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„The Role of PDGF on hepatocellular Epithelial to Mesenchymal Transition“

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For my Family
and Eva

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1. Summary/Zusammenfassung

1.1 Summary

Hepatocellular carcinoma (HCC) is one of the five most common and most deadliest cancers worldwide. A common reason for HCC is the chronic infection with the Hepatitis B virus (HBV). while other risk factors are the infection with Hepatitis C virus (HCV), intoxication by food contaminants (e.g. aflatoxin), alcoholism, obesity or metastases from other organs. These risk factors are able to inflict continuous damage to the liver, which are accompanied by a microenvironment that promotes fibrosis, cirrhosis and finally the formation of HCC.

In the present study, we were able to establish a mouse model for HCC using immortalised $p19^{ARF}$ deficient, Ha-Ras transformed hepatocytes, that conducted epithelial to mesenchymal transition (EMT) upon TGF-β administration. These cells performed a morphological switch, in which they transformed from a polarized, epithelial phenotype to a spindle shaped, fibroblastoid phenotype. The fibroblastoid phenotype exhibited a changed extracellular matrix (ECM) interaction, that advocated motility and invasiveness.

A microarray analyses of gene expression during EMT revealed that PDGF ligand as well as receptor are upregulated, indicating a possible involvement in the transition process. Therefore we constructed a dominant-negative PDGF-α receptor (dnPDGFR-α), to study the effects of upregulated PDGF signalling. We were able to determine the involvement of autocrine PDGF signalling in motility. In addition we could specifically block PDGF signalling using the receptor tyrosine kinase inhibitor STI571.

With the discovery of micro RNAs (miRs) acting as a regulatory network and the insight that PDGF has a miR-140 binding motif in its 3' UTR, we are looking forward to understand and integrate this mechanism of regulation into current cancer models as well as it poses a novel strategy for anti-cancer therapy.

1.2 Zusammenfassung

Das hepatozelluläre Leberkarzinom (HCC) ist eine der fünf verbreitesten und eine der tödlichsten Krebsarten weltweit. Die häufigste Ursache für die Entstehung von HCC ist eine chronische Infektion mit dem Hepatitis B Virus (HBV). Andere, weniger verbreitete Risikofaktoren sind die Infektion mit dem Hepatis C Virus (HBC), Vergiftung durch Nahrungsverunreinigung (z.B. Aflatoxin), Alkoholismus, Fettsucht oder Metastasierung aus anderen Organen. Diese Risikofaktoren können anhaltend die Leber schädigen und so eine Umgebung schaffen, die Fibrose, Zirrhose und letztlich die Entstehung von HCC fördert.

Wir etablierten ein Mausmodell für HCC, bei dem wir immortalisierte $p19^{ARF}$ defiziente, Ha-Ras transformierte Hepatozyten verwendeten, die bei TGF-β Zugabe einen Übergang vom epithelförmigem zum mesenchymalen Zustand (EMT) durchmachen. Diese Zellen erfahren einen morphologischen Wandel, bei welchem sie sich von einem polarisiertem, epitheloiden Phänotyp zu einem fibroblastoiden Phänotyp transformieren. Dieser fibroblastoide Phänotyp besitzt eine veränderte Interaktion mit der extrazellulären Matrix (ECM), welche die Motilität und die Invasivität fördert.

Eine Microarrayanalyse der Genexpression während der EMT ergab, dass PDGF Ligand und Rezeptor hochreguliert sind, was auf eine mögliche Beteiligung an der Transformation schliessen lässt. Wir konstruierten einen dominant-negativen PDGF-α Rezeptor (dnPDGFR-α), um die Auswirkungen von gesteigerter PDGF Signaltransduktion zu untersuchen. Dadurch stellten wir eine Beteiligung von PDGF bei der Motilität fest. Weiters konnten wir durch den Rezeptortyrosinkinaseinhibitor STI571 den PDGF Signaltransduktionsweg blockieren.

Die Entdeckung von micro RNAs (miRs) als regulatives Netzwerk, und die Erkenntnis, dass PDGF ein miR-140 bindendes Motif im 3' UTR besitzt, sind ein aussichtsreicher Ansatz, um diesen Regulationsmechanismus zu verstehen und in bestehende Krebsmodelle, als auch in neue Strategien für die Krebstherapie zu integrieren.

2. Overview of the Liver

2.1 Liver Anatomy

The human liver is the largest internal organ and also represents the largest gland. It consists of 4 different sized and unequally physiological active lobes and it is connected to two large blood vessels: the hepatic artery and the portal vein. The hepatic artery provides oxygen rich blood from the aorta, whereas the hepatic portal vein delivers blood and digested nutrients from the gastrointestinal tract, the spleen and the pancreas into the liver (Benjamin *et al*., 2008). These two main blood vessels divide into capillaries, which lead to functional units called lobules. The liver consists of 50.000 to 100.000 lobules, which are cylindrical structures several millimetres in length and less than one to two mm in diameter and consist of millions of hepatic cells. Each lobule is supplied by capillaries at the periphery, permitting the blood to flow through the sinusoids (Maher, 1997) as shown in Fig. 1.

The sinusoids are channels of engulfing hepatocytes, separated by the space of Disse. They contain immune-system cells called Kupffer cells, that attack bacteria and foreign matter in the blood (Diehl, 1993). While the blood flows through the sinusoids, dissolved substances are released from the blood and exchanged by the surrounding hepatocytes. The exchange rate diminishes along the sinusiod and finally the blood exits through the central vein, a vain located at the centre of the lobule. The veins of several lobules join to form the hepatic veins which exit the liver.

An important duct system originating from within the liver is the "tree branch" like bile duct system. The bile is produced by the hepatocytes and collected in bile canaliculi, which merge in order to form bile ducts. The intrahepatic ducts join to form the right and left hepatic duct, which merge to form the common hepatic duct. The common hepatic duct leaves the liver and thus is the first part of the extrahepatic ducts. It is joined by the cystic duct from the gallbladder to form the common bile duct, which leads to the duodenum. The bile can be stored within the gallbladder or transported to the duodenum directly, where it aids in the digestion of lipids (Netter, 2006)

2.2 Metabolic Functions

The liver performs a wide range of functions and is essentially involved in the synthesis and breakdown of many compounds. It plays central roles in amino acid synthesis, carbohydrate metabolism, protein metabolism, lipid metabolism, the production of coagulation factors and insulin-like growth factor one as well as the excretion of bile. Additionally it is responsible for the breakdown of insulin and bilirubin and the conversion of ammonia to urea as well as the breakdown and modification of toxic substances in general, which is also called drug metabolism (Maher, 1997). The liver also stores glycogen, the vitamins A, -D, -B12, iron and copper and thanks to the Kuppfer cells it is part of the mononuclear phagocyte system. This system is a part of the immune system, that consists of phagocytic cells (Tortora and Derrickson, 2008).

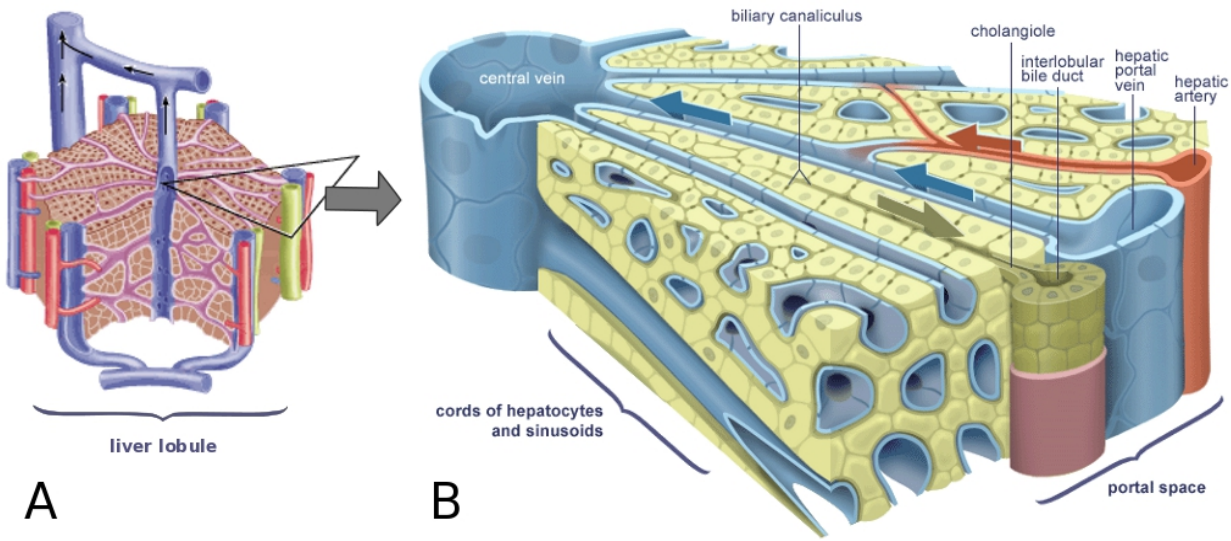

Figure 1

A shows one hexagonal formed liver lobule, while **B** magnifies part of the lobule and shows the bloodflow from the hepatic portal vein and the hepatic artery to the central vein as well as the bile flow from the bile canaliculis to the bile duct (1, 2).

3. Pathology of the Liver

Due to the multiplicity of functions, the liver is susceptible for many disorders and diseases e.g.: hepatitis, cirrhosis or liver cancer, to name the most prominent ones

(Motola-Kuba *et al*, 2006).

3.1 Liver Diseases

Hepatitis is an inflammation of the liver, that can be caused either by viruses, toxins and drugs or auto-immunological reactions (Mormone *et al.*, 2011). The five unrelated viruses named Hepatitis A, -B, -C, -D, -E virus, are accountable for causing the most cases of hepatitis worldwide (Zanetti *et al.*, 2008), followed by alcohol, being the most prominent and most abundant toxin (Bailey and Brunt, 2002). Other viruses and infections like the yellow fever virus, Epstein-Barr virus, Herpes simplex virus, the Cytomegalovirus, leptospira and toxoplasma have the ability to cause hepatitis as well, but are known to be less effective.
In clinical terms, the duration of the inflammation can be distinguished to be either acute (Ryder *et al*., 2001) or chronic (Lok *et al*., 2007). An acute inflammation is lasting shorter than six months and can be caused by the Hepatitis A virus, while a chronic hepatitis lasts for more than six months and can be caused by the Hepatitis C virus.

Another disease of the liver is the non-alcoholic fatty liver disease (NAFLD), which manifests itself with the accumulation of fat within the liver as a consequence to insulin resistance. This can lead to a form af hepatitis, called steatohepatitis (Vuppalanchi *et al.*, 2009), which is believed to possibly cause cirrhosis of unknown origin (Clark *et al*., 2003).

Some diseases of the liver originate because of genetic disorder, that lead to malfunctions in metabolism e.g.: Haematochromatosis, Wilsons disease and Gilberts syndrome. While Haemochromatosis leads to iron accumulation within the body (Feder *et al*., 1996), Wilsons disease causes tissues to retain copper (Thomas, 1995), which both can lead to liver damage. Gilberts syndrome is a malfunction of the bilirubin metabolism, causing unconjugated bilirubin being excreted from the liver and thus elevating its level within the blood (Bosma, 1995).
Two diseases affecting the bile ducts are primary sclerosing colingitis (Duerr, 1991) and primary biliary cirrhosis (Kaplan, 2005). It is believed, that they originate because of autoimmune reactions.

3.2 Cirrhosis

Chronic diseases or continuous exposition to toxins lead to persistent damage of the liver. This ongoing damage leads to the persistent build-up of fibrous connective tissue, scar tissue and regenerative nodules in order to rebuild the damaged tissue. If the build-up is too excessive, then this phenomenon is called fibrosis. Fibrosis can further disrupt the bile ducts and blood vessels, leading to the accumulation of bile within and the stall of blood flow through the organ. This results in a loss of function, which is referred to as cirrhosis.
The most common causes for cirrhosis are chronic viral hepatitis (Monto *et al*., 2001) and alcoholic liver disease (ALD) (Maher, 1997). Cirrhosis is also known to be a major risk factor for the development of hepatocellular carcinoma (HCC), as about five percent of cirrhotic patients develop HCC (Bailey and Brunt, 2002).

3.3 Liver Cancer

Diseases of the liver are not only able to cause cirrhosis, but are also capable of inducing liver cancer. This can happen directly e.g. hemochromatosis, or via the formation of cirrhosis, which is a major risk factor for liver cancer.
The tumours of the liver can be distinguished by their nature as either benign or malign. While benign liver tumours are mostly harmless and do not affect the surrounding tissue, such as hemangioma, malign tumours have the tendency to increase their adverse effect and become worse. They show uncontrolled growth, invasion of nearby tissue and sometimes have the ability to metastasise and can additionally be described as cancerous. Cancers within the liver develop either from liver cells types, called primary liver cancers, or by metastasising cells from other organs. The majority of primary liver cancers are hepatocellular carcinomas (HCC) (Motola-Kuba *et al*., 2006), originating from hepatocytes, followed by cholongiocarcinoma (Landis *et al*., 1998), which is a cancer of the bile ducts. Some of the cancers found within the liver have been metastasising from cancers of other organs. They originate frequently from colon cancers, but also metastases from breast cancer or prostate cancer are found (Chambers, 2002; Bubendorf, 2000).

4. The Epidemiology and Etiology of HCC

Hepatocellular Carcinoma (HCC) is the most prevalent kind of liver cancer. It is one of the five most common and it is among the three deadliest cancers worldwide (El-Serag, 2001). The epidemiology of HCC shows two main patterns: one for the so called western countries, like Europe or Northern America, and one for non-western countries, like Africa or Asia (Motola-Cuba *et al*., 2006).

While in western countries the majority of cancers found within the liver are due to metastases from other organs, the remaining cases of primary liver cancer are caused mostly by alcoholism or metabolic diseases (Kumar, 2003).

Otherwise, in non-western countries, HCC is one of the most frequent cancers, especially in areas where hepatitis is endemic. The most common reason for HCC in Africa and Asia is the Hepatitis B Virus (HBV) infection, followed by much lower cases triggered by the Hepatitis C Virus (HCV) (Tanaka, 2011). HBV and HCV can cause chronic infections, that may generate cirrhosis and thus possibly evolving towards HCC. HBV additionally possesses the feature to cause HCC out of a chronically infected liver without cirrhosis (Bailey and Brunt, 2002) by directly integrating into the host genome.

Another important trigger for HCC are food contaminants, e.g. aflatoxin from Aspergillus flavus, which result because of improper storage conditions. These toxins are transported to the liver, where they are processed to carcinogenous substances e.g. epoxide. If those modified toxins enter the cell nucleus, they can cause DNA adducts which facilitate tumour development. (Lee, 2000; Bressac, 1991)

Other risk factors for HCC are cirrhosis, heamatochromatosis, wilsons disease (Bailey, 2002) and type two diabetes (El-Serag, 2006). In the case of type two diabetes only those people seem to be affected, who have a high circulating insulin concentration.

5. Molecular Events in HCC

The development of cancer is a multistep process, that progressively derails the natural cell cycle progression and routes the cells towards uncontrolled growth.

5.1 The Origin of Malfunction

The first step is a mutation, that is either not noticed by the cellular repair machinery or repairing fails and the mutation gets permanent. In HCC this first step is triggered indirectly by continuous liver damage, either by viral infection or toxic compounds, or directly by the presence of genotoxic substances. Continuous liver damage leads to a constant cycle of damage and repair, which boosts the probability of the repair mechanisms to introduce mistakes. Additionally this poses persisting stress to the cells, which can lead to the overproduction of reactive oxygen species (ROS) (Devasagayam, 2004). ROS has the same ability as genotoxic substances e.g. aflatoxins, they favour directly the formation of DNA adducts (Brooker, 2011; Dragan et al., 1994). This can lead to the overexpression, underexpression and destruction of genes, altering the cellular expression pattern. A factor often found elevated in conjunction with cirrhosis and early HCC development is Transforming Growth Factor-α (TGF-α) (Yeh *et al*., 2007), which normally induces epithelial development. In addition, viruses can integrate within the host genome and thus activate the expression of oncogenes or the shutdown of tumour suppressor genes.

5.2 The Establishment of Growth and Survival

The second step of tumour development is the promotion of mutated cells towards uncontrolled growth and proliferation. This can be achieved by the activation of cell survival pathways, cell cycle deregulation and stable genomic alterations, which result in constantly dividing cells, that lost their ability to go into apoptosis. Regarding HCC, MAPK related genes like c-Ha-Ras, c-Raf, c-Fos, and c-Jun, Protein Kinase C (PKC) isoforms as well as the Signal Transducer and Activator of Transcription-3 (STAT-3) were frequently found to be overexpressed (Feo *et al*., 2007; Calo *et al.*, 2003). The elevated level of MAPK triggering factors leads to its over-reactivity and stimulation of survival pathways, like Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/AKT) pathway and p38 MAPK pathway (Feo et al., 2007). STAT-3 normally acts as an transcription factor and

mediates the expression of genes involved in cell growth and apoptosis.
Furthermore it was noticed, that also cell cyle related genes e.g. C-Myc were found at a higher level (Romach *et al.*, 1997). C-Myc and its targets Igf-2 and Cyclin D1 alter the activation of Cyclin-dependent kinases (Cdk) and thus have a direct effect on cell cycle progression. Other important factors promoting carcinogenesis are the inactivation of tumour suppressor genes (p53, DLC-1) or the downregulation of signalling controlling cell growth and apoptosis (TGF-β) (Feo *et al*., 2007).
All the steps described help a mutated cell to keep dividing whilst avoiding apoptosis.

5.3 Motility and Invasion

In the third step of oncogenic development the mutated, dividing cell gains the ability to invade nearby tissue and to metastasise, not only within the organ but also to distant sites. The cancer cells have to detach from the extracellular matrix (ECM) surrounding the primary tumour and move to a new location. They use the blood or the lymph system for their movement to their new destination. In order for the cells to reattach and continue growth and division, they have to be in a microenvironment, that matches the cell types requirements.
In the case of hepatocytes, which are epithelial cells, they have to change their phenotype and extracellular matrix composition towards a fibroblastoid-like one to get invasive. This process is often described as Epithelial to Mesenchymal Transition (EMT), a process normally taking place during development or wound healing.
Furthermore it has been shown, that angiogenesis is a hallmark of cancer and further invasion (Hanahan *et al*., 2000), as it is crucial for the invading tumour to grow a network of blood vessels for support. It seems to be particularly important, that a subpopulation of cells, called endothelial progenitor cells, are present in order for angiogenesis to be successful.
Thus it was examined, that especially angiogenesis inhibitors, such as platelet factor, angiostatin, endostatin, and vasostatin (Das *et al*., 2007), can prevent or slow down the growth of tumours and metastasis. Additionally it was found, that analysing the gene expression pattern of a primary tumour leads to an insight on the tumours invasive and metastatic potential (van’t Veer *et al*., 2002).

6. Epithelial to Mesenchymal Transition (EMT)

Epithelial to Mesenchymal Transition (EMT) represents the dedifferentiation of a polarized epithelial cell and its conversion to a fibroblastoid-like phenotype as described in Fig. 2. An epithelial cell connected to the basement membrane decomposes its extracellular matrix (ECM) and converts to a mesenchymal phenotype which gains the function to disperse from the epithelial layer.

Hallmarks of EMT are the downregulation of E-cadherin, the disappearance of b-catenin from the cell surface and its accumulation within the nucleus and the upregulation of many ECM components, which are known to be mesenchymal markers (Kalluri *et al*., 2003).

E-cadherin is a cell adhesion molecule located at the adherens junctions within the membrane of epithelial cells. It is able to stabilise intercellular contacts as well as it conserves cell polarity. Markers for an epithelial phenotype, like b-catenin or ZO-1, disappear from the cell boundaries, while markers for fibroblastiod cells e.g. α-sma, fibronectin are found being upregulated. The loss of its intercellular adhesion ability and the acquisition of a mesenchymal phenotype leads to an increased migratory potential.

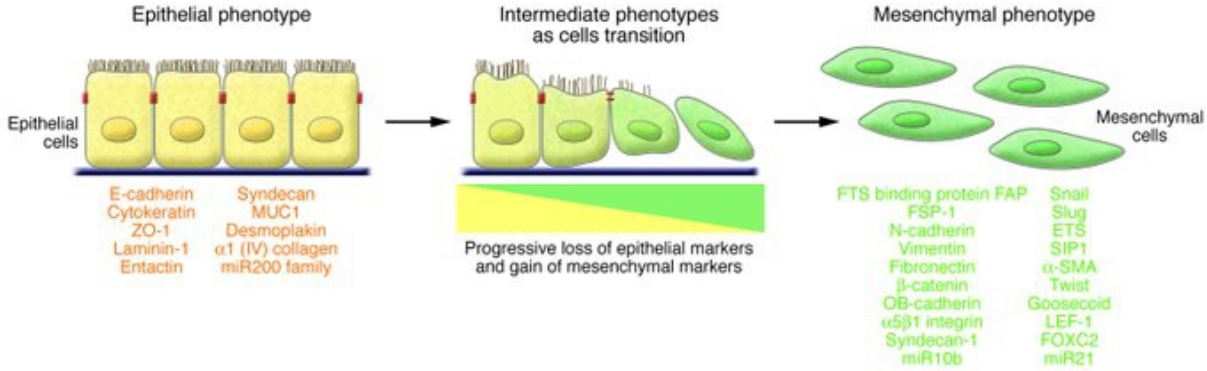

Figure 2

The left image shows the epithelial phenotype with the associated markers written below in orange. At the onset of EMT, the cell acquires mesenchymal attributes and loses the epithelial ones, leading to an intermediate state as seen in the picture in the middle. Finally, the cell acquires a mesenchymal phenotype as seen in the right picture, which is associated with the markers written below in green (3).

At the moment three different subtypes of EMT are distinguishable (Kalluri *et al.*, 2009),

which are defined by their biological context (see Fig. 3). The first subtype (Type I EMT) is seen in embryo formation and development, where repeated processes of EMT and Mesenchymal to Epithelial Transition (MET) take place in order to generate novel cell types and tissues. The second subtype (Type II EMT) is connected to wound healing, tissue regeneration and organ fibrosis. Sustained trauma or injury and the accompanying inflammation initiate the generation of fibroblasts, which are needed to reconstruct and repair damaged tissue. The third subtype (Type III EMT) is observed within neoplastic cells with genetic or epigenetic changes, often affecting oncogenes and/or tumour suppressor genes. The EMT process can boost the invasion capacity of benign neoplasms which may result in a progression towards malignancy.

7. Mechanisms of EMT

7.1 Type I EMT

Type I EMT is being monitored in gastrulation and embryonic development, where it does not cause fibrosis or induction of an invasive phenotype, that would lead to metastatic spreading. Instead this kind of EMT has the ability to create mesenchymal cells, which in turn are able to perform MET to create secondary epithelia.

The first sign of gastrulation is the formation of a primitive streak in the epiblast layer. It facilitates the generation of ecto-, endo- and mesoderm, which will generate all tissue types of the body. The invagination of the primitive streak leads to the generation of the mesendoderm, which separates into mesoderm and endoderm via EMT. The mesoderm consists of spindle shaped cells called fibroblasts, that have the ability to invade ECM, while ecto- and endodermal cells show an epithelial phenotype.

Gastrulation is dependent on canonical Wnt signalling (Skromne *et al*., 2001), which is mediated by TGF-β superfamily proteins e.g. Nodal, Vg1 (Chea *et al*., 2005; Skromne *et al*., 2005). Wnt and fibroblast growth factor (FGF) are inducers of EMT during gastrulation and activate transcription factors like Snail, Eomes and Mesps, which control further EMT progression (Kalluri *et al*., 2009).

For example, the process of mesoderm differentiation is induced by fibroblast-growth-

factor (FGF), which activates the signal transducer Snail. This leads to the transcriptional repression of E-cadherin, an integral component of the epithelial adherence junctions, and thus to EMT.

In embryonic development, epithelial cells of the neuroectoderm start to express genes like Sox, Snail and Slug (Sauka-Spengler *et al*., 2008), which leads to EMT. Thereby these cells become motile and disperse within the embryo, where they undergo further differentiation into different cell types e.g. melanocytes (Kalluri *et al*., 2009). The signalling leading to EMT is transduced via Wnt, FGF, BMP, c-Myb and msh homeobox 1 (Msx-1)(Kalluri *et al*., 2009) and thereby shows some similarities to that of EMT progression during gastrulation.

In neural crest cells it is essential for the occurrence of EMT to have working BMP signalling, because it induces the development towards migration (Burstyn-Cohen *et al*., 2004). Furthermore it is necessary to repress E-cadherin and N-cadherin in order to disrupt their function as cell adhesion molecules (Thiery, 2003).

7.2 Type II EMT

Type II EMT is connected with wound healing, tissue regeneration and organ fibrosis. An inflammatory response aids damaged tissue in order to generate fibroblasts as part of a reconstruction process. Normally, when the tissue is repaired the inflammation abates and the EMT process is terminated. However, if inflammation signals persist and cells continue to respond to the signals in performing EMT, then this leads to organ fibrosis and onward to the destruction of the organ.

Fibroblasts and inflammatory cells release diverse inflammatory signals as well as a complex ECM, containing collagens, laminins, elastins and tenacins (Kalluri *et al*., 2009). This can mediate organ fibrosis in epithelial tissues of lung, kidney, liver and intestine (Potenta *et al*., 2008; Zeisberg *et al*., 2007; Kim *et al*., 2006). Under chronic inflammation, cells of these tissues show epithelial-specific morphology and markers e.g. cytokeratin, E-cadherin, but additionally exhibit mesenchymal specific markers like Fibroblast-specific-protein 1 (FSP1) and α-SMA (Kalluri *et al*., 2009). This represents an intermediate stage of EMT, termed "partial EMT", caused by inflammatory stress. Finally those cells leave the epithelial layer, migrate through the basement layer and congregate in the interstitium

(Okada *et al*., 1996), where they develop their fibroblastoid phenotype.
It has also been shown, that endothelial cells can transform to mesenchymal cells and thus support fibrosis. They use an analogous process called Endothelial to mesenchymal transition (EndMT), a process normally occurring during development and used in heart valve formation (Potenta *et al*., 2008). TGF-β1 is able to induce EndMT in capillary endothelial cells, which manifests with the loss of endothelial markers e.g. CD31 and the acquisition of fibroblast and myofibroblast specific factors, like FSP1, α-SMA, collagen1 and vimentin (Kalluri *et al*., 2009).
The inflammatory response, caused by injury, mobilizes a variety of cells, that are able to trigger EMT, among them macrophages and resident fibroblasts. To evoke EMT, they release growth factors, like TGF-β, PDGF, EGF and FGF2 (Strutz *et al*., 2002) as well as chemokines and MMPs e.g. MMP2, MMP3 and MMP9 (Kalluri *et al*., 2009). This signalling molecules cooperate with inflammatory cells and cause damage to basement membrane of epithelial cells and induce focal degradation of collagen IV and laminin (Strutz *et al*., 2002). As a consequence the epithelial cells are able to migrate to the interstitial area, where they transform to fibroblasts.
In order to avoid fibrosis and to maintain organ functionality, several ways have been shown, that either avoid or revoke EMT. The blocking of MMP-9 expression exhibits the inhibition of epithelial cell recruitment for EMT (Yang *et al*., 2002). Moreover HGF and BMP-7 are known to weaken or disrupt TGF-β signalling, leading to the reversal of TGF-β mediated E-cadherin loss (Yang *et al*., 2002; Zeisberg *et al*., 2003). In the case of BMP-7 it was shown, that systemic administration lead to the reversal of EMT, the repair of damaged epithelial structures and the restoration of organ function.

7.3 Type III EMT

Type III EMT is linked with the development of invasion and metastasis of malignant epithelial cancers. Primary epithelial tumours are characterized by excessive cell proliferation and angiogenesis (Hanahan *et al*., 2000), caused by genetic and epigenetic alterations e.g. oncogenes.
At this stage EMT can assist in the acquisition of an invasive phenotype with increasing motile potential. As a first step it enables the migration through the basement membrane

and with further progression of invasiveness, it is finally leading to the ability to metastasise, which implies the spreading to distant sites. Several experiments have revealed, that cancerous cells transforming to a mesenchymal phenotype also begin to express mesenchymal markers, like α-SMA, FSP1, vimentin and desmin (Yang *et al*., 2008).

However, the secondary tumour colonies found at this sites no longer show this mesenchymal phenotype, but at a histopathological level, they show the same phenotype again as the primary tumour from which they originally emerged. This indicates, that metastatic cancer cells, not only rely on EMT to develop spreading, but also require MET in order to colonise these distant sites (Zeisberg *et al*., 2005). The MET can be triggered by the different microenvironment at the distant sites, which no longer resembles the primary tumour environment, that has lead to the development of EMT (Bissell *et al*., 2002).

The heterotypic signalling in the stroma associated with the primary tumour is heavily suspected to initiate EMT in cancer cells by the presence of factors like HGF, EGF, PDGF and TGF-β (Kalluri *et al*., 2009). They further activate EMT inducing transcription factors, most prominently Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist and FOXC2, which are able to regulate the complex EMT program separately or in conjunction (Kalluri *et al*., 2009). The EMT program contains the modulation of intracellular signalling networks, e.g. ERK, MAPK, PI3K, Akt, Smads, RhoB, β-catenin, lyphoid enhancer binding factor (LEF), Ras and c-Fos, and cell surface proteins e.g. β4 integrins, αVβ6 integrins (Tse *et al*., 2007), as wells as it manages the disruption of cell-cell adherence junctions and cell-ECM adhesions (Yang *et al*., 2008).

TGF-β is one of the most prominent and studied inducers of EMT in cancerous epithelial cells. In epithelial cells it acts as a repressor of proliferation and thus as an important tumour suppressor. However, TGF-β is able to onset EMT in certain transformed cells and hereby contributes to tumour progression. There are two signalling pathways known to be used, involving Smads (Roberts *et al*., 2006) and p38 MAPK (Bhowmick *et al*., 2001).

The first signalling pathway relies on the involvement of Smads, which induces EMT via the ALK-5 receptor (Piek *et al*., 1999). Smads control transcription factors and cytoplasmic kinases and are able to induce an autocrinous loop of TGF-β. In advance,

they are also involved in controlling the action of LEF and β-catenin (Kim *et al*., 2002), which are responsible for the metastatic potential.

The second pathway is connected with MAPK signalling. It was shown, that p38 MAPK and RhoA with the help of integrinβ1 signalling on the one hand, and ERK/MAPK in conjunction with PI3K/Akt and Raf on the other have been identified to be capable of establishing TGF-β induced EMT (Bhowmick *et al*., 2001; Janda *et al*., 2002; Lehmann *et al*., 2000).

The onset of EMT in cancer cells is also connected with the loss of E-cadherin expression (Eger *et al*., 2000).This promotes Wnt signalling and the expression of transcription factors, that advocate a mesenchymal phenotype, such as Snail, Slug and SIP1 (Medici *et al*., 2008; Comijn *et al*., 2001).

In the absence of Wnt induced signalling, E-cadherin is a binding partner for β-catenin, which is a component of the adherens junctions. β-catenin from the cytoplasma is used to charge the extracellular matrix, where it is stably bound, whereas dispensable β-catenin from the cytoplasmic pool is continuously degraded. Therefore it builds a complex with the tumour suppressor Axin, the tumour suppressor protein Adenomatous Polyposis Coli (APC) and with two kinase families, CK1 and GSK3-alpha/beta (Clevers, 2006). These kinases phosphorylate β-catenin, which is then recognized by β-TrCP, a part of a E3 ubiquitin ligase complex, leading to ubiquitinilation and as a consequence to its degradation by the proteasome.

Upon Wnt activated signalling, Axin is inactivated by the Wnt co-receptor LRP. As a consequence, the cytoplasmatic pool of unphosphorylated β-catenin is rising, allowing the transcription factor to enter the nucleus. There it binds to the protein complex TCF/LEF, which leads to the expression of target genes like CyclinD1 and c-myc (Clevers, 2006).

Several studies have confirmed, that the control over E-cadherin and β-catenin/LEF is a key component for the invasive potential of a cell (Bowen *et al*., 2008).

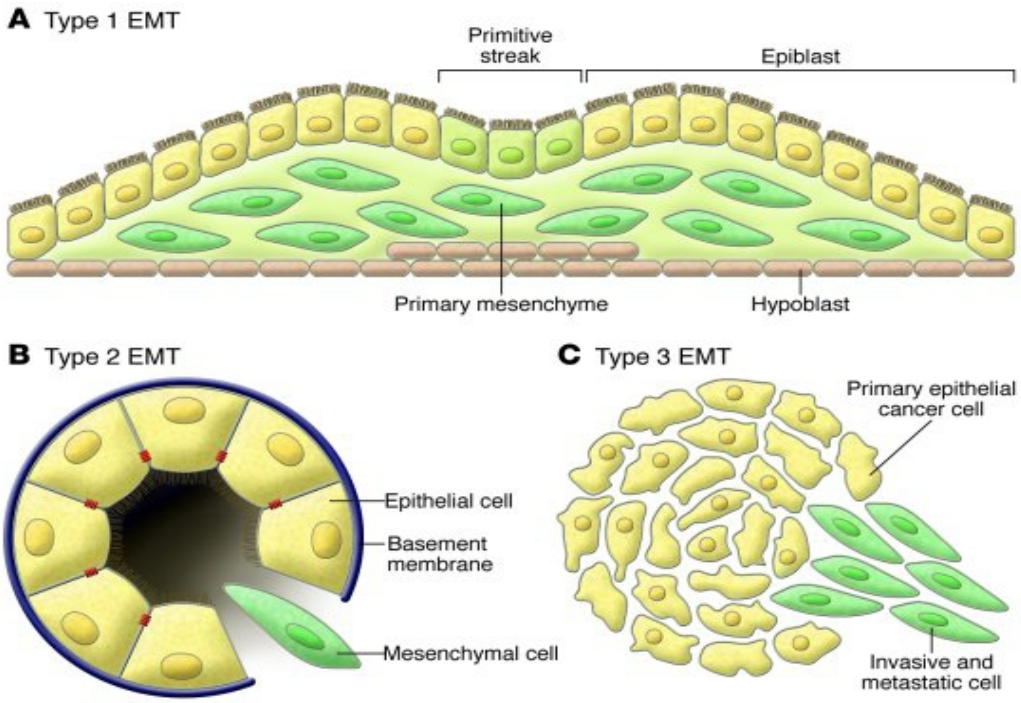

Figure 3

A Type I EMT gives rise to the mesoderm and endoderm during development. The epiblast uses EMT to form the primary mesenchym, which in turn can form secondary epithelia via MET. **B** Type II EMT are present during inflammation anf fibrosis. In contrast to Type I they persist much longer and eventually destroy the organ. **C** Type II EMT is present in the transformation of polarized primary cancer cells towards an invasive and fibroblastoid phenotype (3).

8. The Liver EMT Model

8.1 Models for HCC

In order to study the development and progression of HCC, several models for *in vivo* and *in vitro* analysis have been established.

8.1.1 *In Vivo* Models

The *in vivo* studies are conducted within animal models, which are largely mouse models because of the advantages of its small size, the entirely sequenced genome and the similarities to humans. Mouse models for HCC are either generated by the administration of genotoxic or non-genotoxic carcinogens, the implantation of tissue or are genetically engineered (Leenders *et al*., 2008).
Genotoxic carcinogens cause mutations by forming DNA adducts, which leads to genetic changes within the cell resulting in a preneoplastic state. Non-genotoxic carcinogens do not alter DNA structure, but stimulate the preneoplastic cells to evolve into a malignant neoplasm by manipulating cell proliferation, apoptosis and cell differentiation (Wogan, 2000).
Implantation models are widely used to promote HCC formation in mice for the preclinical evaluation of anticancer agents. This can be obtained by either implanting a HCC cell line or tissue fragment into mice strains from the same origin (Killion *et al*., 1998), or by implanting primary human HCC cell lines or tissue fragments into immune-compromised mice (xenograft models) (Troiani *et al*., 2008).
Genetically engineered mouse models (GEM) for HCCs offer the study of molecular mechanisms involved in hepatocarcinogenesis (Frese *et al*., 2007; Leenders *et al*., 2008). They are used to explore the role(s) or interaction of different genes (e.g. oncogenes and tumour-suppressor genes) as genetic alterations in various cellular pathways (e.g. pathways involved in growth, apoptosis, proliferation and angiogenesis), which are fundamental for the development of HCC.

8.1.2 *In Vitro* Models

The *in vitro* studies are performed with cell lines, which consist of immortalised cells derived mainly from mouse, rat and human HCCs. They offer an increased level of control and examination possibilities compared to animal models (Feo *et al*., 2007). Their advantages are, that their growth can be synchronised, they can be used in high numbers and parameters concerning growth, metabolism and cell death by apoptosis can be evaluated. Additionally, in order to study the role of single genes or the effect of signalling pathways, they pose the option of being engineered. This can be archived by transfection of genes, antisense oligonucleotides, siRNAs or the administration of inhibitory compounds.
One major limitation of cell lines is the tendency, that increasing numbers of *in vitro* passages accelerate the progression of Morris hepatoma, which represents a highly undifferentiated phenotype.
Considering the facts, cell lines are an extremely useful tool for the investigation of molecular mechanisms *in vitro*, however the results of the studies always need to be verified *in vivo*.

8.2 The MIM Hepatocellular Mouse Model

In order to investigate the steps leading to hepatocellular carcinoma, we engineered a inducible hepatocellular EMT model (Gotzmann *et al*., 2002).
We isolated immortalized hepatocytes of p19/alternate reading frame (ARF) knockout mice, and let them grow as monolayers. The ARF protein acts as tumour suppressor by inhibiting ribosome biogenesis and the induction of p53 dependent cell cycle arrest and apoptosis. This is a first step in tumour development, as the cells do not go into cell cycle arrest but keep dividing. However, the cells conduct apoptosis when treated with TGF-ß (1ng/µl). TGF-ß acts as an antiproliferative factor within the cell cycle, induces apoptosis through the SMAD and the DAX pathways and is suspected to be involved in the regulation of the immune system (e.g. regulatory T cells) and blocks the activation of phagocytes. This cell line was named MIM1-4.
The next step was the stable insertion and expression of Ha-ras into MIM1-4 cells,

representing a further step in tumour progression. Ras is a monomeric GTP binding protein, which has GTPase activity and acts as a switch: It is active when GTP is bound and inactive without. Through mutations Ras can lose its GTPase activity which results in permanently bound and thus activated Ras-GTP. Ras phosphorylates and activates microtubole-associated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) through RAF and MEK signalling, which activates transcription factors (e.g C-myc), that are important for cell proliferation and the control of the cell cycle. Additionally Ras is able to activate Akt via PI3K signalling (Downward, 1998), leading to increased cell survival and resistance to apoptosis. As a consequence, permanent Ras activation results in an ongoing stimulus of cell growth.

We could observe, that these cells were growing faster but they still showed a polarized phenotype and formed an epithelial layer on collagen. MIM1-4 cells expressing Ha-ras were called MIM-ras cells.

If we treated MIM-ras with TGF-ß, they did not go into apoptosis but performed a morphogenic switch from epithelial cells to a fibroblastoid phenotype, which we called MIM-rt cells. These cells showed a spindle shaped, dedifferentiated phenotype, they did not need an extracellular matrix to adhere and formed a diffuse polylayer if supplied with TGF-ß. The transformed cells established a weak autocrinous loop of TGF-ß, that promoted the fibroblastoid phenotype for several days, but slowly reverted back to epithelial cells if not treated with TGF-ß.

This resulted in three cell lines in order to explore oncogenous EMT progression. Two of the cells lines acted as a morphogenic switch, depending on the presence of TGF-ß. This gave us the opportunity to compare gene abundance before and after transition, displaying the regulation of the participating pathways.

9. PDGF: A potentative novel therapeutic target for Liver Cancer

In order to determine the involvement and regulation of genes during EMT, we performed a gene expression study of mouse hepatocytes using an Affymetrix Genechip 11k microarray. We used immortalized hepatocytes of the MMH cell line and compared the epithelial phenotype to the mesenchymal state after EMT caused by TGF-β administration. The data of the two different states was clustered and thus revealed the up- and downregulations of genes during the mesenchymal transition. Among the genes connected with EMT, the upregulation of PDGF-α and PDGF-α receptor (PDGFR-α) indicates, that a whole pathway system could be activated to support the mesenchymal phenotype.

9.1 The Involvements of PDGF

Platelet derived growth factor (PDGF) plays an important role in the regulation of cell growth and transformation (Beckmann *et al*., 1988), blood vessel formation (Lindahl *et al*., 1997), wound healing (Pierce *et al*., 1989) and embryonic development (Betsholtz *et al*., 2001). Therefore it is able to regulate the cell cycle (Styles *et al*., 1979), has mitogenic and chemoattractive properties (Ross *et al*., 1986; Heldin, 1992) and, depending on tissue-type, causes apoptosis (Kim *et al*., 1995) or boosts proliferation (Paulsson *et al*., 1987). Additionally it is supposed to take place in ECM tissue remodelling (Yu *et al*., 2003), as it controls the production of collagen (Canalis, 1981), fibronectin (Blatti *et al*., 1988) and collagenase (Chua *et al*., 1985).

9.2 The Form and Function of PDGF

PDGF is a polypeptide with four known ligands (A, B, C, D), that form the homodimers AA, BB, CC, DD and the heterodimer AB by forming disulphide bonds (Heldin *et al*., 2002). These dimers bind to the extracellular domain of the PDGF receptors (PDGFR), which consist of five immunoglobin-like domains and use three of them for ligand recognition (see Fig. 4).

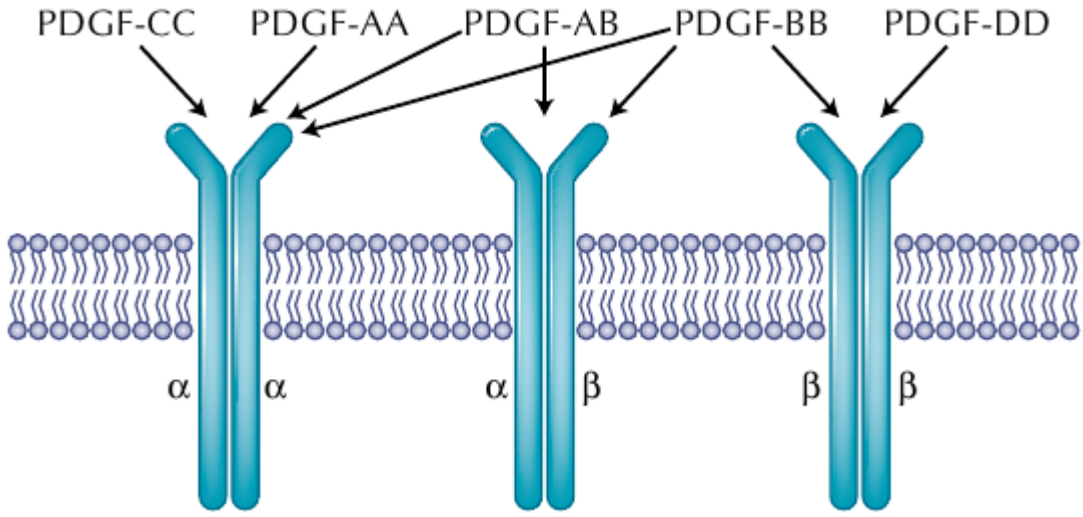

Figure 4

This figure shows the PDGF dimer interactions with PDGF receptors. PDGFR-αα is activated by PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC, PDGFR-αβ is activated by PDGF-AB, PDGF-BB and PDGF-CC and PDGFR-ββ is activated by PDGF-BB and PDGF-DD (4).

The PDGF receptors are present as dimers, consisting of the isoforms PDGFR-α and PDGFR-β, which form the homodimers PDGFR-αα and PDGFR-ββ as well as the less frequent heterodimer PDGFR-αβ. PDGFR-αα binds PDGF-AA, -AB, -BB,-CC, PDGFR-ββ binds PDGF-BB, -DD and PDGFR-αβ binds PDGF-AB, -BB, -CC, (-DD low affinity) (Claesson-Welsh, 1994; Yu *et al*., 2003). The ligands have different binding affinity for the receptors, which are able to specifically control the downstream signalling.

The dimerization upon ligand binding leads to autophosphorylation, a conformational change and the activation of the intracellular receptor tyrosine kinase domain (Yu *et al*., 2003). The activation of the kinase is done by the phosphorylation of a certain tyrosine residue. This enables the kinase to phosphorylate receptor tyrosines of Src homology 2 domains (SH2) of signalling molecules binding to the receptor site, such as phospholipase γ (PLC γ), Ras GTPase-activating protein (Ras-GAP), p85 subunit of phosphatidylinositol 3-kinase (PI-3K), growth factor receptor bound protein 2 (Grb2) and the non-receptor tyrosine kinase family Src (Claesson-Welsh, 1994; Yu *et al*., 2003).

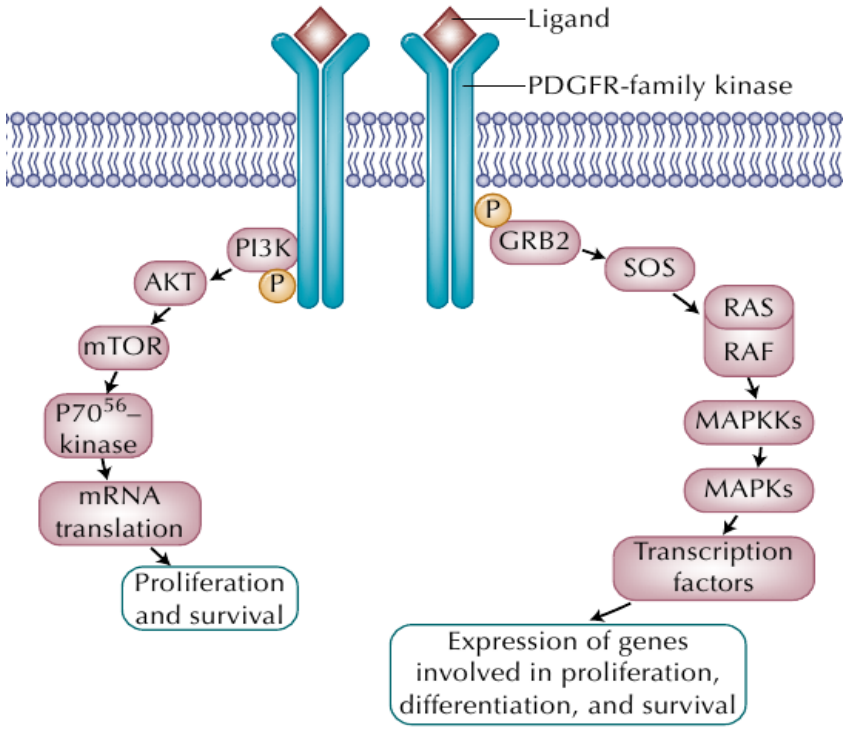

Figure 5

PDGF receptor signalling is able to activate the mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol 3' kinase (PI3K) pathway, leading to cell survival, proliferation and differentiation (4).

9.3 PDGF induced Signalling

The activation of signalling molecules leads to signal transduction and the induction of core downstream signalling components, such as Ras and MAPK signalling as well as the PI3K pathway as outlined in Fig. 5 (Schmahl *et al*., 2007). The MAPK pathway signals through ERK and JNK (Yu *et al*., 2003) and thus regulates gene expression, cell proliferation, differentiation, apoptosis and immunoresponses. The PI3K signals through Akt (Zhang *et al*., 2007) and therefore controls gene expression, cell cycle regulation, survival and motility.

PDGFR-α and PDGFR-β signalling offers some redundancy by activating the same overlapping pathways, however some pathways are exclusively triggered by only one of the receptors (Wu *et al*., 2008). So for instance, only PDGFR-α/β heterodimers activate components of the NFκB and IL-6 pathway, PDGFR-α homodimers activate the C21

steroid hormone biosynthesis and PDGFR-β homodimers activate angiogenesis and epidermal growth factor receptor pathways (Wu *et al*., 2008).

Concerning MAPK signalling, PDGFR-α is able to induce ERK and JNK, whereas PDGFR-β is only able to induce ERK. This gives the PDGFR-α growth stimulation and inhibition properties, leading to positive and negative signalling for cell transformation, while the PDGFR-β mainly has positive signalling for cell transformation. On PDGF-BB administration, which is able to bind both receptors, the signalling balance shifts towards transformation, whereas PDGF-AA administration leads to no transformation, as it only binds to PDGFR-α (Yu *et al*., 2003). However, if the negative signalling for transformation (JNK) of the PDGFR-α is blocked by Bcl-2, then this leads to positive signalling for cell transformation (Kim *et al*., 1994).

The PI3K/Akt activation can be disrupted by mTOR, which negatively regulates the PDGF receptors (Zhang *et al*., 2007).

9.4 PDGF Signalling induced Diseases

Increased PDGF signalling has been observed in pathogenesis of atherosclerosis, restenosis, pulmonary fibrosis, angiogenesis and tumourigenesis (Levitzki, 2004; Wu *et al*., 2008). Tumours are known to be caused by the amplification of the PDGFR-α gene as seen in gliomas (Fleming *et al*., 1992) or activating point mutations and small deletions in PDGFR-α as found in GISTs (Heinrich et al., 2003). Fusion of the PDGF receptor with a protein can lead to its constitutive activation, so constitutive active PDGFR-α is known to cause the idiopathic hypereosinophilic syndrome (Cools *et al*., 2003) whereas constitutive active PDGFR-β leads to chronic myelomonocytic leukaemia (CMML) (Magnusson *et al*., 2001). Translocations lead to the fusion protein of collagen 1A1 gene with PDGF-B which is constitutively produced and further processed to PDGF-BB, leading to dermatofibrosarcoma protuberans (DFSP) (Shimizu *et al*., 1999). Furthermore upregulation of the PDGF ligands and receptors is observed in soft tissue sarcomas and gliomas (Östman *et al*., 2001).

10. Questions and Aims of the Thesis

With the upregulation of PDGFR-α expression during EMT, the question arose, in which way it supported the mesenchymal phenotype. Since it was shown, that blocking of the PDGF pathway by inhibitors leads to the reversal of the mesenchymal phenotype and apoptosis in human mammary carcinoma cell lines (Jechlinger *et al*., 2006), we assumed, that an inhibition using functional genetics would lead to a more studiable system. Thus our aim was to stably introduce and express a dominant-negative PDGFR-α (Yu *et al*., 2000) in MIM-ras and MIM-rt cells, to compete with native PDGF ligand binding and thus block PDGF signalling. With regard to expression level, we used two different promoters: the moderate expression level was driven by an LTR, while the high expression level was archived by the use of glucose regulated protein 78 (grp78) as promoter element. This two differing expression levels would allow us to monitor the impact of PDGF signalling during TGF-ß induced hepatocellular EMT in more detail.

As a next step we studied the changes within the extracellular microenvironment during EMT in conventional and 3D cell culture as well as *in vivo*. As cell surface composition and ECM configuration are important parts in the motile repertoire of cells, we assessed the invasion potential of the cells with disrupted PDGF signalling e.g. by wound healing assays. This gave us an idea, whether PDGF signalling directly influences cell-cell adhesion or ECM composition and thus affects the motility of a transformed cell. By the use of 3D collagen cultures, we addressed the question, if MIM-ras and MIM-dnPDGF-α-ras cells are able to organize in organotypic structures and how those structures are affected by TGF-β administration. With the help of *in vivo* experiments, we observed the tumour forming abilities of MIM-dnPDGF-α-ras and MIM-dnPDGF-α-rt cells and compared them to cell lines with functional PDGF signalling.

Moreover we also monitored the impacts of impaired PDGF signalling on the MAPK and PI3K/Akt signalling pathways. As Ras already activates MAPK signalling in MIM-ras cells, the additional activation of PI3K/Akt pathway through PGDF signalling may play an important role for cell survival during the transition and the subsequent invasion processes. This should give us a picture, whether PGDF signalling is a key player of invasion and if it additionally enhances the survivability of transformed cells.

In addition we employed the receptor tyrosine kinase inhibitor STI571 to block PDGF receptor signalling and evaluate the implications on tumour cell growth and motility. This inhibitor compound is currently used to treat chronic myelogenous leukaemia and gastrointestinal stromal tumours and could be a potential candidate to specifically target the invasiveness of HCC

The mandatory involvement of PDGF ligand and PDGFR-α in EMT would pose a novel and distinct therapeutic target for the treatment of hepatic cancer. This would allow for the specific blocking of the PDGF pathway, resulting in more efficiency and lower side effects. The jamming of invasion and metastasis would be a huge improvement for anticancer therapy, as it would allow for the focused treatment of primary tumours without the threat of spreading tumour cells.

11. The publication "A crucial function of PDGF in TGF-β-mediated cancer progression of hepatocytes"

My personal contribution to the the publication

My contribution to the paper was the *in vitro* analysis of the impaired PDGFR-α signalling during EMT. I was able to establish a MIM-ras cell line expressing the dominant-negative PDGF-α receptor (dnPDGFR-α) together with a red fluorescent protein (RFP), called MIM-dnPDGFR-α-ras. Additionally to the cell line with moderate (LTR driven) expression we constructed and high expression (grp78 driven) vector of dnPDGFR-α, however only the cell line with moderate expression managed to survive and thus was used for further experiments. The cells were sorted for RFP several times, so that I ended up with an approximate percentage of 90% RFP positive cells after sorting. The overexpression of dnPDGFR-α was verified by western blotting analysis. In a next step, I compared the growth and survival rates of MIM-dnPDGFR-α-ras cells to those of MIM-ras cells. Furthermore, I analysed the migration potential of MIM-dnPDGFR-α-ras and MIM-ras cells upon EMT induction through TGF-β stimulation by the use of wound healing assays. Thereby, I measured the ability of the cell lines to close a scratch within a confluent tissue layer over defined time intervals. Similarly, together with Mario Mikula, we analysed and compared the differing tumourigenic properties of MIM-ras and MIM-dnPDGFR-α-ras cells as well as MIM-rt and MIM-dnPDGFR-α-rt cells *in vivo*.
Additionally, I investigated a putative relationship of the PDGFR-α signalling and the activation of PI3K signalling upon EMT by a comprehensive western blotting analysis, but was unable find a direct relationship.

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ORIGINAL ARTICLE

A crucial function of PDGF in TGF-β-mediated cancer progression of hepatocytes

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Polarized hepatocytes expressing hyperactive Ha-Ras adopt an invasive and metastatic phenotype in cooperation with transforming growth factor (TGF)-β. This dramatic increase in malignancy is displayed by an epithelial to mesenchymal transition (EMT), which mimics the TGF-β-mediated progression of human hepatocellular carcinomas. In culture, hepatocellular EMT occurs highly synchronously, facilitating the analysis of molecular events underlying the various stages of this process. Here, we show that in response to TGF-β, phosphorylated Smads rapidly translocated into the nucleus and activated transcription of target genes such as E-cadherin repressors of the Snail superfamily, causing loss of cell adhesion. Within the TGF-β superfamily of cytokines, TGF-β1, -β2 and -β3 were specific for the induction of hepatocellular EMT. Expression profiling of EMT kinetics revealed 78 up- and 235 downregulated genes, which preferentially modulate metabolic activities, extracellular matrix composition, transcriptional activities and cell survival. Independent of the genetic background, platelet-derived growth factor (PDGF)-A ligand and both PDGF receptor subunits were highly elevated, together with autocrine secretion of bioactive PDGF. Interference with PDGF signalling by employing hepatocytes expressing the dominant-negative PDGF-α receptor revealed decreased TGF-β-induced migration *in vitro* and efficient suppression of tumour growth *in vivo*. In conclusion, these results provide evidence for a crucial role of PDGF in TGF-β-mediated tumour progression of hepatocytes and suggest PDGF as a target for therapeutic intervention in liver cancer.

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Introduction

Carcinomas represent the most frequent malignant diseases in the world. Multiple mutations contributing to malignant transformation of epithelial cells have been described and characterized for a variety of tumour types (Hanahan and Weinberg, 2000), but the involvement of such mutated genes to local invasiveness and metastasis remains less well understood.

Epithelial to mesenchymal transition (EMT) is a fundamental process during normal development, inflammation and wound healing. Increasing evidences indicate that EMT represents an *in vivo* correlate to late stages of carcinoma progression (Boyer *et al*., 2000; Thiery, 2002; Grunert *et al*., 2003; Gotzmann *et al*., 2004). During EMT, polarized epithelial cells acquire a fibroblastoid phenotype, display increased motility and an enhanced invasive potential. These alterations are reminiscent of metastatic processes, when tumour cells leave well-organized epithelial structures to migrate to distal locations. Cells undergoing EMT typically lose cell–cell adhesion molecules, change the expression pattern of cell surface molecules such as integrins and show extensive alterations of the extracellular matrix (ECM) composition, including secretion of ECM-remodelling enzymes of the matrix metalloproteinase (MMP) family. Downregulation of the tumour suppressor E-cadherin represents a pivotal event associated with the onset of epithelial dedifferentiation, which contributes to enhanced cell motility and invasiveness and thus correlates with poor prognosis of a variety of carcinomas (Frixen *et al*., 1991; Vleminckx *et al*., 1991; Behrens *et al*., 1992; Perl *et al*., 1998; Conacci-Sorrell *et al*., 2002). The expression of E-cadherin is controlled by transcriptional repressors of the Snail superfamily (Hajra *et al*., 2002; Nieto, 2002), which include the Smad-interacting protein SIP1 (Comijn *et al*., 2001), the basic HLH-protein E12/E47 (Perez-Moreno *et al*., 2001) and the E-box-binding transcription factor delta-EF1/ZEB (Grooteclaes and Frisch, 2000; Eger *et al*., 2005). It was shown that ectopic

overexpression of Snail in different epithelial cells was sufficient to cause a phenotypical conversion to fibroblastoid cells, which was accompanied by loss of E-cadherin expression and acquisition of tumorigenic and invasive properties (Cano *et al*., 2000).

We have recently established a cellular EMT model unique to hepatocytes that is driven by the collaboration of hyperactive Ras and transforming growth factor (TGF)-*β* (Gotzmann *et al*., 2002), a functional synergy also described in breast epithelial cells (Oft *et al*., 1996; Janda *et al*., 2002). TGF-*β* signals through a heteromeric pair of TGF-*β* receptors (T*β*Rs), which phosphorylate intracellular adaptor molecules known as Smads. Upon their nuclear translocation, Smad complexes regulate the transcription of a set of target genes, leading to various changes in the cellular phenotype. On the one hand, TGF-*β* exhibits a tumour-suppressive function since it acts as a potent inhibitor of epithelial cell proliferation by arresting cells in the G1 phase of the cell cycle and by inducing apoptosis (Reynisdottir *et al*., 1995). In several cancer types, the negative interference of this pathway by mutations that lead to nonfunctional Smad4 or T*β*Rs underscores the tumour-suppressive potential of TGF-*β*. On the other hand, TGF-*β* can act in a tumour-promoting manner, particularly if cooperating with other oncogenes (de Caestecker *et al*., 2000; Dumont and Arteaga, 2000; Rossmanith and Schulte-Hermann, 2001; Grunert *et al*., 2003; Gotzmann *et al*., 2004). With respect to hepatocellular carcinoma (HCC), overproduction of TGF-*β* and autocrine stimulation leading to elevated plasma levels in HCC patients correlate with poor prognosis (Ito *et al*., 1991; Shirai *et al*., 1994; Matsuzaki *et al*., 2000).

Ras family proteins are key players in regulating cell homeostasis, and therefore have severe implications in most tumour types, when signalling in an oncogenic manner upon mutation or amplification. Ras exerts its signal through several downstream routes such as the Raf–Erk/MAPK and the phosphatidyl inositol 3 (PI3) kinase–PKB/Akt pathways, both representing the best studied ones. Several, in part, conflicting mechanisms have been described of how Ras can render cells insensitive to growth inhibition by TGF-*β*. Although TGF-*β* alone can be responsible for gross morphological changes occurring during EMT (Bhowmick *et al*., 2001), a cooperative proliferative stimulus is required to fully accomplish maintenance of the invasive phenotype (Janda *et al*., 2002; Grunert *et al*., 2003; Fischer *et al*., 2005).

In the present study, we made use of the previously described, highly synchronous hepatocellular EMT *in vitro* to analyse the sequence of molecular events (Gotzmann *et al*., 2004). Insights into the molecular changes during EMT were obtained by monitoring global expression patterns employing oligo-microarray analysis. Based on this expression profiling, a crucial role of the platelet-derived growth factor (PDGF) pathway was assumed, since PDGF-A ligand and PDGF receptor subunits were highly elevated upon EMT along with a TGF-*β*-induced secretion of PDGF-A. Indeed, interference with PDGF signalling indicated that this pathway is necessary and sufficient to stabilize the invasive phenotype during TGF-*β*-mediated hepatocellular tumour progression.

Results

Loss of cell–cell contacts is orchestrated in a hierarchical manner upon hepatocellular EMT

We recently described a cellular model of late-stage hepatocarcinogenesis that involves the increase in malignancy of neoplastic hepatocytes towards the acquisition of metastatic properties. The change in cell fate of malignant hepatocytes has been characterized by an EMT transition arising from the synergism of TGF-*β*1 and hyperactive Ras signalling (Gotzmann *et al*., 2002). By employing immortalized MMH-D3 hepatocytes (Amicone *et al*., 1997), expression of oncogenic Ha-Ras (referred to as MMH-R) showed malignant transformation along with maintained epithelial characteristics. However, polarized MMH-R hepatocytes rapidly converted to spindle-shaped, fibroblastoid cells upon treatment with TGF-*β*1 and established an invasive phenotype after administration of TGF-*β*1 for more than 21 days (termed MMH-RT). Remarkably, fibroblastoid features could be observed as early as 12 h after TGF-*β*1 treatment, being morphologically completed within 24 h. Importantly, this change in epithelial cell plasticity occurred in a highly synchronous manner (Figure 1). The simultaneous conversion of malignant hepatocytes towards the metastatic phenotype encouraged us to investigate the molecular mechanisms underlying the sequential steps of EMT in a kinetic manner.

A prerequisite for acquiring a spindle-shaped cell type includes loss of cell polarity and adhesion. Comparison of epithelial MMH-R versus fibroblastoid MMH-RT cells revealed that EMT of hepatocytes is accompanied by cytoplasmic redistribution of the polarity marker ZO-1 as well as loss or redistribution of adherens junctions and desmosomal marker proteins such as E-cadherin, *β*-catenin, p120-catenin ($p120^{ctn}$) and desmoplakin, respectively (Gotzmann *et al*., 2002). Kinetic studies during the initial stages of EMT revealed that cytoplasmic accumulation of the tight junction protein ZO-1 preceded apparent changes of adherens junctions family members (Figure 1). Disintegration of tight junction complexes and thus loss of polarity started about 12 h after TGF-*β*1 addition (data not shown) and was nearly completed after 24 h. At this time, only few epitheloid cells retained membrane-associated staining for ZO-1. After 48 h, almost all cells displayed cytoplasmic distribution of ZO-1, as observed for $p120^{ctn}$. Likewise, the tumour suppressor E-cadherin was sparsely retained at cell–cell contacts after 24 h (Figure 1). An accumulation of E-cadherin in vesicle-like structures surrounding the nucleus was observed after 48 h. Prolonged treatment resulted in a continuous reduction of the E-cadherin immunostaining, which was hardly detectable after 3 days (data not shown). During initial stages of EMT, the majority of cells did not completely disintegrate their intercellular adhesion, since a high percentage of cells (>80%) still displayed membrane-localized *β*-catenin. A shift to a cytoplasmic distribution

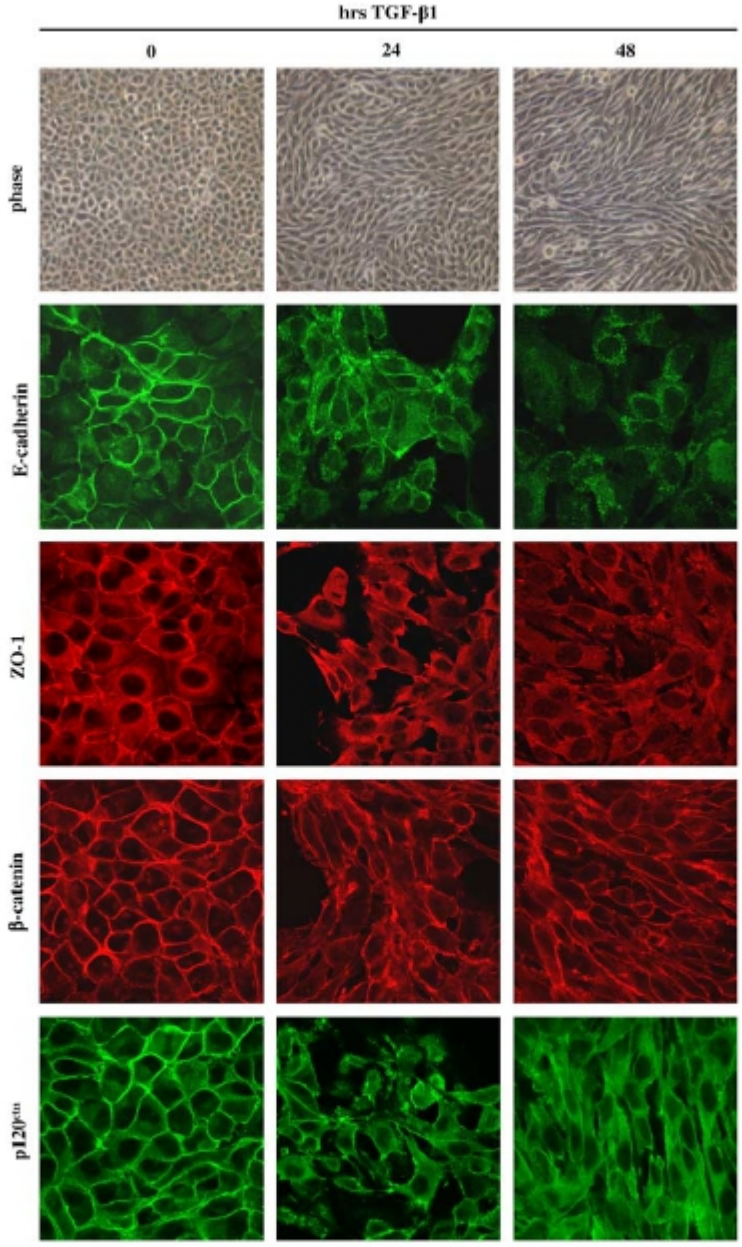


Figure 1 Disintegration of epithelial junctions during hepatocellular EMT. Phase-contrast and confocal immunofluorescence microscopy images after staining of cells with antibodies recognizing E-cadherin, ZO-1, β-catenin and p120^{cas}, respectively. Images were taken from untreated MMH-R cells (0 h) and their fibroblastoid derivatives after administration of 2.5 ng/ml of TGF-β1 for 24 and 48 h, respectively.

of β-catenin became detectable only after 7 days of TGF-β1 treatment (data not shown). In addition, the desmosomal constituent desmoplakin became distributed into the cytoplasm in a dot-like appearance between 24 and 48 h and was undetectable at later stages of EMT (data not shown). These data indicate that epithelial junctions get disintegrated in a hierarchical manner. Loss of polarity (tight junctions) appears to be a

precondition for disintegration and remodelling of other junctional complexes such as desmosomes and adherens junctions, respectively.

The overall amount of the junctional proteins under investigation remained unchanged during the first 24 h, but progressively decreased after prolonged TGF-β1 treatment (Figure 2a), suggesting that the cytoplasmic redistribution was accompanied by a decline in protein abundance. In accordance with immunofluorescence data, the expression of E-cadherin was sharply reduced after 3 days of TGF-β1 treatment, and the 100 kDa isoform of p120^{ctn} (Reynolds *et al*., 1996; Eger *et al*., 2000) was found to be stronger downregulated than the 120 kDa one. Similarly, reduced levels of β-catenin could be detected after 3 days of TGF-β1 treatment.

Recent data on transcriptional downregulation of *E-cadherin* during EMT (Batlle *et al*., 2000; Cano *et al*., 2000; Comijn *et al*., 2001; Poser *et al*., 2001) prompted us to analyse the fate of the transcriptional repressors Snail, SIP1 and delta-EF1. Interestingly, *E-cadherin* transcript levels were already sharply decreased 24 h post-TGF-β1 treatment, thus preceding the decrease in polypeptide expression (Figure 2b). TGF-β1 treatment of MMH-R cells lead to a moderate upregulation of *SIP1* transcripts, whereas the expression of *Snail* was induced immediately after cytokine treatment (Figure 2b). Similarly, expression of *delta-EF1* was rapidly elevated but decreased again to basal levels upon prolonged treatment with TGF-β1. The fast induction of *delta-EF1* suggests it as a potential direct target gene of TGF-β signalling,

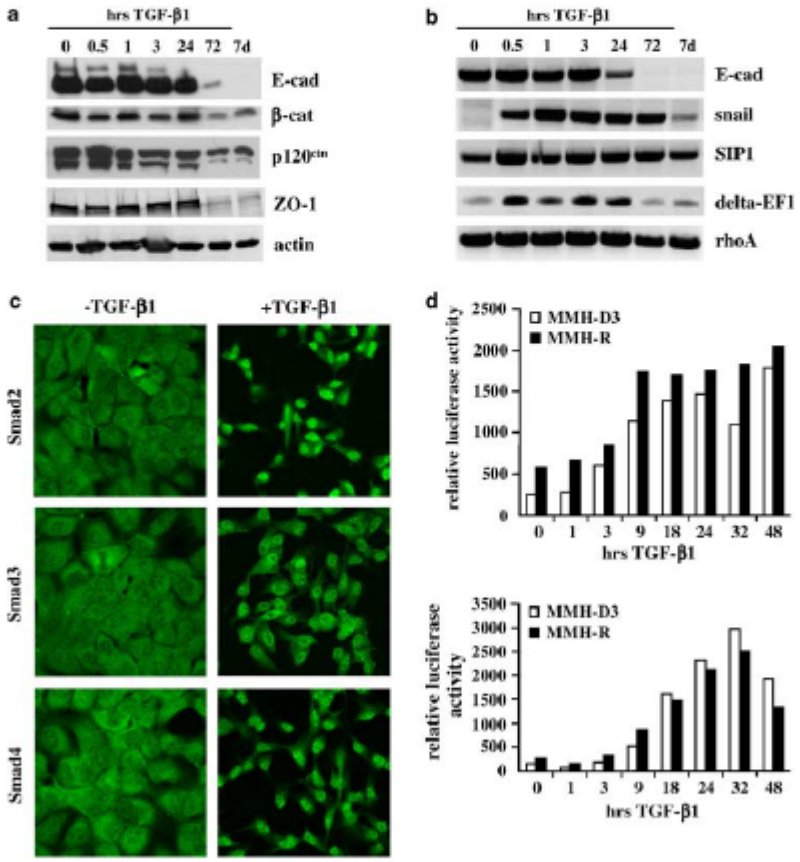


Figure 2 *E-cadherin* repression and TGF-β signalling properties during hepatocellular EMT. (**a**) Expression of the cell adhesion proteins E-cadherin, β-catenin, p120^{ctn} and ZO-1 during EMT as analysed by Western blotting, using constitutively expressed β-actin as loading control. (**b**) Semiquantitative RT–PCR analysis of the transcript levels of *E-cadherin* and the E-cadherin repressors *Snail*, *SIP1* and *delta-EF1*. *RhoA* is shown as loading control. On top of the panels a and b, the time of TGF-β1 treatment (2.5 ng/ml) is indicated. (**c**) Confocal immunofluorescence microscopy of untreated MMH-R hepatocytes (left panel) and cells induced with 2.5 ng/ml of TGF-β1 for 4 h (right panel) after staining with antibodies directed against Smad2, Smad3 and Smad4, respectively. (**d**) Hepatocytes were transiently transfected with reporter constructs responsive to Smad2- (upper panel) or Smad3-mediated transcriptional activation (lower panel, see Materials and methods) and treated with 2.5 ng/ml TGF-β1 for the indicated time points. Relative luciferase activities are standardized to cotransfected β-galactosidase; white bars, MMH-D3; black bars, MMH-R.

resembling TGF-β-driven activation of Snail. These data propose that TGF-β is directly and critically involved in the transcriptional downregulation of *E-cadherin* and the concomitant loss of cell adhesion.

Unperturbed TGF-β signalling during hepatocellular EMT

Ras transformation of MMH-D3 made cells refractory to the antiproliferative activity of TGF-β (Fischer *et al*., 2005). The mechanism how Ras signalling might interfere with the proliferation arrest caused by the TGF-β-Smad pathway, and instead, cooperates with TGF-β to induce EMT is still controversial (Hartsough and Mulder, 1995; de Caestecker *et al*., 1998; Kretzschmar *et al*., 1999; Yue and Mulder, 2000; Saha *et al*., 2001; Janda *et al*., 2002). Thus, we addressed whether changes in Smad signalling, in particular nuclear translocation, in parental versus Ras-transformed MMH-R hepatocytes might account for the escape of growth arrest and the dramatic alterations in epithelial plasticity. After 4 h of TGF-β1 administration to MMH-R cells, Smad2, Smad3 and Smad4 shifted from a cytoplasmic to an almost unambiguous nuclear localization (Figure 2c). When compared to parental MMH cells, no significant variations regarding the percentage or the kinetics of nuclear accumulation of Smads were observed (data not shown).

To further address Smad signalling at the transcriptional level, we performed luciferase reporter assays using constructs that can be specifically activated by Smad2 or Smad3. As shown in Figure 2d, the transactivation properties of both Smad2 (upper panel) and Smad3 (lower panel) remained unchanged irrespective of Ras transformation, although parental MMH-D3 hepatocytes undergo TGF-β-induced cell cycle inhibition and cell death (Gotzmann *et al*., 2002). Together, these data indicate that the escape of Ras-transformed epithelial hepatocytes from tumour-suppressive functions of TGF-β and the acquisition of a fibroblastoid phenotype occurs without overt changes in TGF-β signalling upstream of Smad interactions with other transcription factors.

Specificity and redundancy of TGF-β family members in the induction of hepatocellular EMT

By surveying the functional potential of TβR ligands and other growth factors as inducers of hepatocellular EMT, TGF-β2 and TGF-β3 revealed to be equally effective in executing changes in the plasticity of hepatocytes with kinetics highly comparable to those of TGF-β1 (Figure 3). This observation was underlined by the fact that the immunofluorescence analyses of cells, stained for adhesion molecules as shown in Figure 1, were in accordance with those obtained from TGF-β1 kinetics (data not shown). As found for TGF-β1, parental MMH-D3 cells responded to TGF-β2 and TGF-β3 with growth arrest in G1 and cell death (data not shown). TGF-β2 and TGF-β3 not only elicited nuclear translocation of Smad3 (Figure 3a) and Smad2 (data not shown) but also displayed comparable transactivation

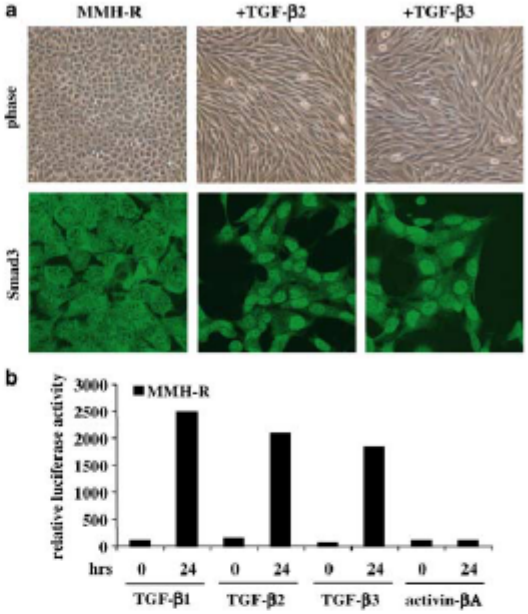


Figure 3 Redundancy and specificity of hepatocellular EMT induction within the TGF-β superfamily. (**a**) Phase-contrast images of MMH-R cells either untreated or administered with 2.5 ng/ml of TGF-β2 or TGF-β3 for 24 h (upper panel). At 4 h after treatment, confocal immunofluorescence analyses were performed to analyse nuclear translocation of Smad3. (**b**) MMH-R cells were transfected with the Smad3-dependent reporter construct, and the promoter activity was assayed by luciferase measurement after respective cytokine treatment at the time points indicated.

potential and kinetics of Smad3- and Smad2-responsive reporters (Figure 3b and data not shown). In sharp contrast, activin βA was neither operative in mediating morphogenetic changes of MMH-R cells nor in activating Smad3 response (Figure 3b), although RNase protection assays revealed that MMH-R cells expressed its cognate activin-like kinase receptors (data not shown). Likewise, members of the bone-morphogenetic protein (BMP) family such as BMP-4 and BMP-7 (administered at 200 or 500 ng/ml, respectively) were unable to elicit EMT, although they efficiently transactivated a BMP-specific reporter (data not shown). Other cytokines such as fibroblast growth factor (FGF)-1, FGF-2, insulin-like growth factor 1 (IGF-I), vascular endothelial growth factor (VEGF), PDGF-A, PDGF-B, and interleukin (IL)-3 failed to induce EMT of MMH-R hepatocytes, neither alone nor in combination (data not shown). Moreover, none of these cytokines and growth factors as well as none of the inducers of hepatocytic differentiation such as the synthetic glucocorticoid dexamethasone or IL-6 was able to inhibit hepatocellular EMT elicited by any of the three TGF-β isoforms (data not shown). These data clearly demonstrate that the induction of hepatocellular EMT can specifically be provoked by TGF-β1, TGF-β2 and

TGF-β3, although a functional redundancy within these TGF-β ligands still persists.

Dynamic changes of gene expression upon hepatocellular EMT

Expression profiling using microarray analysis was performed to obtain a more comprehensive insight into the molecular changes occurring upon hepatocellular EMT. Polysome-bound transcripts were used for comparative hybridization on Affymetrix GeneChips™ containing about 11 000 genes. This approach is superior to identify changes in actively translated mRNA populations by eliminating RNA pools not translated and therefore not representative for the proteome (Mikulits *et al*., 2000; Pradet-Balade *et al*., 2001). RNA fractions were prepared from MMH-R cells treated with TGF-β1 for 24, 72 or 120 h, respectively, and screened against polysome-associated mRNA pools of untreated MMH-R cells as a reference.

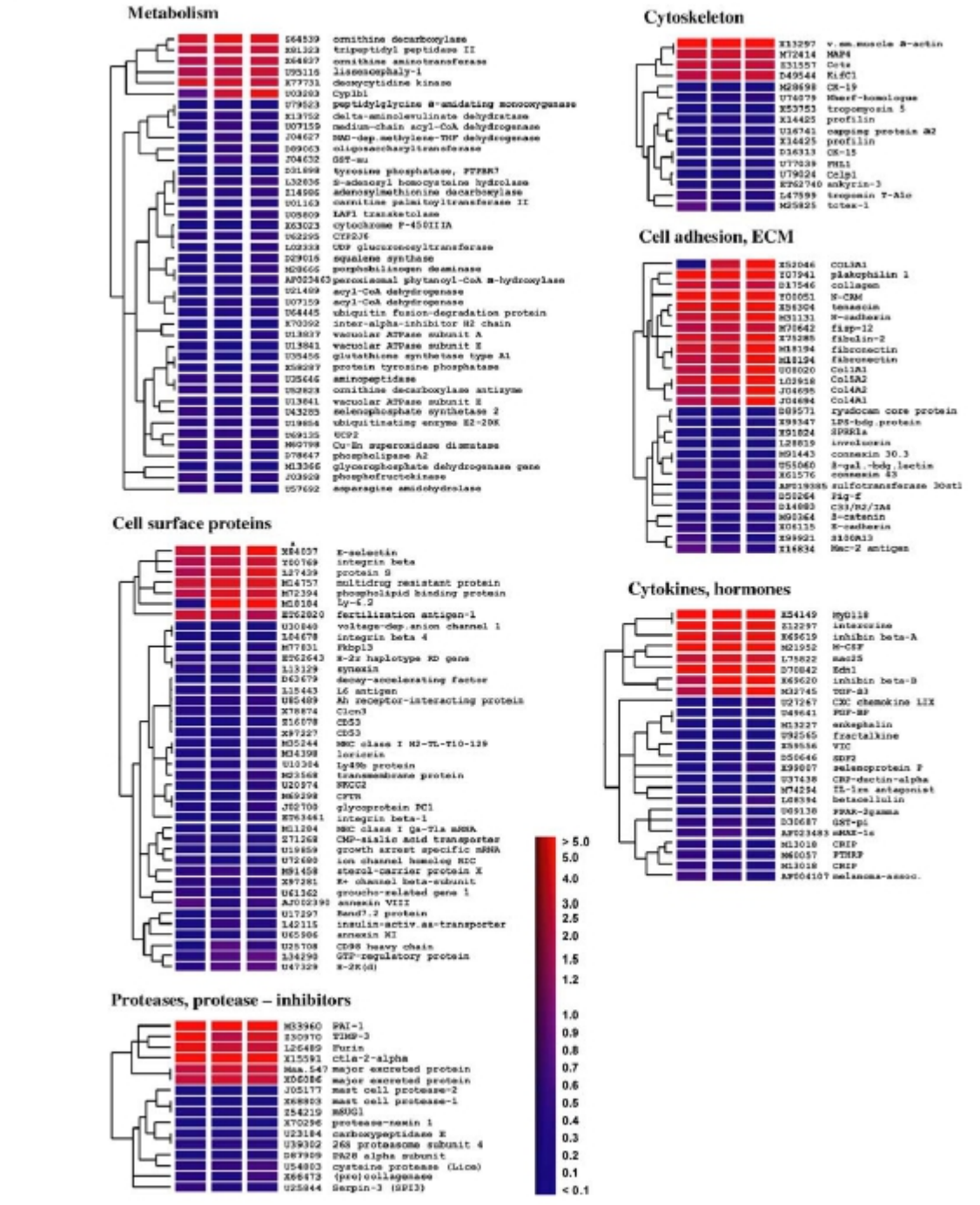
By considering transcript levels induced >2.0-fold or repressed <0.5-fold as significant, we identified in total 917 regulated genes including 313 known genes and 604 (not yet classified) expressed sequence tags (163 upregulated; 441 downregulated). To allow functional classification of regulated genes, we focused on known genes for cluster analysis. The majority of transcripts corresponding to characterized proteins was downregulated (235; 75%), whereas 78 (25%) genes were upregulated in kinetics of hepatocellular EMT. Noteworthy, most of the altered transcripts were strongest regulated 24 h post-TGF-β1 treatment of MMH-R hepatocytes. In order to get a plain overview of the molecular changes during EMT, all differentially regulated genes were arranged into functional subgroups and clustered within their specific characteristics (Figures 4 and 5; whole expression data in tables are available upon request).

In accordance with the morphological changes during EMT, essential genes participating in intercellular adhesion were downregulated, such as the adherens junction components E-cadherin and β-catenin as well as the gap junction proteins connexin-30.3 and connexin-43 (Jiang *et al*., 1995; Cai *et al*., 1998) (Figure 4; see also Figure 2a and b). Furthermore, the adhesion molecules N-CAM (neuronal cell adhesion molecule), plakophilin-1 and N-cadherin became upregulated upon ongoing EMT. Interestingly, N-cadherin, which is also dedicated as mesenchymal cadherin, has been shown to behave as a weak intercellular adhesion component in cells devoid of E-cadherin (Bhowmick *et al*., 2001) and to even enhance the malignant phenotype (Hazan *et al*., 2000).

The interactions between cancer cells and their microenvironment create a context promoting tumour growth, cell invasiveness, metastasis and protection from immune surveillance. Genes remodelling the ECM involved upregulated TGF-β target genes of the collagen family such as *Col5A2*, *Col4A1*, *Col4A2*, *Col1A1* and *Col3A1* (Verrecchia *et al*., 2001) as well as *fibronectin* and *tenascin* during the first 120 h of hepatocellular EMT. In contrast, the differentiation markers *SPRR1a* and *involucrin* were substantially (>5-fold) reduced. In agreement with the acquisition of an invasive phenotype upon EMT, we observed upregulation of ECM remodelling enzymes, such as plasminogen activator inhibitor-1 and the MMP inhibitor TIMP-3. Induction of the TGF-β induced protease furin, which converts latent TGF-β to an active cytokine (Dubois *et al*., 2001), may contribute to the maintenance of the autocrine TGF-β loop. Furthermore, Ly6a.2, a cell surface marker particularly enriched on metastatic cells (Witz, 2000), was sharply increased (>50-fold) after prolonged TGF-β1 treatment. Changes in integrin patterning displayed upregulation of the fibronectin receptor subunit integrin β1 and the concerted decrease of the parenchymal-enriched isoform integrin β4. Hepatocellular EMT is accompanied by extensive reprogramming of the cytoskeleton, primarily of the actin polymer framework (Gotzmann *et al*., 2002). In line with these recent findings, genes for actin-associated (e.g. *profilin*) and actin-capping (*capping protein* α*2*) proteins were found downregulated, as observed for the typical epithelial framework constituents cytokeratin (CK)-19 and CK-15, respectively (Figure 4). In addition, transcripts of the hepatic myofibroblastoid marker proteins fibulin-2 and α-smooth muscle actin were highly increased (Knittel *et al*., 1999; Tsuda *et al*., 2001; Proell *et al*., 2005).

The majority of regulated known genes belong to the class of transcription factors. Increased expression was monitored for *Snail* (see also Figure 2b) and for the orphanic receptor *Coup-TF1*, which is involved in a variety of developmental processes. The dedifferentiation of hepatocytes was reflected by downregulation of the liver-enriched hepatocyte nuclear factor (HNF)-3β. As already observed, the transcript of the immediate early upregulated c-*fos* was vigorously decreased 24 h after induction of EMT, which indicates mandatory repression of the antiproliferative activity provided by c-*fos* in hepatocytes (Mikula *et al*., 2003). The circumvention of triggering cell death is reconciled by downregulation of proapoptotic factors such as c-*myc* and Eya-2.

A special attention in the evaluation of expression profiling was put on cytokines and growth factors. Interestingly, the expression of TGF-β3, which has been shown to induce hepatocellular EMT (Figure 3), was increased with prolonged TGF-β1 administration (Figure 4). Upregulation of TGF-β3, which occurs by translational control (J Gotzmann *et al*., unpublished data), might signal in an autocrine manner and thus contributes to the maintenance of the invasive mesenchymal phenotype, as already shown for TGF-β1 (Gotzmann *et al*., 2002). An explanation for the failure of activin-βA to induce EMT of the MMH-R cells (Figure 3b) might rely on the increase of transcripts of both subtypes of inhibin-β, functionally antagonizing inhibition of liver cell growth by activins in a dominant-negative (dn) manner (Xu *et al*., 1995). The upregulation of inhibin-βA was relatively constant (3.6–4.6-fold) between 24 and 120 h of TGF-β1 treatment, whereas the B-subunit was highly activated at 72 h (9.4-fold) with increased expression after long-term treatment (52-fold in established MMH-RT cells versus MMH-R hepatocytes; J Gotzmann *et al*., unpublished data). The


Metabolism
Cytoskeleton
Cell adhesion, ECM
Cytokines, hormones
Cell surface proteins
Proteases, protease – inhibitors
> 5.0
5.0
4.0
3.0
2.5
2.0
1.5
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
< 0.1

angiogenesis-promoting potential of mesenchymal derivatives is represented by the increased expression of proangiogenic endothelin-1, a crucial cytokine in the complex interplay of neovascularization in metastatic MMH-RT-derived tumours.

Establishment of an autocrine PDGF loop during hepatocellular EMT

Kinetic profiling of hepatocellular EMT revealed induced expression of genes involved in PDGF signalling. In particular, *PDGF-A* transcripts were found highly elevated and forced expression of PDGF-inducible genes encoding the chemokines *MCP-1* and *KC* was monitored (Figure 5). Therefore, semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) was used to analyse the expression of components directly involved in this signalling cascade by employing EMT kinetics and established fibroblastoid MMH-RT cells (Figure 6a). These data confirmed results of microarray analysis, showing a sharp expression peak of PDGF-A at 24 h, which subsequently declined to lower levels at 120 h as compared to the MMH-R reference. A comparable, although fluctuating, upregulated expression of PDGF-α and -β receptors was detected, being highest at 24 h after TGF-β1 treatment and in established MMH-RT cells (Figure 6a). These data on PDGF-α and -β receptor expression during TGF-β1 kinetics could be confirmed at the protein level as analysed by immunoblotting (Figure 6b).

The bioactivity of PDGF secreted by MMH-R and MMH-RT cells was analysed by employing murine FDCP cells expressing human PDGFR-α receptor (von Ruden and Wagner, 1988), which proliferate in a strict PDGF-responsive manner (Figure 6c). Thymidine incorporation assays revealed that fibroblastoid MMH-RT cells, in contrast to epithelial MMH-R cells, secrete amounts of PDGF that significantly induced DNA synthesis rates of FDCP cells, similar to stimulation of proliferation by fetal calf serum (FCS) or recombinant PDGF. Moreover, a neutralizing antibody to PDGF-A – functionally interfering with PDGF-R signalling – reduced the proliferation-inducing activity of fibroblastoid MMH-RT supernatants to basal levels shown by epithelial control supernatants, thus demonstrating that MMH-RT secrete bioactive PDGF. From these data we concluded that the cooperative signalling of Ha-Ras and TGF-β induces autocrine regulation of PDGF-A in invasive hepatocytes.

Interference with PDGF signalling decreases cell migration and suppresses tumour formation

In order to verify and investigate the importance of PDGF signalling, we made use of a recently developed, more physiological murine hepatocellular EMT model based on immortalized $p19^{ARF}$ null hepatocytes that have been referred to as MIM (Mikula *et al*., 2004). The established MIM hepatocytes show a highly differentiated, polarized phenotype, express liver-specific markers, are nontumorigenic and revealed liver reconstituting activity after intrasplenic transplantation into Fas-injured livers of SCID mice (Mikula *et al*., 2004). As shown for MMH hepatocytes, MIM cells were able to undergo EMT upon the collaboration of hyperactive Ha-Ras and TGF-β (Fischer *et al*., 2005). Importantly, the induction of PDGF-A ligand and PDGF-α and -β receptors in TGF-β-treated MIM-R hepatocytes was identical to that observed in MMH-R-cells (compare Figure 6a and Figure 7a). These data demonstrate that the activation of PDGF components during hepatocellular EMT is independent of the genetic background. To obtain genetic evidence for a potential crucial function of PDGF, interference with PDGF signalling was achieved through bicistronic expression of a dn mutant of the PDGF-α receptor (Yu *et al*., 2000) and red fluorescent protein (RFP) in MIM-R hepatocytes (Figure 7c). The functionality of the dn construct in inhibiting the signalling of PDGF-α receptor has already been tested and verified in a variety of cell lines (Yu *et al*., 2000, 2003). The resulting cells, termed MIM-R-PDGFdn, were enriched in only about 80% RFP-positive cells even after two rounds of cell sorting, and showed proliferation kinetics comparable to those of MIM-R hepatocytes *in vitro* (Figure 7b and data not shown).

MIM-R-PDGFdn as well as MIM-R hepatocytes, respectively, were analysed for their migratory abilities by employing wound-healing assays. Immediately after setting the 'wound' in the confluent monolayer, cells were induced to EMT by administration of 2.5 ng/ml TGF-β1. After 48 h, MIM-R cells revealed complete closure of the scratch, whereas MIM-PDGFdn cells were markedly impaired in migration (Figure 8a). Comparable results were obtained after treatment of both cell types with either TGF-β2 or TGF-β3 (data not shown). In line with these observations, pharmacological interference with each TGF-β signalling using SB-431542, and PDGF signalling, employing either the neutralizing antibody for PDGF or the low molecular weight compound STI571, efficiently inhibited the wound closure of MIM-R hepatocytes treated with TGF-β (data not shown).

As recently reported, TGF-β1 pretreatment prior to subcutaneous injections of the cells caused an increase in malignancy resulting in vast tumour formation (Gotzmann *et al*., 2002). To investigate the effect of PDGF signalling on the tumorigenic potential, MIM-R and

Figure 4 Cluster analysis and functional classification of the expression profile of hepatocellular EMT kinetics. The diagrams represent the results of hierarchical clustering using a set of genes significantly altered during hepatocellular EMT. Prefiltering criteria required transcript levels to be altered at least two-fold or 0.5-fold at one of the three time points. Shown is a coloured representation of expression data of annotated genes (no ESTs) with the rows (genes) and columns in cluster order (from left to right: 1st column, MMH-R treated with 2.5 ng/ml TGF-β1 for 24 h compared to untreated MMH-R; 2nd column, 72 h TGF-β1 treatment of MMH-R compared to untreated MMH-R; 3rd column, 120 h TGF-β1 treatment of MMH-R compared to untreated MMH-R). The colour scale inserted represents fold up- (red) and downregulation (blue) of genes as indicated by the vertical numbering. The dendrogram at the left side of the panels represents hierarchical relationships between the genes altered.

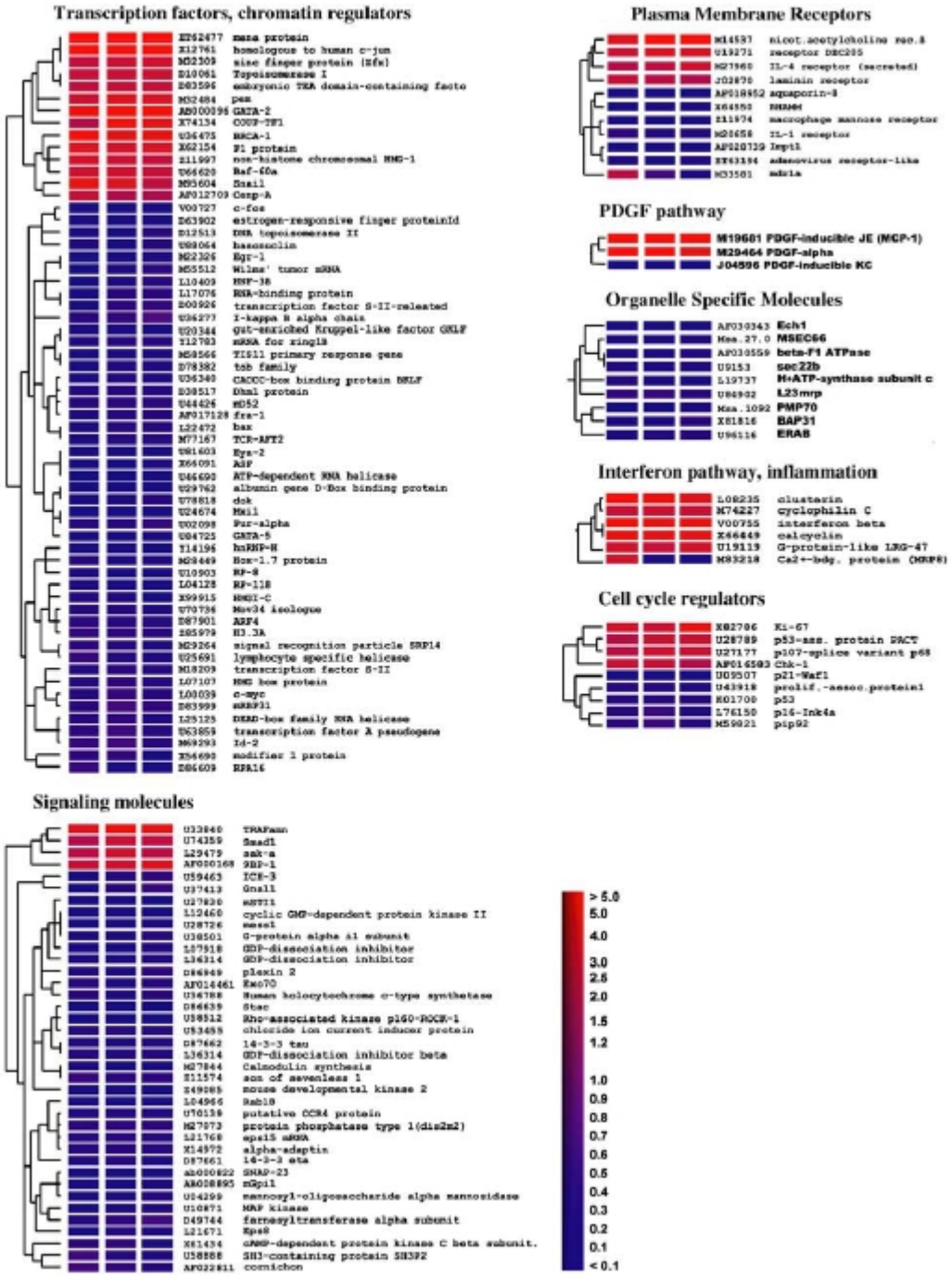


Figure 5 Cluster analysis and functional classification of the expression profile of hepatocellular EMT kinetics. Legend as outlined in Figure 4.

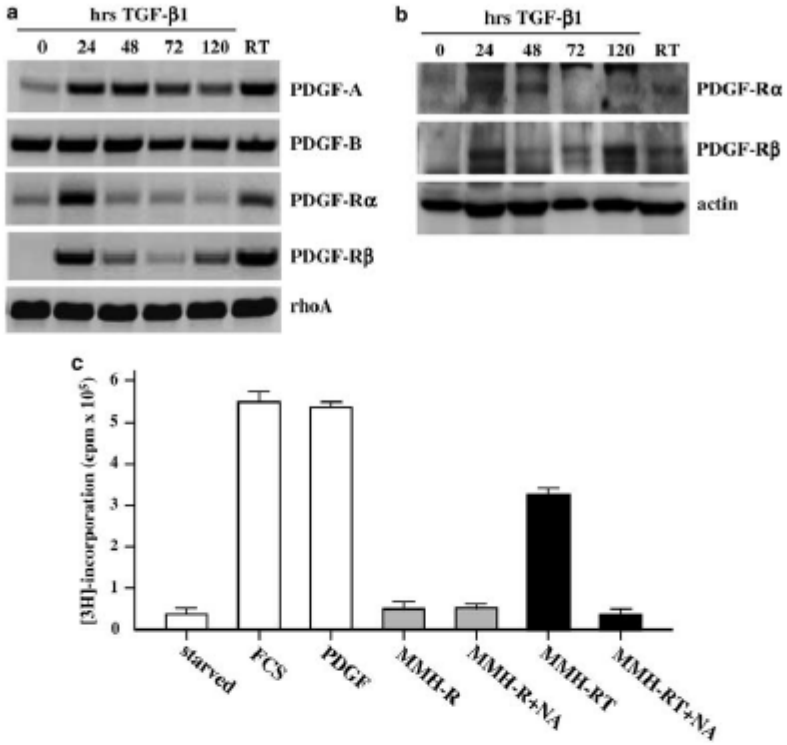


Figure 6 Analysis of PDGF signalling components during hepatocellular EMT. (**a**) Semiquantitative RT–PCR of the transcript levels of ligands (PDGF-A and PDGF-B) and their cognate receptors (PDGF-Rα and PDGF-Rβ). Transcript levels were analysed in MMH-R cells treated with 2.5 ng/ml of TGF-β1 for the indicated time and in long-term (> 21 days) TGF-β1-treated MMH-RT cells. RhoA is shown as loading control. (**b**) Expression of PDGF-Rα and PDGF-Rβ as analysed by Western blotting. The constitutively expressed β-actin is shown as loading control. (**c**) Analysis of autocrine PDGF secretion by thymidine incorporation. PDGF-responsive FDCP cells (expressing human PDGFR-α receptor) were treated with serum-free tissue culture supernatants from MMH-R (grey bars) and MMH-RT (black bars) cells. As positive control, the mitogenic activity was measured by including either supplementation of 10% FCS (FCS) or 2.5 ng/ml of recombinant human PDGF-A/B (PDGF), respectively (white bars). Serum-starved cells served as a negative control. To check the specificity of the proliferative response, a PDGF-A-selective neutralizing antibody (NA) was added at a concentration of 20 μg/ml.

MIM-R-PDGFdn were subcutaneously injected into immunocompromised SCID mice, either untreated or treated with TGF-β1 for more than 21 days. Evidently, interfering with PDGF signalling by expression of the dn PDGF-α receptor significantly decreased the growth of experimental tumours (Figure 8b). The reduction of tumour growth was much more pronounced in the fibroblastoid MIM-RT-PDGFdn versus MIM-RT cells than in the epithelial MIM-R-PDGFdn versus MIM-R cells. Most interestingly, fluorescence microscopy of experimental MIM-R-PDGFdn tumours revealed negative selection for cells expressing the dn PDGF-α receptor (Figure 8c). This led to an accumulation of GFP-positive but RFP-negative cells *in vivo*, probably representing selective outgrowth of the small minority of MIM-R cells, which failed to retain dn PDGF-α expression. These data indicate a crucial role of PDGF signalling in hepatocelluar EMT that cooperates with an autocrine TGF-β activity to enhance proliferation and migration of malignant hepatocytes during late-stage tumour progression.

Discussion

TGF-β represents a potent inducer of epithelial plasticity (Miettinen *et al*., 1994; Bakin *et al*., 2000; Bhowmick *et al*., 2001) and cooperates with oncogenic Ras signalling in the induction and maintenance of hepatocellular EMT, which closely mimics late-stage liver carcinogenesis (Thiery, 2002; Gotzmann *et al*., 2004). Taking advantage of the high synchronicity of EMT in two hepatocellular models, we analysed the molecular changes driven by the cooperative activities of Ha-Ras and TGF-β in a kinetic manner. The functional cooperation of both signalling pathways (Figure 2c

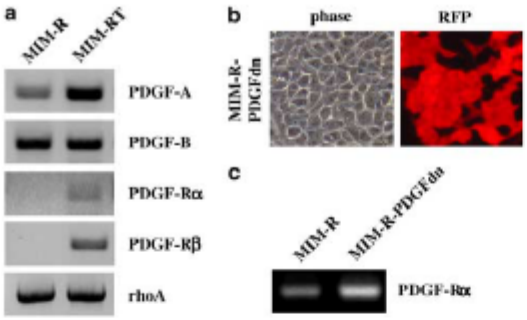


Figure 7 PDGF upregulation during EMT is independent of the genetic background. (**a**) Semiquantitative RT–PCR analysis of PDGF cytokines and receptors, demonstrating that expression of PDGF also increases in MIM hepatocytes upon TGF-*β*1-mediated EMT. (**b**) Phase-contrast and fluorescence microscopy images of cultured MIM-R-PDGFdn prior to subcutaneous injection into SCID mice. (**c**) RT–PCR of the PDGF-Rα comparing MIM-R hepatocytes and those overexpressing mutant PDGF-Rα (MIM-R-PDGFdn).

and d) rather than a previously reported inhibition of Smad transactivation by the Ras–Raf–Mek–Erk pathway (Kretzschmar *et al*., 1999) has been shown to be essential to confer resistance of cells towards TGF-*β*-mediated growth arrest and cell death (Gotzmann *et al*., 2002; Fischer *et al*., 2005). In this study, we therefore focused on the cooperative, tumour-promoting functions of TGF-*β*- and Ras signalling during hepatocarcinogenesis by identifying critical regulators using microarrays and corresponding functional analysis.

Firstly, we demonstrate that in cooperation with Ha-Ras all three isoforms of TGF-*β* cytokines were equally effective in inducing EMT, in sharp contrast to other, noneffective members of the TGF-*β* superfamily, like activin-*β*A, BMP-4 and BMP-7, respectively. Accordingly, Valcourt *et al*. (2005) found that the three TGF-*β* isoforms induced EMT in mammary and lung epithelial cell lines, while displaying a less pronounced morphogenic trigger on keratinocyte lineages. Moreover, activin-*β*A could only elicit scattering in lung epithelial cells, remaining ineffective in the other cells. This observation has been explained by a lack of transactivation of Smad2 target genes (Valcourt *et al*., 2005). Similarly, our data show that activin-*β*A failed to transactivate Smad3-responsive reporter elements (Figure 3b). Ligands of the BMP family completely lacked morphogenic functions, as shown for BMP-7 on various cell types (Valcourt *et al*., 2005). Taken together, the induction of EMT might not only be specific within the TGF-*β* superfamily of cytokines but is indicated to be cell type specific, too.

Despite the specificity within this superfamily of cytokines, a broad spectrum of other ligands (VEGF, PDGF, FGF-1, IL-6, IL-3, IGF-1, HGF, EGF) remained inoperative in eliciting hepatocellular EMT (Figure 3 and data not shown), although some of them have been shown to induce EMT-like characteristics in other cell systems (Gotzmann *et al*., 2004). Noteworthy, TGF-*β*-induced factors, which are involved in autocrine regulation and are considered to be essential for survival of mesenchymal cell types, fail to act as morphogens (VEGF; Bates *et al*., 2003).

Investigating the sequence of morphological alterations in MMH hepatocytes upon EMT revealed that loss of epithelial polarity preceded – and thus appears to be a precondition for – remodelling or disintegration of adhesion and desmosomal junctions, respectively (Figure 1). Transient upregulation of E-cadherin repressors mirrored the kinetics of the cytoplasmic distribution of the tumour suppressor E-cadherin as well as the downregulation of its transcript and protein levels, respectively. *SIP1* and *Snail* have so far been identified as direct TGF-*β* target genes (Peinado *et al*., 2003; Zavadil *et al*., 2004), while Snail and delta-EF1 additionally have been shown to mediate EMT upon ectopic expression by repressing E-cadherin (Batlle *et al*., 2000; Cano *et al*., 2000; Eger *et al*., 2005). The importance of Snail is further emphasized by the fact that Snail knockout mice die early and show defects in EMT during embryonic development (Carver *et al*., 2001). Moreover, SIP1 and delta-EF1 could still act autonomously or in concert with Snail to mediate transcriptional repression of E-cadherin. On the other hand, we observed a sharp reduction in the expression level of WT1 (data not shown), a zinc-finger protein associated with Wilms' tumour, which has recently been demonstrated to be a direct enhancer of E-cadherin expression (Hosono *et al*., 2000). Therefore, we conclude that a complex interplay of transcription factors and associated molecules is instrumental in regulating most cell adhesion properties in the transition from an EMT phenotype in hepatocytes. Disintegration of membrane-localized *β*-catenin and redistribution to the cytoplasm was found to be a late event *in vitro* (Figures 1 and 2), which might facilitate or be accounted as a precondition for nuclear translocation of *β*-catenin, as observed in experimental tumours arising from MIM-RT cells (M Mikula *et al*., unpublished observation).

A variety of expression profiling approaches have been conducted to evaluate target genes for TGF-*β* (Verrecchia *et al*., 2001; Zavadil *et al*., 2001; Coyle *et al*., 2003; Valcourt *et al*., 2005). In our survey of molecular events in hepatocellular EMT, we took advantage of screening the 'active' transcriptome by hybridization of polysome-bound mRNA populations (Mikulits *et al*., 2000; Pradet-Balade *et al*., 2001). The three time points selected covered (i) 24 h TGF-*β*1 treatment, characterized by the induction of Erk signalling and loss of polarity, (ii) 72 h post-TGF-*β*1 treatment representing start of the maintenance phase by upregulation of PI3 kinase, and (iii) 120 h TGF-*β*1 treatment when EMT is essentially completed including adhesive reprogramming (Figures 4 and 5).

Based on these expression profiling data, a crucial role of the PDGF signalling pathway was deduced, since transcripts encoding PDGF-A ligand and both PDGF receptor subunits were found to be highly elevated upon EMT. According to previous reports that TGF-*β*

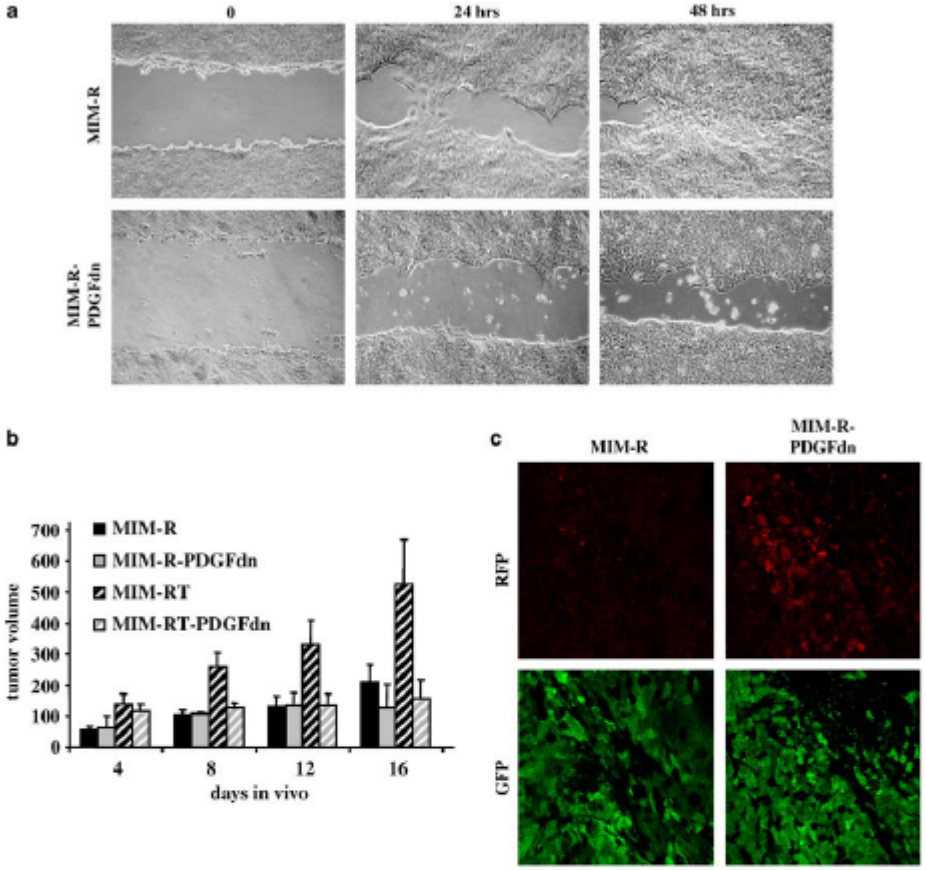


Figure 8 Interference with PDGF signalling constricts motility of TGF-β1-treated MIM-R hepatocytes and reduces tumour growth. (**a**) Wound healing assay of MIM-R and MIM-R-PDGFdn hepatocytes. Representative phase-contrast images at 0, 24 and 48 h after setting the scratch and subsequent treatment of TGF-β1. (**b**) Tumour formation after subcutaneous injection of cells into SCID mice. (**c**) Confocal images taken from paraffin-embedded sections of experimental tumours, induced by injection of MIM-R-PDGFdn cells expressing both RFP and GFP. GFP-positive MIM-R cells were analysed as controls.

induces PDGF-A expression without significantly affecting transcripts for PDGF-B, this ligand was found unchanged in our hepatocellular tumour model (Paulsson *et al*., 1988; Soma and Grotendorst, 1989). Similar upregulation of the entire PDGF signalling pathway was found by expression profiling of Ras-TGF-β-induced EMT in the mammary epithelial cell model EpH4/EpRas (Jechlinger *et al*., 2003). PDGF signalling stimulates various signalling pathways including Ras-MAP kinase, the PI3 kinase, the phospholipase C-γ, the cytoplasmic tyrosine kinase c-Src and different Stats (Heldin *et al*., 1998). Actually, the classical Ras/MAP kinase signalling induced by PDGF has been assigned not only to proliferation but, most interestingly, also to metastasis (MacDonald *et al*., 2001), whereas the PDGF-mediated PI3 kinase activation has been found to exert its effects by protection from apoptosis (Barres *et al*., 1992; Yao and Cooper, 1995). Dysregulation of PDGF ligands and receptors has been found in a variety of diseases such as developmental defects, inflammation, fibrosis and cancer (Heldin and Westermark, 1999). In almost all of these disorders, either the receptors were found to be upregulated or mutated, keeping them constitutively active, or cells within the affected tissue produce PDGF ligands for which they carry the corresponding receptor. The coexpression of PDGF and PDGF receptors suggests an autocrine growth stimulation that has been reported in a considerable number of solid tumours, including gliomas, meningiomas, melanomas, neuroendocrine tumours and carcinomas of the prostate, breast, lung, stomach and pancreas (Ostman and Heldin, 2001). Accordingly, inhibition of the PDGF pathway by imatinib mesylate (also known as STI571 or Gleevec) has entered clinical trials and has already been proved to be successful in therapy of gastrointestinal stromal tumours, brain tumours, prostate cancer and chronic myelogenous leukaemia (Kilic *et al*., 2000; George, 2001; Joensuu, 2002; Croom and Perry, 2003;

Newton, 2003; Deininger, 2004). Noteworthy, imatinib mesylate is a competitive inhibitor of a few tyrosine kinases, including BCR-ABL, c-ABL and c-KIT, in addition to PDGF receptors (George, 2001; Joensuu and Dimitrijevic, 2001).

With regard to liver diseases that give rise to hepatocellular cancer, it is of particular interest that PDGF has been implicated in liver injury and liver cirrhosis (Heldin *et al*., 1991; Pinzani *et al*., 1994; Pinzani *et al*., 1996). In addition, analyses of human HCCs showed a strongly enhanced expression of PDGF receptors in cancerous liver tissue compared to normal liver but not of the corresponding cytokines themselves (Tsou *et al*., 1998; Xu *et al*., 2001; Chen *et al*., 2002). Yet, the functional implication of PDGF activation in human HCC is poorly understood and remains to be elucidated.

Here, we show first evidence that autocrine secretion of PDGF-A is induced in TGF-*β*-mediated liver tumorigenesis. This could be confirmed by employing two different murine models, based on MMH and on MIM immortalized hepatocytes, respectively, indicating independence of the genetic background. Most interestingly, simultaneous blockade of EGF-R and PDGF-R phosphorylation coupled with the administration of paclitaxel induced apoptosis in tumour-associated endothelial cells and significantly suppressed bone metastasis in human prostate cancer patients (Uehara *et al*., 2003; Kim *et al*., 2004). Thus, activation of EGF-R and PDGF-R, both preferentially signalling via Ras, plays a crucial role in migration and generation of the tumour vasculature in prostate cancer and probably as well in HCCs. In Ras-transformed MIM hepatocytes, interference with PDGF signalling through expression of a dn mutant of the PDGF-α receptor strongly reduced tumour growth and selected for loss of dn PDGF-α receptor expression. This suggested an essential, probably cell-autonomous contribution of PDGF to the migratory phenotype during TGF-*β*-mediated hepatocellular late-stage tumour progression. PDGF has been reported to provide migratory abilities through stimulating members of the rho family of small GTP-binding proteins (Hawkins *et al*., 1995; Hooshmand-Rad *et al*., 1997; Rodriguez-Viciana *et al*., 1997). Cooperation of Erk signalling, which is necessary for proliferation and survival of cells during hepatocellular EMT (Fischer *et al*., 2005), and the PDGF pathway has been shown to be a prerequisite for phosphorylation efficiency of FAK, and thus, for enhancing the migration capability (Carloni *et al*., 2001). Moreover, a direct cooperation between PDGF and integrins has been demonstrated, which enhances cell motility and prevention of apoptosis as well as the modulation of the ECM (Sundberg and Rubin, 1996; Schneller *et al*., 1997; Bissell and Radisky, 2001). It is further tempting to speculate that the remodelling of ECM, allowing or enhancing cell movement, is cooperatively driven by autocrine TGF-*β* and PDGF regulation. In this respect, PDGF is known to play an important role in wound healing by stimulating the production of several matrix molecules such as fibronectin, collagen, proteoglycans and hyaluronic acid. At later stages of wound healing, PDGF stimulates the production and secretion of collagenase by fibroblasts (Heldin and Westermark, 1999). Similar to the function of VEGF, PDGF has been shown to be operative as an angiogenic factor, which promotes vascularization of tumours (Risau *et al*., 1992; Battegay *et al*., 1994).

Taken together, our data indicate a crucial role of the PDGF/PDGF-R pathway in supporting tumour progression as well as in promoting motile characteristics of hepatocytes undergoing EMT, thereby maintaining its activity in an autocrine manner. Further functional analyses of PDGF in hepatocellular EMT models will reveal its impact in hepatocarcinogenesis and will contribute to the development of novel auspicious strategies for anticancer therapy.

Materials and methods

Cell culture

Immortalized Met murine hepatocytes (MMH-D3), and those derived from $p19^{ARF}$ null hepatocytes, termed MIM1-4, were cultured on rat tail collagen-coated dishes in RPMI 1640 supplemented with 10% FCS, 40 ng/ml recombinant human TGF-α (Sigma, St Louis, USA), 30 ng/ml recombinant human insulin-like growth factor II (IGF-II, Sigma, St Louis, USA), 1.4 nM insulin (Sigma, St Louis, USA) and antibiotics. MMH-R and MIM-R hepatocytes were generated by retroviral transmission of parental MMH-D3 and MIM1-4 cells with a vector bicistronically expressing constitutive active v-Ha-Ras plus green fluorescent protein (GFP), respectively, and were grown in RPMI 1640 supplemented with 10% FCS and antibiotics (Oft *et al*., 1996). Fibroblastoid derivatives of MMH-R cells, termed MMH-RT, were propagated in RPMI 1640 plus 15% FCS, 1 ng/ml recombinant human TGF-*β*1 (R&D Systems, Minneapolis, USA) and antibiotics as outlined recently (Gotzmann *et al*., 2002). MIM-R-dnPDGF cells resulting from the stable retroviral transmission of MIM-R hepatocytes with pMSCV-dnPDGF-Rα-red, harbouring red fluorescent protein (RFP), were cultured as described for MIM-R cells. All cells were kept at 37°C and 5% CO_2 and routinely screened for the absence of mycoplasma.

Rat tail collagen was prepared as described (Ehrmann and Gey, 1956). Recombinant human TGF-*β*1, TGF-*β*2, TGF-*β*3, PDGF-A/B, activin *β*A, and BMP-4 and -7 (all purchased from R&D Systems, Abingdon, UK) were used at concentrations indicated in the text.

Vector cloning

For construction of the pMSCV-dnPDGF-Rα-red vector allowing bicistronic expression of dnPDGF-Rα and RFP, a *Bgl*II and *Hind*III fragment of pGK obtained from pMSCV-gfp (Clontech, Palo Alto, USA) was ligated into pDsRed-N1 (Clontech, Palo Alto, USA). The resulting plasmid pGKDsRed-N1 was cut with *Bgl*II and *Not*I and blunted to receive the pGKred fragment. The pGKpuro cassette of pMSCV-puro (Clontech, Palo Alto, USA) was further replaced by the pGKred fragment after cutting and blunting *Bgl*II and *Cla*I sites to obtain a pMSCV-red vector (Hawley *et al*., 2001). pMSCV-red was modified to work as destination vector for Gateway™ cloning (Invitrogen, Carlsbad, CA, USA) after ligation of the conversion cassette (RFB, *Eco*RV) into pMSCV-red (*Hpa*I). The dnPDGF-Rα cDNA was amplified by PCR using primers containing the *att*L sites as template from the vector pcDNAI/Neo (Yu *et al*., 2000). The dnPDGF-Rα cDNA was inserted into the pMSCV-RFB by LR reaction

using the Gateway™ technology, resulting in the vector pMSCV-dnPDGF-Rα-red.

Confocal immunofluorescence microscopy

Cells grown on filters (Falcon 353090, Becton Dickinson, Franklin Lakes, USA) or on poly-L-lysine-coated coverslips (Sigma, St Louis, USA) were processed for immunological detection as described recently (Gotzmann *et al*., 2002). Dilutions of primary antibodies used are as follows: anti-E-cadherin (Transduction Laboratories (TL), Lexington, UK), 1:100; anti-β-catenin (TL), 1:125; anti-p120^{ctn} (TL), 1:200; anti-Smad2 (TL), 1:250; and anti-ZO-1 (Zymed Laboratories, South San Francisco, USA), 1:75. Rabbit sera directed against Smad2, Smad3 and Smad4 (1:50) were a kind gift of Dr Peter ten Dijke, The Netherlands Cancer Institute, Amsterdam, The Netherlands (Nakao *et al*., 1997). After application of cye-dye-conjugated secondary antibodies (Jackson Laboratories, West-Grove, USA), cells were imaged with the TCS-SP confocal microscope (Leica, Heidelberg, Germany). Conventional microscopy of cells was performed with a Nikon Eclipse TE300 inverted light microscope (Nikon Corporation, Tokyo, Japan). Since cells themselves show fluorescence due to GFP expression, some microscopic images were pseudo-coloured green or red *in silico* for proper illustration.

Reverse transcription–polymerase chain reaction

Poly(A)$^+$ mRNA was extracted and reverse transcribed employing a mRNA isolation and first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany). Aliquots of the resulting products were used as templates for specific PCR amplification using Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden), as outlined recently (Gotzmann *et al*., 2002). The conditions for PCR reaction were optimized for each primer pair. The following forward and reverse primer were used for specific amplification, respectively: E-cadherin – 5′-GAGCCTGAGTCCTGCAGTCC-3′, 5′-TGTATTGCTGCTTGGCCTCA-3′; PDGF-A – 5′-GGCTTGCCTGCTGCTCCTCG-3′, 5′-CTCCACTTTGGCCACCTTGAC-3′; PDGF-B – 5′-TGCTGAGCGACCACTCCATC-3′, 5′-GATTCTCACCGTCCGAATGG-3′; PDGF-αR – 5′-CAGACTTCGGAAGAG-AGTGCCATC-3′, 5′-CAGTACAAGTTGGCGCGT-GTGG-3′; PDGF-βR – 5′-CCTGAACGTGGTCAACCTGCT-3′, 5′-GGC-ATTGTAGAACTGGTCGT-3′; rhoA – 5′-GCCTTGCATCTGAGAAGT-3′, 5′-AATTAACCGCATGAGGCT-3′; SIP1 – 5′-TCCAAATAGCTTCTCTTCCGAGG-3′, 5′-ACCTGTGATTCATGTGCTGCGA-3′; Snail – 5′-ACCTTCCAGCAGCCCTACGACC-3′, 5′-GTGTGGCTTCGGATGTGCATC-3′; and delta-EF1 – 5′-CCTAGATCAGGACTCAAGAC-3′, 5′-CACAGAAGGCAAGTGCTATC-3′. The amplification products were analysed by electrophoresis on 1% agarose gels and staining with ethidium bromide.

Immunoblotting

The preparation of cellular extracts, separation of proteins by SDS–polyacrylamide gel electrophoresis, and immunoblotting were performed as described recently (Gotzmann *et al*., 2002). Immunological detection of proteins was performed with the SuperSignal detection system (Pierce Chemical Company, Rockford, USA). The primary antibodies were used at the dilutions: anti-E-cadherin (Transduction Laboratories (TL), Lexington, UK), 1:3000; anti-β-catenin (TL), 1:1000; anti-p120^{ctn} (TL), 1:2000; anti-ZO-1 (Zymed Laboratories, South San Francisco, USA), 1:1500; anti-PDGF-αR (Santa Cruz Biotechnology, Santa Cruz, USA), 1:1000; anti-PDGF-βR (Neomarkers, Freemont, USA), 1:1000; and anti-actin (Sigma, St Louis, USA), 1:2500. Horseradish peroxidase-conjugated secondary antibodies (Calbiochem, LaJolla, USA) were used at dilutions of 1:10 000.

Transient transfections and reporter gene assays

MMH-D3 or MMH-R cells were plated at a density of 5×10^4 cells per 12-well plate 1 day before transfection. Lipofectamine Plus was used for transient transfections, as recommended by the manufacturer (Invitrogen, Carlsbad, USA). To analyse the transcriptional response mediated by Smad2 (Germain *et al*., 2000), cells were cotransfected with a luciferase reporter linked to the distal element of goosecoid (DE; a kind gift of Carol Hill, Imperial Cancer Research Fund, London, UK), the Smad2 coactivator Mixer and a β-galactosidase reporter (Eger *et al*., 2000). Smad3-dependent transactivation was determined by cotransfection of cells with (CAGA)12-Luc, containing concatemeric Smad3 consensus binding sites (Dennler *et al*., 1998), and β-galactosidase reporter. After cell lysis, the luciferase activity was determined by a Luminoskan (Labsystems, Farnborough, UK) as described previously (Mikula *et al*., 2003). All assays were performed in triplicate and results represent the average of three independent experiments after normalization to β-galactosidase activities.

RNA isolation and microarray analysis

Polysome-associated mRNA populations were isolated through fractionation of cytoplasmic extracts in sucrose gradients as described (Mikulits *et al*., 2000). Briefly, cellular extracts were prepared and supplemented with dithiothreitol, cycloheximide, heparin and phenylmethylsulfonyl fluoride after removal of nuclei and mitochondria. The resulting supernatants were layered onto 10 ml linear sucrose gradients (15–40% sucrose (w/v)) and centrifuged in an SW41Ti rotor (Beckman, Palo Alto, USA) at 38 000 r.p.m. for 120 min at 4°C. The RNA extracted from 19 harvested fractions were subsequently analysed for integrity and association with (poly)ribosomes (Mikulits *et al*., 2000). Fractions 12–19 of each sucrose gradient containing polysome-associated RNA were pooled and poly(A)$^+$ mRNA were isolated quantitatively using Oligotex-dT beads (Qiagen, Hilden, Germany). Each cRNA preparation for hybridization was performed with 5 μg of polysome-bound RNA, according to the protocol provided by Affymetrix, Santa Clara, USA (Wodicka *et al*., 1997; Mahadevappa and Warrington, 1999). RNA labelled by incorporation of biotinylated CTP and UTP was purified with RNeasy columns (Qiagen, Hilden, Germany) and quantified by a spectrophotometer. Affymetrix Mu11K GeneChips consisting of two individual subarrays (subA and subB), in total containing roughly 11 000 probe sets of oligonucleotides, were subsequently hybridized at 45°C for 16 h. Following washes, probe arrays were scanned three times at 6 μm resolution using the GeneChip system confocal scanner (Hewlett-Packard, Waldbronn, Germany). GeneChip 3.0 (Affymetrix, Santa Clara, USA) was used to scan and quantitatively analyse the scanned image. To correct for minor differences in overall chip fluorescence, intensity values were scaled to a level that the overall fluorescence intensity of each chip of the same type was equivalent. Genes induced > 2.0-fold or repressed < 0.5-fold versus baseline has been considered as significantly regulated and included into interpretation. In EMT kinetics, only those genes displaying significant changes to reference at least at one time point were recorded.

Analysis of PDGF secretion

Mouse hematopoietic FDCP cells exogenously expressing the human PDGF-α receptor were employed to analyse bioactive PDGF-α in tissue culture supernatants (von Ruden and Wagner, 1988). MMH-R and MMH-RT cells were grown in

serum-free RPMI for 48 h, and PDGF-responsive FDCP cells were cultured in RPMI supplemented with 2 ng/ml IL-3 (R&D Systems, Abingdon, UK) for 24 h. Supernatants of MMH-R and MMH-RT cells were added to serum-starved FDCP cells for 20 h. As indicated, a neutralizing anti-PDGF-A antibody (R&D Systems, Abingdon, UK) was employed to interfere specifically with PDGF-A-mediated signalling. To monitor DNA synthesis rates, cells were pulsed with 1 μCi [^{3}H]thymidine (ICN, Irvine, USA) for 2 h (Mikula *et al*., 2003), harvested on glass-fibre filters (Packard, Meriden, USA), and the radioactivity associated with DNA was determined in a microplate scintillation counter (Packard, Meriden, USA). All assays were performed twice in triplicate measurements, and the values determined represent the average of counts (c.p.m.).

In vitro *wound healing assay*

Cells were seeded in six-well culture dishes to grow until confluence. Subsequently, a wound was incised by scratching a path with sterile pipette tips through the confluent layer of cells. The detached cells were removed by careful washing, before adding fresh culture medium supplemented with either 2.5 ng/ml of TGF-β1, TGF-β2 or TGF-β3. For inhibition of signalling pathways, the low molecular weight compounds SB-431542 (Inman *et al*., 2002) and STI571 (a kind gift of Novartis Pharma KG, Basel, Switzerland; Kilic *et al*., 2000) were added to the culture medium at concentrations of 10 μM, whereas PDGF-A-selective neutralizing antibody was used at a concentration of 20 μg/ml. After 24 and 48 h, cells migrating into the wound were monitored by phase-contrast microscopy (Cano *et al*., 2000).

Tumour formation in vivo

Cells of the desired cell type were detached from the tissue culture plate, washed with phosphate-buffered saline and resuspended in Ringer solution. Subsequently, aliquots of 1×10^6 cells in 100 μl Ringer solution were each subcutaneously injected into three immunodeficient SCID/BALB/c recipient mice. Tumour formation was measured periodically by palpation, and the tumour size was determined using a vernier caliper. Tumour volume was calculated from tumour size using the formula diameter × diameter × length/2, as described recently (Gotzmann *et al*., 2002). All experiments were performed twice in triplicate and carried out according to the Austrian guidelines for animal care and protection.

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12. Discussion

Cancer progression is a multistep process, that eventually leads to invasive and metastatic cells at a later stage. In order to pass this step, the tumour cells perform an EMT process and thus gain access to their normally disabled motile potential. In our studies we established a multi-level cell system in order to reproduce how carcinogenesis leads to invasion of far and nearby tissue.

12.0.1 The Prerequisites for EMT

For the first pre-neoplastic step in carcinogenesis we used MIM1-4 $p19^{ARF}$ deleted hepatocytes, which lack the induction of p53 dependent cell cycle arrest and thus lead to abnormal cell survival. These cells needed pre-coated collagen plastic culture dishes, where they grew as a polarized monolayer. On TGF-β administration these cells performed apoptosis, indicating that they were still susceptible to signalling that controls cell proliferation.
As a next step we overexpressed Ha-Ras within these cells, as Ras levels were monitored to be elevated in tumour tissue. Ras activates transcription factors, that are important for cell proliferation and the control of the cell cycle. Mutations lead to permanently active Ras signalling, which continuously invokes cell growth. We found, that cells with the Ras-transformation showed an accelerated cell cycle and seemed to be less sensitive to environmental parameters e.g. they were able to grow at lower growth factor levels than MIM1-4 cells. The cells still formed a monolayer and exhibited a polarized phenotype *in vitro* and formed malign, neoplastic primary tumours *in vivo*.

12.0.2 The Onset of EMT

On TGF-β administration, we got an adverse effect: Instead of going into apoptosis, MIM-ras cells performed a morphological switch in conducting EMT. These cells exhibited a fibroblastoid phenotype and they grew in polylayers *in vitro*. The culture dishes did not have to be collagen coated, as the transformed cells formed their own extracellular matrix. *In vivo,* these cells showed an aggressive tumour growth that was able to invade

nearby tissue. Other known factors known to cause this development are PDGF, Wnt/β-catenin and Notch. We choose TGF-β as EMT promoting factor, as it was found in HCC tissues as well as it was shown to be correlated with carcinogenesis and prognosis of HCC (Okumoto *et al*., 2004; Yim *et al*., 2010).

The treatment with an EMT inducing agent, like TGF-β, triggered the development of a fibroblastoid phenotype. The question arose, why TGF-β promoted the invasive phenotype in transformed cells while it suppressed normal cell growth.
One possible answer might be, that the effects of TGF-β depend on cell cycle status and progression. It has been shown, that cells arrested in the G1/Syntheses phase performed EMT on TGF-β administration, while cells in the G2/Mitoses phase went into apoptosis (Yang *et al*., 2006).

12.0.3 Implications on EMT

Thus, in our case, the activation of Ras seems to influence and alter the cell cycle in order to allow TGF-β to have an growth and de/differentiation promoting effect. Ras invokes ERK, which activates cyclin D1 and thus promotes G1/S phase entry, which could result in an elevated level of cells, that are able to perform EMT on TGF-β induction.
Interestingly ERK can be activated in two different ways: While e.g. FGF treatment triggers sustained activation, PDGF or EGF cause only transient activation of ERK (Yamamoto *et al*., 2006). ERK activation leads to c-fos transcription but only sustained ERK activation induces c-fos hyperphosphorylation and thus its stabilisation, that finally promotes cyclin D1 activation and S phase entry (Murphy *et al*., 2006). Therefore c-fos seems to act as a molecular sensor for ERK levels. Upon sustained activation, it promotes not only cyclin D1 activation, but also downregulates transcription factors for antiproliferative signalling like JunD, Sox6 and MEF2C (Sharrocks, 2006). This triggered response seems to be very cell type specific: While the described proliferative effect happens in fibroblasts, neuronally derived PC-12 cells sustained ERK activation results in differentiation (Marshall, 1995; Murphy *et al*., 2006; Sharrocks, 2006).
As Ras is a major inducer of ERK signalling, these previous findings could lead to the

assumption, that altered levels of ERK signalling also influence the cell cycle entry signalling in our Ras transformed hepatocytes. This alteration seems to be a major prerequisite for the following EMT transformation caused by TGF-β induction. It would be useful to perform a microarray analysis of MIM1-4 cells compared to MIM-ras cells in order to get a picture of the changed transcriptome with regard to pathways concerning the cell cycle.

12.1 The Impacts of PDGF signalling

If we take a look at the different pathways involved in EMT, it shows that TGF-β is a key player, that is connected to most of them. It is able to activate signalling pathways, that assist the changes in differentiation and the following mesenchymal phenotype in an orchestrated way. As we performed gene expression analysis (microarray) and functional analysis (western blot) of transformed vs untransformed cells, we could clearly confirm the downregulation of epithelial markers like E-cadherin, ZO-1, β-catenin and the upregulation of mesenchymal effectors like smooth-musle-actin, fibronectin, N-catherin and vimentin. Moreover we were able to detect an upregulation of factors known to participate in EMT (e.g. Snail) and coexpression of potentially novel factors: PDGF-A ligand in conjunction with both PDGF receptors.
We clearly saw an strong upregulation of PDGF-A expression with an autocrinous loop of bioactive PDGF-A in conjunction with elevated levels of PDGFR-α and PDGFR-β receptor expression. In order to determine the function of PDGF during EMT, we transfected cells with a stable expressed dominant-negative PDGF-α receptor.
We used two vectors to express dominant-negative PDGFR-α at different levels, one contained an LTR as promoter, while the other one additionally contained grp78, which is a strong promoter element. Interestingly, we could not find cells with the additional grp78 promoter, while there were few positive ones containing the dnPDGFR-α transcribed from the weaker LTR promoter. In addition, the ratio of these few positive cells slowly diminished after several cell division cycles indicating a decelerated cell division cycle. We concluded, that cells are affected by the downregulation of PDGF signalling and that a strong overexpression of non-functional PDGFR-α could even send the cells into apoptosis.

12.1.1 PDGF is involved in Cell-Matrix Interactions

We investigated the migratory and invasive potential of MIM-ras and MIM-dnPDGFR-α-ras respectively by a wound healing assay, which clearly indicated the inferior performance of MIM-dnPDGFR-α-ras for closing a gap between cells representing an artificial wound.

A similar finding was obtained from *in vivo* experiments. MIM-dnPDGFR-α-ras cells again showed a diminished tumour growth rate in comparison to MIM-ras cells. Furthermore MIM-dnPDGFR-α-ras cells were able to build spheres when cultivated in 3D culture, however the diameter and size was smaller than that observed from MIM-ras cells. This spheres may represent organotypic structures, as the hepatocytes within the sinusoids form ducts with a villi shaped surface in order to offer a large surface area for the exchange of metabolites from blood to the liver. This indicates that PDGF is needed for the colonisation of a novel area and indicates a role in the migratory and invasive potential of a cell.

When analysing the influence of PDGF during the EMT process, we found that although MIM-dnPDGFR-α-ras cells were able to perform EMT upon TGF-β induction, the switch from the epithelial layer pattern to a fibroblastoid multilayer growth was less intense than that from MIM-ras cells treated with TGF-β. Accordingly, the 3D culture and *in vivo* tumour formation analysis showed slower progression, leading to smaller sized tumours.

We concluded, that PDGF is a key component of cell matrix interactions and thus is an important element regarding the invasive and migratory potential of a cell.

12.1.2 PDGF is linked to β-catenin Accumulation in the Nucleus

A proceeded study showed that PDGF signalling induces PI3 kinase signalling and β-catenin accumulation in the nucleus during EMT (Fischer *et al*., 2007).

It was shown, that the TGF-β induced PDGF signalling during EMT results in an activation of Akt. Since PKB/Akt phosphorylates and thus inactivates GSK3, it provides another possibility to block the cytoplasmatic degradation of β-catenin. However, a direct

connection between Akt signalling and nuclear β-catenin accumulation was not detected, as only MIM-rt cells with the dnPDGFR-α showed an induction of PKB/Akt signalling compared to MIM-ras cells, but both cell types stained for cytoplasmatic β-catenin. Since the authors observed an increase of nuclear β-catenin in MIM-rt cells compared to MIM-ras cells, they concluded that the nuclear β-catenin accumulation is linked to PDGF signalling. However, no direct relation to PKB/Akt activation could be found.
Interestingly, they found, that nuclear β-catenin accumulation prevents cells from anoikis and thus could protect spreading and metastasising cells from cell death. Those MIM-rt cells showed elevated levels of β-catenin, CyclinD1, c-myc and $p16^{INK4A}$ within the nucleus. While CyclinD1 and c-myc have cell cycle promoting capabilities, $p16^{INK4A}$ blocks the cell cycle by inhibiting CDK4 and thus G1 phase cell cycle progression.
This leads to the assumption, that PDGF signalling enables β-catenin accumulation within the nucleus, which results in the expression of cell cycle promoting (CyclinD1 and c-myc) and inhibiting ($p16^{INK4A}$) factors. This could be beneficial for spreading, metastasising cells, as they show protection from anoikis during their detached state, while maintaining a non proliferative attitude. These cells then could have the ability to move along blood vessels, until re-attachment within an adequate microenvironment in order to switch again to a proliferative status.

12.1.3 PDGF maintains EMT at the Tumour Border

In order to get more information about the tumour stroma crosstalk, malignant hepatocytes and myofibroblasts were co-injected into mice and used for 3D collagen gel invasion studies (Zijl *et al*., 2009).
Non-tumourigenic myofibroblasts were derived by long term treatment of activated hepatic stellate cells (HSC) with TGF-β. *In vivo* the authors could detect, that myofibroblasts were able to support the growth of MIM-ras and to a lesser extend MIM-dnPDGFR-α-ras tumours. Immunohistochemical stains of the MIM-ras tumours indicated EMT at the tumour borders by showing the loss of membrane-bound E-cadherin and the nuclear translocation of β-catenin. The tumour centres however, showed a mix of epithelial and mesenchymal phenotypes. The expression of PDGFR-α revealed a similar picture as elevated levels of PDGFR-α at the tumour edges was found, but only little to no

expression at the tumour centres. Interestingly the MIM-dnPDGF-α-ras tumours showed no sign of EMT at the tumour-host border. This indicates, that the overexpression of dnPDGFR-α successfully inhibited PDGF signalling and thus EMT.

The *in vitro* 3D collagen co-culture revealed a similar effect: Here the presence of myofibroblasts enabled malignant hepatocytes to detach from a polarized sphere structure and to invade the nearby gel matrix as a single cell. Thereby, the cell acquired a mesenchymal phenotype, while losing their epithelial markers such as membrane-bound E-cadherin, β-catenin and ZO-1. TGF-β signalling could be blocked by LY02199761, which restored the spheric morphology, however the inhibition of PDGF receptor by STI571 succeeded only in a partial blockage. The cells were still able to invade the nearby gel, but never detached from each other and they maintained their epithelial morphology. Taken together, this results show that TGF-β is important in the initiation of EMT, whilst PDGF signalling is important for maintaining EMT at the tumour border.

The authors could additionally detect elevated CCL-2/MCP-1 secretion from myofibroblasts, which were isolated after a first peak of inflammation. In the case of wound healing, elevated CCL-2/MCP-1 levels could attract macrophages, as an early immune response to tissue damage. Those macrophages respond by secreting metalloproteases and cytokines, like TGF-β, FGF-2 and PDGF. This induces the onset of EMT in neoplastic hepatocytes and the establishment of an autocrinous PDGF production, in order to maintain the EMT. This leads to malignant hepatocytes, that produce TGF-β and PDGF and therefore are able to manipulate the microenvironment in order to generate hepatic myofibroblasts. The hepatic myofibroblasts aid malignant hepatocytes to undergo EMT at the tumour border leading to invasive and metastatic properties.

This finding connects PDGF tumour-stroma crosstalk with immune cell interaction, which states another important feature of the TGF-β/PDGF axis for the development of HCC.

12.2 PDGF Antagonists

As upregulated PDGF signalling is coming along with more invasive tumours, not only in HCCs, one possibility to counteract the spreading would be either by blocking or by downregulation of the pathway. Under physiological conditions, upon ligand binding the PDGF receptors dimerise which induces autophosphorylation Thereupon SH2 domain

containing signalling molecules (e.g. c-src, phospholipase C-gamma, PI3K or GRB2/SOS) are able to bind to phosphorylated tyrosine residues. The cascade can be blocked by three different strategies: Either the use of an antagonist against the extracellular receptor domain, the interference of receptor dimerisation or the block of the receptor tyrosine kinase interaction sites (see Fig. 6).

The extracellular PDGF receptor domain can be blocked by antibodies against the receptor and against the different PDGF isoforms. In animal models, these blockings were successfully used to avoid glomerulonephritis and the growth of tumour xenografts. (Hill *et al*., 2001; Loizos *et al*., 2005)

The function of Systematic Evolution of Ligands by EXponential Enrichment *(*SELEX) aptamers is similar to antibodies, except that their three dimensional structures are made of single stranded DNA or RNA. They bind to PDGF ligands and thereby interfere with receptor-ligand binding. In animal models, it was successfully demonstrated, that its use prevented from glomerulonephritis and reduced the interstitial fluid pressure of tumours (Floege *et al*., 1999; Pietras *et al*., 2001).

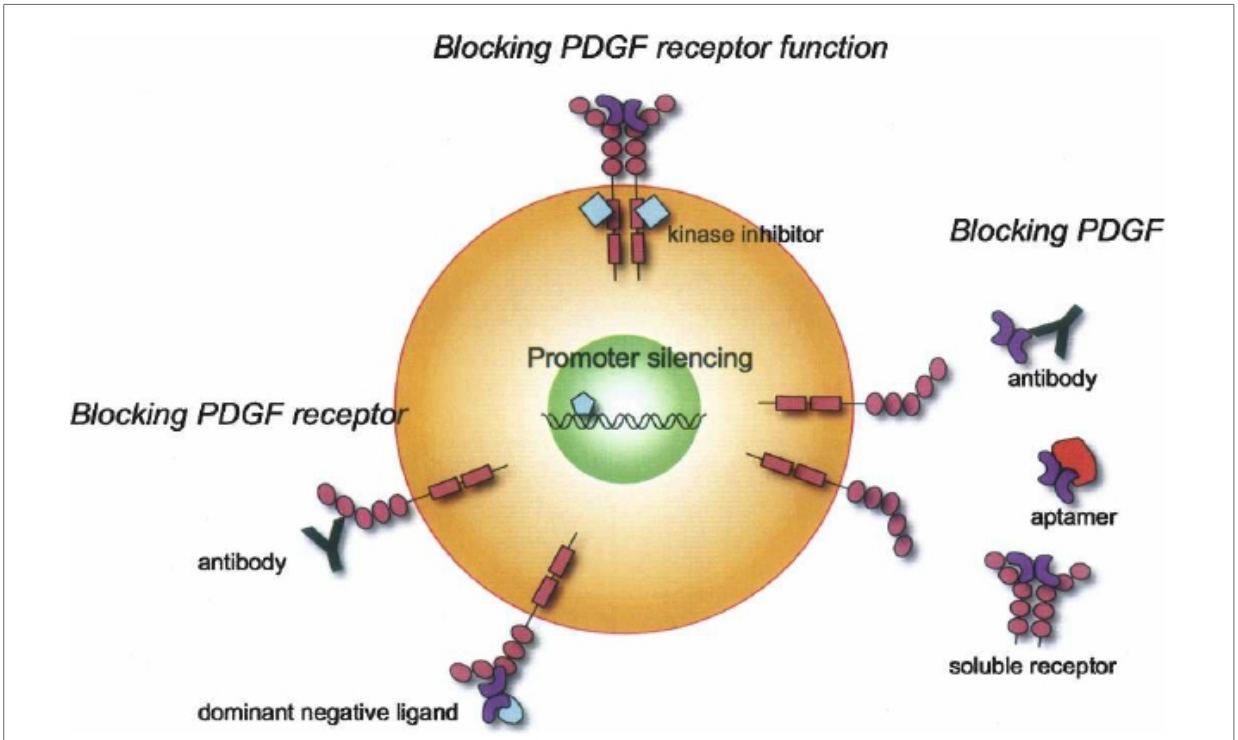

Figure 6

Possible blocking sites of PDGF ligand and receptor. Antibodies and dominant-negative ligands are able to block the PDGF receptor, antibodies and aptamers are able to block the soluble receptor and tyrosine kinase inhibitors are able to block PDGF receptor function (5).

Antagonists, that interfere with receptor-dimerisation monoclonal antibodies which block receptor tyrosine phosphorylation and as a consequence PDGF induced signalling (Shulman *et al*., 1997).

Low molecule weight tyrosine kinase inhibitors such as Imatinib (STI571), Sorafenib (BAY 43-9006), Sunitinib (SU11248), Dasatinib (BMS354825) and AG1296 can bind to the region of functional cytoplasmatic tyrosine residues of the PDGF receptor and thus avoid the binding of receptor tyrosine kinases (Lewis, 2007; Levitzki, 2004). The difficulty in constructing those inhibitors are the rather unpredictable cross reactions and the accompanied cytotoxicity. For example, STI571 binds PDGF receptors very efficiently, but additionally links to the structurally less related c-Abl and the development of SU6668 was discontinued, because its unacceptable toxicity revealed during clinical trials (Manley *et al*., 2002; Homsi and Daud, 2007).

Another possibility is to use genetic approaches to interfere with PDGF receptor signalling, like the above demonstrated dominant-negative PDGF receptor or RNAi interference.

12.2.1 STI571, a Tyrosine Kinase Inhibitor

The compound STI571, also known as Gleevec or Imanitib Mesylate, inhibits the tyrosine kinase properties of PDGF, c-kit, c-Abl and Ang. It is used against chronic myelogenous leukaemia (targeting Bcr-Abl), gastrointestinal stromal tumours (GIST) (targeting c-Kit), c-Abl and myelodysplastic/ myeloproliferative diseases (MDS/MPD) (targeting PDGFR gene arrangements) (Capdeville *et al*., 2002). We could show that STI571 was able to trigger apoptosis in MIM-rt cells, which showed highly elevated PDGF-A ligand and PDGFR-α levels upon EMT. This finding suggests, that hepatocellular carcinoma with upregulated PDGFR could possibly be treated to restrain spreading and metastasising cells.

Besides, Gleevec is able to lower the interstitial pressure of tissue, a major hindrance of drug delivery and uptake of invasive tumours. With its co-application, STI571 boosts the uptake of other tumour targeting substances and thus promotes their potency.

However, certain tumours develop resistance to imanitib mesylate (c-Kit) (Capdeville *et al*., 2002). In order to overcome this issue an alternate compound called SU11248 (sunitib

malate) was developed to target receptor tyrosine kinases, that are resistant to STI571. Furthermore its specificity was changed to include VEGF as target. SU11248 inhibits all PDGF receptors, VEGF, Kit, Ret, CSF-1R and Flt-3. It shows a tolerable toxicity profile and is currently approved for the treatment of clear cell renal carcinoma. Newer compounds in development are Vatalinib, which targets VEGFR-1 and 2 and PDGFR-β, c-kit, and c-FMS at higher doses, and Axitinib, which is designed to block VEGFR-1, -2, and -3, PDGFR-β and c-kit (Lewis, 2007).

The main problems of drugs against cancer cells is their side effects on normal dividing cells and their way of delivery to affected organs or tissue. As for example normal oral intake will deliver the drug to most parts of the body, exposing most cells to the effect of the medication. Therefore, those generally administered substances have to target pathways specifically active in tumour cells to avoid severe side effects in regular tissue. This makes it necessary to treat each kind of tumour with a pool of antagonists, each one blocking a defined or defined set of pathway(s), which are only active in this form within the tumour.

12.3 A Novel Network of Control: Micro RNAs

With the discovery of a novel network of cellular control, which consists of short, non-protein coding RNA fragments, the complexity of cellular interactions reached a new level.

Small RNAs are currently divided in snoRNAs (chemical modification of rRNA and tRNA), miRNA (post-transcriptional mRNA regulation) (Lagos-Quintana *et al*., 2001), siRNA (post-transcriptional gene silencing) (Reinhart and Bartel, 2002) and piRNAs (forms RNA-protein complexes for transcriptional gene silencing) (Aravin *et al*., 2007). Especially miRNAs (for microRNA) or short miR have gained attention recently, as they show a strong sequence conservation among species, which makes them easier to predict. Moreover their location close to the 3'-end of the mRNA allows the scanning of target binding areas. So far several hundreds of miRNAs have been predicted in mammals (Ambros, 2004) of which dozens have been validated experimentally to participate in post-transcriptional gene regulation.

The presence of miRNA has been shown to vary in different tissues as well as in cancer

cells (Liu *et al*., 2004; Lu *et al*., 2005). They are involved in EMT and metastasis formation as well as participating in the TGF-β pathway and PDGFR-α regulation (Kong *et al*., 2008; Cano *et al*., 2008; Korpal *et al*., 2008; Eberhart *et al*., 2008). This findings expect miRNAs to participate in our HCC EMT model as well and thus it is of great necessity for the understanding of the transition process to identify putative involved members.

12.3.1 Occurrence within the Liver

The first liver specific miRNA discovered was miR-122, but many others like miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-146 and the let-7 family were found to be expressed in adult liver (Chen, 2009), others like miR-92a or miR-483 were only present in fetal liver (Chen, 2009), indicating a different expression profile during development. It was also shown that the manipulation of transcription actors affected the expression of miRNAs (e.g. HNF-1alpha influences miR-107 expression, myc can upregulate miR-17-92 cluster while downregulating others) (Ladeiro *et al*., 2008; Aguda *et al*., 2008). Moreover, it was shown that transcription factors (NF-kB) can regulate mi-RNA expression (let-7), while on the other hand also transcription factors (STAT3) can be regulated via micro RNAs (Meng *et al*., 2007; Chen *et al*., 2007).
Regarding hepatocellular carcinoma it was examined, that miR-21, miR-224, miR-34a, miR-221/222, miR-106a, miR-203 were upregulated in malignant hepatocellular carcinomas compared to benign hepatocellular tumours (Chen, 2009). Furthermore miR-122a, miR-422b, miR-145, miR-199a showed downregulation in HCCs compared to non-tumour tissue (Chen, 2009). Several miRNAs also showed a correlation with the degree of transformation, indicating that they also play a role during certain parts of the progression of tumour development (Murakami *et al*., 2006).
It was shown for miR-122, which exhibits downregulation upon HCC, that its potential target genes were N-myc and cyclin G1. Interestingly, N-myc is often rearranged in woodchuck liver tumours whereas cyclin G1 shows an inverse correlation with miR-122 expression. (Gramantieri *et al*., 2007; Jacob *et al*., 2004)
In murine mammary epithelial cells it was even shown, that microRNA actively participate in the regulation of EMT and MET (Korpal *et al*., 2008). In epithelial cells miR-200 family

blocks ZEB1/ZEB2 expression, so ZEB1/ZEB2 cannot block E-cadherin transcription and thus is available for assembling adherens junctions. If cells are stimulated with TGF-β, miR-200 family members are repressed (miR-200b, miR-200c, miR-200a, miR-429), leading to EMT. Within the miR-200 family, there are two gene clusters for target recognition of ZEB1/ZEB2; miR-200b, -200c, -429 and miR-200a, -141. Although all five members can bind to ZEB1 and ZEB2, they exhibit different binding efficiency. The circumstances leading to the varying expression of the 2 clusters remains unknown.

It was shown, that overexpressed miR-200 family members could slow down or even revert the development of a mesenchymal phenotype (Korpal *et al*., 2008). The elevated miR-200 levels lead to MET, reduced in vitro migration and enhanced macroscopic metastases, depending on cell type and miR-200 family members present.

While tumour progression normally causes a downregulation of MiRs, MiR-155, miR-9 and miR-10b on the other hand are examples that are upregulated in tumour metastasis.

TGF-β induces the expression of miR-155 and thus promotes its upregulation during EMT. In addition, miR-155 negatively regulates Rho A, resulting in disrupted tight junction formation and enhanced cell invasion and migration (Kong *et al*., 2008).

MiR-10b has been shown to target HOXD10a, thus inducing the pro metastatic gene RHOC and thereby consequently support metastasis (Ma *et al*., 2007; Ma *et al*., 2010).

Expression of MiR-9 has been shown to suppress E-cadherin in breast cancer cells. Thereby E-cadherin loses its capacity to sequester beta-catenin and potentates the Wnt signalling pathway. Silencing of MiR-9 led to decreased metastasis formation (Almeida *et al*., 2010).

12.3.2 Micro RNA and PDGF

Interestingly PDGFR-α mRNA hosts a binding site for miR-140 within its 3'-UTR. In zebrafish it was shown that miR-140 negatively regulates PDGF signalling during platal development (Eberhart *et al*., 2008). We performed a crosscheck with gallus gallus, rattus norvegicus, mus musculus and homo sapiens PDGFR-α 3'-UTR mRNA and could confirm the presence of conserved miR-140 binding sites.

For the binding of the miRNA to its target it is of great importance, that the first 7 or 8 nucleotides of the miRNA, also called seed sequence, have an exact matching pattern

with the target (Korpal *et al*., 2008; Lewis *et al*., 2005). This perfect match patterns were used by us for the identification of possible targets within the 3'-UTR.
Additionally to the miR-140, we also found, that the same conserved site would allow miR-329 a/b and miR-1988 to bind to this mRNA. However, the significance of this additional binding abilities will have to be evaluated.

12.3.3 MicroRNAs as Therapeutic Targets

Having a widespread impact on the cellular state, miRNAs also pose a novel chance for the development of therapeutic targets and tumour markers. It was shown, that myc induced hepatocellular carcinoma perturbs miR-26a expression. In turn, miR-26a, regulates the expression of cyclins D2 and E2 and induce G1 arrest of human liver cancer cells (Kota *et al*., 2009). A miR-26a construct was transduced into hepatocytes in vivo and in vitro by an adeno-associated virus (AAV) vector, that's envelope is not associated with AAV infections to avoid immunologic cross reactions. Animals with ectopic expression of the miR-26a construct show apoptosis in cancer cells, whereas non-malignant hepatocytes show no effect, because they already have a consistent high level of miRNA. This application indicates, that a single miRNA can have an impact on disease. Thus, the treatment can be refined to only hit a very specific target. This is a remarkable step in maximizing effect and minimizing cross reactions and toxicity effects to other cells.
These new insights will hopefully augment the further understanding of cellular management and the generation of novel strategies for cancer treatment, that can be tuned on a finer scale.
The multiplicity and the distinct functions of miRNA pose a powerful level of control over gene expression in mammalian cells. The above mentioned examples exhibit the close connection of miRNAs with the field of EMT and their possible involvement in tumour invasion and metastasis. The revealing of the crosstalk of cellular signalling poses the unique opportunity to step further in understanding the complexity and significance of gene expression under different conditions or of different tissues. This new network integrates into an existing pattern of regulators and gives us the opportunity to develop new effectors in manipulating cell signalling.

12.4 Summary and Conclusions

We studied the impact of altered PDGF signalling on hepatic cancer cells and could show, that PDGF is involved in the regulation of pro-survival pathways, the protection from anoikis for metastasising cells and the involvement of extracellular components enhancing the potential for migration and invasion. We could examine, that PDGF has a direct or indirect influence on factors like β-catenin, cyclinD1, c-myc, $p16^{INK4A}$ and PKB/Akt and hence with its signalling could actively support the EMT process. Furthermore we could determine, that TGF-β is important for the onset of EMT, while PDGF signalling is mandatory for the maintenance of EMT at the tumour border. It also appears, that upon liver tissue damage, myofibroblasts cooperate with macrophages to release cytokines as TGF-β and PDGF. This changes the microenvironment around the injured site and leads to the onset of EMT in hepatocytes. The transformed hepatocytes in conjunction with myofibroblasts then foster the establishment of autocrinous TGF-β and PDGF expression and extend and maintain their mesenchymal state.

We and others (Pietras *et al*., 2003) showed, that blocking the PDGF pathway by drugs like STI571 is an effective measure, that can slow, arrest or even revert cancer development.

With the discovery of novel regulatory network consisting of miRNAs, that is involved in cellular regulation and transition processes, we could be a step further to understand the basic crosstalk of cells, that leads to cancer and further to invasive cells.

A promising principle in cancer treatment is the analysis of the diversity of activated pathways in tumour cells. This is a necessary step for an individual treatment of each tumour profile with combined inhibitory compounds, that maximize the impact on the tumour but minimize the toxicity to non-involved tissue. For instance, the possibility of miRNA as therapeutic agent introduces a very specific targeting of deranged pathways, that could lead to more effective and less toxic medication.

The analysis of tumour cell expression patterns can be done by microarrays, as we did, or by next generation sequencing (NGS) technologies, whereas the focus of development is centred on NGS. The sequencing methods offer multiple advantages over microarrays, such as increased sensitivity for rare sequences, interrogation of novel genomes without

prior knowledge, single-nucleotide resolution, lower cost and the ability of genome-wide mapping of DNA binding proteins and epigenetic marks (Hurd and Nelson, 2009). However, microarrays may still have their nice as a low cost screening tool or when there are huge amounts of samples, such as in medical tumour tissues analysis.

The development of new screening tools is essential for the detection of novel regulatory mechanisms, that allow us to understand cell signalling and its alterations during cancer progression. So we used microarrays to reveal PDGF and its receptor as a novel potentative player involved in EMT and thus the enhancement of cancer invasiveness. We studied its role during EMT and revealed its impact and significance within the tumour stroma crosstalk. This will hopefully be helpful to develop novel strategies in targeting and inhibiting tumour invasiveness.

13. References

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