Exogenous expression of homeoprotein EGAM1N prevents in vitro cardiomyogenesis by impairing expression of *T* and *Nkx2.5*, but not *Mef2c*, in mouse embryonic stem cells

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Abstract Generation of multiple cell types from embryonic stem (ES) cells and induced pluripotent stem cells is crucial to provide materials for regenerative medicine. EGAM1N has been found in preimplantation mouse embryos and mouse ES cells as a functionally unclassified homeoprotein. Recently, we reported that expression of EGAM1N suppressed the in vitro differentiation of ES cells into progenitor cells that arise in early embryogenesis. To clarify the effect of EGAM1N on terminal differentiation, embryoid bodies (EBs) were prepared from ES cells expressing exogenous *Egam1n*. In EBs expressing *Egam1n*, cardiomyogenesis was inhibited by impairing the expression of crucial transcription factors *Brachyury T* and *Nkx2.5* in the generation of mesoderm and cardiomyocytes, respectively. Expression levels of *Mef2c*, another crucial gene for cardiomyogenesis, were unaffected. Conversely, the expression levels of

Gata6 and *Plat*, markers for the primitive endoderm lineage, and *Cdx2*, a marker for the trophectoderm lineage, were increased. These results suggested that certain cell populations in EBs expressing *Egam1n* preferentially differentiated to such cell lineages. Our results suggest that EGAM1N not only affects the generation of progenitor cells during early embryogenesis, but also the progression of terminal differentiation, such as cardiomyogenesis, in mouse ES cells.

Keywords *Brachyury T* · Cardiomyogenesis · *Crxos1* *tv3* · *Egam1n* · Embryonic stem cells · *Nkx2.5*

Introduction

Mouse embryonic stem (ES) cells are maintained in vitro as a pluripotent self-renewing population in the presence of leukemia inhibitory factor (LIF) (Niwa et al. 2009). In the absence of LIF, ES cells undergo spontaneous differentiation into various cell lineages including mesoderm, primitive endoderm, neuroectoderm, and pluripotent epiblast cells generated during the earlier stages of embryogenesis (Niwa 2007). Hence, ES cells are useful for the characterization of gene functions involved in pluripotency and differentiation. Furthermore, considerable effort has been devoted to generate multiple cell types from ES cells and induced pluripotent stem cells to provide materials for regenerative medicine.

We found the structurally related homeoproteins EGAM1 (GenBank accession No. AB472692, also

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known as CRXOS isoform a), EGAM1N (No. AB472693, CRXOS isoform c), and EGAM1C (No. AB472694, CRXOS isoform b) in both preimplantation mouse embryos and ES cells (Saito et al. 2010). The *Crxos* gene (Gene ID 546024) expresses mRNAs encoding EGAM1 homeoproteins as splice or transcription variants. Upon exogenous expression in trophoblast stem cells, which are stem cells of the trophoctoderm lineage (Tanaka et al. 1998), or mouse ES cells, these homeoproteins can act as positive or negative regulators of differentiation (Iha et al. 2012a, b; Saito et al. 2010; Sato et al. 2015; Soma et al. 2012), cell growth (Iha et al. 2012b; Sato et al. 2015), and induction of gene expression involved in specific cellular functions of differentiated cells (Saito et al. 2011). Because EGAM1 homeoproteins are localized to the nuclei of mouse ES cells (Sato et al. 2013), it is probable that these proteins act as transcription factors. Expression of the *Crxos* gene is detectable not only in early embryogenesis but also in adult organs, such as the eyes, brain, testes, thymus, and ovaries (Saito et al. 2012, 2010).

In this study, we further investigated the functions of EGAM1N. Ectopic expression of EGAM1N inhibited, at least in part, the differentiation of mouse ES cells into progenitor cells that arise in early embryogenesis (Saito et al. 2010; Sato et al. 2015). It is well known that in vitro differentiation of ES cells can give rise to terminally differentiated cell types, including beating cardiomyocytes (Desbaillets et al. 2000; Koike et al. 2005). Although EGAM1N exerted inhibitory effects on the generation of such progenitor cells from mouse ES cells, it remains unclear whether EGAM1N is capable of affecting the progression of terminal differentiation. In addition, our previous studies indicated that the effects of overproduction of EGAM1N resembled those of EGAM1C on cell growth and differentiation of mouse ES cells (Iha et al. 2012b; Sato et al. 2015). As we reported previously in ES cells expressing exogenous *Egam1c* (Iha et al. 2012a), we further examined the effect of EGAM1N on cardiomyogenesis in mouse ES cells.

Materials and methods

Cell culture

Feeder-free mouse ES MG1.19 cells stably expressing EGAM1N (clones N6 and N8) were established by transfection with an *Egam1n* expression vector, as

described previously (Sato et al. 2015). Control transfectants were also established with an empty vector (clones E3 and E12). Transfectants were maintained on gelatin-coated plates in Glasgow modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10 % fetal calf serum (defined for ES cells, Biological Industries, Haemek, Israel), puromycin (2 µg/ml), G418 (250 µg/ml), and recombinant human LIF (prepared in-house (Smith 1991), +LIF medium) at 37 °C with 5 % CO₂. Embryoid bodies (EBs) were prepared in low cell-binding 96-well plates (U bottom, Lipidure-Coat Plate, A-U96, NOF, Tsukuba, Japan) for a 5-day culture period, as reported previously (Iha et al. 2012a). Then, EBs were transferred to gelatin-coated 24 well-plates and allowed to attach and grow without LIF for 7 days (Iha et al. 2012a).

Western blotting and quantitative RT-PCR analyses

EGAM1N, T (also known as BRACHYURY, 1:4000, sc-17745, Santa Cruz Biotechnology, Santa Cruz, CA, USA), NKX2.5 (1:10000, sc-8697, Santa Cruz Biotechnology), MEF2C (1:10000, sc-13266, Santa Cruz Biotechnology), and ACTB (β-ACTIN, 1:20000, IMG-5142A, Imgenex, San Diego, CA, USA) were detected by western blotting as reported previously (Iha et al. 2012a; Sato et al. 2015). Extraction of total RNA, synthesis of cDNA, and quantitative (q) RT-PCR were carried out as reported previously (Iha et al. 2012a, b; Saito et al. 2011). Hydroxymethylbilane synthase (*Hmbs*) was analyzed as a housekeeping gene. Primer sequences are indicated in our previous reports (Iha et al. 2012a, b; Saito et al. 2011). The ratio of the number of cDNA copies for each gene to that of *Hmbs* is indicated as the expression level. The results obtained by qRT-PCR analysis were subjected to the Steel–Dwass test.

Results and discussion

Overproduction of EGAM1N protein suppresses cardiomyogenesis of mouse ES cells

Overproduction of EGAM1N protein was clearly detected in EBs generated from ES cell clones N6 and N8 transfected with the *Egam1n* expression vector

Fig. 1 Effect of exogenous expression of EGAM1N protein on the generation of cardiomyocytes in embryoid bodies. Embryonic stem (ES) cell transfectants were induced to differentiate by generating embryoid bodies (EBs) (E3 and E12: transfectants with an empty vector as a control; N6 and N8: transfectants with an *Egam1n* expression vector). EBs were transferred to attachment culture to promote cardiomyogenesis. **a** Cellular contents of EGAM1N protein. EGAM1N (14 kDa) was detected by western blotting after 4 days of attachment culture. ACTB (also known as β -ACTIN, 42 kDa) is indicated as a loading control. **b** Rates of contracting EBs during attachment culture.

Data represent the sum of two independent trials. In the respective trials, twelve EBs were examined in total. **c** Cell morphology of attachment cultures after a 5-day culture period. Scale bar, 1 mm. **d** Expression levels of transcripts encoding myosin subtypes as cardiomyocyte markers in EBs during attachment culture. The expression levels of myosin light chain (*Myl2*) and myosin heavy chain (*Mhc6*) were evaluated by quantitative (q) RT-PCR after 6 days of attachment culture (D6) and normalized to that of *Hmbs*. Data are expressed as the mean $\pm$ SE ($n = 3$). The mean value of the control E3 and E12 clones was set as 1. * $p < 0.05$

(Sato et al. 2015; Fig. 1a). In EBs prepared from control transfectants, cell contraction was detected at 3 days after attachment culture (Fig. 1b). After 6 days of attachment culture, almost all EBs were contracting. Although no obvious differences in the morphology of EBs were indicated, relatively large EBs were found in *Egam1n* transfectants compared with the controls in low cell-binding 96-well plates (data not shown). In the attachment cultures, larger and thicker cell colonies were found in *Egam1n* transfectants than in the control (Fig. 1c). However, cell contraction was almost undetectable in EBs generated from *Egam1n* transfectants. In addition, the expression levels of myosin light chain (*Myl2*) and myosin heavy chain (*Mhc6*), representative cardiomyocyte markers, were almost undetectable (Fig. 1d). These results

demonstrated that EGAM1N inhibited the generation of cardiomyocytes from mouse ES cells. In fact, expression of *Egam1n* transcripts was undetectable in the heart (Saito et al. 2010).

Obvious decreases in the expression levels of Brachyury *T* and *Nkx2.5* in EBs expressing *Egam1n*

As shown in Fig. 2a, we compared the expression levels of *T*, a crucial transcription factor for the generation of mesoderm, and *Nkx2.5* (Lints et al. 1993) and *Mef2c* (Edmondson et al. 1994), both crucial transcription factors for the progression of cardiomyogenesis (Fig. 2). In *Egam1n* transfectants, the expression levels of *T* and *Nkx2.5* were markedly

Fig. 2 Expression levels of transcription factors in the progression of cardiomyogenesis of mouse ES cells. **a** Expression levels of Brachyury *T*, *Nkx2.5*, and *Mef2c* mRNAs evaluated by qRT-PCR after 4 days (D4) and 6 days (D6) of attachment culture. Data are expressed as mean $\pm$ SE ($n = 3$). Data were processed as described for Fig. 1d. * $p < 0.05$. **b** Cellular

contents of transcription factors *T* (47 kDa) (mesoderm induction), *NKX2.5* (39 kDa) and *MEF2C* (45 kDa) (cardiomyocyte generation) determined by western blotting after 4 days of attachment culture. Note that the data for ACTB are indicated in Fig. 1a. The numbers under the bands represent the relative densities of specific signals normalized to the density of ACTB

down-regulated, whereas those of *Mef2c* and its encoded protein were unchanged. The expression levels of *T* varied in control clones, and the decrease in cellular content of *T* protein was relatively small in *Egam1n* transfectants. However, the reasons for this were unclear. In accordance with the expression levels of *Nkx2.5* transcripts, the cellular contents of *NKX2.5* protein were also decreased. It is well established that *NKX2.5* and *MEF2C* are mutual cofactors for the progression of cardiomyogenesis (Vincentz et al. 2008). We speculated that insufficient expression of *T* and its encoded protein likely resulted in modest maturation of mesoderm, which contains progenitor cells for cardiomyocytes, in EBs expressing *Egam1n*. This insufficiency could further contribute to the impaired expression of *Nkx2.5* and formation of cardiomyocytes. In mouse embryonal carcinoma P19 cells, ectopic expression of *Nkx2.5* or *Mef2c* induces endogenous *T* expression (Skerjanc et al. 1998). In *Egam1n* transfectants, it is likely that the impaired

expression of *Nkx2.5* and *T* resulted in mutually regulated decreases in their expression. The relationship between the function of *EGAM1N* and the regulation of *Mef2c* expression is also intriguing.

Preferential emergence of cell types expressing markers for extraembryonic tissues in EBs expressing *Egam1n*

Finally, we confirmed the differentiation status of EBs expressing *Egam1n*. Expression levels of genes encoding transcription factors after 4 and 6 days of culture are indicated (Fig. 3a). The expression levels of *Pou5f1* (also known as *Oct4*) and *Nanog*, marker genes for the undifferentiated state of ES cells (Niwa 2007), were obviously decreased in both control and *Egam1n* transfectants. Hence, EBs expressing *Egam1n* were differentiating or in a differentiated state. As shown in Fig. 3b, we detected increases in the expression levels of crucial transcription factors *Gata6*

Fig. 3 Expression levels of undifferentiated state and cell lineage-specific markers in embryoid bodies. **a** Expression levels of mRNAs for undifferentiated state markers *Pou5f1* (also known as *Oct4*) and *Nanog* evaluated by qRT-PCR after 4 days (D4) and 6 days (D6) of attachment culture. Data are expressed as mean $\pm$ SE ($n = 3$). Data were processed as described for Fig. 1d. The value in the undifferentiated state was set as 1. $*p < 0.05$. **b** Expression levels of mRNAs for lineage-specific transcription factors and differentiation markers evaluated by qRT-PCR after 4 days (D4) and 6 days (D6) of attachment culture. *Gata6* and *Cdx2* are genes encoding lineage-specific transcription factors for the primitive endoderm and trophoblast, respectively. *Plat*, parietal endoderm marker; *Afp*, visceral endoderm marker; *Fgf5*, pluripotent epiblast marker. Data are expressed as mean $\pm$ SE ($n = 3$). Data were processed as described for Fig. 1d. $*p < 0.05$

for primitive endoderm formation and *Cdx2* for trophoblast formation. As a result, tissue plasminogen activator (*Plat*), a marker gene for parietal endoderm of the primitive endoderm lineage, was relatively higher in *Egam1n* transfectants. In contrast, the expression levels of α -fetoprotein (*Afp*), a marker gene for visceral endoderm of the primitive endoderm

lineage, and fibroblast growth factor 5 (*Fgf5*), a marker for the pluripotent epiblast, were almost undetectable. Based on these results, exogenous EGAM1N expression led to differentiation of EBs into cell types expressing markers for extraembryonic tissues including extra-embryonic trophoblast and parietal endoderm lineages. We considered that long-term induction of differentiation, such as the generation of EBs, resulted in the complete absence of gene expression of pluripotency factors, including *Pou5f1* and *Nanog*, followed by the appearance of EGAM1N for induction of differentiation.

Taken together, we suggest that EGAM1N affects not only the generation of progenitor cell types in early embryogenesis, but also the progression of terminal differentiation in mouse ES cells. Although the homeodomains of EGAM1N and EGAM1C are paired-like type and engrailed-like type, respectively (Saito et al. 2010), these phenotypic changes in ES cells expressing EGAM1N closely resemble those of cells expressing EGAM1C (Iha et al. 2012a, b; Sato et al. 2015). We believe that the dissection of mechanisms underlying gene expression induced by EGAM1N and EGAM1C may provide valuable information about the roles of these homeoproteins as transcriptional regulators.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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