主論文の要旨

A New Tumorsphere Culture Condition Restores Potentials of Self-Renewal and Metastasis of Primary Neuroblastoma in a Mouse Neuroblastoma Model

（神経芽腫原発腫瘍からの自己複製能・転移能を維持した tumorsphere 培養）

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Introduction:

Tumorsphere culture provides important resources for cancer studies, since it enriches and expands tumor cells. However, the efficiency with which long-surviving tumorspheres are established from primary tumors in the nervous system is not satisfactory, whereas tumorspheres are obtained from metastatic tissues with relative ease. NB is the most common pediatric extracranial solid tumor and is derived from sympathetic neurons . Despite intensive multimodal therapy, high-risk NB patients with relapse in bone marrow have less than a 10% chance of survival . To overcome this problem, it is particularly important to verify the mechanisms of tumor initiation and the metastasis of NB, which remain elusive. Here, we have established culture condition for long-surviving tumorspheres from primary NBs. This culture condition restores the potential for self-renewal and metastasis of primary NBs.

Materials and Methods:

To get tumorspheres from the primary tumors, hemizygous MYCN transgenic mice were sacrificed and the tumor tissues were digested with trypsin to get single cell suspension. several medium cocktails were investigated for the tumor sphere formation. ELDA (extreme limiting dilution analysis) was used to evaluate the sphere forming potential. To compare the tumor formation potential, the primary tumor cells, the corresponding primary tumorsphere cells, allograft tumor cells and the corresponding allograft tumorsphere cells were injected subcutaneously into 4 to 5 week-old wild-type mice at different cell numbers and investigated for 1.5 months to evaluate the potential. To evaluate the bone marrow metastasis, tumorsphere cells were labeled with Venus, 10^5 cells were inoculated into syngenic 129/Svj wild type mice. 6 weeks later, the bone marrow cells from both femoral bones were collected and cultured.

Results:

We compared 6 different conditions to culture tumorspheres form MYCN transgenic mice tumor tissues and found tumorspheres formed best in the #4 condition. We also found that FBS and β-mercaptoethanol were indispensable for sphere formation and long-term survival (Fig. 1A-C). We employed ELDA assay to evaluate the sphere-forming potential of both tumor cells freshly prepared from tumors of MYCN hemizygous transgenic mice and tumorsphere cells passaged 4 times in #4 medium. We found that about 5 cells freshly isolated from primary tumors could form a sphere, whereas about 2 cells of tumorspheres grown in #4 medium were enough to form a sphere (Fig. 1D). We call this the culture condition PrimNeuS hereafter. Sphere cells cultured under PrimNeuS had a differentiation property, since radial neurites grew out of these cells under a differentiation condition for 3 days (Fig. 2A). The sphere cells also gave rise to tumors after subcutaneous injection. Furthermore, tumorsphere cells from primary tumors needed less cell number to give rise to tumor as compared with tumor cells from primary tumors, supporting the idea that sphere cells are more tumorigenic (Fig. 2C-D). We next asked whether or not sphere cells from primary tumors of MYCN

Tg mice could metastasize. To answer this, we used spheres from primary tumors after three passages and infected them with lentivirus carrying an EF-promoter-Venus expression vector. Cells were then subcutaneously injected into syngenic 129/SvJ wild-type mice. Bone marrow cells were analyzed 6 weeks later, after subcutaneous tumors were observed. In PrimNeuS medium, Venus-positive cells grew and formed spheres. These spheres could be passaged. In contrast, PrimNeuS did not support sphere formation of normal bone marrow cells. We concluded that sphere cells from primary tumors metastasize to the bone marrow. Consistent with this, we found that PrimNeuS supported sphere formation from the bone marrow of MYCN transgenic mice harboring sympathetic ganglia-derived primary tumors, but not from that of wild-type mice (Fig. 3G, H). These spheres could be passaged under PrimNeuS (Fig. 3H). TH and Snail showed significantly higher expression in both tumorspheres from primary tumor (Primary TS) and tumorspheres from bone marrow (Bone marrow TS) as compared with primary tumors, suggesting that primary TS and bone marrow TS have similar properties

Discussion:

Primary tumors in *MYCN* transgenic mice could be serially transplanted without exhaustion (we called these serially transplanted tumors allograft tumors), suggesting that these primary tumors contain cells with a high self-renewal potential. Nevertheless, primary tumors of *MYCN* transgenic mice did not give rise to tumorspheres in a serum-free neurosphere culture condition, which is commonly used for tumorsphere cultures for neural tumors. In contrast, allograft tumors gave rise to tumorspheres in this condition. These phenomena are reminiscent of those of clinical samples; tumorspheres are rarely obtained from primary or low-grade neural tumors, in contrast to metastatic or high-grade tumors. In this study, we established the new culture condition PrimNeuS, by which tumorspheres could be formed from a primary tumor and passaged indefinitely. Thus, this study has shown the self-renewal capacity of primary NB cells *in vitro*. PrimNeuS has enabled us to estimate self-renewal capacity *in vitro*. Furthermore, PrimNeuS revealed for the first time that tumorspheres from primary tumors (Fig. 3A-F) as well as primary tumors themselves (Fig. 3G-I) have the potential for metastasis to the bone marrow. This is unexpected, since bone marrow metastasis has not been reported in the model of *MYCN* transgenic mice. Personalized therapy has become a realistic concept for cancer treatment. If tumorspheres are obtained from every patient regardless of tumor status, those are precious resources for evaluating the efficacy of the treatment prior to its clinical use. Further evaluation or improvement of the culture condition for human NB will facilitate studies of stem cells of NB. To successfully eradicate tumors, it may be necessary to eliminate both the TICs and their more differentiated progeny. In this context, it was interesting to find in our study that tumorspheres from primary tumors and allograft tumors express distinct markers, but both show tumorigenecity. The success of culturing long-term tumorspheres from primary NB tumors may open new avenues to identify novel stem cell markers for diagnostic and therapeutic NB.

Conclusion:

Here, we successfully established a tumorsphere culture condition for primary neuroblastoma (NB). The newly established culture condition (named PrimNeuS) contained two critical ingredients: fetal bovine serum and β-mercaptoethanol were essential for tumorsphere formation as well as indefinite passages. The spheres could be passaged more than 20 times without exhaustion under this condition, exhibited a property of differentiation and formed tumors in vivo. Unexpectedly, PrimNeuS revealed that the MYCN transgenic mice had bone marrow metastasis. Furthermore, subcutaneous tumors derived from tumorspheres of primary tumors showed bone marrow metastasis. Taken together, PrimNeuS provides resources for the study of NB and can be used as a powerful tool for the detection of minimal residual disease and for in vitro evaluation prior to personalized therapy.