Letter to the Editor

Long noncoding RNA *MALAT1* and cancer metastasis

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Since *Malat1* was identified as a prognostic marker for poor clinical outcomes in non-small cell lung cancer (NSCLC) patients, number of *Malat1* studies have revealed its roles in human cancers (1,2). Recently, we demonstrated unexpected *Malat1* function in suppressing breast cancer metastasis using genetic mouse and cell line models of breast cancer (3). Here, I review the controversies in *Malat1* studies conducted by ourselves and others by comparing research designs and interpreting the results.

Previously, *Malat1* was shown to co-localize with nuclear speckles enriched in pre-mRNA splicing factors. *In vitro* siRNA study revealed that *Malat1* interacts with splicing factors in the nucleus and regulates pre-mRNA splicing (4). *Malat1* genetic knockout mice were generated from independent research groups and exhibited no apparent phenotypic abnormalities (5,6). Surprisingly, unlike *in vitro* results, there were no significant changes in alternative splicing or global gene expression due to genetic loss of *Malat1* (3,6).

Arun *et al.* (7) generated *Malat1* knockout mice by deleting 3 kb genomic locus spanning both upstream and downstream of transcription start site and crossed them to Polyomavirus middle T antigen (PyMT)-induced mouse mammary tumor model. Although *Malat1* loss did not substantially alter primary tumorigenesis, mammary tumors of *Malat1*$^{-/-}$ PyMT mice exhibited cystic phenotype compared to *Malat1*$^{+/+}$ PyMT animals. Furthermore, lung metastasis was significantly suppressed by *Malat1* loss. In addition to genetic ablation of *Malat1*, PyMT mice were treated with antisense oligonucleotides (ASOs). Compared to complete genetic *Malat1* knockout, ASOs led to ~60% reduction in *Malat1* levels but caused stronger suppression in mammary tumor growth and comparable inhibition in lung metastasis. Targeting *Malat1* with less-effective ASOs demonstrated more evident tumor-suppressive outcome, which obviously raises a question of off-target effects of ASOs.

We utilized *Malat1* mouse knockout model generated by targeted insertional inactivation (5) and crossed them to PyMT mice (3). Unexpectedly, however, we observed dramatic induction of lung metastasis in *Malat1*$^{-/-}$ PyMT mice while there was no noticeable difference in mammary tumors compared to *Malat1*$^{+/+}$ PyMT mice; cystic and high-grade tumor areas were similarly found in both groups. Instead, we could see consistent increase in circulating tumor cells from the peripheral blood of *Malat1*$^{-/-}$ PyMT mice, which implies that *Malat1* loss affects certain stage of metastatic process. This surprising result against the current dominant paradigm of *Malat1*'s pro-metastatic function prompted us to restore *Malat1* in *Malat1*$^{-/-}$ PyMT mice using transgenic animals to determine if metastasis-promoting function is attributed to *Malat1* loss. When *Malat1* was re-expressed, metastatic phenotype was dramatically reversed. Furthermore, considering higher *Malat1* levels in *Malat1*-restored PyMT mice compared to *Malat1*$^{+/+}$ PyMT mice, *Malat1*-re-expression resulted in less metastasis than M*alat1*$^{+/+}$, which implies dose-dependent effect of *Malat1* in lung metastasis suppression. Using more aggressive PyMT mice on FVB/N background, we overexpressed *Malat1* by crossing them to *Malat1* transgenic mice on FVB/N and observed that lung metastasis was significantly suppressed by transgenic overexpression of *Malat1*.

In addition, we inactivated *MALAT1* in MDA-MB-231 human breast cancer cells using CRISPR-Cas9 by deleting ~650 bp in 5' genomic locus of *MALAT1*. *MALAT1* expression was completely abrogated unlike siRNAs, shRNAs or ASOs which partially suppressed nuclear-

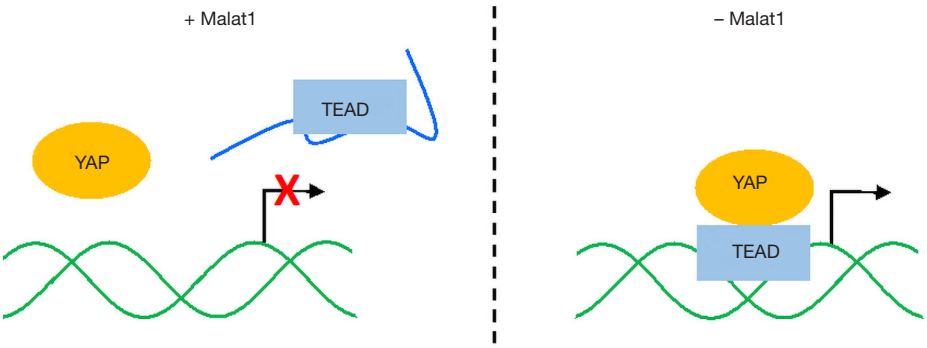

Figure 1 *Malat1* sequesters Tead proteins to prevent them from associating with Yap, a transcriptional coactivator, which inhibits their binding to target gene promoters/enhancers and inactivate transcription.

enriched RNAs. *MALAT1* knockout dramatically elevated metastatic potential, which were reversed by re-expression of *Malat1*. Furthermore, when *Malat1* was overexpressed in highly metastatic 4T1 mouse mammary tumor cells as well as lung metastatic subline of MDA-MB-231 (LM2), metastasis in both cell lines was significantly suppressed.

What possibly causes the substantial difference in studying the same lncRNA *Malat1*? There is one example that inactivating a lncRNA in mice by different strategies exhibited opposite phenotypes. LncRNA *Haunt* expression was inactivated by either inserting transcription stop signal in the downstream of transcription start site or deleting various lengths of genomic locus of *Haunt* (2.3–58 kb) (8). When transcription stop signal was inserted or short genomic region was deleted, expression of *HoxA* gene cluster located in the downstream of *Haunt* was significantly elevated. However, the effect was attenuated when longer genomic regions were deleted. This turned out that *Haunt* genomic locus contains enhancer sequence regulating *HoxA* expression, so minimal deletion/insertional inactivation and long genomic deletion demonstrated opposite gene expression patterns. Moreover, *Haunt* re-expression failed to rescue knockout phenotype driven by deletion of longer genomic locus. This study implicates that deleting genomic locus to inactivate the target lncRNA may disrupt *cis* regulatory elements, and it is essential to restore the lncRNA expression in order to examine if the phenotype driven by lncRNA inactivation is rescued. In fact, *Malat1* inactivation by genomic deletion resulted in significant transcriptional upregulation of its neighbor genes in *cis* (6) while insertional inactivation of *Malat1* did not (1), which suggests that deleted genomic region may contain *cis*-regulatory sequences for *Malat1*-neighboring genes.

To elucidate molecular mechanism by which *Malat1* suppresses breast cancer metastasis, we searched for *Malat1*-interacting proteins in mouse mammary tumors and identified Tead family members, the oncogenic and pro-metastatic transcription factors (Tead1–4). In the subsequent analyses, we found that Tead binding sites are distributed throughout *Malat1*, and *Malat1* could inactivate Tead proteins by sequestering them from interacting with Yap coactivator (*Figure 1*). However, any of previous *Malat1* knockout mouse studies did not report abnormalities in *Malat1*$^{-/-}$ mice due to Tead-Yap hyperactivation (5,6). In human mammary tissue, for example, YAP is highly enriched in the nuclei of breast tumor cells while localized more in the cytoplasm of normal mammary cells (9). Notably, nuclear localization of Yap was prominent in the mammary tumor cells of PyMT mice in our unpublished data. This suggests that *Malat1* inactivation needs to be combined with nuclear translocation of Yap to interact with Tead proteins, which fully enhances their transcriptional potential. For this reason, cooperation model of *Malat1* deficiency and nuclear Yap warrants future studies.

In conclusion, most *Malat1* research including *in vitro* and *in vivo* studies has not been confirmed by rescue experiments which we rigorously conducted recently (3). Especially for finding therapeutic application of RNA interference or ASOs targeting lncRNA *MALAT1* in human cancers, it is critical to exclude any possibility of their off-target effects by performing rescue experiments with accurate controls (10).

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Footnote

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Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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