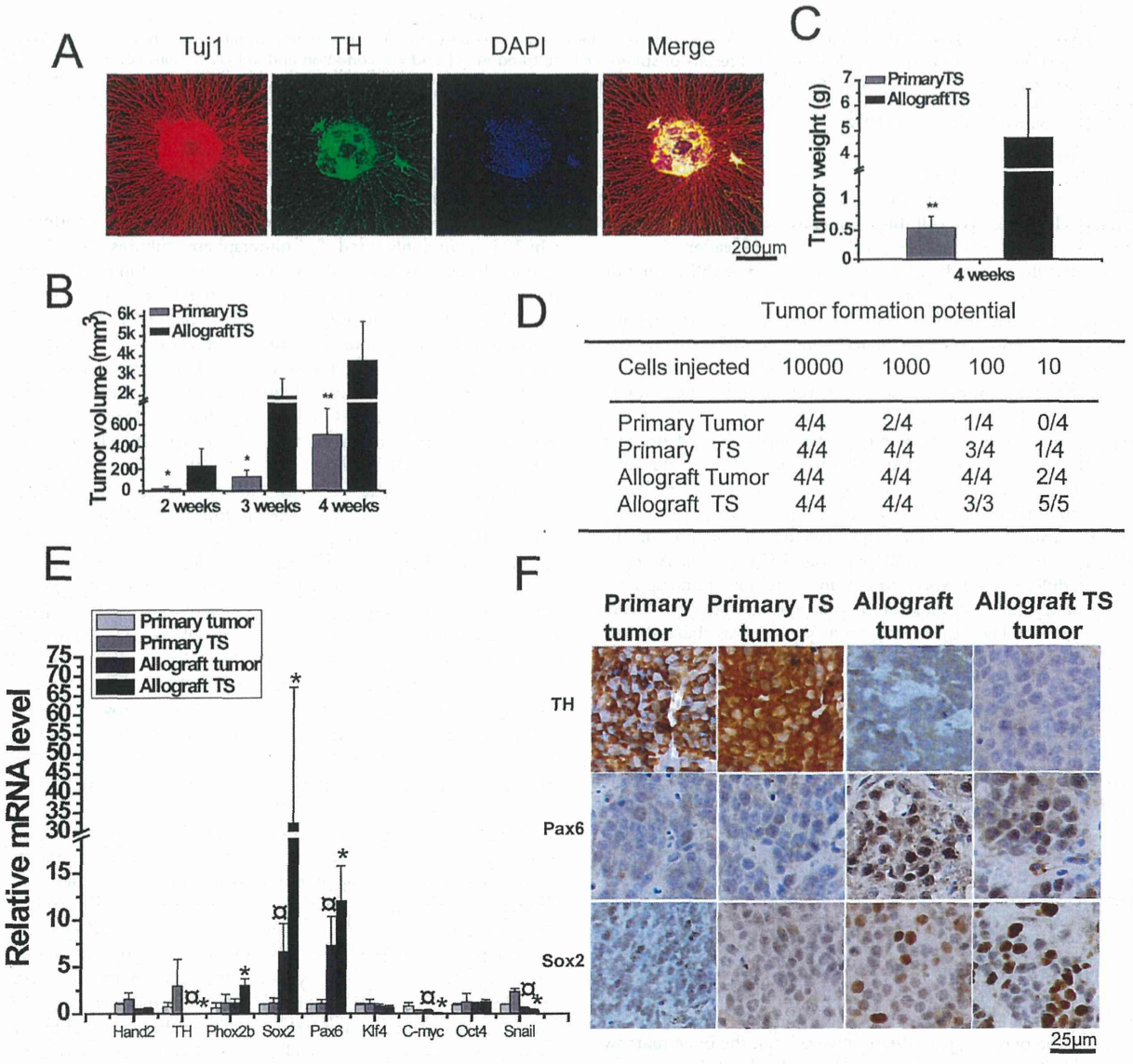

Figure 3. Tumorsphere cells have a differentiation capacity. A, Long radial neurites grew out of tumorspheres under differentiation condition. Representative photos are shown. B, C, Tumorspheres were maintained under PrimNeuS. Totally 10^4 tumorsphere cells together with 30% matrigel were subcutaneously inoculated into wild-type syngenic mice, tumor volumes and weights were monitored. *P<0.05, **P<0.005. D, The primary tumor cells, the corresponding primary tumor sphere cells, allograft tumor cells and the corresponding allograft tumor sphere 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. E, mRNA expression profile of primary tumors, tumorspheres from corresponding primary tumors, allograft tumors and tumorspheres derived from corresponding allograft tumors. The allograft tumorshperes were maintained under the traditional serum-free condition (#1 medium), and the tumorspheres from primary tumors were maintained under PrimNeuS (#4 medium). ¤ P<0.05, primary tumor vs. allograft tumor. *P<0.05, primary TS vs. allograft TS. F, Immunohistochemistry of tumors. Representative photos are shown.
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they differentiate into neurons if FBS is removed from the medium. In our case, the decreased concentration of FBS facilitated the outgrowth of neurites of primary tumor spheres, supporting the idea that FBS strongly inhibits neural differentiation of these cells [7,19,20]. Our results suggest that FBS helps to keep NB cells undifferentiated. In addition, we found that β-mercaptoethanol was critical for tumorsphere formation. If β-mercaptoethanol was removed after several passages, the tumorspheres were no longer passaged. This suggests that β-mercaptoethanol is also essential for indefinite passaging. The mechanisms underlying β-mercaptoethanol's functions remain to be verified.

PrimNeus supported the sphere formation from primary tumors and bone marrow in a neuroblastoma model. In contrast, it did not support the sphere formation from normal bone marrow cells. Therefore, PrimNeus may provide an appropriate culture condition for a subset of tumor cells in neuroblastoma (e.g., tumor initiating cells), but may not be generally applicable to stem cells such as normal bone marrow stem cells. This suggests that an

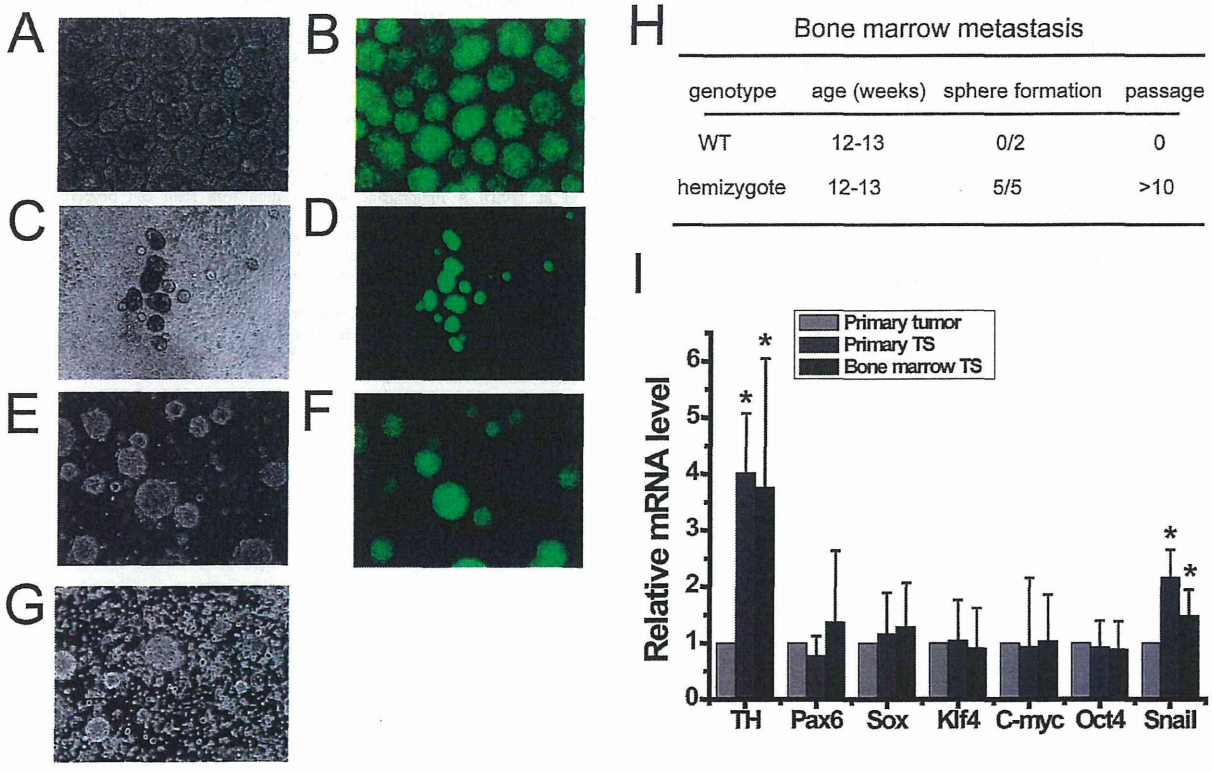

Figure 4. PrimNeuS can support the survival of metastasized cells of neuroblastomas. A–F, Primary tumorspheres were labeled with EF-promoter-Venus by lentivirus, and Venus-positive cells were purified by FACS sorting after 2 passages (A, B). Cells were then subcutaneously inoculated into wild-type mice. One and a half month after inoculation, the bone marrow cells from both femoral bones were cultured under PrimNeuS (C, D). These formed spheres were further expanded under PrimNeuS (E, F). G, The bone marrow of MYCN transgenic mice can form spheres in PrimNeuS medium. H, Bone marrow metastases presented in MYCN transgenic, but not wild-type mice. I, Real-time PCR results of the gene transcripts listed. *P<0.05, primary tumors vs. primary tumorsphere or bone marrow tumorspheres.
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appropriate culture condition may depend on cell types. Indeed, hematopoietic stem cells or progenitor cells require several growth factors, such as insulin, IL-3, IL-6, G-CSF and GM-CSF, but these are dispensable for the culture of tumor initiating cells [21,22,23].

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Author Contributions

Conceived and designed the experiments: DC SD KK. Performed the experiments: DC PH PM. Analyzed the data: DC PM. Contributed reagents/materials/analysis tools: ST MM. Wrote the paper: KK.

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