Molecular imaging: A new way to study molecular processes in vivo

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Abstract

Non-invasive imaging of reporter gene expression using different imaging modalities is increasing its role for the in vivo assessment of molecular processes. Multimodality imaging protocols overcome limitations to a single imaging modality and provide a thorough view of specific processes, often allowing a quantitative measurement and direct visualization of the process in a specific target organ or tissue. The use of the right reporter gene for the development of animal models and the characterization of its expression in different conditions and tissues is fundamental for basic, translational and future pharmacological applications of a given model. This paper summarizes the major steps in the development and evaluation of a specific animal model for in vivo molecular imaging studies and describes the first example of an animal model designed for the in vivo assessment of a specific receptor activity and its possible evolution towards multimodality imaging analysis.

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1. Introduction

The term “molecular imaging” refers to the convergence of approaches from various disciplines (cell and molecular biology, chemistry, medicine, pharmacology, physics, bioinformatics, and engineering) to exploit and integrate imaging techniques in the evaluation of specific molecular processes at the cellular and sub-cellular levels in living organisms.

In the assessment of physical, physiological or metabolic processes, classical imaging technologies can differentiate, under clinical circumstances, pathological from normal conditions but are rather non-specific. Molecular imaging techniques, instead, have led to the development of novel procedures with enhanced specificity. As a result, imaging nowadays focuses on an in-depth understanding of biological processes, early detection and characterization of diseases, and treatment evaluation based on molecular processes assessment (Massoud and Gambhir, 2003).

The advent of genetic engineering has brought about major changes to applied science, including, for example, the drug discovery pipeline. In the same way, the development and exploitation of animal imaging procedures is providing new means for pre-clinical studies (Maggi and Ciana, 2005).

By combining animal engineering with molecular imaging techniques, it has become possible to conduct dynamic studies on specific molecular processes in living animals. This approach could potentially impact on pre-clinical protocols, thus widely changing all aspects of medicine (Maggi et al., 2004a).

2. Molecular imaging applications

The main goal of molecular imaging techniques is to directly assess in living organisms specific processes at the cellular level, including gene expression, protein-protein interaction, dynamic cell tracking throughout the entire organism, and drug action analysis. Molecular imaging techniques may therefore contribute tremendously to our understanding of the physiology of living organisms and provide new means for drug target identification and pre-clinical testing to improve drug discovery. Molecular imaging protocols can achieve these goals non-invasively, rapidly, quantitatively, and repetitively in the same animal, under different conditions and stimuli.

Reporter genes are genetic markers that encode easily detectable proteins. Once located downstream from a specific promoter, these markers become extraordinary tools for reporting on the activity of the specific promoter and the factors regulating its activity. While reporter genes have been widely utilized to study cell physiology, the recent generation of animals ubiquitously expressing a given reporter gene now allows direct in vivo analysis of gene expression and regulation.
3. Imaging modalities

Imaging transgene expression in living animals is principally based on the external detection of bioluminescence (optical imaging) or γ-photon (radionuclide-based imaging) emitted by a reporter located within the body. The reporter can be an enzyme that catalyses a reaction that produces light or that modifies a labeled substrate trapped within the cells, which in turn permits the localization of cells expressing the reporter gene (Massoud and Gambhir, 2003). Otherwise, a reporter can be an acceptor capable of binding a probe (radionuclide tracer or fluorescent molecule) with high affinity (Massoud and Gambhir, 2003; Weissleder, 2002).

3.1. Optical imaging

Optical imaging is based on bioluminescence or fluorescence. Reporter genes, used in bioluminescence imaging protocols such as luciferase, generate photons in the presence of the appropriate substrate, i.e. luciferin, proportionally to the level of luciferase expression. The major limitation to the use of bioluminescent reporters is that the light signal is absorbed and scattered in the tissue volume these low energy photons encounter on their path from the emission site to the detector system. It has been calculated that about 90% of the bioluminescence signal is attenuated per centimeter of tissue depth. So, the amount of photons detected may be not proportional with the reporter expression or insufficient to visualize the enzymatic activity present in the most inner organs of the most common laboratory animals, including mice. Since red light is more efficiently transmitted through tissues than light of other wavelengths, efforts are being made to generate reporters that emit photons at wavelengths above 600 nm. Luciferase activity from Photinus pyralis produces a broad spectrum that peaks at 560 nm with a wide fraction above 600 nm, whereas wild-type green fluorescent protein (GFP) from Aequorea victoria, upon excitation, emits light with a peak at 509 nm. GFP and luciferase mutant variants have been created to shift the emission closer to infrared wavelengths (Matz et al., 2002; Contag and Bachmann, 2002).

Although luciferase enzymatic activity results in the direct emission of photons, GFP needs to be excited by an external light source; therefore, the influence of tissue scattering and absorption is doubled with respect to bioluminescent probes. This limits the use of GFP to very small organisms. At present, optical imaging can be achieved with relatively low-cost instrumentation endowed with good resolution (Table 1), but it lacks three-dimensional (3D) representation of the original photon-emitting source.

3.2. Nuclear-based imaging

An alternative to optical imaging is positron emission tomography (PET) and single photon emission tomography (SPET) technology, by which reporter expression is localized with reporter probes, i.e. radiotracers that bind specifically to the reporter itself. So far, the best reporters developed for PET-based imaging of small animals are the dopamine D2 receptor (Elhrin et al., 1985; Wagner et al., 1983; MacLaren et al., 1999) and the viral thymidine kinase (Massoud and Gambhir, 2003; Gambhir et al., 2000). Efficient radiotracers are available for both approaches (Tjouvar et al., 1995, 1996, 1998; Namavari et al., 2000; MacLaren et al., 1999; Liang et al., 2001). Many other radiotracers have been developed to study cell proliferation, metabolism, apoptosis and other processes as well (Mankoff et al., 2005; Issa et al., 2004; Glaser et al., 2003; Vergote et al., 2001; Delbeke and Martin, 2004). By combining the radionuclide approach with computed tomography techniques 3D images can be generated with a resolution approaching 1 mm³ (using dedicated animal PET scanners) (Chatzioannou et al., 2001), a degree of resolution not yet achievable with optical imaging (Table 1). Conversely, the sensitivity of animal PET remains lower than that of optical imaging. Furthermore, the need of a cyclotron to produce isotopes and the difficulties in developing novel radiotracers still pose major limitations to the use of this technology. The temporal resolution of optical imaging is actually far superior to that of radionuclide techniques and allows better assessment of rapidly cycling processes.

Table 1

<table>
<thead>
<tr>
<th>Radionuclide and optic imaging: pros and cons</th>
<th>Optical imaging</th>
<th>Radionuclide imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation</td>
<td>Visible light or near infrared</td>
<td>γ-rays at high or low energy</td>
</tr>
<tr>
<td>Spatial resolution</td>
<td>About the depth of the object observed (3–5 mm)</td>
<td>1–2 mm</td>
</tr>
<tr>
<td>Depth</td>
<td>1 cm-variabile</td>
<td>No limit</td>
</tr>
<tr>
<td>Temporal resolution</td>
<td>Seconds to minutes</td>
<td>10 s to minutes</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>10⁻¹⁵ to 10⁻³ mol/L bioluminescence; 10⁻¹⁰ to 10⁻⁷ mol/L fluorescence</td>
<td>10⁻¹⁷ to 10⁻¹⁵ mol/L</td>
</tr>
<tr>
<td>Type of probe</td>
<td>Fluorescent or enzymatic activity leading to bioluminescence</td>
<td>Radiolabeled</td>
</tr>
<tr>
<td>Amount of probe needed</td>
<td>Micrograms to milligrams</td>
<td>Nanograms</td>
</tr>
<tr>
<td>Quantitative analysis</td>
<td>Relative quantitation</td>
<td>Absolute quantitation</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Advantages</td>
<td>High sensitivity, rapidity, low-cost, low labour consuming</td>
<td>Simultaneously imaging with multiple probes, high sensitivity, quantitative, three-dimensional images</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>2D images, depth-dependent intensity of signals (tissue absorption and scattering), poorly quantitative</td>
<td>High background, need of a cyclotron, high cost</td>
</tr>
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4. Imaging living animals

The choice of the reporter gene is the most important step in the generation of a new animal reporter model. But candidate reporter genes must satisfy several prerequisites before they can be used for molecular imaging studies. First, they must be detectable by in vivo studies, in other words, their expression must provide information about the location, the magnitude and the persistence of gene expression directly in vivo. Second, their expression must reflect the expression of the gene endogenously driven by the promoter under study. Third, the turnover rate of the reporter proteins must be short enough to allow rapid assessment of changes in the molecular process under investigation occurring in a short time.

Many transgenic animals engineered with different reporters are being employed in the study of molecular processes such as drug metabolism (Zhang et al., 2003; Robertson et al., 2003), genotoxicity (Gossen et al., 1989), and the effects of toxic compounds (Sacco et al., 1997; Wirth et al., 2002). To do this, however, a reporter animal suitable for molecular imaging studies should display specific features. It should provide an ubiquitous expression of the reporter; it should permit rapid assessment of all organs where a given compound is active and an evaluation of drug response after single and repeated administrations; it should be sensitive enough to detect a response to low ligand concentrations; it should also allow the evaluation of the toxicity of the compound under investigation in all tissues and permit the detection of active metabolites and the assessment of their profile of action.

Measured against these requirements, most currently available reporter animals have major limitations for pharmacological application in that some use reporters unsuitable for in vivo imaging or not ubiquitously expressed or with a too long turnover rate to permit dynamic evaluation of the process under study (Sacco et al., 1997; Nagel et al., 2001). These models only provide information about the presence of a specific event in the model before or after a specific treatment, but they do not permit dynamic study or quantification of the specific process.

The first example of a reporter animal developed for in vivo studies for drug development is the ERE-Luc mouse (Ciana et al., 2001).

4.1. ERE-Luc mouse model as a paradigm for molecular imaging studies

Estrogens, by signaling through two nuclear receptors (estrogen receptors, i.e. ERs), ERalpha and ERbeta, can control specific gene networks and modulate target cell activities. In the last decade, cell-based approaches have provided major insights into the transcriptional regulation of ERs at the promoter of target genes, where the interplay between ERs and coactivators and corepressors provides the receptors with tissue specificity of action. Molecular studies have revealed a multiplicity of mechanisms controlling ER transcriptional activation (Ciana et al., 2004). Furthermore, growth factors have been reported to stimulate unliganded ER to induce the transcription of selected target genes (Ciana et al., 2004; Bunone et al., 1996; Kato et al., 1995; Patrone et al., 1996). Activated ERs may cross-talk with other signaling pathways by influencing the activity of other transcription factors like AP-1, STATs, NFkB, SP-1 (Bjornstrom and Sjoberg, 2002; Ciana et al., 2003; McKay and Cidlowsky, 1998; Pasch et al., 1997; Stein and Yang, 1995; Webb et al., 1995; Vegeto et al., 2003) or by interacting with components of different transduction pathways such as Src (Migliaccio et al., 1996), MAPK (Wade et al., 2001) and G-proteins (Wyckoff et al., 2001). Considering the multiplicity of mechanisms and organs involved in estrogen action and the relevance of estrogens in human female physiology, novel approaches are needed to elucidate the exact physiological relevance of these alternative mechanisms in each target tissue.

Since cell systems cannot offer views into the hormonal activity on an entire organism, the ideal model to study the physiology and pharmacology of estrogen and cognate receptor activity is a reporter animal system. A first example of an ER reporter mouse designed to obtain an in-depth view of the physiological mechanisms influencing ER transcriptional capacity and to identify molecules activating ERs in reproductive and non-reproductive organs was recently generated (Ciana et al., 2003).

The model was generated to ensure that: (i) the reporter gene is ubiquitously expressed and responsive to molecules activating ERs in all the mouse cells; (ii) the reporter is a protein not expressed by the mouse, easily measured by quantitative assays, a good antigen to facilitate its cellular localization by immunohistochemistry and visible by non-invasive imaging technologies. For these reasons, the firefly luciferase gene was selected as a reporter gene in the generation of this first animal model. Indeed, the luciferase gene:

1. encodes an insect enzyme not present in mammals, thus ensuring the absence of background due to endogenous product;
2. is easily quantitated by highly sensitive enzymatic assay;
3. permits localization studies with specific, commercially available antibodies;
4. permits optic imaging in live animals (Contag et al., 1997);
5. Most importantly, luciferase has a very short turn-over rate (Thompson et al., 1991); unlike other reporter proteins commonly used in transgenesis, it does not accumulate, thus providing a dynamic view of the state of ERs activity.

In the selection of the estrogen-responsive promoter, the idea of using strong promoters was ruled out because their high activity might have masked hormonal response or would have introduced new estrogen-independent elements for transcription. The prevailing idea was to use a promoter that would ensure a low basal state of reporter expression for highlighting the ubiquitous capacity of the reporter to be expressed in all tissues and hormone-dependent transcription.

Several different estrogen-inducible promoters were generated using deletion mutants of the minimal promoter from the thymidine kinase (tk) gene from herpes simplex virus or the basal TATA box linked to a variable number of estro-
gen receptor-responsive elements (ERE). The promoters were tested in transient transfection assays in different cell lines. The combination of 2 palindromic ERs spaced 8 bp apart located at 55 bp from the constitutive tk promoter (~109 fragment) provided the desired low basal transcription and the highest estrogen-induced reporter expression and was therefore selected (Ciana et al., 2001). In order to bypass position effects and to ensure the ubiquitous expression of the transgene, we adopted the insulator strategy. First described in the 1980s, insulator sequences were reported to increase the chance of obtaining correct transgenic expression in selected tissues (Sun and Elgin, 1999). Insulators are DNA elements that create open chromatin domains permissive to gene expression and constitute a barrier against the influence of distal silencer/enhancer sequences and against acetylation and methylation events, thus preserving the independence of the specific promoter-driven transcription (Burgess-Beausse et al., 2002). For the generation of our constructs we selected two well-characterized insulators from the chicken genome: the β-globin hypersensitive site 4 (HS4, Chung et al., 1993) and the matrix attachment region (MAR, Stief et al., 1989). A comparative analysis of luciferase expression in clones obtained in stable transfection assays carried out with pERE, pMAR and pHS4 plasmids (Fig. 1 A) showed that the presence of insulator sequences increased the number of luciferase expressing clones from 19 to 40–70%, thus enhancing the inducibility of luciferase expression by estrogen treatment (Fig. 1B).

pHS4 and pMAR constructs were employed to carry out transgenesis. Two of the 17 mouse lines generated expressed the transgene ubiquitously (Ciana et al., 2001). Only tissues expressing ERs showed an induction of reporter expression after hormone treatment, thus demonstrating the model’s efficiency in reporting the activity of such receptors. In tissues not expressing ERs or in which they were present only in a small cellular sub-set, hormone treatment did not significantly modify luciferase content.

Immunohistochemistry studies demonstrated the coexpression of luciferase and ERs, thus strengthening our data (uterus, prostate, liver, and lung).

We also demonstrated that luciferase accumulation was directly related to the amount of 17β-estradiol (ER ligand) administered to the mouse and was compatible with the hormone concentration known to regulate receptor activity in vivo; the timing of estrogen-dependent accumulation of luciferase was in line with previous observations made with endogenous estrogen target genes (progestosterone receptor gene); the expression of the reporter was blocked by treatment with ER antagonists, thus demonstrating the specificity of the model in reporting ERs activity but not estrogen-related receptors (ERRs) activity (Giguere (2002)). After having tested these features, we concluded that in the ERE–Luc mouse luciferase expression reflects the state of ER transcriptional activity (Ciana et al., 2001, 2003; Klotz et al., 2002; Di Lorenzo et al., 2002). This animal model allowed us not only to gain new insights into the physiology of ERs but also to demonstrate for the first time that unliganded activation of ERs occurs in vivo (Klotz et al., 2002; Maggi et al., 2004b).

The model has proven to be particularly useful also for the analysis of toxic compounds acting on ERs during pregnancy and breast-feeding (Di Lorenzo et al., 2002). Furthermore, the use of the reporter mouse revealed a differential mechanism of ER activation in reproductive and non-reproductive tissues, leading to the formulation of novel strategies for hormone replacement therapies in post-menopause (Ciana et al., 2003). This result was facilitated by the application of optical imaging technology to dynamically and repeatedly monitor ERs activity (Fig. 2).

5. Development of new models and new strategies

Multimodality imaging strategies could help to improve approaches to visualizing molecular processes in vivo. The ERE-Luc mouse is a paradigmatic model for molecular imaging in pharmacological studies, but it is restricted by limitations intrinsic to optical imaging: bi-dimensional images, tissue scattering and absorption of photons, it is not fully quantitative and is exploitable only in small animal models.

To overcome the shortcomings each imaging modality has, a multimodality approach should be very useful for detecting reporter gene expression. To this aim, several approaches can be taken (Massoud and Gambhir, 2003): (a) vectors containing a reporter gene whose expression can be detected by two different imaging modalities; (b) vectors expressing fusion proteins that can be visualized by two different imaging techniques; (c) bicistronic vectors containing two different reporter genes under the control of the same unique promoter (using internal ribosome entry sites, i.e. IRES sequences). In this case, a single promoter would lead the expression of two different genes placed on the same mRNA but translated into two different proteins that can be detected by two different imaging protocols.

![Fig. 1. Insulator activity prevents position effects in stable transfection experiments (Ciana et al., 2001). (A) DNA vectors used in transgenesis with (pMAR and pHS4) or without (pERE) insulators. (B) MCF-7 cells were cotransfected with the pSV2neo vector expressing the G418 resistance gene. For each construct 40 clones of stably transfected cells were selected and analyzed for luciferase expression in the presence of 1 nM of 17β-estradiol or vehicle. Bars denote the percentage of clones expressing luciferase (open bars) or in which luciferase expression is inducible by estrogen treatment (black bar).]
The dynamics of 17β-estradiol action on its receptors can be followed by analyzing luciferase activity in the same animal at different time points. (A) Luciferase activity is shown in the whole animal; and (B) quantitated in different areas of interest. The estrogen receptor (ER) ligand 17β-estradiol (50 mg/kg) was administered to the mouse by subcutaneous injection and luciferase activity was measured 0, 3, 6 and 24 h later by injecting the animal with the luciferase substrate luciferin (25 mg/kg) 20 min before CCD camera exposure. Because of its short half-life in mammalian cells (3 h), luciferase activity declines rapidly and reaches baseline 24 h after estrogen administration, thus allowing dynamic assessment of the state of ER transcriptional activation. In (A), signal intensity as a result of photon detection is graded from white (highest number of photons) to blue (lowest intensity). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

PET imaging coupled with optical imaging seems to be the most doable technology. In fact, PET technique can provide 3D images of the sample, with a fully quantitative analysis of reporter expression. On the other hand, optical bioluminescent imaging very easily and rapidly produces bi-dimensional images with high sensitivity and resolution. To overcome the limitations intrinsic to single methods we have developed a bicistronic vector for the study of ER activity by both optical and nuclear-based imaging in order to perform multimodality imaging (Fig. 3A,B).

Images acquired with different techniques can be integrated into one single image by using image fusion procedures with specific algorithms to create new parametric images and increase the level of analysis and the amount of information available. Moreover, in the same scanner and in the same mold different small animal imaging instruments are being integrated to achieve...
direct image integration, so that it is now possible to integrate CT (Computed Tomography) and PET scanners to simultaneously obtain morphological and functional images; nevertheless, also optical and radionuclide-based protocols can be integrated in order to provide multimodal images.

6. Conclusions

The studies outlined in this review demonstrate that the ERE-luc mouse is a paradigm of animal model for the in vivo study of a molecular process (ER activity) by imaging techniques. The ability to visualize the state of ER activity in a living animal endows the model with novel, unique features because changes in ER activity can be now evaluated in the same animal at different times, thus allowing a detailed analysis of the dynamic action of physiological and exogenous compounds.

Compared with classical preclinical investigation models, this model has a much broader potential because it can: (a) reveal in which organs or tissues a given compound is active, independently of previously acquired knowledge of the target tissue; (b) assess the effects of and changes in response after repeated administration of a given compound; (c) determine the exact response of each tissue to the administration of a given compound independently of the route of administration; (d) evaluate the minimal drug concentration needed to stimulate ERs independently of the drug’s plasma levels; (e) discover the generation of active metabolites and their profile of action; (f) verify potential sites of drug accumulation during chronic administration; and (g) by combining pharmacological and toxicological studies, it can dramatically shorten the time needed to develop a given compound. With the use of transgenic mice, pharmacological and toxicological studies can be combined, thus decreasing the number of laboratory animals needed for drug development.

Furthermore, the reporter mouse strategy will generate a paradigm shift in future preclinical studies of compounds endowed with potential pharmacological activity.

As with the ERE-luc mice, other reporter mice could be developed for the study of different cell activities by simply substituting the responsive element. We are presently generating animals responsive to ligands specific for peroxisome proliferator activated receptors (PPARs). In addition, by substituting the entire promoter with regulatory regions of genes relevant for selected pathologies, the reporter mouse technology could be extended outside the intracellular receptor field, leading to innovative models for drug development, molecular assessment of physiological and pathological processes. The ability to examine drug action in reporter animals in detail will lead to more efficient and rational drug screening, reducing the time to the development of novel and possibly safer drugs.

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