Original Article
Bioluminescence imaging of estrogen receptor activity during breast cancer progression

Cristina Vantaggiato[1*], Giulia Dell'Omo[1*], Balaji Ramachandran[1], Isabella Manni[2], Enrico Radaelli[3], Eugenio Scanziani[3], Giulia Piaggio[2], Adriana Maggi[1], Paolo Ciana[1]

[1]Department of Pharmacological and Biomolecular Sciences, Center of Excellence on Neurodegenerative Diseases, University of Milan, 20133, Milan, Italy; [2]Department of Research, Advanced Diagnostics and Technological Innovation, Regina Elena National Cancer Institute, Via Elio Chianesi 53, 00144, Rome, Italy; [3]Mouse & Animal Pathology Lab, Fondazione Filarete Viale Ortles, 22/4 - 20139, Milano, Italy. *Equal contributors.

Abstract: Estrogen receptors (ER) are known to play an important regulatory role in mammary gland development as well as in its neoplastic transformation. Although several studies highlighted the contribution of ER signaling in the breast transformation, little is known about the dynamics of ER state of activity during carcinogenesis due to the lack of appropriate models for measuring the extent of receptor signaling in time, in the same animal. To this aim, we have developed a reporter mouse model for the non-invasive in vivo imaging of ER activity: the ERE-Luc reporter mouse. ERE-Luc is a transgenic mouse generated with a firefly luciferase (Luc) reporter gene driven by a minimal promoter containing an estrogen responsive element (ERE). This model allows to measure receptor signaling in longitudinal studies by bioluminescence imaging (BLI). Here, we have induced sporadic mammary cancers by treating systemically ERE-Luc reporter mice with DMBA (9,10-dimethyl 1,2-benzanthracene) and measured receptor signaling by in vivo imaging in individual animals from early stage until a clinically palpable tumor appeared in the mouse breast. We showed that DMBA administration induces an increase of bioluminescence in the whole abdominal area 6 h after treatment, the signal rapidly disappears. Several weeks later, strong bioluminescence is observed in the area corresponding to the mammary glands. In vivo and ex vivo imaging analysis demonstrated that this bioluminescent signal is localized in the breast area undergoing neoplastic transformation. We conclude that this non-invasive assay is a novel relevant tool to identify the activation of the ER signaling prior the morphological detection of the neoplastic transformation.

Keywords: Mammary tumorigenesis, optical imaging, hormone-dependent cancer, chemical carcinogenesis

Introduction

The ER signaling plays a pivotal role during the normal, hyperplastic, dysplastic and neoplastic growth and differentiation of the breast tissue [1]. Receptor activation influences the genetic program of the cell via direct modulation of target genes in the nucleus or by interfering with the signaling of other molecules in the cytoplasm or within the plasmalemma [2]. Modulation of receptor activity may occur through several mechanisms, including alterations of the ER expression levels [3], changes in the ovarian or local production of estrogens [4], post-translational modifications (including phosphorylations, sumoylations, palmytoilations and acetylations) known to influence the receptor signaling [5]. Due to its importance in mammary gland homeostasis, ER serves as a therapeutic target and as a predictive marker of sex hormone sensitivity and is a key factor in the development of hormone responsive breast cancers [6].

In the last three decades in vitro and in vivo methodologies were applied to elucidate the precise role of ER in hormone-dependent carcinogenesis and advanced in silico technologies such as genomics and proteomics characterized the downstream effectors of estrogen signaling in breast carcinogenesis [7-11]. The static nature of these methodologies, however, limits the power of the analysis providing a snapshot of specific phase of tumorigenesis, but not

the dynamic view of the receptor modulation necessary to identify the specific steps where the receptor signaling is indispensible for tumor progression [12].

To overcome these limitations, we developed the reporter mouse technology and applied *in vivo* imaging methodologies suitable to measure the activity of molecular targets in physiological settings [13]. In previous studies, we have extensively validated the ERE-Luc reporter mouse, a transgenic mouse carrying a firefly luciferase reporter system ubiquitously responsive to ER signaling and expressing the reporter proportionally to the state of ER activation [14]. This mouse model has represented a novel, promising, approach to follow in time the dynamics of ER activation in physiology [15] and was hypothesized to provide novel insights on the temporal regulation of ER signaling during tumorigenesis [12].

In this study, we induced a breast carcinoma in the ERE-Luc mouse model with the application of a classical protocol of chemical carcinogenesis based on the systemic treatment with DMBA [16]. Taken together our results demonstrated the possibility to monitor ER signaling during the breast cancer progression to identify the stages at which the receptor signaling might be required for the mammary cancer development.

Materials and methods

Chemicals

DMBA was purchased from Sigma-Aldrich (Pomezia, Italy), ketamine (Imalgene 500) from Merial (Toulouse France), xilazine (Rompun) from Bayer (Shawnee Mission, Kansas, USA), and D-luciferin (Beetle luciferin potassium salt) from Promega (Milan, Italy).

Experimental animals and rodent diets

The ERE-Luc mouse model is a transgenic mouse generated to measure the ER activity in the mouse tissue; the procedure for the generation of the model and its validation has been previously described [14]. Briefly, the construct used for transgenesis consisted of the reporter gene (firefly luciferase) driven by a dimerized ERE and a minimal promoter. Insulator sequences, the matrix attachment region from chicken lysozyme, were use to flank the reporter system in the transgenesis construct to achieve a generalized, hormone-responsive reporter expression. In this transgenic reporter mouse we have showed by biochemical, immunohistochemical, and pharmacological criteria, that luciferase content reflects ER transcriptional activity and thus represents a novel system for the study of the ER dynamics during physiological fluctuations of estrogen and for the identification of SERMs or endocrine disruptors [14].

The current study was carried out using heterozygous 8 weeks old mature female ERE-Luc mice in the C57BL/6 genetic background. Animal colonies were housed according to the Guidelines for Care and Use of Experimental Animals. All animal studies were approved by the Italian Ministry of Research (DM124/20-03-A) and University of Milan after approval by the expert committee at the Department of Pharmacological and Biomolecular Sciences, University of Milan. The animal room was maintained within a temperature range of 22-25°C and relative humidity of 50%±10%. There was a cycle of 12 hrs light/dark (lights on at 07:00 AM). The animals gained free access to AIN93M diet and filtered drinking water.

Study design, in vivo and ex vivo imaging

Before starting the experimental study, the baseline luciferase activity, was measured in all animals by in *vivo* imaging and the mice were allocated to different experimental groups so that the average background luciferase activity at the start was comparable in all groups. In each experimental group, mice were matched for weight and background luciferase. Two different experimental groups (10 animals/group) were assigned: group 1 - vehicle (olive oil) treated group and group 2 - DMBA treated group (1 mg/mice/once a week for 4 weeks by gavage). Mice were maintained in the experiment for up to 24 weeks (32 weeks p.b.). Details of the treatment protocol can be found in **Figure 1**.

BLI sessions were carried out as described before [17] at 0, 6 hrs and 8, 13, 18, 23, 24, 25, 26, 27 weeks p.b. at 10:00 a.m. 80 mg/Kg luciferin (beetle luciferin potassium salt; Promega, Madison, Wisconsin) was administered i.p. to the mice and let to distribute for 15 min before the 5 min acquisition with the CCD-camera. For quantification, photon emission
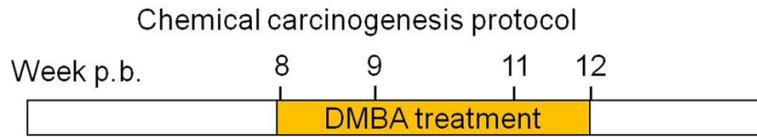
Chemical carcinogenesis protocol

Figure 1. Scheme of the DMBA treatment applied to the ERE-Luc reporter mouse. For the DMBA-induced carcinogenesis, 1 mg of the genotoxic compound or its vehicle was administered by gavage to female ERE-Luc mice weekly for four treatments starting at 8 weeks p.b.; in agreement with the previously published protocol [16], the treatment was suspended for one week to let mice recovering without affecting the overall efficacy in the generation of breast tumors.
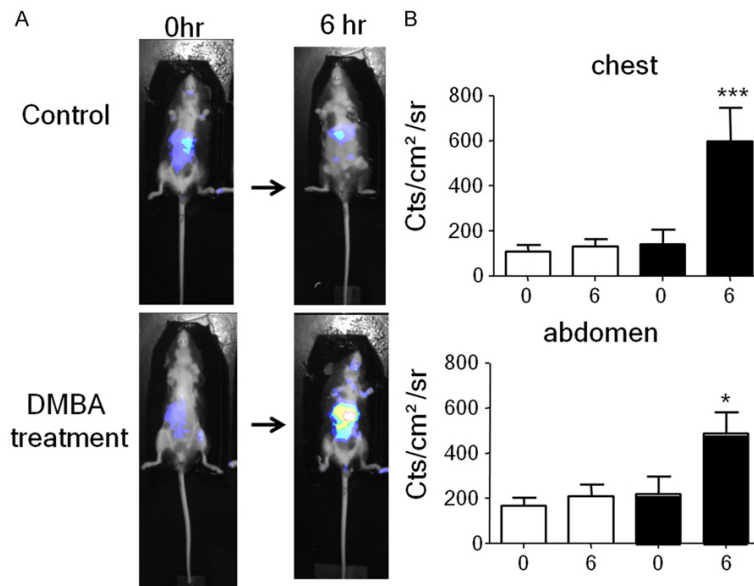

Figure 2. Acute effects of DMBA treatments on ER activation. A. Five female ERE-*Luc* mice were treated by gavage with 1 mg DMBA or olive oil (vehicle) as described in the scheme of **Figure 1**: BLI acquisitions were carried out before and 6 hrs after treatment. Picture of a representative animal per group is shown; photon emission appears to increase in the whole abdominal area in the group of DMBA treated mice. B. Graphs show the quantitative measurement of the photon emission from chest and abdomen. Values are expressed as counts per unit of time and area (cts/cm^{2}s), bars are the means +/- SEM; Student's t test was applied to calculate statistical significance * for p<0.05 and *** for p<0.001 DMBA 6 hrs *versus* control 6 hrs.

was measured in the regions of interest and the signals obtained were integrated from each anatomical area as previously described [17]. Photon emission is defined in this work as the number of counts per second per square centimeter (cts/cm^{2}s). Quantifications were done using WinLight32 imaging software (Berthold Technologies). Normalization was performed using an external source of photons enabling to measure the instrumental efficiency of photon counting (Glowell, Luxbiotech, Edinburgh, UK). At the last day of the experiment, after the *in vivo* imaging procedure, the animals were sacri-

ficed, selected organs were excised and placed in a light-tight chamber for the *ex vivo* measurement of tissue specific signals. Gray-scale images were first taken with dimmed light and then photon emission was registered for 5 min. Merging the signal with the dimmed light pictures enabled to visualize the tissue specific localization of the photon emission signal in individual organs (luciferase signal was transformed in pseudocolors: blue-low, white-high).

After sacrifice, dissected organs were snap frozen in liquid nitrogen for the quantitative analysis of luciferase content in the tissues. Quantitative enzymatic luciferase assay on protein extract from the tissues was carried out as previously described [14].

DMBA treatment

100 ul of olive oil or 1 mg of DMBA in 100 ul of olive oil, was administered to the animals by gavage.

Histochemical analysis

Tissues were fixed in 10% neutral buffered formalin (NBF), routinely processed and embedded in paraffin blocks. Breast tissues were sectioned at 4 μm, and stained with hematoxylin and eosin (HE) for standard histopathologic examination.

Real-time PCR for the quantification of the levels of ERα mRNA transcript

Real-time PCR experiments were done with total RNAs extracted after tissue homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA) as suggested by the manufacturer's instructions. For the preparation of cDNA, 1 μg of RNA was denatured at 75°C for 5 min in the presence of 1.5 μg of random primers (Promega) in
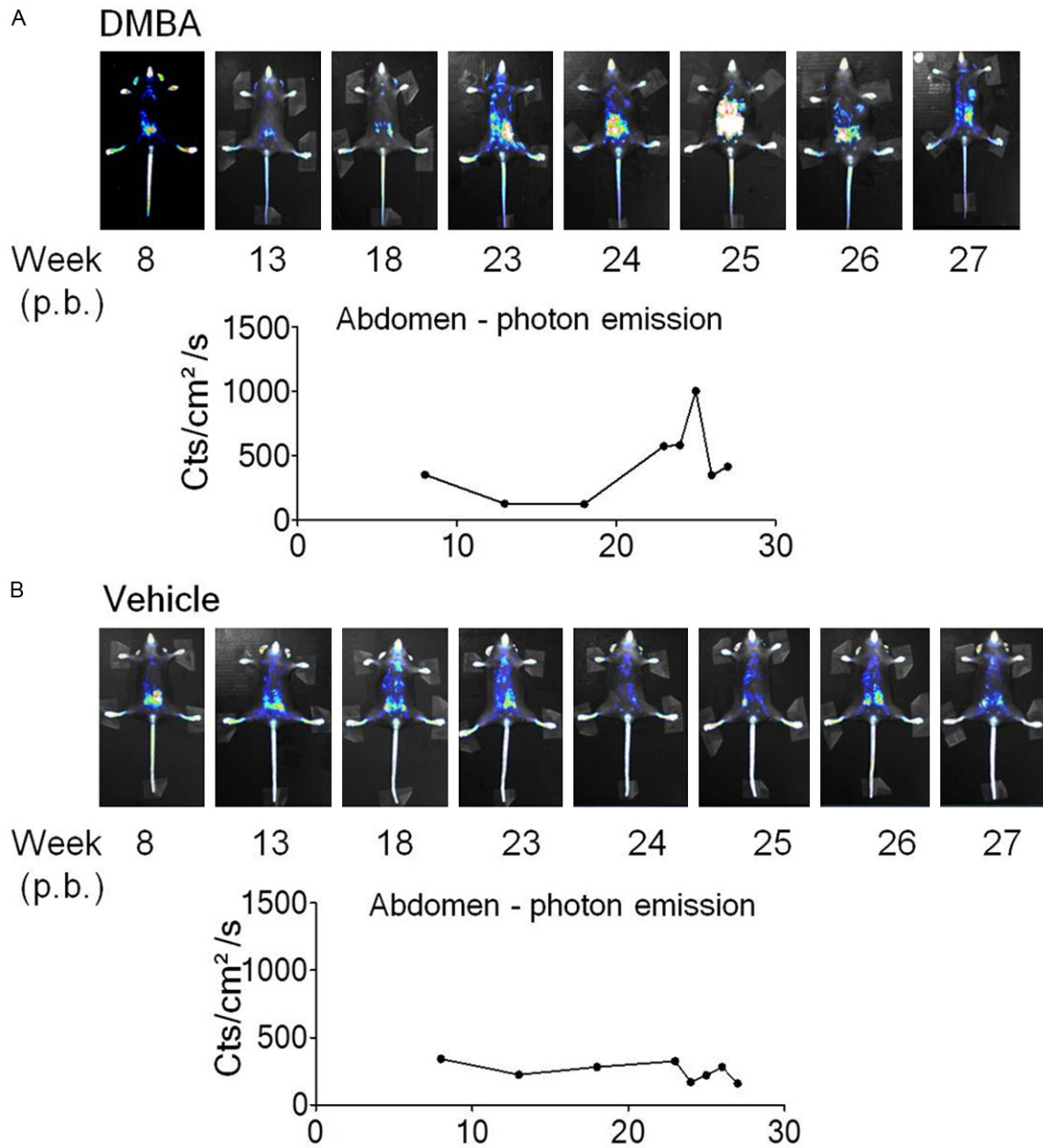
Figure 3. Chronic effects of DMBA treatments on the systemic ER activation. Five female ERE-*Luc* mice were treated as described in the scheme of **Figure 1**; BLI was carried out at the indicated times. Pictures of one representative animal of the DMBA and vehicle groups are shown in (A) or in (B), respectively. Inserted graphs represent the photon emissions (cts/cm²s) from the abdominal area of the corresponding individual showed in the picture.

a 15 µl final volume. Deoxynucleotide triphosphate (GE Healthcare) and Moloney murine leukemia virus reverse transcriptase (RT; Promega) were added at 0.5 mM and 8 U/µl final concentration, respectively, in a final volume of 25 µl. The RT reaction was performed at 37°C for 1 h; the enzyme was inactivated at 75°C for 5 min. Control reactions without addition of the RT enzyme were performed for each sample.

Templates were amplified using the TAQMAN Universal PCR amplification kit (Applied Biosystems) in a thermocycler (ABI Prism 7000, Applied Biosystems). TaqMan Gene Expression Assays was used for ERα (Mm00433149_m1); for 36B4 the following primers and probe were used: forward 5'-ggcgacctggaagtccaact-3', reverse 5'-ccatcagcaccacagccttc-3', probe 5'-atctgctgcatctgcttggagccca-3'; for ERβ the fol-
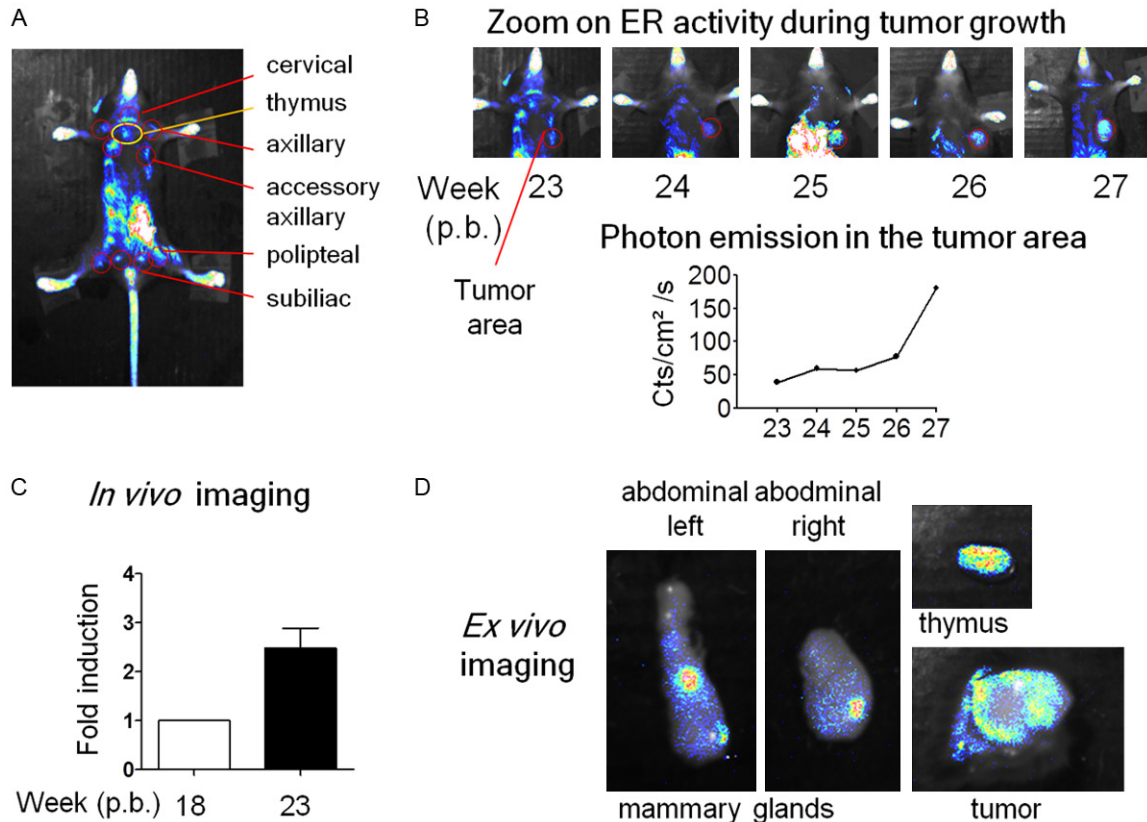
Figure 4. Identification of the source of photon emission by *ex vivo* imaging. (A) Magnification of the picture of the DMBA-treated mouse at week 23 showed in **Figure 3**; the photons emitted from the areas surrounding the thymus (orange area) and the lymph nodes (red area) are highlighted. (B) Pictures show the bioluminescence from the area surrounding the mammary gland, which developed a tumor (red circle); the inserted graph reports the photons emitted from the tumor (cts/cm²s) in time. (C) Fold induction of the photon emission detected at week 23 p.b. *versus* week 18 p.b. in the abdominal area; for each individual, the values detected at week 23 p.b. were normalized over the value detected at week 18 p.b.; bars are the average calculated values (D). *Ex vivo* imaging analysis of photon emission from the tumor, abdominal mammary glands and thymus excised from the DMBA-treated mouse reported in (A).

lowing primers were used forward 5'-aagctggct-gacaaggaactg-3', reverse 5'-caggctgagctcca-caaagc-3'. The reaction was carried out according to the manufacturer's protocol using Applied Biosystems 7000 Sequence Detection System device with the following thermal profile: 2 min at 50°C, 10 min 95°C, 40 cycles (15 s at 95°C, 1 min at 60°C), and data were analyzed using the ABI Prism 7000 SDS Software and the $2^{-\Delta\Delta Ct}$ method. The level of ERα and ERβ mRNA transcripts were normalized on the constitutively expressed gene 36B4.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 (GraphPad Software); we have applied Student's t test analysis for deter-mining statistical significance unless otherwise specified.

Results

To gain novel insights into the dynamics of ER signaling during mammary carcinogenesis, we treated n. 5 females, 8-week old ERE-Luc reporter mice [14] with DMBA according to a previously published protocol [16]: 1 mg DMBA or vehicle (as negative control) was administered by gavage weekly for four weeks with one week interruption between the second and the third week (**Figure 1**). The DMBA treatment was expected to induce adenocarcinoma in about 60% of mice with an average latency of 31 weeks [16]. Shortly after treatment (6 hours) or in the following weeks, we have studied the
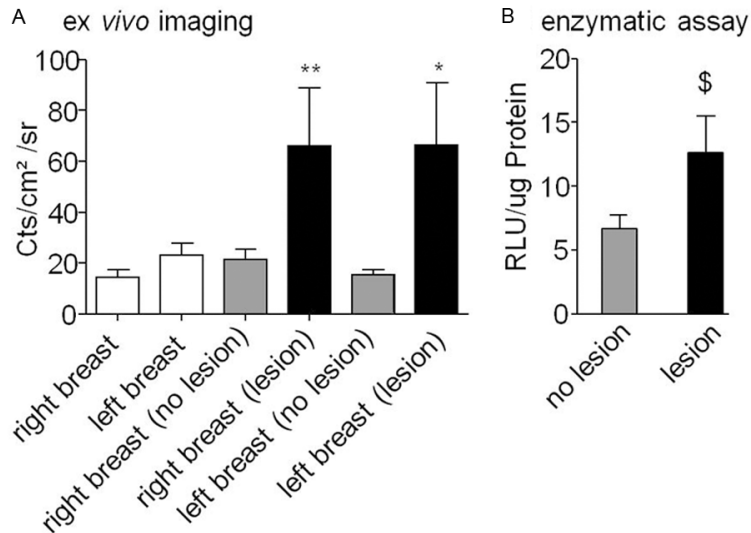
Figure 5. Photon emission and luciferase activity measured from the mammary glands with or without neoplastic lesions. Mammary glands with or without lesions were excised from the DMBA-treated animals and subjected to the *ex vivo* imaging protocol: photon counts (cts/cm²s) were plotted in the graph (A). (B) Luciferase activity was measured in the protein extracts of the same mammary gland reported in (A). Data represents mean +/-SEM (n = 5); asterisks indicate a significant difference from control (vehicle), as assessed by one-way ANOVA plus Bonferroni post hoc test (*p<0.05) (**p<0.01) and dollar indicates a significant difference from control, as assessed by unpaired Student's t-test two tailed ($p<0.05).
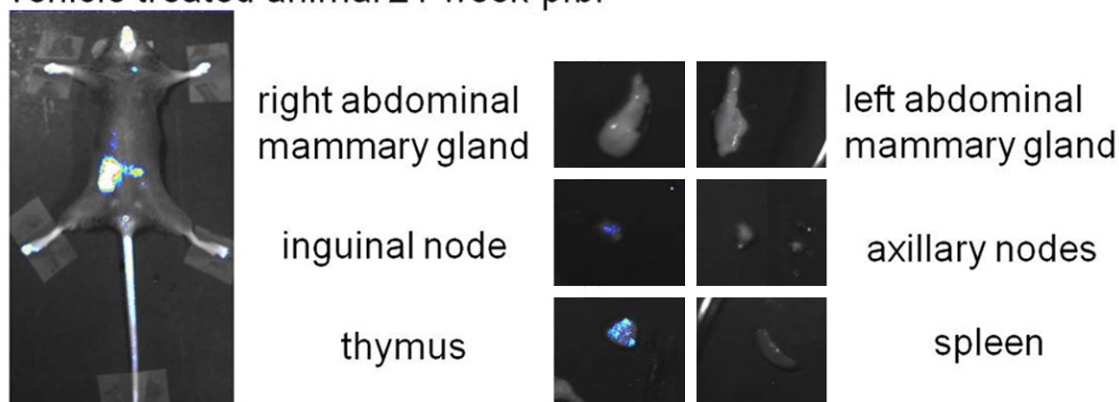
DMBA effects on ER activity by measuring photon emission [15]. In order to minimize the dietary effects shown to influence ER activity [18], animals were fasted the night before each BLI session.

To evaluate the short-term effects, BLI was carried out immediately before and 6 hrs after DMBA treatment. Photon emission was detected for 5 min upon injecting the mice with 80 mg/Kg luciferin. After DMBA administration, bioluminescence increased significantly (more than 2-fold) in the chest and abdominal areas as compared to their baseline or to the bioluminescence measured in vehicle-treated mice (**Figure 2**), suggesting that this genotoxic agent given systemically produces an initial, generalized activation of the receptor in several tissues. This early ER activation returned to baseline one week after the last DMBA treatment (compare **Figure 2** after DMBA treatment with **Figure 3** week 13 p.b.). ER signaling was near baseline at week 13 p.b. and at week 18 p.b. time points; at week 23 p.b., we registered an increased bioluminescence emission (**Figure 3**). The average increase of bioluminescence observed in four (out of five) animals was about 2.5 fold the level of light emission detected at week 18 p.b. (**Figure 4C**). The increased ER activation was not restricted to the mammary glands but spread in the area corresponding to lymph nodes and thymus (**Figure 4A**). From this time point (23 p.b.), we proceeded with a weekly BLI acquisition of the mouse bioluminescence (**Figure 3**). At week 24 p.b. bioluminescence increased in the abdominal area and a novel circular signal was detectable in proximity of the left anterior leg (**Figures 3** and **4B**). The abdominal signal further increased at week 25 p.b. extending to the liver corresponding area, and a tumor became palpable in correspondence of the left anterior leg (**Figure 3**). While the abdominal signal decreased during the following weeks, the tumor-associated signal increased until week 27 p.b., when the mouse was euthanized (**Figure 3**). *Ex vivo* imaging analysis, demonstrated that the tumor was indeed emitting photons (**Figure 4D**), while two spots of ER activation were detected in the abdominal mammary glands possibly associated to lymph nodes or to sites of tissue transformation (left and right). The thymus displayed high luciferase activity confirming that the signals detected on the throat/thoracic area originated from this organ (**Figures 3** and **4D**). Similar, although not identical, dynamics of bioluminescence emission could be detected in different DMBA-treated animals, which lately developed a tumor (4 out of 5 animals in this experiment), and the intensity of the signal was also slightly different in different individuals. The possibility offered by *in vivo* imaging allowed us to highlight longitudinally the increase in the bioluminescence emission in each animal overcaming the intra-individual variability which would have flattened the results, if a classical post-mortem group analysis had been carried out. Control mice were not showing the pattern of activation registered in the DMBA-treated animals (**Figure 3**); fluctuations in bioluminescence in this group of animals were most likely associated to the different phase of the estrus cycle [15].
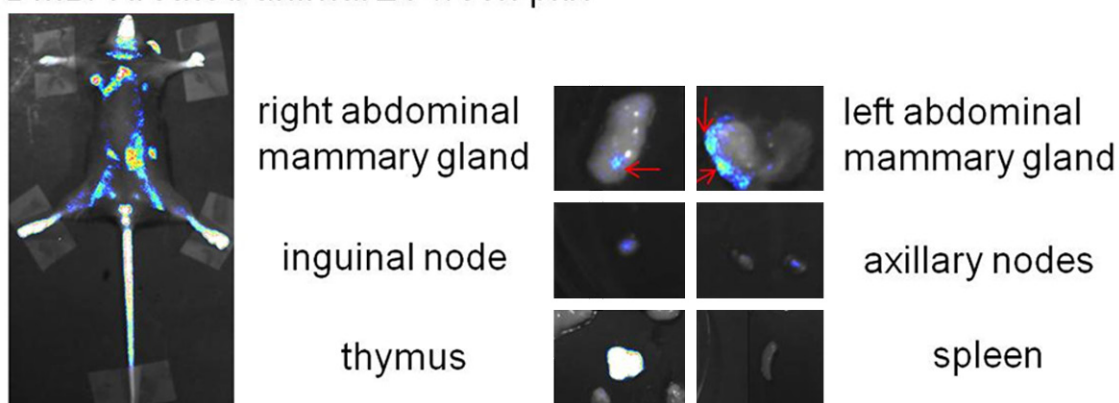
Figure 6. *In vivo* and *ex vivo* imaging analysis of the breast tissue from DMBA-treated mice before tumor appearance. Pictures in the left show *in vivo* imaging analysis of a representative individual of the group of mice treated with vehicle (A) or DMBA (B). Pictures on the right show the photon emission coming from mammary glands, lymph nodes, thymus and spleen explanted from the same mice after sacrifice. An increased photon emission was visible in thymus and in selected spots of the mammary glands form the DMBA-treated mice compared to the control mammary glands.

To demonstrate that ER signaling was indeed ectopically activated in the transformed tissues, both tumor and morphologically untransformed mammary glands were collected at week 27 p.b. for *ex vivo* imaging and luciferase enzymatic assay. Most of the tumors had higher bioluminescent signal and expressed significantly higher luciferase activity compared to the mammary glands without morphological signs of transformation (**Figure 5**); this indicated that most of the DMBA-induced tumors were ER positives as also previously reported [19].

Finally, we tested whether the methodology was able to identify sites of incipient transformation prior detection of a palpable tumor. Thus, in a duplicate experiment, mice from control and DMBA-treated groups (with no palpable tumors) were sacrificed at week 21 p.b. and subjected to *in vivo* and *ex vivo* imaging (**Figure 6**). *Ex vivo* imaging showed specific spots of bioluminescence in the mammary glands of the DMBA-treated group not observed in the vehicle treated animals. These spots were interpreted as possible sites of incipient transformation (see red arrows in **Figure 6B**); indeed, immunohistochemistry revealed the presence of *in situ* hyperproliferation (**Figure 7A**), which is usually preceding the appearance of a primary cancer; the hyperproliferation was not present in the mammary gland of vehicle-treated mice. Interestingly, the ERα mRNA content in hyperplastic tissue was comparable to controls and we recorded only a non significant, increase of the ERβ mRNA in the hyperplastic tissue (**Figure 7B**); this is suggesting that the increased bioluminescence observed in the hyperplasia was only partially due to the increased receptor expression, other mechanisms might also be involved, like for example activation of the ER
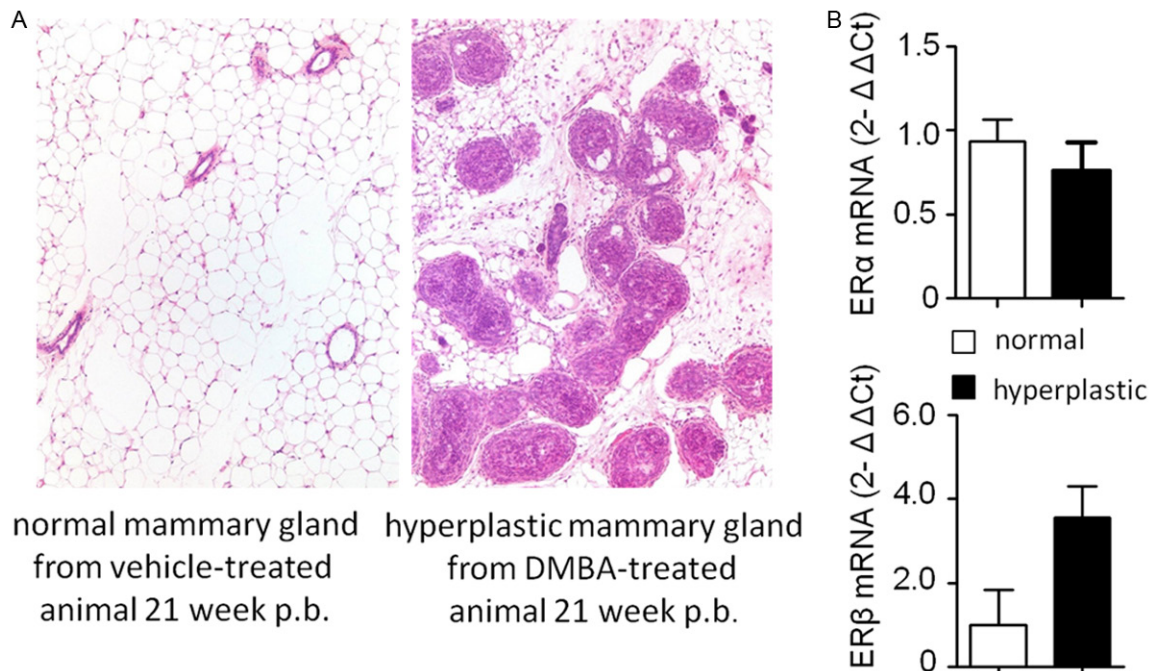
Figure 7. Histochemical analysis of the breast tissues form DMBA- and vehicle-treated mice at 21 weeks of age. A. Eosin/hematoxylin staining shows the presence of *in situ* hyperproliferative foci in the mammary gland of the mice treated with DMBA at this stage of tumorigenesis. B. Real time PCR analysis of ERα and ERβ expression in normal and hyperplastic mammary glands at 21 weeks of age. Bars represent the levels of ERα and ERβ mRNAs normalized on the reference gene 36B4 calculated using the $2^{-\Delta\Delta Ct}$ method.

signaling by local hormone production (liganded activation) or by the cross-talk with other membrane receptor signaling pathways (unliganded activation) [5].

Taken together, our data indicate that imaging luciferase activity in the ERE-Luc reporter mouse may allow a dynamic analysis of receptor activation during breast cancer progression; the possibility, during breast carcinogenesis, to localize the activation of ER signaling in the spatial (position in the body and within the tissue) and temporal dimensions may direct the genomic analysis to the precise site in the breast where neoplastic transformation is occurring, before a palpable tumor become detectable.

Discussion

In this study we aimed at demonstrating the applicability of BLI to the study of ER signaling during breast transformation. The choice to apply the DMBA protocol to induce mammary tumors was dictated by the possibility to generate sporadic, hormone-dependent cancers [19] which closely resemble the human breast

tumors [16]. Tumors were induced in the ERE-Luc reporter mouse; in this model, several pharmacological, molecular and physiological studies carried out in our and other laboratories firmly demonstrated the direct relation between luciferase expression and ER activation in response to physiological [15], pharmacological [20] and dietary estrogens [18]. In the current study, we believe to have demonstrated that the methodology we have developed is suitable for measuring ER signaling during breast transformation in the same animal over time during the progression of the DMBA-induced mammary lesions. This might finally lead to identify from the molecular stand-point where ER is transcriptionally active; this precise *spatio*-temporal localization would enable to identify novel targets for anti-cancer agents. Another added value of this methodology is the possibility to carry out a systemic whole body analysis of ER activation, allowing to highlight other tissues potentially relevant for breast cancer progression (e.g. lymph nodes and thymus).

Furthermore, in our report we show that the pattern of ER activation seems to "label" a

phase which is prodromal to the tumor appearance; this phase is characterized by a 2.5 fold increase of bioluminescence (**Figure 4C**), a threshold increase which can be used in future studies aimed at defining novel steps in which ER signaling might play a role that were not identified by the current analysis (for example in earlier time points between 13 p.b. and 23 p.b). Once identified a step characterized by an increased ER activation, further molecular studies (i.e. transcriptomic, proteomic or metabolomic studies) can be driven by the *ex vivo* imaging analysis on the portion of the mammary gland interested by the transformation event (**Figure 6**).

One limitation of the DMBA-based protocol applied in the current study is that it generates only primary, locally invasive, non metastatic tumors limiting the analysis to the early steps of mammary transformation. Future studies might consider to overcome this limitation, by breeding the ERE-Luc model with GEM which are specifically developing metastatic cancer (e.g. MMTVneu) [21]. In models of this type, there will be the possibility to investigate specific aspects of ER activation in the process of metastasis, which cannot be easily addressed using classical models. For example, we have demonstrate the possibility to study ER activity in the immune system (i.e. see lymph nodes, spleen, thymus **Figure 4**); in a metastatic model, it will be possible to address specific questions regarding the functional role of the receptor signaling in inflammatory cells during the process of metastasis [22].

Conclusions

In conclusion, to our knowledge this is the first report demonstrating ER signal during the entire carcinogenesis process in the same individual: this demonstration might open the possibility to elucidate in the *spatio/*temporal dimension the molecular changes occurring upon ER activation during the process of neoplastic transformation.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

GEM, Genetically engineered mice; p.b., post birth.

Address correspondence to: Paolo Ciana, Department of Pharmacological and Biomolecular Sciences, Center of Excellence on Neurodegenerative Diseases, University of Milan, Via Balzaretti 9, 20133, Milan, Italy. E-mail: paolo.ciana@unimi.it

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