BIOCHEMICAL PHARMACOLOGY 77 (2009) 700–712

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Fitting a xenobiotic receptor into cell homeostasis: How the dioxin receptor interacts with TGFβ signaling

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ARTICLE INFO

Article history:
Received 16 July 2008
Accepted 8 August 2008

Keywords:
Dioxin receptor
TGFβ
LTBP-1
Liver fibrosis

ABSTRACT

As our knowledge on the mechanisms that control cell function increases, more complex signaling pathways and quite intricate cross-talks among regulatory proteins are discovered. Establishing accurate interactions between cellular networks is essential for a healthy cell and different alterations in signaling are known to underline human disease. Transforming growth factor beta (TGFβ) is an extracellular cytokine that regulates such critical cellular responses as proliferation, apoptosis, differentiation, angiogenesis and migration, and it is assumed that the latency-associated protein LTBP-1 plays a relevant role in TGFβ targeting and activation in the extracellular matrix (ECM). The dioxin receptor (AhR) is a unique intracellular protein long studied because of its critical role in xenobiotic-induced toxicity and carcinogenesis. Yet, a large set of studies performed in cellular systems and *in vivo* animal models have suggested important xenobiotic-independent functions for AhR in cell proliferation, differentiation and migration and in tissue homeostasis. Remarkably, AhR activity converges with TGFβ-dependent signaling through LTBP-1 since cells lacking AhR expression have phenotypic alterations that can be explained, at least in part, by the coordinated regulation of both proteins. Here, we will discuss the existence of functional interactions between AhR and TGFβ signaling. We will focus on regulatory and functional aspects by analyzing how AhR status determines TGFβ activity and by proposing a mechanism through which LTBP-1, a novel AhR target gene, mediates such effects. We will integrate ECM proteases in the AhR-LTBP-1-TGFβ axis and suggest a model that could help explain some *in vivo* phenotypes associated to AhR deficiency.

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Abbreviations: AhR, aryl hydrocarbon (dioxin) receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CREB, cAMP responsive element-binding protein; ECM, extracellular matrix; FGM, immortalized mouse fibroblasts; HDAC, histone deacetylase; LTBP-1, latent transforming growth factor-binding protein 1; MEF, mouse embryonic fibroblasts; TGFβ, transforming growth factor β.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.08.032

1. Introduction

Transforming growth factor beta (TGFβ) is a pleiotropic extracellular cytokine that has concentrated much attention due to its prominent role in development, tissue homeostasis and wound healing [1], vasculogenesis [2] and in the progression of frequent diseases such as organ fibrosis, autoimmunity and tumor development [3–5]. Because of these diverse functions, and considering that the response to TGFβ largely depends on the phenotype and the microenvironment of the target cell, a great deal of effort is underway to precisely define the signaling networks and the intermediate molecules that fit the TGFβ response to each individual cell type. It is therefore of great importance to determine the upstream molecular pathways that regulate TGFβ expression and activation and those downstream that propagates the signal to the cell nucleus in a cell-specific context. The dioxin receptor (AhR), a well-conserved transcription factor constitutively expressed in most cell types, has been recently linked to endogenous cell functions that control proliferation, differentiation, migration, development, tissue homeostasis, vasculogenesis and cancer [6–11].

It is remarkable that AhR and TGFβ seem to converge to the regulation of the same cellular processes, which suggest a potential connection between their respective signaling pathways. Indeed, *in vitro* and *in vivo* analyses in cell systems and in mouse models lacking AhR expression ($AhR^{-/-}$) have demonstrated that AhR is relevant to maintain the endogenous levels of TGFβ activity, and that the up-regulation of this cytokine could have a role in producing some of the phenotypes identified in $AhR^{-/-}$ mice. It is therefore reasonable to assume that the transcriptional activity of AhR, whether by a direct effect on TGFβ expression/activation and/or by the regulation of intermediate proteins, contributes to modulate physiological processes involving TGFβ. Equally relevant is the potential implication of AhR in regulating TGFβ activity under pathological conditions. Since the mechanisms controlling TGFβ synthesis and activation in the ECM are largely unknown, and because this cytokine is important for different human diseases, its functional interaction with AhR makes this receptor an interesting partner in TGFβ-dependent functions. On the long term, the study of human diseases in which TGFβ has a relevant role could emphasize AhR as a genetic marker with potential clinical relevance. This review will summarize the current status of the AhR-TGFβ relationship from the regulatory and functional point of views and an attempt will be made to integrate TGFβ in some phenotypes caused by the lack of AhR expression *in vivo*.

2. The players: AhR, TGFβ, LTBP-1 and ECM proteases

2.1. The dioxin receptor in the interphase between toxicity and development

The helix-loop-helix (HLH) family of proteins comprises transcriptional regulators that bind to DNA through a basic domain located at their amino terminus. In metazoans, the so-called basic-helix-loop-helix (bHLH) transcription factors regulate many processes including myogenesis, neurogenesis, hematopoiesis, circadian rhythms, metabolism and organ development [12,13]. bHLH proteins have been grouped in seven classes attending to their sequence similarity, tissue distribution, DNA-binding affinity and dimerization ability [12]. Relevant for this review are the members of the Class VII, which are unique among bHLH proteins for having a PAS domain (Per-ARNT-Sim). The Class VII of bHLH/PAS regulators is formed by type I proteins such as the xenobiotic-inducible aryl hydrocarbon (dioxin) receptor (AhR) and the oxygen sensor hypoxia-inducible factor α (HIF-1α). To be transcriptionally active, AhR and HIF-1α have to heterodimerize with type II nuclear factors such as the aryl hydrocarbon receptor nuclear translocator (ARNT) [8]. The heterodimer AhR-ARNT is well known for its critical role in modulating the toxic and carcinogenic response against environmental contaminants such as dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) and benzo[*a*]-pyrene [14–16]. Nevertheless, the early presence of AhR in metazoans, its high degree of conservation among species, its expression during development and the phenotypes produced by AhR deficiency in mice, have lead to the conclusion that this receptor must have endogenous functions beyond xenobiotic metabolism. Moreover, it has been proposed that the toxic responses mediated by AhR could be in fact reflecting deregulated physiological functions [6,7,9]. To date, different studies have implicated AhR in the homeostasis of the liver [17–19], heart [20–22], immune system [18,23,24] and ovary [25].

The search for mechanistic explanations about the role of AhR in toxicity and physiology has lead to the identification of novel transcriptional targets in addition to classical cytochromes P450 and phase II enzymes [26]. Adding to the large sets of genes identified in microarray experiments as differentially expressed by AhR in the immune system [27] and in liver tissue [28], further studies have shown that AhR up-regulates genes involved in cell proliferation and differentiation such as $p21^{Cip1}$ [29], $p27^{Kip1}$ [30], c-jun and Jun-D [31] and IL-2 [32]. Importantly, AhR also represses the expression of T-cadherin in vascular smooth muscle cells [33], c-Myc in breast tumor [34] and E2Fs in mouse hepatoma Hepa 1 cells [35]. As we will analyze in more detail below, the ability of AhR to constitutively repress gene expression can be a relevant parameter in modulating TGFβ activity and its associated effects on the phenotype of $AhR^{-/-}$ mice. Thus, there is currently enough research data to support the contribution of AhR to normal cell function and to suggest that, at least for some processes, it involves cross-talk with signaling pathways such as the ones regulated by TGFβ.

2.2. TGFβ signaling and AhR

The TGFβ family of proteins comprises many closely related molecules with a major function in the regulation of development, proliferation, apoptosis, differentiation and cell migration [1,36,37]. TGFβ triggers signaling by binding to plasma membrane serine-threonine kinase receptors that will phosphorylate and activate intermediate proteins of the Smad family. Smad protein complexes will enter the cell nucleus and bind to the promoters of target genes to regulate their expression [1,37,38]. Under basal cell conditions, type I TGFβ

receptors remain in an inactive conformation that blocks their kinase domain and distorts the active site [39]. Following TGFβ binding, type II TGFβ receptors phosphorylate and activate type I receptors, thus inducing intracellular signaling to Smad proteins [1]. TGFβs have pleiotropic effects under normal cellular conditions and the perturbation of their signaling appears to be important in human diseases such as fibrosis, cancer and auto-immunity [36,38].

Interestingly, not only TGFβ regulates cell functions that overlap those involving AhR, but also some of its effects are cell type-specific, as for AhR. An illustrative example of such cell-specific effects resides in the control of cell proliferation. Wound healing experiments in Smad3 null-mice revealed that TGFβ stimulated or inhibited cell proliferation depending on whether the target cell was a fibroblast or a keratinocyte [40]. Similarly, AhR cooperated with NF-κB to promote cell proliferation in MCF-7 human breast cancer cells [41] and its over-expression induced hepatic [42] and gastric [43] carcinomas, while it inhibited proliferation via $p27^{Kip1}$ in 5L hepatoma cells [30] and arrested cell cycle in Jurkat T cells [44] and in $CD4^{-}CD8^{-}CD3^{-}$ lymphocytes. It is therefore possible that TGFβ and AhR could functionally converge to the regulation of cell function.

2.3. *Synthesis and extracellular targeting of latent TGFβ: the role of LTBP-1*

Although many key aspects of the signal transduction downstream from TGFβ receptors have been discovered, the extracellular mechanisms controlling TGFβ location and activation in the ECM are critical issues still only partially known. In fact, it has been suggested that the unusual biology of TGFβ regarding its many functions and cell-specific activities, could be due to the complexity of its mechanisms of docking and activation in the ECM [45]. It is becoming increasingly clear that TGFβ receptors represent a mid-point rather than the start of TGFβ-mediated signaling. TGFβ is secreted from producing cells in a latent form that must be activated in the ECM before it becomes able to activate type II TGFβ receptors [45,46]. Three different forms of TGFβ (-1, -2 and -3) are synthesized as latent complexes with a molecular mass of 75 kDa composed of a dimer of TGFβ covalently bound to a dimer of the pro-peptide LAP. After synthesis but before secretion, the interaction between LAP and TGFβ is cleaved between Arg278 and Arg279 by a furin convertase [47] producing the so-called small latent complex (SLC), in which each monomer of TGFβ remains non-covalently bound to LAP [48]. In addition, both the mature TGFβ dimer and the LAP dimer are disulphide bonded [48] (Fig. 1A). Interestingly, the association between TGFβ and its pro-peptide LAP is reversible and the TGFβ1-LAP complex has inhibitory activity against all three TGFβs, suggesting that LAP-mediated latency is not isoform specific [49]. For most cells, however, the efficient secretion of latent TGFβ requires its association to a third protein named latent TGFβ-binding protein (LTBP), thus resulting in the large latent TGFβ complex (LLC) [45,46,50]. The importance of LTBPs in TGFβ function is underlined by the fact that while the SLC is secreted very slowly, its association to LTBPs markedly enhances its release to the extracellular medium [51]. It should be noted, however, that a large fraction

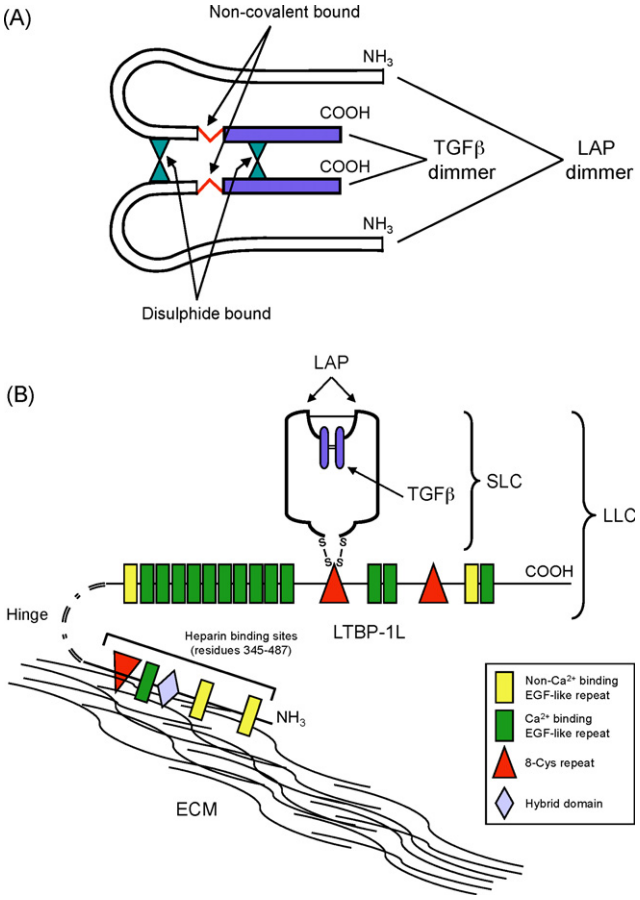

Fig. 1 – (A) Structure of the LAP-TGFβ small latent complex (SLC). Each monomer of TGFβ binds a monomer of LAP through non-covalent bounds while both the mature TGFβ dimer and the LAP dimer are disulphide bonded. (B) Proposed model for the structure of the large latent TGFβ binding protein-1L complex (LLC). LTBP-1L binds to the ECM fibers through its N-terminal domain that harbors non-Ca^{2+} binding EGF-like repeats, a Ca^{2+} binding EGF-like repeat, an 8-Cys repeat and a hybrid domain. The hinge region contains a putative heparin cleavage site in LTBP-1 and -2. The small latent TGFβ complex (SLC) is bound to the LCC by disulphide bridges to the second 8-Cys repeat. A dimer of TGFβ remains attached in the SLC to a dimer of LAP. Redrawn and adapted from references [46,50].

of LTBP does not contain TGFβ, which suggests that LTBPs have additional roles in the ECM [52].

LTBPs are members of the fibrillin family of proteins and, to date, four forms have been isolated from human and rodents that differ in their gene organization, alternative splicing and use of alternative promoters producing cell-specific patterns of expression [53–56]. A long (LTBP-1L) and a short (LTBP-1S) isoforms of LTBP-1 are transcribed in human and mouse from a single gene by the activation of alternative promoters [57]. Fig. 1B depicts a proposed model for the LLC in which LAP-TGFβ remains bound to the ECM though LTBP-1L. A common feature in the four LTBP isoforms is the presence of EGF-like sequences and 8-Cys repeats at their N-termini. LTBP-1L binds

to the extracellular matrix through a tissue transglutaminase (tTGase) site located in its 8-Cys repeat proximal to the N-terminus [58], even if interactions can be also established by the C-terminal domain [59]. In addition, heparin binding sites have been located near the N-terminus between residues 345–487 [60] and 414–425 [61] that presumably have an important role in modulating TGFβ availability in the ECM. The hinge domain also contains proteolysis-sensitive sites that can be cleaved in LTPB-1 and -2 but not in the -3 and -4 isoforms [62,63]. The central part of LTBPs represents about half the size of the protein and contains between 9 and 14 EGF-like repeats that possibly forms a helical rod-like structure [64] able to separate the TGFβ-containing C-terminus from the ECM in order to facilitate the activation of the growth factor [65]. Finally, the TGFβ binding domain is located close to the C-terminus at the 8-Cys3 repeat [66]. Association of LTBP-1 and LAP-TGFβ takes place through a disulphide bond between Cys33 of LAP and a yet partially characterized 8-Cys3 region in LTBP-1 [67]. A dipeptide insertion between Cys6 and Cys7 seems to specify 8-Cys3 as the LAP-TGFβ binding site [67]. Importantly, a previous study has proposed that the connective tissue disease known as the Marfan syndrome, which is caused by mutations in the *Fibrillin-1* gene may have its origin in the disregulation of TGFβ location and activation in the ECM, resulting in increased apoptosis in the developing lung [68]. It is therefore possible that matrix sequestration of cytokines such as TGFβ is critical for their physiological activation and that the alteration of such process can contribute to the development of the disease [68].

Demonstration of the roles of LTBPs in development and organ homeostasis has been provided by animal models in which each murine isoform has been selectively inactivated by gene targeting. Lack of LTBP-1, -3 and -4 altered the local deposition and activation of TGFβ and revealed altered septation of the cardiac outflow tract in *Ltbp-1*$^{-/-}$ [69], bone abnormalities in *Ltbp3*$^{-/-}$ [70] and cardiomyopathy, defective lung development and colorectal cancer in *Ltbp4*$^{-/-}$ mice [71]. LTBP-2 is an integral component of elastin-containing microfibrils and mice with a null mutation in the *Ltbp2*$^{-/-}$ gene showed embryonic lethality between gestational days E3.5 and E6.5. Since LTBP-2 is expressed at the blastocyst stage at E3.5 but not at E6.5, it appears that this protein has a role early in development acting as a structural molecule during implantation rather than as a regulator of elastic fiber homeostasis or TGFβ activation [72]. Importantly, although LTBPs were initially regarded as localization proteins for TGFβ in the ECM, it is now widely assumed that they also contribute to the assembly, secretion and activation of this cytokine [45,73]. ECM components contribute to LTBPs localization and a recent report has shown that fibronectin is critical for the incorporation of LTBP-1 and TGFβ in the ECM of fibroblasts and osteoblasts [74]. A model has been proposed to integrate the processes of TGFβ secretion, localization and activation in the ECM [45]. In such model, the latent TGFβ complex would be an extracellular sensor in which LTBP-1 functions as the localizer, the TGFβ propeptide as the detector and TGFβ as the effector triggering a cell-specific response [45]. Experimental evidence to support such model came from the ability of antibodies raised against LTBP-1 and inhibitors of tTGase [75] to block the activation of latent TGFβ [52,76].

2.4. *Mechanisms of TGFβ activation in the ECM: role of extracellular proteases*

Despite the fact that target cells sense TGFβ only after activation of its membrane receptors, it is clear that important regulatory points also exist upstream from the pre-receptor stage to ensure the release of active cytokine. Latent TGFβ activation in the ECM has proven a complicated process involving protease-mediated and protease-independent mechanisms [77]. To date, this scenario is even more intricate since the activity of some proteases *in vitro* does not seem to reflect their contribution to TGFβ activation *in vivo*. Proteolytic activation of TGFβ can be accomplished in cell culture by diverse extracellular proteases that target three main processes: (i) break of the hinge region of LTBPs; (ii) production of a LLC competent for activation in the ECM and (iii) cleavage of LAP [45].

An important role is attributed to the plasminogen activator (PA)/plasmin system since it can produce active TGFβ in osteoclasts [73] and chondrocytes [78]. Further, plasmin has been suggested to have a role in the vitamin A-mediated increase in TGFβ activity since vitamin A up-regulates PA levels [79]. Additionally, elastase also activates TGFβ in osteoclasts from bone matrix [73]. Metalloproteases such as MMP-2 and MMP-9 can cleave soluble TGFβ in bovine aortic endothelial cells [80] and keratinocytes [81]. Thrombospondin-1 (TSP-1) is an efficient activator of TGFβ *in vitro* [82] and, importantly, *in vivo* by inducing a conformational change in LAP [94]. As will be discussed below, AhR status in mouse embryo fibroblasts (MEF) modulates the activity of proteases such as PA/plasmin, elastase, MMP-2 and TSP-1, thus contributing to increased TGFβ activity. Moreover, LTBP-1 participates in the activation of some of these proteases. A schematic representation of these possible mechanisms of activation of TGFβ in the ECM is shown in Fig. 2. Binding of LLC to the ECM takes place by transglutaminase II activity (TGase II) [83]. Cleavage of the LLC complex at the hinge region of LAP by PA/plasmin, elastase or chymase releases a soluble form of the large latent complex. Active TGFβ is then released from LAP by proteolysis mediated by PA/plasmin, TSP-1, MMPs and TGase II.

3. Functional cross-talk between AhR and TGFβ

AhR has a role in modulating TGFβ expression and activity in cell culture and *in vivo*. However, the regulatory pathway is probably more complex since TGFβ also alters AhR mRNA expression in certain cell lines.

3.1. *Reciprocal regulatory mechanisms*

On the one hand, AhR down-modulation maintains a constitutively increased level of TGFβ expression and/or activity. On the other hand, an increase in TGFβ levels decreased AhR expression and inhibited the AhR-mediated transcriptional response in certain cell lines. Although many aspects of this mutually repressive mechanism remain to be explored, it seems reasonable to assume that it could affect

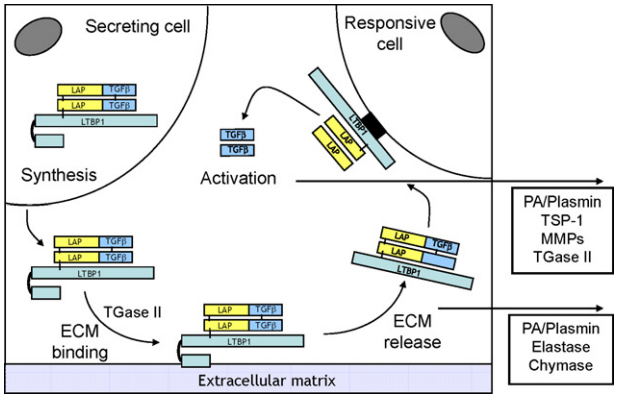

Fig. 2 – Schematic representation of TGFβ activation by ECM proteases. The LCC complex is synthesized and secreted from the producing cell. After being localized in the ECM by TGase II, a soluble form of the LCC is released by cleavage of LTBP-1 at the hinge region. The fraction of TGFβ included in the soluble latent complex will be fully activated and released at the surface of the target cell by further proteolysis.

cellular functions in which AhR or TGFβ have a role (see Sections 2.1 and 2.2). In this context, the contribution of AhR and TGFβ to the control of cell proliferation has driven many studies analyzing the functional cross-talk between both proteins.

Early reports indicated that AhR activation by TCDD down-regulated TGFβ2 transcription in human keratinocytes [84]. Likewise, AhR also regulates TGFβ under normal cell conditions and, thus, recent work using primary fibroblasts (MEF) isolated from mice expressing ($AhR^{+/+}$) or lacking ($AhR^{-/-}$) AhR revealed that these animals have high levels of TGFβ expression due to mRNA stabilization [85] and increased secretion of total and active TGFβ [86,87] (see Section 3.2). These effects are not restricted to mesenchymal cells because microarray expression analysis on smooth muscle cells from aorta of $AhR^{-/-}$ mice showed over-expression of TGFβ3 and of some proteins involved in TGFβ processing [88]. Consistently, long-term treatment of rhesus monkeys with TCDD decreased hepatic AhR levels and simultaneously increased TGFβ protein content [89]. The analysis of liver tumors isolated from mice with a liver-specific inactivation of the retinoblastoma tumor suppressor gene (pRb-negative) or from wild type mice (pRb-positive) showed that AhR was repressed irrespective of pRb status, and that low receptor levels correlated with up-regulation of TGFβ3 in pRb-negative or with TGFβ2 and TGFβ3 in pRb-positive mice [90]. Thus, AhR is able to repress TGFβ expression both in cell culture and *in vivo*.

TGFβ also exerts negative regulation on AhR expression at the mRNA level. Treatment of A549 human lung cancer cells with TGFβ induced a marked reduction not only on AhR expression but also on its transcriptional activity. Since both AhR expression and its transcriptional activity were rescued by the protein synthesis inhibitor cycloheximide, it was suggested that AhR down-regulation by TGFβ requires the *de novo* synthesis of a repressor [91]. Later studies in human lung carcinoma A549 cells and in human hepatoma HepG2 cells showed that TGFβ affects AhR transcriptional activity in a cell-specific manner, inhibiting AhR-dependent gene expression by 50% in A549 cells while increasing transcription rates by three-fold in HepG2. Human AhR promoter analyses uncovered a TGFβ-responsive element containing a Smad binding site that could be modulated by transient expression of Smads 2, 3, and 4 [92]. Furthermore, AhR repression by TGFβ was modulated by the 5′-TG-3′-interacting factor (TGIF), although the mechanism involved remains undefined [92]. Therefore, it is possible that a feed-back mechanism co-regulates the relative levels of AhR and TGFβ on cell-specific bases. Nevertheless, while AhR regulation by TGFβ seems to take place at the transcriptional level, TGFβ regulation by AhR involves at least two complementary processes of mRNA stabilization and cytokine activation in the ECM (see Section 4.2).

3.2. *AhR status modulates TGFβ activity and cell function*

The production of mouse lines lacking AhR expression has greatly contributed to our knowledge about how AhR regulates cell function. A consistent observation is that AhR status modulates TGFβ expression in cultured cells and *in vivo*. Early studies showed that primary hepatocytes from $AhR^{-/-}$ mice produced and secreted increased amounts of TGFβ, which were related to their lower proliferation rates and increased apoptotic numbers [93]. *In vivo*, liver tissue from AhR-null mice also had elevated protein expression for TGFβ1 and TGFβ3 as compared to wild type mice expressing a functional AhR receptor [93]. Later work has confirmed the contribution of AhR in maintaining TGFβ activity since $AhR^{-/-}$ MEFs secreted increased levels of total and active TGFβ and had longer duplication times, decreased proliferation and increased apoptotic rates than wild type cells [86,87]. The causal role of TGFβ on these cellular phenotypes is supported by the fact that addition of exogenous TGFβ to wild type cells blocked cell cycle at the G2/M transition and lowered proliferation rates to values similar to those of $AhR^{-/-}$ MEFs [86]. Thus, TGFβ over-expression is probably a relevant factor in the AhR-dependent control of the cell cycle. Additional studies agree with such hypothesis since MEF cells in which AhR expression was modulated by a stably integrated Tet-Off system revealed that shutting down AhR up-regulated growth-inhibitory genes encoding for TGFβs and cell cycle inhibitors (*Gas6*, *Cgref1*, *Gadd45a* and *TGFβ* receptors) and down-regulated growth promoting genes such as *Cyclin A2*, *B1* and *E1* and Cyclin-dependent kinase *Cdc2* [85]. In agreement to a functional relationship between AhR and TGFβ in cell cycle, Tet-Off $AhR^{-/-}$ MEFs also had lower proliferation rates in absence of exogenous ligand [85]. Our recent data have provided an explanation for the higher levels of TGFβ activity in $AhR^{-/-}$ MEFs: in absence of AhR expression, MEF cells over-express LTBP-1L in their ECM, which could result in increased secretion of latent and active TGFβ and reduced cell proliferation [87]. Indeed, blocking LTBP-1 by a neutralizing antibody increased MEF proliferation to values similar to those obtained after neutralizing TGFβ activity. Therefore, LTBP-1 is constitutively repressed by AhR and represents a link between TGFβ and AhR-dependent control of cell proliferation. The mechanisms by which AhR regulates constitutive LTBP-1

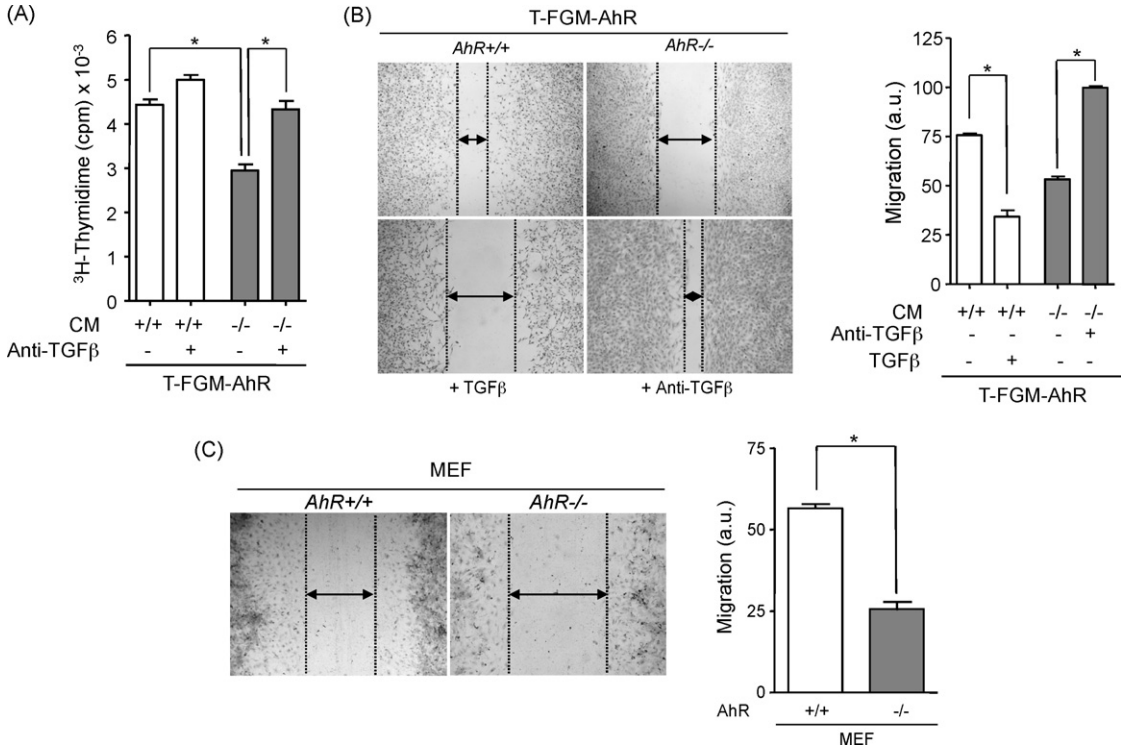

Fig. 3 – AhR status modulates cell proliferation and migration through TGFβ. (A) T-FGM *AhR*$^{+/+}$ and T-FGM *AhR*$^{-/-}$ fibroblasts were cultured for 48 h and their conditioned medium (CM) collected, processed and used in Mv1Lu proliferation assays as indicated [86,87]. In some experiments, 1 μg/ml neutralizing 1D-11 anti-TGFβ antibody (R&D Systems, Abingdon, UK) was added during incubation of the Mv1Lu cells with the CMs. (B) T-FGM *AhR*$^{+/+}$ and T-FGM *AhR*$^{-/-}$ fibroblasts were grown to confluence, starved by serum deprivation and used in wound healing experiments as described [96]. T-FGM *AhR*$^{+/+}$ or T-FGM *AhR*$^{-/-}$ cells were also treated with 5 ng/ml recombinant TGFβ protein (Sigma–Aldrich, St. Louis, MO, USA) or 1 μg/ml neutralizing anti-TGFβ antibody, respectively. (C) MEFs were isolated from *AhR*$^{+/+}$ and *AhR*$^{-/-}$ embryos, grown to confluence and used in wound healing experiments as indicated [96]. Data are shown as mean ± S.E. from triplicate measurements. The experiments were done in three different T-FGM or MEF cultures.

expression and how this protein contributes to TGFβ activation will be discussed (see Sections 4.1 and 4.2).

Additional cell functions can be also regulated by AhR through TGFβ. It has been shown that AhR activation by its ligand TCDD in MCF-7 human breast cancer cells increases the formation of lamellipodia and promotes cell migration in culture [95]. In agreement, immortalized mouse fibroblasts (T-FGM) lacking AhR expression had cytoskeleton alterations, decreased lamellipodia and lower migration rates [96]. The implication of TGFβ in the AhR-dependent control of cell migration has been further characterized by the use of recombinant cytokine or neutralizing antibody. Proliferation assays using the reporter cell line Mv1Lu revealed that culture medium (CM) from T-FGM *AhR*$^{-/-}$ cells contained higher amounts of inhibitory protein(-s) than that obtained from T-FGM *AhR*$^{+/+}$ cells. One such inhibitory protein must be TGFβ since addition of an anti-TGFβ neutralizing antibody (1D11 clone from R&D Systems, Abingdon, UK) to *AhR*$^{-/-}$ fibroblasts restored proliferation to values close to those of wild type cells (Fig. 3A). T-FGM *AhR*$^{-/-}$ cells had lower migration rates than T-FGM *AhR*$^{+/+}$ fibroblasts under basal conditions and this phenotype also involved differences in TGFβ activity (Fig. 3B). Thus, while a recombinant TGFβ protein (Sigma–Aldrich, St. Louis, MO, USA) significantly inhibited the migration of T-FGM *AhR*$^{+/+}$ fibroblasts, the addition of an anti-TGFβ neutralizing antibody to T-FGM *AhR*$^{-/-}$ cultures increased their basal migration to wild type values (Fig. 3B). *AhR*$^{-/-}$ MEF cells also had lower migration rates than wild type MEFs (Fig. 3C), suggesting that AhR is required to maintain migration of cells from fibroblastic origin.

An interesting link between TGFβ and AhR has come from recent studies on the immune system dealing with the differentiation of T_{reg} and T_H17 $CD4^+$ T cell subsets [10,11,97]. AhR directs T_{reg} and T_H17 differentiation in a ligand-specific manner. While TCDD induced T_{reg} differentiation and suppressed autoimmune encephalomyelitis, 6-formylindolo [3,2-*b*] carbazole (FICZ) halted T_{reg} differentiation, promoted T_H17 differentiation and worsened autoimmune encephalomyelitis. On the other hand, TGFβ also induced T_{reg} differentiation, but its combination with IL-6 or IL-21 promoted differentiation of T_H17 T cells. These studies conclude that TGFβ and AhR converge as relevant differentiation factors for T_{reg} and T_H17 cells probably by acting together on a common precursor upstream in the differentiation program for both cell subsets. In addition, since AhR activation stimulated TGFβ secretion by T cells, the specific effects of AhR

on T_{reg} and T_H17 differentiation could involve an autocrine loop of TGFβ activity [10]. These recent findings offer an interesting molecular mechanism that could help explain how environmental contaminants contribute to autoimmune diseases. Since autoimmune diseases are rather complex processes that depend on genetic, hormonal, immune, dietary and environmental factors, the increased prevalence of certain forms of autoimmunity in industrialized countries is probably connected to environmental toxins, and thus, a link between the xenobiotic-dependent transcription factor AhR and alterations in the differentiation program of T_{reg} and T_H17 cells can be proposed. Studies are most probably underway trying to prove such hypothesis.

4. LTBP-1 in the cross-road of AhR and TGFβ signaling

4.1. *AhR as a repressor of constitutive LTBP-1 expression: role of epigenetics*

The human LTBP-1L and -1S isoforms are expressed in a cell-specific manner and genomic sequencing of the human *Ltbp-1* gene promoter revealed that *Ltbp-1L* is produced by alternative splicing to an internal acceptor site in exon 1 of *Ltbp-1S* [53]. An analogous tissue-specific pattern of expression has been described in mouse where LTBP-1L and -1S are produced by two alternatively spliced transcripts from a single *Ltbp-1* gene [57,98]. These studies led to the isolation of the 5′ upstream promoter region and defined the intron–exon organization of the mouse *Ltbp-1* gene. However, its mechanism of transcriptional regulation remains only partially known. Previous work have identified co-activators such as CBP/p300 and NCoA/SRC-1/p160 and co-repressors such as ERα that modulate the AhR/ARNT transcriptional complex by interacting with AhR, ARNT or with both proteins [99–101]. Adding to those complex mechanisms, recent studies also implicate epigenetics as a relevant factor in AhR/ARNT-dependent gene expression. Reports analyzing the inhibitory role of chromium on the induction by benzo-[*a*]-pyrene of the AhR target gene *Cyp1a1*, concluded that chromium inhibited AhR/ARNT transcriptional activity by cross-linking histone deacetylase 1 (HDAC-1)/DNA methyltransferase 1 (DNMT1) to the *Cyp1a1* promoter and by preventing the recruitment of the co-activator CBP/p300 [102,103]. Furthermore, under constitutive (uninduced) cell conditions, HDAC-1 also maintains *Cyp1a1* repression by blocking changes in histone marks (acetylation of lysines 14 and 16 in histones H3 and H4, respectively) that favor gene expression [104].

Recent work using wild type and AhR-null MEF cells and Hepa 1 cell lines defective in AhR-dependent transcription have shown that constitutive *Ltbp-1* repression is AhR dependent. In agreement to the studies mentioned above, AhR status modulates a transcriptional mechanism in which coordinated recruitment of co-activators and co-repressors to the proximal *Ltbp-1* promoter regulates its basal expression [105]. Wild type MEFs had low constitutive levels of *Ltbp-1* expression that could be explained by an increase in HDAC-2 recruitment and by diminished binding of the active form of the transcriptional co-activator CREB-1 (phosphorylated CREB-1, pCREB-1) to the proximal *Ltbp-1* promoter. In $AhR^{-/-}$ MEF cells, constitutive *Ltbp-1* mRNA over-expression was consistent with decreased HDAC-2 recruitment and with increased pCREB-1 binding to the *Ltbp-1* promoter. Histone marks associated to gene expression such as lysine 8 acetylation in histone H4 (AcK8H4) were increased in the proximal *Ltbp-1* promoter in $AhR^{-/-}$ but not in $AhR^{+/+}$ MEFs. HDAC-2 activity was relevant to this epigenetic mechanism since its down-regulation by RNA interference (RNAi) restored *Ltbp-1* expression and recovered AcK8H4 levels in $AhR^{+/+}$ MEF cells [105]. Yet, the regulation of *Ltbp-1* appears more intricate and further experiments are needed to precisely define the nature of the protein complexes that bind to its promoter under physiological conditions. Another relevant question is to determine how AhR modulates the recruitment of HDACs and pCREB-1 (and possibly other proteins) to the *Ltbp-1* promoter, and whether such mechanisms could help explain the tissue- and cell-specific expression of LTBP-1 and TGFβ. The proposed epigenetic mechanism is summarized in Fig. 4. In $AhR^{+/+}$ cells, AhR/ARNT complexes could participate in the coordinated recruitment of co-repressors (HDAC-2) and co-activators (pCREB-1) to XRE/CRE elements located in the proximal *Ltbp-1* promoter, favoring HDAC-2 binding and repressing *Ltbp-1* mRNA expression, which will ultimately decrease TGFβ activity in the ECM (left panel). In $AhR^{-/-}$ cells, the lack of receptor will lead to a situation in which reduced HDAC-2 binding to the *Ltbp-1* promoter would allow recruitment of the transcriptional co-activator pCREB-1 to XRE/CRE elements, which will induce over-expression of *Ltbp-1* and increase the extracellular levels of TGFβ activity (right panel). *Ltbp-1* transcriptional regulation is relevant for two reasons: first, it underlines the role of AhR in constitutive gene repression and supports its relationship to epigenetics, and second, LTBP-1 over-expression provides a plausible explanation for certain phenotypes observed in AhR-null mice (see Section 4.3).

4.2. *AhR as a regulator of TGFβ activity through LTBP-1: role of proteases*

ECM proteases play an important role in the process of TGFβ activation from the latent complexes (see Fig. 2). On the other hand, as a major component of the LLC, LTBP-1 has been proposed as a localization molecule that could also contribute to TGFβ proteolysis and activation in the ECM [51]. Experimental support for such possibility has recently been obtained from AhR-null MEF cells in which constitutive LTBP-1 over-expression in the ECM correlated with TGFβ over-activation and with decreased cell proliferation [86,87,105]. Indeed, LTBP-1 down-modulation by RNAi decreased TGFβ activity in $AhR^{-/-}$ MEFs to levels similar to those obtained by a neutralizing anti-TGFβ antibody [106]. The positive effect of LTBP-1 on TGFβ activity was mediated, at least in part, by ECM proteases because RNAi for LTBP-1 or specific pharmacological inhibitors decreased PA/plasmin, elastase and TSP-1 activities in the conditioned medium of $AhR^{-/-}$ MEF cells [106]. It is therefore possible that, in addition to the localization of latent TGFβ into the ECM, LTBP-1 also serves as a positive modulator of protease activities that release active cytokine into the extracellular medium. Therefore, although much more work

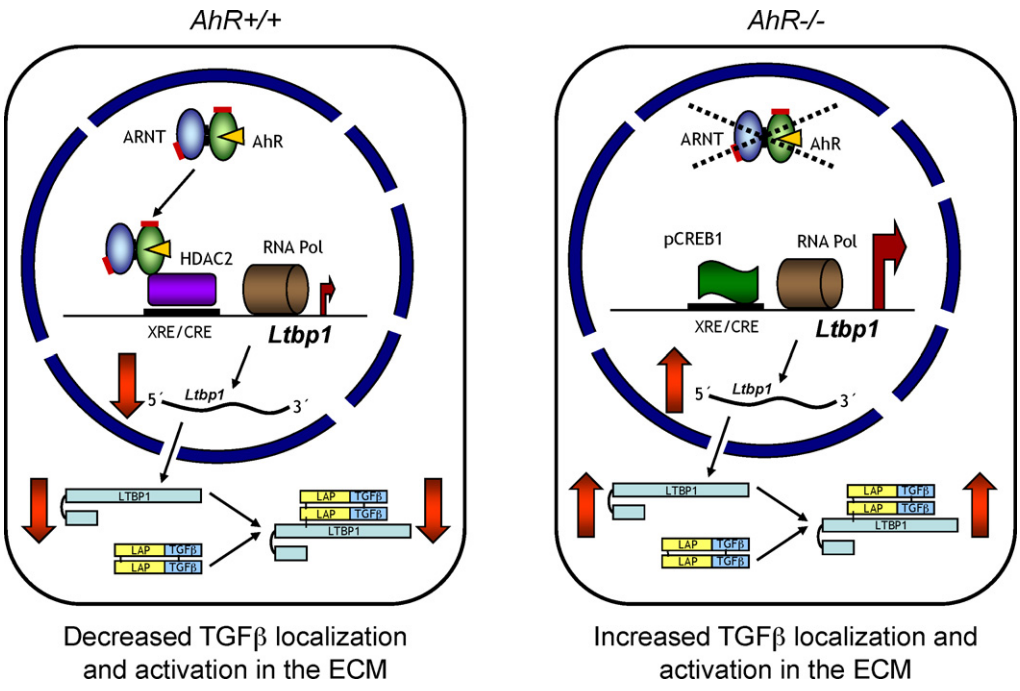

Fig. 4 – Scheme summarizing a potential mechanism for *Ltbp*-1 regulation by AhR/ARNT. By a partially defined process, presence of AhR/ARNT heterodimers determine a differential recruitment of co-repressors (HDAC-2) or co-activators (pCREB-1) to the mouse *Ltbp*-1 promoter. In $AhR^{+/+}$ MEFs, presence of AhR/ARNT complexes favor HDAC-2 binding to XRE/CRE elements in the *Ltbp*-1 promoter, thus maintaining low constitutive *Ltbp*-1 expression and decreasing TGFβ activity in the ECM (left panel). Absence of AhR/ARNT heterodimers in $AhR^{-/-}$ cells impairs HDAC-2 recruitment and increases pCREB-1 binding to XRE/CRE elements in the *Ltbp*-1 promoter, thus resulting in higher constitutive levels of gene expression and increased extracellular TGFβ activity (right panel).

has to be done to precisely explain how LTBP contributes to TGFβ activation, the current available data clearly supports such hypothesis. As a repressor of constitutive LTBP-1 expression (e.g. constitutive TGFβ activity) AhR appears as a relevant target protein to modulate the activity of ECM proteases that ultimately contribute to the modulation of TGFβ-dependent signaling.

4.3. *The AhR-LTBP-1-TGFβ pathway offers a mechanism to explain pathology*

Taken together, the relationship between TGFβ activity and LTBP-1 expression, and their dependence on AhR status, put forward the possibility of an AhR-LTBP-1-TGFβ functional pathway. In fact, this pathway can help explain some phenotypes of AhR-null mice and provides some clues about how TGFβ could be involved in AhR-mediated carcinogenesis.

Lack of AhR expression results in pathological alterations in several tissues [13,20]. The hepatic phenotype of $AhR^{-/-}$ mice has been characterized and includes portal fibrosis [18,19], lipid accumulation [107] and vascular pathology with increased number of small vessels [20] and portosystemic shunting in adult animals [108]. The hepatic fibrosis of $AhR^{-/-}$ mice underlines *in vivo* the functional relevance of the AhR-LTBP-1-TGFβ1 pathway. Remarkably, the fibrosis surrounding the portal areas in $AhR^{-/-}$ mice results from collagen accumulation [17,19] and co-localized with increased levels of LTBP-1 and TGFβ mRNA and protein [17]. TGFβ is a major pro-fibrogenic molecule expressed at low basal levels but significantly increased in fibrotic conditions [109]. It is therefore plausible that the fibrosis present in the portal areas of $AhR^{-/-}$ mice results from TGFβ over-expression. Moreover, the co-localization of TGFβ with LTBP-1 [17], together with the role of LTBP-1 in the activation of TGFβ via ECM proteases *in vitro* [106], suggests that LTBP-1 contributes to TGFβ activation *in vivo*. Thus, under physiological conditions, the repression of LTBP-1 by AhR could maintain low basal levels of TGFβ and blockade of tissue fibrosis. In agreement, AhR activation by TCDD in mouse hepatoma Hepa 1 cells induced plasminogen activator inhibitor-1 (PAI-1), which can decrease TGFβ levels by inhibiting PA/plasmin activity [110].

An additional parameter related to TGFβ that can add to the liver fibrosis of $AhR^{-/-}$ mice is the alteration in the metabolism of retinoic acid (RA). $AhR^{-/-}$ mouse liver has increased RA content and decreased RA metabolism [111] that results from the down-regulation of the 4-hydroxylase CYP2C39 enzyme [112]. High hepatic RA content is relevant to portal fibrosis because an RA-deficient diet abolished portal fibrosis, decreased TGFβ levels and inhibited TGFβ signaling in $AhR^{-/-}$ mice [113]. These effects of RA on TGFβ activation were early observed in bovine aortic endothelial cells where RA induced the activation of latent TGFβ by increasing PA/plasmin activity [114]. Altogether, these studies uncover a novel pathway in

which AhR has a central role in tissue fibrosis by regulating two converging signals: TGFβ activation by the transcriptional regulation of LTBP-1 and RA signaling by the control of the CYP2C39 hydroxylase activity. In addition, RA also modulates TGFβ signaling leading to increased matrix deposition and fibrosis.

Cleft palate is a common birth defect caused by the altered function of signaling molecules that act in a cell-specific manner during embryogenesis. Members of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) are important signaling molecules controlling proper palate development [115]. Likewise, TGFβ- and AhR-dependent signaling are also relevant for palate formation. On the one hand, mice lacking TGFβ3 expression died soon after birth and failed to close the palatal shelves [116]. On the other hand, prenatal treatment of mice with TCDD-induced cleft palate in $AhR^{+/+}$ but not in $AhR^{-/-}$ fetuses [117]. Considering that AhR down-regulates TGFβ expression and activation, a model for cleft palate has been proposed in which AhR activation by TCDD in the fetus will decrease TGFβ3 and thus promote such pathology [115]. Thus, the functional interaction between AhR and TGFβ appears to be relevant not only for liver development and homeostasis but also for xenobiotic-induced teratogenesis.

5. Concluding remarks

An important conclusion that can be extracted from studies dealing with AhR and TGFβ is that both molecules interact, and perhaps converge, to the regulation of common signaling pathways that modulate important cellular functions such as proliferation, differentiation, migration, vasculogenesis, wound healing, fibrosis and tumor development. It is clear that AhR and TGFβ are subjected to a reciprocal regulatory mechanism that mutually represses each other's expression. While this is an important issue, the functional interaction between AhR and TGFβ is probably far more complex and involves additional proteins such as LTBP-1. The constitutive repression of LTBP-1 by AhR is relevant because it requires a novel mechanism in which epigenetics play a prominent role. Nonetheless, equally relevant is the fact that LTBP-1 provides a common link to connect AhR and TGFβ signaling with the homeostasis of the extracellular matrix and, by extension, to common pathologies such as fibrosis and cleft palate. Indeed, considering that both AhR-null [20] and LTBP-1-null [69] mice have phenotypic alterations affecting heart size and structure, it would be of interest to determine whether the simultaneous absence of both proteins in a double knock-out mouse model exacerbates each single phenotype, thus supporting the existence of cross-talk between AhR- and LTBP-1-dependent signaling. AhR-LTBP-1-TGFβ signaling opens new exciting questions about the contribution of this xenobiotic receptor not only to normal cell physiology but also to disease states requiring AhR activity. Despite the fact that much more studies are needed to fully understand the biological functions of AhR, the field is benefiting from intense current investigations that gradually uncover new partner molecules and signaling pathways that extend the number of processes requiring AhR.

The findings about the functional cross-talk between AhR and TGFβ have broad implications in basic research and it is tentative to propose that AhR could have clinical value in the early detection, prognosis and/or therapeutic of human disease. A massive number of studies relate alterations in TGFβ signaling to different human diseases including several types of cancer (whether or not induced by xenobiotics), hematological diseases, diabetes, cardiomyopathy, fibrosis and neurological degeneration. Although AhR is not as well characterized as TGFβ regarding its contribution to human disease, it is reasonable to assume that low or high AhR expression can differentially affect TGFβ levels and disease progression. The mutual regulation between both molecules may reflect the existence of evolutionary conserved mechanisms that could produce different outcomes depending on the cell phenotype. One such example is based on the proposal that TCDD could protect against breast cancer [118]: if we assume that TCDD markedly depletes AhR protein levels *in vivo*, an over-expression of LTBP-1 and TGFβ would be expected that will ultimately produce a decrease in cell proliferation during early stages of the disease. Likewise, if diminished AhR expression could be demonstrated in human breast tumor metastasis, it could be interesting to analyze whether it contributes to the up-regulation of TGFβ activity that has been suggested to drive preferential homing of these cancer cells to lung tissue [119,120]. Therefore, it is tempting to speculate that the co-regulation of AhR and TGFβ could represent a common feature in certain disease states (e.g. cancer cells) and a feasible marker for progression. Major questions on the AhR-TGFβ interaction are why their roles seem to converge to similar cell functions and what does it mean from a biological point of view. Although we do not have clear answers to such questions yet, the available data point to an integrated mechanism rather than to a casual change in expression levels. Detailed analyses of AhR and TGFβ co-expression should be performed during development and in human diseases where both proteins have a significant role. For instance, it will be relevant to determine if the AhR over-expression observed in human breast tumor tissue micro-arrays (TMA) coincides with a decrease in TGFβ expression/activity. If proven to be true, AhR could emerge as a novel marker with clinical value for this malignancy and a potential therapeutic target through the modulation of TGFβ activity. With respect to developmental processes, the coincidence in the cleft palate phenotype produced by either TGFβ3 inactivation or AhR activation again allows us to suggest a converging mechanism with relevance in development. Intense investigations are currently underway to solve these issues and it is expected that the next few years will increase our knowledge about the mechanistic and physiological meaning of the AhR-LTBP-1-TGFβ pathway in health and disease.

Acknowledgments

This manuscript has been funded by Grants SAF2002-0034 and SAF2005-00130 from the Spanish Ministry of Education and Sciences (to P.M.F.-S.). A.G.-D. and J.M.C.-G. were fellows from the Spanish Ministry of Education and Sciences and from the Junta de Extremadura, respectively.

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