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

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Yoshiteru Yano, Naoya Iimura, Nobuhiko Kojima, Hideho Uchiyama[*]

Graduate School of Nanobiosciences, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan

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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.

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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].

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 Bio-medicals), 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
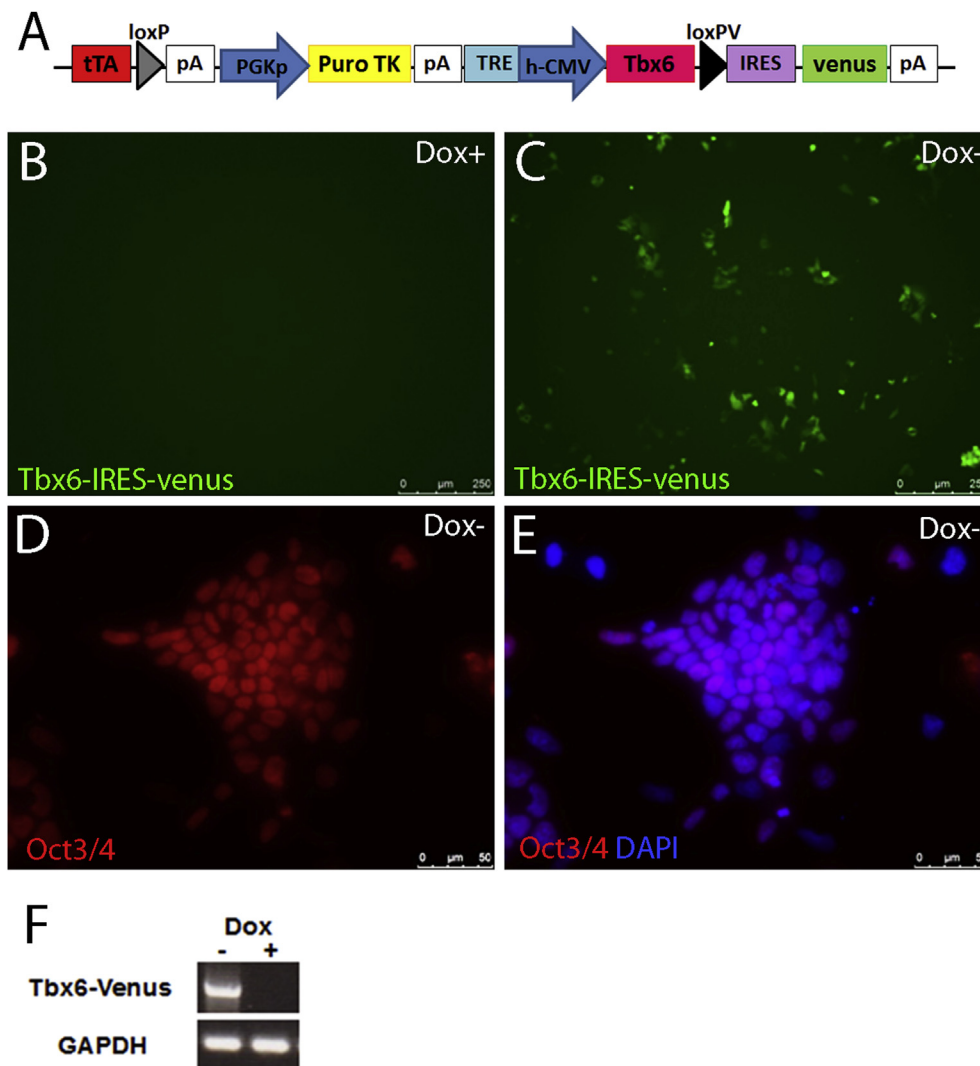

Fig. 1. Establishment of EBRTcPTbx6 cells. (A) Construct of Rosa26 locus in EBRTcPTbx6 cells. (B–C) Response of EBRTcPTbx6 cells to dox. In dox+ conditions, cells showed no fluorescence (B) while in dox– conditions cells were green fluorescent (C). (D–E) Immunostaining with anti-Oct-3/4 antibody showed 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.

dish (TrueLine, Nippon Genetics) that had been plated with MMC (Wako)-treated SNL 76/7 feeder cells (obtained from Riken Bio-resource Center) on the previous day. The cells were passaged every other day, and subjected to isolation from feeder cells using differential adhesive properties to a gelatin-coated cell culture dish before performing differentiation assays as described below.

2.3. Neural differentiation assay

1×10^5 of the isolated EBRTcPTbx6 cells were inoculated to a gelatin-coated 35 mm cell culture dish (TrueLine, Nippon Genetics) and cultured in GMEM, 10% FBS, and 10 ng/mL noggin (MACS Miltenyi Biotec) with or without dox. From day 5, the cells were further cultured without noggin for 10 days with dox. The medium was changed every other day.

2.4. Cardiac differentiation assay

2 mL of calcium-magnesium-free phosphate buffered saline (PBS) was added to a 60 mm cell culture dish, and hanging drops of 1000 isolated EBRTcPTbx6 cells in 20 μL of GMEM containing 10% FBS and dox were made underneath the lid, and cultured for 2 days to make embryoid bodies (EBs).

EBs were transferred to non-treated 60 mm polystyrene dish for suspension culture with the same medium as hanging drops and cultured for 3 days. After this, EBs were attached to a 35 mm cell culture dish (TrueLine, Nippon Genetics) with N2B27 medium [13] (described in Supplementary materials) supplemented with 2% FBS and cultured for further 10 days. The schedule of the addition of dox is shown in Fig. 2A.

2.5. Immunostaining

Cells were fixed in 4% paraformaldehyde (Wako) and 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 30 min at RT. The cells were washed with PBS and subjected to blocking with a blocking solution (1% blocking reagent in PBST, which is 0.1% Tween 20 (Sigma-Aldrich) in PBS) for 1 h at 4 °C. The first antibody in the blocking solution was reacted for 2 h at RT, and then the cells were washed with PBST 3 times for 20 min each. The second antibody in the blocking solution was reacted for 2 h at RT. After 20 min × 3 washes, some of the samples were added with DAPI (Wako) at 5 μg/mL and reacted for 10 min before the cells were observed under Leica DIM6000B fluorescence microscope. For area measurement, tiling was performed with LAS AF software, and the obtained images were subjected to measurement using NIH ImageJ software. Anti-nestin and anti-cardiac Troponin T antibodies were obtained from Developmental Studies Hybridoma Bank (DSHB at the University of Iowa). Further information about the used antibodies is described in Supplementary information.

2.6. RT-PCR

Total RNA was extracted from the cells using RNA iso plus reagent (Takara-bio), and digested with RNase-free DNase I (Nippon Gene). 2 μg RNA was transcribed with 100 U PrimeScript II reverse transcriptase. The obtained cDNA was amplified with GoTaq (Promega). Also, Fast SYBR Green Mix (Applied Biosystems) was used for quantitative PCR. The expression level of each gene was normalized with that of GAPDH, and then compared.

2.7. Statistical analysis

Statistical analyses were done using Student's *t* test.

3. Results

3.1. Responsiveness of EBRTcPTbx6 cells to dox

The puromycin-resistant EBRTcPTbx6 cells were cultured with or without dox for one day and the cells were observed under a fluorescence microscope. The fluorescence of venus was only observed in dox− cultures (Fig. 1B,C). The ratio of venus-positive cells in dox− cultures was around 80%, and this ratio after one week culture with or without puromycin was not different (Supplementary information). A large proportion of EBRTcPTbx6 cells were Oct-3/4-immunoreactive (Fig. 1D,E). The amplification of Tbx6-loxPV-IRES-venus occurred only in dox− cultures in RT-PCR experiments (Fig. 1F). Therefore, the Tet-off regulation of EBRTcPTbx6 cells was confirmed.

3.2. Tbx6 interferes with neural differentiation

EBRTcPTbx6 cells were subjected to neural differentiation assay for 15 days (Fig. 2A) and the cells were stained with anti-β tubulin or anti-nestin antibodies. In Tbx6-off (dox+) conditions, many cells were stained with the two neural marker antibodies (Fig. 2C,G), whereas in Tbx6-on conditions, they were vary scarcely stained (Fig. 2E,I). Although the used anti-β tubulin antibody is not specific to βIII tubulin, strongly stained structures in dox+ cultures were largely process-like, and we could hardly recognize similar structures in dox− cultures. The appearance of cells were not similar, in that the cells in Tbx6-off conditions were packed densely, whereas the cluster of cells in Tbx6-on conditions were small and many cell-free spaces were scattered in the dish.

Next, qRT-PCR experiments for neural and mesodermal markers were performed. The anterior neural and retinal marker Six3, and proneural gene neurogenin1 were significantly downregulated by Tbx6 (Fig. 2J). Conversely, vascular and endothelial marker VE-cadherin and one of Notch ligands and also a vascular marker delta-like 4 (Dll4) were markedly up regulated by Tbx6 (Fig. 2K). Therefore, a temporal Tbx6 expression strongly suppressed neural differentiation.

3.3. Tbx6 expanded and modified cardiac development

The embryoid bodies (EBs) were formed from EBRTcPTbx6 cells through procedures of 2 days hanging drop followed by 3 days suspension culture. RT-PCR of this day5-EBs indicated that endogenous Tbx6 expression weakly occurred even in Tbx6-off conditions (Fig. 3B, Supplementary information).

The EBs were attached to a 35 mm dish and cultured for further 10 days. The cells were stained with anti-cardiac troponin T (cTnT) antibody. The positive signal appeared in a restricted area in the dish both in Tbx6-on and Tbx6-off conditions (Fig. 3C,D). When KY02111, a wnt signal inhibitor, was added in a period of suspension culture, the positive area in the dish greatly expanded; in Tbx6-off conditions, the area increased from 2.3% to 8.1%; and in Tbx6-on conditions, from 3.4% to 22.2% (Fig. 3G). Therefore, the cTnT-positive cells were increased by Tbx6 expression, and the change was prominent under the presence of KY02111.

The myosin type of cells at day 15 was investigated with quantitative PCR (Fig. 3H). MLC (myosin light chain) 2 is ventricular regulatory type; MLC3, ventricular essential type; MLC7, atrial regulatory type and MLC4, atrial essential type, respectively. In Tbx6-on conditions, MLC2 expression increased whereas MLC3 decreased a little as compared with Tbx6-off conditions. More prominently, MLC7 expression was more than 2.5-fold, whereas that of MLC4 was greater than 5-fold as compared to Tbx6-off conditions. Therefore, the expression of atrial myosin types was increased markedly by
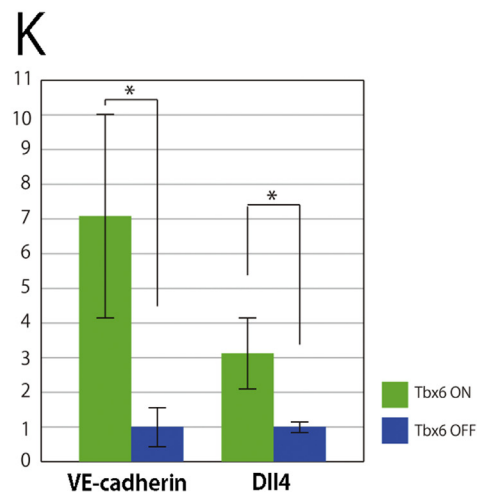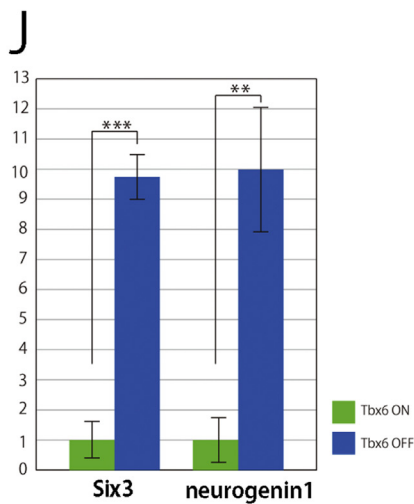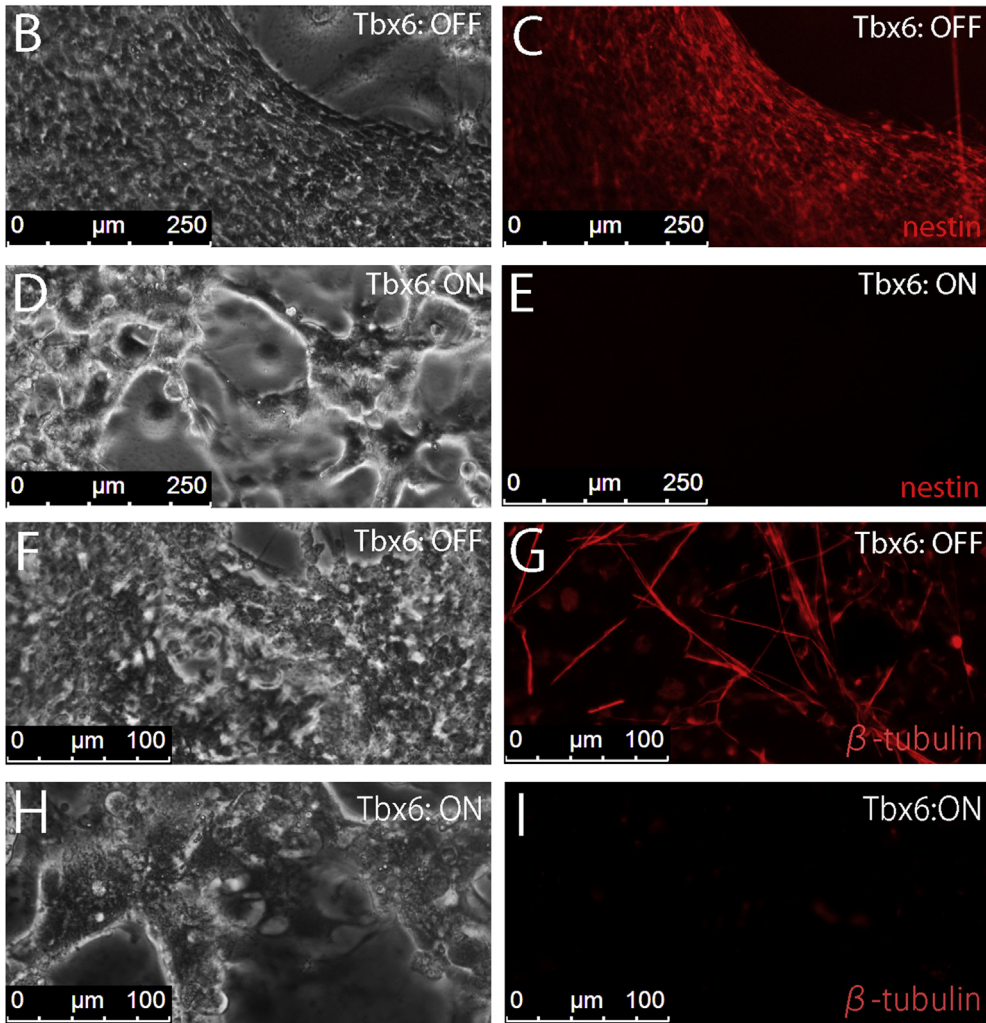
Fig. 2. Neural differentiation of EBRTcPTbx6 cells. (A) Scheme of neural differentiation protocol. (B–I) Four sets of phase contrast and fluorescent images of EBRTcPTbx6 cells at day 15 of culture. The conditions of Tbx6 expression and the used antibody were shown in each panel. (J, K) qRT-PCR experiments for neural (J) and mesodermal (K) genes. Bars in (J, K) are standard deviations ($n = 3$); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
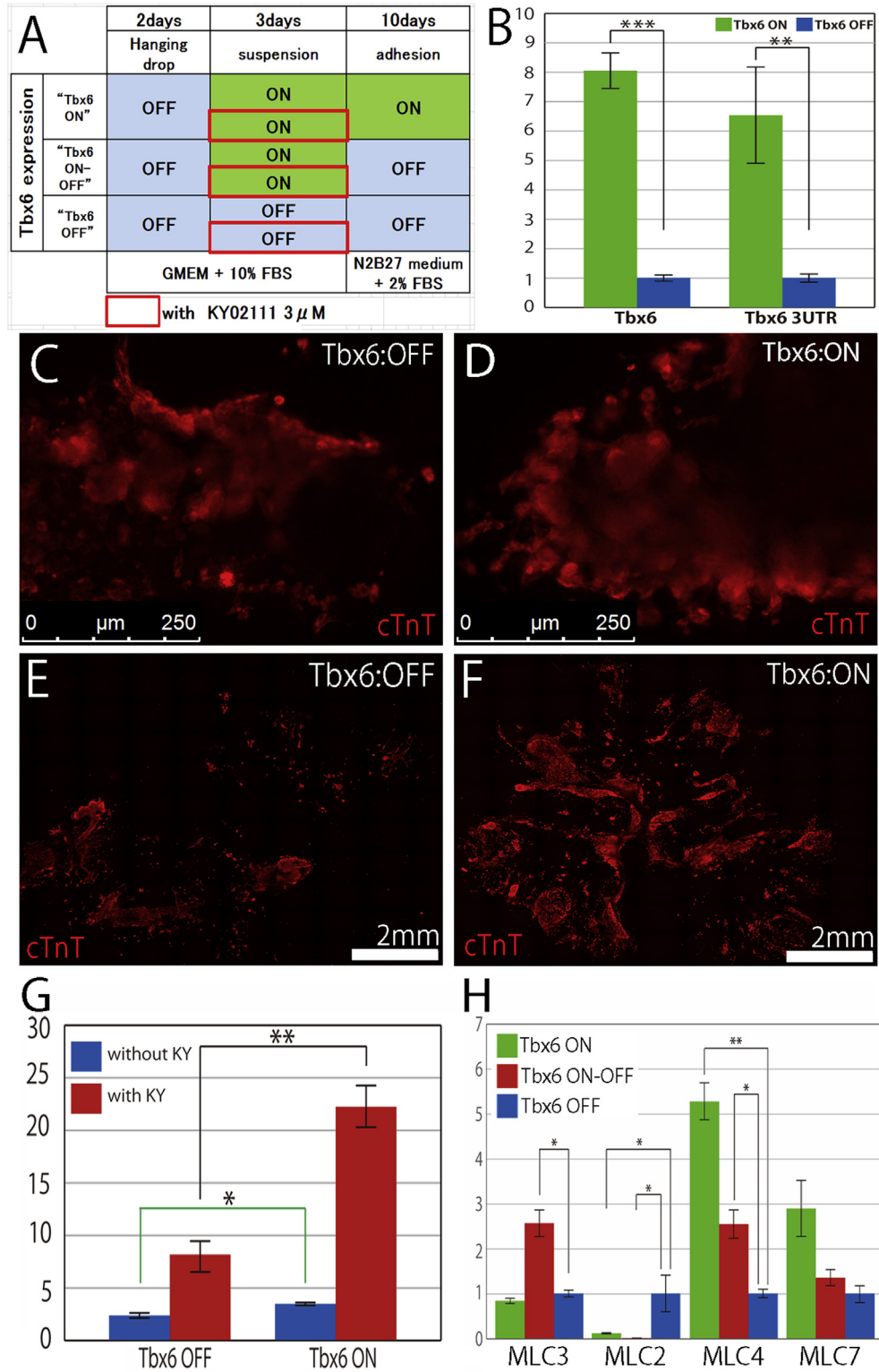
Fig. 3. Cardiac differentiation of EBRTcPTbx6 cells. (A) Scheme of cardiac differentiation protocol. (B) qRT-PCR of Tbx6 coding sequence (Tbx6) and that of 3′UTR at day 5 ($n = 4$). (C, D) Staining with anti-cTnT antibody of cells at day 15 of culture under the presence or absence of dox. (E, F) Wide view of the staining with anti-cTnT antibody of cells at day 15 of culture with KY02111 added during the suspension culture, with (E) or without (F) dox. (G) Rate of cTnT-immunoreactive area in each dish (C−F) was calculated with ImageJ software. (H) Quantitative PCR analysis of the pattern of cardiac myosin light chains of cells at day 15 ($n = 3$). Bars in (B, G, H) are standard deviations; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Tbx6 expression. Even in Tbx6 on−off conditions, the expression of three myosin types increased as compared to Tbx6-off conditions. Thus, Tbx6 expression influenced greatly to the quantity and quality of the cardiac muscle in the culture.

4. Discussion and conclusion

It has been reported that mESCs undergo neural differentiation when noggin was added at the early phase of differentiation culture

[14]. In this study, similar neural differentiation was observed in our assay. The expression of Tbx6 strongly suppressed neural differentiation and conversely promoted several mesodermal genes, coinciding with the function of Tbx6 as a switcher to mesodermal from neural differentiation [3]. We searched for mesodermal genes that are up regulated by overexpression of Tbx6, but found only a limited number of genes. Nevertheless, both of the found genes, VE-cadherin and Dll4 are related to vascular/endothelial markers, suggesting that early mesodermal differentiation by Tbx6 might start from cells that are reminiscent to vascular/endothelial precursors. In *X. laevis*, dorsal injection of Tbx6 mRNA to early embryos caused massive degeneration of the neural tube [6], suggesting that Tbx6 interfere with neural development. At a molecular level, Tbx6 has been shown to inactivate N1 enhancer of Sox2 gene in the caudal lateral epiblast (CLE) and lead mesodermal differentiation in the mouse [15]. Similar mechanisms might have worked in this mESCs culture.

In the cardiac differentiation assay, the expression of exogenous Tbx6 caused marked increase in the number of cTnT-positive cells, especially in the presence of wnt inhibitor KY02111. Eomes has been shown to be an initial activator of Mesp1/2 genes that are involved in cardiac specification, while Tbx6 serves as a second wave activator of Mesp1/2 [10,11]. Our results coincide with these reports. However, it is not clear why Tbx6 and KY02111 synergize in cardiac muscle specification. In *X. laevis*, the injected Wnt8 mRNA blocked cardiogenesis in the dorsal marginal zone explant [16]. Since Wnt8 is one of target genes of Tbx6 in *Xenopus* [17], Tbx6 might upregulate wnt signaling also in mESCs. Interestingly, inhibition of wnt signaling induced heart formation in the chick posterior lateral plate mesoderm [18], suggesting that wnt inhibition might be a general mechanism in heart formation.

There are many Tbx genes expressed during vertebrate heart development. Examples include Tbx1, Tbx2, Tbx3, Tbx4, Tbx5, Tbx18, and Tbx20. Each of these Tbx genes is expressed in a restricted area and is thought to be involved in the compartmentalization of the heart. Tbx6 is not included in this group because its expression is rather early and not restricted to the heart field [19]. Therefore, the result that the expression of atrial myosin subtypes increased in the Tbx6-on group may not directly reflect the normal function of Tbx6 in development. It may be that the prolonged expression of Tbx6 somehow affected the compartmentalization process by substituting function of other Tbx gene(s).

Taken together, this study added insights into the functions in development of a pure mesodermal T-box gene Tbx6 by performing overexpression experiments in mESCs in culture, with special reference to neural or cardiac differentiations.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.reth.2016.02.001.

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