IBRO Neuroscience Reports 17 (2024) 73–82

Contents lists available at ScienceDirect

IBRO Neuroscience Reports

journal homepage: www.sciencedirect.com/journal/IBRO-Neuroscience-Reports

Research paper

Dickopff 1 inhibits cancer stem cell properties and promotes neuronal differentiation of human neuroblastoma cell line SH-SY5Y

Shubham Krishna [a], Bharat Prajapati [a,c], Pankaj Seth [a,*], Subrata Sinha [a,b,**]

[a] National Brain Research Centre, Manesar, Gurugram, India
[b] Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India
[c] Department of Medical Biochemistry and Cell Biology, The Sahlgrenska Academy, Institute of Biomedicine, Gothenburg, Sweden

ARTICLE INFO

Keywords:
Neuroblastoma
DKK1
Wnt signaling
SH-SY5Y cancer stem cell
SH-SY5Y differentiation

ABSTRACT

Neuroblastomas are pediatric tumors arising from undifferentiated cells of neural crest origin with stem cell-like characteristics. Dysregulation of Wnt/β-catenin signaling has been shown to be linked to the development of various tumors. Activated Wnt signaling results in β-catenin accumulation in the nucleus to support pro-neoplastic traits. DKK1, a secreted glycoprotein, is an inhibitor of Wnt signaling, and the addition of DKKI to the culture medium has been used to suppress the Wnt pathway. This study aimed to analyze the role of Dickopff-1 as a potential differentiating agent for the neuroblastoma cell line SH-SY5Y and neurospheres derived from it. The treatment of SH-5Y5Y derived neurospheres by DKK1 resulted in their disintegration and reduced proliferation markers like Ki67, PCNA. DKK1 treatment to the neurospheres also resulted in the loss of cancer stem cell markers like CD133, KIT and pluripotency markers like SOX2, OCT4, NANOG. DKK1 treatment caused reduction in mRNA expression of β-catenin and TCF genes like TCF4, TCF12. When the SH-SY5Y cancer cells were grown under differentiating conditions, DKKI caused neuronal differentiation by itself, and in synergy with retinoic acid. This was verified by the expression of markers like MAPT, DCX, GAP43, ENO2 and also with changes in neurite length. We concluded that Wnt inhibition, as exemplified by DKK1 treatment, is therefore a possible differentiating condition and also suppresses the proliferative and cancer stemness related properties of SH-SY5Y neuroblastoma cells.

1. Introduction

Neuroblastoma is the most common solid childhood cancer originating outside the brain from the primitive sympathetic nervous system, with 90 % of cases occurring in children less than 5 years and is responsible for 15 % of pediatric cancer death (Brodeur, 2003; Spix et al., 2006; Smith et al., 2010). Genetic abnormalities in neural crest cells derived sympathoadrenal cells lead to their rapid proliferation, reduced differentiation, and apoptosis of cells (Otte et al., 2021). Possible molecules that can be used for neuroblastoma therapy along with chemotherapy include those that promote apoptosis, especially by targeting the p53 and MDM pathway (Barbieri et al., 2006), Bcl2 inhibitors (Delbridge and Strasser, 2015) electron transport chain inhibitors (Hirsch et al., 2009; Moreira et al., 2006), autophagy activators especially by mTOR/AKT inhibitors (Berardi et al., 2011; Wang et al., 2011). Limited efficacy and the development of resistance, is, however, a feature of these approaches, indicating the need to develop better methods.

Different neuroblastoma-derived cell lines have been used for studying neuroblastoma. Of these, one very commonly used cell line is SH-SY5Y. It is a subline of the parental line SK-N-SH. SK-N-SH were subcloned to SH-SY, then to SH-SY5, and finally to SH-SY5Y (Kovalevich et al., 2021). The adherent, undifferentiated SH-SY5Y cells tend to grow in clusters and may form clumps, lying one over the other (Kovalevich et al., 2021). They show the expression of proliferative gene markers like PCNA and Ki67. The SH-SY5Y cell line can be differentiated in vitro into mature neuronal-like cells by several methods which include treatment with phorbol esters and retinoic acids (Påhlman et al., 1995). One can

Abbreviations: DKK1, Dickopff-1; FGF, Fibroblast growth factors; EGF, Epidermal growth factors; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum; IFN, Interferon; DHEA, Dehydroepiandrosterone; NSF1, neural survival factor 1; Rspo2, R-spondins.

* Corresponding author.
** Corresponding author at: Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, India.
E-mail addresses: pseth.nbrc@gov.in (P. Seth), sub_sinha@hotmail.com (S. Sinha).

https://doi.org/10.1016/j.ibneur.2024.05.010
Received 31 October 2023; Accepted 24 May 2024
Available online 2 June 2024

also lower the SH-SY5Y aggregation and increase neurite outgrowth of SH-SY5Y cells using dibutyryl cyclic AMP (Sánchez et al., 2004). Retinoic acid has been shown previously used for differentiation therapy in neuroblastoma, but has limited efficacy (Matthay et al., 2009). The MYCN proto-oncogene is an important genetic marker of neuroblastoma tumor aggressiveness. N Myc amplification is seen in aggressive Neuroblastoma (Brodeur et al., 1984). Duffy et al. (2017) show that retinoic acid and TGF β signaling work together to overcome retinoid resistance, which is related to MYCN. Bayeva et al. (2021) describe how synergistic interactions of retinoic acid derivatives like fenretinide and retinoic acid with molecules like IFN or DHEA have differing effects on neuroblastoma cells.

Wnt signaling has been involved in various functions of cell proliferation, stemness, and differentiation. Dysregulation of the Wnt pathway has been linked to the development of various tumors. Wnt-mediated β-catenin activation has both pro and anti-tumor effects in different malignancies There are also differences in the canonical and non-canonical Wnt pathways (Zhan et al., 2017). Zhang et al. (2014) show that the Wnt inhibitory factor (WIF-1) functions as a tumor suppressor in SK-N-SH cells by inhibiting the Wnt/β catenin pathway. Szemes et al. (2018) have shown that Wnt signaling can drive the proliferation of SK-N-AS cells, but also the differentiation of SK-N-BE (2)-C and SH-SY5Y cells. Active MYCN plays a role Wnt mediated proliferation (Szemes et al., 2018). The context-dependent differences due to Wnt activation are well known. Becker and Wilting sum up by saying that the canonical and non-canonical roles of Wnt signaling often interact in a mutually inhibitory manner (Becker and Wilting, 2018). As reviewed by Becker and Wilting, human neuroblastoma reports show both activation and inhibition of the Wnt pathway with contradictory results (Becker and Wilting, 2019). There are reports suggesting inhibition of the Wnt pathway reduces neuroblastoma cell differentiation (Suebsoonthron et al., 2017). Other reports contradict this and suggest that activation of the Wnt pathway is responsible for cancer stemness, leading to chemoresistance linked proliferation (Tang et al., 2020; Cho et al., 2020; Chen et al., 2019; Flahaut et al., 2009). Cancer stem cells are present in primary neuroblastomas, and, as for other tumors, monolayer cultures of neuroblastoma derived cell lines contain a cancer stem cell like population, which is enriched when these are grown as neurospheres under appropriate culture conditions. Aravindan et al. (2019) have reviewed the properties of cancer derived stem cells and their role in therapeutic resistance in neuroblastomas.

DKK1, a secreted glycoprotein, is an inhibitor of Wnt signaling. DKK1 is downregulated by MYCN oncogene. Neuroblastomas show amplification of MYCN oncogene (Koppen et al., 2007). In this study we have used DKK1 to study its effect on stemness, proliferation, and differentiation in the SH-SY5Y cell line. To the best of our knowledge, no study shows an in- vitro assessment of the Wnt pathway inhibitors on stemness-related pathways in neuroblastoma cells. Neurospheres formed from SH-SY5Y cells were treated with DKK 1. This treatment disintegrated the neurosphere, along with a reduction in the expression of stem cell and pluripotency markers. DKK1 also promoted the neuronal differentiation of SH-SY5Y adherent culture, as evident from longer neurites and an increase in neuronal differentiation markers. This effect also synergized with retinoic acid in increasing neuronal differentiation and reducing proliferation.

2. Methods

2.1. Differentiation of SH-SY5Y cells using retinoic acid

DMEM media (Gibco, USA) with 10 % FBS (Thermo fisher scientific, USA) was used to culture the undifferentiated SH-SY5Y cells. 100 mM stock concentration of retinoic acid (Sigma–Aldrich, St. Louis, MO) was prepared in DMSO. Repeated freeze thaw and direct contact with sunlight of RA was avoided. For differentiation experiments, the final concentration of 10 μM of retinoic acid was used in complete DMEM media.

2.2. SH-SY5Y neurosphere formation

Ultra-low attachment flasks (Corning, USA) were used for neurosphere formation. Neurospheres were formed in neurobasal media (Gibco, USA) containing 20 ng/ml EGF (R&D Systems, Minnesota, United States), 25 ng/ml FGF (R&D systems, Minnesota, United States) supplemented with NSF1 (Lonza, Charles City, IA), 4 mM glutamine (Sigma-Aldrich, St. Louis, MO), 0.8 % N2 supplement (Gibco, USA), 2 % penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO), 0.05 mg/ml gentamycin (Gibco, USA) and 0.8 % BSA.

2.3. DKK1 treatment for SH-SY5Y

DKK-1 (Sigma-Aldrich, St. Louis, MO) lyophilized powder was reconstituted in 1X PBS containing 0.1 % BSA. Cells were grown under appropriate conditions till distinct neurospheres had formed. They were then treated to a final concentration of 0.5 μg per ml of recombinant DKK1. In adherent SH-SY5Y culture, cells were seeded, and after 24 hours, cells were treated with DKK1. After every 24 hours, half of the media was changed with fresh media containing DKK1 in both neurospheres and adherent SH-SY5Y cells. 1X PBS containing 0.1 % BSA was used in place of DKK1 in control flasks.

2.4. RNA isolation and cDNA synthesis

The cells were washed with 1 X PBS. 500 μL of trizol reagent (Sigma-Aldrich, St. Louis, MO) was added in to a T25 flask and then cells were scrapped using a scraper. RNA isolation was done according to the lab established protocol using chloroform - isopropanol method. Chloroform and isopropanol were added sequentially. Briefly, 100 μl of chloroform was added and then it was mixed vigorously. Then it was centrifuged and aqueous phase was transferred in to another tube. 250 μl of isopropanol was added. It was then kept on ice for 15 minutes. The tube was then Centrifuged for 30 minutes at 12000 rpm and then supernatant from tube was discarded. The pellet thus formed was washed with ethanol. RNase-free water was then added and heated on the heating block.

1000 ng of RNA were used for cDNA synthesis. cDNA was prepared using a cDNA reverse transcription kit from Applied Biosystems according to their protocol. The cDNA synthesis was done at 25 °C for 10 minutes, 37 °C for 2 hours, and 85 °C for 5 minutes. The sample was then held at 4 °C.

2.5. Immunocytochemistry

The cells were washed with 1X PBS. The cells were fixed in 4 % paraformaldehyde in 1 X PBS. The cells were washed with 1X PBS three times for five minutes. We incubated the cells in 4 % BSA and 0.3 % triton X 100 in 1X PBS for one hour at room temperature. We incubated the cells in primary antibody i.e. Anti-Nestin antibody (Millipore, MAB5326) at 1:500 concentration in a 0.1 % BSA solution at 4 °C overnight. Cells were washed with 1 X PBS five times for five minutes each. Cells were incubated with anti-mouse secondary antibody (Invitrogen, A-11032) at 1:1000 concentration containing 0.1 % BSA for 1 hour at room temperature. Cells were washed with 1 X PBS five times for five minutes each. Cells were once rinsed with milli-Q water. Then the slide was air-dried for 5 minutes. Finally, the slide was mounted with an anti-fade reagent containing DAPI. The chamber slide was left at room temperature overnight. The next day, imaging was taken on an apotome.

2.6. Bright-field Imaging

The cells were imaged on a Nikon bright field microscope at 10X.

2.7. Quantitative Real-Time PCR

For quantitative real-time PCR, 6 μl of SYBR green (Applied Biosystems, USA), 3 μl of water and 1 μl of cDNA were mixed together to form a master mix for a single gene experiment. Experiment was performed in triplicate for a single gene. Then 1 μl of 10 μM primer and 10 μl of master mix were subjected to real time PCR. It was run on Rotor-Gene Q (Qiagen, Germany). The conditions for amplification were followed: denaturation at 95 °C for 5 minutes, annealing at 62 °C for 30 seconds, and amplification at 72 °C for 1 minute. Amplification was done for 40–45 cycles. Primer sequences used in qPCR are given in Table 1. GAPDH was used as a normalizer, and fold changes in expression of each target mRNA was calculated based on the 2-ΔΔCt relative expression formula.

2.8. Statistical analysis

Three independent experiments were performed and each experimental value assessed in triplicates. The figures depict a representative experiment. A student t-test was used to check for a significant difference between the groups. The p-values for significant changes were represented as follows: * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$.

3. Results

3.1. DKK1 treatment fragments the neurospheres and reduces the markers of neuroblastoma stem cell proliferation

SH-SY5Y cell line exists in an undifferentiated state (Fig. 1A). To study the stemness and pluripotency of SH-SY5Y cells, these were grown in a stem cell medium, which resulted in neurosphere formation (Fig. 1B). Expression of the stem cell marker nestin was studied in formed neurospheres on PDL-coated slides (Fig. 1C), which confirmed the stem cell nature of the neurospheres. DKK1 treatment of the neurospheres for 48–72 hours resulted in their fragmentation, with smaller clusters and also some individual cells (Fig. 1D-E). Quantitative real time PCR was used to see differences in gene expression between DKK1 treated and DKK1 control neurospheres. In our study, DKK-1 treatment resulted in a significantly lower expression of proliferation markers (Ki67 and PCNA) in SH-SY5Y neurospheres as compared to DKK1 control SH-SY5Y neurospheres (Fig. 1F-G, *** $p \leq 0.0005$).

3.2. DKK1 treatment reduces the expression of markers of neuroblastoma stemness and pluripotency

When we treated SH-SY5Y neurospheres to DKK1, we observed a decrease in the expression of stem cell markers as compared to control. Quantitative real time PCR was used to measure the expression of cancer stem cell markers. We have found downregulation of cancer stem cell surface markers CD133 and KIT mRNA expression (Fig. 2A-B, * $p \leq 0.05$, *** $p \leq 0.0005$).

SOX2, OCT4, and NANOG are markers of pluripotency; their downregulation results in the perturbation of self-renewable of stem cells. DKK-1 treatment of SH-SY5Y neurospheres resulted in significant downregulation of these three pluripotency markers (Fig. 2C-E, *** $p \leq 0.0005$).

3.3. DKK1 affects β-catenin and TCF mRNA expression

We studied the effect of DKK1 on β-catenin and TCF gene expression. Quantitative real time PCR analysis showed significant difference in mRNA expression of β-catenin and TCF genes among DKK1 treated and control. We found significantly lower mRNA expression of β-catenin, TCF4 and TCF12 after DKK1 treatment as compared to cells without DKK1 treatment (Fig. 3A-C, *$p \leq 0.05$, ***$p \leq 0.0005$).

3.4. DKK1 treatment of adherent SH-SY5Y cells induces neuronal differentiation

We studied the effect of DKK1 on SH-SY5Y neuronal differentiation. We treated the SH-SY5Y adherent cells cultured in complete DMEM media with DKK1 re-constituted in 1X PBS containing 0.1 percent BSA for five days and the control cells were also cultured in complete DMEM media and they were treated with 0.1 percent BSA dissolved in 1X PBS. Morphological differentiation images of SH-SY5Y cells without DKK1 treatment and with DKK1 treatment were captured (Fig. 4A-B). The neurite length was longer, more prominent in DKK1 treated samples as compared to control samples. And, DKK1 treated cells were making neurite to neurite connections more prominently. Equal number of neurites were measured from both DKK1 treated and control samples and average neurite length was calculated from each sample. The neurite lengths of three independent samples without DKK1 treatment and of three independent samples with DKK1 treatment were measured (Fig. 4C, **$p \leq 0.005$). We found significantly higher expression of MAPT (Fig. 4D, * $p \leq 0.05$), DCX, GAP43, and ENO2 (Fig. 4E * $p \leq 0.05$) differentiation markers in DKK1 treated cells as compared to control cells.

3.5. DKK1 synergizes with retinoic acid-induced differentiation of SH-SY5Y

In this experiment, we differentiated the adherent SH-SY5Y cells using retinoic acid with 0.1 percent BSA (dissolved in 1X PBS) for five days as a control (Fig. 5A) and compared this with cells differentiated in media containing retinoic acid and DKK1 (re-constituted in 1X PBS containing 0.1 percent BSA) for five days (Fig. 5B). The number and

Table 1
Primer Sequences.

Gene name	Forward primer sequence	Reverse primer sequence
CD133	TTTGAAACAGAGGAAGCAGAGA	GCATTTAGACATCTTCCTAGGGGT
KIT	CACCGAAGGAGGCACTTACA	CAAACACGAGCCACAACTTT
SOX2	ACACCAATCCCATCCACACT	GCAAACTTCCTGCAAAGCTC
OCT4	GTAGGGTAAAGGAGGGAAGGAGA	ATCTACCTAGCCACCAGACCA
NANOG	TAGCAATGGTGTGACGCAGG	TGTCTGTGACTGGAGTTGTGT
Ki67	GCCTGCTCGACCCTACAGA	GCTTGTCAACTGCGGTTGC
PCNA	GCGTGAACCTCACCAGTATGT	TCTTCGGCCCTTAGTGTAATGAT
β-catenin	TGCAGTTCGCCTTCACTATGGACT	GATTTGCGGGACAAAGGGCAAGAT
TCF4	TGCAAAGCCGAATTGAAGATCG	AGAAGGTCCAATGATTCCATGC
TCF12	CCAGTAGTTATGGCAACCTTCAT	GACTCGTGTTTATGTCTGTTGGT
MAPT	CCAAGTGTGGCTCATTAGGCA	CCAATCTTCGACTGGACTCTGT
DCX	AATCACCAAGCGAGTCCGAG	AAAGCAGACATTCCAGAGCTCAA
GAP43	GGCCGCAACCAAAATTCAGG	CGGCAGTAGTGGTGCCTTC
ENO2	AGGTGCAGAGGTCTACCATAC	AGCTCCAAGGCTTCACTGTTC
GAPDH	ACATCAAGAAGGTGGTGAAGCAGG	TGTCGCTGTTGAAGTCAGAGGAGA

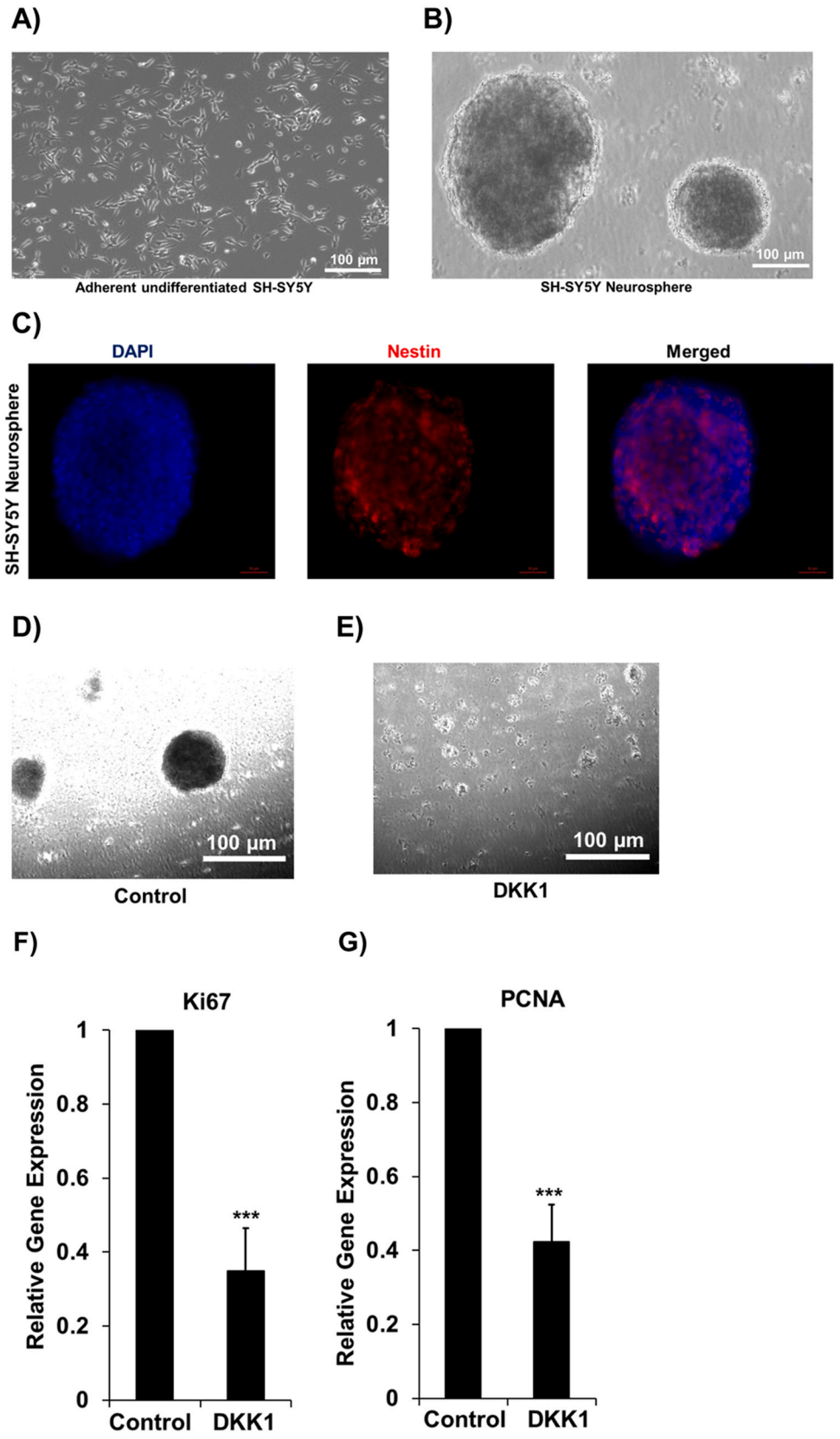

Fig. 1. DKK1 treatment reduces the markers of neuroblastoma stem cells proliferation. A) Bright-field image of undifferentiated adherent SH-SY5Y cells; B) Bright-field image of SH-SY5Y neurosphere. C) Immunocytochemistry images of SH-SY5Y neurosphere showing stem cell marker nestin expression. Scale Bar: A and B) 100 µm, Scale Bar C) 50 µm D) Neurospheres without DKK1 treatment; E) Neurospheres with DKK1 treatment. F-G) Relative mRNA expression of proliferating gene markers Ki67 and PCNA (*** $p \leq 0.0005$). The experiments were done in triplicate and were repeated three times independently with similar results. A representative experiment is depicted in the figure. A student t-test was used to check for a significant difference between the groups.

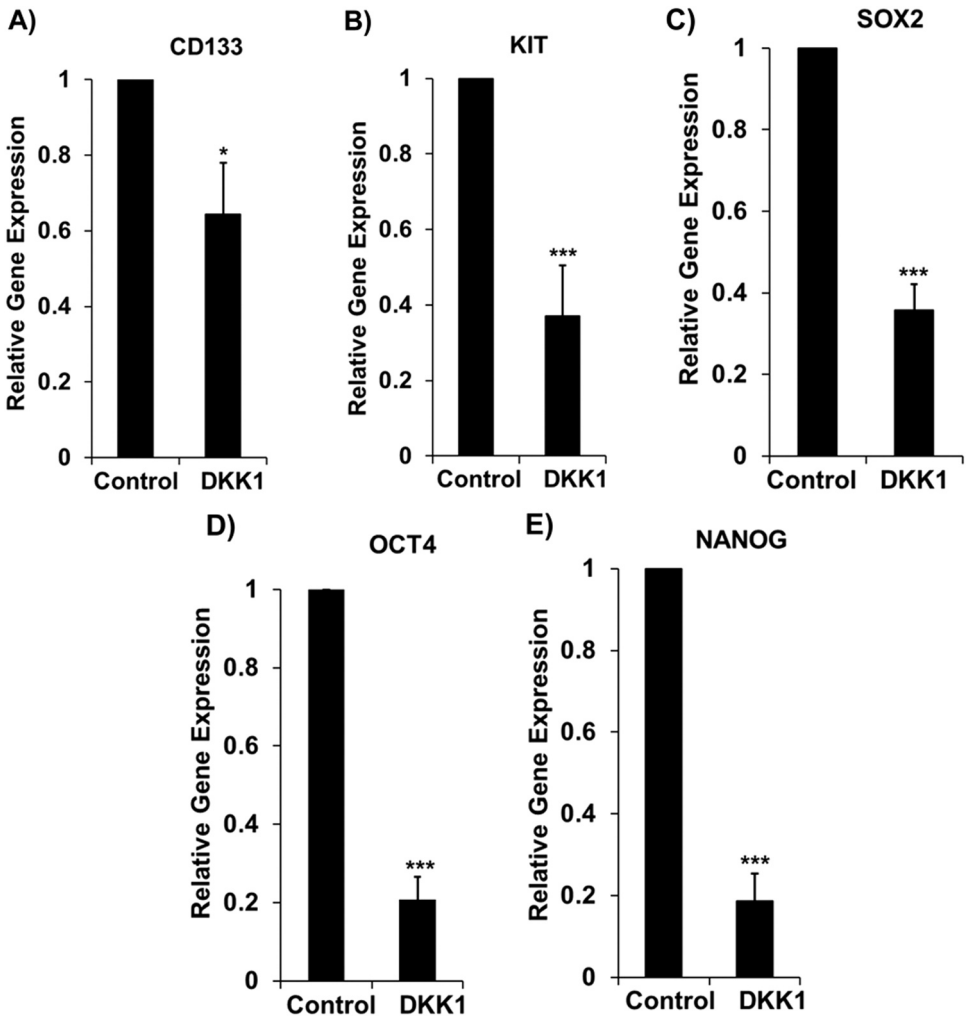

Fig. 2. DKK1 treatment reduces the expression of markers of neuroblastoma stemness and pluripotency (A-B) Relative mRNA expression of cancer stem cell markers CD133 and KIT (*p ≤ 0.05, ***p ≤ 0.0005). (C-E) Relative mRNA expression of pluripotency markers SOX2, OCT4, and NANOG (*** p≤0.0005). The experiments were done in triplicate and were repeated three times independently with similar results. A representative experiment is depicted in the figure. A student t-test was used to check for a significant difference between the groups.

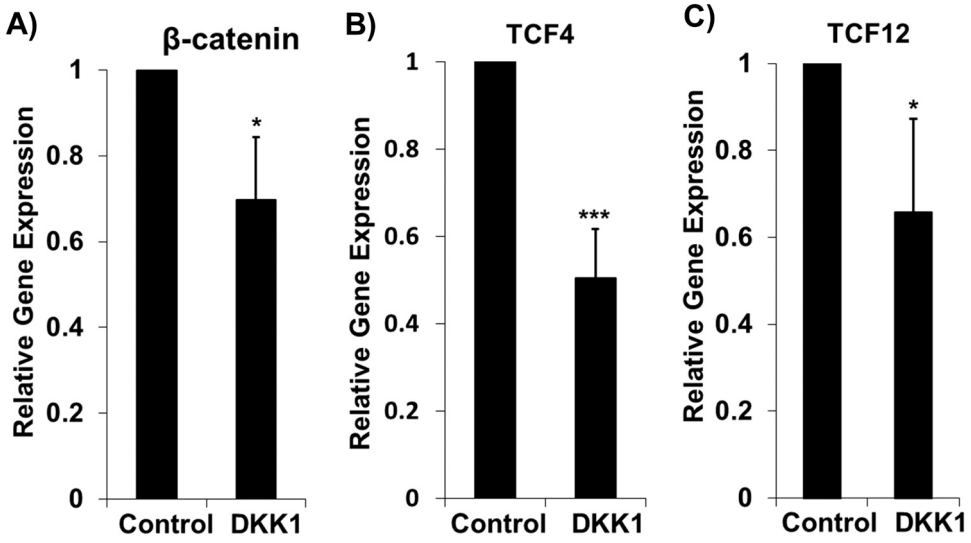

Fig. 3. DKK1 affects β-catenin and TCF mRNA expression. DKK1 treatment affects β-catenin and TCF mRNA expression (A-C) Relative mRNA expression of β-catenin, TCF4, and TCF12 (*p ≤ 0.05, ***p ≤ 0.0005). The experiments were done in triplicate and were repeated three times independently with similar results. A representative experiment is depicted in the figure. A student t-test was used to check for a significant difference between the groups.

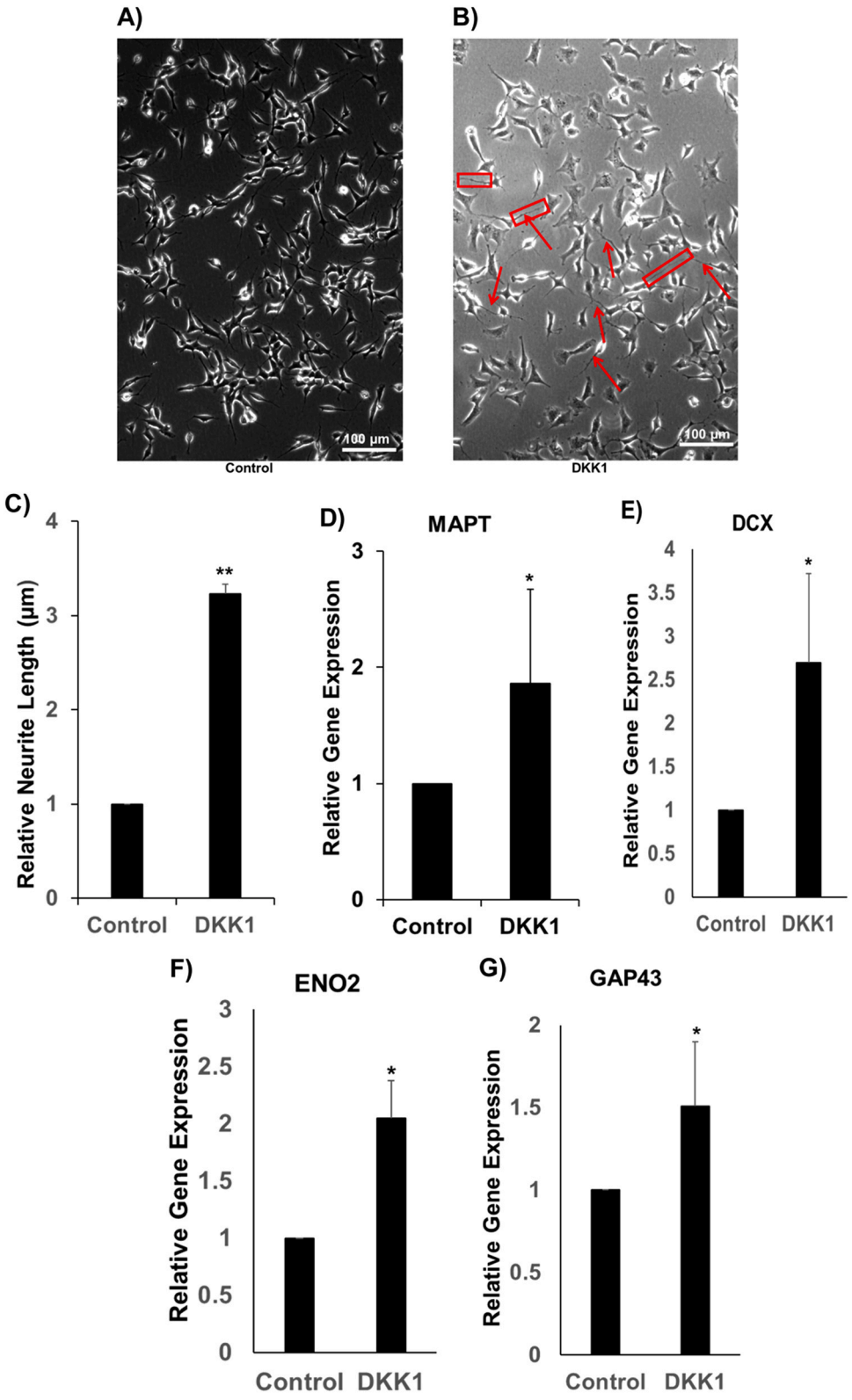

Fig. 4. DKK1 treatment of adherent SH-SY5Y cells induces neuronal differentiation. DKK1 treatment of adherent SH-SY5Y cells induces their neuronal differentiation A-B) Figure showing bright field images of cells without DKK1 treatment and with DKK1 treatment respectively. Scale Bar: A and B) 100 µm. Some of the neurites are shown with red arrow. Red box shows the length of some of the neurites. C) Relative neurite length measured of control sample and DKK1 treated cells using Image J software (**$p \leq 0.005$). We obtain the data as mean ± S.E.M from three samples. A student t-test was used to check for a significant difference between the groups. D) Relative mRNA expression of MAPT E) Relative mRNA expression of DCX F) Relative mRNA expression of GAP43, and G) Relative mRNA expression of ENO2 (*$p \leq 0.05$). The experiments were done in triplicate and were repeated three times independently with similar results. A representative experiment is depicted in the figure. A student t-test was used to check for a significant difference between the groups.

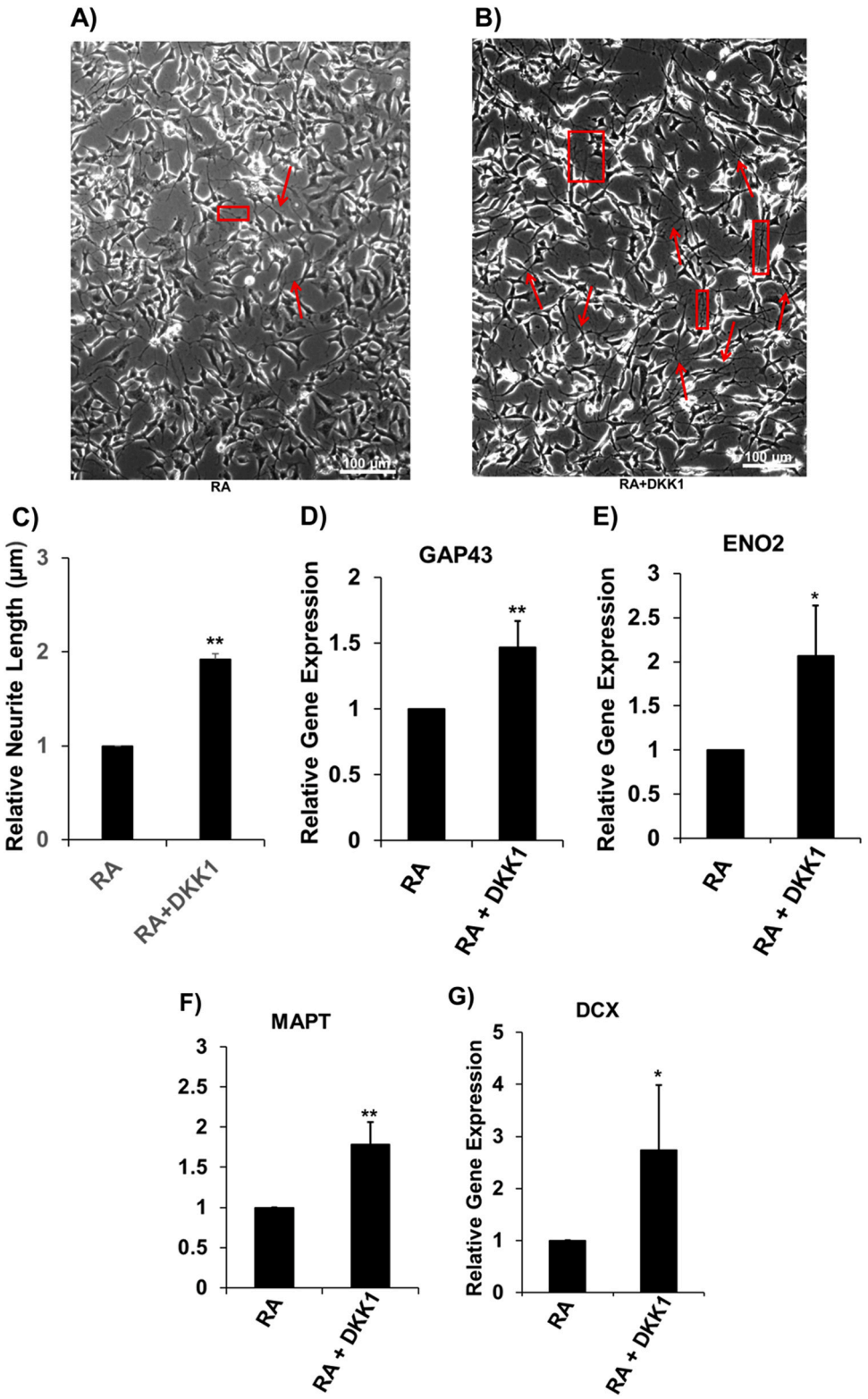

Fig. 5. DKK1 synergizes with retinoic acid-induced differentiation of SH-SY5Y. DKK1 synergizes with retinoic acid-induced differentiation of SH-SY5Y A) Bright field image of adherent SH-SY5Y treated with retinoic acid only and; B) Bright field image of adherent SH-SY5Y treated with retinoic acid and DKK1. Scale Bar: A and B) 100 µm. Red arrow shows the neurites length and their prominence in each of the group. Red box shows some of the area of inter-connectedness among neurites. C) Relative neurite length measured of retinoic acid treated cells and cells treated with combined retinoic acid and DKK1 using Image J software (**$p \leq 0.005$). We obtain the data as mean ± S.E.M from three samples. A student t-test was used to check for a significant difference between the groups. D-G) Relative mRNA expression of neuronal differentiation markers GAP43, ENO2, DCX, and MAPT (*$p \leq 0.05$, **$p \leq 0.005$). The experiments were done in triplicate and were repeated three times independently with similar results. A representative experiment is depicted in the figure. A student t-test was used to check for a significant difference between the groups.

length of neurites per unit area is greater in cells treated with both retinoic acid and DKK1. Cells are more inter-connected in cells treated with both retinoic acid and DKK1. In short, we can say we have found longer neurites, more neurites connected with each other and more differentiated morphology when DKK1 was used with retinoic acid. The relative neurite lengths in samples treated with only retinoic acid and in samples treated with a combination of DKK1 and retinoic acid were measured (Fig. 5C, **p $\leq$ 0.005). Equal number of neurites were measured from samples treated with only retinoic acid and samples treated with a combination of DKK1 and retinoic acid and average neurite length was calculated from each sample. The neurite lengths of three independent samples treated with only retinoic acid and of three independent samples treated with a combination of DKK1 and retinoic acid were measured We have then checked neuronal differentiation markers like GAP43, ENO2, DCX, and MAPT in similar conditions. We have found significantly higher expression of all these differentiation markers in adherent SH-SY5Y cells grown in complete DMEM media containing both retinoic acid and DKK1 as compared to cells grown in complete DMEM media containing only retinoic acid (Fig. 5D-G, ** p $\leq$ 0.005, * $\leq$ 0.05).

4. Discussion

Neuroblastoma is a childhood cancer that is developmental in origin, arising from neural progenitor cells outside the nervous system, often from the adrenal region. It is the third-most common cause of child mortality among pediatric cancers. Early onset and detection in a child of up to 0–1-year results in partial or complete cure, but the outlook is less favorable (Schmidt et al., 2005; Zhang et al., 2023). Different pathways of cellular machinery have been targeted for therapy, but side effects and the development of resistance result in treatment failure. Alk and N-Myc amplification are associated with a poor prognosis (Zhu et al., 2012). The role of the Wnt pathway and Wnt signaling inhibitors is often context-dependent. This is evident at the level of primary tumors as well as in cell lines.

Several neuroblastoma cell lines have been extensively studied. We worked with SH-SY5Y cell line. These lines are grown as adherent cultures, but on the one hand, they can be differentiated into neurons, and on the other, they can form neurospheres with stem cell-like properties under appropriate culture conditions. Cancer stem cell surface markers, such as CD133, KIT, and CD44, have been found in many tumors (Shimokawa et al., 2017; Shibata and Hoque, 2019). CD133, KIT, GPRC5C, NOTCH1, PlGF2, TRKB, and LNGFR are the genes that are elevated in neuroblastoma cells as compared to normal stem cells (Ross et al., 2015). CD133 and KIT are stem cell markers involved in the proliferation of cells (Singh et al., 2004). Overexpression of CD133 is associated with poor clinical outcomes in neuroblastoma and is associated with increased chemoresistance (Tong et al., 2008). And also, expression of target genes SOX2, OCT4, NANOG is more frequently observed in cancer stem cells than in normal stem cells (Basati et al., 2020; Wang et al., 2013). β-catenin is a final effector of the canonical Wnt signaling pathway when affects cell proliferation and differentiation (Becker and Wilting, 2018; Becker and Wilting, 2019). TCF genes, along with β-catenin act as major transcriptional mediators of Wnt signaling pathways (Pai et al., 2017). They determine which genes to be regulated by Wnt signaling. They are responsible for context-dependent interactions of Wnt signaling genes (Pai et al., 2017).

There have been some contradictory reports on the influence of Wnt inhibition or Wnt activation on neuroblastoma cell differentiation, including SH-SY5Y cells. However, on closer look, this appears to be a result of different experimental protocols and the specific actions of different molecules used in the studies. Szemes et al. (2018) have shown that Wnt signaling can drive the differentiation of SH-SY5Y cells. In this case they have treated neuroblastoma cells with Wnt3a/Rspo2. Suebsoonthron et al. (2017) studied the effects of Wnt signaling inhibition by a specific tankyrase inhibitor, XAV939 which leads to the reduction in beta catenin had no effect on cell morphology, survival, and proliferation, though it did enhance chemosensitivity to anti-cancer drugs. It also leads to the reduced ability of the cells to differentiate. These above studies differ from our current protocol, in that only marker expression was studied and the long-term differentiation of SH-SY5Y cells was not carried out respectively. Perhaps, more important in the context of differentiation is the role of the AKT pathway. López-Carballo et al. (2002) states that AKT activation by retinoic acid is required for neuronal differentiation of SH-SY5Y cells. Tankyrase inhibitors act by protein degradation. Kim (2018) states that tankyrase inhibitors stabilize PTEN, which inhibits AKT phosphorylation. Wnt3a/Rspo2 treatments decrease signaling pathways such as Akt and ERK signaling (Vieira et al., 2015). On the other hand, DKK1 inhibits Wnt signaling by binding to LRP5/6 in the membrane (Ahn et al., 2011). Unlike tankyrase inhibitors, it does not inhibit AKT. On the contray, it has been shown to have a role in activating AKT1, though in a different context of mesenchymal stem cells (Chen et al., 2024).

The role of DKK1 has been alluded to in a study by Shakya et al. (2023) who have used a model of impaired differentiation of SH-SY5Y cells when exposed to conditioned medium from an activated microglial cell line, and how this can be reversed by 17 βEstradiol. Conditioned medium from an activated microglial cell line decreased β catenin. 17 βEstradiol activates PI3K-Akt signaling pathway (Guo et al., 2006). There is 17 βEstradiol mediated recovery of WNT1 for SH-SY5Y cell differentiation. This reversal was impaired by DKK1, but this does not decrease the β catenin expression. It showed 17 βEstradiol mediated recovery of WNT1 for SH-SY5Y cell differentiation does not involve β catenin. So, the actual function of Wnt molecule in a particular microenvironment depends on many factors like its interaction with β catenin and other signaling pathways like PI3K-Akt. However, as Wnt signaling is important for both cancer cell proliferation and for neural differentiation, and also, a reduction in cancer cell proliferation accompanies differentiation, the final effects on SH-SY5Y cells would be determined by the experimental protocol. Our model is more likely to reflect the effect of the Wnt inhibitor in neuroblastomas. Our studies therefore indicate that the exact action of the Wnt inhibitor DKK1 is important for its differentiation and its synergism with retinoic acid. Considering the role of Wnt signaling in neuroblastoma, we have studied the effects of a Wnt pathway inhibitor, DKK1, on these cells in terms of their stemness-related properties as well as proliferation and differentiation, either by itself or in combination with retinoic acid. DKK1 levels in neuroblastoma are downregulated by MYCN (Koppen et al., 2007), which further supports the logic of supplementing DKK1 levels.

DKK1 acts as a tumor suppressor in cancers where its expression has found to be very poorly expressed (Zhu et al., 2021). It binds to the Wnt co-receptor LRP5/6. As a result, LRP5/6 is unavailable for interaction with Wnt ligands (Ahn et al., 2011). DKK1 transfected cell lines decreased cell migration and increased cell to cell adhesion in melanoma cell lines. In breast cancers there was inhibition of tumorigenicity with increased expression of DKK1 (Niu et al., 2019). In short, upregulation of DKK1 in melanoma and breast cancer cell lines retards tumor growth. DKK1 upregulation results in increase in apoptosis and decrease in tumor growth in renal cell carcinoma cell lines (Hirata et al., 2011).

DKK1 can convincingly cause the disintegration of preformed neurospheres of SH-SY5Y cells. It is able to lower the expression levels of cancer stem cell markers like CD133 and KIT and pluripotency markers like SOX2, OCT4, and NANOG in SH-SY5Y neurospheres. In addition, proliferation markers like Ki67 and PCNA are lowered. This is a novel finding that indicates that a Wnt inhibitor could have a major role in countering the stem-like properties of neuroblastomas. Retinoic acid derivatives are also in clinical use during the maintenance phase of neuroblastoma therapy to prevent relapse after treatment. They have been shown to promote the neuronal differentiation of neuroblastoma-like cells. However, there are limitations to the use of retinoic acid and its derivatives. Considering the abrogation of stem cell-like properties by DKK1, it was then considered if DKK1 could synergize with

retinoic acid for inducing cell differentiation. Our results, based on morphological studies including neurite formation and the expression of markers, show that DKK1, by itself, is sufficient for neuronal differentiation in SH-SY5Y culture. DKK1 differentiation ability might be due to the activation of AKT pathway. SH-SY5Y cells differentiation using retinoic acid is also through activation of AKT pathway. DKK1 interactions with AKT pathways has not been studied in neuroblastoma and can be studied to better understand the disease. Overall, this study indicates that DKK1 may be considered a candidate molecule by itself or as a prototype molecule for its particular role in the inhibition of Wnt signaling. The combination of stemness reduction and synergism with retinoic acid for neuronal differentiation makes this molecule or pathway a promising candidate for neuroblastoma management.

Funding

NBRC core finances and JC Bose fellowship to Dr. Subrata Sinha supported this study.

CRediT authorship contribution statement

Pankaj Seth: Supervision. **Bharat Prajapati:** Formal analysis. **Subrata Sinha:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Shubham Krishna:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Acknowledgments

We thank P Manish, for his technical assistance. We express our sincere gratitude to the NBRC core fund and Dr. Subrata Sinha thanks funding from JC Bose fellowship and National Science Chair of SERB-DST (India) grant number NSC/2022/000054 for supporting this project. The authors also wish to acknowledge the support of the facilities provided under the Biotechnology Information System Network (BTIS-NET) grant, DBT, India, and computing Facility, NBRC, Manesar, India.

References

Ahn V.E., Chu M.L., Choi H.J., Tran D., Abo A., Weis W.I. Structural basis of Wnt signaling inhibition by Dickkopf binding to LRP5/6. Dev Cell. 2011 Nov 15;21(5): 862-873. DOI:10.1016/j.devcel.2011.09.003.

Aravindan, N., Jain, D., Somasundaram, D.B., Herman, T.S., Aravindan, S., 2019. Cancer stem cells in neuroblastoma therapy resistance. Dec 19 Cancer Drug Resist 2 (4), 948–967. https://doi.org/10.20517/cdr.2019.72.

Barbieri, E., Mehta, P., Chen, Z., Zhang, L., Slack, A., Berg, S., Shohet, J.M., 2006. MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death. Mol. Cancer Ther. 5, 2358–2365. https://doi.org/10.1158/1535-7163.MCT-06-0305.

Basati, G., Mohammadpour, H., Emami Razavi, A., 2020. Association of High Expression Levels of SOX2, NANOG, and OCT4 in Gastric Cancer Tumor Tissues with Progression and Poor Prognosis (Mar). J. Gastrointest. Cancer 51 (1), 41–47. https://doi.org/10.1007/s12029-018-00200-x.

Bayeva, N., Coll, E., Piskareva, O., 2021. Differentiating Neuroblastoma: A Systematic Review of the Retinoic Acid, Its Derivatives, and Synergistic Interactions. Mar 16 J. Pers. Med 11 (3), 211. https://doi.org/10.3390/jpm11030211.

Becker, J., Wilting, J., 2019. WNT Signaling in Neuroblastoma. Jul 19 Cancers (Basel) 11 (7), 1013. https://doi.org/10.3390/cancers11071013.

Becker, J., Wilting, J., 2018. WNT signaling, the development of the sympathoadrenal-paraganglionic system and neuroblastoma (Mar). Cell Mol. Life Sci. 75 (6), 1057–1070. https://doi.org/10.1007/s00018-017-2685-8.

Berardi, D.E., Campodonico, P.B., Diaz Bessone, M.I., Urtreger, A.J., Todaro, L.B., 2011. Autophagy: friend or foe in breast cancer development, progression, and treatment. Int J. Breast Cancer 2011, 595092. https://doi.org/10.4061/2011/595092.

Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E., Bishop, J.M., 1984. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Jun 8 Science 224 (4653), 1121–1124. https://doi.org/10.1126/science.6719137.

Brodeur, G.M., 2003. Neuroblastoma: biological insights into a clinical enigma. Nat. Rev. Cancer 3, 203–216. https://doi.org/10.1038/nrc1014.

Chen, H., Hu, Y., Xu, X., Dai, Y., Qian, H., Yang, X., Liu, J., He, Q., Zhang, H., 2024. DKK1 activates the PI3K/AKT pathway via CKAP4 to balance the inhibitory effect on Wnt/β-catenin signaling and regulates Wnt3a-induced MSC migration. Mar 12 Stem Cells, sxae022. https://doi.org/10.1093/stmcls/sxae022.

Chen, J., Wang, P., Cai, R., Peng, H., Zhang, C., Zhang, M., 2019. SLC34A2 promotes neuroblastoma cell stemness via enhancement of miR-25/Gsk3β-mediated activation of Wnt/β-catenin signaling.. Feb 7 FEBS Open Bio 9 (3), 527–537. https://doi.org/10.1002/2211-5463.12594.

Cho, Y.H., Ro, E.J., Yoon, J.S., Mizutani, T., Kang, D.W., Park, J.C., Il Kim, T., Clevers, H., Choi, K.Y., 2020. 5-FU promotes stemness of colorectal cancer via p53-mediated WNT/β-catenin pathway activation. Oct 21 Nat. Commun. 11 (1), 5321. https://doi.org/10.1038/s41467-020-19173-2.

Delbridge, A.R., Strasser, A., 2015. The BCL-2 protein family, BH3-mimetics and cancer therapy. Cell Death Differ. 22, 1071–1080. https://doi.org/10.1038/cdd.2015.50.

Duffy, D.J., Krstic, A., Halasz, M., Schwarzl, T., Konietzny, A., Iljin, K., Higgins, D.G., Kolch, W., 2017. Retinoic acid and TGF-β signalling cooperate to overcome MYCN-induced retinoid resistance. Feb 10 Genome Med 9 (1), 15. https://doi.org/10.1186/s13073-017-0407-3.

Flahaut, M., Meier, R., Coulon, A., Nardou, K.A., Niggli, F.K., Martinet, D., Beckmann, J. S., Joseph, J.M., Mühlethaler-Mottet, A., Gross, N., 2009. The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway.. Jun 11 Oncogene 28 (23), 2245–2256. https://doi.org/10.1038/onc.2009.80.

Guo, R.X., Wei, L.H., Tu, Z., Sun, P.M., Wang, J.L., Zhao, D., Li, X.P., Tang, J.M., 2006. 17 beta-estradiol activates PI3K/Akt signaling pathway by estrogen receptor (ER)-dependent and ER-independent mechanisms in endometrial cancer cells (Apr). J. Steroid Biochem Mol. Biol. 99 (1), 9–18. https://doi.org/10.1016/j.jsbmb.2005.11.013.

Hirata, H., Hinoda, Y., Nakajima, K., Kawamoto, K., Kikuno, N., Ueno, K., Yamamura, S., Zaman, M.S., Khatri, G., Chen, Y., Saini, S., Majid, S., Deng, G., Ishii, N., Dahiya, R., 2011. Wnt antagonist DKK1 acts as a tumor suppressor gene that induces apoptosis and inhibits proliferation in human renal cell carcinoma. Apr 15 Int J. Cancer 128 (8), 1793–1803. https://doi.org/10.1002/ijc.25507.

Hirsch, H.A., Iliopoulos, D., Tsichlis, P.N., Struhl, K., 2009. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res 69, 7507–7511. https://doi.org/10.1158/0008-5472.CAN-09-2994.

Kim, M.K., 2018. Novel insight into the function of tankyrase (Dec). Oncol. Lett. 16 (6), 6895–6902. https://doi.org/10.3892/ol.2018.9551.

Koppen, A., Ait-Aissa, R., Hopman, S., Koster, J., Haneveld, F., Versteeg, R., Valentijn, L. J., 2007. Dickkopf-1 is down-regulated by MYCN and inhibits neuroblastoma cell proliferation. Oct 28 Cancer Lett. 256 (2), 218–228. https://doi.org/10.1016/j.canlet.2007.06.011.

Kovalevich, J., Santerre, M., Langford, D., 2021. Considerations for the Use of SH-SY5Y Neuroblastoma Cells in Neurobiology. Methods Mol. Biol. 2311, 9–23. https://doi.org/10.1007/978-1-0716-1437-2_2.

López-Carballo, G., Moreno, L., Masiá, S., Pérez, P., Barettino, D., 2002. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. Jul 12 J. Biol. Chem. 277 (28), 25297–25304. https://doi.org/10.1074/jbc.M201869200.

Matthay, K.K., Reynolds, C.P., Seeger, R.C., Shimada, H., Adkins, E.S., Haas-Kogan, D., Gerbing, R.B., London, W.B., Villablanca, J.G., 2009. Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. J. Clin. Oncol. 27, 1007–1013. https://doi.org/10.1200/JCO.2007.13.8925.

Moreira, P.I., Custodio, J., Moreno, A., Oliveira, C.R., Santos, M.S., 2006. Tamoxifen and estradiol interact with the flavin mononucleotide site of complex I leading to mitochondrial failure. J. Biol. Chem. 281, 10143–10152. https://doi.org/10.1074/jbc.M510249200.

Niu, J., Li, X.M., Wang, X., Liang, C., Zhang, Y.D., Li, H.Y., Liu, F.Y., Sun, H., Xie, S.Q., Fang, D., 2019. DKK1 inhibits breast cancer cell migration and invasion through suppression of β-catenin/MMP7 signaling pathway. Jun 24 Cancer Cell Int 19, 168. https://doi.org/10.1186/s12935-019-0883-1.

Otte, J., Dyberg, C., Pepich, A., Johnsen, J.I., 2021. MYCN Function in Neuroblastoma Development. Jan 27 Front Oncol. 10, 624079. https://doi.org/10.3389/fonc.2020.624079.

Påhlman, S., Hoehner, J.C., et al., 1995. Differentiation and survival influences of growth factors in human neuroblastoma. Eur. J. Cancer 31 (4), 453–458. https://doi.org/10.1016/0959-8049(95)00033-f.

Pai, S.G., Carneiro, B.A., Mota, J.M., Costa, R., Leite, C.A., Barroso-Sousa, R., Kaplan, J. B., Chae, Y.K., Giles, F.J., 2017. Wnt/beta-catenin pathway: modulating anticancer immune response. May 5 J. Hematol. Oncol. 10 (1), 101. https://doi.org/10.1186/s13045-017-0471-6.

Ross, R.A., Walton, J.D., Han, D., Guo, H.F., Cheung, N.K., 2015. A distinct gene expression signature characterizes human neuroblastoma cancer stem cells (Sep). Stem Cell Res 15 (2), 419–426. https://doi.org/10.1016/j.scr.2015.08.008.

Sánchez, S., Jiménez, C., Carrera, A.C., Diaz-Nido, J., Avila, J., Wandosell, F., 2004. A cAMP-activated pathway, including PKA and PI3K, regulates neuronal differentiation (Mar). Neurochem Int 44 (4), 231–242. https://doi.org/10.1016/s0197-0186(03)00150-5.

Schmidt, M.L., Lal, A., Seeger, R.C., Maris, J.M., Shimada, H., O'Leary, M., Gerbing, R.B., Matthay, K.K., 2005. Favorable prognosis for patients 12 to 18 months of age with stage 4 nonamplified MYCN neuroblastoma: a Children's Cancer Group Study. Sep 20 J. Clin. Oncol. 23 (27), 6474–6480. https://doi.org/10.1200/JCO.2005.05.183.

Shakya, R., Amonruttanapun, P., Limboonreung, T., Chongthammakun, S., 2023. 17β-estradiol mitigates the inhibition of SH-SY5Y cell differentiation through WNT1 expression (Dec). Cells Dev. 176. https://doi.org/10.1016/j.cdev.2023.203881.

Shibata, M., Hoque, M.O., 2019. Targeting Cancer Stem Cells: A Strategy for Effective Eradication of Cancer. May 27 Cancers (Basel) 11 (5), 732. https://doi.org/10.3390/cancers11050732.

Shimokawa, M., Ohta, Y., Nishikori, S., Matano, M., Takano, A., Fujii, M., Date, S., Sugimoto, S., Kanai, T., Sato, T., 2017. Visualization and targeting of LGR5^{+} human colon cancer stem cells. May 11 Nature 545 (7653), 187–192. https://doi.org/10.1038/nature22081.

Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., Dirks, P.B., 2004. Identification of human brain tumour initiating cells. Nov 18 Nature 432 (7015), 396–401. https://doi.org/10.1038/nature03128.

Smith, M.A., Seibel, N.L., Altekruse, S.F., Ries, L.A., Melbert, D.L., O'Leary, M., Smith, F. O., Reaman, G.H., 2010. Outcomes for children and adolescents with cancer: challenges for the twenty-first century. J. Clin. Oncol. 28, 2625–2634. https://doi.org/10.1200/JCO.2009.27.0421.

Spix C., Pastore G., Sankila R., Stiller C.A., Steliarova-Foucher E.: Neuroblastoma incidence and survival in European children (1978-1997): the Automated Childhood Cancer Information System project report. Eur J Cancer 2006, 42:2081-2091. DOI:10.1016/j.ejca.2006.05.008.

Suebsoonthron, J., Jaroonwitchawan, T., Yamabhai, M., Noisa, P., 2017. Inhibition of WNT signaling reduces differentiation and induces sensitivity to doxorubicin in human malignant neuroblastoma SH-SY5Y cells (Jun). Anticancer Drugs 28 (5), 469–479. https://doi.org/10.1097/CAD.0000000000000478.

Szemes, M., Greenhough, A., Melegh, Z., Malik, S., Yuksel, A., Catchpoole, D., Gallacher, K., Kollareddy, M., Park, J.H., Malik, K., 2018. Wnt Signalling Drives Context-Dependent Differentiation or Proliferation in Neuroblastoma (Apr). Neoplasia 20 (4), 335–350. https://doi.org/10.1016/j.neo.2018.01.009.

Tang, Q., Chen, J., Di, Z., Yuan, W., Zhou, Z., Liu, Z., Han, S., Liu, Y., Ying, G., Shu, X., Di, M., 2020. TM4SF1 promotes EMT and cancer stemness via the Wnt/β-catenin/SOX2 pathway in colorectal cancer. Nov 5 J. Exp. Clin. Cancer Res 39 (1), 232. https://doi.org/10.1186/s13046-020-01690-z.

Tong, Q.S., Zheng, L.D., Tang, S.T., Ruan, Q.L., Liu, Y., Li, S.W., Jiang, G.S., Cai, J.B., 2008. Expression and clinical significance of stem cell marker CD133 in human neuroblastoma (Feb). World J. Pedia 4 (1), 58–62. https://doi.org/10.1007/s12519-008-0012-z.

Vieira, G.C., Chockalingam, S., Melegh, Z., Greenhough, A., Malik, S., Szemes, M., Park, J.H., Kaidi, A., Zhou, L., Catchpoole, D., Morgan, R., Bates, D.O., Gabb, P.D., Malik, K., 2015. LGR5 regulates pro-survival MEK/ERK and proliferative Wnt/β-catenin signalling in neuroblastoma. Nov 24 Oncotarget 6 (37), 40053–40067. https://doi.org/10.18632/oncotarget.5548.

Wang, X., Liu, Q., Hou, B., Zhang, W., Yan, M., Jia, H., Li, H., Yan, D., Zheng, F., Ding, W., Yi, C., Hai Wang, 2013. Concomitant targeting of multiple key transcription factors effectively disrupts cancer stem cells enriched in side population of human pancreatic cancer cells. Sep 11 PLoS One 8 (9), e73942. https://doi.org/10.1371/journal.pone.0073942.

Wang, S.Y., Yu, Q.J., Zhang, R.D., Liu, B., 2011. Core signaling pathways of survival/death in autophagy-related cancer networks. Int J. Biochem Cell Biol. 43, 1263–1266. https://doi.org/10.1016/j.biocel.2011.05.010.

Zhang, Y., Zhang, W.L., Huang, D.S., et al., 2023. Prognostic factors for intermediate- or high-risk neuroblastomas in children in China. BMC Pedia 23, 617. https://doi.org/10.1186/s12887-023-04258-w.

Zhang, J., Zhou, B., Liu, Y., Chen, K., Bao, P., Wang, Y., Wang, J., Zhou, Z., Sun, X., Li, Y., 2014. Wnt inhibitory factor-1 functions as a tumor suppressor through modulating Wnt/β-catenin signaling in neuroblastoma. Jun 28 Cancer Lett. 348 (1-2), 12–19. https://doi.org/10.1016/j.canlet.2014.02.011.

Zhan, T., Rindtorff, N., Boutros, M., 2017. Wnt signaling in cancer (Mar). Oncogene 36 (11), 1461–1473. https://doi.org/10.1038/onc.2016.304.

Zhu, S., Lee, J.S., Guo, F., Shin, J., Perez-Atayde, A.R., Kutok, J.L., Rodig, S.J., Neuberg, D.S., Helman, D., Feng, H., Stewart, R.A., Wang, W., George, R.E., Kanki, J. P., Look, A.T., 2012. Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. Mar 20 Cancer Cell 21 (3), 362–373. https://doi.org/10.1016/j.ccr.2012.02.010.

Zhu, G., Song, J., Chen, W., Yuan, D., Wang, W., Chen, X., Liu, H., Su, H., Zhu, J., 2021. Expression and Role of Dickkopf-1 (Dkk1) in Tumors: From the Cells to the Patients. Jan 25 Cancer Manag Res 13, 659–675. https://doi.org/10.2147/CMAR.S275172.