CRISPR/Cas9-mediated simultaneous knockout of Dmrt1 and Dmrt3 does not recapitulate the 46,XY gonadal dysgenesis observed in 9p24.3 deletion patients

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ABSTRACT

DM domain transcription factors play important roles in sexual development in a wide variety of species from invertebrate to humans. Among seven mammalian family members of DM domain transcription factors, Dmrt1 has been studied in mouse and human for its conserved role in male gonadal identity. Chromosomal deletion of 9p24.3, the region in which DMRT1 is located, is associated with 46,XY gonadal dysgenesis. Dmrt1 knockout (KO) mice also showed male-to-female gonadal reprogramming. However, the phenotype of Dmrt1 KO mouse appears only after birth while 46,XY gonadal dysgenesis occurs during the developmental phase, and the cause behind this difference remained unknown. We hypothesized that in human the function of other Dmrt genes clustered with DMRT1, namely Dmrt3, might also be impaired by the chromosomal deletion, which leads to the gonadal dysgenesis phenotype. Thus, simultaneous loss of multiple DM domain genes in mice could have a more severe impact on gonadal development. To address this issue, we generated double KO mice for Dmrt1 and Dmrt3 via the CRISPR/Cas9 system. Comparing adult and neonatal testes of single and double KO mice, we found that loss of Dmrt1 or Dmrt3, or both, does not have apparent effect on male gonadal formation during embryonic development. Our study demonstrated that the discrepancy between human with 9p24.3 deletion and Dmrt1 KO mouse could not be explained by the simultaneous loss of Dmrt3 gene. CRISPR/Cas9 is a versatile and straightforward approach to elucidate the questions that were otherwise difficult to address with conventional methods.

1. Introduction

DM domain transcription factors are evolutionarily conserved among metazoan species and are involved in the gonadal development in a wide range of species [1–5]. In mammal (mouse and human), seven Ddx- and mab-3-related transcription factor (Dmrt) family genes are identified. Among them, Dmrt1 has been studied for its pivotal role in maintaining male gonad identity [2,6,7] and female germ cell maturation [8,9]. Indeed, human with chromosomal deletion of 9p24.3, the region in which DMRT1 is located, are often associated with 46,XY gonadal dysgenesis [10,11]. In mouse, Dmrt1 deficiency also caused the degeneration and feminization of male gonad, thus partly recapitulating the etiology of human patients of 9p24.3 deletion. However, the gonadal phenotypes observed in Dmrt1 KO mice are apparent only after birth. The embryonic development of male gonad is essentially normal in those mice, which shows stark difference from the etiology of human 9p24.3 deletion in which prenatal feminization is observed. Moreover, despite the phenotypic variation among the patients with 9p24.3 deletion, at least some patients with clear 46,XY gonadal dysgenesis phenotype retain normal DMRT1 exon sequences in their normal chromosome 9, implying haploinsufficiency of the DMRT1 gene in humans [11,12]. On the other hand, heterozygous Dmrt1 mutant mice do not show abnormality [2].

These differences between human and mouse could be due to the distinct roles of DMRT1 in two species, or the other Dmrt family genes with redundant function that are encoded beside the Dmrt1 gene. In particular, Dmrt1, Dmrt3 and Dmrt2 genes are clustered within 200 kb regions in both mouse and human genome, and deleted regions observed in 9p24.3 deletion patients also include DMRT3 and/or DMRRT2 genes [10]. Thus, simultaneous loss of function of DMRT1 and DMRT3 (or DMRT1, DMRT2, and DMRT3) in those patients could have more severe effect on gonadal development than single gene knockout. Accordingly, small deletions that affect only several exons of the DMRT1 gene or regulatory variants of DMRT1 are found in...
patients with milder gonadal dysfunction, such as male infertility [13,14].

Previous studies have shown that while Dmrt2 expression is absent in the gonad, Dmrt3 is expressed in the male gonad in chicken and mouse [15,16]. Moreover, genetic loss of Dmrt2 does not cause any gonadal defect [17], but Dmrt3 deficient mouse is reported to show male sexual developmental abnormality [18]. These studies prompted us to focus on Dmrt3 rather than Dmrt2 to examine if loss of another Dmrt gene in addition to Dmrt1 could cause a more severe phenotype. However, as Dmrt1 and Dmrt3 genes are clustered within 100 kb, it is

Fig. 1. Generation of Dmrt1KO, Dmrt3KO and Dmrt1/3DKO mice. A: A schematic drawing of mouse Dmrt gene loci and the positions of gRNAs for Dmrt1 and Dmrt3. Top panel: A schematic drawing of mouse chromosome 19 with colors correspond to G bands. Dmrt1-2 cluster locus is indicated with the vertical red line. Middle panel: A schematic drawing of the Dmrt cluster in chr19 qB-qC3. Dmrt1, 3, and 2 genes are colored in purple, orange and blue, respectively. Introns, UTRs and coding regions are indicated by lines, narrow boxes and wide boxes, respectively. Bottom panel: Schematic drawings around the first exons of Dmrt1 and Dmrt3 genes. The positions of Dmrt1 gRNA and Dmrt3 gRNA are indicated with blue bars with red bars indicating PAM sequences. B: Sequence alignments of wild-type (WT) and mutant alleles of Dmrt1 and Dmrt3. The target sequences of Dmrt1 and Dmrt3 gRNAs are indicated with blue letters with red letters indicating PAM sequences. The inserted or deleted bases in mutant sequences are indicated in green letter or green bars, respectively. C: The body weight of WT, Dmrt1KO, Dmrt3KO, and Dmrt1/3DKO mice at 6-week old. Values are mean±SD (n > 3). n.s. P > 0.05. D: The body length of WT, Dmrt1KO, Dmrt3KO, and Dmrt1/3DKO mice at 6-week old. Values are mean±SD (n > 3). n.s. P > 0.05.
2. Material and methods

2.1. gRNA synthesis and microinjection

gRNAs were synthesized and microinjected as described previously [21]. Human codon-optimized Cas9 (hCas9) and sgRNA cloning vector a gift from George Church (Addgene plasmid #41815 and #41824, respectively) [20]. Essentially, sequences that recognize target genomic loci by the sequence complementarity and put Cas9 nucleases to introduce double strand breaks (DSBs) to the loci [19,20]. Upon cleavage of genomic DNA, cellular DNA damage response mechanisms repair the DSBs, but small insertions or deletions (indels) could be introduced to the target sites which could cause frame-shift mutations. The CRISPR/Cas9 system could also be applied to mouse mutagenesis via RNA microinjection into the zygotes, and its high efficiency in introducing mutations has been reported [21–23]. In this study, we took advantage of this system to generate single and simultaneous KO mice of Dmrt1 and Dmrt3 genes, and examined their role in male gonad development.

2.2. Identification of mutant allele

Genomic sequences around the target sites of Dmrt1 and Dmrt3 genes were PCR amplified with BIOTAQ DNA Polymerase (Bioline, London, UK) from the genomic DNA extracted from tail tip of the mice. The PCR products were either treated with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and served for the direct sequencing or cloned into pCR4-TOPO cloning vector (Thermo Fisher Scientific, Waltham, MA, USA). Transcribed gRNAs were purified with Megaclear (Thermo Fisher Scientific) and ethanol precipitation and microinjected into the cytoplasm of the fertilized eggs obtained from intercross of F1 hybrid mice. The injected embryos were transferred to the pseudo-pregnant female mice and the obtained pups were examined for the genotypes of both Dmrt1 and Dmrt3 loci. As a result, we found that at least one allele of Dmrt1 locus was mutated in 73.9% of the mice genotyped (Table 1). Similarly, 37.3% of the mice examined had indels in Dmrt3 locus (Table 1). Importantly, 23.2% of the mice had indels in both Dmrt1 and Dmrt3 loci, thus those mice potentially had double KO allele (Table 1). However, since it was impossible to discriminate whether the mutations in Dmrt1 locus and Dmrt3 locus were on the single chromosome (in cis) or on two homologous chromosomes (in trans) at this point, we mated the mice with simultaneous mutations with wild-type (WT) C57BL/6 mice and examined the genotype of F1 generation. As a result, we could identify alleles with indels only in Dmrt1 locus (Dmrt1WT; Dmrt38del), Dmrt3 locus (Dmrt1WT; Dmrt38del) and both loci (Dmrt1WT; Dmrt38del) (Fig. 1B). Dmrt1WT allele has 1 base insertion in the exon 1 of Dmrt1 gene that causes the frame-shift, and the expected protein produced from this allele has premature stop codon and lacked DM domain (Supplementary Fig. S1A). Dmrt38del and Dmrt38del alleles had 8 and 2 base deletions, respectively, in the exon 1 of Dmrt3 gene and both mutations caused the frame-shifts. The expected proteins produced from these alleles were also truncated and lacked DM domain (Supplementary Fig. S1B). As the mice with homozygous Dmrt1ins and Dmrt38del or Dmrt38del alleles reproduced the previously reported phenotypes of Dmrt1 and Dmrt3 KO mice (detailed below), we concluded that we could successfully produce the KO alleles for Dmrt1 and Dmrt3 genes. In this manuscript, we designate mice with homozygous Dmrt1ins, Dmrt38del, and Dmrt1ins; Dmrt38del allele as Dmrt1KO, Dmrt3KO, and Dmrt1/3DKO, respectively.

2.3. Histological examination

Hematoxylin and eosin (H & E) staining and immunofluorescence were performed as described before [24]. Paraffin embedded tissues were sectioned at 7 µm. At least three individuals from one litter, or two individuals from two independent litters were examined for each genotype, and the representative results are shown. The antibodies used were α-Sox9 (Millipore AB5535, 1:1000) (Millipore, Billerica, USA), and TRA98 (Abcam AB82527, 1:1000) (Abcam, Cambridge, UK).

2.4. Statistical analysis

Statistical significances are examined using Student’s t-test.

3. Results

3.1. Generation of Dmrt knockout mice

gRNAs that recognize the exon 1 of Dmrt1 and Dmrt3 genes were designed and cloned into modified gRNA cloning vector (Fig. 1A) [21]. To generate the Dmrt1 and Dmrt3 KO mice, the gRNA sequences were PCR amplified, in vitro transcribed and microinjected with hCas9 mRNA into the fertilized eggs of BDF1 mouse. The injected embryos were transferred to the pseudo-pregnant female mice and the obtained pups were examined for the genotypes of both Dmrt1 and Dmrt3 loci. The summary microinjection targeting the Dmrt1/3 loci is shown in Table 1.

| Microinjection | Survival of zygotes | Result of genotyping |
|---------------|---------------------|----------------------|
| hCas9 mRNA   | Dmrt1 gRNA          | Dmrt3 gRNA           | 2 cell embryo/ |
| 166 ng/μl    | 166 ng/μl           | 166 ng/μl            | injected zygotes |
|               | 192/237             | 192                  | transferred   |
|               |                     | 34/46 (73.9)         | born          |
|               |                     | 25/67 (37.3)         | Dmrt1 mutant/ |
|               |                     | 16/69 (23.2)         | Dmrt3 mutant/ |
|               |                     |                       | genotype (%)  |
|               |                     |                       | genotype (%)  |
|               |                     |                       | mutant (%)    |

Table 1

Summary of microinjection targeting the Dmrt1/3 loci.

Note that some pups were examined only for Dmrt1 or Dmrt3 locus, since we focused to identify double mutant allele.
Fig. 2. Histological analyses of adult testes. A: The testis weight of WT, Dmrt1KO, Dmrt3KO, and Dmrt1/3DKO mice at 6-week old. Values are mean±SD (n > 3). n.s., P > 0.05, ** P < 0.01. B–E: Testes of wild-type (WT), Dmrt1KO, Dmrt3KO, and Dmrt1/3DKO mice isolated from 6-week old males. Bar: 2 mm. F–I: H & E staining of the sections of the testes from WT, Dmrt1KO, Dmrt3KO, and Dmrt1/3DKO mice. Bar: 50 µm. J–M: Hematoxylin and eosin staining of the sections of the epididymis from WT, Dmrt1KO, Dmrt3KO, and Dmrt1/3DKO mice. Bar: 50 µm.
3.2. Analysis of adult testes

Dmrt1KO, Dmrt3KO and Dmrt1/3DKO mice were born in Mendelian ratio without apparent abnormalities (data not shown). However, Dmrt3KO and Dmrt1/3DKO mice exhibited severe malocclusions as they grew (as reported previously in Dmrt3 deficient mice [18]). The phenotype is apparent at the weaning, and most of the mice died around the 6th week if untreated, probably due to feeding impairment. Thus, for further analysis we periodically cut their incisors after the weaning to circumvent this problem. In addition, as our purpose was to examine the role of Dmrt genes in male gonad, hereafter we focused only on the male mice. At the 6th week, Dmrt1KO, Dmrt3KO and Dmrt1/3DKO mice had no significant difference in body length or body weight compared with WT mice, except for the slightly lower body weight of Dmrt3KO mice (Fig. 1C, D). This could be the primary effect of loss of DMRT3 protein on body growth, or could also be caused by the residual feeding impairment as the slight malocclusions remained even after the treatment.

Next, we focused on the male gonad of these mice. The testes of Dmrt1KO mice were smaller and had reduced weight while Dmrt3KO testes had no significant difference compared with the WT testes (Fig. 2A–D). The testes of Dmrt1/3DKO also showed reduced size and weight, but no significant difference was seen between Dmrt1KO and Dmrt3/3DKO testes (Fig. 2A, C, E). Histological examination with H & E staining showed essentially normal testes in Dmrt3KO mice, with properly formed seminiferous tubules that contained differentiating sperms (Fig. 2F, H). Accordingly, fully differentiated sperms were seen in the epididymis (Fig. 2L). In contrast, testes of Dmrt1KO mice were severely degenerated as reported previously (Fig. 2G) [2]. Spermatocyte differentiation was not clear in seminiferous tubules and no mature sperm was observed in the epididymis (Fig. 2G, K). The testes of Dmrt1/3DKO mice also showed degeneration of seminiferous tubules and lack of mature sperm in the epididymis (Fig. 2L, M). These results showed that at the 6th week, loss of Dmrt3 did not affect the male gonad structure or spermatogenesis, and simultaneous loss of Dmrt1 and Dmrt3 did not show additive or synergic effects on the testes degeneration caused by Dmrt1 deficiency.

3.3. Analysis of newborn testes

Given that the adult testes of Dmrt1/3DKO were degenerated as those of Dmrt1KO, we next examined if loss of both Dmrt proteins had any effect on male gonad developmental process during embryogenesis. Testes of the newborn mice of Dmrt1KO, Dmrt3KO, Dmrt1/3DKO and WT mice were fixed, sectioned and histologically analyzed. H & E staining showed that the testes of Dmrt1KO, Dmrt3KO and Dmrt1/3DKO had no apparent abnormalities compared with those of WT; in all genotypes, seminiferous tubules were formed correctly as WT testes (Fig. 3A–D). Furthermore, immunostaining of SOX9 and TRA98 confirmed that Sertoli cells and germ cells differentiate normally in the testes of the mice with all genotypes (Fig. 3E–H). These results suggested that the loss of DMRT1 or DMRT3, or both, did not cause feminization or apparent developmental deficiency to the male gonad during embryonic development.

4. Discussion

In this study, we have generated single and double KO mice for Dmrt1 and Dmrt3 genes using the CRISPR/Cas9 system. While the loss of Dmrt1 gene caused postnatal degeneration of the testes as reported previously [2], no apparent abnormality was observed in the testes lacking Dmrt3 gene (Figs. 2 and 3). Furthermore, simultaneous loss of Dmrt1 and Dmrt3 genes did not cause developmental defect during the embryonic male gonad formation. Our data suggest that Dmrt3 does not play an indispensable role in male gonad development in mouse, and the defective testicular formation phenotype seen in human patient with 9p24.3 deletion could not be recapitulated by the simultaneous loss of Dmrt1 and Dmrt3 genes in mice. Our results indicate that the phenotypic discrepancy between Dmrt1 KO mouse and 9p24.3 deletion, such as the onset of gonadal abnormality or haploinsufficiency, could not be attributed to the associated loss of Dmrt3. The cause of distinct phenotypes in human patient and mouse model remain unclear. This could be due to other genes located around 9p24.3 that might be also impaired, such as Dmrtr2. Alternatively, this may be due to the distinct role of DM domain proteins in the gonadal development between those two species. DM domain transcription factors are known to play indispensable roles in gonadal development in a wide variety of species, but at the same time, their function in the process changes over the course of evolution [25]. Their role is primary sex determination in some species but is a downstream sex differentiation player in other species. Our result implies the possibility of DM domain transcription factors being different between two mammalian species. Of note, discrepancies of phenotypes between human and mouse with similar genetic modifications are observed also in other reports, for example heterozygous Sox9 cause XY gonadal malformation in human while testis of Sox9−/− mouse is histologically normal [26,27]. Recently, identical p. R92W point mutation in Nr5a1 gene are reported to cause distinct phenotypes in XX human and mouse [28,29]. These reports, together with our results, implying that the specific roles of transcription factors during the gonadal development could be distinct between human and mouse.

By microinjecting two gRNAs and hCas9 mRNA into the zygotes, we could successfully generate two single KO and one double KO mice in a single experiment (Table 1). Although we could not directly confirm the loss of DMRT1 and DMRT3 proteins in our KO mice with commercially available antibodies (data not shown), we believe those proteins were depleted from the KO mice, as they exhibited the previously reported phenotypes that are linked with loss of each DMRT proteins; namely, degeneration of adult but not embryonic testes for loss of DMRT1 and severe malocclusion for loss of DMRT3 [2,18]. High efficiency of the CRISPR/Cas9 system enabled us to produce simultaneous KO of two genes located in the vicinity, which was difficult to achieve with the conventional method. Although simultaneous KO of Dmrt1 and Dmrt3 did not show synergic effect, the usefulness of this methodology in answering the questions that were otherwise difficult to address with conventional technique should be stressed.

Dmrt1 deficiency in gonadal somatic cells causes the up-regulation of female gonad specific genes such as Foxl2 in adult XY gonad [6], and hence it may be interesting to assess whether loss of Dmrt1 also causes any feminization of male gonads in the long term. For this purpose, however, gonad-specific KO mouse will be desired to exclude any indirect effect of weight loss caused by feeding impairment. Dmrt3 deficiency has been reported to cause “male sexual developmental abnormality” [18], but in this study we did not observe an apparent phenotype on sexual development or fertility in Dmrt3KO mice. This could be due to the difference in the genetic background or to the methodology of generating KO mice. In addition, severe malocclusion caused by the loss of Dmrt3 prevents the precise and quantitative evaluation of their potential for the reproduction. Thus, tissue-specific KO would be necessary for further detailed investigation. Dmrt1 has both Sertoli cell and germ cell autonomous roles during male gonad development [6,30–32]: the former is to maintain male identity of supporting cells by preventing the expression of female genes such as Foxl2, and the latter is to support the survival and radial migration of male germ cells. Importantly, the role of Dmrt1 in germ cells was revealed only under cell-type specific knockout, because loss of Dmrt1 in Sertoli cell indirectly affects germ cells and covers the relatively minor phenotype of loss of Dmrt1 in germ cells. As our Dmrt3 KO mice are non-conditional and thus affect both Sertoli and germ cells, it will be interesting to examine also germ cell specific role of Dmrt3 in future study.
5. Conclusion

In conclusion, we have generated single and simultaneous KO mice for Dmrt1 and Dmrt3 genes using the CRISPR/Cas9 technique. We found that double knockout of Dmrt1 and Dmrt3 genes does not cause sex reversal or developmental deficiency during the embryonic male gonad formation. Our results imply that the discrepancy between the phenotypes of Dmrt1 KO mice and human with 9p24.3 deletion is not due to the associated impairment of DMRT3 function, and thus it might depend on the distinct genetic programs for the male gonad formation between the two species.

Author contributions

T.K. performed the immunofluorescence, M.T. performed the microinjection, and M.I. performed the rest of the experiments. M.I. and S.T. designed the project and wrote the manuscript.

Competing financial interest

The authors declare no competing financial interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.bbrep.2017.01.001.

References

[1] M.M. Shen, J. Hodgkin, Mah-3, a gene required for sex-specific yolk protein expression and a male-specific lineage in C. elegans, Cell 54 (1988) 1019–1031. http://dx.doi.org/10.1016/0092-8674(88)90117-1.
[2] C.S. Raymond, M.W. Murphy, M.G. O’Sullivan, V.J. Bardwell, D. Zarkower, Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation, Genes Dev. 14 (2000) 2587–2595. http://dx.doi.org/10.1101/gad.834100.
[3] M. Matsuda, Y. Nagahama, A. Shinomiya, T. Sato, C. Matsuda, T. Kobayashi, et al., DMY is a Y-specific DM-domain gene required for male development in the medaka fish, Nature 417 (2002) 559–563. http://dx.doi.org/10.1038/35011804.
[4] J.S. Baker, K.A. Ridge, Sex and the single cell. I. On the action of major loci affecting sex determination in Drosophila melanogaster, Genetics 94 (1983) 383–423.
[5] C.A. Smith, K.N. Roeszler, T. Ohnesorg, D.M. Cummins, P.G. Farlie, T.J. Doran, D.M. Gearhart, A. Minkina, A.D. Krentz, V.J. Bardwell, et al., The avian Z-linked gene DMRT1 is required for male sex determination in D. melanogaster, Genetics 94 (1983) 383–423.
[6] C.K. Matson, M.W. Murphy, A.L. Sarver, M.D. Griswold, V.J. Bardwell, J.R. Kettlewell, R.L. Johnson, Targeted disruption of the DM domain containing transcription factor Dmrt2 reveals an essential role in somite patterning, Dev. Biol. 290 (2006) 200–210. http://dx.doi.org/10.1016/j.ydbio.2005.11.027.
[7] N. Abhinav, Y. Zhu, A. Visel, A. Holt, V. Afral, L.A. Pennacchio, et al., Deletion of ultraconserved elements yields viable mice, Plos Biol. 5 (2007) e234. http://dx.doi.org/10.1371/journal.pbio.0050234.st001.
[8] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, et al., Multiplex genome engineering using CRISPR/Cas systems, Science 339 (2013) 819–823. http://dx.doi.org/10.1126/science.1231143.
[9] P. Mali, L. Yang, X.M. Esveldt, J. Aach, M. Guell, J.E. DiCarlo, et al., RNA-guided human genome engineering via crRNA, Science 339 (2013) 823–826. http://dx.doi.org/10.1126/science.1232903.
[10] M. Inui, M. Miyado, M. Igarashi, M. Tachino, K. Kudo, S. Yamashita, et al., Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system, Sci. Rep. 4 (2014) 5396. http://dx.doi.org/10.1038/srep05396.
[11] M. Inui, M. Miyado, M. Igarashi, M. Tachino, K. Kudo, S. Yamashita, et al., Generation of mutant mice via the CRISPR/Cas9 system using FokI-dCas9, Sci. Rep. 5 (2015) 11221. http://dx.doi.org/10.1038/srep11221.
[12] H. Wang, H. Yang, C.S. Shivalila, M.M. Dawlaty, A.W. Cheng, F. Zhang, et al., One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering, Cell 153 (2013) 910–918. http://dx.doi.org/10.1016/j.cell.2013.04.025.
[13] T. Kato, K. Miyata, M. Sonohe, S. Yamashita, M. Tachino, K. Miura, et al., Production of Sry knockout mouse using TALEN via oocyte injection, Sci. Rep. 3 (2013) 3136. http://dx.doi.org/10.1038/srep03136.
[14] C.K. Matson, D. Zarkower, Sex and the singular DM domain: insights into sexual differentiation, Nat. Rev. Genet. 13 (2012) 163–174. http://dx.doi.org/10.1038/nrg3161.
[15] J. Meyer, P. Südbbeck, M. Held, T. Wagner, M.L. Schmitz, F.D. Bricarello, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[16] W. Bi, W. Huang, D.J. Whitworth, J.M. Deng, Z. Zhang, R.R. Behringer, et al., Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 6698–6703. http://dx.doi.org/10.1073/pnas.101109298.
[17] M. Igarashi, K. Takasawa, A. Hakoda, J. Kanno, S. Takada, M. Miyado, et al., Identification of mouse lines carrying one or more mutant alleles of genes involved in sex determination, Hum. Mutat. 38 (2017) 39–42. http://dx.doi.org/10.1002/humu.23116.
[18] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[19] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[20] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[21] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[22] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[23] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[24] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.
[25] M. Miyado, M. Inui, M. Igarashi, Y. Katoh-Fukui, K. Takasawa, A. Hakoda, et al., Mutational analysis of the SOX9 gene in campomelic dysplasia and autosomal sex reversal: lack of genotype/phenotype correlations, Hum. Mol. Genet. 6 (1997) 91–98.