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THE IMMUNOPHILIN FKBP12: A MOLECULAR GUARDIAN OF THE TGF-β FAMILY TYPE I RECEPTORS

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1. ABSTRACT

FKBP12 as an immunophilin that binds to two well-known immunosuppressive macrolides, FK506 and rapamycin, has attracted immense attention and its role in mediating the immunosuppressive functions of these macrolides has been extensively studied. Since FKBP12 is a well-conserved protein among many species and is also highly expressed in almost all cells, it must play important roles in cellular function in the absence of macrolides. In one such a role, FKBP12 interacts with and regulates the functional state of the ryanodine Ca^{2+} channel receptor by altering protein conformation and coordinating multi-protein complex formation. This review summarizes another physiological role of FKBP12 as an interactor and a regulator of the type I serine/threonine kinase receptors of TGF-β superfamily. Current data, derived from detailed biochemical studies as well as from functional studies in various systems, suggest that FKBP12 functions as a "guardian" for the type I receptors to prevent them from leaky signaling under sub-optimal ligand concentrations, thereby providing a molecular "gradient reader" for TGF-β family morphogens. This aspect of FKBP12 function may be critical for cellular responsiveness to morphogenetic gradients of the TGF-β family members during early development, serving to assure the translation of different ligand concentrations into different signaling readouts.

2. INTRODUCTION

2.1. FKBP12 as a receptor of macrolides

FKBP12, with a molecular mass of 12 kDa, was initially isolated as the cytoplasmic receptor for two immunosuppressant drugs: the macrolides FK506 and rapamycin (1). Like cyclophilin, the cytosolic receptor for cyclosporin A, FKBP12 was shown to be able to catalyze the *cis-trans* isomerization of the proline amide in a tetrapeptide substrate (2,3). FK506 and rapamycin binding

to FKBP12 inhibits its isomerase activity (4,5). Both drugs bind to the same site on FKBP12, as demonstrated by binding competition assays (6) and by structural analysis of the co-crystallized FKBP12/FK506 and FKBP12/rapamycin complexes (1, 7). Although both drugs cause immunosuppression, the molecular mechanisms involved are different. Like cyclosporin A, FK506 mediates immunosuppression by inhibiting the transcriptional activation of cytokines such as interleukin 2 (IL-2), thus preventing T lymphocyte antigen-receptor-induced activation (6), while rapamycin causes direct cell cycle G1 arrest of the T lymphocytes by interfering with IL-2-induced signaling (6). Such a difference is likely due to a different third protein with which the FKBP12/drug complex interacts. The complexes of FK506/FKBP12 were shown to interact with calcineurin, a serine-threonine phosphatase, and to inhibit its phosphatase activity, thus preventing the dephosphorylation of the transcriptional factor NFAT, and its subsequent translocation into the nucleus for activating IL-2 transcription (6,8-10). Another protein, called FRAP (11) or RAFT1 (12), and also called mTOR (mammalian target of rapamycin) (13-22), was isolated as a binding protein of the rapamycin/FKBP12 complex. The molecular mechanisms involved in rapamycin/FKBP12/FRAP-mediated cell cycle G1 arrest have yet to be uncovered. The S6p70 kinase and the p34cdc2 kinase are both inhibited by the rapamycin/FKBP12 complex (23-25). Recently, a nutrient-sensitive complex containing a protein called Raptor was found to be involved in mTOR-mediated phosphorylation of S6p70 kinase (21,22). FKBP12 is highly abundant in almost every cell type, and its structure well conserved from yeast to human, suggesting that it has important cellular functions. Since FK506 and rapamycin are macrolides, not natural molecules present in mammalian cells, it has long been suspected that there are natural ligands for FKBP12.

2.2. FKBP12 as an integral component of the Ca^{2+} channels

There are several in-depth reviews of the role of FKBP12 and its close isoform FKBP12.6 in the regulation of calcium channels and the functional implications of such regulation in heart failure (26-30); therefore, only a brief summary is provided here. Muscular contraction is regulated by intracellular Ca^{2+} levels, which are controlled by the regulation of the activities of Ca^{2+} channels, especially those on the endo/sarcoplasmic reticulum (ER/SR) of the muscle cells. For cardiac muscle cells, there are two types of Ca^{2+} channels, the ryanodine receptor type 2 (RyR2) (31,32) and the inositol 1,4,5-triphosphate receptor IP3R2 (33). For skeletal muscle cells, the predominant Ca^{2+} channel is RyR1 (26). A functional role of FKBP12 in the regulation of RyR1 function was suspected when a peptide of FKBP12 was identified in purified RyR1 (26). Since then, studies have confirmed the physiological and functional interaction between FKBP12 and RyR1. An isoform of FKBP12, FKBP12.6, has been found to be the functional interactor of RyR2. In fact, FKBP12.6 is an integral component of the cardiac Ca^{2+} channels, which include tetrameric RyR/IP3R, each of which binds a set of proteins which include: 1) FKBP12/12.6; 2) PKA catalytic and regulatory subunits; 3) an anchoring protein mAKAP; 4) protein phosphatase 1 (PP1); and 5) protein phosphatase 2A (PP2A).

The interaction between FKBP12 and the RyR1/RyR2 can be blocked by nanomolar concentrations of rapamycin or FK506 (34). An FKBP12.6 binding site was identified within RyR2, containing a proline within the Ile-Pro-Leu motif (35). A similar site (Val-Pro-Leu) was found on RyR1 and the P1 residue Val 2461 was found to be critical for FKBP12 binding (36). As discussed below, a similar FKBP12 binding motif is present on the TGF-β family type I receptors. The proline-targeted binding site is consistent with the known property of FKBP12 as a prolyl-isomerase, although the functional role of the isomerase activity has yet to be defined.

The function of FKBP12/FKBP12.6 within the Ca^{2+} channel has been studied using the immunosuppressive drugs FK506 and rapamycin. One major role of FKBP12 is to modulate RyR1 gating (34). Ryanodine receptors devoid of FKBP12 exhibit subconductance high frequency states and tend to exist in the open state for longer periods than untreated receptors. Lack of FKBP12 binding also sensitizes the receptors to caffeine and Ca^{2+}. Thus, FKBP12 plays a critical role in regulating the Ca^{2+} channel closing kinetics, lack of which cause abnormal responses to agonists (37). Additional roles of FKBP12 are also suggested, such as regulating Excitation-Contraction coupling (38), coupled gating (39), as well as the inactivation of RyR2 (40). In FKBP12 knockout mice, RyR1 exhibits subconductance states (41), confirming the *in vitro* studies (34).

The functional mechanism of FKBP12-mediated regulation of the Ca^{2+} channel is not fully understood. The ability of FKBP12 to regulate protein conformation is thought to play a role. It has been suggested that FKBP12, as a peptidyl prolyl-isomerase, might change the conformation of the bound receptor, thereby coordinating the four receptors for full conductance of the channels, or the interactions between the neighboring RyR/calcium channels to enhance coupled gating (28).

2.3. Initial discovery of FKBP12 as an interactor of the TGF-β family type I receptor

While this subject will be the focus of this review, a brief introduction is given here for the initial discovery of FKBP12 as an unexpected player in the signaling pathways of the Transforming Growth Factor β (TGF-β) superfamily. TGF-β superfamily consists of a large group of structurally related polypeptides including various forms of TGF-β, bone morphogenic proteins (BMPs), activins, growth and differentiation factors (GDFs), and the Mullerian Inhibiting Substance (MIS) or anti-Mullerian hormone (AMH) (42,43). Members of the TGF-β subfamily are molecular organizers for tissue and organ morphogenesis during embryonic development and play key roles in maintaining the homeostasis of various developed systems (44,45). At the cellular level, diverse processes including cell proliferation, differentiation,

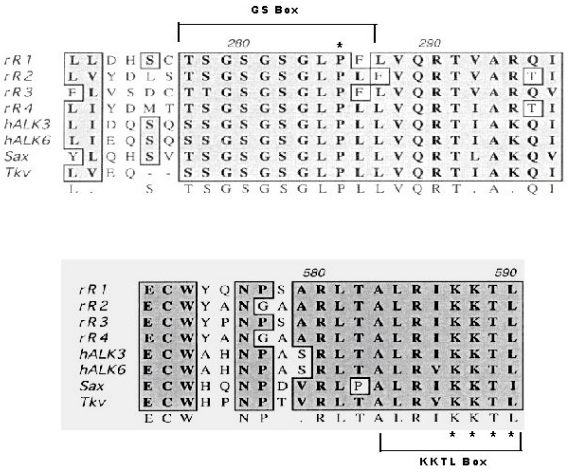

Figure 1. Two highly conserved domains of the TGF-β family type I receptors. "Pretty Box" alignment of the six mammalian members of the TGF-β family type I receptors and two members from *Drosophila*. Both the N-terminus GS box and the C-terminus KKTL box as illustrated, are required for FKBP12 binding in the yeast two-hybrid system (Wang *et al.*, unpublished data). R1, R2, R3 and R4 are described by He *et al.* (50). ALK3 and ALK6 are mammalian BMP type I receptors. Sax: Saxaphone (81); Tkv: thickvein (82).

adhesion, and apoptosis are subjected to TGF-β regulation (46, 47). The intracellular signaling events are initiated upon TGF-β binding to a pair of Ser/Thr kinase receptors known as the Type I receptor (TβR1) and the Type II receptor (TβRII), which are structurally similar but functionally distinct (48,49, 50). In 1992, He *et al.* isolated a family of four membrane receptors named as R1, R2, R3 and R4, through a PCR approach based upon a conserved protein sequence within the kinase domains of the Type II receptors of TGF-β and activin (50). Later these receptors were found to be the Type I receptors of TGF-β family ligands: R1 is the BMP7 and a MIS (Mullerian Inhibiting Substance) type I receptor; R2 is the activin type I receptor; R3 binds both TGF-β and activin and has been found to be a negative regulator of TGF-β in early vasculogenesis, with still unclear functional specificity (51); R4 is the TGF-β type I receptor (48-50,52). To understand the signaling mechanisms of MIS, Wang *et al.* decided to search for the cytoplasmic interactors of the cytoplasmic domain of R1, then considered to be a candidate MIS type I receptor. A modified yeast two-hybrid system called "Protein Trap" was used to identify proteins that directly interact with the cytoplasmic domain of this receptor (53). Screening a rat neonatal heart library yielded more than seventy interactors from screening 10 million transformants, all but one encoded rat FKBP12 (54,55). When a *Drosophila* type I receptor, Thickvein (Tkv), of the *Drosophila* TGF-β family member DPP (Decapentaplegic) was used as the bait to screen a *Drosophila* imaginal disc library, a *Drosophila* homology of the mammalian FKBP12 was isolated as the only interactor (Wang *et al.*, unpublished data). When seven known type I receptors were directly tested for their ability to bind to FKBP12 in the two-hybrid system, all were positive, while three tested type II receptors were all negative (54,55). Although there is significant homology between FKBP12 and several other FKBPs also known to bind to FK506, only FKBP12 was isolated as the interactor of the type I receptor. These data suggested that FKBP12 is a specific interactor for the TGF-β family type I receptors (54,55). Since then, many laboratories have participated in studying the biochemical and functional aspects of the observed interactions. The studies have now revealed an important biological function of FKBP12 as a novel regulator of the TGF-β family type I receptors.

3. FKBP12 AS A SPECIFIC INTERACTOR FOR TGF-β FAMILY TYPE I RECEPTORS

3.1. Domain specific interaction observed in yeast two-hybrid system

The initial studies of the interaction between FKBP12 and the TGF-β family type I receptors were carried out in the yeast two-hybrid system (54-57). The FKBP12 binding domains on two type I receptors, R4 and R1, also known as ALK5 and ALK2 (58) were mapped via detailed deletion studies of the receptors. The deletion mutants of the cytoplasmic domain of both receptors were tested against FKBP12 in the yeast two-hybrid system. Such studies revealed two separate small motifs well conserved among and also distinct for all known type I receptors: the GS domain (a motif containing SGSGSGLPF within the juxta-membrane domain of all type I receptors) and a carboxyl-terminal region of the kinase domain (Figure 1). Within the GS domain, the motif LPF or LPL could be a substrate motif of FKBP12 as a prolyl-isomerase (59). Thus, this proline residue was suspected to play a critical role in mediating the interaction between TGF-β type I receptor and FKBP12. The receptor-binding-domain on FKBP12 was first suggested by the ability of FK506 but not cyclosporin to block the interaction between FKBP12 and the cytoplasmic domain of the TGF-β type I receptor in the yeast two-hybrid system (54). This observation suggested that the macrolide-binding pocket of FKBP12 is somehow involved in FKBP12 interaction with the receptor. Such an involvement of the drug-binding pocket was further indicated by the observation that a point mutation of FKBP12 (D37G) within the drug-binding pocket that abolishes macrolide-binding also disrupted FKBP12 binding to the type I receptor (54). In addition to the drug-binding pocket of FKBP12, other regions on FKBP12 are also found to participate in receptor-binding: deletion of only five amino acids from the C-terminal domain of FKBP12 was sufficient to abolish the ability of FKBP12 to bind to the cytoplasmic domain of R1 and R4 (TβR1) (Wang *et al.*, unpublished data); deletion of the N-terminal five amino acids of FKBP12 abolished its interaction with R1 but not with R4. Since neither N-terminus nor C-terminus of FKBP12 is involved in macrolide binding, these data suggests that *in vivo* interaction between FKBP12 and the type I receptor requires both the drug-binding pocket of FKBP12 as well as separate motifs from the N- and C-termini.

3.2. Domain specific interaction observed in mammalian cells

Several groups independently demonstrated that FKBP12 co-precipitates with the full-length TGF-β type I receptor in mammalian cells, upon overexpression of both

proteins (55,56,57,60). Such an interaction can be abolished by the addition of FK506, or FK506 derivatives, which are deficient in binding to calcineurin (55,60) or rapamycin (60). Mutation of the proline residue to alanine (P194A) on the TGF-β type I receptor effectively abolishes FKBP12 binding (56,60), thus confirming the original observations made in the yeast two-hybrid system. Interestingly, FKBP12 is released from the TGF-β bound type I receptor (55,60). The release of FKBP12 from the type I receptor is dependent upon the presence of an intact type II receptor kinase activity, since mutating the ATP binding site of the kinase domain of the type II receptor prevents the release of FKBP12 from the TGF-β-bound type I receptor (55). Therefore, FKBP12 release is triggered by a phosphorylation event mediated by the type II receptor kinase. It was suspected that the trans-phosphorylation of the type I receptor by the type II receptor within the GS box could be responsible for the release of FKBP12. However, mutation of all five known phosphorylation sites (185TTSGSGSGLPF195) of the GS box did not prevent the release of FKBP12 from the TGF-β-bound type I receptor, suggesting that phosphorylation of these sites, although important for type I receptor activation, is not absolutely required for releasing FKBP12 (55). A possible role of FKBP12 phosphorylation in its release from the type I receptor has also been ruled out, since FKBP12 is not phosphorylated in response to TGF-β (57). Thus, the exact phosphorylation event(s) responsible for releasing FKBP12 in mammalian cells still awaits future studies.

3.3. Structural studies and direct *in vitro* binding studies of the interaction

FKBP12 and a cytoplasmic domain of the TGF-β type I receptor (162-503) have been successfully co-crystallized and their structures have been analyzed (61). FKBP12 binds the GS region of the receptor, with the two residues Leu-195 and Leu-196 on the type I receptor directly binding to the hydrophobic macrolide binding pocket on FKBP12. Leu-195 mimics the hemiketal ring of FK506, while Leu-196 mimics the pipecolinyl moiety on FK506. The proline residue known to be essential for FKBP12 binding does not interact with FKBP12 directly, but appears to stabilize the interaction interface. Two additional residues on FKBP12, H87 and P88, also exhibit direct contacts with the β4 and β5 sheets surrounding a structure called the L45 loop of the type I receptor kinase domain. However, in this crystal structure, unlike what was observed in the interaction tests in the yeast two-hybrid system, the conserved C-terminus of the type I receptor kinase domain as well as the N-terminus or C-terminus of FKBP12 are not shown to be involved in the complex formation between FKBP12 and TGF-β type I receptor. The obvious lack of interaction role of these domains on both proteins in the crystal structure of the complex of FKBP12/TβRIC suggests that additional factors are involved in the complex formation *in vivo*. This point will be further discussed in section 6.2.

Additional *in vitro* binding studies and domain mapping between FKBP12 and TGF-β type I receptor have been elegantly analyzed (62). Using a protein semi-synthetic protocol, a synthetic GS region peptide thioester containing residues 175-195 of the human TGF-β type I receptor with varying phosphorylation of one to all four residues (T185, S187, S189, S191), was made and ligated onto the reminder of the cytoplasmic domain of the receptor (TβRI C), then produced in SF9 cells. Purified GST-FKBP12 binds well to the receptor with the unphosphorylated GS domain (TβRI-0P). The importance of the FKBP12 interaction with the unphosphorylated GS domain was further studied by the crystallization of the TGF-β type I receptor cytoplasmic domain alone in the presence of a small molecule NPC-30345, a quinazo-line-based inhibitor of the type I receptor required for the successful crystal formation of the receptor. Comparison of the structures of TβRI C in the presence and absence of bound FKBP12 reveals that FKBP12 is required to stabilize the GS loop (185-192), which forms an inhibitory wedge that inserts between the regulatory α helix and the N lobe β sheet of the type I receptor kinase domain, blocking the ATP binding site. This data indicates an important functional role of FKBP12 in inhibiting the kinase activity of the type I receptor. Two important effects of the phosphorylation of the four residues within the GS domain were revealed: 1) the kinase activity of the type I receptor was activated by the phosphorylation; 2) FKBP12 exhibits much reduced affinity towards the receptor with the phosphorylated GS domain; 3) GST-Smad2 binds selectively to the type I receptor with phosphorylated GS domain, but not to the one with unphosphorylated GS domain. This data provides a structural rational for the inability of FKBP12 to bind to the type II-receptor phosphorylated type I receptor and reveals phosphorylated GS domain as the binding site for Smad2. However, this data does not imply that FKBP12 is released from the type I receptor upon the phosphorylation of the GS domain by the type II receptor, since the release of FKBP12 is likely a pre-requisite for the phosphorylation of the GS domain as its very binding blocks the phosphorylation sites. Therefore, additional mechanisms are responsible for the release of FKBP12 first, followed by the trans-phosphorylation of the GS domain, which may prevent the re-association of FKBP12 with the phosphorylated GS domain and promote the docking of the signaling protein like Smad2 or Smad3 to the phosphorylated GS domain. The involvement of an additional mechanism in FKBP12 release from the type I receptor was also suggested by an earlier observation that abolishing the phosphorylation sites of the GS domain does not block the release of FKBP12 (55). As discussed below in section 6.2, it is likely that FKBP12 and the cytoplasmic domain of the type I receptor exist in a multimeric complex *in vivo*. Thus, the understanding of how FKBP12 interacts with the type I receptor or how FKBP12 is released from the type I receptor will require study of a more complete receptor complex containing some other proteins.

3.4. Summary of the interaction domains and regulatory mechanisms

In summary, FKBP12 is a specific interactor of the TGF-β type I receptor. The interaction involves separate domains on both FKBP12 and the TGF-β type I receptor. On FKBP12, the macrolide-binding pocket as well as the C-terminal domain of FKBP12 are involved in the interaction with the TGF-β type I receptor in the yeast

two-hybrid system; for MIS and BMP7 type I receptor, the N-terminal domain of FKBP12 is also involved. On the type I receptor of TGF-β, the proline 194 residue within the GS domain serves to stabilize the interaction while the leucine 195 and 196 residues directly mediate the interaction. In addition, a conserved C-terminal region of the kinase domain is also required for FKBP12 binding both in the yeast two-hybrid system and in mammalian cells. The molecular details of the interaction between the C-terminal domain of the receptor and FKBP12 are not completely understood and possibly involve the participation of additional proteins. The interaction between FKBP12 and the type I receptor is abolished in a type II receptor kinase-dependent fashion. The mechanisms involved in releasing FKBP12 are not known, but *in vitro* studies suggested that the transphosphorylation of the GS domain of the type I receptor by the type II receptor kinase likely prevents FKBP12 from re-associating with the activated receptor, thereby exposing the docking site for signal transducers such as Smad2 and possibly also Smad3.

3.5. Questions remaining

A critical issue in the understanding of FKBP12 as an interactor of the TGF-β type I receptor or other members of the type I receptor family is the potential existence of multimeric complex formation of FKBP12 and the type I receptors with additional proteins that co-exist in the complex. Critical insights have been provided by crystallographic analyses of one-to-one interaction between FKBP12 and the type I receptor. Further *in vivo* analyses of how release of FKBP12 from the type I receptor activates the type I receptor as a signaling kinase awaits the elucidation of additional components participating in the receptor/FKBP12 complex.

4. PROGRESS IN THE FUNCTIONAL CHARACTERIZATION OF FKBP12 AS AN INTERACTOR OF THE TGF-β TYPE I RECEPTOR

Since the initial discovery of the interaction between FKBP12 and the TGF-β family type I receptors, several groups have independently examined the functional significance of the interaction.

4.1. Use of non-functional macrolide derivatives to abolish FKBP12 interaction with the type I receptor

The first attempt to discern the functional importance of FKBP12 binding to the type I receptors of the TGF-β family was carried out in 1996, with the aid of two non-functional derivative of FK506, namely the 15-O-desmethyl-FK520 and L685,818 (55). Both compounds bind to FKBP12, but cannot recruit calcineurin; thus, the biological effects can only be ascribed to FKBP12 binding. First it was demonstrated that both compounds were able to abolish FKBP12 interaction with TGF-β type I receptor in transfected and macrolide treated COS1 cells. Subsequently, the compounds were applied in TGF-β-regulated gene response assays in Mv1Lu cells, where they elevated the gene responses induced by low dose TGF-β, but not by high dose of TGF-β. The role of FKBP12 interaction with the putative MIS type I receptor was also examined using these compounds, in a classical organ culture assay system that monitors the biological activity of MIS in inducing the active regression of the Mullerian duct (55). In this assay, the addition of FK506 derivatives significantly sensitized the cells to respond to low dose MIS. Chen *et al.* showed that the TGF-β type II receptor-mediated type I receptor phosphorylation is inhibited by overexpressing FKBP12 and such an inhibition can be abolished by rapamycin (60).

4.2. Use of *in vitro* overexpression systems to enhance FKBP12 effects

Brent R. Stockwell, a graduate student at Stuart Schreiber's laboratory contributed an idea to test whether, by tethering FKBP12 to the cell membrane via attaching a myristylation site at the N-terminus of FKBP12, the inhibitory effect of FKBP12 could persist even in the presence of TGF-β. Such an effect was confirmed; overexpression of the myristylated FKBP12 inhibited transcriptional activation by TGF-β in Mv1Lu cells (55). A potential toxic effect of such an altered form of FKBP12 was ruled out since the overexpressed FKBP12 increases cyclin A expression, which is normally inhibited by TGF-β. This data pointed out that FKBP12 functions as an inhibitor of the type I receptor of TGF-β and suggested that the release of FKBP12 is critical for the activation of the type I receptor signaling activity. Interestingly, a mutant form of FKBP12 defective in its calcineurin-binding site failed to inhibit TGF-β responses, when it was also overexpressed as a myristylated form. Thus, the inhibitory mechanism of FKBP12 might involve the recruitment of additional proteins such as calcineurin, which could mediate dephosphorylation of the type I receptor. Later studies revealed that both the wild type and the mutant form of FKBP12, when myristylated, interact only weakly with the type I receptor (Wang *et al.*, unpublished data). Thus, the observed inhibitory effect of the myristylated form of the wild type of FKBP12 appears to be derived from activities other than direct binding to the type I receptor. Such an observation further strengthens the role of additional proteins bound to FKBP12 in mediating the inhibition of the type I receptor. The inhibitory role of FKBP12 on TGF-β type I receptor as well as the involvement of the macrolide binding domain in such inhibition was also confirmed by Chen *et al.* by directly overexpressing FKBP12 and its mutants that are defective in binding to the type I receptor (F36Y and I90K) (60).

4.3. Test signaling defects of the FKBP12-binding deficient mutant TGF-β type I receptors

Okadome *et al.* demonstrated that several mutant type I receptors defective in signaling were still able to bind to FKBP12 (57). Charng *et al.* created point mutants of the TGF-β type I receptor and demonstrated that the receptor mutant carrying P194A was still capable of signaling and a mutant type I receptor T204D, which is constitutively active, became even more active when the P194A mutation was introduced (63). Both groups concluded that FKBP12 binding is not necessary for the signaling activity of the type I receptor. This is consistent with the observed inhibitory function of FKBP12 (55), since abolishing the binding of an inhibitor is not expected to block the

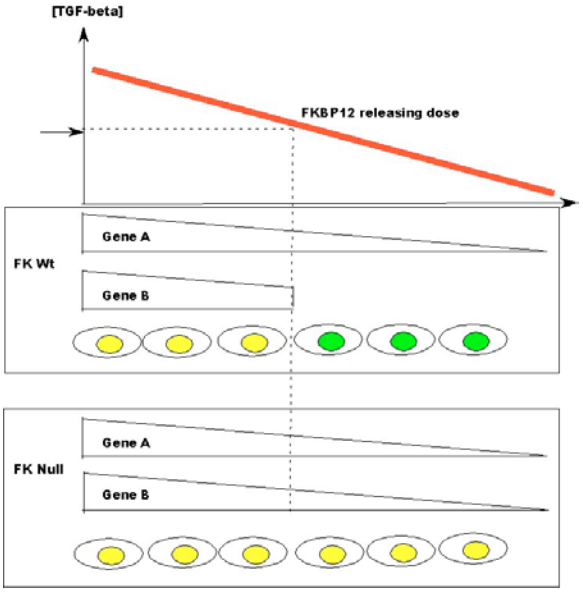

Figure 2. A cartoon to illustrate the role of FKBP12 interaction with the TGF-β family type I receptors for differential signaling along a gradient of a TGF-β family ligand that acts as a morphogen. Along the gradient, two sets of genes, as represented by Gene A and Gene B, are differentially regulated in cells along the gradient, with both sets of genes expressed in cells exposed to concentrations of ligands that are above the FKBP12 releasing dose of the ligand, while only one set of genes are expressed in those that are exposed to ligand dose lower than the releasing dose. The lack of FKBP12 in FKBP12 KO mice could lead to loss of such differential expression of the genes, due to leaky expression of genes below the releasing dose, thereby leading to developmental defects.

signaling, but instead, should assist receptor signaling. Such a property of the mutant receptor was revealed by a set of elegant experiments by Chen *et al.*, who carried out careful functional studies of the interaction using the mutant TGF-β type I receptor (60). Their studies demonstrated that the FKBP12-binding defective type I receptors (P194K, L193G) exhibit much higher (5-8 fold) basal signaling activities when expressed at the Mv1Lu mink lung epithelial cells lacking the wild type type I receptor. When L193G mutant was overexpressed with the type II receptor, it signaled in the absence of TGF-β. The overexpression systems used in these studies could mimic the low-ligand condition *in vivo*. These data suggest that in the absence of FKBP12, the type I receptor would signal upon exposure to low dose ligands, an observation consistent with those from the MIS organ culture assay (55).

In summary, these *in vitro* functional studies indicate that FKBP12 is an inhibitory regulator of the TGF-β type I receptor and MIS type I receptor, which has been recently identified to be ALK2 *in vitro* (64,65) and BMPR1A or ALK3 *in vivo* (66). A separate study also suggests the inhibitory role of FKBP12 in BMP-mediated signaling events (67). The inhibitory interaction of FKBP12 is regulated by the extracellular concentration of TGF-β. The ligand concentration may determine the state of oligomerization of the TGF-β type I and type II receptors. Thus FKBP12 may exhibit different binding properties to the type I receptor in different oligomerization states such that the concentration of the extracellular ligand is monitored from the inside of the cells by FKBP12 binding. Thus, at low dose ligand concentration, FKBP12 binding may keep the type I receptor in an inactive conformation, while at high dose of ligand, FKBP12 is released from the active receptor oligomers to allow them to signal. In this sense, FKBP12 binding functions as a "gradient reader" and could play a role for the morphogenetic activities of members of the TGF-β superfamily. For example, we can imagine the following scenario, as illustrated in the cartoon (Figure 2). Along the gradient of a ligand, cells exposed to a dose of ligand that is equal or above the threshold for FKBP12 release would undergo a full scale of signaling from the type I receptors, while cells more distant from the source of ligand and thereby being exposed to a ligand dose below the threshold for FKBP12 release would fail to be activated. This could lead to different differentiation state of the cells along the gradient. Disruption of such a mechanism could be detrimental to morphogenesis. This prediction is consistent with the phenotypes of the FKBP12 knock-out mice as described below.

4.4. Analysis of FKBP12 KO mice derived cell lines

The most controversial point regarding the physiological role of FKBP12 in regulating TGF-β family receptor signaling was raised by Shou *et al.*, who generated FKBP12 knock-out (KO) mice. Two major developmental abnormalities were found in FKBP12 KO mice, heart defects and failure of neural tube closure (68). Currently the molecular mechanisms involved in these phenotypes are not understood. Based upon the studies of TGF-β responsiveness of the FKBP12-deficient and FKBP12-heterozygous E14.5 fibroblasts, the authors concluded that FKBP12 is not a physiological regulator of the TGF-β family type I receptors since no enhancement of TGF-β signaling was observed in the fibroblast cell line lacking FKBP12. The biochemical data described above, however, point out that abolishing FKBP12 binding leads to an increase of the sensitivity of the type I receptor to respond to low dose ligand, but not to a general enhancement of the signaling capacity of the receptors. This is because FKBP12 is only released when TGF-β concentration is high enough to activate the full signaling capacity of the type I receptor. Therefore the lack of FKBP12 would not alter the signaling readout when ligand levels are high. In other words, the role of FKBP12 was predicted to prevent leaky signaling of the type I receptor at sites where only low level of ligand is present, or sites where high level of type II receptors are expressed. Such a role is likely critical for normal signaling functions of TGF-β family ligands during certain stage of embryonic development when gradient effects of the ligands are crucial, as discussed in the above paragraph. The observed phenotypes of FKBP12 KO mice: cardiac defect and neural closure defects, could well be caused by such a leaky signaling problem from some members of the TGF-β family or BMP family ligands.

Recently another group confirmed constitutive activation of TGF-β signaling in fibroblast cell lines derived from the embryonic day 12.5 of FKBP12 KO mice (69). The FKBP12$^{-/-}$ cells exhibited constitutive cell cycle G1 arrest, which can be rescued by the expression of FKBP12. The G1 arrest was associated with the constitutive elevation in the protein level of the cell cycle inhibitor p21. The TGF-β responsive gene PAI also exhibited increased expression in FKBP12 null cells. The level of phosphorylated p38, which is normally induced upon TGF-β treatment in wild type cells, was also constitutively elevated in FKBP12 null cells. SB203580, a potent and selective inhibitor of p38 catalytic activity, blocked the constitutive elevation of p21 level, suggesting that constitutive activation of p38 in FKBP12 null cells is responsible for increased p21 level and therefore the constitutive G1 arrest. The mRNA level of Smad7, which is known to be turned on as a negative feedback mechanism in response to TGF-β signaling, was found to be constitutively increased in FKBP12 null cells. In aggregate these data are indicative of a constitutively active signaling pathway of TGF-β in FKBP12 null cells. Surprisingly, TGF-β induced phosphorylation of Smad2 was blocked in FKBP12 null cells. This potentially could be due to the presence of large amount of Smad7, which would inhibit Smad2 phosphorylation. Alternatively, the constitutive signaling of TGF-β type I receptor could also accompany the down-regulation of the type I receptor, a possibility yet to be examined.

Taken together, although not fully proven, the *in vivo* studies of FKBP12 KO mice and the embryonic fibroblast cell lines derived from the KO mice provide data that are consistent with a physiological role of FKBP12 in preventing constitutive signaling of the type I receptors of TGF-β family.

4.5. Conclusions

The functional studies of FKBP12 interaction with TGF-β type I receptor in both *in vitro* and *in vivo* systems provide evidence for the role of FKBP12 as a functional regulator of the TGF-β type I receptor. The accumulated data suggest a series of receptor activation steps involving FKBP12 binding and release. FKBP12 binds the type I receptor before TGF-β interaction with the receptors, which are either in monomers or pre-assembled oligomers, as suggested by other studies of the BMP and TGF-β type I receptors (70-72). Upon stimulation of TGF-β, the type II receptor mediates a phosphorylation event which leads to the release of FKBP12, allowing the subsequent phosphorylation of the exposed GS domain, which now no longer can bind to FKBP12, but allows the interaction of signal transducers like Smad2 or Smad3 to approach the receptor kinase. These Smads then become phosphorylated and released from the type I receptor and go on to signal other cytoplasmic and nuclear events. In the absence of FKBP12, the GS domain of the type I receptor is subjected to phosphorylation by the type II receptor without the intermediate step of an FKBP12-releasing event triggered by type II receptor-mediated phosphorylation. Thus, in a cellular environment where a ligand is presented in a gradient, the lack of FKBP12 could significantly alter the cellular responsiveness along the gradient, by reducing the threshold of ligand concentration for GS domain phosphorylation. Therefore, the physiological role of FKBP12 could be an important "gradient reader", or "a type I receptor guardian".

4.6. Questions remaining

It remains to be determined whether FKBP12 is a selective inhibitor of a subset of signaling pathways of the type I receptor, or is a general inhibitor of the activation of the TGF-β type I receptor. The role of TGF-β family signaling leakiness in FKBP12 KO mice needs to be confirmed and other specific physiological roles of FKBP12 in regulating many other TGF-β superfamily members at different developmental stages await future studies.

5.MECHANISTIC STUDIES OF FKBP12-MEDIATED INHIBITION

5.1. The "Docking Inhibitor" model

Wang *et al.* have raised the possibility of FKBP12 docking a third protein to the type I receptor to mediate the inhibitory activity (55). One likely candidate is calcinuerin, since direct interaction between FKBP12 and calcineurin occurs even in the absence of FK506, albeit much weaker (73). The data supporting this possible mechanism is the observation that the myristylated FKBP12 mutant defective in binding to calcineurin is also defective in inhibiting the type I receptor, while the myristylated wild type FKBP12 effectively inhibited the type I receptor. If calcineurin is docked by FKBP12 to the type I receptor, it could mediate inhibition of the type I receptor by dephosphorylating the GS domain.

5.2. The "Blocking type II-mediated type I receptor phosphorylation model"

Chen *et al.* presented data to show that the presence of FKBP12 binding reduces type I receptor phosphorylation, while rapamycin could restore the phosphorylation by releasing FKBP12 (60). The authors thus concluded that the mechanism of inhibition is via blocking type II mediated phosphorylation of the type I receptor. However, the reduction of type I receptor phosphorylation could also be due to FKBP12-mediated docking of an inhibitor of the type I receptor phosphorylation.

5.3. The "Silencing type I receptor kinase activity model"

Experiments combining chemical and biological approaches were carried out to test the inhibitory target of FKBP12 (74). By directly fusing FKBP12 with the cytoplasmic domain of the constitutively activated mutant type I receptor (T204D), which is myristylated and therefore attached on the cell membrane, it was shown that the treatment of cells with FK506 derivatives was sufficient to induce Smad2 phosphorylation and nuclear translocation. This data suggests that FKBP12 can inhibit even a constitutively activated form of the type I receptor; therefore, its inhibitory mechanism does not have to be

mediated by blocking the type II receptor mediated type I receptor phosphorylation. Inhibition of the kinase activity could occur either directly by FKBP12 binding or by another mediator docked by FKBP12.

Therefore, current data points to at least three possible mechanisms for FKBP12-mediated inhibition of the TGF-β type I receptor: 1) FKBP12 binding inactivates the type I receptor kinase activity; 2) FKBP12 brings an inhibitor to the type I receptor to silence the kinase activity; 3) FKBP12 blocks the trans-phosphorylation of the type I receptor by the type II receptor. These mechanisms are not mutually exclusive and could possibly operate in collaboration at different stages.

5.4. Cytoplasmic events inhibited by FKBP12

Several biochemical events along TGF-β signaling pathways are influenced by FKBP12. First, FKBP12 overexpression leads to an inhibition of the type I receptor phosphorylation by the type II receptor in response to TGF-β (60). A recent study revealed the ability of FKBP12 to block the internalization of the TGF-β type I receptor upon TGF-β stimulation (75). In the BMP-mediated signaling pathways, FKBP12 inhibits BMP-induced Smad1 phosphorylation and subsequent proteasomal degradation (76). As suggested by the studies of FKBP12 null fibroblast cells, FKBP12 might also inhibit TGF-β induced p38 phosphorylation, Smad2 phosphorylation, and transcriptional activation of p21 (77).

6. FUTURE PERSPECTIVES

The TGF-β family cytokines/morphogens are well-known for their dynamic and complex biological activities. Many of them are essential for the formation and maintenance of various tissues and organs. The signaling mechanisms used by this family have been gradually unfolding during the past several years. Most of the studies have been focused upon the cellular events after the activation of the TGF-β type I receptor. While it is clear that the type I receptor can activate several different signaling pathways, more intense investigations have been centered around the linear signaling pathways of Smads which at first appeared to be relatively "simple" and "straight-forward". In recent years, however, unexpected findings have made it clear that significant expansion of the current paradigm of TGF-β family signaling mechanisms is inevitable. At the cell membrane, the activation and inactivation of the type I receptor involves not only the type I receptor phosphorylation, but also many other additional events, which could be FKBP12-mediated. Understanding the functional role and mechanism of FKBP12 as a key regulator of type I receptor will lead to a better appreciation of how the various downstream signaling pathways are selectively regulated by varying concentration gradients of extracellular ligands and how the type I receptor can operate dynamically.

6.1. The role of FKBP12 in regulating type I receptor interaction with the type II receptor before, during, and after type I receptor activation

The type I and type II receptors of the TGF-β family are known to form hetero-oligomers before and after ligand binding (70-72) and to activate different signaling pathways from the pre-assembled and ligand-stimulated hetero-oligomers (70). Thus, receptor oligomer formation must play a key role in the assembly and disassembly of the receptor signaling complexes. Furthermore, the down-regulation of the receptor complexes via endocytosis and the subsequent targeting of the receptor complexes to lysosome or proteasome or recycling back to the cell membrane may be also dependent upon the oligomerization state of the receptors.

FKBP12, as a peptidyl-prolyl-isomerase, has been suggested to regulate the conformation of the four subunits of RyR/calcium channels, thereby coordinating the four subunits for full conductance of the channels as well as assisting the interactions between the neighboring RyR/calcium channels to enhance coupled gating (28). Lack of FKBP12 binding to RyR1 channels leads to leaky channels (subconductance states) and contributes to heart failure (26,40,57). Thus, there appears to be a striking similarity between the two known physiological roles of FKBP12, since the lack of FKBP12 binding to the type I receptor also causes leaky signaling from the type I receptor. The cis-trans prolyl-isomerase activity of FKBP12 has yet to be tested, but the conformational role of FKBP12 could be independent from such an activity. One possible scenario, as suggested from studies of another prolyl isomerase, Pin 1 (78), is that FKBP12 regulates the phosphorylation state of the GS domain, by preferably modifying the proline from the cis to the trans conformation after the GS domain is phosphorylated and then allows a PP2A like phosphatase to mediate dephosphorylation of the GS domain. Another candidate phosphatase is calcineurin, which has intrinsic affinity for FKBP12 (73).

6.2. Receptor interaction with other cytoplasmic interactors: prenyltransferases and Caveolin-1

Besides FKBP12, there are multiple other proteins that bind to different type I receptors. Among them is the alpha subunit (FNTA) of the two prenyltransferases, farnesyltransferase (FTase) and geranylgeranyltransferase (GGTase) (79). Domain mapping studies suggest that FNTA interaction with the type I receptor also requires the C-terminal conserved domain (79). Recent studies further indicate that the two β subunits of the prenyltransferases can bind the type I as well as type II receptors under different conditions (Elia A. *et al.*, unpublished data). Thus, the biochemical and functional interactions between FKBP12 and these proteins will be an interesting new territory to explore in the future. Another newfound receptor interactor is the cholesterol-rich membrane microdomain marker protein calveolin-1 (80). Since both FKBP12 and calveolin-1 both are functionally associated with type I receptor endocytosis (75,80), it would be interesting to determine how these two proteins functionally coordinate to regulate receptor endocytosis.

6.3. The mechanism of FKBP12 release upon ligand binding to the type II receptor

As discussed earlier, current studies have not revealed how FKBP12 is released from the activated type I receptor. The structural studies predict that FKBP12

binding to the GS domain would block GS domain phosphorylation. Therefore, the release of FKBP12 has to occur prior to this event. Although the phosphorylated GS domain, when isolated *in vitro*, can no longer bind to FKBP12, this observation does not rule out the possibility of a transient functional interaction between FKBP12 and the phosphorylated GS domain for converting the proline from the cis to the trans conformation.

The release of FKBP12 is known to be dependent upon the kinase activity of the type II receptor. Therefore future studies may focus upon several type II receptor-kinase mediated phosphorylation events, such as type II receptor autophosphorylation, type II receptor-mediated transphosphorylation of the type I receptor at sites other than the GS domain, or phosphorylation of other cytoplasmic proteins that interact with the type I or type II receptor. The phosphorylation could induce a conformational change of the type I receptor, or allow the competitive binding of another protein to FKBP12.

6.4. The physiological function of FKBP12 as a regulator of TGF-β type I receptor

A more direct test for the physiological role of FKBP12 could be to replace the wild-type type I receptor with the type I receptor point mutant, P145A, that is specifically defective in binding to FKBP12 (56,57,60). Tissue-specific and developmental specific knock-in studies will be most informative for a more complete understanding of the physiological role of FKBP12. Different TGF-β family type I receptors can be tested using similar approaches.

7. CONCLUSIONS

The interaction between FKBP12 and the various type I receptors of the TGF-β family ligands may play a key role in allowing the different receptor complexes to recognize and interpret respective ligands gradients and to translate the gradient signals into distinct intracellular signaling responses. Defects in such recognition could dysregulate downstream events that are sensitive to the ligand concentrations and thus contribute to developmental defects. A better understanding of the regulation of FKBP12 interaction with specific TGF-β receptors and other associated intracellular proteins could help to elucidate the downstream elements contributing to tissue and developmental specificity. In the broader context, we see that TGF-β superfamily signaling incorporates many controling steps including multiple levels of extracellular regulators as well as intracellular regulators, involving FKBP12, the prenyltransferases, the inhibitory and activating Smads, as well as members of other downstream pathways. These diverse known and unknown regulatory elements are all needed to assure proper morphogenesis and differentiation.

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9. REFERENCES

1. Schreiber S.L., G.R. Crabtree: The mechanism of action of cyclosporin A and FK506. *Immunol Today* 13, 136-142 (1992)

2. Siekierka J.J., S.H. Hung, M. Poe, C.S. Lin, & N.H. Sigal: A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* 341, 755-757 (1989)

3. Harding M.W., A. Galat, D.E. Uehling & S.L. Schreiber: A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* 341, 758-760 (1989)

4. Lang K., F.X. Schmid: Protein-disulphide isomerase and prolyl isomerase act differently and independently as catalysts of protein folding. *Nature* 331, 453-455 (1988)

5. Van Duyne G.D., R.F. Standaert, P.A. Karplus, S.L. Schreiber & J. Clardy: Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J.Mol.Biol.* 229, 105-124 (1993)

6. Bierer B.E., P.S. Mattila, R.F. Standaert, L.A. Herzenberg, & S.J. Burakoff, Crabtree G, Schreiber SL: Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc.Natl.Acad.Sci.U.S.A* 87, 9231-9235 (1990)

7. Liang J., J. Chio, J. Clardy. Refined structure of the FKBP12-rapamycin-FRB ternary complex at 2.2 A

resolution. Acta Crystallogr. D. Biol. Crystallogr. 55, 736-744 (1999)

8. Liu J, J.D. Farmer Jr., W.S. Lane, J. Friedman, I. Weissman & S.L. Schreiber: Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66, 807-815 (1991)

9. Liu J, M.W. Albers, T.J. Wandless, S. Luan, D.G. Alberg, P.J. Belshaw, P. Cohen, C. MacKintosh, C.B. Klee & S.L. Schreiber: Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry* 31, 3896-3901 (1992)

10. Clipstone NA & G.R. Crabtree: Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 357, 695-697 (1992)

11. Brown EJ, M.W. Albers, T.B. Shin, K. Ichikawa, C.T. Keith, W.S. Lane & S.L. Schreiber: A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* 369, 756-758 (1994)

12. Sabatini DM, H. Erdjument-Bromage, M. Lui, P. Tempst P & S.H. Snyder: RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* 78, 35-43 (1994)

13. Marks AR: Cellular functions of immunophilins. *Physiol Rev.* 76, 631-649 (1996)

14. Cafferkey R, P.R. Young, M.M. McLaughlin, D.J. Bergsma, Y. Koltin, G.M. Sathe, L. Faucette, W.K. Eng, R.K. Johnson & G.P. Livi: Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. *Mol.Cell Biol.* 13, 6012-6023 (1993)

15. Heitman J, N.R. Movva & M.N. Hall: Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* 253, 905-909 (1991)

16. Kunz J, R. Henriquez, U. Schneider, M. Deuter-Reinhard, N.R. Movva & M.N. Hall: Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell* 73, 585-596 (1993)

17. Brunn GJ, C.C. Hudson, A. Sekulic, J.M. Williams, H. Hosoi, P.J. Houghton, J.C. Lawrence Jr. & R.T. Abraham: Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* 277, 99-101 (1997)

18. Burnett PE, R.K. Barrow, N.A. Cohen, S.H. Snyder & D.M. Sabatini: RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc.Natl.Acad.Sci.U.S.A* 95, 1432-1437 (1998)

19. Hara K, K. Yonezawa, M.T. Kozlowski, T. Sugimoto, K. Andrabi, Q.P. Weng , M. Kasuga, I. Nishimoto & J. Avruch: Regulation of eIF-4E BP1 phosphorylation by mTOR. *J.Biol.Chem.* 272, 26457-26463 (1997)

20. Isotani S, K. Hara, C. Tokunaga, H. Inoue, J. Avruch & K. Yonezawa: Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. *J.Biol.Chem.* 274, 34493-34498 (1999)

21. Kim DH, D.D. Sarbassov, S.M. Ali, J.E. King, R.R. Latek, H. Erdjument-Bromage, P. Tempst & D.M. Sabatini: mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110, 163-175 (2002)

22. Wu S, A. Mikhailov, H. Kallo-Hosein, K. Hara, K. Yonezawa & J. Avruch: Characterization of ubiquilin 1, an mTOR-interacting protein. *Biochim.Biophys.Acta* 1542, 41-56 (2002)

23. Chung J, C.J. Kuo, G.R. Crabtree & J. Blenis: Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* 69, 1227-1236 (1992)

24. Kuo CJ, J. Chung, D.R. Fiorentino, W.M. Flanagan, J. Blenis & G.R. Crabtree: Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* 358, 70-73 (1992)

25. Morice WG, G.J. Brunn, G. Wiederrecht, J.J. Siekierka & R.T. Abraham: Rapamycin-induced inhibition of p34cdc2 kinase activation is associated with G1/S-phase growth arrest in T lymphocytes. *J.Biol.Chem.* 268, 3734-3738 (1993)

26. Marks AR: Ryanodine receptors/calcium release channels in heart failure and sudden cardiac death. *J.Mol.Cell Cardiol.* 33, 615-624 (2001)

27. Marks AR: Ryanodine receptors, FKBP12, and heart failure. *Front Biosci.* 7, d970-d977 (2002)

28. Marks AR: Cellular functions of immunophilins. *Physiol Rev.* 76, 631-649 (1996)

29. Schiene-Fischer C & C. Yu: Receptor accessory folding helper enzymes: the functional role of peptidyl prolyl cis/trans isomerases. *FEBS Lett.* 495, 1-6 (2001)

30. Sabatini DM, M.M. Lai & S.H. Snyder: Neural roles of immunophilins and their ligands. Mol.Neurobiol. 15, 223-239 (1997)

31. Brillantes AM, P. Allen, T. Takahashi, S. Izumo & A.R. Marks: Differences in cardiac calcium release channel (ryanodine receptor) expression in myocardium from patients with end-stage heart failure caused by ischemic versus dilated cardiomyopathy. *Circ.Res.* 71, 18-26 (1992)

32. Go LO, M.C. Moschella, J. Watras, K.K. Handa, B.S. Fyfe & A.R. Marks: Differential regulation of two types of intracellular calcium release channels during end-stage heart failure. *J.Clin.Invest* 95, 888-894 (1995)

33. Perez PJ, J. Ramos-Franco, M. Fill & G.A. Mignery: Identification and functional reconstitution of the type 2 inositol 1,4,5-trisphosphate receptor from ventricular cardiac myocytes. *J.Biol.Chem.* 272, 23961-23969 (1997)

34. Brillantes AB, K. Ondrias, A. Scott, E. Kobrinsky, E. Ondriasova, M.C. Moschella, T. Jayaraman, M. Landers, B.E. Ehrlich & A.R. Marks: Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* 77, 513-523 (1994)

35. Marx SO, S. Reiken, Y. Hisamatsu, T. Jayaraman, D. Burkhoff, N. Rosemblit & A.R. Marks: PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* 101, 365-376 (2000)

36. Gaburjakova M, J. Gaburjakova, S. Reiken, F. Huang, S.O. Marx, N. Rosemblit & A.R. Marks: FKBP12 binding modulates ryanodine receptor channel gating. *J.Biol.Chem.* 276, 16931-16935 (2001)

37. Valdivia HH: Modulation of intracellular Ca2+ levels in the heart by sorcin and FKBP12, two accessory proteins of ryanodine receptors. *Trends Pharmacol.Sci.* 19, 479-482 (1998)

38. McCall E, L. Li, H. Satoh, T.R. Shannon, L.A. Blatter & D.M. Bers: Effects of FK-506 on contraction and Ca2+ transients in rat cardiac myocytes. *Circ.Res.* 79, 1110-1121 (1996)

39. Marx SO, K. Ondrias & A.R. Marks: Coupled gating between individual skeletal muscle Ca2+ release channels (ryanodine receptors). *Science* 281, 818-821 (1998)

40. Xiao RP, H.H. Valdivia, K. Bogdanov, C. Valdivia, E.G. Lakatta & H. Cheng: The immunophilin FK506-binding protein modulates Ca2+ release channel closure in rat heart. *J.Physiol* 500 (Pt 2), 343-354 (1997)

41. Shou W, B. Aghdasi, D.L. Armstrong, Q. Guo, S. Bao, M.J. Charng, L.M. Mathews, M.D. Schneider, S.L. Hamilton & M.M. Matzuk: Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. *Nature* 391, 489-492 (1998)

42. Roberts AB & M.B. Sporn: Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors* 8, 1-9 (1993)

43. Roberts AB & M.B. Sporn: Differential expression of the TGF-β isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol.Reprod.Dev.* 32, 91-98 (1992)

44. Hogan BL: Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* 10, 1580-1594 (1996)

45. Whitman M: Smads and early developmental signaling by the TGFbeta superfamily. *Genes Dev.* 12, 2445-2462 (1998)

46. Massague J: TGF-beta signal transduction. *Annu.Rev.Biochem.* 67, 753-791 (1998)

47. Moustakas A, K. Pardali, A. Gaal, C.H. Heldin: Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol. Lett.* 82:85-91 (2002)

48. Wrana JL, L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X.F. Wang & J. Massague: TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 71, 1003-1014 (1992)

49. Massague J, J. Andres, L. Attisano, S. Cheifetz, F. Lopez-Casillas, M. Ohtsuki & J.L. Wrana: TGF-beta receptors. *Mol.Reprod.Dev.* 32, 99-104 (1992)

50. He WW, M.L. Gustafson, S. Hirobe & P.K. Donahoe: Developmental expression of four novel serine/threonine kinase receptors homologous to the activin/transforming growth factor-beta type II receptor family. *Dev.Dyn.* 196, 133-142 (1993)

51. Oh SP, T. Seki, K.A. Goss, T. Imamura, Y. Yi, P.K. Donahoe, L. Li, K. Miyazono, P. ten Dijke, S. Kim & E. Li: Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc.Natl.Acad.Sci.U.S.A* 97, 2626-2631 (2000)

52. Bassing CH, J.M.Yingling, D.J. Howe, T. Wang, W.W. He, M.L. Gustafson, P. Shah, P.K. Donahoe & X.F. Wang: A transforming growth factor beta type I receptor that signals to activate gene expression. *Science* 263, 87-89 (1994)

53. Zervos AS, J. Gyuris & R. Brent: Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. *Cell* 72, 223-232 (1993)

54. Wang T, P.K. Donahoe & A.S. Zervos: Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. *Science* 265, 674-676 (1994)

55. Wang T, B.Y. Li, P.D. Danielson, P.C. Shah, S. Rockwell, R.J. Lechleider, J. Martin, T. Manganaro & P.K. Donahoe: The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell* 86, 435-444 (1996)

56. Charng MJ, P. Kinnunen, J. Hawker, T. Brand & M.D. Schneider: FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-beta receptors. *J.Biol.Chem.* 271, 22941-22944 (1996)

57. Okadome T, E. Oeda, M. Saitoh, H. Ichijo, H.L. Moses, K. Miyazono & M. Kawabata: Characterization of the interaction of FKBP12 with the transforming growth factor-beta type I receptor in vivo. *J.Biol.Chem.* 271, 21687-21690 (1996)

58 ten Dijke P, H. Ichijo, P. Franzen, P. Schulz, J. Saras, H. Toyoshima, C.H. Heldin & K. Miyazono: Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. *Oncogene* 8, 2879-2887 (1993)

59. Schiene-Fischer C & C.Yu: Receptor accessory folding helper enzymes: the functional role of peptidyl prolyl cis/trans isomerases. *FEBS Lett.* 495, 1-6 (2001)

60. Chen YG, F. Liu & J. Massague: Mechanism of TGFbeta receptor inhibition by FKBP12. *EMBO J.* 16, 3866-3876 (1997)

61. Huse M, Y.G. Chen, J. Massague & J. Kuriyan: Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. *Cell* 96, 425-436 (1999)

62. Huse M, T.W. Muir, L. Xu, Y.G. Chen, J. Kuriyan & J. Massague: The TGF beta receptor activation process: an inhibitor- to substrate- binding switch. *Mol.Cell* 8, 671-682 (2001)

63. Charng MJ, D. Zhang, P. Kinnunen & M.D. Schneider: A novel protein distinguishes between quiescent and activated forms of the type I transforming growth factor beta receptor. *J.Biol.Chem.* 273, 9365-9368 (1998)

64. Visser JA, R. Olaso, M. Verhoef-Post, P. Kramer, A.P. Themmen & H.A. Ingraham: The serine/threonine transmembrane receptor ALK2 mediates Mullerian inhibiting substance signaling. *Mol.Endocrinol.* 15, 936-945 (2001)

65. Clarke TR, Y. Hoshiya, S.E. Yi, X. Liu, K.M. Lyons & P.K. Donahoe: Mullerian inhibiting substance signaling uses a bone morphogenetic protein (BMP)-like pathway mediated by ALK2 and induces SMAD6 expression. *Mol.Endocrinol.* 15, 946-959 (2001)

66. Jamin SP, N.A. Arango, Y. Mishina, M.C. Hanks & R.R. Behringer: Requirement of Bmpr1a for Mullerian duct regression during male sexual development. *Nat.Genet.* 32, 408-410 (2002)

67. Gruendler C, Y. Lin, J. Farley & T. Wang: Proteasomal degradation of Smad1 induced by bone morphogenetic proteins. *J.Biol.Chem.* 276, 46533-46543 (2001)

68. Shou W, B. Aghdasi, D.L. Armstrong, Q. Guo, S. Bao, M.J. Charng, L.M. Mathews, M.D. Schneider, S.L. Hamilton & M.M. Matzuk: Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. *Nature* 391, 489-492 (1998)

69. Aghdasi B, K. Ye, A. Resnick, A. Huang, H.C. Ha, X. Guo, T.M. Dawson, V.L. Dawson & S.H. Snyder: FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. *Proc.Natl.Acad.Sci.U.S.A* 98, 2425-2430 (2001)

70. Nohe A, S. Hassel, M. Ehrlich, F. Neubauer, W. Sebald, Y.I. Henis & P. Knaus: The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *J.Biol.Chem.* 277, 5330-5338 (2002)

71. Zwaagstra JC, M. El Alfy & M.D. O'Connor-McCourt: Transforming growth factor (TGF)-beta 1 internalization: modulation by ligand interaction with TGF-beta receptors types I and II and a mechanism that is distinct from clathrin-mediated endocytosis. *J.Biol.Chem.* 276, 27237-27245 (2001)

72. Dore JJ, Jr., M. Edens, N. Garamszegi & E.B. Leof: Heteromeric and homomeric transforming growth factor-beta receptors show distinct signaling and endocytic responses in epithelial cells. *J.Biol.Chem.* 273, 31770-31777 (1998)

73. Cardenas ME, C. Hemenway, R.S. Muir, R. Ye, D. Fiorentino & J. Heitman: Immunophilins interact with calcineurin in the absence of exogenous immunosuppressive ligands. *EMBO J.* 13, 5944-5957 (1994)

74. Stockwell BR & S.L. Schreiber: TGF-beta-signaling with small molecule FKBP12 antagonists that bind myristoylated FKBP12-TGF-beta type I receptor fusion proteins. *Chem.Biol.* 5, 385-395 (1998)

75. Yao D, J.J. Dore, Jr. & E.B. Leof: FKBP12 is a negative regulator of transforming growth factor-beta receptor internalization. *J.Biol.Chem.* 275, 13149-13154 (2000)

76. Gruendler C, Y. Lin, J. Farley & T. Wang: Proteasomal degradation of Smad1 induced by bone morphogenetic proteins. *J.Biol.Chem.* 276, 46533-46543 (2001)

77. Aghdasi B, K. Ye, A. Resnick, A. Huang, H.C. Ha, X. Guo, T.M. Dawson, V.L. Dawson & S.H. Snyder: FKBP12, the 12-kDa FK506-binding protein, is a physiologic regulator of the cell cycle. *Proc.Natl.Acad.Sci.U.S.A* 98, 2425-2430 (2001)

78. Zhou XZ, O. Kops, A. Werner, P.J. Lu, M. Shen, G. Stoller, G. Kullertz, M. Stark, G. Fischer & K.P. Lu: Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. *Mol.Cell* 6, 873-883 (2000)

79. Wang T, P.D. Danielson, B.Y. Li, P.C. Shah, S.D. Kim, & P.K. Donahoe: The p21(RAS) farnesyltransferase alpha subunit in TGF-beta and activin signaling. *Science* 271, 1120-1122 (1996)

80. Razani B, X.L. Zhang, M. Bitzer, G. von Gersdorff, E.P. Bottinger & M.P. Lisanti: Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. *J.Biol.Chem.* 276, 6727-6738 (2001)

81. Xie T, A.L. Finelli & R.W. Padgett: The Drosophila saxophone gene: a serine-threonine kinase receptor of the TGF-beta superfamily. *Science* 263, 1756-1759 (1994)

82. Nellen D, M. Affolter & K. Basler: Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by decapentaplegic. *Cell* 78, 225-237 (1994)

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