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ORIGINAL ARTICLE

Nephronectin is Decreased in Metastatic Breast Carcinoma and Related to Metastatic Organs

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Abstract Breast cancer causes death mostly due to distant metastasis. During metastasis, cancer cells create new conditions in which normal tissue structure can be disturbed. Nephronectin, which is the primary ligand for $\alpha 8\beta 1$ integrin, plays an important role in kidney development. There are conflicting findings regarding its role in cancer progression and metastasis, especially in breast carcinoma. The aim of this study was to determine changes in nephronectin expression in primary tumor tissues and metastatic visceral organs, using metastatic and non-metastatic cell lines in a mouse model of breast cancer. In our study, 4T1-Liver Metastatic and 4T1-Heart Metastatic cells, originally derived from 4T1-murine breast carcinoma, and non-metastatic 67NR carcinoma cells were used. Cancer cells were injected orthotopically into the mammary gland of 8–10 week-old Balb-c mice. Primary tumors, lung, liver tissues were collected on 12th and 25th days after the tumor injection. Immunohistochemistry was used to determine expression of nephronectin in tissues. We also investigated the expression levels of the protein by using western blot technique. We found that lung and liver tissue of control animals (not-injected with tumor cells) expressed nephronectin which was lost in animals bearing metastatic tumor for 25 days. In accordance, nephronectin staining of lung and liver was preserved in animals injected with non-metastatic 67NR tumors. These results demonstrate that loss of nephronectin may play an important role in formation metastatic milieu for cancer cells. This is the first study demonstrating that tumor-induced loss of nephronectin expression in visceral organs in which metastatic growth takes place.

Keywords Nephronectin · Breast cancer · Metastasis · 4TLM · 4THM

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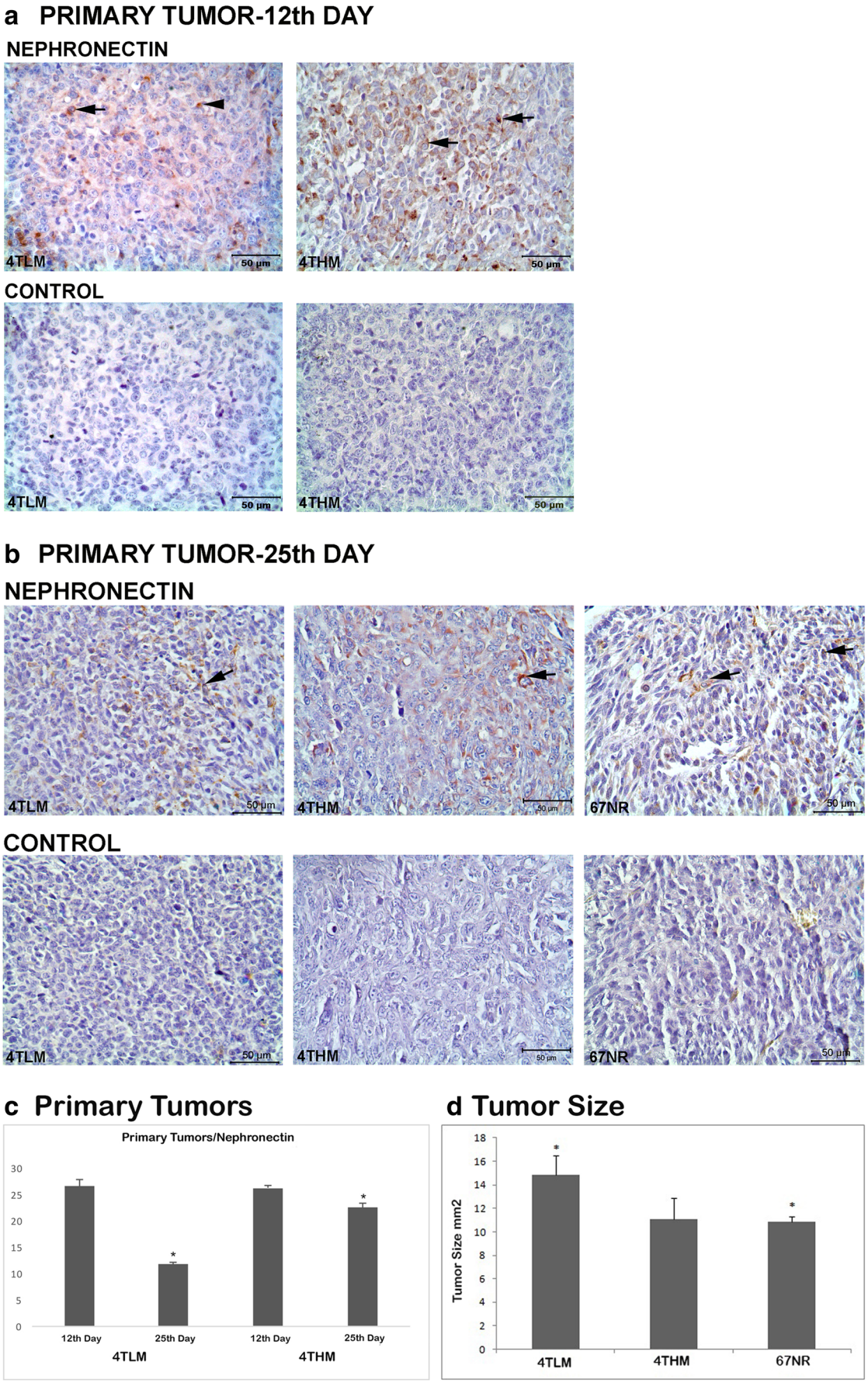
Introduction

Breast cancer is one of the most common malignancies and is the leading cause of cancer deaths in women [1, 2]. The main cause of death in breast cancer is metastasis to distant organs [3, 4]. Breast cancer frequently metastasizes to lymph nodes, lung, liver, bone and brain [5].

Tumor cells can modulate structure and composition of extracellular matrix (ECM) [6–10]. The destruction of the ECM is an important step for tumor cells to escape from their primary site and to invade blood vessels. The ECM and basement membranes also have ECM proteins that release cytokines and growth factors which can increase tumor growth [11].

Invasive breast cancer has a complex microenvironment, which contains different types of cells and ECM proteins, which play important roles in tumor progression, angiogenesis, invasion and metastasis [10, 12, 13]. There are other mechanisms such as synthesis, degradation and cross-linking of the ECM [9, 12, 14] which can modulate cancer cell behaviors [10].

Nephronectin, also known as POEM (preosteoblast epidermal growth factor-like repeat protein with meprin, A5 protein, and receptor protein-tyrosine phosphatase 1 domain), is an extracellular matrix protein. It has been shown that the protein also plays a key role in kidney development.


a PRIMARY TUMOR-12th DAY
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4TLM
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CONTROL
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b PRIMARY TUMOR-25th DAY
NEPHRONECTIN
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4THM
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67NR
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CONTROL
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c Primary Tumors
Primary Tumors/Nephronectin
30
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d Tumor Size
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◀ **Fig. 1** Nephronectin expression in primary tumors formed by 4TLM, 4THM and 67NR cells. Panel (**a** and **b**) nephronectin expression 12 days or 25 days after innoculation of tumor cells orthotopically (into the mammary pad) respectively. *Arrow* shows tumor cells expressing nephronectin (characterized by large nucleus and small cytoplasm). *Arrow head* shows stromal cells expressing nephronectin (likely to be infiltrating immune cells. Majority of the nephronectin positive cells were stromal cells. As seen, majority of tumor tissue (over 95%) was composed of tumor cells with large and/or irregular nucleus and small cytoplasm in 4THM and 4TLM tumors. Majority of cells in 67NR tumor were also cancer cells which had spindle shape and irregular nucleus. Control is a negative-control section. *Scale bars* represent 50 μm. **c** Comparisons nephronectin expression in 4TLM, 4THM and 67NR. $*p < 0, 05$ is a statistically significant difference. **d** Tumor size compared with 67NR, 4TLM and 4THM groups. $*p < 0, 05$ is a statistically significant difference

Nephronectin is associated with α8β1 integrin, which is an extracellular matrix receptor, possesses strong cell adhesion, spreading, and survival-promoting activities through its own RGD motif [15, 16]. Erin et al. reported that nephronectin gene expression decreased in 4THM primary tumors and liver metastasis of 4THM tumors compared with 4T1 primary tumors [17]. 4THM cells were originally obtained from heart metastasis of 4T1 cells [18, 19]. Similarly reduction of nephronectin expression in malignant melanoma in comparison with primary melanocytes were reported [20]. Furthermore wild type nephronectin expression was found to associate with less invasive and migratory potential in comparison with the controls [17].

On the other hand Eckhardt et al. demonstrated increased expression of nephronectin in metastatic breast cancer cells and suggested a role for nephronectin in carcinogenesis and metastasis [21]. Since the role of nephronectin in carcinogenesis is not clear; our goal in this study was to examine changes in nephronectin in metastatic breast carcinoma compared to non-metastatic breast carcinoma.

Factors (chemokine's, cytokines etc.) secreted from primary tumors establish a new microenvironment in distant tissues. For instance, immune cells in this metastatic environment infiltrate visceral tissues before metastasis [22–24]. It is, however, not known how the tumor secreted factors alter extracellular matrix proteins, particularly nephronectin at metastatic sites.

Our second aim was to investigate expression and distribution of nephronectin protein in metastatic organs such as liver and lung by using immunohistochemistry and Western-blot analysis. Our study was the first that attempted to determine nephronectin immuno-localization in primary tumors and metastatic organs such as, lung and liver tissues, which are obtained from animals that have been injected with cells from metastatic and non-metastatic breast carcinoma cell lines.

Material and Methods

Animals

Female Balb/c (Kobay, Turkey) mice were kept under a 12 h light–dark cycle and were fed a standard diet. All experimental protocols conducted on mice were performed in accordance with the standards, which have been established by the Institutional Animal Care and Use Committee at Akdeniz University School of Medicine.

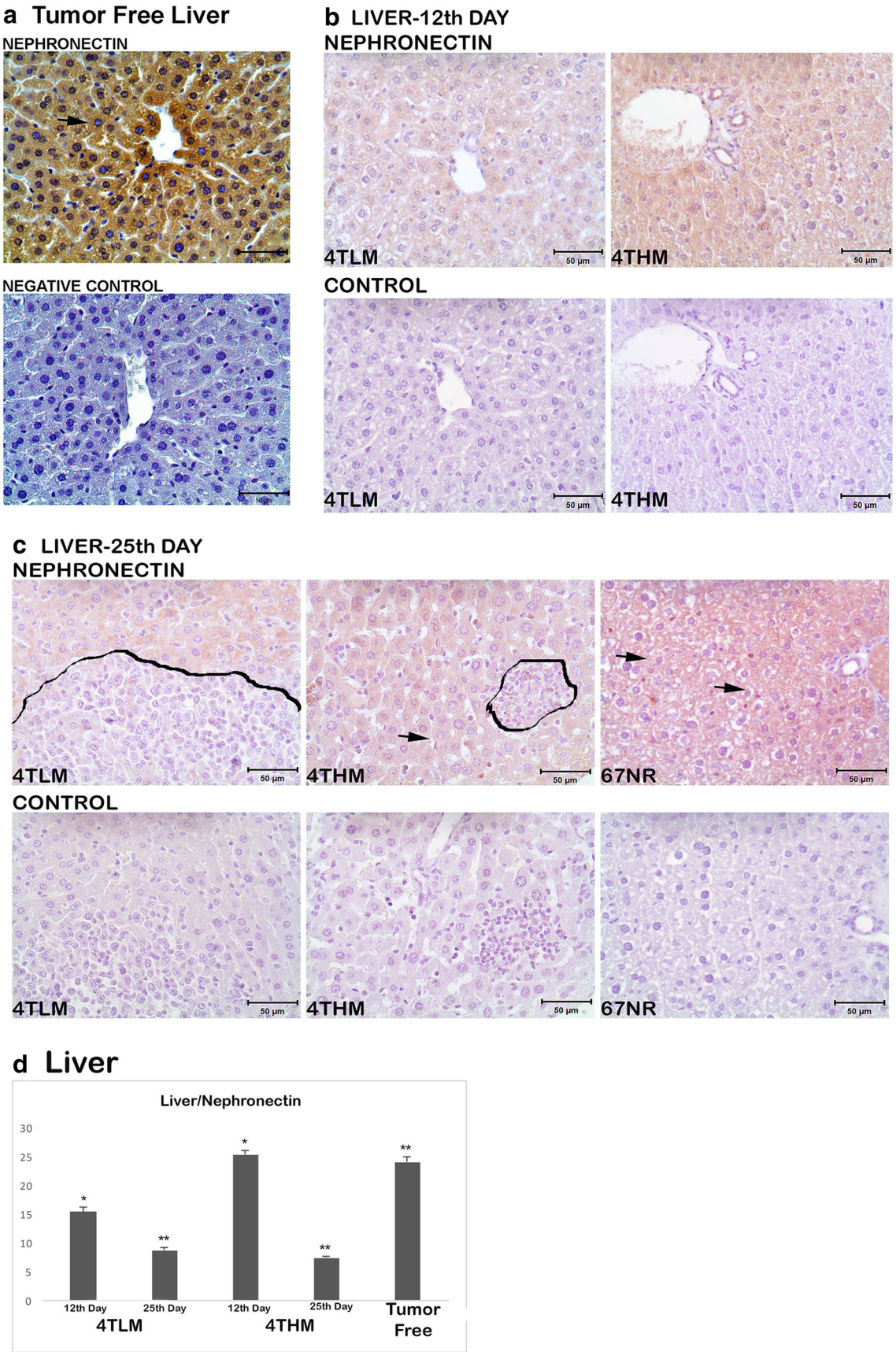
Cell Lines

67NR, 4TLM and 4THM cells were previously derived from spontaneously formed breast tumors in Balb/c mice. When implanted in Balb/c mice, the 67NR cells form mammary tumors that do not metastasize, while the 4T1 mammary tumors are able to spread to distant organs and grow in them [25]. 4THM is derived from heart metastasis of 4T1 metastatic mammary tumors. 4TLM is derived from liver metastasis of mammary tumors formed by 4THM cells [5]. 4TLM and 4THM cells were grown in DMEM-F12 (Invitrogen; #11320074, Waltham, Massachusetts, USA) supplemented with 5% FBS (fetal bovine serum) (Invitrogen; #10270106), 2 mM L-glutamine (Invitrogen; #25030024), 1 mM sodium pyruvate (Invitrogen; #11360039), and 0.02 mM non-essential amino acids (Invitrogen; #11140035) [17].

Metastasis Assay

Mice were divided into three experimental groups as follows: injected orthotopically with 4TLM, 4THM and 67NR non-metastatic breast carcinoma cells. Twelve animals were used for each group. Confluent cells (75–80%) were used for orthotopic transplantation. Equal numbers of 4TLM and 4THM cells in HBSS (10^5 cells per mouse) were injected into the right upper mammary gland just beneath armpit of Balb-c mice under ketamine/xylazine anesthesia (30 mg/kg/10 mg/kg, i.m.). Since formation of 67NR tumors requires implantation of higher numbers of cells, 10^6 cells per mouse were injected orthotopically in 67NR group [18, 19].

Necropsies were performed on 12th (midpoint) and 25th (endpoint) days after injection. 67NR groups of animals only sacrificed 25th days. Therefore, tumor size of 12th days is small and undetectable. We used 6 animals of each techniques in primary tumors and metastatic organs, liver and lung, were removed for immunohistochemistry and Western blotting analysis. At the endpoint time, tumor size was measured before the sacrifice.


a Tumor Free Liver
NEPHRONECTIN
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c LIVER-25th DAY
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◀ **Fig. 2** **a** Nephronectin expression in hepatocytes of control Balb/c mice (tumor-free, not injected with tumor cells). Hepatocytes of tumor-free mice were intensely positive nephronectin staining. **b-c** Nephronectin expression levels in liver tissues 12th and 25th days after the injection of tumor cells. Control is a negative-control section. Metastatic lesions (demarcated) which were detectable 25th days after the injection of tumor cells (at the endpoint time) were largely negative. *Scale bars* represent 50 μm. **d** Image-J analysis of nephronectin expression in hepatocytes of mice injected with 67NR, 4TLM or 4THM cells as well as tumor-free mice. */**$p < 0, 05$ is a statistically significant difference

Immunohistochemistry

Primary tumor, lung and liver tissues were fixed in 10% formalin and embedded in paraffin. Five micrometers-thick sections were cut and were collected onto poly-L-lysine-coated slides. The slides were deparaffinized in xylene, rehydrated in a decreasing gradient of ethanol solutions. An antigen-retrieval procedure was performed by heating the samples with Citric Acid Buffer (pH 6,00) (Merck, 1–00244-1000) in a microwave oven at 804 W for 5 min, and after that cooling in this buffer for 20 min at room temperature. The sections were blocked for endogenous peroxidase activity with methanol containing 3% H_2O_2 for 15 min, and for nonspecific binding with universal blocking reagent (Thermo; TA-125-UB) for 7 min at room temperature. Then the slides were incubated in a humid chamber at 4 °C overnight with primary antibodies such as rabbit anti-nephronectin (Abcam; #ab64419, Cambridge, MA, USA) 1/200 diluted in dilution buffer (Abcam; #ab64211). For negative controls, the primary antibodies were replaced by normal rabbit IgG serum (Vector Lab. Burlingame, CA, USA) at the same concentrations. After several washes in PBS, sections were incubated for 1 h at room temperature with secondary antibody in biotinylated goat anti-rabbit IgG (1/400 dilution Vector Lab; #BA1000). Following washing steps with PBS, sections were incubated by using HRP-streptavidin-peroxidase complex (Invitrogen; #85–9043) for 20 min at room temperature. Antibody-antigen complexes were visualized by incubation of 3,3′-diaminobenzidine (DAB) (Sigma; #D4186) chromogen. Sections were counter-stained with Mayer's hematoxylin (Merck; #1-09240-1000), dehydrated and coverslips with entellan, and examined via a Zeiss-Axioplan (Oberkochen, Germany) microscope.

Immunocytochemistry

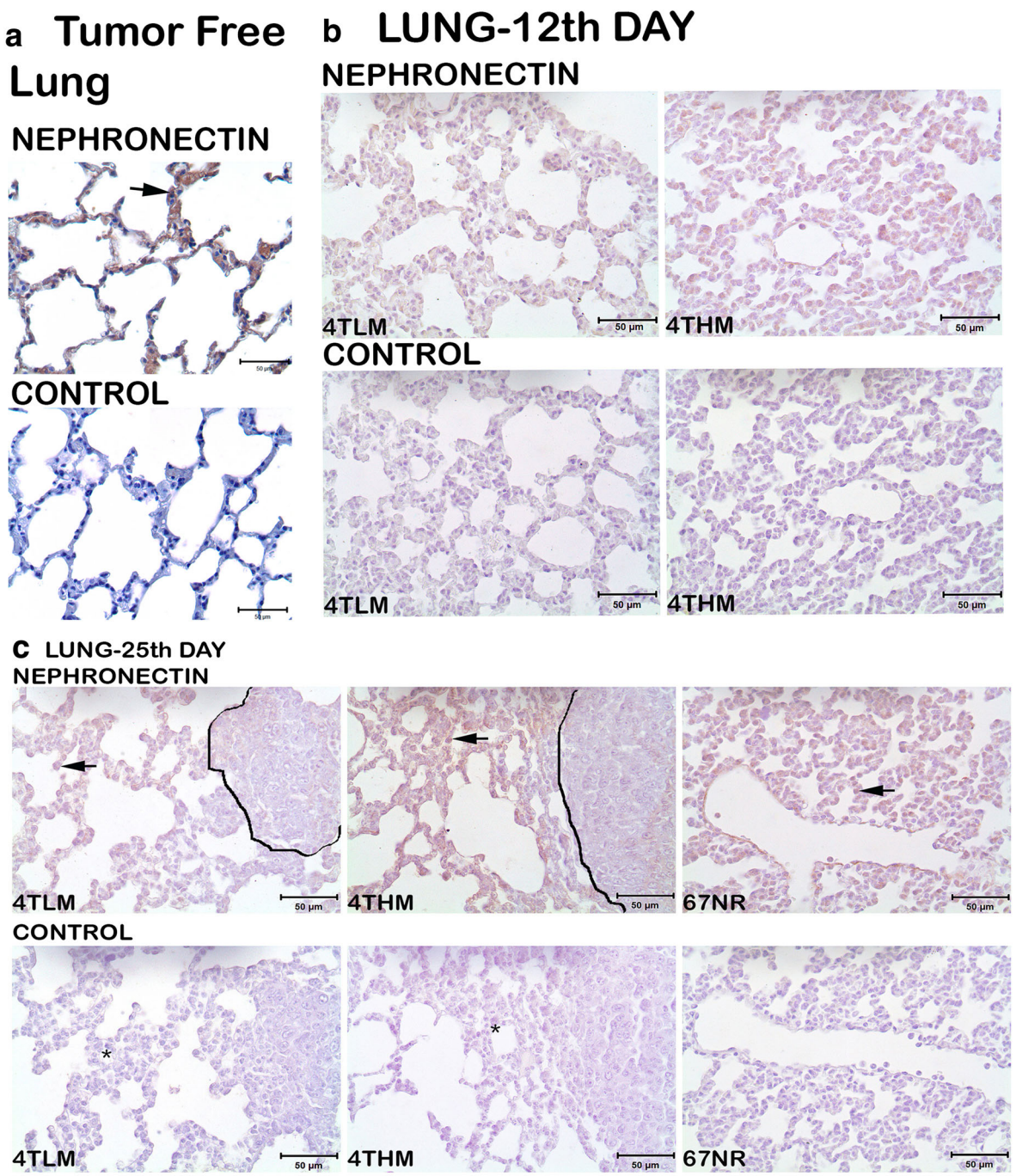
For nephronectin immunocytochemistry, 4THM, 4TLM and 67NR cells were fixed in 4% paraformaldehyde, washed with PBS, permeabilized with 0.5% Triton X-100 (Santa Cruz; #sc-29,112), blocked with blocking solution (1,5 g Bovine Serum Albumin, 0,0375 Glycine, 50 ml PBS) and incubated with rabbit anti-nephronectin (1/50, Abcam; #ab64419 followed by detection with a 1/250 dilution of donkey anti-rabbit immunoglobulin-Alexa Fluor 555 (Invitrogen; #A-21429).

Western Blotting

For Western-blot analysis, total proteins from the primary tumor tissues were extracted in a lysis buffer (10 ml 0.1 M Tris, 0,184 g sodyum-ortovanadate; protease inhibitor cocktail (Sigma; #P8340)). The protein concentrations were determined by using the Bradford Protein Assay kit (BIORAD; #1–800-4). Seventy-five microgram of protein was loaded into each well, separated by SDS-polyacrylamide gel electrophoresis using 10% TRIS-HCl gels, for nephronectin antibody and β-actin. The proteins were blotted onto PVDF membrane (BioRad Laboratories). Membranes were washed twice with PBS-T (0.05% Tween 20 in 1X PBS, pH 7.4) and then blocked with 1% bovine serum albumin (BSA) in TBS-T for 1 h to decrease non-specific binding. Afterwards, the membranes were incubated with anti-nephronectin antibody (1/1000, Abcam; #ab64419) at 4 °C overnight. Following the washing step with 1% PBS-Tween-20, membranes were incubated with a secondary antibody with peroxidase labeled anti-rabbit IgG (1/4000, Vector; #PI-1000 Vector Lab., Burlingame, CA, USA) at room temperature for 1 h. The immunoblots were developed using an ECL Kit (Pierce; #34080 T, Waltham, MA USA) and subsequently, membranes were exposed to BioMax film (Kodak, Rochester, NY, USA). After the membrane was stripped using Stripping solution (Pierce), equal loading of proteins in each lane was confirmed by reprobing the membrane with rabbit monoclonal anti-mouse β-actin antibody (1/5000, Cell Signaling; #4970; Cell Signaling Technology, Inc. MA, USA).

Threshold Analysis of Staining Intensities

Immunohistochemical images of all tissue samples were captured using Spot Imaging software version 4.6 (Diagnostic Instruments, Inc., Michigan) at ×20 magnification. Ten photomicrographs were randomly selected for each group and analyzed by Image-J Version 1.46 (National Institutes of Health, Bethesda, Maryland). All tissue components except background were measured by moving brightness slider until all stained areas were selected and recorded to the excel sheet. To measure the stained areas only, the hue slider was decreased without changing the brightness slider, until only the immunohistochemistry (IHC) stained areas were selected and recorded, as well. Finally, integrated density values of IHC stained areas were normalized to the integrated density values of the total area. The calculated ratio of the immunostained area to total area was graphed.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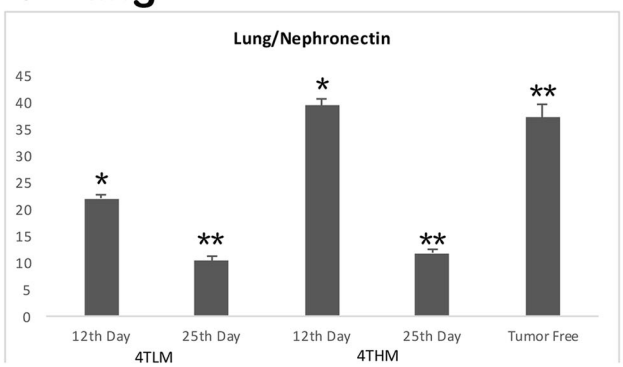
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Tumor Free
4TLM
4THM

◀ **Fig. 3** Nephronectin immunostaining in lung tissues. Normal and after the 12th and 25th days of tumor injection lung tissues to the mice. **a** Nephronectin expression in control mice (tumor-free, not injected with tumor cells). The pneumocytes were intensely positive for nephronectin (*arrow*). **b** Nephronectin expression 12th days after injection of 4THM or 4TLM cells. **c** Nephronectin expression 25th days after injection of 4THM, 4TLM or 67NR cells (*arrows*). Metastatic lesions were demarcated. **d** Image-J analysis of nephronectin expression in pneumocytes of mice injected with 67NR, 4TLM or 4THM cells as well as tumor-free mice. */**$p < 0, 05$ is a statistically significant difference. *Scale bars* represent 50 μm

Statistical Analysis

All these data were analyzed with Sigma Stat software. Analysis of variance (ANOVA) was performed on the tissue on specific midpoint and endpoint days of nephronectin expression parameters. Significance levels were set at $p<0.05$.

Results

Tumor Size

On the 25th day, tumor size was measured with composing stick in each mouse and tumor size was measured (mm^2). 4TLM primary tumors were bigger than 4THM and 67NR primary tumors (Fig. 1d) as reported before [5].

Immunohistochemistry

Distribution of Nephronectin Expression in Primary Tumors

Nephronectin expression was observed in both stromal cells and immune cells as seen in Fig. 1. As seen, majority of tumor tissue (over 95%) was composed of tumor cells with large and/or irregular nucleus and small cytoplasm in 4THM and 4TLM tumors. Majority of cells in 67NR tumor were also cancer cells which had spindle shape and irregular nucleus. Majority of the nephronectin positive cells however were stromal cells which were relatively more abundant in smaller tumors (obtained 12th days after inoculation of tumor cells). In accordance, image J analysis described above demonstrated decreased expression of nephronectin in both 4THM and 4TLM primary tumor tissues at the endpoint compared to midpoint. The change however was more prominent in 4TLM primary tumors (Fig. 1c). The expression pattern suggests that nephronectin expression does not directly correlate with the growth rate of primary tumors (Fig. 1c–d). Negative control immunostainings with normal rabbit IgG confirmed the specificity of nephronectin staining

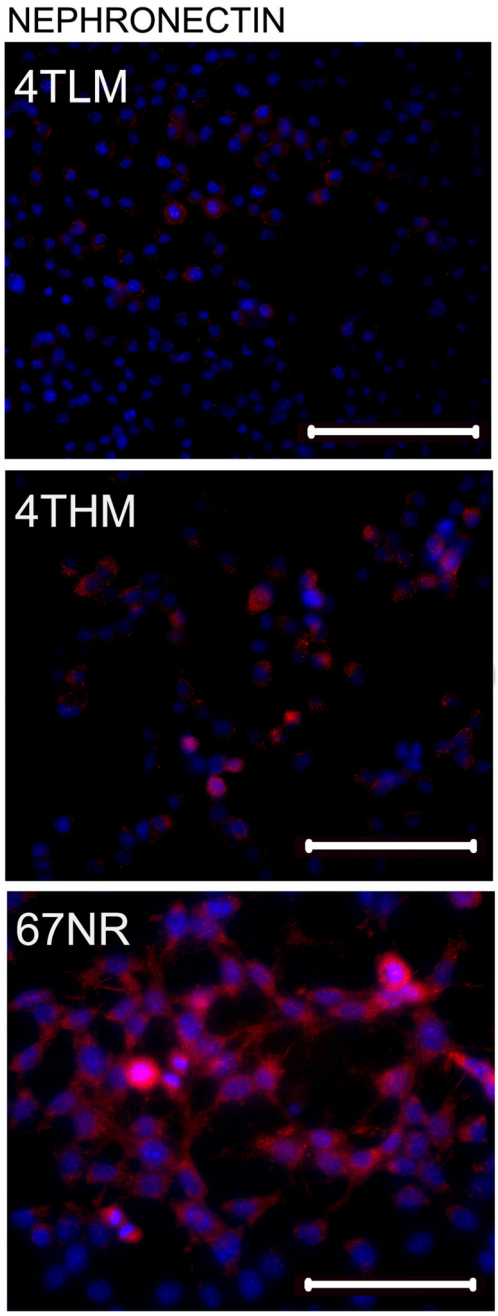

Fig. 4 Immunocytochemistry analysis showed that nephronectin expression were rarely seen in metastatic cell lines as 4TLM and 4THM. 67NR cell lines were strongly expressed in nephronectin. *Red*: Nephronectin expressions. *Blue*: Dapi. *Scale bars* represent 50 μm

patterns in primary tumor tissues at the midpoint and endpoint time (Fig. 1a vs b).

Distribution of Nephronectin Expression in Liver and Lung Tissues

Hepatocytes and pneumocytes displayed a strong immunoreactivity for nephronectin in control animals not injected

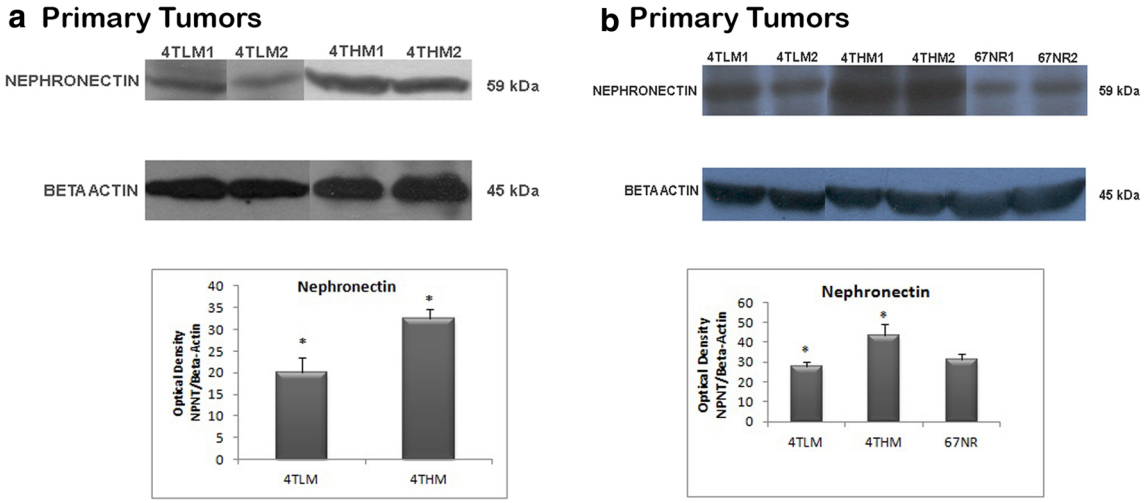

Fig. 5 Western blot analysis of nephronectin in primary tumors at the midpoint (**a**) and endpoint (**b**) times. Bands were detected for nephronectin 59 kDa. The immune expression of β-actin (43 kDa) was used to confirm equivalent amounts of total proteins loaded per lane. The immunoblot bands were quantified by an optical densitometer. The OD (optical density) values of nephronectin bands were normalized to the OD values of β-actin bands. The data in the graph is presented as mean ± SEM. $p < 0, 05$ is a statistically significant difference

with tumor cells (Figs. 2 and 3) which were markedly decreased in animals injected with metastatic breast carcinoma cells (4THM and 4TLM). Spesifically lung tissue of animals bearing tumors (both 4THM and 4TLM) was infiltrated with polymorphonuclear leukocytes (marked with * in unstained control samples) and staining intensity of pneumocytes were markedly decreased compared to non-tumor bearing animals. Infiltrating leucocytes were also moderately immunoreactive for nephronectin. Lung Metastases of both 4THM and 4TLM tumors (demarcated) were largely negative. Similar changes were also observed in liver tissue such that hepatocytes of tumor-free animals were strongly positive for nephronectin which decreased markedly in animals bearing 4THM or 4TLM tumors. Liver metastases of both 4THM and 4TLM tumors were also largely negative (demarcated). Hence nephronectin expression of host tissue decreases markedly during metastatic process which was also evident in image J analysis. We also observed time-dependent decrease in nephronectin expression such that nephronectin expression was significantly lower at endpoint liver and lung tissues compared to tissues obtained at midpoint. (Figs. 2c and 3c). In accordance in animals injected with non-metastatic 67NR cells, nephronectin expression in hepatocytes and pneumocytes were similar to the levels of tumor-free animals.

Distribution of Nephronectin Expression in Cell Lines with Immunofluorescence Analysis

As the immunocytochemistry results shown that 4TLM and 4THM cell lines rarely expressed in nephronectin staining. However, there were clearly expressions in 67NR cell lines cyctoplasmically (Fig. 4).

Western Blot Analysis

Nephronectin expression was analyzed twice, by using Western-blots on primary tumor tissues, one at the midpoint day and the other at the endpoint day (Fig. 5a, b). Blots revealed clear bands for nephronectin 59 kDa. Equivalent amounts of total proteins were loaded per lane as indicated by the immune expression of β-actin corresponding to 45 kDa. Morphological analysis described above demonstrated that majority of the nephronectin positive cells in primary tumors were stromal cells, hence changes observed here mainly reflect the changes in nephronectin positive stromal cells since majority of the tumor cells were negative. In accordance with image-J analysis nephronectin expression was higher in 4THM primary tumors both at the midpoint time and at the end-point (Fig. 5a–b).

Discussion

Cellular adhesion molecules (CAMs) are important in cell–cell interactions and interactions between cells and ECM components. These components have been implicated in a variety of cellular functions including induction of differentiation and maintenance of differentiated state [26]. Nephronectin, ligand for α8β1 integrin receptor, is one of the CAM involved in differentiation [27]. Nephronectin was shown to have a critical role in kidney development, and promotion of osteoblast differentiation [28–30]. Furthermore, neonatal cardiomyocytes were shown to maintain their differentiated status in the presence of Nephronectin under in vitro conditions [31]. Loss of

differentiation is one of the hallmarks of cancer cells that have the potential to metastasize [32]. Here, we demonstrated lung and liver tissue of control mice (not injected with cancer cells) expressed nephronectin which was lost in animals bearing metastatic tumor for 25 days. Nephronectin staining was preserved in animals injected with non-metastatic 67NR tumors. These results demonstrate that loss of nephronectin may promote dedifferentiated status of cancer cells in metastatic milieu.

The metastatic cells used here (4THM and 4TLM) was reported to induce excessive inflammation and have cancer stem-cell properties demonstrating dedifferentiated status [5, 23]. It was shown that TNF-α which is mostly considered as tumor-promoting cytokine by inducing excessive chronic inflammation, inhibits nephronectin expression in both time- and dose-dependent manner [7, 9, 33]. Hence, time-dependent decrease in nephronectin in animals injected with 4THM and 4TBM might be due time dependent increase in TNF-α and other inflammatory cytokines in animals bearing 4THM and 4TBM cells as reported before [23, 34]. Similar findings were reported such that decreased nephronectin expression was found to correlate with tumor progression in malignant melanoma [20].

The role of nephronectin in metastatic disease was further confirmed by the finding obtained by using cell lines with varying metastatic potentials. Loss of nephronectin expression was highest in animals injected with 4TLM cells which have the highest metastatic potential and demonstrate EMT phenotype [5]. Similarly, nephronectin expression was not lost in animals injected with 67NR cells which are considered non-metastatic and do not metastasize to visceral organs.

Although nephronectin expression was observed in primary tumors formed by metastatic cells, the expression was almost always cytoplasmic demonstrating aberrant expression of the protein and loss of function. Hence further studies are required to find the mechanisms of loss membranous nephronectin expression in tumor cells which may provide new perspectives of carcinogenesis.

In conclusion, our results showed that loss of nephronectin in visceral organs might be involved in formation of metastatic niche for metastatic breast cancer cells. Up to our knowledge this is the first report demonstrating tumor-induced loss of nephronectin expression in visceral organs in which metastatic growth takes place. Further studies are required to dissect cellular and molecular pathways of possible anti-metastatic role of nephronectin.

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