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Tbx3 isoforms are involved in pluripotency maintaining through distinct regulation of Nanog transcriptional activity

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ABSTRACT

Tbx3, a member of T-box gene family, has been reported to play critical roles in embryonic development and cell fate determination. In mammalian tissues, Tbx3 is expressed as two isoforms called Tbx3 and Tbx3+2a. However, the differences between the two isoforms in pluripotency maintaining remain obscure. Here we show that both *Tbx3* and *Tbx3+2a* are highly expressed in mouse embryonic stem cells (mESCs) or induced pluripotent stem cells (iPSCs). Overexpression of either Tbx3 or Tbx3+2a could induce the differentiation of mESCs. Mechanistic studies suggest both Tbx3 and Tbx3+2a inhibit the transcriptional activity of pluripotency related transcription factor Nanog. Moreover, Tbx3+2a could directly interact with Nanog while Tbx3 couldn't, indicating the requirement of the 2a domain in Nanog binding. In summary, our results not only reveal the essential roles of Tbx3 and Tbx3+2a in pluripotency maintaining, but also point out the different mechanistic modes between these two isoforms.

1. Introduction

T-box gene family is a group of transcription factors playing essential roles in controlling many aspects of embryogenesis [1–3]. Members of T-box family share a similar T-box DNA binding domain in N terminal. This T-box domain specifically recognizes and targets a unique DNA sequence AGGTG, also called T-sequence [4]. Emerging evidence suggests that the promoter regions of many pluripotency associated transcription factors contain T-sequence and might be the potential downstream targets of T-box genes [5,6].

Tbx3 belongs to T-box genes family and has two transcription products in mouse cells, called *Tbx3* and *Tbx3+2a*. The only difference between Tbx3 and Tbx3+2a is the latter have 20 extra amino acids in T-box domain. The two isoforms regulate different genes or bind different regions of the same promoter. Tbx3 has emerged as key transcriptional regulator during development [7]. Haploinsufficiency of TBX3 causes ulnar-mammary syndrome (UMS) in human [8]. In line with previous studies increased levels of Tbx3 may be a critical step in tumorigenesis, and Tbx3 is overexpressed in several cancer cells including breast cancer, bladder carcinoma and melanoma [9,10]. Recently, Tbx3 was found to improve the reprogramming efficiency of mouse somatic cells and generate iPSCs with higher quality [11]. In addition, knockdown of Tbx3 results in the attenuation of ES self-renewal ability [12].

Although there is a wealth of data suggests the involvements of Tbx3 in development and disorders [7,13,14], little is known about the differences between Tbx3 and Tbx3+2a in maintaining mESCs. In this study, we focused on the roles of Tbx3 and Tbx3+2a in mESCs and showed that both Tbx3 and Tbx3+2a were highly expressed in mouse pluripotent stem cells. We demonstrated that overexpression of both *Tbx3* isoforms induced mESCs differentiation by inhibiting the transcriptional activity of Nanog. Furthermore, we found that only Tbx3+2a, but not Tbx3, directly interacted with Nanog, indicating distinct functions of Tbx3 and Tbx3+2a in maintaining the pluripotency of mESCs.

2. Materials and methods

2.1. Cell culture

mESCs and iPSCs were maintained on feeder layers (mouse fibroblasts were treated with mitomycin C) in DMEM/high glucose supplemented with 15% FBS (GIBCO), glutamine, NEAA, sodium pyruvate, and LIF (Millipore). Puromycin (0.1%) was added to select positive cells after transfection for 3 days.

2.2. Rt-pcr

Cells were cultured on 6 well dishes. Total RNA was isolated with TRIzol (invitrogen). 5 μg RNA was used for reverse

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transcription with RNAce reagent (Toyobo). PCR reaction was performed with QPCR kits (Takara). The forward primer is CCCGAA GAAGAGGTGGAGGACGAC, and the reverse primer is GATGGAGACAGCAGGAGAGGAT.

2.3. Plasmid construction

The full-length mouse *Tbx3*, *Tbx3+2a* and *Nanog* were amplified from R1 mESCs and cloned into PYP vector. *IN4* plasmid is a luciferase expression plasmid with previously identified Nanog binding site in its promoter.

2.4. Luciferase assay

HEK293T cells were cultured on 96 well plates in DMEM high glucose media with 10% FBS. We plated 3000 HEK293T cells each well in transfection experiments. Cells grew for 24 h and were transfected with each plasmid 0.5 μg per well using $CaCl_2$. Cells were lysed 24–48 h later, and processed for luciferase assay using dual-luciferase reporter assay system (Promega).

2.5. Co-immunoprecipitation

Transfected cells were lysed in cell lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% TritonX-100, with protease inhibitor mixture). Whole cell extracts were clarified by centrifugation and the supernatants was immunoprecipitated with Flag or Nanog antibody (Sigma). The eluent were analyzed by Western blotting.

3. Results

3.1. Tbx3 and Tbx3+2a are highly expressed in mouse pluripotent stem cells

Tbx3 and *Tbx3+2a* are broadly expressed in many organs of mouse, such as liver, brain and muscle. To determine the expression of these two isoforms in mouse pluripotent stem cells, we compared the mRNA level of them in several cell lines, including 3 mESCs (R1, CGR8 and OG2) and 2 iPSCs. Our results show that both *Tbx3+2a* and *Tbx3* are highly expressed in all mouse

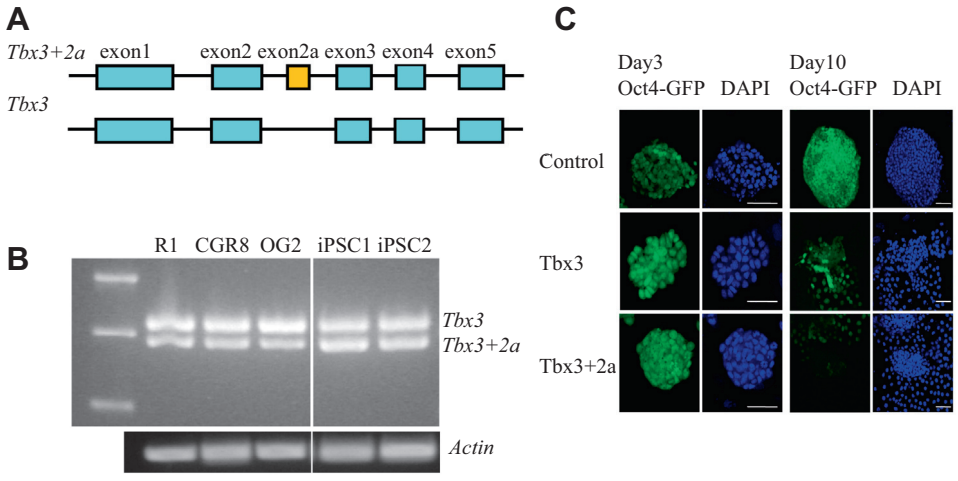

Fig. 1. Overexpression of Tbx3 and Tbx3+2a induce mESCs differentiation. (A) Difference between two isoforms of Tbx3. (B) *Tbx3* and *Tbx3+2a* are highly expressed in mESCs. The expression level of *Tbx3* and *Tbx3+2a* in mESCs and iPSCs were analyzed by RT-PCR. (C) OG2 mESCs with overexpressed Tbx3 or Tbx3+2a were sorted with puromycin and cultured further in the presence of Lif. Morphology of obtained clones was photographed in different days as presented. Bar = 50um.

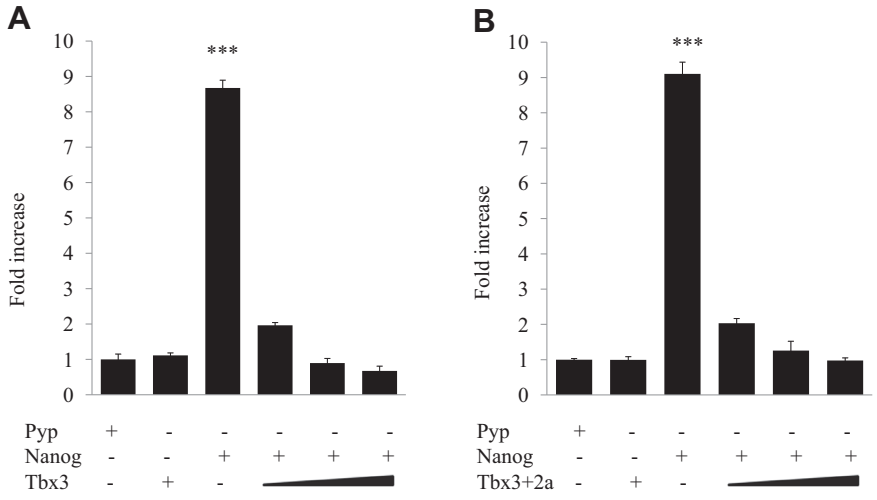

Fig. 2. Tbx3 and Tbx3+2a inhibit the transcriptional activity of Nanog. In4 reporter plasmid and Nanog were co-transfected with Tbx3(A) or Tbx3+2a(B) with increasing doses. Empty vector was also co-transfected with In4 as negative control. The luciferase activity was measured and analyzed as described.

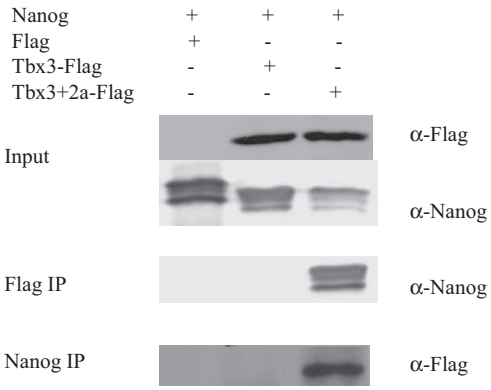

Fig. 3. The interaction between Tbx3 isoforms and Nanog. Nanog was co-transfected with Flag-tagged Tbx3 or Tbx3+2a into HEK293T cells. Empty vector was also co-transfected with Nanog as negative control. Cell lysates were immunoprecipitated with antibodies against Flag or Nanog. Immunoprecipitates (IP) and the total lysates (Input) were blotted with anti-Nanog or anti-Flag antibodies.

pluripotent stem cells (Fig. 1B), suggesting *Tbx3* and *Tbx3+2a* may play essential roles in pluripotency maintaining.

3.2. Overexpression of Tbx3 isoforms induces mESCs differentiation

To better understand the roles of Tbx3 isoforms in mESCs, we overexpressed Tbx3 and Tbx3+2a in OG2 mESCs, respectively. Cells were cultured in mES medium with Lif, and 0.1% Puromycin was added to select positive cells for the first 3 days. Since day10, we observed OG2 mESCs transfected with Tbx3 or Tbx3+2a showed obvious differentiation (Fig. 1C). These results indicate that overexpression of either Tbx3 or Tbx3+2a is sufficient to induce mESCs differentiation.

3.3. Both Tbx3 and Tbx3+2a could inhibit the transcriptional activity of Nanog

Given the above results, we speculated that Tbx3 isoforms might control mES cell fate decision by affecting the activity of mESCs core factors. We chose Nanog as a putative target because Nanog is the gateway to pluripotency and choreographs the emergent gene regulatory network of pluripotency [15,16]. Thus we established a luciferase reporter assay system to measure the transcriptional activity of Nanog. As expected, Nanog could activate the expression and activity of luciferase while Tbx3 isoforms have no effect (Fig. 2). However, when Nanog was co-expressed with Tbx3 or Tbx3+2a, the activity of luciferase reduces sharply (Fig. 2). Furthermore, the activity of luciferase decreases with increasing doses of Tbx3 or Tbx3+2a (Fig. 2). Together, these data demonstrate that both Tbx3 and Tbx3+2a significantly inhibit the transcriptional activity of Nanog in a dose-dependent fashion.

3.4. Tbx3+2a but not Tbx3 directly binds to Nanog

In an effort to explain the suppression effects of Tbx3 isoforms on transcriptional activity of Nanog, we hypothesize that Tbx3 or Tbx3+2a might interact with Nanog. To address this, we fused Tbx3 and Tbx3+2a with Flag tag and performed Co-immunoprecipitation with anti-Flag antibody. Immunoprecipitated proteins were then analyzed with anti-Nanog antibody. Interestingly, Nanog was only detected in Tbx3+2a overexpression cellular lysates, and in contrast, it was absent in lysates with Tbx3 (Fig. 3). The reciprocal Co-IP experiment also showed that only Tbx3+2a, but not Tbx3, can be examined in the Nanog precipitate. These results indicate that Tbx3+2a is a Nanog binding protein while Tbx3 is not. Since the only difference between Tbx3+2a and Tbx3 is in the T-box domain, these results also hint that T-box domain is required for the interaction between Tbx3+2a and Nanog (See Fig. 4).

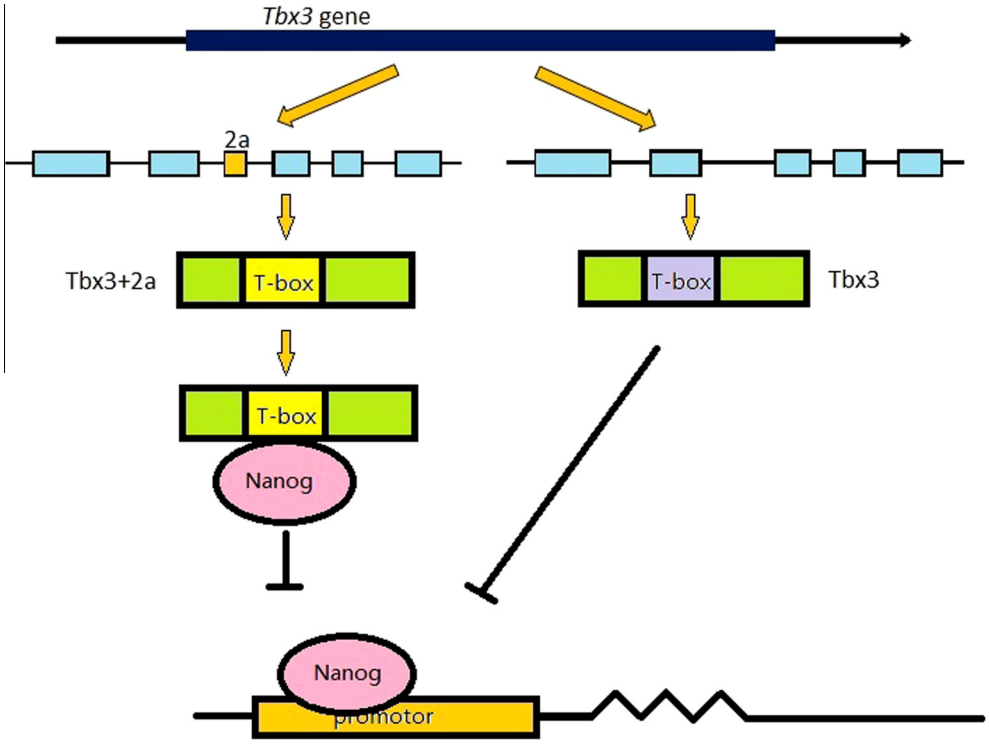

Fig. 4. Model of Tbx3 and Tbx3+2a as regulators of Nanog.

4. Discussion

Tbx3, a member of T genes family, has at least two isoforms in mouse tissues. Tbx3+2a differs from Tbx3 with extra 20 amino acids produced by selective splicing [17]. It is the first time we reported both Tbx3 and Tbx3+2a are highly expressed in mouse pluripotent stem cells, suggesting both isoforms play critical roles in maintaining pluripotency.

Our results reveal overexpression of either Tbx3 or Tbx3+2a is sufficient to induce differentiation of mESCs, which is in agreement with Lu et al.'s report [12]. Forced expression of *Tbx3* induces mESCs differentiation to extraembryonic endoderm while knockdown of *Tbx3* results in ectoderm and traphectoderm differentiation. According to Han et al.'s report [11], Tbx3 could improve the quality of iPSCs by regulating pluripotency-associated and reprogramming factors. These results further indicate Tbx3 controls mESCs fate determination in a dosage-dependent manner, consistent with our data. Notably, a study of TBX3 in human ESCs suggests Tbx3 overexpression has no effect on pluripotency [7]. Thus, Tbx3 may play different roles in mouse and human ESCs.

Nanog is commonly accorded a central position in maintaining pluripotency [16,18]. Published studies have indicated that Nanog and Tbx3 could regulate each other's expression which supported a feedforward circuit of Nanog and Tbx3 may contribute to promote pluripotent stem cell self-renewal [6,19]. Our data presented here indicate both Tbx3 and Tbx3+2a could inhibit the transcriptional activity of Naong. Furthermore, Tbx3+2a could directly interact with Nanog. Taken together, these studies and our findings highlight the intricacy in the regulation network among Nanog, Tbx3 and Tbx3+2a.

Although previous studies suggest multiple roles for Tbx3 in cell fate decision, the function of individual Tbx3 isoforms remains to be clarified. It was reported overexpression of Tbx3 is able to immortalize MEFs, while overexpression of Tbx3+2a accelerate MEFs senescence [17]. It was also indicated that Tbx3, but not Tbx3+2a, is able to target the identified T-box binding site *in vitro* [17]. Combined with our data, 2a domain plays important roles in the function of Tbx3+2a.

In conclusion, our studies not only identified the roles of Tbx3 isoforms in maintenance of pluripotency in mESCs, but also revealed a mechanistic difference between them. These results provided new insight into understanding how mESC balance its self-renew and differentiation.

Acknowledgment

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