PUBLISHABLE SUMMARY

Grant Agreement number : **HEALTH-F2-2008-201439**

Project acronym : **ZF-CANCER**

Project title : **DEVELOPING HIGH-THROUGHPUT BIOASSAYS FOR HUMAN CANCERS IN ZEBRAFISH**

Funding Scheme

Collaborative, Small or medium-scale focused research project

Period covered:

From 1 April 2008 to 31 March 2011

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Final publishable summary report

1.1 An executive summary

ZF-CANCER project (201439) **has established zebrafish as an alternative animal model for cancer drug target discovery and anticancer lead compound selection. The developed platforms enables further integration of these technologies in a robotic setup, thus allowing the full automation of this technology, all the way from tumor cell implantation, drug treatment to bio-imaging and data analysis.** Zebrafish model has the potential for high-throughput application due to the small size of zebrafish embryos, the high numbers with which embryos can be obtained and the choice of high-throughput molecular screening tools established within the project.

ZF-CANCER developed an innovative screening system based on the **implantation of fluorescent human cancer cells into zebrafish embryos**. We have tested representative cell lines of various important human cancer origins e.g. breast, melanoma, intestinal, sarcoma, prostate and lung. An integrated multi-parameter fluorescence-based **bio-imaging platform** for the qualitative and quantitative evaluation of human cancer progression as well as an image data base for automated data acquisition, analysis and excess have been established and will be beneficial for medical imaging. Using 3 sets of non-aggressive versus aggressive cell lines for breast, colorectal, and prostate cancer, we show that correlation with behaviour in long-term mouse models is excellent. For discovery of novel gene targets and compounds the ZF-CANCER combined the power of **genomic tools (RNA interference)** and **chemical libraries** analysis with the advantage of *in vivo* monitoring of tumor progression in a transparent vertebrate model organism.
Various knockdown constructs were generated and transduced into human prostate cancer cells that were imaged in zebrafish. From the screen two novel candidate genes, for which no data on their role in prostate cancer progression/metastasis is available, have been identified. Taken together, this first automated ZF xenograft screen indicates that the automated analysis procedure is compatible with rapid screening for human cancer cell spreading. ZF-CANCER generated a l**iving library of zebrafish with cancers (12 tumor models of melanoma, rhabdomyosarcoma and hemangioma)** that will allow better understanding of cancer progression and design of new intervention strategies. These tumors are different pathologically; reflect the varied stages of tumorigenesis and some represent new genetic combinations that have not been modelled in the mouse. A key finding of this work is that co-operating mutations alter tumor pathology and spectrum. **A new genetic mutation has been identified** to co-operate with BRAFV600E to promote melanoma. The microarray analysis of cancer prone zebrafish to identify pathways important in cancer progression was performed. **Zebrafish cell lines have been established from embryonic mutant lines and from cancer prone** tissues. These cell lines have been allograft in zebrafish embryos and the migratory phenotype has been observed proving the syngenic model. **Screens with the chemical libraries** identified compounds involved in zebrafish and HUVEC angiogenesis, cell migration and tissue morphogenesis defects during gastrulation, melanocyte biology relevant for melanoma progression. Some targets genes were identified.
Finally, to develop a case study on high-throughput platform the **SMEs have set up a high-throughput screening platform** for an automatic anti-angiogenesis screening and image analysis as well an automated high throughput injection of zebrafish larvae with tumor cells. The protocols were successfully validated using set of reference compounds. In addition, the market study and a pilot screening with compounds coming from the companies have been completed. New high-throughput methodologies developed in **ZF-CANCER project enables application of zebrafish model into biomedical screenings in preclinical anticancer drug screening pipelines** *and* thereby ensures European competitiveness in the area of drug discovery. The project's outcome will potentially contributes to cost-effective and more efficient methods in the anti-tumor drug discovery process. Acceleration of drug lead time will benefit economy as well as quality of life of cancer patients.

1.2 A summary description of project context and objectives:

The **ZF-CANCER** project aims to develop clinically relevant **high-throughput bioassays for cancer progression** that will be applicable in preclinical validation pipelines, and to use these bioassays to screen for novel chemical and genetic cancer **targets.** Fluorescently labelled human and zebrafish cancer cells will be implanted (xenogenic and allogenic transplantation) into zebrafish embryos to generate quantitative, **multi-colour fluorescent intravital bio-imaging** of **tumor progression**. Because of the availability of many transgenics and optical transparency zebrafish are a powerful - and the only extant - vertebrate model that allows the simultaneous, *in vivo* imaging of cancer progression hallmarks including cell survival, proliferation, migration and angiogenesis. The visual, non-invasive monitoring of cancer cells in transparent host embryos coupled with **RNA interference technology** will enable the identification of novel gene targets that drive tumor progression in a range of cancers. **Automation** of these fluorescent readouts, and other cancer gene specific readouts, will accelerate the screening process of **chemical libraries** for the discovery of **new compounds** involved in different aspects of cancer progression and inhibition. In our proposed case study, we begin with a select panel of genes and class of compounds and using our established **high-throughput platform aim to identify novel anti-cancer drug leads and gene targets**, relevant for human cancer therapy, with the potential for commercial development.

The **ZF-CANCER** project is a coordinated effort of five research laboratories, one large company and two SMEs, together aiming for two highly interrelated objectives:

Objective 1: Development of high-throughput bioassays for the simultaneous, *in vivo* study of tumor progression hallmarks: survival, growth, migration and angiogenesis.

Objective 2: Discovery of genetic targets and novel lead compounds that control specific aspects of cancer progression using shRNA and chemical libraries.

These objectives will be targeted in different work packages (WPs). The interrelationship between objectives of these WPs is described below and presented as an overview in **Fig.1.**

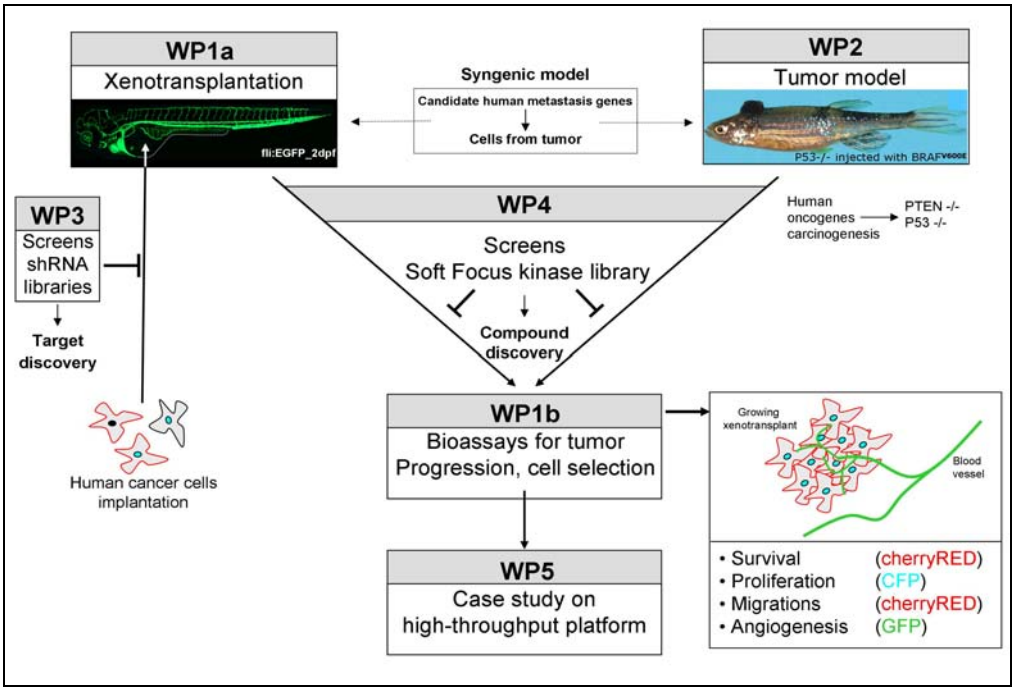


Fig. 1 Overview of ZF-CANCER project.

Remarkably, fluorescently labelled human cancer cells implanted into zebrafish embryos continue their cancerous programme by proliferating, forming tumor masses, migrating and initiating angiogenesis (**Fig. 2**).

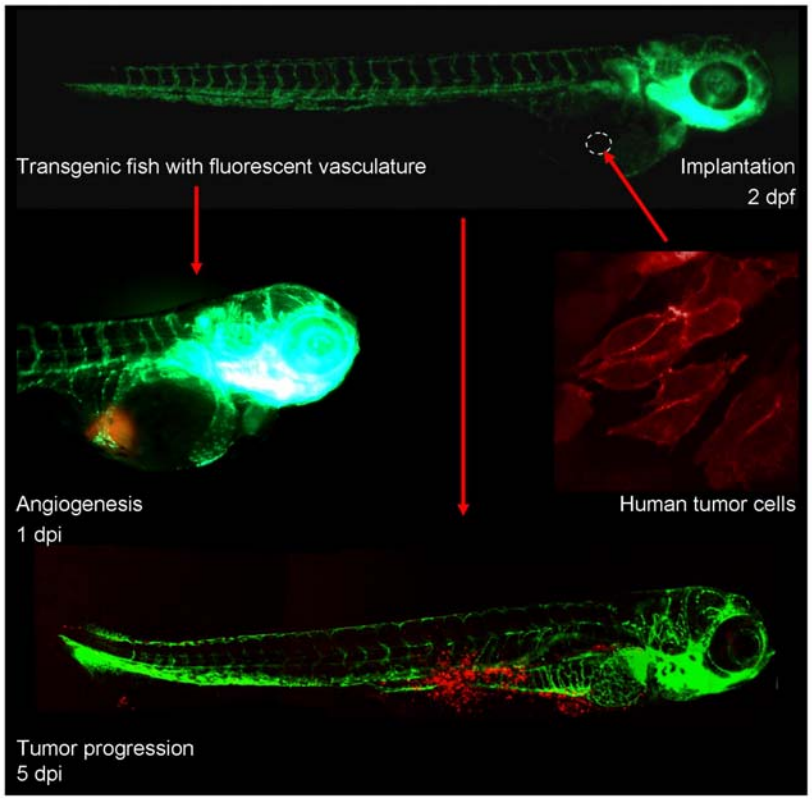


Fig. 2 Work flow of engraftment of human tumor cells into two days old zebrafish embryos.

In **WP1** we will identify a panel of human cell lines that retain their cancer-like phenotypes when implanted into zebrafish embryos. Labelled with fluorescent survival and proliferation markers, we will visualize, in unique cellular detail, human cancer cells proliferating, migrating, and initiating angiogenesis (from the host transgenic embryo expressing a fluorescent vasculature). To support these efforts, **WP1** will establish an automated image acquisition, analysis, and storage programme and database. In addition to human cancer cells, **WP2** will use the zebrafish system to identify new cancer genes *in vivo*, and generate a diverse spectrum of tumors in specific genetic backgrounds. Zebrafish cancer cell lines will be generated, and/or stably transfected with human oncogenes, to provide a wealth of new material for the identification of optimal cancer cell lines for implantation (currently being established by ZF-screens partner 3; patent application WO2006091090). Together, WP1 and WP2 aim to provide detailed, quantitative, automated, multi-colour imaging of the fundamental hallmarks of cancer cells from a diverse tumor spectrum that can be used as a platform for innovative screens for new cancer genes and drug-like leads (**WP5**).
We envisage that a powerful drug discovery programme will be established by combining bio-imaging of cancer progression (WP1, 2) with high-throughput screens for novel cancer genes and anti-cancer drug-like leads using shRNA (**WP3**) and chemical libraries (**WP4**) in zebrafish embryos. Galapagos NV (partner 2) is a leader of applying shRNA technology in target discovery and validation, and will provide expertise and target related chemical compound libraries to the partners of ZF-CANCER consortium to conduct specific phenotypic screens based on tumor progression, as well as the developmental phenotypes reflecting fundamental processes of tumorigenesis. Genomics based target discovery will be implemented in a robotic platform, and using our expertise in software design and image analysis, we will transform our automated screens into efficient, high-throughput assays that are economically competitive tools for pharmaceutical drug

development. Fundamental knowledge, tools and technical expertise gained from ZF-CANCER will be further pursued commercially by our Company partners.

In general, we aim to develop clinically relevant, high-throughput bioassays for cancer progression in zebrafish that will be applicable in preclinical validation pipelines and to use these bioassays to screen for novel chemical and genetic cancer targets.
The ZF-CANCER consortium combines multidisciplinary European experts in this field, and by achieving these objectives, will strengthen the innovative potential of European biotechnology and pharmaceutical industries.

Objectives for the ZF-CANCER:

Objectives		
Work package	**Objectives for the reporting period**	**Beneficiary**
WP1	- Selection of human and zebrafish cell lines for xeno- and all-transplantation in ZF embryos	**UL**/ Galapagos NV/ UEDIN / CMRB/ KNAW
	- Setup of multi-colour microscopy-based screening assays	**UL**/ Galapagos NV/ ZF-Screens/ CMRB/ KNAW/ MPG/ Biobide
	- Development of image data base and automated image Analysis	**UL**/ Galapagos NV/ ZF-creens/ MPG/ Biobide
WP2	- Identification of novel and conserved cancer pathways in ZF	ZF-Screens/ **UEDIN/ KNAW**
	- Generation of transgenic ZF lines to test role of new cancer genes	**UEDIN/** CMRB/ **KNAW/** MPG/ Biobide
	- Generation of tumors in ZF for engraftment into ZF embryos	**UEDIN/ CMRB/ KNAW**
	- Generation of cancer cell-lines for transplantation into embryos	UEDIN/ **CMRB**/ **KNAW**/ UL/ Galapagos
WP3	- Production of positive and negative control viruses	**Galapagos NV**
	- Extension, amplification and quality control of SilenceSelect kinase shRNA collection	**Galapagos NV**
	- Development of Adenoviral transduction protocols	**Galapagos NV/** UL/ ZF-Screens
	- Assay development for RNA interference drug target screening	**Galapagos NV/ UL**
	- A pilot shRNA screen in reference human cell lines to demonstrate that positive control viruses inhibit proliferation, cell survival and migration in *in vitro* and *in vivo* assays	**Galapagos NV/ UL**
	- shRNA-based discovery of novel anticancer kinase drug targets	

	- prioritization of novel anticancer kinase drug targets and inhibitors of kinases	
WP4	- Identification of a set of kinase inhibitors that: - sensitizes *p53* mutant embryos against irradiation induced apoptosis - attenuates pten mutant phenotype in zebrafish embryos - attenuates angiogenesis development in zebrafish embryos - affect embryo morphogenesis during gastrulation - affect melanocytes development and migration - Target validation and mutant analysis	 UEDIN KNAW CMRB/Biobide MPG UEDIN UEDIN/Biobide/CMRB/MPG/ IST
WP5	- Market study, identification and selection of interested Pharmaceuticals - Case study with the panel of genes and pharmaceuticals - Screening of pilot compounds for customer validation - Bioinformatics analysis: study of automatic classification system	ZF-Screens/Biobide

1.3 A description of the main S&T results/foregrounds

A general description of the main S&T results/foregrounds

ZF-CANCER has established zebrafish as an alternative animal model for cancer drug target discovery and anticancer lead compound selection. The developed platforms enables further integration of these technologies in a robotic setup, thus allowing the full automation of this technology, all the way from tumor cell implantation, drug treatment to bio-imaging and data analysis.

Spreading of ~50 cancer lines was analysed following xenotransplantation in the yolk of 2 day old zebrafish embryos. Imaging at higher throughput was successful in 96 well plates using automated microscopy (UL, partner 1). Modification of existing software for automated image analysis has been further developed in a multidisciplinary setting with the operational database (partner 1 (UL), partner 3 (ZF-Screens). Partner 1 (UL) has further **optimized the macro for automated image analyses for quantification of spreading of engraft human tumor cells in ZF**. We have entered representative cell lines of various important human cancer origins into the quantitative imaging pipeline. **Using 3 sets of non-aggressive versus aggressive cell lines for breast, colorectal, and prostate cancer, we show that correlation with behaviour in long-term mouse models is excellent.** We also demonstrate that intra- and inter-experiment variation is low, leading to very good reproducibility of findings. This validation is part of the manuscript submitted in which the fully automated image acquisition and analysis pipeline is described and validated using various human cell lines with known metastatic potential in mouse models. **Adenoviral transduction protocols, of 31 human tumor cell lines, have been established with transduction efficiency of more than 70%** (Galapagos, partner 2). Proliferation and cell survival assays have been generated. In addition, motility and epithelial/mesenchymal transition assays have been achieved. Negative controls have no effect on *in vitro* tumor cell proliferation, cell survival, motility or epithelial/mesenchymal transition. Galapagos has established high content imaging protocols for imaging apoptosis (annexin V staining), cell death (propidium iodide exclusion), migration (scratch wound healing), proliferation (cell titter blue staining of viable cells), and metastatic phenotype (expression of E-cadherin and vimentin). For motility and /mesenchymal transition assays 5 positive controls have been identified that inhibit the phenotype for more than 40%. Thus cells and virus controls are in place to bench mark the screening of the kinase knockdown library in zebrafish models.

A pilot shRNA screen has been performed using human prostate PC3-dsRed cells. It was shown that adenoviral transduction did not affect tumorigenic properties of the cells. Two positive shRNA controls were identified. Knockdown of PI3K, CXCR4, and SYK significantly (40%) reduced metastatic and migratory capacity of PC3 cells in *in vitro* assays. In addition, knockdown of PI3K and CXCR4 resulted in reduced migration of PC3 cells in the zebrafish.
The stable DsRed-PC3 cell line did not remain stable therefore it was decided to revert to transient transduction of the PC3 with an adenoviral DsRed knock-in. Double transduction of AsRed knock-in and postivive control knock-downs were established. However, imaging of AsRed was not sensitive enough. Therefore, it was decided to proceed with the knockdown screen using CM-Dil labelled PC3 which already had been shown to generate high quality images. Various knockdown constructs were generated and transduced into PC3 cells that were imaged in zebrafish. From the screen, which includes **genes highly relevant to prostate cancer based on database searches, 2 novel candidate genes MST1R (Ron) and SYKI**, for which no data on their role in prostate cancer progression/metastasis is available, have been selected for further validation. Taken together, **this first automated ZF xenograft screen indicates that the automated analysis procedure is compatible with rapid screening for human cancer cell spreading.**
Partner 4, 5 and 6 generated a **living library of zebrafish with cancers (12 tumor models)** with 12 genetic combinations that give rise to benign and malignant tumors. The following ZF tumor models were made: BRAFnevi, BRAFp53 melanoma, BRAFmitf melanoma, BRAFmitp53 melanoma, PTEN eye, PTEN intestinal, BRAFpten melanoma, p53 MPNST, p53pten nevi, RAS melanoma, RAS rhabdomyosarcoma and PTEN hemangioma. These tumors are different pathologically and some are new genetic combinations that have not been modelled in the mouse. A key finding in our work is that **co-operating mutations alter tumor pathology** and spectrum. **A new genetic mutation in the MC1R gene has been identified** and in collaboration with Jim Lister (USA) partner 4 (UEDIN) found that mitf mutations co-operate with BRAFV600E to promote melanoma. Partner 4 also generated new mitf transgenic lines using human mitf cancer alleles. **The microarray analysis of cancer prone** zebrafish to identify pathways important in cancer progression (pten, BRAF/MEK, BRAF nevi) were performed. It was found that phosphatases are upregulated in BRAF and MEK mutant embryos and confirmed that the DUSP6 phosphatase can accurately reflect MAPK signalling in zebrafish.
Zebrafish cell lines have been established from embryonic mutant lines and from cancer prone tissues by partner 6 (KNAW). These cell lines have been transferred to WP1, but are also interesting for studying how signalling pathways are altered in pten deficient cells. Zebrafish tumor cells lines have been allograft in resulting in spreading of ZF pten b-/- cells in zebrafish embryos and proving the functionality of the syngenic model.
We **screened the SoftFocus kinase library** for following phenotypes: inhibition of angiogenesis in wild type embryos, cell migration and tissue morphogenesis defects during the first 10 hours of development, melanocyte biology and rescue of irradiation-induced apoptosis in p53 mutant embryos.
Partner 5 (CMRB) and 8 (Biobide) have **identified 6 compounds** from the SoftFocus library using the automated screening platform and **determined the targeted kinases**. Two compounds were shown to inhibit Phosphorylase kinase subunit G1 (PhKG1), Proto-oncogene serine/threonine kinase-1 (PIM1) and Transforming tyrosine kinase A (TrKA). These targets were verified by morpholino knock-down in embryos, confirming that all of **these kinases are important for angiogenesis in zebrafish** during development (partner 5). The kinase targets were further verified by commercial inhibitors (partner 8). Inhibition of PIM1 affects primarily the diameter of vessels whereas inhibition of either TrKA or PhKG1 leads to an inhibition of vessel growth.
Partner 7 and 9 (MPG/new IST) screened the whole library and identified 884 compounds that elicit specific morphogenetic defects during gastrulation. The most interesting phenotypes are **defective convergence and extension movements and arrested development**. The identification of targets and their function in zebrafish gastrulation is ongoing.
Partner 4 (UEDIN) has completed a series of small molecule screens for modulators of melanocyte biology. Two specific compounds **AG1276 and roscovitine reduced melanocytes cell movement and development**, respectively. The kinase profiling study proved that they selectively inhibit KIT and CDK2. Roscovitine causes a reduced number of melanocytes that seems to be rescued by loss of p53. This is an exciting new pathway, as p53 plays an obscure role in melanocyte biology and melanoma. UEDIN has identified **the biochemical target of the nitrofurans that cause melanocyte specific cell death** and found that these compounds directly interact with ALDH2, and that inhibitors of this target completely rescue the

melanocyte phenotype. Partner 4 (UEDIN) has found a panel of compounds that alter melanocyte differentiation by interfering with copper metabolism. The target pathways were identified using yeast genetics screens.
Finally, to develop a case study on high-throughput platform the **SMEs have set up a high-throughput screening platform for an automatic anti-angiogenesis screening and image analysis as well an automated high throughput injection of zebrafish larvae with tumor cells**. HTS protocols were successfully validated using set of reference compounds. The GLP protocols were generated. Biobide developed automated angiogenesis assay. In addition, **the market study and screening of pilot compounds for customer validation have been performed**. A pilot screening with compounds coming from the Spanish Pharmaceuticals company FAES FARMA has been completed in Biobide platform.
Partner 3 (ZF-Screens) **performed proof-of-principle** in which early zebrafish embryos were robotically injected with fluorescently-labelled Mycobacterium marinum and tumor osteosarcoma or melanoma cells, followed by drug treatment and COPAS XL or CLSM endpoint measurement.

A custom combination of the robotic zebrafish embryo injection platform and the COPAS XL Biosorter was presented to GlaxoSmithkline (GSK) as a high-throughput *in vivo* screening system for anti-microbial and anti-tumour compounds. This has resulted in a **contract research agreement** with GSK toward validation of a set of anti-Mycobacterial compounds in the zebrafish larva platform. Further contracts toward anti-tumor screens are expected (partner 3, ZF-Screens).
The robotic setups generated in ZF-CANCER project allow the incorporation of the zebrafish embryo model into the preclinical drug screening pipelines. In conclusion, the ZF-CANCER project made a contribution on reinforcing European competitiveness by generating strategic knowledge in a multidisciplinary research approach. In general this investment in the knowledge-based economy and health care will have a positive impact on social issues. The project's outcome will potentially contributes to cost-effective and more efficient methods in the anti-tumor drug discovery process. Acceleration of drug lead time benefits economy as well as quality of life of cancer patients.

Detail description of the S&T results achieved in the work packages

Work package 1 : High-content multi-color fluorescent bio-imaging of tumor progression/metastasis in human cancer cell to zebrafish xenotransplant models

Lead participant : UL

Task 1.1: Select and validate human cell lines for tumor growth and metastasis in zebrafish embryos.
Spreading of ~50 cancer lines was analysed following xenotransplantation in the yolk of 2 day old zebrafish embryos (**Table 1**) (partner 1, UL) and compatibility of several cell lines with adenoviral infection has been determined (Galapagos). Partner 5 (CMRB) has identified 2 human cell lines that transplant and multiply in zebrafish, SW620 and WM-266-4, and have supplied the SW620 line to partner 1 (UL). Partner 2 (Galapagos) has demonstrated that the human cell lines can be efficiently transduced with a modified adenovirus C20. In addition, partner 2 (Galapagos) has demonstrated that the viral transduction does not interfere with the *in vitro* metastatic capabilities of the tumor cells. Instead of entering all cell lines previously analysed qualitatively (**Table 1**) into the automated analysis pipeline for quantitative analysis, we have entered representative cell lines of various human cancer origins, including melanoma (MV3), prostate cancer (LnCAP, DU145, PC3), breast cancer (MCF7, BT474), colorectal cancer (HT29, SW620), and sarcoma (HT1080) (**Fig. 3**). Fluorescent tagging with dsRed and mCherry followed by FACsorting has been done. Fluorescence was not stable in some lines but we now have stable fluorescent variants of PC3 and MV3. The human melanonoma line MV3 cannot be used for the screen because partner 2 (Galapagos) has found that these cells cannot be efficiently infected with adenoviral constructs. Labelled MV3 has been distributed to partner 8 (Biobide). In the meantime, for the shRNA screen (See task 1.2) we have made use of CM-DiI-labelled PC3 cells. Stable fluorescent variants of this line and MV3 will be used in validation steps. Partner 6 established a stable *ptenb*-

/- and *ptena+/- ptenb-/-* fibroblast cell lines. These cell lines have been shipped to Partner 1 and allograft. The migratory phenotype has been observed and proving the syngenic model.

Table 1. Embryo survival and percentage embryos showing spread cells for all injected cancer cell lines

	Cell line	Cancer type	% surviving embryos 6dpi	% embryos with tumor cells spread to tail region
1	**H460**	**Non small cell lung cancer**	92%	21%
2	**HT29**	**Colorectal carcinima**	100%	21%
3	**BT474**	**Breast carcinoma**	100%	95%
4	**MCF7**	**Breast carcinoma**	97%	20%
5	**LnCAP**	**Prostate adenocarcinoma**	98%	24%
6	**PC3**	**Prostate adenocarcinoma**	97%	70%
7	**DU145**	**Prostate adenocarcinoma**	95%	64%
8	PC3-Untransfected	Prostate adenocarcinoma	98%	52%
9	PC3-siEGFP	Prostate adenocarcinoma	98%	45%
10	PC3-siCXCR4	Prostate adenocarcinoma	96%	37%
11	PC3-siPI3K	Prostate adenocarcinoma	92%	37%
12	4T1 ctr	Mouse mammary carcinoma	97%	54%
14	PC3	Prostate adenocarcinoma	90%	43%
15	DU145	Prostate adenocarcinoma	89%	33%
16	PC3-Untransfected	Prostate adenocarcinoma	93%	47%
17	PC3-siEGFP	Prostate adenocarcinoma	76%	37%
18	PC3-siLuc	Prostate adenocarcinoma	90%	44%
19	PC3-siCXCR4	Prostate adenocarcinoma	90%	36%
20	PC3-siPI3K	Prostate adenocarcinoma	86%	26%
21	PC3-siSrc	Prostate adenocarcinoma	91%	41%
22	PC3	Prostate adenocarcinoma	96%	45%
23	PC3M	Prostate adenocarcinoma	97%	56%
24	**SW620**	**Colorectal carcinima**	94%	52%
25	PC3	Prostate adenocarcinoma	94%	46%
26	PC3-M	Prostate adenocarcinoma	98%	49%
27	SW620	Colorectal carcinima	95%	35%
28	PC3-R	Prostate adenocarcinoma	99%	52%
29	DU145-R	Prostate adenocarcinoma	93%	53%
30	MAT-LYLu	Duning prostate carcinoam(rat)	99%	60%
31	PC3-V	Prostate adenocarcinoma	98%	58%
32	PC3-R	Prostate adenocarcinoma	97%	48%
33	Du145-V	Prostate adenocarcinoma	97%	50%
34	Du145-R	Prostate adenocarcinoma	97%	46%
35	Mtln3	Rat mammary carcinoma	99%	43%
36	Mv3-R+D	Melanoma	99%	60%
38	PC3-V	Prostate adenocarcinoma	98%	65%
39	PC3-R	Prostate adenocarcinoma	96%	45%
40	PC3-G	Prostate adenocarcinoma	95%	48%
41	PC3-M	Prostate adenocarcinoma	91%	50%
42	Mtln3	Rat mammary carcinoma	98%	60%
43	Mtln3-GFP	Rat mammary carcinoma	93%	55%
44	Mtln3-GB	Rat mammary carcinoma	70%	40%
45	**A549**	**Non small cell lung cancer**	60%	20%
46	NCI-H460	Non small cell lung cancer	95%	25%
47	**HT1080**	**Fibrosarcoma**	91%	75%
48	**H1299**	**Non small cell lung cancer**	96%	23%
49	MDA231	Breast carcinoma	92%	20%

Task 1.2: Setup qualitative/quantitative multi-color whole embryo confocal microscopy-based screening assays.

Injection method in the ventral base of the yolk sac is established and compatibility with spreading to distant sites is achieved. Bio-imaging of migration using dsRed is established. Partner 1 (UL) has successfully applied a "short term angiognesis assay" in which a tumor cell implantation into the perivitelline space, between the yolk and the periderm of two days old embryos (2dpf), induced growth of new vascular tubes from subitestinal veins (SIV) 18 hours after implantation (**Fig. 2**). This angiogenesis response is quick and suitable for unique live imaging but requests 1000-2000 cells/embryo, requires matrigel and manual-skillful injection in a different site of the embryo then the yolk implantation suitable for tumor cell migration readout and therefore was not suitable for automated image analysis. Different angiogenesis assay has been developed but was also not visible for throughput screens and automated imaging therefore will be tested with selected shRNAs and chemical compounds (lead chemical entities) identify in WP3 and WP4.

Partner 1 (UL) has further optimized the macro for automated image analysis for quantification of spreading of xenograft human tumor cells in ZF. This macro has been used for characterization of an expanded panel of human tumor cell lines (**Fig. 3**). Using 3 sets of non-aggressive and aggressive lines for breast (MCF7 versus BT474), colorectal (HT29 versus SW620), and prostate cancer (LnCAP versus PC3 and DU145), we show that correlation with behaviour in long-term mouse models is excellent (**Fig. 4**). We also show that intra- and inter-experiment variation is low, leading to very good reproducibility of findings (**Fig. 5**). This validation is part of the manuscript submitted in which the fully automated image acquisition and analysis pipeline is described and validated using various human cell lines with known metastatic potential in mouse models. A primary screen has been performed with CM-DiI labelled PC3 cells. PC3 was chosen because it shows effective spreading and partner Galapagos has demonstrated very good adenoviral transduction in this line. 53 viruses have been tested as collaborative effort of partner 1 and 2 (**Table 3** in WP3**; Fig. 6**). From this screen, ~10 adenoviral constructs significantly inhibited PC3 spreading and we identified 2 genes, MST1R (Ron) and SYK, where 2 independent shRNA constructs showed significant inhibition of spreading and significant inhibition was observed in 2 independent injection experiments. Interestingly, MST1R expression has been observed in prostate cancer whereas no information on SYK levels in prostate cancer is available. Moreover, for neither of these genes a role in prostate cancer progression/ metastasis has been demonstrated. Therefore, these genes may represent interesting novel biomarkers and/or drug targets for prostate cancer progression. PC3dsRed, PC3mCherry, and MV3dsRed are currently being used for subsequent validation steps using additional adenoviral shRNA constructs as well as lentiviral shRNA constructs targeting MST1R and SYK. Taken together, this first automated ZF xenograft screen indicates that the automated analysis procedure is compatible with rapid screening for human cancer cell spreading.

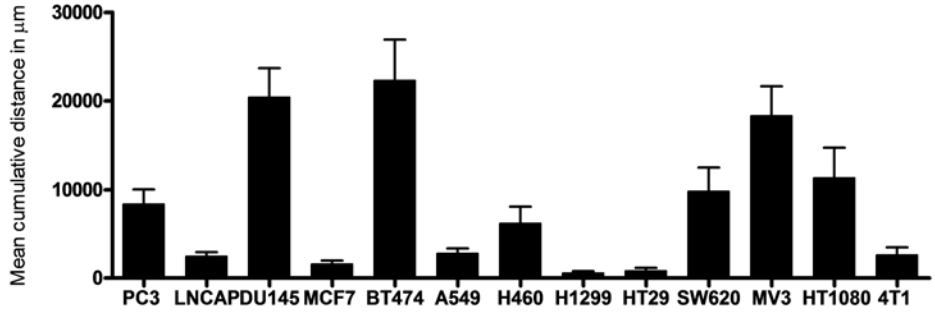


Fig. 3 Comparison of mean cumulative distance in the panel of cancer cell lines.
Data presented as mean ± s.e.m.

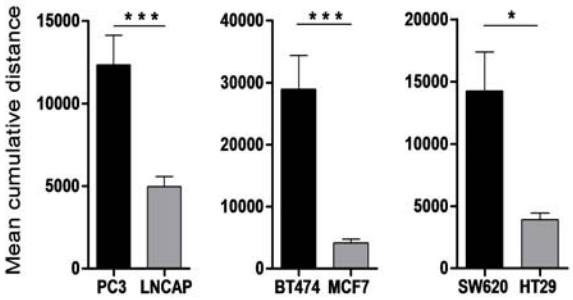


Fig. 4 Automated assay predicts cancer cell aggressiveness. Highly aggressive cancer cell lines (PC3, BT474, SW620) showed significant increased ability to migrate away from the primary tumor mass. Data presented as mean ± s.e.m. *P<0.05, ***P<0.001

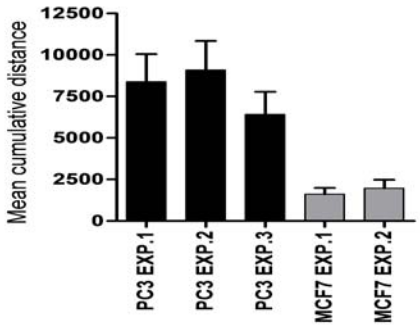


Fig. 5 Automated bioimaging assay showed high reproducibility.
Data presented as mean ± s.e.m.

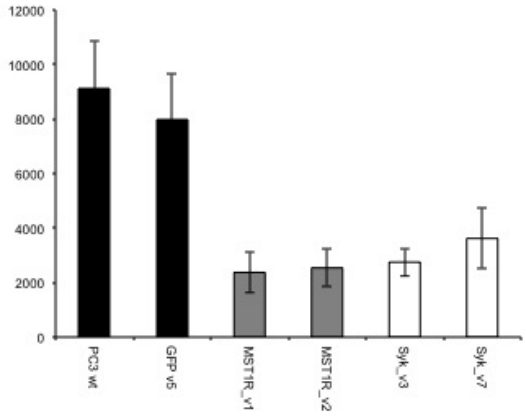


Fig. 6 MSTIR and SYK recognized as novel anti-cancer drug targets. Significant inhibition of PC3 cells migration transduced with MST1R and SYK shRNA adenoviruses. Data presented as mean ± s.e.m

Task 1.3: Develop image data base and automated image analysis.

At partner 1 (UL) collaborations have been initiated between LACDR, Biology, mathematics and IT to create optimal automated image analysis and database storage. Partner 3 (ZS-Screens) with other UL partners established data storage and data sharing of bio-images, which was implemented for our consortium.
Scientific Image Data Base (SIDB) was installed (Nov 2008) and hosted by ZF-screens (adres:https://sidb.zfscreens.com/; username: unileiden and password: Tu9qLL4A).
The software macro that was developed for automated analysis of cell migration has been further optimized and now provides a rapid and reproducible characterization of tumor cell spreading in ZF. This macro has been used for characterization of an expanded panel of human tumor cell lines. Validation of the ZF xenograft model has been expanded with other cancer lines with known metastatic behaviour in mice. Using 3 sets of non-aggressive and aggressive lines for breast, colon, and prostate cancer, we show that correlation with

behaviour in long-term mouse models is excellent. We also show that intra- and inter-experiment variation is low, leading to very good reproducibility of findings. This validation is part of the manuscript submitted in which the fully automated image acquisition and analysis pipeline is described and validated using various human cell lines with known metastatic potential in mouse models.

Results achieved:

- A panel of five human tumor cell lines is selected.
- Optimization of xenotransplantation into ZF embryos is established and assays for migration and angiogenesis are developed. This work is published (Snaar-Jagalska, 2009) and submitted (He et al.).
- A panel of zebrafish tumor cell lines suitable for ZF allotransplantation and bio-imaging is generated.
- PC3 cell line is selected as preferred cell line with which to proceed.
- PC3 has been tagged with more optimal fluorescent marker (dsRed).
- Automated imaging in multiwell plates has been developed for spreading to distant organs.
- Database is developed implemented for ZF-CANCER consortium.
- A pilot experiment with sh-RNA knockdown of PI3K and CXCR4 in PC3 resulted in reduced migration of tumor cells in the zebrafish.
- Human cancer cell migration in ZF xenograft model has been demonstrated to correlate with metastatic capacity in mouse model.
- MV3 melanoma cells have been generated that stably express dsRed or mCherry.
- PC3 prostate cancer cells have been generated that stably express mCherry.
- Automated image analysis for spreading to distant organs has been further optimized and the manuscript on this method is submitted (Ghotra et al.).
- A stable *ptenb*-/- fibroblast cell line has been generated and successfully allograft.
- An adenoviral shRNA screen has been performed divided in 10 independent experiments where mock and control shRNA (GFP) and 6 different tested shRNAs were used. A total of 28 genes, selected for previous correlation with prostate cancer progression, were tested using 25 zebrafish injections per condition. For the majority of genes 2 different shRNAs were tested per gene.
-
- From the screen, which includes genes highly relevant to prostate cancer based on database searches, 2 novel candidate genes, for which no data on their role in prostate cancer progression/metastasis is available, have been selected for further validation.

Work package 2 : The genetics and generation of zebrafish tumors and cell lines

Lead participant : UEDIN

Task 2.1: Identification of novel and conserved cancer pathways in zebrafish.

Three sets of microarrays have been performed in WP2 to identify possible cancer pathways: pten embryonic genetic mutants, BRAF/MEK expressing embryos, and $BRAF^{V600E}$ regenerating nevi. Some have been validated.

1. In the pten mutants, genetic mutants were identified in embryos at specific stages and microarray gene expression analysis was performed. Expression of many genes was altered by microarray, and over 50 have been tested by RNA in situ hybridization, but they do not appear to be obviously different in RT-PCR or by RNA in situ hybridization.
2. BRAF and MEK cancer and CFC developmental human mutations were expressed in the developing zebrafish. A clear series of genes that are up or down regulated in 4 BRAF and 2 MEK mutants have been identified. Some of these are phosphatases that might be upregulated by MAPK signalling to shut-down the MAPK pathway in a feedback loop. The DUSP6-GFP line was used to validate this idea, and indeed altered MAPK signalling can be clearly visualized by the DUSP-GFP line. This line is being crossed into the BRAF melanoma model.
3. $BRAF^{V600E}$ nevi are premaligant tumors of melanocytes. The gene expression signature in nevi and

recurring nevi has been assessed. While many genes are altered there is a clear melanocyte signature, including melanocyte specific enzymes validating this approach.

Task 2.2: Generation of transgenic zebrafish lines to test role of new cancer genes.

To verify key gene targets in tumorigenesis, trangenic lines have been generated from candidate genes identified by expression arrays, and through literature (e.g. kinase drive genes found in human cancer). Genes were expressed either from constitutively active promoters (e.g. CMV) in stable (expressed in all cells), or mosaic (expressed in a random subset of cells) fashion. Some genes induced development abnormalities when expressed in all cells, and were instead expressed in a tissue specific manner using tissue specific promoters (e.g. *mitfa* in melanocytes), or expressed from the heat-shock promoter. Transgenic line generation has recently been optimized in zebrafish using the Tol II transposon system, which allow for a high rate of integration and germ-line transmission. These transgenic lines were crossed into PTEN and p53 mutant lines, and combined with activated BRAF mutations to access their role in specific stages of tumorigenesis.

WP2 has tested four new cancer gene sets in zebrafish. Each of these developments has arisen from identifying new mutations in the literature and testing their function in zebrafish.

1. BRAF mutations: We have tested additional kinase impaired BRAF mutations (also used in the gene expression arrays above). Notably, we found that BRAF cancer mutations that are impaired in vitro are gain of function in vivo, and are capable of forming nevi (bengin tumors of melanocytes).
2. MITF mutations: In collaboration with Jim Lister (USA), we found that *mitf* hypomorphic zebrafish develop melanoma in collaboration with $BRAF^{V600E}$. Building on this work, we have also generated transgenic lines expressing mitf mutations found in human melanomas. Importantly, we find that p53 mutations can alter the tumor spectrum in zebrafish $BRAF^{V600E}$*mitf* melanoma.
3. Kinase drivers: We have generated many transgenic lines expressing predicted melanoma kinase driver lines, but have not seen a change in melanoma potential.
4. Mc1r: We have characterized and confirmed a mc1r insertional mutant line. Mc1r mutations may co-operate with BRAFV600E in humans to drive melanoma progression. The zebrafish crosses have been established, and we are waiting for the outcome.

Task 2.3: Generation of tumors in zebrafish for engraftment into zebrafish embryos.

Genetic combinations of BRAF, p53 and pten lines were generated to test for melanoma disease progression (partner 4, UEDIN); and pten, p53 combinations were assessed for rate of eye tumors, as well as tumor progression and tumor spectrum (partner 6, KNAW). WP2 has created many zebrafish tumors with different genetic and pathological subtypes. These include tumors in PTEN, Ras, BRAF, p53, and Mitf. In addition, we have identified a genetic background that appears to be melanoma prone in the $BRAF^{V600E}$ transgenic background (**Fig. 7**).

Fig. 7 $BRAF^{V600E}$ melanoma co-operating mutations determine pathology.

We have also continued with pathological analysis of Hemangiosarcoma formation in ptena+/-ptenb-/- mutants. We performed immuno-histological and immuno-blotting experiments using various antibodies targeting different signalling pathways, confirming that the cells in the hemangiomas are predominantly endothelial cells (**Fig. 8**). PTEN is an essential tumor suppressor that antagonizes Akt/PKB signaling. The zebrafish genome encodes two *pten* genes, *ptena* and *ptenb*. Zebrafish mutants that retain a single wild type copy of *pten*, $ptena^{+/-}ptenb^{-/-}$ or $ptena^{-/-}ptenb^{+/-}$, are viable and fertile. $Ptena^{+/-}ptenb^{-/-}$ fish developed tumors at a relatively high incidence (10.2%) and most tumors developed close to the eye (26/30). Histopathologically,

the tumor masses were associated with the retrobulbar vascular network and diagnosed as hemangiosarcomas. A single tumor was identified in 42 *ptena*$^{-/-}$*ptenb*$^{+/-}$ fishes that was also diagnosed as hemangiosarcoma. WP2 generated the following tumor types:BRAF nevi; BRAF, p53 melanoma; PTEN eye, intestinal; BRAF, pten melanoma; p53 MPNST; p53, pten nevi (?); RAS melanoma; RAS rhabdomyosarcoma; PTEN hemangioma; *unknown hubrecht mutation* BRAF melanoma; BRAF, mitf melanoma; BRAF, mitf, p53 melanoma

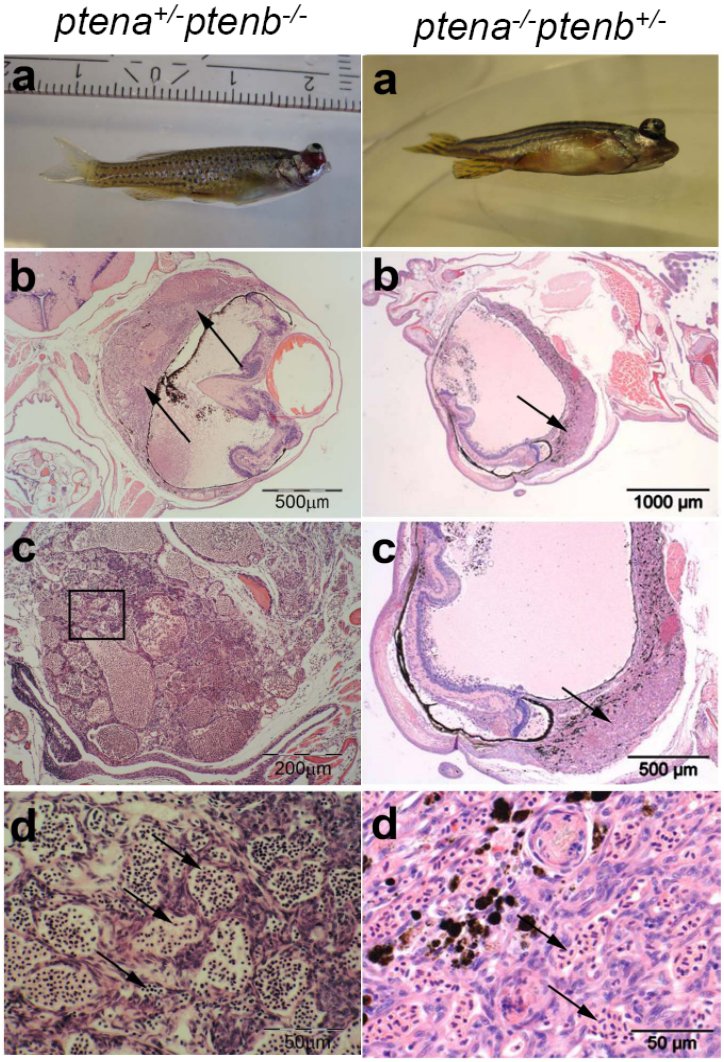


Fig. 8 Hemangiosarcoma formation in *ptena*$^{+/-}$*ptenb*$^{-/-}$ and *ptena*$^{-/-}$*ptenb*$^{+/-}$ mutants.
(a) *Ptena*$^{+/-}$*ptenb*$^{-/-}$ mutant (3 months old) and *ptena*$^{-/-}$ *ptenb*$^{+/-}$ mutant (9 months old) with ocular tumor. The entire intact fish was fixed and embedded in paraffin. (b) Transversal sections were stained with hematoxylin and eosin. Arrows indicate tumor mass, which is associated with the eye bulbs. (c,d) Higher power magnifications of the tumor mass; the tumor consists of cells that form different sizes of blood-filled spaces (arrows in c and d). Scale bars are 500, 200 and 50 µm in (b), (c) and (d), respectively. The tumors were invasive and penetrated into the brain region. The cells had a plump morphology, detached from surrounding tissue and protruded into the vessel lumen, hallmarks of hemangiosarcoma.

Task 2.4: Generation of cancer cell-lines for transplantation into embryos.
Zebrafish cancer cell lines are derived from freshly dissected tumoral tissue and cultured in L15 medium supplemented with 15% foetal calf serum, at 25 °C and atmospheric CO_2 concentration (partner 6, KNAW). Partner 6 (KNAW) established cell lines with different genetic backround. Stable cell lines from embryos (wt, ptena-/-, ptenb-/-, ptena+/-ptenb-/- and ptena-/-ptenb+/-) and pre-stable cell lines (ptena-/-ptenb-/-) are generated and currently grafted in 2dpf embryos to study angiogenic response. Furthermore, stable and pre-stable cell line from a hemangiosarcoma is established. In addition, cell lines from ptenb-/-BRAF, ptena-/-/BRAF and P53/BRAF are generated and prepared for grafting. Some of these lines have been sent to WP1 and successfully engrafted into the yolk of 2 day old zebrafish embryos. The migration of these cells towards tail of the fish was observed 4-5 days after implantation.

Results achieved in WP 2:

- We have generated a living library of zebrafish with cancers (12 tumor models) with at least 12 genetic combinations that give rise to benign and malignant tumors. These tumors are different pathologically, and some are new genetic combinations that have not been modelled in the mouse. These tumors reflect the varied stages of tumorigenesis in some tumor types. While we have not yet identified new genes in these cancers, we have new combinations of genetic mutations that lead to novel cancer models (BRAFpten, and possibly p53 pten nevi formation). A key finding in our work is that co-operating mutations alter tumor pathology and spectrum. Detailed histopathology has been performed for BRAF and PTEN mutant tumors. This work has been published (Patten et al. 2010) and submitted (van Duijn et al.).
- We have identified a new genetic mutation in the MC1R gene. Based on the literature, this gene may collaborate with BRAF to promote melanoma development. This would be the first animal model of this cancer. This mutation has been confirmed, and the early embryonic phenotype clearly established.
- We have generated new zebrafish transgenic and genetic lines based to test the potential of these genes in cancer progression. In collaboration with Jim Lister (USA) we find mitf mutations co-operate with BRAFV600E to promote melanoma. We have also generated new mitf transgenic lines using human mitf cancer alleles. We have also characterized and confirmed the action of a mc1r genetic mutant line, and are testing its contribution towards melanoma progression.
- We have generated microarray analysis of cancer prone zebrafish to identify pathways important in cancer progression (pten, BRAF/MEK, BRAF nevi). We find that phosphatases are upregulated in BRAF and MEK mutant embryos, and confirm that the DUSP6 phosphatase can accurately reflect MAPK signalling in zebrafish. This work has been submitted (Ishizaki et al.)
- Zebrafish cell lines have been established from embryonic mutant lines and from cancer prone tissues. These cell lines have been transferred to WP1, but are also interesting for studying how signalling pathways are altered in pten deficient cells. A key finding of this work is that we have established a robust methodology for generating zebrafish cell lines. This work has been submitted (Choorapoikayil et al.).

Work package 3 : Functional genomics-based kinase target discovery and focused drug discovery

Lead participant : Galapagos

Task 3.1: Assay development for RNA interference drug target screening.

Optimization of adenovirus transduction efficiencies has been achieved. Galapagos has identified 31 human tumor cell lines that can be transduced efficiently (>70% of the cells express adenoviral encoded fluorescent protein) with adenovirus without inducing toxicity. See **table 2** below.

Table 2 Cancer cell lines that can be transduced efficiently with a transduction efficiency of minimum 70% and low viral toxicity

Lung	A-549	NCI-H441	NCI-H460	HCC-78
Prostate	DU-145	PC-3	PC-3M	
Breast	MDA-MB-231	MDA-MB-468	BT-474	MCF-7
Colon	HCT-116	SW-480	SW-620	
Neuroblastoma	SH-SY5Y	SK-N-SH	SK-N-MC	
Liver	Hep-G2	HuH	HuH-7	
Pancreas	MiaPaCa-2			
Melanoma	LOX			

Kidney	HEK293			
Cervix	HeLa			
T lymphoma	Jurkat			
T cell line	SupT1			
Osteosarcoma	U2OS			
myelocytic lymphoma	U937			

Robust proliferation (cell titer blue) and cell survival assays (PI exclusion, annexin V staining) have been established on a high content imager. In addition, the more relevant motility and epithelial/mesenchymal transition assays have been developed.
A set of over 300 control viruses targeting 111 different genes has been generated and a set of various negative controls (empty, scrambled knockdown) has been delivered. Instead of the proliferation/cell survival assays, the positive and negative controls were tested in more relevant assays: motility and epithelial/mesenchymal transition. In each assay control viruses against 5 different targets were selected as relevant positive controls with more than 40% inhibition of motility or epithelial/mesenchymal transition in colon and prostate cancer cell lines.
Effects of negative and positive control virus on tumor cell lines have been assessed. Negative control viruses had no effect on proliferation, survival rates, and *in vitro* migratory capacities of tumor cells. Positive control knockdown constructs targeting various kinases (e.g. SYK, PI3K) decreased *in vitro* metastatic and migratory capacities of tumor cells with at least 40%.

Task 3.2: shRNA-based discovery of novel anticancer kinase drug targets.
All 3000 kinase knockdown constructs have been converted to the C20 library format for screen purpose. Adenovirus had no detrimental effect on transplantation and tumorigenic properties of the cells. Knockdown of PI3K and CXCR4 decreased migratory capacity of PC3 cells in the zebrafish model.
Task 3.2 has been modified duo to lack of automated xeno-transplantation procedure into two days embryos and delay with generation of stable fluorescent PC3 cell line. ZF-screens (partner 3) has developed a prototype automated injector of zebrafish larvae. IThe combination of the automated injector and a COPAS XL Biosorter for the selection and quantification of specific embryos worked successfully to detect drugs that inhibit the proliferation of Mycobacterium marinum. No effect of anti-tumor drugs to inhibit migration or survival of the tumor cells injected in the embryos was found therefore the automated system was not use for xeno-transplantantation of sh-RNA transduced cells.
The steering committee agreed at 3rd project meeting in Barcelona (Apr 2009) that the *in vivo* screen will be performed with selected shRNAs based on their effects in *in vitro* assays and validated with known kinase inhibitors. A screen has been performed with CM-DiI labelled PC3 cells. PC3 was chosen because it shows effective spreading and partner Galapagos has demonstrated very good adenoviral transduction in this line. 53 viruses have been tested in 10 independent xenograft experiments (**Table 3**; **Fig. 6**). In this way, 28 genes have been targeted, for most genes using 2 different viral constructs in two independent experiments. From this screen, ~10 adenoviral constructs significantly inhibited PC3 spreading. For some genes only 1 out of 2 shRNA constructs were effective or results were not consistent between injection dates. However, we identified 2 genes, MST1R (Ron) and SYKl, where 2 independent shRNA constructs showed significant inhibition of spreading and significant inhibition was observed in 2 independent injection experiments. Interestingly, MST1R expression has been observed in prostate cancer whereas no information on SYK levels in prostate cancer is available. Moreover, for neither of these genes a role in prostate cancer progression/ metastasis has been demonstrated. Therefore, these genes may represent interesting novel biomarkers and/or drug targets for prostate cancer progression.

Table 3. Design of adenoviral shRNA screen in PC3 ZF xenografts (>25 embryos were injected per PC3/virus combination in 10 independent experiments testing 5-11 combinations)

EXP.1	**EXP.2**	**EXP.3**
PC3, untransduced	PC3, untransduced	PC3, untransduced
PC3, eGFP_v5	PC3, EGFR_v1	PC3, PGF_v2
PC3, CXCR4_v14	PC3, EGFR_v2	PC3, SRC_v3
PC3, CXCR4_v16	**PC3, SYK_v7**	PC3, PIK3CA_v2
PC3. PIK3CA_v2	PC3, eGFP_v3	PC3, eGFP_v5
PC3. PIK3CA_v4	PC3, FAK_v1	PC3, ITGA5
PC3, Src_v2	PC3, SNAI1_v5	
PC3, Src_v3	PC3, SNAI2_v4	
	PC3, SNAI2_v5	
EXP.4	**EXP.5**	**EXP.6**
PC3, untransduced	PC3, untransduced	PC3, untransduced
PC3, SYK_v7	PC3, NTRK1_v3	PC3, CKS1B_V2
PC3, SNAI2_v4	PC3, PIM1_v3	PC3, GAS6_V2
PC3, eGFP_v5	PC3, PIM1_v1	PC3, VEGFC_V3
PC3, EGFR_v2	PC3, eGFP_v5	PC3, eGFP_v5
PC3, SNAI1_v5	PC3, NTRK1_v20	PC3, CD44_V4
PC3, ITGA5_v2	PC3, SGK_v7	PC3, CDK6_V1
PC3, PGF_v2	PC3, SGK_V6	**PC3, MST1R_V2**
PC3, SRC_v3	PC3, MET_v28	PC3, TWIST1_V5
PC3, eGFP_v5	PC3, ITGA2_v2	PC3, ZFHX1B_V4
EXP.7	**EXP.8**	**EXP.9**
PC3, untransduced	PC3, untransduced	PC3, untransduced
PC3, CD44_V4	PC3, EPHA2_V1	PC3, eGFP_v5
PC3, MST1R_V1	PC3, IGFBP3_V1	PC3, NTRK1_v20
PC3, TWIST1_V5	PC3, IGFBP3_V6	PC3, PIM1_v1
PC3, eGFP_v5	PC3, eGFP_v5	**PC3, SYK_v3**
PC3, CD44_V3	PC3, EPHA2_V3	
PC3, MST1R_V2	PC3, ITGA2_V1	
PC3, TWIST1_V2	PC3, SPHK1_V2	
PC3, ZFHX1B_V2	PC3, SPHK1_V4	
PC3, ZFHX1B_V3	**PC3, SYK_V7**	

EXP.10
PC3, untransduced
PC3, ITGA2_v2
PC3, MET_v25
PC3, MET_v28
PC3, eGFP_v5
PC3, ITGA2_v1
PC3, PTK2_v3
PC3, PTK2_v4
PC3, VEGFC_v3
PC3, VEGFC_v2
PC3, SGK_v7

Task 3.3: Prioritization of novel anticancer kinase drug targets.
This task was discontinued as targets were only identified at end of program. We have identified 4 highly interesting targets whose expression has been correlated with prostate cancer progression while their functional role in prostate cancer metastasis is not known. These represent potential drug targets. Validation experiments are currently ongoing for this set (see WP1). Based on these experiments, compound will be selected aimed at targeting such molecules.

Task 3.4: Screening for pharmacological inhibitors for prioritized novel kinase drug targets.
This task was discontinued as targets were only identified at end of program. The target identification has been delayed due to difficulties in developing a highly effective and reproducible screening platform in WP1. Now that screening is up and running (see WP1) and several promising targets have been identified for prostate cancer, experiments along this line are planned.

Task 3.5: Prioritization of novel pharmacological inhibitors of kinases.
Discontinued as targets were only identified at end of program however experiments are planned to be completed for potential prostate drug targets.

Results achieved:

- A large panel of cell lines has been established suitable for adenoviral transduction. Positive and negative control viruses have been generated which adhere to preset criteria.
- Assays to benchmark cells and viruses for screening in zebra fish have been established and fulfilled all preset criteria.
- We have been able to demonstrate that knock down shRNA viruses targeting kinases and other classes of proteins significantly inhibit *in vitro* metastatic and migratory capacities. These shRNA viruses can be used to benchmark the *in vivo* assays. This has been done in PC3 so far and will also be done in other cell lines.
- The kinase shRNAs have been converted to the adenoviral C20 backbone, a special backbone allowing better transduction of the tumor cell lines
- Two positive control shRNAs targeting PI3K and CXCR4 inhibit migratory capacity of PC-3 cells in the zebrafish. A PC3 cell line stably expressing dsRed allows better tracking of the cells increasing the window of the assay and enhancing the chances of finding novel targets.
- Achieved dual transduction of PC3 cells with AsRed reporter construct in combination with shRNA transduciton
- A total of 135 viruses were produced with an average IU of 2.2x10E7. From this list of knockdown viruses, in total 53 have been screened in zebrafish targeting 25 different genes in 7 batches.
- An adeniviral shRNA screen has been performed divided in 10 independent experiments where mock and control shRNA (GFP) and 6 different tested shRNAs were used. A total of 28 genes, selected for previous correlation with prostate cancer progression, were tested using 25 zebrafish injections per condition. For the majority of genes 2 different shRNAs were tested per gene.
- From the screen 2 novel candidate genes, for which no data on their role in prostate cancer progression/metastasis is available, have been selected for further validation steps.

Work package 4 : High-throughput chemical compound screening

Lead participant : MPG (now IST Austria)

Task 4.1: Chemical screen using kinase-targeted libraries for tumor progression.

Partner 2 (Galapagos) has generated and quality controlled five copies of the SoftFocus Kinase collection of 5000 compounds. These libraries have been shipped together to partner 4 (UEDIN), partner 5 (CMRB), partner 6 (KNAW), partner 7 (MPG) and partner 8 (Biobide) to screen for: (1) inhibition of angiogenesis in wild type embryos ('wild type angiogenesis') and in embryos (partners 5 (CMRB) & 8 (Biobide)); (2) cell migration and tissue morphogenesis defects during the first 10 hours of development (partner 7, MPG); (3) reversal of

the *pten* mutant phenotype at day 5 (partner 6, KNAW) and (4) reversal of irradiation-induced apoptosis in *p53* mutant embryos (partner 4, UEDIN).

(1) inhibition of angiogenesis in wild type embryos ('wild type angiogenesis') (partners 5&8)

Partner 5 (CMRB) has screened 240 compounds from the Galapagos library for inhibition of angiogenesis using the fli1:EGFP transgenic line. The conditions for the large scale screen have been identified based on the results of the small screen (duplicates of 20µM compound treatment at 24hpf, compound removal after 8 hours followed by imaging at 48hpf). Compounds identified as potentially interesting from the first round of screening (i.e. those that showed effects on the vasculature or death) were taken forward for titration. Compounds that were considered to promote inhibition of vasculature development, observed by eye (no automated software has currently been used to assess this), from the second round (titration) have been highlighted as putative "hit" compounds that will be verified by the automated large-scale screen in Biobide (patner 8). Partner 8 (Biobide) manually screened different compounds with known anti-angiogenic effects in zebrafish (KRN633: VEGF receptor tyrosine kinase III inhibitor; AG1478: EGF receptor tyrosine kinase inhibitor); 2-Methoxyestradiol: natural metabolite of 17β-estradiol; Indirrubin-3'-oxime: Cyclin-dependent kinase inhibitor; Curcumin: natural phenolic compound with potent anti-tumor properties). Dose-response curves were done to determine the range of compound action (**Table 4**). These results allowed to learn about the distinct phenotypes that can be detected and to determine the best parameters to quantify the antiangiogenic effect.

Compound	Concentration (µM)										
	0.001	0.01	0.02	0.05	0.1	0.5	1	5	10	20	40
KRN633											
AG1478											
2-ME											
Indirrubin											
Curcumin											

Not tested | No effect | *Moderate effect | *Acute effect | Toxic/ lethal

Table 4. Summary of anti-angiogenic effects detected.

The two parameters that partner 8 has chosen to quantify the antiangiogenesis effect were:

1. Total number of intersegmental vessels present in the trunk. Figure 8 shows a whole embryo (A) and the part of the trunk in which the vessels are quantified (B), that represents the same picture but without the head and the yolk (most bulging part). Intersegmental vessels are shown in blue.
2. Number of intersegmental vessels that are complete, what means that they get to the DLAV (dorsal longitudinal anastomotic vessel) (**Fig. 9B**).

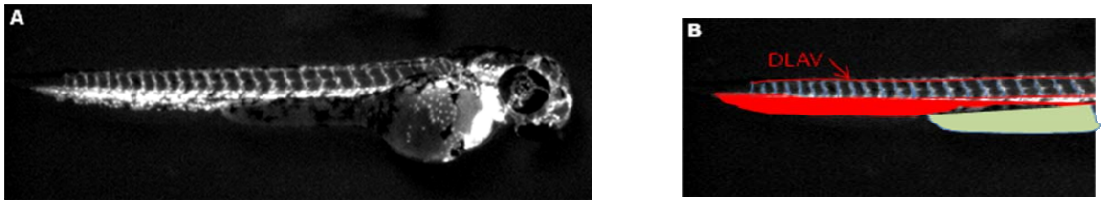


Fig. 9 Quantification of gamogenetic parameters in zebrafish embryo.

Partner 8 (Biobide) used the Galapagos chemogenomic libraries to check for inhibitors of embryonic angiogenesis based on the conditions that both partner 5 and 8 established for this assay. Partner 8 (Biobide) is also currently developing proper image analysis software for the automatic analysis of angiogenic phenotypes focusing on: definition of the parameters to quantify embryonic angiogenesis; establishment of conditions for the automation of the assay. This includes plates, dispensation, image acquisition and software.

Partner 8 (Biobide) has tested on HT platform a VEGF inhibitor (SU5614, Calbiochem) as a positive control for an anti-angiogenic effect and noted an angiogenic phenotype in the presence of 10 uM of this compound.
Partner 8 (Biobide) has partially set up a high-throughput screening platform for angiogenesis.
Partner 5 (CMRB) has identified 6 compounds from the SoftFocus library by the automated screening platform (Partner 8, Biobide) and kinase profiled them to determine the targeted kinases. Two compounds were shown to inhibit Phosphorylase kinase subunit G1 (PhKG1), Proto-oncogene serine/threonine kinase-1 (PIM1) and Transforming tyrosine kinase A (TrKA) (**Fig. 10**). These targets were verified by morpholino knock-down in embryos, confirming that all of these kinases are important for angiogenesis in zebrafish during development (partner 5, CMRB). The kinase targets were further verified by inhibition of kinases TrKA and PIM1 by commercial inhibitors (partner 8). Inhibition of PIM1 affects primarily the diameter of vessels whereas inhibition of either TrKA or PhKG1 leads to an inhibition of vessel growth. Partner 2 performed 3D modeling analysis of the interaction site between target and compound. Space-filling models of BioFocus SoftFocus compounds F10 and F11 from the SFK33 library docked into the catalytic site of PhKG1 were shown. The activity of these compounds combined with a unique binding mode provides a good starting point for compound optimization and further drug discovery efforts.

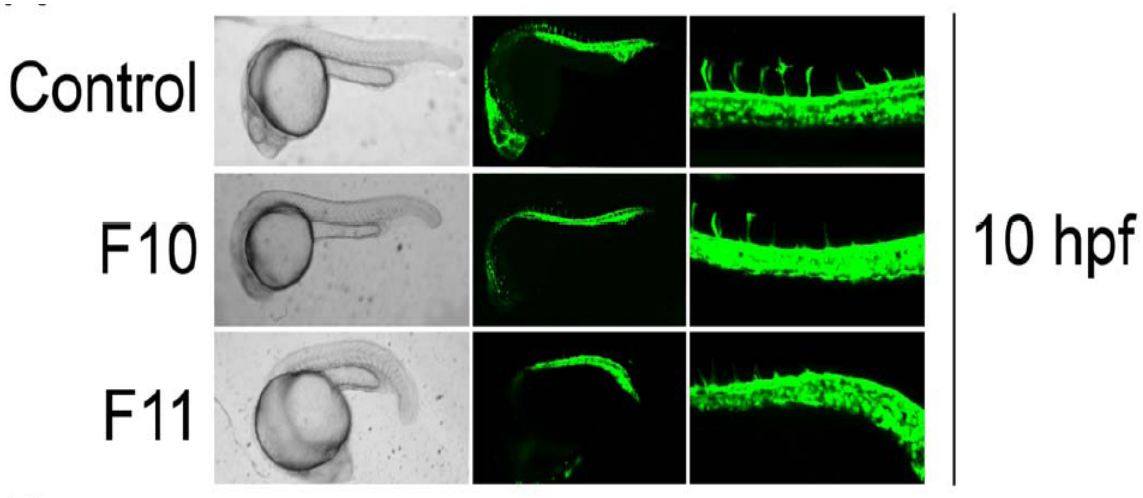


Fig. 10 5uM of either compound F10 or F11, as indicated, was added to 10 hpf embryos. At 24hpf embryos were imaged and the effects on ISV formation and general vascularisation were examined.

(2) cell migration and tissue morphogenesis defects in the first 10 hours of development (partner 7)
Partner 7 (MPG) and partner 9 (IST) has screened the whole Softfocus library and identified 884 compounds that elicit specific morphogenetic defects during gastrulation. The defects can be subdivided into five distinct phenotypic classes: incomplete epiboly, reduced convergence and extension movements; necrosis; and arrested development. By far the largest, and probably also most unspecific phenotypic class is incomplete epiboly (92%), followed by early death (4%), necrosis (2%), defective convergence extension (1%), and arrested development (1%) (**Fig. 11**). The most interesting phenotypes, which we decided to follow up are defective convergence and extension movements and arrested development. We will kinase profile them to determine the targeted kinases and then perform loss- and gain-of-function studies to analyze the function of the respective kinases in zebrafish gastrulation.

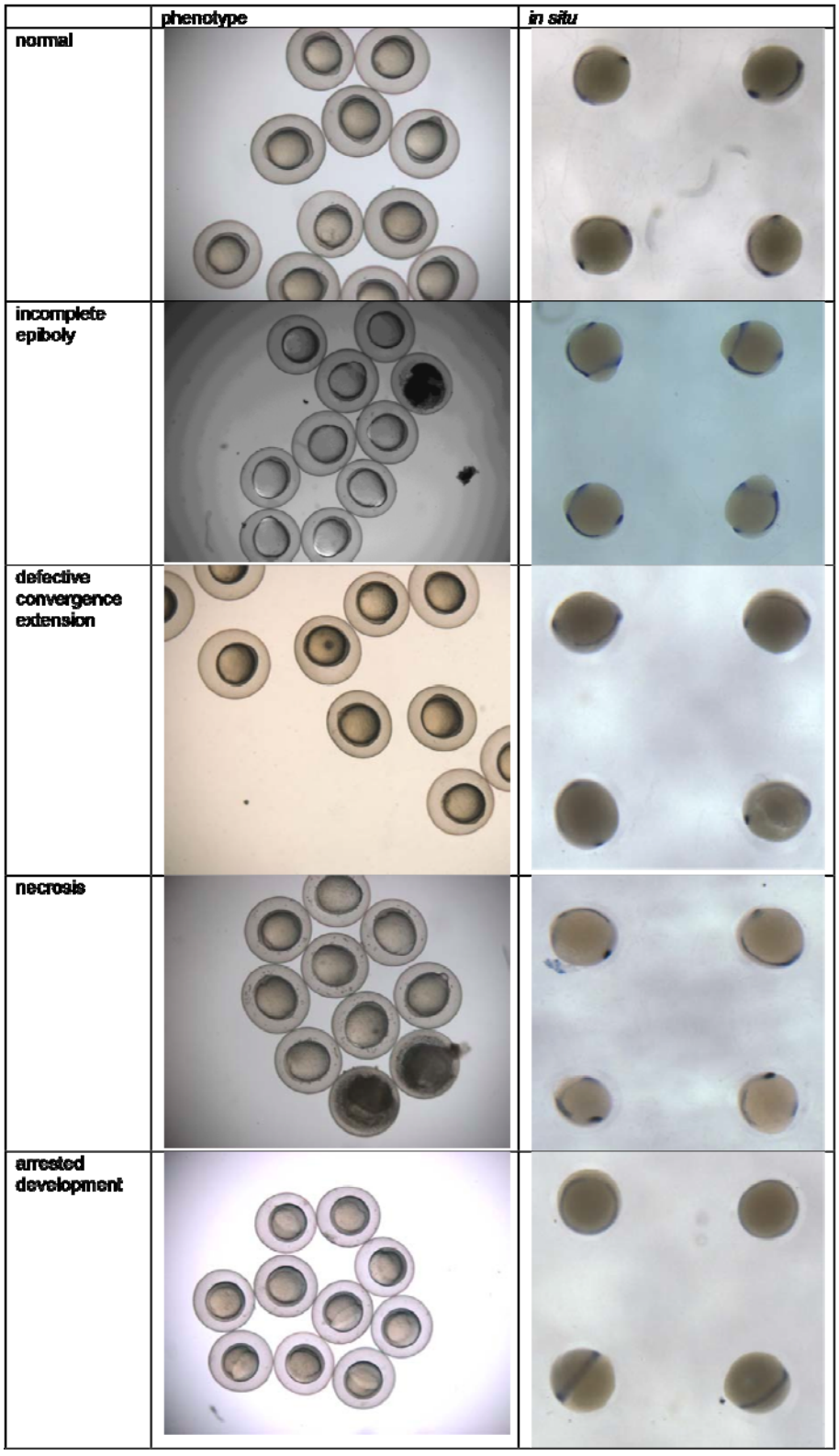


Fig. 11 Initial classification and characterization of the different phenotypes observed in the cell migration and tissue morphogenesis screen

(3) reversal of the pten mutant phenotype at day 5 (partner 6)
Partner 6 (KNAW) decided - in agreement with the Steering Committee during the ZF-CANCER meeting in Utrecht (April, 2010) - not to continue with compound screening using the pten mutants. The complexity of pten genetics turned out not to be feasible to transfer the theoretical aim of screening a large compound library into practical application.

(4) melanocyte biology and reversal of irradiation-induced apoptosis in *p53* mutant embryos (partner 4)
Partner 4 (UEDIN) has completed a series of small molecule screens for modulators of melanocyte biology – cell specification, development, migration, differentiation and survival. Partner 4 (UEDIN) used the "core set" from BioFocus, as well as the libraries from Sigma (LOPAC), and kinase inhibitor and phosphatase inhibitor libraries (ENZOLIFESCIENCE), as well as a subselection of a Maybridge bioactive library. This approach – using multiple panels of inhibitors has been very fruitful, and small molecules that affect many distinct aspects of melanocyte biology have been identified. In addition, partner 4 (UEDIN) has done a first small scale screen of the p53 mutant, and identified a preliminary panel of small molecules that appear to prevent the irradiation-induced apoptosis in *p53* mutant embryos. Some of these compounds directly interact with the p53 pathway. For example, roscovitine causes a reduced number of melanocytes that seems to be rescued by loss of p53. This is an exciting new pathway, as p53 plays an obscure role in melanocyte biology and melanoma. Partner 4 (UEDIN) has confirmed targets using a kinase inhibitor profiling, as well as biochemical target ID, and through yeast genetic screens. Partner 4 (UEDIN) has passed some of these compounds onto WP1 to test in xenograph assays for migration.

Task 4.2: Compound validation and phenotypic characterization.

Partner 4 (UEDIN) has validated the compounds from different screens by reordering the compounds and testing at different concentrations; also by ordering compounds that share target or chemical similarity. Compounds that did not reproduce the original phenotype were not analyzed further. Compounds that did reproduce the phenotype were studied in more detail, often in comparison to the description of genetic mutants.

Partner 4 (UEDIN) has done detailed dose curves and addition of timing for a few specific compounds, with a special interest in roscovitine. Roscovitine is a CDK2 inhibitor, and the "master melanocyte regulator". MITF induces expression of CDK2 in melanoma. Partner 4 (UEDIN) has observed that the cells have an abnormal shape and are reduced in total number. This is an important result as it implies that there is a tissue specific function of CDK2 in melanocyte development. Partner 4 (UEDIN) has also characterized a specific compound AG1276 that alters cell movement phenotypes. Partner 4 (UEDIN) has shown that increasing concentrations of AG1276 causes reduced melanocyte cell movement. As proliferation and movement are key features of melanoma biology, these two compounds appear to be the most interesting to move forward for Task 4.3.

Partners 5 (CMRB) and 8 (Biobide) have identified compounds as inhibitors of angiogenesis during zebrafish development and taken them forward for validation in accepted human in-vitro cell-based assays. Angiogenesis, cell migration and cell proliferation assays were performed in human umbilical vein endothelial cells (HUVEC) assays, which confirmed the efficiency of the compounds in a human context. Partner 7 (MPG) has identified and validated 10 compounds that give rise to specific morphogenetic defects during gastrulation and is currently validating potential targets of these compounds.

Task 4.3: Target validation and mutant analysis.
Partner 4 (UEDIN) has done kinase profiling (Dundee) to examine the target pathway for roscovitine and AG1276, and find that they selectively inhibit CDK2 and KIT. One thing to consider is to make specific CDK mutations and express these in zebrafish. Partner 4 (UEDIN) has confirmed target in various methods:

1. Yeast genetic screens. Compounds that were active in zebrafish were tested for activity in yeast genetic mutants to identify the target pathways.
2. Kinase profiling identified the kinases sensitive to specific kinase inhibitors.
3. Chemical derivatives were made for nitrofurans that specifically kill melanocytes to identify the target by biochemical analysis. The target was confirmed by chemical genetic analysis.
4. Yeast 2-hybrid screen has been started to identify the protein interactors of new protein targets of small molecules

Partners 5 (CMRB) and 8 (Biobide) validated the target kinases identified by profiling of the compounds in zebrafish in terms of their effects on angiogenesis using morpholino knock-down techniques.

Furthermore, the kinase targets of the drugs were validated in zebrafish by rescue of the compound-phenotype using mRNA for one of the kinase targets (PhKG1), confirming that the compounds inhibit PhKG1 in zebrafish and that inhibition of PhKG1 effects angiogenesis (partner 5, CMRB). Partner 7 (MPG)is still in the process of identifying the respective target molecules and validating candidates by morpholino knock-down techniques.

Results achieved:

- Partner 5 (CMRB) has screened a small library and identified 4 compounds that promote reduced/abnormal vasculature.
- Conditions for large scale screen have been determined.
- Partner 8 (Biobide) has partially set up a high-throughput screening platform (**Fig. 12**) and is developing an automatic anti-angiogenesis screening and image analysis (**Fig. 13**).

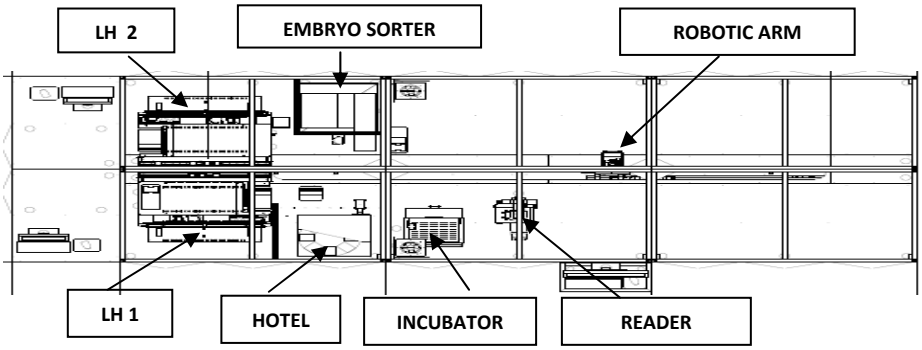


Fig. 12 HTS platform lay-out.

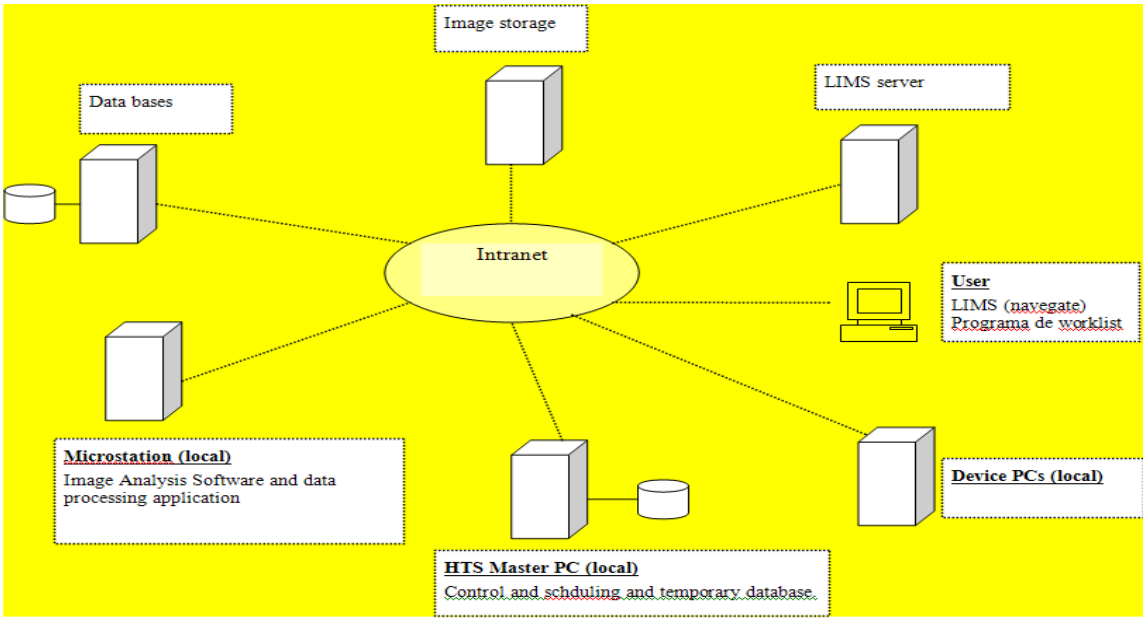


Fig. 13 Schematic representation of HTS databases and applications.

- Partner 7 (MPG) and partner 9 (IST) defined conditions for high throughput screening of cell migration and tissue morphogenesis using the SoftFocus library and identified ~ 20 compounds that affect embryo morphogenesis during gastrulation. The compounds are currently re-screened and the obtained phenotypes characterized.
- Partner 4 (UEDIN) identified two kinase inhibitors from LOPAC library that affect melanocyte development. One compound affects melanocyte development (total number) and another kinase inhibitor affects melanocyte migration.
- Partner 4 (UEDIN) has also characterized a specific compound AG1276 that alters cell movement phenotypes.
- Partner 4 (UEDIN) also studied effects of Roscovitine as a CDK2 inhibitor, and the "master melanocyte regulator". MITF induces expression of CDK2 in melanoma. This is an important result as it implies that there is a tissue specific function of CDK2 in melanocyte development.
- Partner 4 (UEDIN) has done kinase profiling (Dundee) to examine the target pathway for roscovitine and AG1276, and find that they selectively inhibit CDK2 and KIT.
- The results obtained in screens with the BioFocus library for changes in melanocyte development and embryo morphology (Partner 4, UEDIN) were still difficult to interpret as many of the phenotypes were rather pleiotropic. The screen will have to be repeated with lower concentrations of the compounds and different incubations times of the embryos.
- Partners 5 (CMRB) and 8 (Biobide) have identified 6 compounds from the SoftFocus library by the automated screening platform (Partner 8, Biobide) and kinase profiled them to determine the targeted kinases. Two compounds were shown to inhibit Phosphorylase kinase subunit G1 (PhKG1), Proto-oncogene serine/threonine kinase-1 (PIM1) and Transforming tyrosine kinase A (TrKA). These targets were verified by morpholino knock-down in embryos, confirming that all of these kinases are important for angiogenesis in zebrafish during development (partner 5, CMRB). The kinase targets were further verified by inhibition of kinases TrKA and PIM1 by commercial inhibitors (partner 8, Biobide). Inhibition of PIM1 affects primarily the diameter of vessels whereas inhibition of either TrKA or PhKG1 leads to an inhibition of vessel growth. This work has been submitted (Camus et al.)
- Partner 7 (MPG) has begun to characterize the phenotypic classes obtained in their cell migration and tissue morphogenesis screen. The next steps are to kinase profile the most promising and interesting candidates belonging to the defective convergence and extension and arrested development classes and then functionally characterize the targeted kinases using standard gain-and loss-of-function assays in zebrafish. For gain-of-function, mRNA will be injected and for loss-of-function morpholino antisense oligonucleotides will be used.
- Partner 4 (UEDIN) has finished screening the LOPAC and kinase inhibitor libraries for modulators of melanocyte biology. We have identified compounds that alter melanocyte migration, differentiation and number. This work is written into a manuscript that will be submitted shortly (Colanesi et al.)
- Partner 4 (UEDIN) has identified the biochemical target of the nitrofurans that cause melanocyte specific cell death. We find that the compounds directly interact with ALDH2, and that inhibitors of this target completely rescue the melanocyte phenotype. This work has been submitted (Ishizaki et al.)
- Partner 4 (UEDIN) has identified a panel of compounds that alter melanocyte differentiation by interfering with copper metabolism. We use yeast genetics to identify the target pathways, and show that a common MEK inhibitor has an additional target *in vivo*. This work is published (Ishizaki et al., 2010)

Work package 5 : Application and exploitation of high-throughput fluorescent bioassays and computer based image analysis to study tumor progression

Lead participant : Biobide

Task 5.1: Market study, identification and selection of interested pharmaceuticals.

An extensive market study was developed at the beginning of the project to focus on specific Pharmaceutical Companies to offer this market-driven assay once had been finished and properly validated. During 2010 and

2011, Biobide has contacted with different Companies interested in this Angiogenesis Assay. With this objective, Biobide has attended to more than 10 different events in Europe and USA (e.g.: Biopartnering Europe, Bioeurope, Drug Discovery and Development) where this assay has been actively explained and

offered. Moreover, it has been presented in several oral presentations in different events (e.g. SOT 2011) and actively explained in the Headquarters of some of the Top 20 Pharmaceutical Companies.
Specifically, some of this Pharmaceutical and Biotech Companies specialise in the development of anti-cancer drugs have shown big interest. In fact, one assay has been performed by one of the Top 10 Pharmaceutical Company obtaining a satisfactory result.
Finally, different collaborative agreements have been signed with other CRO´s in order to establish a win-win collaboration that would allow increase of our portfolio in the angiogenesis field.

Task 5.2: Case study with the panel of genes and pharmaceuticals.
To validate the angiogenesis assay developed in Biobide´s HTS platform, 18 positive compounds were selected based in their capacity to inhibit known regulators of angiogenesis. On the other hand, 10 compounds previously tested in cellular (HUVECs) and xenopus screenings that did not have an inhibitory action against angiogenesis were used as negative items. The name and target of the compounds tested is shown in the next table:

COMPOUND NAME	TARGET
POSITIVES	
KRN633	VGFR-1-2 and 3
ZD6474 (Vandetanib)	VEGFR2 and EGFR
Sunitinib malate	VEGFR-1-2-3, PDGFRb, c-kit, FLT3, CSF1-R and RET
Sorafenib Tosylate	VEGFR-2-3, PDGFR, c-kit and Raf
PD173074	FGFR-1 and 3
PD166866	FGFR1
AG-1296	PDGFR α and ß and c-kit.
PDGFR tyrosine kinase inhibitor V	PDGFR α y ß
Tie2 Kinase inhibitor	Tie 2
Bosutinib	Abl and Src
AG1478	EGFR
Indirubin-3'-oxime	Cyclin-dependent kinases and GSK-3ß
Fumagillin	Methionine aminopeptidase-2
NS□398	Ciclooxigenase (COX-2)
HIF-1 Inhibitor	HIF-1
NVP-BEZ235	PI3K and mTor
2-Methoxyestradiol	(Metabolite of 17ß-estradiol)
Paclitaxel	Anti-microtubule agent
NEGATIVES	
Tyrphostin AG490	JAK-2
Bestatin	Aminopeptidase

Thioacetamide	(carcinogen)
E64	Cysteine protease
O6-benzylguanine	O6-alkylguanine-DNA alkyltransferase
Cyclosporine A	Calcineurine phosphatase
4-Methylpyrazole hydrochloride	Alcohol dehydrogenase
N-Acetyl-L-cysteine	(Antioxidant)
Amiodarone hydrochloride	Non-selective ion channel blocker
cis-Diammineplatinum(II) dichloride	(Platinum-based antineoplastic agent)

All these compounds were tested in Biobide´s platform following the procedure previously described in WP4 for the Galapagos library screening. A curve of six concentrations (0.01 µM, 0.1 µM, 1 µM, 10 µM, 30 µM and 100 µM) was performed for all the compounds. Only for the items that showed an anti-angiogenic effect, a second curve, with specific concentrations for each compound, was developed to calculate the IC50 for the two parameters (total number and complete number of vessels) used in the quantification of the anti-angiogenic effect.

In the next table a summary of the results that have been obtained, including the presence or absence of an anti-angiogenic effect, the IC50 values when possible or the minimum concentration that showed an effect when not, is shown.

COMPOUND	ANGIOGENESIS INHIBITION	IC50 (µM)		EFECTIVE CONCENTRATION (µM)
		TOTAL VESSELS	COMPLETE VESSELS	
KRN633	Yes	0.035	0.026	-
ZD6474 (Vandetanib)	Yes	-	-	100
Sunitinib malate	Yes	2.6	1.7	-
Sorafenib Tosylate	Yes	0.78	0.53	-
PD173074	Yes	-	-	100
PD166866	Yes	43.9	16	-
AG-1296	Yes	-	-	20
PDGFR tyr kin inhibitor V	Yes	0.19	0.14	-
Tie2 Kinase inhibitor	No	-	-	-
Bosutinib	Yes	-	-	50
AG1478	Yes	22.8	13	-
Indirubin-3`-oxime	Yes	18.3	4.2	-
Fumagillin	No	-	-	-
NS-398	Yes	-	-	30
HIF-1 Inhibitor	No	-	-	-
NVP-BEZ235	Yes	-	-	10
2-Methoxyestradiol	Yes	30.6	10.2	-
Paclitaxel	Yes	-	-	?
Tyrphostin AG490	No	-	-	-
Bestatin	No	-	-	-
Acetamide	No	-	-	-
E64	No	-	-	-
O6-benzylguanine	No	-	-	-
Cyclosporine A	No	-	-	-
4-Methylpyrazole hydrochloride	No	-	-	-

N-Acetyl-L-cysteine	No	-	-	-
Amiodarone hydrochloride	No	-	-	-
cis-Diammineplatinum(II) dichloride	No	-	-	-

15 of the 18 positive compounds were detected as angiogenesis inhibitors in our assay. None of the negative compounds tested showed any anti-angiogenesis effect. Therefore, the values of specificity and sensitivity are 83% and 100% respectively, what indicates that the assay developed allows visualizing anti-angiogenic compounds with high specificity and sensibility in an *in vivo* model.

Results obtained with Tie2 kinase inhibitor agree with previous reports in which a mutant line for Tie2 kinase did not show any defect in ISVs formation. So, even if it is important for vascular development in mammals and in some aspects of heart development in zebrafish, Tie 2 is not essential for ISVs development in zebrafish, at least at the time tested in our assay. In the case of Hif-1 inhibitor, even if Hif-1 factor is essential for angiogenesis induction in the presence of hypoxia (as in tumors) it has never been described its role in developmental angiogensis. Therefore, even if some pathways are not conserved between zebrafish and mammals or not all the regulators of pathologic or developmental angiogensis are the same, we were able to detect inhibitors of many of the main pathways that regulate angiogenesis.
To calculate the repeatability (coefficient of variation, CV), 3 different experiments at one dose close to the IC50 or the effective concentration, have been carried out for some of the positive compounds detected. The results obtained for the total number of vessels parameter are shown in the next table:

COMPOUND	CONCENTRATION TESTED (µM)	CV TOTAL VESSELS
KRN633	0.03	13.6
ZD6474	100	13.9
Sunitinib malate	2	2.2
PD173074	200	12.2
PD166866	30	12.4
AG-1296	20	2.4
Bosutinib	60	0.7
AG1478	20	11.2
Sorafenib Tosylate	1	3.3
Indirrubin-3`-oxime	10	19.3
NS-398	40	3.2
NVP-BEZ235	30	4.0
2-Methoxyestradiol	10	2.6

These results indicate that the assay developed is highly repeatable with CV values always fewer than 20%.
Finally, two clearly different phenotypes were detected for the positive anti-angiogenic compounds described before. In KRN633, ZD6474, Sunitinib, Sorafenib, PD166866, PDGFR tyrosine kinase inhibitor, AG1478, Indirrubin, 2-Methoxyestradiol and Paclitaxel treated embryos the formation of ISVs was perturbed with defective or total absence of sprouting and altered patterns of development. The higher doses tested the more intense phenotype found, with low numbers or absence of vessels and vessels stalled at the boundary between the notochord and the neural tube. However, compounds as PD173074, AG-1296, NS-398, Bosutinib and NVP-BEZ235 showed a moderate effect that did not increase with higher doses, with almost not decrease in the total number of ISVs present but with the presence of thinner vessels (some of them uncompleted) that do not always form the DLAV. While the first group of compounds is probably inhibiting initial essential steps in angiogenesis, the second group is probably more related with targets that participate in the proper maturation of the ISVs vessels or DLAV formation.

Some pictures representative of these different phenotypes are shown next (**Fig. 14**).

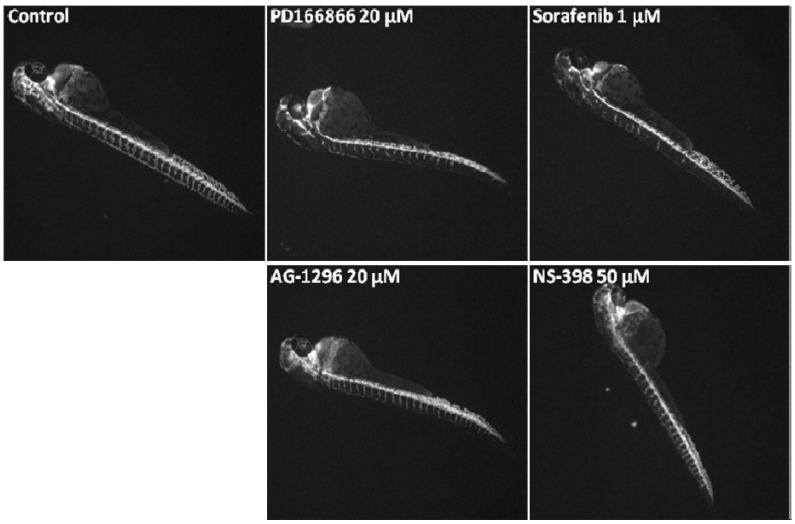


Fig. 14 Examples of different effects induced by different compounds in ISVs development.

While with PD166866 and Sorafenib many of the ISVs are not even developed or grew only halfway, AG-1296 and NS-398 inhibit proper development of the DLAV and the ISVs looks thinner.
The moderate effect of the compounds described has been further demonstrated studding their effects in embryos of 72 hpf, when circulation is already present. PD173074, AG-1296, Bosutinib and NVP-BEZ235 treated embryos showed impaired blood circulation and presented thinner ISVs. No data of NS-398 is presented since this compound is lethal at 72 hpf. Therefore, different phenotypes associated with inhibition of different steps of angiogenesis regulation (sprouting and maturation) can be detected with this method.

Partner 3 (ZF-Screens) discovery that early (up to 1000-cell stage) intrayolk injection of zebrafish embryos with *Mycobacterium marinum* induces highly efficient granuloma formation, prompted us to focus at these early stage embryos for development of a prototype automated injector. The resulting home-built injector is based on a programmable controller linked to a motorized measuring stage and microplate holder, a programmable micromanipulator, a programmable injector, and an external compressor (**Fig. 15**). A custom Python script controls the coordinated activity of the individual components, whereas the injection process can be continuously monitored via a camera mounted underneath the microplate holder. Eppendorf provides custom injection needles with a specifically designed tip diameter and shape.

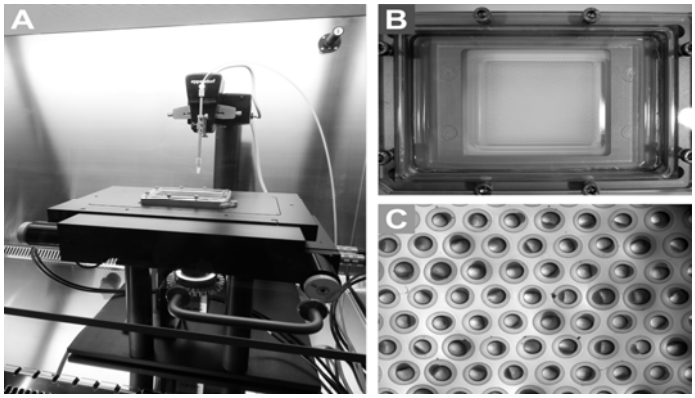


Fig. 15 (A) Home-built robotic device for high- throughput injection of cancer cells and microbes into zebrafish embryos, adapted for use in Downflow-Unit and at Biosafety Level 3.(B) The embryo holder showing the 1024-well agarose grid within the steel support. (C) Regular spacing of embryos on agarose grid.

In order to keep the embryos immobilized throughout the injection procedure, we designed a specific embryo grid mould to prepare embryo grids in agarose on a glass plate. Early stage embryos were transferred to the embryo grid that was attached to the microplate holder via a steel support (Fig. 2).

Extensive testing and optimization of the robotic zebrafish injector has ultimately resulted in a routine injection speed of ~1000 zebrafish embryos per 30 minutes with a success rate of more than 99%!

To test the performance of the automated setup in a proof-of-principle compound screen, we used a panel of drugs that were known to inhibit the proliferation of Mycobacterium marinum. CherryRed-labeled mycobacteria (~20 colony-forming units) were robotically injected into the yolk of 16-512-cell stage embryos. At three days post injection, the COPAS XL Biosorter was used to sort for larvae with equal bacterial load based on CherryRed fluorescence. Of this group of larvae, one half was exposed to a combinatorial drug treatment consisting of Rifampicin and Isoniazid (daily refreshment) and the other half was left untreated. After 2-3 days of drug treatment, the COPAS XL was used again to determine the bacterial load. After 2 days, the bacterial load in the drug-treated group was more than 60% reduced compared with the untreated group, and after 3 days the bacterial load was almost 80% reduced (**Fig. 16**). In conclusion, the combinatorial drug treatment of the robotically injected larvae resulted in successful suppression of Mycobacterium proliferation and granuloma formation.

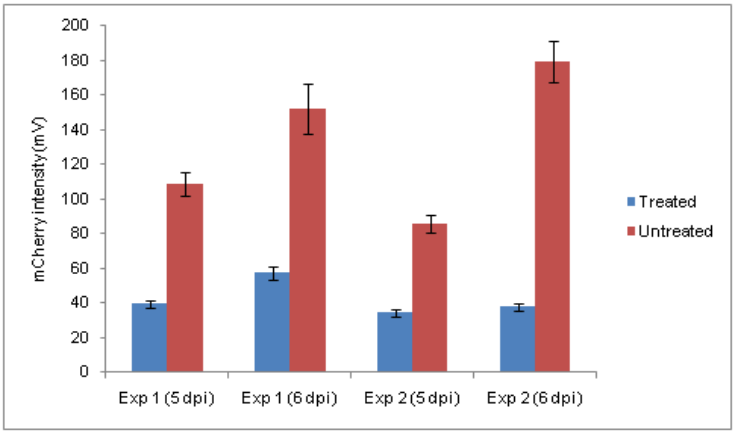


Fig. 16 The effect of combinatorial drug treatment (Rifampicin and Isoniazid) on the amount of Mycobacterium marinum in 5-6 day old larvae after robotic intrayolk injection at the 16-512-cell stage.

Next we tested how the automated setup would perform in an anti-tumor drug screen. Several human tumor cell lines were tested for behaviour in zebrafish larvae after early intrayolk injection, including melanoma, prostate carcinoma and osteosarcoma cells. Since CM-DiI-labeled SJSA-1 human osteosarcoma cells showed reproducible proliferation and migration behaviour inside the larval body (**Fig. 17**), these cells were chosen for this first anti-tumor drug test screen.

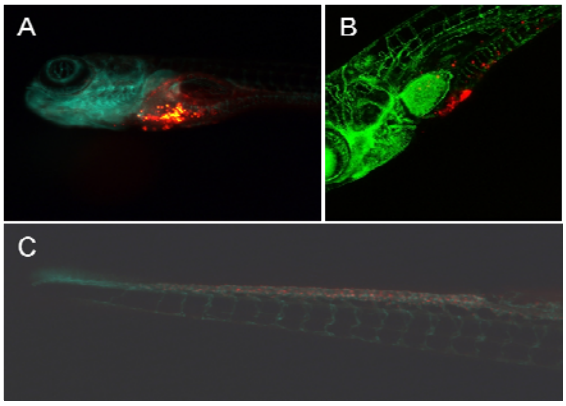


Fig. 17 Migration of CM-DiI-labeled SJSA-1 human osteosarcoma cells from the yolk injection site (A, B) into the tail region (C).

Fli-GFP-labeled embryos (n = 1024) were injected at the 256-cell stage with CM-DiI-labeled SJSA osteosarcoma cells. The success rate was almost 100% (only one failed injection) and the total injection time was 30 minutes. At three days post injection, 650 healthy larvae were sorted using the COPAS XL Biosorter, resulting in a group of 200 larvae with equal tumor cell loads. Of this group, one quarter was left untreated whereas the other three quarters were exposed to one of the following anti-tumor drugs: cisplatin, methotrexate or doxorubicin. Migration of tumor cells to body and head of embryos was clearly visible at 4 days after injection, and angiogenesis was observed around the tumor mass. Despite the absence of vasculature at the time of tumor cell injection, circulating tumor cells were detectable at 4 days post injection, suggesting that the circulating cells infiltrated the blood vessels. COPAS XL-mediated analysis of the tumor cell load at day 5 post injection did not reveal significant differences in CM-DiI fluorescence level between the untreated and treated larvae.

The protocol for cell injection has been further improved. (1) tumor cells are now trypsinized and replated one day before injection to reduce the intercellular aggregation; (2) immediately prior to injection, the trypsinized tumor cells are run through a 50-micron filter to remove cell clumps and ensure a single-cell suspension; (3) the PVP carrier is run through a 0.45-micron filter to remove debris.
In addition to Cm-DiI-labelled SJSA cells, we now also used stably DsRed-transfected MV3 cells for robotic injection rounds (**Fig. 18**). Robotic injection of 1024 embryos at the 256-cell stage with MV3-DsRed cells suspended in 4% PVP resulted in > 99% successful clump-free injections. Post-injection embryo maintenance was done manually (removal of debris, refreshment of water), and at 3 dpf correctly injected embryos were sorted to appropriate microplates using the COPAS XL. Compound treatment started at 3 dpf and lasted until 6 dpf. Tumor load was assessed using the COPAS XL. Imaging-friendly embryo holders for medium-throughput imaging via the BD-Pathway are currently being tested.

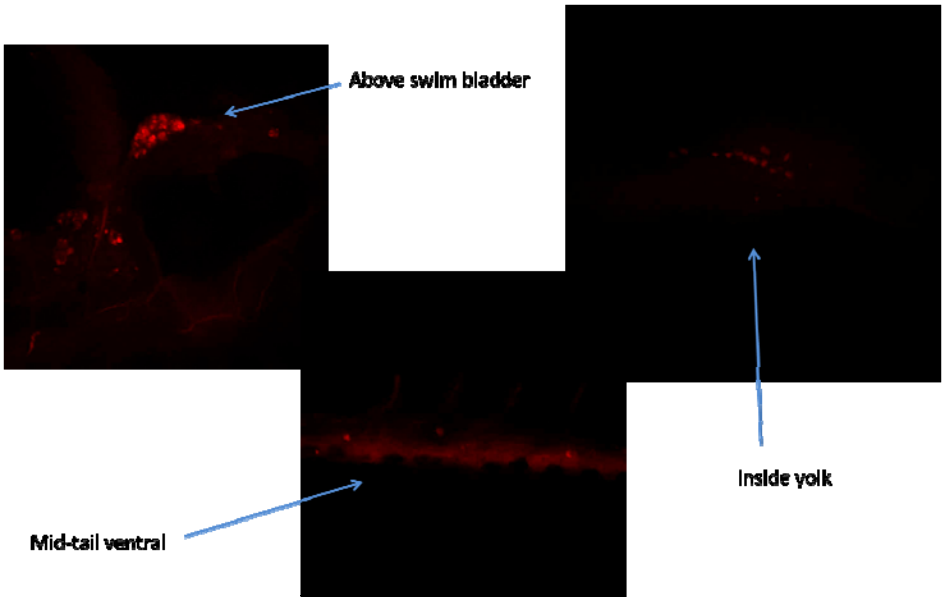


Fig. 18 Migration of stably DsRed-transfected MV3 human melanoma cells from the yolk injection site into the tail region and other parts of the larva.

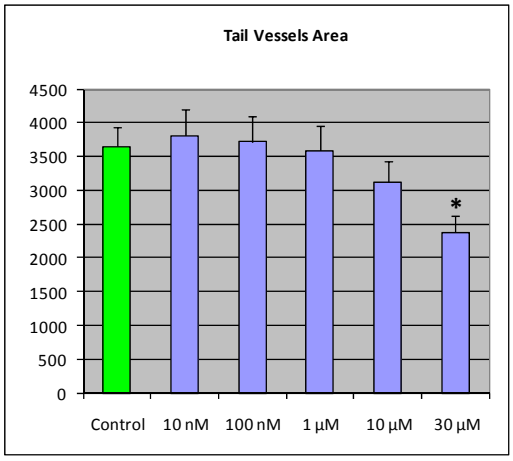
Task 5.3. Screening of pilot compounds for customer validation.
Partner 8 (Biobide) have contacted with **FAES FARMA**, a Spanish Pharmaceuticals company, dealing mainly with the manufacture and marketing of pharmaceutical products (prescription drugs, over-the-counter medications and skin-care treatments) and raw-materials for pharmaceutical use. A pilot screening with 7 compounds for the angiogenesis assay has been performed in our platform. 2 of these compounds showed

anti-angiogenic activity. Information about these products and their angiogenic activity in HUVEC tube formation assays has been obtained to compare and validate our assay.

1. Test item 1

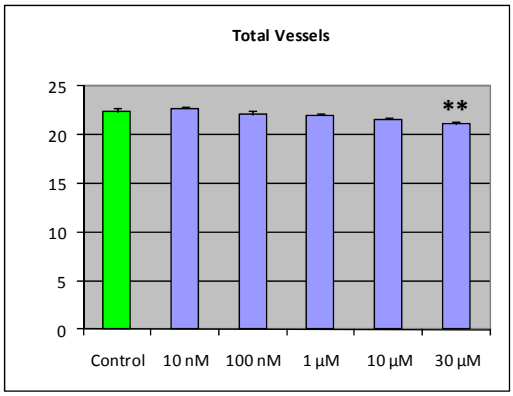
Automatic analysis

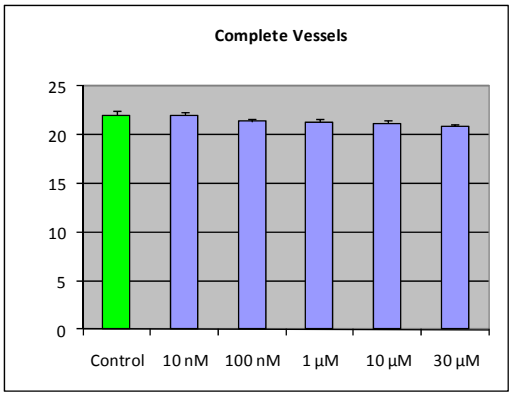
			Test item 1				
		Control	10 nM	100 nM	1 µM	10 µM	30 µM
	Number of cases	10	7	8	10	5	9
Tail Vessels Area	Mean	3649,10	3805,43	3717,00	3589,40	3122,40	2379,33
	SD	904,41	1039,90	1047,50	1133,23	710,93	756,05
	SEM	286,00	393,05	370,35	358,36	317,94	252,02
	p Value	-	1,00	1,00	1,00	0,79	0,03



Manual analysis

			Test item 1				
		Control	10 nM	100 nM	1 µM	10 µM	30 µM
	Number of cases	10	8	8	10	5	9
Total Vessels	Mean	22,40	22,75	22,13	22,00	21,60	21,11
	SD	1,07	0,46	1,13	0,82	0,55	0,60
	SEM	0,34	0,16	0,40	0,26	0,24	0,20
	p Value	-	0,86	0,94	0,75	0,31	<0.01
Complete Vessels	Mean	22,00	22,00	21,38	21,30	21,20	20,89
	SD	1,56	0,93	0,92	0,95	0,84	0,78
	SEM	0,49	0,33	0,32	0,30	0,37	0,26
	p Value	-	1,00	0,63	0,47	0,53	0,11





The anti-angiogenic effect detected with test item 1 was very mild. No important decrease in the number of total or complete ISVs was detected. However there was a decrease in the total fluorescence intensity measured at 30 µM that could be a consequence of defects in DLAV development as is shown in figure 19.

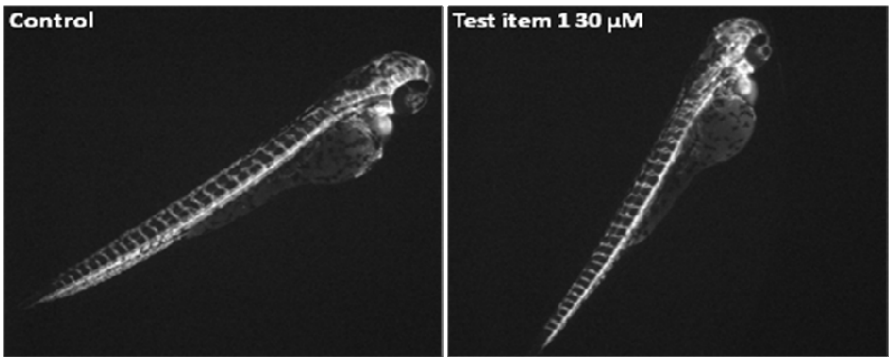


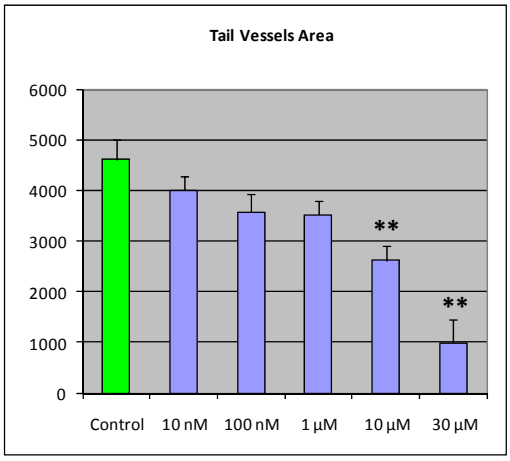
Fig. 19 Representative effect of test item 1 in ISVs and DLAV development.

Higher doses of the test item 1 were toxic and induced 100% of mortality.

2. Test item 2

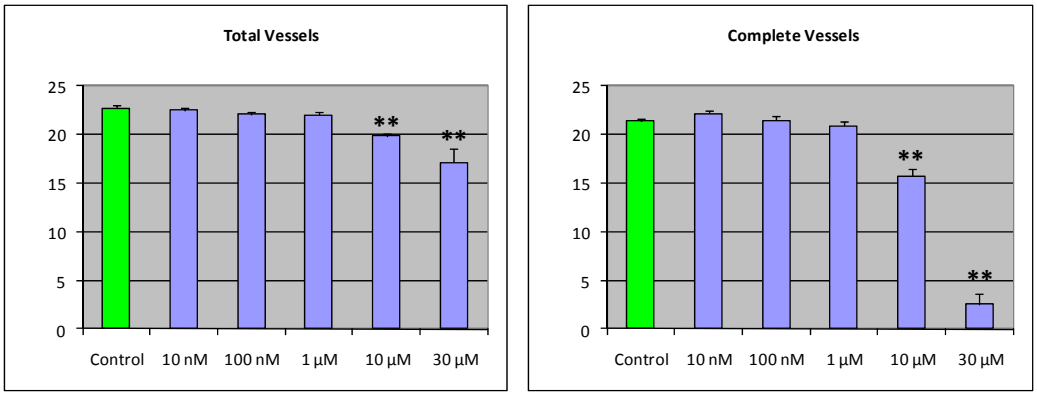
Automatic analysis

		PE-0317 Test item 2					
		Control	10 nM	100 nM	1 µM	10 µM	30 µM
Tail Vessels Area	Number of cases	8	10	10	10	8	7
	Mean	4611,50	4002,00	3572,10	3515,40	2621,00	982,29
	SD	1133,04	831,11	1164,34	841,34	789,69	1271,55
	SEM	400,59	262,82	368,20	266,05	279,20	480,60
	p Value	-	0,57	0,13	0,10	<0.01	<0.01



Manual analysis

			Test item 2				
		Control	10 nM	100 nM	1 µM	10 µM	30 µM
	Number of cases	8	10	10	10	8	7
Total Vessels	Mean	22,75	22,50	22,10	22,00	19,88	17,14
	SD	0,89	0,71	0,88	0,94	0,83	3,80
	SEM	0,31	0,22	0,28	0,30	0,30	1,44
	p Value	-	1,00	0,85	0,76	<0.01	<0.01
Complete Vessels	Mean	21,38	22,20	21,50	20,90	15,75	2,57
	SD	0,92	0,79	1,18	1,29	2,05	2,76
	SEM	0,32	0,25	0,37	0,41	0,73	1,04
	p Value	-	0,69	1,00	0,95	<0.01	<0.01



Test item 2 showed a clear antiangiogenic response mainly detected as a decrease in the number of ISVs that could complete their development and appeared stalled at the boundary between the notochord and the neural tube (**Fig. 20**).

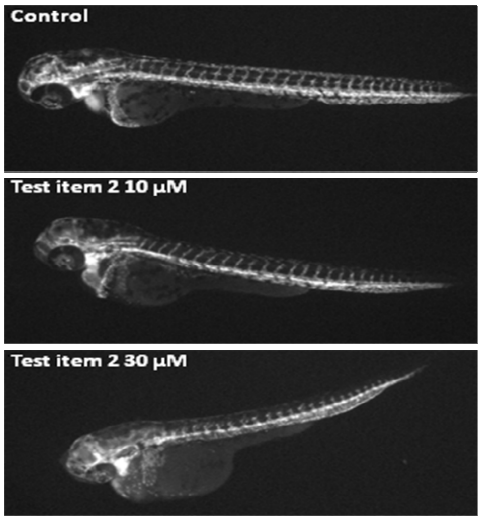


Fig. 20 Embryos representative of the anti-angiogenic effect of test item 2 at 10 and 30 µM

The information available for the compounds tested in this screening that FAES shared with us for the validation of the assay is:

- The seven compounds screened were tested previously for citotoxicity in HUVEC and HAEC (human aortic endothelial cells) cells.
- Only the compounds that showed specific citotoxicity for HUVEC cells (5 of the 7) were later tested in the tube/capillary formation in HUVEC cells angiogenesis assay.
- Four of the five compounds tested in HUVEC tube formation assay did not inhibit angiogenesis. These 4 compounds were detected as negative in our automatic assay.
- One of the five compounds tested in HUVEC tube formation assay inhibited moderately angiogenesis. This is the test item 1 we have described in this section that also lightly inhibited angiogenesis in the assay we have developed.
- Unfortunately, no data of HUVEC angiogenesis assay was available for the other two compounds (included test item 2) tested in our system. However, test item 2, found as positive in our screening, it is an inhibitor of ciclo-oxigenase (COX2). Another inhibitor of the same enzyme (NS-398) was detected as positive in the validation of our assay described in section 5.2.

So, even if not all the results could be compared, for the compounds tested in angiogenesis assays in HUVEC cells and in our system, the same results were obtained.

Task 5.4: Bioinformatics analysis: study of an automatic classification system

Although Biobide have developed an automated HTS platform for angiogenesis assay that includes a software that automatically classified compounds as positive or negative we have not develop a classification system for the phenotypes found. The phenotype detected automatically is the presence of anti-angiogenic effect based in the statistical analysis performed and showed in the excel result sheet (for details see WP4 report). The software of the COPAS XL Biosorter was optimized to allow determination of bacterial or tumour cell load after intrayolk injection and drug treatment of zebrafish embryos/larvae (partner 3).

Task 5.5: Develop a GLP protocol for drug test in zebrafish

Automated angiogenesis assay developed is under the scope of Biobide's GLP since it has been validated and SOP (standard operating procedures) defined for all the steps.

Good Laboratory Practice was implemented in ZF-screens by (1) designing Standard Operating Protocols, (2) planning periodic equipment checkups, (3) maintaining professional laboratory notebooks, (4) obtaining environmental licenses, (5) appointing a dedicated Biological Safety Officer (6) planning a personnel backup program.

Results achieved:

- The market study and selection of interested pharmaceuticals have been completed by partner 8 (Biobide) and partner 3 (ZF-Screens).
- Potential customers were successfully contacted and the list of the biotech companies that are working in cancer related clinical trials has been elaborated.
- Partner 8 (Biobide) has set up a high-throughput screening platform and developed an automatic anti-angiogenesis screening and image analysis (see WP4).
- Partner 8 (Biobide) generated software for the presence or absence of the anti-angiogenic effect.
- Partner 3 (ZF-Screens) with other UL partners established data storage and data sharing of bio-images, which was implemented for ZF-CANCER consortium.
- HTS protocols were successfully validated using set of reference compounds. In order to validate the angiogensis assay developed in the HTS Biobide platform Biobide a panel of 28 different compounds has been selected and tested. The obtained results indicate that Biobide (partner 8) has developed a reliable and robust assay for the detection of compounds with capacity of inhibiting angiogenesis.
- Screening of pilot compounds for customer validation has been performed. A pilot screening with compounds coming from the Spanish Pharmaceuticals company FAES FARMA has been completed in Biobide platform.
- Partner 3 (ZF-Screens) has developed techniques toward automated high throughput injection of zebrafish larvae with tumor cells. To test the performance of the automated setup in a proof-of-principle compound screen, partner 3 (ZF-Screens) used a panel of drugs that are known to inhibit the proliferation of Mycobacterium marinum or anti-tumor drugs to inhibit tumor cells migration or survival. A COPAS XL Biosorter was used for the selection and quantification of specific phenotypes in injected embryos.
- Proof-of-principle experiments were performed in which early zebrafish embryos were robotically injected with fluorescently-labelled osteosarcoma or melanoma cells, followed by anti-tumour drug treatment and COPAS XL or CLSM endpoint measurement.
- A custom combination of the robotic zebrafish embryo injection platform and the COPAS XL Biosorter was presented to GlaxoSmithkline (GSK) as a high-throughput in vivo screening system for anti-microbial and anti-tumour compounds. This has resulted in a contract research agreement with GSK toward validation of a set of anti-Mycobacterial compounds in the zebrafish larva platform. Further contracts toward anti-tumor screens are expected (partner 3, ZF-Screens).
- COPAS XL Biosorter software was optimized for end-point measurements by Zf-Screens.
- The GLP protocols were generated. Partner 8 (Biobide) developed automated angiogenesis assay. Good Laboratory Practice was implemented in partner 3 (ZF-screens).

1.4 The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results (not exceeding 10 pages)

The potential impact

The ZF-CANCER project made a contribution on reinforcing European competitiveness by generating strategic knowledge in a multidisciplinary cancer approaches. The ZF-CANCER objectives were focussed on the incorporation of the zebrafish embryo model into the preclinical drug screening pipelines. The use of mice for *in vivo* monitoring of disease processes such as tumorigenesis and metastasis is limited by costs and throughput level. Introducing a high-throughput zebrafish embryo model will accelerate lead-time of anti-tumor drugs considerably. Even a decrease of a few months will increase the potential revenues of a drug significantly. Furthermore, given that cancer is among the most life-threatening diseases of the century, any new strategies potentially accelerating anti-tumor drug discovery time hold prospects to improve quality of life. The robotic setups generated in ZF-CANCER project allow the incorporation of the zebrafish embryo model into the preclinical drug screening pipelines. The project's outcome will potentially contributes to cost-effective and more efficient methods in the anti-tumor drug discovery process. Acceleration of drug lead time benefits

economy as well as quality of life of cancer patients. In general this investment in the knowledge-based economy and health care will have a positive impact on socio-economic issues. The European SMEs and company involved were co-investing in the ZF-CANCER project and therefore had beneficial effects on employment and training of new personnel. The SMEs and company accelerated the technology transfer and exploitation of results.

Final results and their potential impact and use

- ZF-CANCER developed an innovative screening system based on the implantation of fluorescent tumor cells into zebrafish embryos. This system has the major advantage and makes possible to visualize tumor growth and metastasis in an optically transparent vertebrate model organism. In combination with high-throughput sh-RNA and chemical libraries screens, the system provides a useful tool for drug discovery screens as well as for fundamental research questions (**societal, scientific and economic impact**).
- ZF-CANCER project developed an integrated multi-parameter fluorescence-based bio-imaging platform for the qualitative and quantitative evaluation of human and zebrafish cancer progression (**societal, scientific and economic impact**).
- An image data base for automated data acquisition, analysis and excess are developed and will be beneficial for IT area in medical imaging (**translational impact**).
- ZF-CANCER generated a l**iving library of zebrafish with cancers (12 tumor models)** that will allow better understanding of cancer progression and development of new intervention strategy (**societal, scientific and economic impact**).
- For discovery of novel gene targets and compounds the ZF-CANCER combined the power of genomic tools (RNA interference) and chemical libraries analysis with the advantage of *in vivo* monitoring of tumor progression in a transparent vertebrate model organism.
- It has the potential for high-throughput application due to the small size of zebrafish embryos, the high numbers with which embryos can be obtained and the choice of high-throughput molecular screening tools developed within the project (**societal, scientific and economic impact**).
- The tumor implantation system has been used to generate new knowledge on factors important for tumor growth and metastasis (**societal, scientific impact**).
- ZF-CANCER identified a set of human target genes involved in cancer growth, angiogenesis and metastasis (**societal, scientific, translational and economic impact**).
- ZF-CANCER provided the proof-of-principle for the developed drug discovery screening system for high-throughput application in the anti-tumor drug screening system and with broad usefulness for biomedical research (**scientific, translational and economic impact**).
- New high-throughput methodologies developed in ZF-CANCER project enables application of zebrafish model into biomedical screenings in preclinical anticancer drug screening pipelines *and* thereby ensures European competitiveness in the area of drug discovery (**societal, scientific, translational and economic impact**).

Added value in carrying out the work at a European level

The ZF-CANCER project required a multidisciplinary team of researchers with unique expertise in the field of: human cancer cells and tumor progression (partner 1); zebrafish tumor models (partner 4-6); xeno- and allogenic transplantations (partner 1 (UL), 3 (ZF-Screens)); high-content multi colour intravital imaging (partner 1 (UL), 5 (CMRB), 7 (MPG)); gene expression profiling; imaging database and bioinformatics (partner 3 (ZF-Screens), 4 (UEDIN), 6 (KNAW)); RNA interference for targets and compounds discovery (partner 2); screening for phenotypes affecting cell survival, cell migration, cell proliferation, tissue morphogenesis during early embryogenesis and angiogenesis (partner 3-8) as well as people who are willing to commercialise the tools developed in the project (partner 2 (Galapagos), 3 (ZF-Screens), 8 (Biobide)). ZF-CANCER brought together 8 partners from 5 EU member states. These partners combined forces to direct their expertises to development and validation of novel biomedical screening bioassays. With this multidisciplinary team created the critical mass that was essential to fulfil the project's objectives, as could not be achieved by any single member of the consortium. The scientifical and technological innovation of ZF-CANCER will strengthen the position of the European research community in a highly strategic and economically exciting domain.

Enhancing competitiveness of the European biotechnology sector

The research investments generated with the ZF-CANCER project are expected to be returned by stimulating industrial competitiveness. The project results will directly support the innovation of one company and two SMEs in Europe. Success of these 3 companies will strengthen the competitiveness of the European biotechnology sector. These companies exploited the model by offering it as a pipeline drug screening tool and developed new services within the scope of this project. Particularly in the USA, zebrafish models are now starting to be effectively incorporated into the pipelines for drug discovery. Therefore, it is particularly important that timely and competitive initiatives in Europe are developed.

The main dissemination activities

The Plan for using and disseminating knowledge will be maintained through the lifetime of the project. Updates of the initial plan set out here and a description of the actual achievements will be included in the formal project reports. A **Final Plan for using and disseminating knowledge** is included in the list of project deliverables. This Final Plan will describe the participants' actual achievements in dissemination and their plans at that time for the exploitation of their results - for the consortium as a whole, or for individual participants or groups of participants. It will where appropriate refer back to the interim versions of the Plan for using and disseminating knowledge, indicating how the foreseen activities actually took place, or were modified in the light of circumstances, or where indeed other actions and measures, initially unplanned, were introduced.[1] It will be expressed as much as possible in concrete terms, for example the dissemination strategies, the target groups and the strategic impact of the project in terms of improvement of competitiveness or creation of market opportunities for the participants.
The Plan for using and disseminating knowledge was maintained through the lifetime of the project and is included in the list of project deliverables. This Final dissemination activity described the participants' achievements in dissemination and their plans at that time for the exploitation of their results - for the consortium as a whole, or for individual participants or groups of participants.
Dissemination of results was approved in advance by the Steering Committee to prevent commercially important knowledge being unprotected. Articles or abstracts for conferences were submitted 30 days in advance to the Steering Committee by email. Each WP Coordinator was responsible for the dissemination of knowledge resulting from his WP. The Scientific Coordinator monitored the frequency of the publication of results.
After appropriate IPR protection measures had been taken, **dissemination of the knowledge** generated by ZF-CANCER was effectuated in the following ways:

- **Project flyer:** pdfs from project website were distributed in printed form at congresses.
- **Instalment of a ZF-CANCER website** (see below) with a public domain to provide background information about the project and its objectives, advertise its achievements, and to enhance dissemination of non-commercially sensitive material.
- **Deposition of data into public databases.** After IP protection has been established all data from gene expression profiling analyses was submitted to the EBI databases. We deposited nucleotide sequence information into public databases such as EMBL Bank and GenBank. Information regarding gene expression patterns, mutant phenotype analyses, and morpholino sequences and associated phenotypes was deposited into the Zebrafish Information Network (ZFIN).
- **Presentations at European and other international conferences.** Various lectures presenting results of the project and acknowledging the EU support were given by different project partners at international congresses, national meetings, other research institutes, and meetings of other EU projects. The target audience of most lectures was the research communities, but as indicated in the table lectures aimed at the general public were also given. Results were presented at the annual international zebrafish meetings and at meetings dedicated to advances in cancer research. The coordinator of this project was a co-organizer of the first and second zebrafish meeting

on cancer and infectious disease (sponsored by EMBO) This workshop brought together leading scientists and young investigators that have adopted the zebrafish model for study of molecular mechanisms of disease processes. With a focus on two research areas in which the immune system plays a major role, infectious disease and cancer, this workshop was directly relevant to the research area of the ZF-CANCER project. Partners 1 (UL), 3 (ZF-Screens), 4 (UEDIN) and 6 (KNAW) presented their results and acknowledge the Commission's financial contribution with reference to the contract number **HEALTH-F2-2008-201439.** A follow-up meeting is already scheduled to take place in 2011 after the European Zebrafish Meeting in Edinburgh.

- **Presentations at schools and at open days for the general public** organized at different partners' institutes.
  Partner 1 (UL), 3 (ZF-Screens) and 6 (KNAW) have participated in the 'Open Science Days', organized by the Science Faculty of Leiden University and Hubrecht Laboratory, which are aimed at children in the age of 8-18 and their parents. These Open Days are organized in the context of the Dutch Science Week/Month and usually attracts as much as 3000 visitors.
  A microscopy demonstration showing the transparency of zebrafish embryos always attracts much interest. Similar demonstrations of zebrafish biology are given at Study Festivals and Open Days for High school pupils organized by the University to recruit new students.
- **Contact with Pharmaceutical Companies interested in application of developed platforms.**
  The involved companies have many connections to large pharma companies and presented data to these companies. Since it was the intentions to market new inventions developed in this project via large pharma partners such presentations had great importance. We used our web sites to advertise new results. With the consent of all members involved, ensuring the confidentiality of all the information exposed.
  Partner 2 (Galapagos), 3 (ZF-Screens) and 8 (Biobide) contacted companies interested in application of developed platforms where this assay has been actively explained and offered.
- **Services offered based on technology developed in the project**.
- Availability of **project tools to academia and non-profit instructions** for internal research purposes.
- **Licensing of patented data, tools and technologies**. Patents generated by the ZF-CANCER consortium will be offered to interested industrial parties for commercial exploitation in areas not covered by the SME partners.
- **Participation of members of the consortium in other (inter)national consortia (e.g. national genomics programs and other consortia.**) In particular, the project established a link with the ZF-MODELS IP and ZF-TOOLS. Since two of the participating institutions in ZF-CANCER (UL and ZF-sceens) are also members of these projects, exchange of knowledge was realized easily. Partner 3 (ZF-Screens) was a partner in the EU project ZF-TOOLS. The technology derived from this project includes SAGE (serial analysis of gene expression) and MLPA (multiplex ligation dependent probe amplification) and these tools were used in this project for identification of cancer marker genes and rapid high-throughput screening of cancer marker genes, respectively. The Leiden partner (1) was also involved in the EU network ZF-TOOLS and participates in a large Netherlands genomic network called the Centre for Medical Systems Biology that offers many state of the art genomic facilities that were used in this project. Partner 3 (ZF-Screens) is a member of a network of companies and institutes based in the Netherlands that recently was awarded a subsidy (called Smartmix) by the Dutch government for establishing zebrafish technology using bioinformatics tools.
  Partner 5 (CMRB) is a member of the Spanish Cell Therapy Network, a cooperative association of basic and clinical research centers the goal of which is to promote knowledge exchange between members and the society at large in order to accelerate the research in the use of cell-based therapies. This national network is funded by the Spanish Ministry of Health. In addition, the partner 5 (CMRB) is the scientific coordinator of the European Union Human Embrionic Stem Cell registry (LSSG-CT-2007-037820), aimed to develop a comprehensive, complete, quality controlled and informative repository of available European hESCs, accessible to the wider scientific community.

Partner 7 (MPG) is a part of the EUFP5 ZF-MODELS and EU-FP6 ENDOTRACK consortia.

- **Transfer of knowledge within the project.**
  As the research plan required extensive collaborations between laboratories to achieve different tasks, visits of scientists to other partner's laboratories regularly occurred. This mobility of researchers benefited their personal education, but also stimulated scientific discussion and coherence in the project and added to the spread of know-how and technical expertise among different EU countries.
- **Education.**

  The academic partners (1 (UL), 4 (UEDIN), 5, 6 and 7) teach molecular and cellular biology and genetics courses (lectures and practical) to undergraduate and graduate students (biology, life science and technology, and medicine). Partner 1 also offers guest lessons to high schools and is involved in a course on ethics and technical aspects of animal experimentation, educating students on the use of the zebrafish as a model for human development and disease.
  In addition, during the course of the project several BSc and MSc students of Biology have performed their research training periods in the laboratories of the academic partners under supervision of scientists involved in the ZF-CANCER project. Furthermore, PhD student (He) has successfully defended her theses based on work from the ZF-Cancer project. Second PhD student (Ghotra) will graduate in 2011.
- **Publication of a brief project results accessible to non-specialists.**
- **Publications in high-ranked peer-reviewed scientific journals** in the areas of signalling pathways involved in cancer formation and progression. All publications acknowledged the financial contribution by the Commission.
  Publications that have been published, submitted or that are currently in preparation are listed below. Copies of published and accepted manuscripts are included under Deliverable D 6.9. Copies of publications, posters, newspaper articles and flyer are added as deliverable (D6.9).
  Further details are listed here below.

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The exploitation of results

New high-throughput methodologies developed in ZF-CANCER project enables application of zebrafish model into biomedical screenings in preclinical anticancer drug screening pipelines.
The ZF-CANCER project generated novel platforms for biomedical research and pharmaceutical screenings as well as in technical expertise that will be valuable to a broad range of research topics. These tools and technology will be commercially valuable and will result in the development of intellectual property rights for several partners who will provide a clear route to commercialisation. The involvement of one company and two researches intensive SMEs will ensure that this knowledge is exploited and commercialized. In particular, the company and two SMEs foresee exploitation in the following areas:

Partner 2, Galapagos: The results of this program will have the following impact on the business of Galapagos. Galapagos has extensive experience in functional genomics-based target discovery in, amongst others, the fields of inflammation, bone and joint diseases, diseases of the central nervous system, and cancer. The development of novel target and drug discovery assays in the field of cancer and the incorporation of Galapagos' functional genomics platform in these assays will further expand the expertise of Galapagos in the field of oncology. In addition, the know-how generated in these programs will be used to complement the Chematica knowledge databases of BioFocus DPI. Together, these tools, technologies and know how will be fully incorporated in Galapagos' service arm BioFocus DPI and will contribute to the expansion of the service portfolio of BioFocus DPI that it can offer to the pharmaceutical industry and non-profit patient foundations. Galapagos has presented the expertise developed in the project (*in vitro* cancer models and the zebrafish tumor metastastis model) to the oncology divisions of various large pharmaceutical companies (e.g. Servier, Janssen Pharmaceutica, Human Genome Sciences, etc. It was proposed to use these platforms to identify and/or validate novel targets and/or compounds in collaboration with these Pharma companies. There appeared to be genuine interest but so far no exploitation has come out of it. Galapagos will continue to present the platforms to such Pharma companies.

Partner 3, ZF-Screens: ZF-screens aims to exploit zebrafish tools for anti-tumor drug discovery screens. The current world market for cancer therapeutics is around 20 billion US dollars and this market is still growing due to the increase in population, aging of the population and increased life expectancy of patients undergoing cancer treatment. It is obvious that given the size of the potential market, even a minimal market share in the pipeline for anti-tumor drug screening can be lucrative. The major competing strategy in the area of test animals for cancer screens is the use of rodents, especially for the study of cancer types that are specific for mammalian organisms. Mouse test systems are used frequently in FDA-approved first stage screening strategies. However, major disadvantages of the use of rodents are the high cost per test animal, the ethical constraints for the use of rodents, and the fact that cancer progression, especially metastasis is extremely difficult to monitor at the single cell level. In these three respects the use of zebrafish embryos offers clear advantages. It is therefore envisaged that there is a great potential market for pre-screening therapeutics in zebrafish embryo systems. The outcome of zebrafish embryo-based screens, integrated at an early phase in the drug discovery pipeline, can increase efficiency of further tests in a rodent system. In the U.S.A. there are several companies that are also aiming at this strategy. However, in Europe there are no commercial activities in this field that we are aware of. ZF-screens is in a good position to exploit the zebrafish screening model, because it obtains an exclusive license to a Leiden University patent (application no. 05075490.2 and patent WO/2011/005094), which claims some key elements of the system: methods for zebrafish cell implantations and high throughput method for in vivo screening. ZF-screens intends to strengthen its IP portfolio and expects that the project results will strongly contribute to establishment of the

validity of the zebrafish model in screening of anti-tumor drugs. A custom combination of the robotic zebrafish embryo injection platform and the COPAS XL Biosorter was presented to GlaxoSmithkline (GSK) as a high-throughput *in vivo* screening system for anti-microbial and anti-tumour compounds. This has resulted in a contract research agreement with GSK toward validation of a set of anti-Mycobacterial compounds in the zebrafish larva platform. Further contracts toward anti-tumor screens are expected.

Partner 8, Biobide: The main role of Biobide in the project was the development of Robotic Process of screening from the sorting of embryos to the image acquisition with semi-automatic image analysis.
Currently, the company serves customers from national and international companies and university research departments. Furthermore, Biobide also expects a growing number of customers, since the value of the automated high-throughput screening has been recognized by many research groups, who are often not able

to make the investments to establish this method in their own laboratories. Therefore, Biobide intends to exploit the experience gained from the ZF-CANCER project to add custom services to their portfolio. In addition, Biobide also wants to expand service activities in the direction of cancer and age related diseases, for which it will benefit from the experience gained from the collaboration with expert university research groups in this project. Biobide have contacted with FAES FARMA, a Spanish Pharmaceuticals company, dealing mainly with the manufacture and marketing of pharmaceutical products (prescription drugs, over-the-counter medications and skin-care treatments) and raw-materials for pharmaceutical use. A pilot screening with 7 compounds for the angiogenesis assay has been performed in Biobide platform. Exploitable angiogenesis assay will be applicable in pharmaceutical sector.

1.5 The address of the project public website, if applicable as well as relevant contact details

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SEVENTH FRAMEWORK
PROGRAMME


FP7: ZF-CANCER