Negative Functional Interaction of Retinoic Acid and TGF- β Signaling Mediated by TG-interacting Factor During Chondrogenesis

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Key Words

RAR • TGF- β • Chondrogenesis • All-trans retinoic acid (atRA) • Palatal mesenchyme • TGIF

Abstract

We previously showed that atRA (all-trans Retinoic Acid, atRA) inhibits chondrogenesis by downregulation of TGF- β /Smad signaling. However, the molecular link between RA and TGF- β signaling is unknown. Using a mouse embryo palate mesenchyme micromass cultures (MMCs) system, we explored interactions of RA and TGF- β signaling during chondrogenesis. We found that atRA suppressed chondrogenesis and Smad2/3 phosphorylation regardless of the presence of TGF- β 3. Functional assays indicated that TGF- β 3 treatment or co-transfection of expressing Smad2/3 vectors suppressed atRA-induced RARE-tk-Luc activity. Conversely, atRA or RAR-overexpression repressed TGF- β 3-induced transactivation of the TGF- β -responsive reporter, p3TP-Lux. ChIP assay revealed the binding of the Smad transcriptional co-repressor TGIF (TG-interacting factor, TGIF) to RAR β promoter in control MMCs, but this association was decreased by the addition of RA and increased by TGF- β 3, respectively. Further examinations revealed that TGIF exerted a pivotal role in regulating crosstalk

of RA and TGF- β signaling, since siRNA knockdown of TGIF partially abolished the ability of atRA to suppress TGF- β 3-induced chondrogenesis, whereas forced expression of TGIF blocked the ability of TGF- β 3 to relieve atRA-mediated the suppression of chondrogenesis. Furthermore, we demonstrated that the effects of atRA on TGF- β -dependent gene activation and of TGF- β on RA-dependent gene activation are mediated by TGIF with siRNA to downregulate TGIF. Collectively, these findings indicated a negative functional interplay of RA and TGF- β signaling mediated by TGIF to modulate chondrogenesis in MMCs.

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Introduction

Chondrogenesis is a critical step in palatogenesis. Both TGF- β (β 1, β 2 and β 3) and retinoic acid (RA) signaling are implicated in craniofacial development and play pivotal roles in regulating the proliferation and differentiation of chondrocyte precursors [1-3]. The cellular effects of TGF- β are mediated by the classical Smad pathway. Briefly, TGF- β binds to the TGF- β receptor type II (T β RII) and then activates TGF- β receptor

type I (T β RI), which subsequently activate the R-Smad (Smad2 and Smad3). The phosphorylated R-Smad will bind and activate co-Smad, Smad4, and translocates into the nucleus to regulate the target genes [4]. On the other hand, the biological actions of RA are primarily modulated through heterodimerized forms of retinoid receptors, RARs and RXRs, which are each composed of α , β and γ subtypes with more than one isoform for each receptor subtype [5, 6]. RARs are associated with RXRs to form RAR-RXR heterodimers which bind target RARE. Members of the RAR family recognize two natural stereoisomers of RA, all-trans RA (atRA) and 9-cis RA, whereas the RXR family exclusively recognizes 9-cis RA.

Evidence for a functional linkage between the TGF- β and RA signaling transduction pathways is compelling. RA has been shown to regulate TGF- β expression in numerous systems, both in vitro and in vivo, including cells derived from developing orofacial tissue [7-10]. Indeed, TGF- β activated by RA is thought to mediate the effects of RA on a variety of cell types [11, 12]. Our previous study showed that atRA inhibits chondrogenesis by downregulation of TGF- β /Smad signaling in mouse embryonic palate mesenchymal (MEPM) cells grown in micromass cultures [13]. However, the molecular mechanisms of interaction between TGF- β and RA signaling remain unknown.

TGIF (TG-interacting factor) is a homeodomain containing protein which was first identified by its ability to bind adjacent to a specific retinoic acid response element (RARE) [14]. Function-impaired mutations in human TGIF is associated with holoprosencephaly, a severe genetic disease affecting craniofacial development [15, 16]. TGIF is also a transcriptional corepressor for TGF- β receptor activated Smads [17, 18]. TGIF interacts specifically with Smad2 and Smad3, and is thought to limit the magnitude of the transcriptional response to TGF- β signaling. Since TGIF is involved in both TGF- β and RA signaling, we hypothesized, therefore, that its role was to regulate cross talk between these pathways.

In the present study, we demonstrated an antagonism of RA and TGF- β signaling interaction was mediated by TGIF during chondrogenesis in MEPM micromass cultures (MMCs).

Materials and Methods

Plasmids and reagents

atRA was obtained from Sigma (Sigma-Aldrich, St. Louis, MO). TGF- β 3 was purchased from B&D Bioscience (Bedford, MA). Smad2 (#3103), Smad3 (#9523), phospho-Smad2 (#3101)

and phospho-Smad3 (#9520S) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-TGIF (sc-17800) and anti-actin (sc-1616) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). RAR expressing vector (RAR α and RAR γ) was a kind gift from Dr. Liu (Freie Universität, Berlin). The TGF- β -responsive reporter vector p3TP-Lux, Smad2/3 expression vectors and TGIF expression vectors were kindly provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY) [19, 20].

Preparation of MEPM micromass cultures (MMCs)

MEMC cells were isolated from palate shelves of embryonic day 12 CD1 mouse embryos as described [13]. Briefly, palate shelves were minced and digested with 0.25% trypsin/0.05% EDTA in PBS for 10 min at 37°C with slight shaking. All the cells were filtered through a Cell Strainer (40 μ m; Falcon) to obtain a single cell suspension. The cells were resuspended at a density of 2×10^7 cells/ml before transfections; otherwise, they were resuspended at 1.5×10^7 cells/ml. Droplets were placed in Nunc tissue culture dishes, allowed to adhere to each other and incubated at 37°C for 1 h. Culture medium DMEM/F12 (2:3) supplemented with FBS to 10%; GIBCO BRL) was then carefully added around the cell aggregates. For tests in which 10 ng/ml TGF- β 3 was used, cultures were maintained in serum-free DMEM/F12 medium supplemented with 50 μ g/ml ascorbate phosphate (Sigma) and 50 mg/ml ITS+Premix (Becton-Dickinson; a final concentration of 6.25 μ g/ml bovine insulin, 6.25 μ g/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml bovine serum albumin, and 5.35 μ g/ml linoleic acid). Cultures were maintained at 37°C in a humidified atmosphere of air: CO $_2$, 95:5 (v/v).

Quantitative alcian blue staining of cultures

Alcian blue is a stain specific for cartilage glycosaminoglycans in mature chondrocytes and has been widely used to determine chondrogenic differentiation. Briefly, MMCs were rinsed with phosphate-buffered saline (PBS) and fixed for 10 min with 10% formalin containing 0.5% cetylpyridinium chloride. Spots were washed with 3% acetic acid solution (pH 1.0) and stained overnight with 0.5% Alcian blue 8GX (Sigma) in HCl (pH 1.0). To remove unbound stain, cell layers were washed twice with a 3% acetic acid solution (pH 1.0). The bound stain was extracted with 4 M guanidinium-HCl, 50 mM Tris-HCl, and 0.1% Chaps, pH 7.4, for 2 h at room temperature. Optical density of the extracted dye was evaluated at 600 nm in a microplate reader (Bio-Rad).

Western blot analysis

Cell lysates were prepared from MMCs using 2 x SDS-lysis buffer supplemented with proteases and phosphatases inhibitors (Cell Signaling Technology, Cambridge, MA). Equal amounts of protein extracts were subjected to denatured polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad) by standard blotting procedures. Proteins reactive with primary antibody were visualized with a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence

reagents (Amersham Bioscience, Arlington Heights, IL).

Transient transfection and luciferase assay

MEPM cells were suspended at a density of 2×10^7 cells/ml in DMEM/F12/10% FBS medium. The cells were transiently transfected with a fixed amount (0.1 μ g) of internal control reporter CMV-*Renilla* luciferase construct and either a mix of expression vector RAR (0.7 μ g) + p3TP-Lux (0.7 μ g) or a mix of Smad2 (0.7 μ g)/Smad3 (0.7 μ g) + RARE-tk-Luc (0.7 μ g) using Lipofectamine 2000 reagent (Invitrogen). The blended construct was combined in 100 μ l of Opti-MEM medium (Invitrogen) and then added to an equal volume of Lipofectin/Opti-MEM (6:100) mixture. For transfection purposes, 100 μ l of mesenchymal suspension (2×10^6 cells) was mixed with DNA/Lipofectin complex (2:1 (v/v)). Following incubation for 8 min at room temperature, 15- μ l spots of cell suspension were plated onto 6-well Nunc culture dishes. After incubated at 37°C in a 5% CO₂/95% air atmosphere for 1 h, the cultures were flooded with DMEM/F12/10% FBS medium. 8 h after transfection, test compounds were added and incubated for an additional 2 days. The cultures were then collected and processed for luciferase activity with the Promega dual luciferase assay system, normalized and expressed as mean $\pm$ SE of triplicate from a representative experiment performed at least three times.

For investigating the effect of TGIF on the ability of TGF- β 3 to restrict atRA-induced inhibition of chondrogenesis, TGIF-expressing vector plasmid [pLenti6/V5-D-TOPO vector (Invitrogen)] or empty vector were used for lentivirus production and transduced to MMCs.

TGIF gene silencing using siRNA

The oligonucleotides containing sequences from human TGIF (ATC TGG ACC AAG TAC GAAT) were used to knockdown TGIF expression. The control siRNA is a mutant, which contains three base mutations that do not affect the peptide sequence (ATC TGG ACC CAG TCC AAAT). Briefly, 200 nM siRNA solution was prepared by diluting a siRNA stock (50 μ M) in Richter's Modified Improved MEM medium (Gibco 10373-017) containing Oligofectamine (0.2%) (Invitrogen, Carlsbad, CA). The siRNA media solution was prepared in 2 ml aliquots and 2 ml were used in the 35 mm Nunc tissue culture dishes. After 18 h, the medium of the cultures was changed with culture medium for another 2 days. The cultures were then collected and processed for further analysis.

Chromatin immunoprecipitation (ChIP) assay

Droplets were grown in 100 mm Nunc tissue culture dishes. After various treatments, the cultures were then directly treated with the crosslinking reagent formaldehyde. Nuclei were collected and sheared DNA to between 200 and 1,000 bp in length by Sonication. Supernatants were obtained by centrifugation for 10 min and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). The chromatin was immunoprecipitated with TGIF antibody or with IgG as a negative control. Immunoprecipitates were collected with protein A-agarose beads (Upstate Biotechnology) and washed sequentially with a low-salt and high-salt wash buffer (Upstate

Fig. 1. Effect of atRA versus TGF- β 3 on chondrogenesis and Smad2/3 phosphorylation: The MMCs were treated with 1 μ M atRA, or 10 ng/ml TGF- β 3, or combination of 1 μ M atRA and 10 ng/ml TGF- β 3 for 3 days. The cultures were then collected and processed for quantitative alcian blue staining to evaluate chondrogenesis (A) or for western blot analysis to examine levels of Smad3 and Smad2 phosphorylation (B). Data are presented as mean $\pm$ SEM, $n=3$. **, $P < 0.01$, Significant difference from control values. ##, $P < 0.01$, Significant difference from TGF- β 3. Representative results from three independent experiments with similar results are shown.

Biotechnology). DNA-protein complexes were eluted with 1% SDS and 0.1 M NaHCO₃, heated to 65°C overnight, and deproteinized by treatment with proteinase K at 45°C for 1 h. The DNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1) extractions and precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol using glycogen as a carrier. Immunoprecipitated DNAs were assayed by PCR with the following primers: RAR α -promoter, 5'-GGG AGT TTT TAA GCG CTG TGAG-3' and 5'-GGA GCAGCT CAC TTC CTA CC-3'; and 18S gene, 5'-CGC TAC CAC ATC CAA GGAA-3' and 5'-GCT GGA ATT ACC GCG GCT-3'. The PCR products were separated by electrophoresis through 2.0% agarose gels.

Statistical analysis

Data were presented as mean $\pm$ SEM, from three separated experiments. Statistical differences were evaluated using the one-way ANOVA. Statistical significance was defined as a P value < 0.05 .

Results

Effect of atRA versus TGF- β 3 on chondrogenesis and Smad2/3 phosphorylation

As shown in Fig. 1, atRA inhibited chondrogenesis (Fig. 1A) and the levels of phospho-Smad2/3 (Fig. 1B); whereas, treatment with TGF- β 3 strongly induced phospho-Smad2/3 and chondrogenesis. Additionally, combined treatment with atRA plus TGF- β 3 significantly reduced the levels of phospho-Smad2/3 and repressed chondrogenesis compared with that seen in MMCs treated with TGF- β 3 alone. The expression levels of total Smad2/3 remained unchanged, as shown by re-probing the membranes with anti-Smad2/3 antibodies (Fig. 1B).

Activation of TGF- β /Smad signaling suppressed RAR-mediated gene transcription

To investigate the functional effects of increased TGF- β /Smad activation on RA pathway, the MMCs were co-transiently transfected with Smad2/3 expression vectors and the reporter construct pGL3(RARE)5-tk-luc (RARE-tk-Luc), which contains five DR5-type RAREs from the human RAR β promoter and responds well to RA induction [21]. As shown in Fig. 2, atRA significantly increased the luciferase reporter activity of RARE-tk-Luc. Co-transfection with both Smad2 and Smad3 expression vectors or TGF- β 3 treatment repressed both basal and atRA-induced luciferase activity; and importantly, Smads overexpression plus TGF- β 3 treatment led to a synergistic decrease in RARE-tk-Luc activity compared with that seen in Smad2/3 overexpression or TGF- β 3 treatment alone.

Activation of RA signaling suppressed transactivation of TGF- β -responsive reporter gene

We next tried to determine whether RA-mediated activation would directly affect transactivation of TGF- β -responsive reporter gene. To that end, the MMCs were transfected with a constitutively active RAR α and RAR γ expression plasmid [22] along with p3TP-Lux, which contains elements from the PAI-1 promoter and drives the expression of a luciferase reporter gene in response to TGF- β [19, 23]. After transfection, the cultures were treated 1 μ M atRA, 10 ng/ml TGF- β 3 or in combination for additional 2 days. As illustrated in Fig. 3, TGF- β 3 alone dramatically elevated p3TP-Lux activity; whereas, cotransfection with RAR expression vector, or atRA treatment, or cotransfection of RARs plus atRA decreased both basal and TGF- β 3-induced reporter gene activity.

Fig. 2. Activation of TGF- β /Smad signaling suppressed RAR-mediated gene transcription: MEPM cells were co-transfected with Smad2 and Smad3 expression vectors and RARE-tk-Luc, along with internal control reporter CMV-*Renilla* luciferase construct. 8 h after transfection, the cultures were treated with 1 μ M atRA, or 10 ng/ml TGF- β 3, or 1 μ M atRA plus 10 ng/ml for an additional 2 days. The cultures were then collected and processed for luciferase activity with the Promega dual luciferase assay system, normalized and expressed as mean $\pm$ SEM of triplicate from a representative experiment performed at least three times. *, $P < 0.01$, Significant difference from control values. **, $P < 0.01$, Significant difference from atRA.

Fig. 3. RAR activation suppressed transactivation of TGF- β -responsive reporter gene: MEPM cells were co-transfected with RAR expression vector and p3TP-Lux, along with internal control reporter CMV-*Renilla* luciferase construct. 8 h after transfection, the cultures were treated with 1 μ M atRA, or 10 ng/ml TGF- β 3, or 1 μ M atRA plus 10 ng/ml for an additional 2 days. The cultures were then collected and processed for luciferase activity with the Promega dual luciferase assay system, normalized and expressed as mean $\pm$ SEM of triplicate from a representative experiment performed at least three times. *, $P < 0.01$, Significant difference from control values. **, $P < 0.01$, Significant difference from TGF- β 3.

Fig. 4. The role of TGIF in crosstalk between TGF- β and RA signaling: (A) MMCs were treated with 10 ng/ml TGF- β 3 or 1 μ M atRA for 3 days. Chromatin was cross-linked with formaldehyde and subjected to immunoprecipitation with a TGIF-specific antibody or with IgG as negative control. Precipitates were analyzed by PCR for the presence of a region of the RAR β promoter which spans the RARE. PCR conducted on DNA derived from input chromatin template served as a positive control (100% reference), and that on IgG-precipitated template as specificity control. (B) MEPM cells were transfected with either 200 nM TGIF siRNA or off-target control siRNA. At 18 h after transfection, the cultures were collected and processed for western blot to examine the efficiency of specific siRNA knockdown. (C) The transfected MMCs by TGIF siRNA or control siRNA were further treated with 10 ng/ml TGF- β 3, 1 μ M atRA or in combination as indicated for an additional 2 days. The cultures were then collected and processed for quantitative Alcian blue staining to evaluate chondrogenesis. **, $P < 0.05$ versus control TGIF siRNA; ##, $P < 0.01$ versus TGIF siRNA + TGF- β 3. (D) MEPM cells were transfected with expression vectors encoding TGIF, as described in "Materials and methods". The cultures were treated with 10 ng/ml TGF- β 3, 1 μ M atRA or in combination for an additional 2 days and then processed for quantitative Alcian blue staining to evaluate chondrogenesis. **, $P < 0.05$ versus empty vector; ##, $P < 0.01$ versus TGIF expression vector + TGF- β 3. Data here are mean $\pm$ SEM. Each experiment was performed in triplicate.

The role of TGIF in crosstalk between TGF- β and RA signaling

Since TGIF plays roles in both TGF- β and RA signaling as described previously, we hypothesized, therefore, that TGIF was involved in regulating cross talk between these pathways. To test that assumption, MMCs were treated with 10 ng/ml TGF- β 3 or 1 μ M atRA for 3 days. The cultures were collected and processed for ChIP assay. As shown in Fig. 4A, TGIF was present at the RAR β promoter in solvent control cultures, but this association was clearly decreased by the addition of RA and increased by TGF- β 3.

Next, we used two different approaches to further confirm whether the functional interaction of TGF- β and RA signaling was associated with TGIF during chondrogenesis. In the first approach, we used TGIF siRNA to knockdown TGIF. We observed that TGIF siRNA decreased the expression of endogenous TGIF by 70-80% compared to control (Fig. 4B) and TGIF depletion partially abolished the ability of atRA to suppress TGF- β 3-induced chondrogenesis (Fig. 4C). In the second approach, we employed TGIF expression vectors to examine whether forced expression of TGIF could affect

the ability of TGF- β 3 to restrict atRA-induced inhibition of chondrogenesis. We found that expression of TGIF blocked the ability of TGF- β 3 to attenuate atRA-mediated inhibition of chondrogenesis (Fig. 4D). However, we did not observe that TGIF depletion or increased expression of TGIF had any discernible effect on TGF- β 3- and atRA-mediated chondrogenic differentiation (Fig. 4C and D).

Interaction of TGF- β and RA signaling was mediated by TGIF

The unexpected requirement for TGIF in suppression of TGF- β 3-induced chondrogenesis by atRA implied a novel role for TGIF in coordinating the interactions between RA and TGF- β signaling. Then, we set out to determine whether the negative interplay of these two pathway was mediated through TGIF. For that purpose, we used the same TGIF siRNA as previously tested in the ChIP assay to abolish TGIF in MMCs; we then transfected the MMCs together with RARE-tk-Luc or p3TP-Lux. We found that the effects of atRA on TGF- β -dependent gene activation and of TGF- β on RA-dependent gene activation failed altogether because of the deletion of TGIF (Fig. 5A and Fig. 5B).

Discussion

The interplay between RA and TGF- β signaling has been shown in numerous systems, both *in vivo* and *in vitro*, but the reports from different authors are controversial. In some cell lines, RA increases production of TGF- β 1 and its receptors, leading to growth inhibition [24-27]. RA, however, has also been reported to inhibit endogenous TGF- β signaling in the mesenchyme of the developing inner ear [28] and palate [13] in mice. Disruption of RAR α in mice results in abnormal upregulation of TGF- β 2 in cardiac tissue [29]. A microarray analysis of vitamin A-deficient rat embryos revealed upregulation of TGF- β -dependent activity [30]. By contrast, in the yolk sac, disruption of RA signaling inhibits TGF- β -dependent expression of fibronectin and integrin and disrupts visceral endoderm and vascular development [31]. The discordant data suggest that the way RA and TGF- β interact is strongly influenced by the local milieu and thus depends on the particular system studied.

Our finding of negative regulation of the TGF- β pathway by RA was supported by several lines of observation. First, atRA was able to suppress TGF- β 3-induced Smad2/3 phosphorylation and chondrogenesis. Second, TGF- β 3 treatment or transfection of expressing Smad2/3 vectors suppressed atRA-induced RARE-tk-Luc activity. Third, atRA or RAR-overexpression repressed TGF- β 3-induced transactivation of the TGF- β -responsive reporter, p3TP-Lux. Additionally, our study strongly suggests that the negative functional interaction of RA and TGF- β signaling was mediated by TGIF.

TGIF is a TALE (three amino acid loop extension)-

Fig. 5. Interplay of TGF- β and RA signaling mediated by TGIF: (A) MMCs were transfected with the RARE-tk-Luc reporter together with off-target control siRNA or TGIF siRNA and treated with 10 ng/ml TGF- β 3, 1 μ M atRA or in combination, as indicated, 2 days prior to analysis. Three days after transfection, luciferase activity was assayed. **, $P < 0.05$ versus atRA treatment only; . ##, $P < 0.01$ versus 1 μ M atRA + TGF- β 3. (B) MMCs were transfected with the TGF- β -responsive reporter p3TP-Lux together with control siRNA or TGIF siRNA and treated with 10 ng/ml TGF- β 3, 1 μ M atRA or in combination, as indicated, 2 days prior to analysis. Three days after transfection, luciferase activity was assayed. **, $P < 0.05$ versus TGF- β 3 treatment only; ##, $P < 0.01$ versus 1 μ M atRA + TGF- β 3. Data here are mean $\pm$ SEM. Each experiment was performed in triplicate.

class homeobox transcription factor, and was originally discovered in a screen for retinoid X response element binding proteins [14]. Overexpression of TGIF represses transcription of a retinoid-responsive reporter in human glioma U87 and COS1 cells [14]. Recently, TGIF has been shown to bind retinoid X receptors (RXRs) and less

robustly, retinoic acid (RA) receptors (RARs) in COS1 cells, and repress RA-responsive reporter transcription in HepG2 cells [32]. Overall, these studies suggest that TGIF can function as a repressor to regulate RA-responsive genes. Recently, TGIF was shown to function as a transcriptional corepressor for TGF- β -activated Smads [17]. TGIF interacts with Smad2 and Smad3. It represses Smad-mediated transcriptional activation through recruitment of general corepressors, such as CtBP, mSin3, and histone deacetylases, to a TGF- β -activated Smad complex [17, 33, 34]. The Smad-binding domain in TGIF was mapped to amino acids 138 through 192 [17], and deletion of amino acids 149 through 176 in TGIF disrupts its ability to interact with Smad2 [17]. Another mechanism by which TGIF inhibits TGF- β signaling is by participating in the ubiquitin-dependent degradation of Smad2. TGIF binds to the E3 ubiquitin ligase Tiul1 (TGIF-interacting ubiquitin ligase 1), yet TGIF is not degraded by Tiul1. Instead, TGIF forms a stable complex with Smad2 and Tiul1, which then induces ubiquitination and degradation of Smad2 [35].

As TGIF is involved in both TGF- β and RA signaling, we next set out to determine whether its role was involved in regulating crosstalk between these two pathways. First, we showed by ChIP that endogenous TGIF could associate with a region of the RAR β promoter which contains the canonical DR5 element used in the transcriptional reporters and that this association was clearly decreased by the addition of RA and increased by TGF- β 3, respectively. Further examinations revealed that TGIF exerted a pivotal role in regulating crosstalk of RA and TGF- β signaling, since siRNA knockdown of TGIF partially abolished the ability of atRA to suppress TGF- β 3-induced chondrogenesis, whereas forced expression of TGIF blocked the ability of TGF- β 3 to relieve atRA-mediated the suppression of chondrogenesis. Finally, we provided strong evidence that the crosstalk of TGF- β and RA signaling was mediated by TGIF: the functional antagonism between these two pathways failed altogether when TGIF was deleted by stable siRNA expression (Fig. 5A and 5B). Together these data propose that, as to the role of TGIF in regulating interaction between TGF- β and RA signaling, it might has two choices: to interact with an incoming Smad2/3–Smad4 complex, resulting in the formation of a transcriptional repressor complex, or to bind adjacent to RARE, leading to the repression of RA-induced transcriptional activation. The outcome of this competition is dependent in part on the relative activity balance between RA and TGF- β signaling present in the cell. In summary, we here showed

Fig. 6. Model for the interplay of RA and TGF β signaling through TGIF: The canonical TGF- β signaling involves ligand-mediated receptor activation, R-Smad phosphorylation, and Smad translocation to the nucleus to regulate transcription. The RA signaling is modulated through heterodimerized form of retinoid receptors RAR and RXR, which binds to RARE. as to the role of TGIF in regulating interaction between TGF- β and RA signaling, it might has two choices: to interact with an incoming Smad2/3–Smad4 complex, resulting in the formation of a transcriptional repressor complex, or to bind adjacent to RARE, leading to the repression of RA-induced transcriptional activation. The outcome of this competition is dependent in part on the relative levels of RA and TGF- β signaling present in the cell.

that the mechanism coordinating the antagonism between RAR and TGF- β /Smad signaling during chondrogenesis of MEPM in vitro was mediated through TGIF (Fig. 6).

Abbreviations

atRA(all-trans Retinoic Acid); RAR (retinoic acid receptor); TGF (transforming growth factor); TGIF (TG-interacting factor); MMCs (mesenchyme micomass cultures); T β RI (TGF- β receptor type I); ChIP (chromatin immunoprecipitation); MEPM (mouse embryonic palate mesenchym); RARE (retinoic acid response element).

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