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Change in the Gastro-Intestinal Tract by Overexpressed Activin Beta A

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Originally, activins were identified as stimulators of FSH release in reproduction. Other activities, including secondary axis formation in development, have since been revealed. Here, we investigated the influence of activin β_A on the body, including the gastro-intestinal (GI) tract.

Initially, the activin β_A protein was detected in the serum proportional to the amount of pCMV-rAct plasmid injected. The induced level of activin β_A in muscle was higher in female than male mice. Subsequent results revealed that stomach and intestine were severely damaged in pCMV-rAct-injected mice. At the cellular level, loss of parietal cells was observed, resulting in increased pH within the stomach. This phenomenon was more severe in male than female mice. Consistent with damage of the stomach and intestine, activin β_A often led to necrosis in the tip of the tail or foot, and loss of body weight was observed in pCMV-rAct-injected male but not female mice. Finally, in pCMV-rAct-injected mice, circulating activin β_A led to death at supraphysiological doses, and this was dependent on the strain of mice used.

Taken together, these results indicate that activin β_A has an important role outside of reproduction and development, specifically in digestion. These data also indicate that activin β_A must be controlled within a narrow range because of latent lethal activity. In addition, our approach can be used effectively for functional analysis of secreted proteins.

INTRODUCTION

Activin and inhibin, members of the TGF-β superfamily (Ling et al., 1986), were first identified as gonadal protein hormones that regulate the synthesis and secretion of follicle stimulating hormone (FSH) in the pituitary gland (Ling et al., 1985). Activin and inhibin are generated through the combinatorial assembly of an α subunit and one of two highly related β subunits, β_A or β_B, to generate inhibin A ($\alpha\beta_A$), inhibin B ($\alpha\beta_B$), activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$), and activin AB ($\beta_A\beta_B$). Activin β_C, β_D, β_E chains (Hotten et al., 1995; Oda et al., 1995; Vale et al., 1994), and partially characterized activin AC ($\beta_A\beta_C$) and activin BC ($\beta_B\beta_C$) proteins have also been reported (Fang et al., 1996).

The best known functions of activin are in the reproductive organs. The endocrine function of activin was first inferred from the correlation between high activin and elevated FSH in the mid cycle and luteo-follicular transition period (Muttukrishna et al., 1996). The endocrine function of activin has also been confirmed by the fact that activin β_A induced intramuscularly increases FSH in an endocrine fashion during the estrous cycle (Kim et al., 2008). In addition, the autocrine function within reproductive organs has been inferred from the observation that antibodies to activin B suppress FSH secretion from cultured rat pituitary cells (Corrigan et al., 1991).

Outside the gonads, a main reproductive organ, activin β_A has been reported to be involved in the regulation of the GI tract (Fukamachi et al., 2013; Li et al., 1998) and a GI cancer cell line (Kaneda et al., 2011; Kim et al., 2006; 2009). In inhibin-deficient mice, supraphysiological levels of activins block differentiation of preparietal to acid-producing parietal cells. Activin receptor II mRNA is normally present in pit, parietal, and zymogenic cells (Li et al., 1998). Within the GI tract, activin A regulates growth of GI epithelial cells by mediating epithelial-mesenchymal interaction (Fukamachi et al., 2013). In gastric cancer cell lines, activin inhibits cell growth through apoptosis (Kim et al., 2006; 2009) and vascular endothelial cell growth (Kaneda et al., 2011).

Similar to the cell proliferation in GI tract, functions of activin are also known in other cells. In the ovary, activin is involved in granulosa cell proliferation through Cyp26b1 gene expression and retinoic acid regulation (Kipp et al., 2011). In cancer cells, activin A inhibits the proliferation of breast cancer T47D cells by enhancing the expression of p15 cyclin-dependent kinase inhibitors, and the overexpression of activin A in human prostate cancer LNCaP cells inhibits proliferation, induces apoptosis, and decreases the tumorigenicity of these cells (Burdette et al., 2005; Zhang et al., 1997). Activin A has been reported to be an essential growth factor involved in embryonic stem cell renewal and pluripotency (Jiang et al., 2007; Xiao et al., 2006).

Studies of activin on *in vivo* cell proliferation, however, have been very limited. Initial studies were done intermittently using embryos and *in vitro* culture of eggs. Activin has been reported to be expressed in early pre- and post-implantation mouse embryos (Albano et al., 1993; Manova et al., 1992; Mellor et al.

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2000) and involved in the formation of the mesoderm (Feijn et al., 1994) and secondary body axes in chicks (Thomsen et al., 1990), zebrafish (Mitrani et al., 1990), and amphibians (Schulte-Merker et al., 1992). Activin A increases the rate of morula formation and velocity of embryonic cleavage in mice (Orimo et al., 1996). More recent studies have been done using gene disruption or transgenic animal approaches. However, there are few reports of the overall effects of activin, including cell proliferation, since perinatal lethality and early embryonic lethality have been observed (Matzuk et al., 1995; Tanimoto et al., 1999). In later organogenesis, activin β_A has been reported to be associated with craniofacial development (Matzuk et al., 1995).

Our approach using intramuscular injection of naked plasmid bypasses the fundamental problems that come from perinatal lethality or early embryonic lethality (Ko et al., 2003). In this study, we report distinct activities of activin on the GI tract.

MATERIALS AND METHODS

Animals and experimental design

ICR and BALB/c mice at 2 months of age were purchased from DBL (Korea) and maintained under 14 h light, 10 h dark illumination at 23°C, with food and water available *ad libitum*. Plasmid DNA, pCMV-rAct, a 1.5-kb rat activin cDNA digested with *Eco*RI was cloned into the *Eco*RI site of the pcDNA3 vector (Invitrogen, USA), which contains a CMV early promoter and a bovine growth hormone polyadenylation site (Fig. 1A), as previously described (Kim et al., 2008). The pCMV-rAct plasmid was purified and injected as previously described (Kim et al., 2008; Ko et al., 2003).

The basic protocol involved double injections with a 7-day interval and euthanasia of both female and male mice 4 days later. In females, the first injection was done 10:00 A.M. at diestrus II of the third cycle after confirmation of the two consecutive normal estrous cycles, which normally reveals a lower level of FSH in females. Estrous cycle stages were determined by daily examination of vaginal cytology at 9:30 A.M. To measure activin β_A protein levels, a single injection of 300 μg pCMV-rAct in 50 μl of 10% sucrose in saline was performed at 10:00 A.M. in males, and serum was harvested 4 days after injection (Fig. 1B). For the study of differential expression between the sexes, the site of injection was marked with stitching using cotton thread. In female, a single injection of 300 μg pCMV-rAct in 50 μl of 10% sucrose in saline was performed at 10:00 A.M. on diestrus II after two normal consecutive estrous cycles. The muscle of the marked region was harvested at 10:00 A.M. at diestrus II after 4 days. In males, a single injection of 300 μg pCMV-rAct was performed at 10:00 A.M. and muscle was harvested at 10:00 A.M. after 4 days. After obtaining the muscle, the same quantity of muscle was used for Western blot (Fig. 2). For stomach and liver studies, mice were sacrificed 4 days after the second injection (Figs. 3 and 4; Table 1). For the necrosis study, mice were observed every day after the second injection until 8 months (Fig. 5). For the body weight study, body weight was measured after the second injection until day 15 (Fig. 6). For the survival study, various amounts of plasmid DNA were

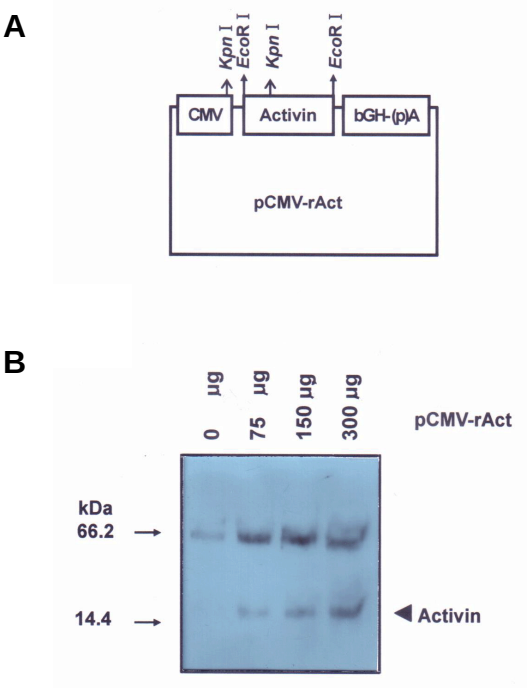

Fig. 1. pCMV-rAct structure and expression of pCMV-rAct. (A) Diagram of the pCMV-rAct construct. Functional elements include the cytomegalovirus (CMV) promoter, the rat activin cDNA, and the human growth hormone (hGH) poly(A) signal. (B) Protein blot analysis was performed as described in the "Materials and Methods". Proteins were obtained after injection of the different doses of pCMV-rAct into mice. The expression of activin β_A was proportional to the injected amount of pCMV-rAct. The Western blot shown is representative of results obtained from four independent experiments. pCMV-rAct: pCMV-rAct-injected mice

Table 1. Changes in pH

	pH	2-3	6-7
Male (N = 34)	Control	97.1	2.9
	pCMV-rAct	2.9	97.1*
Female (N = 13)	Control	100.0	0.0
	pCMV-rAct	15.4	84.6*

pH of the stomach was measured with a Biobasic pH meter (Fisher Scientific Company). Numbers indicate the percentage of mice in each pH category. N means the number of mice tested. *$P < 0.001$.

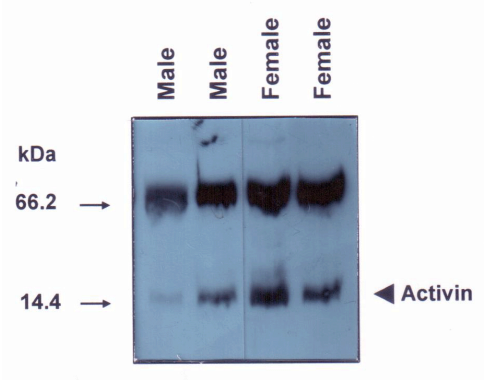

Fig. 2. Level of activin β_A in serum. Proteins were obtained after injection of pCMV-rAct into male or female mice. The Western blot was performed as described in the "Materials and Methods". The protein level was higher in females than males. The results shown are representative of results obtained from four independent experiments.

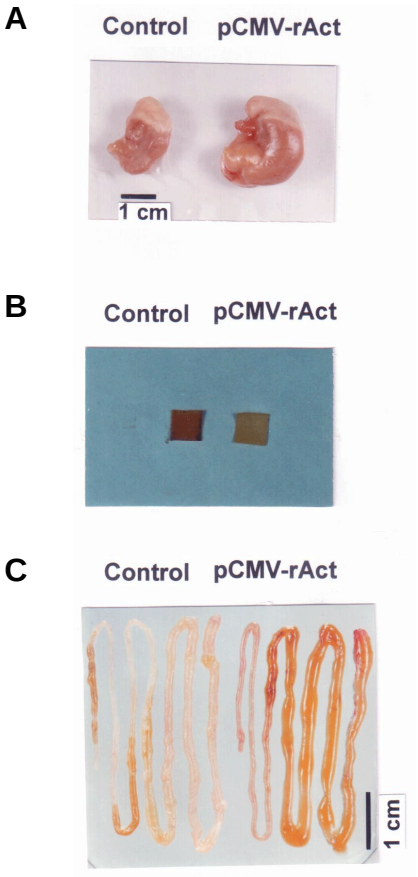

Fig. 3. Morphology of the inflated stomach and intestine. (A) Gross morphology of the stomach. (B) Change in pH of the stomach contents. Red, indicating acidity, was observed in the control mouse, whereas yellow, indicating neutrality, was observed in the pCMV-rAct-injected mouse. (C) Gross morphology of the intestine.

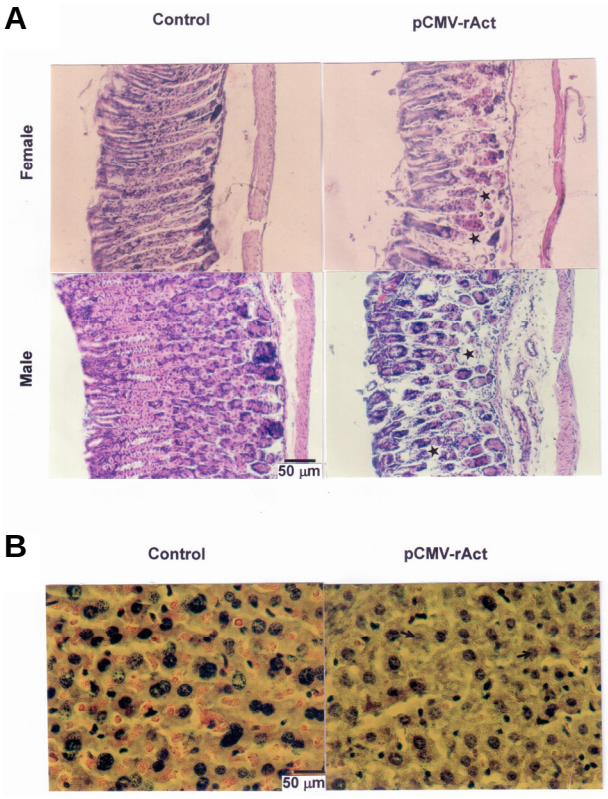

Fig. 4. Histology of the stomach and liver. (A) Histology of the stomach in male and female mice. Tissues were prepared as described in "Materials and Methods" and observed at 40x magnification. Asterisks indicate the region of cell loss (B). Histology of the liver. Tissues were observed at 150x magnification. Note that the less dense nucleus (→) was largely observed in the pCMV-rAct-injected mouse.

injected twice into male mice, and the mice were observed carefully every day at 10:00 A.M. until day 13 (Fig. 7). In the case of the sensitive mouse strain, BALB/c, a single injection of 100 μg of pCMV-rAct was administered, and mobile activities were observed after injection (Fig. 8). All experiments were performed at least four times if not otherwise noted, and representative results are shown.

Protein blot analysis

Muscle tissue was removed, homogenized in 400 μl of protein extraction buffer [0.1 M NaCl, 0.01 M Tris-Cl (pH 7.6), 1 mM EDTA (pH 8.0), 0.1% TritonX-100, 1 μg/ml aprotinin, and 100 ng/ml phenylmethylsulfonyl fluoride], and centrifuged four times. The homogenates were mixed with an equal volume of 2X SDS loading buffer [100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% BPB, and 20% glycerol], placed in boiling water for 10 min, and centrifuged. The supernatants were transferred to fresh tubes. Samples of each extract containing 10 μg of protein were heated at 70°C for 10 min, electrophoresed on a 12% acrylamide gel, and transferred onto Nytran filters in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol). The blots were incubated overnight in blocking solution (5% non-fat dried milk, 0.02% sodium azide, and 0.02% Tween) with shaking at 4°C, followed by exposure to primary activin β_A antibodies (1:400; Serotec, UK) overnight. They were washed in milk-TBS-Tween for 30 min and incubated with secondary anti-rabbit Ig horseradish peroxidase-linked whole donkey antibody (1:100; Amersham Pharmacia Biotech, USA) in azide-free blocking solution [5% non-fat dried milk, 150 mM NaCl, and 50 mM Tris-Cl (pH 7.5)] for 2 h. The secondary antibody-specific signal was detected with an ECL kit (Amersham Pharmacia Biotech). For serum measurement of activin β_A or FSH, one microliter of serum was obtained, electrophoresed, and Western blot analysis was performed, using primary activin β_A antibodies (Serotec) or primary FSH antibodies (1:750; Serotec).

General behavior

Mice were maintained as described in the "Animals and experimental design" section. Their general behavior was observed carefully every day at 10:00 A.M. until 13 days after the single injection of pCMV-rAct. Pictures were captured using a digital camera (Sony DSC-F717, Japan).

Histology

The gross appearance of excised tissues from injected and control mice were examined, and the tissues were immediately fixed in fresh 4% paraformaldehyde in PBS at pH 7.4. Following overnight fixation, tissues were dehydrated in ethanol, embed-

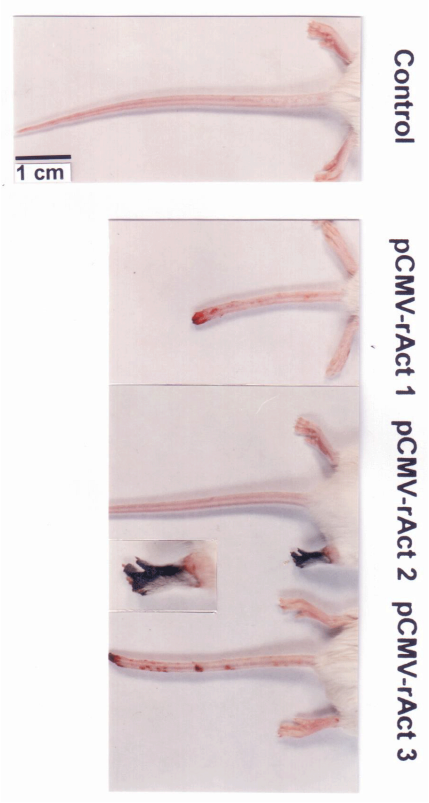

Fig. 5. Necrosis in the pCMV-rAct-injected mice. Necrosis was observed in the tail or foot of the pCMV-rAct-injected mice. Three examples are shown. The foot of the mouse on the left (pCMV-rAct 2) is magnified in the right inset.

ded in paraffin, and sectioned at 7 μm with a microtome (Leica RM2235, Switzerland). The sections were de-paraffinized with xylene, dehydrated in absolute ethanol, and rehydrated in water. Sections were stained with hematoxylin, counterstained with eosin, and observed under a light microscope (Olympus IX70, Japan) or a stereomicroscope (Leica ME Apo, Switzerland).

Measurement of pH

For the pH measurement, the stomach was dissected, placed into a tube, centrifuged at 5000 rpm for 30 sec, and the pH of the supernatant was measured with both a pH meter (Fisher Scientific Company, USA) and alkacid test ribbons (Fisher Scientific Company).

Statistical analysis

For the statistical analysis, Student's *t* test was used for single comparisons at $\alpha = 0.01$. Statistics were performed no less than on four independent experiments.

RESULTS

Induced activin β_A in muscle and blood

In a previous paper (Kim et al., 2008), we reported that activin β_A mRNA was detected in muscle by RT-PCR, and activin β_A protein in blood was detected by Western blot analysis. In this study, we reconfirmed the presence of protein activin β_A in muscle and blood. Initially, we observed that mature activin β_A

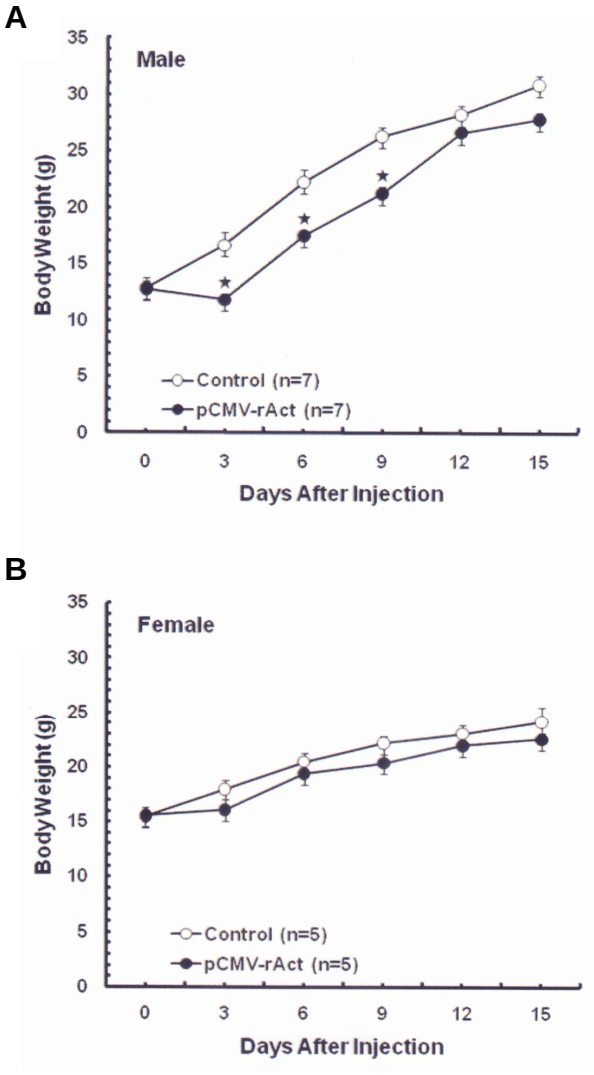

Fig. 6. Changes in body weight. Changes in the body weight of the control and pCMV-rAct-injected male ($n = 7$) (A) and female ($n = 5$) (B) mice. Asterisks denote values that are significantly different from the control mean values (Student's *t*-test at each point, $P < 0.01$). Values shown are means ± the standard deviation.

protein (14 kDa) was synthesized and secreted into the blood proportionally to the injected amount of plasmid (Fig. 1B). This suggests that an adjustable amount of protein in blood can be induced for certain proteins. In the same context, we again detected the expression of activin β_A in muscle of both sexes in order to compare the differential expression between male and female mice. The expression level of activin β_A was higher in female than in male mice after the same dose of pCMV-rAct plasmid was injected into both sexes (Fig. 2).

Damage in the stomach and intestine by induced activin β_A

When the effects of overexpressed activin β_A on various organs were investigated, the stomach of mice revealed substantial and pathological damage (Fig. 3). In males, there was a dysmorphology of the stomach, which appeared to be inflated with gas (Fig. 3A), and an increased stomach pH (Fig. 3B) in pCMV-

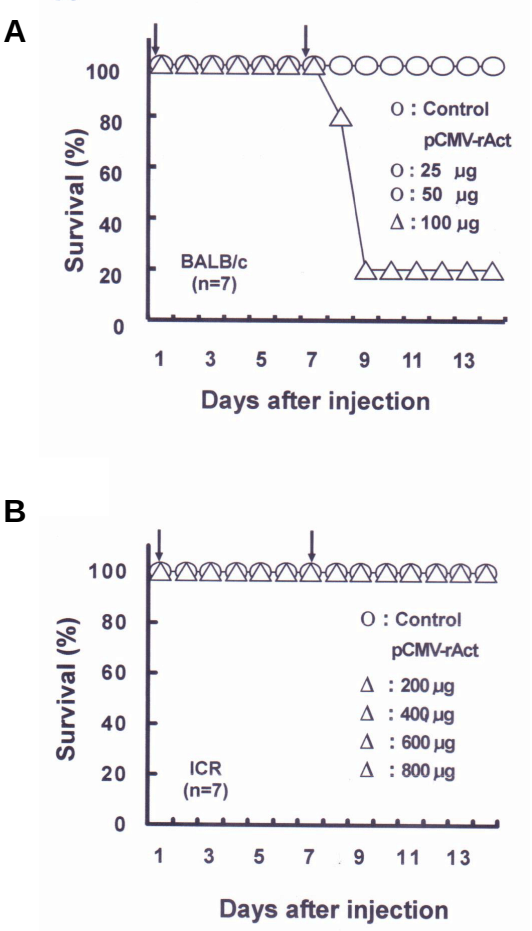

Fig. 7. Survival curves of pCMV-rAct-injected mice. Survival curves for the BALB/c (A) and ICR (B) mouse strains after injection with the indicated amounts of pCMV-rAct at the indicated days. Live mice, starting with seven in each group, were counted every day. ↓: indicates the injection day.

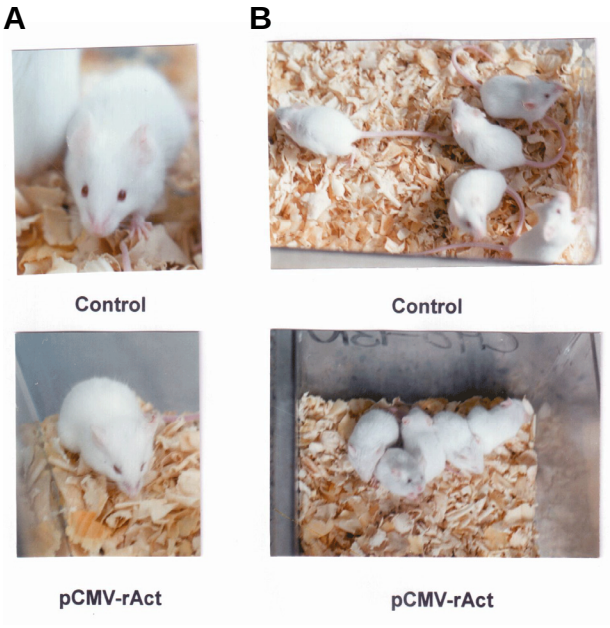

Fig. 8. The macroscopic phenotype of pCMV-rAct-injected mice. Closed eyes were observed in the mouse shown in (A) whereas reduced mobile activity was found in the group of pCMV-rAct - injected mice shown in (B).

rAct-injected mice compared to controls was observed. This pH increase was observed in 97.1% and 84.6% of male and female mice, respectively (Table 1). It was also observed that the taken food remained inside the stomach. Moreover, an inflated intestine with yellow color was observed (Fig. 3C). The intestine was examined because the pH gradient throughout the digestive system is a well-known phenomenon. According to the morphological changes in stomach, we investigated the detailed histology. At the cellular level, loss of parietal cells was observed in the stomach lining, and this was more severe in male than female mice injected with pCMV-rAct (Fig. 4A). When we investigated the effect of activin β_A on the liver, cells in pCMV-rAct-injected mice revealed a seemingly more fragile nucleus (Fig. 4B). However, no significant difference was observed. When we investigated major urine protein (MUP), a physiological marker in the liver, no difference was observed (data not shown).

Necrosis, loss of body weight, and lethality by induced activin β_A

In the context of damage to the internal organs, such as stomach and intestine, research was extended to related changes in the body. Interestingly, necrosis of the foot or the tip of the tail was often observed in pCMV-rAct-injected mice two or three months later (Fig. 5). This phenomenon was wholly unexpected. In the same context, we also investigated body weight changes in both sexes after pCMV-rAct injection. As a result, there was a transient decrease in body weight of pCMV-rAct-injected male mice (Fig. 6A), but not female mice (Fig. 6B), revealing differential effects of activin β_A on body weight between the sexes. Finally, we further investigated the functional consequences of activin β_A overexpression on survival level. When pCMV-rAct at more than 100 μg was used, BALB/c mice died after a double injection of pCMV-rAct (Fig. 7A). However, ICR mice did not die even when 800 μg of pCMV-rAct was injected (Fig. 7B), suggesting that BALB/c mice are much more sensitive than ICR mice in their response to activin β_A. Due to the high sensitivity to activin β_A in BALB/c mice, a single injection of 100 μg of pCMV-rAct was administered. Following a single injection of plasmid, all mice survived. However, the eyes of individual BALB/c mice exhibited abnormalities, with the eyelids being almost closed (Fig. 8A), and groups of mice within the cage congregated in the corner without obvious mobile activity (Fig. 8B).

DISCUSSION

As described previously (Ling et al., 1985), activin was first identified as a gonadal protein hormone that regulates the synthesis and secretion of FSH in the pituitary gland. Activin has also been known to be important in the embryonic development (Matzuk et al., 1995; Tanimoto et al., 1999). However, it is still unclear whether activin really acts in an endocrine fashion in the reproductive axis and in embryonic development. Related

with this, our two reports support the endocrine manner of activin β_A in the reproductive axis and digestive system. The digestive system is a recently elucidated area influenced by activin (Fukamachi et al., 2013; Li et al., 1998). First, our previous report demonstrated that activin β_A influences the estrous cycle, an integral part of reproduction, in females in an endocrine manner (Kim et al., 2008). Second, our present results reveal that activin β_A can exert profound effects on digestion in an endocrine fashion. Overexpressed activin β_A directly and harmfully influences the GI tract, suggesting that activin β_A must be maintained within a narrow physiological range.

When we examined internal organs after pCMV-rAct injection, we observed that the stomach and intestine were severely damaged. It can be inferred that the reduced production of H^+ in the parietal cells induced this change in the luminal environment of the stomach and intestine. This overall change in the shape of the stomach and intestine by activin is expected, since supraphysiological levels of activin (over 10-fold) block the differentiation of multiple gastric epithelial lineages, including parietal cells (Li et al., 1998). However, the change in the GI tract occurred at the overexpressed level of activin β_A (2-3-fold) in our study, which is far below the supraphysiological level, as indicated in Fig.1B. This means that the stomach and intestine are very sensitive to the level of activin β_A.

At the cellular level, the loss of parietal cells was clearly observed. Interestingly, the loss of parietal cells was more severe in males than females in Fig. 4A, although the actual level of activin β_A in muscle and blood was higher in females than males, as indicated in Fig. 2. One possible explanation is that available activin β_A was diminished in females because activin binding proteins such as follistatin might be higher in females than males. Conversely, an abnormality of liver cells was not clearly observed in this study. This is in contrast with results obtained in inhibin α-deficient mice (Matzuk et al., 1994). The reason also appeared to be the lower levels of activin β_A in this experiment. With respect to cell proliferation, activin is known to block the differentiation of gastric epithelial cells (Li et al., 1998) and to inhibit cell growth in normal gastric cells (Fukamachi et al., 2013) and gastric cancer cells (Kaneda et al., 2011; Kim et al., 2006; 2009). Our study revealed that certain cells, but not all cells, are very sensitive to activin β_A.

In contrast to the changes observed in internal organs, external tissue changes, such as tail and foot necrosis, were unexpectedly observed. Necrosis was often observed as diabetic foot ulcers in terminal tissue of the diabetic patient (Jude et al., 2002). In diabetic foot ulcers, it has been shown that TGF-β is involved (Jude et al., 2002). TGF-β and activin β_A belong to the TGF-β superfamily. Although a similar phenotype in foot and tail ulcers was observed, the cause of the ulcers seemed to be different. In our study, severe damage to the stomach and intestine that resulted from activin β_A overexpression is likely to impact food absorption, leading to necrosis of the tail or even the death of these animals. Thus, tail or foot ulcers in our study appear to come from nutrition shortage due to the damaged digestive system, whereas diabetic ulcers result from a damaged insulin system. However, the mechanism would converge at the point of glucose shortage. Another minor possibility is that induced activin β_A might directly hurt the pancreas, and this possibility requires more studies. The ability of activin β_A to cause tissue necrosis or even death is unlikely to be related to its role in enhancing FSH secretion, since FSH-overexpressing transgenic mice do not exhibit any such defects (Kumar et al., 1992; 1999).

In addition to the internal and external changes of the body, integration of every change of the body by activin β_A was investigated at the organism level. First, the effect of activin β_A on the body weight of the ICR strain was sex-dependent. The body weight of males was more affected than female mice (Fig. 6). The loss of body weight was well-correlated with changes in parietal cells (Fig. 4A), suggesting that parietal cell loss leads to a nutritional defect. The greater loss of parietal cells in males was explained in Fig. 4A. The overall loss of body weight is consistent with the result of the previous report in inhibin α-deficient mice, which have a 10-fold elevation of activin β_A levels (Matzuk et al., 1992). Second, the effect of activin β_A on survival was strain-dependent. In terms of survival, BALB/c mice were more sensitive to activin β_A than ICR mice, as observed in Fig. 7. In fact, the body weight of BALB/c mice was about 10 g smaller than the ICR mice (15g) in adults. Thus, the sensitivity to activin β_A was about five-fold higher in BALB/c than ICR mice, when body weight was considered. Activin β_A seemed to be the primary cause of death, because mice died in proportion to the amount of plasmid DNA injected (Fig. 7), which correlated with the amount of activin β_A produced (Fig. 1B). Our results demonstrate that overexpression of activin β_A can cause lethality in adult mice, as might be expected from previous findings (Matzuk et al., 1995). However, the mechanism of lethality might be different, since our transient transgenic mice received less accumulated activin β_A at lower levels than normal transgenic mice. Third, the effect of activin β_A on behavior was also strain-dependent. BALB/c mice showed less mobility than ICR mice after injection of pCMV-rAct (Fig. 8).

These studies provide several important technical advances, as described previously (Ko et al., 2003). Briefly, our approach is relatively simple and rapid, while conventional transgenic approaches require substantial technical skill and time (Cho et al., 2001). Intramuscular injection is convenient, because the expression of targeted gene can be obtained at any time during development or in the adult animal. Intramuscular injection should have wide applicability for the screening of genetically engineered proteins for their therapeutic value or side effects *in vivo*, without the time-consuming production of transgenic mice.

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