Rapid communication

Regulation of the *TMEPAI* promoter by TCF7L2: the C-terminal tail of TCF7L2 is essential to activate the *TMEPAI* gene

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We previously found that TCF7L2 could activate the *TMEPAI* gene efficiently, whereas LEF1 could not nearly augment its transcription. When we comprehended the functional difference(s) between TCF7L2 and LEF1 with respect to the activation of the *TMEPAI* gene, the C-terminal tail of TCF7L2 was needed to reveal its transcriptional activity as well as its interaction with Smad3. Consistently, both TCF7/TCF7L2 and LEF1/TCF7L2 chimeric proteins exhibited an activity similar to TCF7L2 in transcription and Smad3 binding in contrast with LEF1 and TCF7. Our data elaborated on the diverse activity among TCF/LEF family members with respect to the transcriptional regulation of the *TMEPAI* gene.

Keywords: C-clamp/Smad3/TCF/LEF family/TGF- β /TMEPAI.

The Wnt signalling pathways play key roles in regulation of homeostasis during both embryogenesis and adult life. These pathways have also involved in stem cell maintenance and tissue renewal. Thus, dysregulation of Wnt signal transduction is linked to embryonic defects and diseases such as cancer, fibrosis and diabetes (1–5).

The Wnt signalling pathways have been categorized as canonical and non-canonical pathways. The canonical pathway is mediated by β -catenin, which can interact with T-cell factor (TCF)/lymphoid enhance factor (LEF) family members in the nucleus, where the complex between β -catenin and TCF/LEF family members regulates Wnt target genes. On the other hand, the non-canonical pathway, which is alternatively termed the β -catenin-independent Wnt signalling pathway, triggers calcium efflux, ROCK activation, JNK

phosphorylation to control cytoskeleton rearrangement, planar cell polarity and transcriptional events (5). The TCF/LEF family, encompasses TCF7 (also termed TCF1), TCF7L1 (also termed TCF3), TCF7L2 (also termed TCF4) and LEF1 (also termed TCF7L3) in mammals. There are four conserved regions in all TCF/LEFs: the β -catenin binding region in the N terminus, the high-mobility group (HMG) box in the middle region, the basic tail close to the HMG box and the nuclear localization signal (NLS). The HMG box recognizes a specific DNA sequence, (C/G)TTT(C/G)(A/T)N(C/G), together with the basic tail to regulate Wnt/ β -catenin target genes (1, 3, 5). Besides, some isoforms of the TCF/LEF family include the C-clamp in the C-terminal tail, which is known to act as the secondary DNA-binding domain (DBD). The C-clamp, whose four conserved cysteine residues are critical for its function, might target GC-rich sequences in the promoter regions of some genes (1, 6–9).

In a previous study, we showed that the *TMEPAI* gene is transcriptionally regulated by TCF7L2 which has a long C-terminal tail among the alternative splicing forms of TCF7L2. On the other hand, LEF1 could marginally activate the *TMEPAI* gene (10). Because both TCF7L2 and LEF1 can activate the Topflash reporter and interact with Smads (11, 12), we explored in detail the mechanism by which TCF7L2, in contrast to LEF1, dramatically activates the *TMEPAI* gene.

Previously, we did not examine the differences between TCF7L2- and LEF1-induced *TMEPAI* reporter activities upon TGF- β stimulation, although LEF1 had very marginal ability to activate the *TMEPAI* luciferase reporter as compared with TCF7L2 (10). Using the pGL3ti-850 luciferase reporter, whose activity can reflect the transcriptional activation of the *TMEPAI* gene upon TGF- β stimulation (10), we cotransfected either TCF7L2 or LEF1 in HepG2 cells and then subjected them to stimulation with TGF- β . As seen in Fig. 1a, LEF1 could enhance the basal activity of this reporter slightly, whereas TGF- β did not potentiate LEF1-mediated reporter activity. Since LEF1 encodes a shorter protein than TCF7L2, which has an extension of ~200 amino acids at its C terminus (termed the C-terminal tail) (Fig. 1b), we postulated that a chimeric protein LEF1/TCF7L2 might recuperate the ability to activate the pGL3ti-850 reporter upon TGF- β stimulation (Fig. 1b). As expected, the LEF1/TCF7L2 enormously potentiated the basal reporter activity of pGL3ti-850. Moreover, TGF- β further augmented this reporter activity enhanced by LEF1/TCF7L2. When we checked the abilities of the other two TCF/LEF family members, TCF7 and TCF7L1, to activate the pGL3ti-850 reporter, neither one influenced its activity, even though the cells were stimulated with TGF- β (Fig. 1c). Since TCF7L1 also possesses a C-terminal extended region, which has no resemblance to the C-terminal tail of TCF7L2, we speculated that the C-terminal tail of TCF7L2 might provide TCF7L2

Fig. 1 Requirement of C-terminal tail of TCF7L2 for activation of the *TMEMAI* gene. (a) TCF7L2, but not LEF1, can enhance TGF- β -induced *TMEMAI* reporter activity. Either TCF7L2 or LEF1 was transfected in HepG2 cells with pGL3ti850 (15) and pCH110 (GE Healthcare) according to Nakano *et al.* (10). (b) Structures of the examined TCF/LEF family members and their mutants. LEF1/TCF7L2 and TCF7/TCF7L2 were constructed by fusion of LEF1 and TCF7 with TCF7L2 (from Q⁴¹¹ to E⁵⁹⁶), respectively. TCF7L2 Δ C consisted of TCF7L2 lacking the region from Q⁴¹¹ to E⁵⁹⁶. TCF7L2(C463A) indicates replacement of the fourth C⁴⁶³ into A in the C-clamp of TCF7L2. HMG DBD, HMG DNA-binding domain; NLS, nuclear localization signal. (c) Effect of TCF/LEF and its mutants on TGF- β -mediated *TMEMAI* reporter activity. Each expression plasmid was transfected in HepG2 cells with pGL3ti850 and pCH110 according to Nakano *et al.* (10). To observe the protein expression for each construct, western blot analysis was performed with anti-HA (3F10) antibody (Roche) according to Nakano *et al.* (10). (d) The C-clamp is not required for TCF7L2 to activate the *TMEMAI* reporter. HepG2 cells were transfected with Gal4-TATA-Luc (Promega), and either Gal4 DBD alone (Gal4) (16), Gal4 DBD-TCF7L2, Gal4 DBD-C-clamp or Gal4-DBD-C-clamp(C463A) according to Itoh *et al.* (17). Gal4 DBD-C-clamp and Gal4 DBD-C-clamp(C463A) were constructed by fusion of Gal4-DBD with TCF7L2 (from Q⁴¹¹ to E⁵⁹⁶) or TCF7L2(C463A) (from Q⁴¹¹ to E⁵⁹⁶), respectively.

with the ability to activate the pGL3ti-850 reporter. The TCF7 isoform, which does not possess the long C-terminal region of the alternative splicing forms of TCF7, was also fused with the C-terminal tail of TCF7L2 (TCF7/TCF7L2) (Fig. 1b) for investigation of the activity of the pGL3ti-850 reporter. Unlike TCF7, the chimeric protein TCF7/TCF7L2 could activate this reporter upon TGF- β stimulation (Fig. 1c). It has been reported that the alternative splicing form of TCF7 (termed TCF7E), which, like TCF7L2, possesses a C-terminal long tail including the C-clamp, potentiates the activity of LOPTk reporter which includes three copies of E-tail-requiring Wnt response elements (WREs) from the LEF1 promoter instead of three repeats of typical TCF/LEF WREs. That report also showed that the fusion of TCF7E's C-terminal long tail with LEF1 emerges as a transcriptional activator of the LOPTk reporter, although LEF1 loses this activity (7). To confirm that the C-terminal tail of TCF7L2 displays its competence to mediate the activation of the

pGL3ti-850 reporter, we made two mutants, TCF7L2 Δ C and TCF7L2(C463A) (Fig. 1b). Both mutants lost the ability to activate the pGL3ti-850 reporter. Thus, we concluded that the C-clamp in the C-terminal tail of TCF7L2 is necessary for TCF7L2 to potentiate pGL3ti-850 reporter activity upon TGF- β stimulation (Fig. 1c). The possible abilities required by the C-terminal tail of TCF7L2 for activation of the pGL3ti-850 reporter are as follows: DNA binding, interaction with Smad3, or transcriptional activation. When we fused TCF7L2 to the Gal4 DBD (Gal4 DBD), we could observe marginal transactivation of the reporter gene composed of the Gal4-DNA binding sequence although TGF- β stimulation inhibited its action. In contrast, the Gal4 DBD fusion protein with the C-clamp or the C-clamp(C463A) of TCF7L2 suppressed the basal reporter activity. Thus, it is highly possible that the C-terminal region including the C-clamp in TCF7L2 does not possess any ability for transcriptional activation (Fig. 1d). This evidence is

consistent with notion that the N-terminal domain of TCF/LEF family members plays an important role in the activation of canonical Wnt target genes via its association with β -catenin which recruits CBP/p300 (5).

All the TCF/LEF family members are known to bind to specific DNA sequences recognized by their HMG DBD, which consists of both its HMG box and its basic tail. In addition, the C-clamp in the splicing variants of TCF7 and TCF7L2 acts as the secondary DNA-binding region that preferentially recognizes GC-rich sequences (1, 5). Since TCF7L2 is known to bind to TGF- β -responsive TCF7L2-binding element (TTE) within the enhancer of the *TMEPAI* gene in TGF- β -induced *TMEPAI* activation, TTE is a critical *cis*-element for TGF- β to potentiate the transcription of *TMEPAI* together with Smad3 (10). Thus, we examined if the C-terminal tail of TCF7L2 binds to TTE. However, we could not observe any differences between the TCF/LEF family members and their mutants in terms of binding to TTE when a DNA affinity precipitation (DNAP) assay was carried out (Fig. 2a). Therefore, the C-terminal tail of TCF7L2 is not needed for TCF7L2 to bind to TTE. These results are not surprising because the examined TCF/LEF family members and their mutants possess HMG DBD in their structures (Fig. 1b).

TCF7L2 has already been shown to bind to Smad3 (10, 13, 14). Thus, we examined the interaction of Smad3 with TCF/LEF family members and their mutants. Since we have already shown that the Smad3 MH2 domain is involved in the interaction between Smad3 and TCF7L2 (10), we used GST-Smad3 MH2 to pull down the TCF/LEF family members and their mutants expressed in COS7 cells. As seen in Fig. 2b, TCF7L2 and TCF7L2(C463A) could interact with Smad3 MH2, whereas the interaction of TCF7L2ΔC with Smad3 MH2 was reduced. Curiously, both TCF7L2(C463A) and TCF7L2ΔC could not activate the reporter activity (Fig. 1c) in spite of that both of them still bound to Smad3. It is possible that other transcriptional factor(s) and/or co-factor(s) might be needed for TCF7L2 to activate the TMEMPAI gene together with Smad3 via its C-terminal region (or its C-clamp). This possibility will need to be examined in future. Although interaction between LEF1 and Smad3 MH2 could marginally be detected, LEF1/TCF7L2 revealed the ability to associate with Smad3 MH2 obviously. In addition, fusion of the C-terminal tail of TCF7L2 to TCF7 (TCF7/TCF7L2), but not TCF7, exhibited the ability to interact with Smad3 MH2. These results indicate that the C-terminal tail of TCF7L2 is critical for TCF7L2 to associate with Smad3, although its C-clamp is not required for this association.

In conclusion, we found that the C-clamp within TCF7L2 is needed for activation of the *TMEPAI* gene. However, its interaction domain with Smad3 lies in the region of the C-terminal tail other than the C-clamp. We suppose that the mutation of the C-clamp might lead TCF7L2 to a conformational change by which it loses its ability of transcriptional activation. To confirm this, we need to conduct further

Fig. 2 Interaction of C-terminal tail of TCF7L2 with Smad3. (a) Ability of TCF/LEF family members and their mutants to bind to DNA. DNAP was performed according to our previous report (10). Protein binding to DNA using rat anti-HA antibody (3F10) was detected (upper panel). The expression of TCF/LEF family members and their mutants using rat anti-HA antibody (3F10) was measured (lower panel). **(b)** Interaction of Smad3 MH2 with TCF/LEF family members and their mutants. Using GST-Smad3 MH2 (upper and 2nd panels) and GST alone (3rd panel), the pulldown assay was carried out (17). Upper and 2nd panels show photos exposed for short and long-terms, respectively. Total expression of TCF/LEF family members and their mutants was shown (lower panel). Western blot analysis was performed using rat anti-HA antibody (3F10).

experiments. TCF7L2, but not LEF1, can efficiently activate the *TMEPAI* gene together with the TGF- β /Smad3 signalling pathway. The current evidence indicates that interplay between the TCF/LEF family and Smad3 regulates the fine-tuning of TGF- β signalling via *TMEPAI* expression. We are continuing to investigate the importance of the TGF- β -mediated *TMEPAI* gene by TCF/LEF.

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Conflict of Interest

None declared.

References

1. Cadigan, K. M., and Waterman, M. L. (2012) TCF/LEFs and Wnt signaling in the nucleus. *Cold Spring Harbor Perspect. Biol.* **4**, a007906
2. Clevers, H., and Nusse, R. (2012) Wnt/ β -catenin signaling and disease. *Cell* **149**, 1192–1205
3. Mao, C. D., and Byers, S. W. (2011) Cell-context dependent TCF/LEF expression and function: alternative tales of repression, de-repression and activation potentials. *Crit. Rev. Eukaryotic Gene Exp.* **21**, 207–236
4. Krausova, M., and Korinek, V. (2014) Wnt signaling in adult intestinal stem cells and cancer. *Cell. Signal.* **26**, 570–579
5. Lien, W. H., and Fuchs, E. (2014) Wnt some lose some: transcriptional governance of stem cells by Wnt/ β -catenin signaling. *Genes Dev.* **28**, 1517–1532
6. Weise, A., Bruser, K., Elfert, S., Wallmen, B., Wittel, Y., Wohrle, S., and Hecht, A. (2010) Alternative splicing of Tcf7l2 transcripts generates protein variants with differential promoter-binding and transcriptional activation properties at Wnt/ β -catenin targets. *Nucleic Acids Res.* **38**, 1964–1981
7. Atcha, F. A., Syed, A., Wu, B., Hoverter, N. P., Yokoyama, N. N., Ting, J. H., Munguia, J. E., Mangalam, H. J., Marsh, J. L., and Waterman, M. L. (2007) A unique DNA binding domain converts T-cell factors into strong Wnt effectors. *Mol. Cell. Biol.* **27**, 8352–8363
8. Hoverter, N. P., Ting, J. H., Sundaresh, S., Baldi, P., and Waterman, M. L. (2012) A WNT/p21 circuit directed by the C-clamp, a sequence-specific DNA binding domain in TCFs. *Mol. Cell. Biol.* **32**, 3648–3662
9. Ravindranath, A. J., and Cadigan, K. M. (2014) Structure-function analysis of the C-clamp of TCF/Pangolin in Wnt/ β -catenin signaling. *PLoS One* **9**, e86180
10. Nakano, N., Itoh, S., Watanabe, Y., Maeyama, K., Itoh, F., and Kato, M. (2010) Requirement of TCF7L2 for TGF- β -dependent transcriptional activation of the TMEPAI gene. *J. Biol. Chem.* **285**, 38023–38033
11. Van de Wetering, M., Castrop, J., Korinek, V., and Clevers, H. (1996) Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol. Cell. Biol.* **16**, 745–752
12. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) Constitutive transcriptional activation by a β -catenin-Tcf complex in APC^{-/-} colon carcinoma. *Science* **275**, 1784–1787
13. Lei, S., Dubeykovskiy, A., Chakladar, A., Wojtukiewicz, L., and Wang, T. C. (2004) The murine gastrin promoter is synergistically activated by transforming growth factor- β /Smad and Wnt signaling pathways. *J. Biol. Chem.* **279**, 42492–42502
14. Guo, W., Flanagan, J., Jasuja, R., Kirkland, J., Jiang, L., and Bhasin, S. (2008) The effects of myostatin on adipogenic differentiation of human bone marrow-derived mesenchymal stem cells are mediated through cross-communication between Smad3 and Wnt/ β -catenin signaling pathways. *J. Biol. Chem.* **283**, 9136–9145
15. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) Direct binding of Smad3 and Smad4 to critical TGF β -inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* **17**, 3091–3100
16. Sadowski, I. P. M. (1989) A vector for expressing GAL4(1–147) fusions in mammalian cells. *Nucleic Acids Res.* **17**, 7539
17. Itoh, S., Ericsson, J., Nishikawa, J., Heldin, C. H., and ten Dijke, P. (2000) The transcriptional co-activator P/CAF potentiates TGF- β /Smad signaling. *Nucleic Acids Res.* **28**, 4291–4298