TGFβ and LXR signaling in hepatocellular carcinoma

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Abstract
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Hepatocellular carcinoma (HCC) is one of the most prevalent cancer types in the Western world and in the Asia-pacific regions, with its incidence expected to rise up to 22 million cases till 2020. Hepatocellular carcinoma etiology is mainly due to hepatitis B (HBV) and hepatitis C (HCV) infections, and to a lesser extent it is determined by the development of alcohol-driven cirrhosis and non-alcoholic steatohepatitis (NASH). Furthermore, HCC is characterized by a high mortality rate, with poor prognostic expectance and limited therapeutic options currently available in the clinics.

Transforming growth factor beta (TGFβ) is a pleiotropic cytokine with a janus-role in HCC and in other malignancies. TGFβ can in fact elicit either tumor-suppressive and tumor-promoting effects depending on tumor stage, microenvironmental and immunological cues. In HCC specifically, TGFβ determines cytostasis and cellular senescence during the first stages of tumor development, while it enhances HCC malignancy and progression in the later stages due to increased invasiveness, acquired resistance to cytostatic actions and tumor immunotolerance.

Liver X receptors (LXRα/NR1H3 and LXRβ/NR1H2) are transcription factors of the nuclear hormone receptor family, which play an important role in oxysterol metabolism and reverse-cholesterol transport to the liver. Their involvement in malignancies has been studied so far to a limited extend, with evidence of both tumor-suppressive -via cytostatic mechanisms- and tumor-immunotolerance activities. Moreover, the potential crosstalk of LXR and TGFβ pathways has not been yet unraveled in the context of hepatocellular carcinoma.

We have described (Paper I) a high-content imaging platform for the screening of small molecules able to revert the TGFβ-induced epithelial to mesenchymal transition (EMT) in human keratinocytes. This screening allowed us to identify LXR agonists as epithelial plasticity modulators in established terminally differentiated and mouse embryonic fibroblast, as well as in epithelial and mesenchymal HCC cell lines.

We have identified (Paper II) the transcription factor SNAI1 (Snail) as the mediator of the crosstalk between TGFβ and LXRα pathways in epithelial and mesenchymal HCC cell models. LXRα activation diminishes the transcriptional induction of SNAI1 by TGFβ, thus antagonizing the induction of mesenchymal features and the production of reactive oxygen species by TGFβ. However, we have unraveled that LXRα and TGFβ signaling still positively interact in increasing cytostasis in HCC, in order to preserve liver epithelial features.

We have described (Paper III) that LXRα activation counteracts the transcriptional induction of α smooth muscle actin (αSMA), a major hallmark of fibroblast activation, elicited by TGFβ in patient-derived primary liver fibroblasts.

In conclusion, we herein report that the signaling crosstalk between TGFβ and LXRα pathways results in antagonistic effects either on parenchymal and fibroblast cell lines representative of the HCC disease, suggesting the potential future application of LXR agonists as clinical therapeutic options.

Keywords: Hepatocellular Carcinoma, high-throughput imaging, small molecules, epithelial plasticity, LXR, Snail, TGFβ, tumor suppression, reactive oxygen species, cancer-associated fibroblast, myofibroblast, Smad3, αSMA

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Gutta cavat lapidem, non vi sed saepe caedendo
The drop carves the rock not with strength but steadily flowing

Ovidio, Epistulae ex Ponto libro IV,10,5

Ever tried. Ever failed. No matter.
Try Again. Fail again. Fail better.

Samuel Beckett, Wostward Ho

I need…I need…I need to get up in this gig gurrl

Alyssa Edwards

Alla mia famiglia, il mio porto sicuro

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I   Carthy, J.M., Stöter, M., Bellomo, C., Vanlandewijck, M., Heldin, A., Morén, A., Kardassis, D., Gahman, T.C., Shiau, A.K., Bickle, M., Zerial, M., Heldin, C.-H. and Moustakas A. (2016). Chemical regulators of epithelial plasticity reveal a nuclear receptor pathway controlling myofibroblast differentiation. *Scientific Reports*, (6):29868

II   Bellomo, C., Caja, L., Fabregat, I., Mikulits, W., Kardassis, D., Heldin, C.-H., and Moustakas A. (2017). Snail mediates crosstalk between TGFβ and LXRα in hepatocellular carcinoma. *Cell Death and Differentiation. In press.*

III   Morén, A., Bellomo, C., Tsubakihara, Y., Kardassis, D., Mikulits, W., Heldin C.-H., and Moustakas A. (2018). LXRα limits the pro-fibrotic action of TGFβ in liver cancer-associated fibroblasts. *Manuscript.*

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Related Publications

I Mathot, L., Kundu, S., Ljungström, V., Svedlund, J., Moens, L., Adler-teg, T., Falk-Sörqvist, E., Rendo, V., Bellomo, C., Mayrhofer, M., Cortina, C., Sundström, M., Micke, P., Botling, J., Isaksson, A., Moustakas, A., Battle, E., Birgisson, H., Glimelius, B., Nilsson, M. and Sjöblom, T. (2017). Somatic ephrin receptor mutations are associated with metastasis in primary colorectal cancer. *Cancer Research*. 77(7):1730-1740

II Bellomo, C., Caja, L. and Moustakas A. (2016). Transforming growth factor β as regulator of cancer stemness and metastasis. *British Journal of Cancer* 115(7):761-769. *Review article*.

III Caja, L., Bellomo, C., and Moustakas A. (2016). Transforming growth factor β and morphogenetic protein actions in brain tumors. *FEBS Letters* 589(14):1588-1597. *Review article*.

Contents

Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E binding protein 1
8-OHdG	8-hydroxydeoxyguanosine
11β-HSD1	hydroxysteroid 11-beta dehydrogenase 1
αSMA	α smooth muscle actin
ABCG	ATP-binding cassette sub-family proteins
ACC1	acetyl-CoA carboxylase 1
ADAM17	ADAM metallopeptidase domain 17
ADH	alcohol dehydrogenase
AKT	Akt serine/threonine kinase
AMH	anti-Muellerian hormone
ANGPTL3	angiopoietin-like 3
APO	apolipoprotein
APPL	adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper
BMP	bone morphogenetic protein
BRCA	breast and ovarian cancer susceptibility protein
CAFs	cancer associated fibroblasts
CAR	constitutive androstane receptor
CBP	CREB-binding protein
CCL	C-C motif chemokine ligand
CCl_4	carbon tetrachloride
CDC42	cell division control protein 42 homolog
CDK	cyclin dependent kinases
CEBPβ	CCAAT/enhancer-binding protein β
CETP	cholesteryl ester transfer protein
CIN85	SH3 domain containing kinase binding protein 1
CKI	cyclin dependent kinase inhibitor
COX	cycloxygenase
CPT1A	carnitine palmitoyltransferase 1A
CTGF	connective tissue growth factor
DAPK	death associated protein kinase
DAX1	DSS-AHC critical region on the X chromosome protein 1
DAXX	death-associated protein 6

DDR2	discoidin domain receptor tyrosine kinase 2
DEN	diethylnitrosamine
DUOX	dual oxidase proteins
E2F1	E2 transcription factor 1
ECM	extracellular matrix
EGF	epidermal growth factor
ELF	E74-like factor 2
EMT	epithelial mesenchymal transition
EP300	E1A binding protein P300
ERK	extracellular signal-regulated kinases
FAS	fas cell surface death receptor
FASN	fatty acid synthase
FGF21	fibroblast growth factor 21
FOXH1	forkhead box protein H1
FOXO	forkhead box protein
FSP1	S100 calcium binding protein A4
FXR	farnesoid X receptor
GCSF	granulocyte colony stimulation factor
GDF	growth differentiation factor
GEF	guanine nucleotide exchange factor
GFAP	glial fibrillary acidic protein
GLUT	glucose transporter
HAND2	heart and neural crest derivatives-expressed protein 2
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HOX	homeobox protein
HSCs	hepatic stellate cells
I-Smad	inhibitory Smad
ICD	intracellular domain
IDOL	inducible degrader of the LDL-receptor
IGF	insulin-like growth factor
IKK	IκB kinase
IL1β	interleukin 1 beta
IL6	interleukin 6
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal protein kinase
KRAS	Kirsten rat sarcoma 2 viral oncoprotein homolog
LBD	lateral organ boundaries domain
LDL	low density lipoprotein
LIMK2	LIM domain kinase 2

LPL	lipoprotein lipase
LXR	liver X receptor
LXRE	LXR responsive elements
MAP	mitogen activated protein
MAPK	MAP-kinase
MCP	monocyte chemoattractant protein
MEK	MAP kinase kinase
MKK	MAP kinase kinase
MMPs	metalloproteinases
MnSOD	manganese superoxide dismutase
mTOR	mammalian target of rapamycin
MYC	v-myc avian myelocytomatosis viral oncoprotein homolog
NCOR	nuclear receptor corepressor 1
NET1	neuroepithelial cell transforming 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOX	NADPH oxidases
OCLN	occludin
p15	cyclin-dependent kinase inhibitor 2B
p16	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p27	cyclin-dependent kinase inhibitor 1B
p85α	PI3K regulatory subunit 1
p107	Rb transcriptional corepressor like 1
PAK1	p21 (RAC1) activated kinase 1
PAR6	partitioning defective protein 6
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PEPCK1	phosphoenolpyruvate carboxykinase 1
PI3K	phosphatidylinositol 3´ kinase
PKC	protein kinase C
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator activated receptor
PTEN	phosphatase and tensin homolog
PXR	pregnane X receptor
PYDC1	pyrin domain containing 1
R-Smad	receptor-activated Smad
Rab11	Ras-related protein Rab-11A
Raf1	Raf-1 proto-oncogene, serine/threonine kinase
Rb	retinoblastoma-associated protein
RCT	reverse cholesterol transport
RhoA	Ras homolog gene family, member A

ROCK1	Rho associated coiled-coil containing protein kinase 1
ROS	reactive oxygen species
RXR	retinoid X receptor
S6K1	ribosomal protein S6 kinase B1
SARA	Smad anchor for receptor activation
SDF1	stromal cell-derived factor 1
SHP	protein tyrosine phosphatase, non-receptor type 11
SKP2	S-phase kinase associated protein 2
SM22	smooth muscle protein 22-alpha
Smad	Sma and mothers against decapentaplegic homolog
SMRT	nuclear receptor corepressor 2
Smurf	Smad specific E3 ubiquitin protein ligases
SNAI1	Snail family transcriptional repressor 1
SOCS3	suppressor of cytokine signaling 3
SOS1	son of sevenless homolog 1
SOX	SRY (sex determining region Y)-Box
Sp1	specificity protein 1
SRC	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncoprotein homolog
SREBP	sterol regulatory element-binding protein
SULT	sulfotransferase family
SUMO	small ubiquitin-like modifier
TAB	TGFβ-activated kinase 1 binding proteins
TAK1	TGFβ activated kinase 1
TFE3	transcription factor for immunoglobulin heavy-chain enhancer 3
TGFβ	transforming growth factor β
TGFβR	TGFβ receptor
TNFα	tumor necrosis factor alpha
TP53	transformation-related protein 53
TRAF6	TNFα receptor associated factor 6
UCP1	uncoupling protein 1
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
YAP1	yes associated protein 1
WNK	with no lysine protein kinase 1

Introduction

1. TGFβ signaling in cancer

1.1 Overview of TGFβ signaling

The transforming growth factor β (TGFβ) family encompasses 33 members divided in two subfamilies. The first subgroup includes activin, nodal, lefty, myostatin and TGFβ, while the second is represented by bone morphogenetic proteins (BMPs), anti-muellerian hormone (AMH) and growth differentiation factors (GDFs), and the majority of these cytokines exists in different isoforms [1]. The expression of the TGFβ family members is strictly spatio-temporally controlled and they have pleiotropic functions in mammals, ranging from embryonic development to proliferation, apoptosis, differentiation and migration [2, 3]. Upon its discovery, TGFβ challenged the principle that one cytokine has one main function, since the TGFβ signal transduction pathway plays several and diverse roles depending on the cellular context and the microenvironment [2]

TGFβ maintains tissue homeostasis and prevents malignancies either via the tight regulation of cell proliferation, differentiation, survival and adhesion or the modulation of tissue parenchyme-microenvironment communication [1]. During cancer development the inactivation of core TGFβ pathway components (e.g receptor inactivation) and/or alterations of main pathway regulatory components might disable the tumor suppressive arm of TGFβ while enhancing its pro-tumorigenic properties.

TGFβ is characterized by a pleiotropic action, which depends on the wide set of transcription factors interacting with Smad proteins, the main intracellular mediators of TGFβ signaling; depending on this interplay, diversified sets of target genes are modulated in their expression, as represented in figure 1 [1]. Moreover, TGFβ is responsible for the coordinated regulation of different genes, which share conserved enhancer elements recognized by specific and common Smad-cofactor complexes (synexpression groups) [1, 4].
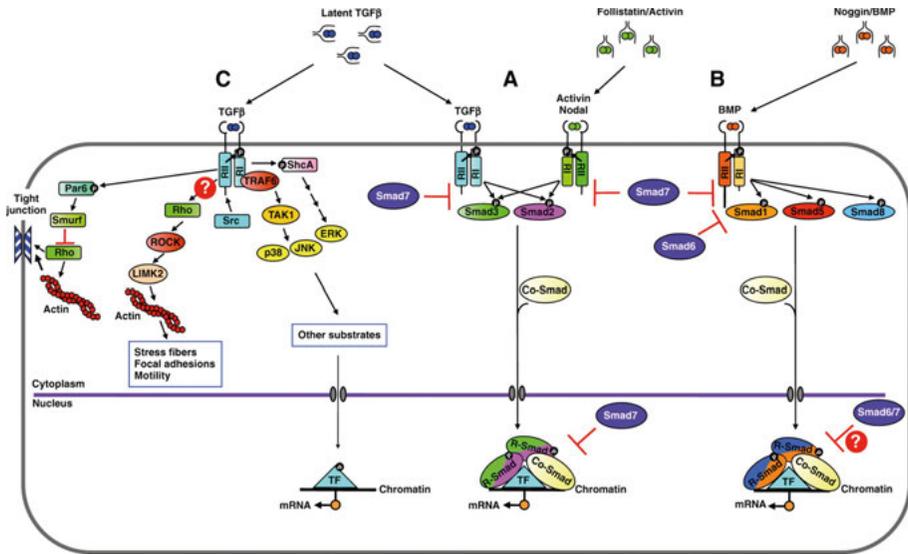
Figure 1. Schematic representation of Smad and alternative TGFβ signaling pathway and principal signaling routes of the TGFβ family members BMP and Activin; adapted from Moustakas, Heldin [5] (Image reproduction allowed by the Creative Commons Attribution 4.0 International License)

1.2 The Smad signaling pathway

TGFβ isoforms (TGFβ1, TGFβ2, TGFβ3) exert their cellular functions by binding to a heterotetrameric complex of type I and type II serine/threonine kinase receptors, in which the constitutively active TGFβ type II receptor (TGFβRII) phosphorylates and activates the type I receptor (TGFβRI) [6, 7]. This event allows the recruitment of the receptor activated Smads (R-Smads), namely Smad2 and Smad3, as well as the activation of several alternative signal transduction routes. R-Smads become phosphorylated by TGFβRI and form a complex with the common mediator (Co-) Smad4, which then translocates to the nucleus and associates with other transcription factors, co-activators or co-repressors to regulate the expression of defined target genes [6, 8]. The Smad signaling pathway can be antagonized by the activity of the inhibitory (I-) Smad7, which is a direct TGFβ target and modulates its activity via feedback mechanisms [6, 8].

R- and Co-Smads are characterized by a Mad homology 1 (MH1) N terminal domain, a linker sequence and a MH2 C-terminal domain, while the I-Smads, Smad6 and Smad7, lack the MH1 domain. Moreover, Smad3 and Smad4 have a β hairpin structure in their MH1 domain that allows the low affinity DNA binding to CAGA and GC-rich DNA motifs, while in the main isoform of Smad2 an inserted exonic sequence in the MH1 domain prevents it from DNA binding [6]. The MH1 domain is designated for interaction

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with other transcription factors, such as Sp1, Jun or TFE3, whose binding helps to stabilize the DNA-bound Smad complex. On the other hand, the α1 loop in the MH2 domain of R-Smads is dedicated to the interaction with the L45 loop in the TGFβRI; in particular, R-Smads contain SSXS residues, which can be phosphorylated by the type I receptor at the MH2 C-terminus [6, 9]. Moreover, the MH2 domain mediates the interaction of R-Smads within the oligomeric complexes with several designated partners, such as the FYVE domain protein SARA (Smad anchor for receptor activation) located in the endosomal membrane, the transcription factor FOXH1, the chromatin remodeling factor CBP and the co-repressor SKI. At last, the MH1-MH2 linker module contains one PY motif, which can bind ubiquitin ligases carrying WW domains, as well as numerous phosphorylation sites targeted by multiple kinases [6]. Thus, the Smad linker mediates regulation of protein stability via ubiquitination and transcriptional activity via phosphorylation.

Smads and receptors are regulated via a plethora of mechanisms, which can modulate their function and, as a consequence, affect TGFβ signaling. Antagonistic phosphorylation in their linker or in their MH1 domain by non-receptor kinases (ERK1/2 and other mitogen activated protein kinases, MAPKs) may occur, prohibiting Smad nuclear translocation and hence function; while other non-receptor kinases, as the WNK family, can agonistically phosphorylate R-Smads and cause Smad signaling enhancement [6]. Ubiquitination, acetylation and sumoylation also affect Smad stability and these events can be modulated by the Smad7 negative feedback mechanisms. As an example, Smad7 recruits the ubiquitin ligases Smurf1 and Smurf2 (Smad ubiquitination regulatory factors) to the receptor and enhances its proteosomal-lysosomal degradation; moreover diverse phosphatases can interact with the TGFβ receptor and the Smads in order to dephosphorylate and thus inactivate them [6, 10, 11].

TGFβ signaling via Smads has a significant effect on cell biology, both under physiological and in malignant conditions. Smad-dependent pathways induce growth arrest or apoptosis, by activation of cell cycle inhibitory or pro-apototic genes, respectively; these pathways are perturbed during malignancy. Moreover, the inactivation or, more frequently, the alteration of Smad signaling can contribute to the switch of TGFβ function from tumor suppression to tumor promotion and it can affect the process of epithelial-mesenchymal transition (EMT) [6].

1.3 Other signaling pathways

The so-called "non-Smad" TGFβ signaling routes are classified according to their mode of action and can be Smad-dependent (first two in the list) or Smad-independent (the last example in the list), as:

- Alternative signaling pathways, which directly modify Smad function.
- Diverse groups of proteins whose function is directly modulated by Smads and which signal to other established pathways, and
- Signaling proteins that directly interact with or become phosphorylated by TGFβRII or TGFβRI, not affecting Smad function [12].

ERK-MAPK pathway

TGFβ induces ERK1/2 activation by loading of GTP on Ras and by the subsequent recruitment of Raf (a MAP kinase kinase kinase, MAP3K) to the plasma membrane, which then leads to the activation of ERK via MEK [13]. Src, a cytoplasmic protein kinase, can also phosphorylate TGFβRI on its Y284 residue [13, 14]. Then, the TGFβRII/RI complex recruits the adaptor protein Grb2, which forms a complex with GEF, SOS1 and Ras [15, 16] The ERK1/2 pathway promotes EMT, since its activation influences the disassembly of adherent junctions and the induction of cell motility; in addition, ERK activation by TGFβ regulates gene sets important in the context of cell-matrix interaction, cell motility and endocytosis [13].

JNK/p38 MAPK pathway

TGFβRI promotes the ring domain ubiquitin ligase TRAF6 (tumor necrosis factor α receptor associated factor 6) activation in a kinase-independent fashion, leading to its K63-linked polyubiquitination. This event allows the recruitment and the binding of TGFβ activated kinase 1 (TAK1) and TAB1/2 protein adaptors to the TGFβ receptor, in order to induce the MAP kinase kinase MKK3, MKK4, MKK6 to activate p38 and Jun N-terminal kinase (JNK) [13, 17].

The TRAF6-mediated polyubiquitination of TGFβRI on K63 residues promotes TGFβRI cleavage by the metallopeptidase ADAM17 in a protein kinase C (PKC ζ) dependent manner. This event causes the release of the TGFβRI intracellular domain (ICD), which interacts with the transcription factor p300 and activates TGFβ-target genes, such as Snail and MMP2 [18]. Interestingly, TGFβRI-ICD nuclear translocation occurs via the endocytic adaptor molecules APPL1 and APPL2, which associate with the type I receptor in a TRAF6-dependent fashion [19]. Moreover, TRAF6 mediates the interaction of TGFβRI to the adaptor protein CIN85 in response to TGFβ stimulation; this event enhances the presence of the type I receptor to the cell

surface due to an increased recycling of the receptor favored by Rab11, a marker of recycling vescicles [20].

Hence, the activation of the TRAF6-TAK1-MKK-JNK/p38 MAPK pathway does not depend on Smads but functions in parallel with them, as described for TGFβ induction of apoptosis –one of the mechanisms through which TGFβ exerts its tumor suppressive role– or during TGFβ-induced EMT. The blockage of p38 activity with specific inhibitors or via the action of MKK3 or p38 dominant negative forms impairs TGFβ-mediated changes in the cell shape and reorganization of the actin cytoskeleton [13, 21].

Rho GTPase pathway

The Rho GTPase family is represented by RhoA, Rac and Cdc42, which control cytoskeletal reorganization, cell motility and gene expression through a variety of effectors [13].

RhoA can be activated by Smad-dependent or -independent routes; it affects actin stress fiber formation and the dissolution of tight junctions during EMT [22]. The rapid and Smad2/3-independent RhoA activation occurs mainly at early stages of EMT and involves the phosphorylation of the polarity protein Par6 by TGFβRII. Par6 then interacts with PKC ζ, the effector of the Par6 complex, which directly binds to Smurf1; Smurf1 then polyubiquitinates RhoA on the K6 and K7 residues, promoting its proteosomal degradation [13, 23]. PKC ζ can also direct Smurf1 localization to active protrusions, allowing a specific and localized RhoA degradation [13]. TGFβ also induces a delayed peak in RhoA activation at later EMT stages, due to the Smad-dependent induction of NET1, a RhoA-specific guanine exchange factor responsible for RhoA activation [13].

TGFβ can indirectly, via RhoA, activate the kinase LIMK2 and its down-stream effector ROCK1; Smad3/Smad4 can cooperate with Rho and p38 signaling to drive the expression of NET1 and Tropomyosin, which are important in stress fibers formation and contractility [12].
Finally, TGFβ receptors can physically interact with the Cdc42/Rac GTPase in order to form a complex with PAK1 and Occludin (OCLN). OCLN recruits TGFβRI to the tight junctions in polarized epithelial cells, in order to guide their local and specific dissolution [13].

PI3K/AKT pathway

TGFβ activates phosphatidylinositol 3´ kinase (PI3K) and AKT protein kinase via the interaction between the p85 subunit of PI3K and TGFβRI, which then leads to the induction of the AKT/mTOR/S6K1 pathway [13].

TGFβ activates the PI3K pathway in a TRAF6-dependent manner. TRAF6 polyubiquitinates p85α, the PI3K regulatory subunit, promoting the formation of the TGFβRI-p85α complex with the subsequent activation of the PI3K/AKT pathway, which does not depend on the kinase activity of the TGFβ type I receptor. Interestingly, AKT also interacts with TGFβRI via a TRAF6-dependent mechanism, suggesting an important role of TRAF6 in the PI3K/AKT pathway [24].

TGFβ indirectly triggers PI3K through TGFβ-induced TNFα expression and the activation of epidermal growth factor receptor (EGFR) receptor signaling. Depending on the cellular context and cell status, TGFβ can also downregulate PI3K/AKT signaling via the Smad-dependent expression of the lipid phosphatase SHIP; this may account for the transient nature of TGFβ-induced AKT phosphorylation [13].

AKT signaling can either synergize with or antagonize the TGFβ pathway in context and cell dependent-manners. As an example, the PI3K/AKT/mTOR pathway positively affects TGFβ-driven EMT, complementing Smad signaling or promoting TGFβ-mediated fibroblast proliferation and activation into myofibroblasts via c-Abl [13, 25]. However, antagonism can also occur; direct interaction between AKT and Smad3 prevents TGFβRI-induced phosphorylation and nuclear localization of Smad3, protecting cells from TGFβ-induced apoptosis.

AKT can also phosphorylate and inhibit the nuclear traslocation of FOXO proteins, resulting in antagonism of TGFβ-induced growth arrest, thus promoting cell survival. Furthermore, the mTORK/S6K1 complex of protein kinases also contribute to antagonism towards TGFβ responses, as it occurs during TGFβ growth arrest due to the involvement of the ribosomal S6 protein kinase 1 (S6K1) [13, 26, 27].

TGFβ enhances mTORC2 transcriptional levels and promotes mTORC2-mediated AKT activation; this favors TGFβ-induced EMT and the induction of TGFβ-dependent EMT transcription factors, such as Snail. Intriguingly, mTORC2 affects actin cytoskeletal reorganization, RhoA activation and cell migration in cell models *in vitro* [28]. TGFβ enhances the activation of mTOR via the PI3K/AKT signaling and favors the phosphorylation of S6K1 and 4E-BP1, major players in protein synthesis. As a result, the activation of the mTOR-S6K1 axis mediates TGFβ-induced cell size increase and protein synthesis, suggesting an important role of TGFβ in the control of cell dimensions and anabolic pathways [29].

1.4 Tumor suppressive role of TGFβ in malignancies

TGFβ suppresses the premalignant progression due to cell growth inhibition and induction of apoptosis [1]. The TGFβ cytostatic action focuses on blocking the cell cycle from G1 to mitotic phase and it involves two main pathways: the mobilization of cyclin dependent kinase (CDK) inhibitors (CKI) and c-Myc blockage. TGFβ activates either the CKI p15^{INK4b}, which blocks the CyclinD/CDK4-6 complex, or p21^{CIP1}, which inactivates the CyclinE-A/CDK2 complex, in a Smad3-dependent fashion [1]. In parallel, TGFβ blunts the expression of c-Myc via Smad3/Smad4-mediated repression; the Smads form a repressor complex with other cofactors, such as p107, E2F4, CEBPβ and hinder the access of the RNA polymerase to the *c-Myc* promoter [1, 30, 31]. Interestingly, CEBPβ acts as the "trait d´union" of these two mechanisms of action; it is both required for *c-Myc* transcriptional inhibition and for *p15^{INK4b}* transcriptional activation in complex with Smad3/Smad4/FOXO3A, allowing a synergic cytostatic response [1, 32].

TGFβ elicits a tumor suppressive effect via the death-associated protein kinase (DAPK), which plays apoptotic roles in hepatoma. The chromatin regulator GADD45β is pro-apoptotic in hepatocytes, while FAS and BIM are apoptotic regulators in gastric cell lines, according to Smad-dependent and -independent routes [33, 34]. Besides the anti-tumorigenic role in epithelial cells, TGFβ antagonizes the stromal contribution to the tumor development. In particular, TGFβ impairs the paracrine and mitogenic stimulation of the epithelial cells by the resident fibroblasts and it exerts an immunosuppressive action on the innate and adaptive immunity, causing a shift of the immune response towards immune suppression and tolerance [1].

1.5 Tumor promoting role of TGFβ in malignancies

TGFβ tumor suppressive mechanisms might become inefficient either due to a defective cytostatic gene response, including lack of p15^{INK4b} induction or c-Myc repression, due to aberrant ID1 expression, or due to a complete loss of the expression of cytostatic genes [1]. In addition, genetic mutations and epigenetic variations in the genes encoding for the main protein components of TGFβ signaling, e.g. TGFβRI, TGFβRII and Smads, have been associated with higher susceptibility to cancer.

As an example, the common variant *TGFβRI*6A* has a 3 alanine deletion in a nine-alanine stretch at the 3'-end of exon 1. This nine-alanine region is part of the signal sequence peptide, which targets the receptor to the membrane and then becomes cleaved at two different sites. It has been reported that the *TGFβRI*6A* variant is indeed deprived of the second cleavage site, which might account for incorrect translocation across the endoplasmic reticulum

and the erroneous localization of the TGFβRI at the membrane [35]. In addition, it has been described that *TGFβRI*6A* might be a tumor susceptibility allele in breast cancer [36].

In colorectal carcinoma, *TGFβRII* has been observed to bear a frameshift mutation in the region *BAT-RII,* a polyadenine tract in exon 3 of *TGFβRII,* which causes a truncated and non-functional receptor, unable to bind to the TGFβ ligands [37]. This genomic event occurs in more than the 90% of microsatellite instable colorectal carcinoma cases and it is propagated, even if it is not linked to any fitness advantage of the tumor [38, 39].

Smad mutations are tumor context-dependent, with mainly Smad2 and Smad4, both mapping on chromosome 18q21, altered in malignancies. Smad2 (*MADR2*) possesses a missense mutations and loss of heterozygocity in colorectal carcinoma; these mutations are inactivating and impair TGFβ signaling [40]. Similarly, Smad4 (*DPC4*) has been reported to undergo homozygous deletion and loss of heterozygosis in ductal pancreatic carcinoma, which are accompanied by mutation in *KRAS* in more than 80% of cases [41]

These events can promote the pro-tumorigenic effects of TGFβ enhancing cancer progression, acquisition of chemoresistance and induction of EMT via Smad-dependent or -independent pathways, which favor the tumor invasion and dissemination providing the cells with a motile, junction-free phenotype [1]. Moreover, TGFβ enhances the autocrine production of mitogens, as it occurs in glioma. In this malignancy, the loss of $p15^{INK4b}$ or the mutational inactivation of the Rb protein induce cell proliferation via autocrine PDGF-BB (platelet-derived growth factor B) production. In this context, PDGF-BB is produced upon TGFβ stimulation with a positive feedback loop and in a manner that depends on the PDGF-BB promoter methylation status [1].

Furthermore, TGFβ acts on the stroma and it enhances the generation of myofibroblasts from mesenchymal precursors. This cellular subpopulation produces matrix metalloproteinases (MMPs), which enhance cell motility, and chemokines, such as CXCL12, in order to promote cell proliferation and migration [1]. TGFβ also aberrantly promotes immune evasion. As an example, TGFβ stimulates the production of cytolytic factors, such as granzyme A and B or perforins, by activated cytolytic CD8$^+$ T lymphocytes; this can be associated with immune-tolerance of the growing tumor mass and its impaired eradication at relatively early stages of the malignancy [1].

2. Hepatocellular carcinoma onset and progression

Hepatocellular carcinoma (HCC) is the fifth and seventh cause of cancer in men and women worldwide, respectively, and the third most common cause of cancer death. Leading causes of HCC are infections with Hepatitis B and, in lower percentage, Hepatitis C, while contributing etiological causes are alcohol-dependent cirrhosis and non-alcoholic steatohepatitis (NASH) [42, 43].

HCC pathogenesis is mainly due to cirrhotic progression associated with hepatic regeneration, the latter being a reparative events aiming at resolving the tissue damage caused by a viral infection. Relevant HCC etiological causes also consist of the exposure to toxins or metabolic determinants (aflatoxins, alcohol) and, in a small percentage, to the occurrence of mutations in certain oncogenes or tumor suppressors [42].

The initial tissue damage triggers a *noxa* (an agent or a condition harmful to the body, from latin *nocēre*) in the liver parenchyma, with necrosis and/or apoptotic signaling activation in the liver epithelial cells (hepatocytes). This event determines the activation of the resident macrophages (Kuppfer cells) and pericytes-related fibroblast populations (stellate cells) in order to promote a concerted wound healing reaction, which either leads to proficient tissue repair in an acute setting or evolves to fibrosis, as it occurs in a chronic scenario. If the *noxa* occurs chronically, e.g. as in the case of alcohol abuse, liver fibrosis cannot be proficiently resolved, and this might possibly lead to a chronic fibrotic condition (cirrhosis), which might in turn predispose the patient to hepatocellular carcinoma [43].

Apoptosis/necrosis of hepatocytes upon damage elicit a cellular response aimed toward damage limitation, removal or repair of damaged cells, wound closure, defense against further infection and, ultimately, tissue repair via liver regeneration [43]. Liver regeneration depends on the oval cells, intrahepatic progenitors (HPCs) with bi-potential capacity residing in the ductules of Hering, which are able to differentiate into the two main epithelial liver populations: the hepatocytes and cholangiocytes. The differentiation into a specific epithelial phenotype depends on the most-damaged cell compartment; moreover, an ineffective proliferation and differentiation of HPCs is related to impaired liver regeneration and rapid hepatic failure [43].

The inflammation occurring during liver damage is induced by the mutual activation of Kuppfer and hepatic stellate cells, which promotes a proflogistic environment and allows a massive infiltration of innate and adaptive immunity cells [43, 44]. The pro-inflammatory environment sustains wound healing and liver repair and allows the physiological deposition of

fibrotic material, the activation of quiescent hepatic stellate cells and resident fibroblasts together with the recruitment of mesenchymal bone marrow-derived precursors, which can locally differentiate into myofibroblasts [43, 45].

In a physiological condition, hepatocyte proliferation occurs and the fibrotic material is degraded once the inflammatory response decays, as sign of the neutralization of the pathogenic determinant. Nonetheless, the continued exposure to a liver damaging factor, as well as an inefficient inflammatory response can cause a persistent deposition of ECM in the liver, i.e. fibrosis. Fibrosis is a reversible physiological event occurring during wound healing, which leads to liver regeneration if the *noxa* is removed.
However, a physiological liver fibrotic response can be disrupted by not yet fully elucidated mechanisms, and this might lead to an irreversible cirrhotic condition. Cirrhosis is characterized by an abnormal, non physiological deposition of fibrotic material, together with hepatic architecture disruption, aberrant hepatocyte regeneration, nodule formation and vascular changes, which might lead to HCC onset [43]. In fact, pre-neoplastic lesions indicative of HCC can be observed in a cirrhotic liver, as nodules formed by foci of dysplastic hepatocytes, abnormal in their morphology [43].

Signaling implicated in the onset and in the progression of HCC encompasses the TGFβ, WNT-β-catenin, IGF-1 (insulin-like growth factor), HGF (hepatocyte growth factor) pathways, with the involvement of the intracellular signaling routes Raf-ERK-MAPK and PI3K-AKT-mTOR [42]. The major sources of TGFβ in the hepatic milieu are the hepatic stellate cells and the Kuppfer cells, which can produce TGFβ upon liver damage, while hepatocytes are able to store TGFβ intracellularly. TGFβ produced in the liver acts on the mesenchymal, epithelial and immune populations in autocrine and paracrine fashions, contributing to the establishment of a proinflammatory environment [43].

3. TGFβ contribution to hepatocellular carcinoma

In HCC, as in other malignancies, TGFβ has a dual role depending on the cell compartment and the microenvironment. TGFβ elicits a tumor suppressive effect via the impairment of hepatocytes proliferation –due to the induction of tumor suppressor genes $p15^{INK4b}$ and $p21^{CIP1}$– and the induction of senescence, differentiation and apoptosis. Moreover, TGFβ tumor suppressive activity also includes the inhibition of mitogens and inflammatory cytokines produced by stromal cells and lymphocytes/macrophages, and the suppression of cancer stem cell populations in the liver [42].

On the other hand, the desensitization and the acquired resistance of hepatocytes towards the tumor suppressive activity of TGFβ account for TGFβ tumor promoting effects [46]. These biological events encompass the promotion of proliferation and migration of HCC cells, the induction of EMT and the TGFβ-driven activation of anti-apoptotic genes. Furthermore, TGFβ elicits tumor-promoting effects either acting on stromal cells –in order to release proteases and inflammatory cytokines– or by enhancing invasion and angiogenesis with the production and release of metalloproteinases (MMP2, MMP9, as example) or pro-fibrotic molecules such as connective tissue growth factor (CTGF) [42].
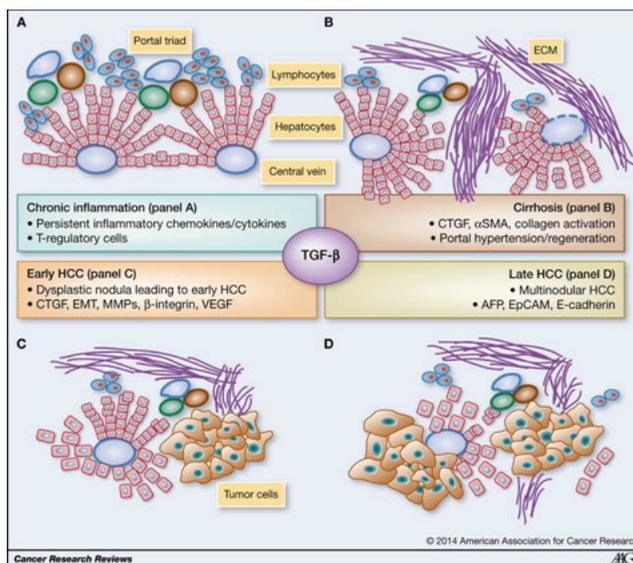

Figure 2. The crucial contribution of TGFβ to liver disease [47] (Image reprinted with AACR permission)

3.1 TGFβ tumor suppressive effect in HCC

TGFβ exerts a cytostatic action on proliferating hepatocytes during physiological liver regeneration [48]. In particular, the Smad-mediated tumor suppressive response induces i) cytostasis due to effects of TGFβ on the CKIs, i.e. the effects of $p15^{INK4b}$ and $p21^{CIP1}$, ii) differentiation via ID1 upregulation and iii) apoptosis, due to BCL2 downregulation and upregulation of BIM and GADD45β [48]. In addition to this, the Smad directed arm affects the induction of EMT and the production of extracellular matrix proteins, cytokines and proteases, while non-Smad signaling pathways have impacts on cell migration, cell shape and cell-cell contacts via JNK/p38 and CDC2/RhoA/Par6 [48].

TGFβ elicits an anti-mitogenic function on hepatocytes at early stages of HCC via the interaction of the R-Smads with the transcription factor Sp1, which induces the expression of the CKI $p21^{CIP1}$, resulting in cell cycle blockage [43, 49].

TGFβ and activin cause hepatocyte apoptosis with a different kinetics of action; TGFβ induces a maximal death response within 1 hour of treatment, while activin significantly induces hepatocytes loss in vitro and in vivo upon a sustained treatment for 24 hours [50]. TGFβ1 and activin A determine hepatocytic death in a similar fashion without competing with each other. Furthermore, activin A acts as an autocrine inhibitor of DNA synthesis [51]. In addition to this, the adaptor protein DAXX mediates in part the TGFβ cytostatic effect on hepatocytes, due to its interaction with the cytoplasmic domain of TGFβRII and its association with the FAS receptor; the resulting TGFβ-induced apoptosis mechanism requires the JNK pathway in the AML12 cell model [43, 52].

Smads play a crucial role in TGFβ-mediated apoptosis also via the induction of DAPK (Death Associated Protein Kinase), which links Smad function to mitochondrial-based proapoptotic events. In vitro inhibition of DAPK hindered the TGFβ-induced mitochondrial release of cytochrome C, as well as the dissipation of the mitochondrial membrane potential, both clear hallmarks of apoptosis [43, 53]. TGFβ also stabilizes the pro-apoptotic protein BIM due to a rapid inhibition of the ERK-MAPK pathway; in particular, TGFβ induces the MAP kinase phosphatase MPK2 via Smad3, which attenuates ERK activation and BIM phosphorylation. This event is crucial to avoid BIM ubiquitination and proteosomal degradation, which are ERK-mediated events [43, 54].

3.2 TGFβ tumor promoting role in HCC

During carcinogenesis epithelial cells become able to evade the tumor suppressive action of TGFβ via different mechanisms, such as mutation of the genes encoding for pathway core components. i.e. TGFβ receptors or Smads, or via the selective inhibition of key pathway elements [46]. As a biological consequence, tumor cells acquire selective resistance to TGFβ cytostatic effects, redirecting the signaling pathway towards tumor progression and metastatic responses [46].

As an example, the genetic loss of βII-Spectrin causes spontaneous formation of HCC tumor *in vivo*. βII-spectrin is an adaptor protein, which mediates the access of R-Smads to TGFβRI, and its genetic impairment is associated with an expedite entry in S and mitosis phases and HCC formation [42]. The disruption of the adaptor protein ELF also impairs the TGFβ-Smad signaling; TGFβ mediates the phosphorylation of ELF, which acts as a scaffold protein with Smad3/Smad4 and allows the complex to translocate into the nucleus. This event is related to the TGFβ cytostatic response involving p15^{INK4b}, p16^{INK4a}, p21^{CIP1} and p57^{Kip2}, as well as the repression of c-Myc, β-catenin, hTERT and IGF2; these biological events are in fact hindered when ELF is not expressed [46, 55-57]. Furthermore, the expression of the E3-dependent ligase Praya1 causes aberrant ELF ubiquitination, negatively affecting R-Smad signal propagation [42]. In particular, the HCC representative cell lines Hep3b and Huh7, which are sensitive to the cytostatic effect of TGFβ, have low levels of Praya1, while in HCC cell lines not sensitive to the TGFβ cytostatic effect Praya1 levels are increased [42, 43].

Smad4 genetic loss acts as a switch from tumor suppressive to tumor promoting effect of TGFβ, while restoring Smad4 expression in Smad4$^{-/-}$ cells re-enables TGFβ tumor suppressor activity. However, TGFβ can also favor the progression of malignancy via EMT and by the induction of SNAI1 expression and E-cadherin transcriptional inhibition [42, 58].

Smad2 and Smad3 also act as a molecular switch from TGFβ cytostatic response towards its fibrinogenic and carcinogenic action in the liver [59]. In particular, under physiological conditions TGFβ induces the C-terminal phosphorylation of Smad3, which impairs hepatocyte proliferation via p21^{Cip1} transcriptional activation. On the other hand, in a stress context (mitogens, alcohol, HCV, HBV) JNK pathway activation leads either to mitogenic signals, or to JNK-mediated phosphorylation of Smad3 in its linker region [60]. The phosphorylation of Smad3 in its linker region (pSmad3L) prevents Smad3 C-terminal phosphorylation by the TGFβRI, thus impairing Smad3-mediated cytostatic signaling. Smad3L phosphorylation might also

determine hepatocyte proliferation, possibly via c-Myc transcriptional induction [46, 60, 61].

In HCC models, a synergism between the inhibition of TGFβ cytostatic signaling and the promotion of oncogenic YAP1 function has been reported. YAP1, a transcriptional regulator of the Hippo pathway, interacts and stabilizes the I-Smad7, increasing its efficiency in blunting TGFβ cytostatic signaling. At the same time, IGFBP3 can mediate the activation of AKT survival signaling, allowing mTOR to interfere with Smad3-mediated signaling [46].

4. Reactive oxygen species (ROS) in malignancies

Reactive oxygen species (ROS) are highly unstable and reactive oxygen radicals, characterized by an unpaired electron in their outer orbital, or valence orbital. Among them, superoxides ($O_2^{\cdot-}$), peroxyl radicals ($ROO^{\cdot}$), hydroxyl radicals (OH), disulfides (RSSR), organic radicals (ROS), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\cdot}$) are the best characterized [62, 63]. ROS function is intimately linked to their concentration, site of production and neutralization by enzymatic and non-enzymatic detoxifying molecules; the balance among these factors accounts for their prosurvival versus proapoptotic roles in physiological and malignant conditions [62].

ROS are produced via a plethora of mechanisms: as byproduct of oxidative phosphorylation (result of metabolic activity), as induced by cytokine and growth factor stimulation (among those, also TGFβ), as a consequence of high peroxisomal oxydase and lypoxigenase activity, and as soluble mediators released by innate immune cells (macrophages, neutrophils) during inflammation [62, 63].

Under physiological conditions, ROS levels are maintained relatively low via detoxifying enzymatic –catalases, superoxide dismutases, peroxidases– and non-enzymatic agents, such as glutathione and flavonoids. In such contexts, ROS mediates intracellular signaling and affects central proliferative routes, e.g. in PI3K-AKT, IKK-NFκB and MAPK-ERK1/2 pathways [62, 63]. However, an increased mitochondrial ROS production and activity is a main event during cell apoptosis and necrosis.

ROS were originally discovered as mediators of TNFα-induced apoptosis and are established key players in FAS- and p53-determined cell death [64-66]. Moreover, superoxide accumulation leads to AMPK-promoted accumulation of the transcription factor E2F1, which is responsible for the transcriptional regulation of proapoptotic genes [67, 68]. Furthermore, cardiolipin oxidation by ROS is described as a central regulator of apoptosis: it mediates the release of cytochrome c from the mitochondrial membrane and it mediates the oxidation of caspase 3 and 7, essential inductors of cell death [69]. In addition, coordinated release of ROS is suggested as an important paracrine signal for a synchronized cellular release of cytochrome c, which is the trigger of the intrinsic apoptotic pathway [70]. In addition, mitochondrial release of H_2O_2 and NO downregulates the antiapoptotic proteins BCL2 and BCL-XL and favors p38-promoted apoptosis at high ROS concentrations [62].

In malignancies, ROS signaling enhances tumor progression over tumor-suppression mechanisms via different means, as represented in figure 3. Cellular proliferation is promoted by the downregulation of manganese superox-

ide dismutase (MnSOD), thus leading to increased superoxide concentration and proliferation in breast cancer [71]. In the same biological context, ROS upregulate the expression of cyclins B2, D3 and E1 promoting G1 to S phase transition, thus cell cycle progression [72]. A high concentration of ROS increases migratory and invasive potential as well as promotes anchorage-independent survival mechanisms, decreasing cell-cell and cell-matrix inter-actions in carcinomas [62]. In addition, ROS promote cancer stem cell survival at low doses, and at moderate concentrations –which are paradoxically caused by the current ionizing radiotherapy approaches– ROS mediate resistance to chemotherapeutic treatments [73, 74].

ROS also positively affect neo-angiogenesis as a response to the hypoxic milieu established in the primary tumor mass, thus enforcing the activity of the pro-angiogenic factors VEGF and HIF-1; in addition, ROS increase the permeability of the newly formed endothelium, which allows the intravasation and the systemic dissemination of metastatic cell subpopulations [75].
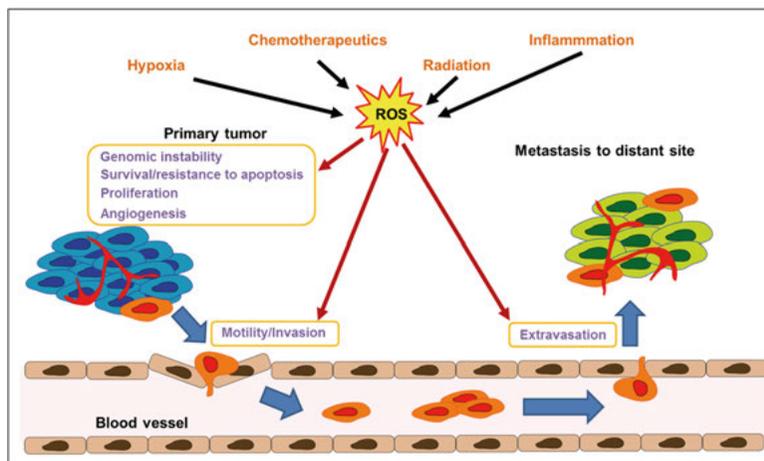

Figure 3. Effect of reactive oxygen species on cancer onset and progression [76] (Image reproduction allowed by the Creative Commons Attribution 4.0 International License).

4.1 NADPH oxidases (Nox) in hepatocellular carcinoma

Alcohol, HBV and HCV infections, the three major causes of HCC, are associated with abnormal production of ROS, suggesting their contribution to hepatocarcinogenesis. As an example, HCV infection is associated with high levels of H_2O_2 and superoxides and with decreased levels of the antioxidant molecule glutathione, secondary to the mobilization of mitochondrial Ca^{2+} [77-79]. ROS induces oxidative DNA damage in HCC, with high incidence of DNA double-stranded breaks and increased concentration of 8-hydroxydeoxyguanosine (8-OHdG), a redox product of DNA damage which

causes G:C to T:A transversion, an event correlating with HCC malignancy [80, 81]. Moreover, liver-specific deletion of the transcription factor Nrf1, involved in the regulation of antioxidant enzymes genes expression, is associated with elevated lipid peroxidation and oxidative DNA damage in hepatocellular carcinoma [82].

NADPH oxidases (Nox) are part of the Nox family, which also encompasses the dual oxidase proteins (Duox), and catalyze the electron transfer from NADPH to O_2 to produce H_2O_2 and superoxides. Nox2 discovery has been reported to have an important role in phagocytes. Upon inflammatory stimulation Nox2 components translocate to the cytosol where the oxidative reaction occurs; this results in increased oxygen consumption, production of superoxides and, ultimately, it causes a respiratory burst responsible for innate immune cell activation [63].

In the liver, the major Nox isoforms are Nox1 and Nox4, which are characterized by six transmembrane domains, an intracellular β loop and an intracellular C-terminal region, containing FAD and NADPH binding sites essential for the catalytic activity. Furthermore, the hepatic Nox4 bears an intracellular D loop, important for protein-protein interaction, and an E loop functional to H_2O_2 production [63].

Nox1 has 60% sequence similarity to the well-characterized Nox2; its subcellular localization has not been univocally clarified and possibly Nox1 resides at caveolin1-positive lipid rafts. Nox1 expression is dependent on the assembly of its subunits and it is tightly regulated at the transcriptional level in liver by PDGF, angiotensin and phorbol esters [83].

Nox1 and Nox2 are activated during hepatic fibrosis, mainly acting in the hepatic stellate cell population. In particular, Nox1 is upregulated by angiotensin II at the mRNA level, thus inducing ROS production and HSC proliferation [84]. Moreover, Nox1 mediates hepatic stellate cells proliferation inactivating PTEN by oxidation, thus inducing the AKT-FOXO4 pathway [85]. Nox1 also promotes hepatic stellate cell proliferation enforcing PDGF signaling via activation of PKC δ [86].

Differently from the other Nox, Nox4 is constitutively active and is mainly regulated at the transcriptional level in hepatocytes; moreover, Nox4 is solely responsible for H_2O_2 production [83]. Interestingly, TGFβ is a potent transcriptional inducer of Nox4 in hepatocytes and hepatic stellate cells, possibly via a Smad-dependent mechanism. In particular, Nox4 is crucial for TGFβ-promoted apoptosis of hepatocytes and its knockdown impairs caspase activation.

Moreover, TGFβ-induced Nox4 favors the expression of proapoptotic proteins BIM and BMF and Nox4 positively affects FAS-mediated cell death [87, 88]. Interestingly, Nox4 is at the crossroad of TGFβ pro-apoptotic and

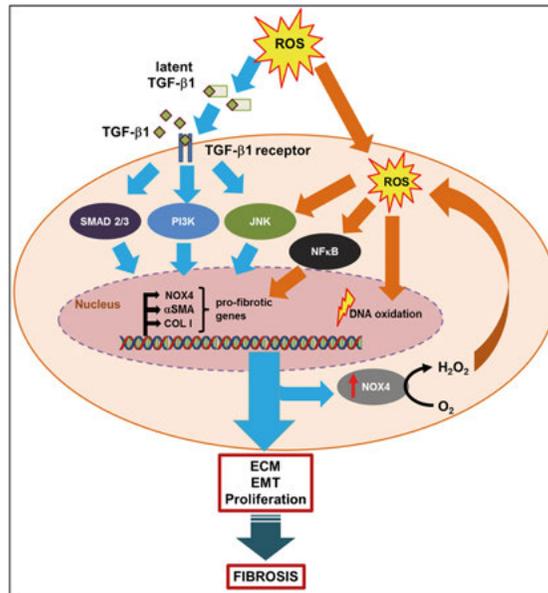EGF-dependent anti-apoptotic events, which antagonize each other in primary rat hepatocytes.

Figure 4 TGFβ and ROS signaling crosstalk in fibrosis [76] (Image reproduction allowed by the Creative Commons Attribution 4.0 International License).

5. Biology of liver X receptors (LXR)

Liver X receptors, namely LXRα and LXRβ, are sterol-activated transcription factors and members of the nuclear hormone receptor family, which includes the pregnane X receptor (PXR), the constitutive androstane receptor (CAR) and the farnesoid X receptor (FXR), among others [89].

LXRs are regulated by the binding of physiological ligands, including oxysterols, metabolically active derivatives of cholesterol, and intermediates of the cholesterol biosynthetic pathway, e.g. desmosterols and 24-(S)-25 epoxycholesterol. Cholesterol levels are sensed by either of two distinct transcription factors: the LXRs and the sterol regulatory element binding proteins (SREBPs). Their pathways are tightly interconnected in modulating sterol homeostasis, but remain distinct and might be targeted independently via pharmacological approaches [90]. LXRα (known as NR1H3) is expressed in tissues with high metabolic rate, such as the liver where LXRα was first discovered, as well as adipose tissue and macrophages, whereas LXRβ (NR1H2) is ubiquitously expressed [89].

LXRs and other nuclear receptors have an amino terminal end (known also as the A/B region) containing a AF-1 transactivation domain of variable length and sequence among the different family members, which is recognized by co-activators and/or other transcription factors [91]. The central DNA-binding domain (DBD) has two zinc finger motifs common to all family members, except for SHP and DAX1. The carboxy-terminal ligand-binding domain (LBD) has a conserved architecture still diverging sufficiently to allow selective ligand recognition. At last, the C-terminal domain contains the ligand-induced activation function (defined as AF2), which is crucially involved in the interaction with transcriptional co-regulators [91].

When LXRs are in an unbound state they form a heterodimeric structure with RXR and other co-repressors, such as SMRT and NCOR1, so that the complex is able to bind LXR responsive elements (LXRE) existing in the promoter region of LXR target genes to inhibit the transcriptional activation [89]. On the other hand, the binding of natural or synthetic LXR agonists, e.g. T0901317 and GW3965 as example of synthetic agonists, induces a conformational change in the heterodimer, which results in the release of the corepressors and the recruitment of coactivators, such as EP300 and PYDC1; this event allows the transcription of specific LXR target genes [89]. Moreover, LXR monomers can alternatively be mono-sumoylated, which trans-represses genes involved in pro-flogistic mechanisms, as described in figure 5 [92].
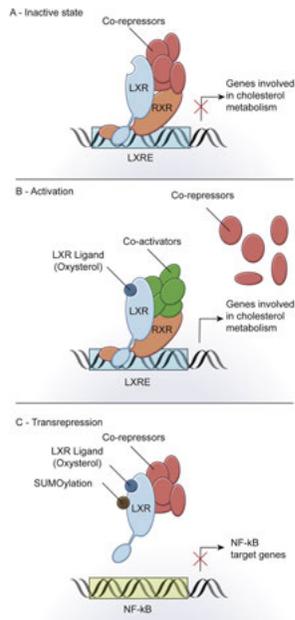
Figure 5. LXR inactive (a), active (b) and transrepressed (c) status in presence of the partner transcription cofactor RXR, LXR ligands or corepressors [93] (Image reprinted with AACR permission)

5.1 Regulation of reverse cholesterol transport

LXRs promote the reverse cholesterol transport (RCT), which returns the excess of cholesterol via high density lipoproteins (HDL) from the peripheral tissues to the liver, where cholesterol is excreted through the bile [94]. The LXR agonists promote the expression of HDL binding proteins and cholesterol transporters, which mediate the mobilization of cholesterol from the periphery; these events increase the concentration of circulating HDL in wild-type LXR mice. In contrast, the removal of cholesterol is severely impaired in LXR$^{-/-}$ models [94, 95].

The first step in RCT is the transfer of cholesterol to lipid-poor molecules in the plasma –such as Apolipoprotein 1 (APO1) and pre-HDL– via the ATP-binding cassette protein ABCA1, a transporter protein whose gene is a direct target of LXR. LXR agonists robustly induce ABCA1 expression in an LXR-dependent manner in macrophages and peripheral tissues; ABCA1 is required for LXR agonists to stimulate cholesterol efflux towards the acceptors APO1 and pre-HDL, thus completing the assembly of HDL [89].

Other well-known LXR targets include:

- ABCG1, intracellular transporter regulating the cholesterol efflux to HDL, which can act synergistically with ABCA1 to promote HDL-mediated cholesterol removal;
- a cluster of apolipoprotein genes, including APOE and APO1, that are essential for the establishment of lipoproteic complexes mediating lipid transport and catabolism and are transcriptionally induced by LXRs via a multienhancer region;
- lipid-remodeling genes, such as *PLTP, CETP* and *LPL*, which hydrolyse tryglycerides to facilitate an efficient RCT from the periphery to the liver;
- SREBP1C and other transcription factors able to induce the expression of downstream prolipidogenic enzymes. SREBP1C directly induces the fatty acid synthase FASN and the acetylCoA carboxylase (ACC) in order to promote lipid synthesis. Moreover, SREBP1C can also be regulated via insulin, suggesting the importance of glucose and lipid metabolism crosstalk in cell homeostasis;
- Angiopoietin-like protein 3 ANGPTL3, which is a key regulator of lipid metabolism in the vascular endothelium. Mouse models with mutated *ANGPTL3* display low levels of plasma triglycerides, cholesterol and non-esterified fatty acids, a condition which is reversed after *ANGPTL3* overexpression [94].

5.2 LXR and cholesterol uptake, absorption and excretion

The LXR and SREBP pathways act in a coordinated and reciprocal fashion to maintain cellular and systemic cholesterol homeostasis: SREBP is activated at low cellular cholesterol levels, while LXR are induced at elevated cellular cholesterol levels [89, 96]. As an example, the SREBP2 pathway promotes LDL receptor expression, thus mediating LDL clearance from the circulation via uptake by the liver and macrophages. LXR negatively regulate the LDL receptor via the activation of the inducible degrader of LDL (IDOL), which acts as an E3 ligase for the LDL receptor and is responsible for its ubiquitination and lysosomal degradation [89, 96].

LXR plays also a role in cholesterol absorption via the induction of the transporters ABCG5 and ABCG8, which heterodimerize and mediate the apical efflux of cholesterol from enterocytes, the intestinal absorbing cells. Genetic loss of *ABCG5* and *ABCG8* is associated with hypercholesterolemia, increased intestinal absorption and reduced binding and excretion of sterols; these effects are reverted with LXR agonist treatment due to the transcriptional induction of other ABCG members, not affected by the genetic aberration, as compensatory mechanism [89, 97].

At last, the intestinal secretion of cholesterol from the enterocytes is stimulated by bile acids and phospholipids, contributing to cholesterol removal; this pathway is dependent on ABCG5 and is induced upon LXR agonistic stimulation [89, 98].

5.3 LXR contribution to glucose metabolism

Besides being important in lipid and cholesterol homeostasis, LXRs are also key regulators in carbohydrate metabolism. LXR agonists hinder the expression of gluconeogenetic enzymes in the liver, where phospho-enolpyruvate carboxykinase (PEPCK), one of the rate-limiting enzymes in gluconeogenesis, is impaired in its expression by LXR agonists, thus leading to a decreased hepatic glucose output [94, 99]. Moreover, LXRα activation increases the basal glucose uptake in adipocytes via GLUT1 transporters; LXR agonists induce transcriptional activation of GLUT1 and the same occurs on the insulin-stimulated glucose transporter GLUT4 in white adipose tissue [94, 99].

Furthermore, LXR agonists hinder the expression of glycolytic enzymes like the 6-phosphofructo-2-kinase –suppressed in white adipose tissue– and induce negative regulators of this pathway, as the pyruvate dehydrogenase kinase 4 [94]. LXR agonists also promote fatty acid β-oxidation and inhibit glucose oxidation in white adipose tissues.

The described mechanisms of action suggest that LXR can mutually affect carbohydrate and lipid metabolism in metabolically active tissues, like the adipose tissue, by promoting glucose uptake, inhibiting glycolysis and inducing gluconeogenesis, and enhancing lipolysis and fatty acid β-oxidation [89, 100, 101].

5.4 Role of LXR in the cellular energy balance

LXRs are able to mediate processes responsible of the nutritional status and the cellular energy balance via leptin and the uncoupling protein UCP1 [94]. Leptin is a hormone involved in the control of the body nutritional status, and lack of leptin or lack of its signaling are linked to obesity. LXR agonists downregulate leptin expression, which eventually causes an increased energy intake [94].

UCP1 is a member of the family of uncoupling proteins and it employs the proton gradient generated in the electron chain to transport protons across the mitochondrial membrane; this event generates heat and is associated with catabolism. LXR agonists decrease UCP1 transcription, causing a decrease in energy expenditure [94]. The inhibitory role of LXR on leptin and UCP1 expression suggests that LXR affects the energy balance by shifting the metabolism to biosynthesis rather than catabolism, which can hinder the potential use of LXR agonists for obesity treatment in the clinic [94].

On the other side, LXR agonists decrease the expression of 11β-hydroxysteroid dehydrogenase1 (11β-HSD1). 11β-HSD1 is a crucial enzyme responsible for the activation of glucocorticoid precursors (cortisone) to active glucocorticoids (cortisol), whose enhanced levels or aberrant activity promote insulin resistance and obesity [94, 102]. LXR agonists decrease the expression of 11β-HSD1, thus antagonizing the effect of glucocorticoids in adipocytes *in vitro* and obese mouse models *in vivo* with an improvement on the overall obesity condition [94, 103].

5.5 LXR activity in the inflammatory response via transrepression mechanisms

LXR activation downregulates the expression of pro-inflammatory mediators, such as cyclooxygenase 2, inducible nitric oxide synthase (iNOS) and interleukin 6 (IL6), as was suggested by transcriptional studies on LPS-activated macrophages [94]. LXR appears to be at the crossroad between inflammation and lipid metabolism and their interplay is dependent on the association of LXR with heteromeric partners –such as peroxisome proliferator-activating receptors (PPAR)– which function as alternative to the canonical RXR subunits, thus leading to the trans-repression of inflammatory genes [92].

LXR antagonizes inflammatory gene expression downstream of TLR4 signaling, such as IL1β and TNFα; vice versa, blocking TLR3 and TLR4 impairs the function of LXR in cholesterol homeostasis, as further evidence of the cross talk between the inflammatory and metabolic pathways [92]. Moreover, LXR inhibits known NFκB-target genes, such as iNOS, IL6 and IL1β, COX1, MMP9 and the pro-flogistic cytokines CCL2 and CCL7, but this effect is not observed on TLR3-downstream genes [92, 104, 105].
In particular, LXR activation recapitulates the function of PPARγ, a major immunosuppressive mediator: LXR sumoylation by SUMO2/SUMO3 ligases allows the preservation of the repressor complex on the promoter of inflammatory genes, inhibiting their transcriptional activation. Moreover, LXR and PPARγ trans-repress different sets of proinflammatory genes, but the mechanism of this parallel and yet diversified regulation remains to be fully elucidated [92, 106].

LXR-mediated reciprocal regulation of lipid synthesis and inflammation is unraveled in macrophages. LXR impairs the expression of iNOS, COX2, IL6, granulocyte colony stimulating factor (GCSF), monocyte chemoattractant protein (MCP) 1, MCP3 and MCP1β in response to bacterial infections or LPS stimulation *in vitro*. This occurs via a LXRα/LXRβ isoform specific mechanism and it is due to antagonism towards the NFκB pathway; in paral-

lel, NFκB antagonizes LXR signaling on lipid synthesis, suggesting a reciprocal negative influence of these two transcriptional routes [105, 107].

6. Liver X receptors in cancer

LXRs have a dual role in cancer biology, either suppressing cancer cell proliferation or inhibiting anti-tumor immunological responses, allowing the tumor escape from immune surveillance [107]. Evidence of a tumor suppressive activity of LXR is provided in prostate cancer, where LXR agonists increase the protein expression of the CDK inhibitor p27; at the same time LXR activation decreases the expression of the ubiquitin ligase S-phase kinase associated protein 2 (SKP2), which targets p27 for degradation.

Similar post-transcriptional effects were observed for the CDK inhibitor p21, but not for p27, in ovarian cancer [108, 109]. Moreover, LXR induction promotes the phosphorylation of Rb protein in T lymphoblasts, while in colon cancer LXR agonists cause the downregulation of c-Myc and of different CDKs [108, 110]. In pancreatic cancer, LXR agonists decrease cell proliferation and diminish SKP2 transcriptional and post-transcriptional levels; this event is associated with downregulation of the expression of EGFR, possibly suggesting a crosstalk between these two signaling pathways [108, 111].

LXR affects metabolic genes also in cancer: LXR ligand treatment of prostate cells increases the levels of the LXR target ABCG1 and alters the cell membrane lipid raft signaling pathway via AKT1, resulting in apoptosis [108, 112]. LXR activation in breast cancer models induces apoptosis, abnormal cholesterol efflux and cell proliferation blockage, mechanisms possibly mediated by the LXR-induced expression of FASN and SREBP1C [108, 113].

Melanoma cells treated with LXR ligands display an increased APOE expression with consequent decrease in invasion and metastasis. At last, LXR signaling in prostate cancer increases the expression of the suppressor of cytokine signaling 3 (SOCS3), promotes apoptosis and impairs cell proliferation, migration and invasion, thus depicting a potential additional link between inflammation and metabolism [108, 114].

The LXR pathway also affects hormone signaling in hormone-dependent malignancies: as an example, the LXR agonist T0901317 weakly binds to the androgen receptor and partially acts as an anti-androgenic molecule. Moreover, the overexpression of LXRα and the treatment with LXR ligand increases the expression of the enzyme SULT2A1 –responsible for androgen inactivation– and diminishes the expression of the steroid sulfatase –an en-

zyme processing androgen precursors into their active form–, thus hindering prostate cancer progression [108].

Similar results were reported for the estrogen sulfotransferase SULT1E1 in liver and breast cancer where treatment with LXR ligands decreases estrogen receptor α (ERα) activity, thus disrupting a major proliferative pathway in breast cancer [108, 115].

Little evidence has been so far provided for the tumor-promoting role of LXR, and this mainly relates to its positive effect on lipidogenesis. As an example, the downregulation of the SREBP1 pathway has been correlated to poor prognosis in HCC patients at 3 years follow-up [116].

7. Cancer associated fibroblasts (CAFs)

Fibroblasts are elongated cells with spindle-like morphology, and constitute the main cellular component of the extracellular matrix, which is located in stromal layers in between functional parenchyma [117]. The origin of fibroblasts has not been unequivocally clarified: they might be generated via endo-EMT events or might derive from mesenchymal stem cells originated in the bone marrow [118]. The absence of specific and *in vivo* reliable fibroblast markers is a major hurdle for their ultimate identification and origin tracing, even if the expression of the protein FSP1 and the classification according to HOX gene transcriptomic signature serve this purpose, respectively [117, 119, 120].

Physiologically, fibroblasts are quiescent –in G0/G1 arrest or in slow self-renewal–, with negligible metabolic and transcriptomic activities and with no migratory and matrix deposition faculties [118]. In a proinflammatory context, resting fibroblast become activated into myofibroblasts, e.g. as result of stimulation by growth factors and cytokines. This event is accompanied by ultrastructural changes –namely the acquisition of an elongated, stellate shape, the organization of actin stress fibers, and the expression of activation markers, such as αSMA, calponin, PDGFRβ and SM22– as well as by proliferation and high metabolic, secretory and transcriptional activities [117, 118].
Moreover, myofibroblasts acquire synthetic and migratory phenotypes in order to depose major connective tissue components, as fibronectin, collagen (especially type I), proteoglycans, tenascin C, and proficiently heal the wound [117].

The activation of fibroblasts into myofibroblasts is a self-resolving and self-limiting event, which physiologically decays once the *noxa* is removed. However, chronic inflammation, due to the persistence or the ineffective removal of the noxious causative agent of inflammation, results in a chronic and stable activation of myofibroblasts, with detrimental effects on tissue homeostasis. This event occurs in malignancies, where abnormally active myofibroblasts –the cancer associated fibroblasts or CAFs– create a reactive tumor stroma, which sustains cancer progression; interestingly a CAF-cancer cells crosstalk occurs, leading to a reciprocal metabolic reprogramming.

Reactive tumor stroma and fibroblast activation occur already at the stage of *in situ* carcinoma, when the primary epithelial tumor is spatially confined, contained by the basal membrane and relies only on the perfusion of nutrients from pre-existing vasculature. One key player in reactive tumor stroma

establishment is the tumor-produced VEGF, which induces fibroblast proliferation and activation. PDGF and fibroblast growth factor 2, FGF2 also have a major role in fibroblast proliferation [121-124]. On the other hand, TGFβ promotes myofibroblast differentiation, contributing to a tumor-permissive extracellular milieu [125].

During the transition from *in situ* to invasive carcinoma the desmoplastic extracellular tissue and the activated CAFs continue to sustain tumor expansion at several levels, e.g. promoting an ECM favorable to cell migration, increasing vascular permeability to enhance systemic dissemination, preserving the cancer stem cell niche, supporting tumor cell metabolism and allowing immune-tolerance towards the malignancy [117]. As an example, YAP1 activation in fibroblasts is linked to ECM stiffening, which enhances cancer cell invasion; at the same time the secretion of MMPs permits cancer cell invasion through the surrounding connective tissue, in order to reach the vessels for systemic dissemination, while the MMP stromelysin 1 allows the cleavage of E-cadherin, loosening cell-cell contacts and favoring cell motility [126-128].

CAFs produce mitogenic and pro-angiogenic factors, such as SDF1, which increases endothelial recruitment to foster neoangiogenesis; notably, SDF1 and TGFβ cooperate to sustain this phenotype in hepatocellular carcinoma [129, 130]. Moreover, Galectin1-expressing CAFs have increased VEGF secretory abilities, which promotes endothelial cell proliferation, migration and tubular assembly *in vitro*. In addition, hypoxia is sensed by CAFs and this modulates VEGF release via a HIF1α/GPER (G protein-coupled estrogen receptor 1) mechanism in breast cancer [131, 132]. Moreover, CAFs contribute to the maintenance of the cancer stem cell nice via production of IGF and periostin, which enhances WNT-driven self-renewal of CSCs [133, 134].

CAFs are characterized by a glycolytic metabolism –which is under the influence of the growth factors TGFβ, PDGF, VEGF, HIF1α- sensed hypoxia and ROS– and high catabolic and autophagic activities, which allow to reuse nutrients. These events underlie the reciprocal influence between CAF-tumor cells and CAF-immune cells [117]. In particular, lactate, ketone bodies and free fatty acids produced and secreted by CAFs positively affect tumor cell proliferation and survival, allowing their metabolic support. On the contrary, a decrease in tryptophan and arginine secretion causes a diminished proliferation of T effector cells and a blockage in their activation, allowing the establishment of tumor immune-tolerance; this event might be also due to a potential metabolic competition occurring between CAFs and immune cells [117].

7.1 CAF contribution to HCC

Liver fibrosis and cirrhosis precede hepatocellular carcinoma, differently from what occurs during the onset of other malignancies. The cirrhotic predisposition illustrates the major microenvironment contribution to the development of liver cancer, distinguishing HCC from other carcinomas, where the desmoplasia may occur once the tumor has already been established, but it is not propaedeutic to it [135]. In accordance to this, the presence of liver cirrhosis is a unifying risk factor with one out of three liver cirrhotic patients predisposed to HCC onset [136].

The pericyte-like liver fibroblasts (hepatic stellate cells, HSC, or Ito cells, as described above) reside in the sinusoidal spaces of Disse, in between liver endothelial sinusoid cells and hepatocytes. Quiescent HSCs are identified by surface markers, either common to the fibroblastic lineage or liver-specific: these markers include PDGFRβ, the enzyme lecithin retinol acyltransferase (LRAT), the glial acidic fibrillary protein GFAP and the transcription factor heart and neural crest derivatives expressed protein 2 (HAND2) [137]. It is worth noting that HSCs also share activation markers with other fibroblastic lineages, such as αSMA, calponin, SM22 and DDR2, a receptor tyrosine kinase activated by collagen type I [137].

Ito cells are known to store vitamin A in droplets, as retinol esters, and the loss of this property is a clear hallmark of their activation. In fact, an elevated expression of the enzymes LRAT –responsible of the conversion of retinol to retinyl esters– and of the alcohol dehydrogenase ADH, which catabolizes retinol to retinal aldehyde, are associated to worse HCC prognosis [138, 139].

HSC activation occurs in response to major proliferative and fibrogenic cytokines, such as VEGF and PDGF, which have both proliferative and pro-migratory effects on the hepatic stellate cells, and CTGF and TGFβ, which elicit major pro-fibrogenic and activation functions [21, 140, 141].

In addition to this, autophagy –linked to endoplasmic reticulum (ER) stress– is considered a major event in HSC activation, which involves the production of free fatty acids after the cleavage of the stored retinyl esters [142]. The induction of the JNK1 pathway following ER stress is also responsible for liver fibroblasts activation into myofibroblasts, leading to fibrosis [143]. Free cholesterol also activates HSCs, as demonstrated by the positive outcome of the use of statins –which antagonize hepatic accumulation of cholesterol– in decreasing NASH, possibly via RhoA and Rac signaling [144]. Finally, also ROS play an important role in Ito cell activation, and the loss of Nox1 and Nox4 is able to attenuate liver inflammation and fibrosis in a CCl_4-induced HCC mouse model [145].

HSC activation into myofibroblasts significantly contributes to liver fibrosis and cirrhosis via a plethora of mechanisms, including abnormal deposition of collagens (especially type I), laminins, fibronectin, syndecans, other glycans and production of MMPs. Moreover, the high expression of lysyl oxidases, responsible to produce crosslinks between ECM components, increases matrix stiffness, allows anchorage-dependent proliferation and migration of HCC cells [135, 137]. An increased stiffness of liver connective tissue is also associated with expansion of liver progenitors (oval cells) and with abnormalities in ductular architecture, leading to dysplasia of the liver parenchyma. In accordance to this, high liver stiffness, measured via elastography, is a clinical predictor of HCC evolution [146, 147].

HSC activation is associated with a decreased immunosurveillance, as for the HSC-derived TGFβ –which enhances tolerant Treg expansion– and induced differentiation of macrophages and neutrophils in their immune-tolerant M2 and N2 subsets, respectively [46, 148].

Ito cell activation also exerts pro-angiogenic effects, either via the production of soluble mediators, as VEGF and angiopoietin I and II, or via a direct influence on vascular remodeling: *in vitro* co-culture studies of HSC and endothelial cells reported a HSC-promoted sprouting of vessels and an organization of these two cellular populations to reproduce the physiological vascular assembly [149, 150].
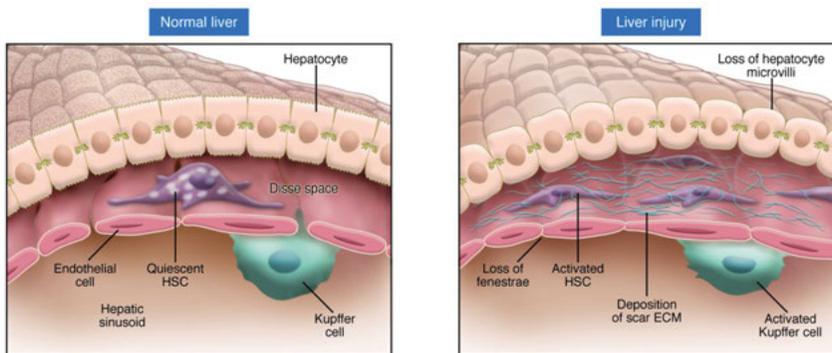

Figure 6. Quiescent and activated hepatic stellate cells in liver physiology and liver injury [151] (Image reproduction allowed by the Creative Commons Attribution 4.0 International License)

Present Investigations

Hepatocellular carcinoma is a malignancy with increasing prevalence worldwide. Currently, no definitive therapeutic approaches are available for HCC treatment; further elucidation of HCC pathogenic and molecular mechanisms is needed, in order to identify future effective clinical treatments. The present study aimed to address:

I. The identification of small molecules able to decrease the mesenchymal properties of established cancer cell lines, including HCC models.

II. The elucidation of the crosstalk between TGFβ and LXRα, its influence by Snail-dependent and -independent pathways and its relevance to hepatocellular carcinoma pathogenesis and treatment.

III. The contribution of TGFβ and LXRα pathway crosstalk on the myofibroblast population involved in HCC progression and malignancy.

The present investigations unraveled the relevance of LXRα in the context of hepatocellular carcinoma and the interplay of this signaling pathway with the TGFβ route, an established contributor to HCC onset and progression.
Our results suggest the potential use of LXR agonists for the treatment of HCC, possibly targeting both the liver cancer parenchyma and the HCC microenvironment.

Paper I: Chemical regulators of epithelial plasticity reveal a nuclear receptor pathway controlling myofibroblast differentiation

Epithelial to mesenchymal transition is an important event in cancer, characterized by multiple transitory cell stages and driven by TGFβ. Sustained TGFβ activity in tissues promotes the accumulation of newly deposited matrix, terminal differentiation of myofibroblasts and chemotaxis of diverse immune cells, all contributing to the fibrotic phenotype [152]. The aim of this study was to identify chemical agents and molecular pathways controlling the terminal stages of TGFβ-induced EMT and myofibroblast differentiation by high throughput screening of a chemical library.

We employed a human keratinocyte cell model, in which TGFβ potently induced epithelial-mesenchymal transition, and a robotic high-content imaging platform to screen chemical compounds with the aim to identify modulators of TGFβ-induced epithelial plasticity. By counter-screening against anti-TGFβ receptor kinase inhibitors, we identified a set of compounds including hydroxycholesterol derivatives, which act as agonists of the nuclear liver X receptor.

Several compounds, among which specific LXR agonists, blocked myofibroblast differentiation and αSMA expression in diverse cell models. While TGFβ promotes myofibroblast terminal differentiation and activation, agonist-dependent LXR activity counteracted such differentiation. This novel role of LXR in myofibroblast differentiation and the protective effect LXR agonists may offer an opportunity to inhibit TGFβ-induced pro-fibrotic effects, and suggests the possibility that anti-lipidogenic therapy may also be relevant in fibrotic disorders and cancer.

Paper II: Snail mediates crosstalk between TGFβ and LXRα in hepatocellular carcinoma

LXR nuclear receptors are involved in a plethora of malignancies and their activation has a significant role in antagonizing cancer progression [110, 153, 154]. The present investigation aimed at defining whether TGFβ and LXRα pathways crosstalk in the context of hepatocellular carcinoma and to elucidate the molecular mechanism responsible for such an interplay. The final aim of this study was to assess if LXR agonists would be a relevant clinical treatment for HCC possibly via inhibition of TGFβ signaling, a well-known signaling route responsible for HCC onset and progression.

We screened a panel of 14 compounds, which scored promisingly in the previous study, in representative epithelial and mesenchymal HCC cell lines, Hep3B and Snu449, respectively. LXR agonists decreased the mesenchymal properties in Snu449, usually associated with higher HCC aggressiveness and worse prognosis in patients. Molecular analysis identified the transcription factor Snai1, involved in EMT, to be a target of the LXRα pathway.
In particular, LXRα activation with specific agonists repressed the expression of Snail at the transcriptional level, thus counteracting Snail induction by TGFβ. This event was biologically significant with respect to the induction of mesenchymal features by TGFβ, which were Snail-dependent and became abrogated in the context of an active LXRα signaling.
On the other side, TGFβ and LXRα pathways positively interacted in a Snail-independent manner in order to promote a cytostatic blockage of HCC

cells: Hep3B proliferation was hindered with no increase in apoptosis, while TGFβ-induced pro-survival signals were further enhanced in the context of active LXRα signaling. These events result in a non-proliferative, non-apoptotic cellular status, which might potentially hinder HCC progression *in vivo*. At last, the TGFβ-promoted production of ROS, which act as signaling molecules at low concentrations also in the context of cancer, was antagonized by active LXRα in a Snail-dependent fashion.

In conclusion, the dual cooperative/antagonistic interaction between TGFβ and LXRα routes, which depends on the biological phenotype, might justify the employment of LXR agonists as cytostatic agents in the contest of HCC. These agents might be relevant not only for inhibition of HCC cell proliferation, but might also simultaneously antagonize ROS-mediated signaling and decrease the expression of mesenchymal features, the latter associated with a poorer HCC clinical outcome.

Paper III: LXRα limits the pro-fibrotic action of TGFβ in liver cancer-associated fibroblasts

LXR signaling is relevant for the treatment of fibrotic conditions associated with diverse pathologies. As an example, LXRs negatively regulate the expression of the fibroblast growth factor 21 (FGF21) in an experimental fatty liver- induced fibrosis model [155-157].

The present investigation aimed at defining whether the LXRα pathway could counteract TGFβ activity in HCC patients derived-fibroblasts, possibly antagonizing their activation into myofibroblasts. The final aim of this study was to assess if LXR agonists could be a relevant treatment of HCC, not only due to their activity on the tumor liver parenchyma, but also because of their antagonistic effect on the activation of the resident fibroblast population, which is known to provide trophic support to the development and sustainability of cancer.

We focused on representative HCC epithelial and mesenchymal cell lines; we identified an inverse correlation between LXRα and αSMA –a major marker of fibroblast activation–, suggesting the potential antagonistic role of LXRα pathway in fibroblast-myofibroblast transition. Moreover, LXRα activation counteracted the expression of TGFβ-induced markers of fibroblast activation, such as calponin, SM22 and αSMA itself.

Transcriptional studies on the *ACTA2* (*αSMA*) promoter elucidated that LXRα decreased its activation in a dose-dependent manner and that this event occurred due to a possible competition between LXRα and Smad3, which is known to activate αSMA in TGFβ stimulated cells, for the binding

to the *ACTA2* promoter. This event might possibly lead to inefficient fibro-blast activation, thus impairing the support function of liver fibroblast in the growth of HCC cells, as observed with growth promoting assays.

In conclusion, LXRα might exert an antagonistic role towards TGFβ also in liver fibroblasts, possibly validating the use of these compounds in HCC due to their antagonistic effects mediated at the tumor microenvironment level.

Future Perspectives

Hepatocellular carcinoma is a "silent" malignancy, where patients arrive to the clinical attention due to symptomatic signs unrelated to HCC itself, e.g. HBV infection or alcohol abuse. In such a scenario, HCC is mainly associated with an irreversible cirrhotic liver phenotype, also characterized by dysplastic and non-functional liver parenchyma. These events determine the difficult and partially effective treatment of HCC, which then relates with the poor clinical outcome and limited survival of patients.

TGFβ is a pleiotropic cytokine with an established role in the onset, progression and metastasis of several malignancies, including HCC. In hepatocellular carcinoma, TGFβ primarily displays cytostatic, tumor suppressive effects in the early phases of tumorigenesis. However, TGFβ is one of the main drivers of HCC progression during the late stages of disease progression, as it diminishes the functionality of liver parenchyma, promotes the microenvironmental support to the cancer tissue and favors tumor immune-tolerance, thus eliciting a clear tumor promoting action.

Paper I

In paper I, we employed a high content imaging screening platform to identify small modulators of epithelial plasticity during short-term TGFβ stimulations of established HCC cell lines, among other *in vitro* models.

It would be of interest to extend the screening of small molecules with a pharmaceutical potential on long-term TGFβ exposed HCC cell lines, in order to elucidate the signaling mechanisms responsible for the switch towards the pro-tumorigenic TGFβ action and how these can be pharmacologically inhibited.
Moreover, it would be clinically relevant to perform such screening on tissue samples representative of HCC pathogenic evolution, from fibrosis to cirrhosis till frank HCC. This analysis would potentially allow finding pharmacological molecules able to inhibit the transition from fibrosis-cirrhosis to HCC, in order to avoid the onset of such malignancy and to possibly treat the patients before it is reached an irreversible clinical condition.

Paper II

In Paper II, we elucidated that the LXRα pathway –involved in the maintenance of the liver epithelial phenotypes and functions– can interact with TGFβ, an established driver of HCC, depending on the biological events.

In particular, TGFβ and LXRα antagonism aims at decreasing the mesenchymal properties of HCC cells, representative of a more aggressive clinical phenotype, in a Snail-dependent fashion. However TGFβ and LXRα routes act cooperatively in a Snail-independent manner to hinder HCC proliferation. This results in a cytostatic blockage of liver cancer cells, which might still hold liver epithelial functions; apoptosis of liver cells is not enhanced when both pathways are co-activated, which might preserve a minor liver function also in HCC.

Thus, the use of LXR agonists for HCC treatment might have relevance as a cytostatic chemotherapeutic approach (to be implemented with radiotherapy in the clinics), which diminishes the aggressiveness –mesenchymal properties– of liver cancer cells without decreasing the liver cell population, thus allowing the maintenance of a limited liver function.

It would be of interest to expand this mechanistic study to *in vivo* HCC models (diethylnitrosamine, DEN, and carbon tetrachloride, CCl$_4$) to validate our results in a more complex organism. At the same time, the assessment of LXRα activation and its correlation to Snail levels and activity might also be important to assess in closer-to-clinic specimens, e.g. HCC biobank tissue sections. In the latter case, the described investigation should be performed either in representative samples of the pathogenic evolution to HCC, as previously discussed, or on HCC samples of different etiology (HCV, HBV, NASH, alcohol abuse), in order to evaluate if the TGFβ-LXRα axis crosstalk differently under discrete HCC causative conditions.

Paper III

In Paper III, we described that TGFβ and LXRα signaling pathways counteract each other in the context of HCC fibroblast-to-myofibroblast activation, a major feature of cancer progression, which correlates with cancer aggressiveness and malignancy. We suggested that this antagonism occurs at the promoter level of *ACTA2* (*αSMA*) gene, a well-known hallmark of fibroblast activation. This event might account for an ineffective growth advantage provided by liver cancer associated-fibroblasts when co-cultured with HCC cell lines in the context of an active LXRα pathway.

Our study suggests that the potential use of LXR agonists in the clinic might thus be relevant not only due to their effect on the liver cancer parenchyma, but also in order to abolish the trophic support provided by the liver malignant microenvironment.

Further investigations are needed to clarify the mechanism behind the LXRα-Smad3 interference to bind to the *ACTA2* promoter and additional experiments are needed to elucidate the biological relevance of this antagonism on the fibroblast population itself, e.g. using collagen contraction assays, as employed in Paper I. This study could also be further expanded to liver fibroblasts derived from patients with different etiologies of HCC; such fibroblast subsets are currently available in our lab.

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No man is an island entire of itself;
every man is a piece of the continent,
a part of the main
John Donne

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