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Original article

Non-neural and cardiac differentiating properties of Tbx6-expressing mouse embryonic stem cells

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ABSTRACT

T-box transcription factors play important roles in vertebrate mesoderm formation. Eomesodermin is involved in the initial step of the prospective mesodermal cells recruited near the primitive streak. Then T or Brachyury gene is responsible for general and axial mesodermal development. Tbx6, on the other hand, promotes paraxial mesodermal development while suppressing neural differentiation. Here, we studied differentiative properties of mouse ES cells (mESCs) with its Tbx6 expression regulated under the Tet-off system. mESCs were treated with noggin to promote neural differentiation. When Tbx6 was simultaneously turned on, later neural differentiation of these cells hardly occurred. Next, mESCs were subjected to formation of the embryoid bodies (EBs). When Tbx6 was turned on during EB formation, the rate of later cardiac troponin T (cTnT)-positive cells increased. If the cells were further treated with a wnt inhibitor KY02111 after EB formation, a synergistic increase of cTnT-positive cells occurred. Tbx6 expression in mESCs influenced the constituent ratio of the cardiac myosin light chain types, such that atrial species markedly increased over ventricular ones. These results are coincident with the function of Tbx6 in normal development, in that Tbx6 strongly suppressed neural differentiation while promoting cardiac development in a cooperative manner with wnt inhibition.

1. Introduction

T-box transcription factors play important roles in early steps of mesoderm formation [1]. Prior to gastrulation, Eomesodermin (Eomes) is involved in the initial step of recruitment of the prospective mesodermal cells near the blastopore or the primitive streak. The nascent mesodermal cells express T or Brachyury gene. Shortly after this, mesodermal cells undergo migration away from the blastopore or the primitive streak and begin expressing paraxial mesodermal genes such as Tbx6. In the mouse, Tbx6 is expressed from 7.5 dpc at the primitive streak and paraxial presomitic mesoderm, and its expression is downregulated when somite segregation begins [2]. Eomesodermin, T, and Tbx6 are all members of the T-box transcription factors.

In Tbx6-knockout mice, somites are changed to neural tube-like structures, indicating that Tbx6 is responsible for fate decision as mesodermal and not neural [3]. Tbx6 is also responsible for formation of metameric structures in the presomitic mesoderm. In the *rib vertebrae* (*rb*) mutant which is a Tbx6-hypomorphic allele, ribs are partially fused, suggesting an incomplete segregation of somites [4]. Similar fusion of somites is also found in zebrafish mutant *fused somites*, the responsible gene of which is Tbx24, a putative ortholog of Tbx6 [5]. In early development of *Xenopus laevis* (African clawed frog), the injection of Tbx6 mRNA in the animal region elicits differentiation of the animal cap to ventral mesodermal tissues [6]. Therefore, Tbx6 is involved in various mesodermal differentiation along with mesodermal vs. neural fate decision.

The vertebrate heart is formed by fusion of the left and right heart anlages that are derived from the splanchnic mesoderm [7]. Deducing from this, the emphasis of mechanisms involved in mesoderm formation may help induce heart formation from mammalian embryonic stem cells. For instance, regulated expression of mesp1, one of the regulatory genes in heart and vascular tissues, induces heart formation in mESCs [8,9]. Eomes, a regulatory gene for mesp1, also elicits heart differentiation in mESCs [10,11].

Abbreviations: mESCs, mouse ES cells; Eomes, eomesodermin; EBs, embryoid bodies; cTnT, cardiac troponin T; PBS, phosphate-buffered saline; dox, doxycycline; FBS, fetal bovine serum.

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In this study, we introduced Tbx6 gene into Rosa-Tet system of mESCs and performed doxycycline (dox)-dependent regulation of Tbx6. The purpose is to gain information about the function of Tbx6 from overexpression experiments in mammalian pluripotent stem cells. We found that the expression of Tbx6 in mESCs caused marked inhibition of neural differentiation. Also, we found that Tbx6 expression increased the cardiac differentiation especially in the presence of a wnt inhibitor, along with modification in the expression pattern of myosin light chain types of the cardiac muscle.

2. Methods

2.1. Establishment of EBRTcPTbx6 cells

Total RNA was extracted from ICR 10.5 dpc mouse embryos and converted to cDNA using PrimeScript II reverse transcriptase (Takara-bio). The coding sequence of mouse Tbx6 gene was isolated by PCR using Tks Gflex DNA polymerase (Takara-bio) from this cDNA. After confirmation of no mutation by DNA sequencing, it was subcloned into pPthc vector. EBRTcH3 mESCs were obtained from Riken Bioresource Center (Tsukuba, Japan), and were co-transfected with pPthc-Tbx6 and pCAGGS-Cre under the presence of dox using Fugene HD transfection regent (Promega), and subjected to selection with puromycin (FOCUS Biomolecules) [12]. The expected structure of rosa-Tet locus of puromycin-selected cells is shown in Fig. 1A. The resultant cell line, EBRTcPTbx6, was used in this study.

2.2. Maintenance of ES cells

Every time a new batch of EBRTcPTbx6 cells was thawed, 1.5 μg/mL of puromycin was added for one week before propagation. EBRTcPTbx6 cells were maintained with Glasgow minimum essential medium (GMEM, Sigma–Aldrich), 10% FBS (MP Biomedicals), 0.1 mM NEAA (Wako), 1 mM sodium pyruvate (Sigma–Aldrich), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich), 10 ng/mL doxycycline (Wako), 100 U/mL penicillin (Meiji Seika Pharma) and 100 μg/mL streptomycin (Meiji Seika Pharma), supplemented with 1000 U/mL mouse LIF (ORF genetics), 3 μM CHIR-99021 (FOCUS Biomolecules), and 0.4 μM PD-0325901 (Adooq Bio Science). 1×10^5 EBRTcPTbx6 cells were inoculated to a 60 mm cell culture

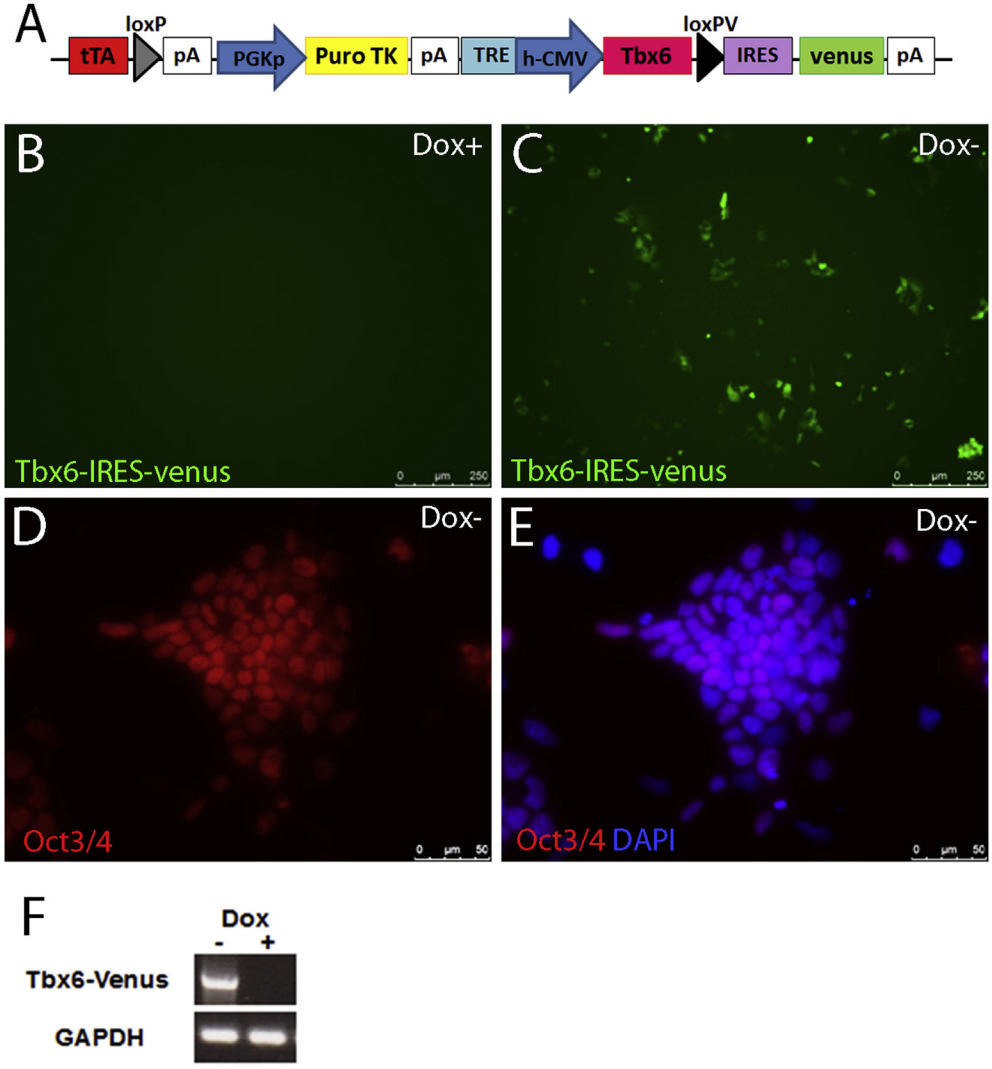

Fig. 1. Establishment of EBRTcPTbx6 cells. (A) Construct of Rosa26 locus in EBRTcPTbx6 cells. (B–C) Response of EBRTcPTbx6 cells to dox. In dox+ conditions, cells showed no fluorescence (B) while in dox− conditions cells were green fluorescent (C). (D–E) Immunostaining with anti-Oct-3/4 antibody showed positive signals in EBRTcPTbx6 cell nuclei under dox+ conditions (D), with merged image with DAPI counterstain (E), (F) RT-PCR of Tbx6-venus and GAPDH genes in EBRTcPTbx6 cells. Tbx6-venus transcript was only detected under dox− conditions.