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Activins and follistatins: Emerging roles in liver physiology and cancer

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Abstract

Activins are secreted proteins belonging to the TGF-β family of signaling molecules. Activin signals are crucial for differentiation and regulation of cell proliferation and apoptosis in multiple tissues. Signal transduction by activins relies mainly on the Smad pathway, although the importance of crosstalk with additional pathways is increasingly being recognized. Activin signals are kept in balance by antagonists at multiple levels of the signaling cascade. Among these, follistatin and FLRG, two members of the emerging family of follistatin-like proteins, can bind secreted activins with high affinity, thereby blocking their access to cell surface-anchored activin receptors. In the liver, activin A is a major negative regulator of hepatocyte proliferation and can induce apoptosis. The functions of other activins expressed by hepatocytes have yet to be more clearly defined. Deregulated expression of activins and follistatin has been implicated in hepatic diseases including inflammation, fibrosis, liver failure and primary cancer. In particular, increased follistatin levels have been found in the circulation and in the tumor tissue of patients suffering from hepatocellular carcinoma as well as in animal models of liver cancer. It has been argued that up-regulation of follistatin protects neoplastic hepatocytes from activin-mediated growth inhibition and apoptosis. The use of follistatin as biomarker for liver tumor development is impeded, however, due to the presence of elevated follistatin levels already during preceding stages of liver disease. The current article summarizes our evolving understanding of the multi-faceted activities of activins and follistatins in liver physiology and cancer.

Key words: Activin; Inhibin; Follistatin; Follistatin-like protein; Transforming growth factor β; Liver cancer

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INTRODUCTION

The activin family

Activins are cytokines belonging to the TGF-β family of growth and differentiation factors[1] and were named according to their first identification as activators of follicle-stimulating hormone (FSH) release from pituitary cells[2,3]. Like TGF-β, activins are formed *via* the covalent dimerization of two subunits[4]. So far, five different subunits participating in the formation of activins have been identified. The subunits activin beta A, beta B, beta

C and beta E were found in humans as well as other mammalian species, while activin beta D has only been identified in Xenopus laevis[5]. The four mammalian beta subunits are each encoded by a single gene, called INHBA, INHBB, INHBC and INHBE respectively[6]. INHBC and INHBE are closely linked in several species and are thought to have arisen from tandem duplication of an ancestral gene[7].

The different activin subunits can form homo- as well as heterodimers. A homodimer of two beta A subunits is called activin A, while a heterodimer of a beta A and a beta B subunit is called activin AB. The nomenclature for dimers of the other subunits follows the same scheme. While activins AB and AC have been described under physiological conditions *in vivo*[8,9], we and others have demonstrated the formation of activins AE, BC and CE after ectopic expression of the respective cDNAs in various cell lines[10-12]. Activin subunits are synthesized as pro-proteins of 350 to 426 amino acids[6]. The proteins are glycosylated in the pro-domain region, but addition of the carbohydrate group seems to be dispensable for secretion. This is in contrast to the related inhibin alpha subunit, a member of the TGF-β family and dimerization partner of activin subunits (see below)[13]. Dimers are created by intermolecular disulphide bond formation between the sixth of nine conserved cysteines in the mature proteins. The other cysteines are involved in the formation of intramolecular disulphide bonds, creating the so-called cysteine knot, typical for members of the TGF-β family and required for their biological activity[14].

Following dimerization, the protein is cleaved by pro-protein convertases of the subtilisin/kexin family in the ER and Golgi, producing a mature peptide chain of 115 or 116 amino acids. While the biologically active protein is secreted as a dimer of the mature peptides only, it has been suggested that the pro-region is required for correct folding, dimer formation and secretion[15]. Unprocessed, dimeric activin A was found to be biologically inactive[16]. Monomers have been reported to retain some affinity for the receptors of dimeric activin A but do not cause activation[17]. In addition to dimerization with another beta subunit, activin beta A and activin beta B can form heterodimers with the inhibin alpha subunit, giving rise to inhibins A and B, both inhibiting FSH release[18]. It remains uncertain if inhibin C exists, as there was evidence for the formation of a dimer between activin beta C and inhibin alpha in some[19] but not all reports[10].

Activin signal transduction

Like other members of the TGF-β family, activins are believed to signal *via* single-pass transmembrane receptors with an intracellular Ser-Thr kinase domain. This has been proven for activins A, B and AB. Activin A first binds to dimers of the type Ⅱ receptors ActR-Ⅱ (aka ACVR2) or ActR-ⅡB (aka ACVR2B), leading to the (preferential) recruitment and phosphorylation of dimers of the type I receptor ALK4 (aka ActR-IB/ACVR1B)[20]. While binding to the same type Ⅱ receptors, activins B and AB preferentially recruit ALK7 (ACVR1C) as type I receptor[21]. Upon ligand binding, receptors are typically internalized[22]. It has been questioned however, if this internalization is generally necessary for signal transduction[23]. As a consequence of activation, receptor-regulated Smads (R-Smads) are recruited to the receptor complex and phosphorylated by the type I receptor. This process is supported by accessory proteins like SARA and the motor protein kinesin-1. Depending on the identity of the receptor, either Smad 2 and Smad 3 (ALK4, ALK5, ALK7) or Smad 1, Smad 5 and Smad 8 (ALK1, ALK2, ALK3, ALK6) are recruited and activated[4]. For TGF-β it has been shown that the ligand can recruit different type I receptors, activating different subsets of Smads depending on the cell type[24]. So far, activins have only been shown to signal through Smad 2 and Smad 3[25]. R-Smads then form complexes with the common mediator Smad 4 and translocate to the nucleus where, together with cofactors, they are directly involved in regulation of gene expression.

In addition, recent evidence suggests Smad independent signaling of activin A *via* MAP kinases ERK 1/2 and p38[26] as well as the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway[27]. Rho and JNK were also found to be stimulated by activin A[28].

ACTIVINS IN HEPATIC FUNCTION AND DYSFUNCTION

Beta A and beta B

Activin A represents the most extensively investigated activin. Multiple biological functions of activin A in a variety of cells and tissues have been described, including involvement in mesoderm induction[29], stem cell biology[30], reproductive biology[31], erythroid differentiation[32], systemic inflammation[33], cell death induction[34], wound healing[35], and fibrosis[36]. Knock-out mice for activin beta A show severe defects in craniofacial development and die shortly after birth[37]. Activin A potently inhibits mitogen-induced DNA synthesis in the liver and induces hepatocyte apoptosis *in vivo* and *in vitro*[38-40]. Activin beta A antisense oligonucleotides stimulated cell proliferation in the human hepatoma cell line HLF suggesting a growth inhibitory function of endogenous activin A[41]. In regenerating liver, activin beta A gene expression was reduced at time points when hepatocyte replication took place and was increased at time points when liver regeneration terminated[42]. Other studies, however, have described increased expression of beta A at earlier time points after partial hepatectomy[43,44].

Beside its effects on DNA synthesis and cell growth, activin A also regulates restoration of liver architecture after partial hepatectomy by stimulating collagen production in hepatic stellate cells (HSC) and tubulogenesis of sinusoidal endothelial cells[45,46]. Stimulation of HSC may also

contribute to liver fibrosis and several investigations have found elevated levels of activin beta A in fibrotic and cirrhotic rat livers[47-50]. In hepatocytes, activin A was also demonstrated to stimulate the expression of connective tissue growth factor (CCTF/CCN2), an important regulator of liver fibrosis[51]. Elevated levels of circulating activin A were found in patients with acute liver failure, chronic viral hepatitis, alcohol induced liver cirrhosis and hepatocellular carcinoma (HCC)[52-57]. Elevated serum activin A was also reported in a study with patients suffering from non-alcoholic fatty liver disease (NAFLD), with particularly high levels in the subgroup with non-alcoholic steatohepatitis (NASH)[58]. These patients also had an increased activin beta A/follistatin mRNA ratio in liver tissue. In the same study activin A was shown in Huh7 hepatoma cells to promote collagen Ⅲ and TGF-β 1 expression, matrix metalloproteinase (MMP) activity, induce mitochondrial beta-oxidation and down-regulate fatty acid synthase (FAS) activity. Together these findings suggest an involvement of activin A not only in fibrosis but also in lipid accumulation. A study from our group in contrast, has found reduced expression of activin beta A transcripts in tumor tissue from chemically-induced rat liver tumors[59]. In addition to a pro-apoptotic and a pro-fibrotic effect, activin A has also been linked to hepatic neoangiogenesis *via* stimulation of VEGF expression in human hepatoma cells[60]. With respect to hepatic differentiation, it has been shown that a gradient of activin/TGF-β signaling controls differentiation of hepatoblasts into hepatocytes and biliary cells in the mouse, with high signaling activity required for development into biliary cells[61]. The contributing activin/TGF-β ligands, however, have not been fully identified. Several studies have used activin A as part of protocols to differentiate human embryonic stem cells (hESC) into hepatocyte-like cells[62-65].

Like activin beta A, the beta B subunit is expressed in multiple tissues and organs[11,66]. Knock-out mice for beta B are viable but show defects in eyelid development and female reproduction[67]. When the coding region of the mature peptide of the beta A subunit gene was replaced with the corresponding region of the beta B subunit, the developmental defects of the beta A knock-out mice were only partially rescued indicating differences in receptor activation or downstream signals[68]. In the liver, the function of the beta B subunit is not well characterized. One reason for this might be the low expression level in normal rat liver, where we observed the beta B subunit to be the only activin subunit undetectable by RNAse protection assay[11]. By immunohistochemistry, however, weak staining of beta B was detected in hepatocytes of normal rat livers and in connective tissue septa in fibrotic livers[47]. Activin beta B mRNA was induced in stellate cells of CCl_4 treated rat livers[47] and exposure to the peroxisome proliferator di-n-butyl phthalate led to a transient surge of beta B mRNA expression 6 h after treatment[69]. With respect to biological activities, recombinant activin B, in contrast to activins A and AB, did not inhibit EGF induced DNA synthesis in primary rat hepatocytes[70]. In contrast to the rat, beta A and beta B transcripts are expressed to similar levels in human liver (Rodgarkia-Dara, unpublished observation). Ectopic expression of ALK7, the preferred type two receptor for activins B and AB induced apoptosis in hepatoma cell lines in a Smad and MAPK- dependent manner[71]. Both activin B and ALK7 have been linked to obesity and diabetes, two well-known risk factors for HCC, *via* participation in regulatory circuits in adipose tissue and the pancreas[72-74].

Beta C and beta E

In contrast to beta A and beta B, whose expression level is the highest in reproductive organs, the liver is the organ where the beta C and the beta E subunit reach by far their highest expression levels. The activin beta C subunit was cloned from liver cDNA and demonstrated to be predominantly expressed in hepatocytes by Northern blot analysis and RNAse protection assays[11,44,75,76]. By immunohistochemistry, significant activin beta C expression has been detected in cells from additional organs, including the prostate, ovary, testes, and pituitary gland[10,77]. After partial hepatectomy, a transient down-regulation of activin beta C expression was observed by several studies[42,44,78,79]. We have found reduced activin beta C expression in HepG2 and Hep3B hepatoma cells versus normal liver tissue[80] and a drop of beta C expression was also described in rat hepatocytes during primary culture with and without EGF treatment[44]. In contrast, increased activin beta C expression was reported in rat liver during the development of CCl_4-induced cirrhosis[48,81] and in response to treatment with the peroxisome proliferator bi-n-butyl phthalate[69]. The functions of the activin beta C subunit are controversial. Activin beta C knock-out mice developed normally and liver regeneration after partial hepatectomy proceeded similar in knock-out animals and wild-type littermates[82]. Studies from our group showed that ectopic expression of activin beta C induced apoptosis in human (HepG2, Hep3B) and rat (H4IIEC3) hepatoma cells and delayed liver regeneration in mice[80,83]. In contrast, in AML12 cells, an immortalized mouse hepatocyte cell line, and in primary rat hepatocytes activin beta C increased DNA synthesis[84]. Adenovirus-mediated expression of activin beta C accelerated liver regeneration after partial hepatectomy in rats[85] and association of activin beta C immunoreactivity with mitotic hepatocytes was observed in regenerating liver after partial hepatectomy[42]. Activin C does not activate activin A-responsive promoters and it was suggested that the beta C subunit down-regulates the levels of bioactive activin A *via* the formation of signaling-incompetent activin AC heterodimers in PC3 human prostate cancer cells[9,86]. In a recent study from the same group, it was shown that homodimeric activin C inhibited activin A-induced Smad2 phosphorylation and growth inhibition, and that activin beta C transgenic mice develop prostate, testis and liver pathologies

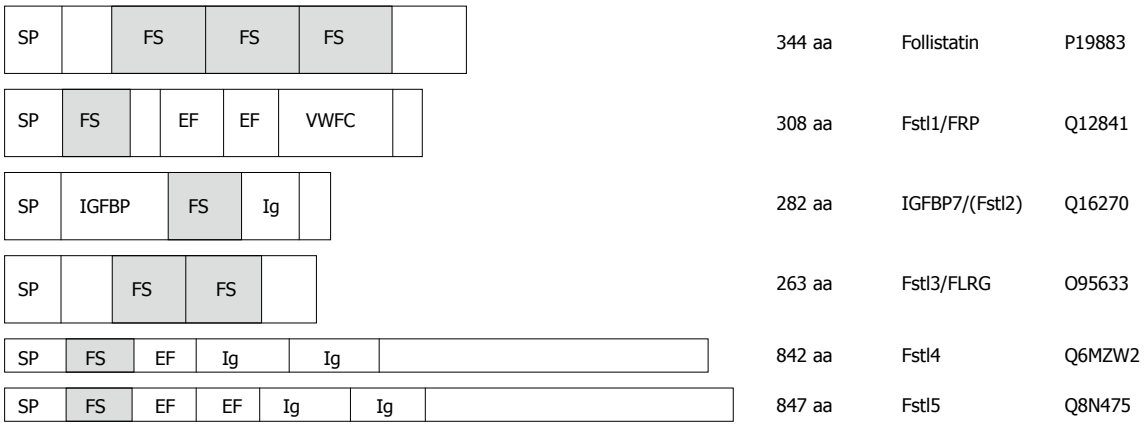

Figure 1 Number and arrangement of follistatin/Kazal-like domains in follistatin-like proteins. SP: Signal peptide; FS: Follistatin/Kazal-like domain; EF: EF-hand domain; VWFC: von Willebrand factor type C repeat; IGFBP: IGF-binding protein N-terminal domain; Ig: Immunoglobulin-like domain. Text to the right shows the number of amino acids (aa), most common name(s) and Uniprot accession number.

suggestive of an activin A antagonistic effect[87]. In line with these observations, elevated beta C immunoreactivity was found in human prostate, testis and liver cancers[87].

Like beta C, also the beta E subunit is highly expressed in the liver, but has been detected at lower levels in several other tissues as well[7,11,88,89]. In the liver of the developing mouse, activin beta E expression could not be detected until the very late stages of embryonic development and peaked at birth[82]. The biological functions and molecular interaction partners of activin beta E remain largely unknown. Like beta C, beta E knock-out (as well as beta C, beta E double knock-out) mice developed normally and showed no impairment of liver function or regeneration[82]. In vitro, overexpression of activin beta E in the human hepatoma cell lines HepG2 and Hep3B, as well as in the murine hepatocyte cell line AML12, caused decreased proliferation and induced apoptosis[12,80]. *In vivo*, transient overexpression of activin beta E inhibited regenerative DNA synthesis in mouse liver[83], while mice constitutively overexpressing the protein showed impaired growth of pancreatic exocrine cells[90]. Following partial hepatectomy, activin beta E mRNA increased rapidly and decreased to near-basal levels after 48 h[82]. We observed a diurnal variation of beta E mRNA depending on food consumption in the rat liver and a surge of beta E expression in response to bacterial lipopolysaccharide (LPS) stimulation was also described[1,89]. Additionally, beta E expression was found and confirmed to be elevated in HepG2 cells as a consequence of phospholipidosis, a lipid storage disorder[91,92]. Expression of activin beta E was also significantly increased in the lung following airway inflammation[93] and in brains of rats infected with Borna disease virus[94]. Interestingly, a neuronal component has also been implied by recent work describing reduced anxiety-related behavior in mice overexpressing activin beta E[95]. Overexpression of the tumor suppressor RASSF1A stimulated expression of beta E, while knock-down of endogenous RASSF1A in nasopharyngeal epithelial cells resulted in beta E downregulation[96]. Finally, in gene chip analysis, mRNA levels from INHBE were found to be altered in HepG2 in response to hypoxia[97]. One possible mode of action for activin beta E was described by Chow *et al*[96], who demonstrated that the expression of Inhibitor of DNA binding 2 (Id2) protein is down-regulated in response to overexpression of activin beta E. Id2 is a known target of TGF-β and a potential oncogene[98]. Large scale analysis identified mutations in the INHBE gene in breast cancer[99]. An evaluation of single nucleotide polymorphisms (SNPs) in genes coding for activins in testicular cancer showed a correlation for the risk of disease and mutations in INHBA but not in INHBB, INHBC or INHBE[100].

FOLLISTATINS AND THEIR ROLE IN ACTIVIN ANTAGONISM AND LIVER DISEASE

Follistatin was discovered as antagonist of activin activity with respect to FSH release from pituitary cells[101]. Sequence analysis of follistatin revealed no homology to the TGF-β family, but the presence of three domains with a similar architecture, namely 10 cysteines spaced in a conserved fashion resulting in a characteristic pattern of intramolecular disulphide bond formation[102]. Accordingly, this domain was termed follistatin domain and bears resemblance to the Kazal domain of serine proteinase inhibitors. Follistatin domains have been identified in a number of additional extracellular proteins and some of these have been filed as follistatin-related proteins or follistatin-like proteins[103-105] (Figure 1). The connection of follistatin and follistatin-like proteins with activin signaling and their involvement in hepatic functions is discussed below. Additional regulation of activin signal transduction takes place at the receptor level by co-receptors, such as cripto, nodal, betaglycan, or BAMBI and intracellularly, for instance by the inhibitory Smad 6 and 7, and has been reviewed elsewhere[106,107].

Follistatin

The biological activities described for follistatin seem to depend largely on its interaction with activins and other members of the TGF-β family. Follistatin, which is expressed in most organs expressing activins[66,108], binds mature secreted activin A with high affinity[109-111]. Complex formation with follistatin completely abolished receptor binding of activin A, thus blocking activin signaling[110,112]. Two follistatin molecules embrace one activin dimer and bury one-third of its residues and its receptor binding sites[113]. Alternative splicing and protein processing of a single follistatin gene results in secretion of three major isoforms containing 288, 303, and 315 amino acids[109]. Of the three follistatin domains present in all follistatin isoforms[114], the first two, but not the third, are necessary for activin A binding[111,115]. Follistatin 288 binds to heparan sulfates, whereas this binding is blocked by an acidic tail in follistatin 315[109]. In addition to binding activins A, B, AB, and E, follistatin was also shown to bind and antagonize myostatin as well as BMPs 2, 4, 6, and 7[88,116-119]. Follistatin administration by intraportal infusion or adenovirus-mediated overexpression caused DNA synthesis and liver growth in the rat, presumably by antagonizing tonic inhibition of hepatocyte proliferation by activin A[120,121]. Following partial hepatectomy, follistatin expression was up-regulated after 24-48 h, the time period in which hepatocyte replication was increased[42]. Administration of exogenous follistatin after partial hepatectomy accelerated liver regeneration but led to impaired restoration of normal tissue architecture and compromised liver function[122-124]. Administration of follistatin in CCl_4-treated rats attenuated the formation of liver fibrosis[125]. These results likely reflect the ability of follistatin to antagonize both growth-inhibitory and pro-fibrotic activities of activin A.

In mouse and rat models of chemically induced liver tumors, we found follistatin expression to be up-regulated in about 60% of tumor tissue samples[59,126]. Moreover, we demonstrated that administration of follistatin stimulated DNA synthesis in preneoplastic rat hepatocytes in an *ex vivo* system, whereas hepatoma cell lines were unresponsive to exogenous follistatin possibly due to autocrine production of follistatin or other activin antagonists[59,126-128]. Knock-out mice for parkin, an E3 ubiquitin ligase implicated in Parkinson's disease and frequently deleted in HCC and hepatoma cell lines[129], develop liver tumors in a follistatin upregulation-dependent fashion[130]. In human HCC elevated follistatin levels were found in the tumor tissue and the circulation of patients[53,59,131]. However, follistatin had no benefit as surveillance biomarker for HCC development in patients with alcoholic and non-alcoholic liver disease (ALD and NAFLD) due to the already elevated levels in the underlying liver pathologies[131]. Whether or not, interaction of follistatin with TGF-β family members other than activins (myostatin, BMP, GDFs) or with angiogenin[132] plays a role in liver tumorigenesis remains to be explored.

Follistatin-like proteins

Follistatin-like 1 (fstl1, also called follistatin-related protein, FRP or Tsc-36) contains only a single follistatin domain and no activin-binding activity has been reported. In fact, the interaction partners of fstl1 on a molecular level have not been identified and its function is far from clear. Fstl1 itself was identified as a TGF-β inducible gene[133] and has been implicated in inflammation and cardioprotection[134-136]. It has been suggested to act as a potential tumor suppressor in epithelial cancers[137-140] but is over-expressed in astrocytic brain tumors[141]. Considering hepatoma cells, we recently demonstrated that the expression of fstl1 is low in HepG2 cells, which show an epithelial morphology/proteome pattern and high in Hep3B cells with fibroblastoid characteristics. These observations suggest fstl1 as potential indicator of epithelial-mesenchymal transition (EMT)[142].

The term follistatin-like 2 (fstl2) is only rarely used. It refers to a protein described to have IGF- (insulin-like growth factor) as well as activin-binding activity and sequence homology with follistatin[143,144]. This protein was also termed mac25 and angiomodulin but is better known as IGFBP7 (IGF binding protein 7) or IGFBP-rP1 (IGF binding protein-related protein 1)[145]. It has been suggested to act as tumor suppressor, because its expression is reduced in neoplastic tissues of different cancer types including liver tumors from SV40T/t antigen transgenic mice[146]. However, the biological relevance of IGFBP7 binding to activin is still unclear. In the course of evolution fish went through whole genome duplication and the term fstl2 has also been used (synonymously with fstl1b) to denote the second zebrafish orthologue of mammalian fstl1.

Among the follistatin like proteins follistatin-like 3 (fstl3), encoded by follistatin-related gene (FLRG), has the highest overall similarity with follistatin and shares its ability to bind TGF-β family proteins, but contains only two instead of three follistatin domains[147]. The FLRG gene was originally identified as a target of chromosomal rearrangement in leukemia[148]. The highest tissue expression of FLRG was found in placenta, whereas highest follistatin expression was found in ovary, testis, and pituitary[147,149]. In HepG2 hepatoma cells, expression of both FLRG and follistatin was induced in response to activin A treatment suggesting that they participate in a feedback loop to restrict activin A signals[150]. FLRG knock-out mice developed increased pancreatic islet number and size, beta cell hyperplasia, decreased visceral fat mass, and hepatic steatosis. This is in line with a physiological role of fstl3 in antagonizing activin and myostatin activity in the pancreas, adipose tissue and liver[151]. Elevated expression of FLRG has been linked to breast cancer[152] and we have found increased FLRG transcript levels in chemically induced rat liver tumors but not in human liver tumor specimens[59].

Recently follistatin-like 4 and 5 were identified as two additional follistatin-related proteins[153], but their expression pattern and function have yet to be worked

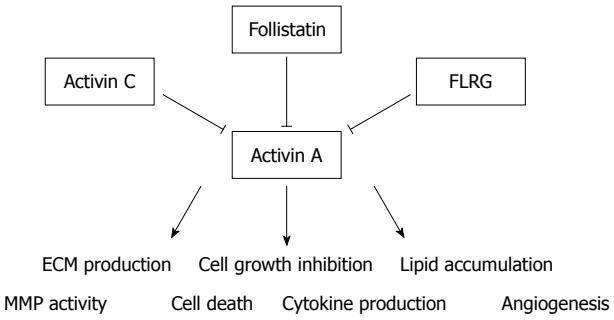

Figure 2 Reported effects of activin A on liver cells and inhibition by activin and follistatin family members. A potential function of the other family members, as agonists or antagonists of hepatic activin signal transduction or as regulators of activin A-independent activities in the liver, remains to be elucidated. ECM: Extracellular matrix; MMP: Matrix metalloproteinase.

out. In addition to the follistatin-like proteins, SPARC, agrin, tomoregulin and others contain one or more follistatin domains, but none of these have so far been connected to activin signal transduction[103,104].

CONCLUSION

Despite the apparent gaps in our knowledge, it is becoming increasingly clear that tightly regulated activin signals are of fundamental importance for the maintenance of liver architecture and cellular homeostasis. While still much has to be learned, especially about the less explored members of the activin and follistatin families, the pace of progress has appreciably sped up in recent years. Deregulated expression of activin A and/or follistatin has been consistently observed in liver cancer in human patients and in a growing number of animal models, and was shown to causally contribute to the inflammatory and fibrotic conditions that promote carcinogenesis (Figure 2). The picture that emerges is that inflammation-associated elevated activin A levels contribute to fibrotic tissue remodelling and cell death of normal hepatocytes, whereas preneoplastic and neoplastic hepatocytes become resistant to activin A-induced growth control, at least in part through overexpression of follistatin. Conditional and liver cell type-specific knock-out of activin beta A and follistatin in mouse hepatocarcinogenesis models could shed further light on the contribution of the activin-follistatin axis to liver cancer development. For the two activin subunits with predominant expression in hepatocytes, namely beta C and beta E, as well as for fstl1, 4 and 5 future efforts should be directed at elucidating their molecular interaction with cell surface receptors or secreted proteins as a prerequisite to better understand their biological activities. Although the complexity of the system may sometimes seem daunting, the hope is well founded that in the not-too-far future, the increasing knowledge on activins and follistatins will translate into improved diagnostic or therapeutic opportunities for patients suffering from chronic liver disease and HCC.

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