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RNAi-mediated Reduction of a Major Ruminant Specific Milk Allergen Using a Transgenic Mouse Model

A thesis

submitted in fulfilment

of the requirements for the degree

of

Doctor of Philosophy

At

The University of Waikato

by

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THE UNIVERSITY OF WAIKATO

Te Whare Wānanga o Waikato

2012

Dedicated

To

My Wonderful Mother

Mrs Anowera Begum

Abstract

Milk from dairy cows is an important human food. It contains the protein beta-lactoglobulin (BLG) which is not present in human milk and thought to be a major allergen in the milk of cows. RNA interference (RNAi), mediated by sequence-specific micro RNAs (miRNAs), provides a new molecular tool to reduce the levels of undesirable proteins.

In this study, I have screened ten miRNAs targeting the BLG mRNA for their potential to knockdown BLG expression using a cell-based *in vitro* assay. I identified four miRNAs which showed substantial bovine BLG (bBLG) (78-97%) and ovine BLG (oBLG) (33-94%) knockdown activity *in vitro*. Tandem miRNA constructs with combinations of these four single miRNAs did not result in significantly increased knockdown efficiency compared to the respective single miRNA constructs in the *in vitro* assay ($P>0.05$). Because targeting of two different regions has the potential for greater knockdown efficiency *in vivo*, I chose a tandem construct which achieved *in vitro* up to 99% bBLG knockdown as compared to 92% and 74% of bBLG knockdown by the single miRNAs. The tandem construct also showed 90% of oBLG knockdown in comparison to 92% and 69% of oBLG knockdown by the single miRNAs in the *in vitro* assay which made it an ideal candidate for the subsequent evaluation in transgenic (Tg) mice. For the *in vivo* studies, the cytomegalovirus (CMV) promoter of the selected miRNA constructs was replaced with the promoter of the mouse milk protein gene for whey acid protein (WAP) which directs the expression of the BLG-specific miRNAs to the lactating mammary gland. The Tg mouse lines generated with the tissue-specific tandem construct and the single miRNAs used for the tandem miRNA construct were then crossed with an oBLG or bBLG expressing Tg mouse line to generate double transgenic (miRNA-BLG) mice for assessing BLG knockdown *in vivo*. Analysis of the milk proteins demonstrated that the tandem miRNA reduced the levels of oBLG and bBLG in milk from the miRNA-BLG mice up to 96% and 46%, respectively. This study validates the mammary gland

specific expression of miRNAs as a promising approach to knockdown allergenic proteins in milk.

Keywords: BLG, miRNA, RNAi, Transgenic mouse, Gene knockdown.

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Abbreviations

Abbreviations	Description
AAV	Adeno-associated virus
AIV	Avian influenza virus
AMD	Age-related macular degeneration
ATCOL	Atelocollagen
BLG	Beta-lactoglobulin
bp	base pairs
BSA	Bovine serum albumin
BVD	Bovine viral diarrhoea
BVDV	Bovine viral diarrhoea virus
cDNA	Complementary DNA
CHS	Chalcone synthase
CMA	Cow milk allergy
CMV	cytomegalovirus
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's minimum essential medium
dNTPs	Deoxynucleoside triphosphates
DMSO	Dimethyl sulphoxide
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	disodium ethylenediaminetetra- acetate
EHF	Extensively hydrolysed formula
FA	Food allergy
FBS	fetal bovine serum
FLP	Flippase recombination enzyme
FMD	Foot-and-mouth disease

FMDV	Foot-and-mouth disease virus
FRT	Flippase recognition target
GFP	Green fluorescent protein
HCV	Hepatitis C virus
HR	Homologous recombination
HRP	Horseradish peroxidise
IgE	Immunoglobulin E
kb	Kilobase
kD	Kilo dalton
L	Litre (s)
M	molar
MCS	Multiple cloning site
MES	Morpholineethanesulfonic acid
miRNA	Micro RNA
ND	Newcastle disease
nt	Nucleotide
OD	Optical density
p	Promoter
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PolII	Polymerase II
PolIII	Polymerase III
PTGS	Post-transcriptional gene silencing
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RSV	Respiratory syncytial virus
RT	room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
TLR3	Toll-like receptor 3
UV	ultraviolet
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
w/v	Weight per volume
WAP	Whey acidic protein
X-gal	5-bromo-4chloro-3-indolyl-beta-D-galactopyranoside

Table of Contents

Abstract ii

Acknowledgements iv

List of Publications and Conference Contributions vii

Abbreviations ix

Table of Contents xii

List of Tables xx

List of Figures xxi

Chapter One: Literature Review 1

1.1 Allergy 1

1.2 Food allergy and allergens 1

1.2.1 Cow milk allergy (CMA) 2

1.2.1.1 BLG 3

1.2.1.1.1 Allergenic properties of BLG 3

1.2.1.1.2 Biological role of BLG 3

1.3 Current strategies to reduce BLG in milk 4

1.4 RNAi 5

1.4.1 Introduction 5

1.4.2 General mechanism of RNAi 9

1.4.3 Recent progress of RNAi in animals 11

1.4.3.1 Delivery of RNAi molecules: from cells to animals 11

1.4.3.2 The use of RNAi for the study of disease-associated genes in livestock 14

1.4.3.2.1 FMD 15

1.4.3.2.2 BVD 16

1.4.3.2.3 AIV 17

1.4.3.2.4 Swine flu 17

1.4.3.2.5 ND 18

1.4.3.2.6 Prion disease 18

1.4.3.3 The use of RNAi for the study of genes associated with livestock improvement 19

1.4.3.3.1 Myostatin 19

1.4.3.3.2 Porcine BLG 20

1.4.3.4 The use of RNAi-based therapeutics for clinical applications 20

1.5 Rationale and Objectives of the Study 22

1.6 Thesis Layout 23

Chapter Two: Materials and Methods 25

2.1 General Materials and Methods 25

2.1.1 General Materials 25

2.1.1.1 General chemicals and reagents 25

2.1.1.2 General biological materials 27

2.1.1.2.1 Molecular biology 27

2.1.1.2.2 PCR reagents 27

2.1.1.2.3 Enzymes 27

2.1.1.3 Oligonucleotides 29

2.1.1.4 Expression vectors 32

2.1.1.5 Kits 35

2.1.1.6 Bacterial cell culture materials 35

2.1.1.6.1 Bacterial strains 35

2.1.1.6.2 Lauria-Bertani (LB) medium 36

2.1.1.6.3 SOC medium....36
2.1.1.6.4 SOB medium....36
2.1.1.7 Mammalian cell culture 36
2.1.1.7.1 Cells 36
2.1.1.7.2 Cell culture medium....36
2.1.1.8 Antibodies used for western blot assay.... 36
2.1.1.9 Buffers and stock solutions.... 37
2.1.1.9.1 General buffers and stock solutions....37
2.1.1.9.2 Antibiotic solutions for cell cultures....37
2.1.1.9.3 Buffers and stock solutions for molecular biology....38
2.1.1.10 Reagents for transfection analysis 39
2.1.1.11 Buffers and reagents for western blot assay.... 39
2.1.1.12 Coomassie blue stain solutions 39
2.1.1.13 Equipment.... 40
2.1.1.13.1 Specific equipment and instruments....40
2.1.1.13.2 Culture vessels 41
2.1.1.13.2.1 Plastic ware 41
2.1.1.13.2.2 Glassware.... 41
2.1.1.13.2.3 Other Consumables.... 42
2.1.1.14 Software 42
2.1.1.15 Animals and Animal Ethics Approval 42
2.1.2 General methods 42
2.1.2.1 Generation of chemically competent cells.... 42
2.1.2.2 Isolation of nucleic acids 43
2.1.2.2.1 Small-scale preparations of plasmid DNA by miniprep 43
2.1.2.2.2 Large-scale preparations of plasmid DNA by maxiprep43

2.1.2.2.3 Isolation of genomic DNA (gDNA) 44
2.1.2.2.4 Isolation and purification of total RNA 45
2.1.2.2.4.1 Handling of RNA 45
2.1.2.2.4.2 Isolation of RNA from mouse mammary tissue 45
2.1.2.3 Quantification of nucleic acid 45
2.1.2.4 Restriction digestion of DNA 46
2.1.2.5 Detection of DNA on agarose gel electrophoresis 46
2.1.2.6 Purification of DNA from agarose gel 47
2.1.2.7 Dephosphorylation of vectors 47
2.1.2.8 Polymerase chain reaction (PCR) 47
2.1.2.8.1 Primer design for PCR 48
2.1.2.9 Synthesis of first-strand cDNA 48
2.1.2.10 Ligation of DNA fragments 49
2.1.2.11 Sub-cloning of the PCR product 49
2.1.2.12 Transformation of *E. coli* competent cells 50
2.1.2.13 DNA sequencing 50
2.1.2.13.1 Sequencing 50
2.1.2.13.2 Sequence analysis 50
2.1.2.14 Glycerol stock 50
2.1.2.15 Mammalian cell culture 51
2.1.2.15.1 Cell culture 51
2.1.2.15.2 Viable cell count 51
2.1.2.15.3 Cryo-preservation of mammalian cells 52
2.1.2.15.4 Recovery of cells from liquid nitrogen storage 52
2.1.2.16 Protein analysis by western blot assay 52
2.1.2.16.1 Preparation of cell lysate 52

2.1.2.16.2 Quantification of protein 53

2.1.2.16.3 SDS polyacrylamide gel electrophoresis (PAGE) 53

2.1.2.16.4 Transfer of protein 53

2.1.2.16.5 Blocking and immune-staining of the blot 54

2.1.2.16.6 Detection of protein 54

2.1.2.17 Statistical analysis 55

2.2 Methods Relating to Chapter Three: *In Vitro* Validation of miRNA Constructs 56

2.2.1 Generation of miRNA expression constructs 56

2.2.1.1 Selection of miRNA sequences for targeting BLG gene 56

2.2.1.2 Generation of single miRNA expression constructs 56

2.2.1.3 Identification of single miRNA inserts 59

2.2.1.4 Generation of multiple miRNA expression constructs 59

2.2.2 Generation of BLG expression constructs 60

2.2.2.1 Construction of a bBLG expression construct 60

2.2.2.2 Construction of an oBLG expression construct 61

2.2.2.2.1 Sub-cloning of oBLG cDNA into a shuttle vector 61

2.2.2.2.2 Cloning of oBLG gene into an expression vector 61

2.2.2.3 *In vitro* expression of BLG 62

2.2.3 Transfection of mammalian cells 62

2.2.3.1 Lipid-based transfection of COS7 cells 62

2.2.3.2 Post-transfection glycerol shock 63

2.2.3.3 FACS analysis of transfected cells 63

2.2.4 *In vitro* knockdown analysis by western blot 64

2.3 Methods Relating to Chapter Four: Generation of Transgenic Mice 65

2.3.1 Generation of Tg mouse 65

2.3.1.1 Generation of tissue specific bBLG construct 65

2.3.1.2 Generation of tissue specific miRNA construct..... 66

2.3.1.2.1 Sub-cloning of WAP promoter into a shuttle vector 66

2.3.1.2.2 Generation of tissue specific miRNA constructs.....66

2.3.1.3 Preparation of DNA constructs for microinjection..... 67

2.3.1.4 Preparation of mice for microinjection 68

2.3.1.5 Screening of mice by PCR genotyping..... 68

2.4 Methods Relating to Chapter Five: Knockdown of BLG *In Vivo* 69

2.4.1 Generation of Tg miRNA-BLG mice 69

2.4.2 PCR genotyping of Tg miRNA-BLG mice..... 69

2.4.3 Milking of mice..... 69

2.4.4 *In vivo* knockdown analysis by coomassie blue staining..... 70

2.4.5 *In vivo* knockdown analysis by western blot 70

Chapter Three: *In Vitro* Validation of MiRNA Constructs..... 72

3.1 Introduction..... 72

3.2 Results..... 73

3.2.1 Optimization of transfection efficiency in COS7 cells..... 73

3.2.2 Screening of miRNAs to suppress BLG expression *in vitro* 78

3.2.3 Comparison of single and tandem BLG specific miRNAs *in vitro* 87

3.3 Discussion..... 92

3.3.1 Transfection efficiency of cells..... 92

3.3.2 MiRNA-mediated knockdown of BLG expression *in vitro*..... 92

Chapter Four: Generation of Transgenic Mice 97

4.1 Introduction..... 97

4.2 Results..... 97

4.2.1 Characterization of bBLG founder mouse lines 97

4.2.2 Identification of transgenic miRNA founder mice 101

4.3 Discussion 108

Chapter Five: Knockdown of BLG *In Vivo* 110

5.1 Introduction 110

5.2 Results 110

5.2.1 Demonstration of miRNA-mediated reduction of BLG expression .. 110

5.3 Discussion 131

Chapter Six: General Discussion and Outlook 135

Appendices 141

Appendix 1: Screening of single miRNA constructs. 141

Appendix 2: Restriction digestion strategy to chain-up miRNAs. 142

Appendix 3: Screening of tandem miRNA constructs. 143

Appendix 4: Restriction digest of pGL 152 and pGL 164 expression constructs. 144

Appendix 5: Analysis of putative pGL 297 expression clones. 145

Appendix 6: Generation of a TOPO-oBLG construct. 146

Appendix 7: Restriction digestion of pGL 164 and TOPO-oBLG constructs. 147

Appendix 8: Analysis of putative pGL 448 clones. 148

Appendix 9: A western blot detection of BLG on a SDS gel. 149

Appendix 10: Generation of a pGEM-T-bBLG construct. 150

Appendix 11: Analysis of putative pGL 449 clones. 151

Appendix 12: Identification of orientation of bBLG in pGL 449 by restriction digestion. 152

Appendix 13: Generation of a pGEM-WAP construct. 153

Appendix 14: Analytical digest of putative tissue specific pGL 298 clones. 154

Appendix 15: Restriction digestion of tandem and single miRNA constructs. 155

Appendix 16: Analytical digest of putative clones of tissue specific pGL 446 and pGL 447. 156

Appendix 17: Preparative gel of the pGL 449 fragment for pronuclear injection..... 157

Appendix 18: Preparative gel of the miRNA fragment and the insulator for pronuclear injection. 158

Appendix 19: miRNA sequences + flanking vector sequences 159

Appendix 20: bBLG cDNA sequences + flanking vector sequences 162

Appendix 21: oBLG cDNA sequences + flanking vector sequences 163

Appendix 22: WAP promoter sequences + flanking vector sequences 164

Appendix 23: Comparison of secondary structures of bBLG and oBLG mRNA sequences. 169

References 170

List of Tables

Table 1-1: Milestone discoveries in the field of RNAi. 9
Table 2-1: A list of restriction enzymes used in this experiment. 27
Table 2-2: RNAi-specific oligonucleotides. 29
Table 2-3: Oligonucleotides used for PCR for direct cloning and genotyping..... 30
Table 2-4: Plasmid DNA expression vectors. 32
Table 2-5: Generation of miRNA duplexes. 56
Table 2-6: A list of reagents to clone ds oligo into the expression vector. 57
Table 3-7: Effect of different transfection conditions on transfection efficiency of COS7 cells. 75
Table 3-1: Effect of Lipofectamine 2000 on co-transfection efficiency of COS7 cells. 76
Table 3-2: Effect of post-transfection glycerol treatment on co-transfection efficiency of COS7 cells. 78
Table 4-1: Germ line transmission in Tg bBLG mice. 99
Table 4-2: Production of Tg founder miRNA mouse lines. 103
Table 4-3: Germ line transmission in miRNA Tg mice. 105
Table 4-4: Germline transmission of transgenic miRNA-bovine BLG mice. 107
Table 4-5: Germline transmission of transgenic miRNA-ovine BLG mice. 108

List of Figures

Figure 1-1: RNAi pathways. 11
Figure 2-1: Binding of Primary and Secondary HRP Antibody and ECL detection. 55
Figure 2-2: A schematic representation of cloning of miRNA duplex into pGL 273..... 58
Figure 3-3: Schematic depiction of plasmid DNA expression constructs for transfection assay. 74
Figure 3-4: Effect of DNA:transfection reagent on transfection efficiency of COS7 cells. 75
Figure 3-5: FACS analysis of co-transfection of COS7 cells using Lipofectamine 2000..... 77
Figure 3-6: Effect of post-transfection glycerol shock on transfection efficiency of COS7 cells. 78
Figure 3-7: Structure of an engineered pre-miRNA sequence on murine miR-155 sequence. 79
Figure 3-8: Position of miRNAs targeting BLG mRNA sequence..... 82
Figure 3-9: Screening of miRNA expression constructs against the expression of bBLG and oBLG. 83
Figure 3-10: Effect of single miRNA constructs against BLG expression *in vitro*. 85
Figure 3-11: Comparison of single miRNA and their combinations with bBLG and oBLG knockdown activity by western blot analysis..... 89
Figure 3-12: Effect of single and tandem miRNA constructs against BLG expression. 90
Figure 3-13: Comparison of GFP expressioin in tandem and single miRNA constructs. 91

Figure 4-1: Schematic depiction of bBLG construct used to generate transgenic bBLG mouse lines. 98
Figure 4-2: PCR genotyping to identify bBLG founder mice. 99
Figure 4-3: Comparison of the expression of bovine BLG protein in different mouse lines. 100
Figure 4-4: Schematic depiction of miRNA constructs used to generate Tg miRNA mouse lines. 102
Figure 4-5: PCR genotyping of putative miRNA mice 104
Figure 4-6: PCR genotyping of putative miRNA-BLG mouse 106
Figure 5-1: Coomassie staining of proteins in milk collected from mice on day 5 of lactation. 111
Figure 5-2: Knockdown of ovine BLG by miRNAs using SDS-PAGE analysis and detection by coomassie blue. 113
Figure 5-3: Comparison of ovine BLG knockdown by miRNA constructs using SDS-PAGE analysis and detection by coomassie blue. 114
Figure 5-4: Analysis of ovine BLG knockdown by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue. 115
Figure 5-5: Knockdown of ovine BLG during the lactation period by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue. 116
Figure 5-6: Western blot analysis of proteins in milk collected from mice on day 5 of lactation. 117
Figure 5-7: Knockdown of ovine BLG by single and tandem miRNAs using western blot analysis. 118
Figure 5-8: Comparison of ovine BLG knockdown by miRNA constructs using western blot analysis. 119
Figure 5-9: Analysis of ovine BLG knockdown by miRNA 6-4 ΔpA construct using western blot analysis. 120
Figure 5-10: Knockdown of ovine BLG around the lactation period by miRNA 6-4 ΔpA construct using western blot analysis. 121
Figure 5-11: Knockdown of bovine BLG by miRNAs using SDS-PAGE analysis and detection by coomassie blue. 123
Figure 5-12: Comparison of bovine BLG knockdown by miRNA constructs using SDS-PAGE analysis and detection by coomassie blue. 124

Figure 5-13: Analysis of bovine BLG knockdown by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue. 125
Figure 5-14: Knockdown of bovine BLG during different lactation time points by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue. 126
Figure 5-15: Knockdown of bovine BLG by single and tandem miRNAs using western blot analysis. 128
Figure 5-16: Comparison of bovine BLG knockdown by miRNA constructs using western blot analysis. 129
Figure 5-17: Analysis of bovine BLG knockdown by miRNA 6-4 ΔpA construct using western blot analysis. 130
Figure 5-18: Knockdown analysis of bovine BLG around the lactation period by miRNA 6-4 ΔpA construct using western blot. 131

Chapter One: Literature Review

1.1 Allergy

Allergy is a misguided hypersensitivity reaction to foreign substances by specific immunologic mechanisms. Hypersensitivities have been divided into four types based on the mechanisms involved and time taken for the reaction as described by Gell and Coombs (1). These types are type I- immediate (anaphylactic) hypersensitivity, type II- antibody-mediated cytotoxic (cytolytic) hypersensitivity, type III- immune complex-mediated hypersensitivity and type IV- cellular-immune (delayed) hypersensitivity. Among the four types of hypersensitivity reactions, type I is the best understood. In this type of allergy, after ingestion, inhalation or injection, the body produces specific IgE antibodies within minutes or hours and releases histamine (2). The symptoms of this type of reaction may involve skin (eczema), eyes (red eyes), nasopharynx (rhinitis) and bronchopulmonary tissues (asthma) (3). Depending on the severity of the reaction, it may cause a range of symptoms from minor inconvenience to death.

1.2 Food allergy and allergens

In the past few decades, food allergy (FA) has become a very severe health problem and there has been a significant increase in the prevalence of FA in infancy and childhood. It has been reported that 25% of adults believe they or their children suffer from FA, however, only approximately 5–8% of children and 1–2% of adults suffer from true FA (4) (5). Over 90% of food allergies in childhood are caused by eight foods: cow's milk, hen's egg, soy, peanuts, tree nuts, wheat, fish and shellfish (6). When allergic individuals consume above-mentioned foods, their immune system becomes stimulated by the specific protein in food and as a consequence specific IgE antibodies are produced. The IgE antibodies react with the allergen that results in the release of histamine and causes the above-mentioned symptoms.

1.2.1 Cow milk allergy (CMA)

Milk is the sole nutrition for young in mammals and contains all the essential nutrients for mammalian growth. In the early years of the last century cow's milk was reported as a cause of diarrhoea and other unexplained problems in infants (7). By the middle of the last century, CMA became known as a cause of child disease due to the decline in breast feeding and increased use of cow milk as infant nutrition in the developed world (7). Although cow's milk is a good food for the young and it has developed into an important human food source, there are at least 18 protein components that may cause allergic reactions in the body (8). Cow milk protein allergy (CMPA) is an immunologically mediated adverse reaction in human due to immune system responding against the foreign milk protein by creating specific antibodies- called immunoglobulin E (IgE) (9). The free-floating IgE molecules specific to the antigen first attach to the cell surface receptors on mast cells and trigger the release of histamine (10), which results in symptoms ranging from mild to moderate to severe such as, irritability, vomiting, wheezing, swelling, hives, other itchy bumps on the skin, and bloody diarrhoea (11). Though most children experience allergenic reactions, for the majority the symptoms remain rather minor. However, symptoms can be severe and 12% of patients who are allergenic to cow milk may have a very serious life-threatening allergic reaction known as anaphylaxis (12). This severe allergic reaction causes swelling of mouth, throat and lungs that results in difficulty in breathing and even it is severe enough to cause death (13). Most of the children develop symptoms before one month of age and allergic symptoms can be seen in the following organ systems: gastrointestinal (50–60%), skin (50–60%) and respiratory tract (20–30%) (14). The symptoms of allergic reactions may vary between individuals and an individual can be allergic to more than one milk protein (15) (16). Usually CMA develops in the first year of infancy (17) and 1.9% to 7.5% of infants suffer from CMA (17) (18). It has been reported that 87% of affected children recover from CMA by the age of three (19). In adults, CMA is rare, only a few case studies reported cow milk proteins as the potential source of allergenic reactions (20) (21). The digestive tract of infants is not fully developed and lacks defense factors (22). So, immune system exerts allergic reactions when milk proteins pass

through their gut (23) (24). The milk is compromised of two main protein fractions, whey and casein, and an individual may be allergenic to either or both. The caseins account for 80% of total milk protein and they can be the major allergenic component of cow's milk to a small fraction of population (25). The non-casein fraction or whey, accounts for 20% of total milk protein and mainly consists of α-lactalbumin and BLG. The antigenicity of BLG was studied in mice and rabbits immunized with bBLG and dominant antigenic regions were recognized by IgG antibodies against BLG in both animals (26).

1.2.1.1 BLG

BLG is the major whey protein of cow's milk at about 2-3 g/l (27) and is also present in the milk of many other mammalian species, such as, goat, sheep, pig, horse, cat and dog (28). However, the milk of rodents and humans does not contain BLG (28).

1.2.1.1.1 Allergenic properties of BLG

Like most of the milk proteins, BLG is produced in mammary gland epithelial cells, under the stimulation of prolactin hormone (29). BLG remains stable in the acidic environment of the stomach and reaches the small intestine mostly unchanged (30) (31). BLG-associated allergic reactions are caused by the presence of specific amino acid sequences (epitopes) of BLG which are recognized by antibodies. Epitopes are amino acid sequences of 8–16 amino acids on the protein surface and act as a potentially allergenic material. At least six epitopes have been found on BLG that are recognized by human IgE from children with CMA (32). The allergenicity of BLG belongs to the type I hypersensitivity. So, when milk is ingested by an infant, BLG resists enzymatic digestion by pepsin in the stomach, allowing it to pass intact through the gut epithelial cells into the blood circulation. The immune system triggers white blood cells to produce BLG-specific allergy antibodies (IgE) and leads to allergy symptoms (33).

1.2.1.1.2 Biological role of BLG

Every year 300 journal articles are published on BLG (28) but the exact physiological function of this whey protein has not been reported (27). BLG

belongs to the lipocalin family of proteins which are generally transporters of small hydrophobic molecules (34). It has been reported that BLG is associated with the transportation of bromododecanoic acid (35), palmitic acid (36) and retinol (27). It has also been linked to hypocholesterolemia (37) and has been ascribed antioxidant properties (38). In the neonatal calf, intestine BLG–retinol complexes have been identified (39) and thus BLG may be involved in transportation and uptake of intact protein directly (40) or indirectly (41). In addition, BLG has also been identified to be an extracellular counterpart of the intracellular fatty acid binding proteins in cow's milk (42). It has been reported that BLG enhances the activity of pre-gastric lipase (42).

1.3 Current strategies to reduce BLG in milk

Cow's milk has become an essential source of food in humans but it contains BLG, a powerful inducer of allergy in early infancy. As a consequence, in the recent years hypoallergenic formulas based on whey protein hydrolysates have been developed to meet the requirements of high risk infants and children. Infant formula companies have produced hypoallergenic formula milk for babies to minimize BLG-related effects. In New Zealand, Nutramigen was the extensively hydrolysed formula (EHF) available that has been replaced by the whey hydrosylate, Pepti-Junior. Nestle has developed an infant formula called NAN HA 1, which is a 100% partially hydrolysed whey protein. Another formula is available in the market known as Neocate which is a synthetic 100% free amino acid-based hypoallergenic formula and is manufactured in a milk protein free environment. It is recommended for infants who are allergic to extensively hydrolysed formula milk (43). The above-mentioned formula milk products are produced by the dairy industries using mainly enzymatic hydrolysis (44). Though enzymatic hydrolysis has become a widely used method of milk processing, it never became popular due to the fact it involves the cleavage of the peptide bond between two amino acid residues that results in breakdown of the intact allergen into smaller peptides. The enzymes used in the hydrolysis step should be specific otherwise one can end up with residual antigenicity by the highest molecular weight peptides (45). Moreover, the degree of enzymatic hydrolysis determines

the breakdown of protein bonds. It has been reported that free (non-protein bound) amino acids such as glutamic acid, aspartic acid and cysteine may act as excitotoxins and can effectively damage brain and nervous system (46). In addition, enzymatic hydrolysis causes unpalatable taste in milk due to the exposure of hydrophobic amino acids such as, serine or glutamate (47) (48). Moreover, the processing technique is very expensive.

Milk components have been targeted for the modification of its nutritional source in order to improve its nutritional value. Two strategies could be used as an alternative tool of expensive post-harvest milk processing to reduce or prevent production of BLG by a more direct approach, RNAi or total deletion of the BLG gene using homologous recombination (HR) (49). It has been reported in mice that complete deletion of another whey protein, α-lactoglobulin, an essential protein component of lactose synthetase resulted in lactose-free milk (50). Lactose controls milk volumes and its absence in milk affects the osmolarity of milk. As a consequence, milk became highly viscous and pups were unable to suck milk from the mammary gland (50). Due to this technical difficulties knockout of BLG in farm animals has not yet been reported yet. Moreover, the efficiency of HR remains very low in farm animals due to low HR in primary somatic cells (49). In comparison to the knockout approach, RNAi could be a straightforward approach to unravel the role of BLG and reduce the allergenicity of cow's milk (51). Moreover, RNAi could allow fine-tuning of BLG expression in particular if some BLG is required for normal milk physiology.

1.4 RNAi

1.4.1 Introduction

RNAi is a phenomenon of mRNA degradation in which short doubled-stranded RNA (dsRNA) is the initiating factor for post-transcriptional gene silencing (PTGS) (52). RNAi plays a vital role in triggering a cascade of events that lead to the degradation or blocking of the corresponding mRNA in a sequence-specific manner (53) and subsequently to a reduction of the level of the corresponding protein (54). RNAi is a conserved biological response to double-stranded RNA

that mediates resistance to both endogenous parasites and exogenous pathogenic nucleic acids (55). In addition, RNAi has also emerged as an important regulator for the expression of endogenous transcripts (54). The power and utility of RNAi and its recent advances has driven its incredibly rapid adaption as a tool for the stable silencing of genes in a variety of different animal species including mammals.

RNA-induced gene silencing was first observed in 1990 when scientists encountered an unexpected suppression of endogenous chalcone synthase (CHS) gene (a pigment producing gene) after introduction of a chimeric CHS gene under the control of a strong promoter into petunia genotypes (56). This phenomenon was initially known as co-suppression where the transgene silenced its expression as well as the expression of the endogenous gene (57). In this reversible co-suppression mechanism RNA polymerase uses sense transcript as a template to produce the dsRNA and triggers RNA silencing. As a result, instead of expected deep purple colour flowers, the flowers became multi-coloured or completely white (56). The co-suppression occurs at the post-transcriptional level and it was defined as PTGS later on (57). The phenomenon of RNAi became clear in 1998 when Fire, Mello and co-workers published their work on *C. elegans*, used as a model animal to study the mechanism of RNAi triggered by dsRNA (52). They injected specific dsRNA molecules or only the sense or antisense RNA strand into *C. elegans* and reported that gene silencing was more effective by dsRNA than sense and antisense strand separately (52). DsRNA-based RNAi was also shown in *Drosophila* and it was reported that dsRNA can silence target gene in the microinjected *Drosophila* embryos (58). Researchers tested specific dsRNAs as a tool to target vertebrate genes using zebrafish and mouse as model animals. It was shown that the silencing efficiency of dsRNA was less efficient than in worms and flies (59). Moreover, dsRNA induced off-target effects in zebrafish, seemingly severely limiting the use of RNAi in the organism (60). Thus, although the use of dsRNA was so successful in worms and flies, it did not seem to work in mammals. As we now know, the reason for the initial failure of the dsRNA-based RNAi in mammals is that long dsRNA molecules elicit an immune response by inducing interferone-linked pathways (61). Long sequences of dsRNA molecules

induce the activation of protein kinase R (PKR), a cellular dsRNA-dependent protein kinase that can be activated either by viral dsRNA intermediates or by the synthetic polyribonucleotide duplexes and can potentially cause non-specific RNA knockdown and ultimately inhibits protein synthesis (62). After the initial disappointment further research showed that small 21 nt long dsRNA molecules (siRNA) can overcome the problem with the unwanted immune responses and cytotoxicity seen for long dsRNAs and work effectively in mammalian cells (63). Soon after this report siRNAs became a popular tool and were successfully used against infectious diseases such as HIV and hepatitis C virus (HCV). It has been reported that synthetic siRNA can specifically inhibit the replication of HIV-I rev transcript in human cells (64) and *in vivo* blocking of HCV gene expression in human liver cells (65). The siRNA approach has shown to be a valid strategy but some specific limitations became apparent such as, the undesired knockdown of non-target transcripts, poor delivery to cells, degradation within a few days and high cost of synthetic siRNAs (66). To resolve these limitations, several groups developed U6 or H1 PolIII promoter-based expression constructs to produce short double-stranded RNA hairpin molecules (shRNA) effective in eliciting gene silencing in mammalian cells (67) (68). Most polIII-based promoters are quite strong and give robust silencing in cell culture systems. However, PolIII promoters are constitutively active and do not allow for restricted expression in a tissue-specific manner. The resulting high level of shRNA expression increases the chances of non-specific gene knockdown and that elicits off-target effects such as interferon response and cytotoxicity (69). These drawbacks of shRNAs have led researchers to improve the design and optimize existing RNAi triggers, in particular miRNA (70). In 1993, Ambros, Ruvkun and co-workers showed that a short non-coding RNA (later called miRNA) *lin-4,* a 22 nt long RNA sequence was partially complementary to multiple sequences in the 3' UTR of the lin-14 mRNA in *C. elegans*. Base paring of the shorter *lin-4* transcript with the lin-14 mRNA transcript knocked down the expression of the gene during development (71). A later study in 2000 reported a second miRNA (*let-7)* that appeared to repress the expression of lin-41, lin-14, lin-28, lin-42, and daf-12 during developmental stage transitions in *C. elegans* (72).

The majorities of the identified endogenous miRNAs are located within the introns of known protein coding genes and regulate endogenous gene function. The primary transcripts of miRNAs give rise to several different mature miRNAs as clusters (73). Generally miRNAs are thought to be involved only in knockdown and/or degradation of target protein coding genes, but recent reports suggested that miRNAs have functions other than knocking down gene expression and blocking protein translation. Vasudevan *et al.*, demonstrated that in human cells (G0-stage), miRNA-369-3p can activate the expression of the tumor necrosis factor-α mRNA through binding to an AU-rich element at the 3' UTR on cell cycle arrest (74). MiRNAs can also bind to the promoter regions of certain genes and up-regulate gene expression (75). In 2002, it was reported that natural and artificially designed miRNAs can inhibit target mRNA in human cells (76). Typically miRNAs are on average 22 nt in length. miRNAs are generally transcribed by RNA polymerase II (77) (78) and result in the degradation or repression of translation of the target mRNA in plants and animals. Recently polII promoter-based miRNA expression vectors have emerged such as-pCDNA6.2GW/EmGFP-miR, Invitrogen. The design of the target specific miRNAs is modeled on the structure of an endogenously expressed miRNA. The miRNAs expressed by the polII promoter get processed by the cell machineries into siRNAs and enter the RNAi pathway for gene silencing. A particularly attractive feature of miRNAs is their potential for tissue-specific or conditional expression *in vivo* and polycistronic strategies (allowing delivery of multiple siRNA sequences) (79) (80). The following Table 1-1 summarizes major discoveries in RNAi field.

Table 1-1: Milestone discoveries in the field of RNAi.

Year	Milestone discovery	Approach	Achievement	Mammalian	Reference
1990	Co-suppression of the target gene	Introduction of chimeric CHS gene	Inhibition of CHS gene expression	Petunia	(56)
1993	Non-coding RNA-mediated knockdown of specific mRNA	Identification of endogenous *lin-4* miRNA	Knockdown of Lin-14 gene	*C. elegans*	(71)
1998	dsRNA as potent and specific cause of RNAi identified	Injection of dsRNA	Suppression endogenous target gene	*C. elegans*	(52)
2001	siRNA duplexes provide a new tool for studying gene function	Transfection of siRNA	Knockdown of endogenous and heterologous genes	Mammalian cells	(63)
2002	shRNA-based knockdown of gene	Transfection of shRNA	Knockdown of firefly luciferase gene	Mammalian cells	(67)
2002	Artificial and natural miRNAs can induce the RNAi pathway	Transfection of miRNA	Sequence specific suppression of a gene	Human cells	(76)

1.4.2 General mechanism of RNAi

RNAi is triggered by dsRNAs in the form of invading RNA viruses or artificial dsRNA molecules. Invading dsRNA molecules (more than 30 bp) are produced by viral replication. The dsRNAs homologous to the target mRNA are converted into 21-23 nt siRNA duplexes by a RNase III enzyme called dicer in the cytoplasm (81) and these siRNA duplexes are assembled into a multi-protein RNA-induced silencing complex (RISC) in the cytoplasm. Functional RISC has helicase, exonuclease and endonuclease activities, and also comprises homology searching domains (82). Within RISC, the siRNA molecule becomes separated and the "sense" strand is degraded. RISC uses the unwound antisense strand as a guide

strand to base-pair with the target mRNA to disrupt the function of the specific gene (63). To protect from the invading virus dsRNAs, host organisms utilize the RNAi pathway to destroy cognate RNAs. On the other hand, miRNAs can be produced from intergenic regions of genome that regulates endogenous gene expression or artificially introduced molecules such as- shRNA or miRNA (sh/miRNA). The hairpin RNAs are first transcribed in the cell nucleus as a long primary transcripts that posses a 5' cap and a poly-A tail (83) (84). The transcribed primary transcript is cleaved in the nucleus by drosha enzyme and produces a 70 nt hairpin transcript (pre-miRNA) (85) (86). The pre-sh/miRNA transcripts are transported to the cytoplasm by exportin 5 through nuclear pores. In the cytoplasm, the pre-miRNA transcripts are cleaved by dicer enzyme and the small ds-transcripts are loaded into RISC complex (81). Hairpin-based RNAi pathway mediates either cleavage or translation termination. A perfect match of mRNA and antisense strand in RISC induces cleavage of target mRNA (most plant miRNAs) (87) (Figure 1-1). In animals, most of the identified miRNAs have partial homology to the target gene encoded protein and causes translational repression. However, miRNAs can also cleave the target mRNA. It has been reported by Yekta *et al.,* 2004 that miRNA-196 had exact complementarity to HOXB8 gene can potentially cleave the message found in mouse embryos (88).

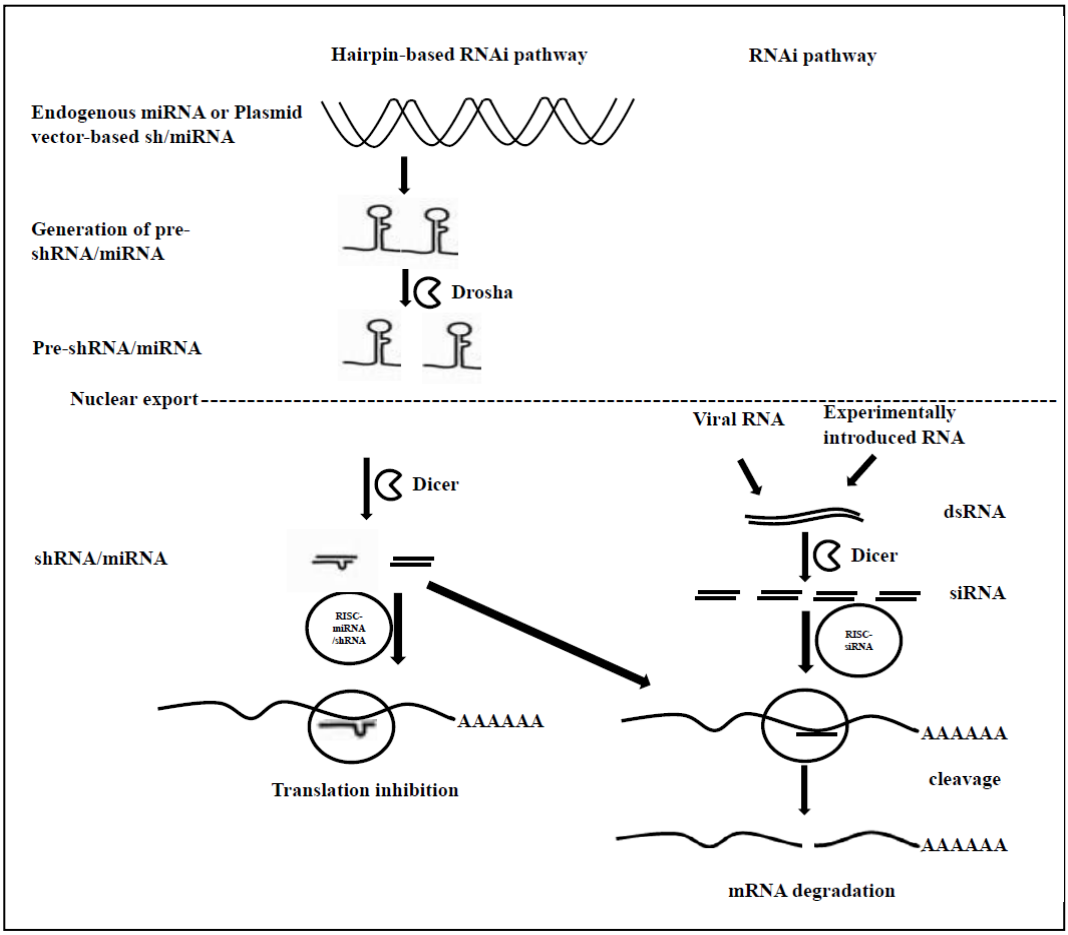


Figure 1-1: RNAi pathways.

Hairpin-based RNAi pathway: Long dsRNA molecules are cleaved by drosha to produce shRNA/miRNA. Hairpin RNA molecules are then transported to the cell cytoplasm and again processed into siRNA molecules by dicer. The siRNA molecules are then incorporated into a RISC. The duplex RNA is unwound leaving the anti-sense strand to guide RISC to complementary mRNA for subsequent translational inhibition.

RNAi pathway: Long dsRNA molecules are cleaved to produce siRNA molecules by dicer. The siRNA molecules are then binds with RISC. The duplex RNA is unwound leaving the anti-sense strand to guide RISC to complementary mRNA for subsequent mRNA degradation.

1.4.3 Recent progress of RNAi in animals

1.4.3.1 Delivery of RNAi molecules: from cells to animals

In the last decade, RNAi has become widely used as a powerful experimental tool and promising biotherapeutic in the field of functional genomics. The

development in the field of RNAi began with the identification of dsRNA in plants and lower organisms targeting specific gene expression. Later on two other small RNAi molecules (synthetic siRNAs and plasmid vector-based shRNAs) were used in mammalian cells to understand gene function. In order to introduce RNAi molecules into cells, several delivery methods such as, retroviral, lentiviral and adenoviral vector systems have been developed to allow efficient and cell type-independent stable RNAi (89). Retroviral vectors are very efficient tools derived from the murine stem cell virus or Moloney murine leukaemia virus and allow stable gene transduction only in dividing cells without transferring any viral gene (90). However, gene transduction by retroviral vectors does not work in non-dividing cells. The major limitation of using retroviral-based gene therapy is the possible transfer of replication competent virus to the patients (91). To address this problem of retroviral vectors, human immunodeficiency virus (HIV)-1-based lentiviral vectors were developed and shown to be efficient to infect both dividing and non-dividing post-mitotic cells, such as, neurons (92). From a biosafety point of view, lentiviruses are acceptable because they are not infectious to human. Adeno-associated virus (AAV) is a small virus and an excellent tool which infects humans and some other primates. It can infect both dividing and non-dividing cells (93) and becomes an attractive tool for gene therapy as this virus has not been reported to cause any disease in human and animals (94). Adenoviral vectors based on AAV have been reported as safer than the other two vectors because adenoviral DNA does not integrate into the genome and remains as an episome in the nucleus. Later on, further improvement on RNAi research was achieved to regulate RNAi externally using conditional RNAi systems. Conditional RNAi-mediated gene knockdown was shown in mice using Cre/loxP for tissue specific control of shRNA expression (95) (96). This procedure of gene expression regulation usually involves the removal of a genetic element that either limits or promotes shRNA expression using the Cre recombinase. Thus it permits to investigate the effect of a gene of interest. Flippase recombination enzyme (FLP)-flippase recognition target (FRT) is another analogous site-specific recombination technique to Cre/loxP that has been applied by Premsrirut *et al*., (97). FLP/FRT involves site-specific recombination between FRT sites mediated by Flp recombinase. Premsrirut, *et al.*, (97) developed a miRNA-30-based shRNA vector

system where shRNA was embedded in the 3′ UTR of a GFP transcript downstream of the inducible TRE-CMV promoter. This vector system showed efficient gene silencing in ES cells and embryonic and adult mouse tissues. These discoveries led to the rapid application of RNAi tools not only limited to cell culture or certain laboratory animals but also make it feasible to apply them in livestock and potentially in drug discoveries. RNAi has helped to better understand gene function in a wide range of organisms. In *C. elegans* and drosophila (*Drosophila melanogaster*), gene manipulation was performed by soaking the animals in a solution containing siRNAs or by adding bacteria expressing siRNAs to the diet (98) (99). RNAi technology has been successfully applied in drosophila to identify the genes with essential functions in biochemical signalling cascades, embryonic development, and other basic cellular processes (100). The whole genome sequencing projects reveals the opportunities to research on novel genes predicted by genome annotation. The biological functions of genes become elucidated using knock-out or knock-down of target genes. In the past, zebrafish (*Danio rerio*) has become a vertebrate model organism to study gene function on embryonic development (101). Soon after the dsRNA-based RNAi study reported in *C. elegans* by Fire *et al.,* (52), RNAi became an effective approach in other eukaryotes, including zebrafish. Microinjection of dsRNAs targeting different genes essential for embryonic development showed specific embryonic defects in embryos (20–30%) (59). Later on, another study showed that a plasmid vector-based dsRNA can potentially knockdown GFP protein (>95%) co-injected in zebrafish embryos (101). The RNAi research in zebrafish was moved forward using siRNA instead of dsRNA because injection of dsRNA has been reported to trigger non-specific effects and toxicity to embryos (102). Liu W-Y *et al.,* (103) reported that siRNA designed against zebrafish no tail gene by *in vitro* transcription can cause specific developmental defects similar to the known no tail mutant. Since these studies, RNAi has become a useful tool for quick and cheap gene knockdown in animals. The delivery of RNAi constructs into model mammalian organism has been achieved by pronucleus injection of mouse zygotes (104) or by introducing shRNAs or siRNAs into adult mice using hydrodynamic injection via tail vein (105) (106) (107).

1.4.3.2 The use of RNAi for the study of disease-associated genes in livestock

Livestock improvements by genetic selection have resulted in considerable progress in livestock production systems worldwide. However, in order to ensure the increasing demands of our expanding population, the use of this strategy is limited as the genetic capabilities and location of the animals are different. Thus the application of RNAi has been evolved to be an attractive method to improve livestock production through disease resistance, production efficiency and nutritional value (108). Livestock production and animal welfare are adversely affected by diseases and they have a great impact on the socio-economic status of farmers and our health. Most of the livestock diseases are caused by viruses, prions, bacteria or parasites. Majority of the bacterial and parasitic diseases can be prevented by proper vaccination and deworming measures. But until today vaccination has remained inefficient to treat viral diseases because viruses use host cell machineries for its replication and thus it becomes difficult to find targets for the drug without interfering with host cells. However, there are a few antiviral drugs with specific antiviral activity on stable viruses but the use of such drugs is limited to those who have already been infected. As a consequence, to control viral diseases, keeping animals in isolation, immunization, vaccination or even killing of infected animals have become routine measures for disease control in livestock. Though using vaccines has been successful in controlling diseases of infected animal population, it has limitation as it has to be delivered by individually injecting each infected animals. Moreover, vaccines are shown to be expensive. Thus, there is a demand for developing new techniques to protect animals from surrounding diseases that have a major impact on our economy and health. Soon after its discovery, RNAi was applied to tackle or at least limit virus shedding and thereby reducing disease spread and the numbers of animals slaughtered despite using the aforesaid measures. RNAi technology has become a new therapeutic tool to study viral cycle and fight against all types of viruses (plus-strand, negative-strand, single stranded and double stranded DNA/RNA) (109). A few examples of economically important viral diseases in livestock such as, foot-and-mouth disease (FMD), bovine viral diarrhoea virus (BVDV), avian

influenza virus (AIV), swine flu (swine influenza), Newcastle disease (ND) and prion disease have been chosen to describe the effectiveness of RNAi to control these diseases.

1.4.3.2.1 FMD

FMD is a highly contagious viral disease in livestock, in particular cattle, sheep, goat, deer and pig and globally it has got the first and foremost priority (110). It is spread by the pathogen, foot-and-mouth disease virus (FMDV) found in infected animals' saliva, milk and dung. The virus can be picked up by the animal or from the direct contact of other infected animals. Moreover, human can be infected by this virus from infected animals. Currently, FMD vaccines based on inactivated virus are available but still carry the risk of incomplete inactivation because complete protection cannot be induced until day seven (111). Thus, development of an alternative strategy became important to resolve the outbreak of FMD. RNAi has been pursued to test its feasibility controlling FMD outbreaks using siRNA to decrease replication of the virus in BHK-21 cells (112). Kahana *et al.*, (112) designed three siRNAs from highly conserved 3B and 3D polymerase gene of FMDV serotype O1 involved in the virus replication. The mixture of siRNAs showed over 90% inhibition of virus replication. Since this result was obtained in cell culture, RNAi has also become a potential tool against FMDV in animal systems. However, the major limitation of this technique is the delivery of the siRNA to the target tissue of an adult organism (113). To deliver RNAi molecules more efficiently in tissues or whole organisms, Chen *et al.,* (114) carried out a study using a plasmid vector-based shRNA targeting 1D and 3D regions of FMDV also involved in virus replication. They designed one specific shRNA for each of the FMDV 1D and 3D regions and tested the effectiveness of the shRNA against the virus replication in swine kidney cells (IBRS-2). They demonstrated complete knockdown of FMDV replication in IBRS-2 delivered by recombinant replication-defective human adenovirus type 5 (rAd5). They also tested these FMDV-specific rAd5 constructs in guinea pig by intramuscular injection with FMDV HKN/2002. Three out of five guinea pigs did not show any clinical signs such as vesicles on foot and mouth or high fever. Other animals developed few

smaller vesicles but recovered quickly afterwards. A mixture of rAd5 constructs was injected in guinea pig but did not show significant antiviral effect.

1.4.3.2.2 BVD

Bovine viral diarrhoea (BVD) is another viral disease in ruminants, including cattle and sheep, affecting herds worldwide and causing significant economic impacts. Many production losses from BVDV occur and as a consequence of persistent infections, milk production and conception rate become low and the animals become susceptible to respiratory disorders and other diseases (115). The BVD virus transmits vertically (before birth) that leads to congenital infections of the fetus and causes abortion or stillbirth. It can also transmit to other healthy animals after birth of the animal. Traditionally, infected cattle are separated or discarded from the other healthy animals to reduce chances of spreading of the virus. Calves are fed high quality colostrum to increase the antibody titer to fight against infections. Final strategy is to use vaccines of BVDV to treat infected animals. Despite the use of one or a combination of these methods, BVDV remains the most prevalent infectious agent in cattle herds all over the world (116). The effect of the disease can cause huge economic losses and that is why the application of RNAi therapeutics has become so important for this disease. It has been reported by Lambeth *et al.*, (116) that siRNA and shRNA can efficiently inhibit the replication of BVDV in Madin-Darby bovine kidney (MDBK) cells. They designed seven siRNAs to inhibit BVDV replication by targeting coding regions (C, NS4B and NS5A proteins) of the BVDV genome required for BVDV replication. The siRNAs were first transfected into MDBK cells and three of seven siRNAs showed significant suppression of BVDV antigen production ($P<0.05$). To compare siRNAs and shRNAs against inhibition of BVDV antigen production, they constructed DNA-based shRNA expression vectors using the most effective siRNA sequences. The efficiency of shRNAs was very low compared to siRNAs and the best siRNA showed >90% inhibition of BVDV antigen, only 50% by the shRNA.

1.4.3.2.3 AIV

Highly pathogenic AIV was one of the first viral diseases described in poultry. H5N1 is a subtype of the influenza A virus also known as "bird flu", which can cause illness in humans and many other animal species. The bird flu virus has already killed millions of birds and forced farmers to dispose hundreds of millions of poultry to stop its proliferation. When H5N1 strain infects humans, it replicates in the lower part of the respiratory tract and ultimately it causes viral pneumonia (117). Vaccines of either killed virus or recombinant surface glycoproteins have been using to prevent the pandemics of the virus. But the use of these vaccines is limited due to the continual mutation of H5N1, rendering the vaccines ineffective against any new virus subtype. So, effective and relatively inexpensive RNAi technique has become a potential anti-influenza therapeutics alternative to the classical vaccinations. Zhang *et al.,* (118) showed siRNA-mediated knockdown of avian influenza virus subtype H5N1 both *in vitro* and *in vivo*. They targeted Polymerase A (PA) gene conserved among different avian influenza virus subtypes and strains, including H5N1. PA protein is the part of the influenza virus RNA polymerase complex which is the crucial component in the viral RNA transcription and replication. For *in vitro* studies they designed three siRNAs specific to the N-terminal region of the PA gene and transfected into Madin Darby canine kidney (MDCK) cell lines infected with the virus. Analysis of the transfected MDCK cells showed a 5-78-fold reduction of virus titers by the siRNAs. The most effective siRNA construct was injected with the virus into mouse. After one day of injection the mouse was sacrificed and the lung homogenates showed significant reduction of virus replication by the siRNA.

1.4.3.2.4 Swine flu

Another type A influenza virus known as swine flu virus is a threat to swine industry. Swine flu is a respiratory disease in pig and it is contagious transmitting from pig to human. Several subtypes of influenza virus A have identified in pigs such as, H1NI, H1N2, H3N2 and H3N1. H1N1 is the most common version and its outbreaks have caused significant monetary damage in the swine industry world wide. Swine flu has similar symptoms to other influenza such as fever, coughing, nasal secretion, headache, vomiting and diarrhoea. The most recent

strain (H1N1) of swine flu virus was found in Mexico in 2009 and it caused 277,607 cases of viral infection including 3205 deaths (as of September 2009) reported by the World Health Organization (WHO) (119). The virus had been found in most of the countries in that year. Commercially available vaccines for swine flu showed limited effect in pigs challenged with the virus (120). For human, four antiviral drugs (oseltamivir, zanamivir, amantadine and rimantadine) are available on the market and they show limited protection against the virus with side effects. The side effects are similar to the other seasonal vaccines, including a mild fever, headache and fatigue. The 2009 swine flu outbreak drew attention to researchers to find an alternative treatment than vaccination as viruses have high mutation rate that makes vaccines ineffective. RNAi-based silencing of swine flu virus has recently been started since its outbreak in 2009. Last year it was reported that the novel H1N1 virus replication can be inhibited using synthetic RNAi molecules *in vitro* (119). They tested 35 synthetic siRNAs specific to the conserved regions of the H1N1 virus mRNA in A549 cells and ten of them showed over 90% inhibition of the virus replication in cells.

1.4.3.2.5 ND

ND is another infectious viral disease of birds caused by the Newcastle disease virus. It affects the respiratory, nervous and digestive systems of the bird. ND does not pose hazard to human health like avian influenza virus, but it causes substantial economic losses in the poultry industry worldwide. Yin *et al.,* (121) targeted Newcastle disease viral matrix protein gene using specific shRNA in cell cultures. *In vitro* studies showed that DNA vector-based shRNA could efficiently block the replication of the virus.

1.4.3.2.6 Prion disease

Another important target of RNAi is prion (PrP) disease. Prions (PrPs) are infectious agents that contain malformed proteins. Prions are responsible for the transmission of spongiform encephalopathies in cattle (also known as mad cow disease) which can be potentially transmitted to humans. Introduction of a prion into a healthy organism induces the pre-existing properly-folded proteins to convert into misfolded proteins. As a consequence, highly stable misfolded

protein accumulates in infected tissue, causing tissue damage and cell death (122). When prions infect central nervous system, they accumulate in infected tissues within the central nervous system and form plaques known as amyloid which disrupt normal tissue structure. Symptoms of such neurodegenerative disease include convulsion, dementia and behavioral changes leading to brain dysfunction and death. In 2006, RNAi-based knockdown of PrP in goat and cattle was reported (123). Golding *et al.,* transfected human embryonic kidney (HEK)-293T cells with PrP mRNA specific shRNAs and firefly luciferase-PrP expression plasmid. Some of the shRNAs showed high PrP mRNA knockdown up to 86% in cell cultures and similar results were revealed in a Tg fetus (123).

1.4.3.3 The use of RNAi for the study of genes associated with livestock improvement

1.4.3.3.1 Myostatin

In order to meet the extra demand for the increasing population, it is important to improve livestock production efficiency and food value of daily consumable food such as cow milk. Production efficiency of animals can be increased by silencing endogenous gene expression, such as the myostatin gene using RNAi. Myostatin is known as growth differentiation factor 8 which inhibits muscle differentiation and growth. Primarily it is secreted from the skeletal muscle cells and acts on skeletal tissues. Animals that produce less myostatin have larger skeletal muscles. In order to increase meat production, scientists have targeted myostatin gene using RNAi to control its expression. The gene encoding myostatin was discovered in 1997 by McPherron *et al.,*(124). They also generated myostatin knockout mice which showed remarkable increase in skeletal muscle mass compared to wild type mice. Soon after this report, research on myostatin became attractive to scientists. In some cattle breeds such as Belgian Blue and Piedmontese, these animals have mutations in their myostatin gene and absence of the functional gene helps in growth and proliferation of muscle cells (125). *In vitro* studies on myostatin expression showed that over expression of this growth factor decreases muscle mass and fiber size in Tg mice (126). After the discovery of RNAi in 1998, it has become a prime tool to prevent the expression of myostatin gene. Magee *et al.,*

(127) showed that plasmid vector-based shRNA silenced mouse myostatin gene expression in cell culture. They also demonstrated *in vivo* suppression of the gene and consequently skeletal muscle mass increase was seen in rat after intramuscular electroporation of the effective shRNA. Another study was reported by Kinouchi *et al.,* (128), they showed the possible use of siRNA as a novel therapeutic strategy to silence the expression of myostatin gene expression in mice. They delivered myostatin-specific siRNA into normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen (ATCOL). Within few weeks of time they observed a remarkable increase in skeletal muscle mass in mice. After these successes RNAi has been applied to inhibit caprine myostatin gene. Four shRNAs were designed based on the bovine and caprine myostatin gene and caprine fibroblast cells were transfected with plasmid vector containing myostatin-specific shRNAs. All of the tested shRNAs showed 22-92% knockdown of the gene (129).

1.4.3.3.2 Porcine BLG

Very recently, a paper has been published on knockdown of porcine BLG using various RNAi molecules (shRNAs and artificial miRNAs) using an *in vitro* system (130). They compared six shRNAs driven by U6 promoter and the expression of the two miRNAs was driven by CMV promoter against the expression of porcine BLG gene. Transient transfection of HEK293 revealed that four shRNAs were efficient to knockdown BLG in the range of 22-74%. MiRNAs were also shown to be effective to knockdown the gene up to 60%.

1.4.3.4 The use of RNAi-based therapeutics for clinical applications

Since its discovery, RNAi has become a versatile tool to understand and control gene function. Researchers have worked on RNAi over a decade to understand its mechanism and its role on gene function. Expression of gene of interest has been studied by using different RNAi molecules both in cell cultures and in animals. In addition, the use of RNAi technology has moved to pre-clinical studies due to advancement made in understanding cellular pathways during states of pathogenesis, gene delivery systems and availability of a wide range of *in vivo* models. From the *in vitro* and *in vivo* studies, RNAi has raised a lot of interest in

developing RNAi-based therapeutics for clinical applications. In order to develop RNAi-based therapeutics, RNAi sequences are first designed and tested against targets in the cell cultures and selected RNAi sequences are then applied to animal models with diseases of interest. Well-performing RNAi sequences may finally be considered for the advance stages of clinical trials. Since the discovery of RNAi, many rapid advances have been achieved but using RNAi as therapeutics is still in its infancy. Currently, few clinical trials are in progress targeting several genes that if knocked down might be expected to improve human health. The most advanced clinical trials are in the treatment of age-related macular degeneration (AMD) which is the common cause of blindness. The genes for vascular endothelial growth factor (VEGF) and the VEGF receptor (VEGFR) were targeted by injecting siRNA into the macula of the eye in order to stop excessive vascularization of the eyes which leads to blindness in preclinical models (131). This research led the therapeutic to test in human clinical trials. Acuity Pharmaceuticals carried out first clinical trials for the treatment of AMD, of which phase I and II were reported with a lack of adverse systemic effects (132). This drug had gone into phase III trials carried out by Opko Health, but unfortunately phase III trials were terminated because it was found that siRNAs could block angiogenesis and activate an immune receptor called toll-like receptor 3 (TLR3) in mouse models of AMD regardless of its sequence (133). Though the effectiveness of RNAi has become questionable after AMD clinical trials, however, some RNAi-based clinic trials that have been published show promising results for other therapeutic target, such as respiratory syncytial virus (RSV) nucelocapsid (N) protein. RSV is a very common virus that causes cold-like symptoms in adults and older healthy children. It can be severe in young babies producing about 10-fold higher mortality rates than influenza in United States (134). Alnylam Pharmaceuticals Inc., Cambridge, MA, USA has made a major advancement for targeting RSV N gene (135). The ALN-RSV01 therapeutics in phase II and it is considered as the most advanced program in the global RNAi pipeline (136). They have used an unmodified siRNA formulated in saline which is administered daily via a nasal spray to patients two days before and three days after inoculation with RSV and which has resulted in 38% decrease in the RSV infected patients (137).

1.5 Rationale and Objectives of the Study

Cow milk is a common food for both infants and adults. Cow milk allergy (CMA) is more prevalent in infants as milk is their primary source of nutrition and it is uncommon in adults and older children. Cow milk evolved as an optimal food for calves, but human milk and nutritional requirements of human are different. So, milk from dairy ruminants has become the subject of intense investigations to improve or modify its quality (138). The composition of milk can be altered either by nutritional management (139) or by alteration of genetic potential of the dairy cows (140). Nutritional management includes feeding of balanced ration to the dairy cows to help to express their genetic potential and for milk production, and milk protein and milk fat composition. Genetic improvement in the dairy cows can be made by selection and breeding of cows with superior genetic merits. Although more efforts have gone into these traditional methods, changes in the milk composition are slow (141) and not too much change has been achieved especially for the traits that are low heritable and difficult to measure by their breeding value (142). Recent advancements in genomic research including whole genome sequencing and high throughput single nucleotide polymorphism (SNP) genotyping has opened the door to identify new genetic variants for milk quality improvement (142). Moreover, identification of SNPs can also play an important role in crop and livestock improvement through breeding programmes. The use of Tg technologies has sparked in livestock improvement. Such improvements could include the modification of the major milk proteins (caseins) to improve the nutritive value of cheese, reducing lactose content in milk to reduce intestinal disorders, production of milk with the over expression of the human form of the antimicrobial protein lyzozyme and human lactoferrin to increase protection against gastro-intestinal infections and removing undesirable, potentially allergic components (e.g., BLG) to create ‘hypoallergic’ milk (51). Reducing the level of those proteins causing allergies could make milk amenable for individuals currently experiencing milk allergies. The most common product of the dairy industry is whole milk and fat-reduced milk, both of which are not modified to mimic human milk. Moreover, infant formula manufacturers are also producing hypoallergenic or hydrolyzed milk, where milk proteins are broken down into

smaller peptides so they are less allergenic than whole proteins. But as a consequence, the taste of milk becomes bitter and almost unpalatable (143).

A number of molecular genetic techniques have been developed to study gene expression and its physiological function. The techniques include gene knockout, and most recently RNAi (144). The gene knockout is a technique in which an endogenous gene of an organism is deleted or mutated to make the gene inoperative. The absence or mutation of the gene can be crucial for the expression of other genes and thus limits the use of this technique (145). Since the discovery of endogenous antiviral defense mechanism mediated by siRNA molecules in plants and animals, RNAi has become an effective alternative tool for gene silencing applications. While RNAi was first demonstrated in nematodes with the introduction of exogenous long dsRNA molecules (52), it soon became clear that RNAi is a conserved strategy utilized by the most eukaryotes to fight viral infections and fine tune the regulation of genes (54).

So, the main aim of this doctoral thesis was to demonstrate the feasibility of reducing the level of BLG in milk by mammary gland-specific expression of miRNAs using a mouse model. The detailed objectives are shown below:

- Evaluation of *in vitro* knockdown efficiency of BLG-specific miRNAs targeting bBLG and oBLG expression.
- Generation of Tg mouse lines for the mammary gland -specific expression of functional BLG–specific miRNAs.
- Generation of Tg mouse lines expressing bBLG in their milk.
- Generation of miRNA-BLG mouse lines expressing bBLG or oBLG and BLG specific miRNAs.
- Evaluation of the miRNA mediated knockdown potential *in vivo*.

1.6 Thesis Layout

The thesis contains six chapters and appendices summarized as follows:

Chapter One: This chapter provides a literature review addressing the effects of cow milk allergy (CMA) on infant health. This chapter particularly emphasizes on BLG and reviews its allergenic and biological properties and current strategies to

handle this milk allergen as decribed in the literature. This chapter introduces RNAi in more detail and presents recent use of this technology to improve livestock production and health or to stop replication of life threatening viruses. This chapter concludes with an outline of five research objectives to conduct this research.

Chapter Two: The general materials and methodologies used to perform this study are written in details in this chapter. This chapter also includes methodologies specificly used for chapter three, four and five.

Chapter Three: This chapter covers results on transfection efficiency optimization of COS7 cells. It also investigates the effect of single and tandem miRNA constructs against BLG expression *in vitro.* The chapter ends with a discussion on comparing the obtained results with the published datas.

Chapter Four: This chapter includes results on generation of miRNA, bBLG and miRNA-o/bBLG mice and characterization of the Tg mice. Finally, a discussion is added at the end of this chapter to compare the results obtained with the published information on Tg mouse production.

Chapter Five: This chapter presents knockdown activity of single and tandem miRNA constructs against bBLG and oBLG expression in mice. A discussion is added which covers the comparision of BLG knockdown ability by single and tandem miRNA constructs using coomassie staining and western blot analysis. It also includes a comparison of BLG knockdown efficiency by the single and tandem miRNA constructs *in vitro* and *in vivo*.

Chapter Six: This chapter presents a general discussion of the thesis with recommendations for future research.

Appendices: Appendices include results obtained from miRNA constructs screening, generation of BLG constructs, generation of tissue-specific miRNA constructs and preparation of tissue specific miRNA and bBLG constructs for pronuclear microinjection. Appendices also include gene sequencing results of miRNA, WAP promoter and BLG gene.

Chapter Two: Materials and Methods

2.1 General Materials and Methods

2.1.1 General Materials

2.1.1.1 General chemicals and reagents

Name	Source
Avertin	Sigma (Germany)
Bovine serum albumin (BSA)	Sigma (Germany)
Bromophenol blue sodium salt	Sigma (Germany)
Chloroform	BDH (UK)
Coomassie Brilliant Blue R-250 crystals	Bio Rad (USA)
Diethyl pyrocarbonate (DEPC)	Sigma (Germany)
Dimethyl sulfoxide (DMSO)	Sigma (Germany)
Dithiothreitol (DTT) (1 M)	Invitrogen (USA)
Dulbeccos Modified Eagles Medium (DMEM)	Invitrogen (USA)
Ethanol (EtOH)	Fisher Scientific (UK)
Ethidium bromide (EB) (10 mg/ml)	Invitrogen (USA)
Ethylenediaminetetraacetic acid (EDTA)	BDH (UK)
Fetal calf serum (FCS)	Gibco (USA)
Formaldehyde	J T Baker (USA)
Formamide	Fisher Scientific (UK)
Glacial acetic acid	J T Baker (USA)
Glycerol	J T Baker (USA)
N-2-ethane-sulphonic acid (HEPES)	Invitrogen (USA)
Human chorionic gonadotropin (hCG)	Intervet (UK)
Hydrochloric acid (HCl)	J T Baker (USA)

Isopropanol	Biolab (AU)
Magnesium chloride ($MgCl_2$)	J T Baker (USA)
Methanol	Fisher Scientific (UK)
3-(N-morpholino)-propane sulfonic acid (MOPS)	Sigma (USA)
OptiMEM	Invitrogen (USA)
Oxytocin	Sigma (USA)
Penicillin	Sigma (USA)
Phenylmethylsulfonyl fluoride (PMSF)	Sigma (USA)
Phosphate buffered saline (PBS)	Oxoid (UK)
Ponceau	Sigma (USA)
Pregnant mare serum gonadotropin (PMSG)	Intervet (UK)
Proteinase Inhibitors cocktail	Sigma (USA)
RNA sample buffer	Invitrogen (USA)
RNase AWAY	Thermo Fisher Scientific Inc (USA)
See blue plus 2 pre-stained marker	Invitrogen (USA)
Sodium bicarbonate ($NaHCO_3$)	Sigma (USA)
Sodium chloride (NaCl)	Sigma (USA)
Sodium dedecyl sulphate (SDS)	Sigma (USA)
Sodium hydroxide (NaOH)	BDH (UK)
Streptomycin	Sigma (USA)
N, N, N', N'- Tetraethylethylenediamine (TEMED)	Sigma (USA)
Tris-base	J T Baker (USA)
Triton-X 100	Sigma (USA)
TRIzol	Invitrogen (USA)
Trypan blue	Sigma (USA)
Trypsin/EDTA	Invitrogen (USA)
Tween 20	Bio Rad (USA)
Whatman 3 MM Paper	Whatman (USA)
Biomax XAR film	Kodak (USA)

Xylene cyanol	Biolab (AU)

2.1.1.2 General biological materials

2.1.1.2.1 Molecular biology

Name	Source
Bacto-agar	BDH (UK)
Bacto-tryptone	Difco Microbiology (USA)
Takara DNA ligation mighty mix	Takara (Japan)
Ultra pure agarose	Axygen (Spain)
Yeast extract	Sigma (USA)
1 kb^+ DNA ladder	Invitrogen (USA)

2.1.1.2.2 PCR reagents

Name	Source
Deoxyribonucleotide triphosphate (dNTP)	Roche (USA)
PCR reaction buffer (10 x)	Roche (USA)
Taq DNA polymerase	Roche (USA)
Expand High Fidelity PCR System	Roche (USA)

All stock solutions were stored at -20 °C.

2.1.1.2.3 Enzymes

Proteinase K Invitrogen (USA)

Restriction endonucleases- following restriction enzymes were purchased from various commercial sources

Table 2-1: A list of restriction enzymes used in this experiment.

Restriction enzyme	Recognition sequence	Source
*Bam*HI	G/GATCC	Roche (USA)
*Bgl*I	GCCNNNN/NGGC	Roche (USA)
*Bgl*II	A/GATCT	Roche (USA)
*Dra*I	TTT/AAA	Roche (USA)

*Eco*RV	GAT/ATC	Roche (USA)
*Fsp*I	TGC/GCA	Roche (USA)
*Hind*III	A/AGCTT	Roche (USA)
*Not*I	GC/GGCCGC	Roche (USA)
*MSc*I	TGG/CCA	Roche (USA)
*Sac*II	CCGC/GG	Roche (USA)
*Sal*I	G/TCGAC	Roche (USA)
Shrimp alkaline phosphatase (SAP) (1 U/µl)		Promega (USA)
SpeI	A/CTAGT	Roche (USA)
*Xho*I	C/TCGAG	Roche (USA)

2.1.1.3 Oligonucleotides

All used oligonucleotides were obtained from Invitrogen and are indicated in Table 2-2 and Table 2-3.

Table 2-2: RNAi-specific oligonucleotides.

Name	Number	Position	Sequence 5´-3´	Application
miRNA 1	GL 705 GL 706	BLG mRNA exon 1	TGCTGATATCCAGGCCCTTCATGGTCGTTTTGGCCACTGACTGACGACCATGAGGCCTGGATAT CCTGATATCCAGGCCTCATGGTCGTCAGTCAGTGGCCAAAACGACCATGAAGGGCCTGGATATC	BLG mRNA knockdown
miRNA 2	GL 707 GL 708	BLG mRNA exon 2	TGCTGTTCTGCAGCAGGATCTCCAGGGTTTTGGCCACTGACTGACCCTGGAGACTGCTGCAGAA CCTGTTCTGCAGCAGTCTCCAGGGTCAGTCAGTGGCCAAAACCCTGGAGATCCTGCTGCAGAAC	BLG mRNA knockdown
miRNA 3	GL 709 GL 710	BLG mRNA exon 2	TGCTGAGCTCCTCCACATACACTCTCGTTTTGGCCACTGACTGACGAGAGTGTGTGGAGGAGCT CCTGAGCTCCTCCACACACTCTCGTCAGTCAGTGGCCAAAACGAGAGTGTATGTGGAGGAGCTC	BLG mRNA knockdown
miRNA 4	GL 711 GL 712	BLG mRNA exon 4	TGCTGTTTGTAGTCGGTGTCCAGCACGTTTTGGCCACTGACTGACGTGCTGGACCGACTACAAA CCTGTTTGTAGTCGGTCCAGCACGTCAGTCAGTGGCCAAAACGTGCTGGACACCGACTACAAAC	BLG mRNA knockdown
miRNA 5	GL 713 GL 714	BLG mRNA exon 5 and 6	TGCTGAGATGTGGCACTGCTCCTCCAGTTTTGGCCACTGACTGACTGGAGGAGGTGCCACATCT CCTGAGATGTGGCACCTCCTCCAGTCAGTCAGTGGCCAAAACTGGAGGAGCAGTGCCACATCTC	BLG mRNA knockdown
miRNA 6	GL 715 GL 716	BLG mRNA exon 3	TGCTGCTTCTGAGCACACTCACCGTTGTTTTGGCCACTGACTGACAACGGTGAGTGCTCAGAAG CCTGCTTCTGAGCACTCACCGTTGTCAGTCAGTGGCCAAAACAACGGTGAGTGTGCTCAGAAGC	BLG mRNA knockdown

miRNA 7	GL 717 GL 718	BLG mRNA exon 3	TGCTGATCTTGAACACCGCAGGGATCGTTTTGGCCACTGACTGACGATCCCTGGTGTTCAAGAT CCTGATCTTGAACACCAGGGATCGTCAGTCAGTGGCCAAAACGATCCCTGCGGTGTTCAAGATC	BLG mRNA knockdown
miRNA 8	GL 719 GL 720	BLG mRNA exon 3 and 4	TGCTGTTCAAGGCATCGATCTTGAACGTTTTGGCCACTGACTGACGTTCAAGAGATGCCTTGAA CCTGTTCAAGGCATCTCTTGAACGTCAGTCAGTGGCCAAAACGTTCAAGATCGATGCCTTGAAC	BLG mRNA knockdown
miRNA 9	GL 721 GL 722	BLG mRNA exon 4	TGCTGCCAGCACAAGGACTTTGTTCTGTTTTGGCCACTGACTGACAGAACAAACCTTGTGCTGG CCTGCCAGCACAAGGTTTGTTCTGTCAGTCAGTGGCCAAAACAGAACAAAGTCCTTGTGCTGGC	BLG mRNA knockdown
miRNA 10	GL 723 GL 724	BLG mRNA exon 4	TGCTGTTTGCTCGGGCTCAGCACTGTGTTTTGGCCACTGACTGACACAGTGCTGCCCGAGCAAA CCTGTTTGCTCGGGCAGCACTGTGTCAGTCAGTGGCCAAAACACAGTGCTGAGCCCGAGCAAAC	BLG mRNA knockdown

*bBLG and oBLG mRNA sequence accession numbers are NM_173929 and NM_001009366, respectively.

Table 2-3: Oligonucleotides used for PCR for direct cloning and genotyping.

Name	Number	Sequence 5´-3´	Application
oBLG_F	GL 356	TGGGCCTCAGAGACCAAGAG	Internal control for genotyping
oBLG_R	GL 357	AACTGTGTCCCACTAACCCC	Internal control for genotyping
miRNA_F	GL 725	TCCCAAGCTGGCTAGTTAAG	miRNA forward sequencing primer
miRNA_R	GL 726	CTCTAGATCAACCACTTTGT	miRNA reverse sequencing primer
oBLG_F	GL 727	ATGAAGTGCCTCCTGCTTGCC	oBLG cDNA forward sequencing primer
oBLG_R	GL 728	CTAGACGTGGCACTGCCCCTC	oBLG cDNA reverse sequencing primer

M13_R	GL 729	CAGGAAACAGCTATGAC	Sequencing
pGTC451_F	GL 730	GATCTCGAGCTCAAGCTTCCT *XhoI*	PCR cloning for generation of pGL 449
pGTC451_R	GL 731	GTTCTCGAGTCACCTAGATGTGGCACTGCT *XhoI*	PCR cloning for generation of pGL 449
pGTC451_F	GL 732	AGCATGGAGTCTTGGACATT	PCR for genotyping bBLG mice
pGTC451_R	GL 733	CTCCTCCACATACACTCTCAG	PCR for genotyping bBLG mice
WAP-MBP_F	GL 734	GTTTACTAGTGCAATTCTTTCACTG *Spe*I	PCR cloning for generation of pGL 298
WAP-MBP_R	GL 735	GTTTGATATCGCAAGTGACTGATGG *Eco*RV	PCR cloning for generation of pGL 298
pGEM-T_WAP_1F	GL 736	GCAATTCTTTCACTGCTAAA	Sequencing of WAP promoter
pGEM-T_WAP_1R	GL 737	GCTGATAGTGAGATTCTGCTG	Sequencing of WAP promoter
pGEM-T_WAP_2F	GL 738	TAGAGTTTCAAGAGGCTGA	Sequencing of WAP promoter
pGEM-T_WAP_2R	GL 739	CCAGAGCTCTGTTCTCTCTA	Sequencing of WAP promoter
pGEM-T_WAP_3F	GL 740	CACACTGTGCTCATCACTTTG	Sequencing of WAP promoter
pGEM-T_WAP_3R	GL 741	CTCTTGCTTGTCCTCTGAGC	Sequencing of WAP promoter
pGEM-T_WAP_4F	GL 742	CTGGAATAGACGAATGTAGA	Sequencing of WAP promoter
pGEM-T_WAP_4R	GL 743	TGCTCCATGTAGAGAGTGTC	Sequencing of WAP promoter
pGEM-T_WAP_5F	GL 744	CAGCAGGACATCTCTTCCTGC	Sequencing of WAP promoter

pGEM-T_WAP_5R	GL 745	TATCGCAAGTGACTGATGGC	Sequencing of WAP promoter
miRNA_F	GL 746	GAGGTAGTGAGTCGACCAGTG	PCR for genotyping miRNA mice
miRNA_R	GL 747	AGTGCGGCCAGATCTGGG	PCR for genotyping miRNA mice

*Underlined sequence represents additional restriction enzyme site

2.1.1.4 Expression vectors

Table 2-4: **Plasmid DNA expression vectors.**

Plasmid	Index number	Functional sequence	Reference or Source
pBS SK	pGL 40		Stratagene (USA)
pGFP-BLG	pGL 152	CMV promoter driven bBLG cassette	Ben Wilson (AgResearch, Ruakura, Hamilton, NZ)
pcDNA5/FRT	pGL 164	CMV promoter driven expression construct	Invitrogen (USA)
pEGFP-C1	pGL 195	CMV promoter driven GFP expression vector	Clontech (USA)
pEPI WAP-MBP	pGL 263	WAP promoter driven MBP cassette	Stefan Wagner (AgResearch, Ruakura, Hamilton, NZ)
pcDNA™6.2-GW/EmGFP miRNA (linearize)	pGL 273	CMV miRNA expression vector containing an GFP expression cassette	Invitrogen (USA)
pGFP-miRNA 1	pGL 274	GFP CMV miRNA expression vector	miRNA oligos GL 705 and GL 706 were annealed and ligated into pGL 273 vector
pGFP-miRNA 2	pGL 275	GFP CMV miRNA expression vector	miRNA oligos GL 707 and GL 708 were annealed and

			ligated into pGL 273 vector
pGFP-miRNA 3	pGL 276	GFP CMV miRNA expression vector	miRNA oligos GL 709 and GL 710 were annealed and ligated into pGL 273 vector
pGFP-miRNA 4	pGL 277	GFP CMV miRNA expression vector	miRNA oligos GL 711 and GL 712 were annealed and ligated into pGL 273 vector
pGFP-miRNA 5	pGL 278	GFP CMV miRNA expression vector	miRNA oligos GL 713 and GL 714 were annealed and ligated into pGL 273 vector
pGFP-miRNA 6	pGL 279	GFP CMV miRNA expression vector	miRNA oligos GL 715 and GL 716 were annealed and ligated into pGL 273 vector
pGFP-miRNA 7	pGL 280	GFP CMV miRNA expression vector	miRNA oligos GL 717 and GL 718 were annealed and ligated into pGL 273 vector
pGFP-miRNA 8	pGL 281	GFP CMV miRNA expression vector	miRNA oligos GL 719 and GL 720 were annealed and ligated into pGL 273 vector
pGFP-miRNA 9	pGL 282	GFP CMV miRNA expression vector	miRNA oligos GL 721 and GL 722 were annealed and ligated into pGL 273 vector
pGFP-miRNA 10	pGL 283	GFP CMV miRNA expression vector	miRNA oligos GL 723 and GL 724 were annealed and ligated into pGL 273 vector.
pGFP-miRNA 11	pGL 285	GFP CMV tandem miRNA expression vector	Tandem construct contains miRNA 2 and miRNA 3.
pGFP-miRNA 12	pGL 286	GFP CMV tandem miRNA expression vector	Tandem construct contains miRNA 2 and miRNA 6.

pGFP-miRNA 13	pGL 287	GFP CMV tandem miRNA expression vector	Tandem construct contains miRNA 3 and miRNA 6.
pGFP-miRNA 14	pGL 288	GFP CMV tandem miRNA expression vector	Tandem construct contains miRNA 2 and miRNA 4.
pGFP-miRNA 15	pGL 289	GFP CMV tandem miRNA expression vector	Tandem construct contains miRNA 6 and miRNA 4.
pGFP-miRNA 16	pGL 290	GFP CMV tandem miRNA expression vector	Tandem construct contains miRNA 3 and miRNA 4.
pcDNA™6.2-GW/miRNA-neg control	pGL 291	CMV miRNA expression vector containing an GFP expression cassette and a scramble miRNA	Invitrogen (USA)
pGTC 451	pGL 293	Goat beta casein promoter, 2 x chicken insulator and beta casein flanking sequences	GTC (USA)
pcDNA5/FRT-bBLG	pGL 297	CMV promoter driven bBLG cassette	
WAP-miRNA 6-4	pGL 298	Mammary tissue specific WAP promoter driven miRNA 6-4 cassette	
WAP-miRNA 4	pGL 446	Mammary tissue specific WAP promoter driven miRNA 4 cassette	
WAP-miRNA 6	pGL 447	Mammary tissue specific WAP promoter driven miRNA 6 cassette	
pcDNA5/FRT-oBLG	pGL 448	CMV promoter driven oBLG cassette	
pGTC451-bBLG	pGL 449	Goat beta-casein promoter driven bBLG cassette	
TOPO-TA		For T/A nucleotide cloning of PCR insert	Invitrogen (USA)
pGEM-T Easy		For T/A nucleotide cloning of PCR insert	Promega (USA)

2.1.1.5 Kits

Kit	Source
BCA protein assay kit	Thermo Fisher Scientific Inc (USA)
Calibrite ™ 3	BD Biosciences (USA)
MinElute PCR Purification Kit	Qiagen (Germany)
pGEM-T Easy cloning kit	Promega (USA)
QIAGEN Plasmid kit (mini)	Qiagen (Germany)
QIAGEN Plasmid kit (maxi)	Qiagen (Germany)
Super Script® III First Strand Synthesis System	Invitrogen (USA)
TOPO TA Cloning kit Invitrogen	Invitrogen (USA)
Wizard® SV gel and PCR clean-up system	Promega (USA)

2.1.1.6 Bacterial cell culture materials

All media and glassware were sterilized by autoclaving at 121°C for 30 minutes.

2.1.1.6.1 Bacterial strains

Strain	Genotype	Source
MAX Efficiency DH5α cells	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen (USA)
One Shot TOP10-cells	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen (USA)
Top 10F *E. coli*	F´{*lac*Iq, Tn*10*(TetR)}c *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen (USA)

2.1.1.6.2 Lauria-Bertani (LB) medium

LB broth for bacteria culture contained 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract and 1% NaCl. The powder was suspended in MilliQ H_2O and sterilized by autoclaving. Culture plates for bacteria colony selection contained 1.5% (w/v) bacto-agar.

2.1.1.6.3 SOC medium

SOC medium contained 20 g bacto tryptone, 5 g bacto yeast extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$, 20 ml of 1 M glucose in 1 litre (L) of MilliQ H_2O. The solution was sterilized by autoclaving and kept at room temperature.

2.1.1.6.4 SOB medium

SOB medium contained 20 g bacto tryptone, 5 g bacto yeast extract and 0.5 g NaCl in 950 ml of MilliQ H_2O.

2.1.1.7 Mammalian cell culture

2.1.1.7.1 Cells

The African green monkey kidney fibroblast-like cell line COS7 was obtained from the American Type Culture Collection (ATCC ® CCL-70). Cells were aliquoted into cryogenic storage vials with 10% of DMSO and stored into liquid nitrogen (see Section 2.1.2.15.3).

2.1.1.7.2 Cell culture medium

The base cell culture medium was purchased from Gibco (USA) and 15.6 g of the powder was dissolved in 850 ml of MilliQ H_2O with 1.2 g of $NaHCO_3$. The pH was adjusted to 7.2 and made up to 1000 ml. The medium was sterilized by filtration through a 0.20 µm disposable filter. The medium was kept at 4°C for up to one month.

2.1.1.8 Antibodies used for western blot assay

Unconjugated rabbit anti-bBLG antibody was purchased from Bethyl Laboratories, USA (1 mg/ml). Anti-GFP rabbit antibody (2 mg/ml) was obtained

from Invitrogen. Horseradish Peroxidase (HRP) conjugated goat anti-rabbit antibody (1 mg/ml) was supplied by Sigma (USA).

2.1.1.9 Buffers and stock solutions

All solutions were made up using deionised MilliQ H_2O from a water purification system (Millipore).

2.1.1.9.1 General buffers and stock solutions

EDTA (0.5 M)	14.6 g EDTA in 100 ml ddH2O, pH 8.0.
PBS (10 x)	80 g of NaCl, 2.0 g KCL, 11.5 g of Na_2HPO_4 and 2.0 g of NaH_2PO_4 in 1 L of MilliQ H_2O, pH 7.2. The solution was sterilized by autoclaving and stored at room temperature.
PBS (1 x)	Stock solution (10 x) was diluted 10-fold in MilliQ H_2O.
PBST	0.05% tween 20 in 1 x PBS, pH 7.2.
Tris base (1 M)	121.1 g of tris base in 1 L of MilliQ H_2O, pH 7.5.
Trypan blue solution	0.5 g of trypan blue and 0.8 g in 100 ml MilliQ H_2O. The stock solution was sterilized by filtration through a 0.20 µm disposable filter. The solution was kept at 4°C.

2.1.1.9.2 Antibiotic solutions for cell cultures

Ampicillin (50 mg/ml)	0.5 g of ampicillin in 10 ml of MilliQ H_2O. The solution was sterilized by filtration through a 0.20 µm disposable filter and was stored at -20°C.
Kanamycin (25 mg/ml)	0.25 g of kanamycin sulphate in 10 ml of MilliQ H_2O. The solution was sterilized by filtration through a 0.20 µm disposable filter and was stored at -20°C.
Streptomycin (50 mg/ml)	0.5 g of streptomycin in 10 ml of MilliQ H_2O. The solution was sterilized by filtration through a 0.20 µm disposable filter and was kept at -20°C.
Spectinomycin (20 mg/ml)	0.2 g of spectinomycin in 10 ml MilliQ H_2O. The

	solution was sterilized by filtration through a 0.20 µm disposable filter was kept at -20°C.
X-gal (20 mg/ml)	0.2 g of X-gal (5-bromo-4-chloro-3-indolyl b-D-galactopyranoside) in 10 ml of DMF (dimethylformamide). The stock solution was stored protected from light at -20°C.

2.1.1.9.3 Buffers and stock solutions for molecular biology

Cell lysis buffer	20 mM Tris-HCL (pH 7.5), 5 mM EDTA, 1% triton X and 1 mM PMSF. PMSF was added fresh to the buffers immediately prior to use.
DEPC-H_2O	100 µl DEPC to 100 ml ddH_2O. Mixed well and let set at room temperature for 1 hour. The solution was sterilized by autoclaving and stored at room temperature.
DNA loading buffer (10 x)	0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol in 30% (v/v) glycerol in MilliQ H_2O.
Glycerol (25%)	25 ml of glycerol in 100 ml of MilliQ H_2O. The solution was sterilized by autoclaving and stored at room temperature.
Inoue transformation buffer	55 mM $MnCl_2.4H_2O$, 15 mM $CaCl_2.2H_2O$, 250 mM KCL and PIPES (0.5 M, pH 6.7).
Lysis buffer for mouse tails	100 mM Tris (pH 8), 5 mM EDTA, 0.1% SDS and 200 mM NaCl
Microinjection buffer	10 mM Tris-HCl, 0.1 mM EDTA and 100 mM NaCl.
10 x MOPS buffer	0.2 M MOPS, 0.05 M sodium acetate, 0.001 M EDTA, pH 7.0. The solution was sterilized by filtration through a 0.20 µm disposable filter and was stored at room temperature.

TAE (20 x)	96 g of tris base, 22.84 ml of glacial acetic acid and 100 ml of 0.5 M EDTA in 1 L of MilliQ H_2O. The buffer was kept at room temperature.
TE buffer	1 ml of 1 M tris-HCl (pH 8.0) and 0.2 ml of 0.5 M EDTA in 100 ml of MilliQ H_2O.

2.1.1.10 Reagents for transfection analysis

Name	Source
FuGENE 6	Roche (USA)
Lipofectamine 2000	Invitrogen (USA)
Lipofectamine LTX plus	Invitrogen (USA)

2.1.1.11 Buffers and reagents for western blot assay

Name	Source
Antibody incubation solution	0.1% BSA (w/v) and 0.05% (v/v) tween
Blocking buffer	1.0% (w/v) PVP, 0.1% BSA (w/v) and 0.05% (v/v) tween
Bis-Tris pre-cast polyacrylamide gel	Invitrogen (USA)
ECL detection reagent A	GE Healthcare (UK)
ECL detection reagent B	GE Healthcare (UK)
SDS-PAGE sample buffer (4 x)	Invitrogen (USA)
SDS-PAGE running buffer (20 x)	Invitrogen (USA)

2.1.1.12 Coomassie blue stain solutions

Destaining solution	50% (v/v) methanol in MilliQ H_2O with 10% (v/v) acetic acid.
Gel fixing solution	50% (v/v) ethanol in MilliQ H_2O with 10% (v/v) acetic acid.
Gel washing solution	50% (v/v) methanol in MilliQ H_2O with 10% (v/v) acetic acid.

Staining solution	0.1% (w/v) of coomassie blue R 350, 20% (v/v) methanol and 10% (v/v) acetic acid in MilliQ H_2O. The stain solution was filtered through a 0.20 µm disposable filter to remove any insoluble material.

2.1.1.13 Equipment

2.1.1.13.1 Specific equipment and instruments

Name	Source
Autoclave	Tuttnauer autoclave (USA)
Bacteria incubator	Sanyo (USA)
Bacteria shaker	Infos HT (Switzerland)
Bench centrifuge	Eppendorf (Germany)
Cell culture incubator	Thermo Forma (USA)
Cell culture microscope	Nikon TMS (Japan)
Cell culture hood	Gelman Sciences (USA)
Computer	HP (USA)
Cryofreezing block	Nalgene™, Nunc International (USA)
Deep freezer (-80°C)	Thermo Fisher Scientific Inc (USA)
Developing cassettes	Kodak (USA)
Developing machine	All-Pro Imazing Corp (USA)
Electrophoresis power supply	E-C Apparatus Corporation (USA)
Electrophoresis unit	Horizon™ (USA)
Becton Dickinson FACS sorter	BD Biosciences (USA)
Gel Doc 1000	Bio Rad (USA)
Heating block	Eppendorf (Germany)
Hemocytometer	Improved Neubauer; Weber, (UK)
iBlot® dry blotting unit	Invitrogen (USA)
Ice machine	A best kitchen (USA)
Liquid nitrogen tank	Chart MVE Biological Systems (USA)
Millipore H2O-prduction unit Milli-Q plus	Bio Lab (AU)

Microwave oven	Sharp (China)
NanoDrop spectrophotometers	Thermo Fisher Scientific Inc (USA)
Olympus BX50 microscope	Olympus (Japan)
pH meter	Mettler Toledo (USA)
PCR	Bio Rad (USA)
Pipettes (p-20, p-200, p-1000)	Biohit group (Finland)
Scale	Sartorius (Germany)
Spectrophotometer	Eppendorf (Germany)
Sonicator (Thyristor Regler TR50)	IKA (USA)
Sorvall refrigerated centrifuge	International MI-SS, Inc (USA)
Vortex	Chiltern Scientific (Australia)
Water bath (37°C, 42°C and 65°C)	Grant Instruments ltd (UK)

2.1.1.13.2 Culture vessels

2.1.1.13.2.1 Plastic ware

Disposable non-treated and sterile cell culture dishes (10 cm), multi-well plates (6-well, 12-well and 24-well) and cryogenic vials were purchased from Nalge Nunc International (Denmark). Sterile plastic centrifuge tubes (15 ml and 50 ml) were purchased from Becton Dickinson lab ware (USA). Single-use disposable sterile pipettes (1 ml, 2 ml, 5 ml, 10 ml and 25 ml) were obtained from Bio Lab (AU). Disposable filter tips with different sizes (10 µl, 20 µl, 200 µl and 1,000 µl) were purchased from Molecular Bio products (USA). Micro centrifuge tubes (1.5 ml and 2.0 ml) were obtained from Bio Lab (AU). PCR-reaction tubes were purchased from Axygen Scientific Inc (USA).

2.1.1.13.2.2 Glassware

Storage bottle with different sizes (100 ml, 250 ml, 500 ml and 1000 ml), volumetric flasks (100 ml, 250 ml, 500 ml, and 1,000 ml), beakers (50 ml, 100 ml, 250 ml, and 400 ml), and measuring cylinder (50 ml, 100 ml, 250 ml and 500 ml) were purchased from Schott Duran (Germany) and Pasteur pipettes were purchased from Poulten & Graf Ltd (UK). All glassware used in this research were washed with detergent, rinsed in water and sterilized by autoclaving at 121°C for 30 minutes.

2.1.1.13.2.3 Other Consumables

Biomax XAR film was purchased from Kodak (USA). iBlot® transfer stack consumables were obtained from Invitrogen (USA).

2.1.1.14 Software

Name	Source
BLOCK-iT™ RNAi Designer tool	Invitrogen (USA)
CellQuest Pro	BD Biosciences (USA)
Chromas Lite version 2.01	Technelysium pty ltd (Australia)
Microsoft Excel 2007	Microsoft (USA)
Quantity One software	Bio Rad (USA)
Vector NTI Advance™ 11	Invitrogen (USA)

2.1.1.15 Animals and Animal Ethics Approval

Pathogen-free mouse colonies were raised in pathogen-free conditions at the SAC, AgResearch, Ruakura, Hamilton, New Zealand. For microinjection and generation of the Tg mouse lines, the FVB and C57BL/6 mice strains were used. Homozygous oBLG expressing mice were kindly provided by Dr. Bruce Whitelaw (Roslin Institute, UK). The study protocol 764 has been approved by the University of Waikato research and ethics committee on December, 2008.

2.1.2 General methods

2.1.2.1 Generation of chemically competent cells

Competent cells of *E. coli* DH5α were prepared by a method described previously (146). In brief, a single colony of *E. coli* was cultured overnight at 37°C with moderate shaking (230 rpm) in 5 ml of LB medium. The bacterial culture was inoculated into a 1 L flask containing 250 ml SOB and incubated at 37^{o}C with shaking. The culture was monitored every 45 minutes until the optical density (OD) reached 0.55 at 600 nm. The cells were harvested by centrifuging at 4,500 rpm (Sorvall GSA rotor) in a 50 ml falcon tube for 10 minutes at 4^{o}C. The supernatant was discarded and the cell pellet was resuspended in 80 ml of ice-cold Inoue transformation buffer. The cell suspension was centrifuged at 4,500 rpm

(Sorvall GSA rotor) for 10 minutes at 4^{o}C to remove traces of SOB medium. The resulting cell pellet was resuspended gently in 20 ml of ice-cold Inoue transformation buffer and 1.5 ml of DMSO was added into the bacterial suspension which was incubated on ice for 10 minutes. The bacterial suspension was aliquoted into chilled, sterile microfuge tubes and snap-frozen by immersing into liquid nitrogen. The ampoules of competent cells were stored at -80^{o}C for future use. The transformation efficiency of the bacteria was measured by transforming a circular plasmid DNA (pGL 40) (see Section 2.1.2.12) and the bacterial preparation producing ~0.8x10^{7} colony forming units (CFU) per µg of plasmid DNA.

2.1.2.2 Isolation of nucleic acids

2.1.2.2.1 Small-scale preparations of plasmid DNA by miniprep

Small-scale plasmid DNA isolation was done using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions. In brief, the bacterial cultures were pelleted by low speed centrifugation at 6,000 rpm for 5 minutes on a bench-top microfuge (Eppendorf). Harvested bacterial cells were resuspended in 250 µl P1 buffer and transferred to a microcentrifuge tube. The cells were lysed by adding 250 µl of P2 buffer and mixed thoroughly by inverting the tube 4–6 times. 350 µl of N3 buffer was then added and mixed immediately as above to neutralize the reaction and centrifuged for 10 minutes at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge. The supernatant was retained by decanting into the QIAprep spin column and centrifuged at 13,000 rpm for 60 seconds. The flow-through was discarded and the QIAprep spin column was washed by adding 0.5 ml of PB buffer followed by centrifuging for 60 seconds. Again the flow-through was discarded and the QIAprep spin column was washed by adding 0.75 ml PE buffer and centrifuged for 60 seconds. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and DNA was eluted in 50 µl EB buffer.

2.1.2.2.2 Large-scale preparations of plasmid DNA by maxiprep

Large-scale plasmid DNA isolation was done using the QIAprep Spin Maxiprep Kit in accordance to the manufacturer's instructions. In brief, a single colony was cultured over night in 500 ml of LB containing the appropriate antibiotic at 37°C

with shaking (225 rpm). The bacterial cells were harvested by centrifugation at 6,000 rpm for 15 minutes at 4°C (Sorvall GSA rotor). All traces of supernatant were removed and the pellet was completely resuspended in 10 ml P1 buffer. The cells were lysed by adding 10 ml of P2 buffer and mixed thoroughly by inverting the tube 4–6 times and incubated at room temperature for 5 minutes. The reaction was neutralized by adding 10 ml of chilled P3 buffer followed by inverting 4-6 times and centrifuged at 18,000 rpm (Sorvall SS 34 rotor) for 30 minutes at 4°C (in Beckman 40 ml polypropylene tubes). The supernatant was poured into a clean centrifuge tube and re-centrifuged at 18,000 rpm (Sorvall SS 34 rotor) for 15 minutes at 4°C. The Qiagen tip was equilibrated by applying 10 ml QBT buffer and by gravity flow. The supernatant was poured into the column and allowed to enter the resin by gravity flow. The tip was washed twice with 15 ml QC buffer and DNA was eluted with 5 ml EB buffer in a 40 ml polypropylene centrifuge tube. The eluted DNA was precipitated by adding 10.5 ml propan-2-ol at room temperature. The DNA was collected by centrifugation at 18,000 rpm (Sorvall SS 34 rotor) for 30 minutes at 4°C. The DNA pellet was washed with 5 ml ice-cold 70% ethanol and centrifuged at 18,000 rpm (Sorvall SS 34 rotor) for 10 minutes at 4°C. Finally, the pellet was air dried at room temperature for 10 minutes and dissolved in 100 µl TE buffer.

2.1.2.2.3 Isolation of genomic DNA (gDNA)

For the genotyping of Tg mice gDNA was obtained from 4 mm of mouse tail biopsies. They were digested with 99 µl of tail digestion buffer with 1 µl of proteinase K (20 mg/ml). The digestion was carried out for 2 hours at 55 ºC and 1,200 rpm on a thermo mixer for complete disruption of the tail. The lysate was centrifuged at 13,000 rpm for 1 minute to spin down the unwanted debris. The supernatant was transferred to a fresh microfuge tube and the enzyme was inactivated by heating at 65ºC for 15 minutes. Finally, the gDNA was diluted in TE buffer (1:20,000 with TE buffer).

2.1.2.2.4 Isolation and purification of total RNA

2.1.2.2.4.1 Handling of RNA

As ribonucleases (RNases) are very stable and active enzymes, special care was taken to isolate total RNA from mouse tissue. RNA isolation place benches were treated with RNase AWAY. All solutions were made in DEPC treated MilliQ H_2O. Sterile, disposable polypropylene tubes and filter pipette tips were used for RNA isolation. Electrophoresis tanks, glassware, spatulas, stirring bars, mortar and pestle were treated with DEPC-treated water to eliminate RNases.

2.1.2.2.4.2 Isolation of RNA from mouse mammary tissue

Total RNA from mammary tissue was carried out in order to isolate mRNA. A mRNA is the transcript of a gene and is a source to synthesize cDNA for research purpose. So, in order to synthesize oBLG cDNA (variant B), total RNA from Tg oBLG mouse mammary tissue was isolated using TRIzol reagent in accordance to the manufacturer's instruction. In brief, tissue samples were homogenized in liquid nitrogen using a pestle and mortar. 1 ml of TRIzol was added per 50-100 mg of homogenized tissue in a pre-chilled 2 ml nuclease free microfuge tube and incubated for 5 minutes at room temperature. Chloroform was added (0.2 ml/tube) and mixed by inverting tube for 4-6 times. The solution was incubated for 5 minutes at room temperature. The sample was centrifuged at 12,000 x *g* for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh microfuge tube. RNA was precipitated by adding 0.5 ml of propan-2-ol and incubated for 5 minutes at room temperature. RNA was pelleted by centrifuging at 12,000 x *g* for 10 minutes at 4°C. The pellet was washed once with 75% ice-cold ethanol and centrifuged at 7,500 x *g* for 5 minutes at 4°C. The pellet was air dried for 10 minutes at room temperature and dissolved in 100 µl DEPC water.

2.1.2.3 Quantification of nucleic acid

The concentration of plasmid DNA and total RNA was measured using spectrophotometry (Nanodrop). Before nucleic acid measurements the machine was calibrated with water followed by blanked it with solutions used to re-suspend DNA or RNA. The concentration of the nucleic acid sample was

measured in ng/µl. The purity of DNA and RNA was determined by dividing the readings of the absorbance at 260 nm and 280 nm. A good quality nucleic acid of 1.7–2.0 at A_{260}/A_{280} absorbance ratio was considered for further experiments.

2.1.2.4 Restriction digestion of DNA

To ensure that the clones contained the target insert, plasmid DNA of the clones were restriction digested. DNA to be digested was diluted in 1 x restriction digestion buffer provided by the suppliers. Endonucleases of 3-5 units were used per µg of DNA. The mixture was mixed by tapping the tube with fingers and briefly centrifuged to remove air bubbles. The reaction was incubated at 37°C for 2 h. The reaction was incubated at 65°C for 15 minutes to de-activate the restriction enzymes.

2.1.2.5 Detection of DNA on agarose gel electrophoresis

An agarose gel was used to separate DNA fragments. The separation of DNA fragments depends on the size of molecules as small fragments run faster than larger fragments on gel metrix. To separate smaller DNA fragments (<200 bp) 2% agarose gel was used. To separate larger DNA fragments (>200 bp) 0.8% agarose gel was used.The gel was prepared by adding 0.8 g of ultra pure agarose powder in 100 ml of 1 x TAE running buffer and heated in a microwave oven until completely melted. 10 µl of SYBR Safe (10,000 x) (Molecular probes, invitrogen) was added to the gel to visualize DNA bands under UV light. The gel was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. A molecular weight ladder of 1 kb^+ (Invitrogen) was added in an adjacent well in order to determine size of the sample DNA bands. Samples containing 6 x DNA loading dye were pipetted into gel wells and run in an electrophoresis tank (Invitrogen) at a constant voltage of 100 volts. The gel was run until the bromophenol blue dye had run to the 2/3 of the length of the gel. The stained DNA was visualized by an ultraviolet transmitter at 350 nm. The image was captured using a camera and analysed using Quantity One software (Bio Rad).

2.1.2.6 Purification of DNA from agarose gel

After electrophoresis, the stained gel was visualized on a UV light box (a trans-illuminator). The desired DNA band was excised using a scalpel blade from the agarose gel under the UV transilluminator with a short period of exposure. The DNA fragments were purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega). In brief, the weight of the individual gel slice was determined and three volumes of membrane binding solution were used followed by incubation of 65°C for 10 minutes to solubilise the agarose. The DNA/gel solution was loaded onto a SV minicolumn. The mixture was centrifuged at 13,000 rpm for 1 minute. The flow through was discarded and the DNA was washed with membrane wash solution to remove residual salts. The column was placed into a sterile 1.5 microfuge tube and the DNA was eluted from the column using elution EB buffer.

2.1.2.7 Dephosphorylation of vectors

Vectors with compatible ends after restriction digest require dephosphorylating in order to prevent re-ligation of the vector to itself. Alkaline phosphatase catalyses the removal of 5' phosphate groups from DNA. In order to prevent the re-ligation of vectors, the 5’ phosphate group of the vector was removed using SAP prior to ligation. The digested DNA was dephosphorylated using 10 units of SAP per µg of vector with 10 x dephosporylation buffer to prevent DNA ligase from re-circularizing of the vector. The mixture was incubated for 15 minutes at 37°C. The SAP was inactivated by incubating at 65°C for 15 minutes.

2.1.2.8 Polymerase chain reaction (PCR)

PCR allows amplification of a particular DNA sequence in a cyclic manner. PCR amplification consists of three steps: Step 1, denaturation of double strand DNA, step 2, primers annealing and step 3, synthesis of new DNA strands (extension). A standard PCR was set up for a final volume of 50 µl.

A PCR reaction mix included:

38 µl MilliQ H_2O, 5 µl 10 x PCR buffer ($MgCl_2$), 0.5 µl dNTP mix (10 mM each dNTP), 0.5 µl sense/forward primer (100 µM), 0.5 µl anti-sense/reverse primer (100 µM), 5 µl template and 1 µl Taq polymerase (5 U).

The PCR cycling condition was:

95 °C	4:00	(minute:second)
95 °C	0:30 ┐	
Tm*	0:30 ├	x 35
72 °C	0:30** ┘	
72 °C	7:00	

*Tm determined by gradient PCR

** Primer extension time dependent of the insert size, as a rule 1 kb = 1 minute

2.1.2.8.1 Primer design for PCR

The PCR primers were designed to amplify the gene of interest. All primers used in this study were designed using Vector NTI (Invitrogen, USA) and the software generated primer sequences were further analysed by Geneious Pro (Version 4.8) (BioMatters Ltd, NZ) to check for secondary structure in the primer sequence. The primers were designed for PCR following with particular specifications. The product length was a minimum of 150 bp to maximum 650 bp, optimum temperature (Tm) of 50-60 °C, primer length of 20-21 nucleotides, 40-50 % GC, avoid complementarity of primes to prevent formation of primer dimmers and avoid GC clamps in sequence. Primers were supplied by Invitrogen (USA) and the lyophilized primers were diluted in 1 x TE buffer adjusted to the final concentration of 100 µM. Although primers were designed according to the rules of primer design, it is sometimes hard to find perfect primer pair for PCR. So, a gradient PCR was run to determine the annealing temperature in order to amplify single product followed by running the products on agarose gel.

2.1.2.9 Synthesis of first-strand cDNA

The synthesis of cDNA involves making copies of DNA from specific mRNA. The reverse transcriptase enzyme uses the oligo dT primer that binds with the polyA tail of the mRNA (3') to start DNA synthesis. For the first strand cDNA

synthesis, the SuperScript III First-Strand Synthesis System (Invitrogen) was used according to the manufacturer's instructions. In brief, 5 µg of total RNA was taken in a 0.5 ml nuclease free microfuge tube. 1 µl of oligo dT (50 µM), 1 µl of dNTP mix (10 mM) and DEPC-dH_2O to a final volume of 10 µl were added into the tube. The mixture was incubated at 65°C for 5 minutes and kept on ice for 1 minute. A cDNA synthesis master mix was prepared by adding each component in the indicated following order. The master mix was added to the RNA/primer mixture and incubated at 50°C for 50 minutes in a thermo mixer. The reaction was terminated by incubating at 85°C for 5 minutes and chilled on ice. Finally, 1 µl of RNase H was added to each tube and incubated for 20 minutes at 37°C to degrade RNA in RNA:DNA hybrids. The first strand cDNA synthesis reaction was immediately used for PCR amplification.

Component	Amount
10 x RT buffer	2 µl
1 M DTT	2 µl
25 mM $MgCl_2$	4 µl
RNaseOUT (40 U/µl)	1 µl
SuperScript III RT (200 U/µl)	1 µl

2.1.2.10 Ligation of DNA fragments

Takara DNA ligation mighty mix was used to ligate the DNA fragments. The digested plasmid vector DNA was combined with the insert DNA fragment with a molar ratio of 1:3. An equal volume of ligation mix was mixed with the DNA mixture and mixed thoroughly. The ligation reaction mix was incubated at 16°C for 30 minutes.

2.1.2.11 Sub-cloning of the PCR product

Sub-cloning of DNA is the transfer of the DNA fragment from one plasmid of interest to another. The TOPO TA (Invitrogen)/pGEM-T Easy (Promega) cloning utilizes the enzyme, DNA topoisomerase I to integrate a PCR product. PCR products were sub-cloned into shuttle vectors before being cloned into appropriate expression vectors. The TOPO vector/pGEM-T vector is a convenient system to sub-clone PCR products. A PCR product generated using *Taq* polymerase

contains compatible 3' A hanging ends. Annealing of the insert and TOPO vector was done in accordance with the manufacturer's instructions. 4 µl of PCR product was mixed with 1 µl of salt solution and 1 µl of TOPO vector. The mixture was incubated at room temperature for 30 minutes. The cloning reaction was transformed into competent cells (see Section 2.1.2.12).

2.1.2.12 Transformation of *E. coli* competent cells

E. coli competent cells were thawed on ice and the ligation reaction was added into 100 µl of competent cells. The ligation reaction was incubated on ice for 30 minutes with competent cells. The DNA and cell mixture was heat shocked at 42°C for 30 seconds and immediately returned to ice and kept on ice for 3 minutes. A fresh aliquot of 250 µl of SOC medium was added to the transformants and incubated at 37°C with shaking at 250 rpm for 1 hour. The cells were plated on LB agar plates containing an appropriate concentration of specific antibiotic and incubated over night at 37°C with shaking at 250 rpm.

2.1.2.13 DNA sequencing

2.1.2.13.1 Sequencing

Plasmid DNA was send to Massey University for sequencing to confirm that there was no introduced point mutation in the DNA sequence and the ligation sites are correct. The sequencing reaction was set up in a final volume of 15 µl, with 300 ng of DNA template and 3.2 pmol of forward and/or reverse sequencing primer.

2.1.2.13.2 Sequence analysis

The sequencing results obtained from Massey University were interpreted by using sequence analysis software Vector NTI (Invitrogen). Any mismatches in the sequenced DNA sequence can be checked by looking at the chromatogram using Chromas Lite (technelysium, Au) software.

2.1.2.14 Glycerol stock

Bacterial culture of the recombinant positive clone was preserved as a 25% glycerol stock by adding 500 µl of an overnight culture to 500 µl of 50% glycerol

in a 2 ml screw top tube. The glycerol stock tube was frozen down at -80^{o}C until required.

2.1.2.15 Mammalian cell culture

The culture of COS7 cells was carried out in a sterile laminar flow hood (Westinghouse Pty Ltd, NSW, Australia). To keep the hood sterile it was routinely wiped with 70% ethanol before and after use. Everything used for cell culture was also wiped with 70% ethanol. Cells were cultured at 37°C and 5% CO_2 in a water jacket incubator (Forma Scientific).

2.1.2.15.1 Cell culture

COS7 cells were seeded into 10 cm^2 sterile culture plate. When cells were grown to log phase, cells were washed gently with sterile PBS to remove dead cells. Cells were trypsinized with pre-warmed (37°C) trypsin-EDTA at 37°C for 5 minutes. The trypsin was deacitivated by adding 5 ml of fresh medium and trypsinized cells were pelleted by centrifuging at 1,000 rpm for 3 minutes. The supernatant was removed by aspiration and 5 ml of pre-warmed culture medium was added to the cell pellet. The cell clumps were broken by pipetting up and down and again pelleted by centrifugation at 1,000 rpm for 3 minutes. The supernatant containing the residues of trypsin was discarded and 1 ml of fresh pre-warmed medium was added to resuspend the cell pellet.

2.1.2.15.2 Viable cell count

The viability of cells was determined by using trypan blue dye. It stains the dead cells by taking up the dye through the damaged cell membrane. In living cells, the cell membrane extrudes the dye because it acts as a selective barrier to foreign compounds to cross cell membrane. Cell suspension of 20 µl was taken into a 1.5 ml of microfuge tube and an equal volume of trypan blue solution was mixed with the cell suspension by inverting the tube 4-6 times. The number of stained cells was counted using a Neubauer hemocytometer using a 20 x inverted phase contrast Olympus CK2 microscope. The cell number was determined by counting four corners of 4 x 4 squares of the hemocytometer. The following formula was used to calculate the number of viable cells:

$$\text{Cell number (cells/ml)} = \frac{\text{Total cell counts}}{4 \times 10^{-4}} \times 2$$

2.1.2.15.3 Cryo-preservation of mammalian cells

When the cells were in excess they were frozen down in the liquid nitrogen for future use. Cultured cells were centrifused at 1,000 rpm and the cells were re-suspended in 1 ml of fresh culture medium. 1 ml of freezing media was added to the cell suspension, which contained 500 µl FCS, 300 µl of DMEM-F12 culture medium and 200 µl DMSO. The cells were then divided into two storage cryo vials and put them into a "Mr Frosty" (Nalgene™ Cryo 1°C Freezing container) that contains isopropanol and placed in a -80°C freezer. The slow freezing of the cells prevents cells from damage from ice crystals. On the following day, the cryo vials were transferred to a liquid nitrogen tank for long term storage.

2.1.2.15.4 Recovery of cells from liquid nitrogen storage

Stocks of surplus cells were kept in cryovials from liquid nitrogen (-196°C) was removed and immediately placed in a 37°C water bath. The thawed cell culture was pipetted out into a sterile 15 ml tube containing 5 ml of fresh medium. The cells were centrifuged at 1,000 rpm for 3 minutes and the supernatant was discarded by aspiration. The cell pellet was re-suspended in pre-warmed fresh medium and precipitated the cell pellet by centrifugation to remove any residues of DMSO. The cell pellet was re-suspended with fresh medium and transferred to a tissue culture flask.

2.1.2.16 Protein analysis by western blot assay

2.1.2.16.1 Preparation of cell lysate

After 48 hours of transfection, cells were washed gently with sterile PBS to remove dead cells. Cells were trypsinized using trypsin-EDTA (100 µl/well) and pelleted at 1,000 rpm for 3 minutes. The cell pellet was washed with 0.5 ml of culture medium to get rid of trypsin residues and centrifuged at 1,000 rpm for 3 minutes. The lysis buffer with protease inhibitor cocktail (1 µl/million of cell) was added to the cell pellet. Sample tubes were vortexed before sonication and bursts

for two 15 seconds at maximum output power on ice using a Misonix Sonicator (U.S.A). Cell lysates was cleared by centrifugation at 13,000 rcf at 4ºC for 15 minutes. The supernatant was removed and analysed for protein content using the BCA (bicinchoninic acid) assay.

2.1.2.16.2 Quantification of protein

The protein concentration of the lysates was measured by BCA protein assay kit in accordance to the manufacturer's guide lines. In brief, a working solution was prepared by mixing 50 parts of reagent A with 1 part reagent B. Protein (albumin) standards (A-H) were made following the manufacturer's guidelines. Sample lysate was mixed with the working solution at 1:20 and incubated in a water bath at 37ºC for 30 minutes. Finally, the protein concentration of the samples was measured using the Nanodrop spectrophotometer at 562 nm.

2.1.2.16.3 SDS polyacrylamide gel electrophoresis (PAGE)

The SDS-PAGE was run using a NOVEX gel apparatus. Protein samples were mixed with 4 x SDS sample loading buffer with 50 mM DTT and boiled at 95°C for 5 minutes before loading. The 10% Bis-Tris pre-cast polyacrylamide gel was assembled into the gel tank and 1 x MES running buffer was poured into the tank up to the top of the gel (~800 ml) and the comb of the gel was removed carefully. The boiled samples were cooled to room temperature and loaded onto the gel with a pre-stained marker. Electrophoresis was carried out at constant voltage (120 V) until the bromophenol blue dye reached to the bottom of the resolving gel. The gel cassette was opened using a spatula to expose the gel. The top and bottom electrode areas of the gel were trimmed off by spatula. The gel was ready for staining with coomassie stain or western blot procedure.

2.1.2.16.4 Transfer of protein

Proteins from gel were transferred to transfer membrane using iBlot gel transfer device (Invitrogen, USA). Once the gel was cut out of the cassette, it was placed on the transfer membrane of the anode stack. The gel was then topped with a pre-soaked (MilliQ H_2O) filter paper and air bubbles were removed using the blotting roller. The cathode stack was topped over the pre-soaked filter paper followed by removing air bubbles using the blotting roller. Finally, a disposable sponge was

placed with the metal contact of the lid. The transfer was performed in 7 minutes. After transfer the membrane was stained with Ponceau for 5 minutes to check efficiency of proteins from gel to membrane followed by destaining with MQ H_2O. The transfer membrane contained the proteins of interest was ready for the western blot procedure.

2.1.2.16.5 Blocking and immune-staining of the blot

The membrane was blocked with blocking buffer (see Section 2.1.1.11) for 2 hours at room temperature on a rocker. Following this, the membrane was incubated with primary antibody (rabbit anti-bBLG, 1:10,000 dilution) or anti rabbit GFP (1:2,000 dilution) in antibody incubation solution against BLG or GFP protein for overnight at room temperature. The membrane was washed three times with an interval of 10 minutes between each wash. The membrane was then incubated with the secondary antibody (goat anti rabbit-HRP conjugated, 1:20,000 dilutions in antibody incubation solution) for 2 hours at room temperature. Finally, three washes of 10 minutes with PBS were done before chemiluminescent detection was made.

2.1.2.16.6 Detection of protein

Detection was made in dark room using enzyme linked chemiluminescence (ECL) detection reagents (A and B) from Amersham (UK). The secondary antibody used was horse radish peroxidase conjugated and it catalyses the break down of luminol in presence of a substrate (alkaline phosphatase) which produces detectable signals (Figure 2-1). ECL reagent A was mixed with ECL reagent B in 1:1 ratio to give sufficient solution to cover the membrane and incubated for 1 minute at room temperature. After the incubation, the excess ECL mixture was aspirated and the membrane was wrapped in plastic and exposed to an X-ray film (KOdak) in a dark room. After developing the film it was scanned and bands on the film were quantified by Quantity One software (Bio Rad).

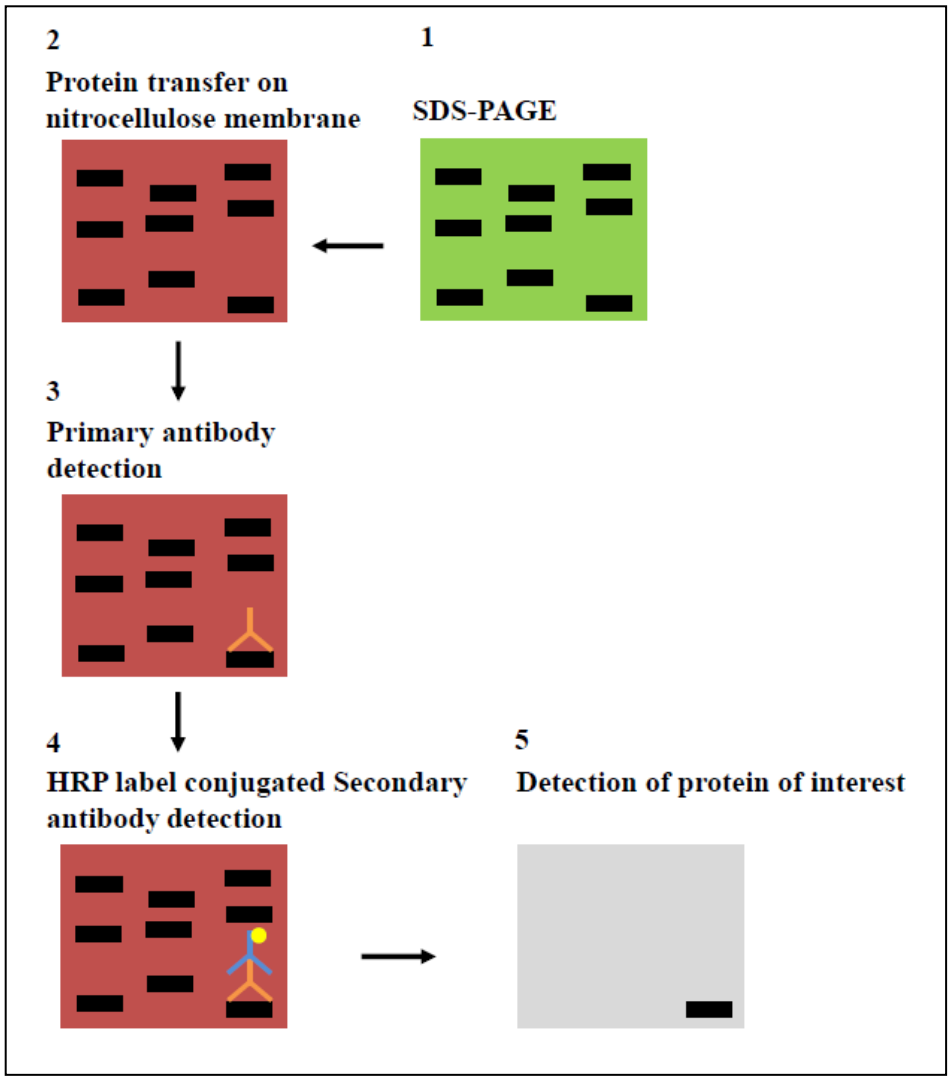


Figure 2-1: Binding of Primary and Secondary HRP Antibody and ECL detection.

2.1.2.17 Statistical analysis

All data were plotted graphically as mean vertical bars representing standard error (S.E.) A Student's *t* test was used to assess differences between two experimental repeats. A probability (P) value of <0.05 was taken as an appropriate level of significance. Statistical analysis was done in Excel.

2.2 Methods Relating to Chapter Three: *In Vitro* Validation of miRNA Constructs

2.2.1 Generation of miRNA expression constructs

2.2.1.1 Selection of miRNA sequences for targeting BLG gene

MiRNA duplexes were designed targeting bBLG gene (accession no: NM_172929, variant B) using BLOCK-iT™ RNAi designer tool (Invitrogen, USA). The BLOCK-iT™ RNAi designer tool utilizes proprietary algorithm to design miRNA sequences. The BLG gene has 7 exons and spans 500 bp. The mRNA sequence of bBLG was retrieved from the NCBI database and 10 BLG specific synthetic miRNA oligonucleotides containing different target sequences were designed (Table 2-2).

2.2.1.2 Generation of single miRNA expression constructs

Both lyophilized single stranded sense and anti-sense miRNA oligos were diluted in TE buffer to a final concentration of 200 µM. Each of the above oligos was mixed to the final concentration of 50 µM. The following annealing procedure (Table 2-5) was set up at room temperature in a 0.5 ml sterile micro centrifuge tube.

Table 2-5: Generation of miRNA duplexes.

Reagent	Amount
Top strand DNA oligo (200 µM)	5 µl
Bottom strand DNA oligo (200 µM)	5 µl
10 x Oligo Annealing Buffer	2 µl
DNase/RNase-Free Water	8 µl
Total volume	20 µl

The annealing reaction was incubated at 95°C for 4 minutes and allowed to cool down to room temperature for 5-10 minutes. The reaction was centrifuged briefly

for 5 seconds and mixed gently. The ds oligo mixture was diluted 100-fold into DNase/RNase-free water (1 µl of 50 µM ds oligo into 99 µl of DNase/RNase-free water) to obtain the final concentration of 500 nM and vortexed gently to mix. The 500 nM of ds oligo was diluted 50-fold into oligo annealing buffer to get a final concentration of 10 nM (1 µl of 500 nM ds oligo into 5 µl of 10 x oligo annealing buffer and 44 µl of DNase/RNase-free water). The pGL 273 expression vector was used to clone and express the ds oligo duplexes under the transcriptional control of CMV promoter. This expression vector leads to a bicistronic expression of GFP with the miRNA (Figure 2-2). For cloning of the ds oligo duplexes into the linearized pGL 273 expression vector a 20 µl ligation reaction was set up at room temperature using the following reagents listed in Table 2-6. The ligation reaction was mixed well by pipetting up and down and incubated for 5 minutes at room temperature. The ligated product was transformed to One Shot® TOP 10F competent cells and selected by spectinomycin (50 µg/ml) resistance (see Section 2.1.2.12). Few colonies from each plate were picked and cultured overnight in LB medium containing 50 µg/ml spectinomycin at 37°C with shaking at 250 rpm.

Table 2-6: **A list of reagents to clone ds oligo into the expression vector.**

Reagent	Sample	Negative control
5 x ligation buffer	4 µl	4 µl
pGL 273 (5 ng/µl)	2 µl	2 µl
miRNA-ds oligo (10 nM)	4 µl	-
DNase/RNase-Free Water	9 µl	13 µl
T4 DNA Ligase (1 U/µl)	1 µl	1 µl
Total volume	20 µl	20 µl

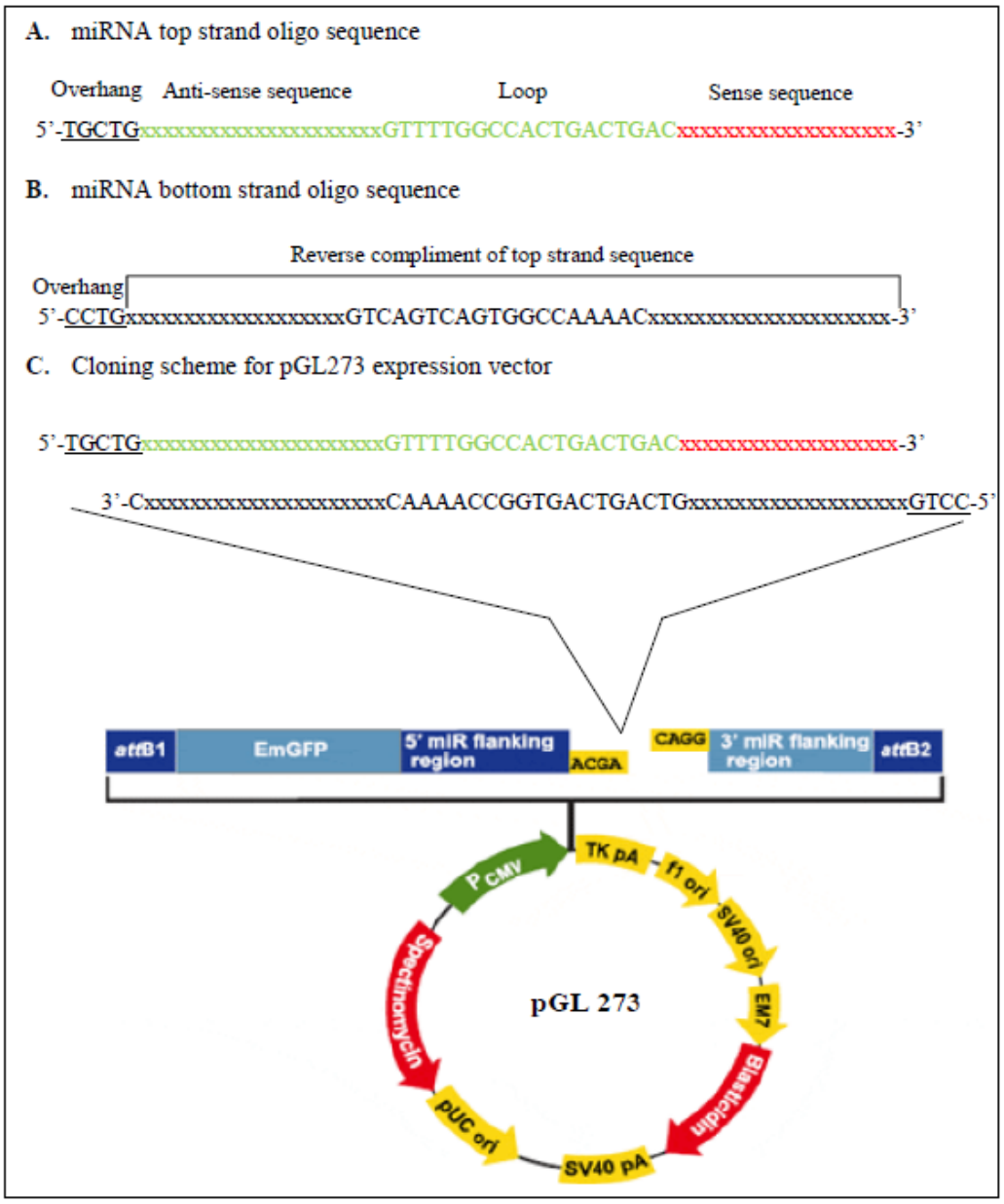


Figure 2-2: **A schematic representation of cloning of miRNA duplex into pGL 273.**

A. miRNA top strand oligonucleotide: the left 5 nt cloning overhang (capitals, underlined) is followed by a 21 nt antisense target sequence (lower case, xx marked in green colour) and a 19 nt loop (capitals) and the sense target sequence (lower case, xx marked in red colour). **B.** represents reverse complement strand of top strand oligo. **C.** Cloning of the annealed top and bottom strand oligonucleotides into pGL 273 results in CMV-GFP-miRNA knockdown vector. The human cytomegalovirus (CMV) promoter is highlighted in green color drives the transcription of GFP and miRNA. The total size of the generated vector is 5759 bp. EmGFP, Enhanced green fluorescent protein; TK pA, TK polyadenylation signal; F1 origin, F1 single strand DNA origin; SV40 early promoter

and origin, SV40 early promoter and origin of replication; EM7 promoter, synthetic prokaryotic promoter; Blasticidin, Blasticidin resistance gene, SV40 pA, SV40 polyadenylation signal; pUC origin, pUC plasmid replication origin and Spectinomycin, Spectinomycin resistance gene.

2.2.1.3 Identification of single miRNA inserts

Screening of single miRNA inserts was done by the plasmid DNA preparation (see Section 2.1.2.2.1) and quantification of DNA (see Section 2.1.2.3) followed by restriction enzyme digest of the plasmid DNA (see Section 2.1.2.4). The miRNA inserts have a unique *Msc*I restriction site and to check the presence of the miRNA inserts, miRNA constructs were digested with *Msc*I restriction enzyme and visualized by gel electrophoresis (see Section 2.1.2.5). Positive clones which were correctly ligated should give rise to bands of 1.4 kb and 4.3 kb. As shown in Appendix 1, all clones (lane 1-10) did give this expected pattern of bands. Finally, the constructed expression vectors were checked by DNA sequencing (see Section 2.1.2.13) using oligonucleotides GL 725 and GL 726 (Table 2-3). The outcome of the sequencing result was compared with the original sequence using Vector NTI Advance™ 11 Invitrogen (USA) and Chromas Lite version 2.01 Technelysium pty ltd (Australia). The DNA sequencing results showed that the miRNA inserts remained unchanged (see Appendix 19). The miRNA expression vector with miRNA inserts were labelled as pGL 274-283 (miRNA 1-10) for future use (Table 2-4).

2.2.1.4 Generation of multiple miRNA expression constructs

To increase the efficiency of single miRNA to down regulate BLG or to see the effect of combination of miRNAs on BLG expression, the single miRNAs that showed consistent knockdown were chained up based on the provider's (Invitrogen, USA) cloning strategy (see Appendix 2). In order to generate tandem miRNA constructs, the insert fragment of pre-miRNA expression cassette 1 and the vector backbone of pre-miRNA expression cassette 2 were cut from the expression vector using restriction enzymes, *Sal*I and *Bgl*II, and *Sal*I and *Bam*HI, respectively (see Section 2.1.2.4). The digests were run on agarose gel (see Section 2.1.2.5) and DNA fragments (insert and vector backbone) were isolated

by gel purification (see Section 2.1.2.6) (see Appendix 2). The insert and the linearized miRNA expression vector were ligated using Takara DNA ligation mighty mix at 16°C for 30 minutes (see Section 2.1.2.10). The ligation products were then transformed into *E.Coli* cells (see Section 2.1.2.12) and plated on spectinomycin selection agar plates. Few positive colonies after overnight incubation at 37°C were selected for plasmid DNA preparation (see Section 2.1.2.2.1). The plasmid DNA was verified by restriction enzyme digestion of *Sal*I and *Xho*I and the digests were run on agarose gel (see Section 2.1.2.5) followed by the gel excision of the DNA fragments before exposure to UV light. The plasmid with the insert had the higher molecular weight (300 bp) than the plasmid without insert (160 bp) after restriction enzyme digestion (see Appendix 3). Finally, the miRNA expression vectors with two different miRNA inserts were labelled as pGL 285 (miRNA 2-3), pGL 286 (miRNA 2-6), pGL 287 (miRNA 3-6), pGL 288 (miRNA 2-4), pGL 289 (miRNA 6-4) and pGL 290 (miRNA 3-4) (Table 2-4) for future use. The recombinant positive clones of tandem constructs were set up a large-scale culture and purify the recombinant plasmid using a Plasmid maxiprep kit (see Section 2.1.2.2.2).The clone was preserved as a 25% glycerol stock at −80 °C (see Section 2.1.2.14).

2.2.2 Generation of BLG expression constructs

2.2.2.1 Construction of a bBLG expression construct

To facilitate bBLG knockdown *in vitro*, a bBLG expression construct was generated using pGL 164 expression vector. The vector expresses the gene under the transcriptional control of CMV promoter. The expression vector was opened up by digesting with *Hind*III and *Bam*HI restriction enzymes. A bBLG cDNA construct (variant A) previous constructed by Ben Wilson (AgResearch, Ruakura, New Zealand) was used for this study (see Appendix 4). The bBLG cDNA was also cut out from an expression construct (pGL 152) using *Hind*III and *Bam*HI restriction digests (see Section 2.1.2.4) (see Appendix 4). Three individual putative clones were picked and cultured overnight at 37°C for small scale plasmid preparation (see Section 2.1.2.2.1). The ligation was examined by *Bgl*II and *Xho*I restriction digestion. As shown in Appendix 5, all three clones displayed

the expected 4.0 kb and 1.64 kb fragments for a correctly integrated bBLG cDNA insert. The clone containing the bBLG insert was labelled as pGL 297 for further use (Table 2-4).

2.2.2.2 Construction of an oBLG expression construct

2.2.2.2.1 Sub-cloning of oBLG cDNA into a shuttle vector

To facilitate oBLG knockdown *in vitro*, an oBLG expression construct was generated using pGL 164 expression vector. Total RNA was isolated from lactating mammary tissues of an oBLG expressing mouse (see Section 2.1.2.2.4). OBLG first-strand cDNA was synthesized as described in Section 2.1.2.9. The first-strand cDNA molecules were PCR amplified (see Section 2.1.2.8) using oligos GL 727 and GL 728 (Table 2-3) amplifying the coding sequence of oBLG. A proof reading polymerase was used to reduce mismatch incorporation of a base by regular *Taq* polymerase. A single band corresponding to the desired PCR product was initially sub-cloned into TOPO vector (see Section 2.1.2.11). The clone containing the oBLG cDNA insert was identified by *Bgl*I restriction enzyme digests (see Appendix 6). Since the oBLG cDNA was generated by PCR, it was necessary to confirm the integration of the insert by nucleotide sequencing. The plasmid DNA containing oBLG cDNA was prepared for sequencing (se Section 2.1.2.13.1) and sent to Massey University, Palmerston North, New Zealand for sequencing using oligos GL 727 and GL 728 (Table 2-3). The sequencing result was aligned with the original sequence of oBLG cDNA using Vector NTI Advance™ 11 Invitrogen (USA) and Chromas Lite version 2.01 Technelysium pty ltd (Australia). The DNA sequencing results revealed that the oBLG cDNA insert remained unchanged (see Appendix 21). The TOPO vector with oBLG cDNA insert was used for construction of oBLG expression vector.

2.2.2.2.2 Cloning of oBLG gene into an expression vector

To test the ability of oBLG cDNA to express at the protein level, an oBLG expression construct was generated using pGL 164 expression vector for knockdown analysis of oBLG *in vitro*. The expression vector was linearized by digesting with *Hind*III and *Not*I restriction enzymes (see Section 2.1.2.4). The oBLG cDNA was also excised from TOPO-oBLG construct using the same

restriction enzymes (see Section 2.1.2.4). The digests were run on agarose gel (see Section 2.1.2.5). The oBLG cDNA fragment was excised from the gel before imaging to avoid introducing any mutation by the UV radiation and both vector and the insert were purified from gel (see Section 2.1.2.6) (see Appendix 7). The insert was directly cloned into the open expression vector (see Section 2.1.2.10) and the ligation was examined by *Hind*III and *Not*I restriction digestion. All clones showed the expected 5.0 kb vector backbone and 650 bp fragments for a correctly integrated oBLG insert (see Appendix 8). The clone containing the oBLG insert was labelled as pGL 448 for further use (Table 2-4).

2.2.2.3 *In vitro* expression of BLG

To test the ability of the pGL 297 and pGL 448 to express BLG at the protein level in eukaryotic cells, a transfection assay was performed (see Section 2.2.3). After 48 hours of transfection, cells were analysed by western blot analysis (see Section 2.2.4). Western blot analysis of the transfected cell pool showed high bBLG and oBLG expression (see Appendix 9).

2.2.3 Transfection of mammalian cells

2.2.3.1 Lipid-based transfection of COS7 cells

For transfection of cells, $2x10^5$ cells were cultured in 24-well tissue culture plate containing 0.5 ml of fresh medium containing 10% FBS. Cells were cultured at 37°C in a CO_2 incubator 12-16 hours prior to transfection (approx. 70-80% confluence). In order to express miRNAs transiently in cells, transfection efficiency of cells was optimized using three different transfection reagents, Lipofectamine 2000, Lipofectamine LTX and FuGENE 6. In this optimization experiment, a GFP expression construct, pEGFP-C1 (Clonetech) (pGL 195) plasmid DNA was used. Cells were transfected at a range of concentrations between 1.0-6.0 µl of transfection reagent with a fixed amount of 1 µg of DNA (condition 1, 1 µl of reagent per µg of DNA; condition 2, 2.5 µl of reagent per µg of DNA and condition 3, 5 µl of reagent per µg of DNA for the Lipofectamine 2000 and Lipofectamine LTX transfection reagents and for the FuGENE 6 reagent, condition 1, 2 µl of reagent per µg of DNA; condition 2, 3 µl of reagent

per µg of DNA and condition 3, 6 µl of reagent per µg of DNA). Except Lipofectamine 2000, Lipofectamine LTX and Plus reagent (0.5 µl) were directly added to the DNA and FuGENE 6 reagent was also added to the DNA and mixed gently. Initially, the transfection reagent was diluted in 50 µL of Opti-MEM medium and incubated for 5 minutes at room temperature. The diluted DNA was dripped into lipid mix, mixed gently and incubated at room temperature for 20 minutes. Meanwhile, cell wells were gently washed with fresh medium to remove dead cells and 0.5 ml of fresh serum free medium was replaced into each well. After the incubation, the DNA and lipid mix was added to the cell well, mixed gently by rocking the plate back and forth and allowed to grow for 48 hours at 37°C in a CO_2 incubator. After 4-6 hours of transfection, the medium was replaced with fresh medium with 10% FBS and Penicillin-Streptomycin liquid (100 x).

2.2.3.2 Post-transfection glycerol shock

To increase transfection efficiency of COS7 cells, cells were subjected to shock using 10% glycerol. After 20 minutes of transfection period fresh medium was replaced in the cell wells. Glycerol was added in the medium and mixed by gentle swirling followed by 3 minutes of incubation period at room temperature. Cells were washed gently with 1 x PBS to get rid of residues of glycerol.

2.2.3.3 FACS analysis of transfected cells

After 48 hours of transfection, medium was changed and cells were washed with PBS to remove dead cells floating on the surface. The remaining cells were harvested by trypsinization and pelleted by centrifugation at 1,000 rpm for 5 minutes. The supernatant was removed by aspiration and the cell pellet was washed with PBS to remove residues of trypsin. Finally, cells were spun down by centrifugation and the pellet was re-suspended with 1 ml of PBS containing 1% FBS. Cells were analyzed on a FACScan™ (Beckton Dickinson, San Jose, CA, USA), with an Argon laser (488 nm) was used. Everytime before using the machine it was calibrated with CaliBRITE™3 beads (BD Biosciences). The calibration helps better separation for forward scatter (FSC), side scatter (SSC), FL1-FL3 channels. The instrument setting was adjusted for COS7 cells. The FSC

and SSC were adjusted to fit maximum events inside the line of FSC/SSC dotplot. In each measurement a total of 10,000 events were acquired and acquired data was analysed using Cell Quest Pro software (BD).

2.2.4 *In vitro* knockdown analysis by western blot

For *in vitro* analysis of BLG knockdown, COS7 cells were co-transfected with plasmid DNA containing single miRNA or double miRNA or negative miRNA control and BLG gene (see Section 2.2.3) (Table 2-4). Transfected cells were prepared for western blot analysis as mentioned in Section 2.1.2.18.1. Cell lysate containing 15 µg of total protein was subjected to SDS-PAGE along with Benchmark™ pre-stained protein ladder (Invitrogen, USA) (see Section 2.1.2.18.3). A transfer sandwich was set up and placed on the iBlot gel transfer device (Invitrogen, USA). Protein transfer was conducted in 7 minutes and efficiency of transfer was checked by Ponceau staining (see Section 2.1.2.18.4). The membrane was then blocked with blocking buffer for 2 hours at room temperature. A rabbit anti-bBLG, (1:10,000 dilution) or anti rabbit GFP (1:2,000 dilution) in antibody incubation solution against BLG or GFP protein was added to the membrane for overnight incubation at room temperature. A goat anti-rabbit HRP conjugate antibody diluted 1:20,000 in antibody incubation solution was added to the membrane and incubated at room temperature for 2 hours (see Section 2.1.2.18.5). The membrane was treated with enzyme linked chemiluminescence (ECL) detection reagents (A and B) from Amersham (UK) and finally, proteins on the membrane were visualised using an X-ray film (KOdak) in a dark room (see Section 2.1.2.18.6).

2.3 Methods Relating to Chapter Four: Generation of Transgenic Mice

2.3.1 Generation of Tg mouse

2.3.1.1 Generation of tissue specific bBLG construct

The tissue specific milk expression vector, pGL 293 contains a unique *Xho*I restriction site to clone the gene of interest. The bBLG cDNA of pGL 152 vector was amplified by PCR with primers GL 730 and GL 731 containing *Xho*I recognition site (Table 2-3) using expand long PCR system (Roche, USA), which contains a thermostable DNA polymerase containing proofreading activity (see Section 2.1.2.8). The PCR product was initially sub-cloned into pGEM-T easy vector (see Section 2.1.2.11). The clone containing the bBLG cDNA was identified by *Xho*I restriction enzyme digest and analysed by agarose gel electrophoresis (see Section 2.1.2.5) (see Appendix 10). One clone was sent to Massey University, Palmerston North, New Zealand sequencing service for sequencing of bBLG (see Section 2.1.2.13) using ologos GL 732 and GL 733 (Table 2-3) to check inclusion of any point mutations introduced by PCR. The sequencing results were aligned with parental sequence and no point mutation was found in the clone sequence (Appendix 20). The bBLG cDNA with *Xho*I hanging ends was cloned into pGL 293 previously opened with *Xho*I restriction enzyme (see Section 2.1.2.4 and 2.1.2.10). Transformants containing pGL 293 vector with bBLG insert were identified by digesting with *Xho*I restriction enzyme and all four clones showed the presence of the bBLG insert (see Section 2.1.2.4) (see Appendix 11). In order to check the orientation of the bBLG insert, the positive clones were further digested with an enzyme set of *Kpn*I and *Sac*I (see Section 2.1.2.4). Two of the clones (clone 3 and 4) showed the expected size of bands on gel electrophoresis (see Appendix 12). The clone containing the bBLG insert was labelled as pGL 449 for further use (Table 2-4).

2.3.1.2 Generation of tissue specific miRNA construct

2.3.1.2.1 Sub-cloning of WAP promoter into a shuttle vector

The WAP promoter of the pGL 263 construct was PCR amplified with oligos GL 734 and GL 735 (Table 2-3) using a proof reading polymerase to reduce mismatch-incorporation of a base by regular *Taq* polymerase (see Section 2.1.2.8). A single band corresponding to the desired PCR product was initially sub-cloned into pGEM-T vector (see Section 2.1.2.11). The clone containing the WAP insert was identified by *Spe*I and *EcoR*V restriction digest (see Section 2.1.2.4). The restriction digests, as analysed by agarose gel electrophoresis revealed that all five clones showed the expected banding pattern on gel (see Appendix 13). One of the clones was send to Massey University for sequencing using five different pairs of oligos (GL 736-745) to confirm that there is no introduced point mutation in the WAP sequence (see Section 2.1.2.13.1). The sequencing results obtained from Massey University were interpreted by using sequence analysis software Vector NTI (Invitrogen) and chromas Lite (technelysium, Au) analysis. The sequence analysis showed that there was no introduced point mutation in the clone (Appendix 22).

2.3.1.2.2 Generation of tissue specific miRNA constructs

The tandem miRNA construct (pGL 289) was digested with *Spe*I and *Dra*I restriction enzymes to excise the CMV promoter and the GFP gene. The WAP promoter was also excised from pGEM-pWAP construct using *Spe*I and *EcoR*V restriction digests (see Section 2.1.2.4). The ligation products were transformed into *E.Coli* (Stbl2) cells (see Section 2.1.2.12). The ligation was examined by *Spe*I and *Sac*II restriction digestion. As shown in Appendix 14, clone 1 and 2 showed the expected 4 kb and 2.8 kb fragments. The tandem miRNA construct with the WAP promoter was labelled as pGL 298 for further use (Table 2-4). To compare the knockdown efficiency of single miRNA constructs (pGL 277 and pGL 279) *in vivo,* mammary tissue specific single miRNA constructs were generated. To generate tissue specific single miRNA constructs, pGL 277, pGL 279 and pGL 289 were digested with *Bam*HI and *Sac*II restriction enzymes (see Section 2.1.2.4). The digests were run on agarose gel (see Section 2.1.2.5) and both vector

backbone of pGL 289 construct and the inserts (miRNA 4 and miRNA 6) were excised from the gel before imaging and DNA was purified from gel (see Section 2.1.2.6) (see Appendix 15). The inserts were directly cloned into the linearized pGL 289, using Takara DNA ligation mighty mix at 16°C for 30 minutes (see Section 2.1.2.10). The ligation products were transformed into MAX Efficiency DH5α cells (see Section 2.1.2.12). Four putative colonies for each of miRNA 4 and miRNA 6 constructs were picked and cultured overnight at 32°C for small scale plasmid preparation (see Section 2.1.2.2.1). The ligation was examined by *Bam*HI and *Sac*II restriction digestion (see Section 2.1.2.4). As shown in Appendix 16, the expected 215 bp fragment was seen for clones 3 and 4 of miRNA 4 and clones 2, 3 and 4 of miRNA 6. The miRNA constructs containing miRNA 4 and miRNA 6 with the mammary tissue specific WAP promoter were labelled as pGL 446 and pGL 447, respectively (Table 2-4).

2.3.1.3 Preparation of DNA constructs for microinjection

To generate Tg bBLG mice, the plasmid DNA of pGL 449 was digested with *Not*I restriction enzyme (see Section 2.1.2.4). The *Not*I restriction enzyme cut two different positions within the construct and excised a 16.3 kb fragment. The resulting fragment contained chicken ß-globin insulator, goat ß-casein promoter, bBLG coding sequence and ß-casein flanking sequence (see Appendix 17). To generate mice carrying the tandem miRNAs (miRNA 4 and 6), the plasmid DNA harbouring miRNAs was digested with *Spe*I and *Ksp*I restriction enzymes (see Section 2.1.2.4). The restriction digest separated WAP promoter with miRNAs fragment that lacks the pA signal and the vector backbone. In order to include the pA signal, the tandem construct (pGL 298) and the other two single miRNA constructs (pGL 446 and pGL 447) were digested with *Spe*I and *Fsp*I restriction enzymes (see Section 2.1.2.4). The restriction enzymes excised fragments of 3.70 kb and 3.2 kb of the pGL 298 and 3.7 kb and 3.0 kb fragments of the pGL 446 and pGL 447. The resulting 3.2 kb/3.0 kb fragment contains the WAP promoter, miRNA 6-4/miRNA 4/miRNA 6 and the SV40 polyA tail. Another plasmid (pBS 2 x insulator) was digested with *Not*I and *Xho*I restriction enzymes to obtain the 2.4 kb fragment of chicken ß-globin insulator (see Section 2.1.2.4) (see Appendix 18). The band of interest was cut out using a clean scalpel blade and the gel slice

transferred into a DNase-free Eppendorf tube. The DNA was purified from the gel using the Qiagen MinElute PCR Purification Kit to remove any unwanted debris which may be toxic to oocytes during pronuclear injection (see Section 2.1.2.6). The recovered DNA was resuspended in filtered microinjection buffer (see Section 2.1.1.9.3). The concentration of DNA was determined using NanoDrop (see Section 2.1.2.3) and the integrity of DNA was assessed by running on agarose gel (see Section 2.1.2.5). Finally, the concentration of DNA was adjusted to 1-2 ng/µl with microinjection buffer just before microinjection.

2.3.1.4 Preparation of mice for microinjection

Mature C57 female mice were superovulated by inducing injection of 5 IU of PMSG (Intervet) and 48 hours latter, 5 IU injection of hCG (Intervet). Female mice were then mated with C57 male mice to yield fertilized embryos for pronuclear microinjection. The embryos were microinjected with the DNA construct into the pronuclei of fertilized mouse embryos and 40-50 normal-appearing 2-cell embryos were then transferred to each pseudopregnant recipient and their pregnancies were monitored. All microinjection and routine care were done by the Small Animal Colony (SAC), AgResearch, Ruakura, Hamilton, New Zealand. The resulting founder Tg pups were separated upon weaning 3-4 weeks after birth.

2.3.1.5 Screening of mice by PCR genotyping

The gDNA was isolated from putative Tg mouse tails and prepared for PCR as described in Section 2.1.2.2.3. Primers (GL 746 and 747) to amplify miRNA, GL 732 and 733 to amplify bBLG and GL 727 and 728 to amplify oBLG insert (Table 2-3) were designed as described in Section 2.1.2.8.1. For each set of oligos a gradient PCR was performed to determine the optimal primers annealing temperature for the PCR reaction. PCR was performed using the diluted gDNA as a template with appropriate primer pairs. A standard PCR reaction was set up as described in Section 2.1.2.8 and PCR products were run on an agarose gel (see Section 2.1.2.5). A 2% agarose gel was used to run miRNA amplicons for better resolution as they are small fragments (160-310 bp). For the larger BLG amplicons (400-650 bp) a 0.8% agarose gel was used (see Section 2.1.2.5). Tg

mice were characterized by the presence of a PCR band corresponding to the junction of the two oligos. Tg mouse lines were established by breeding Tg founders with wild type FVB/C57 mice.

2.4 Methods Relating to Chapter Five: Knockdown of BLG *In Vivo*

2.4.1 Generation of Tg miRNA-BLG mice

The Tg mouse lines expressing single or double miRNAs were crossed with mice expressing bBLG/oBLG to generate Tg miRNA-BLG mice.

2.4.2 PCR genotyping of Tg miRNA-BLG mice

Tail tips from putative Tg miRNA-BLG mice were collected and gDNA was isolated for PCR to identify mice carrying both miRNA and BLG transgenes. The primer pairs GL 746 and 747, GL 732 and 733 and GL 727 and 728, respectively (Table 2-3) were used to identify mice positive for the integration of miRNA, bBLG and oBLG, respectively. A standard PCR reaction was set up as described in Section 2.1.2.8 followd by running miRNA PCR products on 2% and BLG PCR products on 0.8% of agarose gel (see Section 2.1.2.5). The Tg mice showed correct size of band on gel for both miRNA and BLG were kept for crossing with wild type FVB/C57 mice.

2.4.3 Milking of mice

The Lactating Tg miRNA-BLG mice were separated from their litters for 4 hours before milking. Mice were then anesthetized by intraperitoneal injection of 1 ml of avertin (20 mg/ml) immediately prior to milking. After being anesthetized the mice were injected intramuscularly with 0.5 ml of oxytocin (200 IU/ml). Milking was started 2-3 minutes after oxytocin administration and milk was collected manually by gentle massage of teats into capillary tubes and subsequently expelled into eppendorf tubes. After milking the mice were returned gently onto their pups. Mouse milk was stored at -20 ºC until further analysis. Collected milk was diluted to 1:100 with PBS followed by centrifugation at 4°C for 30 minutes at

12,000 rpm. After centrifugation, fat was discarded and the supernatant (defatted milk fraction) was used for further analysis. Milking of mice was done by the SAC, AgResearch, Ruakura, Hamilton, New Zealand.

2.4.4 *In vivo* knockdown analysis by coomassie blue staining

Coomassie blue staining is a simple and reasonably sensitive method to stain protein in SDS-PAGE gels. Mouse milk is highly concentrated and it was diluted in PBS. The concentration of milk protein was measured as described in Section 2.1.2.18.2. Milk samples were prepared as described in Section 2.1.2.18.3. A total milk protein of 10 µg was loaded on SDS gel along with positive and negative controls and Benchmark^{TM} pre-stained protein ladder (Invitrogen, USA) (see Section 2.1.1.1). The SDS-PAGE gel was washed in MilliQ H_2O for 5 minutes. The gel was then fixed in gel-fixing solution (see Section 2.1.1.12) and soaked in that solution for 1 hour. The purpose of this step was to wash the SDS-containing buffers out of the gel and during this fixing step the bromophenol blue dye changed colour from blue to yellow. The gel was rinsed in gel-washing solution (see Section 2.1.1.12) and the fixation of the proteins in the gel continued by incubating for 4 hours at room temperature. The gel was covered with staining solution (see Section 2.1.1.12) and incubated overnight at room temperature with shaking throughout the incubation period. The gel was destained in destaining solution (see Section 2.1.1.12) until the protein bands are seen without background staining of the gel. Thus BLG knockdown can be directly measured from Coomassie-stained SDS-gels. Signal intensities of proteins in the gel were measured by Quantity One software (Bio-Rad). The expression level of BLG was normalized to an internal control protein (α casein) that was well separated on the SDS gel from other proteins (Figure 5-1). Normalization of the expression of the protein of interest improves the accuracy of quantification by taking sample loading differences into account.

2.4.5 *In vivo* knockdown analysis by western blot

Western blot analysis was used as an alternative method to validate the knockdown results observed after coomassie staining of mouse milk. The total

milk protein concentration was measured as described in Section 2.1.2.18.2. Milk Sample preparation for SDS-PAGE was performed as described in Section 2.1.2.18.3. Identical to commassie staining, 10 µg of total milk protein was subjected to SDS-PAGE with controls (positive and negative) and Benchmark™ pre-stained protein ladder (Invitrogen, USA) (see Section 2.1.1.1). After electrophoresis, the proteins were transferred to membrane using iBlot gel transfer device (Invitrogen, USA). The transfer membrane was checked for protein transfer efficiency by staining with Ponceau (see Section 2.1.2.18.4). In order to reduce non-specific binding of the antibodies, the membrane was blocked using blocking buffer for 2 hours at room temperature. The membrane was then incubated with rabbit anti-bBLG, (1:10,000 dilutions with antibody inculation solution) for overnight incubation at room temperature. The antigen-antibody bindings were detected by incubating the membrane with goat anti-rabbit HRP conjugate antibody (1:20,000 dilution with antibody incubation solution) at room temperature for 2 hours (see Section 2.1.2.18.5). Finally, the membrane was incubated with ECL detection reagents (A and B) from Amersham (UK) for 5 minutes at room temperature and protein signals were detected using an X-ray film (KOdak) in a dark room (see Section 2.1.2.18.6).

Chapter Three: *In Vitro* Validation of MiRNA Constructs

3.1 Introduction

The allergenicity of BLG was first discovered in the 1960's. Although the physical and chemical properties of BLG are well characterized, no report has shown yet how the expression of the milk allergen is regulated at the transcript level. Moreover, no study has reported yet how to reduce the presence of BLG in cow's milk except for the dairy industries milk treatments of conventionally denaturing BLG using high temperature short time (HTST) (147) or enzymatic hydrolysis (44). Unfortunately these available treatments affect both macro (protein, carbohydrate and fat) and micro nutrients (vitamins and minerals) in milk and the taste of milk differs from original milk due to exposure of hydrophobic amino acids (47) (48). Moreover, the processing techniques are highly expensive, which led me to use RNAi technique as an alternative way to reduce BLG expression in milk. The discovery of RNAi and its recent advances in gene knockdown in a variety of different animal species including mammals provide us with the opportunity to knockdown BLG in order to improve milk quality. This chapter focuses on the identification of effective miRNAs with high knockdown activity against bBLG and oBLG through screening of ten miRNAs in cell cultures. In order to demonstrate BLG knockdown *in vitro*, ten synthetic miRNA oligonucleotides against BLG mRNA containing different target sequences were designed using available online tools (see Section 2.2.1.1). The algorithms available to select candidate sequences are helpful but they are still not accurate enough to predict a specific miRNA that will indeed knockdown its target mRNA (148). Therefore, it is important to reduce the number of putative miRNAs and identify the most effective candidates on the basis of their knockdown efficiencies in cell culture experiments (149) before applying them in

the *in vivo* system. The vector system used in this study allows assessing the transfection efficiency of cells by monitoring GFP expression using FACS analysis (see Section 2.2.3.3). In this chapter results obtained on establishment of best transfection condition are presented. This chapter also includes evaluation of single miRNA constructs in comparison with tandem miRNAs using the optimized transfection condition to knockdown the expression of bBLG and oBLG *in vitro*.

3.2 Results

3.2.1 Optimization of transfection efficiency in COS7 cells

In RNAi experiments, the transfection of cells plays a major role to identify potential candidates. Cationic liposome-based transfer of transgenes has shown before in cell culture experiments (150) (151) (152). In order to maximize the delivery of the candidate miRNAs and target BLG plasmid DNA into COS7 cells, transfection of COS7 cells was optimized using three commercially available transfection reagents (Lipofectamine 2000, Lipofectamine LTX and FuGENE 6). The success of transfection efficiency depends on the ratio of DNA and liposome and cellular development stages (153). So, initially cells were transfected with GFP expression plasmid DNA (pGL 195) (Table 2-4) (Figure 3-3) using three different ratios of DNA and transfection reagent (condition 1-3) to identify the best transfection reagent and the ratio for further transfection experiments.

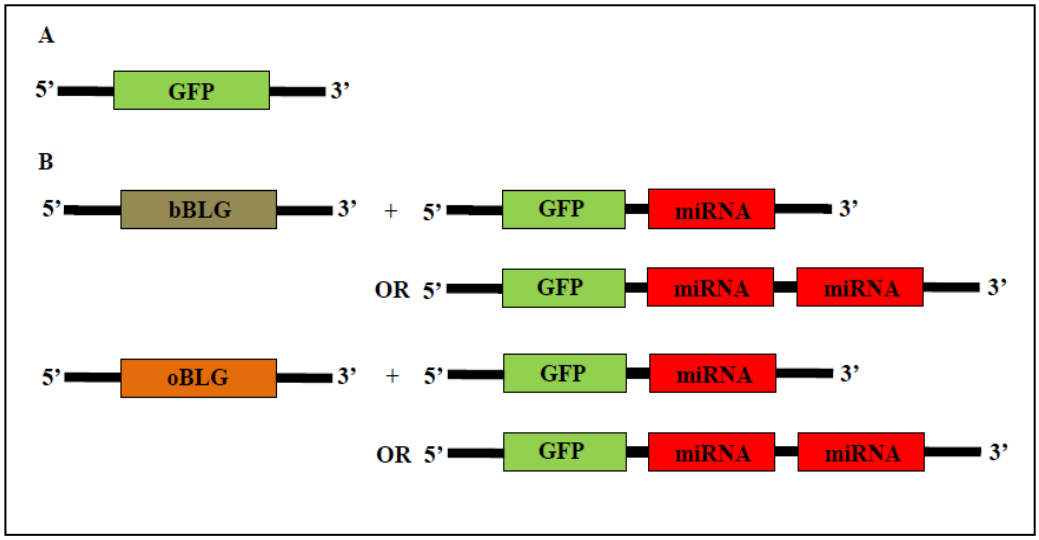


Figure 3-3: Schematic depiction of plasmid DNA expression constructs for transfection assay.

A. A schematic representation of GFP expression plasmid used for transfection optimization experiments. **B.** A schematic representation of bBLG and oBLG expression plasmids and single and tandem miRNA expression plasmids. Single or tandem miRNA constructs were evaluated using co-transfection assay to test their effectiveness against the expression of bBLG/oBLG in cells.

Cells expressing GFP were analysed by FACS analysis and the transfection efficiency of cells was calculated by dividing the number of cells expressing GFP and the total number of viable cells. Overall, the transfection efficiency of COS7 cells ranged from 13-59% among transfection reagents tested (Table 3-7). The transfection assay was done in triplicates and Lipofectamine 2000 resulted in mean transfected cells of 44%±2 (condition 1), 58%±10 (condition 2) and 51%±3 (condition 3), respectively. Lipofectamine LTX resulted in 17%±6 (condition 1), 51%±4 (condition 2) and 58%±4 (condition 3), respectively. FuGENE 6 resulted in mean transfected cells of 36%±6 (condition 1), 30%±1 (condition 2) and 20%±4 (condition 3), respectively (Figure 3-4). Of the three transfection reagents tested, the most efficient reagents were Lipofectamine 2000>Lipofectamine LTX >FuGENE 6. The optimized transfection condition 2 for Lipofectamine 2000 was chosen for the subsequent co-transfection assays.

Table 3-7: Effect of different transfection conditions on transfection efficiency of COS7 cells.

Transfection reagent	Transfection condition	DNA:rearent	DNA (µg)	Reagent (µl)	Transfection efficiency (%)
Lipofectamine 2000	1	1:1	0.8	0.8	42
	2	1:2.5	0.8	2.0	52
	3	1:5	0.8	4.0	49
Lipofectamine LTX	1	1:1	0.5	1.25	13
	2	1:2.5	0.5	3.13	53
	3	1:5	0.5	6.25	59
FuGENE 6	1	1:2	0.4	0.8	40
	2	1:3	0.4	1.2	28
	3	1:6	0.4	2.4	22

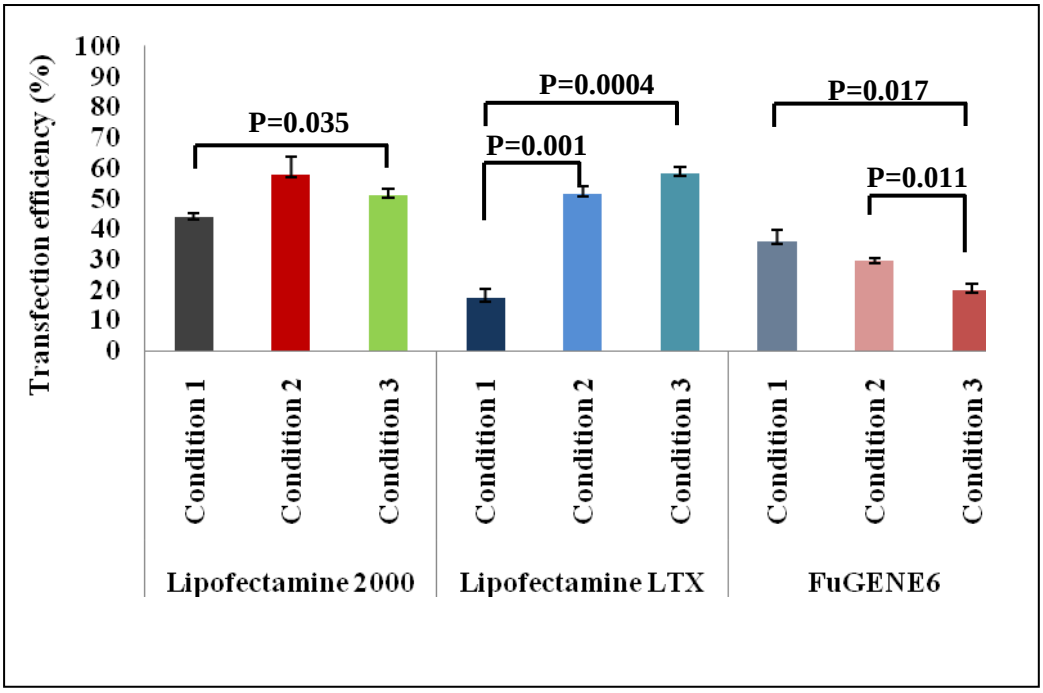


Figure 3-4: Effect of DNA:transfection reagent on transfection efficiency of COS7 cells.

COS7 cells were grown in 24-well plates and transfected with GFP reporter plasmid DNA and different amounts of transfection reagent. The transfection efficiency of cells

was measured by FACS analysis. Each bar represents a different ratio of reagent: DNA tested and mean and standard error of transfection efficiency for each reagent obtained after three independent replicates. Statistical significance among means was determined using a paired two sample *t*-test ($p<0.05$). Error bars represent SEM determined from n = 3 repeats.

Once the optimal transfection condition was determined, the transfection condition was tested in a co-transfection assay by transfecting cells with bBLG (pGL 297) and miRNA expression plasmid 1-3 (pGL 274-276). GFP positive cells were analysed by FACS analysis and the transfection efficiency of cells was measured as mentioned above. The result of the co-transfections is shown in (Table 3-1). The transfection efficiencies were lower than for the single transfection and ranged from 33-40%. The co-transfection assay was done in triplicates and inspection in the GFP channel showed that miRNAs had variability in transfection efficiency of 32%±0.2 (miRNA 1), 33%±7 (miRNA 2) and 35%±4 (miRNA 3). There was no significant difference in transfection efficiency between miRNAs ($P>0.05$) (Figure 3-5).

Table 3-1: Effect of Lipofectamine 2000 on co-transfection efficiency of COS7 cells.

Constructs	Transfection efficiency (%)
bBLG and miRNA 1	33
bBLG and miRNA 2	40
bBLG and miRNA 3	37

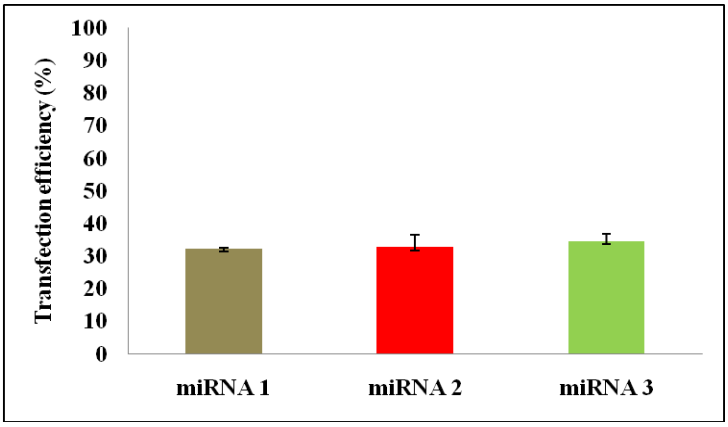


Figure 3-5: FACS analysis of co-transfection of COS7 cells using Lipofectamine 2000.

Cells were transfected with miRNA and bBLG expression plasmid DNA using Lipofectamine 2000 (condition 2). The transfection efficiency of cells was measured by FACS analysis. Each bar represents the transfection efficiency of cells after co-transfection. The mean and standard error of transfection efficiency for each miRNA are calculated from three independent replicates. Error bars represent SEM determined from n= 3 repeats.

As high transfection efficiencies are very important to obtain optimal results in the downstream knockdown experiments and the transfection efficiency of cells after co-transfection was very low, cells were given a post-transfection 10% glycerol shock (v/v) to increase the chances to transfect cells with higher percentage (see Section 2.2.3.2). Cells positive for GFP were analysed by FACS and the transfection efficiency of cells was measured as mentioned above. The glycerol shock played an important role in transfection and increased the transfection efficiency up to 74%. In other words, there was 85 to 112% enhancement in transfection efficiency of cells (Table 3-2). Glycerol acts on disruption of cell membrane and release of DNA into the interior of cells (154). The co-transfection assay with the additional glycerol treatment was done in triplicates and the transfection efficiencies were 72%±3 (miRNA 1), 70%±11 (miRNA 2) and 76%±7 (miRNA 3) (Figure 3-6). There was no significant difference between miRNAs in transfection efficiency of cells using the co-transfection assay with the additional glycerol treatment ($P>0.05$).

Table 3-2: Effect of post-transfection glycerol treatment on co-transfection efficiency of COS7 cells.

Constructs	Transfection efficiency (%)
bBLG and miRNA 1	70
bBLG and miRNA 2	74
bBLG and miRNA 3	70

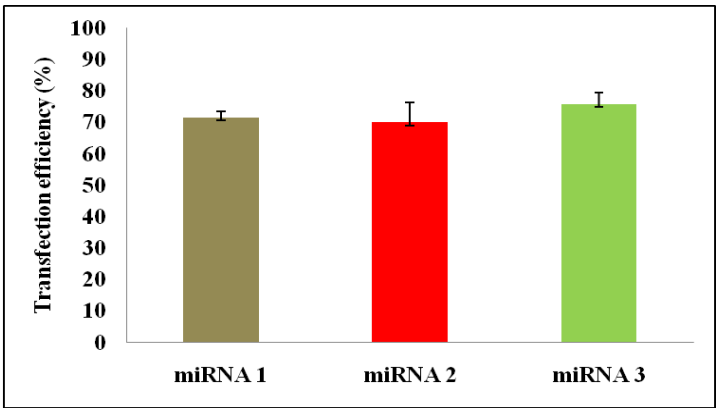


Figure 3-6: Effect of post-transfection glycerol shock on transfection efficiency of COS7 cells.

Cells were co-transfected with miRNA and bBLG expression plasmid DNA using Lipofectamine 2000 (condition 2). The transfection efficiency of cells was measured by FACS analysis. Each bar represents the transfection efficiency of cells after co-transfection and glycerol treatment. The mean and standard error of transfection efficiency for each miRNA are calculated for three independent replicates. Error bars represent SEM determined from n= 3repeats.

3.2.2 Screening of miRNAs to suppress BLG expression *in vitro*

The miRNAs targeting bBLG (variant A) and oBLG (variant B) were designed based on the bBLG mRNA sequence (isoform B). Isoform B of bBLG mRNA is more prevalent and since the ultimate goal is to knockdown BLG in cattle, I designed miRNAs based on the more common B isoform. Both BLG isoforms are highly conserved although a few polymorphisms exist. For the *in vitro*

knockdown of oBLG and bBLG experiments ten candidate miRNA sequences were selected based on the selection criteria set up by Invitrogen miRNA designer's tool. The engineered pre-miRNA sequences were cloed into the miRNA expression vector (pGL273) which is flanked with sequences derived from miR-155 for proper processing of the engineered pre-miRNA sequences (Figure 3-7).

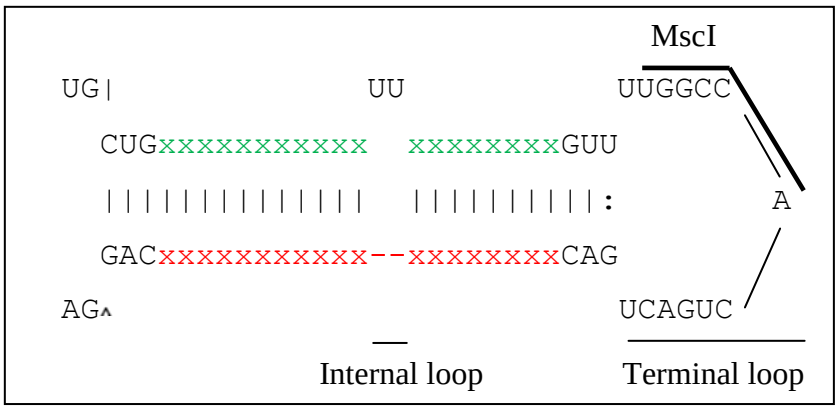


Figure 3-7: Structure of an engineered pre-miRNA sequence on murine miR-155 sequence.

5' 5 nucletides (UGCUG), 3'4 nucletides (CAGG) and 19 nucletides to form a terminal loop are derived from miR-155 transcript. There is an *Msc* I site incorporated in the terminal loop to aid in sequence analysis. The position of antisense and sense target sequences are shown as lower case xx marked in green and red, respectively. Absence of 2 nucleotides at the position of 9^{th} and 10^{th} in the sense target sequence creates an internal loop for better knockdown efficiencies.

Among the designed miRNAs, all miRNAs except miRNA 6 and 8 were perfect matches with bBLG isoform A that was used in the *in vitro* and *in vivo* knockdown studies. Moreover, miRNA 1, 3, 5 and 6 contained one or two nucleotide polymorphisms compared to the oBLG coding sequence and the rest was conserved to the target sequence (Figure 3-8). In order to screen ten miRNAs for BLG knockdown efficiency to identify the most potent miRNAs, ten miRNA constructs were evaluated by co-transfection of COS7 cells with bBLG or oBLG expression plasmid DNAs. A representative western blot analysis of bBLG and oBLG knockdown by the single miRNAs is shown in Figure 3-9. Each experiment was conducted in triplicates and the expression of BLG was detected

by western blot analysis. The intensity of BLG and control GFP signal was measured by Quantity One software (Bio Rad). The expression level of BLG was normalized with the expression of GFP to account for transfection variability between the samples. BLG knockdown by miRNAs was calculated based on the negative scrambled miRNA control which was set as 100% BLG expression (no knockdown). The efficiency of the ten miRNAs (designated miRNA 1-10) against bBLG expression was in the range of -72 to 97%. Seven miRNAs (miRNA 1-6 and 8) showed high knockdown of bBLG ranging from 78-97%. MiRNA construct 10 showed medium bBLG knockdown (70%) compared to the low efficient miRNA 9 (30%). Among the miRNAs only miRNA 7 showed no effect on bBLG expression (Figure 3-10). The statistical analysis of the raw data from three independent experiments showed that candidate miRNAs had variability in bBLG knockdown of 81%±16 (miRNA 1), 92%±12 (miRNA 2), 78%±29 (miRNA 3), 94%±3 (miRNA 4), 80%±23 (miRNA 5), 97%±5 (miRNA 6), -72%±73 (miRNA 7), 78%±13 (miRNA 8), 30%±24 (miRNA 9) and 70%±28 (miRNA 10) (Figure 3-10). Highly effective miRNAs were significantly different to the low effective miRNAs in knocking down bBLG expression ($P<0.05$), with the exception of miRNA 3 and 5 which showed significant difference to miRNA 7 and but not miRNA 9 ($P>0.05$). In almost the same way, medium efficient miRNA 10 was significantly different to miRNA 7 ($P<0.05$) but not miRNA 9 ($P>0.05$). Against oBLG, the ten miRNAs appeared to be effective within a similar range as seen for bBLG with knockdown ranging from -46 to 94%. However, there were some clear differences among miRNAs in silencing of oBLG expression as compared to bBLG. Some of the miRNAs (miRNA 2, 4, 6 and 9) showed high activity against oBLG expression ranging from 78-94%. MiRNA 8 (66%) showed medium knockdown of oBLG compared to those miRNAs were low (miRNA 1, 3 and 5) (12-33%). However, miRNA 7 and miRNA 10 did not show an effect on oBLG expression (Figure 3-10). The statistical analysis of the raw data obtained from three independent experiments showed that candidate miRNAs had variability in oBLG knockdown of 12%±33 (miRNA 1), 94%±4 (miRNA 2), 33%±24 (miRNA 3), 78%±15 (miRNA 4), 31%±28 (miRNA 5), 93%±2 (miRNA 6), -46%±85 (miRNA 7), 66%±13

(miRNA 8), 83%±14 (miRNA 9) and -9%±77 (miRNA 10). Moreover, some of the highly effective miRNAs (miRNA 2 and 6) were significantly different to the low effective miRNAs (miRNA 3 and 5) ($P<0.05$). Similarly, medium efficient miRNA (miRNA 8) was also significantly different to highly effective miRNAs (miRNA 2 and 6) in knocking down oBLG ($P<0.05$) (Figure 3-10).

Figure 3-8: Position of miRNAs targeting BLG mRNA sequence.

A Schematic representation depicts the position of miRNA 1-10 on the BLG mRNA sequence. The miRNA sequences were generated using RNAi design tool, Invitrogen based on the published bBLG mRNA sequence (variant B). Lane 1-3 represents bBLG

variant A, bBLG variant B and oBLG variant B gene sequence, respectively. The inbox bases refer polymorphisms among the BLG mRNA sequences.

Among the ten miRNAs, miRNA 1, 3, 5 and 10 were very specific to bBLG knockdown, whereas, miRNA 9 was very specific to only oBLG. In contrast, miRNA 2, 4, 6 and 8 commonly showed effective knockdown of both bBLG and oBLG. In addition, miRNA 7 had no effect to silence bBLG and oBLG expression. It has been reported that linking up miRNAs targeting different regions of a gene can enhance the knockdown of the target gene (155). To validate this idea, three miRNA constructs which showed good knockdown activity for both bBLG and oBLG (miRNA 2, 4, and 6) were selected along with miRNA 3 that achieved up to 78% of bBLG and 33% of oBLG knockdown, respectively, to generate tandem miRNA constructs in order to compare their BLG knockdown activity using a co-transfection assay in cells.

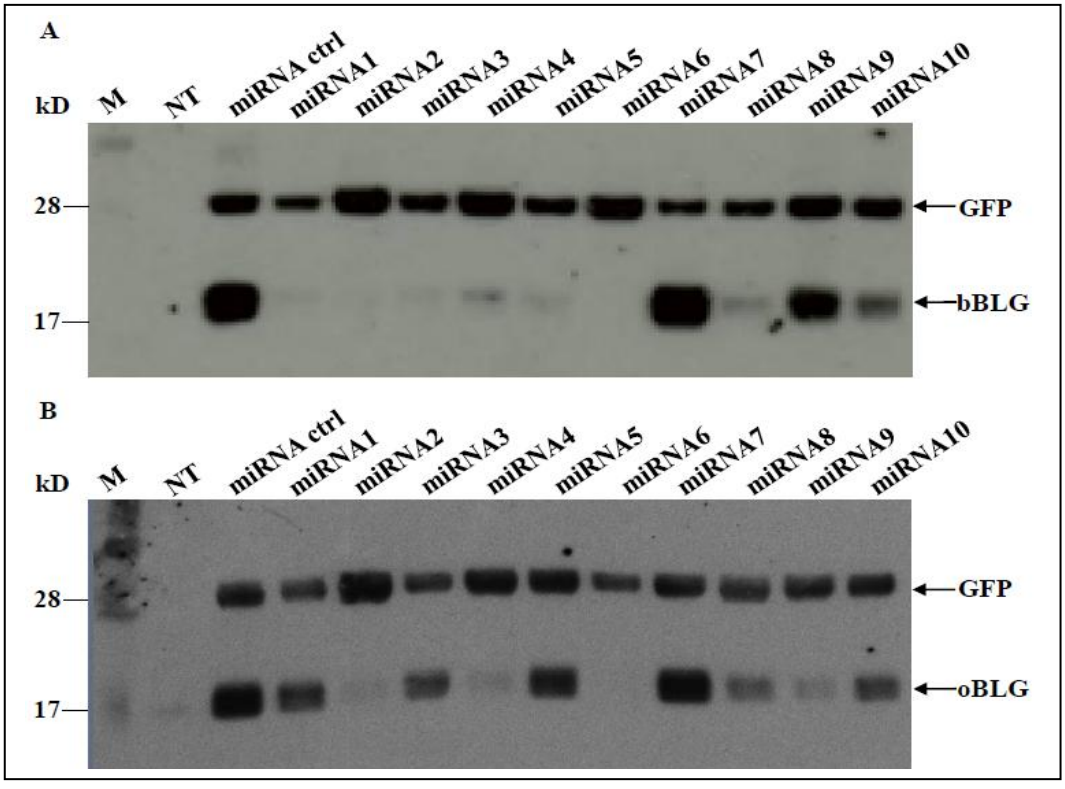


Figure 3-9: Screening of miRNA expression constructs against the expression of bBLG and oBLG.

A. A representative western blot detection of bBLG expression. **B.** A representative western blot detection of oBLG expresssion. The bBLG or oBLG expression plasmids

were transfected with miRNA control or miRNA expression plasmid into COS7 cells. Forty-eight hours after transfection, cells were lysed in lysis buffer and whole cell lysate was run on gel as described in Materials and Methods (Section 2.1.2.18). The western blot was probed as indicated with either rabbit anti bBLG or rabbit anti-GFP as described in Materials and Methods (Section 2.1.2.18). M: pre-stained protein marker (Invitrogen), NT: non-transfected cell, miRNA ctrl: scrambled miRNA control, miRNA 1-10: different single miRNA constructs and kD: kilo daltons.

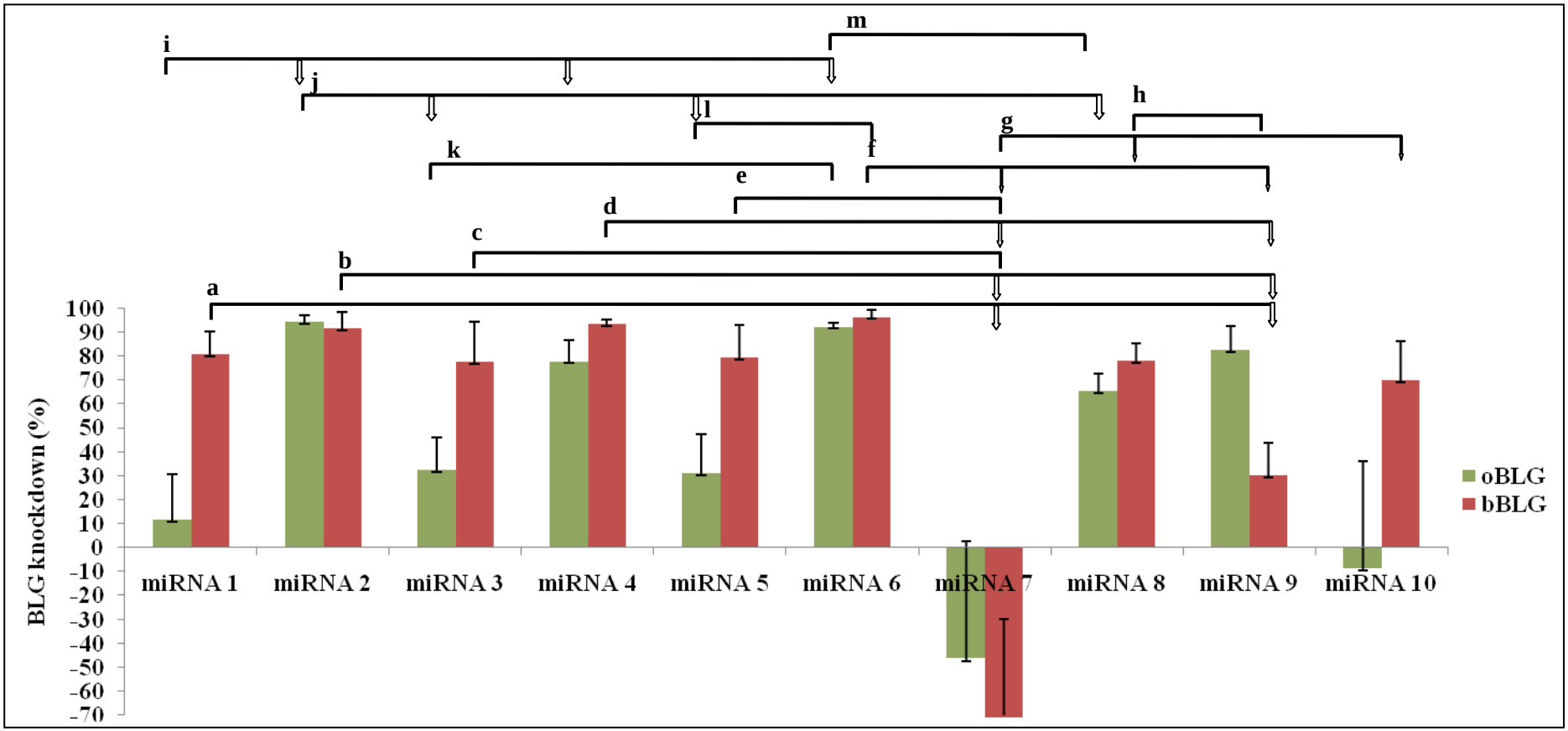


Figure 3-10: Effect of single miRNA constructs against BLG expression *in vitro*.

All data are presented as the percent reduction of bBLG (red bars) or oBLG (green bars) compared with the non-related miRNA control. Knockdown percentages are an average of three independent experiments. Statistical significance among means was determined using a paired two sample *t*-test ($p<0.05$). Error bars represent SEM determined from n= 3 repeats. [a]significant difference between miRNA 1 and 7 and miRNA 1 and 9, [b] significant difference between miRNA 2 and 7 and miRNA 2 and 9, [c]significant difference between miRNA 3 and 7, [d]significant difference between miRNA 4 and 7 and miRNA 4 and 9, [e]significant difference between miRNA 5 and 7, [f]significant difference between miRNA 6 and 7 and miRNA 6 and 9, [g]significant difference between miRNA 7 and 8 and miRNA 7 and 10 and [h]significant difference between miRNA 8 and 9 for bBLG knockdown. For oBLG knockdown- [i]significant difference between miRNA 1 and 2, miRNA 1 and 4 and miRNA 1, and 6, [j]significant difference between miRNA 2 and 3, miRNA 2 and 5 and miRNA 2, and 8, [k]significant difference between miRNA 3 and 6, [l]significant difference between miRNA 5 and 6 and [m]significant difference between miRNA 6 and 8.

3.2.3 Comparison of single and tandem BLG specific miRNAs *in vitro*

The most effective miRNAs were linked up to generate tandem constructs which express two miRNAs targeting different sequences of the BLG mRNA (see Appendix 2). To determine if tandem miRNA transcripts can enhance the knockdown efficiency in cells, single miRNA constructs and their tandem combinations including a scrambled miRNA control expressing plasmids and bBLG or oBLG-expressing plasmids were introduced into COS7 cells by transfection (see Section 2.2.3). The co-transfection experiment was repeated in two other independent experiments and the expression of BLG was analysed by western blot assay. A representative western blot result is shown in Figure 3-11 where the blot shows substantial BLG knockdown by all of the selected single miRNA constructs and their combinations. Some of miRNA constructs showed almost complete BLG knockdown. The intensity of BLG and control GFP signal was measured by Quantity One software (Bio Rad). The expression level of BLG was normalized with the expression of GFP and BLG knockdown by miRNAs was calculated based on the negative scrambled miRNA control (100% BLG expression, no knockdown). The results showed high bBLG knockdown by both single and tandem miRNA constructs ranging from 74-99% (Figure 3-12). The statistical analysis of the raw data showed that tandem and single miRNAs had variability in bBLG knockdown of 98%±1 (miRNA 2-3), 90%±16 (miRNA 2-6), 95%±8 (miRNA 3-6), 95%±2 (miRNA 2-4), 99%±0 (miRNA 6-4), 88%±9 (miRNA 3-4), 96%±4 (miRNA 2), 94%±3 (miRNA 3), 74%±21 (miRNA 4) and 92%±7 (miRNA 6) (Figure 3-12). In general, tandem miRNA constructs showed greater activity (89-98%) than singles (67-92%) to knockdown oBLG, but the differences between single and tandem miRNA constructs were not significant ($P>0.05$). The statistical analysis of the raw data showed that tandem and single miRNAs had variability in oBLG knockdown of 95%±5 (miRNA 2-3), 98%±2 (miRNA 2-6), 93%±7 (miRNA 3-6), 94%±8 (miRNA 2-4), 90%±17 (miRNA 6-4), 89%±11 (miRNA 3-4), 90%±12 (miRNA 2), 67%±29 (miRNA 3), 69%±45 (miRNA 4) and 92%±10 (miRNA 6) (Figure 3-12). This study utilized a

bicistronic expression construct that expresses miRNA and GFP. The GFP expression analysed by western blot analysis showed similar GFP expression for both tandem and single miRNA constructs as expected. A representative blot showing GFP expression analysed by western blot analysis is shown in Figure 3-13. Quantification of GFP expression from three independent experiments showed that for bBLG knockdown experiments miRNA 2-3, miRNA 2-6, miRNA 3-6, miRNA 2-4, miRNA 6-4, miRNA 3-4, miRNA 2, miRNA 3, miRNA 4 and miRNA 6 showed an average GFP expression of 187±59, 162±38, 147±32, 192±90, 235±53, 211±36, 180±64, 169±33, 167±124 and 143±96. In addition, for oBLG knockdown experiments miRNA 2-3, miRNA 2-6, miRNA 3-6, miRNA 2-4, miRNA 6-4, miRNA 3-4, miRNA 2, miRNA 3, miRNA 4 and miRNA 6 showed an average GFP expression of 144±60, 167±50, 148±61, 153±97, 251±60, 191±40, 214±61, 155±58, 191±62 and 249±57. The statistical analysis of the raw data from three independent experiments for GFP expression in BLG gene knockdown experiments revealed that both tandem and single miRNA constructs expressed similar levels of GFP ($P>0.05$) (Figure 3-13). Given this small but statistically insignificant difference in knockdown efficiency between single and tandem miRNA constructs, one tandem construct, miRNA 6-4, was chosen as one of the best constructs for subsequent exeriments *in vivo.* In addition, miRNA 4 and miRNA 6 were also chosen to compare their contribution to knockdown BLG expression with the miRNA 6-4 in the *in vivo* system.

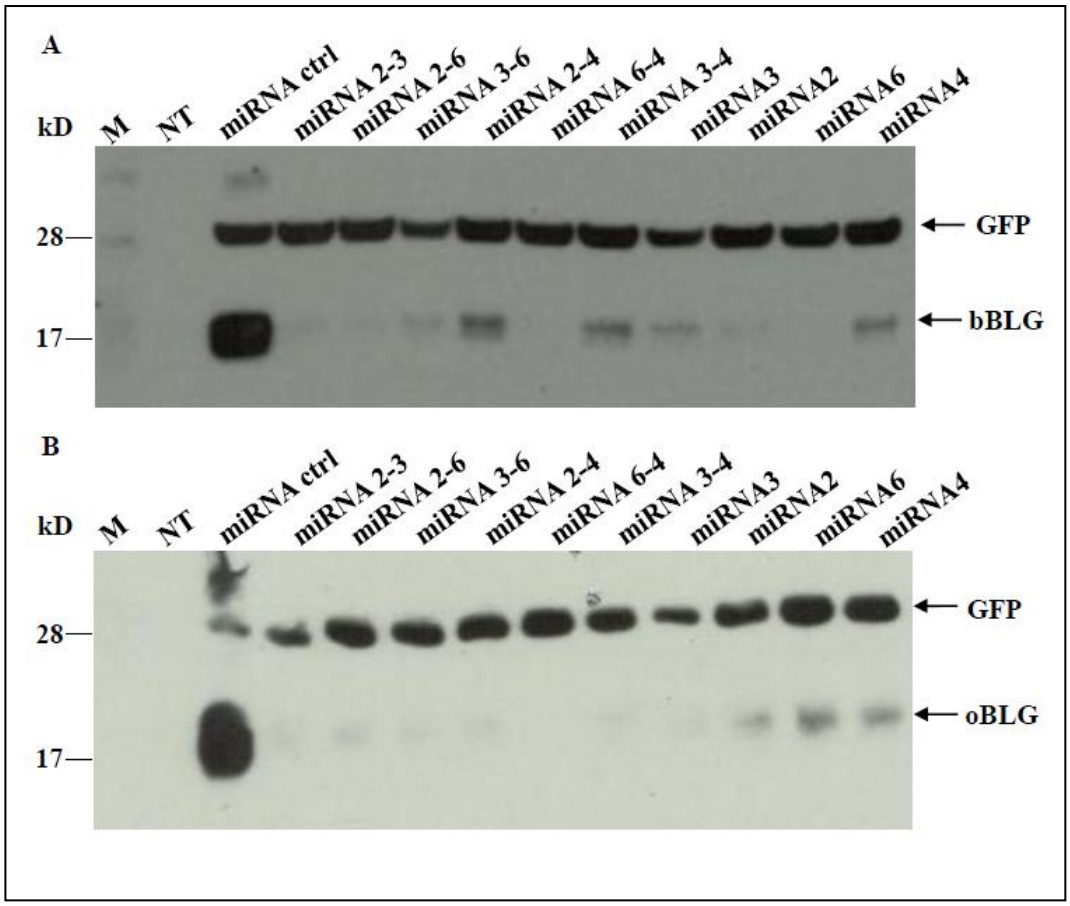


Figure 3-11: **Comparison of single miRNA and their combinations with bBLG and oBLG knockdown activity by western blot analysis.**

A. A representative western blot analysis of bBLG expression. **B.** A representative western blot analysis of oBLG expression. Plasmid DNA of bBLG or oBLG was co-transfected with non-specific miRNA control or single miRNA expression plasmids: miRNA 2, 3, 4 and miRNA 6 or miRNA tandem combinations: miRNA 2-3, miRNA 2-6, miRNA 3-6, miRNA 2-4, miRNA 6-4 and miRNA 3-4 into COS7 cells. Transfected cells were analysed by western blot assay as described in Materials and Methods (Section. 2.1.2.18). The western blot was probed as indicated with either rabbit anti bBLG or rabbit anti-GFP as described in Materials and Methods (Section. 2.1.2.18). M: pre-stained protein marker (Invitrogen), NT: non-transfected cell, miRNA ctrl: non-specific miRNA control, lanes 4-9 represent different tandem miRNA constructs, lanes 10-13 represent different single miRNA constructs and kD: kilo daltons.

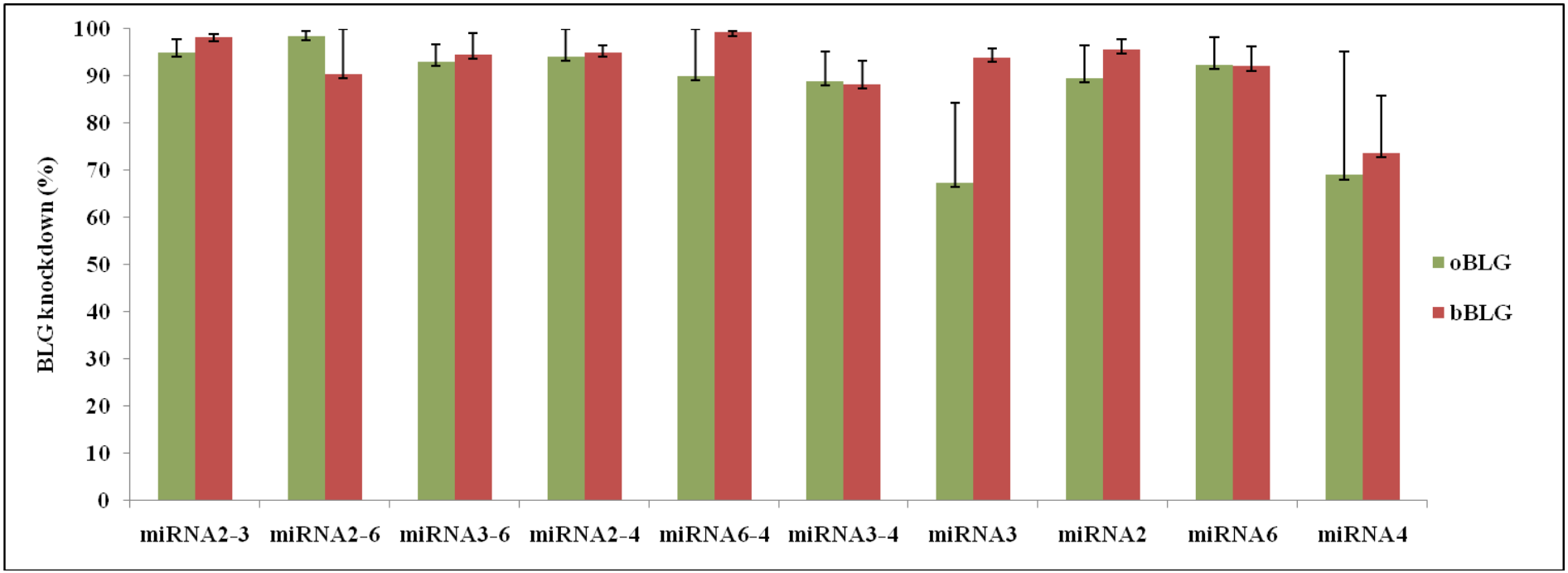


Figure 3-12: Effect of single and tandem miRNA constructs against BLG expression.

All data are presented as the percent reduction of bBLG (red bars) or oBLG (green bars) compared with the non-related miRNA control. Knockdown percentages are an average of three independent experiments. Error bars represent SEM determined from n= 3 repeats.

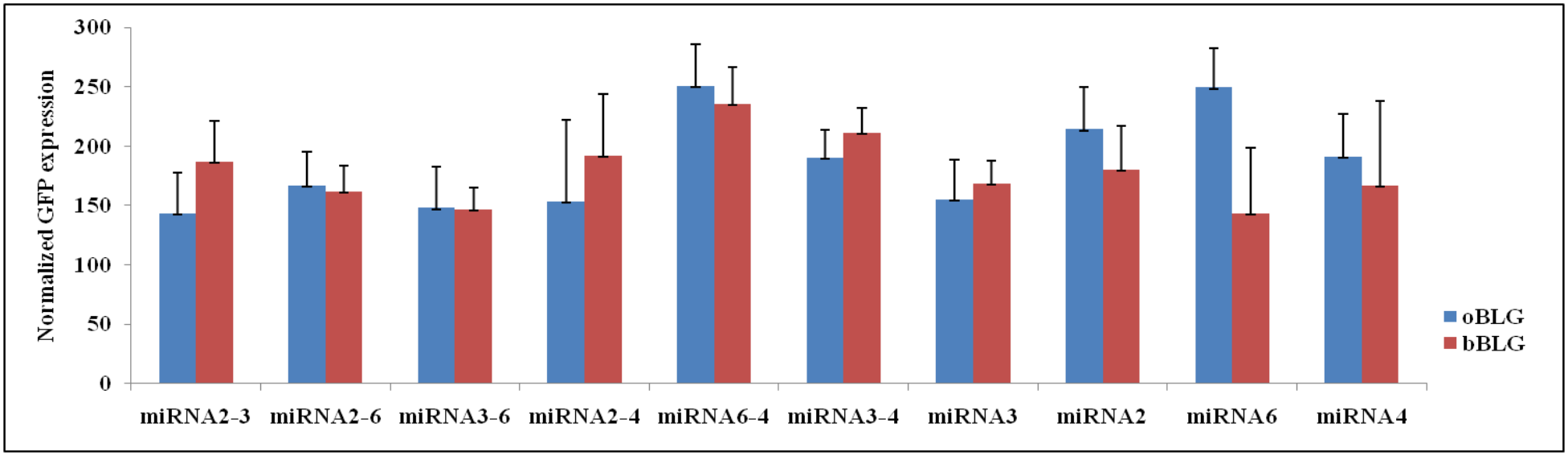


Figure 3-13: Comparison of GFP expressioin in tandem and single miRNA constructs.

All data are presented as the average expression of GFP in bBLG (red bars) or oBLG (blue bars) knockdown experiments. Average values are calculated from three independent experiments. Error bars represent SEM determined from n= 3 repeats.

3.3 Discussion

In this section I will discuss the key findings involved in establishment of transfection condition for BLG knockdown experiments in cells and evaluation of BLG knockdown efficiency of single and tandem miRNA constructs.

3.3.1 Transfection efficiency of cells

The knockdown potential of RNAi molecules is most easily tested by transient transfections. Achieving high transfection efficiency of cells is a prerequisite in order to evaluate selected miRNA constructs and the expression of the gene of interest. This study demonstrated that a small selection of three commercially available lipid-based transfection reagents were able to transfer exogenous genes efficiently to COS7 cells. Cationic liposomes are produced based on different proprietary lipid compositions and designed to be suitable for particular cell types. In my work the most efficient reagent was Lipofectamine 2000, Invitrogen which showed transfection of COS7 cells up to 58% for single plasmid DNA transfection. This is lower than the published 73% of COS7 cell transfection efficiency (156). The transfection efficiency of cells was even lower after co-transfection of cells (up to 35%). A number of reasons can influence transfection efficiency of cells such as use of plasmid vectors of different structure, sizes and purity, cell confluence and vector promoter (157). Reliable evaluation of candidate miRNAs depends on a high transfection efficiency of cells. Higher transfection efficiency of cells increases chances to identify candidate RNAi molecules with high knockdown efficiency against the target gene. To increase the transfection efficiency, an additional post-transfection glycerol shock step was applied. Glycerol helps to disrupt the cellular membrane and to release of DNA into the interior of cells (154) and in this study transfection efficiency of COS7 cells was increased from 35% to 76%.

3.3.2 MiRNA-mediated knockdown of BLG expression *in vitro*

Silencing of gene expression by RNAi has become a widely used tool to understand the function of genes in mammalian cells. RNAi is a sequence-specific

gene silencing mechanism that targets the gene at the mRNA level. Recent advances in the design and delivery of RNAi molecules make it possible to induce a transient knockdown of target gene function within a short period of time in cultured cells. In this study, miRNA sequences targeting BLG gene were designed using Invitrogen's optimized proprietary algorithm. The miRNA sequences were selected based on the coding sequence of BLG avoiding 5′ and 3′ UTRs and starting 100 nt downstream of the BLG start codon (158) assuming that the UTR binding proteins sites and/or translation start sites may interfere with the binding of the RISC to the target gene. Moreover, the UTRs may resemble other transcripts and miRNAs selected based on the UTR sequences may suppress other genes in addition to BLG. MiRNA oligos were cloned into the vector system where the vector-derived transcripts contain a sense and an antisense region that is complementary to the target BLG mRNA sequence. The resulting vectors provide a useful tool for studying the effectiveness of BLG-specific miRNAs in knocking down BLG expression. Rules for selecting RNAi molecules are established and defined. However, despite of numerous efforts on RNAi molecule design, the accuracy of prediction still remains inadequate. Several different parameters contribute to the efficient design of miRNAs. The miRNA design guidelines are not absolute and there is no guarantee that the particular miRNA will work in all systems. So, in any given experiment a number of miRNAs targeting different regions of the target mRNA needs to be tested. Sequence complementary of the siRNA particularly at the centre of the molecule contributes to target recognition in drosophila and any mismatch with the target mRNA may reduce target detection (159). This finding fits with the oBLG knockdown results by miRNA 1, 3 and 5 that have one or more polymorphisms at the centre of the miRNA molecule. Mismatches (up to four nucleotides) in the sequence of siRNAs to the target mRNA at the most 3' terminal positions have been shown to still result in efficient gene knockdown in HeLa cells (160). In my work, miRNA 6 showed high efficacy to silence both bBLG and oBLG expression although the miRNA molecule has one or two nucleotide mismatch (es) at the 3' terminal position. However, three miRNAs (miRNA 7, 9 and 10) showed variable BLG knockdown although they have perfect homology with both bBLG and oBLG mRNA. Among

these miRNAs, miRNA 7 had no effect on both BLG expression and surprisingly miRNA 10 showed medium bBLG knockdown although it had no effect on oBLG expression. Both miRNA 7 and miRNA 10 have a similarity in the sequence that both have three consequitive Cs. It is recommended to avoid strings of 3 or more G/C residues in the siRNA sequence (161) because poly G or poly C sequences may hyperstack to form agglomerates which can potentially interfere miRNA-mediated gene silencing, a criterium that is surprisingly not taken into account by Invitrogen's RNA designer tool. It is unclear as to why miRNA 10 had a moderate effect on bBLG but failed to show any effect on oBLG expression. Another criterium to judge the knockdown efficiency of miRNAs is referred to as 'seed region'. Seed regions consists of 2-8 nucleotides in the miRNA sequence and determine the specificity of the target recognition by the interfering molecules (162). The miRNA 1 and 5 have a single nucleotide mismatch in the seed region of oBLG gene only and that's why they showed low oBLG knockdown in this study. It is reported that the GC content in the miRNA sequence plays an important role for RNAi activity and high GC% has negative effect on RNAi activity. A high GC content slows down the unwinding process of the ds duplexes by the helicase enzyme present in RISC. As a result, the efficiency of the target mRNA recognition and binding of the interfering RNA molecules are reduced (163). However, Amarzguioui *et al.,* (164) reported no correlation between GC content of the siRNA molecule and its functionality. The results of the miRNA induced BLG knockdown data do not support the result showed by Chan *et al.,* (163) on the effect of high GC% of siRNA molecules and their potency on RNAi. A GC content of 35-55% has been recommended by the Invitrogen RNAi designer tool. The majority of the miRNA sequences used in this work had a GC content of above 50%, except miRNA 8 and 9 (<50% GC content). Unfortunately miRNA 8 and 9 were not among the most effective miRNAs though they contain GC content of the recommended level. Moreover, the GC contents of the highly effective miRNA sequences ranged from 52-57%.

In this study, I used a vector system for mammalian RNAi based on miR-155 miRNA precursor. In this vector system the expression of the synthetic miRNA transgenes was directed by CMV promoter and provides effective inhibition of the

target gene *in vitro*. The vector system allows expression of two different synthetic miRNAs from a single transcript for increased inhibition of the target gene. As tandem miRNAs target two separate regions of the target mRNA, induction of increased gene knockdown is expected. In my study, it was found that tandem miRNAs showed greater but non-significantly enhanced BLG knockdown as compared to the respective single miRNA constructs. However, many researches have shown considerably increased knockdown of the target gene by the tandems compared to the respective single miRNAs. Sun, D *et al.,* (165) showed profound reduction of GFP in cells using miRNA-30 based one-, two-, and three hairpin constructs. It was revealed from the research that the efficiency of single miRNA was further improved to near complete knockdown by multimerization with a second hairpin, but addition of a third hairpin only showed modest knockdown improvement. Chung *et al.,* (80) reported that RNAi activity can be increased gradually using up to eight miR-155 based miRNAs against the expression of luciferase reporter gene. McLaughlin *et al.,* (155) showed the increased knockdown efficiency of combined miRNAs targeting different regions within the Abl-coding sequences as a treatment of leukemia. Previously, this group tried to knockdown *in vivo* leukemogenic activity by knocking down the Bcr-Abl gene using shRNAs, but significant level of Bcr-Abl kinase activity remained. Later on, they introduced single and tandem (double and triple) anti-Abl miRNAs using a lentiviral vector system. They found the triple combination of anti-Abl miRNAs was effective to suppress oncogene expression and activation of kinase pathway in order to stop regrowth of leukemic cells *in vitro* and *in vivo*. In contrast to the improvement of gene knockdown by multiple miRNA approach, a report has been published showing negative effect on gene knockdown by the multiple miRNAs compared to singles. Zhou *et al.,* (69) studied the isoform redundancy of cAMP-dependent kinases and they used multiple miR-30 based synthetic miRNAs in a single transcription unit targeting human superoxide dismutase 1 (SOD1) gene. They experienced the completely opposite scenario than expected as the tandem miRNA construct was less effective than the respective single miRNA.

The western blot analysis of single miRNA-based knockdown showed that the level of the reduction of oBLG and bBLG ranged from -46 to 94% and -72 to 97%, respectively. There was some variation between independent experiments as evidenced by relatively high standard deviations for all runs. I also observed considerably different knockdown of oBLG by miRNA 3 while comparing the results from the single miRNA knockdown comparison (miRNA 3 33%±24, Figure 3-10) with that of the single/tandem comparison (miRNA 3 67%±29, Figure 3-12). It has been revealed in this study that tandem miRNAs were not significantly different to the single miRNAs in knocking down both bBLG and oBLG *in vitro*, although tandem constructs showed somewhat greater BLG knockdown over singles. The *in vitro* screening of miRNAs against oBLG and bBLG expression in cells revealed some important answers: difference in the BLG knockdown efficiency among single miRNAs, no significant difference between single and tandem miRNAs to knockdown BLG and variability among experimental repeats.

The following studies (Chapter Four: Generation of Transgenic Mice) are focused on generating transgenic mouse lines that stably express specific miRNAs that can be used to demonstrate BLG knockdown *in vivo*.

Chapter Four: Generation of Transgenic Mice

4.1 Introduction

The use of Tg mice has become a useful model since the first Tg mouse was born in 1980 (166) because mice have a shorter generation interval and are much faster and less expensive to evaluate than large animals. As the cell culture experiments showed substantial BLG knockdown by synthetic miRNAs, it was expected that miRNAs would be also efficient in down regulating BLG expression in mice. Normally however, BLG is not produced in mice. Thus, in order to study miRNA-mediated knockdown in mice, the gene encoding BLG had to first be introduced into mice. Hemizygous mice expressing oBLG were obtained from Roslin Institute, UK and a mouse line expressing bBLG was generated. In order to determine the effect of miRNA expression on production of BLG *in vivo*, several Tg miRNA mice were produced and crossed with BLG mice to generate miRNA-BLG mice which allowed assessing BLG knockdown in milk.

4.2 Results

4.2.1 Characterization of bBLG founder mouse lines

In order to generate a mouse line expressing bBLG, I needed to introduce a bBLG expression construct harbouring bBLG cDNA. The expression of bBLG was intended to be restricted to the mammary gland. Therefore, a vector system designed specifically for mammary gland expression was obtained from GTC Biotherapeutics, USA. The vector system has the unique feature of limiting the transgene expression to the mammary gland by genomic sequences from goat β-casein promoter. It also contains two tandem copies of chicken β-globin insulator sequences located immediately upstream of the β-casein promoter (Figure 4-1). The insulator sequences shield the transgene expression from position effects that

may arise from the transgene integration into a random site in the genome (167). The 3' UTR contains genomic sequences of β-casein (exons and introns) and the pA sequences necessary for transcription termination and polyadenylation of the gene of interest. The bBLG cDNA (variant A) was cloned in between exons 2 and 7 of goat β-casein promoter sequence. The mammary gland tissue-specific bBLG construct was supplied to the SAF, AgResearch for microinjection and production of Tg bBLG founders.

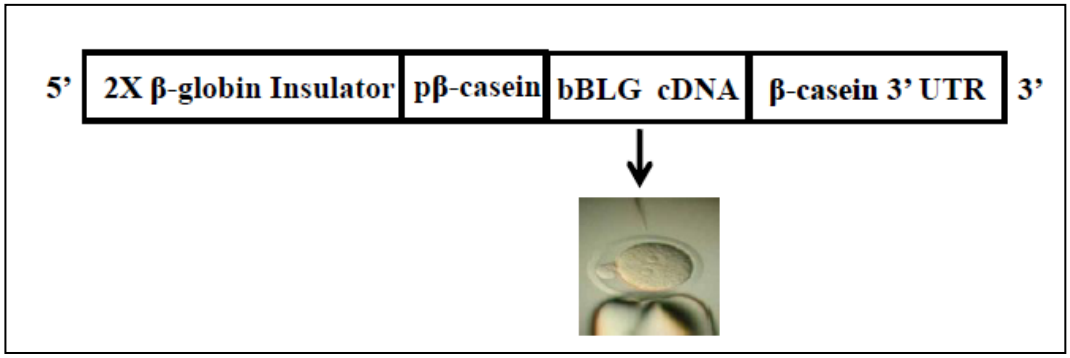


Figure 4-1: Schematic depiction of bBLG construct used to generate transgenic bBLG mouse lines.

The figure shows the sequence of the DNA fragment as represented in boxes used for microinjection into mouse embryos. It consists of 2X chicken β-globin insulator sequences immediately before the β-casein promoter, bBLG cDNA and β-casein 3' UTR sequence, respectively.

PCR genotyping of putative Tg bBLG mice showed that among 16 putative Tg mice 3 founder mice (1 male and 2 females) were positive for the transgene integration (Figure 4-2). Founder animals generated by microinjection may carry the transgenes only in a subset of cells (mosaics) (168) (169) and the transgenes may fail to transmit through the germline (170). To test the germline transmission ability, Tg bBLG founder mouse lines were bred with wild-type mice for the production of F1 generation mice. The bBLG founder lines were able to transfer the transgene to 54% (of 13), 40% (of 10) and 50% (of 12) of offspring, respectively (Table 4-1).

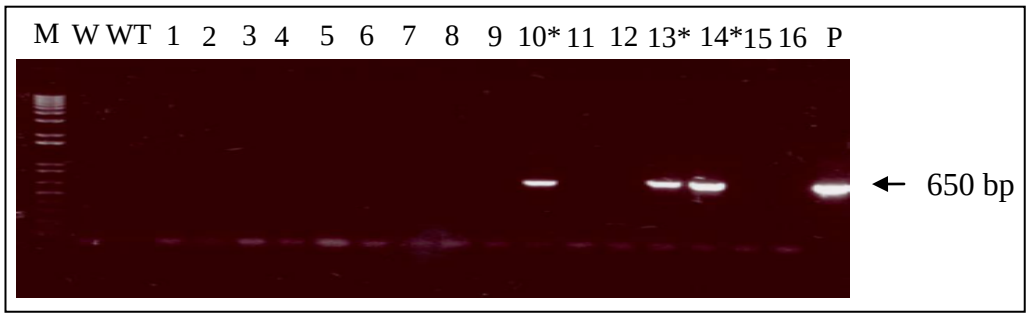


Figure 4-2: PCR genotyping to identify bBLG founder mice.

A band at 650 bp represents an amplified bBLG gene using GL 732 and 733 as compared to a WT mouse thus indicating the presence of the transgene. M represents 1kb^{+} DNA marker (Invitrogen), W: water negative control, WT: wild type mouse gDNA negative control, numbers 1-16 represent individual mouse gDNA sample, P: plasmid DNA positive control and (*), indicates positive bBLG mice. The arrow indicates the position of bBLG.

Table 4-1: Germ line transmission in Tg bBLG mice.

Tg founder lines	No. of born mice	No. of Tg F1 mice (%)
Line 10	13	7 (54)
Line 13	10	4 (40)
Line 14	12	6 (50)

BLG is expressed in milk and for analysis of bBLG expression; bBLG female mice were bred with WT male mice to get females into lactation. To determine BLG expression at the protein level in milk, milk samples were collected by SAF from F1 Tg bBLG mice on day 8 of lactation and analyzed by SDS-PAGE. The coomassie staining of total milk proteins showed high bBLG expression in Tg bBLG line 14 but the other two lines (Tg bBLG line 10 and line 13) failed to demonstrate detectable levels of bBLG (Figure 4-3). The presence of bBLG was confirmed further by a more sensitive assay (western blot assay) using a polyclonal anti-rabbit bBLG antibody and a weak BLG expression was seen in line 10 and no detectable BLG expression was seen in line 13 (Figure 4-3). The results clearly demonstrated that Tg bBLG line 14 produced bBLG as the most abundant milk protein in milk. Moreover, the expressed protein had no adverse

effects on the lactation as Tg females were able to feed their pups with milk containing bBLG. The behaviour of the pups was normal. Thus Tg bBLG line 14 fulfilled the requirements for evaluating bBLG KD *in vivo*.

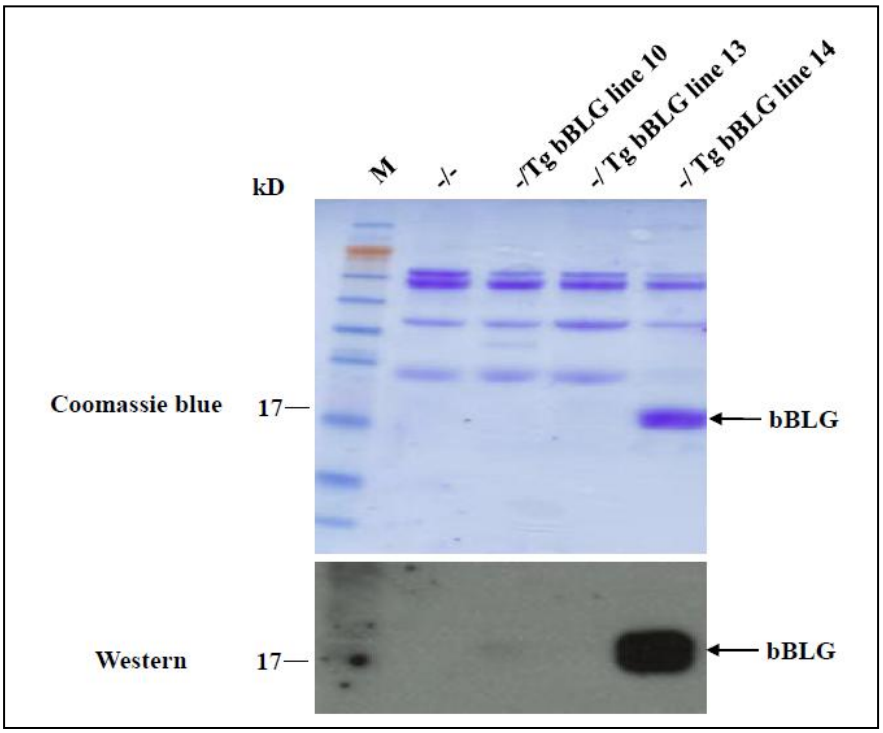


Figure 4-3: Comparison of the expression of bovine BLG protein in different mouse lines.

The top panel of this figure shows the coomassie staining of mouse total milk protein on SDS gel. Equal amount of total milk protein (10 µg) was loaded onto each lane of SDS gel and the gel was stained with 0.4% coomassie stain. The bottom panel of this figure shows a western blot analysis of bBLG expression of Tg mouse milk. Total milk protein (10 µg) from three lines was loaded onto each lane of SDS gel and the bBLG expression was detected using an antibody directed against bBLG (anti-rabbit bBLG) as described in Materials and Methods. M: pre-stained protein maker (Invitrogen), -/-: Wild type mouse, -/Tg bBLG line 10: hemizygous bBLG mouse line 10, -/Tg bBLG line 13: hemizygous bBLG mouse line 13 and -/Tg bBLG line 14: hemizygous bBLG mouse line 14. The arrow indicates the position of bBLG and the number indicates the size of the molecular weight marker in kD.

4.2.2 Identification of transgenic miRNA founder mice

In the *in vitro* experiments, tandem miRNA constructs were equally efficient with single miRNA constructs against BLG expression. One of the tandem constructs (miRNA 6-4) showed insignificant but slightly higher efficiency to knockdown BLG. The miRNA 6-4 construct targets two different regions of BLG gene which can enhance the effectiveness of the construct *in vivo*. So, the tandem construct was chosen to generate Tg mouse lines. The constitute single miRNA constructs (miRNA 4 and 6) were also chosen to generate Tg mouse lines in order to compare their efficiency with the tandem construct *in vivo.* The miRNA constructs used in the *in vitro* experiments were driven by a CMV promoter, a PolII promoter that enables the gene to be expressed constitutively in all tissues of the body. This project aimed to restrict the expression of miRNAs to mammary gland tissues during lactation. The CVM promoter of miRNA constructs was replaced by mouse whey acidic protein promoter (pWAP) in order to minimize potential off target effects by the miRNAs.

In has been reported that the knockdown effect of siRNA can be enhanced by adding a single-stranded poly A (pA) tail to the end of the antisense strand (171). An opposite report was published in the same year by Chung *et al.,* (80) and they reported that SV40 pA is not necessary for efficient production of miR-155. In my study, I also used miR-155-based vector system and I was interested to investigate the knockdown efficiency of miRNA with or without transcription termination signal. So, the miRNA 6-4 construct that showed high efficiency to knockdown both oBLG and bBLG *in vitro* was processed by restriction enzymes to delete the thymidine kinase (TK) pA signal. The miRNA 6-4 construct with or without TK pA (ΔpA) signal was microinjected into mouse embryos. To check the functionality of single miRNAs against BLG expression in mice, the mammary gland tissue-specific single miRNA constructs (miRNA 6 and 4) were processed using restriction digests and linearized miRNA constructs with TK pA signal were microinjected into mouse embryos. Chicken β-globin insulators that shield the genome-integrated miRNAs from potentially silencing heterochromatic regions

were also processed for microinjection (Figure 4-4). The microinjection was performed by SAF, AgResearch.

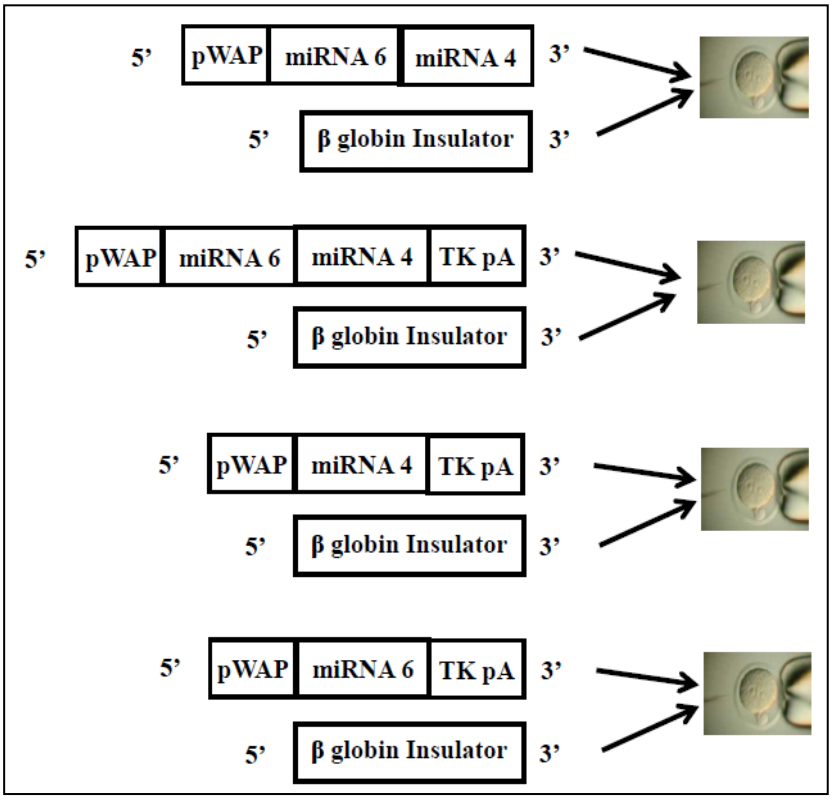


Figure 4-4: Schematic depiction of miRNA constructs used to generate Tg miRNA mouse lines.

The figure shows the DNA fragments as represented in boxes used for microinjection into mouse embryos. The miRNA 6-4 construct was linearized into a fragment containing WAP promoter and miRNA 6-4 sequence with and without (Δ) thymidine kinase (TK) poly A (pA) sequence. The single miRNA constructs (miRNA 4 and miRNA 6) were also processed into fragments including WAP promoter followed by miRNA 4 or miRNA 6 and TK pA tail sequences. Each of the processed miRNA constructs was microinjected into mouse embryos with 1X chicken β-globin insulator sequence.

Offspring produced from microinjected embryos were screened by PCR genotyping to identify Tg founder mice. PCR genotyping of founder miRNA mice revealed that 25% (of 16), of mice were positive for the integration of the miRNA 6-4 ΔpA construct. For miRNA 6-4 construct, 40% (of 5) of mice were positive for the transgene integration and 20% (of 5) and 38% (of 13) of mice were

positive for miRNA 6 and miRNA 4 integration, respectively (Figure 4-5) (Table 4-2).

Table 4-2: Production of Tg founder miRNA mouse lines.

Construct	No. of born mice	No. of Tg mice (%)
miRNA 6-4 ΔpA	16	4 (25)
miRNA 6-4	5	2 (40)
miRNA 6	5	1 (20)
miRNA 4	13	5(38)

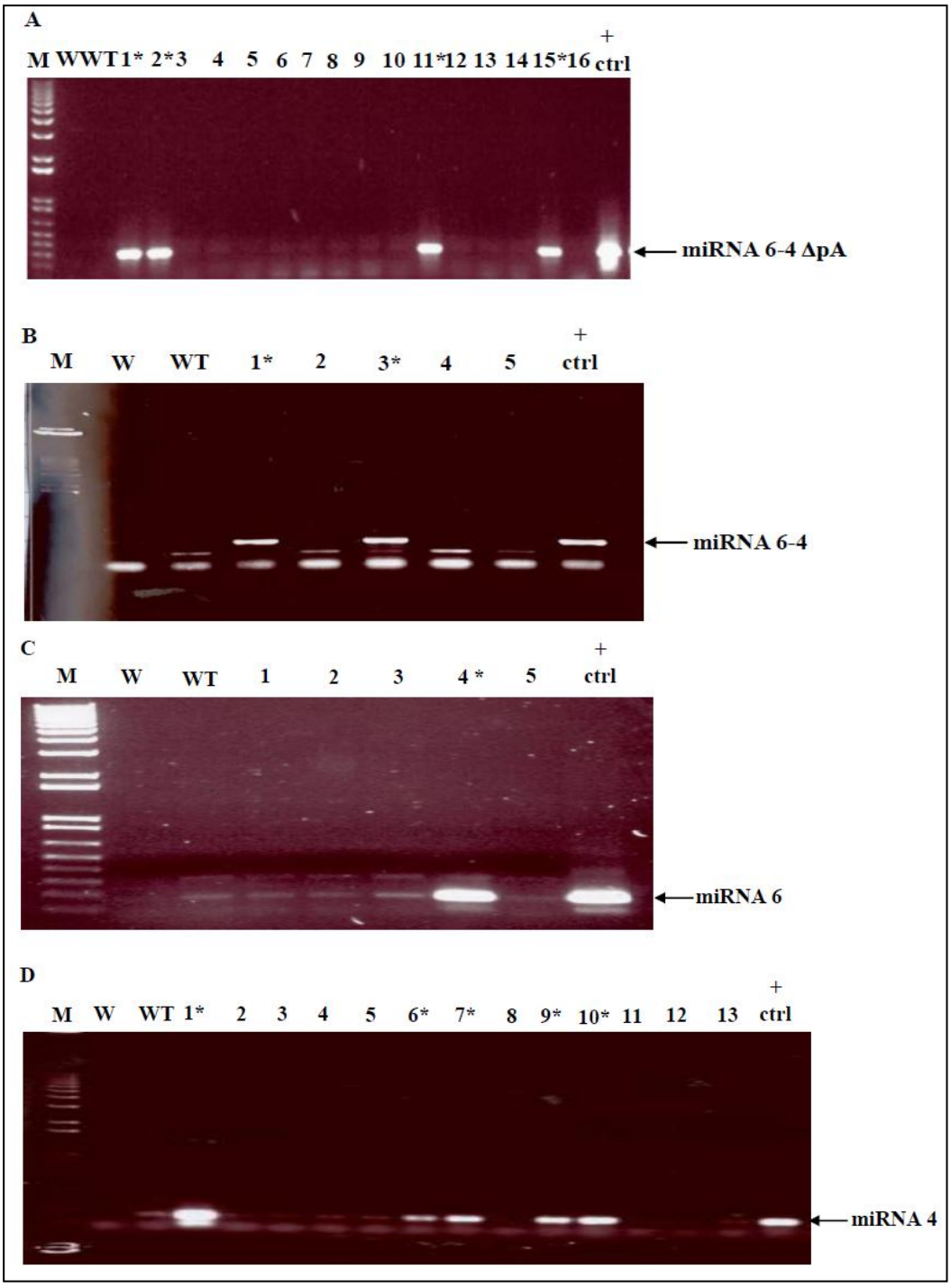


Figure 4-5: PCR genotyping of putative miRNA mice.

A. A PCR gel for genotyping putative Tg miRNA 6-4 ΔpA mice amplifying a band at 310 bp using GL 746 and 747 oligonucleotides containing the miRNA 6-4 sequence. **B.** miRNA 6-4 PCR gel for genotyping putative Tg miRNA 6-4 mice amplifying a band at 310 bp GL 746 and 747 oligonucleotides containing the miRNA sequence. **C.** and **D.** miRNA 6 and miR 4 gel for genotyping putative Tg miRNA mice, amplifying a band at

160 bp GL 746 and 747 oligonucleotides containing the miRNA 6 or miRNA 4 region. M: represents 1kb^{+} DNA marker (Invitrogen, USA), W: water negative control, WT: wild type mouse gDNA negative control, numbers represent individual putative Tg mouse gDNA, +Ctrl: plasmid DNA positive control and (*): indicates all positive Tg mice by PCR.

The Tg founder miRNA mice were bred with wild-type mice to test their ability for germline transmission because the founders can potentially be mosaic and to establish stable Tg miRNA mouse lines. Among the Tg miRNA 6-4 ΔpA mouse line only line 11 transfered the transgene to 80% of the offspring (of 11) while the other three founder lines failed to produce any Tg offspring. The transfer rate of the miRNA 6-4 transgene by line 1 was 36% (of 22) to the offspring. A second line could not be tested for transgene transmission since the respective animal died at four weeks of age. The Tg mouse line 4 carrying miRNA 6 was able to transfer the transgene to 53% (of 19) of offspring. Of the three mouse lines of Tg miRNA 4, 46% (of 24), 24% (of 21) and 43% (of 30) offspring were Tg for line 1, 6 and 7, respectively (Table 4-3).

Table 4-3: Germ line transmission in miRNA Tg mice.

Construct	Tg founder lines	No. offspring	No. Tg (%)
miRNA 6-4 ΔpA	miRNA 6-4 ΔpA line 1	5	0 (0)
	miRNA 6-4 ΔpA line 2	6	0 (0)
	miRNA 6-4 ΔpA line 11	11	9 (80)
	miRNA 6-4 ΔpA line 15	7	0 (0)
miRNA 6-4	miRNA 6-4 line 1	22	8 (36)
miRNA 6	miRNA 6 line 4	19	10 (53)
miRNA 4	miRNA 4 line 1	24	11 (46)
	miRNA 4 line 6	21	5 (24)
	miRNA 4 line 7	30	13 (43)

4.2.3 Identification of miRNA-BLG mice

The final goal of this project was to test the feasibility of the miRNA-mediated knockdown of BLG in mice. That's why a miRNA-BLG mouse model harbouring bBLG or oBLG and miRNA transgenes was generated. In order to generate miRNA-BLG mice, Tg miRNA mouse lines were crossed with hemizygous bBLG or oBLG mice. Offspring from such matings were genotyped by PCR to identify miRNA-BLG mice (Figure 4-6).

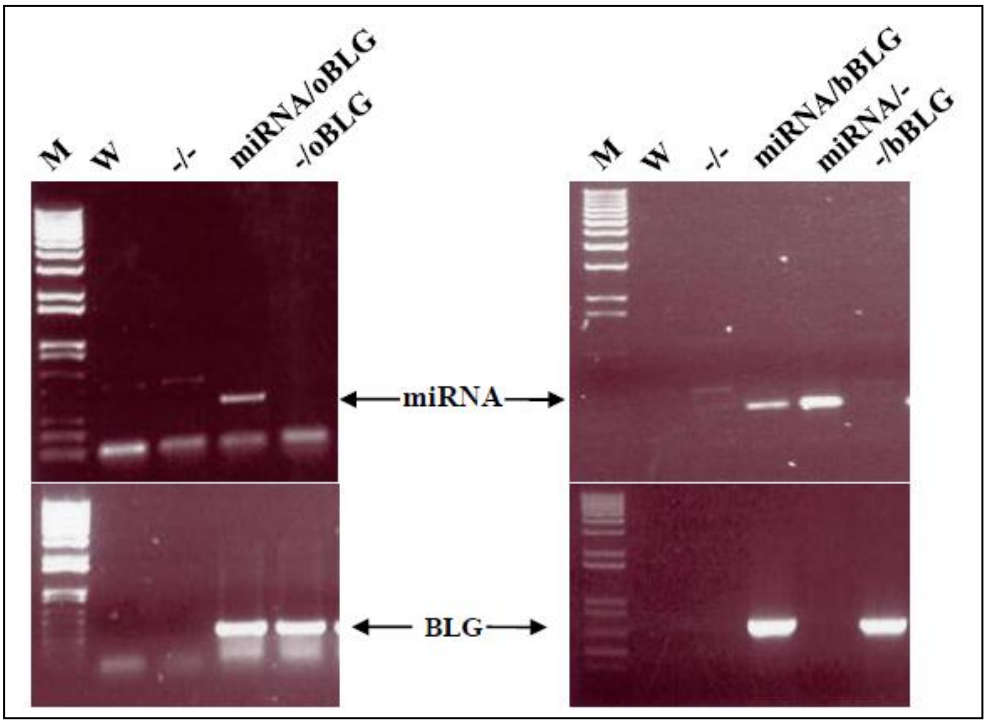


Figure 4-6: PCR genotyping of putative miRNA-BLG mouse.

The representative PCR results of miRNA-BLG mouse show an amplification of miRNA 6-4 as compared to a WT mouse thus indicating the presence of the transgene. A band at 310 bp represents an amplification of miRNA using GL 746 and 747 oligonucleotides as compared to a WT mouse. A band at 650 bp represents an amplification of bBLG gene and a 400 bp band represents an amplification of oBLG using GL 732 and 733 oligonucleotides as compared to a WT mouse thus indicating the presence of the transgenes. M: represents $1kb^{+}$ DNA marker (Invitrogen, USA), W: water negative control, WT: wild type mouse gDNA negative control, miRNA-BLG: a mouse positive for both miRNA and BLG transgenes and -/BLG: a mouse positive for only BLG.

Tail samples of putative miRNA-BLG mice were collected by SAF, AgResearch. PCR genotyping of putative miRNA-BLG mice revealed that 36% (of 14) of mice were positive for both bBLG and miRNA 6-4 ΔpA transgenes (Table 4-4) and 53% (of 15) of mice showed positive for the presence of both oBLG and miRNA 6-4 ΔpA transgenes (Table 4-5). The other tandem construct containing the pA signal showed positive in 17% (of 12) of bBLG mice (Table 4-4) and 30% (of 10) of oBLG mice (Table 4-5). For the single miRNA construct, miRNA 6, 22% (of 9) of mice were positive for bBLG and miRNA 6 transgenes (Table 4-4) and 42% (of 12) of mice showed presence of oBLG and miRNA 6 transgenes (Table 4-5). The presence of miRNA 4 and bBLG transgenes in different mouse lines of miRNA 4 construct ranged from 14-45% (of 11-21) (Table 4-4), whereas, 18-56% (of 9-13) of mice showed positive for both miRNA 4 and oBLG transgenes (Table 4-5).

Table 4-4: Germline transmission of transgenic miRNA-bovine BLG mice.

Construct	Tg miRNA X bBLG mouse	No. offspring	No. Tg (%)
miRNA 6-4 ΔpA	miRNA 6-4 ΔpA X bBLG	14	5 (36)
miRNA 6-4	miRNA 6-4 X bBLG	12	2 (17)
miRNA 6	miRNA 6 X bBLG	9	2 (22)
miRNA 4	miRNA 4 line 1 X bBLG	11	5 (45)
	miRNA 4 line 6 X bBLG	11	3 (27)
	miRNA 4 line 7 X bBLG	21	3 (14)

Table 4-5: Germline transmission of transgenic miRNA-ovine BLG mice.

Construct	Tg miRNA X oBLG mouse	No. offspring	No. Tg (%)
miRNA 6-4 ΔpA	miRNA 6-4 ΔpA X oBLG	15	8 (53)
miRNA 6-4	miRNA 6-4 X oBLG	10	3 (30)
miRNA 6	miRNA 6 X oBLG	12	5 (42)
miRNA 4	miRNA 4 line 1 X oBLG	13	4 (31)
	miRNA 4 line 6 X oBLG	11	2 (18)
	miRNA 4 line 7 X oBLG	9	5 (56)

4.3 Discussion

In order to study the knockdown of BLG in mouse by miRNAs, I needed to generate Tg miRNA mouse lines and a mouse line expressing BLG in milk. Mice expressing oBLG were obtained from Roslin Institute, UK. I also wanted to test the effect of miRNAs to silence bBLG as it is our main target in further studies in cows. I generated bBLG mouse lines in which the expression of bBLG was restricted to the mammary gland. Tg bBLG and miRNA (single and tandem) mice were generated by pronuclear microinjection of linear transgenes for mammary gland specific expression of bBLG and miRNAs into mouse embryos. The restriction in the expression of the miRNA transgenes to the mammary gland provides a tool to achieve gene silencing exclusively during lactation of the double Tg mice and limits potential off-target effects. The microinjection method results in random integration of the transgene into the genome of the embryo and essentially provides no control over the expression level of the transgene. Integration of the transgene into one of the embryonic chromosomes results in animals with a copy of the transgene in every cell only if the transgene integrates at the one cell stage. However, if the transgene fails to integrate into the host genome before doubling of the genetic material, mosaic mice can be produced where many cells do not contain the transgene. So, for the knockdown study the

F1 generation of Tg mice were generated by breeding of founder mouse lines with wild type mice and Tg F1 offsprings because F1 mice are genitically uniform to each other as every cell will contain the transgene.

The survival rate of embryos up to the generation of live pups after pronuclear microinjection of bBLG construct was 16% and transgene integration was only 3%. In 1985, Brinster and colleagues (172) worked on different parameters of pronuclear microinjection methods and they demonstrated that the sticky ended transgene integrated in 6% of the mouse embryos microinjected. Moreover, I also obtained fewer Tg mice than reported for a method using fresh or frozen mouse zygotes (5%) (173). There are several reasons that can cause embryo damage and death of embryos at early embryonic development stage such as the amount and quality of DNA, integration of the transgenes in important gene sites and interruption of essential genes expression. The three confirmed lines of Tg bBLG transmit the transgene to their offspring according to the Mendelian law of inheritance. Most likely, the Tg bBLG mouse lines contain randomly integrated, variable numbers of copies integrated into unknown chromosome locations as they were generated by microinjection. As a consequence, the three Tg bBLG mouse lines showed variable amounts of bBLG expression at the protein level. On the other hand, the efficiency of producing miRNA mouse lines ranged from 1.6-10%. I was able to generate a few Tg miRNA mouse lines for miRNA 6-4 ΔpA and miRNA 4 but for miRNA 6-4 and miRNA 6 contructs only one Tg line was generated. Unexpectedly, three Tg founder lines carrying miRNA 6-4 ΔpA failed to transfer the transgene to their offspring after crossing with a WT mouse. It was observed that the founder Tg mice produced very few live pups compared to other miRNA founders and this suggests that the miRNA construct may somehow be toxic to the embryos and caused embryonic lethality. However, one of Tg miRNA 6-4 ΔpA founder lines transmited the transgene efficiently to 80% of the offspring. The Tg mouse lines carrying miRNA 6-4 and miRNA 4 constructs showed germline transmission ranging from 24-46%. In contrast, Tg miRNA 6 mice showed germline transmission rate of 53% and the small number of mice analysed showed higher germline transmission rate than expected Mendelian ratio.

Chapter Five: Knockdown of BLG *In Vivo*

5.1 Introduction

The generation of a Tg mouse carrying the specific miRNA and the target gene provides us with an opportunity to evaluate the efficiency of the miRNA against the gene expression *in vivo.* The aim of this chapter is to describe the results on testing the feasibility of miRNAs to reduce BLG in milk in a mouse model. In order to demonstrate BLG reduction in milk of miRNA-BLG mice, F1 female miRNA-BLG mice were crossed with wild type mice to get mice into lactation. Milk samples of miRNA-BLG mice were run on SDS gels and coomassie stained to visualize the protein bands and to analyse the effectiveness of the miRNAs to knockdown BLG *in vivo*. In addition, BLG knockdown was also analyzed by western blot analysis. Further, to also determine, whether miRNAs perform knockdown persisitently all over the lactation period, milk of miRNA-BLG mice was analysed on day 5, 10 and 15 by coomassie stain of SDS gels and western blot assays.

5.2 Results

5.2.1 Demonstration of miRNA-mediated reduction of BLG expression

In order to evaluate the efficiency of miRNA constructs against BLG expression *in vivo,* milk from miRNA-BLG mice was compared with milk from mice only expressing BLG but no miRNAs. It has been mentioned earlier that BLG is a ruminant specific protein and does not exist in mouse. The protein banding pattern looks identical for both Tg and non-Tg mouse except for an extra BLG expression band in oBLG and miRNA-oBLG mouse milk (Figure 5-1). Several proteins of

different sizes show up after coomassie staining of SDS gels but fortunately there is no protein visible at the size of BLG protein (18 kD) in wild type and miRNA mouse milk. The activity of miRNAs *in vivo* was calculated based on the BLG expression of the mice only expressing BLG which was set as 100% of BLG expression.

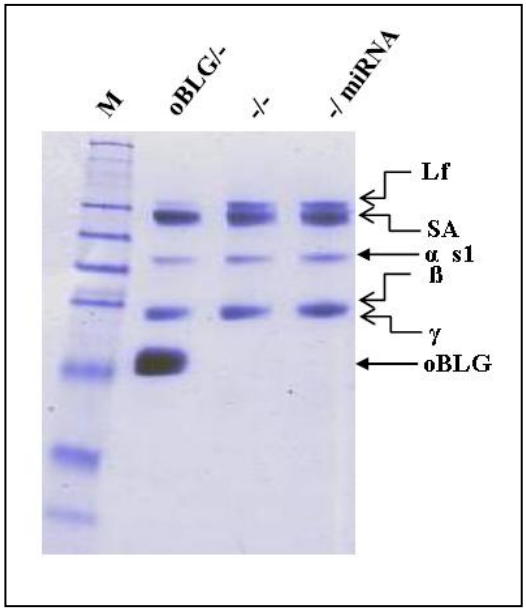


Figure 5-1: Coomassie staining of proteins in milk collected from mice on day 5 of lactation.

Equal amount of total milk protein was loaded onto each lane of gel and the gel was visualized by coomassie stain. M: pre-stained protein maker (Invitrogen), oBLG/-: hemizygous oBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA: miRNA mouse (negative control), Lf: lactoferrin, SA: serum albumin, αs1: α s1-casein, ß: ß-casein and γ: γ-casein.

Initially milk from Tg and non-Tg mouse was collected on day 5 of lactation to get an idea on the effectiveness of the miRNA constructs *in vivo*. Total milk proteins were separated by SDS-PAGE and protein bands were visualized by staining with coomassie blue. Representative coomassie-stained gels show the effect of single and tandem miRNA constructs on the expression of oBLG in milk (Figure 5-2). The expectation for this experiment was to see reduction of oBLG expression in milk of oBLG-miRNA mice at different levels based on the effectiveness of the miRNA constructs. The experiments were repeated three

times to verify their reproducibility. The knockdown calculations were done as mentioned above. The average of the three independent experiments showed that among the miRNAs, the miRNA 6-4 ΔpA construct showed high oBLG knockdown of 96%. The other tandem construct with the pA sequence showed suppression of oBLG by 45%. The oBLG knockdown efficiency by the single miRNA constructs ranged from 43-54%. The knockdown efficiency of miRNA 6 to reduce oBLG expression was 54%. Among the three Tg lines of miRNA 4, line 1, 6 and 7 showed 52, 43 and 48%, respectively of oBLG knockdown. The reduction of oBLG by the miRNA 6-4 ΔpA construct was significantly different from the miRNAs 6-4, miRNA 6 and miRNA 4 (lines 1, 6 and 7) constructs ($P<0.05$) (Figure 5-3). The statistical analysis of the raw data from three independent experiments showed that single and tandem miRNAs had variability in oBLG knockdown of 96%±4 (miRNA 6-4 ΔpA), 45%±23 (miRNA 6-4), 54%±25 (miRNA 6), 52%±16 (miRNA 4 line 1), 43%±7 (miRNA 4 line 6) and 48%±18 (miRNA 4 line 7) (Figure 5-3).

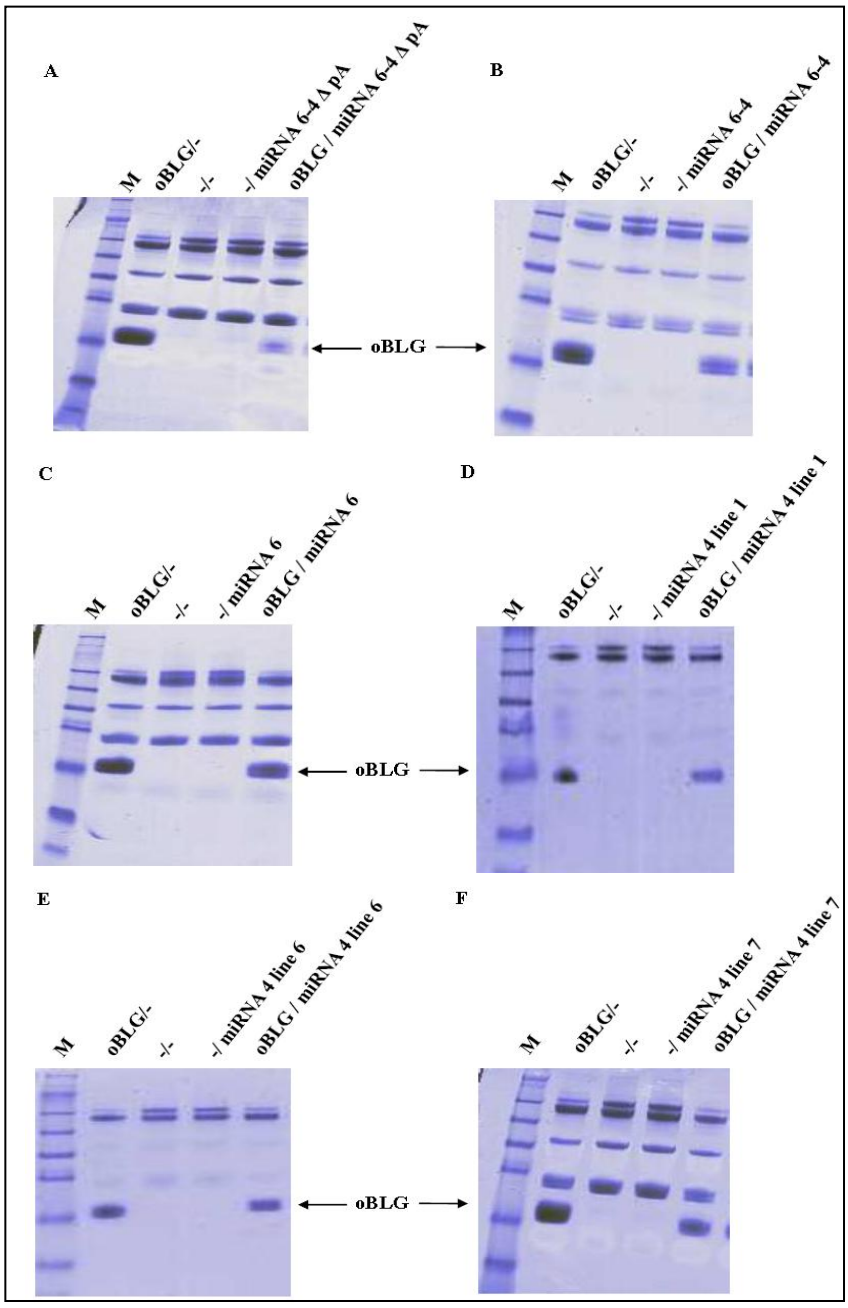


Figure 5-2: Knockdown of ovine BLG by miRNAs using SDS-PAGE analysis and detection by coomassie blue.

This figure shows the coomassie staining of total milk protein of miRNA-BLG mouse containing miRNA and oBLG transgenes. Equal amount of total milk protein was loaded onto each lane of gel and the gel was stained with coomassie stain. A-F represent knockdown of oBLG by miRNA 6-4 ΔpA, miRNA 6-4, miRNA 6, miRNA 4 line 1, miRNA 4 line 6 and miRNA 4 line 7, respectively. M: pre-stained protein maker (Invitrogen), oBLG/-: hemizygous oBLG mouse (positive control), -/-: WT mouse

(negative control), -/miRNA: miRNA mouse (negative control), and oBLG/miRNA: miRNA-BLG mouse positive for both oBLG and miRNA transgenes.

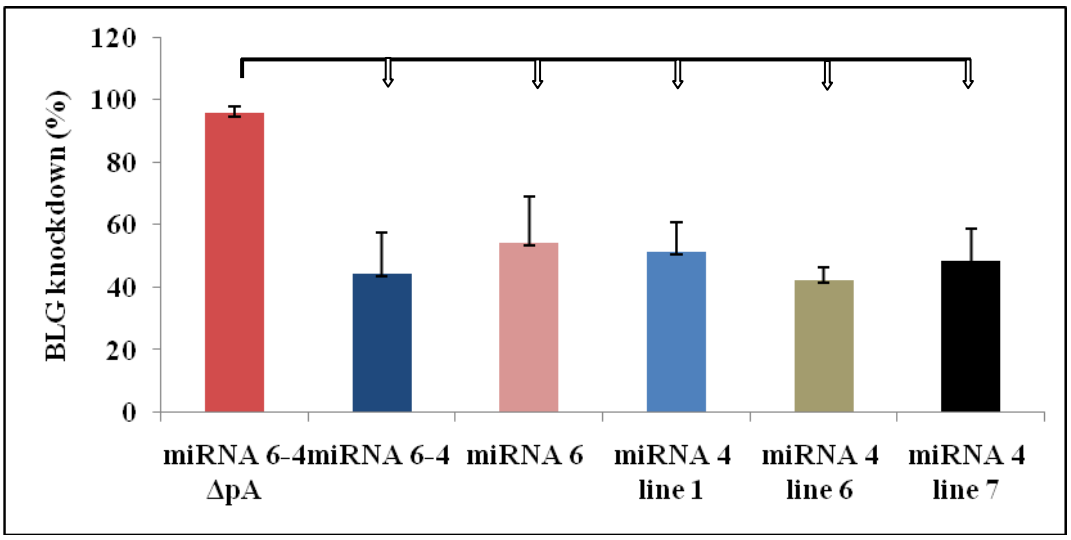


Figure 5-3: Comparison of ovine BLG knockdown by miRNA constructs using SDS-PAGE analysis and detection by coomassie blue.

All data are presented as the percent reduction of oBLG in milk on day 5 compared with the positive control. Experiment results were an average of three independent experiments. Statistical significance among means was determined using a paired two sample *t*-test ($p<0.05$). Error bars represent SEM determined from n= 3 repeats.

Mice have a lactation period of about three weeks. In order to check the stability in the expression of miRNAs and their ability to inhibit oBLG expression over the whole lactation period, milk of miRNA 6-4 ΔpA-oBLG mice was collected on various days of lactation (day 5, 10 and 15) from two mice of the same litter. Expectations from this experiment were that miRNA 6-4 ΔpA would be effective against oBLG expression over the lactation period with no major differences between littermates. A representative gel stained by coomassie blue is shown in Figure 5-4 and firstly reveals that miRNA 6-4 ΔpA construct knocked down oBLG substantially as compared to the mouse expressing only oBLG. Moreover, no major difference in oBLG expression was found during different time points in two littermates. The SDS-PAGE analysis of milk protein was done in three independent experiments and the knockdown efficiency of miRNA 6-4 ΔpA was calculated as mentioned above. One (marked as a in Figure 5-4) of the littermates showed an average of 96%, 97% and 97% of oBLG knockdown on day 5, 10 and

15, respectively. The other (as marked as b in Figure 5-4) showed 97%, 97% and 97% of oBLG knockdown on those days as mentioned above (Figure 5-5). There was no significant difference between littermates in terms of oBLG knockdown on different lactation days (P>0.05). In addition, the difference in oBLG knockdown among day 5, 10 and 15 lactation periods was also insignificant (P>0.05). The statistical analysis of the raw data from three independent experiments showed that miRNA 6-4 ΔpA construct had variability in oBLG knockdown on different days of lactation in both littermates of 96%±4, 97%±2 and 97%±2, respectively (littermate a) and 97%±3, 97%±3 and 97%±1, respectively (littermate b) (Figure 5-5).

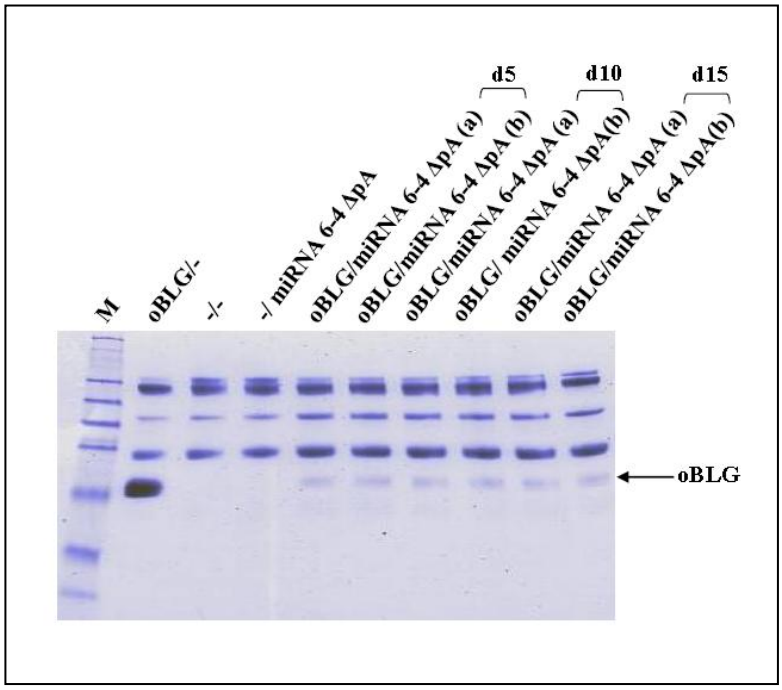


Figure 5-4: Analysis of ovine BLG knockdown by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue.

The figure shows the coomassie staining of total milk protein of Tg and non-Tg mice on day 5, 10 and 15 of lactation. Equal amount of total milk protein was loaded onto each lane of gel and the gel was stained with coomassie stain. M: pre-stained protein maker (Invitrogen), oBLG/-: hemizygous oBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA 6-4 ΔpA: Tg mouse carrying miRNA 6-4 ΔpA (negative control) and oBLG/miRNA 6-4 ΔpA: Tg mouse positive for both oBLG and miRNA 6-4 ΔpA transgenes. a and b represent littermates and both carrying oBLG and miRNA 6-4 ΔpA transgenes.

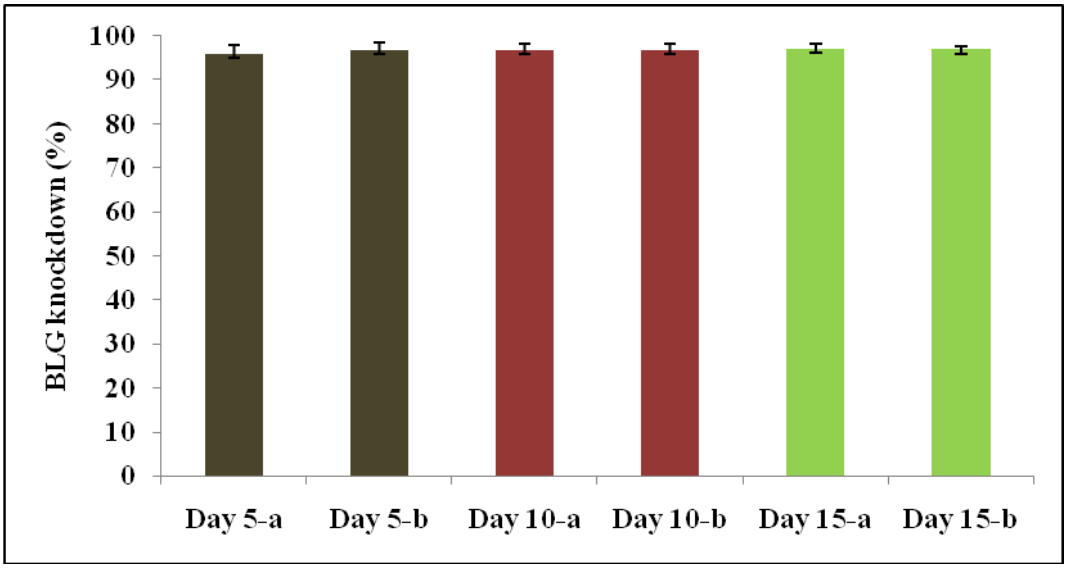


Figure 5-5: Knockdown of ovine BLG during the lactation period by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue.

All data are presented as the percent reduction of oBLG in milk of miRNA 6-4 ΔpA-oBLG mice on day 5, day 10 and day 15 of lactation as compared to the positive control. Experiment results were an average of three independent experiments. Error bars represent SEM determined from n= 3 repeats.

The oBLG knockdown results obtained from the coomassie staining of total milk protein of Tg and non-Tg mice collected on day 5 of lactation were further verified by highly sensitive western blot assay which will allow to compare with the oBLG knockdown results obtained from the coomassie staining. Initially milk from both Tg and non-Tg mouse was analysed by western blot assay for the detection of oBLG and there was no oBLG signal for the negative controls (WT and miRNA mouse) but a bright signal for the positive control (oBLG mouse) as expected (Figure 5-6). The signal intensities of oBLG on exposure film were measured by Quantity One software (Bio-Rad). The efficiency of miRNAs to down regulate the expression of oBLG *in vivo* was calculated based on the BLG expression by the mice only expressing oBLG which was set as 100% of BLG expression.

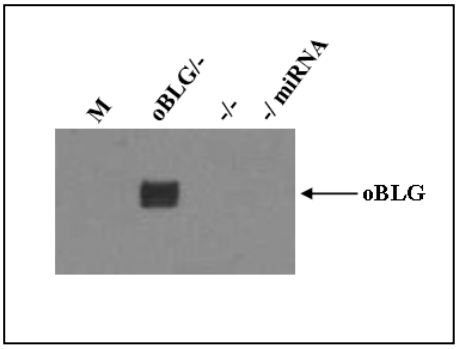


Figure 5-6: Western blot analysis of proteins in milk collected from mice on day 5 of lactation.

This figure shows the western blot analysis of total milk protein on day 5 of lactation of Tg and non-Tg mice. Equal amount of total milk protein was loaded onto each lane of gel and the oBLG expression was detected using an antibody directed against BLG (rabbit anti-BLG) as described in Materials and Methods. M: pre-stained protein maker (Invitrogen), oBLG/-: hemizygous oBLG mouse (positive control), -/-: WT mouse (negative control) and -/miRNA: miRNA mouse (negative control).

Representative western blots showing the effectiveness of single and tandem miRNA constructs against oBLG expression are shown in Figure 5-7. The expectation from this analysis was to see similar reduction of oBLG expression in milk as I found by coomassie staining. The experiments were repeated three times as done for coomassie-stained SDS gels to verify the reproducibility of the western blot assay. The knockdown calculations were done as mentioned above. The western analysis showed that the average oBLG knockdown by the tandem constructs (miRNA6-4 ΔpA and miRNA 6-4) were 88% and 54%, respectively. The single miRNA constructs showed an average oBLG knockdown efficiency ranging from 38-50%. The miRNA 6 construct yielded 38% oBLG knockdown. The three Tg lines (lines 1, 6 and 7) carrying miRNA 4 showed 50%, 38% and 45% of oBLG knockdown, respectively. As seen in coomassie stain knockdown analysis, the miRNA 6-4 ΔpA construct was also significantly different to other miRNA constructs in case of oBLG knockdown ($P<0.05$) (Figure 5-8). The statistical analysis of the raw data from three independent experiments showed that single and tandem miRNAs had variability in oBLG knockdown of 88%±14 (miRNA 6-4 ΔpA), 54%±13 (miRNA 6-4), 38%±17 (miRNA 6), 50%±17

(miRNA 4 line 1), 38%±22 (miRNA 4 line 6) and 45%±12 (miRNA 4 line 7) (Figure 5-8).

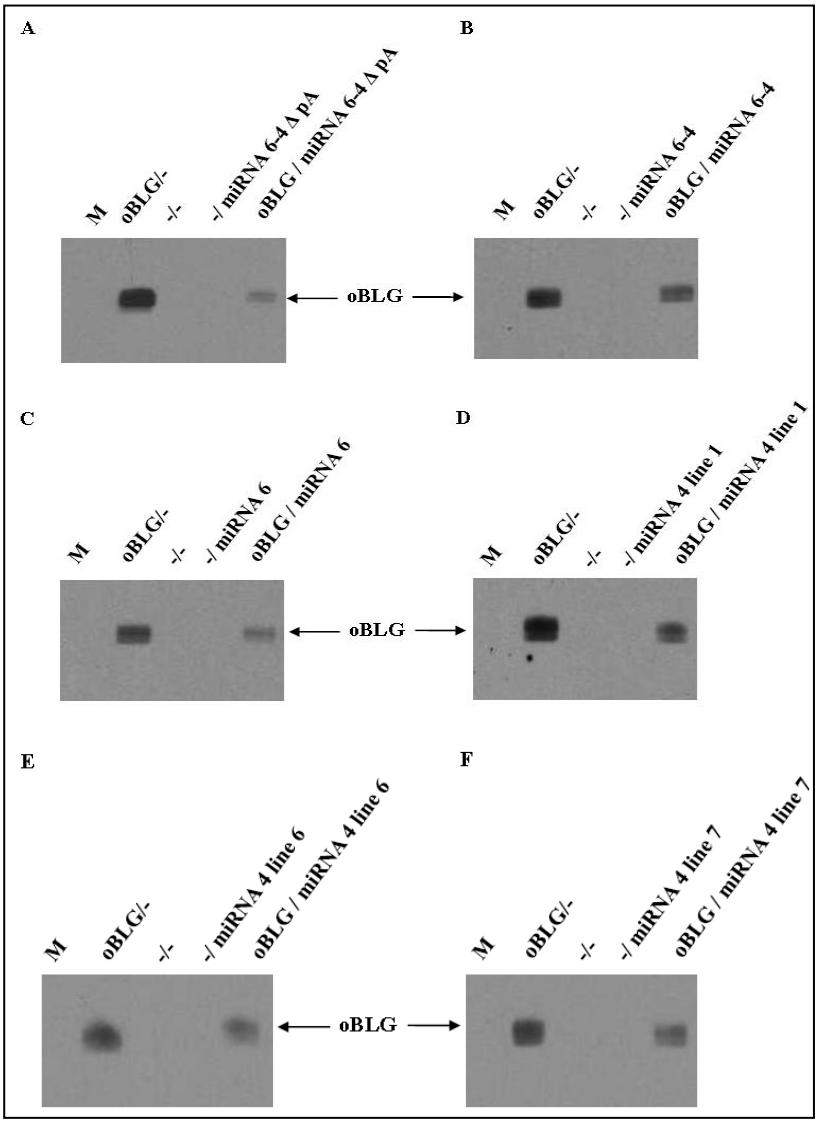


Figure 5-7: Knockdown of ovine BLG by single and tandem miRNAs using western blot analysis.

This figure shows the western blot analysis of total milk protein on day5 of lactation of Tg and non-Tg mice. Equal amount of total milk protein was loaded onto each lane of gel and the oBLG expression was detected using an antibody directed against BLG (rabbit anti-BLG) as described in Materials and Methods. A-F represent knockdown of oBLG by miRNA 6-4 ΔpA, miRNA 6-4, miRNA 6, miRNA 4 line 1, miRNA 4 line 6 and miRNA 4 line 7, respectively. M: pre-stained protein maker (Invitrogen), oBLG/-: hemizygous oBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA: miRNA

mouse (negative control), and oBLG/miRNA: miRNA-BLG mouse positive for both oBLG and miRNA transgenes.

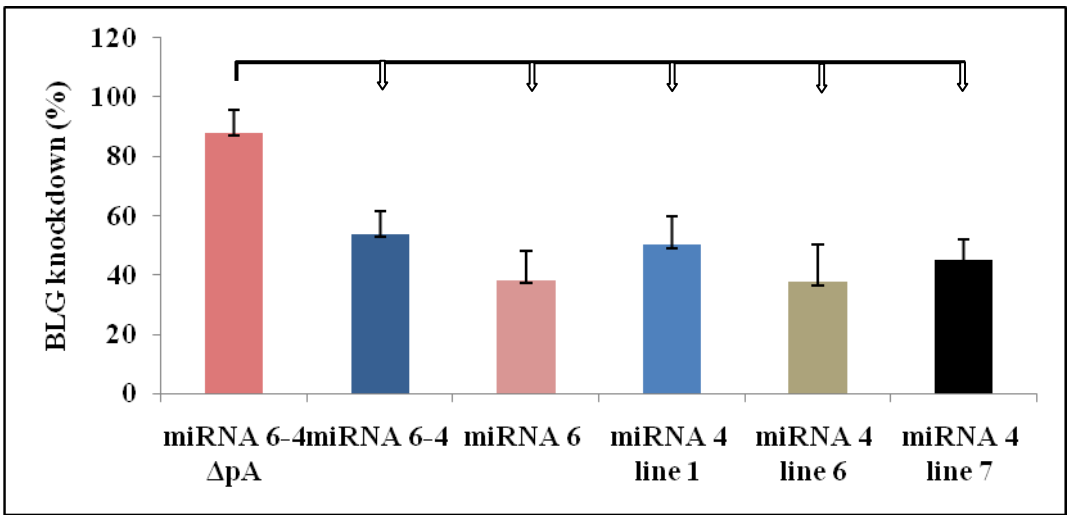


Figure 5-8: Comparison of ovine BLG knockdown by miRNA constructs using western blot analysis.

All data are presented as the percent reduction of oBLG in milk on day 5 compared with the positive control. Experiment results were an average of three independent experiments. Statistical significance among means was determined using a paired two sample *t*-test ($p<0.05$). Error bars represent SEM determined from n= 3repeats.

In order to verify the oBLG knockdown results obtained by coomassie staining during different lactation time points and littermates carrying both miRNA 6-4 ΔpA and oBLG transgenes, milk of miRNA 6-4 ΔpA-oBLG was further analysed by western blot assay. The expectations of this analysis were to see similar knockdown results by the miRNA 6-4 ΔpA construct across the lactation period with no variations between littermates. A representative western blot knockdown analysis by the miRNA 6-4 ΔpA construct is shown in Figure 5-9. The western blot showed a detectable but much weaker oBLG signal than for the control which means miRNA 6-4 ΔpA construct knocked down the gene very efficiently. In addition, the oBLG knockdown ability of the miRNA 6-4 ΔpA construct was consistent over the lactation period and there were no differences in oBLG knockdown between littermates. In order to verify the reliability of the western blot results of oBLG knockdown by miRNA 6-4 ΔpA construct, this experiment was repeated in two other independent experiments and the knockdown efficiency

of miRNA 6-4 ΔpA was calculated as mentioned above. The average of the three experiments showed that miRNA 6-4 ΔpA construct silenced oBLG of 88%, 90% and 93% on day 5, 10 and 15, respectively in one littermate (as marked as a in Figure 5-9). The other littermate (marked as b in Figure 5-9) was equally efficient to its littermate showing oBLG knockdown of 91%, 91% and 95% on day5, 10 and 15, respectively (Figure 5-10). The western blot analysis of oBLG knockdown by the miRNA 6-4 ΔpA construct showed insignificant differences among various lactation days and between littermates (P>0.05). The statistical analysis of the raw data from three independent experiments showed that miRNA 6-4 ΔpA construct had variability in oBLG knockdown on different days of lactation in both littermates of 88%±14, 90%±14 and 93%±8, respectively (littermate a) and 91%±12, 91%±11 and 95%±5, respectively (littermate b) (Figure 5-10).

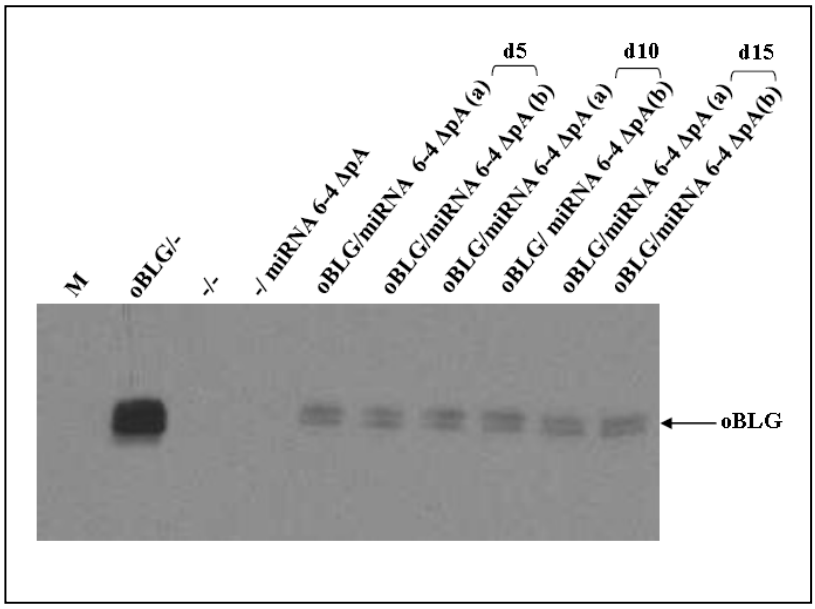


Figure 5-9: Analysis of ovine BLG knockdown by miRNA 6-4 ΔpA construct using western blot analysis.

This figure shows a western blot analysis of oBLG expression in Tg and non-Tg mouse milk on day 5, day 10 and day 15 of lactation. Equal amount of total milk protein was loaded onto each lane of SDS gel and the oBLG expression was detected using an antibody directed against BLG (rabbit anti-BLG) as described in Materials and Methods. M: pre-stained protein maker (Invitrogen), oBLG/-: hemizygous oBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA 6-4 ΔpA: miRNA mouse (negative

control), and oBLG/miRNA 6-4 ΔpA: miRNA-BLG mouse positive for both oBLG and miRNA 6-4 ΔpA transgenes. a and b represent littermates and both carrying oBLG and miRNA 6-4 ΔpA transgenes.

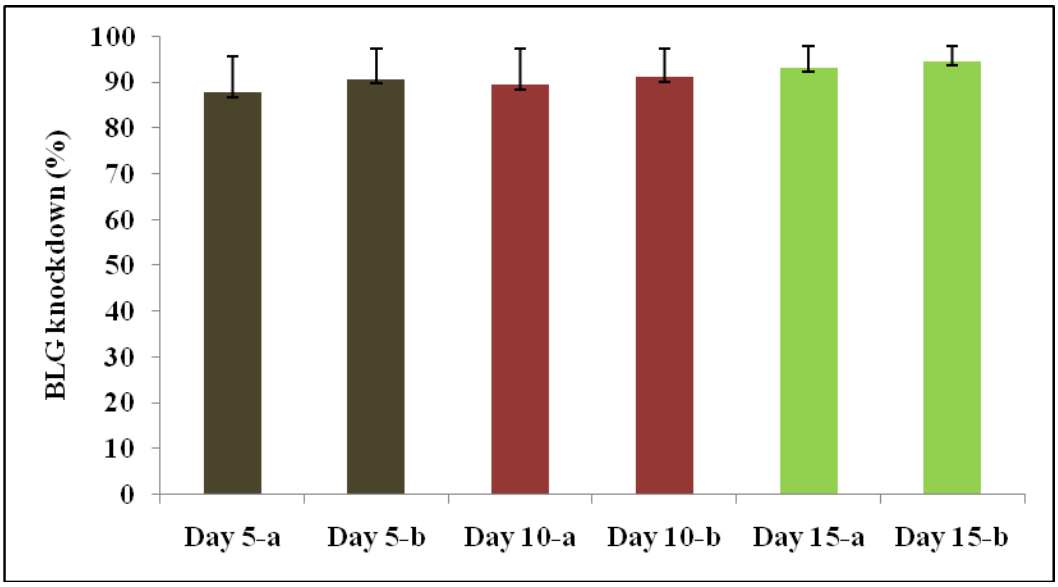


Figure 5-10: Knockdown of ovine BLG around the lactation period by miRNA 6-4 ΔpA construct using western blot analysis.

All data are presented as the percent reduction of oBLG in milk on day 5, 10 and 15 compared with the positive control. Experiment results were an average of three independent experiments. Error bars represent SEM determined from n= 3repeats.

The aim of using two different assay systems (coomassie stain and western blot) was to compare and evaluate miRNAs efficiency against oBLG expression *in vivo*. Theoretically, both assays should produce very similar oBLG knockdown results. Both assay systems showed consistent oBLG knockdown by miRNAs as expected. Moreover, the most effective tandem construct (miRNA 6-4 ΔpA) was found significantly different to other miRNA constructs in both assays.

The knockdown efficiency of miRNA constructs was also tested *in vivo* against the expression of bBLG. The total milk protein pattern of bBLG-miRNA mice was same as oBLG-miRNA mice milk proteins on gels (data not shown). The measurement of signal intensities of coomassie stained milk proteins was done using Quantity One software (Bio-Rad). The internal control protein (α-casein) was considered to normalize the expression of bBLG and the efficiency of

miRNAs to knockdown bBLG was calculated based on 100% of bBLG expression of the bBLG mice.

Milk collected on day 5 of lactation from Tg mice (mice expressing bBLG or miRNA or bBLG and miRNA) and non-Tg mice (WT) was analysed by coomassie stain. A representative coomassie stained gel was shown in Figure 5-11 showing the potential of the miRNAs to inhibit expression of bBLG. The miRNA constructs were expected to reduce bBLG expression at different levels. Milk samples were analysed in three independent experiments using coomassie stain and the intensities of the control protein and bBLG were used for knockdown calculation as mentioned above. Average of the knockdown results obtained from three separate experiments showed that the miRNA6-4 ΔpA construct knocked down only 46% of bBLG which is unexpectedly lower than the singles. The average bBLG knockdown efficiencies were 51%, 71% and 51% by the miRNA 6, miRNA 4 line 1 and line 6, respectively. However, the miRNA 6-4 construct and miRNA 4 line 7 showed a surprising increase in bBLG expression of 15% and 34%, respectively. The miRNA 6-4 and miRNA 4 line 7 had no knockdown effect on bBLG gene and they showed significant difference to other miRNA constructs. In addition, miRNA 6-4 ΔpA construct also showed significant difference to miRNA 4 line 1 ($P<0.05$) (Figure 5-12). The statistical analysis of the raw data from three independent experiments showed that single and tandem miRNAs had variability in bBLG knockdown of 46%±6 (miRNA 6-4 ΔpA),-15%±16 (miRNA 6-4), 51%±15 (miRNA 6), 71%±2 (miRNA 4 line 1), 51%±18 (miRNA 4 line 6) and -34%±41 (miRNA 4 line 7).

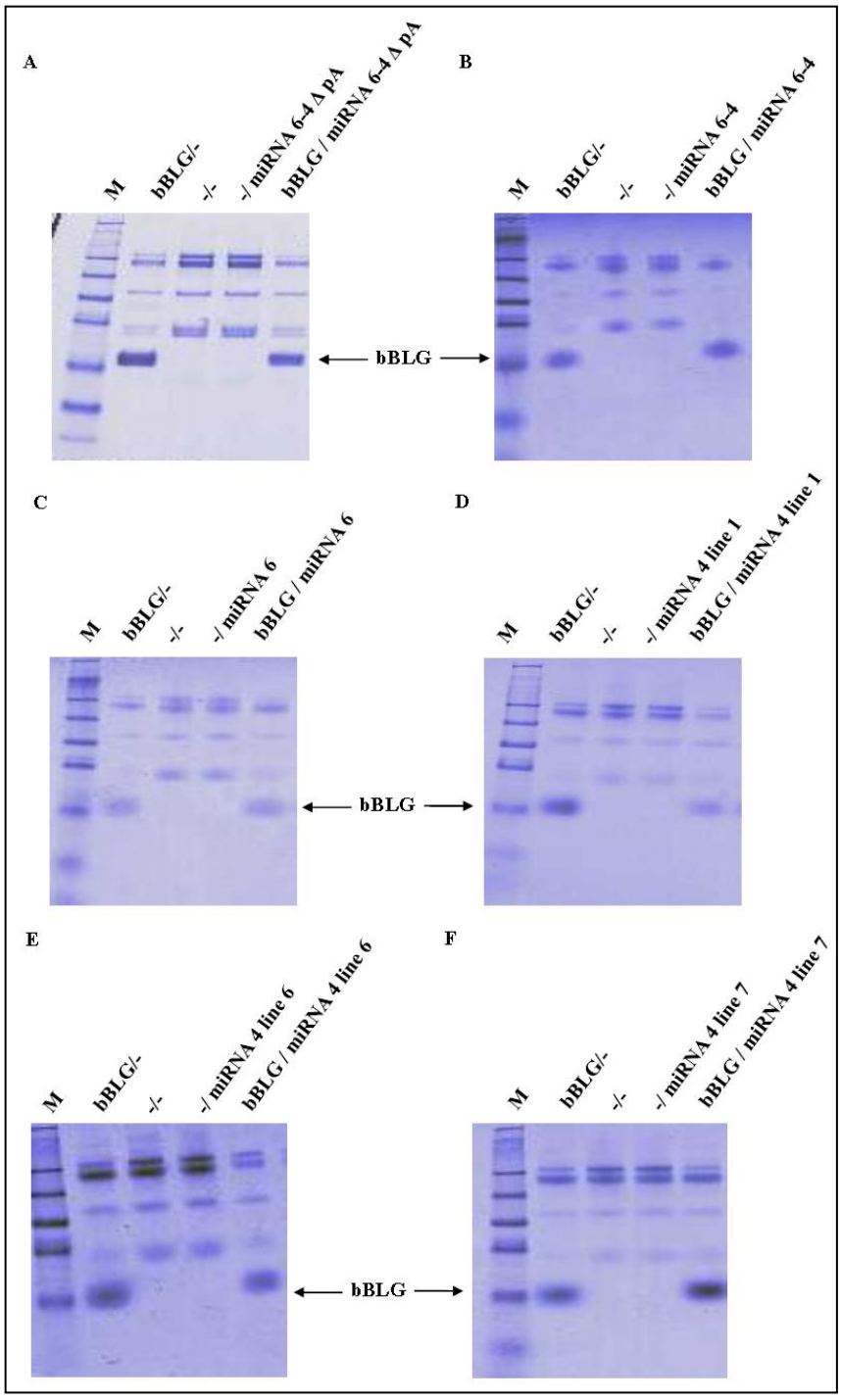


Figure 5-11: Knockdown of bovine BLG by miRNAs using SDS-PAGE analysis and detection by coomassie blue.

This figure shows the coomassie staining of total milk protein of miRNA-BLG mice containing miRNA and bBLG transgenes. Equal amount of total milk protein was loaded onto each lane of gel and the gel was stained with coomassie stain. A-F represent knockdown of bBLG by miRNA 6-4 ΔpA, miRNA 6-4, miRNA 6, miRNA 4 line 1, miRNA 4 line 6 and miRNA 4 line 7, respectively. M: pre-stained protein maker

(Invitrogen), bBLG/-: hemizygous bBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA: miRNA mouse (negative control), and bBLG/miRNA: miRNA-BLG mouse positive for both bBLG and miRNA transgenes.

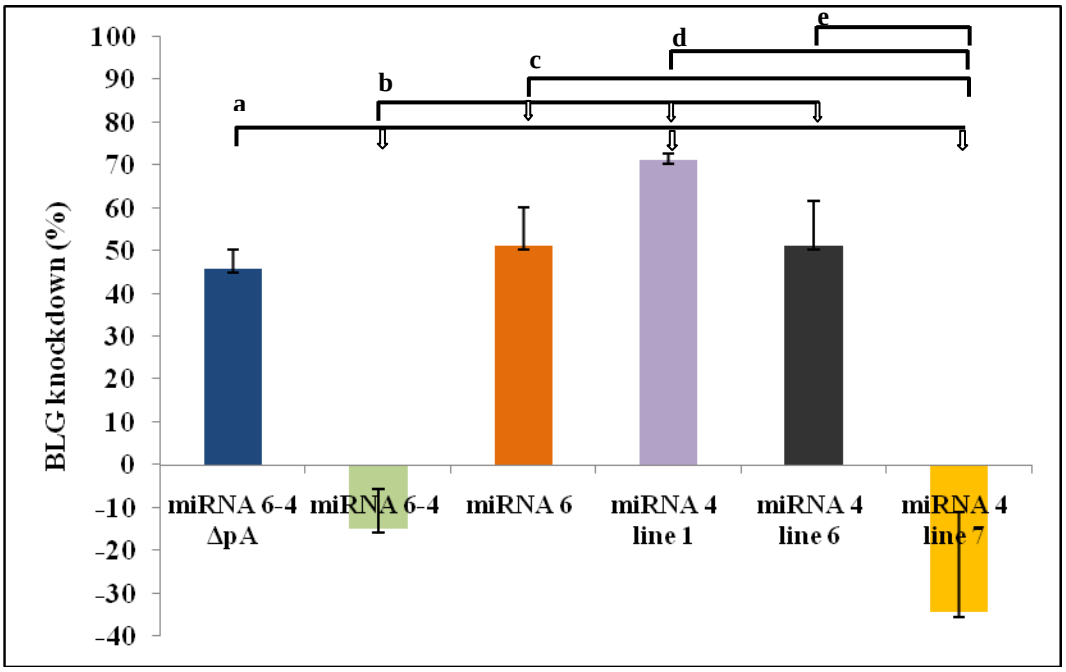


Figure 5-12: Comparison of bovine BLG knockdown by miRNA constructs using SDS-PAGE analysis and detection by coomassie blue.

All data are presented as the percent reduction of bBLG in milk on day 5 compared with the positive control. Experiment results were an average of three independent experiments. Statistical significance among means was determined using a paired two sample *t*-test ($p<0.05$). Error bars represent SEM determined from n= 3repeats.

In the same way as for oBLG knockdown analysis by coomassie staining, milk from miRNA 6-4 ΔpA-bBLG mice was collected and further analysed by coomassie staining in order to compare the potential effect of the miRNA 6-4 ΔpA on bBLG expression on various days of lactation (day 5, 10 and 15) and also to compare the bBLG knockdown efficiency of the miRNA in littermates. Expectations from this experiment were that miRNA 6-4 ΔpA would show reduced expression of bBLG on day 10 and 15 as like as day 5 without any substantial differences in littermates. A representative gel stained by coomassie blue is shown in Figure 5-13 and reveals that miRNA 6-4 ΔpA construct knocked down bBLG equally on different days of lactation with no substantial differences

in littermates as expected. The efficiency of miRNA 6-4 ΔpA to knockdown bBLG was calculated based on three independent experiments as mentioned above. One (marked as a in Figure 5-13) of the littermates showed an average of 46%, 43% and 53% of bBLG knockdown on day 5, 10 and 15, respectively. The other (marked as b in Figure 5-13) showed 56%, 43% and 57% of bBLG knockdown on those days as mentioned above (Figure 5-14). Although there was a slight difference in bBLG knockdown on different days of lactation between littermates, there was no significant difference between littermates and lactation days in terms of bBLG knockdown (P>0.05). The statistical analysis of the raw data from three independent experiments showed that miRNA 6-4 ΔpA construct had variability in bBLG knockdown on different days of lactation in both littermates of 46%±6, 43%±18 and 53%±5, respectively (littermate a) and 56%±4, 43%±15 and 57%±2, respectively respectively (littermate b) (Figure 5-14).

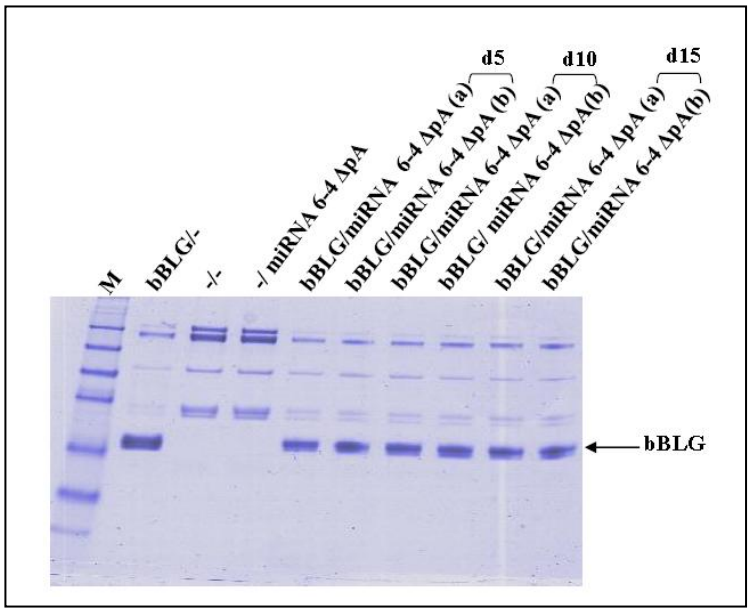


Figure 5-13: Analysis of bovine BLG knockdown by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue.

The figure shows the coomassie staining of total milk protein of Tg and non-Tg mice on day 5, 10 and 15 of lactation. Equal amount of total milk protein was loaded onto each lane of gel and the gel was stained with coomassie stain. M: pre-stained protein maker (Invitrogen), bBLG/-: hemizygous bBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA 6-4 ΔpA: Tg mouse carrying miRNA 6-4 ΔpA (negative control) and bBLG/miRNA 6-4 ΔpA: Tg mouse positive for both bBLG and miRNA 6-4

ΔpA transgenes. a and b represent littermates and both carrying bBLG and miRNA 6-4 ΔpA transgenes.

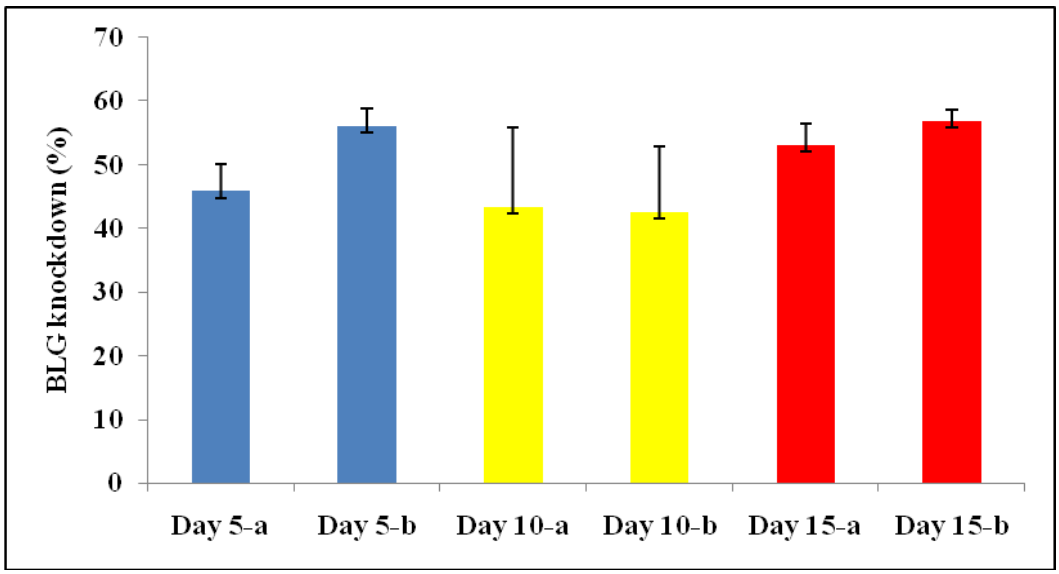


Figure 5-14: Knockdown of bovine BLG during different lactation time points by miRNA 6-4 ΔpA construct using SDS-PAGE analysis and detection by coomassie blue.

All data are presented as the percent reduction of bBLG in milk of miRNA 6-4 ΔpA-bBLG mice on day 5, 10 and 15 of lactation as compared to the positive control. Experiment results were an average of three independent experiments. Error bars represent SEM determined from n= 3repeats.

The bBLG knockdown results obtained from the coomassie staining of total milk protein of bBLG-miRNA mice collected on day 5 of lactation were further verified by highly sensitive western blot assay. Representative western blots showing the effectiveness of single and tandem miRNA constructs against bBLG expression are shown in Figure 5-15. The expectation from this analysis was to see similar effect of miRNAs on the expression of bBLG in milk as observed by coomassie staining. The assay was repeated three times and the efficiency of the miRNAs silencing bBLG was quantified as mentioned before. In an unexpected result, the tandem miRNA constructs (miRNA 6-4 ΔpA and miRNA 6-4) showed up regulation of the bBLG expression by an average of 12% and 7%, respectively. However, miRNA 6-4 ΔpA construct showed 46% of bBLG knockdown by coomassie staining. Moreover, miRNA 6 also showed up regulating bBLG expression by 48% which is the complete opposite of what I observed in

coomassie staining results. The miRNA 4 lines 1 and 6 showed some activity against bBLG expression (2% and 23%, respectively) but the efficiency is much lower than that I observed in coomassie staining results. However, an opposite scenario was found for miRNA 4 line 7 where the construct showed an average bBLG knockdown of 13% by western blot analysis and 34% up regulation of bBLG expression by SDS-PAGE analysis (Figure 5-16). Overall, the knockdown results obtained from western blot analysis were unexpected when compared to the results obtained from coomassie staining. Investigation of the statistical analysis of the bBLG knockdown by miRNA constructs obtained from western blot and coomassie staining assays revealed that there was a high variation among experiment repeats in western blot analysis compared to coomassie staining thus making the western blot knockdown results unreliable. The statistical analysis of the raw data from three independent experiments showed that single and tandem miRNAs had variability in bBLG knockdown of-12%±28 (miRNA 6-4 ΔpA), -7%±51 (miRNA 6-4), -48%±97 (miRNA 6), 2%±50 (miRNA 4 line 1), 23%±49 (miRNA 4 line 6) and 13%±14 (miRNA 4 line 7) (Figure 5-16).

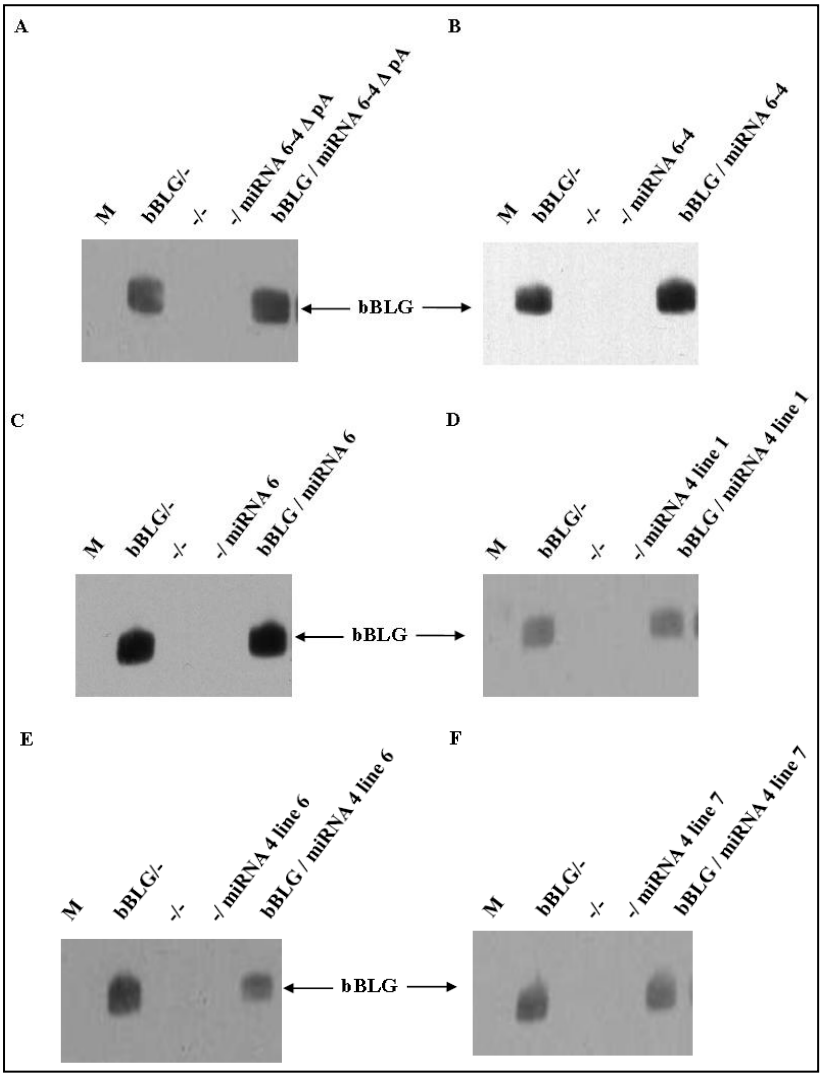


Figure 5-15: Knockdown of bovine BLG by single and tandem miRNAs using western blot analysis.

This figure shows the western blot analysis of total milk protein on day 5 of lactation of Tg and non-Tg mice. Equal amount of total milk protein was loaded onto each lane of gel and the bBLG expression was detected using an antibody directed against BLG (rabbit anti-BLG) as described in Materials and Methods. A-F represent knockdown of bBLG by miRNA 6-4 ΔpA, miRNA 6-4, miRNA 6, miRNA 4 line 1, miRNA 4 line 6 and miRNA 4 line 7, respectively. M: pre-stained protein maker (Invitrogen), bBLG/-: hemizygous bBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA: miRNA mouse (negative control), and bBLG/miRNA: miRNA-BLG mouse positive for both bBLG and miRNA transgenes.

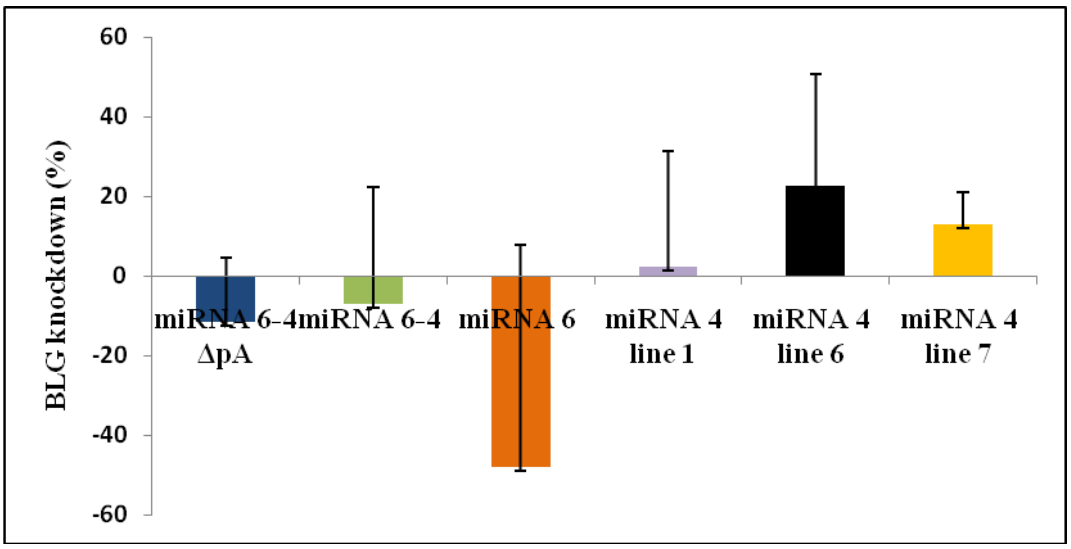


Figure 5-16: Comparison of bovine BLG knockdown by miRNA constructs using western blot analysis.

All data are presented as the percent reduction of oBLG in milk on day 5 compared with the positive control. Experiment results were an average of three independent experiments. Error bars represent SEM determined from n= 3repeats.

The knockdown efficiency of the miRNA 6-4 ΔpA construct analysed by coomassie staining was about 50%. However, no knockdown activity was found by this miRNA construct in day 5 milk sample when analysed by western blot assay. So, I wanted to see the effect of miRNAs on bBLG expression with regard of different stages of lactation and also to compare the results with the results I found in coomassie staining. A representative western blot showing the effect of miRNA 6-4 ΔpA construct on bBLG expression is shown in Figure 5-17. The western blot shows detectable bBLG signals that look alike for both positive control and samples. Moreover, the signals for bBLG look consistent over the lactation period and there are no differences in littermates. This experiment was repeated in three independent experiments and the knockdown efficiency of miRNA 6-4 ΔpA was calculated as mentioned above. The average of the three experiments showed that miRNA 6-4 ΔpA construct increased the expression of bBLG by 12% and 17% on day 5 and 10. However, the construct showed some knockdown activity (20%) on day 15 of lactation in one littermate (marked as a in Figure 5-17). In contrast, the other littermate (marked as b in Figure 5-17) only showed bBLG up regulation on day 5 (16%) and down regulation of bBLG

expression by 6% and 44% on day 10 and 15, respectively. There was no significant difference between different lactation days for littermate a ($P>0.05$). However, a significant difference was found between day 10 and 15 for littermate b ($P<0.05$). Moreover, the effect of miRNA 6-4 ΔpA on day 10 in littermate a was also significantly different to its effect on day 15 in littermate b ($P<0.05$) (Figure 5-18). The statistical analysis of the raw data from three independent experiments showed that miRNA 6-4 ΔpA construct had variability in bBLG knockdown on different days of lactation in both littermates of -12%±28, -17%±19 and 20%±21, respectively (littermate a) and -16%±12, 6%±7 and 44%±32, respectively (littermate b) (Figure 5-18).

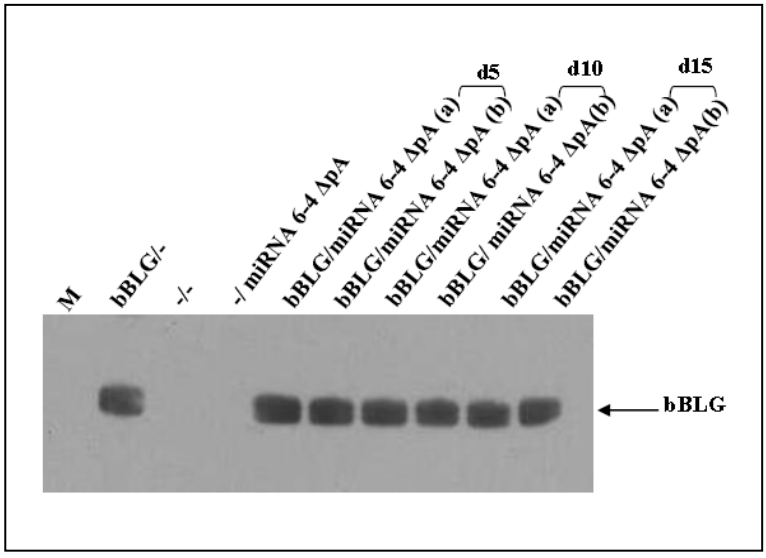


Figure 5-17: Analysis of bovine BLG knockdown by miRNA 6-4 ΔpA construct using western blot analysis.

This figure shows a western blot analysis of bBLG expression in Tg and non-Tg mouse milk on day 5, day 10 and day 15 of lactation. Equal amount of total milk protein was loaded onto each lane of SDS gel and the bBLG expression was detected using an antibody directed against BLG (rabbit anti-BLG) as described in Materials and Methods. M: pre-stained protein maker (Invitrogen), bBLG/-: hemizygous bBLG mouse (positive control), -/-: WT mouse (negative control), -/miRNA 6-4 ΔpA: miRNA mouse (negative control), and bBLG/miRNA 6-4 ΔpA: miRNA-BLG mouse positive for both bBLG and miRNA 6-4 ΔpA transgenes. a and b represent littermates and both carrying bBLG and miRNA 6-4 ΔpA transgenes.

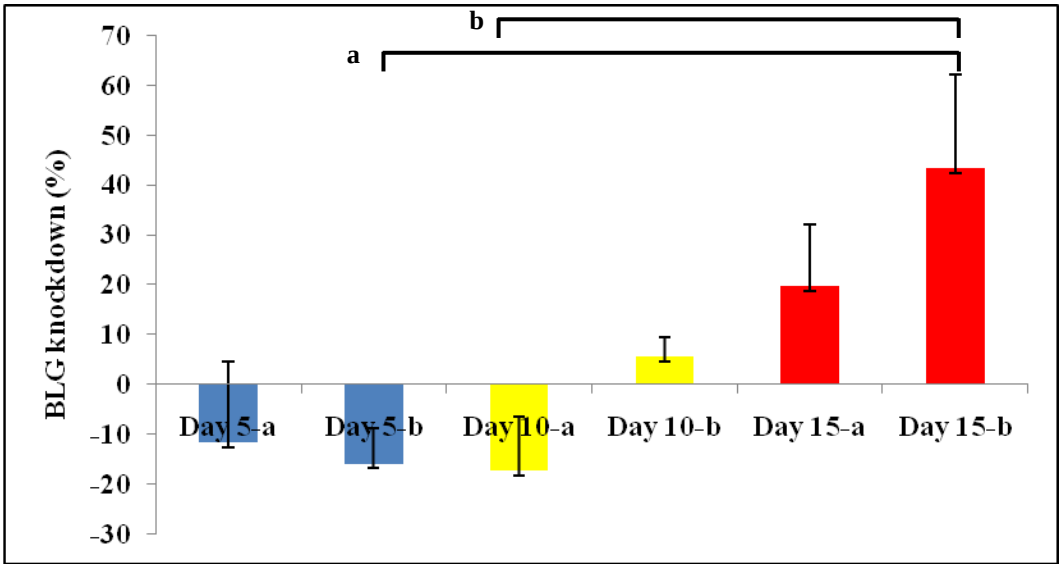


Figure 5-18: Knockdown analysis of bovine BLG around the lactation period by miRNA 6-4 ΔpA construct using western blot.

All data are presented as the percent reduction of bBLG in milk on day 5, day 10 and day 15 compared with the positive control. Experiment results were an average of three independent experiments. Error bars represent SEM determined from n= 3repeats.

5.3 Discussion

Here, I present *in vivo* knockdown of BLG in a mouse model using mammary gland tissue-specific single and multiple BLG specific miRNAs. Several approaches were developed previously to perform BLG knockdown, however, no report has been published yet showing knockdown of bBLG and oBLG in an *in vivo* system. The ultimate goal of this study was to validate the feasibility of reducing BLG levels in milk by expressing a miRNA in the mammary gland under the control of a milk protein promoter. For this, I generated Tg mice that contain BLG and miRNA transgenes that were shown to possess knockdown activity against BLG in an *in vitro* assay. To see whether miRNAs knockdown bBLG and oBLG *in vivo*, milk of mice carrying both BLG and miRNA transgenes were analysed by SDS-PAGE following staining of separated milk proteins with coomassie blue. The western blot analysis was used as an alternative method to validate results obtained from the coomassie stain. The coomassie staining of day 5 total milk protein obtained from double Tg mice showed almost complete abrogation of oBLG by the tandem miRNA 6-4ΔpA construct while the

polyadenylated version of miRNA 6-4 and single miRNAs knocked down BLG levels by about 50%. The Tg miRNA mice were generated by microinjection which contributes random integration of transgenes. As a result the levels of miRNA expression can vary which can potentially affect the knockdown efficiency of miRNAs. Although the processed miRNA 6 has, in relation to the oBLG mRNA, two mismatches at its 3' end it showed effectiveness against oBLG expression (54%). It has been reported that nucleotide mismatches (up to four) at the most 3' terminal positions of siRNAs sequence to the target mRNA have been shown to still result in efficient gene knockdown in HeLa cells (160). In contrast to the oBLG knockdown, the SDS-PAGE analysis of knockdown of the bovine form, bBLG, showed that tandem miRNA 6-4ΔpA construct knocked down bBLG by about 50% while surprisingly the polyadenylated form of miRNA 6-4 construct did not show any knockdown activity on bBLG expression at all. Two of the miRNA 4 Tg lines, line 1 and 6 showed bBLG knockdown activity (71% and 51%, respectively) while line 7 showed unexpected enhancement of bBLG expression by about 35%. The second miRNA forming part of the tandem construct (miRNA 6) showed about 50% knockdown efficiency for bBLG. The observed knockdown difference between miRNA 6-4 with or without pA is in accord with results by Chung *et al.,* who also observed greater Bcl-I (80) knockdown with a non-polyadenylated synthetic miRNA modeled on the murine miRNA-155. The inefficiency of polyadenylated miRNA 6-4 construct to knockdown bBLG is most likely due to the difference in the miRNA expression levels. Moreover the differences in the knockdown efficiencies of bBLG by different miRNAs could be due to the difference in integration site of the transgenes into the genome or could be due to the intrinsic activities of the miRNAs that may affect the levels of miRNA expression and interference with the target mRNA.

Overall, it appears that the miRNAs trigger greater knockdown of oBLG. The reason of the difference in oBLG and bBLG knockdown by the identical miRNAs can be assumed to be possibly due to the the difference in the form of BLG gene (genomic/cDNA) used to generate BLG mice. The Tg oBLG mice were generated from a gDNA construct and bBLG is expressed through a cDNA construct. It has

been reported that transgenes that are composed of a genomic sequence (with natural intron-exon sequence) produce much higher level of protein in Tg animal compared to cDNA-based constructs (174) (175). At similar expression levels of the oBLG and bBLG transgenes, protein levels are likely to differ with oBLG being expected to be produced at higher levels than the bovine variant due to more efficient export of the pre-mRNA from the cell nucleus (176). However, in my work both oBLG and bBLG mice showed high BLG expression in milk. Furthermore, oBLG and bBLG transcripts exhibit very different secondary structures (see Appendix 23) which may affect their accessibility to the RNAi machinery (177) (178) (179) (180). It can also be speculated that the data obtained from genomic construct of oBLG knockdown analysis are more important and meaningful because they are closer to the scenario that we would experience in cows.

The coomassie stain and western blot methods were expected to give fairly similar knockdown results which I indeed observed for oBLG knockdown analysis. Unexpectedly, however, both assays gave different results for most of the bBLG-miRNA mouse lines. Coomassie staining-based BLG knockdown results were much more reliable than western blot results which were hampered by a lack of reproducibility. The bBLG knockdown data obtained by western blot analysis are difficult to interpret because of the large deviations that were observed for experimental repeats. The western blot assay is comparatively a complex and technically challenging procedure. Moreover, I had the opportunity to normalize the BLG knockdown results obtained from coomassie stain to the mouse milk protein α-casein. Although the western blot analysis in this study were not normalized the Ponceau staining of the transfer membrane showed equal protein loading and transfer (results not shown).

It was the ultimate target of this study to demonstrate knockdown of BLG in a mouse model. In the *in vivo* mouse model the expression of the miRNAs and BLG was restricted to the mammary glands, whereas the miRNA and BLG expression was constitutive in the *in vitro* cell culture assay. *In vitro* analysis of miRNA-mediated knockdown showed great effectiveness for both oBLG and bBLG. Although the miRNA 6-4 construct without pA showed about 95% knockdown

oBLG the polyadenylated form of miRNA 6-4 construct showed less effectiveness to downregulate oBLG *in vivo* (about 50%) as compared to the *in vitro* experiments (90%). In contrast, the miRNA 6-4 construct failed to show any knockdown activity on bBLG expression *in vivo*. In addition the miRNA 6-4 ΔpA construct showed some knockdown activity (about 45%) *in vivo*. However, the miRNA 6-4 construct showed great bBLG knockdown (99%) *in vitro*. Despite the nucleotide mismatch (es) between miRNA and target sequence, miRNA 6 proved effective for both BLG genes in *in vitro* experiments. Therefore it was expected that miRNA 6 would achieve similar knockdown efficiencies for both BLG genes *in vivo*. But *in vivo* miRNA 6 showed about 50% of BLG knockdown as compared to the knockdown observed *in vitro.* Moreover, miRNA 4 is perfectly complementary to both BLG genes and the knockdown expectation was also the same as *in vitro.* However, the *in vivo* knockdown results for both BLG genes by the miRNA 4 did not match the expectations as it showed less knockdown activity *in vivo* (about 50%) as compared to *in vitro* (about 85%).

Chapter Six: General Discussion and Outlook

Milk is a basic food for humans; particularly infants because it contains all the nutritions required for growth and development. Moreover, when breast-feeding is not available, cow's milk is used as a substitute. Unfortunately cow milk can lead to immunological problems because it contains a number of milk allergens including the major allergen BLG (181). That's why BLG is an attractive target in order to make milk amenable to everyone. Until today we have been relying on post-harvest processing methods to reduce its presence in milk. Different milk processing methods such as heat treatment and enzymatic treatment have shown to be effective in reducing milk allergens. Such procedures may still lead to presence of residual peptides that can cause allergenicity in children (182). Selective breeding has also been found to be of limited value in altering milk composition (183). Recently, knockout of bBLG with zinc-finger nucleases was attempted (184). Yu *et al.,* introduced short fragment deletions into the BLG gene and generated Tg cattle from cell clones that were shown to be bi-allelic BLG mutants. Unfortunately, the only remaining live animal does not contain BLG knockout alleles but a small in frame deletions and is therefore likely to still produce (slightly shorter) BLG protein variants. Genuine BLG knockout animals have yet to be generated. The research presented in this thesis is the first demonstration of BLG knockdown in an *in vivo* system. Until my study it has only been shown that porcine BLG can be knocked down *in vitro* using synthetic RNAi molecules (130). In this study by Merkl and co-workers, they used the same vector system and targeted similar regions of the BLG gene to my study by artificial miRNAs. The transfection of miRNA showed 74% of porcine BLG knockdown in CHO-K1 cells which is similar to my results. The main objective of my study was to test the feasibility of a tissue-specific miRNA-based RNAi

approach to reduce the expression of a major suspected milk allergen, BLG, in milk using a Tg mouse model.

The algorithms and programs available to predict interfering molecule sequences are helpful but one may have advantages over another. So prediction of a specific miRNA is not commonly reliable and difficult to judge its functionality against the target gene. In this study, miRNAs were predicted by Invitrogen's optimized proprietary algorithm and most of the predicted miRNAs showed functionality against BLG expression. *In vitro* studies revealed that sequence complementarity between miRNA and the target is important for knockdown efficiency and any mismatch can reduce the knockdown efficiency. It becomes clear from the *in vitro* studies that there is still considerable room to improve miRNA design to assess its contribution to silence the target gene. Following the identification of the most effective miRNAs for BLG, I further examined the efficacy of tandem miRNA constructs and their constituent singles against BLG expression. I found tandem miRNA constructs were able to achieve slightly higher BLG knockdown compared to their singles but the differences were small and statistically insignificant. The miRNA constructs used in this study for *in vitro* studies were bicistronic GFP expression constructs. GFP expression correlates with the miRNA expression (one bicistronic construct). Quantification of GFP expression by western blotting showed similar GFP expression for both tandem constructs and their constituent singles (Figure 3-9). Same level of GFP expression thus confirmed that the tandem constructs (eg. miRNA 6-4) produced twice as many RNAi molecules available for the knockdown as compared to single constructs (eg. miRNA 4 and 6). However, I did not see more knockdowns with the tandem constructs and it is indeed likely that there was no synergistic effect. The BLG knockdown efficiency by the tandem miRNA construct might be different *in vivo* as it targets two different regions of the gene which could potentially improve its knockdown efficiency. In order to minimize unwanted off-target effects by the miRNAs, expression of miRNAs was under the control of a milk protein promoter to restrict their activity to the mammary gland. One of the miRNA constructs (miRNA 6-4 ΔpA) showed almost complete oBLG knockdown, while other miRNA constructs knocked down oBLG by about half as compared to mice

expressing only BLG (control). However, the efficiency of the miRNA 6-4 ΔpA construct to reduce bBLG expression was not as great as for oBLG- although the miRNA 6-4 ΔpA construct was expected to show similar knockdown efficiency. It is worthwhile remembering, though, that ovine and bovine transcripts vary considerably. While oBLG is first transcribed as a pre-mRNA from a genomic DNA template, bBLG transcription is driven by a cDNA construct. In this study, it is revealed that the Tg mouse line carrying the tandem miRNAs without the pA signal showed higher BLG knockdown efficiency compared to the tandem miRNA construct with polyadenylation signal. The surprising finding of this work was miRNA 6-4 showed about 50% knockdown for oBLG but slightly enhanced expression (15%) for bBLG in mice. To directly compare the knockdown efficiency of different miRNAs for both BLG genes *in vivo*, it would have been necessary to measure miRNA expression levels to judge the relative knockdown efficiency of various miRNAs. Several methods of RNA detection could be used to detect miRNAs such as, northern blotting, splinted-ligation technique and TaqMan MiRNA Assay system (Applied Biosystems). Northern blotting is considered the standard technique for small RNA expression analysis and tissue-specific miRNA expression patterns have been identified using this technique. However, poor sensitivity and large amount of starting material (total RNA) are the major drawback of Northern blots, especially when analysing the expression of small RNAs such as miRNA. So, to detect miRNAs more efficiently, I used the splinted ligation method described by Maroney *et al.,* for direct labelling and detection of small RNAs present in total RNA (185). The splinted-ligation technique is a DNA:RNA hybridization assay that uses a bridge oligonucleotide with perfect complementary to a target small RNA and a 5'-end-radiolabeled ligation oligonucleotide. It has been reported that this detection technique is ~50 times more sensitive than Northern blotting using DNA probes and requires small amount of total RNA (0.5-5.0 ug) depending on the abundance of the small RNA. Experiments with the DNA and miRNA hybrids to visualize miRNA expression failed to perform as anticipated due to technical problems. A SYBR green based real-time PCR assay has also been used to detect miRNAs. The highly sensitive and sophisticated real-time PCR assay has become a reliable detection technique

for the identification of low abundance RNA molecules (186). Although in this work real-time PCR showed detection of internal control glyceraldehyde-3phosphatedehydrogenase (GAPDH) mRNA, small miRNAs were unable to detect due to its small size and low abundance (results not shown).

The data presented in this study validate the RNAi approach and provide valuable information on silencing BLG expression both *in vitro* and *in vivo* which encourages further research on the improvement of milk quality using tissue-specific miRNA-based models. The next logical step of this study is to knockdown BLG in cows and sheep. RNAi sequences could be either directly administered into the mammary gland, for example by teat injection of siRNAs, or used in fully modified animals that are generated by microinjection or produced via somatic cell nuclear transfer using genetically modified miRNA cells as donors. Currently work is in progress to evaluate the BLG knockdown efficiency of the tandem miRNA construct in cattle. Successful bBLG knockdown in cattle would possibly help us to study the functional implications of BLG on milk characteristics, allergenicity and overall protein secretion and lactation. Cow milk with reduced BLG will also tell us whether it is important for normal growth of calves.

My results demonstrate the feasibility of RNAi for the knockdown of a milk protein and should also open avenues to apply the technique to reduce levels of other milk proteins, for example α-lactalbumin to reduce the amount of lactose, a milk sugar that cannot be converted by a substantial part of the human population or caseins which have been identified as some of the major milk allergens (187). Lactose-intolerant people do not have enough lactase enzyme to break down lactose to glucose and galactose and produce intestinal disorders (188). Lactose is synthesized by lactose synthetase complex which comprises α-lactalbumin gene. Complete disruption of the expression of α-lactalbumin gene resulted in lactose-free milk, but milk became highly viscous and suckling pups were unable to get required nutrition from their mothers (50). In comparison to the gene knockout strategy, RNAi could be an alternative tool to reduce lactose level to an acceptable range in order to retain appropriate amounts of carbohydrate in milk without compromising other milk attributes (51). Although caseins supply essential amino

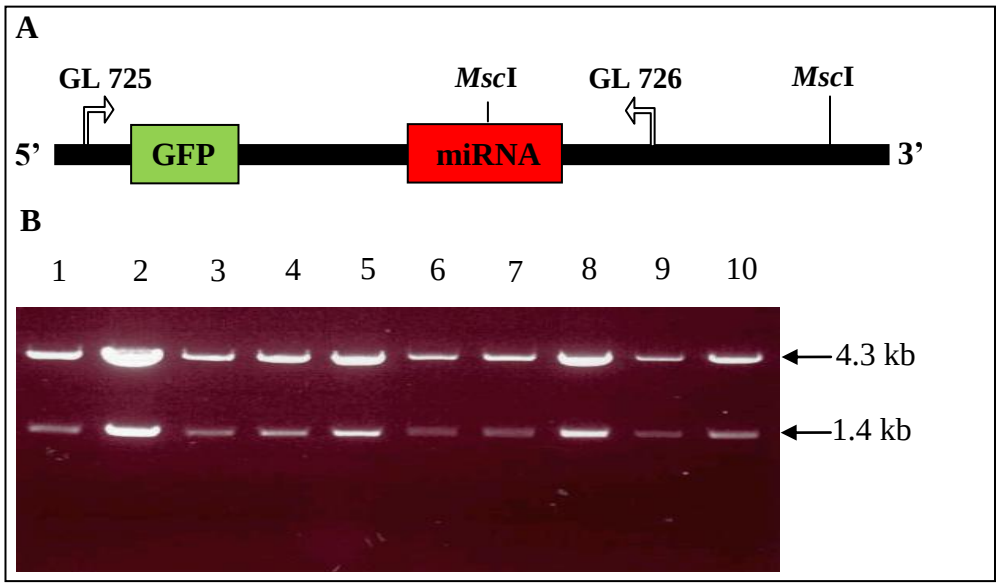
acids, carbohydrates and two essential inorganic nutrients, calcium and phosphorus for bone growth (189), bovine caseins have been identified as major milk allergens (187). Deletion of the κ-casein gene has been performed in mice and showed complete abolishment of milk production (190). Moreover, inactivation of α-casein has showed a significant decrease in other milk proteins that affects the normal growth of pups (191). In contrast, ß-casein has been found to be a non-essential component in milk proteins and showed little effect on milk production and pups body growth after disrupting its function in mice (183). Because the biological fuction of α and κ –casein is crucial for milk production, a complete knockout would not be desirable in farm animals. RNAi could be a useful tool to reduce the presence of α and κ –casein in order to make milk hypoallergenic particularly for those that are allergic to milk casein. There is another milk component in dairy milk such as milk fat that could be targeted by RNAi to improve milk quality. Dairy milk is high in saturated fats with very little unsaturated fatty acids. Too much saturated fat is one of the major risk factors for heart diseases. Reduction of milk fat may reduce the energy requirement of the cows that could reduce feed costs for the cows. As a consequence consumers could get low-fat milk with lower price (192). The major component of fatty acid metabolism in most of the living organisms is acetyl-coenzyme A carboxylases (ACC) and it has become an attractive target in drug discovery against a variety of human diseases (193). It has been reported that ACC can be reduced up to 70% by targeting its mRNA using sequence-specific catalytic RNA molecules (ribozymes) in cells. So far, nobody has reported on the reduction of ACC mRNA in order to reduce fat concentration in milk. Thus it would be interesting to utilize RNAi mechanism to reduce milk fat as this technology has progressed and has been validated in many mammals against various important targets.

In summary, the results presented in this thesis have led to two major results. First, the efficiency of the specific miRNA reducing the expression of the BLG gene can be assessed in a transient assay in mammalian cells and resulted in the identification of effective miRNAs for the proof-of-principle experiment of BLG gene silencing experiments *in vivo.* Secondly, expression of an active miRNA can be directed to the mammary gland to efficiently (>95%) reduce the levels of the

major allergenic milk protein BLG *in vivo*. My data on successful knockdown of bBLG and oBLG in mice are, to the best of my knowledge, the first demonstration of reducing this allergenic protein in the milk of animals and therefore validate the usefulness of the tandem miRNA construct as an effective tool to silence BLG expression in sheep and cattle.

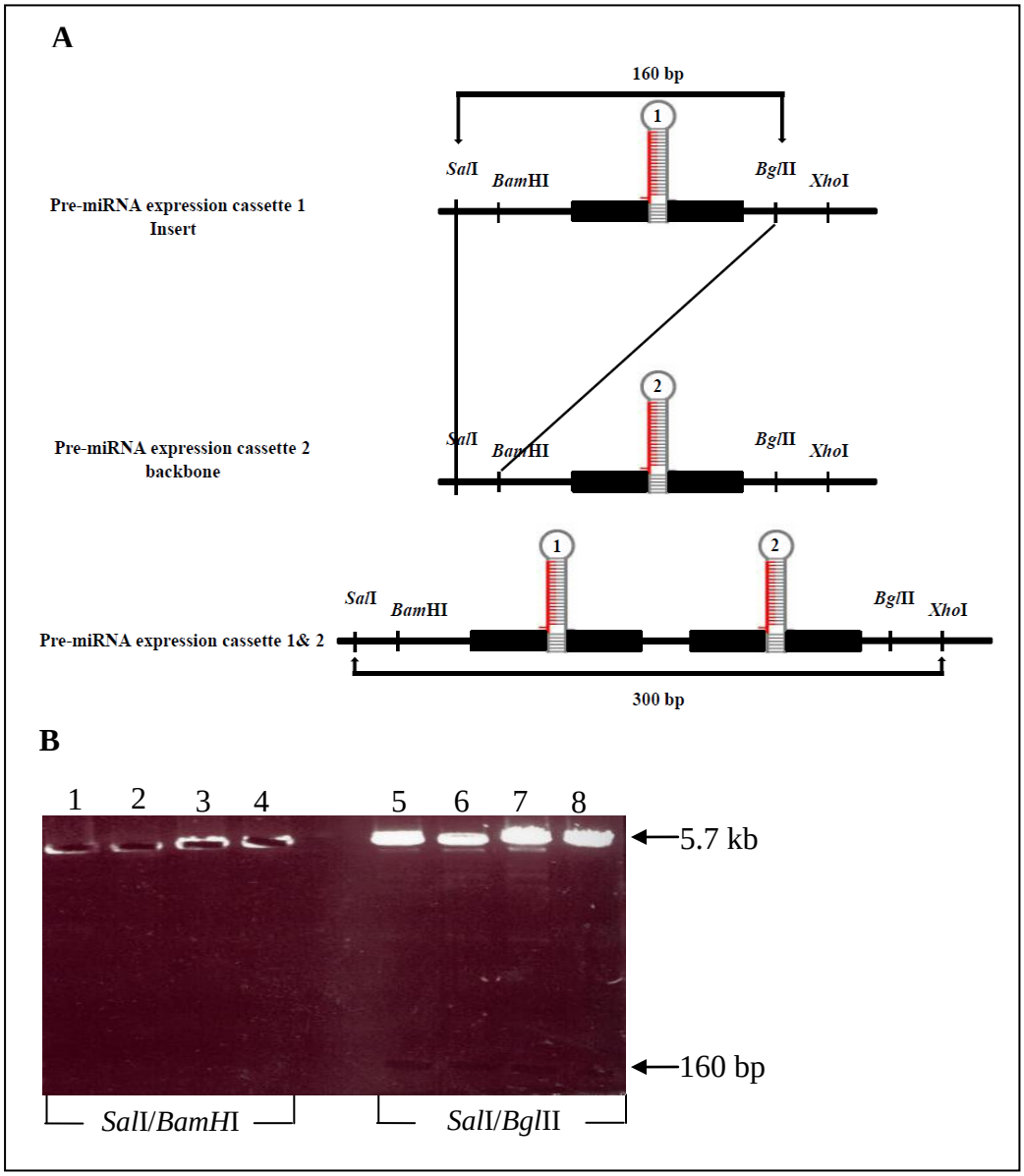
Appendices

Appendix 1: Screening of single miRNA constructs.



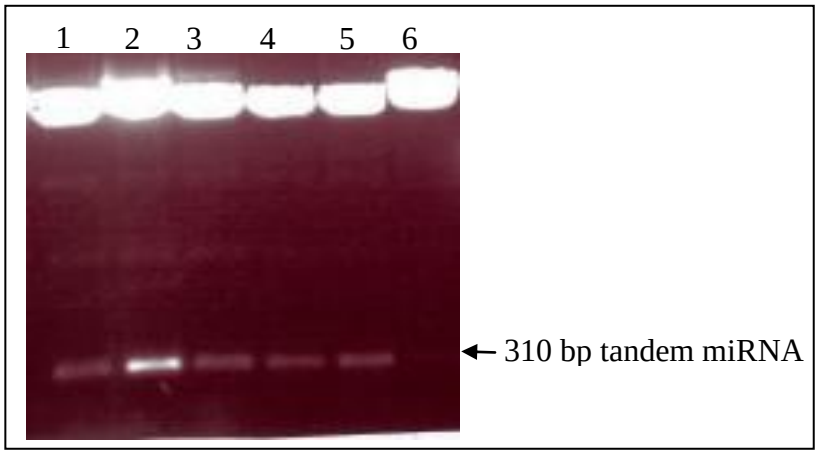
A. A schematic representation of *Msc*I restriction digest and position of the oligos GL 725 and GL 726 to check integration and sequencing of GFP (green box) and single miRNA (red box). **B.** The gel electropherogram in this figure shows the image captured from the restriction digest of pGL 274-283 with *Msc*I restriction enzyme and analysed on agarose gel. The analytical digest of ten miRNA constructs (lanes 1-10) shows the release of the expected 1.4 kb fragment after *Msc*I digest on agarose gel indicated by an arrow. Arrows indicate the expected molecular weight.

Appendix 2: Restriction digestion strategy to chain-up miRNAs.



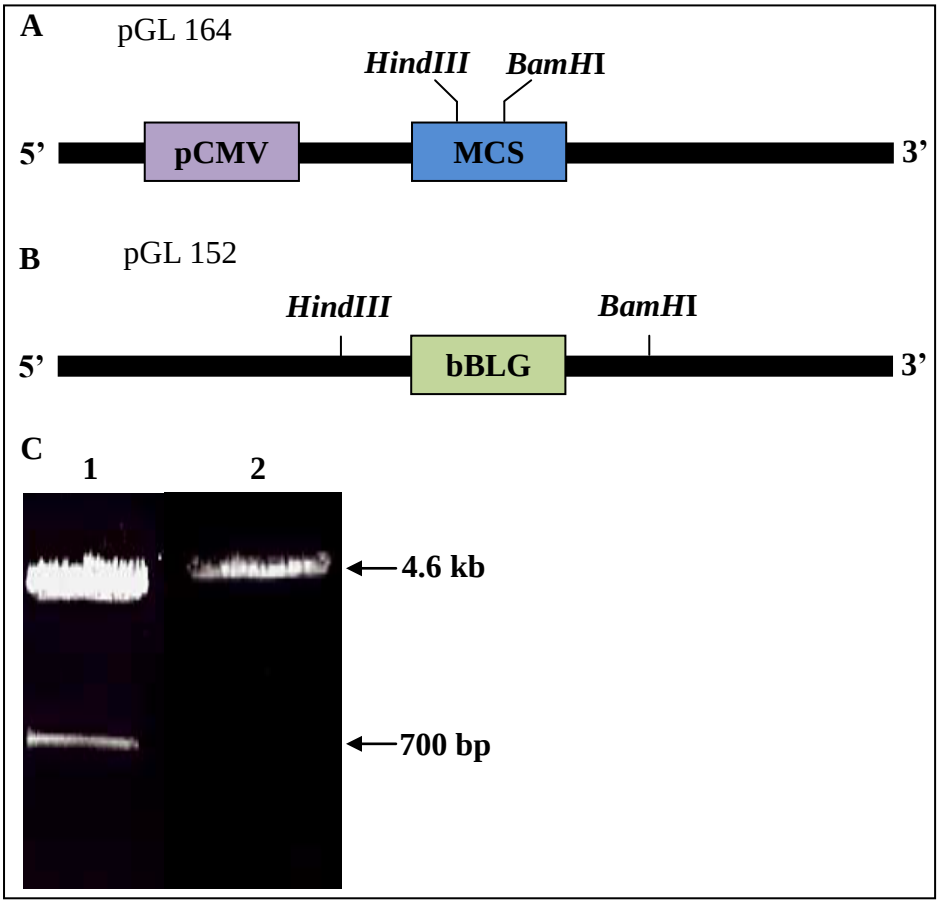
A. A schematic representation of the restriction enzyme digestion of miRNA expression vectors to chain up miRNAs. **B.** The gel electropherogram in this figure shows the image captured from the restriction digest of the pGL 275, pGL 276, pGL 277 and pGL 279 with *Sal*I and *Bam*HI restriction enzymes to open up the vectors and analysed on agarose gel (lanes 1-4). The miRNA inserts were excised from the same expression constructs using *Sal*I and *Bgl*II restriction digest and analysed on agarose gel (lanes 5-8). Arrows indicate the expected molecular weight.

Appendix 3: Screening of tandem miRNA constructs.



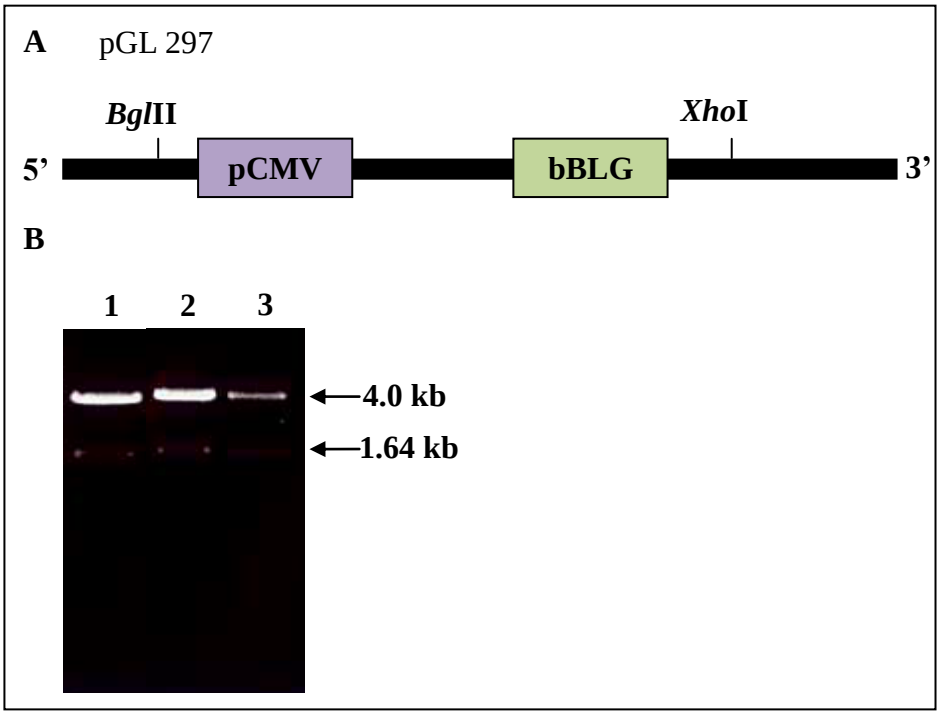
The analytical digest of the putative tandem miRNA clones (pGL 285-290) (lanes 1-6) with *Sal*I and *Xho*I restriction enzymes released the expected 310 bp fragment for the tandem miRNA constructs. Arrows indicate the expected molecular weight.

Appendix 4: Restriction digest of pGL 152 and pGL 164 expression constructs.



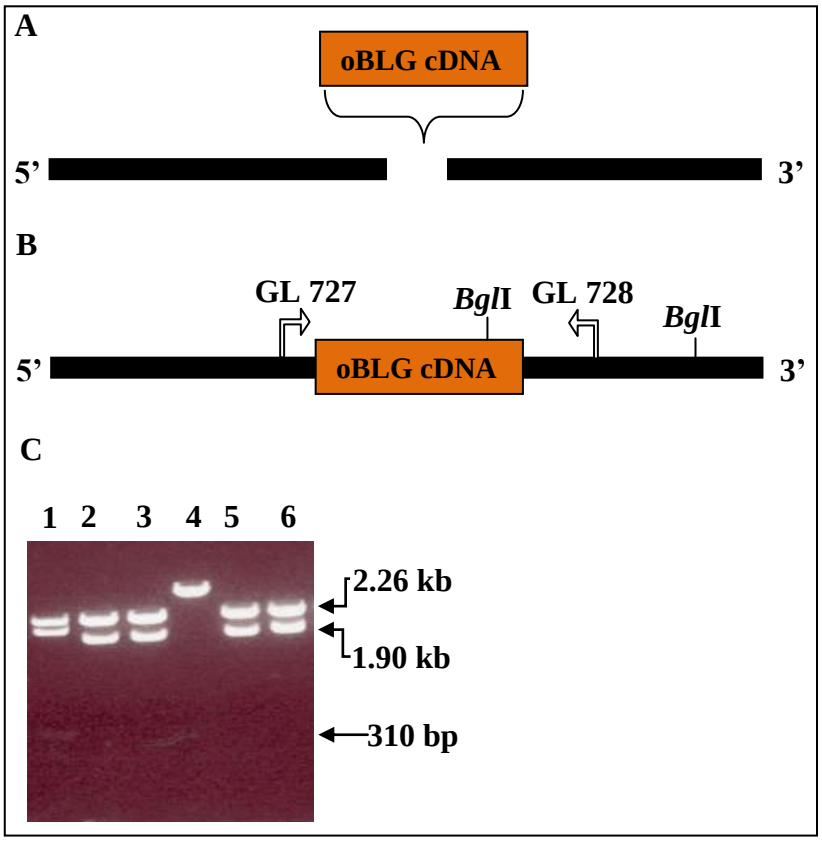
A and **B,** schematic representation of the pGL 164 and pGL 152 expression vectors. **C.** The gel electropherogram shows the restriction digests of the pGL 152 and pGL 164 expression vectors with the *Hind*III and *Bam*HI restriction enzymes. The restriction digest of pGL 152 (lane 1) and pGL 164 (lane 2) excised the expected bands at 700 bp of bBLG cDNA and 4.6 kb linearized pGL 164 expression vector indicated by an arrow. Arrows indicate the expected molecular weight, CMV (purple box), MCS (blue box) and bBLG (green box).

Appendix 5: Analysis of putative pGL 297 expression clones.



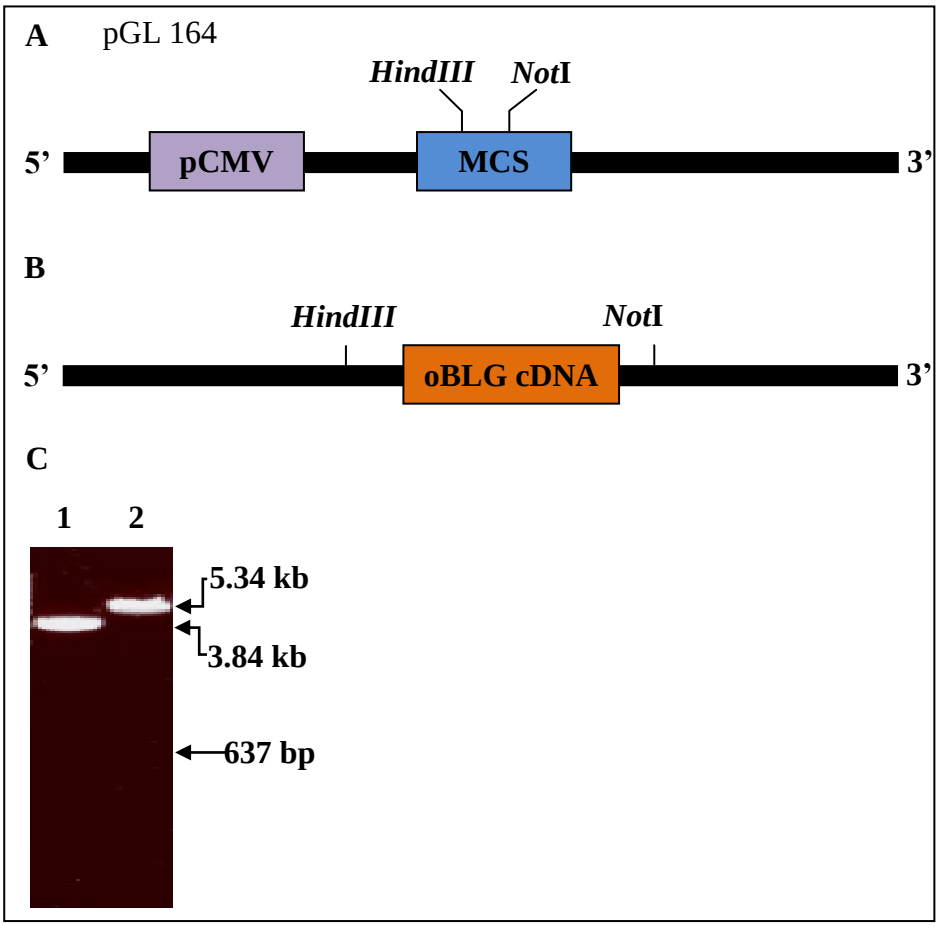
A. A schematic representation of the bBLG expression construct (pGL 297). The inserted bBLG cDNA is highlighted in green colour. **B.** Analytical digest of 3 clones from the bBLG cDNA ligation. Positive clones in lanes 1-3 release the expected 1.6 kb fragment (bBLG cDNA and the CMV promoter) indicated by an arrow, after *Bgl*II and *Xho*I restriction digest. Arrows indicate the expected molecular weight and CMV promoter (purple box).

Appendix 6: Generation of a TOPO-oBLG construct.



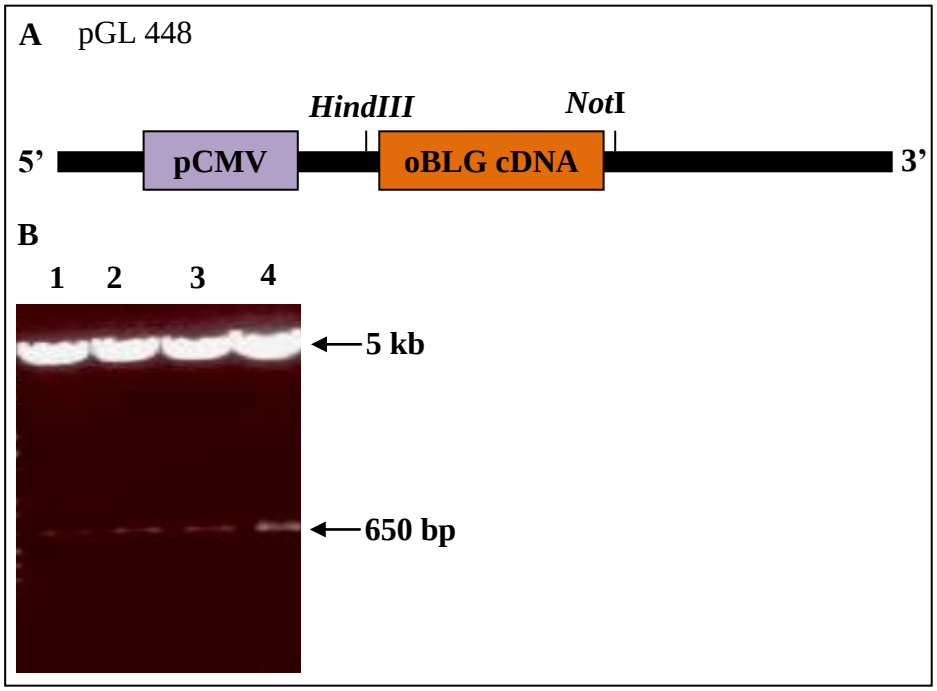
A. A schematic representation of ligation of PCR product of oBLG cDNA (highlighted in yellow) in TOPO vector. **B.** A schematic representation of the TOPO-oBLG construct showing the position of the oligonucleotides GL 727and GL 728 for sequencing of the oBLG sequence. **C.** Analytical digest of 6 clones from the oBLG cDNA ligation. The positive clone 1 in lane 1 releases the expected 310 bp fragment (oBLG cDNA and lacZ), indicated by an arrow after *Bgl*I digest. Clones 2, 3, 5 and 6 respectively (lanes 2, 3, 5 and 6, respectively) showed digested vectors and clone 4 (lane 4) remained linearized. Arrows indicate the expected molecular weight.

Appendix 7: Restriction digestion of pGL 164 and TOPO-oBLG constructs.



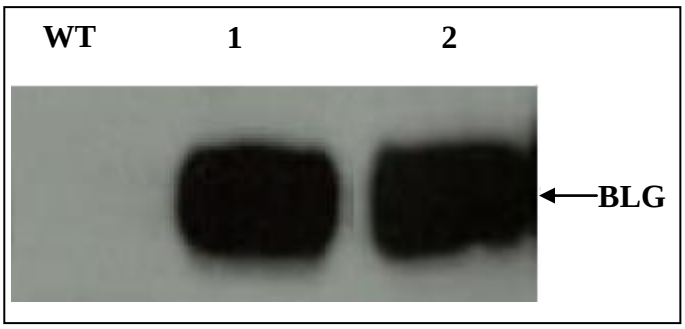
A. and **B.** Schematic representation of the restriction digest of pGL 164 and TOPO-oBLG constructs. **C.** The gel electropherogram shows the restriction digests of the TOPO-oBLG vector and pGL 164 expression vectors with *Hind*III and *Not*I restriction enzymes. The restriction digest of TOPO-oBLG clone (lane 1) and pGL 164 expression vector (lane 2) excised the expected bands at 637 bp of oBLG cDNA and 5.34 kb linearized pGL 164 expression vector indicated by an arrow. Arrows indicate the expected molecular weight, CMV promoter (purple box), MCS (blue box) and oBLG cDNA (yellow box).

Appendix 8: Analysis of putative pGL 448 clones.



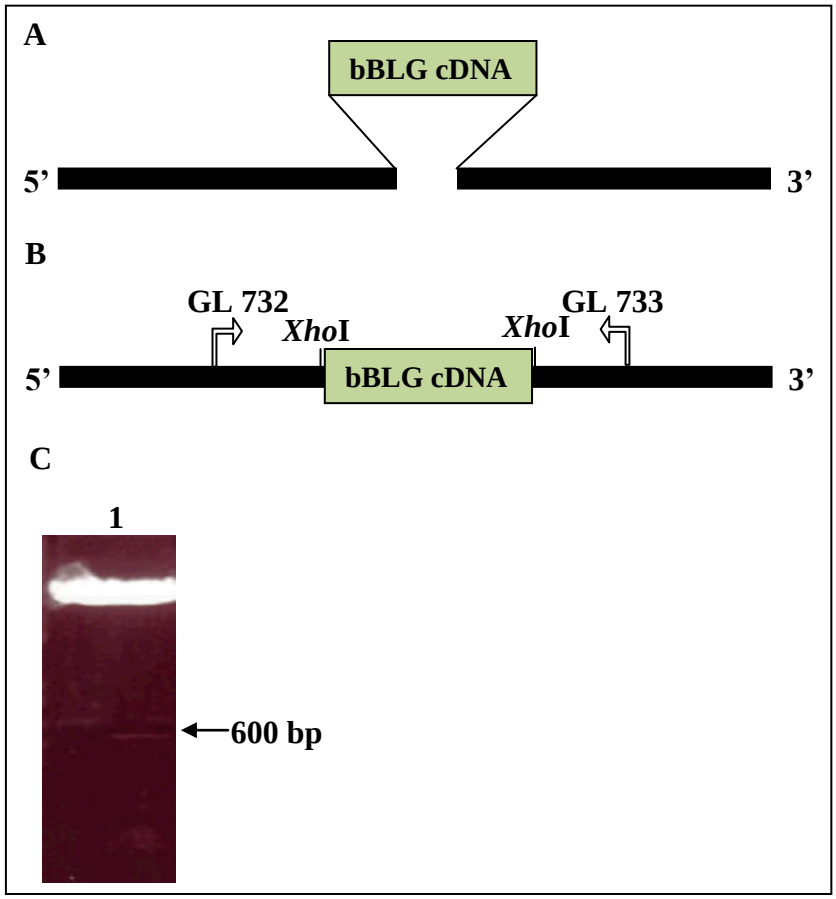
A. A schematic representation of the restriction digest of pGL 448 construct. **B.** Analytical digest of four clones of pGL 448. Positive clones in lanes 1-4 release the expected 650 bp fragment (oBLG cDNA) after *Hind*III and *Not*I restriction digest. Arrows indicate the expected molecular weight, CMV promoter (purple box) and oBLG cDNA (yellow box).

Appendix 9: A western blot detection of BLG on a SDS gel.



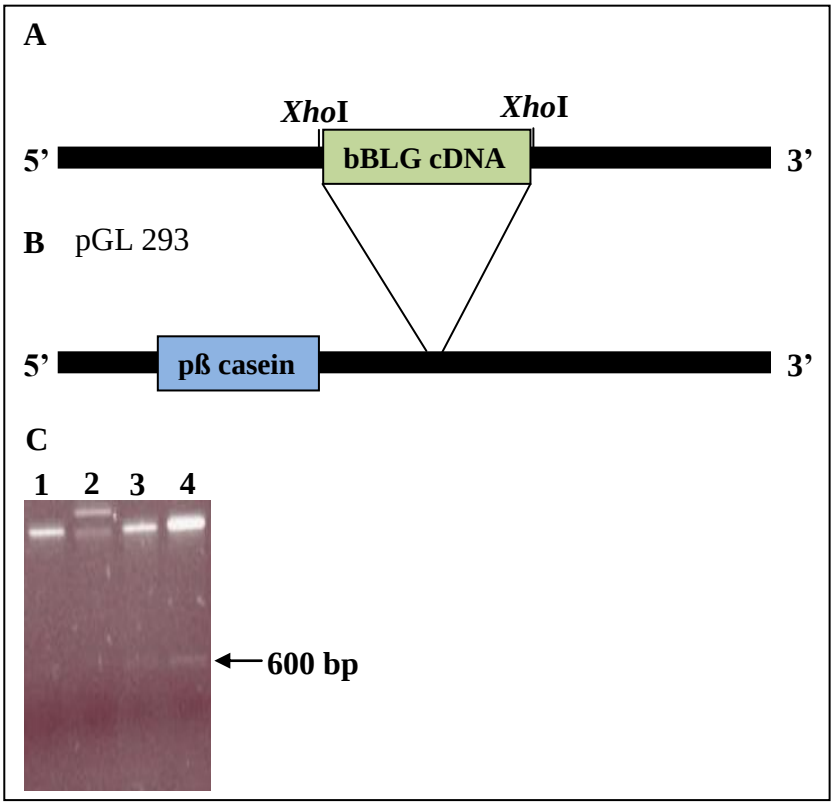
Expression of bBLG and oBLG protein was determined by western blot assay after transfecting COS7 cells with pGL 297 and pGL 448. Equal amounts of total protein for transfected and non-transfected cells were loaded on a gel. The blot was immune-stained using an antibody directed against BLG (rabbit anti-BLG, 1:10,000 dilutions) and visualized using ECL detection reagent (GE Healthcare). WT, represents non-transfected cell as negative control, 1, indicates the expression of bBLG and 2, indicates the expression of oBLG as indicated by an arrow.

Appendix 10: Generation of a pGEM-T-bBLG construct.



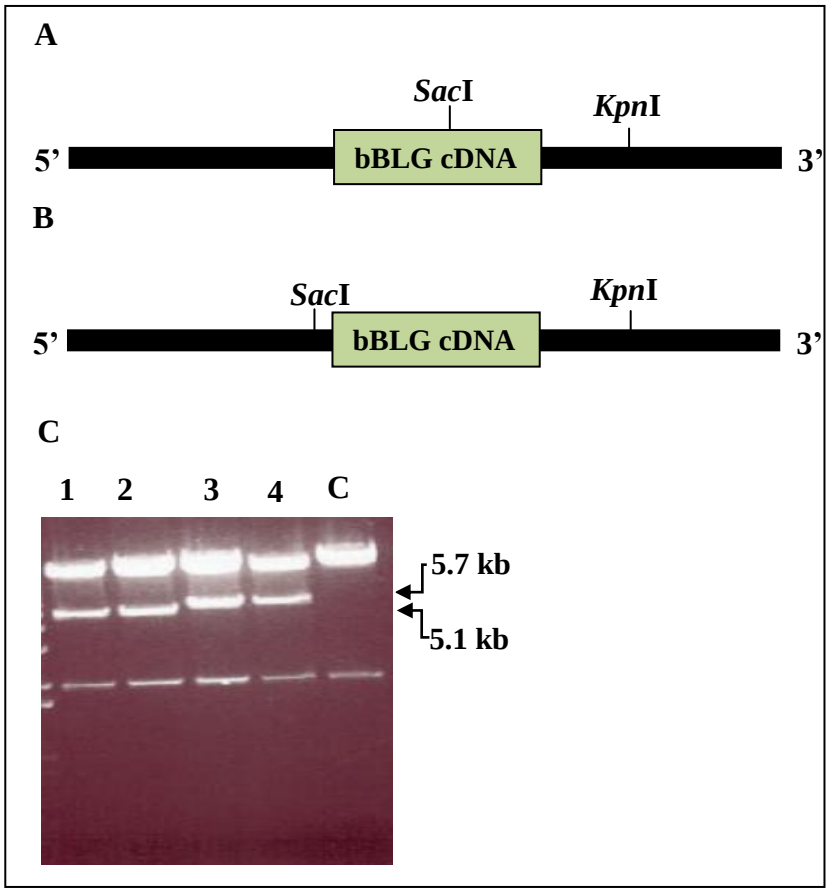
A. A schematic representation of ligation of PCR product of bBLG cDNA (highlighted in green) in pGEM-T vector. **B.** A schematic representation of the pGEM-T-bBLG construct showing the position of the oligos GL 732 and GL 733 for sequencing of the bBLG sequence. **C.** Analytical digest of the clone from the bBLG cDNA ligation. The positive clone in lane 1 releases the expected 600 bp fragment (bBLG cDNA), indicated by an arrow after *Xho*I digest. Arrow indicates the expected molecular weight.

Appendix 11: Analysis of putative pGL 449 clones.



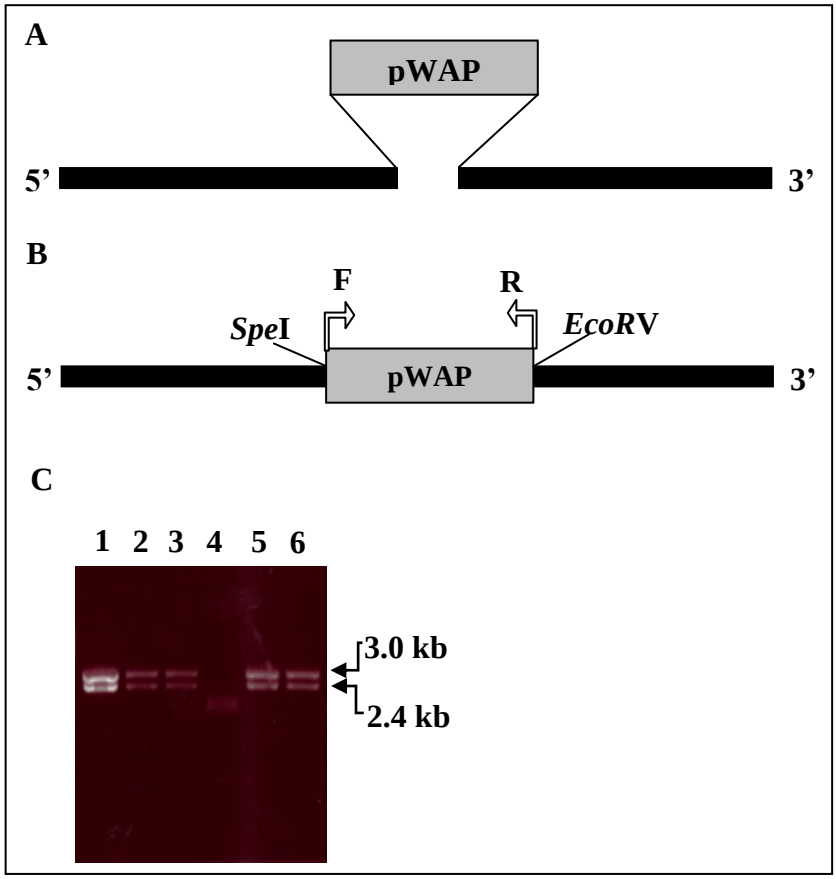
A. and **B.** A schematic representation of the restriction digest of TOPO-bBLG and pGL 293 constructs. **C.** The gel electropherogram shows the restriction digests of the TOPO-bBLG vector and pGL 293 expression vectors with *Xho*I restriction enzyme. The restriction digest of pGL 449 expression vector excised the expected bands at 600 bp of bBLG cDNA indicated by an arrow. Numbers indicate gel lanes and arrow indicates the expected molecular weight and bBLG cDNA (green box).

Appendix 12: Identification of orientation of bBLG in pGL 449 by restriction digestion.



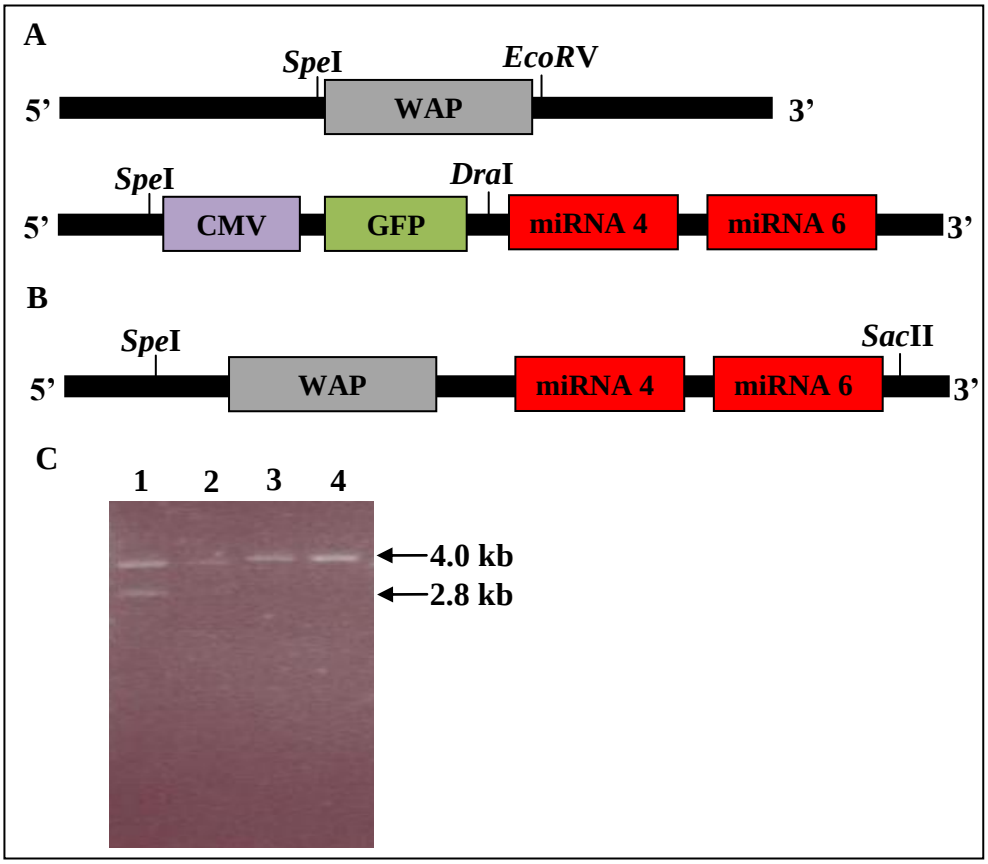
A. and **B.** A schematic representation of the restriction digest of pGL 449 construct. **C.** The gel electropherogram shows the restriction digests of the clones of pGL 449 construct. The restriction digest excised the expected band at 5.7 kb of bBLG cDNA with correct orientation (lanes 3 and 4) and 5.1 kb with incorrect orientation (lanes 1 and 2) as indicated by arrows. Numbers indicate gel lanes, C, represents pGL 293 vector (negative control), arrows indicate the expected molecular weight and bBLG cDNA (green box).

Appendix 13: Generation of a pGEM-WAP construct.



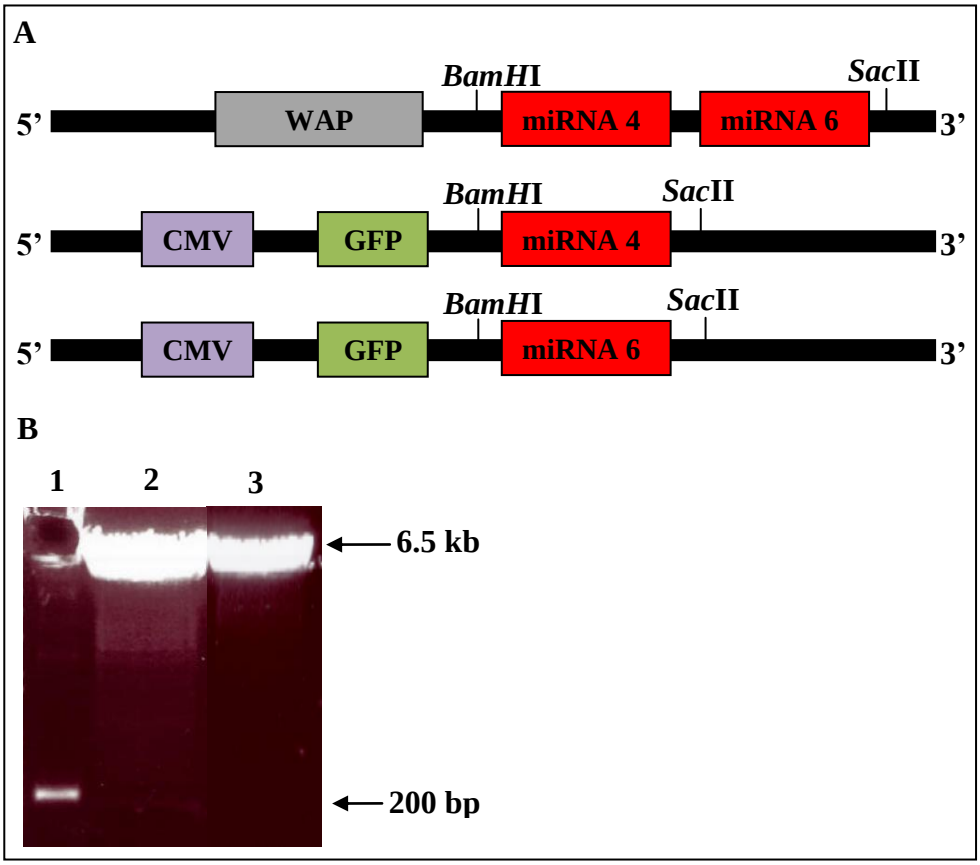
A. A schematic representation of ligation of PCR product of pWAP (grey box) in pGEM-T vector. **B.** A schematic representation of the pGEM-pWAP construct showing the position of the forward (F) and reverse (R) oligos for sequencing of the pWAP sequence. **C.** Analytical digest of the clones from the pWAP ligation. Except clone 4 (lane 4), all of the clones showed the expected 2.4 kb fragment (pWAP) indicated by an arrow after *Spe*I and *Eco*RV digest. Numbers indicate gel lanes and arrows indicate the expected molecular weight.

Appendix 14: Analytical digest of putative tissue specific pGL 298 clones.



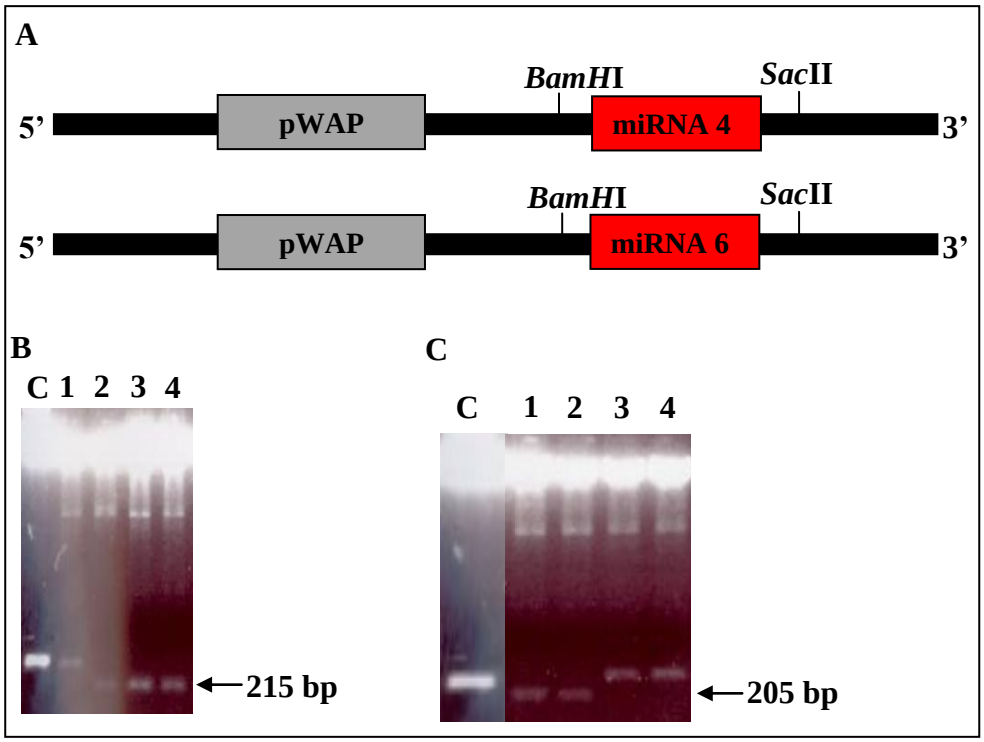
A. A schematic representation of the restriction digest of pGEM-pWAP construct and CMV driven pGL 289 construct. **B.** A schematic representation of the restriction digest of pGL 298 construct. **C.** Analytical digest of the clones from the pWAP replacement. Clones 1 and 2 (lanes 1 and 2) showed the expected 2.8 kb fragment (pWAP) indicated by an arrow after *Spe*I and *Sac*II digest. Numbers indicate gel lane, arrows indicate the expected molecular weight, WAP (grey box), CMV (purple box), GFP (green box) and miRNA (red box).

Appendix 15: Restriction digestion of tandem and single miRNA constructs.



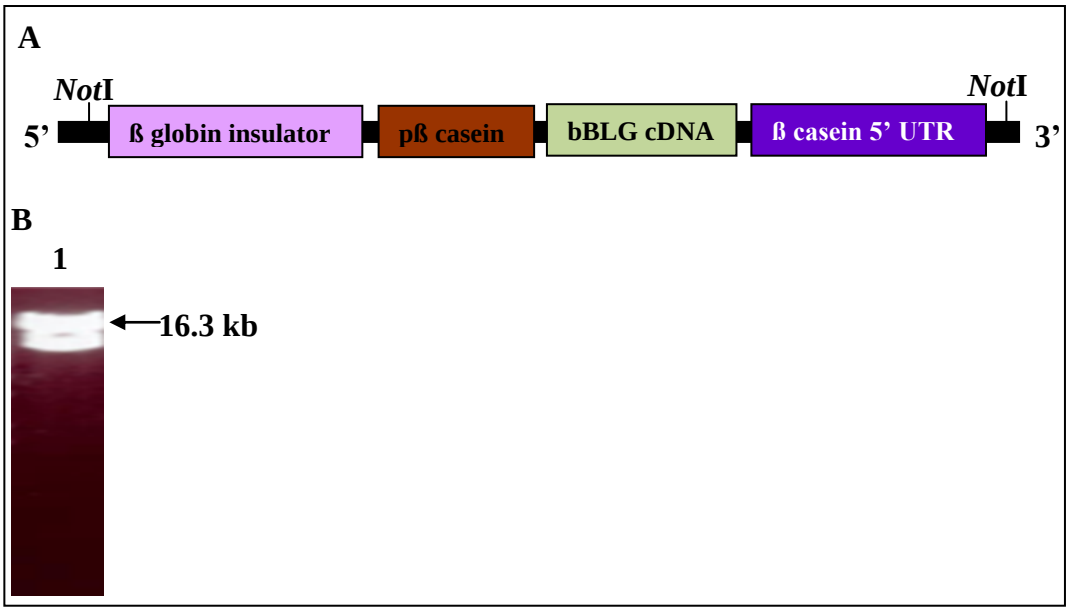
A. A Schematic representation of the pGL 298, pGL 277 and pGL 279 constructs restriction enzyme profile. **B.** This figure shows the image of the restriction digest of the pGL 298 (lane 1), pGL 277 (lane 2) and pGL 279 (lane 3) constructs with *BamH*I and *Sac*II restriction enzymes. The restriction digest excised the expected band at 6.5 kb of vector backbone of pGL 298 and inserts of pGL 277 and pGL 279 constructs as indicated by arrows. Numbers indicate individual digests, arrows indicate the expected molecular weight, WAP (grey box), CMV (purple box), GFP (green box) and miRNA (red box).

Appendix 16: Analytical digest of putative clones of tissue specific pGL 446 and pGL 447.



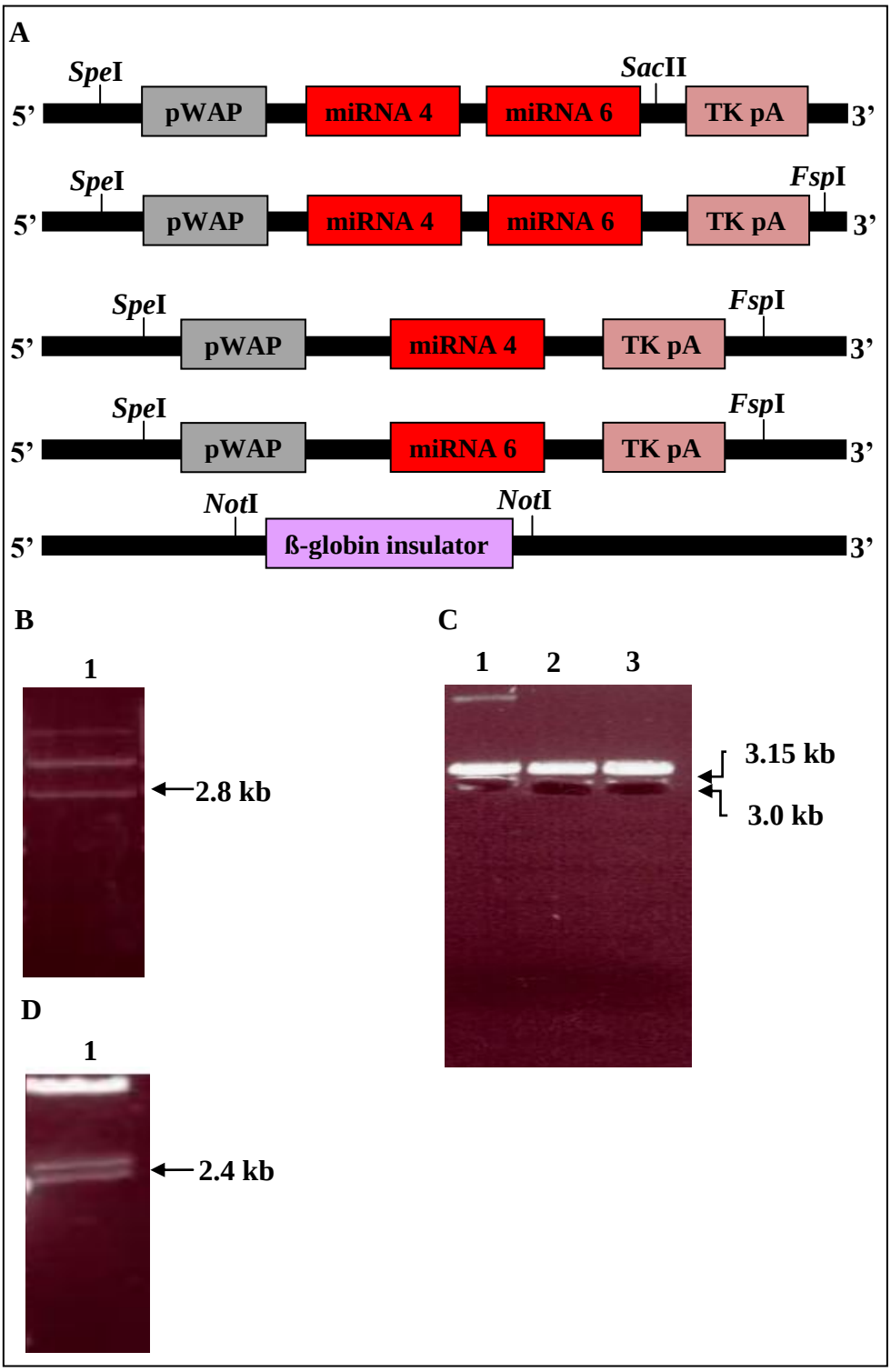
A. A Schematic representation of the tissue specific pGL 446 and pGL 447 constructs restriction enzyme profile. **B.** The figure shows the image captured from the restriction digest of pGL 446 construct with *BamH*I and *Sac*II restriction enzymes. The restriction digest showed that clone 1 (lane 2) failed to swap with pWAP, however, clones 2-4 showed integration of miRNA 4 (lanes 3-5). **C.** The figure shows the image captured from the restriction digest of pGL 447 construct with *BamH*I and *Sac*II restriction enzymes. The restriction digest showed that clones 1-2 (lanes 2 and 3) showed integration of miRNA 6. However, clone 3 and 4 (lanes 4 and 5) failed to swap with pWAP. C, control pGL 298 digest and numbers indicate individual digests, arrows indicate the expected molecular weight, WAP (grey box) and miRNA (red box).

Appendix 17: Preparative gel of the pGL 449 fragment for pronuclear injection.



A. A schematic diagram of the pGL 449 milk expression vector restriction enzyme profile. **B.** The linearised 16.3 kb *Not*I fragment (lane 1), harbouring chicken insulator (purple box), beta-casein promoter (red box) and bBLG cDNA (green box) with beta-casein flanking sequences (blue box) was used for pronuclear injection. Number indicates individual digest and arrow indicates the expected molecular weight.

Appendix 18: Preparative gel of the miRNA fragment and the insulator for pronuclear injection.



A. A schematic diagram of the restriction enzyme profile of pGL 298, pGL 446, pGL 447 and pBS 2 x insulator. **B.** *Spe*I and *Sac*II restriction digestion excised a 2.8 kb fragment

harbouring WAP promoter and the tandem miRNA. **C.** *Spe*I and *Fsp*I restriction digestion of pGL 298, pGL 446 and pGL 447 constructs releasing a fragment of 3.15 kb fragment harbouring WAP promoter and the tandem miRNA with pA sequence and a 3.0 kb fragment encompassing WAP promoter and miRNA 4/6 sequence along with pA sequence. **D.** *Xho*I and *Not*I restriction digestion excised a 2.4 kb fragment harbouring 2 x chicken insulator. Number indicates individual digests, arrows indicate the expected molecular weight, pWAP (grey box), miRNA (red box), TK pA (roan box) and ß-globin insulator (purple box).

Appendix 19: miRNA sequences + flanking vector sequences

Yellow highlighting corresponds to the miRNA sequence. Lane 1 is the vector NTI sequence and Lane 2 is the sequence generated from sequencing.

miRNA 1 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGATATCCAGGCCCTTCATGGTCGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGATATCCAGGCCCTTCATGGTCGTTTTGGCCACTG    117

Lane 1    1553    ACTGACGACCATGAGGCCTGGATATCAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACGACCATGAGGCCTGGATATCAGGACACAAGGCCTGTT    159
```

miRNA 2 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGTTCTGCAGCAGGATCTCCAGGGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGTTCTGCAGCAGGATCTCCAGGGTTTTGGCCACTG    117

Lane 1    1553    ACTGACCCTGGAGACTGCTGCAGAACAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACCCTGGAGACTGCTGCAGAACAGGACACAAGGCCTGTT    159
```

miRNA 3 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75
```

```
Lane 1    1511    TGTATGCTGAGCTCCTCCACATACACTCTCGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGAGCTCCTCCACATACACTCTCGTTTTGGCCACTG    117

Lane 1    1553    CTGACGAGAGTGTGTGGAGGAGCTACAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     CTGACGAGAGTGTGTGGAGGAGCTACAGGACACAAGGCCTGTT    159
```

miRNA 4 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGTTTGTAGTCGGTGTCCAGCACGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGTTTGTAGTCGGTGTCCAGCACGTTTTGGCCACTG    117

Lane 1    1553    ACTGACGTGCTGGACCGACTACAAACAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACGTGCTGGACCGACTACAAACAGGACACAAGGCCTGTT    159
```

miRNA 5 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGAGATGTGGCACTGCTCCTCCAGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGAGATGTGGCACTGCTCCTCCAGTTTTGGCCACTG    117

Lane 1    1553    ACTGACTGGAGGAGGTGCCACATCTCAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACTGGAGGAGGTGCCACATCTCAGGACACAAGGCCTGTT    159
```

miRNA 6 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGCTTCTGAGCACACTCACCGTTGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGCTTCTGAGCACACTCACCGTTGTTTTGGCCACTG    117

Lane 1    1553    ACTGACAACGGTGAGTGCTCAGAAGCAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACAACGGTGAGTGCTCAGAAGCAGGACACAAGGCCTGTT    159
```

miRNA 7 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75
```

```
Lane 1    1511    TGTATGCTGATCTTGAACACCGCAGGGATCGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGATCTTGAACACCGCAGGGATCGTTTTGGCCACTG    117

Lane 1    1553    ACTGACGATCCCTGGTGTTCAAGATCAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACGATCCCTGGTGTTCAAGATCAGGACACAAGGCCTGTT    159
```

miRNA 8 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGTTCAAGGCATCGATCTTGAACGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGTTCAAGGCATCGATCTTGAACGTTTTGGCCACTG    117

Lane 1    1553    ACTGACGTTCAAGAGATGCCTTGAACAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACGTTCAAGAGATGCCTTGAACAGGACACAAGGCCTGTT    159
```

miRNA 9 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGCCAGCACAAGGACTTTGTTCTGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGCCAGCACAAGGACTTTGTTCTGTTTTGGCCACTG    117

Lane 1    1553    ACTGACAGAACAAACCTTGTGCTGGCAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACAGAACAAACCTTGTGCTGGCAGGACACAAGGCCTGTT    159
```

miRNA 10 + flanking sequences

```
Lane 1    1469    AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    1510
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      AGGTAGTGAGTCGACCAGTGGATCCTGGAGGCTTGCTGAAGGC    75

Lane 1    1511    TGTATGCTGTTTGCTCGGGCTCAGCACTGTGTTTTGGCCACTG    1552
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      TGTATGCTGTTTGCTCGGGCTCAGCACTGTGTTTTGGCCACTG    117

Lane 1    1553    ACTGACACAGTGCTGCCCGAGCAAACAGGACACAAGGCCTGTT    1594
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     ACTGACACAGTGCTGCCCGAGCAAACAGGACACAAGGCCTGTT    159
```

Appendix 20: bBLG cDNA sequences + flanking vector sequences

Yellow highlighting corresponds to the bBLG cDNA sequence. Lane 1 is the vector NTI sequence and Lane 2 is the sequence generated from sequencing.

```
Lane 1    613     CTCGAGCTCAAGCTTCCTGCAGAGCTCAGAAGCGTGATCCCG    654
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    34      CTCGAGCTCAAGCTTCCTGCAGAGCTCAGAAGCGTGATCCCG    75

Lane 1    655     GCTGCAGCCATGAAGTGCCTCCTGCTTGCCCTGGCCCTCACC    696
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    76      GCTGCAGCCATGAAGTGCCTCCTGCTTGCCCTGGCCCTCACC    117

Lane 1    697     TGTGGCGCCCAGGCCCTCATCGTCACCCAGACCATGAAGGGC    738
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    118     TGTGGCGCCCAGGCCCTCATCGTCACCCAGACCATGAAGGGC    159

Lane 1    739     CTGGATATCCAGAAGGTGGCGGGGACTTGGTACTCCTTGGCC    780
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    160     CTGGATATCCAGAAGGTGGCGGGGACTTGGTACTCCTTGGCC    201

Lane 1    781     ATGGCGGCCAGCGACATCTCCCTGCTGGACGCCCAGAGTGCC    822
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    202     ATGGCGGCCAGCGACATCTCCCTGCTGGACGCCCAGAGTGCC    243

Lane 1    823     CCCCTGAGAGTGTATGTGGAGGAGCTGAAGCCCACCCCTGAG    864
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    244     CCCCTGAGAGTGTATGTGGAGGAGCTGAAGCCCACCCCTGAG    285

Lane 1    865     GGCGACCTGGAGATCCTGCTGCAGAAATGGGAGAATGATGAG    906
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    286     GGCGACCTGGAGATCCTGCTGCAGAAATGGGAGAATGATGAG    327

Lane 1    907     TGTGCTCAGAAGAAGATCATTGCAGAAAAAACCAAGATCCCT    948
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    328     TGTGCTCAGAAGAAGATCATTGCAGAAAAAACCAAGATCCCT    369

Lane 1    949     GCGGTGTTCAAGATCGATGCCTTGAACGAGAACAAAGTCCTT    990
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    370     GCGGTGTTCAAGATCGATGCCTTGAACGAGAACAAAGTCCTT    411

Lane 1    991     GTGCTGGACACCGACTACAAAAAGTACCTGCTCTTCTGCATG    1032
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    412     GTGCTGGACACCGACTACAAAAAGTACCTGCTCTTCTGCATG    453

Lane 1    1033    GAGAACAGTGCTGAGCCCGAGCAAAGCCTGGTCTGCCAGTGC    1074
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    454     GAGAACAGTGCTGAGCCCGAGCAAAGCCTGGTCTGCCAGTGC    495

Lane 1    1075    CTGGTCAGGACCCCGGAGGTGGACGACGAGGCCCTGGAGAAA    1116
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    496     CTGGTCAGGACCCCGGAGGTGGACGACGAGGCCCTGGAGAAA    537
```

```
Lane 1    1117    TTCGACAAAGCCCTCAAGGCCCTGCCCATGCACATCCGGCTG    1158
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    538     TTCGACAAAGCCCTCAAGGCCCTGCCCATGCACATCCGGCTG    579

Lane 1    1159    TCCTTCAACCCAACCCAGCTGGAGGAGCAGTGCCACATCTAG    1200
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    580     TCCTTCAACCCAACCCAGCTGGAGGAGCAGTGCCACATCTAG    621
```

Appendix 21: oBLG cDNA sequences + flanking vector sequences

Yellow highlighting corresponds to the oBLG cDNA sequence. Lane 1 is the sequence generated from sequencing and Lane 2 is the vector NTI sequence.

```
Lane 1    52      AACGGCCGCCAGTGTGCTGGAATTCGCCCTTATGAAGTGCCT    93
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    264     AACGGCCGCCAGTGTGCTGGAATTCGCCCTTATGAAGTGCCT    305

Lane 1    94      CCTGCTTGCCCTGGGCCTGGCCCTCGCCTGTGGCGTCCAGGC    135
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    306     CCTGCTTGCCCTGGGCCTGGCCCTCGCCTGTGGCGTCCAGGC    347

Lane 1    136     CATCATCGTCACCCAGACCATGAAAGGCCTGGACATCCAGAA    177
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    348     CATCATCGTCACCCAGACCATGAAAGGCCTGGACATCCAGAA    389

Lane 1    178     GGTGGCGGGGACTTGGCACTCCTTGGCTATGGCGGCCAGCGA    219
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    390     GGTGGCGGGGACTTGGCACTCCTTGGCTATGGCGGCCAGCGA    431

Lane 1    220     CATCTCCCTGCTGGATGCCCAGAGTGCCCCCCTGAGAGTGTA    261
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    432     CATCTCCCTGCTGGATGCCCAGAGTGCCCCCCTGAGAGTGTA    473

Lane 1    262     CGTGGAGGAGCTGAAGCCCACCCCCGAGGGCAACCTGGAGAT    303
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    474     CGTGGAGGAGCTGAAGCCCACCCCCGAGGGCAACCTGGAGAT    515

Lane 1    304     CCTGCTGCAGAAATGGGAGAACGGCGAGTGTGCTCAGAAGAA    345
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    516     CCTGCTGCAGAAATGGGAGAACGGCGAGTGTGCTCAGAAGAA    557

Lane 1    346     GATTATTGCAGAAAAAACCAAGATCCCTGCGGTGTTCAAGAT    387
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    558     GATTATTGCAGAAAAAACCAAGATCCCTGCGGTGTTCAAGAT    599

Lane 1    388     CGATGCCTTGAATGAGGACAAAGTCCTTGTGCTGGACGCCGA    429
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    600     CGATGCCTTGAATGAGGACAAAGTCCTTGTGCTGGACGCCGA    641

Lane 1    430     CTACAAAAAGTACCTGCTCTTCTGCATGGAAAACAGTGCTGA    471
                  ||||||||||||||||||||||||||||||||||||||||||||
Lane 2    642     CTACAAAAAGTACCTGCTCTTCTGCATGGAAAACAGTGCTGA    683
```

```
Lane 1    472    GCCCGAGCAAAGCCTGGCCTGCCAGTGCCTGGTCAGGACCCC    513
                 ||||||||||||||||||||||||||||||||||||||||||
Lane 2    684    GCCCGAGCAAAGCCTGGCCTGCCAGTGCCTGGTCAGGACCCC    725

Lane 1    514    GGAGGTGGACAACGAGGCCCTGGAGAAATTCGACAAAGCCCT    555
                 ||||||||||||||||||||||||||||||||||||||||||
Lane 2    726    GGAGGTGGACAACGAGGCCCTGGAGAAATTCGACAAAGCCCT    767

Lane 1    556    CAAGGCCCTGCCCATGCACATCCGGCTTGCCTTCAACCCGAC    597
                 ||||||||||||||||||||||||||||||||||||||||||
Lane 2    768    CAAGGCCCTGCCCATGCACATCCGGCTTGCCTTCAACCCGAC    809

Lane 1    598    CCAGCTGGAGGGGCAGTGCCACGTCTAGAAGGGCGAATTCTG    639
                 ||||||||||||||||||||||||||||||||||||||||||
Lane 2    810    CCAGCTGGAGGGGCAGTGCCACGTCTAGAAGGGCGAATTCTG    853
```

Appendix 22: WAP promoter sequences + flanking vector sequences

Yellow highlighting corresponds to the WAP sequence. Lane 1 is the sequence generated from sequencing and Lane 2 is the vector NTI sequence.

```
Lane 1    1      CAATTCTTTCACTGCTAAAACAGGGCGGGAGGAGTCCAGAGC    41
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1      CAATTCTTTCACTGCTAAAACAGGGCGGGAGGAGTCCAGAGC    41

Lane 1    42     CCTGCCACTGGGTGCAGAACATGAAGACCCCTTGAGTGGAAA    83
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    42     CCTGCCACTGGGTGCAGAACATGAAGACCCCTTGAGTGGAAA    83

Lane 1    84     GGGGTTATACAGCTGGACAGTGGTGGCGCACACCTTTAATCC    125
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    84     GGGGTTATACAGCTGGACAGTGGTGGCGCACACCTTTAATCC    125

Lane 1    126    CAGCACTCGGGAGGCAGAGGCAGACGGATCTCTGAGTTCGAG    167
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    126    CAGCACTCGGGAGGCAGAGGCAGACGGATCTCTGAGTTCGAG    167

Lane 1    168    GCCAGCCTGGTCTACAGAATGAGTTCCGGGACAGCCAGGGCC    209
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    168    GCCAGCCTGGTCTACAGAATGAGTTCCGGGACAGCCAGGGCC    209

Lane 1    210    ACACAGAGAAACTCTTGTCTCGAAAAACCAAAAAAAAACAAA    251
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    210    ACACAGAGAAACTCTTGTCTCGAAAAACCAAAAAAAAACAAA    251

Lane 1    252    AAAGGAAAGGGGTTACACAACAGAGACTCAGGTCACAGCTAC    293
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    252    AAAGGAAAGGGGTTACACAACAGAGACTCAGGTCACAGCTAC    293

Lane 1    294    CCATCACACACAGGATACACATACAAAGGTGTTCACAGGCAG    335
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    294    CCATCACACACAGGATACACATACAAAGGTGTTCACAGGCAG    335
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Lane 1    336    ATGAGGAACGAGGAGAAGGGGCTCAAGCAAGGGCCTAAAGTT   377
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    336    ATGAGGAACGAGGAGAAGGGGCTCAAGCAAGGGCCTAAAGTT   377

Lane 1    598    TCTTTTTTTTTTTTTCTTCTTTTTTTTTTTCCCTGTGGCCTA   419
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    598    TCTTTTTTTTTTTTTCTTCTTTTTTTTTTTCCCTGTGGCCTA   419

Lane 1    420    GAGTTTCAAGAGGCTGAGGACCTAGGCATGAACCAAGAGGGG   461
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    420    GAGTTTCAAGAGGCTGAGGACCTAGGCATGAACCAAGAGGGG   461

Lane 1    462    CCAAACCACTTCAAGAAGCAGGGGGTAGCAGCAGAATCTCAC   503
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    462    CCAAACCACTTCAAGAAGCAGGGGGTAGCAGCAGAATCTCAC   503

Lane 1    504    TATCAGCCTTGAGCACAGCTGGGAAGGAGATCCATGGAAACA   545
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    504    TATCAGCCTTGAGCACAGCTGGGAAGGAGATCCATGGAAACA   545

Lane 1    546    ACCAAGAAAGAGCTGAAAGGGGCTGGAGAGATGGCTCAGCAG   587
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    546    ACCAAGAAAGAGCTGAAAGGGGCTGGAGAGATGGCTCAGCAG   587

Lane 1    588    TTAAGAGCACTGAGTGCTCTTCCGAAGTCCTAGATTCAAATC   629
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    588    TTAAGAGCACTGAGTGCTCTTCCGAAGTCCTAGATTCAAATC   629

Lane 1    630    CCAGCAACCATATGGTGGGTCACAACCATCTGTAATGAGATC   671
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    630    CCAGCAACCATATGGTGGGTCACAACCATCTGTAATGAGATC   671

Lane 1    672    TGATGCCCTCTTCTGGTGTGTCTGAAGACAGCTACAGTGTAC   713
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    672    TGATGCCCTCTTCTGGTGTGTCTGAAGACAGCTACAGTGTAC   713

Lane 1    714    TTATGTATAATAAATAAATAAATCTTTAGAAAGGGAGGGGGG   755
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    714    TTATGTATAATAAATAAATAAATCTTTAGAAAGGGAGGGGGG   755

Lane 1    756    GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA   797
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    756    GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA   797

Lane 1    798    GAGAGAGAGAGAGAGCTGGAAGAGGGAGATCTGGGAAGTCTG   839
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    798    GAGAGAGAGAGAGAGCTGGAAGAGGGAGATCTGGGAAGTCTG   839

Lane 1    840    CTGGCTTTATATGCTGACCATATATAGTCACCTGTGTTTACA   881
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    840    CTGGCTTTATATGCTGACCATATATAGTCACCTGTGTTTACA   881

Lane 1    882    CACTGTGCTCATCACTTTGAAATCTCAGTGGTTTCTTCTTTG   923
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    882    CACTGTGCTCATCACTTTGAAATCTCAGTGGTTTCTTCTTTG   923
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Lane 1    924    AGCCTGTGTCTGTAAGTTCACCAGGAGAGTGGTACATAGGCA    965
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    924    AGCCTGTGTCTGTAAGTTCACCAGGAGAGTGGTACATAGGCA    965

Lane 1    966    AGAATAACAGCCAGTGGGCATAGGACACAGAGTGCATGGGCC    1007
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    966    AGAATAACAGCCAGTGGGCATAGGACACAGAGTGCATGGGCC    1007

Lane 1    1008   CCAGCAAGACTGTAGAGAGAACAGAGCTCTGGCTCCTAAGAC    1049
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1008   CCAGCAAGACTGTAGAGAGAACAGAGCTCTGGCTCCTAAGAC    1049

Lane 1    1050   ATAGGGCCTTCTGGGAAACTCAAGCAGCCAAGCAACCCTAGC    1091
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1050   ATAGGGCCTTCTGGGAAACTCAAGCAGCCAAGCAACCCTAGC    1091

Lane 1    1092   CAGCCCTTTCCTGGTGGCCCTCCTTCTGTTCCAGCAAAGGCG    1133
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1092   CAGCCCTTTCCTGGTGGCCCTCCTTCTGTTCCAGCAAAGGCG    1133

Lane 1    1134   GAAATGGGAACAGGGGTGGAAGCAGAGCATTGGCAGAGCATA    1175
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1134   GAAATGGGAACAGGGGTGGAAGCAGAGCATTGGCAGAGCATA    1175

Lane 1    1176   GGTATGACTTAGTCTTGACTAACACAAGCATGGCAGTAGCCT    1217
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1176   GGTATGACTTAGTCTTGACTAACACAAGCATGGCAGTAGCCT    1217

Lane 1    1218   GACAGTGGCCTAAATGTGGGGATGACTGCCTTAGATGGGGAT    1259
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1218   GACAGTGGCCTAAATGTGGGGATGACTGCCTTAGATGGGGAT    1259

Lane 1    1260   GACTGCCTTAGATGGGCATGACTGCCTTAGATGGGGATGACT    1301
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1260   GACTGCCTTAGATGGGCATGACTGCCTTAGATGGGGATGACT    1301

Lane 1    1302   GCCTTAGATGGGGATGACTGCCTTAGATGGAACAACAAACAT    1343
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1302   GCCTTAGATGGGGATGACTGCCTTAGATGGAACAACAAACAT    1343

Lane 1    1344   CTATGGGCATGCTGTGGAACACTGGCCCACACACGGAACTGA    1385
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1344   CTATGGGCATGCTGTGGAACACTGGCCCACACACGGAACTGA    1385

Lane 1    1386   AGGCACTGGCAATTTCCATAGGGCAGTTAAACCTAAAAGCAT    1427
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1386   AGGCACTGGCAATTTCCATAGGGCAGTTAAACCTAAAAGCAT    1427

Lane 1    1428   GCTCACACTCAACAGGCTGCCGGAATCTCATGAGACACCTGG    1469
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1428   GCTCACACTCAACAGGCTGCCGGAATCTCATGAGACACCTGG    1469

Lane 1    1470   AATAGACGAATGTAGAAACAGAGCAGAGAGTTGGTTGCCAAG    1511
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1470   AATAGACGAATGTAGAAACAGAGCAGAGAGTTGGTTGCCAAG    1511

Lane 1    1512   GTCTGGGGGCTCAGAGGACAAGCAAGAGGCGCGGCTTTCCTT    1553
                 |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1512   GTCTGGGGGCTCAGAGGACAAGCAAGAGGCGCGGCTTTCCTT    1553
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Lane 1    1554    TGGGGCTGGCATGAAAGGAAATATCGAGGTTACAGCCTGAGA    1596
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1554    TGGGGCTGGCATGAAAGGAAATATCGAGGTTACAGCCTGAGA    1596

Lane 1    1597    GGGCTTCCCCTGACACTTCGTATTCAAAGAGGCCATGGGCAC    1638
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1597    GGGCTTCCCCTGACACTTCGTATTCAAAGAGGCCATGGGCAC    1638

Lane 1    1639    CAGTGAAGACAAAGGAGTATGGCCTGCACCACAGGCTGGCGC    1680
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1639    CAGTGAAGACAAAGGAGTATGGCCTGCACCACAGGCTGGCGC    1680

Lane 1    1681    TGACAGTCAGTAAGCACACAGTCACTCTGGGTCATCCCATCC    1722
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1681    TGACAGTCAGTAAGCACACAGTCACTCTGGGTCATCCCATCC    1722

Lane 1    1723    CCTTCCTTGCAAGAGAAATCAAGGAAATGTCCCGAGAACAAT    1764
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1723    CCTTCCTTGCAAGAGAAATCAAGGAAATGTCCCGAGAACAAT    1764

Lane 1    1765    GGGGCACAGTGCCAGCAGGACATCTCTTCCTGCCCATGGCAC    1806
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1765    GGGGCACAGTGCCAGCAGGACATCTCTTCCTGCCCATGGCAC    1806

Lane 1    1807    CCTTTGGCACGGTATGGGCCCTTCTGGGAAGGTGGCCTTCCA    1848
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1807    CCTTTGGCACGGTATGGGCCCTTCTGGGAAGGTGGCCTTCCA    1848

Lane 1    1849    AATTGCTCTGCACAGGCAGCTCCTTTTCAATGTATGCCCGAC    1890
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1849    AATTGCTCTGCACAGGCAGCTCCTTTTCAATGTATGCCCGAC    1890

Lane 1    1891    ACTCTCTACATGGAGCAAGCGCCTCCACACTCTTAGAAGAAT    1932
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1891    ACTCTCTACATGGAGCAAGCGCCTCCACACTCTTAGAAGAAT    1932

Lane 1    1933    TTTAGAAAACTCCAGAAAAGCACCAGGAGAAGTCACCCTCAG    1974
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1933    TTTAGAAAACTCCAGAAAAGCACCAGGAGAAGTCACCCTCAG    1974

Lane 1    1975    ATGTAGCCCGGACTCGAGCCTTGCTCAAAACCTCCTGTCTTG    2016
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    1975    ATGTAGCCCGGACTCGAGCCTTGCTCAAAACCTCCTGTCTTG    2016

Lane 1    2017    TTTTCTATGTGACCTGTACAAATTTGGAGCTCAGAATTGCCT    2058
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    2017    TTTTCTATGTGACCTGTACAAATTTGGAGCTCAGAATTGCCT    2058

Lane 1    2059    TTGTCTGTGATGGGTTCCAACCCAACCACTCAAAGTGACACT    2100
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    2059    TTGTCTGTGATGGGTTCCAACCCAACCACTCAAAGTGACACT    2100

Lane 1    2101    TGTCACATTTGTCACTGATCCTATTTCTTCTTTTTCTGCTCC    2142
                  |||||||||||||||||||||||||||||||||||||||||||
Lane 2    2101    TGTCACATTTGTCACTGATCCTATTTCTTCTTTTTCTGCTCC    2142
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Lane 1   2143   TTCATTTTCTCCGCTTTCATAATAAACAAGTATTACTTTTTA   2184
                |||||||||||||||||||||||||||||||||||||||||
Lane 2   2143   TTCATTTTCTCCGCTTTCATAATAAACAAGTATTACTTTTTA   2184

Lane 1   2185   AGTGGGGGAAAAAATGACCACCCTTACAAAGGACTTTTTAAA   2226
                |||||||||||||||||||||||||||||||||||||||||
Lane 2   2185   AGTGGGGGAAAAAATGACCACCCTTACAAAGGACTTTTTAAA   2226

Lane 1   2227   AATGGCCTCCATTGTGGCCCTTGTTCCTGGCAGCCTGGGCCT   2268
                |||||||||||||||||||||||||||||||||||||||||
Lane 2   2227   AATGGCCTCCATTGTGGCCCTTGTTCCTGGCAGCCTGGGCCT   2268

Lane 1   2269   GCTCTCTCTGTGTGGCCAAGAAGGAAGTGTTGTAGCCCATCT   2310
                |||||||||||||||||||||||||||||||||||||||||
Lane 2   2269   GCTCTCTCTGTGTGGCCAAGAAGGAAGTGTTGTAGCCCATCT   2310

Lane 1   2311   AGAGCTGTGCCAGCCTCTTCCCCCACCCCACCCCCAAAGTCT   2352
                |||||||||||||||||||||||||||||||||||||||||
Lane 2   2311   AGAGCTGTGCCAGCCTCTTCCCCCACCCCACCCCCAAAGTCT   2352

Lane 1   2353   TCCTCCTGTGGGTCCTTTAAATGCATCCCAGACACTCAGACA   2394
                |||||||||||||||||||||||||||||||||||||||||
Lane 2   2353   TCCTCCTGTGGGTCCTTTAAATGCATCCCAGACACTCAGACA   2394

Lane 1   2395   GCCATCAGTCACTTGC   2410
                ||||||||||||||||
Lane 2   2395   GCCATCAGTCACTTGC   2410
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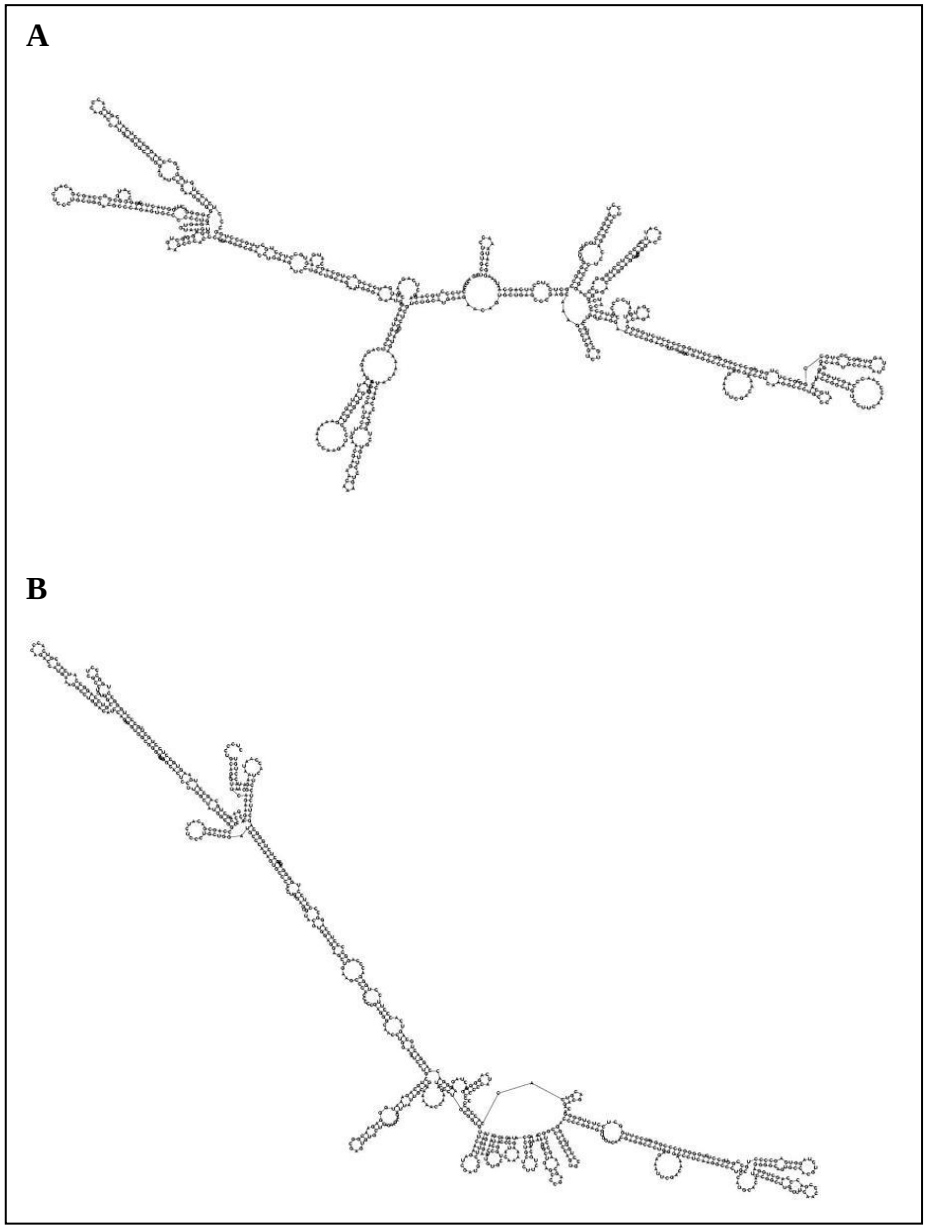
Appendix 23: Comparison of secondary structures of bBLG and oBLG mRNA sequences.



The figure A and B show the secondary structure prediction from running the RNAfold version 2.0.0 on bBLG and oBLG mRNA sequences with accession numbers: NM_173929 and NM_001009366. The program calculates minimum free energy structure of an RNA sequence. The energy parameters are set as described by Mathews DH *et al.,* ((194)). The bBLG and oBLG RNA sequences show -311.10 and -316.80 kcal/mol of free energy, respectively. Both mRNA sequences are almost equally likely to occur since both are similar in minimum free energy values, but the structures are different.

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