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Clinical Implications of Neuroblastoma Stem Cells

Xao X. Tang and Hiroyuki Shimada

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1. Introduction

Neuroblastoma (NB) is a childhood neoplasm and the cause of ~15% of cancer deaths in children. The clinical behavior of NB is highly variable. While some tumors are easily treatable, nearly 50% of the tumors exhibit very aggressive behavior. The latter tumors are classified as high-risk NB and are characterized by widespread tumor dissemination and poor long-term survival. Determining the prognosis of NBs at the time of diagnosis is important because of the clinical heterogeneity of the disease. Current prognostic factors used by the COG (Children's Oncology Group) Neuroblastoma Study for patient stratification and protocol assignment include: Age (<18 months *vs* >18 months), Stage (1, 2, 4S *vs* 3, 4), *MYCN* status (amplification *vs* non-amplification), Ploidy (diploid *vs* hyperdiploid), International Neuroblastoma Pathology Classification (Shimada system: Favorable *vs* Unfavorable Histology), 1pLOH (present *vs* absent), and 11qLOH (present *vs* absent) [1-3]. About half of high-risk NBs exhibit *MYCN* amplification, which is associated with older age, rapid tumor progression, and the worst prognosis [4]. According to the International Neuroblastoma Pathology Classification, NBs exhibiting *MYCN* amplification have unique histologic features, namely, an undifferentiated/poorly differentiated appearance and a high mitosis-karyorrhexis index. Nonetheless, certain NB with these histologic characteristics do not show *MYCN* amplification [5]. A previous report suggests that in non-*MYCN*-amplified unfavorable NB tumors, MYC rather than MYCN expression is responsible for the aggressive phenotype [6].

Current treatment for high-risk NB includes high dosage cytotoxic chemotherapy or myeloablative cytotoxic therapy with autologous hematopoietic stem cell transplantation [7]. Late relapse is often seen in patients with high-risk NB despite achieving a complete clinical remission. A subset of high-risk NBs, which is refractory to current front-line therapy designed for high-risk NB, is termed ultra high-risk NB [8, 9]. These tumors are totally unresponsive to current therapies, and thus reliable diagnostic tools to identify ultra high-risk NB prior to

treatment and innovative and effective therapeutic agents against these NBs are in need of development.

In this article, we will discuss our recent study on neuroblastoma stem cells, histopathological characteristics of these cells, and why the knowledge gained would help improve diagnosis and treatment of children with the most malignant NBs. We have recently reported the establishment of phenotypically stabilized stem cell-like NB cells (refer to as iCSC, see below) by short-term treatments of conventional monolayer NB cell lines with epigenetic modifiers [10]. The study addresses a fundamental problem that has affected a complete success in treating patients with cancers. Cancer stem cells (CSCs) are plastic in nature, a characteristic that hampers cancer therapeutics. To date, two models have been proposed to explain the existence of cancer stem cells in a tumor mass: the stochastic model and the hierarchical model. According to the stochastic model, transformed single cells develop unlimited proliferative capability to cause a tumor. Initially, a single or few transformed cells result in uncontrolled growth. Accumulations of different mutations then occur driving additional tumor growth and resulting in heterogeneous subpopulations within the tumor. These cancer cells are believed to participate in tumor growth, develop resistance, and cause recurrence. Hence, all cells are considered tumorigenic and are targets for treatment. In contrast, the hierarchical or current CSC model states that in a given tumor, there exists a population of cancer cells that have characteristics similar to stem cells. Cancer stem cells have the capacity to renew indefinitely, to initiate tumor formation, and to give rise to multiple non-tumorigenic proge-nies via asymmetric cell division. As a result of this phenotypic drift, an established tumor would always consist of a mixture of CSC and non-CSC. Current anti-cancer therapies are believed to target the more differentiated tumor cells, but not the CSC component, which is ultimately responsible for tumor recurrence. Based on the most current thinking, the two models are not mutually exclusive.

To create phenotypically stabilized stem cell-like NB cells, our approach includes a short-term treatment (i.e., five days) of NB monolayer cell lines (SKNAS, SKNBE(2)C, CHP134, SY5Y) with either an inhibitor of DNA methylation and/or an HDAC inhibitor followed by cell culturing in the sphere-forming medium without the epigenetic modifiers. This strategy not only significantly augments the expression of the Yamanaka reprogramming factors and stem cell markers in the NB spheres generated, but it also captures these spheres in the "totally undifferentiated status" over a long period of time *in vitro* and *in vivo*. To date, known stemness/reprogramming factors include MYC/MYCN, SOX2, OCT4, NANOG, LIN28, and KLF4. These factors were shown to initiate reverse differentiation or reprogramming of somatic cells [11-13]. In addition, several stem cells markers (CD133, CXCR4, ABCG2) [14-16] and neural crest stem cell markers (p75[NTR], SOX9, SOX10, SLUG, Musashi-1, CD24, and HES1) have been reported [17-22].

The stem cell-like NB cells that are created in our recent study are characterized by their high expression of stemness factors, stem cell markers, and their open chromatin structure. We referred to these cells as induced CSC (iCSC) [10]. Our *in vivo* studies show that the NB iCSCs possess a high tumor-initiating ability and a high metastatic potential. SKNAS iCSC and SKNBE(2)C iCSC clones (as few as 100 cells) injected subcutaneously into SCID/Beige mice
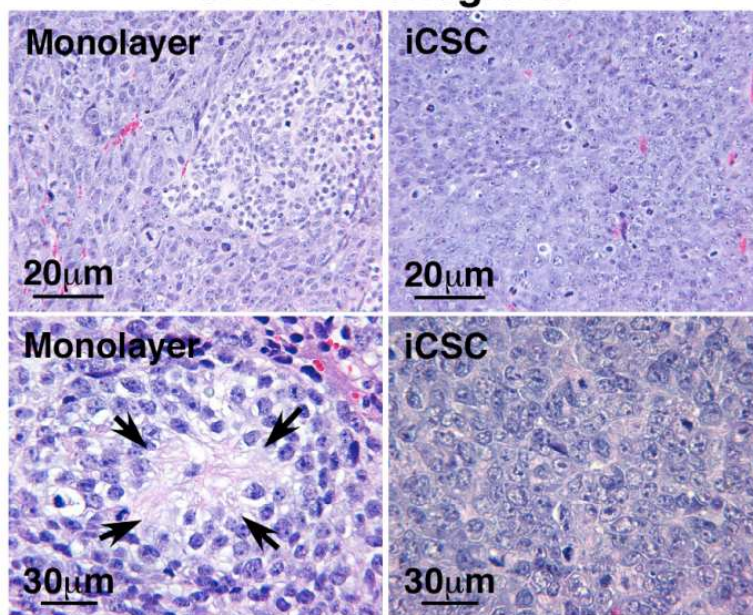
SKNAS Xenografts

Figure 1. Histopathological examinations of SKNAS monolayer cell and iCSC xenografts. The monolayer cell xeno‐
grafts were composed of two distinct components having different cellular morphologies. Tumor cells in the first com‐
ponent were larger cells. Tumor cells in the other component were smaller in both cellular and nuclear size and had
smaller nucleoli. These small tumor cells often produced neurites or neuropils (indicated by the arrows). The monolay‐
er cell xenografts were thus classified as poorly differentiated NB. In contrast, iCSC xenografts were composed of uni‐
formly large cells with vesicular nuclei and one or more prominent nucleoli, and thus were classified as totally
undifferentiated "large-cell" NB. Adapted from Fig. 4 of Ikegaki et al., [10].

formed tumors, and in one case, SKNBE(2)C iCSC metastasized to the adrenal gland, suggest‐
ing their increased metastatic potential [10]. Important histopathological observations were
also made on the NB iCSC xenografts, and highlights of these findings are described in below.

*The NB iCSC xenografts resemble human totally undifferentiated "Large-Cell" NB, the most aggressive
and deadly form of NB.* Histologically, NBs are classically divided into undifferentiated (UD),
poorly differentiated (PD) and differentiating (D) subtypes. However, a unique histological
subset of NBs within the UD and PD subtypes has been identified in the past years [5, 23].
These tumors are uniformly composed of large cells with sharply outlined nuclear membranes
and one to four prominent nucleoli, and are referred to as "Large-Cell Neuroblastomas" or
LCNs. Most importantly, the LCNs are the most aggressive and deadly tumors among the
unfavorable NBs. Patients with the UD neuroblastoma and with the LCN appearance had a
very poor prognosis regardless of age at diagnosis, clinical stage, and DNA index. Surprisingly,
non-*MYCN* amplified UDs behaved significantly worse than *MYCN* amplified UDs [24]. As
described below, our recent study demonstrates that NB iCSC xenografts do in fact resemble
human LCN. In addition, there are histological differences between NB monolayer cell
xenografts and iCSC xenografts.

As shown in Fig. 1, the SKNAS monolayer cell xenografts presented a mosaic pattern and were
composed of at least two distinct components having different cellular morphologies. Tumor
cells in the first component were larger cells. Tumor cells in the other component were smaller
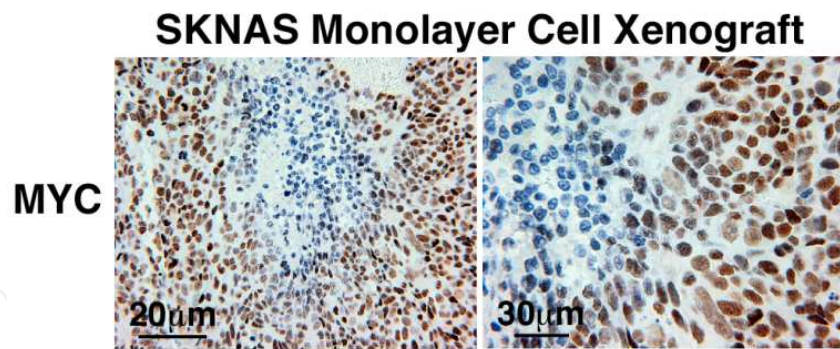
Figure 2. Immunohistochemical examination of SKNAS monolayer cell xenografts for MYC expression. As shown in Fig. 1, the SKNAS monolayer cell xenografts were composed of two distinct components having different cellular morphologies. The smaller tumor cells had reduced activities of mitosis and karyorrhexis (see also text). Accordingly, immunohistochemical examination of SKNAS monolayer cell xenografts with the anti-MYC antibody showed that the smaller tumor cells lacked MYC expression.
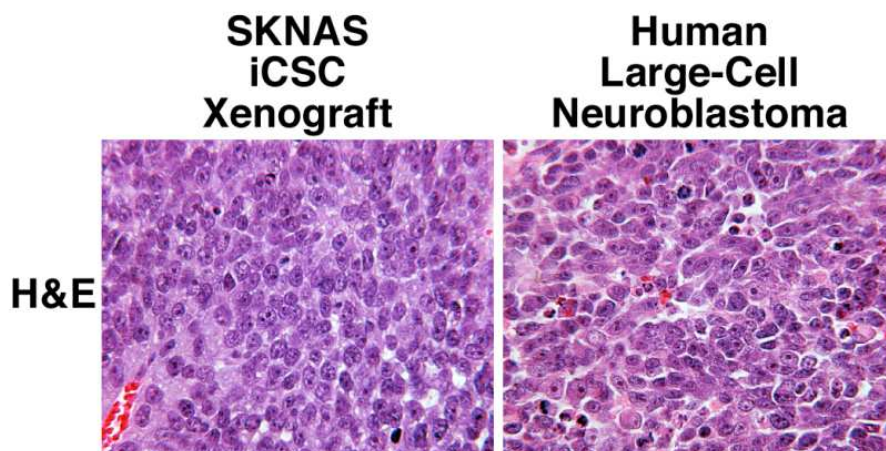

Figure 3. Histopathological examinations of SKNAS iCSC xenografts and the human large-cell" NBs. H&E stained sections showed that the SKNAS iCSC xenografts resembled human undifferentiated "large-cell" NBs histologically. Adapted from Fig. 5 of Ikegaki et al., [10].

in both cellular and nuclear size, and had smaller nucleoli (Fig. 1, upper left panel). Furthermore, these small tumor cells in the second component had reduced activities of mitosis and karyorrhexis (either intermediate MKI of 100~200/5,000 cells or low MKI of <100/5,000 cells) and often produced neurites or neuropils (Fig. 1, lower left panel). In addition, these smaller cells do not express MYC (Fig. 2). The monolayer cell xenografts were thus classified as poorly differentiated NB. In contrast, the SKNAS iCSC xenografts were composed of a diffuse and solid growth of medium-sized, rather uniform cells with a large vesicular nucleus and one or few prominent nucleoli (Fig. 1 right panel). Mitotic and karyorrhectic activities were frequently encountered (either intermediate MKI of 100~200/5,000 cells or high MKI of >200/5,000 cells). The iCSC xenografts were thus classified as totally undifferentiated "large-cell" NB, according to the International Neuroblastoma Pathology Classification [2, 3, 23, 25]. In fact, as reported in our study, all of the other iCSC xenografts from SKNBE(2)C, CHP134, and SY5Y have the
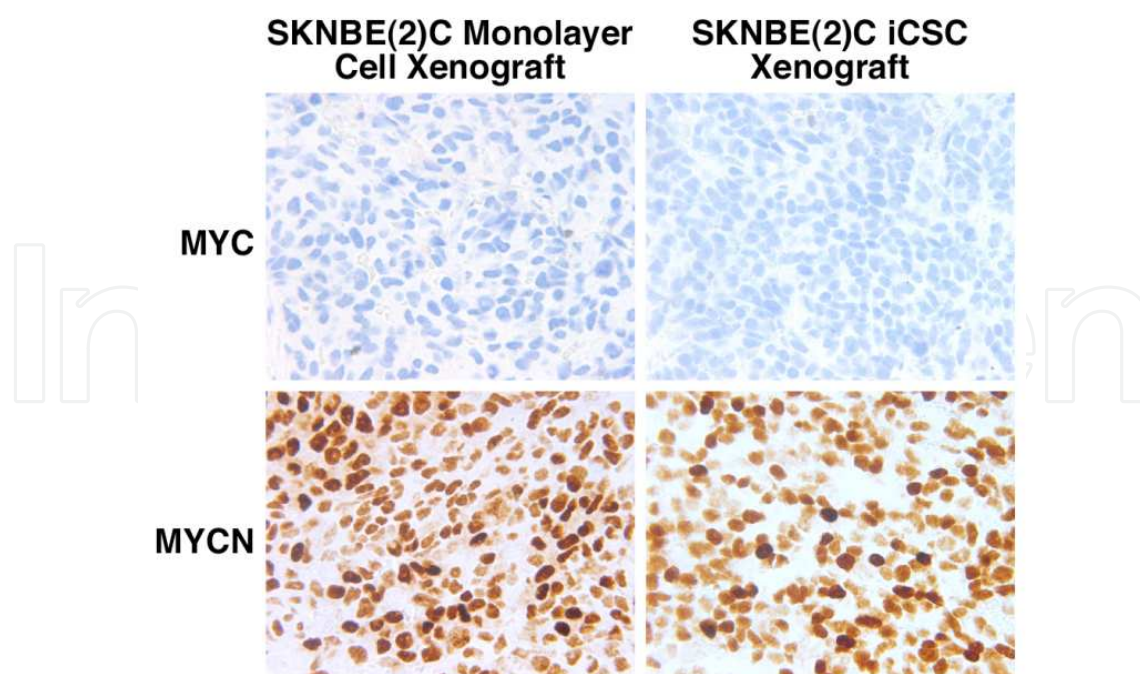
Figure 4. MYCN expression in SKNBE(2)C monolayer cell and iCSC xenografts. SKNBE(2)C are *MYCN*-amplified cells and uniformly expressed high-levels of MYCN in both monolayer cell and iCSC xenografts. MYC expression was examined for comparison. Microscopic magnification of 400X was used for all pictures. Adapted from Fig. 7of Ikegaki et al., [10].

LCN phenotype [10]. Fig. 3 shows a remarkable resemblance of SKNAS iCSC xenografts and human LCN histologically.

MYC/MYCN expression and CXCR4 expression in NB monolayer cell xenografts and iCSC xenografts. Monolayer NB cell lines in culture express high levels of MYC (non-MYCN amplified cells) or MYCN (MYCN amplified cells). In consistent with this, our immunohistochemical analysis demonstrate that all NB monolayer cell xenografts and iCSC xenografts express high levels of MYC (SKNAS, SY5Y) or MYCN (SKNBE(2)C, CHP134)[10]. Fig. 4 shows a representative data of SKNBE(2)C.

In contrast to the consistently high MYC/MYCN expression, among the NB xenografts examined, there is a differential expression of CXCR4 in the SKNAS iCSC xenografts over monolayer cell counterparts (Fig. 5). It should be mentioned that both the larger and smaller cells of the SKNAS monolayer cell xenografts described in Fig. 1 were negative for CXCR4 staining, except some rare cases where a few cells were focally positive for CXCR4 staining (Fig. 5). These observations suggest that the large cells in SKNAS iCSC xenografts had different molecular and biological characteristics from the larger cells in the monolayer cell xenografts. However, the pattern of CXCR4 expression observed among the SKNAS xenografts was not always seen among the other iCSCs. Xenografts from both iCSC and monolayer cells of SKNBE(2)C, CHP134, SY5Y were all positive for CXCR4, but the staining in these cases was not intense and uniform [10].
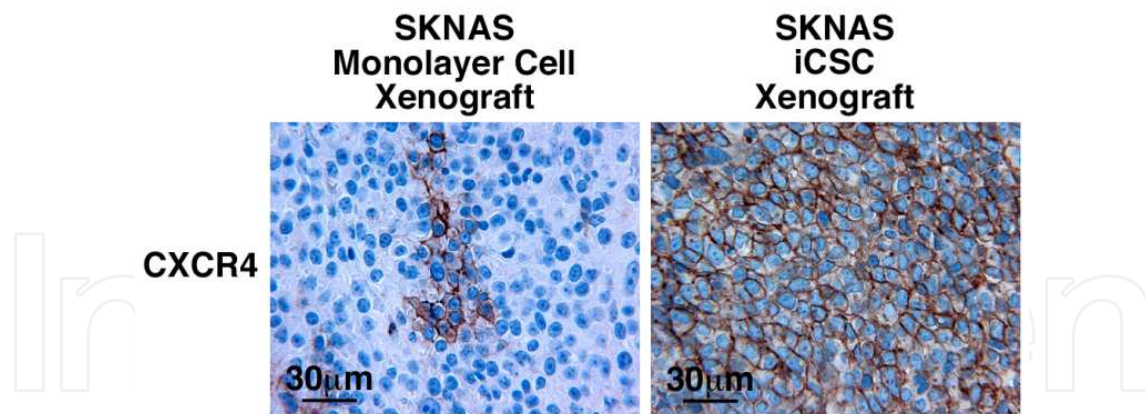
Figure 5. Differential expression of CXCR4 in SKNAS iCSC and monolayer cell xenografts. Immunohistochemical analysis showed that SKNAS iCSC xenografts were uniformly positive for CXCR4. In contrast, SKNAS monolayer cell xenografts were negative for CXCR4 with the exception of some rare cases where a few cells were focally positive for CXCR4 staining. Adapted from Fig. 3 of Ikegaki et al., [10].
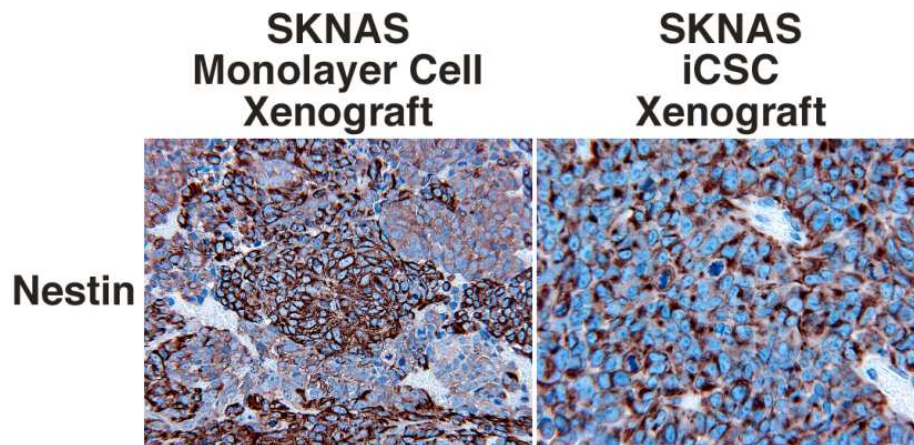

Figure 6. Immunohistochemical examination of SKNAS iCSC and monolayer cell xenografts for nestin expression. Nestin expression was examined with the anti-nestin antibody to determine whether or not nestin could serve as a marker of NB CSCs. Nestin was expressed In both SKNAS iCSC and monolayer cell xenografts. Notably, the smaller tumor cells of the monolayer cell xenograft expressed higher levels of nestin than the larger cells. These smaller cells were in fact negative for MYC expression (see Fig. 2). These observations indicate that nestin expression may not be a specific marker of NB stem cells.

Nestin expression in NB monolayer cell xenografts and iCSC xenografts. Nestin is a type VI intermediate filament protein, and nestin expression has been suggested to be a NB stem cell marker [26, 27]. Nonetheless, our data showed that nestin is expressed in SKNAS iCSC xenografts, and in both the smaller cells and larger cells of SKNAS monolayer cell-xenografts (Fig. 6). This pattern of nestin expression together with the fact that the smaller cells of SKNAS monolayer cell-xenografts are MYC negative (Fig. 2), nestin expression may therefore not serve for a specific marker of NB stem cells.

p75^{NTR} expression in NB monolayer cell xenografts and iCSC xenografts. p75^{NTR} is the low-affinity nerve growth factor receptor and a neural crest stem cell marker [18]. Our in vitro study show
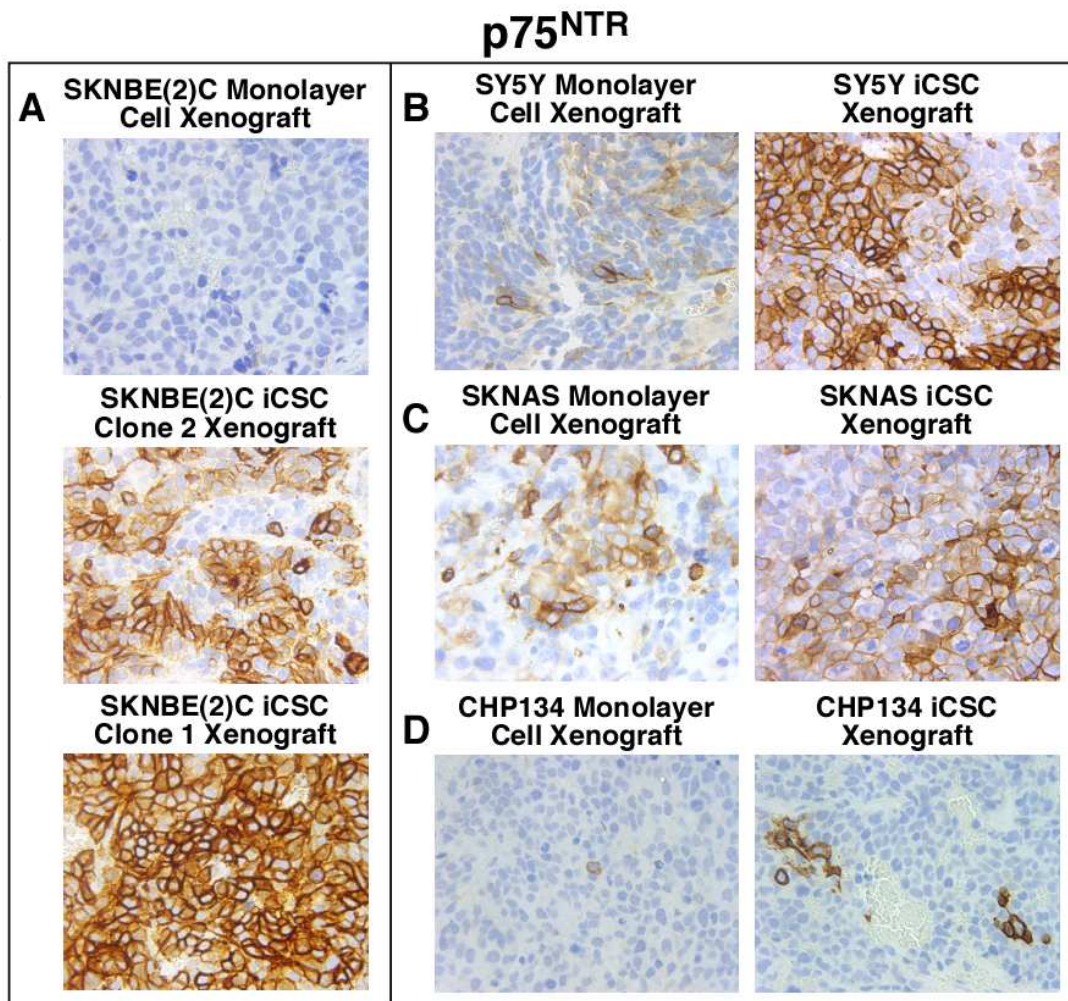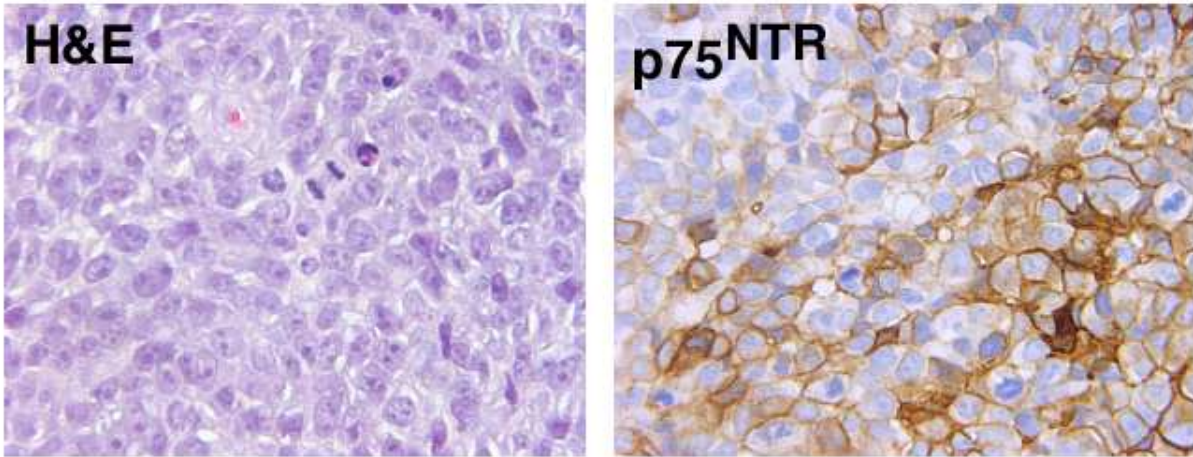
Figure 7. (A) The expression of p75^NTR in xenografts derived from iCSC clones and monolayer cells of SKNBE(2)C. Immunohistochemical examination was performed to assess p75^NTR expression in SKNBE(2)C iCSC clones and monolayer cell xenografts. The SKNBE(2)C monolayer cell xenografts rarely and faintly expressed p75^NTR. In contrast, subcutaneous xenografts of both Clone 1 and Clone 2 expressed high levels of p75^NTR, though Clone 2 xenografts contained cells with positive for p75^NTR and those devoid of p75^NTR staining. Subcutaneous xenografts of Clone 1 are consistently positive for p75^NTR. The expression of p75^NTR in xenografts derived from iCSCs and monolayer cells of (B) SY5Y, (C) SKNAS and (D) CHP134. Immunohistochemical examination was performed to assess p75^NTR expression in xenografts of SY5Y, SKNAS, and CHP134 iCSCs and monolayer cells. SY5Y monolayer cell xenografts expressed low level of p75^NTR, whereas SY5Y iCSC xenografts contained the majority of cells highly positive for p75^NTR and the minority of cells with low p75^NTR expression. The xenografts of SKNAS iCSC contained larger clusters of cells strongly positive for p75^NTR with the surrounding cells of weak p75^NTR staining, whereas the xenografts of SKNAS monolayer cells had medium size clusters of p75^NTR positive cells that were surrounded by p75^NTR negative cells. Only rare and faintly positive cells for p75^NTR were detected in CHP134 monolayer cell xenografts, while CHP134 iCSC xenografts contained small islands of positive cells for p75^NTR. Microscopic magnification of 400X was used for all pictures. Adapted from Fig. S8 of Ikegaki et al., [10].

that SKNAS iCSC, SKNBE(2)C iCSC, and SY5Y iCSC express high levels of p75^NTR [10], and these observations are confirmed by the xenograft data shown in Fig. 7. As described in our study, the expression of p75^NTR in CHP 134 iCSC xenograft was minimal [10]. Interestingly, the pattern of p75^NTR expression in the SKNAS monolayer cell xenografts suggests that p75^NTR expression is not related to neuronal differentiation in NB (Fig. 8).
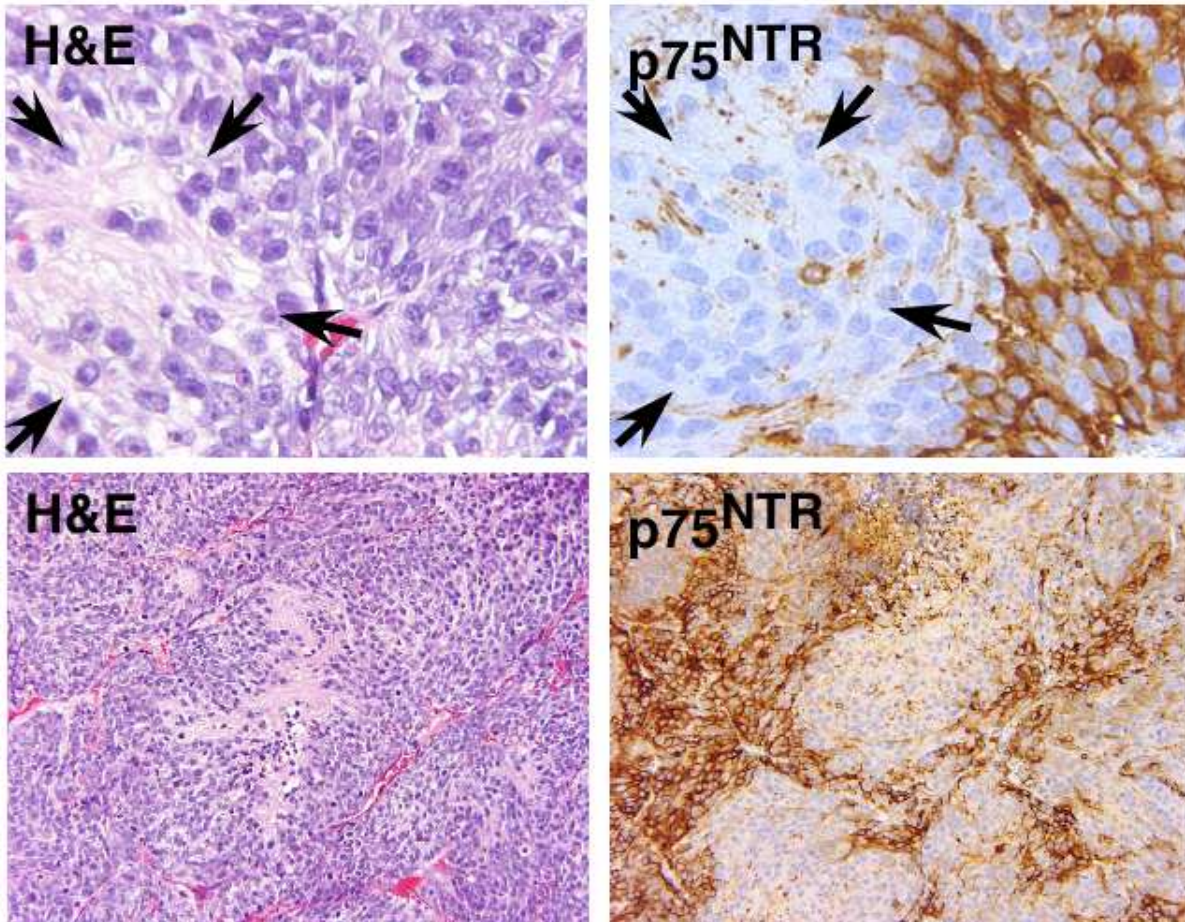
Figure 8. The expression of p75NTR is not related to neuronal differentiation in NB. Varying numbers of cells were positive for p75NTR in SKNAS monolayer cell xenografts. However, in the SKNAS monolayer cell xenografts, the cells with active neuropil formations were negative for p75NTR staining as indicated by arrows. Microscopic magnification of 400X was used for four pictures in the first and second rows, and 100X was used for two pictures in the bottom row. Adapted from Fig. 8 of Ikegaki et al., [10].

2. Conclusion

In conclusion, the xenografts established from the NB iCSCs shared two consistent and common features: the LCN phenotype and high-level MYC/MYCN expression. In addition, our observations suggest that NB cells with large and vesicular nuclei, representing their open chromatin structure, are indicative of stem cell-like tumor cells, and that epigenetic changes may have contributed to the development of these most malignant NB cells. These observations have significant clinical implications. Specifically, one may identify the most malignant and aggressive type of NBs that require immediate innovative therapeutic intervention by examining histological/cytological appearance of the tumor, namely totally undifferentiated large-cell NB with prominent nuclei and high-level expression of MYC and/or MYCN by immunohistochemical analysis. Finally, the availability of the NB iCSCs will serve as useful tools to develop effective anti-CSC agents for NB in vivo and will help improve treatment and cure for children with neuroblastoma.

Acknowledgements

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