Reduced BMP4 abundance in *Gata2* hypomorphic mutant mice result in uropathies resembling human CAKUT

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Constitutive loss of transcription factor GATA-2 leads to embryonic lethality from primitive erythropoietic failure. We serendipitously discovered an essential contribution of GATA-2 to urogenital development when the hematopoietic deficiency of *Gata2* null mutant animals was complemented by a *Gata2* yeast artificial chromosome (YAC) transgene; these mice died from a perinatal lethal urogenital abnormality. Here, we report the generation and analysis of *Gata2* hypomorphic mutant (*Gata2*$^{fGN/fGN}$) mice, which suffered from hydronephrosis and megaureter, as do the YAC-rescued *Gata2* null mutants. *Gata2*$^{fGN/fGN}$ mutants exhibit anteriorly displaced ureteric budding from the Wolffian duct as well as reduced BMP4 expression in the intermediate mesoderm derivatives in a manner that is temporally coincident with ureteric bud emergence. In *Bmp4* mutant heterozygotes, rostral displacement of the initial bud site on the Wolffian duct results in abnormal urogenital development. We show here that *Bmp4* mRNA is reduced approximately two-fold in *Gata2*$^{fGN/fGN}$ mice (as in *Bmp4* null heterozygotes), and that GATA-2 *trans*-activates a *Bmp4* first intron element-directed reporter plasmid in co-transfection assays. These experiments taken together implicate GATA-2 as a direct regulator of *Bmp4* transcription. The pathophysiology described in *Gata2* hypomorphic mutant animals resembles human congenital anomalies of the kidney and urinary tract.

Introduction

The GATA factors belong to an evolutionarily conserved family of transcription factors that play demonstrably critical roles in development (Patient & McGhee 2002). The DNA binding activity of the GATA factors is facilitated by two C4-type zinc fingers, which recognize the consensus sequence 5′ (A/G)GATA(A/T) 3′ (Ko & Engel 1993; Merika & Orkin 1993). In vertebrates, *Gata2* is expressed in numerous cell lineages. Since the cloning of GATA-2 (Yamamoto *et al.* 1990), its functional role in hematopoiesis has been the focus of most investigations, particularly because *Gata2* loss-of-function mutation leads to a reduced number of early hematopoietic progenitor cells, and consequently the *Gata2* null mutants die at embryonic day (E) 10.5 from a failure in primitive erythropoiesis (Tsai *et al.* 1994).

The prominent participation of GATA-2 in urogenital patterning was serendipitously discovered when we attempted to complement the hematopoietic deficiency in *Gata2* null embryos with a transgenic yeast artificial chromosome (YAC) encoding *Gata2* (*Gata2*$^{-/-}$::Tg^{G2YAC}). The 271 kbp d16B YAC, which spans −198 to +73 kbp of the *Gata2* locus, restored full hematopoietic competence in *Gata2*$^{-/-}$::Tg^{G2YAC} embryos, thus overcoming the hematopoietic deficiency and the resultant embryonic lethality attributed to GATA-2 deficit (Zhou *et al.* 1998; Khandekar *et al.* 2004). However, the YAC-rescued compound mutant animals succumbed to a urogenital

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disorder, leading to fully penetrant perinatal lethality. These neonates suffered from megaureter and hydronephrosis due to a developmental failure to generate a patent distal ureter–bladder connection (Zhou *et al.* 1998). Histological analyses reveal that the dilated ureters ended blindly or were aberrantly connected to the seminal vesicle or vas deferens, both Wolffian duct derivatives. More recently, we identified two *Gata2* urogenital enhancers located far 3′ to the *Gata2* structural gene (at +75 and +113 kbp with respect to the translation initiation site), both located beyond the boundaries of the d16B YAC (Khandekar *et al.* 2004). When *cis*-linked to a *lacZ* reporter gene, these enhancers reproduced the mesenchymal expression of endogenous GATA-2 in the developing urogenital system. At E10.5, *Gata2* expression is initiated in the nephrogenic mesenchyme and the cloaca, but is specifically excluded from the epithelial Wolffian duct and mesonephric tubules (Khandekar *et al.* 2004). At E12.5, GATA-2 expression continues in the mesenchymal cells enveloping the Wolffian duct, the branching ureteric bud, the urogenital sinus as well as the ureteric epithelium (Khandekar *et al.* 2004). Later on, GATA-2 expression is activated in the Wolffian duct and its derivatives. This expression pattern remains throughout embryogenesis (Khandekar *et al.* 2004).

To further explore GATA-2 function and regulation *in vivo*, we generated in this study a conditional, marked *Gata2* knock-in allele, so as to facilitate the eventual temporally- and tissue-specific elimination of GATA-2 function. In this *flox-Gata2-Neo* (*Gata2*^{fGN}) conditional allele, a flag-tagged *Gata2* cDNA and a PGK-neomycin cassette were inserted into the murine *Gata2* locus. This insertional disruption of the locus leads to severely diminished *Gata2* transcription in *Gata2*$^{fGN/fGN}$ animals. We found that, of the live *Gata2*$^{fGN/fGN}$ animals born, approximately 70% died within 1 month after birth of urological complications such as hypoplastic kidneys, hydronephrosis and megaureters, clearly resembling the phenotypic spectrum of YAC-rescued *Gata2* null mutants (Zhou *et al.* 1998). We further determined that in the *Gata2*$^{fGN/fGN}$ embryos, the ureteric bud sprouted from a more rostral position on the Wolffian duct in comparison to wild-type littermates. This pre-cocious ureteric budding was accompanied by reduced urogenital BMP4 expression. BMP4, a member of the TGFβ superfamily of secreted signaling ligands, participates in various cell processes (including cell proliferation, differentiation and survival) and is necessary for the morphogenesis of many organs and tissues (Hogan 1996). We further showed that forcibly expressed GATA-2 can activate, albeit weakly, GATA-binding motifs in the *Bmp4* first intron regulatory element. These data are consistent with previous reports of urological aberrations that develop in *Bmp4* heterozygotes and arise from aberrantly localized ureteric bud (Miyazaki *et al.* 2000). Perhaps most importantly, the developmental pathophysiology detected in these *Gata2* hypomorphic mutant animals strongly resembles human congenital anomalies of the kidney and urinary tract (CAKUT). CAKUT accounts for most chronic renal failures in children and encompass diseases with a wide anatomical spectrum, including kidney anomalies (renal agenesis, renal hypoplasia and renal dysplasia), ureteropelvic and ureterovesical junction anomalies, ectopic ureteral orifice, duplicated collecting

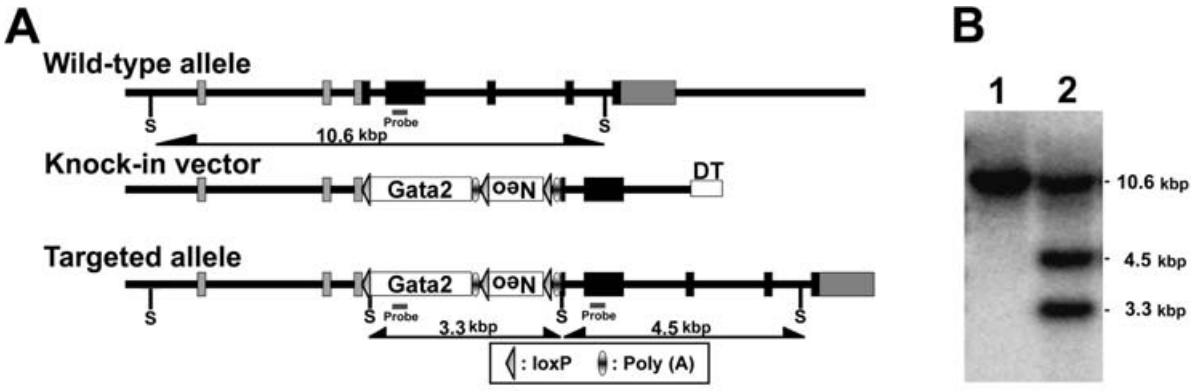

Figure 1 Generation of a *Gata2* conditional knock-in allele. (A) Schematic representations of the mouse *Gata2* locus, the targeting vector and the resultant targeted allele, in which a flag-tagged *Gata2* cDNA and a Neomycin minigene are placed in opposite transcriptional directions. *Gata2* non-coding and coding exons are depicted as gray and black boxes, respectively. *SpeI* restriction fragments detected by the *Gata2* exon 3 probe used in Southern blot analyses are indicated. S, *SpeI*; *Neo*, neomycin cassette; DT, diphtheria toxin. (B) Representative autoradiogram of *SpeI*-digested genomic DNA of wild-type (lane 1) and *Gata2*$^{fGN/+}$ (lane 2) mice hybridized with radiolabeled probe (see panel A).

A

	fGN/fGN	*fGN/+*	*+/+*	Total alive
E14.5	3	7	2	12
E15.5	4	13	6	23
E17.5	3(2)	18	5	26
E18.5	17(4)	49(1)	24	90
P0	11(4)	28	17	56

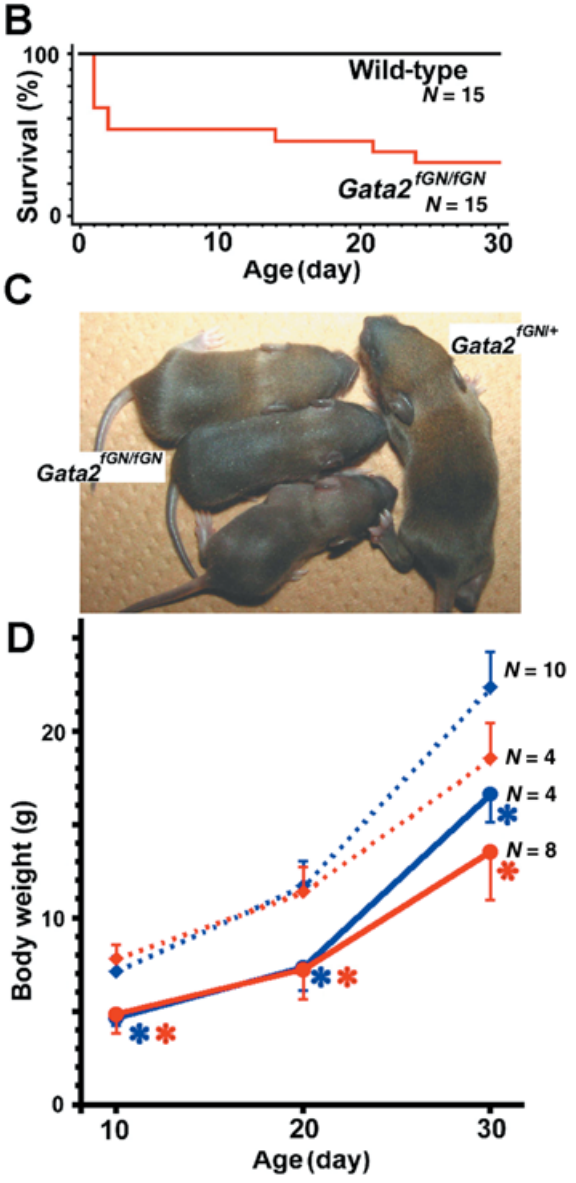

Figure 2 Reduced survival rate and body size in *Gata2*$^{fGN/fGN}$ mice. (A) The table of progeny from *Gata2*$^{fGN/+}$ intercrosses. Numbers in parentheses indicated the dead embryos found at the time of dissection. (B) The survival curve of wild-type (black line; $N = 15$) and *Gata2*$^{fGN/fGN}$ (red line; $N = 15$) mice during the first 4 weeks after birth was plotted. Difference is statistically significant when evaluated by the Log-rank test ($P < 0.0001$). (C) *Gata2*$^{fGN/fGN}$ pups are distinctively smaller than age-matched *Gata2*$^{fGN/+}$ pup at postnatal day (P) 8. (D) The body weights of *Gata2*$^{fGN/fGN}$ (solid line) and *Gata2*$^{fGN/+}$ (broken line) male (blue) and female (red) mice were plotted during the first 30 days after birth. Asterisks show statistically significant differences between the gender- and age-matched *Gata2*$^{fGN/+}$ and *Gata2*$^{fGN/fGN}$ mice ($P < 0.05$; two sample *t*-test).

system and anomalies of the bladder and urethra. Here, we demonstrate that transcription factor GATA-2 and its downstream target, BMP4, are amongst the genes that can give rise to the entire phenotypic spectrum of human CAKUT in mice (Ichikawa *et al.* 2002).

Results

Generation of mice bearing a *Gata2* conditional knockout allele

Previously, it was demonstrated that germ line ablation of *Gata2* led to embryonic lethality at E10.5, a phenotype attributable to defective hematopoiesis in *Gata2* null embryos (Tsai *et al.* 1994). This mid-gestational lethality hampered further exploration of the role of GATA-2 in the many other non-hematopoietic tissues that express GATA-2. In order to investigate GATA-2 contributions to other aspects of murine development, we generated a murine *Gata2* conditional knockout allele. *Gata2* transcription initiates from two alternative promoters (IS and IG) and their associated non-coding first exons (Minegishi *et al.* 1998; Nony *et al.* 1998). While the *Gata2* IG promoter is widely utilized, the IS promoter is used only in hematopoietic and neuronal cells (Minegishi *et al.* 1998). A targeting vector was constructed in which part of exon 2 (containing the translational initiation codon) was replaced by a flag-tagged murine *Gata2* cDNA and a *Neo* transcription cassette that was oriented in reverse of the *Gata2* gene (Fig. 1A). The *Gata2* cDNA and the *Neo* cassette were flanked on either side by loxP sequences to facilitate later selective excision by Cre-mediated homologous recombination (Fig. 1A).

The targeting construct was transfected into E14 embryonic stem (ES) cells, and targeted clones were enriched by positive/negative selection using Diphtheria toxin and growth medium containing G418 (Thomas & Capecchi 1987; Yagi *et al.* 1993). Successful homologous recombination in several ES cell clones was confirmed by Southern blotting (data not shown). Of these, two clones were used for injection into C57Bl/6 blastocysts, which were then implanted into foster mothers. Chimeric mice derived from both clones were intercrossed

A

	+/+	fGN/+	fGN/fGN
Hypoplastic kidney	0	0	2
Megaureter	0	0	*9
Megaureter and hypoplastic kidney	0	0	1
Total	0	0	12

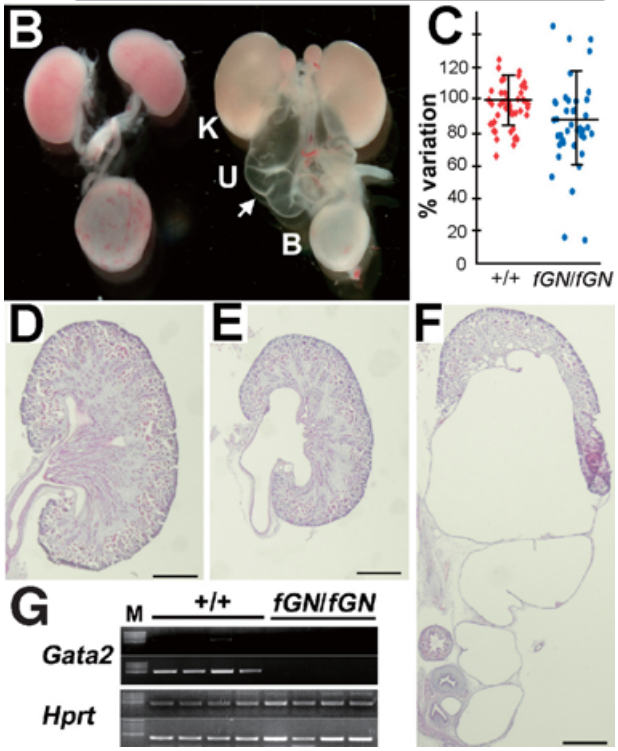

Figure 3 *Gata2*$^{fGN/fGN}$ mice display urological abnormalities. (A) Wild-type ($N = 24$), *Gata2*$^{fGN/+}$ ($N = 57$) and *Gata2*$^{fGN/fGN}$ ($N = 21$) neonatal offspring from *Gata2*^{fGN} intercrosses were examined for urological abnormalities under macroscopic dissection. Observed spectrum of urological abnormality in *Gata2*$^{fGN/fGN}$ neonates as tabulated. *Of these animals, five and four exhibited bilateral and unilateral megaureter, respectively. Two out of nine neonates also suffered from severe hydronephrosis. (B) Urinary systems dissected from P0 wild-type (left) and *Gata2*$^{fGN/fGN}$ (right) pups. The ureters (U, arrow) and kidneys (K) of the *Gata2*$^{fGN/fGN}$ pup (right) are enlarged compared to that of the wild-type neonate (left). (C) Sizes of wild-type (+/+; $N = 25$) and *Gata2*$^{fGN/fGN}$ (*fGN/fGN*; $N = 21$) kidneys are plotted. A pair of kidneys from individual mouse are aligned vertically and shown along abscissa axis; percent variations of the kidney size are shown along ordinate axis. In the latter case, mean value of wild-type kidneys is set to 100%. Note that the kidney sizes vary substantially in the *fGN/fGN* kidneys due to hydronephrosis and hypoplasia/atrophy. The difference of kidney sizes is statistically significant ($P < 0.02$) by Welsh's *t*-test. (D–F) Kidney sections of P0 wild-type (D) and *Gata2*$^{fGN/fGN}$ (E, F) pups stained with hematoxylin and eosin. Kidney size variation in *Gata2*$^{fGN/fGN}$ embryos is directly correlated with the severity of hydronephrosis and range from small, hypoplastic kidney with mild hydronephrosis (E) to extremely enlarged kidney with severe hydronephrosis (F). Scale bar is 0.5 mm. (G) *Gata2* mRNA abundance in the intermediate mesoderm derivatives of four individual E10.5 wild-type and *Gata2*$^{fGN/fGN}$ embryos was detected by semi-quantitative RT-PCR after 27 and 30 cycles of PCR amplification. *Hprt* transcript abundance was used as a control.

with C57Bl/6 mice, and F1 progeny were genotyped by Southern blotting. Using a radiolabeled probe corresponding to *Gata2* exon 3, we detected an expected 10.6 kbp fragment in wild-type animals (Fig. 1B, lane 1) and two additional fragments (3.3 and 4.5 kbp) from the modified *flox-Gata2-Neo* (*Gata2*^{fGN}) allele in heterozygous mutant animals (Fig. 1B, lane 2).

Urologic abnormalities, lower viability and body weight in *Gata2*$^{fGN/fGN}$ mice

Gata2$^{fGN/+}$ mice were born without apparent abnormalities, and reproduced normally. However, a fraction of *Gata2*$^{fGN/fGN}$ pups generated from *Gata2*$^{fGN/+}$ intercrosses died perinatally (Fig. 2A). To determine the onset of lethality, we examined multiple litters of *Gata2*$^{fGN/+}$ intercrosses at gestational ages ranging from E14.5 to E18.5. *Gata2*$^{fGN/fGN}$ embryos began to die during late gestation, beginning around E17.5 (Fig. 2A). When we compared the viability curve of surviving *Gata2*$^{fGN/fGN}$ and wild-type animals during the first 30 days after birth, we found that approximately 50% of *Gata2*$^{fGN/fGN}$ pups died during the first 2 days after birth, and that the viability of *Gata2*$^{fGN/fGN}$ neonates continued to diminish gradually over the next 4 weeks (Fig. 2B).

Compared to wild-type littermates, only 30% of *Gata2*$^{fGN/fGN}$ pups lived more than 1 month beyond birth (Fig. 2B). *Gata2*$^{fGN/fGN}$ pups were generally smaller in size and were easily distinguishable from *Gata2* heterozygous and wild-type littermates even at 1-week old (Fig. 2C). Both male and female *Gata2*$^{fGN/fGN}$ animals continued to show significantly lower body weights compared to their wild-type counterparts during the first 30 days after birth (Fig. 2D).

Previously, we reported that unlike *Gata2* null mutant embryos, YAC-rescued *Gata2* null animals overcame the initial cause of embryonic death at E10.5 due to hematopoietic failure (Tsai *et al.* 1994; Zhou *et al.* 1998). However, the rescued animals succumbed to a urogenital disorder, leading to perinatal lethality with full penetrance (Zhou *et al.* 1998). The compound mutant neonates suffered from megaureters and hydronephrosis as a result of fluid-filled dilatation of the ureters and kidneys (Zhou

et al. 1998). Hence, the *Gata2* YAC transgene was unable to recapitulate endogenous GATA-2 expression in the developing urogenital system (Zhou *et al*. 1998). We therefore wished to determine if the observed lethality in *Gata2*$^{fGN/fGN}$ mice was due to a related fatal urological disorder.

We performed gross examination on wild-type (N = 24), *Gata2*$^{fGN/+}$ (N = 57) and *Gata2*$^{fGN/fGN}$ (N = 21) neonatal littermates from several litters, and found that 57% (12/21) of the *Gata2*$^{fGN/fGN}$ mutants displayed urological anomalies, including hypoplastic kidneys, hydronephrosis and megaureters (Fig. 3A). Although the severity of the urological phenotype was variable amongst the *Gata2*$^{fGN/fGN}$ animals, the most frequently encountered phenotype was uni- or bi-lateral megaureter (Fig. 3B). As with the YAC-rescued *Gata2* null mutants, we did not observe duplicated or bifurcated ureters in *Gata2*$^{fGN/fGN}$ animals. Compared to wild-type animals, the size of kidneys in *Gata2* mutant pups varied over a wide range (Fig. 3C), and was directly correlated with the severity of hydronephrosis, ranging from small, hypoplastic kidneys with mild cases of hydronephrosis (Fig. 3E) to enormously dilated kidneys in severe cases (Fig. 3F). In most instances, the renal parenchyma in the mutant kidneys was thinner than that in normal littermates (Fig. 3D vs. Fig. 3E and F). Hence, *Gata2*$^{fGN/fGN}$ mice suffered from urologic abnormalities that resemble aspects of human CAKUT.

There are multiple examples in the literature describing the disruption of transcription due to the targeted insertion of a selection cassette that resulted in the generation of a hypomorphic allele (e.g. Takahashi *et al*. 1997). We thus explored the possibility that the urogenital hypo/dysplasia observed in *Gata2*$^{fGN/fGN}$ animals could be due to reduced GATA-2 accumulation in the developing urogenital system. We dissected the intermediate mesoderm derivatives (containing the mesonephros, the metanephric anlagen, the Wolffian duct and the developing gonads) *en bloc* from individual E10.5 *Gata2*$^{fGN/fGN}$ and wild-type embryos for total RNA isolation. We then examined the mRNA abundance of *Gata2* and *Hprt*, which served as an invariant control, by semi-quantitative RT-PCR. These data show that urogenital *Gata2* mRNA abundance in E10.5 *Gata2*$^{fGN/fGN}$ mutants (4/4) was reduced to approximately 1.5% of wild-type levels (Fig. 3F and data not shown). We surmise that the *Neo* minigene positive selection cassette, which was cloned in the opposite transcriptional orientation with respect to the inserted *Gata2* cDNA as well as the endogenous *Gata2* allele, embedded in the targeted *Gata2* locus severely reduced *Gata2* transcription, thereby leading to the observed urologic phenotype in *Gata2*$^{fGN/fGN}$ mutants. This hypothesis is supported by the observation that mice hemizygous for a *Gata2-gfp* knock-in null allele (Suzuki *et al*. 2006) and the *Gata2* conditional allele with the *Neo* minigene deleted are normal (R. Shimizu and M. Yamamoto, personal communication).

GATA-2 regulates the urogenital expression of BMP4

The advent of gene targeting technology has dramatically augmented our knowledge of genes that are essential for urogenital patterning. Germ line mutagenesis of *Foxc1/2*, *Bmp4* and *Rar* α/β leads to the formation of hypo/dysplastic kidneys, hydronephrosis and megaureters in mutant mice as a consequence of multiple ureteric buds, anteriorly displaced ureteric buds or defective distal ureter maturation, respectively (Mendelsohn *et al*. 1994, 1999; Kume *et al*. 2000; Batourina *et al*. 2002). The latter process requires the GDNF/c-ret signaling pathway in the caudal Wolffian duct and the urogenital sinus (Batourina *et al*. 2002), where GATA-2 is also expressed (Khandekar *et al*. 2004). We were interested to determine if transcription of any of these genes were affected in *Gata2*$^{fGN/fGN}$ embryos, thereby placing some conceptual constraints on possible epistatic interactions between GATA-2 and these effectors. We prepared total RNA from the intermediate mesoderm derivatives of several individual E10.5 wild-type and *Gata2*$^{fGN/fGN}$ embryos for analysis using semi-quantitative RT-PCR. We found that the mRNAs levels of *Rar* (α, β and γ), *Foxc1*, *Foxc2* and *Gdnf* were unaffected in *Gata2*$^{fGN/fGN}$ embryos (Fig. 4A). This is consistent with the recent observation of continued expression of Foxc1 and Foxc2 in GATA-2-deficient lymphatic endothelial cells (S. Dagenais and K.-C. Lim, personal communication; Khandekar *et al*. 2007).

We also examined *Bmp4* transcript levels in total RNA prepared from the intermediate mesoderm derivatives of E10.5 wild-type and *Gata2*$^{fGN/fGN}$ embryos using semi-quantitative RT-PCR. The abundance of BMP4 mRNA was reduced in *Gata2*$^{fGN/fGN}$ embryos in comparison to wild-type embryos (Fig. 4B). This result was verified by real-time RT-PCR: *Bmp4* transcript level in *Gata2*$^{fGN/fGN}$ embryos was approximately half of that detected in wild-type embryos (Fig. 4C). We also analyzed the mRNA levels of the BMP4 receptor, Alk3, but failed to detect a change (Fig. 4A).

It was previously reported that proper ureteric budding requires the secreted morphogen BMP4, which is expressed in the urogenital mesenchyme surrounding the Wolffian duct and the ureteric stalk (Dudley & Robertson 1997; Miyazaki *et al*. 2000; Michos *et al*. 2007). Anti-GFP immunostaining on transverse sections of

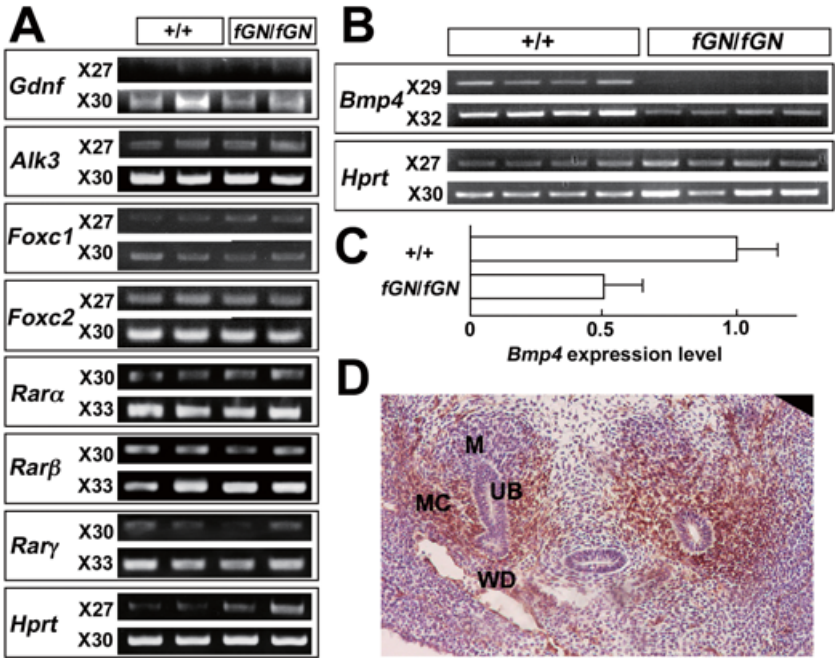

Figure 4 Reduced expression of GATA-2 affects the abundance of BMP4. (A) mRNA abundances of the *Gdnf*, *Alk3*, *Foxc1*, *Foxc2*, *Rarα*, *Rarβ* and *Rarγ* genes were determined in the intermediate mesoderm derivatives of E10.5 *Gata2*$^{fGN/fGN}$ embryos by semi-quantitative RT-PCR. (B) mRNA abundance transcribed from *Bmp4* was determined in E10.5 intermediate mesoderm derivatives from four wild-type and four *Gata2*$^{fGN/fGN}$ embryos by semi-quantitative RT-PCR. Note that the *Bmp4* expression is reduced significantly in the homozygous mutants. Amplification cycles are described in the figure. (C) *Bmp4* mRNA levels in the *Gata2*$^{fGN/fGN}$ embryos were determined by quantitative RT-PCR. The expression levels were normalized to ribosomal RNA in the same cDNA preparation and then the *Bmp4/rRNA* ratio in wild-type mice was arbitrarily set to 1. (D) Immunohistochemical analysis of a transverse section of an E11.0 embryo; staining represents the expression of GFP incorporated into the *Gata2* gene. The GFP reporter is expressed in mesenchymal cells surrounding the Wolffian duct, but not in the epithelium of the Wolffian duct or ureteric stalk. M, metanephric blastema; MC, mesenchymal cells; UB, ureteric bud; WD, Wolffian duct.

E11.0 *Gata2*-GFP knock-in heterozygous embryos (Suzuki *et al.* 2006) indicated that the GFP immunostaining pattern in the urogenital mesenchymal cells surrounding the ureteric bud and the Wolffian duct (Fig. 4D and data not shown; Khandekar *et al.* 2004), resembling that of BMP4. Thus, significant loss of GATA-2 expression in *Gata2*$^{fGN/fGN}$ embryos led to reduced *Bmp4* expression in the urogenital mesenchyme, which in turn resulted in rostrally displaced ureteric outgrowth on the Wolffian duct. Thus, the data presented here implicate GATA-2 as a (direct or indirect) positive regulator of *Bmp4* transcription.

Ectopic ureteric bud position in *Gata2*$^{fGN/fGN}$ mice

The development of the metanephros begins with the outgrowth of a single ureteric bud from the posterior Wolffian duct at E10.5 of mouse embryogenesis (Costantini & Shakya 2006). This positional restriction of ureteral budding to a specific caudal segment of the Wolffian duct is critical as dysregulation of initial ureteric bud formation leads to CAKUT (Ichikawa *et al.* 2002). Previously, it was reported that 53% of *Bmp4*$^{+/-}$ mice exhibited CAKUT symptoms due to the formation of a cranially positioned ureteric bud (Miyazaki *et al.* 2000). We thus investigated whether aberrant ureteral budding occurred in *Gata2*$^{fGN/fGN}$ mice. E11.0 wild-type ($N = 5$), *Gata2*$^{fGN/+}$ ($N = 5$) and *Gata2*$^{fGN/fGN}$ ($N = 10$) embryos were subjected to *in situ* hybridization using *c-ret* and *shh* antisense probes. c-Ret is a receptor tyrosine kinase for the secreted growth factor GDNF, and its expression in the urogenital system is restricted to the epithelial nephric duct and ureteric bud (Dressler 2006), while sonic hedgehog (Shh) is a morphogen that is expressed in numerous tissues, including the somites (Ingham & McMahon 2001).

When we analyzed the embryos after *in situ* hybridization to both probes, we noted that in wild-type and *Gata2*$^{fGN/+}$ embryos, the position of the initial ureteric budding site aligned with the 26th somite (Fig. 5A), as

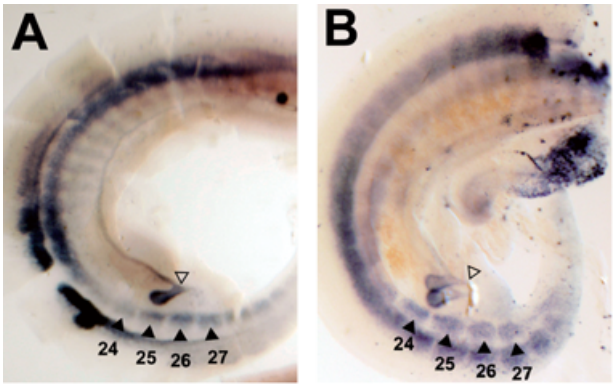

Figure 5 Ectopic ureteric budding in *Gata2*$^{fGN/fGN}$ embryos. *c-ret* and *Shh* whole mount *in situ* hybridizations were performed for wild-type ($N = 5$), *Gata2*$^{fGN/+}$ ($N = 5$) and *Gata2*$^{fGN/fGN}$ ($N = 10$) embryos at E11.0. The somites, which are labeled by the c-ret *in situ* probe, in the caudal region of the embryo are numbered and indicated by black arrowheads. The initial ureteric bud, which is indicated by an open arrowhead, in the wild-type (A) and *Gata2*$^{fGN/+}$ (data not shown) embryos aligns with the 26th somite level, while in 50% (5/10) of the *Gata2*$^{fGN/fGN}$ embryos (B), the ureteric bud sprouts from a more rostral position, at the level of the 25th somite.

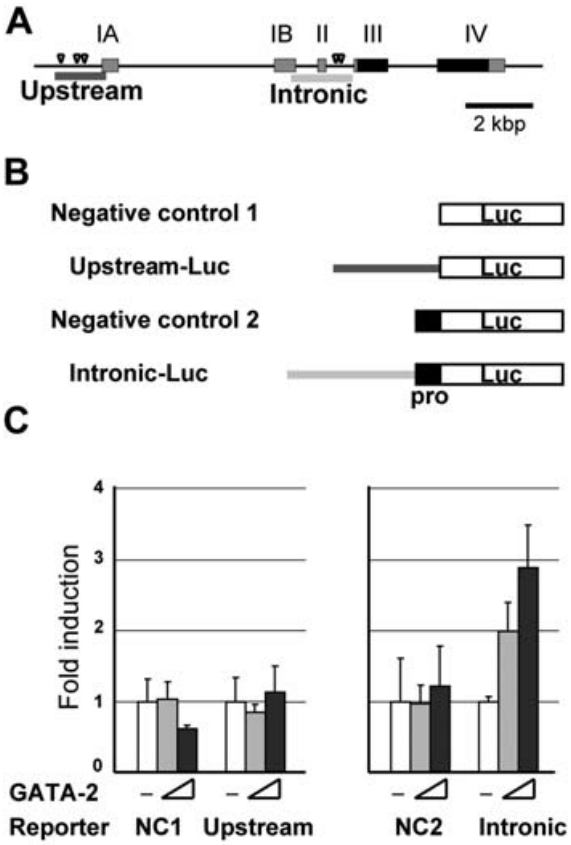

Figure 6 GATA-2 directly activates *Bmp4* transcription. (A) The gray and black boxes represent non-coding and coding exons of the murine *Bmp4* gene, respectively. The positions of the 1.3-kbp upstream and 1.8-kbp intronic (putative regulatory) regions with respect to the *Bmp4* locus are indicated. Consensus GATA-binding sites are indicated by arrowheads. (B) Schematic illustration of the *Bmp4*-luciferase reporter constructs. The *Bmp4* promoter was cloned into a promoter-less luciferase (Luc) reporter plasmid (Basic-Luc) to generate plasmid Upstream-Luc. The 1.8 kbp first intronic region was inserted 5′ of a SV40 promoter-directed luciferase reporter plasmid (Promoter-Luc) to generate plasmid Intronic-Luc. (C) The activity of two putative *Bmp4* regulatory fragments is differentially affected by GATA-2. There is no effect of GATA-2 dose-dependent activity (0, 60 or 180 ng of pEF-GATA-2 expression vector corresponds to open, light grey or dark grey boxes, respectively) on the transcriptional activity of the *Bmp4* promoter, while luciferase activity linked to the first intronic element increases with increasing GATA-2 expression plasmid dosage. NC, negative control.

previously reported (Miyazaki *et al.* 2000). However, in 50% of the *Gata2*$^{fGN/fGN}$ embryos, ureteral budding initiated more anteriorly along the Wolffian duct, at an axial position corresponding to the 25th somite (Fig. 5B). This frequency correlated well with our earlier observation that 57% of *Gata2*$^{fGN/fGN}$ neonates exhibited some uropathy upon gross examination (Fig. 3A). These observations indicate that normal GATA-2 abundance is important for providing positional information for initial ureteric bud outgrowth along the Wolffian duct.

GATA-2 can *trans*-activate a *Bmp4* first intron regulatory element

Several consensus GATA-binding sites present in the *Bmp4* promoter and second intron are conserved across six mammalian species (Nemer & Nemer 2003; Thompson *et al.* 2003; Lugus *et al.* 2007). The *in vivo* occupancy of both elements (as demonstrated by chromatin immunoprecipitation analysis) and their functional importance in the *Bmp4* promoter has been shown in embryoid bodies and cardiomyocytes (Nemer & Nemer 2003; Lugus *et al.* 2007). The *Gata2* hypomorphic mutation could directly or indirectly affect *Bmp4* mRNA levels in *Gata2*$^{fGN/fGN}$ embryos. We attempted to address this question by evaluating GATA-2 activation potential on *Bmp4* transcriptional activity in cell-based transfection assays using a 1.3-kbp *Bmp4* Upstream fragment containing upstream GATA motifs and a part of the IA exon and a 1.8-kbp Intronic fragment containing a part of the IB exon, the entire first intron and II exon, and almost entire second intron in cotransfection transactivation assays (Fig. 6A). The *Bmp4* Upstream

fragment was cloned into a promoter-less luciferase reporter construct to generate Upstream-Luc reporter plasmid, while the *Bmp4* Intronic fragment was inserted 5′ to an SV40 promoter-driven luciferase reporter construct to create Intronic-Luc reporter plasmid (Fig. 6B). Both reporter plasmids as well as their respective base vectors were transfected into QT6 quail fibroblast cells with or without a co-transfected GATA-2 expression vector.

We did not detect any change in luciferase activity associated with the 1.3 kbp *Bmp4* promoter regardless of the presence of exogenous GATA-2 in transfected QT6 cells (Fig. 6C). However, in the presence of forcibly expressed GATA-2, luciferase activity linked to the intronic regulatory element was stimulated threefold over background (Fig. 6C). Thus, GATA-2 indeed activates *Bmp4* transcription in this restricted setting. Taken together, the data support the hypothesis that the *Bmp4* gene is a directly activated target of GATA-2 in the urogenital system and that GATA-2 activates *Bmp4* through a partially characterized first intron element that contains two evolutionarily conserved GATA-binding sites.

Discussion

GATA-2 plays several important roles in the determination and differentiation of numerous cell lineages during embryogenesis and systemic loss of GATA-2 leads to early embryonic lethality due to primitive erythropoietic deficiency (Tsai *et al.* 1994). In this study, we report that most *Gata2* homozygous hypomorphic animals died within 1 month after birth due to urological complications typified by hypoplastic kidneys, hydronephrosis and megaureters, just as was seen in YAC-rescued *Gata2* null animals. We further determined the morphogenetic and molecular events underlying these developmental aberrations.

During early renal morphogenesis, the correct emergence of a single ureteric bud from the posterior Wolffian duct is imperative to the development of a functioning urogenital system later in adulthood. The precision of this process is dictated by the combinatorial actions of both positive and negative regulatory molecules (Costantini & Shakya 2006). Homozygous loss of genes encoding slit2 or its receptor, robo2, or the forkhead/winged helix transcription factor Foxc1 and receptor tyrosine kinase antagonist sprouty1 as well as compound heterozygosity of *Foxc1/2* null mutation lead to formation of supra-numeral ureteric buds as a consequence of expanding the anterior limit of the GDNF-positive mesenchymal domain within the intermediate mesoderm derivatives or of the elimination of inhibitory feedback on GDNF/c-ret signal transduction (Kume *et al.* 2000; Grieshammer *et al.* 2004; Basson *et al.* 2005). Indeed, excess GDNF alone *in vitro* and *in vivo* is sufficient to induce extra ureteral budding (Sainio *et al.* 1997; Brophy *et al.* 2001; Shakya *et al.* 2005). However, the stimulatory effect of GDNF on ureteral budding and branching can be abrogated by BMP4 (Raatikainen-Ahokas *et al.* 2000; Brophy *et al.* 2001). Furthermore, perturbations of BMP level/activity by germ line mutagenesis of BMP4 antagonist Gremlin1 (Michos *et al.* 2004, 2007), transgenic expression of a constitutively active BMP receptor, Alk3 (Hu *et al.* 2003) or haploinsufficiency of *Bmp4* gene in *Bmp4* heterozygous mutants (Miyazaki *et al.* 2000) lead to uropathies in each of these mutants, clearly underscoring the conclusion that homeostasis in BMP activity is critical for proper urogenital patterning. Exposure of cultured metanephric explants to Gemlin-1 or BMP4 does not affect GDNF expression (Miyazaki *et al.* 2000; Michos *et al.* 2007); therefore, how changes in BMP4 activity in the metanephric mesenchyme lead to aberrant ureteric budding *in vivo* remains to be deciphered.

In *Gata2*$^{fGN/fGN}$ mice, we observed that the ureteric bud emerged from an abnormally anterior position along the Wolffian duct; this was commensurate with twofold reduced BMP4 expression in the urogenital region, which is consistent with the report of urological aberrations observed in *Bmp4* heterozygous knockout mice (Miyazaki *et al.* 2000). The ectopic budding of the ureteric bud leads to erroneous ureterovesical junction that later in development manifests as CAKUT (reviewed in Ichikawa *et al.* 2002). We recently ascertained that the same morphogenetic error occurred in YAC-rescued *Gata2*-null mutants (Zhou *et al.* 1998; K.-C. Lim, personal communication). We observed that almost an equivalent number of the *Gata2* hypomorphic mutant animals displayed urologic abnormalities and ectopic ureteric budding (57% and 50%, respectively). In these animals BMP4 expression is reduced in the intermediate mesoderm derivatives. Examination of *Bmp4* heterozygotes revealed a urogenital phenotype that resembles human CAKUT in 53% of the animals (Miyazaki *et al.* 2000). We also showed that GATA-2 could activate expression from a *Bmp4* first intronic regulatory element-directed reporter gene in cell-based transfection assays, again underscoring the possibility that GATA-2 is an upstream regulator of BMP4 expression during urogenital development (Fig. 7). Further experimental evidence addressing this epistatic relationship *in vivo* might be achieved by manipulating BMP4 levels using the recently identified *Gata2* urogenital enhancers UG2/4 (Khandekar *et al.* 2004) to drive BMP4 mesenchymal expression in *Gata2*$^{fGN/fGN}$ mice or by breeding

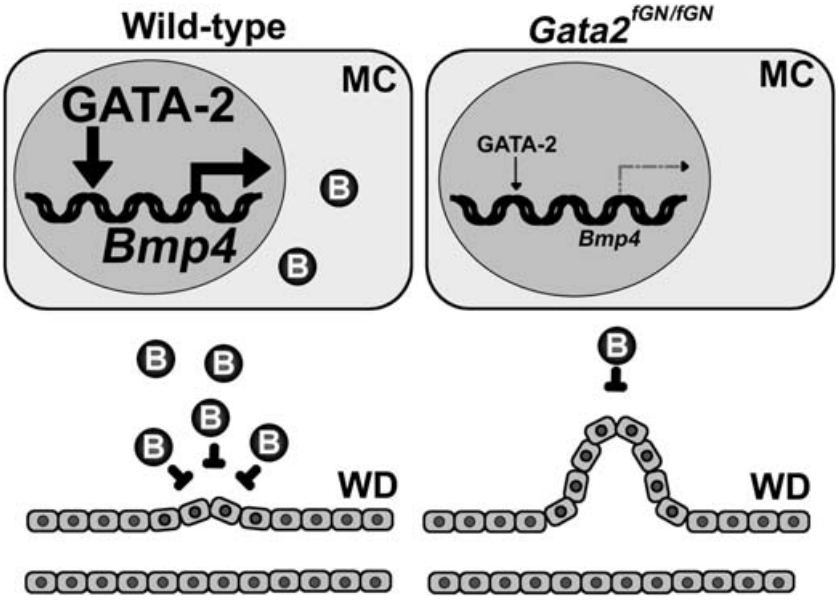

Figure 7 A model for the role of GATA-2 in the ureteral development. GATA-2 activates *Bmp4* transcription in the mesenchymal cells surrounding the Wolffian ducts; consequently, secreted, mature BMP4 suppresses initial ureteric bud emergence (left). In contrast, precocious ureteric budding is detected when there is insufficient metanephric mesenchymal secretion of mature BMP4, as in GATA-2-hypomorphic *Gata2*$^{fGN/fGN}$ mice (right).

the *Gata2*^{fGN} allele into a *Gremlin1*-deficient genetic background (Michos *et al*. 2004).

The *Gata2*$^{fGN/fGN}$ mice generated here also displayed lower body mass. Using a αGSU-Cre transgene to achieve pituitary-specific GATA-2 loss-of-function, Charles *et al*. (2006) revealed reduced gonadotrophic and thyrotropic hormones in pituitary-specific *Gata2* knockout (pitKO) mice. Male, but not female, pitKO mice initially showed lower body mass, but eventually they reached normal size. On the other hand, the growth retardation of the *Gata2*$^{fGN/fGN}$ mice is not sex-specific. This difference between the two *Gata2* mutant mouse lines could be due to the strategy of *Gata2* gene mutation. The αGSU-Cre transgene was shown to effectively convert a floxed SF1 allele to a non-functional allele by E14.5 (Zhao *et al*. 2001), and *Gata2* transcription was reduced to 2%–8% of wild-type levels in pituitary glands of pitKO mice (Charles *et al*. 2006). The reduction of *Gata2* transcripts was more drastic (< 1.5%) and systemic in *Gata2*$^{fGN/fGN}$ mice, and the spatiotemporal timing of GATA-2 loss was dictated by the endogenous targeted *Gata2* locus. While the transient weight reduction in pitKO males was considered to be due to compromised thyrotropic function (Charles *et al*. 2006), the cause of growth retardation in *Gata2*$^{fGN/fGN}$ mice may not be explained solely by thyrotropic dysfunction since GATA-2 is widely expressed. Further experiments will be necessary to elucidate the cause of the growth retardation in *Gata2*$^{fGN/fGN}$ mice.

Previous studies suggested that BMP4 regulates *Gata2* expression. In *Xenopus*, for example, injection of *Bmp4* mRNA promoted *Gata2* activation (Maeno *et al*. 1996). Furthermore, a BMP4 responsive element has been defined in the 5′ upstream region of zebrafish *Gata2* (Oren *et al*. 2005). In contrast, the present data indicate that GATA-2 regulates urogenital BMP4 expression. Other investigators have also reported that other GATA factors (i.e. GATA-4 and GATA-6) regulate *Bmp4* transcription, for example, in cardiomyocytes (Nemer & Nemer 2003; Peterkin *et al*. 2003, 2005). Consistent with this observation, GATA-binding motifs that are evolutionarily conserved across mammalian species have been identified in regulatory sequences controlling *Bmp4* transcription (Nemer & Nemer 2003; Lugus *et al*. 2007). Furthermore, the occupancy of these GATA sites, particularly in the first intron of *Bmp4*, by GATA-2 was determined by chromatin immunoprecipitation assay in differentiating embryoid bodies (Lugus *et al*. 2007). It is interesting to note that the GATA-2/BMP4 relationship was suggested to be non-linear, in which case both factors could reciprocally modulate the expression of one another (Lugus *et al*. 2007). Given that the nearly complete loss of GATA-2 in *Gata2*$^{fGN/fGN}$ mice reduces, but does not abolish, BMP4 expression, we would surmise that GATA-2 is not the sole effector of *Bmp4* transcription, but is required for maintenance of its transcript abundance. Regardless of the specific underlying mechanisms, the regulatory axis formed by GATA-2 and BMP4 clearly appears to be crucial for urogenital development, and elucidation of their roles in this developmental pathway may lead to a better understanding of this extremely common pediatric disease.

Experimental procedures

Animals

This experiment was approved by the Institutional Animal Experiment Committee and by the Regulation for Animal Experiments of the University of Tsukuba

Generation of *Gata2* mice

The targeting construct was electroporated into E14 ES cells and then subjected to a positive and negative selection with DT and neomycin (300 μg/mL). ES cell colonies were isolated, expanded, and screened for homologous recombination by PCR and confirmed by Southern blot analyses. Isolated ES cells were injected into C57BL/6J blastocysts and then surgically implanted into pseudopregnant ICR foster mothers. Chimeric mice bearing the mutant *flox-Gata2-Neo* (*Gata2*^{fGN}) allele were then intercrossed with C57BL/6J mice and the offspring were genotyped by PCR using primers that detect wild-type [5′-tggttcccaagacacagtagtgga-3′ (sense; s) and 5′-agtccctgcctccatcttcttagg-3′ (antisense; as)] or mutant [5′-aagtatccatcatggctgatgc-3′ (s) and 5′-tagccaacgctatgtcttgata-3′ (as)] alleles. *Gata2*$^{fGN/+}$ mice were crossed with wild-type BDF1 mice for maintenance.

Phenotypic analyses

Gata2$^{fGN/+}$ mice were intercrossed and the newborn progeny were analyzed for phenotypes. Dissected newborn kidneys were fixed in phosphate-buffered saline (PBS) containing 10% formaldehyde and then individually measured along their long and short axes using a micrometric grid. Kidneys were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and subsequently were sectioned into 7 μm slices for hematoxylin and eosin staining.

In situ hybridization analysis and immunohistochemistry

In situ hybridization was performed upon the method as described (Wilkinson & Nieto 1993). Briefly, digoxygenin-UTP labeled antisense riboprobes were prepared from *shh* template (generously provided by Dr Noji, University of Tokushima). The murine *c-ret* template was synthesized by PCR from embryonic RNA using primers 5′-acctgagtgcaccaagcttc-3′ (s) and 5′-cagctaagtcccgatgtacaagc-3′ (as). The amplified fragment was cloned into pGEM-T Easy Vector (Promega, Madison, WI), and *c-ret* antisense and sense riboprobes were synthesized using Sp6 and T7 polymerases. For whole mount photography, embryos were cleared in benzyl alcohol–benzyl benzoate (2 : 1).

Embryos (E11.0) were fixed in PBS containing 4% paraformaldehyde for 1.5 h at 4 °C and then equilibrated in 20% sucrose for 4 h prior to embedding in the OCT compound. For antibody staining, thin sections were treated with H_2O_2 and blocked with 2% skim milk, 1% goat serum and 0.25% bovine serum albumin in PBS. Purified rabbit anti-GFP antibody (1 : 1000) was then added and incubated overnight at 4 °C. The sections were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1 : 500) at room temperature for 1 h. Color development was carried out using diaminobenzidine as chromogen.

RT-PCR analyses

Intermediate mesoderm derivatives (containing the mesonephros, the metanephric anlagen as well as the undifferentiated gonads) were dissected *en bloc* from individual E10.5 embryos for total RNA isolation using an Isogen RNA preparation kit (Nippon Gene, Tokyo, Japan). The cDNA samples were synthesized with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). For semi-quantitative analyses, each cDNA was diluted so that the HPRT amplicons are equivalent amongst the test samples. Primers used were: *Gata2* 5′-tcaaccacttcgactcgcag-3′ (s) and 5′-cggtgacttctcttgcatgca-3′ (as); *Bmp4* 5′-ctcccaagaatcatggactg-3′ (s) and 5′-aaagcagagctctcactggt-3′ (as); *Gdnd* 5′-accagataaacaagcggcag-3′ (s) and 5′-tcagatacatccacaccgtttag-3′ (as); *Alk3* 5′-tgactcagctatacacttacatcag-3′ (s) and 5′-caggtctttcagtgattctcc-3′ (as); *Foxc1* 5′-gcggaaattgtaggagttccctag-3′ (s) and 5′-tttggcatctggctcacagg-3′ (as); *Foxc2* 5′-acgagtgcggatttgtaaccag-3′ (s) and, 5′-gtgtttttggaatacccca-gatgg-3′ (as); *Rarα* 5′-cagttccgaagagatagtacc-3′ (s) and 5′-tacaccatgttcttctggatgc-3′ (as); *Rarβ* 5′-tcgagacacagagtaccagc-3′ (s) and 5′-gaaaagcccttgcacccct-3′ (as); *Rarγ* 5′-gcctcctcgggtctacaag-3′ (s) and 5′-atgatacagtttttgtcgcgg-3′ (as); *Hprt* 5′-gctggtgaaaaggacctct-3′ (s), and 5′-cacaggactagaacacctgc-3′ (as).

Co-transfection assay

Upstream and intronic regulatory fragments of *Bmp4* gene were synthesized by PCR using mouse genomic DNA as a template. Primer sequences were 5′-ccctttgagagctacctgaa-3′ (s) 5′-cagagactggatcgctgca-3′ (as) for Upstream fragment and 5′-cggtttctgaaggatctgcttg-3′ (s) and 5′-tttcttcccggtctcaggtatc-3′ (as) for Intronic fragment. We generated two types of reporter plasmids for the luciferase assays. One reporter (Intronic-Luc) was generated by inserting a 1.8-kbp *Bmp4* Intronic fragment (containing the a part of the IB exon, the entire first intron and II exon, and almost entire second intron) into the *Sac*I site of pGL3 (Promega), while the other (Upstream-Luc) was generated by replacing the SV40 promoter of pGL3 with a 1.3-kbp fragment containing the mouse *Bmp4* Upstream fragment (containing upstream GATA motifs and a part of the IA exon). The parental vectors, with or without the SV40 promoter, were used as the controls for Intronic-Luc and Upstream-Luc, respectively. QT6 cells were transfected with 0, 60 or 180 ng of pEF-GATA-2 expression vector together with 10 ng of reporter plasmids using Fugene 6 (Roche, Basel, Switzerland). pRL-TK (Promega) was co-transfected in every well to serve as an indicator of transfection efficiency. The total amount of DNA transfected in each well was adjusted to 200 ng with empty pEF vector. All luciferase assays were executed using the Dual-Luciferase reporter assay system according to the manufacturer's protocol (Promega) and normalized to Renilla luciferase activity.

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