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Characterization of breast cancer skeletal metastasis associated genes by conditional, tetracycline controlled miRNA-mediated RNA interference *in vitro* and *in vivo*

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Combination of the tetracycline-controlled transcription activation system ("Tet-Off system") and RNA interference allowed to initiate and maintain the conditional knockdown of the genes of interest for any intended period of time and to investigate the genes' functions and their mechanisms of action more precisely. The cell clones with conditional miRNA expression, targeting bone sialoprotein (BSP), integrin β3 (ITGB3) or osteopontin (OPN) were generated by recombinase-mediated cassette exchange (RMCE) from a parent cell clone without miRNA. This procedure allowed effective selection of the miRNA containing cell clones. In absence of doxycycline, the tetracycline-dependent transactivator (tTA) was activated, which stimulated miRNA expression, as well as that of the red fluorescent protein mCherry. The mCherry expression differed by three orders of magnitude after cultivating the established cell clones in medium with or without doxycycline and thus revealed good regulative properties of the system in response to doxycycline.
With regard to cell morphology, the specific cell clones showed a change in phenotype and cell number after 6 days of BSP knockdown in comparison to those with normal BSP levels. Phenotypical changes included rounded cells and cell fragments, as indicators of apoptotic events. Less distinct alterations in phenotype were observed in cell clones, which expressed miRNA targeting ITGB3 or OPN. In this regard, only slight detachment was observed in the microscopic pictures taken after 6 days of cultivation of the specific miITGB3 cell clones in media without doxycycline. In response to activated miRNA expression against BSP, the respective protein level was decreased by 86%. As a consequence of this BSP knockdown by a technically novel approach, the proliferation of the respective cell clones was reduced by 90%. Additionally, a significant suppression of colony formation (by 82%) and migration (by 90%) was observed. Following conditional knockdown of ITGB3 or OPN for the same time interval, the respective protein levels were similar to each other (40-50%), but less diminished than the BSP protein level after respective BSP knockdown. In line with this, the effects on cellular properties were less pronounced following conditional inhibition of ITGB3 or OPN translation. For ITGB3, the decreased protein levels were related to significant inhibition of proliferation and spontaneous migration by 22% and 87%, respectively. Similar repression of proliferation was noted in response to the reduced OPN levels. After longer time intervals of miRNA mediated OPN inhibition, more pronounced anti-migratory and anti-clonogenic effects were detected.
The regulation of miRNA against one of the genes of interest by the Tet-Off system allowed predictably to deactivate the expression of the gene and to study its role in various aspects of metastasis, including a nude rat model for site-specific skeletal lesions *in vivo*. A significant

decrease and even complete remission of soft tissue and osteolytic lesions was found by non–invasive and highly sensitive methods for tumor detection such as bioluminescence and magnetic resonance imaging (BLI, MRI), as well as by volume computed tomography (VCT) and thus proved the efficiency of miRNA mediated BSP, ITGB3 or OPN knockdown.

Altogether, these observations were reason to concentrate on the genes and eventual mechanisms related to the conditional, long lasting downregulation of BSP, ITGB3 or OPN, in order to understand and clarify their role in the progression of breast cancer skeletal metastasis. Therefore, the approach was beneficial not only to observe clear in vitro and in vivo results, but also to follow the genes' modulation as consequence of conditional and specific knockdown. A microarray analysis and subsequent western blot gave an overview on those genes, which undergo altered expression after BSP knockdown. The upregulation of the transcription factor c-FOS, of apoptosis related genes (*ATF3, CHOP*), induction of intrinsic and extrinsic apoptotic pathways, upregulation of tumor suppressor genes *EGR1, RASSF1* and *IL24* and the gene related to breast epithelial differentiation (*ID2*), as well as suppression of metastasis associated genes (*CD44* and *IL11*) and of cell proliferation related genes (*CDK2, CDK6, CAPN2*) are an improved basis for understanding the results following BSP knockdown in breast cancer cells in vitro and in vivo. In response to ITGB3 inhibition, the respective expression profiling data revealed decreased levels of genes, which are associated with angiogenesis (*RRM2, NPTN*), adhesion of tumor cells (*PLAU*) and metastatic colonization of the skeleton (*IL11*). Additionally, upregulation of a gene was found, which counteracts uPA and is associated with good prognosis of breast cancer patients (*SERPINB2*). In comparison to that, the conditional OPN knockdown led to upregulation of transcription factor c-FOS and of genes related to apoptosis (*GOS2, IGFBP1*), and downregulation of a gene, correlated with adhesion of tumor cells (*PVRL3*). Interestingly, a similarity was found in the modulation of the *SERPINB2, RRM2* and *IL11* genes after ITGB3 and OPN inhibition. Additionally, OPN knockdown led to a parallel modulation for *c-FOS, SERPINB2* and *IL11* as BSP knockdown. This resemblance of effects, as well as some overlap in function might be explained by the similarity of BSP and OPN, which belong to the same gene family. Taken together, these results suggest that BSP, ITGB3 and OPN are valuable targets in clinical treatment of breast cancer patients with skeletal lesions.