Genes & Diseases (2023) 10, 325–328

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RAPID COMMUNICATION

Microtubule actin crosslinking factor 1 functions as a novel therapeutic target in lung metastasis of osteosarcoma

Lung metastasis is the primary cause of death in osteosarcoma (OS) patients.[1] A better understanding of the molecular mechanisms underlying OS tumorigenesis and metastasis is urgently needed to identify therapeutic targets. Microtubule actin crosslinking factor 1 (MACF1), which belongs to the spectraplakin family of cytoskeletal crosslinking proteins,[2] is critical for cell migration and polarization due to its regulation of the cytoskeleton. Recently, MACF1 was indicated to be involved in the metastatic invasion of some human cancers,[3] but the function of MACF1 in OS is still unclear.

We found that MACF1 was more highly expressed in OS tissues than normal bone in an immunohistochemical microarray (strongly positive in 12/18 OS samples, Table S1 and Fig. 1A; Fig. S1A) as well as an analysis of the GEO database (Fig. S1B). Samples of human OS lung metastasis had even higher MACF1 expression than localized OS tissue (Fig. S1B). In addition, OS cell lines with higher mobility (greater migration and invasion) and proliferation (higher cell viability and single cell colony formation) had higher MACF1 expression (Fig. S2).

To further elucidate the role(s) of MACF1 in OS, siRNA was applied to transiently knock down MACF1 in 143B cells, and three OS cell lines with stable low expression of MACF1 were established through lentiviral transfection (these cell lines were named 143B-, U2OS-, and MG63-shNC and shMACF1; Fig. S3A, B, S4A, B). The OS cell migration and invasion were decreased when MACF1 was silenced (Fig. S3C, D, S4C, D), but no alteration of the proliferation was observed (Fig. S3E, F, S5). Consistently, intra-tibial injection of 143B-shMACF1-luc (a luciferase-tagged cell line) resulted in fewer lung metastasis nodules compared to the injection of 143B-shNC-luc (negative control shRNA) cells in the orthotopic osteosarcoma spontaneous lung metastasis mouse model (Fig. 1B; Fig. S6).

Based on the above results, we speculated that MACF1 might regulate the lung metastasis of OS. Thus, the therapeutic effects of targeting MACF1 were explored *in vitro* and *in vivo*. To provide a more readily translatable approach, bio-engineered recombinant siRNA against MACF1 (r/siMACF1) was manufactured using an *E. coli* system[4] (Fig. S7). The r/siMACF1 was successfully processed to mature siMACF1 in osteosarcoma cells, and significantly silenced MACF1 expression (Fig. S8A, B). The migration and invasion of osteosarcoma cells were efficiently inhibited by r/siMACF1 (Fig. S8C, D). In addition, compared to negative control groups (saline and MSA, Sephadex aptamer-tagged methionyl-tRNA), systemic injection of r/siMACF1 significantly reduced the lung metastasis in mice with xenograft osteosarcoma, as shown by the metastatic nodules on the surface of the lungs and the histopathological analysis of lung tissues (Fig. 1C). However, there were no effects on the xenograft tumor growth in mice (Fig. S9). Notably, r/siMACF1 had no significant liver or kidney toxicity, and was not immunogenic (Fig. S9), suggesting that the efficacy and safety of r/siMACF1 warrant further exploration for the treatment of OS.

Cytokines are essential for promoting tumor metastasis.[5] Therefore, we performed an antibody array to detect the levels of cytokines in three pairs of cell models (143B-, U2OS-, and MG63-shNC *vs.* shMACF1; Fig. S10A). Combined with the results of ELISAs (Fig. S10B), qPCR (Fig. S10C) and a promoter luciferase reporter gene assay (Fig. 1D) revealed that silencing MACF1 decreased the transcription of IL-6 and IL-8 in all three OS cell lines. Next, the transcription factors that co-regulated IL-6 and IL-8 were evaluated based on a screening assay in 143B cells. Compared to 143B-shNC cells, the expression and distribution of NFκB1, NFκB2, RelA, RelB, CREL, NFIL-6, CREB, and Fos (a subunit

Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2022.03.012

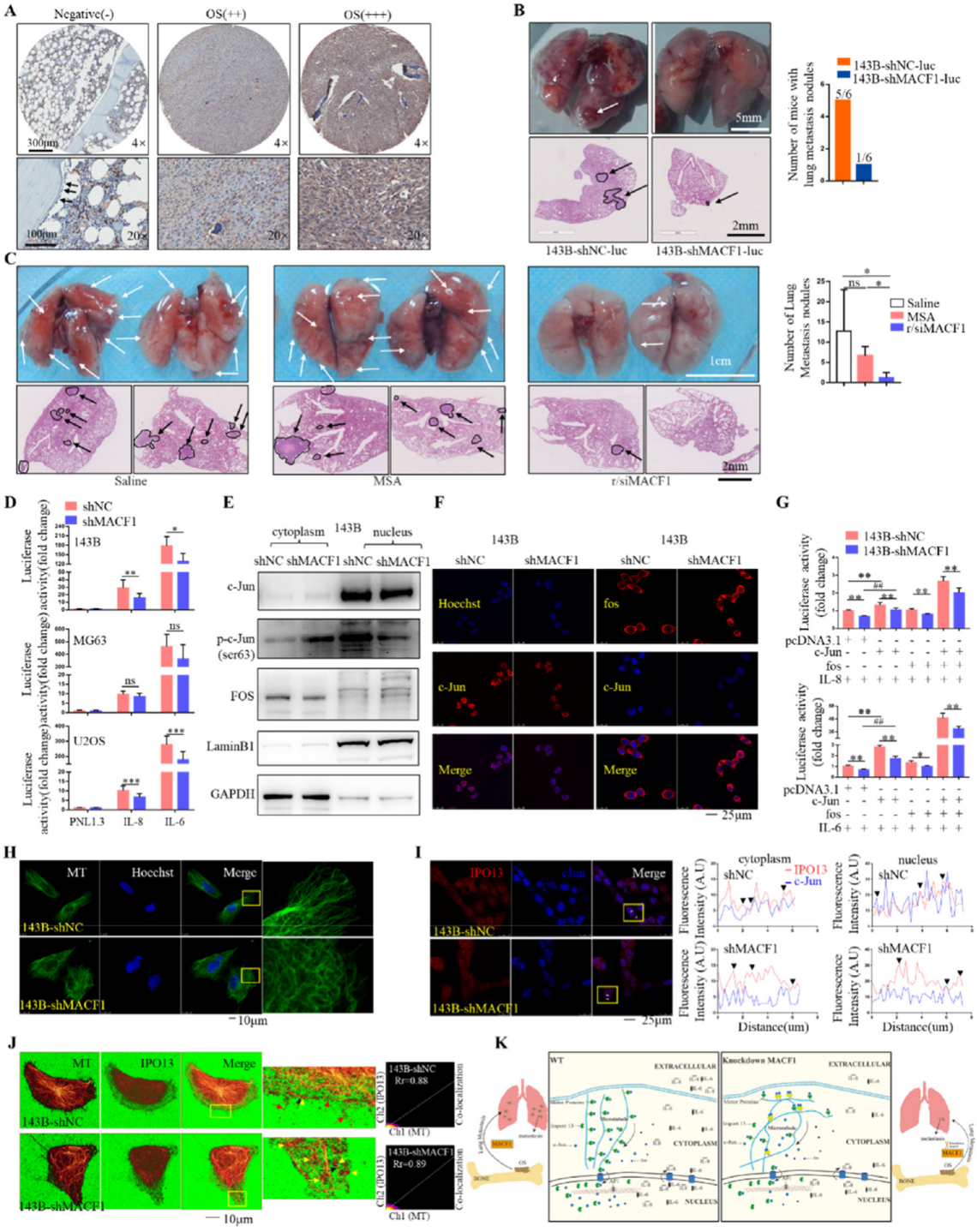

Figure 1 Silencing of MACF1, a highly expressed cytoskeletal crosslinking protein, in OS tissues suppressed the pulmonary metastasis of OS by disturbing the MT dynamics and triggering the formation of more intersections, which led to decreased nuclear translocation of c-Jun and downregulation of IL-6 and IL-8 transcription. **(A)** Representative immunohistochemistry images of MACF1 staining in one OS-negative sample (normal bone) and two positive OS samples from an array (upper, 4 ×); representative sites from each section were magnified (lower, 20 ×). **(B)** The metastatic nodules present in the representative lungs and their corresponding H&E images. The lungs were collected from mice that were intra-tibially injected with 143B-shNC-luc and 143B-

of AP1) did not change significantly in the 143B-shMACF1 cells (Fig. S11; Fig. 1E), but the levels of c-Jun (another subunit of AP1) and p-c-Jun in the nucleus were decreased (Fig. 1E, F; Video S1) without any changes in their mRNA and total protein levels (Fig. S12). In general, c-Jun is phosphorylated in the nucleus and cytoplasm by p-JNK. Although p-c-Jun was increased in the cytoplasm of 143B-shMACF1 cells (Fig. 1E), neither the expression nor distribution of p-JNK or JNK differed between the 143B-shNC and shMACF1 cells (Fig. S11) indicating that the nuclear translocation of c-Jun was blocked without any apparent effect on the phosphorylation.

Supplementary video related to this article can be found at doi:10.1016/j.gendis.2022.03.012

To test whether c-Jun could rescue the reduction in IL-6 and IL-8 transcription caused by MACF1 down-regulation, co-transfection of c-Jun and fos overexpression plasmids was conducted using a luciferase reporter gene assay (Fig. 1G). The co-transfection of the c-Jun plasmid, together with IL-6 and IL-8 promoter luciferase reporter plasmids, led to a significant increase in the luminance of luciferase in both 143B-shNC cells (Fig. 1G; $^{**}P < 0.01$) and 143B-shMACF1 cells (Fig. 1 G; $^{\#\#}P < 0.01$), while co-transfection of the fos plasmid only slightly boosted the signals (Fig. 1G). When both the c-Jun and fos plasmids were co-transfected into 143B cells, dramatic enhancement of the luciferase activity was observed (Fig. 1G). However, the luciferase signals in 143B-shMACF1 cells were lower than those in 143B-shNC cells even when there was plasmid-mediated overexpression of c-Jun and fos. This indicates that c-Jun, but not fos, could rescue the reduction in IL-6 and IL-8 transcription caused by MACF1 loss, and AP1 (c-Jun + fos in this case) strongly increased the transcription of both IL-6 and IL-8. Thus, we speculated that MACF1 regulates the transcription of IL-6 and IL-8 by controlling the nuclear import of c-Jun.

Previous studies suggested that microtubules (MT) and IPO13 were both required for the nuclear translocation of c-Jun. After knocking down MACF1 in 143B cells, the MT dynamics were disturbed (Fig. 1H; Video S2). When MT tracing was performed, it was indicated that the MACF1-GAS2 domain curled up inside the cell and could not be fixed at the cell edge (Fig. S13; Video S3) leading to more intersections, and a loss of the cell's directional movement (Video S4). Our results confirmed that c-Jun and IPO13 were co-localization in the cytoplasm and nucleus (Fig. 1I). IPO13 was distributed with MTs (Fig. 1J). Interestingly, immunofluorescence spots of IPO13 were uniformly distributed along straight MTs (Fig. 1J, red arrow). Larger IPO13 spots, which probably represented the jammed IPO13, were stranded at the MT intersections (Fig. 1J, yellow arrows indicate larger IPO13 spots, dark arrows mark the MT intersections). Therefore, we inferred that silencing MACF1 resulted in disturbed MT dynamics, characterized by the formation of more intersections, which in turn decreased the nuclear translocation of c-Jun carried by IPO13, and thus reduced the transcription of IL6 and IL8. A schematic working model for this interaction is summarized in Figure 1K.

In summary, metastasis is the primary cause of death in OS patients. Cytoskeleton-related molecules and cytokines participate in tumor metastasis.[5] In this study, we found that high expression of MACF1 was closely correlated with the metastasis of OS. Silencing MACF1 reduced the metastasis of OS *in vitro* and *in vivo*. The bioengineered r/siMACF1 was highly effective in inhibiting the pulmonary metastasis of OS, suggesting that MACF1 may represent a novel target for the prevention or treatment of OS metastasis. We also uncovered the mechanism underlying how MACF1 regulates the metastasis of OS, observing that it controls the nuclear translocation of c-Jun through alterations in MT dynamics, thus influencing the transcription of IL6 and IL8.

shMACF1-luc cells after 34 days. The histogram indicates the number of mice with metastatic nodules as indicated by H&E staining, $n = 6$ mice per group. **(C)** 15 days after the intra-tibial injection of 143B-luc cells, the mice were treated with r/siMACF1, MSA (Sephadex aptamer-tagged methionyl-tRNA) or saline through a tail vein once every three days for three weeks. The mice were then sacrificed, and their lungs were extracted and analyzed for the presence of visible lung metastases. Arrows identify visible metastatic nodules and the quantification of metastatic nodules on the lung surface; shown are representative images of H&E staining confirming the lung metastasis. **(D)** The transcription levels of IL-6 and IL-8 in 143B, U2OS and MG63 cells with control or MCAF1 shRNA were measured with a nano luciferase assay. The cells were transfected with 0.5 μg of pnl1.3-IL-6-promoter or pnl1.3-IL-8-promoter or pnl1.3 empty plasmids, respectively. **(E)** The distribution of c-Jun, p-c-Jun and fos in the cytoplasmic and nuclear fractions was evaluated in 143B-shNC and 143B-shMACF1 cells. **(F)** Left: immunofluorescence for c-Jun (red). Hoechst33342 marks cell nuclei. Right: immunofluorescence for c-Jun (blue) and fos (red). The scale bar at the lower right corner is 25 μm. **(G)** The luciferase activity of the IL-8 and IL-6 promoter luciferase reporter plasmid in 143B-shNC and shMACF1 cells co-transfected with pcDNA3.1-c-Jun and pcDNA3.1-fos overexpression plasmids. Upper panel: IL-8; Lower panel: IL-6. **(H)** Immunofluorescence for microtubules (green) and Hoechst33342 (blue) marked cell nuclei in 143B-shNC and shMACF1 cells. The scale bar in the lower right corner is 10 μm. **(I)** Immunofluorescence for importin 13 (IPO13, red) and c-Jun (blue); the curves show the fluorescence intensity for IPO13 and c-Jun in the cytoplasm and nucleus, and the white line denotes the cytoplasm and nucleus, respectively; the scale bar in the lower right corner is 25 μm. **(J)** Immunofluorescence for MT and IPO13, and colocalization images and Pearson coefficients. The pseudo-color, red arrows denote immunofluorescence spots of IPO13 on straight MTs, dark arrows denote MT intersections and yellow arrows denote immunofluorescence spots of IPO13 near the MT intersections. Immunofluorescence images were analyzed using the Image FIJI software. The scale bar in the lower right corner is 10 μm. **(K)** A working model summarizing how MACF1 mediates OS lung metastasis. MACF1 deficiency caused a disturbance of the MT network and led to the formation of more MT intersections, which blocked the c-Jun nuclear translocation mediated by IPO13 and resulted in down-regulation of IL-6 and IL-8 transcription.

Conflict of interests

The authors declare that there are no conflicts of interest with regard to the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81801871), the Shaanxi Provincial Key R&D Program (No. 2021SF-293, 2018KWZ-10, 2018SF-363), the Special Fund for Technological Innovation of Shaanxi Province (No. 2019QYPY-207), the Fundamental Research Funds for the Central Universities (No. 3102018zy053, D5000210746).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.03.012.

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15 January 2022
Available online 15 April 2022

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