# Rescue of K-Casein Knockout Mouse with Echidna K- Casein

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***Abstract: Lactation is one of the traits that distinguish mammals from other vertebrates. Milk is the main source of nutrition for the neonates and provides all the essential nutrients for their growth and development. Caseins constitute more than 80% of the total protein content in the milk. Earlier research on functional analysis of κ casein in mouse showed that in the absence of κ-casein, the mouse fails to lactate because of the destabilization of the casein micelles in milk. Echidna belongs to the subclass prototheria which are the most primitive mammals. Studies on lactation in Echidna κ-casein transgenic female mouse null for mouse κ-casein would show the extent of functional conservation between mouse and echidna κ-caseins. The study included generation of the ES cells transgenic for Echidna κ-casein. 92 ES cell lines were analysed for the integration of Echidna κ-casein cDNA by PCR, out of which 17 lines were showing integration. Northern blot analysis of 17 integrated clones out of which 2 clones showed transcription of Echidna κ-casein.***

***Keywords: Casein, lactate, northern blot.***

## I. INTRODUCTION

Lactation, the nourishment of the young with copious milk secretion via the mammary gland, is an important aspect of mammalian reproductive physiology (5). Indeed, the naming of the class Mammalia by Linnaeus in 1758 (7) refers directly to lactation as the dominant character by which mammalian species can be identified despite the existence of other anatomical characteristics.

### 1.1 Evolution of lactation

Lactation appears to be an ancient reproductive feature that pre-dates the origin of mammals. A cogent theory for the evolution of the mammary gland and lactation has been provided by Olav Oftedal (9). The features of current mammals were gradually accrued through radiations of synapsid ancestors, and the mammary gland is hypothesized to have evolved from apocrine-like glands associated with hair follicles. Oftedal suggests that these glands evolved from providing primarily moisture and antimicrobials to parchment-shelled eggs to the role of supplying nutrients for offspring.

Fossil and molecular evidence point to the appearance of early mammals toward the end of the Triassic period (166 to 210 Mya) (1, 8) on the synaptid branch of the tree of life that separated mammalian ancestors from other living creatures during the Permian period (>320 Mya) (Figure 1). Fossil evidence indicates that some of the therapsids and the mammaliaformes, which were present during the Triassic period more than 200 million years ago, produced a nutrient-rich milk like secretion.

Recent comparative genome analysis has emphasized the ancient origin of the essential components of the lactation system at the molecular level (4, 6). A complex lactation system evolved gradually during the Triassic along the therapsidlineage and was already well established in the the last common ancestor of all living mammals, and probably also in mammaliaformes, of the late Triassic (9). Thus, lactation evolved during the Triassic, probably in the cynodont lineage, concomitant with the appearance of other mammalian characteristics such as the integument, endothermy, and fur.

The evolution of the casein family of milk proteins in particular would provide calcium, phosphate and protein to hatchlings. Fossil records suggest that caseins were present during the Triassic, because the extensive bone and tooth development evident in the relevant species at stages before independent feeding would have required delivery of ample calcium.

Today, after more than 200 My of evolution, the diversity of mammalian species and the extreme variations in their reproductive strategies, affecting in particular the lactation cycle, provide numerous examples of lineage or species-specific adaptations of the lactation system during mammalian evolution (5). The earliest split in the mammalian phylogeny established the Prototheria (monotremes, or Monotremata), which separated from the Theria about 166 (1) to 220 (8) My ago. The Theria later split into the Metatheria (marsupials, or Marsupialia) and Eutheria (Placentalia) lineages approximately 140 My ago. Only two families of monotremes have survived in Australasia: the platypus (Ornithorhynchus anatinus) and echidnas (Tachyglossus and Zaglosus genera). Now a days the Cynodonts and Mammaliaformes are extinct so these egg-laying monotremes are often regarded as representative of early mammals with a more primitive prototherian lactation system.

### 1.1.1 Milk Proteins

The casein family of protein consists of several types of caseins (α-s1, α-s2 , ß, and 6) and each has its own amino acid composition, genetic variations, and functional properties. The caseins are suspended in milk in a complex called a micelle. The caseins have a relatively random, open structure due to the amino acid composition (high proline content). The high phosphate content of the casein family allows it to associate with calcium and form calcium phosphate salts.

### 1.1.2 Casein and it's structure

Caseins account for >80% of the total milk proteins (10).They are the main source of amino acids (AAs), calcium, and phosphate for the suckling infant and also provide several bioactive peptides. Caseins comprise a group of acidic, proline-rich, phosphoproteins that are

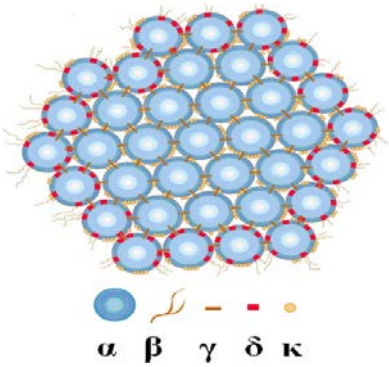


Figure 2: Casein structure

aggregated into large micellar structures in colloidal suspension with calcium in milk. There are 3–4 (depending on the species) evolutionarily related casein genes in mouse: the so-called "Calcium-sensitive" phosphoproteins - caseins *α*-s1 (*CSN1S1* or *Csna*), *β*-(*CSN2* or *Csnb*), and *α*-s2 (*CSN2*S2, A and B, or *Csng* and *Csnd*)) and the physically and functionally linked calcium-insensitive phosphoglycoprotein, *κ*-casein (*CSN3* or *Cnsk*) (10).

In addition to functioning as the primary source of amino acids, one of the key functions of micelles is to sequester large amounts of calcium phosphate from the maternal diet or bodily stores and make it readily available to the newborn (2). The high phosphate content of the casein family allows it to associate with calcium and form calcium phosphate salts. κ-casein is functionally important for stabilizing the Ca-sensitive caseins in the micelle. It is amphiphilic in nature. Milk-clotting proteases act on hydrophilic portion of κ-casein, thus originating an unstable micellar state that results in clot formation. Functional analysis of κ-casein shows that κ-casein null females did not suckle their pups and failed to lactate because of destabilization of the micelles in the lumina of the mammary gland (2).

### 1.1.3 Mouse Casein locus

The analysis of yeast artificial chromosomes (YACs) containing the complete mouse casein gene locus revealed the presence of five casein genes, α-, β-, γ-, δ-, and κ-casein, in this order, in the locus. The α- and β-casein genes are only 10 kb apart and have convergent transcriptional orientations. The distance between the β-casein gene and the αs2-1ike γ-casein gene is about 70kb, and these genes have divergent transcriptional orientations. The γ- and δ-casein genes, both encoding a αs2-1ike casein, are linked within 60 kb and convergently transcribed. The κ-casein gene is located about 100 kb from the δ-gene. Except for the presence of the δ-casein gene, the organization of the mouse casein locus resembles that of the bovine locus, including the transcriptional orientation of the genes. In contrast to the other casein genes, which are strongly induced at mid-lactation, expression of the δ-casein gene is abruptly induced upon parturition. Comparative analysis of αs2-1ike sequences from various species suggests that the ancestral αs2-like gene duplicated around the time of radiation of the rodent and artiodactylid ancestors (11).

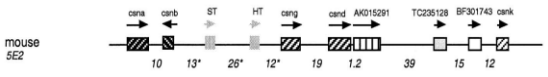


Figure 3: Mouse Casein Locus

### 1.1.4 Echidna Casein Locus

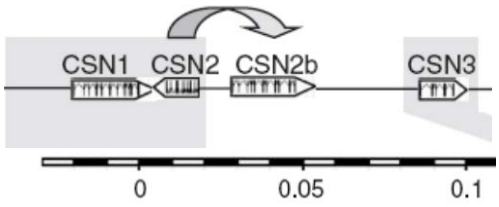


Figure 4: Echidna Casein Locus

A complete gene model was not recoverable from the Echidna draft genome assembly due to unresolved gaps in the coding region. However, at least 14 exons were confirmed. Thirteen exons, including 11 coding exons, a 5'UTR and a 3'UTR (inferred from echidna cDNA matches), could be mapped to the platypus genome sequence and at least one unmapped exon falls into an internal gap in the genome sequence. As in the case of other mammalian *CSN1* genes, the first exon is non-coding, the second exon encodes the leader peptide

and the gene contains many short, in phase exons encompassing as few as seven amino acids (4).

*CSN1 α-casein*

The monotremes CSN1 protein sequences from both species contain three major canonical phosphorylation sites (ser-ser-ser-glu-glu), all following the canonical rule of a splicing event between the glutamic acids (Hobbs and Rosen 1982; Jones *et al.* 1985). Marsupial and eutherian CSN1 generally contain two canonical sites.

*CSN2 β-casein*

Eight coding exons and one 3'UTR could be mapped to the genome while a 47 nucleotide long non-coding 5'UTR likely falling within an unresolved gap of the draft assembly could not be mapped. In the echidna, three splice variants were also identified. Exon V encompassing 30 nucleotides is often absent in the echidna.

*CSN2b: an additional β-like casein in monotremes*

A third casein related to the calcium-binding caseins was identified in both monotreme species. A complete gene model consisting of 15 exons was retrieved from the platypus genome sequence. As generally seen in the casein genes, the first and last exons are non-coding, the second exon encodes the signal sequence, internal exons are often short and all coding exons are in frame. Variations were also observed in monotremes within this gene.

*CSN3 kappa-casein*

The kappa-casein gene (*CSN3*), functionally but not evolutionarily related to other caseins, was also found in monotremes. The gene encompasses five exons including a 5' non-coding UTR, three coding exons and a 3' UTR. The first coding exon, Exon II, encodes the signal peptide and the large Exon IV encodes most of the mature peptide. This gene organisation is similar to other eutherian *CSN3* and confirms the previous observation of stronger evolutionary pressure on the calciuminsensitive kappa-casein gene compared with calcium-sensitive caseins (Rijnkels 2002). The importance of *CSN3* is also illustrated by the fact that *CSN3* gene knock-down mice fail to lactate (Shekar *et al.* 2006) whereas similar deletions of calciumsensitive casein genes has little effect (Kumar *et al.* 1994; Chanat *et al.* 1999), probably due to the redundancy of calcium-sensitive casein genes in many species (Kawasaki and Weiss 2003).

1.1.5 Comparative analysis of the mammalian casein locus

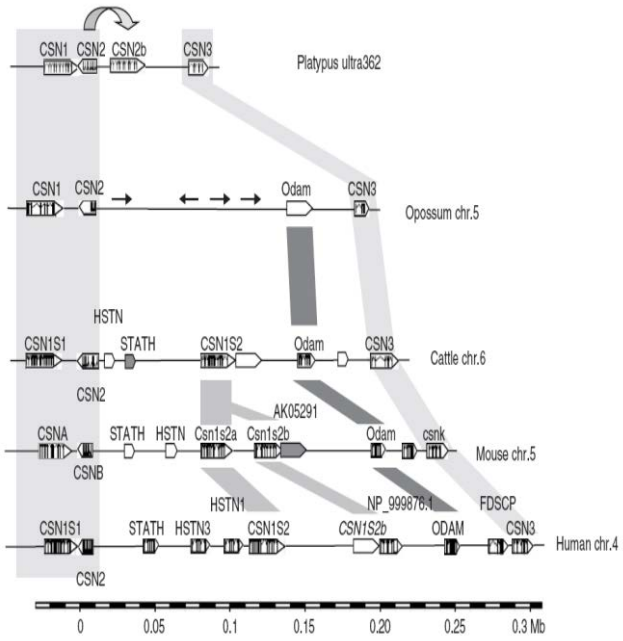


Figure 5: Comparative analysis of the mammalian casein locus, showing the expansion of the casein locus in mammals. Comparison of the casein locus organisation in platypus, opossum, cattle, mouse and human genomes. Drawn to scale and aligned on the β-casein gene (adapted from ensembl and Rijnkels 2002). Genes are represented by a box with a tail arrow pointing in the direction of gene transcription. Gene models for confirmed genes were generated (platypus) or retrieved from ensembl (others) when available. Blank boxes represent putative genes based on similarity, grey boxes represent genes with observed expression. Note the close proximity of α- (CSN1, csna) and β- (CSN2, csnb) casein genes in reverse orientation on the left and the expansion of the region between β- and kappa- (CSN3, csnk) casein on the right. Except for β-casein, all genes are transcribed from left to right. In monotremes, a recent duplication of CSN2 has led to CSN2b, whereas in eutherians, an ancient duplication produced CSN1S2, which has been duplicated in some species to produce CSN1S2b, now a pseudogene in human but not in mouse. In the marsupial locus, there is no casein duplication and the spacing region contains several copies of an invading repetitive element (black arrows) (4).

Replacement of mouse κ-casein with Echidna κ-casein would help to study the functional conservation between mouse and echidna κ-caseins gene. This report contains screening of Echidna κ-casein transgenic ES cell lines by PCR analysis, Northern blot analysis of the transgenic cell lines and screening of κ-casein knockout mice.

## II. MATERIALS AND METHODS

1.2 Materials

All the molecular biology grade reagents, enzymes, buffers and chemicals were purchased from New England bio-labs, Sigma Chemical Co., USA. All the stock and working solutions were prepared in double distilled water and autoclaved at 121°C (15 psi pressure) for 15-20 minutes.

Tissue Culture Equipment, Glassware and Plastic ware – All the cell culture experiments were carried out in horizontal laminar flow cabinets. The other equipments used in tissue culture facility were humidified $CO_2$ incubators set at 5% $CO_2$ and 37°C, an inverted phase contrast microscope (Nikon eclipse TS100), inverted fluorescence microscope (Nikon eclipse TE2000-S with attached camera and imaging software), table top centrifuge (Kubota, with swing bucket rotor). All the glassware was thoroughly cleaned and autoclaved.

1.3 Method

1.3.1 Transformation

In a typical transformation reaction 4-5 µl of ligation mix or 1-2 ng of plasmid DNA was incubated on ice with 50 µl ultra competent cells for 25-30 minutes, followed by a heat shock at 42°C for 2 minutes. The cells were directly plated on to LB agar plate containing 100 µg/ml of ampicillin and incubated overnight at 37°C.

1.3.2 Isolation of Plasmid DNA

Plasmid DNA was prepared by alkali lysis method. Single bacterial colony was inoculated in 5 ml of LB broth containing 100 µg/ml of ampicillin and incubated overnight at 37°C in a shaking incubator (Innova) at 210 rpm. Further QIAprep Spin Miniprep Kit (Qiagen) was used for isolation of plasmid DNA as per the instructions of the manufacturer.

1.3.3 Restriction Digestion of Plasmid DNA

Plasmid DNA was digested with various restriction endonucleases (New England Biolabs) as per the manufacturer's instructions, except that the enzymes were used at 3-8 fold excess of the recommended units.

1.3.4 Isolation of DNA from Agarose Gels

Fragments of DNA (From PCR or restriction digestion reactions) were isolated from the gel using Gel extraction kit (Qiagen) as per the manufacturer's instructions. DNA isolated for the purpose of ligation reactions were isolated using minelute columns in 10 µl of $ddH_2O$.

1.3.5 Ligation Reactions

Ligation reaction was carried out at 25°C for 20-30 minutes with following components.

1.3.6 End-Filling or End-Polishing Reactions

Plasmid DNA digested with appropriate restriction enzymes was precipitated with 0.1 volume 3 M NaOAc (Sodium acetate) and two volumes of absolute alcohol. DNA was pelleted by centrifugation at 13000*g* for 30 minutes and was washed with 70% ethanol. The DNA pellet was dissolved in sterile $ddH_20$.

Cloning with pCAG-IRES-EGFP-Neo vector

pCAG-IRES-EGFP-Neo vector

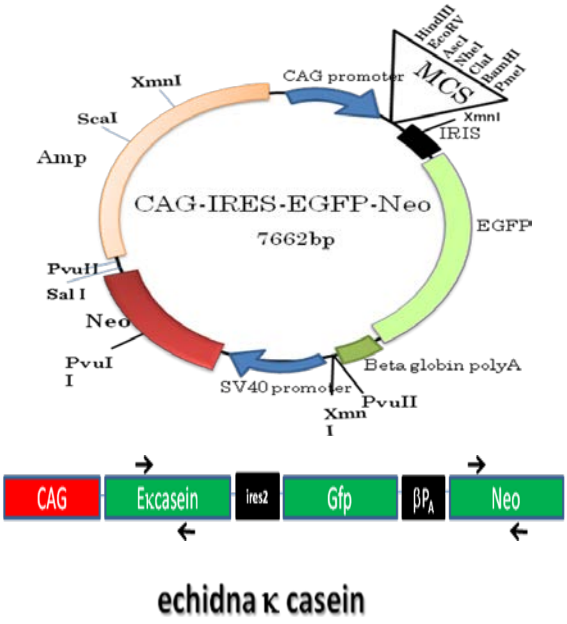


Figure 6: pCAG-IRES-EGFP-Neo vector Cloning Procedure

All cell culture experiments were carried out in horizontal laminar flow cabinet. R1.9 embryonic stem cells (Alex *et al*., 2005) at passages 8-10 were used for electroporation experiments. For other experiments ES cells at passages 14-18 were used. ES cells were cultured under strict aseptic conditions involving regular wiping of the work surfaces with 70% ethanol. *Mycoplasma* infection of cells was monitored by *Mycoplasma* PCR detection kit (sigma), no *Mycoplasma* contaminations were found during the course of this study.

pCAG-IRES-EGFP-Neo vector

↓

Cut with EcoRV restriction enzyme

↓

pCAG-E κ-casein-IRES-GFP-Neo (pEκIGN)
↓
Cut with ScaI restriction enzyme
↓
Linear pEκIGN
↓
Electroporate into R1.9 Cell line
↓
Select in Neomycin 200 µg/ml final concentration for 7 days
↓
Picked 92 clones
↓
PCR

1.3.7 Culture and Genetic manipulation of Embryonic Stem Cells

1.3.8 Electroporation and selection of ES Cells

Growing ES cells from a sub-confluent culture were harvested by trypsinisation, pelleted by centrifugation and resuspended at high density in PBS. The cells were counted using a Neubauer haematocytometer. The cell concentration was adjusted to $10^8$ cells/ml. About 0.8 ml cell suspensions containing 25-40 µg of targeting vector DNA was placed in a Bio-Rad Gene Pulser cuvette (width 0.4 cm). The mixture was given a single pulse of 240 V and 500 µF. The electroporated cells were incubated at room temperature for 10 minutes, transferred into complete medium and plated in suitable dishes containing feeders.

1.3.9 Isolation and Expansion of Individual ES Cell Clones

Following electroporation and selection, individual ES cell colonies with undifferentiated phenotype were picked up using p2-p10 pipette tips while working under inverted microscope in laminar airflow cabinet. Each colony was incubated for a brief period in small drop (50 µl) of 0.05 % trypsin/EDTA. Trypsin activity was neutralized with 200 µl complete medium and colony was disintegrated by trituration with p20-p200 pipette tips. The cell suspension was plated in a well of 24 well plate containing one ml complete medium and cultured in $CO_2$ incubator.

2. Results and Discussion

2.1 Cloning

The pEκIGN recombinant plasmid DNA was inserted into the R1.9 cell lines by electroporation. Clones were selected over 200 µg/ml final concentration of neomycin. Clones were further amplified on PCR to check whether they contain the E κ-casein or not.

2.2 PCR screening

PCR screening of 92 neomycin resistant ES cell clones picked from the Echidna κ-casein integration experiment was carried out using Echidna κ casein forward and reverse primers. These primers bind on Echidna κ-casein cDNA sequence and amplify a 238 bp amplicon. Seventeen clones were found to be transgenic for Echidna κ-casein. There were amplicons from +ve control genomic DNA and plasmid DNA while the –ve controls did not show any amplicon ruling out the possibility that the amplification is nonspecific.

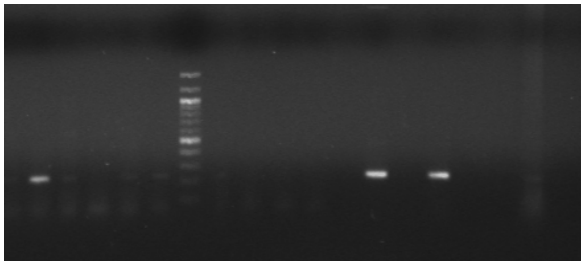

Figure 7. PCR screening of ES cells clone

2.3 RNA Electrophoresis

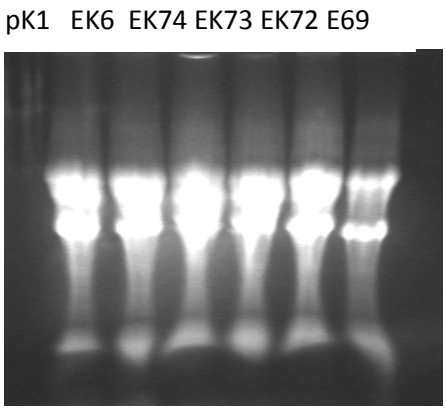


Figure 8: RNA Electrophoresis

2.4 Northern blot analysis of echidna κ-casein transgenic clones

The 620 bp Echidna κ-casein cDNA was used as the probe in Northern hybridization. 17 ES cell clones transgenic for Echidna κ-casein were tested for transcription from the transgene. There were only 1 (EK6) transcription clones. The positive control (pK1) hybridized to the probe indicating that the hybridization reaction worked. When β actin mRNA was used as the loading control it was observed that the EK 73 and Ek 74 lines contain bad quality mRNA.

pK1 EK6 EK 74 EK 73 EK 72 EK 69

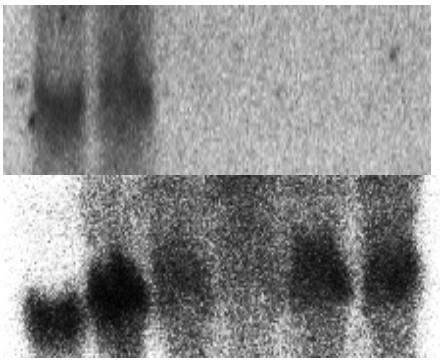


Figure 9. Northern blot of echidna κ-casein transgenic clones

(pK1 – ES cell line transcribing Echidna κ-casein used as positive clone)

(EK 6, EK 74, EK 73, EK 72, EK 69 – clones tested for Echidna κ-casein transcription)

## III. SUMMARY AND CONCLUSION

Functional conservation of κ-casein was studied in mice. 17 R1.9 cell line clones from 92 clones showed amplification and these clones were further tested for transcription of Echidna κ-casein. One R1.9 cell line showed the successful transcription. This cell line aggregates into the blastocyst of female mouse null for mouse κ-casein.

## IV. FUTURE SCOPE

This study will be beneficial for the evaluation of functional conservation of the κ-casein in mammals.

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