Yunlei Zhang Study of the mechanisms underlying the cytotoxic effects of bovine lactoferrin on breast cancer cells

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Universidade do Minho
Escola de Engenharia

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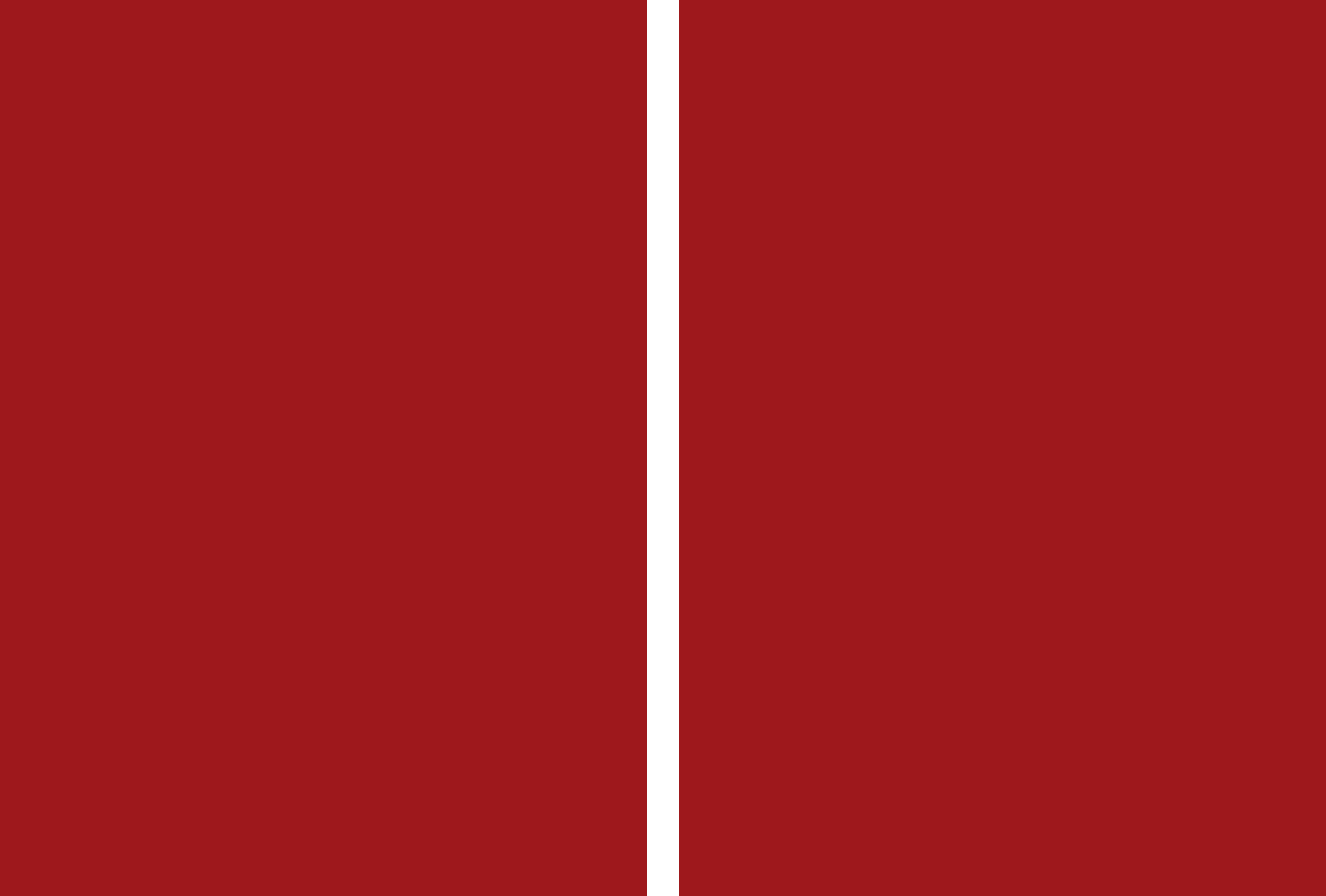

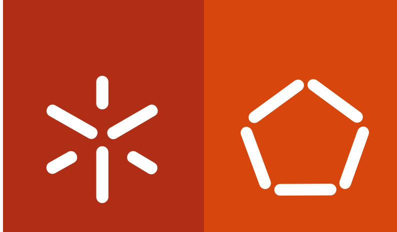

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PhD in Biomedical Engineering

Supervisors:
Professor Lígia Rodrigues
Doctor Cristóvão F. Lima

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ABSTRACT

Study of the mechanisms underlying the cytotoxic effects of bovine lactoferrin on breast cancer cells

Lactoferrin (LF) is an iron-binding protein predominantly found in mammalian secretions. This protein and its variants have been proposed for cancer therapy for many years owing to their tumor-targeting properties. Previous studies showed that LF and its derived peptides inhibit the proliferation of cancer cells. However, the detailed mechanisms by which LF exerts its effect are still fairly unknown. Moreover, there are few reports concerning LF effect on breast cancer cells, which is one of the most common malignant tumors in the World.

In this sense, the present thesis aimed to investigate the cytotoxicity of bovine lactoferrin (bLF) and its variants against several breast cancer cells, namely T-47D, MDA-MB-231, Hs578T and MCF-7 cell lines. The results showed that bLF at concentrations of 1.875 μM, 3.75 μM, 7.5 μM, 15 μM and 30 μM could efficiently inhibit the growth of cancer cells but showed a very low effect on normal breast cells (MCF-10-2A). Moreover, its variants (apo-bLF, holo-bLF and $LfcinB_{17-41}$) were also able to inhibit cancer cells' growth, except for $LfcinB_{26-36}$. Additionally, bLF, apo-bLF and holo-bLF did not promote the proliferation of breast cancer cells at low concentrations (0.25 μM, 0.5 μM and 1 μM) as reported for other cancer cell lines. Simultaneously, the degradation assay excluded the possibility that bLF anticancer effects could be due to its degraded peptides under cell culture conditions. On the other hand, it was found that most of the bLF was blocked outside the cells, despite that a few amount was able to be internalized to the cytoplasm. Its peptide $LfcinB_{17-41}$ also succeeded in penetrating the cell membrane but could not enter the nucleus.

Subsequently, we found that the inhibitory effects of bLF on the breast cancer cells resulted from the cell cycle arrest without effects in cell death by apoptosis. Depending on the cell lines, this prevention of cell cycle progression induced by bLF occurred at different phases. Nevertheless, the MAPK/ERK and PI3K/AKT signaling pathways were not implicated in the cell cycle arrest observed. bLF anticancer effect was associated, however, with an increase of AMPKα phosphorylation and a decrease in the levels of mTOR and its phosphorylation. To our knowledge this is the first time this pathway has been implicated in the mechanisms underlying bLF cytotoxicity against cancer. These findings suggest that

bLF could be a new mTOR-targeting drug in cancer therapy. However, it is important to notice that no apoptotic cells could be found in bLF-treated cancer cells.

The use of higher bLF concentrations (12.5 μM, 50 μM, 125 μM and 175 μM) was expected to exhibit different effects on the breast cancer cells as compared with the low concentrations range. In fact, in the high range of concentrations bLF selectively induced cell death by apoptosis in MCF-7 cells. The mechanisms of bLF-induced apoptosis included the intrinsic pathway since it was observed the mitochondrial membrane depolarization and a decrease in Bcl-2 levels. In addition, bLF also induced significantly the cell cycle arrest of these cells at the G1 phase, while the same concentration of another protein source (bovine serum albumin - BSA) did not affected significantly the cells. This suggests that bLF cytotoxicity is not due to the addition of great amounts of exogenous proteins in the cell microenvironment. The western bolt analysis confirmed that bLF blocked the cell cycle progression by adjusting cell cycle related regulators, such as CDC25c. Additionally, bLF showed a clear inhibitory effect on the MCF-7 cells ability to form colonies, which is one of the favorite features of anticancer drugs for preventing metastasis. We also found that the promoting effect on the migration of MCF-7 cells may be due to the fact that bLF changes cell microenvironment positively for cell migration similarly to BSA.

The results gathered in this thesis demonstrated the potential of bLF as an anticancer agent and provided some new insights on its mechanisms of action. However, further work is still required before bLF can be considered for clinical applications. Being a food-derived protein, bLF is commonly consumed in the daily life, as well as in supplements for health care. Nevertheless, the relation between its consumption and cancer prevention remains to be elucidated.

RESUMO

Estudo dos mecanismos subjacentes aos efeitos citotóxicos da lactoferrina de origem bovina em células de cancro da mama

A Lactoferrina (LF) é uma proteína com alta afinidade para ligação ao ferro, predominantemente encontrada nas secreções dos mamíferos. Esta proteína e as suas variantes têm vindo a ser propostas como agentes interessantes para a terapia do cancro. Estudos anteriores mostraram que a LF e os seus péptidos inibem a proliferação de células cancerígenas. No entanto, os mecanismos detalhados pelos quais a LF exerce o seu efeito são pouco conhecidos. Além disso, há poucos estudos sobre os efeitos da LF em células de cancro da mama, que constitui um dos tumores malignos mais comuns a nível mundial.

Neste sentido, na presente tese pretendeu-se estudar a citotoxicidade da lactoferrina de origem bovina (bLF) e das suas variantes contra várias linhas celulares de cancro da mama, nomeadamente T-47D, MDA-MB-231, Hs578T e MCF-7. Os resultados mostraram que a bLF em concentrações de 1,875 μM , 3,75 μM, 7,5 μM, 15 μM e 30 μM inibe eficientemente o crescimento de células cancerígenas, mas apresenta um efeito muito pouco pronunciado nas células normais da mama (MCF-10-2A). Além disso, as suas variantes (apo-bLF, holo-bLF e LfcinB17-41) também foram capazes de inibir o crescimento das células cancerígenas, exceto o péptido LfcinB26-36. Adicionalmente, a bLF, apo-bLG e holo-bLF não promoveram a proliferação das células cancerígenas a baixas concentrações (0,25 μM , 0,5 μM e 1 μM) tal como foi relatado para outras linhas celulares. Adicionalmente, excluiu-se a possibilidade de que os efeitos anti-cancerígenos da bLF possam ser devidos aos seus péptidos resultantes da degradação da proteína sob as condições de cultura das células. Por outro lado, verificou-se que a maior parte da bLF é bloqueada no exterior das células, apesar de uma pequena quantidade internalizar a célula para o espaço citoplasmático. O péptido LfcinB17-41 também conseguiu penetrar a membrana celular mas não o núcleo.

Subsequentemente, verificou-se que os efeitos inibidores da bLF sobre as células cancerígenas da mama resultaram da paragem do ciclo celular, sem se ter observado um efeito na morte celular por apoptose. Dependendo das linhas celulares, esta paragem do ciclo celular induzida pela bLF ocorreu em diferentes fases. No entanto, não foi possível associar as vias de sinalização MAPK/ERK e PI3K/AKT ao efeito observado no ciclo celular. Por outro lado, o efeito anti-cancerígeno da bLF foi associado a um aumento da

fosforilação da AMPKα e a uma diminuição dos níveis de mTOR e da sua fosforilação. Esta é a primeira vez que esta via foi associada aos mecanismos subjacentes à citotoxicidade da bLF contra células de cancro. Estes resultados sugerem que a bLF poderá ser uma nova droga, cujo alvo é a proteína mTOR, a explorar na terapia do cancro. No entanto, é importante notar que não se observaram células apoptóticas em nenhuma das linhas celulares tratadas com bLF.

Aquando da utilização de concentrações de bLF mais elevadas (12,5 μM, 50 μM, 125 μM e 175 μM) esperava-se que as mesmas exibissem diferentes efeitos sobre as células cancerígenas comparativamente com a gama de baixas concentrações. Na verdade, para concentrações de bLF elevadas observou-se uma indução selectiva de morte celular por apoptose nas células MCF-7. Os mecanismos de apoptose induzida pela bLF incluiram a via intrínseca no sentido em que se detectou a despolarização da membrana mitocondrial e a diminuição dos níveis de Bcl-2. Adicionalmente, a bLF também induziu significativamente a paragem do ciclo celular destas células na fase G1, enquanto que uma concentração similar de outra proteína (albumina do soro bovino - BSA) não afectou significativamente as células. Isto sugere que a citotoxicidade da bLF não é devida ao facto de se adicionarem grandes quantidades de proteína exógena ao microambiente celular. Pela técnica de *Western blot* confirmou-se que a bLF bloqueia a progressão do ciclo celular, tal como mostrou a diminuição dos níveis da proteína CDC25c. Por outro lado, a bLF mostrou um efeito inibidor evidente sobre a células MCF-7 no que se refere à sua capacidade para formar colónias, o que constitui uma das características desejadas em fármacos anti-cancerígenos para prevenir as metástases. O efeito promotor da bLF sobre a migração de células MCF-7 pode ser devido ao facto desta proteína modificar o microambiente celular de uma forma positiva para a migração celular, tal como se observou com a utilização de BSA.

Os resultados obtidos nesta tese demonstraram o potencial da bLF como agente anti-cancerígeno e permitiram esclarecer possíveis mecanismos envolvidos na sua atividade. Todavia, é ainda necessário conduzir mais trabalho de investigação antes que bLF possa ser considerada para aplicações clínicas. Sendo uma proteína derivada de alimentos, a bLF é vulgarmente consumida na alimentação humana, bem como em suplementos para a saúde. No entanto, a relação entre o seu consumo e a prevenção do cancro continua por elucidar.

LIST OF PUBLICATIONS

The thesis is based on the following original articles:

Yunlei Zhang, Cristovao F. Lima, Ligia R. Rodrigues. **Lactoferrin in cancer therapy: underlying mechanisms and future trends**. *Nutrition and Cancer*. *Submitted*. [Chapter 1]

Yunlei Zhang, Cristovao F. Lima, Ana Nicolau, Ligia R. Rodrigues, **"Growth inhibitory potential of lactoferrin and its variants against breast cancer cells"** to be submitted to *British Journal of Nutrition.* [Chapter 2]

Yunlei Zhang, Cristovao F. Lima, Ligia R. Rodrigues. **"Bovine lactoferrin induces cell cycle arrest and inhibits mTOR signaling in breast cancer cells"** to be submitted to *European Journal of Nutrition*. [Chapter 3]

Yunlei Zhang, Cristovao F. Lima, Ligia R. Rodrigues. "**Bovine lactoferrin selectively induces apoptosis of breast cancer cells"** to be submitted to *Nutrition and Cancer.* [Chapter 4]

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Figure 1.1 – Schematic representation of human LF gene. (**A**) Promoter and mRNA structure of human LF gene. The black arrows indicate the LF promoters (LF promoter and ΔLF promoter). The translation start site and termination codon are marked with ATG and TAA, respectively. 1a stands for the first exon in LF, and 1b for ΔLF. The black bars and number indicate the exon size and location. (**B**) Diagram of response modules of human LF gene promoter. The numbers mean the position upstream from the start site of LF promoter 1. Overlapping regions between two different modules are illustrated using the DNA sequence [Yang *et al*., 1996; Liu *et al*., 2003].

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CHAPTER 3

Figure 3.1 – Effect of bLF in cell death by apoptosis. (**A**) Apoptotic cells in T-47D, MDA-MB-231, Hs578T and MCF-7 cell lines were evaluated using the nuclear condensation assay after 72 h incubation with 30 μM bLF. (**B**) Apoptotic cells in T-47D and MDA-MB-231 cell lines were evaluated by the annexin V/ PI assay after 24 h incubation with 30 μM bLF. Results correspond to a representative experiment from three independent experiments with similar results.

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CHAPTER 4

Figure 4.1 - Effect of bLF on breast cancer cells' growth. MCF-7, T-47D, MDA-MB-231 and Hs578T cells were incubated with bLF at several concentrations for 48 h and cell growth evaluated by cell counting of viable cells (not stained with trypan blue). Values are mean ± SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ when compared with the control.

Figure 4.2 - Effect of bLF in the induction of cell death by apoptosis in MCF-7 cells. Cells

were treated with different concentrations of bLF for 40 h or 72 h and apoptosis estimated by the Annexin V/PI and the nuclear condensation assays, respectively. (**A**) Representative images of the nuclear condensation assay where apoptotic cells are shown with arrows: white arrows correspond to examples of early apoptotic cells (condensed DNA); red arrows correspond to examples of late apoptotic cells (condensed and fragmented DNA). Images are provided with a magnification of 400×. (**B**) Apoptosis rate induced by bLF as mean ± SD of three independent experiments, as estimated by the nuclear condensation assay. * $P < 0.05$ and ** $P < 0.01$ when compared with the control. (**C**) Apoptosis assay by Annexin V/PI representative of two independent assays.

Figure 4.3 - Effect of bLF in the cell cycle progression of MCF-7 cells. (**A**) Cells were incubated with bLF at several concentrations for 24 h and cell cycle was analyzed by flow cytometry. Results correspond to a representative experiment from three independent experiments. (**B**) Comparison between the effect of bLF and BSA at the same concentration on the cell cycle progression of MCF-7 cells after 24 h of treatment. The top image is representative of cell morphology for each condition (magnification of the images: 40×).

Figure 4.4 - Effect of bLF on the mitochondrial membrane potential. (**A**) MCF-7 cells were incubated with bLF at several concentrations for 2 h, 24 h and 48 h and mitochondrial membrane potential was analyzed by flow cytometer after labeling cells with JC-1 fluorescent probe. The red/green ratio was calculated from the respective median fluorescent value, where a decrease of this value in comparison to the control is indicative of mitochondrial membrane depolarization. Values correspond to the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.01$ when compared with the control. (**B**) The top image is representative of cells labeled with JC-1 probe, where red cells contain polarized mitochondria and green cells contain depolarized mitochondria (magnification of the images: 400×). The bottom image corresponds to JC-1-labeled MCF-7 cells after 48 h of treatment with positive control (50 μM CCCP) or 175 μM bLF analyzed by flow cytometry. The graphs exhibit the percentages of cells with depolarized mitochondria (green positive/red negative).

Figure 4.5 - Levels of Bcl-2, p53 and CDC25c in MCF-7 cells after treatment with bLF at several concentrations for 48 h, as measured by western blotting. The blots are representative of three independent experiments with similar results. β-Actin was used as

loading control.

Figure 4.6 - Effect of bLF on the MCF-7 cells ability for anchorage-dependent colony formation. (**A**) Cells were seeded at low density and incubated with several concentrations of bLF and DMEM (FBS-free medium) for 24 h. Then, cells were left for 1 week to grow in complete medium to form colonies from single cells. Colonies were fixed, stained and images were captured. (**B**) Quantification of colony formation as mean ± SD of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$, when compared with the control (0 μM bLF).

Figure 4.7 – Effect of bLF on the migration of MCF-7 cells. Cells were incubated with the same concentration of bLF and BSA for 6 h, 12 h and 24 h. (**A**) Representative images of cell migration into the wounded area after 24 h of incubation (Magnification: 40×) (**B**) Relative wound closure as measured by Image J software. Results correspond to the mean ± SD of three independent experiments. * $P < 0.01$ and ** $P < 0.01$ when compared with the control or among each other.

LIST OF ABBREVIATIONS

AKT	Protein Kinase B
AMPK	AMP-activated serine/threonine protein kinase
Apo-LF	Iron-free lactoferrin
BRCA2	Breast cancer susceptibility gene
BSA	Bovine serum albumin
CAPs	Cationic antimicrobial peptide
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
DMBA	Dimethylbenz[a]anthracene
DOX-Lf-PCL	Doxorubicin-loaded Lf-modified procationic liposome
DSBs	DNA double-strand breaks
4E-BP1	4E-binding protein 1
ELP	Elastin-like polypeptide
ER+	Estrogen receptor positive
ER-	Estrogen receptor negative
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
ERK1/2	Extracellular-signal-regulated kinase 1/2
ERα	Estrogen receptor α
ERRα1	Estrogen receptor-related receptor α
FBS	Fetal bovine serum
HBP	Hamster buccal pouch
hLF	Human lactoferrin
HNP1	Human neutrophil peptide-1
Holo-Lf	Iron-saturated Lf
IFN-γ	Interferon-γ
IL-18	Interleukin-18
JNK	c-Jun N-terminal kinase
JNK/SAPK	Stress-activated protein kinase

ΔLF	Cytosolic LF
LfcinB$_{17-41}$	Bovine lactoferricinB17-41
MAPK	Mitogen-activated protein kinase
MPT	Mitochondrial permeability transition
mTOR	Serine-threonine mammalian target of rapamycin
NF-κB	Nuclear factor κB
NPC	Nasopharyngeal cancer
PBS	Phosphate buffered saline
PC12	Rat pheochromocytoma cells
PI	Propidium iodide
PVDF	Hydrophobic polyvinylidene difluoride
RIPA	Radio-Immunoprecipitation Assay
SFRE	Steroid factor 1 response element
S6K1	p70S6 kinase
Th	T helper cells
TNF	Tumor necrosis factors

Preface

The last few decades have been fruitful in great discoveries and advances in medicine. However, cancer is still an unsolved issue in modern medicine. According to the American Association for Cancer Research Progress Report, global cancer incidence is predicted to increase from 12.8 million new cases in 2008 to 22.2 million in 2030. Although more people survive to cancer today than in the past, this trend is confined to some types of cancer. The survival rates of some cancers, such as brain, pancreatic and lung cancer, are still very low. Cancer has become the most important cause of death and morbidity in Europe after cardiovascular diseases.

The search for efficient anticancer drugs has been limited by the scarce understanding of cancer pathogenesis. Hence, preventive measures to reduce cancer incidence have strongly been advocated. Several researchers have been suggesting that some food-derived components exhibit potent inhibitory effects against cancers. If people could prevent the incidence of cancer by changing their diets, a great progress in medicine could be reached with enormous economic benefits. Among the most promising anticancer foods, milk is an essential part of diet in western life style. Current studies have provided enough evidences of the anticancer effects of milk-derived components including TRAIL (TNF-inducing apoptosis ligand), α-lactalbumin and lactoferrin. Today, technology is in place to enable the production of a great amount of potential anticancer agents from milk.

However, prior to clinical application of any drug, even if it is derived from natural sources, we need to figure out the mechanisms underlying its anticancer effects. Since 2007, our group has been dedicated to the study of lactoferrin effects/roles on cancer, as well as its potential for cancer prevention and treatment. The study of the mechanisms underlying its inhibitory effects against cancers has been under investigation. Accordingly, this thesis aimed to unravel the mechanism involved in the lactoferrin effects against breast cancer cells.

CHAPTER 1

General introduction

Abstract

Lactoferrin (LF) has been widely studied over the last 70 years and its role in diverse biological functions is well known and generally accepted by the scientific community. This protein is a key player in the human defense mechanisms against various diseases. The protein response elements have been localized and characterized. Usually, an alteration of the LF gene in cells is associated with an increased cancer incidence. Several studies suggest that exogenous treatment with LF and its derivatives can efficiently inhibit the growth of tumors and reduce cancer susceptibility. However, none of these studies reported a consistent outcome concerning the mechanisms underlying the anticancer effects of exogenous application of LF and its derivatives. In this chapter, the association of LF with cancer is thoroughly discussed from its gene expression to its potential use in cancer therapy. Furthermore, the LF cytotoxicity against several cancers is generalized into four possible mechanisms of action under different conditions: cell membrane disruption, apoptosis induction, cell cycle arrest and immunoreaction. Based on these mechanisms, new strategies to improve the anticancer effects of LF and/or its variants are proposed. Finally, the potential of LF in cancer therapy in the near future is discussed.

1.1 INTRODUCTION

Lactoferrin (LF) is an 80 kDa glycoprotein comprised of a polypeptide chain containing 703 amino acids. The single chain is folded into two homologous lobes in a higher structure, which are connected by a three-turn α-helix [Farnaud＆Evans, 2003]. The two lobes bind and release iron by a substantial conformational change that contributes to the most important functions of the protein. LF can be found in mucosal secretions such as tears, saliva, bile, nasal and bronchial secretions [Rodrigues *et al.*, 2009], and most abundantly in milk and colostrum [Masson＆ Heremans, 1971]. This wide distribution clearly demonstrates the important role of LF in the maintenance of homeostasis.

Many relevant biological functions have been reported for LF, including anticancer, antibacterial, antiviral, antifungal, anti-inflammatory, and immune regulatory activities [Adlerova *et al.*, 2008]. LF has also been suggested as a marker of active diseases [Gibson ＆Bowen, 2011]. Some studies support that LF's iron-binding ability and interaction with its specific receptors are the responsible for the diverse functions that have been demonstrated [Adlerova *et al.*, 2008]. In addition, LF derived peptides, as well as the iron-saturated LF (holo-LF), have also been shown to be efficient anticancer and antibacterial drugs [Rodrigues *et al.*, 2009]. However, the detailed mechanisms underlying the broad range of LF functions still need to be clarified. Though, regarding its role in cancer development and progression, a number of published data allows summing up the possible mechanisms of LF toxicity against cancer cells. For example, some studies showed that the downregulation or silencing of LF or delta LF (ΔLF) genes in cells lead to an increase of malignant tumors [Benaïssa *et al.*, 2010; Hoedt *et al.*, 2010]. On contrary, the restoration of LF gene in cancer cells can efficiently block their proliferation [Breton *et al.*, 2004; Zhou *et al.*, 2008]. Additionally, several studies have shown that LF and/or its derivatives can inhibit the growth of tumors both *in vitro* and *in vivo* [Ushida *et al.*, 1999; Sakai *et al.*, 2005; Chandra *et al.*, 2006; Onishi *et al.*, 2008].

Due to the considerable value that LF and/or its derivatives may have in cancer prevention and treatment, the present chapter aims to provide an overview of their mechanisms of action against cancer, as well as their possible future applications in therapy.

1.2 GENE STRUCTURE OF LACTOFERRIN

LF gene has been localized at the chromosome level in a set of different species (human chromosome 3 and mouse chromosome 9) [Teng *et al.*, 1987]. The full coding region of LF gene varies widely from 2055 to 2190 residues in different species due to the deletions, insertions and mutations that occur in the stop codon [Kang *et al.*, 2008]. However, their transcripts are equally composed of 17 exons that are separated by introns ranging in size from 300 bp to 3.3 kb [Teng *et al.*, 2002b] (**Figure 1.1A**). ΔLF was accidently discovered by positional cloning of the second breast cancer susceptibility gene (*BRCA2*) from a normal breast tissue [Siebert＆Huang, 1997]. It is the product of an alternative (P2) promoter present in the first intron of LF gene. As a result, ΔLF lacks the first 26 amino acids of the secreted LF. The P2 promoter activity can be dramatically enhanced with overexpression of the Ets-1 transcription factor [Liu *et al.*, 2003].

LF gene in the epithelium of the vagina and the isthmus oviduct is constitutively expressed but it is estrogen inducible in the uterus of immature mice and rats [Teng, *et al.*, 2002a]. In fact, LF mRNA synthesis is regulated either directly or indirectly by estrogen, retinoic acid, mitogen, and growth factors. The multiple response elements mainly lie in the three short, but complex, modules within 400 bp from the transcription site of LF gene [Teng *et al.*, 2002b] (**Figure 1.1B**). Two modules of the LF gene that respond to estrogen and growth factor stimulation have been distinctly characterized. The imperfect estrogen response element (ERE) is overlapped with a chicken ovalbumin upstream promoter transcription factor (COUP-TF), which acts as an estrogen-responsive negative regulator (**Figure 1.1B**). Overexpression of the COUP-TF expression vector can block the estrogen-stimulated responses [Liu *et al.*, 1993]. The molecular mechanism of this negative regulation is attributable to the competition between COUP-TF and the estrogen receptor (ER) in the overlapping region. The human LF gene promoter (P1) contains two COUP-TF elements, one of which is overlapped with the ERE, while the other is present within a composite response element adjacent to the ERE [Teng, 1995] (**Figure 1.1B**). These complex modules contribute to the fine-tuning of estrogen regulation of LF gene expression. Steroid Factor 1 Response Element (SFRE), an additional element in the human LF gene, is located 26 bp upstream from the ERE and is also involved in estrogen responsiveness. Mutations at SFRE induce a 50% reduction of the activity of estrogen receptor α (ERα). Also, the estrogen receptor-related receptor (ERRα1) binding to SFRE

affects the transcriptional activity mediated by ERα [Yang *et al*., 1996]. There are other types of ERE-negative regulatory factors, whose absence increases the expression of estrogen-induced LF by up to100-fold [Park *et al*., 2005].

The infection-responsive promoter in LF gene has also been characterized. Lipopolysaccharide (LPS)-responsive regions of the promoter contain one STAT3 site, three AP1 sites and eight nuclear factor κB (NF-κB) sites. LF promoter may respond to infections via the NF-κB site pathway [Zheng *et al*., 2005]. This observation explains the great increase of LF in the mammary secretions when infected by bacteria. The expression of LF is mainly stimulated by bacterial LPS and double-strand RNA (dsRNA) [Li *et al*., 2009]. In addition, LF expression in mammary epithelial cells is mediated by changes in cell shape and actin cytoskeleton [Close *et al*., 1997]. Furthermore, miR-214, expressed in 23 species, was found to be directly involved in the LF expression and the LF-mediated cancer susceptibility inmammary epithelial cells [Liao *et al*., 2010].

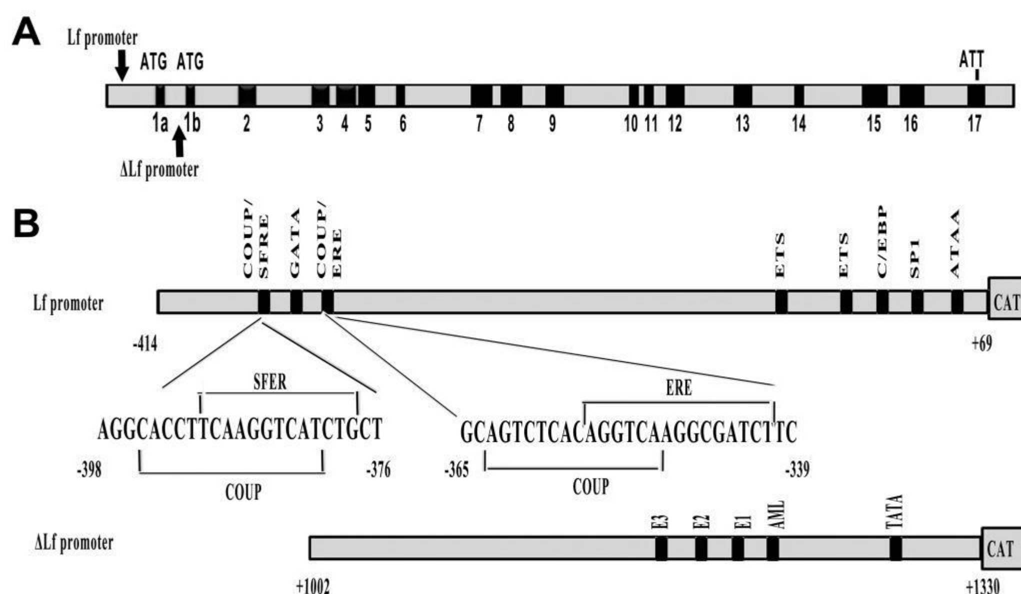

Figure 1.1 – Schematic representation of human LF gene. (**A**) Promoter and mRNA structure of human LF gene. The black arrows indicate the LF promoters (LF promoter and ΔLF promoter). The translation start site and termination codon are marked with ATG and TAA, respectively. 1a stands for the first exon in LF, and 1b for ΔLF. The black bars and number indicate the exon size and location. (**B**) Diagram of response modules of human LF gene promoter. The numbers mean the position upstream from the start site of LF promoter 1. Overlapping regions between two different modules are illustrated using the DNA sequence [Yang *et al*., 1996; Liu *et al*., 2003].

Some of these response elements in the LF gene may be implicated in an increased cancer incidence, such as the estrogen and infection response elements. Estrogen can promote DNA synthesis and cell proliferation by turning on its responsive genes. Exposure to an excess of estrogen will greatly increase the incidence of breast cancers [Muti *et al.*, 2000; Kabat *et al.*, 2006]. On the other hand, it is well known that one out of six cancers develops from infection [de Martel *et al.*, 2012]. The module elements responding to the pathogenic factors are involved in the LF gene expression. This indicates that LF probably acts as a negative regulator in human body to repress the acute reaction to estrogen or to kill virus and bacterial infection by sharply raising the protein expression, which helps to decrease the cancer incidence. The full knowledge on these regulatory modules in the LF gene constitutes a powerful tool for designing novel strategies of cancer treatment.

1.3 STRUCTURE AND PROPERTIES OF LACTOFERRIN AND ITS VARIANTS

LF is a protein that is well-conserved among species [Gerstein *et al.*, 1993]. It contains a simple polypeptide chain, which is folded into two symmetrically globular lobes representing the N- and C- lobes. The two lobes with 33-41% homologous sequences are connected by a short α-helix between the amino acids 333 and 343 [Masson＆Heremans, 1971]. The interactions among the special hinge region mostly consist of hydrophobic effects which provide additional flexibility to the molecule. Both lobes are folded similarly including α-helix and β-pleated sheet structures, which are referred to as N1 and N2, or C1 and C2. The iron binding site is located in the deep cleft of the two domains in each lobe and comprises four protein ligands: 2 Tyr, 1 Asp, and 1 His [Baker, 2009; González-Chávez *et al.*, 2009) (**Figure 1.2A**). It can bind two ions of iron (Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Mn^{2+}) in the synergy with the carbonate ion (CO_3^{2-}), the iron saturation of which is named as holo-LF [Baker, 2009] (**Figure 1.2B(a)**). In addition, the other variant, apo-LF, results from the release of iron from LF (**Figure 1.2B(b)**). The two domains of each lobe in the holo-LF enclose the bound irons, which turns it a closed structure with greater resistance to proteolysis. In contrast, the apo-LF is more flexible due to the absence of a bound metal iron to lock the two domains of each lobe together [Gerstein *et al.*, 1993; Baker, 2009].

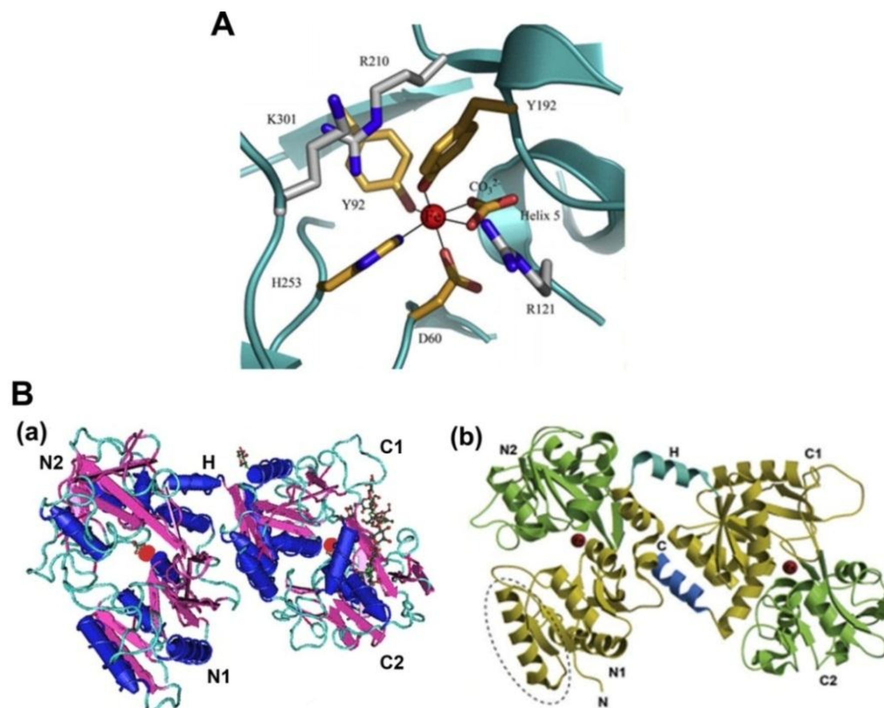

Figure 1.2 – LF structure. (A) Iron binding site. The four protein ligands (two Tyr, one Asp, one His) and the carbonate ion, together with the arginine residue and the N-terminus of helix 5, which help bind the carbonate ion, are shown for the N-lobe of human LF. Two basic residues after the iron site (here Arg210 and Lys301) help modulate iron release [Baker, 2009]. (**B**) Three-dimensional of bi-ferric LF. The N-lobe is on the left and the C-lobe is on the right, with the N- and C-terminus of the polypeptide chain labeled N and C. The four domains are labeled N1, N2, C1, C2, and the helix which joins the two lobes (H). The two iron binding sites are shown with red spheres. (**a**) Bovine lactoferrin [González-Chávez *et al.*, 2009]. **(b)** Human lactoferrin [Baker, 2009].

1.4 BIOLOGICAL FUNCTIONS OF LACTOFERRIN

LF plays an important role in the innate immunity, which responds to external stimulation in an antigen-nonspecific defense mechanism. For example, LF is massively released from neutrophils to blood and inflamed tissues when inflammation occurs [García-Montoya *et al.*, 2012]. More important, LF exhibits strong inhibitory activity against a broad spectrum of bacteria, fungi, yeasts, virus and parasites [Rodrigues *et al.*, 2009]. Moreover, other biological functions have been assigned to LF, such as anticancer, hypoferremia and several enzymatic functions [Farnaud & Evans, 2003] (**Figure 1.3**).

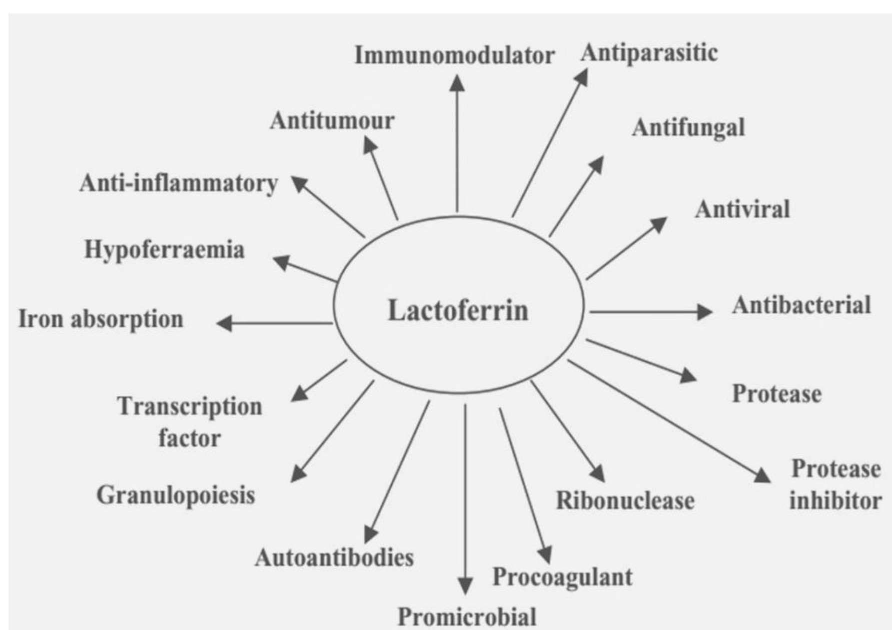

Figure 1.3 – The proposed roles of LF [Farnaud＆Evans, 2003].

LF extensive antimicrobial activities were generally attributed to its iron binding ability, which deprived the bacterium of iron [Adlerova *et al.*, 2008]. However, some reports suggested that the LF bactericidal activities result from the direct interaction between the protein or its derived peptides and the bacterium [Legrand *et al.*, 2008]. Besides, the mechanism underlying LF antiviral activity is determined by two different pathways: binding to viral particles and competing with virus-receptors in host cells [Farnaud＆Evans, 2003]. The mechanisms involved in the other LF actions are also being investigated.

1.5 GENETIC VARIATIION OF LACTOFERRIN GENE IN CANCER CELLS

A relationship between LF expression and breast cancer susceptibility was first proposed by Furmansiki and collaborators [Furmanski *et al.*, 1989]. They found that the LF-associated RNase activity was present at lower levels in the milk obtained from a consanguineous community with a high incidence of breast cancer in India. Recent studies also showed that both P1 and P2 promoter regions of the LF gene are downregulated or silenced in an extensive series of cancer cell lines [Hoedt *et al.*, 2010]. This

downregulation is much more visible for △LF since its expression is either significantly diminished (BT-20 and MCF-7 cells) or practically absent (MDA-MB-231, T-47D and HBL 100 cells) [Benaïssa *et al*., 2005], but it is regularly expressed in normal tissues. Moreover, △LF stable expression or overexpression of LF resulted in a significant inhibition of cancer cells growth, as well as in a weaker in vivo formation potential [Breton *et al*., 2004; Zhou *et al*., 2008].

Besides the distinct LF expression levels in normal or cancer tissues, the evaluation of the LF gene structure showed that the degree and pattern of methylation are notably altered in malignant breast cells [Panella *et al*., 1991; Teng *et al*., 2004]. For instance, an LF cDNA clone extracted from human breast tissues was used to evaluate the restriction fragment length changes and methylation patterns of DNA from normal peripheral blood, leukemia cells from patients, leukemia cell lines and breast cancer cell lines. Methylation patterns of DNA in malignant cells were highly variable and generally less methylated than in normal cells [Liu *et al*., 2002]. In addition, methylation assays conducted in a nasopharyngeal cancer (NPC) model demonstrated that the methylation also occurred in the promoter of the LF gene, which is linked with the decreased LF gene expression. Simultaneously, abnormal migration patterns of the LF gene were also found in cancer cells. More specifically, the alteration generally takes place in the LF exons 4, 5, 13, 14, and 15 [Luczak＆Jagodziński, 1990].

Genetic variation of the LF gene significantly increases cancer susceptibility. The changes in methylation of the promoter or first exon may have the same impact as the effect of mutations of various tumor suppressor genes or proto-oncogenes. Carcinogenesis can result from aberrations in genomic DNA methylation, including hypermethylation and hypomethylation of the promoter or the first exon of cancer-related genes [Luczak＆ Jagodziński, 1990]. Recent studies show that, apparently, a relatively higher genetic polymorphisms, gene mutations, and promoter methylation in the LF gene occur in cancer cells [Panella *et al*., 1991; Liu *et al*., 2002; Teng *et al*., 2004]. All these observations suggest a close relationship between alterations of the LF gene and an increasing incidence of carcinomas. Hence, the use of LF as a novel cancer-specific marker may be useful for diagnosis and prognosis of cancer patients at different stages, or alternatively to be used as a therapeutic agent in the clinical practice. Since the recovery of LF expression successfully depresses the growth of tumors, the LF gene can be further explored as a new targeting site for cancer gene therapy.

1.6 ORAL ADMINISTRATION OF LACTOFERRIN REDUCES CANCER INCIDENCE

To the best of our knowledge, there is no effective drug that can completely overcome cancer, especially for the patients at an advanced stage. This makes the prevention of carcinomas' development a crucial step for decreasing the high cancer mortality. Some epidemiological studies have shown that more than two-thirds of cancers could be prevented through the adoption of an appropriate lifestyle [Haque *et al.*, 2010]. A diet regimen enriched in preventive agents is one of the proposals expected to decrease cancer incidence and other diseases [Cam＆de Mejia, 2012]. Milk-derived LF is a significant functional protein and reaches 7 g/L in colostrum [Masson＆Heremans, 1971]. The multifunctional LF could work as the "magic bullet" in cancer prevention.

Indeed, a large number of reports provide clear evidences that the oral administration of LF can effectively decrease the development of chemically-induced cancers [Tanaka *et al.*, 2000; Tsuda *et al.*, 2000; Chandra *et al.*, 2006]. LF is a relatively stable protein that can survive even after transiting through the gastrointestinal tract as partially degraded fragments [Norrby *et al.*, 2001; Teraguchi *et al.*, 2004]. These fragments with a mass over 20 kDa contain the receptor-binding regions, in addition to the anticancer active regions [Norrby *et al.*, 2001]. An example of these fragments is the bovine lactoferricin B (LfcinB) peptide. Moreover, these fragments can be internalized by specific LF receptors in the intestinal brush border membrane [Suzuki *et al.*, 2001]. The oral administration of 0.2% or 2% bovine lactoferrin (bLF) was found to decrease by 32.5% and 42.5%, respectively, the occurrence of colon carcinogenesis in azoxymethane treated F344 rats [Tsuda *et al.*, 2000]. In a study conducted with the 7, 12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) model, the incidence of carcinogenesis was efficiently reduced by dietary fed of 0.2% bLF [Mohan *et al.*, 2006]. Also, oral administration of bLF was shown to have chemopreventive effects on tongue [Panella *et al.*, 1991] esophagus and lung carcinogenesis in rats [Ushida *et al.*, 1999]. The suggested chemopreventive effects could be due to the multiple LF-induced effects, including stimulation of the immune response [Tanaka *et al.*, 2000; Tsuda *et al.*, 2000], modulation of the carcinogen-metabolizing enzymes and oxidant-antioxidant profile in the target organs [Mohan *et al.*, 2006], as well as inhibition of angiogenesis [Norrby *et al.*, 2001]. The regulation of the immune function may be a key factor in the LF mechanisms of action that are involved in cancer

chemoprevention. Presently, the massive extraction of bLF is an industrial reality. Therefore, it is conceivable that in the near future people could take LF-contained pills or consume LF-enriched products to prevent cancer or delay its onset.

1.7 MECHANISMS OF ACTION OF LACTOFERRIN AGAINST CANCER

Although the exact mechanisms involved in the anticancer activity of LF are still unclear, they can be generally grouped into extracellular effects, intracellular effects, and immunostimulation. The LF extracellular effects are mainly related with its interaction with the cell membrane and membrane receptors [Yang *et al*., 2003; Arseneault *et al*., 2010], while its intracellular effects suggested by most studies dominantly concerns cell apoptosis and cell cycle arrest (**Table 1**). The LF immunostimulation is primarily achieved by activating immune cells to release tumor cytotoxic effectors [Kuhara *et al*., 2000; Iigo *et al*., 2004; Fischer *et al*., 2006; WoLF *et al*., 2007]. The anticancer mechanisms of LF or its derivatives that have been proposed from *in vitro* experiments are diverse (**Figure 1.4**).

1.7.1 TOXIC EFFECTS OF LACTOFERRIN AND ITS DERIVED PEPTIDES ON THE CELL MEMBRANE

LF and its derived peptides were found to be easily internalized by cells [Rochard et al., 1992; Bi et al., 1996]. LF enters cells via receptor mediated endocytosis in Jurkat human lymphoblastic T-cell line. Nevertheless, the efficacy of this uptake pathway is low [Bi *et al*., 1996; Baumrucker *et al*., 2006]. On contrary, LF active peptide LfcinB exhibits higher conformation-dependent uptake efficiency [Duchardt *et al*., 2009]. Increased permeability may produce subtle changes in the membrane’s barrier function that promote cell death. LF can increase cytolysis at a low concentration, while at a high concentration it modulates cytolysis depending on the target cell phenotype [Damiens *et al*., 1998]. Many studies demonstrated the presence of LF receptors on the tumor cell surface, such as heparan sulactoferrinate [Rochard *et al*., 1992; Bi *et al*., 1996; Baumrucker *et al*., 2006], that when removed leads to a reduction of membrane binding and cellular uptake of LfcinB [Duchardt *et al*., 2009]. However, the characterization of these receptors is still under investigation because different cell types appear to express their own specific LF receptors.

Table 1. Mechanisms of action underlying the cytotoxicity of lactoferrin or its derived peptides against different cancer cells

Action mechanism	Cell lines	Effective concentrations (µM)			Ref
		bLF	hLF	LfcinB	
Membrane Disruption	Human neuroblastoma cell lines: Kelly, SK-N-DZ and IMR-32			12.8	Eliassen *et al.*, 2006
	Human leukemia HL-60 cells			*4-31.6	Onishi *et al.*, 2008
Cell cycle arrest	Canine mammary gland adenocarcinoma cell lines: CIPp and CHMp	0.32-6.41			Yamada *et al.*, 2008
	Human breast cancer cell lines: MDA-MB-231 and MCF-7		0.625		Damiens *et al.*2008
	Human keratinizing squamous cell carcinoma (WHOI), CNE1 and undifferentiated carcinoma 5–8F and 6–10B cells		10		Zhou *et al.*, 2008
	Murine squamous cell carcinoma cell line: SCCVII and human cancer cell line: O12 cells		250		WoLF *et al.*, 2007
	Human head and neck cancer cell lines: 011, 012, 019, and 022		10		Xiao *et al.*, 2007
Apoptosis	Human stomach cancer cell line: SGC-7901	50-100			Xu *et al.*, 2010
	Human pheochromocytoma cells (PC12)		8.75		Lin *et al.*, 2005
	Jurkat cells		8.75	64	Mader *et al.*, 2005; Lee *et al.*, 2009
	Human breast cancer cell line: MDA-MB-435			32-64	Furlong *et al.*, 2006; Mader *et al.*, 2005
	Human breast cancer cell line: MCF-7			7.5-64	Mader *et al.*, 2005
	Human breast cancer cell lines: HS578T and T-47D	125			Duarte *et al.*, 2001
	Human leukemia HL-60 cells			**30-126	Onishi *et al.*, 2008; Roy e*t al.*, 2002
	Human tongue-derived squamous cell carcinoma cell line: SAS			640	Sakai *et al.*, 2005

a). bLF**,** Bovine lactoferrin; hLF, Human lactoferrin; LfcinB, Bovine lactoferricin B.

b).*L15 peptide (modified from Lfcin $B_{14\text{-}31}$): P A WR K A R R WARR M K K L AA; **$Lfcin_{17\text{-}38}$.

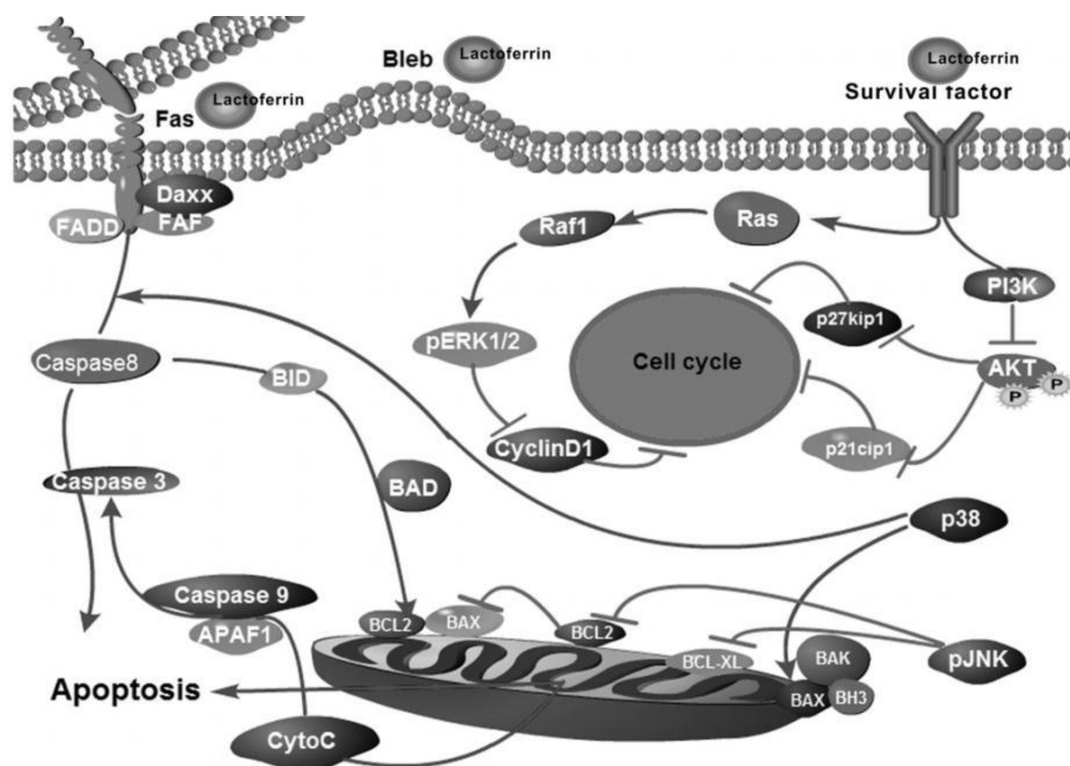

Figure 1.4 – Multiple signaling pathways potentially underlying the cytotoxicity of LF and its derivatives against cancers.

LfcinB revealed a higher potential activity against tumor cells than LF. This may be due to the fact that it is a cationic antimicrobial peptide (CAPs). Some CAPs have shown a selective activity against cancer cells, thus constituting a promising group of novel anticancer agents with a new mode of action and broad spectrum of anticancer activity [Suzuki *et al*., 2001; Duchardt *et al*., 2009; Arseneault *et al*., 2010]. LfcinB is composed of 25 amino acid residues (the residues 17-41 of bLF), containing a disulfide bridge between two Cys residues in positions 19 and 36 [Mohan *et al*., 2006; Liu *et al*., 2011] (**Figure 1.5**). This disulfide bond may not be essential in maintaining its antibacterial activity, but it is very important for the activity against tumor cells [Yang *et al*., 2002; Zhang *et al*., 2010]. In aqueous solution, the peptide loses the α-helical portion of the parent protein, adopting a slightly twisted antiparallel β-sheet amphipathic structure, with nearly all the hydrophobic residues lining up on one face of the peptide [Zhou *et al.*, 2004]. This change makes it bear similarities to several major classes of CAPs, which contain disulfide bridges and antiparallel β-stands, and form amphipathic helices upon binding to membranes.

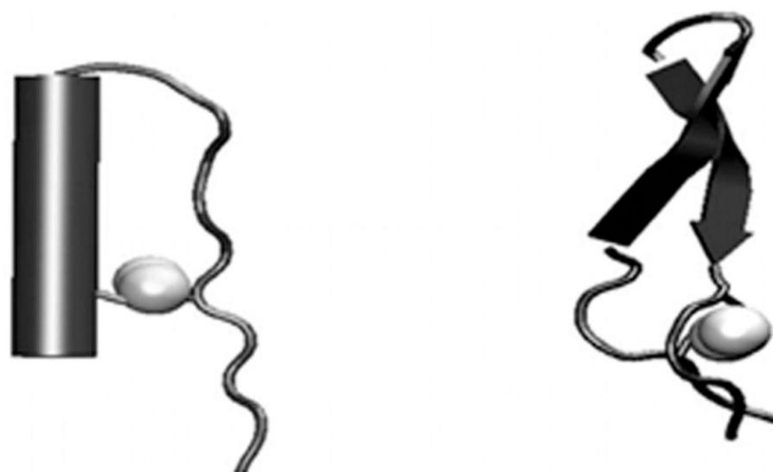

Figure 1.5 – Structure of the LfcinB fragment. Crystal structure on the left and the NMR structure of the solution on the right. The β-sheet and α-helical structures are indicated by arrows and cylinders, respectively. The spheres highlight the two disulfide-linked Cys residues [Cam & de Mejia, 2012].

Simultaneously, LfcinB exists as a α-helix structure when it is still linked to the parent LF, and this is also an important feature of membrane-lytic peptides [Jin *et al.*, 2005; Arseneault *et al.*, 2010]. When CAPs come into contact with the cell membrane, conformational changes occur enabling their association with the membrane, and consequently the cell lyses [Jin *et al.*, 2005]. It is important to notice that this mechanism is still not fully understood. However, the peptide-lipid interactions leading to membrane permeation play a major role in their activity [Arseneault *et al.*, 2010]. Moreover, some CAPs can trigger apoptosis by disrupting the mitochondrial membranes [Ellerby *et al.*, 1999]. Fluorescence-labeled LfcinB has been found in the mitochondrial membrane of neuroblastoma cells [Eliassen *et al.*, 2006]. Additionally, many studies suggest that the membrane may constitute one of the targets for the LF and LfcinB, through which they exert their anticancer effects [Mader *et al.*, 2005; Mohan *et al.*, 2006; Arseneault *et al.*, 2010].

1.7.2 CELL APOPTOSIS AND CELL CYCLE ARREST

Cell apoptosis induced by LF and LfcinB has been described as the pivotal pathway by which they exert their cytotoxicity against various cancer cells. However, the apoptosis pathway that they trigger, extrinsic (Fas and other TNFR receptor family) or intrinsic (mitochondria-associated) pathway, apparently varies depending on the cell type used [Lin *et al*., 2005; Eliassen *et al*., 2006; Lee *et al*., 2009; Xu *et al*., 2010].

Akt (protein kinase B) acts as an anti-apoptotic signaling molecule and it plays a critical role in the function of cancer cells [Testa＆Tsichlis, 2005]. For example, when stomach cancer cells SGC-7901 were treated with LF, the phosphorylated Akt and several key proteins involved in the signaling pathway were decreased, but expression of phosphorylated glycogen synthase kinase-3β (at Ser 256) and phosphorylated caspase-9 (at Ser 196) were increased, which suggests that the apoptosis of stomach cancer cell SGC-7901 induced by LF may be regulated via AKT pathway [Xu *et al*., 2010]. In addition, Akt can induce cell growth arrest by modulating the expression and activity of $p21^{Cip1}$ and $p27^{Kip1}$ [Damiens *et al*., 1999; Xiao *et al*., 2004]. After exposure to LF, the MDA-MB-231 cells stopped at the G1 to S transition phase of the cell cycle, which was associated with a dramatic decrease in the levels of proteins Cdk2, cyclin E and Cdk4, accompanied by an augmented expression of the Cdk inhibitor $p21^{Cip1}$ [Damiens *et al*., 1999]. Cell cycle arrest induced by LF was also demonstrated using NPC cells. The expression of cyclin D1 and phosphorylation of retinoblastoma protein (Rb) was downregulated, while expression of p21 and p27 was enhanced [Breton *et al*., 2004]. Furthermore, LF was also found to induce the mitogen-activated protein kinase (MAPK) pathway. For Jurkat T cells, the JNK (c-Jun N-terminal kinase) associated with Bcl-2 pathway is believed to be the pathway of LF-induced apoptosis. LF treatment induced the activation of caspase-9 and caspase-3 and increased the level of Bcl-2 phosphorylation. When JNK activation was abolished, LF-treated Jurkat cells did not undergo cell death [Lee *et al*., 2009]. Additionally, LF induced apoptosis in the colon mucosa of azoxymethane-treated rats by enhancing Fas expression. After oral administration of LF, the Fas protein expression was augmented more than 2.5 fold, and the active forms of both caspase-8 and caspase-3 were also higher. Immunohistochemical analysis revealed the presence of Fas-positive and apoptotic cells preferentially at the site of LF-mediated tumor inhibition [Fujita *et al*., 2004]. In addition, LF was found to protect PC12 cells from FasL-induced apoptosis at a low concentration (50 μg/ml), while it triggered apoptosis through regulation of the expression of

phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2) at a high concentration (700 μg/ml) [Lin *et al.*, 2005].

Compared to LF, most studies with LfcinB demonstrate that cells undergo the mitochondrial-related apoptosis pathway [Mader *et al.*, 2005; Eliassen *et al.*, 2006; Onishi *et al.*, 2008]. This may be due to its relatively higher efficiency of cell uptake [Bi *et al.*, 1996; Duchardt *et al.*, 2009], which greatly facilitates its interaction with both surface death receptors and intracellular apoptosis-related proteins. An example includes the apoptosis induction in oral squamous carcinoma cells by JNK/SAPK activation using LfcinB. In the process, JNK pathway directly activates the mitochondrial death machinery [Aoki *et al.*, 2002].

1.7.3 IMMUNOSTIMULATION

Immunostimulation is a key factor for the *in vivo* anticancer effects of LF. Both innate and adaptive immunity are involved in the LF or its derivatives induced immunoreaction [Kuhara *et al.*, 2000; Iigo *et al.*, 2004; Fischer *et al.*, 2006; WoLF *et al.*, 2007; Iigo *et al.*, 2009] (**Figure 1.6**). As a result of the cyclic change of LF concentration, the secretory components such as the immunoglobulin (Ig) A and IgG increase sharply at proestrous in the uterus and decline in the other stages [Kaushic *et al.*, 1998]. In cancer cases, LF predominantly functions by activating a strong Th1 response and the release of anticancer killer cells [Fischer *et al.*, 2006]. The recruitment of lymphocytes mainly including CD4+ and CD8+ reaches up to a 20-fold increase within treated animals after oral administration of bLF [WoLF *et al.*, 2007]. The tumor-infiltrating lymphocytes can greatly inhibit the proliferation of cancers [June, 2007]. On the other hand, the expression of interferon-γ (IFNγ) [Iigo *et al.*, 2009], caspase-1 and IL-18, IgM (+) and IgA (+) B cells are also significantly increased in the small intestine after bLF treatment [Wang *et al.*, 2000; Artym *et al.*, 2003]. Actually, a cascade effect exists among them. Caspase-1 is known to cleave pro-IL-18 to generate mature IL-18 [Iigo *et al.*, 2004]. IL-18 is an IFN-γ production-inducing cytokine [Cao *et al.*, 1999]. Further, IL-18 has an important role in the expression of TNF-γ in T cells and NK cells, and as a stimulator of TNF-α and several other cytokines. IL-18 can also enhance Th1 and NK cell responses and generate CD8+ effector T cells [Okamoto *et al.*, 1999]. Systemic and intralesional administrations of IL-18 significantly inhibited the growth of tumors [Cao *et al.*, 1999]. In addition, LF can raise the

effector of NK cells and macrophages at low concentrations and enhance the production of nitric oxide that has been reported to sensitize tumors to chemotherapy [Damiens *et al.*, 1998; Kanwar *et al.*, 2008]. Moreover, oral administrated bLF could systemically restrict $VEGF_{165}$-mediated angiogenesis in rats [Norrby *et al.*, 2001]. The combined effects of these factors succeed in eradicating tumors and blocking pathogens. However, LF iron-free (apo-LF) failed to restrain the growth of tumors [Wang *et al.*, 2000]. LF can flexibly bind and release several kinds of iron related cations, such as Zn^{2+}, Fe^{3+}, Cu^{2+}, Mn^{3+} and Ga^{3+} [Adlerova *et al.*, 2008]. Indeed, LF functions as one of the main iron regulators in human body and it is responsible for keeping the systemic iron homeostasis. The iron balance is a necessary factor for cell growth and enzyme activity. If unbalanced, iron can become a potentially toxic element, leading to the formation of free radicals. These radicals induce a cellular redox imbalance by producing oxidative stress, which may be related to oncogenic stimulation [Valko *et al.*, 2006]. As such, LF may be an effective chelator to keep the balance of iron *in vivo*. Besides, iron chelators have been shown to possess antiproliferative activity both *in vitro* and *in vivo* [Richardson *et al.*, 2009].

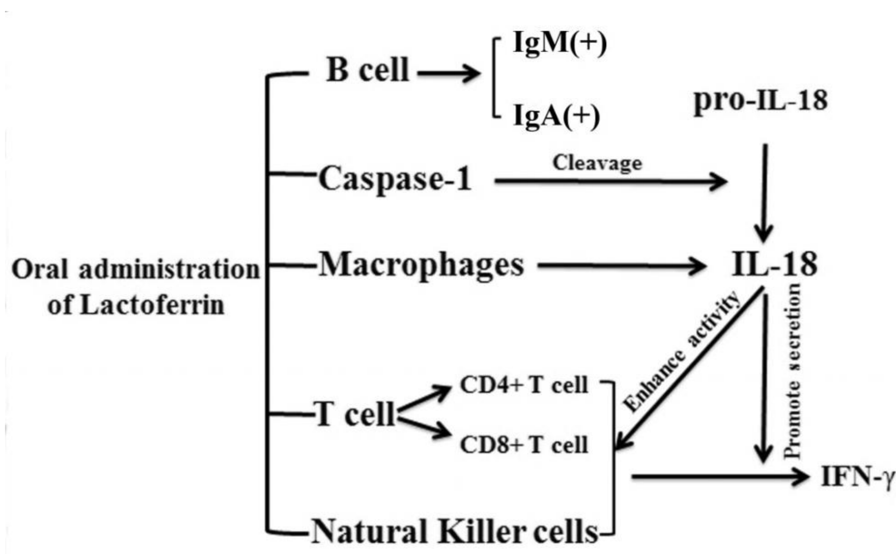

Figure 1.6 – Immunoreaction stimulated by LF or its derivatives *in vivo*.

1.8 ENHANCING THE ANTICANCER EFFECTS OF LACTOFERRIN AND ITS DERIVATIVES

Currently, it is demonstrated and recognized that LF and its derivatives possess anticancer activities. However, their exclusive use in the clinical practice for cancer treatment has not provided the expected results. In contrast, their use in combination with other agents has proved to be highly well succeeded [Furlong *et al*., 2006; McKeown *et al*., 2006; Massodi *et al*., 2009; Roseanu *et al*., 2010]. Therefore, it is expected that LF and its derivatives synergize with other known anticancer agents or delivery systems in order to promote their cytotoxicity against cancer.

The ability to specifically target a tumor is one of the desired properties of an ideal antitumor drug. An appropriate delivery system can be very helpful to target LF and its derivatives to the tumor. Massodi and co-workers [Massodi *et al*., 2009] developed an elastin-like polypeptide (ELP) carrier to deliver the peptide derivative of bLF. The thermally responsive assemblage is soluble in aqueous solutions at 37℃, but aggregates near 41℃, which makes it a perfect carrier for targeting solid tumors in which treatment is focused on hyperthermia. On the other hand, liposomes are an efficient drug delivery system and can greatly enhance the therapeutic potential of the encapsulated compounds. Liposome entrapped iron-free LF (apo-LF) clearly enhanced its inhibitory effect on the growth of B16-F10 cells [Roseanu *et al*., 2010]. In addition, a brain-targeted chemotherapeutical delivery system, doxorubicin-loaded LF-modified procationic liposome (DOX-LF-PCL), efficiently raised the uptake and cytotoxicity of LF against glioma C6 cells [Chen *et al*., 2011]. Furthermore, several studies successfully used adenovirus to target LF to cancers in animal models [Wang *et al*., 2011; Wang *et al*., 2012]. These recombinant adenoviruses could significantly suppress the growth of tumors by releasing LF within these solid tumors.

The combination of LF or its variants with recognized anticancer agents is another effective pathway to increase their anticancer activities. Holo-LF fed tumor-bearing mice could completely eliminate their tumors with a single injection of known chemotherapeutic agents [Kanwar *et al*., 2008]. *In vitro* experiments showed that the cytotoxic effects of LfcinB were greatly augmented when added in combination with C6-ceramide and tamoxifen in breast cancer cells [Furlong *et al*., 2006]. Another interesting study revealed that a combination of human neutrophil peptide-1 (HNP1) and human LF could kill cancer

cells while sparing normal cells. Neither HNP1 nor human LF could individually demonstrate this effect [McKeown *et al*., 2006]. The selective cytotoxicity against cancers is the most important feature of any antitumor drug. Therefore, these successful case studies suggest that the combination of LF or its derivatives with other anticancer therapeutics could be an efficient way to boost their cytotoxicity, and consequently their performance against cancers.

1.9 FUTURE TRENDS IN THE USE OF LACTOFERRIN FOR CANCER THERAPY

Although there is still some uncertainty on the mechanisms underlying the cytotoxicity of LF and its derivatives against cancer, there is no doubt about its potential anticancer activity. Being LF and its derivatives food-derived components, it confers an advantage regarding its potential use as a toxic-free drug for humans. Also, its stability through the gastrointestinal tract is beneficial if oral administration is envisaged. All these features are believed to be essential for an efficient drug. Nonetheless, so far there are no conclusive data that support the clinical application of this protein or its variants as a sole drug in cancer therapy. This is partly due to the paradoxical results from different reports, as well as due to the low cytotoxicity of LF and its derivatives against cancers from *in vivo* experiments. Additionally, immunoreaction stimulated by LF is thought to be a key factor in cancer therapy. Nevertheless, a considerable amount of *in vitro* data showed that LF and its derivatives could greatly restrain the proliferation of various cancer cells [Yang *et al*., 2004; Mader *et al*., 2005; Duarte *et al*., 2011]. This means that their cytotoxicity probably is due to the direct contact between LF and cancer cells. Thus, the direct and indirect actions of LF should be fully explored to get a higher anticancer effect *in vivo* (**Figure 1.7**). The direct action of LF can be achieved by intratumoral or intravenous injection (i.v). Unfortunately, LF could be quickly cleared after i.v administration [Beljaars *et al*., 2002]. In that sense, perhaps a tumor-targeting delivery system is preferable to carry these proteins, which will prevent the protein from degradation before reaching tumor cells. Currently, several carrier vectors, systemic targeting or intracellular targeting, are available to deliver anticancer agents. Either widely used liposomes or rising nanoparticles have proved to be efficient carriers to be used in clinical practice. They may be modified with specific-ligands to target tumor cells, or just to stay longer in circulation, thus providing a

slow release of LF or its variants and so persistently activating the immune system. This approach will greatly reduce any possible adverse effects and will increase their performance against cancer. Also, the combination of LF and its derivatives with known anticancer drugs have shown their added value in cancer therapy and will continue to be further explored. In this approach, the very first use of LF in clinical practice will be as adjuvant in cancer therapy. This will not only take advantage of the immunotherapeutic effects of LF, but will also provide a higher anticancer effect as compared to the drugs' sole use.

However, as previously discussed the best way to overcome cancers is to reduce the probability of their occurrence. LF has been proven to significantly decrease cancer incidence *in vivo* experiments [Iigo *et al*., 2004; Wolf *et al*., 2007]. Therefore, it is feasible to envisage a strategy in which LF can be supplied through diet as a food supplement that can be orally consumed to reduce cancer susceptibility. The oral administration of LF or its derived peptides seems to be an optimal way to prevent or cure gastrointestinal cancers without the occurrence of side effects. In addition, taking into account the strong immunostimulatory effects of LF against several cancers, it could play a key role in developing novel cancer vaccines.

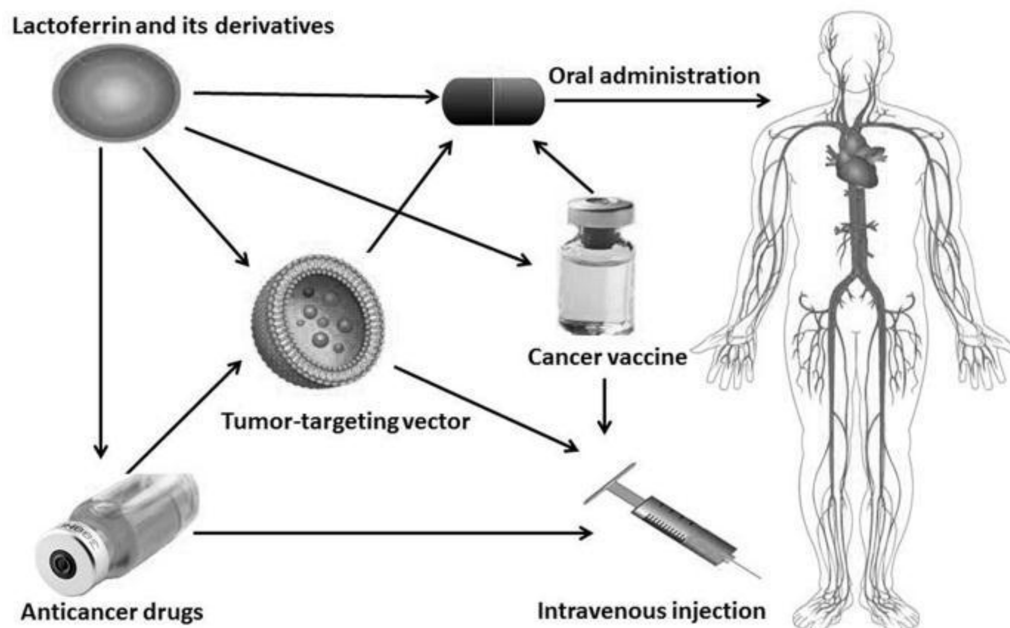

Figure 1.7 – Potential modes of application of LF and its derivatives for cancer prevention/therapy.

1.10 CONCLUSIONS

LF is a multifunctional protein that can be widely found in most mammalian cells. Its expression is regulated by several factors to maintain the homeostasis. Silencing or downregulation of the LF gene in cells usually is related with the occurrence of some diseases, namely with the incidence of carcinomas. Restoration of LF can effectively inhibit the proliferation of cancer cells. The response elements of diseases-related factors in the LF promoter and its adjacent regions could partly explain the vital role of this protein against chronic diseases. Cytotoxic assays also demonstrated the inhibitory effects of LF and its derivatives on the proliferation of multiple cancers, thus suggesting its potential in cancer prevention. However, the mechanisms involved in their cytotoxicity against cancer derived from *in vitro* experiments are not always conclusive. Fortunately, the conclusions drawn from *in vivo* experiments regarding the mechanisms involved are similar, i.e. tumors are eliminated due to the rise of tumor-killer cells that are stimulated by LF. However, the detailed action mechanisms underlying their anticancer effects still need to be further investigated.

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CHAPTER 2

Growth inhibitory potential of bovine lactoferrin and its variants against breast cancer cells

Abstract

Lactoferrin (LF) has long been known as a multifunctional protein and is still an interesting topic of research. Importantly, LF holds a great promise as a potential anticancer agent. Herein, we demonstrated that bovine lactoferrin (bLF) could efficiently inhibit the growth of T-47D, MDA-MB-231, Hs578T and MCF-7 breast cancer cells while showing very low effects against normal breast (MCF-10-2A) cells. Simultaneously, the variants of bLF were also found to inhibit the growth of breast cancer cells. Among them, holo-bLF (iron saturated) exhibited the highest anticancer effects. However, the bLF-derived peptides $LfcinB_{17-41}$ and $LfcinB_{26-36}$ showed low or no, respectively, growth inhibitory activity to the breast cancer cells. In addition, the bLF degradation test indicated that the protein is stable under the cell culture conditions, which excluded the possibility that its effects could be due to its degraded peptides. Furthermore, fluorescent-labeled bLF and $LfcinB_{17-41}$ were found to internalize cancer cells and to remain in the cytoplasm. However, most bLF remained outside the cells, and therefore their effects may result from both the modulation of intracellular and extracellular (through receptor interactions) pathways. The results gathered in this work lay a solid foundation for studying the mechanisms of action underlying the growth inhibitory action of bLF against breast cancer.

2.1 INTRODUCTION

Lactoferrin (LF), an 80 KDa protein with iron-binding ability, was first discovered in mammary secretions, but is synthesized by most mammalian tissues; it is present in high concentrations in human and bovine milk, but in lower concentrations in a variety of secretions derived from epithelial cells and the second granules of neutrophils [Tomita *et al.*, 2002; Rodrigues *et al.*, 2009; Tsuda *et al.,* 2010]. Its expression is estrogen responsive, the LF mRNA and protein expression can be induced several hundred-fold in the reproductive organs by estrogen treatment [Teng *et al.*, 2002a; Teng *et al.*, 2002b]. In normal cycling women, LF gene expression in the endometrium increases during the proliferative phase and diminishes during the luteal phase [Teng *et al.*, 2002b]. Indeed, there are two forms by which LF exists in the cell, secreted LF (LF) and cytosolic LF (ΔLF) that result from the use of alternative promoters: P1 for LF and P2 for ΔLF. Apart from the 5' end, the ΔLF messenger is identical to the LF mRNA and translate from exon 1β leading to a protein devoid of a leader sequence and the first 25 amino acids [Siebert＆Huang, 1997; Liu *et al.*, 2003].

The wide distribution of LF and its special gene structure suggest its crucial role in keeping homeostasis. In fact, previous studies demonstrated that LF is a multifunctional protein and shows a great amount of promising activities including antibacterial, antifungal, anti-inflammatory, antiviral, immunostimulation, among others [Adlerova *et al.,* 2008]. Most importantly, the LF alteration in cells is closely related with the incidence of cancer [Siebert＆Huang, 1997; Hoedt *et al.,* 2010]. For example, LF and ΔLF are significantly downregulated or silenced in an extensive series of cancer cell lines, especially in breast cancer cells [Benaïssa *et al.*, 2005]. However, the recovery of LF or ΔLF could efficiently prevent the proliferation of cancer cells [Breton *et al.*, 2004; Zhou *et al.*, 2008]. Several *in vitro* and *in vivo* assays showed that LF and its peptides present a high potential as anticancer agents [Norrby *et al.*, 2001; Roy *et al.*, 2002; Teraguchi *et al.*, 2004; Sakai *et al.*, 2005; Onishi *et al.*, 2008]. The mechanism of LF action against cancer is not yet fully understood. The *in vitro* experiments showed that LF and its peptides inhibited the growth of cancer cells mainly by inducing cell cycle arrest and/or apoptosis [Damiens *et al.*,1999; Xiao *et al.*, 2004; Mader *et al.*, 2005; Eliassen *et al.*, 2006; Furlong *et al.*, 2006; Wolf *et al.*, 2007; Duarte *et al.*, 2011]. Furthermore, it has been reported that orally administrated LF can survive the passage through the gastrointestinal tract as partially degraded fragments.

The remaining peptides rapidly lead to an increase of anticancer immune factors and decrease the development of chemically-induced cancers [Norrby *et al.*, 2001; Teraguchi *et al.*, 2004]. Although the mechanism underlying the LF and derived peptides cytotoxicity is still under investigation, its anticancer effect is beyond doubt. Indeed, many questions remain unsolved. For example, the possible internalization of LF by the cells and how fast this phenomena can occur. Some potential LF receptors and an indication of the LF internalization process have been reported in several cell lines [Rochard *et al.,* 1992; Fillebeen *et al.*, 1997]. However, more substantial evidences are required to support this conclusion. The elucidation of the LF internalization process will greatly contribute to understand the mechanism behind LF cytotoxicity against cancer.

Based on the close relationship that has been reported between LF and breast cancers, this work aims to comprehensively evaluate the cytotoxicity of bovine lactoferrin (bLF) and its derivatives on normal and breast cancer cells. The results will clarify whether bLF has a selective cytotoxicity to cancer cells and also what is the range of concentrations to be further tested for clinical therapy. Additionally, since a great amount of studies suggest that bLF-derived peptides also have high cytotoxic effects to cancer cells [Roy *et al.*, 2002; Sakai *et al.*, 2005], we will evaluate the possibility that the bLF cytotoxicity against cancer could be due to its degraded peptides. For that purpose, the degradation of the protein subjected to cell culture conditions will be studied. Furthermore, the internalization of bLF and LfcinB will be assessed.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines

T-47D and MDA-MB-231 breast cancer cell lines were kindly provided by IPATIMUP and Medical School from University of Porto (Portugal), respectively. The T-47D cell line was established using a pleural effusion of a 54 year old female patient with an infiltrating ductal carcinoma of the breast and is positive for estrogen receptor (ER+). The MDA-MD-231 epithelial cell line was established from a pleural effusion obtained from a 51 year old female patient with breast cancer and is estrogen receptor-deficiency. Hs578T cells were kindly provided by Life and Health Sciences Research Institute, University of Minho (Portugal). These are fibroblastic cells established from breast tissue of a 74 year

old female patient. MCF-7 and MCF-10-2A were purchased from the American Type Culture Collection. The MCF-7 epithelial cell line was established using a pleural effusion of a 69 year old female patient with adenocarcinoma. MCF-10-2A is epithelial cell from normal breast tissue. The cancer cells (T-47D, MDA-MB-231, Hs578T and MCF-7) were cultured in L-glutamine-containing DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom AG), 100 U/ml penicillin/streptomycin (Sigma-Aldrich, Steinheim, Germany) at 37℃ in a humidified incubator with a 5% CO_2 atmosphere. MCF-10-2A was cultured in DMEM/F12 medium (Biochrom AG) supplemented with 20 ng/ml epidermal growth factor (Gibco), 100 ng/ml cholera toxin (Sigma-Aldrich), 0.01 mg/ml insulin (Sigma-Aldrich) and 500 ng/ml hydrocortisone (Sigma-Aldrich), and 5% horse serum (Biochrom AG) in the same incubator as the cancer cells.

2.2.2 Lactoferrin

Bovine lactoferrin was bought from the company DMV (Veghel, The Netherlands). The purity of the protein is about 80% with 3.5% moisture, and its iron content is around 120 ppm (0.12‰). In other words, the iron saturation of the bLF is 21% iron-content. Purer bLF (≥85%) was purchased from Sigma-Aldrich (Sigma-Aldrich); and peptides, $LfcinB_{26-36}$ (lactoferricin B, fragment 4-14 trifluoroacetate salt) and $LfcinB_{17-41}$, were bought from Sigma-Aldrich and AnaSpec (Catalog #62651) companies, respectively. Except for the fluorescence-labeled experiment in which the higher purity bLF was used, all the other experiments were conducted with bLF from the company DMV. The peptides sequences are described in detail below:

$LfcinB_{26-36}$: Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly (Purity ≥95%).

$LfcinB_{17-41}$: H - Phe - Lys - Cys - Arg - Arg - Trp - Gln - Trp - Arg - Met - Lys - Lys - Leu - Gly - Ala - Pro - Ser - Ile - Thr - Cys - Val - Arg - Arg - Ala - Phe - OH (S - S BOND) (Purity ≥95%).

2.2.3 Preparation of iron-free and iron-saturated lactoferrin

2.2.3.1 Iron-saturated lactoferrin (holo-bLF)

$FeCl_3.6H_2O$ was dissolved in 0.1 M $NaHCO_3$, buffered with 0.1 M sodium citrate at pH 8.0 to obtain a concentration of 20 mM. Meanwhile, bLF was dissolved using the same reagent/buffer to obtain a concentration of 1 mM. Next, both solutions were mixed together by 1:1 and equilibrated for 24 h at room temperature, followed by extensive dialysis against 1 L of 0.1 M sodium bicarbonate to remove free metal ions for 24 h with four bicarbonate changes at 4℃. At last, holo-bLF powder was obtained by freeze-drying [Li *et al.*, 2009].

2.2.3.2 Iron-free lactoferrin (apo-bLF)

Apo-bLF was prepared by dialysis of a 1% bLF solution in water against 30 volumes of 0.1 M citric acid, pH 2.3, containing 500 mg/L di-sodium EDTA, for 30 h at 4 °C. Citrate and EDTA were then removed by dialysis against 30 volumes of ultrapure water at 4℃ for 48 h with four water changes. The solutions containing apo-bLF were freeze-dried [Kanwar *et al.*, 2008].

The iron content in holo-bLF and apo-bLF was determined by spectroscopy. The ratio of A475 nm/A280 nm should be routinely around 0.004 and 0.002 for holo-bLF and apo-bLF, respectively [Hashizume *et al.*, 1987].

2.2.4 MTS assay

Cells at exponential phase were trypsinized and seeded in a 96-well-plate in a volume of 100 μl per well. After 12 h of incubation, the media were replaced with fresh medium containing bLF or its variants at different concentrations. All the stocks of bLF and its variants were prepared by dissolving them in phosphate buffered saline (PBS), respectively. The low range concentrations of bLF were set up at 0.25 μM, 0.5 μM and 1 μM, while the high range concentrations were 1.875 μM, 3.75 μM, 7.5 μM, 15 μM and 30 μM. The cells were incubated with the bLF for 24 h, 48 h or 72 h. At the defined time points, cell viability after treatment with bLF was evaluated using the MTS reagent kit (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) according to the manufacturer guidelines (Promega, Madison, USA). The results of cell viability were recorded at 490 nm using a spectrophotometer. The ability of bLF and its derivatives to inhibit the growth of breast cancer cells were calculated according to the

absorbance values of samples relative to the control (cells incubated with PBS instead of bLF). Each experiment was repeated at least three times.

2.2.5 Lactoferrin degradation assay

For the degradation assay, 15 μM bLF was incubated with cells in a 96-well-plate for 24 h, 48 h or 72 h under cell culture conditions (DMEM media with 10% FBS, 37℃, 80% humidity and 5% CO_2). Afterwards, the media were centrifuged to remove cells, and equal volume of supernatant from each well was analyzed using 10% SDS-PAGE or Tricine-SDS-PAGE to find the possible degradation of bLF under cell culture conditions. Simultaneously, bLF dissolved in PBS, DMEM and DMEM with 10% FBS without incubation in incubator was used as the control. Proteins in the SDS-PAGE and Tricine-SDS-PAGE were visualized by Coomassie blue or silver staining [Schägger, 2006].

2.2.6 Possible uptake of lactoferrin by cells

The disappearance of LF from extracellular medium visualized by SDS-PAGE was used as an indirect indication of LF internalization. For that, a certain number of T-47D cells (10000 cells/well) and MDA-MB-231 (6000 cells/well) or a gradient number of T-47D cells (1200, 6000 and 30000 cells per well) were seeded in a 96-wellplates. 24 h after plating, 3.75 μM bLF was added and incubated with cells, and at a fixed times (24 h, 48 h or 72 h), medium was collected and centrifuged to remove the floating cells. Equal volume of the supernatants was analyzed by 10% SDS-PAGE as described above.

2.2.7 Protein/peptide labeling

The bLF with high purity (≥85%) was dissolved in PBS to get to 1 mg/ml, which is the optimal labeling concentration according to the supplier instructions for the CF^{TM}568 succinimidyl ester labeling kit (Sigma-Aldrich). The bLF solution was mixed with pre-warmed 10× Mix-n-stain reaction buffer at a ratio of 1:10. The mixture was totally transferred to the CF™568 succinimidyl ester-containing vial and was further mixed in a vortex for a few seconds and incubated in the dark for 30 min at room temperature. The final concentration of CF dye-staining bLF solution reached up to 11.25 μM and was then

stored at -20℃.

$LfcinB_{17-41}$ peptide was labeled using the same procedure as the one above described for bLF. Due to the low molecular weight of the peptide [Roy *et al.*, 2002], the final concentration of labeled $LfcinB_{17-41}$ was 213 μM.

2.2.8 Cell internalization of lactoferrin and peptides - confocal analysis

Cells seeded overnight on glass coverslips at 37℃ under 5% CO_2, were rinsed with fresh medium and incubated at 37℃ for 30 min, 1 h or 2 h in pre-warmed medium containing fluorescence–labeled bLF (1.406 μM) or $LfcinB_{17-41}$ (10.65 μM) in a 24-well-plate. After incubation, the coverslips were rinsed 3× with PBS, fixed in 4% paraformaldehyde for 10 min, and washed 3× in PBS. The coverslip was carefully moved to the slides and covered with one drop of Hoechst (0.5 μg/ml) for 15 min in a dark place to counter stain nuclei. Next, the Hoechst was removed and a small drop of mounting media (Glycerol-antifade) was placed on the coverslip. Cellular uptake and distribution were photographed using an inverted confocal laser scanning microscope (Olympus BX61/FLUOVIEW100). Subsequently, the photos were processed by the software of Olympus Fluoview version 3.0.

2.2.9 Statistical analysis

Student's *t* test was used in this study to determine the statistical significance of the experimental results. For *p*-values below 0.05 the differences between experimental groups were considered significant.

2.3 RESULTS

2.3.1 Growth inhibitory effects of lactoferrin against breast cancer cells

In this study, growth inhibition ability of bLF against the breast cancer cells T-47D, MDA-MB-231, Hs578T and MCF-7 was evaluated by the MTS assay. The results showed that bLF could effectively inhibit the growth of all breast cancer cells studied in a

concentration- and time-dependent manner (**Figure 2.1A**). At the highest concentration, the inhibitory rates of bLF at the tested concentrations against T-47D, MDA-MB-231, Hs-578T and MCF-7 exceed by far 50% after 72 h of treatment (**Figure 2.1B**). The four cancer cell lines are representative of different genetic backgrounds (e.g. T-47D and MCF-7 are estrogen-receptor-positive (ER+), while there are no estrogen receptors (ER-) in MDA-MB231 and Hs578T [Mercatante *et al.*, 2002; Zhuang *et al.*, 2008]). However, it was clearly seen that bLF displayed similar growth inhibitory effects on all of them. These results suggest that bLF could be used as a broad-spectrum anticancer agent in cancer therapy, at least for several types of breast cancers. In addition, selectivity is always a desired property of any effective anticancer drug. Therefore, the effect of bLF against MCF-10-2A, a normal breast cell line, was also evaluated in the current work. The results demonstrated that bLF possess a much less pronounced growth inhibitory effect on normal cells as compared to the cancer ones (**Figure 2.1C**). The inhibitory rate was less than 25% after 48 h treatment even for the highest concentration of bLF, while for the breast cancer cells it totally surpassed 40% (**Figure 2.1A＆C**). In summary, the results indicated that bLF broadly inhibited the growth of breast cancer cell lines (T-47D, MDA-MB-231, Hs578T and MCF-7), while it only slightly affected normal cells, and therefore could be considered as a potential selective anticancer drug.

2.3.2 Growth inhibitory effects of lactoferrin variants

Holo-bLF and apo-bLF were prepared by adding or removing Fe^{3+} from bLF using well-known reported methods [Kanwar e*t al.*, 2008; Li *et al.*, 2009]. The holo-bLF was found to be a red powder and the bLF was yellowish. Apo-BLF was a white powder since the colored reagent in bLF, Fe^{3+}, was removed (**Figure 2.2A**). The spectroscopy analysis showed that the ratio A475nm/A280nm was around 0.004 for holo-bLF and 0.002 for apo-bLF. These results confirmed that the iron content of holo-bLF and apo-bLF was around 100% and 0%, respectively [Hashizume *et al.*, 1987].

The bLF cytotoxicity against several cancers has been widely shown in the literature [Roy *et al.*, 2002; Teraguchi *et al.*, 2004; Onishi *et al.*, 2008]. However, there are few reports concerning its derivatives, namely with different iron contents (e.g. holo-bLF, apo-bLF) or its peptides (e.g. $LfcinB_{26-36}$ and $LfcinB_{17-41}$), especially regarding its activity against breast cancer cells. Therefore, the goal of this experiment was to compare the

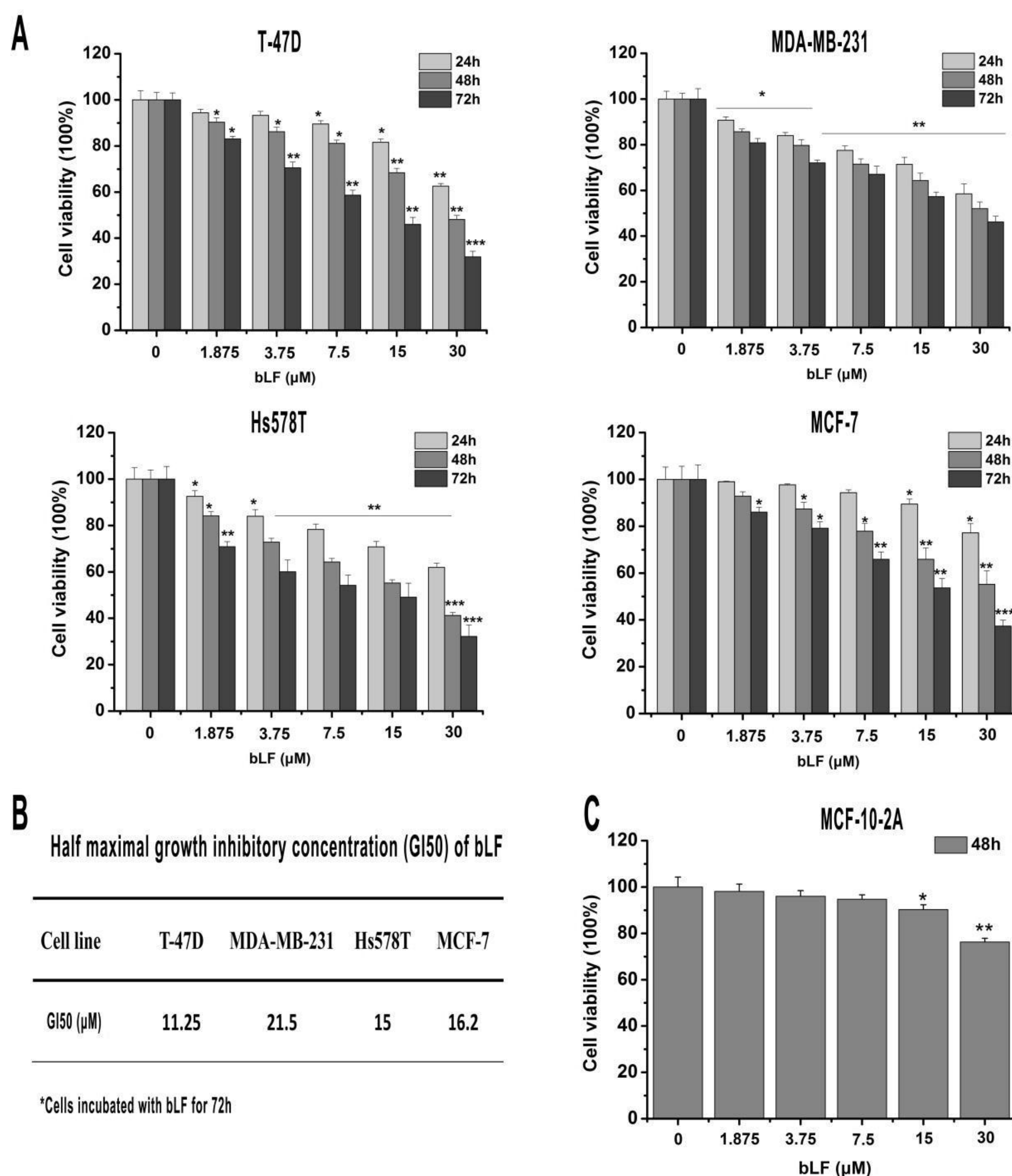

Figure 2.1 – Cytotoxicity of bLF against T-47D, MDA-MB-231, Hs578T, MCF-7 and MCF-10-2A cell lines. Cells were seeded in 96-well-plate and treated with bLF for 24 h, 48 h or 72 h. After exposure to bLF, cell viability was evaluated by the MTS assay. Results correspond to the mean ± SD of three independent experiments. Statistical significance of differences between treated and non-treated groups is given by the *p*-values; **p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001. (**A**) Cell viability of T-47D, MDA-MB-231, Hs578T and MCF-7 breast cancer cells. (**B**) Half maximal growth inhibitory concentration (GI50) of bLF on the viability of T-47D, MDA-MB-231, Hs578T and MCF-7 cells. **(C)** Cell viability of normal breast cancer cells MCF-10-2A after a 48 h exposure to bLF.

anticancer potential between bLF and its variants against several breast cancer cell lines. Cell viability was determined by the MTS assay after treating cells with bLF, holo-bLF, apo-bLF and $LfcinB_{26-36}$ and $LfcinB_{17-41}$ at 15 μM for 48 h. **Figure 2.2B** clearly demonstrated that bLF, holo-bLF, apo-bLF and $LfcinB_{17-41}$ limited the proliferation of breast cancer cells. However, $LfcinB_{26-36}$ did not exert any growth inhibitory effect on these cells. Among the bLF derivatives, holo-bLF showed the highest anticancer potential. Also, the inhibitory rate of bLF was higher than that of apo-bLF on MDA-MB-231 and Hs578T cells. The bLF-derived peptides, in particular the peptide $LfcinB_{17-41}$ was expected to show higher anticancer potential than the protein itself since it is a cationic antimicrobial peptide [Yang *et al.*, 2003] and exhibits a high conformation-dependent uptake efficiency [Duchardt *et al.*, 2009]. However, the peptides showed lower or no inhibitory effects on the growth of cancer cells. Comparing both peptides, it is possible to see that $LfcinB_{26-36}$ sequence is included within the $LfcinB_{17-41}$, but it lacks a disulfide bond [Freiburghaus *et al.*, 2009]. This suggests that the growth inhibitory effect observed for $LfcinB_{17-41}$ could be partly due to the chemical bonding. Indeed, this has been demonstrated to be a key factor for the functioning of the peptides [Yang *et al.*, 2002; Zhang *et al.*, 2002].

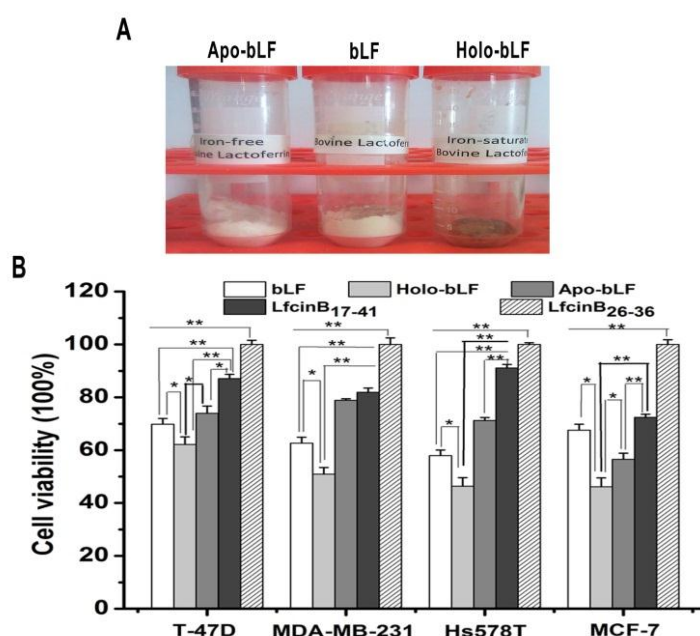

Figure 2.2 – Effect of bLF, holo-bLF, apo-bLF, $LfcinB_{26-36}$ and $LfcinB_{17-41}$ on the viability of T-47D, MDA-MB-231, Hs578T and MCF-7 cells. (**A**) Holo-bLF, bLF and apo-bLF powder appearance. (**B**) Viability of T-47D, MDA-MB-231, Hs578T and MCF-7 cells after treatment with bLF, holo-bLF, apo-bLF, $LfcinB_{26-36}$ and $LfcinB_{17-41}$ at 15 μM for 48 h. Results are expressed as mean ± SD of three independent experiments (*p-value < 0.05, **p-value < 0.01 in paired t-test).

2.3.3 Lactoferrin, apo- and holo- lactoferrin at low range concentrations still inhibited the growth of breast cancer cells

As the above results showed, bLF presented obvious growth inhibitory effects on the studied breast cancer cells at high concentrations. However, some studies had indicated that LF as a food-derived protein could stimulate the growth of cells [Naot *et al.*, 2005; Lee *et al.*, 2009]. Therefore, in this experiment we investigated whether bLF could promote the proliferation of cancer cells at low concentrations. Our results demonstrated that bLF, holo-bLF and apo-bLF inhibited the growth of T-47D (**Figure 2.3A**) and MDA-MB-231(**Figure 2.3B**) cells at the concentrations of 0.25 μM, 0.5 μM and 1 μM. Although the inhibitory effects at the low concentrations were weaker than the ones observed for the high range of concentrations, these results suggest that bLF and its variants (holo-bLF and apo-bLF) do not promote the proliferation of cancer cells, at least of T-47D and MDA-MB-231 cancer cells.

2.3.4 Lactoferrin stability under cell culture conditions

Several studies have demonstrated the cytotoxicity of LF against various cancers [Damiens *et al.*, 1999; Xiao *et al.*, 2004; Furlong *et al.*, 2006]. However, the possibility that LF anticancer effects could result from its major digested peptides have not been

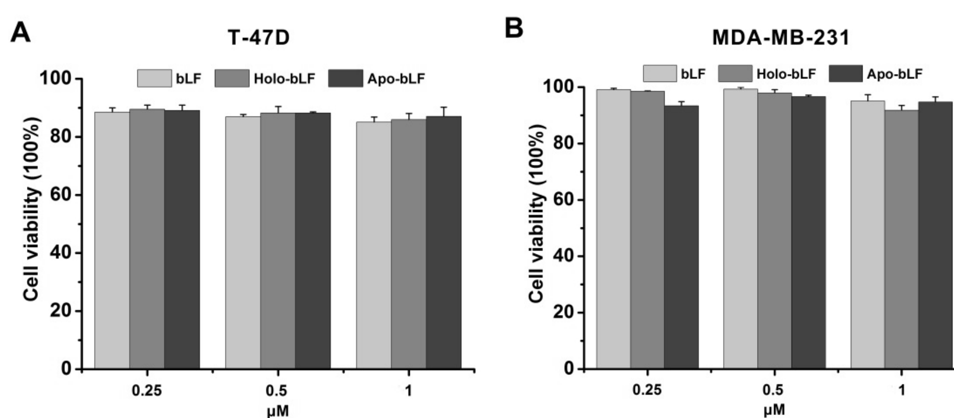

Figure 2.3 – Effect of bLF, holo-bLF and apo-bLF at low concentrations on the cell viability of T-47D and MDA-MB-231 cells. Cells were treated with bLf at 0.25 μM, 0.5 μM and 1 μM for 48 h and cell viability determined by the MTS assay. Results represent the mean ± SD of three independent experiments. No statistical significant differences were found among the different groups.

excluded. In fact, we demonstrated above that $LfcinB_{17-41}$ still possesses growth inhibitory activity. Therefore, the aim of this experiment was to study the possible degradation of bLF when subjected to cell culture medium and cell culture conditions. For that purpose, 15 μM bLF was incubated with T-47D and MDA-MB-231 cells, and culture medium collected at fixed time points to infer about the presence of bLF protein/peptides by SDS-PAGE. To find the potential bLF-derived peptides, 8 μg of bLF (calculated from the amount that was dissolved in the medium at the beginning of the assay) was loaded into each well of the gel. Coomassie blue staining did not show any distinct bands after bLF incubation with cells as compared to control groups (bLF in PBS, DMEM and DMEM with 10% FBS) (**Figure 2.4A**). No pronounced bands corresponding to bLF degradation (≥ 25 KDa) under cell culture conditions could be observed, thus suggesting that the observed activity against cells might be due to the protein itself.

Until now, the published reports on functional bLF-derived peptides regard the $LfcinB_{17-41}$ [Roy *et al.*, 2002; Onishi *et al.*, 2008]. The molecular weight of this peptide is 3.1239 KDa. Tricine SDS-PAGE is a useful method to detect low molecular weight proteins or peptides. To find small bands corresponding to potential digested peptides from bLF, a high concentration of bLF (320 μg) was loaded into each well of a Tricine SDS-PAGE gel after incubation with cells. However, using the Coomassie staining method it was not possible to observe different bands compared with the control groups (**Figure 2.4Ba＆b**). Therefore, the more sensitive silver staining method was used. Nevertheless, still no obvious differences could be observed between the experimental and control groups (**Figures 2.4Bc & d**). Non-specific bLF bands were observed both in the experimental and PBS groups. This was mainly due to the low purity of the bLF used (about 80%). In summary, these results demonstrated that bLF was an extraordinarily stable protein when incubated up to 72 h in culture medium with T-47D and MDA-MB-231 cells at 37℃ in a humidified incubator with a 5% CO_2 atmosphere. Furthermore, the results also suggest that the bLF growth inhibitory effects against cancer cells are due to the presence of the protein itself, rather than from the possible bLF-derived peptides.

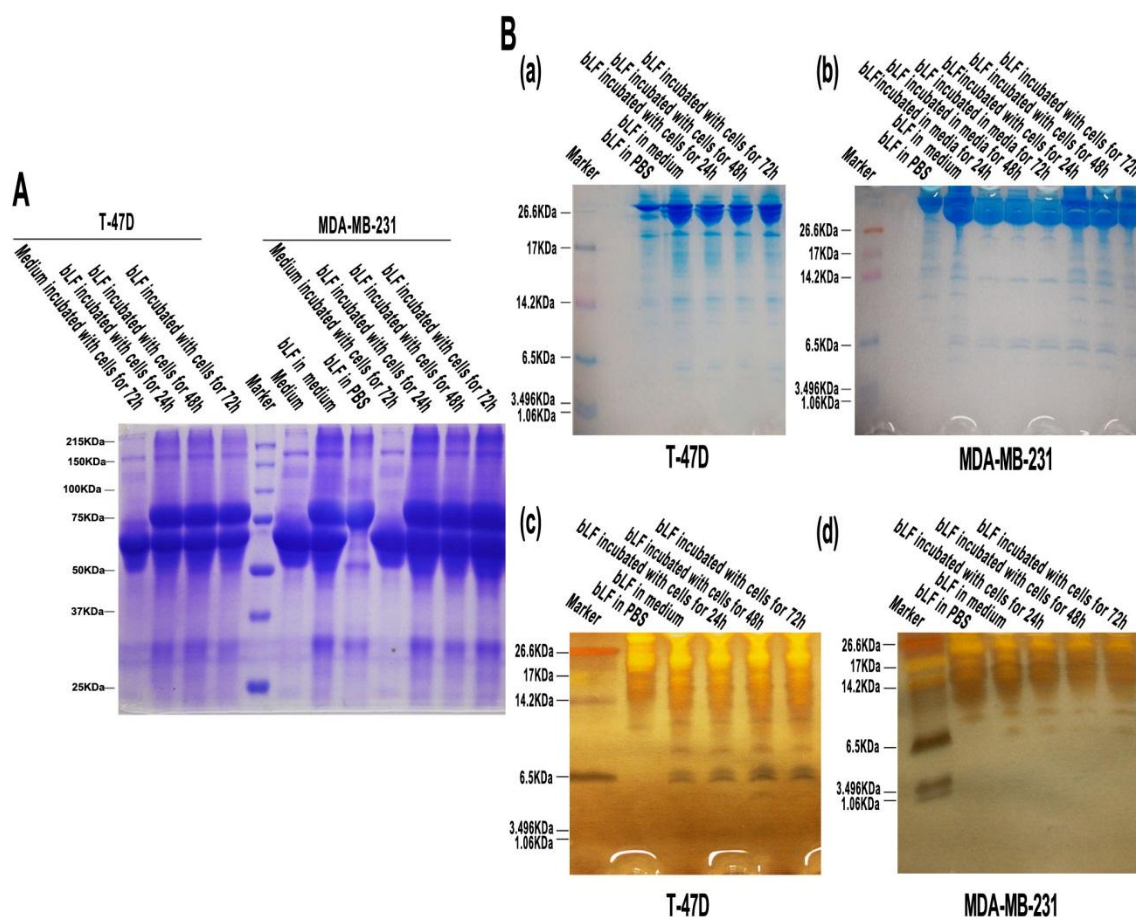

Figure 2.4 – bLF degradation assay. (**A**) bLF was incubated with cells (T-47D and MDA-MB-231) for 24 h, 48 h and 72 h under cell culture conditions. Medium samples containing bLF were loaded in 10% SDS-PAGE and proteins were visualized by Coomassie blue staining. (**B**) bLF degradation assay by Tricine-SDS-PAGE. Cells were treated with bLF for 24 h, 48 h and 72 h. Medium samples containing bLF were loaded in Tricine-SDS-PAGE gels and proteins were visualized by Coomassie blue (**a, b**) and silver staining (**c, d**), respectively. Shown gels are representative of threeindependent experiments.

2.3.5 Lactoferrin was not significantly internalized by T-47D and MDA-MB-231 cells

The previous results had shown the inhibitory effects of bLF on the proliferation of T-47D and MDA-MB-231 cells. According to some reports, bLF mainly exerts its anticancer effects by binding to specific receptors on the cell surface [Rochard *et al.*, 1992; Fillebeen *et al.*, 1999]. Therefore, in this experiment we aimed to evaluate if cells could uptake large amounts of bLF from the culture medium. A 0.2 μg bLF (calculated from the

added bLF concentration at the beginning of the assay) amount from the each sample after incubation under cell culture conditions was loaded onto an SDS-PAGE gel. By loading low amounts of bLF in the gel it is expected to be easier to find noticeable differences of protein content among the groups in which bLF was incubated with the same number of cells for different times, or with different number of cells at the same time point. However, no changes in the amount of protein band corresponding to bLF could be found neither for different incubation times (**Figure 2.5A**) nor for an increased number of cells (**Figure 2.5B**). These results showed that bLF mainly stays outside the cell, but it cannot be excluded the possibility that a small amount of bLF could still be internalized by cells.

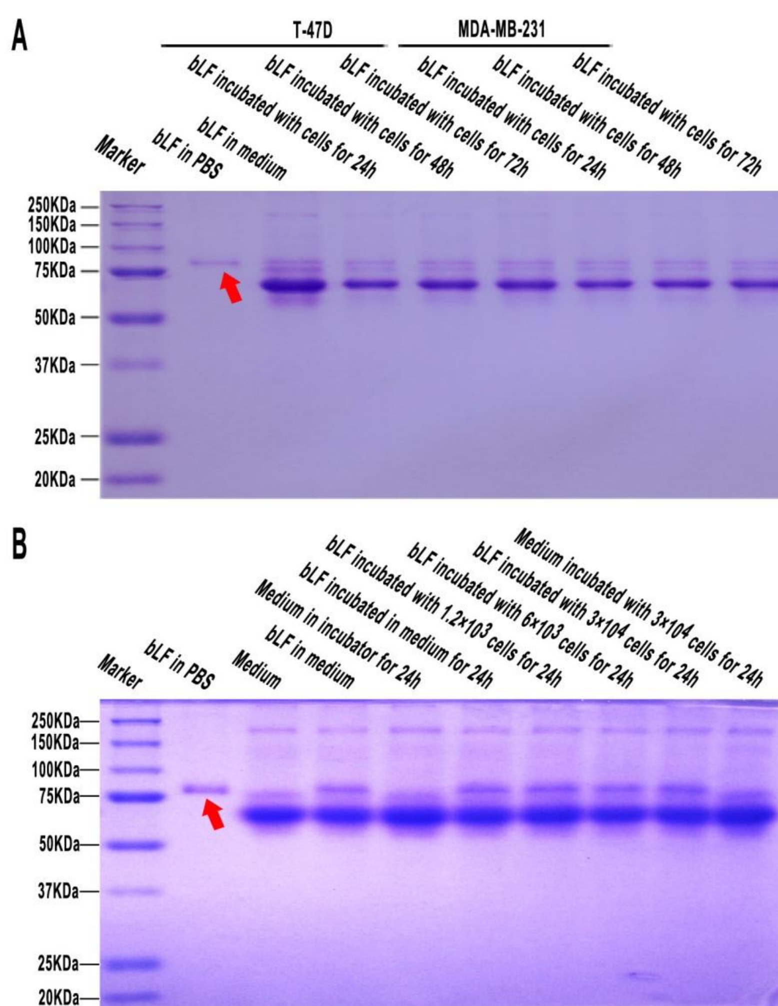

Figure 2.5 – Study of the potential uptake of bLF by T-47D and MDA-MB-231 cells. bLF was incubated with the same number of cells for 24 h, 48 h and 72 h (**A**) or with different number of cells for 24 h (**B**). At a given time point, bLF in the medium was analyzed by SDS-PAGE and stained with Coomassie blue. Shown gels are representative of three independent experiments.

2.3.6 Dye-labeled lactoferrin is rapidly internalized by T-47D and MDA-MB-231 cells

The SDS-PAGE assay showed that bLF (80 KDa) was “blocked” outside the cell membrane. Indeed, it is not expected that such a big protein could be considerably internalized by cells. In order to confirm this assumption we performed an internalization assay using the protein labeled with a fluorescence dye. The results showed that bLF could enter inside T-47D (**Figure 2.6A**) and MDA-MB-231 (**Figure 2.6B**) cells after 1 h incubation and that it accumulates in vesicle-like structures. However, it was found that bLF stays in the cytoplasm and does not enter the nucleus (**Figure 2.6**). These results indicated that an intracellular pathway may be involved in the anticancer effects of bLF.

2.3.7 $LfcinB_{17-41}$ is quickly internalized in T-47D, MDA-MB-231, Hs578T and MCF-7 cells

In the previous section we demonstrated that bLF could enter cells but it did not penetrate the nuclear membrane. $LfcinB_{17-41}$ is a cationic antimicrobial peptide (CAPs) with small molecular weight. These peptides have been shown to have specific membrane-disruption ability [Onishi *et al.*, 2008]. As such, it is expected that it could penetrate cells and also enter the nucleus. Therefore, we evaluated this possibility by incubating the labeled $LfcinB_{17-41}$ with T-47D, MDA-MB-231, Hs578T and MCF-7 cells for 30 min and 2 h. The final concentration of the labeled peptide in the medium was 10.65 μM. The results demonstrated that $LfcinB_{17-41}$ could rapidly internalize cells after 30 min. However, the peptide remained in the cytoplasm even after 2 h incubation and we could not observe the peptide entering the nucleus in any of the four cell lines studied (**Figure2.7**).

2.4 DISCUSSION

In this chapter, we successfully assessed the inhibitory effects of bLF on the growth of four breast cancer cell lines under study (T-47D, MDA-MB-231, Hs578T and MCF-7). bLF could effectively inhibit the growth of all cancer cell lines, while showing a low effect against the normal breast cells. Simultaneously, we demonstrated that bLF variants with

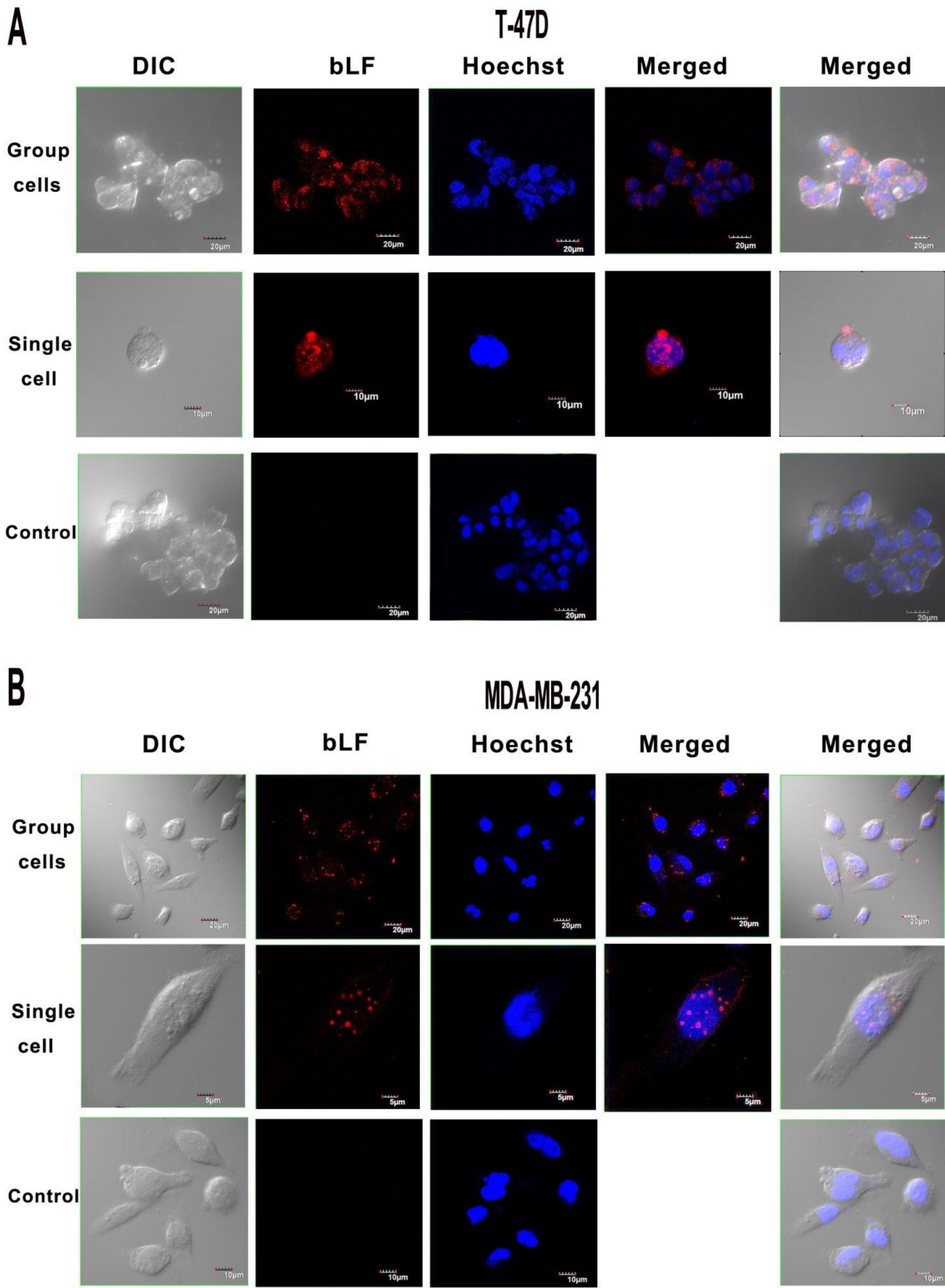

Figure 2.6 – bLF internalization by T-47D and MDA-MB-231 cells. Evaluation of Alexa Fluor 568-labeled bLF penetration into T-47D (**A**) and MDA-MB-231 (**B**) cells after 1 h incubation at 37℃ and 5% CO_2. *Red*, Alexa Fluor-labeled bLF; *blue*, Hoechst-stained nucleus.

different iron contents (holo-bLF and apo-bLF) limited the proliferation of cancer cells. Some studies indicated that holo-bLF presented higher anticancer effects than bLF and apo-bLF on the inhibition of tumors *in vivo* [Kanwar *et al.*, 2008; Sun *et al.*, 2012]. Herein, we also found that holo-bLF exerted the highest growth inhibitory effect on the breast cancer cells as compared with bLF and its other derivatives (apo-bLF, $LfcinB_{26-36}$ and $LfcinB_{17-41}$). Iron-saturation does not lead to special conformational changes in the holo-bLF form except for a "closed" and stable structure when compared with apo-bLF free-iron form [Baker EN＆Baker HM, 2009]. In contrast, the apo-BLF structure is more flexible, and its fully open structure helps it to easily incorporate metal iron in its binding sites [Baker EN＆Baker HM, 2009]. Therefore, the structure differences may not explain their distinct functional activities. Additionally, the iron deprivation from cell culture medium should not account for their cytotoxicity because holo-bLF (100% iron saturation) was more active than bLF (21% iron saturation) and the apo-bLF (0% iron saturation). This indicates that iron content may be responsible for the discrepancy in their anticancer activity against the breast cancer cells. For example, its possible degradation inside the cell may release free iron in quantities that will be toxic for the cells due to promotion of generation of reactive oxygen species. However, it cannot be the sole explanation since even the apo-bLF has significant growth inhibitory activity of the breast cancer cells.

Some studies have reported that bLF can act as a growth stimulant for some cell lines at low concentrations [Naot *et al.*, 2005; Lee *et al.*, 2009]. However, bLF at the concentrations of 0.25 μM (20 μg/ml), 0.5 μM (40 μg/ml) and 1 μM (80 μg/ml) still could inhibit the growth of breast cancer cells. Likewise, the bLF-derived peptides had also been demonstrated to be cytotoxic to cancer cells [Sakai *et al.*, 2005; Eliassen *et al.*, 2006; Furlong *et al.*, 2006]. Therefore, we hypothesized that the bLF cytotoxicity could have been generated from its degraded peptides under cell culture conditions, since these conditions as well as the presence of enzymes potentially released by cells could lead to protein degradation. However, our results from the SDS-PAGE and Tricine-SDS-PAGE experiments could not evidence the presence of any peptides potentially degraded from bLF. This result confirmed that bLF is a very stable protein even under cell culture conditions. As such, the question on how such a big protein exerts its effects against cancer cells, through intracellular or extracellular pathways, remained unclear. Our study showed that at 15 μM most of bLF stayed outside cells, although some fluorescent-labeled bLF could internalize the cells. $LfcinB_{17-41}$ peptide was supposed to be internalized by the

nucleus because of its cationic peptides property [Potocky *et al.*, 2003]. However, similarly to bLF, we only observed the peptide in the cytoplasm and frequently accumulated in vesicle-like structures. Therefore, the internalization results suggest that the bLF anticancer effects may be due to both the modulation of intracellular and extracellular (effects mediated by membrane receptors) targets. Although the protein and its peptide (LfcinB$_{17-41}$) were rapidly internalized by the cells, most of bLF was still in culture medium after 72 h, and therefore, it cannot be excluded that its anticancer effects is potentially mediated in the extracellular milieu by its role in adjusting cell microenvironment or modulating cell receptors-mediated signaling.

Contrarily to most LF-related studies that are only focused on LF or one of its variants

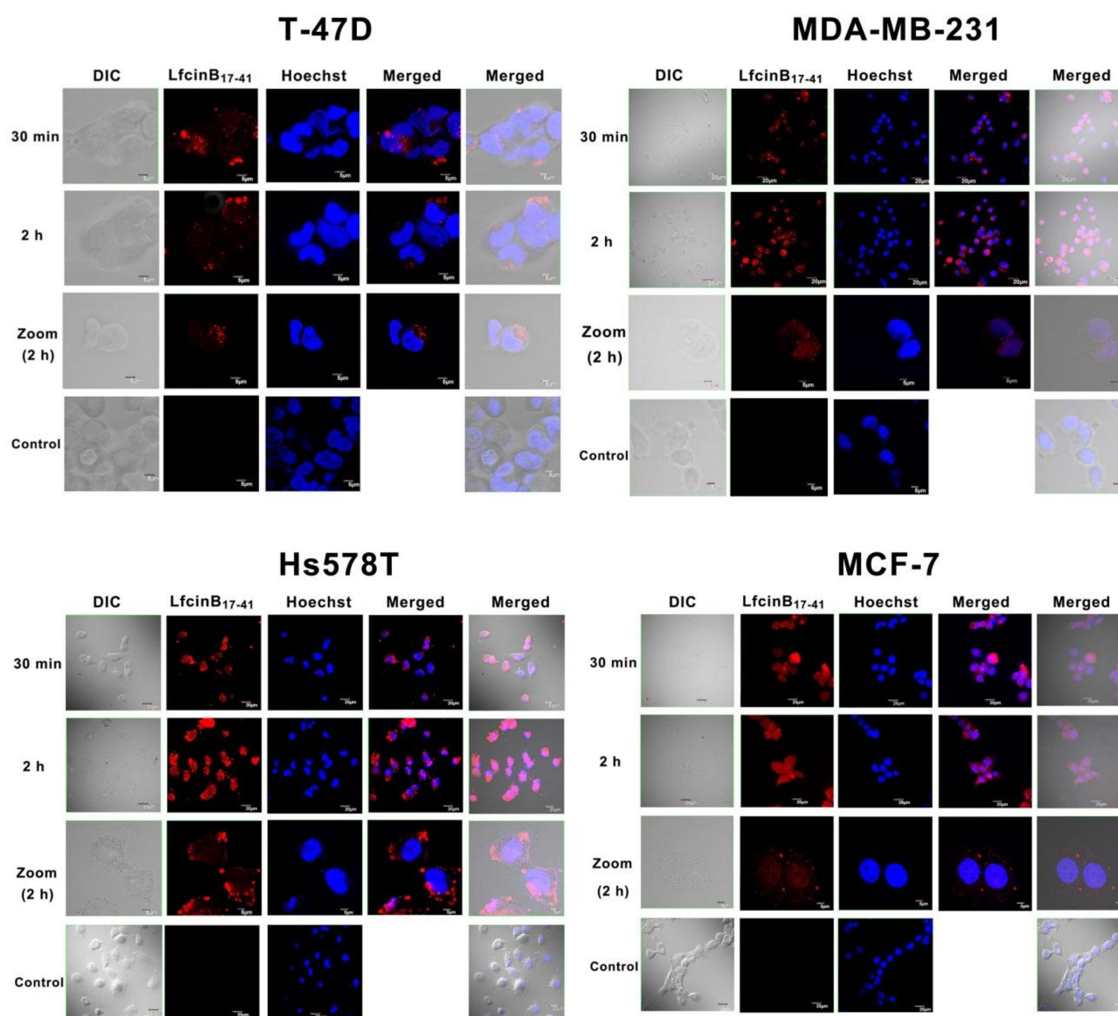

Figure 2.7 – Internalization of LfcinB$_{17-41}$ by breast cancer cells. LfcinB$_{17-41}$ labeled with Alexa Fluor 568 was incubated with T-47D, MDA-MB-231, Hs578T and MCF-7 cells at 37℃ and 5% CO_2 for 30 min and 2 h. *Red*, Alexa Fluor-labeled bLF; *blue*, Hoechst-stained nucleus.

[Damiens *et al.*, 1999; Onishi *et al.*, 2008], the current work was the first report on the systematic evaluation of the growth inhibitory effects of bLF and its variants against multiple breast cancer cell lines. Moreover, a normal human breast cell line, MCF-10-2A, was also used to evaluate the selectivity of bLF, a control procedure that was almost omitted in previous LF studies [Mader *et al.*, 2005; Eliassen *et al.*, 2006; Duarte *et al.*, 2011]. Contrarily to our initial expectations, the bLF-derived peptides did not show higher anticancer effects than the full protein. Additionally, we found that it is unlikely that bLF gets degraded under the conditions used. Therefore, the observed effects are most likely due to the protein itself and not its potential degraded peptides. Considering the specific relationship between LF and breast, we used four human breast cancer cell lines with different genetic backgrounds and characteristics. We found that LF significantly inhibits the growth of all breast cancer cells in a similar magnitude. Hence, this study suggests that bLF could have an efficient broad-spectrum anticancer potential, which can be attained in a near future in cancer therapy by specifically inducing the re-expression of human LF in cancer cells through targeted genetic means.

The current work allowed us to confirm the anticancer effects of bLF and its variants on breast cancer cells through *in vitro* experiments. Their potential therapeutic effects *in vivo* are not known yet. Also, we confirmed that bLF could efficiently and rapidly enter inside cells, although most of it is retained in the extracellular milieu. Therefore, it is crucial to conduct a quantitative analysis of the bLF internalization by cells using other complementary and more accurate techniques. The comparison between the bLF and variants internalization by cells will be another interesting work to be conducted in the future. Although some reports demonstrated that holo- and apo-bLF were internalized by LF receptors via clathrin-mediated endocytosis [Jiang *et al.*, 2011], the full internalization process is still not clear. This knowledge will certainly be very helpful to unravel the mechanisms behind the bLF action.

In conclusion, our data clearly indicated that bLF and some variants could efficiently inhibit the proliferation of the four breast cancer cell lines. The comparison of bLF and its variants cytotoxicity was also evaluated in this study and we observed that the full proteins was more active than some of its peptides. Moreover, we showed that bLF is stable under cell culture conditions and is able to internalize cancer cells. The results gathered in this study build a solid foundation for the future work on the mechanisms involved in the anticancer effects of LF.

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Chapter 3

Bovine lactoferrin induces cell cycle arrest and inhibits mTOR signaling in breast cancer cells

Abstract

The anticancer effects of lactoferrin (LF) on multiple cancers have been reported by several researchers. We previously found that bovine lactoferrin (bLF) exhibited growth inhibitory activity against breast cancer cells without affecting their normal counterparts. In the present study we aimed to uncover the mechanisms of action behind the anticancer effects of bLF. The bLF growth inhibitory effect was associated with cell cycle arrest and not with induction of cell death by apoptosis in all breast cancer cells used (T-47D, MDA-MB-231, Hs578T and MCF-7), even at the highest bLF concentration (30 μM) studied. Depending on the cell line, the treatment with bLF was found to arrest cells at different phases of the cycle. Yet, a marked inhibition of the DNA synthesis (S phase) phase was found to be associated with a decrease of the CDC25c levels. Western blot assays showed the ability of bLF to increase remarkably phospho-AMPKα levels, as well as to downregulate both phospho-mTOR and total mTOR levels, suggesting a novel effect of bLF in breast cancer cells that may be associated with its inhibitory effects on cell growth. This study disclosed an important step in the understanding of the mechanisms by which bLF exerts its anticancer effects that will be of great benefit towards its potential application in cancer therapy.

3.1 INTRODUCTION

Natural products have been the main source of anticancer drugs and will continue to be an attractive source of new therapeutic candidate compounds in view of the tremendous chemical diversity that can be found in millions of species of plants, animals, marine organisms and microorganisms [Milner, 2004; Patel *et al*., 2010]. Indeed, several food components have the potential to be effective chemopreventive agents for different human diseases [Young *et al*., 2011]. The successful characterization of these active agents will generate immense socioeconomic benefits. However, a large fraction of the active molecules present in our diet are still unknown, and the study of their mechanisms of action is rather complex [Milner, 2004]. The milk-derived lactoferrin (LF) is an iron binding protein predominantly found in mammalian secretions [Adlerova *et al*., 2008]. This protein is well known for its multiple biological functions such as antibacterial, antiviral, antitumor, anti-inflammatory and immune regulatory properties [Ushida *et al*., 1999; Kuhara *et al*., 2000; Xiao *et al*., 2004; Chandra *et al*., 2006]. Importantly, LF and its variants have been proposed for cancer therapy for many years owing to their high cytotoxicity and tumor-targeting properties [Tomita *et al*., 2002; Rodrigues *et al*., 2009; Tsuda *et al*., 2010]. Despite the existence of a great deal of reports supporting this conclusion, little is known about the mechanisms against established tumor cell lines and their ability to augment cancer chemotherapy and immunomodulatory mechanisms, by which their effects are thought to be mediated [Roy *et al*., 2002; Sakai *et al*., 2005; Onishi *et al*., 2008].

Actually, the LF receptors are widely dispersed on the cell membrane [Fillebeen *et al*., 1999; Jiang *et al*., 2011], and this protein was shown to penetrate through it [Rochard *et al*., 1992; Bi *et al*., 1996]. For example, in Jurkat human lymphoblastic T-cell, LF was found to enter cells via the receptor mediated endocytosis [Bi *et al*., 1996]. Moreover, holo- and apo-bLF were shown to internalize cells by an LF receptor via clathrin-mediated endocytosis in Caco-2 cells [Fillebeen *et al*., 1999; Jiang *et al*., 2011]. However, an increased permeability may lead to subtle changes in the membrane's barrier function, ultimately promoting cell death. Other authors refer that LF can increase cytolysis at low concentrations while at high concentrations it modulates cytolysis depending on the target cell phenotype [Damiens *et al*., 1998]. This suggests that the LF-receptors aid the internalization of LF but this activity could damage the cell membrane. In addition, cell cycle arrest is thought to be the main mechanism underlying the LF cytotoxicity against

cancers. It generally concerns the upregulation or downregulation of some cell cycle associated proteins, such as ERK, AKT, p21, p27, Cdk2, cyclin E, Cdk4, and cyclin D1, among others [Lin *et al*.,2005; Zhou *et al*., 2008; Xu *et al*., 2010]. For instance, LF was shown to induce the cell cycle arrest of head and neck cancers by decreasing the expression of cyclin E and increasing the expression of the Cdk inhibitor p27. According to the literature, LF usually induces the cell cycle arrest at the G1 phase [Xiao *et al*., 2004]. Simultaneously, ΔLF stable expression in cells could also prevent cell cycle progression and inhibit cell proliferation [Breton *et al*., 2004].

In contrast to the reported cell cycle arrest studies, most apoptosis experiments have been conducted with LF-derived peptides, such as LfcinB [Roy *et al*., 2002; Sakai *et al*., 2005; Onishi *et al*., 2008]. Indeed, there are few reports concerning the LF-induced apoptosis [Mader *et al*., 2005; Furlong *et al*., 2006; Lee *et al*., 2009]. These studies suggest that LF-induced apoptosis in cancer cells is mainly due to a decrease of the expression of the anti-apoptosis protein, Bcl-2 [Lee *et al*., 2009; Duarte e*t al*., 2011]. Furthermore, PARP cleavage and the increase of phospho-p38 and phospho-JNK have also been implicated in the LF-induced apoptosis [Schmid *et al*., 1999]. In fact, it is well-known that JNK and p38 play a key role in the apoptosis induced by various cellular insults. Moreover, several Bcl-2 family proteins, both pro- and anti-apoptotic, are strictly controlled by JNK phosphorylation [Cai *et al*., 2006]. Although these studies demonstrated that LF could induce apoptosis in some cancer cell lines, it is still unknown how signals transfer from LF to pro-apoptotic molecules. LF is mainly produced and secreted by the breast. Interestingly, its downregulation has been associated with an increase of breast cancer incidence [Furmanski *et al*., 1989]. Indeed, the downregulation or silencing of LF expression has been found in an extensive series of cancer cells [Siebert＆Huang, 1997; Hoedt *et al*., 2010]. It has also been shown that the recovery or overexpression of LF can significantly retard cancer cell growth, and also weaken the colony formation [Breton *et al*., 2004; Zhou *et al*., 2008], thus indicating that LF could play an important role in cancer therapy, especially for breast cancers. In a previous work (chapter 2), we also found that LF and its variants possess interesting anticancer potential against a wide array of breast cancer cell lines by inhibiting cell growth, without significantly affecting normal breast cells.

The aim of this study was to evaluate the bLF anticancer mechanisms against several breast cancer cells. Cell lines used included three p53 mutant cell lines (MDA-MB-231, Hs578T and T-47D) and one p53-positive cell line (MCF-7); T-47D and MCF-7 are

estrogen-receptor-positive (ER+), while MDA-MB-231 and Hs578T have negative estrogen receptors (ER-) [Mercatante *et al*., 2002; Zhuang *et al*., 2008].

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and lactoferrin

T-47D and MDA-MB-231 breast cancer cell lines were kindly provided by IPATIMUP and Medical School from University of Porto (Portugal), respectively. Hs578T cells were a kind gift from Life and Health Sciences Research Institute, University of Minho (Portugal). MCF-7 was obtained from the American Type Culture Collection. The cells were cultured in L-glutamine-containing DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG), 100 U/ml penicillin/streptomycin (Sigma-Aldrich, Steinheim, Germany) at 37℃ in a humidified incubator with a 5% CO_2 atmosphere. Bovine lactoferrin was bought from the company of DMV (Veghel, The Netherlands). The purity of the protein is about 80% with 3.5% moisture, and its iron content is around 120 ppm (0.12‰). In other words, the iron saturation of the bLF is about 21% iron-content.

3.2.2 Cell cycle analysis

Cells (T-47D, MDA-MB-231, Hs578T and MCF-7) were seeded on a 100 mm petri dish and cultured for 24 h. Fresh medium with different concentrations of bLF (7.5 μM, 15 μM and 30 μM) was added to cells and incubated for 48 h. Afterwards, cells were fixed with 70% ice-cold ethanol for 15 min. Next, cells were washed twice with phosphate buffered saline (PBS) and then resuspended in 500 μL PBS. A 50 μL volume of RNAse A (200 μg/ml) solution was added to each sample and these were further incubated at 37℃ for 15 min. Finally, 60 μL of propidium iodide (PI) (0.5 mg/ml) was added to the samples and these were incubated for 30 min at room temperature prior to flow cytometry analysis (Coulter Epics XL flow cytometer, Beckman Coulter Inc., Miami, FL, USA). For cell cycle analysis, at least 30,000 single cells per sample were used, and fitting to the cell cycle phases was done using the mathematical Watson Pragmatic model with the FlowJo Analysis Software (Tree Star, Inc., Ashland, OR, USA).

3.2.3 Assessment of apoptosis by nuclear condensation assay

Cells (T-47D, MDA-MB-231, Hs578T and MCF-7) at exponential phase were collected and seeded in 6-well-plates. Cells were grown for 24 h before bLF treatment at several concentrations (7.5 μM, 15 μM and 30 μM). After 72 h, both floating and attached cells were collected and fixed with 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature. Next, cells were washed and attached into a polylysine-treated slide using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA), and nuclei were stained with Hoechst (5 μg/ml) for 10 min in the dark. Fluorescent-dyed cells were observed and images were captured using an Olympus IX71 microscope (Hamburg, Germany). More than 400 cells per sample were counted using different fields and the apoptosis rate was calculated according to the following equation (**Equation 3.1**):

$$\textbf{\% Apoptosis} = \frac{\text{Number of cells with nuclear condensation}}{\text{Total number of cells (stained blue)}}$$

Equation 3.1

3.2.4 Assessment of apoptosis by Annexin V / PI assay

T-47D and MDA-MB-231 cells were treated with 30 μM bLF for 24 h. Subsequently, cells were washed with PBS and resuspended in 1X Binding Buffer (0.01 M Hepes - pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. A 100 μl volume of each sample was stained with 5 μl FITC Annexin V (BD Pharmingen, BD, Franklin Lakes, NJ, USA) and 10 μl PI (50 μg/ml). Afterwards, the samples were gently mixed in a vortex and further incubated at room temperature for 15 min in the dark. Finally, 400 μl of 1X Binding Buffer was added to each sample and these were analyzed by flow cytometry.

3.2.5 Histone extraction

Total histone extraction was used to measure the levels of phospho-histone H2A.X as a marker of DNA damage. For that, T-47D cells were incubated with 15 µM and 30 µM bLF for 1 h, 6 h and 12 h. Incubation of cells with 50 µM etoposide was used as positive control for DNA damage. Cells were collected and washed with ice-cold PBS. Next, cells were resuspended in Triton Extraction Buffer (TEB: PBS with 0.5% Triton X-100 (v/v), 2 mM phenylmethylsulfonyl fluoride, 0.02% (w/v) NaN_3) at a cell density of 1×10^7 cells/ml. The cell suspension was left on ice for 10 min with gentle stirring, nuclei was centrifuged at 6,500 xg for 10 min at 4 ℃ and washed once with half volume of TEB. Subsequently, the nuclei pellet was resuspended in 0.2 N HCl at a density of 4×10^7 nuclei/ml to perform an acid extraction of histones overnight at 4℃. After centrifugation, the supernatant containing histone protein was used for further analysis or otherwise stored at -80℃.

3.2.6 Total protein extraction

T-47D, MDA-MB-231, Hs578T and MCF-7 cells were grown in 6-well-plates and further treated with bLF at several concentrations (7.5 µM, 15 µM and 30 µM) for 16 h and 48 h. Furthermore, to detect the effects of bLF on the phosphorylation of AKT and ERK in a short term, T-47D and MCF-7 cells were treated for 30 min, 1 h, 2 h, 3 h and 6 h without refreshing the culture medium. At a definite time, cells were washed twice with PBS and lysed in RIPA (Radio-Immunoprecipitation Assay) buffer (50 mM Tris-HCl pH=7.5, 1% (v/v) NP-40, 150 mM NaCl, 2 mM EDTA, and added fresh 20 mM Na_3VO_4, 20 mM NaF, 1 mM PMSF and protease inhibitory cocktail (Roche)) on ice for 15 min. Subsequently, the cell homogenate was centrifuged at 10,000 xg at 4 ℃ to pellet the cell debris. The supernatant was collected and stored at -80℃ for further analysis of different protein levels by western blot.

3.2.7 Quantification of protein and Western blot

The concentration of the protein was determined by the detergent compatible DC Protein

assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for total protein extracts, while the histone protein extracts were quantified using the Bradford reagent from Sigma-Aldrich. Bovine serum albumin (BSA) was used as protein standard. For the SDS-PAGE, 20 μg or 30 μg of protein from each sample was mixed with Laemmli's buffer [Nirmalan *et al.*, 2009] and these were held in boiling water for 5 min for protein denaturation. Next, after separating proteins in the SDS-PAGE gel, they were electroblotted to a hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Bucks, UK), and the membranes were blocked in TPBS (PBS with 0.05% (v/v) Tween-20) containing 5% (w/v) nonfat dry milk. After washing in TPBS, membranes were incubated overnight at 4 ℃ with a specific primary antibody of interest, followed by washing and incubation with a secondary antibody for 1 h at room temperature. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as loading control. The antibodies used were the following: anti-phospho-AMPKα (thr172), anti-AMPKα, anti-CDC25c, anti-phospho-JNK and anti-JNK acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-mTOR, anti-mTOR, anti-phospho-AKT, anti-AKT, anti-phospho-ERK, anti-ERK, anti-phospho-p38 and anti-p38 bought from Cell Signaling (Danvers, MA, USA); anti-β-actin was from Sigma-Aldrich; antibody against phospho-histone H2A.X (Ser139, clone JBW301) was from Millipore.

3.3 RESULTS

3.3.1 Lactoferrin failed to induce apoptosis in breast cancer cells

Apoptosis-inducing agents are generally considered the most favorable anticancer drugs since apoptosis is a process of programmed cell death and does not lead to an undesirable inflammatory response. Some previous studies showed that bLF could induce apoptosis in cancer cells [Mader *et al.*, 2005; Furlong *et al.*, 2006; Xu *et al.*, 2010]. The cell viability results (Chapter 2) indicated that bLF could efficiently inhibit the growth of T-47D, MDA-MB-231, Hs578T and MCF-7 cells. Herein, we aimed to evaluate whether inhibition of cell growth of those cell lines by bLF could be associated with the induction of cell apoptosis. By the nuclear condensation assay, no apoptotic cells could be found for all the

studied cell lines after a 72 h period of treatment with bLF at 7.5 μM, 15 μM and 30 μM (**Figure 3.1A** (this figure only shows results for the highest bLF concentration tested)). Moreover, we also performed the Annexin V/PI assay since it is a useful tool to assess early apoptosis events. These experiments were done with T-47D and MDA-MB-231 cells to confirm the nuclear condensation results. Likewise, no apoptotic cells (Annexin V positive/PI negative cells) could be observed for both cell lines subjected to a 24 h treatment with 30 μM bLF (**Figure 3.1B**). The results suggest that the previous observed bLF effects on the cell growth of the four breast cancer cells is not due to cell death by apoptosis but can be due to inhibition of cell proliferation. In fact, by microscopic observations (*data not shown*), cells treated with the highest concentration of bLF (30 μM) showed evident inhibition of cell proliferation (lower cell number) without presenting increased number of floating cells (dead cells).

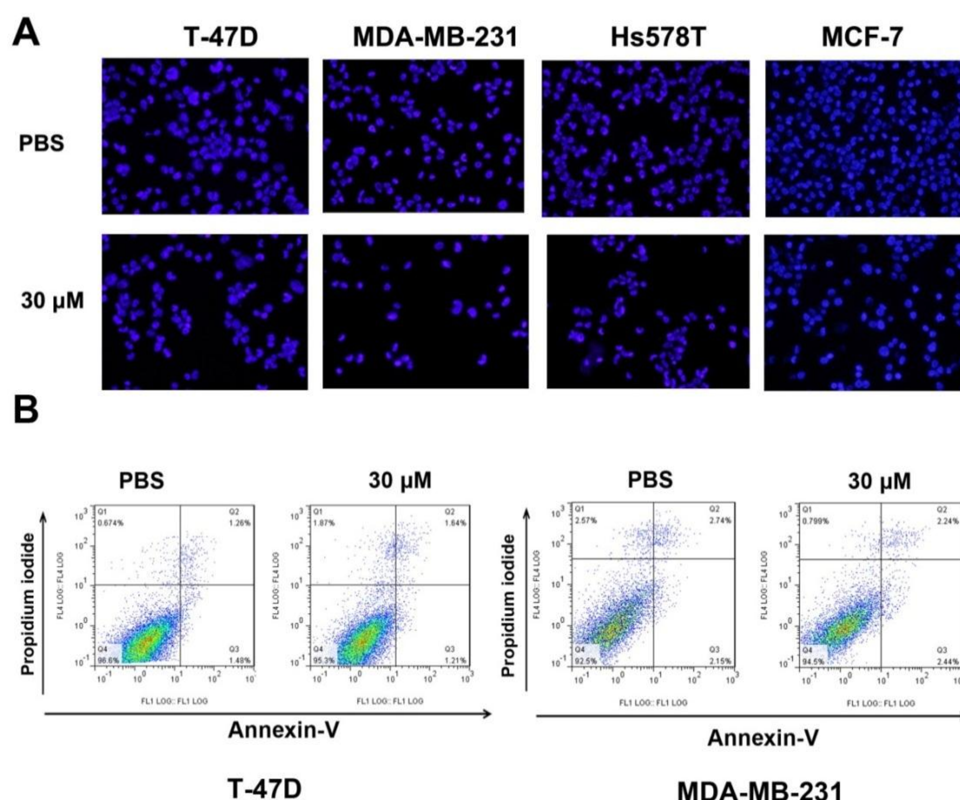

Figure 3.1 – Effect of bLF in cell death by apoptosis. (**A**) Apoptotic cells in T-47D, MDA-MB-231, Hs578T and MCF-7 cell lines were evaluated using the nuclear condensation assay after 72 h incubation with 30 μM bLF. (**B**) Apoptotic cells in T-47D and MDA-MB-231 cell lines were evaluated by the annexin V/ PI assay after 24 h incubation with 30 μM bLF. Results correspond to a representative experiment from three independent experiments with similar results.

3.3.2 Apoptosis-associated JNK and p38 pathways were not affected by bovine lactoferrin

It is well known that the persistent activation of JNK and p38 stress-induced kinases is a key factor for cells to undergo apoptosis [Cai B *et al*, 2006]. Therefore, their involvement on bLF effects in breast cancer cells were evaluated by western blot, measuring their phosphorylation levels as an indication of signal activation. As can be seen in **Figure 3.2**, no differences could be found in the expression of phospho-JNK and phospho-p38 between bLF-treated groups and control group in the cancer cells. Furthermore, no differences could be observed among the different bLF concentrations studied. These results indicate that both JNK and p38 signaling pathways are not involved in the anticancer effects of bLF, and further confirm that apoptosis is not involved in growth inhibition induced by bLF.

3.3.3 Lactoferrin induced cell cycle arrest in breast cancer cells

Since apoptosis was not observed, we then tested whether cell growth inhibition induced by bLF (Chapter 2) could be due to the retardation of cell division. For that purpose, the

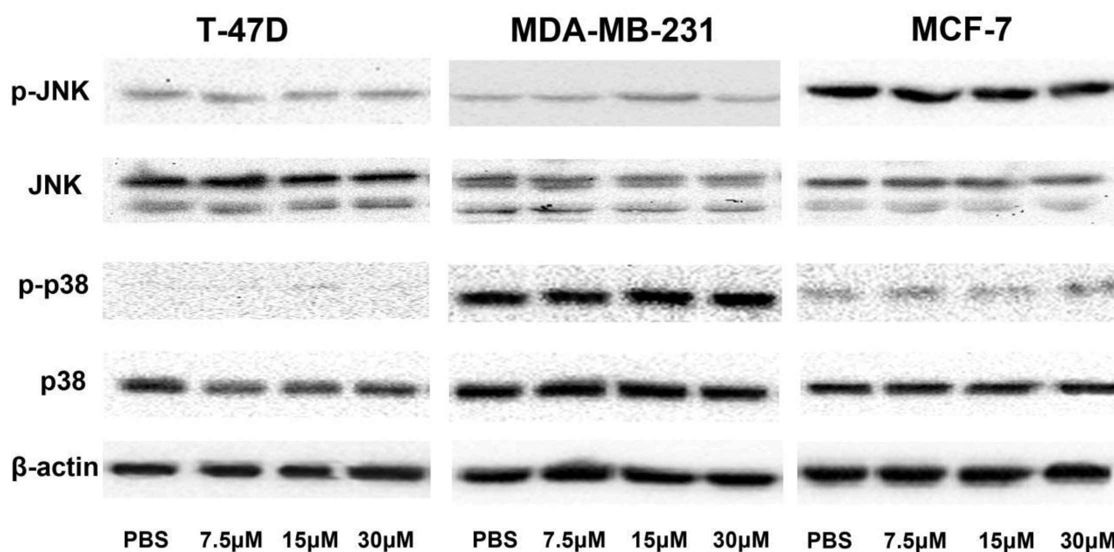

Figure 3.2 – Expression of JNK and p38 pathways in breast cancer cells exposed to bLF. T-47D, MDA-MB-231 and MCF-7 cells were incubated with different bLF concentrations for 48 h. The protein level of JNK and p38 in these cells were evaluated by western blot. The blots are representative of three independent experiments with similar results. *β*-actin was used as loading control.

effect of bLF on cell cycle progression was investigated by flow cytometry. After exposure to bLF for 48 h, it could be clearly observed a very strong inhibitory effect on the cell cycle progression for the MDA-MB-231 and MCF-7 breast cancer cells. Curiously, it was found that the bLF-induced cell cycle arrest was cell-type dependent (**Figure 3.3**). For the MDA-MB-231 cell line, bLF induced cell cycle arrest at the G2 phase. The proportion of cells at G2 phase increased with increasing bLF concentrations. In MCF-7 cells, a different behavior of cell cycle as a result to the bLF treatment was observed. At lower bLF concentrations a strong G1 arrest was observed, but a higher concentration (30 μM) was also accompanied by a G2 arrest. The cell cycle inhibition in T-47D and Hs578T cell lines was much less evident. Using 30 μM of bLF, a G2 arrest was found in T-47D cells, whereas a G1 arrest occurs for Hs578T cells. Interestingly, with the exception of T-47D cells, in all the other 3 cell lines bLF induced a marked decrease in the number of cells at the S phase, indicating that DNA synthesis was being inhibited in the presence of bLF.

These results show that bLF inhibits cell growth of breast cancer cells by interrupting the cell cycle progression, but induces cell cycle arrest at distinct checkpoints depending on the cells used. For MCF-7 cell line, it was found that different bLF concentrations lead to distinct effects on the cell cycle progression.

3.3.4 DNA damage assay

DNA damage is one of the main events that lead to cell cycle arrest [Dasika *et al.*, 1999]. Since it was shown by us (chapter 2) and others [Bi e*t al.*, 1996; Jiang *et al.*, 2011] that cells can internalize bLF, it is plausible to think that bLF may arrest the cell cycle by inducing DNA damage. A very early step in the cellular response to DNA double-strand breaks (DSBs) is the phosphorylation of a histone H2A variant, H2A.X, at the sites of DNA damage [Burma *et al.*, 2001]. Therefore, the phosphorylation levels of histone H2A.X can be used as a marker of DNA damage. As shown in **Figure 3.4**, exposure of T-47D cells to bLF concentrations of 15 μM and 30 μM did not induce phosphorylaton of histone H2A.X, contrarily to the positive control etoposide. Therefore, these results demonstrate that the induction of cell cycle arrest by bLF was not due to DNA double-strand breaks.

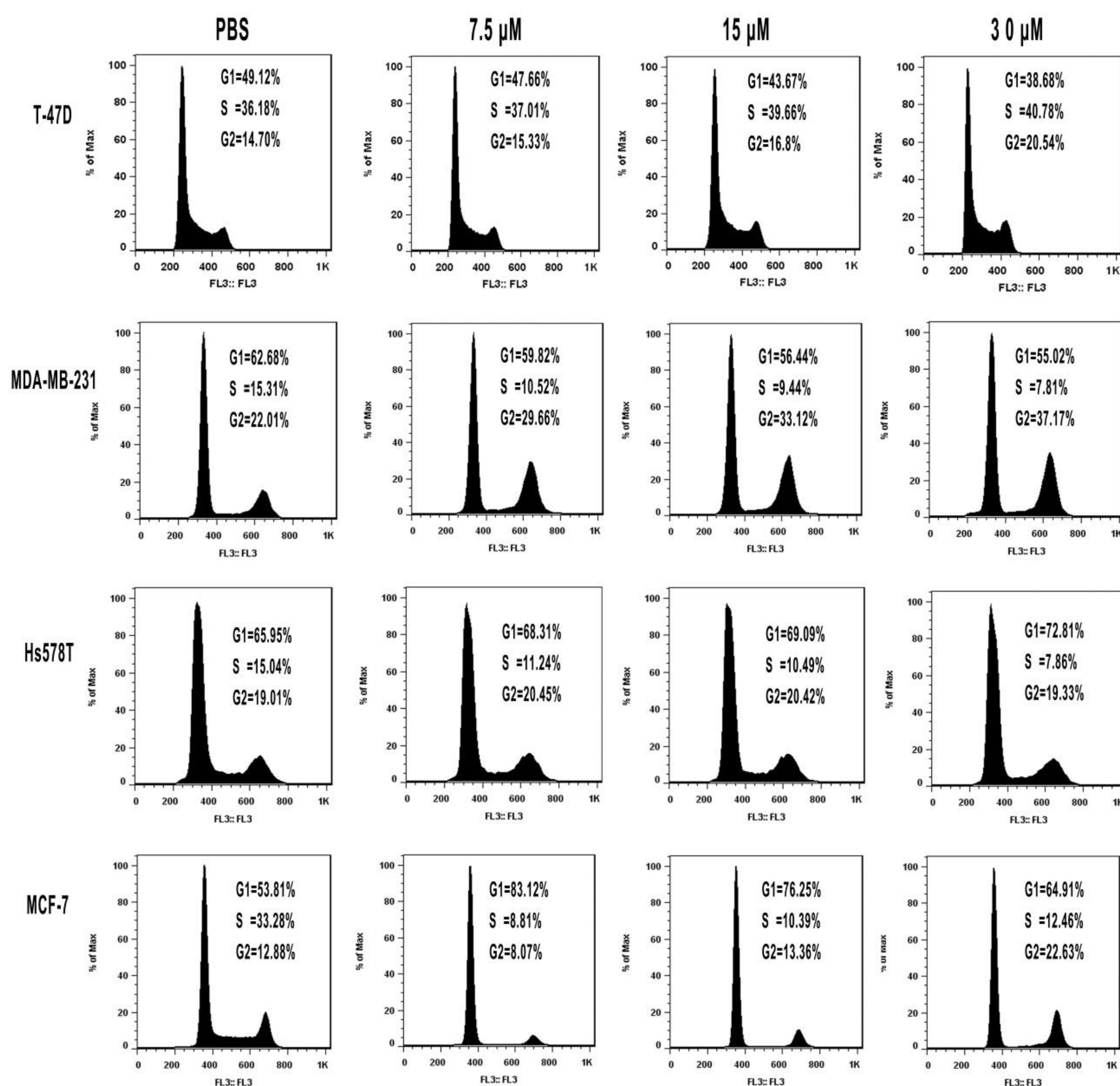

Figure 3.3 – Cell cycle progression in breast cancer cells exposed to different bLF concentrations for 48 h. T-47D, MDA-MB-231, Hs578T and MCF-7 cells were incubated with different concentrations of bLF for 48 h, and the cell cycle of cells were analyzed by flow cytometry. These are representative results from three independent experiments with similar results.

3.3.5 Lactoferrin did not inhibit ERK and AKT pathways

ERK and AKT pathways play an important role in cell proliferation and cell cycle progression. ERK regulates the phase transitions G1/S and G2/M in cell division, while AKT is a key regulator in the G1/S transition [Worster *et al*., 2012]. In previous reports, downregulation of phospho-ERK (p-ERK) and/or phospho-AKT (p-AKT) was associated with bLF-induced cell cycle arrest [Damiens *et al*., 1999; Xu *et al*., 2010]. In this study, we

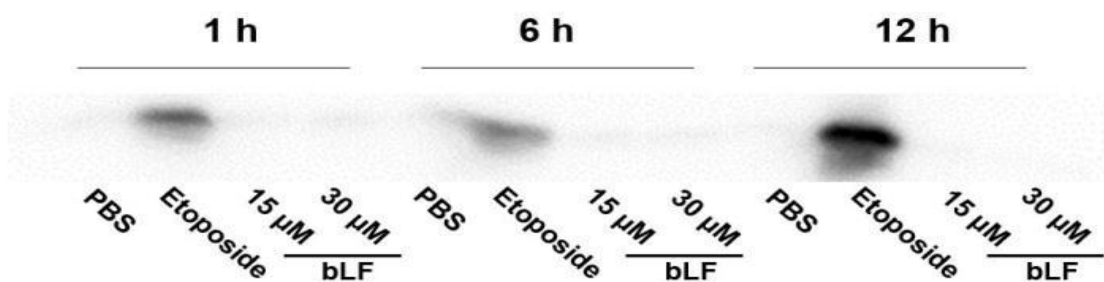

Figure 3.4 – Effect of bLF in the ability to induce DNA double-strand breaks, as measured by the phospho-histone H2A.X levels. This was evaluated in T-47D cells by western blot after incubation with bLF at 15 µM and 30 µM for different time points. Etoposide at 50 µM was used as positive control. The image is the representative bots of three independent experiments.

also measured the activity of the MAPK/ERK and PI3K/AKT signaling pathways by measuring through western blot the phosphorylation levels of ERK and AKT, respectively. However, contrarily to previous studies in other cell lines, we did not found any significant difference in the levels of p-ERK and p-AKT after 16 h of bLF treatment in both T-47D and MDA-MB-231 cells (**Figure 3.5**). Phospho-AKT and phospho-ERK were not present in T-47D and MDA-MB-231 cells, respectively. These results indicate that the molecular mechanisms underlying the bLF-induced cell cycle arrest in the breast cancer cells does not involve the ERK and AKT pathways.

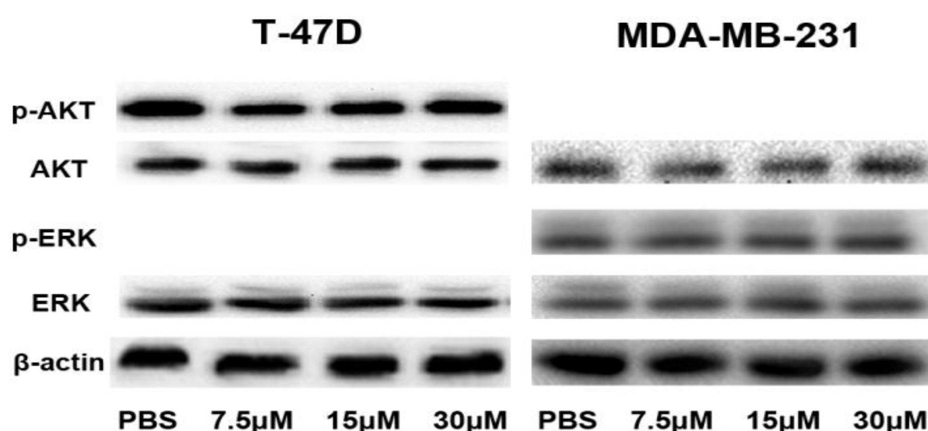

Figure 3.5 – Expression of ERK and AKT pathways in breast cancer cells exposed to different bLF concentrations for 16 h, as assessed by western blotting. This experiment was repeated three times with similar results. β-actin was used as control.

3.3.6 Lactoferrin increased the phosphorylation of ERK and AKT at an early stage

As shown above, no obvious inhibition of the ERK and AKT pathways could be observed due to cells treatment with bLF for 16 h. However, this does not exclude the possibility that the bLF could be affecting these pathways in a shorter exposure time. Therefore, several assays were conducted at times ranging from 30 min to 6 h, in which bLF was added to cells without refreshing the medium to exclude any possible influence from fresh FBS (containing mitogenic components) on these signaling pathways. As shown in **Figure 3.6**, bLF at 15 μM greatly increased the phosphorylation of ERK and AKT in a short period (30 min) of exposure time, for T-47D (**Figure 3.6A)** and MCF-7 (**Figure 3.6B)** cells, respectively. Afterwards, a decrease of phosphorylation was observed from 1 h to 6 h. However, phospho-AKT in T-47D and phospho-ERK in MCF-7 was not detected in a short term. These results indicate that bLF has a mitogenic potential at the early stages, but then by an unknown mechanism this protein affects the growth of breast cancer cells by inducing cell cycle arrest. This mitogenic potential of bLF at an early stage may explain why the cell cycle arrest was not very pronounced after 24 h of bLF treatment at the different concentrations tested (*data not shown*).

3.3.7 Lactoferrin decreased the phosphorylation and expression of mTOR

Although the short-term effects of bLF were contradictory with the observed bLF cell growth inhibition against the breast cancer cells, this finding led us to focus the research on the study of nutrient-related factors that could possibly be involved in the cell cycle arrest. Recent studies had demonstrated that the serine-threonine mammalian target of rapamycin (mTOR) is an important nutrient/energy sensor. Indeed, mTOR can integrate multiple signals and serves as a central regulator in cell survival, metabolism and cell proliferation [Tokunaga *et al.*, 2004; Morgensztern & McLeod, 2005]. Therefore, the levels of phospho-mTOR and total mTOR in the breast cancer cells treated with bLF were evaluated by western blot. The results showed that 30 μM bLF efficiently inhibit the phosphorylation of mTOR after 2 h and 20 h treatment (**Figure 3.7A**). Furthermore, it was also found that bLF not only decreased the phosphorylation of mTOR, but also decreased the total levels

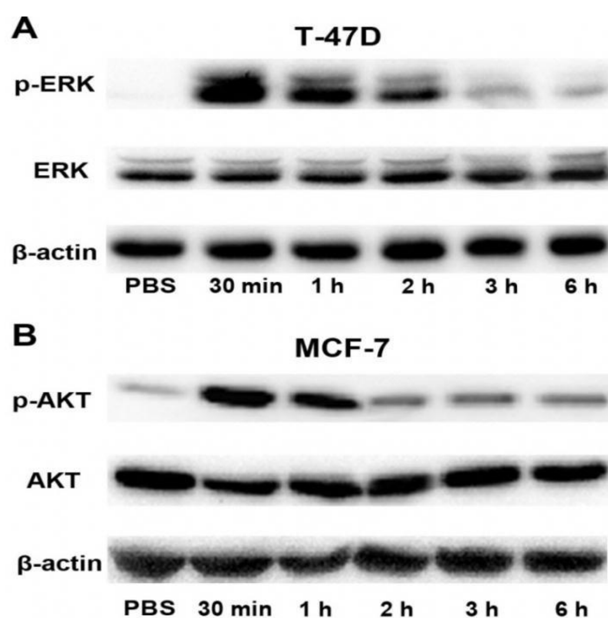

Figure 3.6 - Short-term effects of bLF on the phosphorylation of ERK and AKT. Cells were incubated with 15 μM bLF for 30 min, 1 h, 2 h, 3 h and 6 h (there were no medium change when bLF was added). The protein level of p-ERK and p-AKT in T-47D (**A**) and MDA-MB-231 (**B**), respectively, was analyzed by western blot. *β*-actin was used as loading control. This image is representative of three independent experiments with similar results.

of mTOR. In addition, the phospho-mTOR and mTOR decreased with increasing concentrations of bLF after 16 h incubation, which indicated that the bLF inhibitory effects on mTOR signaling acted in a dose-dependent manner (**Figure 3.7B**). A simultaneous decrease of mTOR and phospho-mTOR is also observable in other studies with other agents such as the mTOR kinase inhibitor rapamycin [Vollenbröker *et al.*, 2009; Johnson *et al.*, 2009]. These results show that bLF downregulates the mTOR pathway, which may inhibit the phosphorylation of p70S6 kinase (S6K1) and 4E-binding protein 1 (4E-BP1), affecting protein translation, autophagy survival pathway and cell cycle progression [Noh *et al.*, 2004].

3.3.8 Lactoferrin increased the phosphorylation of AMPKα

The AMP-activated serine/threonine protein kinase (AMPK), a sensor of cellular energy status, can be activated by low intracellular ATP following some stresses such as nutrient

deprivation or hypoxia [Zhuang&Miskimins, 2008]. Several studies demonstrated that mTOR is one of the major downstream signaling pathways regulated by AMPK [Shackelford&Shaw, 2009]. AMPK induces the rapid suppression of mTOR by directly phosphorylating TSC2 tumor suppressor and the critical mTORC1 binding subunit raptor [Tokunaga *et al.*, 2004]. Therefore, in the current study, the phosphorylation levels of AMPKα were evaluated by western blot. As shown in **Figure 3.8**, bLF induced an increase in the levels of phospho-AMPKα in a dose-dependent manner after 48 h of treatment in all breast cancer cell lines studied. Therefore, activation of AMPK by bLF may explain the inhibition of mTOR signaling leading cancer breast cells to stop growing. To confirm cell cycle inhibition, the levels of CDC25c, a crucial player in the G2/M phase transition of the cell cycle [Tamura et al., 2000], was also measured and we found that it was clearly decreased in bLF-treated cells. Moreover, the bLF inhibitory effects on the cells CDC25c expression were found to be in concentration-dependent manner.

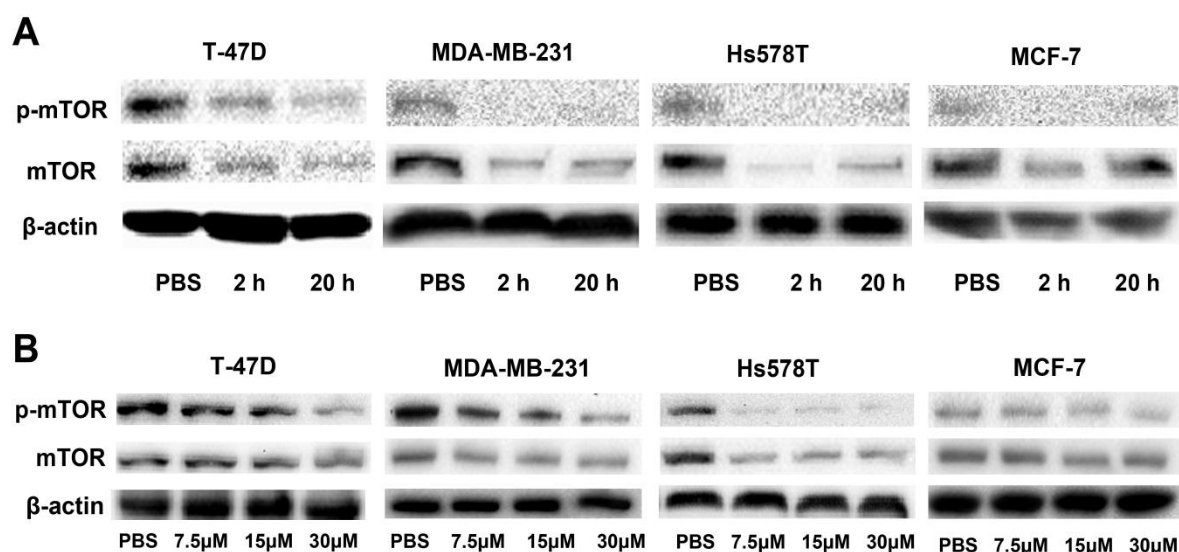

Figure 3.7 – Evaluation of bLF effect in the expression of phospho-mTOR and mTOR levels in several breast cancer cell lines. Cells were incubated with the same concentration of bLF for 2 h and 20 h (**A**) or treated by different concentrations of bLF for 16 h (**B**). The relative protein levels in the respective cells were measured by western blot. The blots are representative of three independent experiments with similar results. *β*-actin was used as loading control.

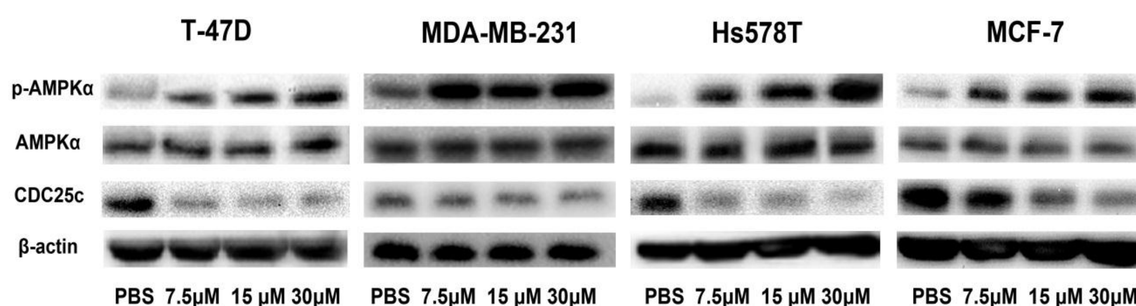

Figure 3.8 – Evaluation of the bLF effect on the expression of AMPKα and CDC25c in breast cancer cells. Cells were incubated with different concentrations of bLF for 48 h and the protein level of phospho (p) - AMPKα, AMPKα and CDC25c were evaluated by western blot. Shown bots are representative of three independent experiments with similar results. *β*-actin was used as loading control.

3.4 DISCUSSION

Multiple functions have been described for LF including its anticancer effects, which regarding breast cancer is corroborated by the association between LF expression and cancer risk [Furmanski *et al.*, 1989]. In a previous study we found that bLF is able to be internalized by cells and induces growth inhibition of several breast cancer cell lines (chapter 2). In the present study, we aim to unravel the mechanisms of action involved in the anticancer potential of bLF against breast cancer cell lines with different genetic backgrounds. We have found that the anticancer effect of bLF is associated with cell cycle arrest and not with induction of cell death by apoptosis. However, many studies reported that bLF is able to induce apoptosis in several cancer cell lines [Xu *et al*., 2010; Furlong *et al*., 2006; Duarte *et al*., 2011], which indicates that the effects may be dependent on the cancer type and/or concentration of bLF used. Yet, the majority of these studies used LF extracted from human milk to show its apoptosis-inducing activity [Damiens *et al*., 1999; Lin *et al*, 2005; Lee *et al*., 2009], which may also explain the different outcome between their results and ours. In our study, the lack of apoptosis in the presence of bLF may also be due to the resistance of the cell lines to this type of cell death. As it is known, one of the hallmarks of cancer cells is their potential to escape from cell death by apoptosis [Igney & Krammer, 2002]. For example, p53 is a well-known pro-apoptotic protein, but mutations in the p53 gene that affect its normal functions as tumor suppressor occur in approximately

50% of sporadic human tumors [Gasco *et al*., 2002; Meulmeester & Jochemsen, 2008]. The cell lines T-47D, MDA-MB-231 and Hs578T all possess a somatic mutation in the p53 gene locus [Mercatante, *et al*., 2002]. Although p53 is wild type in MCF-7 cells, this cell line is also known as resistant to apoptosis, probably due to the lack of expression of the apoptotic effector caspase-3 [Jänicke, 2009].

In this work, a systematic evaluation of bLF effects on cell cycle was done by flow cytometry in multiple breast cancer cells. The results demonstrated the ability of bLF to induce cell cycle arrest in T-47D, MDA-MB-231, Hs578T and MCF-7 cells. However, the phase of cell cycle in which the cells were arrested differed from cell line to cell line, probably due to their different genetic backgrounds. Nevertheless, a decrease in the number of cells synthesizing DNA (cells at S phase) and inhibition of CDC25c levels was a common feature in the effect of this protein in cell cycle progression, corroborating therefore the ability of bLF to inhibit cell proliferation. Other studies also found that LF is able to induce cell cycle arrest in cancer cells [Xiao *et al.*, 2004; Damiens *et al*., 1999; Yamada *et al*., 2008]. For example, human lactoferrin (hLF) induced cell cycle arrest at G1 to S transition in MDA-MB-231 cells by decreasing Cdk2 and Cdk4 activity and inducing the expression of p21 [Damiens *et al*.,1999]. Likewise in our study, bLF induced cell cycle arrest in MDA-MB-231 cells but this occurred at G2/M phase, indicating that the anticancer mechanism of action of LF may be dependent on the protein species source. Since induction of DNA damage is also correlated with cell cycle arrest [Dasika *et al*., 1999], we tested herein whether inhibition of cell growth by bLF would be dependent on DNA double strand breaks. However, this hypothesis was discarded since bLF did not induce phosphorylation of the histone H2A.X.

Many signaling pathways, such as MAP kinases and PI3K/AKT, are commonly altered in cancer affecting cell proliferation and mechanisms of cell death by apoptosis [Roy *et al*., 2010]. Some studies have shown the involvement of AKT in the anticancer effects of LF against stomach cancer cells [Xu *et al*., 2010], whereas JNK was required for the apoptosis induced by bLF [Lee *et al*., 2009]. In our study, bLF did not induce the stress-induced kinases JNK and p38, and therefore these results are in accordance with the lack of apoptosis. On the other hand, the MAPK/ERK and PI3K/AKT signaling pathways were not inhibited by bLF indicating that they were not implicated in the cell cycle arrest observed in all breast cancer cells used. However, in the short-term bLF induced both the phosphorylation of AKT and ERK, but they return to normal levels after few hours. This

effect is associated with a signal for growth and proliferation as it occurs due to mitogenic factors present in FBS [Worster *et al*., 2012]. Although this effect is contrary to the inhibition of cell proliferation observed here, bLF may have other cellular effects that surpass this earlier event. Interestingly, a previous study also found that hLF had a bi-directional action on apoptosis and ERK pathway depending on the dose used in PC12 cells [Lin *et al*., 2005]. Herein, a bi-directional action of bLF in cell proliferation events seems to depend on time.

To further advance in the search for the possible mechanisms behind the growth inhibition of bLF, the nutrient/energy sensor mTOR kinase that integrates multiple signals and known to regulate cell survival (autophagy), metabolism and cell proliferation [Tokunaga *et al*., 2004; Kim *et al*., 2011], was studied. Interestingly, we observed that bLF induced in all breast cell lines a fast downregulation of this signaling pathway, in view of both the decrease of total and phosphorylated forms of mTOR. The upstream effector of mTOR, AKT [Morgensztern＆McLeod, 2005], probably was not responsible for this effect since it was not downregulated by bLF and, on the contrary, it was even induced in the first hours of incubation. The energy sensor AMPK is another upstream effector of mTOR [Shackelford＆Shaw, 2009], and here a remarkable activation of phospho-AMPK in breast cancer cells was observed in the presence of bLF. Therefore, a downregulation of mTOR through activation of AMPK may explain the ability of bLF to inhibit cell growth and to induce cell cycle arrest. Inhibition of protein synthesis in cells through mTOR is expected to markedly retard cell proliferation since many cell cycle-related proteins need to be coordinately synthesized and degraded at different phases for cell cycle to progress [Tokunaga *et al*., 2004; Kim *et al*., 2011]. As far as we know, this is the first report showing the effects of bLF in AMPK and mTOR kinases, which may be relevant in the fight against breast cancer cells since this signaling pathway is considered an important target for cancer treatment [Noh *et al*., 2004]. However, more studies are needed to confirm the involvement of AMPK/mTOR axis in the growth inhibition potential of bLF, in particular to explain how a high concentration of the protein bLF in cell culture medium induces a starvation-like state in breast cancer cells.

bLF has been largely produced at an industrial scale and is currently used as a food supplement with health benefits [Y.Z. *et al*., 2008; Manzoni *et al*., 2009]. bLF, as a milk-derived protein, is relative more safe than other anticancer agents, especially when it shares 70% sequence homology with human LF [Nozaki A *et al*., 2003]. Therefore, this

protein may have a great potential for application in cancer therapy. However, the *in vivo* evaluation of bLF will be essential if a clinical application is foreseen. Nevertheless, some previous reports succeeded to show the LF potential as antitumor agent *in vivo* using animal models [Fischer *et al*., 2006; Wolf *et al*., 2007].

In summary, this study reported that bLF possesses an interesting anticancer activity against breast cancer cells. This activity was found to be associated to cell cycle arrest but not to cell death by apoptosis. Some insights on the mechanisms behind the bLF growth inhibitory effects in the four breast cancer cells were also provided. The observed effects were not associated with modulation of the signaling pathways JNK, p38, MAPK/ERK and PI3K/AKT. Interestingly, cell cycle arrest induced by bLF was found to be associated with a downregulation of mTOR signaling probably due to AMPK activation. We believe that the direct anticancer effects of bLF plus its immunostimulatory effects will lead to a pronounced anticancer activity *in vivo*, and therefore that this protein could be envisaged as a powerful agent for cancer therapy in a near future.

3.5 References

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Chapter 4

Bovine lactoferrin selectively induces apoptosis of breast cancer cells

Abstract

Apoptosis is a mechanism that has been reported to be involved in the lactoferrin (LF) anticancer effects. We previously showed that bovine lactoferrin (bLF) at concentrations lower than 30 μM inhibits the growth of breast cancer cells by induction of cell cycle arrest without notorious effects in cell death by apoptosis. In this study, higher concentrations of bLF were used against the same breast cancer cell lines under study (T-47D, MDA-MB-231, Hs578T and MCF-7 cells) and the anticancer mechanisms were evaluated. The results showed that bLF at concentrations ranging from 12.5 μM to 175 μM for 48 h induced growth inhibition in a concentration-dependent manner in all breast cancer cells. However, bLF only induced apoptosis in MCF-7 cancer cells. Moreover, the mechanism underlying the bLF cytotoxicity against MCF-7 cells also included cell cycle arrest at G1/G0 phase. The bLF-induced apoptosis was associated with mitochondria membrane depolarization and with a decrease of Bcl-2 levels, which suggests that bLF induced apoptosis by the intrinsic apoptotic pathway. Induction of cell cycle arrest was also corroborated by a significant decrease in the expression of the cell cycle regulator CDC25c. Besides, it was excluded the possibility that the bLF anticancer effects could be due to the addition of a high concentration of exogenous protein to the culture media, since the same concentration of bovine serum albumin failed to affect the cell cycle in the same magnitude as bLF. Furthermore, we also showed that bLF clearly restrained the colony formation by MCF-7 cells, although promoted cell migration. This later effect was, however, related to the presence of a high concentration of exogenous protein. The results gathered in this work indicated that the bovine milk-derived protein LF could be viewed in the near future as an efficient agent for cancer chemoprevention.

4.1 INTRODUCTION

Due to the safety concerns on the use of pharmaceuticals, as well as their regulatory limitations, dietary agents are becoming attractive alternatives as cancer chemopreventive agents [Mignogna *et al*., 2004; Scott *et al*., 2009]. Some dietary agents, such as selenium, vitamin D and green tea polyphenols have been described as potentially applied in the chemoprevention of prostate cancer [Syed *et al*., 2007]. Lactoferrin (LF), a milk-derived glycoprotein, is widely distributed in several secretion fluids of mammalian cells but it is extremely abundant in milk. This protein has been assigned with multiple biological functions, including antibacterial, antiviral, antifungal and immune regulatory activities [Adlerova *et al*., 2008]. More interestingly, it has been found that LF downregulation in breast cells could greatly increase the incidence of breast cancers [Furmanski *et al*., 1989]. On the other hand, the exogenous application of LF and its variants were reported to efficiently inhibit the cancer growth both *in vitro* and *in vivo* [Xu *et al*., 2010; Yamada *et al*., 2008; Damiens *et al*., 1999].

Despite its great potential, the mechanisms underlying the LF and its variants cytotoxicity against cancer cells are still fairly unknown. The genetically-induced overexpression of human lactoferrin (hLF) in MCF-7 cells was reported to promote cellular apoptotic activities [Liao *et al*., 2010]. Also, recombination adenovirus-mediated hLF cDNA led to cell cycle arrest and stimulated apoptosis in MCF-7 cells associated with a decrease of Bcl-2 [Wang *et al*., 2012]. Furthermore, the restoration of delta-lactoferrin (ΔLF) in HEK 293 cells resulted in cell cycle arrest and growth retardation [Breton *et al*., 2004]. Importantly, exogenous hLF could efficiently inhibit the growth of cancer cells and induce cell cycle arrest at G1 phase [Damiens *et al*., 1999; Yamada *et al*., 2008; Zhou *et al*., 2008]. This effect occurred through a p53-independent pathway, and the key G1 regulatory proteins played an important role in the process [Damiens *et al*., 1999; Yamada *et al*., 2008]. Additionally, bovine lactoferrin (bLF) was found to induce apoptosis of SGC-7901 human stomach cancer cells by inhibiting the Akt activation and regulating its downstream signals [Xu *et al*., 2010]. However, hLF-induced apoptosis in PC12 cells (rat pheochromocytoma cells) was observed to be associated to a decrease in the expression of the phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2) and Bcl-2 [Lin *et al*., 2005]. In addition, hLF was reported to induce apoptosis of Jurkat T cells via the regulation of c-Jun N-terminal kinase (JNK) activity [Lee *et al*., 2009]. Furthermore, the main bLF-derived peptide, LfcinB, also showed similar effects as its parent protein.

Nevertheless, LfcinB inhibited the proliferation of cancer cells mainly by activating apoptosis-inducing pathways [Roy *et al.*, 2002; Sakai *et al.*, 2005; Furlong *et al.*, 2006; Onishi *et al.*, 2008]. JNK/SAPK (stress-activated protein kinase) activation, downregulation of Bcl-2, mitochondria swelling and release of cytochrome c were believed to be the mechanisms underlying the LfcinB cytotoxicity against cancer cells [Mader *et al.*, 2005; Sakai *et al.*, 2005]. All these reports suggest that the mechanisms behind LF and LfcinB activities against cancer cells are greatly dependent on the cell type. Additionally, the potential of LF for application in cancer therapy was further confirmed *in vivo*. LF exerts the *in vivo* anticancer ability mainly through activating both the innate and adaptive immune responses and stimulating the proliferation and differentiation of T helper cells (Th) into type 1 helper cell (Th1) or type 2 helper cell (Th2) phenotypes, which release tumor-killing cytokines (Tumor necrosis factors (TNF), interferon-γ (IFN-γ), caspase-1 and interleukin-18 (IL-18)) in the intestine and tumor [Fischer *et al.*, 2006; de la Rosa *et al.*, 2008].

Therefore, considering the correlation between LF and breast cancer, and the fact that exogenous LF has been reported to efficiently inhibit cancer growth both *in vitro* and *in vivo*, we have been studying recently the anticancer effects of bLF in breast cancer cells [Duarte *et al.*, 2011]. We showed previously the bLF potential against breast cancer cells without affecting the normal counterparts (chapter 2). At concentrations below 30 μM, growth inhibitory effects of bLF was shown to be mainly due to cell cycle arrest associated with effects at the AMPK-mTOR axis (chapter 3). In the present work, we aimed to investigate the bLF cytotoxicity against breast cancer cells at higher concentrations and to propose the possible mechanisms involved in its anticancer effect. Evidences provided by the *in vitro* experiments will be of great value towards a future clinical use of bLF in cancer therapy.

4.2 MATERIAL AND METHODS

4.2.1 Cell lines and lactoferrin

T-47D and MDA-MB-231 breast cancer cell lines were a kind gift from IPATIMUP and Medical School from University of Porto (Portugal), respectively. Hs578T cells were donated by Life and Health Sciences Research Institute, University of Minho (Portugal).

MCF-7 was bought from the American Type Culture Collection. The cells were cultured at 37℃ in a humidified incubator with a 5% CO_2 atmosphere in L-glutamine-containing DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, Steinheim, Germany). Bovine lactoferrin was purchased from the company of DMV (Veghel, The Netherlands). The purity of the protein is about 80% with 3.5% moisture and 21% iron-saturated according to the manufacture.

4.2.2 Cell proliferation assay

The trypan blue staining was used to estimate cell growth and cell death induced by the treatment with bLF. For that, breast cancer cells (MCF-7, T-47D, MDA-MB-231 and Hs578T) were seeded in 24-well plates overnight. Afterwards, bLF at several concentrations (12.5 μM, 50 μM, 125 μM and 175 μM) was added to each well. After a 48 h treatment period, both floating and attached cells were collected and resuspended in medium to adjust to an adequate cell concentration. A 50 μl volume of suspended cells were mixed with 50 μl of trypan blue 0.04% (w/v) (Sigma-Aldrich) and were incubated for 5 min before counting viable and non-viable cells using a haemocytometer under a light microscope. Cells that exclude trypan blue (viable cells) were used to estimate cell growth, where control condition was set as 100%. Cell viability was expressed in percentage and calculated according to the following equation: Number of viable cells/total number of cells ×100.

4.2.3 Nuclear condensation assay

The ability of bLF to induce cell death by apoptosis was estimated by the nuclear condensation assay. In brief, cells at exponential phase were collected and seeded in 6-well plates. After 24 h of incubation, cells were treated for 72 h with bLF at the concentrations of 12.5 μM, 50 μM, 125 μM and 175 μM. Then, both floating and attached cells were collected and washed with phosphate buffered saline (PBS). Next, cells were immersed in 4% (w/v) paraformaldehyde for 20 min at room temperature. Afterwards, cells were washed once again with PBS and they were fixed to a polylysine-treated slide using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA). Nuclei were stained with

Hoechst (5 μg/ml) fluorescent dye for 10 min in the dark. Fluorescent cells were observed and photographed using a fluorescent microscope (Olympus IX71, Hamburg, Germany). Five different fields from each sample were counted, in order to reach at least 400 cells. The apoptosis rate was calculated as the number of cells presenting nuclear condensation divided by the total number of cells (stained in blue).

4.2.4 Annexin V/Propidium iodide (PI) staining

Annexin V/PI staining was also used to estimate cell death by apoptosis. For that, MCF-7 cells were seeded in 6-well plates for 24 h. Subsequently, cells were treated with 12.5 μM, 50 μM, 125 μM and 175 μM bLF. After 48 h of bLF treatment, the cells were washed twice with ice-cold PBS and resuspended in 1× Binding Buffer (0.01 M Hepes - pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) to get a final cell concentration of 1×10^6 cells/ml. A 100 μl volume of each sample was then stained with 5 μl FITC Annexin V (BD Pharmingen, BD, Franklin Lakes, NJ, USA) and 10 μl PI (50 μg/ml). The samples were gently vortexed and incubated at room temperature for 15 min in the dark. In the end, 400 μl of 1× Binding Buffer was added to each sample, which were then analysed by flow cytometry using a Coulter Epics XL flow cytometer (Beckman Coulter Inc., Miami, FL, USA).

4.2.5 Cell cycle analysis

MCF-7 cells were seeded on 100 mm petri dish and cultured for 24 h. Fresh medium with bLF at the concentrations of 12.5 μM, 50 μM, 125 μM and 175 μM was added to cells and incubated for 24 h. In the end, cells were collected and washed with PBS. Further, cells were fixed with 70% (v/v) ice-cold ethanol for 15 min on ice. After that, cells were washed twice and resuspended in 500 μL PBS. 50 μL RNAse A (200 μg/ml) was added to each sample and incubated at 37℃ for 15 min. At last, 60 μL PI (0.5 mg/ml) was added to samples and incubated for 30 min before analysing on a flow cytometer (Beckman Coulter Inc.). For cell cycle analysis, at least 30,000 single cells per sample were used and fitted to the cell cycle phases using the mathematical Watson Pragmatic model with the FlowJo Analysis Software (Tree Star, Inc., Ashland, OR, USA).

4.2.6 Mitochondrial membrane potential assay

MCF-7 cells were incubated with different concentrations of bLF (12.5 μM, 50 μM, 125 μM and 175 μM) for 2 h, 24 h and 48 h. A positive control was included, containing carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma-Aldrich) at a 50 μM and incubated for 30 min at 37℃ before cell collection. Subsequently, cells were resuspended in 1 ml of warm-PBS, and 10 μl of 200 μM JC-1 (Molecular Probes, Life Technologies, Eugene, OR, USA) was added to each sample and incubated for 15 min under cell culture conditions. At last, the samples were analysed by flow cytometry (Beckman Coulter Inc.).

4.2.7 Western blot assay

MCF-7 cells were seeded in 6-well pates 24 h before incubating cells with bLF at the concentrations of 12.5 μM, 50 μM, 125 μM and 175 μM for 48 h. After cell washing with PBS, total protein was extracted using RIPA (Radio-Immunoprecipitation Assay) buffer (50 mM Tris-HCl – pH 7.5, 1% (v/v) NP-40, 150 mM NaCl, 2 mM EDTA, 20 mM Na_3VO_4, 20 mM NaF, 1 mM PMSF, 1× protease inhibitory cocktail). Protein content in each sample was determined by the detergent compatible DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using BSA as standard. 20 μg or 30 μg protein from the samples were loaded into 10% SDS-PAGE and transferred into PVDF membranes (GE Healthcare, Bucks, UK). After membrane blocking with TPBS (PBS with 0.05% (v/v) Tween-20) containing 5% (w/v) nonfat dry milk, the levels of chosen proteins were detected using specific primary antibodies incubated overnight with membranes followed by incubation with associated secondary antibodies. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-actin was used as loading control. The antibodies anti-CDC25c, anti-Bcl-2 and anti-p53 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2.8 Colony formation assay

MCF-7 cells were trypsinized and pipetted repeatedly to disperse them until single cells

account for 95% of the total number of cells. Next, the cells were counted and then seeded in 6-well-plates to a concentration of 1×10^3 cells per well and incubated for 24 h. Subsequently, the cells were treated with bLF at several concentrations (12.5 μM, 50 μM, 125 μM and 175 μM), while DMEM without FBS was used as a positive control. After 24 h incubation with bLF, the media in all wells was replaced with fresh media (DMEM with 10% FBS). After 7 to 10 days, the cells were fixed with 70% methanol for 15 min and were dried in air. Giemsa was used to stain cells for 10 min and was subsequently washed carefully with water. Colonies containing at least 50 cells were counted. Colony formation was calculated as the ratio between the number of colonies in the experimental samples and the number of colonies in the control sample.

4.2.9 Migration assay

The bottom of 6-well-plates was marked with 5-10 straight lines with 0.2 cm interval among the lines. Then, 5×10^5 MCF-7 cells/well were seeded in 6-well plates for growing until cell confluence reached over 85%. Then, a scratch wound across each well of the 6-well plates was made using a pipette tip. Next, cells were washed twice with PBS and FBS-free DMEM containing 175 μM bLF or BSA was added. Cells were photographed at the exact same point (marked with straight line) in the 6-well plates after 0 h, 6 h, 12 h and 24 h. The cell migration ratio was calculated by comparing the wound area at 6 h, 12 h and 24 h to the wound area at 0 h in each group.

4.2.10 Statistical analysis

Statistical significance of the experimental results was determined by the Student's *t* test. For *p*-values below 0.05 the differences between experimental groups were considered significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Lactoferrin possess growth inhibitory effects against breast cancer cells

In a previous study, bLF at relatively low concentrations presented interesting growth inhibitory effects against a panel of breast cancer cells, as shown by the MTS assay

(Chapter 2, Figure 2.1). This effect was associated with cell cycle arrest but not with cell death (Chapter 3). In the present study, we have studied higher concentrations of bLF in order to test whether the protein is able to induce apoptosis in breast cancer cells. For that purpose, the trypan blue staining method was firstly used since it provides information on growth inhibitory activities and total cell death. Considering the number of viable cells in each well, as it is observed in **Figure 4.1**, bLF significantly inhibited the growth of breast cancer cells after 48 h of treatment in a concentration-dependent manner. The magnitude of growth inhibition was identical in all cell lines, which indicates that the effect is not cell type specific. When comparing these results with the ones obtained in Chapter 2 at 48 h, it was found, as expected, that the degree of inhibition of cells' growth was remarkably increased when using higher bLF concentrations, reaching about 70% to 80% inhibition at 175 μM (**Figure 4.1**). In the case of the Hs578T cell line, as compared with non-treated cells, cell death increased about 30% due to exposure to 175 μM of bLF. However, few dead cells were observed in the other cell lines (*data not shown*). These results suggest that the inhibitory effect of bLF on the breast cancer cells is mostly associated with the inhibition of cell proliferation.

4.3.2 Lactoferrin selectively induces apoptosis in MCF-7 cells

Considering that some cell death was present in the Hs578T cells, we then performed a nuclear condensation assay to estimate the potential induction of apoptosis. However, at 48 h, no evident nuclear condensation was present in all cell lines used (*data not shown*), which corroborate the lack of massive cell death shown above. Since we previously have shown that bLF presents some mitogenic effects in the first hours of incubation (Chapter 3), we hypothesise that the cell death by apoptosis could be a more delayed event. Therefore, induction of apoptosis was evaluated then 72 h after incubation of cells with bLF. The results demonstrated that bLF failed to induce apoptosis in all breast cancer cell lines under study (*data not shown*), except for the MCF-7 cells (**Figure 4.2**). Cells presenting nuclear condensation are shown with arrows in **Figure 4.2.A**. Nuclear condensation was found to increase in a dose-dependent way in MCF-7 cells, reaching a ratio of about 35% after 72 h of treatment with 175 μM bLF (**Figure 4.2.B**). This assay indicated that bLF could selectively induce apoptosis in MCF-7 cells among the tested breast cancer cells, probably due to their different genetic backgrounds. For example, the p53 gene plays an important

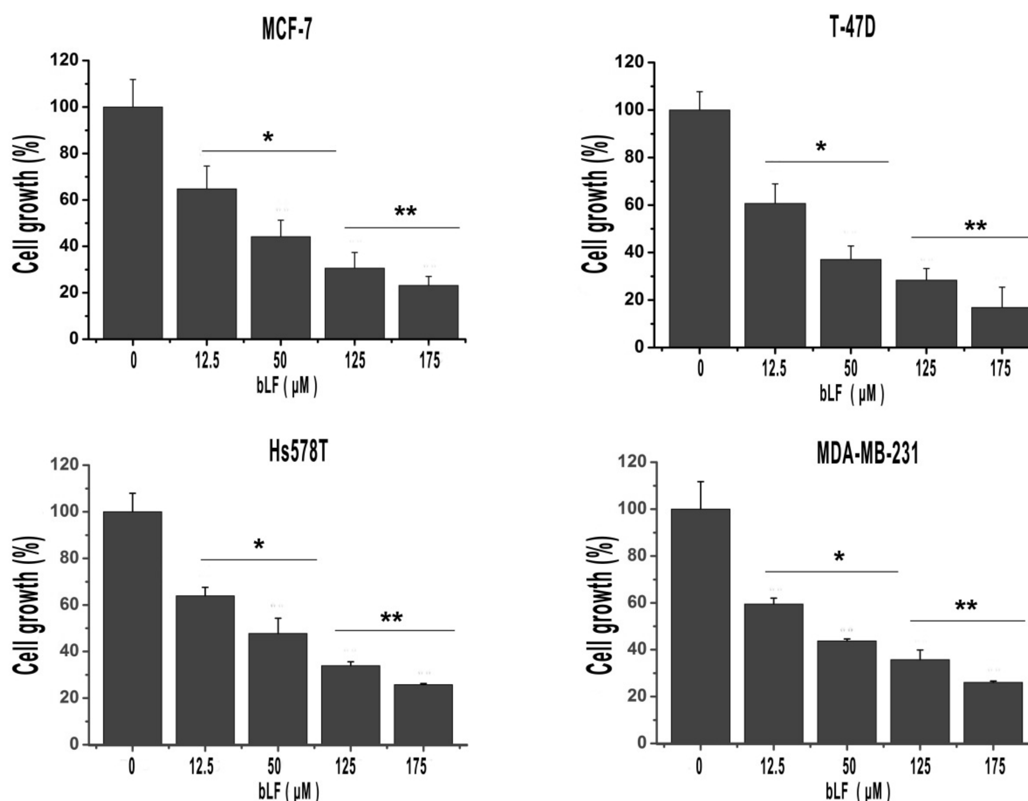

Figure 4.1 - Effect of bLF on breast cancer cells' growth. MCF-7, T-47D, MDA-MB-231 and Hs578T cells were incubated with bLF at several concentrations for 48 h and cell growth evaluated by cell counting of viable cells (not stained with trypan blue). Values are mean ± SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ when compared with the control.

role in cell apoptosis [Meulmeester & Jochemsen, 2008]. Whereas T-47D, MDA-MB-231 and Hs578T cells harbour a mutated p53 gene, MCF-7 cells possess a wild-type p53 [Xu *et al*., 2007; Jänicke, 2009]. Therefore, the resistance of T-47D, MDA-MB-231 and Hs578T cells to undergo apoptosis in the presence of bLF may be due to the lack of the normal functions of p53. However, the role of p53 in the induction of apoptosis by bLF needs to be confirmed in further experiments. In fact, some studies previously reported the ability of LF to induce apoptosis in cancer cells [Lin *et al*., 2005; Xu *et al*., 2010; Duarte *et al*., 2011; Wang *et al*., 2011]. Herein we show that this effect may depend on the genetic background of the cell line used, such as the presence of a functional p53 protein.

To confirm the induction of apoptosis by bLF in the MCF-7 cells, the annexin V/PI assay was performed after 40 h of incubation, since the translocation of phosphatidylserine

for the outer leaflet of plasma membrane is an apoptotic event that occurs earlier than the DNA fragmentation [Elmore, 2007]. The results showed that the amount of non-apoptotic cells (Q4 region: annexin V negative/PI negative) decreased in a concentration-dependent manner compared to the non-treated group (**Figure 4.2.C**). In contrast, the number of cells in the early stage (Q3 region: annexin V positive/PI negative) and later stage of apoptosis (Q2 region: annexin V positive/PI positive) was considerably increased by bLF in a concentration-dependent manner (**Figure 4.2.C**). Although the percentage of apoptotic cells in bLF-treated groups estimated by the Annexin V/PI assay was less than that by the nuclear condensation assay, this assay confirmed the ability of bLF to induces apoptosis in MCF-7 cells.

4.3.3 Lactoferrin inhibits cell cycle progression in MCF-7 cells

In a previous work, we have shown that induction of cell cycle arrest in the four breast cancer cell lines was the major mechanism of cell growth inhibition by bLF at concentrations below 30 μM (Chapter 3). Considering that apoptotic cell death was only observed in MCF-7 cells, in this work we tested only for this cell line the effects of bLF at higher concentrations in the cell cycle progression. As shown in **Figure 4.3A**, bLF clearly induced cell cycle arrest of MCF-7 cells at the G1 phase after 24 h of treatment, and this effect increased in a dose-dependent manner. This was accompanied with a remarkable decrease in the percentage of cells at the S phase (cells synthesising DNA), therefore corroborating the previous results (Chapter 3). These findings are also consistent with reports on the LF action against other cancer cell lines [Damiens *et al*., 1999; Yamada *et al*., 2008; Zhou *et al*., 2008]. Specifically regarding to MCF-7 cells, previous studies demonstrated that re-expression of LF or ΔLF using several delivery vectors induced cell cycle arrest [Liao *et al*., 2010; Wang *et al*., 2012]. In the current work, we showed that exogenous bLF presented a similar effect by inhibiting cell proliferation.

Since the concentration of bLF being used is relatively high (175 μM), reaching a quantity of 14 g/L in the culture media, we questioned whether the observed effects could be due to this considerable amount of exogenous protein added, which would disturb the cellular microenvironment and prevent cell division. Therefore, a parallel assay using bLF and bovine serum albumin (BSA) at similar quantities (14 g/L) on the MCF-7 cell cycle progression was conducted. As shown in **Figure 4.3B**, BSA also induced cell cycle arrest

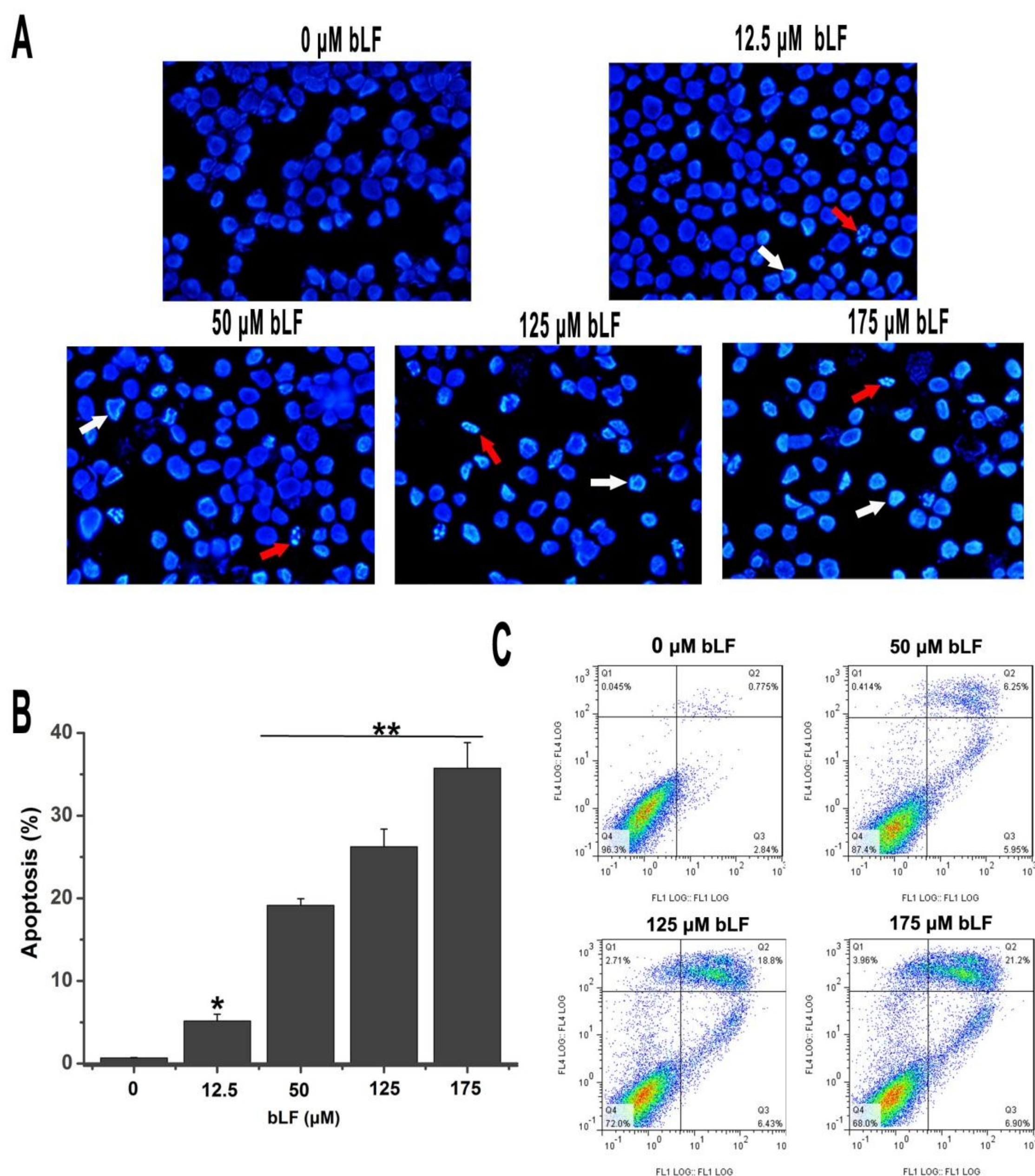

Figure 4.2 - Effect of bLF in the induction of cell death by apoptosis in MCF-7 cells. Cells were treated with different concentrations of bLF for 40 h or 72 h and apoptosis estimated by the Annexin V/PI and the nuclear condensation assays, respectively. (**A**) Representative images of the nuclear condensation assay where apoptotic cells are shown with arrows: white arrows correspond to examples of early apoptotic cells (condensed DNA); red arrows correspond to examples of late apoptotic cells (condensed and fragmented DNA). Images are provided with a magnification of 400×. (**B**) Apoptosis rate induced by bLF as mean ± SD of three independent experiments, as estimated by the nuclear condensation assay. * $P < 0.05$ and ** $P < 0.01$ when compared with the control. (**C**) Apoptosis assay by Annexin V/PI representative of two independent assays.

of MCF-7 cells at the G1 phase. However, this effect was found to be much lower when compared to bLF. Moreover, cells incubated with bLF presented a distinct morphology, while the morphology of the BSA treated group was very similar to the control one (**Figure 4.3B**). The results gathered in this work suggest that bLF could significantly inhibit the MCF-7 cell growth through cell cycle arrest due to a specific biological function.

4.3.4 Lactoferrin decreases mitochondrial membrane potential

Mitochondrial depolarization is considered as an early event in apoptosis that is usually associated to the intrinsic apoptosis pathway [Ly *et al.*, 2003]. In this study, we used the JC-1 probe to assess the modification of the mitochondrial membrane potential in cells after treatment with bLF for 2 h, 24 h and 48 h. After 2 h of bLF treatment at several concentrations (12.5 µM, 50 µM, 125 µM and 175 µM), it could be observed a significant decrease of the red to green ratio, which represents the decrease of the mitochondrial membrane potential. Furthermore, the depolarization effects of bLF on mitochondria increased in a dose-dependent way (**Figure 4.4A**). The mitochondrial depolarization occurred in all bLF-treated groups after 24 h incubation, although not in a dose-dependent manner. Contrarily, after 48 h of bLF treatment, mitochondria depolarization was found to be more pronounced and to be dependent on the bLF concentration (**Figure 4.4A**). From the cell distribution in the dot plot graph, it was observed that treatment of MCF-7 cells with 175 µM bLF for 48 h led to a significant shift of the number of cells with red to green fluorescence, as compared with the non-treated cells (0 µM bLF) (**Figure 4.4B**). In fact, the percentage of cells with depolarized mitochondria after 48 h of bLF treatment (30%) is similar to the apoptosis rate observed at 72 h (35%). When cells were observed under a fluorescent microscope it was confirmed that bLF induced the depolarization of mitochondria in a significant part of the cell population, in contrast to the positive control CCCP that basically depolarized the mitochondria of all cells (**Figure 4.4B**).

The mitochondrial intrinsic pathway has been reported as the main mechanism of LfcinB-induced apoptosis [Sakai *et al.*, 2005; Eliassen *et al.*, 2006]. Our results demonstrated that mitochondrial depolarization was also an event associated with the bLF-induced apoptosis. Depolarization of the mitochondria membranes was kept during the time period ranging from 2 h to 48 h, and no reversible change could be observed. The

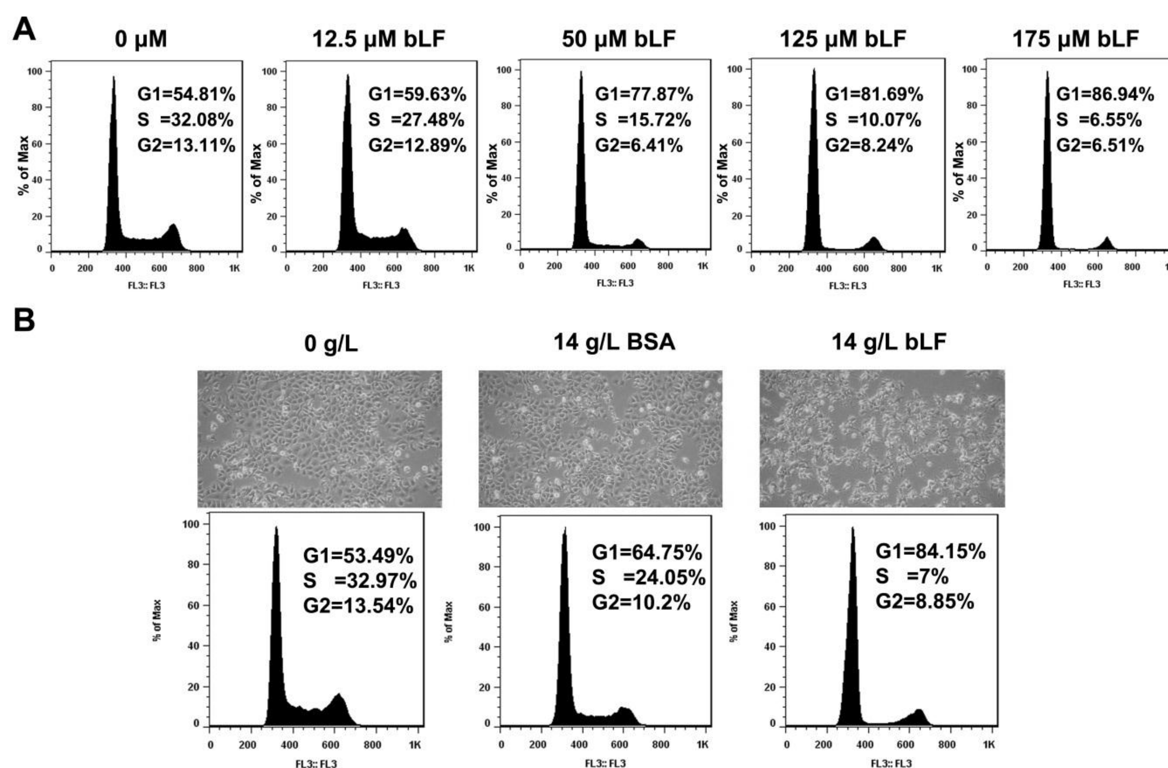

Figure 4.3 - Effect of bLF in the cell cycle progression of MCF-7 cells. (**A**) Cells were incubated with bLF at several concentrations for 24 h and cell cycle was analyzed by flow cytometry. Results correspond to a representative experiment from three independent experiments. (**B**) Comparison between the effect of bLF and BSA at the same concentration on the cell cycle progression of MCF-7 cells after 24 h of treatment. The top image is representative of cell morphology for each condition (magnification of the images: 40×).

changes in the membrane potential presumably stand for the opening of the mitochondrial permeability transition pore, which allows the release of cytochrome c from the mitochondria. This is a key event in the initiation of apoptosis [Gottlieb *et al.*, 2003]. Therefore, these findings suggest that depolarization of membrane potential is a crucial factor in the bLF-induced apoptosis of MCF-7 cells.

4.3.5 Downregulation of Bcl-2 is involved in the lactoferrin-induced apoptosis

Considering that mitochondria membrane depolarization was associated with the apoptosis induced by bLF in MCF-7 cells, the expression of Bcl-2 was also studied by

western blot. As shown in **Figure 4.5,** bLF significantly decreased in a concentration-dependent manner the expression of anti-apoptotic protein Bcl-2. This is totally consistent with the former reports that downregulation of Bcl-2 is associated with the LF- and LfcinB-induced apoptosis [Lin *et al.*, 2005; Mader *et al.*, 2005; Xu *et al.*, 2010; Wang *et al.*, 2012]. This decrease of Bcl-2 is considered an important event to the opening

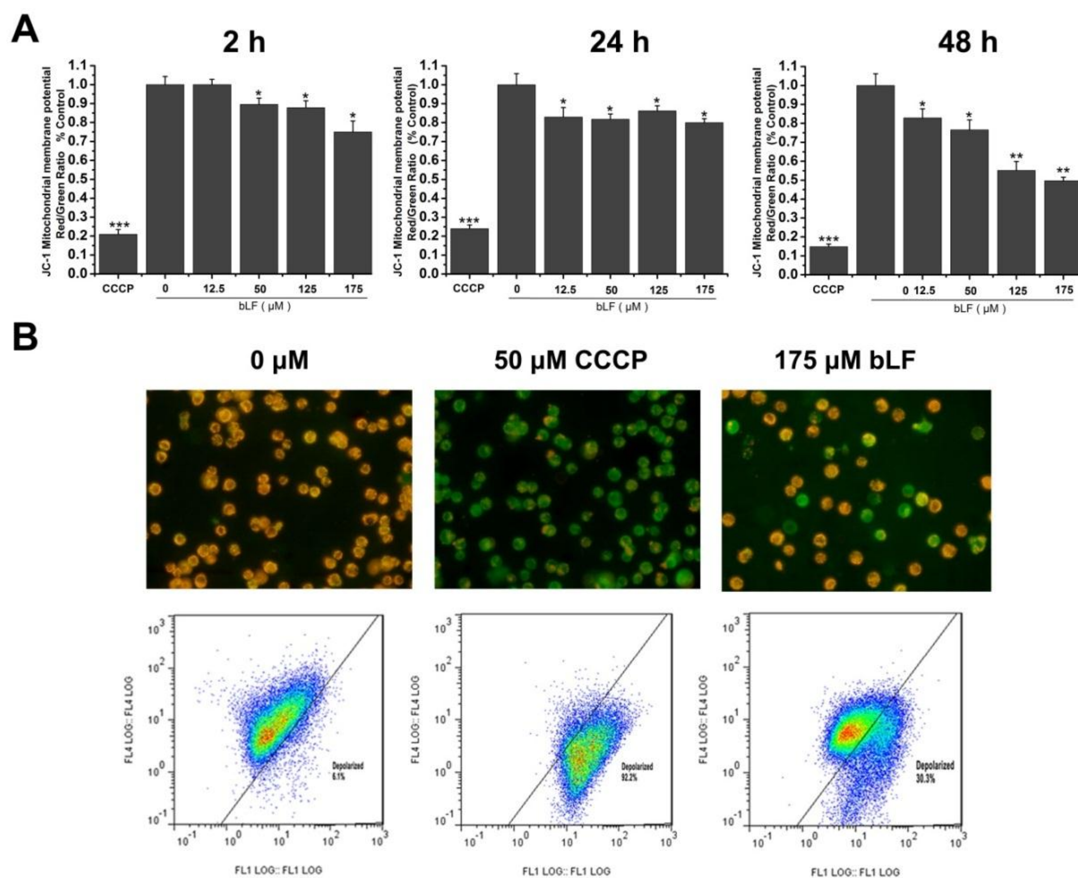

Figure 4.4 - Effect of bLF on the mitochondrial membrane potential. (**A**) MCF-7 cells were incubated with bLF at several concentrations for 2 h, 24 h and 48 h and mitochondrial membrane potential was analyzed by flow cytometer after labeling cells with JC-1 fluorescent probe. The red/green ratio was calculated from the respective median fluorescent value, where a decrease of this value in comparison to the control is indicative of mitochondrial membrane depolarization. Values correspond to the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.01$ when compared with the control. (**B**) The top image is representative of cells labeled with JC-1 probe, where red cells contain polarized mitochondria and green cells contain depolarized mitochondria (magnification of the images: 400×). The bottom image corresponds to JC-1-labeled MCF-7 cells after 48 h of treatment with positive control (CCCP 50 μM) or 175 μM bLF analyzed by flow cytometry. The graphs exhibit the percentages of cells with depolarized mitochondria (green positive/red negative).

of the mitochondrial permeability transition (MPT) pore and loss of the mitochondrial membrane potential, for the release of cytochrome c and other pro-apoptotic proteins characteristic of the intrinsic apoptosis pathway [Gottlieb *et al.*, 2003]. It is known that the tumour suppressor gene p53 has a critical role in the regulation of Bcl-2 and other proteins of the Bcl-2 family [Meulmeester＆Jochemsen, 2008]. As we previously discussed, the p53 has probably a key role in the selective apoptosis induction in MCF-7 cells as compared with the other breast cancer cells used. However, by western blot analysis we did not observed significant changes in the total levels of p53 in the bLF-treated groups after 48 h of treatment (**Figure 4.5**). This does not exclude, however, that p53 may have an important function in the bLF-induction of apoptosis in MCF-7 cells, for example through its phosphorylation. A previous study with hLF showed, however, that induction of apoptosis in cancer cells occurred through a p53-independent mechanism [Damiens *et al.*, 1999].

Related with inhibition of cell proliferation, the levels of CDC25c were measured by western blot and shown to clearly decrease after bLF treatment for 48 h (**Figure 4.5**). Although it is not a key regulator in the G1/S transition of cell cycle, the downregulation of CDC25c suggests that bLF induced cell cycle arrest through the regulation of key factors in the cell cycle progression. This would not be dependent on DNA damage, as we previously observed (Chapter 3).

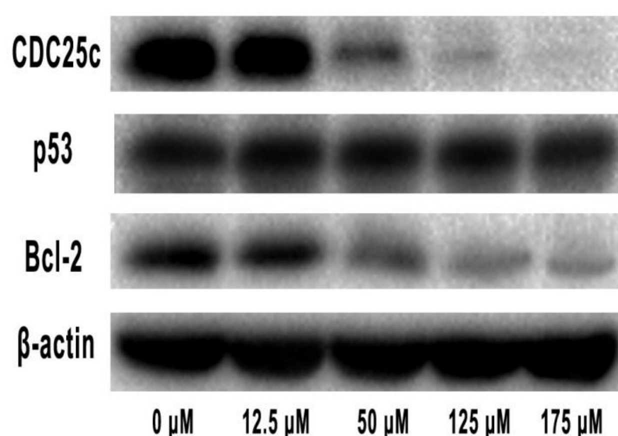

Figure 4.5 - Levels of Bcl-2, p53 and CDC25c in MCF-7 cells after treatment with bLF at several concentrations for 48 h, as measured by western blotting. The blots are representative of three independent experiments with similar results. β-Actin was used as loading control.

4.3.6 Lactoferrin significantly inhibits the colony formation

Considering that bLF inhibited cell growth of MCF-7 cells due to both cell cycle arrest and induction of apoptosis, we then performed a functional assay to determine the effectiveness of the bLF anticancer effect. Therefore, an anchorage-dependent colony formation assay was performed, which essentially tests the ability of a single cell to grow into a colony by undergoing cell division. Compared with the control (0 μM bLF), the bLF-treated cells exhibited a much lower cloning efficiency (**Figure 4.6A**). The number of colonies in the condition treated with 175 μM bLF could only reach 2.6% of the control (***P<0.001; **Figure 4.6B**). Additionally, DMEM media without FBS used as a positive control also inhibited the colony formation (**Figure 4.6A&B**). These findings are in accordance with other reported studies; for example, hLF was previously shown to significantly inhibit the colony formation of nasopharyngeal carcinoma cells [Zhang *et al.*, 2011]. The results gathered in the current work demonstrated that bLF could efficiently inhibit the colony formation of MCF-7 cells, thus suggesting that bLF could have a potential antitumor effect *in vivo*.

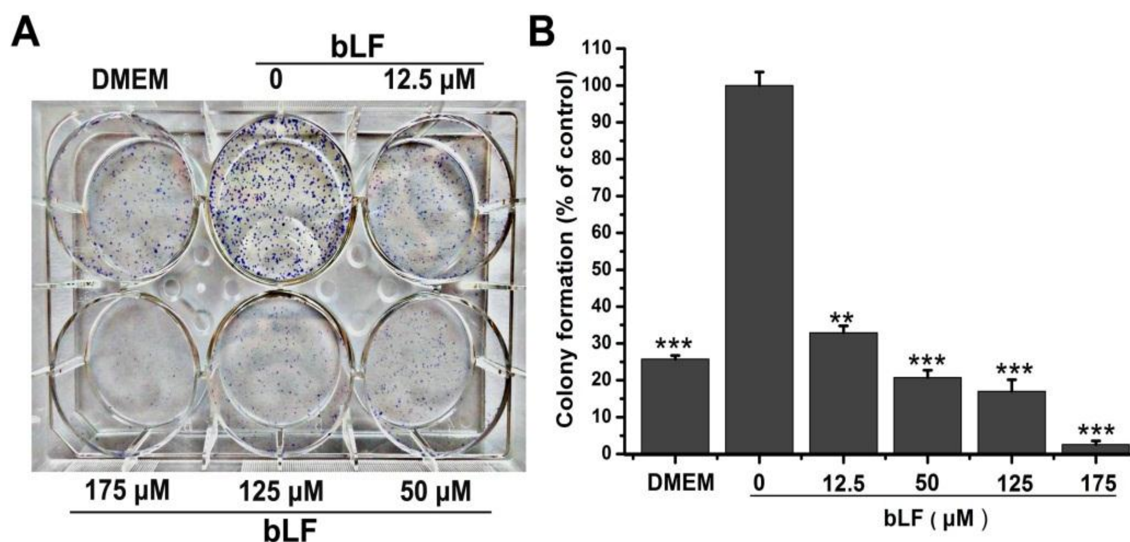

Figure 4.6 - Effect of bLF on the MCF-7 cells ability for anchorage-dependent colony formation. (**A**) Cells were seeded at low density and incubated with several concentrations of bLF and FBS-free medium (DMEM) for 24 h. Then, cells were left for 1 week to grow in complete medium to form colonies from single cells. Colonies were fixed, stained and images were captured. (**B**) Quantification of colony formation as mean ± SD of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$, when compared with the control (0 μM bLF).

4.3.7 Induction of cell migration by bovine lactoferrin in MCF-7 cells is not specific for this protein

Metastasis is the primary cause of mortality in most cancer patients. The repression of the metastatic process has been used as an important strategy in cancer therapy. Cell migration and invasion are the two key factors involved in the metastasis formation [Kramer *et al.*, 2013]. LF was previously described as a promoting factor for the migration of MCF-7 cells [Ha *et al.*, 2011]. Herein, we also tested the effect of bLF in cell migration and we indeed observed that this protein promotes the migration of MCF-7 cells at 175 μM [**Figure 4.7**]. To test if this effect was only due to the presence of a higher concentration of protein in the extracellular milieu, we also compared the results with a condition with the same concentration of BSA. As shown in **Figure 4.7**, cell migration was also greatly increased when cells were treated with BSA. Actually, BSA appeared to be more efficient in promoting cell migration than the bLF after 6 h of treatment. However, no significant differences were found between BSA- and bLF-treated groups after 12 h and 24 h of incubation. This result showed that the ability of bLF to induce cell migration is an unspecific property and it may result from a positive change of cell microenvironment due to the presence of a high concentration of exogenous protein.

4.4 CONCLUSIONS

In this chapter, bLF at high concentrations (up to 175 μM) was found to selectively induce apoptosis in MCF-7 cells among the four breast cancer cells under study. At low concentrations, no obvious bLF effect on apoptosis could be found (Chapter 3). This suggests that a certain amount of bLF is essential to stimulate the apoptotic event in MCF-7 cells. Moreover, apoptosis was found to be a delayed event that could only be observed at 48 h with mitochondria depolarization and at 72 h with DNA condensation and fragmentation. Furthermore, bLF significantly decreased the anti-apoptotic Bcl-2 protein, and in addition to the mitochondrial depolarization, it indicates that is the intrinsic pathway that may be involved in the apoptosis induction by this milk protein. The bLF inhibitory effect on the MCF-7 cells growth was shown to be associated with the cell cycle arrest at the G1 phase. Finally, the bLF anticancer potential against breast cancer cells was confirmed by its pronounced ability to inhibit the colony formation. Moreover, bLF promoted the migration of MCF-7 cells, but was shown to be an unspecific effect to this

protein, and probably not relevant in the *in vivo* context in view of the high concentration of proteins in the biological fluids.

Interestingly, since the mechanism by which bLF exerts its effect against MCF-7 cells was found to be similar to the one reported for hLF [Damiens *et al*., 1999; Lin *et al*., 2005; Yamada *et al*., 2008], bLF could be considered as a suitable substitute for hLF with the advantage that it can be produced in great amounts from bovine milk. On the other hand, the oral administration of bLF has been suggested as a good choice for chemoprevention strategies [Kuhara *et al*., 2000; Tanaka *et al*, 2000; Norrby *et al*., 2001]. Therefore, bLF could be developed into an efficient nutraceutical to cure or prevent cancer. Our findings provide further evidences that bLF could be considered for cancer therapy.

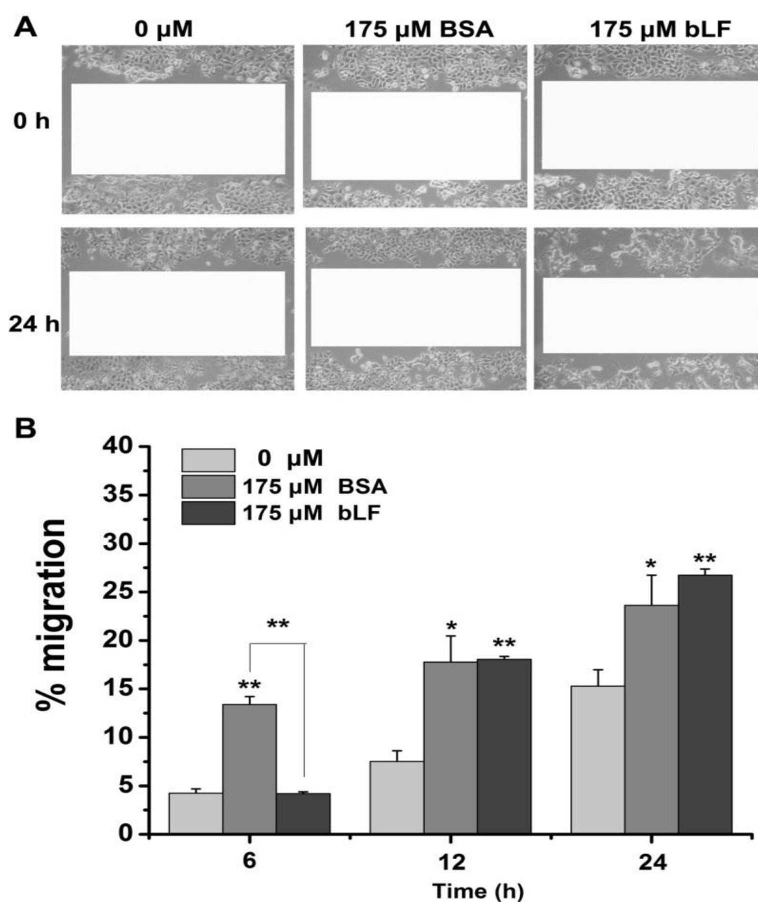

Figure 4.7 – Effect of bLF on the migration of MCF-7 cells. Cells were incubated with the same concentration of bLF and BSA for 6 h, 12 h and 24 h. (**A**) Representative images of cell migration into the wounded area after 24 h of incubation (Magnification: 40×) (**B**) Relative wound closure as measured by Image J software. Results correspond to the mean ± SD of three independent experiments. * $P < 0.01$ and ** $P < 0.01$ when compared with the control or among each other.

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Chapter 5

Conclusions and future perspectives

5.1 CONCLUSIONS

Four breast cancer cell lines (T-47D, MDA-MB-231, Hs578T and MCF-7) with different characteristics were used to evaluate the potential anticancer effects of bovine lactoferrin (bLF) and its derivatives. Similar anticancer effects could be found for the bLF extracted from milk and its variants as compared to the ones reported for human lactoferrin (hLF). The MTS results showed that bLF at the concentrations of 1.875 μM, 3.75 μM, 7.5 μM, 15 μM and 30 μM could efficiently inhibit the growth of the four breast cancer cell lines under study but showed very low effect to the normal breast cells (MCF-10-2A). Furthermore, comparing the results obtained for the bLF variants we found that holo-bLF possessed the highest growth inhibitory activity, while bLF and apo-bLF showed higher inhibitory effects than $LfcinB_{17-41}$ and $LfcinB_{26-36}$. However, no significant differences in the effects of LF and apo-bLF were found in T-47D and MCF-7 cells. In addition, bLF, holo-bLF and apo-bLF at low concentrations (0.25 μM, 0.5 μM and 1 μM) still inhibited the growth of T-47D and MDA-MB-231 cells. All these results suggest that the iron saturation of LF has an effect on its anticancer activity depending on the cell line, and provided some guidelines for the study of bLF and its variants in the future. Regarding the possibility of bLF cytotoxicity resulting from its degraded peptides, we used SDS-PAGE and Tricine-SDS-PAGE to evaluate the presence of peptides from LF degradation under cell culture conditions. The results showed that bLF was a very stable protein even after incubation with cells for 72 h. Moreover, bLF and $LfcinB_{17-41}$ labeled with a fluorescent dye could enter cells and were found to remain in cytoplasm. However, most of the protein stayed outside the cells, which indicated that the inhibitory effects of bLF on the breast cancer cells may also be due to the interaction between the protein and cell membrane or to the unfavorable adjustment of the cell microenvironment.

The mechanisms underlying the bLF cytotoxicity against cancer cells have not always been consistent among different reports. In the current work we found that bLF exerted its effects by preventing the cell cycle progression. However, bLF at concentrations lower than 30 μM could not induce apoptosis in the cancer cells, which was further confirmed by the lack of effect in the JNK and p38 pathways after bLF treatment. Additionally, bLF was found to increase the phosphorylation of ERK and AKT, respectively, in T-47D and MCF-7 cells in the short term. However, this increase in the signaling of these mitogenic kinases was not enough to counteract the inhibitory effects of bLF on the cells' proliferation. Also, bLF-induced cell cycle arrest did not result from double-DNA-strand breaks. Nevertheless,

the bLF treatment significantly decreased the phospho-mTOR and total mTOR expressions. Furthermore, a key regulator upstream the mTOR pathway, phospho-AMPKα, was clearly increased after bLF treatment compared to the control. These effects were found to be dose responsive. The AMPKα/mTOR pathway plays an important role in the cells protein synthesis and metabolism. The results herein gathered suggest that bLF exerted its growth inhibitory effects through the regulation of the AMPKα/ mTOR pathway.

Based on the above results, we hypothesized that, at higher concentration, bLF could exhibit different effects on the breast cancer cells. Interestingly, bLF at the concentrations of 12.5 μM, 50 μM, 125 μM and 175 μM induced significantly apoptosis in MCF-7 cells. However, no apoptotic cells were found for the remaining breast cancer cell lines used. In addition, in the case of the MCF-7 cells, bLF at high concentrations led to an obvious cell cycle arrest at the G1 phase. This was probably due to the regulation of the key proteins in the cell cycle progression, such as CDC25c. It is also important to mention, that we could confirm that bLF effects at high concentrations were not due to the interference of massive exogenous proteins in cell culture media. Besides, the induced apoptosis by bLF observed for MCF-7 cells was related with the mitochondrial membrane depolarization and the decrease of Bcl-2 levels. Moreover, the effect of bLF on the colony formation by MCF-7 cells indicated that it can be a potential drug to inhibit metastasis *in vivo*. Simutaneously, the promoting effects of bLF on the migration of MCF-7 cells should not be attributed to its biological function.

In short, bLF efficiently inhibited the growth of breast cancer cells through preventing cell cycle progression. Although it only induced apoptosis in MCF-7 cells, the growth inhibition of T-47D, MDA-MB-231 and Hs578T cannot be ignored. Due to its dietary characteristics, we believed that bLF could be used as an efficient anticancer drug or adjuvant in cancer therapy and prevention.

5.2 FUTURE PERSPECTIVES

Prior to this study, LF has already been pointed as an efficient anticancer agent against other cancer cell lines. Likewise, the current work succeeded in demonstrating the bLF cytotoxicity against breast cancer cells, besides providing some insights on the probable mechanisms involved in such effect. However, there are still many unknowns that remain to be elucidated and that can be recommended as future research topics.

In this study we conducted a comparison between the bLF variants growth inhibitory activities, and found that holo-bLF showed the most pronounced effect. Therefore, it would be interesting to evaluate the anticancer effects of this bLF variant against other cancer cell lines. On the other hand, since it seems that iron saturation of the bLF is involved in the protein anticancer activity, it would be relevant to clarify the mechanisms underlying the bLF variants effects against cancer cells. Furthermore, although the bLF-derived peptides herein studied showed lowest anticancer effects as compared to the protein itself, in further work these peptides could be modified to exert higher cytotoxic effects, since peptides are advantageous over proteins for clinical applications.

Additionally, the current work highlighted the probable mechanisms of bLF action against the breast cancer cells at low and high concentrations. Moreover, we found that bLF mainly stayed outside cells, thus probably functioning via both intracellular and extracellular pathways. The extracellular pathway was supposed to be the dominant one in the bLF anticancer activity. However, the results were not conclusive enough to confirm if the initial target of bLF is the cell membrane or microenvironment. The unknown signals lying in the upstream or downstream of the proven pathways in this study need to be further elucidated in the future. Moreover, both this work and most of the former reports were mainly conducted *in vitro*. Drugs usually have different performances *in vivo* since they have to go through complex steps before targeting the cancer cells, such as absorption, circulation in the body, metabolism and dilution in the blood. Therefore, the bLF effects against breast cancer have to be further evaluated through *in vivo* experiments.

The results from *in vitro* experiments gave good indications about the bLF anticancer effect, but understanding its mechanism of action is crucial before a clinical application can be foreseen. Nevertheless, being a dietary protein there should be no limitations in its use at least as a preventive agent, and later in cancer therapy. We believe that its relatively low cytotoxic effect to cancer cells as compared with known potent anticancer drugs, as

well as the unspecific mechanisms regarding the bLF action, is hampering its clinical use in cancer treatment. LF and its derivatives could be an efficient anticancer drug if combined with other therapeutics, or encapsulated in carriers after appropriate modifications. It is interesting to notice that some studies already began to use these approaches to strengthen the cytotoxic effects of LF or its derivatives. While combination therapy is becoming more and more popular today, the food-extracted protein will exhibit its potential value as an adjuvant or cancer drug in cancer treatment.

In summary, the results gathered in this thesis made an important contribution to the elucidation of the mechanisms underlying the bLF effects against breast cancer cells *in vitro*. Although many issues remain to be solved before bLF is used in the clinical practice, the suggestions herein made represent a step forward towards that reality. Furthermore, considering that there are still no efficient treatment strategies for most of the cancers, bLF being a dietary-derived protein should be an interesting topic of research in cancer chemoprevention.