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GATA2 functions in adrenal chromaffin cells

Tomomi Watanabe-Asaka[1] | **Moyuru Hayashi**[1] | **James Douglas Engel**[2] | **Yoshiko Kawai**[1] | **Takashi Moriguchi**[3]

[1]Division of Physiology, Tohoku Medical and Pharmaceutical University, Sendai, Japan

[2]Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, USA

[3]Division of Medical Biochemistry, Tohoku Medical and Pharmaceutical University, Sendai, Japan

Correspondence
Takashi Moriguchi, Division of Medical Biochemistry, Tohoku Medical and Pharmaceutical University, 1-15-1 Fukumuro, Miyagino-ku, Sendai 983-8536, Japan.
Email: moriguchi@tohoku-mpu.ac.jp

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Abstract

Catecholamine synthesized in the sympathoadrenal system, including sympathetic neurons and adrenal chromaffin cells, is vital for cardiovascular homeostasis. It has been reported that GATA2, a zinc finger transcription factor, is expressed in murine sympathoadrenal progenitor cells. However, a physiological role for GATA2 in adrenal chromaffin cells has not been established. In this study, we demonstrate that GATA2 is specifically expressed in adrenal chromaffin cells. We examined the consequences of *Gata2* loss-of-function mutations, exploiting a *Gata2* conditional knockout allele crossed to neural crest-specific Wnt1-Cre transgenic mice (*Gata2* NC-CKO). The vast majority of *Gata2* NC-CKO embryos died by embryonic day 14.5 (e14.5) and exhibited a decrease in catecholamine-producing adrenal chromaffin cells, implying that a potential catecholamine defect might lead to the observed embryonic lethality. When intercrossed pregnant dams were fed with synthetic adrenaline analogs, the lethality of the *Gata2* NC-CKO embryos was partially rescued, indicating that placental transfer of the adrenaline analogs complements the lethal catecholamine deficiency in the *Gata2* NC-CKO embryos. These results demonstrate that GATA2 participates in the development of neuroendocrine adrenaline biosynthesis, which is essential for fetal survival.

KEYWORDS

catecholamine, chromaffin cells, *Gata2*, neural crest

1 | INTRODUCTION

Catecholamines, including dopamine, noradrenaline and adrenaline, play a crucial role in the development and maintenance of the cardiovascular system. In sympathoadrenal cells, tyrosine hydroxylase (TH) converts tyrosine to L-DOPA, which is converted to dopamine by L-aromatic amino acid decarboxylase (AADC) (Zhou, Quaife, & Palmiter, 1995; Rahman, Nagatsu & Kato,, 1981). Subsequently, the enzymatic activity of dopamine β-hydroxylase (DBH) produces noradrenaline (Thomas, Matsumoto, & Palmiter, 1995). In adrenal chromaffin cells, phenylethanolamine N-methyltransferase (PNMT) converts noradrenaline to adrenalines (Martin, Begun, McLeish, Caine, & Grunewald, 2001). Mice deficient in either TH or DBH have diminished catecholamine levels and exhibit midgestational lethality due to cardiovascular failure (Kobayashi et al., 1995; Thomas et al., 1995; Zhou et al., 1995).

Early embryonic neural crest cells differentiate into sympathoadrenal progenitor cells in response to instructive signaling, including bone morphogenetic protein secreted from the dorsal aorta (Goridis & Rohrer, 2002). Sympathoadrenal progenitors subsequently migrate to their final destinations, including the sympathetic ganglia and the adrenal medulla, which in turn give rise to sympathetic neurons and adrenal chromaffin cells, respectively (Goridis & Rohrer, 2002).

Over the years, a series of transcriptional regulators have been identified that play a role in the development of adrenal chromaffin cells (Unsicker, Huber, Schütz, & Kalcheim, 2005). Among these transcriptional regulators is GATA3, which belongs to the GATA family of transcription factors containing two C_4 zinc fingers that serve as its DNA binding domain and recognize the cognate consensus motif (A/T)GATA(A/G) (George et al., 1994; Ko & Engel, 1993; Lakshmanan et al., 1999; Lim et al., 2000; Yamamoto et al., 1990). *Gata3*-deficient embryos die from noradrenaline deficiency around embryonic day 10.5 (e10.5) (Lim et al., 2000; Moriguchi et al., 2006; Pandolfi et al., 1995). Homozygous mutant embryonic lethality can be rescued to the perinatal stage by feeding heterozygous intercrossed pregnant dams with catecholamine intermediates, underscoring that GATA3 plays a critical role in catecholamine biosynthesis, which itself is essential for midgestational survival (Lim et al., 2000). The drug-rescued *Gata3* null-deficient perinatal embryos had far fewer adrenal chromaffin cells, indicating the essential requirement of GATA3 for chromaffin cell development (Lim et al., 2000).

A separate study has shown that GATA2 could be required for early sympathetic neuronal development in chick embryos (Tsarovina et al., 2004). However, the expression pattern of GATA2 and the roles played by GATA2 in adrenal chromaffin cell development in mammals has not been determined. In this study, we demonstrate that GATA2 is specifically expressed in murine adrenal chromaffin cells and that conditional deletion of GATA2 in adrenal medullary cells by Wnt1-Cre reduces the number of chromaffin cells. Consequently, the GATA2 conditionally mutant mice expired at around e14.5. Notably, this embryonic lethality was partially rescued by feeding pregnant dams synthetic adrenaline analogs. These results demonstrate that GATA2 participates in sympathetic catecholamine biosynthesis.

2 | RESULTS

2.1 | GATA2 is specifically expressed in adrenal medullary cells

We have previously demonstrated that GATA3 is specifically expressed in TH-positive adrenal chromaffin cells and plays a crucial role in the development and maintenance of chromaffin cells (Lim et al., 2000; Moriguchi et al., 2006). Therefore, we examined whether GATA2 and GATA3 are co-expressed in adrenal chromaffin cells. For this purpose, we employed *Gata2* GFP knock-in ($Gata2^{G/+}$) and *Gata3* LacZ knock-in ($Gata3^{Z/+}$) mice, in which GFP and LacZ reporter genes were inserted at the translational initiation site of the *Gata2* and *Gata3* genes, respectively (van Doorninck et al., 1999; Suzuki et al., 2006). The $Gata2^{G/+}$ and $Gata3^{Z/+}$ mice were intercrossed, and the recovered $Gata2^{G/+}$::$Gata3^{Z/+}$ compound heterozygous mutant mice were examined by anti-GFP and anti-β-gal co-immunohistochemical analysis. We found that GFP (GATA2) and β-galactosidase (GATA3) expression were clearly colocalized in adrenal chromaffin cells (Figure 1). This result indicates that GATA2 and GATA3 are co-expressed in adrenal chromaffin cells, implying that GATA2 could play a functional role in adrenal chromaffin cells.

2.2 | Neural crest-specific GATA2 deletion results in embryonic lethality

Gata2 null mice die at around e9.5 due to severe hematopoietic failure (Lim et al., 2012; Tsai et al., 1994), which hampers the analysis of adrenal chromaffin cells that only develop at later stages of embryogenesis. To circumvent this limitation and to address possible physiological functions of GATA2 in adrenal chromaffin cells, we employed Wnt1-Cre transgenic mice that direct Cre recombinase activity specifically in neural crest-derived cells (Wilson, Richards, Ford-Perriss, Panthier, & Murphy, 2004). When the Wnt1-Cre mice were crossed with the ROSA26 *LacZ* reporter strain, β-gal activity was specifically detected in chromaffin cells of the adrenal medulla, confirming chromaffin cell-specific Wnt1-Cre activity (Figure 2b). We then crossed the Wnt1-Cre transgene into $Gata2^{GFP/+}$ heterozygous deficient mice (Figure 2a). Thereafter, $Gata2^{GFP/+}$::Wnt1-Cre mice were crossed to $Gata2^{f/+}$ heterozygous floxed mutant mice to recover $Gata2^{GFP/f}$::Wnt1-Cre mutants (hereafter referred to as *Gata2* NC-CKO, for *Gata2* neural crest-specific conditional knockout).

No *Gata2* NC-CKO mice were recovered at weaning (175 two-week-old mice were genotyped from 32 litters; Table 1), suggesting that GATA2 deficiency in neural crest-derived tissue leads to peri- or prenatal lethality. Therefore, we conducted timed-breeding experiments and embryos were collected at e11.5, e12.5, e14.5, e16.5 and e18.5. *Gata2* NC-CKO embryos were recovered at approximately Mendelian expectation until e14.5, while no further live *Gata2* NC-CKO mutant embryos were detected from e16.5 onward (Table 1). These results indicate that most of the *Gata2* NC-CKO embryos expired between e14.5 ~ 16.5. Gross observation of the e12.5 *Gata2* NC-CKO embryos showed an almost normal gross physiological appearance, indicating that fetal hematopoiesis was largely unaffected in the *Gata2* NC-CKO embryos and that another defect likely accounted for the observed mid-embryonic lethality from defective neural crest-derived tissue (Figure 2c,d).

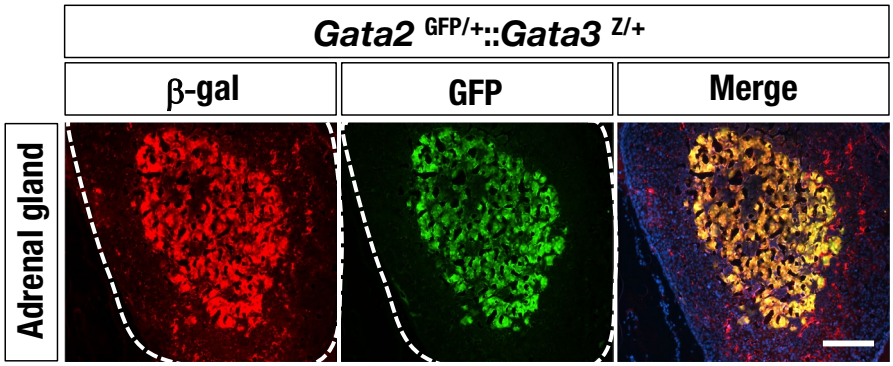

FIGURE 1 GATA2 and GATA3 expression in the adrenal medulla. The GFP (i.e., GATA2) and β-galactosidase (i.e., GATA3) expression were colocalized in the adrenal medulla cells of *Gata2*$^{GFP/+}$::*Gata3*$^{Z/+}$ compound heterozygous mutant mice (2-week-old). Scale bar: 100 μm

2.3 | Reduced size of adrenal gland in Gata2 NC-CKO

Gata3-null mutant embryos succumb to noradrenaline deficiency during the mid-gestational period (Lim et al., 2000). Given the colocalization of GATA2 and GATA3 in chromaffin cells (Figure 1), we speculated that *Gata2* NC-CKO mice might also die from similar catecholamine deficiency. To address this hypothesis, we histologically assessed the adrenal glands of *Gata2* NC-CKO embryos at e14.5. In the HE-stained adrenal gland sections, *Gata2* NC-CKO had a reduced number of basophilic medullary cells (Figure 3b,d), while littermate controls had more abundant medullary cells (Figure 3a,c). Chromogranin A, a neuroendocrine marker for adrenal chromaffin cells, was decreased in the *Gata2* NC-CKO embryos in comparison with the controls (Figure 3e,f). Consequently, the size of the adrenal gland was reduced in the *Gata2* NC-CKO embryos compared with the control embryos (compare Figure 3a,b). These results suggest that GATA2 plays a role in the development and/or maintenance of adrenal chromaffin cells.

2.4 | Diminished adrenal chromaffin cells in Gata2 NC-CKO

Tyrosine hydroxylase (TH) serves as the rate-limiting enzyme in the catecholamine biosynthetic pathway and is specifically expressed in adrenal chromaffin cells (Unsicker et al., 2005). We found that TH-positive chromaffin cells were significantly decreased in the adrenal gland of *Gata2* NC-CKO embryos, while the control littermates had more abundant TH-positive chromaffin cells (Figure 4a-d). Quantification of the TH-immunoreactive surface area and

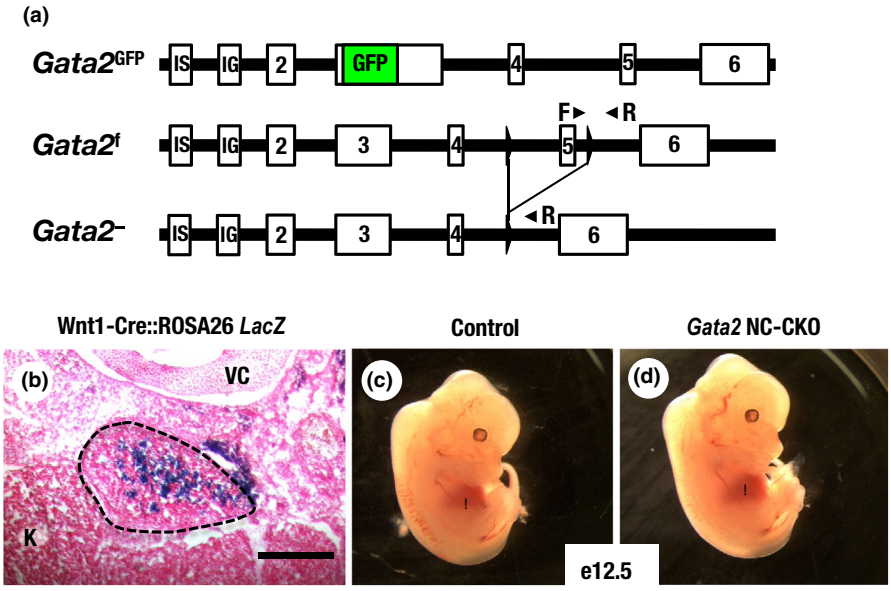

FIGURE 2 (a) Schematic diagram of *Gata2*^{GFP}, *Gata2*^{f} and recombined *Gata3*$^{-}$ null alleles. *Gata2*^{GFP} functions as a null allele. Primers used to detect the *Gata2*^{f} allele are depicted. (b) Activity of Wnt1-Cre indicated by LacZ expression (blue signal) is detected in the adrenal chromaffin cells of Wnt1-Cre::ROSA26 *LacZ* e12.5 embryos. The dotted line indicates the outer margin of the primordial adrenal gland. VC; vertebral cartilage; K, kidney. (c and d) Gross observation of Gata2$^{GFP/f}$::Wnt1-Cre (*Gata2* NC-CKO) and control littermate embryos at e12.5. *Gata2* NC-CKO embryos show normal outer appearance. Asterisks indicate fetal liver. Representative images from analysis of three e12.5 embryos in each group are depicted. Scale bar: 100 μm

the immune-fluorescence intensity in serial sections of these embryonic adrenal glands showed a significant reduction in the *Gata2* NC-CKO embryos in comparison with the control littermates (Figure 4e). These results suggest that GATA2 is important for the development of chromaffin cells and maintenance of the TH expression in the mouse adrenal gland.

TABLE 1 Genotyping frequency of *Gata2* NC-CKO progenies from *Gata2*$^{G/+}$::Wnt1-Cre and *Gata2*$^{f/+}$ intercrosses

Stage	Number of *Gata2* NC-CKO observed (expected)	Number of littermates
E11.5	2 (2)	16
E12.5	3 (2.5)	20
E14.5	3 (4)	32
E16.5	0 (1.9)	15
E18.5	0 (2.25)	18
P14	0 (21.9)	175

Note: The observed and expected number of *Gata2* NC-CKO embryos and the total number of littermates are denoted. The expected number of *Gata2* NC-CKO embryos was estimated as one-eighth of the total number of littermates.

2.5 | Adrenaline feeding partially rescues the lethality of the Gata2 NC-CKO embryos

We next asked whether the embryonic lethality of the *Gata2* NC-CKO embryos was due to a presumptive adrenaline deficiency. To this end, we fed L-phenylephrine (α-adrenoreceptor agonist) and isoproterenol (β-adrenoreceptor agonist) in the drinking water of pregnant intercrossed dams from approximately e6.5 onwards. Subsequently, we analyzed the numbers of recovered mutant embryos at embryonic stages. We found that feeding these adrenaline analogs to the pregnant dams efficiently extended the lifespan of the *Gata2*-NCKO embryos beyond the e14.5 stage up to the perinatal period (Table 2). This pharmacological adrenaline complementation rescue allowed us to examine gene expression profile in the dissected adrenal glands of *Gata2* NC-CKO embryos at later stages. As anticipated, GATA2 mRNA expression level was significantly diminished in the adrenal gland of the e16.5 *Gata2* NC-CKO embryos, confirming efficient

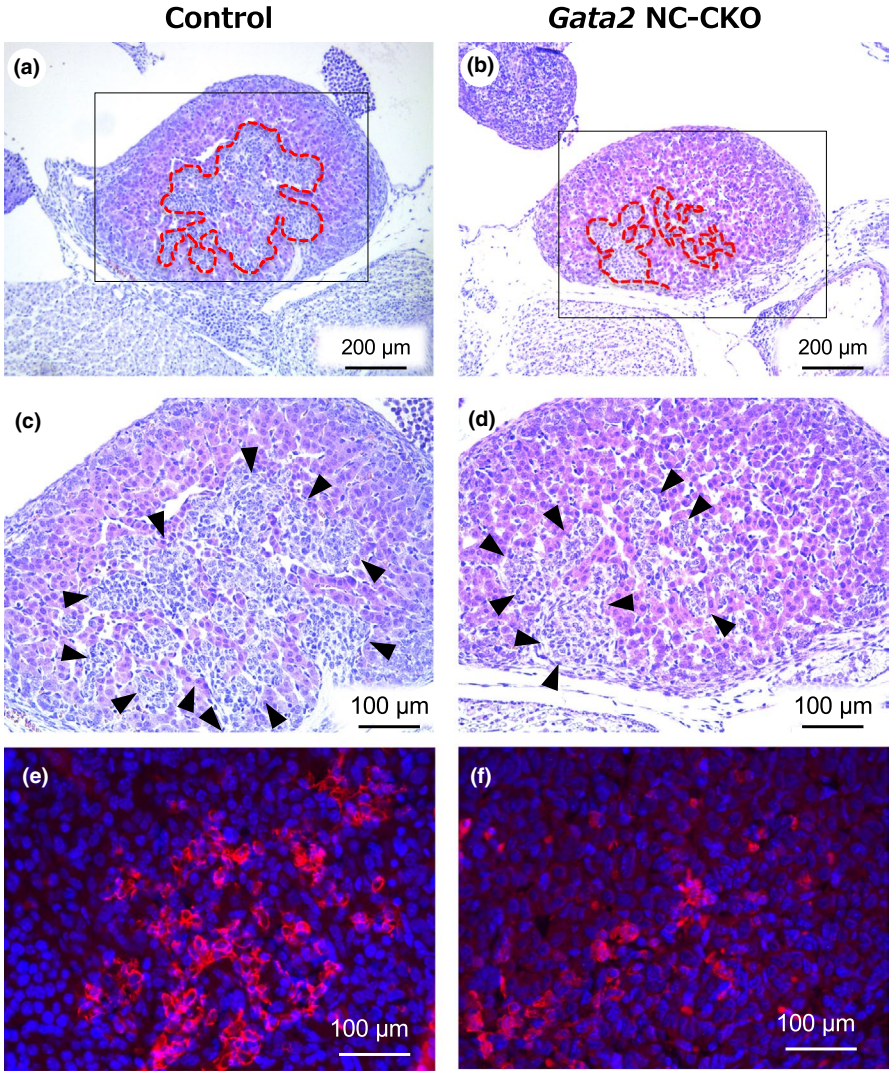

FIGURE 3 (a and b) The size of the adrenal medullary region (encircled with red dotted lines) was smaller in the *Gata2* NC-CKO than in the littermate control embryos at e14.5 stage. *Gata2* NC-CKO showed a reduced population of basophilic medullary cells, while the control embryos showed more abundant medullary cells (arrowheads in c and d; large magnification of the rectangles in a and b). (e and f) Chromogranin A-positive adrenal chromaffin cells were decreased in the *Gata2* NC-CKO in comparison with the control embryos. Representative images from three e14.5 embryos in each group are depicted. Scale bars are 200 μm (a and b) and 100 μm (c, d, e and f)

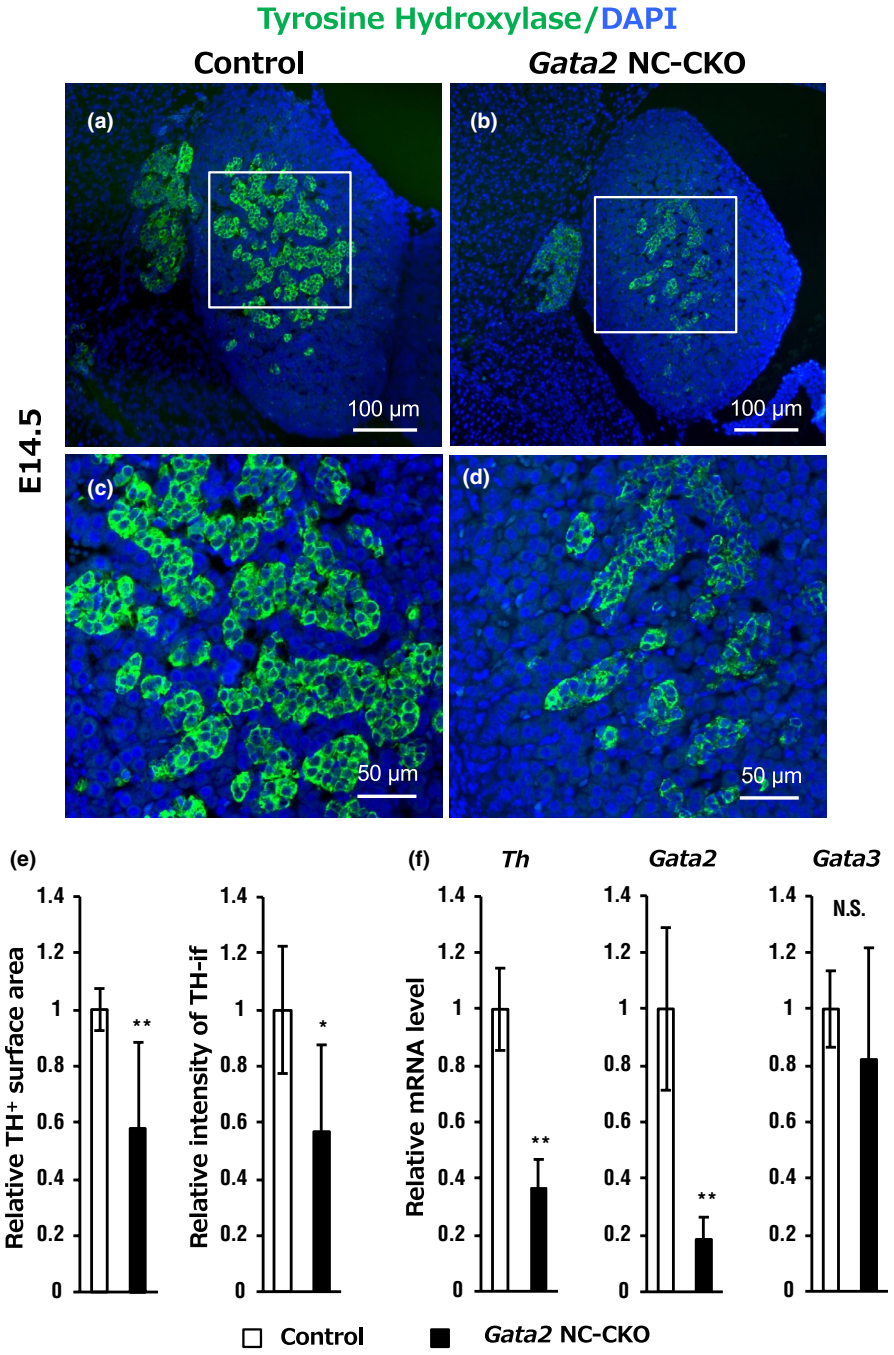

FIGURE 4 Tyrosine hydroxylase (TH)-positive chromaffin cells were significantly reduced in the adrenal gland of *Gata2* NC-CKO embryos at e14.5 (b and d). The control littermates showed more abundant TH-positive cells (a and c). Representative images from analysis of three embryos in each group are depicted. Scale bars are 100 μm (a and b) and 50 μm (c and d). e, Quantification of relative TH-positive surface area and intensity of TH immunofluorescence (TH-if). Data are presented as mean ± *SD* ($n = 3$ in each group). f, mRNA levels in the adrenal gland of individual e16.5 embryos (normalized to GAPDH mRNA) were assayed by RT-qPCR. Data are presented as mean ± *SD* ($n = 5$ in each group). The statistical significance of the differences between *Gata2* NC-CKO and the control is indicated (*$p < .05$, **$p < .01$; Student's *t* test). N.S.; not significant

TABLE 2 Genotyping frequency of *Gata2* NC-CKO progenies from breeding *Gata2*$^{G/+}$::Wnt1-Cre and *Gata2*$^{f/G}$ intercrosses with catecholamine feeding

Stage	Drug[a]	Number of *Gata2* NC-CKO observed (expected)	Number of littermates
E14.5	(+)	4 (3)	18
E15.5	(+)	7 (5.8)	35
E16.5	(+)	5 (6)	36
E18.5	(+)	5 (5.4)	32

Note: The expected number of *Gata2* NC-CKO embryos was estimated as one-sixth of the total number of littermates.

[a]Drug; L-phenylephrine and isoproterenol feeding.

deletion of *Gata2* in the adrenal glands (Figure 4f). TH mRNA expression level was diminished less than 37% in the *Gata2* NC-CKO adrenal gland consistent with the reduced TH immunoreactivity (Figure 4e,f). We found the GATA3 mRNA expression was relatively maintained in the adrenal glands of the *Gata2* NC-CKO embryos, suggesting that GATA3 was not under intense regulatory influence of GATA2 in the adrenal chromaffin cells (Figure 4f). These results indicate that GATA2 plays a role for maintenance of the TH expression in the adrenal gland and that the embryonic lethality of *Gata2* NC-CKO mice can be attributed, at least partially, to adrenaline deficiency in mid-gestational embryos.

TABLE 3 Sequence of primers used for genotyping

Gene	Sense primer	Antisense primer	Assay
Gata2 flox	TCCGTGGGACCTGTTTCCTTAC	GCCTGCGTCCTCCAACACCTCTAA	Genotyping
GFP	CTGAAGTTCATCTGCACCACC	GAAGTTGTACTCCAGCTTGTGC	Genotyping
Wnt1-Cre	CAG CGC CGC AAC TAT AAG AG	CAT CGA CCG GTA ATG CAG	Genotyping
ROSA26 *LacZ*	AAAGTCGCTCTGAGTTGTTAT	GCGAAGAGTTTGTCCTCAACC	Genotyping
Th	AGTTCTCCCAGGACATTGGACTT	ACACAGCCCAAACTCCACAGT	RT-qPCR
Gata2	ACCTGTGCAATGCCTGTGGG	TTGCACAACAGGTGCCCGCT	RT-qPCR
Gata3	GGTGGACGTACTTTTTAACATCGA	CCCTGACGGAGTTTCCGTAG	RT-qPCR
Gapdh	CCTGCACCACCAACTGCTTA	TCATGAGCCCTTCCACAATG	RT-qPCR

3 | DISCUSSION

In the present study, we discovered that GATA2 is specifically expressed in adrenal chromaffin cells. The vast majority of *Gata2* NC-CKO embryos died *in utero* and showed a decrease in TH expression in adrenal chromaffin cells, which possibly led to a decrease in catecholamine in the embryos. GATA2 is known as an important regulator in the hematopoietic system; thus, *Gata2* null- or hematopoietic cell-specific deficiency leads to early (before e9.5) embryonic lethality primarily due to hematopoietic defects (Lim et al., 2012; Tsai et al., 1994). In contrast, *Gata2* NC-CKO embryos did not exhibit anemia (this study). Given these findings, we concluded that the fetal lethality observed in the *Gata2* NC-CKO embryos is not due to hematopoietic deficiency.

Catecholamine plays a crucial role in cardiovascular development in mouse embryos. Catecholamine deficiencies in both TH- or DBH-deficient mice lead to embryonic lethality due to cardiovascular failure, which can be pharmacologically rescued by feeding catecholamine analogs (Kobayashi et al., 1995; Thomas et al., 1995; Zhou et al., 1995). Since the midgestational lethality of the *Gata2* NC-CKO embryos was partially rescued by the catecholamine feeding, we hypothesized that the lethality of the *Gata2* NC-CKO embryos could be attributable at least in part to catecholamine deficiency. We did not detect apparent cardiovascular defects in the *Gata2*-NC CKO mice by histological examination (data not shown). We therefore speculate that subtle changes in cardiovascular function, such as inability to maintain sufficient heart rate or contractility, may affect the viability of the *Gata2*-NC CKO embryos.

We have previously reported that *Gata3* homozygous deficient embryos showed early embryonic lethality (e10.5) with a significant decrease in sympathetic neurons and adrenal chromaffin cells (Lim et al., 2000; Moriguchi et al., 2006; Pandolfi et al., 1995). In contrast, the *Gata2* NC-CKO embryos exhibited somewhat later embryonic lethality (e14.5) with a modest decrease in chromaffin cells. These results suggest that GATA3 plays a dominant role to GATA2 in the maintenance or function of adrenal chromaffin cells. It has been previously shown that GATA2 mRNA expression was decreased in *Gata3*-deficient sympathetic progenitor cells, indicating that GATA2 expression might be directly or indirectly regulated by GATA3 (Tsarovina et al., 2004). In contrast, in the present study, GATA3 expression was not significantly affected in GATA2-deficient chromaffin cells. These results suggest that GATA2 may function hierarchically downstream of GATA3. The sustained GATA3 might account for the remaining TH expression in the *Gata2*-NC CKO adrenal gland. Further elucidation of the regulatory relationships between GATA2 and GATA3 and their distinct and cooperative functions in adrenal chromaffin cells would be of particular value.

The results of this study suggest that GATA2 is involved in the development of adrenal chromaffin cells. Recent studies have demonstrated that GATA2 and GATA3 are highly expressed in noradrenaline-producing human primary neuroblastoma and neuroblastoma cell lines and likely play a role in the proliferation of neuroblastoma cells (Boeva et al., 2017). Thus, GATA2 could be a promising therapeutic target for neuroblastoma. Precise elucidation of the physiological roles played by GATA2 during the development and maintenance of the sympathoadrenal system may help to elucidate how GATA2 participates in the tumorigenesis of chromaffin cells.

4 | EXPERIMENTAL PROCEDURES

4.1 | Mutant mice

Gata2 eGFP knockin (*Gata2*$^{GFP/+}$) and *Gata3 LacZ* knockin (*Gata2*$^{LacZ/+}$) mice carry eGFP and *LacZ* reporter genes, which are inserted into the translational initiation site of the *Gata2* and *Gata3* alleles, respectively (Figure 2a) (van Doorninck et al., 1999; Suzuki et al., 2006). The generation and characterization of the *Gata2*^{flox} conditional allele was described previously (Lim et al., 2012). Wnt1-Cre transgenic mice and the ROSA26 *LacZ* reporter strain of mice were supplied by Jackson Laboratory (Chai et al., 2000; Soriano, 1999). *Gata2*

NC-CKO were generated by mating *Gata2*$^{GFP/+}$::Wnt1-Cre mice and *Gata2*$^{f/+}$ or *Gata2*$^{f/G}$ mice. Offsprings genotyped as *Gata2*$^{+/+}$::Wnt1-Cre or *Gata2*$^{f/+}$::Wnt1-Cre were used as controls. Genotyping primers for *Gata2*^{GFP}, *Gata2*^{f}, Wnt1-Cre and ROSA26 *LacZ* are listed in Table 3. These mice were maintained in the C57BL/6J genetic background. To pharmacologically complement catecholamine deficiency, pregnant dams were given freshwater daily containing 100 μg/ml L-phenylephrine (α-adrenoreceptor agonist) (Sigma P6126), 100 μg/ml isoproterenol (β-adrenoreceptor agonist) (Sigma I5627) and 2 mg/ml ascorbic acid (Sigma A0278) at e 6.5 onward (Lim et al., 2000). All mice were handled according to the regulations of the Standards for Human Care and Use of Laboratory Animals of Tohoku Medical and Pharmaceutical University and the Guidelines for the Proper Conduct of Animal Experiments from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. All animal experiments were approved by the Tohoku Medical Pharmaceutical University Animal Experiment Committee (registration number: 18034-cn).

4.2 | Histological analysis

Embryonic tissues were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and then processed for immunostaining as frozen or paraffin-embedded sections. For hematoxylin and eosin (HE) staining, serial paraffin sections (4 μm) were subjected to a regular protocol. For immunofluorescence analysis, mouse anti-tyrosine hydroxylase (TH) (SIGMA T1299) antibody and M. O. M. Fluorescein Kit (VECTOR FMK-2201) were used. For detection of adrenal chromaffin cells, rabbit anti-chromogranin A antibody (Abcam ab15160) was used. The colocalization of β-galactosidase and GFP was performed using a Cy3-conjugated rabbit anti-β-galactosidase antibody (Moriguchi et al., 2006) and rabbit anti-GFP antibody (Molecular Probes). Fluorescence was visualized using a Leica DM inverted microscope. Separate images were taken and merged using OpenLab software. Section X-gal staining of embryos was performed as previously described (Lakshmanan et al., 1999).

4.3 | Quantitative real-time RT-PCR

Total RNA was extracted from the fetal adrenal gland using Sepasol-RNA I Super G (Nacalai Tesque). cDNA was synthesized using ReverTra Ace (TOYOBO). Quantitative real-time PCR (RT-qPCR) was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) on CFX96 TouchTM Detection System (Bio-Rad Laboratories). The primers used in the RT-qPCR are listed in Table 3.

4.4 | Morphometric analysis

Tyrosine hydroxylase immunoreactivity-positive surface area and the immunofluorescence intensity of the equally thresholded area were morphometrically quantified using ImageJ 1.53a software (https://imagej.nih.gov/ij/download.html). Four sections of bilateral adrenal glands from five embryos from each genotype were subjected to analysis. Measurements from wild-type littermate control mice were arbitrarily set at 100%.

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ORCID

Takashi Moriguchi https://orcid.org/0000-0002-5341-8932

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