Contract no.: LSHC-CT-2006-037297

Project acronym: MCSCs

Migrating cancer stem cells in breast and colon cancer

Instrument: Specific Targeted Research or Innovation Project (STREP)

Thematic Priority: Sixth Framework Programme Priority 1
(Exploring the patient's cancer stem cell as a novel therapeutic target)

Final Activity Report for MCSCs

Period covered:
from 1 November 2006 to 30 April 2010

Date of preparation:
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Duration:
42 months

Project coordinator name: Prof. Dr. R. Fodde
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II. INTRODUCTION TO MCSCs

Cancer stem cells (CSC) have received much attention in the recent scientific literature as they are thought to maintain and propagate human malignancies and as such represent an attractive therapeutic target. Nevertheless, CSC are poorly defined by their tumor-initiating capacity when transplanted into a recipient (immuno-deficient) animal, a feature that on its own does not encompass other essential characteristics of these cells, e.g. their capacity to self-renew and differentiate, and to detach and migrate away from the primary site and invade distal organs. This more functional view of resident (located in the primary mass) and *migrating* cancer stem cells (MCSCs) is of clinical relevance and is likely to have important consequences on the clinical management of cancer patients. The CSC concept is also intimately bound to another feature of neoplastic diseases, namely tumour heterogeneity. By asymmetric cell division CSCs may give rise to new stem cells together with more differentiated cells, thus continuously fuelling the tumor mass providing with proliferating but progressively differentiating cells, but also retaining their multipotency (i.e. "*stemness*") and preventing their own exhaustion.

Recent experimental evidences point out that cancer stem cells are key factors not only tumor onset, growth, local invasion, and distant metastasis but also in the development of drug resistance, thus posing a major challenge towards the development of novel tailor-made cancer therapies directed against CSCs. Yet, the increasing knowledge of the structure and regulation of the mouse and human genomes together with the awareness that (migrating) cancer stem cell could be the ultimate target for effective therapies offer unprecedented research opportunities.

The MCSCS consortium was originally designed to seize these opportunities and has been focusing on understanding the function, regulation and evolution of (M)CSCs in a multicellular organism. To this end our original plan was to identify and isolate breast and colon (M)CSCs by developing and taking advantage of unique reagents, animal models, and technical approaches, and translate the results on large collections of human cancers, disseminating cancer cells, and metastases. The ultimate goal being the characterization and functional analysis of the MCSCs and their micro-environment (*the MCSC niche*) and define a "MCSC signature", instrumental for the development of future tailor-made therapeutic approaches.

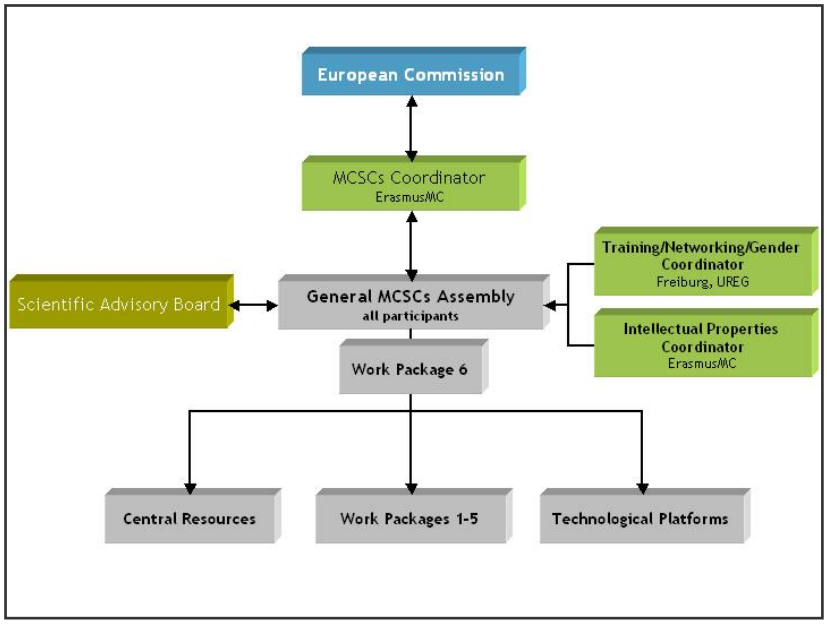
The goals of MCSCs

- To isolate intestinal and breast cancer stem cells from both experimental mouse models and cancer patients.
- To analyze the genetic and protein profiles of the above cancer stem cells and their micro-environment.
- To establish high-throughput compound screens based on (isogenic) cell models.
- To develop new models for breast and colon cancer that closely reproduce the natural history of cancer stem cells and their progression towards malignancy and metastasis.
- To generate pre-clinical animal models for (M)CSCs-targeted drug intervention.
- To develop diagnostic, prognostic, and therapeutic tests for the early detection of (M)CSCs and the prediction of metastases in breast and colon cancer patients.

To address these objectives the MCSCs consortium comprises five participants from four different EU member states that form a multidisciplinary and highly-competitive community of European research institutes dedicated to the isolation and analysis of cancer stem cells in breast and colon cancer. These are:

Participant organisation name	Participant organization short name	Scientific team leader	Town	Country
Erasmus Medical Centre	Erasmus MC	Riccardo Fodde (Coordinator)	Rotterdam	Netherlands
University of Torino	UniTo	Alberto Bardelli	Turin	Italy
University of Freiburg	UKL-FR	Thomas Brabletz	Freiburg	Germany
Spanish National Cancer Centre (till December 31,2008)	CNIO	Manel Esteller	Madrid	Spain
University of Regensburg	UREG	Christoph Klein	Regensburg	Germany

The consortium has been designed with great care based on the scientific insight, expertise, discipline, and available reagents of the participants, with the aim to develop MCSC-based diagnostic and therapeutic approaches. Also, most members of the consortium are already involved in existing collaborations, which will greatly facilitate the proposed research.



In more detail, the MCSCs consortium encompasses different research institutions including 2 cancer research organizations (CNIO; IRCC) and 3 University-based research groups (University of Erlangen; LMU; ErasmusMC). One SME (Horizon Discovery Ltd; http://www.horizondiscovery.com) has been sub-contracted to carry out specific tasks relative the screening of anti-cancer compounds on cell-based assays.

The general organization of the MCSCs consortium, depicted in the flowchart here above, was meant to maximally enhance communication and to reduce the management burden to a minimum. This is also why the MCSC project has been designed as a relatively small consortium with a deliberately limited number of highly qualified and high-profile stem cell and cancer scientists. To assist the consortium in ensuring scientific progress, a **Scientific Advisory Board** (**SAB**) has been assembled composed by

Prof. Daniel Louvard, PhD; Research Director Institute Curie, Paris, France; dr. Frank Barry, PhD, Scientific Director of the Regenerative Medicine Institute of the National Centre for Biomedical Engineering Science (NCBES), Galway, Ireland; Prof. Chris Potten, PhD, professor of Stem Cell Biology at Manchester University and Chairman of EpiStem Limited, Manchester, UK.

The SAB has played a central role in evaluating our scientific progresses and in providing advice for the continuation of the experimental plans. We have deliberately chosen for a small board composed by some of the most prestigious stem cell experts and tumor cell biologists. Their track-record and experience in providing guidance to excellent European scientific institutions like the Curie Institute in Paris and the Regenerative Medicine Institute of the NCBES in Galway, represents an extra-bonus for their capacity of providing advice also on other matters such as management, coordination of the efforts, intellectual properties, etc,. The specific activities and tasks of the SAB include: to oversee, guide and advice direction of the scientific work programme; and to evaluate the scientific-technological progress based on written reports and oral presentations at the annual meetings and write a scientific report on a yearly basis.

Each MCSCs participant was involved in one or more work packages (**WP**) with specific research questions and goals to be achieved with the unique reagents and analytical tools (technical platforms) shared within the consortium. Also, each member of the consortium brings specific competences to the consortium to be adequately integrated via efficient coordination and management activities that form a very important part of the proposal as it stands. In the project, each partner has contributed with specific technologic platforms, *in vitro* and *in vivo* models, resources, and expertise to the whole project to facilitate and enhance the collaborative structure of our research effort. The partners are therefore complimentary to each others as depicted in the tables and flowchart here below.

Table 1. Technical platform list

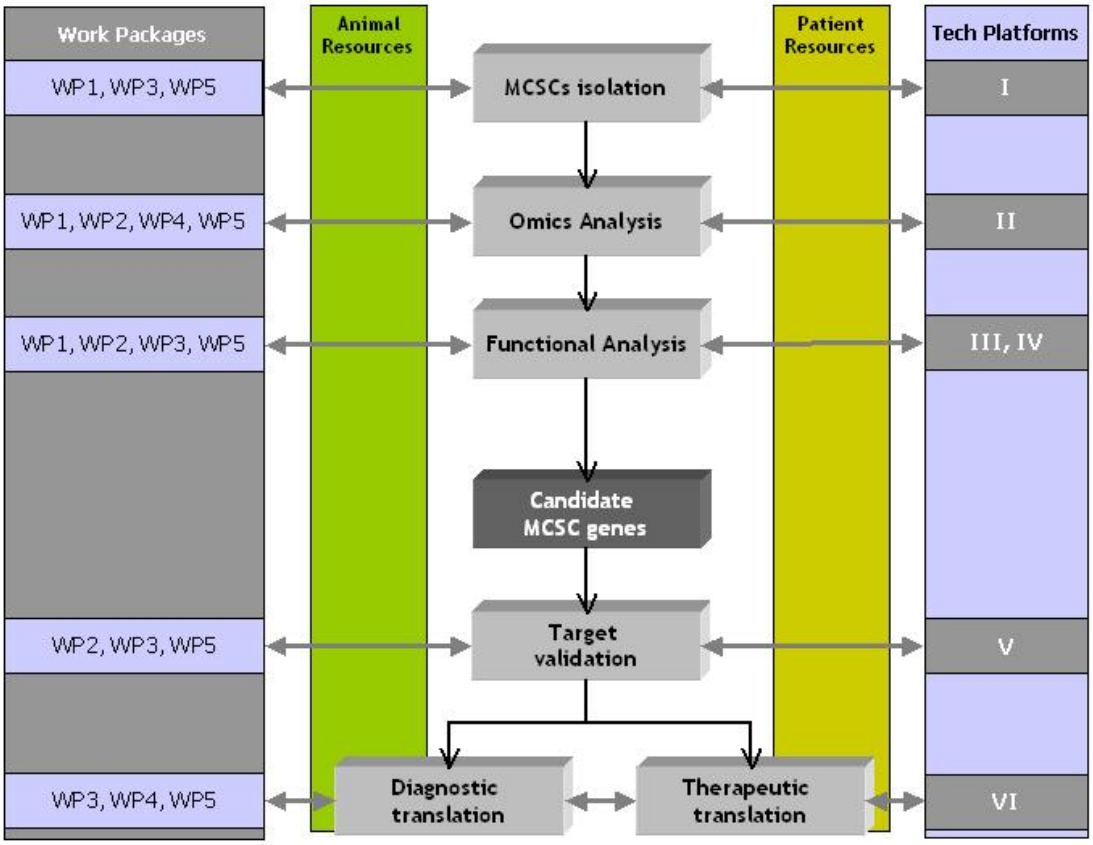
TP No	Technical platform title	Participant No
I	MCSCs isolation	P1
II	Omics analysis	P1
III	*In vitro* functional interference approaches	P1
IV	*In vitro* functional interference approaches	P2
V	Target validation	P2
VI	Diagnostic and therapeutic platforms	P2

Table 2. Work package list (full duration of project)

WP No	Workpackage title	Participant No.
1.1	Role of KRAS mutation and ß-catenin tyrosine phosphorylation in intestinal and mammary MCSCs progression and metastasis.	P1
1.2	Transcriptome and kinome analysis of mouse and human MCSCs and their micro-environment.	P1
1.3	*In vivo* functional analysis of cell-autonomous and micro-environmental modifiers of MCSCs behavior in intestinal and mammary tumorigenesis.	P1
2.1.1	Mutational profiling of kinase genes in MCSCs.	P2
2.1.2	Evaluation of the relevance and prevalence of the identified mutations	P2
2.1.3	Gene content analysis of of kinase genes in MCSCs	P2
2.2.1	Effects of mutations on the biochemical properties of kinase proteins	P2
2.2.1	Establishment of isogenic cells models carrying specific genetic alterations	P2
3.1	Identification of potential stem cell and niche factors.	P 3
3.2	Characterisation of expression and clinical relevance of selected factors	P 3
3.3	Isolation of cancer stem cells from human colorectal carcinomas	P 3
3.4	Functional analyses in cell culture and animal models	P 3
4.1	Epigenetic analysis of candidate MCSC markers in mouse models of colorectal and breast cancer.	P 4
4.2	Genome-wide epigenetic analysis of markers for disseminated CSCs in mouse and human intestinal and breast cancers.	P 4
4.3	Validation of identified targets of epigenetic inactivation in bone marrow and lymph node samples from colorectal and breast cancer patients.	P 4
4.4	Functional study of epigenetic targets of metastatic progenitor cells in colorectal and breast cancer.	P 4
4.5	Test of epigenetic drugs in disseminated tumour stem cells in preclinical mouse models.	P 4
5.1	Identification of markers for disseminated CSCs in mouse models of colorectal and breast cancer.	P 5
5.2	Validation of identified candidate proteins in bone marrow and lymph node samples from colorectal and breast cancer patients.	P 5
5.3	Transcriptome analysis of putative metastatic stem cells isolated from human samples.	P 5
5.4	Functional evaluation of MCSC genes.	P 5
5.5	Specific therapeutic targeting of disseminated tumor stem cells in preclinical mouse models.	P 5

Technical platforms and work packages were fully integrated in the MCSCs structure as depicted in the flowchart below.

Graphic representation of the MCSCs work packages and their interdependency with the major "phased milestones", technical platforms, and animal/patient resources



To address and achieve the consortium main objective, namely to combat prevalent human diseases such as cancer and to bring basic knowledge through to application, our consortium has been focusing on the identification, isolation and characterization of breast and colon cancer stem cells and the elucidation of the underlying signalling pathways that drive their onset, expansion and evolution. To achieve these goals, we developed cellular and mouse models that faithfully reproduce the natural history of CSCs in human tumors (WP1, WP3, WP5). These models are likely to represent unique tools to validate cancer targets of potential benefit for cancer patients. Specifically, we planned to use the multi-disciplinary and complementary expertise of the participants to develop *in vitro* and *in vivo* tumor models that faithfully reproduce the mechanistic and pathological changes observed in the two forms of human solid cancer, namely breast and colon cancer, with the highest incidence in the Western World.

To this end, genes known to contribute to the natural history of (M)CSCs have been searched. These genes are likely to be specific for different stages of CSC onset and progression in the parenchymal (tumor) cell (WP1), its direct micro-environment (WP3), and when disseminating throughout the body (WP5). The ultimate models will consist of compound *in vitro* cellular systems and mutant mice carrying specific combinations of targeted mutations in "MCSC genes" designed following the sequence of cell-autonomous, micro-environmental, genetic and epigenetic changes observed to occur in human breast and colon cancers (WP2, WP3, WP4, WP5).

The study is also likely to have important implications for the establishment and improvement of cancer diagnosis and prognosis as the above cellular and animal models will help in evaluating the metastatic potential of migrating and disseminating CSCs (WP1, WP3, WP5). In the long run, the identification of genes playing rate-limiting roles in MCSC activation and metastasis will lay the basis for the development of screening methodologies to detect these cells in cancer patients and target post-surgical chemotherapy. Also, the recognition of micro-environmental factors that modulate the invasive behavior of MCSCs (WP3) will open the way to novel prognostic approaches towards the prediction of the cancer patient's clinical course based on genetic background (e.g. functional polymorphisms in stromal genes) rather than on tumor-specific expression profiles.
This consortium also planned to generate cellular and animal models to validate "drugable" targets, that is, molecules considered of potential interest as therapeutic targets, based on their inhibitory action in the above models. Target validation can be carried out through genetic and genomic analysis. Specifically, we originally proposed to use different experimental approaches aimed at the kinome/phospatome, as kinase inhibitors have been shown to represent promising anti-cancer drugs. The same is true for epigenetic changes involving a broader group of relevant MCSC-related genes, potentially modulated by compounds that inhibit epigenetic processes such as DNA methyltransferase and histone deacetylase inhibitors. Strains of mice carrying conditional mutations in genes encoding for kinases, phosphatases, and epigenetic factors (WP2, WP4) of potential therapeutic interest have been employed to obtain direct genetic evidence *in vivo* for their requirement in (M)CSC onset and progression towards malignancy and thus forecast the therapeutic value that selective inhibitors may have in the clinic. Since most inhibitors do not completely block the biological activity of their targets, the use of *in vitro* and *in vivo* models for conditional and tissue-specific down-regulation by RNA interference of targets of interest will provide a more realistic perspective of what to expect in terms of therapeutic benefit upon down-regulation of the expression of a specific target in tumor cells or their micro-environment.

Additionally, we have included a SME subcontractor to screen for anti-cancer compounds on 'isogenic' cell-based assays. This isogenic cell-based model allows high-throughput screening of large number of compounds on genetically engineered somatic cell lines.

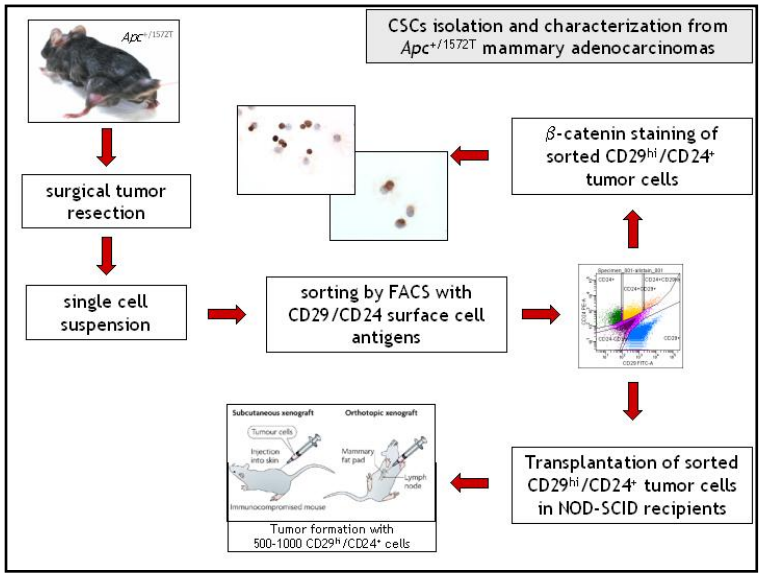
The above technological advances will improve the quality and speed of drug testing while using fewer animals, thereby contributing to the three "R"s (reduction, refinement, replacement).

III. THE RESULTS OF MCSCS

Progress in year 1

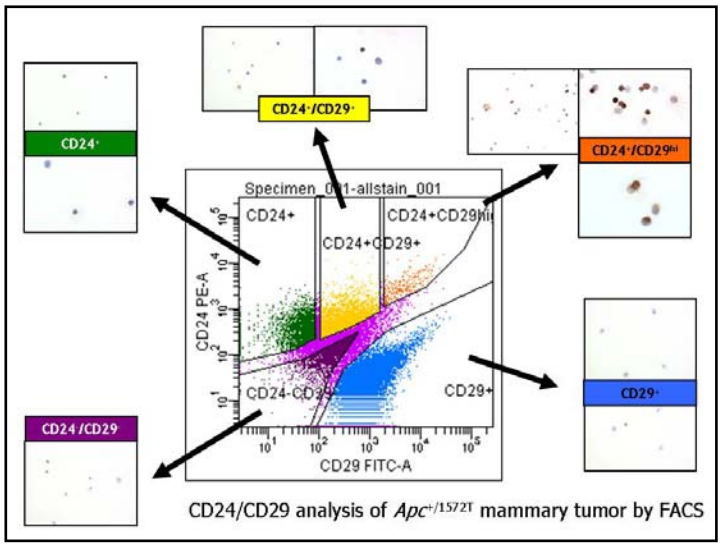
WP1 *Intracellular β-catenin accumulation as a CSC determinant in intestinal and mammary tumorigenesis.*

One of the main working hypothesis of our consortium consists in the role of intracellular □-catenin accumulation as a functional determinant of cancer stemness not only in our murine models for mammary and gastro-intestinal cancer but also in human breast and colorectal cancer. Proof of principle for this fundamental concept has been now delivered for the *Apc*-driven mouse model (*Apc*$^{+/1572T}$) of mammary cancer and distant (pulmonary) metastasis. Expression of the surface antigen markers CD29^{hi}CD44^{+} earmarks mammary CSCs (MaCSCs) characterized by cytoplasmatic and nuclear accumulation of □-catenin. The same cells were shown to underlie lung metastasis formation in these mice. As far as the *Apc*-mutant intestinal cancer mouse models are concerned, the preliminary results indicate that expression of the surface antigen markers CD29^{+}CD24^{hi} identifies a subpopulation of cancer cells highly enriched in nuclear ß-catenin. These cells are now being transplanted in immune-deficient animals to test their tumor-initiating capacity and CSCs identity. The same is true for a yet limited number of human colorectal cancers and liver metastases analyzed to date.



Isolation and omics analysis of mouse mammary and intestinal MCSCs characterized by intracellular ß-catenin accumulation

The discovery according to which the CD24/CD29 surface antigen markers allow the isolation of subsets of mammary and intestinal cancer cells with intracellular ß-catenin accumulation and tumor-initiating capacities has been pivotal for the achievement of our primary objective in the first project year. In short, sorting of *Apc*1572T mammary tumours for the CD24^{+}CD29^{hi} surface antigens results in the isolation of a relatively small (1-2%) subpopulation of cancer cells earmarked by intracellular ß-catenin accumulation (see Figure). The CD24^{+}CD29^{hi} cells are able to recapitulate tumorigenesis when transplanted in



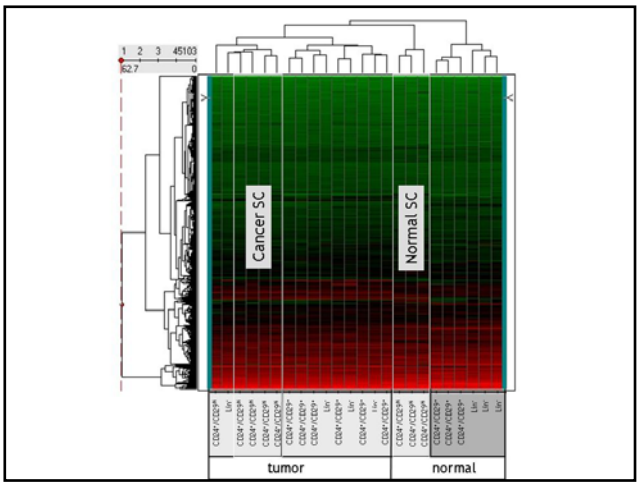
CD24/CD29 analysis of *Apc*$^{+/1572T}$ mammary tumor by FACS

NOD-SCID recipient animals at low multiplicities. These results and the vast amount of information being derived from them will lay a solid basis for the remaining MCSCs project years and provide unique material for our own and the other work packages. Our results underlie the importance of specific dosages of Wnt signalling activation in conferring predisposition to organ-specific tumorigenesis. Moreover, we show that intracellular ß-catenin accumulation, previously shown to earmark a small and non-randomly distributed subpopulation of tumour cells in Wnt-driven epithelial cancers and to predict poor prognosis in breast cancer in man, underlies cancer stemness and metastatic behaviour in the mouse mammary gland. Hence, the primary objective has been achieved and completed for the mammary tumors and pulmonary metastases from *Apc*$^{+/1572T}$ mice, whereas they are still being pursued with the *Apc*$^{+/1638N}$/*KRAS*^{G12D} models and the patient-derived colorectal cancers and metastases. To reinforce this observation, we also set up an *in vivo* protocol to isolate label-retaining cells (LRCs) from normal adult stem cell niches and in particular in the mammary gland and the intestinal crypt, and from the correspondent mammary and intestinal tumors from the *Apc*-mutant mouse models. The latter will be further pursued during the second year.

Omics analysis of mouse mammary cancer stem cells

To elucidate the molecular and cellular mechanisms underlying stemness in mammary homeostasis and cancer, MaSCs and MaCSCs earmarked by CD29^{hi}CD24^{+} were analyzed by genome-wide expression profiling. Single cell suspensions were prepared from normal (*Apc*$^{+/+}$) mammary glands and from *Apc*$^{+/1572T}$ mammary adenocarcinomas and from each of the Lin^{-}, CD29^{+}CD24^{+} and CD29^{hi}CD24^{+} FACSorted subpopulations and their total RNA analyzed by Affymetrix microarrays. Unsupervised two-dimensional hierarchical clustering of the profiling data clearly resolved the normal CD29^{hi}CD24^{+} cells within the same dendogram as the tumor-derived samples (see Figure), thus suggesting that normal MaSCs are more similar to cells from *Apc*$^{+/1572T}$ mammary adenocarcinomas than to their own differentiated (Lin^{-}) progenies. In fact, normal and cancer CD29^{hi}CD24^{+} cells differ by approx. 600 genes (n=592, FDR<0.01), which is considerably less than when comparing other differentiated subpopulations between the normal mammary gland and adenocarcinomas from *Apc*$^{+/1572T}$ animals. Further bioinformatic analysis revealed that normal and cancer mammary stem

cells are more similar to each other in terms of canonical Wnt/□-catenin, Notch and TGF-□ signaling. The latter resembles the tight control of these signaling pathways in the intestinal epithelium where their coordinated activity promotes stem cell renewal. Similar analysis will be repeated upon isolation of intestinal CSCs from the *Apc*$^{+/1638N}$/*KRAS*^{G12D} models and the patient-derived colorectal cancers and metastases.



Analysis of the role of oncogenic KRAS activation in modulating MCSC invasive and metastatic behaviour in intestinal tumors.

The development and characterization of a compound *Apc*$^{+/1638N}$/villin-*KRAS*^{G12D} mouse model (Janssen KP, et al. *Gastroenterology*. 2006 Oct;131(4):1096-109) have shown that this combination of tumor suppressor and oncogene mutations result in enhanced intestinal tumor initiation and an accelerated adenoma-carcinoma sequence. Moreover, evidence was found for the presence of micrometastases in the liver. Also, the relative number of alleged intestinal CSCs earmarked by ß-catenin nuclear accumulation was significantly increased in the compound *Apc*$^{+/1638N}$/villin-*KRAS*^{G12D} animals when compared with *Apc*$^{+/1638N}$ mice. This is likely to be due to ß-catenin Tyr-phosphorylation which results in its release from E-cadherin and the consequent increase of the intracellular and nuclear ß-catenin signalling pool. To elucidate which Tyr-kinase downstream of oncogenic KRAS is responsible for ß-catenin phosphorylation we are applying PepChips Kinase Arrays by Pepscan Systems (www.pepscan.nl), a proteomics platform that encompass the substrate of all known kinases. The results with mouse cell lines carrying specific Apc and Kras mutations have pinpointed a number of Ser-Thr kinase known to make integral part of the Wnt cascade which serves as a validation of the technical approach. However, the constitutive villin-driven *KRAS* mutation results in such an enhancement of the upper GI tumor phenotype that the animals have to be sacrificed before they can develop full-blown metatsases.

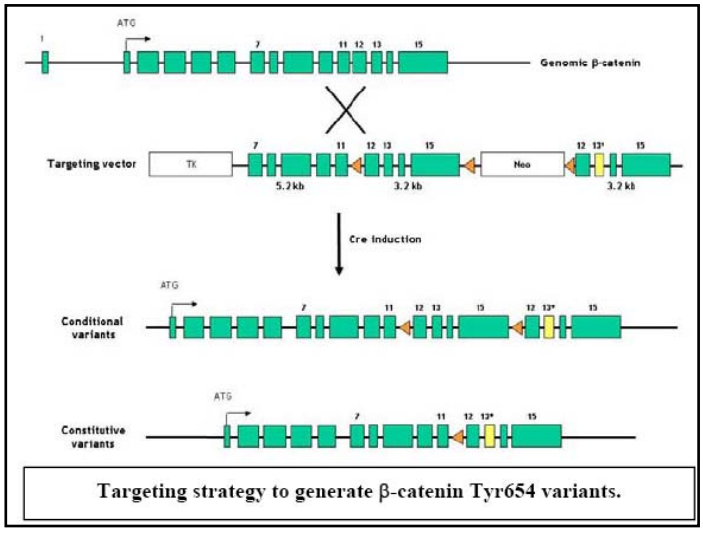
Analysis of the role of ß-catenin tyrosine phosphorylation in modulating MCSC invasive and metastatic behavior

Our own previously published results (Janssen et al., *Gastroenterology*. 2006 Oct;131:1096-109) have indicated that the synergistic effect of oncogenic *KRAS* activation in enhancing ß-catenin nuclear translocation resides in the activation of downstream tyrosine kinases.

Increased ß-catenin tyrosine phosphorylation releases ß-catenin from E-cadherin at the adherens junction thus augmenting the intracellular pool available for Wnt signaling. Based on these observations, we have successfully completed the generation of two conditional knock-in mouse models encoding for:

1. the ß-catenin Tyr654Y-F variant, mimicking Tyr-dephosphorylation of ß-catenin and thus a stronger E-cadherin association, and
2. the ß-catenin Tyr654Y-E variant, mimicking Tyr-phosphorylation of ß-catenin and thus a weaker E-cadherin association.

Both models have been obtained by knocking in the single residue change in the endogenous ß-catenin mouse gene and are therefore driven by the promoter (see figure). The phenotypic characterization of these novel models is ongoing and thus far, no evidence of susceptibility to cancer was observed, though the animals are still relatively at young age. Also, homozygosity for the above point mutations does not appear to result in *in utero* lethality.



Targeting strategy to generate β-catenin Tyr654 variants.

As planned in the MCSCs grant, we are breeding the ß-catenin knock-in models with the already available *Apc*1638N and *Apc*1572T mutant mice in order to investigate the effects of β-catenin Tyr-phosphorylation on MCSCs progression and invasion potential.

- ***WP2*** *Mutational and functional analysis of metastatic progenitor cells in colorectal and breast cancer.*

Understanding the genomic landscape of cancer stem cells is one of the priorities of this consortium. As planned we begun to tackle this issue by focusing on the generation of an effective protocol for the mutational analysis of genomic DNA isolated from MCSC. As a test case we set up conditions for the analysis of cancer genes such as BRAF, KRAS, EGFR and PI3KCA that are known to be mutated in breast and colorectal cancer cells. The approach that we have developed is capable of amplifying and sequencing individual exons from very limited amount of genomic DNA and can therefore be applied to analyze the mutational profile of cancer stem cells that are typically isolated in limited amounts.

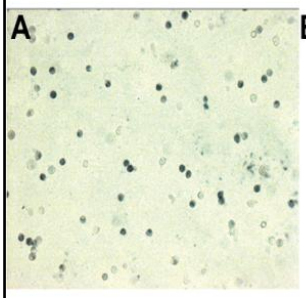
- ***WP3*** *Identification and functional characterisation of stem cell- and niche-factors from human colorectal mucosa and colorectal cancers.*

We have identified a subset of transcriptional repressors (e.g. *ZEB1*) capable of activating epithelial-to-mesenchymal transitions (EMTs). These novel genes represent important promoters of tumor progression and their functional analysis is central to the MCSCs project. Also, ZEB1 expression appears to be relevant for self renewal in CSCs in view of the role it plays in asymmetric cell division.

- ***WP5*** *Identification, genomic and functional characterization of metastatic progenitor cells in colorectal and breast cancer.*

Disseminated tumor cells from 11 week-old transgenic Her2 mice form bone marrow carcinosis in wild type recipients.

A



B

Donor			Recipient		
Identifier	CK^+ cells/ 0.5×10^6 BM cells	Absolute number of transplanted tumor cells	Identifier	Weeks after transplantation	Percentage of CK^+ cells in BM
D-1	4	80	BMT9	19	31.40
		80	BMT10	19	28.01
		80	BMT11	19	10.39
D-2	11	154	BMT1	20	*
		154	BMT2	20	*
		154	BMT12	20	*
		154	BMT5	22	19.71
D-3	12	240	BMT13	20	*
		240	BMT6	22	22.21
		240	BMT7	22	30.20
		240	BMT8	22	15.51
C-1 (wildtype)	0	0	C1-1	19	0
		0	C1-2	28	0
		0	C1-3	43	0

* not available because mice were found dead before analysis

We performed gene expression analysis of single DTCs (disseminated tumor cells) isolated from mouse models and patients. For breast cancer patients, sample acquisition is almost complete and we are currently hybridizing reference samples, such as normal breast glands and reference bone marrow cells. In the mouse models, sample acquisition is not yet completed. DTCs isolated from lungs are still missing, DTCs from bone marrow are almost complete.

Selected MCSCs publications in year 1

- Fodde R, Brabletz T. Wnt/beta-catenin signaling in cancer stemness and malignant behavior. ***Curr Opin Cell Biol***. 2007 Apr;19(2):150-8.
- Gaspar C, Cardoso J, Franken P, Molenaar M, Morreau H, Möslein G, Sampson J, Boer JM, de Menezes RX, Fodde R Cross-species comparison of human and mouse adenomatous polyps reveals conserved mechanisms in *APC*-driven intestinal tumorigenesis. ***Am J Pathol*** 2008 *in press*.
- Le H, Franken P, Fodde R. Tumour-stroma interactions in colorectal cancer: converging on ß-catenin activation and cancer stemness. ***BJC*** 2008 *in press*.
- S. Spaderna, O. Schmalhofer, M. Wahlbuhl, A. Dimmler, K. Bauer, A. Sultan, F. Hlubek, A. Jung, D. Strand, A. Eger, T. Kirchner, J. Behrens, T. Brabletz. The transcriptional repressor ZEB1 promotes metastasis and a loss of cell polarity in cancer. ***Cancer Res***., *in press*.
- Hüsemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, Forni G, Eils R, Fehm T, Riethmüller G, Klein CA. Systemic spread is an early step in breast cancer. ***Cancer Cell***, *in press.*
- Benvenuti S, Frattini M, Arena S, Zanon C, Cappelletti V, Coradini D, Grazia Daidone M, Pilotti S, Pierotti MA, Bardelli A. PIK3CA cancer mutations display gender and tissue specificity patterns. Hum Mutat. 2007 Nov 16
- Balakrishnan A, Bleeker FE, Lamba S, Rodolfo M, Daniotti M, Scarpa A, van Tilborg AA, Leenstra S, Zanon C, Bardelli A. Novel somatic and germline mutations in cancer candidate genes in glioblastoma, melanoma, and pancreatic carcinoma. **Cancer Res**. 2007 Apr 15;67(8):3545-50.

Progress in year 2

• ***WP1*** *Intracellular β-catenin accumulation as a CSC determinant in intestinal and mammary tumorigenesis*

Major progresses have been scored in this specific area. Overall, all the lines of investigations seem to confirm the initial working hypothesis: intracellular β-catenin accumulation underlies cancer stemness both at the primary tumor site and in metastases. This has been shown to be true in the *Apc*1572T model for mammary adenocarcinomas and pulmonary metastases; in the intestinal cancer model *Apc*1638N/*KRAS*^{G12V}; and in human colon cancer.

1. *Apc*$^{+/1572T}$ mice have proven a unique model for the validation of the CSC model. After having prospectively isolated CSCs from the primary mammary carcinomas by employing the CD29^{hi}CD24^{+} combination of surface antigen markers, we characterized them extensively both by IF and IHC analysis with specific epithelial and mesenchymal markers, and by microarray analysis and compared with normal mammary stem cells. The latter analysis revealed that the differences between MaSCs and MaCSCs are mainly limited to the Sonic Hedgehog pathway, thus offering novel opportunities to test CSC-targeted therapy by Cyclopamin and Hh Antag, two known antagonist of the SHH pathway. Moreover, tail vein injections of different subpopulations of tumor cells has revealed that only the CSCs are able to efficienly form metastases in a broad spectrum of distant organs, whereas the same type of lesion were never observed when equal amounts of bulk tumor cells where injected into the blood stream. These preliminary data may also provide the first piece of evidence for the very existence of migrating CSCs.

Collaborations with Partners 4 and 5 have been established for the epigenetic and genetic molecular analysis of the CSCs and MCSCs isolated from the *Apc*1572T mouse model.

2. We have now identified a novel combination of CD markers, namely CD29^{+}/CD24^{hi} which highly enrich for tumor initiating cells or CSCs from the intestinal adenocarcinomas from *Apc*$^{+/1638N}$/*KRAS*^{G12V} animals. As for the *Apc*$^{+/1572T}$ mammary adenocarcinomas, these cells are enriched in nuclear β-catenin and can form tumors when as few as ~1000 cells are transplanted in NOD-SCID mice. These alleged CSCs have self-renewal capacity as assessed by two rounds of serial transplantations through which the resulting tumors retain the heterogeneity of differentiated lineages observed in the primary tumors.

As for *Apc*1572T, collaborations with Partners 4 and 5 have been established for the epigenetic and genetic molecular analysis of the CSCs and MCSCs isolated from the *Apc*$^{+/1638N}$/*KRAS*^{G12V} animals.

3. By employing the CD133+ and CD166+/CD44+ combination of CD markers previously published for the prospective isolation of colorectal cancer stem cells from human patients,

we have shown that also these subpopulations of tumor cells are earmarked by intracellular β-catenin accumulation.
In collaborations with Partner 3, these prospectively isolated human CSCs are being analyzed for expression of ZEB1 and various miRNA's belonging to the miR-200 family.

• ***WP2*** *Mutational and functional analysis of metastatic progenitor cells in colorectal and breast cancer*

Understanding the genomic landscape of cancer stem cells is one of the priorities of this consortium. As planned in collaboration with Partner 5 we tackled this issue by focusing on the genetic analysis (genomic content and mutational analysis) of the disseminated MCSCs isolated in patients**.**
Also, we have generated human cell models recapitulating some of the most common molecular alterations found in human cancers. As described in details in the PMR we refer to these as cell based '*genetic matrix*' models. The functional characterization of such models with respect to tumorigenicity and stemness is in progress.
In more details, during the 1st year we focused on the completion of this line of investigation in collaboration with Partner 5 (Dr. Christoph Klein) and, as reported in the planning, we started the work regarding the second part of our work package, achieving important progresses.
After isolation of disseminated MCSCs, a collaboration with Partner 5 for the evaluation of the genome content of the isolated cells has been established, in order to identify genes specifically deleted or amplified in MCSCs. Technology for the evaluation of genome content of isolated disseminated cancer cells is being developed in Partner 5's laboratory and it is based on a CGH approach. The read-out of these experiments could give us genetically altered genes important for the function of the MCSCs.
As a parallel approach, we are collecting gDNA samples coming from isolated MCSCs of human patients. As soon as a sufficiently large panel of samples will be collected, the sequencing project of candidate oncogenes will start. The initial list of genes to be sequenced includes EGFR, KRAS, BRAF and PIK3CA. These genes are frequently mutated in human cancers and amplification and sequencing primers have already been designed and tested on different tumor samples.
Integration of data coming from these two approaches will give us a novel map of potential genetic determinants of the MCSCs' phenotype.
This work package includes the generation of human cell models with specific genetic alterations resembling mutations found in human cancers. These models could be employed to investigate how oncogenes and oncosuppressor genes frequently altered in human cancers influence the development of the MCSCs' phenotype and determine the proceeding of the metastatic cascade for which MCSCs seem to be responsible. Therefore,

the work performed during the last year mainly focused on the generation of isogenic human cell models expressing commonly mutated alleles under the control of their own promoters.

In particular we focused on the following alleles: EGFR (delE746-A750), KRAS (G13D), BRAF (V600E) and PIK3CA (E545K and H1047R), that are found in multiple human cancers and that will be first target of investigation in the sequencing experiments on isolated MCSCs. These specific mutations have already been successfully knocked-in in human cells by rAAV-mediated homologous recombination.

As recipient cells, two human non transformed epithelial cell lines of breast (MCF10A, hTERT-HME1) origin have been employed. These cell lines can be propagated indefinitely in vitro, but are not tumorigenic, which makes them a suitable model to underlie functional cancer-oriented effects induced by the mutated alleles introduced.

Oncosuppressor genes frequently inactivated in human cancers have also been integrated in these cellular models, as we permanently knocked-down the expression of TP53, PTEN or Rb1 in isogenic cell models harbouring the above mentioned activating mutations on EGFR, KRAS, BRAF or PIK3CA. The result is a genetic matrix combining specific oncogene activating mutations with specific oncosuppressor genes inactivation. Importantly, the genetic matrix includes also the matched wt isogenic cell line (harboring no activating mutations and in which a scramble control shRNA have been introduced) to be compared with the KI/knock-down models described.

Models included in the genetic matrix have been validated verifying the presence at the genomic level and the expression at the RNA level of the corresponding Knocked-in mutations, while the knocking down of oncosuppressor genes have been assessed at the protein level.

Biochemical characterization of the models is in progress, to assess the effect of specific combined genetic alterations on the biochemical network of non-transformed cells.

Also appropriate functional assays to evaluate the role of specific genetic alterations in cellular transformation, motility and invasion are ongoing.

Oncogenes and oncosuppressor genes frequently altered in human cancers could influence the development of the MCSCs phenotype by affecting the fraction and the behavior of the stem-like side population. Therefore we are optimizing the probing and the FACS –based isolation of the stem cell-like population in each KI/Knock down model. Our aims are:

- evaluating differences in the fraction of stem-like population in different genetic models;
- characterizing by a biochemical and functional point of view isolated side populations.

• ***WP3*** *Identification and functional characterisation of stem cell- and niche-factors from human colorectal mucosa and colorectal cancers*

After the characterization of the EMT-inducer ZEB1 as a crucial promoter of malignant tumor progression, including tumor invasion and metastasis, we further elucidated its molecular mechanisms and its role in the formation of migrating cancer stem cells within the second reporting period: by mRNA and microRNA expression profiling of the stable ZEB1 knockdown clones (generated in the first reporting period) we were able to identify several ZEB1 target genes as potential candidate genes involved in tumor progression and cancer stem cell malignant behavior. We identified a specific miRNAs cluster (miR-200 family) as a strong inducer of differentiated epithelial phenotype, which is repressed by ZEB1. This repression of miR-200 expression results is enhanced tumor cell migration and invasion. In cooperation with P4 we performed a DNA methylation array to identify patterns of target genes hyper-and hypomethylated by ZEB1. Data from the mRNA expression and methylation arrays were compiled to select for the most promising candidate genes for further functional analyses. We already constructed Tetracyclin inducible cell clones for some of the selected candidate mRNAs and miRNAS and started to xenograft these cell clones in immunodeficient mice.

In more details, after the characterisation of the EMT-inducer ZEB1 as a crucial promoter of malignant tumor progression, including tumor invasion and metastasis, we further elucidated its molecular mechanisms and its role in the formation of migrating cancer stem cells within the second reporting period:

By using stable ZEB1 knockdown clones, constructed in the first reporting period, for mRNA and microRNA expression arrays we could identify many ZEB1 target genes as potential candidate genes involved in tumor progression and cancer stem cell accumulation. Thereby, we identified a cluster of microRNAs (miR-200 family) as a strong inducer of a differentiated epithelial phenotype, and which is repressed by ZEB1. We could show that miR-200c and miR-141, two members of the miR-200 family induce epithelial differentiation in undifferentiated breast and colorectal cancer cell lines by upregulation of epithelial genes, like E-cadherin. This correlated with reduced migration and invasion activity of the tumor cells. Vice versa inhibition of these microRNAs using anti-microRNAs in differentiated cell lines induced an EMT by upregulation of mesenchymal genes like vimentin. We could identify the molecular mechanism of these differentiation changes by showing that ZEB1 is the best target mRNA suppressed by these microRNAs. Moreover in addition to ZEB1 miR-141 also inhibited expression of TGFbeta, another strong EMT inducer. These results indicate that ZEB1 triggers a microRNA-mediated positive feedback-loop, which stabilizes EMT and promotes invasion of cancer cells.

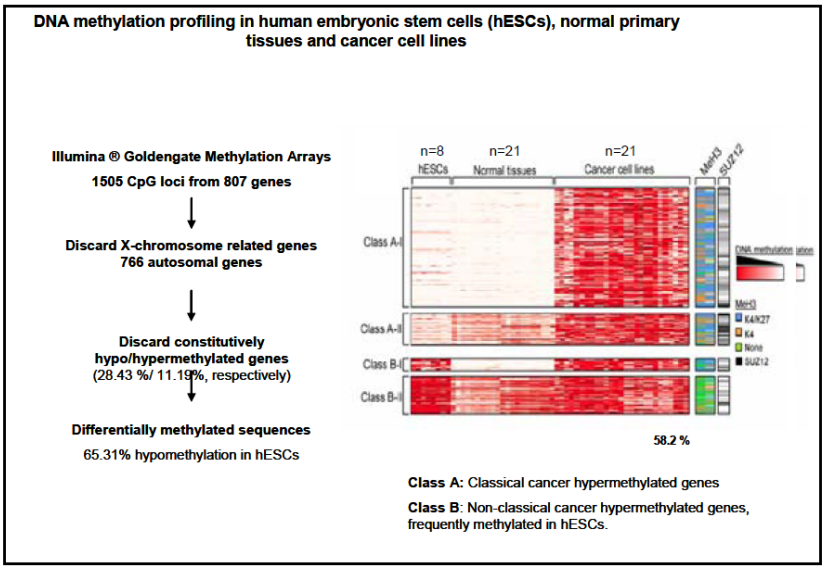
The expression of miR-200 family members in human tumors and its clinical relevance was investigated in human breast cancers. We could show that both the expression of miR-141 and of miR-200c is strongly reduced in the aggressive basal type of breast cancer,

compared to the common ductal invasive type of breast cancer. Currently an in situ hybridisation protocol for expression of these miRNAs is established to proof if miR-200 family members are reduced in the putative migrating cancer stem cells at the invasive front of ductal invasive breast cancers and colorectal cancers. In cooperation with R. Fodde (partner 1), cancer stem cells isolated from human colorectal cancer (within WP1) are currently analysed for expression of ZEB1, miR-200 family members and other candidate genes identified in WP3.

Using mRNA expression arrays, we identified potential target genes of ZEB1, These genes could explain its tumor- and metastasis-promoting function. In cooperation with M. Esteller (WP4) we could show that ZEB1 induces specific epigenetic changes in colorectal and breast cancer cells. Using the Illumina methylation array we identified a distinct pattern of genes hypomethylated or hypermethylated at CpG islands within their promoters. By compiling these array data with data from our expression array we could show that hypomethylation largely correlated with overexpression, and hypermethylation with reduced expression of putative ZEB1-target genes. This compilated list of target genes was used to further select the most promising candidate genes (upregulated by ZEB1: FGF5, Wnt5a, IGFBP7, CTGF, TGFbeta 1, TIMP3, CDH11, Slit2, Il6, Il11; down by ZEB1: Casp2, NTSR1, Serpin5, ABCC2, FGF2, IGF2, NES, NTSR1, PLAGL1) for further analyses for their role in regulating tumor progression and cancer stemness. We already constructed Tetracyclin inducible cell clones for ZEB1, selected candidate mRNAs and miRNAS and started to xenograft these cell clones.

• ***WP4*** *Characterization of epigenetic markers of metastatic progenitor cells in colorectal and breast cancer*

We performed genome-wide DNA promoter methylation studies, including more than 800 cancer-related genes, from embryonic stem cells, somatic stem cells, different types of terminally differentiated adult tisssues and cancer cell lines. Results- provide a list of differentially methylated genes that allow establishing a specific epigenetic signature of stem cells during ontogenic development. Taking into account this information we performed the same methylation arrays in mesenchymal stem cells (MSC) derived from adult breast tissues and in *in vitro* induction of differentiated cells from MSCs. Methylation signals allow the classification of each sample within each group of samples, confirming the existence of a specific methylation signature in stem cells. Furthermore, we observed that the profiles of promoter methylation in *in vitro* differentiated mesenchymal stem cells strongly depend on the tissue specifity.



In more detail, we had obtained the promoter DNA methylation signature of embryonic stem cells (hESC) and different types of terminally differentiated adult tisssues (NPT) and cancer cell lines (CCL). For the investigation of genome-wide methylation signatures in the different tissue types we have performed the powerful Goldengate Methylation Arrays from Illumina®. With this platform we were able to quantitatively investigate a set of 1505 CpG loci covering 807 genes at single-nucleotide resolution. Selection of the genes was performed from the well-annotated NCBI CCDS database (Genome Build 36) and is supplemented with cancer-related genes with a role in cell fate and differentiation described in published literature.

Unsupervised clustering of the samples exclusively using the methylation signals of the autosomal genes (1,421 sequences) contained in the arrays enabled the correct classification of each sample within its corresponding group (hESC, NPT, or CCL). Then, contrary to expectation, we found that a substantial proportion (around 20%) of cancer methylated genes is also frequently hypermethylated in hESCs. To demonstrate further that the differentiation of hESCs is associated with less DNA methylation at the promoter region of certain genes, we induced the *in vitro* differentiation of the hESC line Shef-1 in two cell lineages (fibroblast-like cells and neural precursors) and then used methylation arrays to identify a substancial proportion of the genes (13%) became hypomethylated during differentiation.

Having demonstrated that some cancer genes are hypermethylated and repressed in hESCs and that they can lose methylation during in vitro differentiation of hESCs, we investigated whether this phenomenon is restricted to embryonic development or, conversely, is an epigenetic mechanism associated with stemness status regardless of the ontogenetic stage of the cell. We used the same methylation arrays to identify genes hypermethylated in CD34+ somatic stem cell progenitors compared with peripheral blood lymphocytes and neutrophils, two types of adult primary cells derived from the CD34+ hematopoietic progenitors and we conclude that hypermethylation of cancer genes can occur in stem cells in an ontogenetic stage-independent manner.

To test the CpG promoter hypermethylation on specific genes (*i.e. RUNX3, DLC1, AIM2,MGMT, PYCARD,* between others) bisulfite genomic sequencing of multiple clones using primers spanning the CpG island around the transcriptional start site had been performed in all tissues analysed. Selection of the genes will attend to their role in cell growth and proliferation. Finally, to understand better their role in promoter hypermethylation of our selected specific genes, we use quantitative-PCR to measure their expression in all sample groups.

Once we had optimized the approach for determining the promoter DNA hypermethylation profile in embryonic stem cells, we extend the studies to different mesenchymal stem cells (MSCs) and their corresponding counterparts in *in vitro* induced-differentiated cells. Mesenchymal stem cells had been derived from adult fat tissue (from breast) and all the markers for the characterization of the multipotent stem cells had been performed. *In vitro* differentiated osteoblasts, chondrocites, neuronal and muscle cells have been obtained from the aforementioned MSCs, being performed all the tissue-specific markers for the differentiation process. Again, we were able to establish a comprehensive DNA methylation signature of mesenchymal stem cells and differentiated cells. Furthermore, we observed that the profiles of promoter methylation in *in vitro* differentiated mesenchymal stem cells strongly depend on the tissue specifity. Actually we are validating the promoter hypermethylation-regulated gene expression on specific genes.

• ***WP5*** *Identification, genomic and functional characterization of metastatic progenitor cells in colorectal and breast cancer*

Based on the results of the first year, we proceeded in the analysis of the Balb/NeuT model. First profiling experiments identified at least 3 subpopulations of DTC in the bone marrow of the animals. These cells differ for their expression of CSC associated markers.

Functional experiments have therefore been initiated in which these cells are individually isolated and injected to test their growth potential.

For analysis of DTC gene expression profiles we developed several models and classifiers. These include the separation of EpCAM+ cells from patients with and without breast cancer, in order to identify unambiguously EpCAM+ DTC; second, a classifier to separate DTC from primary tumours; third, a classifier to assess the differentiation state of a single cell; fourth, a classifier to assess the proliferation state of a single cell. All these classifiers are based on specific gene expression signatures.

Expression profiling of single disseminated cancer cells isolated from bone marrow of mouse models (Her2, Apc1638N, Apc1572T) and definition of a list of candidate MCSC markers.

Sample acquisition for expression profiling of HER2 mouse model is complete. As mentioned in first activity report we added single cells isolated from lungs for comparison with bone

marrow-derived DTCs. In first limited expression analysis of cDNA samples of single $EpCAM^+$ DTCs we found that stem cell markers, namely Sca-1 and the combination $CD24^+/CD44^+$, are expressed in EpCAM+ DTC. Furthermore, in isolated single $Her2^+$ DTCs we found no expression of these markers. Because of these obviously existing subpopulations of DTCs in bone marrow we performed double-staining against the antigens Her2 and EpCAM. As expected, we detected single cells differentially expressing epithelial markers. We found and isolated $EpCAM^+/Her2^+$, $EpCAM^+/Her2^-$ and $EpCAM^-/Her2^+$ cells. Therefore, we added also single cells of each subpopulation from bone marrow of Her2 model for expression profiling. Data analysis and validation has been started.

In addition to expression profiling of single DTCs we performed bone marrow transplantation experiments to define the subpopulation, in which potential cancer stem cells are enriched . As read-out we used an assay leading to bone marrow carcinosis in recipients observed in Husemann et al. 2008. For this approach we isolated bone marrow cells of Her2-transgenic mice. After following enrichment of certain subpopulations the cells were injected into tail vein of Nod/Scid mice. Only 6 weeks after transplantation of EpCAM-enriched cells and we observed a massive proliferation of cells in bone marrow of Nod/Scid-recipients expressing epithelial markers, CK 8/18 and especially Her2. Further transplantation experiments with other subpopulations, namely $EpCAM^+/Her2^-$, $EpCAM^-/Her2^+$ and $Her2^+$, have already been started.

Expression profiling of single disseminated cancer cells isolated from BM or LN samples of breast and colorectal cancer patients and definition of a list of candidate MCSC markers.

Expression analysis of breast cancer cells is complete and bioinformatics evaluation is performed. Currently, we are performing experiments to evaluate different normalization strategies for single-cell expression profiling. These experiments are crucially before we proceed with patient data analysis.

Establishment of a mouse model of minimal residual disease to allow drug testing in an adjuvant setting.

The mouse model has been developed. Breeding is already established and the preparation of mice (i.e. transplantationof transgenic mammary tissue, waiting for tumor development and resection of primary tumours) for systemic treatment has already been started and is still in process. Here, we transplanted orthotopically mammary tissue before transformation from Her2-transgenic mice into wildtype recipients (Balb/c). Drugs for therapy, antibodies against EpCAM and Her2, have been produced by Micromet and purified. The evaluation of dosage and application for mice is in process and close to be finished. Taken together, the therapy

study will be started at the beginning of next year. It is planned that the study not only includes the comparison of treatment of recipients after resection of graft at different stages of tumor progression, i.e. atypical hyperplasia versus invasive cancer, but also includes the comparison of different treatment models, anti-EpCAM- versus anti-Her2-antibody.

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Progress in year 3

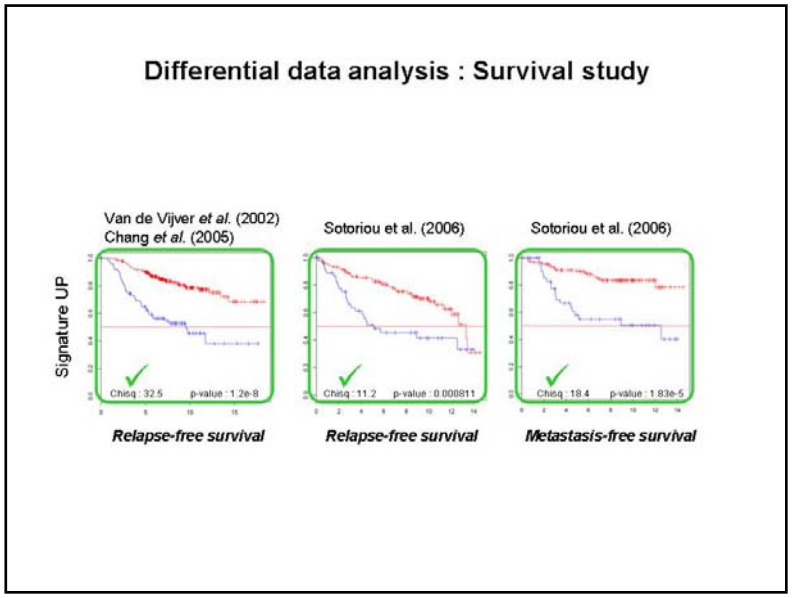
- ***WP1*** *Intracellular β-catenin accumulation as a CSC determinant in intestinal and mammary tumorigenesis*

Mammary and intestinal CSCs earmarked by β-catenin intracellular accumulation have been enriched, characterized and profiled from the tumors of our two main mouse models namely *Apc*$^{+/1638N}$/*KRAS*^{V12G} (intestinal cancer) and *Apc*$^{+/1572T}$ (mammary cancer). The corresponding profiles have been analyzed to extract CSC-specific signatures and elucidate the underlying molecular and cellular mechanisms responsible for CSC maintenance and malignant behaviour.

Isolation and omics analysis of mouse mammary and intestinal MCSCs characterized by intracellular ß-catenin accumulation.

Significant progresses have been scored in these specific areas. As also stated in the previous progress report, the initial working hypothesis according to which intracellular β-catenin accumulation underlies cancer stemness both at the primary tumor site and in metastases has been confirmed and validated. This has been shown to be true in the Apc1572T model for mammary adenocarcinomas and pulmonary metastases and in the intestinal cancer model Apc$^{+/1638N}$/KRAS^{G12V}.

1. Apc$^{+/1572T}$ mice have proven a unique model for the validation of the CSC model. After having prospectively isolated CSCs from the primary mammary carcinomas by employing the CD29^{hi}CD24^{+} combination of surface antigen markers, we characterized them extensively both by IF and IHC analysis with specific epithelial and mesenchymal markers, and by microarray analysis and compared with normal mammary stem cells. The latter analysis revealed that the differences between MaSCs and MaCSCs are very limited and possibly restricted to few pathways. Moreover, tail vein injections of different subpopulations of tumor cells have confirmed that only the CSCs are able to efficiently form metastases in a broad



spectrum of distant organs, whereas the same type of lesion were never observed when equal amounts of bulk tumor cells where injected into the blood stream.
These results are also being validated with clinical data sets from breast and colon cancer cohorts. In the particular case of *Apc*$^{+/1572T}$ mammary tumors, the signature derived from the CSCs is able to predict breast cancer survival in man (see figure above).

2. As for the Apc$^{+/1638N}$/KRAS^{G12V} model, we have identified a combination of CD markers, namely CD29^{+}/CD24^{hi} which highly enrich for tumor initiating cells or CSCs from the intestinal adenocarcinomas. These cells are enriched in nuclear β-catenin and can form tumors when as few as ~1000 cells are transplanted in NOD-SCID mice. These alleged CSCs have self-renewal capacity as assessed by two rounds of serial transplantations through which the resulting tumors retain the heterogeneity of differentiated lineages observed in the primary tumors. Expression profiling analysis is currently being carried out and the preliminary data seems to indicate that a specific gene signature earmarks and distinguishes CSCs of adenocarcinomas from the corresponding adenoma (benign) cells.
To address the clinical relevance of the above observations, we have been employing the CD133^{+} and CD166^{+}/CD44^{+} combination of CD markers for the prospective isolation of colorectal cancer stem cells from human patients. Due to a number of technical and practical problems (availability of freshly resected specimens, etc) we decided to initiate an alternative strategy based on the use of colo- and mammo-spheres, i.e. suspensions cultures of self-renewing cancer stem cells derived from colon and breast cancer patients. We are now analyzing whether these subpopulations of tumor cells are earmarked by intracellular β-catenin accumulation. The colo- and mammo-spheres have been made available by prof. Fiorgio Stassi (University of Palermo, Italy) and dr. Onno Kranenburg (University of Utrecht).

Analysis of the role of oncogenic KRAS activation in modulating MCSC invasive and metastatic behavior.

This objective has been hampered by a major technical limitation encountered during the implementation of the work plans, namely the lack of reliability and reproducibility of the pepchip platform to identify novel kinases downstream of oncogenic KRAS. Nevertheless, the CSC-specific pattern expression of the villin- KRAS^{G12V} constructs indicate the rate-limiting role played by this oncogene in activating and modulating malignant behavior of the intestinal CSCs in the Apc$^{+/1638N}$/KRAS^{G12V} model, and will be the main subject of future investigations.

Analysis of the role of ß-catenin tyrosine phosphorylation in modulating MCSC invasive and metastatic behavior.

Recently, tyrosine (Y) 654 phosphorylation of β-catenin by activation of receptor tyrosine kinases was shown to increase Wnt signaling *in vitro*. To investigate the *in vivo* relevance of β-catenin Y654 phosphorylation, we generated a conditional knock-in mouse model encoding a modified β-catenin where the endogenous tyrosine at residue 654 is replaced by glutamic acid (E), thus mimicking a phosphorylated tyrosine. Using this model, we have provided *in vivo* evidence that β-catenin^{E654} is characterized by reduced affinity for cadherins and increased signaling activity. Homozygosity for the *β-catenin^{E654}* targeted allele caused anterior truncation resulting in embryonic lethality. Heterozygosity for the *β-catenin^{E654}* allele was sufficient to initiate intestinal tumor development, and to enhance *Apc*-driven intestinal tumorigenesis associated with increased nuclear accumulation of β-catenin. Moreover, we uncovered a thus far unknown mechanism in which Y654 phosphorylation of β-catenin facilitates additional phosphorylation at Serine 675 by PKA. Together, our data provide a novel regulatory network between receptor tyrosine kinases, PKA and β-catenin which modulates Wnt signaling, thereby contributing to intestinal tumorigenesis. We are now evaluating whether the effects of the mutant □-catenin allele are of relevance for the CSCs/MCSCs.

- ***WP2*** *Mutational and functional analysis of metastatic progenitor cells in colorectal and breast cancer*

The implementation of this work package has suffered from delays in the isolation and purification of sufficient amounts of (M)CSCs to allow their mutational and functional analysis. Although the isolation and enrichment of the above mentioned cancer stem cells was successfully achieved, it has been difficult to collect them in the numbers requested for these analyses. Also, the degree of purification obtained (1:500/1500), although sufficient for transcriptome (expression) analysis, was considered insufficient for sequencing analysis. Nevertheless, considerable time and effort was put in the establishment and optimalization of the protocols necessary for these analyses. Also, the consortium plans to implement these plans even if outside the official project time.

Protocols for the isolations of MCSC were developed and further optimized by Partner 5. Protocols for the mutational analysis of the MCSC were developed in Year 1 as previously reported. Also in this case, further optimalization was achieved in the last year. As described in the last report during the final year Partner 5 focused on the completion of WP2.1 by performing molecular and mutational profiling of the MCSC.

In the previous year we generated isogenic human cell models expressing commonly mutated alleles under the control of their own promoters. Specifically we developed isogenic cell lines carrying the following alleles EGFR (delE746-A750), KRAS (G13D), BRAF (V600E) and PIK3CA (E545K and H1047R). These alleles are found in multiple human cancers and have already been successfully knocked-in in human cells by rAAV-mediated homologous recombination. Oncosuppressor genes frequently inactivated in human cancers have also been integrated in these cellular models, as we permanently knocked-down the expression of TP53, PTEN or Rb1 in isogenic cell models harbouring the above mentioned activating mutations on EGFR, KRAS, BRAF or PIK3CA. The result is a genetic matrix combining specific oncogene activating mutations with specific oncosuppressor genes inactivation. Importantly, the genetic matrix includes also the matched wt isogenic cell line (harboring no activating mutations and in which a scramble control shRNA have been introduced) to be compared with the KI/knock-down models described.

These innovative cells models recapitulate the genetic alterations found in primary tumors and likely also in MCSCs. Next the cellular models included in the genetic matrix were validated by verifying the presence at the genomic level and the expression at the RNA level of the corresponding Knocked-in mutations, while the knocking down of oncosuppressor genes have been assessed at the protein level.

As originally planned we focused next on setting up functional assays of the mutated alleles as well as the screening of genotype-specific drugs. Both approaches were successful.

On one side we performed biochemical characterization of the models to assess the effect of specific combined genetic alterations on the biochemical network of non-transformed cells. This led to the definition of the role of specific genetic alterations in cellular transformation, motility and invasion.

At the same time we conducted multiple pharmacogenomic analysis to identify genotype-drug specific relationships. Among other findings we defined oncogenic variants of *PIK3CA*, *PTEN* and *KRAS* as determinants of response to the mTOR inhibitor everolimus. Cancer cells carrying alterations in the PI3K pathway were responsive to everolimus, both in vitro and in vivo, except when *KRAS* mutations were occurring concomitantly or were exogenously introduced. In double mutated (*PIK3CA* and *KRAS*) cancer cells, genetic ablation of mutant *KRAS* reinstated response to the drug. Metabolic profiling showed that *PIK3CA*, but not *KRAS*, mutant cells display everolimus sensitive translation. We then validated the approach by demonstrating that the identified biomarkers have predictive value in patients treated with the same drug. In a cohort of metastatic cancer patients, the presence of oncogenic *KRAS* mutations was associated with lack of benefit after everolimus therapy. Thus, alterations in the *KRAS* and *PIK3CA* pathways may represent biomarkers to optimize treatment of patients with mTOR inhibitors.

In light of the fact that the PIK3CA-PTEN-mTOR pathway is thought to play a central role in controlling the stem-like properties of cancer cells these finding might have relevant implications for targeting tumor stem cells.
Within the frame of the project the isogenic cellular models were also assessed in joint effort between us and partner 5. Dott. Davide Zecchin who developed the isogenic cells in our laboratory spent several months in the laboratory of Partner 5 were he performed in vitro and in vivo studies using the knock-in cell lines to assess the effect of specific oncogenic mutations on mammary stem cells. Dr. Zecchin established mammosphere cultures and in-vitro differentiation techniques as well as orthotopic transplantation of mammary tissue in immunocompromised mice. He then returned to our laboratory were he successfully transferred the in vivo approach.

- ***WP3*** *Identification and functional characterisation of stem cell- and niche-factors from human colorectal mucosa and colorectal cancers*

In the last year we further investigated the role of ZEB1 and miR-200 on stem cell properties and could demonstrate that ZEB1 increases tumorigenicity and the number of potential cancer stem cells by increasing the expression of stem cell factors like Bmi1. This increase was due to a dinished repression of Bmi1 by the miR-200 family.
In cooperation with P4 we performed a DNA methylation array to identify patterns of target genes hyper-and hypomethylated by ZEB1. The epigenetic regulation of candidate genes was now further analysed in particular for histone modifications. This is an ongoing project and will lead to the establishment of an epigenitc map of an EMT/stemness phenotype.
In cooperation with partner 4 the role of the EMT inducer ZEB1 in the differentiation of breast epithelial cells is investigated. Analyses of ZEB1 in disseminated cancer cells was performed. This work is ongoing.

- ***WP4*** *Characterization of epigenetic markers of metastatic progenitor cells in colorectal and breast cancer*

During this third year, WP4 has been mainly centred on the functional studies of epigenetic targets in different experimental systems. Basically, it could be summarized as follows:

1. Evaluation of epigenetic changes after knock in of well-characterized oncogenes in breast cancer cells. This objective has been performed in collaboration with P2.
2. Since a key role for microRNA regulation has been recently proposed in cancer development, we propose to analyse the effect of microRNA machinery impairment on cancer stem cells, using a colon cell line as model.
3. We had developed a new tool for the CpG genome-wide analysis of samples from mouse.

Evaluation of epigenetic changes after knock in of well-characterized oncogenes in breast cancer cells. The laboratory of Dr. Bardelli (P2) has generated stable knock-ins (KI) of well-known oncogenes that are disrupted in colon and breast cancer. Mutations of these genes are well documented in literature, as well as their correlation with epigenetic alterations in cancer. The genes included in the study were: EGFR, PIK3CA, BRAF, KRAS and □-catenin. Mutations were induced in two types of samples: 1) normal breast cell line, cells are immortalized by telomerase activity; 2) in a colon cancer cell line. The role in our laboratory consists on analyzing whether these individuals mutations could affect the epigenome of the cells. In order to asses this issue we studied the status of 27578 CpG covering all the genome. Interestingly, we observed the morphology of the cells of the WT and KI cells in the normal breast cell line really differs depending of the mutation. Different clones for each mutation were available and the morphology was dependent of the mutation. However, cancer cell lines did not change the morphology after the KI (Figure 1)

Figure 1. Morphological aspect of *in vitro* WT and KI normal breast cells

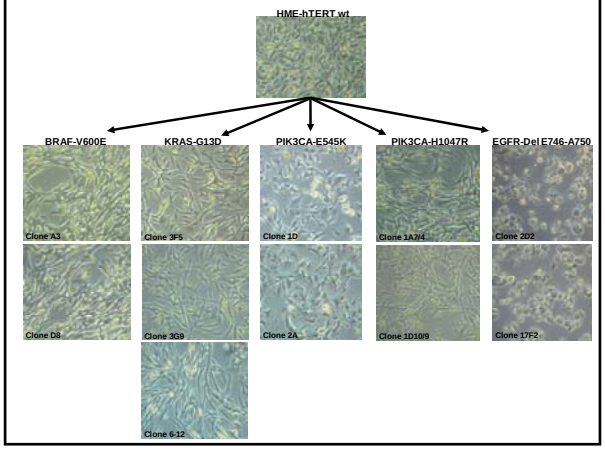
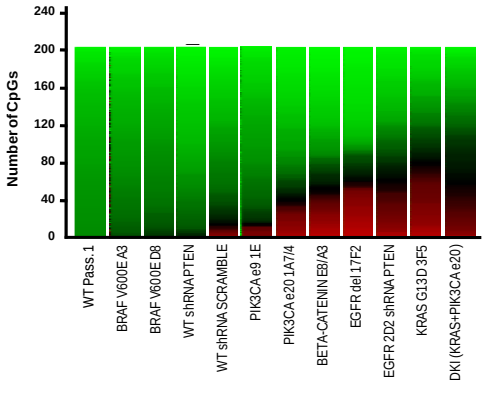


Figure 2. Different oncogenes induce different numbers of hypermethylation.



Data from 208 CpGs hypermethylated (beta >0.75) in at least 1 cell line are shown

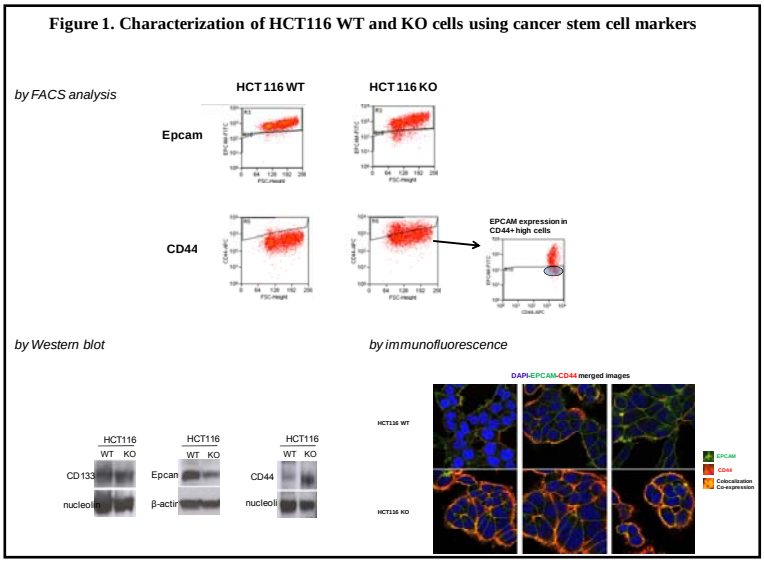
The first observation that we could extract from methylation array analysis as a result of the clustering of the samples is that the mutational effect varies depending of the gene. There were mutations that did not strongly affect the epigenome (i.e., BRAF), in contrast to some KI (i.e., EGFR or catenin) that strongly affect the CpG epigenetic profile. In a second step, we centered our attention in sequences that were hypermethylated after KI on a cancer-related gene because the normal situation in the cell is promoter unmethylation. Again, we could conclude that KRAS, EGFR and B- catenin were the oncogenes that altered mostly the methylation status. Indeed, BRAF does not seem to induce any aberrant hypermethylation (Figure 2). It is important to mention that differentially methylated sequences in KIs from seem to affect at certain some metabolic pathways. There

is not a random representation on the biological function of epigenetically regulated genes after KI. Indeed, there is a noticeable enrichment of homeobox and development-related genes, and also an enrichment of transmembrane receptor and extracellular matrix componets was obtained.

In conclusion, hypermethylation event of CpG promoters as a response of mutation in oncogenes is guided to specific metabolic pathways. In the last step of the research, we have selected specific and well-known cancer-related genes (i.e.,TP73) for bisulfite sequencing confirmation and we could confirm that alterations of CpG promoter methylation are correlated with gene repression. Furthermore, treatment with a demethylation drug (Aza) is associated with a restoration of gene expression.

Effect of microRNA machinery impairment on cancer stem cells. Included in this deliverable, we also performed a study dealing with microRNA epigenetic alterations in cancer stem cells from colon cancer models. Cancer is associated with specific profiles of microRNAs, in general terms tumour showed an overall downregulation of microRNAs. Explanations of this reduction included several failures in post-translational regulation. In our laboratory we also described that the transcriptional silencing of microRNAs in human cancer was associated with hypermethylation of CpG island promoters. Recently we found that a mutation in one enzyme involved in miRNA processing pathway was also responsible of this aberrant profile. But little is known about the role of microRNA and cancer stem cells. As experimental system we selected a colon cancer cell line (HCT116) and the knock-out form for Dicer (a member of the RNAse III family of double-stranded RNAses). After testing the decrease of Dicer we observed that this mutation really has an effect on the miRNAs processing. We want to assess whether the suppression of microRNA production could affect the tumourogenic properties of the cancer stem cells. We first need to isolate the cancer stem cell population of these two cell lines and we had done it by FACS analysis with classical surface markers, such as CD133, CD166, Epcam or CD44. There is a noticeable decrease of Epcam after KO of Dicer. On the contrary, Dicer KO cells showed higher levels of CD44 marker. If we analyzed both the RNA expression levels by qRTPCR and the protein levels by immunofluorescence and western blotting we could conclude that Epcam expression we could conclude that the

Figure 1. Characterization of HCT116 WT and KO cells using cancer stem cell markers



increase of CD44 in KO cells is correlated to lower levels of Epcam surface marker (see Figure 1 above).

We want also to assess the *in vivo* growth suppression properties of KO Dicer cells. Immunosuppressed nude mice were injected with WT cells and KO dicer cells. Suppression of microRNa machinery is accomplished by a tumour reduction. It is not clear which are the pathways underlying this process. It could be hypothesized that CD44 overexpression could contribute to metastasis in the KO mice. However, after 4 weeks, no metastatic nodules in the inner organs were found. Collection of tissues samples were done in order to performed more detailed analysis of possible micrometastais in different tissues.
In the last year, we have developed a custom array for this purpose. With this tool we are actually able to analyze the methylation status of 768 CpG spanning more than 400 genes. The design is represented in the figure. First we collected all the genes that were hypermethylated in different tissue-types of tumours, including breast, colon, pancreas, liver or bladder. The well-known genes that are hypermethylated in, at least, one of this tumour type had been selected. Then, we search for mouse homologues. We select genomic sequences between -750 and +250 bp, that is to say, regulatory regions. With this information, Illumina designs probes and we choose those with the better score values. At the end we have a platform that allows the study of methylation status of 400 mouse genes in 96 samples.

- ***WP5*** *Identification, genomic and functional characterization of metastatic progenitor cells in colorectal and breast cancer*

The focus of the last funding year has been on the evaluation of the microarray data and the development of culture conditions of DTCs from the Balb/NeuT model. Regarding the first aspect, we have developed a novel method to assign functional phenotypes to single DTCs (together with R. Spang, Institute for Bioinformatics, University of Regensburg). While this approach works nicely for single cells, it is linked to the disadvantage that the functional phenotypes are derived from in-vitro models of cell function (e.g. proliferating cells; label-retaining cells etc.). Thus, for each phenotype a new model needs to be established, which is partially ongoing work, since all models are based on the analysis of single cells and not on published signatures. Once all signatures from the models are generated, we will apply classifiers to assign the signature to the DTCs.
To uncover the metastastic potential of DTCs in the Balb/NeuT model, we first tested whether DTC subpopulations can be detected in the bone marrow and found that indeed expression of Cytokeratins, EpCAM, Her2 and Sca-1 is seen in different subsets of cells. To uncover, which cells are able to grow into metastasis, we reasoned that sphere forming ability may serve as functional surrogate marker. Interestingly, DTCs are not able to grow into mammospheres under standard mammosphere assays. However, we were able to establish conditions for

DTCs from the Balb/NeuT bone marrow. First experiments indicate that separation of the surface marker-defined subpopulations enables the identification of the sphere-forming subpopulation. Confirmation and validation experiments are ongoing. In addition to the usefulness of the culture conditions to explore the nature of Balb/NeuT DTCs, we hope that adaptation to human cells will enable controlled generation of human DTC cell lines.

Selected MCSCs publications in year 3

- Fodde R, Tomlinson I. Nuclear beta-catenin expression and Wnt signalling: in defence of the dogma. ***J Pathol.*** 2010 Jul;221(3):239-41.
- Monteiro J, Fodde R. Cancer stemness and metastasis: therapeutic consequences and perspectives. ***Eur J Cancer***. 2010 May;46(7):1198-203.
- Robanus-Maandag EC, Koelink PJ, Breukel C, Salvatori DC, Jagmohan-Changur SC, Bosch CA, Verspaget HW, Devilee P, Fodde R, Smits R. A new conditional Apc-mutant mouse model for colorectal cancer. ***Carcinogenesis***. 2010 May;31(5):946-52.
- Gaspar C, Franken P, Molenaar L, Breukel C, van der Valk M, Smits R, Fodde R. A targeted constitutive mutation in the APC tumor suppressor gene underlies mammary but not intestinal tumorigenesis. **PLoS *Genet***. 2009 Jul;5(7):e1000547.
- Fodde R. The stem of cancer. ***Cancer Cell***. 2009 Feb 3;15(2):87-9.
- Roth S, Franken P, van Veelen W, Blonden L, Raghoebir L, Beverloo B, van Drunen E, Kuipers EJ, Rottier R, Fodde R, Smits R. Generation of a tightly regulated doxycycline-inducible model for studying mouse intestinal biology. ***Genesis***. 2009 Jan;47(1):7-13.
- U. Wellner, Schubert J., Burk U., Schmalhofer O., Zhu F., Sonntag A., Waldvogel B., Vannier C., Darling D., zur Hausen A., Brunton V.G., Morton J., Sansom O., Schüler J., Stemmler M.P., Herzberger C., Hopt U., Keck T., Brabletz S., T. Brabletz. The EMT activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. ***Nature Cell Biol*****,** 11, 1487-95 (2009)

IV. CONCLUSIONS

The results of the work conducted within the MCSCs consortium can be summarized into 5 main points:

- Intracellular ß-catenin accumulation represents a functional marker of CSCs and MCSCs. This is reflected in the activation of the canonical Wnt signaling pathway in these cells, known to elicit self-renewing and EMT while inhibiting differentiation. This is true for *Apc*-driven mouse models of intestinal and mammary tumorigenesis, for the majority of colon cancer cases in man, and for a relatively small group of Wnt-driven breast cancers.

- The generation of isogenic human cell models carrying targeted mutations in (endogenous) genes known to play rate-limiting roles in CSCs represent a powerful tool to conduct multiple pharmacogenomic analyses and identify genotype-drug specific relationships. Given that CSCs and MCSCs are thought to underlie onset and growth of primary cancer and their distant metastases, the development and use isogenic cell lines will be of great relevance in the development of CSC-targeted therapies.

- The ZEB1 gene and the miR-200 non-coding RNA play rate-limiting roles in CSCs. Expression of ZEB1 increases tumorigenicity by increasing the expression of stem cell factors like Bmi1. This increase is due to a diminished repression of Bmi1 by the miR-200 family.

- Specific epigenetic profiles at both the mRNA and miRNA levels characterizes CSCs. The elucidation further characterization of these changes will open novel therapeutic avenues to the use of epigenetic drugs (e.g. methylation- and acetylation-affecting agents) in the clinic.

- Single-cell genomic and expression profiling of DTCs (disseminated tumor cells) coupled with the analysis of their cell surface antigens is likely to lead to the elucidation of the functional heterogeneity that characterize these cells and the primary lesions they are derived from.

V. Dissemination of knowledge

Overview table

Planned/ actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
March 2007 and ongoing	Project web-site: www.mcscs.eu	General public / Scientists	worldwide	variable	All partners
November 2007 and ongoing	Publications in peer-reviewed journals	Scientists	worldwide	variable	All partners
November 2007 and ongoing	Various presentations	General Public / Scientists	Worldwide	Variable	All partnes
November 2007 and ongoing	Various publications	General Public / Scientists	Worldwide	Variable	All partnes
November 13, 2009	Conference: The Stem of Cancer	Scientists	worldwide	350	All partners
November 2007 and ongoing	Posters presentations	Scientists	Worldwide	Variable	All partners

Publications in peer-reviewed journals

1. Alberici P, Gaspar C, Franken P, Gorski MM, de Vries I, Scott RJ, Ristimäki A, Aaltonen LA, Fodde R. Smad4 haploinsufficiency: a matter of dosage. ***Pathogenetics*** 2008 Nov 3;1(1):2.
2. Arena S, Bardelli A. Understanding how kinase-targeted therapies work (2008) ***Cell Cycle***. Mar 30;7(11)
3. Balakrishnan A, Bleeker FE, Lamba S, Rodolfo M, Daniotti M, Scarpa A, van Tilborg AA, Leenstra S, Zanon C, Bardelli A. Novel somatic and germline mutations in cancer candidate genes in glioblastoma, melanoma, and pancreatic carcinoma. ***Cancer Res.*** 2007 Apr 15;67(8):3545-50.
4. Balakrishnan A, Penachioni JY, Lamba S, Bleeker FE, Zanon C, Rodolfo M, Vallacchi V, Scarpa A, Felicioni L, Buck M, Marchetti A, Comoglio PM, Bardelli A, and Tamagnone L Molecular Profiling of the 'Plexinome' in melanoma and pancreatic cancer (2009) Human Mutation. Aug; 30(8):1167-74.
5. Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer.(2010) ***Journal Of Clinical Oncology***. Mar 1;28(7):1254-61. Review
6. Benvenuti S, Frattini M, Arena S, Zanon C, Pierotti M, and Bardelli A. PIK3CA cancer mutations display gender and tissue specificity patterns (2008) ***Hum Mutat***. Feb;29(2):284-8
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13. Di Nicolantonio F, Arena S, Gallicchio M, Zecchin D, Martini M, Flonta SE, Stella GM, Lamba S, Cancelliere C, Russo M, Geuna M, Appendino G, Fantozzi R, Medico E and Bardelli A. Replacement of normal with mutant alleles in the genome of normal human cells unveils mutation-specific drug responses. ***Proc Natl Acad Sci U S A***. 2008 Dec 30;105(52):20864-9. Epub 2008 Dec 23.
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17. Flonta SE, Arena S, Pisacane A, Michieli P and Bardelli A. Expression and Functional Regulation of Myoglobin in Epithelial Cancers. (2009) ***American Journal of Pathology*** . Jul;175(1):201-6.
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28. Monteiro J, Fodde R. Cancer stemness and metastasis: therapeutic consequences and perspectives. ***Eur J Cancer***. 2010 May;46(7):1198-203.
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clinical resistance to EGFR-targeted monoclonal antibodies. (2009) ***Cancer Research***. Mar 1;69(5):1851-7. Epub 2009 Feb 17.
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Various publications

1. Fodde, Riccardo. Stem Cells and Cancer. In: Stem Cell Research. Status, Prospects, Prerequisites. EMBO. April 2006: 35-38.
2. Fodde, Riccardo. Cellule staminali e tumori. In: Stem Cell Research. Status, Prospects, Prerequisites. EMBO. April 2006: 35-38.
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5. Interview with Dr. Thomas Brabletz:
   - o Die tödlichen Ableger des Tumors, in: Handelsblatt, 11.10.2007
   - o Mobile Cancer Stem Cells– The real bad guys?, in: Medical News Today, 26.03.2008
   - o Das Geheimnis der Metastasen, in: Focus Online, 28.03.2008
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Various presentations

1. Brabletz T. Malignant progression in colorectal cancer: EMT, ß-Catenin & cancer stem cells. Paul Basset Memorial Cancer meeting, Strasbourg, France, January 30, 2009.
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3. Brabletz T. Migrating Cancer Stem Cells. XII ASEICA Cancer Meeting, Madrid, Spain, May 7,2009.
4. Brabletz T. Malignant tumor progression: EMT, ß-Catenin & cancer stem cells. Karolinska Institute, Stockholm, Sweden, May 29. 2009.
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6. Brabletz T. EMT and Cancer Stem Cells in Tumor Progression. Cancer Research Centre, Univ. of Edinburgh, UK, June 30. 2009.
7. Brabletz T. Malignant tumor progression: EMT, ß-Catenin & cancer stem cells. FOR942 Workshop 'Wnt-associated networks in tumor progression and development', Univ. Göttingen, Germany, Sept. 1. 2009.
8. Brabletz T. Tumor invasion and Metastasis: EMT, & cancer stem cells. EMBO Workshop 'Invasive Growth: a genetic programme for stem cells and Cancer', Turin, Italy, Sept. 11. 2009.
9. Brabletz T. Tumor Invasion and Metastasis: EMT, & cancer stem cells. Meeting 'Cellular and molecular mechanisms of tumor progression and metastasis', Seeon, Germany, Sept. 21. 2009.
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18. Fodde R, "Wnt/beta-catenin signaling in intestinal and mammary cancer stemness". "Invasion and Metastasis" , Max Delbrück Center for Molecular Medicine (MDC), Berlin-Buch, March 26 - 29, 2008.
19. Fodde R, **"**Wnt signaling and cancer stemness: a matter of dosage**".** Scientific Meeting "Advances in colorectal cancer" Spanish National Cancer Centre (CNIO), Madrid, April 14, 2008.
20. Fodde R, " Specific levels of beta-catenin signalling underlie organ-specific cancer stemness and malignant behaviour". Oncology seminar, MAASTRO clinic, Maastricht, The Netherlands, May 30, 2008.
21. Fodde R, "Cancer Stemness and Wnt/beta-catenin Signaling: A Matter of Dosage". Seminar MD Anderson Cancer Center, Department of Experimental Therapeutics, Houston, Texas, USA, September 2, 2008.
22. Fodde R, "Stem Cells and Colorectal Cancer". Cleveland Clinic Colorectal Cancer Summit, Cleveland, Ohio, USA, September 3-4
23. Fodde R, "Wnt/beta-catenin signaling dosage and cancer stemness". 2nd DKFZ-NCI International Conference on Stem Cells and Cancer and 5th International Heinrich F.C. Behr-Symposium jointly organized with Leopoldina National Academy of Science, German Cancer Research Center, Heidelberg, Germany, October 26-28,2008.
24. Fodde R. 8th International Conference: The Biology of Stem Cells, Paris, France**,** 27- 28 November 2008
25. Fodde R. "Cancer Stem Cells: are you a Feyenoord or Ajax supporter?". Netherlands Cancer Institute, institutional seminar, Amsterdam, The Netherlands, 13 February 2009
26. Fodde R. "Cancer Stem Cells: are you a Feyenoord or Ajax supporter?". NKI-AVL Amsterdam, The Netherlands, 15 April 2009, Seminar in Pathology.
27. Fodde R. "Wnt/ß-catenin signaling and cancer stemness: a matter of dosage". XII National Congress of ASEICA, Madrid, Spain, 7 May 2009.
28. Fodde R. Seminar VIB Department for Molecular Biomedical Research, UGent. Gent, Belgium, 15 May 2009.
29. Fodde R. 21st Meeting of the European Society for Animal Cell Technology **(**ESACT), "Cellular Solutions for Clinical Challenges "Cancer stem cells". Dublin, Ireland, 8 June 2009.
30. Fodde R. "The Colon Cancer Stem Cell and Aspirin". InSiGHT 2009, 3rd Biennial Meeting of the International Society for Gastrointestinal Hereditary Tumours. Dusseldorf, Germany, 25 June 2009.
31. Fodde R. "Intestinal label-retaining cells in homeostasis and cancer". EMBO Workshop, Wnt Signalling in Development and Disease, Arolla, Switzerland, 26-29 August 2009.

32. Fodde R. Keynote lecture: "Cancer Stem Cells: are you a VfL Wolfsburg or Bayern München supporter?". FOR 942 Workshop, Wnt-associated signaling networks in tumor progression and in development, University of Göttingen Göttingen, Germany, 31 August – 2 September 2009.
33. Fodde R. FP6 Strokemap Symposium, Stem Cells: Biology and use in Vascular Brain Disorders Leuven, Belgium, 17 September 2009.
34. Fodde R. Lecture University of Bari. Bari, Italy, 20 October 2009.
35. Fodde R. "Stem cells & Label Retaining cells in Intestinal Homeostasis & Cancer".Josephine Nefkens Symposium: The Stem of Cancer, Rotterdam, The Netherlands, 13 November 2009.
36. Fodde R. "Label retaining and stem cells in homeostasis and cancer". Master Class/ Minisymposium: "Stem Cell Biology incl. immunobiology of (stem) cell therapy", GROW – School for oncology and Developmental BiologyMaastricht, The Netherlands, 20 November 2009.
37. Fodde R. "Intestinal quiescent stem cells in homeostasis and cancer". San Raffaele Scientific Institute (DIBIT), Milan, Milan, Italy, 14 December 2009.
38. Fodde R. "Cancer Stem Cells: are you a Chelsea or Man United supporter?". Newcastle University, Public lecture, Newcastle, UK, 14 January 2010
39. Gaspar, Claudia. Apc1572T: a mouse model encoding for Wnt/b-catenin signaling dosages affecting mammary but not intestinal stem cell homeostasis and malignancy. Presentation at International Workshop on Cancer Stem Cells 2nd edition. IFOM-IEO, SEMM, Milan, Italy, December 1-3, 2007.
40. Klein CA, Will large scale screening lead to the identification to novel therapy targets ESMO 2008.
41. Klein CA, Mutation and selection in cancer progression, International Symposium on Cancer genotypes and Cancer phenotypes, Firenze 2008.
42. Klein CA, From early malignant lesions to manifest metastasis, Barcelona BioMed Confer-ence on Metastasis and Genes Functions Barcelona 2008.
43. Klein CA, Mutation and selection of disseminated tumor cells in cancer progression, Gordon Research Conference 2008
44. Klein CA, Genome and transcriptome analysis of single disseminated cancer cells. ECCO 2007
45. Klein CA. IMPAKT Breast Cancer Conference, Bruessels, Belgium, 2009.
46. Klein CA. International Meeting of the SPP 1190 "Cellular and molecular mechanisms of tumor progression and metastasis", Kloster Seeon, Germany, 2009.
47. Klein CA. ECCO15-34 ESMO, Berlin, Germany, 2009.
48. Klein CA. AACR Breast Cancer Conference, San Diego, USA , 2009.
49. Klein CA. Josefine Nefkens Symposium "The Stem of Cancer", Rotterdam, 2009.
50. Klein CA. The Miami Winter Symposium, Florida, USA, 2010.
51. Klein CA. AACR Translational Cancer Medicine 2010, Amsterdam, Netherlands, 2010.
52. Klein CA. ENBDC annual meeting, Weggis, Switzerland, 2010.
53. Klein CA. 6th International Symposium on Translational Oncology, Barcelona, 2010.
54. Smits, Ron. Wnt/b-catenin Signaling Dosage and Tumor Formation. Presentation at AACR Advances in Colon Cancer Research. Hyatt Regency Cambridge Cambridge, Massachusetts, USA. November 14 - 17, 2007.

Poster presentations

1. Monteiro, Joana. Apc1572T: a mouse model for Wnt-driven mammary cancer Stemness. Poster presented at Advances in Breast Cancer Research AACR Special Conference, October 17-20 October, 2007, San Diego, California.
2. Ghazvini, Mehrnaz. Intracellular b-catenin accumulation and cancer stemness in intestinal tumors. Poster presented at AACR Advances in Colon Cancer Research. Hyatt Regency Cambridge Cambridge, Massachusetts, USA. November 14 - 17, 2007. Scholar in training award (AACR-Astrazeneca award).
3. Roth S, "Isolation and characterization of intestinal label-retaining cells: A quiescent stem cell population? " Dutch Stem Cell Meeting, April 24, 2008, Haren, The Netherlands.
4. Monteiro J, Gaspar C, Franken P, Molenaar L, Breukel C, Van der Valk M, Smits R, Fodde R. "Intracellular b-catenin accumulation underlies cancer stemness and metastatic behaviour in the mammary gland." 2nd DKFZ-NCI International Conference on Stem Cells and Cancer and 5th International Heinrich F.C. Behr-Symposium jointly organized with Leopoldina National Academy of Science, German Cancer Research Center, 26- 28 October, 2008, Heidelberg, Germany.

5. Schmalhofer O, Spaderna S, Schubert J, Burk U, Wahlbuhl M, Strand D, Eger A, Behrens J, Brabletz T. The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. March 17 – March 20, 2008 EMT Meeting Cold Spring Harbor, USA
6. Schmalhofer O, Burk U, Schubert J, Wellner U, Spaderna S, Brabletz T. ZEB1 promotes EMT and invasion of cancer cells by inducing a micro RNA mediated positive feedback loop. March 17 – March 20, 2008 EMT Meeting Meeting Cold Spring Harbor, USA
7. Schubert J, Burk U, Wellner U, Schmalhofer O, Spaderna S, Brabletz T. The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. March 26 – March 29, 2008 Invasion and Metastasis, MDC Meeting Berlin.
8. Burk U, MDC Schubert J, Burk U, Wellner U, Schmalhofer O, Spaderna S, Brabletz T. ZEB1 promotes EMT and invasion of cancer cells by inducing a micro RNA mediated positive feedback loop. March 26 – March 29, 2008 Invasion and Metastasis, MDC Meeting Berlin.
9. Burk U, MDC Schubert J, Burk U, Wellner U, Schmalhofer O, Spaderna S, Brabletz T. ZEB1 promotes EMT and invasion of cancer cells by inducing a micro RNA mediated positive feedback loop. Sept 17 – Sept 20 2008, Meeting Comprehensice Cancer Center, Stanford University, Stanford USA .
10. Schubert J, Burk U, Wellner U, Schmalhofer O, Spaderna S, Brabletz T. The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. Nov 3 – Nov 5 2008, Stem Cells in Development and Cancer.Meeting, Amsterdam, Netherlands.