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# Lactoferrin and cancer in different cancer models

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## 1. ABSTRACT

Lactoferrin (Lf) is a multifunctional protein and an essential element of innate immunity. Cancer is a major killer in today's world accounting for around 13% of all deaths according to the World Health Organisation (W.H.O.). The five most common forms of cancer include lung, colorectal, stomach, liver and breast cancer. Lactoferrin is a natural forming iron-binding glycoprotein with antibacterial, antioxidant and anti-carcinogenic effects. It is produced in exocrine glands and is secreted in many external fluids as a first line of defence. Lactoferrin also has the capacity to induce apoptosis and inhibit proliferation in cancer cells as well as restore white and red blood cell levels after chemotherapy. This review focuses on the therapeutic effect bovine sourced lactoferrin has on various forms of cancer in various models. It also focuses on the benefits of 3D *in vitro* cell culture. 3D cell culture has vast advantages over 2D models including demonstration of realistic therapeutic results and heightened resistance that 2D models fail to display.

## 2. INTRODUCTION

Current treatment for cancer involves dramatic therapies such as chemotherapy and radiation, as well as surgery all of which have heavy side effects and potential risks. The establishment of new therapeutic techniques is highly desirable and important. The fight against cancer involves many directions and aspects of treatment including the treatment of side effects from therapies like chemotherapy. Complimentary treatments are also necessary components. The use of lactoferrin as a natural supplement is already a mainstream immune boosting application. Lactoferrin however also has the potential for further anti-carcinogenic applications and has many advantages as a safe, natural compound with limited proven side effects.

## 3. LACTOFERRIN, CANCER AND CULTURE MODELS

### 3.1. Lactoferrin

Lactoferrin is an 80 kDa, transferrin-like, single chained glycoprotein found in purified milk capable of binding iron. It is also present in many exocrine excretions such as tears, nasal mucous, saliva, bronchial mucous, gastrointestinal fluids and cervico-vaginal mucous and seminal fluids (1-3.) Due to its presence in secretions that form a link with the external and internal environments, lactoferrin acts as an important first line immune defence (1). Produced and released by neutrophils, lactoferrin is an important anti-microbial, anti-

viral, anti-fungal, anti-parasitic and most interestingly anti-tumorigenic protein that is present in high concentrations in human colostrum as well as in that of other animals (1, 4-6).

### 3.2. Molecular structure and mechanism

Lactoferrin's iron binding capacity is due to two globular subunits each of which can tightly yet reversibly bind one ferric ion ($Fe^{3+}$) giving it potential antioxidant properties (3). This binding also involves the simultaneous binding of carbonate ions, $CO_3^{2-}$ (7). The two lobes are called the N and C lobe, each of which has two domains referred to as N1, N2, C1 and C2 respectively. Within each is of these domains lies the iron-binding site. The two lobes are connected by a 10-15 residue, 3-turn, alpha helical peptide. A feature that is unique to lactoferrin amongst the transferrin family. Also unique to lactoferrin is its ability to remain bound to iron at lower pH (~3) where transferrin will release its bound ions at a higher pH (~5.5) (7, 8).

In its natural state, lactoferrin is only partially saturated with iron allowing it to seek out iron from the external environment (3). There are two names for the states of Lf, apo-Lf and holo-Lf. Apo-Lf, or the closed structure is characterized by two bound iron molecules, in each of the two lobes, holo-Lf is therefore the open, iron free structure (9). Lactoferrin has a high affinity for iron due to 4 protein ligands that provide 3 negative charges to bind $Fe^{3+}$ and a helix N-terminus and Arg side chain to balance the carbonate anion (9). This means that iron may be tightly bound, released only by receptors or at pH levels low enough to destabilise the protein. In addition to $Fe^{3+}$ binding, Lf can bind other metals including trivalent ions such as $Ga^{3+}$ and $Al^{3+}$ and some anions (9).

### 3.3. Expression

Retinoic acid, growth factors, nutrition and especially estrogen are all transcription cues for the lactoferrin gene. Lactoferrin genes are responsive to both estrogen and estrogen-like receptors, with an estrogen response site close to the genes promoters allowing estrogen control of transcription (10). Like most protein synthesis, lactoferrin transcription is also dependant on chromatin modification and co-regulator recruitment (10).

### 3.4. Lactoferrin and immunity

Reacting with the mucosa in the small intestine, orally administered lactoferrin stimulates cytokine production, increases natural killer cell, macrophage and cytotoxic T cell activity as well as inducing an immunological cascade (1, 11, 12). Orally administered bovine Lf (bLf) stimulates an intestinal immune function where NK cell numbers and cytotoxicity increases, as does the production of interferon-γ (IFN-y) (12). This was demonstrated in a study in 2006 where bLf was administered orally to mice where after treatment, NK cell levels and interleukin-18 (IL-18) levels all rose as well as IFN-y. In IL-18 knockout mice, the increase in NK cell levels did not occur indicating a link between the two. Also using gene knockout mice, Iigo demonstrated an immune pathway involving lactoferrin where orally administered bLf activated IFN-y and caspase 1 expression that in turn induced IL-18. This pathway was present in normal mice yet not in GKO mice (1) Orally ingested lactoferrin has also been shown to increase the levels of cytokines and Th1 (T-helper cells), which generate $CD8^+$ T cells via the activation of IL-18 (1, 13). Increasing the levels of these factors continues to boost the levels of NK cells and immune cell proliferation. The expression of caspase 1 may also lead to apoptosis, as it is a pro-apoptotic enzyme.

A study into the immune systems of healthy males was conducted where lymphocyte counts, T cell activation, NK cell cytotoxicity, serum cytokine levels and serum capacity were monitored closely during a trial with orally ingested bLf. 100mg and 200mg supplementation for 7 days each was carried out where afterwards, immune systems of the males showed significant activation of various components including T cell activation and hydrophilic antioxidant capacity indicating that lactoferrins role as an immuno-modulator includes boosting immune surveillance (4).

### 3.5. Lactoferrin and digestion

When ingested orally, pepsin in the stomach digests lactoferrin to a peptide termed lactoferricin which has shown great antimicrobial properties especially the bovine form which is more potent than the human form (2). Lactoferricin has been shown to decrease the growth rate and cell proliferation of a colon cancer cell line CaCo-2. When treated with varying concentrations of lactoferricin, CaCo-2 cells demonstrated changes in the cell cycle (elongation of the S phase due to lower level of the protein cyclin responsible for its transition) resulting in reduced cell proliferation (14) In 2009, Iigo demonstrated also that lactoferricin in particular increased the level of cytokine production and increased the level of immune surveillance allowing for anti-carcinogenesis properties due to the heightened capacity to remove tumour cells. It has been demonstrated however that the majority of lactoferrin, around 60%, resists degradation in the intestine and unless chronically ingested, is absorbed readily in an antigenic form directly into the blood stream (15-16).

### 3.6. Oral lactoferrin

For *in vivo* studies, lactoferrin is administered orally and is thought to activate immune cells and associated lymphoid tissues in the small intestine (11). In the intestine, the human lactoferrin receptor is capable of binding both human and bovine lactoferrin and allows absorption of iron attached to lactoferrin, especially in infants fed from breast milk in their first 6 months (17). As well as in the gastrointestinal tract, lactoferrin receptors are also found on leukocytes, macrophages, platelets and some bacteria (15)

Most cells are capable of binding Lf, including immune cells such as macrophages, monocytes and dendritic cells (18). This direct binding to immune cells is what allows lactoferrin immuno-modulation and regulation. Through this interaction, lactoferrin encourages migration,

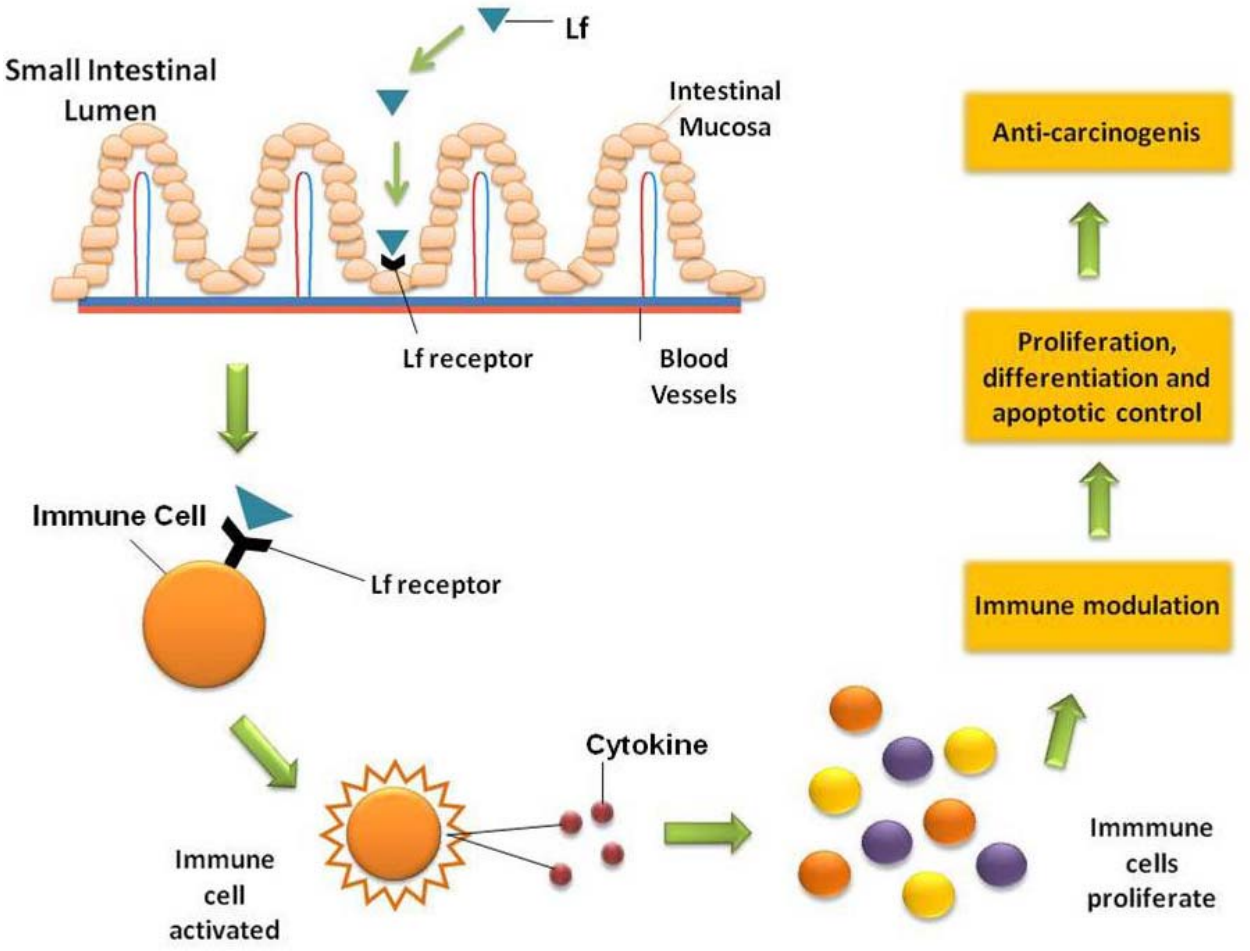


**Figure 1.** Lactoferrin enters the body through the intestinal mucosa either directly through to cells or via lactoferrin receptors. Once through, Lf reacts with proteoglycan-chain receptors on immune cells such as macrophages, monocytes and dendritic cells. This increases immune cell proliferation and cytokine production through cytokines such as IL-18 and IFN-y to increase activity, cytotoxicity and abundance of NK cells, cytotoxic T-cells and macrophages. This increased immunity increases surveillance and the potential to remove malignant cells and cause cell death (1, 18, 19)

activation and proliferation of immune cells as well as the expression of cytokines and effector molecules (18). Sulphated proteoglycan chains on the surface of cells are responsible for low affinity, high density binding as well as some lipoproteins (19). These protein chains are the lactoferrin receptors (LfRs), and have been found on mammalian cells and human monocytes as well as binding sites on dendritic cells.

Oral lactoferrin supplied in the diet of chemotherapeutically treated, tumour-containing mice showed both increased immune and anti-tumour properties. Iron-saturated lactoferrin was used and shown to have a greater effect. The outcome in the mice was restoration from chemotherapeutic damage by increasing both red and white blood cell levels, increase of Th1 and Th2 cytokine such as TNF and IFN-y (Figure 1). In addition, Fe-bLf induced anti-tumour properties such as increased anti-tumour cytotoxicity, tumour apoptosis and infiltration of tumours by leukocytes (20).

### 3.7. Lactoferrin and apoptosis

Lf has been shown to activate both extrinsic and intrinsic apoptotic pathways through activation of different caspases. Via the activation of caspase 8 in the extrinsic pathway, apoptosis was initiated in colon cancer cells (21) and studies on both colon cancer and leukemia cells have shown treatment with Lf to lead to the initiation of both caspases 3 and 9 (22). Caspase 9 is an initiator caspase in the intrinsic pathway. The latter studies into the intrinsic pathways also demonstrated that these caspases where increased without increasing Bcl-2, a known anti-apoptotic protein (22). Through the activation of initiator caspases 8 and 9, the caspase cascade may be started, activating effector caspase 3, which induces the final step of apoptosis. Apoptosis is a controlled form of cell death, leaving surrounding cells intact and undamaged, a desirable trait when combating cancer cells to minimize or eliminate damage to healthy tissues.

Further apoptotic properties of Lf have been demonstrated. It is thought that lactoferrin increases the

expression of Fas, a known tumour necrosis factor relative that also leads to apoptosis of tumour cells. This was shown in the colon mucosa of tumour-induced rats with heightened levels of both apoptotic cells and the Fas protein (21) In addition to apoptosis of cancer cells, early studies on lactoferrin also indicated that it may also lead to inhibition of angiogenesis, an important step in tumorigenesis (23). This was demonstrated in an *in vitro* study using mice and rat cells.

Recent studies however have shown that both selenium and iron saturated bLf (Se-bLf and Fe-bLf) have had greater success in inducing apoptosis in colon cancer cells. Due to the presence of caspase 9 and absence of caspase 8, the apoptosis was induced via intrinsic pathway (24). Caspase 3 was also detected along with Bax, a known pro-apoptotic protein that counters the effect of Bcl-2, an anti-apoptotic protein.

**3.8. Lactoferrin and cancer**

An annual study conducted by the American Cancer Society indicated that cancer prevalence overall is on the decrease. This being the case it was still predicted that over 1.4 million new cases would present and over 500,000 deaths would occur rendering the disease still a major concern (25). In 2004, the WHO estimated that cancer caused around 13% of all deaths around the world, around 7.4 million (26). WHO also projected that by the year 2030, cancer will kill 12 million people per year. In 2006, American statistics indicated that cancer accounted for up to 23% of deaths (27), highlighting the significance of cancer in the western world. The top five most common forms of cancer are lung, colorectal, stomach liver and breast with the most common forms of cancer in men being prostate and breast in woman (25).

For many treatments and pharmaceutical applications, natural products and bioactive molecules have strong advantages due to the relative abundance and availability, suitability, ease of administration via the oral route, cost effectiveness and immuno-compatibility with the host (3). Lactoferrin as a natural compound has the capacity to boost the immune system as well as potential as a chemo-preventative agent against carcinogenesis. *In vivo* studies have shown that Lactoferrin has inhibited colon, esophagus, lung and bladder cancer in rats and has induced apoptosis in colon epithelial cancer cells and leukemia in mice (3) (Table 1).

Many studies have been carried out into the effect of lactoferrin on colorectal cancer. One example in 2009 showed that colorectal polyps, potential adenomas, were greatly retarded in human participants who ingested either 1.5 or 3.0 g capsules of bLf daily in a double blind, placebo controlled experiment. Polyps where monitored by regular colonoscopy's and it was suggested that ingestion of bLf could inhibit colorectal carcinogenesis and serve as an alternative to the attempt to remove polyps (28).

Lactoferrin has shown potential in the treatment of head and neck squamous cell carcinoma as a chemotherapeutic. A study in 2007 focusing this form of carcinoma showed that lactoferrin has the potential to induce $G_1$-$G_0$ growth arrest as well as deter the release of pro-inflammatory and pro-metastatic cytokines, increasing the immune systems activity. Mice were administered human recombinant lactoferrin orally after tumour implantation and demonstrated up to 75% tumour reduction and 20-fold increase in lymphocytes (29).

An *In vitro* study on canine mammary gland tumour cells incubated with bLf demonstrated positive results in the anti-proliferation of cancer cells. Two cell lines where grown and both demonstrated growth arrest in the G1 stage, an effect not observed in normal, controlled canine mammary gland cells (30). Although it is not known the exact mechanism of lactoferrins anti-tumorigenic functions, it has shown potential in chemo-preventative and anti-carcinogenic therapeutics.

**3.9 *In vivo m*odels**

*In vivo* or animal models provide the most realistic results for the effects of therapeutics. They can demonstrate systemic effects and can demonstrate side effects which are common in chemotherapies. *In vivo* models allow determination of targeted effects especially if the therapeutic is taken orally and needs to be transported to a particular organ, tissue or cell as in the case of lactoferrin.

**3.10 *In vivo* models, lactoferrin and cancer**

*In vivo* studies on cancer and lactoferrin treatment have been conducted in mice, rats and humans. Studies on humans tend to be clinical trials generally after animal and *in vitro* studies have proven successes. The human subjects are therefore individuals with already diagnosed forms of specific cancers. Extensive studies on mice and rats have been carried out in all aspects of scientific and medical research including cancer. Mice and rats used in the experiments are induced with tumours. One method is by implantation as seen in a study by Wolf in 2007 where cancer cells where injected into healthy mice. Tumours developed in both the head and neck squamous cells and were observed after 5 and 10 days, with and without treatment with lactoferrin (29). A second method for tumour induction involves treating healthy animals with a tumour forming chemical. Azoxymethane is a commercially available chemical that induces colon tumours used in various studies including those using lactoferrin (21, 31-37). Once the tumours have developed, therapies can be administered and observed.

*In vivo* models are an extensive research model yet are restricted as they take longer to conduct, set up and maintain as well as involve ethics approval and considerations. They do however provide a more realistic platform for study and are essential in the clinical trial phase of therapeutic approval.

**3.11. *In vitro m*odels**

*In vitro* models are the most abundant and easiest models for studying cancer cells. Cancer cells are grown either in tissue or cell culture in laboratory settings and behave quite differently to normal cells. Cancer cell lines

**Table 1.** Effect of lactoferrin on various cancer types

| Cancer | Model | Organism | Description | Reference |
|---|---|---|---|---|
| Colon | *In vivo*, *In vitro* | Rat, Human | Inhibition of colon carcinogenesis | (14, 35, 47) |
| Colorectal Polyps | *In vivo* | Human | 3.0g oral dose daily retarded growth of polyps, potential adenomas | (28) |
| Lung & Esophagus | *In vivo* | Rat | Dose dependant tumour inhibition, 0.2% to 2% bLf in diet | (48) |
| Bladder | *In vivo* | Rat | Dose dependant tumour inhibition, 2% bLf in diet | (49) |
| Head and Neck Squamous | *In vivo*, *In vitro* | Mice | Dose dependant tumour inhibition and cancer cell growth arrest | (29) |
| Mammary Gland | *In vitro* | Canine | Cancer cell growth arrest | (30) |
| Lymphoma | *In vivo*, *In vitro* | Mice | Iron-saturated bLf a potent natural adjuvant for augmenting cancer chemotherapy such as paclitaxel, doxorubicin, epirubicin or flurouracil | (20) |
| Lewis lung carcinoma | *In vivo*, *In vitro* | Mice | Iron-saturated bLf a potent natural adjuvant for augmenting cancer chemotherapy such as paclitaxel, doxorubicin, epirubicin or flurouracil | (20) |
| Melanoma | *In vivo*, *In vitro* | Mice | Iron-saturated bLf a potent natural adjuvant for augmenting cancer chemotherapy such as paclitaxel, doxorubicin, epirubicin or flurouracil | (20) |

Form sof cancer, organism type and specific effect of lactoferrin on both *in vitro* and *in vivo* models.

are transformed and immortal. They can continue to divide indefinitely compared to normal cells, which have a limited number of replications. In terms of cancer both 2D and 3D models are very important each with their own advantages.

### 3.12. 2D models

A good starting point for cancer cell research is in 2D monolayer cell culture. Immortal cell lines derived from cancer tumours in respective diseased organisms are used and maintained using this model. Cell lines themselves however can be difficult to establish depending on the organ of origin. For example, melanoma cells are tougher form to establish (38).

Cells are grown in single layers on flat plastic or glass surfaces such as on slides, plates or in wells or flasks. They require specific nutrient medium depending on the cell type, typically containing fetal serum (38). Due to their nature, cells adhere to these surfaces and continue to replicate in a uniform layer. Through addition of substances to the media, cells can be manipulated and treated with therapeutics and observed/studied to determine effects. 2D models provide simple, easy methods to observe preliminary effects in cancer cells. Often cells to be used for 3D or even *in vivo* methods are maintained and transplanted from 2D models (29, 39). In terms of lactoferrin, all *in vitro* studies to date have been performed on 2D monolayer's including studies on colon, melanoma and mammary gland cells (3, 30).

### 3.13. 3D models

3D models provide an important link between 2D cell culture and *in vivo* models. Without the requirement to have the whole host or animal present, 3D models simulate the *in vivo* environment including demonstration of proliferation, differentiation, morphology, drug and chemical response, apoptosis, gene expression and cell signaling (40, 41). Drug and chemical responses demonstrated in 3D models are often different to those demonstrated in 2D models due to 2D cells being forced to exhibit unrealistic, flat structure, reducing their normal functions (42). The advantages of 3D models over 2D models include the capacity to demonstrate more real time physiological reactions and the observation of more realistic structure (39) In addition, 3D models exhibit a microenvironment including stroma, an element lacking in 2D cultures. Stroma components, such as fibroblasts (40), can also be included in 3D cultures increasing the realism of the microenvironment (41). Transport, well defined environment, architecture, multiple cell phenotypes and stromal-tumour, stromal-cell interactions are further advantages of 3D cultures (41).

Unlike *in vivo* models, 3D models are best for short-term use and struggle to show progression of conditions and some cell-cell interactions including immune functions and vasculature. This model system however can be used to study stem cells, development of organs, tissues and tumours at a closer, molecular level (39). The mechanism and characteristic of cancer genes and tumorigenesis can be studied and both cell-cell and cell-matrix interactions can be observed (43). Cells used to grow in 3D models can be taken from a number of sources including cell implantation following propagation in 2D models (common with cell lines) or microscopic tumours from embryos or tissue samples (39). Even though 3D models don't show the complete molecular effects demonstrated in animal models, they can still give a stronger result to that shown in 2D models and can provide an excellent starting point in a relatively short period of time before commencing studies *in vivo*.

### 3.14. Cell matrix

3D models require a layer of matrix for the cells to grow on, acting as the basement membrane. This encourages the cells to form their destined structure such as lobular, milk producing hollow balls in the case of mammary gland epithelial cells (43). The presence of the ECM in 3D models changes the dynamic of the cell growth and interactions in relation to transport and drug, hormone and growth factor responses (41). The matrices used in 3D models can consist of many substances including reconstituted basement membrane from animals, collagen, fibrin or polyacrylamide gels (43, 44). The source of the

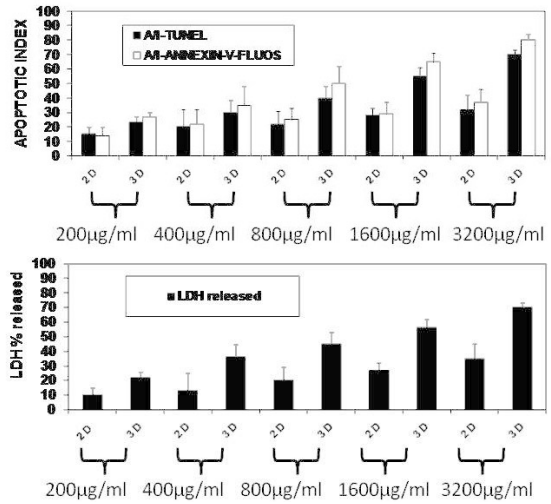


**Figure 2.** Comparison of apoptosis (A) and cytotoxicity (B) in colon cancer cells (CaCo-2) in 2D and 3 D models after treatment with Fe-bLf (20, 50) Cell death (apoptosis; % apoptotic index) and cytotoxicity in CaCo-2 cells after 24 hour treatments with bLf Cell cytotoxicity assay (LDH release assay) results show CaCo-2 cells after treatments with Fe-bLf at concentrations of 400, 800, 1600, 2400 and 3200 µg/ml. Mean percentages were calculated from the range between high (100%) and low (0%) controls in each assay. (A) Apoptosis indices (AI) after 24hour incubation with the Fe-bLf treatments. (B) Cytotoxicity levels after 24 hour incubation. Data expressed as mean percentages (±SE) obtained from 6 repeat wells in each group.

ECM is very important especially in tumour studies and in studies where tumour and stroma elements are both incorporated, ECM is also produced by some stromal cells (41).

### 3.15. 3D models and cancer

There are many methods and culture systems that may be used to study cancer. Due to the extensive pathways that carcinogenesis can take; it can be a lengthy period for advanced or invasive tumours to develop. This means that it advantageous to find a model that allows relatively fast results without compromising the realism of the study.

3D culture systems have been useful in studying tumorigenesis and cancer gene relations within the microenvironment. Tumorigenesis is determined by the interaction between pluripotent tumour stem cells and the microenvironment (39) Phenotypic differences in cells have been demonstrated in studies with known cancer genes in 3D conditions including invasion properties of cells (42) An important element of cancer development is the stroma. If abnormal stroma is present, it has the capacity to induce or enhance the level of tumorigenesis in normal cells (39, 41). Tumour-stroma interactions also enhance the level of carcinogenesis and can increase the size of tumours. This mechanism is due to changes in matrix metalloproteinases, inflammatory cell recruitment and alteration of stromal signals (39, 41).

When grown in 3D, epithelial tumour cells tend to form aggregate structures called spheroids. These clusters of cells can consist all of one type of cell or can be made up of many types including tumour, stromal or immune cells. Due to the nature of these spheroids, some cells are closer to the surface and hence more oxygenated while others become hypoxic, deep within the cluster where they can secrete tumour cytokines (39). Also within these spheroids, proliferating and non-proliferating cells exist. In epithelial cell cancers, the characteristic hollow lumen often becomes filled with cells in the initial stages. This process was studied using 3D culture that identified apoptotic cells responsible for forming the lumen as targets for oncogenes (42).

In 2010, Li. concluded that 3D models have the potential to model the resistance to cytotoxic drugs that tumour cells display, where 2D models do not. This multi-cellular resistance shows a much closer link to *in vivo* studies. It was also demonstrated that 3D models may also hold the key to identifying oncogenic pathways and inhibitors that may lead to therapeutic advances (45-49) Initial *in vitro* experiments on lactoferrin were done on 2D cell culture models. However 3D models are closer and reliable to *in vivo* experiments. We compared the anti-cancer activities of lactoferrin in 3D model and compared with 2D cell culture model and found significant anti-cancer activities as for apoptosis and cytotoxicity is concerned. The complete anti-cancer activities/mechanism of lactoferrin on cancer cells in 3D models is yet to be elucidated however indications from our initial experiments and other studies on other therapeutics would suggest that using lactoferrin in 3D models would provide excellent insight into the predicted effects *in vivo*. We prepare 3D adhesion substrates and cells were suspended in 2% alginate solutions (50). The apoptotic and cytotoxicity levels of the various treatments with Fe-bLf were determined using kits (Roche) as described (20). These 3D *in vitro* models can be used in the large screening tests in research and development of anti-cancer drugs (Figure 2).

### 3.16. 3D models and breast cancer

Breast cancer is the leading form of cancer in amongst women in the western world. It is as common as 1 in 8 and can take between 5 to 30 years to develop hence why *in vivo* studies are not ideal as they can take up to a decade 3D models are therefore highly favorable (41, 43).

When grown in three-dimensional systems, mammary epithelial cells assume a particularly distinct architectural structure. Also termed mammospheres, epithelial cells form hollow, spheroid balls capable of producing milk (43). In *in vivo* conditions, such structures are called acini and form the functional part of the mammary gland. Spheroids consist of a layer of polarized epithelial cells and grow either on or within a matrix such as the commercially available Matrigel$^{TM}$ (42)

An important feature in epithelial cell cancer is cellular polarity. Polarity refers to the defined domains of epithelial cells, one basal the other apical (46). Disorganisation of cellular polarity can lead to

tumorigenesis and cell proliferation (41) Polarity is regulated by the stroma which highlights the importance for its presence in 3D cultures and why 2D cultures, when studying tumorigenesis and cancer development, has disadvantages(41). As with all therapeutics, pre-clinical and clinical trials are essential. All levels of cell culture and animal model research are important in this long-term process. Research must begin at the cellular level with 3D models providing a more solid grounding to represent the cell culture stage of therapeutic development. If cancer treatments can be shown to be effective in 3D models, they will have a much higher chance of being successful in the further stages of development and even approval.

## CONCLUSION

Lf possesses multiple roles which turn it into either a weapon or a shield in the host defense system. The beneficial effects of Lf administered in prevention or treatment of infectious pathologies and have emphasized the importance of Lf in the regulation of immunity. The data reviewed here establishes bLf as a natural compound which has anti-carcinogenesis activity. Ingestion of bLf is both effective and safe. Experimental animal models suggest that a likely mode of action of ingested bLf is enhancement of immune function, in particular the immune function of the gut-associated lymphoid tissue. bLf or more likely its proteolytic peptide fragments induces expression of various cytokines. These cytokines would in turn function in immune physiology. Interferons inhibit cell proliferation and induce the expression of numerous apoptotic effector proteins. IL-18 inhibits angiogenesis, resulting in inhibition of tumorigenesis. Other bLf induced cytokines with the activation of immune effector cells and are involved in several of the signaling pathways associated, resulting in an enhanced immune response against transformed cancer cells. Due to the great potential 3D models have to offer research, it would be ideal to develop functioning models and systems to represent real scenario diseases. With cancer being so prevalent in society among other diseases, the benefits of developing therapies in this area are obvious. It is expected that researches in this field will lead to further progress. In the future, novel effects of orally administered bLf and mechanisms of action will be shown. New generation nanodelivery systems will be devised to make it more efficient delivery and bioavailability to diseased organs. Results of these studies will open up new application opportunities of bLf for various human diseases including cancer. 3D *in vitro* models has the potential for rapid and valid *in vitro* model to screen chemotherapeutic drugs with a feature to mimic *in vivo* models.

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