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CONNEXIN36 IS A NEGATIVE REGULATOR OF DIFFERENTIATION IN HUMAN NEUROBLASTOMA CELLS

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CONNEXIN36 IS A NEGATIVE REGULATOR OF DIFFERENTIATION IN HUMAN NEUROBLASTOMA CELLS

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Abstract

Gap junction channels permit intercellular communication through the passage of small molecules and metabolites between adjacent cells and are involved in regulating cellular processes such as homeostasis, differentiation, and proliferation. Connexins (Cx) are the constituent proteins of gap junctions and the neuronal connexin, Cx36, is highly expressed in the developing nervous system. In the present study, the effect of Cx36 on differentiation was investigated by manipulating Cx36 expression levels and inducing differentiation of SH-SY5Y cells. Following retinoic acid induced differentiation of SH-SY5Y cells, Cx36 mRNA expression increased, while Cx45 mRNA expression was unaffected. Cx36 overexpression resulted in diminished retinoic acid induced neuritogenesis, accompanied by decreased expression of differentiation markers, Gap43 and NPY, while knockdown of Cx36 had the reverse effect. Accordingly, overexpression of Cx36 resulted in increased proliferation of SH-SY5Y cells while Cx36 knockdown decreased cell proliferation. In conclusion, Cx36 is a transcriptional target of retinoic acid signaling and acts as a negative regulator of differentiation in SH-SY5Y cells.

Keywords: Cx36, Cx45, SH-SY5Y, neuroblastoma, retinoic acid, differentiation, aggregation, proliferation

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List of Abbreviations

4-HPR	N-(4-hydroxyphenyl) retinamide
APL	Acute promyelocytic leukemia
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BDNF	Brain derived neurotrophic factor
β	Beta
BMP4	Bone morphogenic protein 4
CNS	Central nervous system
Cx	Connexin
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
Gap43	Growth associated protein 43
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GJIC	Gap junctional intercellular communication
HBSS	Hank's buffered saline solution
Hz	Hertz
ID2	Inhibition of Differentiation/ DNA binding 2
kDa	Kilodalton
KO	Knockout
NC	Neural Crest
NGF	Nerve growth factor

mRNA	Messenger ribonucleic acid
µl	Microliter
µm	Micrometer
µM	Micromolar
ml	Milliliter
ng	Nanogram
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYCN	Myc Myelocytomatosis Viral Related Oncogene
NCAM	Neural cell adhesion molecule
NPY	Neuropepetide Y
PCR	Polymerase chain reaction
pH	Power of hydrogen
PKA	Protein kinase A
PKC	Protein kinase C
PPIA	Peptidylprolyl isomerase A (cyclophilin A)
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
rpm	Revolutions per minute
RXR	Retinoid X receptor
S.E.	Standard error
shRNA	Small hairpin ribonucleic acid

TPA	12-O-tetradecanoylphorbol-13-acetate
Trk	Neurotrophin tyrosine kinase receptor
ZO	Zona occludens

CHAPTER ONE:

INTRODUCTION

1.1 Gap Junctions

1.1.1 Gap Junction Function

Gap junctions are important for intercellular communication and are present within most adult mammalian cells (Sohl et al., 2005; Laird, 2006). They consist of channels that allow electrical and chemical messaging between adjacent cells via the passage of ions, second messengers and metabolites less than 1kDa in size (Saez et al., 1989; Goldberg et al, 1999; Alexander & Goldberg, 2003). Adenosine triphosphate, cyclic adenosine monophosphate, and inositol triphosphate are examples of molecules that are able to pass through gap junction channels (Goldberg et al, 1999). Within neurons, gap junctions are responsible for the formation of bidirectional electrical synapses, allowing synchronization of electrical activity between many cells (Connors and Long, 2004). Gap junctional intercellular communication (GJIC) allows for the regulation of processes such as homeostasis, cell growth, differentiation, and synchronization (Vinken et al., 2006; Churko et al., 2010; Prost et al., 2008; Ionta et al., 2009; Omori et al., 2001).

1.1.2 Structure, Synthesis and Regulation of Gap Junctions

Connexins (Cx) are the substituent proteins of gap junction channels (Figure 1.1). They consist of four membrane-spanning domains, 2 extracellular loops, and 3 cytoplasmic components, including a cytoplasmic loop, and amino (N)- and carboxy (C)-terminals (Laird, 1996; Mese et al., 2007). The diversity of connexins arises from the cytoplasmic loop and the C-terminus, whereas the transmembrane domains, N-terminus, and extracellular loops are highly conserved among connexin family members (Duffy et

al., 2002). The nomenclature of connexins is based on the predicted molecular mass of the connexin protein; for example Cx36 is named according to its predicted molecular mass of 36 kDa (Condorelli et al., 2000).

During the synthesis of gap junctions, connexins are co-translationally inserted into the endoplasmic reticulum (ER) where they fold and translocate to the Golgi apparatus. Connexins oligomerize in the Golgi to form hexamers known as connexons (Falk et al, 1994; Musil & Goodenough, 1993). However, some connexins appear to form hexamers in the ER rather than the Golgi (Koval, 2006). These connexons are then transported in a closed state, within vesicles to the cell membrane. Connexons dock with connexons of the apposing cell membrane to form gap junction channels. The channels diffuse through the lipid bilayer to form gap junction plaques consisting of hundreds of individual gap junction channels (Laird et al., 1995; Laird, 1996).

Connexins are dynamic and tightly regulated proteins, as indicated by their short half-lives of 1-2 hours. This allows connexins to be rapidly trafficked to and be removed from the cell surface depending on the metabolic needs of the cell (Laird et al., 1991; Laird et al., 1995). Connexins associate with several binding partners, including the zona occludins (ZO), β catenin, tubulin, and the cadherins, which may serve to regulate connexin function (Toyofuku et al., 1998; Ai et al., 2000; Geipmans et al., 2001; Geipmans et al., 2001). For example, ZO-1 acts as a chaperone to facilitate efficient trafficking of Cx43 to the cell membrane, while N-cadherin mediates gap junction plaque formation (Duffy et al., 2002; Wei et al., 2005). Connexins are also regulated by several kinases, for example, the phosphorylation of Cx43 by Protein Kinase C (PKC) results in channel closure (Solan & Lampe, 2005).

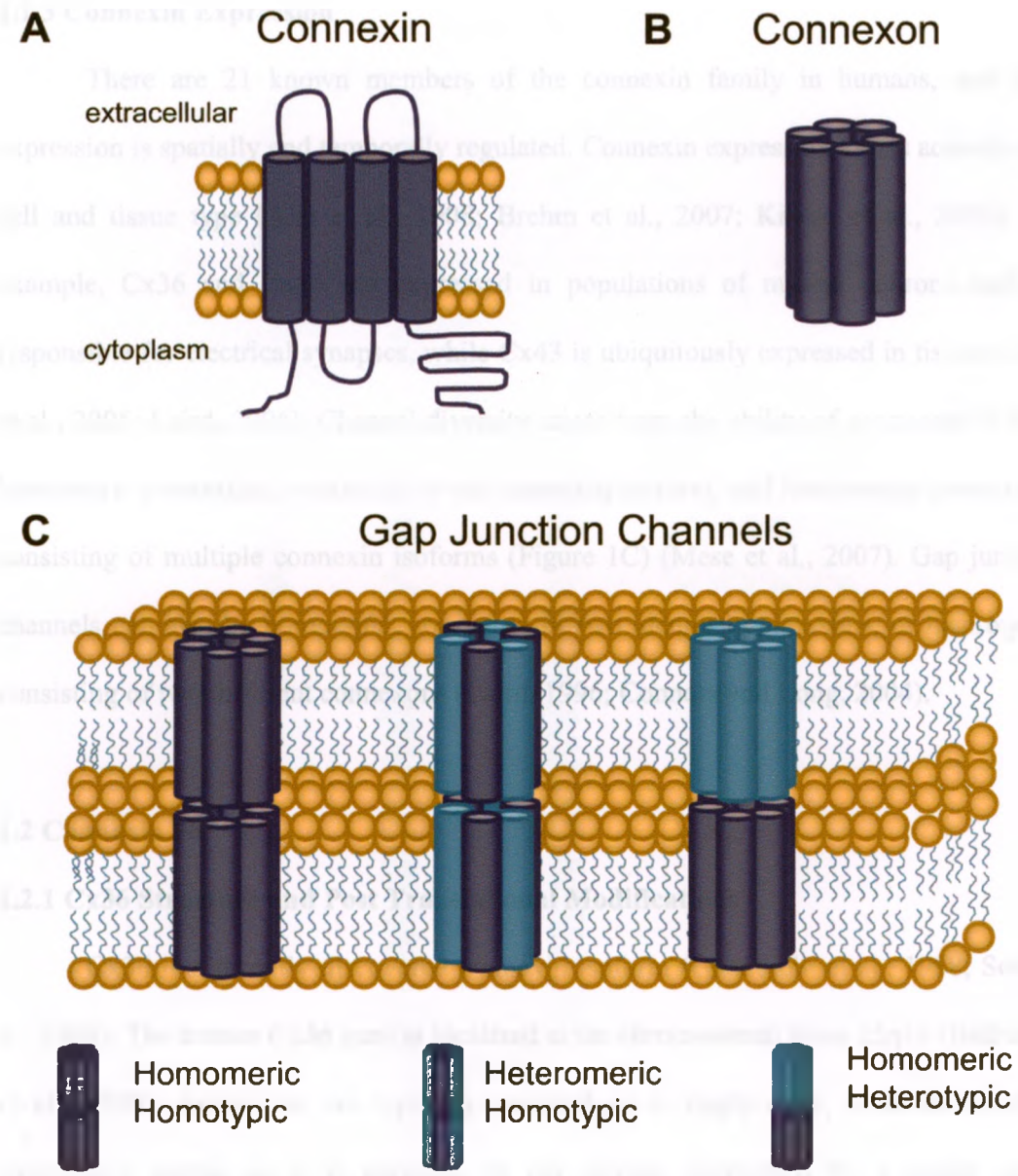

Figure 1.1. The Structure of Gap Junctions.

Gap junctions are comprised of connexin subunits. Each connexin consists of 4 transmembrane domains, 2 extracellular loops, a cytoplasmic loop, and amino and carboxy termini (A). Six connexins oligomerize to form a homomeric or heteromeric connexon (B), which docks with a connexon from an adjacent cell to form a homotypic or heterotypic gap junction channel (C).

1.1.3 Connexin Expression

There are 21 known members of the connexin family in humans, and their expression is spatially and temporally regulated. Connexin expression varies according to cell and tissue type (Ahn et al., 2008; Brehm et al., 2007; Kihara et al., 2006). For example, Cx36 and Cx45 are expressed in populations of mature neurons and are responsible for electrical synapses, while Cx43 is ubiquitously expressed in tissues (Sohl et al., 2005; Laird, 2006). Channel diversity arises from the ability of connexins to form homomeric connexons, consisting of one connexin isoform, and heteromeric connexons, consisting of multiple connexin isoforms (Figure 1C) (Mese et al., 2007). Gap junction channels can also be homotypic, consisting of two identical connexons, or heterotypic, consisting of two different connexons (Laird, 1996; Connors and Long, 2004).

1.2 Connexin 36

1.2.1 Cx36 Structure and Post Translational Modifications

Cx36 is a recently discovered connexin isoform (Condorelli et al., 1998; Sohl et al., 1998). The human Cx36 gene is localized at the chromosomal locus 15q14 (Belluardo et al., 1999). Connexins are typically encoded on a single exon, however Cx36 is structurally unique as it is encoded on two exons, interrupted by a single intron (Belluardo et al., 1999). This places Cx36 in a novel delta subgroup of the connexin family (Condorelli et al., 2000). The Cx36 protein consists of 321 amino acid residues, and is characterized by a large cytoplasmic loop and short C-terminus (Figure 1.2) (Condorelli et al., 1998; Condorelli et al., 2000; Claros & von Heijne, 1994).

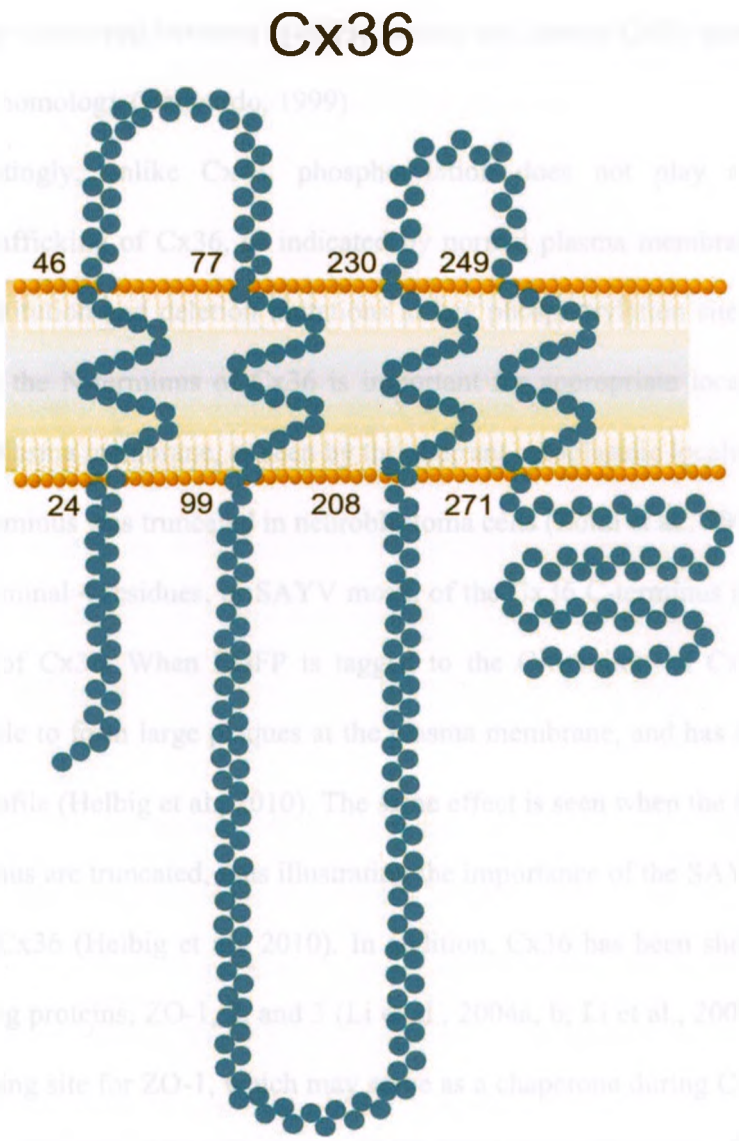

Figure 1.2. Topology of Cx36

Connexin36, like other connexins, consists of four transmembrane domains, 2 extracellular loops (EL), a cytoplasmic loop (CL), and intracellular amino (NH_2) and carboxy termini (COOH). This Cx36 structural model was created using specifications from TopPred 2 software. Cx36 is characterized by a large cytoplasmic loop, consisting of 111 amino acids, and a short carboxy terminus, comprised of only 51 amino acids.

Cx36 is highly conserved between species; human and mouse Cx36 share 98% amino acid sequence homology (Belluardo, 1999).

Interestingly, unlike Cx43, phosphorylation does not play a key role in intracellular trafficking of Cx36, as indicated by normal plasma membrane localization following substitution and deletion mutations at key phosphorylation sites (Zoidl et al., 2002). Rather, the N-terminus of Cx36 is important for appropriate localization of the protein at the plasma membrane, as seen by the aberrant cytoplasmic localization of Cx36 when the N-terminus was truncated in neuroblastoma cells (Zoidl et al., 2002).

The terminal 4 residues, or SAYV motif, of the Cx36 C-terminus also play a role in trafficking of Cx36. When EGFP is tagged to the C-terminus of Cx36, the fusion protein is unable to form large plaques at the plasma membrane, and has an intracellular distribution profile (Helbig et al., 2010). The same effect is seen when the final 4 residues of the C-terminus are truncated, thus illustrating the importance of the SAYV motif in the trafficking of Cx36 (Helbig et al., 2010). In addition, Cx36 has been shown to interact with scaffolding proteins, ZO-1, 2, and 3 (Li et al., 2004a, b; Li et al., 2009). The SAYV motif is a binding site for ZO-1, which may serve as a chaperone during Cx36 trafficking to the plasma membrane. Thus, disruption of the ZO-1 binding site may be the cause of the trafficking defects seen in the Cx36-EGFP fusion protein and SAYV truncated mutant (Helbig et al., 2010; Li et al., 2004a).

Although not required for trafficking, phosphorylation has an effect on Cx36 channel function, as protein kinase A (PKA) mediated phosphorylation of Cx36 reduces intercellular communication (Urschel et al., 2006). Conversely, Kothmann et al (2009) have shown that dopamine-mediated stimulation of PKA induces dephosphorylation of

Cx36 through activation of protein phosphatase 2A, resulting in intercellular uncoupling. Cx36 has also been shown to be phosphorylated by calcium/calmodulin dependent kinase II (Alev et al., 2008).

1.2.2 Cx36 Channel Properties

Cx36 channels behave differently from those of other connexins, particularly in response to pH. For example, Cx36 channels close in response to alkalosis, and show minor increases in coupling during acidosis. Furthermore, the alkalosis gating mechanism is abolished in certain N and C-terminus mutants, thus demonstrating the role of these domains in pH gating of Cx36 channels in response to alkalosis (Gonzalez-Nieto et al., 2008).

Cx36 is unable to pair with other connexins to form heteromeric connexons, nor is it able to form heterotypic channels with connexins such as Cx32 or Cx26 (Al-Ubaidi et al., 2000). One study showed that Cx36 is able to form heterotypic channels by docking with connexons comprised of Cx45 (Al-Ubaidi et al., 2000). However, a more recent study showed that Cx36 is unable to form heterotypic channels with Cx45, however homotypic Cx36 and Cx45 channels were recruited to the same plaque (Li et al., 2008). Although some connexins, including Cx43 and some other connexins such as skate Cx35, form hemichannels to communicate with the extracellular environment, Cx36 does not show any hemichannel activity in single Xenopus oocytes (Al-Ubaidi et al., 2000). However, hemichannel activity has been reported under pathological conditions such as ischemia (Schock et al., 2008; Orellana et al., 2009).

1.2.3 Cx36 Expression and Function in Neurons

Cx36 is expressed in neurons, microglia, as well as pancreatic ß cells (Degen et al., 2004; Dobrenis et al., 2005; Serre-Beinier et al., 2009). Cx36 is highly expressed in neurons during development, but is down-regulated in many adult tissues (Belluardo et al., 2000; Gulisano et al., 2000). In adults Cx36 is expressed in neural tissues such as the inferior olive, hippocampus, retina, and sympathetic preganglionic motor neurons where it forms gap junctions on the soma and dendrites of neurons and occasionally on axons (Connors & Long, 2004; Marina et al., 2008). During development, Cx36 is expressed in tissues such as the spinal cord, thalamus, and hypothalamus, although it is absent in these regions or restricted to specific subregions in the adult nervous system. Cx36 is also highly expressed in the developing rat cerebral cortex, but expression is lost by the second postnatal week (Belluardo et al., 2000). This downregulation of Cx36 expression may be due in part to replacement of electrical synapses by chemical synapses, as the latter begin to form and mature postnatally (Lee et al., 2010).

In neurons, Cx36 is responsible for forming electrical synapses, thereby regulating neural synchronization and gamma frequency oscillations (Connors & Long, 2004). Gamma frequency oscillations are waves of electrical activity occurring at approximately 40 Hz, that contribute to attention, learning and memory (Kaiser & Lutzenberger, 2003). Cx36 preferentially couples inhibitory interneurons, as demonstrated by studies with a Cx36 knockout mouse model where the Cx36 gene is replaced with β galactosidase and placental alkaline phosphatase reporters (Deans et al., 2001). In the thalamic reticular nucleus, electrical synapses synchronize action potentials between adjacent cells, and allow action potentials to propagate to adjacent neurons, even

in the absence of chemical synapses (Landisman et al., 2002). Interestingly, Cx36 has been implicated in epileptogenesis, as its expression is increased during the early phases of epileptic activity. Since seizures arise during bouts of abnormally high and widespread neural synchronization, Cx36 may contribute to epileptogenesis due to its capacity to synchronize neurons (Beheshti, 2010).

Therefore, Cx36 is preferentially expressed during development, present in some adult structures, and has also been linked to pathological conditions of the nervous system.

1.2.4 Characterization of Cx36 Knockout Mice

Cx36 knockout (KO) mice do not present any obvious physical defects. However, electrical synapses in the neocortex are largely ablated. Specifically, the synchronization and power of gamma oscillations of inhibitory neurons, and therefore large-scale synchronization of inhibitory postsynaptic potentials were diminished (Deans et al., 2001; Hormuzdi et al., 2001; Buhl et al., 2003). Cx36 KO mice exhibit learning and memory impairments, in particular for increasingly complex tasks, such as Y-maze and object memory tasks (Frisch et al, 2005). Similarly, ablation of Cx36-mediated coupling in the inferior olive disrupts learning-dependent motor timing while not affecting motor performance (Kistler et al., 2002; Van Der Giessen et al, 2008). Cx36 deficient mice also experience a disruption in circadian rhythms due to ablation of electrical synapses between neurons of the suprachiasmatic nucleus (Long et al, 2005). Interestingly, these mice exhibited increased apoptosis following retinal injury compared to wild type littermates, introducing a role for Cx36 in cell survival following trauma (Striedinger et

al., 2005). Thus, although Cx36 is not vital, it is required for successful execution of a variety of neural tasks ranging from learning and memory to cell survival.

1.3 Connexin 45

1.3.1 Cx45 Expression

Cx45 is found in cardiomyocytes and vascular smooth muscle cells, as well as other tissues such as bone and skin; however it is also present in neurons (Kumai et al., 2000; Sohl et al., 2005; von Maltzahn et al., 2009). Cx45 is essential for cardiogenesis, as Cx45 knockout mice are embryonic lethal due to conduction blocks, and thus heart failure (Kumai et al., 2000). Cx45 expression is upregulated in several brain regions during development, including the cortex, hippocampus, and thalamus, and declines postnatally (Maxeiner et al., 2003; von Maltzahn et al., 2009). Cx45 may also be involved in neuronal response to apoptosis as it is expressed in apoptotic regions of the CNS, such as the hippocampus, where it is not normally present in the adult (Condorelli et al., 2003).

1.4 Gap Junctions and Cancer

1.4.1 Gap Junctions are Perturbed in Cancer

Disturbances in GJIC are implicated in many disease processes, including cancer (Kelsell et al., 2001). Dysfunctional GJIC may occur between cancer cells, or between cancer cells and healthy adjacent cells (Mesnil et al., 2005). Numerous studies have shown that GJIC is decreased or absent in tumor cells due to decreased connexin expression, or aberrant localization within the cytoplasm rather than at the plasma membrane, thus

limiting coupling between cells (Mesnil et al., 2005 Leithe et al., 2006; Czyz, 2008). Cancer cells exhibit dedifferentiation and proliferation, as indicated by lack of growth control and specialization (Mesnil et al., 2005). The fact that tumor promoting agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA) have GJIC inhibiting properties further illustrates the importance of GJIC in the carcinogenic process (Naus, 2002). Furthermore, Cx32 knockout mice and Cx43 hemizygous mice are more susceptible to chemically induced tumorigenesis in the liver (Temme 1997; King & Bertram, 2005).

1.4.2 Connexins as Tumor Suppressors

Overexpression of connexins results in tumor suppression of various cancer types. For example, Mao et al (2000) have shown that Cx43 has a growth suppressive effect on the neuroblastoma cell line, Neuro-2A, following overexpression of Cx43 by retroviral infection. In addition, McLachlan et al (2006) showed that inducing over-expression of Cx26 and Cx43 suppresses tumor growth and promotes differentiation in breast cancer cells. Furthermore, connexins display a differential ability to suppress tumor growth, depending on connexin and tumor type. For example, Cx26 and Cx43 suppress HeLa cell proliferation, while other connexins do not affect HeLa cell growth (Mesnil, 2002). Additionally, Cx26 and Cx43 are more potent suppressors of glioma than Cx32 since they are expressed in healthy astrocytes (Jimenez et al., 2006; Bond et al., 1994). Thus, it is postulated that connexins preferentially suppress tumors originating from tissues where the connexin is normally expressed. This differential ability of connexins to act as tumor suppressors may be due to differences in permeability to transjunctional compounds. For example, Cx43 overexpressing C6 glioma cells allow glutamate and ATP to pass through

gap junctions more readily than Cx32 overexpressing cells (Goldberg et al., 2002). However, connexin-mediated growth suppression is not necessarily due to increased channel function since connexins have been shown to decrease proliferation without increases in intercellular communication (Qin et al., 2003; Ionta et al., 2009; Sato et al., 2007). The role of connexins in growth regulation may also be due to the effects on connexin binding partners, such as ZO-1. Regarding the specific neuronal gap junction proteins, there have been no published investigations into the tumor suppressive properties of Cx36 while Cx45 has decreased expression in transformed nasopharyngeal tissue and various carcinomas (Xiang et al., 2002; Fan et al., 2003).

1.5 Neuroblastoma

1.5.1 Etiology of Neuroblastoma

Neuroblastoma is an embryonically derived cancer, originating from precursor cells that deviate from the developmental pathway evading differentiation (Figure 1.3). While many cancer types originate from terminally differentiated cells that undergo a transition to an immature phenotype, neuroblastoma involves progenitor cells of the neural crest (Anderson, 1993a,b). The neural crest (NC) arises during neurulation, when the notochord induces pinching off of the neural tube from the overlying ectoderm. A variety of structures arise from the NC, including facial bones, melanocytes, connective tissue, glia, the dorsal root ganglia, sympathetic neurons, and adrenal chromaffin cells. The NC is divided into cranial, trunk, sacral, and cardiac components, depending on localization and the structures they derive. The trunk NC cells migrate to the dorsal aorta, where environmental cues, such as bone morphogenic protein 4 (BMP4) induce

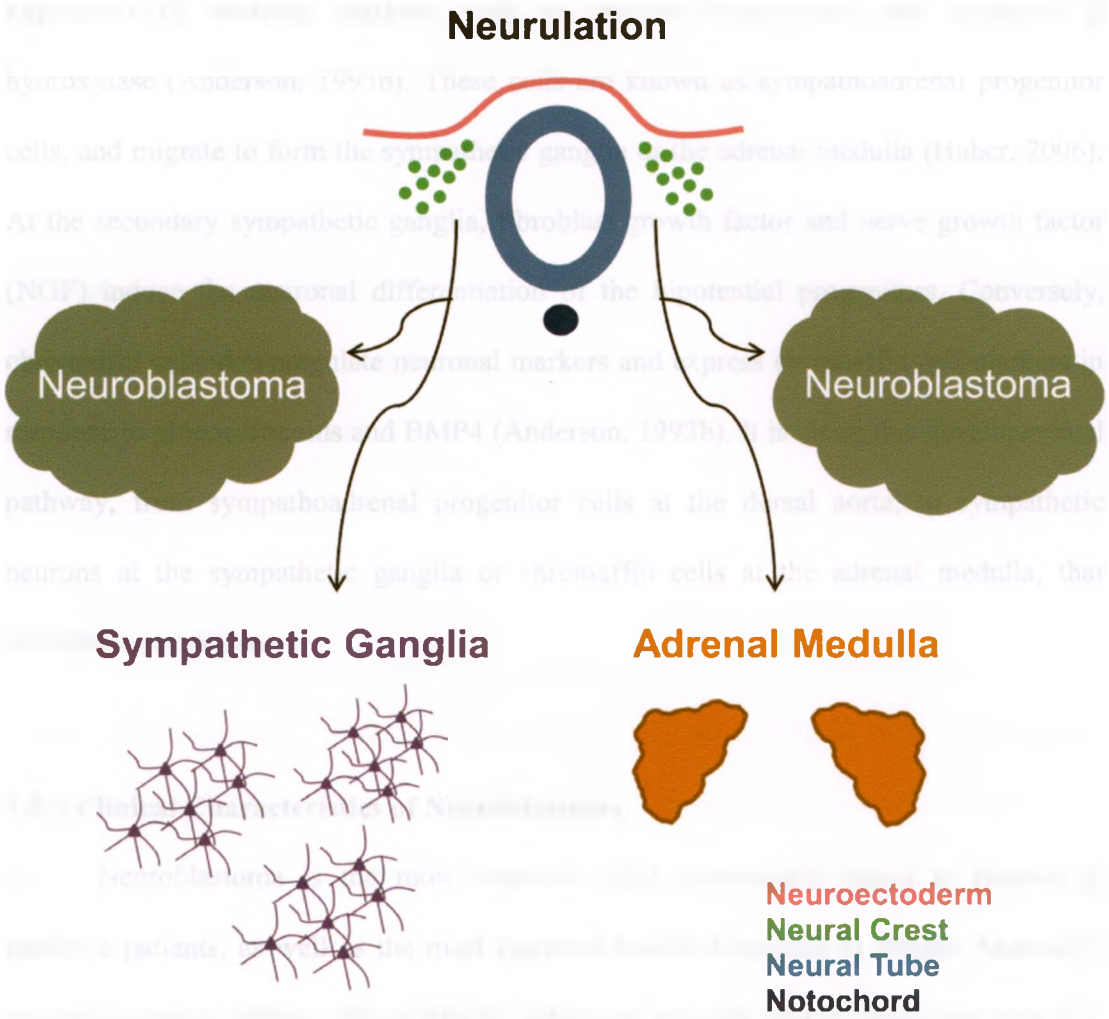

Figure 1.3. Etiology of Neuroblastoma

Neuroblastoma arises from bipotential sympathoadrenal progenitor cells of the neural crest. Cells of the trunk neural crest migrate to the dorsal aorta, where they are exposed to environmental cues to form either the sympathetic ganglia or adrenal medulla. These bipotenial progenitors then migrate to their sites of terminal differentiation. However, neuroblastoma arises when these progenitors become tumorigenic, and bypass the differentiation stage to form tumors in the chest, abdomen, and pelvis.

expression of neuronal markers, such as tyrosine hydroxylase and dopamine β hydroxylase (Anderson, 1993b). These cells are known as sympathoadrenal progenitor cells, and migrate to form the sympathetic ganglia or the adrenal medulla (Huber, 2006). At the secondary sympathetic ganglia, fibroblast growth factor and nerve growth factor (NGF) induce the neuronal differentiation of the bipotential progenitors. Conversely, chromaffin cells downregulate neuronal markers and express chromaffin cell markers in response to glucocorticoids and BMP4 (Anderson, 1993b). It is along this developmental pathway, from sympathoadrenal progenitor cells at the dorsal aorta, to sympathetic neurons at the sympathetic ganglia or chromaffin cells at the adrenal medulla, that neuroblastoma arises.

1.5.2 Clinical Characteristics of Neuroblastoma

Neuroblastoma is the most common solid, extracranial tumor to present in pediatric patients, as well as the most common cancer diagnosed in infants. Metastatic neuroblastoma is present within 60% of diagnosed patients, and is associated with a 5-15% survival rate (Brodeur, 2003). Forty percent of neuroblastoma patients fall within a high risk category and only 40% of this group are effectively cured with multi-modal therapy (Wagner & Danks, 2009). Neuroblastoma is characterized by clinical heterogeneity, with high risk aggressive and metastatic tumors, differentiated tumors that are relatively benign, and tumors that spontaneously regress to ganglioneuromas. Interestingly, neuroblastoma has one of the highest rates of spontaneous regression of any cancer type (Maris, 2010). Poor neuronal differentiation is associated with aggressive tumors, whereas highly differentiated tumor cells are associated with a more favorable

prognosis. Tumors usually arise within the adrenal medulla or the paraspinal sympathetic ganglia in the chest, abdomen, or pelvic ganglia (Brodeur, 2003).

Treatment of high risk neuroblastoma involves multi-modal therapy including chemotherapy, radiation, and surgery. These treatments are highly toxic to the patient and are often not well tolerated (Laverdiere et al., 2005). 13-*cis* retinoic acid is well tolerated with minimal side effects, and has now been implemented during remission following standard therapy to eradicate minimal residual disease (Wagner & Danks, 2009).

1.5.3 Markers of Neuroblastoma

There are several prognostic markers for neuroblastoma, such as myc myelocytomatosis viral related oncogene (*MYCN*) amplification, and neurotrophin tyrosine kinase receptor (Trk) A and B expression, which influence the aggressiveness of the tumor (Brodeur et al, 2009; Schwab, 2004; Bell et al., 2010). TrkA is a receptor for the neurotrophin, NGF, and its expression is associated with decreased expression of angiogenic factors (Barbacid, 1995; Brodeur et al., 2009). Since angiogenesis is vital to tumor progression and metastasis, the down-regulation of angiogenic factors such as vascular endothelial growth factor is associated with patient survival (Eggert et al., 2002). Conversely, TrkB is a receptor for brain derived neurotrophic factor (BDNF) and associated with increased proliferation and poor prognosis (Barbacid, 1995; Schramm et al, 2005).

1.5.3.1 MYCN

MYCN is amplified in 20% of all neuroblastomas. MYCN belongs to the MYC

family of transcription factors. MYCN heterodimerizes with MAX and binds to E-box sequences to activate transcription. MYCN can also repress transcription by recruiting DNA methyltransferases to target genes (Bell et al., 2010). *MYCN* is an oncogene, and its overexpression results in increased proliferation by regulation of genes involved in cell cycle progression. Specifically, activation of the MYCN signaling pathway is strongly correlated with poor prognosis (Bordow et al., 1998; Fredlund et al., 2008). Transgenic mice overexpressing *mycn* under a tyrosine hydroxylase promoter, which targets cells of neuroectodermal lineage, spontaneously develop neuroblastoma within months after birth (Weiss et al, 1995).

1.6 Differentiation Therapy

1.6.1 Differentiation Therapy

Neoplastic cells acquire an immature phenotype characteristic of undifferentiated cells, displaying increased proliferation and invasiveness. Differentiation therapy is based on the concept that exposing cancer cells to differentiating agents will cause arrest of proliferation and promote differentiation, which sensitizes cells to cancer drugs. For example, SH-SY5Y human neuroblastoma cells are more prone to undergo apoptosis in response to flavonoids when they are pretreated with the differentiating agent, retinoic acid (Das et al., 2009). The efficacy of differentiation therapy has been established in multiple cancers such as melanoma, acute promyelocytic leukemia (APL), and neuroblastoma. 13-*cis* retinoic acid has been clinically employed in differentiation therapy of aggressive neuroblastoma following chemotherapy (Reynolds 2003). All-trans-retinoic acid (ATRA) has been used to effectively treat APL (Leszczyniecka, 2001).

13-*cis* retinoic acid is now implemented in standard therapy during the first remission of high risk neuroblastoma patients. Furthermore, the efficacy of other derivatives of retinoic acid, including the synthetic retinoic, N-(4-hydroxyphenyl) retinamide (4-HPR or fenretinide), is currently being investigated. (Maris, 2010; Wagner & Danks, 2009). Specifically, 4-HPR is now being tested in Phase I clinical trials and shows promise due to minimal toxic effects and tolerance by patients (Wagner & Danks, 2009).

1.6.2 Retinoic Acid

Retinoic Acid (RA) is a derivative of Vitamin A. It exists in many isomers, including ATRA, 13-cis retinoic acid, and a synthetic derivative, 4-HPR (Reynolds 2003). Retinoic acid acts by binding to retinoic acid receptors (RAR) or retinoid X receptors (RXR), which are members of the steroid/thyroid hormone family of transcription factors (Glass et al., 1991). Specifically RAR binds ATRA and 13-cis retinoic acid, whereas RXR only binds 13-cis RA. RAR and RXR are bound to nuclear corepressors until retinoic acid binds the receptors and causes a conformational change to release the corepressors (Linney, 1992). RAR and RXR then bind retinoic acid response elements in the promoter regions of target genes, and activate transcription (Reynolds 2001). Retinoic acid is endogenously synthesized during development and in adults. Lack of Vitamin A or RA during development is lethal. In vitro, ATRA diminishes MYCN expression, arrests proliferation, and induces differentiation of neuroblastoma cells (Thiele et al., 1985). The mechanism of ATRA mediated differentiation may be through TrkA and TrkB activation, sensitizing them to NGF and BDNF respectively (Clagett-Dame et al., 2006). Conversely, 4-HPR induces cell death of neuroblastoma cells,

including cells that are resistant to 13-*cis* retinoic acid and ATRA (Reynolds et al., 2003). In relation to gap junctions, RA increases GJIC and Cx43 expression (Bertram & Vine, 2005; Stahl & Sies, 1998; Long et al., 2010; King & Bertram, 2005). Interestingly, the mouse *Cx36* gene contains an RXR binding motif and a MYCN binding site, indicating that *Cx36* is susceptible to transcriptional regulation by RA and MYCN (Cicirata et al., 2000). No studies have explored the effects of retinoic acid on Cx36 or Cx45 in neuroblastoma or any other cancer cell lines thus far.

1.6.3 Neuritogenesis

Neurites are cytoplasmic extensions of the neuron cell body, forming the precursors of axons and dendrites. The growth cone is the mobile and dynamic front of the neurite. (Clagett-Dame et al., 2006). It guides the neurite to other neurons or nutrients. Neuritogenesis is a dynamic process involving reorganization of the cytoskeleton, specifically of actin and microtubules. Actin filaments comprise the peripheral domain of the growth cone, where they organize in a loose meshwork to form lamellopodia, and tight bundles to form filopodia (Bouqet & Nothias, 2007; Clagett-Dame et al., 2006). The central domain is filled with microtubules that stabilize the neurite (Bouqet & Nothias, 2007). Neuritogenesis is a function of neuronal differentiation and thus is often used as a measure to quantify the extent of differentiation (Belliveau et al., 1997; Belliveau et al., 2006; Encinas et al., 2000; Pahlman et al., 1990).

1.6.3.1 Gap43

Growth associated protein 43 (Gap43) is found at very high concentrations in the

1.7 Rationale, Hypothesis and Aims

1.7.1 Rationale

Gap junctional intercellular communication is often disrupted in cancer cells. Many connexins have been shown to be downregulated in neoplasms, and the overexpression of these connexins results in tumor suppression. Connexin family members also enhance differentiation in cells of the neuroectodermal lineage. In addition, Cx43 is shown to be responsive to retinoic acid signaling and *Cx36* has an identical binding site for RXR; however the effect of retinoic acid on Cx36 has not yet been investigated. Although Cx36 expression patterns have been extensively investigated, the expression and role of Cx36 in cancer cells have not been assessed. Since Cx36 is predominantly a neuronal connexin, and the ability of connexins to act as tumor suppressors is specific to tissues in which they are normally expressed, the effect of Cx36 on the neuronal tumor, neuroblastoma, was investigated. In this study, I explored the effects of the differentiating agent, all-trans-retinoic acid, on *Cx36* expression in the SH-SY5Y, human neuroblastoma cell line. The SH-SY5Y cell line has been utilized as a model system for neural diseases, as well as in many studies of differentiation. Furthermore, I investigated the role of Cx36 on tumor cell characteristics by manipulating Cx36 expression and assessing proliferation and features of differentiation, such as neuritogenesis and differentiation marker expression of human neuroblastoma cells. My study is the first to explore the role of Cx36 in the tumor cell phenotype.

1.7.2 Hypothesis

Cx36 regulates the differentiation and proliferation of human neuroblastoma cells.

1.7.3 Aims

1. Determine the changes in connexin gene expression following retinoic acid induced differentiation of neuroblastoma.
2. Investigate the effects of Cx36 overexpression on differentiation and proliferation of human neuroblastoma cells,
3. Investigate the effects of knocking down Cx36 on differentiation and proliferation of human neuroblastoma cells.

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CHAPTER TWO:

Connexin36 is a Negative Regulator of Differentiation in Human Neuroblastoma

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2.1 Introduction

Neuroblastoma is the most common extracranial solid tumor to present in children (Brodeur, 2003). Forty percent of neuroblastoma patients fall within a high risk category, and only 40% of this group are effectively cured with multi-modal therapy (Wagner & Danks, 2009). Neuroblastoma arises from bipotential sympathoadrenal progenitor cells of the trunk neural crest. In normal development, sympathoadrenal progenitors differentiate to form the sympathetic ganglia and adrenal medulla, however in neuroblastoma they form tumors at these sites instead (Anderson, 1993; Huber, 2006; Brodeur, 1994).

Retinoic acid is used as an adjunct to multi-modal therapy to treat high-risk neuroblastoma and eradicate minimal residual disease (Reynolds, 2003; Wagner & Danks, 2009). Retinoic acid is a Vitamin A derivative that induces differentiation in several cell types (Das et al., 2009; Reynolds, 2003; Reynolds, 2001; Melino et al., 1997). For example, all trans retinoic acid (ATRA) diminishes MYCN oncogene expression, arrests proliferation, and induces differentiation of neuroblastoma cells (Thiele et al., 1985; Sidell et al., 1983).

Gap junctional intercellular communication (GJIC) and connexins (Cx) have been implicated in carcinogenesis and differentiation. GJIC is often perturbed and connexins are typically downregulated or aberrantly localized in cancer cells (Ozawa et al., 2007; Arnold et al., 2005; Naus, 2002; Czyz, 2008; Li et al., 2007). Many connexins have been identified as tumor suppressors when overexpressed in cancer cells (Jimenez et al., 2006; Zhang et al., 1998; Temme et al., 1997; Sato et al., 2007; McLachlan et al., 2006). For example, overexpression of Cx43 resulted in growth suppression of communication deficient Neuro-2A murine neuroblastoma cells (Mao et al., 2000). In addition, connexins

have been shown to enhance differentiation in several cancer cell lines (Vinken et al., 2006; Banks et al., 2007; Brehm et al., 2007; Mao et al., 2000). For example, overexpression of Cx32 and Cx43 resulted in enhanced nerve growth factor induced neurite outgrowth in PC12 cells (Belliveau et al., 2006).

Gap junctions are membrane channels that allow intercellular communication between adjacent cells (Laird, 1996). GJIC involves the passage of ions, second messengers, and metabolites less than 1kDa in size between cells (Goldberg et al., 1998; Goldberg et al., 1999; Saez et al., 1989). Gap junctions and their constituent proteins, connexins, regulate cellular processes such as homeostasis, growth, and differentiation (Vinken et al., 2006; Churko et al., 2010; Prost et al., 2008; Ionta et al., 2009; Omori et al., 2001). Gap junctions are formed from the docking of two connexons or hemichannels contributed by adjacent cells. Each connexon in turn is comprised of a hexamer of connexin subunits (Laird, 1996). There are 21 known members of the human connexin family, most with unique spatial and temporal expression patterns (Ahn et al., 2008; Belluardo et al., 2000; Boucher & Bennett, 2003; Cina et al., 2007; Kihara et al., 2006).

Cx36 is a recently identified connexin that is expressed in neurons and pancreatic beta cells (Sohl et al., 1998; Condorelli et al., 1998; Serre-Beinier et al., 2000; Wellerhaus et al., 2008). Cx36 is highly expressed in the developing nervous system, however its expression is decreased or lost by the second post-natal week in many structures (Belluardo et al., 2000; Gulisano et al., 2000). In adults, Cx36 is the predominant connexin involved in electrical synapses (Connors & Long, 2004). It is primarily responsible for large-scale synchronization of gamma frequency oscillations in inhibitory interneurons (Deans et al., 2001).

The differentiating agent, retinoic acid, increases gap junctional intercellular communication and Cx43 expression in multiple cell types (Bertram & Vine, 2005; Stahl & Sies, 1998; Long et al., 2010). Interestingly, the mouse *Cx36* gene contains a retinoid X receptor binding motif indicating that *Cx36* may be susceptible to transcriptional regulation by retinoic acid (Cicirata et al., 2000).

In this study, I explored the effect of the neuronal connexin, Cx36, on the differentiation of SH-SY5Y human neuroblastoma cells. SH-SY5Y cells are often utilized as a model system for studies of neural disease, as well as in studies of differentiation (Encinas et al., 2002; Agholme et al., 2010; Cheung et al., 2009). The effect all-trans-retinoic acid on *Cx36* expression was first examined in SH-SY5Y cells. Furthermore, the effects of Cx36 on proliferation and features of differentiation including neuritogenesis and molecular differentiation marker expression were investigated by manipulating Cx36 expression.

2.2 Methods

2.2.1 Cell Culture

The neuroblastoma cell line, SH-SY5Y was obtained from American Type Culture Collection (Manassas, VA). SH-SY5Y is a proliferative cell line with a documented doubling time of 48 hours. SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) with high glucose, and supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin and streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen). For cell passage, cells were washed with Hank's Buffered Salt Solution (HBSS, Sigma), treated with 0.25% trypsin and incubated for 5 minutes at 37°C to disperse the cells. Five ml of growth medium was added to the cells, and they were resuspended in a 10 cm dish in fresh growth medium after pipetting several times.

2.2.2 Generation of Stable Cell Lines

2.2.2.1 Overexpression

Cells were plated at a density of $1x10^6$ cells per well in a 6-well dish. The following day, 4 µg of Cx36myc-DDK (Origene, Rockville, MD) or EGFP pcDNA3.1 were diluted in OPTI-MEM (Invitrogen) and mixed with 2.5 µl of lipofectamine 2000 (Invitrogen) in OPTI-MEM. The DNA and lipofectamine were mixed and the resulting complexes added to the cells after 20 minutes, which were also in OPTI-MEM. The cells and complexes were incubated for 6 hours and then returned to normal growth medium. The following day, transfected cells were passaged onto 10 cm dishes. Kill curves were performed on wild-type cells to determine optimal antibiotic concentrations. For stable cell lines, 800 µg/ml G418 was added to the cells, and all viable cells were passaged to a 10 cm dish

following two weeks of selection, and G418 was reduced to 200 µg/ml. However, cells were treated with 800 µg/ml G418 monthly to ensure purity of the culture.

2.2.2.2 Knockdown

shRNA knockdown: Cells were plated at a density of $5x10^5$ cells per well in a 6-well dish. The following day, 1 µg of four distinct shRNAs targeting Cx36 or non-effective (scrambled) control plasmids co-expressing GFP (Origene, Rockville, MD) were diluted in OPTI-MEM and mixed with 1.5 µl of lipofectamine 2000 in OPTI-MEM. The cells were then maintained as per the overexpression protocol. For stable cell lines, 800 ng/ml puromycin was added to the cells, and all viable cells were passaged to a 10 cm dish following two weeks of selection, and puromycin was further increased to 1.5 µg/ml. In addition, cells were treated with 2.5 µg/ml puromycin monthly to maintain purity of the culture due to the occasional appearance of non-fluorescent cells.

2.2.3 Differentiation Assay

SHSY-5Y cells were plated on rat-tail collagen coated 6-well or 60 mm plates, at a density of 10 000 cells/cm^2 in growth medium. The following day, cells were treated with 10 µM of all trans-retinoic acid (Sigma) for 4 days, with medium and treatment refreshed every 2 days. Cells were then washed with HBSS and treated with 20 ng/ml of brain derived neurotrophic factor (BDNF) in serum-free DMEM for 6 days, with medium and BDNF replaced every 2 days (modified from Encinas et al., 2000). Medium for transfected cells contained the appropriate antibiotic. RNA and protein were extracted at Days 0, 2, 4 and 10 of differentiation, and connexin and differentiation marker expression

were analyzed. Images were taken at each timepoint with a Leica inverted epifluorescent microscope. The images were analyzed with the NeuronJ plugin for ImageJ (E. Meijering et al., 2004; Abramoff et al., 2004). For each image, neurites within the 0.16 mm^2 field of view were traced. Neurites were classified as cell projections longer than one cell body length. For analysis of average neurite length, the mean length of all neurites in the field of view was computed. In order to calculate maximum neurite length, the length of the longest neurite in the field of view was analyzed. Finally, for neurite density, the number of neurites was divided by the number of cell bodies in the field of view.

2.2.4 Real-Time PCR

RNA was extracted and purified using the Qiagen RNeasy mini kit, and 0.5 µg of RNA was reverse transcribed using the qScript cDNA synthesis kit (Quanta Biosciences). Connexin primers were designed using Primer-Blast yielding the following sense and anti-sense sequences: Cx36, 5'-AAG GCA TCT CCC GCT TCT ACA - 3' and 5'- GCC AAC CAG GAA CCC AAT TT- 3'; Cx45, 5'-CTG GAG GCT CTG CAG CGG GA- 3' and 5'-TCT CCC GGG GAC CAT GAG GG- 3'; Cx43, 5'-GGT TAC ACT TGC AAA AGA GAT C- 3', and 5'-GAG CAG CCA TTG AAA TAA GC- 3'. Differentiation markers: Neuropeptide Y (NPY), 5'- TCC AGC CCA GAG ACA CTG ATT-3' and 5'-AGG GTC TTC AAG CCG AGT TCT-3'; Growth associated protein 43 (Gap43), 5'-ACG ACC AAA AGA TTG AAC AAG ATG-3' and 5'-TCC ACG GAA GCT AGC CTG AA-3' (Origene). Adhesion molecule: Neural cell adhesion molecule (NCAM), 5'-CAT CAC CTG GAG GAC TTC TAC C-3' and 5'- CAG TGT ACT GGA TGC TCT TCA GG- 3' (Origene). Values were normalized to those of the PPIA reference gene:

cyclophillin A (PPIA), 5'-AGA CAA GGT CCC AAA GAC-3' and 5'ACC ACC CTG ACA CAT AAA-3'. All primer pairs were tested using standard curves with 10-fold serial dilutions, and selected only if the effiencies were within the 95-110% range over a minimum of 3 points. Three technical repeats were included for each biological replicate. The qPCR was performed in the CFX96 Real-Time PCR Detection System (Bio-Rad) using Perfecta Sybr Green Fastmix (Quanta Biosciences), and the data analyzed with CFX Manager software (Bio-Rad).

2.2.5 Western Blot Analysis

Protein was extracted using 18 mM Tris, 123mM NaCl, 10% glycerol, 1% NP40, and protease inhibitor cocktail (Calbiochem). Cell lysates were kept on ice for 5 minutes with frequent agitation, sonicated for 10 seconds, and lysates were cleared in a cold centrifuge at 10,000 rpm for 10 minutes. The supernatant was removed and used for western blotting. Protein concentration was measured using the BCA Protein Assay (Thermo Scientific, Rockford, IL). Thirty µg of protein were electrophoresed on 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked for one hour in 3% bovine serum albumin (BSA) in phosphate buffered saline with 0.05% Tween-20 (PBST), followed by incubation with primary antibodies: mouse monoclonal Gap43 (Invitrogen) 1:500 overnight at 4°C; mouse monoclonal N-cadherin (BD) 1:2500 2 hours at room temperature; mouse monoclonal GAPDH (Millipore) 1:5000 for 2 hours at room temperature. Membranes were washed with PBST and incubated with secondary antibodies for one hour: horseradish peroxidase conjugated goat anti-mouse at a 1:5000 dilution (Thermo Scientific, Rockford, IL). Membranes were

then washed with PBST and developed using enhanced chemiluminscence (Thermo Scientific, Rockford, IL). Protein expression data could not be collected for Cx36 due to unreliable antibodies.

Protein expression was quantified with Quantity One software (Bio-Rad) by densitometric analysis. Protein levels were normalized to GAPDH.

2.2.6 MTT Proliferation Assay

Cells were plated at 20, 000 cells per well in a 48-well plate. At 0, 2, 4, and 6 days, 20 µl of MTT (Invitrogen) solution was added to each well and incubated for 4 hours. 200 µl of SDS-HCl was then added to each well and incubated for 14 hours. The absorbance was measured at 570 nm on a µQuant Biomolecular spectrophotometer (Bio-Tek). Increased absorbance indicates higher cell numbers. Four technical repeats were included in each biological replicate.

2.2.7 Statistical Analysis

Student's t-test was conducted for aggregate and neuritogenesis analysis between EGFP and Cx36 overexpressing cells. One-way analysis of variance (ANOVA) was conducted for the following experiments: gene expression analysis between wild-type cells, and neuritogenesis analysis between non-effective shRNA and Cx36 shRNAs. Tukey's multiple comparison post-hoc test was used to assess differences in means between groups.

Two-way ANOVA was conducted for the following experiments: All gene and protein expression analysis and proliferation assays between transfected cells

(overexpression and knockdown). Bonferroni's post-hoc test was conducted to assess differences in means between groups. $p<0.05$ was considered significant for all tests. All statistical analysis was performed with GraphPad Prism 4.0 software.

2.3 Results

2.3.1 Retinoic Acid Induces Differentiation and Upregulates Cx36 Expression of SH-SY5Y Neuroblastoma Cells

SH-SY5Y cells were exposed to retinoic acid and BDNF treatment to induce cell differentiation. SH-SY5Y cells were treated with 10 μM retinoic acid in growth medium for 4 days, followed by 20 ng/ml BDNF for 6 days to induce differentiation. Neurite outgrowth was observed in treated cells beginning at day 2 (Figure 2.1C). Neuritogenesis continued through day 4, and within 10 days extensive neurite networking was observed (Figure 2.1 E & G). Untreated cells began to cluster and form aggregates by day 2, and continued to form large aggregates by day 10 (Figure 2.1 B, D, F). Thus, retinoic acid and BDNF treatment induces the morphological differentiation of SH-SY5Y cells.

In order to determine whether retinoic acid regulates the expression of neuronal connexins, the effect of retinoic acid treatment on *Cx36* and *Cx45* transcript expression was examined. *Gap43* transcript expression was also assessed to confirm differentiation at the molecular level. RNA was isolated from untreated and treated cells at 0, 2, 4 and 10 days. Real-time PCR was used to quantify gene expression. *Cx36* mRNA was increased in treated cells at 2 (2.6-fold), 4 (1.8-fold), and 10 (13.9-fold) days compared to untreated cells (Figure 2.2A). *Cx45* mRNA, however, did not increase in treated cells (Figure 2.2B). Messenger RNA for the growth cone marker, *Gap43*, was upregulated at days 4 (2-fold) and 10 (2.8-fold) of treatment compared to untreated cells (Figure 2.2C). The drop in *Gap43* expression following day 0 appears to be an artifact, possibly due to its initial mRNA upregulation following cell seeding. It does not appear in western blots in

Figure 2.1. Retinoic acid induces neuritogenesis in SH-SY5Y cells.

Cells were seeded on rat-tail collagen coated dishes (A) and treated with 10 μM of retinoic acid the following day. During days 5-10, cells were treated with 20 ng/ml of BDNF in serum-free medium. Untreated cells formed increasingly larger aggregates over time (B, D, F). Neurite outgrowth (arrows) was observed beginning at days 2 and 4 of treatment (C, E), and dense neurite networks were formed following 10 days of treatment (G). Scale bar = 100 μm.

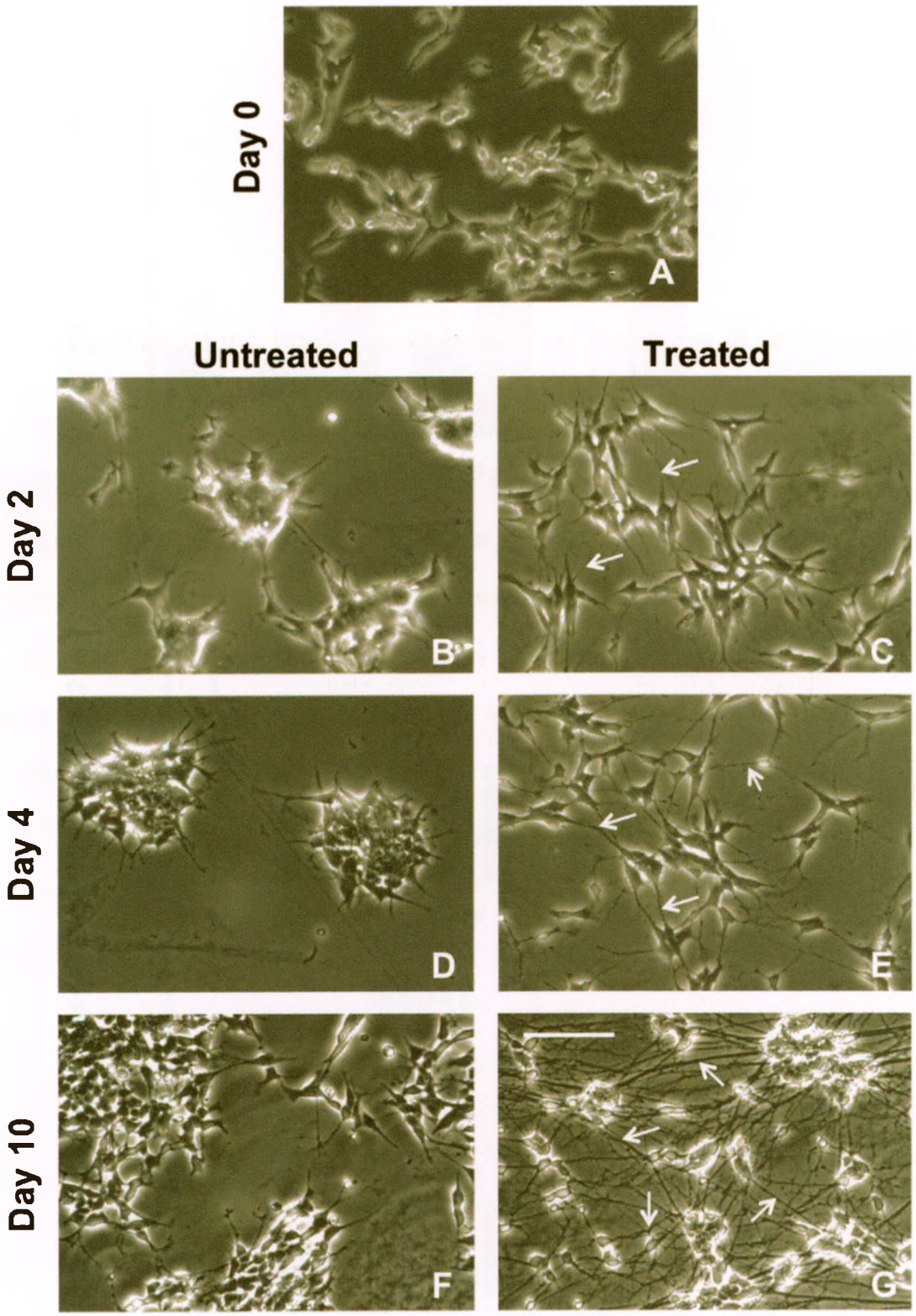
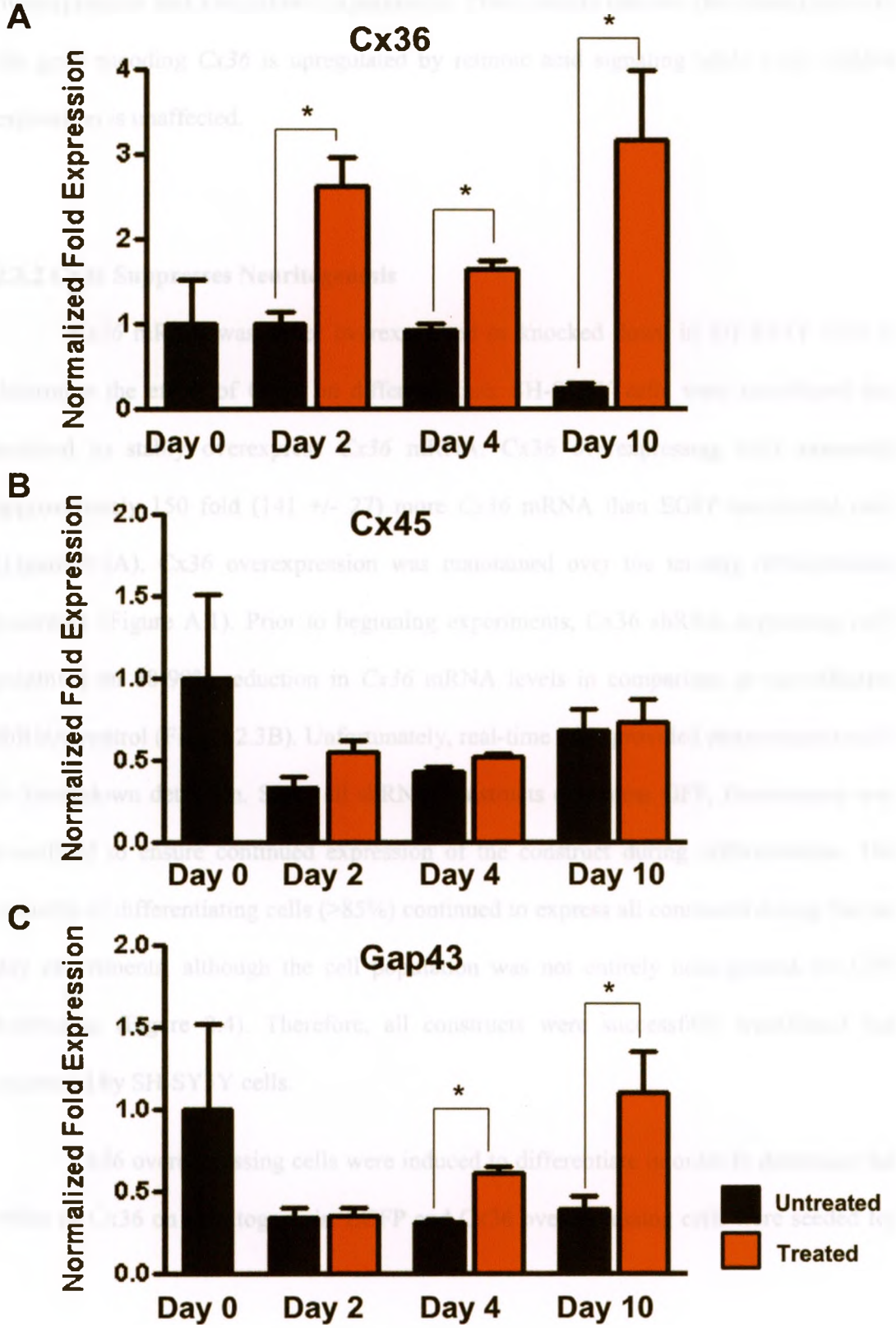

Day 0
A
Untreated
Treated
Day 2
B
C
Day 4
D
E
Day 10
F
G

Figure 2.2. *Cx36* and *Gap43* are transcriptionally upregulated following retinoic acid induced differentiation of SH-SY5Y cells.

RNA was isolated from SH-SY5Y cells at days 0, 2, 4, and 10 of treatment. Real-time PCR was used to quantify mRNA expression. *Cx36* expression increased following 2, 4 and 10 days of treatment, as compared to untreated cells (A). *Cx45* expression did not increase in either treated or untreated cells (B). *Gap43* expression increased following 4 and 10 days of treatment (C). mRNA levels were normalized to *PPIA*. N=3 (Day10, N=5). Asterisks denote statistical significance. $p<0.05$. Bars show mean +/- S.E.


A
Cx36
Normalized Fold Expression
4
3
2
1
0
*
*
*
Day 0
Day 2
Day 4
Day 10
B
Cx45
Normalized Fold Expression
2.0
1.5
1.0
0.5
0.0
Day 0
Day 2
Day 4
Day 10
C
Gap43
Normalized Fold Expression
2.0
1.5
1.0
0.5
0.0
*
*
Day 0
Day 2
Day 4
Day 10
Untreated
Treated

overexpression and knockdown experiments. These results indicate that transcription of the gene encoding *Cx36* is upregulated by retinoic acid signaling while *Cx45* mRNA expression is unaffected.

2.3.2 Cx36 Suppresses Neuritogenesis

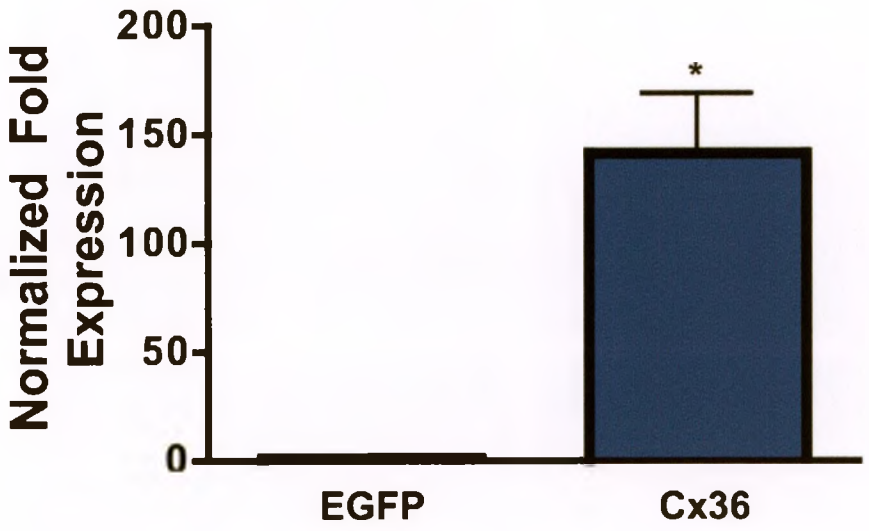
Cx36 mRNA was either overexpressed or knocked down in SH-SY5Y cells to determine the effect of Cx36 on differentiation. SH-SY5Y cells were transfected and selected to stably overexpress *Cx36* mRNA. Cx36 overexpressing cells expressed approximately 150 fold (141 +/- 27) more *Cx36* mRNA than EGFP transfected cells (Figure 2.3A). Cx36 overexpression was maintained over the ten-day differentiation treatment (Figure A.1). Prior to beginning experiments, Cx36 shRNA expressing cells exhibited an 88-99% reduction in *Cx36* mRNA levels in comparison to non-effective shRNA control (Figure 2.3B). Unfortunately, real-time PCR provided inconsistent results in knockdown detection. Since all shRNA constructs coexpress GFP, fluorescence was visualized to ensure continued expression of the construct during differentiation. The majority of differentiating cells (>85%) continued to express all constructs during the ten day experiments, although the cell population was not entirely homogenous for GFP expression (Figure 2.4). Therefore, all constructs were successfully transfected and expressed by SH-SY5Y cells.

Cx36 overexpressing cells were induced to differentiate in order to determine the effect of Cx36 on neuritogenesis. EGFP and Cx36 overexpressing cells were seeded for

Figure 2.3. Stable transfection of SH-SY5Y cells with a construct encoding Cx36 results in *Cx36* mRNA overexpression and transfection with shRNA results in *Cx36* mRNA knockdown.

SH-SY5Y cells were transfected and selected to stably overexpress Cx36 or EGFP as a transfection control. In addition, cells were transfected and selected to express four distinct Cx36 shRNA's and a non-effective shRNA transfection control. RNA was isolated to determine expression levels prior to seeding cells for experiments. Cx36 overexpressing cells expressed increased transcript levels of *Cx36* compared to EGFP control (A). Cx36 shRNA expressing cells had decreased *Cx36* transcript levels (B). RNA expression levels were normalized to *PPIA* mRNA. N=3 (B, N=1). Asterisk denotes statistical significance compared to EGFP. $p<0.05$. Bars show mean +/-S.E.

A


Overexpression of Cx36
Normalized Fold Expression
200
150
100
50
0
*
EGFP
Cx36

B

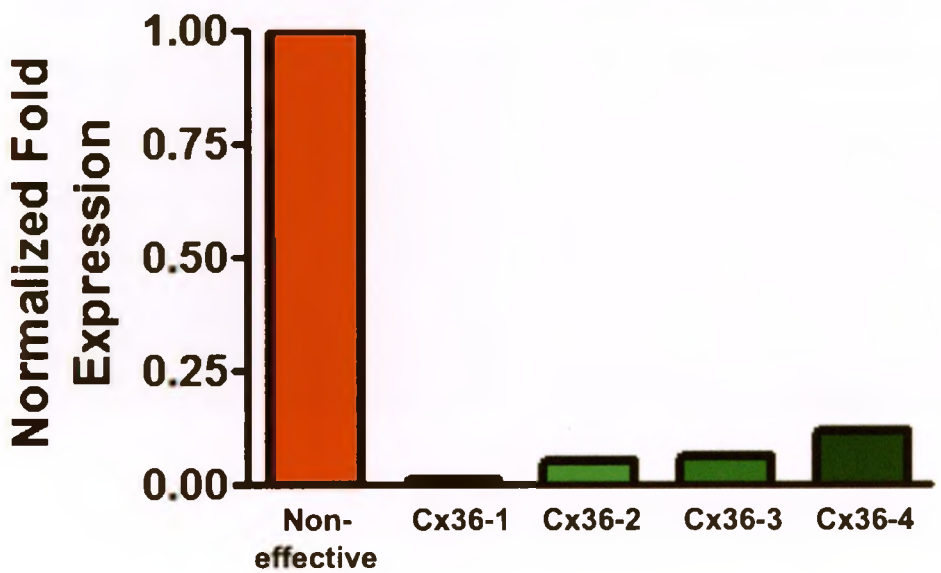
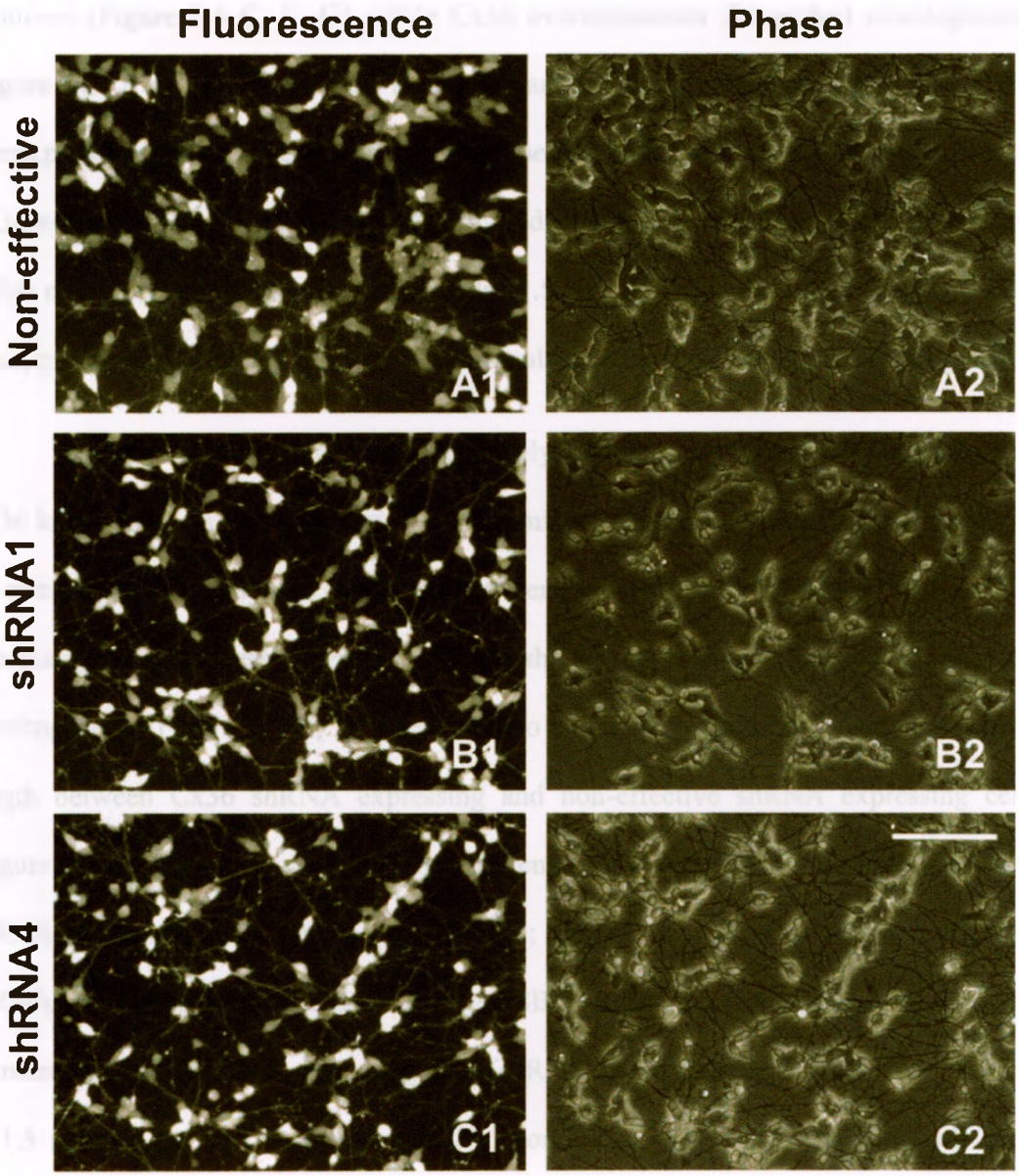

shRNA Knockdown of Cx36
Normalized Fold Expression
1.00
0.75
0.50
0.25
0.00
Non-effective
Cx36-1
Cx36-2
Cx36-3
Cx36-4

Figure 2.4. Expression of shRNA constructs is maintained throughout differentiation.

SH-SY5Y cells were stably transfected to express Cx36 shRNA or non-effective shRNA control constructs that coexpress GFP. Most of the cell population expressed GFP through ten days of differentiation treatment (A1, B1, C1), however some non-fluorescent cells were present within the culture (A2, B2, C3). Scale bar = 100 μm.


Fluorescence
Phase
Non-effective
shRNA1
shRNA4
A1
A2
B1
B2
C1
C2

differentiation, and imaged at 0, 2, 4, and 10 days following differentiation treatment. EGFP expressing cells showed intense neurite networking by day 10 of differentiation treatment (Figure 2.5 C, E, G), while Cx36 overexpression diminished neuritogenesis (Figure 2.5 J, L M). There was no significant difference between EGFP and Cx36 overexpressing cells in average or maximum neurite length (Figure 2.6 A, B). However, Cx36 overexpressing cells had significantly reduced neurite density (1.0 neurite per cell body) compared to EGFP expressing cells (1.5 neurites per cell body) (Figure 2.6C). Thus, my findings indicate that Cx36 downregulates retinoic acid induced neuritogenesis.

In order to verify that Cx36 negatively regulates neuritogenesis, the effect of Cx36 knockdown on neuritogenesis was determined. An identical experimental design as the overexpression study was conducted between non-effective shRNA and Cx36 shRNA expressing cells. In these experiments, Cx36 shRNA expressing cells showed enhanced neuritogenesis (Figure 2.7 H, J). There was no significant difference in average neurite length between Cx36 shRNA expressing and non-effective shRNA expressing cells (Figure 2.8A). However, maximum neurite length was significantly increased in Cx36 shRNA expressing cells (shRNA 1: 217 μm; shRNA 4: 263 μm) over non-effective shRNA expressing cells (163 μm) (Figure 2.8B). Neurite density was also significantly increased in Cx36 shRNA expressing cells (shRNA 1: 1.8 neurites per cell body; shRNA 4: 1.5 neurites per cell body) compared to non-effective shRNA expressing cells (0.9 neurites per cell body) (Figure 2.8C). Therefore, knockdown of Cx36 enhanced retinoic acid induced neuritogenesis. These findings confirm that Cx36 downregulates retinoic acid induced neuritogenesis of SH-SY5Y cells.

Figure 2.5. Cx36 overexpression increases cell clumping and diminishes neuritogenesis in SH-SY5Y cells.

Stably transfected EGFP and Cx36 overexpressing SH-SY5Y cells were treated with retinoic acid and BDNF. Untreated Cx36 overexpressing cells formed numerous small cell clumps (I, K, M) whereas EGFP expressing cells formed larger aggregates (B, D, F). EGFP expressing cells (C, E, G) formed denser neurite networks than Cx36 overexpressing cells following treatment (J, L, M). Arrows identify neurites. Scale Bar = 100 μm. Insert scale bar= 50 μm.

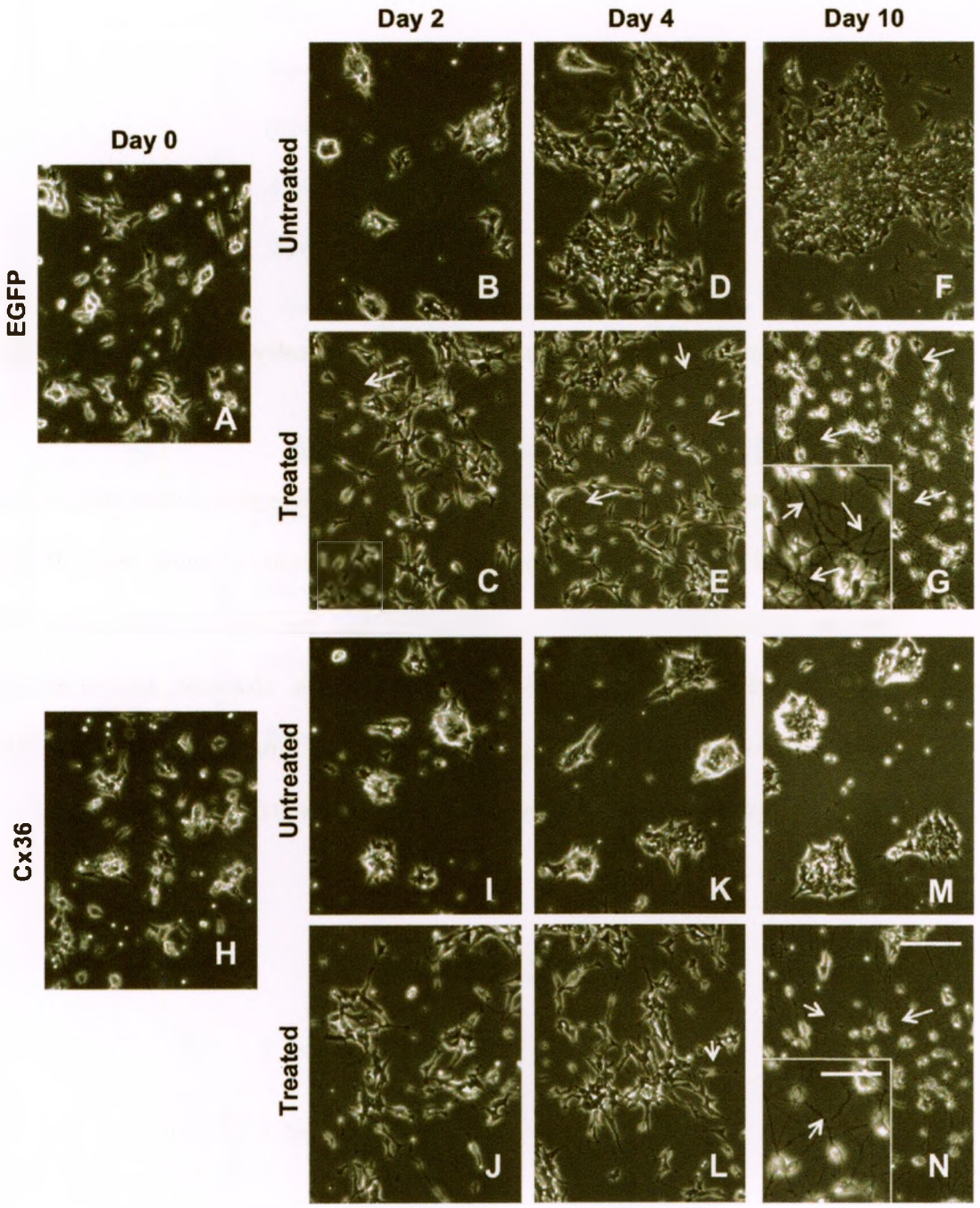

Day 2
Day 4
Day 10
Day 0
Untreated
Treated
EGFP
Cx36
A
B
C
D
E
F
G
H
I
J
K
L
M
N

Figure 2.6. Overexpression of Cx36 diminishes neurite density in differentiating SH-SY5Y cells.

Cells were seeded and subjected to 10 days of differentiation treatment. Cells were imaged and neurite length and density were measured using Neuron J. There was no significant difference in average (A) and maximum neurite length (B) between EGFP control and Cx36 overexpressing cells. However, neurite density was lower in Cx36 overexpressing cells than in EGFP control cells. N=3. Asterisk denotes statistical significance compared to EGFP. $p<0.05$. Bars show mean +/- S.E.

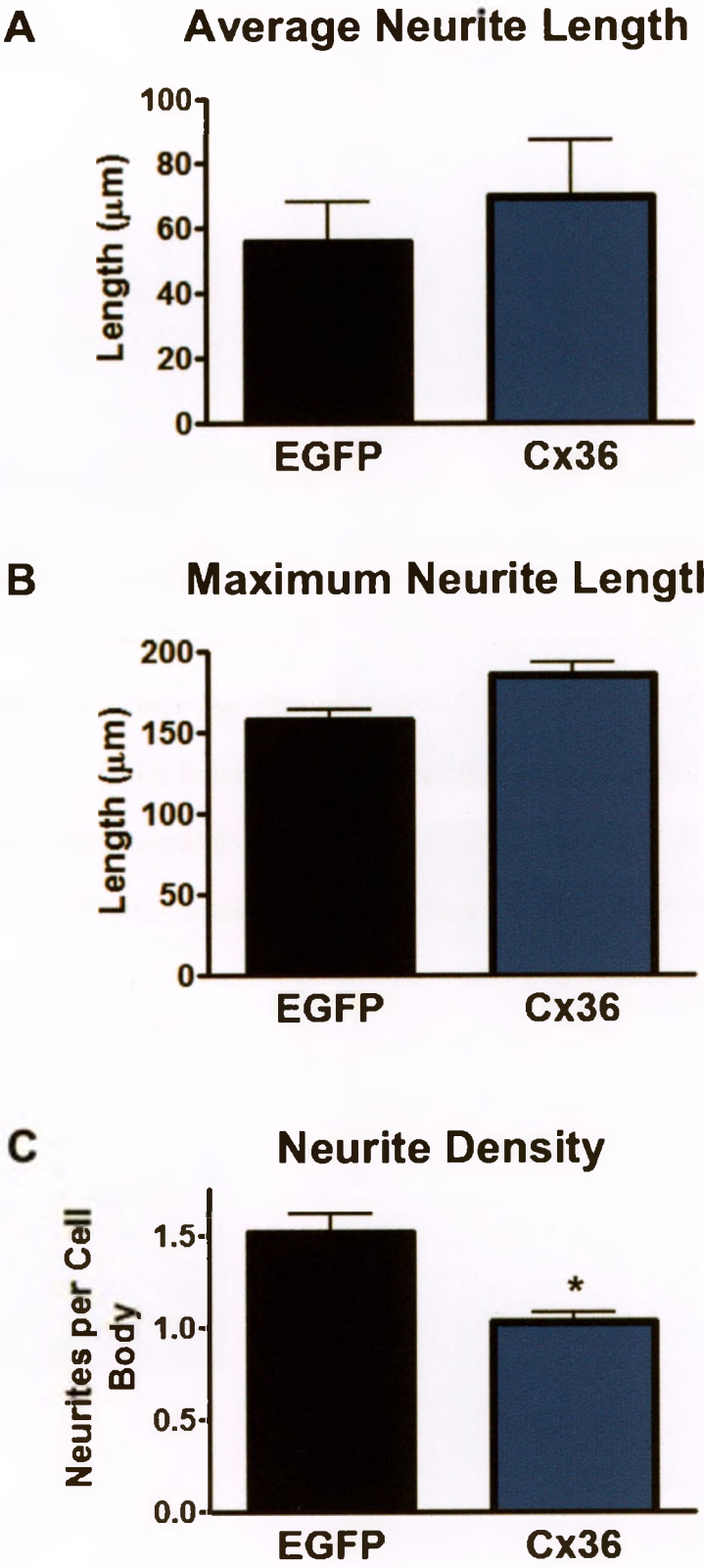

A
Average Neurite Length
Length (µm)
100
80
60
40
20
0
EGFP
Cx36
B
Maximum Neurite Length
Length (µm)
200
150
100
50
0
EGFP
Cx36
C
Neurite Density
Neurites per Cell Body
1.5
1.0
0.5
0.0
*
EGFP
Cx36

Figure 2.7. Cx36 knockdown suppresses aggregate formation and enhances neuritogenesis in SH-SY5Y cells.

Cells were plated and subjected to differentiation treatment over 10 days. Untreated non-effective shRNA expressing cells aggregate on collagen (B, D), while Cx36 shRNA expressing cells remain dispersed (G, I). At Day 10, more neurites (white arrows) were observed in the differentiating Cx36 shRNA expressing cells (J) than in non-effective shRNA control cells (E). Scale bar = 100 μm. Insert scale bar = 50 μm.

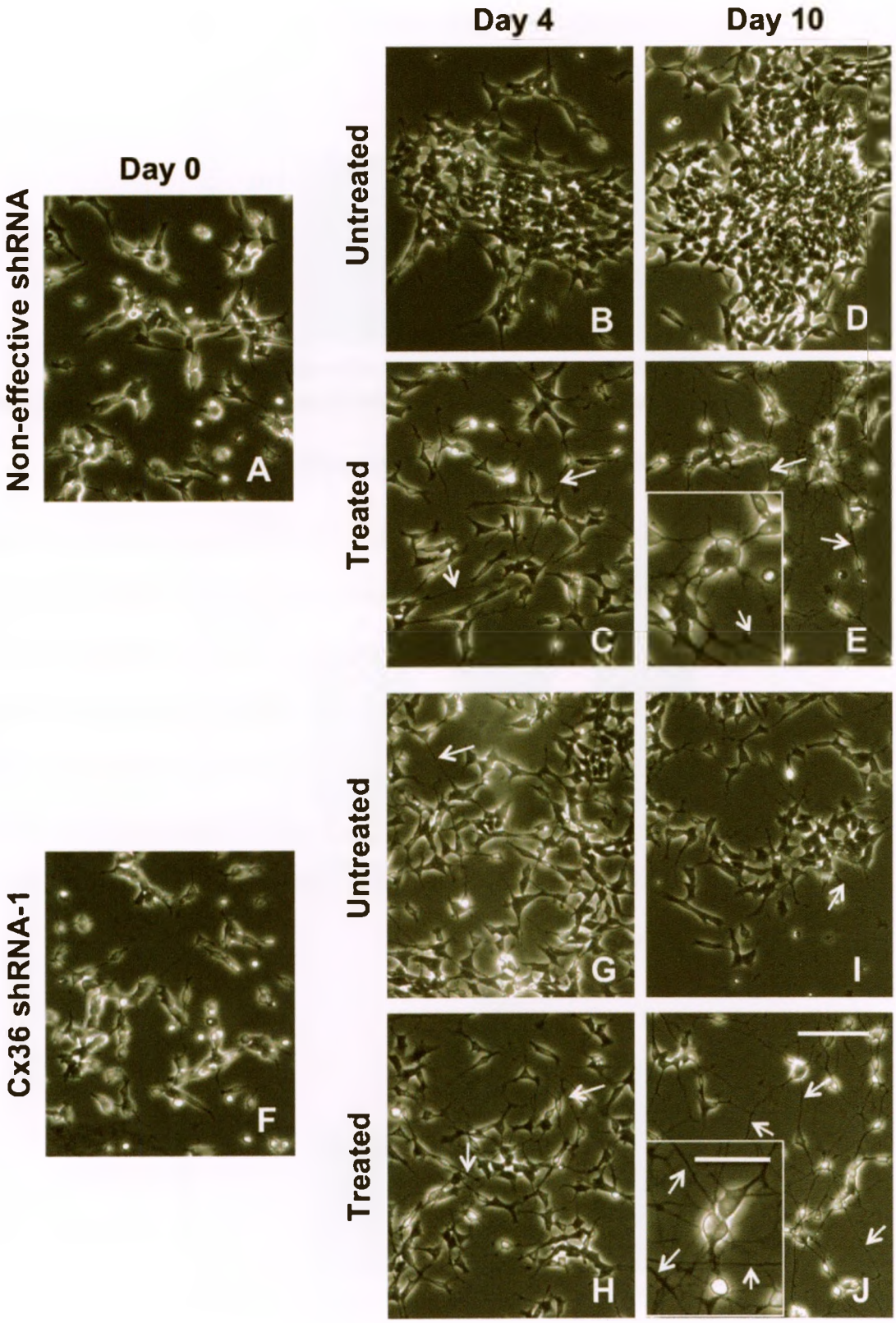
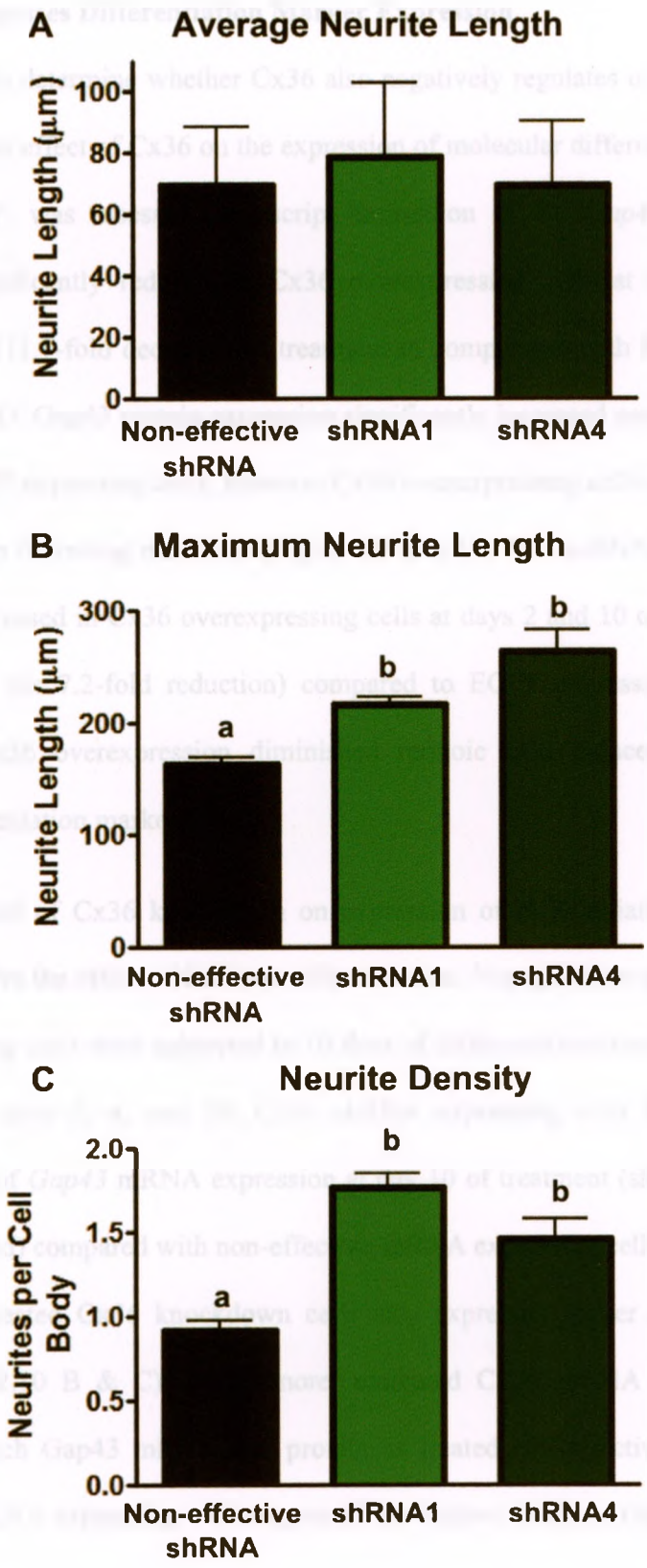

Day 0
Day 4
Day 10
Non-effective shRNA
Cx36 shRNA-1
Untreated
Treated
Untreated
Treated
A
B
C
D
E
F
G
H
I
J

Figure 2.8. Cx36 knockdown results in increased maximum neurite length and neurite density in differentiating SH-SY5Y cells.

Cells were seeded and subjected to 10 days of differentiation treatment. Cells were imaged and neurite length and density were measured using Neuron J software. There was no significant difference in average neurite length between non-effective shRNA expressing control and Cx36 shRNA expressing cells (A). However, maximum neurite length (B) and neurite density (C) were higher in Cx36 shRNA expressing than control cells. N=3. Bars represented by letters indicate a significant difference from bars represented by a different letter. $p<0.05$. Bars show mean +/- S.E.


A
Average Neurite Length
Neurite Length (µm)
100
80
60
40
20
0
Non-effective shRNA
shRNA1
shRNA4
B
Maximum Neurite Length
Neurite Length (µm)
300
200
100
0
a
b
b
Non-effective shRNA
shRNA1
shRNA4
C
Neurite Density
Neurites per Cell Body
2.0
1.5
1.0
0.5
0.0
a
b
b
Non-effective shRNA
shRNA1
shRNA4

2.3.3 Cx36 Suppresses Differentiation Marker Expression

In order to determine whether Cx36 also negatively regulates other indicators of differentiation, the effect of Cx36 on the expression of molecular differentiation markers, Gap43 and NPY, was assessed. Transcript expression of the *Gap43* differentiation marker was significantly reduced in Cx36 overexpressing cells at days 2 (18-fold decrease) and 4 (11.7-fold decrease) of treatment in comparison with EGFP expressing cells (Figure 2.9A). Gap43 protein expression significantly increased upon differentiation treatment in EGFP expressing cells, however Cx36 overexpressing cells failed to increase Gap43 expression following treatment (Figure 2.9 B & C). *NPY* mRNA levels were also significantly decreased in Cx36 overexpressing cells at days 2 and 10 of treatment (Day 2: 24-fold; Day 10: 7.2-fold reduction) compared to EGFP expressing cells (Figure 2.9D). Thus, Cx36 overexpression diminished retinoic acid induced expression of molecular differentiation markers.

The impact of Cx36 knockdown on expression of differentiation markers was assessed to confirm the effect of Cx36 on differentiation. Non-effective shRNA and Cx36 shRNA expressing cells were subjected to 10 days of differentiation treatment, and RNA was isolated at days 0, 4, and 10. Cx36 shRNA expressing cells had significantly increased levels of *Gap43* mRNA expression at day 10 of treatment (shRNA1: 6.9-fold; shRNA 4: 1.6-fold) compared with non-effective shRNA expressing cells (Figure 2.10A). Untreated and treated Cx36 knockdown cells also expressed higher levels of Gap43 protein (Figure 2.10 B & C). Furthermore, untreated Cx36 shRNA expressing cells expressed as much Gap43 mRNA and protein as treated non-effective control, while treated Cx36 shRNA expressing cells expressed the highest levels of Gap43. In addition,

Figure 2.9. Cx36 overexpression diminishes retinoic acid induced Gap43 and *NPY* expression.

RNA was isolated from cells at day 0, 2, 4, and 10 of differentiation treatment, and real-time PCR was used to quantify changes in mRNA expression. Protein was extracted from cells at 0 and 10 days of treatment and western blot analysis was used to quantify protein expression. *Gap43* expression was diminished in Cx36 overexpressing cells at days 2 and 4 of treatment (A). Gap43 protein expression did not significantly differ between Cx36 overexpressing and EGFP expressing cells. Gap43 expression significantly increased upon treatment in EGFP cells, however it failed to increase in treated Cx36 overexpressing cells (B & C). *NPY* expression is diminished at days 2 and 10 of treatment in Cx36 overexpressing cells (D). mRNA levels were normalized to *PPIA* and protein levels to GAPDH. N=3. Asterisks indicate statistical significance. Bars represented by different letters denote a statistically significant difference. $p<0.05$. Bars show mean +/- S.E.

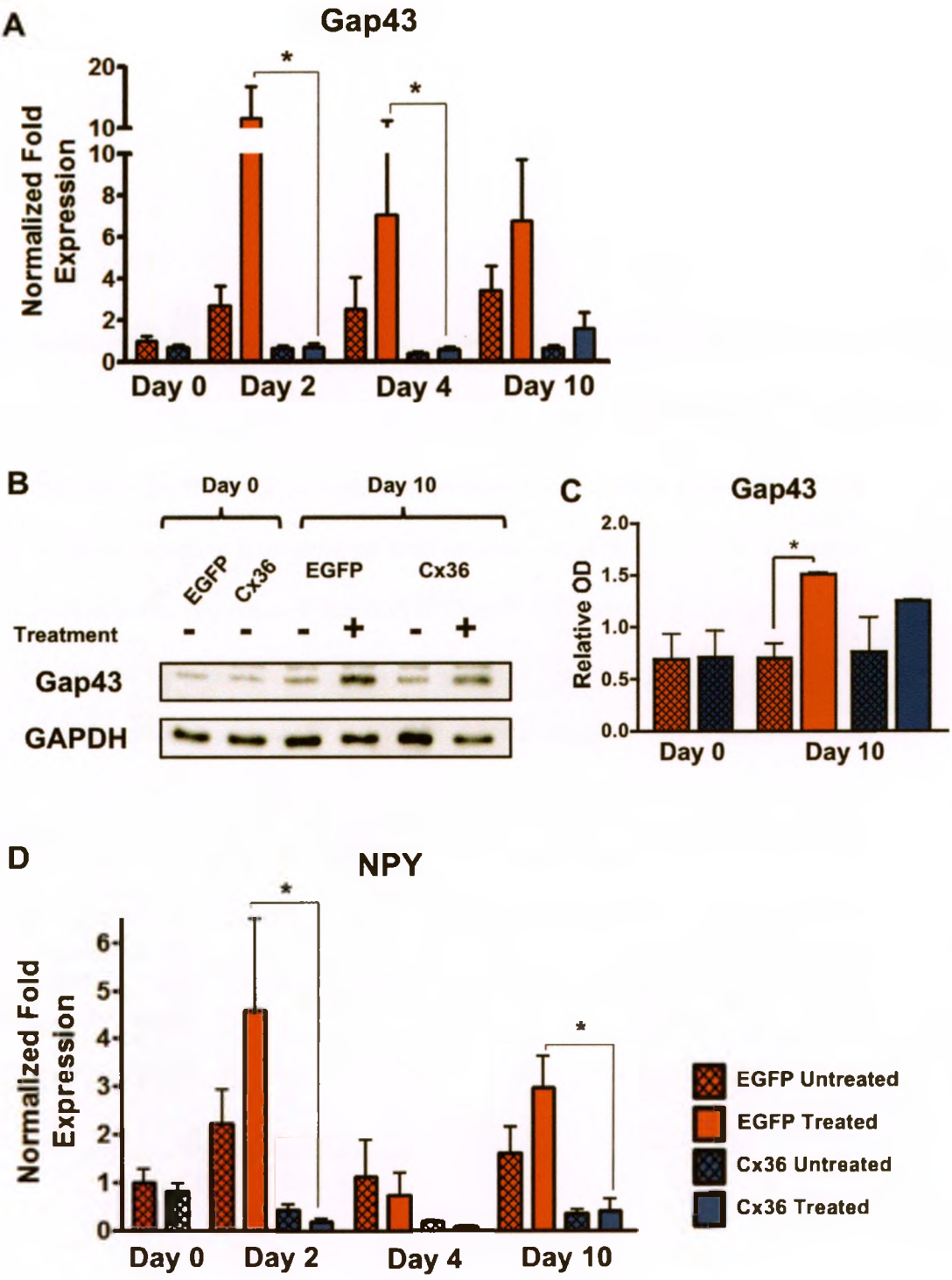
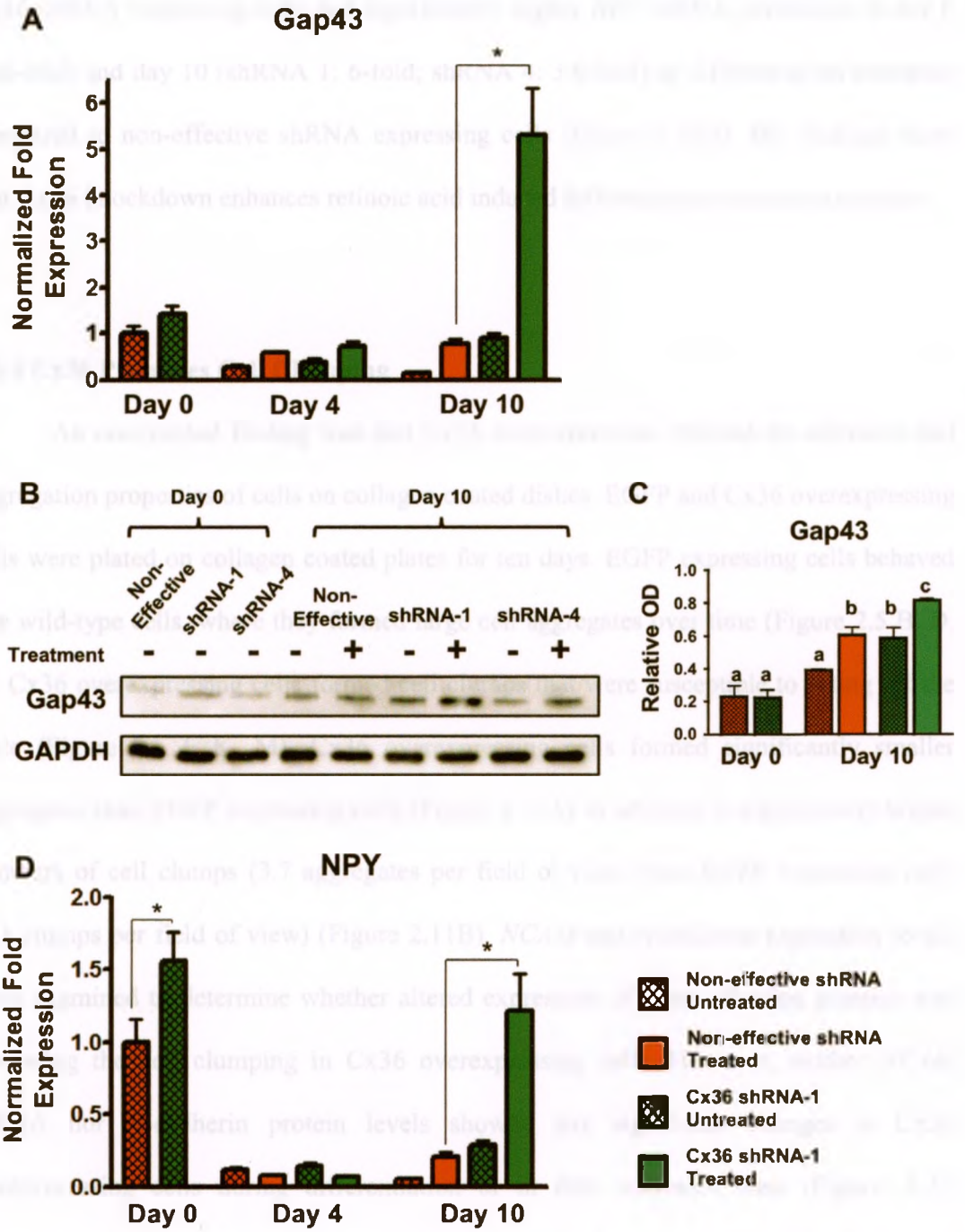

A
Gap43
Normalized Fold Expression
20
10
10
8
6
4
2
0
*
*
Day 0
Day 2
Day 4
Day 10
B
Day 0
Day 10
EGFP
Cx36
EGFP
Cx36
Treatment
- - - + - +
Gap43
GAPDH
C
Gap43
Relative OD
2.0
1.5
1.0
0.5
0.0
*
Day 0
Day 10
D
NPY
Normalized Fold Expression
6
5
4
3
2
1
0
*
*
Day 0
Day 2
Day 4
Day 10
EGFP Untreated
EGFP Treated
Cx36 Untreated
Cx36 Treated

Figure 2.10. Cx36 knockdown enhances retinoic acid induced Gap43 expression and upregulates *NPY* expression

RNA and protein were isolated from cells at days 0, 4, and 10 of differentiation treatment. Real-time PCR and western blotting were used to quantify mRNA and protein expression respectively. Gap43 expression is increased at the mRNA (A) and protein (B & C) levels in Cx36 shRNA expressing cells at day 10 of treatment. *NPY* expression is increased at days 0 and 10 of treatment in Cx36 shRNA expressing cells compared to non-effective shRNA expressing cells (D). mRNA levels were normalized to *PPIA* and protein normalized to GAPDH. N=3. Asterisks denote statistical significance. Bars represented by different letters denote a statistically significant difference. $p<0.05$. Bars show mean +/- S.E.


A
Gap43
Normalized Fold Expression
0
1
2
3
4
5
6
*
Day 0
Day 4
Day 10
B
Day 0
Day 10
Non-Effective
shRNA-1
shRNA-4
Non-Effective
shRNA-1
shRNA-4
Treatment
- - - - + - + - +
Gap43
GAPDH
C
Gap43
Relative OD
0.0
0.2
0.4
0.6
0.8
1.0
a a a b b c
Day 0
Day 10
D
NPY
Normalized Fold Expression
0.0
0.5
1.0
1.5
2.0
*
*
Day 0
Day 4
Day 10
Non-effective shRNA Untreated
Non-effective shRNA Treated
Cx36 shRNA-1 Untreated
Cx36 shRNA-1 Treated

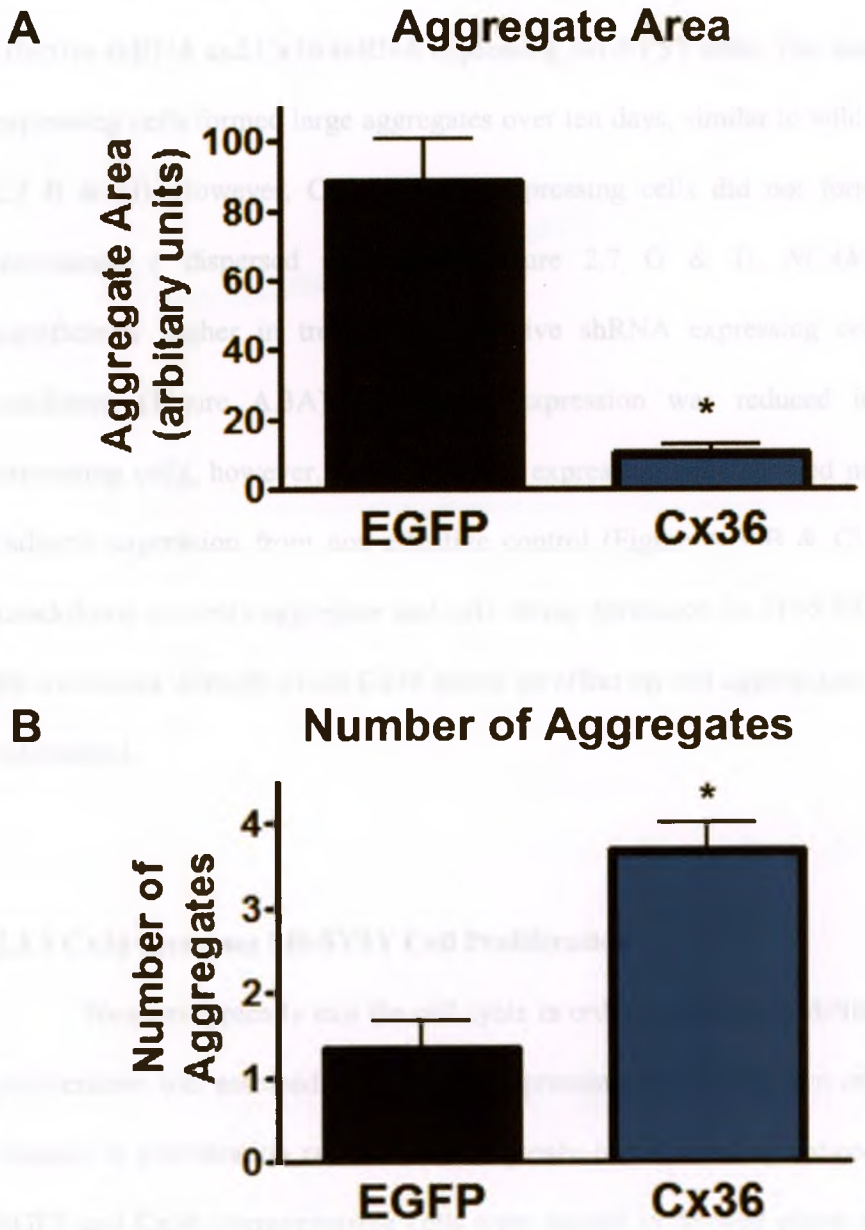
Cx36 shRNA expressing cells had significantly higher *NPY* mRNA expression at day 0 (1.6-fold) and day 10 (shRNA 1: 6-fold; shRNA 4: 3.6-fold) of differentiation treatment compared to non-effective shRNA expressing cells (Figure 2.10D). My findings show that Cx36 knockdown enhances retinoic acid induced differentiation marker expression.

2.3.4 Cx36 Promotes Cell Clumping

An unexpected finding was that Cx36 overexpression affected the adhesion and aggregation properties of cells on collagen coated dishes. EGFP and Cx36 overexpressing cells were plated on collagen coated plates for ten days. EGFP expressing cells behaved like wild-type cells, where they formed large cell aggregates over time (Figure 2.5 B, D, F). Cx36 overexpressing cells formed cell clumps that were susceptible to lifting off the plate (Figure 2.5 I, K, M). Cx36 overexpressing cells formed significantly smaller aggregates than EGFP expressing cells (Figure 2.11A) in addition to significantly higher numbers of cell clumps (3.7 aggregates per field of view) than EGFP expressing cells (1.3 clumps per field of view) (Figure 2.11B). *NCAM* and N-cadherin expression levels were examined to determine whether altered expression of these adhesion proteins was mediating the cell clumping in Cx36 overexpressing cells. However, neither *NCAM* mRNA nor N-cadherin protein levels showed any significant changes in Cx36 overexpressing cells during differentiation or in their untreated state (Figure A.2). Therefore, Cx36 overexpression resulted in increased formation of small cell clumps, although the adhesion molecules involved in this process have not yet been identified.

Figure 2.11. Cx36 overexpression increases the number of small cell clumps.

EGFP and Cx36 overexpressing SH-SY5Y cells were plated on collagen coated dishes. Following 10 days in culture, images were taken and aggregate area and number were measured. Cx36 overexpressing cells formed aggregates with significantly smaller area (A), however developed more numerous aggregates per field of view (B) than EGFP expressing cells. N=3. Asterisks indicate statistical significance compared to EGFP. $p<0.05$. Bars show mean +/- S.E.


A
Aggregate Area
Aggregate Area (arbitary units)
100
80
60
40
20
0
*
EGFP
Cx36
B
Number of Aggregates
Number of Aggregates
4
3
2
1
0
*
EGFP
Cx36

In order to confirm the effect of Cx36 on cell clumping, the effect of Cx36 knockdown on aggregate formation was assessed in a comparable study between non-effective shRNA and Cx36 shRNA expressing SH-SY5Y cells. The non-effective shRNA expressing cells formed large aggregates over ten days, similar to wild-type cells (Figure 2.7 B & D). However, Cx36 shRNA expressing cells did not form aggregates, and maintained a dispersed phenotype (Figure 2.7 G & I). *NCAM* expression was significantly higher in treated non-effective shRNA expressing cells than all other conditions (Figure A.3A). N-cadherin expression was reduced in Cx36 shRNA4 expressing cells, however, Cx36 shRNA1 expressing cells showed no difference in N-cadherin expression from non-effective control (Figure A.3 B & C). Therefore, Cx36 knockdown prevents aggregate and cell clump formation in SH-SY5Y cells. However, the molecules through which Cx36 exerts its effect on cell aggregation have not yet been determined.

2.3.5 Cx36 Increases SH-SY5Y Cell Proliferation

Neurons typically exit the cell cycle in order to terminally differentiate. Thus, cell proliferation was assessed in Cx36 overexpressing and knockdown cells to determine if changes in proliferation rate are a consequence of Cx36 manipulation of differentiation. EGFP and Cx36 overexpressing cells were seeded in 48-well plates and assessed using the MTT proliferation assay. Absorbance was measured at 0, 2, 4, and 6 days in culture. At day 6, Cx36 overexpressing cells had significantly higher absorbance than EGFP expressing cells implying enhanced cell proliferation in these cells (Figure 2.12).

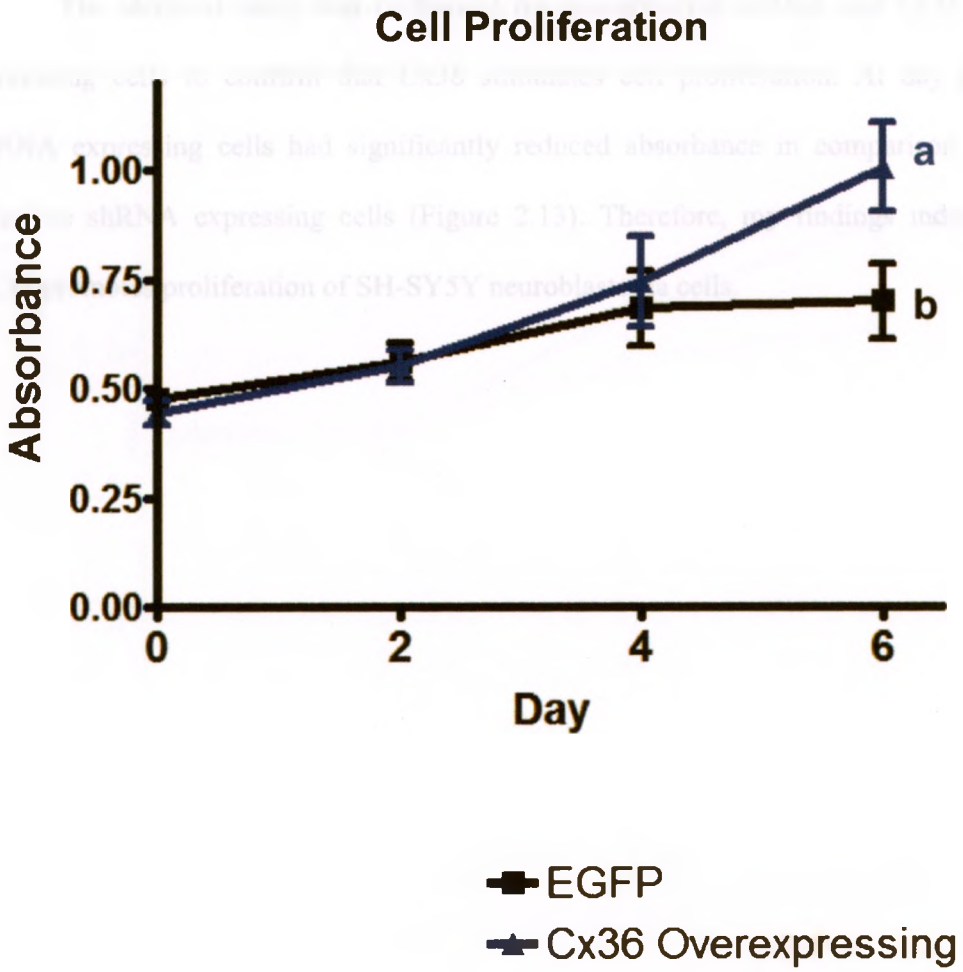


Figure 2.12. Cx36 overexpression increases SH-SY5Y cell proliferation.

Cells were seeded at 20,000 cells per well in 48-well plates and the MTT proliferation assay was conducted according to manufacturer's guidelines. Following 6 days in culture, Cx36 overexpressing cells had a significantly higher proliferation rate than EGFP control cells. Different letters indicate significant difference. $p < 0.05$. N=3. Points show mean +/-S.E.

The identical assay was performed for non-effective shRNA and Cx36 shRNA expressing cells to confirm that Cx36 stimulates cell proliferation. At day 6, Cx36 shRNA expressing cells had significantly reduced absorbance in comparison to non-effective shRNA expressing cells (Figure 2.13). Therefore, my findings indicate that Cx36 promotes proliferation of SH-SY5Y neuroblastoma cells.

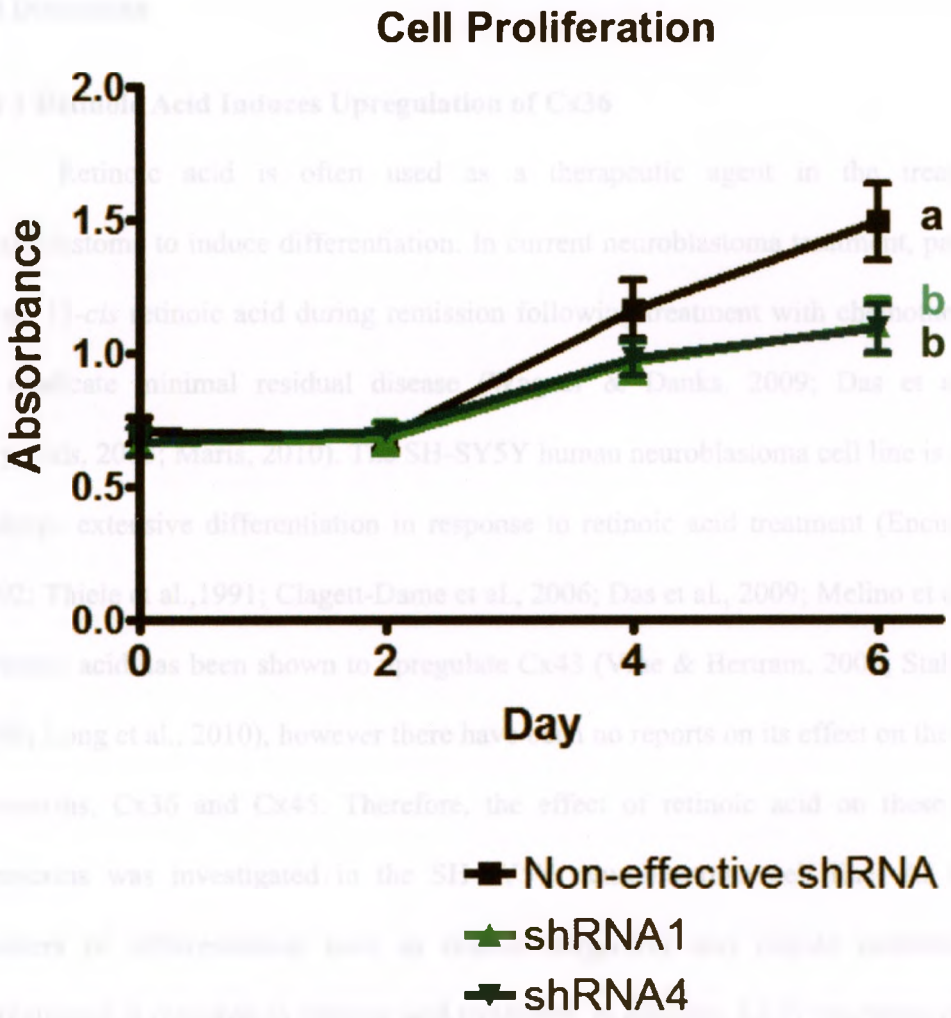


Figure 2.13. Cx36 knockdown decreases proliferation of SH-SY5Y cells.

Cells were seeded at 20,000 cells per well in 48-well plates and the MTT proliferation assay was conducted according to manufacturer's guidelines. Following 6 days in culture, Cx36 shRNA expressing cells had a significantly lower rate of proliferation than non-effective shRNA expressing control cells. Different letters indicate significant difference. $p<0.05$. N=3. Points show mean +/-S.E.

2.4 Discussion

2.4.1 Retinoic Acid Induces Upregulation of Cx36

Retinoic acid is often used as a therapeutic agent in the treatment of neuroblastoma to induce differentiation. In current neuroblastoma treatment, patients are given 13-*cis* retinoic acid during remission following treatment with chemotherapeutics to eradicate minimal residual disease (Wagner & Danks, 2009; Das et al., 2009; Reynolds, 2003; Maris, 2010). The SH-SY5Y human neuroblastoma cell line is known to undergo extensive differentiation in response to retinoic acid treatment (Encinas et al., 2002; Thiele et al.,1991; Clagett-Dame et al., 2006; Das et al., 2009; Melino et al., 1997). Retinoic acid has been shown to upregulate Cx43 (Vine & Bertram, 2005; Stahl & Sies, 1998; Long et al., 2010), however there have been no reports on its effect on the neuronal connexins, Cx36 and Cx45. Therefore, the effect of retinoic acid on these neuronal connexins was investigated in the SH-SY5Y neuroblastoma cell line. As expected, markers of differentiation such as neurite outgrowth and *Gap43* expression were upregulated in response to retinoic acid treatment. In addition, *Cx36* was transcriptionally upregulated in response to retinoic acid induced differentiation, whereas *Cx45* expression levels did not increase in differentiating cells. Therefore, *Cx36* appears to be downstream of retinoic acid signaling, since it is upregulated upon retinoic acid induced differentiation and *Cx36* contains a retinoid X receptor binding motif similar to that of the *Cx43* gene (Cicirata et al., 2000). Conversely, *Cx45* does not appear to be regulated by retinoic acid. Thus, I have identified a potential transcriptional target, *Cx36*, in retinoic acid signaling. In addition, since *Cx36* expression increased upon retinoic acid induced

differentiation, *Cx36* gene expression was manipulated to ascertain its effects on differentiation and proliferation of SH-SY5Y cells.

2.4.2 Cx36 Negatively Regulates SH-SY5Y Cell Differentiation

SH-SY5Y cells stably transfected with either EGFP or Cx36 were induced to differentiate with retinoic acid treatment. EGFP expressing cells formed neurites within two days of differentiation and formed extensive neurite networks by ten days. Interestingly, Cx36 overexpressing cells formed sparse neurites by two and four days of differentiation treatment. At ten days of differentiation, Cx36 overexpressing cells formed limited neurite networks, indicated by decreased neurite density, compared with EGFP expressing cells. Cx36 overexpressing cells also experienced diminished Gap43 and *NPY* transcript expression in response to differentiation treatment.

Conversely, SH-SY5Y cells stably expressing Cx36 shRNA projected neurites within four days of differentiation treatment and formed extensive neurite networks within ten days of differentiation. Cx36 knockdown resulted in significantly increased neurite density compared to non-effective shRNA expressing cells. In addition, knockdown of Cx36 resulted in increased Gap43 transcript and protein levels in response to retinoic acid. Cx36 knockdown also resulted in increased Gap43 protein expression in untreated cells. It was also observed that Cx36 shRNA expressing cells had increased levels of *NPY* transcript prior to treatment and following ten days of differentiation treatment.

Cx36 appears to negatively regulate differentiation of SH-SY5Y human neuroblastoma cells. Accordingly, knockdown of Cx36 enhances differentiation. This appears to be in contradiction with the upregulation of Cx36 upon retinoic acid induced differentiation. However, since I was unable to measure protein expression, it is possible that although *Cx36* mRNA is upregulated in response to retinoic acid, Cx36 protein expression is unaffected. However, this discrepancy may be due to the requirement of an optimal level of Cx36 for proper differentiation. Accordingly, the small increase in Cx36 expression induced by retinoic acid in wild-type cells does not appear to interfere with differentiation. However, since Cx36 overexpressing cells exhibit diminished differentiation and Cx36 knockdown enhances differentiation, it is apparent that substantial changes in Cx36 expression alter the process of differentiation.

The effect of Cx36 on differentiation is in contrast to other connexins, which are known to enhance differentiation (Vinken et al, 2006; Asazuma-Nakamura et al., 2009; Banks et al., 2007; Brehm et al., 2007; Churko et al., 2010; Mao et al., 2000). For example, overexpression of Cx43 and Cx32 enhanced nerve growth factor induced differentiation of PC12 cells (Belliveau et al., 2006). Although connexin overexpression usually enhances differentiation, Todorova et al (2008) have shown that GJIC and Cx43 are required to maintain pluripotency of embryonic stem cells. Inhibition of GJIC or Cx43 knockdown resulted in reduced proliferation and stem cell marker expression, with a concomitant increase in differentiation marker expression (Todorova et al., 2008). Thus, in certain environments, as with my Cx36 overexpression study, connexin expression is required to maintain the stem cell phenotype. My finding of Cx36 knockdown enhancing differentiation in fact parallels the natural expression patterns of

Cx36, where it is highly expressed in many neural structures of the developing fetus but is subsequently downregulated during postnatal differentiation and maturation of the nervous system (Belluardo et al., 2000; Gulisano et al., 2000). This downregulation may be due in part to replacement of electrical synapses with chemical synapses (Lee et al., 2010), or it may indicate that Cx36 is actually detrimental in the process of terminal differentiation of certain structures, as was shown in this study.

2.4.3 Cx36 Promotes Clumping of SH-SY5Y Cells

A surprising finding was that altering Cx36 expression dramatically changed the adhesion and aggregation properties of SH-SY5Y cells. EGFP expressing cells formed large aggregates similar to wild-type cells following growth on collagen over ten days. Interestingly, Cx36 overexpressing cells did not form these large adherent aggregates. In contrast, Cx36 overexpressing cells formed small cell clumps. These clumps did not adhere well to the dish, and were susceptible to lifting from the dish and growing in suspension. This type of growth is similar to that of neurospheres, which are suspended clusters of neural stem cells (Bez et al., 2003). In fact, it is characteristic of the neuroblast subpopulations of SH-SY5Y cells to aggregate, lift off, and grow in suspension (Ross et al., 1983; Biedler et al., 1978). Interestingly, this neuroblastic phenotype appears to be stimulated by overexpression of Cx36, which is in accordance with my findings of Cx36 induced downregulation of differentiation.

Non-effective shRNA expressing cells formed large aggregates in a manner similar to wild-type and EGFP expressing cells, however, Cx36 shRNA expressing cells

did not form aggregates of any type when grown on collagen. Rather, knockdown of Cx36 resulted in a dispersed phenotype with some neuritogenesis. This is in contrast to non-effective shRNA expressing cells which extend few or no neurites.

NCAM and N-cadherin expression were assessed as potential mediators of the differential aggregate formation in Cx36 overexpressing and knockdown cells. Cx43 is known to colocalize with N-cadherin and regulate its localization at the cell surface (Wei et al., 2005). Although there have been no documented reports of Cx36 and N-cadherin interaction, I investigated whether manipulation of Cx36 expression resulted in changes to N-cadherin expression. There was no difference between EGFP and Cx36 expressing cells in N-cadherin protein expression. Non-effective shRNA and Cx36 shRNA-1 expressing cells also did not differ in N-cadherin protein expression. In addition, I explored the effect of Cx36 expression on *NCAM* mRNA expression, as NCAM is upregulated in poorly differentiated and aggressive neuroblastomas (Jensen & Berthold, 2007; Bourne et al., 1991). *NCAM* mRNA expression was not significantly altered with Cx36 overexpression, however *NCAM* expression was reduced in Cx36 knockdown cells compared with differentiating non-effective control. Therefore, although *NCAM* may be mediating some of the adhesion properties related to cell aggregation, from this study, it is most likely not the primary adhesion molecule involved in this process as it was unaffected by Cx36 overexpression. While Cx36 expression has a dramatic effect on cell aggregation, the adhesion molecules mediating this process are yet to be identified.

2.4.4 Cx36 Increases Cell Proliferation

There is a substantial linkage between connexin expression and cell growth and proliferation (Vinken et al., 2006; Kardami et al., 2007). My findings, however, were unexpected as Cx36 overexpression increased cell proliferation, while knockdown of Cx36 diminished cell proliferation. This is in contrast to other connexins which typically act as tumor suppressors, specifically by inhibiting proliferation of tumor cells (Mao et al., 2000; McLachlan et al., 2006; Jiminez et al., 2006; Omori et al., 2001; Zhang et al., 1998). However, in certain environments, connexins and gap junctional intercellular communication are required to maintain the proliferative ability of cells. For example, extravillous trophoblasts lost the ability to proliferate when GJIC was inhibited by a gap junction blocker or Cx40 antisense cDNA (Nisimura et al., 2004).

Additionally, Cx36 has been implicated in retinal cell survival following injury (Striedinger et al., 2005). In light of the role of Cx36 in cell survival and its high expression during development when neural precursors are proliferating, it is not surprising to find that Cx36 serves to increase proliferation. It is also important to consider that Cx36 is regulated in a different manner from other connexins. For example, Cx36 channels show differential pH gating and experience very low sensitivity to voltage gating (Gonazelz-Nieto et al., 2008; Srinivas et al., 1999). Specifically, Cx36 channels close in response to alkalosis and open slightly in response to acidosis (Gonazelz-Nieto et al., 2008). This pH gating may be important under pathological conditions such as ischemia, where Cx36 expression has been shown to be upregulated and involved in cell survival (Oguro et al., 2001). Therefore, the differential regulation mechanisms of Cx36

channels may suggest a different role for Cx36 in key cellular processes from that of other connexins.

In relation to differentiation, as neurons do not retain a capacity to divide, cells must exit the cell cycle and stop proliferating in order to terminally differentiate into neurons. Retinoic acid also exerts part of its differentiation inducing effects by inhibiting cell proliferation (Voigt & Zintl, 2003). Therefore, the enhanced differentiation observed in Cx36 shRNA expressing cells may be partially due to their decreased proliferative ability.

This study is the first to determine the effects of Cx36 in the process of differentiation. I have identified *Cx36* as a novel potential transcriptional target of retinoic acid signaling. However, further research is required to determine whether *Cx36* is a direct target of retinoic acid signaling. In addition, Cx36 appears to negatively regulate differentiation, which is in contrast to the effects of other connexins on differentiation. Furthermore, it appears that Cx36 promotes a neural stem cell like phenotype based on the diminished differentiation, enhanced clustering and increased proliferation in overexpressing cells, and the reversal of this phenotype in cells where Cx36 is knocked down.

However, it is unknown whether Cx36 mediates these changes in a gap junctional intercellular communication dependent manner or through interaction of Cx36 with binding partners, and therefore downstream signaling pathways. Therefore, further research is required to elucidate the mechanisms underlying the regulation of differentiation by Cx36.

2.4.4 Acknowledgements

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CHAPTER THREE:

GENERAL DISCUSSION

3.1 Summary and Conclusions

Objective One: Determine the changes in connexin gene expression following retinoic acid induced differentiation of neuroblastoma

Cx43 is known to be upregulated in response to retinoic acid (Stahl & Sies, 1998; Vine & Bertram, 2005; Long et al., 2010). However, the effect of retinoic acid treatment on Cx45 and Cx36 has not been explored. In this study, I investigated whether Cx45 or Cx36 were involved in retinoic acid signaling. Retinoic acid treated cells formed neurites and had increased levels of the differentiation marker, *Gap43*. *Cx45* expression did not increase in retinoic acid treated cells. Conversely, *Cx36* mRNA expression was upregulated in response to retinoic acid treatment, indicating that *Cx36* may be a target of retinoic acid signaling.

Objective Two: Investigate the effects of Cx36 overexpression on differentiation and proliferation of human neuroblastoma cells

Although many connexins have been shown to regulate differentiation and proliferation, the effect of Cx36 on these processes has not been explored. EGFP and Cx36 were stably transfected in SH-SY5Y human neuroblastoma cells. Cx36 overexpressing cells had diminished neuritogenesis in response to retinoic acid and BDNF differentiation treatment. These differentiated cells also had decreased expression of differentiation markers, Gap43 and *NPY*. Interestingly, untreated Cx36 overexpressing cells formed abundant clumps when grown on collagen. These cells were also susceptible to lifting from the dish and growing in suspension. Conversely, EGFP expressing cells

formed large, adherent aggregates on collagen. In addition, Cx36 overexpressing cells were more proliferative than EGFP expressing cells. Thus, overexpression of Cx36 diminishes neuronal differentiation and promotes cell clumping and proliferation.

Objective Three: Investigate the effects of knocking down Cx36 on differentiation and proliferation of human neuroblastoma cells

SH-SY5Y human neuroblastoma cells were stably transfected with non-effective shRNA, or shRNA targeting *Cx36*. Cx36 shRNA expressing cells had enhanced neuritogenesis in response to differentiation treatment. In addition, differentiated cells expressed higher levels of Gap43 and *NPY*. Cx36 shRNA expressing cells exhibited a dispersed morphology when grown on collagen and rarely formed clumps or aggregates. Finally, Cx36 shRNA expressing cells had decreased proliferation compared to non-effective shRNA expressing cells. Therefore, knockdown of *Cx36* promotes neuronal differentiation and decreases cell proliferation and aggregation.

3.2 Contributions of the Research

3.2.1 General Significance

Cx36 is one of the most recently discovered members of the connexin family (Belluardo et al., 1999; Sohl et al., 1998). The Cx36 literature is heavily focused on spatial and temporal expression analysis, as well as some trafficking and channel function studies (Srinivas et al., 1999; Gulisano et al., 2000; Zoidl et al., 2002; Gonzalez-Nieto et

al., 2008; Wellerhaus et al., 2008). Therefore, I investigated the effect of manipulating Cx36 expression on neuronal differentiation. In this study, I identified *Cx36* as a novel potential target of retinoic acid signaling. In addition, Cx36 was found to act as a negative regulator of neuronal differentiation. This study is the first to explore the effects of manipulating Cx36 expression levels to investigate the process of differentiation. Since most connexins enhance differentiation and suppress proliferation (Temme et al., 1997; Zhang et al., 1998; Belliveau et al., 2006; Jimenez et al., 2006;), it would be intriguing to determine which distinguishing characteristic of Cx36 leads to its unique effects on these cellular processes.

3.2.2 Cx36 Negatively Regulates Differentiation of SH-SY5Y Cells

Cx36 overexpressing cells exhibited diminished neuritogenesis as well as Gap43 and *NPY* expression in response to retinoic acid treatment, whereas Cx36 shRNA expressing cells displayed enhanced neuritogenesis and Gap43 and *NPY* expression in response to retinoic acid. Cx36 shRNA-4 did not have an effect on Gap43 expression, however this was most likely due to the lower knockdown efficiency of this shRNA.

These studies have revealed Cx36 as a down-regulator of differentiation, while knockdown of Cx36 enhances differentiation. As stated in Section 3.2.1, this corresponds to the temporal expression patterns of Cx36. In the adult brain Cx36 is expressed in many structures including the inferior olive, olfactory bulb, retina, and hippocampus (Gulisano et al., 2000; Belluardo et al., 2000; Weickert et al., 2005; Placantonakis et al., 2006; Pan et al., 2010). Cx36 is highly expressed in the first two postnatal weeks in many neuronal

structures, including the spinal cord, ventral thalamic nuclei and the majority of the cerebral cortex. However, these structures either lose or limit Cx36 expression to specific subregions following the second postnatal week (Belluardo et al, 2000). In light of my findings, potentially detrimental effects of Cx36 on differentiation of certain structures may necessitate the tight temporal regulation of Cx36 expression.

3.2.3 Cx36 Promotes Cell Clumping

Unexpectedly, Cx36 overexpressing cells formed numerous small clumps when grown on collagen, whereas EGFP expressing cells grew to form large adherent aggregates. The clumps formed by Cx36 overexpressing cells were prone to lifting off the dish and growing in suspension in a manner similar to neural progenitor cells that form neurospheres (Bez et al., 2003). Conversely, SHSY-5Y cells expressing Cx36 shRNA did not form aggregates on collagen, and rather, maintained a dispersed phenotype.

It appears that Cx36 may regulate the expression of one or more adhesion molecules involved in both cell-matrix and cell-cell adhesion. The cell-matrix adhesion would influence the ability of the cells to lift off and grow in suspension, while cell-cell adhesion would mediate their ability to form clumps or aggregates. Both N-cadherin and neural cell adhesion molecule (NCAM) mediate cell-cell adhesion. Since Cx43 is known to regulate N-cadherin localization, the effect of Cx36 overexpression and knockdown on N-cadherin expression was investigated (Wei et al., 2005). However, manipulation of Cx36 expression did not result in changes in N-cadherin protein expression. NCAM is associated with aggressive and poorly differentiated neuroblastoma tumors (Jensen &

Berthold, 2007; Bourne et al., 1991), and thus its expression was also examined in response to Cx36 expression manipulation. *NCAM* mRNA expression did not change in response to Cx36 overexpression, however it was decreased in Cx36 shRNA expressing cells. The NCAM protein has three different isoforms due to alternative splicing and a post-translational modification to incorporate polysialic acid, which is the form that is associated with the aggressive cancer phenotype (Jensen & Berthold, 2007). Since NCAM protein expression was not assessed, I cannot conclude with certainty that it is not a mediator of the clumping effect seen in Cx36 overexpressing cells. Therefore, although there is an obvious change in clumping and aggregation in response to manipulating Cx36 expression, the adhesion molecule involved in this process has yet to be identified.

3.2.4 Cx36 Increases Proliferation of SH-SY5Y Cells

In addition to its effect on differentiation and aggregation, Cx36 also regulates proliferation. Cx36 overexpression in SH-SY5Y cells increased cell proliferation, while knockdown of Cx36 reduced proliferation. As with the effect of Cx36 on differentiation, this result is in accordance with the high expression of Cx36 during early postnatal development when neural cells are proliferating. In adulthood, neurons do not divide, and thus downregulation of Cx36 may be necessary to exit the cell cycle. In addition, Cx36 promotes cell survival in retinal cells following injury and in the hippocampus during ischemia (Oguro et al., 2001; Striedinger et al., 2005; Orellana et al., 2009). In light of my results, it is likely that Cx36 promotes cell proliferation in addition to its established role in cell survival.

3.3 Limitations and Future Studies

3.3.1 Limitations of In Vitro Model

My studies were limited to the SH-SY5Y neuroblastoma cell line. Unfortunately, these cells were unsuitable for immunocytochemical uses. Cells aggregated on glass coverslips and permanox chamber slides and lifted off the slides during washing or fixing. In addition, the remaining cells exhibited disrupted cell morphology. Several different fixatives were applied, however only one of these maintained the integrity of the cells: 2% paraformaldehyde supplemented with 150 mM sodium chloride, 120 mM sucrose, and 0.1% glutaraldehyde. However, even this fixative resulted in few cells remaining on the coverslip and inconsistent labelling, and thus immunocytochemical studies were abandoned. Immunocytochemistry would have allowed me to assess the localization patterns of endogenous Cx36, as well as the effects of Cx36 overexpression and knockdown on its localization. Furthermore, the effect of Cx36 on the subcellular localization patterns of N-cadherin could have been investigated, as the effect of Cx43 on N-cadherin is on localization rather than expression (Wei et al., 2005).

SH-SY5Y cells had intermediate expression levels of Cx36 and thus allowed for both overexpression and knockdown studies. However, only a single cell line was utilized and therefore I cannot generalize to other cell lines or tissues. Originally, I had the use of three neuroblastoma cell lines, however, due to unsuccessful transfection and differentiation, I could not pursue my studies with the other cell lines. This study would benefit from the use of additional neuroblastoma cell lines, especially retinoic acid responsive and *MYCN* amplified cell lines, such as LAN-5 and SK-N-BE (Negroni et al., 1991; Cervello et al., 2000; Suenaga et al., 2009). In addition, it would be ideal to analyze

human neuroblastoma tumor samples for *Cx36* expression at various stages of differentiation. Furthermore, assessing *Cx36* expression in the transgenic mouse model that spontaneously develops neuroblastoma would supplement my work at the cellular level with tissue, and thus ensure the generalizability of my studies (Weiss et al., 1997).

3.3.2 Cx36 Expression Analysis Tools

Cx36 is a relatively recently discovered connexin, thus limiting the availability of reliable antibodies for its detection by western blot. Several antibodies were tested including Zymed 366400, which is the most cited Cx36 antibody in the literature (Li et al., 2004a,b; Rezende et al., 2007; Helbig et al., 2010). However, none of these antibodies provided a consistently detectable signal, despite the use of several different cell lysis buffers. Two custom polyclonal antibodies were then designed; one was targeting the carboxy terminus and the other targeting the cytoplasmic loop. However, neither of these antibodies has provided consistent detection when both positive and negative control tissues were included for testing. Further characterization of these antibodies is required. At this point, however, real-time PCR is the most consistent and reliable method of *Cx36* expression analysis. This method only detects transcript levels, which is usually but not always correlated with protein expression (Guo et al., 2008). The knockdown studies would have greatly benefited from Cx36 protein expression data due to my inconsistent detection of the *Cx36* transcript. shRNA knockdown inhibits translation by targeting the transcript for the protein of interest for degradation (Paddison et al., 2002). However, in real-time PCR, the amplicon is very small (less than 200bp long), and thus a region of the transcript that has not been degraded may be amplified and mask the effect of the shRNA

knockdown (Shepard, Jacobson & Clark., 2005; Holmes et al., 2010). Therefore it would be ideal to quantify Cx36 expression at the protein level; however this is not feasible with the currently available antibodies.

3.3.3 Future Studies

Although I have identified *Cx36* as a potential target of retinoic acid signaling, it is yet to be determined whether this occurs through a direct mechanism. I would undertake either of two approaches in order to determine whether *Cx36* is a direct transcriptional target of retinoic acid. Transfection of cells with a plasmid encoding the promoter region of *Cx36* and the firefly luciferase reporter, followed by measuring reporter activity after retinoic acid treatment would demonstrate that *Cx36* is directly regulated by retinoic acid. Alternatively, adding retinoic acid following treatment with the protein synthesis inhibitor, cycloheximide, and detecting *Cx36* upregulation within four hours would indicate that *Cx36* is a direct target of retinoic acid. Either of these experiments would allow me to determine whether there is a direct interaction between retinoic acid and *Cx36* expression.

The most immediate concern involves determining the identity of the adhesion molecules that are regulated by Cx36. N-cadherin and NCAM are unlikely to be the primary facilitators of the clumpy and dispersed phenotype exhibited by Cx36 overexpressing and knockdown cells respectively. Integrin beta 1 and CD147 are other possible candidates that are involved in differentiation and cell aggregation (Rozzo, Chiesa & Ponzoni, 1997; Garcia et al., 2009). The intregrins are prime candidates since

they are responsible for cell-matrix adhesion and Cx36 overexpressing cells appear to be compromised in their ability to adhere to collagen-coated culture dishes. However further studies must be conducted to determine whether expression patterns of these adhesion molecules are altered by Cx36 manipulation.

Furthermore, due to the immature phenotype of Cx36 overexpressing cells, including increased proliferation and clustering as well as decreased cell differentiation, these cells may be reverting to a more progenitor cell phenotype. To further explore this possibility, Notch and Inhibition of Differentiation/ DNA binding 2 (ID2) expression should be investigated (Ferrari-Toninelli et al., 2009; Lopez-Carballo et al., 2002). Notch is a stem cell marker that has been implicated in neuroblastoma. Notch activation results in morphological remodeling of differentiated cells and its inhibition results in growth arrest and increased differentiation of SH-SY5Y cells (Ferrari-Toninelli et al., 2009; Ferrari-Toninelli et al., 2010). In addition, ID2 is expressed in neural crest cells and is also known to increase proliferation and inhibit differentiation of neuroblastoma cells (Lofstedt et al., 2004). ID2 is a transcriptional target of MYCN and is highly expressed in *MYCN* amplified neuroblastoma cell lines (Lasorella et al., 2000; Lasorella et al., 2002). Therefore, since Notch and ID2 are highly expressed in the neural crest and in dedifferentiated neuroblastoma cells, it would be of interest to ascertain whether Cx36 overexpression upregulates these markers.

The Cx36 and Cx50 specific channel blocker, mefloquine, or dominant-negative mutant of Cx36 could be used to supplement the shRNA knockdown component of this study (Allison et al., 2006; Cruikshank et al., 2004). In particular, if mefloquine has the same effect on differentiation as the knockdown, it would suggest a gap junction

intercellular communication dependent function of Cx36. Conversely, if mefloquine successfully blocks Cx36 channel function without effecting differentiation then I may conclude that knockdown of Cx36 has a GJIC independent effect on differentiation. In this event, to further elucidate the mechanism behind the effect of Cx36 on differentiation, the impact of transfecting a dominant negative mutant in wild type (WT) and Cx36 overexpressing cells would be investigated. The dominant negative mutant of Cx36 blocks WT Cx36 from trafficking to the plasma membrane and prevents GJIC in multiple cell types (Placantonakis, Cicirata & Welsch, 2002). If the mutant has similar effects to Cx36 shRNA, I may conclude that the effect of Cx36 on differentiation is due to its interaction with binding partners. The interaction of Cx36 with known binding partners occurs during trafficking to the cell membrane or at the cell membrane itself (Li et al., 2004a, b; Li et al., 2009), thus this interaction would be limited since the mutant prevents Cx36 trafficking and traps it intracellularly.

The SH-SY5Y human neuroblastoma cell line is not *MYCN* amplified (Edsjo et al., 2003). It would be of interest to broaden my studies to include other human neuroblastoma cell lines that are retinoic acid responsive and *MYCN* amplified, such as the LAN-5 and SK-N-BE cell lines (Negroni et al., 1991; Cervello et al., 2000; Suenaga et al., 2009). In particular, this will allow us to determine whether manipulation of Cx36 expression has an effect on MYCN expression, and whether Cx36 knockdown has a stronger effect on differentiation and proliferation in a more aggressive and less differentiated neuroblastoma cell line.

In addition, in order to take this study another step forward, the effect of Cx36 on the tumor cell phenotype should be investigated. For example, the migration, invasion,

and anchorage independent growth properties of Cx36 overexpressing and knockdown cells should be studied. The anchorage independent growth studies would be of particular interest due to the clumping and growth in suspension of Cx36 overexpressing cells. To assess differences in tumorigenicity, Cx36 overexpressing and knockdown cells along with their respective controls could be subcutaneously injected into athymic (Balb/c nude) mice to evaluate the sizes of the tumors formed (Zhao et al., 2008).

As mentioned in Section 3.3.1, analysis of murine or human tumor samples would allow us to broaden the implications of my work from the cellular to the tissue and organism level. Based on my results, it would be interesting to determine whether Cx36 is more highly expressed in less differentiated and therefore, more aggressive neuroblastoma tumors. The Weiss Laboratory has been contacted regarding murine samples, however we are still awaiting a response (Weiss et al., 1997). We are also currently preparing a proposal to acquire human patient samples from the Children's Oncology Group.

Finally, the susceptibility of Cx36 overexpressing and knockout mice to chemically induced tumorigenesis could be explored in the future. Cx43 and Cx32 knockout mice are prone to chemically induced tumorigenesis (Temme 1997; King & Bertram, 2005). However, since Cx36 knockdown at the cellular level results in enhanced differentiation and suppressed proliferation, it is more likely that Cx36 overexpressing mice are more susceptible to chemically induced tumorigenesis. Unfortunately, although Cx36 knockout mice are available (Deans et al., 2001; Guldenagel et al., 2001), Cx36 overexpressing mice have not been generated. Thus, this study must wait until the necessary tools become available.

This is the first study to report the effects of manipulating Cx36 expression on cellular processes such as differentiation and proliferation. Thus, it provides a baseline from which to expand into other processes including the hallmarks of cancer, such as migration, invasion, anchorage independent growth and tumorigenicity. Furthermore, the use of animal models would also expand this study from the cell to the tissue and organism level. In conclusion, while this study provides an insight into the effect of Cx36 on key cell processes, it remains a novel area of research open to further investigation.

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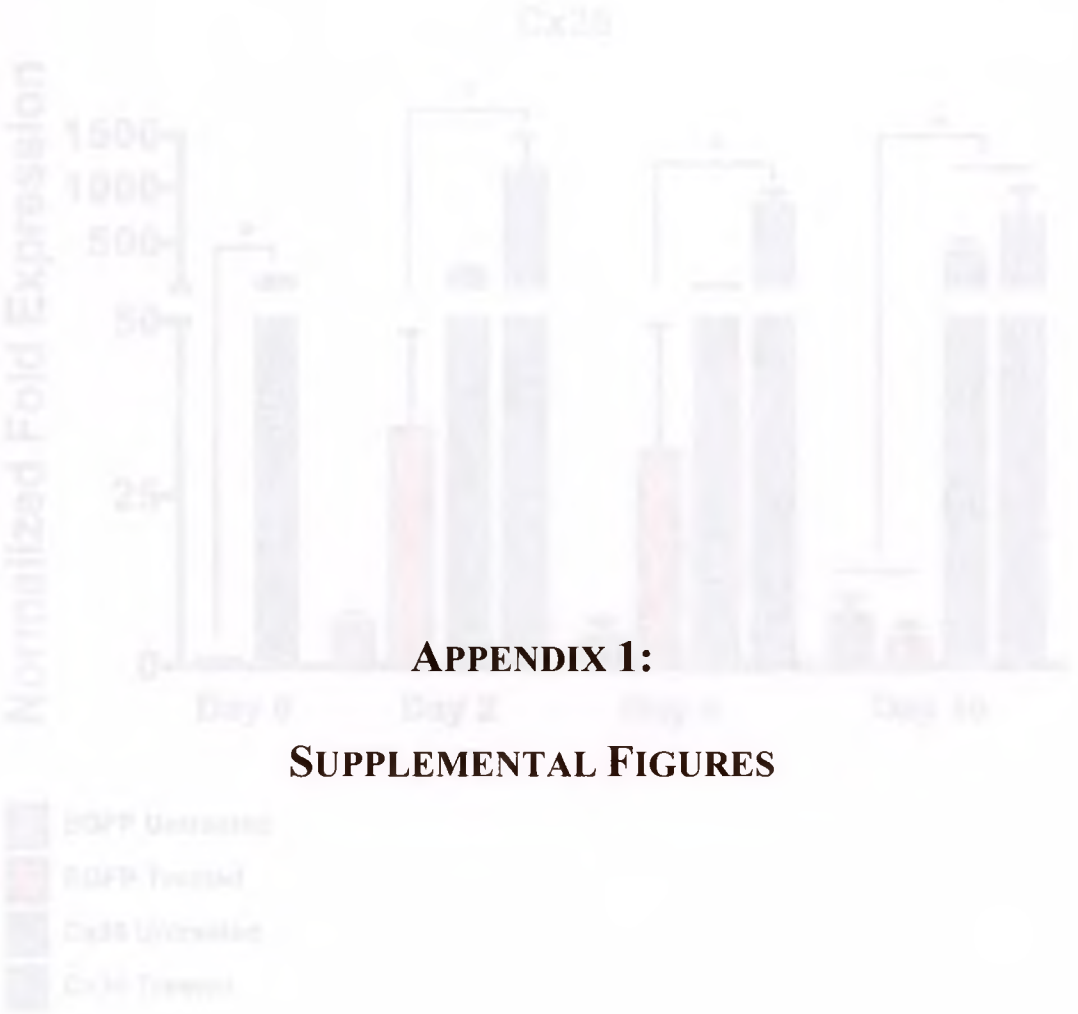


APPENDIX 1:

SUPPLEMENTAL FIGURES

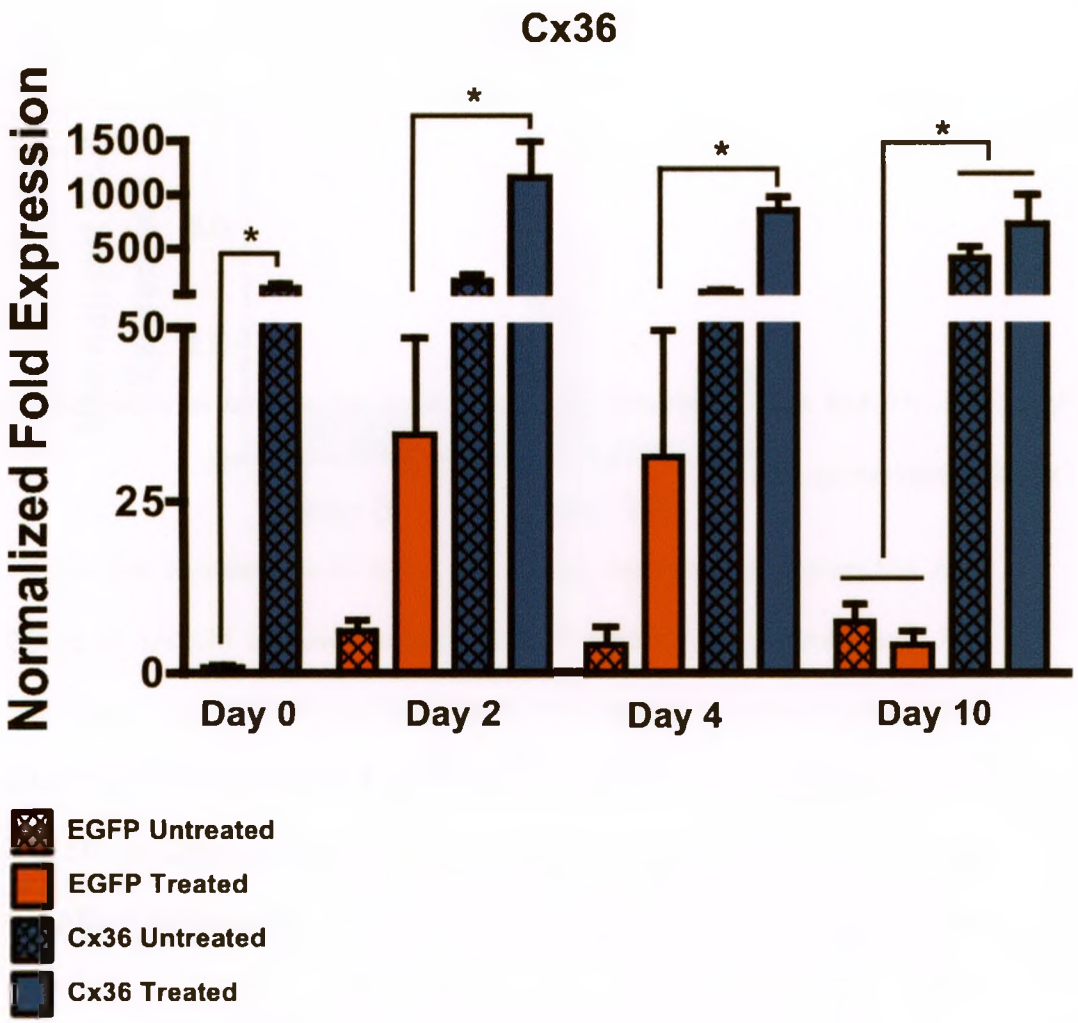


Figure A.1. *Cx36* overexpression is maintained over ten days of differentiation treatment.

RNA was isolated from cells at day 0, 2, 4, and 10 of treatment, and real-time PCR was used to quantify mRNA expression. *Cx36* mRNA expression was significantly higher in Cx36 overexpressing cells at day 0 and 10 in untreated cells, and days 2, 4, and 10 in treated cells. mRNA levels were normalized to *PPIA* mRNA. *$p < 0.05$. N=3. Bars show mean +/- S.E.

Figure A.2. NCAM and N-cadherin expression were not altered in differentiating Cx36 overexpressing cells.

RNA and protein isolated from cells at day 0 and 10 of treatment, and real-time PCR was used to quantify mRNA expression and western blotting to quantify protein expression. *NCAM* mRNA expression levels were not significantly different between any condition (A). N-cadherin protein expression levels did not change between differentiation treatment or overexpression conditions (B & C). mRNA levels were normalized to *PPIA* mRNA and protein levels to GAPDH. N=3. Bars show mean +/- S.E.

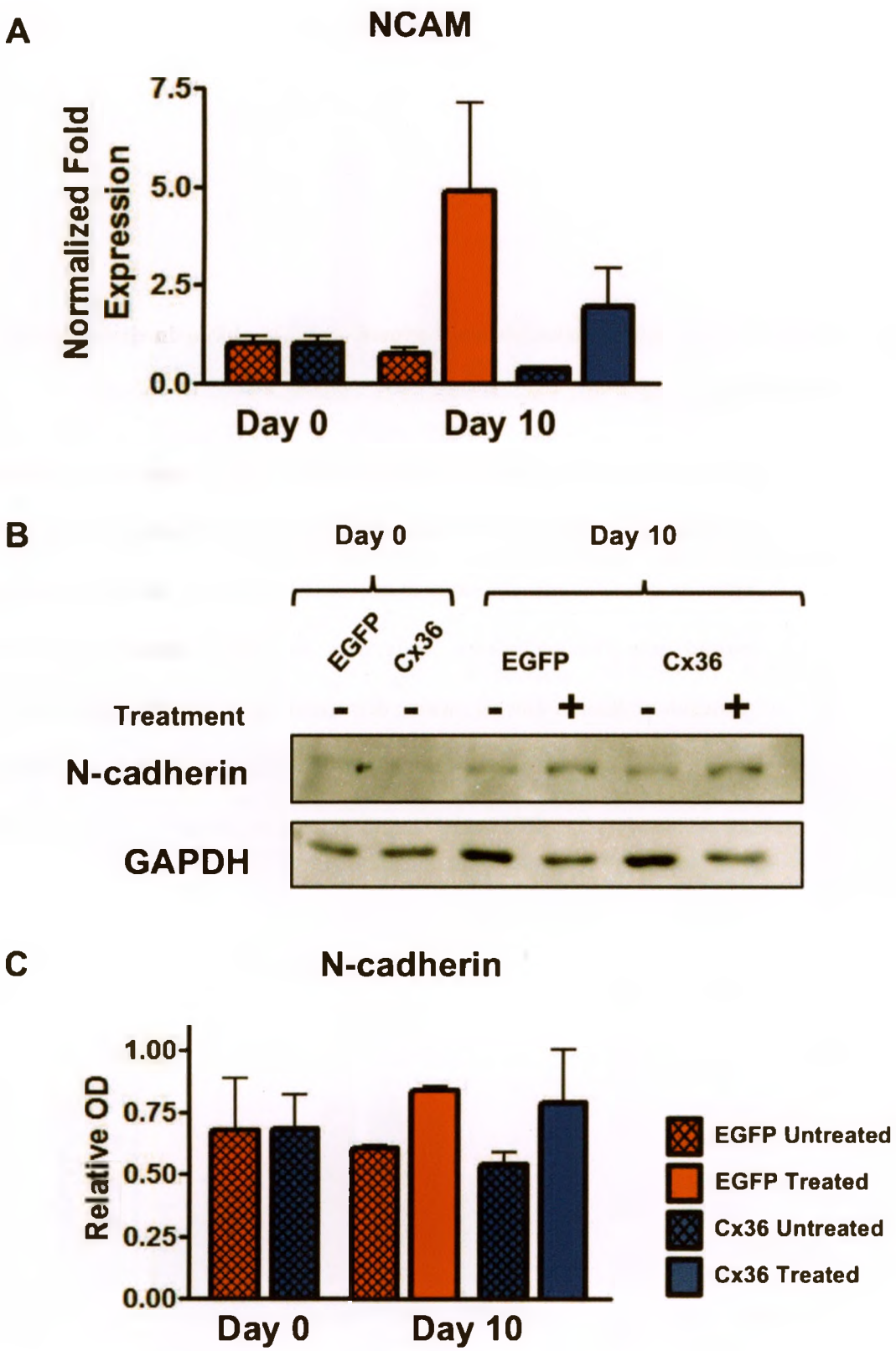
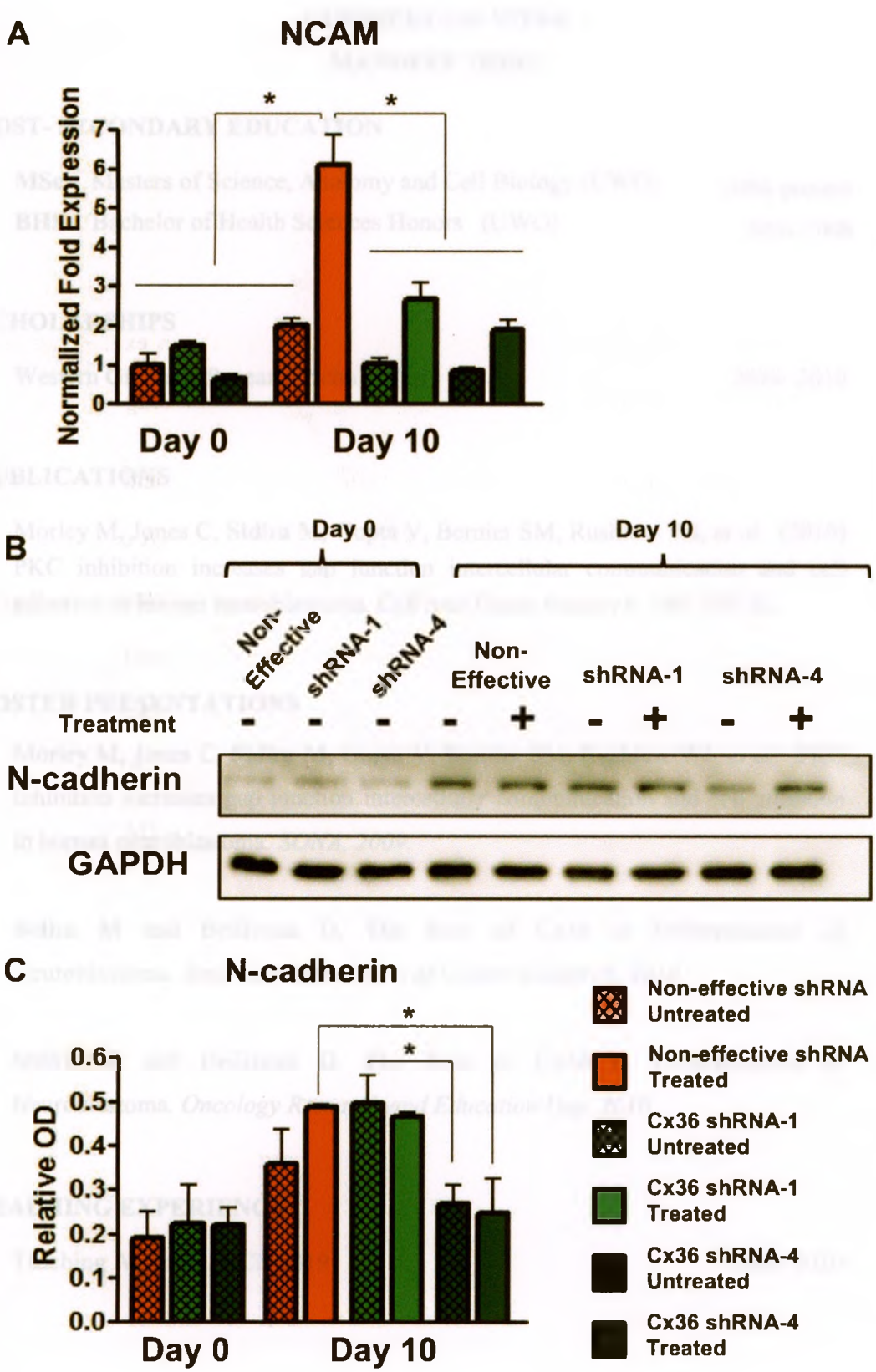

A
NCAM
Normalized Fold Expression
7.5
5.0
2.5
0.0
Day 0
Day 10
B
Day 0
Day 10
EGFP
Cx36
EGFP
Cx36
Treatment
- - - + - +
N-cadherin
GAPDH
C
N-cadherin
Relative OD
1.00
0.75
0.50
0.25
0.00
Day 0
Day 10
EGFP Untreated
EGFP Treated
Cx36 Untreated
Cx36 Treated

Figure A.3. NCAM expression was reduced in Cx36 shRNA expressing cells and N-cadherin expression was only decreased in shRNA-4 expressing cells.

RNA and protein were isolated from cells at day 0 and 10 of differentiation treatment, and real-time PCR was used to quantify mRNA expression and western blots to quantify protein expression. *NCAM* mRNA expression was higher in treated non-effective control cells than all other conditions (A). N-cadherin protein expression levels were decreased in shRNA-4 but not shRNA-1 expressing cells in comparison to non-effective control (B & C). mRNA levels were normalized to *PPIA* mRNA and protein levels to GAPDH. *$p < 0.05$. N=3. Bars show mean +/- S.E.


A
NCAM
Normalized Fold Expression
0
1
2
3
4
5
6
7
*
*
Day 0
Day 10
B
Day 0
Day 10
Non-Effective
shRNA-1
shRNA-4
Non-Effective
shRNA-1
shRNA-4
Treatment
- - - - + - + - +
N-cadherin
GAPDH
C
N-cadherin
Relative OD
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*
*
Day 0
Day 10
Non-effective shRNA Untreated
Non-effective shRNA Treated
Cx36 shRNA-1 Untreated
Cx36 shRNA-1 Treated
Cx36 shRNA-4 Untreated
Cx36 shRNA-4 Treated