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Response to Veitia *et al*

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Reply to: **RA Veitia *et al***
See also: **E Shin *et al***

Previously, we provided experimental evidence demonstrating the discriminative miRNA targeting of a mutated site within the coding sequence of a variant allele (c.402C>G; p.C134W) of the tumor-suppressor gene *FOXL2* that results in lower expression levels of the variant *FOXL2* mRNA (Shin *et al*, 2020). The central concern described in the correspondence from Veitia *et al* is with regard to the biological relevance of the allelic imbalance that we observed in adult-type granulosa cell tumors (AGCTs) and AGCT-derived KGN cell line. Their concern largely stems from two experimental data, specifically the electropherograms from Sanger sequencing of cDNA containing the FOXL2 mutational locus in KGN cells (fig 1 of Veitia *et al*) and the RNA sequencing results of four AGCTs reported by Shah *et al* (2009).

We disagree with the claims of Veitia *et al* based on the simple interpretation of the height of the electropherograms obtained from the Sanger sequencing analysis (fig 1of Veitia *et al*). When we performed Sanger sequencing of the cDNA derived from KGN cells, our results revealed a major peak for wild-type (WT) and a much weaker peak for the mutant *FOXL2*, and both peaks were almost equally visible when the genomic DNA from KGN cells was sequenced (Fig 1A). Moreover, we performed additional pyrosequencing analyses (Fig 1B) and primer extension assays using the 5′-end ^{32}P-radiolabeled primers (Fig 1C) on KGN cells, and the results revealed a much lower expression of the variant *FOXL2* mRNA than that of the WT mRNA. The allele proportion of the WT to that of the variant was 76.5:23.5 in KGN cells according to two independent pyrosequencing experiments (Fig 1B). Pyrosequencing analysis of COV434 cells (Fig 1B), a juvenile-type GCT (JGCT)-derived cell line that lacks the c.402C>G mutation, and a primer extension analysis of cDNAs synthesized from *FOXL2* mRNA in KGN cells that ectopically express the mutant *FOXL2* mRNA (Fig 1C) demonstrated that these methods can reliably measure the relative abundance of the WT to that of the variant *FOXL2* mRNA. We acknowledge that quantitation of the pyrogram peak at 402G requires additional consideration, as the *FOXL2* mutant locus possesses a consecutive GGG (Levebratt & Sengul, 2006).

In 2009, Shah *et al* observed that the RNA sequencing of four AGCTs was not indicative of a consistent lack of allelic imbalance, but rather, variable ratios (9:1, 1:1, 4:6, and 6:4) of the variant and WT *FOXL2* mRNA levels, and we have previously discussed this in detail (Shin *et al*, 2020).

We also disagree with the comment by Veitia *et al* regarding the limited effect of miR-1236 on the destabilization of variant *FOXL2* mRNA *in vivo*. Our *in vivo* genome-editing results clearly demonstrate that miR-1236 is an endogenous functional miRNA that specifically downregulates variant *FOXL2* mRNA (Shin *et al*, 2020).

As Veitia *et al* mentioned, the purity of the AGCT tumor cells that were analyzed may be important. As *FOXL2* is an exon-containing gene, exome sequencing cannot be applied to assess tumor purity. Alternatively, when we analyzed the genomic DNA levels of both alleles in all AGCTs, similar levels of both alleles were detected (Shin *et al*, 2020). Although this result suggests a minimal presence of normal cells, further studies performed using techniques such as single-cell RNA sequencing from fresh AGCTs are required to ensure the clinical implications of allelic imbalance of *FOXL2*.

Materials and Methods

Sanger sequencing

Genomic DNA (gDNA) was extracted from KGN cells using an Intron G-DEX^{TM} Genomic DNA Extraction Kit (Intron, Seongnam, Korea) according to the manufacturer's protocol. Total RNA from KGN cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Following the confirmation of the quality and quantity of extracted total RNA samples using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA), cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). *FOXL2* fragments were amplified by PCR (35 cycles) from both gDNA and cDNA using the 273F primer (5′-CGAGAAGAATAAGAAGGGCT-3′) and the 488R primer (5′-CGGGCTGGAAGTG CGCGGGC-3′) in combination with 2X HOT Master Mix with Dye (Mgmed, Seoul, Korea). The amplified PCR fragments were purified using the LaboPassTM Gel Extraction Kit (Cosmogenetech, Seoul, Korea) and sequenced using the 273F primer.

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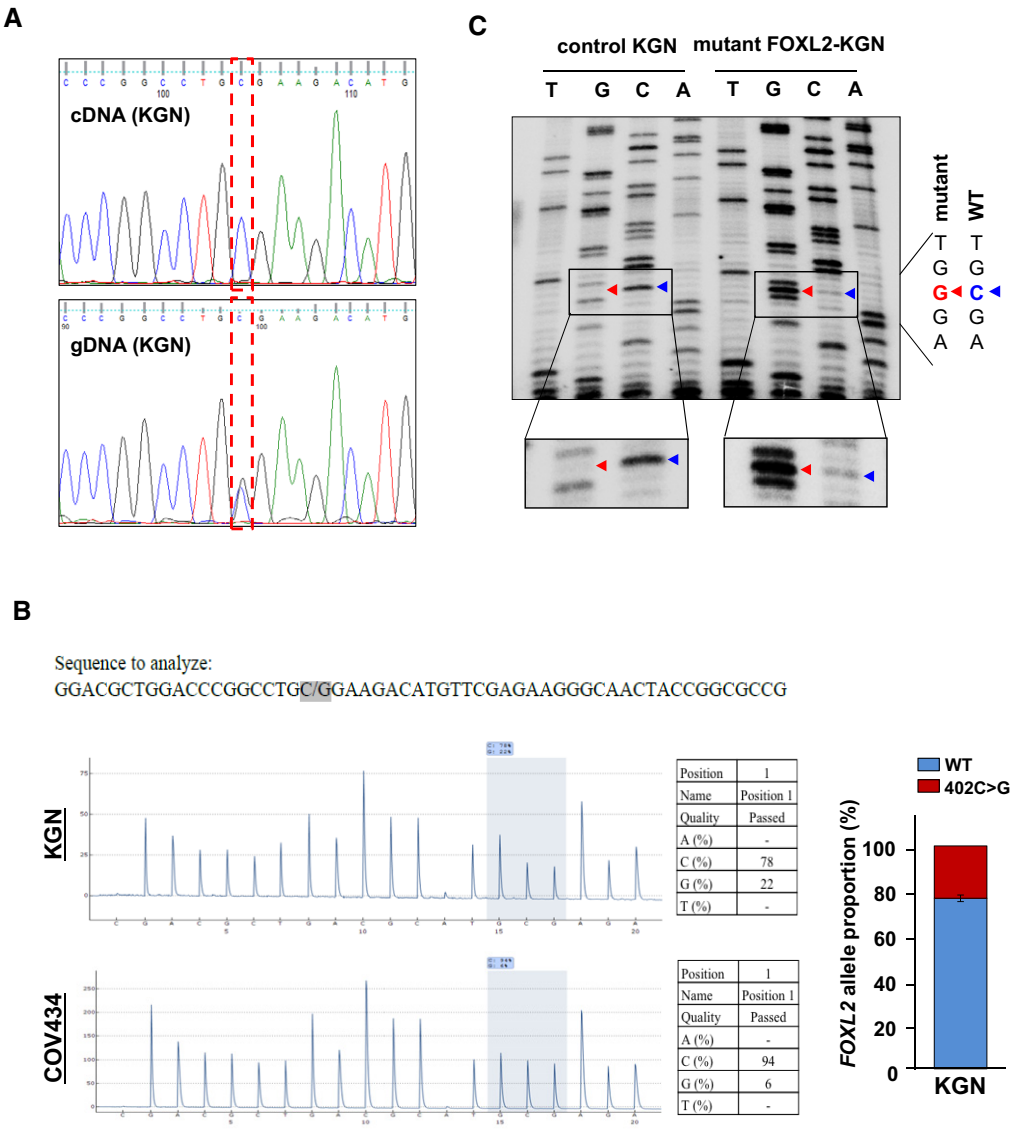

Figure 1. Allelic imbalance of heterozygous *FOXL2* transcripts in KGN cells.
(A) Electropherograms from the Sanger sequencing of the locus of c.402C>G of *FOXL2* from cDNA and gDNA from KGN cells are presented. The presented electropherograms are representative of eight independent experiments. The red dotted boxes indicate the 402C>G position. (B) Actual pyrograms and PyroMark Q48 software-generated allele proportions of WT and variant *FOXL2* mRNAs in KGN and COV434 cells from pyrosequencing experiments are presented. The data are provided as the mean ± SEM from two independent experiments. (C) Primer extension analysis performed on total RNA extracted from KGN cells transfected with an empty vector (control) or mutant (402G) FOXL2-encoding plasmid. The expected WT (C) or mutated (G) nucleotides at position 402 are indicated by blue or red text and arrowheads, respectively. Enlarged images of signals from the 402 regions of *FOXL2* transcripts are presented below.

Pyrosequencing

The PyroMark PCR Kit (Qiagen, Hilden, Germany) was utilized for pyrosequencing using a forward primer (Pyro-FOXL2-F: 5′-AGAAGGGCTGGCAAAATAGCATC-3′) and reverse biotinylated primer (Pyro-FOXL2-R: 5′-CCGGAAGGGCCTCTTCAT-3′). The PCR products were purified using streptavidin Sepharose HP beads (GE Healthcare, Buckinghamshire, UK) and then hybridized with the sequencing primers (FOXL2-seq F: 5′-CGCAAGGGCAACTACT-3′), as described in the PyroMark Q48 vacuum workstation guide (Qiagen). The sequencing data were analyzed using PyroMark Q48 software (Qiagen). Pyrosequencing was performed and analyzed by Macrogen (Seoul, Korea).

Primer extension

Reverse transcription of the total RNA was performed using poly(dT) and random hexamers according to the manufacturer's protocol (Invitrogen). First-strand cDNA was amplified using the forward primer F (5′-CTAGAATTCAAATGATGGCCAGCTACCCC-3′) and reverse primer R (5′- CTACTCGAGTCAGAGATCGAGGCGCGAATG-3′) and purified using the QIAquick Gel Extraction Kit (Qiagen). The reverse primer R3 (5′- GCGCCGGTAGTTGCCCTTCTC-3′) was labeled at the 5′ end using [γ-^{32}P] ATP (PerkinElmer, Waltham, MA, USA) and T4 polynucleotide kinase (TaKaRa Bio, Kyoto, Japan). The extension reaction was performed using the AccuPower® DNA

Sequencing Kit (Bioneer, Daejeon, Korea), and the reaction products were resolved on a 10% denaturing polyacrylamide gel and subsequently visualized by autoradiography.

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