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鈣網蛋白與血管內皮生長因子於神經母細胞瘤分化調控之研究

Study of Calreticulin and VEGF-A on the Regulation of Neuronal Differentiation in Neuroblastoma

翁妏謹

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本論文係翁奴謹君（學號 D97B41013）在國立臺灣大學生命科學系所完成之博士學位論文，於民國一零四年一月二十八日承下列考試委員審查通過及口試及格，特此證明

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誌謝

在鍵盤上敲下誌謝的這一刻，才真實感受到博士班的生涯即將告一段落，回首過去，研究的生涯一度面臨家庭與工作必須同時兼顧的痛苦掙扎，也曾萌生放棄的念頭，但我很慶幸這一路上有許多的貴人相助，讓我能夠堅持到這一刻，在此由衷獻上我最誠摯的感謝。

首先最要感謝的是李心予老師對於我的諄諄教誨與悉心關懷。李老師不僅在實驗上給予專業的指導與充分的支援，更在我人生遇到各種困境與挫折時不斷鼓勵我，幫助我，常讓我在沮喪之餘，再度重燃信心。老師對於研究與教育的熱忱與風範，都讓我永誌不忘，深刻影響著我日後的工作和生活。此外，還要感謝李旺祚醫師，他在臨床工作與研究上對於我的指導與建議，讓我少走許多冤枉路，讓我這些年來無論在工作或研究上都獲益匪淺。另外，要特別感謝許文明醫師，他對於晚輩們總是慷慨且毫不保留地提供重要的意見與實質的協助，在辛苦工作之餘仍不忘與我討論實驗的方向，是這篇論文能夠完成的重要推手。再者，很榮幸可以邀請到廖永豐老師和黃敏銓老師擔任我的口試委員，給予我寶貴的建議與指導，讓我的論文可以更臻完善。

李老師的研究室是一個溫暖且歡樂的大家庭，雖然我是研究室中年齡最大的學姊，但在許多實驗的技術上常常需要學弟妹的幫忙。特別感謝貫浤、沛翊、柏堅、Milky、易謙、予農，以及許多曾經一起打拼的學弟妹們，謝謝你們在實驗上的協助，也很喜歡跟你們相處的日子，讓我常常覺得自己年輕了十歲，也讓我以身為實驗室的一分子為榮。

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中文摘要

神經母細胞瘤是幼兒期最常見的惡性腫瘤，其腫瘤形成的分子機制依然不清楚，有可能是胚胎交感神經系統發育異常使神經母細胞無法分化或無法凋亡而造成。我們過去的文獻回顧發現內質網伴護蛋白包括鈣網蛋白(Calreticulin, CRT)與葡萄糖調節蛋白(GRP)為神經系統的胚胎發過育過程所必需。我們的斑馬魚研究亦顯示 CRT 在神經系統的胚胎發育過程極具重要性。過去的研究發現 CRT 是神經母細胞瘤一項重要的預後指標，CRT 的大量表現與神經母細胞瘤的分化程度呈正相關性，病人並有較佳的預後表現。因此，CRT 也在神經母細胞瘤的分化上扮演了重要的角色。

血管內皮生長因子(VEGF-A)與其引導的血管新生現象亦被證實在神經母細胞瘤的形成具有重要的角色。而在胃癌研究發現 CRT 與 VEGF-A 具有相關性。因此，本研究希望藉由細胞實驗與動物實驗探討 VEGF-A 是否參與在 CRT 對於神經母細胞瘤的分化調控，並進一步探討 VEGF-A 對於人類神經母細胞瘤的分化調控與臨床意義。我們過去的研究結果發現在三種不同的神經母細胞瘤細胞株實驗，CRT 的大量表現皆會正向調控 VEGF-A 與其上游調控分子缺氧誘導因子 HIF-1α的表現量，並增加 VEGF-A 的蛋白質分泌。反之，利用 shRNA 抑制 CRT 的表現亦會造成 VEGF-A 及 HIF-1α的表現下降。而在本研究中，我們進一步發現 CRT 的大量表現不會影響細胞凋亡，但會促

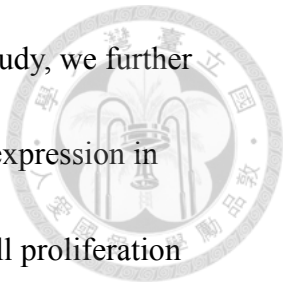
進細胞分化並抑制細胞增生。此外，我們利用 VEGF 接受器抗體去抑制 VEGF-A 的作用，則神經母細胞瘤的神經分化指標包括 GAP43、NSE、NFH 及 TrkA 亦會受到抑制。以上結果顯示 VEGF-A 的確在 CRT 誘導的神經母細胞分化扮演重要角色。然而，我們利用神經母細胞瘤細胞株進行實驗發現 CRT 大量表現會促進細胞之神經分化無法增生，因此經由四環黴素誘導 CRT 表現之 stNB-V1 神經母細胞瘤細胞株來進行動物實驗。我們接著利用腫瘤異體移植實驗進一步證實 CRT 對於 VEGF-A 及 HIF-1α的正向調控，實驗亦發現誘導 CRT 的表現可以顯著抑制腫瘤體積並促進腫瘤分化。除此之外，我們發現在病人腫瘤裡 CRT 的表現和 VEGF-A 的表現呈現顯著正相關。更重要的是，從病患的病理切片染色發現具有 VEGF-A 表現的病患其預後亦較佳。VEGF-A 的表現與腫瘤的分化程度呈正相關，與 MYCN 的表現呈負相關，但與內皮血管形成無相關性，顯示 VEGF-A 可能透過與內皮血管新生無關之機轉來調控神經母細胞瘤的形成與分化。

總之，本研究證實在神經母細胞瘤中，CRT 可正向調控 VEGF-A 表現並促進腫瘤的神經分化，且 VEGF-A 確實參與此神經分化調控。我們亦首次證實 VEGF-A 是神經母細胞瘤的一個獨立預後因子，具有 VEGF-A 表現的病患其預後亦較佳。我們的研究為神經母細胞瘤的腫瘤形成開啟嶄新的機轉，同時也有助於對神經母細胞瘤新治療的發展。

關鍵詞：鈣網蛋白、血管內皮生長因子、神經母細胞瘤、神經分化、血管新生

Abstract

Neuroblastoma (NB) is the most common malignant tumor of infancy. The tumorigenesis of NB could be a divergence of the embryonic development of sympathetic nervous system. ER chaperones including calreticulin (CRT) and GRP78 are suggested to participate during embryonic development in our previous review. Our present study in zebrafish also revealed that CRT is essential for embryonic and neuronal development. Previous study has identified CRT as an independent favorable prognostic factor which is related to differentiated histologies in NB. Taken together, CRT could play an important role in neuronal differentiation of NB. Recently evidence has suggested that vascular endothelial growth factor (VEGF)-A, a key regulator of physiological and pathologic angiogenesis, participates in the behavior of NB. Furthermore, recent studies have found a correlation between CRT and VEGF-A in gastric cancers. In the present study, we aimed to determine whether the CRT expression in NB was associated with the VEGF-A pathway and to determine the role of VEGF-A in regulating NB behavior focusing on angiogenesis and neuronal differentiation *in vitro* and *in vivo*. Our previous study clearly demonstrated that in different NB cell lines, CRT over-expression increases the expression and secretion of VEGF-A and HIF-1α, a major positive regulator of VEGF-A. In contrast, knockdown



of CRT decreases VEGF-A and HIF-1α expression. In the present study, we further demonstrated that NB cell apoptosis was not affected by CRT over-expression in stNB-V1 cells. Nevertheless, over-expression of CRT suppressed cell proliferation and enhanced cell differentiation in stNB-V1 cells, whereas blockage of VEGFR-1 markedly suppressed the expression of neuron specific markers including GAP43, NSE and NFH as well as TrkA, a molecular marker indicative of NB cell differentiation. These results indicate an essential role of VEGF-A in CRT-related neuronal differentiation in NB. However, constitutive over-expression of CRT led to NB cell differentiation without proliferation. Thus, we used an inducible-CRT stNB-V1 cell line by a tetracycline-regulated gene system for further animal experiments. The mice xenograft models further confirmed the positive regulation of CRT on VEGF-A and HIF-1α, as well as the role of CRT in enhancing neuronal differentiation and suppressing tumor growth in NB. Furthermore, we have demonstrated a significantly positive correlation between CRT and VEGF-A expression in human NB tumors. Most important of all, we verified that VEGF-A expression predicts a favorable outcome in NB patients and are associated with differentiated histology and normal MYCN expression, both of which are favorable prognostic factors. On the other hand, there was no correlation between the expression of VEGF-A and CD34, a marker of endothelial cells, suggesting a novel mechanism

of VEGF-A participating in NB formation through angiogenesis-independent pathway.

In conclusion, our study indicated that CRT-dependent VEGF-A up-regulation is critical for NB differentiation and VEGF-A is involved in CRT-related neuronal differentiation in NB. For the first time, we have demonstrated that VEGF-A is an independent prognostic factors and predicts favorable outcomes in NB patients with tight relationship with differentiated histology and MYCN status. Our findings also delineate a novel mechanism of VEGF-A in the biology of NB. This study provides important information that is needed for deciphering the crucial role of CRT and VEGF on the regulation of NB differentiation. Furthermore, our findings will shed light to a novel therapeutic strategy to improve the outcome of NB patients in the future.

Keywords: Calreticulin, VEGF-A, neuroblastoma, neuronal differentiation, angiogenesis

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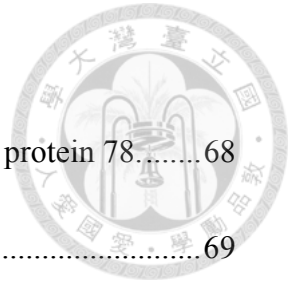

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Chapter I.
Introduction

1. ER stress and ER-resident chaperone proteins

1.1 ER stress and UPR

Endoplasmic reticulum (ER) is the principle cellular organelle in which secretory and membrane proteins are properly folded and modified. ER also functions as a major intracellular calcium store and is responsible for biosynthesis of steroids and cholesterol. Moreover, ER is the site for N-linked glycosylation, which is also important for correct protein folding [1]. Proteins that cannot be correctly folded would be eradicated via proteasome-mediated ER-associated degradation (ERAD) pathway [2]. The accumulation of unfolded or misfolded proteins in the ER lumen has referred to as ER stress and in eukaryotes it would induce adaptive coordinated responses, which are described as unfolded protein response (UPR) [3]. A number of cellular stress conditions, such as disruption of calcium homeostasis, altered glycosylation level, secretory protein mutations, and abnormal cholesterol level, can activate the UPR and contribute to a wide range of human diseases. Three ER-resident transmembrane proteins function as stress sensors and are involved in the UPR: the inositol requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticular kinase (PERK) transducers, which transduce the unfolded proteins signal cross the ER membrane and lead to the activation of UPR [4] (Fig. 1). If these adaptive coordinated responses cannot eliminate inappropriate

folding proteins during prolonged and severe ER stress, the UPR would elicit pro-apoptotic pathway and trigger apoptotic cell death [5].

1.2 Multifunctional roles of ER-resident chaperone proteins

To assist and regulate the correct folding of proteins, ER contains abundant proteins known as molecular chaperons including the 78-kDa glucose-regulated protein 78/immunoglobulin-binding protein (GRP78/BiP), GRP75 and calreticulin (CRT). These chaperone proteins contain ER stress elements (ERSE) in their promoters and are upregulated during ER stress [6]. Other than as regulators of UPR, several ER chaperones are involved in many cellular processes For example, GRP78, a member of heat shock protein 70 (HSP70) family, also referred to as BiP or Hspa5, has been found to regulate important biological functions including calcium homeostasis, anti-apoptosis, autophagy and as cell surface receptor [4] (Fig. 1).

1.3 Calreticulin

1.3.1 Fundamental information of calreticulin

Calreticulin (CRT) is another important chaperone protein primarily localized to the endoplasmic reticulum and highly conserved in diverse species [7]. It is composed of three structurally and functionally distinct domains, including N-, P-, and

C-domain [8]. The N-terminal domain is responsible for chaperon functions of CRT [9]. The proline-rich P-domain contains two sets of three repetitive regions [10]. These repeated amino acid sequences form the lectin-like chaperone structures which are responsible for protein-folding function of CRT. The C-domain of CRT is a highly acidic region which is important for Ca^{2+}-buffering functions. It is known that Ca^{2+} binding to this region play a critical role in the interaction with other chaperone proteins in ER [8, 11].

Apart from highly enriched within the ER lumen, CRT has also been identified in cytosol [12] and on cell surface [13]. Cell-surface CRT is found to be associated with phagocytic uptake and immunogenicity of cells [14]. The evidence provides more insights for CRT as a multifunctional protein. The multi-functional roles of CRT in protein chaperoning, Ca^{2+} homeostasis, cell transduction, modulating cell adhesion, and gene expression disclose its major involvement in various physiologic and pathologic conditions [7, 15].

1.3.2 Regulation of calreticulin expression

The human calreticulin gene (*CALR*) is located on chromosome 19p13.2 with nine exons. Calreticulin promoter region contains several binding site for reputed transcriptions factors and many of these factors have been identified as important modulators of CRT expression including NKx2.5, MEF2C, COUP-TF1, GATA6,

Evi-1, and PPAR factors [16]. In addition, calcium depletion and ER stress were shown to be important activators of *CALR* transcription [17]. Recently, studies have also revealed that nerve growth factor (NGF) can also up-regulate CRT expression in both ovarian cells and neuronal differentiation [18, 19]. These results further suggested an involvement of CRT expression in various biological and pathological processes.

2. Roles of calreticulin in neuroblastoma

2.1 ER chaperones in cancer development

More and more evidences demonstrated that ER-resident chaperone proteins play critical roles in many pathological illnesses and a variety of human diseases [20]. Whether the involvement of ER-resident chaperone proteins is the primary cause or a secondary consequence in diseases is yet to be determined. Most findings of ER chaperones in human diseases have been focused on carcinogenesis and tumor progression. For example, our recent reviews showed that GRP78 is involved in cancer progression and drug resistance [4].

Moreover, the correlation between CRT expression levels and tumorigenesis has been extensively studied in various cancers and most reports have revealed that tumor tissues express significant higher levels of CRT compared to normal tissues [21].

Up-regulation of CRT presented in a variety of cancer cell lines, solid tumors, and human cancer cells, including oral cancer [22], breast ductal carcinoma [23, 24], colorectal cancer [25], prostate cancer [26], and vaginal carcinoma [27]. Tumor invasion, metastasis and recurrence were also positively correlated to increased CRT expression while the patients' survival rate were inversely correlated to increased CRT expression in gastric cancer [28], breast cancer [29], bladder cancer [30], pancreatic cancer and esophageal squamous cell carcinoma [31, 32]. These results indicate that increased CRT expression might play a crucial role in cancer progression.

Another important role for CRT exposed on the cell surface, which is relevant for destruction of cancer cells, is via induction of the immune response [14, 33]. Results from several laboratories have demonstrated that cell-surface CRT facilitates the phagocytic uptake of apoptotic and cancer cells [34-36]. Obeid *et al.* demonstrated that drug treatments (anthracyclins) caused tumor cell to expose a surface pro-phagocytic protein, CRT, which induced immunogenic cell death [37]. It is becoming clear that surface exposure of CRT is required for phagocytosis on dying tumor cells. CRT expressed on the cell surface is considered as an "eat-me" signal and promote phagocytic uotake of cancer cells by immune system for multiple human cancers.

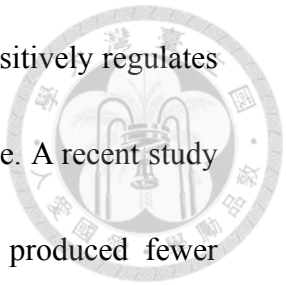
On the other hand, the roles of CRT in ovarian cancer progression are

inconclusive. Compared with primary tumors and solid metastases, reduced CRT expression was observed in malignant effusions of high-grade ovarian carcinoma along disease progression [38]. Besides, CRT expression levels may be associated with better response to chemotherapy while the survival was not related to CRT expression [38]. Very recently, mutations in calreticulin gene (*CALR*) were detected in a majority of myeloproliferative neoplasms [39-41]. Furthermore, in neuroblastoma, increased CRT expression is found to be associated with better prognosis and differentiated histologies [42, 43]. Therefore, the impact of CRT on tumor formation and progression may depend on different cell types and clinical stages.

2.2 Calreticulin in regulating cancer cell proliferation

Cancer formation and progression is characterized by rapid proliferation of mutant cells. Increasing evidence have revealed that manipulation of CRT expression had obvious effects on cell proliferation in various cancers. In pancreatic cells, overexpressed CRT enhanced cell growth, while knockdown of CRT had the opposite effect on cell growth [31]. In addition, reduction of CRT caused cell cycle arrest at the G0/G1 phase which resulted in significantly suppressed growth rate, colony-formation capacity, and anchorage-independent growth in oral cancer cell [22]. In bladder cancer, knockdown of CRT is also found to suppress cell growth

[15]. Although most studies have suggested that CRT expression positively regulates cell growth, other studies provided conflicting evidence on this issue. A recent study demonstrated that prostate cancer cells with higher CRT levels produced fewer colonies as well as inhibition of tumor growth both *in vitro* and *in vivo* [44]. According to these reports, whether CRT promotes or suppresses cell proliferation may rely on different cell types.



2.3 The biology of Neuroblastoma

Neuroblastoma (NB) is a common childhood tumor and the most frequently diagnosed malignancy in infancy, with more than 96% of patients diagnosed at the age of <10 years old [45, 46]. It is derived from the sympatho-adrenal lineage of embryonic neural crest cells [47]. Children with NB have a broad spectrum of clinical diversity that is highly associated with age at onset and genetic, biological, and pathological characteristics [47]. As a result, 50% of the NB patients die from this disease that continues to be one of the most challenging tasks among pediatric tumors.

The mechanism underlying its tumorigenesis remains obscure, despite the identification of several clinically relevant prognostic markers. Previous studies suggest that incomplete development and failure of differentiation or apoptosis of neuroblastic cells is critical in its development [48]. Previous studies have shown that

NB cells exhibit a capacity of differentiating into mature cells or spontaneous regression by apoptosis [49, 50]. Studies also demonstrated that NB can be forced to differentiate upon the treatment of retinoic acid [51]. On the other hand, NB with better prognosis often express molecular markers indicative of cell differentiation, such as TrkA [52]. Furthermore, the expressions of apoptosis-related genes including p53, Bcl-2, and Bax have been demonstrated in NB and are correlated with favorable prognosis [53]. However, the factors contributing to the regulation of NB cell differentiation or apoptosis are still unclear.

Genetic studies have postulated MYCN as one of the most important molecules that is closely related to the pathogenesis including differentiation, proliferation and apoptosis of NB. MYCN is a proto-oncogene normally expressed in the developing central and peripheral nervous systems [54]. Its expression is regulated by several signaling pathways, such as E2F1 [55]. As a bHLH transcription factor, MYCN dimerizes with MAX and binds to the E-box sequence (CACGTG) of the promotor region of target genes [56]. The major gene family that is directly regulated by MYCN is called the N-MYC Downstream Regulate Gene (NDRG) [57]. Through the transcriptional regulation of these genes, MYCN regulates the proliferation, growth, differentiation and survival of cells in the developing nervous system. In NB tumors, amplification of MYCN is closely associated with metastasis, advanced disease stages

and poor outcome [58].

2.4 ER chaperones and calreticulin in neuroblastoma

Though extensive exploration of ER chaperones in tumorigenesis, the roles of ER chaperones in neuroblastoma have not carefully addressed. Recently, Hsu et al. have demonstrated that several chaperones including CRT, GRP75 and GRP78 are independent favorable prognostic markers in NB [42, 59, 60]. Hsu et al. found that the percentage of positive GRP78 immunostaining increased as the tumor histology of NB became differentiated ($p=0.001$) [60]. GTP78 expression as a significant factor for predicting favorable outcome has also been found in other studies of olfactory NB [61]. In addition, Hsu et al. have also found that positive GRP75 immunostaining is strongly correlated with differentiated histologies ($p<0.001$) and as an independent favorable prognostic factor [59].

Moreover, evidences also suggest that CRT may play an essential role in the biology of NB. Previous studies reveal that CRT is on the surface of NB cells and is essential for neurite formation when NB cells are induced to differentiate [62, 63]. Another *in vitro* study using NB cell line reveals that increased CRT expression is correlated with the differentiation of NB cells [64]. Recently, CRT has been identified by Hsu et al. as an independent favorable prognostic marker in

NB [42]. They have found that increased CRT expression in NB positively correlates with tumor differentiation and therefore predicts favorable outcome. In conclusion, CRT may play an essential role in the differentiation of NB cells, as well as in the differentiation of other neural progenitor cells or neural stem cells. However, how CRT affects the differentiation of NB warrants further clarification.

3. Roles of calreticulin in embryonic and neuronal development

3.1 ER chaperones in embryonic and neuronal development

A number of molecules normally expressed during embryonic development, including HNK-1, neuropeptide Y, tyrosine hydroxylase, TrkA and CD44, are found in NB [48, 65], suggesting that the tumorigenesis of NB could be a divergence of the embryonic development of the sympathetic system. During embryogenesis a number of coordinated events occur to ensure orderly generation of the diverse cell types leading to proper organ system development including nervous system development. One of these events is apoptosis that has been shown to widely occur during central nervous system development [66]. Apoptosis may be induced by a number of different stimuli including ER stress [67]. Recently, a study found evidence that ER stress involved in the development of the central nervous system in the mouse models

[68]. Several ER chaperones, including CRT and GRP78 were expressed at higher levels in embryonic brain and retina than in adult tissues. Apart from the roles in human diseases, converging evidences suggested that ER-resident chaperone proteins also participate during embryonic development and physiological conditions [4, 20].

3.2 Roles of calreticulin in neuronal development and neuronal differentiation

Neuroblastoma is derived from the sympathoadrenal lineage of embryonic neural crest cells and results from incomplete development and differentiation of precursor cells [45]. A recent study using CRT knockout (KO) mouse model exhibited lethality with significant defects in heart, brain and body wall, suggesting that CRT is essential in the embryonic development of nervous system [69]. Moreover, a recent study using PC-12 cells expressing mutant CRT lacking a Ca^{2+}-buffering domain (C domain), suggested that the Ca^{2+}-regulating capacity of CRT is essential for NGF-elicited neuronal differentiation [19]. In NB, previous studies have demonstrated that the expression levels of CRT positively correlated with the differentiation of NB both *in vitro* and *in vivo* [42, 43]. In addition, it has been reported that surface CRT is crucial for neurite formation when NB cells are induced to differentiate [62]. Accordingly, these findings suggest that CRT may play an important role in nervous system development, neuronal differentiation. as well as the tumorigenesis (focusing on

differentiation) of NB.

3.2 Zebrafish as a model in studying embryonic development

The zebrafish is vertebrae with transparent embryos and identifiable, stereotyped neurons. As a consequence of the widespread use of zebrafish in developmental biology studies, an extensive array of experimental tools and techniques has also been assembled in the analysis of human neurodegenerative and neurodevelopmental diseases.

4. VEGF-A and angiogenesis in neuroblastoma

4.1 Fundamental information of VEGF-A

Vascular endothelial growth factor (VEGF)-A (also referred to as VEGF), which belongs to a family of secreted homodimeric disulfide-bound glycoproteins, is a key regulator of physiologic and pathologic angiogenesis and highly conserved between animals [70, 71]. In mammals, this family includes VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). The human VEGF-A gene contains eight exons and alternative splicing of VEGF-A gene gives rise to at least six different transcripts [72].

The biological functions of VEGF-A in regulating endothelial cell proliferation, migration, vascular permeability, secretion and other endothelial functions were

mediated by two related receptor tyrosine kinase (RTK), VEGFR-1(Flt-1) and VEGFR-2 (KDR/Flk-1) [73]. Both VEGFR-1 and VEGFR-2, which differ in signaling properties, contain seven immunoglobulin-like extracellular domains, a single transmembrane region and a consensus tyrosine kinase sequence. The VEGF-A also binds to two non-tyrosine kinase receptors of the neuropilin (NRP) family, NRP1 and NRP2, which are thought to be modulators for the VEGF-VEGFRs signaling.

VEGF-A expression is mainly regulated by oxygen tension [74]. The VEGF-A gene contains hypoxia-responsive enhancer elements (HREs) and the mRNA expression of the VEGF-A gene induced by hypoxia is mediated by binding of the transcription factor hypoxia-inducible transcription factor 1 (HIF-1) to the HRE. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. Under hypoxic situations, the HIF-1α is up-regulated because the proline hydroxylation is inhibited and then enhanced the transcription of VEGF-A [71]. In addition, several growth factors and inflammatory cytokines, including TGF-α, TGF-β, platelet-derived growth factor, fibroblast growth factor, insulin-like growth factor-1, interleukin-1 and interleukin-6 can also upregulate VEGF-A expression [70].

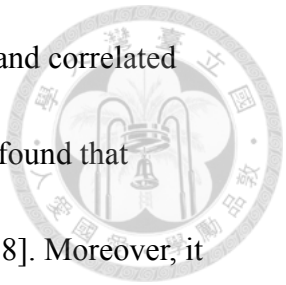
4.2 Angiogenesis in neuroblastoma

NB tumors are highly vascular and a large variety of pro-angiogenic factors,

such as VEGF, FGF-2,IL-8, TGF-α, PDGF-A, angiopoietins and erythropoietin have been found to play crucial roles in the induction of angiogensis in advanced-stage NB tumors [75, 76]. Although it is still debatable, the high vascular index, as expressed by numbers of vessels per square millimeter of tissue area, has been shown to correlate with the adverse prognosis of NB patients, suggesting a close relationship between aggressive tumor behavior and active angiogenesis [77, 78]. However, another study reveals that tumor vascularity is not correlated with prognosis and tumor stage in NB patients [79]. Moreover, it has been shown that there is no tumor growth difference in NB xenograft mice treated with angiogenesis inhibitor [80]. In a recent study, investigators have explored the essential role of angiogenesis in the maturation phase of NB [81]. Therefore, the role of angiogenesis in NB tumorigenesis and outcome is complicated and needs further investigation.

4.3 VEGF-A in neuroblastoma

Apart from its role in embryonic vascular development, more and more studies reveal that VEGF-A plays an important role in the progression and metastasis of cancer cells [82]. In NB, angiogenesis is an essential mechanism regulating NB tumorigenesis, while VEGF-A-driven angiogenesis plays a critical role in the pathogenesis of NB formation and metastasis [83-85]. Both in human and in



experimental NB, VEGF-A over-expression has been demonstrated and correlated with a high-risk phenotype [86, 87]. In contrast, recent studies have found that VEGF-A is not related to tumor progression and metastasis in NB [88]. Moreover, it has been shown that there is no tumor growth difference in NB xenograft mice treated with or without Anti-VEGF antibody [89]. Thus, the roles of VEGF-A in the tumorigenesis of NB remain obscure and need further clarification.

5. The relationship between calreticulin and VEGF-A-driven angiogenesis

Some studies have found a correlation between CRT and VEGF-A in human cancers. In gastric cancers, CRT has been found to up-regulate VEGF expression and enhance angiogenesis, leading to poor prognosis [28]. However, it has also been reported that exogenous CRT and the CRT fragment, vasostatin, are considered as anti-angiogenic factors and inhibit VEGF-induced endothelial cell proliferation [90, 91]. According to these reports, whether CRT promotes or suppresses VEGF-A-driven angiogenesis remains elusive and needs further exploration.

6. Rationales

The tumorigenesis of NB could be a divergence of the embryonic development of sympathetic nervous system. ER chaperones including CRT and GRP78 are found to participate during embryonic development. Studies in CRT knockout mice which demonstrated the essential role of CRT for embryogenesis have suggested the important role of CRT in nervous system development as well as the biology of NB. As we mentioned above, calreticulin (CRT) has been previously correlated with the differentiation of NB tumors, implying a favorable prognostic factor. However, how CRT affects the neuronal differentiation and of NB remains unclear.

VEGF-A and VEGF-A-driven angiogenesis have been reported to participate in the behavior of NB. However, the roles of VEGF-A in NB progression and prognosis are complicated and controversial. Furthermore, recent studies have found a correlation between CRT and VEGF-A in gastric cancers. Nevertheless, the relationship between CRT and VEGF-A in NB has never been studied. **Thus, in this study, we proposed to investigate the association of CRT and VEGF-A in regulating NB tumorigenesis focusing on angiogenesis and neuronal differentiation *in vitro* and *in vivo*.**

Chapter II.
Materials and Methods

Ethics statement

All zebrafish and mouse experiments were performed after approval from the Institutional Animal Care and Use Committee at National Taiwan University. The clinical evaluation and use of tumor tissues for this study were approved by the Institutional Review Board of National Taiwan University Hospital. Written informed consent was obtained from the patients before sample were collected.

Fish breeding and embryo collection

Breeding fish will be maintained at 28.5 °C on a 14-h light/10-h dark cycle in a certified fish facility. Embryos will be collected by natural spawning, raised in 0.3X Danieau's buffer (by diluting 1X Danieau's buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5.0 mM HEPES (pH 7.6), with double distilled water) until observation or fixation. Embryos will be staged according to Kimmel et al. [92], and stages are given as hours post-fertilization.

Sequence analysis of calreticulin

The homologous calreticulin genes of human, mouse and zebrafish were identified from the NCBI database. The genomic sequence alignment and phylogenetic tree were carried out using Ensembl and MEGA4.3, respectively.

Total RNA isolation and RT-PCR in zebrafish

A reverse-transcription polymerase chain reaction (RT-PCR) will be performed on total RNA extracted from embryos at the designated times using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA will be subsequently treated with DNase I (Invitrogen), and first-strand cDNA will be synthesized. cDNA will be generated from total RNA of zebrafish larvae using the SMART kit (Clontech, Mountain View, CA) according to the manufacturer's instructions.

Morpholino oligonucleotide (MO) microinjections

Antisense MOs will be purchased or custom made by Gene Tools, LLC (Philomath, OR). A standard control MO (stdMO, 5'-CCTCTTACCTCAGTTACAATTTATA-3') with no sequence homology to any known zebrafish sequences will be used. To knock down the target genes activity, we used two different MOs targeting the translation initiation site and the splicing donor site, respectively. The MOs will be dissolved in sterile double-distilled water to 1 mM and stored at -20 °C.MOs will be diluted to the desired working concentrations in 1X Danieau's buffer with 0.5% phenol red and stored at 4 °C before being used.

Thin-wall (1 mm (o.d.) × 0.75 mm (i.d.)) glass capillaries with filaments (A-M Systems, Carlsborg, WA) will be pulled using a horizontal puller (P-97, Sutter Instrument, Navato, CA). Embryos at desired stages will be immobilized at an injection trough on a 100-mm 2% agar plate. MOs will be prepared as described at designated concentrations. An injection pipette will be forced into the chorion and the yolk cells to reach the junction between the yolk cells and blastodisc where the solution will be ejected by using a pressure injector (IM-300, Narishige, Japan). After injection, embryos will be recovered from the injection troughs and cultured in 0.3X Danieau's buffer at 28.5 °C until being examined.

Cell culture

The NB cell lines SH-SY5Y (ATCCH CRL-2266TM) and SK-N-DZ (ATCCH CRL-2149TM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), while the stNB-V1 was kindly provided by Dr. Yung-Feng Liao of Academia Sinica. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/High glucose medium (Biowest) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). The cells were grown in a humidified atmosphere containing 5% CO2 and 95% air at 37°C. Using a hemocytometer and trypan blue, cells were sub-cultured in 10 cm plates at a density

of 10^6 cells per plate.

Transfection

The cells were seeded in 3.5 cm plates at density of $3*10^5$ cells and were transfected with construct plasmids using Lipofectamine 2000 (Invitrogen). Every plate was transfected with 4 μg of the plasmid and 10 μL of the Lipofectamine 2000 in serum-free DMEM. After 8 hours of transfection, the medium was changed into DMEM with 10% FBS. The cells were harvested after 48 h.

Cells transfected with a vector, without the insert gene, were used as vehicle control and cells treated with ddH_2O were used as negative control. The construction of CRT expression vector pEGFP-C1-CRT and CRT shRNA vector pCR3.1-CRTshRNA was as previously described [28].

Construction of stable cell lines

Tetracycline-inducible CRT expression vector pLKO_AS3.1-p5CRT-HY was constructed. pLKO_AS3.1 was the control vector of the inducible vector. CRT shRNA expression vector was purchased from the National RNAi Core Facility Platform, Academia Sinica (Taipei, Taiwan). The shRNA target sequence was: 5'-CCAGTATCTATGCCTATGATA-3' (shCRTa, TRCN0000019989), 5'-

CGTCTACTTCAAGGAGCAGTT-3' (shCRTb, TRCN0000019991). pLKO.1 was the control vector of the shRNA plasmid.

Lentiviral stocks were produced by calcium phosphate transfection. Around 30-40% confluent 293T cells in T25 flasks were prepared and transfected with a DNA mixture containing 7.5 g packaged plasmids and 7.5 g lentivector of the target gene for 16 h. The transfected condition medium was replaced with 12 ml fresh DMEM containing 10 mM sodium butyrate. After 24 h, all conditioned medium were harvested and treated with the stNB-V1 cells for infection. Cells were selected by 1 μg/μL puromycin (InvivoGen, USA).

RNA isolation and reverse-transcription (RT)

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen). Complementary DNA was synthesized with 1 μg total RNA using a Toyobo RT-polymerase chain reaction (PCR) kit (Toyobo, Osaka, Japan).

Quantitative real-time PCR

The real-time PCR with the mixture reagent KAPA SYBR-Green as the fluorescent dye (Bio-Rad) was conducted on a Mini-Opticon real-time detection system (Bio-Rad, Hercules, CA, USA). Gene-specific primers were used and the

specificity was confirmed by single melting-curve after real-time PCR. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

For quantification, the target gene was normalized to the GAPDH to act as an internal control for SH-SY5Y and SK-N-DZ cells and heat shock protein 60 (HSP 60) for stNB-V1. P53, Bcl-2 and Bax were used as apoptotic markers [93]. Primers for the real-time PCR were: GAPDH (F-5'-AAG GTG AAG GTC GGA GTC-3' and R-5'-TGT AGT TGA GGT CAA TGA AGG-3'); HSP60 (F-5'- CA CCG T AA GCC TTT GGT CAT-3' and R-5'- CTT GAC TGC CAC AAC CTG AA-3'); CRT (F-5'-CC TCC TCT TTG CGT TTC TTG-3' and R-5'-CAG ACT CCA AGC CTG AGG AC); HIF-1α (F-5'-CAT AAT GTG AGT TCG CAT CT-3' and R-5'-ATA TCC AAA TCA CCA GCA TC); VEGF-A (F-5'-GGC ACA CAG GAT GGC TTG AAG-3' and R-5'-GGC ACA CAG GAT GGC TTG AAG-3'); p53 (F-5 ´ -TGA CTG TAC CAC CAT CCA CTA-3 ´ and R-5 ´ -AAA CAC GCA CCT CAA AGC-3 ´); Bax ((F-5 ´ -TGC TTC AGG GTT TCA TCC AG-3 ´ and R-5 ´ -GGC GGC AAT CAT CCT CTG-3 ´); Bcl-2 (F-5 ´ -AGG AAG TGA ACA TTT CGG TGA C-3 ´ and R-5 ´ -GCT CAG TTC CAG GAC CAG GC-3 ´); GAP43 (F-5 ´ -TCC GTC GAC ACA TAA CAA -3 ´ and R-5 ´ -CAG TAG TGG TGC CTT CTC C-3 ´); neuron-specific enolase (NSE) (F-5 ´ -TGT CTG CTG CTC AAG GTC AA-3 ´ and R-5 ´ -CGA

TGA CTC ACC ATG ACC C-3´); neurofilimant-H (NFH) (F-5´-CCG ACA TTG CCT CCT ACC-3´ and R-5´-GAG CCA TCT TGA CAT TGA GCA-3´); TrkA (F-5´-TTG GCA TGA GCA GGG ATA TCT-3´ and R-5´-ACG GTA CAG GAT GCT CTC GG-3´).

Western blot analysis

Total proteins were extracted from cells using lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM Na3VO4, and 10% glycerol) with 10% protease inhibitor cocktail. The cells were lysed for 15 min on ice and then spun at 4°C and 13,000 rpm for 15 min. The supernatant was then collected for Western blotting. A Bio-Rad protein assay kit was used to measure protein concentration. Concentration-normalized lysates were boiled at 100°C in an SDS sample buffer for 5 min. Proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (80 volts for 30 min in stacking gel and 120 volts for 1.5 h in running gel) and transferred to nitrocellulose membranes (100 Volts for 60 min).

The membranes were blocked with 5% BSA in TBS-T (0.1% Tween 20 in TBS), followed by overnight incubation at 4°C with appropriate dilutions of primary antibody in 1% TBS-T. After three washes with TBS-T (5 min each), the membranes were incubated with the appropriate secondary antibody coupled with horseradish

peroxidase. Immuno-complexes were visualized using an enhanced chemi-luminescence (ECL) kit according to the manufacturer's instructions.

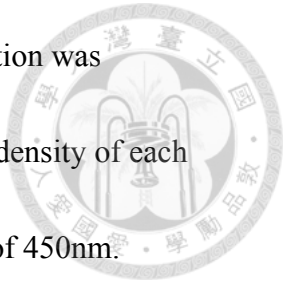
The antibodies used were as follows: rabbit polyclonal anti-CRT antibody (Upstate Biotechnology, Lake Placid, NY), rabbit monoclonal anti-VEGF-A antibody (Santa Cruz, CA, USA), goat polyclonal anti-β-actin (Santa Cruz, CA, USA), and goat monoclonal anti-GAPDH antibody (Genetex, USA).

Enzyme-linked immuno-sorbent assay (ELISA) for VEGF-A secreted proteins in the conditioned medium

The cells were seeded in six-well plates at $3x10^5$/well. The conditioned medium was collected after 48 h of transfection and analyzed by ELISA specific for human VEGF (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. The conditional medium was previously centrifuged to remove cells and other unnecessary particles. Samples and VEGF-A standards were added into micro-plates that were pre-coated with anti-human VEGF-A capture antibody. The micro-plates were incubated at room temperature on a horizontal orbital shaker for 2 hours. After incubation, samples and standards were discarded and washed with wash buffer 4 times.

After aspiration of conjugates and a further wash, the substrate solution was

added to each well, which were incubated for 30 min. After the reaction was completed, stop solution was added to end the reaction. The optical density of each well was measured with an ELISA plate reader set to a wavelength of 450nm.



Cell Proliferation Assay

Cells were seeded in the 96-well plate at density of 10^3/100 μL, 5x10^3/100 μL and 10^4/100 μL respectively. Cells cultured in serum-free medium were used as negative control. After tetracycline induction for 48 hours, MTT reagent (Sigma) was added to each well to a final concentration of 0.05% for reaction. After incubation at 37℃ for 4 hours, MTT-containing medium were removed and 50 μL dimethyl sulfoxide (DMSO) were added for 20 minutes at 37℃ to dissolve formazan. Reactions were monitored by 96-well ELISA plate reader at 595nm.

Apoptosis Detection Assay

10^6 cells were seeded in 10 cm plate. Cells treated with 1 μg/μL actinomycin-D (Sigma, St.Louis, MO) were used as positive control. After tetracycline induction for 24 hours, stNB-V1 cells were harvested and washed by cold PBS twice. Cell apoptosis rate was detected by using fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit (BD, Pharmingen, San Diego, CA). 10^5

cells were suspended in 100 μL of 1 x binding buffer. Harvested cells were then stained by 10 μL of FITC-conjugated Annexin V antibody and propidium iodide for 15 minutes. The stained cells were analyzed by BD FACSCanto2 cell flow cytometry.

VEGFR-1 Blockade

$2x10^5$ stNB-V_1 cells were cultured in 6-well plate. Cells were treated with 1μg/mL goat polyclonal anti-human VEGF R1 antibody to block the VEGF-A signaling. Normal goat IgG was used as the negative control.

Patients and sample Preparation

A cohort of histologically proven NB patients with complete follow-up were enrolled in this study. Tumor samples were obtained during surgery and imeditely frozen in liquid nitrogen. The categorization of tumor histology was based on the International Neuroblastoma Pathology Classification scheme [94].

Immunohistochemical staining

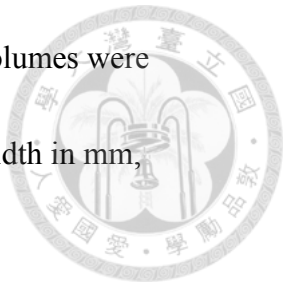
A total of 69 tumor specimens collected before chemotherapy were fixed and embedded in paraffin. The expression of CRT, VEGF-A and CD34 was assayed using an avidin–biotin complex immunoperoxidase staining technique on archival

paraffin-embedded tissue specimens obtained before chemotherapy. Tissue sections (5mm) of tumors were deparaffinized and rehydrated in a routine manner. After microwave pretreatment, the CRT, VEGF-A and CD34 antibodies were then applied at a dilution of 1:150 overnight at 48C. The N-Histofine Simple Stain MAXPO (Nichirei, Tokyo, Japan) was then applied for 30 min at room temperature. Diaminobenzidine was used for visualization and nuclei were counterstained with hematoxylin. One ganglioneuroma tumor with consistent CRT expression by immunohistochemistry was used as a positive control in each staining. Non-immunized rabbit serum was used as a negative control. Tumors with various differentiating histologies were included in each staining. The immunoreactivity of CRT, VEGF-A and CD34 was assessed by a single pathologist who was blinded to the clinical background of the patients.

Mouse xenograft studies with inducible CRT stNB-V1 cells

$5x10^6$ CRT inducible stNB-V1 cells were injected subcutaneously into four-week old female athymic nude mice with matrigel (BD Bioscience). Mice were randomized into two treatment groups. Tumor-bearing mice were treated with doxycycline in their daily drinking water (2g/L) or vehicle alone (sucrose) for 21 days. The growth ability of xenografted tumors on animals was measured according to the metric measurement

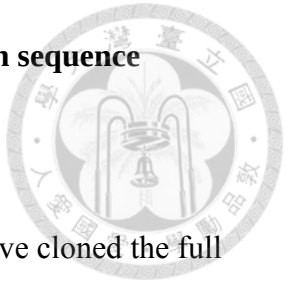
of tumor size. Tumor diameters were measured with calipers, and volumes were calculated as $LxW^2x0.5$, where L and W are the tumor length and width in mm, respectively.



Statistical analysis

The correlation between CRT and VEGF-A mRNA expression level were analyzed using non-parametric Wilcoxon rank-sum test and Spearman's correlation test. Other data analyses were performed using one-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference (LSD) test (StatView; Abacus Concept, Berkeley, CA, USA). Each result was obtained from at least three independent experiments and expressed as mean ± standard deviation. Statistical significance was set at $p<0.05$.

Chapter III.
Results



Cloning and gene analysis of zebrafish CRT (zCRT) showed high sequence homology

To investigate the role of CRT in zebrafish development, we have cloned the full length transcript of zCRT..The zebrafish zCRT are highly homologous to their human and mouse counterparts with protein sequence similarity close to 90% as shown in the calreticulin sequence alignment graph (Fig. 2A). The evolutional distances of zCRT to other species' CRT are shown in the phylogenetic trees (Fig. 2B). To further investigate the genetic conservation of *CRT* in vertebrate including zebrafish, mouse and human, we compared their chromosome location maps from the Ensemble database. The results showed that all *CRT* genes are located near *rad23a*. The gene orientation of *zCRT* is the same as that of mouse *CRT* but is opposite to that of human CRT (Fig. 2C).

Expression profiles of CRT in embryonic development of zebrafish

To evaluate the temporal and spatial expression analysis of *zCRT*, the quantitative RT-PCR (qRT-PCR) was performed. Our studies revealed that zebrafish CRT could be detected very early and reduced gradually during the segmentation and pharyngula stages (Fig. 3). The whole mount in situ hybridization (WISH) analysis of zCRT from ZFIN showed that zCRT was highly expressed before 5 dpf, compatible

with our expression analysis of qRT-PCR. Besides, the spatial analysis from ZIFN revealed that zCRT was expressed mainly in hatching gland, floor plate, chordo-neural hinge and lateral line in embryos. However, in adult fish (older than three months), the zebrafish CRT were detected in all organs by qRT-PCR analyses (Fig. 4).

Knockdown of zCRT protein expression by morpholinos cause an embryonic defect and developmental retardation

Microinjection of morpholinos targeting the ATG start site into embryos effectively abolished the expression of zCRT protein. Knockdown of zCRT expression resulted in severe embryonic lethality within 3 dpf. The surviving morphants showed developmental retardation, such as slow growth, reduced brain size and heart edema compared to the wild type fish and standard control-MO fish (Fig. 5A). The severe embryonic defect of the zCRT knockdown prevented us from studying its impact on locomotive movement. Both heart edema and phenotype change are morpholino concentration-dependent (Fig. 5B).

CRT positively regulated VEGF-A and HIF-1α expressions

To investigate the relationship between CRT and VEGF-A, CRT was

over-expressed using pEGFP-C1-CRT expression vector via Lipofectamine 2000 transfection system in SK-N-DZ and SH-SY5Y cells, which was performed by Kuan-Hung Lin. According to real-time PCR analysis, the expression vector significantly enhanced CRT mRNA expression in SK-N-DZ and SH-SY5Y to 1800- and 1400-folds higher, respectively, compared to the negative control (none) and vector control (pEGFP-C1) (Fig. 6A). This CRT over-expression at the protein level was also confirmed by western blotting (Fig. 6B).

To elucidate the effects of CRT on VEGF-A and HIF-1α, VEGF-A mRNA expression was analyzed in transiently CRT-over-expressing NB cells. The over-expression of CRT increased VEGF-A mRNA expression in both SK-N-DZ and SH-SY5Y cells (Fig. 6C&D). The mRNA expression level of HIF-1α, a well-known up-regulator of VEGFs in NB, also positively correlated with CRT level [95]. Elevated HIF-1α expression suggested that HIF-1α might be involved in the CRT-dependent VEGF-A up-regulation.

To further clarify the relationship between CRT and VEGF-A, CRT was transiently knocked-down using shRNA in SK-N-DZ and SH-SY5Y cells. According to real-time PCR analysis, the CRT mRNA expression levels were significantly inhibited by the pCR3.1-CRT-shRNA in both SK-N-DZ and SH-SY5Y cells (Fig. 7A). The knockdown of CRT was further confirmed at the protein level by western

blotting (Fig. 7B).

To examine the effect of CRT knockdown on VEGF-A and HIF-1α, VEGF-A and HIF-1α mRNA expressions were analyzed in transiently CRT-knocked down NB cells. The VEGF-A and HIF-1α mRNA expression were lower in SK-N-DZ and SH-SY5Y cells with CRT knockdown (Fig. 7C&D). The studies about the CRT knockdown were also performed by Kuan-Hung Lin. The results further confirmed that CRT could regulate VEGF-A and its up-regulator, HIF-1α expression in NB cells.

Effects of CRT on VEGF-A protein expression and secretion level in NB cells

Whether the CRT expression affected the VEGF-A protein expression in NB cells was further determined. Results showed that VEGF-A protein expression was up-regulated by CRT over-expression both in SK-N-DZ and SH-SY5Y cells (Fig. 8A). Moreover, knockdown of CRT decreased the protein expression of VEGF-A (Fig. 8A). The results demonstrated that CRT positively regulated VEGF-A both at the mRNA and protein level in different NB cells.

The VEGF family members, including VEGF-A, are known to be secreted polypeptides. The VEGF-A secretion level was then examined in the conditioned media. Results showed that the CRT over-expression up-regulated VEGF-A secretion in the conditioned media of SK-N-DZ and SH-SY5Y cells (Fig. 8B). On the other

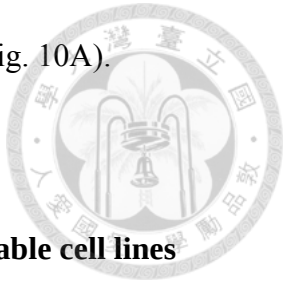
hand, CRT knockdown suppressed the VEGF-A protein secretion in conditioned media (Fig. 8B). These results further supported the effects of CRT on VEGF expression and secretion in different NB cells. The results about the VEGF-A protein expression and secretion level were provided by Kuan-Hung Lin.

Establishment of stable cell lines

In order to further clarify the effects of CRT on NB cell lines, CRT over-expression and knockdown stable cell lines were selected. However, constitutive over-expression of CRT led to NB cell differentiation without proliferation. Thus, kindly provided by Dr. Yung-Feng Liao and Dr. Bo-Jeng Wang, we received an inducible-CRT stNB-V1 cell line by a tetracycline-regulated gene system. To induce CRT expression, cells were treated with tetracycline. After 1 μg/μL tetracycline induction for 24, 48, and 72 h, CRT mRNA expression was elevated by 3-, 6-, and 4-folds higher, respectively, than non-induced cells (Fig. 9A). As such, 48 h induction was chosen as the best condition for subsequent experiments. By western blotting, the protein expression of CRT was also enhanced by tetracycline induction (Fig. 9B).

For knockdown stable cell lines, stNB-V1 NB cells were transfected with a CRT-shRNA plasmid via lentiviral system and then selected by respective antibiotics. After selection by puromycin, CRT-shRNA cells were generated (19989 and 19991)

and CRT knockdown efficiency was confirmed by real-time PCR (Fig. 10A).



CRT positively regulated VEGF-A and HIF-1α expressions in stable cell lines

The expressions of VEGF-A and HIF-1 were examined in inducible-CRT stNB-V1cells. After 48 h of tetracycline treatment, the mRNA expressions of VEGF-A and HIF-1 were significantly augmented (Fig. 9C). The positive regulation of CRT on VEGF-A protein expression was also demonstrated in inducible-CRT stNB-V1cells (Fig. 9B), revealing that CRT positively regulated VEGF-A and HIF-1α expressions in different NB cells.

The VEGF-A and HIF-1α mRNA expression levels were further examined in CRT knockdown stable cell lines (stNB-V1, 19989) and revealed lower mRNA expressions (Fig. 10B). These results further confirmed that CRT regulated the VEGF-A and HIF-1α expression levels in different NB cells.

Apoptotic rate of NB cells was not affected by CRT over-expression

To clarify the impact of CRT over-expression on NB cell apoptosis, we use Annexin V/propidium iodide staining to determine the apoptotic cells in inducible-CRT stNB-V1 cells. Actinomycin-D, a potent inducer for apoptosis in several cell lines, was used as positive control. After tetracycline treatment, the

fluorescence intensity of apoptotic cells was not different from cells without tetracycline treatment (Fig. 11A), which indicated that CRT over-expression did not affect apoptosis in stNB-V1 cells. The mRNA expression levels in several apoptotic markers including p53, Bcl-2 and Bax were further determined by real-time PCR [93]. As shown in Figure 11B, no difference in p53, Bcl-2 and Bax mRNA expressions was observed after tetracycline induction in inducible-CRT stNB-V1 cells. These results further confirmed that over-expression of CRT did not induce NB cell apoptosis.

Cell proliferation rate is lower in CRT over-expressing NB cells

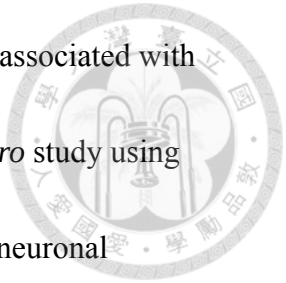
We previously found that constitutive over-expression of CRT led to NB cell differentiation without proliferation in SK-N-DZ and SH-SY5Y cells. Thus, we have generated an inducible-CRT stNB-V1 cell line. In order to further confirm the effect of CRT over-expression on cell proliferation, we used MTT assays to evaluate the cell proliferation rate in stNB-V1 cells. Cells cultured in serum-free medium were used as negative control. After tetracycline induction, the MTT readings were significantly decreased (Fig. 12), indicating that up-regulation of CRT suppressed proliferation of NB cells.

VEGF-A is involved in CRT-induced NB cell differentiation

To further clarify the roles of CRT-dependent VEGF-A up-regulation on NB cells differentiation, the expressions of neuron specific markers including GAP43, neuron-specific enolase 2 (NSE2) and neurofilimant-H (NFH) as well as TrkA, a molecular marker indicative of NB cell differentiation, were examined in inducible-CRT stNB-V1cells [96-98]. After tetracycline induction, the mRNA expression levels of GAP43, NSE, NFH and TrkA were significantly augmented (Fig. 13), which is compatible with our previous study suggesting that CRT is critical for NB differentiation.

We next used anti-VEGFR-1 antibodies to examine whether up-regulated VEGF-A is involved in CRT-related neuronal differentiation in NB. VEGF-A binds to two related receptor tyrosine kinases, VEGFR-1 and VEGFR-2, and regulates downstream biological signaling. Antibodies against VEGFR-1 could partially block the VEGF-A signaling. As shown in Fig. 13, after treatment with anti-VEGFR-1 antibodies, the mRNA expressions of GAP43, NSE2, NFH and TrkA were suppressed significantly in both CRT over-expression (treated with tetracycline) and controlled (treated with ddH2O) stNB-V1 cells. These results implied the involvement of VEGF-A in neuronal differentiation in NB.

CRT expression suppressed NB tumor growth in mice xenograft model



In previous studies, increased CRT expression was found to be associated with differentiated histologies in NB tumors. Moreover, our present *in vitro* study using NB cells indicated that CRT could up-regulate VEGF-A and induce neuronal differentiation in NB cells. To further investigate the roles of CRT-dependent VEGF-A up-regulation on NB tumor growth, we established mice xenograft models of NB using inducible-CRT stNB-V1 cells transplantation. To induce CRT expression, mice were treated with doxycycline in their daily drinking water after tumor cells injection. During daily doxycycline treatment, the tumor growth was significantly suppressed compared to xenograft mice without doxycycline treatment (Fig. 14). These results further suggested the essential role of CRT in the tumor progression of NB.

VEGF-A is upregulated in CRT-induced NB cell differentiation in mice xenograft model

Our *in vitro* study using NB cells indicated that CRT could up-regulate VEGF-A and induce neuronal differentiation in NB cells. In order to confirm the effect of CRT *in vivo*, we have established the mice xenograft models. Tumor inoculated mice were treated with doxycycline in their daily drinking water (2g/L) to induce CRT expression. Tumor inoculated mice were sacrificed after 21-days doxycycline treatment for CRT induction and the tumor was removed for experiments. The mRNA

expression levels of VEGF-A and HIF-1α were significantly increased, suggesting the positive regulation of CRT on VEGF-A in xenograft NB tumors (Fig. 15). To further confirm the roles of CRT-dependent VEGF-A up-regulation on NB cell differentiation *in vivo*, the expressions of neuro-specific markers, GAP43, was examined. After doxycycline induction, the mRNA expression level of GAP43 was significantly augmented (Fig. 15), which is compatible with our *in vitro* study suggesting that CRT is critical for NB differentiation.

VEGF-A expression was positively correlated with CRT and differentiated histology in human NB tumors

Both our *in vivo* and *in vitro* studies have demonstrated the positive regulation of CRT on VEGF-A. To further verify the correlation between CRT and VEGF-A, the expressions of CRT and VEGF-A in 56 primary NB tumor were determined by real-time PCR. The results revealed a significantly positive correlation between CRT and VEGF-A (Fig. 16, Spearman's $\rho = 0.648$, $p < 0.001$). Furthermore, from 31 primary NB tumors analysis, we found that differentiated NB exhibited higher CRT and VEGF-A expression levels than undifferentiated NB (Fig. 17A). The CRT and VEGF-A expression were significantly increased in differentiated NB (Fig 17B&C). These results further supported the critical roles of CRT and VEGF-A in neuronal

differentiation in NB tumorigenesis.

VEGF-A expression and clinicopathologic and biological factors

In order to investigate the clinical significance of VEGF-A as well as its relationship with CRT and other clinicopathologic factors in NB, we examined the protein expression of CRT, VEGF-A and CD34 in 69 NB tumors by immunohistochemical staining. Both CRT and VEGF-A were commonly found in more differentiated NB than in undifferentiated NB (Fig. 18). Our immunohistochemical data showed that a positive expression of VEGF-A could be detected in 27 of 69 NB tumors. Positive VEGF-A protein expression was significantly associated with favorable biomarker of CRT expression ($P = 0.001$). Furthermore, we found that positive VEGF-A expression was significantly correlated to differentiated tumor histology ($P = 0.013$). There was also an inverse association between VEGF-A expression and MYCN amplification ($P = 0.034$). Interestingly, there was no correlation between the protein expressions of VEGF-A and CD34, a marker of endothelial cells ($P = 0.808$). We found that CD34, but not VEGF-A, is highly expressed in undifferentiated NB (Fig. 18). Other adverse prognostic factors, including older patient age at diagnosis and advanced clinical stage were not associated with VEGF-A expression in our study. The relationship between VEGF-A

protein expression and the clinicopathologic and biological variables of NB tumors was summarized in Table 1.

VEGF-A expression in NB tumors predicts a favorable outcome

To further clarify the relationship between the expression of VEGF-A and clinical outcome, Kaplan-Meier analysis was used to predict the five-year survival rate. The survival analysis revealed that patients with positive VEGF-A expression have a significantly higher survival rate compared to patients with negative VEGF-A expression (Fig 19; $P = 0.013$, Log-rank test), suggesting that positive VEGF-A expression could predict a favorable outcome in NB patients.

Chapter IV. Discussions

The tumorigenesis of NB could be a divergence of the embryonic development of sympathetic nervous system. ER chaperones including CRT and GRP78 are suggested to participate during embryonic development in our previous review. CRT, a well-known multi-functional protein, is located not only in the cytosol but also on the cell surface and in the extracellular environment to modulate a number of physiologic and pathologic conditions [7, 16, 63]. Here, we found CRT is highly conserved across species, with an amino acid identity of approximately 90% from the invertebrate to mouse and human [7]. In the human and mouse genomes, CRT is known to be a single-copy gene [10]. In zebrafish, CRT also appears to be a single copy gene. The high sequence homology implies that CRT may be functionally conserved in zebrafish.

Studies in CRT knockout mice which demonstrated the essential role of CRT for embryogenesis have suggested the important role of CRT in nervous system development as well as the biology of NB [69]. Our temporal and spatial analysis of CRT expression in zebrafish found that CRT is highly expressed in early embryonic development and is mainly in hatching gland, floor plate, chordo-neural hinge and lateral line in embryos, supporting the crucial role of CRT in neuronal development. Furthermore, our study used anti-sense morpholino oligonucleotide to knock down of zCRT protein expression at different levels. The results showed a

concentration-dependent phenotype change including slow growth, reduced brain size and heart edema with increasing lethality. These results confirmed that CRT is essential for embryonic and neuronal development.

. In some cancers, CRT is found to be up-regulated in tumor tissues compared to normal tissues [21]. Our previous studies have also found that increased CRT expression is associated with tumorigenesis and metastasis of gastric cancer and bladder cancer [15, 28]. Conversely, in NB, increased CRT expression is associated with better prognosis and differentiated histologies [42]. The present study demonstrates that constitutive over-expression of CRT in different NB cells lead to NB cell differentiation without proliferation and make it difficult to establish stable cell lines. It has been found that CRT is essential for neurite formation when NB cells are induced to differentiate [62, 63]. Available evidence is consistent with the results here showing that CRT is critical for NB differentiation. Thus, an inducible-CRT cell line via tetracycline-regulated gene system is necessary as a stable cell line for further experiments.

Converging evidence showed that NB cells exhibit a capacity of differentiating into neuron-like cells or regression by apoptosis [49, 50]. In previous studies, increased CRT expression was found to be associated with differentiation histology in NB [64]. The present studies clearly demonstrated that CRT could suppress cell

proliferation and enhance cell differentiation whereas apoptosis was not altered in NB cells, implying CRT as an important favorable prognostic factor in NB. Our *in vivo* study using mice xenograft model further proved that CRT enhanced neuronal differentiation and suppressed tumor growth in NB. Furthermore, we also confirmed that the human NB tumors exhibit higher CRT expression in differentiated NB compared to undifferentiated NB. These results were compatible with our previous study revealing that CRT expression correlates with differentiation of NB and predicts favorable survival in NB patients [42].

Studies have shown that CRT can regulate VEGF (also referred to as VEGF-A) expression in gastric cancers [28]. The present studies in NB also demonstrate that CRT can regulate VEGF-A expression. VEGF-A is a well-recognized pro-angiogenic factor and a key regulator of physiologic and pathologic angiogenesis [70]. In gastric cancers, CRT has been found to up-regulate VEGF expression and enhance angiogenesis, leading to poor prognosis [28]. However, it has also been reported that exogenous CRT and the CRT fragment vasostatin are anti-angiogenic factors that directly target endothelial cells and suppress tumor growth [90, 91]. The role of CRT in angiogenesis remains elusive. In this study, CRT over-expression increased HIF-1α expression with subsequent up-regulation of VEGF-A in three different NB cells as well as in mice xenograft models. Knockdown of CRT suppressed HIF-1α expression

and downstream VEGF-A transcription, translation, and further secretion. Moreover, it has been previously shown that increased CRT expression is associated with favorable outcome in NB patients. Therefore, it is postulated that the CRT-up-regulated VEGF-A expression does not necessarily promote angiogenesis in NB. The effect of CRT on angiogenesis may depend on different cell types and stages. Further investigation is warranted to clarify the correlation between CRT and angiogenesis in NB.

RNA stability which is regulated through interaction between specific sequences (*cis*-acting elements) in 3′-UTR of mRNA and specialized RNA-binding proteins (*trans*-actin factor), is a key regulator of gene expression [99, 100]. Previous studies have suggested the role of CRT in regulating RNA stability. In 2002, Nickenig *et al.* first indicated CRT as a novel mRNA binding protein that destabilizes type I angiotensin II receptor mRNA by binding to AU-rich region in 3′-UTR [101]. Totary-Jain *et al*. also reported that CRT also binds to specific element in 3′-UTR of glucose transporter-1 mRNA and destabilizes the mRNA under high-glucose conditions [102]. However, other studies showed that CRT could stabilize mRNA via direct or indirect interaction [103-106]. There are three classes of mRNA according to the characteristics of *cis*-acting elements and the most common one is AU-rich element (ARE) identified in 3′-UTR. Here we showed that CRT could positively

regulate both mRNA and protein expression of VEGF-A *in vivo* and *in vitro*. In addition, we have demonstrated a significantly positive correlation between CRT and VEGF-A mRNA and protein expression in human NB tumors. From the ARE database (http://brp.kfshrc.edu.sa/ARED/), VEGF-A is confirmed to have an ARE on the 3′-UTR region of its mRNA, suggesting that ARE element is important for regulating VEGG-A mRNA stability [107]. Consequently, we can postulate that CRT may regulate VEGF-A expression by influencing VEGF-A mRNA stability in ARE region. Further investigation is needed to verify the mechanism about the regulation of VEGF-A by CRT.

In NB, angiogenesis is an essential mechanism regulating NB tumorigenesis, while VEGF-A-driven angiogenesis plays a critical role in the pathogenesis of NB formation and metastasis [83-85]. Studies have revealed that the high vascular index has been shown to correlate with the adverse prognosis of NB patients, suggesting a close relationship between aggressive tumor behavior and active angiogenesis [77, 78]. However, another study reveals that tumor vascularity is not correlated with prognosis and tumor stage in NB patients [79]. It has also been shown that there is no tumor growth difference in NB xenograft mice treated with angiogenesis inhibitor [80]. The anti-angiogenic therapies in NB also revealed limited and modest success [47, 108]. As such, the role of VEGF-A-driven angiogenesis in NB tumorigenesis and

outcome is complicated and needs further investigation. In the present study, VEGF-A is shown to be involved in CRT-related neuronal differentiation of NB both *in vivo* and *in vitro*. The VEGF-A expression was also increased in differentiated NB of patients. Taken together, it can be surmised that VEGF-A-mediated angiogenesis may play a role in the differentiation and maturation of NB [64]. In a recent study, investigators explored the role of angiogenesis in the maturation phase of NB [81] and found VEGF expression in differentiating neuroblastic cells of patients as well as in SH-SY5Y cells during differentiation after retinoic acid treatment. These findings are consistent with the results here. VEGF-A-associated angiogenesis is not only associated with tumor aggressiveness but also plays an essential role for NB differentiation and maturation, which is similar to the physiologic events bringing about the maturation of the vasculature in normal neuronal development. Further studies about the relationship between angiogenesis and NB differentiation will help to clarify the role of angiogenesis in NB differentiation and maturation.

HIF-1α is a major positive regulator of VEGF-A and is activated during tissue hypoxia. Previous studies have revealed that HIF-1α up-regulation is associated with tumor aggressiveness [109]. In our study, for the first time, we demonstrated that CRT could also up-regulate HIF-1α and we postulated that CRT up-regulating VEGF-A might be HIF-1α-dependent. HIF-1α may also play a crucial role during the

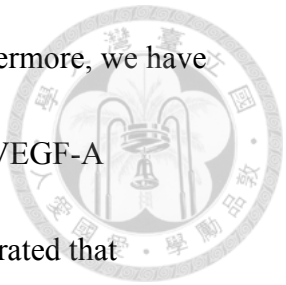
differentiation of NB by itself or via angiogenesis activated by VEGF-A. Further study is warranted to clarify the role of HIF-1α in NB cell differentiation.

Studies in the role of VEGF-A in NB tumorigenesis have unveiled inconsistent results. Both in human and in experimental NB, over-expression of VEGF-A has been demonstrated and correlated with a high-risk phenotype [86, 87, 110]. In contrast, recent studies have found that VEGF-A is not related to tumor progression and metastasis in NB [88]. Moreover, it has been shown that there is no tumor growth difference in NB xenograft mice treated with or without Anti-VEGF antibody [89]. In our study, for the first time, we demonstrated that VEGF-A expression predicts a favorable outcome in NB patients. Furthermore, we also found that positive VEGF-A expression was significantly correlated to differentiated histology and was inversely correlated with MYCN amplification, both of which are favorable prognostic factors. Interestingly, there was no correlation between the expression of VEGF-A and CD34, a marker of endothelial cells, suggesting a novel mechanism of VEGF-A participating in NB formation through angiogenesis-independent pathway. Apart from its major role in angiogenesis, VEGF-A also has a direct effect on neurons and modulates various neuronal functions, including neuronal proliferation, migration, survival, axon guidance, and differentiation [111, 112]. Studies, both *in vitro* and *in vivo*, have shown that VEGF up-regulation can promote neuronal differentiation, while VEGF

down-regulation inhibits neuronal differentiation in stroke and hypoxia models [113-116]. In the present study, partial blockage of VEGF-A signaling via anti-VEGFR-1 antibodies significantly suppressed the expressions of GAP43, NSE2, NFH and TrkA, which are molecular markers indicative of neuronal differentiation in NB. These results clearly demonstrated the involvement of VEGF-A in neuronal differentiation in NB. Thus, based on current studies, CRT-dependent VEGF-A up-regulation is critical for NB differentiation. Besides, VEGF-A may participate in the CRT-mediated NB differentiation via both angiogenesis-dependent and angiogenesis-independent pathways and predicts favorable survival in NB patients.

Chapter V.
Concluding remarks and future perspectives

Recent advances in understanding of the genetics and biology of NB have allowed risk-adapted therapeutic strategies [46, 117]. Previous study has identified CRT as an independent favorable prognostic marker which is related to differentiated histologies in NB. Our study in zebrafish revealed that CRT is essential for embryonic and neuronal development. This study was designed to determine whether the CRT expression in NB was associated with the VEGF-A pathway and to determine the role of VEGF-A in regulating NB behavior focusing on angiogenesis and neuronal differentiation *in vitro* and *in vivo*. In the present study, an inducible-CRT NB cell line is established for further experiments because constitutive CRT over-expression leads to NB differentiation without proliferation. Our previous and present studies clearly demonstrates that CRT over-expression increases the expression and secretion of VEGF-A and HIF-1α, a major positive regulator of VEGF-A in NB cells. In contrast, knockdown of CRT decreases VEGF-A and HIF-1α expression. Furthermore, we confirmed that NB cell apoptosis was not affected by CRT over-expression, while over-expression of CRT suppressed cell proliferation and enhanced cell differentiation in NB. Blockage of VEGF-A signaling markedly suppressed the expressions of GAP43, NSE, NFH and TrkA, indicating an essential role of VEGF-A in CRT-related neuronal differentiation in NB. The mice xenograft models further confirmed the positive regulation of CRT on VEGF-A, as well as the role of CRT in enhancing



neuronal differentiation and suppressing tumor growth in NB. Furthermore, we have demonstrated a significantly positive correlation between CRT and VEGF-A expression in human NB tumors. Most important of all, we demonstrated that VEGF-A expression predicts a favorable outcome in NB patients and are associated with differentiated histology and normal MYCN expression, both of which are favorable prognostic factors. On the other hand, there was no correlation between the expression of VEGF-A and CD34.

In conclusion, our study indicated that VEGF-A participates in CRT-related neuronal differentiation in NB with summary of schematic illustration in Fig. 20. For the first time, we have demonstrated that both CRT and VEGF-A are independent prognostic factors and predicts favorable outcomes in NB patients with tight relationship with differentiated histology and MYCN status. Our findings also delineate a novel mechanism of VEGF-A in the biology of NB. This study provides crucial information needed for developing a new therapeutic strategy in NB patients. Further studies are warranted to elucidate several unresolved issues. First, if CRT regulates VEGF-A expression by influencing VEGF-A mRNA stability in ARE region or through another pathway needs further investigation. Second, the relationship between CRT and angiogenesis in NB tumors remains obscure. Third, the association between angiogenesis and differentiation in NB tumors is also unclear. Fourth,

previous studies had suggested anti-VEGF antibody for possible treatment of NB. However, according to our present study, we postulate that anti-VEGF antibody is not suitable in the treatment of NB, which needs further investigation. Finally, the associated gene changes and molecular mechanism during the processes of VEGF-A involved neuronal differentiation in NB needs to be determined. To decipher the crucial role of CRT and VEGF on the regulation of NB differentiation will shed light to a novel therapeutic strategy to improve the outcome of NB patients in the future.

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Tables

Table 1. VEGF-A expression and clinicopathologic and biologic characteristics of neuroblastomas.

	Cases	Positive VEGF expression (%)	*P* value*
Age at diagnosis			
≤ 1.5 year	22	11 (50.0)	0.290
> 1.5 year	47	16 (34.0)	
Sex			
Male	37	13 (35.1)	0.621
Female	32	14 (43.8)	
Clinical stage			
1, 2, 4S	24	13 (54.2)	0.075†
3, 4	45	14 (31.1)	
Histology			
Undifferentiated	34	8 (23.5)	0.013
Differentiated	35	19 (54.3)	
MYCN			
Amplified	15	2 (13.3)	0.034
Non-amplified	54	25 (46.3)	
CRT expression			
Positive	31	19 (61.3)	0.001
Negative	38	8 (21.1)	
CD34			
High	35	13 (37.1)	0.808
Low	34	14 (41.2)	

*Chi-square test
†Stages 1, 2, and 4S versus stages 3 and 4

Figures

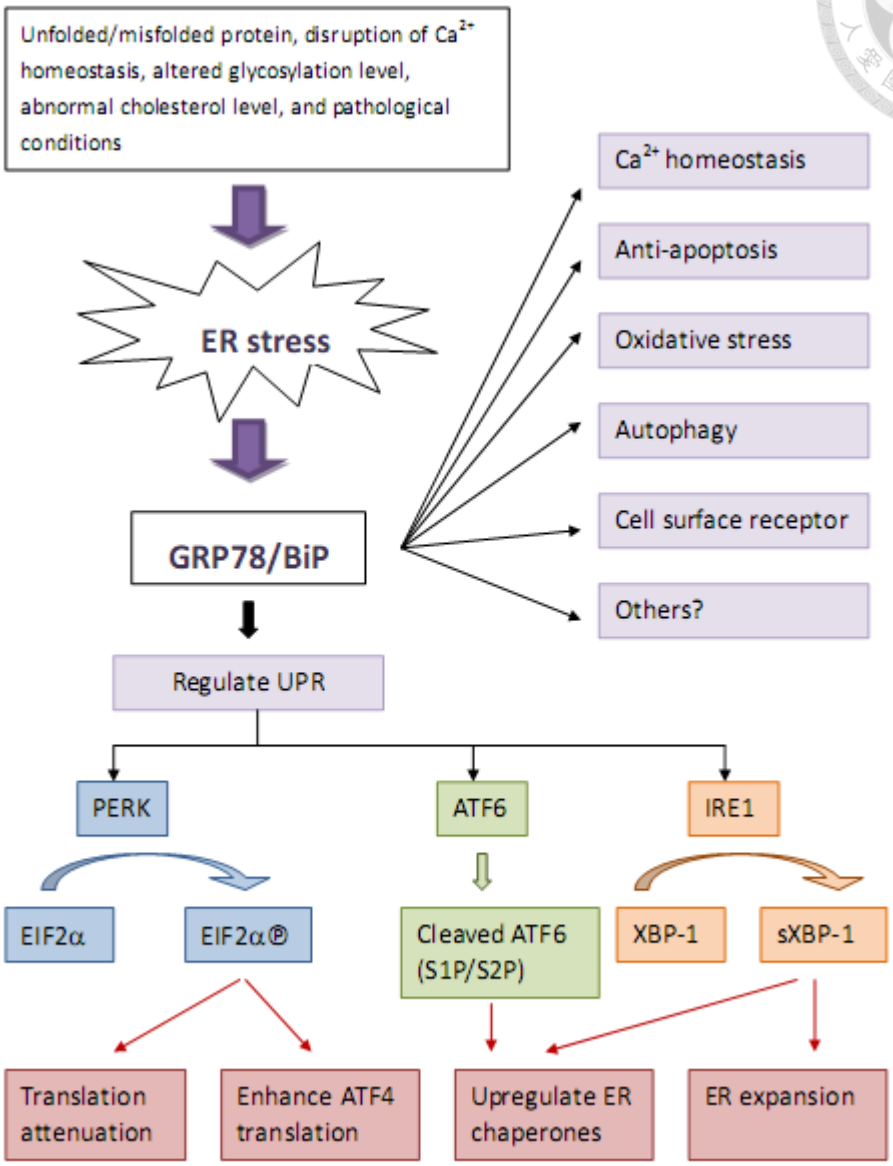

Figure 1. Summary of physiological functions of glucose-regulated protein 78.

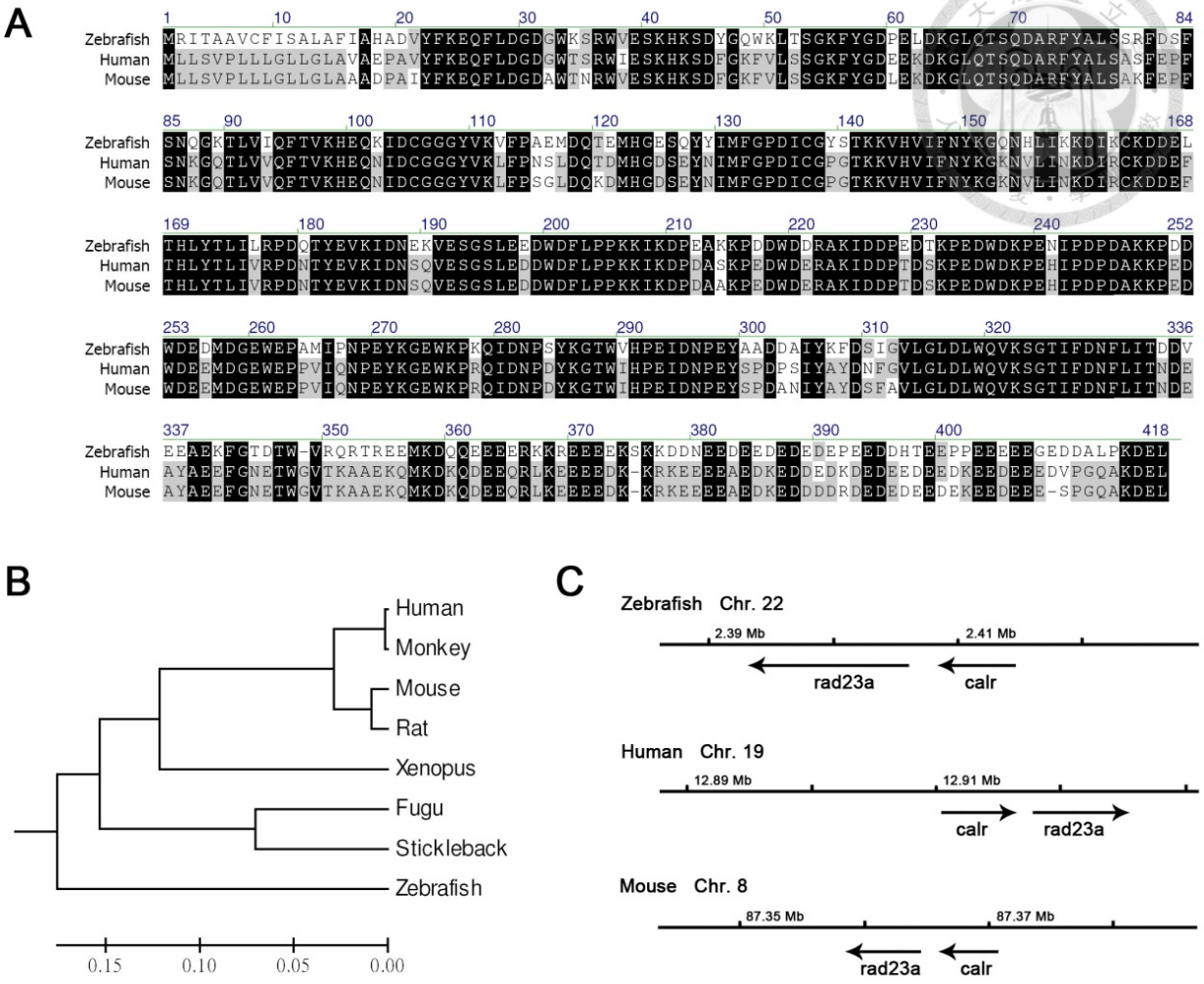

Figure 2**. Sequence conservation analysis of zebrafish CRT.**

(A) protein sequence alignments of CRT in the zebrafish, human and mouse. Identical amino acids across all and some species are marked in black and gray respectively. (B)Phylogenetic tree analysis of CRT. The horizontal length indicates the estimated time that the sequence diverged from related family members. (C) Chromosomal map of CRT in zebrafish, human and mouse. The orientation and comparable length of each gene are shown by arrows. Gene neighboring CRT are also shown. Chr, chromosome; Mb, megabase.

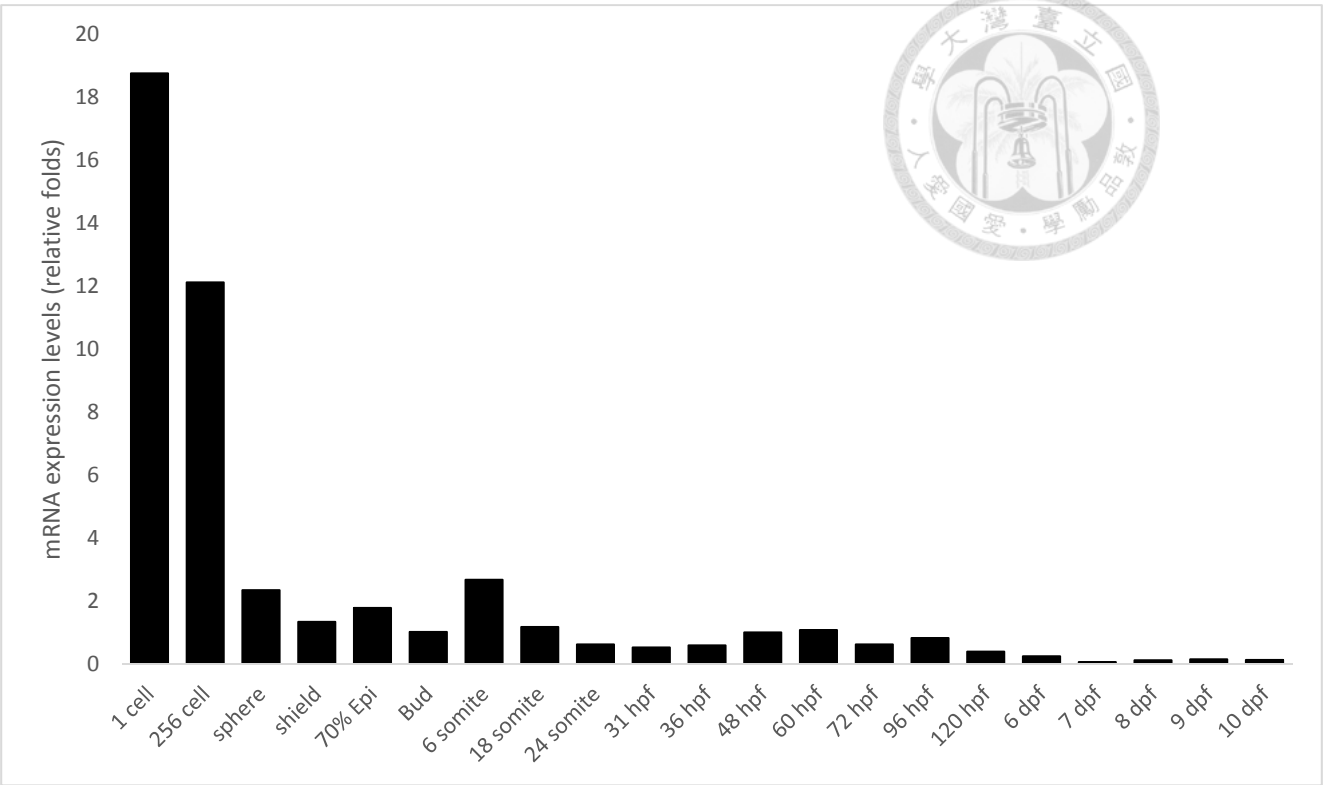

Figure 3. Temporal expression pattern of CRT in developing zebrafish embryos.

The mRNA expression level of zebrafish CRT appeared in 1-cells-stage embryos and was remarkably decreased during the segmentation and pharyngula stages. The zebrafish CRT mRNA was normalized to the internal control Actin.

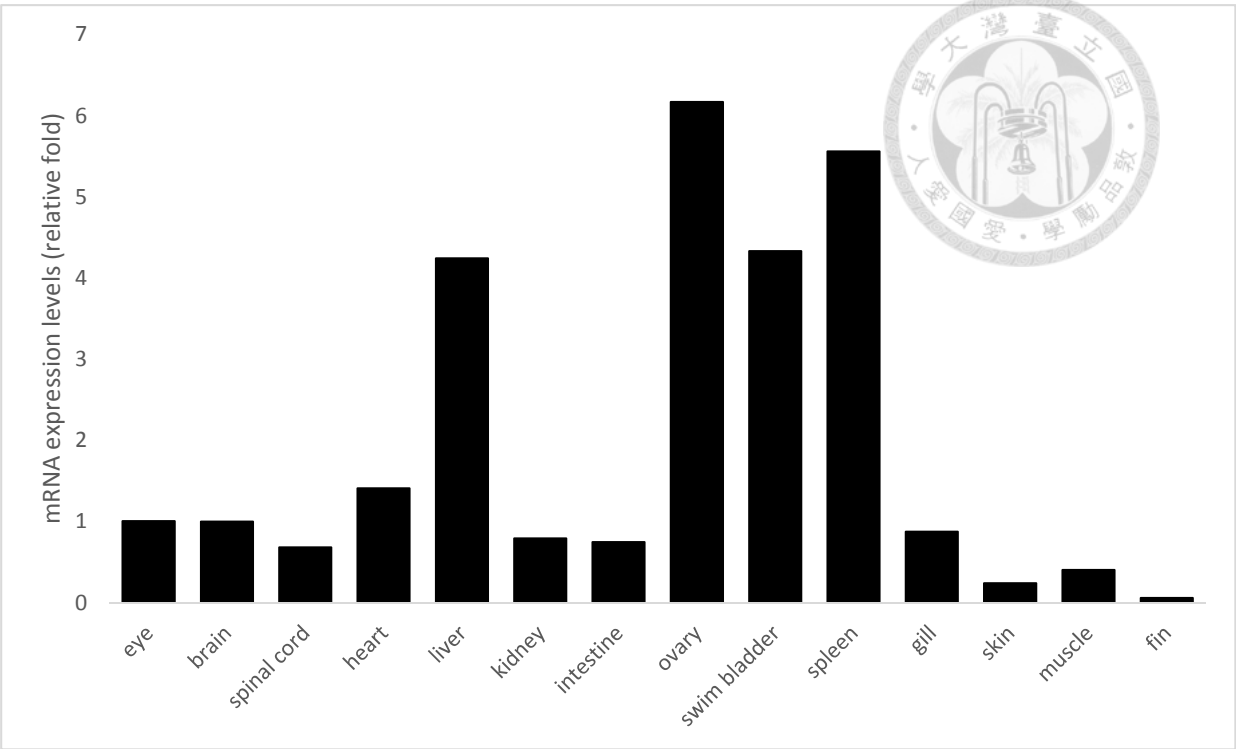

Figure 4. Spatial expression patterns of CRT in adult fish.

The mRNA expression level of zebrafish CRT appeared in all adult tissues. The zebrafish CRT mRNA was normalized to the internal control Actin.

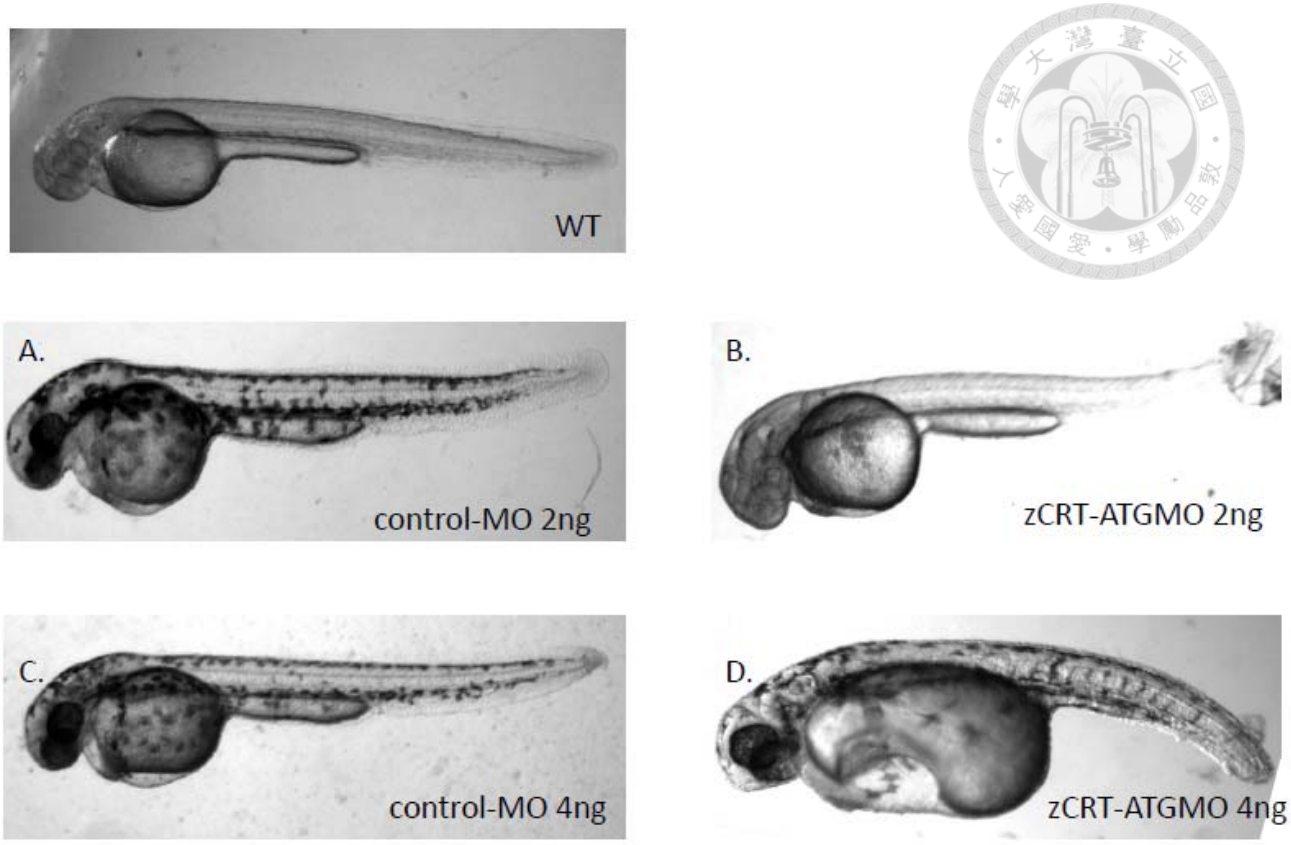

Figure 5. The zCRT-ATGMO morphants at 2dpf showed severe developmental retardation with concentration-dependent effects.

(B) (D) Slow growth, decreased brain size and heart edema were observed in the morphants. A&B: dosage of MO: 2 ng; C&D: dosage of MO: 4 ng

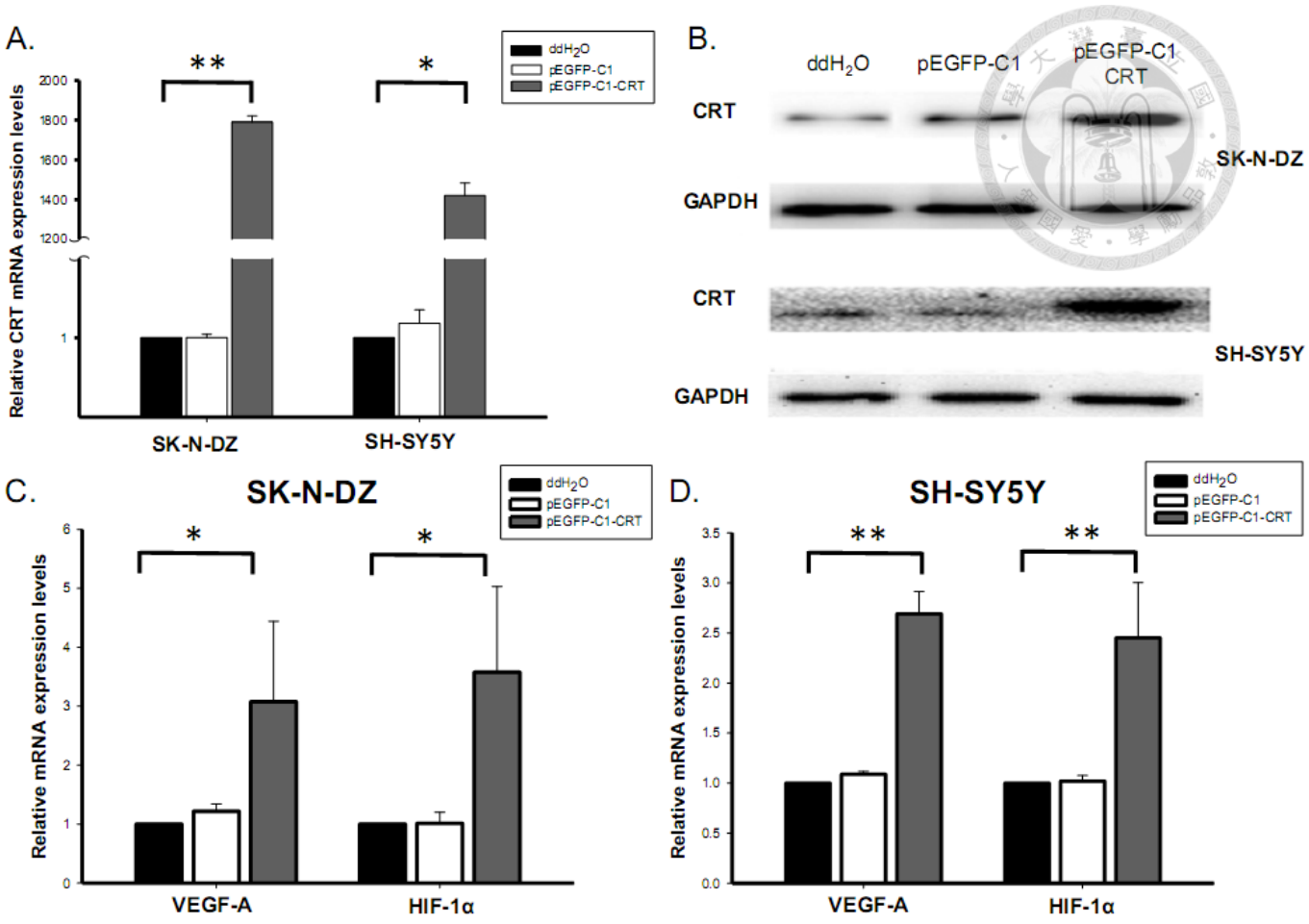

Figure 6. CRT over-expression up-regulated VEGF-A and HIF-1α in SK-N-DZ and SH-SY5Y NB cells.

CRT was over-expressed via Lipofectamine 2000 transfection in SK-N-DZ and SH-SY5Y cells. The CRT mRNA expression level was confirmed by real-time PCR and CRT mRNA was normalized to the internal control GAPDH. **(B)** Western blot analysis showed CRT protein expression in SK-N-DZ and SH-SY5Y cells. The human GAPDH level was used as a loading control. **(C)** The mRNA expression of VEGF-A and HIF-1α were up-related both in SK-N-DZ and SH-SY5Y cells with

CRT over-expression. These results were confirmed by at least three independent experiments. Each bar of the histogram represents quantified results and shown as the mean ± SD. Statistical differences were compared with the control level. *p<0.05; **p<0.01; ***p<0.005

(These results were provided by Kuan-Hung Lin)

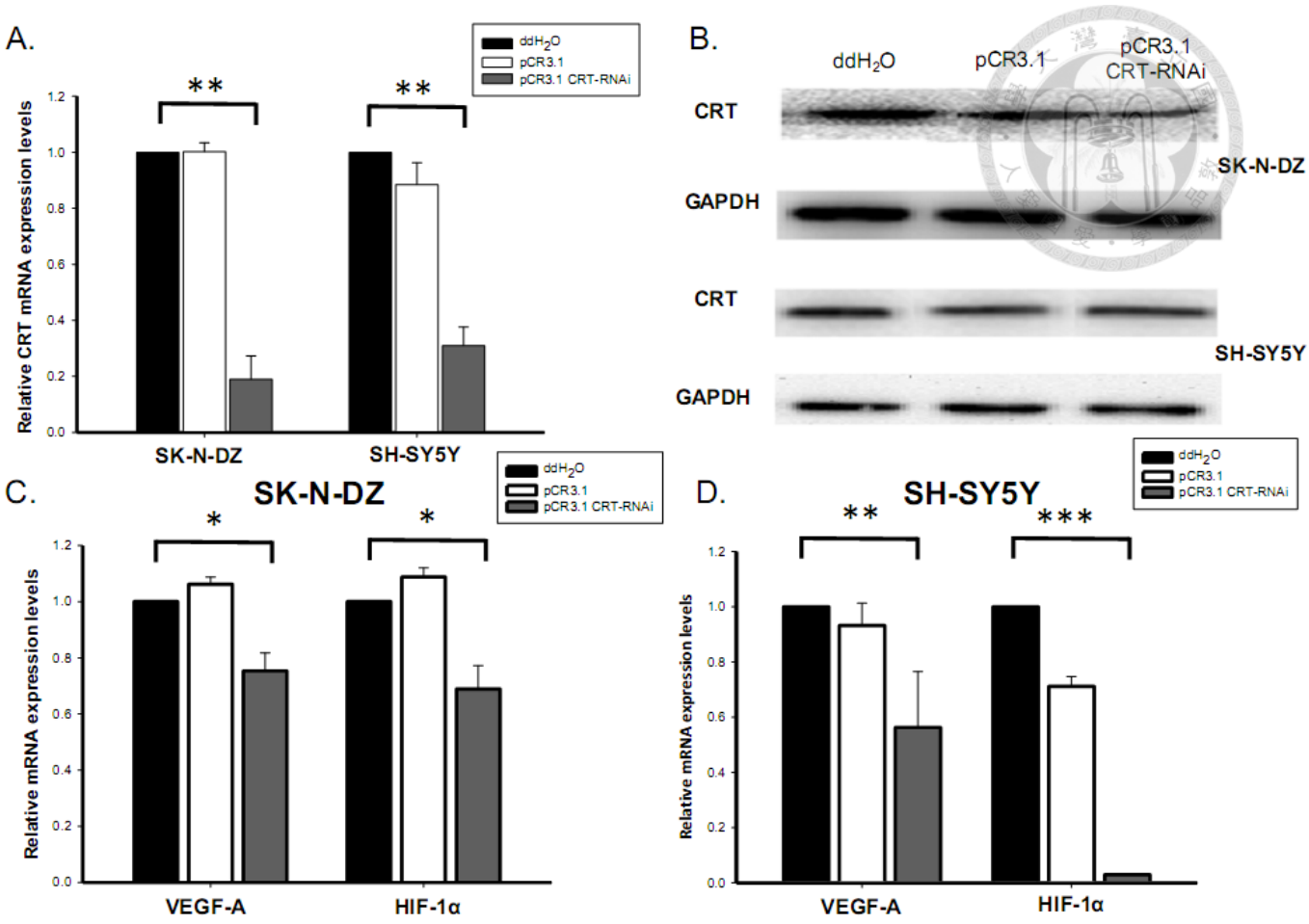

Figure 7. Knockdown of CRT down-regulated VEGF-A and HIF-1α in SK-N-DZ and SH-SY5Y NB cells.

(A) CRT was knocked down via Lipofectamine 2000 transfection in SK-N-DZ and SH-SY5Y cells. The CRT mRNA expression level was confirmed by real-time PCR and CRT mRNA was normalized to the internal control GAPDH. **(B)** Western blot analysis showed CRT protein expression in SK-N-DZ and SH-SY5Y cells. The human GAPDH level was used as a loading control. **(C)** The mRNA expression of VEGF-A and HIF-1α were down-regulated both in SK-N-DZ and SH-SY5Y cells with CRT knockdown. These results were confirmed by at least three independent

experiments. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared with the control level.

*p<0.05; **p<0.01; ***p<0.005

(These results were provided by Kuan-Hung Lin)

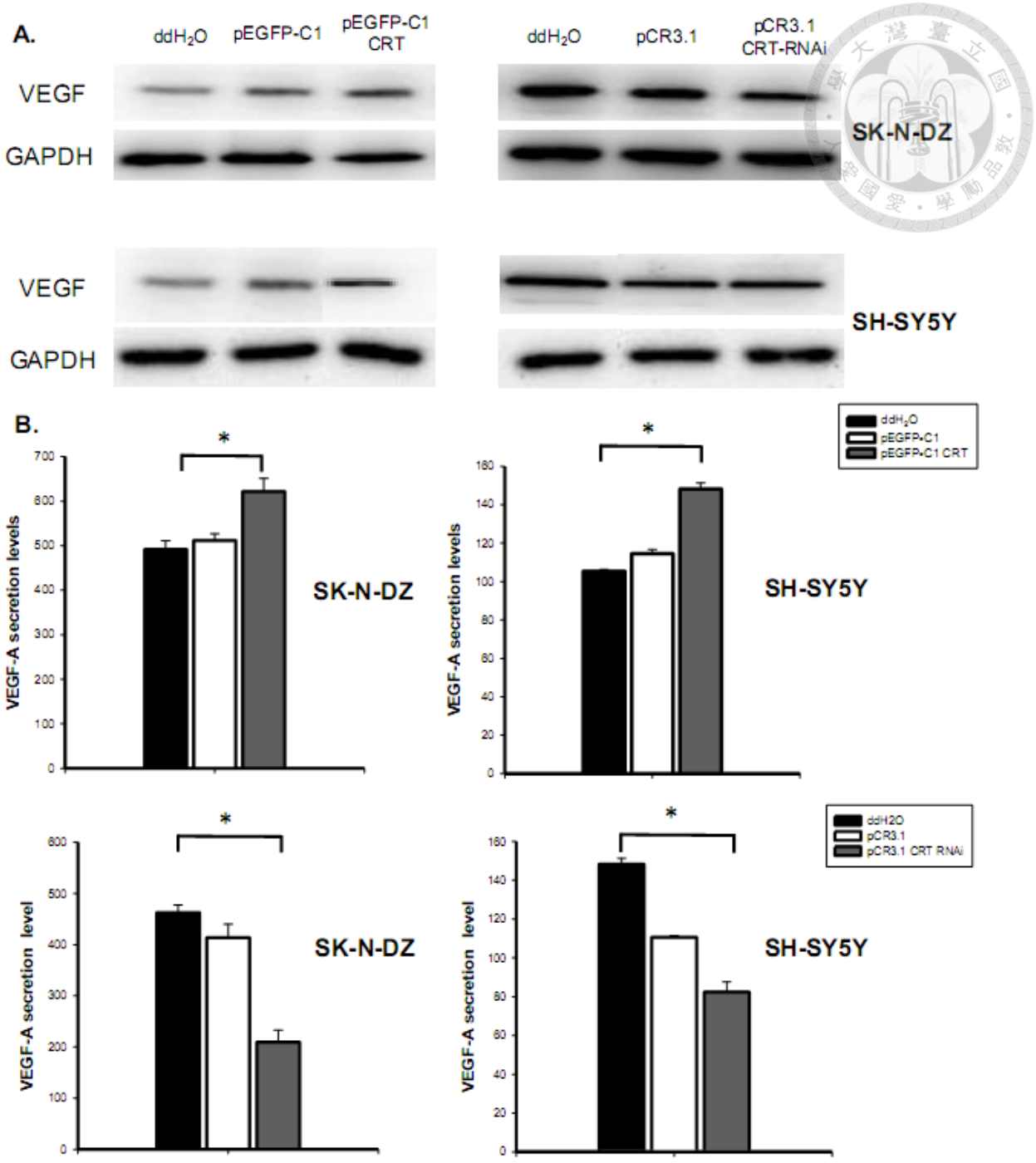

Figure 8. VEGF-A protein expression and secretion level were up-regulated in SK-N-DZ and SH-SY5Y NB cells with CRT over-expression, but decreased by the knockdown of CRT in SK-N-DZ and SH-SY5Y NB cells.

(A) Western blot analysis showed VEGF-A protein expression in SK-N-DZ and SH-SY5Y cells. The human actin level was used as a loading control. In both cell lines, VEGF-A was up-regulated in pEGFP-C1 CRT cells and down-regulated in pCR3.1 CRT-RNAi cells. **(B)** VEGF-A secretion level in the conditioned media was higher by CRT over-expression and lower by CRT knockdown, as analyzed by ELISA. All of the results were repeated in at least three independent experiments. Statistical differences were compared with the control level. *$p<0.05$

(These results were provided by Kuan-Hung Lin)

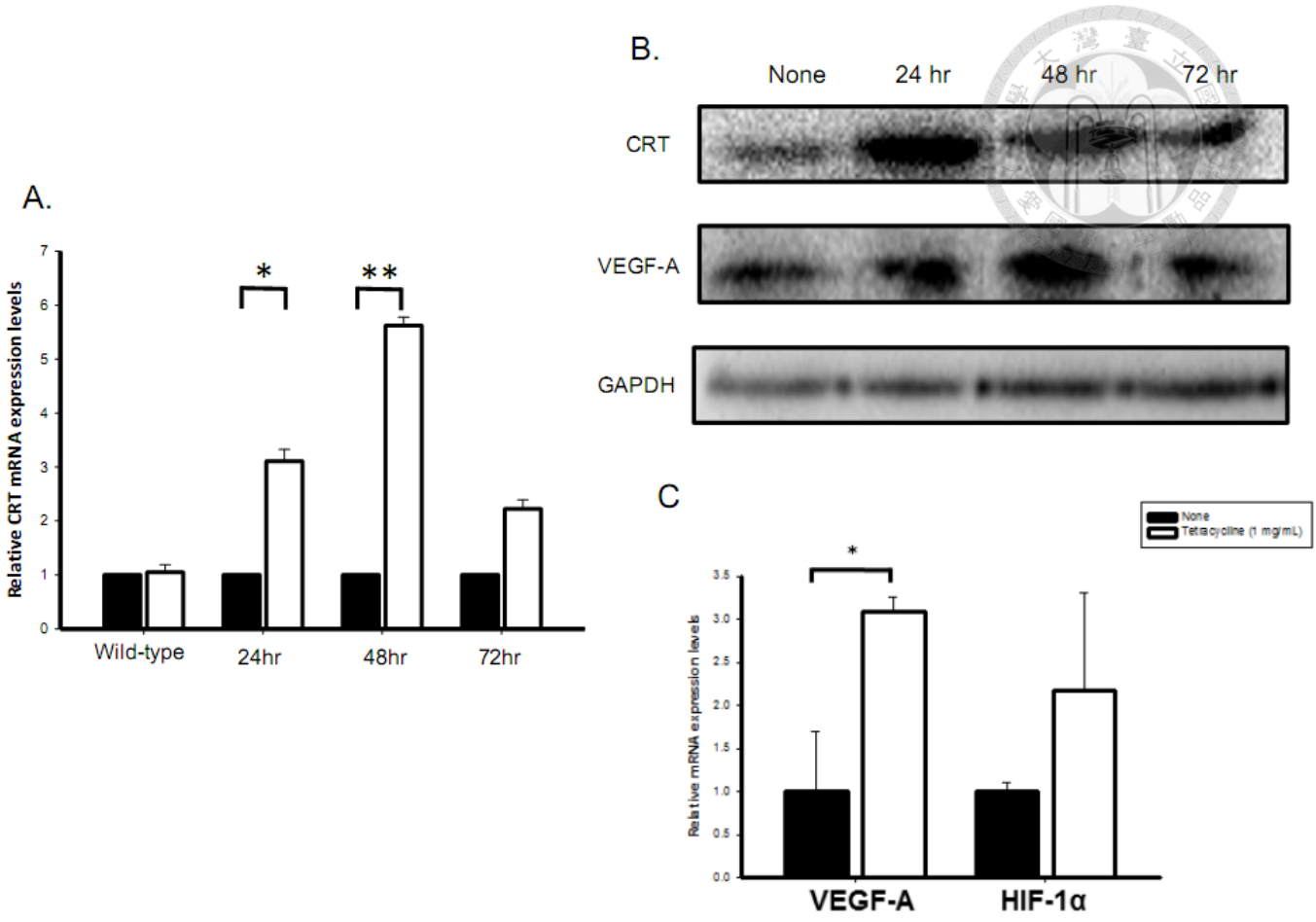

Figure 9. The VEGF-A and HIF-1α expressions were regulated by CRT expression induced by tetracycline treatment in stNB-V1 cells.

(A) stNB-V1 NB cells were stimulated with 1 μg/ml tetracycline to induce CRT expression. The CRT mRNA expression level was normalized to the internal control HSP60 and was highest 48 h after tetracycline treatment. **(B)** The CRT protein expression after tetracycline-induced was also confirmed by western blot analysis. The human GAPDH level was used as a loading control. **(C)** stNB-V1 NB cells were stimulated with 1 μg/ml tetracycline to induce CRT expression. The VEGF-A and

HIF-1 mRNA expression levels were normalized to the internal control HSP60 and were increased after tetracycline treatment. These results were examined in at least three independent experiments. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared with the control level. *p<0.05

(These results were provided by Kuan-Hung Lin)

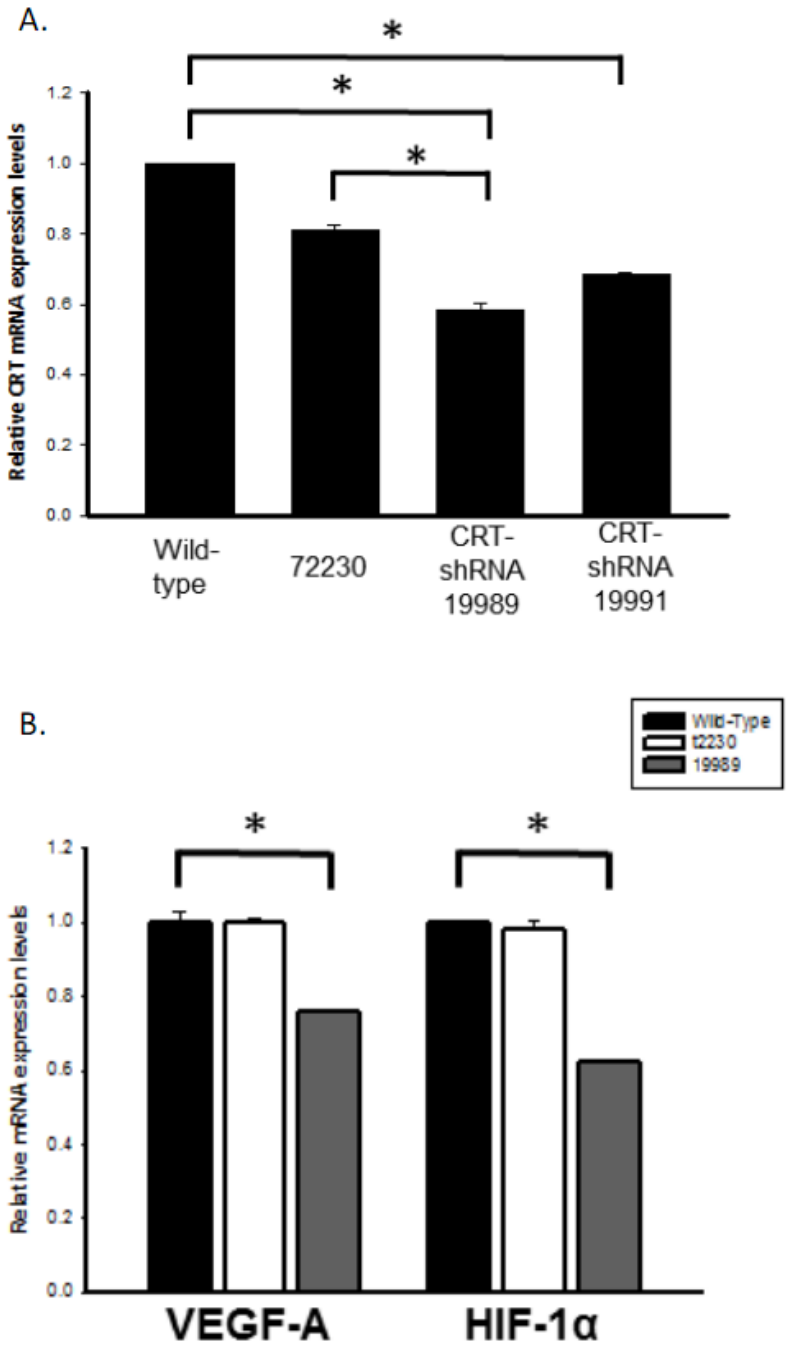

Figure 10. The VEGF-A and HIF-1α mRNA expressions were lower in CRT knockdown stable stNB-V1 cells.

(A) The CRT mRNA expression level was confirmed by real-time PCR in CRT knockdown stable cell lines (19989 and 19991). The CRT mRNA expression level

was normalized to the internal control HSP60. **(B)** The VEGF-A, VEGF-C, and HIF-1α mRNA expressions were confirmed by real-time PCR in CRT knockdown stable cell lines (19989). The mRNA expression level was normalized to the internal control HSP60. All of the results are repeated in at least three independent experiments. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared with the control level. *p<0.05

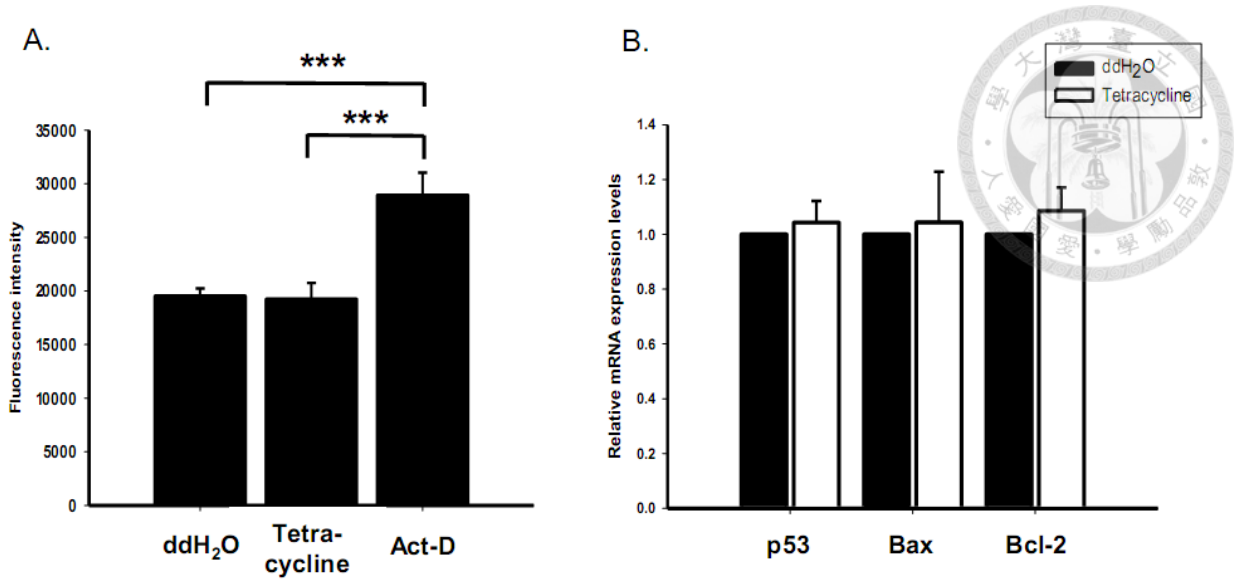

Figure 11. Apoptosis was not affected by CRT expression induced by tetracycline treatment in stNB-V1 cells.

stNB-V1 NB cells were stimulated with 1 μg/ml tetracycline to induce CRT expression. **(A)** Apoptosis was evaluated by Annexin V/propidium iodide staining. Treatment of actinomycin-D was used as positive control. The fluorescence intensity of stained cells were analyzed by flow cytometry and were no differences after tetracycline treatment. **(B)** The p53, Bcl-2 and Bax mRNA expressions were normalized to the internal control HSP60 and showed no differences after tetracycline treatment. All of the results were examined in at least three independent experiments (***$p<0.005$).

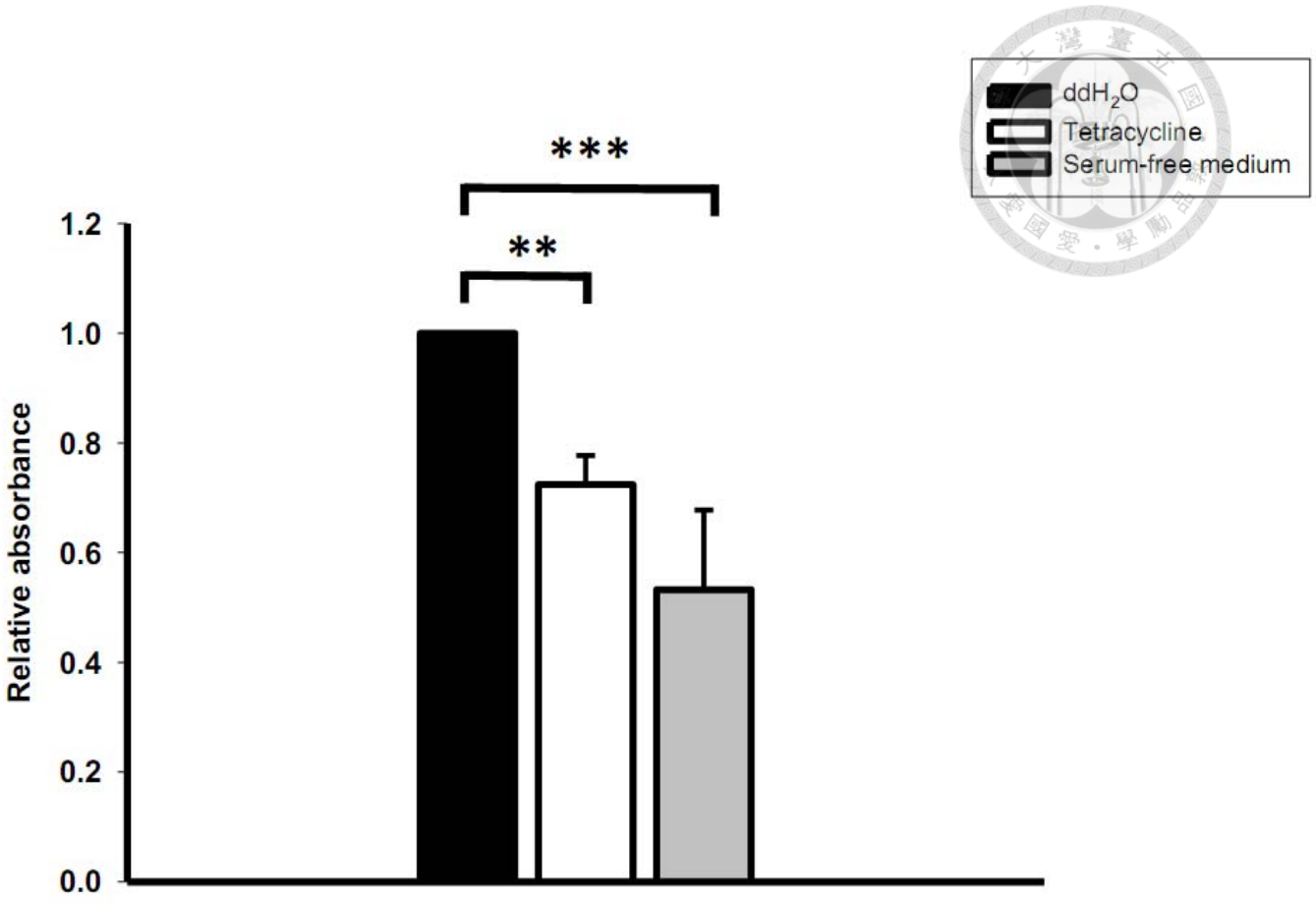

Figure 12. Over-expression of CRT reduced the proliferation rate of stNB-V1 cells.

stNB-V1 NB cells were stimulated with 1 μg/ml tetracycline to induce CRT expression. Cells cultured in serum-free medium were used as negative control. Cell proliferation was evaluated by MTT assay. Cells were seeded in the 96-well plate (10000 cells/well). Cell numbers were determined by measuring the absorbance at 595nm and were significantly lower after tetracycline treatment. These results were confirmed by at least three independent experiments (**p<0.01; ***p<0.005).

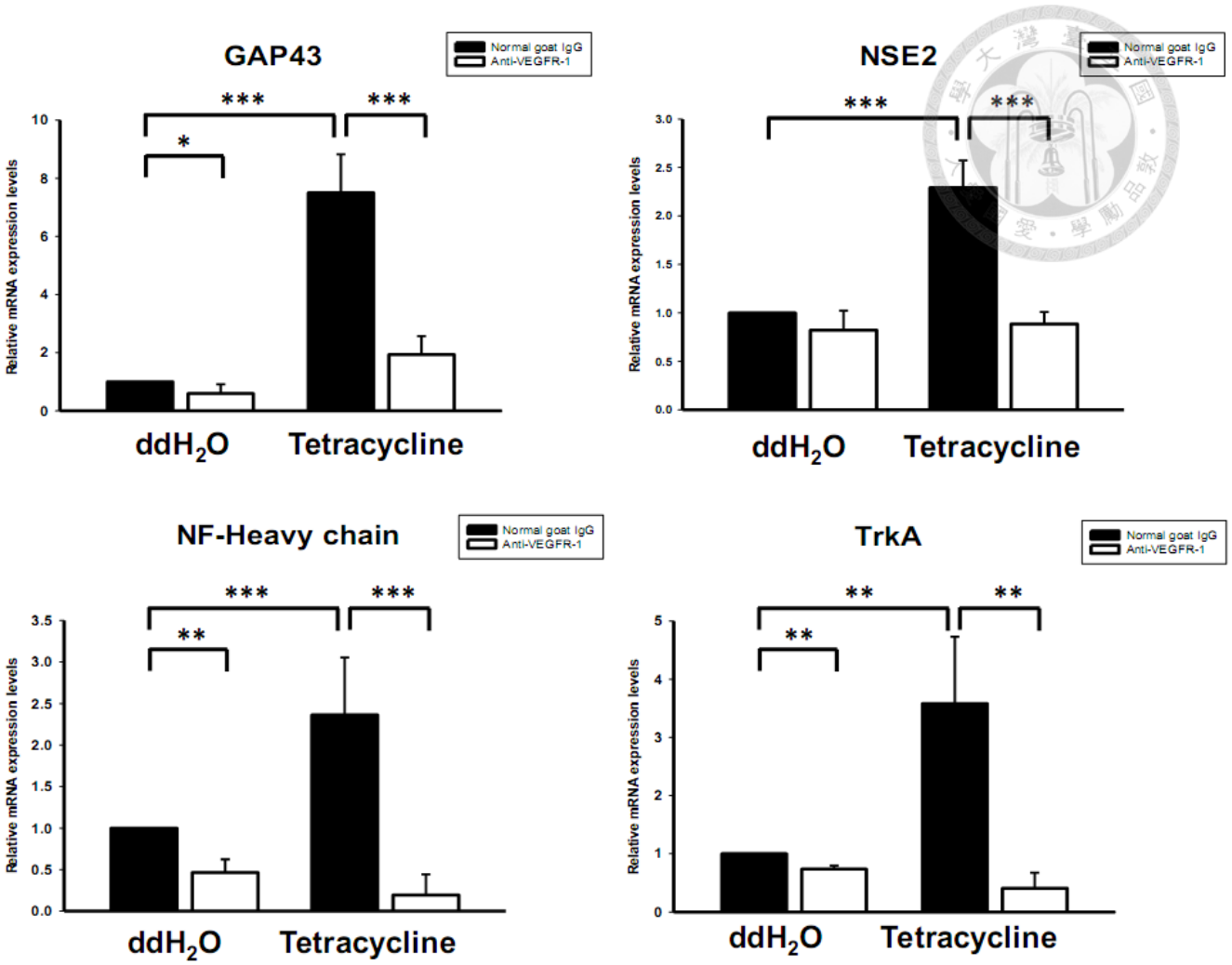

Figure 13 CRT-induced neuronal markers expression in NB cells is VEGF-A dependent.

stNB-V1 NB cells were stimulated with 1 μg/ml tetracycline to induce CRT expression. The mRNA expression levels of GAP43, NSE, NFH and TrkA were confirmed by real-time PCR and were increased after tetracycline treatment (dark bar). The mRNA expression level was normalized to the internal control HSP60. After treatment with anti-VEGFR-1 antibody, the mRNA expressions of GAP43, NSE,

NFH and TrkA were suppressed significantly in both CRT over-expression (treated with tetracycline) and controlled (treated with ddH2O) stNB-V1 cells. These results were examined in at least three independent experiments (*p<0.05; **p<0.01; ***p<0.005).

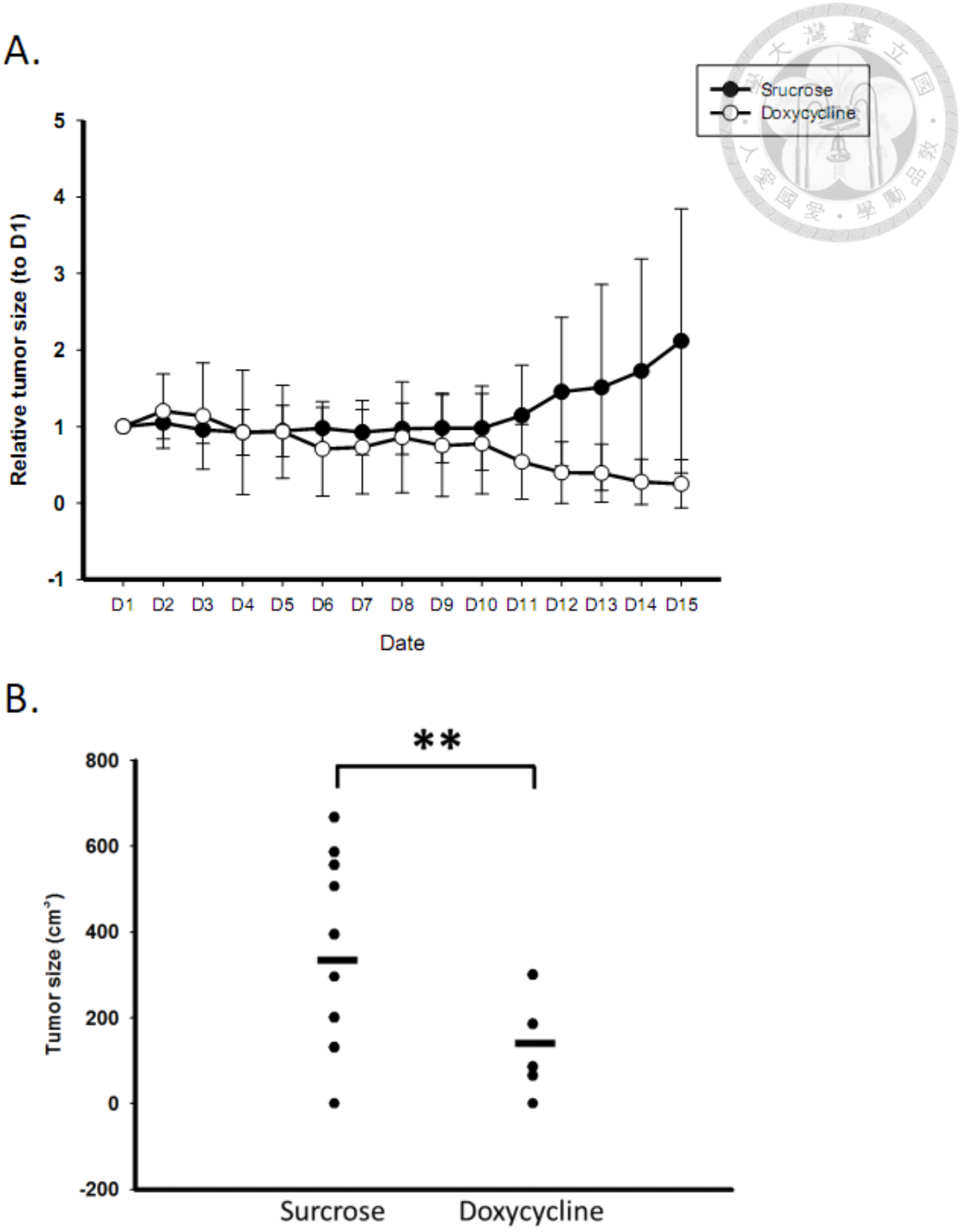

Figure 14. CRT expression suppressed NB tumor growth in mice xenograft models.

(A) inducible-CRT stNB-V1 cells were employed in mice xenograft models. Tumor inoculated mice were treated with doxycycline in their daily drinking water (2g/L) to induce CRT expression. The growth of tumor was measured for 15 days. **(B)** Tumor inoculated mice were sacrificed after 21-days treatment and the tumor was removed to measure the tumor size. Statistical differences were compared with the control level. (*p<0.05; **p<0.01)

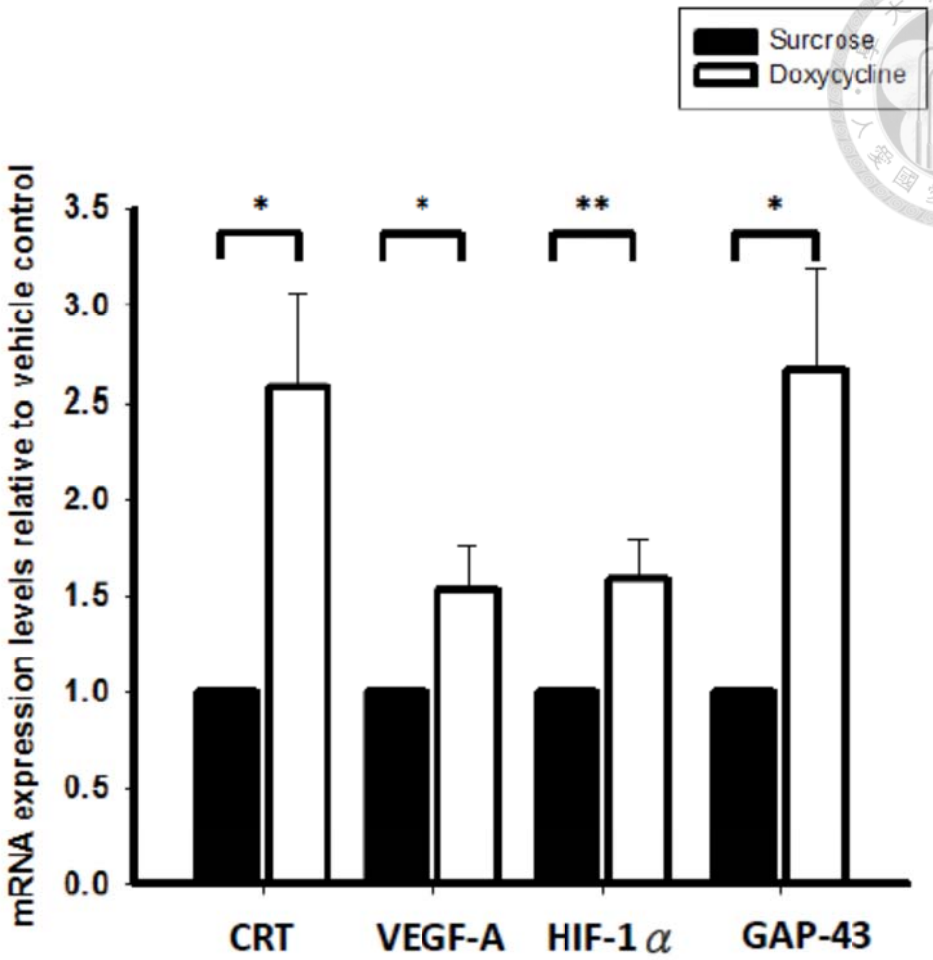

Figure 15. The VEGF-A, HIF-1α and GAP43 expressions were up-regulated by CRT expression in mice xenograft models.

Inducible-CRT stNB-V1 cells were injected subcutaneously into nude mice. Tumor inoculated mice were treated with doxycycline in their daily drinking water (2g/L) to induce CRT expression. Mice were sacrificed after 21-days treatment and the tumor was removed for experiments. The mRNA expression levels of VEGF-A, HIF-1α and GAP43 confirmed by real-time PCR and were increased after doxycycline treatment.

The mRNA expression level was normalized to the internal control HSP60. Each bar of the histogram represents quantified results and is shown as the mean ± SD. Statistical differences were compared with the control level. (*p<0.05; **p<0.01)

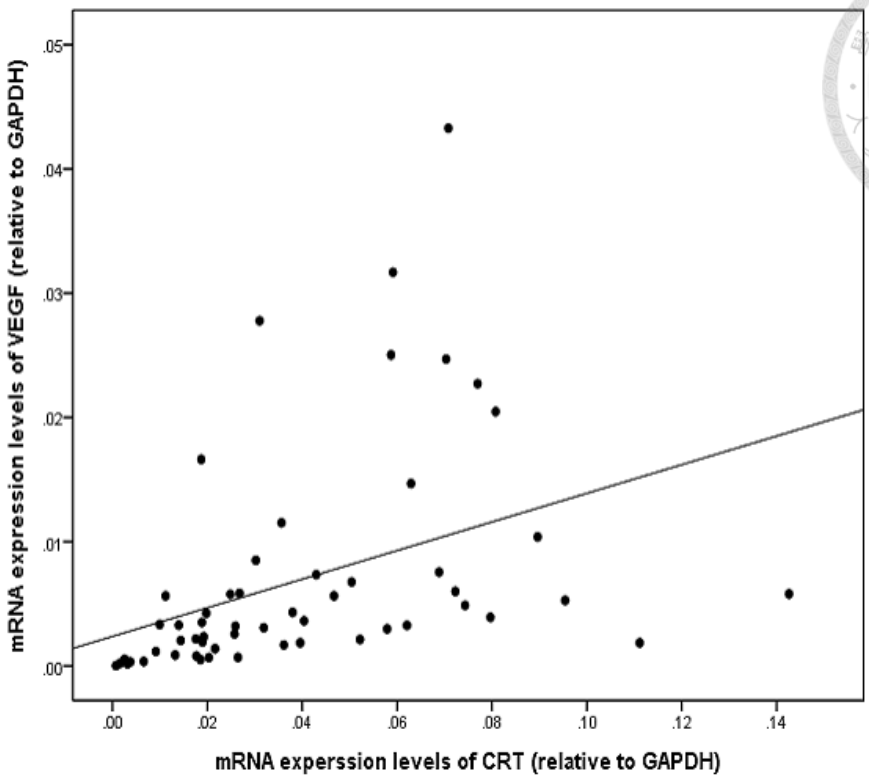

Figure 16. VEGF-A mRNA expressions were positively correlated with CRT expression in primary NB tumors.

The CRT and VEGF-A mRNA expressions were determined by real-time PCR in 56 human NB tumors. The correlation between expression levels of CRT (*x a*xis) and VEGF-A (*y* axis) was analyzed by Spearman's correlation test (Spearman's $\rho = 0.648$, $p < 0.001$).

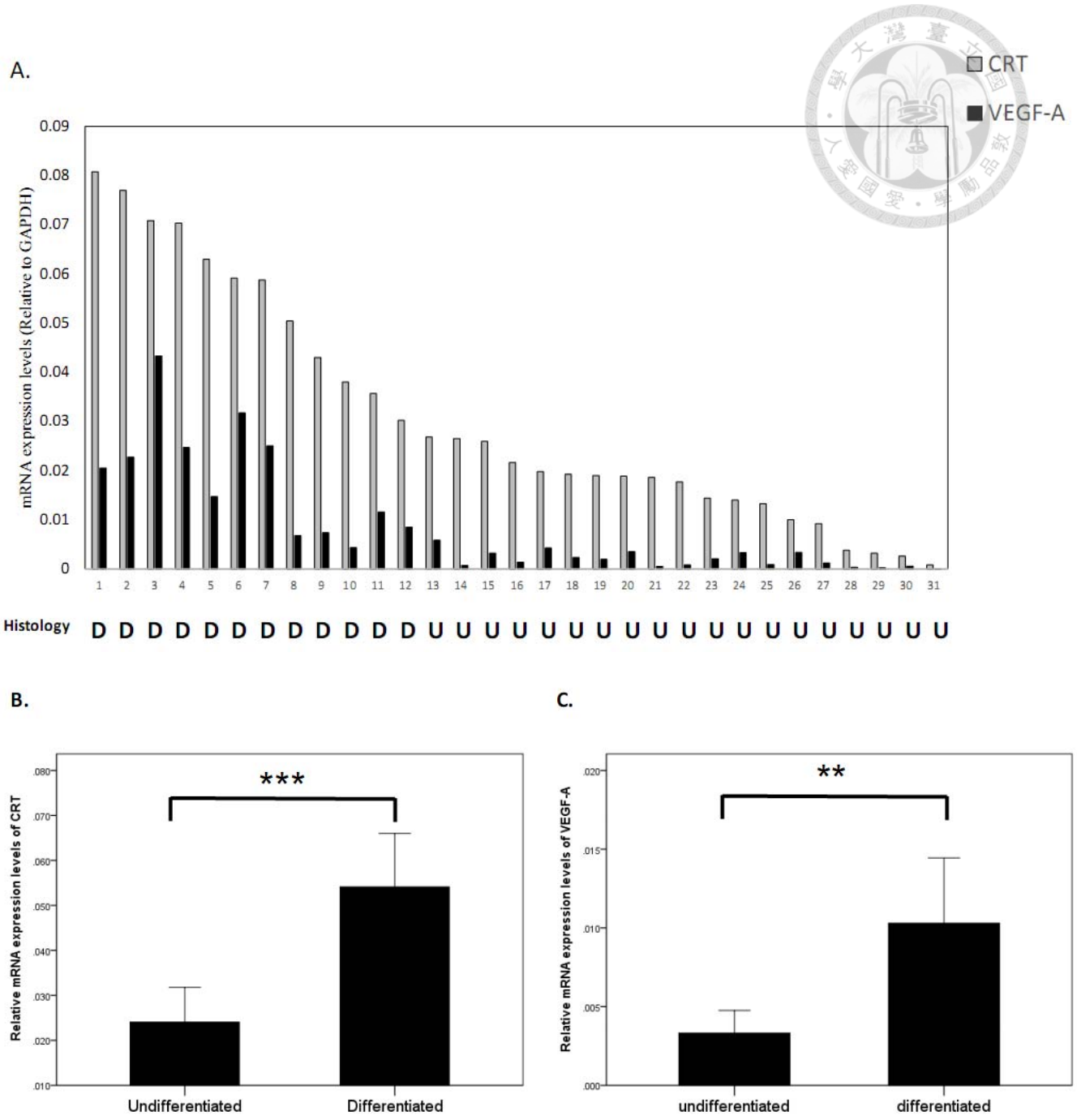

Figure 17.The expressions of CRT and VEGF-A are enhanced in primary NBs with differentiated histology.

(A) CRT and VEGF-A mRNA levels in 31 NB tumors were evaluated by real-time PCR. The levels of CRT (shaded bar) and VEGF-A (solid bar) are higher in

differentiated NB than in undifferentiated NB. D: differentiated NB, U: Undifferentiated NB. **(B)** The CRT mRNA expressions were determined by real-time PCR in 56 human NB tumors and presented as the mean (± SEM) of each group, undifferentiated NB (n=27) versus differentiated NB (n=29). **(C)** The VEGF-A mRNA expressions were determined by real-time PCR in 56 human NB tumors and presented as the mean (± SEM) of each group, undifferentiated NB (n=27) versus differentiated NB (n=29). (**p<0.01; ***p<0.005).

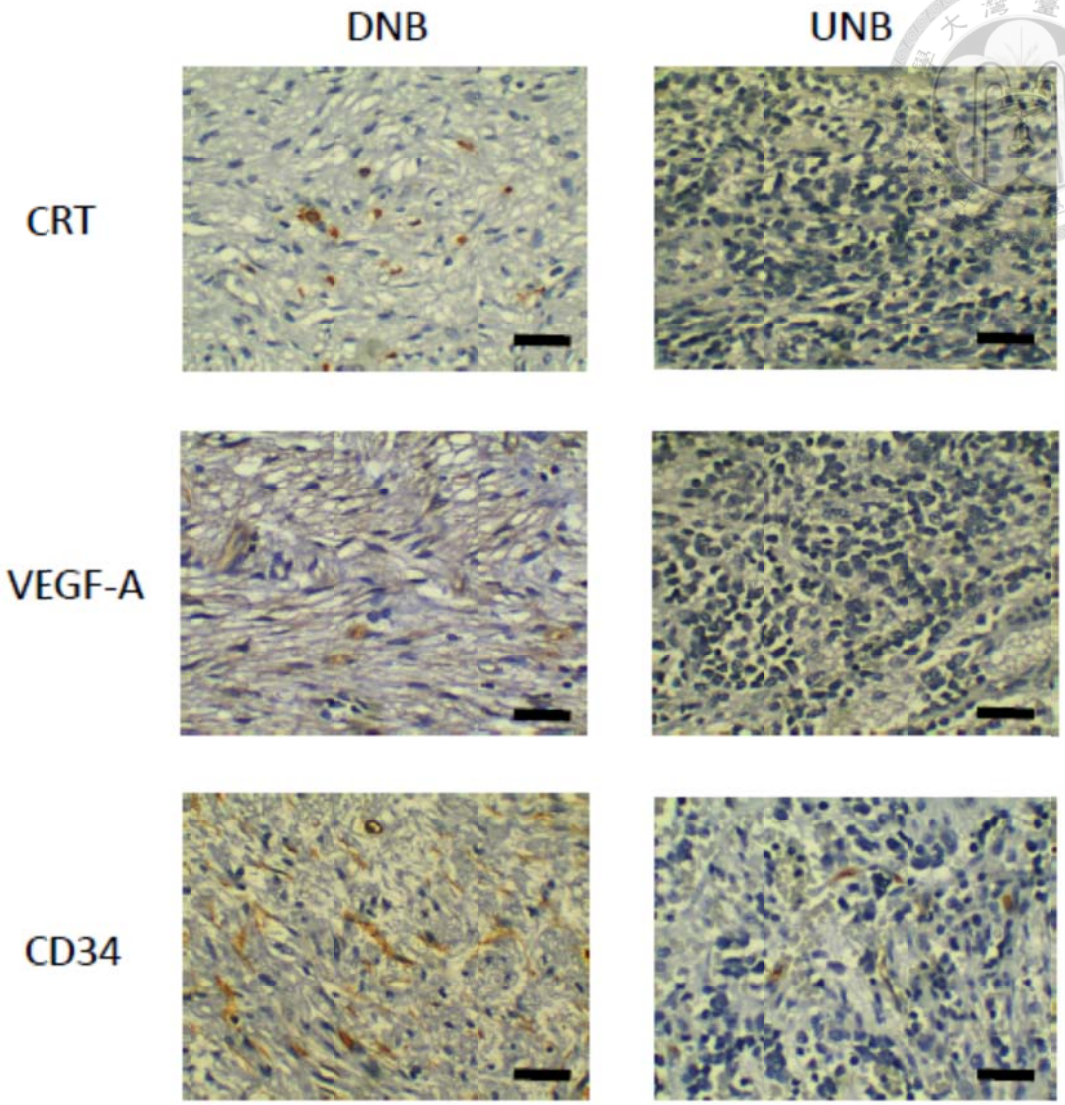

Figure 18. Immunohistochemical study of CRT, VEGF-A and CD34 expression in NB tumors.

Immunohistochemical histochemical staining of CRT, VEGF-A and CD34 in two representative patients with differentiated NB (DNB) and undifferentiated NB (UNB). Brown color represents positively stained cells. The tumor from a DNB patient exhibited strong immunostaining of CRT, VEGF-A and CD34. The UNB tumor with

immature histology displayed negative staining of CRT and VEGF-A, concomitant with positive CD34 immunostaining. Scale bar, 100 μm.

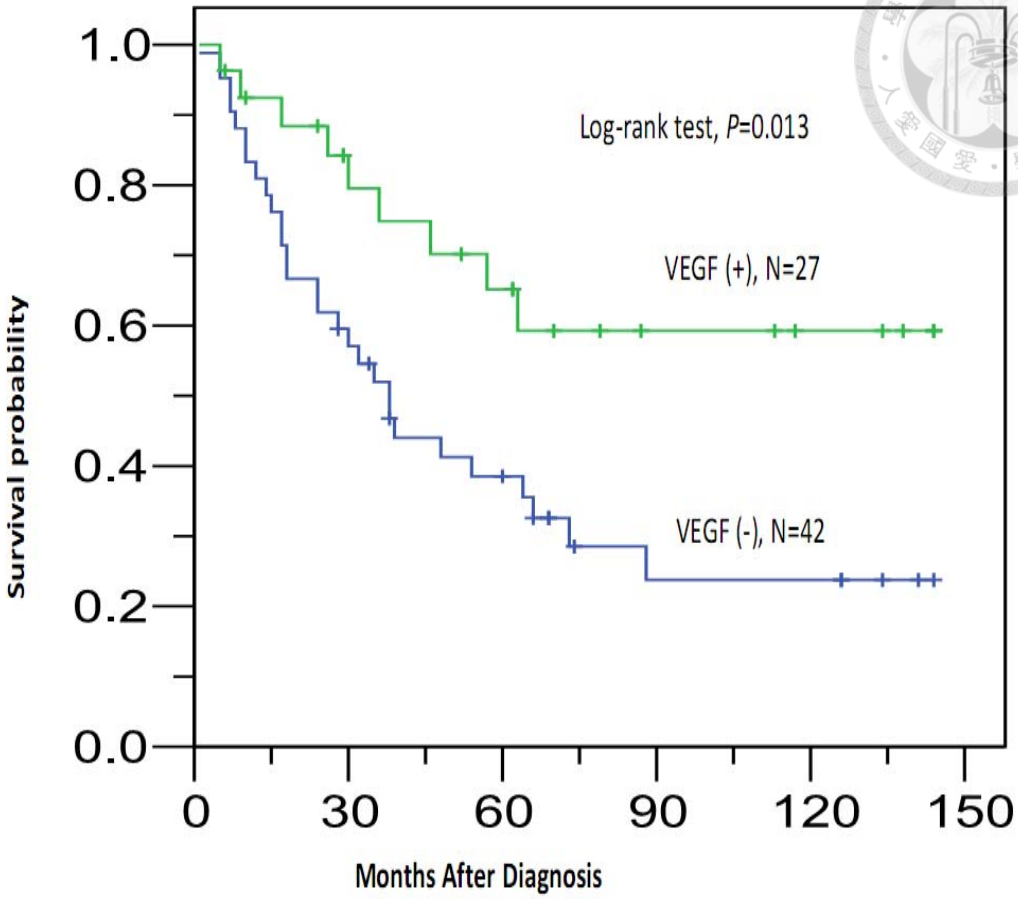

Figure 19. VEGF-A protein expression is correlated with favorable outcome in NB patients.

Kaplan-Meier survival analysis according to the expression of VEGF-A (VEGF) in a cohort of 69 NB patients.

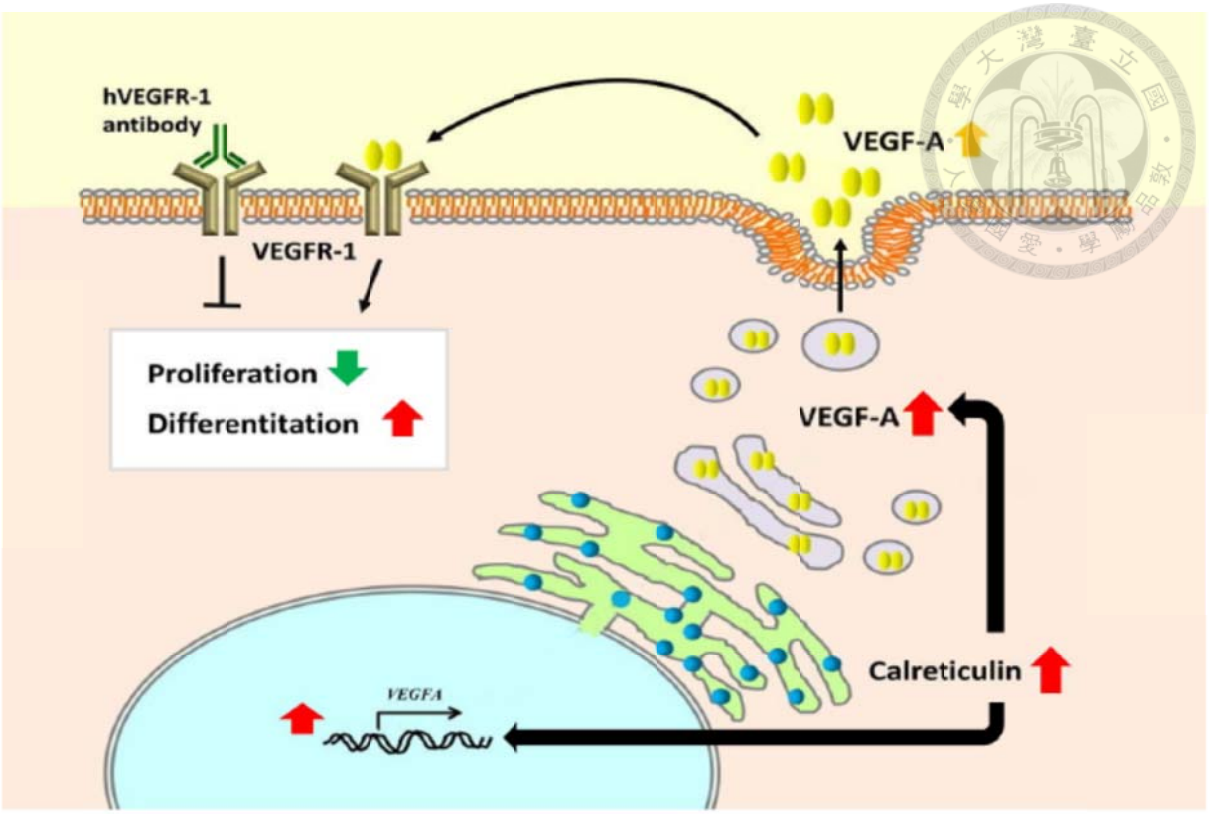

Figure 20. Schematic illustration summarized the role of VEGF-A in CRT-related neuronal differentiation in NB.

Appendix I: Role of glucose-regulated Protein 78 in embryonic development and neurological disorders

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Review Article

Role of Glucose-regulated Protein 78 in Embryonic Development and Neurological Disorders

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Glucose-regulated protein 78 (GRP78) is an important chaperone protein that is predominantly expressed in the endoplasmic reticulum. The multifunctional roles of GRP78 in protein folding, endoplasmic reticulum calcium binding, cytoprotection, and anti-apoptosis, as well as its function as a receptor on the cell surface, disclose its major involvement in physiological and numerous pathological conditions. Recent advances in mouse models targeting GRP78 allele have revealed the essential roles of GRP78 in development and neurological disorders, as well as accurate neural migration and neuroprotection. This review of correlation between GRP78 and embryogenesis and neurological disorders provides further directions for investigation, as well as potential therapeutics for clinical use.

Key Words: embryonic development, endoplasmic reticulum stress, glucose-regulated protein 78, neurological disorders, unfolded protein response

Endoplasmic reticulum (ER) is the principle cellular organelle in which secretory and membrane proteins are properly folded and modified. In addition, ER also functions as a major intracellular calcium store, and is responsible for biosynthesis of steroids and cholesterol. Moreover, ER is the site for N-linked glycosylation, which is also important for correct protein folding.[1] Proteins that cannot be correctly folded are eradicated via proteasome-mediated ER-associated degradation.[2] The accumulation of unfolded or misfolded proteins in the ER lumen is referred to as ER stress, and in eukaryotes it induces adaptive responses that are described as the unfolded protein response (UPR).[3] A number of cellular stress conditions, such as disruption of calcium homeostasis, altered glycosylation level, secretory protein mutations, and abnormal cholesterol level, can activate the UPR and contribute to a wide range of human diseases.[3] To assist and regulate the correct folding of proteins, ER contains abundant proteins known as molecular chaperones, including the 78-kDa glucose-regulated protein (GRP)78/immunoglobulin-binding protein (BiP),

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GRP94, calreticulin, calnexin, protein disulfide isomerase (PDI), oxidoreductase, and ER protein 57.[4] It has been demonstrated that GRP78 plays crucial roles in diabetes, cancer progression and therapy, heart diseases, and neurodegenerative diseases. Whether the involvement of GRP78 is the primary cause or a secondary consequence in diseases is yet to be determined. Besides, GRP78 and UPR also participate during embryonic development, and under physiological conditions such as development and differentiation of plasma cells and pancreatic β cells.[5] This review focuses on the physiological roles of GRP78/BiP, as well as the recent advances in the study of crucial roles of GRP78/BiP in embryonic development and neurological disorders.

ER Protein Quality Control, UPR, and ER-resident Chaperone Proteins

The protein quality control in ER is important for cell survival and ER chaperones are crucial for the normal function of ER quality-control systems.[6] Many genetic and environmental insults disturb the normal functions of ER and induce ER stress. ER stress is triggered by accumulation of unfolded and misfolded proteins in the ER lumen by the induction of UPR processes in eukaryotes.[7] Three ER-resident transmembrane proteins function as stress sensors and are involved in the UPR: inositol requiring kinase 1 (IRE1), activating transcription factor (ATF)6 and PKR-like (RNA-activated protein kinase-like) endoplasmic reticular kinase (PERK) transducers, which transduce the unfolded protein signal across the ER membrane and lead to the activation of the UPR (Figure).[7] Upon ER stress, the first response is the activation of the PERK pathway, which leads to the attenuation of general protein translation by phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α).[8] However, the phosphorylated eIF2α can selectively enhance the translation of mRNAs containing inhibitory upstream open reading frames in their 5′ untranslated region, such as ATF4.[9] In addition, under ER stress, ATF6 becomes an active transcription factor by transiting to the Golgi complex, where it is cleaved by site-1 and site-2 proteases.[10] The active cleaved form of ATF6 translocates to the nucleus and binds to the promoter of UPR-inducible gene to upregulate proteins that adjust ER protein folding, especially ER chaperones and X-box-binding protein (XBP)-1.[11] The last activated pathway with response to the ER stress is the IRE1 pathway. Activated IRE1 acts as an endoribonuclease, which brings about the unconventional splicing of XBP-1 mRNA and subsequent translation of an active transcription factor.[8,11] The active transcription factor then promotes the expression of ER-resident chaperones, which facilitate protein folding in the ER.[8,11] If these adaptive coordinated responses cannot eliminate inappropriately folded proteins during prolonged and severe ER stress, the UPR elicits a pro-apoptotic pathway and triggers apoptotic cell death.[5,12]

In the ER lumen, several chaperone proteins are involved in the UPR to regulate protein quality control, including glucose-regulated proteins (GRPs), calreticulin, calnexin, PDI, oxidoreductase, and ER protein 57. These chaperone proteins contain ER stress elements in their promoters and are upregulated during ER stress.[13] Some chaperones, including GRP78, GRP94 and PDI have been identified to form a multimeric protein complex to bind to and interact with the unfolded proteins.[4,14] GRP78, a member of the heat shock protein 70 family, also referred to as BiP or heat shock protein A5, is one of the major ER chaperones capable of regulating UPR signaling, through a bind-and-release mechanism with the ER stress sensors and transducers (PERK, ATF6 and IRE1).[15] GRP78 is composed of three domains: the ATPase domain, the peptide-binding domain, and the C-terminal domain.[16] Upon ER stress, GRP78 dissociates from the ER transmembrane transducers to bind to the unfolded proteins in the ER, which leads to the activation of the PERK, ATF6 and IRE1 pathway (Figure).[15,17] The cycles of binding and release of GRP78 are mediated by its N-terminal ATPase activity.[18] GRP78 also binds to unfolded proteins when GRP78 is associated with ATP. DnaJ

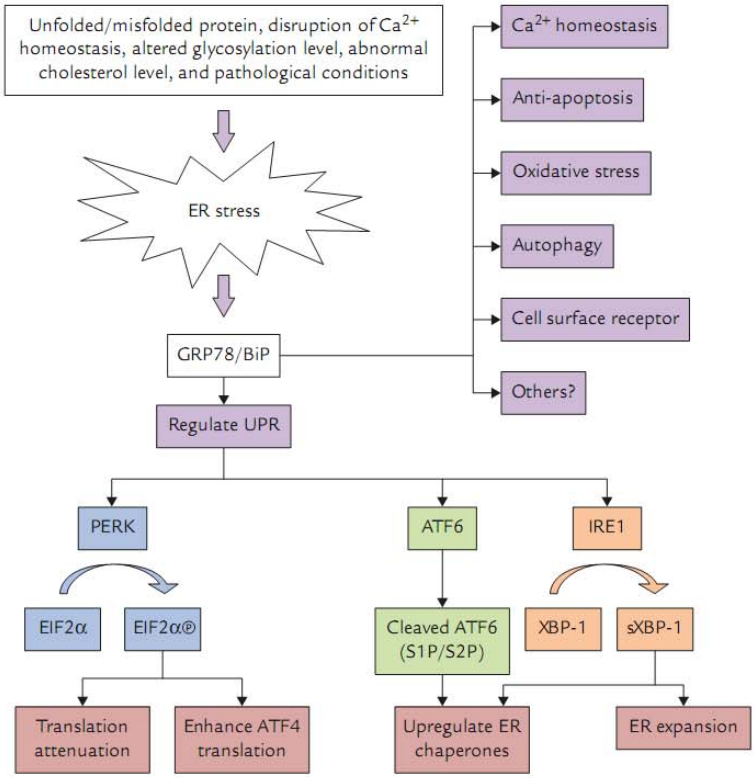

Figure. Summary of physiological functions of glucose-regulated protein 78.

family members ERdj1–5 are induced by ER stress and serve as cofactors of GRP78, which bind to the GRP78 complex and stimulate the ATPase activity of GRP78 to hydrolyze ATP to ADP.[19–21] Hydrolysis of ATP to ADP results in conformational change of GRP78 and augments the binding ability of GRP78 to unfolded protein. Subsequently, BiP-associated protein, a nucleotide exchange factor, converts ADP to ATP, thereby facilitating the release of GRP78 from the proteins.[22] The cycles of GRP78 bind-and-release help the correct folding of proteins and maintain the quality control of the ER.

Multifunctional Roles of GRP78

GRP78 is involved in many cellular processes other than as a major regulator of UPR (Figure). With its Ca^{2+} binding capacity, GRP78 assists in maintaining cytosolic calcium homeostasis by sequestering Ca^{2+} within the ER.[23] Overexpression of GRP78 has been found to increase the Ca^{2+} storage capacity of the ER. Conversely, diminished expression of GRP78 increases the intracellular Ca^{2+} levels induced by oxidants.[24,25] In addition, recent studies have revealed that GRP78 has cytoprotective and anti-apoptotic properties.[24–27] Some studies have demonstrated that GRP78 forms a complex with caspase-7 and caspase-12 on the cytosolic side of the ER membrane, thereby blocking the main apoptosis-related machinery, without directly binding to caspase-3.[27,28] Other studies have shown that reactive oxygen species generated in hypoxic or ischemic preconditioning lead to upregulation of GRP78 expression. After GRP78 is induced, cells are protected from apoptosis via suppression of

oxidative stress.[29] However, the detailed mechanisms by which GRP78 protects cells against cell death under stressful and pathological conditions remain to be explored.

Recently, it has been shown that ER stress could induce autophagy for cell survival in SK-N-SH neuroblastoma cells.[30] Autophagy is a catabolic process of cytosolic protein recycling, and plays important roles in intracellular protein degradation. In mammals, autophagy has been shown to be related to ER stress and the UPR.[31,32] More recently, studies have shown that the ER structure is disrupted, and autophagosome formation induced by ER stress and nutrient starvation is blocked in cells in which GRP78 is knocked down by small interfering RNA (siRNA). These results imply an essential role of GRP78 in ER-stress-induced autophagy.[33]

Knockdown of GRP78 by siRNA in cells causes compensatory upregulation of GRP94, suggesting that some functions of GRP78 can be compensated by other homologous chaperones, but it cannot be replaced totally because of its unique multifaceted subcellular location.[34] Besides, GRP78 has been identified on the cell surface or cytosolic surface of the ER membrane.[27,35] The physiological function of cell-surface GRP78 appears to be a multifunctional receptor that allows it to regulate a variety of signaling pathways that are involved in cell proliferation and cell death regulation.[35]

Roles of GRP78 in Embryonic Development and Nervous System Development

Although many studies have described the crucial roles of GRP78 for modulating the physiological function of eukaryotic cells, few have described how essential GRP78 is for development. The amino acid sequence of GRP78 is highly conserved from yeast to humans, thereby implying its crucial function for eukaryotes.[36] One study using two-dimensional gel electrophoresis has found that GRP78 is abundant at the blastocyst stage of the developing mouse embryo, suggesting an essential role of GRP78 in embryonic development.[37] It has also been shown that GRP78 is highly expressed in the heart, neural tube, gut endoderm, somites, and surface ectoderm of mouse embryos during early organogenesis.[38] To investigate the direct role of GRP78 for growth and development *in vivo*, heterozygous and knockout (KO) GRP78 mouse models have been created.[39] The GRP78 KO mice exhibit lethality beyond the peri-implantation stage, with obvious defects in cell proliferation and massive apoptosis in the inner cell mass. By contrast, the heterozygous GRP78^{+} mice, which express half of the GRP78 proteins compared with wild-type adults, are viable, phenotypically normal, and respond normally to ER stress. The heterozygous GRP78 cells upregulate other ER chaperones, including GRP94 and PDI at both the transcript and protein levels. However, the compensatory responses cannot rescue the GRP78 KO mice from lethality, suggesting that GRP78 is essential for embryonic development.

However, the roles of GRP78 in neuronal development and underlying mechanisms for ER stress during embryogenesis are still unclear. During embryogenesis, a number of coordinated events occur to ensure orderly generation of the diverse cell types leading to proper development of organs. One of these events is apoptosis, which occurs widely during development of the central nervous system.[40] Apoptosis can be induced by a number of different stimuli, including ER stress.[41] Recently, it has been shown in mouse models that ER stress is involved in the development of the central nervous system.[42] Several ER chaperones, including GRP78, are expressed at higher levels in embryonic brain and retina than in adult tissues. The indicators of the UPR, including unphosphorylated and phosphorylated forms of eIF2α, spliced XBP-1 mRNA, and partially glycosylated ATF6, are detected predominantly in embryonic brain compared with adult tissues. Caspase-7 and -12 are found more abundantly in embryonic brains than in adult tissues, which suggests that ER stress induces apoptosis during embryonic development of the central nervous system.[42]

More recently, a mouse model of GRP78 conditional KO in Purkinje cells (PCs) was established.[43] The GRP78 PCs-specific KO mice have demonstrated accelerated PC degeneration and cerebellar atrophy, with growth retardation and severe defects in motor coordination. Besides, in GRP78-null PCs, the other ER chaperones, GRP94, PDI and GADD34 (growth arrest and DNA damage-inducible protein 34), as well as the induction of CHOP (C/EBP homologous transcription factor), an ER-stress-inducible transcriptional factor, are upregulated. However, the phosphorylated eIF2α and apoptotic cell death are suppressed through feedback. An association between GRP78 depletion and reduction in cytosolic ubiquitin has also been demonstrated, suggesting a requirement for GRP78 in protein ubiquitination.

The available evidence supports the notion that GRP78 plays a crucial role in embryogenesis and development of the nervous system. However, the underlying mechanisms and participation of GRP78 in embryogenesis are yet to be determined.

GRP78 and Human Diseases

Increasing evidence suggests that ER stress and the UPR are involved in a variety of human diseases, including diabetes, cancer progression, cardiovascular diseases, renal diseases, and neurological disorders.[23,44–47] Most findings of ER stress and the UPR in human diseases have been focused on carcinogenesis and tumor progression. Studies have demonstrated that the microenvironment of tumor cells is often nutrient-deprived and hypoxic, which mimics the physiological ER stress and causes activation of the UPR.[48] Besides, the apoptotic pathways are often suppressed for immortalization of cancer cells. With the anti-apoptotic function, GRP78 has been implicated in cancer progression and drug resistance.[27,34] Upregulation of GRP78 is present in a variety of cancer cell lines, solid tumors, and human cancer cells, including lung cancer, prostate cancer, gastric carcinoma, hepatocellular carcinoma and breast cancer.[34,49–51] Tumor invasion, metastasis and recurrence are also positively correlated with increased GRP78 expression, whereas patient survival rate and chemosensitive response are inversely correlated with increased GRP78 expression.[49–51]

With regard to neural tumors, the level of GRP78 expression is significantly higher in malignant glioma specimens and cell lines compared with the low expression level in normal adult brain tissues.[52] The expression of GRP78 is positively correlated with cell proliferation. Besides, knockdown of GRP78 by siRNA or treatment with GRP78 inhibitors augments the effectiveness of chemotherapeutic agents, including temozolomide, a prevalent chemotherapeutic agent for malignant glioma, thereby suggesting the crucial role of GRP78 in chemoresistance and potential combination therapy.[52,53] In addition, human brain endothelial cells, derived from blood vessels of malignant glioma tissues, constitutively overexpress GRP78 and are more resistant to chemotherapeutic agents, which supports a crucial role for the vasculature in tumor growth and survival.[53] Nevertheless, Hsu et al have demonstrated that GRP78 is an independent favorable prognostic marker in neuroblastoma (NB).[54] NB is a childhood tumor derived from sympathoadrenal lineage of the neural crest progenitor cells, and is the most common malignant disease of infancy.[55] The molecular mechanism underlying the tumorigenesis of NB remains elusive, and it has been postulated that the pathogenesis of NB is due to the failure of differentiation or apoptosis of neuroblastic cells. Recent evidence suggests that NB cells exhibit a capacity to differentiate into mature cells and can be regressed spontaneously by apoptosis.[56,57] Hsu et al have found that increased GRP78 expression in NB tumors positively correlates with tumor differentiation, and therefore predicts a favorable outcome.[54] GRP78 expression as a significant factor for predicting favorable outcome has also been found in other studies of olfactory NB.[58] An *in vitro* study using the PC12 cell line has revealed that increased GRP78 expression is correlated with promotion of neurite outgrowth in the presence of nerve growth factor (NGF).[59] Overexpression of GRP78 by adenoviral gene transfer

promotes NGF-induced neurite outgrowth, and antisense nucleotides of GRP78 block the promotion of neurite outgrowth.[59] Thus, GRP78 might play an essential role in the differentiation of NB cells, as well as in the differentiation of other neural progenitor or stem cells.[60]

GRP78 in Neurological Disorders

Neurons are vulnerable to different genetic and environmental insults that affect the homeostasis of ER function via the accumulation of misfolded proteins. Therefore, it is not surprising that a number of studies have demonstrated that ER stress and misfolded protein aggregation are present in several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, bipolar disorders, amyotrophic lateral sclerosis, spinocerebellar ataxia, and Lafora disease.[61–69] ER stress has also been implicated in other neurological diseases, such as methamphetamine-induced neurotoxicity, sleep disorders, cerebral ischemia, and demyelinating diseases.[44,70–72] A spontaneous recessive mutant mouse model, woozy mouse, has provided a direct connection between ER dysfunction and neurodegeneration.[73] The woozy mutant mice develop adult-onset ataxia with selective loss and death of PCs. Genetic studies have identified that the woozy mutation disrupts the Sil1 gene, which encodes a nucleotide exchange factor for GRP78. GRP78 is upregulated in degenerative PCs. Sil1-deficient mice possess a nonlethal phenotype, suggesting the existence of other co-chaperones that might serve as alternative nucleotide exchange factors for GRP78 function. As previously mentioned, a mouse model of GRP78 conditional KO in PCs has been created recently, and confirms the crucial roles of GRP78 in neurodegeneration.[43]

Humans with Sil1 mutation develop Marinesco–Sjogren syndrome, which is characterized by cerebellar ataxia, cataract, myopathy, and psychomotor retardation.[74,75] However, the physiological and pathological roles of GRP78 in neuronal development and neurologic disorders are yet to be recognized. A knock-in mouse model expressing mutant GRP78 has been created recently, and has helped to clarify the effects of defective GRP78 function in neuronal development.[76] The mutant mice that express low levels of GRP78 in the ER die after birth due to respiratory failure. The brain of mutant mice displays abnormal layer formation in the cerebral cortex and cerebellum, which results in a neurological phenotype of reeler-mutant-like malformation, which is linked to schizophrenia.[76,77] The size of the brain is also reduced and the expression of reelin, which is secreted by Cajal–Retzius cells, is markedly reduced. These findings imply that mutant GRP78 and aberrant ER quality control are involved in improper neuronal migration and maturation, which results in neurological disorders.

In humans, a recent study has demonstrated increased expression of GRP78 in the cortex and hippocampus of patients with Alzheimer's disease, as well as in anterior horn motor neurons from patients with amyotrophic lateral sclerosis, which suggests that ER stress induces activation of the UPR in Alzheimer's disease and amyotrophic lateral sclerosis.[69,78,79] By contrast, reduced level of GRP78 has been illustrated in the cortex of Alzheimer's disease patients and in aging brain.[80] Reduced GRP78 in neurodegenerative diseases has also been observed in the cell model of spinocerebellar ataxia, suggesting that GRP78 plays an important role in neuroprotection.[68]

It has been shown that selective induction of GRP78 plays an important role in the apoptotic death of differentiated PC12 cells deprived of NGF, as well as rat sympathetic neurons.[81] In addition, it has been demonstrated that the ER-stress induced cell death pathway in differentiated PC12 cells was characterized by up-regulation of GRP78, calpain expression and processing of caspase-12.[82] In another study, induction of GRP78 by ischemic preconditioning was found to reduce ER stress and postpone neuronal cell death.[83] Moreover, GRP78 participates in the neuroprotective mechanism induced by hypothermic treatment in reducing brain damage caused by traumatic, hypoxic and ischemic injury.[84] The reduction of GRP78

Table. Roles of glucose-regulated protein 78 in embryonic development and human diseases

Conditions	Manifestations
Embryonic development	GRP78 is highly expressed in developing embryo[37,38] GRP78 KO mice exhibit lethality[39]
Neuronal development	GRP78 is expressed at higher levels in embryonic brain[42] GRP78 expression is positively correlated to neurite outgrowth in PC12 cells[59] GRP78 PC-specific KO mice demonstrated cell deaths[43]
Cancers	GRP78 is involved in cancer progression and drug resistance[27,34,48–53]
Neuroblastoma	GRP78 is an independent favorable prognostic marker[54,58]
Neurological disorders	GRP78 is involved in Alzheimer's disease, amyotrophic lateral sclerosis, and spinocerebellar ataxia[68,69,78–80] A knock-in mouse model expressing mutant GRP78 displayed reeler mutant-like malformation and phenotype mimicking schizophrenia[76,77] Upregulation of GRP78 is involved in cell death in PC12 cells[81,82] Upregulation of GRP78 expression could prevent neuronal death both *in vitro* and *in vivo*[83–87]
Others	GRP78 is involved in diabetes, cardiovascular diseases, and renal diseases[45–47]

GRP = glucose-regulated protein; KO = knockout.

expression in ischemic brain is restored by hypothermic treatment that enhances neuronal survival. More recently, several studies have demonstrated that upregulation of GRP78 expression could prevent neuronal death both *in vitro* and *in vivo*.[84–87] Finally, the discovery that valproic acid (a common drug for treatment of epilepsy and bipolar disorder) enhances expression of GRP78 further suggests a neuroprotective effect of GRP78.[88] These results shed new light on the possible mechanisms of valproic acid in the treatment of neurological diseases.

Conclusions and Future Directions

Over the years, evidence has shown that GRP78 is unique and essential for embryogenesis, and contributes to a wide range of neurological diseases (Table). However, little is known about the mechanisms by which GRP78 protects eukaryotes from cell death. Moreover, whether the involvement of GRP78 is a primary cause or a secondary consequence in disease evolution has yet to be determined. Considering the potential importance of GRP78 in neuronal development and neurological disorders, further studies are needed to evaluate the detailed participation of GRP78 in embryogenesis and nervous system development, as well as its effects on neural differentiation and neurological disorders. Additionally, the discovery of cell-surface GRP78 implies a novel function in disease that differs from its chaperone activity. Further investigations on the crucial roles of GRP78 in neurological diseases will provide a solid foundation for development of potential therapeutics to modulate and treat disease progression.

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Appendix II. Calreticulin Regulates VEGF-A in Neuroblastoma Cells

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Calreticulin Regulates VEGF-A in Neuroblastoma Cells

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Abstract Calreticulin (CRT) has been previously correlated with the differentiation of neuroblastoma (NB), implying a favorable prognostic factor. Vascular endothelial growth factor (VEGF) has been reported to participate in the behavior of NB. This study investigated the association of CRT and VEGF-A in NB cells. The expressions of VEGF-A and HIF-1α, with overexpression or knockdown of CRT, were measured in three NB cells (SH-SY5Y, SK-N-DZ, and stNB-V1). An inducible CRT NB cell line and knockdown CRT stable cell lines were also established. The impacts of CRT overexpression on NB cell apoptosis, proliferation, and differentiation were also evaluated. We further examined the role of VEGF-A in the NB cell differentiation via VEGF receptor blockade. Constitutive overexpression of CRT led to NB cell differentiation without proliferation. Thus, an inducible CRT stNB-V1 cell line was generated by a tetracycline-regulated gene system. CRT overexpression increased VEGF-A and HIF-1α messenger RNA (mRNA) expressions in SH-SY5Y, SK-N-DZ, and stNB-V1 cells. CRT overexpression also enhanced VEGF-A protein expression and secretion level in conditioned media in different NB cell lines. Knockdown of CRT decreased VEGF-A and HIF-1α mRNA expressions and lowered VEGF-A protein expression and secretion level in conditioned media in different NB cell lines. We further demonstrated that NB cell apoptosis was not affected by CRT overexpression in stNB-V1 cells. Nevertheless, overexpression of CRT suppressed cell proliferation and enhanced cell differentiation in stNB-V1 cells, whereas blockage of VEGFR-1 markedly suppressed the expression of neuron-specific markers including GAP43, NSE2, and NFH, as well as TrkA, a molecular marker indicative of NB cell differentiation. Our findings suggest that VEGF-A is involved in CRT-related neuronal differentiation in NB. Our work may provide important information for developing a new therapeutic strategy to improve the outcome of NB patients.

Keywords Calreticulin · VEGF · Neuroblastoma · Neuronal differentiation

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Introduction

Neuroblastoma (NB) is a common childhood tumor and the most frequently diagnosed malignancy in infancy, with more than 96 % of patients diagnosed at the age of <10 years old [1, 2]. It is derived from the sympathoadrenal lineage of embryonic neural crest cells [3]. The mechanism underlying its tumorigenesis remains obscure, and previous studies suggest that failure of differentiation or apoptosis of neuroblastic cells is critical in its development [4]. Children with NB have a broad spectrum of clinical diversity that is highly associated with age at onset and genetic, biological, and pathological characteristics [3].

Previous studies have shown that NB cells exhibit a capacity of differentiating into mature cells or spontaneous regression by apoptosis [5, 6]. On the other hand, NB with better prognosis often express molecular markers indicative of cell differentiation, such as TrkA [7]. Furthermore, the expressions of apoptosis-related genes including p53, Bcl-2, and Bax have been demonstrated in NB and are correlated with favorable prognosis [8]. However, the factors contributing to the regulation of NB cell differentiation or apoptosis are still unclear.

Calreticulin (CRT) is an important chaperone protein in the endoplasmic reticulum. It is highly conserved across species [9]. Its multifunctional roles in chaperoning, Ca^{2+} homeostasis, cell transduction, modulating cell adhesion, and gene expression disclose its major involvement in physiologic and pathologic conditions [9, 10]. Evidences suggest that CRT may play an essential role in the biology of NB. Previous studies reveal that CRT is on the surface of NB cells and is essential for neurite formation when NB cells are induced to differentiate [11, 12]. Another in vitro study using NB cell line reveals that increased CRT expression is correlated with the differentiation of NB cells [13]. Recently, CRT has been identified as an independent favorable prognostic marker in NB [14]. Studies suggest that increased CRT expression in NB positively correlates with tumor differentiation and therefore predicts favorable outcome. However, how CRT affects the differentiation and apoptosis of NB warrants further clarification.

Vascular endothelial growth factor (VEGF)-A (also referred to as VEGF), which belongs to a family of homodimeric disulfide-bound glycoproteins, is a key regulator of physiologic and pathologic angiogenesis [15]. In mammals, this family includes VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). More and more studies reveal that VEGF-A plays an important role in the progression and metastasis of cancer cells [16]. Both in human and in experimental NB, VEGF-A overexpression has been demonstrated and correlated with a high-risk phenotype [17, 18]. In contrast, recent studies have found that VEGF-A is not related to tumor progression and metastasis in NB [19]. Thus, the roles of VEGF-A in the tumorigenesis of NB remain obscure and need further clarification.

The present study aimed to investigate the correlation between CRT and VEGF-A in NB cells through establishing NB cell lines with stable CRT overexpression and knockdown.

Materials and Methods

Cell Culture

The NB cell lines SH-SY5Y (ATCCH CRL-2266TM) and SK-N-DZ (ATCCH CRL-2149TM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), while the stNB-V1 was kindly provided by Dr. YF Liao of Academia Sinica. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/high glucose medium (Biowest) containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (Invitrogen). The cells were grown in a humidified atmosphere containing 5 % CO_2 and 95 % air at 37 °C. Using a hemocytometer and trypan blue, cells were subcultured in 10-cm plates at a density of 10^6 cells per plate.

Transfection

The cells were seeded in 3.5-cm plates at density of 3×10^5 cells and were transfected with construct plasmids using Lipofectamine 2000 (Invitrogen). Every plate was transfected with 4 μg of the plasmid and 10 μL of the Lipofectamine 2000 in serum-free DMEM. After 8 h of transfection, the medium was changed into DMEM with 10 % FBS. The cells were harvested after 48 h.

Cells transfected with a vector, without the insert gene, were used as vehicle control and cells treated with double-distilled water (ddH_2O) were used as negative control. The construction of CRT expression vector pEGFP-C1-CRT and CRT-shRNA vector pCR3.1-CRT-shRNA was as previously described [20].

Construction of Stable Cell Lines

Tetracycline-inducible CRT expression vector pLKO_AS3.1-p5CRT-HY was constructed. pLKO_AS3.1 was the control vector of the inducible vector. CRT-shRNA expression vector was purchased from the National RNAi Core Facility Platform, Academia Sinica (Taipei, Taiwan). The shRNA target sequence was 5′-CCAGTATCTATGCCTATGATA-3′ (shCRTa, TRCN0000019989), 5′-CGTCTACTTCAAGG AGCAGTT-3′ (shCRTb, TRCN0000019991). pLKO.1 was the control vector of the shRNA plasmid.

Lentiviral stocks were produced by calcium phosphate transfection. Around 30–40 % confluent 293T cells in T25 flasks were prepared and transfected with a DNA mixture

containing 7.5 g packaged plasmids and 7.5 g lentivector of the target gene for 16 h. The transfected condition medium was replaced with 12-mL fresh DMEM containing 10 mM sodium butyrate. After 24 h, all conditioned media were harvested and treated with the stNB-V1 cells for infection. Cells were selected by 1 μg/μL puromycin (InvivoGen, USA).

RNA Isolation and RT

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen). Complementary DNA was synthesized with 1 μg total RNA using a Toyobo reverse transcription (RT)-polymerase chain reaction (PCR) kit (Toyobo, Osaka, Japan).

Quantitative Real-Time PCR

The real-time PCR with the mixture reagent KAPA SYBR-Green as the fluorescent dye (Bio-Rad) was conducted on a Mini-Opticon real-time detection system (Bio-Rad, Hercules, CA, USA). Gene-specific primers were used, and the specificity was confirmed by single melting curve after real-time PCR. Cycling conditions were 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

For quantification, the target gene was normalized to the GAPDH to act as an internal control for SH-SY5Y and SK-N-DZ cells and heat shock protein 60 (HSP60) for stNB-V1. P53, Bcl-2, and Bax were used as apoptotic markers [21]. Primers for the real-time PCR were as follows: GAPDH (F-5′-AAG GTG AAG GTC GGA GTC-3′ and R-5′-TGT AGT TGA GGT CAA TGA AGG-3′), HSP60 (F-5′-CA CCG T AA GCC TTT GGT CAT-3′ and R-5′-CTT GAC TGC CAC AAC CTG AA-3′), CRT (F-5′-CC TCC TCT TTG CGT TTC TTG-3′ and R-5′-CAG ACT CCA AGC CTG AGG AC), HIF-1α (F-5′-CAT AAT GTG AGT TCG CAT CT-3′ and R-5′-ATA TCC AAA TCA CCA GCA TC), VEGF-A (F-5′-GGC ACA CAG GAT GGC TTG AAG-3′ and R-5′-GGC ACA CAG GAT GGC TTG AAG-3′), p53 (F-5′-TGA CTG TAC CAC CAT CCA CTA-3′ and R-5′-AAA CAC GCA CCT CAA AGC-3′), Bax (F-5′-TGC TTC AGG GTT TCA TCC AG-3′ and R-5′-GGC GGC AAT CAT CCT CTG-3′), Bcl-2 (F-5′-AGG AAG TGA ACA TTT CGG TGA C-3′ and R-5′-GCT CAG TTC CAG GAC CAG GC-3′), GAP43 (F-5′-TCC GTC GAC ACA TAA CAA-3′ and R-5′-CAG TAG TGG TGC CTT CTC C-3′), neuron-specific enolase 2 (NSE2) (F-5′-TGT CTG CTG CTC AAG GTC AA-3′ and R-5′-CGA TGA CTC ACC ATG ACC C-3′), neurofilimant-H (NFH) (F-5′-CCG ACA TTG CCT CCT ACC-3′ and R-5′-GAG CCA TCT TGA CAT TGA GCA-3′), and TrkA (F-5′-TTG GCA TGA GCA GGG ATA TCT-3′ and R-5′-ACG GTA CAG GAT GCT CTC GG-3′).

Western Blot Analysis

Total proteins were extracted from cells using lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 % NP-40, 1 mM Na_3VO_4, and 10 % glycerol) with 10 % protease inhibitor cocktail. The cells were lysed for 15 min on ice and then spun at 4 °C and 13,000 rpm for 15 min. The supernatant was then collected for Western blotting. A Bio-Rad protein assay kit was used to measure protein concentration. Concentration-normalized lysates were boiled at 100 °C in an sodium dodecyl sulfate (SDS) sample buffer for 5 min. Proteins were fractionated by SDS polyacrylamide gel electrophoresis (PAGE) (80 V for 30 min in stacking gel and 120 V for 1.5 h in running gel) and transferred to nitrocellulose membranes (100 V for 60 min).

The membranes were blocked with 5 % BSA in 0.1 % Tween 20 in TBS (TBS-T), followed by overnight incubation at 4 °C with appropriate dilutions of primary antibody in 1 % TBS-T. After three washes with TBS-T (5 min each), the membranes were incubated with the appropriate secondary antibody coupled with horseradish peroxidase. Immunocomplexes were visualized using an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions.

The antibodies used were as follows: rabbit polyclonal anti-CRT antibody (Upstate Biotechnology, Lake Placid, NY), rabbit monoclonal anti-VEGF-A antibody (Santa Cruz, CA, USA), goat polyclonal anti-β-actin (Santa Cruz, CA, USA), and goat monoclonal anti-GAPDH antibody (Genetex, USA).

ELISA for VEGF-A Secreted Proteins in the Conditioned Medium

The cells were seeded in six-well plates at 3×10^5/well. The conditioned medium was collected after 48 h of transfection and analyzed by ELISA specific for human VEGF (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. The conditional medium was previously centrifuged to remove cells and other unnecessary particles. Samples and VEGF-A standards were added into microplates that were precoated with anti-human VEGF-A capture antibody. The microplates were incubated at room temperature on a horizontal orbital shaker for 2 h. After incubation, samples and standards were discarded and washed with wash buffer four times.

After aspiration of conjugates and a further wash, the substrate solution was added to each well, which were incubated for 30 min. After the reaction was completed, stop solution was added to end the reaction. The optical density of each well was measured with an ELISA plate reader set to a wavelength of 450 nm.

Cell Proliferation Assay

Cells were seeded in the 96-well plate at density of 10^3/100 μL, 5×10^3/100 μL, and 10^4/100 μL, respectively. Cells cultured in serum-free medium were used as negative control. After tetracycline induction for 48 h, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma) was added to each well to a final concentration of 0.05 % for reaction. After incubation at 37 °C for 4 h, MTT-containing medium were removed, and 50 μL dimethyl sulfoxide (DMSO) were added for 20 min at 37 °C to dissolve formazan. Reactions were monitored by 96-well ELISA plate reader at 595 nm.

Apoptosis Detection Assay

Cells (10^6) were seeded in 10-cm plate. Cells treated with 1 μg/μL actinomycin-D (Sigma, St. Louis, MO) were used as positive control. After tetracycline induction for 24 h, stNB-V1 cells were harvested and washed by cold PBS twice. Cell apoptosis rate was detected by using fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit (BD, Pharmingen, San Diego, CA). Cells (10^5) were suspended in 100 μL of 1× binding buffer. Harvested cells were then stained by 10 μL of FITC-conjugated Annexin V antibody and propidium iodide for 15 min. The stained cells were analyzed by BD FACSCanto2 cell flow cytometry.

VEGFR-1 Blockade

stNB-V_1 cells (2×10^5)were cultured in six-well plate. Cells were treated with 1 μg/mL goat polyclonal anti-human VEGF R1 antibody to block the VEGF-A signaling. Normal goat IgG was used as the negative control.

Statistical Analysis

Data analyses were performed using one-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference (LSD) test (StatView; Abacus Concept, Berkeley, CA, USA). Each result was obtained from at least three independent experiments and expressed as mean ±standard deviation. Statistical significance was set at $p<0.05$.

Results

CRT Positively Regulated VEGF-A and HIF-1α Expressions in CRT Overexpressing NB Cells

To investigate the relationship between CRT and VEGF-A, CRT was overexpressed using pEGFP-C1-CRT expression vector via Lipofectamine 2000 transfection system in SK-N-DZ and SH-SY5Y cells. According to real-time PCR analysis, the expression vector significantly enhanced CRT messenger RNA (mRNA) expression in SK-N-DZ and SH-SY5Y to 1,800- and 1,400-fold higher, respectively, compared to the negative control (none) and vector control (pEGFP-C1) (Fig. 1a). This CRT overexpression at the protein level was also confirmed by Western blotting (Fig. 1b).

To elucidate the effects of CRT on VEGF-A and HIF-1α, VEGF-A mRNA expression was analyzed in transiently CRT-overexpressing NB cells. The overexpression of CRT increased VEGF-A mRNA expression in both SK-N-DZ and SH-SY5Y cells (Fig. 1c). The mRNA expression level of HIF-1α, a well-known upregulator of VEGFs in NB, also positively correlated with CRT level [22]. Elevated HIF-1α expression suggested that HIF-1α might be involved in the CRT-dependent VEGF-A upregulation.

VEGF-A and HIF-1α Were Downregulated in CRT Knockdown NB Cells

To further clarify the relationship between CRT and VEGF-A, CRT was transiently knocked down using shRNA in SK-N-DZ and SH-SY5Y cells. According to real-time PCR analysis, the CRT mRNA expression levels were significantly inhibited by the pCR3.1-CRT-shRNA in both SK-N-DZ and SH-SY5Y cells (Fig. 2a). The knockdown of CRT was further confirmed at the protein level by Western blotting (Fig. 2b).

To examine the effect of CRT knockdown on VEGF-A and HIF-1α, VEGF-A and HIF-1α mRNA expressions were analyzed in transiently CRT-knocked down NB cells. The VEGF-A and HIF-1α mRNA expressions were lower in SK-N-DZ and SH-SY5Y cells with CRT knockdown (Fig. 2c). The results further confirmed that CRT could regulate VEGF-A and its upregulator, HIF-1α expression in NB cells.

Effects of CRT on VEGF-A Protein Expression and Secretion Level in NB Cells

Whether the CRT expression affected the VEGF-A protein expression in NB cells was further determined. Results showed that VEGF-A protein expression was upregulated by CRT overexpression both in SK-N-DZ and SH-SY5Y cells (Fig. 3a). Moreover, knockdown of CRT decreased the protein expression of VEGF-A (Fig. 3a). The results demonstrated that CRT positively regulated VEGF-A both at the mRNA and protein level in different NB cells.

The VEGF family members, including VEGF-A, are known to be secreted polypeptides. The VEGF-A secretion level was then examined in the conditioned media. Results showed that the CRT overexpression upregulated VEGF-A secretion in the conditioned media of SK-N-DZ and SH-

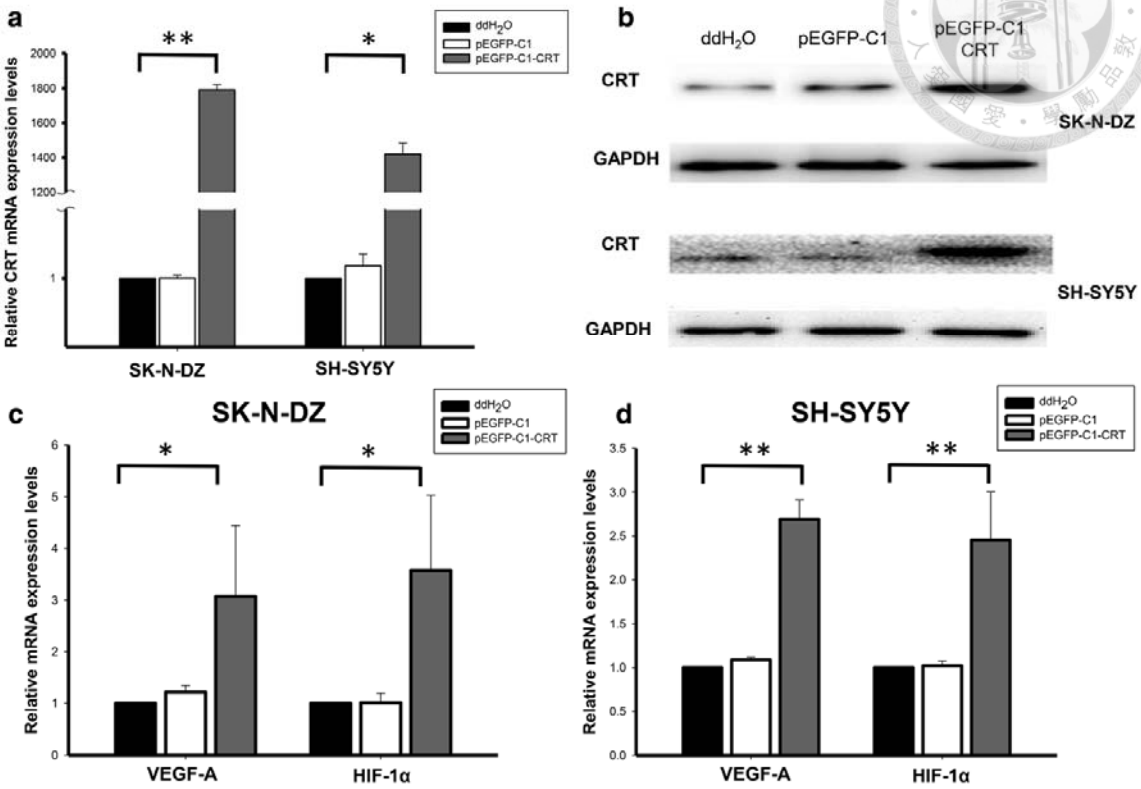

Fig. 1 CRT overexpression upregulated VEGF-A and HIF-1α in SK-N-DZ and SH-SY5Y NB cells. **a** CRT was overexpressed via Lipofectamine 2000 transfection in SK-N-DZ and SH-SY5Y cells. The CRT mRNA expression level was confirmed by real-time PCR, and CRT mRNA was normalized to the internal control GAPDH. **b** Western blot analysis showed CRT protein expression in SK-N-DZ and SH-SY5Y cells. The human GAPDH level was used as a loading control. **c** The mRNA expressions of VEGF-A and HIF-1α were upregulated both in SK-N-DZ and SH-SY5Y cells with CRT overexpression. These results were confirmed by at least three independent experiments. Each bar of the histogram represents quantified results and shown as the mean±SD. Statistical differences were compared with the control level. *$p<0.05$; **$p<0.01$; ***$p<0.005$

SY5Y cells (Fig. 3b). On the other hand, CRT knockdown suppressed the VEGF-A protein secretion in conditioned media (Fig. 3b). These results further supported the effects of CRT on VEGF expression and secretion in different NB cells.

Establishment of Stable Cell Lines

In order to further clarify the effects of CRT on NB cell lines, CRT overexpression and knockdown stable cell lines were selected. However, constitutive overexpression of CRT led to NB cell differentiation without proliferation (data not shown). Thus, an inducible CRT stNB-V1 cell line was generated by a tetracycline-regulated gene system. To induce CRT expression, cells were treated with tetracycline. After 1 μg/μL tetracycline induction for 24, 48, and 72 h, CRT mRNA expression was elevated by 3-, 6-, and 4-fold higher, respectively, than non-induced cells (Fig. 4a). As such, 48-h induction was chosen as the best condition for subsequent experiments. By Western blotting, the protein expression of CRT was also enhanced by tetracycline induction (Fig. 4b).

For knockdown stable cell lines, stNB-V1 NB cells were transfected with a CRT-shRNA plasmid via lentiviral system and then selected by respective antibiotics. After selection by puromycin, CRT-shRNA cells were generated (19989 and 19991) and CRT knockdown efficiency was confirmed by real-time PCR (Fig. 4c).

CRT Positively Regulated VEGF-A and HIF-1α Expressions in Stable Cell Lines

The expressions of VEGF-A and HIF-1 were examined in inducible-CRT stNB-V1cells. After 48 h of tetracycline treatment, the mRNA expressions of VEGF-A and HIF-1 were significantly augmented (Fig. 5). The positive regulation of CRT on VEGF-A protein expression was also demonstrated in inducible CRT stNB-V1cells (Fig. 4b), revealing that CRT

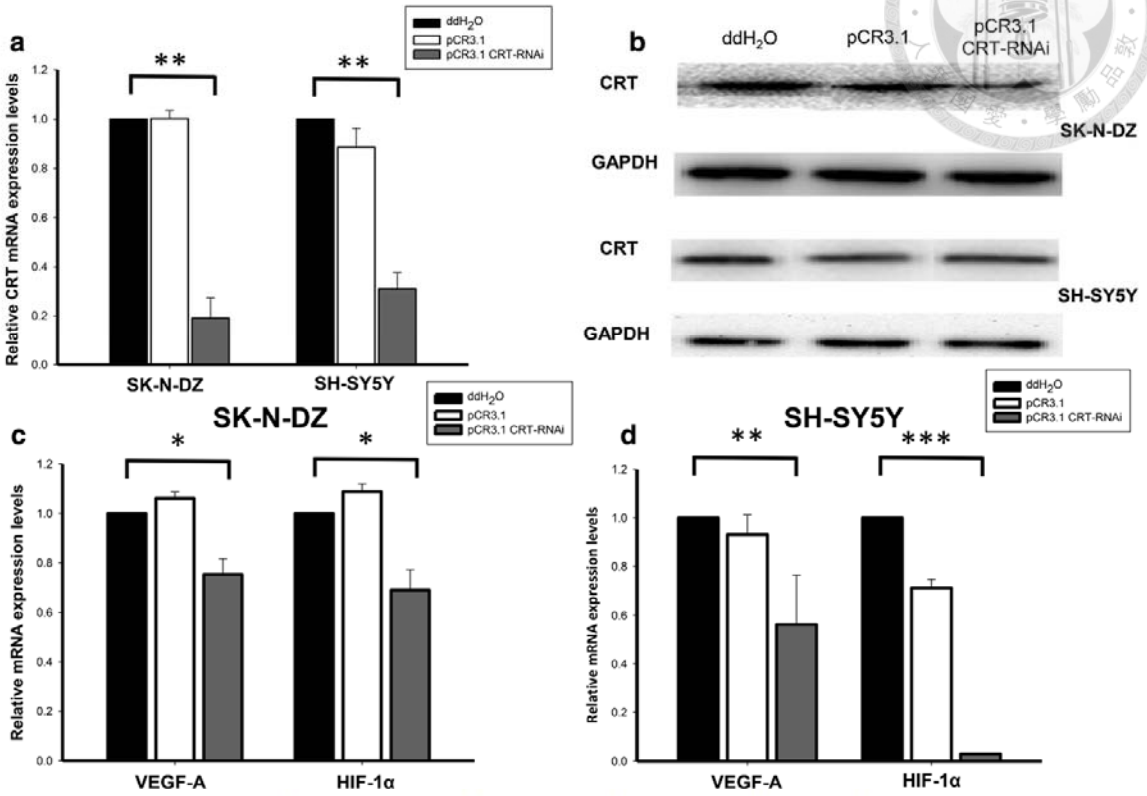

Fig. 2 Knockdown of CRT downregulated VEGF-A and HIF-1α in SK-N-DZ and SH-SY5Y NB cells. **a** CRT was knocked down via Lipofectamine 2000 transfection in SK-N-DZ and SH-SY5Y cells. The CRT mRNA expression level was confirmed by real-time PCR, and CRT mRNA was normalized to the internal control GAPDH. **b** Western blot analysis showed CRT protein expression in SK-N-DZ and SH-SY5Y cells. The human GAPDH level was used as a loading control. **c** The mRNA expression of VEGF-A and HIF-1α were downregulated both in SK-N-DZ and SH-SY5Y cells with CRT knockdown. These results were confirmed by at least three independent experiments. Each bar of the histogram represents quantified results and is shown as the mean±SD. Statistical differences were compared with the control level. *$p<0.05$; **$p<0.01$; ***$p<0.005$

positively regulated VEGF-A and HIF-1α expressions in different NB cells.

The VEGF-A and HIF-1α mRNA expression levels were further examined in CRT knockdown stable cell lines (stNB-V1, 19989) and revealed lower mRNA expressions (Fig. 6). These results further confirmed that CRT regulated the VEGF-A and HIF-1α expression levels in different NB cells.

Apoptotic Rate of NB Cells Was Not Affected by CRT Overexpression

To clarify the impact of CRT overexpression on NB cell apoptosis, we use Annexin V/propidium iodide staining to determine the apoptotic cells in inducible CRT stNB-V1 cells. Actinomycin-D, a potent inducer for apoptosis in several cell lines, was used as positive control. After tetracycline treatment, the fluorescence intensity of apoptotic cells was not different from cells without tetracycline treatment (Fig. 7a), which indicated that CRT overexpression did not affect apoptosis in stNB-V1 cells. The mRNA expression levels in several apoptotic markers including p53, Bcl-2, and Bax were further determined by real-time PCR [21]. As shown in Fig. 7b, no difference in p53, Bcl-2, and Bax mRNA expressions was observed after tetracycline induction in inducible CRT stNB-V1 cells. These results further confirmed that overexpression of CRT did not induce NB cell apoptosis.

Cell Proliferation Rate is Lower in CRT Overexpressing NB Cells

We previously found that constitutive overexpression of CRT led to NB cell differentiation without proliferation in SK-N-DZ and SH-SY5Y cells. Thus, we have generated an inducible CRT stNB-V1 cell line. In order to further confirm the effect of CRT overexpression on cell proliferation, we used MTT assays to evaluate the cell proliferation rate in stNB-V1 cells.

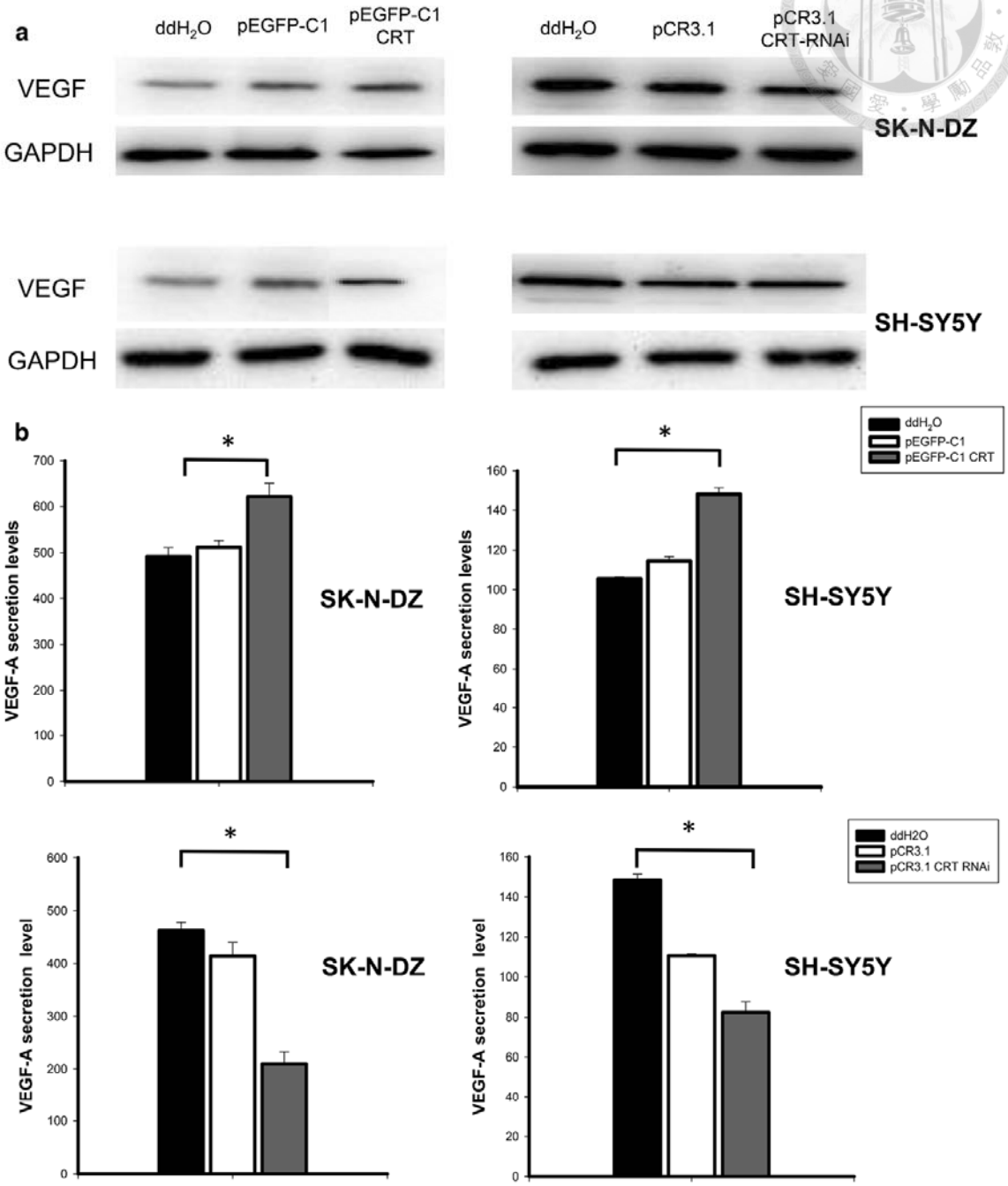

Fig. 3 VEGF-A protein expression and secretion level were upregulated in SK-N-DZ and SH-SY5Y NB cells with CRT overexpression but decreased by the knockdown of CRT in SK-N-DZ and SH-SY5Y NB cells. **a** Western blot analysis showed VEGF-A protein expression in SK-N-DZ and SH-SY5Y cells. The human actin level was used as a loading control. In both cell lines, VEGF-A was upregulated in pEGFP-C1 CRT cells and downregulated in pCR3.1-CRT-RNAi cells. **b** VEGF-A secretion level in the conditioned media was higher by CRT overexpression and lower by CRT knockdown, as analyzed by ELISA. All of the results were repeated in at least three independent experiments. Statistical differences were compared with the control level. $*p<0.05$

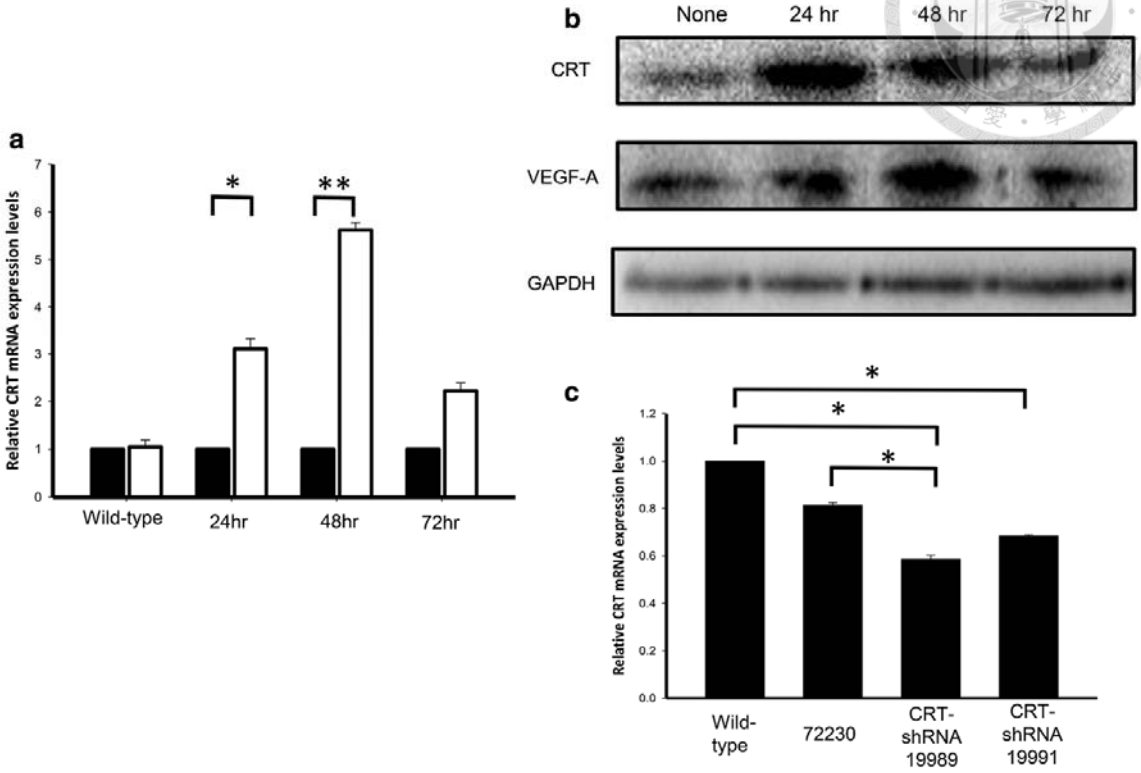

Fig. 4 stNB-V1 NB cells were stably transfected with CRT overexpressing and shRNA plasmids. **a** stNB-V1 NB cells were stimulated with 1 μg/mL tetracycline to induce CRT expression. The CRT mRNA expression level was normalized to the internal control HSP60 and was highest 48 h after tetracycline treatment. **b** The CRT protein expression after tetracycline-induced was also confirmed by Western blot analysis. The human GAPDH level was used as a loading control. **c** The CRT mRNA expression level was confirmed by real-time PCR in CRT knockdown stable cell lines (19989 and 19991). The CRT mRNA expression level was normalized to the internal control HSP60. All of the results are repeated in at least three independent experiments. Each bar of the histogram represents quantified results and is shown as the mean±SD. Statistical differences were compared with the control level. *$p<0.05$; **$p<0.01$

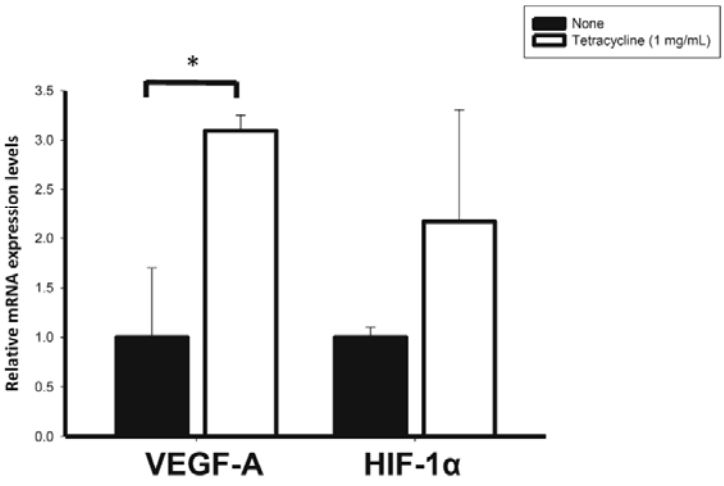

Fig. 5 The VEGF-A and HIF-1α expressions were regulated by CRT expression induced by tetracycline treatment in stNB-V1 cells. stNB-V1 NB cells were stimulated with 1 μg/mL tetracycline to induce CRT expression. The VEGF-A and HIF-1 mRNA expression levels were normalized to the internal control HSP60 and were increased after tetracycline treatment. These results were examined in at least three independent experiments. Statistical differences were compared with the control level. *$p<0.05$

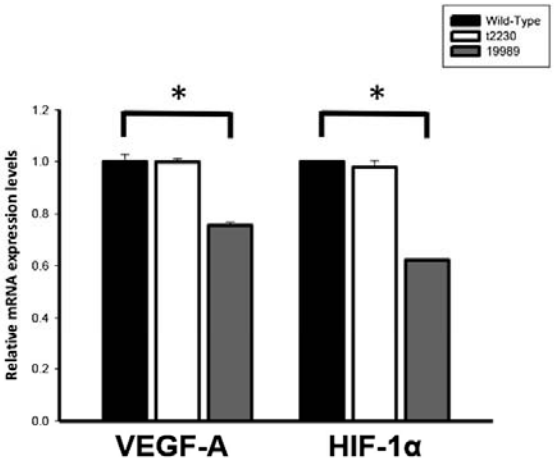

Fig. 6 The VEGF-A and HIF-1α mRNA expressions were lower in CRT knockdown stable stNB-V1 cells. The VEGF-A and HIF-1α mRNA expressions were confirmed by real-time PCR in CRT knockdown stable cell lines (19989). The mRNA expression level was normalized to the internal control HSP60. All of the results are repeated in at least three independent experiments. Statistical differences were compared with the control level. *$p<0.05$

Cells cultured in serum-free medium were used as negative control. After tetracycline induction, the MTT readings were significantly decreased (Fig. 8), indicating that upregulation of CRT suppressed proliferation of NB cells.

VEGF-A is Involved in CRT-Induced NB Cell Differentiation

To further clarify the roles of CRT-dependent VEGF-A upregulation on NB cell differentiation, the expressions of neuron-specific markers including GAP43, neuron-specific enolase 2 (NSE2), and neurofilimant-H (NFH), as well as TrkA, a molecular marker indicative of NB cell differentiation, were examined in inducible CRT stNB-V1cells [23, 24]. After tetracycline induction, the mRNA expression levels of GAP43, NSE2, NFH, and TrkA were significantly augmented (Fig. 9), which is compatible with our previous study suggesting that CRT is critical for NB differentiation.

We next used anti-VEGFR-1 antibodies to examine whether upregulated VEGF-A is involved in CRT-related neuronal differentiation in NB. VEGF-A binds to two related receptor tyrosine kinases, VEGFR-1 and VEGFR-2, and regulates downstream biological signaling. Antibodies against VEGFR-1 could partially block the VEGF-A signaling. As shown in Fig. 9, after treatment with anti-VEGFR-1 antibodies, the mRNA expressions of GAP43, NSE2, NFH, and TrkA were suppressed significantly in both CRT overexpression (treated with tetracycline) and controlled (treated with ddH2O) stNB-V1 cells. These results implied the involvement of VEGF-A in neuronal differentiation in NB.

Discussion

Recent advances in understanding of the genetics and biology of NB have allowed risk-adapted therapeutic strategies [2]. Our previous study has identified CRT as an independent favorable prognostic marker which is related to differentiated histologies in NB [14]. This study was designed to determine whether the CRT expression in NB was associated with the VEGF-A pathway. In the present study, an inducible CRT NB

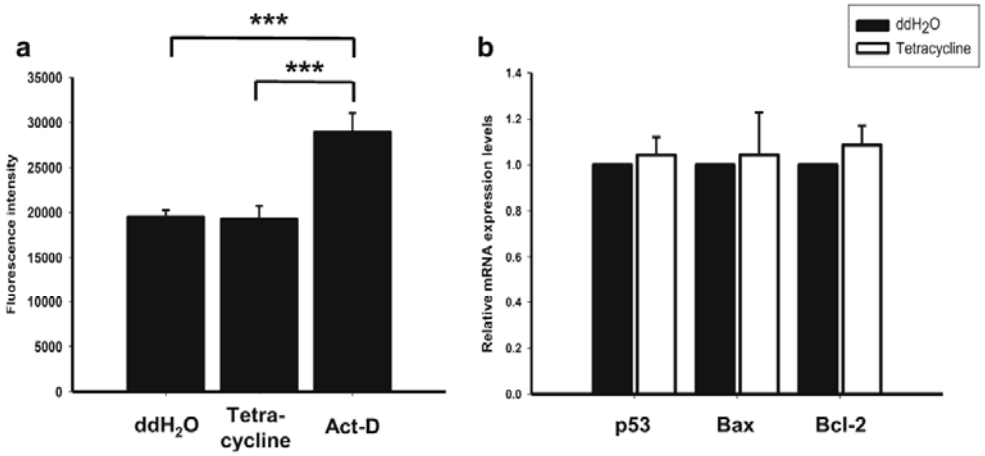

Fig. 7 Apoptosis was not affected by CRT expression induced by tetracycline treatment in stNB-V1 cells. stNB-V1 NB cells were stimulated with 1 μg/mL tetracycline to induce CRT expression. **a** Apoptosis was evaluated by Annexin V/propidium iodide staining. Treatment of actinomycin-D was used as positive control. The fluorescence intensity of stained cells were analyzed by flow cytometry and were no differences after tetracycline treatment. **b** The p53, Bcl-2, and Bax mRNA expressions were normalized to the internal control HSP60 and showed no differences after tetracycline treatment. All of the results were examined in at least three independent experiments. ***$p<0.005$

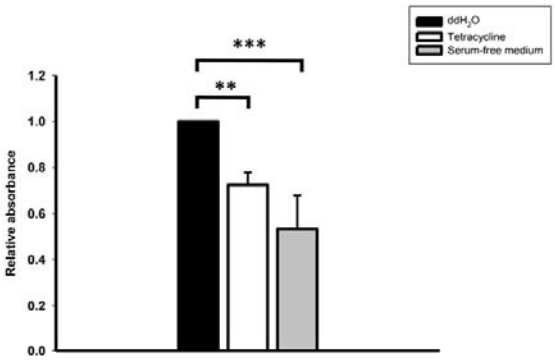

Fig. 8 Overexpression of CRT reduced the proliferation rate of stNB-V1 cells. stNB-V1 NB cells were stimulated with 1 μg/mL tetracycline to induce CRT expression. Cells cultured in serum-free medium were used as negative control. Cell proliferation was evaluated by MTT assay. Cells were seeded in the 96-well plate (10,000 cells/well). Cell numbers were determined by measuring the absorbance at 595 nm and were significantly lower after tetracycline treatment. These results were confirmed by at least three independent experiments. $^{**}p<0.01$; $^{***}p<0.005$

cell line is established because constitutive CRT overexpression leads to NB differentiation without proliferation. This study, for the first time, clearly demonstrates that in different NB cell lines, CRT overexpression increases the expression and secretion of VEGF-A and HIF-1α, a major positive regulator of VEGF-A. In contrast, knockdown of CRT decreases VEGF-A and HIF-1α expressions. Furthermore, we confirmed that NB cell apoptosis was not affected by CRT overexpression, while overexpression of CRT suppressed cell proliferation and enhanced cell differentiation in NB. Blockage of VEGF-A signaling using anti-VEGFR-1 antibodies markedly suppressed the expressions of GAP43, NSE2, NFH, and TrkA, indicating an essential role of VEGF-A in CRT-related neuronal differentiation in NB.

CRT, a well-known multifunctional protein, is located not only in the cytosol but also on the cell surface and in the extracellular environment to modulate a number of physiologic and pathologic conditions [9, 12]. In some cancers, CRT is found to be upregulated in tumor tissues compared to normal tissues. Previous studies have also found that increased CRT expression is associated with tumorigenesis and metastasis of gastric cancer and bladder cancer [10, 20]. Conversely, in NB, increased CRT expression is associated with better prognosis and differentiated histologies [14]. The present study demonstrates that constitutive overexpression of CRT in different NB cells lead to NB cell differentiation without proliferation and make it difficult to establish stable cell lines. It has been found that CRT is essential for neurite formation when NB cells are induced to differentiate [11, 12]. Available evidence is consistent with the results here showing that CRT is critical for NB differentiation. Thus, an inducible CRT cell line has been generated via tetracycline-regulated gene system and CRT overexpression is initiated by tetracycline treatment.

Converging evidence showed that NB cells exhibit a capacity of differentiating into neuron-like cells or regression by apoptosis [5, 6]. In previous studies, increased CRT expression was found to be associated with differentiation histology in NB [13]. The present studies clearly demonstrated that CRT could suppress cell proliferation and enhance cell differentiation whereas apoptosis was not altered in NB cells, implying CRT as an important favorable prognostic factor in NB. These results were compatible with our previous study revealing that CRT expression predicts favorable survival in NB patients [14].

Studies have shown that CRT can regulate VEGF (also referred to as VEGF-A) expression in gastric cancers [20]. The present studies in NB also demonstrate that CRT can regulate VEGF-A expression. VEGF-A is a well-recognized pro-angiogenic factor and a key regulator of physiologic and pathologic angiogenesis [15]. In gastric cancers, CRT has been found to upregulate VEGF expression and enhance angiogenesis, leading to poor prognosis [20]. However, it has also been reported that exogenous CRT and the CRT fragment vasostatin are anti-angiogenic factors that directly target endothelial cells and suppress tumor growth [25, 26]. The role of CRT in tumorigenesis remains elusive. In this study, CRT overexpression increased HIF-1α expression with subsequent upregulation of VEGF-A in three different NB cells. Knockdown of CRT suppressed HIF-1α expression and downstream VEGF-A transcription, translation, and further secretion. Moreover, it has been previously shown that increased CRT expression is associated with favorable outcome in NB patients. Therefore, it is postulated that the CRT-upregulated VEGF-A expression does not necessarily promote angiogenesis in NB. The effect of CRT on angiogenesis may depend on different cell types and stages. Further investigation is warranted to clarify the correlation between CRT and angiogenesis in NB.

In NB, angiogenesis is an essential mechanism regulating NB tumorigenesis, while VEGF-A-driven angiogenesis plays a critical role in the pathogenesis of NB formation and metastasis [27–29]. Both in human and in experimental NB, overexpression of VEGF-A has been demonstrated and correlated with a high-risk phenotype [17, 18]. However, another study reveals that tumor vascularity is not correlated with prognosis and tumor stage in NB patients [30]. Moreover, it has been shown that there is no tumor growth difference in NB xenograft mice treated with or without anti-VEGF antibody [31]. As such, the role of VEGF-A in NB tumorigenesis and outcome is complicated and needs further investigation. In the present study, CRT can upregulate VEGF-A expression in three different NB cells. Taken together with a previous study showing the increased CRT expression correlates with differentiation in NB patients, it can be surmised that VEGF-A-

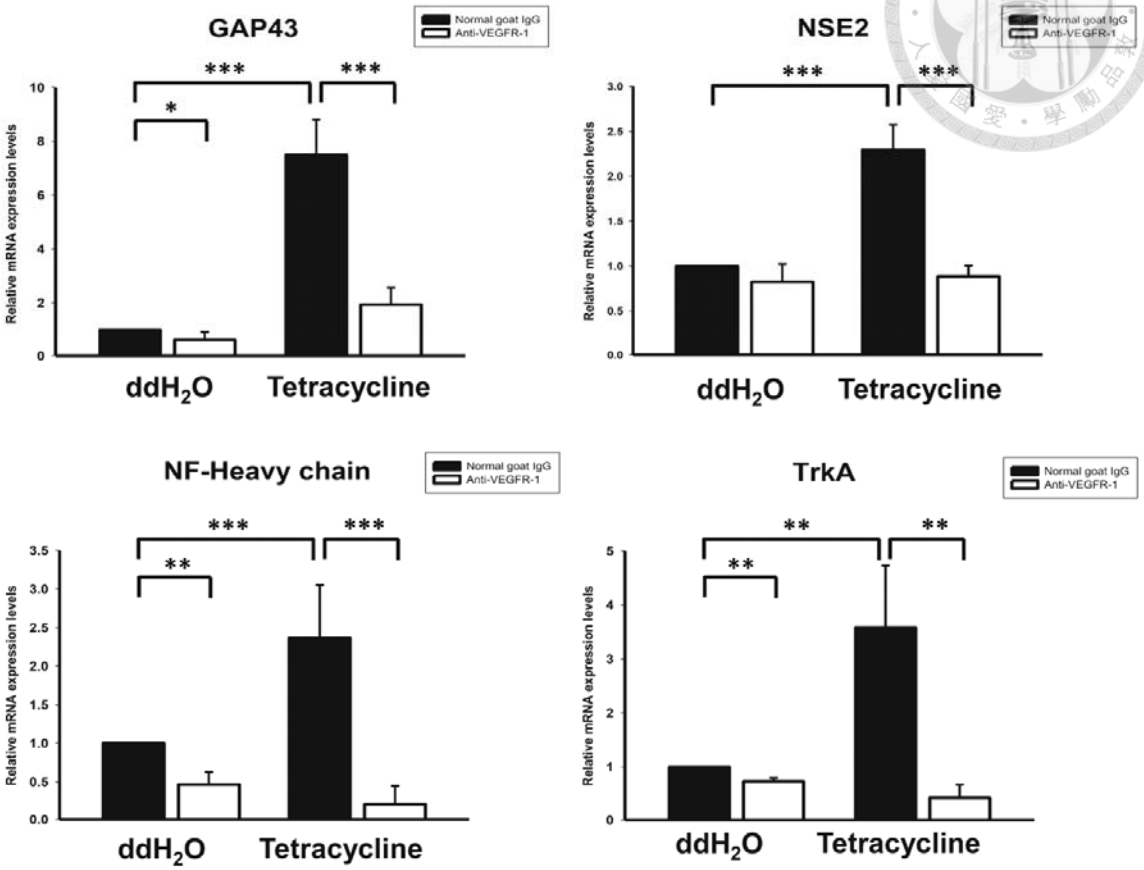

Fig. 9 CRT-induced neuronal markers expression in NB cells is VEGF-A dependent. stNB-V1 NB cells were stimulated with 1 μg/mL tetracycline to induce CRT expression. The mRNA expression levels of GAP43, NSE2, NFH, and TrkA were confirmed by real-time PCR and were increased after tetracycline treatment (*dark bar*). The mRNA expression level was normalized to the internal control HSP60. After treatment with anti-VEGFR-1 antibody, the mRNA expressions of GAP43, NSE2, NFH, and TrkA were suppressed significantly in both CRT overexpression (treated with tetracycline) and controlled (treated with ddH_2O) stNB-V1 cells. These results were examined in at least three independent experiments. $*p<0.05$; $**p<0.01$; $***p<0.005$

mediated angiogenesis plays a role in the differentiation and maturation of NB [13]. In a recent study, investigators explored the role of angiogenesis in the maturation phase of NB [32] and found VEGF expression in differentiating neuroblastic cells of patients as well as in SH-SY5Y cells during differentiation after retinoic acid treatment. These findings are consistent with the results here. VEGF-A-associated angiogenesis is not only associated with tumor aggressiveness but also plays an essential role for NB differentiation and maturation, which is similar to the physiologic events bringing about the maturation of the vasculature in normal neuronal development.

HIF-1α is a major positive regulator of VEGF-A and is activated during tissue hypoxia. Previous studies have revealed that HIF-1α upregulation is associated with tumor aggressiveness [33]. In our study, for the first time, we demonstrated that CRT could also upregulate HIF-1α and we postulated that CRT upregulating VEGF-A might be HIF-1α-depedent. HIF-1α may also play a crucial role during the differentiation of NB by itself or via angiogenesis activated by VEGF-A. Further study is warranted to clarify the role of HIF-1α in NB cell differentiation.

Apart from its major role in angiogenesis, VEGF-A also has a direct effect on neurons and modulates various neuronal functions, including neuronal proliferation, migration, survival, axon guidance, and differentiation [34, 35]. Studies, both in vitro and in vivo, have shown that VEGF upregulation can promote neuronal differentiation, while VEGF downregulation inhibits neuronal differentiation in stroke and hypoxia models [36–39]. In the present study, partial blockage of VEGF-A signaling via anti-VEGFR-1 antibodies significantly suppressed the expressions of GAP43, NSE2, NFH, and

TrkA, which are molecular markers indicative of neuronal differentiation in NB. These results clearly demonstrated the involvement of VEGF-A in neuronal differentiation in NB. Thus, based on current and previous studies, CRT-dependent VEGF-A upregulation is critical for NB differentiation. Besides, VEGF-A may participate in the CRT-mediated NB differentiation via both angiogenesis-dependent and angiogenesis-independent pathways.

This study examines the role of VEGF-A in CRT-related NB differentiation in different NB cells. An inducible CRT NB cell line is established because CRT overexpression leads to NB differentiation without proliferation. This study shows that CRT can upregulate VEGF-A expression and enhance NB cell differentiation. Blockage of VEGF-A signaling can suppress neuronal differentiation in NB cells. In conclusion, VEGF-A participates in CRT-related neuronal differentiation in NB. This study provides crucial information needed for developing a new therapeutic strategy to improve outcome in NB patients. Further studies are warranted to clarify the role of CRT and VEGF-A in NB behavior and biology in vivo.

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Conflict of Interest The authors declare that they have no conflict of interest.

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