Publishable summary

Mathematical modelling of β-catenin and ras signalling in liver and its impact on proliferation, tissue organization and formation of hepatocellular carcinomas

1.1 Summary description of the project objectives

The goal of the CancerSys project is to establish a three-dimensional integrative mathematical multi-level model capable of explaining fundamental processes in the formation of hepatocellular cancer. On the tissue level the development of a tumor is modelled from the precursor cell until the tumor extends over several lobules. The three-dimensional architecture of each liver lobule during tumor development is resolved in time and space (Fig. 1). Within each lobule sinusoids, the portal triads and central veins, the hepatocytes and hepatocyte-derived tumor cells, and other important cell types are explicitly modelled. The individual cells are parameterized within single-cell based models by cell-biophysical and cell-biological parameters. The cell-biological parameters include the cell proliferation status and rate, the death rate, the cell micro-motility, the cell polarity etc. They are tracked experimentally during the tumor growth process together with the size of the tumors, the distribution of vessels etc.. This set of parameters – which we name ``process parameters´´ - are used to quantitatively characterize the tumor development process on the cell and tissue scale. In a later step dynamic models of the beta-catenin and ras core modules and their interactions are integrated into each model cell in order to gain insights into processes controlling single cell decisions. Predictions obtained by the mathematical model will be validated by inducible transgenic mice, in which beta-catenin and ras signalling can be manipulated in hepatocytes. In an iterative process the model will be validated and adjusted to the in vivo situation (Fig.1). We will start the description of our main results with SP4 which includes the spatial temporal model. From there we will move on to SP1 – SP3 describing the signalling modules which should later be integrated into the spatial-temporal model and will finally summarize the model validation in vivo.

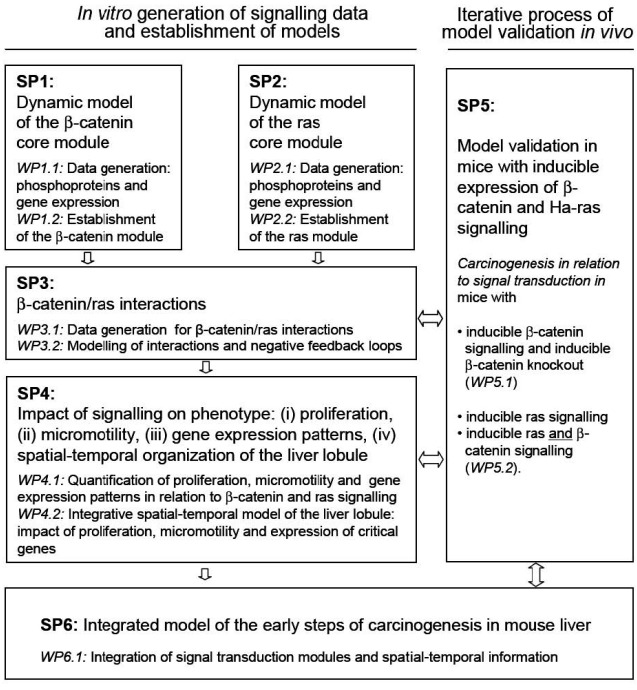


Fig1: *Graphical presentation of the subprojects (SP) and work packages (WP) of CancerSys*

1.2 Establishment of a spatial-temporal model of the liver lobule including proliferation, micro-motility and gene expression **(SP4; WP4.1, WP4.2; SP5; WP5.1, WP5.2)**.

1.2.1 Reference situation

A precondition for modelling of hepato-carcinogenesis at the lobule level is knowledge about the healthy tissue which was needed as a starting situation. Hepatic parenchyma is organised in repetitive functional units called liver lobules, which besides its main constituents, hepatocytes, consists of endothelial cells, Kupffer, stellate and bile duct cells. Branches of the hepatic artery and portal vein guide blood to the periportal regions of the lobules (Fig. 2A). From there it flows through microvessels, the sinusoids along hepatocyte columns that are lined with endothelial cells (generally known as sinusoidal cells), and drains into the

central vein. We established a three-step procedure based on confocal laser scans visualizing hepatocytes and sinusoidal cells (Fig. 2B), image processing and 3 D tissue reconstruction (Fig. 2C-E), and quantitative mathematical modeling (Hoehme et al., PNAS, 2010). This enabled us to extract quantitative information on tissue microarchitecture that would otherwise be inaccessible, such as the 3D spatial-temporal proliferation pattern of hepatocytes and the contact area between hepatocytes and sinusoids (Fig. 2F). The image processing chain presented in Fig. 2 for a single lobule has been extended for many lobules and components have been developed for image analysis of bright field micrographs. So far we have reconstructed the static situation of the healthy liver. In order to include the dynamic behaviour of liver cells into the model we disturbed the situation by induction of pericentral liver damage using the prototypical hepatotoxic compound CCl_4. To quantify the reaction to pericentral necrosis we experimentally determined a number of process parameters after CCl_4 induced necrosis that were measured in a time-resolved manner for up to 16 days. The process parameters include (i) the spatial temporal pattern of cell proliferation, (ii) the average lobule hepatocyte density, (iii) the area of the necrotic lesion, and (iv) the liver lobule microarchitecture, namely, the hepatocyte-sinusoid contact area (Hoehme et al., PNAS, 2010; open access under http://www.pnas.org/content/early/recent). The resulting model can be visualized as a movie which is available at the CancerSys website; http://www.ifado.de/cancersys/publications/index.html. The model unambiguously predicted a so-far unrecognized mechanism as essential for the reference situation, whereby daughter hepatocytes align along the orientation of the closest sinusoid, a process we named “hepatocyte-sinousoid alignment” (HSA). Without considering this mechanism realistic modelling of hepatocarcinogenesis as described in the next paragraph would have been impossible

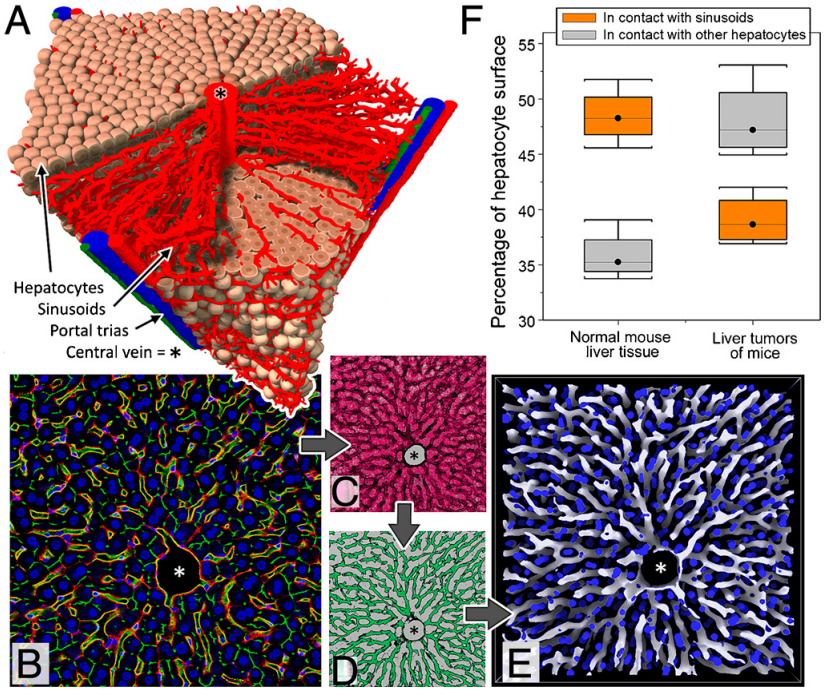


Fig. 2. (A) *Concrete liver lobule inferred from experimental data by the image processing chain shown in (B)–(E) and successive image analysis. Reconstructed lobules served as an initial state for the mathematical model.* ***(B)*** *A typical image obtained by confocal microscopy after adaptive histogram equalization filtering. Blue: DAPI (hepatocyte nuclei); yellow: ICAM þ DPPIV (sinusoids); red: ICAM; green: DPPIV.* ***(C)*** *Effect of generalized erosion filtering (all red pixels will be removed).* ***(D)*** *Effect of generalized dilatation filtering (all green pixels are added).* ***(E)*** *Result of image processing chain in three dimensions. Blue: Hepatocyte nuclei; white: sinusoids. Note the complex architecture that links the periportal zone with the central vein in the middle of the lobule. (F) Fraction of the surface area of hepatocytes in contact with sinusoids (orange) and other hepatocytes (gray) in normal liver tissue and liver carcinomas (from Hoehme et al.,PNAS,2010).*

In order to infer parameter identifiability and to support the experimental design, we developed a new method for structural and practical identifiability analysis (Raue et al., Bioinformatics 2009), which is applied thoughout the project.

1.2.2 Modelling of hepatocellular carcinogenesis **(SP4; WP4.1, WP4.2; SP5; WP5.1, WP5.2), SP6, WP6.1).**

Based on the above described “reference situation”, we next modified parameters in the model that might be relevant for hepatocarcinogenesis. Modelling led to three predictions: (i) Elimination or reduction of the physiological process HSA leads to microstructural alterations by which the healthy liver microarchitecture characterised by columns of hepatocytes is compromised and replaced by hexagonal cell structures typically found in tumors (see video on website: http://www.ifado.de/cancersys/publications/index.html). (ii) As expected tumor-like structures were obtained if the proliferation rate of an individual hepatocyte was increased. However, spatial-temporal modelling demonstrated that it is highly relevant whether increased proliferation occurs in hepatocytes with compromised or normal cell polarity. If polarity is compromised a tumor structure with cell clusters as shown in Fig. 3 will result. (iii) If cell polarity remains unaltered a completely different cell structure will result that resembles scrunched up columns of hepatocytes (see video on website) We outline these simulated scenarios, because they could later be validated in vivo (see **SP5**).

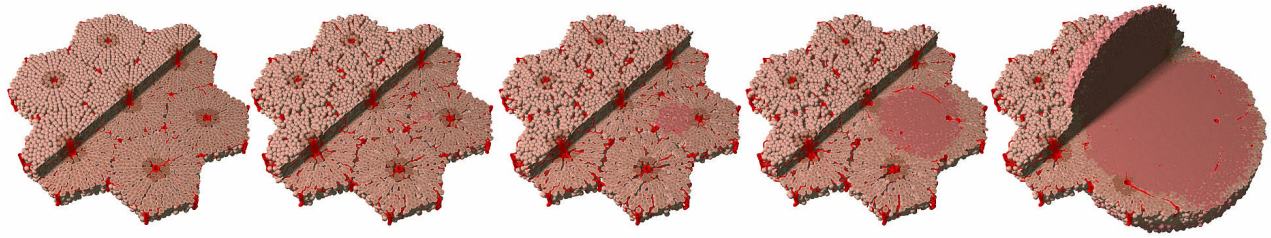
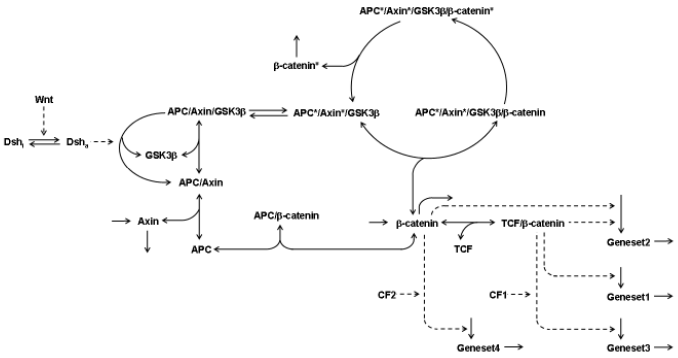


Fig 3: *Spatial-temporal model of hepatocarcinogenesis. The simulation shows tumor growth of unpolarized cells. The corresponding video and details are available at the CancerSys website. Our current software also permits to take into account the irregular size of the individual liver lobules, see report of WP 4.2.*

1.2.3 The signalling modules β-catenin and ras and their interactions **(SP1; WP1.1, WP1.2, SP2; WP2.1, WP2.2, SP3; WP 3.1; WP3.2.)**



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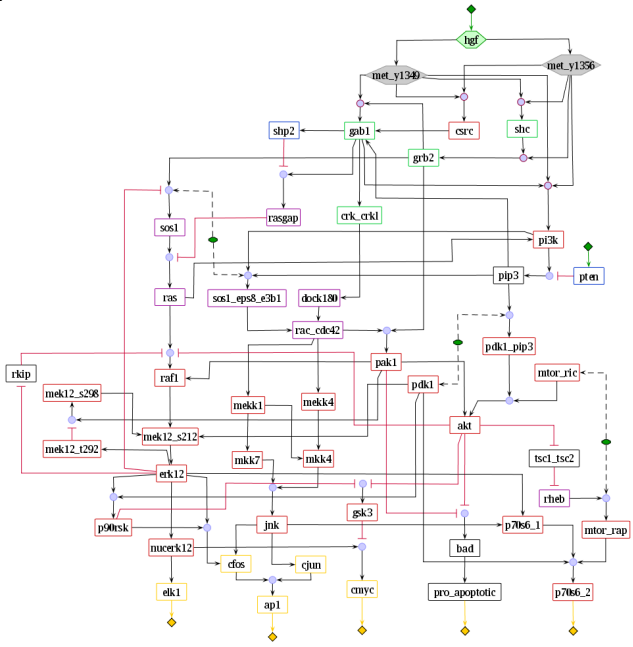

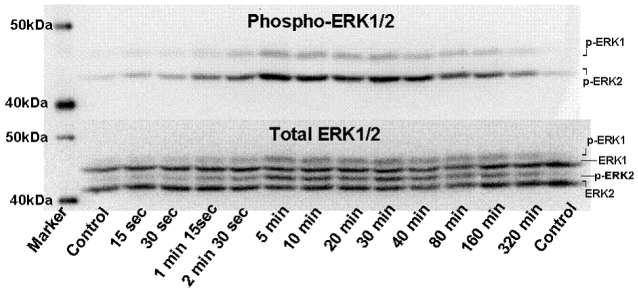

4C 4B

Fig. 4 (A) *Model structure of Wnt/β-catenin siganling relevant for driving target gene expression(see CancerSys website).* ***(B)*** *A manually curated HGF signalling pathway map for mouse primary hepatocytes. There are 84 nodes and 114 edges. The red arrows are the inhibitory interactions. (see CancerSys website)* ***(C)*** *Time resolved immunoblot showing activation of ERK after stimulation by HGF*

Signalling core modules of the β-catenin (Fig. 4A) and the ras (Fig. 4B) networks were established based on literature data and on quantitative immunoblotting. A representative example of time resolved analysis of HGF induced ERK1/2 activation by quantitative immunoblotting is shown in Fig 4C. Modelling with whole genome expression profiling from hepatocytes with activated beta-catenin or activated ras signalling identified negative interactions between both pathways. Signalling through β-catenin leads to attenuation of expression of genes that are positively regulated by the ras signaling module and vice versa. The model predicted several DUSP proteins as probable candidates for the negative β-catenin/ras interactions. Currently, validation experiments with siRNA knockdown of the candidate DUSP proteins are performed. To generate time-resolved data on Ras/MAP kinase and PI3 kinase signalling pathways we have established functional fluorescently-tagged variants of components of the signalling cascades and expressed these variants in primary hepatocytes. The mCherry-tagged AKT is functional and shows time and dose dependent phosphorylation and membrane recruitment upon HGF stimulation in (normal) primary hepatocytes as well as in a hepatoma cell line. Together with SP4;5 the members of SP1-3 have studied the influence of MAP-kinase and β-catenin signalling on the hepatocyte phenotype in vitro (SP4) and in vivo (SP5). Overexpression of a constitutively active form of ras causes massive ERK 1/2 phosphorylation, features of epithelial to mesenchamal transition and enhanced S-phase entry (Godoy et al., 2009). However, when we overactivated MAP kinase signalling in livers of mice this did not lead to immediate proliferation of the hepatocytes. A similar in vivo/in vitro discrepancy was obtained for β-catenin signalling. When β-catenin is overexpressed in hepatocytes in vivo as a consequence of an inducible APC knockout this leads to proliferation and hepatomegaly within one week. However, in vitro siRNA knockdown of APC (also leading to overexpression of β-catenin dependent genes) was not sufficient to induce proliferation. Therefore, we first have to understand the reasons for the obtained in vitro/in vivo discrepancies and to improve the corresponding signalling core modules, before we can integrate the latter into a multi-scale spatial-temporal model of liver carcinogenesis.

1.2.4 Model validation in vivo **(SP5; WP5.1, WP5.2)**

For model validation of β-catenin signalling we used a tamoxifen inducible transgenic mouse, where knockout of exon 14 of the APC gene causes overexpression of β-catenin (Fig.5A).

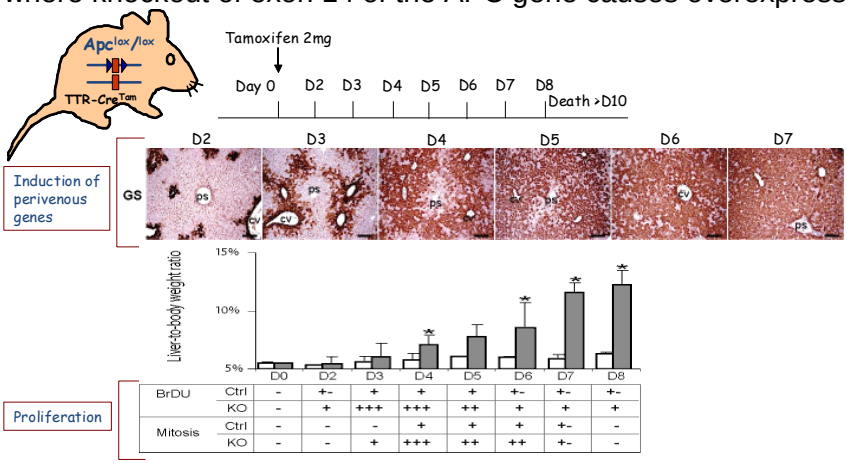

Figure 5A: *Kinetic analysis of the consequences of Apc loss in the mouse liver*

When β-catenin overexpression is induced in virtually all hepatocytes this leads to proliferation and hepatomegaly within 7 days. A spatial-temporal model of this scenario has been established (see CancerSys website). When, in contrast, the APC knockout is induced in a small fraction of hepatocytes this leads to hepatocellular carcinomas within 2 to 6 months. We observed two histologically distinct types of hepatocellular carcinomas, namely poorly and well-differentiated tumors (Fig. 5B).

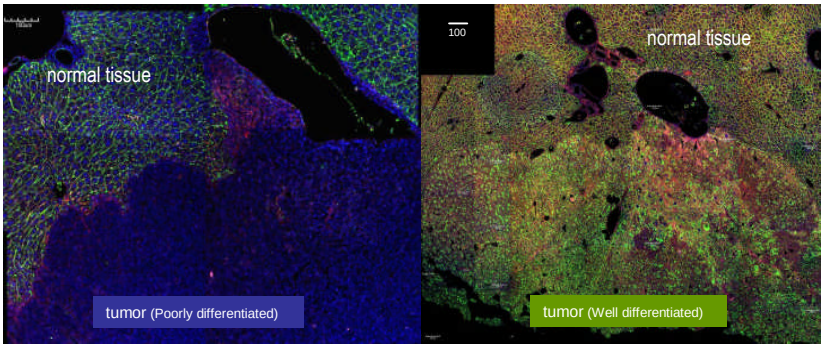

Figure 5B: *Comparison of DPPIV staining (green) and ICAM staining (red) in poorly and well differentiated hepatocellular carcinomas. (DAPI, blue).*

Interestingly the poorly differentiated carcinomas correspond to the model simulation with compromised hepatocyte polarity, whereas the well differentiated tumor type, resembles the "crunched up column" architecture obtained by the model simulation with intact hepatocyte polarity (see chapter 1.1.2). Therefore, we immunostained both tumor types for hepatocyte polarity markers. Indeed DPPIV as a bile canalicular polarity marker was expressed in the well-differentiated tumors, which showed a polar cell morphology, in contrast to the poorly differentiated carcinomas where DPPIV was found to be completely deleted (Fig.5B)

References:

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