Chapter 6

Modulation of Gene Expression During Stages of Liver Colonization by Pancreatic Cancer in a Rat Model

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Additional information is available at the end of the chapter

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is known for its early spreading of tumor cells into the liver. The aim of this study was to investigate the modulated gene expression of PDAC cells during liver colonization. To that purpose, ASML rat pancreatic cancer cells marked with enhanced green fluorescent protein were inoculated into the portal vein of isogenic BDX rats and reisolated from livers by fluorescence-activated cell sorting sorting at early (1, 3 days), intermediate (9 days), advanced (15 days), and terminal (21 days) stages of liver colonization. Reisolated ASML cells were used for total RNA isolation and subsequently their gene expression was investigated by Illumina chip array for mRNA and miRNA species, followed by Ingenuity Pathway Analysis (IPA). Following reisolation, 7–20% of genes and 10% of miRNA species were modulated significantly in expression during the early stage of liver colonization and continuously thereafter. These overall changes led to distinguish certain categories and processes participating in cancer progression. The knowledge of these alterations in gene expression will suggest targets, which could be used for new diagnostic procedures as well as for combating liver metastasis successfully.

Keywords: pancreatic cancer, liver metastasis, ASML cells, isogenic rat model, modulation of gene expression, stages of liver colonization

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive cancer [1, 2], as derived from the respective 5-year survival rate of about 6% [3, 4]. This poor prognosis results, at least in part, from a delayed diagnosis of the disease. All therapeutic efforts during the past 50 years,

such as surgery, radiation therapy, chemotherapy, or combinations thereof, have shown little impact on the course of this aggressive neoplastic disease. It is hoped, however, that a full understanding of the molecular biology of pancreatic cancer will help in diagnosing, preventing, and treating this cancer.

A most recent study on pancreatic cancer patients describes genetic details, which have been detected through full genome sequencing and copy number analyses in 100 PDACs. Genetic drivers known for pancreatic cancer, such as K-ras, TP53, SMAD4, and mutations in CDKN2A, were confirmed, whereas newly identified mutations, such as in the KDM6A gene, highlighted the role of chromatin modifications [5]. Based on familial clustering studies, it was estimated that 10% of PDAC cases are linked to an inherited predisposition [4, 6, 7]. Germ line mutations were especially found in tumor suppressor genes, such as INK4A, BRCA2, and LKB1, the mismatch repair gene MLH1, and the cationic trypsinogen gene PRSS1 [8, 9]. These germ line mutations are responsible for onset and penetrance of PDAC, but they likely contribute more to the malignant progression of precursor lesions than to cancer initiation [10, 11]. Of course, exogenous factors, such as cigarette smoking, constitute a measurable risk, as well [12].

In the early stage of PDAC, the genetic mutations of oncogenes like K-ras and suppressor genes like TP53—or both—lead to constitutive activation of transcription factors, but in the late stage stress factors such as acidosis and hypoxia, which are frequently encountered in the tumor microenvironment, further deregulate the expression of metastasis-related proteins. PDAC metastasis is a progressive, debilitating disease that is characterized by pain, asthenia, anorexia, and cachexia. The formation of metastasis is a complex and progressive process, including four basic steps: dissociation of cells from the primary tumor, existence and survival in the circulatory system, break down and degrading of endothelium and basement membranes in target organs, and (d) establishment of a colony of metastatic cells [13].

Currently, it is hoped that new findings related to K-ras, the tumor's unique metabolic needs, and how the stroma and immune system affect the PDAC, will change the overall situation for the better [14].

In order to better understand the PDAC metastatic process, a liver metastasis model was developed by our group [15]. This model focused on the final steps of metastasis, which are related to organ colonization. To that purpose, ASML rat PDAC cells, which had been marked by luciferase ($ASML^{luc}$ [15]) were implanted intraportally into isogenic BDX rats for mimicking the final phase of liver metastasis. This process of liver colonization was related to the size of the liver and the remaining life span of a given animal. Thus, for reisolation of tumor cells from rat liver, four periods were selected and classified as early (days 1, 3), intermediate (day 6), advanced (day 15), and terminal (day 21) stages. Also, tumor cells were recultivated in vitro after their reisolation at 21 days for three additional periods (3, 6, and 9 days) to define the microenvironment effect on gene expression after residing 21 days in rat liver. Reisolated ASML tumor cells were used for total RNA isolation and then investigated by Illumina chip array for genes and miRNAs, which show modulation of their expression during liver colonization. These modulations were evaluated by Ingenuity Pathway Analysis (IPA) as described in the study of Al-Taee et al. [16].

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The aim of this study was to establish a rat model for identifying and understanding pathophysiological processes during metastatic liver colonization, as well as for finding new and specific tumor markers as tools for diagnostic and therapeutic approaches. Here, we report on the modulation of gene expression during stages of liver colonization by ASML pancreatic cancer cells in rat liver.

2. Gene expression modulation in ASML rat PDAC cells during liver colonization

2.1. Reisolation of tumor cells from liver tissue

ASML cells, which had been implanted to BDX rats, were reisolated after various periods. The purity of these cells was based on a procedure, which involved gradient centrifugation as first step followed by FACS sorting of eGFP-labeled tumor cells. Pending on the number of tumor cells, the final grade of purity varied from 90% (day 1) to 99% (day 15) based on 1.6 to 52% of tumor cells present in the cell suspension after gradient centrifugation.

2.2. mRNA modulation of ASML cells colonizing rat liver

2.2.1. Fold changes in mRNA expression

In order to analyze the mRNA modulation, which occurs in ASML pancreatic cancer cells colonizing the rat liver, the gene expression profiles of ASML cells were determined by microarray in five batches of tumor cells that had been reisolated from rat liver after 1, 3, 6, 15, and 21 days post injection into the portal vein. For each of the five batches, representing different stages of liver colonization, 23,400 genes were analyzed in total. In an attempt to categorize the observed changes in gene expression, two parameters were chosen initially: the fold change in mRNA expression over that of respective genes in control ASML cells growing in vitro, and the time until reisolation of tumor cells from rat liver. The gene expression profile from cells isolated after days 1 and 3 was classified as reflecting early colonization, as no tumor burden was visible with naked eye. On day 6, the ASML cells showed signs of infiltrative growth in the rat liver, visible as white spots of 1–2 mm in diameter and were discernible macroscopically. The corresponding gene profile was classified as intermediate colonization. At 15 days post injection, the ASML cells colonized about 40% of the rat liver and the tumor spot size increased to ~5 mm in diameter. The respective gene expression profile was considered as advanced colonization. At 21 days post injection, ASML cells almost completely infiltrated the rat liver and the corresponding gene expression profile was classified as terminal stage. An overview for the two categories, the fold changes in mRNA expression and the respective stage of liver colonization, is shown in **Figure 1**.

The observed modulation ranged from more than 2-fold to more than 20-fold, with a similar distribution pattern regarding decreased and increased levels, thus resulting in a curve resembling a normal (Gaussian) distribution. This distribution was similarly bell shaped over

all stages, thus indicating little overall variation between early and late colonization stages. There was one exception, however, regarding mRNAs with more than 20-fold increased or decreased levels: the former were distinctly more prevalent than the latter at the early stage, thus indicating a possible need for the strong activation of genes initially after tumor cell implantation.

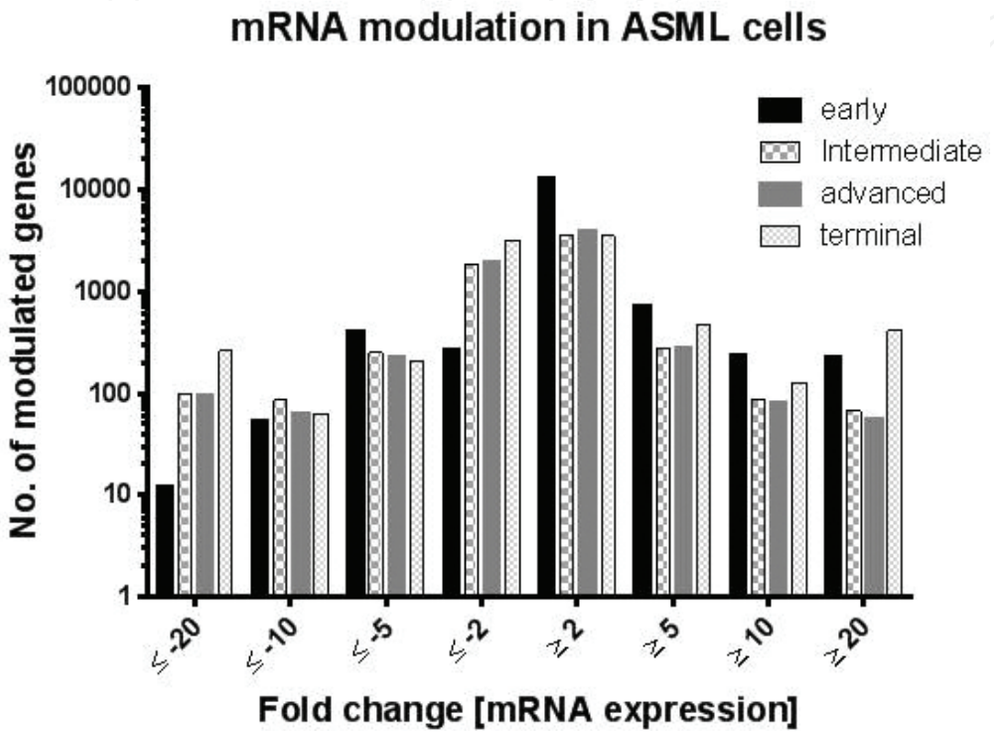

Figure 1. mRNA modulation in rat pancreatic cancer cells colonizing rat liver: Given is the fold change of mRNA expression of reisolated ASML cells during early, intermediate, advanced, and terminal stages of liver colonization relative to control cells growing in vitro.

2.2.2. Types of mRNA modulation

On a single gene level, several types of modulation were found during the different stages of colonization. Some examples are shown in **Figure 2**.

These included a steady increase in expression of certain genes, as seen for matrix metalloproteinase-2 (MMP-2) and ephrin type-A receptor 8 (Epha8), as well as a steady decrease, as observed for cadherin 11 (Cdh11) and 5-hydroxytryptamine (serotonin) receptor 5B (Htr5b) (see **Figure 2A**).

Another type, typical of an immediate increase or decrease in gene expression followed by a persistent plateau was observed as well. This type of modulation was seen for the expression levels of claudin-1 (Cldn1) and insulin-like growth factor 2 (IGF2), which showed a sustained increase, whereas transforming growth factor alpha (TGF-α) and MDM2 proto-oncogene/E3 ubiquitin protein ligase (Mdm2) showed a sustained decrease (see **Figure 2B**).

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A third type of altered gene expression was classified as early type responsive gene (e.g. insulin-like growth factor binding protein 2 (Igfbp2) and keratin, type I cytoskeletal 42 (Krt42)) or late type responsive gene (e.g. lipolysis-stimulated lipoprotein receptor (Lsr) and family with sequence similarity 132, member A (Fam132a)), which was either activated greatly in the early stages of colonization and later showed a somewhat reduced expression or they started with a mildly increased expression level and showed intensely increased expression only at the end of the colonization period (see **Figure 2C**).

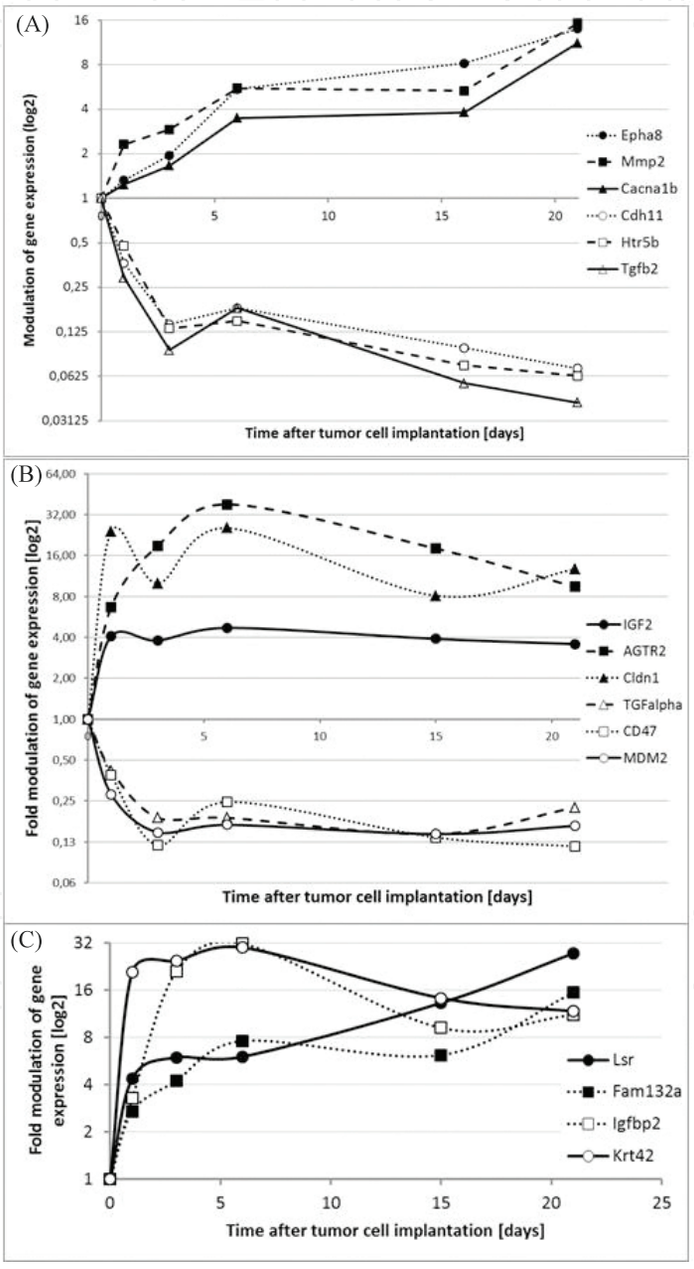

Figure 2. Types of gene expression modulation found during the different stages of liver colonization: given are examples of steady increases in expression (**A**), immediate increases or decreases in gene expression followed by a persistent plateau (**B**), and early or late type responsive genes (**C**).

Categories	Cellular movement, Hematological system, Development & function, Immune cell trafficking, Inflammatory response		Cellular development, Growth & proliferation, Connective tissue / Nervous system develop-ment & function, Tissue development		Tissue development	Lipid metabolism, Small molecule biochemistry
Diseases or Functions Annotation	movement of mononuclear leukocytes	migration of phagocytes	proliferation of fibroblasts	proliferation of neuronal cells	growth of connective tissue	fatty acid metabolism
No of genes	121	80	93	77	158	158
Early stage	Increased	Increased	Increased	Increased	Increased	Increased
p-Value	4,96E-23	6,82E-20	7,67E-19	1,30E-08	1,09E-24	1,65E-25
Activation z-score	2,812	2,563	2,436	2.461	3,143	2,751
Intermediate stage	-	not significant	not significant	Decreased	decreased	not significant
p-Value				6,24E-13	8,00E-14	
Activation z-score				-2.949	-2,034	
Advanced stage	not significant	not significant	not significant	not significant	Decreased	-
p-Value				5,47E-14	5,93E-16	
Activation z-score				-1,098	-2,159	
Final stage	Increased	Increased	Increased	-	Increased	Increased
p-Value	5,40E-30	3,21E-23	1,69E-14		1,26E-20	2,63E-05
Activation z-score	3,614	3,623	2,319		2,623	2.733

Categories	Cell cycle	Hematological system development & function, Tissue morphology	Inflammatory response	Cell-to-cell interaction, Cellular function , Inflammatory response	Cancer, organismal injury & abnormalities, Tumor morphology
Diseases or Functions Annotation	M phase of tumor cell lines	quantity of myeloid cells	immune response of cells	phagocytosis of cells	invasion of tumor
No of genes	26	78	107	56	51
Early stage	Increased	Increased	Decreased	not significant	Decreased
p-Value	5,68E-10	2,34E-16	3,56E-13	8,14E-13	2,04E-14
Activation z-score	2,123	2,498	-2,231	-2,583	-2,156
Intermediate stage	Increased	Increased	Decreased	Decreased	not significant
p-Value	1,07E-12	2,34E-16		2,83E-16	
Activation z-score	2.486	2,498		-2.574	
Advanced stage	-	Increased	Decreased	Decreased	Decreased
p-Value		3,57E-15	1,74E-14	4,25E-15	1,52E-10
Activation z-score		2,084	-3,068	-2.406	-2.301
Final stage	-		not significant	not significant	not significant
p-Value					
Activation z-score					

Table 1. Some categories and related diseases or functions annotations.

2.2.3. Categories of the tumor cell genome showing modulation

By Ingenuity Pathway Analysis (IPA), a more comprehensive way of categorizing altered gene expression was realized. In this analysis, several possibilities of grouping or ordering genes were followed. Initially, some general cellular categories were chosen, which encompass a limited number of more specific disease or function annotations. These, in turn, are characterized by a distinct number of genes participating in the disease or function. The overall status

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of these genes (i.e. increased in expression or decreased in expression) is used to coin an activation z-score, which is considered significant if higher than 2 (i.e. activated) or lower than −2 (decreased). In addition, a p value indicates the significance of the alteration of the respective process (**Table 1**).

Based on this analysis, the category "cellular movement" with the two function annotations "cell movement of mononuclear leukocytes" and "migration of phagocytes" with 121 and 80 genes, respectively, was significantly increased at early and terminal stages, but not significantly altered at intermediate and advanced stages. This is surprising, as it shows that movement or migration of cells is not equally important over the whole colonization period. Although this category of genes is increased initially, soon thereafter it is deactivated and reactivated only for the terminal stage (**Table 1**, top part, column 1).

Another example is the category of genes related to proliferation. As could be expected, genes belonging to this category were augmented in expression initially, as derived from the significantly increased activation z-score (2.44), but failed to show activation at intermediate and advanced stages. At these stages, genes related to neuronal cell proliferation were even decreased in expression or showed no activation. At the terminal stage, there was activation of genes related to the proliferation of fibroblasts, but not of genes related to proliferation of neuronal cells (**Table 1**, top part, column 2).

Genes related to the growth of connective tissue, fatty acid metabolism, M-phase of tumor cell lines, and quantity of myeloid cells were mainly upregulated but not throughout the whole liver colonization process. In addition, genes related to the immune response, phagocytosis, and invasion were decreased in activation (**Table 1**, top and bottom parts, columns 3–9).

2.2.4. Modulation of acute phase signaling chain genes

Subsequently, Ingenuity Pathway Analysis (IPA) was used for overlaying the findings of this study onto scaffolds of known genetic relationships. This included a scaffold for the genes of the acute phase signaling chain, which was overlaid with gene expression levels obtained at intermediate (day 6 after tumor cell implantation, **Figure 3A**) and terminal (day 21 after tumor cell implantation, **Figure 3B**) stages.

These figures show a differentiation of genes according to cellular compartments as well as to colors as indicator of expression levels. Symbols, which have been placed to the specific cellular or extracellular sites of the respective proteins, are used to report on a specific gene expression. A colored border line of the respective symbol indicates that data from the present analysis were available to describe the status of the gene. The colors used for filling the symbol denote differences in expression: red color indicates increased expression and green color indicates decreased expression, with less intense colors hinting to less pronounced increased or decreased expression.

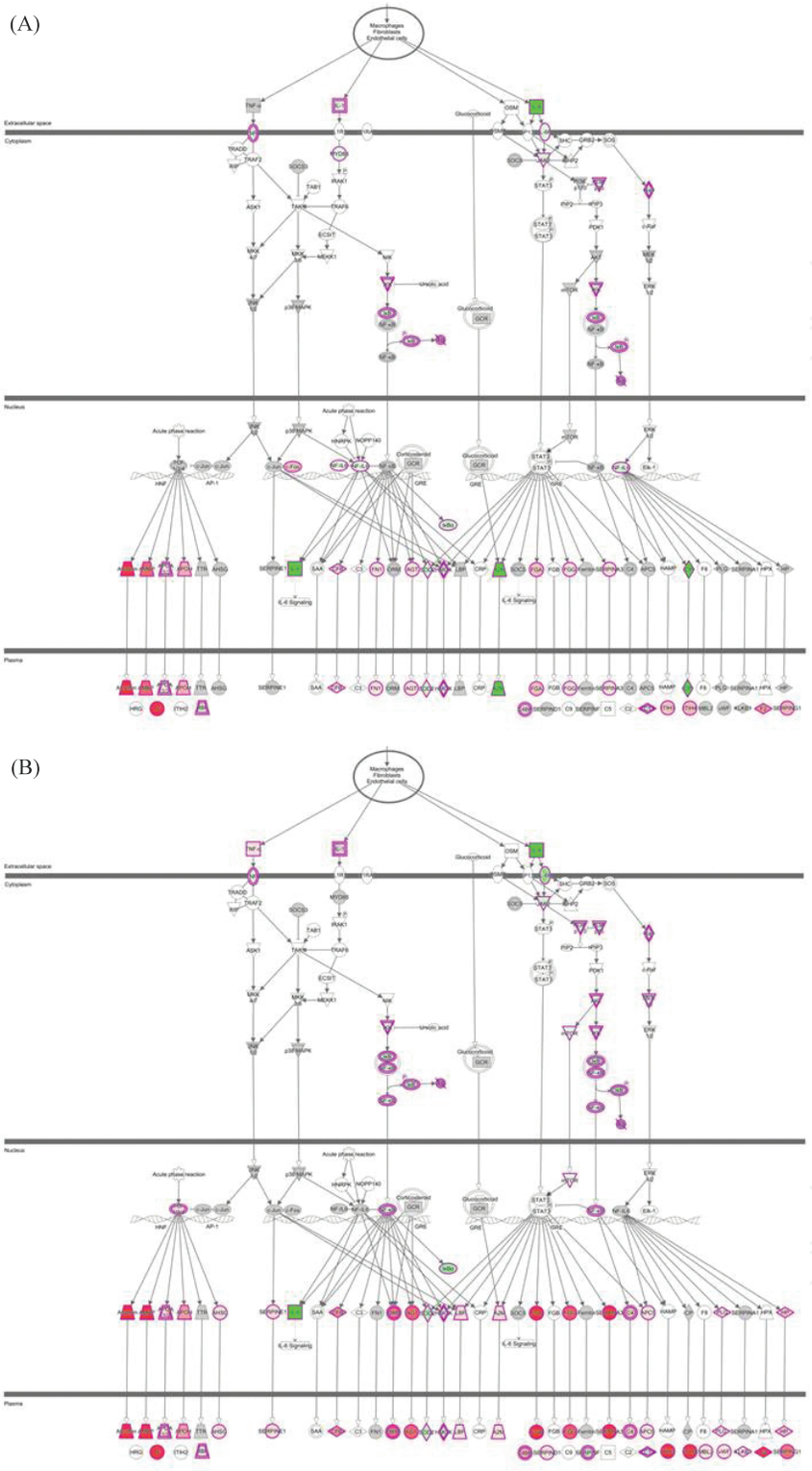

Figure 3. A) Overlay of ASML cell gene expression (day 6) on acute phase signaling chain. **B**) Overlay of ASML cell gene expression (day 21) on acute phase signaling chain.

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On day 6 after tumor cell implantation, two genes (albumin and transferrin) of the acute phase signaling chain are highlighted in intense red color and three genes (interleukin 6, α-2-macroglobulin, and ceruloplasmin) in intense green color. Fifteen more genes are shown by red colors of lower intensity, indicating submaximally increased expression levels. In addition, 14 genes are highlighted in a green color of reduced intensity, indicating less than maximally decreased expression (see **Figure 3A**).

At the terminal stage, eight genes (albumin, transferrin, α-1-microglobulin, fibrinogen alpha chain, serpin family A member 3, inter-alpha-trypsin inhibitor heavy chain H3, inter-alpha-trypsin inhibitor heavy chain H4, and coagulation factor II (thrombin)) were highlighted in the most intense red color and 21 more genes in less intense red colors. Concomitantly, only 1 gene (interleukin 6) was highlighted in intense green color and 15 in less intense green colors (see **Figure 3B**), indicating a shift from reduced expression of acute phase signaling genes at the intermediate stage to increased expression at the terminal stage of liver colonization.

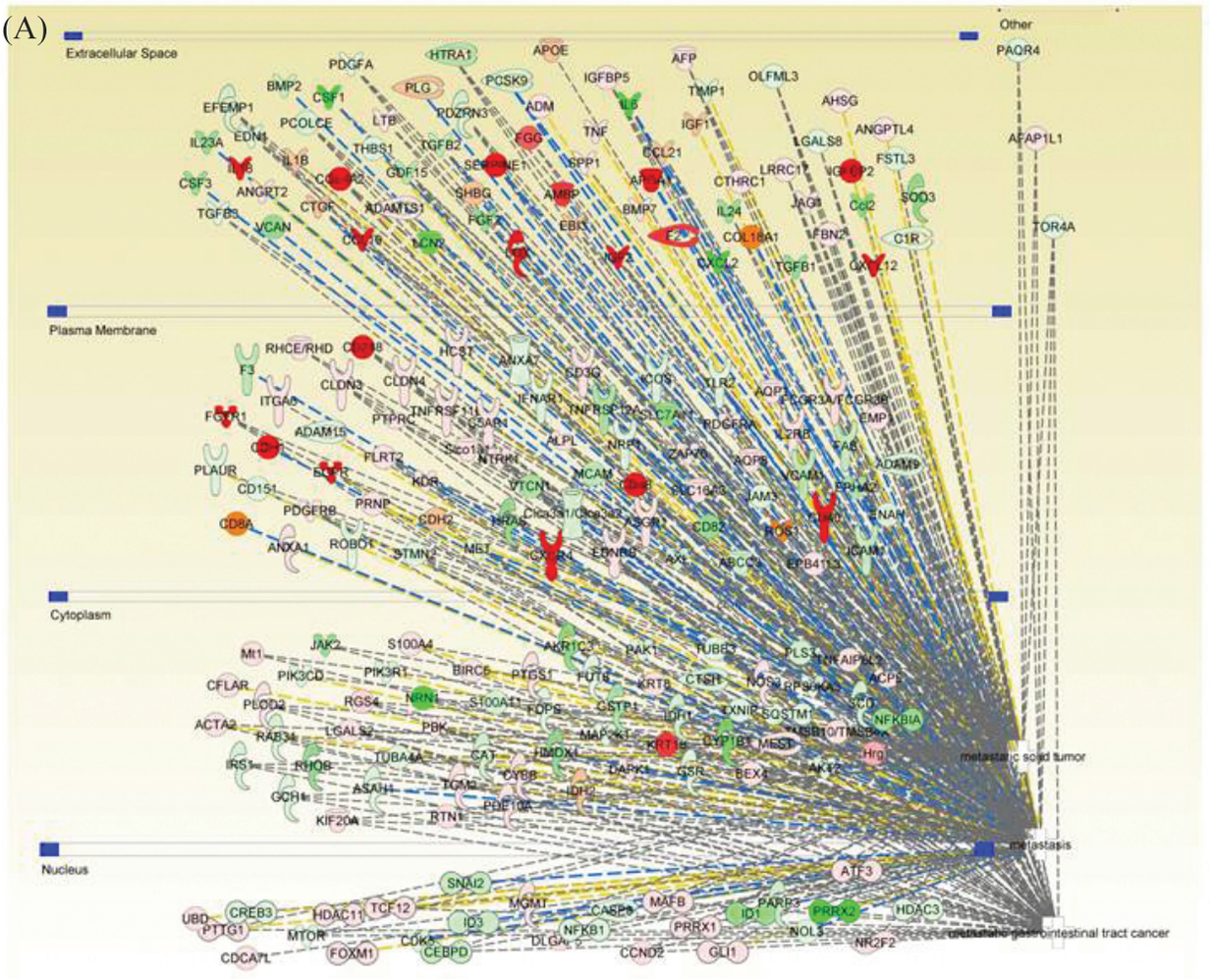
2.2.5. Modulation of metastasis associated genes

In addition to using genes of the acute phase signaling as scaffold, another overlay of was done by IPA analysis at the terminal stage of liver colonization for the three signaling cascade scaffolds known as "metastatic solid tumor," "metastasis," and "metastatic gastrointestinal tract cancer" (**Figure 4**). Because of the large overlap between the corresponding genes, all three scaffolds were combined in one scheme, resulting in a picture, in which the three scaffold designations are connected with their respective genes by straight lines. For clarity, the gene symbols are arranged according to their cellular site, i.e. extracellularly, in the plasma membrane, in the cytoplasm, or in the nucleus. In addition to the red/green color scheme known from **Figure 3A** and **B**, the straight lines are colored by yellow and blue colors, which indicate activation or inhibition of the respective gene products activity. Thus, very often a gene highlighted in a reddish color, which indicates increased expression over control, will be connected with its scaffold designation by a yellow line, indicating activation of the respective group of genes as given for the scaffold designation "metastatic solid tumor" and the gene expression of collagen, type IV, alpha 2 (Col4a2), or of coagulation factor II, thrombin (F2). This means that the increased expression of Col4a2 or F2 predicts activation of a significant share of related genes. Conversely, a gene highlighted in green color, which indicates decreased expression versus control, is sometimes connected with its scaffold designation by a straight blue line, indicating inhibition of the respective group of genes, as given for the scaffold designation "metastatic solid tumor" and the gene expression of colony stimulating factor 1 (Csf1) or interleukin 6 (IL-6). This means that the decreased expression of Csf1 or Il6 predicts inhibition of a significant share of related genes.

In addition, a gene symbol colored reddish can be connected to the scaffold designation by a yellow line, indicating that its increased expression is related to inhibition of the corresponding gene group, as for the genes apolipoprotein A1 (Apoa1) and serpin family E member 1

(Serpine1). This means that the increased expression of APOA1 or Serpine1 predicts inhibition of a significant share of related genes. Finally, a gene symbol colored greenish can be connected to the scaffold designation by an orange line, as for the genes superoxide dismutase 3 (Sod3) and TIMP metallopeptidase inhibitor 1 (Timp1). This implies that the decreased expression of Sod3 or Timp1 predicts activation of a significant share of related genes (**Figure 4A**).

As **Figure 4A** gives an overview on a multitude of genes being connected to the three signaling cascade scaffolds known as "metastatic solid tumor," "metastasis," and "metastatic gastrointestinal tract cancer," which allowed to show less details, a more complete description of a selection of metastasis genes is given in **Figure 4B**. This figure shows the respective fold changes in mRNA levels versus control as positive or negative numbers below the corresponding gene symbols.



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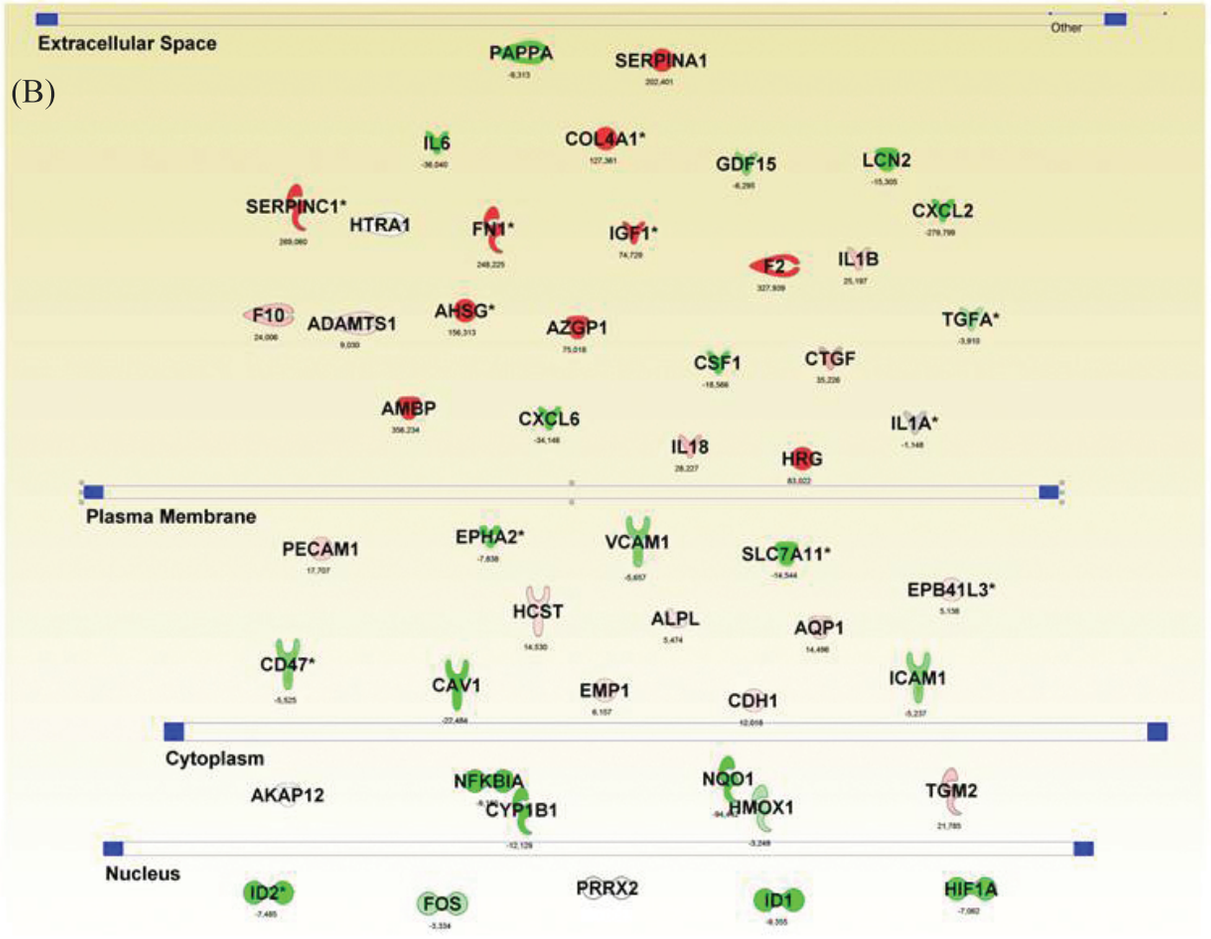

Figure 4. A) Overlay of ASML cell gene expression (day 21) on the three signaling cascade scaffolds termed "metastatic solid tumor," "metastasis," and "metastatic gastrointestinal tract cancer." The three scaffold designations are connected with their respective genes by straight lines. For clarity, the gene symbols are arranged according to their cellular site, i.e. extracellularly, in the plasma membrane, in the cytoplasm, or in the nucleus. In addition to the red/green color scheme known from **Figure 3A** and **B**, the straight lines are colored by yellow and blue colors, which indicate activation or inhibition of the respective related genes. **B**) More detailed description of a selection of metastasis genes. The respective fold changes in mRNA levels versus control are given as positive or negative numbers below the corresponding gene symbols.

2.2.6. Role of upstream regulators

The third IPA analysis concentrated on upstream regulators, which showed a significant modulation in expression and therefore altered the activation state of a whole series of downstream proteins.

From the early stage of liver colonization, six genes were selected for detailed analysis. These included member RAS oncogene family-like 6 (Rabl6), which was increased in expression and caused significant activation of downstream target genes, as well as Ras association domain family member 1 (Rassf1), nuclear protein, transcriptional regulator, 1 (Nupr1), and jun proto-oncogene (Jun), which also showed increased expression but caused inhibition of the corresponding downstream molecules (**Table 2**). Finally, the genes interleukin 1 alpha (Il1a) and

inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (Ikbkg) showed decreased expression and caused inhibition of their respective downstream molecules (see **Table 2**).

Upstream regulator (early stage)	Exp. fold change	Molecule type	Predicted activation state	Activation z-score / p-value of overlap	Target molecules in Ingenuity Pathway Analysis dataset
Rabl6	1,97	other	Activated	4,899 / 5,42E-14	AURKB,BUB1B,CCNA2,CCNB1,CCNE2,CENPF,CKS1B,CX3CL1,DUT,H2AFX,HMMR,HMOX1, KIF23, MAD2L1, MCM7,MELK, NDC80, PARP2, PBK,PLK1, POLD1, POLE2, PRC1,RFC3
Rassf1	2,78	other	Inhibited	-2,002 / 4,10E-08	APOE,CAV1,CCNA2,CCND2,CPE,EFEMP1,EMP1,FGF2,FOS,GDF15,LITAF,MDK,MGMT,MYL9, PAK1, SAMD5, SEPP1,SPP1, SRPX, TGM2
Nupr1	4,05	Trans-cription regulator	Inhibited	-3,543 / 5,06E-11	ARHGAP11A,ASPM,ATF3,ATP6V0A1,BUB1B,CAMK2N1,CASC5,CCDC77,CCNA2,CCNF,CDCA2, CDCA3, CDCA8, CEBPB,CENPI,CKAP2L,COL3A1,CXCL3, DHCR24,EME1, ERCC6L, ESPL1, FANCD2, FCHSD2, FHL2, FUCA1, FZD8, GBP2,GCH1,GDF15, GINS1, GMEB1, IL13RA1, IL6R, KIF11, KIF18A, KIF20A, KIF23, KIF2C, KIFC1, MAFG, MAN2B2, MKI67, MYD88, NAAA, NAPEPLD, NEIL3, NUPR1, PIK3R1, PLK1, PM20D2, POLE2, PRNP,RAB20, RAB32,SAMD4A, SERF1A/SERF1B, SGK223, SHCBP1, SHPK, SHROOM3, SKP2, SLC6A6, SPAG5, SPC25,TRIB3, TRIM16,UNC5B
Jun	3,35	Trans-cription regulator	Inhibited	-3,047 / 4,20E-20	A2M,Abcb1b,ALAS1,ANXA2,APOE,ATF3,BCL3,BIRC3,CAMP,CAV1,Ccl2,CCNA2,CCND2,CDC20,CDK1,CGA,CMTM5,CSF1,CTGF,CX3CL1,CXCL16,CXCL3, DIO2, EHD4, F3, FBLN5,FGF2,FLNC,FN1, FOS, FOSL1, FTH1, GCLC, GJA1, GSTA5, GSTP1, HES1, HMOX1, HPGD, ICAM1, IGFBP2, IL6,ITGB4,JUN, LGALS3, MDM2, MYLPF, NCAM1, NEFH, NFKBIA,NFKBIZ, Nppb, NQO1, NR0B2, PADI4, PLA2G4A, PLAUR, Prl2c2, PTX3,RGN, S100A10, SGK1, SLC6A6, SMAD7, SOD2, SPP1, STAR, STMN1, SULF2, TGFB1, TIMP1, TK1, TNC, UGT2B15, VCAM1, WNT4
Il1a	-1,82	cytokine	Inhibited	-4,061 / 1,46E-11	ADORA2A,BCL3,BIRC3,Ccl2,CCL20,CD83,CSF1,CXCL2,CXCL3,CXCL6,CYP1A1,CYP3A4,FGF2,FOS,FTH1,GCH1, HMOX1,HSD11B2,ICAM1,IGFBP5,IL11, IL18,IL6, JUN, MCAM, NFKB1, NFKBIA, NFKBIZ, NPY, NR3C1, P2RY6, PLA2G4A, PTX3, SERPINA3, SOD2, SPP1, TGFB1, TK1, TLR2, VCAM1,VEGFC
IKBKG	-1,31	kinase	Inhibited	-2,414 / 1,32E-12	AMPD3,Ccl2,CEBPB,CEBPD,CP,CSF1,CTSB,CX3CL1,CXCL2,CXCL3,CXCL6,CYP7B1,ENPP2,FOS, GBP2,GCH1, ICAM1, IGFBP6,IKBKE,IL11,IL6,Mt1,NFKB2, Nppb, Prl2c2, PTX3, RGS16, SEMA3C, SGK1, SOD3, TMEM176B

Rabl6, member RAS oncogene family-like 6; Rassf1, Ras association domain family member 1; Nupr1, nuclear protein, transcriptional regulator, 1; Jun, jun proto-oncogene; Il1a, interleukin 1 alpha; and Ikbkg, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma.

Table 2. Shortlist of significantly modulated upstream regulators during the early stage of ASML cell rat liver colonization.

From the intermediate stage of liver colonization, also six genes were selected for detailed analysis. These genes included colony stimulating factor 2 (Csf2) and NK2 homeobox 3 (Nkx2-3), which showed decreased expression, but activated their respective downstream molecules. The transcription regulator forkhead box M1 (Foxm1) was increased in expres-

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sion and activated the corresponding downstream genes. The genes cytochrome P450, family 2, subfamily e, polypeptide 1 (Cyp2e1), angiotensinogen (Agt), and secreted phosphoprotein 1 (Spp1) showed increased expression but inhibited their corresponding downstream genes (see **Table 3**).

Upstream regulator (intermediate stage)	Exp. fold change	Molecule type	Predicted activation state	Activation z-score / p-value of overlap	Target molecules in Ingenuity Pathway Analysis dataset
Csf2	-2,94	cytokine	Activated	3,114 / 7,97E-35	ABCA1,ANLN,ANXA1,ARG1,AURKA,BCL3,BIRC3,BIRC5,BUB1B,CASP1,Ccl2,CCL3L3,CCNA2, CCNB1, CCNF, CD14, CD74, CDC20,CDCA2,CDCA3, CDCA5, CDCA8, CDK1, CDKN2C, CHAF1A, CHAF1B, CKS1B, COL8A1, CSF1, CSF3, CXCL2, CXCR4, DSCC1,E2F8, EDN1,ERCC6L, F3, FANCA,FAS, FBXO5, FCGR2B, FOS,FOSL1,FOXM1,GCH1,GDF15,HDC,HLA-DQB1, ICAM1, ID3, IER3, IGF1, IL1B, IL3RA,IL6, INHBA, IRAK3, JAK2, KIF11,KNTC1, LAMP2, LY96,MCM5, MDM2, MKI67, MNS1, MYC, NCAPD2, NFE2, NFKBIA,NOX1, NR4A1,NUSAP1, PIM3, PLK1, POLD1, POLE,PRC1, PRNP,PTK2B, RACGAP1, REC8, RECQL4,SGK1,SKA1,SLC11A2, SLC1A5, SOD2,SPAG5, SPC25, SPP1, SQSTM1, STMN1, TGFB1, TGM2, TLR2, TNFRSF1A, TNFRSF1B, TNFRSF9,TRIP13,UHRF1,UPP1,VTCN1
Nkx2-3	-1,27	Transcription regulator	Activated	2,351 / 5,76E-09	ADM,ALPL,ANGPTL4,ANKRD37,ANLN,ARHGDIB,BMP2,CASP1,CEP55,CSRNP1,CX3CL1, CXCL16,EDN1,FAM198B, FGF2, FLRT2, GBP2,GCH1, GDF15, GIMAP7, GNG12, HIST2H2AA3 / HIST2H2AA4, HIST2H2AC,MFNG,MYD88,NR2F1, NT5DC2, PAPSS2, PIM3,PLA1A, PSAT1, PTPRE, RTP4, SHMT2, SRPX, VCAM1
Foxm1	2,91	Transcription regulator	Activated	3,840 / 2,40E-17	ANXA1,AURKB,BIRC5,BRIP1,BUB1B,CCNA2,CCNB1,CCND2,CCNF,CDC20,CDC25B,CDK1, CENPA,CENPF,CKS1B, FOXM1, FZD1, HSD11B2, IGF1, IL6, KDR, KIF20A, MCM8, MYC, PECAM1, PLAUR, PLK1, PLK4,PRC1,PTCH1,SKP2,STMN1, VCAN, VIM
Cyp2e1	3,93	enzyme	Inhibited	-2,762/ 7,73E-06	CAT,Ccl2,CD14,Cyp4a14,GCLC,HMOX1,IL6, MGST1
Agt	4,22	growth factor	Inhibited	-2,332 / 3,85E-24	ADAM23,ADIPOQ,ADM,AGT,AGTR2,ANGPT1,ATF3,ATP1B1, BGN,Calm1 (includes others), CAT, Ccl2, CCL3L3, CCL5, CCND2, CDK1,CIT, COL3A1, COL4A1, CP, CTSS, CXCL2, Cyp2c44, CYP2E1, Cyp4a14,DHFR,EDN1,F3,FDX1,FGF13, FGF2,FN1, FOS,GCH1,GJA1, GSS,GSTA3, GSTA5, HES1, HIF1A, HMGCS1,HMOX1,HSPA1A/HSPA1B,ICAM1,ID3,IDH1, IDI1,IGF1, IGFBP5,IL18, IL1B, IL6, LDLR, LSS,MAD2L1, MAPT, MGST1, MSMO1, MYC, NOX1, Nppb, NPR3,NPY,NR4A1, NRG1, PIK3R1, PPFIBP1, PPP1R3C,PTGS1,RGS2, RRAS,SGK1, SOD2, SOD3, SPP1, STAR, STC1, TGFB1, TGFB2, TIMP1, TNFRSF12A, TNFRSF1B, TUBB3, UGCG, VCAM1, VEGFC
SPP1	-4,35	cytokine	Inhibited	-2,527 / 9,62E-06	ADIPOQ,ANGPT1,ARG1,AURKA,CCL20,CCL5,CDC20,CXCL2, FN1,FOS,HMOX1,ICAM1,IL1B,IL6,KRT18,MYC,PIK3R1, S100A4, S100A6,SEPW1, SPP1,TGFB1,TIMP1, TM7SF2,VAT1, VIM

Csf2, colony stimulating factor 2; Nkx2-3, NK2 homeobox 3; Foxm1, forkhead box M1; Cyp2e1, cytochrome P450, family 2, subfamily e, polypeptide 1; Agt, angiotensinogen; and Spp1, secreted phosphoprotein 1.

Table 3. Shortlist of significantly modulated upstream regulators during the intermediate stage of ASML cell rat liver colonization.

From the advanced stage of liver colonization, four genes were selected for detailed analysis. These genes included Versican (Vcan), which showed a reduced expression but activated its downstream genes, as well as myelocytomatosis oncogene (Myc), which exhibited increased expression and also activated a considerable number of downstream genes. The genes CCAAT/enhancer-binding protein beta (Cebpb) and transforming growth factor, beta 1 (Tgfb1), on the other hand, were reduced in expression and as a result inhibited their respective genes (**Table 4**).

Upstream regulator (advanced stage)	Exp. fold change	Molecule type	Predicted activation state	Activation z-score/ p-value of overlap	Target molecules in Ingenuity Pathway Analysis dataset
Vcan	-4,25	other	Activated	2,823 / 5,37E-18	ADM,AEBP1,ANG,ASS1,C1R,C1S,Calm1, CASP1, Ccl2, CCL20,CEBPB, CIDEA,CPE,CXCL2,CYBA,FABP5,FAS, FST, ICAM1,LGALS9B, Mecom, METRNL,NFKB1, NFKBIA,PLA2G2A, PRDX5, RBP1, SERPING1, Slpi, SOD2, TGFB1, TIMP1, VCAM1, VCAN,VEGFA, Wfdc17/Wfdc18
Myc	2,57	trans-cription regulator	Activated	2,907 / 3,06E-20	ADK,ADM,ALB,ALDH18A1,ARG1,ASS1,AURKB,BCL6,BIN1,BIRC5, CASP1, CCNA2,CCNB1,CCND2,CD151,CD19, CDC20,CDCA7, CEBPD, CITED1, CKS2,COL2A1,COL3A1,COL5A2,CPD,CPT1A,CSPG4,CSRP2,CTSB,CTSD,D DIT3, DDX39B,DHFR,DUSP5,EBI3,EDN1,EFEMP1, EMP1, EPHA2, FABP5, FAS,FGFR1,FMOD,FOS, FOXE1, FTH1,G6PD,GAA,GADD45B, GADD45G, GAMT,Gar1,GBP2,GBP4,GCLC, GJA1,H19,HES1,HIVEP2,HLA-A, HMOX1, ICAM1, ID1, IDH1, IDH2, IER3, INHBA, ITGA6, JUN, KDR,KLF10, KLF4, KLF6,KRT7,Ptma,LUM,LYZ,MCM7,MGP,MKI67,Mt1,MYC,MYL9, MYLPF, NFKBIA, Nppb, NPY, OPRK1, PDCD4, PLA1A, PLK1, PLP1, PLS3, POLD1, PPL,Ppp1r15a,PSAT1,PTN,RBP1,SCAMP5,SHMT1,SHMT2, SLC22A4, SLC7A5, SMN1/SMN2, SOD2,SPN,STMN1, TAF1D, TF, TGFB1, THBS1, TIMP1,TMSB10/TMSB4X,TXNRD1,VCAM1,VEGFA,VEGFC, VIM, WISP1
Cebpb	-2,51	trans-cription regulator	Inhibited	-3,299 / 1,84E-21	Abcb1b,AKR1B10,AKR1C3,ALB,APLN,ARG1,BLNK,CCL2,Ccl2,CCL3L3 CCNA2,CCND2,CD14,CDC45,CDH3,CEBPB,CEBPD,COL5A2, CP, CPT1A, CSF3,CTSC,CXCL2,CXCR4,CYP11A1,CYP1A1,CYP27B1,DDIT3, DGAT2, DHFR, FAS, FBLN1, FGFR2,FHL2,FOS,FZD1,HDC,HLA-A, HP, ICAM1, ID1,IER3,IFITM3,IKBKE,IL1B,IL6,JUN, KRT18,MGP,MYC,NDRG4, NFKBIZ, NUPR1,PDK4,PEA15, PLA2G4A,PLAC1,PTGS1, SCD, SLC12A2, TF,TGFB1, TMEM176A, TNFAIP6, TNFRSF1A, TRIB3, TUBB3, VCAN, VIM, XDH
Tgfb1	-2,48	growth factor	Inhibited	-2,346 / 6,57E-59	ABCA1,ADAMTS4,ADK,ADM,ADORA1,AGTR2,ALB,ALDH18A1,APLN,AP OC2,AQP9,ARG1,ASS1,AXL,B3GALT2,BARD1, BCL3,BCL6, BGN, BIN1, BIRC5,BMP7,C1QA,C1QB,C1S,Calm1, CALML4, CAMP,CARS, CASP1, CAT,CBR3,CCL2,Ccl2,CCL20, CCL3L3,CCNA2,CCNB1, CCND2, CD14, CD36, CD68, CDC20, CDH11, CEBPB, CENPF,CITED2, CKS2, CLDN4, CMTM5, COL18A1, COL2A1, COL3A1, COTL1, CREB3, CSF1, CSPG4, CSRP2, CST3, CTGF,CTSB, CTSC, CTSD,CTSS,CX3CR1, CXCL2, CXCR4, CYBA,CYP11A1,CYP27B1, CYP3A7, DFNA5, DNMT3A, EDN1, ELF3, ELK3, ENPP1, EPHA2, ESPL1, EXT1, FABP5, FAM110B, FAS, FCER1G, FDXR, FGF2, FGF21, FGFR1,FGFR2,FOS,FSTL3,FTH1, FXYD5, FZD1, GADD45B, GATA3,GBP2,GCLC,GDF15,GJA1,GLCE,GLI1, Gsta4, GSTA5, GTPBP1, H6PD,HEBP1,HES1,HLA-DQB1, HMOX1, HPGD, HSPA1A / HSPA1B, HTRA1, ICAM1, ID1, IER3, IFRD1,IGF2, IGFBP2, IGFBP5, IGFBP6, IL11, IL13RA2, IL18,IL1B, IL33,IL6,IL6R,INHA, INHBA, IRAK3, ITGA1, ITGA6, ITGB6, JUN, KDR, KLF10,KLF15, KLF4, KRT18,KRT7,LITAF,LOXL1, LPAR1, MAOA,MFAP2,MGP, MKI67, MMP12, MSMO1, MTHFD2, MYBL1, MYC, MYD88,MYLK,MYLPF, MYO10, NAB2, NDC80, NDRG4, NFKBIA, NNMT, NOV, NPR1, NR4A1, NR4A3, NT5E,NUPR1,OSR2,P2RY6,PAPPA, PARP3, PDCD4,PDE4D,PGRMC1,PLA1A, PLA2G4A, PLAGL1, PLS3, PLXNC1, PNOC, POLD1, PPFIBP2, PPP1R13B, PPP1R3C, PRC1,PTGS1, PTK2B, PTPN6,RAB31, RACGAP1, RAPGEF3, RASGRP3, RASL11B,RASSF1, RBPMS, RHOD, S100A11, S100A4, SBNO2, SCD, SDC4, SEMA3A, SEMA3B, SFTPC, SHMT1, SHOC2, SLC7A5, SMC4, SNAI2,SOD2, SOD3, SPC25,SPEG,SPHK1,SRFBP1,TBX3,TCF12,TGFB1, TGM2, THBS1, TIMP1, TJP2, TLR2, TLR4,TNFAIP6, TNFRSF12A,Tpm2,TUBB2A,TUBB3, TXNRD1, VCAM1, VCAN,VEGFA, VEGFC,VIM, WFS1, WISP1,WNT11, WT1, XDH

Vcan, Versican; Myc, myelocytomatosis oncogene; Cebpb, CCAAT/enhancer-binding protein beta; and Tgfb1, transforming growth factor, beta 1.

Table 4. Shortlist of significantly modulated upstream regulators during the advanced stage of ASML cell rat liver colonization

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Upstream regulator (terminal stage)	Exp. fold change	Mole-cule type	Predicted activation state	Activation z-score / p-value of overlap	Target molecules in Ingenuity Pathway Analysis dataset
Csf2	-2,81	Cyto-kine	Activated	2,752 / 3,11E-35	See Table 3
Foxm1	2,54	Transcri ption reg.	Activated	2,751 / 2,02E-11	See Table 3
Tsc2	3,08	other	Inhibited	-2,551 / 1,78E-12	A2M,ACSS2,ANXA1,ATF3,B3GALT2,BIRC5,Ccl2,CCND2,CCND3,DDIT3, DYNC1I1,EGR1,GPNMB, HMGCS2,HMOX1,IRS1,MGP, NSDHL, PDGFRA, PDGFRB,PRELP,RASSF4,S100A11,S100A6,SCD,SLC2A1, SMPDL3A, SOD2, TAGLN, TMEM176A, TMEM176B, VAMP8
Tnf	3,53	Cyto-kine	Inhibited	-2,209 / 3,74E-86	A2M,A4GALT,Abcb1b,ABCC3,ACP5,ACSL1,ADAMTS4,ADM,ADORA1,AD ORA2A,AEBP1,AGT, AGTR1, AKR1B1, AKR1B10,ALB,ANGPT2, ANGPTL4, ANXA1,AOC3,APCS,APOA1,APOC2,APOC4,APOE,AQP1,AQP3,AQP9, ARG1,ARHGDIB,ARL6IP5,ARRDC3,ASGR1,ASS1,ATF3,AXIN2, B4GALNT1, B4GALT1, BACE1, BCL2A1, BCL3, BCL6, BID, BIK, BIRC5, BMP2, BTG2, BUB1B, C5AR1,CASP8,CAT, CCDC80,CCL19, CCL2,Ccl2, CCL20, CCL3L3, CCL4, CCL5,Ccl6,CCND2,CCND3,CCR1,CCR5,CCR6,CD14,CD163, CD247, CD40,CD82, CDH1, CDH11, CDH2, CEBPD, CFB, CFD, CFLAR, CH25H, CLDN4, COL1A2,COL3A1, COTL1, CPT1A,CREB3, CREB3L3, CSF1, CSF3, CTGF, CTSK, CTSS, CTSZ, CXCL12, CXCL13, CXCL2, Cxcl9, CXCR4, CYBA, CYBB,CYLD, CYP1A1, CYP1B1, CYP26B1,CYP2C8,CYP2E1,CYP7B1, CYTIP, DCBLD2, DCHS1, DCN, DDIT3,DENND2D, DPP4,DPYS,DUSP1, DUSP2, DUSP5, DUSP9,EBI3,EDN1,EDNRB, EFNA1, EGFR,EGR1, EGR2, EIF2AK2, ELF3, ELK3, EMP1, ENPP3, ENTPD5, EPHA2, ERG, ESM1,EXT1, F3,FABP1, FABP4, FADS1, FAM132A, FAM198B, FAS, FCER1G,FCGR2B, FDPS, FGF2, FGFR1, FGG, FOSL1,FOXE1,FRMD6,FRZB, G0S2,GADD45B,GCH1, GCLC, GDF15,GDNF, GPD1, GPR176, GPRC5B,GPX1, Gsta4, GSTP1, H19, HDC, HERC1,HES1,HID1,HK3,HLA-DRA,HMOX1,HP,HPGD,HSD11B1, HSD17B7, HSPA1A/ HSPA1B,ICAM1, ICAM2, ICOS, ID1, ID3,IDH2, IER2, IER3, IFIH1, IFIT3, IGF1, IGF2, IGFBP1, IGFBP2, IGFBP3, IGFBP5, IKBKE, IL11, IL16, IL18, IL1B, IL1R2, IL23A, IL24,IL3RA, IL6,INHA,INPP5D,IRAK3,IRF5, IRS1, ITGA6, ITGB2, ITGB6,JAG1, KDR, KIF20A, LAMC1,LBP,LCAT,LCN2, LDLR, LGALS8, LOX, LPAR6, LPL, LSS, LTB, LXN,LY96, LYVE1, MAFF, MALL, MAN1C1,MAP3K8,MBL2, MCAM, MEF2C,MET,MFHAS1, MGMT, MGP, MGST1, MMD, MMP12, MSLN, MST1, Mt1,MVP, Mx1/Mx2, MYLK, NCF1, NEFH, NFKB1,NFKB2, NFKBIA, NFKBIZ, NINJ1, NNMT, NOS3, NOTCH4, NOX1, NPM3, Nppb, NQO1, NR0B2, NR1I2, NR4A1, NRP1, OAS1, OCLN, OLR1, OPTN, ORM1, P2RX5,PCK1, PCSK6,PDE2A, PDGFA, PDGFRA, PDIA4, PENK, PER2, PIM3, PLA2G3, PLA2G4A, PLAUR, PLK2, PLK3, PLOD2, PLP1, PLVAP, POMC, POSTN,PPARG, PPP1R3C, Prl2c2, PRNP, PRSS23, PSMB9, PTGS1, PTPRC, PTPRN,RAB32,RARRES2, RBP1, RFTN1,RGCC, RGS1, RGS2, RGS20, RGS4, RGS5, RGS9, RND1, ROBO1, S100A8, S100A9,SCD,SDC2,SDC4, SEMA3C,SEPP1,SERPINA3, SERPINB1, SERPIND1,SERPINE1,SERPINE2,SGK1,SHBG,SLC10A1,SLC11A1, SLC11A2, SLC1A4, SLC22A4, SLC27A5, SLC2A1, SLC2A2, SLC7A2,SLC7A8, Slco1a1, Slco1a4, SMPD1, SMPD2, SMPDL3A, SNN,SOAT1,SOD2, SOD3, SPARC, SPHK1, SPP1, SPSB1, SQSTM1,ST3GAL6,STMN1,SYNPO,TAGLN, TBXAS1, TF,TGFB1,TGFB2,TGFB3,TGM2,THBS1,THBS2,TIMP1,TK1,TLR2, TLR3, TMEM176B, TNF, TNFRSF11B, TNFRSF1A,TNFRSF21, TNNC1, TPST1, TRAF3,TRPC6,TXNIP,TXNRD1,UBD,VCAM1,VMP1,XIAP, ZC3H12A

Csf2, colony stimulating factor 2; Foxm1, forkhead box M1; Tsc2, tuberous sclerosis 2; and Tnf, tumor necrosis factor.

Table 5. Shortlist of significantly modulated upstream regulators during the terminal stage of ASML cell rat liver colonization.

From the terminal stage of liver colonization, again four genes were selected for detailed analysis. These included two genes, which had been listed before (Csf2 and Foxm1) that caused the same pattern of expression and resulting influence as in the intermediate stage of liver

colonization (decreased expression but subsequent activation in the case of Csf2 and increased expression resulting in subsequent activation in the case of Foxm2). The other two regulators (tuberous sclerosis 2 (Tsc2) and tumor necrosis factor (Tnf)) were both increased in expression and were predicted to inhibit a large number of downstream genes (see **Table 5**).

2.3. Changes in mRNA expression after recultivation in vitro

For assessing the influence of the environment on ASML tumor cells, ASML cells were also recultivated in vitro for 3, 6, and 9 days after they had been reisolated from rat liver after a colonization period for 21 days. As a result, genes were found to be downregulated in vitro or upregulated at varying degrees. Some examples are given in **Figure 5**. As can be seen, the genes desmoglein 4 (Dsg4), tRNA methyltransferase 10A (Trmt10a), and stathmin 2 (Stmn2) were decreased more than 1000-fold, more than 100-fold, and more than 3-fold, respectively. In contrast, the genes apolipoprotein C-II (Apoc2), ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1), and Keratin 18 (Krt18) were increased more than 3-fold (Apoc2 and Enpp1) and more than 100-fold (Krt18). The differences between the cultivation periods of 3, 6, and 9 days were very low and thus negligible.

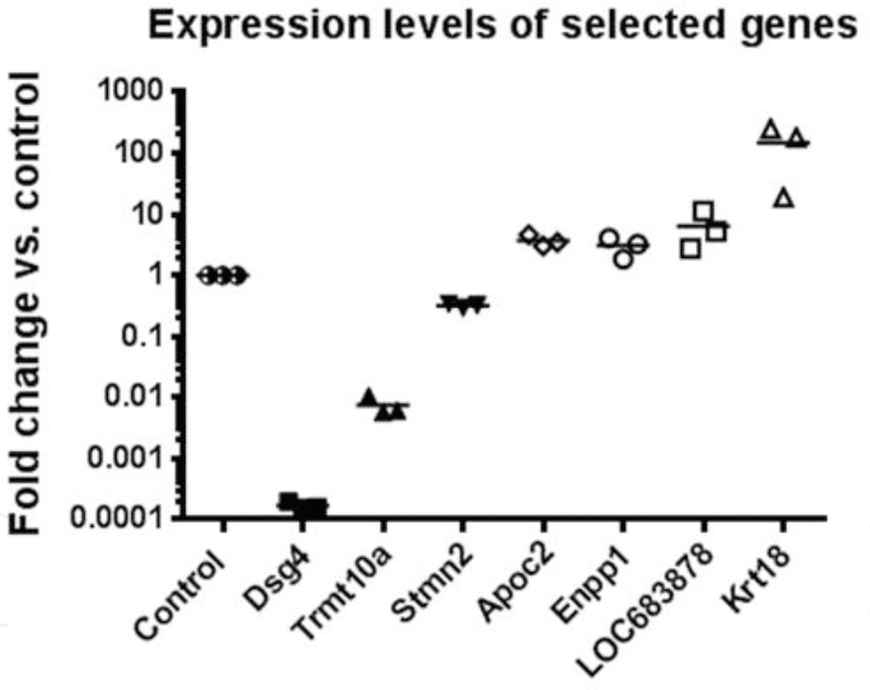

Figure 5. Changes in mRNA expression after recultivation in vitro: ASML cells were also recultivated in vitro for 3, 6, and 9 days after they had been reisolated from rat liver after a colonization period for 21 days. As a result, some genes were downregulated or upregulated in vitro, at varying degrees. Seven examples are given in comparison to control.

2.4. miRNA modulation of ASML cells colonizing rat liver

Concomitantly with the mRNA species, all known miRNA molecules were analyzed in the respective batches of reisolated ASML cells. As for the distribution of mRNAs, the modulation

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of miRNA expression was detailed for the two same categories, i.e. the fold changes in miRNA expression and the respective stage of liver colonization, as shown in **Figure 6**.

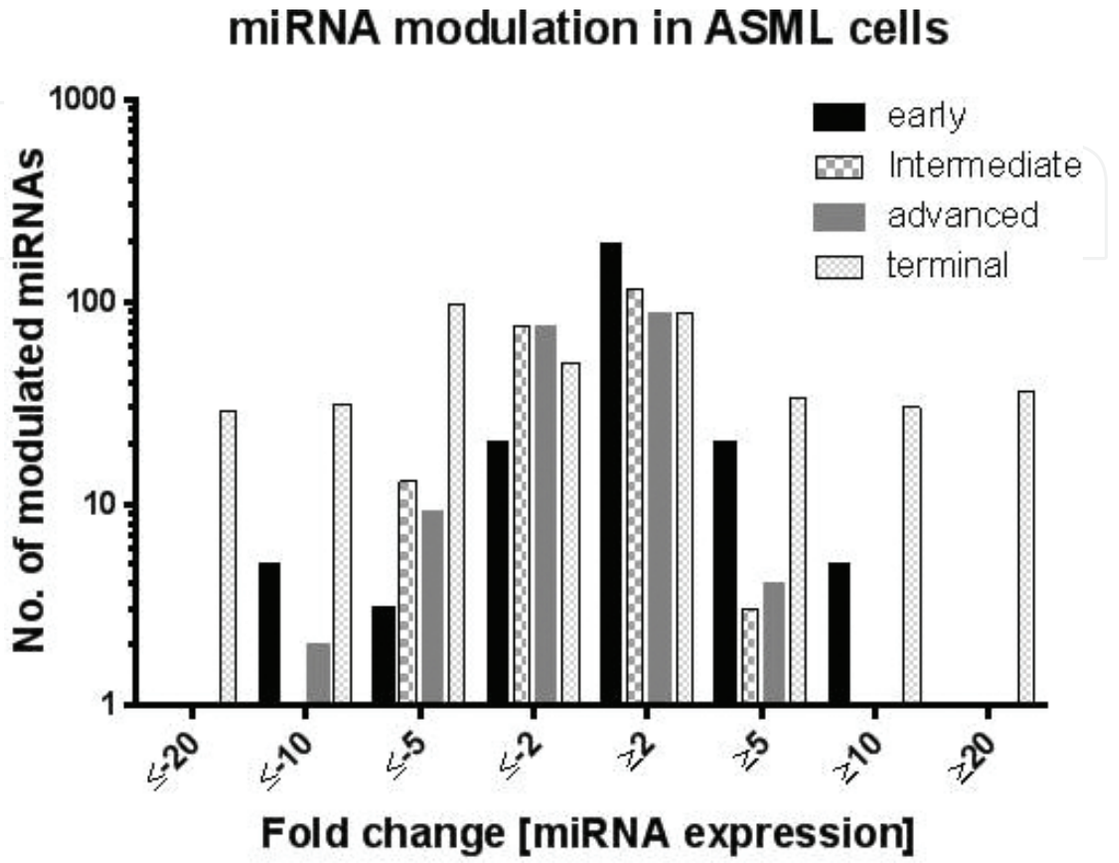

Figure 6. miRNA modulation of rat pancreatic cancer cells colonizing rat liver: Given is the fold change of mRNA expression of reisolated ASML cells during early, intermediate, advanced, and terminal stages of liver colonization relative to control cells growing in vitro.

Because of the lower number of existing miRNAs as compared to mRNAs, the distribution looks not as bell shaped as for mRNAs in **Figure 1**. This is also due to a considerable number of miRNAs, which show a more than 20-fold regulation (both, positive and negative) in the terminal stage of liver colonization.

An example for the influence of miRNAs is given in **Figure 7**. The miRNA 29b-3p is known for its effect on extracellular matrix proteins, as on collagens [17]. Downregulation of miRNA 29b-3p was observed in ASML cells during the whole period of liver colonization. This is shown in **Figure 7A**, with the nadir of miRNA 29b-3p expression appearing on day 3 (less than 10-fold of control level), and some recovery thereafter to a plateau of expression ranging from fivefold to threefold reduced levels, as related to control expression in vitro. This reduction was associated with distinctly increased expression of the collagens Col4a2 (collagen, type IV, alpha 2), Col3a2, Col1a2, Col1a1, and Col4a1. Col5a3 showed only a minor increase in expression, but Col5a2 (collagen, type V, alpha 2) showed reduced expression, as was true for Tgfb3 (transforming growth factor, beta 3) (**Figure 7B**).

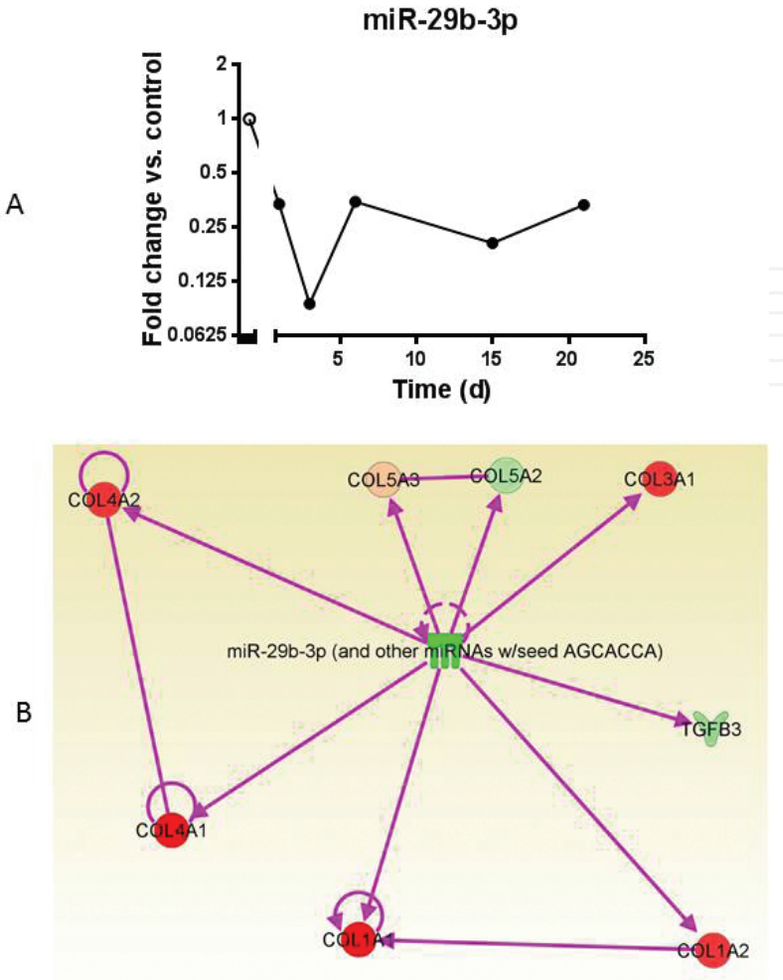

Figure 7. Influence of miRNA 29b-3p on gene expression: Shown is the expression of miRNA 29b-3p in ASML cells during liver colonization (**A**). In addition, the influence on mRNAs is given in terms of upregulation or downregulation (**B**). For color code, see **Figure 3**.

This analysis shows that certain extracellular matrix proteins, as the collagen family, are highly regulated by their respective miRNA. Further experiments are needed to reveal the mechanistic role of these proteins during liver colonization and to address the question whether they can be diagnostic or therapeutic targets.

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