BMB
reports

Geft is dispensable for the development of the second heart field

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Geft is a guanine nucleotide exchange factor, which can specifically activate Rho family of small GTPase by catalyzing the exchange of bound GDP for GTP. Geft is highly expressed in the excitable tissue as heart and skeletal muscle and plays important roles in many cellular processes, such as cell proliferation, migration, and cell fate decision. However, the *in vivo* role of Geft remains unknown. Here, we generated a Geft conditional knockout mouse by flanking exons 5-17 of Geft with loxP sites. Cre-mediated deletion of the Geft gene in heart using Mef2c-Cre transgenic mice resulted in a dramatic decrease of Geft expression. Geft knockout mice develop normally and exhibit no discernable phenotype, suggesting Geft is dispensable for the development of the second heart field in mouse. The Geft conditional knockout mouse will be a valuable genetic tool for uncovering the *in vivo* roles of Geft during development and in adult homeostasis. (BMB reports 2012; 45(3): 153-158)

INTRODUCTION

The Rho family of small GTPases, including RhoA, Rac1, and Cdc42, controls a wide range of cellular processes (1-4). The activation of Rho-GTPases requires the exchange of GDP for GTP, a process catalyzed by the Dbl family of guanine nucleotide exchange factors (GEFs) (5). It was well documented that the Rho family of small GTPases plays important roles in cardiac development. In mice, inhibition of RhoA, Rac1 and Cdc42 results in embryonic lethality, cardiac morphogenesis disruption, incompleted looping, loss of chamber demarcation and a reduction in cell proliferation (6). In addition, cardiac-specific overexpression of Rac1 in mice results in either a lethal neonatal dilated cardiomyopathy or a resolving transient cardiac hypertrophy (7). Recently, some evidence shows that GEFs play an essential role in cardiogenesis (8, 9). For example, knockdown of Obscurin A, a Rho-GEF, shows cardiac abnormalities ranging from mild cardiac dilatation to severe cardiac hypoplasia in zebrafish (8). Mice null of AKAP13, an AKAP-GEF, display deficient sarcomere formation and thin-walled hearts, and died at E10.5-11.0 (9).

Geft (a new guanine nucleotide exchange factor) is identified as a Rho-family-specific GEF, which is highly expressed in the excitable tissue such as brain, heart and skeletal muscle from the early embryonic stage (10). It was reported that Geftcomprises primarily the Dbl homology domain (DH domain) and the pleckstrin homology domain (PH domain). The catalytic guanine nucleotide exchange activity resides in DH domain, whereas PH domain plays important regulatory roles by serving as protein-protein interaction module (11). Previous evidence has showed that Geft regulates cell proliferation and migration (10, 12), neurite outgrowth (13), and mesenchymal cell fate decision (12), through interacting with and catalysing GDP/GTP exchange at RhoA, Rac1 and Cdc42. However, the *in vivo* function of Geft remains elusive.

In this study, we investigated whether Geft participated in heart development. We generated a Geft conditional knockout mouse, and deleted Geft gene in early embryonic heart using Mef2c-Cre transgenic mice. Cadiac-specific Geft knockout mice developed normally and exhibited no discernable phenotype. Our work, for the first time, provided insight into the *in vivo* role of Geft in heart development. Moreover, the Geft conditional knockout mice will be a valuable genetic tool for uncovering the *in vivo* roles of Geft during development and in adult homeostasis.

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http://dx.doi.org/10.5483/BMBRep.2012.45.3.153

Received 23 September 2011, Revised 20 October 2011, Accepteed 29 October 2011

Keywords: Conditional null allele, Cre-loxP, FLPe-Frt, Geft, Heart development

RESULTS AND DISCUSSION

Generation of the Geft conditional null allele

The murine Geft gene consists of 17 exons spanning ~7.4 kb on chromosome 10. Geft comprises primarily the Dbl homology (DH) and pleckstrin homology (PH) domains with short N- and C-terminal sequences (14,15). To completely knockout the Geft activity through Cre-mediated excision, we decided to target the genomic region from exon 5 to exon 17, which encodes the entire DH and PH domains. To make the Geft conditional targeting construct (Fig. 1A), we used an efficient recombineering-based system mediated by the λ phage Red proteins (16). Following subcloning of a 12.1 kb DNA fragment containing the whole Geft sequences, a PGKneo selection cassette, flanked by Frt sites and one loxP site at its 3′ region, was introduced into intron 4, and a second loxP was placed into a

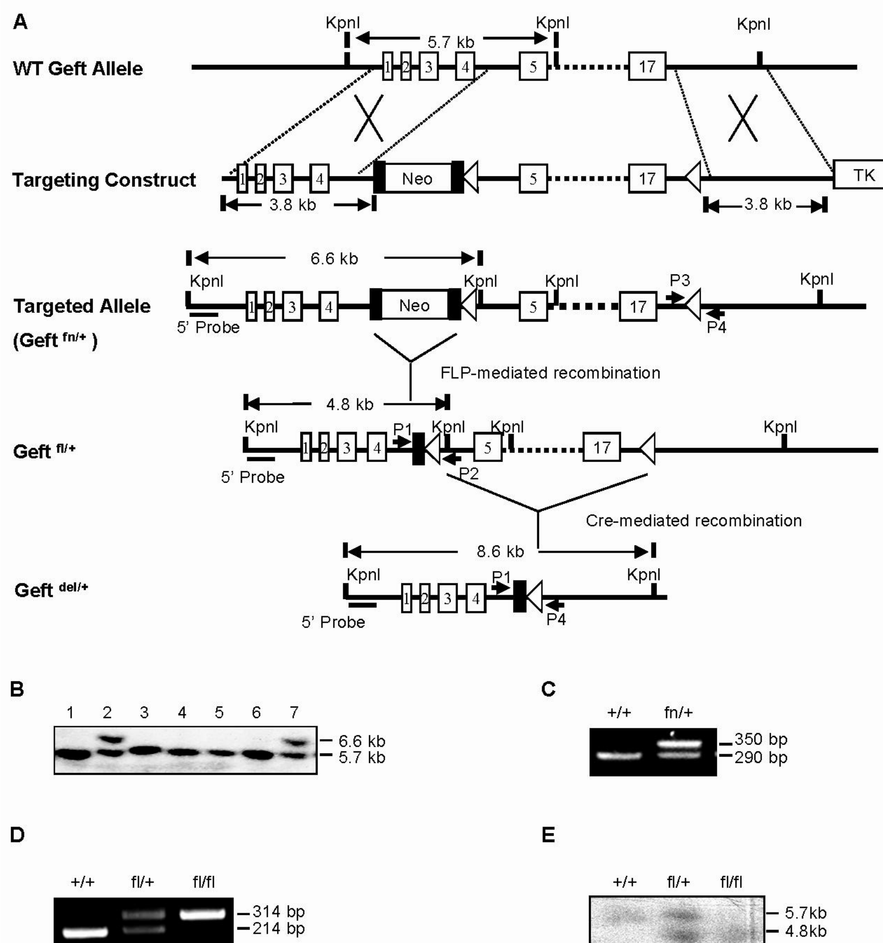

Fig. 1. Generation of the Geft conditional null allele. (A) Schematic representation of the wild type Geft allele, the Geft1^{fn} targeting vector, the targeted Geft1^{fn} (Geft1$^{fn/+}$) allele, the Geft1^{fl} (Geft1$^{fl/+}$) allele, and Geft1^{del} (Geft1$^{del/+}$) allele. The targeting region consisted of a 3.8 kb 5′ arm of homology, a 4.5 kb core region containing exons 5-17, and a 3.8 kb 3′ arm of homology. The Frt-PGKneo-Frt-LoxP cassette was inserted into intron 4 and the second LoxP site was inserted 530 bp downstream of exon 17 of the Geft gene. A TK cassette was inserted dowmstream of 3′ arm of homology. FLPe-mediated excision of Frt-PGKneo-Frt-LoxP cassette from the Geft1^{fn} allele resulted in the Geft1^{fn} allele, and then Cre-mediated excision of LoxP sites led to Geft1^{del} allele. The open boxes numbered 1-17 represented the exon 1 to exon 17. Open triangles represented the loxP sequence, and black boxes indicated Frt sites. The bars represented the 5′ external probe for Southern blot analysis and the arrows indicated the position for primers P1, P2, P3, and P4. (B) Southern blot analysis of targeted ES cell clones. Genomic DNA digested by Kpn I was hybridized with 5′ probe indicated in (A), yielding 5.7 kb wild type and 6.6 kb targeted bands. (C) PCR genotyping of mice carrying Geft$^{+/+}$ and Geft$^{fn/+}$ alleles using the primer pair P3 and P4. (D) PCR genotyping of mice carrying Geft$^{+/+}$, Geft$^{fl/+}$ and Geft$^{fl/fl}$ alleles using the primer pair P1 and P2. (E) Southern blot analysis of mice carrying Geft$^{+/+}$, Geft$^{fl/+}$ and Geft$^{fl/fl}$ alleles.

site 530 bp downstream of exon 17. The design of the $Geft^{fn}$ (fn denotes floxP-neo) allele permitted removal of the PGKneo cassette to generate the floxed Geft allele ($Geft^{fl}$) by using a Flpe deleter mouse line (17), or removal of exons 5-17 of Geft gene to generate the $Geft^{del}$ allele by mating to a Cre deleter line (Fig. 1A).

Targeted ES cells with correct homologous recombination were identified by Southern blot analysis using a 5′ flanking probe (Fig. 1B), and were injected into C57BL/6J blastocysts. Chimeras were bred with C57BL/6J female mice, and germline transmission was confirmed by PCR analysis (Fig. 1C). $Geft^{fn/+}$ heterozygous mice were bred with a deleter line expressing the Flpe recombinase to excise the Frt-flanked pGKneo cassette and generate the Geft floxed heterozygous mice ($Geft^{fl/+}$). Deletion of this cassette was confirmed by PCR detection of the loxP-Frt sites that remained after Flpe-mediated recombination (Fig. 1D) and Southern blot analysis using the above 5′ probe (Fig. 1E). Both $Geft^{fl/+}$ and $Geft^{fl/fl}$ mice were viable, fertile, and presented no genotype dependent differences in gross morphological abnormalities, indicating that the floxed Geft allele was a functional Geft allele.

Generation of the heart-specific Geft-null mice

To assess the *in vivo* excision of the Geft conditional knockout allele, the $Geft^{fl/fl}$ mice were bred with Mef2c-Cre transgenic mice, where Cre recombinase is expressed in the second heart field during heart development after E7.5 (18). Cells from the second heart field eventually contribute to form right ventricle and interventricular myocardium (19).

Mef2c-Cre-mediated deletion of the loxP-flanked exons 5-17 of Geft was confirmed by PCR analysis using a primer set that spanned exons 5-17, which showed a novel 400 bp product specifically in the $Geft^{fl/fl}$; Mef2c-Cre heart tissue (Fig. 2A, lane 3), but not in the $Geft^{fl/+}$; Mef2c-Cre tail tissue where Cre recombinase does not express (Fig. 2A, lane 2). Furthermore, Southern blot analysis verified the correct excision in the heart tissue of $Geft^{fl/+}$; Mef2c-Cre mice (Fig. 2B). Real-time PCR analysis revealed that homozygous Geft mutant heart expressed 36 $\pm$ 8% of control Geft transcripts (Fig. 2C). Consistently, Western blot analysis also revealed that Geft expression was dramatically decreased in the homozygous Geft mutant heart compared with control heart (Fig. 2D). A small amount of Geft RNA and protein were detected in the homozygous Geft mutant heart, possibly due to the presence of cells did not come from second heart field. These data demonstrated that this Geft allele was recombined to delete exons 5-17 in heart tissue.

Geft is dispensable for mouse second heart field development

Previous study indicates that Geft is highly expressed in adult heart tissue (10). RT-PCR analysis revealed that Geft was detected in the second heart field at E11.5 and E12.5 when cardiac chamber forms (Fig. 3A).

$Geft^{fl/fl}$ mice were mated with $Geft^{fl/+}$; Mef2c-Cre mice to obtain Geft knockout mice ($Geft^{fl/fl}$;Mef2c-Cre). This mating resulted in 4 genotypes ($Geft^{fl/fl}$, $Geft^{fl/+}$, $Geft^{fl/fl}$ Cre and $Geft^{fl/+}$ Cre) of offsprings. $Geft^{fl/fl}$; Mef2c-Cre mice were born with an expected Mendelian ratio (~25%, Fig. 3B), and survived until adulthood without visible abnormalities.

In morphology the size of the heart in Geft knock out mice were normal compared to control (Fig. 3C), and hematoxylin and eosin staining showed a normal structure of right ventricle and interventricular myocardium in Geft knock out heart compared with controls (Fig. 3C). We detected no significant changes in heart to body weight and tibia length ratios compared with control mice at the age of one month (Fig. 3D), suggesting no cardiac hypertrophy in Geft mutant heart.

These results demonstrated that Geft is dispensable for the development of the second heart field giving rise to the right ventricle and outflow tract. A reasonable explanation was that a gene redundancy mechanism existed in mouse heart development compensating for the loss of Geft function. In fact, an accumulating body of evidence indicated that other GEFs such as AKAP13 (10), Scambio (20), p114RhoGEF (21), Clg (22) and Trio (23) are abundant in heart. Previous studies have demonstrated that in adult, the Rho family of small GTPases play critical role in cardiac hypertrophy (24). We speculated that under the conditions of various heart stresses, Geft deletion might

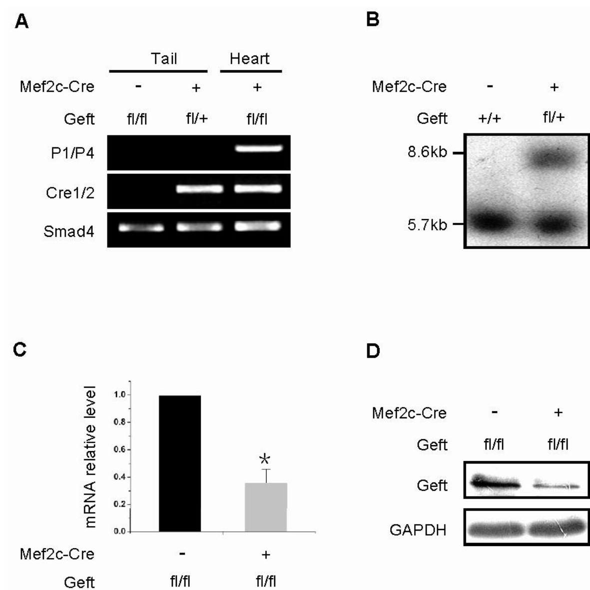

Fig. 2. Generation of the heart-specific $Geft1^{del}$ mice. (A) PCR analysis of the tail or heart tissues from $Geft^{fl/fl}$, $Geft^{fl/+}$; Mef2c-Cre and $Geft^{fl/fl}$; Mef2c-Cre; and mice carrying $Geft^{fl/fl}$ and Mef2c-Cre(-) developed normal. (B) Southern blot analysis of the heart tissues from $Geft^{fl/fl}$ and $Geft^{fl/+}$; Mef2c-Cre mice. (C) Real-time PCR result showed a significant decrease of Geft transcripts in $Geft^{fl/fl}$ Mef2c-Cre heart. *$P < 0.05$. (D) Western blot analysis revealed that Geft expression was dramatically decreased in the homozygous Geft mutant heart compared with control heart.

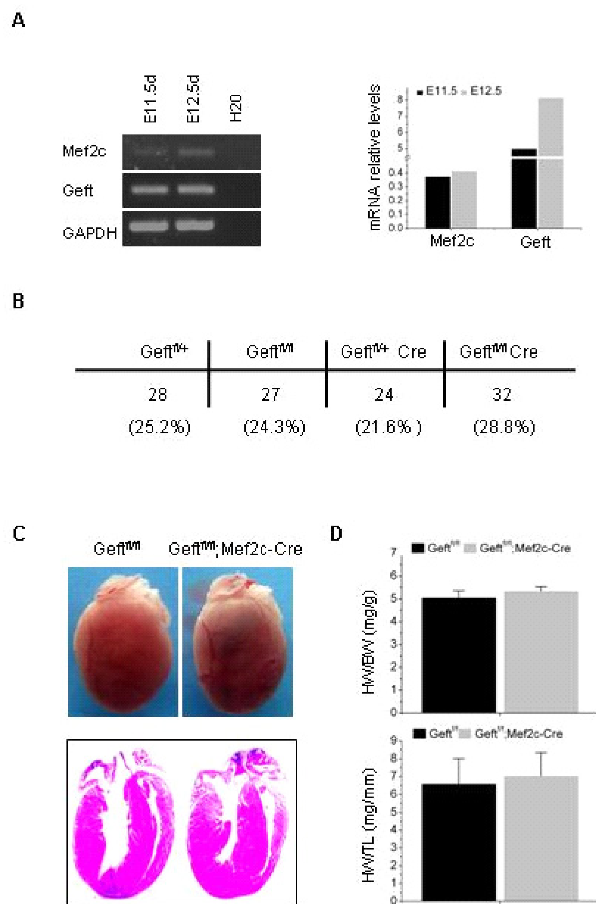

Fig. 3. Geft is dispensable for the development of second heart field (A) RT-PCR (left) and Real-time PCR (right) showed Geft highly expressing in the second heart field at E11.5 and E12.5. (B) Mating of Geft$^{fl/fl}$, Geft$^{fl/+}$; Mef2c-Cre resulted in offspring of four genotypes at normal Mendelian ratios. (C) Top: photographs of Geft$^{fl/fl}$, Geft$^{fl/+}$; Mef2c-Cre were taken at the age of one month (originally magnification: 10×). Bottom: Hematoxylin and eosin staining shown a normal structure in one month age heart tissue of Geft$^{fl/fl}$, Geft$^{fl/+}$; Mef2c-Cre (originally magnification: 10×). (D) Heart weight to body weight and tibia length ratios were obtained from adult male Geft$^{fl/fl}$, Geft$^{fl/+}$; Mef2c-Cre at age of one month. SD, n = 5, P > 0.2, HW (heart weight), BW (body weight), TL (tibia length)

lead to abnormal response to these stresses, which need to be further investigated. Moreover, Geft is also highly expressed in the brain, skeletal muscle and lens. Former study found that gene transfer of Geft into cardiotoxin-injured mouse tibialis anterior muscle exerts a powerful promotion of skeletal muscle regeneration *in vivo* and inhibits insulin-induced adipogenesis *in vitro* (10, 12). Previous study also examined the role that Geft plays in the regulation of dendritic spine morphogenesis and neurite outgrowth (13). Through the thoughtful use of tissue specific Cre-expressing mouse strains, deletion of Geft in brain and skeletal muscle tissue will offer further insight into the roles of Geft in above issues.

In summary, we found that Geft was dispensable for the development of the second heart field in mouse. The Geft conditional knockout mouse will be valuable for the investigation of Geft function in development and tissue homeostasis.

MATERIALS AND METHODS

Construction of the Geft^{fn} allele

The Geft^{fn} conditional gene-targeting vector was constructed using recombineering-based system mediated by the λ phage Red protein as previously described (16). A 12.1 kb fragment containing the whole Geft sequences was subcloned from the BAC clone bMQ-117G10 into the plasmid pL253, a modified vector containing a thymidine kinase (TK) negative selection cassette. The 3′ loxP site was inserted into 530 bp downstream of exon 17 by bacterial recombineering. A Frt-PGKneo-Frt-loxP cassette with a KpnI site for Southern blot screening was inserted into intron 4 by bacterial recombineering.

Electroporation of ES cells and generation of Geftfn mice

The Geft^{fn} targeting vector was linearized with NotI, and electroporated into ES cells (25). ES cells were subjected to selection with G418- and gancyclovir-resistant clones were screened for homologous recombination by Southern blot using KpnI digestion and the 5′ external probe with a 5.7 kb WT band and a 6.6 kb targeted band. A 500 bp genomic fragment used as the 5′ external probe was amplified by using the primer set Geft5ps/5pa (Geft5ps: 5′ ACCTGACTAGGGTGCAAGA3′; Geft 5pa: 5′ CAAAGACAGACACGGGAGA 3′). The primer set P3/P4 (p3: 5′TCCTTTGGTTCAGACTCC3′; p4: 5′CTTGCCTGTCT CTGTTCC3′) for screening the correct insertion of 3′ loxP site yielded a 290 bp WT band and 350 bp floxed band. Nine colons showed the correct homologous recombination, and two colons were microinjected into C57BL/6J blastocysts. Five chimeras were bred with C57BL/6 female mice to obtain germ line transmission. The Geft^{fn} mice were genotyped by PCR using the primer pairs P3/P4 and Southern blot using the above 5′ probe.

Generation of Geft^{fl} and Geft^{del} mice

Geft^{fn} heterozygous mice were mated to Flpe transgenic to delete the Frt-flanked pGKneo cassette to produce the Geft^{fl} allele. Geft^{fl} mice were genotyped with the primer pairs P1/P2 (P1: TTCTGGTCTCTGTGGGTA; P2: GGACTTGTTTGTAGGG TC) spanning the remained loxP-Frt sites with 214 bp WT band and 314 bp floxed band. Southern blot analysis using the above 5′ probe showed a 4.8 kb band in Geft^{fl} allele. Geft^{del} mice were generated by crossing the Geft^{fl} mice with Mef2c-Cre transgenic mice (18) to delete exons 5-17 of Geft in the heart. Geft^{del} mice were genotyped using the primer pairs P1/P4 spanning the entire deleted region, yielding a 400 bp

band. Southern blot analysis using the above 5′ probe showed a 8.6 kb band in Geft^{del} heart. All PCR reactions were carried out under the following conditions: 94°C for 5 min, 30 cycles at 94°C for 15 s, 60°C for 30 s, 72°C for 60 s, and an extension step at 72°C for 5 min.

mRNA isolation and real time PCR

Total RNA was extracted from heart tissue using Trizol reagent (Invitrogen). Real-time PCR was performed by a SYBR Green assay with Roche Light Cycler 2.0 system as previously described (26). Expression values were normalized to GAPDH expression. The primer sequences were as follows: RtGe^{ft}: 5′-CAACGCATTACAGTCACC-3′ and 5′-ACAGCACTCTGGAC ACCT-3′; GAPDH: 5′-TGCCCAGAACATCATCCCT-3′ and 5′-GGTCCTCAGTGTAGCCCAAG-3′; Mef2c: 5′-CCCCTTCGAGA TACCCACAA-3′ and 5′-GAAGGTCTGGTGAGTCCAATGG-3′.

Western blot

The protein from heart was collected at days 30 after birth. The tissue lysates were separated by 12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). Antibodies for Western blot analysis: Geft (sc-161639 ; Santa Cruz) at a 1 : 200 dilution and GAPDH (Sigma) at a 1 : 4,000 dilution as previously described (27).

Histology

The hearts were fixed in 4% PFA overnight, then dehydrated through an increasing ethanol series and transferred to paraffin. Paraffin blocks were 6 μm-thick sectioned and transferred onto superfrost slides. Hematoxylin and eosin staining were perform as previously described (28).

Acknowledgements

We thank Xiang Gao for kindly providing the phage-based homologous recombination system and FLPe transgenic mice, and Zhong-Zhou Yang for Mef2c-Cre transgenic mice. This study was supported in part by the National Natural Science Foundation of China (Nos. 30930054, 30930054, 30970425, 30971105, 30900851, 31171402).

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