Isolation and characterization of a new cell line from spontaneous mouse mammary tumour, MBL-6, for *in vivo* cancer studies

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Abstract

In search for treatments against breast cancer, cell lines are one of the basic resources, particularly as *in vitro* models. Additionally, animal models of cancer are used as the successive step in therapeutics research. In this regard, human breast cancer cell lines provide fundamental models *in vitro*. However, *in vivo* studies require immunodeficient mice, which lack the influence of other *in vivo* factors such as the native microenvironment and the immune system. There are few standard models to study the pathogenic mechanism at molecular level and cell signaling pathway of breast cancer. In this study, a new mouse breast cancer cell line, MBL-6, was successfully established and characterized from tissues of a spontaneous mammary tumor. The cell line had epithelial morphology, formed adherent monolayer, maintained continuously *in vitro* and was able to form new tumors when injected subcutaneously in syngeneic mice. The growth pattern and metastasis evaluations revealed a considerable *in situ* duration before invading distant organs. Real time polymerase chain reaction (PCR) analysis showed the expression of ER-, PR- and Her-2 receptors. The chromosome analysis showed numerous chromosomal abnormalities. Aggressive tumorigenecity in tumorigenesis test and the IC50 to cyclophosphamide (CTX), celecoxib (CLX) and cisplatin (CPN) was also evaluated. The numerous tests performed on the new MBL-6 cell line suggest that it is in good quality and may be used in animal models of breast cancer studies.

Introduction

Breast cancer is one of the leading causes of cancer related deaths in women worldwide. 235,030 new cases and 40,430 deaths have been estimated in 2014. The chance of developing breast cancer is one in eight for women in the United States.[1-3] Many researches are conducted in hope of finding new therapies and preventions of breast cancer and cell lines are the principle working material. Many human and animal cell lines have been isolated for *in vitro* and *in vivo* studies. A number of human cell lines are routinely used as breast cancer models, such as MCF-7, BT20, MDA-MB-231, MDA-MB-435s, and T-47D, which have been well-known in culture for over 30 years.[4] Although studies begin with *in vitro* cultures, they proceed to *in vivo* and animal studies before extending into clinical trials.[5,6] The process of *in vivo* studies with human cell lines requires nude mice as the host for growth. However, the findings may only be applicable to the effects on the cancer cells only, and the role of immune system and the related microenvironment signals are omitted. In this regard, mouse cell lines, as a genetically close species, can be utilized in anti-cancer studies. In this regard, many animal models of cancer are developed either by carcinogens or spontaneously formed tumors are preserved by routine subcutaneous transplantations.

Spontaneous Mouse Mammary Tumor (SMMT) is an invasive ductal carcinoma formed in the breast tissue of Balb/c female mice. This tumor is able to form new tumors when transplanted subcutaneously. This model has the advantage of spontaneous formation without the addition of carcinogen. It has a durable amount of in situ time before metastasizing to lung, liver, spleen and lymph nodes. Therefore, it is an ideal model to study the cancer biology and novel therapeutic agents *in vivo*. However, the transplantation of tumor tissue is not an accurate model and requires surgery on the recipient host. Thus, we attempted to isolate the cancerous cells, which can be useful for pre-*in vivo* evaluations of various agents and monitor the serial growth of tumor stages through to metastasis and the various influential factors in this process.

Materials and Methods

Isolation

A tumor bearing female mouse was sacrificed and tumor tissue was aseptically isolated. SMMT tumor specimen was washed five times in Hanks' balanced saline and minced with scalpel into 1–3 mm^3 pieces. Explants of SMMT were cultured in Dulbecco minimum essential medium (DMEM, Gibco) with 30% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified incubator containing 5% carbon dioxide. After 2 weeks, fibroblastic colonies were formed and the explants were moved to a new container in which out growths of epithelial cells were then evident. Tissues were removed and the media was supplemented with selective mammary epithelial media containing DMEM/F12, 5% FBS, 5μg/mL insulin, 1μg/mL hydrocortisone and 10 ng/mL EGF. Cell growth was monitored and gradually, the growth factors were removed from the culture media within a week. The ability to grow in serial passages was evaluated by trypsinization at 80% confluence or bi-weekly intervals and retrieval after cryopresevation.

Characterization

In vitro population doubling time

Population doubling time is a measure of tumor aggressiveness, which serves to prognosticate, measure therapeutic success, and quantify tumor kinetics and growth rate. In this regard, passage 10 MBL-6 cells were counted and cultured at the starting number of 5000 cells in 24 well plates. At 80% confluence, cells were trypsinized, counted and re-plated. The PDT evaluation was repeated three times from passage 10 cells and the average cell number was used for the final calculations. This process was repeated for nine passages (30 days) and *in vitro* doubling time was calculated using the exponential curve equation.

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In vitro tumorigenesis

Tumor cells show independence to anchorage signals for growth, which is accounted as one of the hallmarks of cancer and is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells. The anchorage-independent growth (or soft agar tumorigenesis assay) was performed using a previously established method. Briefly, 1% agar (merk) was melted by microwave and kept warm in 56°C water bath. The melted agar was diluted with 2X DMEM and 0.5 mL was dispensed in 24 well plates. The bottom agar was left to cool under sterile conditions. MBL-6 Cells were harvested and pipette well into single-cell suspension in complete culture media (10% FBS DMEM) at 2500 cells/mL To make the top layer, equal amounts of melted 0.7% agarose (Fermentase) and 10% 2X DMEM was mixed and a concentration of 2500 cell/mL was added. Gently, 0.5 mL of top layer was added onto the bottom layer and was left to set under cell culture safety cabinet. 0.25 mL media was added and incubated in 37°C. The media was exchanged every other day for 3 weeks. Then the colonies were fixed and stained with 0.005% crystal violet. Photographs of the stained colonies were taken and counted using imageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016.).

Karyology

Sub-confluent cells at passage 23 were used for chromosome analysis. Cells were treated with colcemid (0.1 μg/mL) and metaphase cells were collected after 4-hour exposure. Afterwards, cells were incubated in 0.075 M KCl hypotonic solution at 37°C for 35 min. After fixing with a mixture of methanol (Merk) and glacial acetic acid (Merk) (3:1, v/v), cell suspensions were spread onto cold slides. The slides were dried in 70ºC and stained with 3% Giemsa for morphological and chromosomal analysis.

Quantitative relative real-time polymerase chain reaction

The expression of Her2, progesterone (PR) and estrogen (ER) receptors, E-cadherin and VEGF was evaluated using real-time polymerase chain reaction (PCR). Real-time PCR was performed on Corbett thermal cycler with rotor-gene 6000 software (Qiagen). Briefly, Cells were plated at 2 × 10^6 in six-well plates 24 h prior to harvest. After 24 h cells were rinsed twice with PBS, and RNA was prepared using a Trizol extraction method (Invitrogen, CA, USA). cDNA was synthesized from 5 μg of total RNA with 10 mm of random hexamer primer (Metabion, CA, USA) and RevertAid™M-MuLV Reverse Transcriptas (Invitrogen, CA, USA), according to manufacturer's instructions. PCR reactions were performed in triplicate with a 25μL final reaction volume consisting of 4 μL DNA, 1×TaqMan® Universal PCR Master, AmpErase® UNG, 500 μm. For real-time PCR studies, cDNA samples were diluted 10-fold and PCR amplification and analysis were performed with StepOnePlus Real Time PCR System (Applied Biosystems, CA, USA). The SYBR® GreenER™ qPCR SuperMix (Invitrogen, CA, USA) was used for all reactions. Primers used were the following: HER2 primers (FW: ATCAAACGAAGGCGACAG, RV: ATCCAGATGCCCTTGTAGAC), PR primers (FW: AAATCTCTGCCAGGTTTCC, RV: AAATATAGCATCTGTCCACTGAC), ER receptors (FW: TGGAGATTCTGATGATTGGTC RV: CATTTACCTTGATTCCTGTCC), E-cadherin primers (FW:TCGAAACATGCAGTTCTGCCAGAGG, RV: TCATGCAGTTGTTGACCGTCCCTTC) VEGF primers (FW: AACTTTCTGCTCTCTTGGG, RV: CTTCGCTGGTAGACATCC). A standard PCR condition was used. Beta Actin was used as the house keeping gene (FW:CTTCTT GGGTATGGAATCCTG, RV:GTGTTGGCATAGAGGTCTTTAC).

Mycoplasma contamination

The cell line cultures were routinely tested for the presence of Mycoplasma by PCR. Briefly, cells were trypsinized and 10^6 cells were washed twice with PBS. The pellet was suspended in 1 mL Trizol (Cinagen) lysis buffer and transferred into a 1.5 mL eppendorf tube. Of chloroform 250 μL (Merk) were added and mixed vigorously. After 15 min incubation in room temperature, cell lysate was centrifuged at 12,000 g for 15 min at 4°C. Nucleic acid layer was transferred to a new 1.5 mL Eppendorf tube and precipitated with 600 μL isopropanol, kept at -20°C for 30 min and centrifuged for 15 min at 4°C. Pellet was washed with 70% ethanol, air dried and re-suspended in 20 μL DW. RNA content was determined using Eppendorf AG biophotometer (Thermo Scientific, MA, USA) and cDNA was synthesized as described above. 100 ng of sample DNA and 1 ng of positive control (genomic DNA from *Mycoplasma orale*) were used for PCR amplification at an annealing temperature of 55°C. PCR primers used were as follows: FW: 5 -TGCGTAGATATWWGGAAGAACAC-3 ; RV: 5 -RGATGTCAAGAGTGGGTAAGG-3 .

Drug sensitivity

Sensitivity to common chemotherapy agents can be a useful tool in novel experimental anti cancer agents as controls. Briefly, 10^4 cells were seeded in a 96-well plate. After 24 hours, cells were treated with serial dilutions of cyclophosphamide (CTX), celecoxib (CLX) and cisplatin (CPN). After 48 hours, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. A standard cell-MTT curve was used to estimate the number of remaining cells. The number of remaining cells was plotted to find the cell responses and the IC50 dose.

In vivo tumorigenesis, growth and metastasis

SMMT is a tumor of Balb/C origin. Therefore, Balb/C female were used as the host to examine the ability of MBL-6 tumorigenesis in vivo. 10^6 MBL-6 cells were inoculated subcutaneously into the right flank of 6-week-old female Balb/c mice (Pasteur Institute, Tehran, Iran). Mice were monitored until the tumor was palpable. Then the tumor size was measured 3 times a week using a digital vernier caliper (Mitutoyo, japan) and tumor volume was calculated using the following equation: where V= volume, L= length, W= width and H= height, until they reached a lethal volume. The lethal volume was assumed as the volume bearing more than 5×10^9 cells. The number of tumor cells was estimated using the following formula: where *D*=tumor diameter and *d*= tumor cell diameter. Here the tumor cell diameter was assumed as 10 μm. The animal protocol was reviewed and approved by the Animal Care and Research Committee of the Stem cell technology research center, Tehran, Iran. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. Mice reaching the lethal volume were sacrificed and various organs including liver, lung, spleen and lymph nodes were isolated, fixed and were paraffin-embedded. Of sections 5 μm were stained with H&E and analyzed for traces of tumor colonies by a pathology specialist.

Results

Isolation

The cultured cell line was checked regularly for changes in morphology and growth pattern. The newly established cells grew as adherent monolayer with characteristic epithelial morphology. The cultured cells maintained consistent morphology from the primary culture to the subsequent subculture passages. Cells have shown continuous growth for over 9 months and have undergone more than 40 passages. They appeared to be permanent cell lines since growth continued after recovery from multiple cryopreservation (Figure 1).

Population doubling time

The growth kinetics of MBL-6 was studied at

passage 10. Figure 2A represents the growth curve in 10 subsequent passages. Cells were trypsinized, counted and replated accordingly. Cell count was plotted against time and the curve equation was used to calculate the population doubling time. The *in vitro* population-doubling time of MBL-6 was calculated as 23 hours.

Additionally, 10^6 MBL-6 cells were inoculated in the right flank of 8 female mice. After 2 weeks, palpable tumors appeared. When the tumors reached a measurable size, the growth was monitored. MBL-6 was able to induce a stable growing tumor in the host. All eight mice developed tumors with similar growth rate and morphology. The average tumor size was plotted against time and the curve formula was used to calculate the population doubling time. The mean *in vivo* doubling time was calculated as 70 hours (Figure 2B).

Anchorage independent growth

Anchorage-independent growth is one of the characteristics of cancer cells and a hallmark of cancer. Soft agar growth was assessed in 3% agarose. The cultured cells were able to form colonies in the agarose layer. Colonies of MBL-6 were evident at the end of week one and at the end of three weeks the colonies were fixed with 4% paraformaldehye and stained with 0.005% crystal violet (Figure 3). Colonies were counted manually using an inverted microscope and the percent rate of colony formation was 10.4% (130±12 colonies from 1250 seeded cells).

Karyology

Chromosomal analysis was performed on MBL-6 cells in their logarithmic phase of growth. Methaphase cells were fixed and standard Giemsa staining was used for chromosomal analysis. The chromosome number ranged from 34-77 with a median of 56-60 (Figure 4). The karyotypes were mostly multiploid and many chromosomal abnormalities such as fractured chromosomes were observed.

Gene expression analysis and Mycoplasma contamination

The melting curve analysis was performed for verifying the correct product according to its specific melting temperature (Tm). We investigated the expression of HER-2, PR and ER receptors, VEGF and E-cadherin genes in MBL-6 cell line. Statistical analysis of Real-Time PCR results with REST® software illustrated that the MBL-6 cells expressed HER-2, PR and ER receptors, VEGF and E-cadherin mRNAs. Additionally, PCR analysis showed that the cell lines were negative for the presence of Mycoplasma (Figure 5).

Drug sensitivity

Cells were treated with various concentrations of three routine anti cancer chemotherapies and the growth inhibition curve was used to calculate the IC50. As shown in Figure 6, the IC50 for CTX, CLX and CPN was 0.776676 mg/mL, 2.520542 µm and 7.158757 µm respectively.

In vivo metastasis

Figure 7 shows the H&E stains of collected organs from tumor bearing mice at the late stages. As evident, colonies of MBL-6 cancer cell had spread to the regional lymph nodes, liver, spleen and the lung of the tumor-bearing mouse.

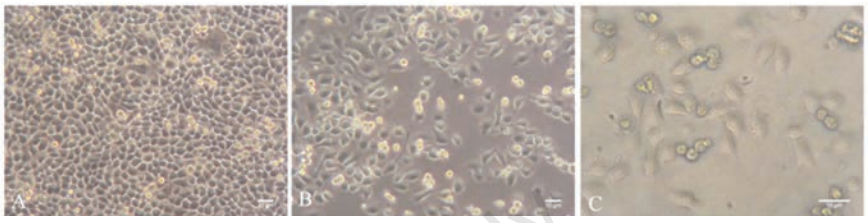

Figure 1. Morphology of isolated MBL-6 cells from explants and the subsequent cultures. Cells possessed an epithelial morphology and formed adherent monolayer in culture. A) 10x; confluent cells with polygonal morphology is evident; scale bar: 20 µm. B) 20x; sub confluent culture; scale bar: 10 µm. C) 40x; sub confluent culture; scale bar: 10 µm.

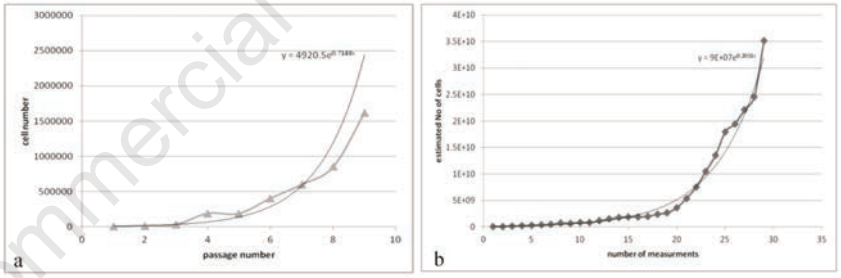

Figure 2. *In vivo* tumorigenesis passage 10 cells were counted and plated; 80% confluent cells were trypsinized, counted and plated. After 10 passages cell count was plotted and population-doubling time was calculated using curve equation, which was equal to 23 hours; 5×10^9 passages 21 cells were subcutaneously inoculated. Then the tumor size was measured 3 times a week and *in vivo* tumor growth was plotted and the doubling time was calculated as 70.8 hours using the curve equation.

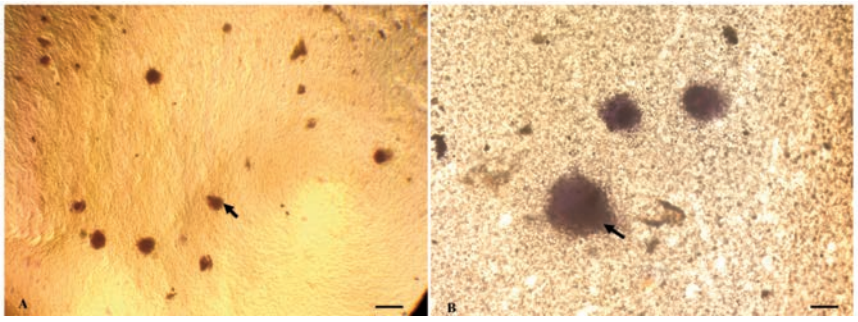

Figure 3. Soft agar tumorigenesity assay. MBL-6 cells were cultured in 3% agarose and the ability to form a colony (anchorage independent growth) was evaluated. Three weeks later, colonies were fixed and stained. A) 4x, scale bar: 100 µm. B) 20x, scale bar: 20µm.

Discussion and Conclusions

Breast cancer is one of the major culprits for women's death worldwide.[2] Many researches are conducted to elucidate the basis and finding new therapies. In this regard, well-characterized cancer cell lines are important resources for studying anti-cancer therapies and are a tool for elucidating the cancer cell biology.[4] A number of advantages count for their wide use; easy to handle, infinite number of cells, readily available from frozen stocks and high homogeneity. However, they have the disadvantage of genotypic and phenotypic drifts and the heterogeneity of the primary tumor is lost in cell culture.[7]

In this study, we report the establishment and characterization of a new mouse breast cancer cell line derived from a spontaneously developed invasive ductal carcinoma. The established cell line had an epithelial morphology and grew as adherent monolayer in tissue culture and formed colonies in soft agar. Cells showed features of an established cell line, the ability to endure continuous passage for more than 40 passages over 9 months and recovery after cryopreservation. The primary tumor was obtained from spontaneous tumor growth without the addition of carcinogens or from transgenic animals. This brings the advantage of a cell line with a more native background and lowest level of manipulation, providing a more close simulation of breast cancer initiation in human. The moderate growth rate and the delay in metastasis compared to other murine breast cancer cell lines, makes MBL-6 an attractive model for studying breast cancer progression and for new therapeutic strategies *in vitro* and *in vivo*.

Gene expressions analysis showed that the MBL-6 cell line expressed ER, PR and Her2 receptors. As most spontaneous human tumors are,[8-10] this triple positive cell line may be used in hormone therapy studies. Gene expression study also showed the expression of E-cadherin, which is an indicator of epithelial origin.[11] Additionally, the expression of VEGF suggests that this model also can be utilized in anti-angiogenesis studies.

The immune system is an essential component of the tumor microenvironment.[12] Many studies have shown the role of immune system in tumor progression and regression.[13-16] Established in immune-competent mice, the in vivo model of this cell line can be used as a basic study tool for immunotherapy and immune-modulation studies.

The ability to invade distant organs is one of the hallmarks of cancer.[17] It additionally characterizes the mode of cell growth. The current model showed that it has the ability to metastasize to lymph nodes, lung, spleen and liver at later stages. This study revealed that in early

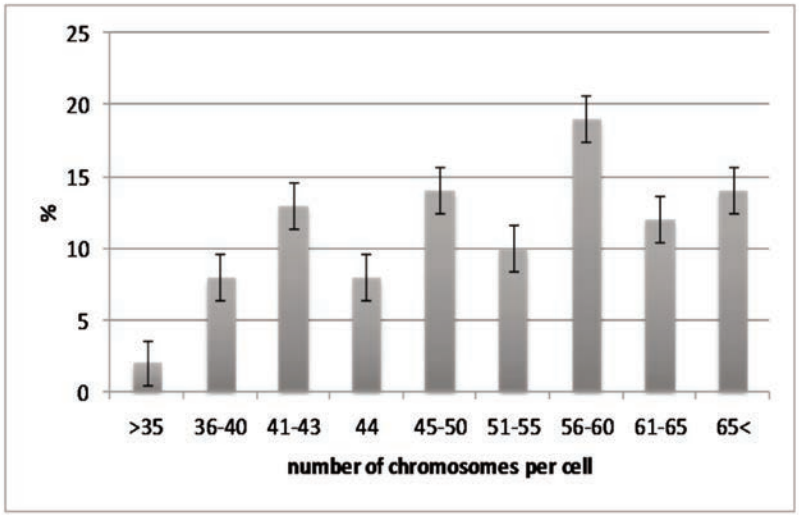

Figure 4. Distribution of chromosome numbers in MBL6 cell line. Cells in their logarithmic growth phase were treated with calcemide, fixed and stained with Giemsa. Chromosomes were counted in 1000 cells and were analyzed for chromosomal abnormalities.

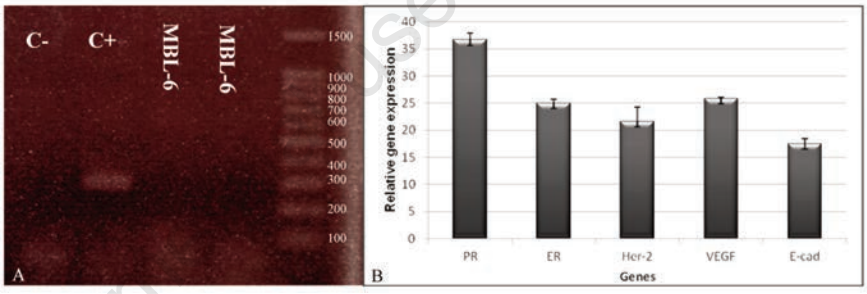

Figure 5. Mycoplasma contamination and relative gene expression were evaluated using RT-PCR and real-time PCR. A) RNA extracts were used for PCR reactions. Genomic DNA from *Mycoplasma orale* was used as positive control. A reaction without DNA template was used as negative control. PCR results of MBL-6, showed no evidence of mycoplasma contamination. B) the relative expression of PR, ER, Her2, VEGF and E-cad genes were analyzed using real-time PCR. Results indicated a significant expression in PR, ER and Her2 mRNAs. Also the cell line had high amounts of VEGF and E-cad mRNA.

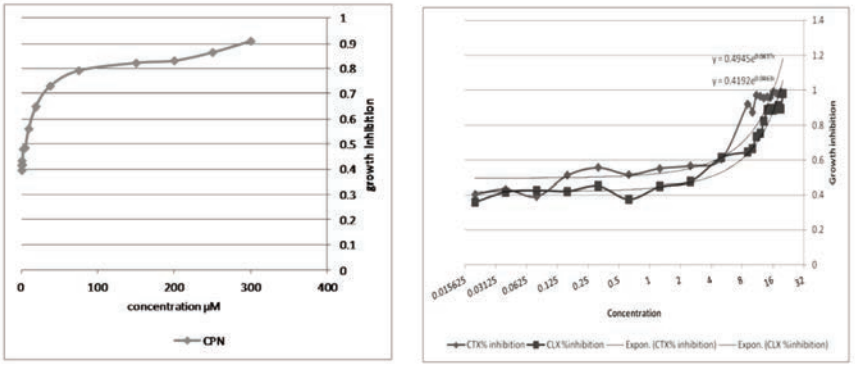

Figure 6. Drug sensitivity assay. Sensitivity to cisplatin (CPN), cyclophosphamide (CTX) and celecoxib (CLX) was evaluated using MTT assay. After 48 hours, the number of remaining cells was plotted to find the cell responses and the IC50 dose. According to the growth inhibition curve, the IC50 for CTX, CLX and CPN was 0.776676 mg/mL, 2.520542 µM and 7.158757 µM respectively.

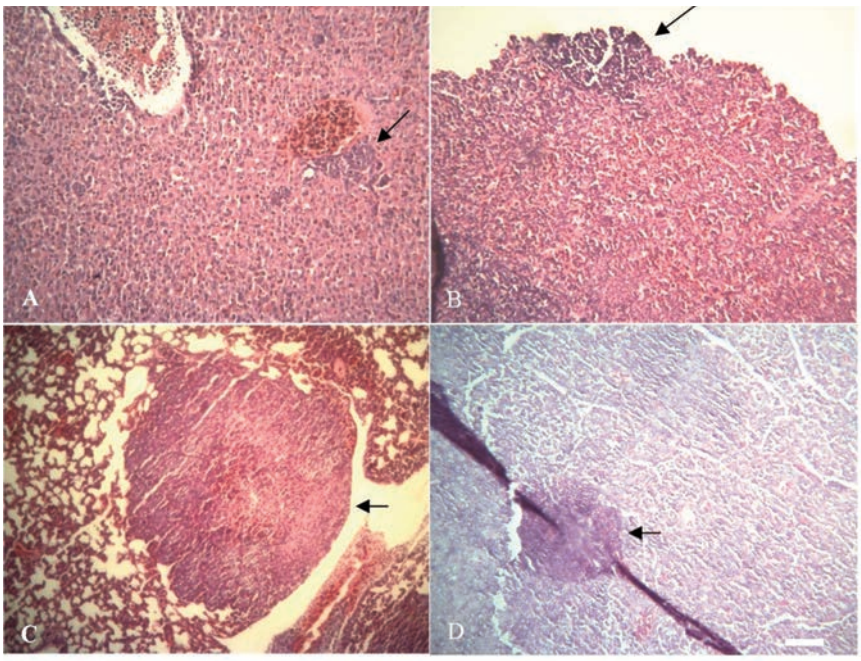

Figure 7. Distance organ metastasis of MBL-6. A) liver, B) spleen, C) lung, D) lymph node. Tumor sizes were monitored and when they reached the lethal size (containing 5×109 cells) mice were sacrificed and various organs were fixed. H&E sections were analyzed for traces of malignant growth. All four organs showed growth of tumor cells. Scale bar: 100μm.

stages cells primarily invade the draining lymph nodes and later on move to other organs. Metastasis via lymph node is one of the properties of breast cancer lines. The fact that this line has a durable amount of *in situ* growth prior to invasion makes it an ideal tool for studying the biology of cancer cells and the mechanisms of and therapeutic strategies against metastasis.

References

1. Berterö C, Wilmoth MC. Breast cancer diagnosis and its treatment affecting the self: a meta-synthesis. Cancer Nurs 2007;30:194-202.
2. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9-29.
3. DeSantis C, Ma J, Bryan L, Jemal A. Breast cancer statistics, 2013. CA Cancer J Clin 2014;64:52-62.
4. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004;83:249-89.
5. Boone CW, Kelloff GJ, Malone WE. Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: a review. Cancer Res 1990;50:2-9.
6. Begley CG, Ellis LM. Drug development: raise standards for preclinical cancer research. Nature 2012;483:531-3.
7. Burdall SE, Hanby AM, Lansdown M, Speirs V. Breast cancer cell lines: friend or foe? Breast Cancer Res 2003;5:89-95.
8. Engel RH, Kaklamani VG. HER2-positive breast cancer. Drugs 2007;67:1329-41.
9. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. Clin Med Res 2009;7:4-13.
10. Parise CA, Bauer KR, Brown MM, Caggiano V. Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999–2004. Breast J 2009;15:593-602.
11. Oka H, Shiozaki H, Kobayashi K, et al. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Res 1993;53:1696-701.
12. Allavena P, Mantovani A. Immunology in the clinic review series; focus on cancer: tumour associated macrophages: undisputed stars of the inflammatory tumour microenvironment. Clin Exp Immunol 2012;167:195-205.
13. Kerkar SP, Restifo NP. Cellular constituents of immune escape within the tumor microenvironment. Cancer Res 2012;72:3125-30.
14. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. Annu Rev Immunol 2011;29:235-71.
15. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat rev cancer 2006;6:24-37.
16. Colotta F, Allavena P, Sica A, et al. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 2009;30:1073-81.
17. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.