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Acetylcholine receptors regulate gene expression that is essential for primitive streak formation in murine embryoid bodies

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ABSTRACT

Muscarinic acetylcholine receptors (mAchRs) are critical components of the cholinergic system, which is the key regulator of both the central and peripheral nervous systems in mammals. Interestingly, several components of the cholinergic system, including mAchRs and choline acetyltransferase (ChAT), have recently been found to be expressed in mouse embryonic stem (ES) cells and human placenta. These results raise the intriguing possibility that mAchRs play physiological roles in the regulation of early embryogenesis. Early embryogenesis can be mimicked *in vitro* using an ES cell-based culture system in which the cells form a primitive streak-like structure and efficiently develop into mesodermal progenitors. Here we report that chemical inhibitors specifically targeting mAchRs suppressed the expression of genes essential for primitive streak formation, including *Wnt3*, and thereby blocked mesodermal progenitor differentiation. Interestingly, mAchR inhibitors also reduced the expression of *Cyp26a1*, an enzyme involved in the catabolism of retinoic acid (RA). RA is an important regulator of *Wnt3* signaling. Our study presents evidence indicating that mAchRs influence RA signaling necessary for the induction of the primitive streak. To our knowledge, this is the first report showing that mAchRs have important functions not only in adult mammals but also during early mammalian embryogenesis.

1. Introduction

Muscarinic acetylcholine receptors (mAchRs) are expressed in virtually all organs, tissues and cell types of adult mammals, and play key roles in neuronal systems. In the central nervous system, mAchRs regulate locomotor activity and cognitive functions. In peripheral parasympathetic nervous systems, acetylcholine released by vagal nerve endings stimulates mAchRs, thereby inducing muscle contraction and gland secretions [1]. Intriguingly, recent work has shown that components of the cholinergic system, including mAchRs and enzymes of acetylcholine metabolism, are also expressed in murine embryonic stem (ES) cells [2]. In addition, the enzyme that synthesizes acetylcholine, choline acetyltransferase (ChAT), has been detected in human placenta [3]. These studies suggest that mAchRs play physiological roles during early development as well as at the adult stage. However, the molecular mechanisms by which mAchRs are involved in early embryonic development are unclear.

In mammalian embryos, a single layer of epithelial cells called the epiblast generates the three germ layers – the mesoderm, endoderm and ectoderm – through the primitive streak. The primitive streak induces differentiation of mesoderm and endoderm at the posterior pole of the embryo [4], whereas the anterior epiblast expressing *SRY-box containing gene 2* (*Sox2*) differentiates into neuroectodermal derivatives [5]. Primitive streak formation is regulated by many extracellular signals but particularly by those mediated via the "wingless-related MMTV integration site 3" (Wnt3) pathway [6]. Retinoic acid (RA) also plays a key role in primitive streak formation, and RA abundance in a mammalian embryo is determined by a balance between RA synthesis by retinaldehyde dehydrogenase (RALDH) and RA degradation mediated by CYP26 [7,8].

To investigate the roles of mAchRs in early embryogenesis, we used a murine system in which ES cells derived from the inner cell mass of the blastocyst are induced to aggregate in culture and form an embryoid body (EB) [9]. EB formation mimics early embryogenesis *in vivo*, in that the ES cells can differentiate into the usual three germ layers. In the presence of Wnt3 signaling, a primitive streak-like region is established in EBs that generates mesodermal progenitor cells in this region [10]. In this study, we have used the EB system to demonstrate that mAchRs are required for the expression of genes essential for primitive streak and mesoderm formation.

Abbreviations: ChAT, choline acetyltransferase; DH, dicyclomine hydrochloride; EB, embryoid body; mAchR, muscarinic acetylcholine receptor; RA, retinoic acid.

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2. Materials and methods

2.1. Reagents and antibodies

Aprofene (InterBioScreen Ltd., Bio-0805), dicyclomine hydrochloride (Sigma, D7909), and retinoic acid (Sigma, R2625) were purchased from the indicated suppliers. Antibodies (Abs) recognizing the following proteins were used in this study: β-tubulin III (Tuj-1, Covance, MMS-435P), sarcomeric α-actinin (Abcam, ab9465), GAPDH (Millipore, MAB374), synaptophysin (Invitrogen, 18–0130), and SOX2 (Santa Cruz Biotechnology, sc-17320).

2.2. ES cell culture and differentiation

Embryoid bodies (EBs) were prepared as described previously [11,12]. Briefly, undifferentiated ES cells were dissociated into single-cell suspensions and cultured in hanging drops to induce embryoid body (EB) formation. Initial cell density (on Day 0) was 3000 cells per drop (25 μl) of differentiation medium without LIF. After two days in hanging drop culture in the absence or presence of aprofene (final concentration 10 μM), the resulting EBs were transferred to non-coated culture dishes (Day 2). On Day 6, the EBs were plated in plastic gelatin-coated dishes and cultured until Day 12. Culture medium was changed every 2 days. EBs were left untreated, or treated with 10 μM aprofene or 10 μM DH or 1 pM RA during days 1–6.

2.3. Immunostaining

Immunostaining was performed as described [11,12]. EBs were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA)/0.1% Triton X-100. Fixed EBs were incubated with blocking solution [5% bovine serum albumin (BSA)/PBS/0.1% Triton X-100] for 30 min at room temperature (RT). Blocked EBs were incubated overnight with primary Ab (1:1000 dilution) at 4 °C followed by two washes in PBS/0.1% Triton X-100. Washed EBs were incubated for 1 h at RT with AlexaFluor568- or Cy3-conjugated secondary Ab (1:1000 dilution) plus 8 μM Hoechst 33342. Stained EBs were washed three times in PBS/0.1% Triton X-100.

2.4. Immunoblotting

Immunoblotting was performed as described [13]. ES cells or EBs were homogenized in RIPA buffer [150 mM NaCl, 5 mM ethylenediamine tetra-acetic acid (EDTA), 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.5% deoxycholic acid, and 50 mM Tris–HCl pH 8.0] containing protease inhibitor mixture tablets. Lysates were clarified by centrifugation for 5 min at 12,000*g*, and protein concentrations of supernatants were equalized using the Pierce BCA Protein Assay Kit (Thermo). Supernatants were fractionated by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred by electroblotting onto polyvinylidene difluoride membranes. Membranes were blocked with 2% nonfat milk or Blocking One (Nacalai Tesque) and incubated overnight at 4 °C with primary antibody. Blots were then incubated with the appropriate secondary antibody and developed with the ECL detection system (Amersham Biosciences).

2.5. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR)

qRT-PCR and RT-PCR were performed as described [11–13]. Total RNA extraction was carried out using Tri Reagent (Molecular Research Center) according to the manufacturer's instructions. Total RNA (4 μg) was reverse-transcribed into cDNA using Superscript III RNase H Reverse Transcriptase (Invitrogen) and 500 ng Oligo-d(T) primers. Each quantitative real-time RT-PCR reaction was performed using the Chromo4 real-time detection system (Bio-Rad). For a 20 μl PCR reaction, 10 μl containing cDNA template mixed with the appropriate primers to a final concentration of 200 nM was combined with 10 μl Eva Green (Biotium). The reaction was incubated at 95 °C for 3.5 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. PCR primers are listed in Supplementary Table S1.

3. Results

3.1. Inhibition of mAchRs alters gene expression patterns associated with EB differentiation

To investigate the role of mAchRs in early embryogenesis, we used two specific inhibitors of mAchRs, aprofene [14] and dicyclomine hydrochloride (DH) [15], and an *in vitro* ES cell-based system in which ES cells can be induced to efficiently differentiate into mesodermal progenitors through a primitive streak-like structure. These progenitors normally differentiate into cardiomyocytes that soon commence cardiac "beating", but inhibition of this cardiomyogenesis results in the induction of neurogenesis [12]. When we used either mAchR inhibitor to treat differentiating EBs, no "beating" ES cells could be detected upon examination on day 12 (Fig. 1A), indicating that cardiomyocyte differentiation was completely blocked. *In vitro*, both mAchR inhibitors efficiently suppressed the mRNA expression of the cardiac-associated gene *a-cardiac Myosin heavy chain* (*Mhc*) (Fig. 1B). However, mRNA expression of the neuronal lineage gene *Microtubule-associated protein 2* (*Map2*) was increased in these cells (Fig. 1C). Immunostaining of EB outgrowths in culture revealed positive staining for β-tubulin III, a neuron-specific marker (Fig. 1D). The expression in our EB system of mRNAs for mAchRs [*cholinergic receptor, muscarinic* (*Chrm*) *1–5*], as well as *Choline acetyltransferase* (*Chat*), the enzyme that synthesizes acetylcholine, was confirmed by RT-PCR. *Chrm2*, *3*, *5*, *and Chat* were detected in both ES cells and EBs (Fig. 1E). *Chrm4* expression was found only in EBs. *Chrm1* was not expressed in either ES cells or EBs. These results suggest that mAchRs promote cardiomyocyte differentiation during murine embryogenesis.

3.2. Inhibition of mAchRs during days 3–4 of EB differentiation decreases cardiomyocyte differentiation

To determine precisely when mAchRs influence ES cell differentiation, we treated EBs with aprofene or DH for various time periods. As shown in Fig. 2A and B, treatment with either aprofene or DH significantly decreased the proportion of EBs containing beating foci (the "beating ratio") only when the inhibitor was applied between days 3–4. Treatment for any other period did not reduce the beating ratio. Next, we measured the mRNA expression and protein levels of cardiomyocyte and neuronal markers by immunoblotting and qRT-PCR. Our immunoblotting analysis indicated that expression of the cardiac-specific protein sarcomeric-actinin was reduced in EBs treated with aprofene during days 3–4 compared to untreated controls, or compared to EBs treated with aprofene for other periods (Fig. 2C). In contrast, the expression of the neuron-specific protein synaptophysin was sharply induced by aprofene treatment during days 3–4 (Fig. 2C). qRT-PCR analysis confirmed that the mRNA expression of the cardiac-specific gene *Mhc* was suppressed by mAchR inhibition during days 3–4 (Fig. 2D), but that mRNA expression of the neuron-specific gene *Map2* was induced by this treatment (Fig. 2E). Thus, the critical

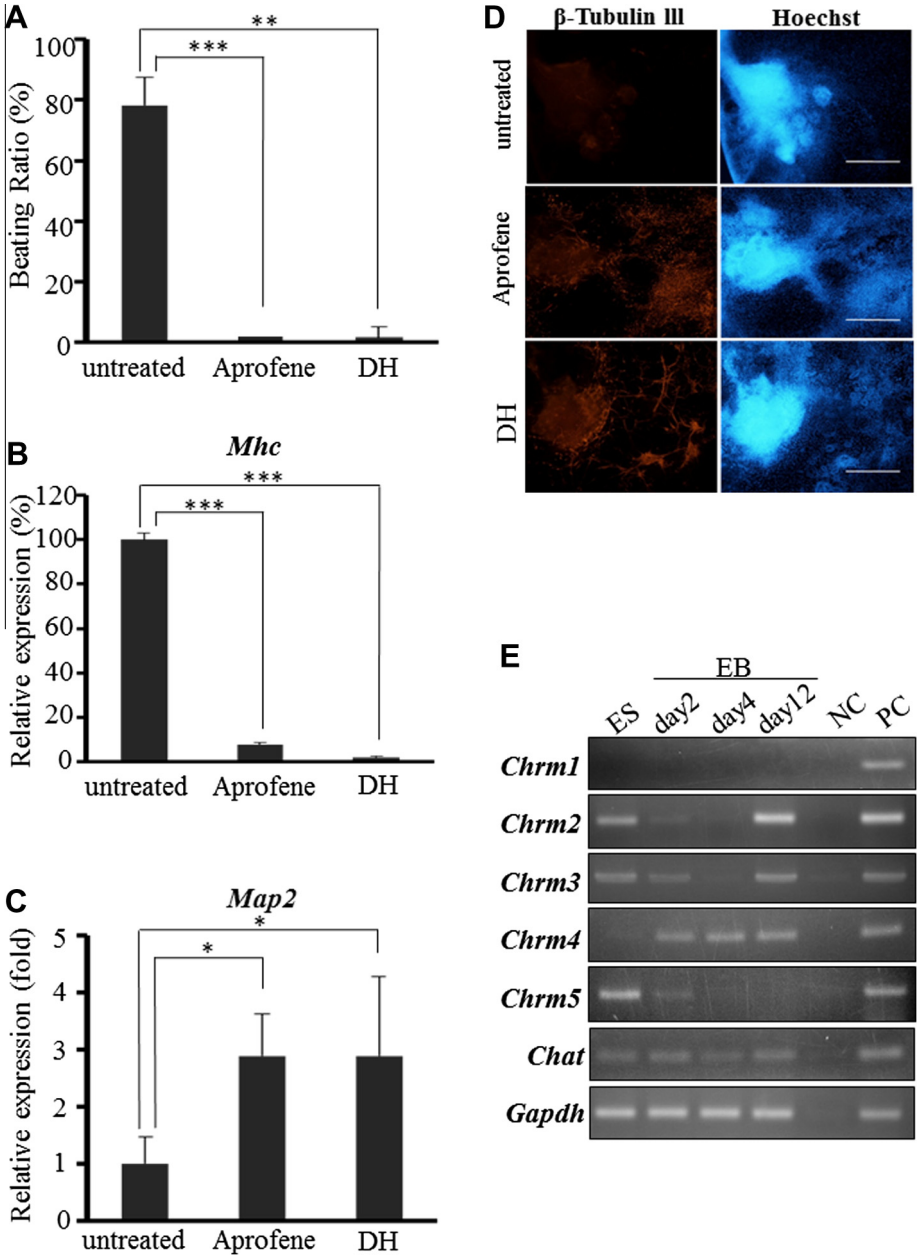

Fig. 1. Effects of mAchR inhibitors on gene expression patterns during EB differentiation. (A) Decreased cardiomyocyte differentiation. EBs were left untreated, or treated with 10 μM aprofene or 10 μM DH during days 1–6, and cultured for a total of 12 days. The number of EBs containing beating foci were counted on day 12. Data are the mean ratio ± SD of EBs with beating foci among total EBs plated, expressed as a percentage. For all figures, results are representative of at least 3 independent trials. (B, C) Decreased cardiac-specific but increased neuron-specific gene expression. mRNA was extracted from the EBs in (A) on day 12 and analyzed by qRT-PCR to detect transcripts of the cardiac-specific gene *Mhc* (B) and the neuron-specific gene *Map2* (C). Data were normalized to *Gapdh* mRNA levels and are expressed as the relative mean ± SD. (D) Increased neural outgrowths. EBs were left untreated, or treated with 10 μM aprofene or 10 μM DH dicyclomine hydrochloride (DH) during days 2–6, and outgrowths in culture were examined on day 12. Neuronal lineage cells within EB outgrowths were detected by immunostaining with anti-β-tubulin III antibody. Nuclear were stained with hoechst 33342. Data are representative of 3 cultures examined per condition. Scale bar, 500 μm. (E) Confirmation of mAchR and ChAT mRNA expression. mRNA was extracted from ES cells and untreated EBs on days 2, 4 and 12, and subjected to RT-PCR analysis to detect *ChrmR1–5* (encoding mAchR1–5) and *ChAT* mRNAs. *Gapdh*, loading control. Negative control (NC): without reverse transcriptase. Positive control (PC): adult mouse brain. $^{*}P < 0.05$, $^{**}P < 0.0001$, $^{***}P < 1 \times 10^{-5}$.

period of mAchR influence on cardiomyogenesis is days 3–4 of EB differentiation.

3.3. Inhibition of mAchRs reduces primitive streak gene expression

The expression of genes essential for mesoderm formation has been previously shown to increase from day 3 and peak at day 4 *in vitro* ES cell-based systems [16,17]. We confirmed this pattern in our system by examining the expression of *T-brachyury* (*T*), a gene required for the generation of mesodermal progenitors. When we treated EBs with aprofene during days 1–4, *T* expression was dramatically decreased (Fig. 3A). In contrast, expression of the neuroectodermal gene *Sox2*, which normally drops to a low level in untreated EBs by day 4, did not decrease in aprofene-treated EBs (Fig. 3B). Immunoblotting analysis confirmed that aprofene treatment blocked the expected reduction in SOX2 protein on day 4 (Fig. 3C). Because these data suggested that primitive streak formation was impaired in aprofene-treated EBs, we examined the mRNA expression of a variety of primitive streak genes, including *Wnt3*, *Wnt3a*, *LIM homeobox protein 1* (*Lhx1*), *Wnt8a*, and *fibroblast*

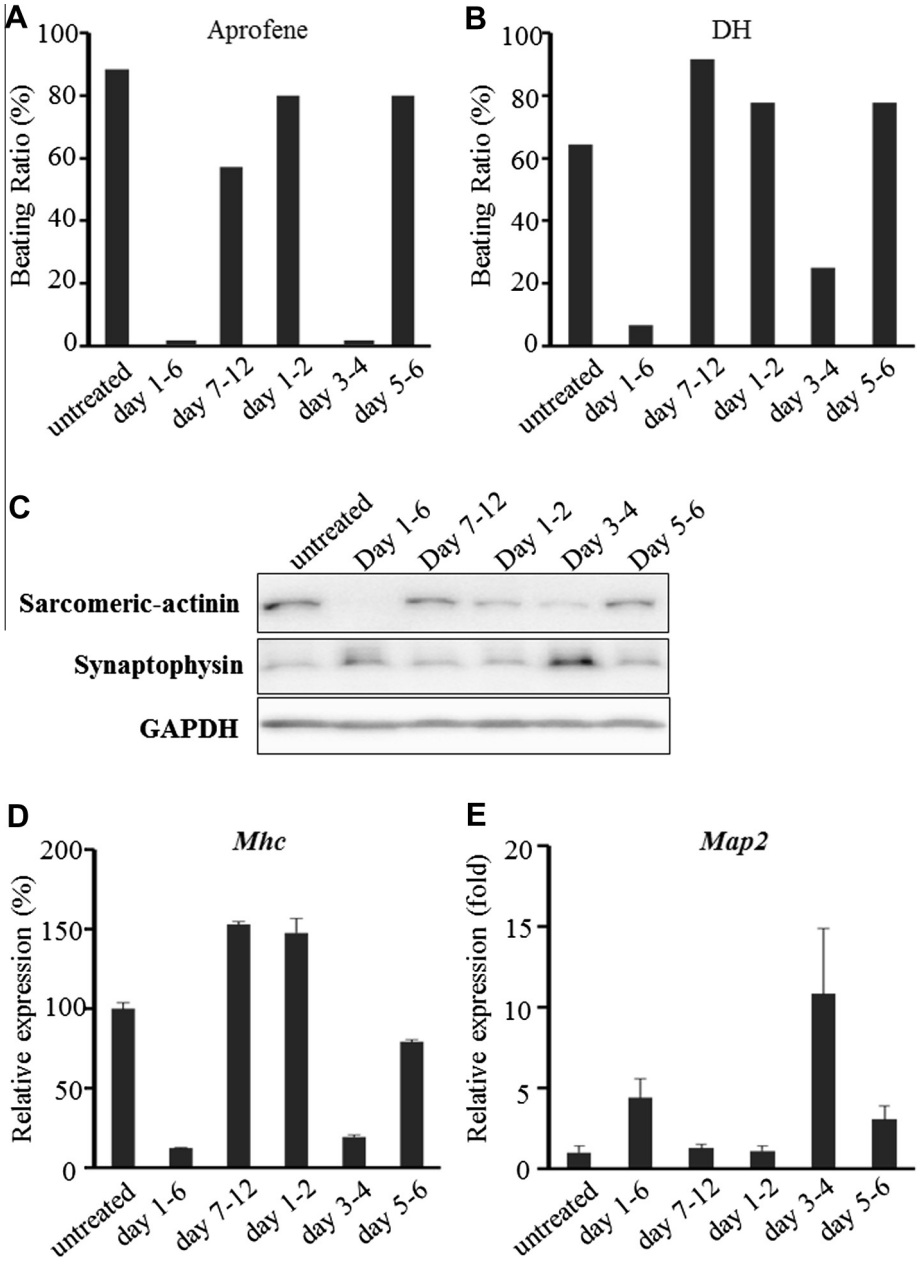

Fig. 2. Inhibition of mAchRs on days 3–4 of EB differentiation decreases cardiomyocyte differentiation. (A, B) Narrow window of mAchR influence. EBs were left untreated or treated with 10 μM aprofene (A) or 10 μM DH (B) for the indicated time periods. On day 12, cardiomyocyte differentiation was determined as for Fig. 1A. (C) Altered lineage-specific proteins. Extracts from the EBs in (A) were analyzed by immunoblotting to detect cardiac-specific sarcomeric α-actinin and neuron-specific synaptophysin. GAPDH, loading control. Results are representative of at least 3 trials. (D, E) Altered lineage-specific mRNAs. Extracts from the EBs in (A) were subjected to qRT-PCR to detect mRNA expression levels of the cardiac-specific gene *Mhc* (D) or the neuron-specific gene *Map2* (E). Results were analyzed as for Fig. 1B.

growth factor 8 (*Fgf8*). Transcript levels of all of these genes were significantly reduced in aprofene-treated EBs on day 4 compared to untreated EBs (Fig. 3D). These data indicate that mAchRs regulate gene expression associated with primitive streak formation and the subsequent differentiation of mesodermal progenitors.

3.4. mAchRs influence primitive streak gene expression through effects on RA

As noted above, RA signaling plays a key regulatory role in primitive streak formation by controlling Wnt3 signaling [8]. We therefore examined the expression of primitive streak and mesodermal genes in EBs treated with RA during days 1–4. We found that mRNA levels of *Wnt3*, *Wnt3a*, *Lhx1* and *T* were all significantly decreased in RA-treated EBs (Fig. 4A). To investigate whether the effects of mAchRs on EB differentiation were due to modulation of RA signaling, we measured the mRNA expression of genes encoding enzymes involved in RA metabolism. Expression of *aldhyde dehydrogenase 1* (*Aldh1*), an enzyme required for RA synthesis, was comparable between untreated and aprofene-treated EBs (Fig. 4B). However, mRNA levels of *cytochrome P450*, *family 26*, *subfamily a*, *polypeptide 1*(*Cyp26a1*), which is critical for RA catabolism, were markedly decreased by aprofene treatment (Fig. 4C). These findings indicate that mAchRs support primitive streak formation leading to mesodermal progenitor generation and cardiomyocyte differentiation by increasing *Cyp26a1* expression, thereby promoting RA degradation and allowing Wnt3 signaling to proceed.

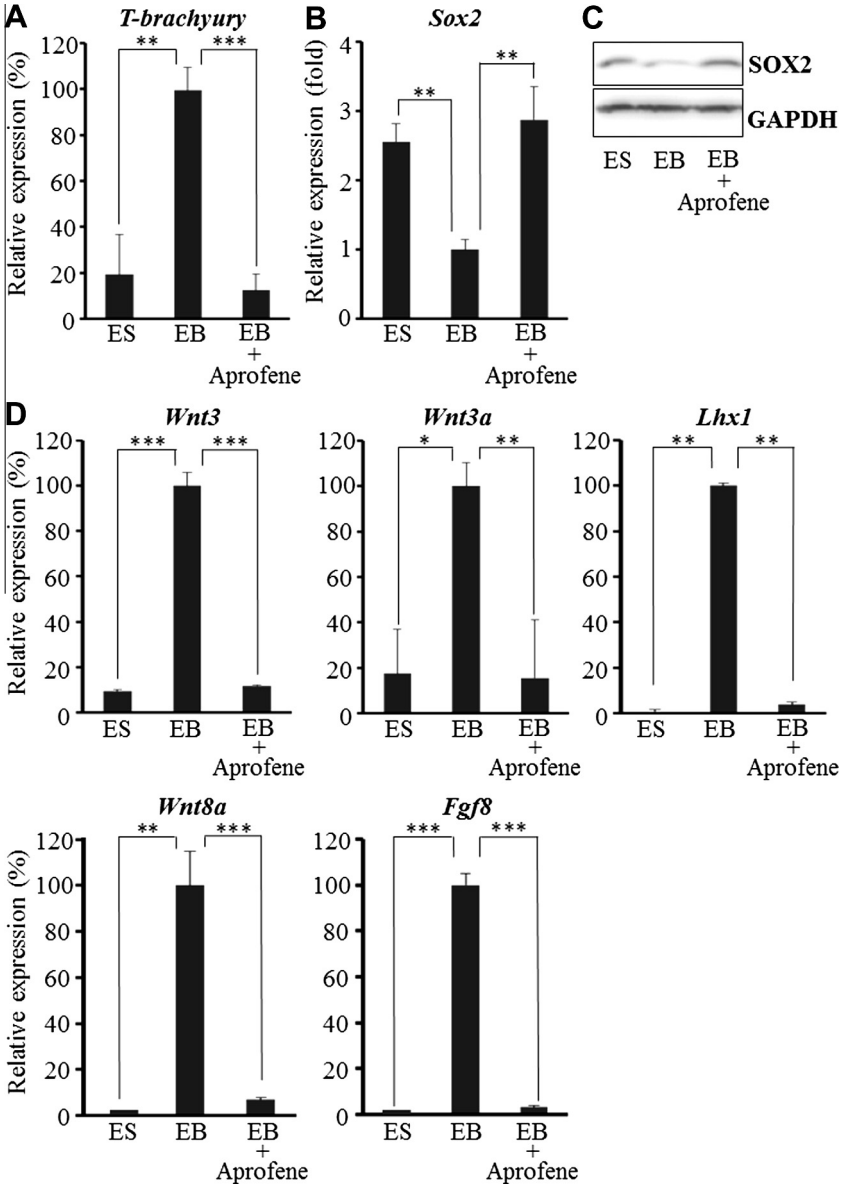

Fig. 3. Inhibition of mAchRs reduces primitive streak gene expression. (A) Decreased mesodermal gene expression. EBs were left untreated or treated with 10 μM aprofene during days 1–4. mRNA was extracted from control ES cells, or untreated or treated EBs on day 4, and analyzed by qRT-PCR to detect mRNA levels of *T-brachyury* (*T*). (B, C) Resistant *Sox2* expression. The extracts in (A) were analyzed by qRT-PCR (B) or immunoblotting (C) to detect *Sox2* mRNA or protein, respectively, as for Fig. 2B and C. (D) Decreased expression of primitive streak genes. The extracts in (A) were analyzed by qRT-PCR to detect mRNA levels of the indicated primitive streak genes. For A, B and D, results were analyzed as for Fig. 1B. $^{*}P < 0.05$, $^{**}P < 0.0001$, $^{***}P < 1 \times 10^{-5}$.

4. Discussion

In this study, we investigated the function of mAchRs during early murine embryogenesis. As depicted in Fig. 4D, our data suggest a model in which mAchRs promote the expression of *Cyp26a1*, a gene encoding an enzyme involved in RA catabolism. This moderation of RA concentration then enables Wnt3 to induce primitive streak genes, which in turn promote the differentiation of ES cells into mesodermal progenitors and ultimately cardiomyocytes, at the expense of neuronal lineages.

In early embryos, RA abundance is mainly determined by degradation mediated by CYP26 [18]. Previous studies have demonstrated that *Cyp26a1* is highly expressed during early embryonic patterning, and that depletion of maternal RA by embryonic CYP26 is required for proper primitive streak formation. Accordingly, *Cyp26a1/b1/c1* knockout mice show abnormalities in primitive streak formation. Our data shown in Fig. 4 are consistent with these reports.

Our results also show that mAchR inhibition increased the expression of ectodermal genes and promoted neurogenesis (Figs. 1 and 3). Knockout mice deficient for primitive streak genes, such as *Wnt3*$^{-/-}$ mice, exhibit similar phenotypes of decreased mesoderm formation and an expanded ectodermal region [19]. Taken together, these observations suggest that inhibition of primitive streak formation may enhance neuroectodermal differentiation *in vivo* and *in vitro*.

There are five mAchR subtypes, mAchR1–5. It has been previously reported that neither single knockout mice for any of these five genes, nor various double knockout mutants (mAchR1$^{-/-}$ and mAchR3$^{-/-}$, mAchR1$^{-/-}$ and mAchR4$^{-/-}$, mAchR1$^{-/-}$ and mAchR5$^{-/-}$, mAchR2$^{-/-}$ and mAchR3$^{-/-}$, mAchR2$^{-/-}$ and mAchR4$^{-/-}$), show severe developmental abnormalities [20].

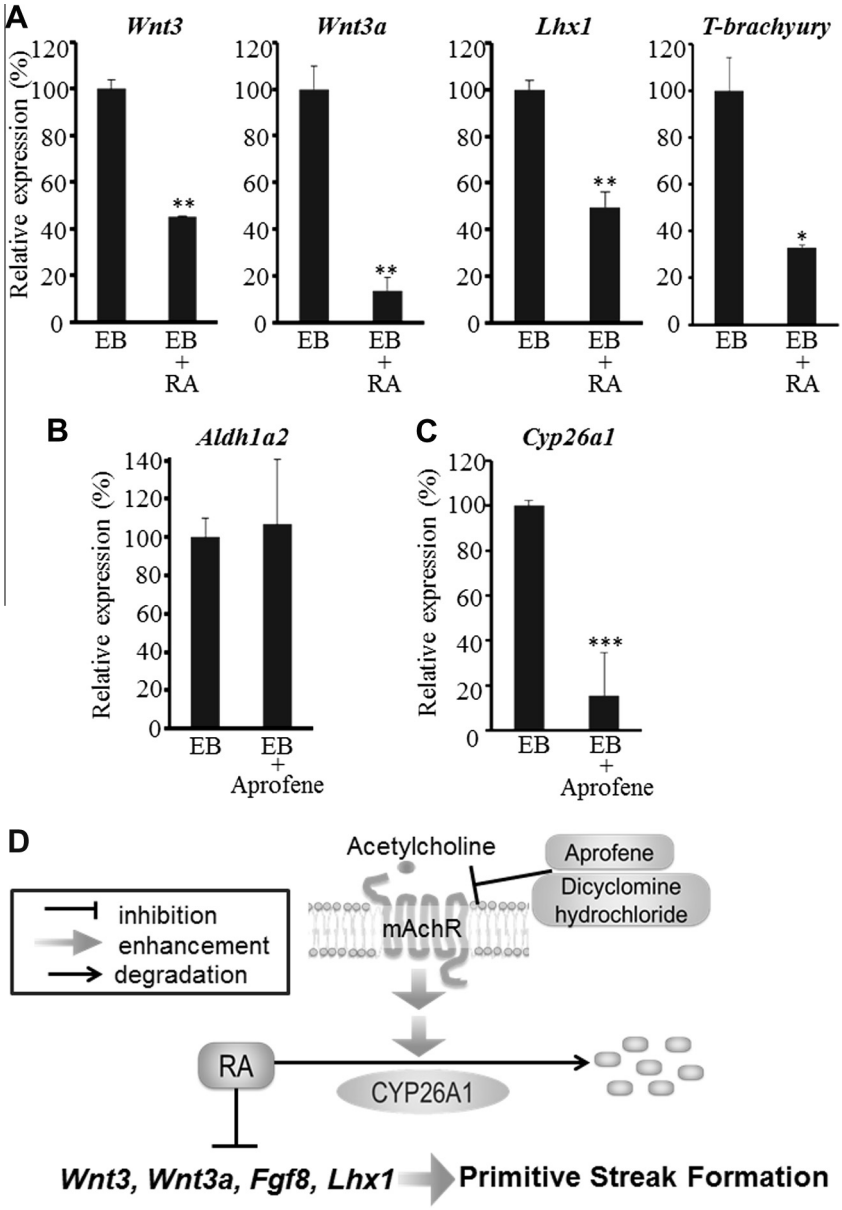

Fig. 4. mAchRs influence primitive streak gene expression through effects on RA. (A) RA reduces primitive streak gene expression. EBs were left untreated or treated with 1 pM RA during days 1–4 from day 1 and extracts were analyzed by qRT-PCR on day 4 to detect mRNA levels of the indicated primitive streak genes. Data were analyzed as for Fig. 1B. (B, C) mAchR inhibition reduces *Cyp26a1* expression. EBs were left untreated or treated with 10 μM aprofene during days 1–4 from day 1 and extracts were analyzed by qRT-PCR on day 4 to detect mRNA levels of the RA anabolic enzyme Aldh1a2 (B) and the RA catabolic enzyme Cyp26a1 (C). Data were analyzed as for Fig. 1B. (D) A model depicting a putative mechanism by which mAchRs can influence primitive streak formation. RA normally inhibits the expression of a variety of primitive streak genes, including Wnt3. RA abundance is controlled by degradation mediated by the catabolic enzyme Cyp26A1, whose expression is enhanced by mAchR activity. $^{*}P < 0.05$, $^{**}P < 0.0001$, $^{***}P < 1 \times 10^{-5}$.

However, these normal phenotypes may have been due to the redundancy of mAchR genes. We have shown, using two chemical inhibitors that specifically block five mAchRs [21–23], that embryonic development patterns are clearly altered in the absence of mAchR function. Thus, although the mechanism by which mAchRs control *Cyp26a1* expression has yet to be clarified, our work has revealed a novel physiological function for mAchRs in influencing gene expression required for embryogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.006.

References

[1] M.P. Caulfield, N.J. Birdsall, International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors, Pharmacol. Rev. 50 (1998) 279–290.

[2] L.E. Paraoanu, G. Steinert, A. Koehler, I. Wessler, P.G. Layer, Expression and possible functions of the cholinergic system in a murine embryonic stem cell line, Life Sci. 80 (2007) 2375–2379.

[3] I. Wessler, R. Michel-Schmidt, C. Brochhausen, C.J. Kirkpatrick, Subcellular distribution of choline acetyltransferase by immunogold electron microscopy in non-neuronal cells: placenta, airways and murine embryonic stem cells, Life Sci. 91 (2012) 977–980.

[4] N. Ramkumar, K.V. Anderson, SnapShot: mouse primitive streak, Cell 146 (2011) 488.
[5] H.B. Wood, V. Episkopou, Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages, Mech. Dev. 86 (1999) 197–201.
[6] P. Liu, M. Wakamiya, M.J. Shea, U. Albrecht, R.R. Behringer, A. Bradley, Requirement for Wnt3 in vertebrate axis formation, Nat. Genet. 22 (1999) 361–365.
[7] R.K. Kam, Y. Deng, Y. Chen, H. Zhao, Retinoic acid synthesis and functions in early embryonic development, Cell Biosci. 2 (2012) 11.
[8] N. Engberg, M. Kahn, D.R. Petersen, M. Hansson, P. Serup, Retinoic acid synthesis promotes development of neural progenitors from mouse embryonic stem cells by suppressing endogenous, Wnt-dependent nodal signaling, Stem Cells 28 (2010) 1498–1509.
[9] T.C. Doetschman, H. Eistetter, M. Katz, W. Schmidt, R. Kemler, The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium, J. Embryol. Exp. Morphol. 87 (1985) 27–45.
[10] D. ten Berge, W. Koole, C. Fuerer, M. Fish, E. Eroglu, R. Nusse, Wnt signaling mediates self-organization and axis formation in embryoid bodies, Cell Stem Cell 3 (2008) 508–518.
[11] N. Shimizu, H. Watanabe, J. Kubota, J. Wu, R. Saito, T. Yokoi, T. Era, T. Iwatsubo, T. Watanabe, S. Nishina, N. Azuma, T. Katada, H. Nishina, Pa6–5a promotes neuronal differentiation of murine embryonic stem cells, Biol. Pharm. Bull. 32 (2009) 999–1003.
[12] J. Wu, J. Kubota, J. Hirayama, Y. Nagai, S. Nishina, T. Yokoi, Y. Asaoka, J. Seo, N. Shimizu, H. Kajiho, T. Watanabe, N. Azuma, T. Katada, H. Nishina, P38 mitogen-activated protein kinase controls a switch between cardiomyocyte and neuronal commitment of murine embryonic stem cells by activating myocyte enhancer factor 2C-dependent bone morphogenetic protein 2 transcription, Stem Cells Dev. 19 (2010) 1723–1734.
[13] Y. Uchida, T. Osaki, T. Yamasaki, T. Shimomura, S. Hata, K. Horikawa, S. Shibata, T. Todo, J. Hirayama, H. Nishina, Involvement of stress kinase mitogen-activated protein kinase kinase 7 in regulation of mammalian circadian clock, J. Biol. Chem. 287 (2012) 8318–8326.
[14] H. Leader, A.D. Wolfe, P.K. Chiang, R.K. Gordon, Pyridophens: binary pyridostigmine-aprophen prodrugs with differential inhibition of acetylcholinesterase, butyrylcholinesterase, and muscarinic receptors, J. Med. Chem. 45 (2002) 902–910.
[15] J.W. Downie, D.A. Twiddy, S.A. Awad, Antimuscarinic and noncompetitive antagonist properties of dicyclomine hydrochloride in isolated human and rabbit bladder muscle, J. Pharmacol. Exp. Ther. 201 (1977) 662–668.
[16] A.L. Evans, T. Faial, M.J. Gilchrist, T. Down, L. Vallier, R.A. Pedersen, F.C. Wardle, J.C. Smith, Genomic targets of brachyury (T) in differentiating mouse embryonic stem cells, PLoS ONE 7 (2012) e33346.
[17] D.G. Wilkinson, S. Bhatt, B.G. Herrmann, Expression pattern of the mouse T gene and its role in mesoderm formation, Nature 343 (1990) 657–659.
[18] M. Uehara, K. Yashiro, K. Takaoka, M. Yamamoto, H. Hamada, Removal of maternal retinoic acid by embryonic CYP26 is required for correct nodal expression during early embryonic patterning, Gene. Dev. 23 (2009) 1689–1698.
[19] P.P. Tam, D.A. Loebel, Gene function in mouse embryogenesis: get set for gastrulation, Nat. Rev. Genet. 8 (2007) 368–381.
[20] J. Wess, Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications, Annu. Rev. Pharmacol. Toxicol. 44 (2004) 423–450.
[21] H. Leader, R.K. Gordon, J. Baumgold, V.L. Boyd, A.H. Newman, R.M. Smejkal, P.K. Chiang, Muscarinic receptor subtype specificity of N, N-dialkylamino)alkyl 2-cyclohexyl-2-phenylpropionates: cylexphenes (cyclohexyl-substituted aprophen analogues, J. Med. Chem. 35 (1992) 1290–1295.
[22] N.J. Buckley, T.I. Bonner, C.M. Buckley, M.R. Brann, Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells, Mol. Pharmacol. 35 (1989) 469–476.
[23] M. Eltze, M. Galvan, Involvement of muscarinic M2 and M3, but not of M1 and M4 receptors in vagally stimulated contractions of rabbit bronchus/trachea, Pulm. Pharmacol. 7 (1994) 109–120.