Supplementary Information

ROR2 regulates the survival of murine osteosarcoma cells in lung capillaries.

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Supplementary Methods

Gene mapping

Differentially expressed genes were selected from the microarray data of LM8 sublines and subjected to pathway analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG). The analysis was performed with the clusterProfile package using R/Bioconductor[1]. The reference gene set used was the Wnt signaling pathway (mm0431) set.

Hypotonic assay

LM8-H and H/*Ror2*-KO (1×10^4) were seeded in 24-well plates and cultured overnight. The medium was then replaced with either hypotonic solution (12.5% PBS) or isotonic solution (100% PBS) and incubated for 30 minutes. Live and dead cells were identified by trypan blue staining and then counted.

Endothelial transmigration assay

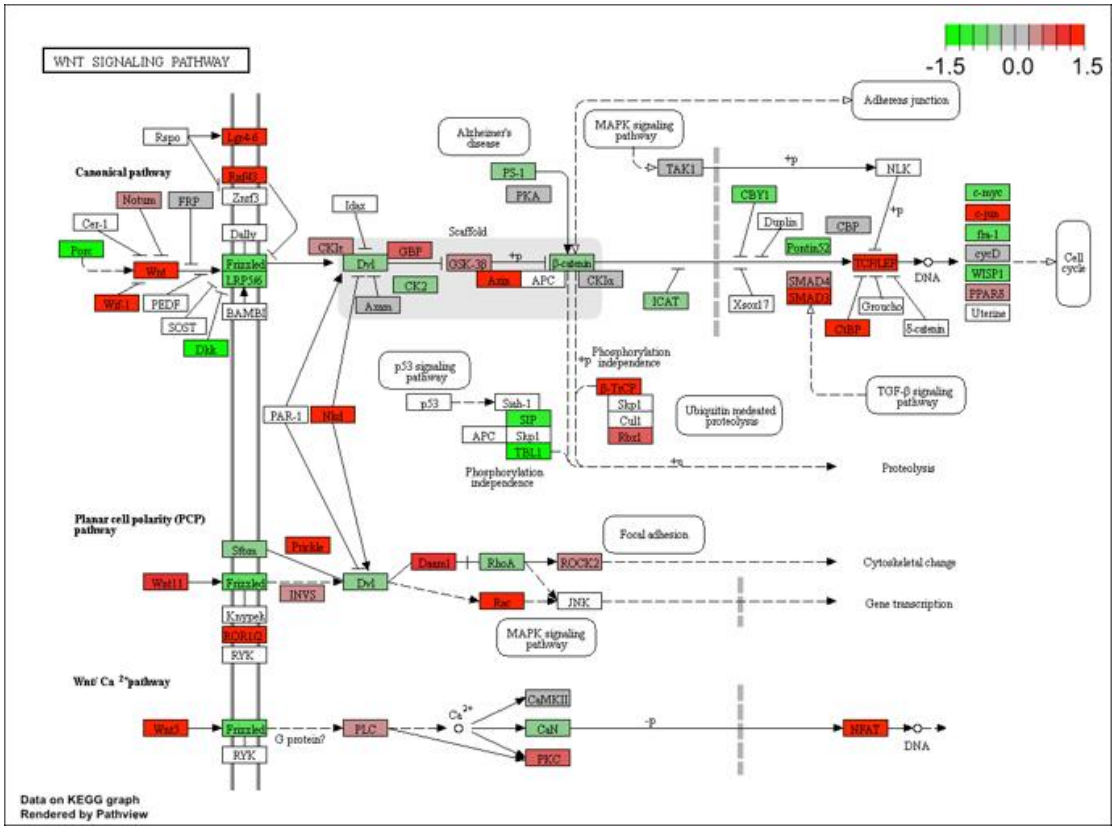
bEnd.3 cells (1 × 105) were seeded onto a top filter with an 8 μm pore in a 24-well Transwell® plate (Corning) and grown until the monolayer became confluent. The LM8 sublines were labeled with 25 μM CellTracker® Green for 30 minutes. After washing with PBS, the cells (5 × 104) were seeded onto the bEnd.3 monolayers. After 24 hours of incubation, the untransmigrated cells were wiped off with a cotton swab, and the filter was fixed with 4% paraformaldehyde for 20 minutes. Then, the transmigrated cells on the bottom of the filter were observed under a fluorescence microscope. Partial fluorescent images of each filter were combined, and the number of transmigrated cells were counted using BZ-X analyzer software. For analysis of

Wnt5a function in LM8 transmigration, recombinant Wnt5a (R&D System) was dissolved in PBS and used at a final concentration of 5 μg/mL.

REFERENCE

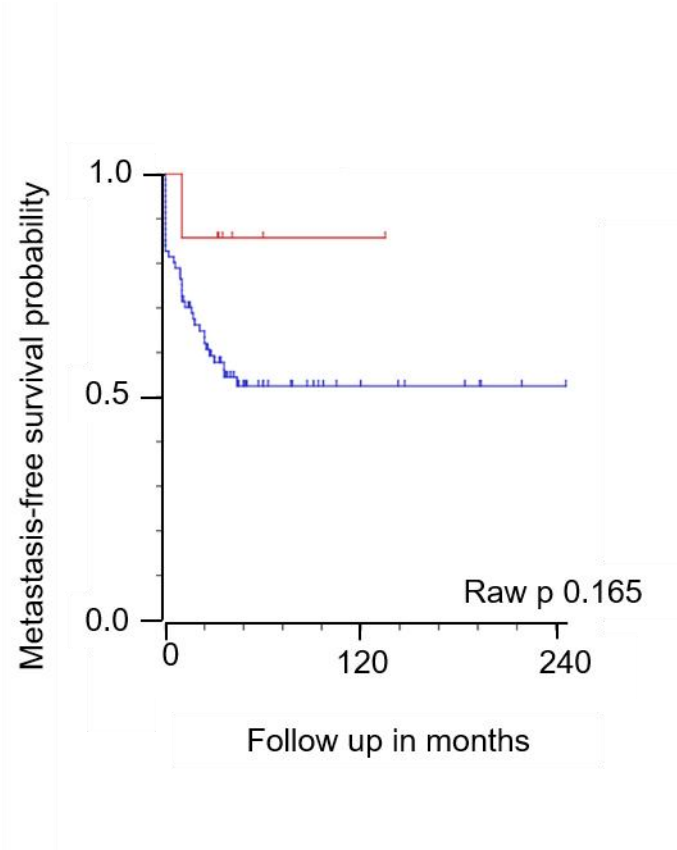
1. Yu G, Wang LG, Han Y, et al. An R package for comparing biological themes among gene clusters. Omi A J Integr Biol. 2012;16:284–287.

Figure legend



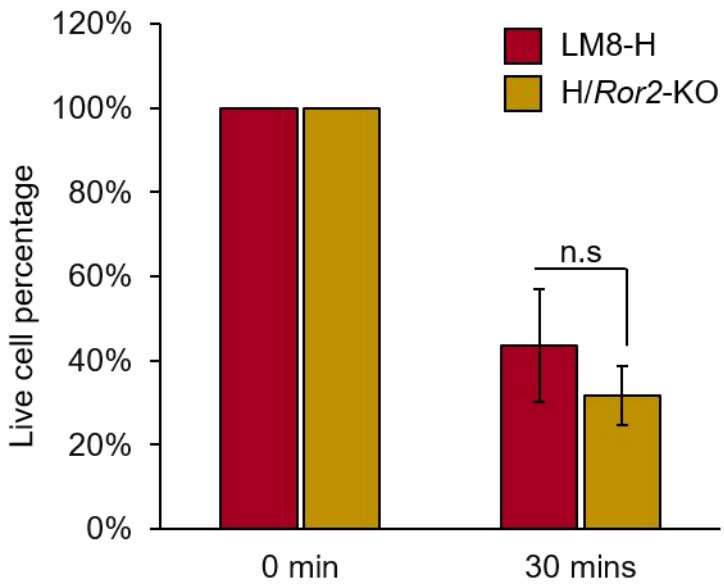
Supplementary Figure 1: Wnt-related gene expression analysis of LM8

The Wnt signaling pathway in LM8. A putative Wnt signaling pathway of LM8 was constructed based on KEGG mapping. The color of the boxes with gene names corresponds to their mRNA levels from increasing (red) to decreasing (green), as per the color scale shown in the upper right of the figure.



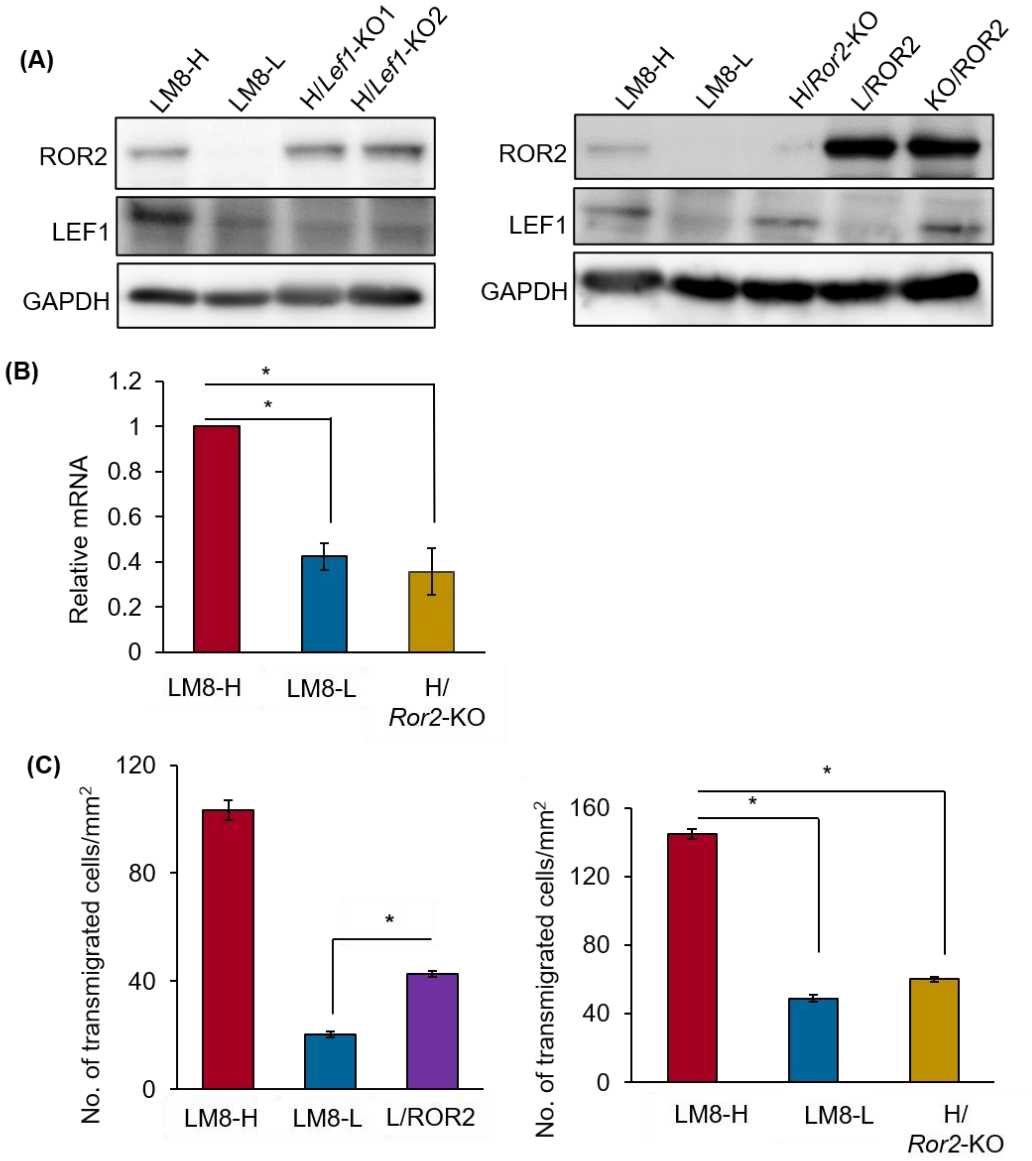
Supplementary Figure 2: Correlation of *ROR2* expression with metastasis-free survival of OS patient

Kaplan-Meier metastasis-free survival curves were constructed from data sets of the R2 database (osteosarcoma-Kuijjer-127-vst-ilmnhwg6v2). The red and blue lines show the survival rates of OS patients with low and high *ROR2* expression, respectively.



Supplementary Figure 3: ROR2 function is not involved in mechanical stress

LM8-H and H/*Ror2*-KO were incubated for 30 minutes in an extremely hypotonic (12.5% PBS) solution, and then live and dead cells were identified by trypan blue staining. The viability in hypotonic buffer was normalized by the viability in isotonic buffer (100% PBS). The live cell percentage is shown as the percentage of the viability of cells treated with isotonic buffer. n = 3, ***p < 0.001.

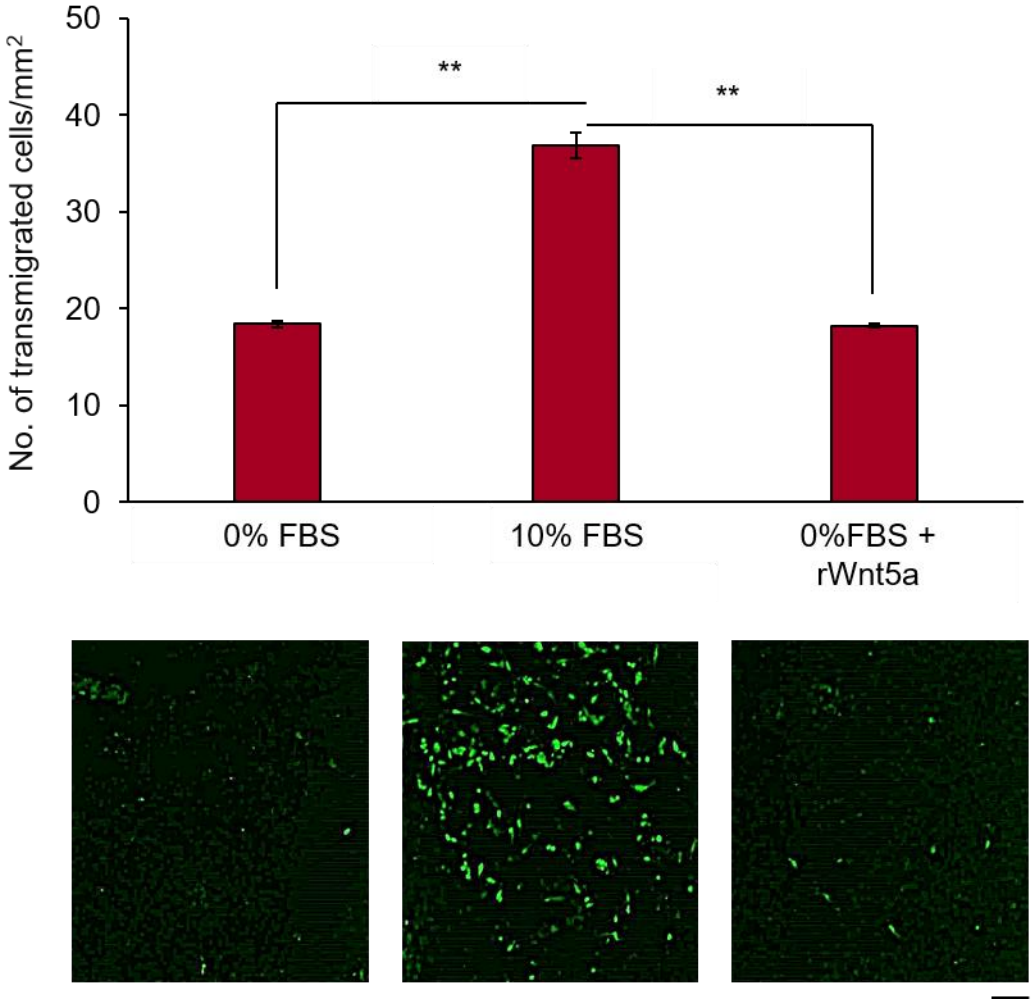


Supplementary Figure 4: Effect of ROR2 on *Cygb* expression and transmigration activity in LM8

(A) The ROR2, LEF1, and GAPDH protein levels in the indicated LM8 sublines examined by western blotting. H/*Lef1*-KO1 and H/*Lef1*-KO2 are independent Lef1-knocked-out LM8-H sublines.

(B)Relative *Cygb* mRNA levels in LM8-H, LM8-L, and H/*Ror2*-KO evaluated by qPCR.

(C)The migration activity of LM-H, LM8-L, and H/*Ror2*-KO or of transiently ROR2-expressing LM8-L (L/ROR2) examined by endothelial transmigration assay. Data are shown as the mean ± SD of migrated cells. n = 3,*p < 0.05.



Supplementary Figure 5: Effect of Wnt5a on the transmigration ability of LM8

The number of transmigrated LM8-H cells treated with 5 µg/mL recombinant Wnt5a (rWnt5a) or PBS examined by an endothelial transmigration assay in serum-free (0% FBS) medium. Data with medium containing 10% FBS is shown as a positive control. The photos below the graph are fluorescent images of the transmigrated LM8-H. Data are shown as the mean ± SD of migrated cells. n = 3, **p < 0.01. Scale bar: 100 µm.