11 Noninvasive Real-Time In Vivo Bioluminescent Imaging of Gene Expression and of Tumor Progression and Metastasis

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11.1 General Introduction

In 2003, the human genome project ended with the completion of the entire human genetic sequence. Now that most genes have been identified, the big challenge for the future is to try to discover where the genes are expressed, how they are regulated, and what is their exact function in the homeostasis of specific cell types. This so-called functional genomics research will lead to a better understanding of the molecular, cellular, and biological processes involved in normal physiology and pathology in a variety of diseases. It will also allow us to find new drug targets and, consequently, better management of diseases.

In order to study the location and regulation of gene expression in vitro as well as in vivo, reporter genes (e.g., β-galactosidase, CAT, green fluorescent protein, luciferase) have been widely used and are essential tools. They are also used for developing gene delivery systems for gene therapy and for tracking the fate of cells injected systemically in small experimental animals. Until recently, the proteins translated from the reporter gene-constructs were either visualized in histological tissue sections or detected biochemically in tissue extracts after sacrifice of the animal. However, bioluminescent imaging (BLI) coupled to luciferase as reporter gene currently allows very sensitive image recording of the topographical expression of this enzyme noninvasively and repetitively in the living animal.

11.2 Principles of Bioluminescent Imaging

Bioluminescence refers to the enzymatic generation of visible light by living organisms. The most commonly used bioluminescent reporter gene for research purposes has been luciferase from the North American firefly *Photinus pyralis*. The enzyme catalyzes the transformation of its substrate D-Luciferin into oxyluciferin in an oxygen and ATP-dependent process, leading to the emission of photons. The first report of real-time in vivo imaging of gene expression using firefly luciferase as a reporter was from Millar and coworkers in plants, in which they used a low-light video imaging system to establish that luciferase bioluminescence in vivo accurately reports the

temporal and spatial regulation of cab2 transcription in single seedlings (Millar et al. 1992).

Biological sources of light (bioluminescence) have sufficient intensity to cross animal tissues, provided that the endogenous light has a wavelength >550 nm. Above this wavelength the tissues do not absorb photons. The firefly luciferase/luciferin reaction emits photons at wavelengths from 500 to 620 nm and is therefore suitable for external detection. Apart from fulfilling this criterion, another important feature is that the substrate luciferin diffuses within a few minutes from intravenous or peritoneal injection throughout the entire body of small animals and is rapidly taken up by cells (Contag et al. 1997, 1998). Besides firefly luciferase, Renilla luciferase can also be used as a reporter in combination with its specific substrate coelenterazine. Although this reaction will emit mainly blue light, the emission spectrum is very broad and a small part also exceeds the wavelength of 580 nm, making it suitable for BLI (Bhaumik and Gambhir 2002).

Recent technical advances for imaging weak visible light sources using cooled charged coupled device (CCCD) cameras, peltier-cooled detectors and microplate channel intensifiers allow detection of bioluminescent emission from inside the tissues of the animal (see Table 1 for available BLI systems).

This technical development made it possible to monitor gene expression in live animals via a *luc*-reporter gene linked to specific pro-

Table 1. Several currently available BLI systems

Company	System name	BLI technology
Xenogen	IVIS-100	Liquid nitrogen-cooled CCD camera
Berthold	Nightowl	Peltier-cooled CCD camera
Hamamatsu + Improvision	VIM Camera model C2400-47	Intensified CCD camera
Roper Scientic	ChemiPro	Cryogenic-cooled CCD camera
Biospace	photoImager	Intensified CCD camera

moters (Carlsen et al. 2002; Zhang et al. 2001, 2003, 2004; Ciana et al. 2003) or to follow in real time the fate of luciferase (*luc*) transfected tumor cells (Edinger et al. 1999, 2003; Sweeney et al. 1999; Contag et al. 2000; Wetterwald et al. 2002; El Hilali et al. 2002) or immune cells (Hardy et al. 2001; Mandl et al. 2002) injected in living animals. One of the many advantages of this methodology is that it is non-invasive and therefore allows investigations in the same animal at different time points. Accordingly, the number of the animal groups in the experimental setting is greatly reduced.

There are further important advantages of using luciferase as a reporter.

1. The luciferase protein, in contrast to other reporter proteins, has a relatively short half-live of about 1–1.5 h. This feature makes it ideally suited for kinetic and dynamic analysis of gene expression within short time frames and, therefore, to identify circadian or even infradian rhythms of gene expression.
2. There is no background activity, making it very specific and sensitive. This in contrast to β-galactosidase activity, which is also found endogenously in some tissues or GFP signals which are disturbed by auto-fluorescence of some tissues.
3. Luciferase activity can easily and sensitively be quantitatively assayed in tissue extracts.
4. Luciferase protein can also be detected in tissue section using immunohistochemistry.

We will discuss examples in the two major areas of application of the bioluminescent technology: gene expression in transgenic *luc*-reporter mice and cancer research where it allows early detection and reliable quantification of primary tumor growth, and of micro- and macrometastatic tumor spread.

11.3 Transgenic *Luc*-Reporter Mice to Study Specific Gene Expression

Transgenic mice offer one of the most efficient and elegant ways to determine gene function in vivo. However, gene function is usually assessed by time-consuming and sometimes difficult phenotypic and

biochemical assays performed ex vivo. In contrast, detection of the bioluminescent activity in vivo of the luciferase enzyme as a transcriptional reporter facilitates rapid and repetitive screening for both the presence and function of transgenes in intact living mice. Using transgenic ERE-*luc* reporter mice, we were able to image transcriptionally active estrogen receptors (Ciana et al. 2003) in vivo. Using transgenic mice expressing luciferase under the control of the human osteocalcin (OC) promoter we could show skeletal sites of active bone formation/remodeling since osteocalcin is a bone-specific gene expressed by the bone-synthesizing cells, the osteoblasts.

11.3.1 Monitoring Estrogen Receptor Transcriptional Activity In Vivo

It is now well established that estrogens control not only reproductive functions, but also modulate other important physiological processes such as cognitive brain function, adipogenesis, lipid metabolism, blood pressure, and bone turnover. Estrogen deficiency related to menopause is clearly associated with increased incidence of neurodegenerative disorders, like Alzheimer's disease, and of obesity, cardiovascular disease, and postmenopausal osteoporosis. Identification of compounds with tissue-selective activity (SERMs, selective estrogen receptor modulators) will lead to new drugs mimicking the beneficial effects of estrogen on the prevention of osteoporosis, and cardiovascular or neurodegenerative diseases, while avoiding its detrimental proliferative effects which can lead to cancer. For this purpose the group of Adriana Maggi in Milan (Ciana et al. 2001, 2003) engineered a transgenic mouse that ubiquitously expresses a luciferase reporter gene in all known estrogen target tissues. The construct used for transgenesis consisted of the reporter gene driven by a dimerized estrogen-responsive element (ERE) and a minimal promoter. The key to the successful realization of this model was most probably the use of insulators. It is well known that the expression of transgenes driven by weak promoters is heavily influenced by enhancers/silencers surrounding the regions of insertion. In addition, methylation may gradually extinguish their transcriptional activity. Insulators have been successfully used to counteract these effects in

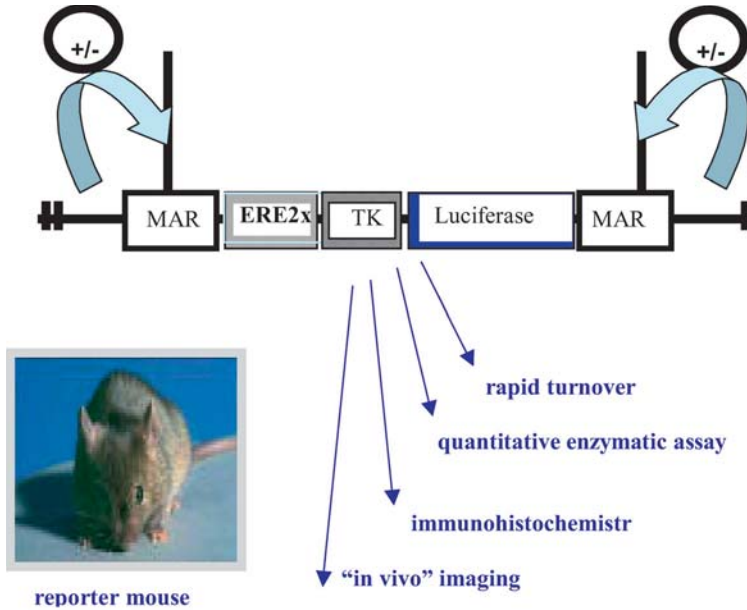

Fig. 1. The constructs for transgenesis consist of the reporter gene driven by a dimerized estrogen-responsive element (*ERE*) and a minimal TK promoter. The construct is flanked by matrix attachment region (*MAR*) insulator sequences to achieve a generalized, hormone-responsive luciferase expression. The advantage of using this kind of luciferase construct to make transgenics is indicated: rapid turnover of the luciferase protein, luciferase activity can be quantitatively assayed, luciferase can be detected using immunohistochemistry, luciferase activity can be used for BLI

specific tissues. In the transgenic animals used for BLI, the matrix attachment region (MAR) sequence was used as an insulator to flank the construct and, thus, to achieve a uniform and consistent, hormone-responsive luciferase expression in all target tissues (see Fig. 1).

Apart from the advantage of using insulators to obtain a uniform, hormone-responsive luciferase expression, the use of luciferase offered the additional and important advantages of this reporter listed above. Accordingly, the ERE-*luc* mouse allowed us to monitor, in a

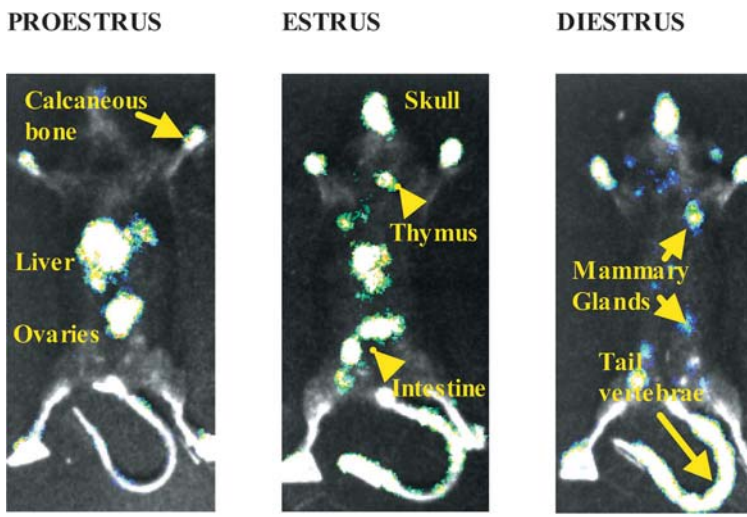

Fig. 2. In the three *upper panels* BLI of ER transcriptional activity is shown at 3 consecutive days. In the *lower panel* plasma estradiol levels in cycling mice is shown

spatiotemporal manner, the transcriptional activity of ER genes in intact or hormonally manipulated mice.

Figure 2 shows the ER transcriptional activity measured by BLI during the 5 day estrous cycle of female ERE-*luc* mice. The phase of the estrous cycle was determined by vaginal smears. For 3 consecutive days the mice were anaesthetized at a fixed time point in the morning (9:00 a.m.) and injected with 100 µl D-luciferin (25 mg/ml) 10 min before imaging. In these experiments the imaging system consisted of an intensified charge-coupled device camera (VIM camera, model C2400-47; Hamamatsu Photonics, Hamamatsu, Japan) fitted to a light-tight chamber (Hamamatsu Photonics) and equipped with an 80 mm/f 0.9 f Schneider lens. The images are generated on an Argus-20 image processor (Hamamatsu Photonics) and transferred via SCSI using a software module (Openlab; Improvision, Coventry, UK) to a computer (Macintosh G4; Apple Computer, Cupertino, CA) and processed by image analysis software (Openlab; Improvision, Coventry, UK).

As shown in Fig. 2, the level of estradiol is highest at pro-estrus and at this time point the highest photon emission was observed in the liver and ovaries with very little activity in limbs and tail (vertebrae) and none in the area corresponding to the brain. The next morning, in estrus, when the same mouse was imaged again, a very different pattern of luciferase/luciferin-related photon emission, showing much lower emission in areas corresponding to liver and ovaries than the previous day, was observed. Now photon emission was clearly seen in areas corresponding to the head (=brain+bone), bone (ankles, wrists, and tail) and intestine and thymus. The following day when the mouse was in diestrus-1, there was much less signal coming from liver and ovaries, but the strongest signals were now coming from the head, which includes brain and skull bones, the mechanically loaded ankle and wrist bones and tail vertebrae (note the increase over time in the tail vertebrae). At the same time point there was also detectable expression in some of the mammary glands. This pattern of ER transcriptional activity as detected by BLI during the estrous cycle was very reproducible and indicated that there are tissues with either estrogen (ligand)-dependent or -independent activation of ER transcriptional activity. This was confirmed by biochemical measurement of luciferase enzyme activity in the same tissues at different phases of the cycle. What was extremely interesting is the fact that in tissues with reproductive functions, like the ovaries, uterus, mammary glands, hypothalamus, and liver, the activity was highest during pro-estrus, when estrogen levels were also the highest. In the nonreproductive tissues like bone, brain, intestine, kidney, lungs, and thymus, maximum luciferase activity was found at di-estrus, when estradiol levels are low. In line with this was the finding that progesterone receptor content, a direct downstream ER target, was maximal in liver at pro-estrus and maximal in bone at di-estrus. These results suggest that ER transcriptional activity in reproductive tissues is essentially dependent on the presence of the ligand, while in nonreproductive tissues it is not.

Further evidence for estrogen-independent activation of ER transcriptional activity came from the observations that in situations where gonadal estrogen synthesis is absent, as in 10-day-old immature female mice and 3 weeks after ovariectomy (OVX), luciferase activity could still be detected. In the immature mice, luciferase ac-

tivity was even higher in the nonreproductive tissues like bone and brain compared to adult mice, whereas in the estrogen-dependent liver, ER transcriptional activity, as expected, was lower in the immature mice compared to adult mice. Similarly, 3 weeks after OVX both the imaging and biochemical detection of luciferase expression showed appreciable activity under basal conditions in bone, brain, and the intestine, being the activity in bone and brain 50%–60% of the maximal activity measured during the cycle. As expected, the ligand-dependent tissues, liver and uterus, had 13% and 7% of the highest activity as measured during pro-estrus of normal cycling female mice.

Although these data strongly indicate a different regulation of estrogen receptor transcriptional activity in the liver and reproductive organs compared to the nonreproductive organs, when estrogen is administered exogenously to 3-weeks ovariectomized mice, all tissues tested responded by an increase in ER transcriptional activity. However, the response to 5 μg/kg 17β-estradiol, which is comparable to levels at pro-estrus, was much higher in liver and uterus (eight- and sixfold) compared to brain and bone (about twofold). This difference can be explained by a difference in basal activity of ERE-*luc* after OVX, which, as mentioned earlier, is higher for bone and brain compared to liver and uterus. These findings show that the observed differences in ER transcriptional activation are not due to their ability to respond to circulating estrogen per se. Furthermore, administration of the specific ER-antagonist ICI182,780 to either immature mice or to adult female mice resulted in a strong reduction in luciferase activity in all organs, although the relative decrease was stronger in liver compared to brain and bone. This suggests that even in the absence of estrogen the luciferase activity strongly depends on activated ERs.

All together, the findings described above and described in more detail elsewhere (Ciana et al. 2003) strongly suggest that both in immature and adult cycling mice ER can be activated by estrogen-independent mechanisms in nonreproductive tissues. Since it has been shown both in vitro and in vivo that ER activity can also be modulated independently of estrogen by signaling pathways downstream from growth factors and neurotransmitters, these are likely candidates involved in the estrogen-independent activation (Kato 2001).

In fact, it has been shown that IGF-1 administration to OVX ERE-*luc* mice resulted in an increase in luciferase activity in the uterus and in nonreproductive organs (Klotz et al. 2002). Preliminary data in the ERE-luc mice using an inhibitor of IGF-1 signaling indicate that it mainly blocked the activation in the nonreproductive organs, suggesting that indeed IGF-1 is a major player in this process (A. Maggi, personal communication). This is also in agreement with the knowledge that during the estrous cycle in mammals circulating IGF-1 levels increase at estrus and decline during the luteal phase (Hashizume et al. 2002). Since most of the circulating IGF-1 is derived from the liver, it is very possible that during pro-estrus, when estrogen levels are high, estrogen activates ER, leading to enhanced IGF-1 production as has been shown in other organs and cell types (Kassem et al. 1998; Shingo et al. 2003). This in turn would lead to activation of the ER in the nonreproductive organs.

These new exciting findings may have great implications for hormone replacement therapy (HRT) and were initially discovered due to transgenic mice in which recording of gene expression was greatly facilitated by BLI. The search for new tissue selective modulators of ER activity (SERMs), either synthetic or natural, will be made much easier using these kind of transgenic reporter animals in combination with BLI.

11.3.2 Osteocalcin-*luc* Reporter Mice to Monitor Bone Formation and Remodeling

Osteocalcin, a noncollagenous bone protein synthesized by the osteoblast, has been suggested as a good biochemical marker for bone turnover or bone formation (Young 2003). Osteocalcin is one of the most abundant noncollagenous proteins in bone and expressed by mature osteoblasts, osteocytes, and to some extent in odontoblasts, and hypertrophic chondrocytes. Osteocalcin is a bone-specific protein that binds calcium and has strong affinity for hydroxyapatite, and its production by osteoblasts adjacent to mineralizing surfaces coincides with mineralization of developing bone. In vitro experiments in developing osteoblast cultures has shown that osteocalcin starts to be expressed at the onset of mineralization. Moreover, the

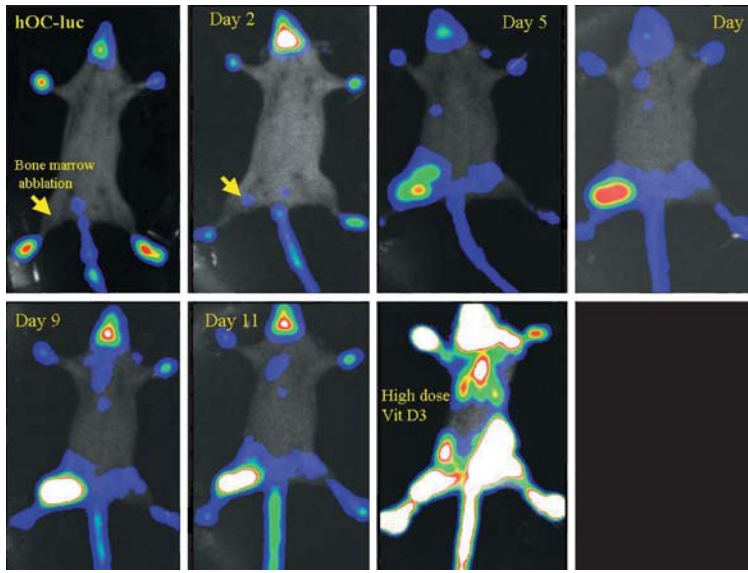

Fig. 3. Bone formation model. BLI of a hOC-*luc* mouse under basal conditions and after bone marrow ablation, which is known to result in very strong and local bone formation during the first 10–12 days after the ablation. In the *last frame* a mouse is shown after stimulation with 1.25(OH)2D3, a known stimulator of human osteocalcin expression. The mice were monitored every morning at the same time and BLI was performed with the animal lying in the same position on its back

human and rat osteocalcin gene contains a vitamin D response element (VDRE) and, therefore, its expression is regulated by 1.25(OH)2D3 (Desai 1995).

Clemens and coworkers (Clemens et al. 1997) has generated transgenic mice in which the luciferase (*luc*) reporter gene was under the regulation of the human osteocalcin (hOC) promoter. Using these hOC-*luc* mice in combination with BLI, one can study the human osteocalcin gene expression in vivo noninvasively and in principle detect changes in bone formation and/or remodeling.

As shown in Fig. 3, under basal conditions the hOC-*luc* mouse strongly expresses the gene at all sites of the body with high mechanical loading, like the ankle and wrist bones, the jaw, and the tail

vertebrae. It is known from studies in humans that especially the calcaneus (ankle) bones are undergoing high mechanical forces during mechanical loading due to walking, running, and jumping (Lehtonen-Veromaa et al. 2001). Since mice are walking on 4 feet, all the calcaneus bones show high luciferase activity. The luciferase expression shown in the bones of the jaw is the result of the high remodeling of alveolar bone as a result of the constant growth of the incisor teeth, which is characteristic of the rodents. In the hOC-*luc* mice the expression in the jaw varied during the day most probably consequent to the known circadian rhythm of the incisor growth and mastication due to feeding schedule (Ohtsuka and Shinoda 1995). Finally, the tail vertebrae of mice are mechanically loaded by continuous tail movements.

Depending on the physical activity of the mice and food intake, the gene expression of the human osteocalcin gene varied, most probably due to differences in mechanical loading. This was in line with our observations that mice that have been inactivated for a while show much less activity in calcaneus bones and tail vertebrae.

Femoral bone marrow ablation in rodents has been shown to lead to a strong but temporary increase in new bone formation with a peak around 10–12 days after the ablation (Bab 1995). During this period, the total marrow space is completely filled with new bone trabeculae. Hereafter, the osteoclast will come in and completely resorb the new trabecular bone back to its original size within 8–10 days. Thus, in this model bone formation and resorption are nicely separated in time. When a bone marrow ablation was performed on the femur of the right hind leg of the hOC-*luc* mouse an increase of OC-*luc* expression could be visualized by BLI 2 days after the ablation and peak activity was obtained at day 9 (see Fig. 3). These data are in line with the recent findings of Iris and coworkers (Iris et al. 2003). When after 3 weeks the expression was back to normal, the mouse received one single high dose of 1.25(OH)2D3 which, as expected, resulted in an enormous increase in luciferase activity all over the entire skeleton due to increased OC-*luc* expression in almost all skeletal elements since the human OC-promotor is known to be activated by vitamin D3 (Clemens et al. 1997).

These findings show that the hOC-*luc* mouse model in combination with BLI is nicely suited to study effects of mechanical loading and drugs like vitamin D and related analogs.

These studies with transgenic *luc*-reporter mice show that BLI allows rapid and convenient assessment of changes in gene expression in time and place noninvasively. It also allowed us to make new, unexpected discoveries that are not easily made with conventional technologies.

11.4 Tumor Progression and Bone/Bone Marrow Metastasis of Breast and Prostate Cancer

11.4.1 Skeletal Metastasis and Minimal Residual Disease

Metastatic disease is the major cause of death in cancer patients. Bone marrow is a preferential site of metastasis in a variety of cancers. However, only a restricted number of solid cancers, especially breast and prostate cancer, are responsible for the majority of skeletal metastases. According to the "Seed and Soil" theory, this propensity to metastasize to bone is related to chemoattraction, survival, and growth factor support, specific for these cancers, that is provided by the bone marrow/bone microenvironment. Experimental evidence suggests that this support mechanism is active during bone resorption. Micrometastatic spread is often present in bone marrow of cancer patients devoid of clinically evident metastases (minimal residual disease) and represent the pathophysiological basis for cancer relapse as overt bone metastases (see Fig. 4). Established micrometastases can stay dormant in G_0-status for a long time, but can be activated through a local mesenchymal stroma reaction, usually cytokines or growth factors. Dormancy also explains the failure of chemotherapy or radiotherapy, which aims at inhibition of proliferation. Once the micrometastases starts growing into a macrometastases it can grow until a size of 2–3 mm^3 without the need of new blood vessels by co-option with the existing blood vessels. When this size is reached, hypoxia will occur, leading to induction of VEGFs and angiogenesis. Until now, there has been no good animal model to study minimal residual disease because the detection limit of tumor

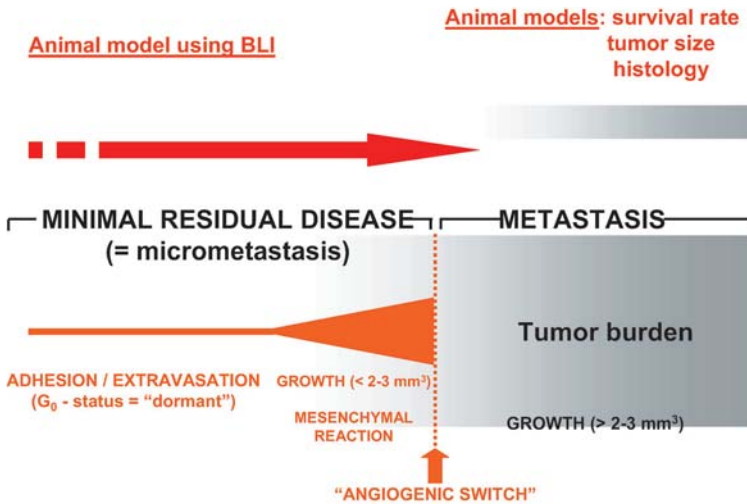

Fig. 4. An animal model for studying minimal residual disease using BLI

cells is not sensitive enough. In most current models tumor volume of subcutaneously transplanted tumors, survival rate, and histology are still used to monitor therapeutic effects. However, BLI now offers the possibility to measure tumor development, progression, and metastasis in a very rapid and time-sensitive manner.

An emerging concept identifies the growth support provided by a specific organ microenvironment as the principal target for adjuvant therapies aiming to prevent progression of micrometastases to overt metastases. The tumor-host interface in bone metastasis represents a unique opportunity to test this hypothesis, since, differently from other organs, therapeutic means that lower bone resorption and, consequently, tumor growth support are available.

Recently, we developed a BLI-based, highly sensitive method to monitor, continuously and reliably, development and progression of experimental bone metastases in living animals (Wetterwald et al. 2002). After intracardiac injection of the tumor cells, very small amounts of tumor cells can be detected in bone marrow/bone, mimicking micrometastatic spread. Therefore, this model is also a nice model for minimal residual disease and effectively allows sensitive

localization and growth monitoring of minimal metastatic deposits in the bone marrow at a stage largely preceding tumor-induced osteolysis. Below, the BLI models will be discussed and we will show, as an example, how they can be used to investigate the effects of a potent nitrogen-containing bisphosphonate (Olpadronate) on the development and progression of osteolytic metastases.

Our investigations may help to better identify situations at risk for bone metastasis and novel therapeutic strategies that could be extended to the clinical reality.

11.4.2 Bone Metastasis of Breast and Prostate Cancer

Because of the progress made in early detection and surgical treatment of the primary tumor, mortality in cancer patients is increasingly linked to metastatic disease. Bone is the second most frequent site of metastasis. However, only a restricted number of solid cancers, especially breast cancer (BCa) and prostate cancer (PCa), are responsible for the majority of skeletal metastases. These are a major cause of morbidity, characterized by severe pain and high incidence of skeletal and hemopoietic complications (fractures, spinal cord compression, and bone marrow aplasia) requiring hospitalization. They represent a relevant problem for health care because the patients may show a long clinical course. Furthermore, the quality of life of these patients and their families is greatly affected.

Despite the frequency of skeletal metastases in BCa and PCa, the molecular mechanisms for their propensity to colonize bone are poorly understood. This is also reflected by the fact that treatment options for bone metastasis, namely chemotherapy, radiotherapy, hormonal ablation, and surgery, are unsatisfactory.

Metastasis is a multistep process characterized sequentially by loss of intercellular cohesion, and induction of cell migration, angiogenesis, access to the systemic blood circulation, and subsequent extravasation at distant organs. In animal models, it has been estimated that $3–4\times10^6$ cancer cells/g of tumor can reach the bloodstream per day (Butler and Gullino 1975). However, according to the “Seed and Soil” theory (Paget 1889), for the final development of a metastasis it is essential that the metastatic cells can adapt, and therefore sur-

vive and grow, to the specific organ environment that they colonize. Genetic instability within the cancer cell population(s) of the primary tumor is responsible for the acquisition of these new properties. However, acquisition of "all" these properties is a relatively rare event and, consequently, the metastatic process is highly inefficient and only a restricted minority of cancer cells reaching the blood will survive and grow at the distant sites (Fidler 1970). It derives that therapeutic interference with the ultimate survival/growth step, targeting a small minority of cancer cells, should be far more efficient than inhibition of later steps of the metastatic process, which seems to be accomplished by a much greater number of cancer cells.

11.4.3 Bone Turnover

Bone is a highly dynamic tissue that is continuously remodeled by microscopic patches of bone resorption and subsequent bone formation. The result is replacement of old bone with new bone, thus maintaining structural integrity of the skeleton throughout adult life. These morphological entities, together with their cellular components, are called "basic multicellular units" (BMU; Eriksen et al. 1994). The number and the activity of these BMU determine the rate of bone turnover and they are under the control of mechanical stress, cytokines, and hormones. During the bone remodeling process, osteoblasts and osteoclasts, the cellular components of the BMU, secrete paracrine growth factors. Furthermore, there is experimental evidence that growth factors are exposed from the bone matrix and activated during bone resorption (Mundy and Yoneda 1996; see Fig. 5). Direct evidence that tumor cells do not have intrinsic ability to resorb bone and that osteoclasts are required for tumor-induced osteolysis and tumor growth was provided in osteoclast-deficient mice (Clohisy and Ramnaraine 1998). As described in the next section (see also legend to Fig. 5), increased bone resorption, especially near the site of tumor seeds, favors the growth of breast cancer cells. Furthermore, there is some clinical evidence that increased bone resorption is associated with a higher incidence of breast cancer bone metastasis.

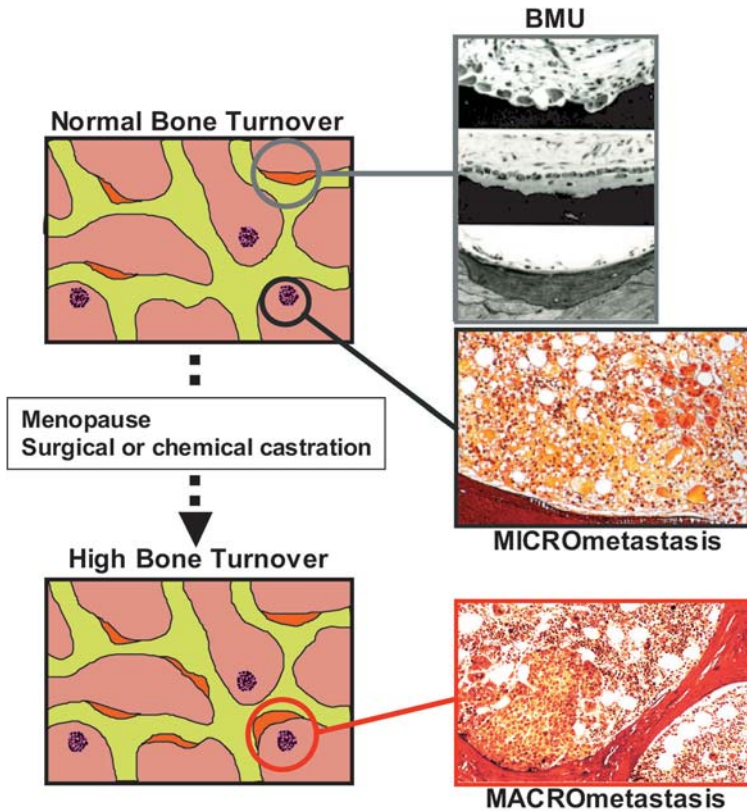

Fig. 5. Role of bone turnover in bone metastasis. During normal bone turnover, several BMUs are opened in which bone remodeling (bone resorption followed by bone formation) takes place. In this area, the bone marrow stroma is locally activated by cytokines to induce osteoclast and osteoblast formation from bone-marrow derived progenitors. If only a few BMUs are present, the statistical chance of activating a new BMU near a dormant micrometastases is relatively small. This changes dramatically when the number of new BMUs are opened, leading to a much higher risk of activating the dormant tumor cells and the formation of macrometastases

11.4.4 "Vicious Cycle" Hypothesis of Bone Metastasis

The bone/bone marrow microenvironment may contribute to development of metastasis at this site through chemoattraction, adhesion, survival- and growth-promoting molecules locally released during bone resorption/formation. The primary epithelial tumor cells lose their cell-cell adherence (E-Cadherin) and acquire an invasive phenotype (increase in proteolytic enzymes, i.e. cathepsins and MMPs). They increase the production of angiogenic factors (i.e., VEGF, FGF) and intravasate into the blood vessels. Recent findings show that chemokine receptor CXCR4 and its ligand SDF-1 (stromal derived factor 1), which is also produced by bone marrow stroma cells, play a critical role in metastasis to bone marrow (Muller et al. 2001). We have shown that β-integrins play an important role in adhesion to bone marrow stroma. Secretion of parathyroid hormone-related protein (PTHrP) by tumor cells present in the bone microenvironment activates osteoblasts to produce receptor activator of nuclear factor-κB ligand (RANK-L) and to downregulate osteoprotegerin (OPG). As a result, osteoclast recruitment and activity are stimulated, and bone resorption is increased. The consequent release of growth factors, such as transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), and other cytokines stimulates tumor growth and further secretion of PTHrP and VEGFs, thus establishing a "vicious cycle" (Guise et al. 1996; Guise 1999; Mundy 2002; see Fig. 6). Accordingly, pharmacological suppression of bone turnover (i.e., bisphosphonates, BP) should interfere with its growth support and, thus, prevent development and progression of bone metastases.

11.4.5 Micrometastases and Minimal Residual Disease

Due to improved and earlier diagnosis, an increased proportion of cancer patients, including those affected by BCa and PCa, shows no macroscopic metastasis at the moment of first diagnosis. Nevertheless, 30% of these patients will develop macroscopic metastasis in the following 5 years. It is now clear that already at the moment of the original diagnosis these patients have microscopic deposits of

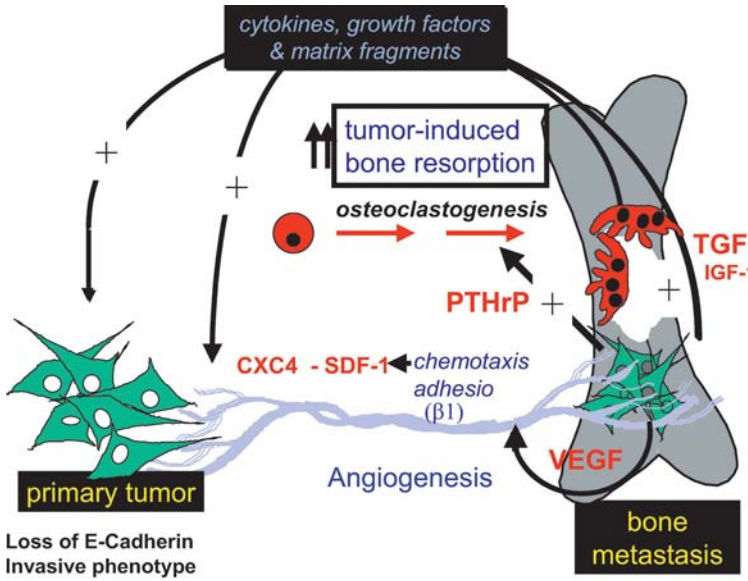

Fig. 6. The "vicious cycle" hypothesis of bone metastasis

cancer cells in various tissues that cannot be identified by current diagnostic methods. In clinical studies, micrometastases have been detected in a vast proportion of the bone marrow aspirates of subjects affected by mammary and prostate carcinoma with no radiological or scintigraphic evidence of bone metastases (Braun et al. 2000; Mansi et al. 1987; Melchior et al. 1997; Gao et al. 1999). This has led to the hypothesis that most cancer patients harbor neoplastic cells in their bone marrow at the time of diagnosis (Lange and Vasella 1999). However, analysis of their cell cycling status shows that most of these cancer cells are dormant (G_0-status; Pantel et al. 1993). After resection of the primary tumor, the proportion of bone marrow aspirates that are positive for cancer cells dramatically declines and, eventually, revert to a negative status within 6–9 months (Lange and Vasella 1999). This indicates that the presence of micrometastases is a relatively early event in the disease process and it is due to the continuous supply of blood circulating cancer cells as a

result of cell shedding from the primary tumor. The G_0-status of these micrometastatic cells may explain failure of chemotherapy and suggests that alternative adjuvant therapies, aiming to repress microenvironmental growth support during the period following surgery, are a better therapeutic rationale.

11.5 Use of Bioluminescent Imaging in Animal Models of Skeletal Metastasis

11.5.1 Breast Cancer Bone Metastasis Model

In 1988, Arguello and coworkers showed that injection of cancer cell lines into the left cardiac ventricle of immunodeficient (*nu/nu*) mice will lead to colonization of specific sites of the skeleton. This model is now a widely used animal model of bone/bone marrow metastasis. We and others have applied this model to study human breast cancer metastasis (Arguello et al 1988, 1992; Sasaki et al. 1995; Yin et al. 1999; van der Pluijm et al. 2000, 2001, 2002a, b; Wetterwald et al. 2002). For this we use the highly malignant human breast carcinoma cell line MDA-MB-231, that metastasizes to bone marrow and will rapidly cause multiple osteolytic lesions that can be detected using X-ray radiography. However, radiological-evident osteolytic/osteosclerotic metastases are a late and macroscopic event in the development of metastatic bone disease. Thus, the model lacks the sensitivity that would be necessary to dissect the initial processes essential for tumor progression. Furthermore, the radiological detection of osteolytic (or osteosclerotic, as seen in prostate cancer) metastases is an indirect measure of the tumor burden. Therefore, we developed a more sensitive method to detect and monitor directly metastatic growth in bone marrow/bone using whole animal BLI (Wetterwald et al. 2002).

To use BLI to monitor osteolytic bone metastasis of breast cancer, we use a stably *luc*-transfected, bone-seeking sub-clone (MDA-231-B) derived from four sequential cycles of intracardiac inoculation in vivo and expansion in vitro of the cell population recovered from the resulting bone metastases. Using BLI in this model has the following advantages:

1. Early detection of minimal bone metastatic sites (=0.3 mm^3 volume/approximately 5,000 cells; when the metastases are not yet osteolytic), as compared to conventional radiography detecting almost exclusively the osteolytic sites (2–3 weeks vs. 5 weeks)
2. Much higher precision and reliability, as compared to radiography
3. The possibility to quantify the tumor burden for each metastatic site
4. The possibility to follow the kinetics of tumor growth in the same animal
5. Targeted dissection of all the metastatic sites for end-point histological and/or molecular analysis
6. Detect possible nonosteolytic and soft tissue metastases.

11.5.1.1 Intracardiac Injection Model

Figure 7 shows the principle of the intracardiac injection model.

11.5.1.2 Intraosseous Implantation Model

Figure 8 depicts the principle of the intraosseous implantation model.

11.5.2 Bisphosphonates

Bisphosphonates (BP) are nonhydrolysable pyrophosphate analogs that exert a strong inhibitory effect on osteoclastic bone resorption (Fleish 1998; Rodan 1998). Typically, BP show a very high affinity to bone mineral and, consequently, in vivo they exclusively accumulate in bone (Fleish 1998). Nitrogen-containing BP inhibit the prenylation (mevalonate pathway) of guanidine triphosphate-binding proteins required for osteoclast development, function, and survival (Luckman et al. 1998; van Beek et al. 1999a, b; Russell et al. 1999). Nonnitrogen containing BP, such as Clodronate, acts prevalently by generation of cytotoxic ATP analogs (Frith et al. 1997).

Bisphosphonates have been reported to exert direct antiproliferative and proapoptotic effects on cancer cells in vitro (Shipman et al. 1997; Seneratne et al. 2000; Lee et al. 2001), to interfere with cancer cell adhesion to bone matrix proteins (van der Pluijm et al. 1996; Boissier et al. 1997), and to inhibit matrix metalloproteinases (Heikkila et al. 2002; Teronen et al. 1999) and cancer cell migration

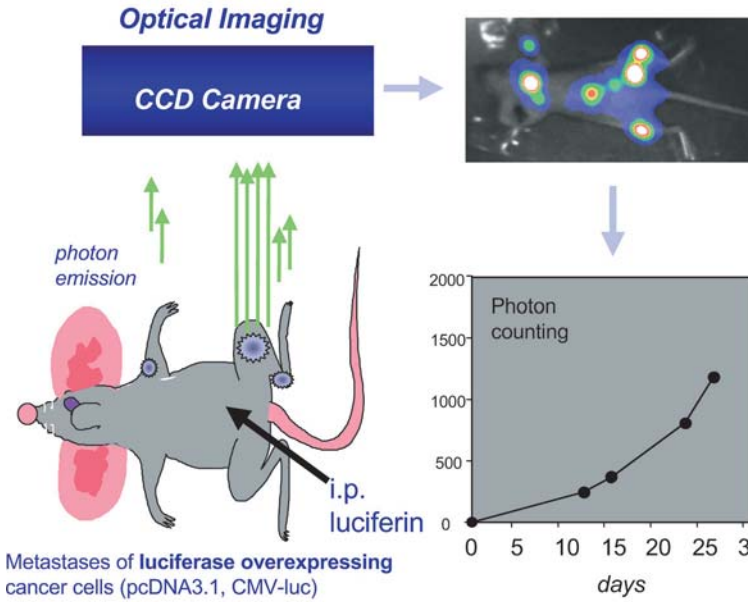

Fig. 7. Intracardiac injection model. Injection of 100,000 MD-231 B-*luc* cells into the left heart ventricle of 8-week-old female nude mice causes multiple skeletal metastases. At any given time point the animals can be monitored using BLI. For this the animals were anesthetized and 25 µl of a 250-mmol/l aqueous solution of luciferin was injected intraperitoneally 10 min before beginning photon recording. Mice were placed in the light-tight chamber and a gray-scale image of the animal was first recorded with dimmed light. Photon emission was then measured and recorded as pseudo-color images. For colocalization of the bioluminescent photon emission on the animal body, gray-scale and pseudo-color images were merged by using imaging software. The progression of each individual metastases can be monitored in time as indicated

and invasion (Boissier et al. 2000). An antiangiogenic effect has also been described (Wood et al. 2002; Fournier et al. 2002). All these effects are possibly relevant for cancer disease (Clezardin et al. 2003; Green 2002). However, in most of the cases they have been obtained at concentrations that are too high to be reached in vivo in the extracellular fluid.

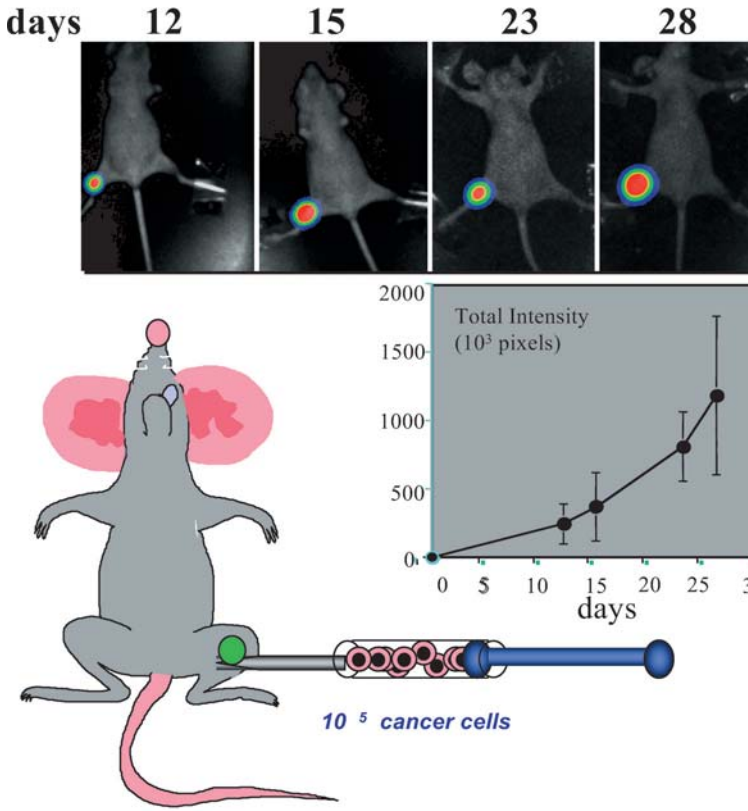

Fig. 8. Intraosseous implantation model. For direct intraosseous implantation of MDA-231B-*Luc* cells, two holes, 4–5 mm distant from each other and each with a diameter of 0.35 mm, were drilled through the bone cortex of the upper third of the right tibia with the aid of a dental drill. Space in the marrow cavity was created by flushing out the bone marrow of the proximal segment of the bone shaft. The upper hole was sealed with surgical bone wax and a single cell suspension of MDA-231B-*Luc* cells (1×10^5/25 µl of PBS) slowly inoculated via a 30-gauge needle inserted through the lower hole. Finally, the lower hole was sealed with surgical bone wax and the cutaneous wound was sutured. Local tumor development was monitored with BLI

In animal models of bone metastasis, BP have been reported to reduce the tumor burden in established bone metastases, but not in soft tissue and visceral metastases. This effect was suggested to be mediated by induction of apoptosis in the cancer cells (Hiraga et al. 2001). Prophylactic treatment with BP, given prior to the development of evident metastasis, resulted in a marked reduction of the number of bone metastases (Hall and Stoica 1994; Sasaki et al. 1995). However, in one preventive study it was reported that BP pretreatment leads to an increased tumor burden in the developing bone metastases, but not in extraosseous metastases (Kostenuik et al. 1993). Nevertheless, these results further supported the concept that BP inhibition of osteoclastic bone resorption could be a useful adjunctive therapy for the treatment of cancers that have colonized bone.

In clinical practice, BP have been widely used to control the skeletal complications (bone metastasis and humoral hypercalcemia of malignancy) in various neoplastic diseases. In established bone metastases, BP reduce the number of skeletal-related events (Elomaa et al. 1983; Berenson et al. 1996, 1998; van Holten-Verzantvoort et al. 1993; Hortobagyi et al. 1996). Other clinical studies have also shown that BP may prevent development of new bone metastatic foci in patients either with bone metastasis already present or with no bone metastasis at the beginning of the treatment (Kanis et al. 1996; Conte et al. 1996). However, a similar study failed to demonstrate a reduction in the number of metastases (van Holten-Verzantvoort et al. 1996). Three clinical trials have primarily focused their investigation on the possibility that BP may prevent the development of metastases in women affected by mammary carcinoma, but free of bone metastases at the moment of diagnosis. In two of these studies (Powles et al. 2002; Diel et al. 1998), the preventive administration of BP reduced significantly the number of patients developing bone metastases and, in those who developed bone metastases, the number of the bone metastases. In one study (Diel et al. 1998) there was also a significant reduction of the number of visceral metastases. However, in the third study (Saarto et al. 2001) BP treatment did not prevent the development of bone metastases and seemed even to increase the development of nonskeletal metastases. In prostate cancer similar prevention trials are ongoing, but the definitive results are not yet known.

The contradictory results reported above still leave some open questions concerning the efficacy of BP as adjuvant therapy for preventing bone metastasis in cancer disease. First of all, it is unresolved whether BP act directly on cancer cells or indirectly, via inhibition of bone resorption and, thus, release of growth factor support for cancer cells. Second, the stage of metastatic disease and the bone turnover status for which BP should find their best indication are not yet defined.

11.5.2.1 Treatment with the Potent Antiresorptive Bisphosphonate Olpadronate Inhibits Osteolysis But Does Not Inhibit Tumor Progression of Established MDA-B-*luc* Tumors in Bone

In order to determine if bisphosphonate treatment also effects tumor progression of breast cancer tumors in bone, either directly or indirectly via inhibition of bone resorption, the following experiments were performed. One hundred thousand MDA-B-*luc* tumor cells were inoculated directly into the bone marrow space and 14 days later, when small photon-emitting tumors had formed, daily Olpadronate treatment (1.6 μM/kg/day subcutaneous) started and lasted 28 days. Tumor-induced osteolysis was determined using X-ray radiography and depicted as lytic area (mm^2). Changes in tumor volume were determined using BLI and depicted as total intensity (number of pixels).

In the left upper panel of Fig. 9, it is shown that Olpadronate treatment strongly inhibits tumor-induced osteolysis. However, as shown in the right upper panel, Olpadronate treatment does not have an inhibitory effect on tumor progression as measured by BLI. This was confirmed by histological examination of the bones. It is clear that in the vehicle-treated animal, the bone (in green) is completely lost and fractured and that the tumor is now also growing outside of bone. In the Olpadronate-treated animal, the bone collar is still intact and the bone is not fractured. However, the tumor is also growing outside of bone. The reason for this is that due to inhibition of bone resorption the tumor cell can not grow any further within the bone marrow compartment and will migrate outside of the bone marrow space via the vascular channels. These data show that Olpadronate has no direct antitumor effect and that inhibition of bone re-

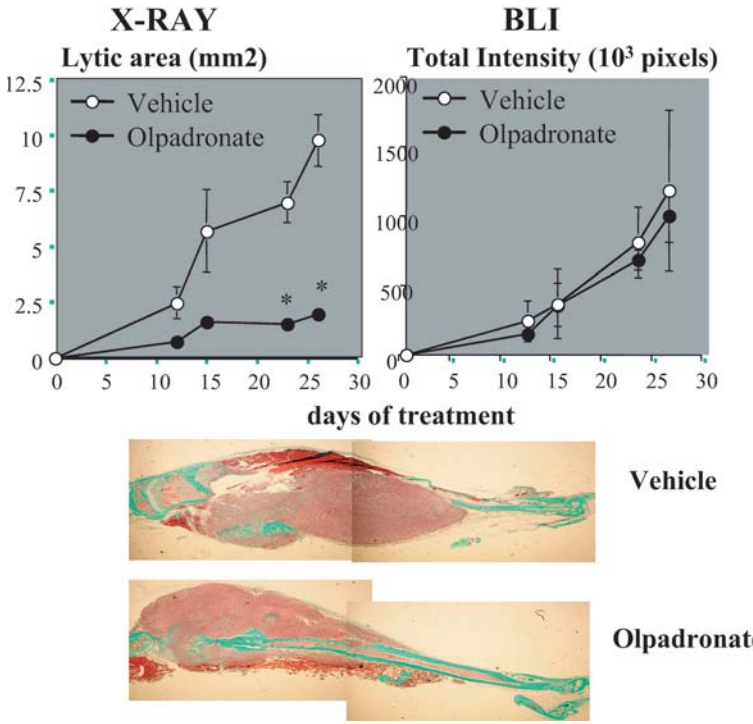

Fig. 9. Effect of the potent antiresorptive bisphosphonate Olpadronate on established MDA-B-*luc* tumors growing in bone. For this purpose, 100,000 MDA-B-*luc* cells were intraosseously inoculated and daily treatment with Olpadronate started 14 days after inoculation, when small tumors were established. Osteolysis was measured using X-ray radiography and tumor progression was measured using BLI

sorption does not lead to a slower progression of already-established tumors within bone.

11.5.2.2 Olpadronate Inhibits the Formation of New Bone Metastases

To test whether prophylactic Olpadronate treatment could reduce the number of metastases formed, the following experiments were performed. Two days before intracardiac injection of the MDA-B-*Luc*

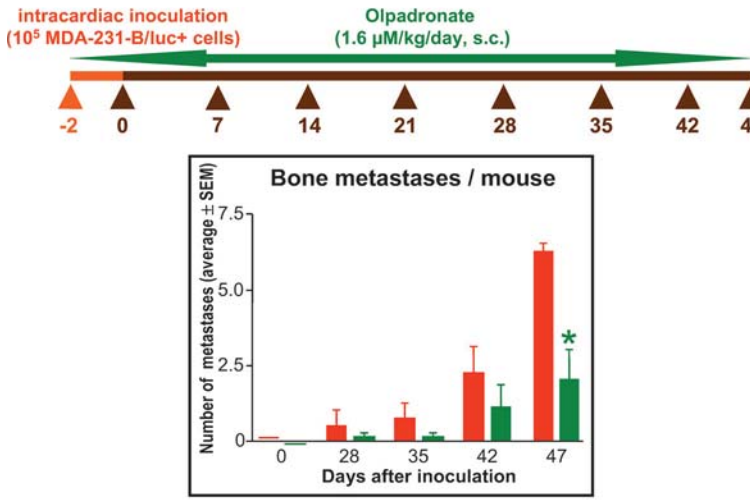

Fig. 10. Effect of prophylactic Olpadronate treatment on the development of bone metastases after intracardiac inoculation of MDA-B-*luc* breast cancer cell

cells, the daily treatment with Olpadronate (1.6 µM/kg/day subcutaneous) started and lasted 47 days. The number of metastatic sites in the whole animal was determined using BLI (see also Fig. 7). As depicted in Fig. 10, when Olpadronate was given daily starting 2 days before the inoculation of the tumor cells, there was a significant reduction in the number of metastases formed.

These findings lead us to conclude that bisphosphonates, via their antiresorptive activity, can reduce breast cancer metastasis to bone, most probably by reducing bone remodeling, leading to a decrease of local factors that are normally released during the resorption process and that are involved in activation of micrometastases. However, our data also suggest that once a micrometastases has turned into a macrometastases or small tumor, it becomes independent of local bone turnover for its growth and, therefore, bisphosphonate treatment will not slow down tumor progression. We also did not find any evidence for a direct effect of Olpadronate on tumor growth. It might still be possible that, dependent on the structure of

the bisphosphonate used, compounds with lower bone affinity that are also slowly released from the bone surface can have direct effects on the nearby tumor itself. In our preliminary experiments we did not find an increase in soft tissue metastases. However, it is important to note that our studies were performed with a selected clone of MDA-MB-231 cells which almost exclusively give rise to bone metastases. Other studies are needed to investigate this in more detail.

11.6 Conclusions and Future Perspectives

It is clear from the work presented in this chapter and from work by others that BLI is perfectly suited to monitor gene expression in transgenic reporter mice and to detect and follow small numbers of cells noninvasively. As we have shown, it also enables the quantification of tumor cells within internal organs in animal models of cancer. BLI is a powerful tool in functional genomics of cancer development, progression, and metastasis and will allow us to identify in vivo molecular targets of cancer and their metastasis. The application of BLI in combination with new animal models for cancer will allow us to study very rapidly and conveniently the efficacy of new therapeutic approaches such as gene therapy stem cell therapy and antiangiogenic therapy, and when successful can be a first step towards clinical application. Furthermore, the development of new smart luciferase-based reporter constructs as well as new possibilities to create transgenic animals containing these reporter constructs will make noninvasive in vivo BLI also a powerful new tool in other small animal models of human biology and disease.

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