Generation of a Mouse Model for a Conditional Inactivation of Gtf2i Allele

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Summary: The multifunctional transcription factor TFII-I encoded by the Gtf2i gene is expressed at the two-cell stage, inner cell mass, trophectoderm, and early gastrula stages of the mouse embryo. In embryonic stem cells, TFII-I colocalizes with bivalent domains and depletion of Gtf2i causes embryonic lethality, neural tube closure, and craniofacial defects. To gain insight into the function of TFII-I during late embryonic and postnatal stages, we have generated a conditional Gtf2i null allele by flanking exon 3 with loxP sites. Crossing the floxed line with the Hprt-Cre transgenic mice resulted in inactivation of Gtf2i in one-cell embryo. The Cre-mediated deletion of exon 3 recapitulates a genetic null phenotype, indicating that the conditional Gtf2i line is a valuable tool for studying TFII-I function during embryonic development. genesis 54:407–412, 2016. © 2016 Wiley Periodicals, Inc.

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TFII-I is a ubiquitously expressed transcriptional factor involved in both basal transcription and signal-induced transduction activation or repression (Roy, 2007, 2012). The GTF2I gene encoding TFII-I is the prime candidate responsible for cognitive abnormalities in the Williams-Beuren syndrome and autism spectrum disorders (Malenfant et al., 2012; Sakurai et al., 2011). In developing mouse embryos, TFII-I exhibits distinct and dynamic expression pattern (Enkhmandakh et al., 2004; Fijalikowska et al., 2010; Ohazama and Sharpe, 2007). Gtf2i is often subjected to alternative splicing, which generates TFII-I isoforms with different activities and distinct biological roles (Makeyev and Bayarsaihan, 2009).

Previously, we identified a set of developmental genes as direct TFII-I targets revealing the complexity of TFII-I-mediated processes associated with distinct regulatory networks (Chimge et al., 2008; Enkhmandakh et al., 2009; Makeyev and Bayarsaihan, 2011; Makeyev and Bayarsaihan, 2013). Genome-wide mapping studies revealed TFII-I occupancy at multiple genomic sites in mouse embryonic stem (ES) cells and embryonic tissues (Bayarsaihan et al., 2012; Makeyev et al., 2012). Moreover, many TFII-I-bound regions colocalize with H3K4me3/K27me3 bivalent chromatin in the promoters of lineage-specific genes (Adamo et al., 2015; Bayarsaihan, 2013; Makeyev et al., 2012). Interestingly, TFII-I appears to have specific roles in distinct phases of the mammalian cell cycle (Ashworth and Roy, 2009).

TFII-I plays an inhibitory role in regulating genes that are essential in osteogenesis and moreover, it has the ability to intersect with the bone-specific transcription factor Runx2 and the retinoblastoma protein Rb (Lazebnik et al., 2009). Activation of PI3K/Akt signaling, which is sufficient to maintain the pluripotency of mouse ES cells, results in down-regulation of Gtf2i (Chimge et al., 2012). Moreover upregulation of Gtf2i leads to activation of a specific group of developmental genes during ES cell differentiation.

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The mouse Gtf2i gene is composed of 34 exons spanning approximately 77 kb region on chromosome 5G2 (Bayarsaihan et al., 2002). A targeting vector for the generation of a Gtf2i conditional allele was constructed following a procedure described elsewhere (Liu et al., 2003). Briefly, one loxP site was inserted into intron 2, while a second loxP site along with the PGK neo selectable marker gene flanked by two Frt sites were introduced into intron 3 (Fig. 1a).

The final targeting vector has the 5' end long homology arm of approximately 4.3 kb in size, spanning from intron 1 to exon 2, and a 3' short arm of 3.3 kb, spanning from intron 3 to exon 4 including the HSV TK negative selectable marker (Fig. 1a). The removal of exon 3 will result in complete loss of Gtf2i allele that deletes the entire TFII-I protein because exon 3 is common to all known transcript variants (Makeyev and Bayarsaihan, 2009). The truncated protein product is very short and lacks important functional regions including nuclear localization signal and DNA-binding domains.

The purified vector was electroporated into the D2 embryonic stem (ES) cell line and colonies resistant to G418 and ganciclovir were screened by long-range PCR screening of the long and short arms to identify properly targeted clones (Fig. 1c). Ten correctly targeted clones were identified from several dozen G418-resistant colonies. Two-independent ES clones were used to generate mouse chimeras by morula aggregation with CD1 wild-type embryos. Chimeric males were bred with ROSA26-Flpe females to test for germline transmission and removal of the PGKneo cassette in the targeted allele. F1 mice were genotyped initially by PCR analysis using tail genomic DNA samples for the presence of the targeted Gtf2i allele. Southern blots containing DNA samples of positive clones were rehybridized with the probe from intron 1 to verify the expected integration of a loxP-neo-loxP cassette in the third intron (Fig. 1b). Heterozygous mice were intercrossed and genotyping of F2 progeny a week after birth detected homozygous Gtf2i null mice at the expected Mendelian ratio. The homozygous Gtf2i null mice exhibited a normal life span and fertility. The resultant Gtf2i null mice were intercrossed to generate homozygous Gtf2i null progeny with no detectable abnormalities. To test for Cre-mediated inactivation of the Gtf2i conditional allele in vivo, we crossed the Gtf2i null mice with Hprt-Cre mice, which express Cre in one-cell embryo causing deletion of loxP-flanked sequences in most tissues, including cells of the developing germ line. The resultant Gtf2i null mice were normal, whereas analysis of embryos from Gtf2i null heterozygous intercrosses revealed that the Gtf2i null mutant embryos died before birth. The Gtf2i null embryos exhibit the same phenotype as the previously reported Gtf2i null embryos (Enkhmandakh et al., 2009). Depletion of Gtf2i resulted in embryonic lethality before E10.5 (data not shown). Collectively, these results indicate that Cre-mediated deletion of exon 3 abolished the function of Gtf2i gene.

**FIG. 1.** The generation of a conditional Gtf2i knockout mouse model. (a) The schematic representation of the targeting strategy for producing the Gtf2i conditional allele. The region including exon 3 of the wild-type Gtf2i locus is shown, and the approximate sites of the 5' and 3' flanking probes used for screening ES cell clones. The loxP sites and Frt sites are marked by triangles and gray ovals, respectively. (b) Southern blot analysis of targeted ES clones after Sacl digestion was screened for homologous recombinants. The Southern probe hybridizes to the wild-type fragment of 5.6 kb, floxed fragment of 5.8 kb, and a fragment of 3.6 kb from the correctly targeted clone. (c) PCR genotyping of a litter of a correctly targeted ES clone from intercross of a pair of Gtf2i null mice. A PCR product of 319 bp produces from wild-type allele, whereas a PCR product of 424 bp was amplified from the Gtf2i null allele.
Chromatin immunoprecipitation tiling assays were used to confirm that ES cells derived from \textit{Gtf2i} embryos did not produce a functional TFII-I protein. Previously, we demonstrated that TFII-I binds to the regulatory elements containing either the core RGATTR motifs or the E-boxes within the promoters of key developmental genes (Makeyev et al., 2012). While TFII-I occupies its cognate promoters in wild-type ES cells, no such binding was observed in the \textit{Gtf2i} depleted cells (Fig. 2). Next, we investigated the expression pattern of a number of developmental genes affected by \textit{Gtf2i} inactivation. Whereas some genes such as \textit{Gata4} and \textit{Esrrb} were expressed at significantly lower level, the expression of \textit{Nodal} and \textit{Pou5f1} was elevated in the \textit{Gtf2i} cells (Fig. 3). Therefore, the generated \textit{Gtf2i} conditional line can be a valuable mouse model for dissecting the mechanisms by which TFII-I transcription factor regulates cell differentiation and tissue-specific gene expression.

**MATERIALS AND METHODS**

**Generation of the \textit{Gtf2i} Conditional Allele**

After the sequence confirmation by NotI digestion, the purified vector was electroporated into the D2 embryonic stem (ES) cell line derived from F1 (C57BL/6jx129SV) embryos. Colonies resistant to G418 and ganciclovir were screened by long-range PCR screening.
of the long and short arms to identify properly targeted clones. The primer Gtf2iScrF1 lying outside of the 5′ homology arm and the primer PL452-loxp-sc1R specific to the 5′ loxP site were used to detect the proper integration of a long homology arm. The primer Gtf2i Scr1 resides outside of the 3′ homology arm and the primer PL452-3′-sc1F, which is specific to the 3′loxP site were used to detect the 3′arm. Ten correctly targeted clones were identified among 48 G418-resistant colonies. Two-independent ES clones were used to generate mouse chimeras by morula aggregation with CD1 wild-type embryos. Resulting chimeric males were bred with ROSA26-Flpe females (Jax stock no: 009086) to test for germine transmission and removal of the PGKneo cassette in the targeted allele. Both clones resulted in germ-line transmission and removal of the PGKneo cassette in the targeted allele. Southern blots containing DNA samples of positive clones were rehybridized with the probe from intron 1 to verify the expected integration of a loxP-neo-loxP cassette in the third intron. Heterozygous mice were intercrossed and genotyping of F2 progeny a week after birth detected the 3′ integration of a long homology arm. The primer Gtf2i Scr1 (5′-GGCGTGCCCCGGGACTAGATG3′ (forward) and 5′-GGCGTTACTGTGGCCGAGCC-3′ (reverse). Fifteen micrograms of SacI-digested genomic DNA was run by PCR from wild-type genomic DNA using primers PL452-3′-sc1F (5′-AGC-3′) and Frt gtR (5′-AGC-3′), PL452-loxp-sc1R (5′-GAGGGACCTAATAACTTCGT-3′). Southern Blot and PCR Genotyping

The 1084 bp repeat-free fragment from intron 1 was selected as a probe for a Southern blot and amplified by PCR from wild-type genomic DNA using primers 5′-GCCGTGCCCGGGACTAGATG3′ (forward) and 5′-GGCGTTACTGTGGCCGAGCC-3′ (reverse). Fifteen micrograms of SacI-digested genomic DNA was run using agarose gel electrophoresis and after the transfer to a positively charged nylon membrane BrightStar-Plus (Ambion), DNA was hybridized with the biotinylated probe (NEBlot Phototope Kit, NEB) according to the manufacturer’s instructions. The hybridization signals were registered using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and ChemiDoc MP gel imaging system (Bio-Rad) equipped with CCD camera.

For the PCR genotyping, we used Lox gtF (5′-CCA AGG TTT CAG CTT TGG AG-3′) and Frt gtR (5′-AGC CAC TCC AAC AGT TAC CG-3′) primers. The PCR product of 319 bp was detected in wild-type cells, while in Gtf2iF3/ES cells a 424 bp fragment was amplified as expected. Additional primers used in the study were: Gtf2iScrF1 (5′-CTCAGAAATTCACCTGCTG3′), PL452-loxp-sc1R (5′-GAGGGACCTAATAACTTCGT3′), Gtf2i Scr1 (5′-GCAAGGAGAGAATGCTGCTG3′), PL452-3′-sc1F (5′-CTTCTGAGGGAGAAGACCA3′).
qRT-PCR Profiling

Change in the expression of selected genes derived Gtf2irKO/D (TFII-I KO) and normal (WT) mouse ES cells at early stage of differentiation (3 d after LIF withdrawal) was analyzed by qRT-PCR using Mouse Stem Cell RT2 Profiler PCR Array PAMM-405A (Qiagen) containing ESC differentiation markers (Fig. 3). Total RNA was isolated from cells using RNeasy Kit (Qiagen) and cDNA was prepared using RT2 First Strand Kit (Qiagen). Real-time measurements were performed using ABI 7900HT instrument (Applied Biosystems) and RT2 SYBR Green ROX qPCR Mastermix (Qiagen).

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed essentially as it was described in Makeyev et al. (2012) using ChIP-IT kit (Active Motif, CA). Chromatin was immunoprecipitated from $2 \times 10^6$ ES cells with rabbit anti-TFII-I polyclonal antibodies 4562 (Cell Signaling Technology, MA). After reverse cross-linking and purification on QIAquick spin columns (Quagen, CA), immunoaffinity-enriched DNA fragments (IP) and the input samples were amplified using whole-genome GenomePlex Complete WGA kit (Sigma-Aldrich, MO). IP and input samples were labeled in separate reactions with Cy5 and Cy3, respectively, and then were cohybridized to Mouse 385K RefSeq Promoter Arrays (Roche NimbleGen, WI). The data were extracted using NimbleScan software. Peaks were detected by searching for four or more probes with a signal above a cut-off value using a 500 bp sliding window. A log2 ratio of the IP versus input samples was calculated and the transcription start site mapping was performed using SignalMap software (Roche NimbleGen, WI).

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LITERATURE CITED


