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Identification of the mechanism for physiological hepatocyte polyploidization

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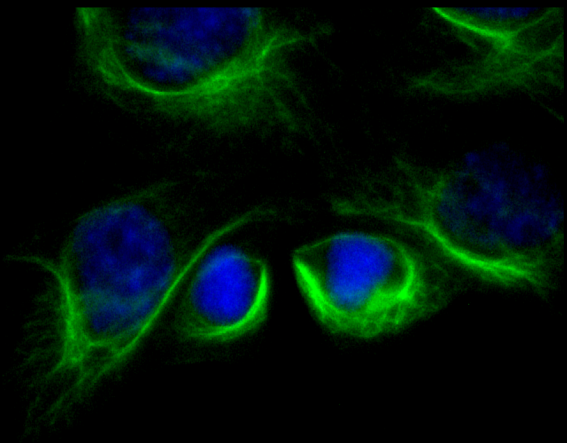

Ph.D. program: “Human Biology and Genetics”
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Beyond the rules, beyond the protocols

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ABSTRACT

Polyploidy is the condition in which a cell possesses more than two sets of homologous chromosomes. Although polyploidy is not common in mammalians, some specific tissues including heart, muscle cells, megakaryocytes, trophoblast giant cells and parenchyma liver cells are physiologically polyploid. In liver an extensive polyploidization occurs during the weaning period between the 15^{th} and 40^{th} day after birth, leading to the formation of tetraploid and octapoloid cells with one or two nuclei. Polyploid hepatocytes retain an highly proliferative potentiality; they can undergo multipolar mitosis due to extra centrosomes generating 3 or 4 aneuploid daughter cells. Hepatic aneuploidy was proposed to be a substrate for selection of more resistant clones during liver injury. In fact it has been demonstrated that specific aneuploid injury resistant nodules emerged after chronic liver damage. However, how polyploidization is triggered, established and regulated remains an open question. Several data suggest that micro-environmental elements (mainly soluble factors), rather than cell autonomous mechanisms, play a direct role in the appearance of polyploidy. For example, insulin and T3

hormone were identified as positive regulators and TGFα as a negative regulator of the binucleation. We screened the effects of a set of soluble factors involved in liver development and/or mass liver control on a murine hepatocyte cell line. Within the screening, we identified the cytokine TGFβ as a strong inducer of hepatocyte binucleation. Time lapse video microscopy highlighted that binucleated cells originated from a mechanism of cytokinesis failure without affecting the kinetics of the mitosis; this suggests that binucleation is a physiological alternative program of hepatocyte division. Very interestingly, *in vivo* experiments performed with the oral administration of a TGFβ receptor chemical inhibitor, during days 18-32 post-birth in mice, produced a significant decrease of binucleated hepatocytes, confirming the crucial role played by this cytokine in the polyploidization of the liver cells. *In-vivo* data showed that the hepatocytes cellular model recapitulated the physiology of the whole organism. Analysis of TGFβ downstream elements, known to be involved in cytoskeleton rearrangement, showed that Src kinase activity has a role in the polyploidization process of hepatocytes, most probably through the control of the GTPase protein RhoA, an actin cytoskeleton regulator, crucial for the cytokinesis. In TGFβ treated cells, in

fact, together with the activation of Src, we observed a delocalization from the mid-body structure of the active form of RhoA. TGFβ is well known to trigger Epithelial to Mesenchymal Transition (EMT) in hepatocytes. The TGFβ-induced binucleated cells, in fact, showed a fibroblastoid morphology with upregulation of mesenchymal markers and downregulation of epithelial ones. TGFβ withdrawal was also performed and it produced an increase in ploidy levels with the appearance of an octaploid population and giant nuclei. Together with a ploidy increase, TGFβ withdrawal triggered the Mesenchymal to Epithelial Transition (MET) characterized by upregulation of ephitelial markers and downregulation of mesenchymal markers and generating a full epithelial polyploid population. In conclusion, TGFβ seems to be one of the major stimuli for hepatocyte binucleation; it acts through Src kinases and involves the activity of RhoA during cytokinesis. Moreover, intriguing is the relationship between EMT/MET and polyploidization that might reveal a new biological process in which these transdifferentiation mechanisms are involved and thus, requires further study.

CHAPTER 1. INTRODUCTION

1. Epithelial-to-mesenchymal transition in the liver

1.1 The liver

The liver is the largest solid organ of the body. It is provided with a dual blood supply. Approximately 75% of the blood entering the liver is venous blood filled with nutrients and toxic substances from the portal vein, drained from the spleen, gastrointestinal tract, and its associated organs. Liver plays a pivotal role in metabolic conversion of nutrients and xenobiotics and release them into the blood and bile. Hepatic parenchyma is made up of two cell types: hepatocytes (about 70-75% of hepatic cells) and cholangiocytes (10-5%). Both derive from the anterior portion of the definitive endoderm, which is established in the embryo during gastrulation. The stromal compartment of the liver includes hepatic sinusoidal endothelial cells, hepatic stellate cells (HSCs), and Kupffer cells (KC). Sinusoids are the first blood vessels to form during hepatogenesis, they develop by angiogenesis from existing vessels in the septum trasversum mesenchyme. Sinusoidal

endothelial cells, supplied of multiple fenestrations, facilitate transfer of factors between the sinusoidal lumen and the surface of the hapatocytes (Si-Tayeb et al. 2010). Kupffer cells are resident macrophages on the surface of hepatic sinusoidal endothelial cells. They represent 15% of the liver cell population and 50% of resident macrophages in the body (Figure 1). Thus, the liver is composed of both mesenchymal and epithelial cells and a process called ephitelial-to-mesenchymal transition play a crucial role during liver development/morphogenesis and chronic degenerative processes.

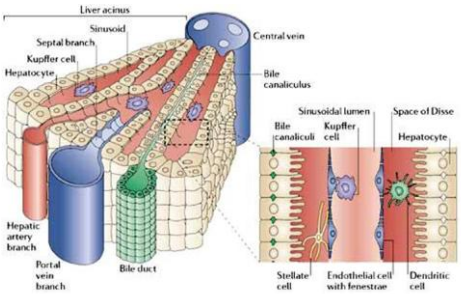

Figure 1 Architecture of hepatic parenchyma. Liver comprises about 60-80% of hepatocytes arranged in an epithelial monolayer called hepatic cord. The basolateral hepatocyte surface is lined with a fenestrated endothelium, formin sinusoidal vessels. The apical face of adjacent hepatocytes forms bile canaliculus. Other intrahepatic cell populations

include biliary cells, liver sinusoidal endothelium cells (LSECs), resident macrophages called Kupffer cells and hepatic stellate cells, located in the Disse space between hepatocytes and LSECs. From Adams and Eksteen 2006.

1.2 Epithelial-to-Mesenchymal and Mesenchymal-to-Epithelial transitions.

Epithelial cells are adherent cells that closely attach to each other, forming coherent layers in which cells exhibit apico-basal polarity. In contrast, mesenchymal cells are non-polarized cells, capable of moving as individual cells because they lack intracellular connection. An **epithelial-mesenchymal transition (EMT)** is a biological process that allows a polarized epithelial cell which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. Moreover, a new transcriptional program is induced to maintain the mesenchymal phenotype (Grünert at al. 2003). EMT is a complex, extreme manifestation of cellular plasticity that involves an orchestrated series of events. The key to EMT is the reduction of cell-cell adhesion by transcriptional repression of cadherins (adherent junctions), occludin and claudin (tight junctions) and desmoplakin

(desmosomes). Repression of E-cadherin transcription is accompanied by induction of N-cadherin (neural cadherin) or cadherin-11 expression, that is known as the **cadherin switch**. Other major changes in cytoskeleton organization and composition are the replacement of F-actin fibers by the Rho-mediated network of the stress fibers at the tip of which ECM adhesion molecules localize, including integrins and integrin linked kinase (Fuchs et al 2008). Those changes are sufficient for the cells to separate, lose apico-basal polarity and gain a spindle shape (Hugo et al. 2007) (Figure 2). A hallmark of EMT is its reversibility. The phenotypic plasticity afforded by an EMT is revealed by the occurrence of reverse process, a **mesenchymal-epithelial transition (MET)**, which involves the conversion of mesenchymal cells to epithelial derivatives (Thiery et al., 2009).

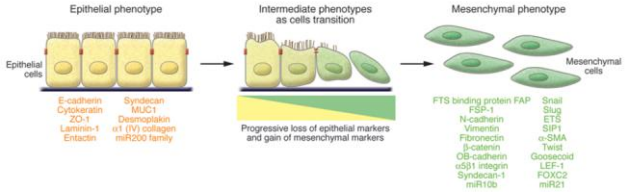

Figure 2 Epithelial-to-mesenchymal transition. The transition encompasses a broad spectrum of phenotypic changes from a transient loss of cell polarity to a total cellular reprogramming. Transitional cells which display an intermediate phenotype (coexistence of both epithelial and mesenchymal markers) have also been detected. Epithelial and mesenchymal markers commonly modulated during the EMT/MET transition are listed. From Kalluri and Weinberg 2009

1.3 Physio-pathological situations associated with EMT/MET

EMT and the reverse process MET are strongly involved in embryogenesis and development. However, it is important to emphasize that EMT/MET refer to changes in cell shape and adhesive properties. Cell fate (lineage) is specified by other mechanisms (Choi and Diehl 2009). Hence, EMT/MET are merely manifestations of the **inherent plasticity** (Kalluri 2009). EMT during embryogenesis is the formation of the primitive streak, which leads in turn to the formation of the

three germ layers, including the formation of mesenchymal cells and mesoderm that generate all tissue types of the body. Indeed, mesodermal and endodermal cells can undergo MET to generates secondary epithelia that than undergoes further rounds of EMT/MET to form various organs (Figure 3). Moreover, the formation of placenta, somites, heart valves, neural crest, uro-genital tract and secondary palate, and branching morphogenesis of multiple different organs all involve EMT (Savagner 2001, Nieto et al. 2009). In this scenario EMT/MET are fundamental processes parallel to differentiation and/or associated with the building or remodelling tissues. It is used whenever high motility and loss of polarity is needed. It has been shown that also adult cells are capable to undergo EMT/MET. Liver adult cells such as hepatocytes, choloangiocytes and hepatic stellate cells (HSC) are best known to undergo in EMT/MET. In fact, several groups including us have demonstrated in-vitro that treating primary murine hepatocytes or non-tumorigenic hepatocytes cell lines with TGFβ cytokine causes them to down regulate expression of epithelial genes and up regulate expression of mesenchymal genes (Spagnoli et al. 2000, Kaimori at al. 2007, Zeisberg et al. 2007). In-vivo EMT/MET of adult liver cells are

associated with the replacement of dead or damaged epithelial cells with healthy new epithelial cells (Figure 3).

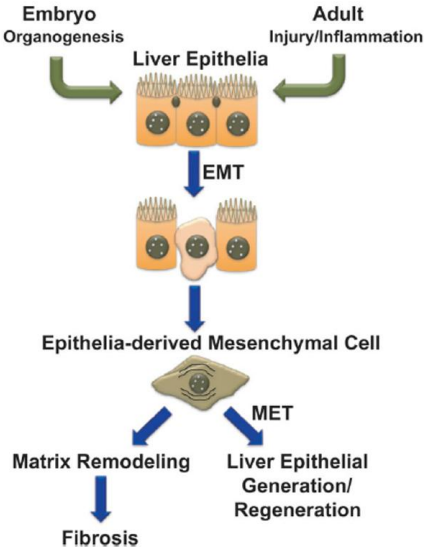

Figure 3 EMT can occur when tissues are built during embryogenesis/development and during adult tissue remodeling responses such as injury. During adult liver injury, EMT promotes liver repair. EMT facilitates transient acquisition of a mesenchymal phenotype by certain types of liver epithelial cells. Epithelia-derived mesenchymal cells may contribute to liver fibrosis or they can revert to epithelial cells by MET process. EMT/MET is an intermediate step to form new hepatocytes and cholangiocytes. Recent fate-mapping studies in transgenic mice suggest that MET may have a role in hepatocyte regeneration. From Choi and Diehl 2009

The best known example of EMT/MET of liver adult cells is the response to liver injury. The normal mammalian response to injury occurs in three overlapping but distinct stages: inflammation, new tissue formation, and tissue remodelling (Gurtner et al. 2008). The latter two processes are known as **liver regeneration**. For example, after 2/3 hepatectomy, most hepatic cells rapidly enter the cell cycle and undergo an average of approximately 1.6 cycles of replication per cell to completely restore the original liver mass (Taub 2004). The regenerative response is mostly mediated by two groups of components: growth factors and cytokines, i.e. interleukin-6 (IL-6), tumor necrosis factor (TNF), epidermal growth factor (EGF) and transforming growth factor (TGFβ). TNF binds its type I receptor, leading to nuclear factor kappa B activation in Kuppfer cells, which produce IL-6 and TNF; IL-6 is subsequently released into the serum and binds to its receptor to activate the STAT-3 signaling pathway to initiate hepatocyte regeneration (Fausto et al. 2006). EGF and TGFβ have been identified as promoters of EMT (Figure 4) (Pagan et al. 1997, Pagan et al. 1999, Valdes et al. 2002). Indeed, TGFβ that is mostly released from hepatic stellate cells (HSC) can directly activate adjacent hepatocytes through Smad signaling and

induce EMT. Based on the intensity and/or persistence of the injury a partial and complete EMT are possible. In the partial EMT (or EMT-like) the cells coexpress both epithelial and mesenchymal markers (such as cytokeratins, albumin, E-cadherin as epithelial genes and vimentin, collagen-I as mesenchymal genes) (Kalluri and Weinberg 2009). The MET process on these EMT-like cells is associated with gain of regeneration and production of new hepatocytes (Figure 4). Nevertheless, a continuous insult can lead to a persistence of EMT-like cells in a mesenchymal phenotype that accumulate. In these condition a complete EMT occurs together with the upregulation of Snail transcription factor and deposition of extracellular matrix (ECM) collagen 1/3 and elastin that triggers the **fibrotic process** (Figure 4). In fibrotic tissues, myofibroblasts accumulate and secrete an excessive amount of collagen that is deposited as fibers, thereby compromising organ function and leading to its failure. In conclusion, the balance between EMT and MET modulates the outcomes of liver injury. When EMT activity exceeds MET, repair is mainly fibrogenic, causing fibrosis. Conversely, predominance of MET favours more normal liver regeneration (Choi and Diehl 2009).

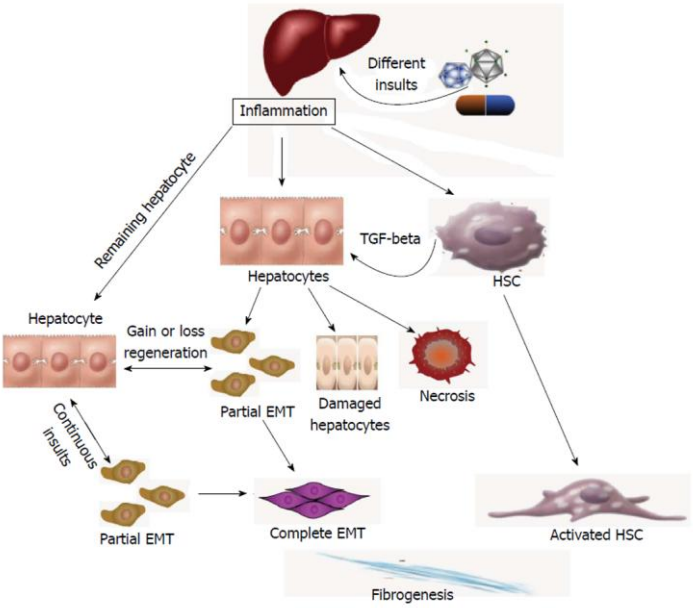

Figure 4 Liver regeneration and EMT. Different insults initiate inflammation and then cause hepatocyte stellate cells (HSCs) activation and hepatocyte damage. HSCs secrete a key cytokine TGFβ that affects normal hepatocytes and promote EMT. Partial EMT followed by MET produces new healthy hepatocytes (regeneration). Continuous insult shifts partial EMT cells to complete EMT cells and finally myofibroblasts, the main producer of extra cellular matrix, leading to fibrotic process and loss of regenerative capacity . Modified from Xue et al 2013

1.4 Molecular signalling in EMT: TGFβ

Multiple extracellular signals can initiate an EMT program, and there is a significant crosstalk among the downstream intracellular signaling pathways and transcription factors that orchestrate this complex process. Growth factors acting through tyrosine kinase receptors (EGF, FGF2, IGF-II, HGF and PDGF), TGFβ, Wnt, Notch, integrins, endothelin A receptor (ETAR) appear to be responsible for the induction or functional activation of a series of EMT-inducing transcription factors, such as Snail, Slug, zinc finger E-box binding homeobox1 (ZEB1), Twist, Goosecoid, and FOXC2 (Moustakas and Heldin 2007). Members of the TGFβ superfamily are the major and the best characterized inducers of EMT. The **TGFβ superfamily** is composed of the TGFβ isoforms (TGFβ1, 2 and 3), Activin, Nodal, bone morphogenetic proteins (BMPs), growth and differentiation factor (GDF), and Mullerian inhibitory factor (MIF). The active form of TGFβ is a 25kDa dimer in which the two polypeptides interact via a disulfide bond and hydrophobic interactions. Once activated, TGFβ family members initiate signaling by interacting with and leading to the activation of two receptor serine/threonine kinases referred to as the type I and type II receptors, forming a heterotetrameric active

complex. There are seven known mammalian type I receptors termed ALK1-7 (activin receptor-like kinase) and five type II receptors. Due to the multipliclicity of receptor class combinations, no cross link between different ligands and the same receptor has been identified (Ehrlich et al. 2011). TGFβ utilizes ALK5 (TβR-I) and TβR-II receptors in the vast majority of cell types (Rahimi et al. 2007). The most well characterized mechanism whereby TGFβ initiate signal transduction is via the phosphorylation and activation of the Smad 2/3 and 4 proteins. This transduction pathway is known as **canonical TGFβ pathway.** Phosphorylated Smad proteins translocate to the nucleus where they accumulate and collaborate with other transcription factors to regulate gene expression. This pathway converges to the induction of the E-cadherin transcriptional repressors such as SNAIL1, SLUG, ZEB2 and E47, which directly represses E-cadherin transcription (Peinado et al. 2003) (Figure 5). In particular, Smad3 has been shown to upregulate the zinc-finger transcription factor Snail, which represses the transcription of E-cadherin (Cho et al. 2007). These transcriptional factors act as "EMT master genes", since their overexpression in epithelial cells not only results in loss of E-cadherin but also in a total

cellular reprogramming towards a mesenchymal state, through the downregulation of adhesion molecules (cytokeratins, integrins, occludins and ZO proteins) and the induction of mesenchymal features in a coordinated manner (Peinado et al. 2007).

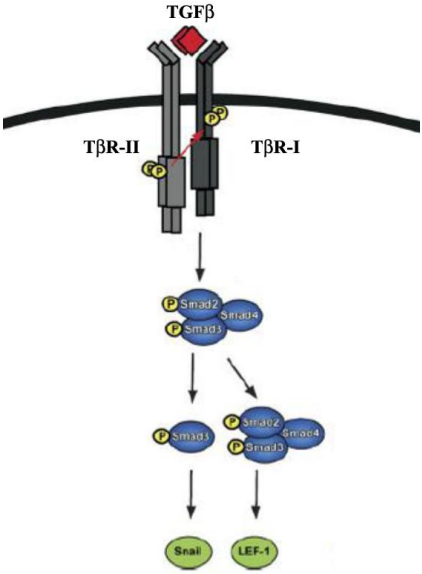

Figure 5 The smad-dependent TGFβ downstream pathway. Activation of tetrameric complex 2X TβR-1/TβR-II causes the phosphorylation of Smad2/3 which then forms a complex with Smad4. Activated Smad 2/3 and 4 complex translocates into the nucleus and drives the transcription of Snail that is regarded to be a master gene of the EMT process.

Other downstream pathways activated by TGFβ are referred to as **non-Smad pathways**, which also play an important role to trigger the EMT process.

Erk/MAPK pathway: Activation of TGFβ receptor causes the loading of GTP on Ras oncogene (Mulder and Morris 1992). In its GTPbound state, Ras can bind Raf and activate a MAPK (Mitogen-activated protein kinase) cascade that includes MEK and Erk (Hartsough and Mulder 1995). Activation of this pathway is necessary to trigger the EMT in combination with the canonical pathway. Indeed, Erk activation is required for disassembly of cell adherents junctions and induction of cell motility by TGF-β. The activated form of Erk can also interact with Smad proteins such as Smad 2/3 and 7 and regulate their activities (Matsuura et al. 2005). Inhibitory phosphorylation of Smads by Erk was proposed as a mechanism to explain how oncogenic Ras overrides TGF-β-mediated growth arrest in cancer cells (Kretzschmar et al. 1999) (Figure 6).

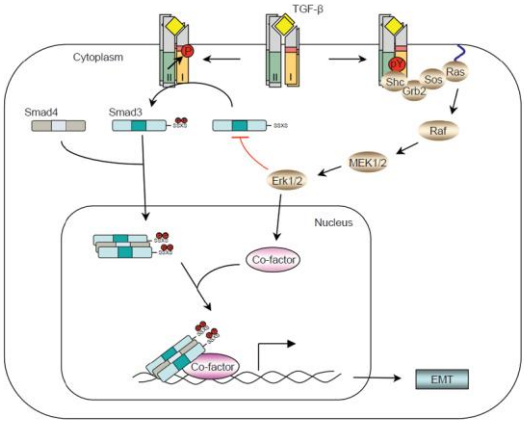

Figure 6 The Erk non-Smad pathway. TGF-β can induce phosphorylation of tyrosine residues on both type I and type II receptors. The phosphorylated tyrosines are capable to activate Erk through Ras, Raf, and their downstream MAPK cascades. Erk then regulates target gene transcription through its downstream transcription factors in conjunction with Smads to control EMT. Erk can also inhibit Smad activities through phosphorylation of Smads. From Zhang 2009.

PIP3/Akt pathway: TGFβ is known to cause the activation of the phosphoinositide 3-kinase (PI3K)-Akt, through the regulatory subunit p85 associated to the TβR-I (Edlund et al. 2005, Yi et al. 2005). Despite the fact that the molecular mechanism is not well characterized, recent studies have demonstrated that TGFβ regulates the mammalian target of rapamycin (mTOR) kinase in an Akt-dependent manner. Activation of this pathway regulates metabolism, migration,

and invasion (Lamouille and Derynck 2007, 2011) features of the EMT process. Moreover it was shown that the PI3K/Akt pathway antagonizes Smad-mediated effects. For example, activation of PI3K or Akt protects cells from TGF-β-induced apoptosis and growth inhibition (Chen et al. 1998, Song et al. 2006). This protection has been suggested to result from a physical interaction between Akt and Smad3 that prevents TβRI-mediated phosphorylation (Remy et al. 2004) (Figure 7).

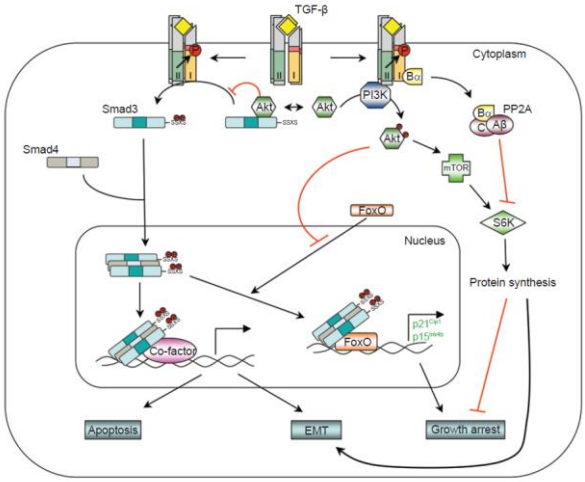

Figure 7 The PI3K/Akt non-Smad pathway. TGF-β can activate PI3K and Akt, possibly by inducing a physical interaction between the p85 subunit of PI3K and the receptors. The activated PI3K/Akt pathway then controls translational responses through mTOR, which collaborates with Smad-mediated transcriptional responses during EMT. Akt is also

capable of antagonizing TGF-β-induced apoptosis and growth arrest by sequestering Smad3 in the cytoplasm and by inhibiting the activity of FoxO transcription factor. From Zhang 2009.

Rho-like GTPase pathway: Rho-like small GTPase, including RhoA, Rac and Cdc42 plays a fundamental role in cytoskeleton rearrangement, organization and cell motility. It has been reported that TGFβ-dependent modulation of this pathway is independent of Smad 2/3 protein activation and it produce a short term response after the TGFβ stimulation (Bhowmick et al. 2001). Activation of this pathway mediates a short term response to TGFβ because they do not directly comprise regulation of gene expression. TGF-β rapidly activates RhoA-dependent signaling pathways to induce stress fiber formation and mesenchymal characteristics in epithelial cells and primary keratinocytes (Bhowmick et al. 2001). Indeed, RhoA is a common effector of different mechanisms involving cytoskeleton arrangement. Different mechanisms may use different GTPase regulators conferring specificity: the inhibitors are GTPase-activating proteins (GAPs) and the activators are guanine nucleotide exchange factors (GEFs). In the EMT process, RhoA regulation involves at least two different GEFs, Net1 (Shen et al. 2001, Papadimitriou et al.

2012) and Vav2 (Papadimitriou et al. 2011). However, downregulation of RhoA protein levels at tight junctions by TGF-β has also been reported. TβR-II phosphorylates Par6 that recruits Smarf1. The complex Par6/Smurf1 drives localized ubiquitination and turnover of RhoA at cellular protrusions (Ozdamar et al. 2005). Thus, downregulation activity of RhoA is restricted to particular subcellular sites. In conclusion, rapid activation and localized degradation of RhoA are parallel effects of TGFβ stimulation and both appear to be necessary for EMT (Figure 8).

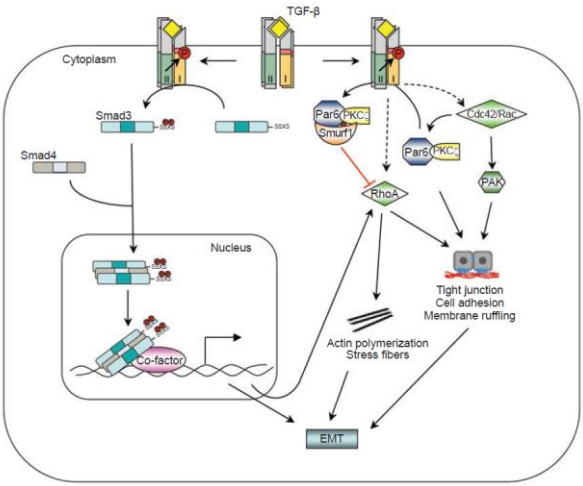

Figure 8 The Small GTPase non-Smad pathway. RhoA can be activated by TGF-β via either Smad-dependent or independent routes to induce actin stress fiber formation during EMT. TGF-β also induces the dissolution of tight junctions during EMT by recruiting Cdc42 to the receptor complex, and by triggering degradation of RhoA at cellular protrusions. TGF-β-induced RhoA degradation requires phosphorylation of Par6 by TβRII and the Smurf1 E3 ligase. From Zhang 2009.

2. Liver polyploidy

2.1 Polyploidy among mammalian organisms

Polyploidy is the state in which cells possess more than two sets of homologous chromosomes. Polyploidy is a widespread physiological phenomenon observed particularly in plants, fungi, insect, fish and amphibians (Otto 2007). Although polyploidy is not common in mammalians, some specific tissues are physiologically polyploid. Indeed, polyploidy seems to be part of the developmental program resulting in the formation of highly differentiated cells, as it has been reported for megakaryocytes (Winkelmann at al. 1987), cardiomyocytes (Brodsky et al. 1980), trophoblast giant cells (Ullah et al. 2008), Purkinje neurons (Lapham 1968), retinal ganglion cells (Morillo et al. 2010) and finally for hepatocytes of the liver parenchyma (Gupta 2000). Despite the importance of polyploidy in mammalians, the knowledge about its functions is very poor. The biological significance of polyploidy has been explained as gene amplification. A double genome simply doubles gene expression without modulating the transcriptional machinery (Storchova et al. 2006). Moreover, increased DNA content also increases cellular size. It was hypothesized that

cell size might provide a metabolic benefit (Comai 2005). Polyploid cells have an intrinsic gene redundancy within the genome. Having multiple copies of the same allele might, in principle, protect polyploid cells from deleterious mutations (Comai 2005). For example, loss of a tumor suppressor on one or both homologs in a diploid cell could have disastrous consequences. Finally, it was demonstrated that polyploid cells are a substrate for the formation of aneuploidy. This aspect will be described in more detail in the next sections.

2.2 Routes to polyploidy

In physiological and pathological conditions polyploid cells can arise by at least four well-known processes with different outcomes. During **cell-cell fusion** genesis of polyploid cells may occur independently of cell proliferation. In this process, mononucleated cells fuse their plasma membranes, leading to the formation of a binucleated cell with a double number of centrosome (Figure). Cell-cell fusion has been observed during physiological development of osteoclast and skeletal muscle cells (Vignery 2000, Taylor 2002). Certain virusses can induce cell-cell fusion with as side effect the fusion of different cell types together, leading to pathological states.

Endoreduplication is another mechanism for the genesis of polyploid cells. During this process proliferative cells alternate S (DNA replication) and G phases of the cell cycle without performing mitosis (Figure 9). The result is a mononucleated cell with a high grade of DNA content that depends of the number of the endoreduplication rounds (Lilly and Durino 2005). The number of centrosomes is also doubled as replication of centrosomes takes place in conjunction with the replication of DNA during the S phase (Figure 9). The best characterized example of endoreduplication occurs during placenta development, when trophoblast stem cells that are deprived of fibroblast growth factor 4 (FGF4) differentiate into trophoblast giant cells (TGCs). This switch is dependent of the inhibition of cyclin dependent kinase 1 (CDK1), that is required for mitosis, and on the activity of CDK2 which promotes S phase (Ullah et al. 2008). **Endomitosis** is a variant of mitosis and is well known to characterize the cell cycle of the megakaryocytes (Ravid et al. 2002). These cells enter in and progress through mitosis until the metaphase but never fully separate sister chromatids in anaphase or undergo cytokinesis. It results in mononucleated globule polyploid cells (Figure 9). It was shown that endomitosis in megakaryocytes is

promoted by the downregulation of CylcinB kinase activity, necessary for the metaphase/anaphase transition, and the upregulation of Cyclin E which promotes the progression in G1 phase (Zhang et al. 1998, Eliades et al. 2010).

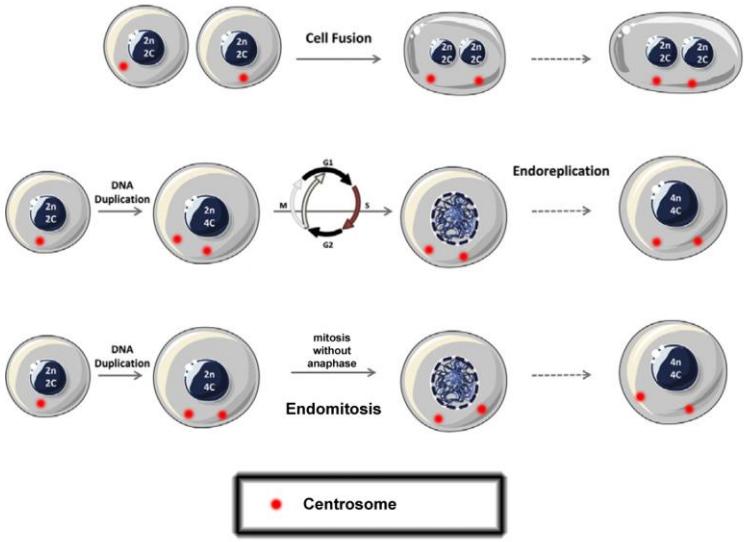

Figure 9 Different mechanisms for the genesis of polyploid cells. Cell-cell fusion results in a binucleated cell with double number of centrosomes. Endoreduplication: the cell proceeds into cell cycle without mitosis stage resulting in the formation of a mononucleated tetraploid cell with double number of centrosomes. Endomitosis: mitosis stage is performed without separation of sister chromatids and cytokinesis resulting in a mononucleated tetraploid cell. From Gentric and Desdouets 2013

The last mechanism that leads to the formation of polyploid cells involves cytokinesis. **Cytokinesis** is the physical separation of one cell into two daughter cells and it is the last step of the cell cycle. It is often referred as C phase of the cell cycle (Canman et al. 2000) because it is highly coordinated with the other phases and it uses both specific and shared molecular machineries. The cytokinesis onset is marked by cleavage plane specification in which proteins required for furrowing accumulate at the equator cortex in a microtubule-dependent manner (Eggert et al. 2006). Specification of the cleavage plane depends on RhoA GTPase that is necessary for completion of cytokinesis (Melendez et al. 2011). RhoA activity is regulated by specific RhoA GEFs and GAPs that mediate the turnover of GTP and GDP. The best known are the GEF Ect2 (epithelial transforming sequence 2) and the GAP McgRacGAP (male germ cell Rac GAP). Ect2 and McgRacGAP form a complex with the mitotic kinesin-like protein MKLP1 that is responsible of the contractile ring localization of the whole complex (Yuce et al. 2005, Kamijo et al. 2006, Nishimura and Yonemura 2006) (Figure 10). Indeed, localized activity of RhoA GTPase at the equatorial membrane provides both filamentous actin assembly and myosin-II

recruitment forming the actomyosin ring structure. Contraction of this structure is also mediated by the activity of RhoA and leads to the furrow ingression (Pienkny et al. 2005). When the cleavage furrow has fully ingressed it compresses the midzone and an intercellular bridge called midbody is created (Figure 10).

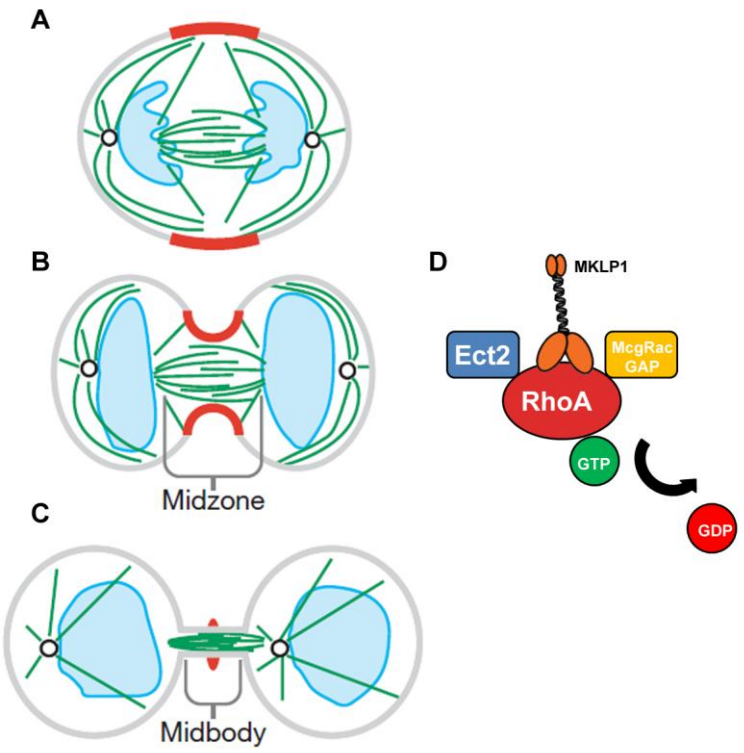

Figure 10 Stages and main actors of cytokinesis. A) Specification of the cleavage plane. Red marker identifies the equatorial cortex where actin and myosin II are recruited to form the contractile ring. Contraction of the actomyosin ring depends on the localized activity of RhoA. B) Contraction of the actomyosin ring leads to the ingression of the cleavage furrow. C) Last stage of cytokinesis in which cells are connected with a cytoplasmic bridge called mid-body. D) RhoA forms a complex with MKLP1 necessary for the

localization to the equatorial zone, the GEF Ect2 and the GAP McgRacGAP that regulates the activity of RhoA. A, B, C Modified from Eggert et al 2006

Completion of cytokinesis occurs when the intercellular bridge is resolved creating two daughter cells. Aurora B kinase has an important role during the cytokinesis completion (abscission). This kinase localizes to the midzone associated with the microtubule and equatorial cortex, accumulating ultimately at the midbody (Carmena et al. 2009). It is a key regulator of the abscission timing if unsegregated chromatin is trapped at the furrow ingression site in human cells. In cells with chromatin bridges between daughter nuclei, active Aurora B persists in the intercellular bridge stabilizing the bridge and delaying the abscission (Steigemann et al. 2009). **Cytokinesis failure** can occur following dysfunction of anyone of the necessary proteins described above. Depending on which molecular mediator is compromised, the cytokinesis stage can show a partial furrowing followed by regression of the cleavage furrow or a full absence of contracting activity (Eggert et al 2006). Moreover, the chromatin bridge also leads to regression of the cleavage furrow and cytokinesis failure in an Aurora B dependent manner (Steigemann et al. 2009). In this case the

cytokinesis stage is often strongly delayed, aborted and finally the cell progresses into the next G1 phase after few hours due to degradation of Cyclin B. Irrespective of the molecular mechanism involved, cytokinesis failure produces a tetraploid binucleated cell. Increase in cellular DNA content is associated with two centrosomes in the same cytoplasm (Storchova and Pellman 2004) (Figure 11).

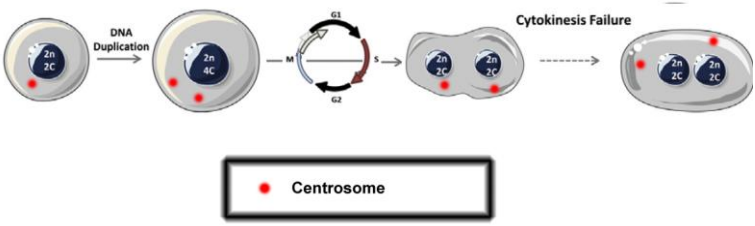

Figure 11 Cytokinesis failure mechanism. Diploid cells undergo a normal cell cycle and progress into all the phases of mitosis. However cytokinesis is impaired with the formation of tetraploid binucleated cells with double centrosome number. Modified from Gentric and Desdouets 2013

2.3 Polyploidy in liver

Hepatic development is an extended process that continues through early post-natal life. In murine liver, at 14 days post-coitus most of the hepatoblasts are bipotent with the ability to renew into hepatoblast or differentiate into biliary cells. However, by 15 days post-coitus most hepatoblasts are committed to the hepatocytes lineage. During the last period of gestation and the first post-natal month, hepatoblasts differentiate in hepatocytes (Germain et al. 1988, Shiojiri et al 1991, Gentric et al 2012). In parallel with hepatocyte lineage specification begins a process to increase ploidy . Mammalian liver at birth is fully composed of mononucleated diploid hepatocytes. However, during the first 4 weeks after birth an extensive polyploidization takes place in the parenchyma in which tetraploid (4n) and octaploid (8n) hepatocytes accumulate. Kinetics of liver polyploidization has been analyzed on liver slice by quantitative fluorescent microscopy, a technique that allows discriminating number of nuclei and relative DNA content. After weaning (day 17 post-birth), the proportion of diploid hepatocytes started to fall significantly, with the successive appearance of binucleated tetraploid and

then also mononucleated tetraploid hepatocytes (Guidotti et al. 2003) (Figure 12).

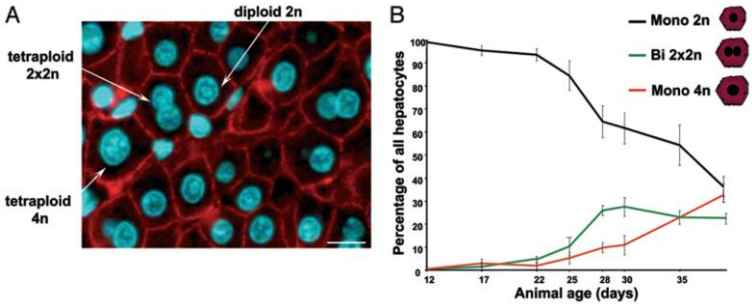

Figure 12 Kinetics of liver binucleation. A) Quantitative fluorescent analysis of DNA stain (blue) allows to discriminate diploid nuclei from tetraploid nuclei. B-catenin stain (red) allows to discriminate mononucleated form binucleated cells. B) Percentage of diploid mononucleated, tetraploid mononucleated and tetraploid binucleated hepatocytes during the first month of development in mouse. From Celton-Morizur et al. 2010

The hepatocyte ploidy levels reaches a plateau at maturity with binucleated and mononucleated octaploid hepatocytes appearing during the second month after birth (Seglen 1997). In the adult healthy liver the percentage of polyploid hepatocytes is variable between mammals. For example 80% of adult rat hepatocytes are polyploid, whereas the average in human is 50% (Seglen et al. 1997, Toyada et al. 2005).

Polyploid hepatocytes in the adult organism are equally distributed in the hepatic lobule, in fact similar proportions of binucleated hepatocytes are present in both periportal and perivenous areas (Margall-Ducos et al. 2007). During the adult age ploidy levels remain stable among the healthy lifespan. However certain pathological conditions increase ploidy levels. In the adult liver hepatocyte turnover occurs slowly in which less than 0.1% of hepatocytes are cycling at any given time (Fausto and Campbell 2003). Although hepatocytes are mostly quiescent in non-injury situations, these cells retain high regenerative potential in response to liver injury. For example, rats regenerate their livers in response to seven sequential 50% partial hepatectomies (Stocker at al. 1973). During the regenerative process after partial hepatectomy quiescent hepatocytes are able to enter in the cell cycle and restore the liver mass in response to mitogenic signals (Michalopoulos 2007). It was demonstrated that during this process the ratio of binucleated cells decreases to less than 5% while there is an increase in tetraploid and octaploid mononucleated hepatocytes (Sigal et al. 1999) (Figure 13). Another pathological condition that leads to an increase in polyploidy is the metabolic overload that induces liver lesions (Gentric at al. 2011). For example

long Evans Cinnamon (LEC) rats, a murine model that shares the clinical characteristics of human Wilson's disease, show excess of iron and cupper in the liver. In this model liver polyploidy is increased and giant mononucleated polyploid hepatocytes are present in the liver mass (Muramatsu et al. 2000, Troadec et al. 2006) (Figure 13). Finally, oxidative damage to the liver is also associated with pronounced increase of polyploid hepatocytes (Gorla et al. 2001) (Figure 13). In line with this data it was demonstrated that overexpression of antioxidant enzymes (i.e. glutathione peroxidise) in transgenic mice decreases hepatocytes ploidy (Nakatani et al. 1997).

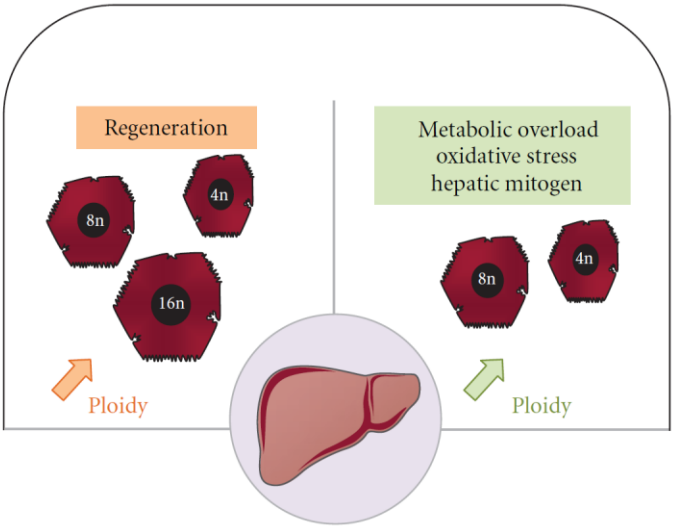

Figure 13 Pathological states such as liver regeneration and metabolic overload/oxidative stress that causes liver lesions are associated with an increase of polyploidy. Mitogenic stimuli are also known to induce an increase of liver polyploidy. From Gentric et al. 2012

2.4 Establishment of liver polyploidy

Since in mammal organisms the liver at the birth is completely composed of mononucleated diploid cells, one of the most fascinating questions is how polyploidization is established during the post-natal development. This issue represented a mystery and only in the last decade some studies have shed light on the cellular and molecular mechanisms. However our

knowledge about liver polyploidization is still poor. Two different cellular mechanisms were identified that contribute to the genesis of polyploid hepatocytes. Initially, cell-cell fusion was identify as principal mechanism. It has been shown that bore-marrow derived hepatocytes transplanted in recipient mice with fumarylacetoacetate hydrolase ($Fah^{-/-}$) deficiency can repopulate the liver and correct their liver disease (Vassilopoulos et al 2003). DNA analysis on repopulating hepatocytes in the liver showed that they were heterozygous for alleles unique to the donor marrow, in contrast to the original homozygous donor cells, indicating homotypic hepatocyte fusion occured (Wang at al 2003). Moreover, rare events of heterotypic cell fusion were also identified in the liver between hepatocytes and myelomonocytic cells, presumably liver resident macrophages known as Kuppfer cells (Wang et al. 2002). Other interesting studies conducted on primary hepatocytes identified failure of cytokinesis as a second mechanism for hepatocytes binucleation. Indeed, cultured hepatocytes isolated from 10 days old rats progressed normally through mitosis with a successfull cytokinesis and gave rise solely to diploid progenies. Conversely, cultured hepatocytes from 25 days old rats did not complete cytokinesis

producing a binucleated progeny cell (Margal-Ducos et al 2007). Moreover characterization of incomplete cytokinesis in-vivo and in cultured hepatocytes showed a defect in actomyosin ring formation and delocalization of the cytokinesis regulators McgRacGAP and RhoA at the equatorial cell cortex (Margal-Ducos et al 2007). The stimuli that drive hepatocytes binucleation were also investigated by different groups. As mentioned above mitogenic stimulation, often mediated by soluble factors, is in relation with the ploidy levels (Michalopoulos 2007). Studies conducted on TGFα overexpressing mice pointed towards the TGFα cytokine as a negative regulator of polyploidization. Indeed, 5 week old mice livers were analysed for ploidy levels by cytofluorimetry. While in normal mice 4n and 8n hepatocytes were prevalent, in transgenic TGFα overexpressing mice 2n and 4n cells were prevalent without an 8n hepatocyte population (Webber et al 1994) (Figure 13). In another interesting paper methimazole (MMI)-induced hypothyroid rats were analysed for polyploidy levels. Two month old MMI treated rats showed lower levels of tetraploid liver nuclei than their corresponding untreated controls (Torres et al. 1999). Moreover, they found that T_3 thyroid hormone injection in rats produces an increase in

tetraploid liver cells (Torres et al. 1999) (Figure 13). These data suggested that T_3 hormone acts as a positive regulator of liver polyploidization. More recent works demonstrated that insulin blood levels are positively related with liver binucleation. In this work selective destruction of pancreatic β cells by streptozotocin in 4 week old mice led to a drastic reduction in binucleated tetraploid hepatocytes (Celton-Morizur et al 2009, 2010). Conversely, increased insulin blood levels stimulated the genesis of binucleated tetraploid hepatocytes. (Celton-Morizur et al 2009, 2010). Insulin stimulated binucleation by activation of the PIP3/Akt signaling pathway regulated the cytokinesis process (Celton-Morizur et al 2009, 2010) (Figure 13). In conclusion, liver polyploidization during the postnatal development seems to be a multifactor process due to soluble factors (cytokines, hormons) involved in proliferation and cell cycle regulation.

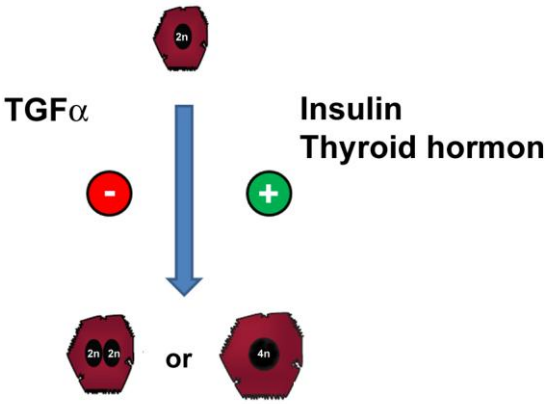

Figure 14 Formation of polyploid hepatocytes during post-natal development. Insulin blood levels and thyroid hormone are positively related to the percentage of tetraploid hepatocytes. Whereas transgenic mice overexpressing TGFα showed a decrease of tetraploid hepatocytes.

2.5 Functions of polyploidy hepatocytes

Polyploidy is not the only feature of hepatocytes. It was shown by FISH that both normal human and mouse liver were composed of aneuploid cells (Faggioli et al. 2011, Duncan et al. 2012). Entire chromosomes were gained and/or lost, and structural rearrangements were rarely seen. Chromosome counts clustered around the expected number for the different ploidy classes: diploid (near 40 chromosomes), tetraploid

(near 80 chromosomes) and octaploid (near 160 chromosomes). Aneuploidy in wild type mice occurred randomly as all chromosomes were affected equally (Duncan et al 2012). Frequency of liver aneuploidy is age-related and it was estimated between 25% in 3 week old mice to 70% in 4-15 month old mice (Duncan et al. 2010). The origin of hepatic aneuploidy was demonstrated injecting octaploid hepatocytes isolated from wild type in the recipient liver. Ploidy levels were examined upon complete liver repopulation by donor hepatocytes. As expected, the liver was repopulated with donor-derived octaploid hepatocytes but also tetraploid and diploid hepatocytes (Duncan et al. 2010). These important experiments demonstrated that hepatocytes were able to undergo ploidy reversal. The ploidy reduction can arise from a cellular division with a successfull cytokinesis, however centrosome number significantly affects the outcome of cell division. The four centrosomes of a binucleated cell orient along three or four distinct poles, forming a multipolar spindle. In a multipolar cell division chromosomes segregate to distinct poles randomly, forming three or four genetically different nuclei (Duncan et al 2010) (Figure 15). Random chromosome distribution would generate highly aneuploid daughters that are

likely non-viable. However, it does not seem the case of the liver in fact live cell imaging shows that progeny resulting from tripolar divisions are, indeed, viable and mitotically active. Moreover multipolar spindles can be transient. Pairs of centrosomes rapidly cluster, leading to the formation of two discrete poles. Nuclear segregation then proceeds in a bipolar manner to generate two tetraploid nuclei. However, mitotic segregation events such as lagging chromosomes are very frequent in this type of cell division and lead to the loss of entire chromosomes (Duncan et al 2010) (Figure 15).

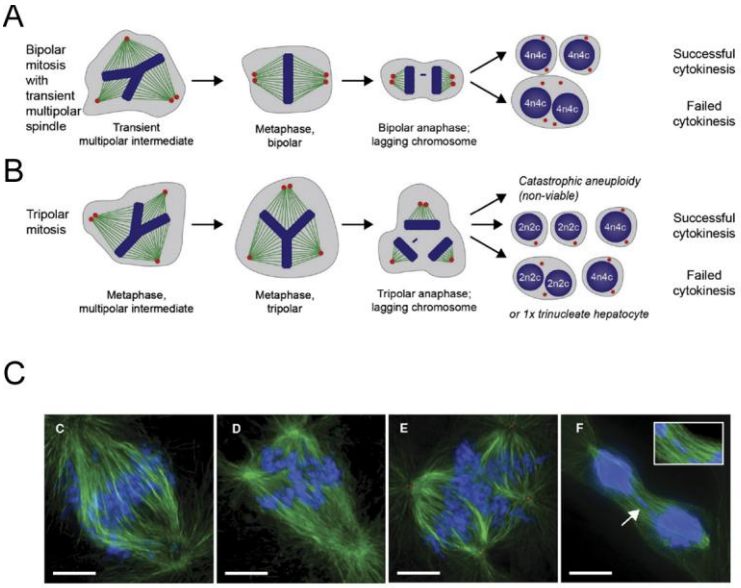

Figure 15 Mechanisms of formation of aneuploid hepatocytes. Proliferative polyploid hepatocytes with extra centrosomes can produce multipolar mitosis. A) A transient multipolar spindle is resolved in a bipolar spindle by centrosome clustering. However, segregation errors such as chromosome lagging are frequent. Subsequently a functional cytokinesis produces aneuploid progeny cells. B) In a multipolar cellular division chromosomes segregate in a random manner, moreover lagging chromosomes can also occurs at high frequency. In both functional and failing cytokinesis aneuploid cells are generated. Examples of multipolar mitosis and segregation errors identified in liver cells. (microtubule: green, DNA: blue, centrosomes: red) c) bipolar mitosis with clustered centrosomes d) tripolar mitosis e) tetrapolar mitosis f) lagging chromosome at late telophase. From Duncan et al 2012.

The function of aneuploid hepatocytes was studied with elegant experiments in Fah−/− mice. This animal model is defective in the tyrosine catabolic pathway. Loss of the FAH gene leads to the accumulation of toxic metabolites that damage the liver and cause death. However, Fah−/− mice can survive if supplemented with 2-(2-nitro-4-trifluoro- methylbenzoyl)-1,3-cyclohexanedione (NTBC) that blocks the pathway upstream of FAH or if they are knocked-out for homegentisic acid dioxygenase (HGD), an enzyme involved in the tyrosine metabolism. Fah−/− Hgd+/− mice were maintained by NTBC supplementation until adulthood, and then the drug was removed. Normally NTBC withdrawal leads to liver failure and death within 2–4 weeks. However after several months of NTBC withdrawal normal liver function was restored. Analysis of healthy liver tissue in rescued mice showed a complete loss of Hgd. Comparative genome hybridization analysis showed that that the liver was repopulated with a chromosome specific aneuploid population: monosomy of chromosome 16, in which the locus Hgd is present (Duncan et al. 2012). In conclusion this excellent experiments lead to the idea that liver polyploidy is a substrate for the development of aneuploid cells. Wide spread aneuploidy is a way to generate genetic variation in a

tissue that is continuously subject to different potential damages such as in the liver. Specific aneuploid injury-resistant clones are selected under the selective pressure of chronic liver damage and they are useful to restore liver functions (Figure 16)

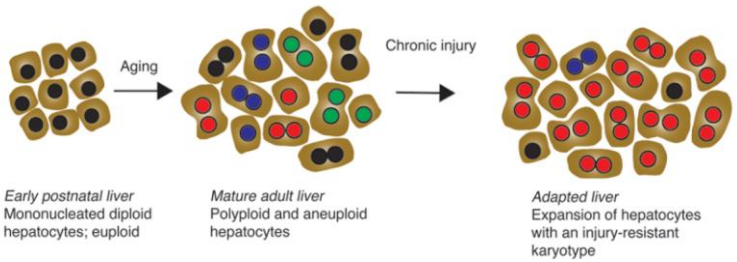

Figure 16 Model for development of liver aneuploidy. At birth the mammalian liver is completely composed of diploid hepatocytes. During the post-natal development and with ageing liver is enriched of polyploid and aneuploid hepatocytes. During chronic injury, specific aneuploid clones are selected to restore the liver functions. Modified from Duncan et al 2012.

3. Relation between EMT/MET and cytokinesis

It is clear from the previous paragraphs, EMT/MET and cytokinesis processes share common effectors that are evolutionary conserved among the species. Indeed RhoA GTPase is necessary in both processes. During EMT/MET, RhoA activity is fundamental in cytoskeleton rearrangements, organization and cell motility. In cytokinesis, RhoA is necessary for cleavage furrow ingression and completion of cytokinesis. Interesting studies in *Drosophila* demonstrated that also the Rho regulatory factors GEF and GAP are implicated in both process. The GEF Pebble (ECT2 mammalian homologue) is required for EMT during the *Drosophila* mesoderm migration. A point mutation in Pebble affects the migration during the EMT without cytokinesis problems (Schumacher et al. 2004). The migration impaired phenotype was rescued by a Pebble truncated protein (missing the amino-terminal BRCT domain) however binucleated cells were formed at high ratio, demonstrating that Pebble/ECT2 is necessary in both EMT and cytokinesis (Smallhorn et al 2004) (Figure 17). As mentioned in the paragraph 2.2 (Routes to polyploidy), RhoA physically interacts at the equatorial cortex

with Ect2, McgRacGAP and the kinesin MKLP1. This tetrameric complex is necessary for completion of cytokinesis in different organisms including fly, worms and mammals (Figure 17). Other studies in the nematode *C.elegans* demonstrate that ZEN-4 (MKLP1 mammalian homologue) and CYK-4 (McgRacGAP mammalian homologue) are necessary in polarizing cells undergoing MET during pharynx morphogenesis (Portereiko et al. 2004) (Figure 17). All these observations lead to the interesting hypothesis that the same molecular machinery is used in distinct ways, but as a similar unit, in cytokinesis and in EMT/MET. Alternatively, regulation of Ect2 and McgGapGAP by phosphorylation may drive the specificity for the processes (Labouesse 2004)

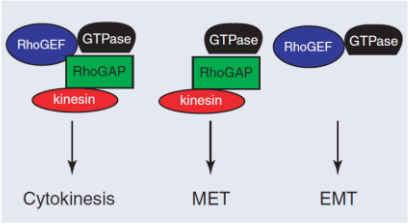

Figure 17 Common effectors in cytokinesis and EMT/MET
From Labouesse 2004

CHAPTER 2. AIM OF THE WORK

Although polyploidy has been described in the liver for over 100 years, hepatocyte polyploidization during the post natal development has remained a mystery in the last century. Only in the last decade few studies have partially investigated this topic indicating the involvement of soluble factors like cytokines, hormones and growth factors as main promoters of liver polyploidization. TGFα, Thyroid hormone and insulin, which have deeply different functions in the organism have been demonstrated *in-vivo* to be involved in the liver polyploidy regulation (Webber et al 1994, Torres et al. 1999, Celton-Morizur et al 2009). Thus, polyploidization seems to be a multifactorial phenomenon and quite difficult to investigate in the whole organism. This work aimed to identify the major stimuli for hepatocyte polyploidization by initially investigating the influence of soluble factors on ploidy levels in cell culture in which treatment conditions can be standardized. We took advantage of MMH-E14, a hepatocyte cellular model developed in our laboratory. Subsequently, results obtained from the hepatocyte cell line culture were translated *in-vivo* in wild-type mice. *In-vivo* analysis was compared with data from

cell culture to assess if our experimental cellular model was representative for the organisms' physiology. Once the stimulus for liver polyploidization was identified, the molecular mechanism(s) through which it acts were also analyzed in the MMH-14 cell line.

CHAPTER 3. MATERIAL AND METHODS

Cellular model

Met Murine Hepatocytes (MMH-E14) are immortalized, non-tumorigenic cells. This cell line was derived from explants of 14 days post-coitus fetal liver obtained from transgenic mice expressing a constitutively active truncated human Met receptor (cyto-Met) under the control of the human α1-antitrypsin transcription unit (Amicone et al. 1997). MMH-E14 cells are polarized, epithelial hepatocytes which express all the hepatic functions. Making use of the MMH-E14 cell model we demonstrated that TGFβ acts as an inducer of EMT also in hepatocytes. TGFβ treated MMH-E14 epithelial cells show typical features normally associated with an epithelial-to-mesenchymal transition such as: a fibroblastoid phenotype, downregulation of membrane adhesion molecules (E-cadherin, ZO-1), downregulation of epithelial differentiation markers (HNFs) and liver function gene expression (Albumin), activation of the master gene of EMT Snail transcription factor (Spagnoli et al. 1998). Furthermore the TGFβ-induced EMT results completely reversible after the cytokine withdrawal,

showing that these cells represent a good model to study EMT-MET dynamics in hepatocytes.

Cell culture and treatments

The non-tumorigenic murine hepatocytes MMH-E14 (Spagnoli et al. 1998) cell line was grown in RPMI (Gibco, USA) medium supplemented with 10% FBS, 10 µg/ml Insulin and 50 ng/ml EGF, 30 ng/ml IGF-2 growth factors, L-glutamine and penicillin/streptomycin on collagen I (Transduction Laboratories, Lexington, UK) coated dishes (Falcon-BD, Franklin Lakes, NJ, USA). Mesothelial Met-5A cells were grown in DMEM (Gibco, USA) supplemented with 10% FBS, L-glutamine and penicillin/streptomycin on plastic dishes. For TGFβ treatments normal medium was supplemented with 10 ng/ml of this cytokine (Preprotech) for 72 h with or without the following inhibitor: 2µM pyrazolopyrimidine PP2 (Calbiochem, Merck Chemicals Ltd. Beeston, Nottingham, UK), 10 µM Src inhibitor, 5 µM LY294002 PI2K inhibitor. For TGFβ withdrawal, medium was removed from 72h TGFβ treated cells and they were washed once with PBS and after normal cell medium was added for further 72h.

Transfection and RNA interference

MMH-E14 cells were transfected with siRNA oligos against mouse Snail (sense 50 -GCUCCUUCGU CCUUCUCCU-30 ; antisense: 50 -AGGAGAAGGACGAAGGACG-30 ; Sigma-Aldrich), or GFP by Lipofectamine 2000 (Invitrogen). After 6 hours of transfection, cells were split and seeded for further treatments.

Analysis of binucleated cells

Cells were washed in PBS and fixed in glacial methanol for 5 min at -20°C. After fixation, samples were stained with 5% Giemsa water solution for 20 min at room temperature, rinsed with water and analyzed for the presence of binucleated cells by optical microscopy.

Immunostaining

Cells were rinsed in PBS, fixed for 10 min with 4% formaldehyde in PBS and permeabilized for 5 min with 0.1% Triton-X100. After fixation, coverslips were blocked in PBS containing 3% bovine serum albumin for 30 min at 37°C, before being processed for immunofluorescence. Antibody

dilution was as follows: anti-RhoA-GTP (NewEast Biosciences 1:400), anti-ECadherin (1:50), anti-Snail (1:50). Secondary antibodies conjugated to Alexa-488 or Alexa-594 (Molecular Probes) were used as recommended by the supplier. DNA was counterstained with 0.1 μg/ml 4'-6'-Diamidino-2-phenylindole (DAPI, Sigma). All stained sample were analyzed under a Nikon Microphot-FXA microscope equipped with a CCD camera and digital images were acquired with Nikon NIS-elements software.

Fluorescence microscopy and image analysis

All preparations were examined under an Nikon Eclipse fluorescent microscope equipped with a 20X (0.9 NA) or 40X (1.35 NA) oil immersion objective and a CCD camera (Nikon Inc.) equipped with a specific filter set. Colour-encoded images were acquired using NIS elements software (Nikon Inc.). Midbody structures and cell morphology were identified and analyzed by phase contrast image.

In-vivo time-lapse video microscopy

TGFβ treated or untreated MMH-14 cells were seeded on a glass-button collagen I coated micro-dish (Ibidi, Germany) and

after 48 h were mounted on a micro-incubator stage (OKO Lab, Italy) at 37°C and 5% CO2 for time-lapse analysis. Time-lapse video microscopy was performed using a Nikon Ti Eclipse microscope equipped with a 40X PlanFluor objective starting from cells holding single nuclei . Differential interference contrast images were acquired every 8 min for 24 h. Image series acquired were analyzed with NIS Elements (Nikon) and ImageJ (rsbweb.nih.gov/ij/) softwares.

Flow Cytometry Analysis

Cells were fixed in glacial ethanol for 5 min, washed twice with PSB and finally stained with 0.1 µg/µl propidium iodide (PI) for 10 min at 37°C. Ten thousand events were acquired for each sample using a FACStar Plus fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) flow cytometer and analyzed by FACS WinMDI software. The amplification scale was linear for FSC-H and FL2-A parameters. Photomultiplier tension was set as to place the peak corresponding to 2C DNA content (G0/G1) at channel 200 in the FL2-H histogram. Cell aggregates were carefully gated out using FL2-W versus FL2-A bivariant graphs.

SDS–PAGE and Western blots

Cells were lysed in RIPA buffer containing protease inhibitors (Roche, Monza, IT). Protein concentrations were determined with the Bio-Rad assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by SDS–PAGE and transferred to nitrocellulose membranes (Millipore, MA, USA). Blots were blocked in 5% non-fat milk prepared in TBST and incubated overnight with the primary antibody. Antibodies against phospho-Src (Tyr416) (Cell Signaling, 1:400) and α-tubulin (Santa Cruz Biotechnology, CA, USA, 1:1000) were used. Blots were then incubated with HRP-conjugated species-specific secondary antibodies (Bio-Rad, Hercules, CA, USA), followed by enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL).

RNA extraction, reverse transcription and RT–qPCR

Total RNA was extracted with NucleoSpin® RNA II kit (Macherey–Nagel, Germany) and reverse transcribed with MMLV-reverse-transcriptase (Promega, MI, Italy). cDNA was amplified by qPCR reaction using BioRad Miniopticon with KAPA SYBR® Green FAST qPCR mix

(KAPABIOSYSTEMS, Woburn, MA, USA), and the following specific primers: mouse *Mmp9* forward 5′-CGCTACCACCTCGAACTTTG-3′ and reverse 5′-GCCATTCACGTCGTCCTTAT-3′; mouse *e-cadherin* forward 5′-ATGCCCCGGAACTCCTTTTC-3′ and reverse 5′-CAACAGGCAGGCAGCTTTAT-3′; mouse *snail* forward 5′-ATGGCTTCTCATCGTCTGCT-3′ and reverse 5′-GCTCCTCATTCCTTGGGATT-3′; mouse *HNF4α* forward 5′-ATCTTCTTTGATCCAGATGCCA -3′ and reverse 5′-GTTGATGTAATCCTCCAGGC-3′

Animal care and treatment and sample preparation.

Animal care and experiments were carried out according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' of the National Academy of Sciences, published by the National Institutes of Health (NIH publication 86–23 revised 1985). Wild-type C57BL6 mice (Charles-River Laboratory) were treated by LY2109761 (Cat. S2740, Selleckchem) on the days 18-32 post-birth twice daily at the dose of 100 mg/Kg body weight. LY2109761 in 1% carboxymethyl cellulose (CMC) UPS (Sigma) was orally administeed by gavage with specific needle and sham-treated

mice received 1% CMC UPS as a control group. At day 33 post-birth the mice ware sacrificed by cervical dislocation and livers were collected and embedded in paraffin. Livers were sliced (7µm) by a microtome and attached to polarized glass slides.

Hematoxylin eosin staining on liver tissue slices

Liver tissue slices were deparaffinized by two xylene washes for 10 min and rehydrated by 5 min washes with an alcohol series (100%, 95%, 80%, 70% and 50% ethanol). Samples were rinsed in distilled water and stained in 1% Harris Hematoxylin (Sigma) for 6 min. Samples were then rinsed in running tap water for 20 min and quickly destained in acid alcohol, rinsed in tap water for 5 min and immersed in Lithium Carbonate for 3 sec. Slides were rinsed in tap water for 5 min and counterstained with 1% Eosin (Sigma). Finally, slides were dehydrated by 3 min washes in 95% and 100% ethanol.

Ki-67 immunostaining on liver tissue slices

Liver tissue slices were deparaffinized by two xylene washes for 10 min and rehydrated by 5 min washes of 100%, 95%, 80%, 70% and 50% ethanol. Antigen retrieval was performed

in citrate buffer (10mM Sodium citrate, 0.05% Tween20, pH=6) for 20 min at 100°C. Liver slices were permeabilized with 0.2% Triton X-100 for 5 min and blocked in 10% Bovine Serum Albumin in PBS for 30 min at 37°C. After the blocking phase, slices were incubated with mouse monoclonal anti-Ki67 antibody (1:25, BD Bioscience Pharmigen, CA, USA) in 1% BSA overnight. Samples were washed 3 times in PBS for 5 min and incubated with the anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen, CA, USA). Samples were washed 3 time in PBS and counterstained with 0.1 µg/ml 4,6-diamino-2-phenylindole (Sigma) for 5 min at room temperature. Slices were mounted with 70% glycerol and analyzed. Ten randomly chosen different fields were digitally acquired for each mouse and the number of Ki-67 positive cells/ number of total cells were manually counted with Adobe Photoshop software.

CHAPTER 4. RESULTS

TGFβ induces cytokinesis failure and binucleation in MMH-E14 cells

To investigate a possible involvement of soluble factors in liver polyploidization we selected 6 cytokines and growth factors known to be involved in liver development and/or in mass liver control: EGF, IGF2, FGF1, FGF-2, Wnt3a and TGFβ. MMH-E14 cells were treated for 72h , giemsa stained and analyzed for binucleated cells content (Figure 18A). We decided to limit our investigation to the cells with two different nuclei, even if binucleated cell analysis represents an underestimation of polyploid cells and polyploid mononucleates may also form. This because it is virtually impossible to discriminate a tetraploid G1 cell cycle phase from a diploid G2 cell cycle phase by both microscopy analysis and propidium iodide incorporation. We found that TGFβ induces binucleated cells at a high ratio versus the untreated cells (23,3±3% for TGFβ- treated versus 2,1±1,8% for untreated, $P<0,01$) (Figure 18B and 18C). Moreover, TGFβ treatment of MMH-E14 cells produces a morphological change

in which cells acquire spindle shape morphology (Figure 18C). In fact we have already shown that TGFβ triggers EMT in MMH-E14 (Spagnoli et al. 1999, 2000). In order to assess the hepatospecificity of TGFβ-induced binucleation, we perform the same treatment on a non -tumorigenic mesothelial cell line Met-5A. Also these cells are well known to undergo in EMT in response to TGFβ stimulation. However, TGFβ stimulation did not induce binucleated cells in Met-5A (Figure 18D). This data suggest that the TGFβ-induced binucleation may have a tissue specific basis conceivably triggered by different downstream pathways.

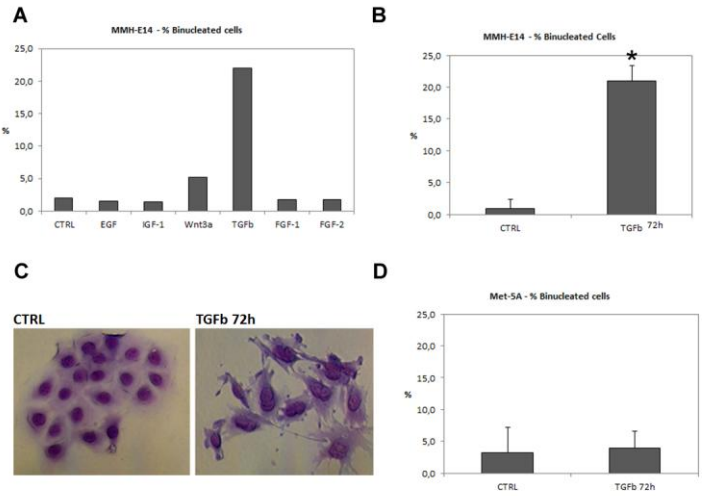

Figure 18. Analysis of binucleation in MMH-E14 cells. (A) Percentage of binucleated cells after 72 h treatment with EGF, IGF2, FGF1, FGF-2, Wnt3a and TGFβ. (mean ± s.e.m. of 4 independent experiments) (B) Percentage of binucleates after 72h TGFβ treatment (mean ± s.e.m. of 4 indipendent experiments) (* $P(\chi^2)<0.01$). (C) Acquisition of mononucleated ephitelial untreated cells andbinucleated spindle cell shape after 72 h treatment with TGFβ. Magnification 20x (D) Percentage of binucleates after TGFβ-treatment in Met-5A cells (mean ± s.e.m. of 3 independent experiments).

Two different mechanisms have been suggested to contribute to binucleation in hepatocytes: the cell-cell fusion and cytokinesis failure (Duncan et al. 2009, Margall-Ducos et al. 2007) . In order to analyze which mechanism was involved in

the production of binucleated cells during TGFβ stimulation we performed in-vivo time lapse video microscopy. The last 24 hours of a 72 hours TGFβ treatment of single mononucleated cells have been recorded. Video analysis revealed that TGFβ treated cells were able to progress into the different stages of mitosis with kinetics comparable to untreated cells. However, the latest cytokinesis stage was incomplete and a regression of the cleavage furrow was observed. Cytokinesis failure produced a binucleated cell at the next G1 cell cycle phase (Figure 19). As mentioned in the introduction, it is well know that abortive cytokinesis may arise from structural defects such as chromatin trapping in the cleavage furrow zone. This type of cytokinesis failure often produces a strong delay in the mitosis outcome. Cells may progress in the next G1 phase after a long block in cytokinesis stage due to Cyclin B degradation. In our experiment, comparable mitosis kinetics within untreated and TGFβ treated cells suggest that TGFβ-induced cytokinesis failure is a specific cell division program rather than an abortive mitosis.

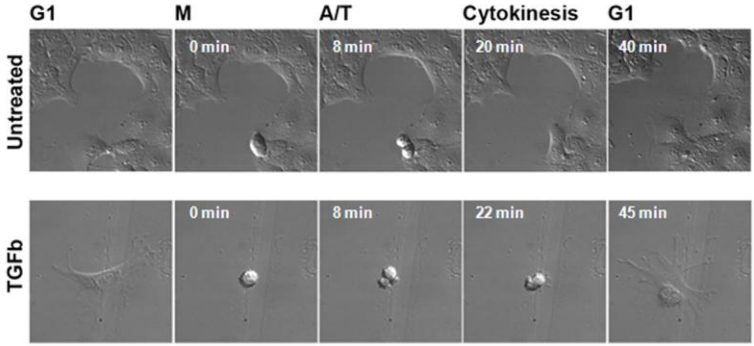

Figure 19. In-vivo time lapse video microscopy in the last 24 hours of 72 hours TGFβ treatment (lower panels) starting from single mononucleated cells compared to untreated controls (upper panels). Differential interference contrast was acquired and kinetic of mitosis was monitoring starting from metaphase plate formation. Magnification 40X

Inhibition of TβR class I and II prevent binucleation in mice

To validate data obtained in our experimental cellular model as representative of the organisms' physiology, we analyzed the role of TGFβ in liver polyploidization in C57BL6 mice by pharmacological inhibition of the TGFβ receptor (TβR). LY2109761 is a novel specific inhibitor of both TβR class I and II. This inhibitor can be orally administrated; moreover, pharmacodynamics/pharmacokinetics and toxicity data are well

known (Melisi et al. 2008; Zhang et al. 2011, Flechsig et al. 2012). Consistently with previously reported data about the weaning timeframe for liver binucleation (Guidotti et al. 2003), LY2109761 was in-vivo administered during the days 18-32 post-birth twice daily at the dose of 100 mg/Kg body weight (Figure 20).

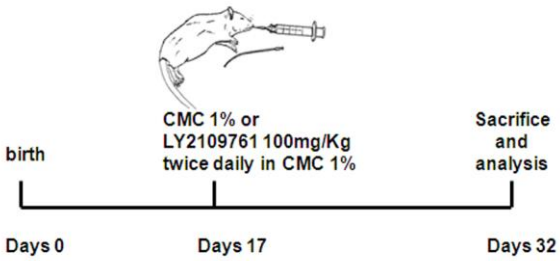

Figure 20. Schematic representation of the in-vivo treatment schedule in C57BL6-mice. 17days after birth mice were treated with 1% CMC (sham-treated group) or 100 mg/kg body weight LY2109761in 1% CMC. Mice were sacrificed 32 days after birth. CMC, carboxymethyl cellulose.

Four mice were treated with LY2109761 dissolved in 1% carboxymethyl cellulose (CMC) and other four mice received 1% CMC as a control group. At the 32nd day mice were sacrificed and liver tissue slices were scored for binucleated cells by microscopical analysis after hematoxylin and eosin

staining. LY2109761 treated mice showed a highly significant decrease of binucleated cells versus the sham-treated mice (8,7 ±0,23 %versus 26,9 ± 0,99 % respectively; P<0,01) (Figure 21). TGFβ-mediated cytokinesis failure observed in the MMH-E14 cell line requires cellular proliferation. To assess a possible side effect of LY2109761 on the hepatocytes proliferation we performed immunostaining against Ki-67 on the liver sections. It is well known that the Ki-67 antigen is expressed during all cell cycle phases but it is not present during the G0 phase. Ki-67 positive cells in sham-treated mice were found to be comparable to Ki-67 positive cells in LY2109761-treated mice (Figure 22). The proliferative index was around 1%, which is the value reported in literature for wild type mice. Overall these data indicate that decrease in binucleization ratio in LY2109761 treated mice is due to direct effect of TβR inhibition rather than inhibition of liver cells proliferation. Moreover, ploidy response to TGFβ cell line is representative of the organism' physiology.

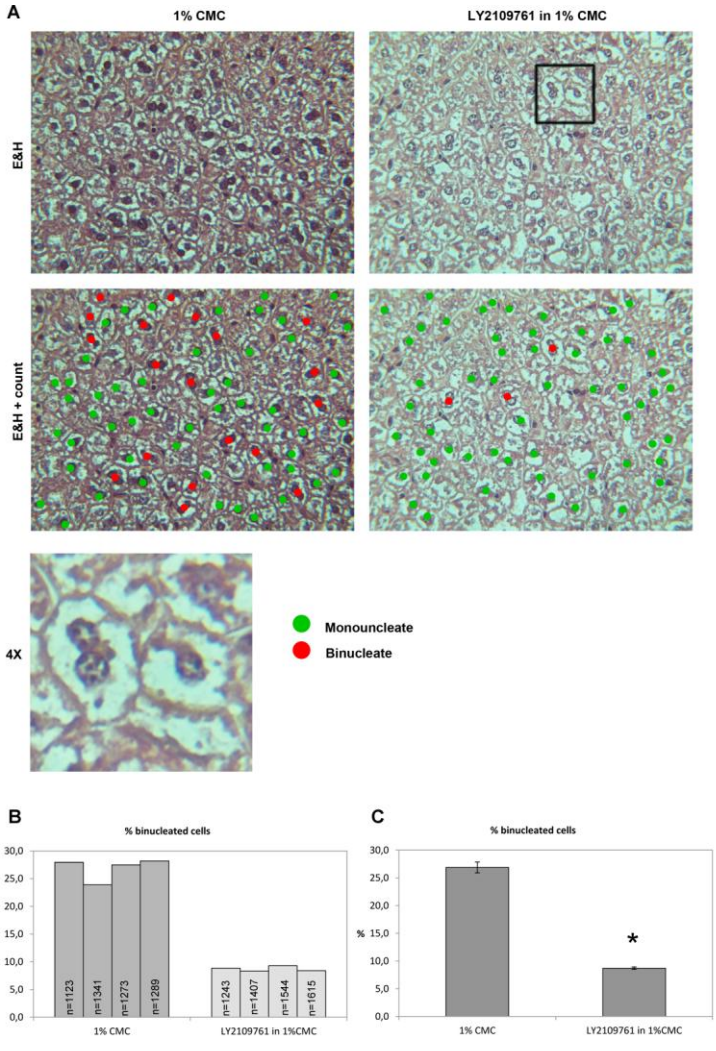
A
1% CMC
LY2109761 in 1% CMC
E&H
E&H + count
4X
Mononucleate
Binucleate
B
% binucleated cells
30,0
25,0
20,0
% 15,0
10,0
5,0
0,0
n=1123
n=1341
n=1273
n=1289
n=1243
n=1407
n=1544
n=1615
1% CMC
LY2109761 in 1%CMC
C
% binucleated cells
30,0
25,0
20,0
% 15,0
10,0
5,0
0,0
*
1% CMC
LY2109761 in 1%CMC

Figure 21. Analysis of binucleated cells in liver tissue sections after eosin and hematoxylin (E&H) staining. (A) Microscopical images of liver tissue sections of sham-treated mice (1% CMC) (left panels) and mice treated with LY2109761 in 1% CMC (TβR inhibitor) (right panels). Green dots represent mononucleates, red dots represent binucleates. Magnification 20X (B) Percentage of binucleated cells per mouse. N corresponds to the number of cells analyzed. (C) Average of the percentage of binucleated cells per treatment group (* $P(\chi^2)<0.01$).

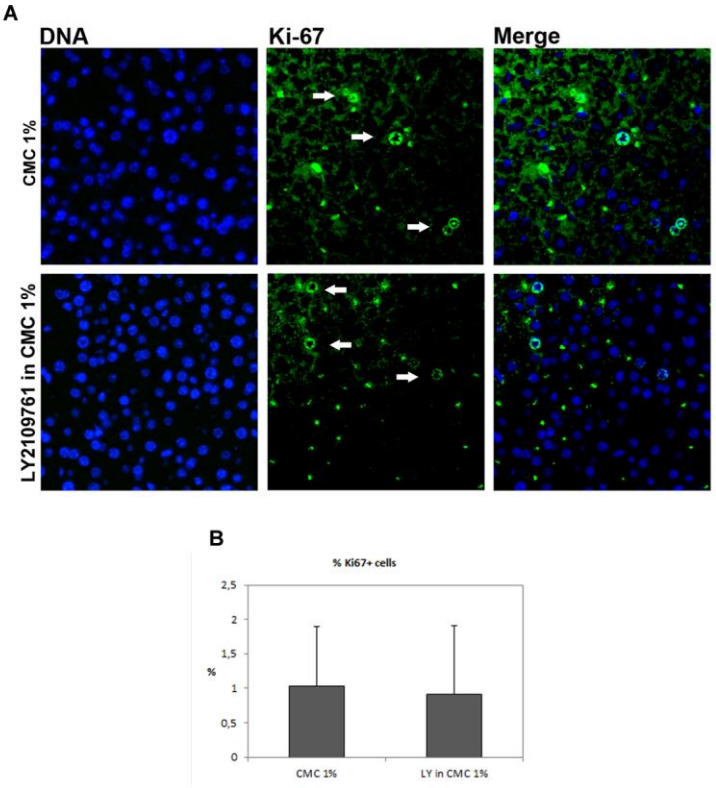

Figure 22. (A) Analysis of proliferation in liver tissue sections of sham-treated mice (1% CMC) (upper panels) and mice treated with LY2109761 in 1% CMC (TβR inhibitor) (lower panels). White arrows indicates Ki-67 positive cells. Magnification 20X(B) Percentage of Ki67-positive cells and (C) average number of cells per field in liver tissue sections of sham-treated mice (1% CMC) and mice treated with LY2109761 in 1% CMC.

TGFβ mediated binucleation is Src kinase dependent

In order to analyze which TGFβ downstream pathways were involved in binucleation we used pharmacological inhibition of two kinases, known to be activated from TGFβ, PI3K and Src. To this purpose, double treated TGFβ / LY294002 (PI3K inhibitor) and TGFβ / PP2 (Src inhibitor) cells were analyzed for binucleated cell formation. While inhibition of PI3K does not affect the ratio of binucleated cells, inhibition of Src kinase largely rescued the binucleated phenotype observed in TGFβ treated cells (Figure 23A). At the dose of 2μM PP2 was able to inhibit Src activity. Western blot analysis of TGFβ-, PP2- and double –treated cells demonstrated a clear decrease in Src phosphorylation at tyrosin 416 after double treatment compared to TGFβ-treatment alone(Figure 23B and 23D). Double band showed in Figure 23B is due to cross reaction of the antibody to different isoforms of the same protein as mentioned in the product datasheet and in Yamanashi et al 1989. As mentioned above, cytokinesis failure requires cell proliferation. In order to analyze possible side effects of Src inhibition on cellular proliferation, cells were counted at the end of each treatment. 72 h TGFβ/PP2 treated cells showed

comparable cell numbers of 72 h TGFβ treated cells (Figure 23C).

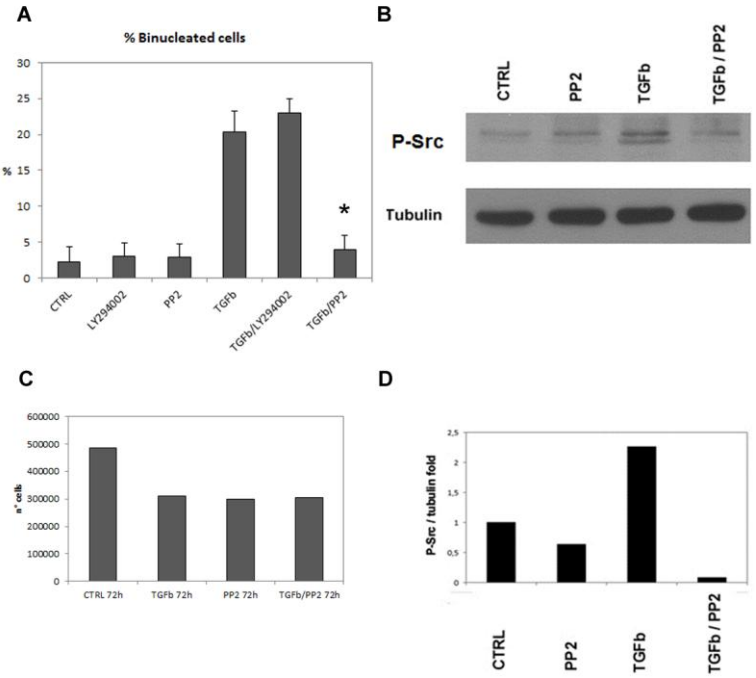

Figure 23. (A) Percentage of binucleated cells after treatment with LY294002 (PI3K inhibitor), PP2 (Src inhibitor), TGFβ, LY294002/ TGFβ and PP2/ TGFβ compared to untreated controls.(mean ± s.e.m of 3 independent experiment) (*TGFβ versus TGFβ/PP2 $P(\chi^2)<0.01$) (C) Cell number after PP2-, TGFβ- and PP2/TGFβ-treatment compared to untreated controls. (B, D) Levels of phosphorylation of Src at Tyr416 in

PP2-, TGFβ- and PP2/TGFβ-treated cells compared to untreated controls.

These data suggest the involvement of Src kinase in TGFβ-dependent binucleation. As said, a parallel effect of TGFβ stimulation in MMH-E14 cells is the triggering of EMT. Activation of Smad dependent TGFβ downstream pathways leads to EMT process. However, other non-Smad pathways may contribute to EMT. To investigate on relation between binucleation and the EMT process, characterization of the cell populations upon different treatments was performed by qPCR analysis. . Gene expression analysis of control cells as expected revealed that E-cadherin and HNF4 (epithelial markers) are downregulated while Snail and metalloproteinase 9 (MMP9) (mesenchymal markers) are upregulatedupon TGFβ treatment.. TGFβ and the Src inhibitor PP2 double treated cells showed a similar transcript profile. (Figure 24).

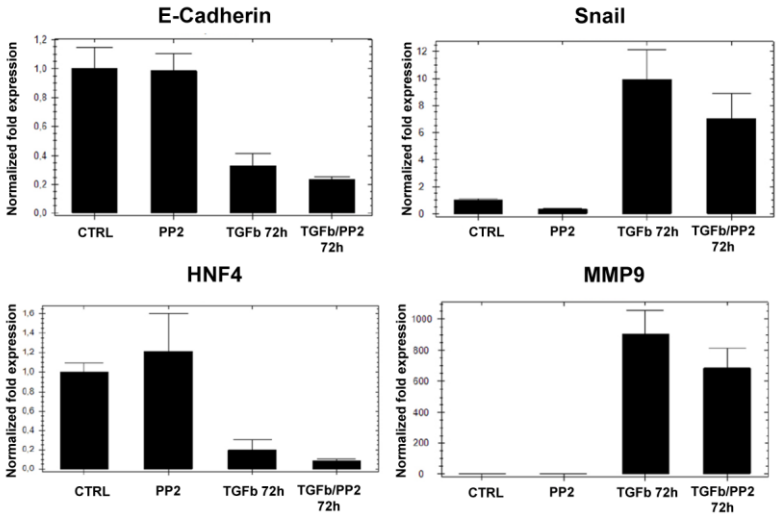

Figure 24. Gene expression analysis of epithelial markers, E-Cadherin and HNF4, and mesenchymal markers, Snail and MMP9 after treatment for 72 h with PP2, TGFβ and PP2/TGFβ. Expression levels are normalized against the housekeeping gene L32.

Immunostaining against E-cadherin in untreated, TGFβ-, PP2-, and double-treated cells reveals an E-cadherin switch between untreated and TGFβ treated cells. As in TGFβ-treated cells, E-cadherin is also delocalized from the plasma membrane in double-treated cells with TGFβ / PP2 . Moreover no difference of E-cadherin localization was found between mononucleated and binucleated cells, indicating that both cellular

subpopulations are able to undergo in EMT (Figure 25). Taken all together, this data indicate that Src activity is not required for EMT while it is necessary for the binucleation process

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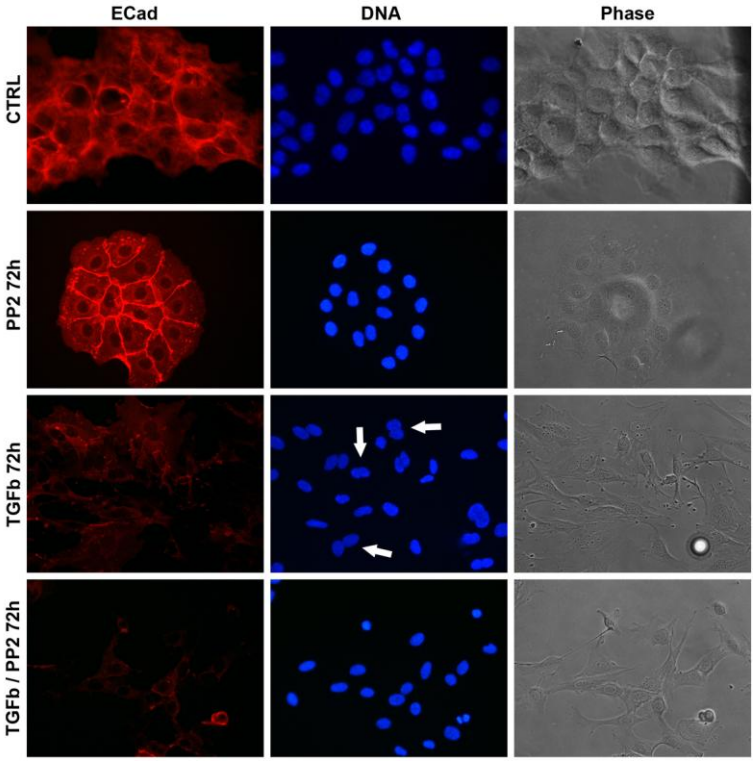


Figure 25. Localization of E-Cadherin (red) in PP2-, TGFβ- and PP2/ TGFβ double-treated cells (left panels). Arrows indicate binucleate cells. DAPI DNA staining is shown in the middle panels. Phase contrast images are shown in the right panels. Magnification 20X.

Binucleation is associated with RhoA GTPase activity

RhoA GTPase is one of the well known non-Smad downstream effectors of TGFβ that is responsible for actin cytoskeleton rearrangements. As mentioned in the introduction RhoA may be differently regulated in different subcellular compartments and was shown to be activated by the Src kinase. Moreover, it is known to be necessary for cytokinesis completion, acting at the midbody structure. In order to investigate the involvement of RhoA in TGFβ-mediated binucleation the localization of the active GTP bound RhoA was analyzed by fluorescence microscopy during cytokinesis. In these experiments cells in cytokinesis and subcellular mid-body structures were identify by phase contrast microscopy. TGFβ treated cells showed a higher percentage of delocalization of RhoA-GTP from the midbody structure against the control cells. Conversely, the ratio of delocalization in double treated TGFβ/PP2 cells was comparable to the untreated cells (Figure 26). These data suggest that RhoA activity at the midbody is Src dependent and it is associated with binucleation in MMH-14 cells.

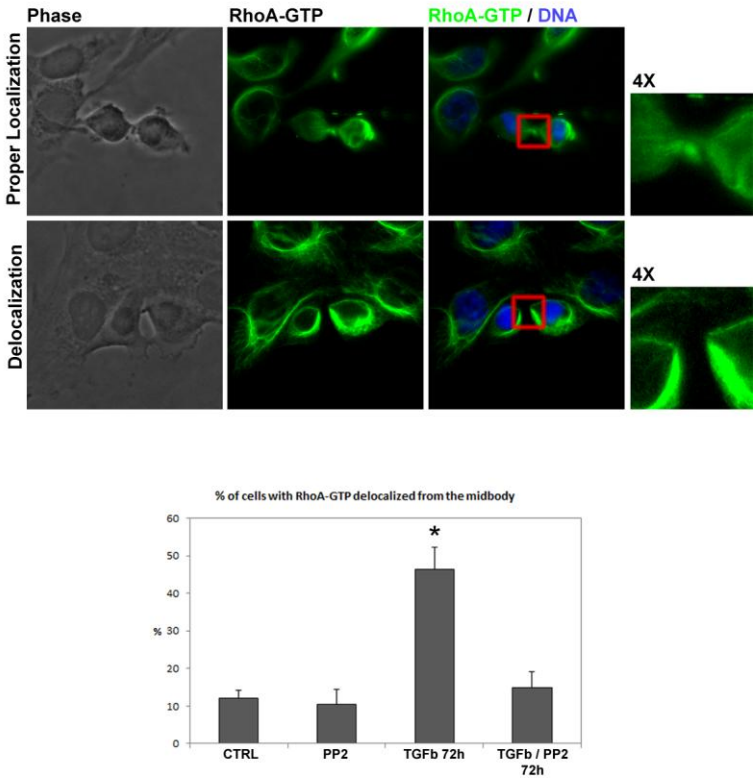


Figure 26. Localization of Rho A-GTP at the midbody structure. The upper panels show proper localization while the lower panels demonstrate delocalization of Rho A-GTP (green) from the midbody. Nuclei are stained with DAPI (blue). The graph represents the percentage of cells with Rho A-GTP delocalization after treatment during 72 h with PP2, TGFβ and PP2/TGFβ (mean ± s.e.m of 3 independent experiments) (*CTRL versus TGFβ $P(\chi^2)<0.01$).

Snail-induced EMT is necessary for binucleation

As mentioned in the introduction, the Snail transcription factor is the master gene of the EMT process and is well known to be induced by TGFβ. To assess the relation between EMT and binucleation, genetic knockout of Snail by RNA interference has been performed in TGFβ treated cells. Green fluorescent proteins (GFP) RNA interference was used as a control. Preliminary data demonstrated that after 72 h of TGFβ stimulation, Snail-RNAi cells showed a decrease in the formation of binucleated cells compared to the TGFβ treated cells (6,6 % versus 19,3 % respectively). However, a slight decrease has also been observed in TGFβ treated GFP RNAi cells. (Figure 27A). Efficiency of RNA interference was assessed by the determination of Snail protein levels. Western blot analysis of TGFβ / Snail RNAi cells demonstrated a decrease in Snail compared to TGFβ-treated and TGFβ / GFP RNAi transfection control cells (Figure 27 B). Gene expression analysis of TGFβ / Snail RNAi cells revealed that E-cadherin is upregulated compared to the TGFβ –treated and TGFβ / GFP RNAi. Moreover, TGFβ / Snail RNAi cells showed an epithelial gross morphology (data not shown), indicating that

this cell population is epithelial and that it did not undergo EMT despite the TGFβ treatment. These preliminary data may suggest that the EMT process is a mandatory step for TGFβ-mediated binucleation, however, further analysis are necessary.

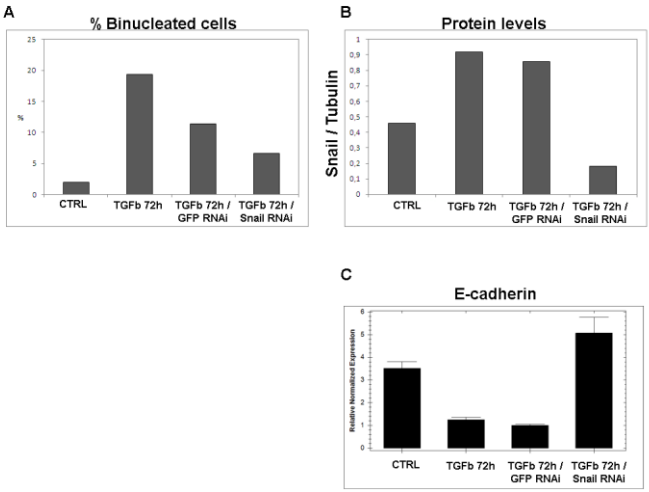
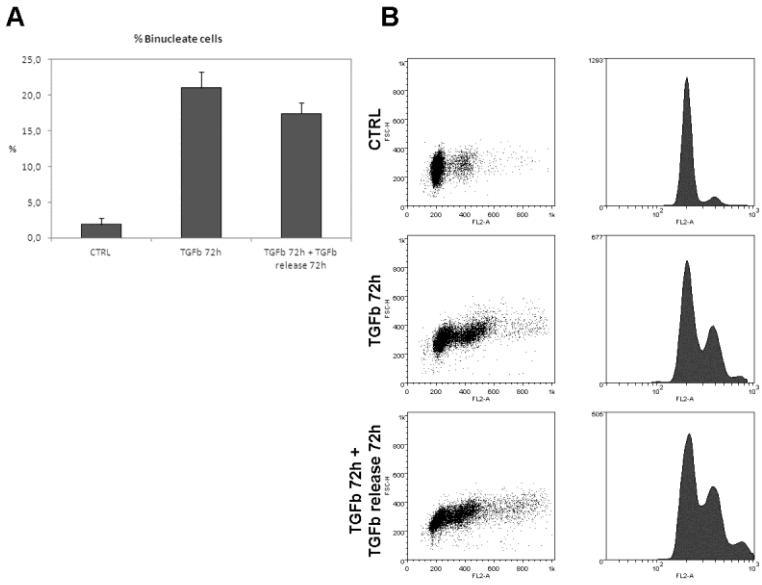


Figure 27 (A) Percentage of binucleated cells after 72h TGFβ treatment in Snail RNAi and GFP RNAi transfection control compared to TGFβ treated cells and untreated cells. (B) Dosimetry of western blot analysis of Snail transcriptional factor in TGFβ, TGFβ/Snail RNAi and TGFβ/GFP RNAi compared to untreated control cells. (D) Gene expression analysis of epithelial marker, E-Cadherin after treatment for 72 h with TGFβ in Snail RNAi and GFP RNAi cells. Expression levels are normalized against the housekeeping gene L32.

TGFβ withdrawal increases ploidy grade in MMH-E14 cells

It was shown that primary polyploid hepatocytes spontaneously reduce their ploidy grade in-vitro, a phenomenon called "ploidy conveyor" (Duncan et al. 2010). In our experimental system TGFβ works as inducer of polyploidy. We investigated ploidy changes after TGFβ withdrawal. MMH-E14 cells were treated with TGFβ for 72 h and they were released for further 72 h followed by the analysis of binucleation and ploidy. Ploidy was assessed by cytofluorimetric analysis of propidium iodide incorporation TGFβ released cells showed a decrease in ratio of binucleated cells compared to the TGFβ treated cells. However, a significant percentage of binucleated cells is still retained in this population (17,4 ± 1,5%) (Figure 28A). In TGFβ-released cells an increased population with 4C DNA content (tetraploid) versus the untreated cells was seen. The 4C DNA content peak corresponds to both diploid G2 phase and tetraploid G1 cell cycle phase, that are undistinguishable by flow cytometry. Moreover, PI incorporation highlighted the appearance of a population with 8C DNA content (octaploid)

(10,4 %) that is virtually absent in both untreated and TGFβ-treated cells (Figure 28B and 27C).



C

Treatment	% Cells with DNA content near 8C
CTRL	0,92
TGFb 72h	2,83
TGFb 72h + TGFb release 72h	10,1

Figure 28. (A) Percentage of binucleated cells after treatment with TGFβ and TGFβ withdrawal compared to untreated controls. (mean ± s.e.m of 3 independent experiment). (B) Cytofluorimetric analysis of PI incorporation in TGFβ and TGFβ withdrawal compared to untreated controls. (C)

Quantification of subpopulation with DNA content near 8C for the experiment shown in “B”

Characterization of the epithelial and mesenchymal phenotype was also performed. mRNA levels of the whole population showed upregulation of the ephitelial marker E-cadherin and downregulation of the mesenchymal marker Snail in TGFβ-released cells compared to TGFβ-treated cells (Figure 29).

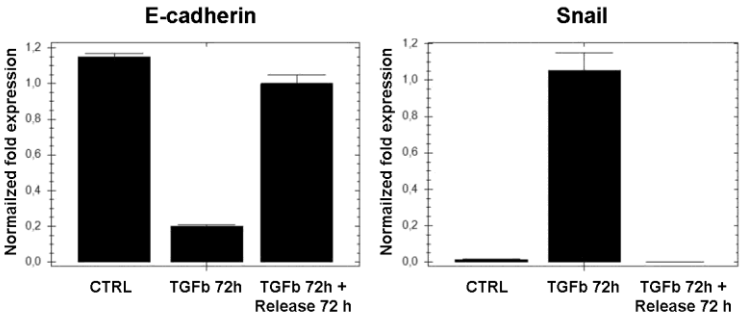
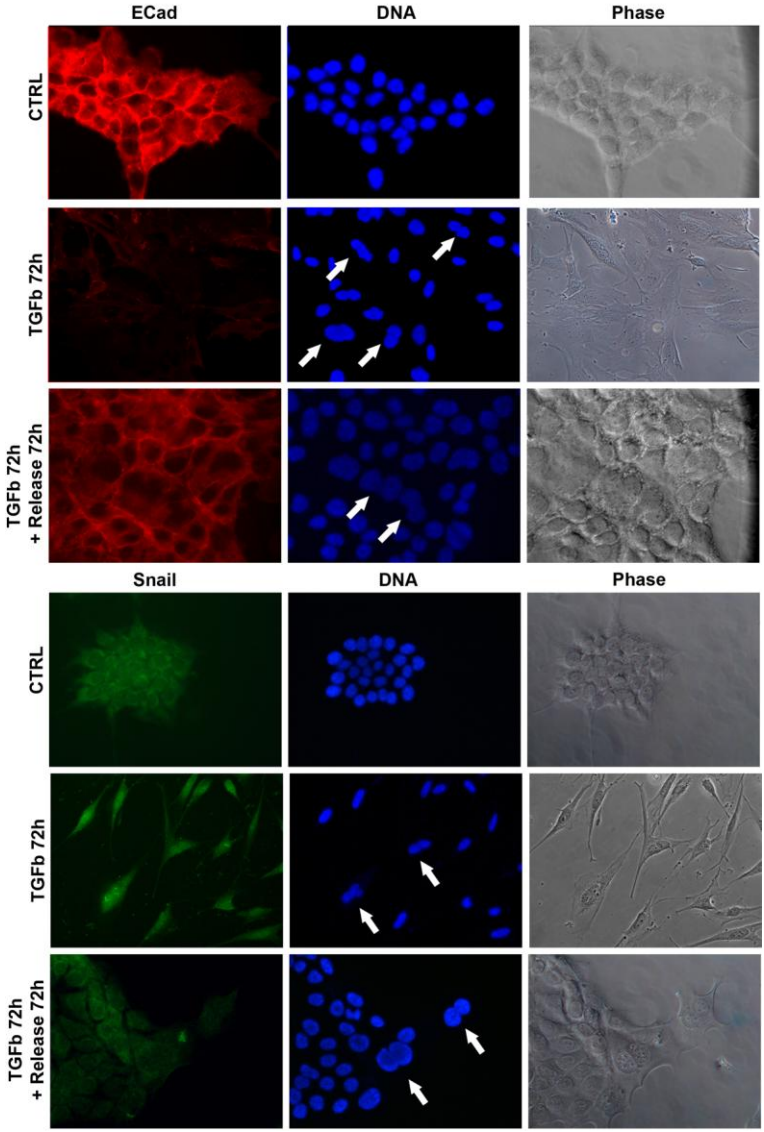


Figure 29. Gene expression analysis of E-Cadherin and Snail in TGFβ-treated and TGFβ-released cells. Expression levels are normalized for the housekeeping gene L32.

This gene expression data indicated that TGFβ-released cells are epithelial cells. This observation was confirmed by immunostaining analysis of E-cadherin and Snail transcription

factors. In TGFβ-released cells E-cadherin localizes to the plasma membrane and Snail nuclear stain was negative. Moreover, phase contrast micrographs showed epithelial gross morphology. No difference in E-cadherin localization and Snail nuclear stain was found between the mononucleated and binucleated cellular subpopulations (Figure 30).

Figure 30. Localization of E-Cadherin (upper panels) and Snail (lower panels) in TGFβ-treated and TGFβ-released cells. Arrows indicate binucleated cells. Magnification 20X


ECad
DNA
Phase
CTRL
TGFb 72h
TGFb 72h
+ Release 72h
Snail
DNA
Phase
CTRL
TGFb 72h
TGFb 72h
+ Release 72h

These data suggested that MMH-E14 cells pretreated with TGFβ are committed to polyploidy and they can proliferate increasing ploidy grade. Moreover, pretreatment of TGFβ generates a polyploidy full epithelial hepatocytes population.

CHAPTER 5. DISCUSSION

The aim of this study was to indentify the major stimulus inducing liver polyploidization and to elucidate the pathways involved in the regulation of this physiological process. We collected evidence indicating TGFβ to be a major inducer of polyploidization in hepatocytes. We showed, in fact, that this cytokine induces binucleation in the hepatocyte cell line and that chemical inhibition of its receptor impairs the physiological hepatocyte polyploidization *in vivo.* Thus in our cellular and animal systems TGFβ seems to be the main stimulus for hepatocyte binucleation (Figure 31). The MMH-E14 cell line recapitulates the organisms' physiology. Data obtained with a non-hepatic epithelial cell line (i.e. mesothelium), moreover, indicates this is a largely tissue-specific effect of TGFβ.

TGFβ is a pleiotropic cytokine that regulates proliferation, differentiation, adhesion, migration, and other functions in many cell types. The role of TGFβ as a main inducer of EMT in a large variety of epithelial cells, including hepatocytes, is well known. In the liver, its role has been well described during the regeneration process when it is released

from activated hepatic stellate cells (Kalluri and Weinberg 2009, Xue et al 2013). TGFβ paracrine stimulation induces a partial EMT in hepatocytes, useful for cell migration and organ remodelling, and appears essential for the control of liver mass. Afterwards transitional hepatocytes can undergo MET producing new healthy tissue (Kalluri and Weinberg 2009, Xue et al 2013). The regeneration process, as mentioned in the introduction, is also associated with increased ploidy levels (Gentric et al. 2012). Nevertheless, nothing has been known about the role of TGFβ in the hepatocytes polyploidization, that physiologically occurs during the first month after birth. Here, we demonstrated this role and, in addition, we revealed i) cytokinesis failure as the main mechanism used by TGFβ to induce hepatocyte polyploidization, ii) a Src-dependent signalling as the molecular mechanism that hepatocytes use to transduce the cytokine signal, iii) the delocalization of the GTPase RhoA from the mid-body during last phase of the hepatocyte mitosis as a probable main cause of cytokinesis failure and iv) the EMT as an instrumental process for polyploidization (Figure 31). Particularly intriguing is the correlation between EMT/MET and polyploidization because it could reveal a novel process in which transdifferentiation

events of hepatocytes are involved. Our preliminary data regarding interference of the EMT master gene Snail indicate that the EMT process is mandatory in TGFβ- mediated hepatocyte binucleation.

Several literature data are in line with this hypothesis. RhoA is a common effector of EMT/MET and cytokinesis. During cytokinesis RhoA forms a complex with the GEF Ect2, the GAP McgRacGAP and with the kinesin MKLP1. During EMT RhoA was found to be associated with the Ect2 while during MET it is associated with McgRacGAP and MKLP1. It was hypothesized that the same molecular machinery is used in distinct ways in cytokinesis and EMT/MET (Labousse et al. 2004). Thus, the relation between cytokinesis failure and EMT/MET may be found in the regulation of these shared regulatory units (GAP and GEF). In fact, it is well known that activities of both Ect2 and McgRacGAP are regulated by phosphorylation (Tatsumoto et al. 1999, Minoshima et al. 2003). Differential phosphorylation states could present a possible mechanism to enable regulation of both distinct processes, EMT/MET and cytokinesis. The hepato-specificity of TGFβ-mediated binucleation was analyzed by stimulating with the same cytokine the mesothelial non tumorigenic cell

line Met-5A. In this cell line, TGFβ treatment does not induce binucleation demonstrating hepatospecificity. We speculate that also the hepatospecificity may be a consequence of differential regulation of the main complex RhoA/Ect2/McgRacGAP in different cell types.

By TGFβ withdrawal we were able to obtain a full epithelial cellular population. These cells, however, have increased levels of tetraploid and octaploid classes compared to the mesenchymal TGFβ-treated cells, together with the presence of binucleated and mononucleated giant cells (Figure 31). In this scenario TGFβ stimulation uses EMT/MET processes as intermediate steps towards a polypoid status (Figure 31). This hypothesis is in line with the role that EMT/MET plays in building and modeling tissues.

Recent excellent studies have demonstrated that liver polyploidy represents a substrate for the development of aneuploid cells and increased genetic variability. Subsequently, specific aneuploid injury-resistant clones are selected under the selective pressure of chronic liver damage (Duncan et al 2010, 2012) whereas in all other solid tissues polyploidy has been associated to a pre-transformed state (Storchova and Pellman 2004). Intrinsic numerical chromosome instability (CIN), due

to the extra centrosome number in tetraploid cells, triggers the formation of a wide-spread aneuploidy that is associated with tumor progression and development of a more aggressive phenotype (Godhino et al 2009). Indeed, aneuploidy is a hallmark of solid tumors (Rajagopalan and Lengauer 2004). Under this point of view, liver polyploidy/aneuploidy represent a paradox. During the development of hepatocellular carcinomas (HCC), a growth shift to a non-polyploidizing pattern and expansion of the diploid hepatocytes population has been observed in neoplastic nodules (Seglen, 1997). Moreover, it has been shown that heterozygous TbR-II mice have increased susceptibility to HCC (Im at al. 2001) and that foci of transformed hepatocytes *in vivo* express low or null rate of TGFβ receptor (Amicone et al 2002) This data suggested that whereas the liver polyploid fraction is functional after liver injury, unregulated TGFβ-mediated ploidy changes could contribute to tumorigenesis. We believe that this work and further analysis about TGF-mediated hepatocyte ploidy changes could contribute not only to gaining insight in liver physiology but also to a better understanding of hepatocarcinoma formation and progression.

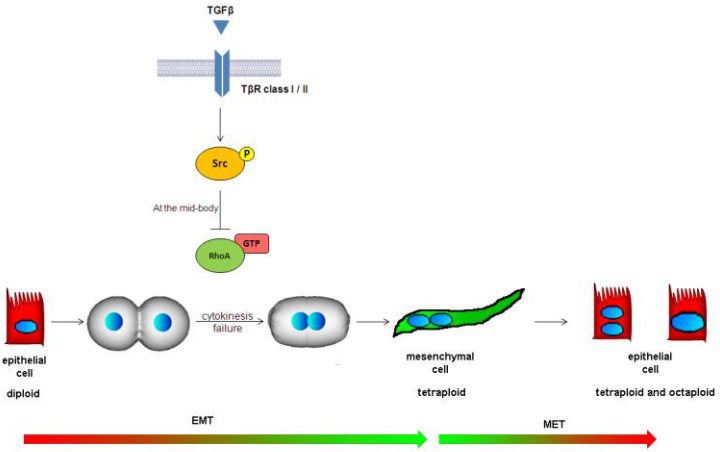


Figure 31 Proposed model for TGFβ-dependent binucleation and polyploidization in hepatocytes.

REFERENCES

Adams, D. H. and B. Eksteen (2006). Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel disease. Nat Rev Immunol 6(3): 244-51.

Amicone, L., F. M. Spagnoli, G. Spath, S. Giordano, C. Tommasini, S. Bernardini, V. De Luca, C. Della Rocca, M. C. Weiss, P. M. Comoglio and M. Tripodi (1997). Transgenic expression in the liver of truncated Met blocks apoptosis and permits immortalization of hepatocytes. EMBO J 16(3): 495-503.

Amicone L, Terradillos O, Calvo L, Costabile B, Cicchini C, Della Rocca C, Lozupone F, Piacentini M, Buendia MA, Tripodi M (2002) Synergy between truncated c-Met (cyto-Met) and c-Myc in liver oncogenesis: importance of TGF-beta signalling in the control of liver homeostasis and transformation.Oncogene 21(9):1335-45

Bhowmick, N. A., M. Ghiassi, A. Bakin, M. Aakre, C. A. Lundquist, M. E. Engel, C. L. Arteaga and H. L. Moses (2001). Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell 12(1): 27-36.

Brodsky, W. Y., A. M. Arefyeva and I. V. Uryvaeva (1980). Mitotic polyploidization of mouse heart myocytes during the first postnatal week. Cell Tissue Res 210(1): 133-44.

Canman, J. C., D. B. Hoffman and E. D. Salmon (2000). The role of pre- and post-anaphase microtubules in the cytokinesis phase of the cell cycle. Curr Biol 10(10): 611-4.

Carmena, M., S. Ruchaud and W. C. Earnshaw (2009). Making the Auroras glow: regulation of Aurora A and B kinase

function by interacting proteins. Curr Opin Cell Biol 21(6): 796-805.

Celton-Morizur, S. and C. Desdouets (2009). [Cellular polyploidy in hepatic tissue: new role of insulin]. Med Sci (Paris) 25(6-7): 651-3.

Celton-Morizur, S. and C. Desdouets (2010). Polyploidization of liver cells. Adv Exp Med Biol 676: 123-35.

Celton-Morizur, S., G. Merlen, D. Couton and C. Desdouets (2010). Polyploidy and liver proliferation: central role of insulin signaling. Cell Cycle 9(3): 460-6.

Celton-Morizur, S., G. Merlen, D. Couton, G. Margall-Ducos and C. Desdouets (2009). The insulin/Akt pathway controls a specific cell division program that leads to generation of binucleated tetraploid liver cells in rodents. J Clin Invest 119(7): 1880-7.

Chen, R. H., Y. H. Su, R. L. Chuang and T. Y. Chang (1998). Suppression of transforming growth factor-beta-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway. Oncogene 17(15): 1959-68.

Cho, H. J., K. E. Baek, S. Saika, M. J. Jeong and J. Yoo (2007). Snail is required for transforming growth factor-beta-induced epithelial-mesenchymal transition by activating PI3 kinase/Akt signal pathway. Biochem Biophys Res Commun 353(2): 337-43.

Choi, S. S. and A. M. Diehl (2009). Epithelial-to-mesenchymal transitions in the liver. Hepatology 50(6): 2007-13.

Comai, L. (2005). The advantages and disadvantages of being polyploid. Nat Rev Genet 6(11): 836-46.

Duncan, A. W., A. E. Hanlon Newell, W. Bi, M. J. Finegold, S. B. Olson, A. L. Beaudet and M. Grompe (2012). Aneuploidy as a mechanism for stress-induced liver adaptation. J Clin Invest 122(9): 3307-15.

Duncan, A. W., A. E. Hanlon Newell, L. Smith, E. M. Wilson, S. B. Olson, M. J. Thayer, S. C. Strom and M. Grompe (2012). Frequent aneuploidy among normal human hepatocytes. Gastroenterology 142(1): 25-8.

Duncan, A. W., R. D. Hickey, N. K. Paulk, A. J. Culberson, S. B. Olson, M. J. Finegold and M. Grompe (2009). Ploidy reductions in murine fusion-derived hepatocytes. PLoS Genet 5(2): e1000385.

Duncan, A. W., M. H. Taylor, R. D. Hickey, A. E. Hanlon Newell, M. L. Lenzi, S. B. Olson, M. J. Finegold and M. Grompe (2010). The ploidy conveyor of mature hepatocytes as a source of genetic variation. Nature 467(7316): 707-10.

Edlund, S., S. Y. Lee, S. Grimsby, S. Zhang, P. Aspenstrom, C. H. Heldin and M. Landstrom (2005). Interaction between Smad7 and beta-catenin: importance for transforming growth factor beta-induced apoptosis. Mol Cell Biol 25(4): 1475-88.

Eggert, U. S., T. J. Mitchison and C. M. Field (2006). Animal cytokinesis: from parts list to mechanisms. Annu Rev Biochem 75: 543-66.

Ehrlich, M., D. Horbelt, B. Marom, P. Knaus and Y. I. Henis (2011). Homomeric and heteromeric complexes among TGF-beta and BMP receptors and their roles in signaling. Cell Signal 23(9): 1424-32.

Eliades, A., N. Papadantonakis and K. Ravid (2010). New roles for cyclin E in megakaryocytic polyploidization. J Biol Chem 285(24): 18909-17.

Faggioli, F., P. Vezzoni and C. Montagna (2011). Single-cell analysis of ploidy and centrosomes underscores the peculiarity of normal hepatocytes. PLoS One 6(10): e26080.

Fausto, N. and J. S. Campbell (2003). The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 120(1): 117-30.

Fausto, N., J. S. Campbell and K. J. Riehle (2006). Liver regeneration. Hepatology 43(2 Suppl 1): S45-53.

Flechsig, P., M. Dadrich, S. Bickelhaupt, J. Jenne, K. Hauser, C. Timke, P. Peschke, E. W. Hahn, H. J. Grone, J. Yingling, M. Lahn, U. Wirkner and P. E. Huber (2012). LY2109761 attenuates radiation-induced pulmonary murine fibrosis via reversal of TGF-beta and BMP-associated proinflammatory and proangiogenic signals. Clin Cancer Res 18(13): 3616-27.

Fuchs, B. C., T. Fujii, J. D. Dorfman, J. M. Goodwin, A. X. Zhu, M. Lanuti and K. K. Tanabe (2008). Epithelial-to-mesenchymal transition and integrin-linked kinase mediate sensitivity to epidermal growth factor receptor inhibition in human hepatoma cells. Cancer Res 68(7): 2391-9.

Gentric, G., S. Celton-Morizur and C. Desdouets (2012). Polyploidy and liver proliferation. Clin Res Hepatol Gastroenterol 36(1): 29-34.

Gentric, G., C. Desdouets and S. Celton-Morizur (2012). Hepatocytes polyploidization and cell cycle control in liver physiopathology. Int J Hepatol 2012: 282430.

Germain, L., M. J. Blouin and N. Marceau (1988). Biliary epithelial and hepatocytic cell lineage relationships in embryonic rat liver as determined by the differential expression of cytokeratins, alpha-fetoprotein, albumin, and cell surface-exposed components. Cancer Res 48(17): 4909-18.

Godinho, S. A., M. Kwon and D. Pellman (2009). Centrosomes and cancer: how cancer cells divide with too many centrosomes. Cancer Metastasis Rev 28(1-2): 85-98.

Gorla, G. R., H. Malhi and S. Gupta (2001). Polyploidy associated with oxidative injury attenuates proliferative potential of cells. J Cell Sci 114(Pt 16): 2943-51.

Grunert, S., M. Jechlinger and H. Beug (2003). Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol 4(8): 657-65.

Guidotti, J. E., O. Bregerie, A. Robert, P. Debey, C. Brechot and C. Desdouets (2003). Liver cell polyploidization: a pivotal role for binuclear hepatocytes. J Biol Chem 278(21): 19095-101.

Gupta, S. (2000). Hepatic polyploidy and liver growth control. Semin Cancer Biol 10(3): 161-71.

Gurtner, G. C., S. Werner, Y. Barrandon and M. T. Longaker (2008). Wound repair and regeneration. Nature 453(7193): 314-21.

Hartsough, M. T. and K. M. Mulder (1995). Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells. J Biol Chem 270(13): 7117-24.

Hugo, H., M. L. Ackland, T. Blick, M. G. Lawrence, J. A. Clements, E. D. Williams and E. W. Thompson (2007). Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. J Cell Physiol 213(2): 374-83.

Im, Y. H., H. T. Kim, I. Y. Kim, V. M. Factor, K. B. Hahm, M. Anzano, J. J. Jang, K. Flanders, D. C. Haines, S. S. Thorgeirsson, A. Sizeland and S. J. Kim (2001). Heterozygous mice for the transforming growth factor-beta type II receptor gene have increased susceptibility to hepatocellular carcinogenesis. Cancer Res 61(18): 6665-8.

Kaimori, A., J. Potter, J. Y. Kaimori, C. Wang, E. Mezey and A. Koteish (2007). Transforming growth factor-beta1

induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro. J Biol Chem 282(30): 22089-101.

Kalluri, R. (2009). EMT: when epithelial cells decide to become mesenchymal-like cells. J Clin Invest 119(6): 1417-9.

Kalluri, R. and R. A. Weinberg (2009). The basics of epithelial-mesenchymal transition. J Clin Invest 119(6): 1420-8.

Kamijo, K., N. Ohara, M. Abe, T. Uchimura, H. Hosoya, J. S. Lee and T. Miki (2006). Dissecting the role of Rho-mediated signaling in contractile ring formation. Mol Biol Cell 17(1): 43-55.

Kretzschmar, M., J. Doody, I. Timokhina and J. Massague (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. Genes Dev 13(7): 804-16.

Kurinna, S., S. A. Stratton, Z. Coban, J. M. Schumacher, M. Grompe, A. W. Duncan and M. C. Barton (2013). p53 regulates a mitotic transcription program and determines ploidy in normal mouse liver. Hepatology 57(5): 2004-13.

Labouesse, M. (2004). Epithelium-mesenchyme: a balancing act of RhoGAP and RhoGEF. Curr Biol 14(13): R508-10.

Lamouille, S. and R. Derynck (2007). Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J Cell Biol 178(3): 437-51.

Lamouille, S. and R. Derynck (2010). Emergence of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin axis in transforming growth factor-beta-induced epithelial-mesenchymal transition. Cells Tissues Organs 193(1-2): 8-22.

Lapham, L. W. (1968). Tetraploid DNA content of Purkinje

neurons of human cerebellar cortex. Science 159(3812): 310-2.

Lilly, M. A. and R. J. Duronio (2005). New insights into cell cycle control from the Drosophila endocycle. Oncogene 24(17): 2765-75.

Maeda, M., Y. Shintani, M. J. Wheelock and K. R. Johnson (2006). Src activation is not necessary for transforming growth factor (TGF)-beta-mediated epithelial to mesenchymal transitions (EMT) in mammary epithelial cells. PP1 directly inhibits TGF-beta receptors I and II. J Biol Chem 281(1): 59-68.

Margall-Ducos, G., S. Celton-Morizur, D. Couton, O. Bregerie and C. Desdouets (2007). Liver tetraploidization is controlled by a new process of incomplete cytokinesis. J Cell Sci 120(Pt 20): 3633-9.

Matsuura, I., G. Wang, D. He and F. Liu (2005). Identification and characterization of ERK MAP kinase phosphorylation sites in Smad3. Biochemistry 44(37): 12546-53.

Melendez, J., K. Stengel, X. Zhou, B. K. Chauhan, M. Debidda, P. Andreassen, R. A. Lang and Y. Zheng RhoA GTPase is dispensable for actomyosin regulation but is essential for mitosis in primary mouse embryonic fibroblasts. J Biol Chem 286(17): 15132-7.

Melisi, D., S. Ishiyama, G. M. Sclabas, J. B. Fleming, Q. Xia, G. Tortora, J. L. Abbruzzese and P. J. Chiao (2008). LY2109761, a novel transforming growth factor beta receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. Mol Cancer Ther 7(4): 829-40.

Meserve, J. H. and R. J. Duronio (2012). Atypical E2Fs drive atypical cell cycles. Nat Cell Biol 14(11): 1124-5.

Minoshima, Y., T. Kawashima, K. Hirose, Y. Tonozuka, A.

Kawajiri, Y. C. Bao, X. Deng, M. Tatsuka, S. Narumiya, W. S. May, Jr., T. Nosaka, K. Semba, T. Inoue, T. Satoh, M. Inagaki and T. Kitamura (2003). Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. Dev Cell 4(4): 549-60.

Morillo, S. M., P. Escoll, A. de la Hera and J. M. Frade (2010). Somatic tetraploidy in specific chick retinal ganglion cells induced by nerve growth factor. Proc Natl Acad Sci U S A 107(1): 109-14.

Moustakas, A. and C. H. Heldin (2007). Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 98(10): 1512-20.

Mulder, K. M. and S. L. Morris (1992). Activation of p21ras by transforming growth factor beta in epithelial cells. J Biol Chem 267(8): 5029-31.

Muramatsu, Y., T. Yamada, D. H. Moralejo, H. Mochizuki, K. Sogawa and K. Matsumoto (2000). Increased polyploid incidence is associated with abnormal copper accumulation in the liver of LEC mutant rat. Res Commun Mol Pathol Pharmacol 107(1-2): 129-36.

Nakatani, T., M. Inouye and O. Mirochnitchenko (1997). Overexpression of antioxidant enzymes in transgenic mice decreases cellular ploidy during liver regeneration. Exp Cell Res 236(1): 137-46.

Nieto, M. A. (2009). Epithelial-Mesenchymal Transitions in development and disease: old views and new perspectives. Int J Dev Biol 53(8-10): 1541-7.

Nishimura, Y. and S. Yonemura (2006). Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. J Cell Sci 119(Pt 1): 104-14.

Otto, S. P. (2007). The evolutionary consequences of

polyploidy. Cell 131(3): 452-62.

Ozdamar, B., R. Bose, M. Barrios-Rodiles, H. R. Wang, Y. Zhang and J. L. Wrana (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science 307(5715): 1603-9.

Pagan, R., I. Martin, M. Llobera and S. Vilaro (1997). Epithelial-mesenchymal transition of cultured rat neonatal hepatocytes is differentially regulated in response to epidermal growth factor and dimethyl sulfoxide. Hepatology 25(3): 598-606.

Pagan, R., A. Sanchez, I. Martin, M. Llobera, I. Fabregat and S. Vilaro (1999). Effects of growth and differentiation factors on the epithelial-mesenchymal transition in cultured neonatal rat hepatocytes. J Hepatol 31(5): 895-904.

Papadimitriou, E., D. Kardassis, A. Moustakas and C. Stournaras (2011). TGFbeta-induced early activation of the small GTPase RhoA is Smad2/3-independent and involves Src and the guanine nucleotide exchange factor Vav2. Cell Physiol Biochem 28(2): 229-38.

Papadimitriou, E., E. Vasilaki, C. Vorvis, D. Iliopoulos, A. Moustakas, D. Kardassis and C. Stournaras (2012). Differential regulation of the two RhoA-specific GEF isoforms Net1/Net1A by TGF-beta and miR-24: role in epithelial-to-mesenchymal transition. Oncogene 31(23): 2862-75.

Paulk, N. K., K. Wursthorn, A. Haft, C. Pelz, G. Clarke, A. H. Newell, S. B. Olson, C. O. Harding, M. J. Finegold, R. L. Bateman, J. F. Witte, R. McClard and M. Grompe (2012). In vivo selection of transplanted hepatocytes by pharmacological inhibition of fumarylacetoacetate hydrolase in wild-type mice. Mol Ther 20(10): 1981-7.

Peinado, H., D. Olmeda and A. Cano (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 7(6): 415-28.

Peinado, H., M. Quintanilla and A. Cano (2003). Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. J Biol Chem 278(23): 21113-23.

Piekny, A., M. Werner and M. Glotzer (2005). Cytokinesis: welcome to the Rho zone. Trends Cell Biol 15(12): 651-8.

Portereiko, M. F., J. Saam and S. E. Mango (2004). ZEN-4/MKLP1 is required to polarize the foregut epithelium. Curr Biol 14(11): 932-41.

Rahimi, R. A. and E. B. Leof (2007). TGF-beta signaling: a tale of two responses. J Cell Biochem 102(3): 593-608.

Rajagopalan, H. and C. Lengauer (2004). Aneuploidy and cancer. Nature 432(7015): 338-41.

Ravid, K., J. Lu, J. M. Zimmet and M. R. Jones (2002). Roads to polyploidy: the megakaryocyte example. J Cell Physiol 190(1): 7-20.

Remy, I., A. Montmarquette and S. W. Michnick (2004). PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. Nat Cell Biol 6(4): 358-65.

Savagner, P. (2001). Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 23(10): 912-23.

Schumacher, S., T. Gryzik, S. Tannebaum and H. A. Muller (2004). The RhoGEF Pebble is required for cell shape changes during cell migration triggered by the Drosophila FGF receptor Heartless. Development 131(11): 2631-40.

Seglen, P. O. (1997). DNA ploidy and autophagic protein degradation as determinants of hepatocellular growth and survival. Cell Biol Toxicol 13(4-5): 301-15.

Shen, X., J. Li, P. P. Hu, D. Waddell, J. Zhang and X. F. Wang (2001). The activity of guanine exchange factor NET1 is essential for transforming growth factor-beta-mediated stress fiber formation. J Biol Chem 276(18): 15362-8.

Shiojiri, N., J. M. Lemire and N. Fausto (1991). Cell lineages and oval cell progenitors in rat liver development. Cancer Res 51(10): 2611-20.

Sigal, S. H., P. Rajvanshi, G. R. Gorla, R. P. Sokhi, R. Saxena, D. R. Gebhard, Jr., L. M. Reid and S. Gupta (1999). Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events. Am J Physiol 276(5 Pt 1): G1260-72.

Si-Tayeb, K., F. P. Lemaigre and S. A. Duncan Organogenesis and development of the liver. Dev Cell 18(2): 175-89.

Smallhorn, M., M. J. Murray and R. Saint (2004). The epithelial-mesenchymal transition of the Drosophila mesoderm requires the Rho GTP exchange factor Pebble. Development 131(11): 2641-51.

Soeda, S., Y. Nakayama, T. Honda, A. Aoki, N. Tamura, K. Abe, Y. Fukumoto and N. Yamaguchi (2013). v-Src causes delocalization of Mklp1, Aurora B, and INCENP from the spindle midzone during cytokinesis failure. Exp Cell Res 319(10): 1382-97.

Song, K., H. Wang, T. L. Krebs and D. Danielpour (2006). Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. Embo J 25(1): 58-69.

Spagnoli, F. M., L. Amicone, M. Tripodi and M. C. Weiss (1998). Identification of a bipotential precursor cell in hepatic cell lines derived from transgenic mice expressing cyto-Met in the liver. J Cell Biol 143(4): 1101-12.

Spagnoli, F. M., C. Cicchini, M. Tripodi and M. C. Weiss

(2000). Inhibition of MMH (Met murine hepatocyte) cell differentiation by TGF(beta) is abrogated by pre-treatment with the heritable differentiation effector FGF1. J Cell Sci 113 (Pt 20): 3639-47.

Steigemann, P., C. Wurzenberger, M. H. Schmitz, M. Held, J. Guizetti, S. Maar and D. W. Gerlich (2009). Aurora B-mediated abscission checkpoint protects against tetraploidization. Cell 136(3): 473-84.

Stocker, E., H. K. Wullstein and G. Brau (1973). [Capacity of regeneration in liver epithelia of juvenile, repeated partially hepatectomized rats. Autoradiographic studies after continous infusion of 3H-thymidine (author's transl)]. Virchows Arch B Cell Pathol 14(2): 93-103.

Storchova, Z., A. Breneman, J. Cande, J. Dunn, K. Burbank, E. O'Toole and D. Pellman (2006). Genome-wide genetic analysis of polyploidy in yeast. Nature 443(7111): 541-7.

Storchova, Z. and D. Pellman (2004). From polyploidy to aneuploidy, genome instability and cancer. Nat Rev Mol Cell Biol 5(1): 45-54.

Tatsumoto, T., X. Xie, R. Blumenthal, I. Okamoto and T. Miki (1999). Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. J Cell Biol 147(5): 921-8.

Taub, R. (2004). Liver regeneration: from myth to mechanism. Nat Rev Mol Cell Biol 5(10): 836-47.

Taylor, M. V. (2002). Muscle differentiation: how two cells become one. Curr Biol 12(6): R224-8.

Thiery, J. P., H. Acloque, R. Y. Huang and M. A. Nieto (2009). Epithelial-mesenchymal transitions in development and disease. Cell 139(5): 871-90.

Torres, S., B. P. Diaz, J. J. Cabrera, J. C. Diaz-Chico, B. N. Diaz-Chico and A. Lopez-Guerra (1999). Thyroid

hormone regulation of rat hepatocyte proliferation and polyploidization. Am J Physiol 276(1 Pt 1): G155-63.

Toyoda, H., O. Bregerie, A. Vallet, B. Nalpas, G. Pivert, C. Brechot and C. Desdouets (2005). Changes to hepatocyte ploidy and binuclearity profiles during human chronic viral hepatitis. Gut 54(2): 297-302.

Troadec, M. B., B. Courselaud, L. Detivaud, C. Haziza-Pigeon, P. Leroyer, P. Brissot and O. Loreal (2006). Iron overload promotes Cyclin D1 expression and alters cell cycle in mouse hepatocytes. J Hepatol 44(2): 391-9.

Ullah, Z., M. J. Kohn, R. Yagi, L. T. Vassilev and M. L. DePamphilis (2008). Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity. Genes Dev 22(21): 3024-36.

Valdes, F., A. M. Alvarez, A. Locascio, S. Vega, B. Herrera, M. Fernandez, M. Benito, M. A. Nieto and I. Fabregat (2002). The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes. Mol Cancer Res 1(1): 68-78.

Vassilopoulos, G., P. R. Wang and D. W. Russell (2003). Transplanted bone marrow regenerates liver by cell fusion. Nature 422(6934): 901-4.

Vignery, A. (2000). Osteoclasts and giant cells: macrophage-macrophage fusion mechanism. Int J Exp Pathol 81(5): 291-304.

Wang, X., E. Montini, M. Al-Dhalimy, E. Lagasse, M. Finegold and M. Grompe (2002). Kinetics of liver repopulation after bone marrow transplantation. Am J Pathol 161(2): 565-74.

Wang, X., H. Willenbring, Y. Akkari, Y. Torimaru, M. Foster, M. Al-Dhalimy, E. Lagasse, M. Finegold, S. Olson and M.

Grompe (2003). Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422(6934): 897-901.

Webber, E. M., J. C. Wu, L. Wang, G. Merlino and N. Fausto (1994). Overexpression of transforming growth factor-alpha causes liver enlargement and increased hepatocyte proliferation in transgenic mice. Am J Pathol 145(2): 398-408.

Winkelmann, M., P. Pfitzer and W. Schneider (1987). Significance of polyploidy in megakaryocytes and other cells in health and tumor disease. Klin Wochenschr 65(23): 1115-31.

Xue, Z. F., X. M. Wu and M. Liu Hepatic regeneration and the epithelial to mesenchymal transition. World J Gastroenterol 19(9): 1380-6.

Yi, J. Y., I. Shin and C. L. Arteaga (2005). Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. J Biol Chem 280(11): 10870-6.

Yuce, O., A. Piekny and M. Glotzer (2005). An ECT2-centralspindlin complex regulates the localization and function of RhoA. J Cell Biol 170(4): 571-82.

Zeisberg, M., C. Yang, M. Martino, M. B. Duncan, F. Rieder, H. Tanjore and R. Kalluri (2007). Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 282(32): 23337-47.

Zhang, M., S. Kleber, M. Rohrich, C. Timke, N. Han, J. Tuettenberg, A. Martin-Villalba, J. Debus, P. Peschke, U. Wirkner, M. Lahn and P. E. Huber (2011). Blockade of TGF-beta signaling by the TGFbetaR-I kinase inhibitor LY2109761 enhances radiation response and prolongs survival in glioblastoma. Cancer Res 71(23): 7155-67.

Zhang, Y., Z. Wang, D. X. Liu, M. Pagano and K. Ravid (1998). Ubiquitin-dependent degradation of cyclin B is accelerated in polyploid megakaryocytes. J Biol Chem 273(3): 1387-92.

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