needle for several times prior use, to separate out aggregates.

In vitro transfection of mammary epithelial cells with HNF-V: For transfection, mammary epithelial cells were grown until they reached at 60–70% of confluence in monolayer culture before treatment with HNF-V. HNF-V suspension was diluted in various concentrations (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg) in 0.22 μm filter sterilized MEBM. Cells were treated with HNF-V in serum-free MEBM medium for 4 h at 37 °C in 5% CO_2 after which cells were rinsed with HBSS and replenished by MEBM supplemented with 10% FBS, 10 ng/ml EGF, 2.5 μg/ml ITS, 1 μg/ml hydrocortisone, 5 μg/ml prolactin, and 1% antibiotic antimitotic compounds.

Intraductal perfusion delivery of HNF-V in mammary gland of mice: For in vivo delivery of HNF-V suspension in the mammary gland, we adopted intraductal perfusion delivery technique (Krause et al. 2013). We delivered four different doses of HNF-V (25, 50, 75, and 100 μg) suspended in 20 μl of sterile Dulbecco's phosphate buffer saline (DPBS), directly in the mammary glands of mice. Heat-inactivated HNF-V (100 μg) with entrapped transgene and only transgene suspended in sterile DPBS, in excess (300 ng), were used as controls. Mammary duct injection was given in five different reproductive age groups (group A: 40–45 days old, group B: 60–65 days old, group C: 3–4 days of pregnancy, group D: 17–19 days of pregnancy, and group E: 2–4 days of lactation) in female FVB/J mice (Table S1). Among these five groups, animals of group A belonged to late stage of puberty, group B belonged to virgin adult, group C belonged to early stage of pregnancy, group D belonged to advanced stage of pregnancy, and group E belonged to early stage of lactation. Female mice were put for mating and were checked daily for vaginal plug formation. Number of days of pregnancy was calculated considering the day of vaginal plug formation as day 0. Days of lactation was calculated, considering the day of parturition as day 0. Intraperitonial injection (100 μl) of ketamine hydrochloride (45 mg/kg) and xylazine hydrochloride (8 mg/kg) was delivered to anesthetize the animal. Mice were stretched on pieces of autoclaved blotting sheet. The suspension was delivered either in abdominal mammary gland and/or in inguinal mammary gland. Abdominal hair of the mice was removed occasionally by use of hair remover or was trimmed using a trimmer. After hair removal, abdominal part was wiped with providone iodine solution twice followed by wiping with 70% alcohol once. Suspension was loaded in to a 10-μl Hamilton Gastight Syringe and was ready for delivery. Mice were then transferred on a stage of Stereozoom Microscope under appropriate objective. Teat of the mammary gland was held using sterile blunt tip forceps while needle of Hamilton Gastight Syringe was inserted into the teat canal through the teat (Fig. S3). Once inserted, the suspension was released slowly and the needle was taken out (Fig. S4). Once completed, the whole abdominal region was again wiped with providone iodine solution and mice were left under heating lamp until consciousness was regained (Fig. S5). All HNF-V delivered mice were housed until they reached lactating phase after successful mating with male FVB/J mice (Fig. S6). Mice of group E (mice delivered with HNF-V at 2–

4 days of lactation) were housed until they reached 5th day of lactation (Fig. S6). Animals from all groups were analyzed for transgene (EGFP) expression on different days, as described in Fig. S7.

Observation of direct in vivo EGFP fluorescence in mammary glands: The alveolar mass of lactating mammary glands was observed under SMZ-1500 stereo zoom microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) with UV filters to detect native in vivo EGFP expression. Mammary glands administered with HNF-V along with wild type and non-injected control mammary glands were observed on various lactating days.

Preparation of tissue sections: Mammary tissues were dissected out and washed briefly in PBS followed by fixation in 4% paraformaldehyde for 18–20 h. Fixed tissues were then put either into gradient of sucrose (10, 20, and 30%) for embedding in optimal cutting temperature compound (OCT) medium (Leica Jung, Germany) or alcohol (50, 70, and 100%) for embedding in paraffin. Tissue sections (5 to 7 μm) were generated using Shandon Cryotome E (Thermo Electron Corporation) for OCT-embedded tissue block and Reicher Jung, 2040; Microtome (Leica Jung, Germany) for paraffin-embedded tissue block.

Hematoxylin and eosin staining: Hydrated tissue sections were stained with Harris's hematoxylin for 10 min at room temperature. Excess stain was removed with 1% acid ethanol (1% HCl in 95% ethanol) and washed briefly under running tap water. For staining with eosin, the sections were processed in ascending grades of ethanol (50, 70, and 100%), incubating the sections at each solvent for 10 min, and then stained with 1% eosin (1% ethanolic eosin) for 5 min. Excess stain was removed with ethanol and the slides were treated with xylene for 5 min. After mounting in DPX mountant, the sections were observed under bright field illumination with Nikon Eclipse TE2000-S inverted microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) attached to a DS-5 M camera assisted by Digital Sight DS-L1 software for capturing the images.

Detection of EGFP expression and cellular marker in mammary gland or cultured cells

Immunohistochemistry was performed on paraffin-embedded section as per methods described previously by Robertson et al. (2008) or on cryo sections as per protocol described by Ganguli et al. (2015) and immunocytochemistry was performed on cultured cells as per protocol described by Ganguli et al. (2015) using antibodies described below. For detection of LEC, rabbit polyclonal Cyto-Keratin18 antibody was procured from Santa Cruz Biotechnology CA, USA (1:100, dilution). Rabbit FC region specific IgG raised in goat and tagged with alexaflour546 (Molecular Probes, Invitrogen, USA) were used as secondary antibody (1:500, dilution). For detection of EGFP protein, mouse monocleonal anti-GFP antibody was procured from Clontech, Mountainview, USA, and was used as primary antibody (1:250, dilution). Goat-raised anti-mouse IgG conjugated to Alexa fluor 488 (Molecular Probes, Invitrogen, USA) used as secondary antibody (1:500, dilution). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). Sections were analyzed under bright field and ultraviolet illumination (with FITC, TRITC and BLUE/CYAN filter) using a Nikon Eclipse TE2000-S inverted microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) attached to a DS-5 M camera assisted by Digital Sight DS-L1 software for capturing the images.

RNA isolation and RT-PCR analysis: RNA was extracted from mammary tissue using TRIzol (TRI) reagent (Sigma Aldrich, USA) following manufacturer's guidelines. Reverse transcription (RT) reaction was carried out using a two-step Reverse transcription kit (Promega, USA) using guidelines provided by the manufacturer. Briefly, for cDNA synthesis, 1 μg RNA in 5 μl RNase-free water was denatured at 70 °C for 10 min and then immediately transferred to ice and incubated for 5 min. To the same tube, 5 mM $MgCl_2$, 1× RT buffer, 1 mM each dNTPs, 1 U/μl RNasin, 0.5 μg oligo dT per mg RNA, and 15 U/μg AMV Reverse Transcriptase were added. cDNA was synthesized at 42 °C for 1 h and the reaction was denatured at 94 °C for 5 min on a Cycler with heated lid (Biorad, USA). PCR was carried out from cDNA using TaqDNA Polymerase (Genei, India) using forward and reverse primers specific for hIFN-γ (F:TGTCCAACGCAAAGCAATACA; R: CTGGGATGCTCTTCGACCTC) and Cyclophilin A, as expression control (F:GTCTCTTTTCGCCGCTTGCT R:TCTGCTGTCTTTGGAACTTTGTCTG (Bar et al. 2003).

Western blot analysis: Total protein was isolated from mammary gland along with various other organs using ripa lysis buffer. Western blot was performed using standard protocol, described by Ganguli et al. (2015) utilizing antibodies mentioned below. Mice raised monoclonal anti EGFP antibody was procured from Santa Cruz Biotechnology, CA, USA, to detect EGFP in western blot. Monoclonal antibody against human IFN-γ raised in mice was procured from Cell Signaling Technology, USA. Primary antibody for mice β-actin raised in rabbit was procured from Cell Signaling Technology, USA. HRP conjugated anti-mouse IgG and HRP conjugated anti-rabbit IgG both were procured from Thermo Scientific, USA.

FACS analysis: Mice mammary epithelial cells were isolated and single cells suspension was generated as described before through enzymatic digestion. Cells were then passed through

sterile 40 μm filter to get rid of unwanted cells along with clumps of epithelial cells. All the processing steps were performed on ice maintaining aseptic condition. Neutral pH of every solution was maintained to prevent quenching of EGFP fluorescence. Cell suspension was collected in a closed cap FACS tube and was run in BD-FACS Aria III. Initially, live cell population was gated out from the dead cell population followed by gating of EGFP positive cells out of the total live cell population.

Slot blot analysis: Slot blot analysis was performed as per method described by Ganguli et al. (2016). The template DNA for Probe preparation was generated by digesting the pBuCSN2-IRES2-EGFP vector by *Bam*HI and *Not*I resulting a fragment which spans the IRES-EGFP region (Fig. S1). The probe was generated by radioactive labeling through random priming using High Prime DNA Labeling kit from Roche, Basel, Switzerland, following the manufacturer's instructions. Briefly, in a 20-μl reaction, 80 ng of denatured DNA was labeled using 4 μl of the Hi-Prime mix and 5 μl (50 Ci) of 32P dCTP at 37 °C for 35 min and immediately chilled on ice. The labeled probe was diluted with MilliQ water to 100 μl. One hundred microliters of 10 mg/ml sheared and denatured salmon sperm DNA was added to the diluted labeled probe and was precipitated using sodium acetate and ethanol. The probe was finally dissolved in 100 μl of sterile MilliQ water and radioactive counts were taken in a counter (Beckman LS 5801, Beckman Inst., Irvine, CA, USA). Typical specific activity obtained was in the range of 1–2 × 10^9 cpm/μg with percentage incorporation of 30–55%. Yield of gDNA was quantitated spectrophotometrically, and slot blot analysis was carried out using 0.5 to 2 μg of gDNA with appropriate positive and negative control. Loading volume for each sample was 15 μl along with dye. DNA was denatured at 95 °C for 10 min prior blotting on to Hybond N^+ (Amersham Pharmacia Biotech, England) membrane in a slot blot apparatus (Cleaver Scientific Co., England) under vacuum. The membrane was UV cross-linked (Church and Gilbert 1984) at 12×10^4 μJ/cm^2 energy in a CL-1000 Ultraviolet crosslinker (UVP, Upland, CA, USA). The cross-linked membrane was subjected to pre-hybridization at 42 °C for 4 h in a hybridization bottle with 7 ml hybrisol (Millipore, USA) pre-warmed at 45 °C in a hybridization oven (Amersham, UK). The labeled probe was denatured at 95 °C for 10 min, chilled on ice-bath, and added to the hybridization reaction. Fifty microliters of the labeled probe was typically added per blot and hybridization was carried out overnight at 60 °C. The blots were washed twice for 5 min each, at room temperature, in wash solution I (2× saline sodium citrate buffer and 0.1% SDS), followed by another washing in wash solution II (0.1× saline sodium citrate buffer and 0.1% SDS, pre-warmed at 65 °C) for 10 min at 65 °C. The hybridization signals were detected by autoradiography.

Isolation of milk from mice mammary gland: Mammary glands were dissected out from the HNF-V-treated mice along with untreated control mice. Prior to dissection, mice were separated from their pups for about 4–6 h. Dissected glands were then put on the side wall of a micro centrifuge tube and incubated on ice for 4 h. Milk, diffused out and collected at the bottom, were pipetted out and used for further analysis.

ELISA for detection of hIFN-γ expression: Total protein was isolated from the mammary tissue by lysing with ripa lysis buffer. To isolate total milk proteins, milk samples were centrifuged at 5000 rpm for 10 min to separate the fat, which forms a distinct separate layer on top after centrifugation. Milk serum containing total protein was collected from the bottom and used for analysis. Enzyme-linked immunosorbent assay (ELISA) was performed as per instruction of BD optEIA human IFN-γ ELISA Set (BD Biosciences Pharmingen, USA). Total protein was estimated and loaded into wells of microtitre plate with initial concentration of 640 μg/ml diluting up to 5 μg/ml. Absorbance was read at 450 nm with λ correction at 570 nm in Bio Tek Power Wave XS, ELISA reader (Bio Tek Instruments, Inc., USA). Graph was generated by *t* test analysis using Graph Pad Prism Software.

Results

Assessing transgene delivery efficiency in vitro Primary mammary epithelial cells of mice were cultured in vitro which contained both LEC as well as myoepithelial cells (MEC) population. Cells were transfected with buffalo β-casein promoter (BuCSN2)-driven *egfp* (*pBuCSN2-IRES2-EGFP*) entrapped HNF-V. Among various doses, transfection with 40 μg of HNF-V resulted in strong EGFP expression in LEC but not in MEC as confirmed by co-localization of EGFP and Cytokeratin 18, a marker of LEC (Fig. S8). No EGFP expression was observed in the cells, treated with heat-inactivated HNF-V or only transgene cassette dissolved in PBS (data not shown).

Assessing transgene delivery efficiency in vivo: For assessing the efficacy of delivery system in vivo, *pBuCSN2-IRES2-EGFP* entrapped HNF-V was delivered in mammary gland of mice of different reproductive ages followed by housing them, until they reach lactating phase (Fig. S6) and were analyzed (Fig. S7) for assessing the transgene (EGFP) expression.

Initially, EGFP expression was assessed on day 1 of lactation. To assess this, three mice each from all the age groups for four different doses in two inguinal and two abdominal mammary glands were delivered with HNF-V as well as one mice each from all age group were used for controls. Mammary glands of animals from group "D" which received 75 or 100 μg of HNV-V dose showed prominent EGFP expression

on day 1 of lactation. No EGFP expression was observed in the heat-inactivated HNF-V delivered mammary gland (Fig. 1a). Twenty-five micrograms or 50 μg of HNF-V delivered mammary gland also failed to express EGFP efficiently (Fig. S9). Immunohistochemistry of tissue sections of such mammary glands also resembled this observation (Fig. 1b and Fig. S10). Western blot analysis of total protein isolated from these mammary glands also showed lack of expression of EGFP in 25 μg of HNF-V delivered glands along with mammary glands delivered with heat-inactivated HNF-V or with transgene alone. Mammary glands delivered with 50 and 75 μg of HNF-V showed lower level of EGFP expression as compared to mammary glands of age-matched transgenic female mice. However, with increased dose HNF-V (100 μg), level of EGFP expression in mammary gland was similar to that found in mammary glands of age-matched transgenic animals (Fig. 1). Mammary glands of animals from other age groups ("A," "B," "C," and "E") failed to express the transgene efficiently (data not shown).

EGFP expression was assessed further on day 7 of lactation, in mammary glands of group D animals. Three different mice were delivered with HNF-V for this purpose. Immunohistochemistry of mammary tissue section revealed an increase in level of EGFP expression on day 7 of lactation as compared to day 1, in 75 μg of HNF-V delivered gland (Fig. 2a). We also observed a decrease in EGFP expression in 100 μg of HNF-V delivered gland. The mammary alveolar architecture was also found to be disturbed in 100 μg of HNF-V delivered gland (Fig. 2a). Hematoxylin and eosin staining of mammary gland showed normal alveolar architecture in 75 μg of HNF-V delivered gland at day 7 of lactation (Fig. 2b). One hundred micrograms and 150 μg of HNF-V delivered gland revealed disturbed tissue architecture due to loss of epithelial cells in mammary alveoli (Fig. 2b). We also observed that higher dose of HNF-V (150 μg) caused severe degeneration of mammary fat pad as compared to 75 μg of HNF-V delivered gland (Fig. S11). In western blot analysis, we observed comparable level of expression of EGFP in 75 μg of HNF-V delivered glands with respect to mammary glands of age-matched control (Fig. 2c). One hundred micrograms of HNF-V delivered gland showed a little decrease in EGFP expression (Fig. 2c). Direct in vivo observation of EGFP fluorescence revealed robust EGFP expression all over the mammary gland delivered with 75 μg of HNF-V as compared to that of non-injected control mammary gland of same animal (Fig. 2d and Fig. S12).

Assessing the tissue and cell type specificity of the delivered transgene: Western blot analysis of total protein, isolated from 75 μg HNF-V delivered mammary gland along with tissues of various other organs (heart, liver, kidney, spleen, brain, abdominal skin) of same mice, confirmed no leaky expression of delivered transgene in any other organs (Fig. 3a). Absence of EGFP expression in the inner side of the abdominal skin adjoining mammary gland in mice confirmed that expression of transgene followed by direct delivery of HNF-V is specific to mammary gland only (Fig. 3a and Fig. 2d). Immunohistochemistry of mammary gland further showed that expression of delivered transgene was confined in the LEC only with no leaky expression in MEC (Fig. 3b). We sorted EGFP positive cell population using FACS from such mammary gland. Immunocytochemistry analysis of these cells showed expression of Cytokeratin 18, in all sorted EGFP positive cells, confirming their identity as LEC (Fig. 3c).

Assessing sustained expression of delivered transgene: To check the ability for sustained expression of the delivered transgene, mice which was delivered with 75 μg HNF-V at advanced stage of pregnancy was housed until 4th consecutive term of lactation. Four different mice were delivered with the HNF-V and four different litters from the same female mice were produced and at the 4th lactation term the original female was found to express the exogenous protein EGFP. In comparative analysis of protein isolated from mammary glands, we observed declined EGFP expression at day 7 of 4th term of lactation as compared to that of day 7 of 1st term of lactation. EGFP expression at day 18 of lactation in both the lactating term also followed the same pattern (Fig. 3d). Immunohistochemical analysis of such mammary glands showed presence of prominent EGFP expressing LEC scattered in different mammary alveoli (Fig. 3e). LEC lacking EGFP expression was also found in several alveoli, residing adjacent to the EGFP expressing LEC (Fig. 3e). We performed slot blot analysis using the gDNA isolated from the mammary gland of such mice. In slot blot, sustained presence of integrated transgene in the genome was detected until 4th term of lactation (Fig. S13).

Establishing expression of human therapeutic protein in mammary glands: Use of this approach for expressing valuable human therapeutic protein in milk was tested using *pBuCSN2-hIFN-γ-IRES2-EGFP* construct where hIFN-γ was expressed under control of pBuCSN2. Three different mice were utilized for this purpose. In RT-PCR analysis, high level of hIFN-γ mRNA expression was detected in mammary glands which received 75 μg of HNF-V as opposed to wild type and non-injected mammary glands (Fig. 4a). This was further confirmed by western blot analysis which showed expression of hIFN-γ in 75 μg HNF-V delivered mammary glands (Fig. 4b). We performed ELISA from mammary tissue lysate and extracted milk (Fig. 4c) of 75 μg HNF-V delivered mammary gland. Approximately, 22 ng of hIFN-γ/mg of total protein was detected by ELISA in the tissue lysate of mammary glands on day 7 of lactation (Fig. 4d). Secretion of hIFN-γ was also detected in the milk (~6.6 ng of hIFN-γ/mg of total milk protein) of mice (Fig. 4d).

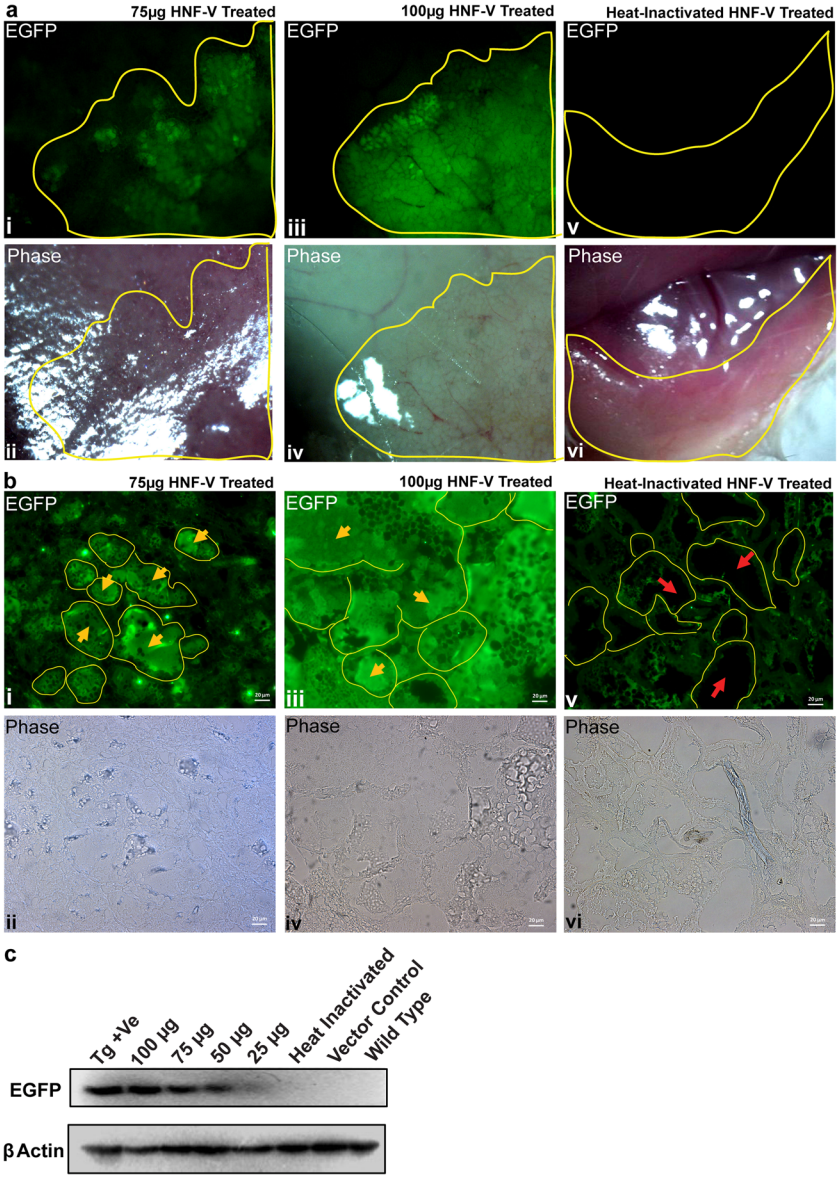

Fig. 1 Analysis of transgene expression on day 1 of lactation in mice, delivered with pBuCSN2-IRES2-EGFP entrapped HNF-Virosomes at advanced stage of pregnancy. **a** Observation of in vivo EGFP fluorescence in inner alveolar mass of mammary gland under stereozoom microscope. Images i and iii show expression of EGFP in mammary alveoli of mice, delivered with 75 and 100 μg of HNF-V, respectively. EGFP expression level was found to be higher in 100 μg of HNF-V delivered gland. Image v shows lack of EGFP expression in mammary alveoli delivered with 100 μg of heat-inactivated HNF-V. Images ii, iv, and vi show respective bright field images. ii, iv, and vi—the yellow line indicate the alveolar tissue of breast gland. **b** Immunohistochemical analysis of cross section of the mammary glands for detection of EGFP expression. Images i and iii show expression of EGFP in the epithelial cells and milk space of mammary gland, delivered with 75 and 100 μg of HNF-V, respectively. EGFP expression level was found to be higher in 100 μg of HNF-V delivered gland. Image v shows lack of EGFP expression in mammary gland of mice delivered with 100 μg of heat-inactivated HNF-V. Images ii, iv, and vi show respective phase contrast images. Yellow and red arrows indicated the presence and absence of EGFP expression in milk space of mammary alveoli, respectively. Yellow line marks the periphery of mammary alveoli. Scale bar = 20 μm. **c** Western blot analysis of total protein for detecting EGFP expression in mammary glands, delivered with various doses of HNF-V. There is a gradual decrease in the level of EGFP expression from 100 μg of HNF-V-treated mammary gland to 50 μg of HNF-V-treated gland. No EGFP expression is observed in the 25 μg of HNF-V-treated gland. EGFP expression level is relatively equal in 100 μg of HNF-V-treated mammary gland as compared to that of Tg + Ve mammary gland. There is no EGFP expression in heat-inactivated and vector control delivered mammary gland. Tg + Ve denotes mammary gland of transgenic female mice carrying functional cassette of *pBuCSN2-IRES2-EGFP* construct as transgene; 100 μg, 75 μg, 50 μg, and 25 μg denote mammary gland administered with 100, 75, 50, and 25 μg of HNF-V, respectively. Heat Inactivated denotes mammary gland in which 100 μg heat-inactivated HNF-V was delivered. Vector Control denotes mammary gland in which only transgene (*pBuCSN2-IRES2-EGFP*) suspended in DPBS was delivered. βActin expression is measured as the loading control. Pictures have been cropped to size for suitable presentation

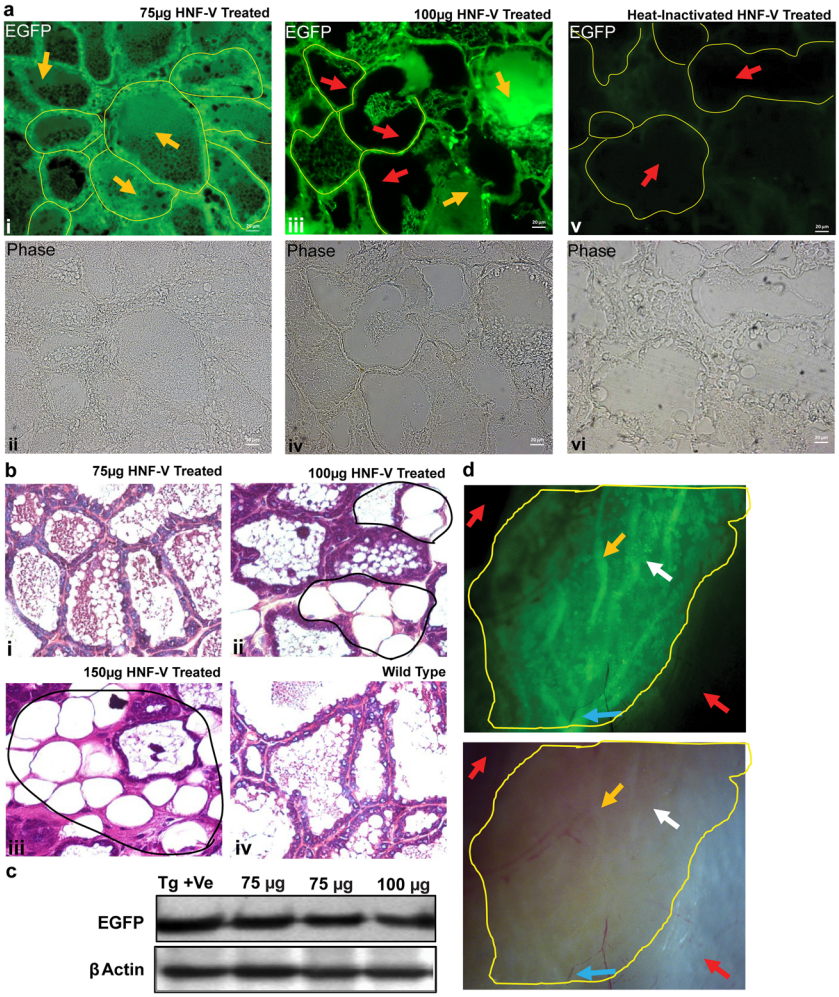

Fig. 2 Analysis of transgene expression on day 7 of lactation in mice, delivered with pBuCSN2-IRES2-EGFP entrapped HNF-Virosomes at advanced stage of pregnancy. **a** Immunohistochemical analysis of cross section of the mammary glands for detection of EGFP expression. Images i and iii show the expression of EGFP in the epithelial cells and milk space of the mammary gland delivered with 75 and 100 μg of HNF-V, respectively. One hundred micrograms of HNF-V treated gland also shows absence of EGFP expression in several alveoli. Image v shows the lack of EGFP expression in 100 μg of heat-inactivated HNF-V delivered mammary gland. Images ii, iv, and vi show the respective phase contrast images. Yellow line marks the periphery of mammary alveoli. Yellow arrows mark the presence of EGFP expression in the milk space. Red arrows mark the absence of EGFP expression in the milk space. Scale bar = 20 μm. **b** Hematoxylin and eosin staining of mammary gland section for observation of mammary tissue architecture. Images i, ii, and iii show mammary gland delivered with 75, 100, and 150 μg of HNF-V, respectively. One hundred micrograms and 150 μg HNF-V delivered gland show disturbed mammary alveoli due to increased epithelial cell death. Image iv shows the tissue architecture of wild type gland. Scale bar = 20 μm. **c** Western blot analysis of total protein to compare level of EGFP expression in 75 and 100 μg of HNF-V delivered mammary glands along with mammary gland of transgene positive female mice. EGFP expression is found to be declined in 100 μg of HNF-V delivered gland. Tg + Ve denotes mammary gland of transgenic female mice carrying functional cassette of *pBuCSN2-IRES2-EGFP* construct as transgene; 75 μg denotes mammary gland administered with 75 μg HNF-V; 100 μg denotes mammary gland administered with 100 μg HNF-V. βActin expression was measured as the loading control. Pictures have been cropped to size for suitable representation. **d** Image showing in vivo EGFP fluorescence in inner alveolar mass of mammary gland delivered with 75 μg of HNF-V under stereozoom microscope. Image i shows the expression of EGFP in the mammary alveoli. Image ii shows the respective bright field image. The Yellow line indicates the inner alveolar mass. Blue arrow marks the teat canal, yellow arrow marks the milk duct, and white arrow marks the branched alveoli. Red arrows mark the inner side of the abdominal skin

Discussion

We tested the feasibility of using virosomes derived from membrane of Sendai virus to breach the cell membrane of the epithelial cells of mice mammary gland, in vivo for delivering transgene entrapped within. HNF-V comprised both the functional receptor of Sendai virus, HN, and F, projecting from outer surface of virosome, which was devoid of any viral nucleic acid. HNF-V-mediated delivery of transgene is non-specific as HN interacts with terminal sialic acid residue present abundantly on surface of several types of cells exerting membrane fusion. For this drawback, HNF-V have never been used successfully as an in vivo gene delivery vehicle before. To overcome this caveat of non-specificity and for ensuring

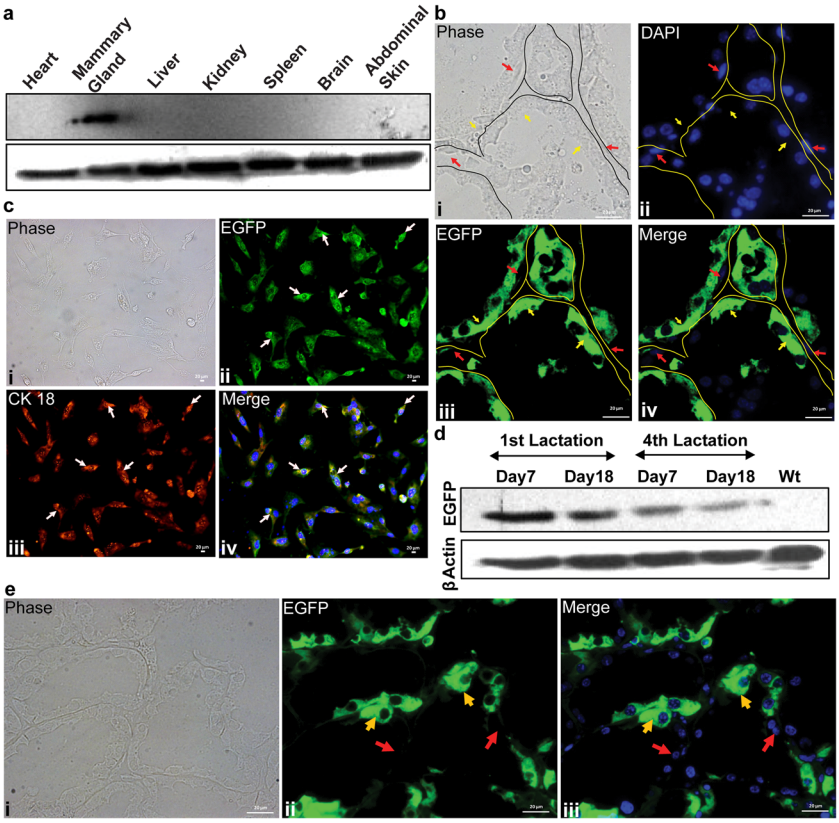

Fig. 3 Analysis of mammary gland specific and sustained expression of transgene in mice delivered with 75 μg of HNF-V. **a** Western blot analysis of total protein isolated from HNF-V treated mammary gland along with various other organs on day 7 of lactation for detection of EGFP expression. EGFP expression is detected in the mammary gland only. βActin expression is measured as the loading control. Pictures have been cropped to size for suitable representation. **b** Immunohistochemical analysis of mammary gland for detection of EGFP expression in luminal epithelial cell. Image i shows the phase contrast view of cross section of mammary gland. Image ii shows the nucleus stained with 4′, 6-diamidino-2-phenylindole (DAPI). Image iii shows presence of EGFP expression in luminal epithelial cells and absence of EGFP expression in myoepithelial cells**.** Image iv shows the merged view. Presence of EGFP expression specifically in luminal epithelial cells is marked by yellow arrow. Absence of EGFP expression in myoepithelial cells is marked with red arrow. Black and yellow lines mark the periphery of the mammary alveoli. Scale bar = 20 μm. **c** Immunocytochemistry analysis of FACS sorted EGFP positive cells isolated from mammary gland. Image i shows the phase contrast view of cells. Image ii shows presence of EGFP expression. Image iii shows presence of CK18 expression**.** Image iv shows the merged view. Cells show co-expression of EGFP as well as Ck18 (white arrow**)**. Scale bar = 20 μm. **d** Western blot analysis of total protein from mammary glands on day 7 and day 18 of 1st and 4th term of lactation for detection of sustained EGFP expression. EGFP expression is detected in 4th consecutive term of lactation after delivery of HNF-V. On day 18th, EGFP expression is found to be declined in both the lactating term. Wt denotes mammary gland of wild type mice. βActin expression is measured as the loading control. Pictures have been cropped to size for suitable representation. **e** Immunohistochemical analysis of mammary gland for detection of sustained EGFP expression on day 7 of 4th term of lactation. Image i shows phase contrast view of cross section of mammary gland. Image ii shows EGFP expression in luminal epithelial cells of mammary gland. Image iii shows EGFP expression in luminal epithelial cells along with nucleus stained by 4′, 6-diamidino-2-phenylindole (DAPI). EGFP expression in luminal epithelial cells, scattered in mammary alveoli is observed (marked by yellow arrow). Red arrow marks the luminal epithelial cells which lack EGFP expression. Scale bar = 20 μm

LEC specific expression of delivered transgene, we used construct in which expression of the gene was driven by a promoter of buffalo β-casein gene (BuCSN2) which ensured LEC specific expression (Ganguli et al. 2015). Different promoters of milk protein gene, like goat β-casein, mouse and rabbit whey acidic protein, *ovine* β-lactoglobulin, and goat α-lactalbumin, have been isolated previously and genes coding for proteins of therapeutic interest were cloned downstream of these promoters to guide their expressed products in the milk of various species showing ability of these promoters to work across the species (Rudolph 1999; Eric et al. 2006). Caseins comprise 80% of the total milk protein and are divided into four groups, among which β-casein is the most abundantly expressed protein found in buffalo milk (Feligini et al. 2009). Therefore the buffalo β-casein promoter is predicted to be strong one and used in the study instead of using mice own promoter for β-casein gene, to drive high level of expression of exogenous protein in the milk. Moreover, for practical purpose, large animals will have to be used for generating therapeutic proteins; hence, the present study also tested ability of buffalo β-casein promoter to drive human gene in milk, by this technique.

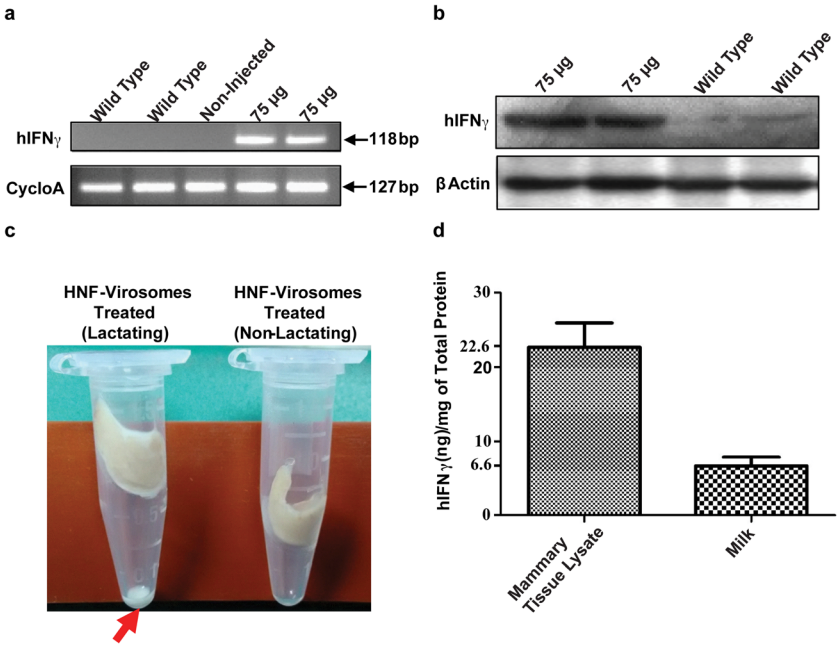

Fig. 4 Expression of therapeutic protein (human IFN-γ) in mammary gland and milk of mice delivered with pBuCSN2-hIFN-γ-IRES2-EGFP entrapped HNF-Virosomes (75 μg). **a** Detection of hIFN-γ transcript in mRNA isolated from mammary glands by RT-PCR on day 7 of lactation. hIFN-γ transcript is detected in 75 μg of HNF-V delivered gland as compared to wild type and non-injected gland. Cyclophilin A expression is estimated as the house keeping gene control; 75 μg denotes mammary gland administered with 75 μg HNF-V. Non-Injected denotes mammary gland in which no HNF-V was delivered. Wild type denotes mammary gland of wild-type mice. Pictures have been cropped to size for suitable representation. **b** Western blot analysis of total protein extracted from mammary glands on day 7 of lactation for detection of hIFN-γ expression. hIFN-γ expression is detected in the mammary gland delivered with 75 μg HNF-V as compared to wild-type mammary gland. βActin expression is measured as the loading control; 75 μg denotes mammary gland administered with 75 μg HNF-V. Wild type denotes mammary gland of wild type mice. Pictures have been cropped to size for suitable representation. **c** Image showing isolation of milk from HNF-V delivered lactating mice mammary gland as compared to non-lactating control mammary gland. Red arrow indicates the isolated milk at the bottom of tube. **d** Graph showing level of hIFN-γ expression (in ng) as detected by ELISA from mammary tissue lysate and milk, respectively. Bioactive hIFN-γ protein was detected in both the samples

BuCSN2 was isolated and characterized by us showing its LEC specific expression (Ganguli et al. 2015). To test the hypothesis, in vitro transfection of primary mammary epithelial cells of mice was performed. Strong EGFP expression observed specifically in LEC, but not in MEC, as a result of transgene delivery specifically through fusion between virosome and epithelial cells. These observations were the basis for attempting to use this system for in vivo transgene delivery into mammary glands.

In experiments of in vivo gene delivery, we desired to (1) target maximum number of LEC for transgene delivery, (2) deliver transgene in LEC when they are in active phase of division, facilitating permanent integration of transgene, (3) avoid delivery of transgene in MEC, and (4) ensure delivery of HNF-V in mammary glands in a safe way without disturbing its internal cellular architecture.

First round of major division and differentiation of mammary epithelial cells within mammary gland start at puberty and is completed by the time a female reaches adulthood. During this phase, ductal branching and formation of alveoli takes place. Architecturally, the LEC form the lumen of a mammary alveoli and MEC give structural support by forming a fence like structure, lying behind LEC layers. Basement membrane lies behind the layer of MEC. The next phase of mammary epithelial cell division occurs during early stage of pregnancy until advanced stage of pregnancy. In this stage, inflation of the alveoli takes place due to massive proliferation of LEC, which eventually fill up the complete mammary fat pad followed by regression at the time of involution. LEC lining milk duct secrete milk and express milk proteins like β-casein during lactation and undergo repetitive cycle of regeneration and regression in each pregnancy-lactation cycle (Richert et al. 2000; Sternlicht 2006; Macias and Hinck 2012).

To serve our purpose of delivering transgene to maximum number of LEC followed by permanent integration through HNF-V-mediated delivery, we chose five different phases of mammary gland development in which LEC are actively dividing, as dividing cells are more susceptible for such event. To ensure safe delivery of HNF-V in the mammary glands, facilitating maximum fusion of HNF-V with LEC, we chose intraductal perfusion delivery of HNF-V in the mammary glands. This is a well-practiced process for perfusion of antibiotic in mammary glands of farm animals affected with microbial infection (Stockler et al. 2009). Through this delivery

system, the transgene loaded HNF-V was released in the milk duct which eventually accessed LEC facilitating maximum fusion of HNF-V with LEC (Fig. 5a–c). In spite of such strategy, if the virosome gets fused with MEC, recombinant protein will not be expressed as we used LEC specific buffalo β-casein promoter to drive the transgene expression.

For assessing the efficacy of such delivery system in vivo, mice from various age groups were housed as described in the Fig. S6 and sacrificed on various days (Fig. S7) of lactation for evaluating the extent of transgene expression.

Initially, expression of delivered transgene (EGFP) was analyzed on day 1 of lactation for mice of groups A, B, C, and D, and on day 3 of lactation for group E. The data obtained is compiled in Table S2. Mammary glands of mice of group D in which HNF-V were delivered during advanced stage of pregnancy showed EGFP expression in graded fashion, depending on the amount of HNF-V administered. Mammary glands which were administered with 75 or 100 μg HNF-V showed relatively high EGFP expression in comparison to other doses. We did not investigate the reason behind absence of EGFP expression in animals of groups A and B as well as inefficient expression of EGFP in groups C and E animals. We feel that very low rate of proliferation of the LEC in groups A and B animals did not allow to incorporate the gene permanently and hence did not show EGFP expression. Whereas, animals of group C showed expression of EGFP because LEC proliferation gets initiated in this age group. However, this was best shown in animals from group D (Table S2). The hindrance created by secreting milk in post parturition gland might be responsible for limited transfection of LEC resulting into very low level of expression of EGFP in group E animals.

Observing efficient EGFP expression in mammary glands of group D animals at day 1 of lactation, we further analyzed the EGFP expression in this age group only. Observation at day 7 of lactation revealed an increase in EGFP expression in 75 μg HNF-V delivered gland, while 100 μg HNF-V delivered gland showed decrease in EGFP expression as well as disturbed mammary alveolar architecture. Such observation was further confirmed when we analyzed hematoxylin-eosin-stained section of mammary glands, delivered with three different HNF-V doses. Mammary gland which received highest dose (150 μg) showed severe degeneration of mammary fat pad. This observation indicated that higher dose of HNF-V might have induced cell death followed by degeneration of mammary alveoli as well as decrease in EGFP expression (Table S3). At day 7 of lactation, level of EGFP expression in 75 μg HNF-V delivered mammary gland was found to be similar to that of mammary gland of age-matched transgenic female mice, and distributed all over the mammary ductal system. But all the mice/mammary glands which received a specific dose of HNF-V did not express the protein at same level. There was variability of expression in different glands receiving same dose. We have not measured the expression of EGFP quantitatively but did comparative analysis through Western blot with regard to positive control (mammary gland of transgene positive female mice generated through germline integration). No apparent issues related to immune response were observed in any of the treated animals as all mouse remained healthy and we followed them for long duration to observe presence of exogenous proteins in subsequent lactational periods (sometimes up to 4th lactation). With these observations, we concluded that 75 μg of HNF-V dose was most effective in terms of expression of delivered transgene in mammary gland of female FVB/J mice without disturbing the normal tissue architecture of mice mammary gland.

Being satisfied by the level of expression of the delivered transgene in mammary gland without disrupting the tissue architecture, we further determined the tissue and cell type specificity of delivered transgene. We found that the expression of transgene was specific to mammary gland. In the mammary gland, expression of transgene was further confined in the LEC only. This was presumably due to effect of method of delivery and regulation of transgene expression by β-casein promoter.

We further analyzed the sustained expression of delivered transgene in consecutive lactating term. We found that the expression of the transgene in 75 μg HNF-V delivered glands sustained until 4th term of lactation, although the level of expression declined. The decline in expression indicated that the expression of transgene was narrowed down to lineage of those LEC in which the transgene was integrated after delivery (Fig. 5d, e), at advance stage of pregnancy, before 1st lactation. Integration of delivered transgene in the somatic genome of the LEC was facilitated by the event of massive cell division at advanced stage of pregnancy.

Finally, we tested this combinatorial approach for expression of human therapeutic protein in milk. We detected the expression of human therapeutic protein interferon-γ (hIFN-γ) in the mammary gland of mice. The secretion of bioactive hIFN-γ was also confirmed in the mammary tissue lysate as well as in secreted milk.

This combinatorial approach of using HNF-V along with mammary gland LEC specific buffalo β-casein promoter, for driving LEC specific expression of transgene, led us to achieve sustained expression of delivered transgene only in the mammary glands. HNF-V have never been used previously for in vivo gene delivery in spite of its robustness because it interacts non-specifically with any cell types leading to random delivery of transgene. Utilization of LEC specific expressional ability of buffalo β-casein promoter-driven expression vector helped us to achieve high level of LEC specific expression of transgene in mammary gland through membrane fusion-mediated virosomal transfection. This helped us to exploit the transfection efficiency of HNF-V while restricting the undesired leaky expression of transgene in any place other than mammary LEC lining the milk duct. Adhering to

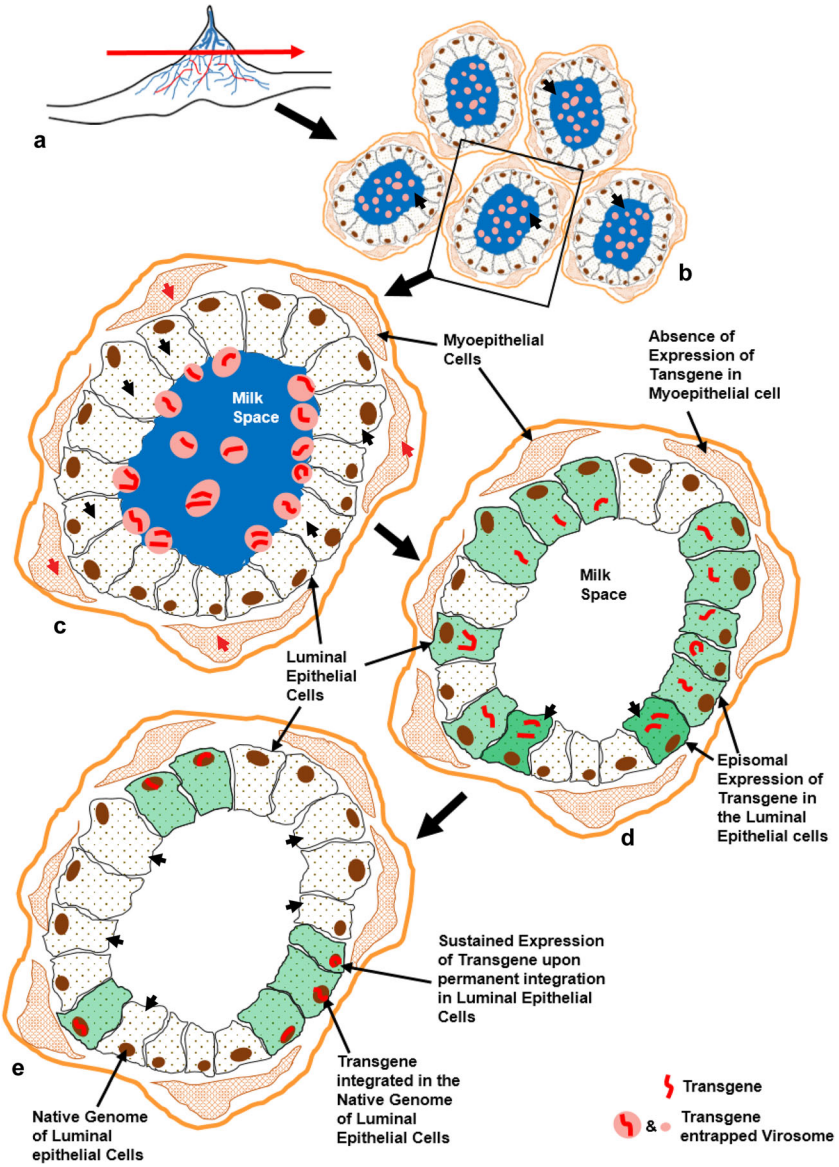

Fig. 5 Graphical representation of the mechanism of the transgene delivery through HNF-Virosomes followed by expression. **a** Image showing mammary ductal system after HNF-V delivery. Blue color in mammary ductal system is formed due to use of trypan blue dye in the HNF-V solution. Red arrow marks the direction of incision. **b** Image showing cross section of mammary gland depicting presence of HNF-V in the milk space (black arrow). Bluish color in the milk space is formed due to addition of trypan blue dye in the HNF-V suspension. **c** Image showing fusion of HNF-V with luminal epithelial cells (black arrow) preferentially, as they reside barely in the milk duct and confront first with the HNF-V while myoepithelial cells (red arrow) reside behind layers of luminal epithelial cells and fail to come in contact with HNF-V. **d** Image showing luminal epithelial cell specific episomal expression of delivered transgene in early stage of 1st lactation. Increase number of transgene delivery in cells (black arrow) result in to high level of protein expression. **e** Image showing sustained expression of delivered transgene upon integration in the native genome of the luminal epithelial cells, in late stage of 1st lactation followed by continuation of expression through several successive lactating term. Black arrows mark the cells which do not show transgene expression in later stage due to loss of transgene by various cellular degradation processes without having it integrated permanently in the native genome

"intraductal perfusion delivery method" for delivery of transgene entrapped in HNF-V further enhanced the LEC (surrounding ductal region) specific delivery of transgene by reducing the chance of interaction of HNF-V with any other cell type of mammary glands. Efficacy of this combinatorial method in obtaining high level expression of human therapeutic protein in mammary epithelial cells as well as in the milk was established for the first time. The level of expression of transgene on 1st lactating term in mammary glands transfected using HNF-V was found to be comparatively equal to the level of expression found in mammary glands of age-matched lactating transgenic animals. This generates a hope of replacing animal bioreactors generated through germline gene integration with the presently developed animal

bioreactor bypassing germline gene integration. The presently available method of generation of animal bioreactor through germline gene integration has several drawbacks related to the productivity, the production period, and the cost associated with it. The time involved in production of an animal bioreactor through germline gene integration is very high with very less efficiency (Rudolph 1999). One of the major drawback of the existing process is the time required for such an animal to attain the lactating phase is very high depending on the species, like for a cow it takes 15 months to be sexually mature and 9 months of gestation to enter in lactating phase so the cost involved in rearing of animals in existing method is very high which eventually increase the cost of the final product (Wall and Seidel 1992; Wall et al. 1997; Rudolph 1999; Montesino and Toledo 2006). This newly developed approach also prevented the chances of leaky expression of the gene of interest in any other organs. The declining level of transgene expression in successive lactating term could easily be restored by redelivery of HNF-V during each cycle of pregnancy. Repeated delivery of HNF-V may also contribute in increased transgene expression. The valuable therapeutic protein thus expressed can be isolated and purified as described previously depending on the type of protein which is being expressed. It has also been shown previously that proteins expressed in the milk retain their biological activities (Velander et al. 1992; Edmunds et al. 1998). Such evidence lead us to believe that the biotherapeutics produced by this newly developed method will also be of equal potential for therapeutic use.

This pilot-scale study has generated the knowledge of the developmental time when mammary glands can be transfected at best by HNF-V and the dose of HNF-V to be delivered for generating optimum level of transgene expression without disturbing the normal tissue architecture. This knowledge can be used for producing therapeutic proteins in milk of the farmed animals. This newly developed method of generating animal bioreactor based on non-germline gene integration will also bypass the period required for birth of transgenic progeny and their sexual maturation, prior to lactation, thereby remarkably reducing the cost of the therapeutics.

Mammary glands can now be utilized as a bioreactor for producing valuable therapeutic proteins without germline gene integration. Animal bioreactors generated based on germline gene integration are considered to be potentially hazardous because genomic alteration by random transgene integration may have long-term implications which may affect the health of normal herds, in case of unrestrained germline dispersal (Muir and Howard 1999; Devlin et al. 2006). In contrast, our present approach of gene delivery will help in generating safe animal bioreactors without any potential environmental hazard posed by animal bioreactor generated through germline gene integration.

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Author contribution The experiments were conceived and designed by NG1 (Nirmalya Ganguli) and SSM. Experiments were performed by NG1, NG2 (Nilanjana Ganguli) MC, and SC. The virosomes were generated by DPS, NG1, and SC. The data presented in the manuscript were analyzed by NG1, NG2, DPS, and SSM. The manuscript was prepared by NG1 and SSM and reviewed by DPS and NG2.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval Experiments with mice were carried out according to the guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA), India. Protocols were approved by the Institutional Animal Ethics Committee of National Institute of Immunology.

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