

Doublecortin, Caspase-1, and Interleukin-18 mRNA Expression in Neuroblastoma Cell lines and the Detection of an Alternatively Spliced Transcript

Katherine Reid
Department of Biological Science
Fordham University, Bronx, New York

Abstract

DCX is a X-linked gene that codes for the microtubule-associated protein doublecortin and is highly expressed in migrating neurons. RT-PCR was performed to test for DCX expression on N-type, I-type, and S-type cells. DCX expression was found in N-type and I-type cells, but not in S-type cells. RT-PCR also yielded a novel alternatively spliced mRNA transcript missing exon 6. To investigate the effect of retinoic acid and BUdR (which induce N-type and S-type phenotypes from I-type cells, respectively) on DCX expression, RT-PCR was performed on untreated, RA, and BUdR (5-bromo-2'deoxyuridine) treated cells. DCX exhibited increased expression in RA treated cells when compared to untreated and BUdR cells. In addition, mRNA expression of caspase-1 and interleukin-18 were also examined in these cell lines. Both CASP-1 and IL-18 were expressed only in the SH-EP1 S-type cell line. BUdR treatment of the BE(2)-C I-type cell line induced IL-18 and CASP-1 expression. Based on these results, the mRNA expression of these transcripts in neuroblastoma cells merit further investigation.

Introduction

Neuroblastoma (NB) is a cancer of the sympathetic nervous system that arises from neural crest cells (Walton et. al. 2004). NB accounts for approximately 9% of pediatric cancers with about 700 new cases reported each year in the US (Theiele, 1999). NB cell lines express a great deal of heterogeneity and frequently contain three phenotypically and biochemically distinct cell types: neuroblastic (N), substrate-adherent (S) and intermediate (I) (Walton et. al. 2004). I-type cells display the greatest malignancy, followed by N-type, while S-type cells are considered nonmalignant due to their lack of ability of anchorage-independent growth in soft agar (Walton et. al. 2004). It is suggested that the I-type cell is a neural crest stem

cell from which the other two cell types may arise based on its ability to express specific enzymatic markers of both N-type and S-type cells, namely neuronal and melanocytic enzyme markers (Walton et. al. 2004). Although the cause of neuroblastoma remains unclear, many attempts toward effective treatments are currently being pursued. Agents that induce apoptosis, which destroy aberrant cells and thereby inhibit tumor progression, and induce cellular differentiation, are current theories behind treatment. One line of cancer treatment involves the administration of retinoids. While the details behind the mechanism of retinoids remain elusive, they have shown to modulate cellular differentiation and apoptosis (Sidell et. al. 1983).

Doublecortin is a 40kDa microtubule-associated protein that is expressed in migrating and differentiating neurons in both the central and peripheral nervous systems (LoTurco, 2004). DCX is also a reliable and commonly used molecular marker for neurogenesis (Couillard-Depres, S. et al. 2005). Mutations in this gene lead to neuronal migratory disorders such as lissencephaly in males and subcortical band heterotopia in heterozygous females (Polloneal et. al. 2006). Such individuals lack the gyri and sulci of the normal brain and suffer epilepsy and mental retardation (Polloneal et. al 2006). DCX contains microtubule-binding domains that direct migration of microtubules and increase their stability. However, the direct mechanism of how DCX aids in neuronal migration is poorly understood (Polloneal et. al 2006). DCX has also been recently proposed to be a tumor marker for minimal residual disease in neuroblastoma. A previous study has also recently reported that retinoic acid treatment decreases DCX expression in the SK-N-SH cell line (Oltra et. al. 2005; Messi et. al. 2008). This offers valuable insight into another cellular mechanism in which retinoic acid may help to combat neuroblastoma.

Caspases are a family of cytokine proteases that play an integral role in the apoptotic and proinflammatory processes in the cell. Alternative splicing events result in five different isoforms: alpha, beta, gamma, delta, and epsilon. A new isoform, zeta, has recently been discovered (Feng et. al. 2004). Caspase-1 cleaves and, thereby, activates the proinflammatory cytokines interleukin-1-beta and interleukin-18. Although caspase-1 is generally thought to be involved in

proinflammatory responses, caspase-1 does induce cell death in many systems (Feng et. al. 2004).

After proteolytic processing of caspase-1, the proinflammatory cytokine interleukin-18 induces the production of IFN-gamma. IFN-gamma has been shown to have anti-tumor effects in cells and has also been suggested as a treatment approach in neuroblastoma (Feng et. al. 2004). It was demonstrated that IFN-gamma increased the rate of apoptosis by up-regulating caspase-8 activity in neuroblastoma cell lines (Feng et. al. 2004).

Due to the lack of information on the expression of DCX in neuroblastoma cell lines and its possible response to retinoic acid treatment, we have investigated DCX expression in three neuroblastoma cell lines. We observed that DCX is expressed in only N-type and I-type cells. DCX expression was also observed in retinoic acid treated and BUdR (5-bromo-2' deoxyuridine) treated I-type cells. RA treatment increased the expression of DCX, while BUdR treatment showed a down regulation of DCX expression. These findings suggest that DCX is exclusively expressed in N-type and I-type cells and may be linked to cellular invasiveness in neuroblastoma.

Due to the potential anti-cancer role that caspase-1 and IL-18 may play, we have investigated CASP-1 and IL-18 expression in three neuroblastoma cell lines and their expression in untreated, RA treated, and BUdR treated cells. We show here that both CASP-1 and IL-18 were only expressed in S-type cells. CASP-1 and IL-18 were also only expressed when I-type cells were induced over to the S-type phenotype by BUdR treatment. These findings are important in that they demonstrate that CASP-1 and IL-18 are restricted to the non-malignant S-type cell in the cell lines studied.

Materials and Methods

Neuroblastoma cell lines and RNA purification

The cell lines used in this study: KCN-83n, BE(2)-C, and SH-EP1 were generously provided by Dr. Robert Ross. The KCN-83n, BE(2)-C and SH-EP1 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum at 37 °C. The treated BE(2)-C cells were cultured in DMEM/F12 supplemented with

10% fetal bovine serum in the presence of either 1 μ M of retinoic acid (RA) or 10 μ M 5-bromo-2'-deoxyuridine (BUdR) for 21 days. RNA was purified using RNeasy[®] Plus Mini Kit (QIAGEN), according to the manufacturer's instruction. The RNA was diluted to 10 ng/ μ l.

Primers

Primers (Table 1.) were diluted to 10pmol/ μ l. Primers span at least one intron.

RT-PCR

Each RT-PCR reaction contained 5x RT-PCR buffer (5 μ l), dNTP (1 μ l), enzyme mix (1 μ l), forward primer (1 μ l), reverse primer (1 μ l), and 13.5 μ l of dH₂O. 2.5 μ l of 10ng/ μ l RNA was added to each reaction. Amplification temperatures were as follows: 50° C for 30 minutes, 95° C for 15 minutes, 50 cycles of: [94° C (30 sec) 57° C (30 sec) 72° C (30 sec)], 72° C for 7 minutes, and 4° C ∞ . Three RT-PCR trials were performed for each set of primers.

Table 1.

Gene		Sequence 5'-----3'	Position
DCX	F1	CTCTGTCTGACAACATCAACC	684-704
	R1	CTTCTGTGATATCAGTGAGGAC	1043-1064
	F2	GTGTTTATTGCCTGTGGTCCTG	1157-1178
	R2	CATGGAATCACCAAGCGAGTC	1475-1495
Caspase-1	F1	ATCCGTTCCATGGGTGAAGG	57-76
	R1	GCATTGTCATGCCTGTGATGTC	570-591
	F2	GTGCAGGACAACCCAGCTATG	366-386
	R2	CCTGGATGATGATCACCTTCG	847-867
	F3	CACAAGACCTCTGACAGCAC	687-706
	R3	GTCAAAGTCACTCTTTCAGTGG	1180-1201
Interleukin-18	F1	GCTGCTGAACCAGTAGAAGAC	224-244
	R1	CTGGGACACTTCTCTGAAAG	631-650
	F2	GACAATTGCATCAACTTTGTGG	242-263
	R2	GTTCTCACAGGAGAGAGTTG	543-562

Gel Electrophoresis

RT-PCR products containing 5 μ l of loading dye were fractionated on a 1% agarose gel (1.2 g agarose, 120ml 1X TBE, 9.9 μ l ethidium bromide). Gels were visualized in BioRad UV trans-illuminator.

Gel Purification, PCR Purification, and Sequencing

PCR products were purified from the 1% agarose gel using QIAquick Gel Extraction Kit[®] following the manufacturer's instructions. Non-gel extracted PCR products were purified using the PCR Purification Kit[®] (QIAGEN) according to manufacturer's protocol. All samples were sent out for sequencing containing 80ng of DNA and 1 μ l of each primer. Sequencing results were confirmed by BLAST.

Results

DCX is expressed in N-type and I-type cells, but not in S-type cells.

RT-PCR results show DCX expression in N-type and I-type cells only (Fig. 1A). This result supports previous findings of DCX expression in only the more invasive phenotypes in the SK-N-SH cell line (Messi et. al. 2008). Therefore, DCX expression may be correlated to the tumorigenicity of neuroblastoma cell types.

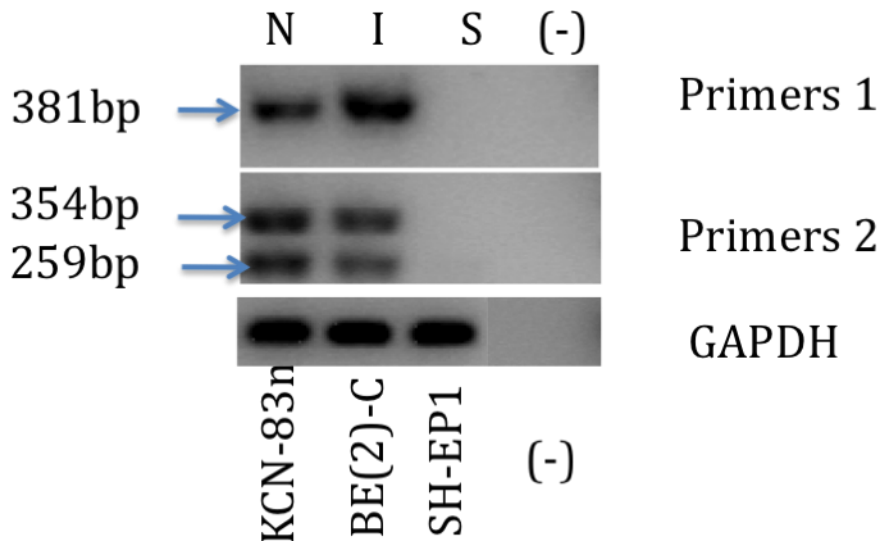


Figure 1A. RT-PCR DCX products using two different primers showing DCX expression in only N-type and I-type cells of the cell lines tested. Band at 259bp is the result of an alternative splicing event. Arrows indicate the number of base pairs. GAPDH was used to monitor mRNA levels.

Alternative splicing event detected in DCX in N-type and I-type cells

The results for DCX showed an alternative splice variant 95 bp shorter than the expected product (Fig1A). This band was detected by primer pair 2, which spans exon 4 to exon 7 (Fig.1.B). Sequencing results of the splice variant confirmed both the identity of the band and the deletion of exon 6. It also confirmed a weak splice consensus sequence on the 5' end in intron 6. This splice variant has never before reported.

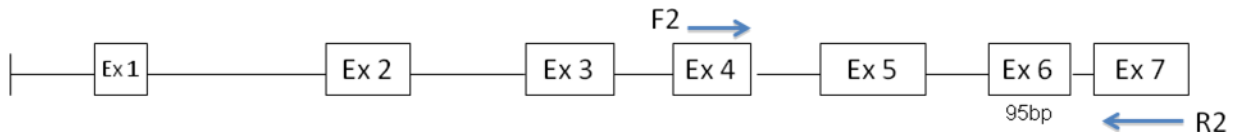


Figure 1B. Primer pair 2 spanning DCX gene. Expected product size is 354bp.

DCX is expressed in differentiated I-type cells by retinoic acid and BUdR treatment

DCX expression was up-regulated when I-type cells were induced into the N-type phenotype by RA treatment (Fig.2). In contrast to RA treatment, BUdR down-regulated DCX expression when compared to untreated cells. Figure 3 depicts the morphologies of I-type untreated, RA, and BudR treated cells. RA treated cells exhibit neuroblastic phenotypes, such as neurites, while BUdR treated cells are flat and contact inhibited as in S-type cells. (Walton et. al 2004)

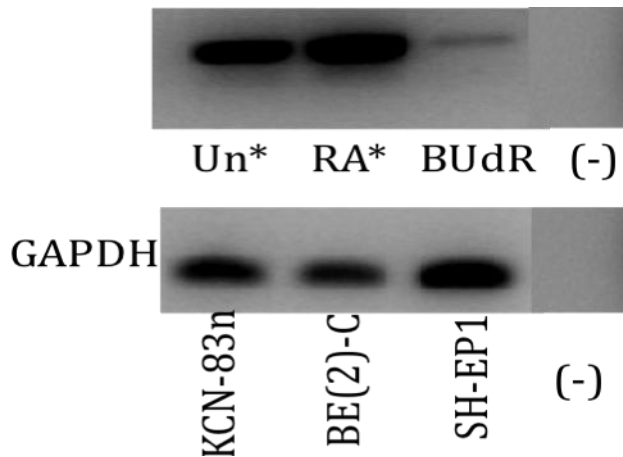


Figure 2. Impact of retinoic acid and BUdR treatment of I-type BE(2)-C cell line on DCX expression. Shown here is RT-PCR products using DCX primer 1 on treated cells. DCX expression is up-regulated in RA treated cells, while down-regulated in

BUdR treated cells. GAPDH was used to monitor mRNA levels. *Un=Untreated; *RA=retinoic acid.

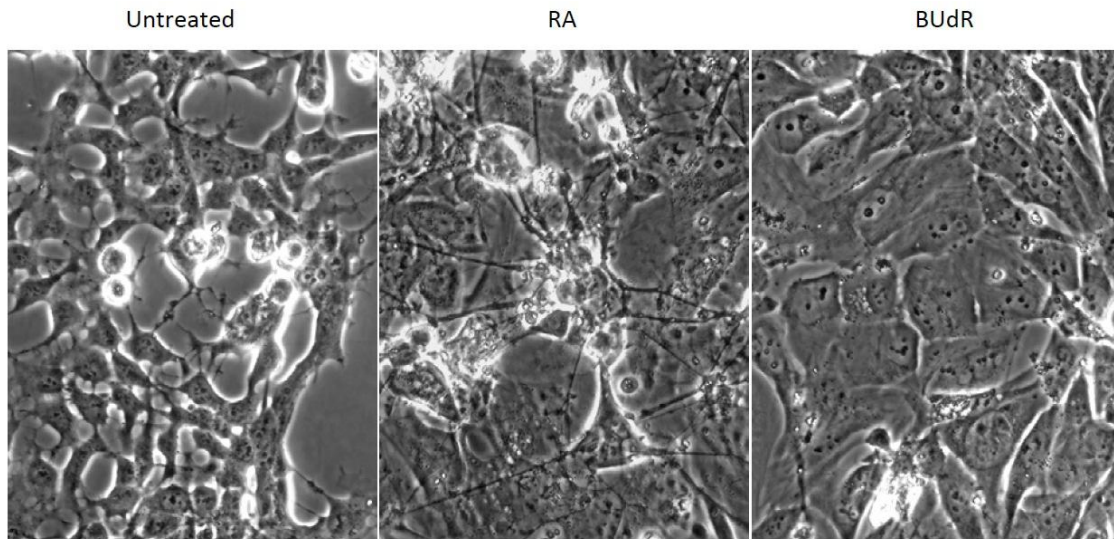


Figure 3. Depiction of different cellular morphologies: untreated, retinoic acid, and BUdR treated BE(2)-C cell line. Cells were treated with 1 μ M of retinoic acid or 10 μ M 5-bromo-2'-deoxyuridine for 21 days. (From Ross et. al. Fordham University, NY)

Caspase-1 and interleukin-18 are only expressed in S-type cells and are expressed in differentiated I-type cells by BUdR treatment

Results show that both interleukin-18 and its activator caspase-1 are only expressed in S-type cells (Fig 4.) and are expressed when I-type cells are induced to differentiate into the S-type cell. Caspase-1 and interleukin gene expression is not seen when I-type is induced to N-type by RA. (Fig. 5) This is consistent with the finding that caspase-1 is not expressed in N-type cells.

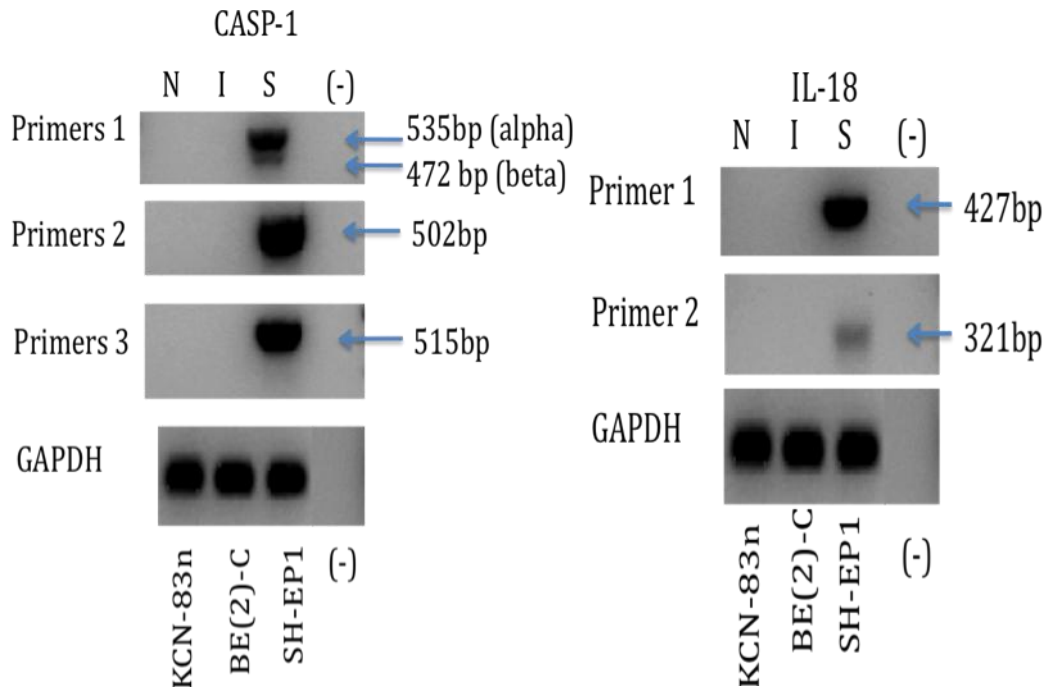


Figure 4. RT-PCR products using three different primers showing caspase-1 and interleukin 18 expression in only S-type cells of the cell lines tested. Arrows indicated the number of base pairs. Note that primer pair one detects two CASP-1 isoforms: alpha and beta. Beta is missing exon 3. GAPDH was used to monitor mRNA levels

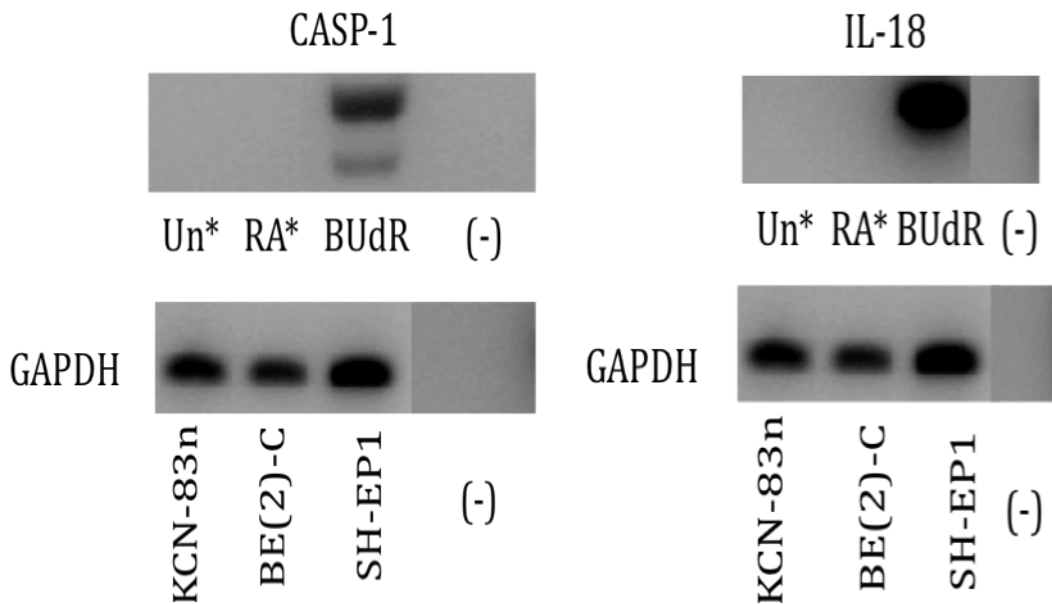


Figure 5. Impact of retinoic acid and BUdR treatment of I-type BE(2)-C cell line on caspase-1 and interleukin-18 expression. Shown here are the RT-PCR products using caspase-1 primer 1 on the I-type treated cells. GAPDH was used to monitor mRNA levels. *Un=Untreated; *RA= retinoic acid.

Discussion

This study demonstrated the differential expression of DCX in N-type, I-type, and S-type neuroblastoma cells. DCX is expressed in N-type and I-type cells, and not the non-tumorigenic S-type cells, which confirms previous reports (Messi et. al. 2008). DCX expression has also been reported to respond to retinoic acid treatment; a cancer treatment used to promote cellular differentiation and cell death (Sidell et. al 1983). We have found here that DCX expression was higher when I-type cells of the BE(2)-C were induced toward a more neuronal phenotype. Considering the pivotal role that DCX plays in neuronal migration, it is not surprising that agents inducing differentiation of I-type toward N-type would subsequently express higher levels of DCX, while S-type showed down regulation. However, it is noteworthy to mention that neural crest stem cells are highly migratory cells (Walton et. al. 2004). Thus, the stem cell-like I-type cells and S-type cells are likely to express DCX. It is feasible to predict that RA treatment would have little to no effect on DCX expression in I-type cells induced to N-type. However, it is shown here that DCX expression is upregulated when I-type is induced to N-type cells by retinoic acid treatment. In order to better understand DCX expression, real time PCR should be used to detect if DCX mRNA is more prevalent in N-type or I-type cells.

BUdR treatment resulted in a down regulation of DCX, which is consistent due to the non-neuronal, non-invasive properties of S-type cells, and the fact that DCX was not expressed in S-type cells. These findings suggest that DCX may be linked to cellular invasiveness in neuroblastoma and warrants further investigation to understand its presence and regulation in this cancer. A stronger correlation of DCX expression and cell-type should be confirmed.

Serendipitously, a novel splice variant of DCX was found in this study. In order to see if this variant codes for a new isoform of DCX, doublecortin protein should be analyzed in N-type and I-type cells. It may be unique to this particular tumor, or it may be a universal splice variant among malignant neuronal cells.

Interleukin-18, after its activation by caspase-1, induces the production of IFN-gamma (Yang et. al. 2004). IFN-gamma has a paramount role in anti-tumor responses in the cell. This evidence comes predominantly from animal models where IFN-gamma insensitive mice developed MCA-induced sarcomas (which are 3-methylcholanthrene-induced fibrosarcoma cells of BALB.c mice) 3 to 5 fold more

frequently than wildtype (Dunn et. al 2006). Also, Yang (2004) has shown that IFN-gamma increases caspase-8 expression (a key caspase in the TRAIL apoptotic pathway) in neuroblastoma cells. Apoptosis is an important cellular mechanism for destroying aberrant cells and thereby inhibiting tumor progression. Thus, caspase-1, leading to interleukin-18 activation, will presumably lead to production of IFN-gamma and help to decrease tumorigenicity of invasive cell types.

Our results show caspase-1 and interleukin-18 were expressed in S-type cells, and not the invasive cell-types. In addition, BUdR-treated I-type cells, but not retinoic acid or the untreated I-type cells, showed both caspase-1 and interleukin-18 mRNA expression. These results strongly suggest that these genes are exclusively expressed in S-type cells of the cell lines studied. However the role they play in S-cells is unknown. It may be that CASP-1 and IL-18 are constitutively expressed in S-type cells due to the fact that S-type cells are not under cellular stresses that would typically induce the presence of these two cytokines. In addition, since both apoptosis and IFN-gamma are mechanisms against cancer development, their absence in the two tumorigenic cell-types may be of significance in the progression of neuroblastoma.

However, it should be noted that albeit IFN-gamma has anti-tumor effects, high levels of IL-18 have negative implications and is correlated with cancer progression, specifically by inducing angiogenesis (Arnerd et. al 2008). However, other studies have reported that IL-18 is an angiogenic and tumor suppressor (Cao et. al 1999). Due to the seemingly complex and baffling role IL-18 plays in cancer, it may be inferred that IL-18 activity depends on the nature and stage of the cancer. There is little to no information on caspase-1 and interleukin-18 in neuroblastoma cells lines, but given that they are consistently expressed in only the S-type cell in the cell lines studied, further insight into their role should be pursued.

Altogether, these findings merit further investigation on why CASP-1 and IL-18 are only expressed in the non-invasive S-type cell and on the correlation of DCX expression with the invasiveness and progression of this cancer.

Acknowledgements

I would like to thank Dr. Robert Ross for generously supplying the cells. I would like to thank Bo Liu for his unyielding patience and guidance throughout this project. Also I would like to give a special thanks to Leleesha Samaraweera for her advice

and support. In addition, I would like to thank the support and encouragement that my classmates have given me over the course of this project. And finally, I would like to offer a sincere thank you to Dr. Berish Rubin for his guidance and making this project possible.

References

Arend, W. P., Palmer, G. Gabay, C., IL-1, IL-18, and IL-33 families of cytokines. (2008) *Immunol. Rev.* 223: 20-38

Cao, R., Farnebo, J., Kurimoto, M., Cao, Y. Interleukin-18 acts as an angiogenesis and tumor suppressor. (1999). *FaseB J.* 13: 2195-2202.

Couillard-Depres, D. Schaubeck, W. B., Aigner, R. Vroemen, M. Weidner, N. Bogdahn, U., Winkler, J., Kuhn, H. G., Aigner, L. (2005) Doublecortin expression levels in adults brain reflect neurogenesis. *Eur J Neurosci.* 21:1-14.

Dunn, Gp., Koebel, C. M., Schreiber, R. D. (2006) Interferons, immunity, and cancer immunoediting. *Nature Immunol.* 6: 836-846.

Feng, Q., Li, Peixiang., Leung, P. C.K., Auersperg, N. Caspase-1 zeta, a new splice variant of the caspase-1 gene. (2004) *Genomics* 84: 587-591.

LoTurco, J. (2004) Doublecortin and a tale of two serines. *Neuron.* 41:175-177.

Messi, E., Florian, M C., Caccia, C. Zanisi, M., Maggi, R. (2008) Retinoic acid reduces human neuroblastoma cell migration and invasiveness: effects on DCX, LIS1, neurofilaments-68 and vimentin expression. *BMC Cancer* 8:30.

Oltra S, Martinez F, Orellana C, Grau E, Fernandez JM, Canete A, Castel V. (2005) The Doublecortin Gene, A New Molecular Marker to Detect Minimal Residual Disease in Neuroblastoma. *Diagn Mol Pathol.* 14:53-57.

Polloneal, C. J., Dizon, M. L.V., Shin, L., Szele, F. G. Doublecortin is necessary for the migration of adult subventricular zone cells from neurospheres. (2006). *Mol. Cell. Neurosci.* 33: 126-135.

Sidell, N., Altman, A., Haussler, M. R., Seeger, R. C. Effects of retinoic acid on the growth and phenotypic expression of several human neuroblastoma cell lines. (1983) *Exp. Cell Res.* 143: 21-30.

Tanaka, T., Serneo, F. F., Tseng, Huang-Chun., Kulkarni, Ashok, B., Tsai, L., Geeson, J. G. (2004) Cdk5 phosphorylation of doublecortin Ser297 regulates its effect on neuronal migration. *Neuron* 41: 215-227.

Thiele, C. J. (1999). Neuroblastoma cell lines. In J. Masters (Ed.), *Human cell culture* (pp. 21-53). Lancaster, UK: Kluwer Academic Publishers.

Walton, J. D., Kattan, D. R., Thomas, S. K., Spengler, B. A., Guo, H., Biedier, J. L., Cheung, N. V., Ross, R. A. (2004) Characteristics of stem cells from human neuroblastoma cell lines and in tumors. *Neoplasia*. 6: 838-845.

Yang, X., Merchant, M. S., Romero, M. E., Tsokos, M. Wexler, L. H., Kontny, U., Mackall, C. L., Thiele, C. J. (2003) Induction of caspase 8 by interferon gamma renders some neuroblastoma (NB) cells sensitive to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) but reveals that a lack of membrane TR1/TR2 also contributed to TRAIL resistance in NB. *Cancer Res*. 63: 1122-1129.