NANOMA

FP7-ICT-2007-2
224594

Nano-Actuators and Nano-Sensors for Medical Applications

D 7.3

In-vivo testing in mouse cancer models

Version 0.3

Strep Project : **NANOMA**

Duration : June 2008 to September 2011
Co-ordinator: Antoine Ferreira
Contract no.: 224594
Contacts : Université d' Orléans
UORL

Project Partners : UNOL, UORL, ETHZ, Pius Hospital, FemtoTools, UCY, BRF

IST Project Number	FP7-ICT-2007-2	**Acronym**	NANOMA
Full Tile	Nano-Actuators and Nano-sensOrs for Medical Applications		
Project URL	http://www.nanoma.eu		
Document URL			
EU Project Officer	Thomas Sommer		

Deliverable	**Nr°**	D 7.3	**Title**	In-vivo testing in mouse cancer models
Workpackage	**Nr°**	WP 7	**Title**	Integration

Date of delivery	**Contractual**		**Actual**	
Status	Version 0.4,		Final	
Nature	Report			
Dissemination Level	Consortium			

Authors (Partner)	BRFAA		
Responsible Author	Apostolos Klinakis	**Email**	aklinakis@bioacademy.gr

Abstract (for dissemination)	Genetically-modified mice are attractive systems for the in vivo preclinical testing of promising drugs and also for the evaluation of pioneering therapeutic approaches such as the one proposed in this application. .	
Keywords	MRI imaging, magnetic artifacts, tracking, propulsion, software, hardware, integration	
Version Log/Date	Final	**Authors**
Version 0.3		Apostolos Klinakis

Versions and Changes

Version	*When*	*What*	*Who*
0.1	01.10.2011	First version	BRFA
0.2	8.10.2011	Accepted	UORL

Table of Contents

Executive Summary

"Cancer" is a general term used to characterize many complex diseases sharing common features. A variety of factors are involved in cancer development, during which the controls that usually regulate growth and maintain homeostasis are disrupted. Cancer is often perceived as a disease of deregulated cellular signalling typically involving several somatic mutations that lead to abnormal proliferation and reduction of apoptosis. Engagement of deregulated signalling networks controlling these processes eventually culminates to full fledged malignancy. Today, in addition to cultured cells, mouse models of cancer play crucial roles as experimental systems used for gaining some understanding in causal mechanistic relationships between components of oncogenic signalling processes. Importantly, such mouse studies dissecting the combinatorial involvement of abnormal control mechanisms in tumorigenesis can lead to the identification of candidate drug-targets. In addition, genetically-modified mice are attractive systems for the *in vivo* preclinical testing of promising drugs and also for the evaluation of pioneering therapeutic approaches.

1. Introduction

"Cancer" is a general term used to characterize many complex diseases sharing common features. A variety of factors are involved in cancer development, during which the controls that usually regulate growth and maintain homeostasis are disrupted. Cancer is often perceived as a disease of deregulated cellular signalling typically involving several somatic mutations that lead to abnormal proliferation and reduction of apoptosis. Engagement of deregulated signalling networks controlling these processes eventually culminates to full fledged malignancy. Today, in addition to cultured cells, mouse models of cancer play crucial roles as experimental systems used for gaining some understanding in causal mechanistic relationships between components of oncogenic signalling processes. Importantly, such mouse studies dissecting the combinatorial involvement of abnormal control mechanisms in tumorigenesis can lead to the identification of candidate drug-targets. In addition, genetically-modified mice are attractive systems for the in vivo preclinical testing of promising drugs and also for the evaluation of pioneering therapeutic approaches such as the one proposed in this application.

Description

The BRF beneficiary has developed a mouse model of carcinogenesis that entails the conditional overexpression of activated Kras (Kras*), which can be used advantageously in the context of this proposal (Klinakis A *et al*., 2007). In this model, a mouse Kras cDNA encoding an oncogenic protein with a glycine to aspartate acid amino replacement in codon 12 (G12D) has been placed into the first intron of the highly expressed Ef1alpha1 locus (Elongation factor 1alpha1; Ef1/Kras). The mutant form of Kras, which is a key cell signaling molecule, is constitutively active and promotes uncontrolled cell growth leading to tumorigenesis. The expression of Kras* in our mouse model is prevented by a floxed neo-triple polyA cassette acting as a transcriptional block. When this mouse is mated with mouse lines designed to express with tissue specificity the Cre recombinase, the block is removed and a fusion transcript composed of the first, non-coding, intron of the Ef1alpha gene and the Kras* cDNA is produced leading to the formation of a tumor in only the tissue expressing Cre.(Figure 1).

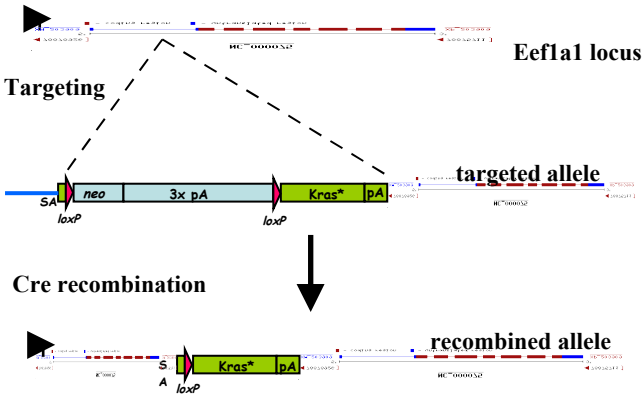

Figure 1. The Ef1/Kras "knock-in" strategy
The Kras* cDNA is targeted into the highly expressed Eef1a1 locus (eukaryotic translation elongation factor 1 alpha 1). A promoterless neo/triple poly(A) cassette placed downstream of a splice acceptor site (SA) blocks the transcription of the cDNA, but allows selection of targeted ES clones. Upon Cre-mediated recombination, the blocking sequence is excised and the cDNA is juxtaposed to the (SA), and its expression is driven by the powerful promoter of the locus.

These results confirmed that the Ef1/Kras* is a versatile tool, which can be used to study tumorigenesis in any tissue of choice. For the purpose of this proposal we will focus on the mammary gland. To activate Kras* in the mammary epithelial cells we crossed the Ef1/Kras* mice with WAPcre mice (Ludwig *et al*., 2001). These mice carry a knock-in Cre transgene into the WAP (Whey Acidic Protein) locus which is expressed in luminal secretory cells during late pregnancy and lactation. The tumors developing in female animals (see below) turned out to correspond to human basal-like breast cancers. Molecular classification of human breast cancers by gene

expression profiling showed that there are three major subtypes: luminal, an estrogen receptor (ER) positive category, and two ER-negative classes, one of them overexpressing ERBB2 (HER2/NEU) and the other ("basal-like") exhibiting phenotypic features of basal/myoepithelial cells (Perou et al., 2000; Sorlie et al., 2001, 2003). In general, basal-like cancers not only lack ER, but also progesterone receptor (PR) and ERBB2 (ER-/PR-/ERBB2- "triple negative breast cancers"), while they frequently express EGFR and basal markers, such as cytokeratins 5/6 and/or 14 and p63 (Nielsen et al., 2004; Reis-Filho et al., 2006). The category of basal-like breast cancers (15-20% of the total; recently reviewed by Yehiely et al., 2006; Finnegan and Carey, 2007) includes high proportions of BRCA1-associated, medullary and metaplastic subtypes, the latter group being heterogeneous and consisting of squamous and spindle cell carcinomas and other forms. Basal cancers appear to have extremely bad prognosis (Sorlie et al., 2001), especially in the early years of follow-up after diagnosis and treatment. It is the histopathological heterogeneity and the aggressiveness of the basal-like cancers that led investigators to postulate that they come from stem/progenitors cells. Consistent with this hypothesis is the observation that mouse mammary stem cells, like basal-like tumor cells, do not express the luminal markers ERa, PR and Erbb2, while they are positive for a number of basal markers such as Egfr, Cytokeratin 14 and p63. Because they do not express ER or ERBB2, basal-like cancers cannot be treated with targeted therapies with antiestrogens (Tamoxifen) or an anti-ERBB2 antibody (trastuzumab), while there is no clear choice for chemotherapeutic intervention.

Table1. Kras*-induced neoplasms in bitransgenic mice expressing the Ef1/Kras* and a Cre transgene

Cre line	**Type of neoplasm**	**Latency**
WAP/cre	Mammary carcinomas, (adenocarcinoma, squamous Cell carcinoma, sarcomatous carcinoma)	2 days-2 months (T_{50}=9 days)
MMTV/cre	Mammary carcinomas, Salivary adenocarcinoma, T-cell lymphoma, splenic sarcoma	~2-3 months
Villin/cre	Adenomatous polyposis Colon adenocarcinoma	~2 months ~8 months
Pdx/cre	Pancreatic adenocarcinoma, pancreatoblastoma	At birth
Ngn/cre	Pancreatic cyst adenoma	A few days
Probasin/cre	Penile squamous cell carcinoma Prostate adenocarcinoma Endometrial carcinoma	~2 months ~6 months ~8 months

In-Vivo Testing

For in vivo testing, we generated Ef1/Kras*; WAPcre mice. Upon pregnancy/lactation (essential for Cre recombinase expression), the mice will be monitored for the development of mammary tumors. The advantage of breast as a target tissue is that the tumor size can be assessed early by palpation and can be followed and measured with the use of a caliper throughout this process. Considering that mice have five pairs of mammary glands, while Kras*-induced tumors are multifocal and affect all of them, multiple tumors can be studied at the same time in a single experimental animal. This allow us to introduce additional parameters, such as initial tumor size, in our study evaluating treatment efficacy. Mammary tumors can be palpated at early stages and when fully developed usually reach a weight of at least 400 mg. This corresponds to 1/50 of the body weight for the average mouse (~20 grams).

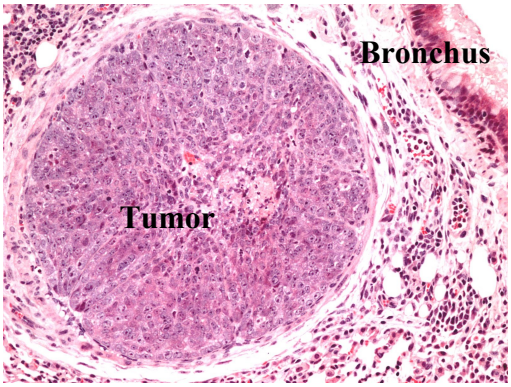

Figure 2. Lung invasion of Kras* cells injected in the tail vein of immuno-compromised mice.

The equivalent of that in humans would be a tumor weighing over 1 kg. Considering the fact that our mice will be carrying multiple such tumors, the tumor burden in mice would correspond to a very advanced stage of the disease in humans. It is notable in this regard, that with the exception of surface or easily accessible from the surface organs such as the skin and the mammary gland, tumors of that size would be almost impossible to visualize in internal organs of the abdominal or the chest cavity. This could be challenging in the study of lung metastases, whether they are derived from the primary tumor in our mouse model or from tail vein injections using our cell line (Figure 2). This will allow us to assess the response of the metastases to the therapy by measuring their size during the course of the experiment.

Because the Ef1/Kras* transgene can be activated by Cre recombination in any anatomical site, studies can be extended in other organs, if need arises. As shown in Table 2, by simple crossing of the Ef1/Kras* mice with different Cre transgenic lines, we can easily generate mouse cancer models at practically anatomical site. Our preliminary expression profiling analysis has shown that there is a Kras "signature" referring to a group of genes that are overexpressed in Kras*-induced tumors in comparison to normal tissues independently of the tissue in which the tumor appears. Genes which are part of the "signature" can be used as cell surface markers for the delivery of the nanoparticles.

We were prepared to deal with this potential problem with the use of the animal MRI equipment available at the Foundation of Biomedical Research in Athens. However, as decided during the 2nd Review meeting, the integration hardware and software MRI platform has been performed on the 3T Siemens MRI setup at Pius-Hospital in Oldenburg (Germany).

As stated during the 2nd review meeting, the software and hardware integration were not compatible with the BRFAA equipment dedicated to small animals (7T micro-MRI system). That is why, deliverable 7.3 was impossible to provide at this stage for the following reasons:

1. The absence of MRI guidance, the nanocapsules will be "lost" everywhere in the mouse body and no clear statement could be made on the efficacy of drug targeting.

2. The amounts required for in vivo testing greatly exceed the amounts obtained from the NANOMA consortium.

3. In the absence of nanoparticles functionalized with the antibody for tumor cell recognition it is impossible to target tumor cells which was the essence and novelty of this proposal.

For all these reasons "*in vivo*" experiments are impossible to perform. This is exactly the reason why during the 2nd year review it was agreed upon that "*in vivo*" testing was not feasible and necessary and therefore, deliverable 7.3 should be ommited and replaced by Deliverable: 7.4 "Evaluation of toxicity of the nanocapsules".

Table 2. Expression profile of normal mammary gland and Kras*-induced mammary tumors

	Normal mammary gland				*Carcinoma*			
	V	P	L	I	Dunn A/B	PCC	SCC	SRC
1. Luminal markers								
Ck18 (Krt18)	+++	+++	+++	+++	+++	++	++	++
ER	ND	ND	ND	ND	++	-	-	-
PR	ND	ND	ND	ND	++	-	-	-
GATA3	+++	+++	+++	+++	+++	++	-	-
2. Basal markers								
Ck14 (Krt14)	+++	+++	+++	+++	-	+/-	+++	+++
SMA (Actg2)	+++	+++	+++	+++	-	-	-	+/-
p63 (Trp63)	+++	+++	+++	+++	-	+	+++	-
3. "Stem cell" markers								
Itga6	++	+/-	+/-	+/-	-	+/-	++	-
Ck6 (Krt6)	++	+/-	+/-	+/-	-	++	+++	+/-
Nestin	+/-	+/-	-	+/-	-	+++	+	++
Annexin VIII	-	+/-	-	+/-	-	+++	+++	+
Sca1	+/-	+/-	-	+/-	-	++	+++	-
4. Signaling markers								
pErk	+/-	++	-	-	++	+++	+++	+++
pAkt1	+	++	-	+	-	+++	++	++
pS6	+	+++	-	++	-	+++	+++	+
Igf1r	+/-	+	+	+/-	++	+++	++	+/-
Igf1	+/-	-	+	-	-	-	-	-
Igf2	-	-	-	-	-	-	-	-
Egfr	++	+	+	+	+	++	+++	-
Erbb3	-	-	-	-	+	++	+++	+++
Notch1	++	++	+++	+/++	+	+++	++	+
Jagged	-	-	-	-	-	+++	+++	+++
Myc	++	+++	-	++	-	+++	++	+++
Fosl1	+	+	+	+	-	+/-	+++	+++
5. Other markers								
E-cadherin	+++	+++	+++	+++	+++	+++	+++	-
Vimentin	-	-	-	-	-	+/-	+	+++
β-catenin (nuclear)	-	-	-	-	-	-/++	-	-
β-catenin (cell surface)	+++	+++	+++	+++	+++	++	+++	-

References

[1A] Klinakis A, Szabolcs M, Chen G, Efstratiadis A. Modeling and treatment of basal-like breast cancer in mice (to be submitted)

[1] Engelman JA, Zejnullahu K, Mitsudomi T et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316:1039-43.

[2] ***Artavanis-Tsakonas S, Rand MD, Lake RJ.*** Notch signaling: cell fate control and signal integration in development. Science. 1999;284:770-6.

[3] Lai EC. Notch signaling: control of cell communication and cell fate. Development. 2004;131:965-73.

[4] Allenspach EJ, Maillard I, Aster JC, Pear WS. Notch signaling in cancer. Cancer Biol Ther. 2002;1:466-76.
[5] Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer. 2003;3:756-67.
[6] Brennan K, Brown AM. Is there a role for Notch signalling in human breast cancer? Breast Cancer Res. 2003;5:69-75.
[7] Politi K, Feirt N, Kitajewski J. Notch in mammary gland development and breast cancer. Semin Cancer Biol. 2004;14:341-7.
[8] Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.
[9] Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98:10869–74.
[10] Allenspach EJ, Maillard I, Aster JC, Pear WS. Notch signaling in cancer. Cancer Biol Ther. 2002;1:466-76.
[11] Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer. 2003;3: 756-67.
[12] Brennan K, Brown AM. Is there a role for Notch signalling in human breast cancer? Breast Cancer Res. 2003;5:69-75.
[13] Politi K, Feirt N, Kitajewski J. Notch in mammary gland development and breast cancer. Semin Cancer Biol. 2004;14:341-7.
[14] Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.
[15] Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98:10869–74.