Galectin-1 enhances the generation of neural crest cells

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ABSTRACT Neural crest (NC) cells are multipotent cells that emerge from the dorsal region of the neural tube. After delaminating from the neural tube, NC cells migrate throughout the developing embryo and differentiate into various cells: neurons and glial cells of the peripheral nervous system, melanocytes of skin, and skeletal elements of the face and head. We previously analyzed the gene expression profile of a NC subpopulation isolated from Sox10-IRES-Venus mice and found that the carbohydrate-binding protein, *Galectin-1 (Gal-1)* was strongly expressed in generating NC cells. In the present study, we identified GAL-1 as a factor that promotes NC cell generation. *Gal-1* was significantly expressed in NC cells generated in explanted neural tubes. The presence of GAL-1 enhanced the generation of NC-like cells from mouse embryonic stem (ES) cells. In the differentiation of ES cells into NC-like cells, GAL-1 enhanced neurogenesis in the early stages and facilitated NC-like cell generation in the later stages. GAL-1 also enhanced the generation of NC cells from explanted neural tubes. These results suggest that GAL-1 plays a facilitative role in NC cell generation.

KEY WORDS: *neural crest cell, galectin-1, mouse embryonic stem cell*

NC cells emerge from the dorsal region of the neural tube and migrate throughout the embryo. During their migration or at target tissues, they react with various environment factors and differentiate into a wide variety of derivatives including neurons and glial cells of peripheral sensory and autonomic ganglia, melanocytes, endocrine cells, smooth muscle cells, and skeletal and connective tissue cells of the craniofacial complex (Le Douarin and Kalcheim, 1999). Notwithstanding their critical roles in embryogenesis, the molecular mechanisms responsible for NC cell generation have not yet been elucidated in detail due to the difficulties associated with isolating and manipulating these cells. NC cells emerge as a continuous cell population, progressively disperse, and invade neighboring tissues; therefore, the separation and isolation of these cells is very challenging. The utilization of genetically modified animals has an impact on NC cell investigations. These animals express a reporter gene or Cre recombinase under the control of the regulatory elements of genes known to be essential for NC cell development, one of which is *Sox10*.

SOX10 is a NC cell specifier; its expression starts in premigratory NC cells and continues in migrating NC cells and NC cell derivatives (Herbarth *et al.*, 1998, Mollaaghababa and Pavan, 2003, Paratore *et al.*, 2001, Sonnenberg-Riethmacher *et al.*, 2001, Southard-Smith *et al.*, 1998). Transgenic mice in which the complete open reading frame of *Sox10* was replaced by *lacZ* sequences were reported and the mice precisely marked the NC cells (Britsch *et al.*, 2001). We also generated mice designed to express green fluorescence protein under the control of *Sox10* (Sox10-IRES-Venus mice) (Motohashi *et al.*, 2011) and analyzed the gene expression profile of a pure NC subpopulation isolated from Sox10-IRES-Venus mice. In the study, we demonstrated that the expression of *Gal-1* was significant in generating NC cells (Motohashi *et al.*, 2016).

GAL-1 is a carbohydrate-binding protein with an affinity for β-galactosides, is differentially expressed by various tissues, and

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appears to be functionally polyvalent with a wide range of biological activities (Camby *et al.*, 2006). GAL-1 is widely distributed in the developing embryo and plays diverse roles in early gestation, embryogenesis, organ structural development, and cell differentiation (Camby *et al.*, 2006). Previous studies showed that GAL-1 promoted the proliferation of hematopoietic stem cells (HSCs) and neural stem cells (NSCs) in *in vitro* cultures (Sakaguchi *et al.*, 2006, Vas *et al.*, 2005). GAL-1 was also expressed in a cohort of cells presumed to be NC cells during the formation of organ primordia in early chick embryos (Zalik *et al.*, 1994), suggesting that GAL-1 plays roles in the generation of NC cells.

We herein described the expression and functions of GAL-1 in mouse NC cell development. The expression of *Gal-1* was significant in mouse NC cells delaminated from explanted neural tubes. GAL-1 enhanced the generation of NC-like cells from ES cells, and NC cells from explanted neural tubes. GAL-1 had two functions in the context of NC cell generation: the enhancement of neurogenesis and induction of NC cell generation. These results support a facilitative role for GAL-1 in NC cell generation.

Results

GAL-1 is expressed in generating neural crest cells

We previously used a gene array analysis to show that the expression of *Gal-1* was significant in generating NC cells (Motohashi *et al.*, 2016), and we reconfirmed its expression using a RT-PCR analysis. We isolated generating NC cells from the trunk regions of Sox10-IRES-Venus embryos 9.0 and 9.5 days post coitum (E9.0, E9.5) with a flow cytometer and the expression of *Gal-1* was analyzed. The isolated SOX10-positive (SOX10+) NC cells expressed *Gal-1* in E9.0 and E9.5 embryos, showing that GAL-1 is expressed in developing NC cells (Fig. 1A). In an immunostaining analysis of E9.5 embryos, GAL-1 was expressed in migrating NC cells (Fig. 1B). GAL-1 was expressed along the NC cell migration pathway, suggesting that GAL-1 functions in NC cell generation (Fig. 1B).

In order to quantify the expression of *Gal-1* during the generation of NC cells, we utilized a mouse Sox10-IRES-Venus ES cell culture system for NC-like cells that recapitulates NC cell generation (Motohashi *et al.*, 2007, Motohashi *et al.*, 2011). We isolated SOX10+ NC-like cells from day 9 to 12 of the Sox10-IRES-Venus ES cell culture and analyzed the expression of *Gal-1* with microarrays. The expression of *Gal-1* was maintained in generating NC-like cells and its expression level remained unchanged during their generation (Fig. 1C).

GAL-1 enhances the generation of NC-like cells

In order to investigate the function of GAL-1 in generating NC cells, we cultured Sox10-IRES-Venus ES cells in a culture system in the presence of GAL-1 and analyzed the rate of generation of SOX10+ NC-like cells using flow cytometry. The presence of 1.0 ng/ml of GAL-1 increased the rate of SOX10+ NC-like cells on day 9 of the culture, by which time NC-like cells began to generate in the system (Fig. 2A). On day 12 of the culture, by which time NC-like cells had fully generated, the presence of 0.1 ng/ml or 1.0 ng/ml of GAL-1 significantly increased the rate of Sox10+ NC-like cells over that in the absence of GAL-1 (Fig. 2A). A high dose of GAL-1 (10 ng/ml) did not increase the rate of SOX10+ NC-like cells (Fig. 2A). The SOX10+ NC-like cells that generated in the presence of GAL-1 expressed the following NC cell marker genes: *Sox10*,

P75, *Pax3*, and *Snail*, and differentiated into neurons, glial cells, and melanocytes, similar to SOX10+ NC-like cells in the absence of GAL-1 (Supplementary Fig. S1A, S1B, S1C). These results suggest that GAL-1 enhances the generation of NC-like cells from ES cells without affecting their properties.

In order to investigate the presence of a sensitive culture period for GAL-1 during the induction of NC-like cells in the culture, we added 1.0 ng/ml of GAL-1 to the culture at various time points, as shown in Fig. 2B. The presence of GAL-1 on days 0-2, days 2-4, and days 10-12 enhanced the generation of SOX10+ NC-like cells. These results suggest that GAL-1 enhances NC-like cell generation at the early and late stages of generation.

We cultured Sox10-IRES-Venus ES cells in the presence of a GAL-1 antagonistic antibody and analyzed the rate of generation of SOX10+ NC-like cells. No significant differences were observed
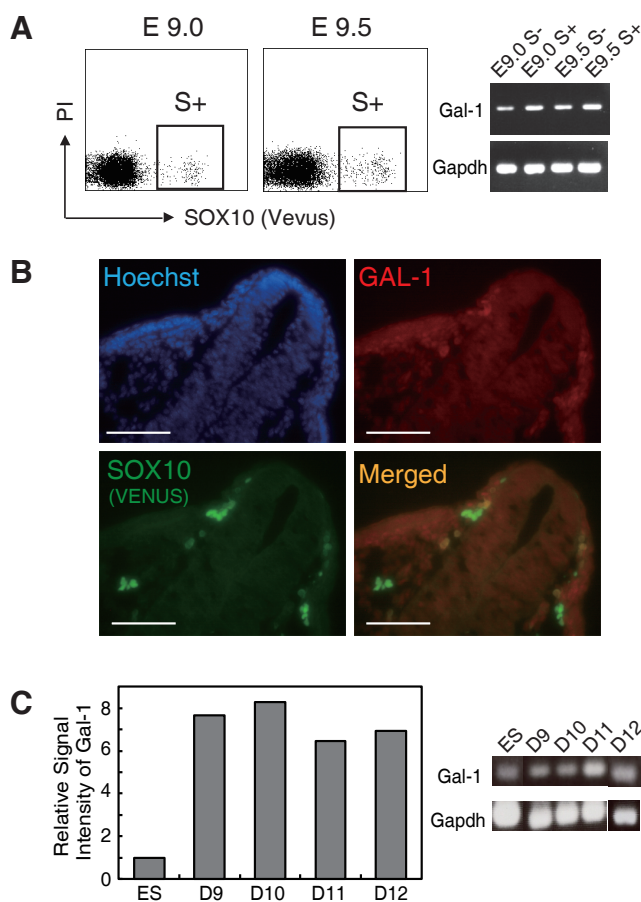

Fig. 1. GAL-1 expression in Sox10+ neural crest (NC) cells in embryos.
(A) *SOX10-positive (S+) and negative (S-) cells were isolated from trunk neural tubes and their adjacent areas in E9.0 and E9.5 Sox10-IRES-Venus embryos (left). S+ and S- cells were analyzed for the expression of* Gal-1 *by RT-PCR (right).* **(B)** *Cross section of an E9.5 embryo. The trunk region of E9.5 Sox10-IRES-Venus embryos was stained with antibodies for GAL-1 (red) and GFP (green). Nuclei were stained with Hoechst 33258 (blue). Scale bar, 100 μm.* **(C)** *SOX10+ NC-like cells on days 9 to 12 of the Sox10-IRES-Venus embryonic stem (ES) cell culture were analyzed for the expression of* Gal-1 *with microarrays (left). Signal intensity of* Gal-1 *expression was normalized with respect to that of* Gal-1 *expression in uncultured ES cells. The expression of* Gal-1 *was analyzed by RT-PCR (right).*

in the rate of generation of SOX10+ NC-like cells between cultures conducted in presence and absence of anti-GAL-1 (Fig. 2C). Therefore, other Galectin family proteins may compensate for the absence of Galectin-1.

GAL-1 effectively enhances neural ectoderm differentiation at the early stage of NC-like cell generation

NC cells are induced in the neural ectoderm adjacent to the neural plate and emerge from the region by epithelial-to-mesenchymal transition during embryogenesis. We hypothesized that the presence of GAL-1 on days 0-2 or days 2-4 of the culture effectively

induced neural ectoderm differentiation from undifferentiated ES cells. We cultured ES cells that stably expressed GFP (D3-GFP ES cells) in the culture system for NC-like cell generation in the presence of GAL-1, isolated ES-derived cells on days 2-9 of the culture, and investigated the expression of the following neural markers: *Hoxb9*, *Ncam*, *Nestin*, and the NC marker, *Sox10* using quantified-PCR (qPCR) (Mizuseki *et al.*, 2003). The expression levels of *Ncam* remained unchanged in the presence of GAL-1, while those of the neural ectoderm marker *Nestin* significantly increased on day 6, and those of *Hoxb9* were approximately 2.5-fold higher than control levels on day 8 of the culture (Fig. 3). These results
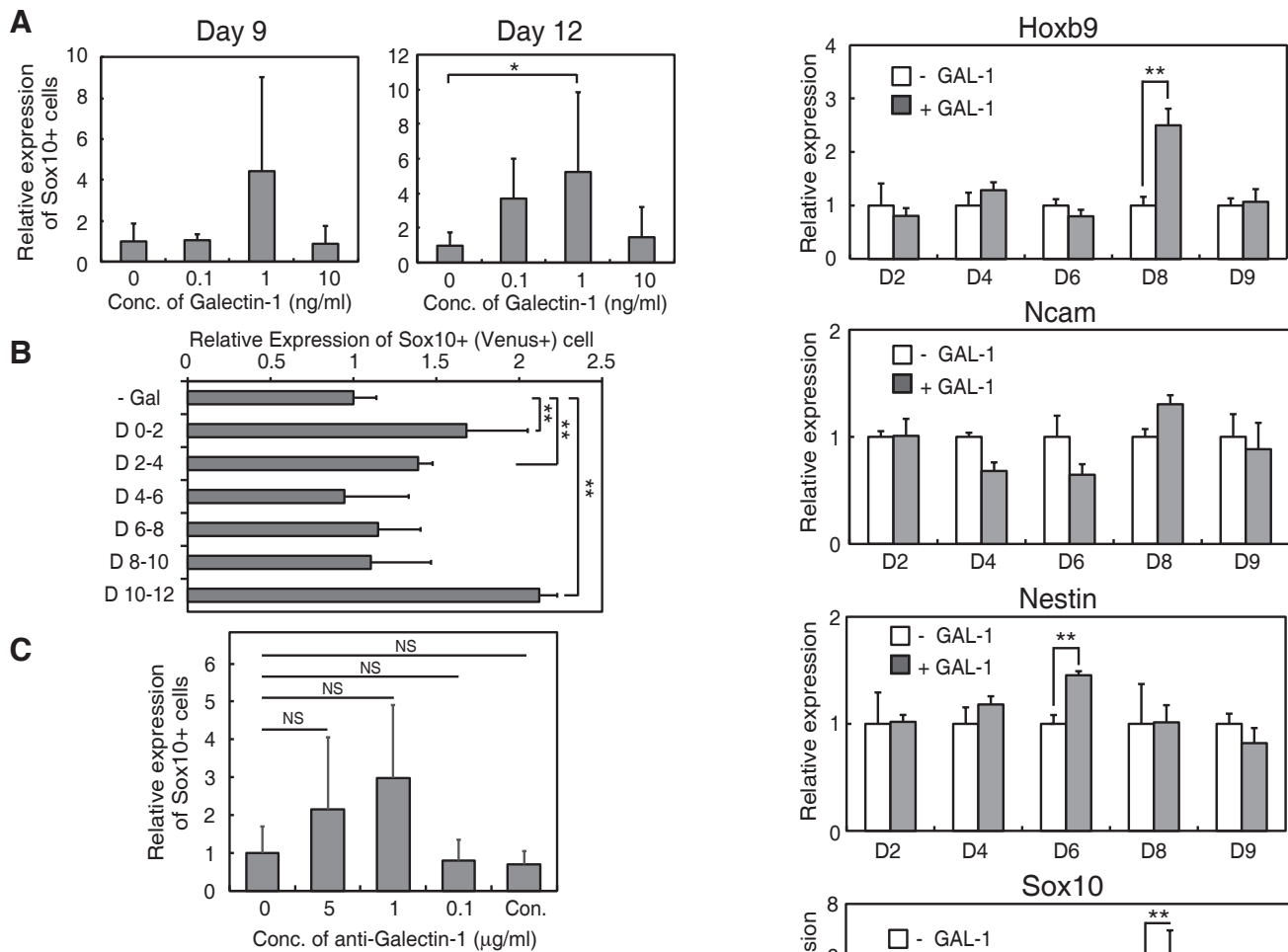

Fig. 2 (left). The presence of GAL-1 promotes the generation of Sox10+ NC-like cells from embryonic stem (ES) cells. (A) *Sox10-IRES-Venus ES cells differentiated into NC-like cells in the presence of GAL-1 at the indicated concentration. On days 9 and 12 of the culture, the rate of SOX10+ cells was analyzed by flow cytometry. The rate of SOX10+ cells was normalized with respect to that of SOX10+ cells without GAL-1. Error bars represent the SD of experiments performed 5 (Day 9) or 6 times (Day 12). *p < 0.05.* **(B)** *Sox10-IRES-Venus ES cells differentiated into NC-like cells in the presence of GAL-1 in the indicated manner. On day 12 of the culture, the expression of SOX10+ cells was analyzed by flow cytometry. Error bars represent the SD of experiments performed in triplicate. **p < 0.01.* **(C)** *Sox10-IRES-Venus ES cells differentiated into NC-like cells in the presence of antagonistic anti-GAL-1 or a control antibody (Con). On day 12 of the culture, the expression of SOX10+ cells was analyzed by flow cytometry. Error bars represent the SD of experiments performed in triplicate. NS, not significant.*

Fig. 3 (right). GAL-1 promotes the expression of neurogenic genes. *D3-GFP ES cells were cultured on ST2 stromal cells with or without GAL-1. GFP+ cells were isolated on days 2, 4, 6, 8, and 9 of the culture and the expression of the neurogenic genes* Hoxb9, Ncam, *and* Nestin, *and the NC marker gene* Sox10 *was analyzed by qPCR. Error bars represent the SD of experiments performed in triplicate. **p < 0.01.*

suggest that GAL-1 effectively induces the neural ectoderm from ES cells at the early stages of the culture system. On day 8 of the culture, the expression levels of *Sox10* were 7-fold higher than control levels, confirming that the effective induction of neural ectoderm cells results in enhanced NC-like cell induction (Fig. 3).

The carbohydrate-binding activity of GAL-1 is not involved in the generation of NC-like cells

GAL-1 is a homodimer with a subunit molecular mass of 14.5 kDa and exhibits carbohydrate-binding activity. However, oxidative environments induce the formation of intra-molecular Cys-Cys bonds, which change the structure of GAL-1 and result in the complete loss of its carbohydrate-binding activity (Horie and Kadoya, 2004, Inagaki *et al*., 2000). In order to examine the roles of carbohydrate-binding activity in the generation of NC-like cells, oxidized recombinant human GAL-1 (rhGAL-1/Ox) and a GAL-1 mutant (CSGAL-1) that exhibits constitutive carbohydrate-binding activity by preventing intra-molecular Cys-Cys bonds (Inagaki *et al*., 2000) were added to the culture at 1.0 ng/ml, and the rate of SOX10+ NC-like cell generation was analyzed. Although both types of GAL-1 increased the rate of SOX10+ NC-like cells over that in the control culture, significant differences were not observed between rhGAL-1/Ox-and CSGAL-1-containing cultures (Fig. 4A). These results suggest that the carbohydrate-binding activity of GAL-1 is not important for the induction of NC-like cells. The addition of rhGAL-1/Ox or CSGAL-1 did not change the expression of the following NC cell-related genes: *Sox10*, *P75*, *Pax3*, and *Snail*

A

B

Fig. 4. GAL-1 promotes the generation of SOX10+ neural crest (NC)-like cells regardless of their carbohydrate-binding activity. (A) *Sox10-IRES-Venus embryonic stem (ES) cells differentiated into NC-like cells in the presence of rhGAL-1/Ox or CSGAL-1. On day 12 of the culture, the expression of SOX10+ cells was analyzed by flow cytometry and was normalized to that of SOX10+ cells without GAL-1 (-GAL-1).* **(B)** *Sox10-IRES-Venus ES cells differentiated into NC-like cells in the presence of rhGAL-1/Ox in the indicated manner. On day 12 of the culture, the expression of SOX10+ cells was analyzed by flow cytometry and normalized to that of SOX10+ cells without GAL-1 (-rhGAL-1/Ox). Error bars represent the SD of experiments performed in triplicate. NS, not significant. *p < 0.05, **p < 0.01.*

in SOX10+ cells, or the differentiation potency of SOX10+ cells (Supplementary Fig. S1B, S2). The treatment with rhGAL-1/Ox or CSGAL-1 also did not promote differentiation. An investigation of the sensitive culture period for rhGAL-1/Ox showed that the presence of rhGAL-1/Ox on days 2-4 and days 10-12 enhanced the generation of SOX10+ NC-like cells, similar to the native form of GAL-1 (Fig. 4B).

GAL-1 promotes the generation of NC cells in explanted neural tubes

We further investigated whether GAL-1 enhances the generation of *in vivo* NC cells. We extirpated neural tubes from E9.5 Sox10-IRES-Venus embryos, cultured them in the presence of rhGAL-1/Ox for 1 day, and analyzed the rate of SOX10+ cells generated using flow cytometry. The presence of rhGAL-1/Ox in the culture of explanted neural tubes increased the rate of SOX10+ cells (Fig. 5A). We then examined the expression of genes related to NC cell generation in cultured neural tubes by qPCR. The expression of *Sox9*, *Sox10*, *Foxd3*, and *Snail* increased in the presence of rhGAL-1/Ox than in the absence of GAL-1 (Fig. 5B). We also analyzed the expression of NC cell-related genes in E10.5 neural tubes in the culture with rhGAL-1/Ox. The expression of *Sox9*, *Sox10*, and *Snail* also increased in cultured E10.5 neural tubes with rhGAL-1/Ox (Fig. 5C). However, the difference observed in relative expression levels in the presence or absence of rhGAL-1/Ox was smaller in E10.5 explants than in E9.5 explants (Fig. 5B, C); the expression levels of *Sox9*, *Sox10*, *Snail*, and *Foxd3* were up to 13-, 8-, 9-, and 1.25-fold higher, respectively, than control levels in E9.5 explants (Fig. 5B). However, the expression levels of *Sox9*, *Sox10*, and *Snail* were 3.5-, 1.25-, and 1.5-fold higher, respectively, than control levels, and no significant difference was noted in *Foxd3* expression levels between the culture with rhGAL-1/Ox and the control culture in E10.5 explants (Fig. 5C). This result suggests that rhGAL-1/Ox promotes NC cell generation in the early stages of NC cell development. In contrast to rhGAL-1/Ox, the presence of CSGAL-1 slightly enhanced the expression of *Sox10* exclusively in E9.5 explants. This result shows that the carbohydrate-binding activity of GAL-1 has no role in *in vivo* NC cell generation.
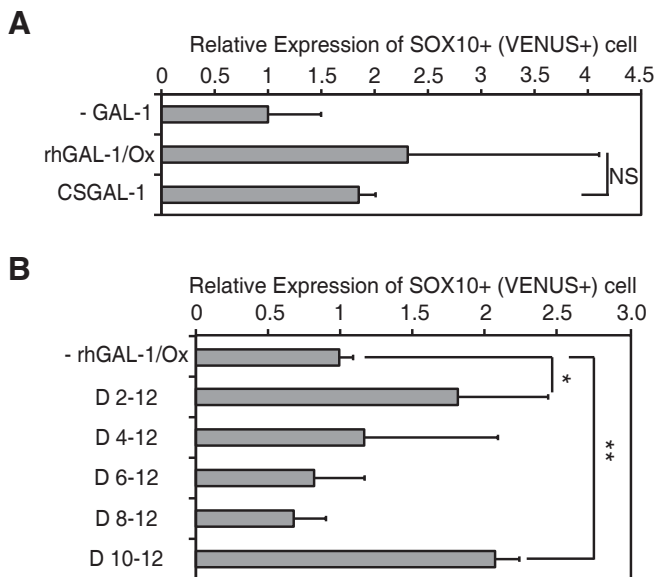
Discussion

GAL-1 is expressed in a cohort of cells presumed to be NC cells in chick embryos (Zalik *et al*., 1994). We also reported that it was significantly expressed in generating mouse NC cells (Motohashi *et al*., 2016) and reconfirmed its expression in embryos (Fig. 1), suggesting a possible role in NC cell generation. In the present study, we found that GAL-1 enhanced NC cell generation.

The generation of NC-like cells appeared to be enhanced at low concentrations of GAL-1 (Fig. 2A). A previous study demonstrated that while high doses of recombinant GAL-1 (more than 1 μM) inhibited cell proliferation independently of GAL-1 sugar-binding activity, low doses (less than 1 nM) exhibited mitogenic activity in fibroblasts and hematopoietic progenitor cells (Adams *et al*., 1996, Vas *et al*., 2005). Furthermore, GAL-1 in its oxidized form, a form that lacks lectin activity, enhanced axonal regeneration in peripheral and central nerves, even at relatively low concentrations (picoM range) (Horie and Kadoya, 2000, Kadoya *et al*., 2005, McGraw *et al*., 2005). These findings indicate that GAL-1 also
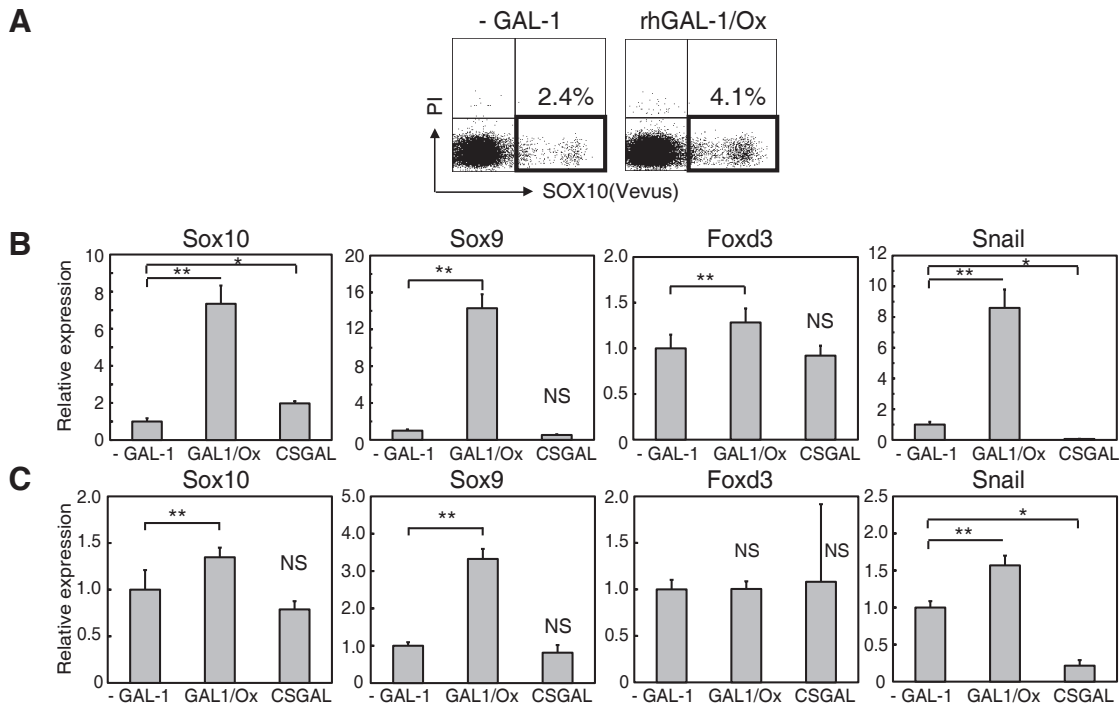
Fig. 5. The presence of GAL-1 promotes the generation of SOX10+ neural crest (NC) cells from explanted neural tubes. (A) *Neural tubes were isolated from E9.5 Sox10-IRES-Venus embryos and cultured with or without rhGAL-1/Ox. On day 1 of the culture, the expression of SOX10+ cells was analyzed.* **(B,C)** *Neural tubes isolated from E9.5* **(B)** *or E10.5* **(C)** *Sox10-IRES-Venus embryos were cultured with 25 ng/ml rhGAL-1/Ox (GAL1/Ox) or CSGAL-1 (CSGAL). On day 1 of the culture, the expression of the neural crest marker genes* Sox10, Sox9, Foxd3, *and* Snail *was analyzed by qPCR. Error bars represent the SD of experiments performed in triplicate. NS, not significant. *p < 0.05, **p < 0.01.*

functions at a relatively low concentration in NC cell generation.

The mechanisms by which the concentration of GAL-1 affects NC cell generation have not yet been elucidated. However, a biphasic effect that depends on GAL-1 concentrations has been reported in hematopoietic cells. While a large amount of GAL-1 reduces the growth of committed blood-forming progenitor cells, a low amount increases the formation of colony-forming units for granulocytes/macrophages (CFU-GM) and erythroid colonies (BFU-E) and the frequencies of cobblestone area-forming cells (CAFCs) (Vas *et al.*, 2005). These findings suggest that a large amount of GAL-1 functions as a classical proapoptotic factor for hematopoietic cells and that growth inhibition correlates with apoptotic death in hematopoietic cells (Vas *et al.*, 2005). These paradoxical positive and negative effects of GAL-1 on cell growth are not unique and its seemingly contradictory effects are strongly dependent on the type and activation state of cells (Sacchettini *et al.*, 2001). Our results are essentially consistent with those of previous studies using other cells isolated from different tissues. Although GAL-1 promotes the generation of NC cells, it may also act as a proapoptotic factor for other cells in NC cell development.

The effects of GAL-1 may exhibit developmental stage dependency in NC generation. The presence of GAL-1 on days 0-4 or days 10-12 of the culture more efficiently induced SOX10+ NC-like cells from ES cells (Fig. 2B). qPCR showed that the expression of the early neuronal marker *Nestin* increased on day 6 of the culture, while that of the spinal cord marker *Hoxb9* and NC cell marker *Sox10* increased on day 8 of the culture in the presence of GAL-1 (Fig. 3). These results demonstrated that GAL-1 affects the generation of NC cells at early and late developmental

stages: GAL-1 enhanced the generation or proliferation of neural ectoderm cells at the early stages, and promoted the generation of NC cells themselves from the neural ectoderm at the late stages. The enhancement of neural ectoderm cell generation is supported by previous studies showing that GAL-1 is expressed in neural stem cells in the subependymal zones of the brain and promotes the proliferation of neural stem cells in the adult brain (Sakaguchi *et al.*, 2006).

Our results also showed that GAL-1 was more effective at the very early stage of NC cell generation. Fig. 5B and 5C show that the expression of the NC specifiers *Foxd3*, *Sox9*, and *Sox10* was markedly increased in E9.5 neural tube explants cultured with GAL-1, while their expression did not significantly change between E10.5 neural tube explants cultured with or without GAL-1, suggesting that GAL-1 promotes the generation of NC cells at the early stage. The expression of *Snail*, a marker of epithelial-mesenchymal transition (EMT), was markedly increased in explanted E9.5 neural tubes cultured with GAL-1 (Fig. 5B). It is possible that GAL-1 also promotes EMT at the early stage of NC generation.

In order to examine the role of the carbohydrate-binding activity of GAL-1 in the generation of NC cells, we added lectin-incompetent rhGAL-1/Ox and lectin-competent CSGAL-1 to the culture for NC-like cell generation. rhGAL-1/Ox and CSGAL-1 enhanced the generation of SOX10+ NC-like cells; however, no significant difference was observed between them (Fig. 4). These results suggest that the lectin activity of GAL-1 is not important for the generation of NC cells. Oxidized GAL-1, which lacks lectin activity, promoted axonal regeneration at lower concentrations than those maintaining lectin activity (Outenreath and Jones, 1992, Puche *et al.*, 1996).

Oxidized GAL-1 has been suggested to act on nervous system tissues not as lectin, but as a cytokine (Sango *et al.*, 2004). GAL-1 may also play a role in NC cell generation as a cytokine. On the other hand, a recent study showed that a GAL-1 N46D mutant, which attenuated glycan-binding activity, had similar functions to wild-type GAL-1 in mammary gland branching (Bhat *et al.*, 2016). The nuclear localization of the GAL-1 mutant was required for branching; however, the molecular mechanisms responsible for the nuclear localization of GAL-1 remain unknown. In order to enhance NC cell generation, GAL-1 may enter the cytoplasm and function in the nucleus. An analysis of the localization of GAL-1 in cells is needed in order to elucidate the mechanisms underlying it functions in NC cell generation. In contrast, CSGAL-1 did not alter NC cell generation in explanted neural tube cultures (Fig. 5B, C). The explanted neural tube consists of various types of cells, and is a more heterogeneous environment than the ES cell culture system. In the neural tube culture, CSGAL-1 may be sequestered by extracellular matrix (ECM) glycoproteins and cell-surface carbohydrates in a heterogeneous environment before inducing NC cell generation. CSGAL-1 may also be exhausted by the differentiation of other cells, not by NC cell generation. A previous study reported that reduced GAL-1, the intra-molecular Cys-Cys bonds of which were inhibited similar to CSGAL-1, induced astrocyte differentiation in a carbohydrate-dependent manner (Sasaki *et al.*, 2004).

Homozygous mutant animals that lack *Gal-1* are viable, fertile, and develop normal NC cell derivatives (Poirier and Robertson, 1993). The absence of any NC cell abnormalities in mutant animals suggests that other factors potentially compensate for the absence of GAL-1. Fifteen mammalian Galectins have been identified to date (Camby *et al.*, 2006). The results of our inhibition experiment on GAL-1 in the ES cell culture appear to support this idea (Fig. 2C). Although GAL-1 may not play an essential role in NC cell development, further studies on the mechanisms underlying its functions in NC cells may open new avenues in basic developmental biology research.

Materials and Methods

Mice

Sox10-IRES-Venus mice (Motohashi *et al.*, 2011) were maintained in our animal facility. Noon of the day the vaginal plug was detected was designated day 0.5 of gestation (E0.5). The developmental stages of embryos were judged by their morphological appearance, as described in "The Mouse" (Rugh, 1990). All animal experiments were performed in accordance with the Regulations of Animal Experiments in Gifu University.

Culture of embryonic stem cells

The maintenance of ST2 cells and ES cells and differentiation of NC-like cells from ES cells were described previously (Motohashi *et al.*, 2007). The day when ES cells were seeded onto ST2 monolayers was defined as day 0. GAL-1 (Abcam) or rhGAL-1/Ox and CSGAL-1 (gifted from Dr. Kadoya) was also present in each experiment. Regarding GAL-1 inhibition, anti-GAL-1 (gifted from Dr. Kadoya) or control IgG (Biolegend) was used for each culture.

Culture of neural tubes

E9.5 and E10.5 embryos were incubated in 0.75 mg/ml collagenase (Wako) at room temperature for 20 min. After washing, each embryo was dissected from the region corresponding to the end of the branchial arches and the cranial region was discarded. The embryo was dissected rostral to the forefeet with fine scissors. The neural tube was removed from the trunk region of the embryos, and directly inoculated into dishes coated with poly-D-Lysine (Biomedical Technologies Inc.) and human plasma fibronectin (GIBCO) and containing a 5:3 mixture of DMEM-low: neuro-basal medium (GIBCO) supplemented with 15% Chick Embryo Extract (CEE), 1% N2 supplement (GIBCO), 2% B27 supplement (GIBCO), 50 μM 2-mercaptoethanol (Sigma), 35 ng/ml all-trans retinoic acid (Sigma), 20 ng/ml IGF-1 (R&D systems), and 20 ng/ml bFGF (R&D Systems) (Morrison *et al.*, 1999). After 1 day of the culture in 5% CO_2 at 37°C, 25 ng/ml rhGAL-1/Ox or CSGAL-1 was added to the culture, and after one more day, flow cytometry or a gene expression analysis was performed.

Flow cytometry

A flow cytometric analysis and cell sorting were performed as previously described (Motohashi *et al.*, 2007, Motohashi *et al.*, 2011). Briefly, ES cells cultured for 9-12 days or explanted neural tubes were dissociated with 0.05% trypsin/EDTA (GIBCO). After washing with staining medium (SM: PBS containing 3% FSC), cells were suspended in SM containing 3 μg/ml propidium iodide (PI) (Calbiochem) to eliminate dead cells. All cell sorting and analyses were performed using a FACS Vantage (Becton-Dickinson).

Immunohistochemical analysis

An immunohistochemical analysis was performed as previously described (Motohashi *et al.*, 2011). In the immunostaining of embryos, E9.5 embryos were fixed in 4% paraformaldehyde/PBS at 4 °C for 3 h, and subsequently immersed in 30% sucrose/PBS at 4 °C overnight. Embryos were embedded in OCT (Sakura Finetechnical), sectioned at a thickness of 10 μm, and placed on tissue-adhering slides. The antibodies used for immunohistochemistry were as follows: rabbit anti-galectin-1 (1:200, gifted from Dr. Kadoya), rat anti-GFP (1:200, Nacalai), mouse anti-mouse neuronal class III β-tubulin (1:500; TuJ-1, COVANCE), rabbit anti-mouse glial fibrillary acidic protein (GFAP, 1:500; Z0334, DakoCytomation), Texas Red-conjugated anti-mouse IgG (1:500; Molecular Probes), Texas Red-conjugated anti-rabbit IgG (1:1000; Life Technologies), Alexa Fluor 488-conjugated anti-rat IgG (1:1000; Invitrogen), and Alexa Fluor 488-conjugated anti-rabbit IgG (1:500; Molecular Probes).

RT-PCR and qPCR analyses

The purification of total RNA, first-strand cDNA synthesis, and PCR reactions were performed as previously described (Motohashi *et al.*, 2011). qPCR was performed under standard conditions using a Thermal cycler Dice Real time system (TAKARA). Samples were run in triplicate for each probe and quantification was based on ΔΔCT calculations. Samples were normalized to β-actin as loading controls and calibrated to wild-type levels. Primers are listed in Supplementary data.

Microarray analysis

Total RNA was prepared as duplicate sample sets from isolated SOX10+ NC-like cells using Trizol-LS Reagent (Invitrogen). RNA samples from Sox10-IRES-Venus ES cells without a differentiation culture were used as the control. SOX10+ NC-like cells were sorted twice with FACS from the Sox10-IRES-Venus ES cell culture. Total RNA prepared from SOX10+ NC-like cells, and the synthesis of aRNA were performed using the Amino Allyl aRNA kit (Ambion) from total RNA. aRNAs were labeled with Cy5 using Cy5-Mono-Reactive Dye (Amersham) and hybridized with the mouse oligo chip 24K 3D-Gene (Toray) using the supplier's protocol. Detected signals were scanned using ScanArray Lite (Perki-nElmer) and the signal for each gene was normalized by the global normalization method.

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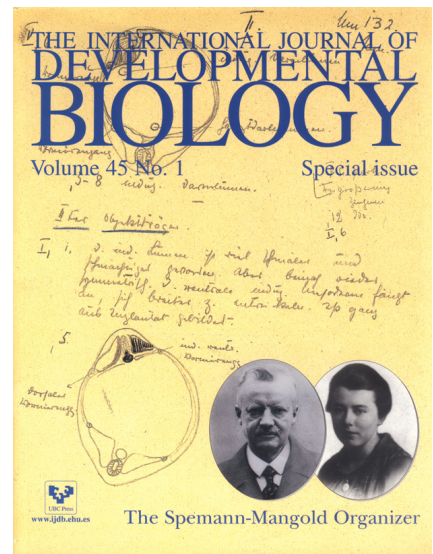**Matrigel supports neural, melanocytic and chondrogenic differentiation of trunk neural crest cells**
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