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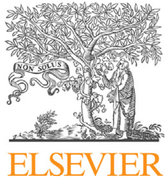

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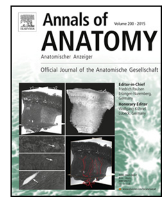

A bioluminescent mouse model of proliferation to highlight early stages of pancreatic cancer: A suitable tool for preclinical studies

Luisa de Latouliere[a], Isabella Manni[a,**], Carla Iacobini[b], Giuseppe Pugliese[b], Gian Luca Grazi[c], Pasquale Perri[c], Paola Cappello[d,e], Franco Novelli[d,e], Stefano Menini[b], Giulia Piaggio[a,*]

[a] Department of Research, Advanced Diagnostics and Technological Innovation, Regina Elena National Cancer Institute, Via Elio Chianesi 53, Rome 00144, Italy
[b] Department of Clinical and Molecular Medicine, Sapienza University of Rome, Via di Grottarossa 1035-1039, Rome 00189, Italy
[c] Department of Experimental Oncology, Regina Elena National Cancer Institute, Via Elio Chianesi 53, Rome 00144, Italy
[d] Department of Molecular Biotechnologies and Health Sciences, University of Turin, Via Nizza 52, Torino 10126, Italy
[e] Center for Experimental Research and Medical Studies, Città della Salute e della Scienza di Torino, Via Santena 5, Torino 10126, Italy

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SUMMARY

Transgenic mouse models designed to recapitulate genetic and pathologic aspects of cancer are useful to study early stages of disease as well as its progression. Among several, two of the most sophisticated models for pancreatic ductal adenocarcinoma (PDAC) are the *LSL-Kras*$^{G12D/+}$;*Pdx-1-Cre* (KC) and *LSL-Kras*$^{G12D/+}$;*LSL-Trp53*$^{R172H/+}$;*Pdx-1-Cre* (KPC) mice, in which the Cre-recombinase regulated by a pancreas-specific promoter activates the expression of oncogenic *Kras* alone or in combination with a mutant *p53*, respectively. Non-invasive *in vivo* imaging offers a novel approach to preclinical studies introducing the possibility to investigate biological events in the *spatio*/temporal dimension. We recently developed a mouse model, *MITO-Luc*, engineered to express the luciferase reporter gene in cells undergoing active proliferation. In this model, proliferation events can be visualized non-invasively by bioluminescence imaging (BLI) in every body district *in vivo*. Here, we describe the development and characterization of *MITO-Luc*-KC- and -KPC mice. In these mice we have now the opportunity to follow PDAC evolution in the living animal in a time frame process. Moreover, by relating *in vivo* and *ex vivo* BLI and histopathological data we provide evidence that these mice could represents a suitable tool for pancreatic cancer preclinical studies. Our data also suggest that aberrant proliferation events take place early in pancreatic carcinogenesis, before tumour appearance.

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is incurable with patients often presenting with metastatic disease resistant to therapy. The disease progresses through pancreatic intraepithelial neoplasia (PanIN) lesions to PDAC. Histologically, PanINs are divided into four stages, PanIN-1A, PanIN-1B, PanIN-2 and PanIN-3, defined by increasing degrees of cellular architectural atypia. PDAC is further characterized by a massive stromal reaction and inflammation. Genetically, activating mutations in *Kras* gene seem to be an ubiquitous event, although mutations in *p53* or other genes are also common (Hidalgo, 2012). Early detection is the key to improving survival in PDAC. However several factors contribute to make diagnosis of pancreatic cancer difficult at early stages: the anatomical location of the pancreas, the symptoms that are typically associated with advanced disease and the low resolution of conventional imaging modalities. Thus, early detection strategies of sporadic pancreatic cancer are urgently needed.

Genetically engineered mouse models (GEMMs) of cognate human diseases allow the identification of molecular mechanisms of disease pathogenesis (Tuveson and Hanahan, 2011). Several GEMMs that accurately mimic the pathophysiological features of human PDAC have been described (Tuveson and Hingorani, 2005; Cappello and Novelli, 2013). In particular, a mouse model (KC), in which a "*lox–stop–lox*" *Kras*^{G12D} allele is expressed in murine pancreatic progenitor cells using a pancreas-specific Cre recombinase

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; KC, *LSL-Kras*$^{G12D/+}$, *Pdx-1-Cre*; KPC, *LSL-Kras*$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, *Pdx-1-Cre*; PanIN, pancreatic intraepithelial neoplasia; BLI, bioluminescence imaging.

* Corresponding author. Tel.:+39 06 5266 2458; fax: +39 06 4180 526.
** Corresponding author. Tel.:+39 06 5266 2585; fax: +39 06 4180 526.
E-mail addresses: manni@ifo.it (I. Manni), piaggio@ifo.it (G. Piaggio).

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and that recapitulates all human features developing both preneoplastic and invasive PDAC, has been characterized (Hingorani et al., 2003). Moreover, in a mouse model that incorporates a *p53* mutation corresponding to the *Trp53*^{R172H} hotspot mutation in human cancers (KPC), it has been demonstrated that the expression of an endogenous *Kras*^{G12D} allele cooperates with that of a concomitant *p53* mutation to closely recapitulate the human disease at the pathophysiological and molecular level showing accelerated disease onset and metastasis (Hingorani et al., 2005). Although these models have been useful for therapeutic vaccination and pharmacological treatment in several preclinical studies (Cappello et al., 2013; Cappello and Novelli, 2013; Capello et al., 2013), the current methodological approaches are static and restrict the analysis to a specific phase of tumourigenesis or to a particular snapshot of time, therefore, they do not provide a dynamic view of carcinogenesis useful to identify early steps for which the environment is compulsory for tumour progression.

Non-invasive bioluminescence imaging (BLI) is a powerful tool for studying molecular events over time in a living organism (Signore et al., 2010). When combined with cancer models, this technology offers an unprecedented opportunity to investigate molecular events resulting in neoplastic development and progression in the entire organism. We have recently developed a reporter mouse model, the *rep*TOP^{TM} *mito*IRE (*MITO-Luc*), in which it is possible to measure physiological and/or aberrant proliferation in any body tissue by BLI (Goeman et al., 2012; Spallotta et al., 2013; Oliva et al., 2013; Rizzi et al., 2015). In these mice, the transcription of the firefly luciferase reporter gene is selectively induced during the cell cycle by the transcription factor NF-Y, a trimeric transcription activator composed of NF-YA, NF-YB, and NF-YC subunits, all required for DNA binding. The NF-Y complex exerts its activity only in proliferating cells regulating basal transcription of regulatory genes responsible for cell cycle progression (Farina et al., 1999; Manni et al., 2001; Sciortino et al., 2001; Gurtner et al., 2003, 2008, 2010; Di Agostino et al., 2006; Manni et al., 2008).

Deregulation of cell cycle and consequent aberrant proliferation have been implicated in the early steps of the carcinogenic process, including PDAC (Hezel et al., 2006). Thus, we hypothesized that the visualization of hyperproliferation occurring during early carcinogenesis could allow us to identify the first stages of tumour development. Based on this hypothesis we crossed KC and KPC mice, in which the somatic activation of *Kras*^{G12D} (KC) and mutant *Trp53*^{R172H} (KPC) is mediated by *Pdx-1-Cre* (Hingorani et al., 2003, 2005), with *MITO-Luc* mice (Goeman et al., 2012) generating MKC and MKPC mouse models. By correlating BLI and histopathological analysis of the pancreas from these mice we provide evidence that we are able to follow tumour evolution in terms of cell proliferation in a time frame manner. Of note, we have identified early steps of pancreatic carcinogenesis thus making these models useful for preclinical pharmacological studies.

2. Material and methods

2.1. Mouse strains

All animal studies were approved by the Institutional Animal Care of the Regina Elena National Cancer Institute and by the Government Committee of National Minister of Health and were conducted according with EU Directive 2010/63/EU for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm *LSL-Kras*$^{G12D/+}$;*LSL-Trp53*$^{R172H/+}$ mice and *Pdx-1-Cre* transgenic mice (Hingorani et al., 2003, 2005) were interbred with *MITO-Luc* reporter mice (Goeman et al., 2012) to obtain *MITO;LSL-Kras*$^{G12D/+}$;*Pdx-1-Cre* and *MITO;LSL-Kras*$^{G12D/+}$;*LSL-Trp53*$^{R172H/+}$;*Pdx-1-Cre*, respectively. The *LSL-Kras*$^{G12D/+}$ and *LSL-Trp53*$^{R172H/+}$ lineages were maintained in the heterozygous state.

2.2. Genotyping of transgenic mice

After genomic DNA extraction of tail biopsies, the positive founder animals were identified by PCR using the following primers specific for the transgenes:

MITO:	oligonucleotide up:	5′-TGTAGACAAGGAAACAACAAA-GCCTGGTGGCC;
	oligonucleotide down:	5′-GGCGTCTTCCATTTTACCAACAG-TACCGG.
Kras:	oligonucleotide 1:	5′-GTCTTTCCCCAGCACAGTGC;
	oligonucleotide 2:	5′-CTCTTGCCTACGCCACCAGCTC;
	oligonucleotide 3	5′-AGCTAGCCACCATGGCTTGAGT-AAGTCTGCA.
Trp53:	oligonucleotide 1:	5′-AGCTAGCCACCATGGCTTGAGT-AAGTCTGCA;
	oligonucleotide 2:	5′-TTACACATCCAGCCTCTGTGG;
	oligonucleotide 3:	5′-CTTGGAGACATAGCCACACTG.
Pdx-1-Cre:	oligonucleotide up:	5′-ATGCTTCTGTCCGTTTGCCG;
	oligonucleotide down:	5′-TGAGTGAACGAACCTGGTCG.

2.3. Histology

Tissues were fixed in 10% neutral buffered formalin (Sigma) for 24 h, dehydrated through a graded series of ethyl alcohol solutions and paraffin embedded. Pancreatic tissue was cut into 5-μm sections and stained with haematoxylin and eosin (H&E) for morphological assessment. Four non serial pancreatic sections were examined and images were acquired using a Nikon Eclipse E600 light microscope equipped with Olympus C-3030 digital camera.

2.4. In vivo *and* ex vivo *BLI*

For *in vivo* BLI, mice were anesthetized and 75 mg/kg of D-luciferin (Caliper, PerkinElmer company) was injected intraperitoneally. Ten minutes later, quantification of light emission was acquired for 5 min. Signal was detected using the IVIS Lumina II CCD camera system and analysed with the Living Image 2.20 software package (Caliper Life Sciences). Photon emission was measured in specific regions of interest (ROIs). Data were expressed as photon/second/cm^2/steradiant (p/s/cm^2/sr). The intensity of bioluminescence was colour-coded for imaging purposes; the scale used in each experiment is reported in each figure. For *ex vivo* BLI experiments, animals were sacrificed after *in vivo* BLI sessions and immediately subjected to a BLI session. Images of organs were detected as for the live animals.

3. Results and discussion

3.1. Generation of bioluminescent PDAC mouse models

A mouse model has been previously described in which the expression of *Trp53*^{R172H}, concomitantly with that of *Kras*^{G12D}, leads to the development of invasive and metastatic PDAC that recapitulates clinical, histopathological, and genomic features of the cognate human disease (Hingorani et al., 2005). In order to visualize proliferation events during pancreatic tumour evolution we crossed these mice with the *MITO-Luc* mice that we recently described as a useful tool for tracking proliferation events *in vivo* in longitudinal studies in any body region. To this end, we first

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